An Insight into the Epidemiology, *fimH* Gene Polymorphisms and Regulation of Phase Variation in Type 1 Fimbriae Operon of Drug-Resistant Uropathogenic *Escherichia coli* Isolated from Asymptomatic and Symptomatic Hospitalized Patients of Kolkata, West Bengal, India- A Molecular Perspective

A Thesis submitted for the Degree of
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By

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This is to certify that the thesis entitled **"An Insight into the Epidemiology, *fimH* Gene Polymorphisms and Regulation of Phase Variation in Type 1 Fimbriae Operon of Drug-Resistant Uropathogenic *Escherichia coli* Isolated from Asymptomatic and Symptomatic Hospitalized Patients of Kolkata, West Bengal, India- A Molecular Perspective"** submitted by **Smt. Arunita Ghosh** who got her name registered on 4th May, 2016, for the award of **Ph.D. (Science)** degree of **Jadavpur University**, Kolkata, India, is absolutely based upon her own work under the supervision of **Dr. Mandira Mukherjee** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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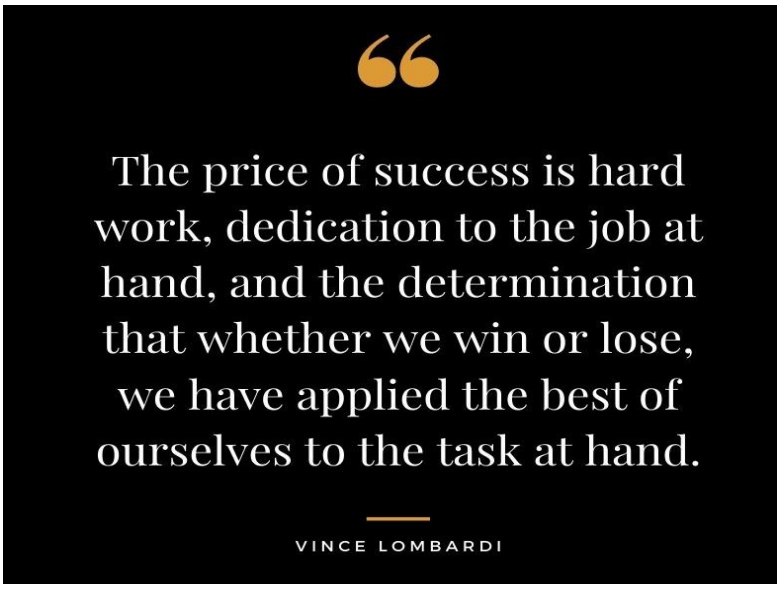
DEDICATION



This thesis is dedicated to:

The God Almighty my creator, my source of wisdom, knowledge, and perception,

My great parents, who have always loved me unconditionally, never stopped giving of themselves in infinite ways and whose commitment and values of life shaped the person I am today. My beloved mother has always encouraged and inspired me with her fullest and truest attention to accomplish my work with truthful self-confidence. My dear father has been a wonderful supporter and motivator until my research was completed,

My beloved elder brother and my sister-in-law, who have nicely been my mental supporters,

My loving husband who has been a part of my life in the last two years of my doctoral research which has been one of the toughest period of my life. I am truly thankful to God for having him in my life. Despite living miles apart, he has always encouraged me to pursue my dreams and finish my research. He has always made sure that I give it all it takes to finish that which I have started. He is himself a scientist by profession and has led me through the valley of darkness with the light of hope and support,

My gracious and reverent in-laws, especially to my beautiful grandmother-in-law for being so empathetic and for giving me moral support during the last years of my doctoral research, and,

Last but not the least the newest and the most special member in our family, who is none other than my adorable nephew.

THE SCIENTIFIC PLEDGE

"A dream is not that which you see while sleeping, it is something that does not let you sleep."

- APJ Abdul Kalam

"The secret of getting ahead is getting started." *- Mark Twain*

"If there has been any success in my life, that was built on the unshakable foundation of failure..."

-Jagadish Chandra Bose

"Research is seeing what everybody else has seen and thinking what nobody else has thought."

- Albert Szent-Györgyi

"Innovation distinguishes between a leader and a follower." *- Steve Jobs*

"Science is fun. Science is curiosity. We all have natural curiosity. Science is a process of investigating. It's posing questions and coming up with a method. It's delving in. " *-Sally Ride*

"Scientific advancement should aim to affirm and to improve human life. " *-Nathan Deal*

"Believe in yourself and all that you are. Know that there is something inside you that is greater than any obstacle."

- Christian D. Larson

"Without ambition, one starts nothing. Without work, one finishes nothing. The prize will not be sent to you. You have to win it."

- Ralph Waldo Emerson

This research focuses on understanding the molecular epidemiology, pathogenic potential, type 1 fimbrial phase variation and adhesive properties of clinical asymptomatic and symptomatic UPECs. This study is also expected to shed light on the regulatory interplay of different cellular factors controlling the inversion of phase switch, thereby guiding the adherence to the host and expediting the invasion process among ABU, in comparison to the symptomatic UPECs.

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LIST OF ABBREVIATIONS

ABU: Asymptomatic Bacteriuria
***E. coli*:** *Escherichia coli*
UPEC: Uropathogenic Escherichia coli
UTI: Urinary Tract Infection
IMViC test
a) I = Indole test
b) M = Methyl red test
c) V = Voges-Proskauer test
d) C = Simmons Citrate test
TSI: Triple Sugar Iron test
EMB agar: Eosin methylene blue agar
L.B: Luria Bertani
M.H: Mueller Hinton
SDW: Single Distilled Water
DDW: Double Distilled Water
OD: Optical Density
CC: Clonal Complex
CFU: Colony Forming Unit
CLSI: Clinical and Laboratory Standard Institute
AK: Amikacin
GEN: Gentamicin
TOB: Tobramycin
CAZ: Ceftazidime
CTX: Cefotaxime
IPM: Imipenem
CIP: Ciprofloxacin
LE: Levofloxacin
COT: Cotrimoxazole
NIF: Nitrofurantoin
CAC: Ceftazidime-Clavulanic acid
CEC: Cefotaxime- Clavulanic acid
CV: Crystal Violet
MDR: Multi-drug resistance
ESBL: Extended Spectrum β-lactamase
BLIR:-β-lactamases Inhibitor Resistance
MSHA: Mannose-Sensitive Haemagglutination
SNP: Single Nucleotide Polymorphisms
NSM: Non-Synonymous Mutation
SDS: Sodium Dodecyl Sulphate
EDTA: Ethylenediaminetetraacetic acid
PCR: Polymerase Chain Reaction
DNA: Deoxyribonucleic Acid

dNTPs: Deoxynucleotide Triphosphates
EtBr: Ethidium Bromide
ERIC: Enterobacterial Repetitive Intergenic Consensus
UPGMA: Unweighted Pair Group Method Using Arithmetic Averages
VFs: Virulence Factors
PAIs: Pathogenicity Islands
MGE: Mobile Genetic Element
IS: Insertion Sequence
IDSA: The Infectious Disease Society of America
MSA: Multiple Sequence Alignment
MLST: Multilocus Sequence Typing
MST: Minimum Spanning Tree
RPM: Revolutions Per Minute
SEM: Standard Error of Mean
ST: Sequence Types
TAE: Tris-Acetate EDTA

ABSTRACT

Uropathogenic *Escherichia coli* (UPECs), the predominant cause of all urinary tract infections (UTIs), can cause both asymptomatic Bacteriuria (ABU) and symptomatic UTIs. Symptomatic UTIs should be treated with antimicrobials to assuage symptoms and further complications, whereas ABU generally does not warrant treatment. However, the emergence of multidrug resistance (MDR) among pathogenic variants of *E. coli* isolated from asymptomatic and symptomatic patients represents a massive public health concern. Hence, the identification and characterization of ABU UPECs and their further comparison with symptomatic UPECs are an absolute necessity in the present era, for clinicians and microbiologists to devise strategies to combat their increasing pathogenicity.

In this study, 95% (p-value ≤ 0.05) of the ABU UPECs and 100% (p-value ≤ 0.05) of the symptomatic UPECs were MDR. Moreover, 80% (p-value ≤ 0.05) of the isolates from both groups were ESBL (Extended Spectrum β-lactamase) producers. The distribution of certain pathogenicity island markers, virulence factor genes, β-lactamase genes, mobile genetic elements (MGEs) and novel phylotype property (NPP) were also found to be significant (p-value ≤ 0.05) among both groups. Clonal heterogeneity and predominance of ST940 (CC448) were evidenced among ABU and symptomatic UPECs; however zoonotic transmission was observed only in the former group. A close association between ABU UPECs with known and unidentified STs having NPPs with isolates that belonged to phylogroups Clade I, D, and B2 was observed. This study for the first time ventured to introduce a new approach to ascertain the phylotype property of the unassigned UPECs. Pathoadaptive FimH mutations, especially hot spot mutation V27A and mannose-sensitive haemagglutination (MSHA) was significantly prevalent among ABU UPECs, mostly reported in the symptomatic ones worldwide. The clinical ABU UPECs with remarkable adhesive capacity, unlike the prototype ABU strain but similar to symptomatic UPECs, highlighted the incidence of bladder epithelial cell adherence in the case of individuals with ABU without manifestation of symptoms. Withal, this is the first study that attempted to investigate the regulatory interplay of three cellular factors; H-NS, IHF, Lrp on *fimB* and *fimE* recombinases that further domineered the inversion of *fim* switch and adhesive capacity among ABU UPECs. An association of V27A, N70S, and S78N FimH mutations with higher adhesive capability and type 1 fimbrial expression was identified among both groups. Highly adherent UPECs regardless of their asymptomatic and symptomatic nature were mostly ESBL producers, harboured MGEs, and possessed NPP. These comparable characteristics of ABU and symptomatic UPECs might be attributed to the genome plasticity caused due to the deleterious effect of MDR.

Therefore, this study displayed the fact that ABU, although generally not considered as a clinical condition, their increased recognition, proper understanding, and characterization together with appropriate therapeutic measures when necessary is the need of the era which otherwise might lead to serious complications in the vulnerable population.

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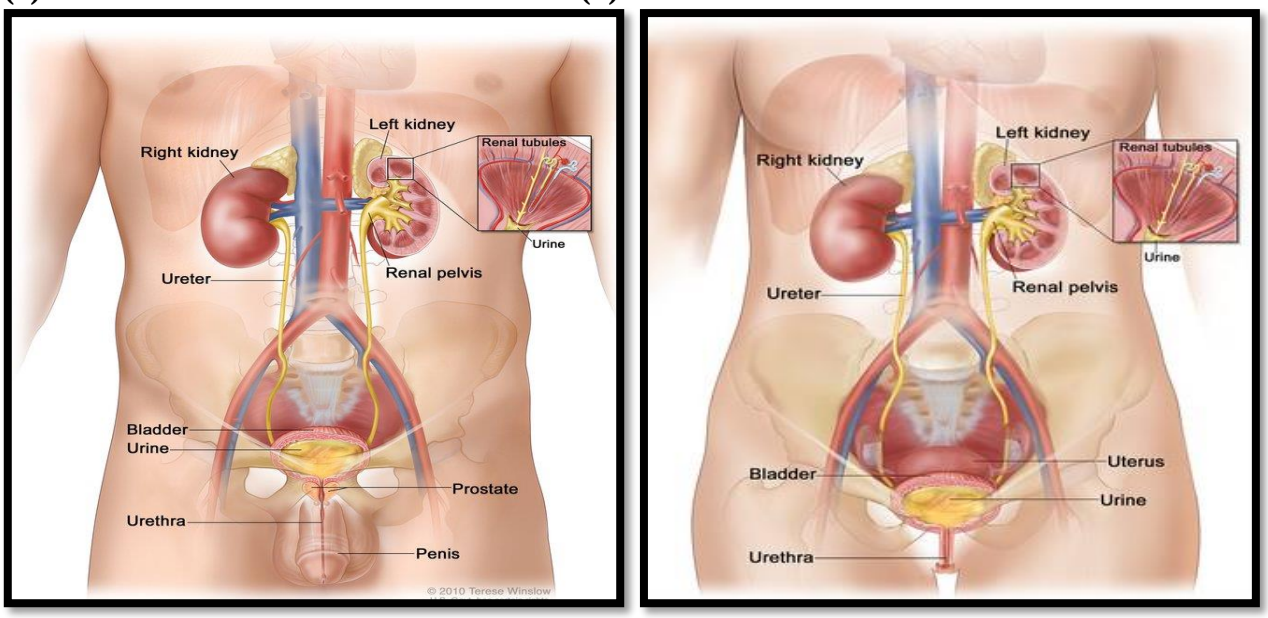
CHAPTER 1.

General Introduction

1.1 General Epidemiology of Urinary Tract Infection

The urinary tract subsumes the kidneys, ureters, bladder, and urethra **(Fig. 1.1)** and, with the noninclusion of the urethra, the maximum part of the tract is observed to be sterile. The defense from microbial colonization is facilitated by different factors that are secreted into urine and by structural barriers, for instance, the glycoprotein plaque uroplakins **(Wu et al. 2009; Abraham and Miao 2015**) and a coating of hydrated mucus **(Grist and Chakraborty 1994; Abraham and Miao 2015)**. Additionally, epithelial cells and a range of inhabitant immune cells line the urinary tract that further guard against infection. The aforementioned barriers avert the pathogens from inflowing the urinary tract and from establishment of persistent infection **(Abraham and Miao 2015)**.

(a) **(b)**



https://visualsonline.cancer.gov/details.cfm?imageid=9754

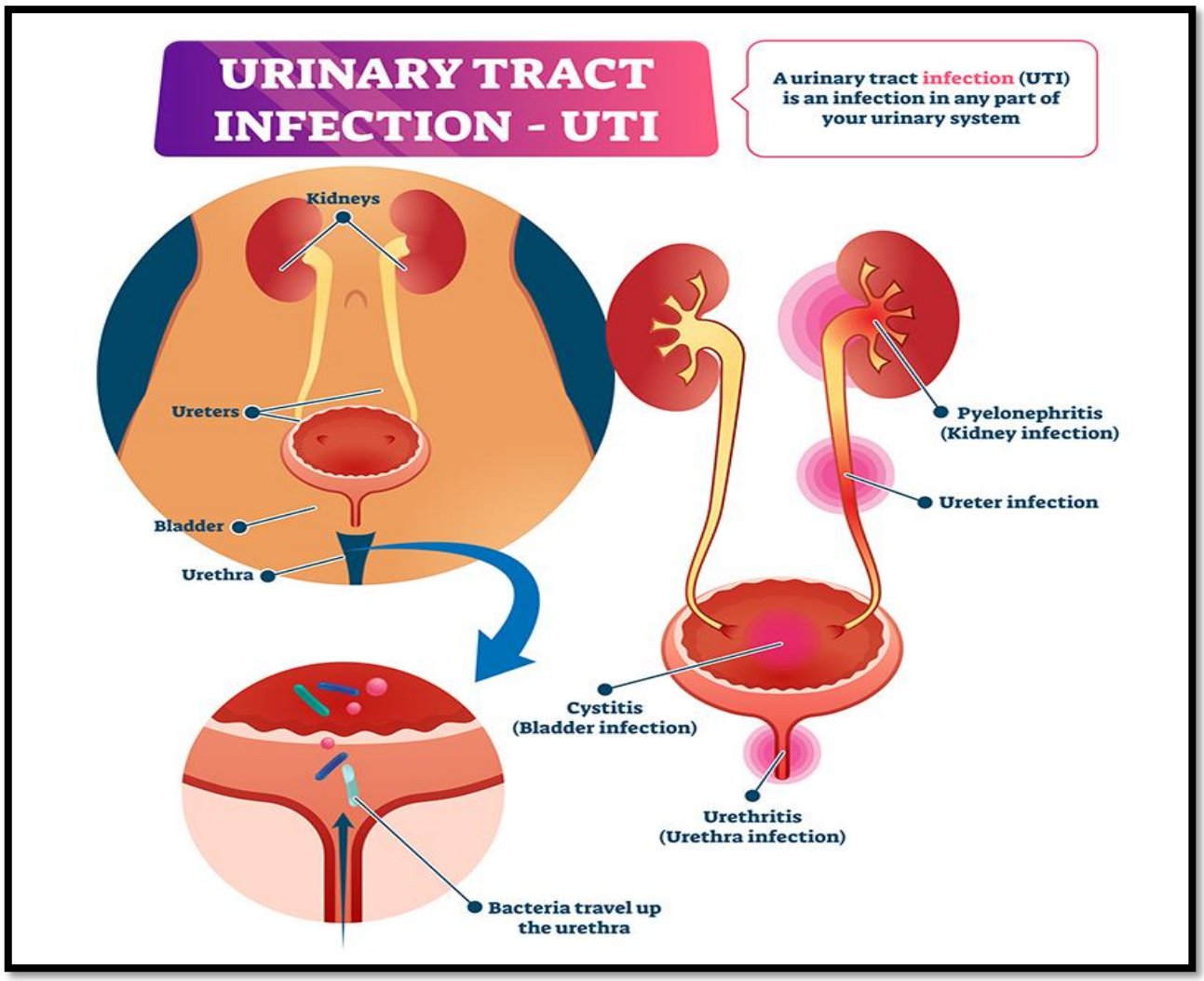
Fig. 1.1: Schematic representation of the urinary system of (a) male and (b) female.

Nonetheless, urinary tract infections (UTIs) **(Fig. 1.2)** are the most common bacterial infections affecting 150 million people from all age groups each year worldwide **(Flores-Mireles et al. 2015; Sewify et al. 2016)**. UTIs account for noteworthy causes of morbidity and mortality worldwide **(Schwan 2011; Hailay et al. 2020)**.

1.1.1 Classification of UTIs

UTIs may be classified into three types based on the part of the urinary tract affected **(Yeruham et al. 2006; Lane and Takhar 2011)**. They are:

(a) Urethritis (Urethra infection): Inflammation **(Fig. 1.3)** of the tube carrying urine from the bladder to the outside of the body. https://stanfordhealthcare.org/medical-conditions/womens-health/urinary-tract-infection/types.htmL



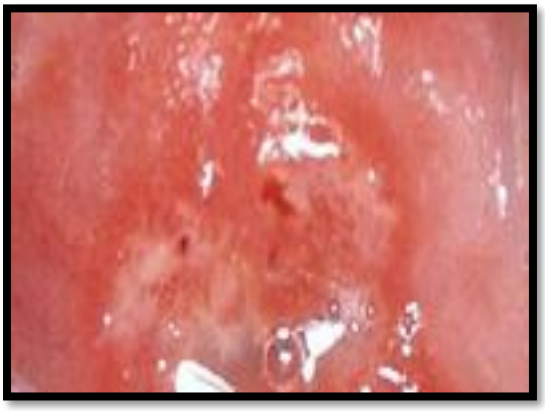
https://myobmd.org/gynecology/urinary-tract-infections-uti-symptoms-diagnosis-and-treatment/

Fig. 1.2: Schematic representation of UTI.

Signs and symptoms:

- Burning with urination
- Discharge

https://www.mayoclinic.org/diseases-conditions/urinary-tract-infection/symptoms-causes/syc-20353447



https://www.healthline.com/health/pain-in-urethra

Fig. 1.3: Pictorial representation of urethritis.

(b) Cystitis (Bladder infection): Infection caused by bacteria in the bladder that has moved up from the urethra. **https://stanfordhealthcare.org/medical-conditions/womens-health/ urinary-tract-infection/types.htmL**

Signs and symptoms:

- A strong, persistent urge to urinate
- A burning sensation when urinating
- Passing frequent, small amounts of urine
- Blood in the urine (haematuria)
- Passing cloudy or strong-smelling urine
- Pelvic discomfort **(Fig. 1.4)**

https://pacificcross.com.vn/cystitis/

Fig. 1.4: Pictorial representation of pain or pressure in the lower abdomen due to cystitis.

- A feeling of pressure in the lower abdomen **(Fig. 1.4.)**
- Low-grade fever

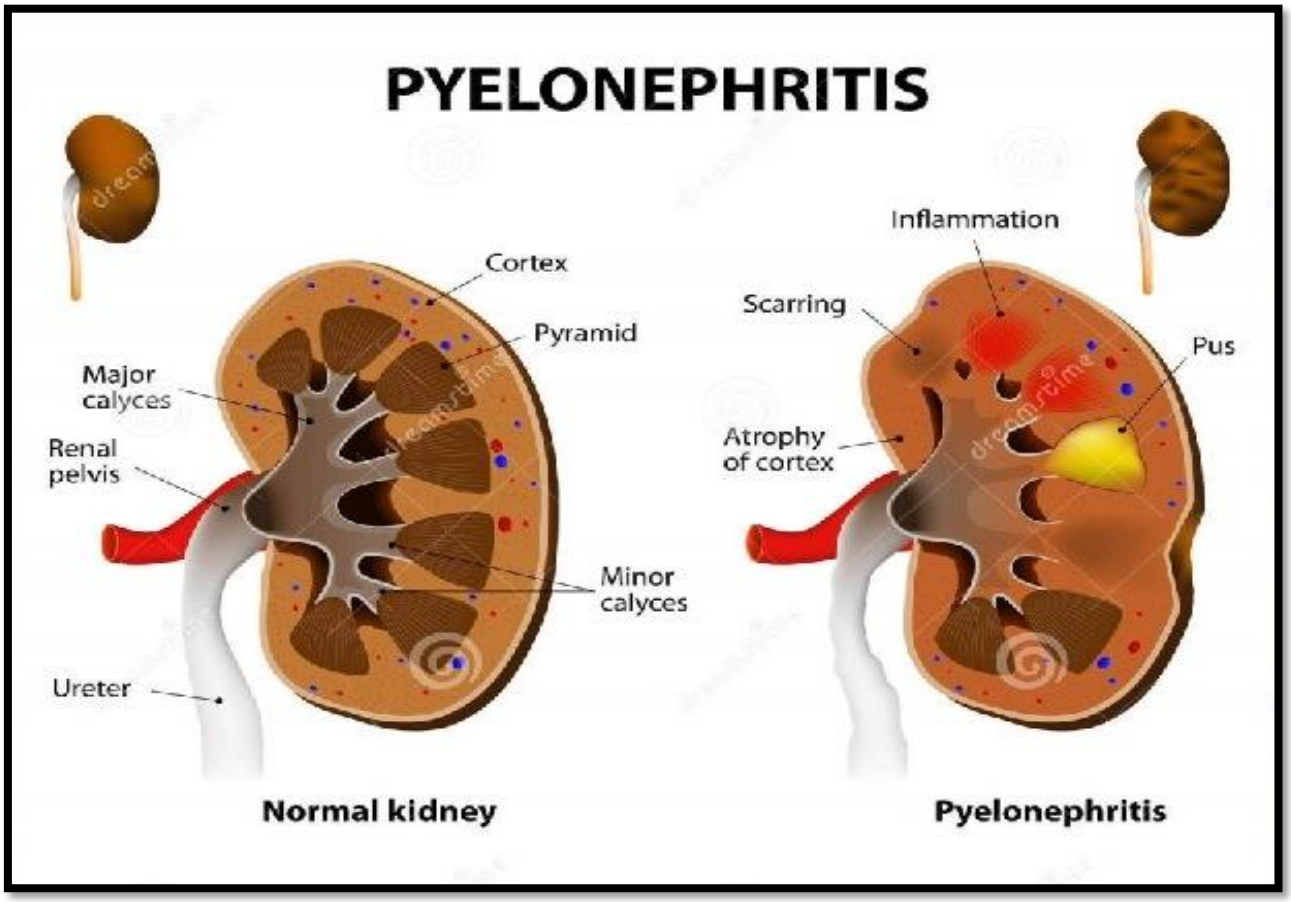
https://www.mayoclinic.org/diseases-conditions/cystitis/symptoms-causes/syc-20371306

(c) Pyelonephritis (Kidney infection): An infection of the kidneys **(Fig. 1.5)** that is typically a consequence of an infection that has spread up the tract, or from an impediment in the urinary tract. An impediment in the urinary tract causes the urine to backflow into the ureters and kidneys. **https://stanfordhealthcare.org/medical-conditions/womens-health/urinary-tract-infection/types.htmL**

Signs and symptoms:

- Fever **(Fig. 1.6)**

- Chills **(Fig. 1.6)**



https://slidetodoc.com/pyelonephritis-dr-kiran-h-s-assistant-professor-ymc/

Fig. 1.5: Schematic representation of normal kidney and pyelonephritis.

- Back, side (flank), or groin pain **(Fig. 1.6)**

https://www.medindia.net/patientinfo/pyelonephritis.htm

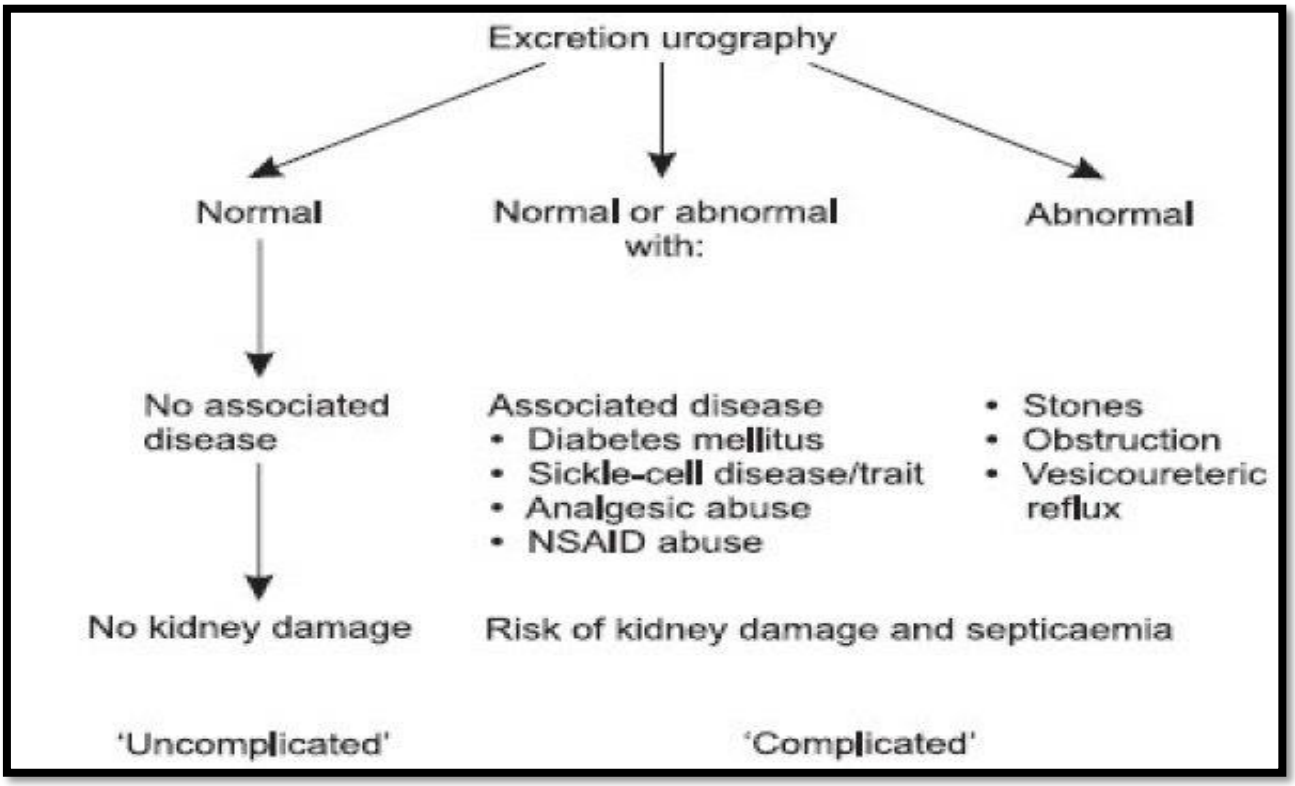
Fig. 1.6: Pictorial representation of major symptoms of pyelonephritis.

- Night sweats.
- Extreme fatigue
- Excessive thirst
- Abdominal pain
- Frequent urination
- Strong, persistent urge to urinate
- Burning sensation or pain when urinating
- Nausea and vomiting **(Fig. 1.6)**
- Pus or blood in your urine (hematuria)
- Urine that smells bad or is cloudy

https://www.mayoclinic.org/diseases-conditions/kidney-infection/symptoms-causes/syc-20353387

UTIs are often classified into two types based on the factors that trigger the infection **(Vasudevan 2014)**. They are as follows:

(a) Uncomplicated UTIs: An uncomplicated UTI **(Fig. 1.7)** is usually cystitis or pyelonephritis happening in a normal host who has no structural or functional abnormalities, is not pregnant, or who has not been instrumented (for example, with a catheter) **(Vasudevan 2014; Johnson 2017)**.



https://www.researchgate.net/publication/42339505_Approach_to_urinary_tract_infections/figures?lo=1 **(Najar et al. 2009)**

Fig. 1.7: Classification of complicated and uncomplicated urinary tract infections.

(b) Complicated UTIs: They are those that bear a higher risk of treatment failure, and usually require longer antibiotic courses and often additional workup. These infections comprise those that occur: in males, in pregnant females, as a consequence of obstruction, hydronephrosis, renal tract calculi, or colovesical fistula, in immunocompromised patients or the elderly, due to unusual organisms, after instrumentation or in conjunction with medical equipment such as urinary catheters, in renal transplant patients, in patients with impaired renal function, or after prostatectomies or radiotherapy. As well, urinary tract infections that reappear in spite of sufficient treatments are complicated (**Fig. 1.7) (Sabih and Leslie 2021)**.

UTIs are often classified into two types based on the nature of occurrence **(Vasudevan 2014)**. They are as follows:

(a) Primary UTI: Primary UTI is generally associated with first manifestations of infections in the lower part of the urinary tract. The majority of patients diagnosed with lower UTI in primary care settings receive same-day empirical antibiotics with little diversity in the choice of agent. However, the re-prescription rates of antibiotics are low **(Pujades-Rodriguez et al. 2019)**.

(b) Recurrent UTI: Recurrent UTI is usually defined as having three or more episodes of UTI's with symptoms within a 12-month-period after the first presentation or two or more episodes within six months. The frequencies of recurrent UTIs differ depending on the source of data, with the occurrence being lower in the primary care departments, and higher in the emergency and referral settings. Chronic symptoms like abdominal pain, nausea, vomiting, and fatigue may be caused by recurrent UTIs. Nonetheless, the most formidable complication of recurrent UTI is renal scarring, which leads to the development of chronic renal failure and hypertension. Moreover, recurrent UTI may be predisposed by underlying structural abnormalities, together with bowel dysfunction or vesicoureteral reflux disease. However, children with no reflux or any other predisposing condition such as structural abnormalities (calculi, infected cysts, renal or bladder abscesses, pyelonephritis, spinal cord injury catheters) and bowel dysfunction were also found to be at threat for the development of recurrent UTI **(Doğan and Ipek 2020)**.

UTIs can also be classified into two types based on the manifestation of symptoms **(Vasudevan 2014; Aamir et al. 2021)**. They are as follows:

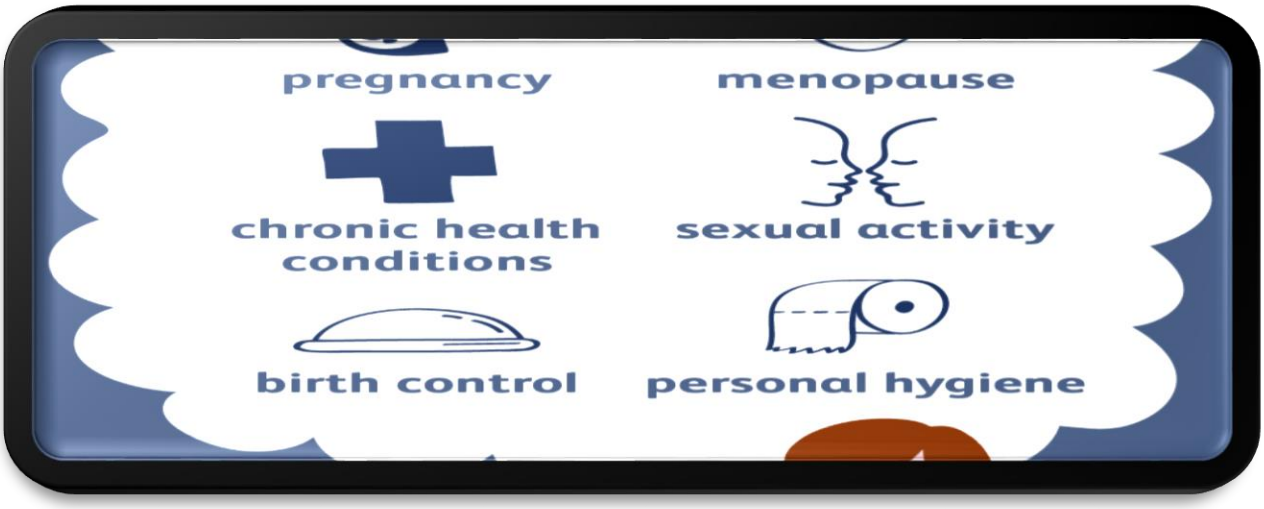
(a) Asymptomatic Bacteriuria (ABU): ABU is the occurrence of bacteria in the appropriately collected urine of a patient who has no signs or symptoms of a UTI. ABU is exceedingly prevalent in clinical practice and its frequency increases with age (**Givler and Givler 2021)**. The current guidelines by the Infectious Diseases Society of America (IDSA) defined ABU as the existence of 1 or more species of bacteria growing in the urine at stated quantitative counts ($\geq 10^5$ colony-forming units [CFU]/mL or $\geq 10^8$ CFU/L) **(Nicolle et al. 2019)**.

(b) Symptomatic UTI: This includes cystitis, pyelonephritis, urosepsis, septic shock, or all combined **(Mody and Juthani-Mehta 2014)**. It is defined by the Centers for Disease Control and Prevention as a clean-catch urine culture growing $\geq 10^5$ CFU/mL of no more than 2 species of a uropathogen in a patient with symptoms of a UTI. An alternative definition projected by the centre includes 2 of the following: fever (>38°C [fever is usually not seen in uncomplicated cystitis]), dysuria, urgency, frequency, or suprapubic pain, and at least 1 of the following: positive Gram stain, pyuria ≥10 white blood cells per high-power field in centrifuged urine, positive leukocyte esterase and/or nitrite by dipstick method, or 2 positive urine cultures with the same uropathogen ($\geq 10^2$/ μL) in a non-voided sample **(Matthews and Lancaster 2011)**.

1.1.2 Determinants

A range of parameters are linked to UTI that consist of parity, age, gravidity, and association of diseases that augment the condition of the infection **(Fig. 1.8)**. Bacteria are the leading executor accountable for conferring the infection among humans but the part played by certain fungi and viruses cannot be disregarded. However, the occurrence of UTI as a result of viral or fungal infection is considered to be rare phenomenon **(Vasudevan 2014; Ali et al. 2018; Ahmed et al. 2019)**. Major determinants of UTIs are as under:

(a) Risk factors of UTI: Risk factors for urinary tract infections (UTIs) may be behavioural, anatomical, or genetic in nature **(Fig. 1.9)**, and will fluctuate depending on the population being considered and the form of UTI. Fleeting conditions such as pregnancy may incline to UTI or upsurge the peril of serious complications from an infection. In permanent conditions such as neurogenic bladder malfunction, due to spinal cord injury, the surfacing nature of the patient's requirements and therapeutic interventions mean that the risk of UTI changes over time **(Storme et al. 2019)**.



https://www.verywellhealth.com/urinary-tract-infections-causes-and-risk-factors-4161060

Fig. 1.8: Range of parameters related to the development of UTI.

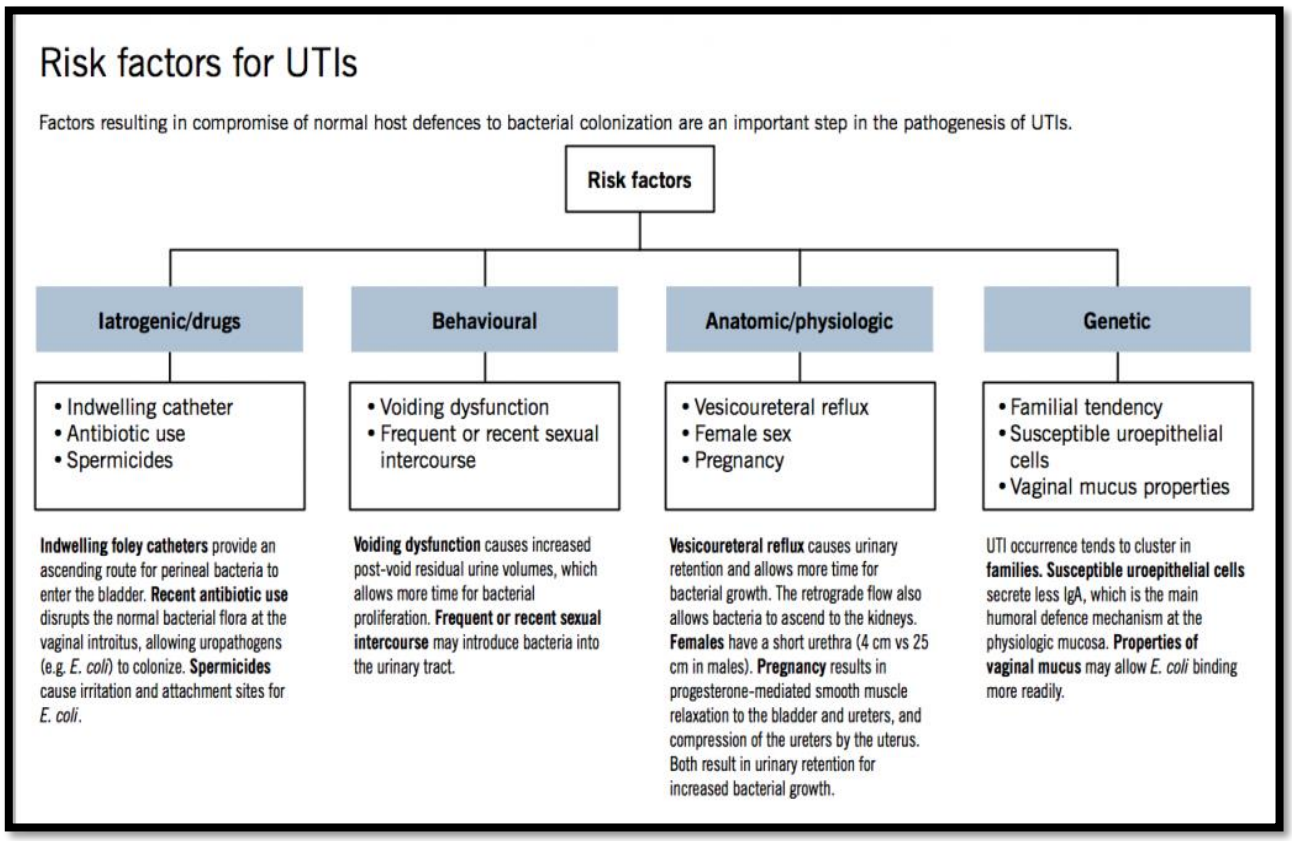
In general, the risk factors of UTIs are as follows:

- **Gender:** Due to certain anatomical factors, women visage a much higher risk of UTIs (compared to men). This is because females have a shorter urethra, which permits bacteria to get to and infect the bladder effortlessly. Moreover, the opening to the urethra in women is notably nearer to the rectum, where UTI-causing bacteria are identified to reside.
- **Pregnancy:** UTIs are also recognized to be more common during gestation (especially from week six through week 24). The increase in size and weight of the uterus during preganancy may prevent the complete drainage of urine from the bladder, which can make pregnant women more prone to UTI.
- **Menopause:** Females going through menopause may also have a greater risk of urinary tract infections, probably due to hormonal changes that might affect the beneficial bacteria accountable for fighting off dangerous microorganisms in the urinary tract.
- **Health Conditions:** Chronic health problems may augment UTI risk as well. These consist of conditions associated with defective immune response (such as diabetes), which can weaken your body's ability to stave off bacteria. Age-related diseases like Alzheimer's disease may also factor into UTI risk, since they may meddle with personal hygiene.

  Additionally, the following people are expected to develop urinary tract infections:
  - those with spinal cord injuries or nerve damage around the bladder, which can disallow complete emptying of the bladder
  - those with kidney stones, inflated prostate, or any other issue that blocks the normal flow of urine and encourages bacterial growth
  - those with vesicoureteral reflux (VUR) or other abnormalities of the urinary tract
  - those who have lately used a urinary catheter
  - those with bowel incontinence
- **Genetics:** Genetics may play a role in the development of UTIs. For example, research states that **(Ragnarsdóttir et al. 2011; Godaly et al. 2015)** genetic variation in immune response may either influence the severity of UTIs or protect against infection.
- **Lifestyle Risk Factors:** Various factors may add to the development of urinary tract infections.
- **Sexual Activity:** One of the most common lifestyle risk factors for UTIs is sexual activity predominantly for women. Sexual intercourse may transfer bacteria from the genitals and anus into the urethra and, consecutively, lead to infection. For men, unguarded sexual activity involving women with a vaginal infection may increase the risk of UTIs.

- **Birth Control:** The use of certain types of birth control (such as diaphragms or spermicide) may also elevate risk of UTIs in women.
- **Personal Hygiene:** Numerous personal hygiene habits are also considered risk factors for UTIs. These habits include:
  - use of douches and feminine hygiene sprays or powders,
  - wiping from back to front after urination or having a bowel movement, especially in the case of women,
  - retaining urine for an abnormally prolonged period (i.e. "holding it in"),
  - extended periods of immobility (such as during recovery from an injury or illness).

https://www.verywellhealth.com/urinary-tract-infections-causes-and-risk-factors-4161060

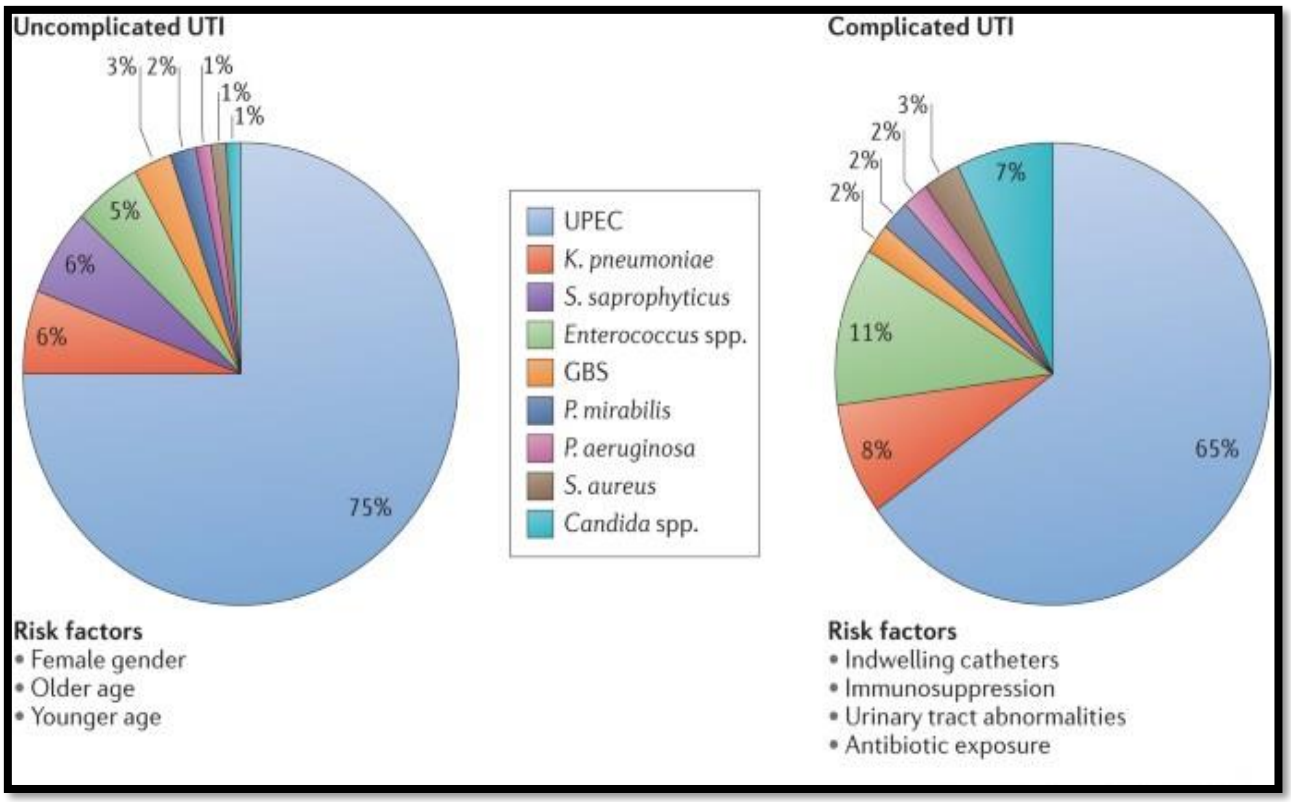


http://www.pathophys.org/uti/uti-riskfactors/

Fig. 1.9: Risk factors of UTI.

(b) Causative agents of UTI: UTIs are caused by both Gram-negative and Gram-positive bacteria in addition to by certain fungi. The most predomiant causative agent for both uncomplicated and complicated UTIs **(Fig. 1.10)** is uropathogenic *Escherichia coli* (UPEC) **(Ronald 2003; Jacobsen et al. 2008; Nielubowicz and Mobley 2010; Fisher et al. 2011; Kline et al. 2011; Levison and Kaye 2013; Chen et al. 2013; Foxman 2014; Flores-Mireles et al. 2015)**. Moreover, UPECs are largely known to be associated with both ABU and symptomatic UTI **(Rowe and Juthani-Mehta 2013; Bien**

et al. 2012). In the case of uncomplicated UTIs, prevalence of UPEC is followed by *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, group B *Streptococcus* (GBS), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida* spp **(Ronald 2003; Nielubowicz and Mobley 2010; Kline et al. 2011; Foxman 2014; Flores-Mireles et al. 2015)**. Nonetheless, in the case of complicated UTIs, UPEC is the most common causative agent, followed by *Enterococcus* spp., *K. pneumoniae*, *Candida* spp., *S. aureus*, *P. mirabilis*, *P. aeruginosa* and GBS **(Jacobsen et al. 2008; Fisher et al. 2011; Levison and Kaye 2013; Chen et al. 2013; Flores-Mireles et al. 2015)**.



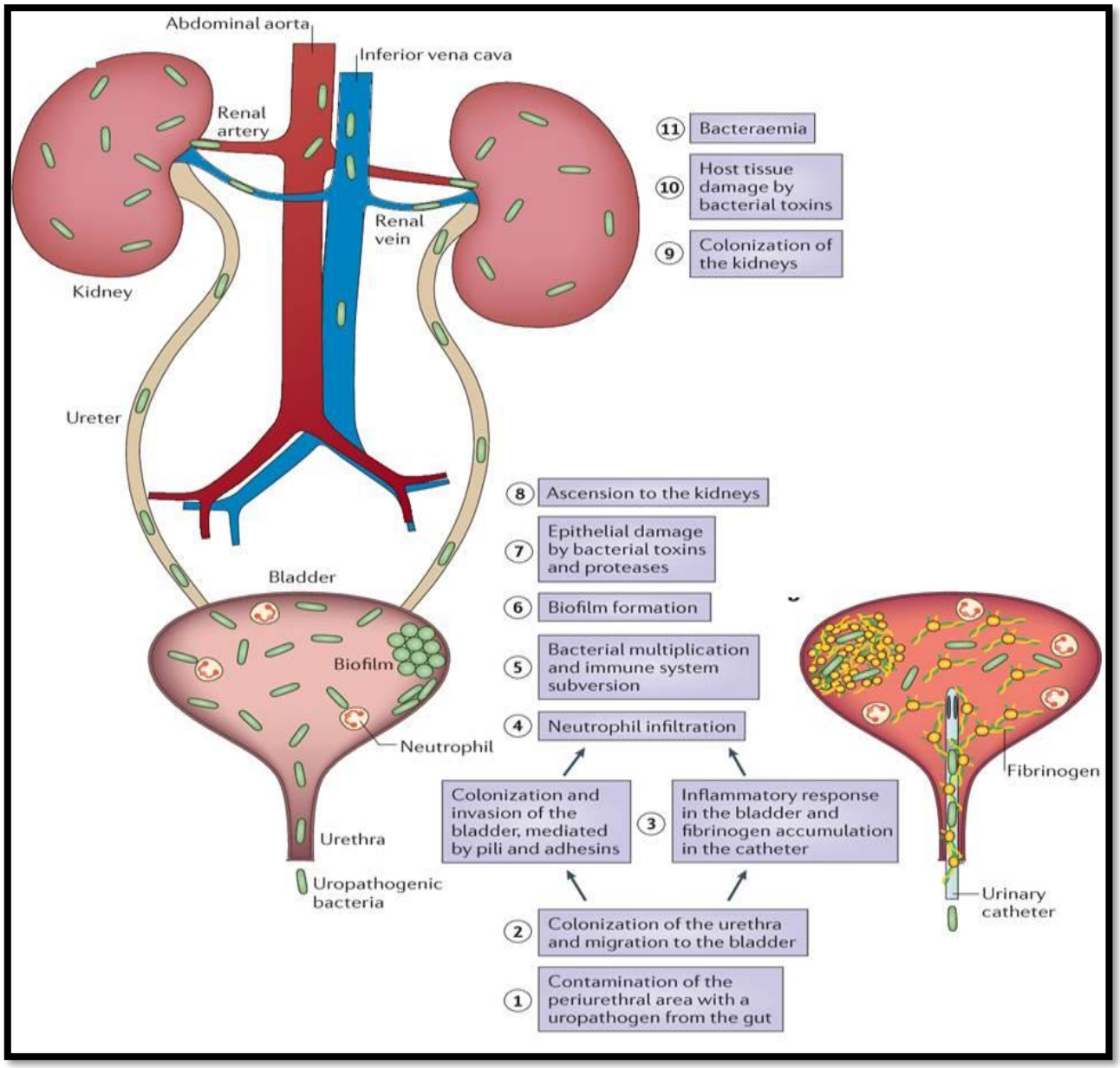
https://www.nature.com/articles/nrmicro3432 **(Flores-Mireles et al. 2015)**

Fig. 1.10: Causative agents of uncomplicated and complicated UTI.

(c) Pathogenesis of UTI: The interactions between the uropathogen and host casue UTIs and their pathogenesis involve numerous processes. Firstly, the uropathogen binds to the epithelial surface and it consequently colonizes and spreads all over the mucosa instigating tissue damage. Following the primary colonization period, ascension of pathogens into the urinary bladder is observed, thereby resulting in symptomatic UTI or ABU. Further progression leads to pyelonephritis and renal damage. Explicit virulence factors inhabiting the uropathogen's membrane are accountable for bacterial resistance to the usually active host's defence mechanisms **(Davis and Flood 2011)**.

Adherence and colonization: Adherence is the most important event initiating to each step in UTI pathogenesis. A UTI usually starts when the uropathogen staying in the gut causes periurethral

contamination followed by colonization of the urethra and consequent movement of the pathogen to the bladder. This event requires certain appendages such as pili and flagella **(Fig. 1.11)**. The consequences of complex host-pathogen interactions in the bladder eventually govern whether uropathogens are efficacious in colonization or are eliminated.



https://www.semanticscholar.org/paper/Urinary-tract-infections%3A-epidemiology%2C-mechanisms-Flores-Mireles-Walker/5653705a64b0996847cd6bb295fb78f30fad16c1/figure/2

(Flores-Mireles et al. 2015)

Fig. 1.11: Pathogenesis of UTI.

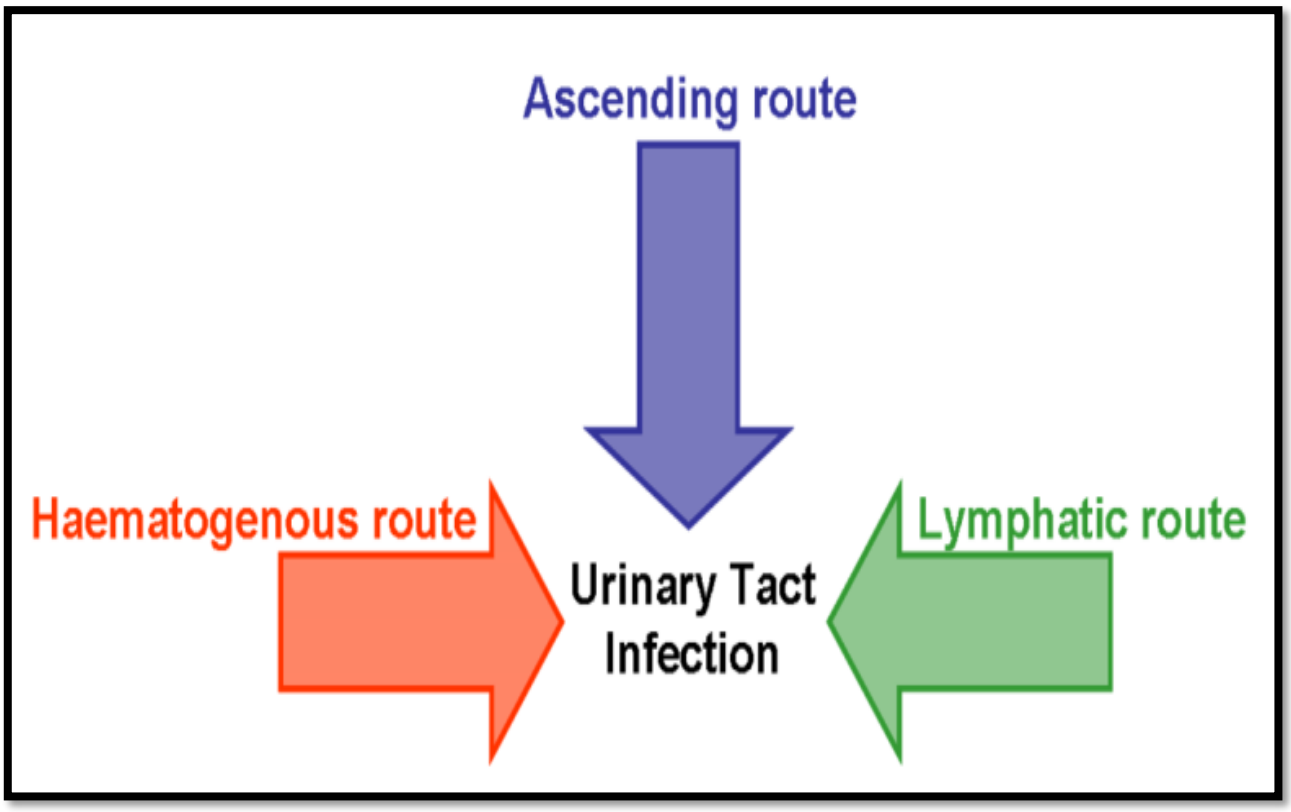
Numerous bacterial adhesins identify receptors on the the uroepithelium and intercede colonization. Uropathogens like UPECs invades the bladder epithelium to survive, producing toxins and proteases thereby releasing nutrients from the host cells, and synthesizing siderophores to acquire iron **(Fig. 1.11)**. These uropathogens can consequently ascend to the kidneys via adhesins or pili to colonize the renal epithelium thereby producing tissue-damaging toxins after overcoming host immune

surveillance **(Fig. 1.11)**. Subsequently, the uropathogens are competent to cross the barrier of tubular epithelium to access the bloodstream, thereby initiating bacteraemia.

UPEC, *K. pneumoniae, S. saprophyticus* and the other uropathogens that cause uncomplicated UTIs, can bind straight to the bladder epithelium that is composed of the umbrella cells, intermediate cells, and basal cells. UPEC and *K. pneumoniae* bind to uroplakins, which are the primary protein components of the apical membrane of umbrella cell and that form a crystalline array shielding the mammalian bladder tissue from harmful agents of urine. Additionally, uroplakins, $\alpha_3\beta_1$ integrins that are expressed at the uroepithelial cells surface, can also dole out as receptors for UPEC. However, complicated UTIs are initiated when the bacteria bind to a urinary catheter, a kidney stone, or a bladder stone, or when they are retained in the urinary tract by a physical obstruction. Few pathogens (for example, UPEC) can cause both uncomplicated and complicated UTIs. Nonetheless, others such as *P. mirabilis, P. aeruginosa,* and *Enterococcus* spp. mostly cause complicated UTIs **(Fig. 1.11) (Flores-Mireles et al. 2015)**.

Routes of infection: UTIs mainly occurs via three major routes **(Fig. 1.12)**: They are:

- **Ascending:** Ascending routes of infection are most frequent among patients with an established UTI **(Davis and Flood 2011)**. This occurs when bacteria colonizing the urethra consequently travel upwards, or ascend, the urethra to the bladder and cause cystitis and continue to ascend to the ureters to cause pyelonephritis.
- **Haematogenous:** The haematogenous route of infection involves the seeding of the kidney in the setting of a bloodstream infection. This may also engross the seeding of the urinary tract with pathogens carried by the blood supply. *S. aureus* bacteremia can cause renal abscesses via the haematogenous route. *E. coli* and *P. aeruginosa* are less likely to seed the kidneys via haematogenous spread.
  https://eopcw.com/assets/stores/Integerated%20Therapeutics%20IV/lecturenote_288026489Chapter%203-%20Urinary%20Tract%20Infection.pdf
- **Lymphatic:** On uncommon occasions, bacteria from adjacent organs may penetrate the urinary tract via the lymphatics. Conditions associated with the lymphatic route are retroperitoneal abscesses and severe bowel infections **(Davis and Flood 2011)**.



https://www.intechopen.com/chapters/19318 **(Davis and Flood 2011)**

Fig. 1.12: Routes of UTI.

1.1.3 Frequency, pattern, specified populations and related countries and states

Several reports from different parts of the world as countries like Italy, Saudi Arabia, India, Singapore, and the United States of America, to name a few, over the last 12 years stated UTIs, both symptomatic UTI and ABU as the common contagion among both men and women but the prevalence is quite high among women due to their anatomy and reproductive physiology **(Salvatore et al. 2011; Al-Badr and Al-Shaikh 2013; Vasudevan 2014; Tan and Chlebicki 2016; Moreno 2016; Scaglione et al. 2021; Givler and Givler 2021)**. **Givler and Givler (2021)** stated that although most women have transitory bacteriuria after sexual intercourse, however, few among them develop symptomatic infections because the body's normal defense mechanisms prevent symptomatic infection in most cases. A recent report from Italy **(Scaglione et al. 2021)** indicated that around 50% of all women suffer from symptomatic UTIs at least once in their lifetime; 20–40% of them experience recurrent episodes. However, Vasudevan (**2014)** from India displayed a comparatively higher incidence of UTI among pregnant women. Nonetheless, an earlier study from Italy **(Salvatore et al. 2011)** indicated the peak to be between the ages 16 to 35 years and also stated that around 27% of females with a first episode of UTI record a recurrence within 6 months, and 48% within the first year. However, reports from Germany and Uruguay **(Tandogdu and Wagenlehner 2016; Medina and Castillo-Pino 2019)** respectively stated a higher prevalence of uncomplicated UTI among women over 65 years of age. However, according to Givler and Givler **(Givler and Givler 2021)** less than 0.5%

of infants and toddlers have ABU but the occurrence increases with age. The rate is 5% or less among healthy premenopausal women, up to 15% or more in women and men age 65-80 years, and it continues to hike after age 80 to as high as 40% to 50% in the case of long-term care residents. Nonetheless, **Sabih and Leslie, (Sabih and Leslie 2021)** from New Zealand stated that the incidence of complicated UTI is associated with specific risk factors like there is a daily risk of developing bacteriuria in 10% individuals with indwelling bladder catheters, and equal to a 25% risk that bacteriuria will advance to a UTI. Bacteriuria ensues in up to 14% of diabetic females but does not have a propensity to occur with a higher frequency in diabetic males. The incidence of ABU in pregnant females is akin to that in nonpregnant females (2% to 7%) but tends to progress to symptomatic UTI in as many as 40% of pregnant women. ABU also inclines to increase with age in females and exists in up to 80% of the aged female population. It is unusual among younger healthy males but can be present in up to 15% of elderly males. UTIs are the most prevalent infections in renal transplant patients. Likely, 25% of these patients will progress to having a UTI within the first year after a transplant. Augmented incidence of UTI has been specified in patients using Dapagliflozin (SGLT2i).

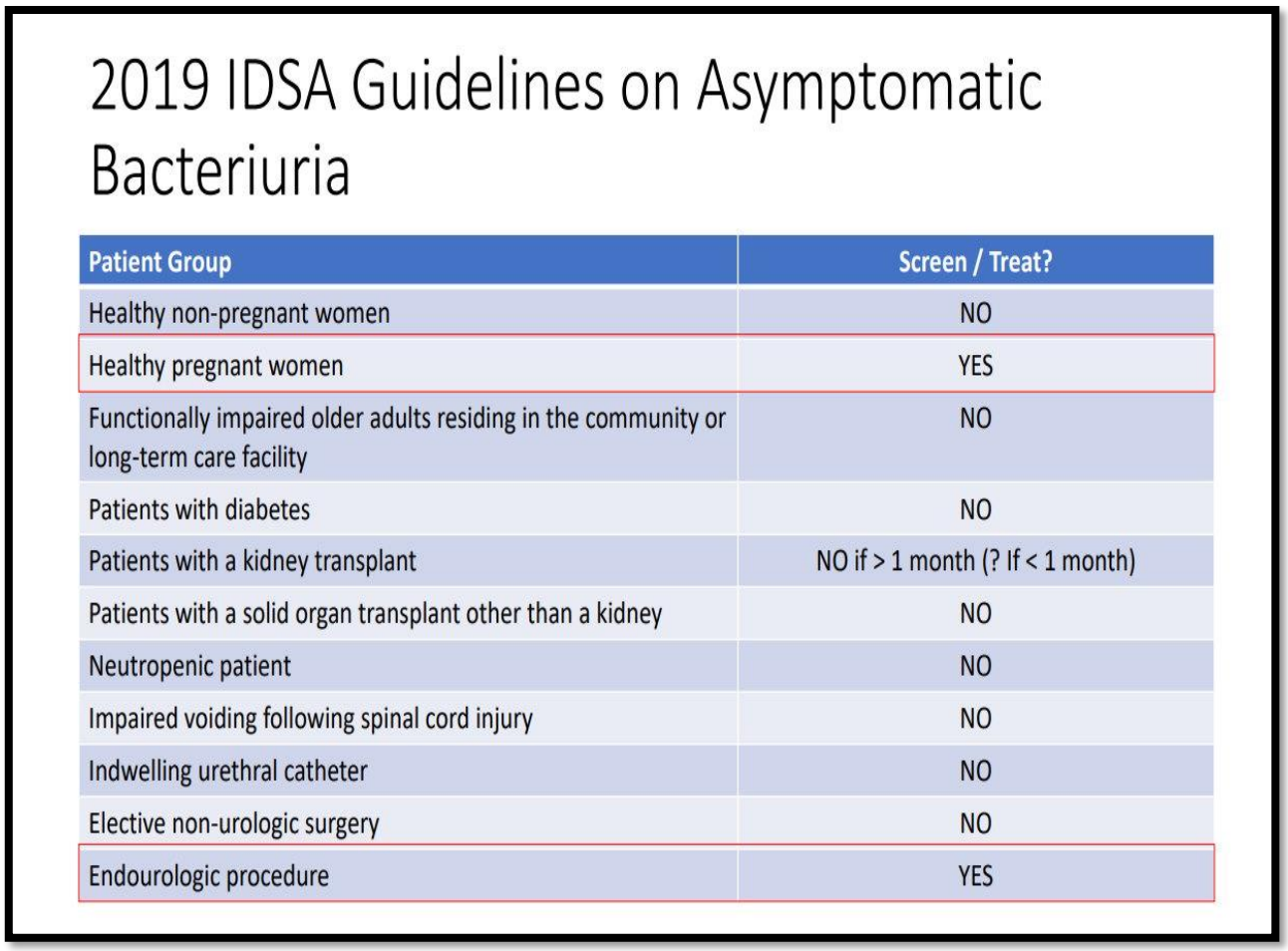
Moreover, a recent report from Spain **(Hernández-Hernández et al. 2021)** indicated that ABU is more frequent in diabetic patients than in healthy controls (17% vs. 10%), and it can progress to symptomatic UTI in up to 20% of them within 6 months, especially if glycemic control is suboptimal. Nonetheless, in patients with neurogenic lower urinary tract dysfunction (NLUTD), rates of ABU vary between 42 and 91% depending on the type of bladder emptying method **(Hernández-Hernández et al. 2021).** In the recent past several studies conducted in different states of India like Tamil Nadu, Uttar Pradesh, Haryana and Telangana indicated notable incidence of symptomatic UTI among people of all age groups **(Christy et al. 2019; Chooramani et al. 2020; Malik et al. 2021)**, however higher incidence was reported among the female population with regard to UTIs with classical symptoms (**Christy et al. 2019; Malik et al. 2021; Faraz et al. 2021)**. Nevertheless, Srivastava et al. **(Srivastava et al. 2016)** from Uttar Pradesh reported incidence of culture-positive urine samples among healthy non-pregnant women between age groups of 18- 50 years and without any classical symptoms of UTIs. Furthermore, reports from various parts of the world (Denmark, Poland, United States of America) **(Roos et al., 2006; Bien et al. 2012; Givler and Givler 2021)** stated that *E. coli* is one of the most common uropathogen, responsible for more than 80% of all urinary tract infections (UTIs) and can cause both ABU and symptomatic UTI.

1.1.4 Diagnosis

The diagnosis with regard to ABU and symptomatic UTI are different according to the international guidelines **(de Cueto et al. 2017; Nicolle et al. 2019)** and as described in previous

studies **(Schmiemann et al. 2010; Hernández-Hernández et al. 2021)**.

(a) ABU: Identification of ABU is based on the presence of positive cultures ($\geq 10^5$ cfu/mL) in the absence of clinical symptoms of UTI. Two consecutive positive cultures from appropriately collected samples of midstream urine are required in women. However, a single positive detection is passable for men **(Schmiemann et al. 2010; Hernández-Hernández et al. 2021)**. Moreover, according to the recently updated (2019) guideline from the IDSA, ABU should be screened for and treated only in pregnant women or in individuals expected to undergo invasive urologic procedures **(Fig. 1.13) (Nicolle et al. 2019)**.



2019 IDSA Guidelines on Asymptomatic Bacteriuria

Patient Group	Screen / Treat?
Healthy non-pregnant women	NO
Healthy pregnant women	YES
Functionally impaired older adults residing in the community or long-term care facility	NO
Patients with diabetes	NO
Patients with a kidney transplant	NO if > 1 month (? If < 1 month)
Patients with a solid organ transplant other than a kidney	NO
Neutropenic patient	NO
Impaired voiding following spinal cord injury	NO
Indwelling urethral catheter	NO
Elective non-urologic surgery	NO
Endourologic procedure	YES

https://www.grepmed.com/images/12207/treatment-indications-asymptomatic-management-idsa **(Nicolle et al. 2019)**

Fig. 1.13: The current guidelines from the Infectious Diseases Society of America on ABU.

- **Pregnancy:** Presently, ABU in pregnancy betokens screening and treatment according to most international guidelines (for example, EAU, AUA, US Preventive Services Task Force, IDSA), but the level of evidence for the aforementioned recommendation is low due to a variety of reasons. First, there is a lack of absolute perceptions of the mechanisms linking ABU, pyelonephritis, and perinatal complications. Moreover, most available studies have a high risk of bias and were published between the 1960s and 1980s, making it difficult to compare them

with current health protocols and services **(Wingert et al. 2019; Hernández-Hernández et al. 2021)**.

- **Postmenopausal women:** The guideline from the IDSA advises against screening and treatment of ABU in healthy postmenopausal women **(Nicolle et al. 2019; Hernández-Hernández et al. 2021).**
- **Elderly and Frail Patients:** The IDSA recommends inspecting other causes of delirium in older patients with functional or cognitive impairment and ABU who do not present with systemic signs of infection or genitourinary symptoms **(Nicolle et al. 2019; Colgan et al. 2020; Hernández-Hernández et al. 2021).**
- **NLUTD:** The prevalence of ABU in NLUTD is high, but only a low percentage of these bacteriuric patients actually develop symptomatic UTI even after invasive investigations such as urodynamics **(Tornic et al. 2020; Hernández-Hernández et al. 2021).** The IDSA advises against screening and treating ABU in NLUTD patients **(Nicolle et al. 2019)**.
- **Transplant and Immunosuppressed Patients:** The occurrence of ABU in kidney transplant recipients is between 5 and 27% **(Coussement et al. 2019; Kotagiri et al. 2017; Hernández-Hernández et al. 2021)**, and current guidelines do not recommend screening for ABU, at least in the first month after surgery **(de Cueto et al. 2017; Nicolle et al. 2019).** No recommendations for the first/second months after kidney transplantation can be made **(Nicolle et al. 2019)**.
- **Catheter Associated:** As per rule, patients with suprapubic, transurethral, or nephrostomy catheters should be considered bacteriuric. It is vital to distinguish between catheter-associated UTI, which requires antibiotic treatment, and catheter-associated bacteriuria, which does not. Transurethral and suprapubic catheter exchanges or placements in patients with ABU do not require antibiotic treatment or prophylaxis; however, in the case of ABU and nephrostomy tube or ureteral stent manipulation, treatment of ABU prior to the procedure is advised (https://uroweb.org/guideline/urological-infections/) **(Hernández-Hernández et al. 2021).** However, "The IDSA" has made no recommendation for or against screening or treatment of ABU in patients with indwelling catheters **(Nicolle al. 2019)**.
- **Periprocedural and Perioperative Investigation of Bacteriuria:**
  - **Urological Procedures (UDS, Endourological Procedures, Prosthesis, etc.):** Strong recommendations have been made by the IDSA **(Nicolle et al. 2019)** and "The Spanish Society of Clinical Microbiology and Infectious Diseases **(de Cueto et al. 2017)** for screening and treating ABU in patients prior to endourologic procedures to avoid the serious postoperative complication of sepsis, which is a substantial risk for patients undergoing invasive endourologic procedures in the presence of bacteriuria **(Nicolle et al. 2019)**.

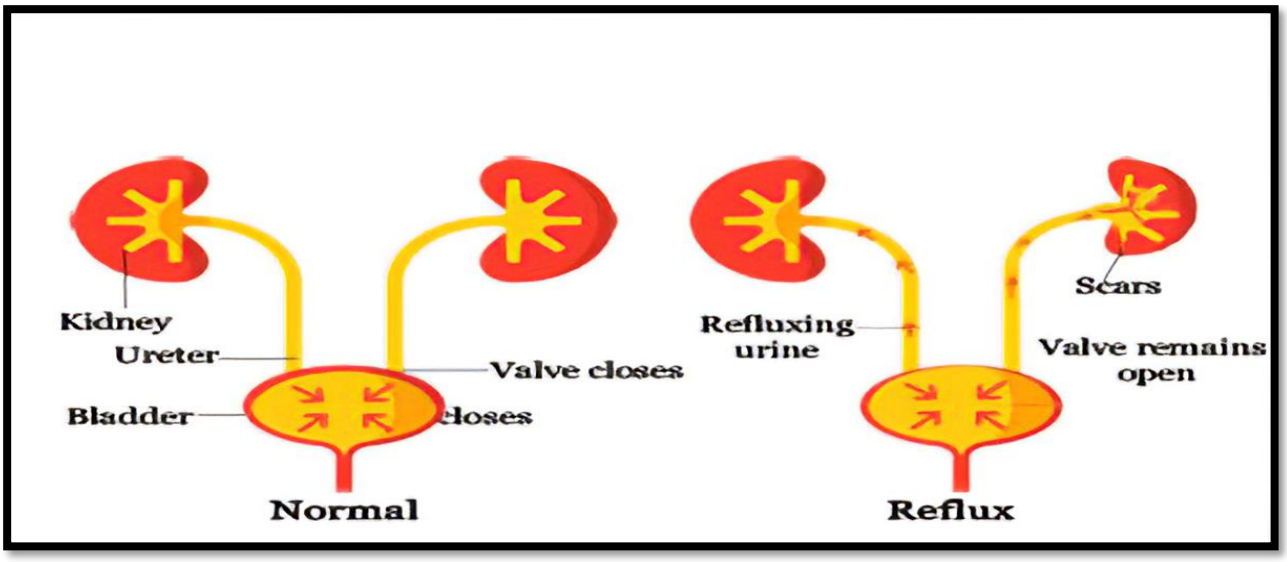
- **Orthopaedics and other surgeries (vascular and so on):** The peri-operative ITU is known to raise the risk of prosthetic infection **(Yassa et al. 2017; Hernández-Hernández et al. 2021);** however**,** this is not the case with non-urological prosthetic infection in patients with ABU **(Hellinger et al. 2020; Rodríguez-Pardo et al. 2021)**. Additionally, previous studies have found that bacteria causing the prosthetic joint infection are not alike to those producing ABU **(Sousa et al. 2019; Partridge et al. 2020; Hernández-Hernández et al. 2021)**. Thus, most societies advocate against screening for or treating ABU in these patients (**de Cueto et al. 2017; Nicolle et al. 2019)**. However, "The Spanish Society of Clinical Microbiology and Infectious Diseases" only recommends screening and treating ABU in neurogenic or incontinent patients, as well as those with indwelling urine catheters, prior to prosthetic spinal surgery to avoid Gram-negative surgical site infections **(de Cueto et al. 2017)**.

(b) Symptomatic UTI: The clinical diagnosis of an UTI is essentially based on the medical history. Explicit data may either increase the likelihood of an infection of the urinary tract (↑) or decrease it (↓). The following factors have been established from clinical studies **(Schmiemann et al. 2010)**:

- Dysuria, pollakisuria, nycturia (↑)
- Present or increased incontinence (↑)
- Macrohematuria (↑)
- Suprapubic pain (↑)
- "Offensive" smell, turbid urine (↑)
- Prior infections of the urinary tract (↑)
- Changed or new discharge, vaginal irritation (↓).

In addition, risk factors are known which increase the probability of UTI. These include:

- Sexual intercourse within the preceding two weeks
- Contraception with a vaginal diaphragm or spermicide
- Contraception with DMPA (depot medroxyprogesterone acetate)
- Antibiotic administration within the preceding two to four weeks
- Special anatomical features or restrictions (for example, from vesicoureteral reflux **(Fig. 1.14)**, neuropathic bladder, mechanical or functional obstruction)
- Diabetes mellitus. **(Schmiemann et al. 2010)**



https://www.medindia.net/patientinfo/reflux-nephropathy.htm

Fig. 1.14: Vesicoureteral reflux.

- **Urine testing:** Urine testing is an important element in diagnostic testing.
  - **Urine collection:** A variety of studies have dealt with the essentiality of collecting midstream urine and of cleaning the perineum and vulva or glans penis **(Baerheim and Laerum 1990; Lifshitz and Kramer 2020**). However, for an initial urine investigation with a dipstick, a fresh spontaneous urine sample can be taken rather than midstream urine and it is not always compulsory to clean the genitals **(Schmiemann et al. 2010)**. Withal, according to https://www.mayoclinic.org/tests-procedures/urinalysis/about/pac-20384907, one might collect a urine sample at home or at one's health care provider's office. Providers typically give out containers for urine samples. One might be asked to collect the first urine in the morning when the urine is more concentrated.

    One might be instructed to collect the sample midstream, using a clean-catch method. This procedure involves the following steps:

    - ✓ Cleaning the urinary opening is required usually. Women must spread the labia and clean from front to back. Men should wipe the tip of their penis.
    - ✓ Then beginning to urinate into the toilet.
    - ✓ Passing the collection container into one's urine stream.
    - ✓ One must urinate at least 1 to 2 ounces (30 to 60 mL) into the collection container.
    - ✓ Finish urinating into the toilet.
    - ✓ Delivering the sample as directed by one's health care provider is also required.
    - ✓ If one can't deliver the sample to the designated area within 60 minutes of collection, refrigerate the sample, unless your provider has told you otherwise.

- **Practical test methods:** The gold standard for a urine test is to carry out a bacteriological urine culture, with the detection of the pathogen, with quantification and sensitivity testing. For investigating, whether the patient has a UTI at all or not, orientating indirect methods are often used in practice to detect the bacteria or inflammation (dip sticks). The bacterial count may be assessed by urine microscopy and immersion culture media **(Schmiemann et al. 2010)**.
  - **Dip sticks:** One of the most widely used instruments for diagnostic testing when there is clinical proof that a patient is suffering from UTI is the Urine dipsticks test. A dipstick is a thin, plastic stick with strips of chemicals on it which is placed in the urine. The chemical strips cause alteration in color if certain constituents are present or if their levels are directly above typical levels. A dipstick test checks for:
    - **Acidity (pH):** The pH level specifies the amount of acid in urine. The pH level might also specify a kidney or urinary tract ailment.
    - **Concentration:** The measurement of concentration displays the number of concentrated particles in one's urine. A higher-than-normal concentration often is a consequence of not drinking enough fluids.
    - **Protein:** Normally urine has low level of proteins. A small increase in protein in the urine usually should not be a cause for concern, but greater amounts might specify a kidney problem.
    - **Sugar:** The glucose (sugar) amount in urine is normally too low to be perceived. Any detection of sugar on this test generally is suggestive of the follow-up testing for diabetes.
    - **Ketones:** Just like sugar, any number of ketones detected in one's urine could be a mark of diabetes and necessitates follow-up testing.
    - **Bilirubin:** Bilirubin is a product obtained from red blood cell breakdown. Generally, bilirubin is passed in the blood and further passes into one's liver, where it's separated and becomes part of bile. Bilirubin in urine might specify liver damage or disease.
    - **Evidence of infection:** Either nitrites or leukocyte esterase which is a product of white blood cells when present in one's urine might indicate a UTI.
    - **Blood:** Additional testing is required if one finds blood in urine. This might indicate certain infection, kidney damage, kidney or bladder stones, kidney or bladder cancer, or blood disorders.

    https://www.mayoclinic.org/tests-procedures/urinalysis/about/pac-20384907

- **Urine microscopy:** Methodological limitations may contribute to the low sensitivity in diagnosing UTI with $<10^5$ cfu/mL by gram-stained microscopy. Studies have found that skilled workers can attain improved diagnostic accuracy than with urine culture **(Schmiemann et al. 2010)**. Microscopy when performed as part of a urinalysis, requires viewing drops of concentrated urine; urine which is spun in a machine under a microscope. If any of the following levels are above average, one might need more tests:
  - **White blood cells** (leukocytes) might indicate an infection.
  - **Red blood cells** (erythrocytes) might indicate a blood disorder, kidney disease, or another underlying medical condition, such as bladder cancer.
  - **Bacteria, yeast, or parasites** might be a sign of an infection.
  - **Casts**- These are tube-shaped proteins which can be a result of kidney disorders.
  - **Crystals** that form from chemicals in urine might indicate kidney stones.

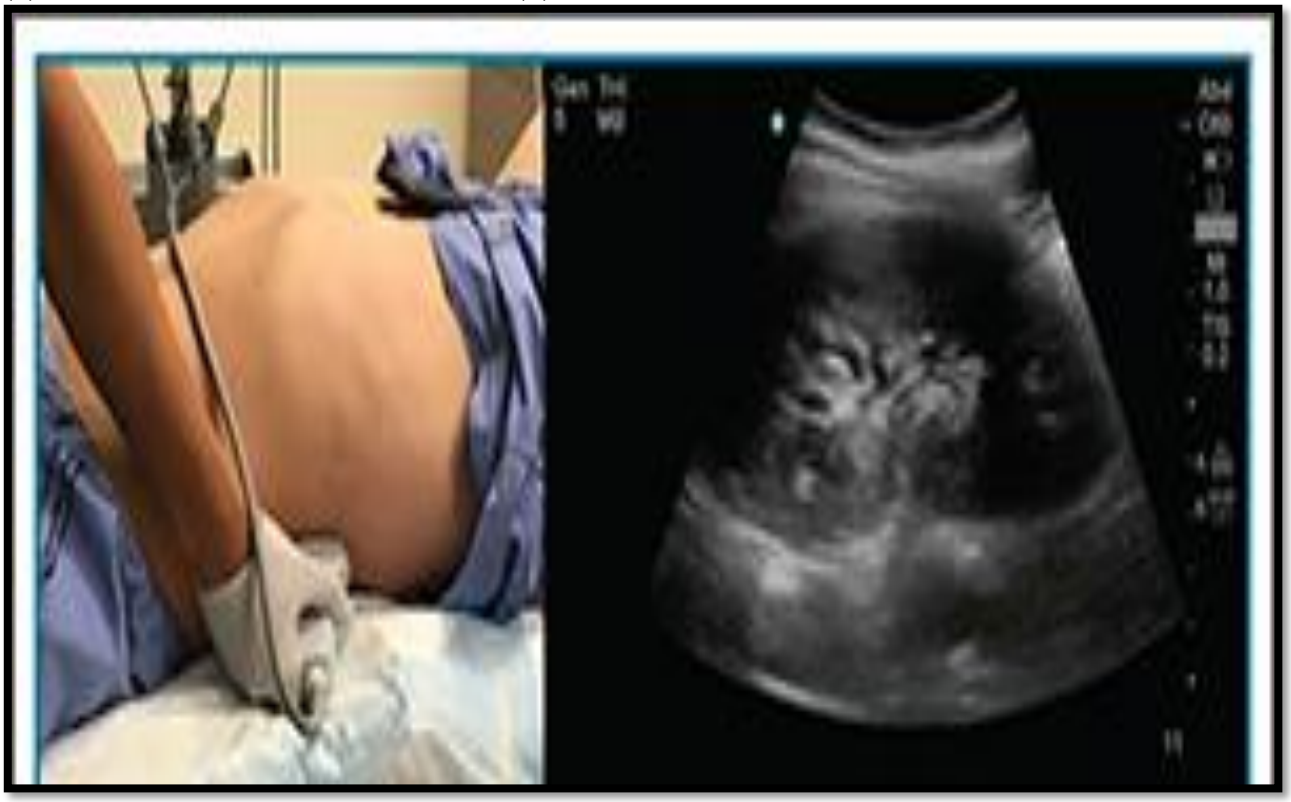
  https://www.mayoclinic.org/tests-procedures/urinalysis/about/pac-20384907
- **Immersion culture media:** This test uses a plastic rod layered with culture medium which is mainly a combination of CLED agar and MacConkey agar and requires 24 h culture. The standards for sensitivity and specificity obtained in the laboratory cannot be reproduced under the conditions of primary care. This method does not allow the reliable detection of $<10^4$ cfu/mL **(Schmiemann et al. 2010)**.
- **Diagnosing testing in special patient groups:** In the (reasonably frequent) case of uncomplicated UTI, it is typically sufficient to diagnose UTI solely based on these indirect test methods. Nonetheless, for all therapy-resistant and complicated infections of the urinary tract, an effort should generally be made to execute a urine culture to identify the causative organisms and their antimicrobial susceptibility **(Schmiemann et al. 2010)**.
  - **Pregnant women:** The treatment of ABU in pregnant females reduces the incidence of pyelonephritis and possibly also damage to the child **(McDermott et al. 2001; S O'Neill et al. 2003)**. On the other hand, the dip stick test used in present antenatal care testing is rather insensitive. Immersion culture media when generally used would provide a identification rate for asymptomatic bacteriuria comparable to that with the urine culture **(Mignini et al. 2009; Schmiemann et al. 2010)**.
  - **Female geriatric patients:** The prevalence of ABU evidently increases in this group. Thus, the occurrence in residents of homes for the elderly is 25% to 50%, even rising to 100% in catheterized patients. However, neither dip sticks nor urine culture is beneficial in confirming the clinical diagnosis of UTI in the latter group. Only a negative urine

culture can rule out an infection. An American consensus conference **(Loeb et al. 2001)** has inducted special diagnostic criteria for this group, which have led to a diminution in antibiotic prescriptions in nursing homes **(Schmiemann et al. 2010)**.

- **Female patients with diabetes mellitus:** Female patients with medically treated diabetes commonly display a UTI or ABU. The spectrum of bacteria and resistance rates are not distinct in this group **(Boyko et al. 2005; Meiland et al. 2006)**.

- **Imaging:** If a patient suffers from recurrent UTIs and does not respond to treatment, the doctor may order other tests to determine the underlying cause. The rationale behind imaging is to identify an underlying structural abnormality (if any), such as hidden obstruction from a stone or an abscess. Although renal ultrasonography **(Fig. 1.15)** and magnetic resonance imaging are occasionally used, computed tomography with contrast media is considered the imaging modality of choice mainly in the case of nonpregnant women **(Colgan et al. 2011)**

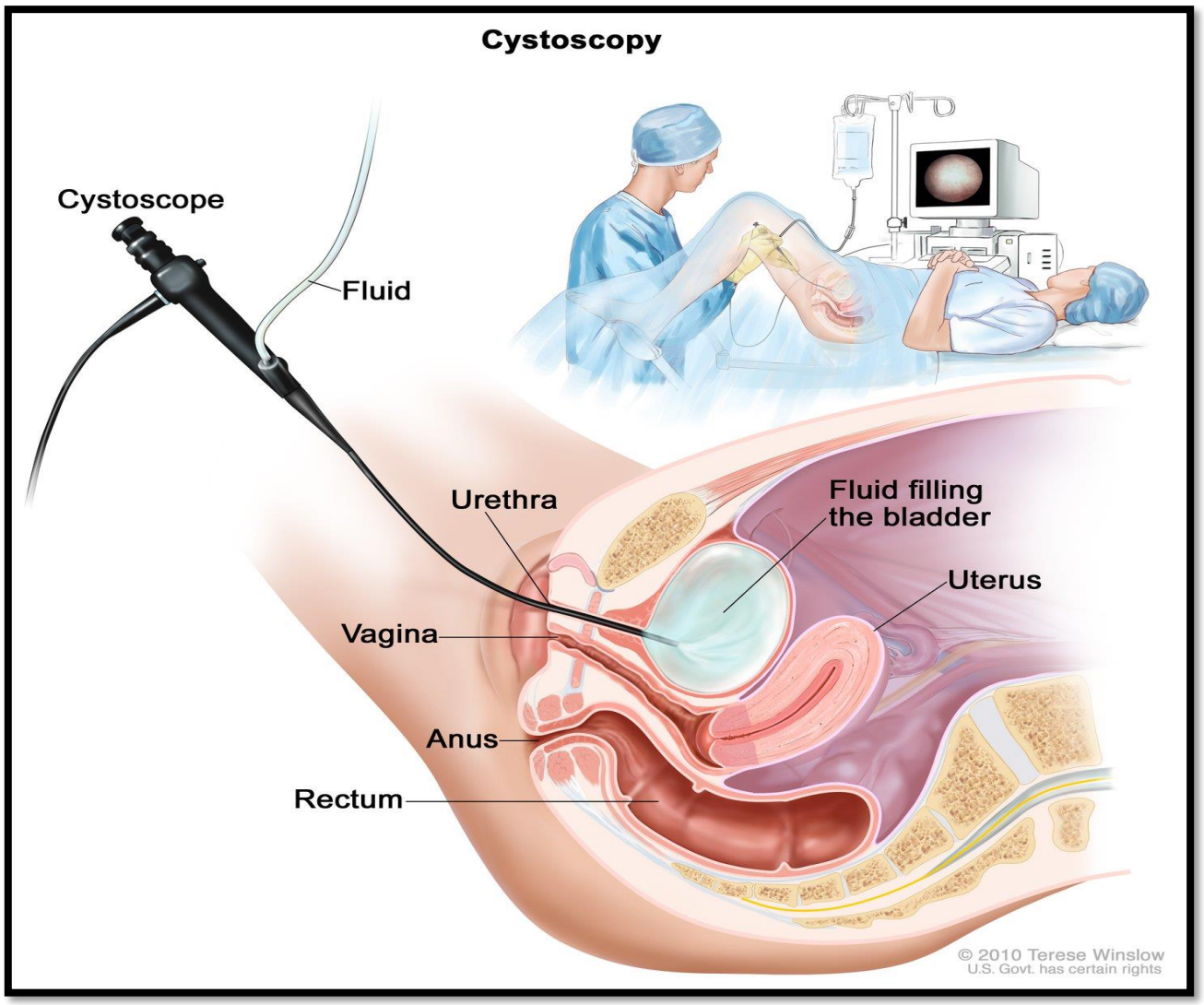
(a) **(b)**



https://cdn.mdedge.com/files/s3fs-public/images/RTEmagicC_em047120553_f1.jpg.jpg

Fig. 1.15: Renal ultrasonography (a) Photo demonstrates the correct probe position to obtain the longitudinal view of the kidneys and (b) Longitudinal ultrasound image of the kidney

- **Cystoscopy:** If one has recurrent UTIs, the doctor may perform a cystoscopy **(Fig. 1.16)**, using a long, thin tube with a lens (cystoscope) to see inside his/her urethra and bladder. The cystoscope is inserted in the urethra of the patient and passed through to the bladder.



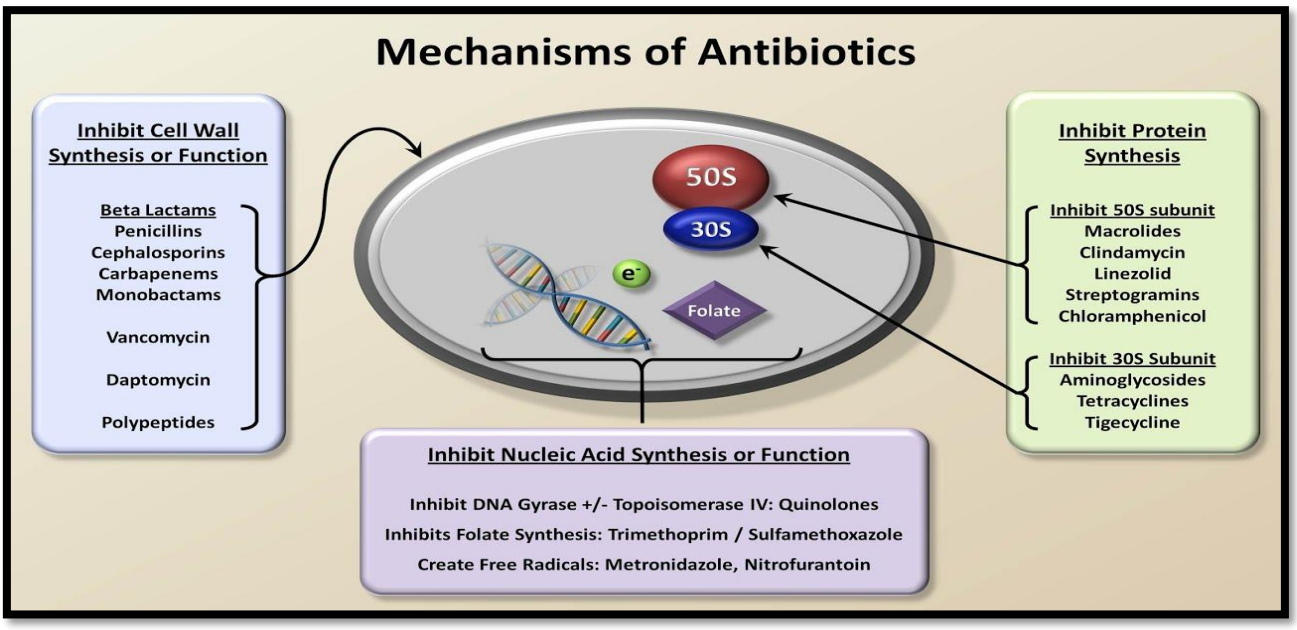
https://www.niddk.nih.gov/health-information/diagnostic-tests/cystoscopy-ureteroscopy

Fig. 1.16: The process of cystoscopy.

1.1.5 Treatment

UTIs are associated with significant morbidity and mortality, resulting in considerable economic and public health burdens and also largely affecting the life quality of the distressed individuals **(Flores-Mireles et al. 2015; Bazzaz et al. 2021)**. Although the mainstay of treatment of UTIs is antimicrobial therapy, however the use of combination therapies, nonantimicrobial therapy, nutrition therapy, and vaccines are also evident nowadays **(Abou Heidar et al. 2019; Scherberich et al. 2021; Bazzaz et al. 2021)**.

(a) Antimicrobial therapies: The bedrock of treatment of any bacterial infection, including a UTI is antimicrobial therapy. UTIs are very common worldwide, therefore controlled use of antibiotics **(Fig. 1.17)** must be initiated for treatment. It is the role of the clinician to treat his/her patients satisfactorily while practicing proper antibiotic stewardship by adhering to relevant practice guidelines **(Abou Heidar et al. 2019)**.



https://www.grepmed.com/images/4725/pharmacology-sites-action-antibiotics-bacteria

Fig. 1.17: Different antibiotics used to treat UTI and their mode of action.

- **Overview of antibacterial agents:** The classification of different groups of antibiotics mainly used to treat symptomatic UTI on the basis of their mode of action **(Fig. 1.17)** are written as under:

➢ **Antibiotics targeting cell wall synthesis:** The bacterial cell is covered by peptidoglycan (PG, or murein) layers, a covalently cross-linked polymer matrix composed of peptide-linked β-(1–4)-*N*-acetyl hexosamine. The mechanical strength allowed by this layer of the cell wall is imperative to a bacterium's capability to survive environmental conditions that may change prevailing osmotic pressures. Moreover, the PG cross-linking degree can be linked with the structural integrity of the cell. The PG layer maintenance is adepted by the activity of transglycosylase and transpeptidase enzymes, which append disaccharide pentapeptides to extend the glycan strands of existing PG molecules and cross-link adjacent peptide strands of immature PG units, respectively **(Kohanski et al. 2010)**.

The specific steps in homeostatic cell wall biosynthesis are inhibited by the certain classes of antibiotics such as β-lactams and glycopeptides. The changes to size cell and shape, induction cellular stress responses, and termination of cell lysis is a consequence of the successful treatment with a cell wall synthesis inhibitor. **β-lactams (penicillins, carbapenems and cephalosporins)** obstruct the cross-linking of PG units by impeding the peptide bond formation reaction catalyzed by transpeptidases that are also known as penicillin-binding proteins (PBP). This inhibition is accomplised by penicilloylation of a PBP's transpeptidase active site, the β-lactam drug molecule (containing a cyclic amide ring) which is an analog of the terminal D-alanyl-D-alanine dipeptide of PG. This acts a substrate for the enzyme in the acylation phase of

cross-link formation that disables the enzyme due to its incapability to hydrolyze the bond created with the now ring-opened drug **(Kohanski et al. 2010)**.

Nonetheless, **glycopeptide antibiotics (e.g., vancomycin)** prevent PG synthesis through binding with PG units (D-alanyl-D-alanine dipeptide) and by the blockage of transglycosylase and transpeptidase activity. Moreover, antibiotics that obstruct the synthesis (e.g., **Fosfomycin**) and transport (e.g., **Bacitracin**) of individual PG units are also presently in use, like lipopeptides (e.g., **daptomycin**) that affect structural integrity through their capability to insert into the cell membrane and cause depolarization **(Kohanski et al. 2010)**.

- **Penicillins:** Penicillin G; Penicillin V etc.
- **Aminopenicillins:** Ampicillin; Amoxicillin.
- **Penicillinase-resistant-penicillins:** Methicillin; Nafcillin etc.
- **Antipseudomonal penicillins:** Carbenicillin; Piperacillin etc.
- **Cephalosporins:** 1st generation- Cefazolin; Cephalexin etc.
  2nd generation- Cefoxitin; Cefuroxime etc.
  3rd generation- Cefotaxime; Ceftazidime; Ceftriaxone etc.
- **Carbapenems:** Imipenem; Meropenem; Doripenem etc.
- **Glycopeptides:** Vancomycin; Telavancin etc.
- **β-lactamase inhibitors:** Clavulanic acid; Tazobactam; Sulbactam.
- **Others:** Fosfomycin; Bacitracin etc.

https://www.orthobullets.com/basic-science/9059/antibiotic-classification-and-mechanism

- **Antibiotics targeting protein synthesis:** Various enzymes and structural change in organisms are involved in the long process of protein synthesis. Different antibacterial classes inhibit bacterial protein synthesis by causing interference in the 30s or 50s subunit. Antibiotics target three specific steps which include initiation, formation of the 70s, and elongation process of making polypeptides. Antibiotics that inhibit or meddle with bacterial protein synthesis include; Aminoglycosides, Macrolides, Tetracycline, Oxazolidinone, and Chloramphenicol. Protein synthesis inhibitors **(Fig. 1.18)** typically act at the ribosomal level in the translation process of protein synthesis which comprises initiation, elongation, and termination. Primarily, tRNA attaches to the three sites of mRNA complex; A-site or aminoacyl site, Peptidyl site or P-site, and E site or Exit site (https://microbenotes.com/protein-synthesis-inhibitors/)

  - **Anti-30S ribosomal subunit: Aminoglycosides-** Amikacin; Gentamicin; Tobramycin etc.
    Tetracyclines- Tetracycline; Doxycycline; Minocycline etc.

- **Anti-50S ribosomal subunit: Macrolides-** Erythromycin; Azithromycin; Clarithromycin etc.

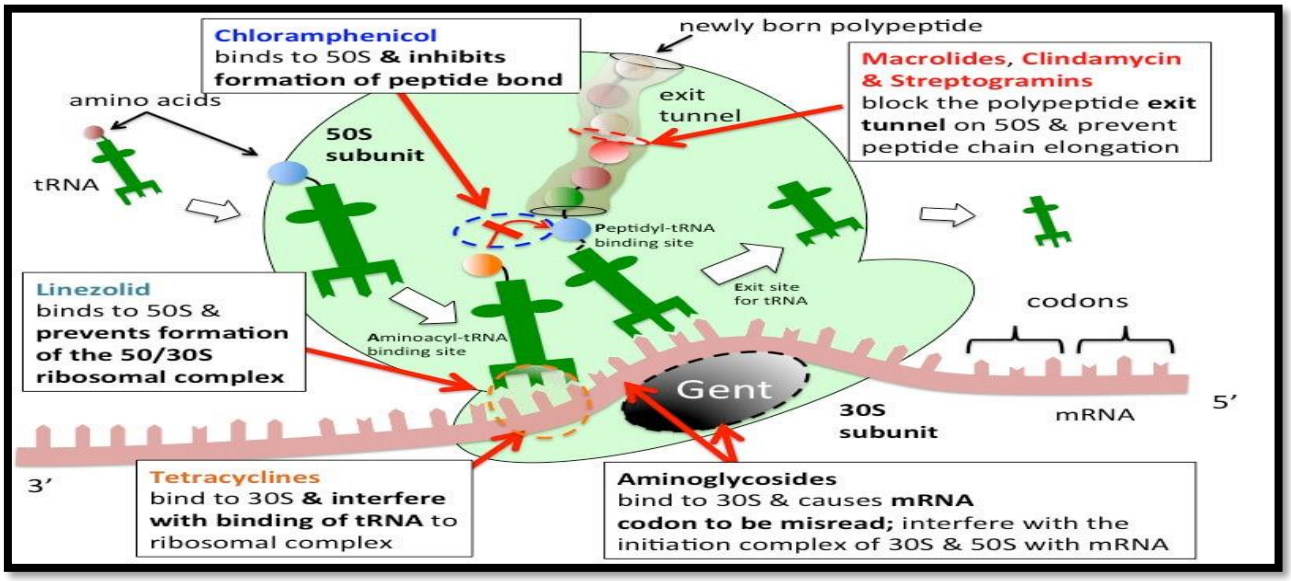
  Chloramphenicol- Chloramphenico.l

  Lincosamide- Clindamycin.

  Linezolid- Linezolid.

  Streptogramins- Quinupristin; Dalfopristin.

https://www.orthobullets.com/basic-science/9059/antibiotic-classification-and-mechanism



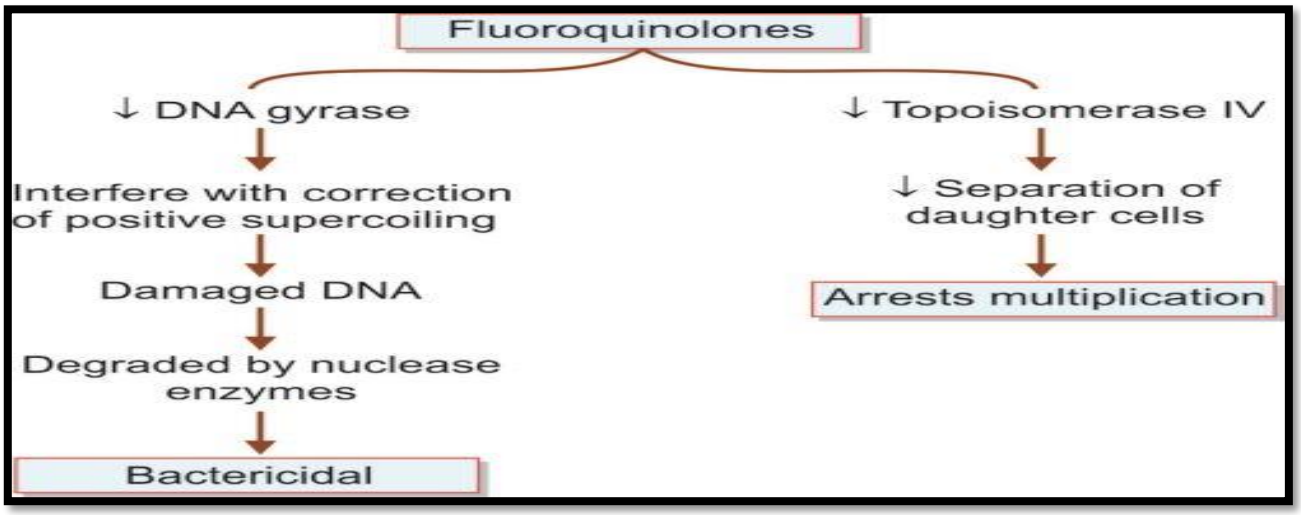
https://microbenotes.com/protein-synthesis-inhibitors/

Fig. 1.18: Sites of action of antibiotics that interfere with bacterial protein synthesis.

- **Antibiotics targeting DNA synthesis:** DNA synthesis occurs at chromosomes which require substrates, primers, templates, and enzymes. Various drugs are used in UTI by inhibiting DNA synthesis in different manners. Antibacterial activity of drugs targeting DNA gyrase enzyme follows one of two mechanisms. They either impede the catalytic activity of the enzyme by binding to the active site or they even out the covalent enzyme-DNA complex which is formed during the reaction. The latter said mechanism makes a more efficacious antibiotic. Inhibition of the ATPase activity of the gyrase enzyme is caused by the antibiotic Novobiocin. Ciprofloxacin, a fluoroquinolone, works by stabilizing the enzyme-DNA complex and thus interrupting the religation step. The first-generation drugs that include the first quinolone antibiotic nalidixic acid achieves only minimal serum concentration and so is not of much use. Later generation quinolones have good tissue penetration, can reach high serum levels, and have broader spectrum of activity. Second generation quinolone antibiotics counting norfloxacin and ciprofloxacin are cogent against gram-negative bacteria. levofloxacin is the third generation quinolone which is effective against both gram-negative and gram-positive bacteria while fourth generation quinolones that include trovafloxacin, moxifloxacin, and gemifloxacin and have the

broadest spectrum of activity. Aminocoumarins, which include novobiocin, clorobiocin, and coumermycin A1, are natural products isolated from Streptomyces species and can hinder the supercoiling activity of DNA gyrase. Few antibiotics such as the nitroheterocycles function by cleaving DNA and thereby inhibiting its replication to synthesize new DNA, unlike the quinolones and fluoroquinolones that inhibit DNA synthesis. Two types of antibiotics belonging to this nitroheterocyles category of antibiotics are the nitroimidazoles (e.g., Metronidazole) and nitrofurans (e.g., Nitrofurantoin). Metronidazole is different from that of most other antibiotics because it works by degrading DNA by a chemical reaction that is not catalyzed by an enzyme. It is considered that in order to be efficacious, the nitrofurans need to be first activated by being reduced. One of the intermediates formed during the reduction of the nitro group is supposed to be responsible for the antibiotic activity of nitrofurans **(Bhattacharjee, 2016).**

- **Fluoroquinolones (Fig. 1.19):** 1st generation- Nalidixic acid.
  2nd generation- Norfloxacin; Ciprofloxacin.
  3rd generation- Levofloxacin.
  4th generation- Trovafloxacin; Moxifloxacin, and Gemifloxacin.
- **Other fluoroquinolone derivatives:** Ofloxacin.
- **Aminocoumarins:** Novobiocin; Clorobiocin and Coumermycin A1.
- **Other DNA inhibitors:** Nitroimidazoles: Metronidazole.
  Nitrofurans: Nitrofurantoin.



https://www.jaypeedigital.com/book/9789386056856/chapter/ch34

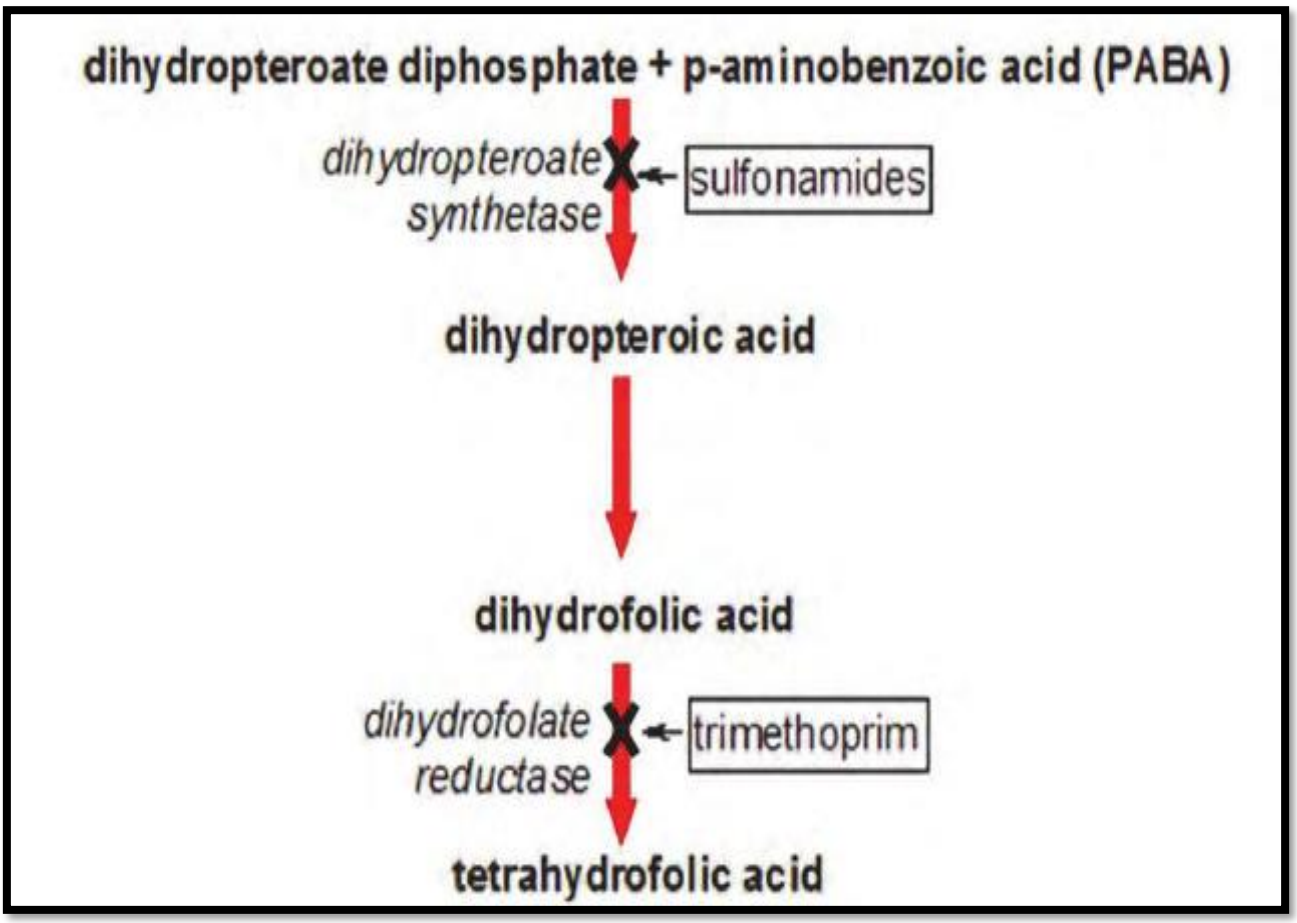
Fig. 1.19: Mechanism of action of fluoroquinolones.

- **Antibiotics targeting RNA synthesis:** RNA denote Ribonucleic acid that is a polymer of ribonucleotides. DNA is transcribed into RNA with the help of the enzyme RNA polymerase by a process known as transcription. Transcription encompasses three steps; elongation, initiation,

and termination. RNA polymerase in RNA synthesis is inhibited by the Rifamycins group of antibiotics. Mechanism of action of Rifamycin displays the binding of antibacterial to the DNA-dependent RNA polymerase by binding to their beta-subunits (α1, β, β1, and Ϭ). The binding site for rifampicin is the B-subunit that is encoded by the gene *rpoB*. It inhibits the initiation of mRNA transcription. Ribosomal and transfer RNA are also similarly affected as mRNA. It also prevents the translation of polypeptides (https://microbenotes.com/rna-synthesis-inhibitors/). The microbiologic and pharmacokinetic properties of Rifamycin group of antibiotics appears apt for the treatment of UTIs **(Brumfitt et al. 1983)**

- **Rifamycin:** Rifampicin; Rifaximin etc.

- **Antibiotics targeting folic acid synthesis:** Folic acids are the enzymes that are indispensable for the bacterial protein synthesis and synthesis of amino acids. Trimethoprim and Sulfonamides are the antimicrobial classes that meddle with the folic acid synthesis at diverse levels and are bacteriostatic. Trimethoprim and Sulfonamides are used as combination therapy in treating UTI, owing to their synergistic mechanisms. They are structurally correspondent to PABA (para-aminobenzoic acid) that impedes dihydropterate synthetase. Sulfonamides are the substances that substitute PABA that marks in the blocking of enzymes that are crucial for the biosynthesis of metabolic reactions for the RNA formation. Growth and replication inhibition occurs that cannot use dietary folate which shows bacteriostatic activity. Trimethoprim is bactericidal that inhibits dihydrofolate reductase enzyme. Dihydrofolate reductase is the most important enzyme that catalyzes the formation of THF (Tetrahydrofolic acid). Trimethoprim binds with dihydrofolate reductase enzyme interfering biosynthesis of nucleic acids and proteins causing bacterial lysis (https://microbenotes.com/folic-acid-synthesis-inhibitors/) **(Fig. 1.20)**.
    - **Trimethoprim:** Bactrim
    - **Sulfonamides:** Sulfadiazine; Sulfamethoxazole; Sulfadoxine
    - **Trimethoprim/ Sulfonamides:** Trimethoprim/ Sulfamethoxazole (Cotrimoxazole).



https://www.researchgate.net/publication/221917930_Drugs_and_Hypoglycemia/figures?lo=1

Fig. 1.20: Mechanism of action of Sulfonamides and Trimethoprim.

(b) Nonantimicrobial therapy: The raison detre behind nonantimicrobial therapy stems from two major drawbacks of the antimicrobial prophylaxis for UTI. The emergence of resistant strains in the urine and failure to fully eradicate microorganisms are always crucial to take into consideration for any antimicrobial therapy **(Abou Heidar et al. 2019).** Therefore, the most accepted alternative therapeutic options for treating UTI **(Fig. 1.21)** are written as under:

- **Urinary alkalization:** Urinary alkalization has been projected as an intercession to decrease recurrent UTI **(Abou Heidar et al. 2019)**. Urinary alkalization with potassium citrate/bicarbonate **(Fig. 1.20)** is a well tolerated and highly effective treatment of UTI **(Trinchieri et al. 2009)**. It raises poison elimination by the administration of intravenous sodium bicarbonate to produce urine with a pH > or = 7.5. The term urine alkalization asserted that urine pH management rather than a diuresis is the prime goal of treatment; the terms forced alkaline diuresis and alkaline diuresis should therefore be discontinued. Urine alkalinization increases the urine elimination of chlorpropamide, 2,4-dichlorophenoxyacetic acid, diflunisal, fluoride, mecoprop, methotrexate, phenobarbital, and salicylate **(Proudfoot et al. 2004).**

https://www.pinterest.com/pin/544654148659157785/

Fig. 1.21: Nonantimicrobial therapeutic options for treating UTI.

- **Probiotics:** Probiotics **(Fig. 1.21)** have been publicized to be effective in varied clinical trials for long-term preventions of recurrent UTI. The use of probiotics such as *Lactobacillus* spp. has been proved to be valuable in treating UTI by the formation of healthy vaginal flora acting as barrier to pathogenic bacteria **(Gupta et al. 2017; Abou Heidar et al. 2019)**.
- **Cranberry juice (Fig. 1.21):** Cranberry (*Vaccinium macrocarpon* Ait.) is a berry that can be found in North America. In last few years, the use of cranberry has increased in the prophylactic approach of UTI. The major efficacy is related to the antiadherence properties of cranberry **(Ahuja et al. 1998; Liu et al. 2006; Loubet et al. 2020)** due to the A-type proanthocyanidin (PAC-A) that has been shown to be a significant inhibitor of Type-I fimbriae *E. coli* adhesion to uroepithelial cells. Some *in vitro* and *in vivo* studies verified the ability of the cranberry to reduce the adhesion of bacteria to the cells **(Ermel et al. 2012; Rafsanjany et al. 2015; Liu et**

al. 2019; Loubet et al. 2020). Cranberry is found to have a negative impact on the swarming of *Pseudomonas aeruginosa* and *P. mirabilis* **(Chan et al. 2013)** and on the biofilm formation of *E. faecalis*, *P. aeruginosa* and *E. coli* **(Ulrey et al. 2014; Rodríguez-Pérez et al. 2016; Wojnicz et al. 2016; Loubet et al. 2020)**.

- **Hydrotherapy:** Hydrotherapy **(Fig. 1.21)** is the use of water as treatment of UTIs. Drinking more water than usual while suffering from UTI can help increase the frequency of urination, thereby flushing away the infection-causing bacteria with every visit to the bathroom, and thereby delaying their breeding. (https://www.medindia.net/homeremedies/urinary-tract-infection.asp)
- **Vitamin C:** Vitamin C **(Fig. 1.21)** (ascorbic acid) is known to possess antioxidant and antimicrobial activities. Like all other microbial infections, UTIs also cause reactive oxygen species (ROS) release by phagocytes; vitamin C is helpful in the limitation of infection through deactivation of microorganism killing. However, ROS may also cause damage to the host cells; therefore, the level of ROS released by phagocytes should be reduced directly after infection **(Liu et al. 2018; Loubet et al. 2020).** Vitamin C is a vital co-enzyme in the oxidative stress pathways, capable of ROS removal. Previous studies suggested **(Habash et al. 1999; Loubet et al. 2020)** that vitamin C reduced the adhesion and microorganisms' colonization of the biomaterials used in diagnostic/treatment procedures involving the urinary tract.
- **Hyaluronic Acid:** The urinary bladder epithelium is composed of urothelial cells which carry specific sensors and properties, thus forming the first barrier to pathogens. Therefore, to maintain this capability to fight infections, these cells produce sulfated polysaccharide glycosaminoglycan (GAG) which covers the epithelium and forms a non-specific anti-adherence factor. Hyaluronic acid (HA) and chondroitin sulfate (CS) are the major constituents of the GAG layer of the bladder. Virulence factors (secreted by UPECs for example) damage the GAG layer to prepare UPEC's adhesion. Thus, management of UTI can be based on the re-establishment of the GAG layer of the bladder epithelium with intravesical instillations of HA alone or in combination with CS **(Loubet et al. 2020)**.
- **Topical estrogens:** Topical vaginal estrogens help to reduce UTI incidence with the protective mechanisms being multifaceted and involving change in the vaginal pH and microbiota composition, the strengthening of the bladder epithelial barrier and thus the enhanced antimicrobial capacity of the urothelium **(Stanton et al. 2020).**

(c) Nutrition therapy: Nutrients can be used as an integral part of the management, prevention, and treatment of UTIs (**Bazzaz et al. 2021)**.

- **Vitamins:** Vitamin C possesses antimicrobial activities and has already been mentioned before as a popular nonantimicrobial alternative for treating UTI. Nevertheless, it is also regularly used as a vital supplement to antibiotic therapy for UTI. Vitamin C is considered as a non-enzymatic antioxidant that bogs down the production of free radicals and oxidation, which leads to strengthening the immune system and the deficiencies of vitamin C can place the persons at risk for infections owing to the harmful impacts on immune function. The role of vitamin A or E supplementation in the prevention and treatment of UTI has also been reported in recent studies **(Sobouti et al. 2013; Bazzaz et al. 2021)**. Various mechanisms have been indicated with regard to vitamin D on the management of UTI. It has been revealed that tight junction proteins play imperative roles in stopping the bacterial invasion of the epithelial barrier. The supplementation with vitamin D can fortify the urinary bladder lining and mend the bladder epithelial integrity. Likewise, vitamin D can act as a local immune response mediator in the case of UTI. However, on the other hand, increasing vitamin D levels leads to modify the innate immune system and delivers a protective response to infection **(Hertting et al. 2017; Bazzaz et al. 2021).**

- **Minerals:** The lower levels of zinc are said to be associated with susceptibility to UTI, and therefore, zinc administration has been recommended. Moreover, selenium-containing analogs of L-proline and L-cystine are found to be efficacious in the UTI treatment. Withal, copper supplementation in drinking water has been advised as a potent approach to reduce *E. coli* colonization in the urinary bladder of the animal model **(Bazzaz et al. 2021)**.

(d) Vaccines: Uro-Vaxom, or OM-89, is an oral vaccine, comprising 18 diverse strains of lyophilized lysates of *E. coli*. A meta-analysis on Uro-Vaxom has confirmed its usefulness for the treatment of recurrent UTI **(Naber et al. 2009).** The administration protocol approved is typically 1 capsule per day for three months as induction treatment, then stopped for the next 90 days. Then when proposed as consolidation treatment, 1 capsule per day will be given for the first ten days of every month, for 3 successive months. Vaginal vaccines are not yet in clinical practice due to the lack of adequate evidence **(Smith et al. 2018; Abou Heidar et al. 2019)**.

Types of UTIs and their treatment regimens: The mainstay of treatment for UTI is antibiotics. Different antibiotic regimens for treating various types of UTIs are written as under:

- **ABU:** According to the recently updated (2019) guidelines from the IDSA, ABU, an identifiable contributor to inapt antimicrobial use thereby promoting antimicrobial resistance, should be treated only in the case of pregnant women or in individuals expected to undergo invasive urologic procedures **(Nicolle et al. 2019).** ABU, ranging from 27% to 44%, has frequently been reported in chronic kidney diseases (CKD) and haemodialysis (HD) patients **(Dalrymple et al.**

2012; **Scherberich et al. 2021)**. In patients with advanced renal disease, those receiving immunosuppressive agents, ABU should probably be treated. The enhanced rates of renal transplant pyelonephritis and acute rejection episodes in patients with ABU have been reported. Antibiotic treatment of bacteriuria is suggested in CKD and HD patients (with residual urine volume excretion) and diabetes mellitus, where leucocytosis, peripheral neuropathy, and elevated C-reactive protein (CRP) accompanies ABU. However, normal stable patients with ABU should not be generally treated with antibiotics **(Scherberich et al. 2021)**.

- **Uncomplicated UTIs:** Uncomplicated UTIs can be treated based on the severity of symptoms. The recommended empiric treatment has been listed in **Table 1.1**.
- **Complicated UTIs:** Complicated UTIs are not easy to treat and usually requires more belligerent evaluation, treatment and follow-up. This may require identifying and addressing the underlying complication **(Wagenlehner et al. 2013).** The recommended empiric treatment has been listed in **Table 1.1**.
- **Pyelonephritis:** Pyelonephritis is treated more aggressively than a simple bladder infection using either a longer course of oral antibiotics or intravenous antibiotics **(Colgan et al. 2011)**.

Table 1.1: Recommended empiric treatment for different types of UTI.

Condition	1st line therapy	2nd line therapy
Acute uncomplicated UTI	SMX/TMP po X3 days or TMP X3 days po or Nitrofurantoin po X7days	Fluroquinolone po X3 days Cephalexin po X7 days. Fosfomycin single dose
Pyelonephritis (Mild/Moderate)	Fluroquinolone po (10-14d)	Amox/Clav (10-14d) or SMX/TMP (10-14d) or TMP (10-14d)
Pyelonephritis (Severe)	Aminoglycoside iv ± Ampicillin iv (10-14d)	Fluroquinolone iv (10-14d) or 3rd Gen. Cephalospiron iv ± Aminoglycoside iv (10-14d)
Complicated UTI (Mild/Moderate)	Fluroquinolone po (7-10d) SMX/TMP po (7-10d) TMP po (7-10d) Nitrofurantoin po (7-10d)	Amox/Clav (7-10d)
Complicated UTI (Severe)	Aminoglycoside iv ± Ampicillin iv (10-14d)	Fluroquinolone iv (10-14d) or 3rd Gen. Cephalospiron iv

https://www.slideshare.net/abahnassi/uti-40556157

1.2 UPECs- The Leading Cause of UTIs

E. coli **(Fig. 1.22)** that cause the majority of UTIs are thought to represent only a subset of the strains that colonize the colon **(Mobley et al. 2009)**. The German-Austrian paediatrician Theodor Escherich discovered this organism (after whom it's named) in the faeces of healthy individuals in 1885. He called it *Bacterium coli commune* because it is found in the colon. *E. coli* is a Gram-negative, facultative anaerobe, nonsporulating coliform bacterium. *E. coli* cells are typically rod-

shaped, and are 1.0- 2.0 μm long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm^3. They are known to grow best at 37◦ C (https://en.wikipedia.org/wiki/Escherichia_coli).

E. coli: **Scientific classification:** Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *coli*.

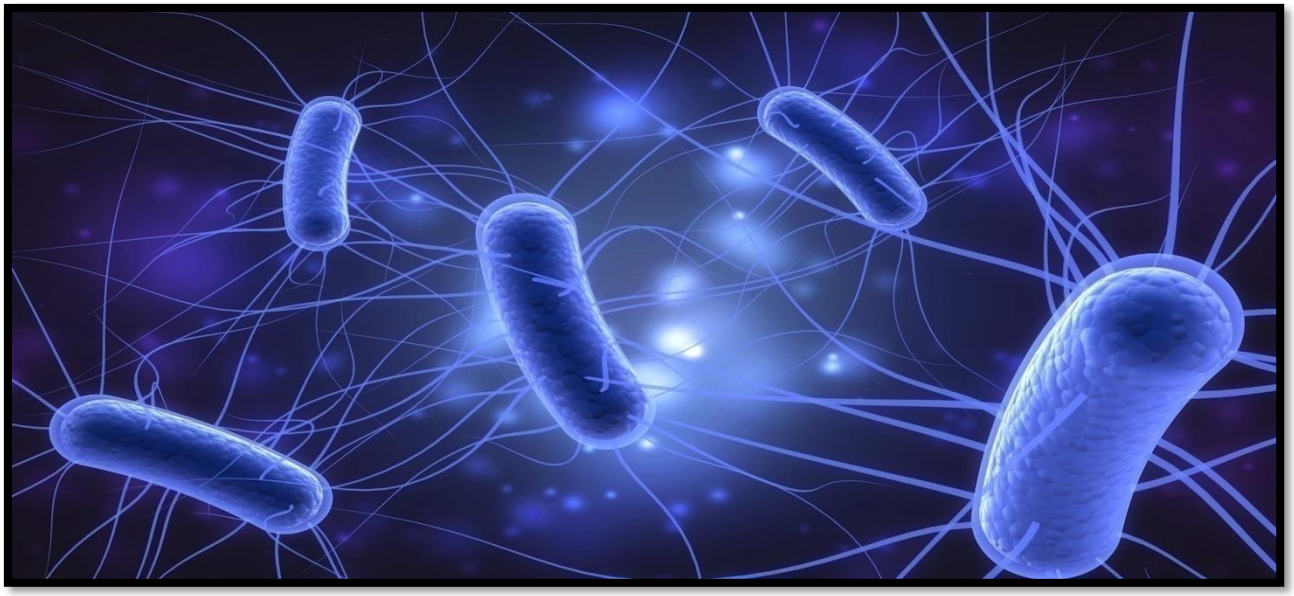


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Fig. 1.22: The pictorial representation of *E. coli* bacteria.

Pathogenic *E. coli* are generally classified into two categories and they are enteric/ diarrheagenic *E. coli* and extraintestinal *E. coli* (ExPEC). Enteric/ diarrheagenic *E. coli* are further divided into six pathovars which are written as under:

i. Enteropathogenic *E. coli* (EPEC),
ii. Enterohaemorrhagic *E. coli* (EHEC),
iii. Enterotoxigenic *E. coli* (ETEC),
iv. Enteroinvasive *E. coli* (EIEC; including Shigella),

v. Enteroaggregative *E. coli* (EAEC) and

vi. Diffusely adherent *E. coli* (DAEC)

However, ExPECs are divided into two pathovars. They are:

i. uropathogenic *E. coli* (UPEC) and

ii. neonatal meningitis *E. coli* (NMEC) **(Croxen and Finlay 2010)**

E. coli strains that cause UTIs are termed UPECs **(Mobley et al. 2009)**. UPECs are the causative agent in the vast majority of urinary tract infections (UTIs) that includes asymptomatic as well as symptomatic (cystitis and pyelonephritis) infections and their subsequent infectious complications, which may result in acute renal failure in healthy individuals as well as in renal transplant patients **(Bien et al. 2012)**. Moreover, UPECs account for about 80% of uncomplicated UTIs, 95% of community-acquired infections, and 50% of hospital-acquired infections **(Tabasi et al. 2016; Kot 2019)**. UPEC also remains the most predominant pathogen in complicated UTIs **(Bartolett et al. 2016; Kot 2019)**.

1.2.1 Types of UPECs

UPECs are largely known to be associated with both asymptomatic bacteriuria (ABU) and symptomatic UTI **(Roos et al. 2006; Bien et al. 2012)**.

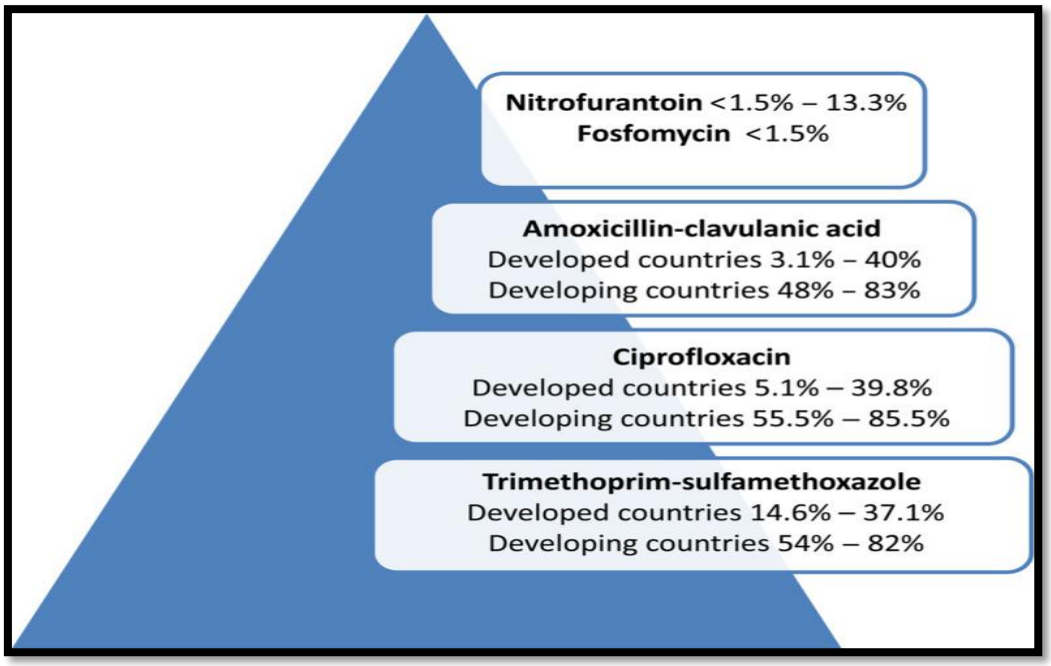
(a) ABU UPECs: UPECs are the most common organisms associated with asymptomatic bacteriuria (ABU). In contrast to UPECs that cause symptomatic UTI, very little is known about the mechanisms by which these strains colonize the human urinary tract **(Roos et al. 2006)**. The prototype ABU *E. coli* strain 83972 was originally isolated from a young Swedish girl with ABU who had carried it for at least 3 years without any symptoms of UTI **(Andersson et al. 1991; Roos et al. 2006)**. It is well suited for growth in the human urinary tract, where it establishes long-term bacteriuria. Deliberate colonization with *E. coli* 83972 has for example been shown to lessen the frequency of UTI in patients with spinal cord injury and neurogenic bladder, and the strain can prevent catheter colonization by bacterial and fungal uropathogens **(Wullt et al. 1998; Roos et al. 2006)**. The mechanism of bladder colonization by *E. coli* 83972 is not known and the mechanisms underlying its ability to keep other strains away are not known either. Moreover, it was found that *E. coli* 83972 is incapable of expressing functional type 1 and P fimbriae. This explained to a great degree the reason behind the fact that the aforementioned strain does not cause symptoms in the host **(Klemm et al. 2006; Roos et al. 2006)**.

(b) Symptomatic UPECs: The symptomatic strains of UPEC, which colonize the urinary tract, may ascend towards bladder to cause cystitis, which is typically associated with the classical symptoms of UTIs, that is, frequency (frequent urination), pain (painful urination), and urgency (sudden compelling desire to urinate). Among Gram-negative bacteria, *E. coli* is the most frequent pathogen inducing acute renal failure. Likewise, urological complications, for instance after renal transplantation, are often

associated with UTIs and UPEC is the most common clinical isolate causing such complications. Acute allograft injury in the renal transplant patient is associated with UPEC and clinical finding of upper UTIs (**Bien et al. 2012)**.

1.2.2 Antibiotic resistance in UPECs nationwide and worldwide

Antimicrobial resistance (AMR) has emerged as one of the major public health problems of the 21st century that impends the successful prevention and treatment of an ever-increasing range of infections that are caused by bacteria, parasites, viruses and fungi which are no longer susceptible to the common medicines used to treat them **(Prestinaci et al. 2015)** due to their overuse. Moreover, antibiotic resistance in UPECs and the dissemination of the multi-drug resistant (MDR) UPECs is presently a global public health concern **(Malik et al. 2020; Zalewska-Piątek and Piątek 2020).** MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories **(Magiorakos et al. 2012)**. The rising frequency of MDR UPEC, especially in developing countries, results in excessive use of broad-spectrum antibiotics such as fluoroquinolones, cephalosporins, and aminoglycosides that has elevated the cost of treatment and hospitalization **(Kot 2019)**. Furthermore, a study by **Kot (2019)** has shown the time- and area-related variability regarding antimicrobial resistance in UPECs with regard to various countries (**Fig. 1.23)**. **Fig. 1.23** illustrates the resistance percentage of different antibiotics against UPECs in various developed and developing countries.



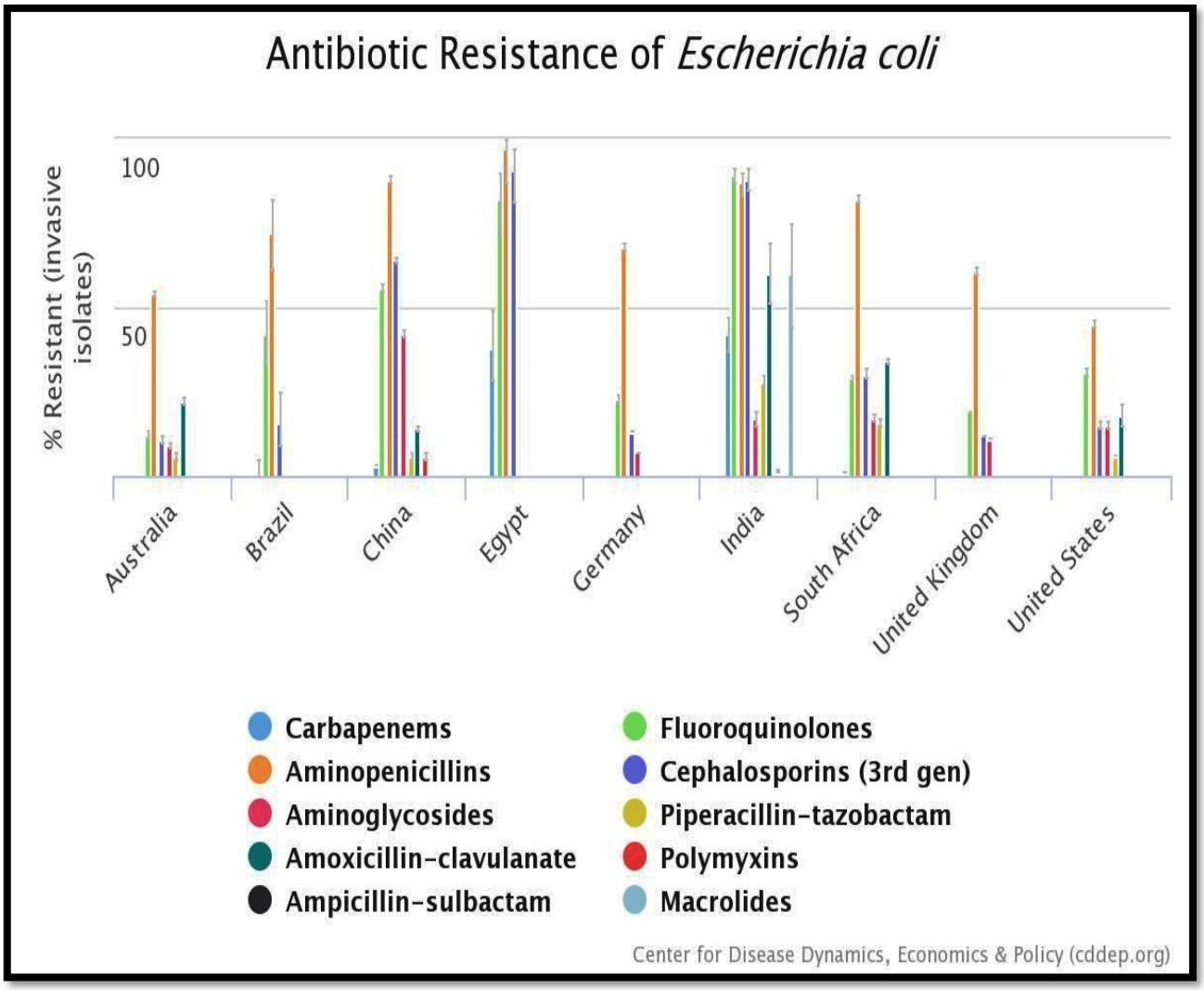
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7260639/figure/fig2/?report=objectonly

Fig. 1.23: Resistance of UPECs to different antibiotics.

Amoxicillin-clavulanic acid**; the developed countries (USA, 3.1–40%; Germany, 5.3%; Poland, 13.9%; England, 30%; France, 37.6%), developing countries (Nepal, 48%; Pakistan, 71%; Jordan,**

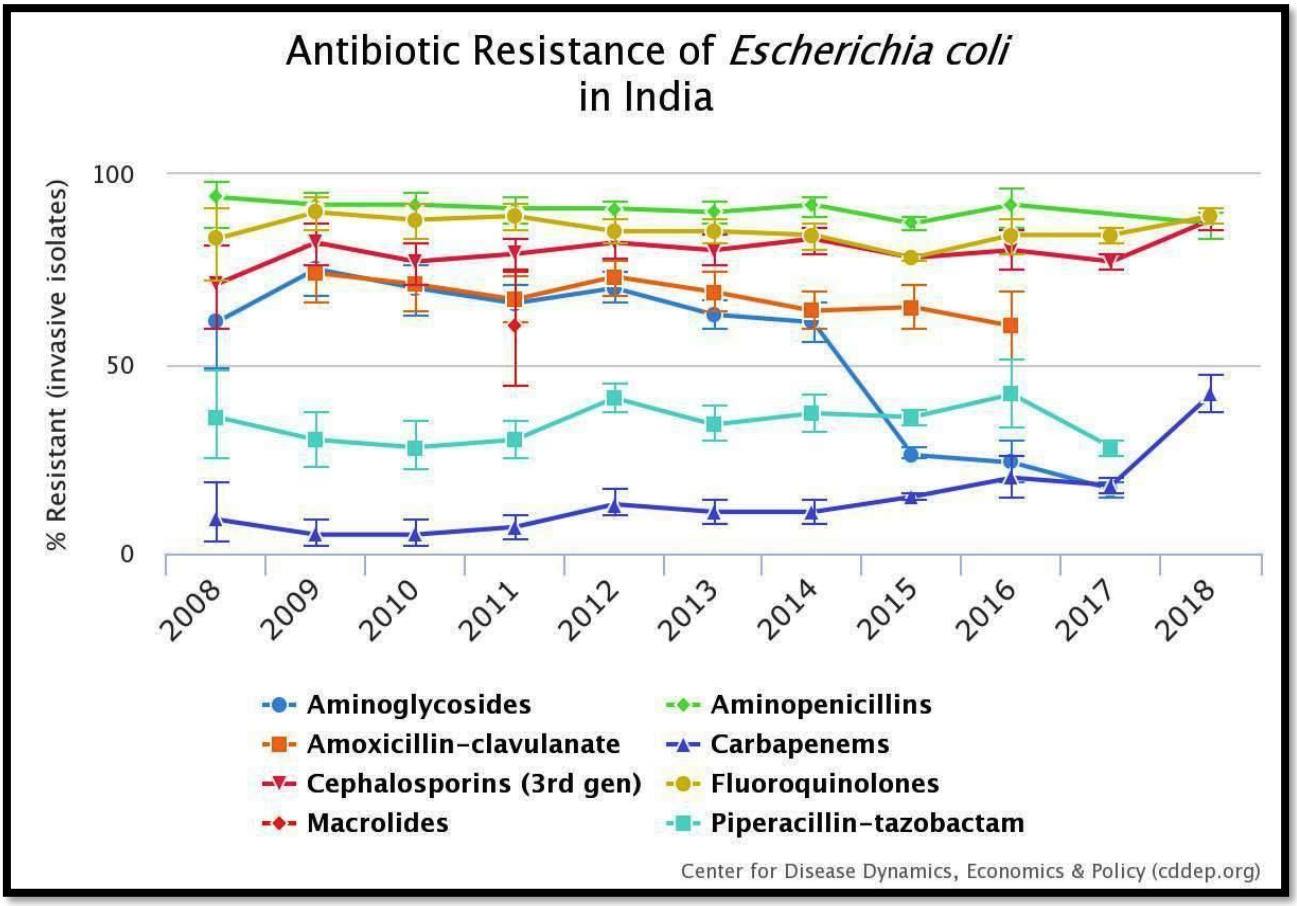
83%). Ciprofloxacin**; developed countries (USA, 5.1–12.1%; Belgium, 12.9%; Germany, 10.5–17.3%; Switzerland.17.4%; England, 20.4%; France, 24.8%; Spain, 39.8%), developing countries (Jordan, 55.5%, Mongolia, 58.1%; Pakistan, 60.8%; Nepal, 64.6%; Ethiopia, 85.5%).** Trimethoprim-Sulfamethoxazole**; developed countries (Belgium, 14.6%; USA, 17.4%; Germany, 18.45%; Poland, 21.4%; Switzerland, 24.5%; Spain, 30.9%; France, 37.1%), developing countries (Iran, 54%; Mexico, 66%; Ethiopia, 68.5%; Mongolia, 70.9%; Jordan, 73,1%; Pakistan, 82%).**

Nonetheless (**Fig. 1.24)** illustrates the distribution of antibiotic resistance in *E. coli* in different popular countries of different parts of the world like East Asia and Pacific, Europe and Central Asia, Latin America and Caribbean, Middle East and North Africa, North America, South Asia and Sub-Saharan Africa. Withal, (**Fig. 1.25)** illustrates the trend of resistance of *E. coli* to different groups of antibiotics in India from 2008-2018.



https://resistancemap.cddep.org/AntibioticResistance.php

Fig. 1.24: Distribution pattern of resistance of *E. coli* to different groups of antibiotics across different countries of the world.

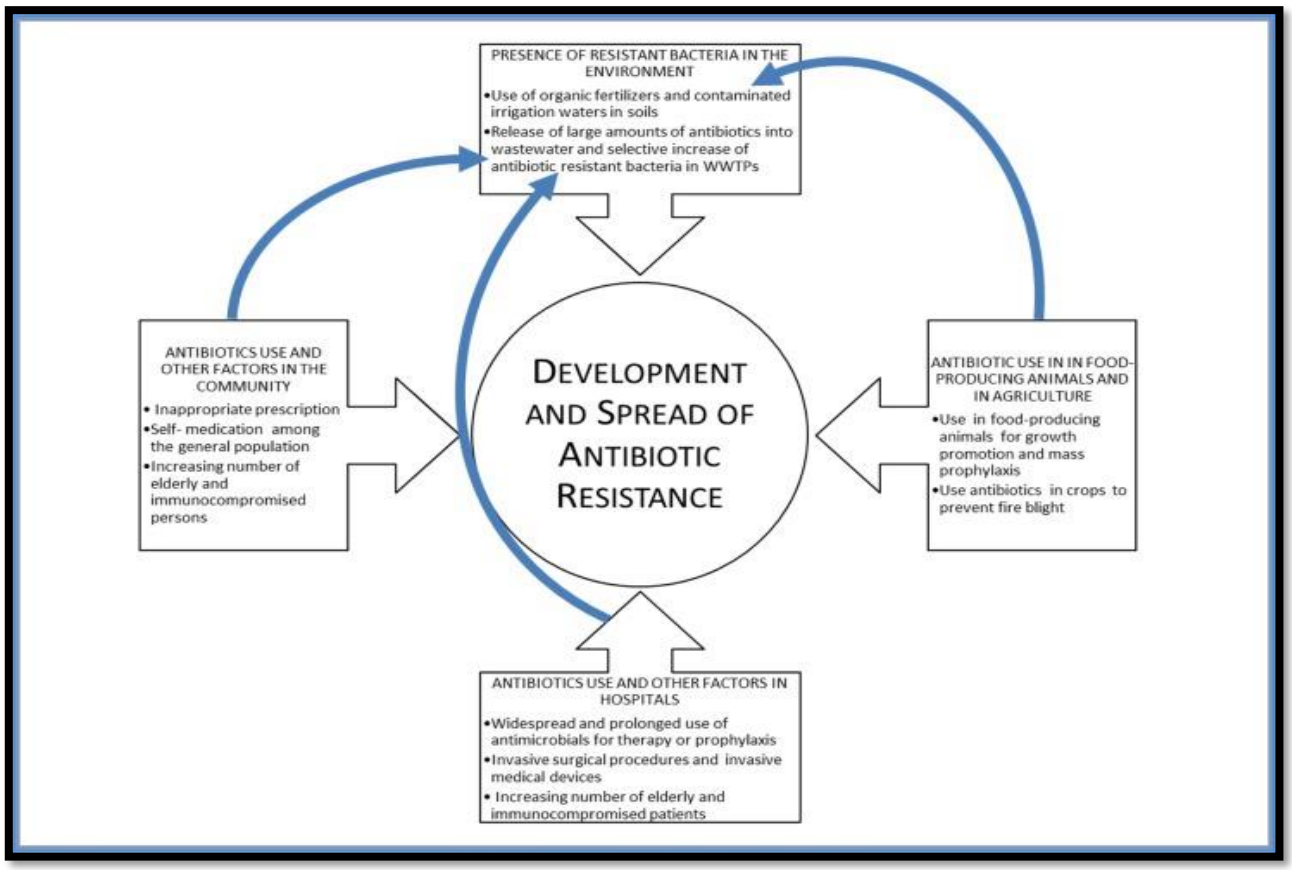


https://resistancemap.cddep.org/AntibioticResistance.php

Fig. 1.25: The trend of antibiotic resistance of *E. coli* in India from 2008-2018.

(a) Factors contributing to the emergence of antibiotic resistance: A natural phenomenon such as antibiotic resistance happens when microorganisms especially bacteria are exposed to antibiotic drugs. The susceptible bacteria are killed or inhibited under the selective pressure of antibiotics, however, bacteria that are naturally resistant or which have developed antibiotic-resistant traits have an improved chance to survive and multiply. The overuse of antibiotics together with the inapt use (inappropriate choices, inadequate dosing, poor adherence to treatment guidelines) contribute to the increase of antibiotic resistance **(Fig. 1.26) (Prestinaci et al. 2015)**.

(b) Modes and mechanism of antibiotic resistance: There are four general antimicrobial resistance mechanisms that bacteria including UPECs use. These are restrictive uptake of the drug, modifying the target of the drug, inactivating the drug, and active efflux of the drug. These mechanisms may be positioned on the bacterial chromosome and occur naturally in all members of a species (intrinsic) or come from other bacteria, usually via a plasmid (acquired). Intrinsic resistance genes may be expressed constitutively (usually at a low level) or be incited by the presence of antimicrobial drugs. Gram-negative bacteria like *E. coli* widely use all four of these mechanisms and are proficient of horizontal transfer of resistance elements **(Reygaert 2017)**. **Table 1.2** shows the resistance mechanisms associated with resistance to various antimicrobial groups of drugs.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4768623/figure/F1/ **(Prestinaci et al. 2015)**

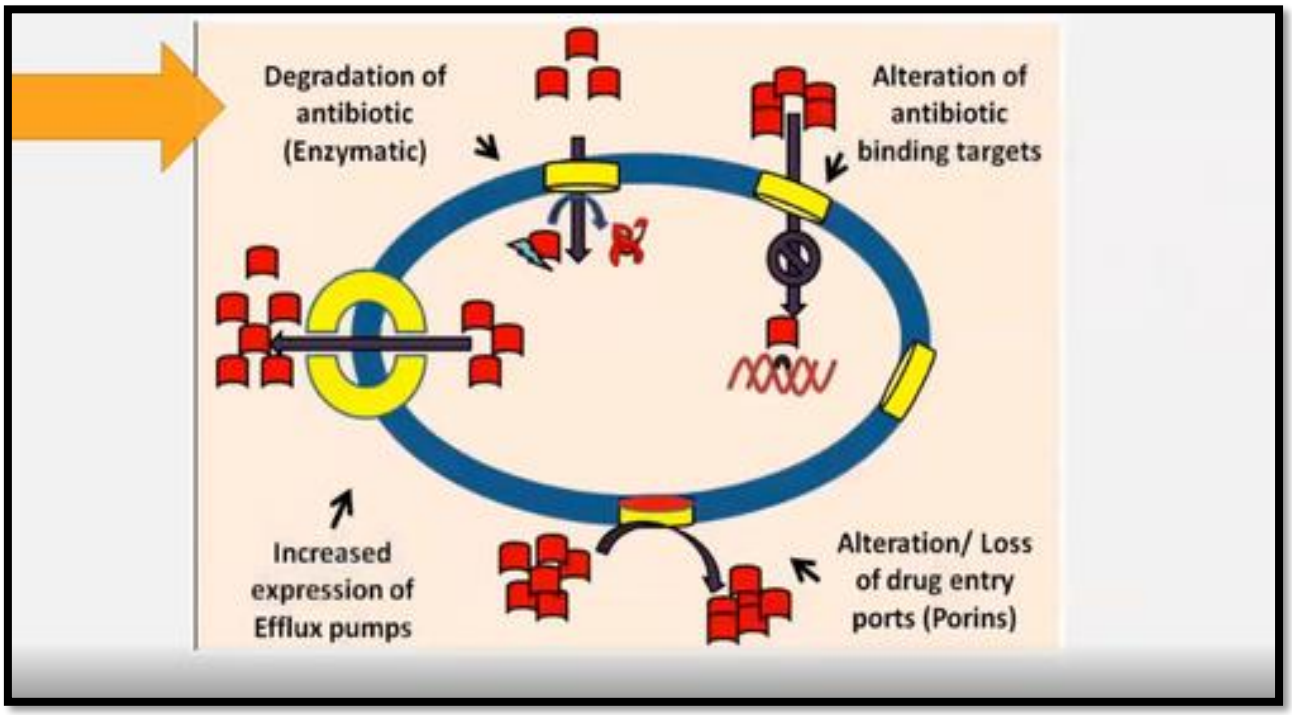
Fig. 1.26: The factors involved in the spread of antibiotic resistance.

- **Mechanism of resistance to β-lactams:** Resistance to β-lactams (**Fig. 1.27)** is associated with the production of different types of β-lactamase enzymes. Among the genes often positioned on plasmids are those coding multiple types of β-lactamases (*bla* genes) **(Adamus-Białek et al. 2018; Kot 2019)**. The amide bond of the four- membered β-lactam ring of β-lactam antibiotics (penicillin, cephalosporin, monobactams, and carbapenems) is hydrolyzed by the β-lactamases **(Noyal et al. 2009)**. ESBL are enzymes that confer resistance to β-lactam antibiotics (all penicillins, cephalosporins, and monobactams), except for carbapenems, cephamycins, and β-lactamase inhibitors such as tazobactam, sulbactam, and clavulanic acid **(Baudry et al. 2009; Kot 2019).** Nonetheless, in the recent years, rampant use of the drug drug-inhibitor combinations has increased number of β-lactamase variants including extended-spectrum (ESBL) and inhibitor-resistant that is β-lactamase inhibitor resistant (BLIR) phenotypes. Studies from different European countries stated that inhibitor resistant TEM β-lactamases are the primary cause of BLIR phenotypes that first emerged in the 1990's and are mostly found in urine isolates **(Cantón et al. 2008; Mukherjee et al. 2018).** Moreover, in addition to resistance to β-lactam antibiotics, ESBL-producing *E. coli* isolates are also found to be resistant to other antimicrobial agents, such as aminoglycosides, tetracycline, and trimethoprim/Sulfamethoxazole (**Kot 2019)**

Table 1.2: Different resistance mechanims adopted by Gram negative bacteria like *E. coli* against different groups of antibiotics.

Antibiotic class	Resistance type	Resistance mechanism
Aminoglycoside	Decreased uptake	Changes in outer membrane
	Enzymatic modification	AGE's
Beta-lactams	Altered PBP	PBP 2a
	Enzymatic degradation	Penicillinase which are classified as per ambler classification
Glycopeptides	Altered target	D-alanyl-alanine is changed to D-alanyl-D-lactate
Macrolides	Altered target	Methylation of ribosomal active site with reduced binding
	Efflux pumps	Mef type pump
Oxazolidinones	Altered target	Mutation leading to reduced binding to active site
Quinolones	Altered target	Mutation leading to reduced binding to active site(s)
	Efflux	Membrane transporters
Tetracyclines	Efflux	New membrane transporters
	Altered target	Production of proteins that bind to the ribosome and alter the conformation of the active site
Chloramphenicol	Antibiotic inactivation	Chloramphenicol acetyl transferase
	Efflux pump	New membrane transporters
Sulfa drugs	Altered target	Mutation of genes encoding DHPS

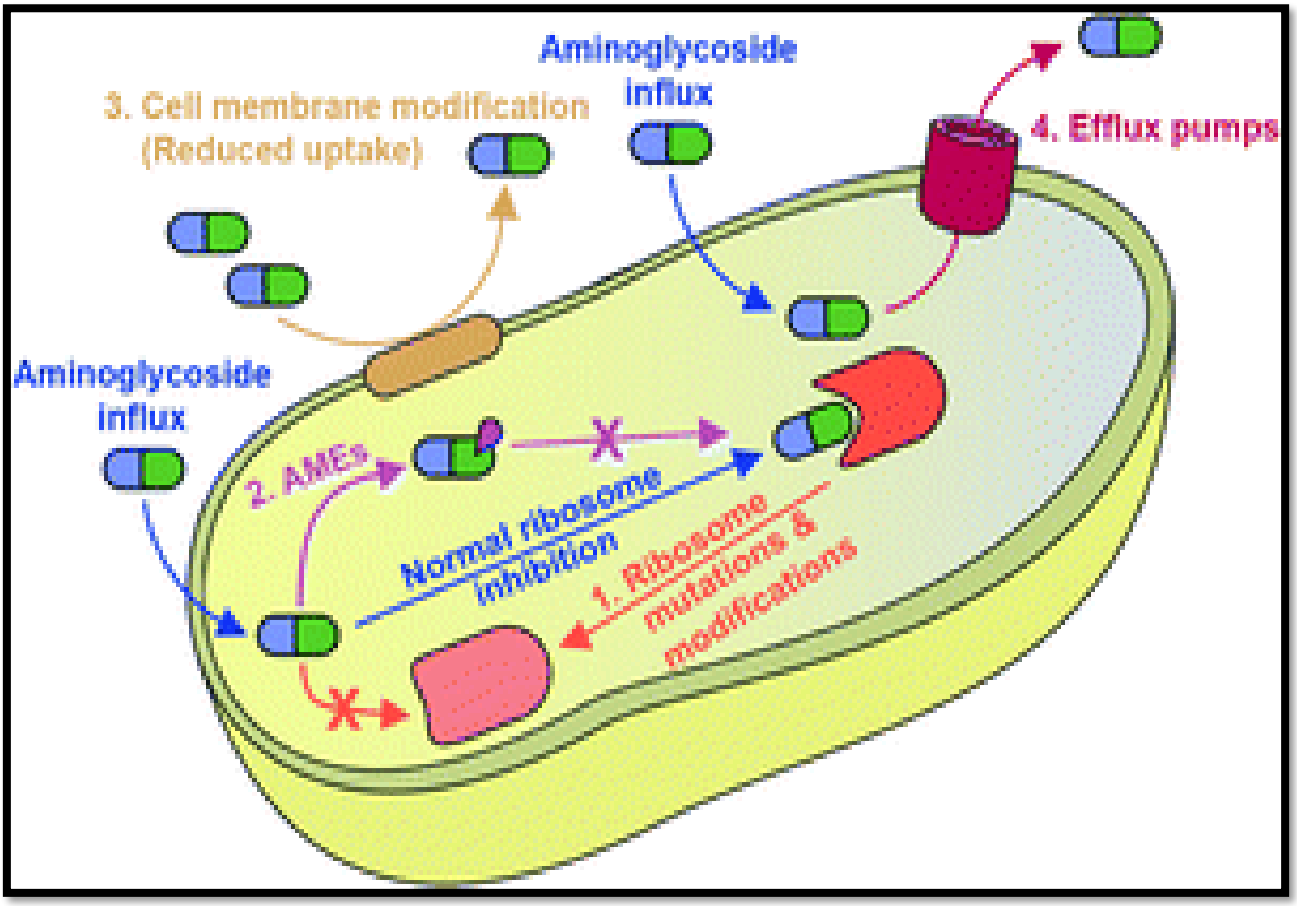
AGE's-Aminoglycoside modifying enzymes; DHPS-Dihydropteroate synthase, PBP-penicillin-binding protein **(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5672523/table/T1/) (Kapoor et al. 2017)**.



https://quizlet.com/302204276/day-3-objective-2-describe-the-mechanism-of-resistance-of-betalactams-flash-cards/

Fig. 1.27: Mechanism of resistance of UPECs to β-lactam antibiotics.

- **Mechanism of resistance to glycopeptides:** D-alanyl-alanine is altered to D-alanyl-lactate which inhibits the cross-linking of glycopeptides thereby causing resistance. Seven van genes are accountable for causing vancomycin resistance. These genes encode dehydrogenases that form lactate that is imperative for the formation of unmodified peptidoglycan (https://microbenotes.com/cell-wall-synthesis-inhibitors/).
- **Mechanism of resistance to aminoglycosides:** Aminoglycosides resistance **(Fig. 1.28)** is normally caused by the methylation of 16s rRNA. The mechanisms of aminoglycosides include modification in the structures by various enzymes. The three classes of enzymes are aminoglycoside nucleotidyltransferases, aminoglycoside phosphotransferases, and aminoglycoside acetyltransferases. Modification in structures causes resistance to antibiotics due to the steric or electrostatic interactions (https://microbenotes.com/protein-synthesis-inhibitors/).



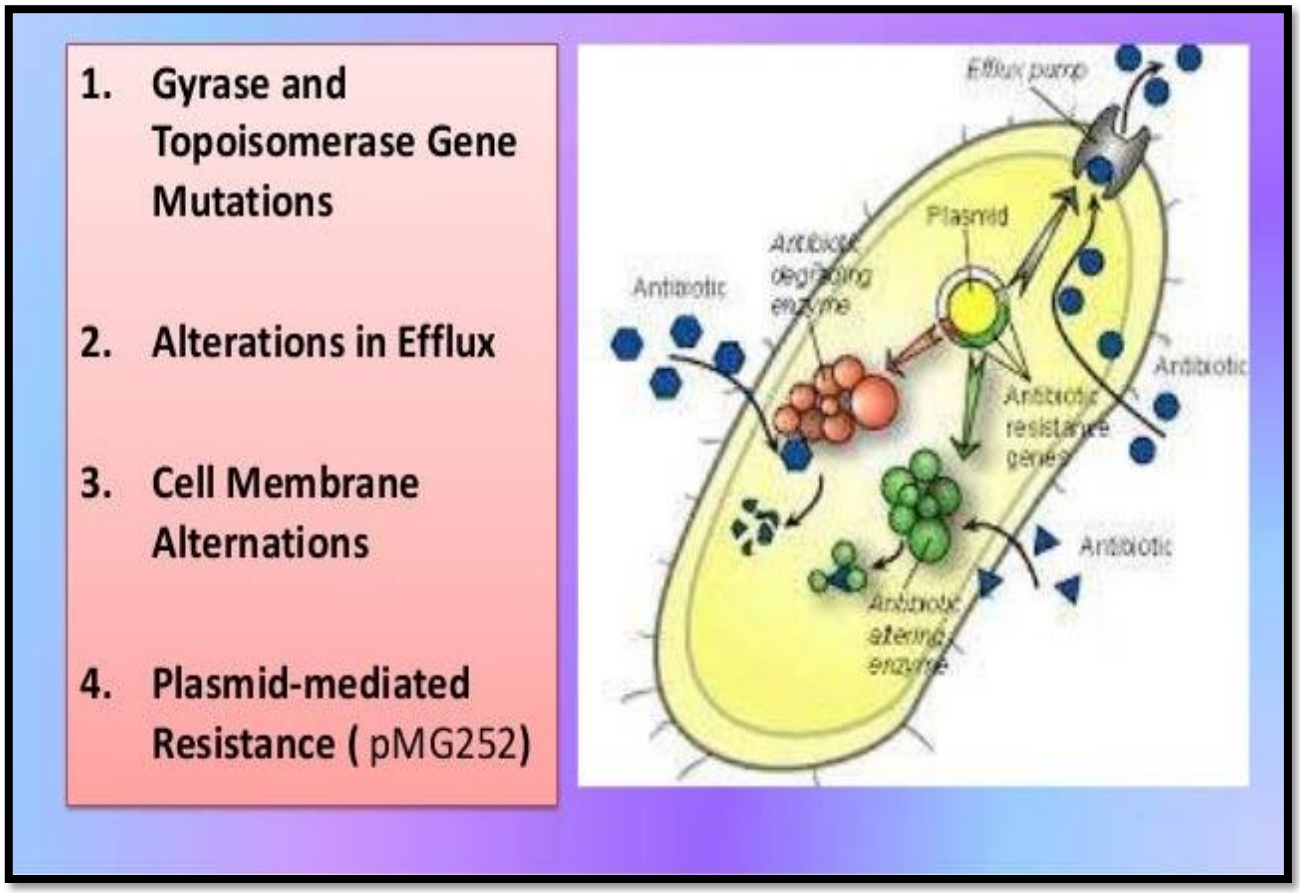
https://pubs.rsc.org/en/content/articlelanding/2016/md/c5md00344j

Fig. 1.28: Mechanism of resistance of UPECs against aminoglycosides

- **Mechanism of resistance to tetracyclines:** Resistance to tetracyclines can be cause by efflux pump, a method that removes active antibiotics from the cell causing antibiotics to pump from the cell because of trans membrane proteins. This mechanism transfers the molecule with magnesium ions generating a lower concentration of antibacterials inside the cells. Tetracycline

resistance is also caused by the cytoplasmic proteins that guard the ribosome from tetracycline (https://microbenotes.com/protein-synthesis-inhibitors/).

- **Mechanism of resistance to quinolones and fluoroquinolones:** Quinolones and fluoroquinolones are extensively used worldwide in the treatment of UTIs and their indiscriminate use led to increased resistance in UPECs. The mechanism of fluoroquinolone action is based on binding to and impeding the action of topoisomerase II (DNA gyrase) and topoisomerase IV (parC and parE) **(Komp Lindgren et al. 2003)**. Moreover, DNA gyrase is encoded by the *gyrA* and *gyrB* genes **(Pourahmad Jaktaji and Mohiti 2010).** The resistance of *E. coli* to quinolones **(Fig. 1.29)** often results from a mutation in the *gyrA* and *gyrB* genes that catalyze DNA supercoiling. The point mutations in gyrA protein N-terminal sequence (amino acids 67 (Ala-67) to 106 (Gln-106)) strongly correlate with phenotypic resistance to quinolones and fluoroquinolones, and this particular sequence is termed a quinolone resistance-determining region (QRDR) **(Friedman et al. 2001)**.



https://pt.slideshare.net/SushmitaJha1/plasmid-mediated-quinolone-resistance/7

Fig. 1.29: Mechanism of resistance of UPECs to quinolones and fluoroquinolones.

- Analysis of mutations in codons 83 and 106 of the *gyrA* gene in UPECs in Iran presented the significant relationship between mutations in the *gyrA* gene and quinolone and fluoroquinolone resistance pattern of UPEC isolates **(Shenagari et al. 2018)**. The other genes accountable for

the resistance to quinolones and fluoroquinolones are the *qnr* genes (*qnrA, qnrB,* and *qnrC*), being the most important PMQR (plasmid-mediated quinolone resistance) genes that incite antibiotic resistance by inhibition of binding of quinolones to DNA gyrase and topoisomerases **(Shahbazi et al. 2018)**. Resistance of *E. coli* to quinolones and fluoroquinolones are related to the incidence of efflux pumps and decreased uptake of the antibiotics due to changes in the outer membrane porin proteins **(Asadi Karam et al. 2019)**. Abdelhamid and Abozahra **(Abdelhamid and Abozahra 2017)** showed that the augmented expression of the efflux pump-coding genes *acrA* and *mdfA* is related to the growing resistance to levofloxacin, which affirms that efflux pump systems contribute to fluoroquinolone resistance in urinary *E. coli* isolates.

- **Mechanism of resistance to sulfonamides and trimethoprim:** Sulfonamide resistance is mainly due to the genes that are positioned either chromosomally or in the plasmid. Alteration of the protein is the common mechanism that causes resistance which reduces the affinity of drugs. Mutation in the dihydrofolate reductase is cause by plasmid-mediated genes and chromosomally mediated genes cause resistance in dihydropterate synthetase. Cross-resistance between sulfonamides and excessive production of PABA that inhibits dihydropterate synthetase are also some reasons for sulfonamides resistance. Resistance to trimethoprim is caused due to different reasons like alteration of the bacterial cell wall, overproduction of dihydrofolate reductase (https://microbenotes.com/folic-acid-synthesis-inhibitors/).
- **Mechanism of resistance to nitrofurans:** The resistance of UPEC to nitrofurantoin is usually very low. Resistance to nitrofurantoin did not progress as fast as to other drugs because of this antimicrobial act at multiple targets in the bacterial cell **(Shakti and Veeraraghavan 2015)**. **Sandegren et al. (Sandegren et al. 2008)** identified mutations causing nitrofurantoin resistance and found that the frequency of mutation is approximately 10^{-7}/cell in *E. coli*. The mutations in the *nsfA* and *nfsB* genes that encode oxygen-insensitive nitroreductases were accountable for resistance against nitrofurantoin. It was also found that the growth of bacterial cells in the presence of nitrofurantoin at therapeutic concentrations was wholly reduced in nitrofurantoin-resistant mutants. It may indicate that resistant mutants in the presence of nitrofurantoin were possibly unable to establish an infection **(Sandegren et al. 2008; Kot 2019)**.

1.2.3 Pathogenicity island markers (PAIs) in UPECs

PAIs are mobile genetic elements (MGEs) made up of huge blocks of DNA (> 10 kb) inserted adjacent to tRNA genes, and generally flanked by short direct repeats. PAIs contain insertion sequences, integrases and transposases, and have a G + C content that differs from the host bacterial genome. The virulence factors of UPECs are usually encoded on PAIs, providing a mechanism for synchronized horizontal transfer of virulence genes. PAIs are detected in a 93% of UPECs **(Sabaté et al. 2006)**. Eight

PAIs (PAI I536, PAI II536, PAI III536, PAI IV536, PAI ICFT073, PAI IICFT073, PAI IJ96 and PAI IIJ96) are found to be associated with pathogenic *E. coli* isolates and PAI IV536 is also termed High-Pathogenicity Island (HPI) **(Sabaté et al. 2006; Najafi et al. 2018).**

1.2.4 Virulence characteristics of UPECs

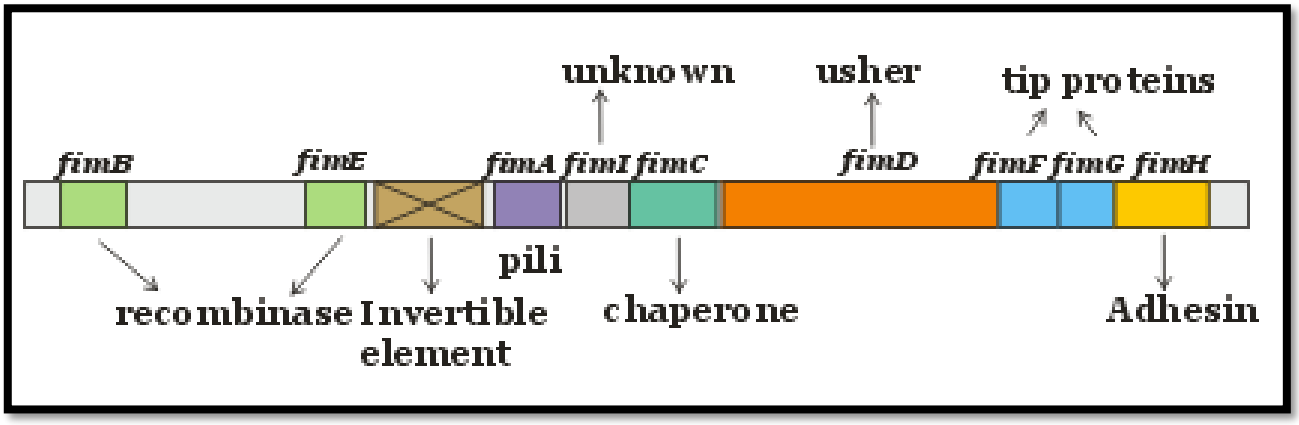
UPECs encode a number of virulence factors, which facilitate them to colonize the urinary tract and persist in face of highly effective host defense. UPECs display a high degree of genetic diversity due to the possession of specialized virulence genes located on MGEs called PAIs. Virulence factors of *E. coli* that have been potentially implicated as imperative to establish UTIs can be divided into two groups:

(a) Virulence factors associated with the surface of bacterial cell and

(b) Virulence factors, which are secreted and exported to the site of action

(a) Surface virulence factors: UPEC's surface virulence factors include a number of different types of adhesive organelles (fimbriae), which facilitate bacterial attachment to host tissues within the urinary tract. The production of adhesive molecules (adhesins) by UPEC is the most important determinant of pathogenicity. UPEC adhesins can contribute to virulence in various ways: (i) directly triggering host and bacterial cell signaling pathways, (ii) facilitating the delivery of other bacterial products to host tissues, and (iii) promoting bacterial invasion **(Bien et al. 2012)**.

- **Type 1 fimbriae:** Type 1 fimbriae have been reported as the key mediator of attachment of UPECs to uroepithelial cells in the urinary tract of humans, further expediting the process of successful establishment of infection **(Schwan 2011; Bien et al. 2012)**. Type 1 fimbriae are produced from a conterminous DNA segment, labeled the *fim* operon **(Fig. 1.30)**, which encodes the genes necessary for their synthesis, assembly, and regulation **(Schwan 2011)**.



https://www.researchgate.net/publication/303840594_Investigating_the_Virulence_Potential_of_the_Multidrug_Resistant_Uropathogenic_Escherichia_coli_ST131_clone/figures?lo=1

Fig. 1.30: Type 1 fimbriae operon of UPECs.

- **Why type 1 fimbriae of UPECs are so important?**

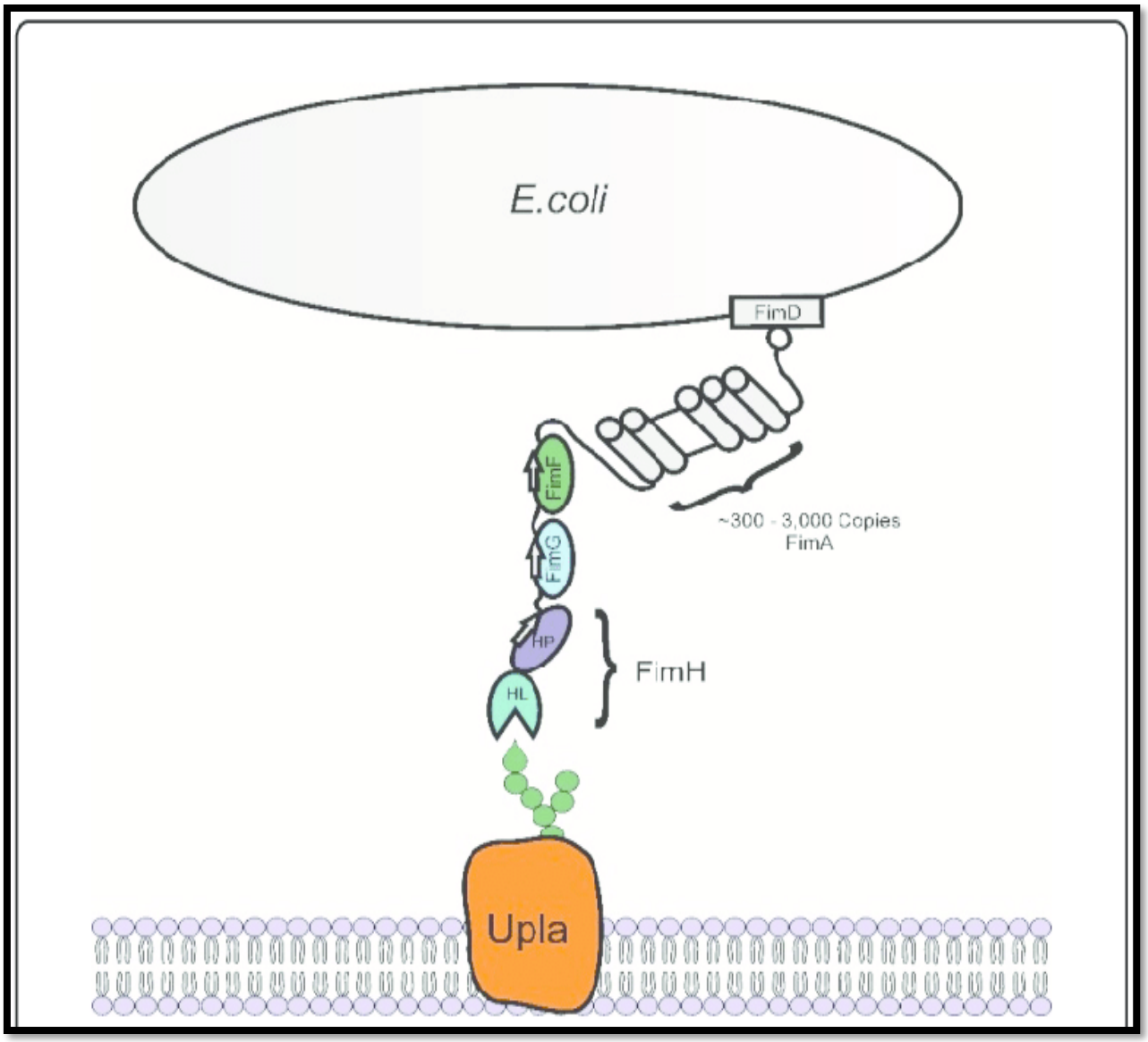
  This is because:

  - UPEC's foremost virulence factors are hair-like surface structures called type 1 fimbriae.
  - Adherence -one of the first steps in the initiation of infection, and UPEC that is generally accomplished by type 1 fimbriae.
  - The type 1 fimbriae bind to the urothelial mannosylated glycoproteins uroplakin Ia and IIIa (UPIIIa) via the adhesin subunit FimH, located at the fimbrial tip.
  - This interaction leads to molecular phosphorylation events, which are requisite for stimulation of signalling pathways, involved in invasion and apoptosis and may also contribute to elevation of the intracellular Ca^{2+} level in urothelial cells.
  - Type 1 fimbriae have been shown to enhance bacterial survival, to stimulate mucosal inflammation, and to promote invasion and growth as a biofilm **(Schwan 2011)**.

- **Key characteristics of Type 1 fimbriae, which is the key mediator of attachment of UPECs to uroepithelial cells are**:

  - Type 1 fimbriae were originally associated with adhesive and pellicle-promoting actions which are impeded by D-Mannose, that is, they are mannose sensitive.
  - They are produced from a contiguous DNA segment, labelled the *fim* operon.
  - Nine genes have been identified, each having distinct functions in the attachment.
  - Two of them, *fimB* and *fimE*, code for recombinases involved in the regulation of type 1 pilus expression.
  - The adjacent gene encodes the main structural pilus subunit FimA.
  - Immediately upstream of the *fimA* gene is a 314-bp invertible DNA element called *fimS*, which contains the promoter for *fimA*.
  - Downstream of *fimA*, the *fimI* gene is found encoding a protein that shows high homology to FimA.
  - Adjacently, two genes are located, *fimC* and *fimD*, coding for proteins that are not part of the pilus but have a vital role in pilus assembly.
  - FimC, the periplasmic pilus chaperone facilitates subunit incorporation into the pilus, whereas FimD, a pore forming protein, enables the translocation of newly formed type 1 pili through the outer membrane and anchors them in the bacterial membrane.
  - On the distal part of the gene cluster three genes are located, *fimF*, *fimG* and *fimH* encoding the minor subunits of type 1 pili including the mannose specific adhesin FimH **(Schwan 2011; Terlizzi et al. 2017)**.

- **FimH of UPECs-Its characteristics and importance:** The FimH **(Fig. 1.31)** subunit of type 1–fimbriated UPECs have been drawn in as an important determinant of bacterial adherence and colonization of the urinary tract since the 20th century **(Thankavel et al. 1997)**.



https://www.researchgate.net/publication/309361632_Exploitation_of_Glycobiology_in_Anti-Adhesion_Approaches_against_Biothreat_Agents/figures?lo=1

Fig. 1.31: FimH adhesin of UPECs.

The key characteristics of FimH adhesin are as follows:

- FimH is the adhesive subunit (protein of approx. 32kDa) **(Dreux et al. 2013)** of type 1 fimbriae of UPECs **(Tchesnokova et al. 2008)**.
- It is located at the organelle tip in a short fibrillum and also additionally intercalated along the fimbrial shaft (**Schembri et al. 2001)**.
- The FimH protein is produced as a precursor of 300 amino acids (aa) and is processed into a mature form of 279 aa (**Schembri et al. 2001)**.

- FimH is a two-domain protein (**Fig. 1.32)**, composed of an N-terminal, mannoside-binding lectin domain and a C-terminal pilin domain which are connected by a short tetrapeptide loop of 4aa (aa residues 157 to 159) **(Dreux et al. 2013; Sauer et al. 2016).**

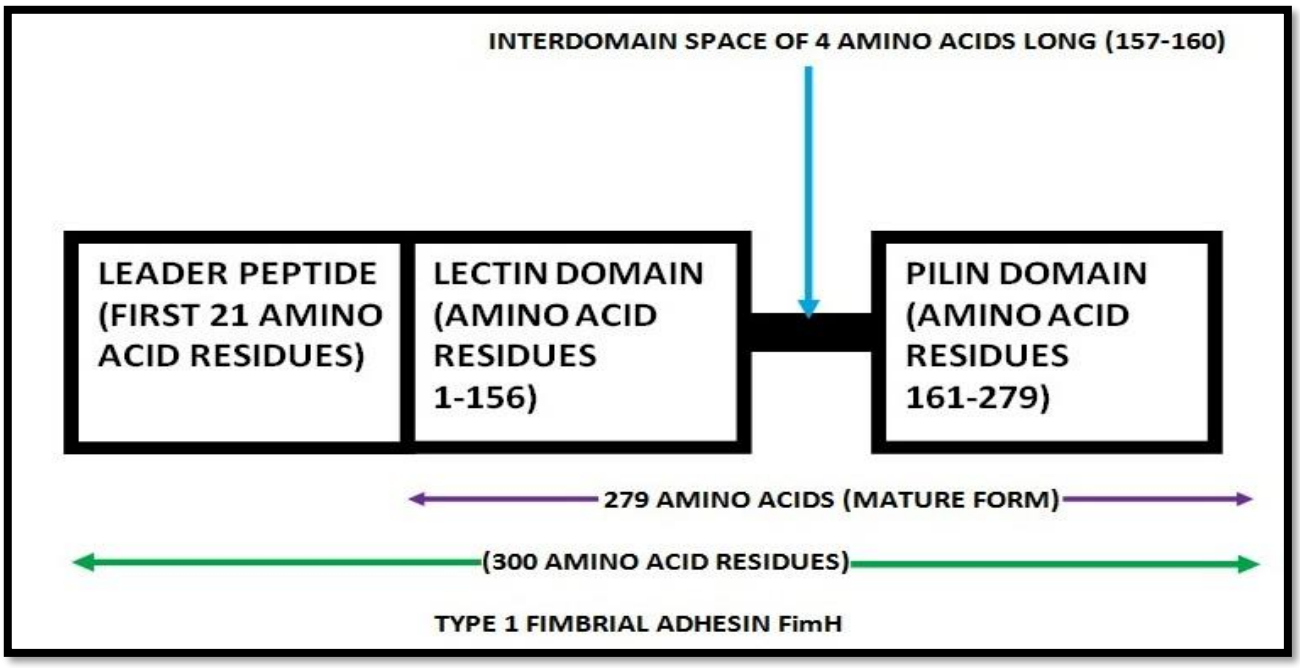
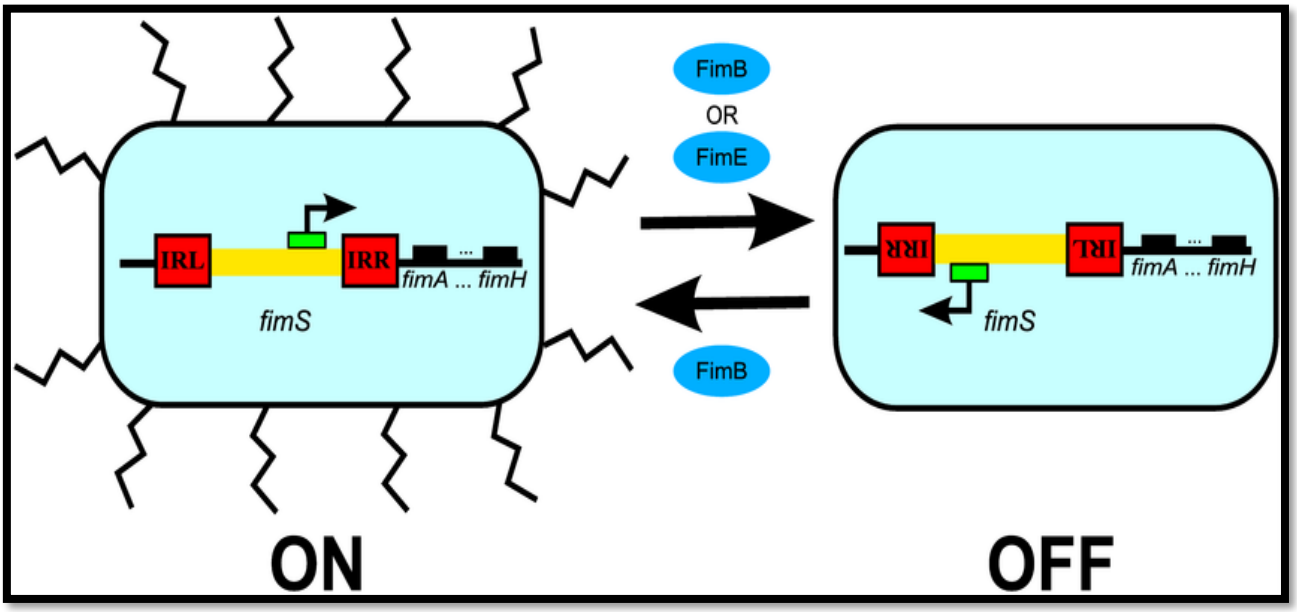


Fig. 1.32: FimH adhesin of UPECs consist of two domains.

However, the importances of FimH adhesin in UPECs are written as under:

- The FimH protein is the receptor recognizing constituent of type 1 fimbriae (**Schembri et al. 2001)**.
- FimH is essential during urinary tract infection for mediating colonization and invasion of the bladder epithelium and establishment of intracellular bacterial communities.
- The high binding ability of FimH can result in increased bacterial binding to target cells and increased pathogenicity of UPECs **(Hojati et al. 2015).**
- The FimH adhesin at the fimbrial tip specifically binds in a catch-bond mode to terminal α-D-linked mannoses of N-linked glycans of the receptor uroplakin 1a on uroepithelial cells. On account of its important role in establishing infection, FimH is an attractive target for the development of anti-adhesive drugs for UTI treatment **(Sauer et al. 2016)**.
- Arbitrary point mutations in *fimH* genes that increase binding of the adhesin to mono-mannose residues, structures abundant in the oligosaccharide moieties of urothelial glycoproteins, confer increased virulence in the mouse urinary tract **(Sokurenko et al. 1998**).

- **Type 1 fimbrial phase variation- Its regulation and its importance:** UPECs adhere to uroepithelial cells via type 1 fimbriae that undergo phase variation (**Fig. 1.33)** where a 314-bp *fimS* DNA element, flanked by two 9-bp inverted repeats, that contain the *fimA* promoter, flips between Phase-ON and Phase-OFF. When the invertible element (*fimS*)is in the "Phase ON" orientation, the promoter is in the direction of the structural *fim* gene (*fimA*), thus allowing transcription, whereas transcription is abolished in the inverted "Phase OFF" orientation **(Schwan 2011)**.



https://www.researchgate.net/publication/42834252_Temperature_Control_of_Fimbriation_Circuit_Switch_in_Uropathogenic_Escherichia_coli_Quantitative_Analysis_via_Automated_Model_Abstraction/figures?lo=1

Fig. 1.33: Phase variation in type 1 fimbriae operon of UPECs.

The phase switching of the 314-bp *fimS* invertible element is controlled by the products of two recombinase genes, *fimB* and *fimE*, positioned upstream of *fimA*. The *fimB* and *fimE* gene products are site-specific recombinases that influence the positioning of the *fimS* region. FimE appears to promote inversion of the promoter-containing *fimS* element from the Phase-ON to Phase-OFF orientation, whereas FimB promotes switching in both directions but with a switching bias toward the Phase-ON orientation. Several global regulators are involved in the proper modulation of the expression of type 1 fimbriae by environmental conditions. An appropriate supercoiling state of the DNA and the incidence of accessory proteins, like the DNA binding proteins Lrp and IHF, are the vital features that affect the recombination process and find out whether the cell is fimbriated (Phase OFF/ Phase ON) or not. Additionally, H-NS, the global regulator is known to influence type 1 fimbriation both by controlling the recombinases expression and by directly interacting with the invertible element (*fimS*) **(Schwan 2011; Schwan 2017)**.

- **P fimbriae:** P fimbriae are the second most common virulence factor of UPEC, which plays an indispensable role in the pathogenesis of ascending UTIs and pyelonephritis in humans. They play dependable for adhesion to mucosal and tissue matrix and for the production of cytokines. These fimbriae recognize kidney glycosphingolipids carrying the Gal α (1–4) Gal determinant on the renal epithelia via its *papG* adhesion. Attachment of P fimbriae to this receptor leads to the release of ceramide that acts as an agonist of toll-like receptor 4 (TLR4), a receptor involved in the activation of the immune cell response which in turn, leads to the development of the local inflammation and pain associated with UTIs. P fimbriae consist of heteropolymeric fibres composed of different protein subunits, encoded by the *pap*A-K gene operon **(Fig. 1.34) (Bien et al. 2012)**.

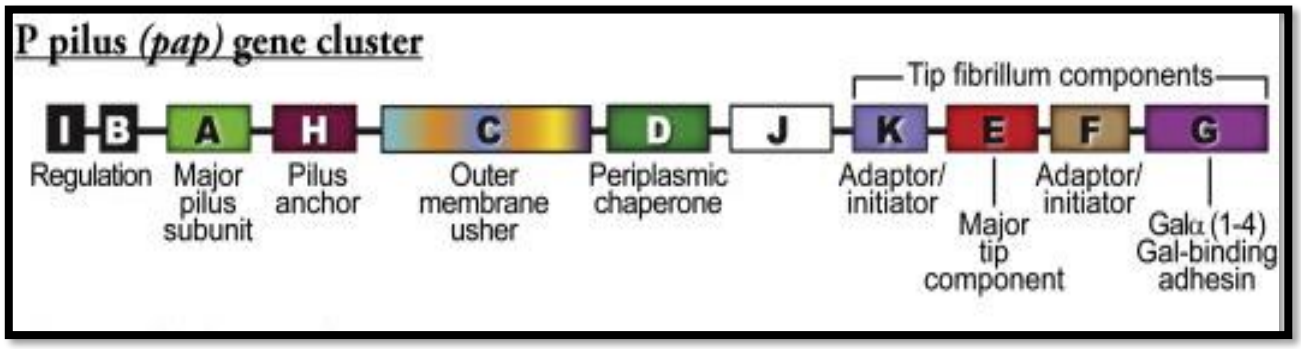


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Fig. 1.34: P fimbriae operon of UPECs.

Six different subunits that are ordered into two separate subassemblies (the tip fibrillum and the pilus rod) form the P pilus. At the distal end, the tip fibrillum is composed of the major adhesin PapG adhesin followed by PapF (adaptor/initiator) and PapE (major tip component) subunits. The pilus rod is made by more than thousand copies of the PapA subunit. The adaptor subunit PapK connects the above subunits to the PapA rod, which is a superhelical structure at the base of the pilum (**Terlizzi et al. 2017**). PapD, a conserved chaperone molecule with an Ig-like domain, is necessary to passage several pilus subunits from the cytoplasmic membrane to the outer membrane (OM). PapD-subunit complexes are directed to the PapC outer membrane usher that forms a pore through which the pili are transported across the OM.

- **S fimbriae and F1C fimbriae:** S fimbriae and F1C fimbriae encoded by *sfa* and *foc* genes respectively are drawn in, in the process of UTIs. These types of fimbriae display binding to

endothelial and epithelial cell lines that originated from the lower human urinary tract and kidney. Nevertheless, S fimbriae are known to ease bacterial dissemination within host tissues and are frequently associated with UPECs that cause sepsis, meningitis, and ascending UTIs **(Bien et al. 2012)**. Receptors containing sialic acid sugar moieties are places where S fimbriae binds. The sialic acid residues are accessible on UP3, one of four integral membrane uroplakin proteins. However, F1C fimbriae binds to the GalNAcβ1-4Galβ sequence of glycolipids, i.e., asialo-GM_1 and asialo-GM_2 with high affinity an additional binding to carbohydrate structures GlcNAcβ1-3Galβ, Galβ1-4Glc, Gal, and Glc of glycolipids may indicate functional low-affinity receptor sites. F1C fimbriae are genetically homologous to S fimbriae, but differ in receptor specificity **(Mitsumori et al. 1998)**.

- **Dr fimbriae and afimbrial adhesins:** Dr fimbriae and afimbrial adhesin encoded by *dra* and *afa* genes respectively are associated with UTIs, especially, with gestational pyelonephritis and recurring cystitis. These adhesins are expressed by those UPEC strains that have a unique renal tissue tropism **(Bien et al. 2012)**.

- **Capsule and the lipopolysaccharides (LPS):** Virulence factors located on the bacterial surface also consist of the capsule and the lipopolysaccharide (LPS). The capsule is primarily a polysaccharide structure casing and shielding the bacterium from the host immune system. The capsule provides protection against phagocytic engulfment and complement-mediated bactericidal effect in the host **(Bien et al. 2012; Parvez and Rahman 2018)**. The LPS is an indispensable component of the cell wall of Gram-negative bacteria. LPS is known to initiate host response and to induce nitric oxide and cytokine production. LPS of UPEC is imperative in activation of proinflammatory response in uncomplicated UTIs; however, it is not clear whether LPS plays a role in interceding a renal failure and acute allograft injury in patients with ascending UTIs **(Bien et al. 2012)**.

(b) Secreted virulence factors: UPEC's secreted virulence factors primarily include toxins and siderophores which may cause inflammatory response and modulate host cellular pathways.

- **Toxins:** Toxins are vital virulence factors in a variety of *E. coli*-mediated UTIs **(Bien et al. 2012)**. Toxins have the ability to modify the host cell signaling cascade and modulate inflammatory responses. Several *in vitro* and *in vivo* studies showed that toxins also contribute to the stimulation of the host cell death and releasing of necessary nutrients that provide the ability to access deeper tissues within the urinary tract **(Parvez and Rahman 2018).** Different toxins secreted by UPECs are as follows:

  - **α-hemolysin:** *α*-haemolysin (HlyA), a lipoprotein is most important secreted virulence

- factor of UPECs and is known to be commonly associated with upper UTIs such as pyelonephritis. HlyA is a pore-forming toxin encoded by *hlyA* genes, which belongs to the family of RTX (repeats in toxin) toxins and causes inducible nitric-oxide-synthase (iNOS)-mediated cell membrane injury and apoptosis. Nevertheless, HlyA can lyse nucleated host cells and erythrocytes at high concentration by a process allowing UPECs that may harm the host immune effector cells for acquring improved access to the host nutrients and iron stores. However, when the concentration is low, HlyA can induce the apoptosis of target host cells and promote the exfoliation of bladder epithelial cells. Withal, Moreover, HlyA has the role in the increased production of IL-6 and IL-8 by inducing Ca^{2+} oscillations in renal epithelial cells **(Bien et al. 2012; Parvez and Rahman 2018)**.

- **Cytotoxic necrotizing factor 1 (CNF1):** About one-third of all pyelonephritis strains produce CNF1 and may also be involved in the invasion of kidney. The aforementioned protein is secreted by UPECs and stimulates actin stress fibers formation and membrane ruffle formation in a Rho GTPase-dependent manner, resulting in the entry of the bacteria into the cells. *In vivo*, CNF1 may lead to bladder cell exfoliation and better bacterial access to underlying tissue **(Bien et al. 2012; Parvez and Rahman 2018)**.

- **Cytolethal distending toxin (CDT):** CDT is a toxin secreted by UPECs that has the ability to arrest the cell cycle and contributes to the pathogenesis of UTIs. CDT is an operon product encoding three proteins comprising CdtA, CdtB, and CdtC proteins that are encoded by the *cdtA*, *cdtB*, and *cdtC* genes, respectively. CDT has DNase I-like enzymatic activity and attacks DNA. This sole property of attacking DNA damages the target cell DNA that results in progressive cell distending leading to the cell death **(Parvez and Rahman 2018)**.

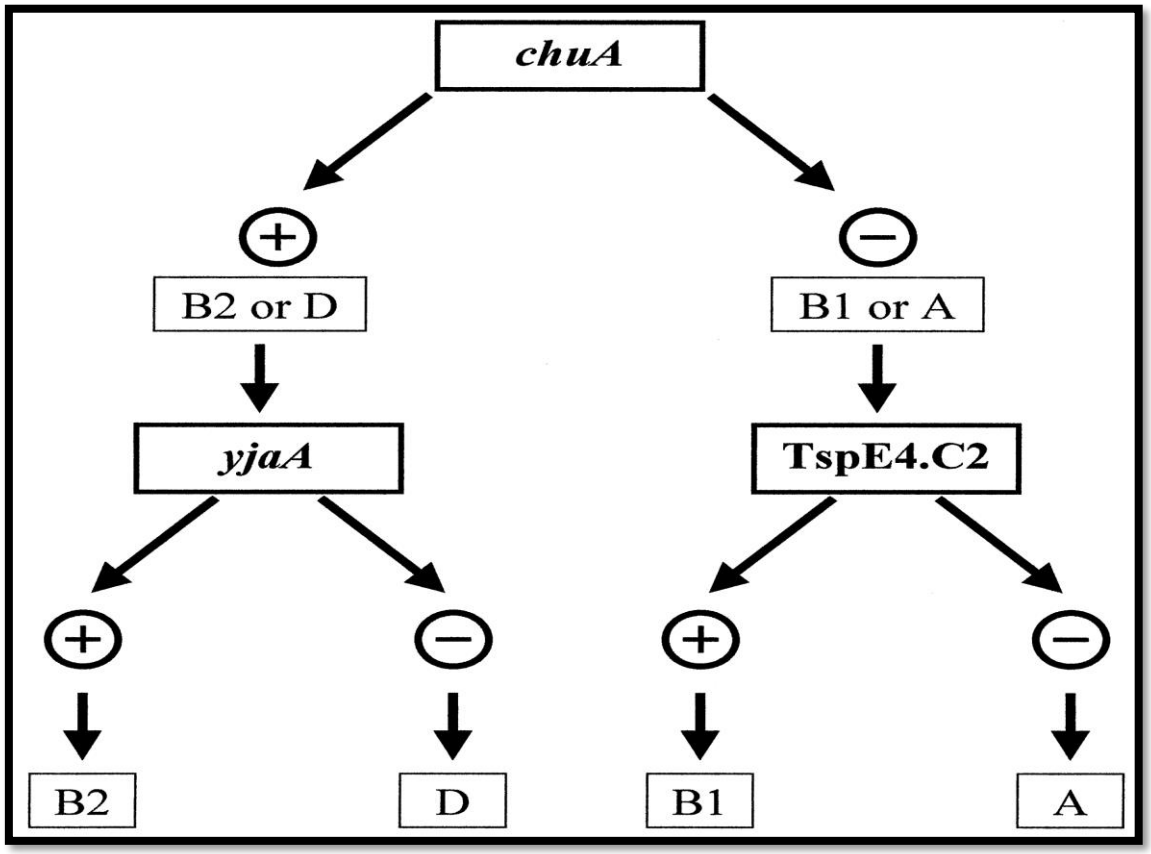
- **Secreted autotransporter toxin (SAT):** SAT is mostly known to be associated with UPECs causing pyelonephritis. A serine protease autotransporter, SAT that falls within one subgroup of autotransporters which is recently classified as the SPATE (serine protease autotransporters of *Enterobacteriaceae*) family. SAT have the cytopathic activity that results in the damage of the host tissue and may also increase the propagation ability of the UPECs. However, this toxin may even facilitate the entry of pyelonephritogenic UPEC strains into the bloodstream resulting from specific damage to the glomeruli and proximal tubules **(Bien et al. 2012; Parvez and Rahman 2018)**.

- **Siderophores:** UPECs possess some multiple functionally redundant systems that intercede iron uptake by secreting low-molecular-weight Fe^{3+}-chelating molecules which are widely known as siderophores. Iron utilization, mediated by these siderophores, is critical for colonization of the urinary tract by UPECs. Four distinct siderophore systems are found

in UPECs such as, aerobactin, yersiniabactin, enterobactin, and salmochelin. These systems also comprise some genes like the *iuc* genes encoding aerobactin, *ent* genes encoding enterobactin, and *iro* genes encoding an ent-like system. Aerobactins encoded by *iuc* genes are most common siderophores secreted by UPECs. They are low-weight molecules and hydroxamate siderophores with higher Fe^{3+}-binding stability in acidic environments and are maximally produced at low pH. Aerobactins extract Fe^{3+} from host iron-binding proteins and are taken up through an outer membrane receptor protein. Moreover, aerobactins have many advantages over other siderophores and are formed from the condensation of two lysine molecules and one citrate catalyzed by an enzyme named aerobactin synthase **(Parvez and Rahman 2018)**.

1.2.5 Phylogenetic background of UPECs

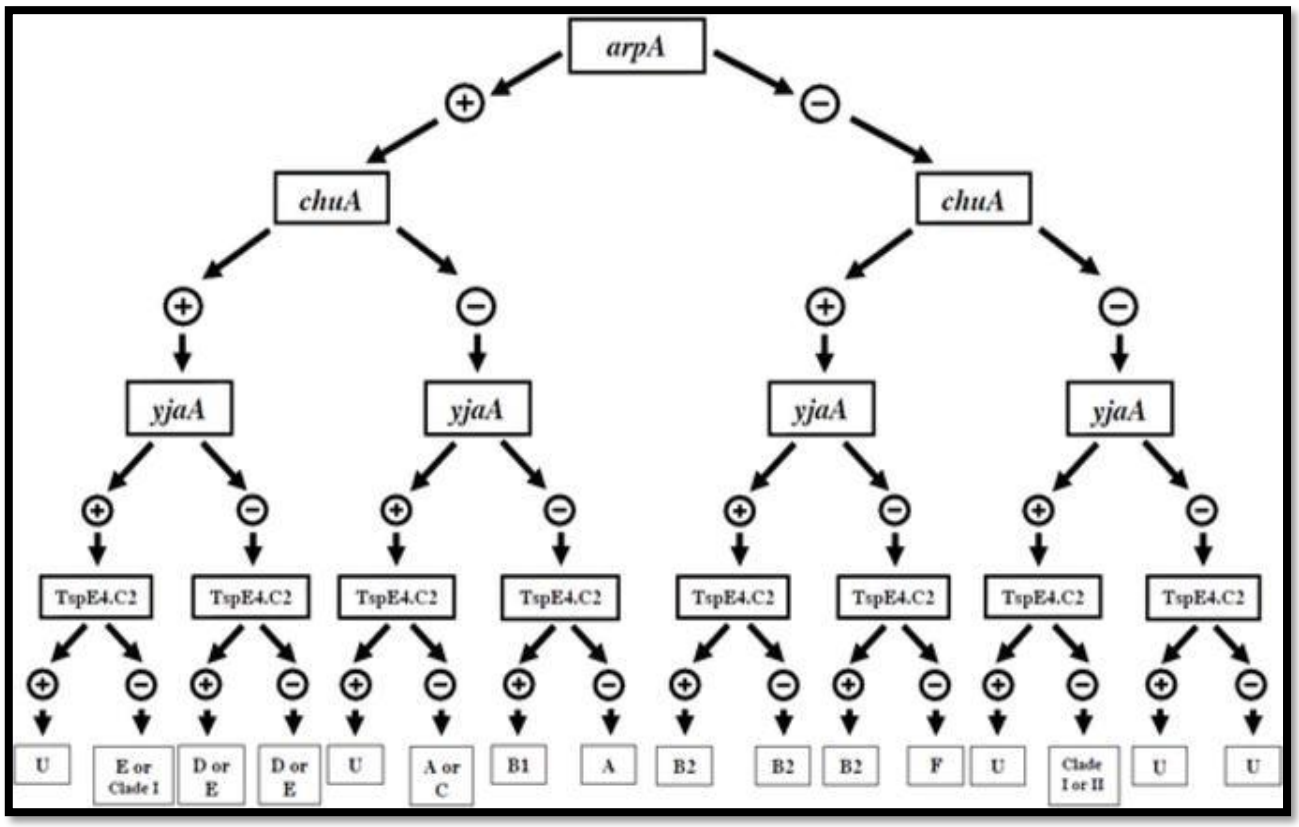
Initially **Clermont et al. (2000)** developed a triplex PCR based assay that enabled strains of *E. coli* to be assigned to one of the four main phylogroups that is A, B1, B2 or D **(Fig. 1.35)** based on the presence or absence of two genes *chuA* (a gene required for haem transport in enterohemorrhagic O157:H7 *E. coli*) and *yjaA*(a gene initially identified in the recent complete genome sequence of *E. coli* K-12, the function of which is unknown) and a DNA fragment (TspE4.C2), later characterized as a putative lipase esterase gene **(Gordon et al. 2008)**.



https://journals.asm.org/doi/10.1128/AEM.66.10.4555-4558.2000 **(Clermont et al. 2000)**

Fig. 1.35: Phylogenetic groups of UPECs according to triplex PCR-based assay.

Moreover, Gordon et al**. (Gordon et al. 2008)** also reported the existence of five main phylogenetic groups (A, B1, B2, and D and E), after further confirmation by multi-locus sequence typing and later studies found Enterohemorrhagic *E. coli* O157:H7(EHEC O157:H7) to be the best-known member of the aforesaid phylogroup E (**Clermont et al. 2013)**. Another phylogroup, F was also documented to consists of strains that form a sister group to phylogroup B2 **(Jaureguy et al. 2008; Clermont et al. 2011; Clermont et al. 2013).** Phylogroup C was also proposed for a group of strains closely related to, but distinct from phylogroup B1 **(Moissenet et al. 2010; Clermont et al. 2011**). A report by Luo et al. **(Luo et al. 2011)** revealed that cryptic clade I should also be considered as a phylogroup of *E. coli* depending on the extent of recombination detected between strains belonging to clade I and *E. coli*. A new PCR (Quadruplex) based assay was developed (**Clermont et al. 2013**) by adding an additional gene target *arpA* to existing triplex PCR based assay **(Clermont et al. 2000)** that enabled *E. coli* strains belonging to phylogroup F, formerly misidentified as D strains (*chuA*+, *yjaA*-, TspE4.C2), to be distinguished because *arpA* is present in all *E. coli* except those strains belonging to phylogroups B2 and F. This is because a previous study **(Clermont et al. 2004)**, revealed that *arpA*, a gene of unknown function was absent from all phylogroup B2 and most of the phylogroup D meningitis strains, while it was present in all of the phylogroup B1 and A strains, thereby concluding the fact that this gene was most likely acquired by avirulent strains after separation of phylogroups A and B1 from B2 and D. Therefore the appropriate assignment of *E. coli* isolates to any one of the aforesaid phylogroups needed the application of extended quadruplex method by the usage of two allele specific primer pairs in addition to those specific for the four genes (*arpA*, *chuA*, *yjaA*,TspE4.C2), that helped to identify the *E. coli* strains belonging to phylogroup C and E with certainty and as reported 95% of the *E.coli* strains could be correctly assigned, using this extended quadruplex method **(Clermont et al. 2013)**. Therefore, according to **Clermont et al. (Clermont et al. 2013),** there are eight recognized phylogroups of *E. coli*, with seven belonging to *E coli sensu stricto* (A, B1, B2, C, D, E and F) and one corresponding to *Escherichia* cryptic clade I**. Fig. 1.36** illustrates the phylogenetic groups of UPECs based on the extended quarduplex phylotyping method as described by Clermont and colleagues **(Clermont et al. 2013)**.



(Clermont et al. 2013)

Fig. 1.36: Phylogenetic groups of UPECs according to extended quadruplex PCR-based assay. "**U" stands for "unknown" phylogroup.**

1.2.6 Genetic diversity among drug-resistant UPECs

On the whole, the dissemination of ESBL producing MDR UPECs have increased dramatically in the recent years, becoming a serious worldwide threat (**Kot 2017; Pérez-Etayo et al. 2018; Qasemi et al. 2021).** Various genetic mechanisms have been involved in the acquisition and dispersion of antimicrobial resistances. Moreover, MGEs play an important role in the dissemination of the aforementioned antibiotic resistant strains among human and environmental sources **(Pérez-Etayo et al. 2018)**. Susceptible UPECs may attain resistance through mutations or the transfer of resistance genes located on MGEs **(Lavakhamseh et al. 2015)**. The rapid spread of β-lactamases resistance, led by MGEs, among susceptible bacteria and acquisition of plasmid-mediated β-lactamases such as ESBL, and class C plasmid-mediated AmpC β-lactamases (ABL) among UPECs are well documented. Moreover, increase Metallo-β-lactamases (MBL) producing UPECs; have also further led to limitations in the treatment options. However, out of the 10 β-lactamase genes, four belonged to ESBL (*TEM*, *SHV*, CTX-M, and *OXA*); three to MBL (*NDM*-1, *IMP*, and *VIM*); and three to ABL (*ACT*, *DHA* and *CMY*) class of genes **(Singh et al. 2019)**. β-lactamase genes have been known to be associated with plasmids, transposons, integrons and insertion sequences **(Kurpiel and Hanson 2011)**. Integrons are naturally efficient recombination and expression systems that are able to capture genes

as part of genetic elements known as gene cassettes **(Salem et al. 2010)**. Moreover, integrons are usually composed of two conserved segments (termed 5′-conserved region (5′-CS) and 3′-conserved region (3′-CS)) separated by a variable region that contains the gene cassettes. The 5′-CS end includes: (i) the *int* gene coding for an integrase, which belongs to a discrete family of the tyrosine-recombinase (ii) a primary recombination site (*attI*); and (iii) a promoter (Pc) that certifies the transcription of the cassette genes. However, the 3′-CS region is formed by (i) a truncated gene of resistance to quaternary ammonium compounds (*qacEΔ1*); (ii) a sulfonamide resistance gene (*sul1*); and (iii) an unknown function sequence (orf5) **(Pérez-Etayo et al. 2018)**. Class 1 (often called IntI1 as they encode the integron-integrase gene *intI1*) and class 2 (often called IntI2 as they encode the integron-integrase gene *intI2*) integrons are the most commonly involved in antibiotic resistances while limited work has shown the presence of class 3 (often called IntI2 as they encode the integron-integrase gene *intI3*) in Enterobacteriaceae **(Pérez-Etayo et al. 2018; de Los Santos et al. 2021)**. Antibiotic resistant genes located on integrons like structures are being increasingly reported worldwide **(Gillings et al. 2008; Salem et al. 2010)**. Furthermore, MGEs such as insertion sequences (ISs) are the smallest transposable elements (<2.5 kb) that are classified into families according to their different characteristics, with transposases (enzymes that catalyze the IS movement) being the major classification system used **(Pérez-Etayo et al. 2018)**. Withal, ISs such as IS5, IS*26*, IS*Ecp1* in association with class 1 integrons, are the most involved elements in the antimicrobial resistance to β-lactamics **(Cattoir et al. 2008; Kurpiel and Hanson 2011; Pérez-Etayo et al. 2018)**.

Moreover, Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) typing was earlier reported **(Durmaz et al. 2015**) as one of the most powerful tools to determine the genetic relationships between different bacterial isolates. The ERIC sequences were known as intergenic consensus sequences, found at different loci within a huge number of bacterial genomes, counting Enterobacteriaceae family members *E. coli*. They were identified as 127bp imperfect palindromes that occurred in multiple copies within the genomes and were generally identified in the transcribed areas in association with the intergenic consensus **(Wilson et al. 2006; Hellmuth et al. 2017; Ranjbar et al. 2017)**. The ERIC-PCR method was earlier reported to use these exact intergenic repeated sequences as primer sites for amplifications of the regions between them. The number and location of the aforementioned sequences were revealed to vary from strain to strain and the electrophoretically determined amplified fragment was identified to form a distinct DNA fingerprint **(Gibreel et al. 2011).** Likewise, Durmaz et al. **(Durmaz et al. 2015)** from Turkey confirmed the genetic diversity among symptomatic *E. coli* isolates using the aforementioned method of typing.

Further, the multilocus sequence-typing (MLST) technique is extensively used to study ExPEC lineages, most important of which are UPECs **(Kot 2019)**. MLST, in which internal portions of multiple housekeeping genes are sequenced to define clonal diversity, has emerged as a most

prevailing tool to describe the genetic structure of bacterial populations **(Jaureguy et al. 2008)**. Moreover, MLST has been widely regarded as a useful system for phylogenetic and epidemiological studies of MDR *E. coli*. Furthermore, although three (Michigan State University, Warwick Medical School and Pasteur Institute) distinct MLST schemes exist for *E. coli*, but the most widely used is Mark Achtman's (Warwick Medical School) set of 7 housekeeping genes **(Fig. 1.37) (Kaas et al. 2012; Ahmed et al. 2016)**. Sequence types (STs) 10, 69, 73, 95, 127, and 131 identified by MLST are isolated as pandemic clones of ExPEC from human infections, most predominantly UTIs **(Tartof et al. 2005; Riley 2014)**. **Gibreel et al. (Gibreel et al. 2012)** and **Alghoribi et al. (Alghoribi et al. 2015)** reported that UPEC isolates from patients in the Northwest region of England and Saudi Arabia which belonged to lineage ST131 displayed higher levels of antibiotic resistance when compared to ST127 isolates which were the most widely susceptible to antibiotics**.** The UPEC strains belonging to ST 95, 127, 73, 69, 131, and 10 are found to be responsible for 56% of UTI cases in 1999–2000. However, during the period 2016–2017, the same STs caused 64% of the UTI cases **(Kot 2019)**.

Genes	Gene product
adk	Adenylate kinase
fumC	Fumarate hydratase
gyrB	DNA gyrase
icd	Isocitrate/isopropylmalate dehydrogenase
mdh	Malate dehydrogenase
purA	Adenylosuccinate dehydrogenase
recA	ATP/GTP binding motif

(Wirth et al. 2006)

Fig. 1.37: *E. coli* Achtman MLST scheme.

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CHAPTER 2.

Asymptomatic & symptomatic uropathogenic *E. coli* - Isolation & identification with special reference to the patients' characteristics; Antibiogram analysis

2.1 Background study

UTI is a major public health predicament in terms of morbidity and mortality worldwide. It ranks as the number one infection that leads to an antibiotic prescription after a physician's visit **(Hailay et al. 2020; Bazzaz et al. 2021)**. Moreover, it accounts for the majority of the reasons for hospital visit globally. Therefore, profound knowledge of different factors associated with UTI may allow judicious intervention that can easily bring the disease under control. UTIs can be community acquired or nosocomial. Community-acquired UTIs (CA-UTIs) are the infection of the urinary system that takes place in one's life in the community setting or in the hospital environment within 48 hours of admission. CA-UTIs is the second most commonly encountered microbial infection in the community setting. Nosocomial UTIs (N-UTIs) are the infections of the urinary tract that occurs after 48 hours of hospital admission, and the patient is not incubating at the time of admission or within 3 days after discharge **(Odoki et al. 2019)**. Furthermore, UTIs may be asymptomatic, acute or, chronic, and complicated or uncomplicated, and the clinical manifestations of UTIs vary on the portion of the urinary tract involved, the etiologic organisms, the severity of the infection, and the patient's ability to mount an immune response to it **(Olowe et al. 2015; Odoki et al. 2019).** Moreover, although according to the recent report by Nicolle et al. **(Nicolle et al. 2019)** asymptomatic bacteriuria (ABU) should be screened for and treated only in pregnant women or in patients expected to undergo invasive urologic procedures, however, ABU besides symptomatic UTI is also indicated to pose a serious threat to public health care, thereby reducing the quality of life and resulting into work absenteeism **(Olowe et al. 2015; Odoki et al. 2019)**.

Furthermore, globally the most frequent cause of UTI is *E. coli*, a gram-negative pathogen and member of the family *Enterobacteriaceae* **(Bunduki et al. 2021)**. However, *E. coli* is one of the most genetically diverse bacterial species that can occur in varied forms in nature such as a probiotic, commensal or as a harmful intestinal and/or extraintestinal pathogen affecting humans and animals by causing a wide array of diseases like urinary tract infections, diarrhoea, septicaemia and neonatal meningitis **(Clermont et al. 2000; van Elsas et al. 2010; Clermont et al. 2011; Do et al. 2017)**. Nonetheless, Uropathogenic *E. coli* (UPECs) are among the most common extra-intestinal pathogenic *E. coli* (ExPEC) **(Bunduki et al. 2021)** encountered in the vast majority of asymptomatic bacteriuria (ABU) and symptomatic UTIs, including cystitis and pyelonephritis **(Bien et al. 2012)**.

Generally, symptomatic UTIs should be treated with antimicrobials to assuage symptoms and further complications, whereas ABU generally does not avouch treatment as reported by Abbo and Hooton **(Abbo and Hooton 2014)**. However, there is contrariety to this analysis that indicated prevalence of high proportion of multidrug resistant (MDR) ABU among the healthy individuals **(Onanuga and Selekere 2016).** Moreover, Phillips et al. **(Phillips et al. 2012)** stated that ABU is

frequently misdiagnosed as UTI leading to improper antimicrobial use. Although current guidelines suggest screening for ABU and treating it in particular circumstances such as during pregnancy or before invasive urologic procedures, antibiotic overuse for ABU seems to be irresistible in clinical practice, as supported by several studies reporting that 20–80 % of cases of ABU being inappropriately treated (**Cope et al. 2009; Khawcharoenporn et al. 2011**).

Nonetheless, antimicrobial resistance in UPECs, especially symptomatic and the spreading of MDR UPECs in recent decades is a clinical problem. Moreover, although worldwide incidence of MDR is mainly reported in symptomatic UPECs, a relatively recent study **(Mukherjee et al. 2015)** from India indicated incidence of MDR among asymptomatic UPECs isolated from pregnant women. Moreover, studies conducted in the recent past from the countries like Ethiopia **(Belete et al. 2020)** and Iran **(Naziri et al. 2020)** indicated low and moderate incidences of extended-spectrum β-lactamase (ESBL) production among the MDR ABU and symptomatic UPECs respectively**.** The increasing frequency of MDR symptomatic UPECs, especially in developing countries, results in excessive use of broad-spectrum antibiotics such as cephalosporins, aminoglycosides and fluoroquinolones that raise the cost of treatment and hospitalization **(Bartoletti et al. 2016; Sanchez et al. 2016; Kot 2019)**. A relatively recent report from Ethiopia **(Gashe et al. 2018)** displayed high resistance of symptomatic UPECs to third generation cephalosporins like (ceftriaxone or ceftazidime). Moreover, low to high incidence of resistance of symptomatic UPECs to third generation cephalosporins was been reported from various parts of the world like England **(Abernethy et al. 2017)**, Pakistan **(Ali et al. 2016)** and India **(Basu and Mukherjee 2018; Malik et al. 2021)**. However, mostly low resistance to carbapenems was reported from studies conducted worldwide **(Bonkat et al. 2017; Shahbazi et al. 2018; Kot 2019)**, thereby recommending them as suitable antimicrobials for the treatment of acute uncomplicated pyelonephritis, complicated UTI, and urosepsis. Moreover, two relatively recent studies from India indicated moderate to high level of resistance of symptomatic UPECs to aminoglycosides like tobramycin, gentamicin **(Basu and Mukherjee 2018)**, neomycin and amikacin **(Mir et al. 2016).** However, worldwide incidence of resistance against aminoglycosides especially, amikacin was quite low **(Dehbanipour et al. 2016; Ali et al. 2016; Ramírez-Castillo et al. 2018)**. Furthermore, the increasing emergences of UPECs resistant to fluoroquinolones were reported worldwide, and it has emerged probably due to the excessive use of these antibiotics **(Kot 2019)**. Withal, earlier studies **(Basu and Mukherjee 2018; Prasada et al. 2019)** from India reported a -high rate of fluoroquinolone resistance in symptomatic UPECs with maximum against the 2nd generation drug ciprofloxacin. To boot, high incidence of resistance of symptomatic UPECs to folic acid synthesis inhibitors like trimethoprim/Sulfamethoxazole was reported from various parts of the world like Mexico **(Ali et al. 2016)**, Pakistan **(Ramírez-Castillo et al. 2018)**, Mongolia **(Munkhdelger et al. 2017)**, Ethiopia **(Regasa Dadi et al. 2018)** and India **(Basu and Mukherjee 2018)** in the relatively recent past.

However, very low level of resistance of UPECs to nitrofurantoin of the nitrofuran group was reported worldwide, hence regarded as a suitable antibiotic for treatment of uncomplicated UTIs (**Bonkat et al. 2017; Kot 2019)**. Therefore, antibiotic overuse in treatment of bacterial infections has several adverse effects, including the emergence of (MDR) microbes causing increased costs of health care.

Moreover, till date, the incidence of ABU UPECs isolated from males and non-pregnant females and their antibiogram profile have not been investigated from Kolkata, an eastern region of India, with regard to the symptomatic ones. So, this study for the first time aimed to identify ABU UPECs among hospitalized patients of Kolkata, India and provide a detailed analysis on their clinical characteristics, susceptibility pattern against different groups of antibiotics, ESBL phenotype and further compared to the symptomatic UPECs to implement proper prescription policies for appropriate therapeutic interventions.

2.2 Objectives

- Isolation of culture-positive urine samples from the patients admitted to the Carmichael Hospital for Tropical Diseases without any symptoms of UTI (asymptomatic) as well as from patients with symptoms of UTI (symptomatic) along with their appropriate clinical details.
- Identification of UPECs by Gram staining and various biochemical tests and their evaluation with the patient demographics.
- Antibiogram analysis and determination of ESBL/BLIR phenotype of the isolated asymptomatic and symptomatic UPECs by Kirby-Bauer disc diffusion method to explore effective treatment options by understanding their antibiotic-susceptibility profile.

2.3 Materials

(a) Equipments:

- Laminar Air Flow [B.D Instrumentation]
- Shaker – Incubator [ICT]
- Autoclave [PrimeSurgicals]
- Compound binocular microscope [Magnus, India]
- Spectrophotometer [Bio-Rad, India]
- Hot air oven [Digisystem Laboratory Instruments Inc.]
- Freezer (-20°C) [Celfrost]
- Inoculation loop
- Staining rack

- Glass spreaders
- Spirit Lamp
- 90mm Glass petri dish [Borosil]
- Glass culture tubes [TOUFF, Borosil]
- Glass slides
- Test tube racks [Tarsons]
- Micropipettes (0.5-10µl, 2-20µl, 20-200µl, 200-1000µl) [Corning, P'fact, Microlit, Biohit]
- Micro tips (0.5-10µl, 2-20µl, 20-200µl, 200-1000µl) [HiMedia]
- Eppendorf Tubes (1.5 mL, 2 mL) [Tarsons]
- Cotton [Bengal Surgicals Limited] [Lakshmi Healthcare Products (P) Ltd]
- Surgical Gloves [PriCARE, HiMedia]
- Cryogenic Tubes (1.5mL) [Tarsons]
- Wash bottles

(b) Reagents:

- Luria Bertani (LB) media [SRL Chemicals India]
- Mueller Hinton (MH) media [SRL Chemicals India]
- Agar Agar [Merck]
- Tryptone broth [HiMedia]
- MR-VP Medium (Glucose Phosphate broth) [HiMedia]
- Simmon's citrate media [HiMedia]
- Triple Sugar Iron Agar (TSI) media [HiMedia]
- MacConkey agar [HiMedia]
- Eosin Methylene Blue EMB agar [HiMedia]
- Barium chloride [Merck]
- Sulphuric acid [Hospital Store]
- Crystal Violet [Stanbio Reagents Pvt. Ltd.]
- Safranin [Stanbio Reagents Pvt. Ltd.]
- Gram's Iodine [Stanbio Reagents Pvt. Ltd.]
- 70% Ethanol [Bengal Chemical]
- 95% Ethanol [HiMedia]
- Acetone [Hospital Store]
- 87% Glycerol [SRL Chemicals India]
- Single Distilled water (SDW) [Hospital Store]

- Double distilled water (DDW) [Laboratory distillation plant]
- Antibiotic Discs [HiMedia]

2.3.1 Preparation of reagents

- **LB broth:** 10gms of LB broth powder was dissolved in 500mL of SDW (Conc.- 20gms/lit). Then it was thoroughly mixed, dispensed into culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes (file:///C:/Users/Arunita%20Ghosh/Downloads/M575.pdf).
- **LB agar:** 10gms of LB broth powder was dissolved in 500mL of SDW (Conc.- 20gms/lit). Then to the aforesaid mixture 7.5gm of agar agar (Conc. 1.5%) was added, thoroughly mixed, and autoclaved at 15 psi pressure at 121°C for 15 minutes. After autoclaving, the sterile medium was distributed into different 90mm petriplates, cooled and solidified for future use.
- **Nutrient agar:** 14gms of the nutrient agar was dissolved in 500 mL of SDW (Conc.-28gms/lit). The media was then autoclaved at 15 psi pressure at 121°C for 15 minutes. Then the sterile medium was distributed in 90mm glass petri dish by pouring method to get cooled and solidified for future use (file:///C:/Users/Arunita%20Ghosh/Downloads/M001.pdf).
- **MH broth:** 10.5gm of MH broth powder was dissolved in 500mL of SDW (Conc.-21gms/lit). Then it was thoroughly mixed, dispensed into culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes (file:///C:/Users/Arunita%20Ghosh/Downloads/M391.pdf).
- **MH agar:** 10.5gm of MH broth powder was dissolved in 500mL of SDW (Conc.-21gms/lit). Then to the aforesaid mixture 7.5gm of agar agar (Conc. 1.5%) was added, thoroughly mixed, and autoclaved at 15 psi pressure at 121°C for 15 minutes. After autoclaving, the sterile medium was distributed into different 90mm petriplates, cooled and solidified for future use
- **Tryptone broth:** 7.5gms of tryptone broth was dissolved in 500mL of SDW (Conc.-15gms/lit). The aforesaid mixture was thoroughly mixed and the pH was checked to be around 7.6. Then the mixture distributed into different culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes (file:///C:/Users/Arunita%20Ghosh/Downloads/M463.pdf).
- **MR-VP medium (Glucose Phosphate broth):** 8.5gms of glucose phosphate broth was dissolved in 500mL of SDW (Conc.-17gms/lit). The aforesaid mixture was thoroughly mixed and the pH was checked to be around 7.0. Then the mixture distributed into different culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes (https://himedialabs.com/TD/M070.pdf).
- **Simmon's citrate agar:** 12.14gms of Simmon's citrate agar was dissolved in 500mL of SDW (Conc.-24.28gms/lit). The aforesaid mixture was thoroughly mixed, distributed into different culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes. Then using a ring stand

and clamp, the rack was clamped so that the tubes (with liquid medium in them) have a 3 cm slant with a 2-3 cm butt. It was cooled until solid and then incubated for 48 hours at 37ºC to guarantee sterility (https://himedialabs.com/TD/M099.pdf).

- **TSI agar:** 32.25gms of TSI agar was dissolved in 500mL of SDW (Conc.-64.5gms/lit). The aforesaid mixture was thoroughly mixed, distributed into different culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes. Then using a ring stand and clamp, the rack was clamped so that the tubes (with liquid medium in them) have a 3 cm slant with a 2.5 cm butt. It was cooled until solid and then incubated for 48 hours at 37ºC to guarantee sterility (https://himedialabs.com/TD/M021.pdf).
- **MacConkey Agar:** 25gms of the media was dissolved in 500 mL of SDW (Conc.-50gms/lit). The media was then autoclaved at 15 psi pressure at 121°C for 15 minutes. Then the sterile medium was distributed in 90mm glass petri dish by pouring method to get cooled and solidified for future use (https://himedialabs.com/TD/M081Bpdf).
- **EMB agar:** 18gms of the medium was dissolved in 500 mL of SDW (Conc.-36gms/lit). The medium was then autoclaved at 15 psi pressure at 121°C for 15 minutes. Then the sterile medium was distributed in 90mm glass petri dish by pouring method to get cooled and solidified for future use (https://himedialabs.com/TD/M317.pdf).
- **McFarland solution:** 0.05mL of 1% Barium chloride ($BaCl_2$) was added to 9.95mL of 1% Sulphuric acid (H_2SO_4) to make 0. McFarland 5 standard solution. Its O.D. is in the range of 0.08 - 0.1 (https://en.wikipedia.org/wiki/McFarland_standards).

2.4 Experimental methods

2.4.1 Sample collection

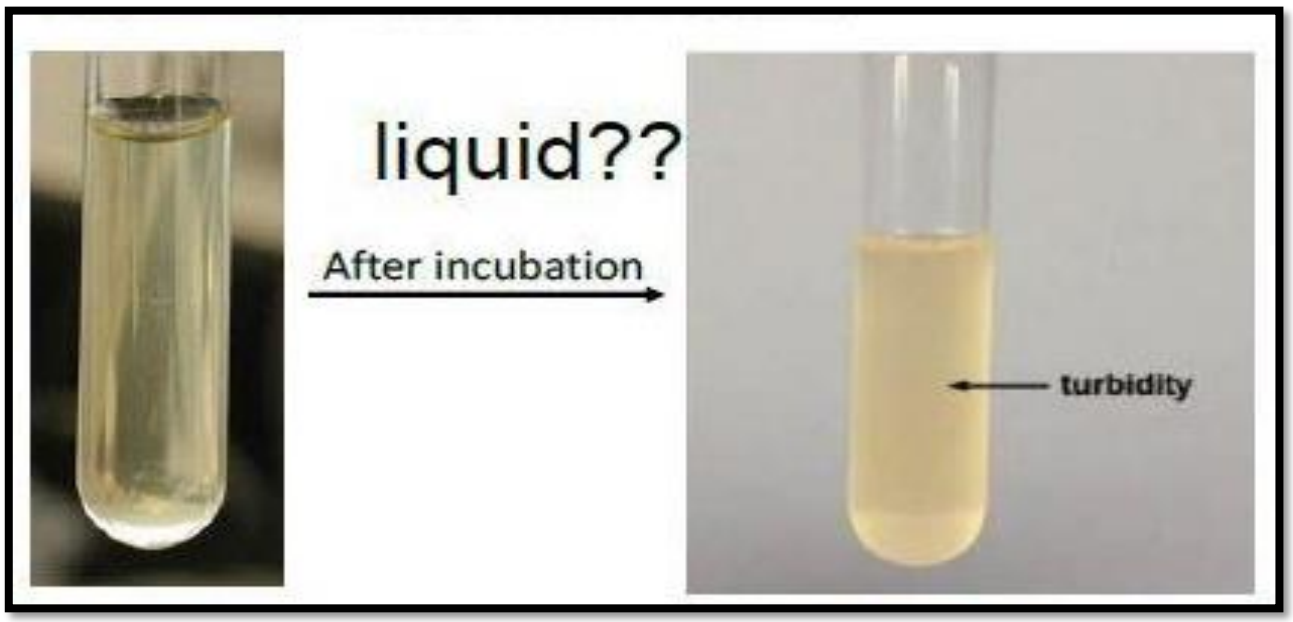
This study has been carried out on urine samples collected from patients admitted to the Carmichael Hospital for Tropical Diseases without any symptoms of UTI (asymptomatic) as well as from patients with classical symptoms of UTI (symptomatic). A total of 200 urine samples were collected from asymptomatic individuals as well as symptomatic patients during the time period of 2016-2018. The study protocol was approved by the institutional ethical committee. The Informed consent was obtained from all patients for being included in this study.

2.4.2 Processing of clinical samples

200 fresh midstream urine samples were aseptically collected in sterile containers. Then from each container 1mL of samples were taken & mixed with 2mL of LB broth in separate tubes and kept in a shaker incubator for overnight at 37°C for optimum microbial growth. The samples which showed

significant microbial growth (≥10^5 cfu/mL) after overnight incubation **(Fig. 2.1)** were selected for this study. The growth positive samples were carefully spread on nutrient agar plates using glass spreader and incubated for overnight at 37°C. Different single bacterial colonies were picked from the nutrient broth agar plates using inoculating loop. Then from them glycerol stocks of the samples were made and kept at -20°C for further analysis. Moreover, each individual isolate was subjected to Gram staining for identification of Gram-negative bacteria.

(a) **(b)**



https://www.chegg.com/homework-help/questions-and-answers/transfer-bacteria-broth-broth-liquid-incubation-turbidity-nutrient-broth-inoculated-e-coli-q65428265

Fig. 2.1: LB (a) Control; before inoculation and (b) After inoculation; showing microbial growth.

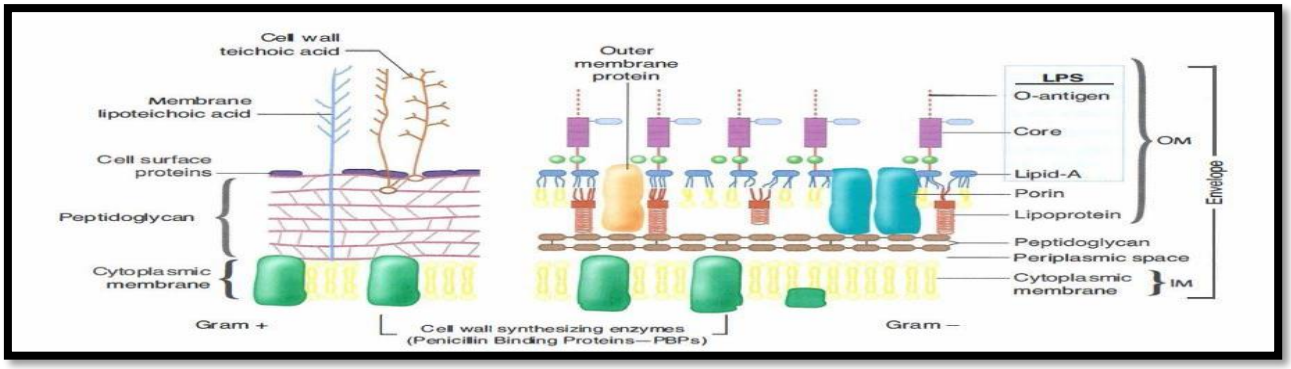
2.4.3 Gram staining

Gram staining, also called Gram's method, is a procedure of staining used to categorize bacterial species into two large groups: Gram-positive bacteria and Gram-negative bacteria. The name comes from the Danish bacteriologist Hans Christian Gram, who had developed the aforementioned technique in 1884. Gram staining in most cases the initial step in the preliminary identification of a bacterial organism. Gram staining is a valued diagnostic tool in both clinical and research settings (https://en.wikipedia.org/wiki/Gram_stain).

(a) Principle of Gram staining: The differences in cell wall structure and composition of bacteria are the basis of Gram staining and differentiation. Decolourization of primary stain and appearance of violet or purple colour will be resisted by bacteria having cell walls with a thick peptidoglycan layer. However, bacteria having a thin layer of peptidoglycan with lesser cross-linkage lose primary stain during decolorizing and gain counter stain thereby appearing pink or red.

The molecules of the dye crystal violet in aqueous solution dissociate into CV^+ and Cl^- ions. These ions effortlessly infiltrate the cell wall components of both gram-positive and negative bacteria. The CV^+ ion intermingles with negatively charged cell wall components. However, when the mordant, Gram's Iodine is added, the iodine (I^- or I^{-3} ion) intermingles with CV^+ ion forming the CV-I complex inside the cytoplasm and cell membrane along with the cell wall layers.

Then addition of the decolorizing solution (ethanol or a mixture of ethanol and acetone) causes interaction with lipids in the cell wall. The peptidoglycan layer is then revealed after the outer membrane of the Gram-Negative bacterial cell wall is dissolved. This peptidoglycan layer is typically thin with a smaller amount of cross-linking in the case of Gram-Negative cell wall, thereby becoming leaky. This causes cells' CVI complexes to lose mostly. However, in the case of the Gram-Positive bacteria, there is absence of outer membrane, and the peptidoglycan layer is also thick with the higher cross-linkage which causes the decolorizing solution to dry up the peptidoglycan layer. This traps all the CVI complexes within the cell wall and the bacteria hold the purple or violet color of crystal violet dye (https://microbenotes.com/gram-stain-principle-reagents-procedure-and-result-interpretation/).



https://microbeonline.com/wp-content/uploads/2013/08/Bacterial-cell-wall.jpg

Fig. 2.2: Principle of Gram staining

However, after addition of the counterstain, that is positively charged safranin, it interacts with the free negatively charged components in Gram-Negative cell wall and membrane and bacteria becomes pink/red. Nonetheless, there is no space to pass within the dehydrated Gram-Positive cell wall due to CVI complex and dehydration. Hence, safranin cannot stain them pink or red and Gram-Positive bacteria expose the purple or violet colour **(Fig. 2.2)** (https://microbenotes.com/gram-stain-principle-reagents-procedure-and-result-interpretation/).

(b) Gram staining reagents: Gram staining procedure uses different chemicals and dyes that can be grouped as under:

- **Primary stain (Crystal violet):** This is a deeply purple-coloured organic compound chemically called triphenylmethane dye. This is also known as hexamethyl pararosaniline chloride or methyl violet 10B or gentian violet. Its colour hinge on the pH of the dissolving medium such

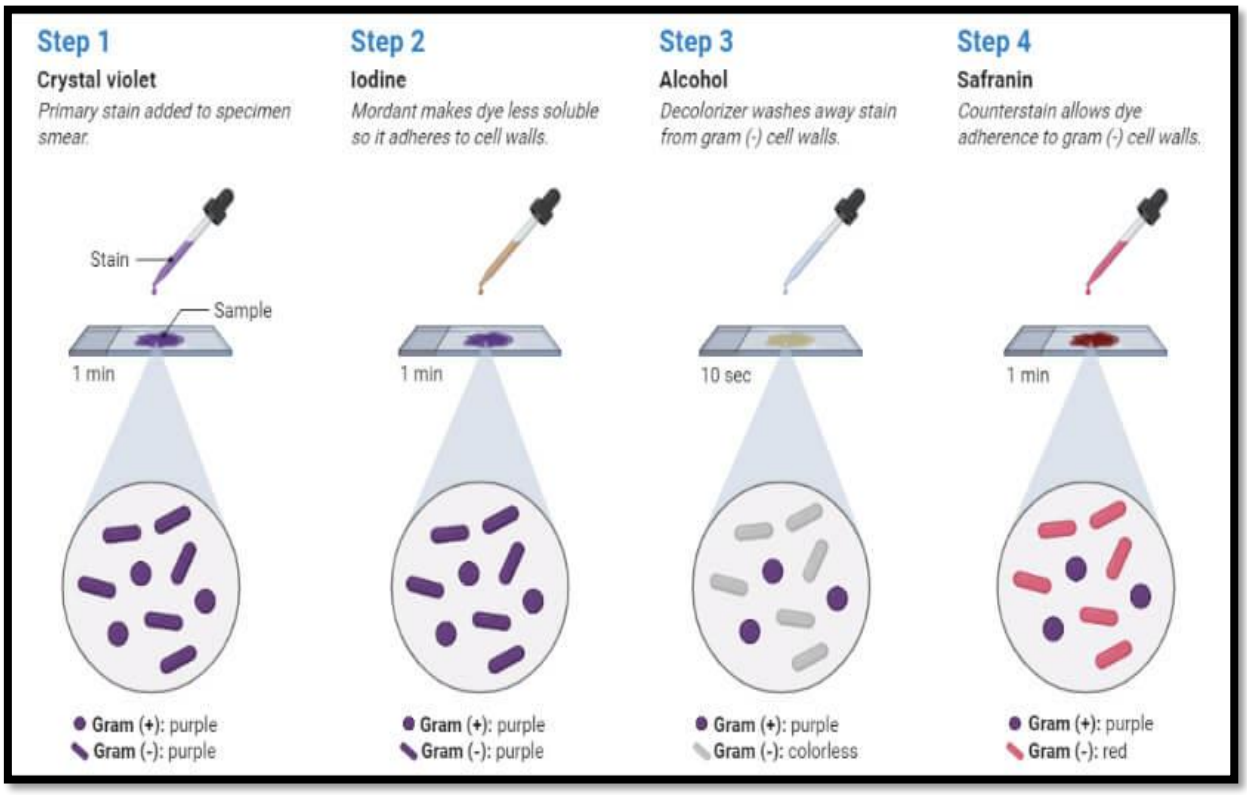
as, at pH -1.0 or less, it seems yellow, and at acidic pH of 1 to 2 it appears green, at neutral pH, it looks purple (deep blue-violet), and at very basic pH it appears colourless.

Staining of textiles, papers, and fibers, in ball pens, and chemicals like detergents, fertilizers, etc are done using CV. This is used for staining histological slide, bacteria, staining, DNA staining, etc. in microbiology and molecular biology. CV also displays antibacterial and antifungal properties, so used in sterilization and disinfection. In Gram Staining, it is used as a basic dye in the ionized form of CV+ and Cl-. It provides violet colour to Gram-Positive bacteria **(**https://microbenotes.com/gram-stain-principle-reagents-procedure-and-result-interpretation/)

- **Mordant (Gram's iodine):** This is an aqueous solution of iodine and potassium iodide used in Gram staining. It intermingles with CV+ and a CVI complex is formed that gets trapped in the parched peptidoglycan layer of the Gram-Positive cell wall.
- **Decolorizing solution:** Either acetone or ethanol (95%) is used. A mixture of acetone and ethanol in ratio 1:1 by volume is also used. This solution dissolves raises the permeability of the outer membrane of the Gram-Negative cell wall by dissolving its lipid content. However, in the case of the Gram-Positive cell wall the decolourizer desiccates the peptidoglycan layer and traps the CVI complex inside the cell.
- **Counter stain (Safranin):** This is a red-colored counter stain required to stain decolorized Gram-Negative cells. It is a basic dye that interacts with negatively charged cell wall and cell membrane components. In addition to safranin, dilute carbol fuchsin solution is also used as a counter stain (https://microbenotes.com/gram-stain -principle- reagents-procedure-and-result-interpretation/).

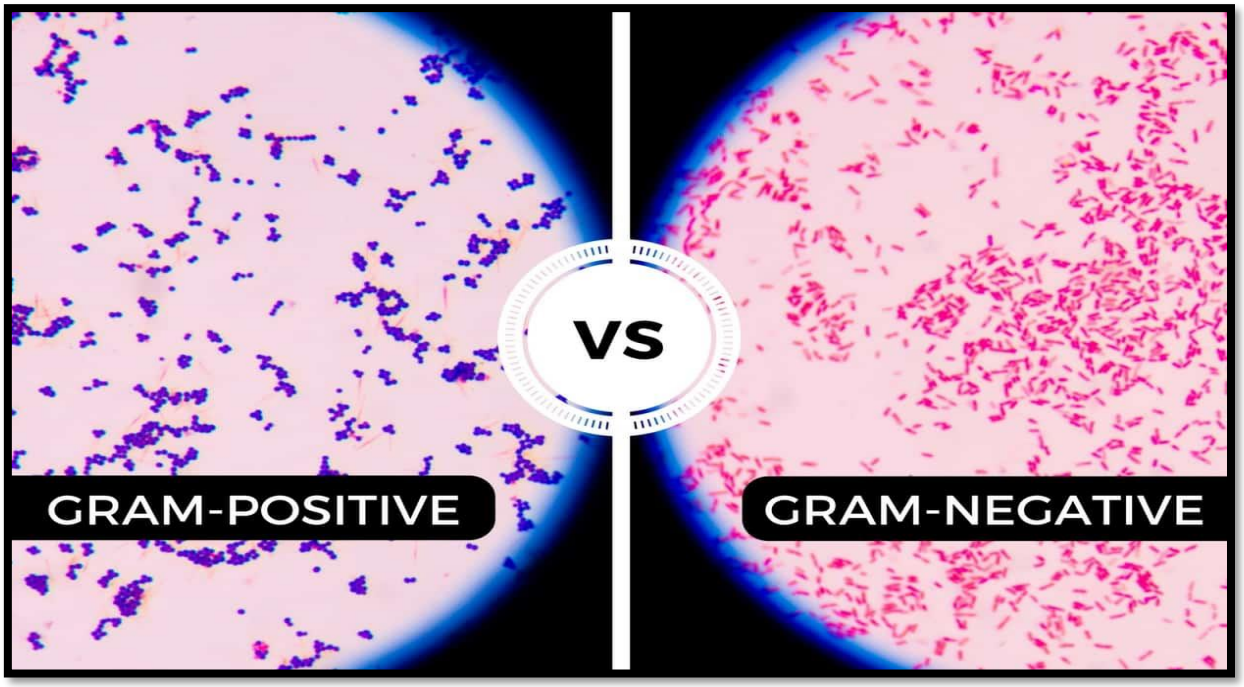
(c) Procedure of Gram staining: Gram staining procedure **(Fig. 2.3)** was performed with the single colony isolated from the nutrient broth agar plate to affirm the presence of Gram-negative bacteria in the case of each of 200 isolated samples. A small drop of the respective bacteria cultures was placed at the edge of each slide. Then a smear was drawn with an inoculating loop to create a thin film on the slide and the suspension was allowed to dry. The smear drawn was neither too thick nor too thin. The slide was heat-fixed over a spirit lamp flame. Then the entire smear was covered with Crystal violet stain for 1 min followed by washing of the stain under gentle running tap water. Then the smear was covered with Gram's iodine for 2 mins and the slide was rinsed with 95% ethanol for 10-30 seconds and further the slide was rinsed with water to stop further decolourization. The smear was then counterstained with safranin for 30 seconds to 1 min. The slide was rinsed with water and dried. The remaining water was blotted with tissue paper and then the slide was observed under a compound binocular microscope to see if Gram-negative bacteria were present among the collected samples. A

blue or purple-colored sample is indicative of Gram-positive bacteria while a red or pink colored sample is indicative of Gram-negative bacteria **(Fig. 2.4)**.



https://microbenotes.com/gram-stain-principle-reagents-procedure-and-result-interpretation/

Fig. 2.3: Procedure of Gram staining.



https://www.onlinebiologynotes.com/difference-between-gram-positive-and-gram-negative-bacteria/

Fig. 2.4: Microscopic view of gram-positive and gram-negative bacteria.

2.4.4 Growth on MacConkey agar plates

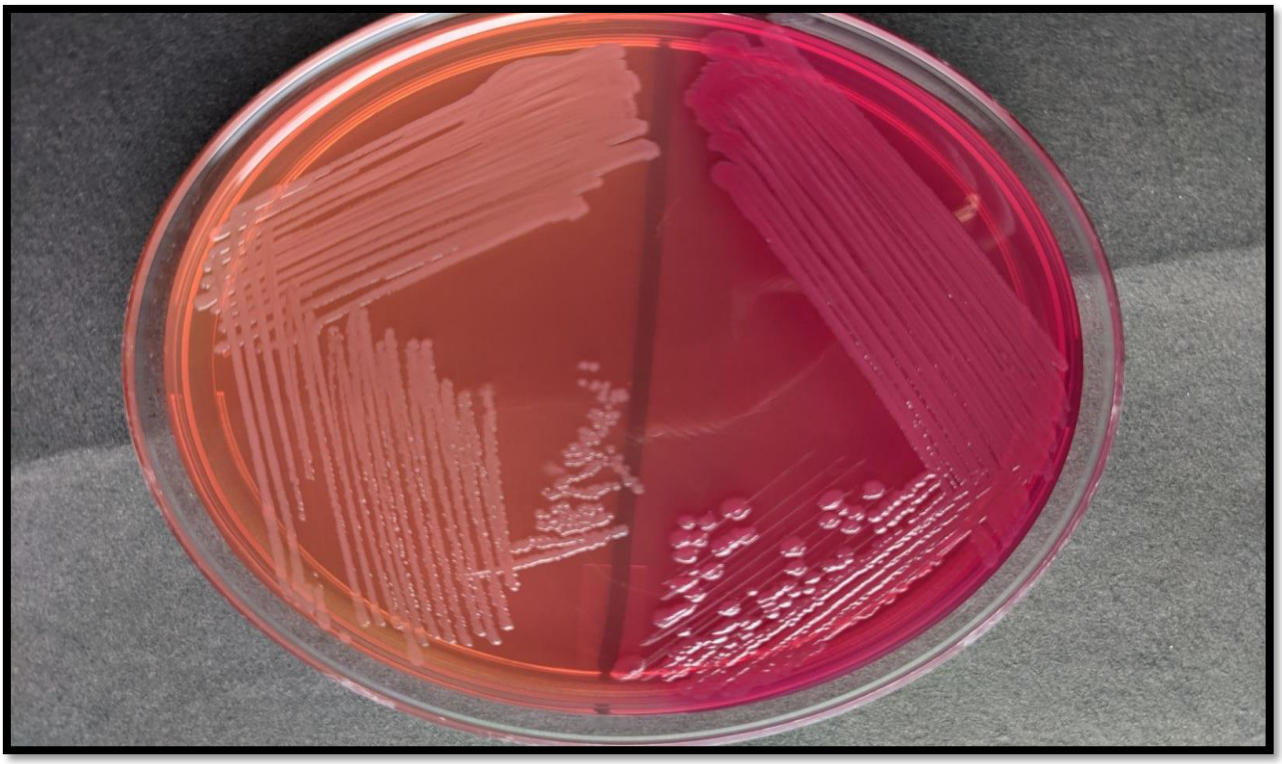
(a) Principle of MacConkey agar: MacConkey agar contains four main ingredients (lactose, bile salts, crystal violet, and neutral red) making it a selective and differential media. Selective agents such as bile salts and crystal violet inhibit the growth of Gram-positive organisms, and proliferate the selective growth of Gram-negative bacteria. Lactose serves as a source of carbohydrate. Lactose-fermenting bacteria produce pink-red colonies **(Fig. 2.5)**, after fermenting the lactose to acids and dropping the pH of the indicator **(**neutral red**)** present in the medium. Since, non-fermenters can't utilize lactose, colonies look colourless or transparent **(Fig. 2.5) (https://microbeonline.com/macconkey-agar-mac-composition-preparation-uses-and-colony-characteristics/#:~:text=Pink%2Dred%20%20colonies%3A%20Pink%2D,%2C%20Citrobacter%2C%20Enterobacter%2C%20etc).**

Other ingredients such as enzymatic digest of gelatin, casein, and animal tissue provide nitrogen, vitamins, minerals, and amino acids vital for growth. Sodium chloride offers osmotic balance and supplies important electrolytes for transport. Agar is unified as the solidifying agent. Gram-negative enteric bacteria which grow on MacConkey agar are differentiated by their capability to ferment lactose. The production of the acid drops the pH of the media if the lactose is fermented by the bacteria. The drop in pH is specified by the change of neutral red indicator to pink (neutral read appears pink at pH's below 6.8).

The sufficient acid which causes precipitation of the bile salts around the growth is produced by the strongly lactose fermenting bacteria. It looks like a pink halo neighboring colonies or areas of confluent growth. Pink halo is not seen around the colonies of weaker lactose fermenting bacteria. Gram-negative bacteria that grow on MacConkey agar but do not ferment lactose appear colourless on the medium and the agar surrounding the bacteria remains relatively transparent. (https://microbeonline.com/macconkey-agar-mac-composition-preparation-uses-and-colony-characteristics/#:~:text=Pink%2Dred%20%20colonies%3A%20Pink%2D,%2C%20Citrobacter%2C%20Enterobacter%2C%20etc)

- **Pink-red colonies:** Pink-red colonies on MacConkey agar indicate the presence of lactose fermenting bacteria. Examples include *Escherichia coli, Klebsiella* spp, *Citrobacter* spp, *Enterobacter* spp, etc.
- **Colourless colonies/pale colonies (colonies similar to the colour of the media):** Colourless or pale colonies indicate that the test organism is a non-lactose fermenter. Examples include species of *Salmonella* spp, *Shigella* spp, *Proteus* spp, *Providencia* spp, *Pseudomonas* spp, *Morganella* spp, etc https://microbeonline.com/macconkey-agar-mac-composition-preparation-uses-and-colony

characteristics/#:~:text=Pink%2Dred%20%20colonies%3A%20Pink%2D,%2C%20Citrobacter%2C%20Enterobacter%2C%20etc



https://microbeonline.com/macconkey-agar-mac-composition-preparation-uses-and-colony-characteristics/

Fig. 2.5: Growth of Gram-negative bacteria on MacConkey agar plates. Pale colonies on the left-hand side demonstrate non-lactose fermenters and pinkish red colonies on the right hand side indicate lactose fermenters.

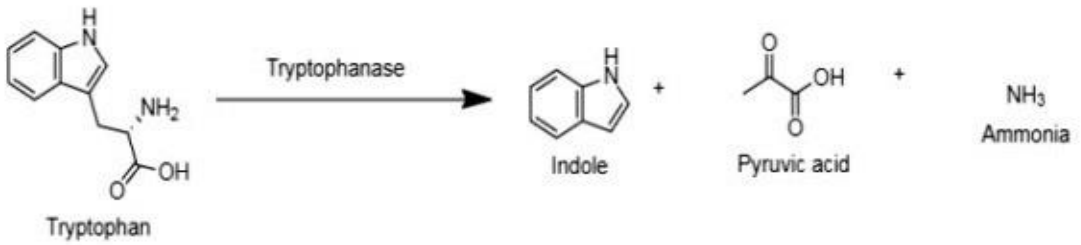
(b) Procedure of growth on MacConkey agar plates: The culture positive isolates that showed presence of Gram-negative bacteria were subjected to growth on MacConkey agar plates. The single colonies isolated from nutrient broth agar plate (that grew after overnight incubation in LB broth and which contained Gram-negative bacteria as confirmed by Gram Staining) were subjected to streaking on MacConkey agar plates. This was done by using a sterile inoculating loop dipped in each culture and then streaking on MacConkey agar plates. The plates were then incubated overnight at 37°C and the plates were observed for appearance of pinkish red colonies indicative of Gram-negative lactose fermenting bacteria which could possibly be *E. coli*, *Klebsiella* spp, *Citrobacter* spp, *Enterobacter* spp, etc. Then plates with pinkish colonies were sealed with parafilm to prevent contamination and stored at 4°C for future analyses. The pinkish red colonies with flat, dry, pink, non-mucoid structure with a surrounding darker pink area of precipitated bile salts mainly indicate *E. coli* and colonies that typically appear large, mucoid, and pink, with pink- red pigment, usually diffusing into the surrounding agar indicate *Klebsiella* spp. However, further tests are required for confirmation a particular type of bacterium.

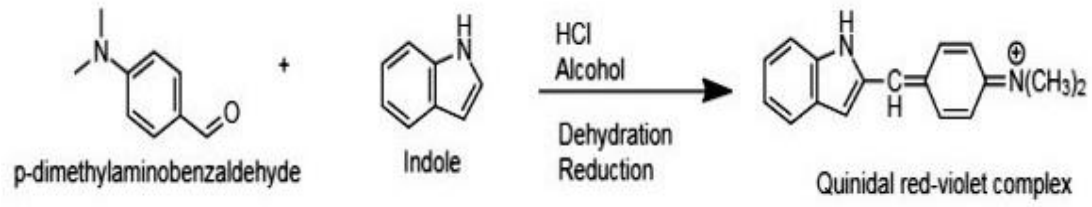
2.4.5 Biochemical analysis

In this study, the particular biochemical tests used to identify Gram-negative lactose fermenting bacteria *E. coli* were performed. Therefore, this study used IMViC and TSI as biochemical tests for confirmed identification of *E. coli*.

(a) IMViC: IMViC reactions are a set of four valuable reactions that are commonly used in the identification of members of family Enterobacteriaceae. Each of the letters in "IMViC" stands for one of these tests. **"I"** is for indole; **"M"** is for methyl red; **"V"** is for Voges-Proskauer, and **"C"** is for citrate, lowercase **"i"** is added for the ease of pronunciation (https://microbeonline.com/imvic-tests-principle-procedure-and-results/).

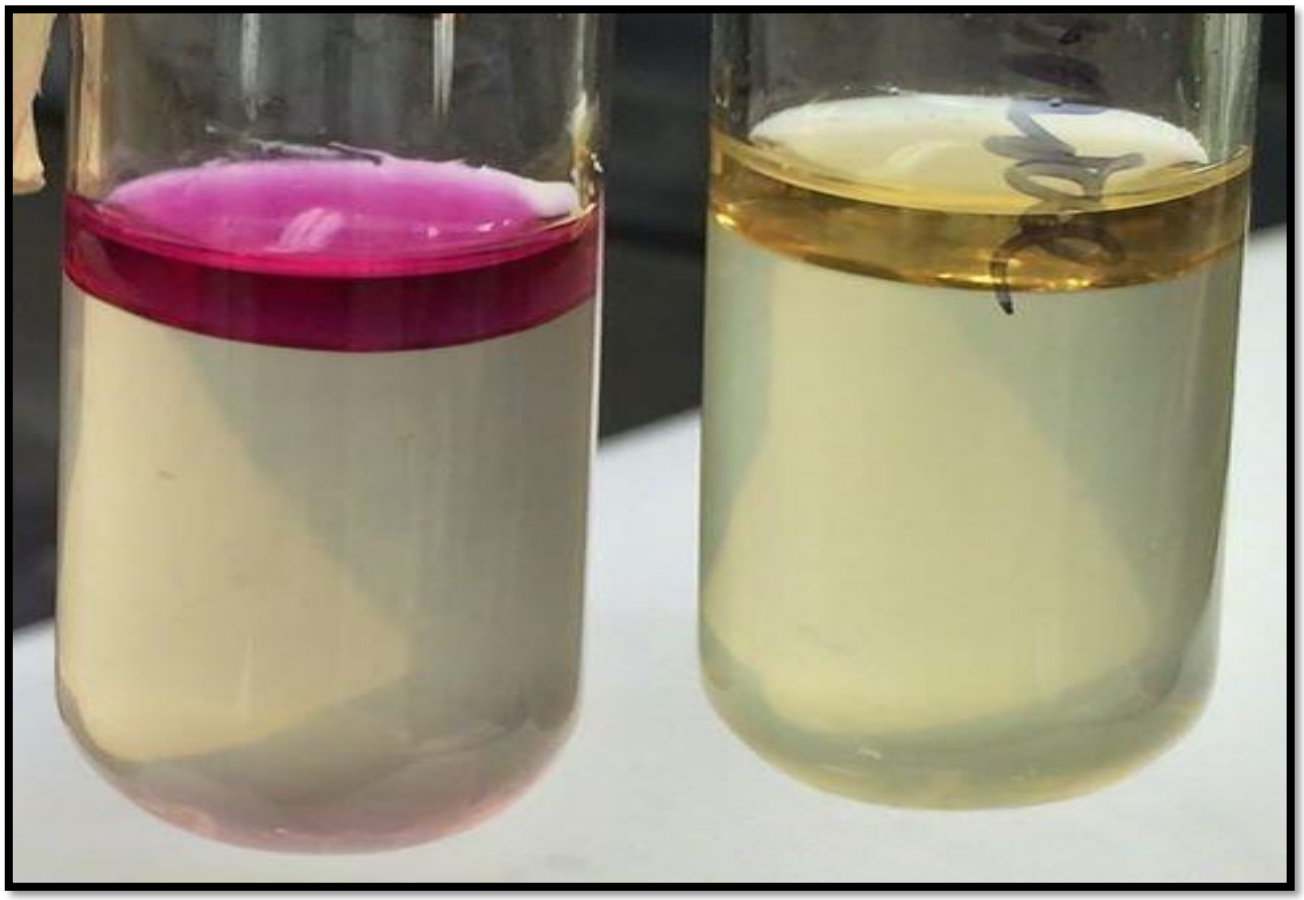
- **Indole test:** Indole test is a biochemical test steered on bacterial species to detect their ability to produce indole from tryptophan in the presence of a group of enzymes called 'tryptophanase'.
  - **Principle:** The capability of an organism to split indole from the amino acid tryptophan is due to the presence of tryptophanase. Amino acid tryptophan undergoes deamination and hydrolysis in the presence tryptophanase enzyme. Reductive deamination of tryptophan causes the production of indole via the intermediate molecule indole pyruvic acid. The tryptophanase catalyzes the removal of the amino group ($-NH_2$) from the tryptophan molecule during the deamination process. The enzyme needs pyridoxal phosphate as a coenzyme. The ultimate products of the catalysis reaction are indole, pyruvic acid, ammonium (NH_4^+), and energy. Indole, when present, associates with the aldehyde in the reagent to yield a pink to red-violet quinoidal compound (benzaldehyde reagent) or a blue to green color (cinnamaldehyde reagent). The indole is combined with Kovac's reagent (hydrochloric acid and p-dimethylaminobenzaldehyde in amyl alcohol) solution to form a yellow or cherry red coloration. The water-insoluble amyl alcohol forms a red-colored oily layer at the top of the broth. In the rapid spot test, indole is spotted straight from a colony growing on a medium rich in tryptophan. The indole associates with the p-dimethylaminocinnamaldehyde (DMACA) existing on the filter paper at an acid pH to produce a blue to the blue-green compound (https://microbenotes.com/indole-test-objective-principle-media-procedure-and-results/).





(http://dspace.bracu.ac.bd/xmLui/bitstream/handle/10361/11077/14146032_PHR.pdf?sequence=1&isAllowed=y).

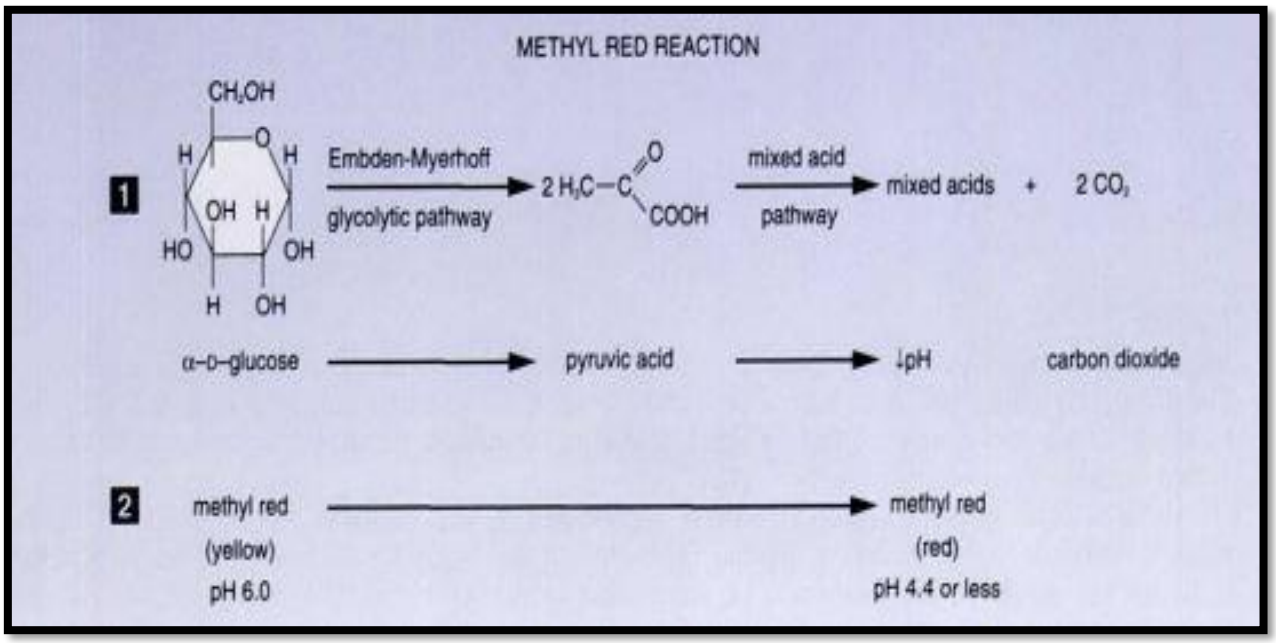
- **Procedure:** 5 µl of those samples that showed Gram negative property and also gave pinkish red colonies in MacConkey Agar plates were inoculated in 1 mL LB broth separately and incubated overnight at 37°C for optimum bacterial growth. 30 µl from each sample culture was inoculated in 2mL of tryptone broth and incubated at 37°C for overnight. After incubation, 100µl of Kovac's reagent was added to each test tube. Appearance of cherry red colour ring at the top of the tube indicated a positive result. A negative result appeared as yellow.
- **Examples:** *E. coli*: Positive; *Klebsiella pneumonia* (*K. pneumonia*)*:* Negative **(Fig. 2.6)**.



https://microbiologie-clinique.com/indole-test-en.htmL

Fig. 2.6: Indole positive and negative results. Left (+) and Right (-).

- **Methyl red (MR) test:** Methyl Red (MR) test governs whether an organism makes mixed acid fermentation and produces stable acid end products. MR indicator is used to actuate the pH after an enteric Gram-negative rod has fermented glucose to completion.
  - **Principle:** Three acids (acetic, lactic, and succinic/formic) are formed in substantial amounts in mixed acid fermentation, decreasing the pH of the medium below 4.4. This is apprehended by using a pH indicator, methyl red (p-dimethylaminoazeobenzene-O-carboxylic acid) pH indicator that is red at pH ≤ 4.4, and yellow colour at pH 5.8. The pH at which methyl red senses acid is noticeably lesser than the pH indicators used in bacteriologic culture media. Thus, to produce a colour change, the test organism must yield great amounts of acid from the provided carbohydrate source (https://microbeonline.com/methyl-red-mr-test-principle-procedure-results/) **(Fig. 2.7)**.



https://microbeonline.com/methyl-red-mr-test-principle-procedure-results/

Fig. 2.7: Principle of MR test.

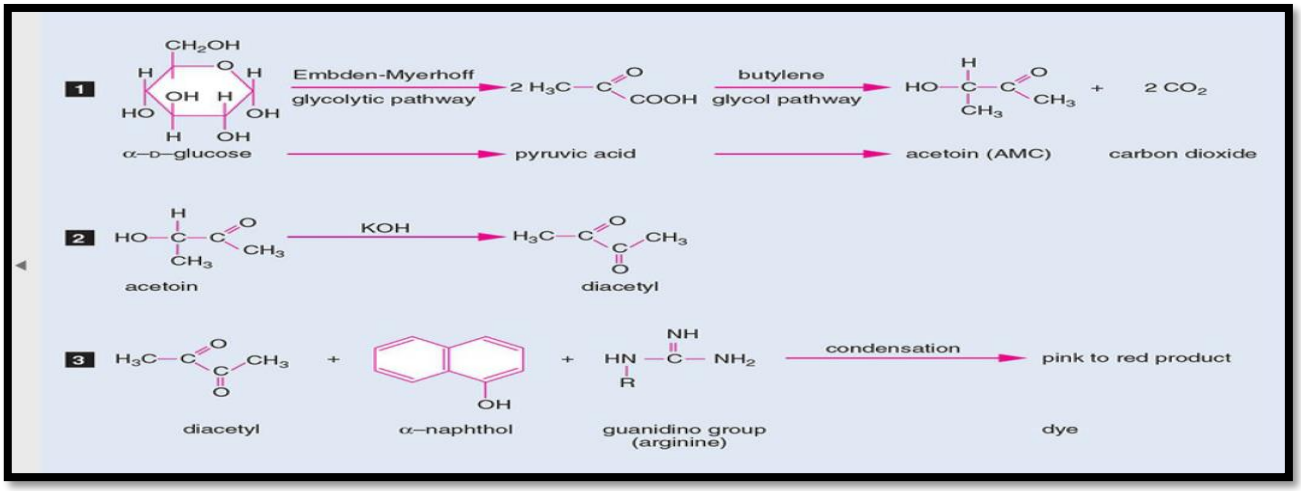
- **Procedure:** 5 µl of those samples which showed positive Gram-negative property and gave pinkish red colonies in MacConkey Agar plate were inoculated in 1 mL LB broth and incubated overnight at 37°C for optimum bacterial growth. Then 30 µl from each sample culture was inoculated in 2mL of glucose phosphate broth separately and incubated at 37°C for overnight. After incubation, few drops of methyl red indicator were added to the respective test tube. Appearance of red colour indicated acid production by the organism and thus a positive result. Presence of yellow colour or no colour change indicated a negative result.

➢ **Examples:** *E. coli*: Positive; *K. pneumonia*: Negative **(Fig. 2.8)**.

https://microbenotes.com/methyl-red-mr-test-objectives-principle-media-used-procedure-result-interpretation-limitations-and-examples/

Fig. 2.8: MR test positive and negative results.

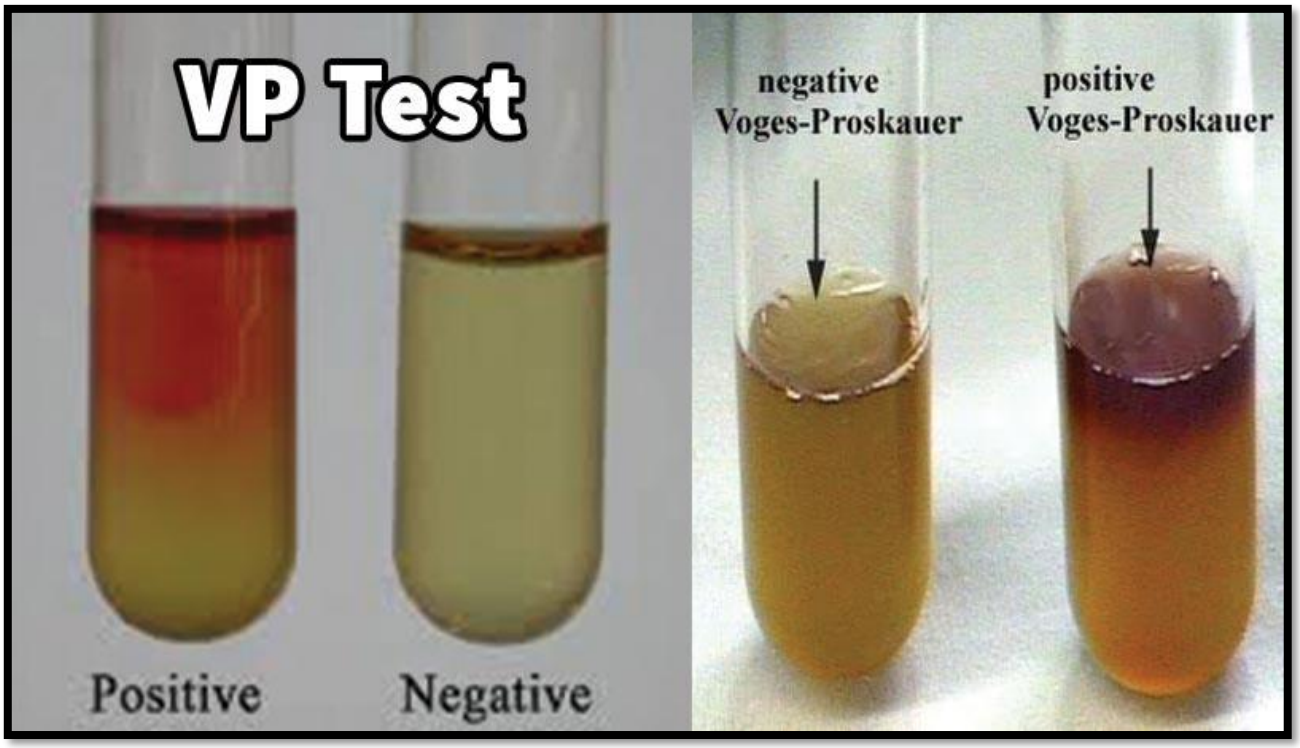
- **Voges-Proskauer (VP) test:** Voges and Proskauer, in 1898, first detected the production of a red colour after adding of potassium hydroxide to cultures grown on specific media. Harden later stated that the build-up of the red colour was a result of acetyl-methyl carbinol production. The test is made more sensitive in 1936 by Barritt by adding alpha-naphthol to the medium before adding potassium hydroxide (https://microbiologyinfo.com/voges-proskauer-vp-test-principle-reagents-procedure-and-result/).
  - ➢ **Principle:** VP test is used to find out if an organism produces acetyl methyl carbinol (acetoin) from glucose fermentation. If produced, acetyl methyl carbinol (acetoin) is changed to diacetyl in the presence of ∝- naphthol, strong alkali **(**40% KOH), and atmospheric oxygen. The original procedure did not have ∝-naphthol but was found to act as a colour intensifier by Barritt and should be added first. The diacetyl and quanidine-containing compounds identified in the peptones of the broth was found to condense to form a pinkish red polymer (https://microbiologyinfo.com/voges-proskauer-vp-test-principle-reagents-procedure-and-result/) **(Fig. 2.9)**.



https://microbeonline.com/voges-proskauer-test-principle-procedure-results/

Fig. 2.9: Principle of VP test.

- **Procedure:** 5 µl of those samples which showed positive Gram-negative property and gave pinkish red colonies in MacConkey Agar plate were inoculated in 1 mL LB broth and incubated overnight at 37°C for optimum bacterial growth. Then, 30 µl from each sample culture was inoculated in glucose phosphate broth and incubated at 37°C for overnight. After incubation, 0.6 mL of alpha naphthol (Barritt's reagent A) and 0.2 mL of 40% KOH (Barritt's reagent B) were added and the tubes were kept for 1 hr for maximum colour development. Appearance of pinkish red colour gave a positive result. The lack of pink-red colour gave negative result.
- **Examples:** *E. coli*: Negative; *K. pneumonia*: Positive **(Fig. 2.10)**.



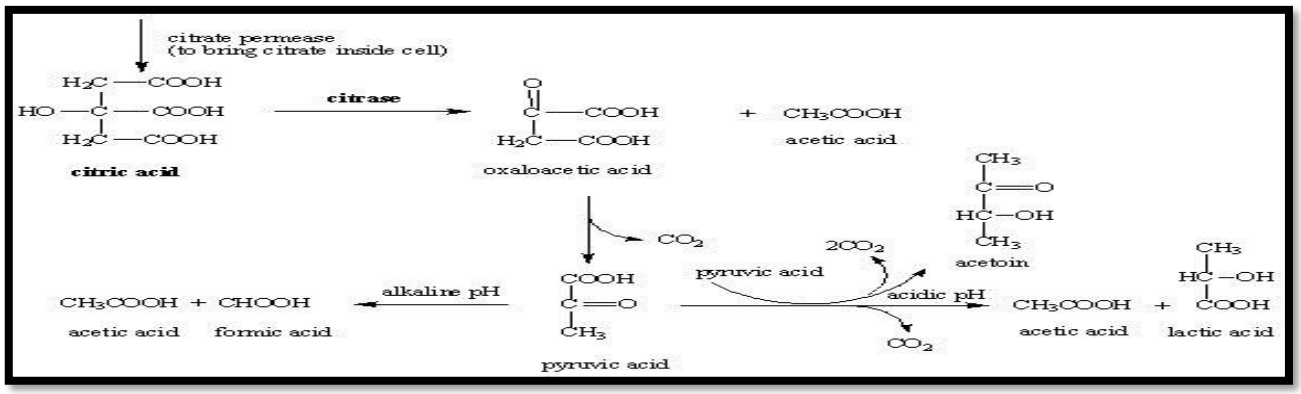
https://microbenotes.com/voges-proskauer-vp-test/

Fig. 2.10: VP test positive and negative results.

- **Citrate utilization test:** The citrate test is done with the other IMViC tests to distinguish Gram-negative bacilli of the Enterobacteriaceae family. It is an imperative test that allows the species-level detection of the members of the Enterobacteriaceae family. The test utilizes Simmon's citrate agar, therefore, also called Simmon's citrate test as it that contains citrate as the major source of energy (https://microbenotes.com/citrate-utilization-test-principle-procedure-and-result-interpretation/).
  - **Principle:** An organism's ability to utilize citrate as a source of energy is tested using Citrate agar. The medium contains citrate as the single carbon source and inorganic ammonium salts (NH4H2PO4) as the only source of nitrogen**.**

Bacteria growing on this medium produce an enzyme, citrate-permease, capable of performing citrate to pyruvate conversion. Pyruvate can then go into the organism's metabolic cycle for the energy production. Growth is suggestive of consumption of citrate, an intermediate metabolite in the Krebs cycle.

The ammonium salts are broken down to ammonia when the bacteria metabolize citrate which increases alkalinity. The shift in pH converts the bromthymol blue indicator in the medium from green to blue above pH 7.6 (https://microbiologyinfo.com/citrate-utilization-test-principle-media-procedure-and-result/) **(Fig. 2.11)**.



https://microbiologyinfo.com/citrate-utilization-test-principle-media-procedure-and-result/

Fig. 2.11: Principle of citrate utilization test.

- **Procedure:** 5 µl of those samples which showed positive Gram-negative property and gave pinkish red colonies in MacConkey Agar plate were inoculated in 1 mL LB broth and incubated overnight at 37°C for optimum bacterial growth. Then, an inoculation loop was dipped into LB overnight culture, followed by streaking onto Simmon's citrate agar slants. The tubes were inoculated at 37°C for overnight. Presence of growth with colour change from green to intense blue along the slant indicated a positive result whereas, no growth and no colour change that is the slant remaining green indicated a negative result.
- **Examples:** *E. coli*: Negative; *K. pneumonia*: Positive **(Fig. 2.12)**.

https://microbiologyinfo.com/citrate-utilization-test-principle-media-procedure-and-result/

Fig. 2.12: Citrate utilization test positive and negative results.

IMViC test interpretations of different Enterobacteriaceae were depicted in **Table 2.1**.

Table 2.1: IMViC test interpretations of different Enterobacteriaceae.

	I	M	Vi	C
Escherichia coli	+	+	−	−
Edwardsiella tarda	+	+	−	−
Proteus vulgaris	+	+	−	−
Klebsiella pneumoniae	−	−	+	+
Klebsiella oxytoca	+	−	+	+
Enterobacter spp.	−	−	+	+
Serratia marcescens	−	−	+	+
Citrobacter freundii	−	+	−	+
Citrobacter koseri	+	+	−	+

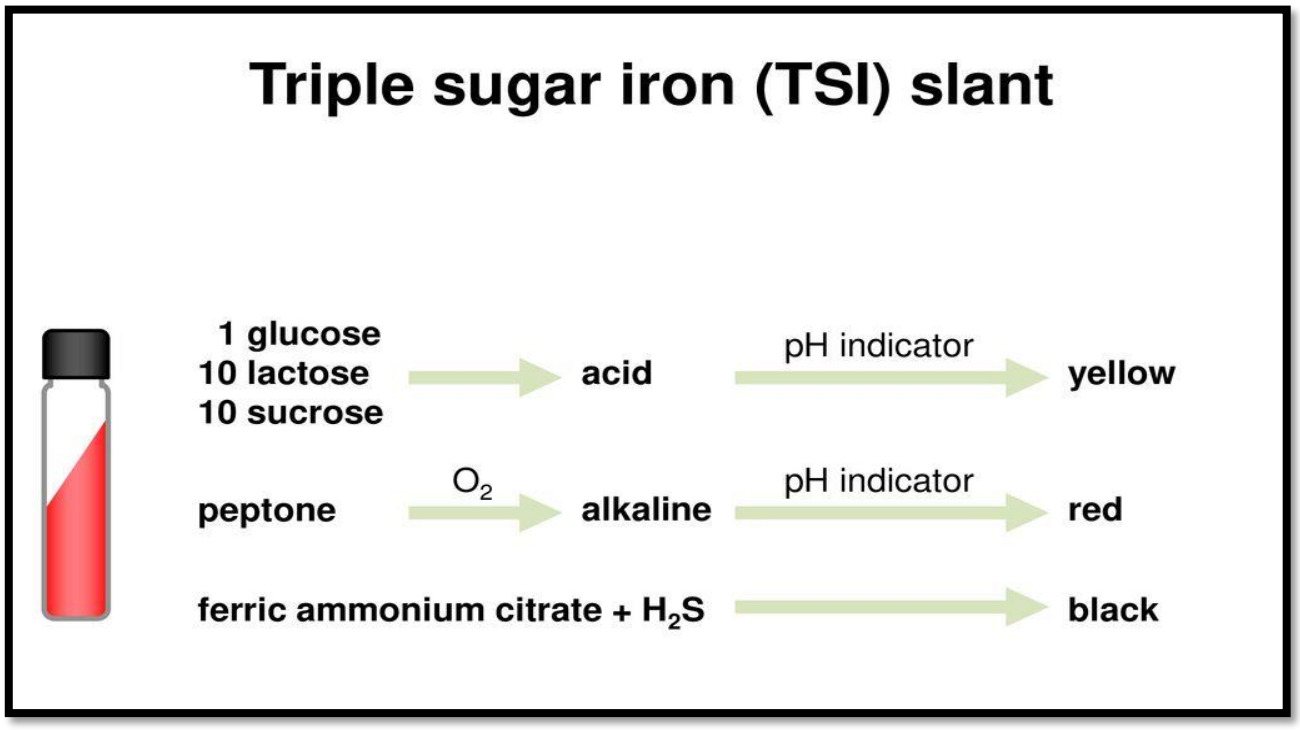
https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fthebiotechnotes.files.wordpress.com%2F2019%2F07%2F07da8a97-2267-4f44-be0b-d067f5561764.jpg%3Fw%3D616&imgrefurl=https%3A%2F%2Fthebiotechnotes.com%2F2019%2F07%2F05%2Fimvic-tests%2F&tbnid=uckIQVBLnBp82M&vet=12ahUKEwiMosnLk6j2AhUf73MBHXa_DmEQMygGegQIARAp..i&docid=6-EG-2852c9a7M&w=616&h=616&q=positive%20isolates%20were%20speciated%20by%20IMViC%20test%20observations&ved=2ahUKEwiMosnLk6j2AhUf73MBHXa_DmEQMygGegQIARAp#imgrc=uckIQVBLnBp82M&imgdii=mFZEBMzNpvi3ZM

(b) TSI test: The ability of a microorganism to ferment sugars and to produce hydrogen sulphide (H_2S) is tested using the Triple Sugar Iron (TSI) test. TSI agar was developed by Sulkin and Willet in 1940 and is a modification of Kliger's Iron agar (https://en.wikipedia.org/wiki/TSI_slant#:~:text=The%20Triple%20Sugar%20Iron%20(TSI,bacteria%20including%20Salmonella%20and%20Shigella.).

- **Principle:** The TSI test using Triple Sugar Iron Agar is considered to differentiate among organisms on the basis of the differences in carbohydrate fermentation patterns and hydrogen sulfide production. Carbohydrate fermentation is specified by the production of gas and a variation in the colour of the pH indicator from red to yellow.

  In order to ease the observation of carbohydrate utilization patterns, TSI Agar comprises three fermentative sugars, lactose and sucrose in 1% concentrations and glucose in 0.1% concentration. The pH falls due to the building of acid during fermentation. The detection of carbohydrate fermentation that is specified by the change in colour of the carbohydrate medium from orange red to yellow in the presence of acids is done by incorporating the acid base indicator Phenol red. In the case of oxidative decarboxylation of peptone, the pH rises due to build up of the alkaline products. This is specified by the alteration in colour of the medium from orange red to deep red. The production of hydrogen sulfide is detected by the Sodium

thiosulfate and ferrous ammonium sulfate present in the medium. This is indicated by the black colour in the butt of the tube **(Fig. 2.13)**.



https://slideplayer.com/slide/17423304/

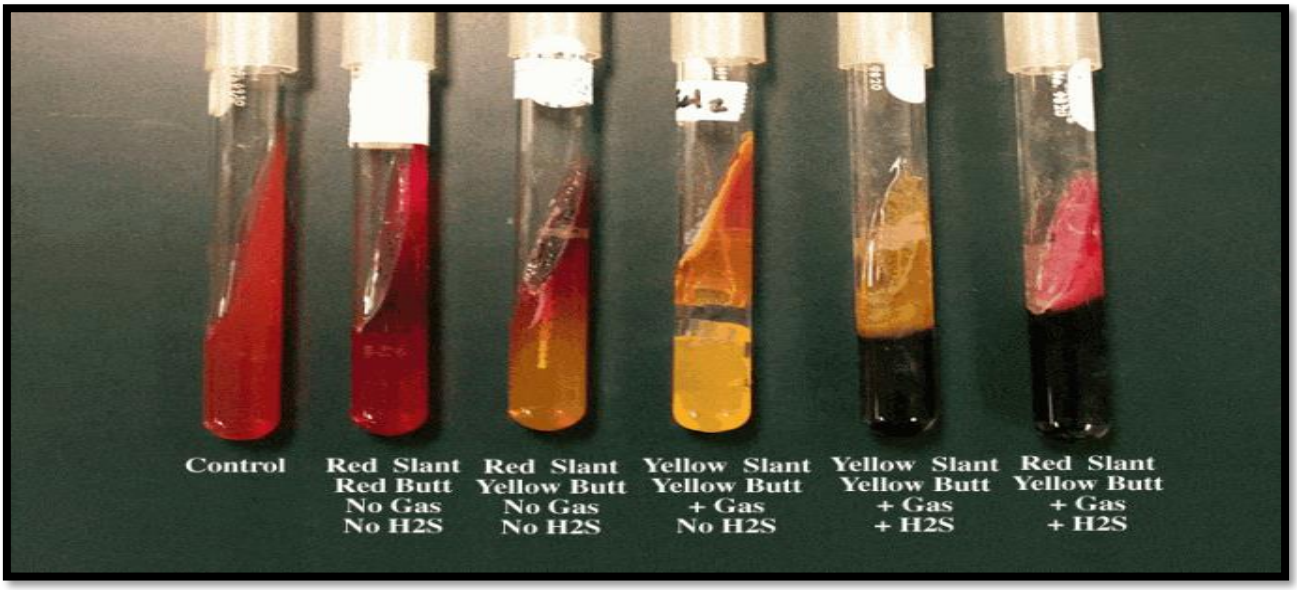
Fig. 2.13: Principle of TSI test.

To enable the finding of organisms that only ferment glucose, the glucose concentration is made one-tenth the concentration of lactose or sucrose. The inadequate amount of acid production in the slant of the tube throughout glucose fermentation oxidizes quickly, causing the medium to keep on orange red or return to an alkaline pH. However, the acid reaction (yellow) is sustained in the butt of the tube as it is below lower oxygen tension. After exhaustion of the limited glucose, organisms capable of doing so will begin to use the lactose or sucrose. To increase the alkaline condition of the slant, free exchange of air should be allowed by closing the tube cap loosely (https://microbiologyinfo.com/triple-sugar-iron-tsi-test/).

- **Procedure:** 5 µl of those samples which showed positive Gram-negative property and gave pinkish red colonies in MacConkey Agar plate were inoculated in 1 mL LB broth and incubated overnight at 37°C for optimum bacterial growth. Then, an inoculation loop was dipped into LB overnight culture, followed by stabbing into the butt and streaking onto the slant of TSI agar test tube. The test tubes were then incubated overnight at 37°C. Only dextrose-fermenting organisms produced an alkaline (red) slope and an acid (yellow) butt. However, acid (yellow) slant and acid (yellow) butt were produced by organisms capable of digesting dextrose, lactose, and/or sucrose. The H2S produced and the resulting black precipitate could sometimes hide the acidity reaction. However, to avoid this, the color reaction could be evaluated early in the incubation

period (after18 hours). Strict aerobes, such as *Pseudomonas aeruginosa*, would only grow on the slant of the tube rather than the butt, resulting in no change in the color of the tube's butt. Gas would be produced as a by-product of several metabolic cycles during carbohydrate and peptone degradation, and it appeared as gas bubbles or fractures in the medium. Some organisms, such as *E. coli*, might release an excessive amount of gas (CO2 & H2), causing the media to be fully displaced to the tube's top; hence, caution should be exercised when handling these tubes. Another technique of distinction was based on the generation of hydrogen sulfide (H2S). A black precipitate (ferrous sulfide) in the medium or a black ring around the tube's top indicated a positive H2S reaction

- **Expected Results:** Different expected observations **(Fig. 2.14)** are illustrated below.
- **Examples:** TSI test result interpretation of different Enterobacteriaceae were tabulated **Table 2.2** as under:



https://microbeonline.com/triple-sugar-iron-agar-tsi-principle-procedure-and-interpretation/

Fig. 2.14: TSI test positive and negative results.

Table 2.2: TSI test interpretations.

Organisms	Growth
Salmonella enterica	**Growth; red slant, yellow butt, gas positive, black-butt (H_2S produced)**
Escherichia coli	**Growth; yellow slant, yellow butt, gas positive, no H_2S produced**
Pseudomonas aeruginosa	**Growth; red slant, red butt, no gas, no H_2S produced**

Shigella sonnei	**Growth; red slant, yellow butt, no gas, no H_2S produced**
Citrobacter freundii	**Yellow slant, yellow butt, gas production; positive reaction for H_2S Blackening of medium**
Enterobacter aerogenes	**Yellow slant, yellow butt, gas production; no H_2S produced**
Klebsiella pneumoniae	**yellow slant, yellow butt, gas positive, no H_2S produced**
Proteus vulgaris	**Red slant, yellow butt, no gas production; H_2S produced**
Salmonella Paratyphi A	**Red slant, yellow butt, gas production; no H_2S produced**
Salmonella Typhi	**Red slant, yellow butt, no gas production; H_2S produced**
Salmonella Typhimurium	**Red slant, yellow butt, gas production; H_2S produced**
Shigella flexneri	**Red slant, yellow butt, gas negative, H_2S not produced**

https://microbenotes.com/triple-sugar-iron-tsi-agar/

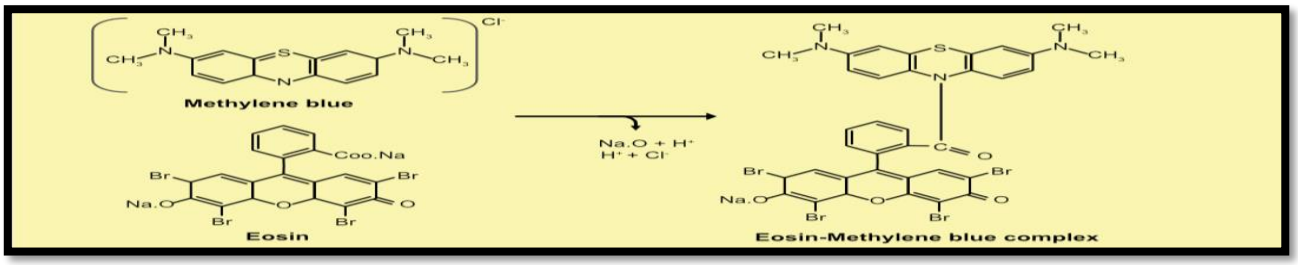
(c) EMB agar test: Eosin Methylene Blue (EMB) agar is a differential microbiological medium that to some extent inhibits the growth of Gram-positive bacteria and offers a color indicator differentiating between organisms that ferment lactose (e.g., *E. coli*) and those that do not (e.g., *Salmonella* spp, *Shigella* spp). Holt-Harris and Teague originally devised EMB agar and was further modified by Levine. It is therefore a combination of the Levine and Holt-Harris and Teague formulae that encompasses a peptic digest of animal tissue and phosphate as suggested by Levine and two carbohydrates as recommended by Holt-Harris and Teague (https://microbenotes.com/eosin-methylene-blue-emb-agar/).

- **Principle:** EMB agar comprises sucrose and lactose, used as fermentable carbohydrates substrates which boost the growth of some gram-negative bacteria, especially fecal and non-fecal coliforms. Differentiation of enteric bacteria is likely due to the presence of the sugars lactose and sucrose in the EMB agar and the capacity of certain bacteria to ferment the lactose in the medium (https://asm.org/ASM/media/Protocol-Images/Eosin-Methylene-Blue-Agar-Plates-Protocol.pdf?ext=.pdf). The medium is acidified by the lactose-fermenting gram-negative bacteria, which reduces the pH, and the dye produces a dark purple complex generally associated with a green metallic sheen. This metallic green sheen is indicative of vigorous lactose and/or sucrose fermentation capacity, typical of fecal coliforms **(Fig. 2.15)**

  Organisms that are slow lactose-fermenters yield less acid, and the colonies look brown-pink. Non-lactose fermenters upsurge the medium's PH by proteins deamination and produce colourless or light pink colonies.

  Eosin Y and methylene blue are pH indicator dyes that associate to form a dark purple

precipitate at low pH; they also assist to impede the growth of most Gram-positive organisms. Peptic digest of animal tissue acts as a source of carbon, nitrogen, and other vital growth nutrients. The medium is buffered by phosphate. (https://www.austincc.edu/microbugz/eosin_methylene_blue_agar.php#:~:text=Eosin%20methylene%20blue%20agar%20(EMB,of%20most%20Gram%20positive%20organisms.)

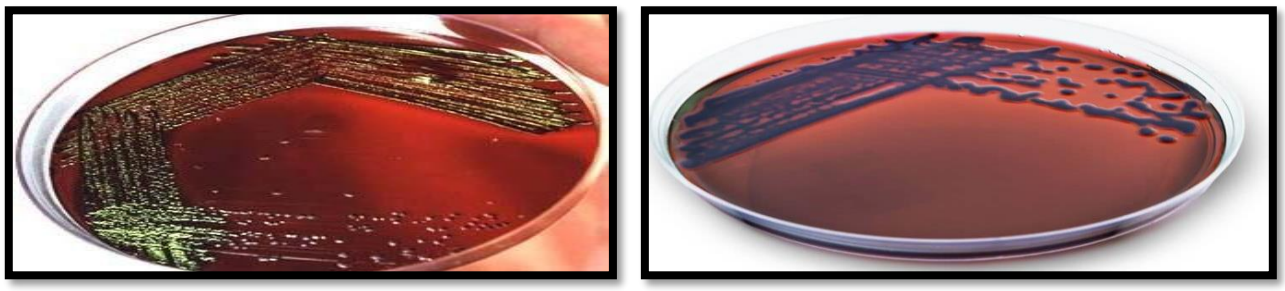


https://sharebiology.com/emb-agar-eosin-methylene-blue-agar/#gs.s4q621

Fig. 2.15: Principle of EMB agar test in strong lactose fermenters.

- **Procedure:** 5 µl of those samples which showed Gram-negative property and gave pinkish red colonies in MacConkey Agar plate, were inoculated in 1 mL LB broth and incubated overnight at 37°C for optimum bacterial growth. Then, an inoculation loop was dipped into LB overnight culture, followed streaking onto the EMB agar plates and incubated at 37°C. The results were analyzed after 18 to 24 hours of incubation.
- **Examples:** *E. coli*: Blue-black bull's eye; may have a green metallic sheen; *K. pneumonia*: Pink-purple mucoid colonies **(Fig. 2.16)**.

(a) **(b)**



https://www.researchgate.net/publication/306020855_Prevalence_of_Salmonella_and_Escherichia_coli_contamination_in_shrimp_Penaeus_monodon_farms_depots_and_processing_plants_in_different_areas_of_Bangladesh/figures?lo=1https://paramedicsworld.com/klebsiella-https://paramedicsworld.com/klebsiella-pneumoniae/morphology-culture-characteristics-of-klebsiella-pneumoniae/medical-paramedical-studynotes

Fig. 2.16: EMB agar test results (a) *E. coli* (b) *K. pneumonia*.

2.4.6 Storage of confirmed UPECs

UPECs were identified using the aforementioned tests from the culture positive urine samples collected from patients with ABU and symptomatic UTI admitted to the Carmichael Hospital for

Tropical Diseases in Kolkata. Then the selected UPEC isolates were again streaked on to Mac-Conkey-agar plates and incubated overnight at 37°C. Then the very next day single colonies were taken from each of the Mac-Conkey-agar plates using an inoculating loop and dipped into separate 2mL of LB broth and incubated for overnight at 37°C. 300 µl of the overnight cultures were mixed with 100 µl of 87% glycerol each (culture: glycerol = 3:1 ratio) to make glycerol stocks which were further stored at -80°C in 1.5mL cryogenic tubes for long-term storage.

2.4.7 Antibiotic susceptibility testing

Susceptibility of the isolated UPECs to different antibiotics were tested using the Kirby-Bauer disk diffusion method using Muller Hinton agar (Hi-Media, India) against regimen of 10 selected antibiotics; Ceftazidime (CAZ; 30µg), Cefotaxime (CTX; 30µg), Imipenem (IMP; 10µg), Amikacin (AK; 30µg), Gentamicin (GEN; 10µg), Tobramycin (TOB;10µg), Ciprofloxacin (CIP; 5µg), Levofloxacin (LE; 5µg), Co-trimoxazole (COT; 30µg), Nitrofurantoin (NIF; 300µg). All antibiotic discs were purchased from Hi-Media, India. The sensitivity test was standardized using *E. coli* ATCC 25922 strain. Inhibition zone size was interpreted using standard recommendation of Clinical and Laboratory Standards Institute (**CLSI 2018) Table 2.3**. Resistance against three or more than three groups of drug was designated as MDR **(Mukherjee et al. 2015)**.

Table 2.3: The range of zone of inhibition for different antibiotics.

Antibiotics	Diameter of zone of Inhibition (mm)		
	S	I	R
Ceftazidime (CAZ; 30µg)	≥ 21	18–20	≤ 17
Cefotaxime (CTX; 30µg)	≥ 26	23–25	≤ 22
Imipenem (IPM; 10µg),	≥ 23	20–22	≤ 19
Amikacin (AK; 30µg)	≥ 17	15-16	≤14
Gentamicin (GEN; 10µg)	≥15	13-14	≤12
Tobramycin (TOB;10µg)	≥15	13-14	≤12
Ciprofloxacin (CIP; 5µg)	≥21	16-20	≤15
Levofloxacin (LE; 5µg)	≥17	14-16	≤13
Co-trimoxazole (COT; 30µg)	≥16	11-15	≤10
Nitrofurantoin (NIT; 300µg)	≥ 17	15-16	≤14

[R – resistance; S – Susceptible and I – Intermediate] **(CLSI 2018)**

2.4.8 Phenotypic detection of ESBL production

All 40 UPECs selected for this study irrespective of their asymptomatic or symptomatic nature was found to be resistant to ceftazidime and cefotaxime. So, all the aforementioned isolates were screened for ESBL or BLIR phenotypes. 5µl of glycerol stock from each of the UPEC positive sample were inoculated in 2mL of the LB broth and incubated for overnight at 37°C. 150µl of the overnight culture was added to 3mL of MH broth and incubated at 37°C for 45 minutes to one hour to obtain the log phase growth of the microbes (over day culture). OD was measured at 600nm against 0.5 McFarland standards. From the over day cultures 10^7 CFU /mL of culture were spread in MH agar Petri plates and were kept for drying for 10 min. Phenotypic confirmatory test for ESBL producers were determined using drug and drug-inhibitor combinations; ceftazidime (CAZ) and ceftazidime-clavulanate (CAC; 30+10µg), cefotaxime (CTX) and cefotaxime-clavulanate (CEC; 30+10µg) disks. *E. coli* ATCC 25922 was used as a negative control. A ≥5 mm increase in the zone diameter of the drug-drug inhibitor combination marked the isolates to be ESBL producers. A difference of less than 5 mm between the zone of inhibition of a single disk and in combination with clavulanic acid (inhibitor) was assigned as BLIR **(CLSI 2018; Basu and Mukherjee 2018; Mukherjee et al. 2018)**.

2.4.9 Statistical analysis

The data were statistically analyzed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The chi-square test and the Fisher exact test were applied to compare categorical variables in terms of their resistance to different tested antibiotics. P values ≤ 0.05 were considered to be statistically significant **(Iranpour et al. 2015; Najafi et al. 2018)**. Paired t-test was performed using GraphPad Prism version 9 (GraphPad Software, La Jolla California USA) to determine the difference in the prevalence of men and women population among ABU and symptomatic patient with UPECs. P values ≤ 0.05 were considered to be statistically significant. Moreover, GraphPad Prism version 9 (GraphPad Software, La Jolla California USA) was also used to statistically compute the Mean with SEM (Standard Error of Mean) to determine the variability in zone of inhibition of different antibiotics from the population mean in both the studied group of isolates. Furthermore, the correlation coefficient was determined using the Prism software package (GraphPad Prism version 9) **(Parra et al. 2017)** and also further validated using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) (**Yadav et al. 2018**) to find the degree of association between the incidences of resistances against different antibiotics of 40 UPECs that comprised of 20 asymptomatic and symptomatic isolates respectively with regard to their antibiogram profile were also analyzed. Low (>0.3 to 0.5), moderate (>0.5 to 0.7), and high (> 0.7 to 1) positive correlations between antibiotics among the aforementioned group of isolates were also ascertained as indicated by **Yadav et al** (**Yadav et al. 2018**). Moreover, previous reports **(Yadav et al. 2018)** stated values ≤ 0.3 as negligible or poor correlation. Nevertheless, according to SPSS version

21.0, correlation coefficient values < 0.2 were found to be statistically non-significant. Therefore, correlation coefficient values ≤ 0.2 were not considered when ascertaining the highest and lowest correlations. However, values between 0.2 and 0.3 were only considered for analysis if they were found to be significant at ≤ 0.05 level. Furthermore, correlation graphs were constructed from the correlation matrices using the GraphPad Prism version 9 (GraphPad Software, La Jolla California USA).

2.5 Results

2.5.1 Relevant bacteriology

Out of the total 200 urine samples collected from hospitalized patients of Kolkata, West Bengal, India, 100 were asymptomatic (collected from patients with no classical symptoms of UTI) and 100 were symptomatic (collected from patients clinically with UTI) **(Fig. 2.17)**. Significant microbial growth ($\geq 10^5$ cfu/mL) **(Fig. 2.18a)** was observed in 107 out of the 200 urine samples **(Fig. 2.19)** isolated from asymptomatic (45/107) **(Fig. 2.20)** and symptomatic (62/107) individuals respectively **(Fig. 2.20)**. The presence of Gram-negative bacteria was observed among 30 and 33 of the urine culture-positive asymptomatic **(Fig. 2.21)** and symptomatic isolates respectively **(Fig. 2.21)**. Finally, biochemical detection revealed *E. coli* **(Fig. 2.18b)** in 20 out of the 45 **(Fig. 2.22)** of the culture-positive and 20 out of the 30 **(Fig. 2.23)** isolates with Gram-negative property collected from asymptomatic individuals respectively. Moreover, biochemical detection also revealed *E. coli* **(Fig. 2.18b)** in 20 out of the 62 **(Fig. 2.22)** of the culture-positive and 20 out of the 33 **(Fig. 2.23)** isolates with Gram-negative property collected from symptomatic patients respectively. Among 20 ABU UPECs, 10 and 10 were isolated from male and non-pregnant female individuals respectively **(Fig. 2.24)**. However, among symptomatic UPECs, 6 and 14 were isolated from male and non-pregnant female individuals respectively **(Fig. 2.24)**. The incidence of female patients with UPECs was found to be significantly higher (p-value ≤ 0.05) than males with UPECs. Furthermore, the mean age distribution was 44.8 years (range 22–82 years) and 48.2 years (range 7- 82years) among isolated ABU **(Fig. 2.25)** and symptomatic UPECs respectively **(Fig. 2.25)**. Withal, 12 out of the 20 symptomatic isolates were found to be associated with patients suffering from acute and/or chronic cystitis. The remaining 8 were isolated from patients suffering from pyelonephritis **(Table 2.4)**.

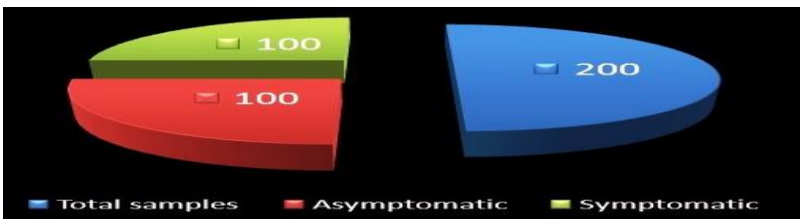


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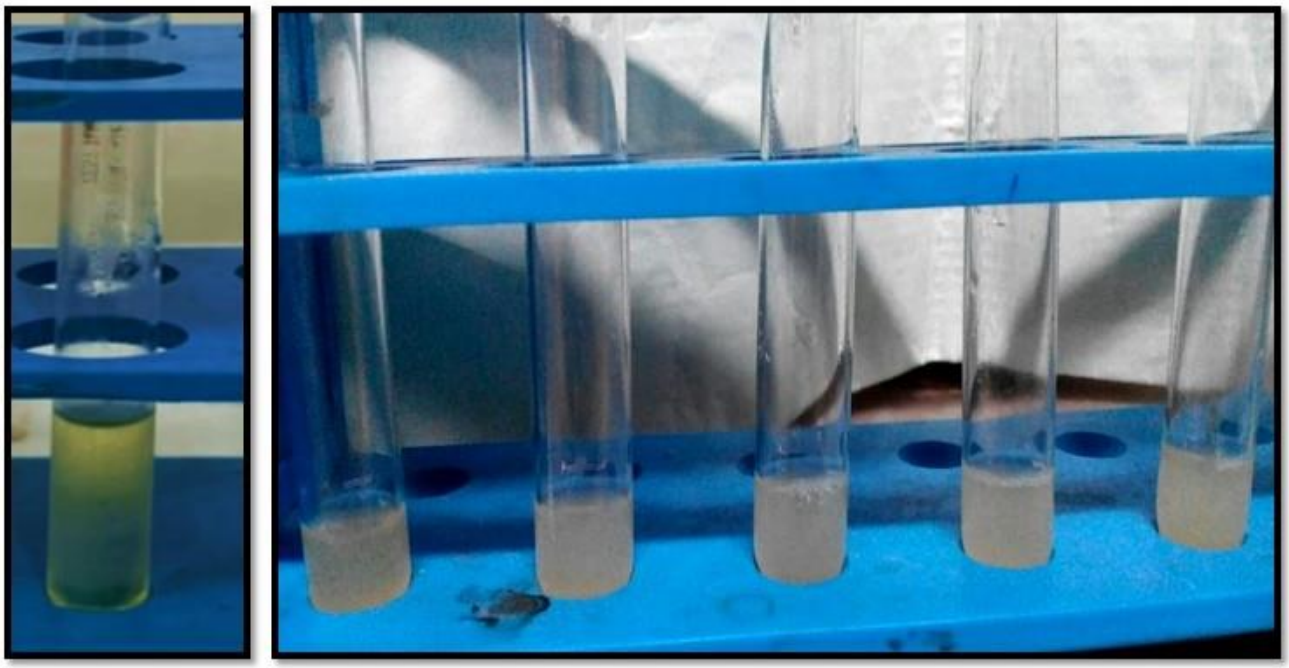
Fig. 2.17: Numbers of asymptomatic (100/200) and symptomatic samples (100/200) among the total (200) collected urine samples.

(a)



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(b)

(This study)

Fig. 2.18: Representative picture of (a) Left-control (blank media); Right (Urine culture-positive isolates) and (b) Presence of *E. coli* detected biochemically [From left- VP-Negative; MR-Positive; Citrate-Negative; Indole-Positive]

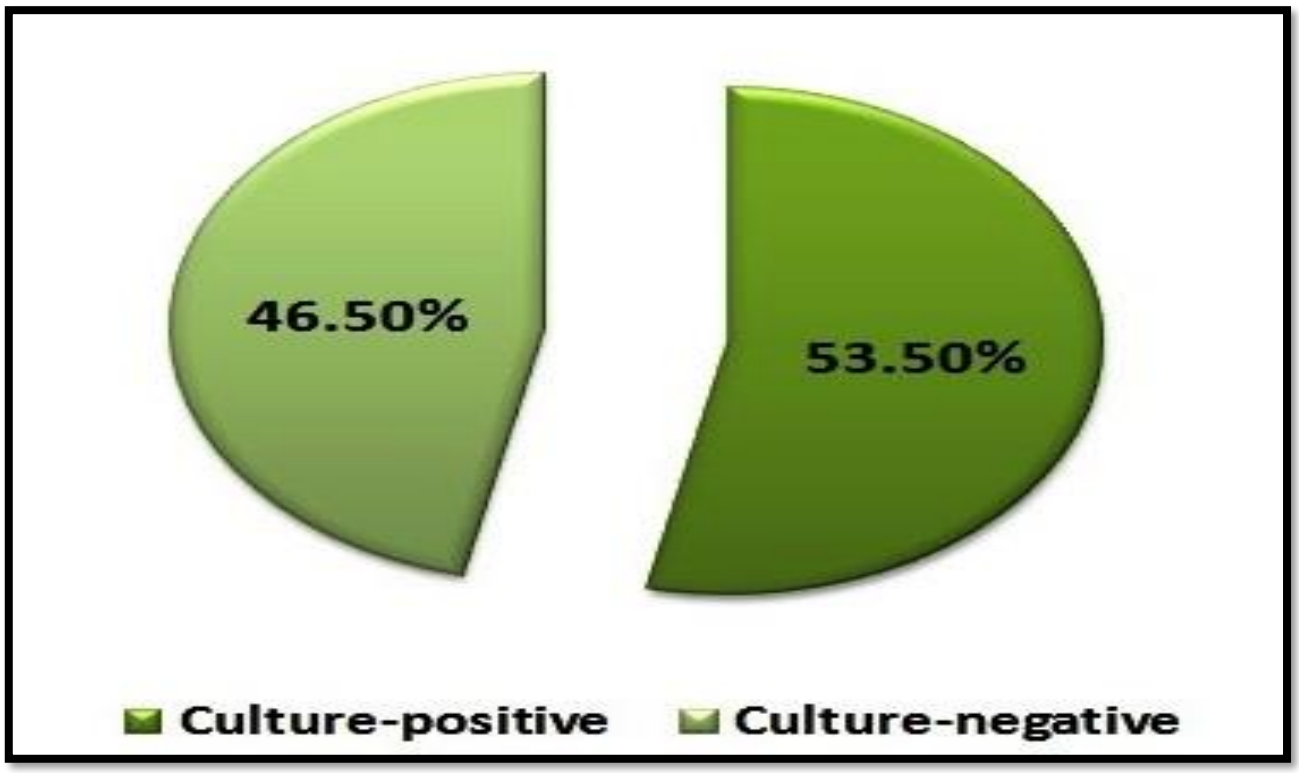

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Fig. 2.19: Percentage distribution of culture positive and culture-negative urine samples among the total samples collected.

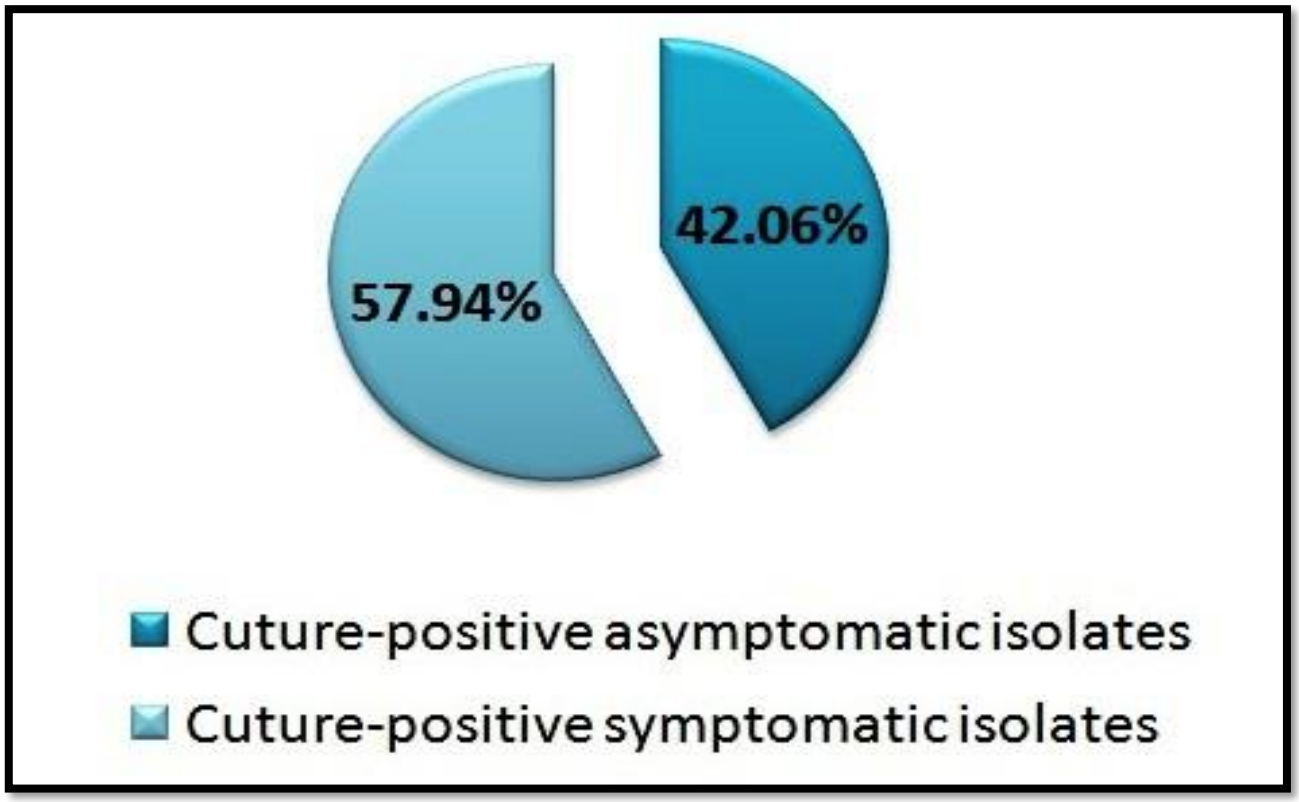

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Fig. 2.20: Percentage distribution of culture positive urine samples among asymptomatic and symptomatic patients.

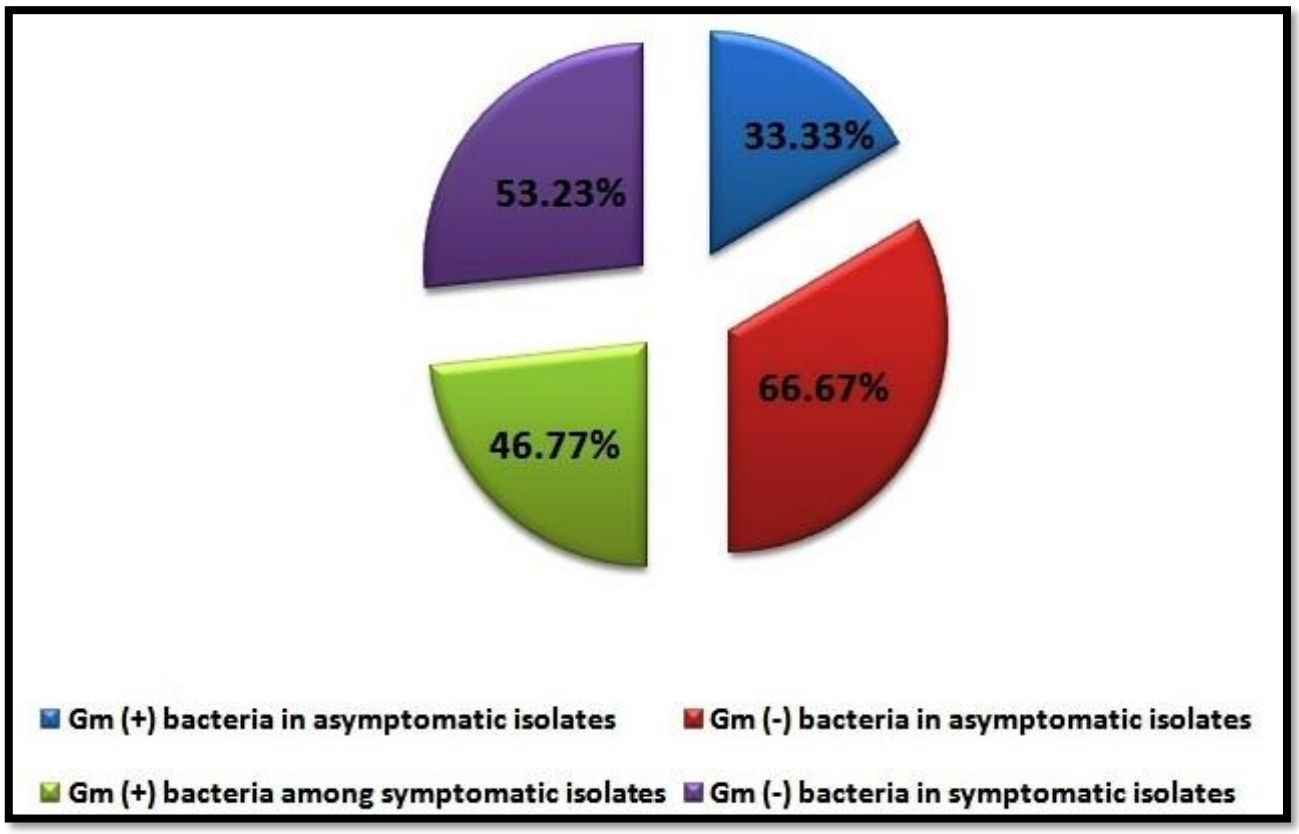

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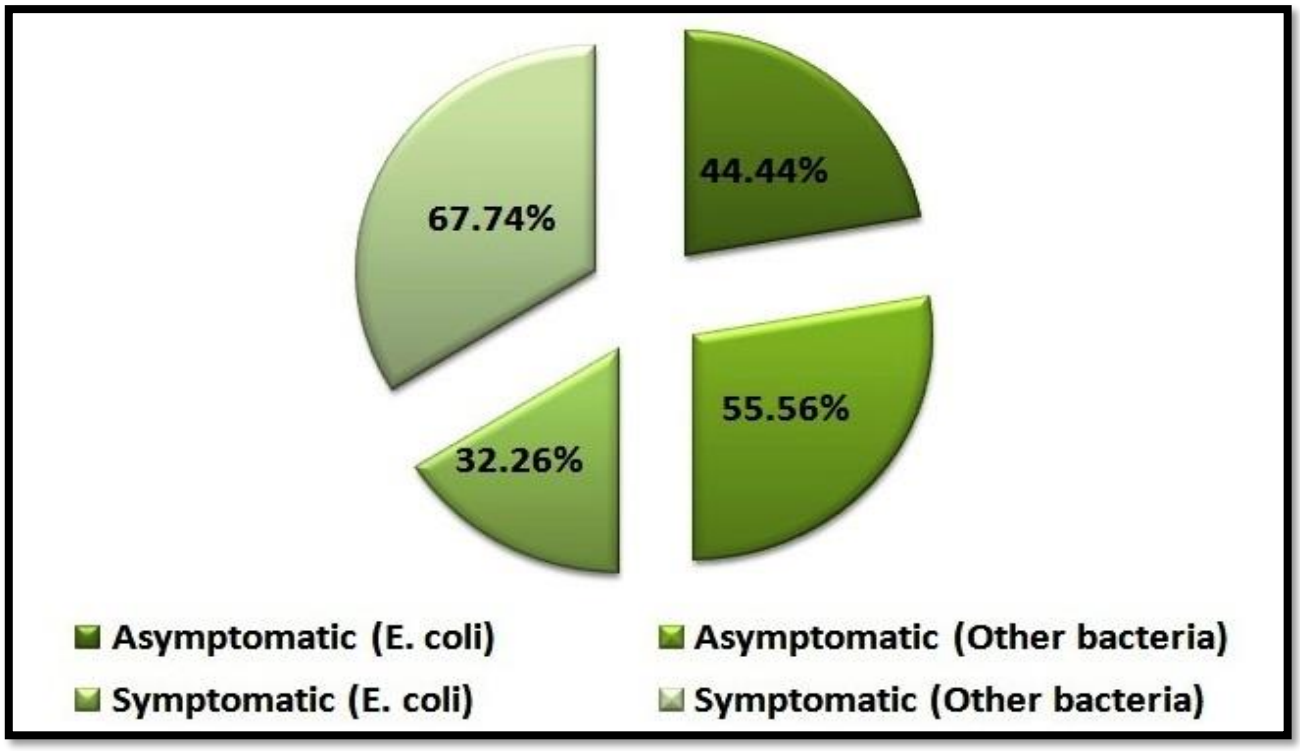
Fig. 2.21: Percentage of Gram-positive and Gram-negative bacteria among culture positive urine samples isolated from asymptomatic and symptomatic patients.



(This study)

Fig. 2.22: Percentage of *E. coli* among culture positive urine samples isolated from asymptomatic and symptomatic patients.

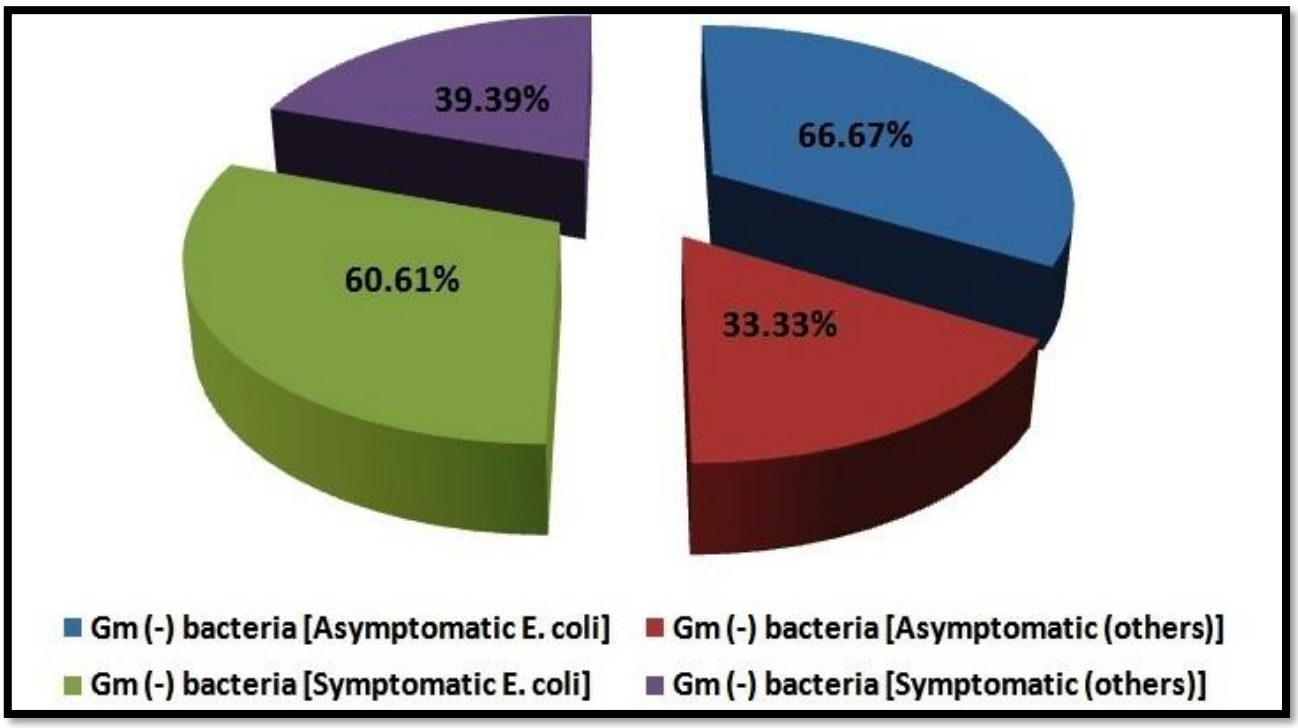


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Fig. 2.23: Percentage of *E. coli* among Gram-negative bacteria isolated from asymptomatic and symptomatic patients.

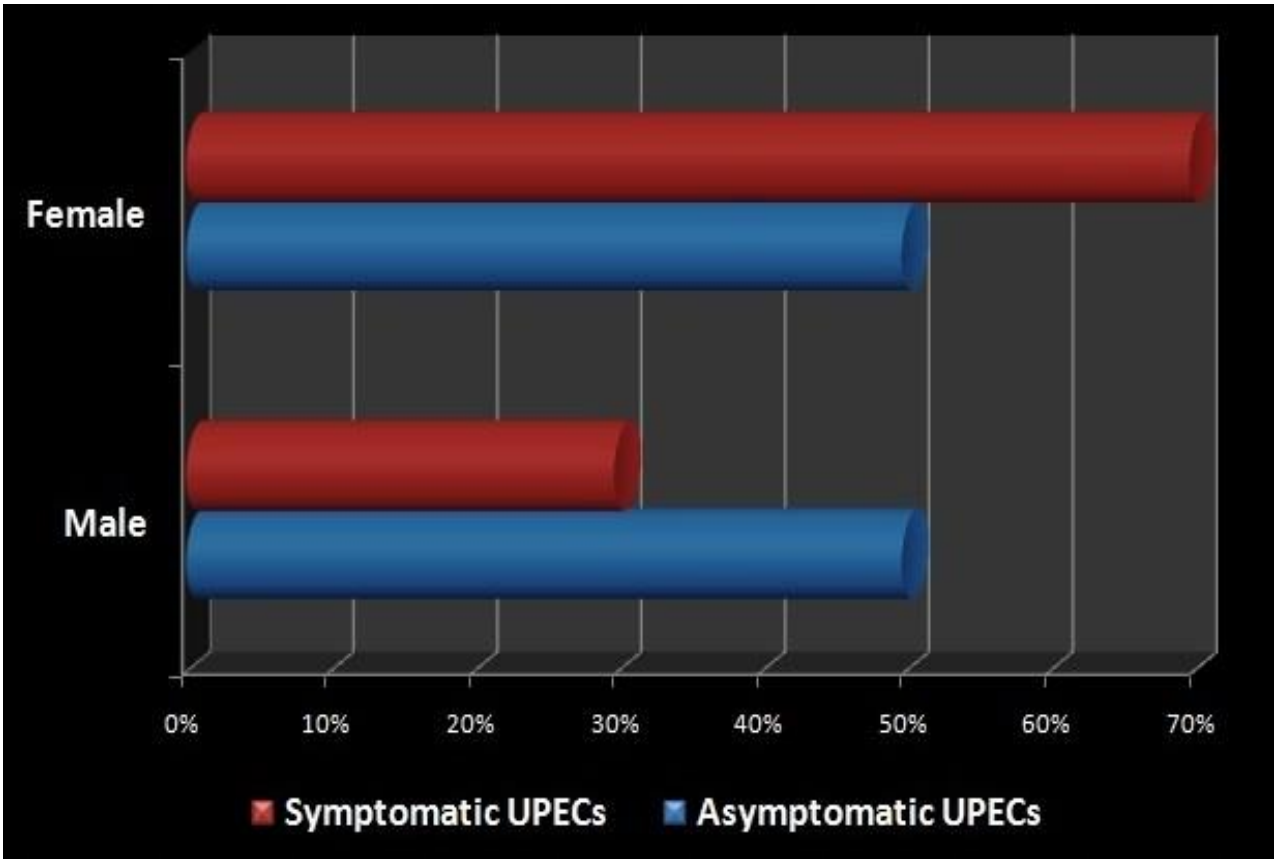

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Fig. 2.24: Percentage distribution of male and non-pregnant females among UPEC positive asymptomatic and symptomatic patients.

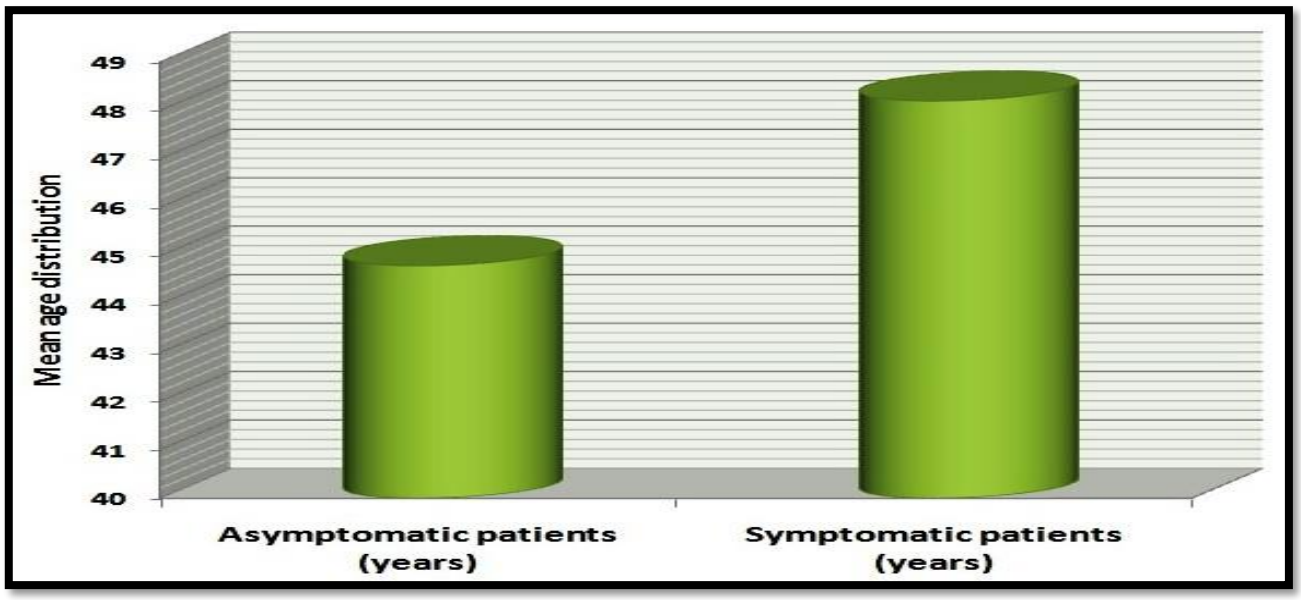

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Fig. 2.25: Mean age distribution among UPEC positive asymptomatic and symptomatic patients.

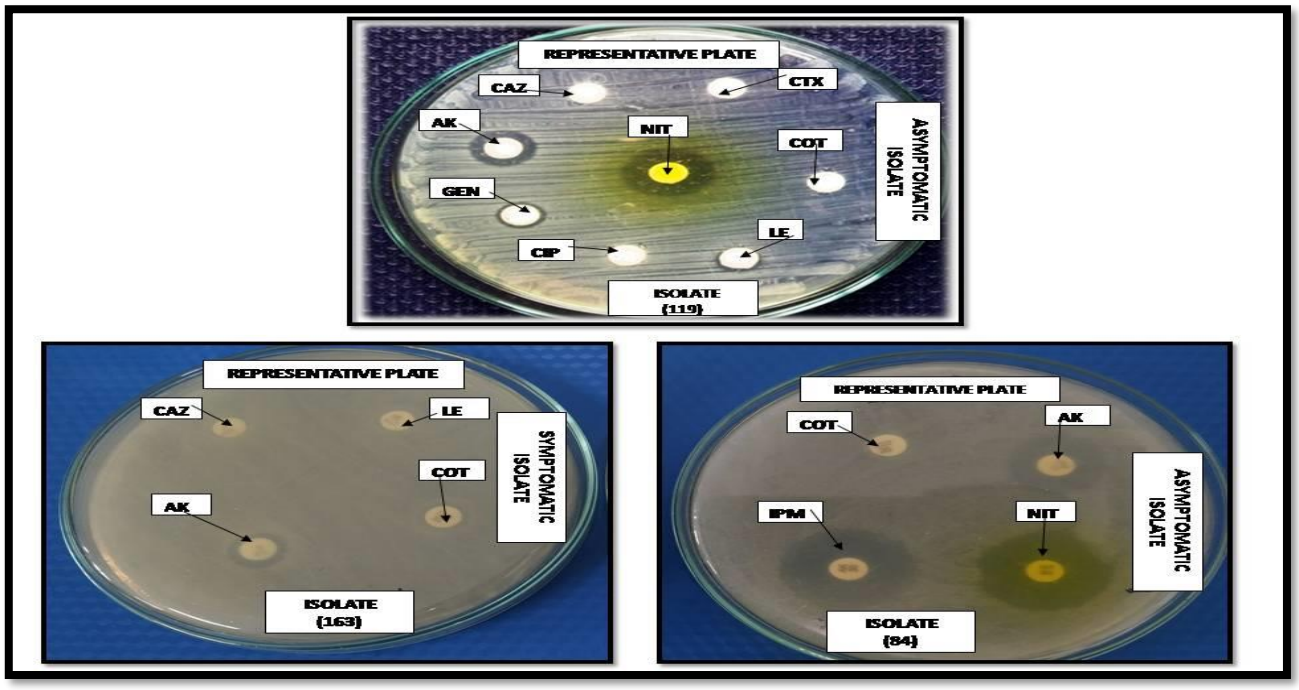
Table 2.4: Patient characteristics of different isolated symptomatic UPECs.

Sl. no.	Symptomatic isolates (Sample no.)	Cystitis/Pyelonephritis
1	9	Cystitis
2	17	Cystitis
3	46	Cystitis
4	79	Pyelonephritis
5	82	Pyelonephritis
6	86	Pyelonephritis
7	94	Pyelonephritis
8	101	Pyelonephritis
9	109	Pyelonephritis
10	111	Cystitis
11	112	Cystitis
12	130	Cystitis
13	137	Cystitis
14	145	Cystitis
15	147	Cystitis
16	161	Pyelonephritis
17	162	Pyelonephritis
18	173	Cystitis
19	184	Cystitis
20	196	Cystitis

2.5.2 Antibiogram study

A varied antibiotic resistance pattern was observed among the isolated 20 ABU and 20 symptomatic UPECs respectively against a regimen of 10 selected antibiotics from 6 different groups (cephalosporin, fluoroquinolone, trimethoprim/ sulfamethoxazole, aminoglycoside, carbapenem and nitrofuran) of drug (the representative picture of the antibiotic susceptibility test had been illustrated in **Fig. 2.26**). The highest resistance was observed against Ceftazidime, Cefotaxime (100%; 100%) and Ciprofloxacin, Levofloxacin, Cotrimoxazole (95%; 100%), moderately high against Tobramycin (70%; 70%), intermediate against Amikacin (45%; 50%) and Gentamicin (55%; 60%) and least against Imipenem (30%; 35%) and Nitrofurantoin (10%; 25%) among the ABU and symptomatic isolates respectively**.** The inferential statistics displayed the sampling distribution of the ABU (**Fig. 2.27a)** and symptomatic (**Fig. 2.27b)** UPECs with regard to the level of resistance against 10 different antibiotics. Moreover, the resistances towards different antibiotics (Ceftazidime, Cefotaxime, Ciprofloxacin, Levofloxacin, Cotrimoxazole, Gentamicin and Tobramycin) individually tested were significant (p value ≤ 0.05) among the ABU and symptomatic UPECs. However, significant (p-value ≤ 0.05) positive correlations that varied from low to high was perceived among both the asymptomatic (**Fig. 2.28a)** and symptomatic (**Fig. 2.28b)** UPECs with regard to their distribution of resistance against 10 different antibiotics except nitrofurantoin in the case of ABU UPECs. Among, ABU UPECs, high correlations were perceived in the resistance against ceftazidime with cefotaxime; tobramycin; ciprofloxacin; levofloxacin; cotrimoxazole, cefotaxime with; ceftazidime; tobramycin; ciprofloxacin; levofloxacin; cotrimoxazole, amikacin with gentamicin; tobramycin, tobramycin with ceftazidime; cefotaxime; amikacin; ciprofloxacin; levofloxacin; cotrimoxazole, ciprofloxacin with ceftazidime; cefotaxime; tobramycin; levofloxacin; cotrimoxazole, levofloxacin with ceftazidime; cefotaxime; tobramycin; ciprofloxacin; cotrimoxazole and cotrimoxazole with ceftazidime; cefotaxime; tobramycin; ciprofloxacin; levofloxacin respectively **(Fig. 2.28a)**. Moreover, moderate correlations regarding resistance were observed in the cases of ceftazidime and cefotaxime with amikacin; gentamicin, imipenem with gentamicin; tobramycin, amikacin with ceftazidime; cefotaxime; ciprofloxacin; levofloxacin; cotrimoxazole, gentamicin with ceftazidime; cefotaxime; imipenem; tobramycin; ciprofloxacin; levofloxacin; cotrimoxazole, tobramycin with imipenem; gentamicin, ciprofloxacin, levofloxacin and cotrimoxazole with amikacin; gentamicin (**Fig. 2.28a)**. Significant low positive correlations were observed in the cases of resistance against ceftazidime and cefotaxime with imipenem, imipenem with ceftazidime; cefotaxime; amikacin; ciprofloxacin; levofloxacin; cotrimoxazole, amikacin with imipenem **(Fig. 2.28a)**. Similar kind of observations was also observed in the case of symptomatic UPECs **(Fig. 2.28b).** Moreover, significant positive correlations (p-value ≤ 0.05) were also observed between isolates of asymptomatic and symptomatic groups with regard to their zone of inhibition (mm) signifying varied level of resistances against 7 (Ceftazidime, Cefotaxime, Ciprofloxacin, Levofloxacin, Cotrimoxazole, Gentamicin and Tobramycin), of the 10 different aforementioned antibiotics tested (**Fig. 2.29)**. However, significant (p-value $\leq$ 0.05) negative

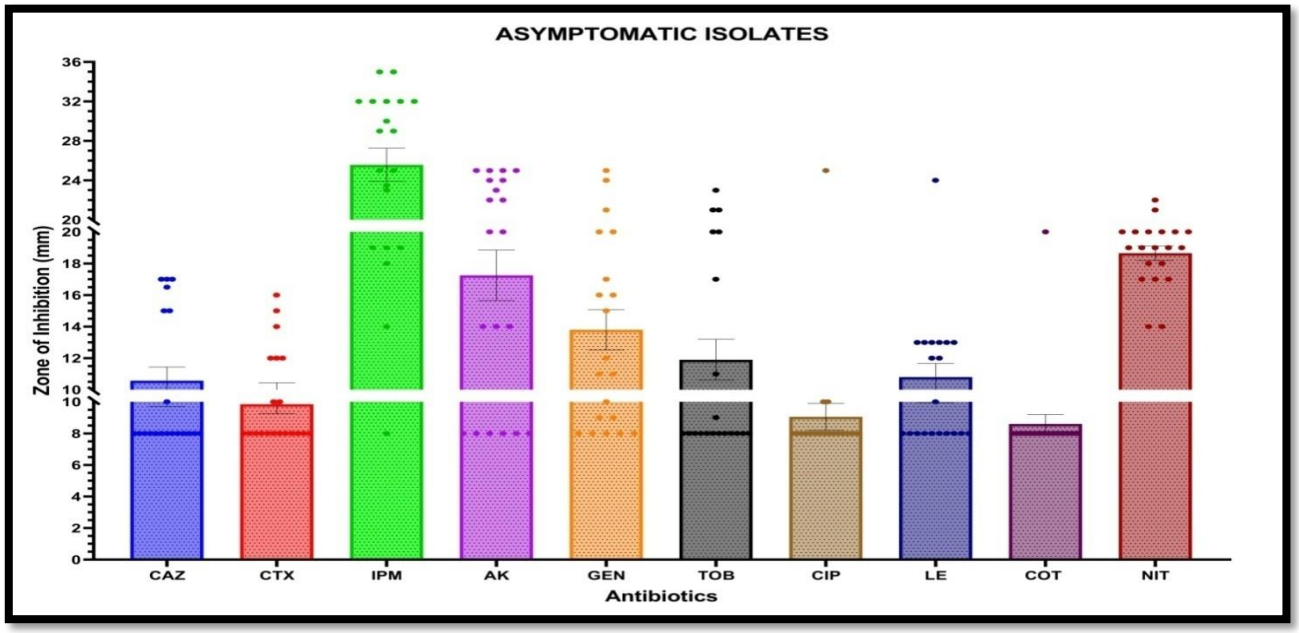
correlations were observed between isolates of asymptomatic and symptomatic groups with regard to their zone of inhibition (mm) signifying varied level of resistances [(Imipenem with that Ceftazidime, Cefotaxime, Ciprofloxacin, Levofloxacin, Cotrimoxazole, Amikacin, Gentamicin and Tobramycin), (Amikacin with Ceftazidime, Cefotaxime, Cotrimoxazole, Nitrofurantoin), (Nitrofurantoin with Ceftazidime, Cefotaxime, Ciprofloxacin, Levofloxacin, Gentamicin and Tobramycin)]. Ninety-five percent of the asymptomatic and 100% symptomatic isolates were MDR and this incidence was found to be significant (p-value ≤ 0.05) among both groups.



(This study)

Fig. 2.26: Representative pictures of the antibiogram study of ABU and symptomatic UPECs.

(a)



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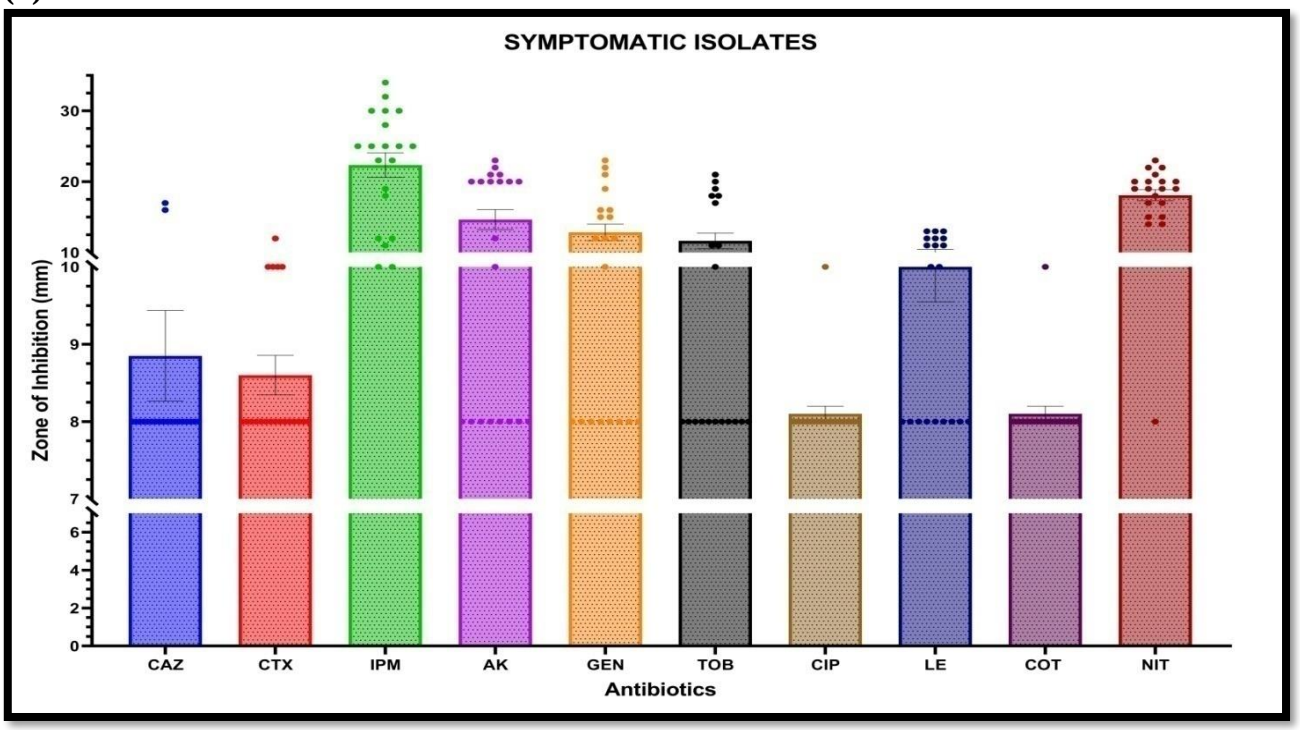
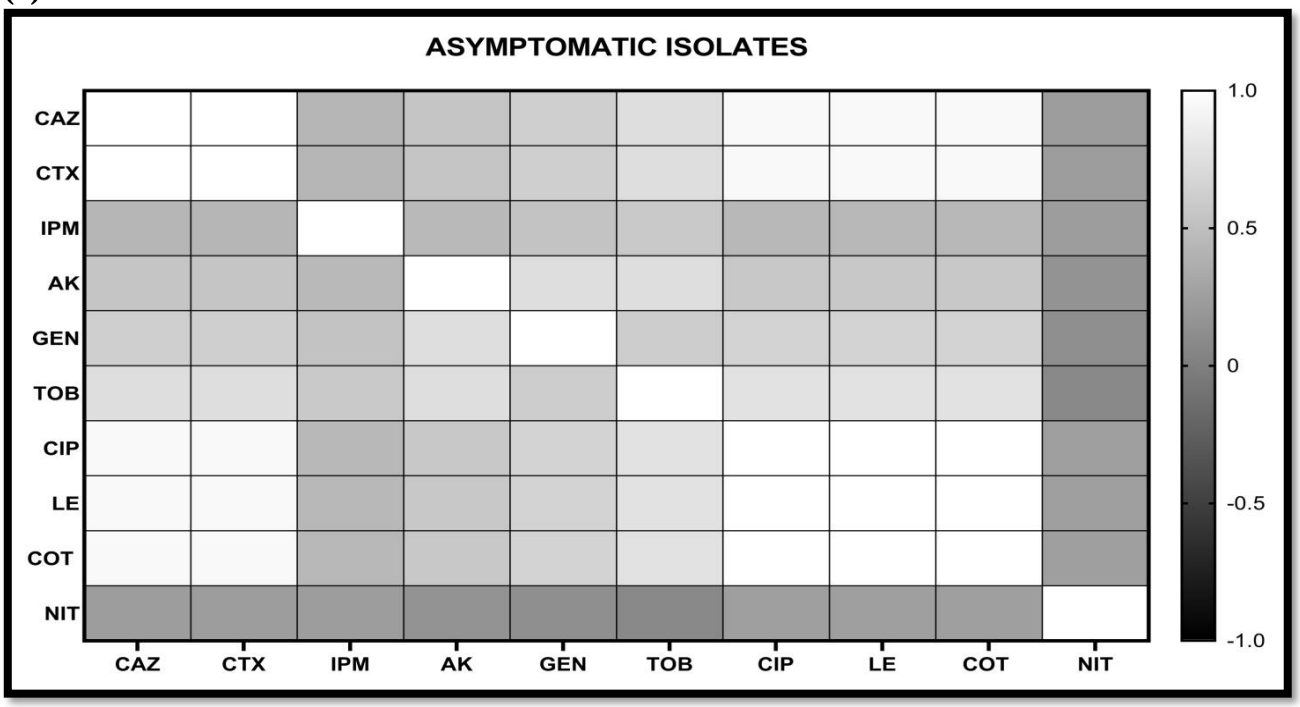
(b)



(This study)

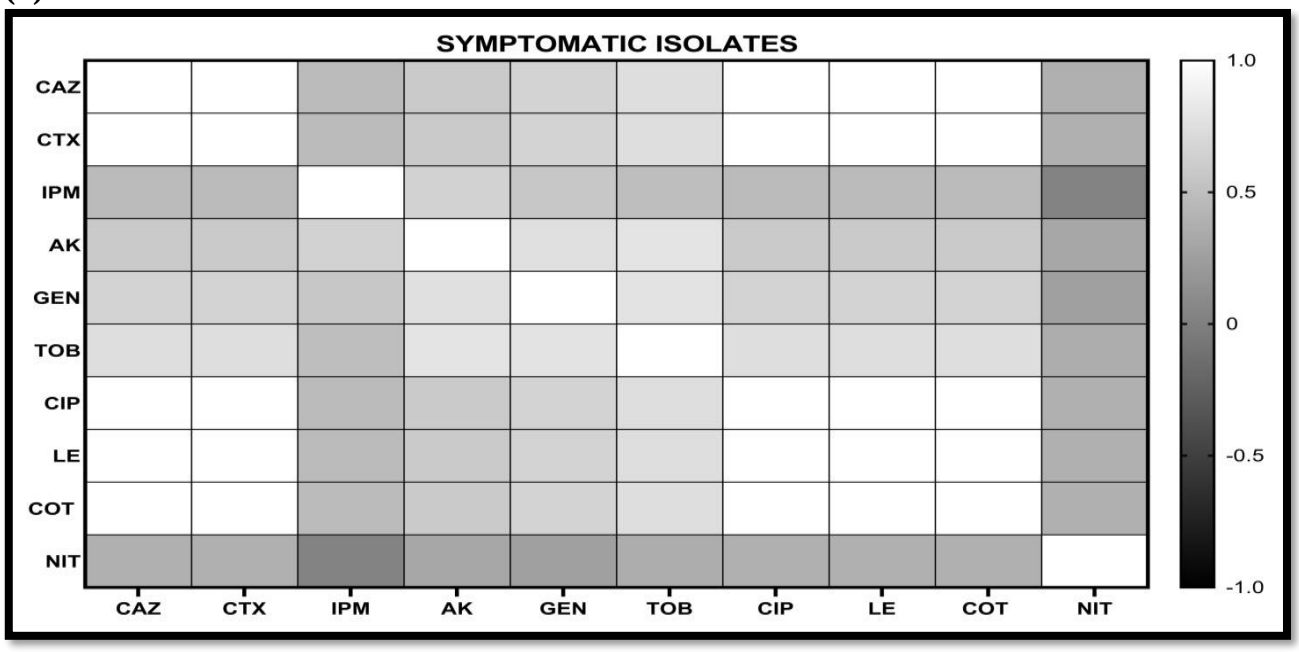
Fig. 2.27: Graphical representation of the mean with standard error of mean (SEM) values of 10 different antibiotics (CAZ, CTX, IPM, AK, GEN, TOB, CIP, LE, COT, NIT) [a] asymptomatic UPECs (N=20) [b] symptomatic UPECs (N=20), based on their zone of inhibition (mm) generated using GraphPad Prism version 9 (Prism software package). Different antibiotics were represented by scatter dot plots with varied colours. Error bars indicated the spread of data in case of each of the individual antibiotics.

(a)



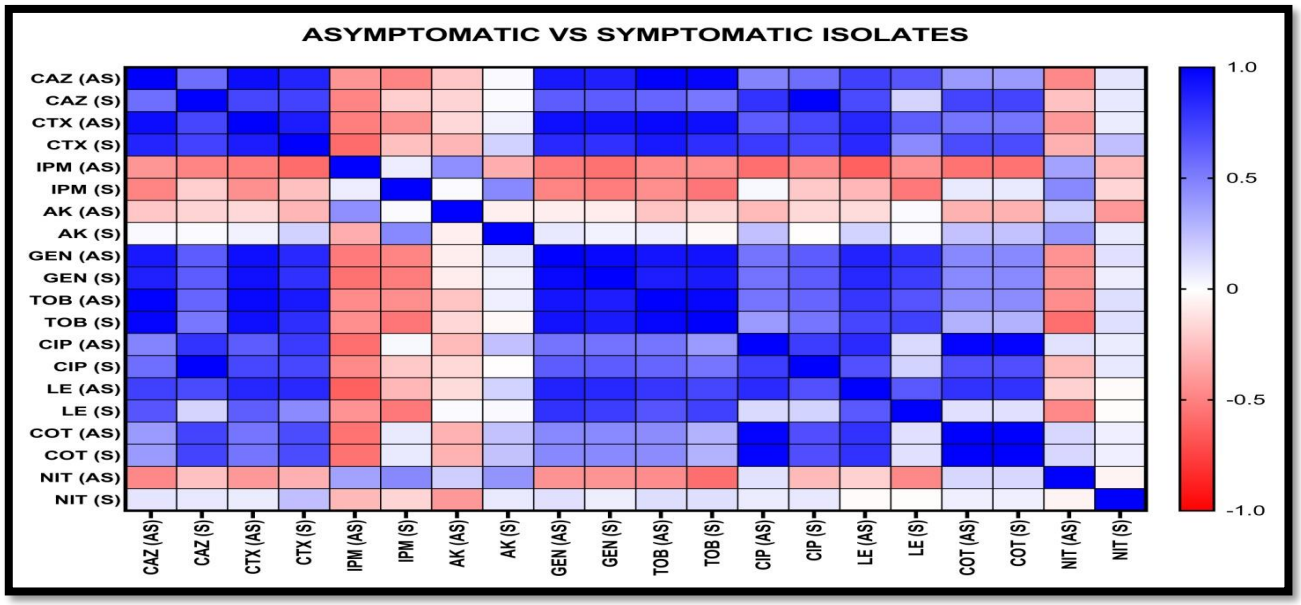
(This study)

(b)



(This study)

Fig. 2.28: Statistical representation of correlations between the incidence of resistance against 10 different antibiotics (CAZ, CTX, IPM, AK, GEN, TOB, CIP, LE, COT, NIT) when individually computed using GraphPad Prism version 9 (Prism software package) separately among (a) asymptomatic (b) symptomatic UPECs. Heat maps were generated on the correlation coefficient values represented by the color keys that ranged from (-) 1 (black) to (+) 1 (white).



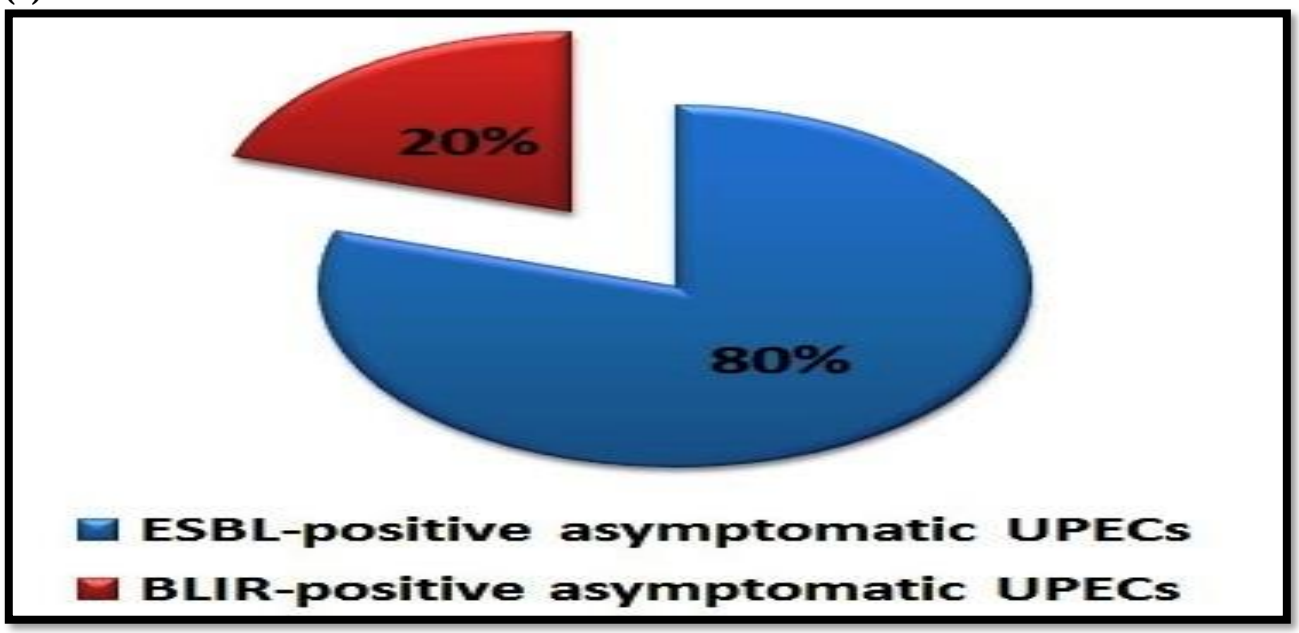
(This study)

Fig. 2.29: Statistical representation of correlations between the asymptomatic (AS) and symptomatic (S) UPECs with regard to their zone of inhibition (mm) signifying varied level of resistances against 10 different antibiotics (CAZ, CTX, IPM, AK, GEN, TOB, CIP, LE, COT, NIT) computed using GraphPad Prism version 9 (Prism software package). Heat maps were generated on the correlation coefficient values represented by the color keys that ranged from (-) 1 (red) to (+) 1 (blue).

2.5.3 Identification of ESBL Producers

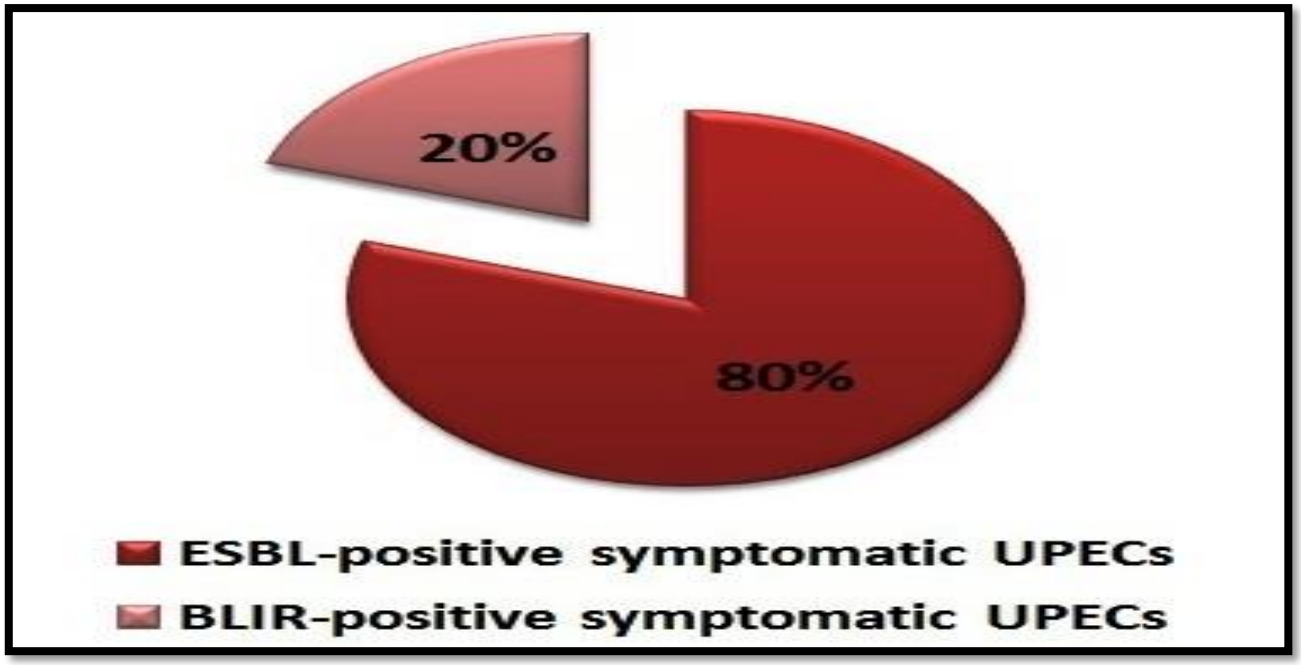
Thirty-two (80%) out of the 40 UPECs (Asymptomatic=16; Symptomatic=16) selected for this study were ESBL producers **(Fig. 2.30a)** and the 8 [(20%); Asymptomatic=4; Symptomatic=4] **(Fig. 2.30b)** remaining isolates were BLIR **(Table 2.5)**. The aforementioned incidence of ESBL producers was found to be statistically significant (p-value ≤ 0.05) unlike the non-significant (p-value ≥ 0.05) occurrence BLIR phenotype respectively among both groups.

(a)



(This study)

(b)



(This study)

Fig. 2.30: Percentage of ESBL and BLIR positive isolates among (a) asymptomatic and (b) symptomatic UPECs respectively.

Table 2.5: Incidence of ESBL and BLIR production among asymptomatic and symptomatic uropathogenic *E. coli* isolates.

Sl. No	Asymptomatic isolates (Sample no.)	ESBL phenotype	BLIR phenotype	Symptomatic isolates (Sample no.)	ESBL phenotype	BLIR phenotype
1	74	+	−	9	+	−
2	75	+	−	17	+	−
3	77	+	−	46	−	+
4	80	+	−	79	+	−
5	83	+	−	82	+	−
6	84	+	−	86	−	+
7	91	−	+	94	+	−
8	93	+	−	101	−	+
9	96	−	+	109	+	
10	99	+	−	111	+	−
11	102	+	−	112	+	−
12	104	+	−	130	+	−
13	107	−	+	137	+	−
14	110	+	−	145	+	−
15	113	+	−	147	+	−
16	114	+	−	161	−	+
17	119	+	−	162	+	−
18	133	−	+	173	+	−
19	138	+	−	184	+	−
20	158	+	−	196	+	−

(+)=Positive ESBL /BLIR production; (-) = No/negative ESBL/BLIR production

2.6 Discussion

In this study, urine-culture analysis showed microbial growth in 53.5% **(Fig. 2.19)** of the urine samples collected from hospitalized patients, which was comparatively higher (28.35%; 18.24%; 8.06%; 12.1%; 17.1%) than various studies conducted in India **(Pai and Nair 2012; Jitendranath et al. 2015)** and other parts of the world like Iran **(Khoshbakht et al. 2013)**, Portugal **(Linhares et al. 2013)** and South Korea **(Kim et al. 2021)**. Moreover, this study revealed that among the culture-positive urine samples, 42.06% and 57.94% **(Fig. 2.20)** were collected from asymptomatic and symptomatic individuals respectively. This high incidence of growth positive isolates was mostly in agreement with the previous studies conducted on asymptomatic samples from Canada **(Silver et al. 2009)** and India **(Vaijanathrao et al. 2015)**. However, the percentage of culture-positive symptomatic isolates was comparatively higher than the studies conducted previously from the United States of America (33%) **(Muder et. al., 2006)** and Canada (25%) **Silver et al. 2009).** Nonetheless, the aforementioned observation was in accordance with the findings observed by Vaijanathrao et al. **(Vaijanathrao et al. 2015)** from India. The variation observed in this study when compared with the others mentioned, might be due to the differences in the environments, social habits, socioeconomic

status, standards of living and education of the individuals, included in the studies conducted worldwide. The differences in the findings might also be due to the variation in sample sizes.

Moreover, 44.44% **(Fig. 2.22)** and 32.26% **(Fig. 2.22)** of the culture positive isolates obtained from asymptomatic and symptomatic patients respectively were found to be *E coli* positive. The percentage of ABU and symptomatic UPECs respectively among culture-positive isolates was found to be comparatively higher and lower than two previous reports from two of India states (Tamil Nadu; Kerala) by Jayachandran et al. **(Jayachandran et al. 2016)** and Kulkarni et al **(Kulkarni et al. 2017)** respectively. Moreover, *E. coli* was the most frequently espied uropathogen in the present study among the culture positive urine isolates obtained irrespective of their asymptomatic and symptomatic nature. This was mostly in conformity with studies conducted in different places of the world like Ethiopia **(Demilie et al. 2012)**, India **(Raval et al. 2015; Venkatesan et al. 2017)**, Kenya **(Ayoyi et al. 2017)** and South Korea **(Kim et al. 2021)**.

The present study indicated equal prevalence of men and women among the ABU patients infected with UPECs that was discordant to an earlier report from Australia that displayed the higher incidence females (**Mabbett et al. 2009)**. However, among symptomatic patients with UPECs, significantly higher prevalence of females was observed which was in congruity with several reports from various parts of the world like Nepal **(Shah et al. 2019)**, Uganda **(Odongo et al. 2020)** and Iraq **(Assafi et al. 2022).**

Among, individuals infected ABU UPECs, the mean age was observed to be 44.8 years which was found to be nine years lower than an earlier report from Australia conducted on ABU UPECs **(Mabbett et al. 2009)**. However, among symptomatic patients with UPECs, mean age group was 48.2 years which was mostly in accordance with an earlier report from Australia **(Mabbett et al. 2009)**, but was higher than that recently reported from Taiwan (**Lin et al. 2021)**. Moreover, the present study indicated higher incidence of cystitis compared to pyelonephritis **(Table 2.4)** among patients with symptomatic UPECs quite discordant to a previous report from Australia (Mabbett et al. 2009).

There is a burgeoning concern regarding antimicrobial resistance worldwide, especially to UPECs, a dominant causative agent of UTI, as these resistant bacteria are making the treatment options very limited **(Bartoletti et al. 2016; Sanchez et al. 2016; Kot 2019)**. UPECs obtained in this study irrespective of their asymptomatic or symptomatic nature were resistant to most of the antibiotics tested except for imipenem, amikacin and nitrofurantoin. Moreover, majority of UPECs irrespective of their asymptomatic **(Fig. 2.26a)** and symptomatic **(Fig. 2.26b)** nature were found to be highly resistant (zone of inhibition $\leq$ 10mm) to different antibiotic groups like third generation cephalosporins (ceftazidime; cefotaxime), aminoglycoside especially tobramycin, both second (ciprofloxacin) and third generation (levofloxacin) fluoroquinolones and trimethoprim/sulfamethoxazole (cotrimoxazole). However, although among aminoglycosides group of antibiotics, moderate to moderately high resistance was

observed against gentamicin and tobramycin respectively, but about half of the UPECs from both groups were found to be sensitive to amikacin. These observations were mostly in concurrence with previous studies conducted from India **(Wani et al. 2009; Nalini et al. 2014**, **Rath and Padhy 2015)**. However, various other studies conducted from different parts of the world (Netherlands, India and Kenya) suggested that the second-generation fluoroquinolone; especially ciprofloxacin can be used as a drug of choice to treat *E. coli* associated asymptomatic or symptomatic bacteriuria due to its high level of sensitivity to this particular drug (**den Heijer et al. 2012; Vaijanathrao et al. 2015; Ayoyi et al. 2017)**. Moreover, the present study indicated that UPECs were most sensitive to Nitrofurantoin (Asymptomatic=90%; Symptomatic=75%). This was in agreement with the studies conducted from Egypt on ABU UPECs **(Abdel-Aziz Elzayat et al. 2017)** and from Nigeria on symptomatic UPECs **(Okonko et al. 2009)** but in disagreement with the findings obtained from another part of India (**Vaijanathrao et al. 2015)** where UPECs were mostly sensitive to Amikacin. Moderate to high level of sensitivity to Amikacin and Imipenem was also observed in the present study.

Moreover, among ABU and symptomatic UPECs incidence of nitrofurantoin resistance was found to be negligibly **(Fig. 2.28a)** and very weakly **(Fig. 2.28b)** correlated respectively to all other tested antibiotics. Additionally, among both the groups **(Fig. 2.28a-b)**, incidence of imipenem resistance revealed low correlations with 7 of the 9 other tested antibiotics. To boot, among both the aforementioned groups **(Fig. 2.28a-b)** incidence of amikacin resistance was found to be weakly or moderately correlated to 7 out of the 9 studied antibiotics. Withal, significant correlations (p-value $\leq$ 0.05) were observed between ABU and symptomatic UPECs with regard to their zone of inhibition (mm) signifying moderately high to very high level of resistances against 7 (Ceftazidime, Cefotaxime, Ciprofloxacin, Levofloxacin, Cotrimoxazole, Gentamicin and Tobramycin), different antibiotics (**Fig. 2.29)**. However, significant negative correlations (p-value $\leq$ 0.05) were observed between ABU and symptomatic UPECs with regard to resistances against imipenem with the antibiotics of cephalosporin, fluoroquinolone, aminoglycoside and trimethoprim/ sulfamethoxazole groups, amikacin with drugs of cephalosporin, trimethoprim/ sulfonamides and nitrofuran groups and nitrofurantoin with the antibiotics of cephalosporin, fluoroquinolone, aminoglycoside groups (**Fig. 2.29)**. Therefore, the aforementioned observations suggested that nitrofurantoin, and to some extent imipenem and amikacin can be used as a drug of choice to treat ABU as well as patients suffering from symptomatic UTI, infected with UPECs **(Ghosh and Mukherjee 2019)**.

Several studies conducted worldwide reported, at least 50% of the asymptomatic UPECs to be susceptible to most of the antibiotics tested **(Ayoade et al. 2013, Abdel-Aziz Elzayat et al. 2017; Venkatesan et al. 2017)**, however, in this study 95% of ABU UPECs were found to be MDR. This significantly (p-value $\leq$ 0.05) high incidence of multidrug resistance among ABU UPECs was really alarming and this trend needs to be watched closely. Moreover, a statistically significant (p value $\leq$

0.05) incidence of ESBL producers among both ABU and symptomatic UPECs perceived in this study was comparable to the studies reported on symptomatic UPECs from India and Upper Egypt **(Basu and Mukherjee 2018; Hassuna et al. 2020)** but on contrary to the recent studies conducted on asymptomatic and symptomatic UPECs from Ethiopia **(Belete et al. 2020)** and Iran **(Naziri et al. 2020).** Therefore, the aforementioned high incidence of MDR **(Ghosh and Mukherjee 2019)** along with significant occurrence of ESBL production justified the need to cease the spread of antibiotic resistance by sagacious use of antibiotics, especially in a resource-poor country like, India.

2.7 Conclusion

The present study provided a detailed insight into the clinical characteristics and antibiogram profile of the ABU UPECs besides, symptomatic ones, isolated from hospitalized patients of Kolkata, an eastern region of resource poor country India. The equal prevalence of male and female population in the case of ABU UPECs, discordant to the higher incidence of women patients in the case of symptomatic UPECs was alarming. This displayed the probable incidence of sexually transmitted diseases with or without the prostate problems in the case of males and thus must be watched closely. Among both the groups, mean age distribution was below 50 years which betokened the need for screening of UTI among both male and female population, even in absence of symptoms, on or before the age of 50 years. The extremely high incidence of fluoroquinolone resistance, both to the 2nd and the third-generation drugs along with the high occurrences of resistance to the third generation cephalosporins and other commonly administered antibiotics, indicated the solemn need for the routine antibiotic susceptibility tests in the laboratories to design proper prescription policies along with their appropriate implementation to prevent further resistance development. This study also indicated that ABU in our population can be successfully treated with nitrofurantoin and to some extent by imipenem and amikacin, similar to symptomatic UTIs**.** Furthermore, the extremely high incidence of MDR and ESBL producers among ABU UPECs comparable to the symptomatic ones might be due to the indiscriminate use of antibiotics to treat extraintestinal infections. Therefore, this study on ABU and symptomatic UPECs justified the need to cease dissemination of antibiotic resistance by proper implementation of prescription policies especially in a resource poor country, India.

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CHAPTER 3.

β-lactamase producing drug-resistant asymptomatic & symptomatic uropathogenic *E. coli* - Genotypic characterization relating to phylogenetic background, distribution of pathogenicity islands and virulence factor genes

3.1 Background study

Uropathogenic *E. coli* (UPECs) are one of the most common uropathogen, responsible for more than 80% of all UTIs and can cause both asymptomatic bacteriuria (ABU) and symptomatic UTI **(Bien et al. 2012; Ghosh and Mukherjee 2019)**. Moreover, the recent reports from China and India declared a high incidence of ABU UPECs among individuals with asymptomatic UTI **(He et al. 2018; Ghosh and Mukherjee 2019)**. Furthermore, a study from India (**Ghosh and Mukherjee 2019)** had indicated similar MDR profiles among the asymptomatic and symptomatic UPECs with very high incidences of resistance against cephalosporins, fluoroquinolones, trimethoprim/ sulfamethoxazole which were generally reported in the case of symptomatic UPECs from various parts of the world like Pakistan **(Ali et al. 2016)**, India **(Basu and Mukherjee 2018)**, Mexico **(Ramírez-Castillo et al. 2018)** and Poland **(Kot 2019)**. Nonetheless, reports from the recent past from Mexico **(Paniagua-Contreras et al. 2017)** and Iran **(Malekzadegan et al. 2018)** highlighted the fact that the incidence of MDR in symptomatic UPECs is often associated with increased urovirulence including a high degree of heterogeneity among them. However, earlier studies on symptomatic UPECs from Japan **(Kawamura-Sato et al. 2010)** and India (**Basu et al. 2013**) had reported a correlation between antibiotic resistance and reduction in virulence factor genes and suggested that quinolone resistance may be directly associated with the loss of virulence. Nevertheless, the clinically benign nature of ABU was initially explained by a lack of virulence, since many ABU UPEC strains lack adhesins commonly associated with virulence **(Beatson et al. 2015)**. There is disparity in this interpretation as a previous study by Watts et al. **(Watts et al. 2010)** from Australia demonstrated that quite a high percentage of ABU strains expressed functional fimbrial adhesins. Moreover, earlier reports from Australia **(Mabbett et al. 2009; Watts et al. 2010)** stated that many ABU strains are phylogenetically related to the virulent symptomatic UPEC strains.

Additionally, an earlier study from India **(Srivastava et al. 2016)** had indicated similar pathogenic profiles among the ABU and symptomatic UPECs. However, Mabbett et al. **(Mabbett et al. 2009)** from Australia indicated that ABU UPECs possessed fewer virulence factor genes compared to the symptomatic ones. Moreover, the distribution of the virulence genes such as fimH (mannose-specific adhesin of type I fimbriae), papC; papEF; papG (usher; tip component/ adaptor-initiator; adhesin respectively of P-fimbriae), sfa/foc (S−/F1c-fimbriae), afa (afimbrial adhesin), toxins encoded by hlyA (α-hemolysin), cnf1 (cytotoxic necrotizing factor 1) and iron acquisition factors produced by iucD (aerobactin iron transport system) which resides on pathogenicity islands (PAIs) were mostly attributed to pathogenicity of *E. coli* (**Kryger et al. 2015; Samei et al. 2015; Najafi et al. 2018)**. Nonetheless, previous studies worldwide (Germany, Australia, Sweden and India) had also reported

that there were different factors responsible to designate a bacterial population as asymptomatic (**Zdziarski et al. 2008; Mabbett et al. 2009; Lutay et al. 2013; Srivastava et al. 2016)**. Moreover earlier studies conducted from Poland **(Bien et al. 2012)** and Iran (**Naderi et al. 2016)** stated that pathogenic *E. coli* was evolved from commensal E. coli through horizontal gene transfer (HGT)**.** Therefore, characterizing the ABU with respect to pathogenicity may help the microbiologists and clinicians to understand the exigencies under which these patients are more likely to experience symptomatic UTIs.

The results and interpretation drawn from several of these aforesaid studies conducted worldwide have been inconsistent, revealing either avirulent nature of ABU unlike symptomatic strains or somewhat similar virulence repertoire like the symptomatic ones. Gaining, a deep insight into the molecular characteristics by which ABU UPECs are able to establish infections and survive in the high flow environment of human urinary tract just like symptomatic ones but without actually developing any proper symptoms unlike the later, may be particularly helpful in case of individuals who are unable to promulgate any symptoms. Moreover, the aforementioned reports together with the high incidence of MDR and ESBL production among ABU UPECs in our study population (**Ghosh and Mukherjee 2019)** demanded their genotypic characterization especially with respect to their phylogenetic background, distribution PAIs and virulence factor genes. However, till date, the incidence of ABU UPECs which were isolated from males and non-pregnant females and their molecular characteristics have not been investigated from Kolkata, an eastern region of India. So, this is the first study of its kind from Kolkata, India that aimed to characterize the drug-resistant UPECs isolated from urine samples of asymptomatic hospitalized patients in Kolkata, India with respect to their phylogenetic background, distribution of PAIs, virulence factor genes acquisition and distribution, which were further compared with that of the symptomatic ones. This study is expected to add relevant information to the existing knowledge in this field and also serve as a means to control the spread of healthy individuals to vulnerable ones in future.

3.2 Objectives

- The genotypic characterization of the isolated ABU and symptomatic UPECs with respect to their phylogenetic background, distribution of PAIs and major virulence factor genes.

3.3 Materials

(a) Equipments:

- Laminar Air Flow [B.D Instrumentation]
- Shaker – Incubator [ICT]

- Autoclave [PrimeSurgicals]
- Hot air oven (Digisystem Laboratory Instruments Inc.)
- Freezer (-20°C) [Celfrost]
- Freezer (-80°C) [Remi]
- Thermal cycler [ABI Instruments Private Limited, Model-Veriti Thermal Cycler]
- Horizontal gel electrophoresis apparatus [Genei]
- Power pack [Genei]
- Gel Documentation system [BIO-RAD]
- Inoculation loop
- Glass spreaders
- Spirit Lamp
- 90mm Glass petri dish [Borosil]
- Glass culture tubes [TOUFF, Borosil]
- Test tube racks [Tarsons]
- Micropipettes (0.5-10µl, 2-20µl, 20-200µl, 200-1000µl) [Corning, P'fact, Microlit, Biohit]
- Micro tips (0.5-10µl, 2-20µl, 20-200µl, 200-1000µl) [HiMedia]
- Eppendorf Tubes (1.5 mL, 2 mL) [Tarsons]
- PCR tubes (0.2 mL) [Tarsons]
- Cotton [Bengal Surgicals Limited] [Lakshmi Healthcare Products (P) Ltd]
- Surgical Gloves [PriCARE, HiMedia]
- Wash bottles

(b) Reagents:

- Luria Bertani (LB) media [SRL Chemicals India]
- Mueller Hinton (MH) media [SRL Chemicals India]
- Agar Agar [Merck]
- Barium chloride [Merck]
- Sulphuric acid [Hospital Store]
- 70% Ethanol [Bengal Chemical]
- Chloroform [Hospital Store]
- 95% Ethanol [HiMedia]
- Single Distilled water (SDW) [Hospital Store]
- Double distilled water (DDW) [Laboratory distillation plant]
- Primers [GCC Biotech(I) Pvt.Ltd]

- dNTP [Invitrogen]
- Taq DNA Polymerase and buffer [Invitrogen]
- 50mM MgCl2 [Invitrogen]
- DNA ladders [HiMedia]
- Tris Base [SRL Chemicals India]
- 6X Gel loading buffer [HiMedia]
- Hydrogen chloride [Hospital Store]
- Agarose [HiMedia]
- Ethidium bromide [SRL Chemicals India]

3.3.1 Preparation of reagents and compositions of solutions used

- **LB broth:** 10gms of LB broth powder was dissolved in 500mL of SDW (Conc.- 20gms/lit). Then it was thoroughly mixed, dispensed into culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes (file:///C:/Users/Arunita%20Ghosh/Downloads/M575.pdf).
- **50X TAE (Tris-Acetate EDTA) buffer [1 litre]:** 242 gm of Tris-base (MW = 121.14 g/mol) was dissolved in approximately 700 mL of DDW. Then 57.1mL of 100 % glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0) was added to the aforesaid mixture. The solution was adjusted to a final volume of 1L. The pH of this buffer was then adjusted to 8.5 using potassium hydroxide (KOH). Then stock solution was stored in a Borosil container at room temperature for future use (https://www.protocols.io/view/recipe-for-50x-tae-buffer-gtvbwn6?step=3).
- **1X TAE (Tris-Acetate EDTA) buffer [500mL]:** This was prepared using 49 parts of DDW water with 1 part of 50X TAE (Tris-Acetate EDTA) buffer. The pH of the final solution was checked to be at 8.5.

3.4 Experimental methods

3.4.1 Bacterial culture

The forty (Asymptomatic= 20; Symptomatic=20) non-duplicate UPECs obtained from our previous study conducted on 200 hospitalized patients (**Ghosh and Mukherjee 2019)** were considered in the present study. Twelve out of the 20 symptomatic isolates were found to be associated with patients suffering from acute or chronic cystitis. The remaining 8 were isolated from patients suffering from pyelonephritis (Chapter 2) (**Ghosh and Mukherjee 2019)**. All of the aforesaid isolates were MDR except one asymptomatic isolate (**Ghosh and Mukherjee 2019)**. Sixteen UPECs from each of the asymptomatic and symptomatic groups were ESBL producers and the remaining isolates showed BLIR phenotype (Chapter 2). Bacterial cultures were prepared from the glycerol stocks of each of the

40 samples stored at the -80°C at the Department of Biochemistry and Medical Biotechnology, School of Tropical Medicine, Kolkata, using previous protocols as described by Zhang and Poh **(Zhang and Poh 2018)**, but with minor modifications. *E. coli* cultures were grown in absence of any antibiotics. This study protocol was approved by the institutional ethical committee.

3.4.2 Isolation of bacterial total DNA

Total DNA was prepared from each of the 40 isolated UPECs by boiling method as described by Basu et al. (**Basu et al. 2013)** with certain modifications. Briefly, bacterial cells from 2mL of overnight culture were harvested by centrifugation at 8000 rpm for 6 mins. The cell pellet was then resuspended in 100µL of double distilled water and lysed by heating at 100°C for 10 mins, and chilled on ice for 5 mins. Cellular debris was removed by centrifugation at 10,000 rpm for 30 mins at 4°C. Equal volume of chloroform was added to the supernatant collected and centrifuged at 10,000 rpm for 10 mins at 4^{o}C. The aqueous layer was used as template for various genotypic studies.

3.4.3 Phylogenetic background analysis

The new quadruplex PCR assay as described by Clermont et al. **(Clermont et al. 2013) (Table 3.1)** was used to assign the UPECs to one of the eight phylogenetic groups: A, B1, B2, C, D, E, F and clade I. Each of the PCR assay was performed in 20µl reaction volume containing 1 µl of the total DNA as template, 0.5 µM of each primer (GCC biotech, India), 2.0 µl 10 X PCR buffer (Invitrogen, Thermo Fisher Scientific), 150 µM of dNTPs (Invitrogen, Thermo Fisher Scientific), 1.5 mM of the MgCl2 (Invitrogen, Thermo Fisher Scientific) and 1 U of the Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific). Amplicons generated were separated by 1.5% agarose gel electrophoresis and visualized using Ethidium bromide by Gel documentation system (BIO-RAD, USA) as described in section 3.4.5. The target phylogeny genes with their respective primer sequences were depicted below and marked in green. The primers and PCR conditions used for this study were shown in **Table 3.2**.

Table 3.1: Quadruplex genotypes and steps required for assigning UPECs isolates to different phylogroups.

Sl No.	*arpA* (400 bp)	*chuA* (288 bp)	*yjaA* (211 bp)	TspE4.C2 (152 bp)	Phylogroup	Next step (Clermont et al. 2013)
1	+	−	−	−	A	
2	+	−	−	+	B1	
3	−	+	−	−	F	
4	−	+	+	−	B2	

5	**−**	**+**	**+**	**+**	**B2**	
6	**−**	**+**	**−**	**+**	**B2**	
7	**+**	**−**	**+**	**−**	**A or C**	**Screening with C-specific primers. If C+ then C, else A.**
8	**+**	**+**	**−**	**−**	**D or E**	**Screening with E-specific primers. If E+ then E, else D.**
9	**+**	**+**	**−**	**+**	**D or E**	**Screening with E-specific primers. If E+ then E, else D.**
10	**+**	**+**	**+**	**−**	**E or Clade I**	**Screening with E-specific primers. If E- then Clade I**
11	**−**	**−**	**+**	**−**	**Clade I or II**	**Screening with cryptic Clade I and II specific primers.**
12	**−**	**476 bp***	**−**	**−**	**Clade III, IV or V**	**Screening with cryptic Clade III, IV and V specific primers.**
13	**−**	**−**	**−**	**+**	**Unknown**	**Performing MLST.**
14	**−**	**−**	**+**	**+**	**Unknown**	**Performing MLST**
15	**+**	**−**	**+**	**+**	**Unknown**	**Performing MLST**
16	**+**	**+**	**+**	**+**	**Unknown**	**Performing MLST**
17	**−**	**−**	**−**	**−**	**Unknown**	**Confirming *Escherichia* identification using *uidA* or *gadA*/*B*, if positive screening using cryptic clade primers and/or performing MLST**

(*) The quadruplex PCR reaction will result in strains belonging to cryptic clade III, IV or V yielding a 476 bp PCR product. If this outcome eventuates then such strains should be screened using the cryptic clade detection primers **(Clermont et al. 2011b)**.

The target phylogeny genes with their respective primer sequences are written as under:

***arpA* [400 bp (*aceK*)]**

***Escherichia coli* UMN026 chromosome, complete genome**

GenBank: CU928163.2

GenBank Graphics

>CU928163.2: 4707218-4708954 *Escherichia coli* UMN026 chromosome, complete genome

ATGCCGCGTGGCCTGGAATTACTGATTGCACAAACCATTTTGCAAGGTTTCGACGCTCAGTATGGTCGATTCCTCGAAGTG

ACTTCCGGGGCGCAGCAGCGTTTCGAACAAGCCGACTGGCACGCTGTCCAGCAGGCGATGAAAAACCGTATCCATCTTTAC

GATCATCACGTGGGTCTGGTCGTGGAGCAACTGCGCTGCATTACCAACGGCCAAAGCACGGACGCGGCATTTTTACTGCGC

GTCAAAGAGCATTACACCCGGCTGTTGCCGGATTACCCGCGCTTCGAGATTGCGGAGAGCTTTTTTAACTCCGTGTACTGT
CGGTTATTTGACCACCGCTCGCTTACTCCCGAGCGGCTTTTTATCTTTAGCTCCCAGCCAGAGCGCCGCTTTCGTACCATT
CCCCGCCCGCTGGCGAAAGACTTTCACCCCGATCACGGCTGGGAATCTCTGCTGATGCGCGTTATCAGCGACCTGCCGCTG
CGCCTGCGCTGGCAGAATAAAAGCCGTGACATTCATTACATCGTTCGCCATCTGACGGAAACGCTGGGGACAGACAACCTC
GCGGAAAGTCATTTACAGGTGGCGAACGAACTGTTTTACCGCAATAAAGCCGCCTGGCTGGTAGGCAAACTGATCACGCCT
TCCGGCACATTGCCATTTTTGCTGCCGATCCACCAGACGGACGACGGCGAGTTATTTATTGATACCTGCCTGACGACGACC
GCCGAAGCGAGCATTGTTTTTGGCTTTGCGCGTTCTTATTTTATGGTTTACGCGCCGCTGCCCGCAGCACTGGTCGAGTGG
CTACGGGAAATTCTGCCAGGTAAAACCACCGCTGAATTGTATATGGCTATCGGCTGCCAGAAGCATGCCAAAACCGAGAGC
TACCGCGAATATCTCGTTTATCTACAGGGCTGTAATGAGCAGTTCATTGAAGCGCCGGGTATTCGTGGAATGGTGATGTTG
GTGTTTACGTTGCCGGGTTTTGATCGAGTATTCAAAGTCATCAAAGACAAGTTCGCGCCGCAGAAAGAGATGTCTGCCGCT
CACGTTCGTGCCTGCTATCAATTGGTGAAAGAGCACGATCGCGTGGGCCGAATGGCGGACACCCAGGAGTTTGAAAACTTT
GTGCTGGAGAAGCGGCATATTTCCCCGGCATTAATGGCATTACTGCTCCAGGAAGCAGCGGAAAAAATCACCGATCTCGGC
GAACAAATTGTGATTCGCCATCTTTATATTGAGCGGCGGATGGTGCCGCTCAATATCTGGCTGGAGCAAGTGGAAGGTCAG
CAGTTGCGCGATGCCATTGAAGAATACGGTAACGCTATTCGCCAGCTTGCCGCTGCTAACATTTTCCCTGGCGACATGCTG
TTTAAAAACTTCGGTGTCACCCGTCACGGGCGTGTGGTGTTTTATGATTACGATGAAATTTGCTACATGACGGAAGTGAAC
TTCCGCGACATCCCGCCGCCGCGCTACCCGGAGGACGAACTTGCCAGCGAACCGTGGTACAGCGTCTCGCCGGGCGATGTT
TTCCCGGAAGAGTTTCGCCACTGGCTATGCGCTGACCCCCGCATTGGGCCACTCTTTGAAGAGATGCACGCCGACCTGTTC
CGCGCTGATTACTGGCGCGCGCTACAAAACCGTATCCGTGACGGGCATGTGGAAGATGTTTATGCGTATCGGCGCAGGCAA
AGATTTAGCGTACGGTATGGGGAGATGCTTTTTTGA

(https://www.ncbi.nlm.nih.gov/nuccore/CU928163.2?report=graph)

chuA

Escherichia coli chuA gene, complete cds

GenBank: U67920.1

GenBank Graphics

>U67920.1:491-2473 *Escherichia coli* ChuA gene, complete cds

ATGTCACGTCCGCAATTTACCTCGTTGCGTTTGAGTTTATTGGCCTTAGCTGTTTCTGCCACCTTGCCAACGTTTGCTTTT
GCTACTGAAACCATGACCGTTACGGCAACGGGGAATGCCCGTAGTTCCTTCGAAGCGCCTATGATGGTCAGCGTCATCGAC
ACTTCCGCTCCTGAAAATCAAACGGCTACTTCAGCCACCGATCTGCTGCGTCATGTTCCTGGAATTACTCTGGATGGTACC
GGACGAACCAACGGTCAGGATGTAAATATGCGTGGCTATGATCATCGCGGCGTGCTGGTTCTTGTCGATGGTGTTCGTCAG
GGAACGGATACCGGACACCTGAATGGCACTTTTCTCGATCCGGCGCTGATCAAGCGTGTTGAGATTGTTCGTGGACCTTCA
GCATTACTGTATGGCAGTGGCGCGCTGGGTGGAGTGATCTCCTACGATACGGTCGATGCAAAAGATTTATTGCAGGAAGGA
CAAAGCAGTGGTTTTCGTGTCTTTGGTACTGGCGGCACGGGGGACCATAGCCTGGGATTAGGCGCGAGCGCGTTTGGGCGA
ACTGAAAATCTGGATGGTATTGTGGCCTGGTCCAGTCGCGATCGGGGTGATTTACGCCAGAGCAATGGTGAAACCGCGCCG
AATGACGAGTCCATTAATAACATGCTGGCGAAAGGGACCTGGCAAATTGATTCAGCCCAGTCTCTGAGCGGTTTAGTGCGT
TACTACAACAACGACGCGCGTGAACCAAAAAATCCGCAGACCGTTGGGGCTTCTGAAAGCAGCAACCCGATGGTTGATCGT
TCAACAATTCAACGCGATGCGCAGCTTTCTTATAAACTCGCCCCGCAGGGCAACGACTGGTTAAATGCAGATGCAAAAATT
TATTGGTCGGAAGTCCGTATTAATGCGCAAAACACGGGGAGTTCCGGCGAGTATCGTGAACAGATAACAAAAGGAGCCAGG
CTGGAGAACCGTTCCACTCTCTTTGCCGACAGTTTCGCTTCTCACTTACTGACATATGGCGGTGAGTATTATCGTCAGGAA
CAACATCCGGGCGGCGCGACGACGGGCTTCCCGCAAGCAAAAATCGATTTTAGCTCCGGCTGGCTACAGGATGAGATCACC
TTACGCGATCTGCCGATTACCCTGCTTGGCGGAACCCGCTATGACAGTTATCGCGGTAGCAGTGACGGTTACAAAGATGTT

GATGCCGACAAATGGTCATCTCGTGCGGGGATGACTATCAATCCGACTAACTGGCTGATGTTATTTGGCTCATATGCCCAG
GCATTCCGCGCCCCGACGATGGGCGAAATGTATAACGATTCTAAGCACTTCTCGATTGGTCGCTTCTATACCAACTATTGG
GTGCCAAACCCGAACTTACGTCCGGAAACTAACGAAACTCAGGAGTACGGTTTTGGGCTGCGTTTTGATGACCTGATGTTG
TCCAATGATGCTCTGGAATTTAAAGCCAGCTACTTTGATACCAAAGCGAAGGATTACATCTCCACGACCGTCGATTTCGCG
GCGGCGACGACTATGTCGTATAACGTCCCGAACGCCAAAATCTGGGGCTGGGATGTGATGACGAAATATACCACTGATCTG
TTTAGCCTTGATGTGGCCTATAACCGTACCCGCGGCAAAGACACCGATACCGGCGAATACATCTCCAGCATTAACCCGGAT
ACTGTTACCAGCACTCTGAATATTCCGATCGCTCACAGTGGCTTCTCTGTTGGGTGGGTTGGTACGTTTGCCGATCGCTCA
ACACATATCAGCAGCAGTTACAGCAAACAACCAGGCTATGGCGTGAATGATTTCTACGTCAGTTATCAAGGACAACAGGCG
CTCAAAGGTATGACCACTACTTTGGTGTTGGGTAACGCTTTCGACAAAGAGTACTGGTCGCCGCAAGGCATCCCACAGGAT
GGTCGTAACGGAAAAATTTTCGTGAGTTATCAATGGTAA

(https://www.ncbi.nlm.nih.gov/nuccore/U67920.1?report=graph)

yjaA

***Escherichia coli* UTI89, complete genome**

GenBank: CP000243.1

GenBank Graphics

>CP000243.1:4461423-4461842 *Escherichia coli* UTI89, complete genome

TTGCATATCAATTATTTGCATGATGAAGGGAATCTCATGTCAGTTCTGTATATCCAAATTCGTCGTAATCAAATTACTGTT
CGCGATCTTGAAAGCAAACGTGAAGTGTCAGGAGATGCTGCCTTCAGTAACCAGCGCCTGTTAATCGCCAATTTCTTTGTT
GCAGAAAAAGTTCTGCAAGATCTTGTTCTGCAACTCCACCCACGTTCAACCTGGCATTCTTTTTTGCCAGCAAAACGTATG
GATATTGTTGTGAGCGCGCTGGAAATGAATGAGGGCGGTTTGTCACAGGTTGAGGAACGCATTCTTCATGAAGTGGTCGCA
GGGGCAACGTTAATGAAATATCGCCAGTTCCACATCCATGCGCAATCAGTGGTACTCAGTGATAGTGCGGTCCTGGCAATG
TTTAAGCAGAAATAA

(https://www.ncbi.nlm.nih.gov/nuccore/CP000243.1?report=graph)

TspE4.C2

***Escherichia coli* UTI89, complete genome**

GenBank: CP000243.1

GenBank Graphics

>CP000243.1:4357883-4358913 *Escherichia coli* UTI89, complete genome

TTACTTGACTGAAAAGGTTTTCAATCTGAAAACCTTTTCTGGGCCCTGGTCCGGAGTTACCTTCCCGCTCTCCAGGCAACA
TCCCGTAGCGCCGATCGCGCAGCCTCACTGATACCACCTAACTGAAAAAATCCGTGGATAACGCCAAGGTAGCGTTGGCAG
GTGCATTGCACTCCTTGCCCTGTCATGCGGTGATACAACGCTTCACCTTCATCGCAAAGTGGGTCGTATTCAGCGGTAATG
ATATGTACAGGTGGCAAGCCATTGAAATCATTGCGCCAAATAGGGCTGGCTTCAGGATGCCTGCGTTCAATGCCTGGCAGG
TACATCTCAAAGCCGCTCAGTAAAGTGTCGCGTGTGATGATGTAATCGTGACCATTGCGTATGTAGCTTTCGAAATGCGCA
GTCGCATCAAGCATAGGATAAATTAAGATGAGTTGTGCTGGTTGCCACTCGCCAGCACGCTTCAATCGCAAAGCAGTTACC
AGCGCCAGATGCCCTCCCGCACTGTCACCGGCAAGAGTTATTCGTTGTTTATCAACGCCGAGTCGTTCGGCGTGTTGCCGG
ACTAATTCTGCACCTCGTTGTGCATCATCATGGGCGGCGGGGAAAGTATGTTCCGGTGCCAGCCGATACTGGACGGCAATA
ATCCGACATTGCCCGTAATAGGCTAACTGGCGCAGCTGGTTGTCGTGAGTTGCGAACCCGCCGCTAACAAAACAGCCACCG
TAGTAATAAATTATCGTCGGGAGTAATGTCGGGGCATTCAGCGGTGACACTATTCGTAAGGTCATCCCTTCAAGTTCGATA

GTCTGAATATCTACCCGCGTTTCTGTCTCACCCGCAAGGACAGCGCTGGCGATATAGCCCTCTCTGCGCTGCGTAATACTT
TGTTGGCGCGATGAGGGGCGACCCGCAGCGATAAACTCTTCGACTAACTCTGCAATTCCTTTTTCCAGTGCCATAGTAGAA
CTCATTTTGCTGTATGAATGTACAGTTTTATACACCCGTTTTTGCTGGATGGATACAGG

(https://www.ncbi.nlm.nih.gov/nuccore/CP000243.1?report=graph)

arpA [301bp (*arpAgpE*- group E specific)]

Escherichia coli UMN026 chromosome, complete genome

GenBank: CU928163.2

GenBank Graphics

>CU928163.2:4708923-4711109 *Escherichia coli* UMN026 chromosome, complete genome

TTAGCGTACGGTATGGGGAGATGCTTTTTTGAGTAAAGCTTCCATATAATTTTTCTCCGCAATGTATCGAGGGTTATCCGT
AAAGCCAAAGCTTTCAGCCATCTTATTTATCGTATTAAGGATTAATTCAGCAATAACCCGGTGATCCAATTCAAAAGCCAA
CTCAAAGGCAGAGTATTTTTGTGGCGCTTTGTGTTGCCAAAAATCCATAATATCTTCAGCAGTAAATCCAAACAGGCGTGC
ATGGTCAGATAAAGCAAGATAAACCGTCTCTACAACGTTTTGTTGTTTATGCTGTATCGCTGAAAACAAACCGGGATATTC
ATTAGAGTTATTTGCCAGGAGGAGGGGCTTCATATTTTTTTTATCGAATTTAAACGTATTAAACAGAGTGGGTAATACGTT
AAAAATAGTCTTAATAACGTTCATATGTCCGCGCTGCATGGCCATAAACAAACCCGTGTCGCGCGCAAGACTTTTAGCGGT
CAGAAGATCGACAATATCGGAAGCTGAAATGTTAATTTCCTGGGCCAGACAGGGTAATGCTTCGAGAATAACTTTCACGAT
ATCGCTATGTCCATTTTGCATCGCCAGGTATAGTCCTGGGCAACCATAAAAATCCTTTGCCTTCAGGAGATCGAGTACCTG
TTCTTTAGTCAAATGACATGTGCGAATTAACAAAGGTAACGCGTCCAAAACAATTTTCAGCATGTCGGCATCACCATTCGC
CATAACATGGTATAAAACATGGCTGGACGTTCTATTTTTGGCACTCAGGAATTTATACACCTGTTCATTATCTAAATGATG
TGTTGCGGCCAGTTTAGGTAAGACGTTCAGAATCGAGGTTACAACATTTTTATCCTTACGTGATATCGCTAAAAATAATCC
AGAAAAACCATTTTTATCTTTTGCCTCCAGAATATGCATGAGATTTTTTTTCGAGAGTAACCCTTCATATCCTGGCTCTGA
CAATGAATTGAAAATAGTCTCAACGATATCCGCATTACCATAATTTATAGCCAAATATAAACCGGGGAGGTTAATACTATT
ATAGGCAGTCAACATTTCTGTTCTATGTAGTTCTTGCATTTTCTGGAGTTGAGTCATCAGTTGCGTAAGTTGATGATTCTG
ACCAATTGCCATTAGCAAATTCATCGTTGCAGGTGATAATGGAGGGGTTATTTCATCCACAGATCCTTTTAGCATCAATAG
CTGTTCTTTCGGAAGTGCTGGAATTATTGCAACAGATTGGTTAATAACATGATCATTTGACCATTTCAGGAGGCTATAATC
TACATTCATAAAGTCCATCAATGAATAGTGTTTAATATCCTCTTTACTTTCGCTCATGATTCTTATTTTATCGTTAGTTAC
ATTTGGATCATAAACCGAAACCACGTAATGGGTACATCCTTCAGTTGTGTTCTTTATTCTTAGCCGGACCGTCAAAGCATG
GTTATCCACCAATAATATGGCTGCCATAACACTGATGCCATTTGATGCCATCTTGTCAAAATATGCTTTCATAACTAGCCC
GAAATCACCGCAGGCAGCAACGCAACGCCCCTCAGGACGGCTGGAAATCAACGCCAAAGTCTGGTTTTTGCATTTTTTGTT
GAAGTGAGTGAGAAATTTTTCAACTGAAGAGTAATCATCTTTATGAGGTTTGTAGTCAACATTCTGATAAATATCATTAAT
ATATTGTGCAGCAATGTGTCGGCATACTATACGGTTTTGGGGTCGCCCCGGAAAATAACATTGACCATTAAGTTTAATTCT
TGGAAATTCTTTTTCTTTTCTATCTGATTTTAGATTTAATTTTTGTTTATCAAGAGCGTCTGAGCGTTGAGGTAAAATTTC
CATCAGGTATTCTTCAAATATTGGAGCAATGGTTTGAGTGAGTGTCGTGTTTTCATTTTTATTAATTATCGTAATTTCTTT
TTTATCAGTCTGTAAGACGGACGTGGAAAACTTAATGACAAAATCCTTGTTACTAAAGATATGACGACCATTTTGTCTACA
GTTCTCTGAGAAGCTTTTTAATAGAGGCGTCGCCAGGTCCTTGCCAGAAAATTTATCCTCGAGTTCTTTATAAAACAATTC
ACTCAGGGTTTGGTGTTCATTTGTCCGGGCTGTATTATTAATATTTGCAGAGAAAGAACTACGAGGAATACGAGTAATCAT

(https://www.ncbi.nlm.nih.gov/nuccore/CU928163.2?report=graph)

trpA [219bp (*trpAgpC*- group C specific)]

Escherichia coli UMN026 chromosome, complete genome

GenBank: CU928163.2

GenBank Graphics

>CU928163.2:1568249-1569055 *Escherichia coli* UMN026 chromosome, complete

ATGGAACGCTACGAATCTCTGTTTGCCCAGTTGAAGGAGCGCAAAGAAGGCGCATTCGTTCCTTTCGTCACGCTCGGTGAT
CCGGGCATTGAGCAGTCGCTGAAAATTATCGACACGCTAATTGAAGCCGGTGCTGACGCGCTGGAGTTAGGTATCCCCTTC
TCCGACCCACTGGCGGATGGCCCGACGATTCAAAACGCCACACTGCGTGCCTTTGCGGCAGGTGTGACCCCGGCACAGTGC
TTTGAGATGCTGGCACTGATTCGCCAGAAGCACCCGACCATTCCCATCGGCCTTTTGATGTATGCCAACCTGGTGTTTAGC
AAAGGCATTGATGAGTTTTATGCCCAGTGCGAGAAAGTCGGCGTCGATTCGGTGCTGGTTGCCGATGTGCCAGTGGAAGAG
TCCGCTCCCTTCCGCCAGGCCGCGTTGCGTCATAATGTCGCACCTATCTTTATTTGCCCGCCGAATGCCGACGATGATTTG
CTGCGCCAGATAGCCTCCTACGGTCGTGGATACACCTATTTATTGTCACGTGCGGGCGTGACCGGCGCAGAAAACCGCGCC
GCGTTACCCCTCAATCATCTGGTCACGAAGCTGAAAGAGTACAATGCTGCGCCTCCATTGCAGGGCTTTGGTATTTCCGCC
CCGGATCAGGTAAAAGCAGCGATTGATGCAGGAGCTGCAGGCGCGATTTCAGGTTCAGCCATTGTTAAAATCATCGAGCAA
CATATTAATGAGCCAGAGAAAATGCTGGTGGCACTGAAAGCTTTTGTACAACCGATGAAAGCGGCTACGCGCAGTTAA

(https://www.ncbi.nlm.nih.gov/nuccore/CU928163.2?report=fasta&from=1568249&to=1569055)

trpA[489bp]

Escherichia coli UMN026 chromosome, complete genome

GenBank: CU928163.2

GenBank Graphics

>CU928163.2:1568127-1569055 *Escherichia coli* UMN026 chromosome, complete genome

GTGGTTAACCTTTCCGGTCGCGGCGATAAAGACATCTTCACCGTTCACGATATTTTGAAAGCACGAGGGGAAATCTGATGG
AACGCTACGAATCTCTGTTTGCCCAGTTGAAGGAGCGCAAAGAAGGCGCATTCGTTCCTTTCGTCACGCTCGGTGATCCGG
GCATTGAGCAGTCGCTGAAAATTATCGACACGCTAATTGAAGCCGGTGCTGACGCGCTGGAGTTAGGTATCCCCTTCTCCG
ACCCACTGGCGGATGGCCCGACGATTCAAAACGCCACACTGCGTGCCTTTGCGGCAGGTGTGACCCCGGCACAGTGCTTTG
AGATGCTGGCACTGATTCGCCAGAAGCACCCGACCATTCCCATCGGCCTTTTGATGTATGCCAACCTGGTGTTTAGCAAAG
GCATTGATGAGTTTTATGCCCAGTGCGAGAAAGTCGGCGTCGATTCGGTGCTGGTTGCCGATGTGCCAGTGGAAGAGTCCG
CTCCCTTCCGCCAGGCCGCGTTGCGTCATAATGTCGCACCTATCTTTATTTGCCCGCCGAATGCCGACGATGATTTGCTGC
GCCAGATAGCCTCCTACGGTCGTGGATACACCTATTTATTGTCACGTGCGGGCGTGACCGGCGCAGAAAACCGCGCCGCGT
TACCCCTCAATCATCTGGTCACGAAGCTGAAAGAGTACAATGCTGCGCCTCCATTGCAGGGCTTTGGTATTTCCGCCCCGG
ATCAGGTAAAAGCAGCGATTGATGCAGGAGCTGCAGGCGCGATTTCAGGTTCAGCCATTGTTAAAATCATCGAGCAACATA
TTAATGAGCCAGAGAAAATGCTGGTGGCACTGAAAGCTTTTGTACAACCGATGAAAGCGGCTACGCGCAGTTAA

(https://www.ncbi.nlm.nih.gov/nuccore/CU928163.2?report=fasta&from=1568127&to=1569175)

Table 3.2: Primer sequences and PCR conditions used for the amplification of the phylogeny genes.

Sl no.	Target genes	Primer ID with sequences (5'-3')	PCR conditions (Time)	No. of cycles	Amplicon size (bp)	References
1	*arpA*	*aceK* F.P- AACGCTATTCGCCAGCTTGC *arpA* R.P- TCTCCCCATACCGTACGCTA	95°C (30 sec) 50°C (30 sec) 72°C (1min)	30	400	Clermont et al. 2013
2	*chuA*	*chuA* F.P- ATGGTACCGGACGAACCAAC *chuA* R.P- TGCCGCCAGTACCAAAGACA	95°C (30 sec) 62°C (30 sec) 72°C (1min)	30	288	Clermont et al. 2013
3	*yjaA*	*yjaA* F.P- CAAACGTGAAGTGTCAGGAG *yjaA* R.P- AATGCGTTCCTCAACCTGTG	95°C (30 sec) 58°C (30 sec) 72°C (1min)	30	211	Clermont et al. 2013
4	TspE4.C2	TspE4.C2 F.P- CACTATTCGTAAGGTCATCC TspE4.C2 R.P- AGTTTATCGCTGCGGGTCGC	95°C (30 sec) 51°C (30 sec) 72°C (1min)	30	152	Clermont et al. 2013
5	*arpA* [group E]	*arpAgpE* F.P- GATGCCATCTTGTCAAAATATGC *arpAGpE* R.P- GAAAAGAAAAAGAATTTCCAAGA	95°C (30 sec) 53°C (30 sec) 72°C (1min)	30	301	Clermont et al. 2013
6	*trpA* [group C]	*trpAgpC* F.P- AGTTTTATGCCCAGTGCGAG *trpAgpC* R.P- TCTGCGCCGGTCACGCCC	95°C (30 sec) 58°C (30 sec) 72°C (1min)	30	219	Clermont et al. 2013
7	*trpA* [internal control]	*trpA* F.P- CGGCGATAAAGACATCTTCAC *trpA* R.P- GCAACGCGGCCTGGCGGAAG	95°C (30 sec) 60°C (30 sec) 72°C (1min)	30	489	Clermont et al. 2013

3.4.4 Agarose gel electrophoresis

On the basis of the sizes of the DNA fragments (phylogeny genes) to be separated, the concentration of agarose (0.8% or 1% or 1.5%) was weighed for gel electrophoresis of the PCR products. Then after weighing agarose, DDW and 1X TAE buffer was added and mixed by heating in a microwave oven until the agarose was dissolved completely. 5 µL of the stock EtBr (10mg/mL) solution was added to the gel and the entire mixture was poured into the gel tray (EtBr was used with caution as it is a known carcinogen.). The gel casting tray was previously prepared for the above-mentioned procedure using combs of different sizes according to the requirements (8 wells, 13 wells, 26 wells or 52wells). Any bubbles if formed after pouring the gel was removed and allowed gel to cool for 30 min at room temperature. Then after the gel got solidified, combs were removed and the casting tray was placed in electrophoresis tank (chamber) filled with 1X TAE buffer. PCR products and DNA

ladders were mixed with 6X gel loading buffer and loaded in the different wells of the solidified gel. The entire set up was then connected to the power supply and electrophoresed at 100V for 40 -50mins.. After separation of the DNA fragments, gel bands (DNA fragments) were analyzed and documented using gel documentation system (https://www.thermofisher.com/in/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-electrophoresis/agarose-gel-electrophoreis/agarose-gel-electrophoresis-protocols-e-gel-ex-agarose-gel-and-ultrapure-agarose.html).

3.4.5 PAI marker detection by Multiplex PCR assay

All 40 UPECs irrespective of their asymptomatic or symptomatic nature were analyzed by two multiplex PCR based assay to detect the presence of eight most investigated PAIs in UPECs: PAI I536, PAI II536, PAI III536, PAI IV536, PAI ICFT073, PAI IICFT073, PAI IJ96 and PAI IIJ96. All the PAI markers used in this study were evaluated using primers **(Table 3.3)** as described by Sabate et al. **(Sabate et al. 2006)**. PCR conditions used in this study were as described by Sabate et al. **(Sabate et al. 2006)** and Najafi et al. **(Najafi et al. 2018)** but with minor modifications. Briefly, both the multiplex PCR reaction A (PAI III536; PAI IV536; PAI IICFT073) and multiplex PCR reaction B (PAI I536; PAI II536; PAI ICFT073; PAI IJ96; PAI IIJ96) contained 20 µl reaction volumes comprising of 2 µl of the total DNA as template, 250 µM dNTPs (Invitrogen), 1.5 mM MgCl2 (Invitrogen), 0.5 µM of each primer (GCC Biotech, India) 1U Taq DNA polymerase (Invitrogen) and 2.0 µl 10 X PCR buffer (Invitrogen). Initially, the nucleotide sequence of an amplified PCR product of each of the PAIs, representing a single isolate, was determined using ABI 3100 automated genetic analyzer. Once a PCR product for an individual PAI marker was confirmed, the DNA from this isolate was used as a positive control for all subsequent PCRs. Both PCRs were tested using UPEC strains 536 and J96 as PAI marker controls. The cycling conditions for each multiplex reaction were as follows: Multiplex A- Initial denaturation at 95 °C for 5 mins and 30 cycles each of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, amplification at 72 °C for 60 s and final extension at 72 °C for 10 min. Multiplex PCR B- Initial denaturation at 95 °C for 5 mins and 30 cycles each of denaturation at 95 °C for 60 s, annealing at 55 °C for 60 s, amplification at 72 °C for 60 s and final extension at 72 °C for 10 min. In majority of the cases multiplex PCR B could not amplify PAI I536, so separate uniplex PCR assay was performed for the same. The PCR products obtained after amplification were analyzed and viewed by the procedure of agarose gel electrophoresis as described in the section 3.4.4.

The target PAI markers and the respective primer sequences were depicted below and marked in green.

PAI I536

***Escherichia coli* UTI89, complete genome**

GenBank: CP000243.1

GenBank Graphics

>CP000243.1:4806146-4808046 *Escherichia coli* UTI89, complete genome

TAATGCCGGAGATTCATTGTCATTATTTAAACGTATGCTAATACTCTTTTGAGTCGCCGGATAAACAACACGGGTTCCCAT
GATAACTACACTACCCTGAACAACTGCAGATACAGATAGAGTAAAAAAAAACAGCACAAACCTTAGCATGGTATCTCCAGA
AGAAAACAGGGCAGTATTTCCTGCCCAGAAATACAAAAGAGTAAAAAGTTTTTTATTCGTAGGCAATGGTATAATGGACCG
TCGCTTTTACATCCCCTGCGGTAGATTGTCCTGTTGCATAATACTGGGCCATATAACGCAATGTGGCACTTCCATCAGATC
CGATTGTCTCAGACTGGACATCTTGCCCATTATCCGCTTCCCCCAAAAGAATAGTTGAAGTTCCATTACTATTTAGCAACT
GAATCTGTACATTATCCGCTTTAGTTGGAGTAGCAGTATTTTTTAAATTATGTGTAGCTAAGTCAATGTTACTTGAAGGCT
CAAAGTACGCTTTAACATTCTGAGCACTATTCATACCAGTTGCACAACCAGTTAAACGAATGGCAAAGGGTGTCAGCCCTG
ACGTAGCAGCATTGTCTTTTAGAGATGAAGTGGCAACAGTGGGTAAAGTTACTGCTAAGTCTTTGTCGCTTGTATTAACTG
TGCAGGTCTGAGCTACAACTTTACCGGTAAACGTAATTGTTCCGTCATAGGCCATAGCTGAACCAGCAAACACAGCAGAAA
CAAATGTAGCCAATGCTATAACTTTTATTTTCATAAAATGAATTCCTGTTTAATTCCAGTATTGATCATTTGTTCAGCAAT
CATCCCCAACAAAACAATCATTTTCAAAATGTTTTTACCGATCGATAACCAGCGTATGATAGATTGCACCTATCATGATTG
CTAAAACGATCGGGAAAAACGATCAAAAACCATATTTATTGTGTTGGTAATGACAAAAGATATGCTTTACCCTGAAATGAG
CGACCTATTCATGAAAATATGTAGGTCTGTATTTGATTACTATCATTGCTATATTTCCACTATCCAATTTATATTTCATGA
TTAAAATATACCTTTTTACACTATTATTTATTTGTTGCAGCTTGCCTGGCTTTATCTTATTCCGACTATTTTATGGTAGAT
ACAGAATACAATTAATTAAACTTATTTAAAGATTTTATAAATACCATATTGGAGTTGACCGATAGATACCTACTAACAAGA
GCAATCACCACCACCCCATGAGGTGGTTTAGGAATACAATCAATAAACAACATCCATGCCCGGCGACGTACATACCTGTTT
GCTATGATATCTGTTACGCTACGCTTGCTAATTTACTGAAACTCAGCGTCTGTCGACGGAGATTCGTCCGGGCCCTGATAC
AACAAGGGCAAGAAAACCACCCGAAATACAGATATTCTTATAAAAATGGATCATATTTCCATGTGCAAGTTCAGCTGGCAT
CGTCCAGAATGCGTGTCCAAGAAATGAAGCAAGCACGGTATACAGGCACAGAATAATGCTCACTGGCCGGGTGAAAAAACC
AAAAACAATCATTAATGCTCCAACGATTTCGACAAGGACCACTATTGCTGCAGTAATCGCCGGAAATATAAGCCCAAGAGA
GGCCATTTTATCGATAGTGCCAGTGAATGATAGCAGCTTGGGAACGCCGGATATCATATAAAGGCATGCCAGCATCAGACG
GGCAAGGAGCAACAATGCCGACGTGTAATTTCCCATATTAAAATACCTGATTTTATCCACTATCAATGCTCAGTCTCCTTG
TTTCTGATAAAGCCCTGAGCCAAATCCTTAAGTGTACGAGCACCACTCAGTAACATTGCCGTCCTCAGTTCCGTCTTCAGG
TGCTCAATGACACCGGCAACGCCCCCGACACCACCTGC

(https://www.ncbi.nlm.nih.gov/nuccore/CP000243.1?report=graph)

PAI II536

***Escherichia coli* UTI89, complete genome**

GenBank: CP000243.1

GenBank Graphics

>CP000243.1:4840603-4841653 *Escherichia coli* UTI89, complete genome

CATGTCCAAAGCTCGAGCCTTTGTTCTTATTTTAGCCATATATCTATGAATCCTTATTAGTACAATTTTCTATGAGATGTA
GCCCAAATAGTCTAGCGAGTTCGCAAGGTACAGCATTGCCGATTTGCTTTGCCATTGAATTCAGCGAACCTTTAAAAACAT
AGCTTAAAGGAAATGTTTGTAATCTTGATGCTTCTCTTATGCTAATTGCTCTATGTTGAGTGGGGTCAGGATGCCCAAAAC
GACCATTGGAGTAACTATTACATTTCGTCGTAAGTGTAGGCGCAGGCTTATCCCAACTCATTCTTCCATAAGTATCTGTGT

GGCCATCATAATTTTTATGGCATTTATTAACTAACTCTTCTGGCCAATTTCTTCTATCCCCTCCTTCTGGAGTGTGCATAA
TTCTTTTTAGGTTAAGAGGGCTCAGTGTTCCAGCCCTATGTAAAGGATCTTTGGGGTCGGTTTCTCCTGAACATAACTTTG
TGAAGTCCTGGATATAATCTCGTACAGTTTTGAATGGGATTTTATTTTTACCATGGGTTATCTCTGGTAGGGTAACTTTAC
CTACTCGACTAGCTAAGAGCACGAGTCTTTTTCTTCTTTGGGGAATCCCATAGTTCTCAGCATTGGCTATAAAAGATATAT
AGTTATACTCTAACTCTTTAAGTAGCTTAATAAACTCCTGAAATGGGCCTTCTTTTTCTTCATCAATTTTTTGCATTCCAG
GAACATTTTCAAGCATAATATATTCAGGAAGAAGTTCTCTAATAAAACGATGAGTTTCATTTAGTAGATTTCTCCTTGAGT
CGTCACTAGTTTTATTTTTATTCTGTTGCGAAAATGGTTGACATGGTGCACATGCACTCAGTAACAAAGGCCGTTTAGCTT
TAATATCAATGATGTCGGAGATATCTTGAGGTTCGATTTTCCTAATATCATCTTGGATGAATTTTGCATCAGGGAAATTAG
CTTTAAATGTTTCTGATGCTTGTTGGTCAATATCTAATCCAAGCTCGATATCAAAGCCAGCCTGACGTAGCCCTTCACT

(https://www.ncbi.nlm.nih.gov/nuccore/CP000243.1?report=graph)

PAI III536

Escherichia coli

GenBank: X16664.4

GenBank Graphics

>X16664.4:17230-17530 *Escherichia coli* encoding determinant *sfa(I)*

CGGGCATGCATCAATTATCTTTGCAGGAAATGTTATTGCTACACACAATGATGTGCTGTCTCTACAGAATAGTGCTGCAGG
TAGTGCAACAAATGTAGGTATTCAGATATTGGATCATACAGGTACTGCAGTTCAATTTGACGGAGTGACTGCATCTACACA
ATTTACATTAACAGATGGCACCAATAAAATTCCTTTCCAGGCAGTTTATTATGCAACAGGTAAGTCAACGCCTGGTATTGC
CAACGCCGACGCCACCTTTAAAGTTCAGTACCAGTAATATCAGAACAGTGTAACGGAT

PAI IV536

Escherichia coli UTI89, complete genome

GenBank: CP000243.1

GenBank Graphics

>CP000243.1:2078978-2079328 *Escherichia coli* UTI89, complete genome

TCGGTAAGACGTGCCATCAGGAGGAAGAATGATTTCTGGCGCACCATCTCAGGATTCGCTGTTACCGGACAACCGCCACGC
GGCTGATTACCAACAATTACGCGAGCGGCTCATACAGGAACTGAATTTAACGCCGCAGCAGTTACATGAAGAGAGCAACCT
GATCCAGGCCGGCCTGGATTCCATAAGATTGATGAGATGGTTACACTGGTTTCGTAAAAATGGCTACCGCCTTACCCTTCG
CGAGCTGTATGCCGCCCCCACGCTGGCGGCATGGAACCAGTTAATGCTCAGCCGGTCGCCGGAGAACGCGGAAGAAGAAAC
GCCGCCCGACGAATCATCCTGGCCGAA

(https://www.ncbi.nlm.nih.gov/nuccore/CP000243.1?report=graph)

PAI ICFT073

Escherichia coli CFT073, complete genome

GenBank: AE014075.1

GenBank Graphics

>AE014075.1:3253167-3254995 *Escherichia coli* CFT073, complete genome

TGTCGGATATTTGAATGTCGGCTTGAAAAATAAGCCGACCATCTCTTTAATTAAGCACAATTCCGTGTAATGTATACGC
ATTAGTTAATCATCTTGTAACTGTTAAATCAGGCAAGGCAATGTTTGAAGTAGTTATTACTTCTGACGTGCCTTGCCTTTT
TTTTTGGAGCCATGGAATGATCATCGAAAAAGTCATGAACAATAATTGTGTGCAGGCATCGATGAATGGACAGGAGGTTAT

CATTTCTGGGCCTGGCGTCGGTTACAACAAAAAATATGGAATGTTGGTCCCTGAGCATCCGGCTAACCGGATTTTTTATGT
CAGAAATGAACAAAAAAACAAACTCTATAAATTGATTGAACATGTAGATATTGAGTATGTGTTTGTTGCCGAAAAAATAGT
GCAATATGCGGAGAAAAATCTCGAAAAAAATCTCAATCCATCGCTACTATTGATTCTTGCGGATCACATTTCGAATGCAAT
ATCCCGAGTCGTTTCAGGTATACAAATTAATAATGTTTTCCTTGATGAAATCAAAGCGTTGTACAAAGCAGAGTATGCGAT
AAGTCGCGATGCATTAACTATCATTAATGAGCAATTCAGCGTTCAACTTCCTGATGATGAGATTGGTTTTATAGCATTGCA
TATTTTAAATAATTATGAAAATTCAGTTGATTATGAATCAGTACGGATTATTGAGTTGTCGCAAATAATCACGGAGCTTAT
TGAAGTTGTTTATAACAGAAAGGTGGACAGAAGTTCATTTAACTATTCCAGATTTATGATGCACCTTAAATATTTTTCAAG
TCGCGTGTTATGCAATGAAAAAATAAAACAGAAAGATATTGGTGATATCTATGAACAGTTTCTTGAAAAGGACATCCTGTT
ACAGCGCGCAATTCATGAAATTGAACGGTATCTGTATGCCACTTTTAAATATGAATTAATTTTAGAAGAAAAACTATATCT
CTCTATTCGTACCAAAGTATTAATGGACTAATTATATATAACATTTATATGAGATAACCCACATGAAACAAAAGAAAGCCT
GGAGTTTTTTTCAGAGCCTGGGGAAGGCATTTATGTATCCCATTGCTCTGCTAAGTGTATGTGGCATGATGCTAGGGCTGG
GAAGTGGTTTAGCCAGTGATGATATGGCAAAGTTAATTCCATTTCTGGCTATTCCAATAATTAAAACCATACTTGATTTCA
TTGTTAGTCTTGGTTTGTTTGCCTTTGTTAATTTACCTGTATTGTTTGCGATAGCGATTCCCTTAGGATTATTAAAAGATA
AAGAGGATAAAGCCTATGGTGCTTTTTCTGGCTTAATTGGTTTTATGGCGATGCATCTGGGAACGAACTTTTATCTTAAAC
AGCACGACTTATTGGTCGTTGCTGACCAAATGTCGACACATGGGCAAACCATCATTCTGGGGATCCAGTCCTACAATACCA
GCGTGTTGGGGGGAATTGTTGCTGGGTTATTAGTCGCCAGCATGTATAAAAAGATCGTTAATTTACGCATTCCTGAATCGT
TAGGTTTTTATAGCGGCCCACGTCTGGTGCCTATCATTACACTGATTGTGATGAGTGGATTTGGTCTGATCATTCCTTTTA
TCTGGCCGCCGTTTTTCAATCTTTTCATGCTCATTGGACACTGGATTTCAACTTCCGGTCCTGTTGGTTATTTCTTCTATG
CAGTTGCCGAACGCGTGACGATTCCTTTTGGCTTAAACCATCTGGTGACGTCAGTTTTCCGCTTTACGCCAATCGGCGGTT
CGGCTGTGATTGGTGGCGAAGAATATTACGGCACCCTGAACATGTTTAT

(https://www.ncbi.nlm.nih.gov/nuccore/AE014075.1?report=graph)

PAI IICFT073

Escherichia coli CFT073, complete genome

GenBank: AE014075.1

GenBank Graphics

>AE014075.1:278252-279188 Escherichia coli CFT073, complete genome

ATGAGCGGGCTTACGATTAATGCGCTGTGCGCCGGTTACGGCAAACGGCGGATTATTGAGCATCTGTCGATTTCTACGCTG
CCGCGCGGCGAAGTCACTGTACTGCTGGGGCCTAATGGCTGCGGTAAATCAACGCTACTGCGCGCCCTGGCCGGGCTAAAT
CGCGCCAGTGGCGAAGCCTGGCTGAATGAAGAGAATCTCTTATCGCTGCCGTTTGCTCGTCGGGCTGAAAAAGTTGTGTTC
CTGCCGCAGTCCCTGCCGCAGGGCGTGCATTTACAGGTGCTGGAGTCGGTGGTTGTCGCCCAGCGCGCCTCCGGCGCCGGG
CAAAATCAGGCGCAGGCTATAGCGCTGCTCGAAGAGCTAGGCATCGCACATCTGGCAATGAACTACCTCGATAGCCTGTCC
GGCGGTCAGAAGCAGCTGGTGGGGCTTGCACAGTCGCTTATTCGCCGCCCCGCATTATTATTGCTGGATGAACCACTGAGC
GCGCTGGATCTCAATTATCAGTTCCATGTGATGGATGTTGTATCGCGCGAAACGCGAAGACGCAATATGGTTACGCTGGTC
GTCTTACACGATATCAATATCGCACTGCGGCATGCCGCCCAGGTCATCATGCTGAAAGAGGGGAAACTTATCGACAGCGGC
GACCCGCAAACGGTGATCCATGCAGAGAGCCTTGCGCAGGTATACGGCGTACGCGGGAGAGTTGAACGTTGTGCTCAGGGA
AGATCGATGGTGATAGTGGATGGTGCAATCGAAAAATAGTCGGATTAACGTGGTTGCTTCAGCCTCAGGTAAAGATCAATA
GGAAGTCTGTAATGCAACATATAGACCGCCTTAATGTCATTAAAGCACTTGTGCTTCTAGAAGATGAGCAGATTGTTCGTT
TTAACATCGCTGCGAACGATAACGCCTCGCAGATCCACATGCTCGT

(https://www.ncbi.nlm.nih.gov/nuccore/AE014075.1?report=graph)

PAI IJ96

***Escherichia coli* J96**

GenBank: M20146.1

GenBank Graphics

>M20146.1 complete cds, isolate J96

ATGATGCTTTAGCTGGATGGCACAATGTCATGTTTTATGCTTTTAACGACTATTTAACTACAAATGCTGGTAATGTTAAGG
TTATTGACCAACCTCAGCTATATATACCCTGGAATACAGGCTCTGCTACAGCAACTTATTATTCGTGCTCAGGTCCGGAAT
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TACAGCACCATTACTATGATCTCTGGCAGGATCATTATAAAATGCCTTACGATCAGATTAAGCAGCTACCTGCCACTAATA
CATTGATGTTATCATTCGATAATGTTGGGGGATGCCAGCCGTCAACACAAGTACTTAATATAGACCATGGGAGTATTGTGA
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TCAGAAATACACCACCAATATACAATAATAATAAATTTTCGGTTGGGTTAGGTAATGGCTGGGATTCGATAATATCTCTTG
ATGGGGTTGAACAGAGTGAGGAAATATTACGCTGGTACACAGCCGGCTCAAAAACAGTAAAGATTGAGAGCAGGTTGTATG
GTGAAGAGGGAAAGAGAAAACCCGGGGAGCTATCTGGTTCTATGACTATGGTTCTGAGTTTCCCCTGAATAAGATGATGGA
TTATCTGACTGGCTGTTCATCAGTCGGATAATGATGAAAACTGATGAGCAACAGGTTGTCGGGCAATGTCAGGATCC

(https://www.ncbi.nlm.nih.gov/nuccore)

Table 3.3: Primer sequences used for amplification of the PAI markers.

Sl no.	Target PAI markers	Primer sequences (5'-3')	Amplicon size (bp)	References
1	PAI I536	F.P- TAATGCCGGAGATTCATTGTC	1810	Sabate et al. 2006
		R.P- AGGATTTGGCTCAGGGCTTT		
2	PAI II536	F.P- CATGTCCAAAGCTCGAGCC	1042	Sabate et al. 2006
		R.P- CTACGTCAGGCTGGCTTTG		
3	PAI III536	F.P- CGGGCATGCATCAATTATCTTTG	162	Sabate et al. 2006
		R.P- TGTGTAGATGCAGTCACTCCG		
4	PAI IV536	F.P- AGGATTCGCTGTTACCGGAC	286	Sabate et al. 2006
		R.P- TCGTCGGGCGGCGTTTCTTCT		
5	PAI ICFT073	F.P- GGACATCCTGTTACAGCGCGCA	922	Sabate et al. 2006
		R.P- TCGCCACCAATCACAGCCGAAC		
6	PAI IICFT073	F.P- ATGGATGTTGTATCGCGC	421	Sabate et al. 2006
		R.P- ACGAGCATGTGGATCTGC		
7	PAI IJ96	F.P- TCGTGCTCAGGTCCGGAATTT	461	Sabate et al. 2006
		R.P- TGGCATCCCCCAACATTATCG		
8	PAI IIJ96	F.P- GGATCCATGAAAACATGGTTAATGGG	2300	Sabate et al. 2006
		R.P- GATATTTTTGTTGCCATTGGTTACC		

3.4.6 Virulence factors genotyping

All 40 UPECs irrespective of their asymptomatic or symptomatic nature were examined by individual PCR based assay for presence of following twelve virulence factor genes: *fimH, papC, papEF, papGI, papGII, papGIII, sfa, afa, hlyA, iucD, cdtB,* and *cnf1*. The first 10 aforesaid genes were amplified using gene specific primers and PCR conditions **(Table 3.4)** as described by Johnson and Stell **(Johnson and Stell 2000),** Tiba et al. **(Tiba et al. 2008)** and Basu et al. **(Basu et al. 2013)**. However *iucD and cdtB genes* were amplified with primers and PCR conditions as described in this study **(Table 3.4)**. Each of the virulent gene was detected by individual PCR in 20 µl of the reaction volume containing 1 µl of the total DNA as template, 150 µM of dNTPs (Invitrogen), 1.5 mM of MgCl2 (Invitrogen), 0.5 µM of each primer (GCC biotech, India), 1 U of Taq DNA polymerase (Invitrogen) and 2.0 µl 10 X PCR buffer (Invitrogen). The sequences, PCR conditions and the amplicon sizes of the desired PCR product for the specific target genes were shown in **(Table 3.4).** DNA sequencing was used to confirm the identity of the amplified PCR products and to establish positive controls initially. The nucleotide sequence of an amplified PCR product of each virulence gene, representing a single isolate, was determined using ABI 3100 automated genetic analyzer. Once a PCR product for an individual virulence gene was confirmed, the DNA from this isolate was used as a positive control for all subsequent PCRs. A reaction mixture containing DNA template from DH5α was used as a negative control in each PCR assay. The PCR products obtained after amplification were analyzed and viewed by the procedure of agarose gel electrophoresis as described in the section 3.4.4. The target virulence factor genes with their respective primer sequences were depicted below and marked in green. Cluster analysis on the prevalence and distribution of virulence factor genes were performed on the basis of Heat maps generated using R software package (version 3.2.5).

fimH

***Escherichia coli* UTI89, complete sequence**

NCBI Reference Sequence: NC_007946.1

GenBank Graphics

>NC_007946.1:4913555-4914457 *Escherichia coli* UTI89, complete sequence

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GGTGCGGCTTATGGCGGCGTGTTATCTAGTTTTTCCGGGACCGTAAAATATAATGGCAGTAGCTATCCTTTCCCTACTACC
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TTCCAGTTTGTGTGGAATATTTACGCCAATAATGATGTGGTGGTGCCCACTGGCGGCTGCGATGTTTCTGCTCGTGATGTC
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GTACAGTTGACGCGCAACGGTACGATTATTCCAGCGAATAACACGGTATCGTTAGGAGCAGTAGGGACTTCGGCGGTAAGT
CTGGGATTAACGGCAAATTACGCACGTACCGGAGGGCAGGTGACTGCAGGGAATGTGCAATCGATTATTGGCGTGACTTTT
GTTTATCAATAA

(https://www.ncbi.nlm.nih.gov/nuccore/NC_007946.1?report=fasta&from=4913419&to=4914591)

papC

E. coli papABCDEFGHIJK genes for F13 P-pili proteins

GenBank: X61239.1

GenBank Graphics

>X61239.1:3030-5540 *E.coli papABCDEFGHIJK* genes for F13 P-pili proteins

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GTTGAGTTTAATACAGATGTACTTGACGCAGCGGACAAGAAAAATATTGACTTCACCCGTTTTTCAGAAGCCGGCTATGTT
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CGTCCTGATACCGGAGCGGGCGTATTACGCATCAATATGCCGCAGGCCTGGCTTGAGTATTCTGATGCCACCTGGCTGCCT
CCCTCACGCTGGGACGACGGCATTCCCGGACTGATGCTGGATTATAACCTCAACGGGACGGTTTCCCGTAATTATCAGGGA
GGAGACTCTCATCAGTTCAGTTATAACGGGACTGTGGGGGGGAATCTGGGGCCCTGGCGCCTGCGGGCTGACTATCAGGGA
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CTGGACAGTTCAGTTCGCGGACGTCTTGATGTTGAGGTTATTGAACAGAACGGACGGAAGAAAACCTTTCAGGTCGATACG
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GGAACGGGCGTGGTGACTGACATCAGCAGTTATTACCGGAATACAACCTCTGTTGACCTGAAGCGCTTACCGGATGATGTG
GAAGCAACCCGTTCTGTTGTGGAATCGGCGCTGACAGAAGGTGCCATTGGTTACCGGAAATTCAGCGTGCTTAAAGGGAAA
CGTCTGTTTGCAATACTGCGTCTTGCTGATGGCTCTCAGCCCCCGTTTGGTGCCAGTGTAACCAGTGAAAAAGGCCGGGAA
CTGGGCATGGTGGCCGACGAAGGCCTTGCCTGGCTGAGTGGCGTGACGCCGGGGGAAACCCTGTCGGTAAACTGGGATGGA
AAAATACAGTGTCAGGTAAATGTACCGGAGACAGCAATATCTGACCAGCAGTTATTGCTTCCCTGTACGCCTCAGAAATAA

(https://www.ncbi.nlm.nih.gov/nuccore/X61239.1?report=fasta&to=12537)

papEF

E. coli papABCDEFGHIJK genes for F13 P-pili proteins

GenBank: X61239.1

GenBank Graphics

>X61239.1:7536-8635 *E.coli papABCDEFGHIJK* genes for F13 P-pili proteins

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AATAATGCAGATAAAACTATATCACTTCATGCCAAACTTGGATATAAAGGGAATATGCAGAATTTGATAGCCGGTCCTTTC
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TGGCTGATGTGCAGATTAACATCAGGGGGAATGTTTATATCCCCCCATGCACCATTAATAACGGGCAGAATATTGTTGTTG
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ATTTTGGTATAGCGCTGTATCAGGGAAAAGGAATGTCAACACCTCTTATATTAGGTAATGGTTCAGGAAATGGTTACGGAG
TGACAGCAGGTCTGGACACAGCACGTTCAACGTTCACCTTTACTTCAGTGCCCTTTCGTAATGGCAGCGGGATACTGAATG
GCGGGGATTTCCAGACCACGGCCAGTATGAGCATGATTTATAACTGA

(https://www.ncbi.nlm.nih.gov/nuccore/X61239.1?report=fasta&from=7536&to=8635)

papGI

E. coli papG genes, complete cds

GenBank: M20146.1

GenBank Graphics

>M20146.1:1222-2229 *E. coli papG* genes, complete cds

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CTCAATCTTTACTGTTTTTGAGCCGGCTGTGTACCAGCGTAATATTTCCTCACTCTGTTCAACCCCATCAAGAGATATTAT
CGAATCCCAGCCATTACCTAACCCAACCGAAAATTTATTATTATTGTATATTGGTGGTGTATTTCTGAGCAGAGATATTTT
TACACTAACTGGTACATCGCAATAAATTGAAAGCGTCTGACTTGCAATATTTCCGTTAGCACGATCAATCACAATACTCCC
ATGGTCTATATTAAGTACTTGTGTTGACGGCTGGCATCCCCCAACATTATCGAATGATAACATCAATGTATTAGTGGCAGG
TAGCTGCTTAATCTGATCGTAAGGCATTTTATAATGATCCTGCCAGAGATCATAGTAATGGTGCTGTATTCCACGTATATA
TCTCACAGGAAAATTATAATGTCCCTTGGGGAGATCTACTGGCAAAAGAACCCTGAATACTAAATCATTAAATTTATTGTT
CAACTGCTTCATATTTCCAGGATAGAAACATATACGGGCACCATTATTTGTCCATGTATCCCATTCATAACCATTAACAAA
GAAGTAAAAATCTTTGTCATTTTCATTCTCCATAGACCAACCATATTTGCTCTGAACATCAAGAAATATATTAAACCCCTC
ATTAGTATAGACATGTTTAGGAACAACCATCCAGGCCAGATACTCCTGAAAATACACTCCACTCGCAAATTCCGGACCTGA
GCACGAATAATAAGTTGCTGTAGCAGAGCCTGTATTCCAGGGTATATATAGCTGAGGTTGGTCAATAACCTTAACATTACC
AGCATTTGTAGTTAAATAGTCGTTAAAAGCATAAAACATGACATTGTGCCATCCAGCTAAAGCATCATTACCGCCTGACAG
GGATAAAAATAAAAAAGCAGGGAACCATTTTTTCAT

(https://www.ncbi.nlm.nih.gov/nuccore/147089)

papGII

E. coli papE, papF, and papG genes, complete cds, isolate IA2

GenBank: M20181.1

GenBank Graphics

>M20181.1: 1222-2235 *E.coli papE, papF,* and *papG* genes, complete cds, isolate IA2

ATGAAAAAATGGTTCCCAGCTTTGTTATTTTCCTTGTGTGTGTCTGGTGAGTCCTCTGCATGGAATCACAATATTGTCT
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GCCCGGGCATTGCTACGGTAACCTGGAATCAGTGTAATGGTCCTGAGTTCGCTGATGGCTCCTGGGCTTACTACAGGGAGT
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CTGCAGATCTTCCTTTAGGGGATTATTCTGTTACAATTCCATACACTTCCGGCATACAGCGTCATTTCGCGAGTTACTTGG
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GCGGATGCCGTCCTTCTGCACAGTCTCTGGAAATAAAGCATGGTGATCTGTCTATTAATAGCGCTAATAATCATTATGCGG
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ATGGTAAGAAATTTTCGGTTGGTCTGGGGCATGGCTGGGACTCCATTGTTTCAGTTAACGGGGTGGACACAGGAGAGACAA
CGATGAGATGGTACAAAGCAGGTACACAAAACCTGACCATCGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAACCAG
GAGTACTATCTGGTTCAGCAACGCTGCTCATGATATTGCCATAA

(https://www.ncbi.nlm.nih.gov/nuccore/M20181.1?report=fasta&to=2287)

papGIII

Escherichia coli strain APEC 14 PapGIII (*papGIII*) gene, complete cds

GenBank: AY212281.1

GenBank Graphics

>AY212281.1 *Escherichia coli* strain APEC 14 PapGIII (*papGIII*) gene, complete cds

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TCACTTGATGCAGAAGATAATGATAATTACTATCTTACCAAGGGATTTGCATGGGATGAAGCAAACACATCTGGACGGACA
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CCTGAGGGGGATTATACGTTTCCAGTTAAGTTCTTACGTGGCATTCAGCATAATAATTATGATTATATTGGTGGACGCTAC
AAAATTCCTTCCTCGTTAATGAAAACATTTCCTTTTAATGGTACATTGAATTTCTCAATTAAGAATACCGGAGTATGCCGT
CCTTCTGCACAGTCTCTGGAAATAAATCATGGTGATCTGTCGATTAATAGCGCTAATAATCATTATGCGGCTCAGACTCTT
TCTGTGTCTTGCGATGTGCCTACAAATATTCGTTTTTTCCTGTTAAGCAATACAGCTCCGGCATACAGTCATGGTCAGAAG
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TACAGAGCAGGTACACAAAACCTGACCATCGGCAGTCGCCTCTATGGTGAATCTTCAAAGATACAACCAGGAGTACTATCT
GGTTCAGCAACGCTGCTCATGATATTGCCATAA

(https://www.ncbi.nlm.nih.gov/nuccore/AY212281.1?report=fasta&to=1005)

afa

E. coli afaF, afaA, afaB, afaC, afaD, afaE-3, int and *rep* genes

GenBank: X76688.1

GenBank Graphics

>X76688.1 *E. coli afaF, afaA, afaB, afaC, afaD, afaE-3, int* and *rep* genes

ATGAGGGAGCGATATCTGTATCTTGCTGACACCCCTCAGGGGATACTGATGTCCGGTCAGGTGCCGGAAATCAGTTCTGGT
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AGGACGGAACGCCGCTGCCGTTTGGTGCGGTGGTGACAGTTGAGGGCGAACGGGGTCAGGCTGCGGGATCAGCCGGTGTGG
TGGGAGACCGTGGTGAGGTGTACCTGAGCGGGCTGAAGGAAAGCGGTAAGCTGAAGGCGCAGTGGGGAGAGAACAGTCTGT
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(https://www.ncbi.nlm.nih.gov/nuccore/X76688.1?report=fasta&to=9163)

sfa

Escherichia coli encoding determinant sfa(I)

GenBank: X16664.4

GenBank Graphics

>X16664.4:17500-18900 *Escherichia coli* encoding determinant sfa(I)

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(https://www.ncbi.nlm.nih.gov/nuccore/X16664.4?report=fasta&from=17500&to=18900)

hlyA

Escherichia coli ATCC 25922, complete genome

GenBank: CP009072.1

GenBank Graphics

>CP009072.1:1761835-1764909 *Escherichia coli* ATCC 25922, complete genome

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(https://www.ncbi.nlm.nih.gov/nuccore/CP009072.1?report=fasta&from=1761373&to=1765369)

iucD

Escherichia coli ATCC 25922, complete genome

GenBank: CP009072.1

GenBank Graphics

>CP009072.1:1721483-1722820 *Escherichia coli* ATCC 25922, complete genome

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(https://www.ncbi.nlm.nih.gov/nuccore/CP009072.1?report=fasta&from=1721402&to=1722899)

cdtB

Escherichia coli IHE3034, complete sequence

NCBI Reference Sequence: NC_017628.1

GenBank Graphics

>NC_017628.1:2105808-2106875 *Escherichia coli* IHE3034, complete sequence
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(https://www.ncbi.nlm.nih.gov/nuccore/NC_017628.1?report=fasta&from=2105808&to=2106875)

cnf1

Escherichia coli cytotoxic necrotizing factor 1 (*cnf1*) gene, complete cds

GenBank: U42629.1

GenBank Graphics

>U42629.1: 858-3902 *Escherichia coli* cytotoxic necrotizing factor 1 (*cnf1*) gene, complete cds
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(https://www.ncbi.nlm.nih.gov/nuccore/U42629.1?report=graph)

Table 3.4: Primer sequences and PCR conditions used for the amplification of the virulence factor genes.

Sl no.	Target genes	Primer sequences (5'-3')	PCR conditions (Time)	No. of cycles	Amplicon size (bp)	References
1	*fimH*	F.P- TCGAGAACGGATAAGCCGTGG R.P- GCAGTCACCTGCCCTCCGGTA	95°C (30 sec) 60°C (30 sec) 72°C (1min)	30	506	Tiba et al. 2008
2	*papC*	F.P- GACGGCTGTACTGCAGGGTGTGGC R.P- ATATCCTTTCTGCAGGGATGCAATA	95°C (30 sec) 63°C (30 sec) 72°C (1min)	30	328	Tiba et al. 2008
3	*papEF*	F.P- GCAACAGCAACGCTGGTTGCATCAT R.P- AGAGAGAGCCACTCTTATACGGACA	95°C (30 sec) 55°C (30 sec) 72°C (1min)	30	336	Tiba et al. 2008
4	*papGI*	F.P- CAACCTGCTCTCAATCTTTACTG R.P- CCTGGATGGTTGTTCCTAAACAT	95°C (30 sec) 63°C (30 sec) 72°C (1min)	30	692	Tiba et al. 2008
5	*papGII*	F.P- GGAATGTGGTGATTACTCAAAGG R.P- TCCAGAGACTGTGCAGAAGGAC	95°C (30 sec) 52°C (30 sec) 72°C (1min)	30	562	Tiba et al. 2008
6	*papGIII*	F.P- CATGGCTGGTTGTTCCTAAACGT R.P- TCCAGAGACTGTGCAGAAGGAC	95°C (30 sec) 52°C (30 sec) 72°C (1min)	30	421	Tiba et al. 2008
7	*afa*	F.P- GGCAGAGGGCCGGCAACAGGC R.P- CCCGTAACGCGCCAGCATCTC	95°C (30 sec) 60°C (30 sec) 72°C (1min)	30	592	Basu et al. 2013
8	*sfa*	F.P- CGGAGGAGTAATTACAAACCTGGCA R.P- CTCCGGAGAACTGGGTGCATCTTAC	95°C (30 sec) 58°C (30 sec) 72°C (1min)	30	408	Tiba et al. 2008
9	*hlyA*	F.P- AACAACGATAAGCACTGTTCTGGCT R.P- ACCATATAAGCGGTCATTCCCATCA	95°C (30 sec) 63°C (30 sec) 72°C (2min)	30	1177	Tiba et al. 2008
10	*iucD*	F.P- TACCGGATTGTCATATGCAGACYGT R.P- AATATCTTCCTYCAGTCCGGAGAAG	95°C (30 sec) 55°C (30 sec) 72°C (1min)	30	602	This study
11	*cdtB*	F.P- GCAACCTGGAATTTGCAGG R.P- GATCAGAGGCAATTTGCCCTC	95°C (30 sec) 50°C (30 sec) 72°C (1min)	30	706	This study
12	*cnf1*	F.P- AAGATGGAGTTTCCTATGCAG R.P- TCAGAGTCCTGCCCTCATTAT	95°C (30 sec) 54°C (30 sec) 72°C (1min)	30	495	Tiba et al. 2008

3.4.7 Statistical analysis

The data were statistically analyzed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The chi-square test and the Fisher exact test were applied to compare categorical variables. P values <

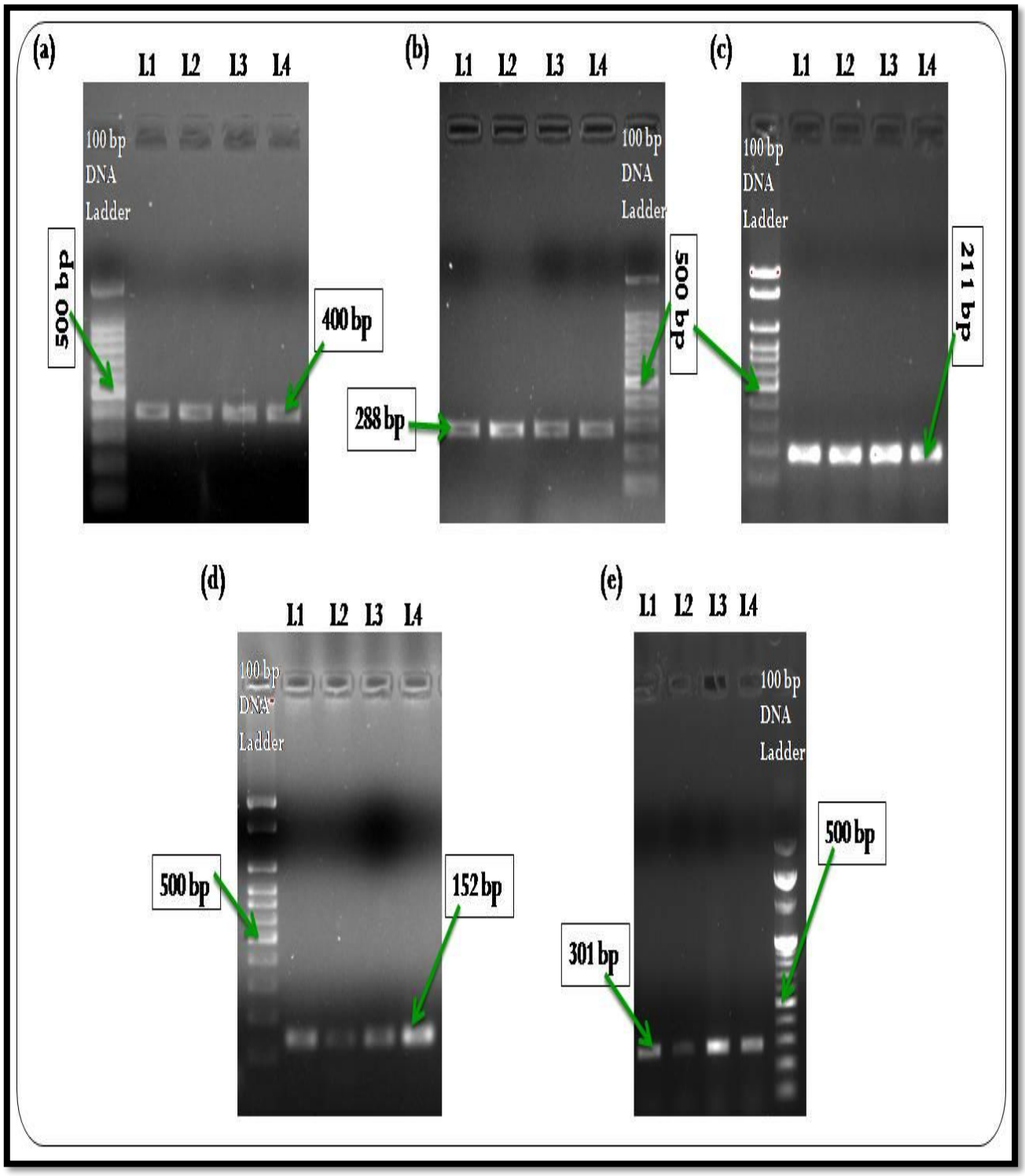
0.05 were considered to be statistically significant **(Najafi et al. 2018)**. Likewise, the statistical significance of the data analyzed by SPSS version 21.0 was further validated by using the chi-square test in the Prism software package (GraphPad Prism version 9). Furthermore, the correlation coefficient was determined using the Prism software package (GraphPad Prism version 9) **(Parra et al. 2017)** and also further validated using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) (**Yadav et al. 2018**) to find the degree of association between the distribution of different PAIs and major virulence factor genes among each of the aforementioned group of isolates individually. Withal, the degrees of correlation obtained between the incidences of the above-mentioned factors among asymptomatic UPECs were also compared to that obtained in the case of symptomatic UPECs. Low (>0.3 to 0.5), moderate (>0.5 to 0.7), and high (> 0.7 to 1) positive correlations between different PAIs and virulence factor genes among the aforementioned group of isolates were also ascertained as indicated by **Yadav et al** (**Yadav et al. 2018**). Nevertheless, according to SPSS version 21.0, correlation coefficient values < 0.2 were found to be statistically non-significant. Therefore, correlation coefficient values ≤ 0.2 were not considered when ascertaining the highest and lowest correlations. The values between 0.2 and 0.3 were only considered for analysis if they were found to be significant at ≤ 0.05 level. Furthermore, correlation graphs were constructed from the correlation matrices using the GraphPad Prism version 9 (GraphPad Software, La Jolla California USA) **(Ghosh et al. 2021).**

3.5 Results

3.5.1 Phylogenetic background analysis

UPECs that could not be assigned into any of the eight phylogroups, thus designated as 'Unknown' was significantly predominant in each (Asymptomatic=70%, p value≤0.05; Symptomatic=85%, p value ≤ 0.05) group of isolates compared to the incidence of isolates that belonged to phylogroup E [Asymptomatic=15% (p value > 0.05) , Symptomatic=10% (p value > 0.05)], Clade I [Asymptomatic=10% (p value > 0.05), Symptomatic=0% (p value > 0.05)], phylogroup D [Asymptomatic=5% (p value > 0.05), Symptomatic=0%) and phylogroup B2 [Asymptomatic=0%, Symptomatic=5% (p value > 0.05)] respectively that were non-significant among the asymptomatic and symptomatic groups. Moreover, none of the isolates from both groups belonged to the phylogroups A, B1, C or F respectively. The representative gel pictures had been depicted in the **Fig. 3.1.** However, significant (p-value ≤ 0.05) positive correlations were neither perceived among the asymptomatic (**Fig. 3.2a)** nor among the symptomatic (**Fig. 3.2b)** UPECs with regard to their phylogroup distribution. However, as earlier mentioned, among all the phylogroups investigated, significant numbers of isolates from both groups were undesignated, thus assigned to the "Unknown" phylogroup. So, when the degree of correlation with regard to the incidence of isolates belonging to the Unknown phylogroup

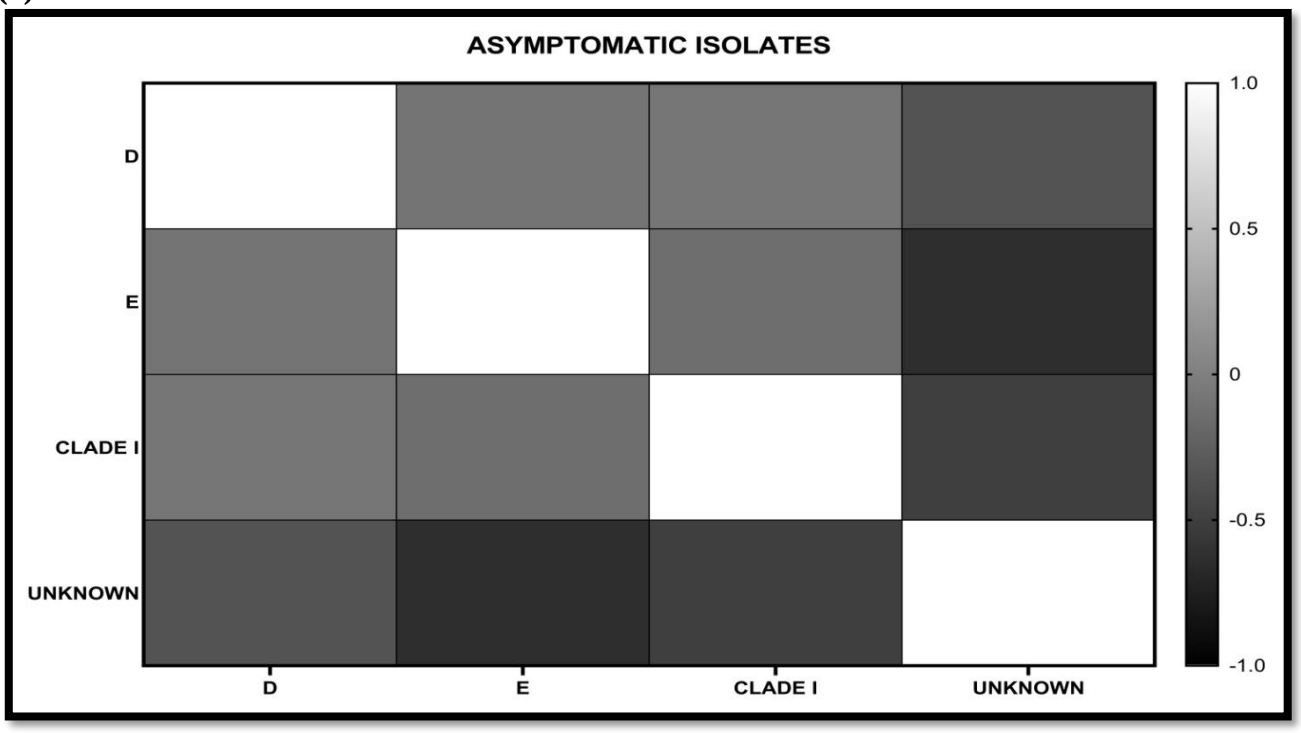
was compared between the asymptomatic and symptomatic groups, significant moderately high positive correlations (p-value ≤ 0.05) were observed (**Fig. 3.3)**.



(This study)

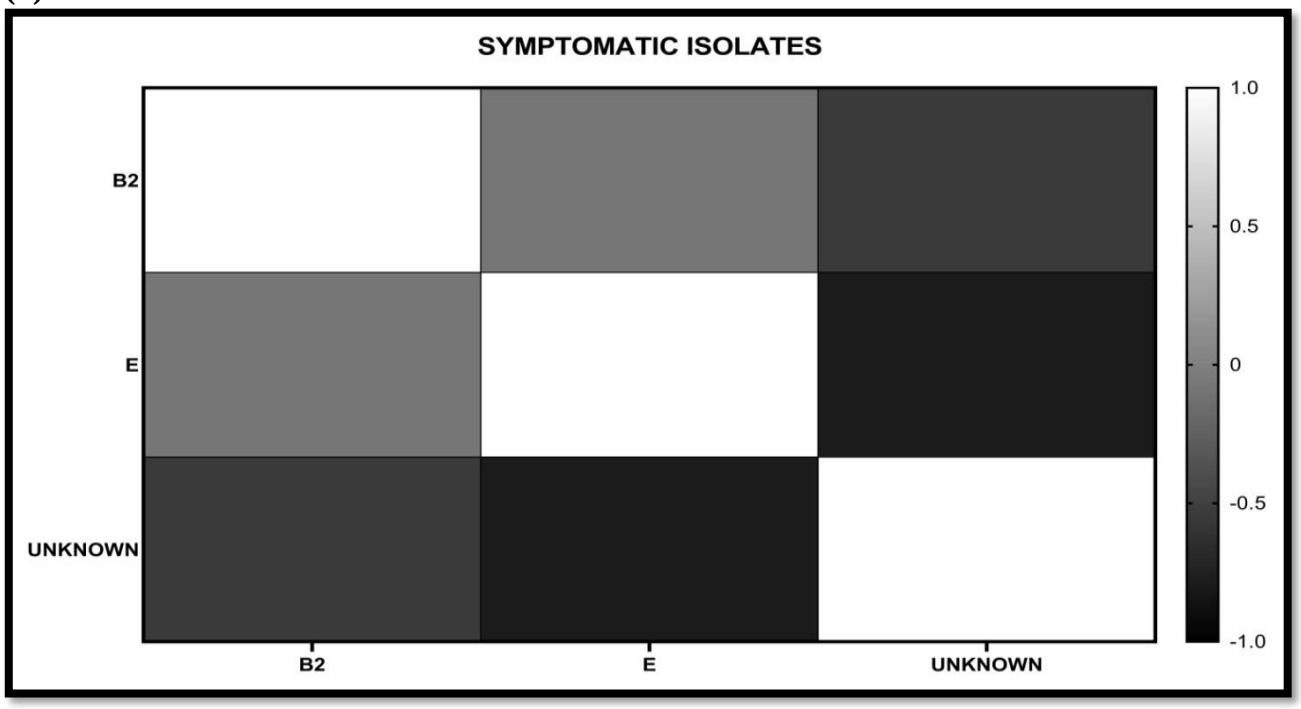
Fig. 3.1: The representative gel pictures of the phylogenetic background study [a] *arpA* (400bp) [b] *chuA* (288bp) [c] *yjaA* (211bp) [d] TspE4.C2 (152bp) and [e] *arpAgpE* (301bp) of ABU and symptomatic UPECs.

(a)



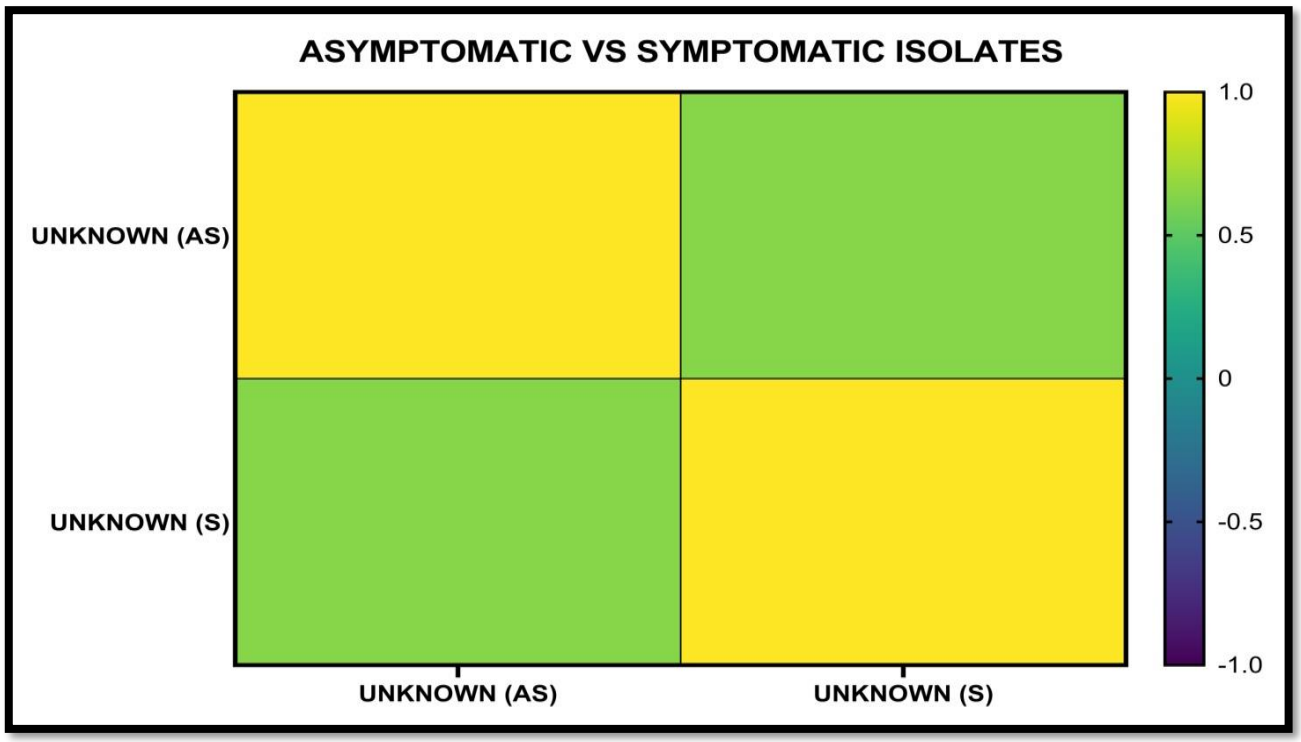
(This study)

(b)



(This study)

Fig. 3.2: Statistical representation of correlations between the distribution of 4 (D, E, CladeI and Unknown) and 4 (B2, E, Unknown) phylogroups among (a) asymptomatic (b) symptomatic UPECs respectively. Correlations were individually computed among asymptomatic and symptomatic UPECs using GraphPad Prism version 9 (Prism software package). Heat maps were generated on the correlation coefficient values represented by the color keys that ranged from (-) 1 (black) to (+) 1 (white)



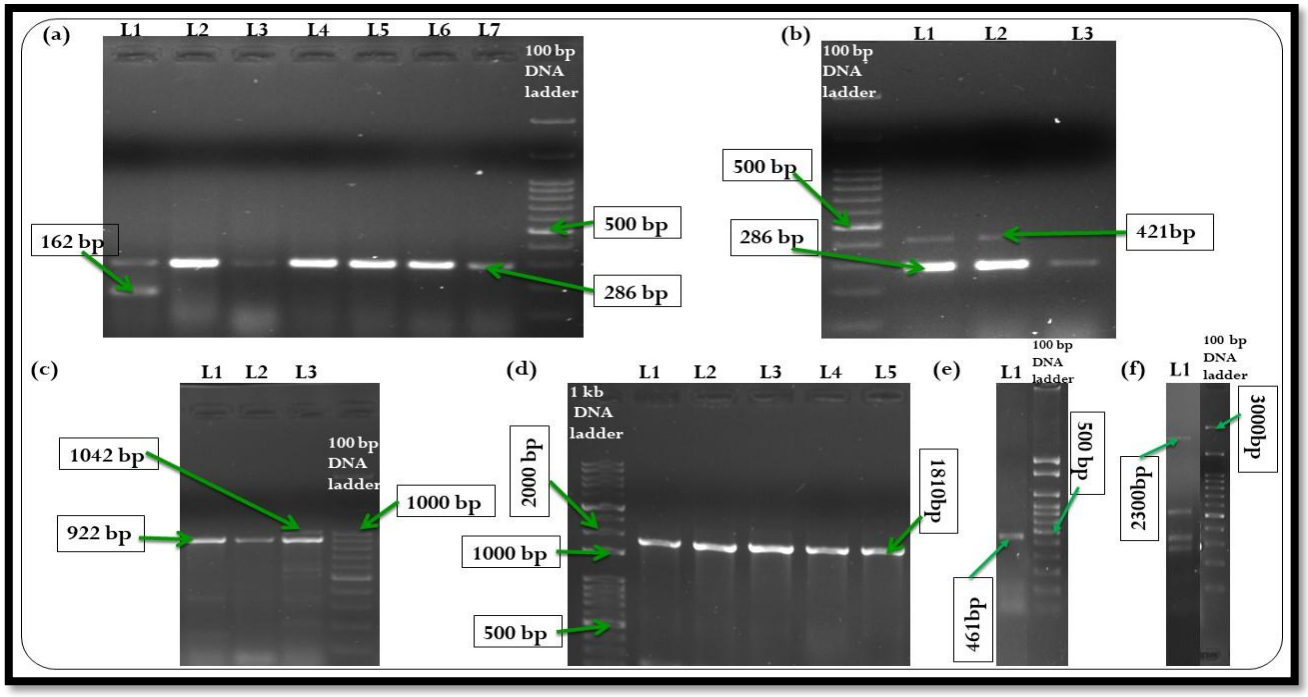
(This study)

Fig. 3.3: Statistical representation of correlations between the asymptomatic (AS) and symptomatic (S) UPECs relating to the distribution of Unknown phylogroup computed using GraphPad Prism version 9 (Prism software package). Heat maps were generated on the correlation coefficient values represented by the color keys that ranged from (-) 1 (purple) to (+) 1 (yellow).

3.5.2 Distribution of PAI markers

Overall, 38 (95%) of the 40 UPECs (Asymptomatic=90%; Symptomatic=100%) selected for this study carried at least 1 of the 8 PAI markers. The prevalence of 4 [PAI I536 (25%), PAI II536 (20%), PAI IV536 (90%) and PAI ICFT073 (55%)] and 6 [PAI I536 (55%), PAI II536 (15%), PAI III536 (5%), PAI IV536 (100%), PAI ICFT073 (60%) and PAI IICFT073 (20%)] of the 8 PAI markers were found in case of asymptomatic and symptomatic isolates respectively (the representative gel pictures of the PAI markers investigation had been depicted in **Fig. 3.4)**. Moreover, in the case of both groups, PAI IV536 [Asymptomatic (p value= <0.0001); Symptomatic (p value= <0.0001)] followed by PAI ICFT073 [Asymptomatic (p value= 0.006); Symptomatic (p value= 0.0034)] was found to be the significantly predominant with complete absence of PAI IJ96 and PAI IIJ96 among the studied isolates. Nonetheless, significant prevalence (p value= 0.006) of PAI I536 was found in case of symptomatic UPECs unlike the asymptomatic ones. However, significant (p-value $\leq$ 0.05) positive correlations that varied from low to high was perceived among both the asymptomatic (**Fig. 3.5a)** and symptomatic (**Fig. 3.5b)** UPECs with regard to their distribution of 4 and 6 PAI markers respectively. Among, ABU UPECs, high and moderate correlations were perceived in the distribution of PAI I536; PAI II536 and PAI I536; PAI ICFT073, PAI II536; PAI ICFT073, PAI 1V536; PAI ICFT073

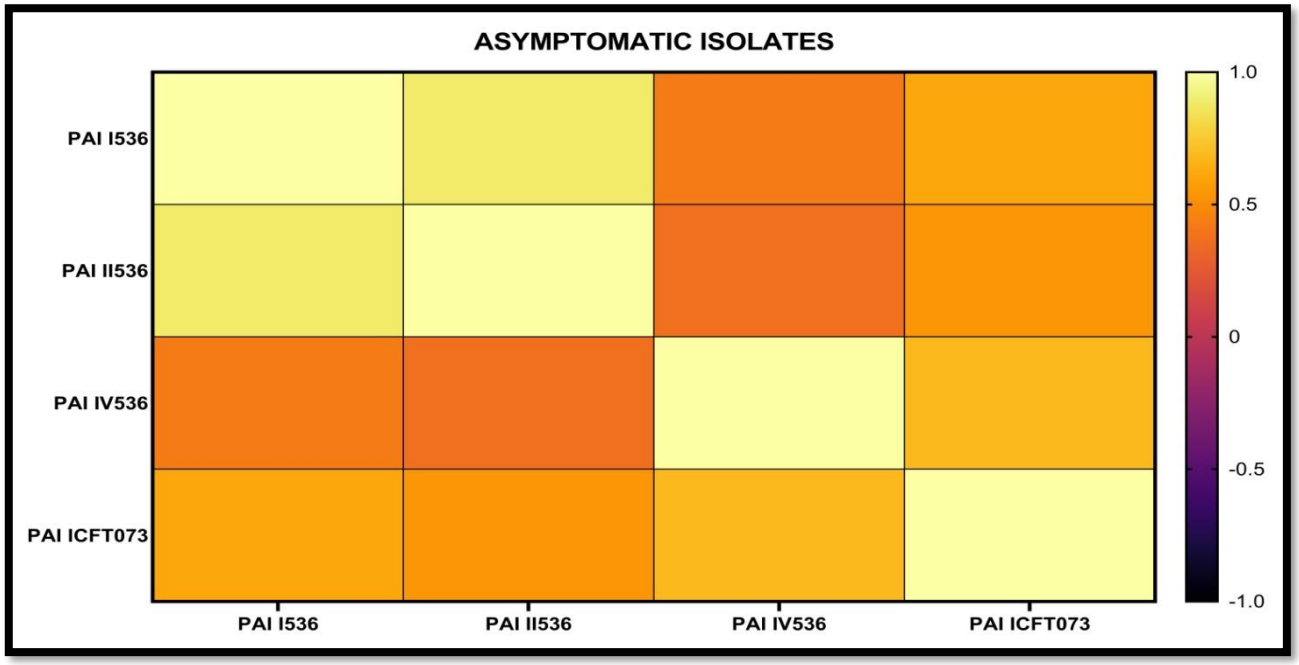
respectively (**Fig. 3.5a)**. However, in the case of symptomatic UPECs, although high correlations could not be observed in the distributions of any of the PAI markers studied, moderate correlations were observed in the distribution of PAI I536 with PAI IV536; PAI ICFT073, PAI II536 with PAI III536, PAI IV536 with PAI ICFT073(**Fig. 3.5b)**.



(This study)

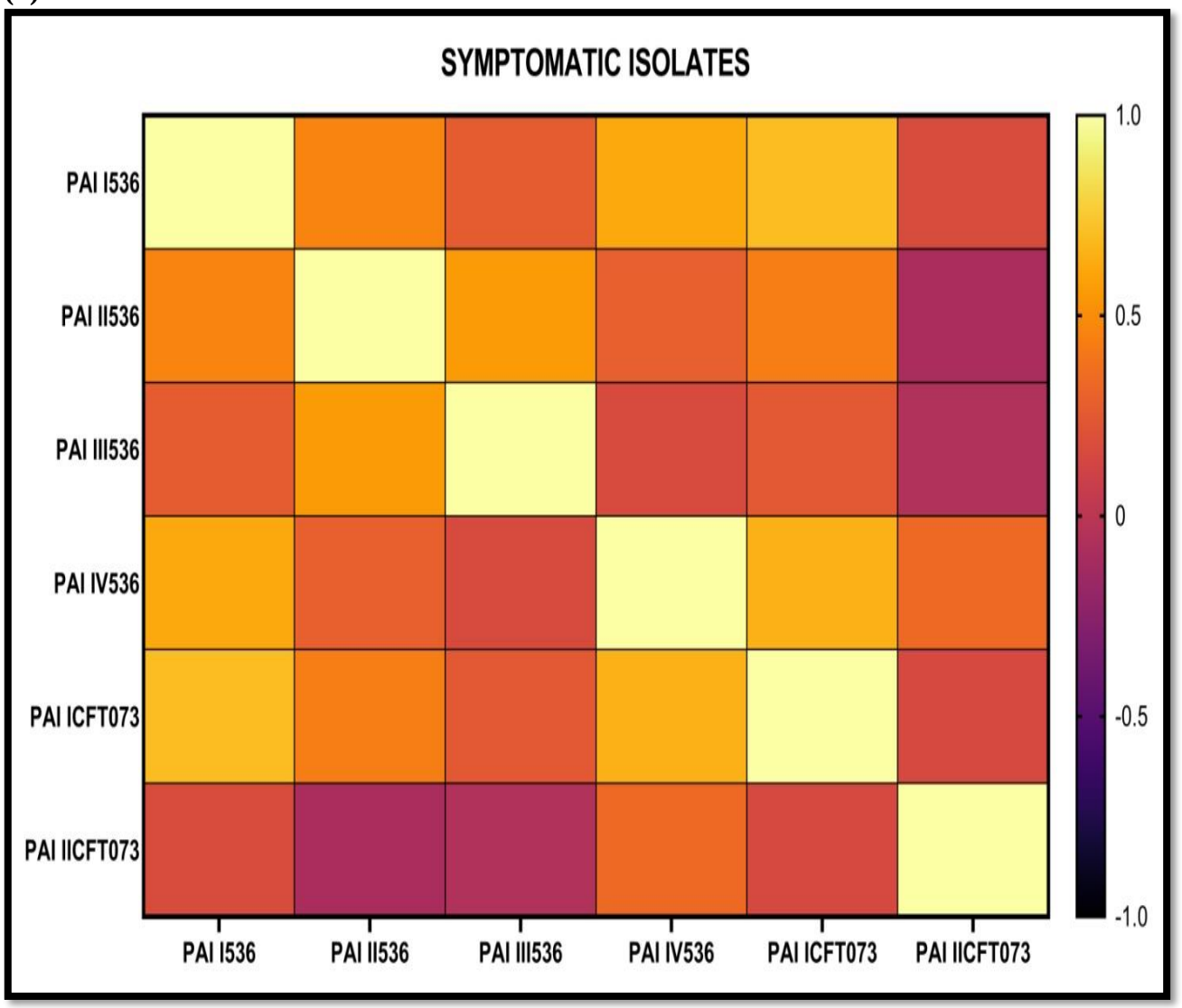
Fig. 3.4: The representative gel pictures of the PAI markers study [a] PAI III536 (162bp); PAI IV536 (286bp) [b] PAI IV536 (286bp); PAI IICFT073 (421bp) [c] PAI ICFT073 (922bp); PAI II536 (1042bp) and [d] PAI I536 (1810bp) of ABU and symptomatic UPECs; [e] PAI IJ96 (461bp) of control sample and [f] PAI IIJ96 (2300bp) of control sample.

(a)



(This study)

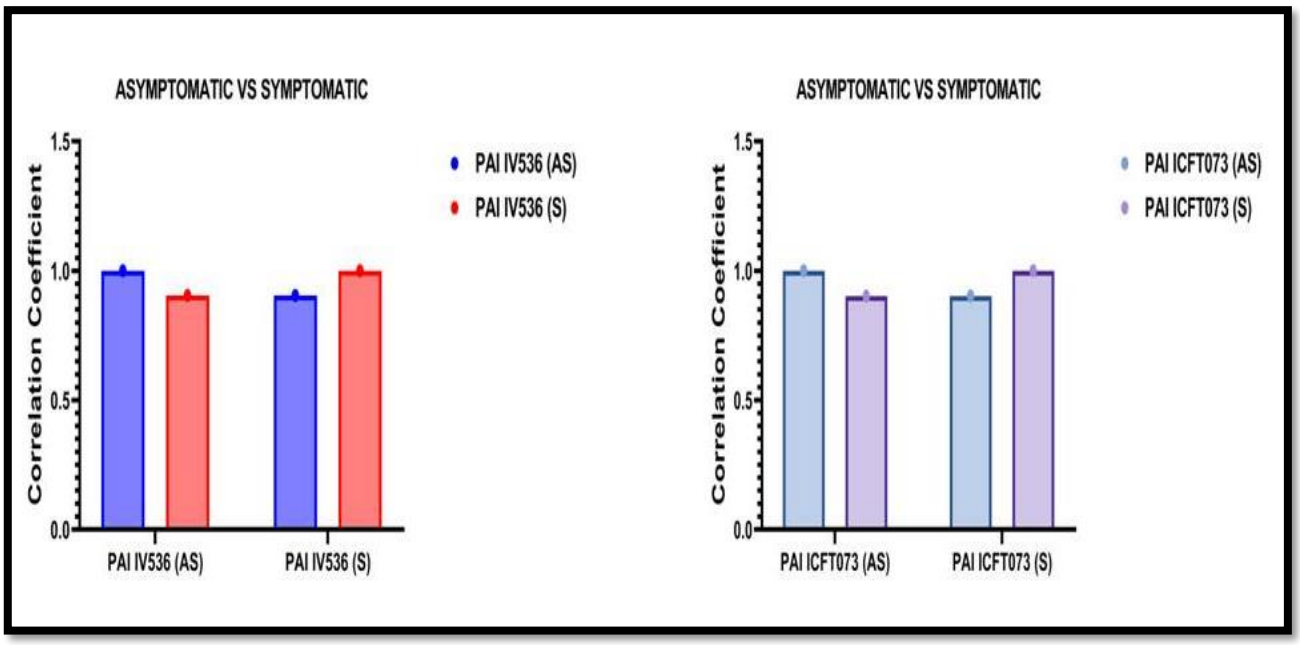
(b)



(This study)

Fig. 3.5: Statistical representation of correlations between the distribution of 4 (PAI I536, PAI II536, PAI IV536 and PAI ICFT073) and 6 (PAI I536, PAI II536, PAI III536, PAI IV536, PAI ICFT073 and PAI IICFT073) PAI markers among (a) asymptomatic (b) symptomatic UPECs respectively. Correlations were individually computed among asymptomatic and symptomatic UPECs using GraphPad Prism version 9 (Prism software package). Heat maps were generated on the correlation coefficient values represented by the color keys that ranged from (-) 1 (black) to (+) 1 (cream).

However, when correlations in the incidence of the studied PAI markers were compared between the entire set of isolates of the asymptomatic and symptomatic groups, significant high positive correlations (p-value ≤ 0.05) observed in the case of PAI IV536 and PAI ICFT073 (6 of other studied PAIs were not considered in the correlation analysis which was based on the incidence as either they were totally absent or their presence were non-significant among one or both groups) (**Fig. 3.6)**.

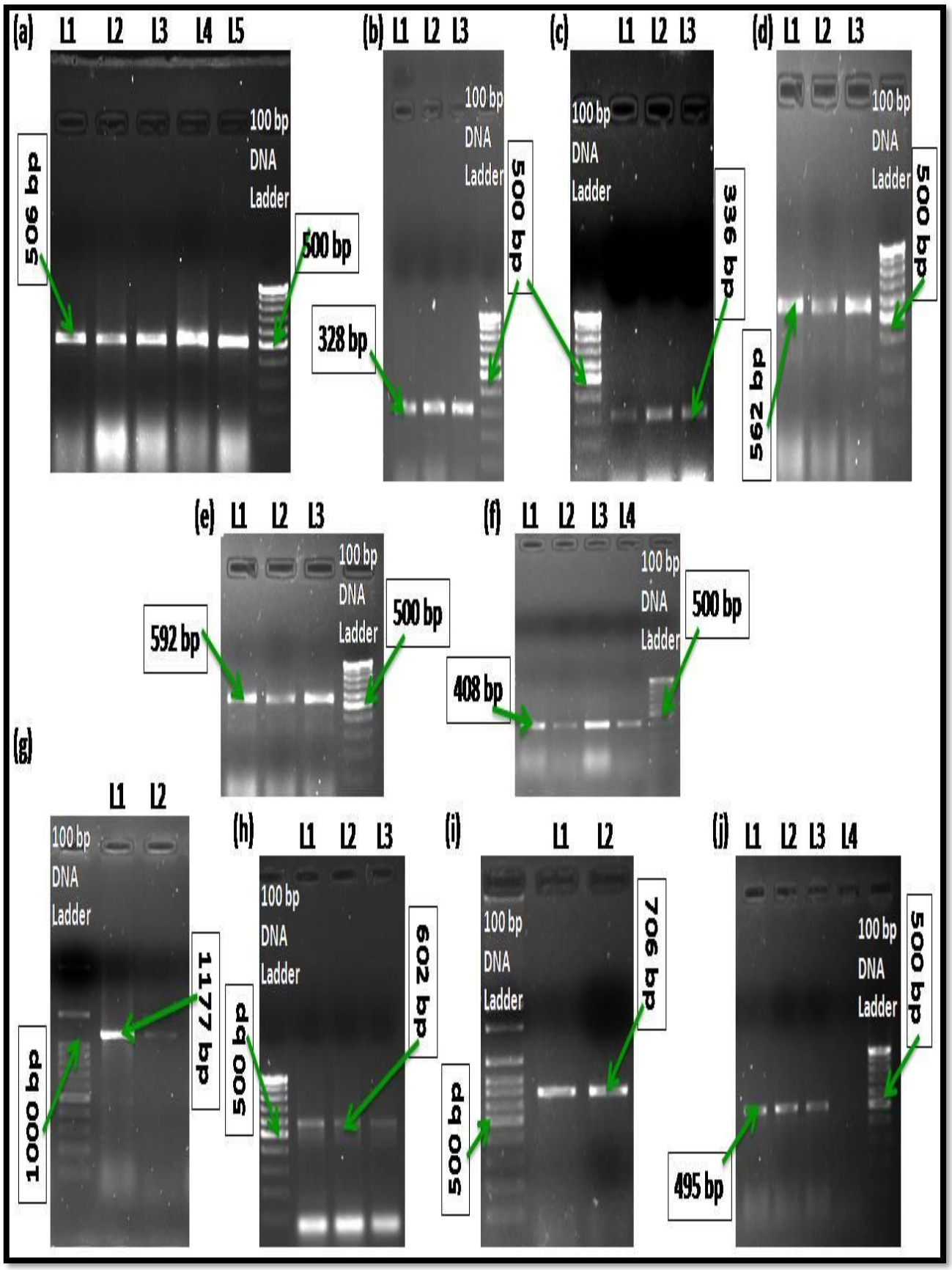


(This study)

Fig. 3.6: Statistical representation of correlations between the asymptomatic (AS) and symptomatic (S) UPECs relating to the incidence isolates from both groups harbouring two (PAI IV536 and PAI ICFT073) PAIs computed using GraphPad Prism version 9 (Prism software package). Different PAIs were represented by bar graphs with varied colours.

3.5.3 Distribution of virulence factor genes

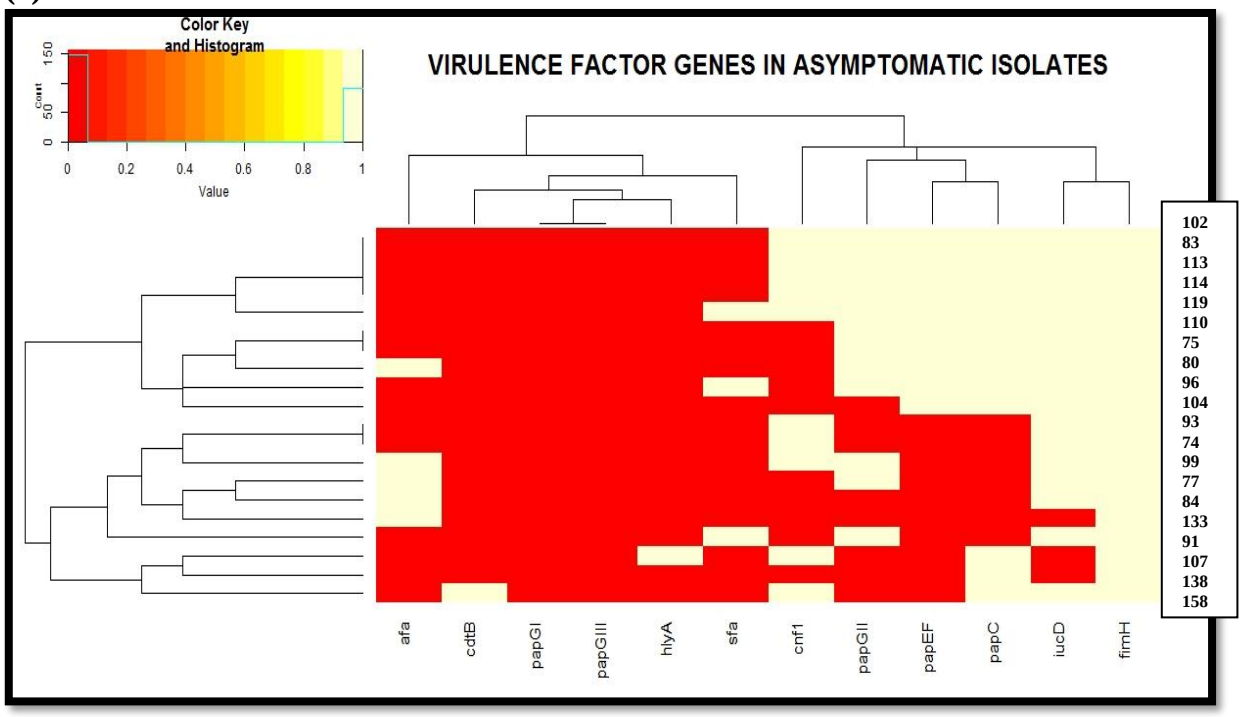
Two heat maps were constructed based on individual distribution of 12 virulence associated genes (the representative gel pictures of the virulence factor genes had been depicted in **Fig. 3.7**) in asymptomatic **Fig. 3.8a** and symptomatic **Fig. 3.8b** isolates, to understand genetic associations related to virulence that imparts an important role in pathogenicity. Two major clusters could be distinguished, on the basis of significant distribution pattern of 5 virulence factor genes (*cnf1, papEF, papGII, papC, iucD*) in case of asymptomatic isolates. Cluster 1 and 2 comprised of 10 isolates each. Type 1fimbrial gene (*fimH*) was found to be evenly distributed between both the clusters **(Fig. 3.8a)**. However discreet clusters could be observed in case of symptomatic isolates in spite of the significant distribution pattern of 5 virulence factor genes (*cnf1, papEF, papGII, papC, iucD*) **(Fig. 3.8b)**. Nonetheless, type 1 fimbrial gene (*fimH*) was found to be universally distributed among all the clusters. Furthermore, among both the aforementioned groups the 6 virulence factor genes *fimH* [Asymptomatic (p value= <0.0001); Symptomatic (p value= <0.0001)], *papC* [Asymptomatic (p value= 0.0019); Symptomatic (p value= 0.0005)], *papEF* [Asymptomatic (p value= 0.01); Symptomatic (p value= 0.01)], *papGII* [Asymptomatic (p value= 0.0034); Symptomatic (p value= 0.0019)], *iucD* [Asymptomatic (p value= 0.0001); Symptomatic (p value= <0.0001)] and *cnf1* [Asymptomatic (p value= 0.01); Symptomatic (p value= 0.001)] were found to be significantly prevalent.



(This study)

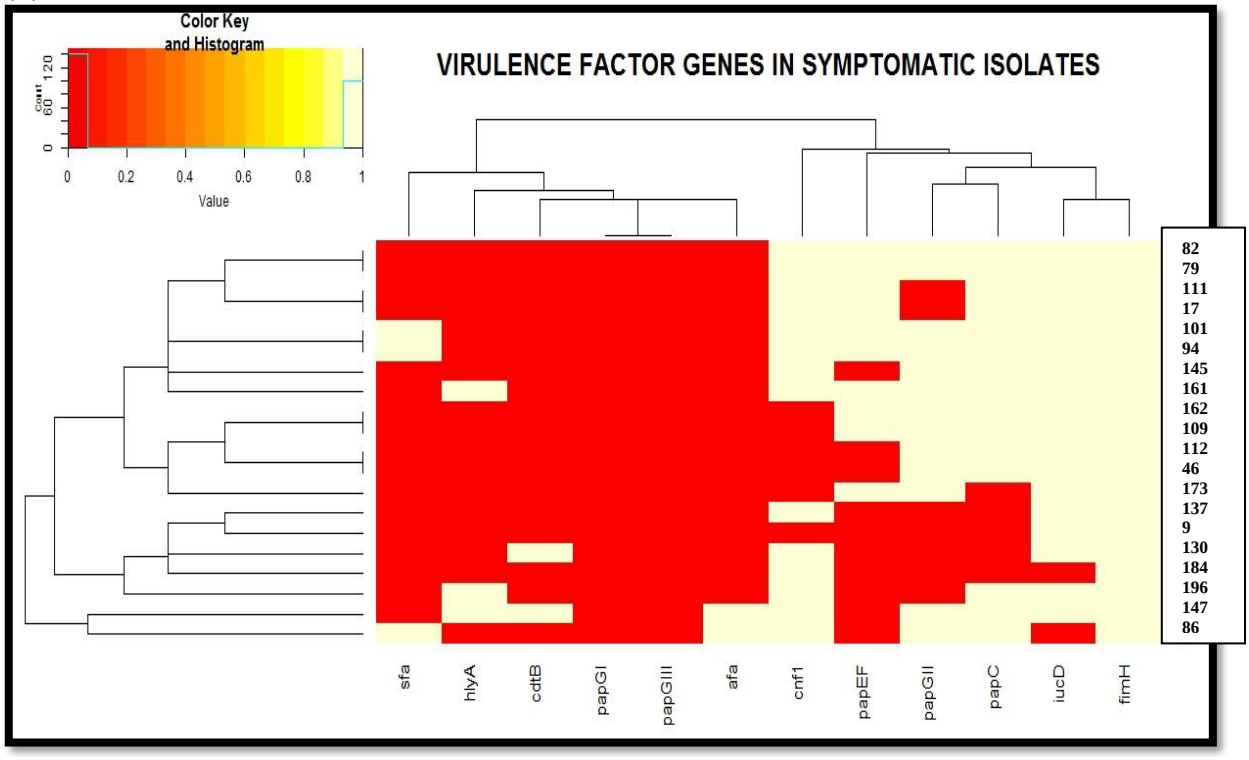
Fig. 3.7: The representative gel pictures of the virulence factor genotypic study [a] *fimH* (506bp) [b] *papC* (328bp) [c] *papEF* (336bp) [d] *papGII* (562bp) [e] *afa* (592bp) [f] *sfa* (408bp) [g] *hlyA* (1177bp) [h] *iucD* (602bp) [i] *cdtB* (706bp) and [j] *cnf1* (495bp) of ABU and symptomatic UPECs.

(a)



(This study)

(b)

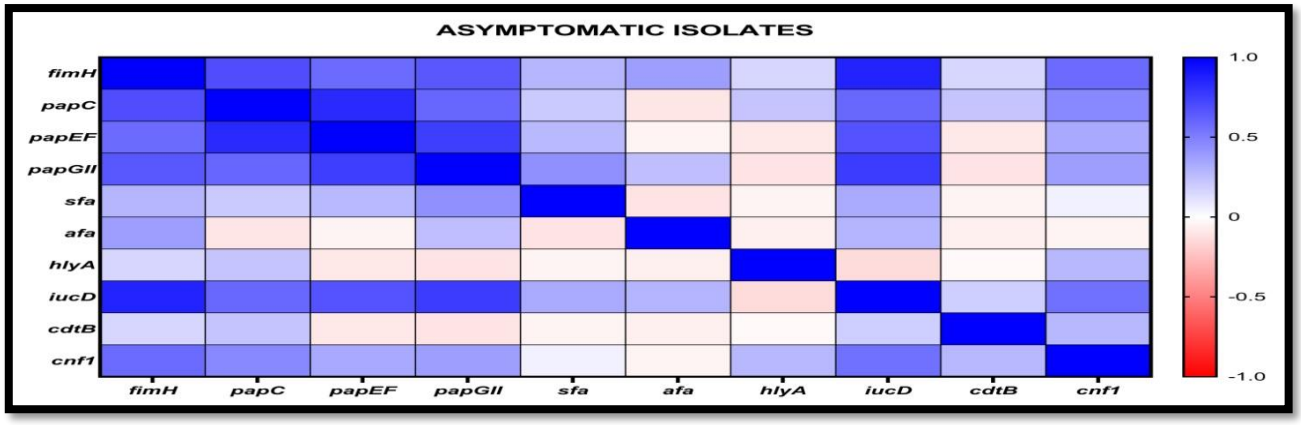


(This study)

Fig. 3.8: Cluster analysis performed on Heat maps generated using R software package (version 3.2.5), based on the presence and absence twelve different virulence factor genes in individual isolate of (a) Asymptomatic UPEC group and (b) Symptomatic UPEC group. Numbers in the text box provided on the righthand side represents sample ID of the *E. coli* isolates considered in each group. Colour key represents the variation in colours from deep red to white illustrating the complete absence of a particular gene to its complete presence respectively.

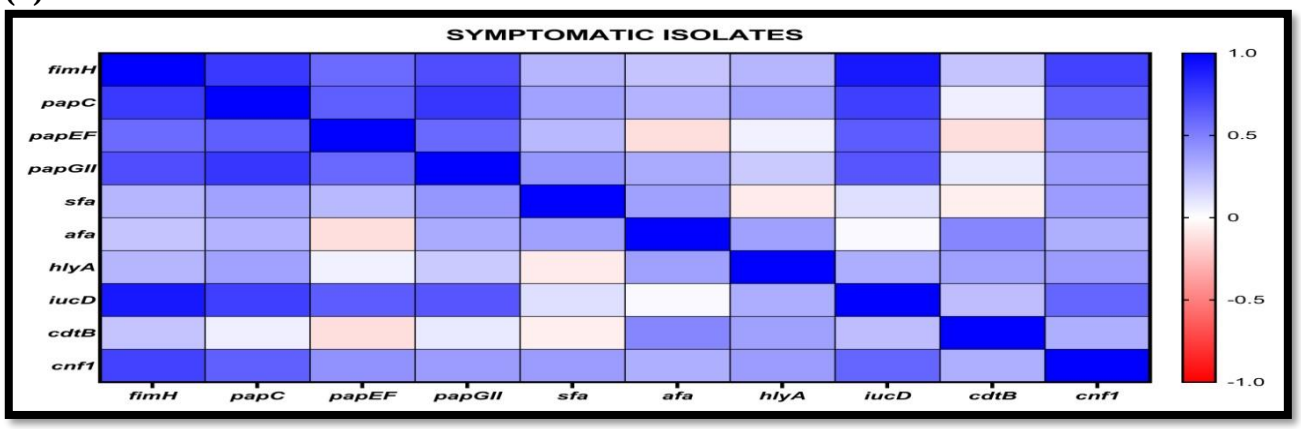
However, significant (p-value ≤ 0.05) positive correlations that varied from low to high was perceived among both the asymptomatic (**Fig. 3.9a)** and symptomatic (**Fig. 3.9b)** UPECs with regard to their distribution of 10 out of 12 investigated virulence factor genes (*papGI* and *papGIII* were not considered in the analysis due to their complete absence among both the studied groups). Among, ABU UPECs, high and moderate level of correlations were perceived in the distribution of *fimH* with *iucD, papC with papEF*, *papEF* with *papGII, papGII with iucD and fimH with papC, papEF; papGII; cnf1, papC with papGII; iucD, cnf1 with iucD* respectively (**Fig. 3.9a)**. However, in the case of symptomatic UPECs *fimH* with *papC*; *iucD; cnf1* and *papC* with *papGII*; *iucD* were found to be highly correlated (**Fig. 3.9b)**. Moderate correlations were observed in the case of distribution of *fimH* with *papEF*; *papGII*, *papC* with *papEF*; *cnf1*, *papEF* with *papGII*; *iucD, papGII* with *iucD, iucD* with *cnf1*

(a)



(This study)

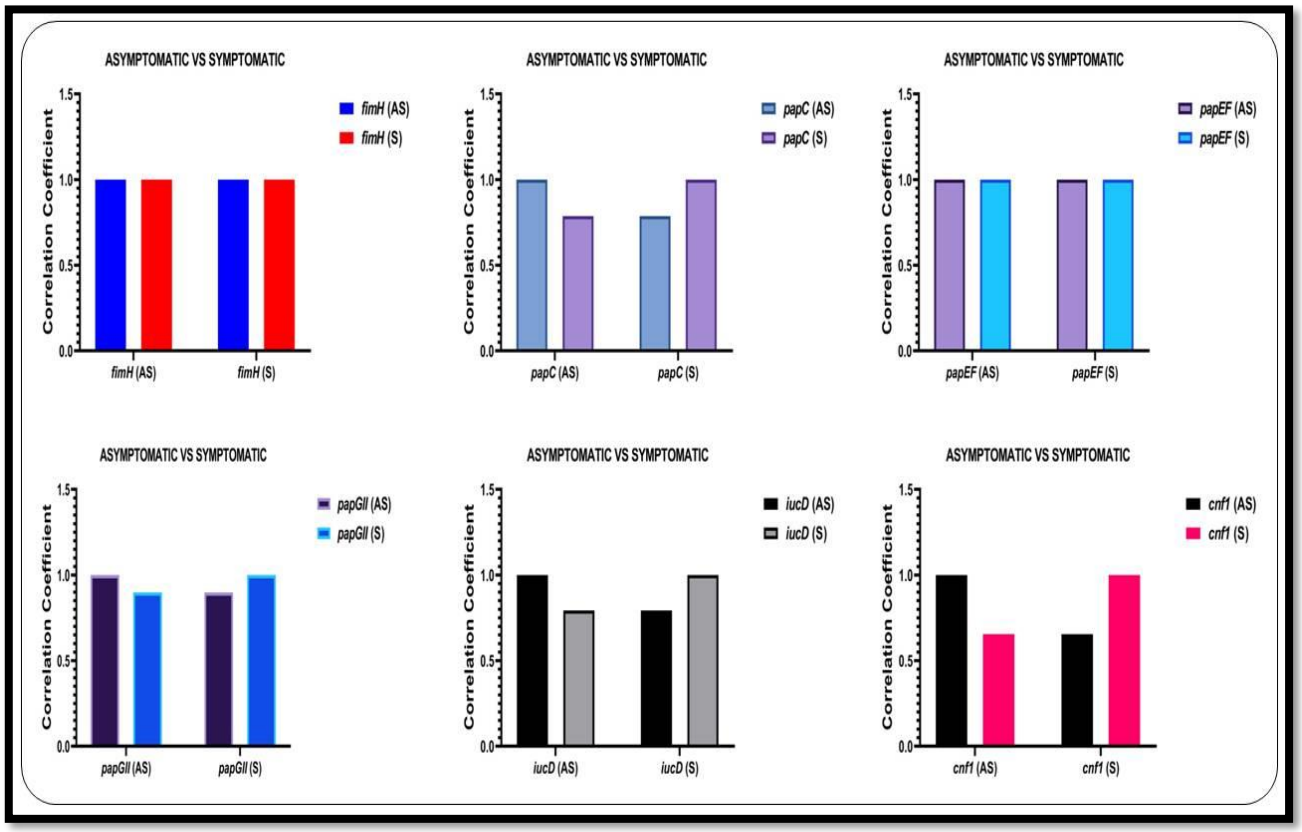
(b)



(This study)

Fig. 3.9: Statistical representation of correlations between the distribution 10 virulence factor genes (*fimH, papC, papEF, papGII, sfa, afa, hlyA, iucD, cdtB, cnf1*) among (a) asymptomatic (b) symptomatic UPECs respectively. Correlations were individually computed among asymptomatic and symptomatic UPECs using GraphPad Prism version 9 (Prism software package). Heat maps were generated on the correlation coefficient values represented by the color keys that ranged from (-) 1 (black) to (+) 1 (cream).

However, when correlations in the incidence of the 6 (*fimH*, *papC*, *papEF*, *papGII*, *iucD* and *cnf1*) studied virulence factor genes were compared between the total isolates of asymptomatic and symptomatic groups, significant high positive correlations (p-value ≤ 0.05) were observed in all except *cnf1* which showed moderate correlation (6 of other studied virulence factor genes were not considered in the correlation analysis which was based on the incidence as either they were totally absent or their presence were non-significant among both groups) (**Fig. 3.10)**.



(This study)

Fig. 3.10: Statistical representation of correlations between the asymptomatic (AS) and symptomatic (S) UPECs relating to the incidence of 6 (*fimH, papC, papEF, papGII, iucD, cnf1*) virulence factor genes individually computed using GraphPad Prism version 9 (Prism software package). Different PAIs were represented by bar graphs with varied colours.

3.6 Discussion

In the present study, new quadruplex method of phylogenetic grouping revealed a significant (p-value ≤ 0.05) incidence of the ABU UPECs that belonged to 'Unknown' phylogroup as they could not be assigned to any of the eight known phylogroups as also observed in the case of symptomatic isolates. This was antithetical to studies conducted from Iran in the relatively recent past **(Iranpour et al. 2015; Najafi et al. 2018)** where majority of the UPECs were found to belong to phylogroup B2. Moreover reports from Paris **(Clermont et al. 2013)** and Iran **(Iranpour et al. 2015; Najafi et al. 2018)** revealed that 1%, 27.1% and 27.1% of the *E. coli* isolates respectively remained unclassified after analyzing the

isolates by the new quadruplex method of phylogrouping that was contrary to the present study where most of the isolates of both asymptomatic and symptomatic group remained unclassified. The aforementioned observation could be imputed to the presence of recombination events between two different and/or extremely rare phylogroups (**Clermont et al. 2013; Iranpour et al. 2015).** Withal, the significant (p-value $\leq$ 0.05) moderately high positive correlations (p-value $\leq$ 0.05) between asymptomatic and symptomatic groups regarding the isolates that belonged to the "Unknown" phylogroup **(Fig. 3.3)** further avowed the fact that the ABU isolates analyzed in this study might have originated from the symptomatic population. Likewise, this is the first study that utilized the new quadruplex PCR method for phylotyping of ABU UPECs isolated from males and non-pregnant females **(Ghosh and Mukherjee 2019)**.

Previous reports demonstrated that extraintestinal *E. coli* strains might harbour various virulence factors, usually encoded on PAIs, providing a mechanism for coordinated horizontal transfer of virulence genes, known to contribute to bacterial pathogenesis and survival in a specific environment **(Sabate et al. 2006; Kryger et al. 2015)**. In the present study high predominance of PAI IV536, termed as High-Pathogenicity Island (HPI) (**Sabate et al. 2006)** followed by PAI ICFT073 was evident in case of both ABU and symptomatic isolates with overall higher prevalence of the PAI markers in symptomatic ones. The predominance of the two aforesaid PAI markers was found to be statistically significant (p-value $\leq$ 0.05) and was mostly in accordance with the studies conducted on commensal, symptomatic and ABU UPECs in different parts of the world **(Sabate et al. 2006; Kryger Set al. 2015; Samei et al. 2015; Najafi et al. 2018).** Moreover, significant (p-value $\leq$ 0.05) moderate to strong level of correlations were perceived between the distribution and incidence of PAI IV536 and PAI ICFT073 among ABU and symptomatic UPECs when evaluated individually (**Fig. 3.5a-b)** and also in combination **(Fig. 3.6)** respectively. However, this PAI IV536 (HPI) and PAI ICFT073, known to contain certain toxin, P fimbrial and iron uptake system encoding genes, are imperative in efficacious colonization and successful survival of *E. coli* strains in the human urinary tract **(Najafi et al. 2018).** Therefore the aforesaid observations among both asymptomatic and symptomatic groups implied that the asymptomatic isolates included in this study might have rendered potent colonization capability and efficient pathogenic potential to these ABU UPECs, which might have originated from the symptomatic isolates and this condition was highly alarming **(Ghosh and Mukherjee 2019)**.

Virulence factors are conspicuous determinants of bacterial pathogenesis and are often encoded within the PAIs **(Sabate et al. 2006; Najafi et al. 2018)**. The degree of virulence is generally related directly to the capability of a particular organism to cause disease regardless of host resistance mechanisms **(Peterson 1996)**. In this study, the overall prevalence of the 12 studied virulence factor genes (*fimH, papC, papEF, papGI, papGII, papGIII, sfa, afa, hlyA, cnf1, iucD* and *cdtB*) in the ABU UPECs was comparable to those in symptomatic ones, very similar to a report from northern India

(Srivastava et al. 2016). Earlier studies conducted on asymptomatic, commensal and symptomatic *E. coli* isolates, from Korea **(Lee et al. 2010)** and India **(Srivastava et al. 2016)** had confirmed varied incidence of different virulence factor genes**;** with significant incidence of the independent predictors of pathogenicity; *hlyA, iutA, fyuA and tratT* (**Lee et al. 2010**) and *hlyA, cnf1, fyuA, ibeA* and *KpsMII* virulence factor genes (**Srivastava et al. 2016**) respectively. However, this study indicated the significant incidence of 6 virulence factor genes (*fimH, papC, papEF, papGII, iucD* and *cnf1*) among isolates of both asymptomatic and symptomatic groups. Moreover, significant (p-value ≤ 0.05) moderate to high positive correlations were observed in the distribution of all the six aforementioned virulence factor genes in various pairs and combinations among both the asymptomatic **(Fig. 3.9a)** and symptomatic groups **(Fig. 3.9b)** when analyzed individually. Furthermore, comparison of degree of correlations between isolates of asymptomatic and symptomatic groups revealed moderate to high positive correlations with regard to the incidence of the 6 aforementioned virulence factor genes **(Fig. 3.10)**. These observations advocated that ABU and the symptomatic isolates were similar with respect to their virulence potential. Nevertheless, the significant distribution patterns of virulence factor genes in ABU **(Fig. 3.8a)** and symptomatic **(Fig. 3.8a)** UPECs, proffered the fact that process of their acquisition might have been different under indiscriminate drug pressure, further indicating the possibility that accretion of these virulence genes in different PAIs by mobile genetic elements and their subsequent horizontal gene transfer might have occurred in a more organized way in case of asymptomatic isolates unlike to the randomized acquisition in the case of symptomatic ones **(Ghosh and Mukherjee 2019)**.

Therefore, this part of the present study along with that described in Chapter 2 displayed the significant correlation between isolates of asymptomatic and symptomatic groups with regard to their resistances against 7 different antibiotics (Ceftazidime, Cefotaxime, Ciprofloxacin, Levofloxacin, Cotrimoxazole, Gentamicin and Tobramycin), incidence of 'Unknown' phylogroup, pathogenicity islands (HPI and PAI ICFT07) and acquisition of 6 virulence factor genes (*fimH, papC, papEF, papGII, iucD, cnf1*) respectively which implied a direct relationship among the asymptomatic and symptomatic UPEC isolates. However, more detailed epidemiologic, molecular characterization and adherence experiments must be conducted on ABU UPECs considered in this study and should be compared to the symptomatic ones to assess the potency of the isolates from the former group **(Ghosh and Mukherjee 2019)**.

3.7 Conclusion

The results presented in this study provided a thorough insight into the genotypic characteristics of ABU UPECs isolated from hospitalized patients of Kolkata, an eastern region of resource-poor country India. The sharing of characteristics (phylogenetic background, PAIs, virulence genes) between

the ABU and symptomatic isolates suggested that the selection of the former might be from the latter group due to unchecked use of unprescribed antibiotics that might have resulted from mutations or aberrant gene expressions. Moreover, to the best of our knowledge, this is the first study from India that identified the prevalence of 'Unknown' phylogroup, PAI markers in the asymptomatic UPECs isolated from males and non-pregnant female hospitalized patients. This condition was highly distressing which should invoke the attentions of clinicians and microbiologists as it suggested the emerging pathogenic potential of ABU UPECs that might colonize and persist in human urinary tract without actually initiating any symptoms rendering the host as a reservoir of pathogenic microbes in this country.

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CHAPTER 4.

β-lactamase producing drug-resistant asymptomatic & symptomatic uropathogenic *E. coli* - Genetic diversity analysis

4.1 Background study

Escherichia coli (*E. coli*), the epitome of most variegated bacterial species, although well known to encompass harmless residents of gastrointestinal tract of humans, their pathogenic variants represent a massive public health concern **(Clermont et al. 2000; Van Elsas et al. 2011; Clermont et al. 2011)**. Moreover, *E. coli* is one of the most widespread uropathogen associated with asymptomatic bacteriuria (ABU) and symptomatic urinary tract infections (UTIs) **(Bien et al. 2012; Ghosh and Mukherjee 2019)**.

The recent reports from China **(He et al. 2018)** and eastern India **(Ghosh and Mukherjee 2019)** stated a high incidence of UPECs among individuals with ABU. Moreover, a current study from central part of India **(Kande et al. 2021)** indicated predominance of UPECs among isolates collected from diabetic patients with ABU. Furthermore, previous studies from different regions of India had specified similar MDR and pathogenic profiles among the asymptomatic and symptomatic UPECs **(Srivastava et al. 2016; Ghosh and Mukherjee 2019)**. A relatively recent report from the United States of America **(Cortes-Penfield et al. 2017)** indicated the unwarranted use of antibiotics for the treatment of ABU**.** Nonetheless, Venkatesan et al. **(Venkatesan et al. 2017)** from India highlighted the devoir for diagnosis and proper management of ABU, especially among diabetic patients which or else might lead to severe problems. Therefore, not merely the identification of ABU UPECs but also their intricate phenotypic, molecular, genetic and epidemiological analysis with respect to the symptomatic ones is an absolute necessity of the present era.

Several studies conducted on MDR microbes from the worldwide diverse pathogenic pool suggested that the globalization which expedited the movement of people also promoted the dissemination of these MDR strains around the world **(Allcock et al. 2017; Cohen et al. 2019)**. The exudation of these MDR strains had been reported to be caused due to acquisition of the MDR genes that led to certain genome alterations. These included mutations as well as chromosomal rearrangements that alluded to the highly plastic nature of these bacterial genomes **(Hoeksema et al. 2018).** Further, MDR and ESBL production among UPECs and presence and/ or expression of β-lactamase genes had often been cognated with mobile genetic elements (MGEs) like plasmids, integrons, and insertion sequences (ISs) from various parts of the world like the United States of America (**Kurpiel et al. 2011)**, Iran **(Lavakhamseh et al. 2016)**, India **(Basu and Mukherjee 2018)** and Spain **(Pérez-Etayo et al. 2018)**. Hence, it is essential to isolate ABU UPECs from various geographical locations and perform global epidemiological and periodic regional studies to comprehend their pathogenic potential which might prevent the spread of infections to vulnerable populations from the asymptomatic carriers.

The earlier studies from different parts of the world like the United States of America **(Johnson and Stell 2000)** and France **(Clermont et al. 2000)** indicated a clear relationship between the

distribution of phylogroups in *E. coli* strains and their extent of pathogenicity. Previous reports from Germany **(Salvador et al. 2012)** and India **(Srivastava et al. 2016)** demonstrated moderate to high incidences of ABU UPECs that belonged to the pathogenic phylogroup B2 (**Clermont et al. 2000**). Moreover, the enterohemorrhagic *E. coli* O157:H7, the best-known member of the phylogroup E **(Clermont et al. 2013)** was mostly documented as one of the highly pathogenic MDR *E. coli* strain worldwide **(Carone et al. 2014; Safwat Mohamed et al. 2018)**. Over and above that, studies from different regions of the world like France **(Li et al. 2009)** and Denmark **(Larsen et al. 2012)** had demonstrated the preponderance of bacterial strain typing for diagnosis, treatment, and epidemiological control of bacterial infections, especially the ones that are pathogenic and drug-resistant. Nonetheless, a very few studies worldwide had explored the sequence types (STs) of ABU (**Salvador et al. 2012; van der Mee-Marquet et al. 2016)** and symptomatic **(Giufre et al. 2012; Liu et al. 2015; Gauthier et al. 2018**) UPECs. Moreover, the worldwide dissemination of the *E. coli* ST131 clone, mostly associated with multidrug resistance and majority of which belonged to phylogroup B2 **(Giufre et al. 2012; Ali et al. 2019)** also justified the need of accurate strain typing of bacterial isolates together with the identification of their clonal and phylogenetic character.

Therefore the history of emergence and propagation of *E. coli* belonging to different phylogenetic groups, their clonality and incidence of rapid emergence of multidrug resistance amongst this pathogen, demands intricate analysis at the molecular level with respect to their phylogenetic background, sequence types (STs), clonal complexes (CCs) and Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) profile as a strain's ecological background and propensity to cause a disease vary extensively with its phylogenetic origin and clonal character . An earlier study **(Ghosh and Mukherjee 2019)** from our laboratory also showed the significant incidence of MDR UPECs that could not be grouped into any of the eight known phylogenetic groups **(Clermont et al. 2013)** identified till date and also failed to characterize the circulated isolates intricately. Moreover, the aforementioned isolates showed either ESBL or BLIR phenotype (Chapter 2). Withal, to the best of our knowledge, till date, no studies have compared MDR ABU and symptomatic UPECs relating to the acquisition of MGEs, genetic diversity, STs, CCs, and evolutionary relationships. So, this is the first study of its kind that aimed to characterize the drug-resistant UPECs isolated from urine samples of asymptomatic hospitalized patients in Kolkata, an eastern region of resource-poor country, India with respect to their acquisition of β-lactamase genes and MGEs, ERIC-banding pattern, STs, CCs, evolutionary relationships and Minimum spanning tree (MST) profile which were further compared with that of the symptomatic ones. Moreover, ABU and symptomatic UPECs with "Unknown" phylogroups were also subjected to more intricate phylotype analysis to understand their epidemiology.

4.2 Objectives

- Investigation of ABU and symptomatic UPECs on acquisition of β-lactamase genes and MGEs, to get an insight into the dissemination of resistant determinants.
- Multi-locus sequence typing, intricate phylotype property analysis and determination of clonal character of the isolated ABU and symptomatic UPECs in order to get an insight into their epidemiology and clonality. `
- Determination of evolutionary and /or phylogenetic and quantitative relationships between ABU and symptomatic UPECs.

4.3 Materials

(a) Equipments:

- Laminar Air Flow [B.D Instrumentation]
- Shaker – Incubator [ICT]
- Autoclave [PrimeSurgicals]
- Spectrophotometer [Bio-Rad, India]
- Hot air oven [Digisystem Laboratory Instruments Inc.]
- Thermal cycler [ABI Instruments Private Limited, Model-Veriti Thermal Cycler]
- Horizontal gel electrophoresis apparatus [Genei]
- Power pack [Genei]
- Gel Documentation system [BIO-RAD]
- Inoculation loop
- Glass spreaders
- Spirit Lamp
- 90mm Glass petri dish [Borosil]
- Glass culture tubes [TOUFF, Borosil]
- Test tube racks [Tarsons]
- Micropipettes (0.5-10µl, 2-20µl, 20-200µl, 200-1000µl) [Corning, P'fact, Microlit, Biohit]
- Micro tips (0.5-10µl, 2-20µl, 20-200µl, 200-1000µl) [HiMedia]
- Eppendorf Tubes (1.5 mL, 2 mL) [Tarsons]
- Cotton [Bengal Surgicals Limited] [Lakshmi Healthcare Products (P) Ltd]
- Surgical Gloves [PriCARE, HiMedia]
- Wash bottles

(c) Reagents:

- Luria Bertani (LB) media [SRL Chemicals India]
- Mueller Hinton (MH) media [SRL Chemicals India]

- Agar Agar [Merck]
- Barium chloride [Merck]
- Sulphuric acid [Hospital Store]
- 70% Ethanol [Bengal Chemical]
- Isopropanol [Hospital Store]
- Phenol [Hospital Store]
- Chloroform [Hospital Store]
- 95% Ethanol [HiMedia]
- Single Distilled water (SDW) [Hospital Store]
- Double distilled water (DDW) [Laboratory distillation plant]
- Primers [GCC Biotech(I) Pvt.Ltd]
- dNTP [Invitrogen]
- Taq DNA Polymerase and buffer [Invitrogen]
- 50mM MgCl2 [Invitrogen]
- DNA ladders [HiMedia]
- Tris Base [SRL Chemicals India]
- 6X Gel loading buffer [HiMedia]
- EDTA [Sigma-Aldrich]
- Glacial Acetic Acid [Merck]
- Glucose [HiMedia]
- Hydrogen chloride [Hospital Store]
- Potassium acetate [HiMedia]
- RNaseA [HiMedia]
- Potassium hydroxide [Hospital Store]
- Sodium hydroxide [Hospital Store]
- Sodium dodecyl sulphate
- Lysozyme [Sigma Aldrich]
- Proteinase K [HiMedia]
- Agarose [HiMedia]
- Ethidium bromide [SRL Chemicals India]

4.3.1 Preparation of reagents and compositions of solutions used

- **LB broth:** 10gms of LB broth powder was dissolved in 500mL of SDW (Conc.- 20gms/lit). Then it was thoroughly mixed, dispensed into culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes (file:///C:/Users/Arunita%20Ghosh/Downloads/M575.pdf).
- **MH broth:** 10.5gms of MH broth powder was dissolved in 500mL of SDW (Conc.-21gms/lit). Then it was thoroughly mixed, dispensed into culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes (file:///C:/Users/Arunita%20Ghosh/Downloads/M391.pdf).
- **MH agar:** 10.5gms of MH broth powder was dissolved in 500mL of SDW (Conc.-21gms/lit). Then to the aforesaid mixture 7.5gm of agar agar (Conc. 1.5%) was added, thoroughly mixed, and autoclaved at 15 psi pressure at 121°C for 15 minutes. After autoclaving, the sterile medium was distributed into different 90mm petriplates, cooled and solidified for future use
- **50X TAE (Tris-Acetate EDTA) buffer [1 litre]:** 242 gm of Tris-base (MW = 121.14 g/mol) was dissolved in approximately 700 mL of DDW. Then 57.1mL of 100 % glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0) was added to the aforesaid mixture. The solution was adjusted to a final volume of 1L. The pH of this buffer was then adjusted to 8.5 using potassium hydroxide (KOH). Then stock solution was stored in a Borosil container at room temperature for future use (https://www.protocols.io/view/recipe-for-50x-tae-buffer-gtvbwn6?step=3).
- **1X TAE (Tris-Acetate EDTA) buffer [500mL]:** This was prepared using 49 parts of DDW water with 1 part of 50X TAE (Tris-Acetate EDTA) buffer. The pH of the final solution was checked to be at 8.5 (http://2009.igem.org/TAE_Buffer) .
- **Buffers and solutions used:** The composition and the protocols used for different buffers and solutions used in this study are written as under-
  - **Alkaline lysis solution I (Resuspension buffer):** 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0), DDW. Protocol followed-

  (i) 1M glucose stock solution (50 mL) - 9.0 gm of glucose was dissolved in 50 mL sterilized DDW. Then the aforementioned solution was Filter sterilized using membrane millipore (0.20 µM and stored at 4°C for preservation.

  (ii) 1 M Tris-HCl stock solution (50 mL) - 6.057 gm of the Tris base was dissolved in 50 ml of sterilized DDW. pH was adjusted the to the desired value of 8.0 by adding concentrated HCl.

  (iii) 0.5 M EDTA stock solution (100 mL) - 14.612 gm of EDTA was dissolved in 100 ml of sterilized DDW. pH was adjusted the to the desired value of 8.0 by adding concentrated

NaOH. Solution I was prepared from the standard stocks in batches of approx. 100 ml and autoclaved for 15 minutes at 15 psi and stored at 4°C.

Alkaline lysis solution 1	Volume
1M Glucose	5 mL
1 M Tris-HCl buffer	2.5mL
0.5 M EDTA	2mL
DDW	90.5mL
Total volume	100mL

- **Alkaline lysis solution II (Lysis buffer):** 0.2 N NaOH, 1% (w/v) SDS, DDW. Protocol followed-

(i) 10 N NaOH stock solution (50 mL) - 20 gm of NaOH was dissolved in 50 ml of sterilized DDW.

(ii) 1% (w/v) SDS stock solution (30 mL) - 0.3 gm of SDS was dissolved in 30 ml of sterilized DDW. Solution II was freshly for every single use and stored at room temperature.

Alkaline lysis solution II	Volume
10N NaOH	200µL
1% SDS	1mL
DDW	8.8mL
Total volume	10mL

- **Alkaline lysis solution III (Neutralization buffer):** 5 M potassium acetate, glacial acetic acid, DDW. Protocol followed-

(i) 5 M potassium acetate stock solution (100 mL) - 49.071 gm of potassium acetate was dissolved in 100 ml of sterilized DDW. The solution was stored at 4°C and transferred to an ice bucket just before use.

Alkaline lysis solution III	Volume
5 M Potassium acetate	60ml
Glacial acetic acid	11.5ml
Double distilled water	28.5ml
Total volume	100ml

(https://static.igem.org/mediawiki/2015/3/30/ITB_INDONESIA_IGEM2015_DNA_Plasmid_isolation.pdf f).

4.4 Experimental methods

4.4.1 Bacterial culture

The forty (Asymptomatic= 20; Symptomatic=20) non-duplicate UPECs obtained from the previous part of this study conducted on 200 hospitalized patients were considered in the present analysis. The patient characteristics, various phenotypic and genotypic features of the isolated UPECs

had been documented in the previous chapters (Chapters 2 and 3) of this thesis. This study protocol was approved by the institutional ethical committee.

4.4.2 Plasmid DNA extraction

Plasmid DNA was extracted from all the 40 ABU and symptomatic UPECs using protocols as described by Basu and Mukherjee (**Basu and Mukherjee 2018),** but with minor modifications. Briefly, a single colony of each of the UPEC was inoculated from MacConkey agar plate into the 2ml of LB broth and incubated overnight in shaking condition at 37º C. The cells from the overnight culture were harvested by centrifugation at 10,500 rpm for 5 min. Plasmid DNA were isolated by alkaline lysis method. Resuspension of the isolated cells were performed with the resuspension buffer. Then after resuspension, the bacterial cells were lysed by the lysis buffer. After lysis, the plasmid DNA was reannealed by neutralizing buffer. Finally, the Phenol-chloroform extraction followed by ethanol precipitation was performed. The plasmid pellet was then washed with 70% ice cold ethanol air dried and resuspended in 20µl of 1x TAE (40mM tris, 20mM acetic acid, 1mM EDTA) buffer and kept at 4˚C and -80˚C for short-term and long-term storage respectively.

4.4.3 Genomic DNA extraction

The bacterial genomic DNA was isolated from 40 non-duplicate UPECs irrespective of their asymptomatic and symptomatic nature using the protocol as described by Wright et al. **(Wright et al. 2017)**. The extracted genomic DNA was quantified and stored at − 20 °C up till further use.

4.4.4 Identification of β-lactamase genes

The β-lactamase genes, *blaTEM, blaCTXM* **(Mukherjee et al. 2011)** and *blaOXA* **(Basu and Mukherjee 2018)** were detected by PCR using gene-specific primers. Separate PCR reactions for each of the aforesaid genes were carried using both the extracted plasmid and genomic DNA as template. All the aforementioned PCR assays were performed in 20 µl reaction volume that contained 10 ng of plasmid DNA/genomic DNA, 0.5 µM of each primer (GCC Biotech, India), 150 µM dNTPs (Invitrogen), 1.5 mM MgCl2 (Invitrogen), 1 U of the high fidelity Taq DNA polymerase (Invitrogen, "Platinum™ *Taq* DNA Polymerase High Fidelity") and 2.0 µl 10X PCR buffer (Invitrogen) **(Mukherjee et al. 2011; Basu and Mukherjee 2018)**. Amplicons generated were separated by 1.5% agarose gel electrophoresis and visualized using Ethidium bromide by Gel documentation system (BIO-RAD, USA) as described below. The target β-lactamase genes with their respective primer sequences were depicted below and marked in green. The primers and PCR conditions used for this study were shown in **Table 4.1**. Cluster analysis on the prevalence of the β-lactamase genes were performed based on Heat maps generated using the R software package (version 3.2.5) as described by Ibrahim et al. **(Ibrahim et al. 2016)**.

The target β-lactamase genes with their respective primer sequences are written as under:

blaTEM

Escherichia coli plasmid beta-lactamase (*blaTEM*) gene, complete cds

GenBank: KR872626.1

GenBank Graphics

>KR872626.1 *Escherichia coli* plasmid beta-lactamase (*blaTEM*) gene, complete cds

AAAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGT
CAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCA
TGAGACAATAACCCTGGTAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTTCGTGTCGCCCTTA
TTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGT
TGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTC
CAATGATGAGCACTTTTAAAGTTCTGCTATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCC
GCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAG
AATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCTGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGC
TAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAA
ACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAG
CTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCT
GGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCT
CCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCT
CACTGATTAAGCATTGGTAA

(https://www.ncbi.nlm.nih.gov/nuccore/KR872626.1?report=fasta)

blaCTXM

Escherichia coli plasmid beta-lactamase (*blaCTX-M*) gene

GenBank: AY156923.1

GenBank Graphics

>AY156923.1 *Escherichia coli* plasmid beta-lactamase (*blaCTX-M*) gene, complete cds

ATGGTGACAAAGAGAGTGCAACGGATGATGTTCGCGGCGGCGGCGTGCATTCCGCTGCTGCTGGGCAGCGCGCCGCTTTAT
GCGCAGACGAGTGCGGTGCAGCAAAAGCTGGCGGCGCTGGAGAAAAGCAGCGGAGGGCGGCTGGGCGTCGCGCTCATCGAT
ACCGCAGATAATACGCAGGTGCTTTATCGCGGTGATGAACGCTTTCCAATGTGCAGTACCAGTAAAGTTATGGCGGCCGCG
GCGGTGCTTAAGCAGAGTGAAACGCAAAAGCAGCTGCTTAATCAGCCTGTCGAGATCAAGCCTGCCGATCTGGTTAACTAC
AATCCGATTGCCGAAAAACACGTCAACGGCACAATGACGCTGGCAGAACTGAGCGCGGCCGCGTTGCAGTACAGCGACAAT
ACCGCCATGAACAAATTGATTGCCCAGCTCGGTGGCCCGGGAGGCGTGACGGCTTTTGCCCGCGCGATCGGCGATGAGACG
TTTCGTCTGGATCGCACTGAACCTACGCTGAATACCGCCATTCCCGGCGACCCGAGAGACACCACCACGCCGCGGGCGATG
GCGCAGACGTTGCGTCAGCTTACGCTGGGTCATGCGCTGGGCGAAACCCAGCGGGCGCAGTTGGTGACGTGGCTCAAAGGC
AATACGACCGGCGCAGCCAGCATTCGGGCCGGCTTACCGACGTCGTGGACTGTGGGTGATAAGACCGGCAGCGGCGGCTAC
GGCACCACCAATGATATTGCGGTGATCTGGCCGCAGGGTCGTGCGCCGCTGGTTCTGGTGACCTATTTTACCCAGCCGCAA
CAGAACGCAGAGAGCCGCCGCGATGTGCTGGCTTCAGCGGCGAGAATCATCGCCGAAGGGCTGTAA

(https://www.ncbi.nlm.nih.gov/nuccore/AY156923.1?report=fasta)

blaOXA

***Escherichia coli* strain ZR23 plasmid beta-lactamase (*OXA*) gene, partial cds**

GenBank: KX171194.1

GenBank Graphics PopSet

>KX171194.1 *Escherichia coli* strain ZR23 plasmid beta-lactamase (*OXA*) gene, partial cds

AGGAACTGAAGGTTGTTTTTTACTTTACGATGCATCCACAAACGCTGAAATTGCTCAATTCAATAAAGCAAAGTGTGCAAC
GCAAATGGCACCAGATTCAACTTTCAAGATCGCATTATCACTTATGGCATTTGATGCGGAAATAATAGATCAGAAAACCAT
ATTCAAATGGGATAAAACCCCCAAAGGAATGGAGATCTGGAACAGCAATCATACACCAAAGACGTGGATGCAATTTTCTGT
TGTTTGGGTTTCGCAAGAAATAACCCAAAAAATTGGATTAAATAAAATCAAGAATTATCTCAAAGATTTTGATTATGGAAA
TCAAGACTTCTCTGGAGATAAAGAAAGAAACAACGGATTAACAGAAGCATGGCTCGAAAGTAGCTTAAAAATTTCACCAGA
AGAACAAATTCAATTCCTGCGTAAAATTATTAATCACAATCTCCCAGTTAAAAACTCAGCCATAGAAAACACCATAGAGAA
CATGTATCTACAAGATCTGGATAATAGTACAAAACTGTATGGGAAAACTGGTGCAGGATTCACAGCAAATAGAACCTTACA
AAACGGATGGTTTGAAGGGTTTATTATAAGCAAATCAGGACATAA

(https://www.ncbi.nlm.nih.gov/nuccore/KX171194.1?report=fasta)

Table 4.1: Primer sequences and PCR conditions used for the amplification of the β- lactamase genes

Sl no.	Target genes	Primer sequences (5'-3')	PCR conditions (Time)	No. of cycles	Amplicon size (bp)	References
1	*blaTEM*	F.P- ATGAGTATTCAACATTTTCGTG R.P- TTACCAATGCTTAATCAGTGAG	95°C (30 sec) 52°C (30 sec) 72°C (1min)	30	861	Mukherjee et al. 2011
2	*blaCTXM*	F.P- ATGTGCAGYACCAGTAAAG R.P- ATATCRTTGGTGGTGCCRT	95°C (30 sec) 53°C (30 sec) 72°C (1min)	30	536	Mukherjee et al. 2011
3	*blaOXA*	F.P- CACTTATGGCATTTGATGCGGA R.P- TGCTGTGAATCCTGCACCAG	95°C (30 sec) 52°C (30 sec) 72°C (1min)	30	432	Basu and Mukherjee 2018

4.4.5 Agarose gel electrophoresis

On the basis of the sizes of the DNA fragments (β- lactamase genes) to be separated, the concentration of agarose (0.8% or 1% or 1.5%) was weighed for gel electrophoresis of the above-mentioned PCR products. Then after weighing agarose, DDW and 1X TAE buffer was added and mixed by heating in a microwave oven until the agarose was dissolved completely. 5 µL of the stock EtBr (10mg/mL) solution was added to the gel and the entire mixture was poured into the gel tray (EtBr was used with caution when as it is a known carcinogen.). The gel casting tray was previously

prepared for the above-mentioned procedure using combs of different sizes according to the requirements (8 wells, 13 wells, 26 wells or 52wells). Any bubbles if formed after pouring the gel was removed and allowed gel to cool for 30 min at room temperature. Then after the gel got solidified, combs were removed and the casting tray was placed in electrophoresis tank (chamber) filled with 1X TAE buffer. PCR products and DNA ladders were mixed with 6X gel loading buffer and loaded in the different wells of the solidified gel. The entire set up was then connected to the power supply and electrophoresed at 100V for 40 -50mins. After separation of the DNA fragments, gel bands (DNA fragments) were analyzed and documented using gel documentation system (https://www.thermofisher.com/in/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-electrophoresis/agarose-gel-electrophoreis/agarose-gel-electrophoresis-protocols-e-gel-ex-agarose-gel-and-ultrapure-agarose.html).

4.4.6 Identification of MGEs

The integrons class1 (*intI1*) and class2 (*intI2*) **(Salem et al. 2010)** and insertion element *IS5* **(Kurpiel et al. 2011)** were detected by PCR using gene-specific primers**.** However, presence of insertion elements *ISEcp1* (406bp) and *IS26* (590bp) were investigated by PCR using gene specific primers. All the aforementioned PCR assays were performed in 20 µl reaction volume that contained 10 ng of plasmid DNA, 0.5 µM of each primer (GCC Biotech, India), 150 µM dNTPs (Invitrogen), 1.5 mM MgCl2 (Invitrogen), 1 U of the high fidelity Taq DNA polymerase (Invitrogen "Platinum™ *Taq* DNA Polymerase High Fidelity") and 2.0 µl 10X PCR buffer (Invitrogen) **(Basu and Mukherjee 2018)**. Amplicons generated were separated by 1.5% agarose gel electrophoresis and visualized using Ethidium bromide by Gel documentation system (BIO-RAD, USA) as described in section 4.4.5. The target MGEs with their respective primer sequences were depicted below and marked in green. The primers and PCR conditions used for this study were shown in **Table 4.2**. Cluster analysis on the prevalence of MGEs was performed based on Heat maps generated using the R software package (version 3.2.5) as previously described by Ibrahim et al. **(Ibrahim et al. 2016)**.

The target MGE genes with their respective primer sequences are written as under:

intI1

***Escherichia coli* In848 integron IntI1 (*intI1*), AacA4 (aacA4), VIM-1 (*blaVIM-1*), AadA1 (*aadA1*), CatB2 (*catB2*), and QacEdelta1 (*qacEdelta1*) genes, complete cds**

GenBank: KC417377.1

GenBank Graphics

>KC417377.1:1-1014 Escherichia coli In848 integron IntI1 (*intI1*), AacA4 (*aacA4*), VIM-1 (*blaVIM-1*), AadA1 (*aadA1*), CatB2 (*catB2*), and QacEdelta1 (qacEdelta1) genes, complete cds

ATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTG
CATTACAGCTTACGAACCGAACAGGCTTATGTCCACTGGGTTCGTGCCTTCATCCGTTTCCACGGTGTGCGTCACCCGGCA
ACCTTGGGCAGCAGCGAAGTCGAGGCATTTCTGTCCTGGCTGGCGAACGAGCGCAAGGTTTCGGTCTCCACGCATCGTCAG
GCATTGGCGGCCTTGCTGTTCTTCTACGGCAAGGTGCTGTGCACGGATCTGCCCTGGCTTCAGGAGATCGGAAGACCTCGG
CCGTCGCGGCGCTTGCCGGTGGTGCTGACCCCGGATGAAGTGGTTCGCATCCTCGGTTTTCTGGAAGGCGAGCATCGTTTG
TTCGCCCAGCTTCTGTATGGAACGGGCATGCGGATCAGTGAGGGTTTGCAACTGCGGGTCAAGGATCTGGATTTCGATCAC
GGCACGATCATCGTGCGGGAGGGCAAGGGCTCCAAGGATCGGGCCTTGATGTTACCCGAGAGCTTGGCACCCAGCCTGCGC
GAGCAGCTGTCGCGTGCACGGGCATGGTGGCTGAAGGACCAGGCCGAGGGCCGCAGCGGCGTTGCGCTTCCCGACGCCCTT
GAGCGGAAGTATCCGCGCGCCGGGCATTCCTGGCCGTGGTTCTGGGTTTTTGCGCAGCACACGCATTCGACCGATCCACGG
AGCGGTGTCGTGCGTCGCCATCACATGTATGACCAGACCTTTCAGCGCGCCTTCAAACGTGCCGTAGAACAAGCAGGCATC
ACGAAGCCCGCCACACCGCACACCCTCCGCCACTCGTTCGCGACGGCCTTGCTCCGCAGCGGTTACGACATTCGAACCGTG
CAGGATCTGCTCGGCCATTCCGACGTCTCTACGACGATGATTTACACGCATGTGCTGAAAGTTGGCGGTGCCGGAGTGCGC
TCACCGCTTGATGCGCTGCCGCCCCTCACTAGTGAGAGGTAG

(https://www.ncbi.nlm.nih.gov/nuccore/KC417377.1?report=fasta&to=1014)

intI2

Escherichia coli strain 8157 class II integron, partial sequence, IntI2 (*intI2*), dihydrofolate reductase (*dfrA14*), and putative lipoprotein signal peptidase (*lsp*) genes, complete cds, and putative outer membrane lipoprotein (lip) pseudogene, complete sequence

GenBank: EU780012.1

GenBank Graphics

>EU780012.1:864-1841 *Escherichia coli* strain 8157 class II integron, partial sequence, IntI2 (*intI2*), dihydrofolate reductase (*dfrA14*), and putative lipoprotein signal peptidase (*lsp*) genes, complete cds, and putative outer membrane lipoprotein (lip) pseudogene, complete sequence

ATGTCTAACAGTCCATTTTTAAATTCTATACGCACGGATATGCGGCAAAAAGGTTATGCGCTGAAAACTGAAAAAACTTACC
TGCACTGGATTAAGCGTTTTATTCTGTTTCACAAAAAACGTCATCCTCAGACCATGGGCAGTGAAGAGGTCAGGCTGTTTTT
ATCCAGCTTAGCAAACAGCAGACATGTAGCCATAAACACGCAGAAAATCGCTTTAAATGCCCTAGCTTTTTTGTACAACAGG
TTTTTACAACAGCCGTTGGGCGATATTGATTATATCCCTGCAAGCAAGCCTAGACGGCTACCCTCTGTTATCTCTGCAAATG
AAGTGCAACGCATTTTGCAGGTTATGGATACTCGCAACCAAGTAATTTTTGCGCTGCTGTATGGTGCAGGTTTGCGCATTAA
TGAATGCTTGCGTTTGCGGGTTAAAGATTTTGATTTTGATAATGGCTGCATCACTGTGCATGACGGTAAGGGTGGGAAAAGC
AGAAACAGCCTACTGCCCACGCGCCTAATCCCAGTAATAAAACAACTCATTGAGCAAGCGCGGCTTATTCAGCAAGACGACA
ACTTACAAGGCGTAGGGCCATCGCTGCCTTTTGCTTTAGATCGCAAATACCCTTCTGCTTATCGACAAGCGGCGTGGATGTT
TGTCTTTCCCTCCAGCACGCTCTGCAACCACCCGTATAACGGCAAATTATGCCGCCATCATCTGCATGACTCCGTTGCGCGA
AAGGCATTGAAGGCAGCCGTACAAAAAGCAGGCATCGTTAGCAAGCGTGTCACTTGTCATACATTTCGTCACTCGTTTGCTA

CGCATCTATTACAAGCGGGGCGTGATATTCGCACTGTGCAAGAACTCTTAGGGCATACCGATGTTAAGACCACGCAAATCTA
TACGCATGTGTTGGGTCAGCATTTTGCCGGCACCACCAGTCCTGCGGATGGACTGATGCTACTTATCAATCAGTAA

(https://www.ncbi.nlm.nih.gov/nuccore/EU780012.1?report=fasta&from=864&to=1841)

ISEcp1

Escherichia coli strain HV295 plasmid pHV295, complete sequence

GenBank: KM377240.1

GenBank Graphics

>KM377240.1:85184-86446 *Escherichia coli* strain HV295 plasmid pHV295, complete sequence

ATGATTAATAAAATTGATTTCAAAGCTAAGAATCTAACATCAAATGCAGGTCTTTTTCTGCTCCTTGAGAATGCAAAAAGC
AATGGGATTTTTGATTTTATTGAAAATGACCTCGTATTTGATAATGACTCAACAAATAAAATCAAGATGAATCATATAAAG
ACCATGCTCTGCGGTCACTTCATTGGCATTGATAAGTTAGAACGTCTAAAGCTACTTCAAAATGATCCCCTCGTCAACGAG
TTTGATATTTCCGTAAAAGAACCTGAAACAGTGTCACGGTTTCTAGGAAACTTCAACTTCAAGACAACCCAAATGTTTAGA
GACATTAATTTTAAAGTCTTTAAAAAACTGCTCACTAAAAGTAAATTGACATCCATTACGATTGATATTGATAGTAGTGTA
ATTAACGTAGAAGGTCATCAAGAAGGTGCGTCAAAAGGATATAATCCTAAGAAACTGGGAAACCGATGCTACAATATCCAA
TTTGCATTTTGCGACGAATTAAAAGCATATGTTACCGGATTTGTAAGAAGTGGCAATACTTACACTGCAAACGGTGCTGCG
GAAATGATCAAAGAAATTGTTGCTAACATCAAATCAGACGATTTAGAAATTTTATTTCGAATGGATAGTGGCTACTTTGAT
GAAAAAATTATCGAAACGATAGAATCTCTTGGATGCAAATATTTAATTAAAGCCAAAAGTTATTCTACACTCACCTCACAA
GCAACGAATTCATCAATTGTATTCGTTAAAGGAGAAGAAGGTAGAGAAACTACAGAACTGTATACAAAATTAGTTAAATGG
GAAAAAGACAGAAGATTTGTCGTATCTCGCGTACTGAAACCAGAAAAAGAAAGAGCACAATTATCACTTTTAGAAGGTTCC
GAATACGACTACTTTTTCTTTGTAACAAATACTACCTTGCTTTCTGAAAAAGTAGTTATATACTATGAAAAGCGTGGTAAT
GCTGAAAACTATATCAAAGAAGCCAAATACGACATGGCGGTGGGTCATCTCTTGCTAAAGTCATTTTGGGCGAATGAAGCC
GTGTTTCAAATGATGATGCTTTCATATAACCTATTTTTGTTGTTCAAGTTTGATTCCTTGGACTCTTCAGAATACAGACAG
CAAATAAAGACCTTTCGTTTGAAGTATGTATTTCTTGCAGCAAAAATAATCAAAACCGCAAGATATGTAATCATGAAGTTG
TCGGAAAACTATCCGTACAAGGGAGTGTATGAAAAATGTCTGGTATAA

(https://www.ncbi.nlm.nih.gov/nuccore/KM377240)

IS5

Escherichia coli strain CUMC-201 plasmid CMY-2 (*blaCMY-2*) gene, complete cds; insertion sequence *IS5* complete cds

GenBank: HQ680722.1

GenBank Graphics

>HQ680722.1:1559-2753 *Escherichia coli* strain CUMC-201 plasmid CMY-2 (*blaCMY-2*) gene, complete cds; insertion sequence IS5, complete sequence

GGAAGGTGCGAACAAGTCCCTGATATGAGATCATGTTTGTCATCTGGAGCCATAGAACAGGGTTCATCATGAGTCATCAAC
TTACCTTCGCCGACAGTGAATTCAGCAGTAAGCGCCGTCAGACCAGAAAAGAGATTTTCTTGTCCCGCATGGAGCAGATTC
TGCCATGGCAAAACATGGTGGAAGTCATCGAGCCGTTTTACCCCAAGGCTGGTAATGGCCGGCGACCTTATCCGCTGGAAA
CCATGCTACGCATTCACTGCATGCAGCATTGGTACAACCTGAGCGATGGCGCGATGGAAGATGCTCTGTACGAAATCGCCT
CCATGCGTCTGTTTGCCCGGTTATCCCTGGATAGCGCCTTGCCGGACCGCACCACCATCATGAATTTCCGCCACCTGCTGG
AGCAGCATCAACTGGCCCGCCAATTGTTCAAGACCATCAATCGCTGGCTGGCCGAAGCAGGCGTCATGATGACTCAAGGCA
CCTTGGTCGATGCCACCATCATTGAGGCACCCAGCTCGACCAAGAACAAAGAGCAGCAACGCGATCCGGAGATGCATCAGA

CCAAGAAAGGCAATCAGTGGCACTTTGGCATGAAGGCCCACATTGGTGTCGATGCCAAGAGTGGCCTGACCCACAGCCTGG
TCACCACCGCGGCCAACGAGCATGACCTCAATCAGCTGGGTAATCTGCTGCATGGAGAGGAGCAATTTGTCTCAGCCGATG
CCGGCTACCAAGGGGCGCCACAGCGCGAGGAGCTGGCCGAGGTGGATGTGGACTGGCTGATCGCCGAGCGCCCCGGCAAGG
TAAGAACCTTGAAACAGCATCCACGCAAGAACAAAACGGCCATCAACATCGAATACATGAAAGCCAGCATCCGGGCCAGGG
TGGAGCACCCATTTCGCATCATCAAGCGACAGTTCGGCTTCGTGAAAGCCAGATACAAGGGGTTGCTGAAAAACGATAACC
AACTGGCGATGTTATTCACGCTGGCCAACCTGTTTCGGGCGGACCAAATGATACGTCAGTGGGAGAGATCTCACTAAAAAC
TGGGGATAACGCCTTAAATGGCGAAGAAACGGTCTAAATAGGCTGATTCAAGGCATTTACGGGAGAAAAAATCGGCTCAAA
CATGAAGAAATGAAATGACTGAGTCAGCCGAGAAGAATTTCCCCGCTTATTCGCACCTTCC

(https://www.ncbi.nlm.nih.gov/nuccore/HQ680722.1?report=fasta&from=1559&to=2753)

IS26

Escherichia coli strain CDF8 plasmid pCDF8, complete sequence

GenBank: MF175191.1

GenBank Graphics

>MF175191.1:8609-9313 *Escherichia coli* strain CDF8 plasmid pCDF8, complete sequence

ATGAACCCATTCAAAGGCCGGCATTTTCAGCGTGACATCATTCTGTGGGCCGTACGCTGGTACTGCAAATACGGCATCAGT
TACCGTGAGCTGCAGGAGATGCTGGCTGAACGCGGAGTGAATGTCGATCACTCCACGATTTACCGCTGGGTTCAGCGTTAT
GCGCCTGAAATGGAAAAACGGCTGCGCTGGTACTGGCGTAACCCTTCCGATCTTTGCCCGTGGCACATGGATGAAACCTAC
GTGAAGGTCAATGGCCGCTGGGCGTATCTGTACCGGGCCGTCGACAGCCGGGGCCGCACTGTCGATTTTTATCTCTCCTCC
CGTCGTAACAGCAAAGCTGCATACCGGTTTCTGGGTAAAATCCTCAACAACGTGAAGAAGTGGCAGATCCCGCGATTCATC
AACACGGATAAAGCGCCCGCCTATGGTCGCGCGCTTGCTCTGCTCAAACGCGAAGGCCGGTGCCCGTCTGACGTTGAACAC
CGACAGATTAAGTACCGGAACAACGTGATTGAATGCGATCATGGCAAACTGAAACGGATAATCGGCGCCACGCTGGGATTT
AAATCCATGAAGACGGCTTACGCCACCATCAAAGGTATTGAGGTGATGCGTGCACTACGCAAAGGCCAGGCCTCAGCATTT
TATTATGGTGATCCCCTGGGCGAAATGCGCCTGGTAAGCAGAGTTTTTGAAATGTAA

(https://www.ncbi.nlm.nih.gov/nuccore/MF175191.1?report=graph)

Table 4.2: Primer sequences and PCR conditions used for the amplification of the MGE genes

Sl no.	Target genes	Primer sequences (5'-3')	PCR conditions (Time)	No. of cycles	Amplicon size (bp)	References
1	*intI1*	F.P- GGTCAAGGATCTGGATTTCG R.P- ACATGCGTGTAAATCATCGTC	95°C (30 sec) 50°C (30 sec) 72°C (1min)	30	483	Salem et al. 2010
2	*intI2*	F.P- CACGGATATGCGGCAAAAAGGT R.P- GTAGCAAACGAGTGACGAAATG	95°C (30 sec) 51°C (30 sec) 72°C (1min)	30	789	Salem et al. 2010
3	*ISEcp1*	F.P- CTGCGGTCACTTCATTGGC R.P- GATCATTTCCGCAGCACCG	95°C (30 sec) 50°C (30 sec) 72°C (1min)	30	406	Basu and Mukherjee 2018

4	***IS5***	**F.P- CATGCTACGCATTCACTGC** **R.P- GAACTGTCGCTTGATGATGC**	**95°C (30 sec)** **52°C (30 sec)** **72°C (1min)**	**30**	**682**	**Kurpiel et al. 2011**
5	***IS26***	**F.P- CGCTGGTACTGCAAATACGGC** **R.P- GCTGAGGCCTGGCCTTTG**	**95°C (30 sec)** **52°C (30 sec)** **72°C (1min)**	**30**	**590**	**Basu and Mukherjee 2018**

4.4.7 Phylotype property analysis

Phylogroup assignment of the *E. coli* to any of the eight established phylogroups (A, B1, B2, C, D, E, F, and clade I) by the new quadruplex PCR method was based on the identification of the *arpA* gene (400bp) along with the original gene targets *chuA* (288bp), *yjaA* (211bp), TspE4.C2(152bp) **(Clermont et al. 2013)**. In this study, intricate phylotype property analysis was performed on the UPECs isolates using group E specific primer sets targeting *arpA*; 301bp **(Clermont et al. 2013)**, to develop a modified quadruplex PCR method to analyze the phylogenetic properties of the isolates that could not be assigned to any of the eight known phylogroups (results obtained from section 3.4.1) **(Ghosh and Mukherjee 2019)** by the established quadruplex PCR method. This was because *aceK* and *arpA* genes share a common region **(Clermont et al. 2004)** which had been illustrated below. Moreover, for confirmatory analysis, the aforementioned isolates were also investigated using *trpA*; 219 bp (group C) and *trpA*; 489 (internal control) specific primers **(Clermont et al. 2013)**. All the PCR assays were performed in triplicates using a high fidelity Taq DNA polymerase (Invitrogen, "Platinum™ *Taq* DNA Polymerase High Fidelity"). The PCR conditions and the primer used were mentioned in the section 3.4.3; Table 3.2 of the Chapter 3. Amplicons generated were separated by 1.5% agarose gel electrophoresis and visualized using Ethidium bromide by Gel documentation system (BIO-RAD, USA) as described in section 4.4.5. The selected amplicons were purified and sequenced by Bioserve Biotechnologies (India) Private Limited (Hyderabad, India) using protocols as described by Wang et al. **(Wang et al. 2020)**

***Escherichia coli* UMN026 chromosome, complete genome**

GenBank: CU928163.2

GenBank Graphics

>CU928163.2:4707218-4711109 *Escherichia coli* UMN026 chromosome, complete genome

ATGCCGCGTGGCCTGGAATTACTGATTGCACAAACCATTTTGCAAGGTTTCGACGCTCAGTATGGTCGATTCCTCGAAGTG
ACTTCCGGGGCGCAGCAGCGTTTCGAACAAGCCGACTGGCACGCTGTCCAGCAGGCGATGAAAAACCGTATCCATCTTTAC
GATCATCACGTGGGTCTGGTCGTGGAGCAACTGCGCTGCATTACCAACGGCCAAAGCACGGACGCGGCATTTTTACTGCGC
GTCAAAGAGCATTACACCCGGCTGTTGCCGGATTACCCGCGCTTCGAGATTGCGGAGAGCTTTTTTAACTCCGTGTACTGT
CGGTTATTTGACCACCGCTCGCTTACTCCCGAGCGGCTTTTTATCTTTAGCTCCCAGCCAGAGCGCCGCTTTCGTACCATT
CCCCGCCCGCTGGCGAAAGACTTTCACCCCGATCACGGCTGGGAATCTCTGCTGATGCGCGTTATCAGCGACCTGCCGCTG
CGCCTGCGCTGGCAGAATAAAAGCCGTGACATTCATTACATCGTTCGCCATCTGACGGAAACGCTGGGGACAGACAACCTC
GCGGAAAGTCATTTACAGGTGGCGAACGAACTGTTTTACCGCAATAAAGCCGCCTGGCTGGTAGGCAAACTGATCACGCCT

TCCGGCACATTGCCATTTTTGCTGCCGATCCACCAGACGGACGACGGCGAGTTATTTATTGATACCTGCCTGACGACGACC

GCCGAAGCGAGCATTGTTTTTGGCTTTGCGCGTTCTTATTTTATGGTTTACGCGCCGCTGCCCGCAGCACTGGTCGAGTGG

CTACGGGAAATTCTGCCAGGTAAAACCACCGCTGAATTGTATATGGCTATCGGCTGCCAGAAGCATGCCAAAACCGAGAGC

TACCGCGAATATCTCGTTTATCTACAGGGCTGTAATGAGCAGTTCATTGAAGCGCCGGGTATTCGTGGAATGGTGATGTTG

GTGTTTACGTTGCCGGGTTTTGATCGAGTATTCAAAGTCATCAAAGACAAGTTCGCGCCGCAGAAAGAGATGTCTGCCGCT

CACGTTCGTGCCTGCTATCAATTGGTGAAAGAGCACGATCGCGTGGGCCGAATGGCGGACACCCAGGAGTTTGAAAACTTT

GTGCTGGAGAAGCGGCATATTTCCCCGGCATTAATGGCATTACTGCTCCAGGAAGCAGCGGAAAAAATCACCGATCTCGGC

GAACAAATTGTGATTCGCCATCTTTATATTGAGCGGCGGATGGTGCCGCTCAATATCTGGCTGGAGCAAGTGGAAGGTCAG

CAGTTGCGCGATGCCATTGAAGAATACGGT**AACGCTATTCGCCAGCTTGC**CGCTGCTAACATTTTCCCTGGCGACATGC

TGTTTAAAAACTTCGGTGTCACCCGTCACGGGCGTGTGGTGTTTTATGATTACGATGAAATTTGCTACATGACGGAAGTGA

ACTTCCGCGACATCCCGCCGCCGCGCTACCCGGAGGACGAACTTGCCAGCGAACCGTGGTACAGCGTCTCGCCGGGCGATG

TTTTCCCGGAAGAGTTTCGCCACTGGCTATGCGCTGACCCCCGCATTGGGCCACTCTTTGAAGAGATGCACGCCGACCTGT

TCCGCGCTGATTACTGGCGCGCGCTACAAAACCGTATCCGTGACGGGCATGTGGAAGATGTTTATGCGTATCGGCGCAGGC

AAAGAT**TTAGCGTACGGTATGGGGAGA**TGCTTTTTTGAGTAAAGCTTCCATATAATTTTTCTCCGCAATGTATCGAGG

GTTATCCGTAAAGCCAAAGCTTTCAGCCATCTTATTTATCGTATTAAGGATTAATTCAGCAATAACCCGGTGATCCAATTC

AAAAGCCAACTCAAAGGCAGAGTATTTTTGTGGCGCTTTGTGTTGCCAAAAATCCATAATATCTTCAGCAGTAAATCCAAA

CAGGCGTGCATGGTCAGATAAAGCAAGATAAACCGTCTCTACAACGTTTTGTTGTTTATGCTGTATCGCTGAAAACAAACC

GGGATATTCATTAGAGTTATTTGCCAGGAGGAGGGGCTTCATATTTTTTTTTATCGAATTTAAACGTATTAAACAGAGTGGG

TAATACGTTAAAAATAGTCTTAATAACGTTCATATGTCCGCGCTGCATGGCCATAAACAAACCCGTGTCGCGCGCAAGACT

TTTAGCGGTCAGAAGATCGACAATATCGGAAGCTGAAATGTTAATTTCCTGGGCCAGACAGGGTAATGCTTCGAGAATAAC

TTTCACGATATCGCTATGTCCATTTTGCATCGCCAGGTATAGTCCTGGGCAACCATAAAAATCCTTTGCCTTCAGGAGATC

GAGTACCTGTTCTTTAGTCAAATGACATGTGCGAATTAACAAAGGTAACGCGTCCAAAACAATTTTCAGCATGTCGGCATC

ACCATTCGCCATAACATGGTATAAAACATGGCTGGACGTTCTATTTTTGGCACTCAGGAATTTATACACCTGTTCATTATC

TAAATGATGTGTTGCGGCCAGTTTAGGTAAGACGTTCAGAATCGAGGTTACAACATTTTTATCCTTACGTGATATCGCTAA

AAATAATCCAGAAAAACCATTTTTATCTTTTGCCTCCAGAATATGCATGAGATTTTTTTTCGAGAGTAACCCTTCATATCC

TGGCTCTGACAATGAATTGAAAATAGTCTCAACGATATCCGCATTACCATAATTTATAGCCAAATATAAACCGGGGAGGTT

AATACTATTATAGGCAGTCAACATTTCTGTTCTATGTAGTTCTTGCATTTTCTGGAGTTGAGTCATCAGTTGCGTAAGTTG

ATGATTCTGACCAATTGCCATTAGCAAATTCATCGTTGCAGGTGATAATGGAGGGGTTATTTCATCCACAGATCCTTTTAG

CATCAATAGCTGTTCTTTCGGAAGTGCTGGAATTATTGCAACAGATTGGTTAATAACATGATCATTTGACCATTTCAGGAG

GCTATAATCTACATTCATAAAGTCCATCAATGAATAGTGTTTAATATCCTCTTTACTTTCGCTCATGATTCTTATTTTATC

GTTAGTTACATTTGGATCATAAACCGAAACCACGTAATGGGTACATCCTTCAGTTGTGTTCTTTATTCTTAGCCGGACCGT

CAAAGCATGGTTATCCACCAATAATATGGCTGCCATAACACTGATGCCATTT**GATGCCATCTTGTCAAAATATGCC**TT

TCATAACTAGCCCGAAATCACCGCAGGCAGCAACGCAACGCCCCTCAGGACGGCTGGAAATCAACGCCAAAGTCTGGTTTT

TGCATTTTTTGTTGAAGTGAGTGAGAAATTTTTCAACTGAAGAGTAATCATCTTTATGAGGTTTGTAGTCAACATTCTGAT

AAATATCATTAATATATTGTGCAGCAATGTGTCGGCATACTATACGGTTTTGGGGTCGCCCCGGAAAATAACATTGACCAT

TAAGTTTAAT**TCTTGGAAATTCTTTTTTCTTTTTC**TATCTGATTTTAGATTTAATTTTTGTTTATCAAGAGCGTCTGAG

CGTTGAGGTAAAATTTCCATCAGGTATTCTTCAAATATTGGAGCAATGGTTTGAGTGAGTGTCGTGTTTTCATTTTTATTA

ATTATCGTAATTTCTTTTTTATCAGTCTGTAAGACGGACGTGGAAAACTTAATGACAAAATCCTTGTTACTAAAGATATGA

CGACCATTTTGTCTACAGTTCTCTGAGAAGCTTTTTAATAGAGGCGTCGCCAGGTCCTTGCCAGAAAATTTATCCTCGAGT

TCTTTATAAAACAATTCACTCAGGGTTTGGTGTTCATTTGTCCGGGCTGTATTATTAATATTTGCAGAGAAAGAACTACGA

GGAATACGAGTAATCAT

(https://www.ncbi.nlm.nih.gov/nuccore/CU928163.2?report=fasta&from=4708923&to=4711109)

Note: The aforementioned sky (sequence) region depicted the *aceK* gene with the forward primer (for *arpA* 400bp) marked in red. The yellow region illustrated the sequence region shared by both the *aceK* and *arpA* genes with reverse primer (for *arpA* 400bp) marked in red. The green (sequence) region depicted the *arpA* gene with the forward and the reverse primer (*arpAgpE*; 301bp) marked in purple.

4.4.8 Molecular typing by ERIC-PCR

ERIC-PCR was performed on genomic DNA as described by Dhanashree and Mallya **(Dhanashree and Mallya 2012)** but with minor modifications. Briefly, PCR was performed on 25µL reaction volume containing 10ng of genomic DNA, 400µM dNTPs (Invitrogen), 5 mM MgCl2 (Invitrogen), 2.5 µM of each primer (GCC Biotech, India), 2 U Taq DNA polymerase (Invitrogen, "Platinum™ *Taq* DNA Polymerase High Fidelity") and 2.5 µl of 10X PCR buffer (Invitrogen). The primers and PCR conditions used were shown in **Table 4.3**. The amplicons generated were separated by 1.5% agarose gel electrophoresis and visualized using Ethidium bromide by Gel documentation system (BIO-RAD, USA) as described in section 4.4.5. A dendrogram was generated by hierarchical cluster analysis method using Dice coefficient and UPGMA algorithm in the SPSS version 21.0 software **(Basu and Mukherjee 2018).**

Table 4.3: Primer sequences and PCR conditions used ERIC-PCR typing

Sl no.	Target gene	Primer sequences (5'-3')	PCR conditions (Time)	No. of cycles	References
1	ERIC	F.P- ATGTAAGCTCCTGGGGATTCAC R.P- AAGTAAGTGACTGGGGTGAGCG	95°C (30 sec) 52°C (1min) 72°C (4min 30sec)	35	Dhanashree and Mallya 2012

4.4.9 Multi-locus sequence typing (MLST)

All 40 UPECs were ascribed to multilocus sequence types by the classical seven gene approach (Achtman MLST scheme) as previously described (**Wirth et al. 2006).** Following the primer sequences and the protocols (PCR conditions) specified at the *E. coli* MLST website **(https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html),** PCR was performed on *E. coli* genomic DNA using the seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) (**Wirth et al. 2006**). All the PCR amplifications were performed using a high fidelity Taq DNA polymerase (Invitrogen, "Platinum™ *Taq* DNA Polymerase High Fidelity"). The consensus region of the seven housekeeping genes used for sequence typing a particular *E. coli* isolate

had been depicted below using the allele template as *E. coli* strain MG1655. Moreover, the target MLST genes with their original sequence, the consensus region (yellow) and the respective primer (green) sequence in the case of the *E. coli* strain MG1655 were depicted and marked below as described (https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html). The amplicons were purified and sequenced by Bioserve Biotechnologies (India) Private Limited (Hyderabad, India) using protocols as described by Wang et al. **(Wang et al. 2020)**. Allele numbers for the seven gene fragments of each *E. coli* isolate were acquired by comparing with corresponding alleles available at the *E. coli* MLST database **(https://enterobase.warwick. ac.uk/species/ecoli/ allele_st_search) (Zheng et al. 2019).** Sequences of the seven genes were concatenated for each isolate using **(https://www.bioinformatics.org/sms2/combine_fasta.html)** and ST of each isolate was identified on the basis of the aforesaid concatenated sequence data (combining seven allelic profiles) using **(https://cge.cbs.dtu.dk/services/MLST/) (Larsen et al. 2012).** CCs of the identified STs were obtained via the *E. coli* MLST database **(http://enterobase.warwick.ac.uk/species/ecoli/search_strains?query=st_search) (Zheng et al. 2019)**. The consensus regions of the seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) as found in the *E. coli* MLST (https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html) database are depicted below (**Wirth et al. 2006).**

Allele template

Allelic profile of *E. coli* strain MG1655 (GenBank accession no.- NC_000913.3)

adk (536 bp): [Allele no. 10]

GGGGAAAGGGACTCAGGCTCAGTTCATCATGGAGAAATATGGTATTCCGCAAATCTCCACTGGCGATATGCTGCGTG
CTGCGGTCAAATCTGGCTCCGAGCTGGGTAAACAAGCAAAAGACATTATGGATGCTGGCAAACTGGTCACCGACGAA
CTGGTGATCGCGCTGGTTAAAGAGCGCATTGCTCAGGAAGACTGCCGTAATGGTTTCCTGTTGGACGGCTTCCCGCG
TACCATTCCGCAGGCAGACGCGATGAAAGAAGCGGGCATCAATGTTGATTACGTTCTGGAATTCGACGTACCGGACG
AACTGATCGTTGACCGTATCGTCGGTCGCCGCGTTCATGCGCCGTCTGGTCGTGTTTATCACGTTAAATTCAATCCG
CCGAAAGTAGAAGGCAAAGACGACGTTACCGGTGAAGAACTGACTACCCGTAAAGATGATCAGGAAGAGACCGTACG
TAAACGTCTGGTTGAATACCATCAGATGACAGCACCGCTGATCGGCTACTACTCCAAAGAAGCAGAAGCGGGTA

fumC (469 bp): [Allele no. 11]

CGAGCGCCATTCGTCAGGCGGCGGATGAAGTACTGGCAGGACAGCATGACGACGAATTCCCGCTGGCTATCTGGCAG
ACCGGCTCCGGCACGCAAAGTAACATGAACATGAACGAAGTGCTGGCTAACCGGGCCAGTGAATTACTCGGCGGTGT
GCGCGGGATGGAACGTAAAGTTCACCCTAACGACGACGTGAACAAAAGCCAAAGTTCCAACGATGTCTTTCCGACGG
CGATGCACGTTGCGGCGCTGCTGGCGCTGCGCAAGCAACTCATTCCTCAGCTTAAAACCCTGACACAGACACTGAAT
GAGAAATCCCGTGCTTTTGCCGATATCGTCAAAATTGGTCGTACTCACTTGCAGGATGCCACGCCGTTAACGCTGGG
GCAGGAGATTTCCGGCTGGGTAGCGATGCTCGAGCATAATCTCAAACATATCGAATACAGCCTGCCTCACGTAGCGG
AACTGGC

gyrB (460 bp): [Allele no. 4]

GGTCTGCACGGCGTTGGTGTTTCGGTAGTAAACGCCCTGTCGCAAAAACTGGAGCTGGTTATCCAGCGCGAGGGTAA
AATTCACCGTCAGATCTACGAACACGGTGTACCGCAGGCCCCGCTGGCGGTTACCGGCGAGACTGAAAAAACCGGCA
CCATGGTGCGTTTCTGGCCCAGCCTCGAAACCTTCACCAATGTGACCGAGTTCGAATATGAAATTCTGGCGAAACGT
CTGCGTGAGTTGTCGTTCCTCAACTCCGGCGTTTCCATTCGTCTGCGCGACAAGCGCGACGGCAAAGAAGACCACTT
CCACTATGAAGGCGGCATCAAGGCGTTCGTTGAATATCTGAACAAGAACAAAACGCCGATCCACCCGAATATCTTCT
ACTTCTCCACTGAAAAAGACGGTATTGGCGTCGAAGTGGCGTTGCAGTGGAACGATGGCTTCCAGGAAAACATCT

icd (518 bp): [Allele no. 8]

CGACGCTGCAGTCGAGAAAGCCTATAAAGGCGAGCGTAAAATCTCCTGGATGGAAATTTACACCGGTGAAAAATCCA
CACAGGTTTATGGTCAGGACGTCTGGCTGCCTGCTGAAACTCTTGATCTGATTCGTGAATATCGCGTTGCCATTAAA
GGTCCGCTGACCACTCCGGTTGGTGGCGGTATTCGCTCTCTGAACGTTGCCCTGCGCCAGGAACTGGATCTCTACAT

CTGCCTGCGTCCGGTACGTTACTATCAGGGCACTCCAAGCCCGGTTAAACACCCTGAACTGACCGATATGGTTATCT
TCCGTGAAAACTCGGAAGACATTTATGCGGGTATCGAATGGAAAGCTGACTCTGCCGACGCCGAGAAAGTGATTAAA
TTCCTGCGTGAAGAGATGGGCGTGAAGAAAATTCGCTTCCCGGAACATTGCGGTATCGGTATTAAGCCGTGTTCTGA
AGAAGGCACCAAACGTCTGGTTCGTGCAGCGATCGAATACGCAATTGCTAACGATC

mdh (452 bp): [Allele no. 8]

GGCGTAGCGCGTAAACCGGGTATGGATCGTTCCGACCTGTTTAACGTTAACGCCGGCATCGTGAAAAACCTGGTACA
GCAAGTTGCGAAAACCTGCCCGAAAGCGTGCATTGGTATTATCACTAACCCGGTTAACACCACAGTTGCAATTGCTG
CTGAAGTGCTGAAAAAAGCCGGTGTTTATGACAAAAACAAACTGTTCGGCGTTACCACGCTGGATATCATTCGTTCC
AACACCTTTGTTGCGGAACTGAAAGGCAAACAGCCAGGCGAAGTTGAAGTGCCGGTTATTGGCGGTCACTCTGGTGT
TACCATTCTGCCGCTGCTGTCACAGGTTCCTGGCGTTAGTTTTACCGAGCAGGAAGTGGCTGATCTGACCAAACGCA
TCCAGAACGCGGGTACTGAAGTGGTTGAAGCGAAGGCCGGTGGCGGGTCTGCAACCCTGTCTATGGG

purA (478 bp): [Allele no. 8]

ATAACGCGCGTGAGAAAGCGCGTGGCGCGAAAGCGATCGGCACCACCGGTCGTGGTATCGGGCCTGCTTATGAAGAT
AAAGTAGCACGTCGCGGTCTGCGTGTTGGCGACCTTTTCGACAAAGAAACCTTCGCTGAAAAACTGAAAGAAGTGAT
GGAATATCACAACTTCCAGTTGGTTAACTACTACAAAGCTGAAGCGGTTGATTACCAGAAAGTTCTGGATGATACGA
TGGCTGTTGCCGACATCCTGACTTCTATGGTGGTTGACGTTTCTGACCTGCTCGACCAGGCGCGTCAGCGTGGCGAT
TTCGTCATGTTTGAAGGTGCGCAGGGTACGCTGCTGGATATCGACCACGGTACTTATCCGTACGTAACTTCTTCCAA
CACCACTGCTGGTGGCGTGGCGACCGGTTCCGGCCTGGGCCCGCGTTATGTTGATTACGTTCTGGGTATCCTCAAAG
CTTACTCCACTCGTGT

recA (510 bp): [Allele no. 2]

CGCACGTAAACTGGGCGTCGATATCGACAACCTGCTGTGCTCCCAGCCGGACACCGGCGAGCAGGCACTGGAAATCT
GTGACGCCCTGGCGCGTTCTGGCGCAGTAGACGTTATCGTCGTTGACTCCGTGGCGGCACTGACGCCGAAAGCGGAA
ATCGAAGGCGAAATCGGCGACTCTCACATGGGCCTTGCGGCACGTATGATGAGCCAGGCGATGCGTAAGCTGGCGGG
TAACCTGAAGCAGTCCAACACGCTGCTGATCTTCATCAACCAGATCCGTATGAAAATTGGTGTGATGTTCGGTAACC
CGGAAACCACTACCGGTGGTAACGCGCTGAAATTCTACGCCTCTGTTCGTCTCGACATCCGTCGTATCGGCGCGGTG
AAAGAGGGCGAAAACGTGGTGGGTAGCGAAACCCGCGTGAAAGTGGTGAAGAACAAAATCGCTGCGCCGTTTAAACA
GGCTGAATTCCAGATCCTCTACGGCGAAGGTATCAACTTCTACGGCGA

The target MLST genes with their original sequences, the consensus regions (yellow) and the respective primer (red) sequences in the case of the *E. coli* strain MG1655 is depicted and marked below.

adk

Escherichia coli str. K-12 substr. MG1655, complete genome

NCBI Reference Sequence: NC_000913.3

GenBank Graphics

>NC_000913.3:497175-497819 *Escherichia coli* str. K-12 substr. MG1655, complete genome

ATGCGTATC**ATTCTGCTTGGCGCTCCGGG**CGCGGGGAAAGGGACTCAGGCTCAGTTCATCATGGAGAAATATGGTATTCCG
CAAATCTCCACTGGCGATATGCTGCGTGCTGCGGTCAAATCTGGCTCCGAGCTGGGTAAACAAGCAAAAGACATTATGGAT
GCTGGCAAACTGGTCACCGACGAACTGGTGATCGCGCTGGTTAAAGAGCGCATTGCTCAGGAAGACTGCCGTAATGGTTTC
CTGTTGGACGGCTTCCCGCGTACCATTCCGCAGGCAGACGCGATGAAAGAAGCGGGCATCAATGTTGATTACGTTCTGGAA
TTCGACGTACCGGACGAACTGATCGTTGACCGTATCGTCGGTCGCCGCGTTCATGCGCCGTCTGGTCGTGTTTATCACGTT
AAATTCAATCCGCCGAAAGTAGAAGGCAAAGACGACGTTACCGGTGAAGAACTGACTACCCGTAAAGATGATCAGGAAGAG
ACCGTACGTAAACGTCTGGTTGAATACCATCAGATGACAGCACCGCTGATCGGCTACTACTCCAAAGAAGCAGAAGCGGGT
AATACC**AAATACGCGAAAGTTGACGG**CACCAAGCCGGTTGCTGAAGTTCGCGCTGATCTGGAAAAAATCCTCGGCTAA

(https://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3?report=fasta&from=497175&to=497819)

fumC

Escherichia coli str. K-12 substr. MG1655, complete genome

NCBI Reference Sequence: NC_000913.3

GenBank Graphics

>NC_000913.3:1685185-1686588 *Escherichia coli* str. K-12 substr. MG1655, complete genome

ATGAATACAGTACGCAGCGAAAAAGATTCGATGGGGGCGATTGATG**TCCCGGCAGATAAGCTGTGG**GGCGCACAAACTCAA
CGCTCGCTGGAGCATTTCCGCATTTCGACGGAGAAAATGCCCACCTCACTGATTCATGCGCTGGCGCTAACCAAGCGTGCA
GCGGCAAAAGTTAATGAAGATTTAGGCTTGTTGTCTGAAGAGAAAGCGAGCGCCATTCGTCAGGCGGCGGATGAAGTACTG
GCAGGACAGCATGACGACGAATTCCCGCTGGCTATCTGGCAGACCGGCTCCGGCACGCAAAGTAACATGAACATGAACGAA
GTGCTGGCTAACCGGGCCAGTGAATTACTCGGCGGTGTGCGCGGGATGGAACGTAAAGTTCACCCTAACGACGACGTGAAC
AAAAGCCAAAGTTCCAACGATGTCTTTCCGACGGCGATGCACGTTGCGGCGCTGCTGGCGCTGCGCAAGCAACTCATTCCT
CAGCTTAAAACCCTGACACAGACACTGAATGAGAAATCCCGTGCTTTTGCCGATATCGTCAAAATTGGTCGTACTCACTTG
CAGGATGCCACGCCGTTAACGCTGGGGCAGGAGATTTCCGGCTGGGTAGCGATGCTCGAGCATAATCTCAAACATATCGAA
TACAGCCTGCCTCACGTAGCGGAACTGGCTCTTGGCGGTACAGCGGTGGGTACTGGACTAAATACCCATCCGGAGTATGCG
CGTCGCGTAGCAGATGAACTGGCAGTCATTACCTGTGCACCGTTTGTTACCGCGCCGAACAAATTT**GAAGCGCTGGCGACC**
TGTGATGCCCTGGTTCAGGCGCACGGCGCGTTGAAAGGGTTGGCTGCGTCACTGATGAAAATCGCCAATGATGTCCGCTGG
CTGGCCTCTGGCCCGCGCTGCGGAATTGGTGAAATCTCAATCCCGGAAAATGAGCCGGGCAGCTCAATCATGCCGGGGAAA
GTGAACCCAACACAGTGTGAGGCATTAACCATGCTCTGCTGTCAGGTGATGGGGAACGACGTGGCGATCAACATGGGGGGC
GCTTCCGGTAACTTTGAACTGAACGTCTTCCGTCCAATGGTGATCCACAATTTCCTGCAATCGGTGCGCTTGCTGGCAGAT
GGCATGGAAAGTTTTAACAAACACTGCGCAGTGGGTATTGAACCGAATCGTGAGCGAATCAATCAATTACTCAATGAATCG
CTGATGCTGGTGACTGCGCTTAACACCCACATTGGTTATGACAAAGCCGCCGAGATCGCCAAAAAAGCGCATAAAGAAGGG
CTGACCTTAAAAGCTGCGGCCCTTGCGCTGGGGTATCTTAGCGAAGCCGAGTTTGACAGCTGGGTACGGCCAGAACAGATG
GTCGGCAGTATGAAAGCCGGGCGTTAA

(https://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3?report=fasta&from=1685185&to=1686588)

gyrB

Escherichia coli str. K-12 substr. MG1655, complete genome

NCBI Reference Sequence: NC_000913.3

GenBank Graphics

>NC_000913.3:3877705-3880119 Escherichia coli str. K-12 substr. MG1655, complete genome

ATGTCGAATTCTTATGACTCCTCCAGTATCAAAGTCCTGAAAGGGCTGGATGCGGTGCGTAAGCGCCCGGGTATGTATA**TC**
GGCGACACGGATGACGGCACCGGTCTGCACCACATGGTATTCGAGGTGGTAGATAACGCTATCGACGAAGCGCTCGCGGGT
CACTGTAAAGAAATTATCGTCACCATTCACGCCGATAACTCTGTCTCTGTACAGGATGACGGGCGCGGCATTCCGACCGGT
ATTCACCCGGAAGAGGGCGTATCGGCGGCGGAAGTGATCATGACCGTTCTGCACGCAGGCGGTAAATTTGACGATAACTCC
TATAAAGTGTCCGGCGGTCTGCACGGCGTTGGTGTTTCGGTAGTAAACGCCCTGTCGCAAAAACTGGAGCTGGTTATCCAG
CGCGAGGGTAAAATTCACCGTCAGATCTACGAACACGGTGTACCGCAGGCCCCGCTGGCGGTTACCGGCGAGACTGAAAAA
ACCGGCACCATGGTGCGTTTCTGGCCCAGCCTCGAAACCTTCACCAATGTGACCGAGTTCGAATATGAAATTCTGGCGAAA
CGTCTGCGTGAGTTGTCGTTCCTCAACTCCGGCGTTTCCATTCGTCTGCGCGACAAGCGCGACGGCAAAGAAGACCACTTC

CACTATGAAGGCGGCATCAAGGCGTTCGTTGAATATCTGAACAAGAACAAAACGCCGATCCACCCGAATATCTTCTACTTC
TCCACTGAAAAAGACGGTATTGGCGTCGAAGTGGCGTTGCAGTGGAACGATGGCTTCCAGGAAAACATCTACTGCTTTACC
AACAACATTCCGCAGCGTGACGGCGGTACTCACCTGGCAGGCTTCCGTGCGGCGATGACCCGTA**CCCTGAACGCCTACATG**
GACAAAGAAGGCTACAGCAAAAAAGCCAAAGTCAGCGCCACCGGTGACGATGCGCGTGAAGGCCTGATTGCGGTCGTTTCC
GTGAAAGTGCCGGACCCGAAATTCTCCTCCCAGACCAAAGACAAACTGGTTTCTTCTGAGGTGAAATCGGCGGTTGAACAG
CAGATGAACGAACTGCTGGCAGAATACCTGCTGGAAAACCCAACCGACGCGAAAATCGTGGTTGGCAAAATTATCGATGCT
GCCCGTGCCCGTGAAGCGGCGCGTCGCGCGCGTGAAATGACCCGCCGTAAAGGTGCGCTCGACTTAGCGGGCCTGCCGGGC
AAACTGGCAGACTGCCAGGAACGCGATCCGGCGCTTTCCGAACTGTACCTGGTGGAAGGGGACTCCGCGGGCGGCTCTGCG
AAGCAGGGGCGTAACCGCAAGAACCAGGCGATTCTGCCGCTGAAGGGTAAAATCCTCAACGTCGAGAAAGCGCGCTTCGAT
AAGATGCTCTCTTCTCAGGAAGTGGCGACGCTTATCACCGCGCTTGGCTGTGGTATCGGTCGTGACGAGTACAACCCGGAC
AAACTGCGTTATCACAGCATCATCATCATGACCGATGCGGACGTCGACGGCTCGCACATTCGTACGCTGCTGTTGACCTTC
TTCTATCGTCAGATGCCGGAAATCGTTGAACGCGGTCACGTCTACATCGCTCAGCCGCCGCTGTACAAAGTGAAGAAAGGC
AAGCAGGAACAGTACATTAAAGACGACGAAGCGATGGATCAGTACCAGATCTCTATCGCGCTGGACGGCGCAACGCTGCAC
ACCAACGCCAGTGCACCGGCATTGGCTGGCGAAGCGTTAGAGAAACTGGTATCTGAGTACAACGCGACGCAGAAAATGATC
AATCGTATGGAGCGTCGTTATCCGAAAGCAATGCTGAAAGAGCTTATCTATCAGCCGACGTTGACGGAAGCTGACCTTTCT
GATGAGCAGACCGTTACCCGCTGGGTGAACGCGCTGGTCAGCGAACTGAACGACAAAGAACAGCACGGCAGCCAGTGGAAG
TTTGATGTTCACACCAATGCTGAGCAAAACCTGTTCGAGCCGATTGTTCGCGTGCGTACCCACGGTGTGGATACTGACTAT
CCGCTGGATCACGAGTTTATCACCGGTGGCGAATATCGTCGTATCTGCACGCTGGGTGAGAAACTGCGTGGCTTGCTGGAA
GAAGATGCGTTTATCGAACGTGGCGAGCGTCGTCAGCCGGTAGCCAGCTTCGAGCAGGCGCTGGACTGGCTGGTGAAAGAG
TCCCGTCGCGGCCTCTCCATCCAGCGTTATAAAGGTCTGGGCGAGATGAACCCGGAACAGCTGTGGGAAACCACTATGGAC
CCGGAAAGTCGTCGTATGCTGCGCGTTACCGTTAAAGATGCGATTGCTGCCGACCAGTTGTTCACCACGCTGATGGGCGAC
GCCGTTGAACCGCGCCGTGCGTTTATTGAAGAGAACGCCCTGAAAGCGGCGAATATCGATATTTAA
(https://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3?report=fasta&from=3877705&to=3880119)

icd

Escherichia coli str. K-12 substr. MG1655, complete genome

NCBI Reference Sequence: NC_000913.3

GenBank Graphics

>NC_000913.3:1195123-1196373 *Escherichia coli* str. K-12 substr. MG1655, complete genome

ATGGAAAGTAAAGTAGTTGTTCCGGCACAAGGCAAGAAGATCACCCTGCAAAACGGCAAACTCAACGTTCCTGAAAATCCG
ATTATCCCTTACATTGAAGGTGATGGAATCGGTGTAGATGTAACCCCAGCCATGCTGAAAGTGGTCGACGCTGCAGTCGAG
AAAGCCTATAAAGGCGAGCGTAAAATCTCCTGGATGGAAATTTACACCGGTGAAAAATCCACACAGGTTTATGGTCAGGAC
GTCTGGCTGCCTGCTGAAACTCTTGATCTGATTCGTGAATATCGCGTTGCCATTAAAGGTCCGCTGACCACTCCGGTTGGT
GGCGGTATTCGCTCTCTGAACGTTGCCCTGCGCCAGGAACTGGATCTCTACATCTGCCTGCGTCCGGTACGTTACTATCAG
GGCACTCCAAGCCCGGTTAAACACCCTGAACTGACCGATATGGTTATCTTCCGTGAAAACTCGGAAGACATTTATGCGGGT
ATCGAATGGAAAGCAGACTCTGCCGACGCCGAGAAAGTGATTAAATTCCTGCGTGAAGAGATGGGGGTGAAGAAAATTCGC
TTCCCGGAACATTGTGGTATCGGTATTAAGCCGTGTTCGGAAGAAGGCACCAAACGTCTGGTTCGTGCAGCGATCGAATAC
GCAATTGCTAACGATCGTGACTCTGTGACTCTGGTGCACAAAGGCAACATCATGAAGTTCACCGAAGGAGCGTTTAAAGAC
TGGGGCTACCAGCTGGCGCGTGAAGAGTTTGGCGGTGAACTGATCGACGGTGGCCCGTGGCTGAAAGTTAAAAACCCGAAC
ACTGGCAAAGAGATCGTCATTAAAGACGTGATTGCTGATGCATTCCTGC**AACAGATCCTGCTGCGTCC**GGCTGAATATGAT

GTTATCGCCTGTATGAACCTGAACGGTGACTACATTTCTGACGCCCTGGCAGCGCAGGTTGGCGGTATCGGTATCGCCCCT
GGTGCAAACATCGGTGACGAATGCGCCCTGTTTGAAGCCACCCACGGTACTGCGCCGAAATATGCCGGTCAGGACAAAGTA
AATCCTGGCTCTATTATTCTCTCCGCTGAGATGATGCTGCGCCACATGGGTTGGACCGAAGCGGCTGACTTAATTGTTAAA
GGTATGGAAGGCGCAATCAACGCGAAAACCGTAACCTATGACTTCGAGCGTCTGATGGATGGCGCTAAACTGCTGAAATGT
TCAGAGTTTGGTGACGCGATCATCGAAAACATGTAA

(https://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3?report=fasta&from=1195123&to=1196373)

mdh

Escherichia coli str. K-12 substr. MG1655, complete genome

NCBI Reference Sequence: NC_000913.3

GenBank Graphics

>NC_000913.3:3383330-3384268 *Escherichia coli* str. K-12 substr. MG1655, complete genome

ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGGTATTGGCCAGGCGCTTGCACTACTGTTAAAAACCCAACTGCCTTCAGGT
TCAGAACTCTCTCTGTATGATATCGCTCCAGTGACTCCCGGTGTGGCTGTCGATCTGAGCCATATCCCTACTGCTGTGAAA
ATCAAAGGTTTTTCTGGTGAAGATGCGACTCCGGCGCTGGAAGGCGCAGATGTCGTTCTTATCTCTGCAGGCGTAGCGCGT
AAACCGGGTATGGATCGTTCCGACCTGTTTAACGTTAACGCCGGCATCGTGAAAAACCTGGTACAGCAAGTTGCGAAAACC
TGCCCGAAAGCGTGCATTGGTATTATCACTAACCCGGTTAACACCACAGTTGCAATTGCTGCTGAAGTGCTGAAAAAAGCC
GGTGTTTATGACAAAAACAAACTGTTCGGCGTTACCACGCTGGATATCATTCGTTCCAACACCTTTGTTGCGGAACTGAAA
GGCAAACAGCCAGGCGAAGTTGAAGTGCCGGTTATTGGCGGTCACTCTGGTGTTACCATTCTGCCGCTGCTGTCACAGGTT
CCTGGCGTTAGTTTTACCGAGCAGGAAGTGGCTGATCTGACCAAACGCATCCAGAACGCGGGTACTGAAGTGGTTGAAGCG
AAGGCCGGTGGCGGGTCTGCAACCCTGTCTATGGGCCAGGCAGCTGCACGTTTTGGTCTGTCTCTGGTTCGTGCACTGCAG
GGCGAACAAGGCGTTGTCGAATGTGCCTACGTTGAAGGCGACGGTCAGTACGCCCGTTTCTTCTCTCAACCGCTGCTGCTG
GGTAAAAAC**GGCGTGGAAGAGCGTAAATC**TATCGGTACCCTGAGCGCATTTGAACAGAACGCGCTGGAAGGTATGCTGGAT
ACGCTG**AAGAAAGATATCGC**CCTGGGCGAAGAGTTCGTTAATAAGTAA

(https://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3?report=fasta&from=3383330&to=3384268

purA

***Escherichia coli* str. K-12 substr. MG1655, complete genome**

NCBI Reference Sequence: NC_000913.3

GenBank Graphics

>NC_000913.3:4404687-4405985 *Escherichia coli* str. K-12 substr. MG1655, complete genome

ATGGGTAACAACGTCGTCGTACTGGGCACCCAATGGGGTGACGAAGGTAAAGGTAAGATCGTCGATCTTCTGACTGAACGG
GCTAAATATGTTGTACGCTACCAGGGCGGTCACAACGCAGGCCATACTCTCGTAATCAACGGTGAAAAAACCGTTCTCCAT
CTTATTCCATCAGGTATTCTCCGCGAGAATGTAACCAGCATCATCGGTAACGGTGTTGTGCTGTCTCCGGC**CGCGCTGATG**
AAAGAGATGAAAGAACTGGAAGACCGTGGCATCCCCGTTCGTGAGCGTCTGCTGCTGTCTGAAGCATGTCCGCTGATCCTT
GATTATCACGTTGCGCTGGATAACGCGCGTGAGAAAGCGCGTGGCGCGAAAGCGATCGGCACCACCGGTCGTGGTATCGGG
CCTGCTTATGAAGATAAAGTAGCACGTCGCGGTCTGCGTGTTGGCGACCTTTTCGACAAAGAAACCTTCGCTGAAAAACTG
AAAGAAGTGATGGAATATCACAACTTCCAGTTGGTTAACTACTACAAAGCTGAAGCGGTTGATTACCAGAAAGTTCTGGAT
GATACGATGGCTGTTGCCGACATCCTGACTTCTATGGTGGTTGACGTTTCTGACCTGCTCGACCAGGCGCGTCAGCGTGGC

GATTTCGTCATGTTTGAAGGTGCGCAGGGTACGCTGCTGGATATCGACCACGGTACTTATCCGTACGTAACTTCTTCCAAC
ACCACTGCTGGTGGCGTGGCGACCGGTTCCGGCCTGGGCCCGCGTTATGTTGATTACGTTCTGGGTATCCTCAAAGCTTAC
TCCACTCGTGTAGGTGCAGGTCCGTTCCCGACCGAACTGTTTGATGAAACTGGCGAGTTCCTCTGCAAGCAGGGTAACGAA
TTCGGCGCAACTACGGGGCGTCGTCGTCGTACCGGCTGGCTGGACACCGTTGCCGTTCGTCGTGCGGTACAGCTGAACTCC
CTGTCTGGCTTCTGCCTGACTAAACTGGACGTTCTGGATGGCCTGAAAGAGGTTAAAC**TCTGCGTGGCTTACCGTATG**CCG
GATGGTCGCGAAGTGACTACCACTCCGCTGGCAGCTGACGACTGGAAAGGTGTAGAGCCGATTTACGAAACCATGCCGGGC
TGGTCTGAATCCACCTTCGGCGTGAAAGATCGTAGCGGCCTGCCGCAGGCGGCGCTGAACTATATCAAGCGTATTGAAGAG
CTGACTGGTGTGCCGATCGATATCATCTCTACCGGTCCGGATCGTACTGAAACCATGATTCTGCGCGACCCGTTCGACGCG
TAA

(https://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3?report=fasta&from=4404687&to=4405985)

recA

***Escherichia coli* str. K-12 substr. MG1655, complete genome**

NCBI Reference Sequence: NC_000913.3

GenBank Graphics

>NC_000913.3:2822708-2823769 *Escherichia coli* str. K-12 substr. MG1655, complete genome

ATGGCTATCGACGAAAACAAACAGAAAGCGTTGGCGGCAGCACTGGGCCAGATTGAGAAACAATTTGGTAAAGGCTCCATC
ATGCGCCTGGGTGAAGACCGTTCCATGGATGTGGAAACCATCTCTACCGGTTCGCTTTCACTGGATATCGCGCTTGGGGCA
GGTGGTCTGCCGATGGGCCGTATCGTCGAAATCTACGGACCGGAATCTTCCGGTAAAACCACGCTGACGCTGCAGGTGATC
GCCGCAGCGC**AGCGTGAAGGTAAAACCTGTG**CGTTTATCGATGCTGAACACGCGCTGGACCCAATCTACGCACGTAAACTG
GGCGTCGATATCGACAACCTGCTGTGCTCCCAGCCGGACACCGGCGAGCAGGCACTGGAAATCTGTGACGCCCTGGCGCGT
TCTGGCGCAGTAGACGTTATCGTCGTTGACTCCGTGGCGGCACTGACGCCGAAAGCGGAAATCGAAGGCGAAATCGGCGAC
TCTCACATGGGCCTTGCGGCACGTATGATGAGCCAGGCGATGCGTAAGCTGGCGGGTAACCTGAAGCAGTCCAACACGCTG
CTGATCTTCATCAACCAGATCCGTATGAAAATTGGTGTGATGTTCGGTAACCCGGAAACCACTACCGGTGGTAACGCGCTG
AAATTCTACGCCTCTGTTCGTCTCGACATCCGTCGTATCGGCGCGGTGAAAGAGGGCGAAAACGTGGTGGGTAGCGAAACC
CGCGTGAAAGTGGTGAAGAACAAAATCGCTGCGCCGTTTAAACAGGCTGAATTCCAGATCCTCTACGGCGAAGGTATCAAC
TTCTACGGCGAACTGGTTGACCTGGGCGTAAAAGAGAAGCTGATCGAGAAAGCAGGCGCGTGGTACAGCTACAAAGGTGAG
AAGATC**GGTCAGGGTAAAGCGAATGCG**ACTGCCTGGCTGAAAGATAACCCGGAAACCGCGAAAGAGATCGAGAAGAAAGTA
CGTGAGTTGCTGCTGAGCAACCCGAACTCAACGCCGGATTTCTCTGTAGATGATAGCGAAGGCGTAGCAGAAACTAACGAA
GATTTTTAA

(https://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3?report=fasta&from=2822708&to=2823769)

4.4.10 Determination of evolutionary and/or phylogenetic relationships among different UPECs and their STs

The concatenated MLST sequence data of each of the 40 tested UPECs (Asymptomatic=20; Symptomatic=20) was aligned using **(https://www.ebi.ac.uk/Tools/msa/clustalo/)** and their evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model in Molecular Evolutionary Genetics Analysis (MEGA) **(Liu et al. 2015)** version 7.0. software. Moreover, the phylogenetic relationships between different STs irrespective of their asymptomatic or

symptomatic nature were generated using the goeBURST algorithm **(Wang et al. 2020)** in PHYLOViZ 2.0 software.

4.4.11 Determination of quantitative relationships among different STs by MST

MST was generated from the allelic profiles of the studied asymptomatic and symptomatic UPECs using BioNumerics version 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium) **(Wang et al. 2020)**.

4.4.12 Statistical analysis

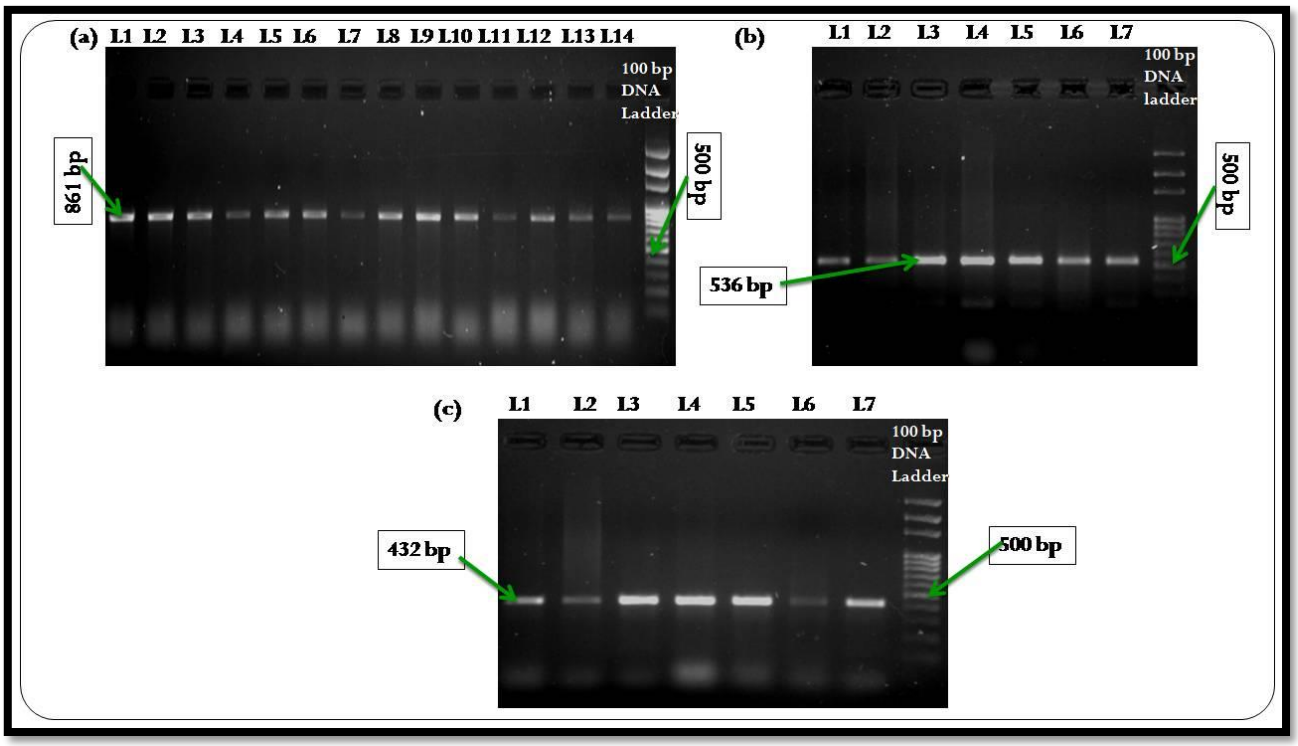
The data obtained were statistically analyzed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The chi-square test using goodness of fit and the Fisher exact test were applied to compare categorical variables. P values ≤ 0.05 were considered to be statistically significant **(Najafi et al. 2018)**. Moreover, the statistical significance of the data analyzed by SPSS version 21.0 was further validated by using the chi-square test in the Prism software package (GraphPad Prism version 9). Additionally, the correlation coefficient was determined using the Prism software package (GraphPad Prism version 9) **(Parra et al. 2017)** and also further validated using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) (**Yadav et al. 2018**) to find the degree of association between different MGEs and also between various β-lactamase genes among 40 UPECs that comprised of 20 asymptomatic and symptomatic isolates respectively. Low (>0.3 to 0.5), moderate (>0.5 to 0.7), and high (> 0.7 to 1) positive or negative correlations between different MGEs and β-lactamases among the aforementioned group of isolates were also ascertained as indicated by **Yadav et al** (**Yadav et al. 2018**). Nevertheless, according to SPSS version 21.0, correlation coefficient values < 0.2 were found to be statistically non-significant. Therefore, correlation coefficient values ≤ 0.2 were not considered when ascertaining the highest and lowest correlations. The values between 0.2 and 0.3 were only considered for analysis if they were found to be significant at ≤ 0.05 level. Furthermore, the correlation graphs were constructed from the correlation matrices using the GraphPad Prism version 9 (GraphPad Software, La Jolla California USA) **(Ghosh et al. 2021)**.

4.5 Results

4.5.1 Distribution of β-lactamase genes

The forty of (100 %) of the 40 UPECs (Asymptomatic=100%; Symptomatic=100%) considered for this study harboured at least 1 of the 3 β-lactamase genes in both their plasmid and chromosomal DNA. The representative pictures of the 3 β-lactamase genes had been depicted in the **Fig. 4.1**. All ESBL producers (Chapter 2) irrespective of their asymptomatic or symptomatic nature carried atleast 2 of the 3 tested β-lactamase genes in their plasmid DNA. Nonetheless, BLIR isolates from both groups

harboured either *blaTEM* or *blaOXA* genes or both together in their plasmid and genomic DNA. The statistically significant predominance of *blaOXA*, followed by *blaTEM* and *blaCTX-M* genes was perceived in isolates that displayed distribution of the β-lactamase genes in both their plasmid and genomic DNA together in case of asymptomatic and symptomatic isolates respectively **(Table 4.4)**. However, a statistically significant prevalence of *blaTEM* followed by *blaOXA* and *blaCTX-M* genes was observed in the plasmid DNA of UPECs of both the asymptomatic and symptomatic groups. Nevertheless, *blaOXA* gene showed the highest statistical significance in the genomic DNA of both the aforementioned groups **(Table 4.4).**



(This study)

Fig. 4.1: The representative gel pictures of the β-lactamase genes investigation study [a] *blaTEM* (861bp) [b] *blaCTX-M* (536bp) [c] *blaOXA* (432bp) of ABU and symptomatic UPECs.

Moreover, two heat maps each for plasmid and genomic DNA respectively were constructed based on the individual distribution pattern of 3 β-lactamase genes among asymptomatic (**Fig. 4.2a; Fig. 4.3a)** and symptomatic **(Fig. 4.2b; Fig. 4.3b)** isolates, to understand their role in the dissemination of resistance determinants. Two major clusters could be distinguished, on the basis of the significant distribution pattern of 3 β-lactamase genes (*TEM*, *CTXM* and *OXA*) in the plasmid DNA of asymptomatic **(Fig. 4.2a)** and symptomatic **(Fig. 4.2b)** isolates respectively. Cluster 1 and 2 comprised of 16 and 4 isolates respectively in each of the aforementioned groups. All 16 isolates that formed cluster1 carried *blaOXA* gene in their plasmid DNA unlike the complete absence of the aforementioned β-lactamase gene among the 4 isolates that were part of the cluster 2 in both the

Table 4.4: Prevalence of β-lactamase genes and mobile genetic elements among Uropathogenic *E. coli* isolates from asymptomatic and symptomatic groups.

	Groups				
	Asymptomatic(n=20)	**P value (Asymptomatic)**	**Symptomatic(n=20)**	**P value (Symptomatic)**	**Total(n=40)**
β-lactamase genes in both plasmid and genomic DNA					
blaTEM	**15(75)**	**0.0005**	**14(70)**	**0.001**	**29(72.5)**
blaCTX-M	**07(35)**	**0.04**	**10(50)**	**0.01**	**17(42.5)**
blaOXA	**16(80)**	**0.0003**	**16(80)**	**0.0003**	**32(80)**
β-lactamase genes only in plasmid DNA					
blaTEM	**20(100)**	**<0.0001**	**17(85)**	**0.0001**	**37(92.5)**
blaCTX-M	**8(40)**	**0.03**	**12(60)**	**0.0034**	**20(50)**
blaOXA	**16(80)**	**0.0003**	**16(80)**	**0.0003**	**32(80)**
β-lactamase genes only in genomic DNA					
blaTEM	**15(75)**	**0.0005**	**16(80)**	**0.0003**	**31(77.5)**
blaCTX-M	**15(75)**	**0.0005**	**14(70)**	**0.001**	**29(72.5)**
blaOXA	**20(100)**	**<0.0001**	**20(100)**	**<0.0001**	**40(100)**
Mobile Genetic Elements					
intI1	**14(70)**	**0.001**	**16(80)**	**0.0003**	**30(75)**
intI2	**12(60)**	**0.0034**	**04(20)**	**ns**	**16(40)**
ISEcp1	**17(85)**	**0.0001**	**18(90)**	**<0.0001**	**35(87.5)**
IS5	**11(55)**	**0.006**	**12(60)**	**0.0034**	**23(57.5)**
IS26	**19(95)**	**<0.0001**	**18(90)**	**<0.0001**	**37(92.5)**

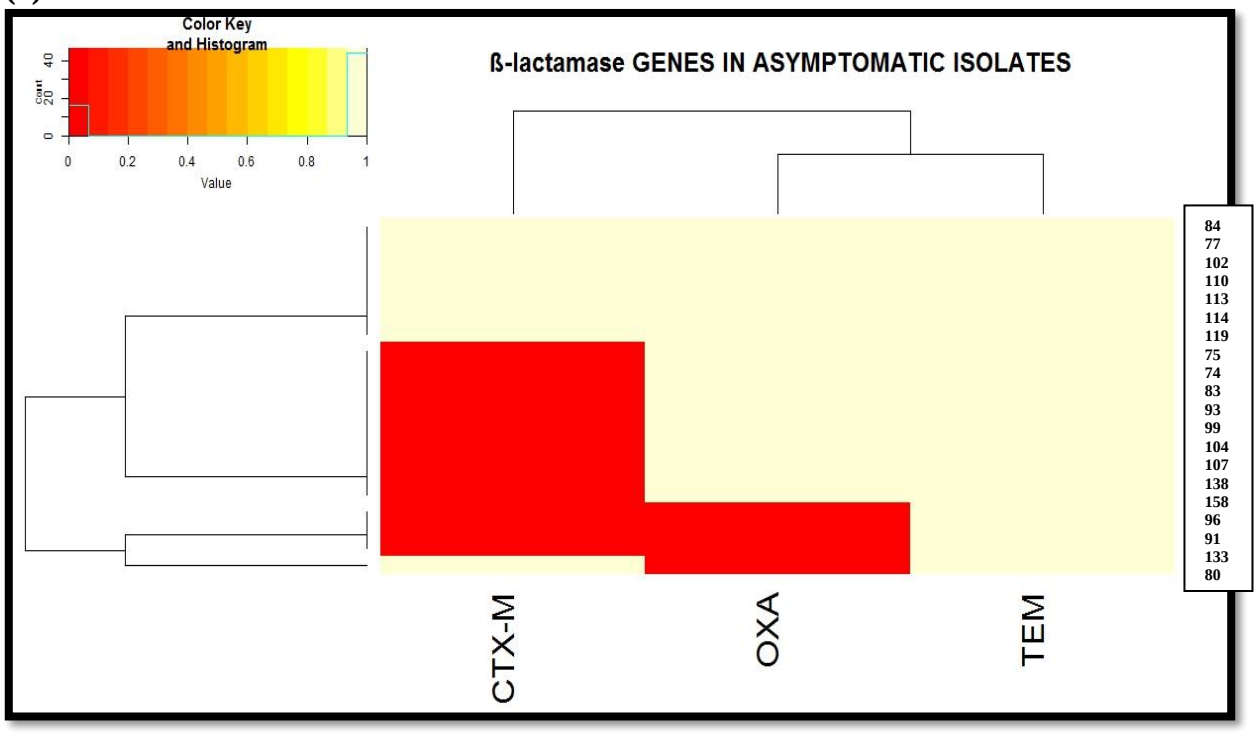
[#] Percentage in parentheses; ns=not significant.

groups. In the case of asymptomatic isolates, cluster 1 that contained 16 isolates could be divided into two groups that comprised 7 and 9 isolates respectively. All three tested β-lactamase genes were universally present in all the 7 isolates that formed Group 1. All 9 isolates that were part of Group 2 carried *blaTEM* and *blaOXA* genes. Three isolates that were part of cluster 2 carried only *blaTEM* and the remaining isolate carried both *blaTEM* and *blaCTX-M* genes **(Fig. 4.2a).** However, in the case of symptomatic isolates cluster 1 could be sub-divided into two sub-clusters that comprised of 11 and 5 isolates respectively. All 11isolates that formed sub-cluster 1 contained all three tested β-lactamase genes except two in which *blaTEM* gene was absent. Four out of 5 isolates that formed sub-cluster 2 carried *blaTEM* and *blaOXA* genes. Nevertheless, 3 isolates that were part of cluster 2 carried only *blaTEM* β-lactamase and the remaining isolate carried both *blaTEM* and *blaCTXM* genes **(Fig. 4.2b)**

Two major clusters could be distinguished, on the basis of the significant distribution pattern of 3 β-lactamase genes (*TEM, CTXM* and *OXA*) in the genomic DNA of asymptomatic **(Fig. 4.3a)** and symptomatic **(Fig. 4.3b)** isolates respectively. In the case of asymptomatic isolates, Cluster 1 and 2 comprised of 15 and 5 isolates respectively. All 15 isolates that constituted cluster 1 harboured CTX-M β-lactamase contrary to its complete absence among the isolates that formed cluster 2. Cluster 1 that encompassed 15 isolates were divided into two groups that contained 11 and 4 isolates respectively on the basis of the universal presence of all three β-lactamases and presence of CTX-M and OXA β-lactamases respectively. Nevertheless, all isolates that were part of cluster 2 carried both TEM and OXA β-lactamases except 1 that harboured only OXA β-lactamase. However, in the case of symptomatic isolates cluster 1 and 2 consisted of 16 and 4 isolates respectively. All 16 isolates that composed cluster 1 carried TEM β-lactamase opposite to its total absence among the isolates that formed cluster 2. Cluster 1 that comprised of 16 isolates formed two groups that contained 12 and 4 isolates respectively depending on the universal presence of all three β-lactamases and the presence of TEM and OXA β-lactamases respectively. Moreover, 4 isolates that constituted cluster 2 were also found to be divided into two groups on the basis of the presence of CTX-M; OXA and only OXA β-lactamases respectively.

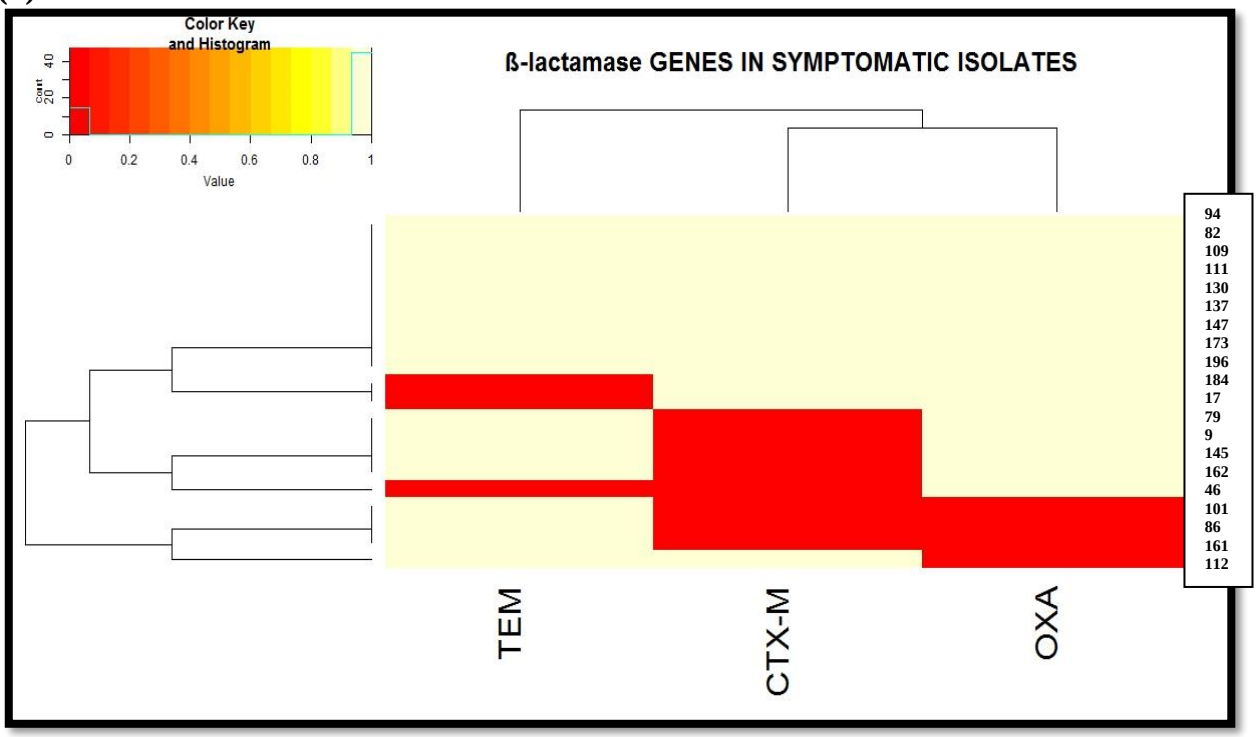
Moreover, a low to a high level of positive correlation was observed in the incidence of the 3 tested β-lactamase genes in the plasmid DNA of the asymptomatic **(Fig. 4.4a)** UPECs. A high and a low level of correlation was observed between *blaTEM*; *blaOXA* and *blaCTX-M*; *blaOXA* genes respectively. However, *blaTEM* and *blaCTX-M* genes were found to be moderately correlated **(Fig. 4.4a)**. Nevertheless, in the case of plasmid DNA of symptomatic isolates, only a moderate level of correlation was perceived between *blaTEM*; *blaCTX-M*, *blaTEM*; *blaOXA* and *blaCTX-M*; *blaOXA* respectively **(Fig. 4.4b)**.

(a)



(This study)

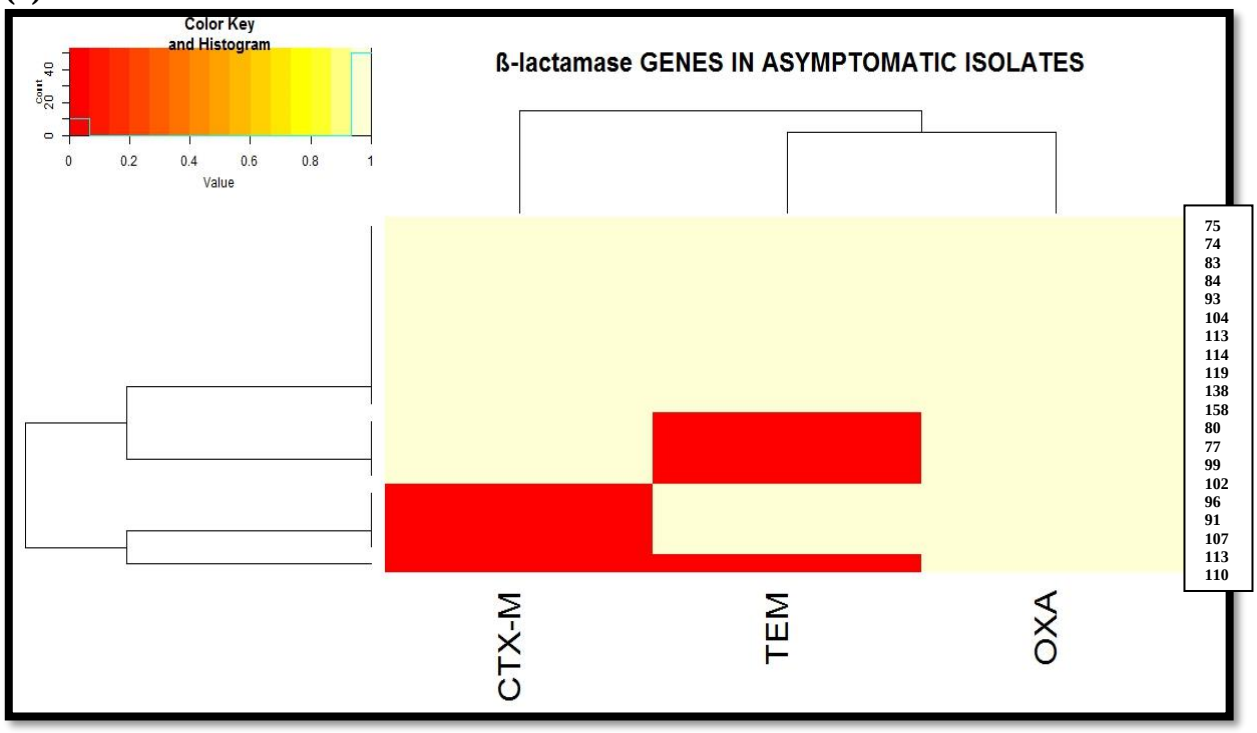
(b)



(This study)

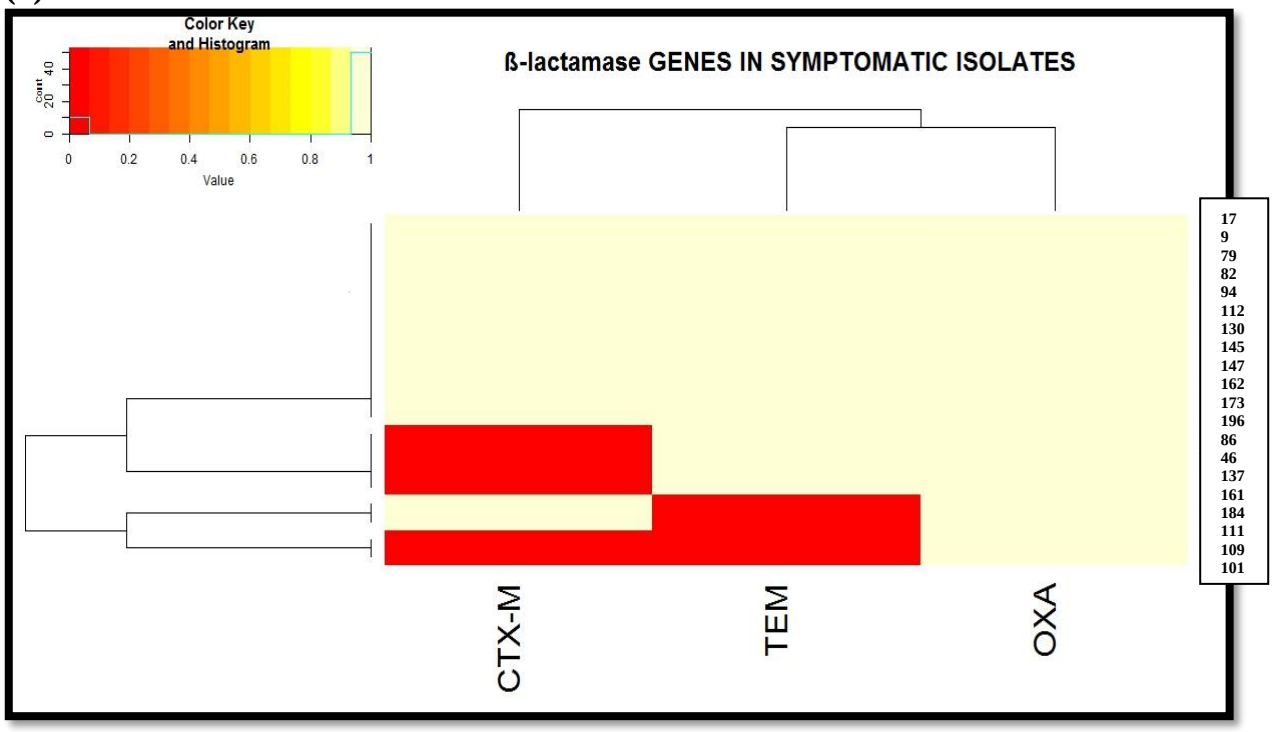
Fig. 4.2: Cluster analysis performed on Heat maps generated using R software package (version 3.2.5)., based on the presence and absence three β-lactamase genes in the plasmid DNA of each of the individual isolate from (a) Asymptomatic UPEC group and (b) Symptomatic UPEC group. Numbers in the text box provided on the righthand side represented sample ID of the UPECs considered in each group. Colour key represented the variation in colours from red to white illustrating the complete absence of a particulargene to its complete presence respectively.

(a)



(This study)

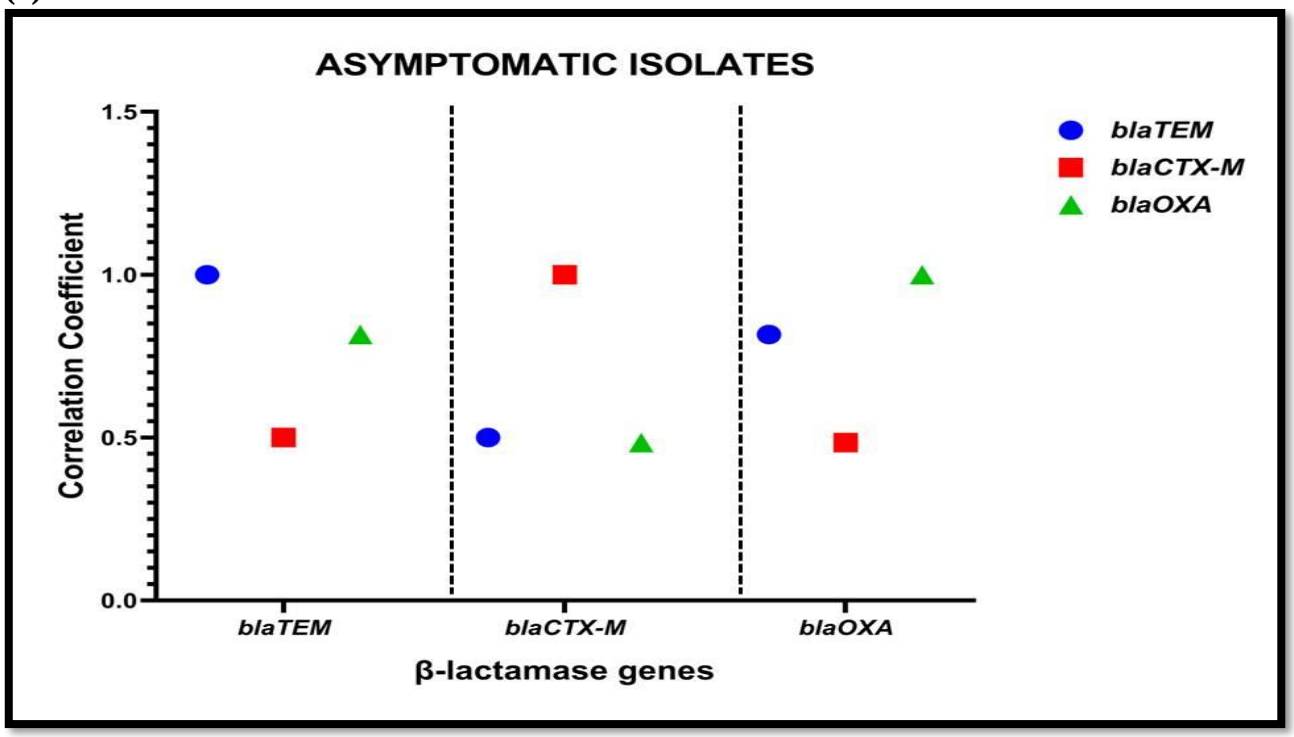
(b)



(This study)

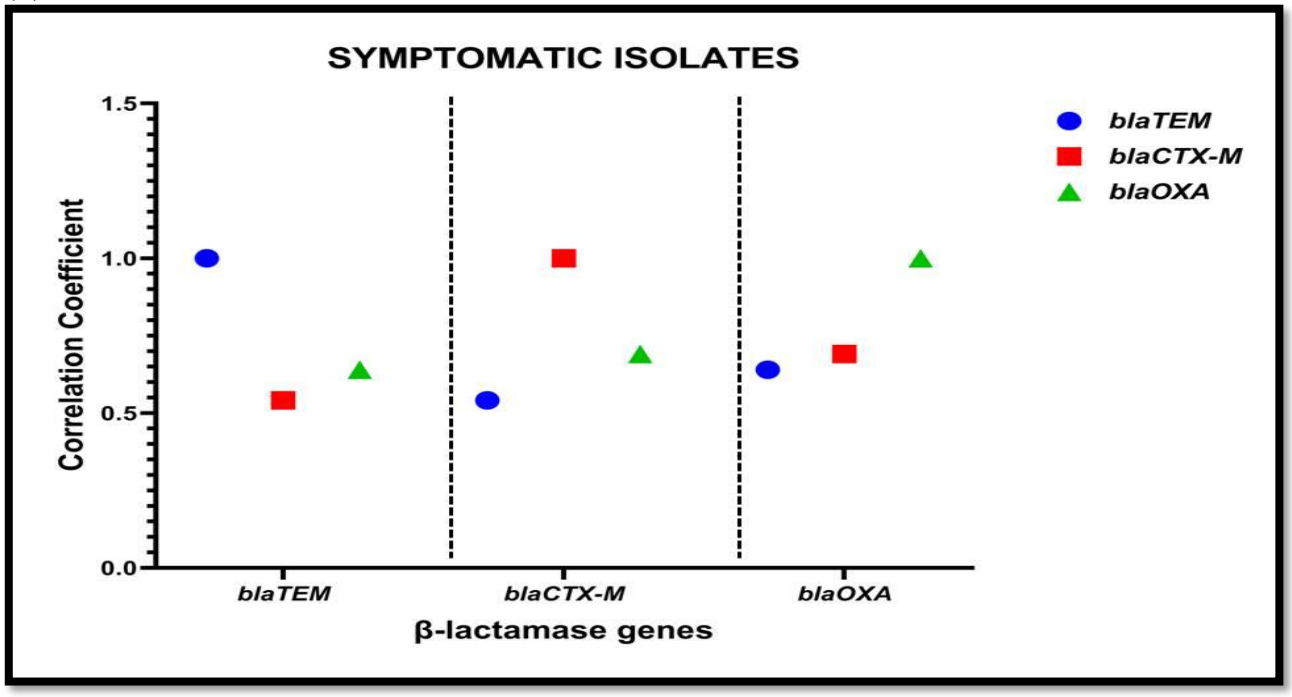
Fig. 4.3: Cluster analysis performed on Heat maps generated using R software package (version 3.2.5)., based on the presence and absence three β-lactamase genes in the genomic DNA of each of the individual isolate from (a) Asymptomatic UPEC group and (b) Symptomatic UPEC group. Numbers in the text box provided on the righthand side represented sample ID of the UPECs considered in each group. Colour key represented the variation in colours from red to white illustrating the complete absence of a particular gene to its complete presence respectively.

(a)



(This study)

(b)

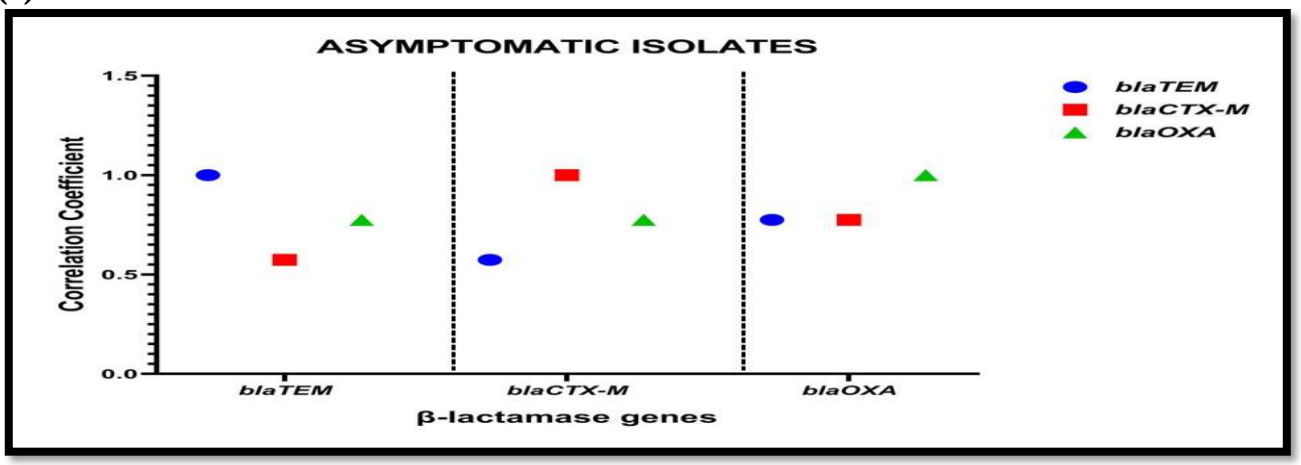


(This study)

Fig. 4.4: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation among three different β-lactamase genes in the plasmid DNA of each of individual (a) asymptomatic and (b) symptomatic UPECs. Different β-lactamases were represented by interleaved symbols with varied colours. Dotted lines were introduced to differentiate correlations of each of the β-lactamase gene with two others.

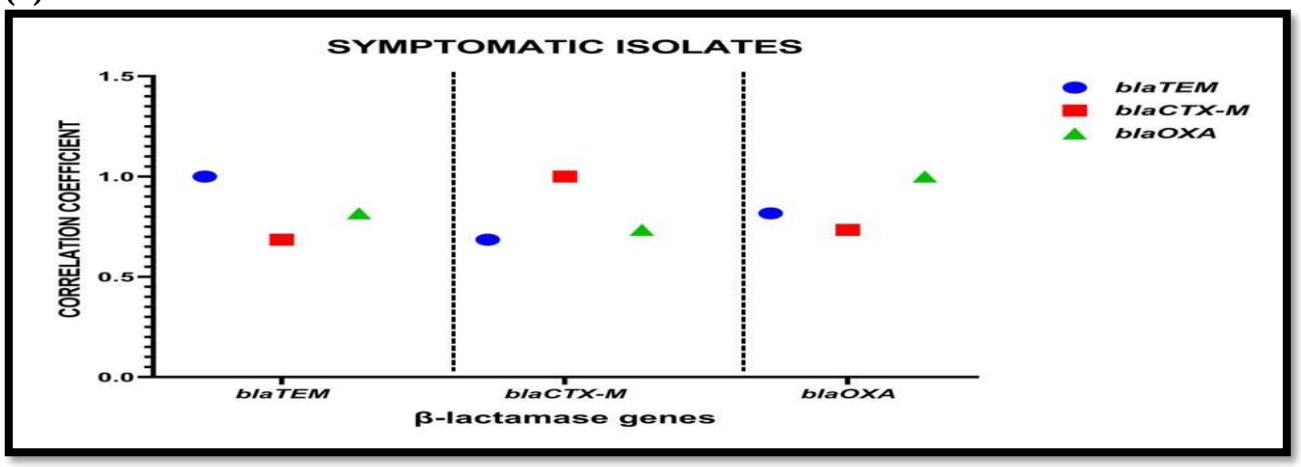
Likewise, a moderate to a high level of positive correlation was perceived in the incidence of the 3 tested β-lactamase genes in the genomic DNA of both the asymptomatic **(Fig. 4.5a)** and symptomatic **(Fig. 4.5b)** group of isolates. Among both asymptomatic **(Fig. 4.5a)** and symptomatic **(Fig. 4.5b)** UPECs, *blaOXA* gene was found to be highly correlated to *blaTEM* and *blaCTX-M* genes. However, a moderate correlation was observed in the incidence of *blaTEM* and *blaCTX-M* genes **(Fig. 4.5a-b)**.

(a)



(This study)

(b)



(This study)

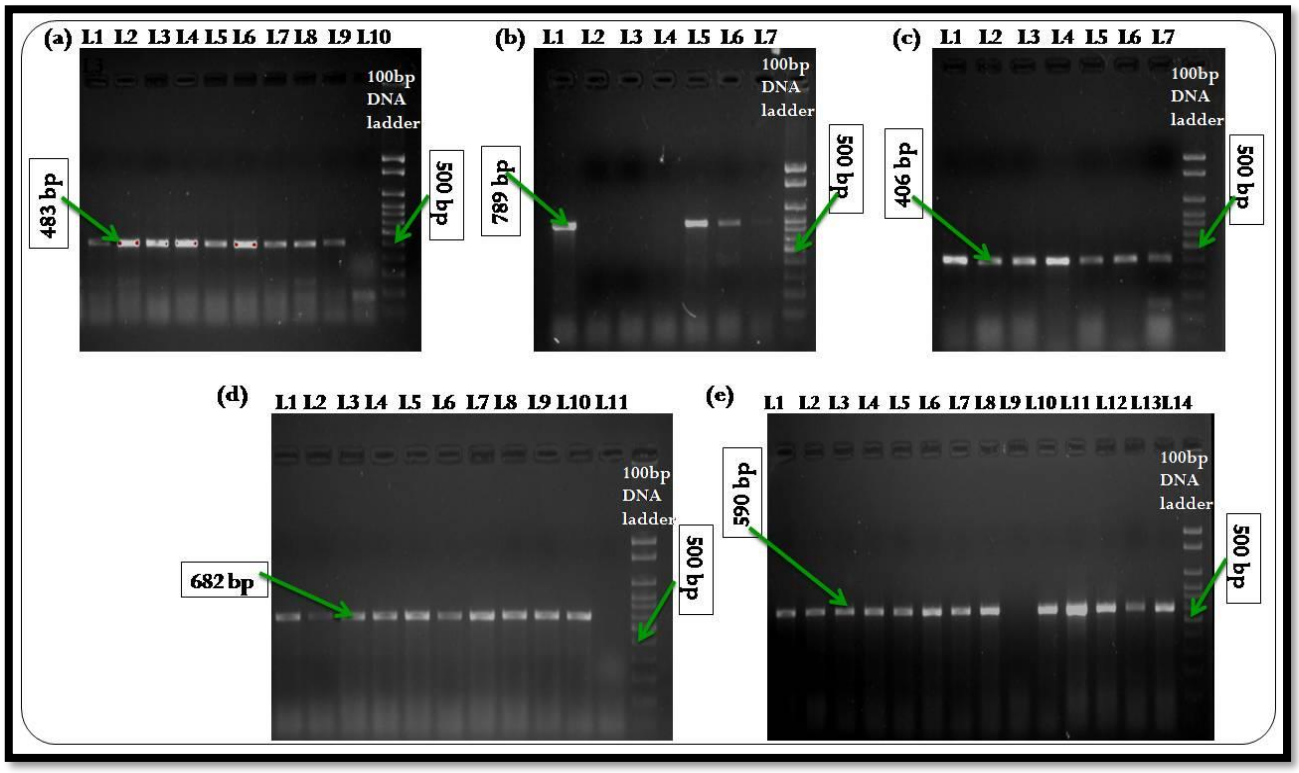
Fig. 4.5: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of three different β-lactamase genes in the genomic DNA of each of individual (a) asymptomatic and (b) symptomatic UPECs. Different β-lactamases were represented by interleaved symbols with varied colours. Dotted lines were used to differentiate correlation of each of the β-lactamase gene with two others.

4.5.2 Distribution of MGEs

On the whole 40 (100 %) of the 40 UPECs (Asymptomatic=100%; Symptomatic=100%) selected for this study carried at least 1 of the 5 MGEs. The representative pictures of the 5 MGE genes of had been depicted in the **Fig. 4.6**. The statistically significant predominance of all the MGEs was descried in the case of asymptomatic isolates. However, in the case of symptomatic isolates, the

significant prevalence of all the tested MGEs except the class II integrase gene was perceived. Nevertheless, the most prevalent MGE was *IS26* followed by *ISEcp1* with regard to both groups of isolates **(Table 4.4)**.

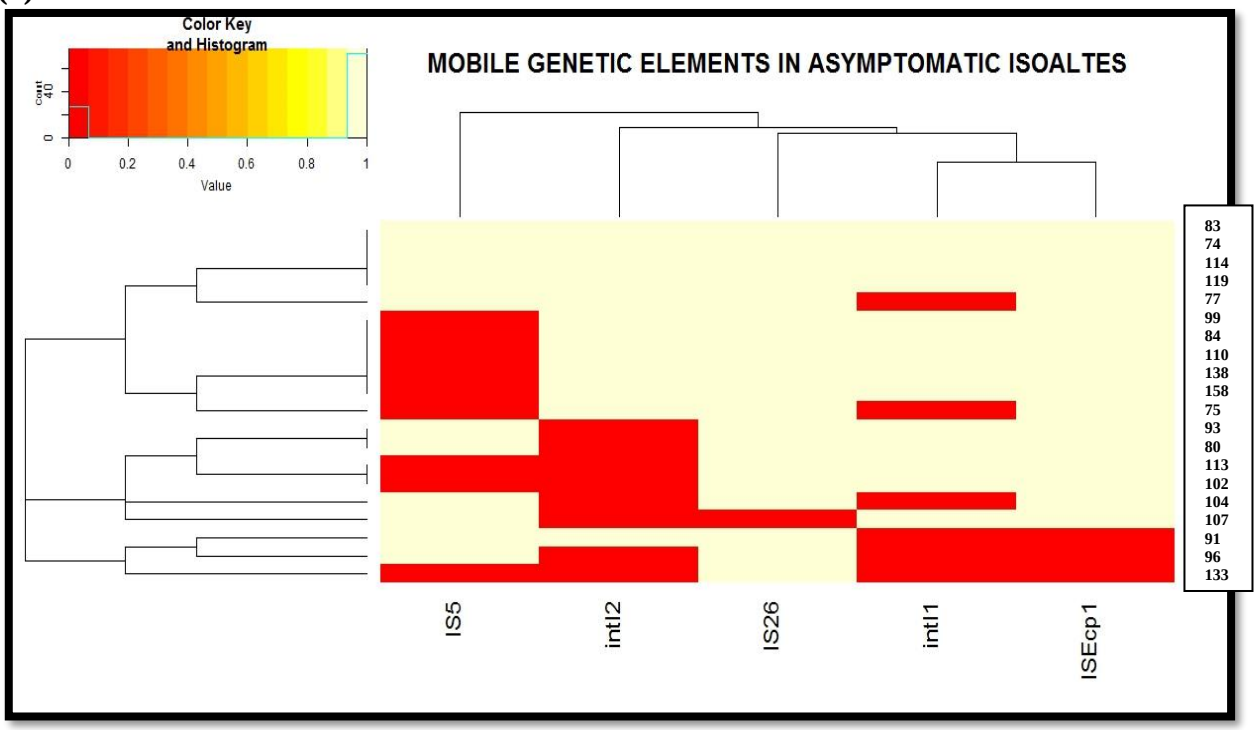
Two heat maps were constructed based on the individual distribution pattern of 5 MGEs in asymptomatic **(Fig. 4.7a)** and symptomatic **(Fig. 4.7b)** isolates, to comprehend their role in genome plasticity. Three major clusters could be distinguished, on the basis of the significant distribution pattern of 5 MGEs (*intI1, intI2, ISEcp1, IS5* and *IS26*) in the case of asymptomatic isolates. Cluster 1, 2, and 3 comprised of 11, 6 and 3 isolates respectively. Three (*intI2, ISEcp*1, *IS26*) of tested MGEs were universally present in all the 11 isolates that formed cluster1. All the 6 isolates that were part of cluster 2 carried *ISEcp1, intI1* and *IS26,* except two isolates in which *intI1* (1 isolate) *IS26* (1 isolate) was absent respectively. *IS26* was universally present in all the 3 isolates that formed cluster 3 **(Fig. 4.7a)**. However, in the case of symptomatic UPECs, only two major clusters could be observed. Cluster 1 and 2 comprised 15 and 5 isolates respectively. Universal presence and absence of *intI1* and *intI2* respectively was observed in all the 15 isolates that constituted cluster 1. All the 5 isolates that formed cluster 2 carried *ISEcp1,* and *IS26* **(Fig. 4.7b)**.



(This study)

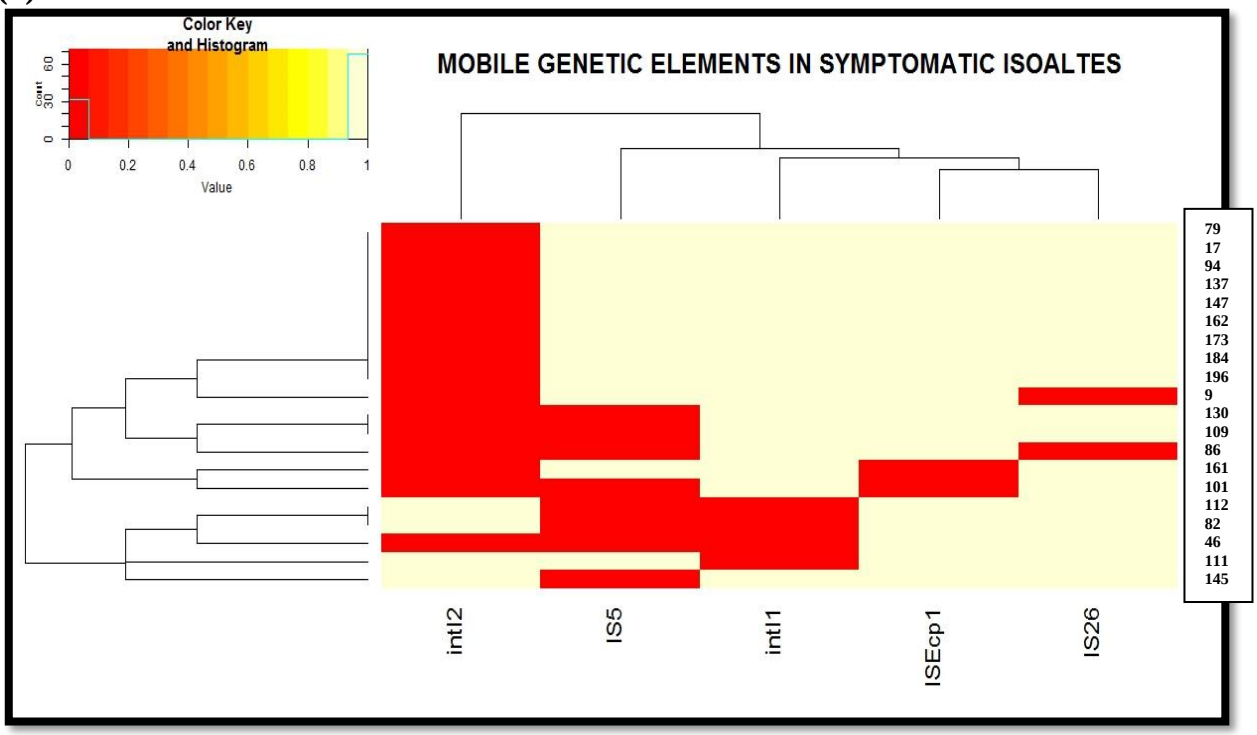
Fig. 4.6: The representative gel pictures of the MGE investigation study [a] *intI1* (483bp) [b] *intI2* (789bp) [c] *ISEcp1* (406bp) [d] *IS5* (682bp) and [e] *IS26* (590bp) of ABU and symptomatic UPECs.

(a)



(This study)

(b)

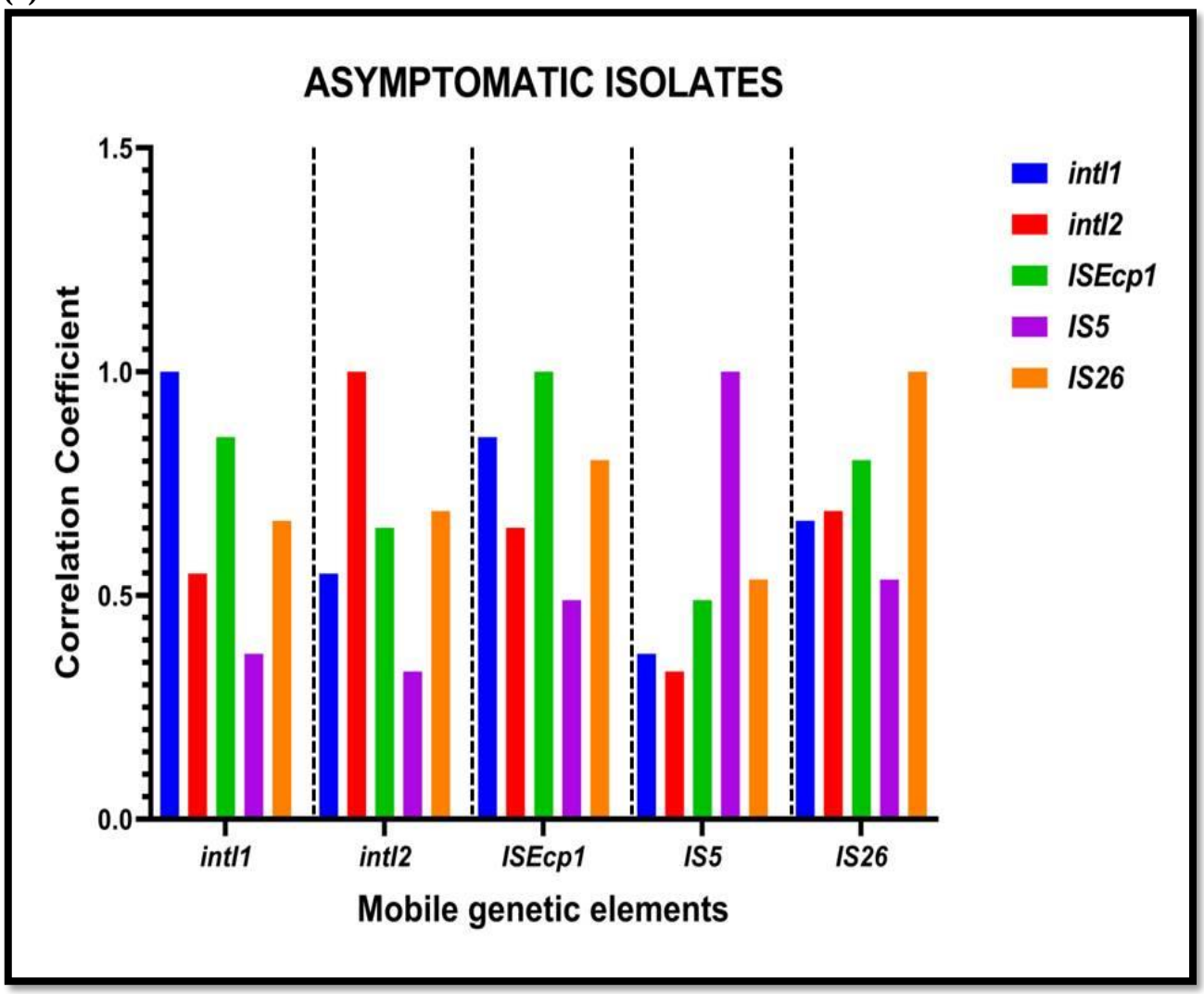


(This study)

Fig. 4.7: Cluster analysis performed on Heat maps generated using R software package (version 3.2.5)., based on the presence and absence five mobile genetic elements (MGEs) in each of the individual isolate from (a) Asymptomatic UPEC group and (b) Symptomatic UPEC group. Numbers in the text box provided on the righthand side represented sample ID of the *E. coli* isolates considered in each group. Colour key represented the variation in colours from red to white illustrating the complete absence of a particular gene to its complete presence respectively.

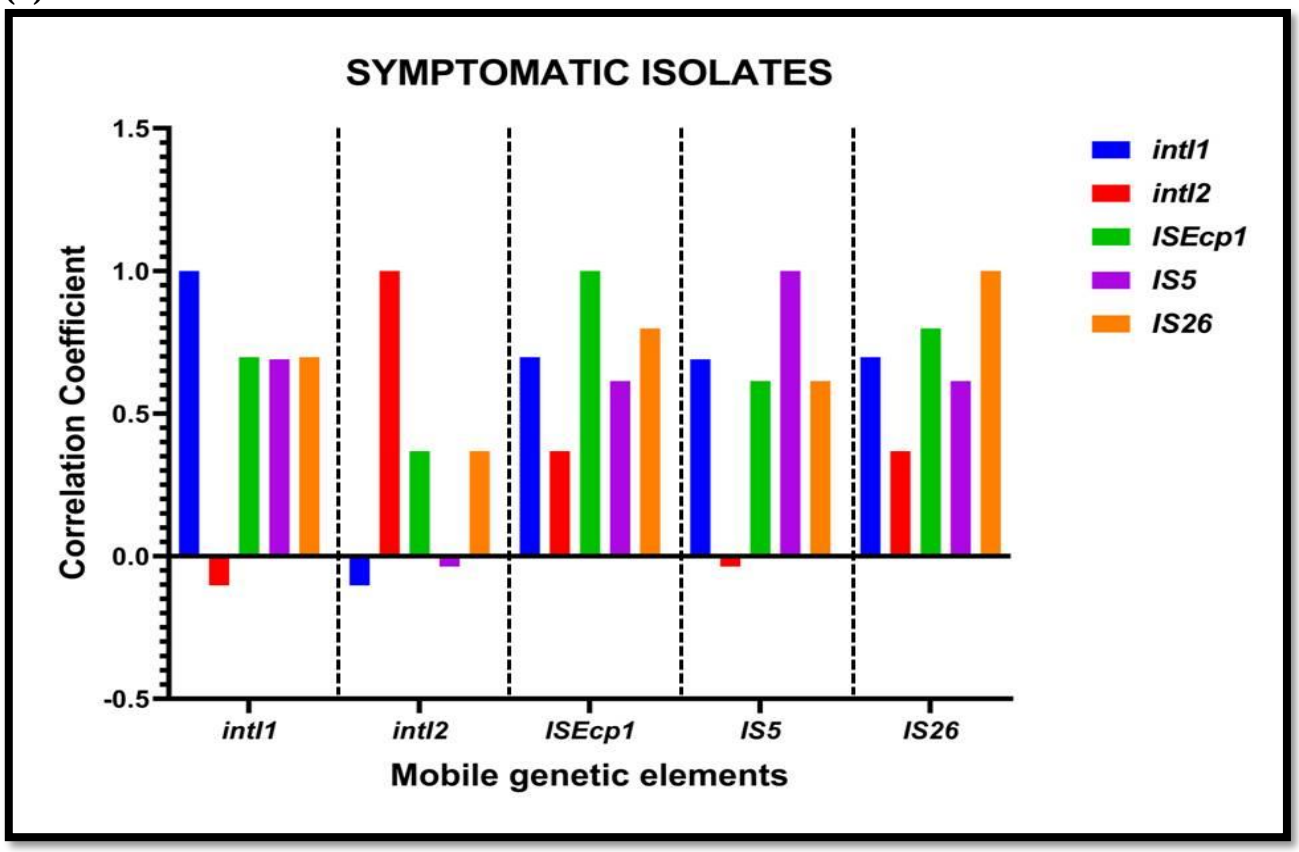
A low to a high level of positive correlation was perceived in the occurrence of the 5 tested MGEs among the asymptomatic **(Fig. 4.8a)** and symptomatic **(Fig. 4.8b)** UPECs respectively. However, a negative correlation in the incidence of 3/5 MGEs (*intI1*, *intI2* and *IS5*) was observed only in the case of the symptomatic UPECs. Among asymptomatic UPECs incidence of *intI1*; *ISEcp1* and *ISEcp1*; *IS26* was found to be highly correlated. Moderate to moderately high correlation was observed in *intI1* (with *intI2* and *IS26*), *intI2* (with *intI1*; *ISEcp1*; *IS26*), *ISEcp1* (with *intI2*), IS5 (with *IS26*), and *IS26* (with *intI1*; *intI2*; *IS5*) respectively. Low correlation was perceived in the incidence *int5* when associated with *intI1*, *intI2 and ISEcp1* respectively **(Fig. 4.8a)**. However, among symptomatic UPECs, the only incidence of ISEcp1and IS26 was found to be highly correlated. Moderate to moderately high correlation was observed in *intI1* (with *ISEcp1*; *IS5* and *IS26*), *ISEcp1* (with *intI1*; *IS5*), *IS5* (with *intI1*; *ISEcp1*; *IS26*) and *IS26* (with *intI1*; *IS5*). Low correlation was perceived among *intI2*; *ISEcp1* and *intI2*; *IS26* respectively. Furthermore among the symptomatic UPECs tested incidence of *intI2* was found to be negatively correlated to *intI1* and *IS5* **(Fig. 4.8b)**.

(a)



(This study)

(b)



(This study)

Fig. 4.8: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation coefficient values of five different mobile genetic elements (MGEs) in each of individual (a) asymptomatic and (b) symptomatic UPECs. Different MGEs were represented by interleaved bars with varied colours. Dotted lines were introduced within the generated graph to differentiate correlation of each of the five MGEs.

4.5.3 Phylotype distribution

The intricate phylotype analysis revealed that 14 out of the 14 (100%) ABU UPECs **(Table 4.5a)** and 15 out of 17 (88.2%) symptomatic UPECs **(Table 4.5b)** from a pool of 40 drug-resistant UPECs (Chapter 3) **(Ghosh and Mukherjee 2019)**, with undesignated (Unknown) phylogroup, showed distinctly different phylotype property(Unknown+E) by the modified quadruplex PCR method of typing using phylogroup E specific primer (*arpA*; 301bp). These isolates were assigned into a class entitled **"novel phylotype property" (NPP).** None of the isolates showed the presence of *trpA*; 219. The representative picture of the PCR based assay **(Fig. 4.9)** and sequencing **(Fig. 4.10)** of the individual genes have been depicted below.

The aforesaid "NPP" (Unknown+E) was found to be significantly predominant in both (Asymptomatic=70%, p value= 0.001; Symptomatic=75%, p- value= 0.0005) groups of isolates.

Table 4.5a: Phylotype property of Asymptomatic Uropathogenic *E. coli* isolates (n=20).

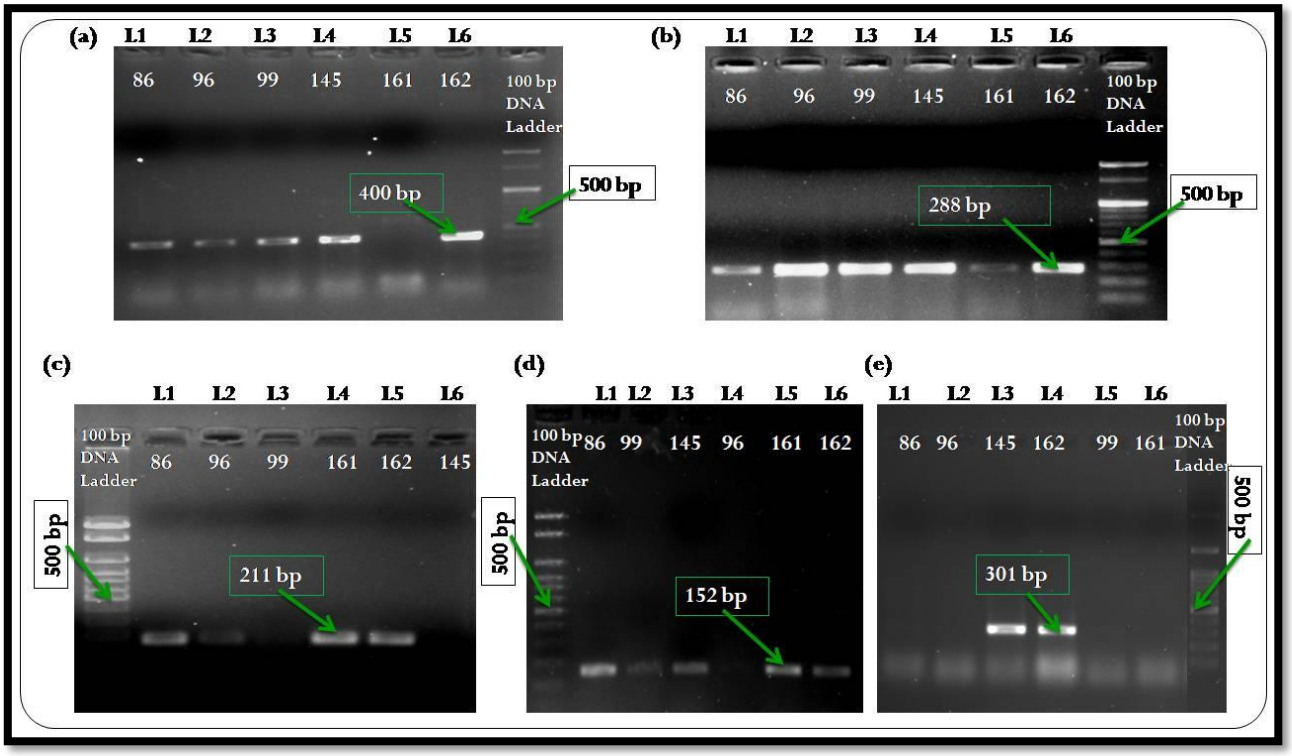
Sl. No.	Sample No.	*arpA* (400bp)	*chuA* (288bp)	*yjaA* (211bp)	TspE4.C2 (152bp)	Phylogenetic Grouping By Quadruplex PCR	Targeting Group E (*arpA*;301bp)	Modified Phylogroup (This Study)
1	74	+	+	+	+	Unknown	+	E variant(NPP)
2	75	+	+	+	+	Unknown	+	E variant(NPP)
3	77	+	-	+	+	Unknown	+	E variant(NPP)
4	80	+	+	+	+	Unknown	+	E variant(NPP)
5	83	+	+	-	+	D or E	+	E
6	84	+	+	+	+	Unknown	+	E variant(NPP)
7	91	+	+	+	+	Unknown	+	E variant(NPP)
8	93	+	+	+	+	Unknown	+	E variant(NPP)
9	96	+	+	+	-	E or CladeI	-	CladeI
10	99	+	+	-	+	D or E	-	D
11	102	+	+	+	+	Unknown	+	E variant(NPP)
12	104	+	+	+	+	Unknown	+	E variant(NPP)
13	107	+	+	+	+	Unknown	+	E variant(NPP)
14	110	+	+	+	+	Unknown	+	E variant(NPP)
15	113	+	+	+	+	Unknown	+	E variant(NPP)
16	114	+	+	+	+	Unknown	+	E variant(NPP)
17	119	+	+	+	+	Unknown	+	E variant(NPP)
18	133	+	+	+	-	E or CladeI	-	CladeI
19	138	+	+	-	+	D or E	+	E
20	158	+	+	-	+	D or E	+	E

NPP=Novel phylotype property.

Table 4.5b: Phylotype property of Symptomatic uropathogenic *E. coli* isolates (n=20).

Sl. No.	Sample No.	*arpA* (400bp)	*chuA* (288bp)	*yjaA* (211bp)	TspE4.C2 (152bp)	Phylogenetic Grouping By Quadruplex PCR	Targeting Group E (*arpA*;301bp)	Modified Phylogroup (This Study)
1	9	+	+	+	+	Unknown	+	E variant(NPP)
2	17	+	+	+	+	Unknown	+	E variant(NPP)
3	46	+	+	+	+	Unknown	-	Unknown
4	79	+	+	-	+	D or E	+	E
5	82	+	+	+	+	Unknown	+	E variant(NPP)
6	86	+	+	+	+	Unknown	-	Unknown
7	94	+	+	+	+	Unknown	+	E variant(NPP)
8	101	+	+	+	+	Unknown	+	E variant(NPP)
9	109	+	+	+	+	Unknown	+	E variant(NPP)
10	111	+	+	+	+	Unknown	+	E variant(NPP)
11	112	+	+	+	+	Unknown	+	E variant(NPP)
12	130	+	+	+	+	Unknown	+	E variant(NPP)
13	137	+	+	+	+	Unknown	+	E variant(NPP)
14	145	+	+	-	+	D or E	+	E
15	147	+	+	+	+	Unknown	+	E variant(NPP)
16	161	-	+	+	+	B2	-	B2
17	162	+	+	+	+	Unknown	+	E variant(NPP)
18	173	+	+	+	+	Unknown	+	E variant(NPP)
19	184	+	+	+	+	Unknown	+	E variant(NPP)
20	196	+	+	+	+	Unknown	+	E variant(NPP)

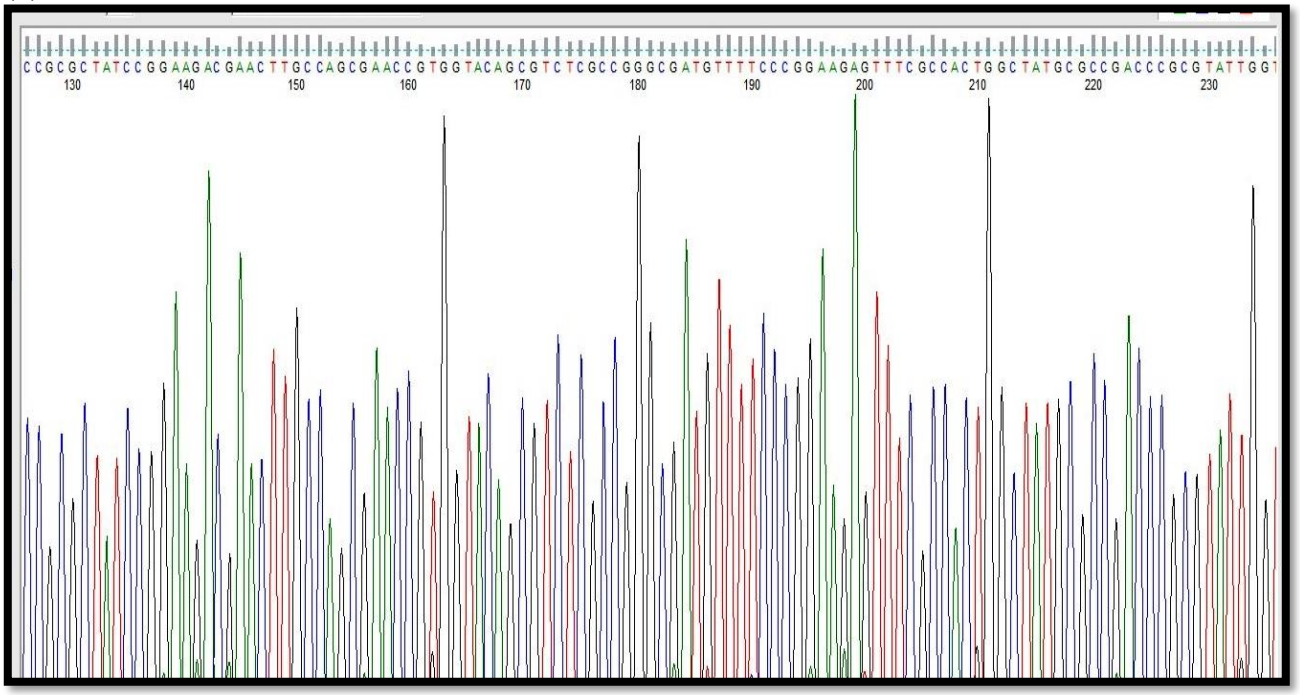
NPP=Novel phylotype property



(This study)

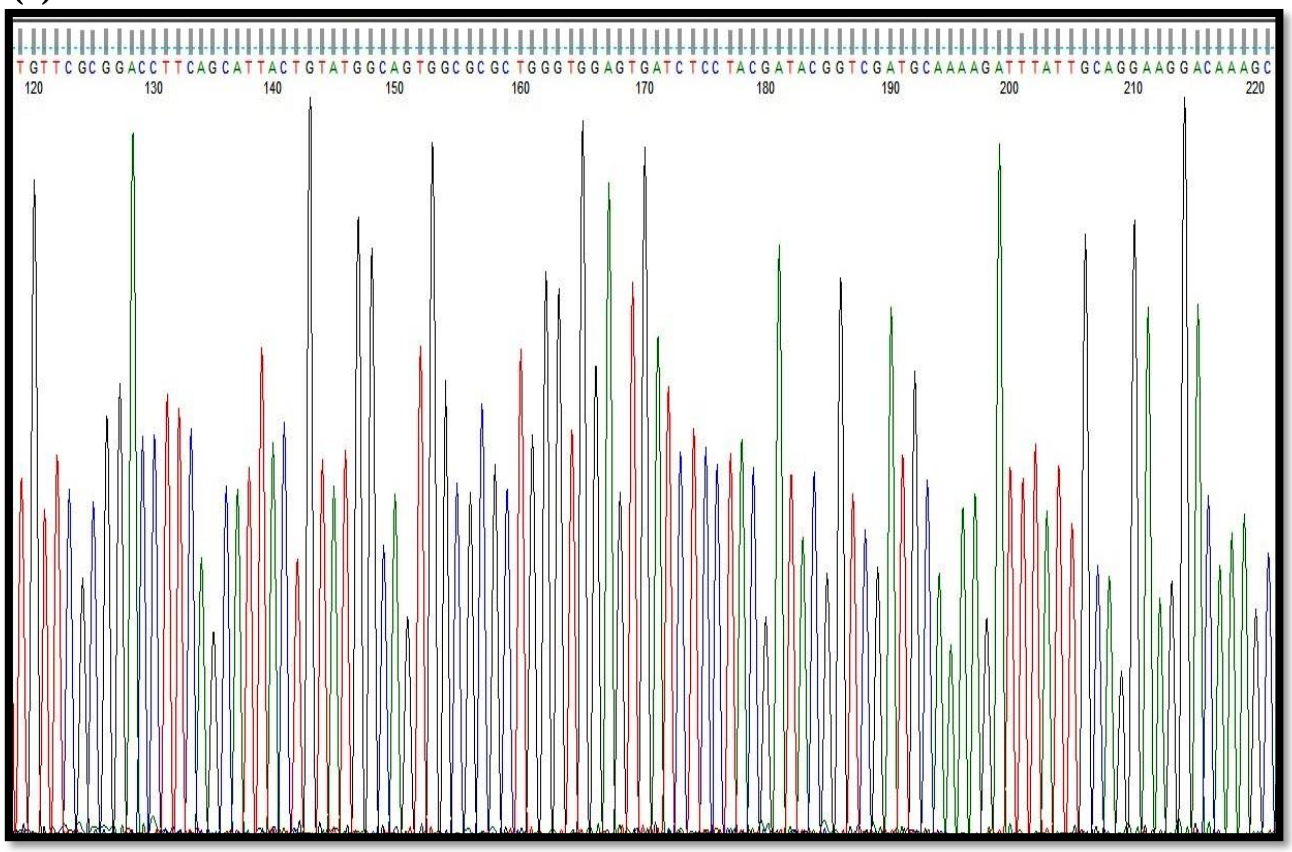
Fig. 4.9: The representative gel pictures of the intricate phylotyping study 5 randomly selected UPECs from ABU or symptomatic group [a] *arpA* [(400bp); the entire 5 representative isolates harboured *arpA* gene except 161 [b] *chuA* [(288bp); the entire 5 representative isolates harboured *chuA* gene [c] *yjaA* [(211bp); except isolates 99 and 145, all harboured the *yjaA* gene [d] TspE4.C2 [(152bp); all harboured TspE4.C2 except isolate 96 and [e] *arpAgpE* [(301bp); isolates 145 and 162 harboured *arpAgpE* genes. The intricate phylotype properties of the selected isolates have been tabulated earlier.

(a)



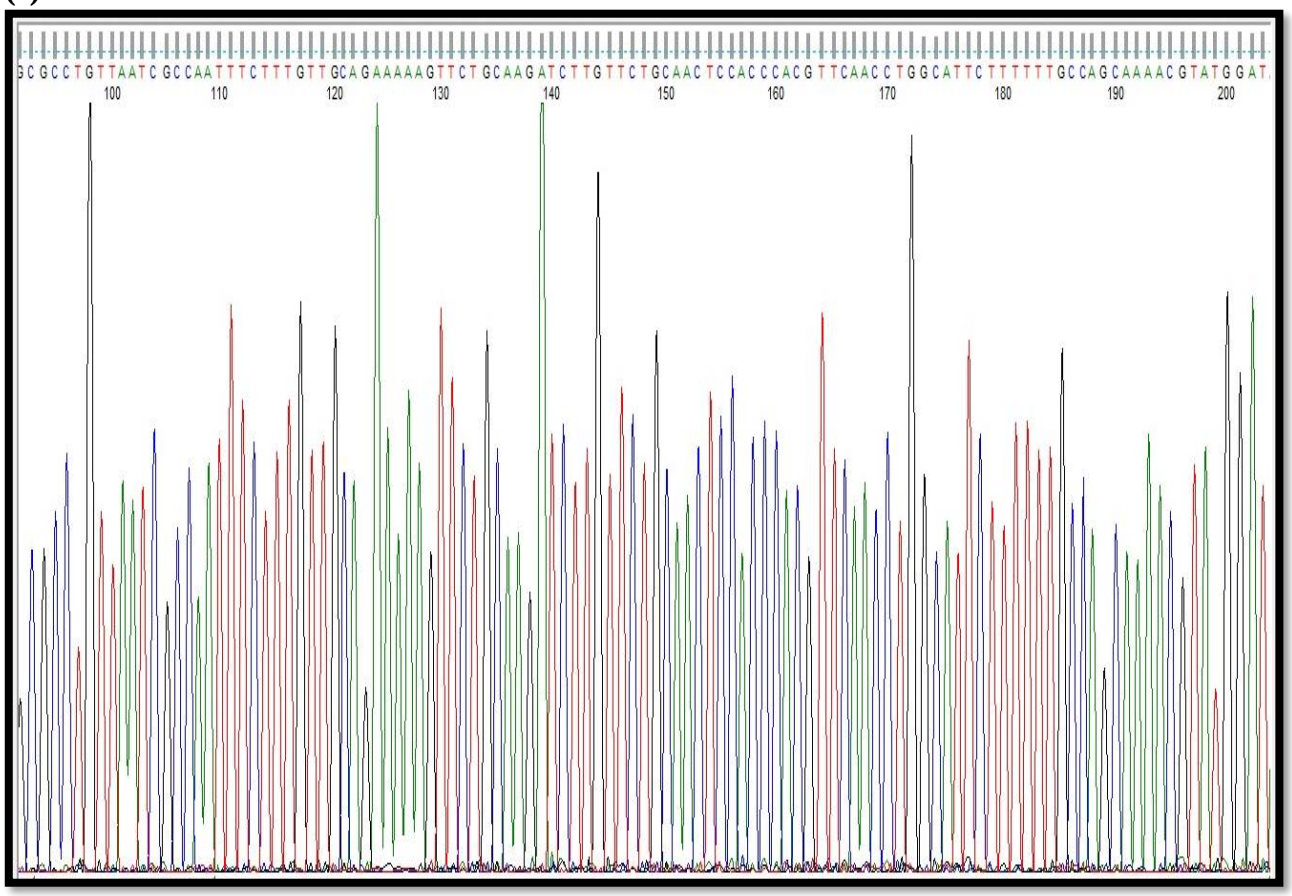
(This study)

(b)



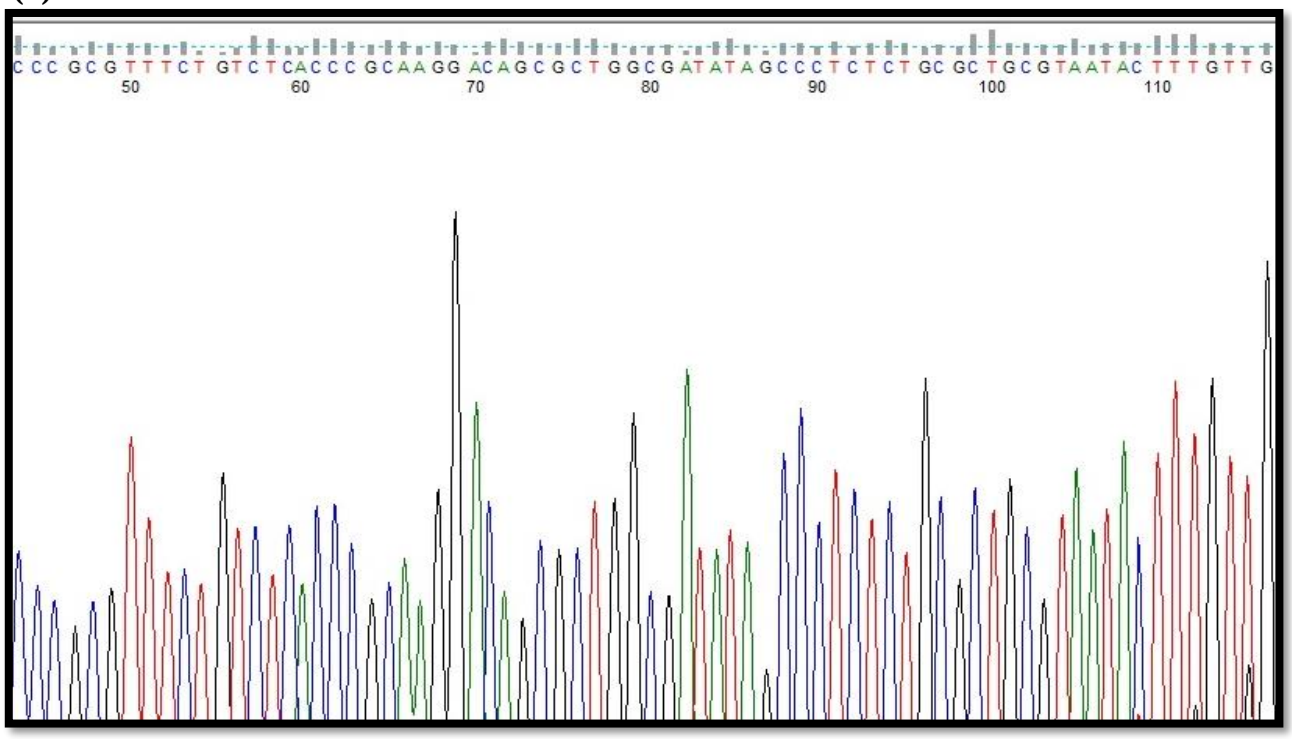
(This study)

(c)



(This study)

(d)



(This study)

(e)

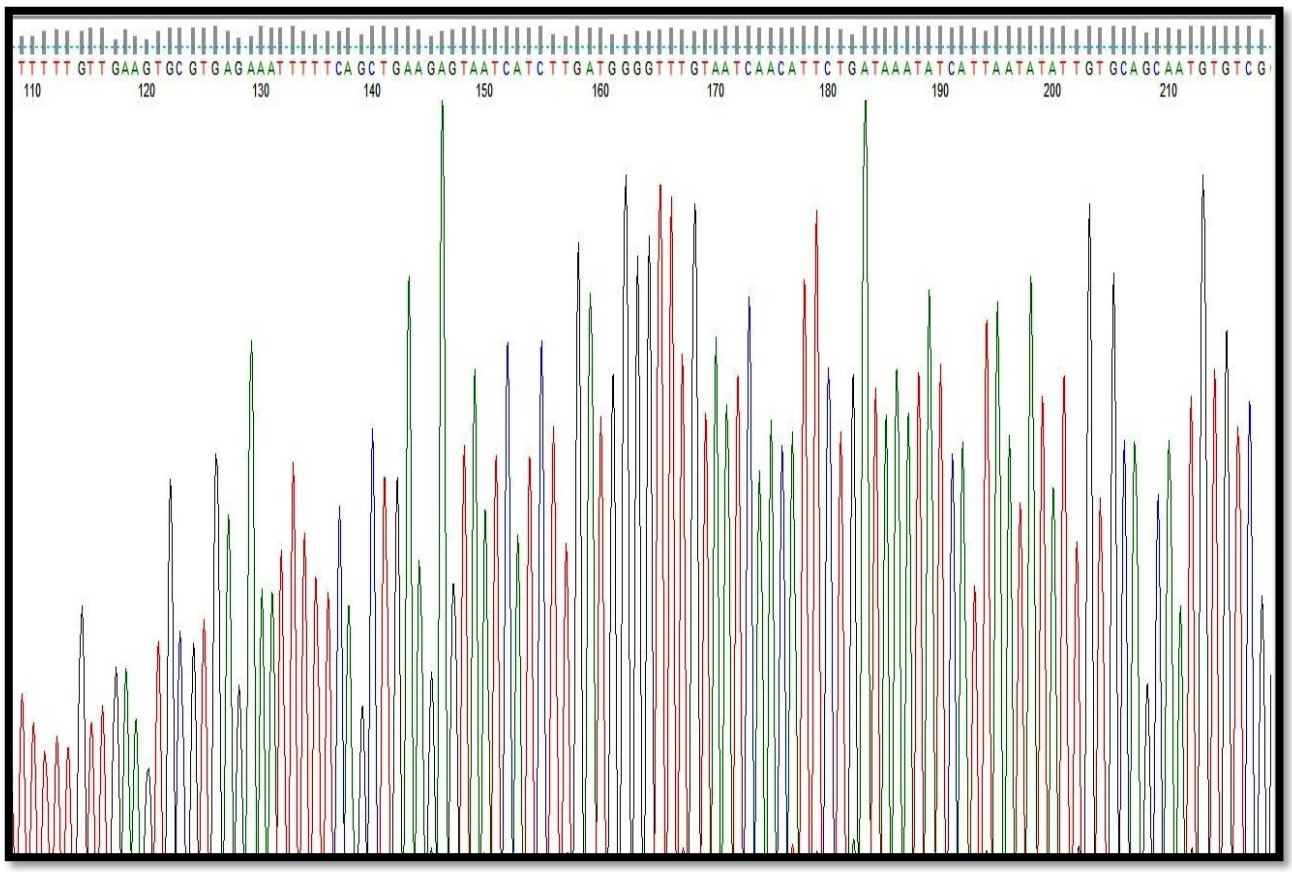


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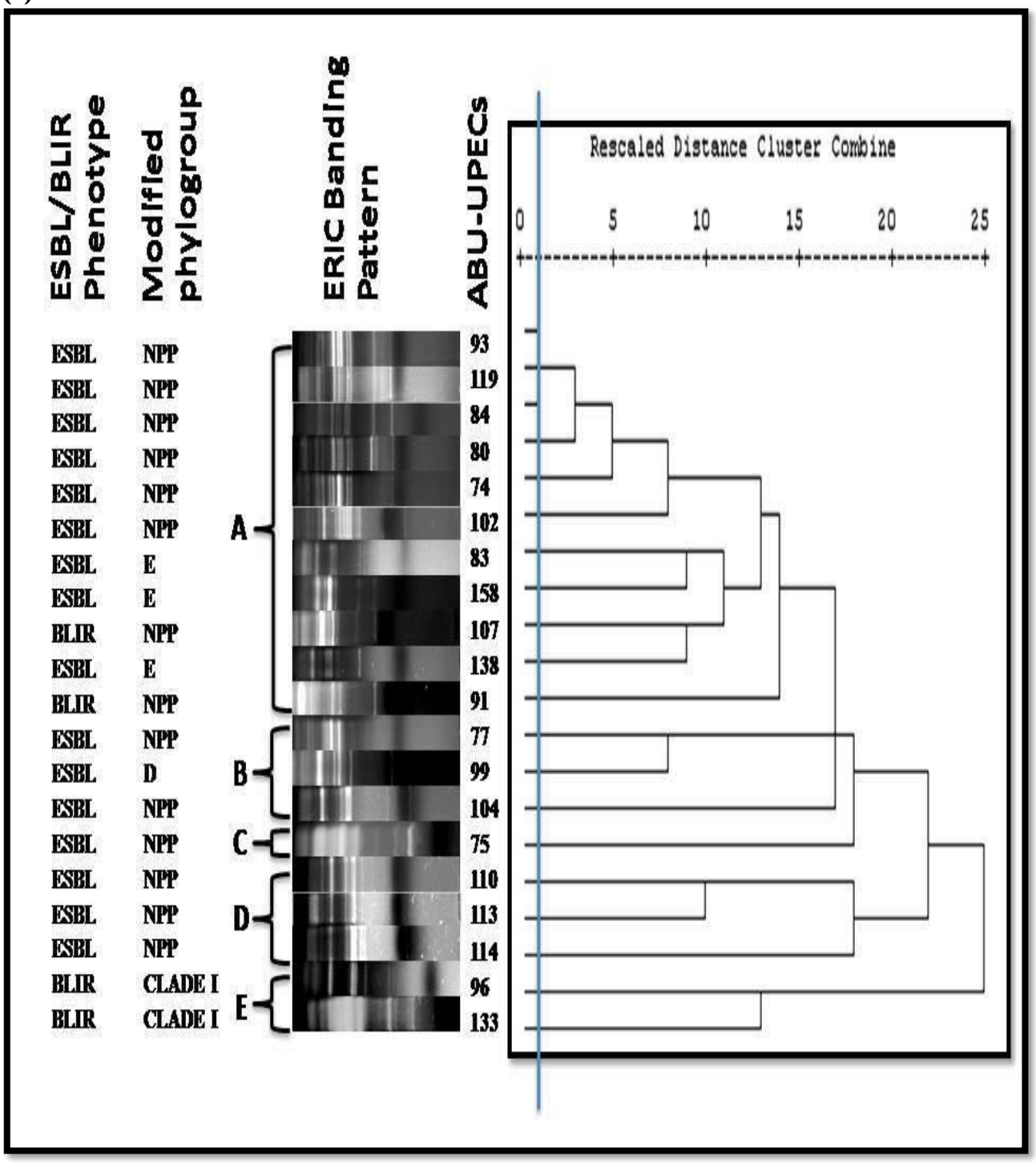
Fig. 4.10: The representative chromatograms of different phylogeny genes obtained after sequencing (a) *arpA* (400bp) (b) *chuA* (288bp) (c) *yjaA* (211bp) (d) TspE4.C2 (152bp) (e) *arpAgpE* (301bp).

4.5.4 Genetic diversity analysis

The ERIC-PCR typing showed a high degree of genetic heterogeneity among the UPECs, irrespective of their asymptomatic **(Fig. 4.11a)** and symptomatic **(Fig. 4.11b)** nature, at a similarity level of ≥ 96%. The presence of diverse clonal groups was espied among both the ABU (5groups; A-E) **(Fig. 4.11a)** and symptomatic (6 groups, A-F) **(Fig. 4.11b)** UPECs irrespective of their ESBL/BLIR phenotype (documented in Chapter 2) and phylotype property.

(a)



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(b)

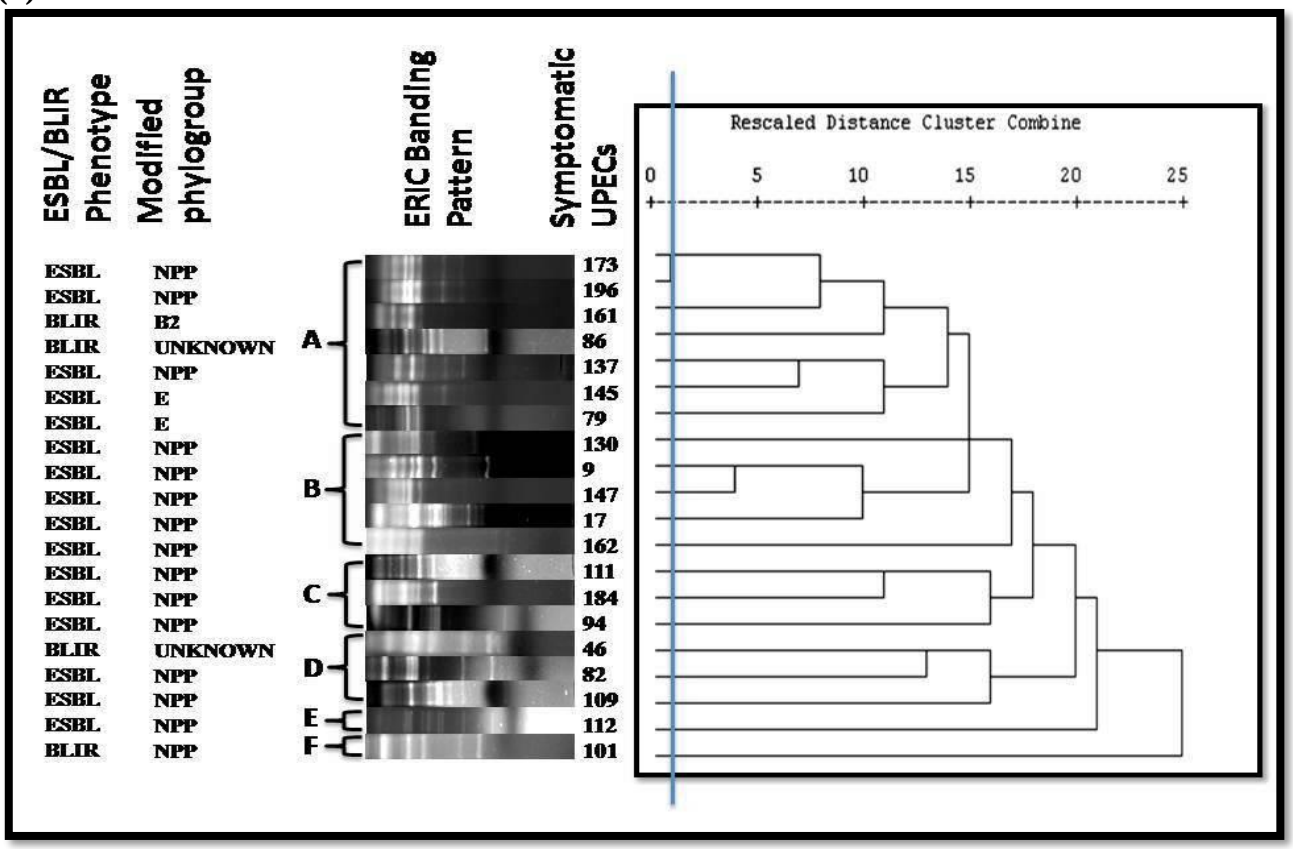

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Fig. 4.11: Dendogram generated on ERIC-PCR profiles of (a) asymptomatic (n=20) and (b) symptomatic (n=20) UPECs. Dice similarity coefficient values were used to generate the dendogram by UPGMA method of clustering using SPSS version 21.0 software. Isolates were distinctly unrelated at a coefficient of similarity value ≥ 96% (indicated by a solid line). ERIC-banding pattern of 20 each of ABU and Symptomatic UPECs respectively were represented as grouped individual lanes that contained the amplicons of each isolate. Five and six clonal groups were identified based on the cluster analysis of the individual ERIC profiles of asymptomatic and symptomatic isolates respectively. The extreme left column represented respective ESBL /BLIR phenotype of the isolates. Immediately adjacent to that was the phylogroup of the isolates.

4.5.5 MLST analysis

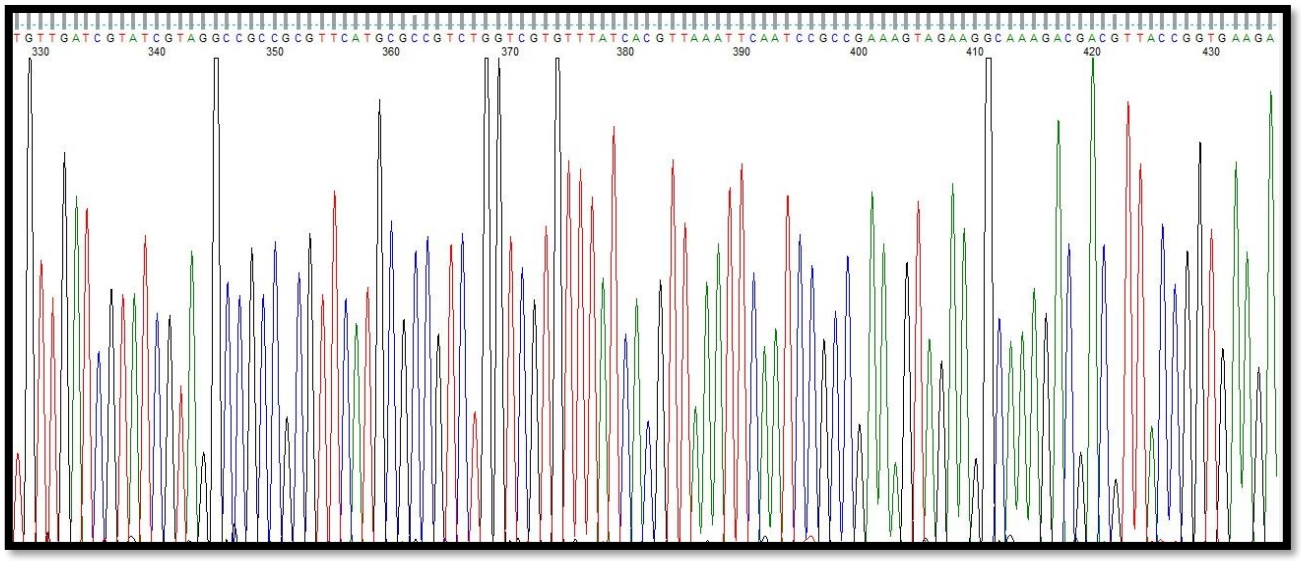
Numerous alleles of the seven gene housekeeping genes were perceived from the MLST analysis performed on the 40 (Asymptomatic=20; Symptomatic=20) drug resistant UPEC isolates. Two representative isolates (Asymptomatic=1; Symptomatic=1) and their respective chromatograms (part of individual genes) of each of the seven housekeeping genes indicating their respective alleles had been depicted below in **Fig. 4.12** and **Fig. 4.13** respectively. The number of alleles observed for each of the seven housekeeping genes were: *adk*= 9 [Asymptomatic= 2 (22.22%); Symptomatic=2 (22.22%); Common=5 (55.56%)], *fumC*= 9 [Asymptomatic= 0 (0%); Symptomatic= 2 (22.22%); Common=7 (77.78%)], *gyrB*= 12 [Asymptomatic= 1 (8.33%); Symptomatic= 3 (25%);Common= 8

(66.67%)], *icd*= 10 [Asymptomatic=1 (10%); Symptomatic= 3(30%); Common=6 (60%)], *mdh*= 10 [Asymptomatic= 1 (10%); Symptomatic= 3 (30%); Common=6 (60%)], *purA*= 11 [Asymptomatic= 2 (18.18%); Symptomatic= 3(27.27%); Common= 6 (54.54%)], and *recA*= 10 [Asymptomatic= 0 (0%); Symptomatic= 4 (40%); Common=6 (60%)]. Among ABU UPECs, the allele that most frequently occurred at each of the seven locus were *adk* 6 [n=9], *fumC* 6[n=6], *gyrB* 22[n=5], *icd* 16[n=6], *mdh* 11[n= 7], *purA* [n= 5] and *recA* 7[n= 10]. However, the most frequently occurring alleles among symptomatic UPECs were *adk* 6 [n=8], *fumC* [4; n=4, 6; n=4, 40; n=4], *gyrB* 47 [n=5], *icd* 8 [n=4], *mdh* 36[n= 5], *purA* 28 [n= 6] and *recA* 7[n= 7].

Withal, the aforementioned MLST analyses performed on the 40 drug-resistant UPECs revealed that out of 26 discrete STs, 12 (46 %) belonged to an unassigned class and were designated as unidentified STs (USTs). Among the 14 known STs, 5(35.7%) and 5(35.7%) were uniquely distributed among 6 asymptomatic **(Table 4.6a)** and 5 symptomatic **(Table 4.6b)** isolates respectively. Nonetheless, 4 (28.6%) were common in both the asymptomatic (9 isolates) **(Table 4.6a)** and symptomatic (6 isolates) **(Table 4.6b)** groups. Nevertheless, among the 12 USTs, 3(25%) and 7(58%) were uniquely distributed among 3 asymptomatic and 7symptomatic isolates respectively. However, 2 (17%) were common in both the aforementioned groups with 2 isolates in each group. ABU and symptomatic *E. coli* isolates with 5 known STs each were successfully grouped into 4 and 4 CCs respectively. Moreover, 4 STs that were common among both the groups were also distributed among 4 CCs. Three and 2 CCs were unique to asymptomatic and symptomatic isolates respectively. ST 2346 (symptomatic isolate) did not belong to any of the designated CCs. The most frequent ST among ABU UPECs was ST940 (N=5; 25%). However, among symptomatic UPECs the most frequent STs were ST410 (n = 2; 10%) and ST940 (n = 2; 10%). Three and two isolates respectively from the asymptomatic and symptomatic group with ST940 were found to belong to phylogroup E. On the other hand, two ABU UPECs isolates with ST940 and 2 symptomatic UPECs with ST410 were found to be distributed among the *E. coli* phylotype variants grouped into class entitled as **novel phylotype property (NPP) (Table 4.6a-b)**.

Furthermore, results obtained from the MLST analysis indicated that 9 out of 14 ABU UPECs with NPP were from 7 discrete CCs and 7 STs respectively. However, the remaining 5 isolates could not be assigned to known *E. coli* STs and CCs. Nevertheless, 4 out of those 5 ABU-UPECs with USTs could be grouped into 3 nearest clonal complexes (NCCs; clonal complexes that differed by 1 allele) **(Table 4.6a)**. Among 15 symptomatic UPECs with NPP, 6 were from 5 discrete CCs and STs respectively. However, 3 out of the remaining 9 isolates with USTs were grouped into 3NCCs. However, incidences of both identical and varied STs of the same CCs were also found to be distributed among the isolates with NPP **(Table 4.6b).**

(a)



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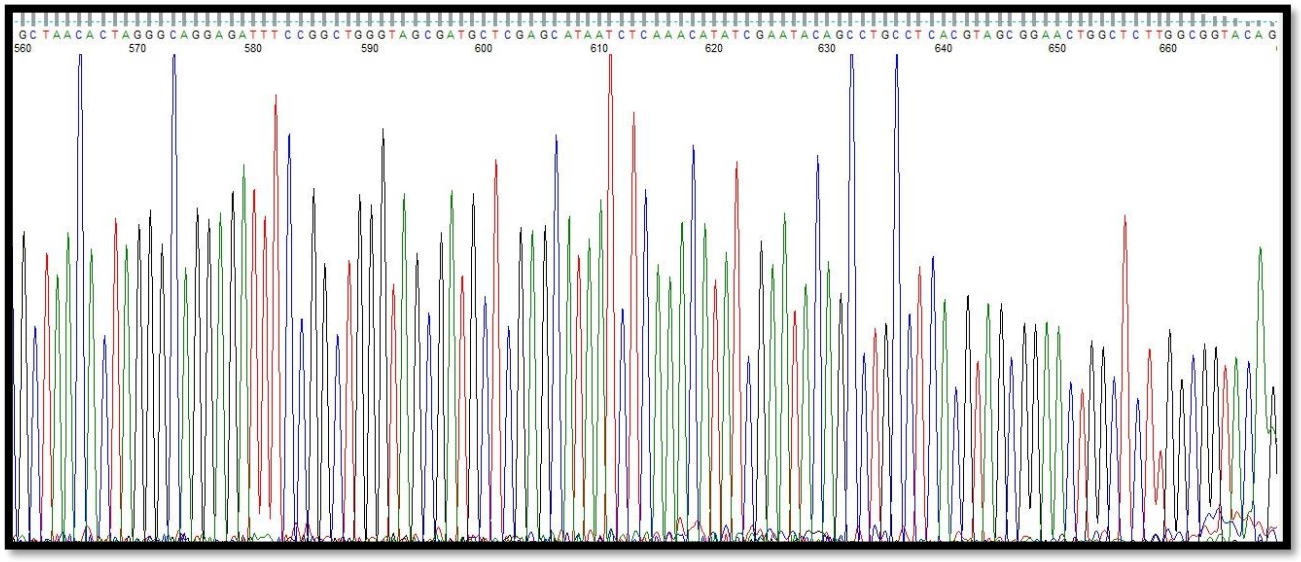
(b)



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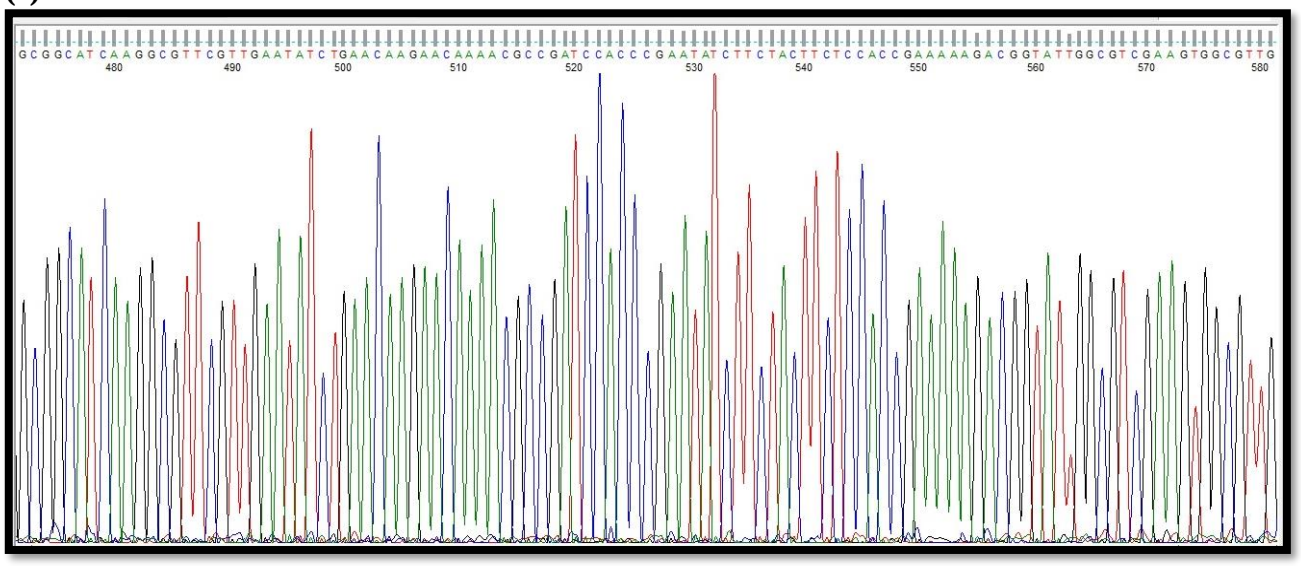
(c)



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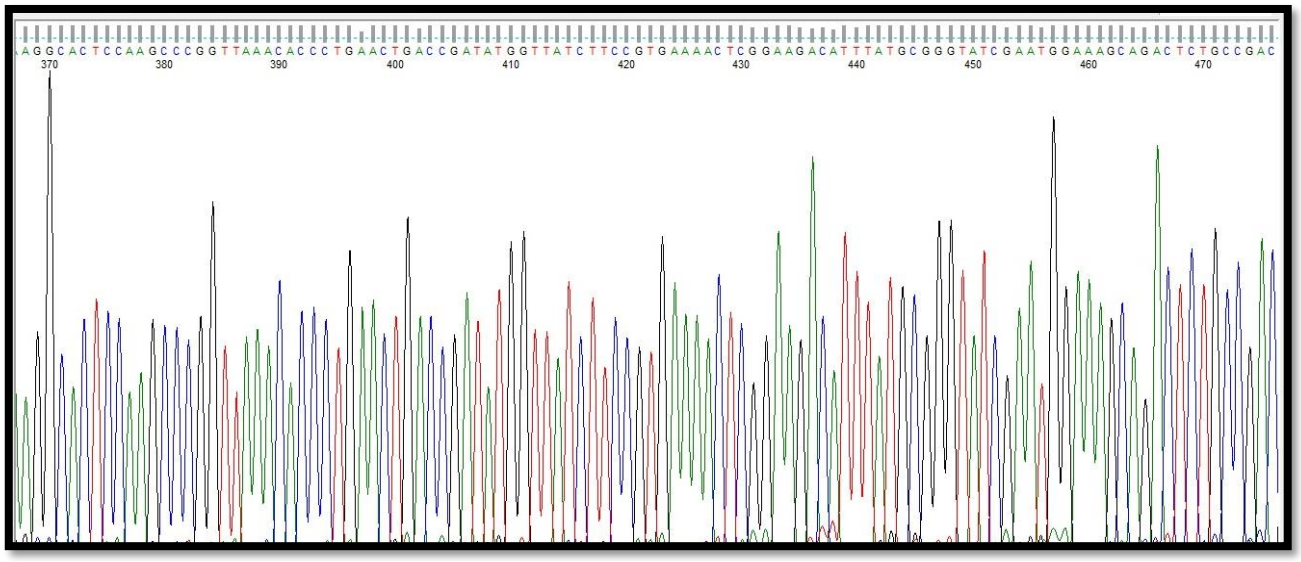
(d)



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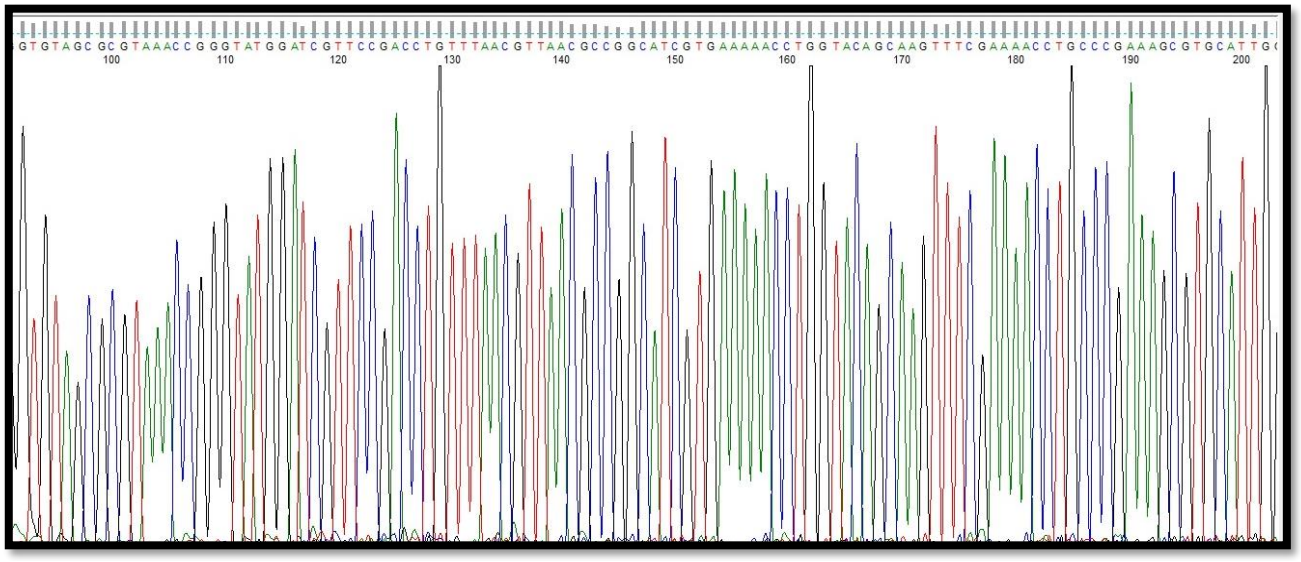
(e)



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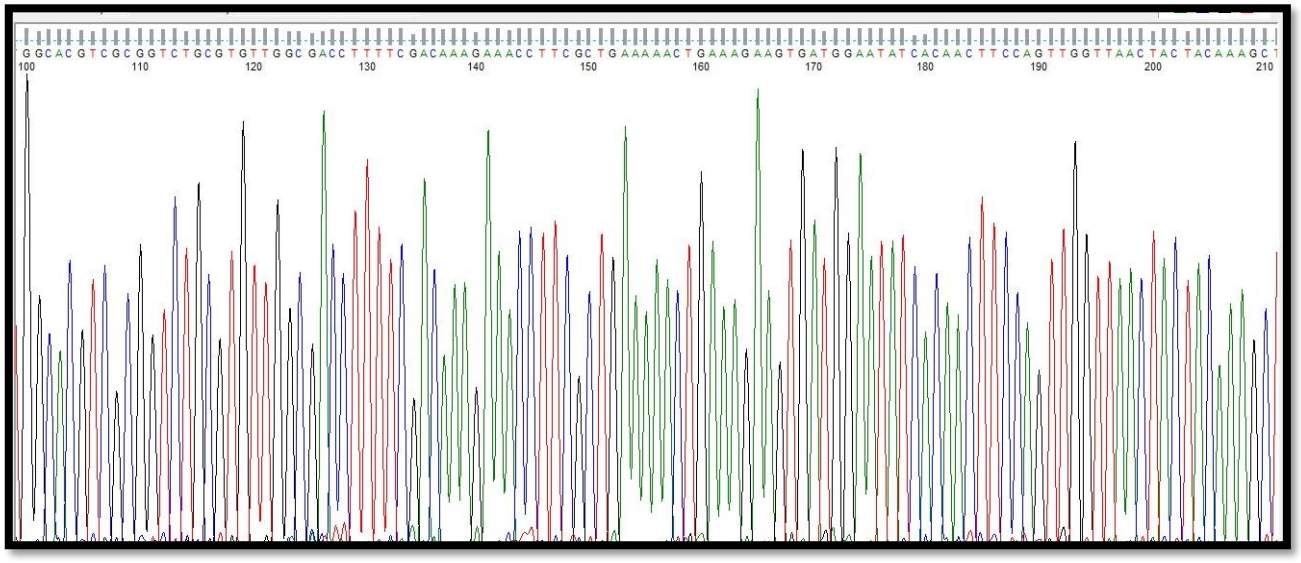
(f)



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(g)

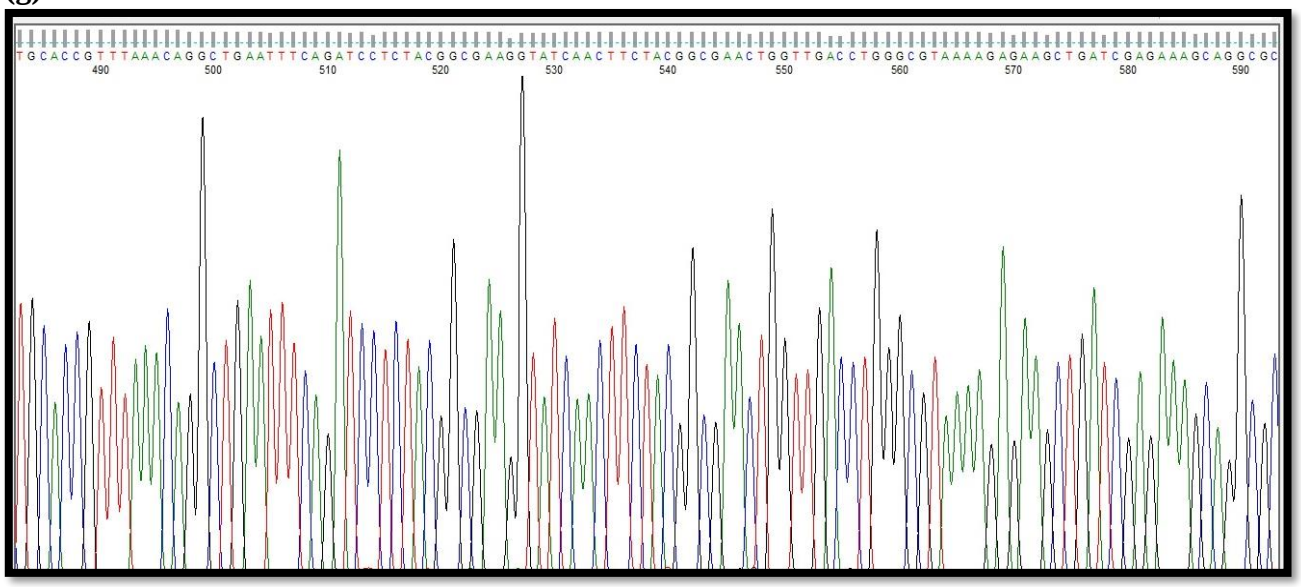

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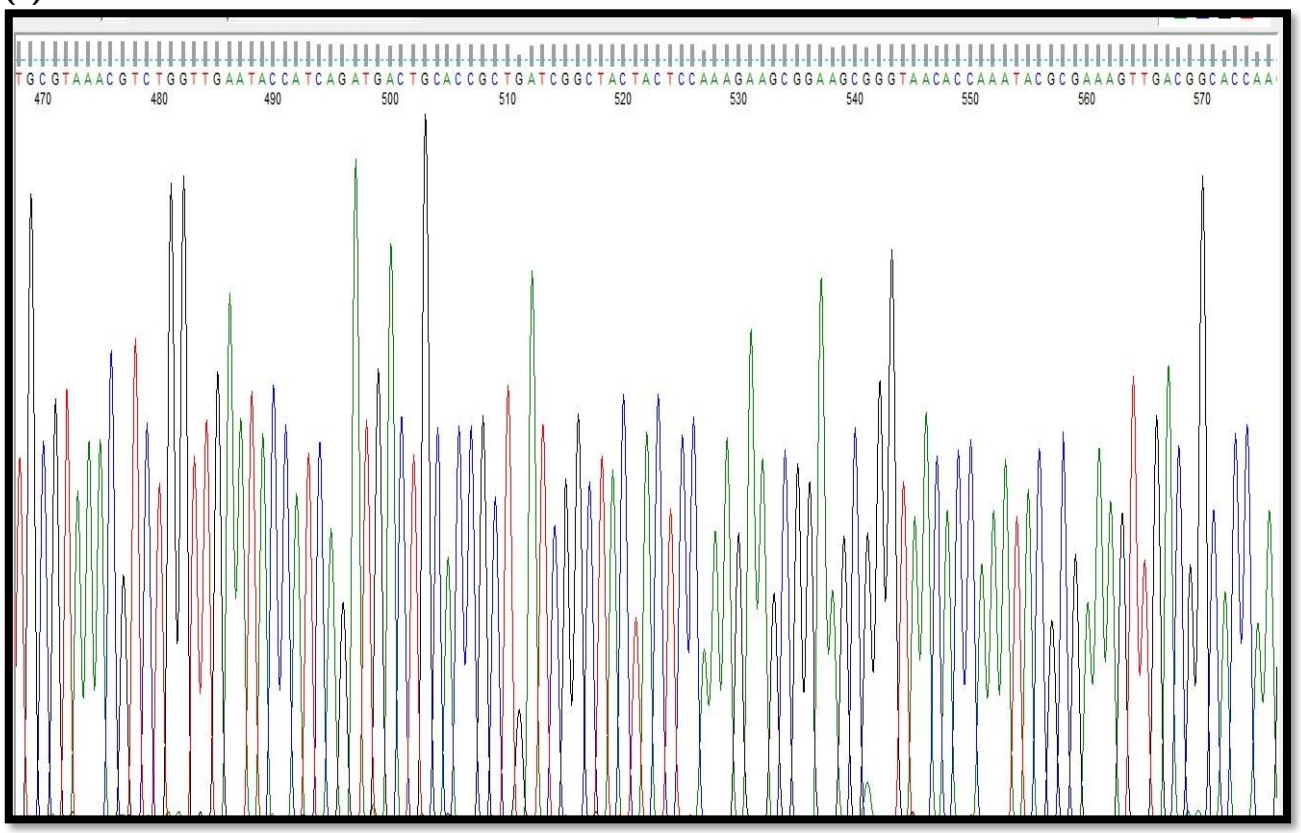
Fig. 4.12: The representative chromatograms of seven different housekeeping genes obtained after sequencing of asymptomatic isolate 83 (a) *adk* [(536bp); Allele no. 6] (b) *fumC* [(469bp); Allele no. 6] (c) *gyrB* [(460bp); Allele no. 22] (d) *icd* [(518bp); Allele no. 16] (e) *mdh* [(452bp); Allele no. 11 (f) *purA* [(478bp; Allele no. 1] and (g) *recA* [(510bp); Allele no. 7]. A part of the entire chromatogram of the respective genes had been depicted above. Allele numbers were obtained from the MLST data analysis mentioned "experimental methods" section.

(a)



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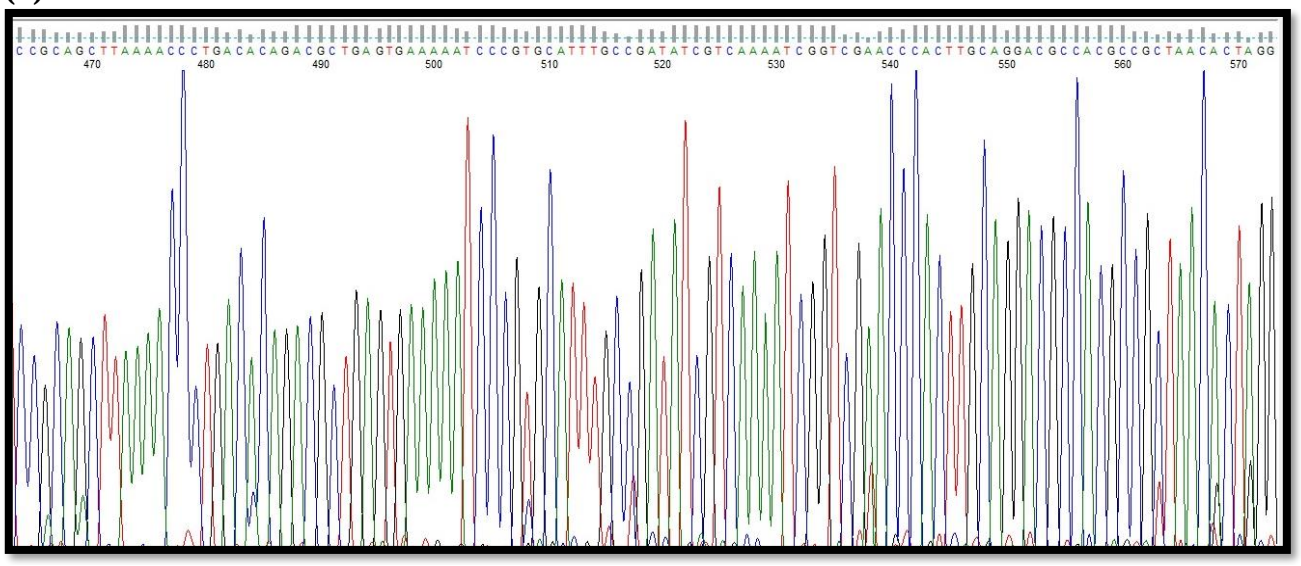
(b)



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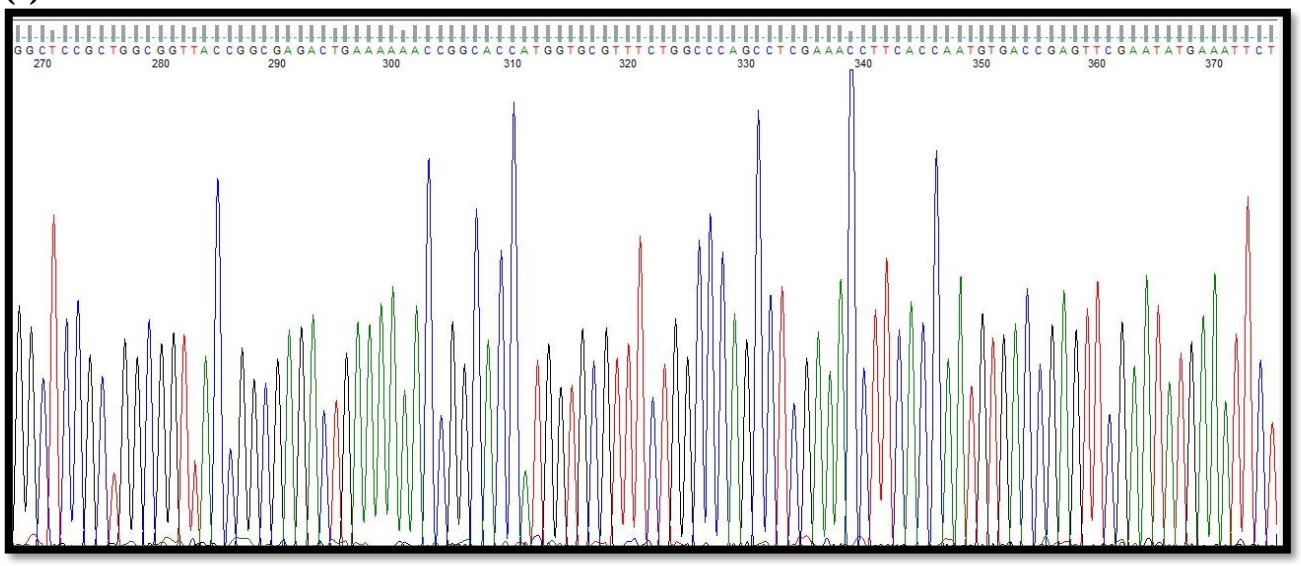
(c)



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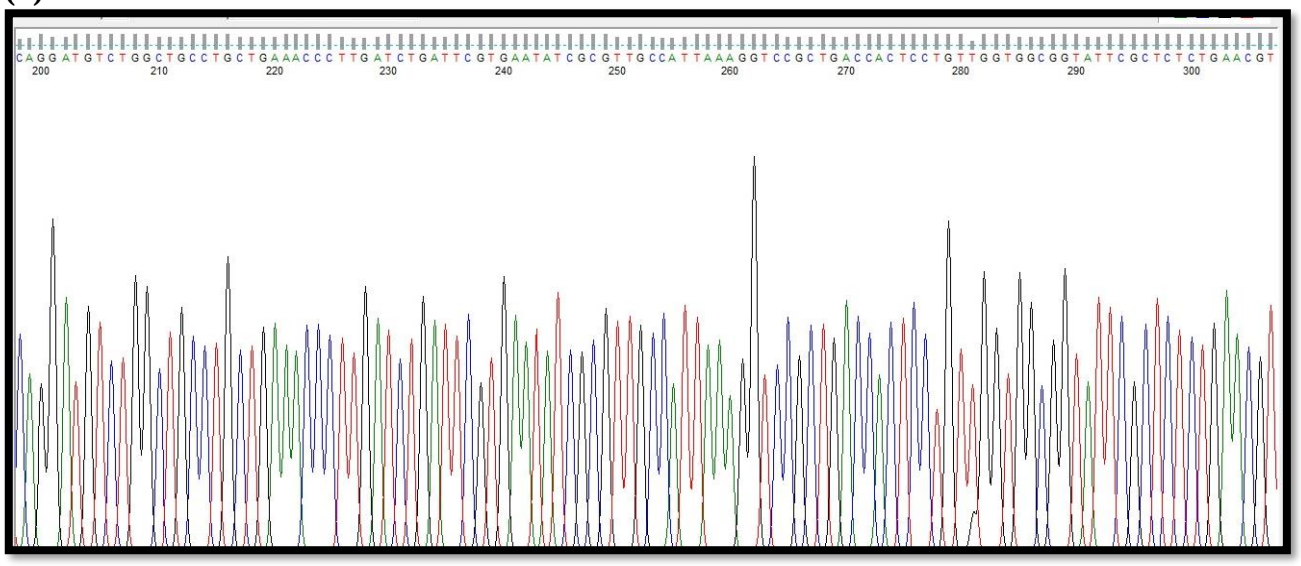
(d)



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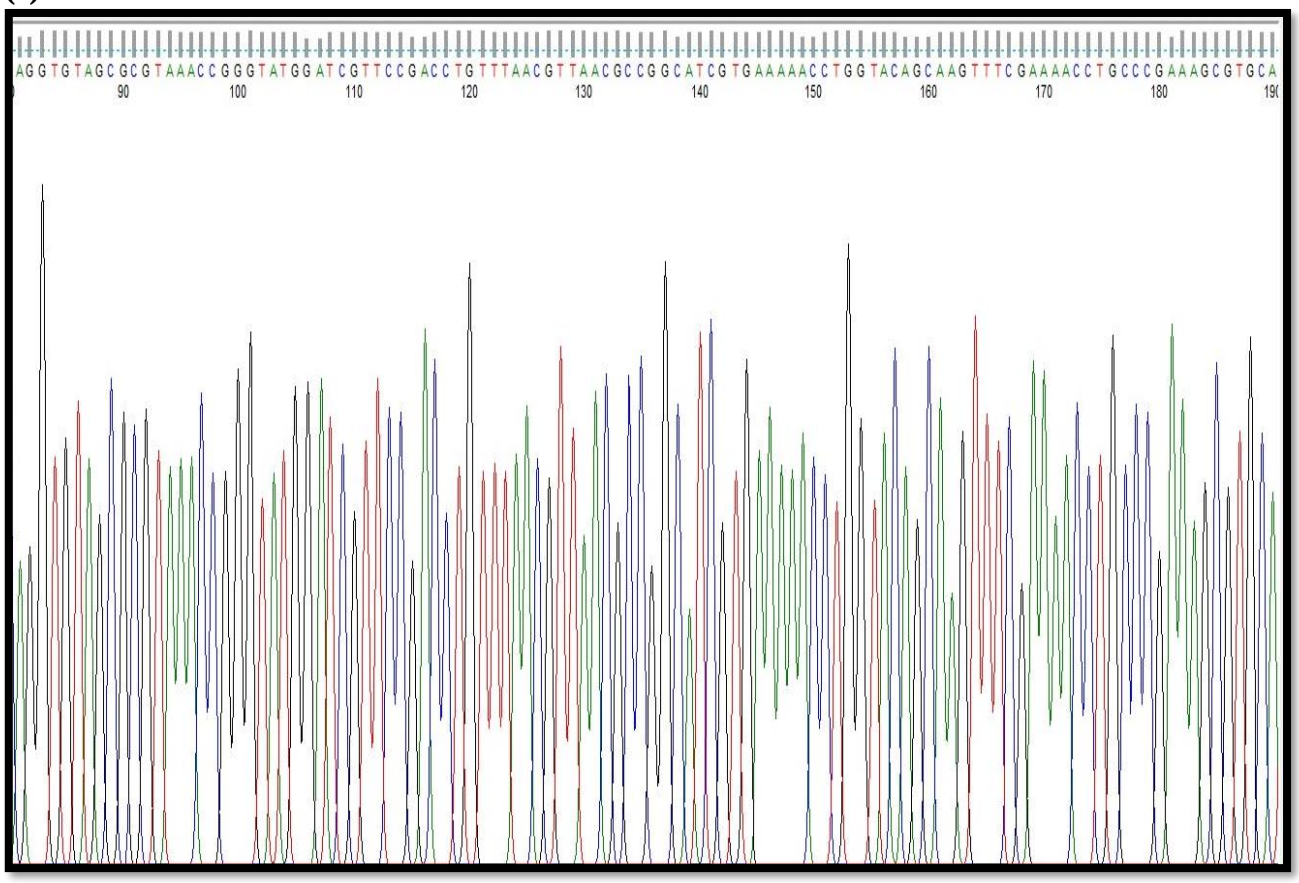

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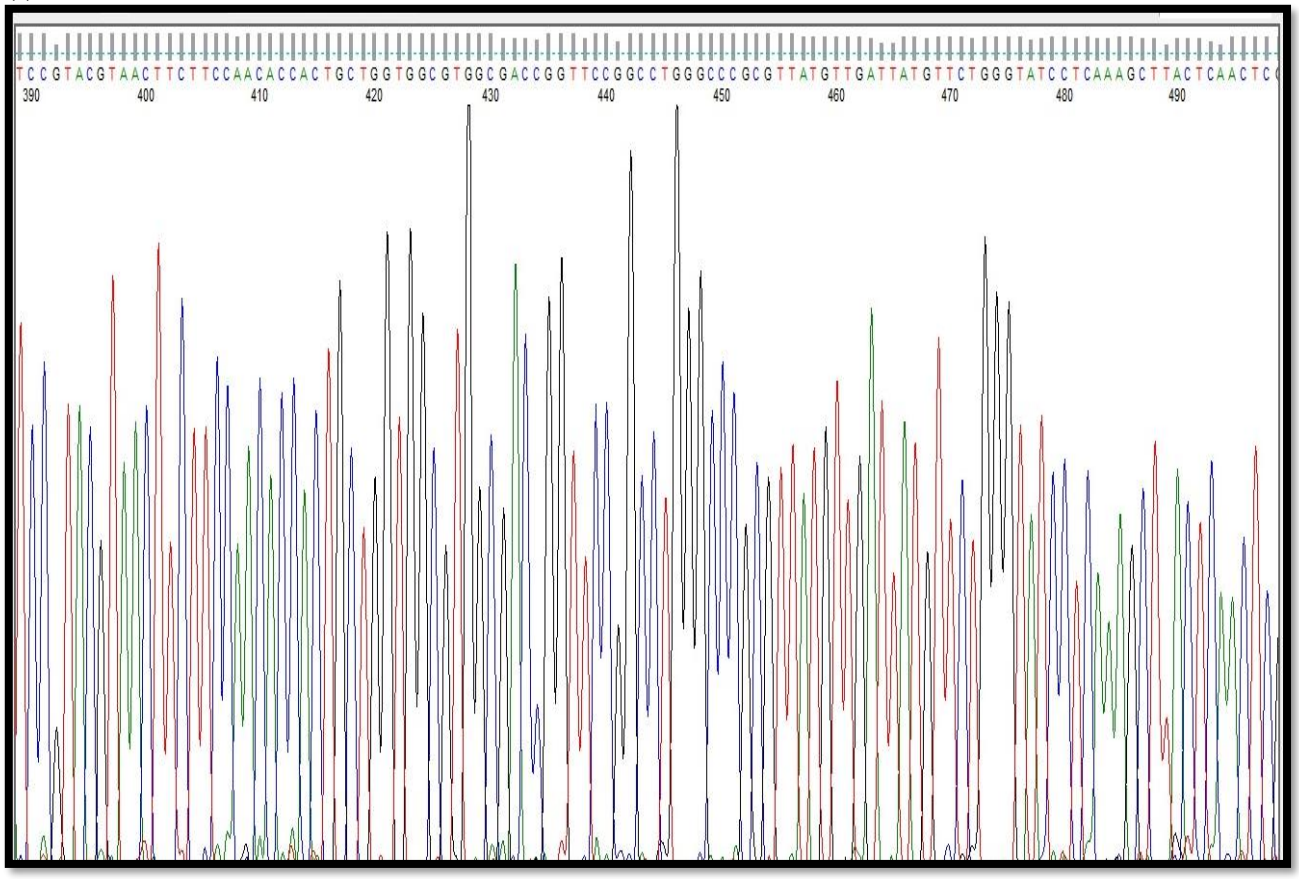
(f)



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(g)

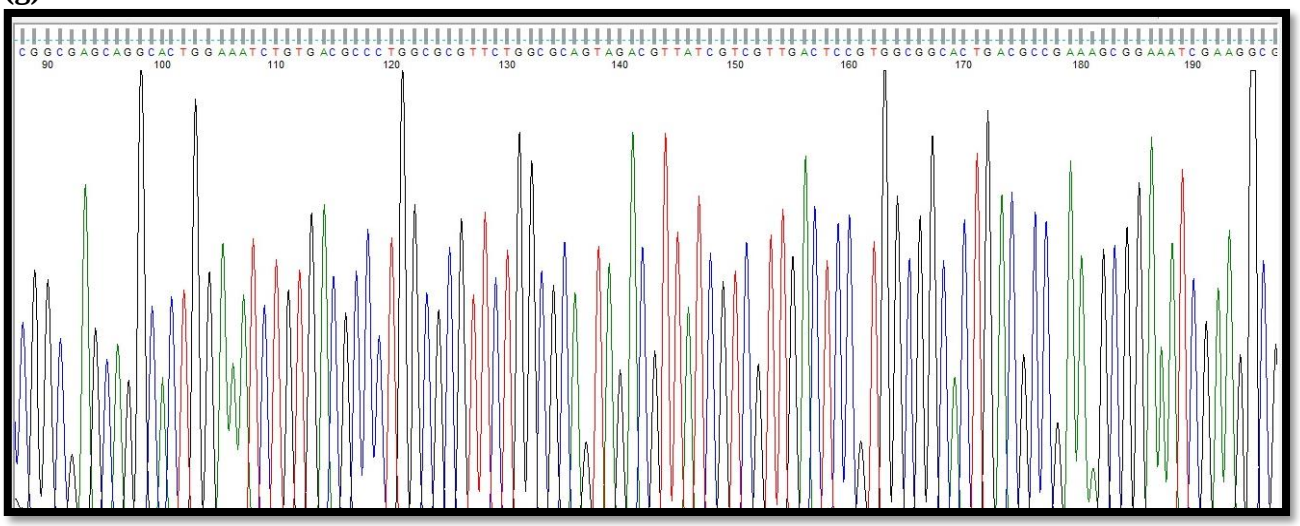

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Fig. 4.13: The representative chromatograms of seven different housekeeping genes obtained after sequencing of symptomatic isolate 147 (a) *adk* [(536bp); Allele no. 43] (b) *fumC* [(469bp); Allele no. 41] (c) *gyrB* [(460bp); Allele no. 15] (d) *icd* [(518bp); Allele no. 18] (e) *mdh* [(452bp); Allele no. 11 (f) *purA* [(478bp; Allele no. 7] and (g) *recA* [(510bp); Allele no. 6]. A part of the entire chromatogram of the respective genes had been depicted above. Allele numbers were obtained from the MLST data analysis mentioned "experimental methods" section.

Table 4.6a Sequence types and clonal complexes in the asymptomatic uropathogenic *E. coli isolates* (n=20).

Sl no.	Sample no.	Phylotype property	Sequence types (STs)	Clonal Complexes (CCs)
1	74	NPP	ST167	CC10
2	75	NPP	ST38	CC38
3	77	NPP	UST3	NCC-CC405
4	80	NPP	ST101	CC101
5	83	E	ST940	CC448
6	84	NPP	ST410	CC23
7	91	NPP	UST4	NCC-CC131
8	93	NPP	UST6	NCC-CC23
9	96	CladeI	ST648	CC648
10	99	D	ST405	CC405
11	102	NPP	UST8	NCC-CC131
12	104	NPP	ST2112	CC648
13	107	NPP	UST9	NRMD
14	110	NPP	ST1195	CC131
15	113	NPP	ST1195	CC131
16	114	NPP	ST940	CC448
17	119	NPP	ST940	CC448
18	133	CladeI	ST648	CC648
19	138	E	ST940	CC448
20	158	E	ST940	CC448

UST: Unidentified sequence type; NRMD: Not registered in the MLST database;

NPP: Novel phylotype property

Table 4.6b: Sequence types and clonal complexes in the symptomatic uropathogenic *E. coli* isolates (n=20).

Sl no.	Sample no.	Phylotype property	Sequence types (STs)	Clonal Complexes (CCs)
1	9	NPP	UST1	NRMD
2	17	NPP	UST2	NRMD
3	46	Unknown	ST40	CC40
4	79	E	ST940	CC448
5	82	NPP	UST4	NCC-CC131
6	86	Unknown	UST5	NRMD
7	94	NPP	ST1195	CC131
8	101	NPP	UST7	NRMD
9	109	NPP	UST10	NCC-CC131
10	111	NPP	UST3	NCC-CC405
11	112	NPP	UST11	NRMD
12	130	NPP	ST2346	NRMD
13	137	NPP	ST448	CC448
14	145	E	ST940	CC448
15	147	NPP	ST101	CC101
16	161	B2	ST131	CC131
17	162	NPP	ST2659	CC38
18	173	NPP	ST410	CC23
19	184	NPP	UST12	NRMD
20	196	NPP	ST410	CC23

UST: Unidentified sequence type; NRMD: Not registered in the MLST database;

NPP: Novel phylotype property

4.5.6 Evolutionary and /phylogenetic relationships among different UPECs and their STs

The evolutionary relationships among different UPECs included in this study irrespective of their asymptomatic or symptomatic origin had been illustrated in the **Fig. 4.14**. Moreover, according to the *E. coli* MLST database all known STs, except one (**Fig. 4.14,** red font) articulated in this study were found to be associated with different sources like animals, birds, foods, poultry, livestock and environment, with or without humans (**Fig. 4.14; pink, sky, ash, green, orange and purple fonts)**.

Furthermore, taking into consideration only the single locus variants (SLVs) links, application of the goeBURST algorithm to the entire set of STs, irrespective of their asymptomatic or symptomatic origin resolved the 26 STs into 5 clonal complexes (CCs) and 13 singletons (**Fig. 4.15)**. Among the 5 CCs, 4 contained 2 STs each. The largest clonal complex contained 4 isolates with 2 known STs [ST131 (1symptomatic isolate); ST1195 (Asymptomatic=2; Symptomatic=1)] that belonged to CC131 and 4USTs (Asymptomatic=2; Symptomatic=2) with NCCs-CC131 [(**Fig. 4.15)** ;**(Table 4.6a-b)]**.

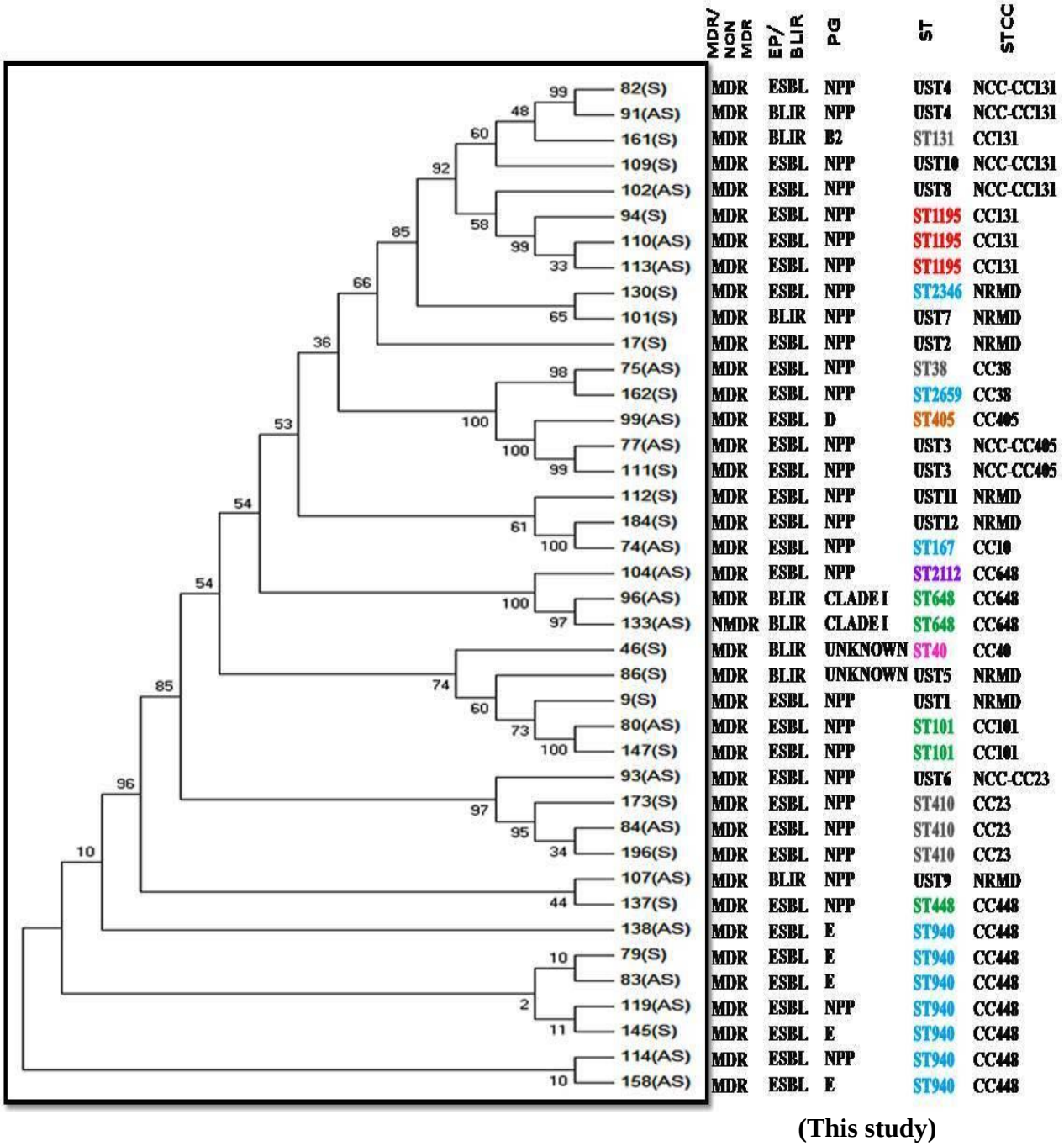

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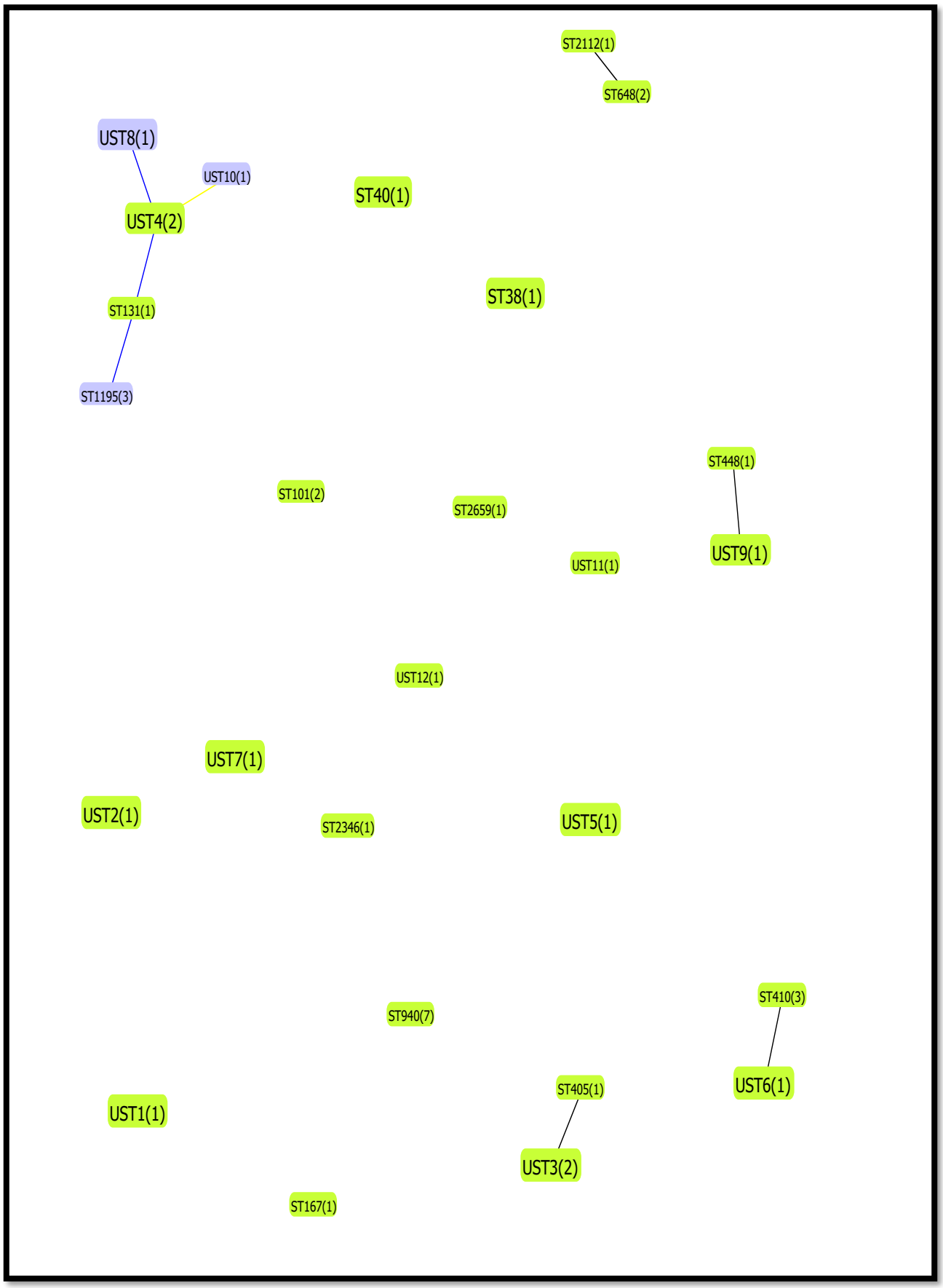
Fig. 4.14: Maximum likelihood tree constructed using MEGA 7.0 on the basis of the nucleotide sequences of the seven classical housekeeping genes of *E. coli* depicted the evolutionary relationships among 40 UPECs (Asymptomatic=20; Symptomatic=20). Multi-drug resistant (MDR) or Non Multi-drug resistant (NMDR) type , ESBL phenotype (EP), β-lactam-β-lactamase inhibitor resistant (BLIR), Phylogenetic group (PG), Sequence type (STs), Sequence type clonal complex as obtained from MLST database (STCC), NRMD (Not registered in the MLST database) of the individual UPECs were represented right of the dendogram. The STs highlighted in different colours represented their varied sources of isolation as found from the *E. coli* Enterobase [red (only humans); pink (humans, animals, birds, environment); sky (humans, animals, environment); ash (humans, animals, birds, poultry, foods); green (humans, animals, poultry, livestock); orange (humans, environment); purple (only animals)]. "AS" and "S" denoted asymptomatic and symptomatic isolates respectively.

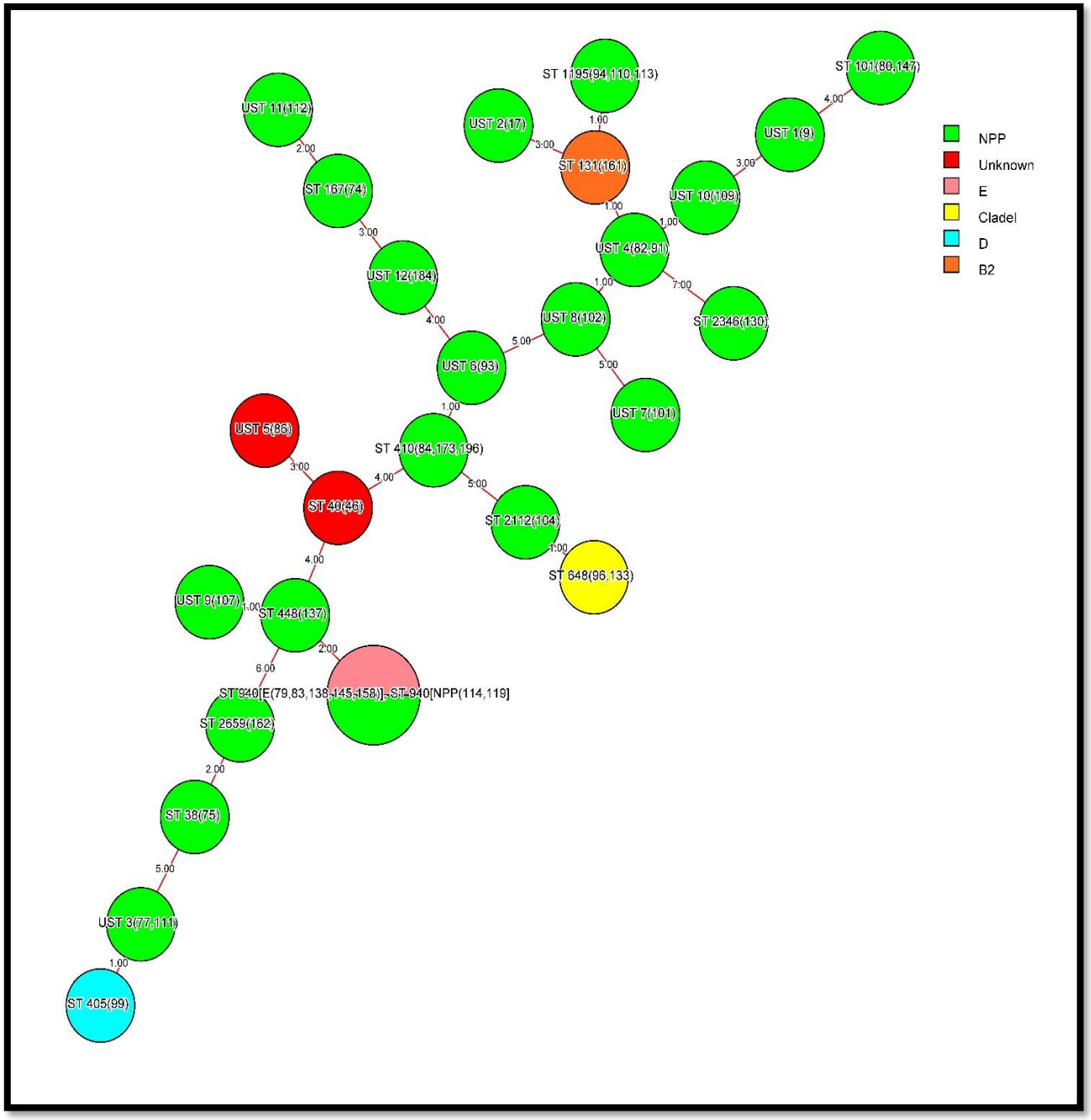


(This study)

Fig. 4.15: The twenty six distinct STs analyzed using the goeBURST algorithm in the PHYLOViZ 2.0 software. 13 singletons and 5 clonal complexes (CCs) were identified.

4.5.7 Quantitative relationship amongst the varied STs

MST analysis brought to light an overall clonal diversity among the UPECs irrespective of their asymptomatic or symptomatic nature (**Fig. 4.16)**.



(This study)

Fig. 4.16: Minimum spanning tree constructed on 26 varied sequence types (STs) of 40 uropathogenic *E. coli* isolates (Asymptomatic=20; Symptomatic=20). Detected STs, obtained from allelic profiles of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) by MLST using BioNumerics version 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium) were coloured according to their phylogenetic groups. Unidentified sequence types were represented as USTs. Numbers in the first brackets represented the sample ID of each the UPECs. Allele differences in the STs and USTs were marked as integers (1-7) indicated by their branch lengths

On the other hand, except for 2 asymptomatic UPECs, similar known STs were found to belong to a particular phylogroup. Nevertheless, one ABU UPEC (ST2112) with NPP was found to be intimately related to two other ABU UPECs that belonged to phylogroup Clade I. Additionally, a close association of 3 USTs that were distributed among the isolates with NPP was observed with the ones that belonged to the known phylogroups. UST3 (2 isolates; Asymptomatic=1; Symptomatic=1), UST4 (2 isolates; Asymptomatic=1; Symptomatic=1), UST8 (Asymptomatic=1) and UST10 (Symptomatic=1) was found to be closely related to phylogroup D (ST405) and phylogroup B2 (ST131) respectively. Short branch lengths indicated their close association (**Fig. 4.16)**.

4.6 Discussion

The current study evinced the fact that either *blaTEM* or *blaOXA* alone or their combinations were pivotal contributors to the ESBL or BLIR phenotypes. *blaTEM* gene was found to be statistically most predominant among plasmid DNA of both the asymptomatic and symptomatic groups **(Table 4.4)** of isolates regardless of their ESBL or BLIR phenotype (Chapter 2; Table 2.5). The aforementioned observation was mostly in agreement with the studies conducted on symptomatic isolates from India **(Mukherjee et al. 2018; Basu and Mukherjee 2018)** as well as from other countries of the world like Egypt **(Abd El Tawab et al. 2016)** and China **(Xiao et al. 2019).** However, *blaOXA* gene was most predominant **(Table 4.4)** in the genomic DNA of the studied isolates, irrespective of their asymptomatic or symptomatic nature which was discordant to the recent report from Upper Egypt **(Hassuna et al. 2020)** that demonstrated the predominance of *blaTEM* gene. Nonetheless, the statistically significant (p value ≤ 0.05) occurrence of sharing of these β-lactamases in between the plasmid and genomic DNA of the studied UPECs **(Table 4.4)** was mostly in accordance with the previous studies reported on symptomatic UPECs isolated from the human **(Mukherjee et al. 2011)** and avian population (**Abd El Tawab et al. 2016)** from the countries like India and Egypt respectively**.** Howbeit, a combination of all three (TEM, CTX-M and OXA) β-lactamase genes among both the plasmid and genomic DNA was more predominant among the symptomatic UPECs **(Fig. 4.2b; Fig. 4.3b)** as compared to the asymptomatic (**Fig. 4.2a; Fig. 4.3a)** ones. Moreover, a high correlation was perceived in the incidence of the *blaTEM* and *blaOXA* genes among both the plasmid **(Fig. 4.4a-b)** and genomic DNA **(Fig. 4.5a-b)** of the ABU and symptomatic UPECs except in the plasmid DNA of the symptomatic isolates **(Fig. 4.4b)** in which moderate correlation was observed. The aforementioned observations were mostly in conformance with an earlier report from India (**Basu and Mukherjee 2018).** However, it was in dissension to the report by Mukherjee et al. **(Mukherjee et al. 2018)** from India that exhibited the highest co-existence of *blaTEM* and *blaCTX-M* genes. Nevertheless, the incidence of distinctly different distribution patterns of the aforesaid β-lactamase

genes especially in the plasmid DNA of the ABU (**Fig. 4.2a**) and symptomatic (**Fig. 4.2b)** UPECs projected the fact that process of their acquisition might have been dissimilar under indiscriminate drug pressure. This further betokened the likelihood of plasmid-mediated procuration of these β-lactamases due to horizontal gene transfer that might have occurred incongruously in the ABU and symptomatic UPECs respectively. Therefore, the high incidence of ESBL production and β-lactamase genes among ABU UPECs was exceedingly alarming which highlighted the detestable consequences of unrestrained drug usage in a resource-poor country like India **(Ghosh et al. 2022)**. The extraneous use of the newer and potent antibiotics in the last few years might have expedited the selection of novel β-lactamase variants that had further developed complications in health care management.

Earlier reports from the United States of America (**Kurpiel et al. 2011)** and India **(Basu and Mukherjee 2018)** had stated the association of MGEs like integrons and ISs with ESBL encoding genes especially in MDR *E. coli***.** In this study, a statistically significant (p value ≤ 0.05) incidence of MGEs (*intI1*, *ISEcp1*, IS5, IS26) was evident among both ABU and symptomatic UPECs **(Table 4.5)** with an overall maximal prevalence of *IS26* in both groups of isolates. Additionally, the present study for the first time reported the incidence of MGEs among ABU UPECs with the predominance of ESBL production. Till date the incidence of MGEs were reported in symptomatic UPECs from Egypt **(Salem et al. 2010)**, Spain **(Pérez-Etayo et al. 2018)** and India **(Basu and Mukherjee 2018).** To boot, in this study, *ISEcp1* alone or in combination with *IS26* was found to be present among all ABU UPECs and 15 out of 16 symptomatic UPECs that were ESBL producers akin to reports from France **(Cattoir et al. 2008)** and Australia **(Harmer et al. 2019)** during different time periods which revealed that ISs like *ISEcp1* and *IS26* were primarily associated with dissemination of resistance determinants with the former being associated with high-level expression of β-lactamase genes among the *E. coli* isolates. Nonetheless, this study demonstrated that except for one asymptomatic and one symptomatic isolate *IS26* was present among all UPECs that showed BLIR phenotype regardless of their asymptomatic or symptomatic nature. Thus, IS26 might be a discernible contributor to the aforementioned phenotype **(Ghosh et al. 2022)**. Furthermore, a significantly (p value ≤ 0.05) remarkable prevalence of *intI2*, among ABU UPECs than in the symptomatic isolates indicated a strong association of *intI2* with ABU **(Table 4.4)** than with the symptomatic population. However, a low incidence of *intI2* among symptomatic UPECs was in conformity with a report from Iran (**Lavakhamseh et al. 2016).** Withal, significant distinctiveness in the distribution pattern of 5 distinct MGEs in ABU **(Fig. 4.7a)** and symptomatic **(Fig. 4.7b)** UPECs proposed the fact that the course of acquisition of MGEs might have been different due to the pressure exerted by the indiscriminate drug usage. This further pointed out the possibility that accretion of these MGEs might have occurred in a more randomized way in the case of ABU UPECs unlike the apparently organized acquisition in case of the symptomatic ones

(Ghosh et al. 2022). *ISEcp1*and IS26 was perceived to be highly positively correlated (p value ≤ 0.05) among both ABU **(Fig. 4.8a)** and symptomatic **(Fig. 4.8b)** UPECs, similar to the earlier studies conducted from France **(Cattoir et al. 2008)** and Australia **(Harmer et al. 2019).** Notwithstanding, *ISEcp1* was found to be present in all ESBL or BLIR positive UPECs irrespective of their asymptomatic or symptomatic nature that carried more than one β-lactamase gene **(Fig. 4.7a-b; Fig. 4.2a-b)**. The aforesaid observation was mostly in accordance with an earlier report from France **(Cattoir et al. 2008)** that propounded a predominant role of *ISEcp1* in the acquisition and expression of β-lactamase genes. Therefore, the predominance of MGEs in the MDR and ESBL or BLIR producing UPECs, especially in the ABU UPECs, together with their diversity in the distribution pattern among both groups advocated that acquisition of MGEs and their dissemination might have resulted from unmethodical drug pressure. This further alluded to the plausibility that avirulent, susceptible ABU UPECs might have acquired resistance through mutations or transfer of resistance genes associated with MGEs **(Ghosh et al. 2022)**.

The previous reports from Spain **(Alonso et al. 2017)** and Iran **(Najafi et al. 2018)** indicated that the majority of the commensal and/ or pathogenic *E. coli* isolated from varied sources mostly belonged to phylogroup B2. Moreover, studies from France **(Clermont et al. 2013)** and Iran **(Najafi et al. 2018)** also intimated a rare or low incidence of *E. coli* isolates that could not be assigned to any of the eight known phylogroups. Nevertheless, the aforementioned reports were discordant to the observation reported earlier which indicated the statistically significant (p value ≤ 0.05) incidences of both asymptomatic and symptomatic UPECs that belonged to the "Unknown" phylogroup (Chapter 3) **(Ghosh and Mukherjee 2019).** It had been stated earlier that isolates that were designated to belong to phylogroup E showed the presence of both *arpA*; 400bp (obtained from amplification between *aceK* and part of *arpA*) and *arpA*; 301bp **(Clermont et al. 2013; Clermont et al. 2004)**. Hence, in this study, it might be assumed that ABU and symptomatic UPECs which harboured of all the four target genes *arpA* (400 bp) *chuA* (288 bp) *yjaA* (211 bp) TspE4.C2 (152 bp) along with *arpA* (301bp) might have originated from phylogroup E that displayed the novel phylotype property (NPP). The incidence of NPP observed in 100% and 88.2% of ABU and symptomatic UPECs respectively that belonged to "Unknown" phylogroup further substantiated the effect of MDR in the ABU and symptomatic UPECs circulated in Kolkata, an eastern region of India. Moreover, the emergence of ABU isolates with NPP was highly disquieting and their incidence might be accredited to the genome instability due to the selection of random mutations through the movement of mobile genetic elements and/or chromosomal rearrangements in response to unbridled drug usage. Therefore, this study for the first time embarked to introduce a new approach to ascertain the phylotype property of unassigned *E. coli* isolates **(Ghosh et al. 2022)**.

Moreover, in this study, a high degree of genetic heterogeneity in ERIC-PCR profiles with diverse clonal groups among both the ABU **(Fig. 4.11a)** and symptomatic **(Fig. 4.11b)** UPECs regardless of their ESBL phenotype and phylotype property, quite similar to an earlier study **(Durmaz et al. 2015)** conducted on symptomatic *E. coli* from Turkey. This adumbrated the clonal unrelatedness of the isolates along with a possibility of transmission and dissemination of resistance among the members of independent groups **(Ghosh et al. 2022)**.

Various studies from different parts of the world like China **(Liu et al. 2015)**, Spain **(Ojer-Usoz et al. 2017)** and France **(Gauthier et al. 2018)** demonstrated MLST as the most reliable method for unambiguous characterization of different bacterial species with respect to their epidemiology and evolutionary dynamics**.** In this study, ABU UPECs that belonged to ST940 and CC448 (designated as ST complex in *E. coli* Enterobase) were most predominant **(Table 4.6a)** which was discordant to the report from Germany **(Salvador et al. 2012)** that indicated the predominance of ST73 and CC73 respectively. Withal, among symptomatic UPECs **(Table 4.6b)** ST410 and ST940 were found to be equally predominant, although the most predominant CC was CC448, observations quite dissimilar from previous reports from France (**Gauthier et al. 2018)** and China **(Wang et al. 2020)** which demonstrated the predominance of CC10. UPECs that belonged to ST940 and CC448 were found to be associated mostly with phylogroup E, less with NPP. This observation was on contrary to earlier reports from Poland **(Izdebski et al. 2013)** and Lebanon **(Dagher et al. 2018)** that stated the incidence of ST940 among phylogroup B1 and D respectively. Moreover, this study showed the incidence of USTs, among both ABU and symptomatic UPECs with NPPs **(Table 4.6a-b),** although a much higher incidence was observed in the latter group **(Ghosh et al. 2022)**.

The study on evolutionary relationships among different UPECs and their STs showed that MDR and ESBL positive isolates had a greater ST diversity irrespective of their asymptomatic or symptomatic nature **(Fig. 4.14)**, which was partly in conformity with a study from China on symptomatic UPECs isolated from cats **(Liu et al. 2015).** In the present study, strikingly ST2112 that was previously found to be only associated with animals was identified in a UPEC isolated from an asymptomatic individual **(Fig. 4.14)**. Additionally, 86% of the known STs identified in this study irrespective of their asymptomatic or symptomatic origin were previously isolated from different animals, birds, livestock, poultry, and the environment besides humans **(Fig. 4.14)**. The aforementioned findings threw spotlight on the incidence of zoonotic transmission and presently zoonotic diseases are major public health threats. Likewise, the aforementioned incidence of asymptomatic transmission was inordinately worrisome which further indicated the exigency to include asymptomatic individuals in the testing programs **(Ghosh et al. 2022)**. Moreover, serious and deliberate measures must be taken to stop the spread of zoonotic diseases which might otherwise contribute to the growing economic burden,

especially in a low-income country like India. Furthermore, on analyzing the genetic relationships among all the UPECs selected for this study despite their asymptomatic or symptomatic nature using goeBURST, the most predominant CC was CC131 that contained 8 isolates with the equal prevalence of asymptomatic and symptomatic UPECs **(Fig. 4.15)**. This was antithetical to the report from China **(Wang et al. 2020)** that stated the dominance of CC10. Withal, earlier reports from Brazil **(da Cruz Campos et al. 2019)** and Iraq **(Al-Guranie et al. 2020)** demonstrated *E. coli* ST131 clone as a major global public health threat due to its high virulence and MDR profile. They were also often found to be associated with complicated UTIs. Hence, the incidence of CC131 especially, among the ABU UPECs in our study population was highly dismaying and it indicated the successful global spread of the aforementioned highly virulent MDR strain which might be imputed to the transmission and acquisition of resistance genes through lateral gene transfer facilitated by MGEs **(Ghosh et al. 2022)**.

A previous report from India **(Biswas et al. 2016)** stated MST as a tree of minimum weight spanning all the vertices of a weighted, undirected and connected graph, where the weight of the tree corresponded to the sum of weights of its edges. Moreover, an earlier report from Germany **(Wirth et al. 2006)** described MST as a graphical tool used to link allelic designation obtained from the MLST data analysis. Furthermore, MST was known for years as an impeccable graphical tool for displaying the quantitative relationships between STs and their CCs among isolates, measured by the number of shared alleles, determined by branch thickness and branch lengths (**Wirth et al. 2006; Wang et al. 2020).** So, in the current study MLST analysis showed ABUUPEC with known ST and both ABU and symptomatic UPECs with USTs, having NPPs was to be closely related to isolates that belonged to phylogroups Clade I, D and B2 respectively **(Fig. 4.16).** Therefore, the MLST and MST analysis avowed the fact that isolates with the NPPs might not have particularly originated only from phylogroup E as presumed but emanated as isolates with novel phylotype property, which might be a result of certain recombination events between isolates from different phylogroups. The incidence of high-frequency recombination events among UPECs irrespective of their asymptomatic or symptomatic nature was highly gruesome as it indicated the generation of novel strain types in the future which might be with enormous genetic diversity and capricious changes in pathogenicity that might put clinicians and microbiologist under new therapeutic challenge **(Ghosh et al. 2022)**.

Nevertheless, this study together with the previous analysis (Chapter 3) provided a comprehensive view of the resistance profile, virulence repertoire, molecular characteristics, STs, genetic diversity and evolutionary relationships of the ABU-UPECs circulated in Kolkata, an eastern region of the resource-poor country like India, and also compared them to the symptomatic ones **(Ghosh et al. 2022)**.

4.7 Conclusion

The present study for the first time declared the high incidence of MGEs among the MDR and ESBL producing ABUUPECs that highlighted the strong association between indiscriminate use of antibiotics, dissemination, and the emergence of antimicrobial resistance through the acquisition of MGEs. Additionally, the emergence of NPP among ABU UPEC was highly appalling. This could be attributed to the genome plasticity caused due to acquisition of resistance genes that led to the selection of random mutations through the movement of MGEs or chromosomal rearrangements pertaining to rampant drug usage. Moreover, this study also led to the prolegomenon of a new aspect of exploring the phylotype properties in MDR UPECs that could not be assigned to any of the eight known phylogroups. This might provide a better perception of their chromosomal candor in view of different environmental conditions depending on various geographical locations. Additionally, ERIC-PCR typing, MLST, MEGA, and MST analysis betokened a high degree of genetic heterogeneity among the ABU UPECs which further gave an insight into their epidemiology and evolutionary origin. Therefore, the aforementioned analysis ratified the detrimental consequences of MDR among these pathogenic microbes that threw spotlight on the exigency for the implementation of antimicrobial stewardship. This also connoted the need to intervene in the alternative therapeutic strategies. However, this study also displayed the fact that ABU, although generally not considered as a clinical condition, their increased recognition, proper understanding, and characterization together with appropriate therapeutic measures when necessary is the need of the era which otherwise might lead to serious complications in the vulnerable population.

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CHAPTER 5.

β-lactamase producing drug-resistant asymptomatic & symptomatic uropathogenic *E. coli - fimH polymorphisms* & type 1 fimbrial phase variation *in vitro;* Regulatory interplay of cellular factors in the phase variation post adherence to human uroepithelial cells

5.1 Background study

Uropathogenic *Escherichia coli* (UPECs) are prodigiously known to be cognated with both asymptomatic bacteriuria (ABU) and symptomatic UTI **(Bien et al. 2012; Ghosh and Mukherjee 2019; Ghosh et al. 2022)** former known to be distinguished from the latter by the absence of clinical signs and symptoms evocative of UTI **(Bien et al. 2012)**.

Recently, various reports from different parts of the world like Belgium **(Biggel et al. 2019)**, India **(Ghosh and Mukherjee 2019)** and the United States of America **(Hooton et al. 2021)** stated a remarkable incidence of UPECs among individuals with ABU. Nonetheless, according to the currently restructured (2019) guideline from the Infectious Diseases Society of America **(Nicolle et al. 2019),** ABU, a discernable contributor to inapt antimicrobial use thereby facilitating antimicrobial resistance, should be screened for and treated only in pregnant females or in individuals expected to undergo invasive urologic procedures. However, assessment for other causes rather than antimicrobial treatment was recommended in older patients with functional and/or cognitive impairment **(Nicolle et al. 2019)**. Withal, a very recent report **(Tauseef et al. 2021)** from the United States of America, specified the determinants like chronic kidney disease, benign prostate hyperplasia, diabetes, hypertension, and female gender as potential risk factors for developing UTI and also suggested the absolute need for ABU screening and initiation of antibiotic regimen in individuals with the evidence of two or more of the aforesaid risk factors, which otherwise might lead to uncompromising complications in the vulnerable population. Moreover, Venkatesan et al. **(Venkatesan et al. 2017)** from India articulated the need for diagnosis and proper management of ABU, especially among diabetic patients which otherwise might turn out to be the potent contributor to their morbidity and/or mortality by causing pyelonephritis or other diabetic complications like nephropathy.

Notwithstanding, another earlier report from India **(Srivastava et al. 2016)** exhibited ABU UPECs with virulence profiles, similar to symptomatic ones. Moreover, recent reports from India **(Ghosh and Mukherjee 2019; Ghosh et al. 2022)** displayed very high incidences of multidrug resistance (MDR), pathogenicity, strain types with genetic diversity, and clonal heterogeneity among ABU UPECs, very similar to symptomatic ones. Furthermore, the capability of UPECs to cause symptomatic UTIs was often found to be linked with adhesive molecules, especially highly functional type1 fimbriae, irrefutably a vital determinant of pathogenicity **(Roos et al. 2006; Bien et al. 2012)**. Additionally, the previous studies from the United States of America **(Kariyawasam and Nolan 2009; Tchesnokova et al. 2011)** and United Kingdom **(Li et al. 2009)** showed that the expression of type 1 fimbriae among symptomatic *E. coli* can be determined by mannose sensitive haemagglutination (MSHA). These reports also demonstrated that FimH adhesin of type 1 fimbriae is necessary for this MSHA (phenotypic) property of type1 fimbriae operon. Nevertheless, another earlier study conducted

symptomatic UPECs from the United States of America **(Weissman et al. 2007)** stated that FimH variants are 99% identical at protein level but evolutionary analysis indicated that mutations occur in this adhesin very frequently and this adhesin consists of the lectin and pilin domains, the lectin domain being responsible for mannose specific adhesion of the bacteria to the host uroepithelial cells. Likewise, the earlier reports from France **(Hommais et al. 2003)** and the United States of America **(Sokurenko 2016)** indicated that Pathoadaptive mutations in mutations in FimH adhesin are sometimes known to increase the pathogenicity of *E. coli* and also known to offer significant advantage upon bacteria during bladder colonization. However, previous reports from the United States of America **(Hull et al. 1999)** and Denmark **(Roos et al. 2006)** declared absence of MSHA *in vitro* and limited uroepithelial cell adherence of prototype ABU strain *E. coli* 83972. Nonetheless, the potentiality of deliberate long-term bladder colonization (**Roos et al. 2006; Stork et al. 2018)** was reported previously from Denmark and Germany**.** To boot, several studies conducted worldwide threw the spotlight on the nonadherent nature of different ABU strains **(Roos et al. 2006; Mabbett et al. 2009; Bien et al. 2012)**. Thus, not only identification of ABU UPECs and perception of their molecular epidemiology but also understanding of their adhesive capacity, in comparison to symptomatic ones is of utter necessity as adherence to host epithelial cells is the pivotal step in the pathogenesis of UPECs **(Schwan, 2011)** and successively the initiation of infection.

Type 1 fimbriae were reported as the essential mediator of attachment of UPECs to uroepithelial cells in the urinary tract of humans, further facilitating the process of successful establishment of infection **(Schwan, 2011; Bien et al. 2012)**. This attachment was known to be expedited by the expression of type 1 fimbriae (phase ON) and ceased by the loss of expression (phase OFF) **(Schwan 2011; Schwan and Ding 2017)**. Moreover, the earlier reports the United States of America **(Schwan 2011; Schwan and Ding 2017)** stated the expression of FimA, the major structural subunit of the type 1 fimbriae encoded by the *fimA* gene as a intriguing contributor to the ON-OFF switching process that allows individual *E. coli* cells to alternate between piliated (Phase-ON) and non-type 1 piliated states (Phase-OFF). This phase switching was reported **(Schwan 2011; Schwan and Ding 2017)** to be due to the inversion of a 314-bp *fimS* invertible DNA element containing the promoter for the *fimA* gene. The piliated cells were known to bind to the urothelial mannosylated glycoproteins uroplakin Ia and IIIa (UPIIIa) via the adhesin subunit FimH, located at the fimbrial tip **(Bien et al. 2012),** subsequently their attachment to uroepithelial cells being inhibited by the exogenous application of the natural sugar D-mannose (**Li K et al. 2009; Scribano et al. 2020; Scaglione et al., 2021)**. The phase switching phenomenon, affirmed to be controlled by two site-specific recombinases, *fimB*, and *fimE*, that influence the positioning of the *fimS* were known to promote the inversion in both directions with a switching bias toward the Phase-ON orientation (*fimB*), and from the Phase-ON to Phase-OFF

orientation (*fimE)* respectively **(Schwan and Ding 2017)**. Earlier reports from Ireland (**Corcoran and Dorman 2009)** and the United States of America **(Schwan 2011)** demonstrated that the aforementioned recombinases that control the phase switch were in turn regulated by mainly three global regulatory factors like histone-like nucleoid structuring protein (H-NS), leucine responsive protein (LRP), and Integration host factor (IHF). H-NS was mainly known to repress transcription of the recombinases thereby favouring the Phase OFF orientation. LRP was known to aid the ON orientational bias, further noted to be maintained by IHF and H-NS would bind to favor alternate orientational bias when none of the other two regulators were present at adequate levels to maintain the phase ON state (**Corcoran and Dorman 2009; Schwan 2011)**. Moreover, the multitudinous *in vitro*, *in vivo*, or cell line-based studies conducted from different parts of the world like the United States of America **(Lim et al. 1998; Bryan et al. 2006; Greene et al. 2015; Schwan and Ding 2017)** and Denmark **(Struve and Krogfelt et al. 1999)** focused on the evaluation of phase states (*fim* switch) of clinical and prototype symptomatic *E. coli*, unlike a study from the United Kingdom **(Graham et al. 2001)** that reported phase state of *E. coli* isolated from women with bacteriuria during pregnancy. Furthermore, earlier studies **(Ghosh and Mukherjee 2019; Ghosh et al. 2022)** from our laboratory enunciated the significant incidences of MDR, extended-spectrum β-lactamase (ESBL) producers, certain pathogenicity island markers (PAIs) and virulence factor genes, pathoadaptive FimH mutations, mobile genetic elements (MGEs), novel phylotype property (NPP) among ABU UPECs. ABU UPECs in our study population were also found to be genetically diverse and clonally heterogenic **(Ghosh et al. 2022).** This demanded their further characterization as the ability of a bacterium to cause disease may vary with their adherence potential.

Nevertheless, to the best of our knowledge, till date, no studies have compared drug-resistant ABU (isolated from males and non-pregnant females) and symptomatic UPECs relating to the presence or absence of MSHA, incidence of type 1 fimbrial phase variation and synonymous and non-synonymous FimH mutations and *in vitro*. To boot, no studies have compared drug-resistant clinical ABU UPECs with the prototype ABU strain *E. coli* 83972 and symptomatic UPECs, based on the phase sate (*fim* switch) and expression levels of the aforesaid recombinases and regulatory factors. Hence, this is the first study of its kind that aimed to comprehend the drug-resistant UPECs isolated from urine samples of asymptomatic hospitalized patients in Kolkata, India relating to their MSHA phenotype, incidence of type 1 fimbrial phase variation and FimH mutations *in vitro* and further compared to the symptomatic UPECs. Moreover, the aforementioned ABU UPECs were also evaluated based on their adhesive properties, type 1 fimbrial phase variation, echelon of expression of type1 fimbrial genes, the recombinases and regulators controlling them post adherence to uroepithelial cells which were further compared with that of the prototype ABU strain and symptomatic UPECs. Furthermore, the adherence inhibition assay was also performed on isolates of both groups using different concentrations of D-

mannose. Furthermore, this study also put through ABU and symptomatic UPECs to more intricate analysis to establish a prospective association (if any) between their adhesive capacity and the ascertained pathoadaptive FimH mutations, phylotype property, ESBL phenotype, and acquisition of MGEs (Chapter 4) **(Ghosh et al. 2022)** in order to get an insight into their interdependence.

5.2 Objectives

- MSHA assay of ABU and symptomatic UPECs for the phenotypic confirmation of type 1 fimbrial expression *in vitro*.
- Exploration of *fim*-switch orientation of the ABU and symptomatic UPECs *in vitro* to understand the phase state of the isolates. `
- Investigation of pathoadaptive mutations among ABU and symptomatic UPECs *in vitro* by the *fimH* gene polymorphisms analysis.
- Understanding the adhesive capacity of ABU and symptomatic UPECs using T24 human uroepithelial cells.
- Exploration of *fim* switch orientation of adherent ABU and symptomatic UPECs to identify their phase states and further compare them to that observed in case of the *in vitro* study.
- Understanding the regulatory interplay of cellular factors in phase variation of the adherent UPECs by the evaluation of the echelon of expression of the ype1 fimbrial genes, the recombinases and regulators controlling them post adherence to uroepithelial cells.
- Understanding the extent of inhibition of ABU and symptomatic UPECs to human uroepithelial cells by the adherence inhibition assay using different concentration of D-mannose.

5.3 Materials

(a) Equipments for Microbiological part of experiments:

- Laminar Air Flow [B.D Instrumentation]
- Shaker – Incubator [ICT]
- Autoclave [PrimeSurgicals]
- Spectrophotometer [Bio-Rad, India]
- Hot air oven [Digisystem Laboratory Instruments Inc.]
- Cooling centrifuge [Remi]
- Refrigerator [Godrej]
- Dry Bath [Remi]
- Thermal cycler [ABI Instruments Private Limited, Model-Veriti Thermal Cycler]

- Horizontal gel electrophoresis apparatus [Genei]
- Power pack [Genei]
- Gel documentation system [BIO-RAD]
- Inoculation loop
- Glass spreaders
- Spirit lamp
- 90mm glass petri dish [Borosil]
- Glass culture tubes [TOUFF, Borosil]
- Test tube racks [Tarsons]
- Micropipettes (0.5-10µl, 2-20µl, 20-200µl, 200-1000µl) [Corning, P'fact, Microlit, Biohit]
- Micro tips (0.5-10µl, 2-20µl, 20-200µl, 200-1000µl) [HiMedia]
- Eppendorf Tubes (1.5 mL, 2 mL) [Tarsons]
- Cotton [Bengal Surgicals Limited] [Lakshmi Healthcare Products (P) Ltd]
- Surgical gloves [PriCARE, HiMedia]
- Wash bottles

(b) Equipments for cell culture part of experiments:

- Laminar Air Flow Hood
- CO2 Incubator [Thermo Fisher Scientific]
- Cooling centrifuge [Remi]
- Room temperature bench top centrifuge [Remi]
- Refrigerator (Godrej)
- Freezer (-20°C) []
- Freezer (-80°C) [Remi]
- Aspiration pump [Thermo Fisher Scientific]
- Pipette controller [Tarsons]
- Cryo storage container with boxes [HiMedia]
- Haemocytometer [Sigma-Aldrich]
- Water Bath [Borosil]
- Light microscope
- Inverted microscope [Thermo Fisher Scientific]
- pH meter [Hitech Lab India]
- Spirit lamps
- 10 mL syringes [Dispovan]

- PVDF syringe-driven filters (0.22µ) [HiMedia]
- T25 Cell culture flasks [Tarsons]
- Cell culture bottles [Borosil]
- 96 and 24 well tissue culture plates [HiMedia]
- Sterile autoclavable 15 mL and 50 mL centrifuge tubes [HiMedia]
- Centrifuge tube holders [Tarsons]
- Micropipettes (0.5-10µl, 2-20µl, 20-200µl, 100-1000µl) [Corning, Biohit]
- Barrier tips (0.5-10µl, 2-20µl, 20-200µl, 200-1000µl) [HiMedia]
- Serological pipettes (1 mL, 2 mL, 5 mL, 10 mL, 25 mL, 50 mL) [Tarsons]
- Eppendorf Tubes (1.5 mL, 2 mL) [Tarsons]
- Cotton [Bengal Surgicals Limited] [Lakshmi Healthcare Products (Pvt.) Ltd]
- Tissue paper [Hospital store]
- Nitrile gloves [HiMedia]
- Wash bottles

(c) Reagents:

- Luria Bertani (LB) media [SRL Chemicals India]
- Agar Agar [Merck]
- McCoy's 5A medium [HiMedia]
- Fetal bovine serum (FBS) [HiMedia]
- Gentamicin powder [HiMedia]
- Trypsin [HiMedia]
- Hank's balanced salt solution [HiMedia]
- Dimethyl sulfoxide (DMSO) cell culture [HiMedia]
- 1X Phosphate buffer saline (PBS) [HiMedia]
- 0.4% Trypan blue solution [HiMedia]
- D-mannose [HiMedia]
- MTT dye [HiMedia]
- Dimethyl formamide (DMF) [HiMedia]
- Dulbecco's Phosphate Buffered Saline (DPBS) [HiMedia]
- TRIzol [Thermo Fisher Scientific]
- Barium chloride [Merck]
- Sulphuric acid [Hospital Store]
- 70% Ethanol [Bengal Chemical]

- Isopropanol [Hospital Store]
- Phenol [Hospital Store]
- Chloroform [Hospital Store]
- Triton X-100 [SRL chemicals]
- 95% Ethanol [HiMedia]
- Diethylpyrocarbonate (DEPC) water [HiMedia]
- Molecular biology grade nuclease free water [HiMedia]
- Single Distilled water (SDW) [Hospital Store]
- Double distilled water (DDW) [Laboratory distillation plant]
- Primers [GCC Biotech(I) Pvt.Ltd]
- dNTP [Invitrogen]
- Taq DNA Polymerase and buffer [Invitrogen]
- 50mM MgCl2 [Invitrogen]
- cDNA reverse transcription kit [Takara]
- DNA ladders [HiMedia]
- 6X Gel loading buffer [HiMedia]
- Hydrogen chloride [Hospital Store]
- Sodium chloride [Hospital Store]
- Agarose [HiMedia]
- Ethidium bromide [SRL Chemicals India]

5.3.1 Preparation of reagents and compositions of solutions used

- **LB broth:** 10gms of LB broth powder was dissolved in 500mL of SDW (Conc.- 20gms/lit). Then it was thoroughly mixed, dispensed into culture tubes and autoclaved at 15 psi pressure at 121°C for 15 minutes (file:///C:/Users/Arunita%20Ghosh/Downloads/M575.pdf).

- **LB agar:** 10gms of LB broth powder was dissolved in 500mL of SDW (Conc.- 20gms/lit). Then to the aforesaid mixture 7.5gm of agar agar (Conc. 1.5%) was added, thoroughly mixed, and autoclaved at 15 psi pressure at 121°C for 15 minutes. After autoclaving, the sterile medium was distributed into different 90mm petriplates, cooled and solidified for future use. (https://asm.org/getattachment/5d82aa34-b514-4d85-8af3-aeabe6402874/LB-Luria-Agar-protocol-3031.pdf)

- **1X TAE (Tris-Acetate EDTA) buffer [500mL]:** This was prepared using 49 parts of DDW water with 1 part of 50X TAE (Tris-Acetate EDTA) buffer as described in the section 4.3.1. The pH of the final solution was checked to be at 8.5 (http://2009.igem.org/TAE_Buffer).

- **MTT stock solution (5mg/mL):** MTT stock solution (5 mg/ml) was prepared by dissolving 50 mg of MTT powder in 10 mL of (DPBS) at pH 7.4. This solution was filtered and sterilized through a 0.2-µm filter into a sterile and light-protected container. MTT solution was stored at –20°C until analysis or at 4°C for immediate use and was kept away from the light as it is light-sensitive **(Kamiloglu et al. 2020).**

5.4 Experimental methods

5.4.1 Bacterial culture

The 40 non-duplicate UPECs (Asymptomatic= 20; Symptomatic=20) obtained from our earlier part of the study conducted on 200 hospitalized patients (Chapter 2) (**Ghosh and Mukherjee 2019)** were considered in the present analysis. The widely used prototype ABU strain *E. coli* 83972 was procured from BEI Resources, USA. This study protocol was accepted by the institutional ethical committee.

5.4.2 Mannose sensitive haemagglutination assay (MSHA)

All UPECs (Asymptomatic= 20; Symptomatic=20) and the prototype ABU strain were grown in pilus-inducing conditions. The log-phase cultures of each of the aforementioned UPECs were considered for this analysis. Type 1 pilus expression was assessed by MSHA of chicken and guinea pig erythrocytes (**Kariyawasam and Nolan, 2009).** MSHA was performed in the absence and presence of 2% w /v of D-Mannose, as described by **Tabasi et al. (Tabasi et al., 2015)**, using fresh chicken and guinea pig erythrocytes**.** *E. coli* (ATCC 25922) was used as a positive control **(Desai et al. 2013).** Wells with only the suspension of erythrocytes with or without D-mannose served as negative control **(Tabasi et al. 2015).** Agglutination of erythrocytes and the inhibition of agglutination in the presence of D-mannose confirmed the presence of type 1 fimbriae **(Li et al. 2009).**

5.4.3 Genomic DNA extraction

The bacterial genomic DNA was isolated from 40 non-duplicate UPECs irrespective of their asymptomatic and symptomatic nature using the protocol as described by Wright et al. **(Wright et al. 2017)**. The extracted genomic DNA was quantified and stored at − 20 °C up till further use.

5.4.4 Assay for *fim* switch orientation *in vitro*

The *fim* switch is phase variable (PHASE OFF and PHASE ON) which is mediated by the inversion of a 314-bp invertible chromosomal element *fimS* that contains a promoter for *fimA* **(Schwan**

2011). So, all 40 UPECs irrespective of their asymptomatic or symptomatic nature were examined for their *fim* switch orientation (Phase states) after growth at static and shaking conditions and at different temperature conditions (28°C, 37°C and 42°C). The nucleotide sequence for *E. coli* type 1 fimbrial genes of various reference strains [K12-MG1655 (GenBank accession no.: U00096.3); UT189 (GenBank accession no.: CP000243.1); ATCC-25922 (GenBank accession no.: CP009072.1); CFT073 (GenBank accession no.: AE014075.1)] was obtained from NCBI database and *fimS* region was identified. Then the orientation of the -10 and -35 region for proper transcription of the structural gene *fimA* (PHASE ON) and also that when the transcription is silenced (PHASE OFF) was identified using bacterial promoter hunt (http://www.softberry.com/berry.phtml?topic =bprom&group= programs& subgroup=gfindb). The phase ON and OFF sequence including their respective -10 and -35 regions was found to exactly match to that reported by Abraham et al. **(Abraham et al. 1985)** and had been illustrated below. Three separate primer pairs (Primers and PCR conditions were depicted in **Table 5.1**) were designed to amplify the Phase ON and Phase OFF *fim* switch respectively. All the aforementioned PCR assays were performed in 20 µl reaction volume that contained 10 ng of genomic DNA, 0.5 µM of each primer (GCC Biotech, India), 150 µM dNTPs (Invitrogen), 1.5 mM MgCl2 (Invitrogen), 1 U of the high fidelity Taq DNA polymerase (Invitrogen "Platinum™ *Taq* DNA Polymerase High Fidelity") and 2.0 µl 10X PCR buffer (Invitrogen). Amplicons generated were separated by 1.5% agarose gel electrophoresis and visualized using Ethidium bromide by Gel documentation system (BIO-RAD, USA) as described in section 4.4.5. The amplified products (598bp) that consisted of a part of the *fimE* gene, the entire *fimS* region, and a part of the *fimA* gene were subjected to SnaBI (New England Biolabs) digestion, known to cut amplicons asymmetrically at one location to reveal the orientation of the promoter element. The cut site for each of the phase ON and phase OFF sequences were determined using NEB cutter V2.0 (https://www.neb.com/products/r0130-snabi#Product%20Information). The "ON" orientation was indicated by fragments of 398 and 200 bp, and the "OFF" orientation was indicated by fragments of 444 and 154 bp respectively. The digested products were electrophoresed on 2% agarose gel and visualized under a UV transilluminator. The aforementioned sequences with the primer pairs and the digested fragments had been illustrated below. The nature of the phase state of the asymptomatic and symptomatic isolates was also confirmed using, two separate primer pairs **(Table 5.1)** designed to amplify the Phase ON and Phase OFF *fim* switch (433 bp) respectively. Individual isolates with Phase ON and Phase OFF orientation were also sequenced using the aforementioned primer pairs for further corroboration of their orientation.

The target *fim* switch region with their respective primer sequences are written as under:

***fimS* [*fim switch* (OFF) orientation as found from the NCBI database]**. Promoter regions found in OFF orientation were underlined below.

Escherichia coli str. K-12 substr. MG1655, complete genome

GenBank: U00096.3

GenBank Graphics

>U00096.3:4542037-4543663 *Escherichia coli* str. K-12 substr. MG1655, complete genome

GTGAGTAAACGTCGTTATCTTACCGGTAAAGAAGTTCAGGCCATGATGCAGGCGGTTTGTTACGGGGCAACGGGAGCCAGAGATTATTGT
CTTATTCTGTTGGCATATCGGCATGGGATGCGTATTAGTGAACTGCTTGATCTGCATTATCAGGACCTTGACCTTAATGAAGGTAGAATA
AATATTCGCCGACTGAAGAACGGATTTTCTACCGTTCACCCGTTACGTTTTGATGAGCGTGAAGCCGTGGAACGCTGGACCCAGGAACGT
GCTAACTGGAAAGGCGCTGACCGGACTGACGCTATATTTATTTCTCGCCGCGGGAGTCGGCTTTCTCGCCAGCAGGCCTATCGCATTATT
CGCGATGCCGGTATTGAAGCTGGAACCGTAACGCAGACTCATCCTCATATGTTAAGGCATGCTTGCGGTTATGAATTGGCGGAGCGTGGT
GCAGATACTCGTTTAATTCAGGATTATCTCGGGCATCGAAATATTCGCCATACTGTGCGTTATACCGCCAGTAATGCTGCTCGTTTTG
CCGGATTATGGGAAAGAAATAATCTCATAAACGAAAAATTAAAAAGAGAAGAGGTTTGATTTAACTTATTGATAATAAAGTTAAAAAA
ACAAATAAATACAAGACAATTGGGGGCCAAACTGTCCATATCATAAATAAGTTACGTATTTTTTCTCAAGCATAAAAATATTAAAAAACG
ACAAAAAGCATCTAACTGTTTGATATGTAAATTATTTCTATTGTAAATTAATTTCACATCACCTCCGCTATATGTAAAGCTAACGTTT
CTGTGGCTCGACGCATCTTCCTCATTCTTCTCTCCAAAAACCACCTCATGCAATATAAACATCTATAAATAAAGATAACAATAGAATATT
AAGCCAACAAATAAACTGAAAAAGTTTGTCCGCGATGCTTTCCTCTATGAGTCAAAATGGCCCCAAATGTTTCATCTTTTGGGGGAAAA
CTGTGCAGTGTTGGCAGTCAAACTCGTTTACAAAACAAAGTGTACAGAACGACTGCCCATGTCGATTTAGAAATAGTTTTTTGAAAGGAA
AGCAGCATGAAAATTAAAACTCTGGCAATCGTTGTTCTGTCGGCTCTGTCCCTCAGTTCTACAGCGGCTCTGGCCGCTGCCACGACGGTT
AATGGTGGGACCGTTCACTTTAAAGGGGAAGTTGTTAACGCCGCTTGCGCAGTTGATGCAGGCTCTGTTGATCAAACCGTTCAGTTAGGA
CAGGTTCGTACCGCATCGCTGGCACAGGAAGGAGCAACCAGTTCTGCTGTCGGTTTTAACATTCAGCTGAATGATTGCGATACCAATGTT
GCATCTAAAGCCGCTGTTGCCTTTTTAGGTACGGCGATTGATGCGGGTCATACCAACGTTCTGGCTCTGCAGAGTTCAGCTGCGGGTAGC
GCAACAAACGTTGGTGTGCAGATCCTGGACAGAACGGGTGCTGCGCTGACGCTGGATGGTGCGACATTTAGTTCAGAAACAACCCTGAAT
AACGGAACCAATACCATTCCGTTCCAGGCGCGTTATTTTGCAACCGGGGCCGCAACCCCGGGTGCTGCTAATGCGGATGCGACCTTCAAG
GTTCAGTATCAATAA

https://www.ncbi.nlm.nih.gov/nuccore/U00096.3?report=fasta&from=4542037&to=4543663

***fimS* [*fim* switch (ON) orientation]** and the promoter regions marked (underlined) were found to be in appropriate orientation for the transcription of the main structural gene *fimA* as reported by **Abraham JM et al. 1985].**

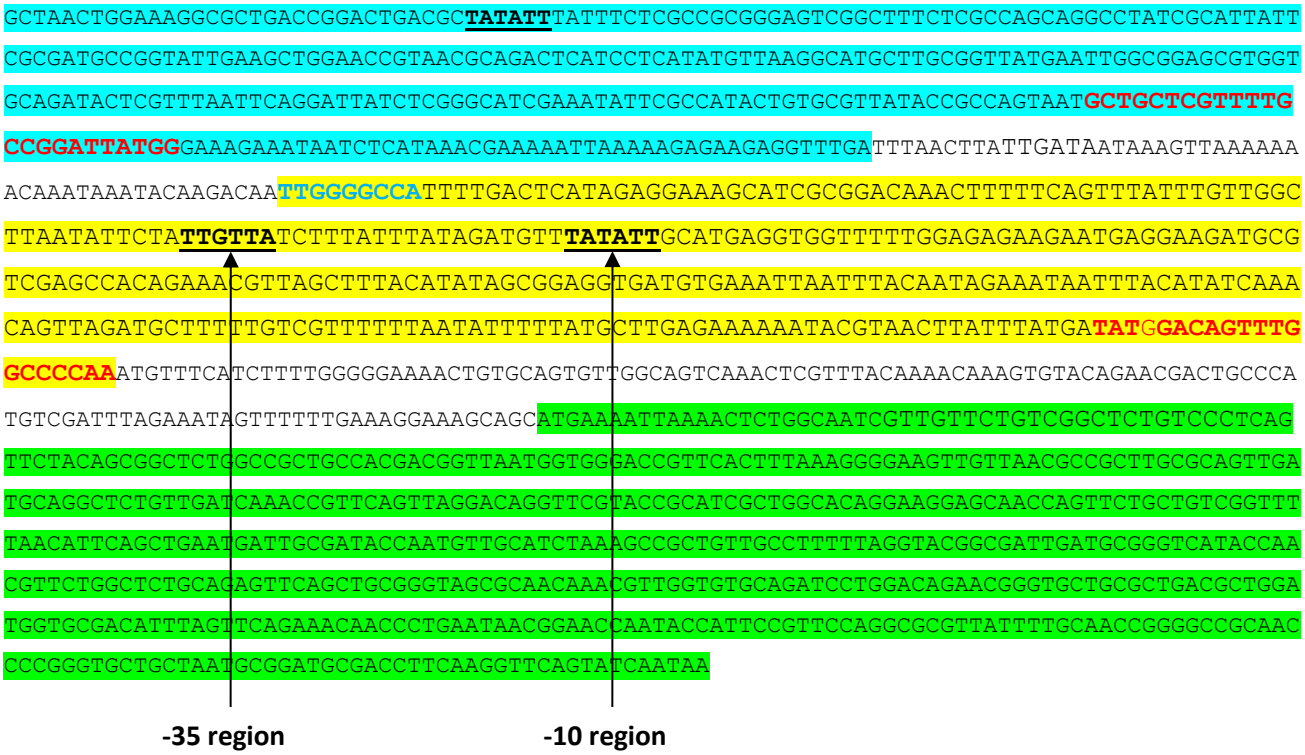
Escherichia coli str. K-12 substr. MG1655, complete genome

GenBank: U00096.3

GenBank Graphics

>U00096.3:4542037-4543663 Escherichia coli str. K-12 substr. MG1655, complete genome

GTGAGTAAACGTCGTTATCTTACCGGTAAAGAAGTTCAGGCCATGATGCAGGCGGTTTGTTACGGGGCAACGGGAGCCAGAGATTATTGT
CTTATTCTGTTGGCATATCGGCATGGGATGCGTATTAGTGAACTGCTTGATCTGCATTATCAGGACCTTGACCTTAATGAAGGTAGAATA
AATATTCGCCGACTGAAGAACGGATTTTCTACCGTTCACCCGTTACGTTTTGATGAGCGTGAAGCCGTGGAACGCTGGACCCAGGAACGT



Note: Sky-*fimE* sequence; yellow- *fimS* [(314bp) invertible DNA element in off/on orientation)]; -10 and -35 region promoter region were underlined in black; green - *fimA* region. Primer sequences were marked in red.

The target *fim* switch region with their respective primer sequences and fragments after digestion with SnaBI of a representative strain *E. coli* K12 are written as under:

***fimS* [*fim switch* (OFF) orientation as found from the NCBI database]**

***Escherichia coli* str. K-12 substr. MG1655, complete genome**

GenBank: U00096.3

GenBank Graphics

>U00096.3:4542037-4543663 *Escherichia coli* str. K-12 substr. MG1655, complete genome

GCTGCTCGTTTTGCCGGATTATGGGAAAGAAATAATCTCATAAACGAAAAATTAAAAAGAGAAGAGGTTTGATTTAACTTATTGATAAT
AAAGTTAAAAAAACAAATAAATACAAGACAATTGGGGCCAAACTGTCCATATCATAAATAAGTTACGTATTTTTTCTCAAGCATAAAA
ATATTAAAAAACGACAAAAAGCATCTAACTGTTTGATATGTAAATTATTTCTATTGTAAATTAATTTCACATCACCTCCGCTATATGT
AAAGCTAACGTTTCTGTGGCTCGACGCATCTTCCTCATTCTTCTCTCCAAAAACCACCTCATGCAATATAAACATCTATAAATAAAGATA
ACAATAGAATATTAAGCCAACAAATAAACTGAAAAAGTTTGTCCGCGATGCTTTCCTCTATGAGTCAAAATGGCCCCAAATGTTTCATC
TTTTGGGGGAAAACTGTGCAGTGTTGGCAGTCAAACTCGTTTACAAAACAAAGTGTACAGAACGACTGCCCATGTCGATTTAGAAATAGT
TTTTTGAAAGGAAAGCAGCATGAAAATTAAAACTCTGGCAATCGTTGTTCTGTCGGCTCTGTCCC

1st Fragment: 155bp

GCTGCTCGTTTTGCCGGATTATGGGAAAGAAATAATCTCATAAACGAAAAATTAAAAAGAGAAGAGGTTTGATTTAACTTATTGATAAT
AAAGTTAAAAAAACAAATAAATACAAGACAATTGGGGCCAAACTGTCCATATCATAAATAAGTTAC

2nd Fragment: 444bp

GTATTTTTTCTCAAGCATAAAAATATTAAAAAACGACAAAAAGCATCTAACTGTTTGATATGTAAATTATTTCTATTGTAAATTAATT
TCACATCACCTCCGCTATATGTAAAGCTAACGTTTCTGTGGCTCGACGCATCTTCCTCATTCTTCTCTCCAAAAACCACCTCATGCAATA
TAAACATCTATAAATAAAGATAACAATAGAATATTAAGCCAACAAATAAACTGAAAAAGTTTGTCCGCGATGCTTTCCTCTATGAGTCAA
AATGGCCCCAAATGTTTCATCTTTTGGGGGAAAACTGTGCAGTGTTGGCAGTCAAACTCGTTTACAAAACAAAGTGTACAGAACGACTG
CCCATGTCGATTTAGAAATAGTTTTTTGAAAGGAAAGCAGCATGAAAATTAAAACTCTGGCAATCGTTGTTCTGTCGGCTCTGTCCC

Note: Sky- part of *fimE* sequence; yellow part- *fimS* [(314bp) invertible DNA element in off/on orientation)]; -10 and -35 region promoter region were underlined in black; green – part of *fimA* region. Primer sequences were marked in red. SnaBI sequence and cut site had been marked in pink and pointed with black arrow. In case of *E. coli* K12 there is an extra adenine at the 102 of the above sequence, not found in case of *E. coli* UTI89, *E. coli* CFT073 *and E. coli* ATCC 25922, so except for *E. coli* K12 others have first fragment as 154bp (shown below).

***fimS* [*fim* switch (ON) orientation** and the promoter regions marked (underlined) were found to be in appropriate orientation for the transcription of the main structural gene *fimA* as reported by **Abraham JM et al. 1985].**

***Escherichia coli* str. K-12 substr. MG1655, complete genome**

GenBank: U00096.3

GenBank Graphics

>U00096.3:4542037-4543663 Escherichia coli str. K-12 substr. MG1655, complete genome

GCTGCTCGTTTTGCCGGATTATGGGAAAGAAATAATCTCATAAACGAAAAATTAAAAAGAGAAGAGGTTTGATTTAACTTATTGATAAT
AAAGTTAAAAAAACAAATAAATACAAGACAATTGGGGCCATTTTGACTCATAGAGGAAAGCATCGCGGACAAACTTTTTCAGTT
TATTTGTTGGCTTAATATTCTATTGTTATCTTTATTTATAGATGTTTATATTGCATGAGGTGGTTTTTGGAGAGAAGAATG
AGGAAGATGCGTCGAGCCACAGAAACGTTAGCTTTACATATAGCGGAGGTGATGTGAAATTAATTTACAATAGAAATAATT
TACATATCAAACAGTTAGATGCTTTTTGTCGTTTTTTAATATTTTTATGCTTGAGAAAAAATACGTACTTATTTATGATA
TGGACAGTTTGGCCCCAAATGTTTCATCTTTTGGGGGAAAACTGTGCAGTGTTGGCAGTCAAACTCGTTTACAAAACAAAGTGTACAG
AACGACTGCCCATGTCGATTTAGAAATAGTTTTTTGAAAGGAAAGCAGCATGAAAATTAAAACTCTGGCAATCGTTGTTCTGTCGGCT
CTGTCCC

1st Fragment: 399bp

GCTGCTCGTTTTGCCGGATTATGGGAAAGAAATAATCTCATAAACGAAAAATTAAAAAGAGAAGAGGTTTGATTTAACTTATTGATAAT
AAAGTTAAAAAAACAAATAAATACAAGACAATTGGGGCCATTTTGACTCATAGAGGAAAGCATCGCGGACAAACTTTTTCAGTT
TATTTGTTGGCTTAATATTCTATTGTTATCTTTATTTATAGATGTTTATATTGCATGAGGTGGTTTTTGGAGAGAAGAATG
AGGAAGATGCGTCGAGCCACAGAAACGTTAGCTTTACATATAGCGGAGGTGATGTGAAATTAATTTACAATAGAAATAATT
TACATATCAAACAGTTAGATGCTTTTTGTCGTTTTTTAATATTTTTATGCTTGAGAAAAAATAC

2nd Fragment: 200bp

GTAACTTATTTATGATATGGACAGTTTGGCCCCAAATGTTTCATCTTTTGGGGGAAAACTGTGCAGTGTTGGCAGTCAAACTCGTT
TACAAAACAAAGTGTACAGAACGACTGCCCATGTCGATTTAGAAATAGTTTTTTGAAAGGAAAGCAGCATGAAAATTAAAACTCTGGCAA
TCGTTGTTCTGTCGGCTCTGTCCC

The target *fim* switch region with their respective primer sequences and fragments after digestion with SnaBI of a representative strain *E. coli* UT189 are written as under:

fimS [*fim switch* (OFF) orientation as found from the NCBI database]

Escherichia coli UTI89, complete genome

GenBank: CP000243.1

GenBank Graphics

>CP000243.1:4907305-4907902 Escherichia coli UTI89, complete genome

GCTGCTCGTTTTGCCGGATTATGGGAAAGAAATAATCTCATAAACGAAAAATTAAAAAGAGAAGAAGCTTGATTTAACTAA
TTGATAATAAAGTTAAAAAACAAATAAATACAAGACAATTGGGGCCAAACTGTCTATATCATAAATAAGTTACGTATTTTT
TCTCAAGCATAAAAATATTAAAAAACGACAAAAAGCATCTAACTGTTTGATATATAAATTATTTCTCTTGTAAATTAATTT
CACATCACCTCCGCTATATGTAAAGCTAACGTTTCTGTAGCTCGACGCAACTTCCTCATTCTTCTCTCCAAAAACCACCTC
ATGCAATATAAAAAACTGCAAATAAAGATAACTATAGAACATTAAGCCAACAAATAAACTGAAAAAGTTTGTGCGCGATGC
TTTCCTCTATGAGTCAAAATGGCCCCAAATGTTTCATCTTTTGGGGGAAAACTGTGCAGTGTTGGCAGTCAAACTCGTTGA
CAAAACAAAGTGTACAGAACGACTGCCCATGTCGATTTAGAAATAGTTTTTTTAAAGGAAAGCAGCATGAAAATTAAAACT
CTGGCAATTGTTGTTCTGTCGGCTCTGTCCC

1st Fragment: 154bp

GCTGCTCGTTTTGCCGGATTATGGGAAAGAAATAATCTCATAAACGAAAAATTAAAAAGAGAAGAAGCTTGATTTAACTAA
TTGATAATAAAGTTAAAAAACAAATAAATACAAGACAATTGGGGCCAAACTGTCTATATCATAAATAAGTTAC

2nd Fragment: 444bp

GTATTTTTTCTCAAGCATAAAAATATTAAAAAACGACAAAAAGCATCTAACTGTTTGATATATAAATTATTTCTCTTGTAA
ATTAATTTCACATCACCTCCGCTATATGTAAAGCTAACGTTTCTGTAGCTCGACGCAACTTCCTCATTCTTCTCTCCAAAA
ACCACCTCATGCAATATAAAAAACTGCAAATAAAGATAACTATAGAACATTAAGCCAACAAATAAACTGAAAAAGTTTGTG
CGCGATGCTTTCCTCTATGAGTCAAAATGGCCCCAAATGTTTCATCTTTTGGGGGAAAACTGTGCAGTGTTGGCAGTCAAA
CTCGTTGACAAAACAAAGTGTACAGAACGACTGCCCATGTCGATTTAGAAATAGTTTTTTTAAAGGAAAGCAGCATGAAAA
TTAAAACTCTGGCAATTGTTGTTCTGTCGGCTCTGT

fimS [*fim* switch (ON) orientation]

Escherichia coli UTI89, complete genome

GenBank: CP000243.1

GenBank Graphics

>CP000243.1:4907305-4907902 Escherichia coli UTI89, complete genome

GCTGCTCGTTTTGCCGGATTATGGGAAAGAAATAATCTCATAAACGAAAAATTAAAAAGAGAAGAAGCTTGATTTAACTAA
TTGATAATAAAGTTAAAAAACAAATAAATACAAGACAATTGGGGCCATTTTGACTCATAGAGGAAAGCATCGCGCACAAAC
TTTTTCAGTTTATTTGTTGGCTTAATGTTCTATAGTTATCTTTATTTGCAGTTTTTTATATTGCATGAGGTGGTTTTTGGA
GAGAAGAATGAGGAAGTTGCGTCGAGCTACAGAAACGTTAGCTTTACATATAGCGGAGGTGATGTGAAATTAATTTACAAG
AGAAATAATTTATATATCAAACAGTTAGATGCTTTTTGTCGTTTTTTAATATTTTTATGCTTGAGAAAAAATACGTAACTT
ATTTATGATATAGACAGTTTGGCCCCAAATGTTTCATCTTTTGGGGGAAAACTGTGCAGTGTTGGCAGTCAAACTCGTTGA

CAAAACAAAGTGTACAGAACGACTGCCCATGTCGATTTAGAAATAGTTTTTTTAAAGGAAAGCAGCATGAAAATTAAAACT
CTGGCAATTGTTGTTCTGTCGGCTCTGTCCC

1st Fragment: 398bp

GCTGCTCGTTTTGCCGGATTATGGGAAAGAAATAATCTCATAAACGAAAAATTAAAAAGAGAAGAAGCTTGATTTAACTAA
TTGATAATAAAGTTAAAAAACAAATAAATACAAGACAATTGGGGCCATTTTGACTCATAGAGGAAAGCATCGCGCACAAAC
TTTTTCAGTTTATTTGTTGGCTTAATGTTCTATAGTTATCTTTATTTGCAGTTTTTTATATTGCATGAGGTGGTTTTTGGA
GAGAAGAATGAGGAAGTTGCGTCGAGCTACAGAAACGTTAGCTTTACATATAGCGGAGGTGATGTGAAATTAATTTACAAG
AGAAATAATTTATATATCAAACAGTTAGATGCTTTTTGTCGTTTTTTAATATTTTTATGCTTGAGAAAAAATAC

2nd Fragment: 200bp

GTAACTTATTTATGATATAGACAGTTTGGCCCCAAATGTTTCATCTTTTGGGGGAAAACTGTGCAGTGTTGGCAGTCAAAC
TCGTTGACAAAACAAAGTGTACAGAACGACTGCCCATGTCGATTTAGAAATAGTTTTTTTAAAGGAAAGCAGCATGAAAAT
TAAAACTCTGGCAATTGTTGTTCTGTCGGCTCTGTCCC

Note: Note: Sky- part of *fimE* sequence; yellow part- *fimS* [(314bp) invertible DNA element in off/on orientation)]; -10 and -35 region promoter region were underlined in black; green – part of *fimA* region. Primer sequences were marked in red. SnaBI sequence and cut site had been marked in pink and pointed with black arrow. In case of *E. coli* K12 there is an extra adenine at the 102 of the above sequence, not found in case of *E. coli* UTI89, *E. coli* CFT073 *and E. coli* ATCC 25922 etc, so except for *E. coli* K12 others have first fragment as 398bp

Table 5.1: Primer sequences and PCR conditions used for the amplification of the *fimS* gene.

Sl no.	Target gene	Primer sequences (5'-3')	PCR conditions (Time)	No. of cycles	Amplicon size (bp)	References
1	*fimS*-SnaBI	F.P-GCTGCTCGTTTTGCCGGATTATGG R.P-GGGACAGAGCCGACAGAACAAC	95°C (30 sec) 57°C (30 sec) 72°C (1min)	35	598	This study
2	*fimS-ON*	F.P- GCTGCTCGTTTTGCCGGATTATGG R.P- TTGGGGCCAAACTGTCYATA	95°C (30 sec) 53°C (30 sec) 72°C (1min)	35	433	This study
3	*fimS-OFF*	F.P- GCTGCTCGTTTTGCCGGATTATGG R.P- TTGGGGCCATTTTGACTCAT	95°C (30 sec) 53°C (30 sec) 72°C (1min)	35	433	This study

5.4.5 *fimH* polymorphism study

fimH (903bp) gene of each of the 40 clinical UPECs regardless of their asymptomatic or symptomatic nature was amplified by PCR and sequenced using two sets of primers (seq1*fimH* and seq2*fimH*) **(Table 5.2) (Dreux et al. 2013).** The target *fimH* genes with their respective primer sequences were depicted below and marked in green and yellow respectively. Amino acid sequences were deduced using ExPASy tool (https://www.expasy.org/). Single Nucleotide Polymorphisms

(SNPs) of *fimH* and its amino acid variants were identified by a multiple sequence alignment program (https://www.genome.jp/tools-bin/clustalw) with respect to the FimH encoding gene (GenBank accession no.: U00096.3) and the protein (GenBank accession no:AAC77276.1) of *E. coli* K12 strain respectively.

The target *fimH* gene with their respective primer sequences are written as under:

Escherichia coli str. K-12 substr. MG1655, complete genome

GenBank: U00096.3

GenBank Graphics

>U00096.3:4548808-4549710 *Escherichia coli* str. K-12 substr. MG1655, complete genome

ATGAAACGAGTTATTACCCTGTTTGCTGTACTGCTGATGGGCTGGTCGGTAAATGCCTGGTCATTCGCCTGTAAAACCGCC
AATGGTACCGCTATCCCTATTGGCGGTGGCAGCGCCAATGTTTATGTAAACCTTGCGCCCGTCGTGAATGTGGGGCAAAAC
CTGGTCGTGGATCTTTCGACGCAAATCTTTTGCCATAACGATTATCCGGAAACCATTACAGACTATGTCACACTGCAACGA
GGCTCGGCTTATGGCGGCGTGTTATCTAATTTTTCCGGGACCGTAAAATATAGTGGCAGTAGCTATCCATTTCCTACCACC
AGCGAAACGCCGCGCGTTGTTTATAATTCGAGAACGGATAAGCCGTGGCCGGTGGCGCTTTATTTGACGCCTGTGAGCAGT
GCGGGCGGGGTGGCGATTAAAGCTGGCTCATTAATTGCCGTGCTTATTTTGCGACAGACCAACAACTATAACAGCGATGAT
TTCCAGTTTGTGTGGAATATTTACGCCAATAATGATGTGGTGGTGCCTACTGGCGGCTGCGATGTTTCTGCTCGTGATGTC
ACCGTTACTCTGCCGGACTACCCTGGTTCAGTGCCAATTCCTCTTACCGTTTATTGTGCGAAAAGCCAAAACCTGGGGTAT
TACCTCTCCGGCACAACCGCAGATGCGGGCAACTCGATTTTCACCAATACCGCGTCGTTTTCACCTGCACAGGGCGTCGGC
GTACAGTTGACGCGCAACGGTACGATTATTCCAGCGAATAACACGGTATCGTTAGGAGCAGTAGGGACTTCGGCGGTGAGT
CTGGGATTAACGGCAAATTATGCACGTACCGGAGGGCAGGTGACTGCAGGGAATGTGCAATCGATTATTGGCGTGACTTTT
GTTTATCAATAA

https://www.ncbi.nlm.nih.gov/nuccore/U00096.3?report=fasta&from=4548808&to=4549710

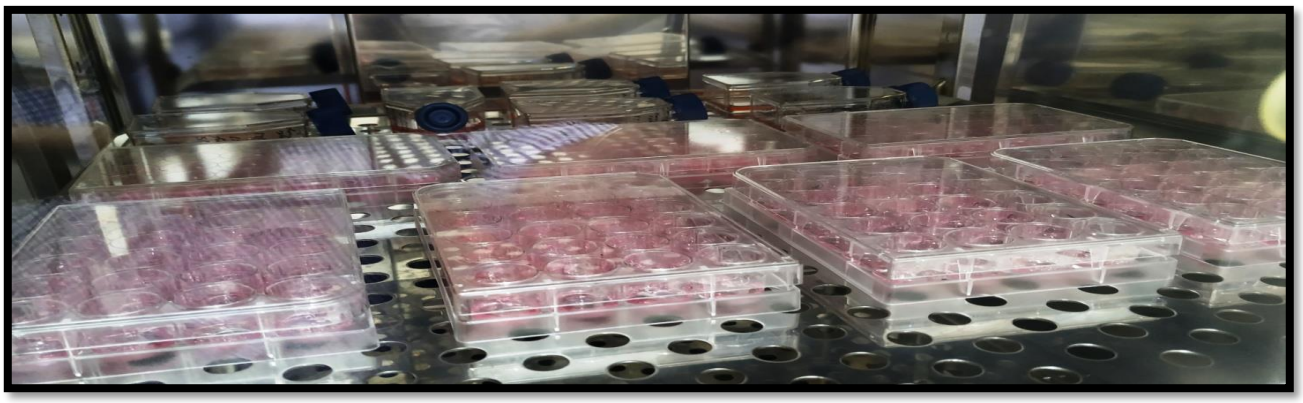
Table 5.2: Primer sequences and PCR conditions used for the sequencing of the *fimH* gene

Sl no.	Target gene	Primer sequences (5'-3')	PCR conditions (Time)	No. of cycles	Amplicon size (bp)	References
1	***Seq1fimH***	F.P- ATGAAACGAGTTATTACCCTGTTTG R.P- GCCAGTAGGCACCACCACATCATT	95°C (30 sec) 52°C (30 sec) 72°C (1min)	30	540	This study
2	***Seq1fimH***	F.P- TCGAGAACGGATAAGCCGTGG R.P- TTATTGATAAACAAAAGTCACGCCA	95°C (30 sec) 52°C (30 sec) 72°C (1min)	30	552	This study

5.4.6 Cell culture

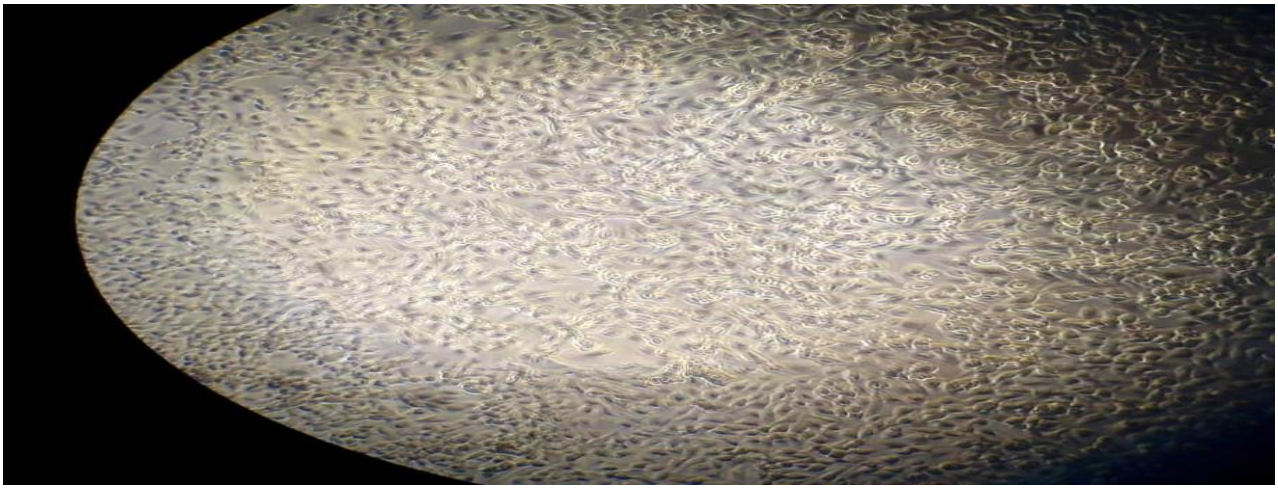
This study used T24 cells (ATCC®HTB4™) which is an uroepithelial cell line derived from transitional bladder carcinoma of a female patient in 1973. This is because the aforementioned cell line was reported to display similarity to the primary human bladder epithelial cells in earlier studies conducted worldwide **(Hilbert et al. 2008; De Llano et al. 2015)**. T24 cells were cultured using protocols as described by Geerlings et al. **(Geerlings et al. 2002)** and De Llano et al. **(De Llano et al.**

2015), but with minor modifications. T24 uroepithelial cells were grown and maintained in McCoy's 5A medium (HiMedia), supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) (HiMedia) and gentamicin (0.05 mg/mL) (HiMedia) in T25 cell culture flasks (Tarsons). T25 flasks with cells were maintained at 37 °C in an atmosphere of 5% CO2/95% air with constant humidity. Two to three days prior to the individual experiments, cells were removed from the cell culture flasks by Trypsin (0.05%; 10min) treatment and were subsequently seeded into 96 and/or 24-well tissue culture plates **(Fig. 5.1)**. They were grown for 24 to 72hrs to facilitate cell attachment and to obtain a confluent cell monolayer **(Fig. 5.2).**



(This study)

Fig. 5.1: The representative picture for seeding of T24 uroepithelial cells into 24-well tissue culture plates and maintaining them at 37 °C in an atmosphere of 5% CO2/95% air with constant humidity.



(This study)

Fig. 5.2: The representative picture of confluent T24 uroepithelial cells at 20X magnification.

5.4.7 Cell concentration and viability assay

The concentration and viability of T24 cell suspension were determined by 0.4% trypan blue staining using protocols as described by Lv et al. **(Lv et al. 2019).** Live (viable) cells that excluded the dye maintained a regular shiny appearance. However, dead cells that took up the stain appeared blue and swollen under the light microscope.

5.4.8 Adherence assay

Adherence of the asymptomatic (n=20) and symptomatic (n=20) UPECs to T24 uroepithelial cells were performed by the procedure as described in the earlier studies **(Geerlings et al. 2002; De Llano et al. 2015; Stork et al. 2018)**, but with minor modifications. A day or two before the adherence assay, 3X10^5 T24 uroepithelial cells were seeded into each well of the 24 well tissue culture plates and kept in a C02 incubator maintained at 37 °C in an atmosphere of 5% CO2/95% air with constant humidity, until they produced a confluent monolayer. A night before the experiment they were starved in a serum-free medium to synchronize all cells to the same cell cycle phase. On the day of the experiment, the planktonic medium was aspirated out and washed thrice with PBS. Each of the 40 clinical UPECs (asymptomatic and symptomatic) along with the prototype ABU strain *E. coli 83972*, *E. coli* ATCC 25922, and *E. coli* 536-21 at the log phase and the concentration of 1X10^8 CFU/mL was added to each well and incubated for two different time periods (1 and 3hrs) respectively. Assay for each isolate was performed in triplicates. After, each of the aforementioned time durations; the planktonic medium was aspirated out and the infected monolayers were washed thrice with PBS to remove unattached extracellular bacteria. T24 cells with adherent bacteria were lysed by the addition of 200 ml of 1% Triton X-100 in PBS for 10 min. For confirmation of measurement of adherence, without internalization gentamicin protection assay as described by Sheng et al. **(Sheng et al. 2011)** was performed. The aforementioned assay differentiated between adherent and internalized bacteria. Adherent bacterial viable counts were determined by serial dilution of samples followed by plating onto LB agar plates. The number of adherent bacteria was formulated as a percentage of the total number of bacteria added at the beginning of the incubation period (10^8 CFU/mL). *E. coli* ATCC 25922 and *E. coli strain* 536-21served as a positive and negative control respectively as described previously **(Stork et al. 2018).**

5.4.9 Adherence inhibition assay

In the current study, the adherence inhibition assay was performed using T24 uroepithelial cell line using procedures as previously described with a different cell line (**Scribano et al. 2020)**, but with modifications. Different concentrations (1.5 to 2%) of D-Mannose (HiMedia) were used as inhibitors to test the degree of inhibition in UPECs attachment to T24 uroepithelial cells in each case. The adherence assay protocol used was as previously described section 5.4.8. The effect of different dosages of D-mannose on viability and growth of human T24 uroepithelial cells was also assessed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) prepared in DPBS (**Scribano et al. 2020)**. The confluent T24 monolayers were treated with D-mannose at a final concentration of 1.5% and 2%. Confluent cell monolayers of T24 were infected with UPECs grown in the absence and presence of the different aforementioned concentrations of D-Mannose (1X10^8 CFU/mL) and

incubated at 37 °C in an atmosphere of 5% CO2/95% air with constant humidity for two different time periods (1 and 3hrs) respectively. Then cell monolayers were profusely washed with PBS to remove the unbound bacteria. T24 cells with adherent bacteria as described by Stork et al. **(Stork et al. 2018)** were lysed by the addition of 200 ml of 1% Triton X-100 in PBS for 10 min. Without, D-Mannose isolates served as the untreated control to each individual isolate **(Van der Bosch et al. 1980)**. Similar experiments were also performed with the prototype ABU strain *E. coli* 83972 and the control strain *E. coli* ATCC 25922. Viable bacterial counts were determined by serial dilution of samples followed by plating onto Luria Bertani (LB) agar plates. The number and percentage of adherent bacteria were calculated and determined as described above. Moreover, the percentage of reduction of adherent bacteria on exposure to D-mannose relative to without mannose as control was also calculated.

5.4.10 Preparation of DNA template post adherence

The preparation of DNA template was performed using the procedure mentioned below. A day or two before the adherence assay, 3X10^5 T24 uroepithelial cells were seeded into each well of 24 well tissue culture plates and kept in a C02 incubator maintained at 37 °C in an atmosphere of 5% CO2/95% air with constant humidity, until they formed a confluent monolayer. Adherent assay performed for this experiment was as described above. Each of the 20 clinical asymptomatic 20 symptomatic UPECs along with the prototype ABU strain *E. coli* 83972 and *E. coli* ATCC 25922 at the concentration of 1X10^8 CFU/mL was added to each well and incubated for two different time periods (1 and 3hrs) respectively. Assay for each isolate was performed in triplicates. After, each of the aforementioned time durations; the planktonic medium was aspirated out and the infected monolayers were washed thrice with PBS to remove unattached extracellular bacteria. T24 cells with adherent UPECs were lysed by the addition of 200 ml of 1% Triton X-100 in PBS for 10 min. For each *E. coli*, 6 wells of the 24 well tissue-culture plate were used to get enough pool of each adherent bacterium. DNA template was also prepared from adherent UPECs exposed to different concentrations of D-Mannose. DNA was purified for PCR amplification for all adherent samples by ethanol precipitation as described by Lim et al. **(Lim et al. 1998)** and was stored at − 20 °C until further use.

5.4.11 Assay of *fim* switch orientation of adherent UPECs

Quantification of the percentage of the adherent bacterial population in each of the aforementioned phase-state in the case of isolated ABU and symptomatic UPECs was performed using the protocol as described by Lim et al. **(Lim et al. 1998)**, but with certain modifications. The aforementioned analysis was also performed on adherent UPECs exposed to different concentrations of D-Mannose. The phase ON and OFF sequence including their respective -10 and -35 regions (illustrated in section 5.4.4) was found to exactly match to that reported by Abraham et al. **(Abraham**

et al. 1985). Three pairs of primer sets [listed in earlier section of this study (5.4.4) **Table 5.1**] were used in this study to amplify the *fimS* invertible region and confirm the presence of each phase state in the studied adherent bacterial population. PCR was performed using gene-specific primers (Primers with PCR conditions were listed in **Table 5.1**) and template DNA prepared above. The aforementioned PCR assays were performed in 20 µl reaction volume that contained 10 ng of genomic DNA, 0.5 µM of each primer (GCC Biotech, India), 150 µM dNTPs (Invitrogen), 1.5 mM MgCl2 (Invitrogen), 1 U of the high fidelity Taq DNA polymerase (Invitrogen "Platinum™ *Taq* DNA Polymerase High Fidelity") and 2.0 µl 10X PCR buffer (Invitrogen). Amplicons generated were separated by 1.5% agarose gel electrophoresis and visualized using Ethidium bromide by Gel documentation system (BIO-RAD, USA) as described in section 4.4.5The amplified products (598bp) that consisted of a part of the *fimE* gene, the entire *fimS* region, and a part of the *fimA* gene were subjected to SnaBI (New England Biolabs) digestion, known to cut amplicons asymmetrically at one location to reveal the orientation of the promoter element. The cut site for each of the phase ON and phase OFF sequences were determined using NEB cutter V2.0 (https://www.neb.com/products/r0130-snabi#Product%20Information). The "ON" orientation was indicated by fragments of 398 and 200 bp, and the "OFF" orientation was indicated by fragments of 444 and 154 bp respectively. The digested products were electrophoresed on 2% agarose gel and visualized under a UV transilluminator. The aforementioned sequences with the primer pairs and the digested fragments had been illustrated in the section 5.4.4. The nature of the phase state of the adherent samples was also confirmed using, two separate primer pairs **(Table 5.1)** designed to amplify the Phase ON and Phase OFF *fim* switch respectively. Individual isolates with Phase ON and Phase OFF orientation were also sequenced using the aforementioned primer pairs for further corroboration of their orientation.

5.4.12 Total RNA extraction and cDNA preparation post UPECs' adherence

The adherent assay executed for this experiment for two different durations (1 and 3hrs) was the same as narrated previously. The total RNA was extracted from all the 40 adherent non-duplicate UPECs along with the *E. coli* ATCC 25922 and prototype ABU strain *E. coli* 83972 using TRIzol (Invitrogen) method as described previously **(Chen et al. 2018)** with few incorporations. Briefly, after the completion of the required infection duration and the necessary protocols followed for removal of the unattached bacteria, TRIzol (Invitrogen) was added to each well of the 24 well tissue culture plates and kept for 20mins. For each of the UPECs, 6 wells of the 24 well tissue-culture plate were used to get enough pool of each adherent bacterium. This was followed by addition of 300 µl of chloroform per 1.5 mL of trizol with adherent bacteria and vortexing for 15 secs. Then the mixture was kept and room temperature for 5mins and centrifuged at 13000 rpm for 15mins. Then after transferring the aqueous phase to a fresh eppendorf tube, 750 µl of Isopropanol was added and kept at room temperature

for 10mins and again centrifuged at 13000 rpm for 10mins. Then the supernatant was removed and the RNA pellet was washed with75% ethanol prepared in DEPC water. The above mixture was further vortexed and centrifuged at 10500 rpm for 5 mins at 4°C. This was further followed by discarding the supernatant and air drying the pellet. The dried pellet was then resuspended in 30 µl of DEPC water and incubated at 60 °C for 10mins. Total RNA was also prepared from asymptomatic and symptomatic adherent UPECs exposed to different concentrations of D-mannose. The concentration and purity of the extracted total RNA was confirmed spectrophotometrically. The O.D at A260 and A280 was measured and the ratio between A260 /A280 ~2.0 was considered. cDNA was prepared from the extracted total RNA using the reverse transcription kit (Takara; India) following the manufacturer's instructions **(Chen et al. 2018; Monroy-Perez et al. 2020)**.

5.4.13 Quantitative real-time PCR (RT-qPCR) assay

Real-Time PCR assay was performed to determine quantitative expression (transcription level) of seven genes: *fimH, fimA, fimB, fimE, hns, himA, lrp* using gene-specific primers **(Table 5.3)** on the cDNA prepared from the total extracted RNA from the aforementioned adherent samples. The sequences of the seven aforementioned target genes and their respective primers were depicted below and marked in green. Primer specificity was ensured by the PCR product run on agarose gel and product melting, assessed at the end of the reaction to verify the specificity of the reaction **(Chen et al. 2018).** Each RT-qPCR test was subsequently performed in 20 µL of reaction mix that included 10µL of SYBR Green Master Mix (Thermo Fisher Scientific), 0.5uL (0.25 uM) each of forward and reverse primers, 2µL of the cDNA (100 ng) from the adherent isolates, and 7µL molecular grade H2O (RNase-free water). Amplification was executed with an initial denaturation at 95°C for 10 mins, followed by 40 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s and extension at 72°C for 30s. Transcript measurements were carried out in triplicates **(Dona et al. 2019).** Relative quantification (level of expression) of target genes in each of the tested time points (1 and 3hrs of infection) was calculated with the $2^{-\Delta\Delta Ct}$ method using 16srRNA gene as reference **(Bandyopadhyay et al. 2020)** and *E. coli* ATCC 25922 strain as a calibrator **(Tracz et al. 2005).** No template reactions were used as negative control **(Monroy-Perez et al. 2020).** The relative change (increase or decrease) in the level of expression of each of the aforementioned genes was also calculated from the individual expressions at 1 and 3hrs. Different column, bar graphs and scatter plots were prepared using GraphPad Prism version 9 (GraphPad Software, La Jolla California USA) to illustrate the expression levels of various genes expressed within the adherent UPECs.

The sequences of the seven aforementioned target genes and their respective primers are written as under:

fimH

Escherichia coli ATCC 25922, complete genome

GenBank: CP009072.1

GenBank Graphics

>CP009072.1:2590260-2591162 *Escherichia coli* ATCC 25922, complete genome

ATGAAACGAGTTATTACCCTGTTTGCTGTACTGCTGATGGGCTGGTCGGTAAATGCCTGGTCATTCGCCTGTAAAACCGCC
AATGGTACCGCTATTCCTATTGGCGGTGGCAGCGCTAATGTTTATGTAAACCTTGCGCCTGCCGTGAATGTGGGGCAAAAC
CTGGTCGTAGATCTTTCGACGCAAATCTTTTGCCATAACGATTATCCGGAAACCATTACAGACTATGTCACACTGCAACGA
GGCTCGGCTTATGGCGGCGTGTTATCTAATTTTTCCGGGACCGTAAAATATAGTGGCAGTAGCTATCCATTTCCGACTACC
AGCGAAACGCCGCGGGTTGTTTATAATTCGAGAACGGATAAGCCGTGGCCGGTGGCGCTTTATTTGACGCCTGTGAGCAGT
GCGGGTGGGGTGGCGATTAAAGCTGGCTCATTAATTGCCGTGCTTATTTTGCGACAGACCAACAACTATAACAGCGATGAT
TTCCAGTTTGTGTGGAATATTTACGCCAATAATGATGTGGTGGTGCCTACTGGCGGCTGCGATGTTTCTGCTCATGATGTC
ACCGTTACTCTGCCGGACTACCCTGGTTCAGTGCCAATTCCTCTTACCGTTTATTGTGCGAAAAGCCAAAACCTGGGGTAT
TACCTCTCCGGCACAACCGCAGATGCGGGCAACTCGATTTTCACCAATACCGCGTCGTTTTCACCAGCGCAGGGCGTCGGC
GTACAGTTGACGCGCAACGGTACGATTATTCCAGCGAATAACACGGTATCGTTAGGAGCAGTAGGGACTTCGGCGGTAAGT
CTGGGATTAACGGCAAATTACGCACGTACCGGAGGGCAGGTGACTGCAGGGAATGTGCAATCGATTATTGGCGTGACTTTT
GTTTATCAATAA

https://www.ncbi.nlm.nih.gov/nuccore/CP009072.1?report=fasta&from=2590260&to=2591162

fimA

Escherichia coli ATCC 25922, complete genome

GenBank: CP009072.1

GenBank Graphics

>CP009072.1:2584567-2585115 *Escherichia coli* ATCC 25922, complete genome

ATGAAAATTAAAACTCTGGCAATCGTTGTTCTGTCGGCTCTGTCCCTCAGTTCCGCAGCGGCTCTGGCCGATACTACGACG
GTAAATGGTGGGACCGTTCACTTTAAAGGGGAAGTTGTTAACGCCGCTTGCGCAGTTGATGCAGGCTCTGTTGATCAAACC
GTTCAGTTAGGCCAGGTTCGTACCGCTAGCCTGAAGCAGGCTGGAGCAACCAGCTCTGCCGTTGGTTTTAACATTCAGCTG
AATGATTGCGATACCACTGTTGCCACAAAAGCCGCTGTTGCCTTCTTAGGTACGGCAATTGATGCTACGCATACTGATGTA
CTGGCTCTGCAGAGTTCAGCTGCGGGTAGCGCAACAAACGTTGGTGTGCAGATCCTGGACAGAACGGGTGCTGCGCTGGCG
CTGGACGGTGCGACATTTAGTTCAGAAACAACCCTGAATAACGGAACCAACACCATTCCGTTCCAGGCGCGTTATTTTGCA
ACCGGTGCCGCAACCCCGGGTGCTGCTAATGCGGATGCGACCTTCAAGGTTCAGTATCAATAA

https://www.ncbi.nlm.nih.gov/nuccore/CP009072.1?report=fasta&from=2584567&to=2585115

fimB

Escherichia coli ATCC 25922 chromosome, complete genome

NCBI Reference Sequence: NZ_CP009072.1

GenBank Graphics

>NZ_CP009072.1:2580519-2583014 *Escherichia coli* ATCC 25922 chromosome, complete genome

ATGAAGAATAAGGCTGATAACAAAAAAAGGAACTTCCTGACCCATAGTGAAATCGAATCACTCCTTAAAGCAGCAAATACC
GGGCCTCATGCAGCACGTAATTATTGTCTGACTTTGCTTTGTTTTATTCATGGTTTCCGGGCGAGTGAAATTTGTCGATTG
AGGATTTCGGATATTGATCTTAAGGCAAAGTGTATATATATGGGTCTTCCCCGATCATGGTGGGAAGACTCAGTACGCCAT
ATTCAGTTTCCCATAGTGGAACATAACTCCCAGTTTAAAGCGTTCCCGGTTTCGGTATCCCCTGGCCTTTATCCTCAGCAG
TCTGATCTTGCTGTTCAGCGCCTCCGCATTTCCATTCGAGACGCGATGACGCATTGCATTCAGTATTCCGTATAACCGTTT
TTTAATGGTTTTCGCTGCGTTTTTCATCATCGGCACATCACATTCAGACGCCAGCGATATCCACTGCAACCAGTCATTCCT
GCGTTCTGTGCTCCAGGGGCGATCCCAGATATTTTTTGCCAGCTCTTTCAGTGTCCAGCACTGGCTTGTCTGTTGCATCTG
TTCCCGCAACCACATCAGCTTTTCCTGCCGGGACTCAGTCATCCATTTGTCGCTGTACTGCCACAGGAAGCGGGTACCTTT
GGCCTGACGACGACTTTCAACAGGGAGGTGCGGATGTTCATTCTGGCGGGTTTTATCAACCACTTCGCCCAGTTGCTTCGC
CACATGGAAGCGATCGAAGGCGATTTTCTCGACCGCATTGGGTAAATGGATACGCGCTGCTCTTATATAGCCCGCATTCAT
GTCCATCGACAGTGTTTTGATGGCCAACAACTGACTGTCAGTAAGCGAACGGAGATAGCTGGCAAGACTCTCTGTGCCACG
ATCATCGGTTAATGCCAGTGCCCGCCCGTCGCGATCAGATACCACGGTTATATACCTATGCCCTTTTTTAAAGGCGACTTC
GTCTACATTCATATGACGCACTGATAAAGGCTTTTTAATCCGCGACAGGCCTCGCTTAACCGCGCGGGTCATGATGCCGTC
AACGGCATTCCAGCTAAGTTTAAGTTGTTTTCTGACCGCATCAACGGTGCTGATTTTAAGCCATGAGAGCACGAACGATTC
GAACAGCAGAGTGTACCGACTGCCGGAACCTGCCCACGGTACAGGCAGAGTCTGGCAGCCATGCTCCGGGCACATAACGCG
GGGAACATCGGTTTCTACTAATGTCATGAACTGGCAGGTATCAAGGTGGCGCCATTTACGATGTCGGTGATCGTGAACAGA
ACAGGATTTCCTGCAGGTCGGACAGGTTAACTGAGTATTTTCAGCAATTCCGACTGTAACAGTAACGGAACCTGCATTTTC
ATCGAGGGTAAGGGATTTAACCTGCCATGGTGCAGTCAGGTTAAGGATATGGGCATAGAGGGATTTTTCGTCCATATAATT
TCATTCTGAAGCTTACTTTAAACTGATATTATTATTTTTTGCCCACTTTCTTAATCTTCCATTAGCGTTGGCGTAAGGCGA
AGATGTGTTAAATGAGATCATTCTCCCCATCGTCCATTTTGTGTACCATGGTCTTCCATACAAAATATCATCGGTACGATC
ATTAATAAGGTTCACAATCTCATTTTTTACAGTCTGAAGTTCAGCTACTAACAACTCATAACTTAACTCACTGTAATCTGA
GTAAAATTTTTGAGCAAGAAGGCCAAGCTGATTCCATTTATAGCCAGTTTCCGGAAAATCGACAGGCAGACCTTTAGCATC
AGAAGCGATCCACTTTACAACAAGAGCATTCCATCCAAGCAGATACGAAACGAGATCACGAACACTCATCTCCGTTCCTTT
GGCGTGTCCGTCCATTGATTTATCTGAAGTAATTTCTGGTGGGATTGTGTTGAGGTAACTAATTAATTTACTAAAATTTTT
ATCAATAGCTAAAAGCAGTTCAGCTTTTGTTTGCGGCACACTCATAAGAATTCCCTCTCATGTAAAAAGAGACACCATAAA
GGTACTGTGTGGTGTTTGAAAATCACTCAATTATGTACCACCCACCACAACAGGGGAAGACCCATATATATCCATCGATTA
AAAAAAGGCTTTTCAACAACGCATCCGCTATTGAACAAAGAAGTTCAGGCTTTAAAAAACTGGTTGAGTATCCGTACTTCG
TACCCGCATGCTGAGAGCGAGTGGGTATTTTTATCTCGTAAGGGAAATCCGCTTTCTCGGCAACAGTTTTACCATATTATC
TCGACTTCCGGTGGTAATGCCGGGTTGTCACTGGAGATTCATCCGCACATGTTACGCCATTCGTGTGGTTTTGCTTTGGCG
AATATGGGAATAGATACGCGACTTATCCAGGATTATCTTGGGCATCGCAATATTCGTCATACTGTCTGGTATACCGCCAGC
AATGCAGGGCGTTTTTACGGCATCTGGGATAGAGCCAGAGGACGACAGCGTCACGCTGTTTTATAG

https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP009072.1?report=fasta&from=2580519&to=2583014

fimE

Escherichia coli ATCC 25922, complete genome

GenBank: CP009072.1

GenBank Graphics

>CP009072.1:2583492-2584088 Escherichia coli ATCC 25922, complete genome

GTGAGTAAACGTCGTTATCTTACCGGTAAAGAAGTTCAGGCCATGATGCAGGCGGTTTGTTACGGGGCAACGGGAGCCAGA
GATTATTGTCTTATTCTGTTGGCATATCGGCATGGGATGCGTATTAGTGAACTGCTTGATCTGCATTATCAGGACCTTGAC
CTTAATGAAGGTAGAATAAATATTCGCCGACTGAAGAACGGATTTTCTACCGTTCACCCGTTACGTTTTGATGAGCGTGAA
GCCGTGGAACGCTGGACCCAGGAACGTGCTAACTGGAAAGGCGCTGACCGGACTGACGCTATATTTATTTCTCGCCGCGGG

AGTCGGCTTTCTCGCCAGCAGGCCTATCGCATTATTCGCGATGCCGGTATTGAAGCTGGAACCGTAACGCAGACTCATCCT
CATATGTTAAGGCATGCTTGCGGTTATGAACTGGCGGAGCGTGGTGCAGATACTCGTTTAATTCAGGATTATCTCGGGCAT
CGAAATATTCGCCATACTGTGCGTTATACCGCCAGTAATGCTGCTCGTTTTGCCGGATTATGGGAAAGAAATAATCTCATA
AACGAAAAATTAAAAAGAGAAGAGGTTTGA

https://www.ncbi.nlm.nih.gov/nuccore/CP009072.1?report=fasta&from=2583492&to=2584088

hns

Escherichia coli ATCC 25922, complete genome

NCBI Reference Sequence: NZ_CP009072.1

GenBank Graphics

>NZ_CP009072.1:3800271-3800684 *Escherichia coli* ATCC 25922, complete genome
ATGAGCGAAGCACTTAAAATTCTGAACAACATCCGTACTCTTCGTGCGCAGGCAAGAGAATGTACACTTGAAACGCTGGAA
GAAATGCTGGAAAAATTAGAAGTTGTTGTTAACGAACGTCGCGAAGAAGAAAGCGCGGCTGCTGCTGAAGTTGAAGAGCGC
ACTCGTAAACTGCAGCAATATCGCGAAATGCTGATCGCTGACGGTATTGACCCGAACGAACTGCTGAATAGCCTTGCTGCC
GTTAAATCTGGCACCAAAGCTAAGCGTGCTCAGCGTCCGGCAAAATATAGCTACGTTGACGAAAACGGCGAAACTAAAACC
TGGACTGGCCAGGGCCGTACTCCAGCTGTAATCAAAAAAGCAATGGATGAGCAAGGTAAATCCCTCGACGATTTCCTGATC
AAGCAATAA

https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP009072.1?report=fasta&from=3800271&to=3800684

himA

Escherichia coli ATCC 25922, complete genome

NCBI Reference Sequence: CP009072.1

GenBank Graphics

>NZ_CP009072.1:3339963-3340262 *Escherichia coli* ATCC 25922, complete genome
ATGGCGCTTACAAAAGCTGAAATGTCAGAATATCTGTTTGATAAGCTTGGGCTTAGCAAGCGGGATGCCAAAGAACTGGTT
GAACTGTTTTTCGAAGAGATCCGTCGCGCTCTGGAAAACGGCGAACAGGTGAAACTCTCTGGTTTTGGTAACTTCGATCTG
CGTGATAAGAATCAACGCCCGGGACGTAACCCGAAAACGGGCGAGGATATTCCCATTACAGCACGGCGCGTGGTGACCTTC
AGACCCGGGCAGAAGTTAAAAAGCCGGGTCGAAAACGCTTCGCCCAAAGACGAGTAA

lrp

Escherichia coli ATCC 25922, complete genome

NCBI Reference Sequence: NZ_CP009072.1

GenBank Graphics

>NZ_CP009072.1:4336187-4336681 *Escherichia coli* ATCC 25922, complete genome
ATGGTAGATAGCAAGAAGCGCCCTGGCAAAGATCTCGACCGTATCGATCGTAACATTCTTAATGAGTTGCAAAAGGATGGG
CGTATTTCTAACGTCGAGCTTTCTAAACGTGTGGGACTTTCCCCAACGCCGTGCCTTGAGCGTGTGCGTCGGCTGGAAAGA
CAAGGGTTTATTCAGGGCTATACGGCGCTGCTGAACCCCCATTATCTGGATGCATCACTTCTGGTATTCGTTGAGATTACT
CTGAATCGTGGCGCACCGGATGTGTTTGAACAATTCAATACCGCTGTACAAAAACTTGAAGAAATTCAGGAGTGTCATTTA
GTATCCGGTGATTTCGACTACCTGTTGAAAACACGCGTGCCGGATATGTCTGCTTACCGTAAGTTACTGGGGGAAACCCTG
CTGCGTCTGCCTGGCGTCAATGACACACGGACATACGTTGTTATGGAAGAAGTCAAGCAGAGTAATCGTCTGGTTATTAAG
ACGCGCTAA

https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP009072.1?report=fasta&from=4336187&to=4336681

Table 5.3: Primer sequences used for real-time PCR amplification.

Sl no.	Genes	Primer sequences (5'-3')	Amplicon size (bp)	References
1	*fimH*	F.P- TCGAGAACGGATAAGCCGTGG	180	This study
		R.P- CACCACCACATCATTATTGGCG		
2	*fimA*	F.P- GTTGTTCTGTCGGCTCTGTC	130	This study
		R.P- CAACAGAGCCTGCATCAACTG		
3	*fimB*	F.P- GCCGGGTTGTCACTGGAGATTC	171	This study
		R.P- CCAGATGCCGTAAAAACGCCC		
4	*fimE*	F.P- CGCCGACTGAAGAACGGA	162	This study
		R.P- GGCCTGCTGGCGAGAAAG		
5	*hns*	F.P- CGTGCGCAGGCAAGAGAATG	162	This study
		R.P- GTCAGCGATCAGCATTTCGCG		
6	*himA*	F.P- CAAGCGGGATGCCAAAGAAC	151	This study
		R.P- CTCGCCCGTTTTCGGGTTAC		
7	*lrp*	F.P- GTGTGGGACTTTCCCCAACG	165	This study
		R.P- GTTCAAACACATCCGGTGCGC		

5.4.13 Statistical analysis

The data obtained from the ON and OFF *fim* switch analysis and adherence assay were statistically analyzed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The chi-square test and the Fisher exact test were applied to test categorical variables. P values ≤ 0.05 were considered to be statistically significant **(Najafi et al. 2018; Ghosh et al. 2022)**. Moreover, the statistical significance of the data analyzed by SPSS version 21.0 was further validated by using the chi-square test using the goodness of fit in the Prism software package (GraphPad Prism version 9) **(Ghosh et al. 2022)**. Furthermore, the correlation coefficient of the data obtained from the various aforementioned analyses were determined using the Prism software package (GraphPad Prism version 9) **(Parra et al. 2017)** and also further validated using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) (**Yadav et al. 2018**) to find the degree of association between different tested genes, their combinations and adhesive capacity among 20 asymptomatic and 20 symptomatic isolates respectively. Low (>0.3 to 0.5), moderate (>0.5 to 0.7), and high (> 0.7 to 1) positive correlations between different genes and their

combinations among the aforementioned group of isolates were also ascertained as indicated by **Yadav et al** (**Yadav et al. 2018**). Nevertheless, according to SPSS version 21.0, correlation coefficient values < 0.2 were found to be statistically non-significant. Therefore, correlation coefficient values ≤ 0.2 were not considered when ascertaining the highest and lowest correlations. The values between 0.2 and 0.3 were only considered for analysis if they were found to be significant at ≤ 0.05 level. Furthermore, correlation graphs were constructed from the correlation matrices using the GraphPad Prism version 9 (GraphPad Software, La Jolla California USA) **(Ghosh et al. 2022).** Withal, GraphPad Prism version 9 (GraphPad Software, La Jolla California USA) was also used to statistically compute the Mean with SEM (Standard Error of Mean) to determine the variability of expression of different genes from the population mean in both the studied group of isolates. To boot, paired t-test was performed using GraphPad Prism version 9 (GraphPad Software, La Jolla California USA) to determine whether or not the expression level of each of the tested genes within the adherent UPECs significantly differed between 1 and 3hr post-infection among both ABU and symptomatic UPECs. P values ≤ 0.05 were considered to be statistically significant. Furthermore, statistical difference between untreated, 1.5% and 2% D-Mannose treated adherent UPECs respectively was analyzed using one-way ANOVA test GraphPad Prism version 9 (GraphPad Software, La Jolla California USA). P values ≤ 0.05 were considered to be statistically significant **(Scribano et al. 2020).**

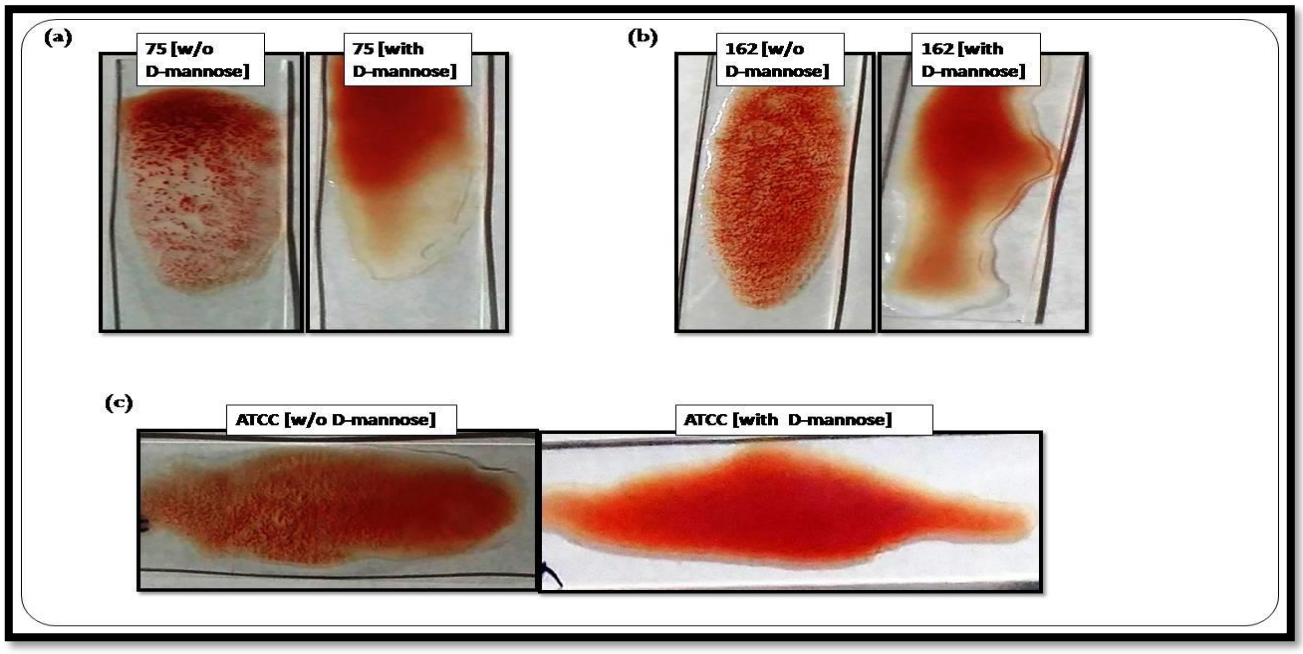
5.5 Results

5.5.1 Mannose sensitive haemagglutination (MSHA)

On the whole, 40 (100%) of the 40 UPECs (Asymptomatic=100%; Symptomatic=100%) considered for this study including the control strain *E. coli* ATCC 25922 showed MSHA atleast weakly. The representative picture of the MSHA assay had been depicted in **Fig. 5.3**. Moreover, the MSHA was found to be statistically significant among isolates of both the aforementioned groups (Asymptomatic-p value=<0.0001; Symptomatic-p value=<0.0001). Detectable MSHA could not be observed in the case of ABU strain *E. coli* 83972.

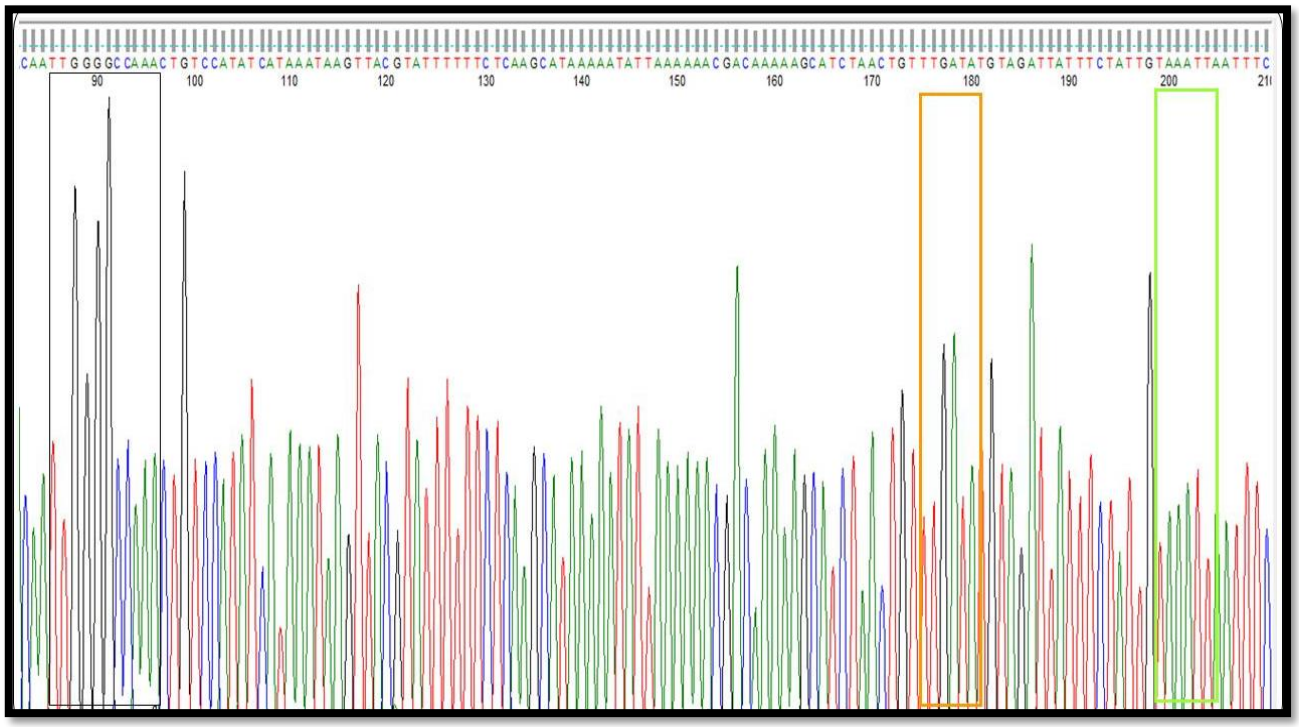
5.5.2 Analysis of *fim* switch orientation of UPECs studied *in vitro*

The results obtained from the investigation of *fim* switch orientation *in vitro* study was not consistent. However, majority of the results obtained from the triplicate analysis of the *fim* switch orientation on different durations, temperature and growth conditions revealed "phase OFF" orientation of *fim* switch among all 40 UPECs irrespective of their asymptomatic and symptomatic nature. Nonetheless, in certain instances "phase ON" orientation was also observed among the studied isolates, although they were inconsistent. The representative chromatogram indicating -10 and -35 region as found in the case of the "phase OFF" orientation was depicted in **Fig. 5.4**.



(This study)

Fig. 5.3: The representative pictures of the MSHA study [a] representative asymptomatic UPEC [b] representative symptomatic UPEC [c] control strain *E. coli* ATCC 25922.

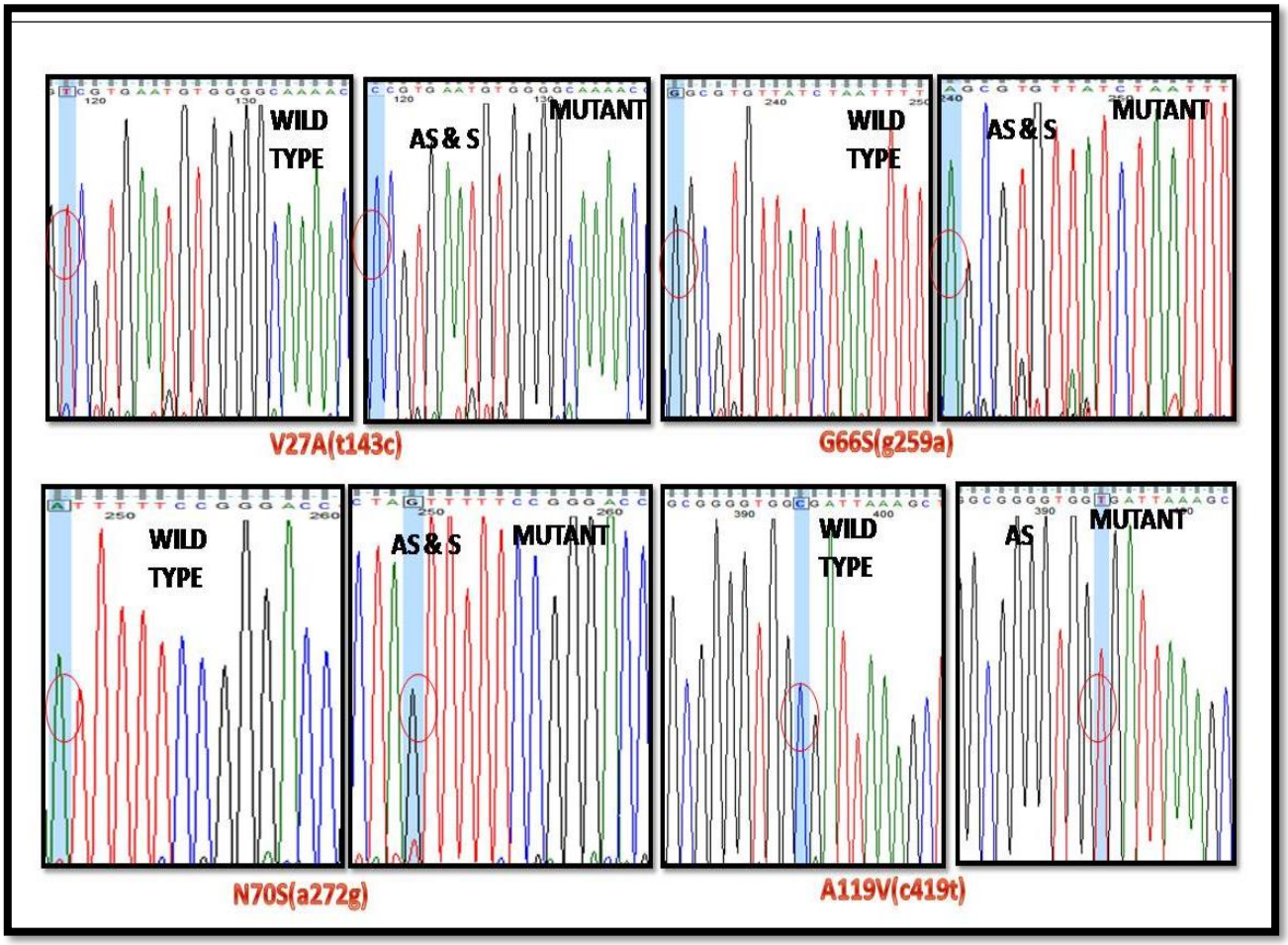


(This study)

Fig. 5.4: The representative chromatograms of Phase OFF orientation of *fim* switch obtained from sequencing of the particular *fimS* region. Black box, yellow box and green box regions indicated the 9bp inverted repeat, -35 and -10 regions in the case of "OFF" orientation of phase switch respectively.

5.5.3 *fimH* polymorphisms analysis

In entirety, a total of 30 varied synonymous single nucleotide polymorphisms (SNPs) were observed at 29 different polymorphic sites in the *fimH* gene in 16 asymptomatic isolates (representative chromatogram depicted in **Fig. 5.5**) **(Table 5.4a; Table 5.5a)**, whereas 31 different synonymous SNPs were observed at 29 different polymorphic sites in the *fimH* gene in 16 symptomatic isolates **(Table 5.4b; Table 5.5b)** (representative chromatogram depicted in **Fig. 5.5**). However, 6 different non synonymous mutations (NSMs), (V27A, G66S, N70S, S78N, P102S, A119V) in the lectin domain and 3 different NSMs (R166H, A202V, Q269K) in the pilin domain of FimH were found in 18 and 11 asymptomatic isolates respectively **(Table 5.4a; Table 5.5a)**. Moreover, 9 different NSMs (V27A, Q41K, G66S, G66V, N70S, S78N, P102S, V118G, V128M) in lectin domain and 3 different NSMs (V163A, H166R, A202V) in the pilin domain were found in 17 and 9 symptomatic isolates respectively **(Table 5.4b; Table 5.5b)**. Moreover, the incidence of NSM V27A was found to be statistically significant among isolates of both the aforementioned groups (Asymptomatic-p value= 0.0003; Symptomatic-p value= 0.0003).



(This study)

Fig. 5.5: The representative chromatograms with nucleotide change in case some pathoadaptive FimH mutations.

Table 5.4a: Synonymous and Non synonymous mutations in the Lectin domain of the FimH of ABU UPECs (n=20).

STRAINS/ ISOLATES	MUTATIONS																										
	fimH(NUCLEOTIDE POSITIONS)																										
E.coli K12 (U00096.3)	87	90	96	108	117	141	143	171	207	225	246	259	272	296	312	315	318	321	327	339	367	411	414	419	429	489	534
	t	c	c	t	c	c	t	g	t	c	c	g	a	g	a	t	t	c	c	c	c	c	g	c	t	c	t
	LECTIN DOMAIN(AMINO ACID POSITIONS)																										I.D[a]
	8	9	11	15	18	26	27	36	48	54	61	66	70	78	83	84	85	86	88	92	102	116	117	119	122	142	157
	G	T	I	G	A	P	V	V	Y	D	G	G	N	S	P	F	P	T	S	R	P	G	G	A	A	F	P
74	c						c		c	t							g		t	g				t			c
	-						A		-	-							-		-	-				V			-
75						t	c		c		t		g	a	t	c		t		g		g	a		a		c
						-	A		-		-		S	N	-	-		-		-		-	-		-		-
77																					t						
																					S						
80							c					a														t	
							A					S														-	
83			t		t	t	c	a									g	t		g		t					
			-		-	-	A	-									-	-		-		-					
84							c					a														t	
							A					S														-	
91		a			t		c	a																			c
		-			-		A	-																			-
93							c					a														t	
							A					S														-	
96			t		t	t	c	a									g	t		g		t					
			-		-	-	A	-									-	-		-		-					
99																					t						
																					S						
102			t		t	t	c	a									g	t		g		t					
			-		-	-	A	-									-	-		-		-					

104																												
107		**a**			**t**	**t**	**c**	**a**									**g**											
		-			**-**	**-**	**A**	**-**									**-**											
110			**t**	**g**	**t**	**t**	**c**	**a**									**g**	**t**		**g**		**t**						
			-	**-**	**-**	**-**	**A**	**-**									**-**	**-**		**-**		**-**						
113			**t**		**t**	**t**	**c**	**a**									**g**	**t**		**g**		**t**						
			-		**-**	**-**	**A**	**-**									**-**	**-**		**-**		**-**						
114							**c**											**t**		**g**		**t**						
							A											**-**		**-**		**-**						
119							**c**					**a**														**t**		
							A					**S**														**-**		
133		**a**			**t**		**c**	**a**																				**c**
		-			**-**		**A**	**-**																				**-**
138																												
158		**a**			**t**		**c**	**a**																				**c**
		-			**-**		**A**	**-**																				**-**

[a]: Interdomain space between lectin and pilin domain of FimH adhesin.

Capital letters-Amino acids

[A=Alanine=Aspartic Acid; F=Phenylalanine; G=Glycine; I=Isoleucine; N=Asparagine; P=Proline; R=Arginine; S=Serine; T=Threonine; V=Valine; Y=Tyrosine]

Small letters-Nucleotides

[a=Adenine; t=Thymine; g=Guanine; c=Cytosine]

Table 5.4b: Synonymous and Non synonymous mutations in the Lectin domain of the FimH of Symptomatic UPECs (n=20).

STRAINS/ ISOLATES	MUTATIONS																													
	fimH(NUCLEOTIDE POSITIONS)																													
E.coli K12 (U00096.3)	90	93	96	117	141	143	171	184	207	225	246	259	260	272	296	312	315	318	321	327	339	367	411	414	416	420	429	445	489	534
	c	t	c	c	c	t	g	c	t	c	c	g	g	a	g	a	t	t	c	c	c	c	c	g	t	g	t	g	c	t
	LECTIN DOMAIN(AMINO ACID POSITIONS)																													I.D[a]
	9	10	11	18	26	27	36	41	48	54	61	66	66	70	78	83	84	85	86	88	92	102	116	117	118	119	122	128	142	157
	T	A	I	A	P	V	V	Q	Y	D	G	G	G	N	S	P	F	P	T	S	R	P	G	G	V	A	A	V	F	P
9	a			t	t	c	a	a										g								a				
	-			-	-	A	-	K										-								-				
17			t	t	t	c												g			g		t							
			-	-	-	A	a											-			-		-							
46				t	t	c	-											g			g									
				-	-	A												-			-									
79																						t								
																						S								
82			t	t	t	c	a											g	t		g		t							
			-	-	-	A	-											-	-		-		-							
86					t	c			c		t	a		g	a	t	c		t		g		g	a						c
					-	A			-		-	S		S	N	-	-		-		-		-	-						-
94			t	t	t	c	a											g	t		g		t							
			-	-	-	A	-											-	-		-		-							
101																														
109			t	t	t	c	a											g	t		g		t							
			-	-	-	A	-											-	-		-		-							
111																														
112																														

130		**a**			**t**	**c**			**c**	**t**										**t**	**g**							**a**		
		-			**-**	**A**			**-**	**-**										**-**	**-**							**M**		
137	**a**			**t**	**t**	**c**	**a**											**g**												
	-			**-**	**-**	**A**	**-**											**-**												
145			**t**	**t**	**t**	**c**	**a**											**g**	**t**		**g**		**t**							
			-	**-**	**-**	**A**	**-**											**-**	**-**		**-**		**-**							
147	**a**			**t**	**t**	**c**	**a**	**a**										**g**									**a**				
	-			**-**	**-**	**A**	**-**	**K**										**-**									**-**				
161					**t**	**c**			**c**		**t**		**t**	**g**	**a**	**t**	**c**		**t**		**g**		**g**	**a**			**a**			**c**
					-	**A**			**-**		**-**		**V**	**S**	**N**	**-**	**-**		**-**		**-**		**-**	**-**			**-**			**-**
162					**t**	**c**			**c**		**t**			**g**	**a**	**t**	**c**		**t**		**g**		**g**	**a**			**a**			**c**
					-	**A**			**-**		**-**			**S**	**N**	**-**	**-**		**-**		**-**		**-**	**-**			**-**			**-**
173						**c**						**a**																**t**		
						A						**S**																**-**		
184						**c**																			**g**				**t**	
						A																			**G**				**-**	
196						**c**						**a**																	**t**	
						A						**S**																	**-**	

[a]: Interdomain space between lectin and pilin domain of FimH adhesin.

Capital letters-Amino acids

[A=Alanine; =Aspartic Acid; F=Phenylalanine; G=Glycine; I=Isoleucine; K=Lysine; N=Asparagine; M=Methionine; P=Proline; Q=Glutamine; R=Arginine; S=Serine; T=Threonine; V=Valine; Y=Tyrosine]

Small letters-Nucleotides

[a=Adenine; t=Thymine; g=Guanine; c=Cytosine]

Table 5.5a: Synonymous and Non synonymous mutations in the Pilin domain of FimH adhesin of ABU UPECs (n=20).

STRAINS/ ISOLATES	MUTATIONS										
	fimH(NUCLEOTIDE POSITIONS)										
E.coli K12 U00096.3)	546	560	603	639	668	714	717	795	807	831	868
	c	g	a	c	c	t	a	g	g	t	c
	PILIN DOMAIN(AMINO ACID POSITIONS)										
	161	166	180	192	202	217	218	244	248	256	269
	C	R	P	N	A	P	A	G	V	Y	Q
74			g						a	c	
			-						-	-	
75			g						a	c	
			-						-	-	
77											
80				t	t	a	g		a	c	
				-	V	-	-		-	-	
83		a				a	g		a	c	
		H				-	-		-	-	
84				t	t	a	g		a	c	
				-	V	-	-		-	-	
91	t					a	g	a	a	c	a
	-					-	-	-	-	-	K
93				t	t	a	g		a	c	
				-	V	-	-		-	-	
96		a				a	g		a	c	
		H				-	-		-	-	
99											
102		a				a	g		a	c	
		H				-	-		-	-	
104											
107									a	c	
									-	-	
110		a				a	g		a	c	
		H				-	-		-	-	
113		a				a	g		a	c	
		H				-	-		-	-	
114						a	g		a	c	
						-	-		-	-	
119				t	t	a	g		a	c	
				-	V	-	-		-	-	
133	t					a	g		a	c	
	-					-	-		-	-	
138											
158	t					a	g	a	a	c	a

	-					-	-	-	-	-	**K**

Capital letters-Amino acids

[A=Alanine; C=Cysteine; G=Glycine; H=Histidine; K=Lysine; N=Asparagine; P=Proline; Q=Glutamine; R=Arginine; V=Valine; Y=Tyrosine]

Small letters-Nucleotides

[a=Adenine; t=Thymine; g=Guanine; c=Cytosine]

Table 5.5b: Synonymous and Non synonymous mutations in the Pilin domain of FimH of Symptomatic UPECs (n=20).

STRAINS/ ISOLATES	**MUTATIONS**										
	***fimH* (NUCLEOTIDE POSITIONS)**										
***E.coli* K12 (U00096.3)**	**546**	**551**	**560**	**577**	**603**	**639**	**668**	**714**	**717**	**807**	**831**
	c	**t**	**g**	**c**	**a**	**c**	**c**	**t**	**a**	**g**	**t**
	PILIN DOMAIN (AMINO ACID POSITIONS)										
	161	**163**	**166**	**172**	**180**	**192**	**202**	**217**	**218**	**248**	**256**
	C	**V**	**R**	**L**	**P**	**N**	**A**	**P**	**A**	**V**	**Y**
9	**t**				**g**					**a**	
	-				**-**					**-**	
17			**a**								
			H								
46								**a**	**g**	**a**	**c**
								-	**-**	**-**	**-**
79											
82			**a**					**a**	**g**	**a**	**c**
			H					**-**	**-**	**-**	**-**
86	**t**	**c**		**t**	**g**			**c**	**g**	**a**	**c**
	-	**A**		**-**	**-**			**-**	**-**	**-**	**-**
94			**a**					**a**	**g**	**a**	**c**
			H					**-**	**-**	**-**	**-**
101											
109			**a**					**a**	**g**	**a**	**c**
			H					**-**	**-**	**-**	**-**
111											
112											
130					**g**			**c**	**g**	**a**	**c**
					-			**-**	**-**	**-**	**-**
137										**a**	**c**
										-	**-**
145			**a**					**a**	**g**	**a**	**c**
			H					**-**	**-**	**-**	**-**
147	**t**				**g**					**a**	
	-				**-**					**-**	
161					**g**			**c**	**g**	**a**	**c**
					-			**-**	**-**	**-**	**-**

162					**g**			**c**	**g**	**a**	**c**
					-			**-**	**-**	**-**	**-**
173						**t**	**t**	**a**	**g**	**a**	**c**
						-	**V**	**-**	**-**	**-**	**-**
184						**t**	**t**	**a**	**g**	**a**	**c**
						-	**V**	**-**	**-**	**-**	**-**
196						**t**	**t**	**a**	**g**	**a**	**c**
						-	**V**	**-**	**-**	**-**	**-**

Capital letters-Amino acids

[A=Alanine=; C=Cysteine; G=Glycine; H=Histidine; L=Leucine; N=Asparagine; P=Proline; R=Arginine; V=Valine; Y=Tyrosine]

Small letters-Nucleotides

[a=Adenine; t=Thymine; g=Guanine; c=Cytosine]

5.5.4 Cell concentration and viability of T24 cell line

The concentration of T24 cell suspension as identified by Trypan blue staining was 638cells/µL. Withal, the cell viability was 95.0±1.25%.

5.5.5 Adherence of UPECs to T24 uroepithelial cell line

On the whole, the 40 (100 %) UPECs, selected for this study regardless of their asymptomatic and symptomatic nature successfully adhered to T24 uroepithelial cells. Post 1hr of infection, 21 [(52.5%); (Asymptomatic=35%; Symptomatic=70%)] and 19 [(47.5%); (Asymptomatic=65%; Symptomatic=30%)] of the 40 UPECs exhibited greater and lesser adhesive capacity respectively compared to the control strain *E. coli* ATCC 25922. None of the isolates showed similar adherence potential to that of the control **(Table 5.6a)**.

Withal, 15 [(37.5%); (Asymptomatic=25%; Symptomatic=50%)] and 24 [(60%); (Asymptomatic=75%; Symptomatic=45%)] of the 40UPECs exhibited higher and lower adhesive capacity respectively compared to the control post 3hrs of infection. However, only 1 symptomatic isolate showed exactly similar adherence potential compared to the control **(Table 5.6b)**. The representative picture of ABU and symptomatic UPEC showing their adhesive potential had been depicted in **Fig. 5.6**. Nevertheless, although **Table 5.7** displayed significant predominance of lesser and greater adhesive capacity among ABU and symptomatic UPECs respectively, both post 1and 3hrs of infection respectively, compared to the control but statistically significant incidence of greater adhesive capacity compared to control was also observed among ABU UPECs post 1hr of infection. Among ABU UPECs, isolates 74, 75, 80, 84, 93, and 119 displayed marked adhesive capacity after both 1 and 3hrs of infection. Very low adherence potential was observed in the case of isolates 138 followed by 104 at both above-mentioned time durations. Nevertheless, among symptomatic isolates, the majority showed remarkable adhesive capacity post 1and 3hrs of infection. However, among them, highly remarkable were isolates 86, 161, 162,173, 184, and 196. Low adhesive capacity was perceived

in the case of isolates 112 followed by 111after both 1 and 3hrs of T24 cell infection. ABU strain *E. coli* 83972 showed detectable but limited T24 uroepithelial cell adherence.

Table 5.6a: Percentage change in adhesive capacity of asymptomatic and symptomatic uropathogenic *E. coli* isolates (n=40) to T24 uroepithelial cells, compared to control strain *E. coli* ATCC 25922 after 1hr of infection incubation.

SL. NO	ASYMPTOMATIC ISOLATES (SAMPLE NO.)	% CHANGE	SYMPTOMATIC ISOLATES (SAMPLE NO.)	% CHANGE
1	74	61.33G	9	180G
2	75	240G	17	241.33G
3	77	62.67L	46	184G
4	80	185.33G	79	65.33L
5	83	4L	82	58.67L
6	84	192G	86	1128G
7	91	44L	94	208G
8	93	157.33G	101	34.67L
9	96	72L	109	64G
10	99	54.67L	111	70.67L
11	102	44L	112	72L
12	104	89.33L	130	77.33G
13	107	34.67L	137	44L
14	110	85.33L	145	32G
15	113	80L	147	13.33G
16	114	81.33L	161	912G
17	119	173.33G	162	1232G
18	133	44L	173	869.33G
19	138	97.33L	184	936G
20	158	6.67G	196	301.33G

G: % Greater than ATCC 25922 post 1hr of Infection;

L: % lesser than ATCC 25922 post 1hr of Infection.

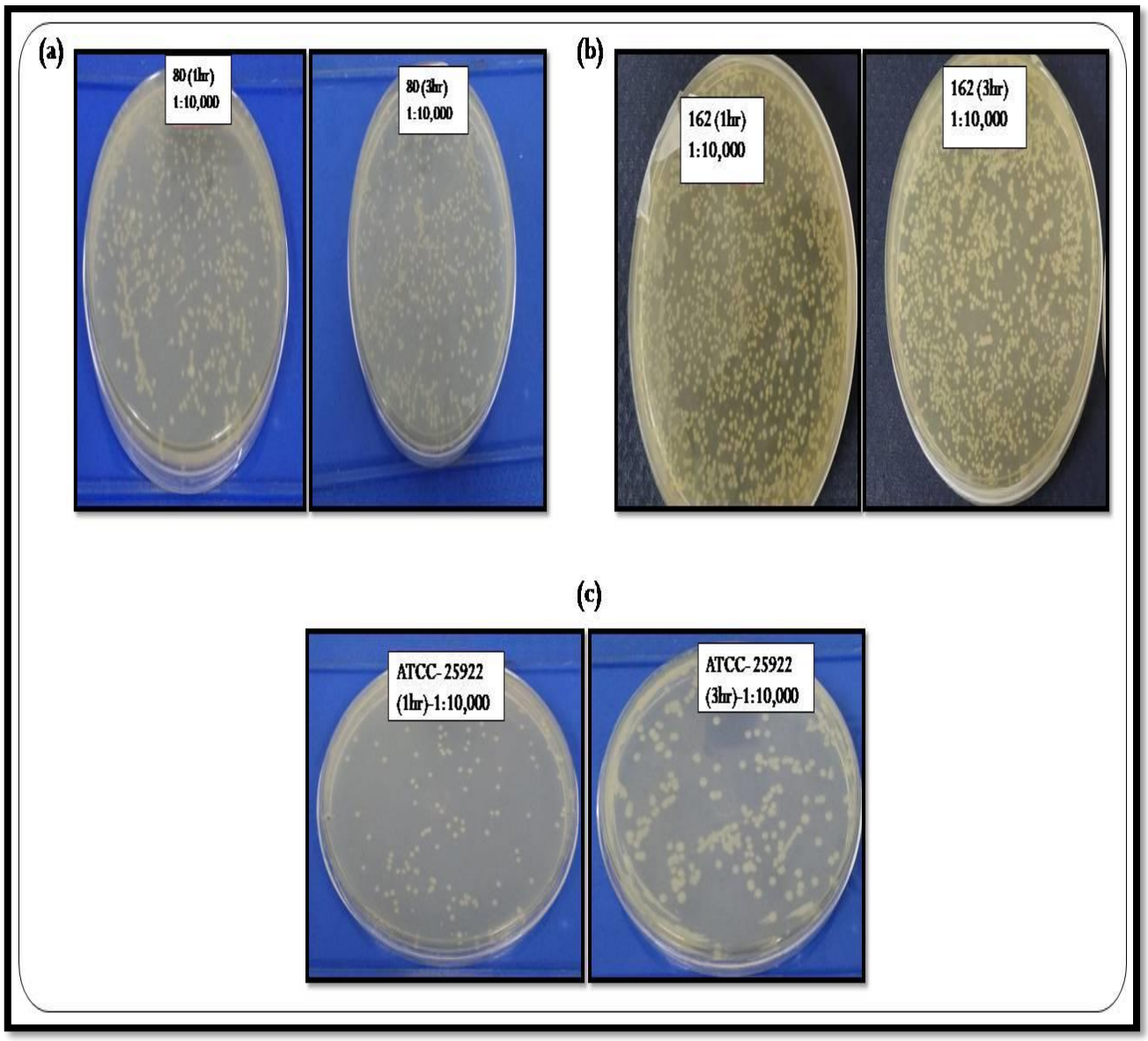
Table 5.6b Percentage change in adhesive capacity of asymptomatic and symptomatic uropathogenic *E. coli* isolates (n=40) to T24 uroepithelial cells, compared to control strain *E. coli* ATCC 25922 after 3hrs of infection incubation.

SL. NO	ASYMPTOMATIC ISOLATES (SAMPLE NO.)	% CHANGE	SYMPTOMATIC ISOLATES (SAMPLE NO.)	% CHANGE
1	74	5.3L	9	59.09G
2	75	155.3G	17	133.33G
3	77	84.09L	46	90.91G
4	80	62.12G	79	87.88L
5	83	43.94L	82	74.24L
6	84	73.48G	86	680.3G
7	91	68.18L	94	112.88G
8	93	102.27G	101	65.91L
9	96	88.6L	109	6.82L
10	99	75L	111	87.88L

11	**102**	**68.18L**	**112**	**92.42L**
12	**104**	**97.73L**	**130**	**0**
13	**107**	**46.21L**	**137**	**75L**
14	**110**	**96.21L**	**145**	**25L**
15	**113**	**93.94L**	**147**	**22.73L**
16	**114**	**89.4L**	**161**	**498.49G**
17	**119**	**90.91G**	**162**	**596.21G**
18	**133**	**68.18L**	**173**	**405.3G**
19	**138**	**99.85L**	**184**	**779.55G**
20	**158**	**13.64L**	**196**	**193.94G**

G: % Greater than ATCC 25922 post 3hrs of Infection;

L: % lesser than ATCC 25922 post 3hrs of Infection.



(This study)

Fig. 5.6: The representative pictures of the adherence assay (a) asymptomatic UPEC (b) symptomatic UPEC (c) control strain *E. coli* ATCC 25922.

Table 5.7: Adhesive capacity and *fim* switch orientation among Uropathogenic *E. coli* isolates from asymptomatic and symptomatic groups after their attachment to T24 human uroepithelial cells

	Groups				
	Asymptomatic(n=20)	**P value (Asymptomatic)**	**Symptomatic(n=20)**	**P value (Symptomatic)**	**Total(n=40)**
Adhesive capacity compared to *E. coli* ATCC 25922, post 1hr of infection					
Greater	**07(35)**	**0.04**	**14(70)**	**0.001**	**21(52.5)**
Lesser	**13(65)**	**0.002**	**6(30)**	**ns**	**19(47.5)**
Same	**0(0)**	**ns**	**0(0)**	**ns**	**0(0)**
Adhesive capacity compared to *E. coli* ATCC 25922, post 3hrs of infection					
Greater	**5(25)**	**ns**	**10(50)**	**0.01**	**15(37.5)**
Lesser	**15(75)**	**0.0005**	**9(45)**	**0.02**	**24(60)**
Same	**0(0)**	**ns**	**1(5)**	**ns**	**1(2.5)**
Orientation of *fim* switch at 1hr post infection					
ONLY ON	**0(0)**	**ns**	**5(25)**	**ns**	**5(12.5)**
BOTH OFF AND ON	**19(95)**	**<0.0001**	**15(75)**	**0.0005**	**34(85)**
ONLY OFF	**1(5)**	**ns**	**0(0)**	**ns**	**1(2.5)**
Orientation of *fim* switch at 3hr post infection					
ONLY ON	**0(0)**	**ns**	**4(20)**	**ns**	**4(10)**
BOTH OFF AND ON	**12(60)**	**0.0034**	**12(60)**	**0.0034**	**24(60)**
ONLY OFF	**8(40)**	**0.03**	**4(20)**	**ns**	**12(30)**

Percentage in parentheses (); ns=not significant

5.5.6 Analysis of *fim* switch orientation of adherent UPECs

The *fimS* (314-bp invertible DNA element) either in phase OFF or phase ON or both phase ON and OFF orientation was detected in the entire 40 (100 %) adherent UPECs (Asymptomatic =100%; Symptomatic=100%) considered for this study, both post 1 and 3hrs infection to T24 uroepithelial cells respectively **(Table 5.8 a-b)**. The representative picture had been depicted in **Fig. 5.7**. The only "ON" orientation of *fim* switch could only be detected in symptomatic UPECs **(Table 5.8b)**; however, the incidence was found to be non-significant **(Table 5.7)**. Nevertheless, the incidence of both phases ON and OFF orientation of *fim* switch after both 1hr [34 (85%); (Asymptomatic=95%; Symptomatic=75%)] and 3hrs [24 (60%); (Asymptomatic=60%; Symptomatic=60%)] of infection of T24 uroepithelial cells by ABU and symptomatic UPECs **(Table 5.8a-b)** were found to be statistically predominant **(Table 5.7)**. Both phases ON and OFF orientations were also perceived in the case of *E. coli* ATCC 25922. Furthermore, among all isolates, post 1hr [1 (2.5%); (Asymptomatic= 5%; Symptomatic= 0%)] and 3hrs [12(30%); (Asymptomatic=40%; Symptomatic=20%)] hrs of infection **(Table 5.8a-b)**, significant incidence of phase OFF orientation was observed only among adherent ABU UPECs post 3hrs of incubation **(Table 5.7)**. However, the *fimS* invertible region could not be observed in the case of ABU strain *E. coli* 83972.

Table 5.8a: Orientation of *fim* Switch in the adherent asymptomatic (n=20) uropathogenic *E. coli* isolates.

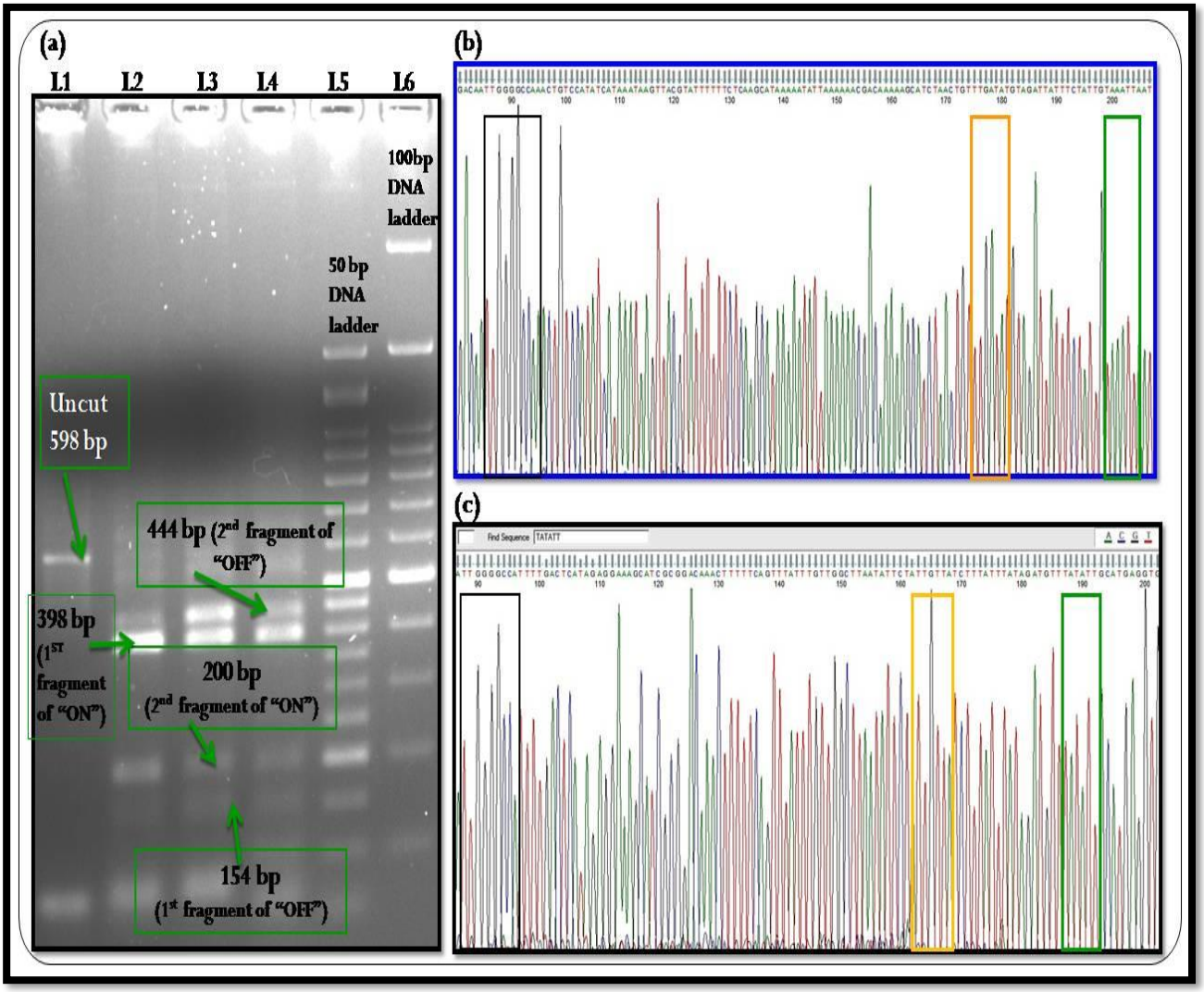
Sl no.	Asymptomatic Isolates (Sample no.)	Infection Duration (In Hours)	Orientation of *fim* Switch in Adherent Fraction
1	74	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
2	75	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
3	77	1	BOTH OFF AND ON
		3	ONLY OFF
4	80	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
5	83	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
6	84	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
7	91	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
8	93	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
9	96	1	BOTH OFF AND ON
		3	ONLY OFF
10	99	1	BOTH OFF AND ON
		3	ONLY OFF
11	102	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
12	104	1	BOTH OFF AND ON

		3	ONLY OFF
13	107	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
14	110	1	BOTH OFF AND ON
		3	ONLY OFF
15	113	1	BOTH OFF AND ON
		3	ONLY OFF
16	114	1	BOTH OFF AND ON
		3	ONLY OFF
17	119	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
18	133	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
19	138	1	ONLY OFF
		3	ONLY OFF
20	158	1	BOTH OFF AND ON
		3	BOTH OFF AND ON

Table 5.8b: Orientation of *fim* Switch in the adherent symptomatic (n=20) uropathogenic *E. coli* isolates.

Sl no.	Symptomatic Isolates (Sample no.)	Infection Duration (In Hours)	Orientation of *fim* Switch in Adherent Fraction
1	9	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
2	17	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
3	46	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
4	79	1	BOTH OFF AND ON
		3	ONLY OFF
5	82	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
6	86	1	ONLY ON
		3	ONLY ON
7	94	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
8	101	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
9	109	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
10	111	1	BOTH OFF AND ON
		3	ONLY OFF
11	112	1	BOTH OFF AND ON
		3	ONLY OFF
12	130	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
13	137	1	BOTH OFF AND ON
		3	ONLY OFF
14	145	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
15	147	1	BOTH OFF AND ON
		3	BOTH OFF AND ON
16	161	1	ONLY ON
		3	ONLY ON

17	162	1	ONLY ON
		3	ONLY ON
18	173	1	ONLY ON
		3	ONLY ON
19	184	1	ONLY ON
		3	BOTH OFF AND ON
20	196	1	BOTH OFF AND ON
		3	BOTH OFF AND ON



(This study)

Fig. 5.7: The representative pictures of the *fim* switch orientation of the adherent UPECs (a) gel pictures showing all 4 fragments of both phase OFF and phase ON orientation (b) chromatogram showing phase OFF orientation (c) chromatogram showing phase ON orientation.

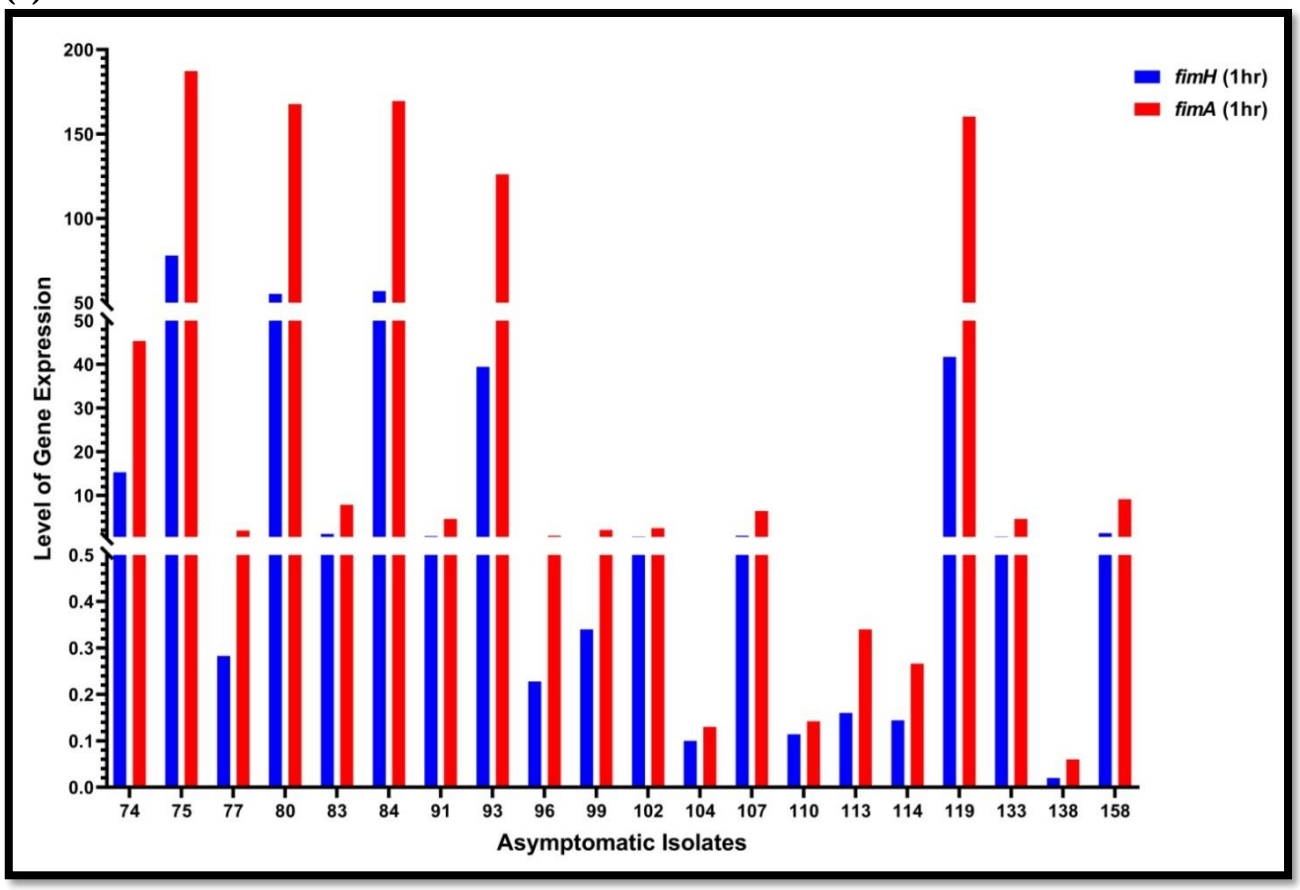
5.5.7 Gene expression analysis of adherent UPECs

The expression levels of the *fimH*, *fimA*, *fimB*, *fimE*, *hns*, *himA* encoding IHF, and *lrp* were determined to get a detailed insight into their interplay. A wide-ranging level of expressions of *fimH*; *fimA*, *fimB*; *fimE and hns*; *himA*; *lrp* were observed among both ABU and symptomatic UPECs after 1 **(Fig. 5.8a-c; Fig. 5.9a-c)** and 3 **(Fig. 5.8d-f; Fig. 5.9d-f)** hrs of T24 uroepithelial cell infection

respectively. The relative change in the expression level of the aforementioned genes among adherent ABU **(Fig. 5.8g-i)** and symptomatic **(Fig. 5.9g-i)** UPECs from 1 to 3hrs of infections also varied. Markedly high and low expressions of *fimH; fimA* [(74, 75, 80, 84, 93, 119); (104, 110, 113, 114, 138)] and *fimB* [(74, 75, 80, 84, 93, 107, 119); (99, 104, 138)] genes respectively was observed in asymptomatic isolates with highest and lowest being observed in case of isolate 75 and 138 respectively post 1hr of infection **(Fig. 5.8a-b)**. However, expression of the *fimE* recombinase gene was found to be comparably lower than *fimB* in the majority of the adherent ABU UPECs studied post 1hr of infection **(Fig. 5.8b).** Moreover, distinctly high expression of type 1 fimbrial genes, especially of the *fimA* was perceived in the case of isolates 74, 75, 80, 84, 93, and 119 (highest being in case of isolate 84) **(Fig. 5.8d).** Nevertheless, unlike at 1hr post-infection, at 3hrs post-infection, a significant (p-value ≤ 0.05) number of adherent asymptomatic UPECs were found to express *fimE* recombinase gene more than *fimB* **(Fig. 5.8e)**. The majority of the adherent symptomatic UPECs expressed *fimH, fimA, fimB* and *fimE* genes much more than the asymptomatic ones, at both aforesaid time durations. Withal, post 1hr of infection, exceedingly high and low expression levels of *fimH; fimA* [86, 161, 162,173, 184, 196 (highest -162); 111, 112 (lowest -112)] **(Fig. 5.9a)** and *fimB* [17, 46, 86, 94, 161, 162,173, 184, 196 (highest -162); 82, 112 (lowest-112)] (**Fig. 5.9b)** genes respectively were perceived in the case of symptomatic UPECs. However, expression of the *fimE* recombinase gene was found to be relatively lower than *fimB* in the entire adherent symptomatic UPECs studied post 1hr of infection **(Fig. 5.9b).** *fimH, fimA* **(Fig. 5.9d)** and *fimB* **(Fig. 5.9e)** expressions of all the symptomatic isolates including the aforementioned ones decreased at 3hrs post-infection, however, noticeably high expression levels were observed in the case of isolate 86 followed by 162 and lowest in the case of 112. Moreover, discordant to that observed at 1hr post-infection, at 3hrs post-infection significant (p-value ≤ 0.05) incidence of higher expression of *fimE* gene compared to *fimB* among symptomatic UPECs was perceived **(Fig. 5.9e)**.

Additionally, the variegated levels of regulatory factor genes (*hns, himA,* and *lrp*) expressions were also observed among both ABU **(Fig. 5.8c; Fig. 5.8f)** and symptomatic **(Fig. 5.9c; Fig. 5.9f)** UPECs after 1 and 3 hrs of T24 uroepithelial cell infection respectively. Detectable *fimH* expression was noticed in the case of ABU strain *E. coli* 83972, but *fimA* expression was found to be negligible. However, imperceptible *fimB* and *fimE* expressions but the detectable intensity of expression of all the regulatory factors could be perceived in the case of adherent ABU strain *E. coli* 83972 at both the studied durations of infection.

(a)



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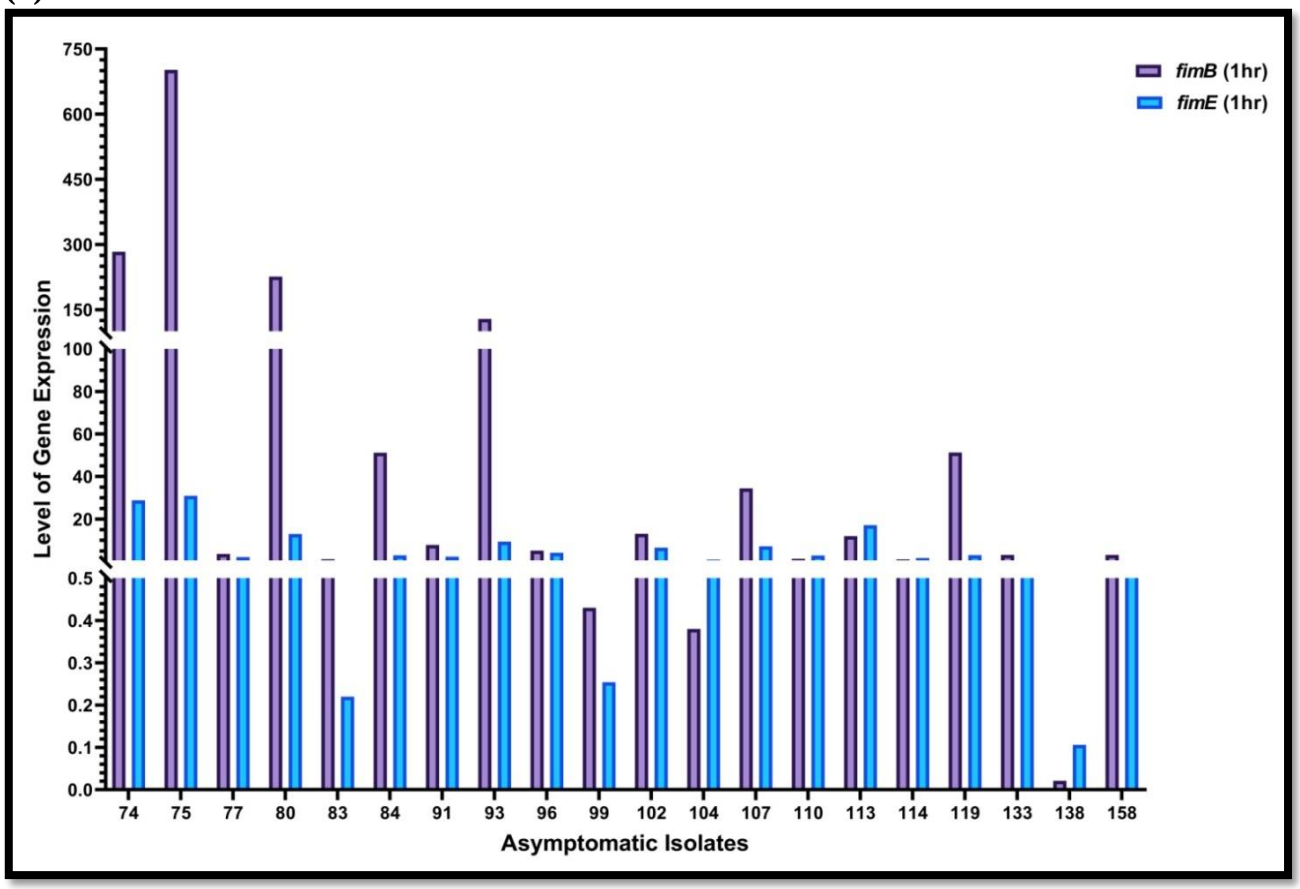
(b)



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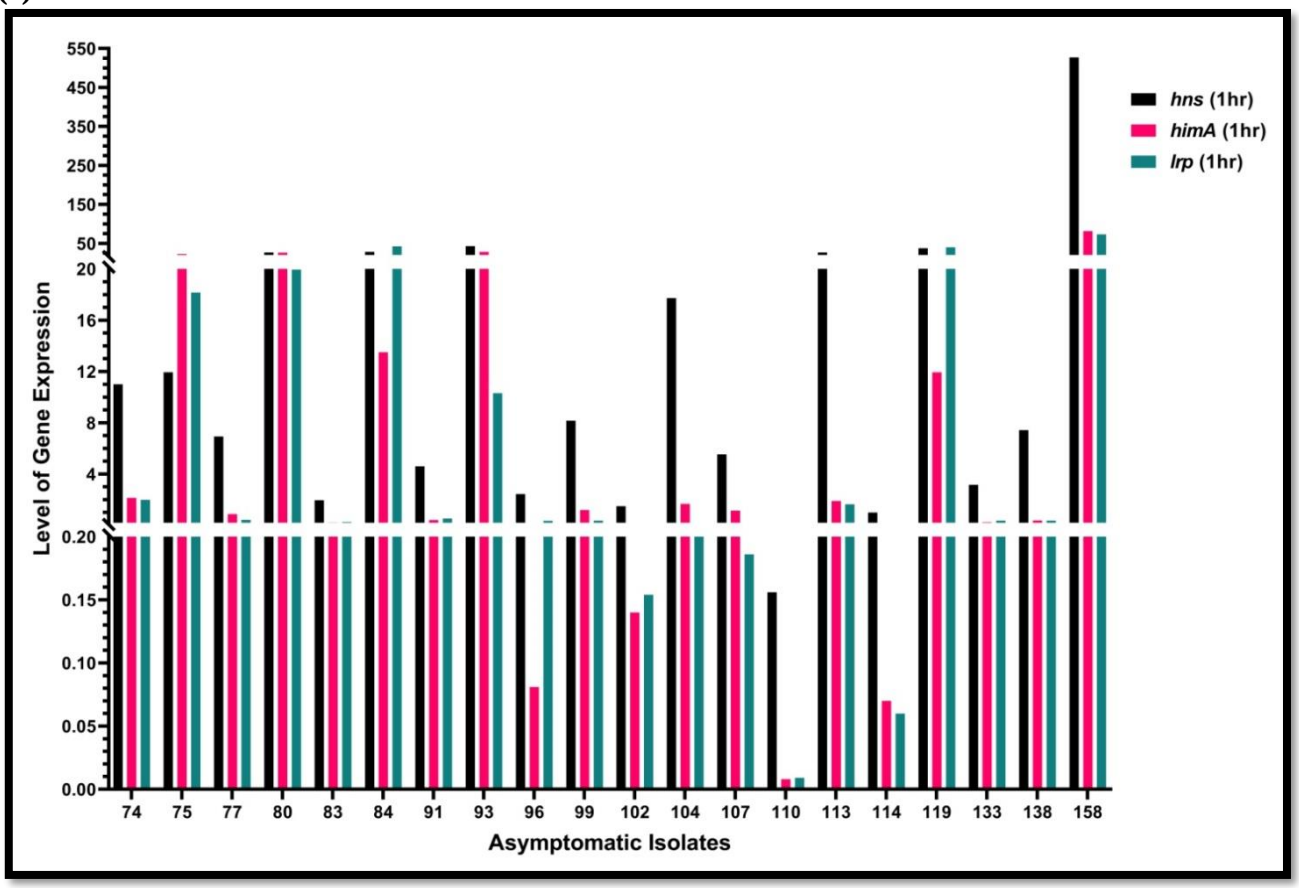

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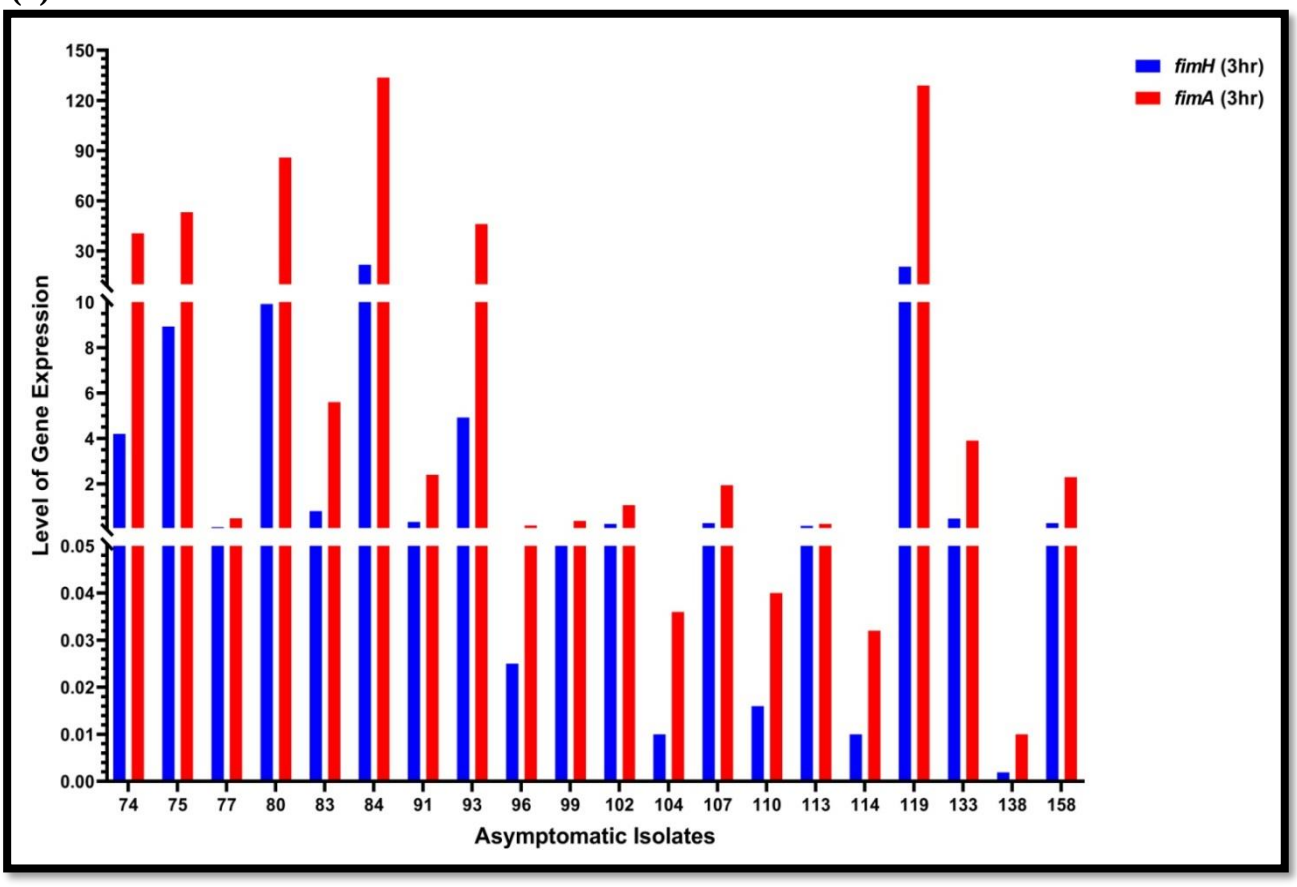
(d)



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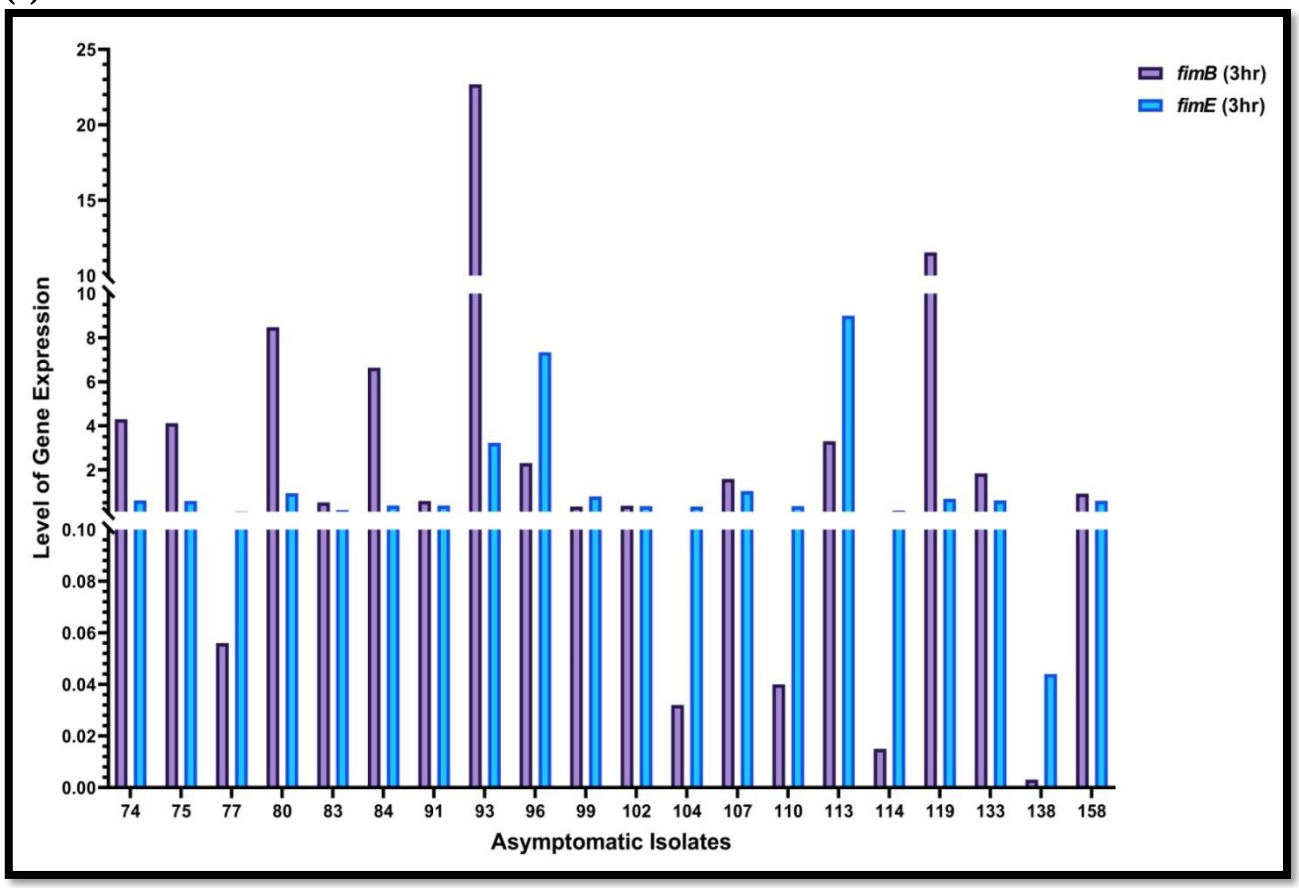
(e)



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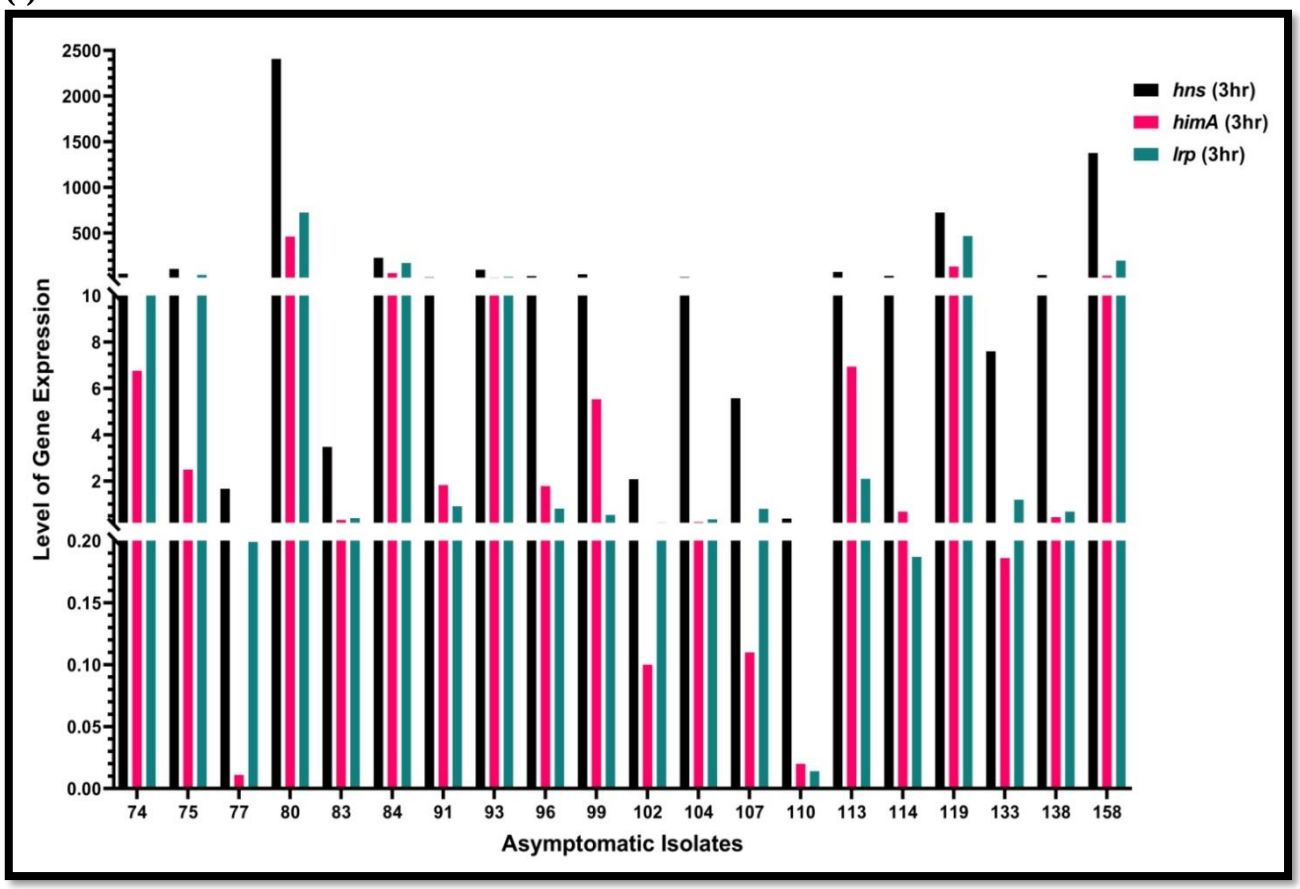
(f)



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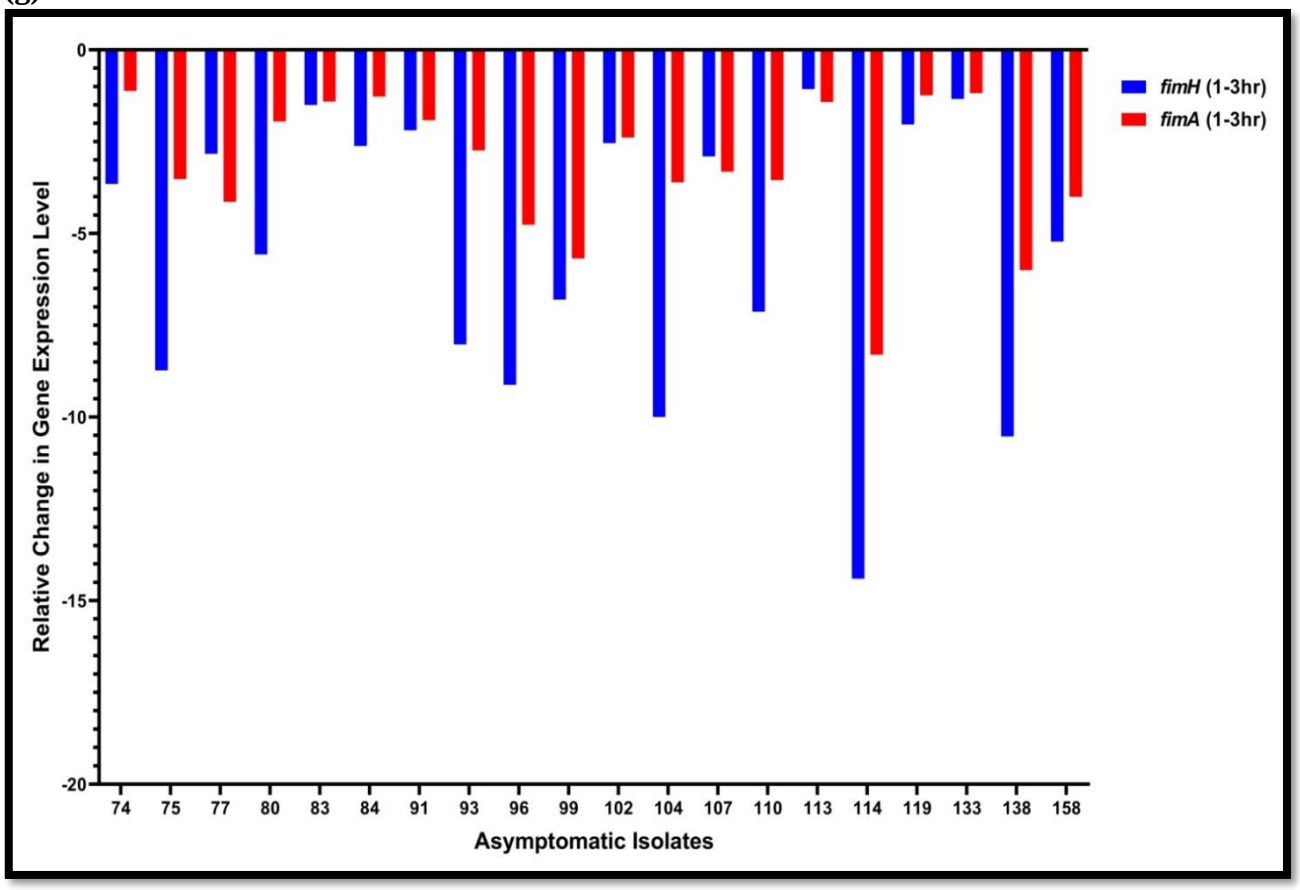
(g)



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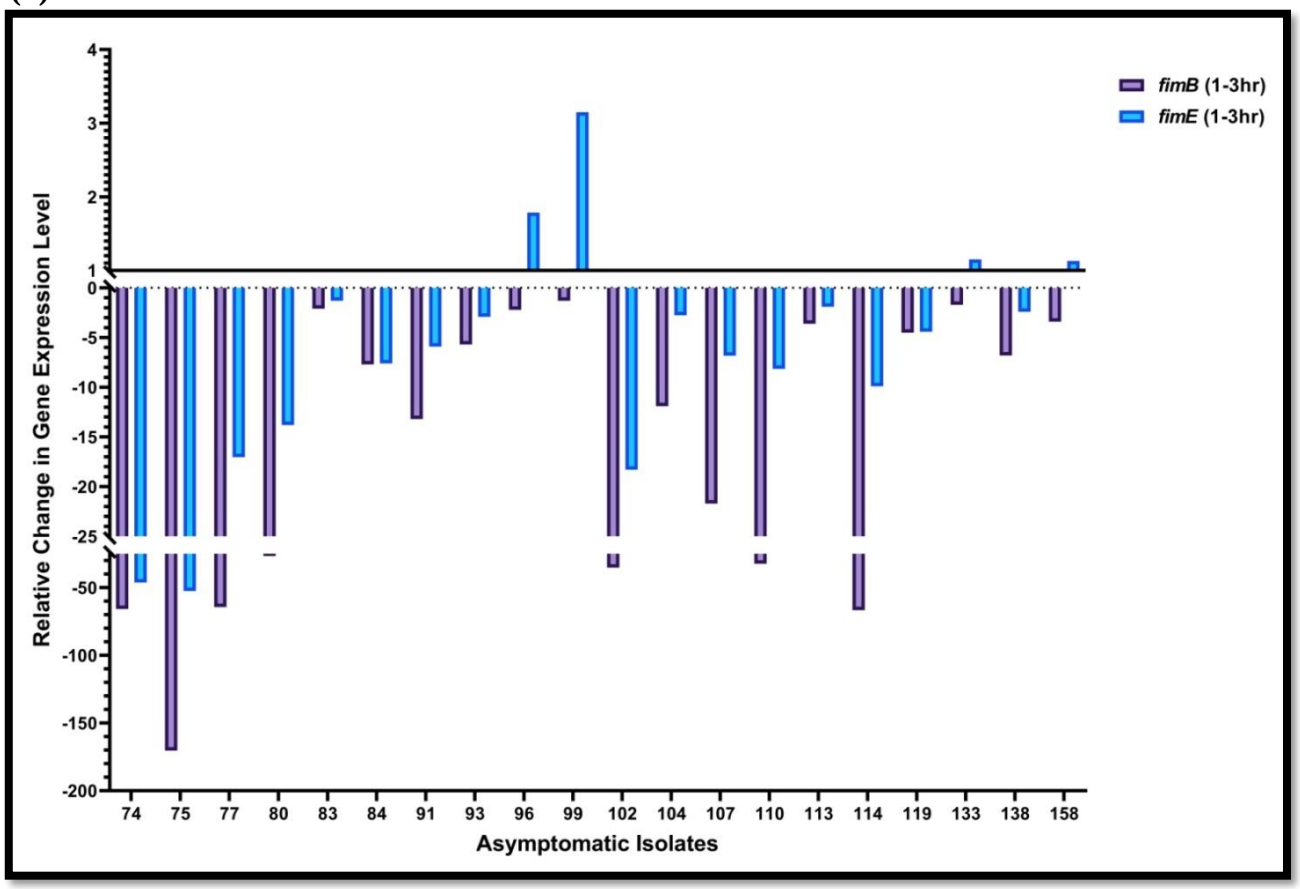

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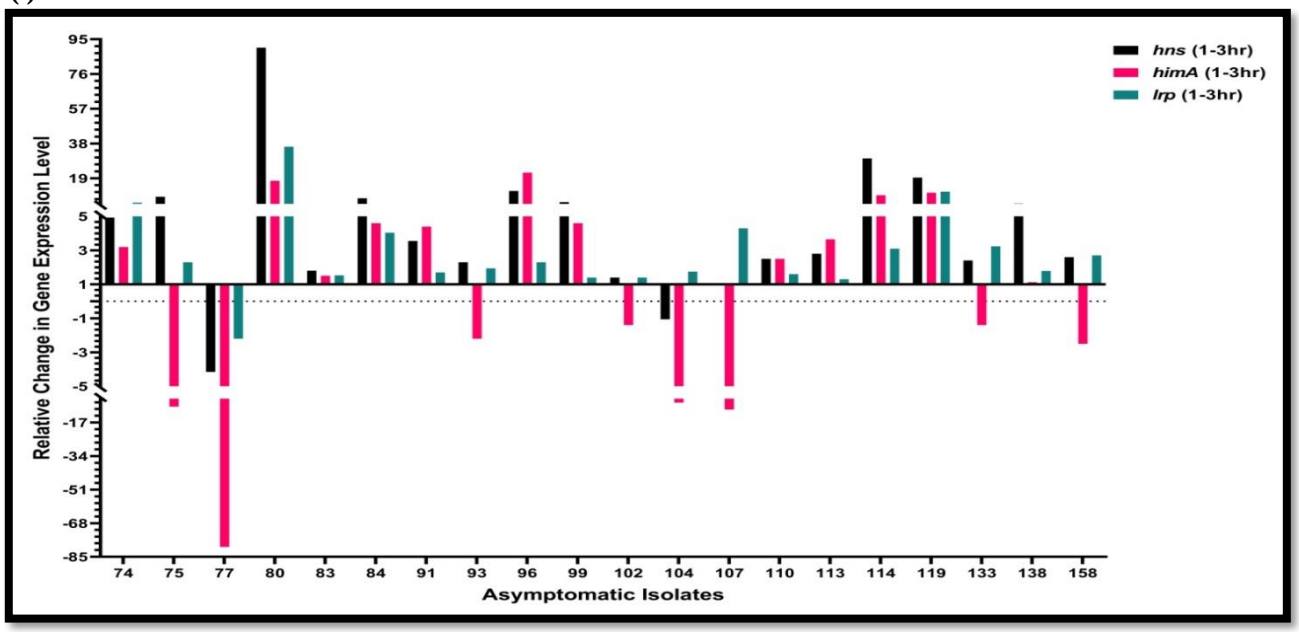
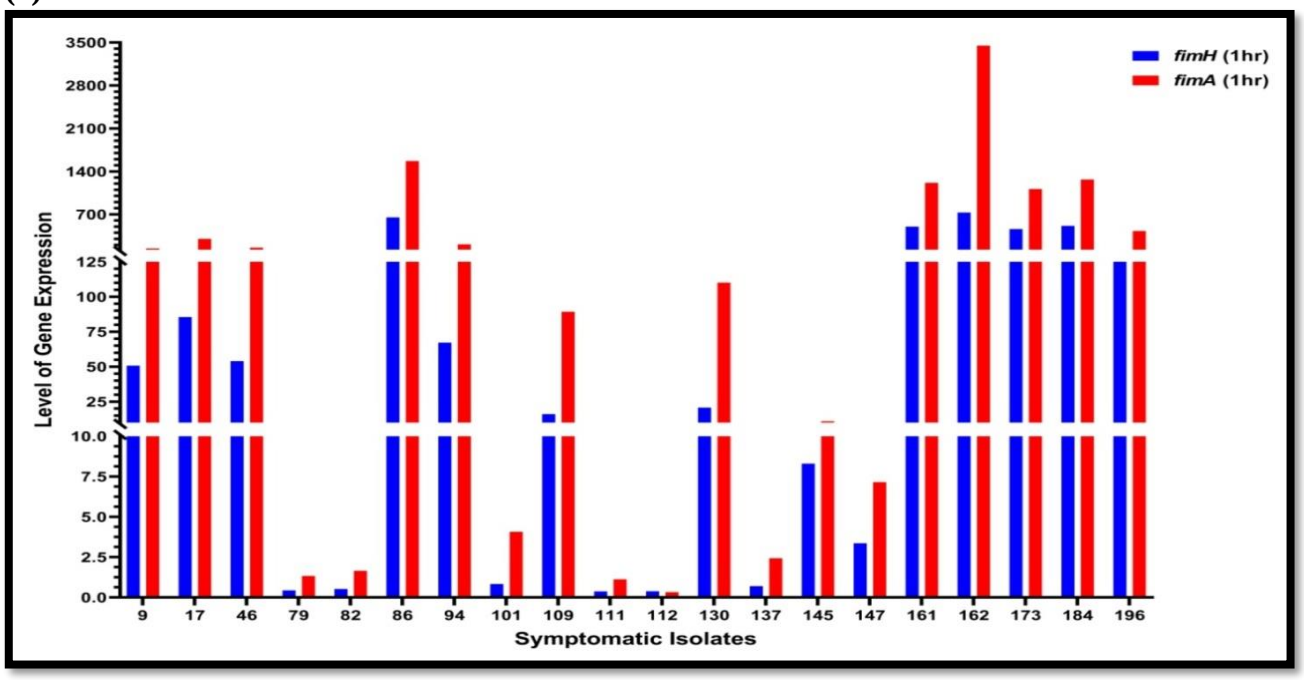


(This study)

Fig. 5.8: Graphical representation of the level of quantitative expression of different target genes at different studied infection durations and also their relative changes (a) *fimH* (1hr); *fimA* (1hr) (b) *fimB* (1hr); *fimE* (1hr) (c) *hns* (1hr); *himA* (1hr); *lrp* (1hr) (d) *fimH* (3hr); *fimA* (3hr) (e) *fimB* (3hr); *fimE* (3hr) (f) *hns* (3hr); *himA* (3hr); *lrp* (3hr) (g) *fimH* (1 to 3hrs relative changes); *fimA* (1 to 3hrs relative changes) (h) *fimB* (1 to 3hrs relative changes); *fimE* (1 to 3hrs relative changes) and (i) *hns* (1 to 3hrs relative changes); *himA* (1 to 3hrs relative changes); *lrp* (1 to 3hrs relative changes) in the adherent asymptomatic UPECs (n=20), computed with the $2^{-\Delta\Delta Ct}$ method using 16srRNA gene as reference and *E. coli* ATCC 25922 as a calibrator generated using GraphPad Prism version 9 (Prism software package). Different genes were represented by bar graphs with varied colours.

(a)



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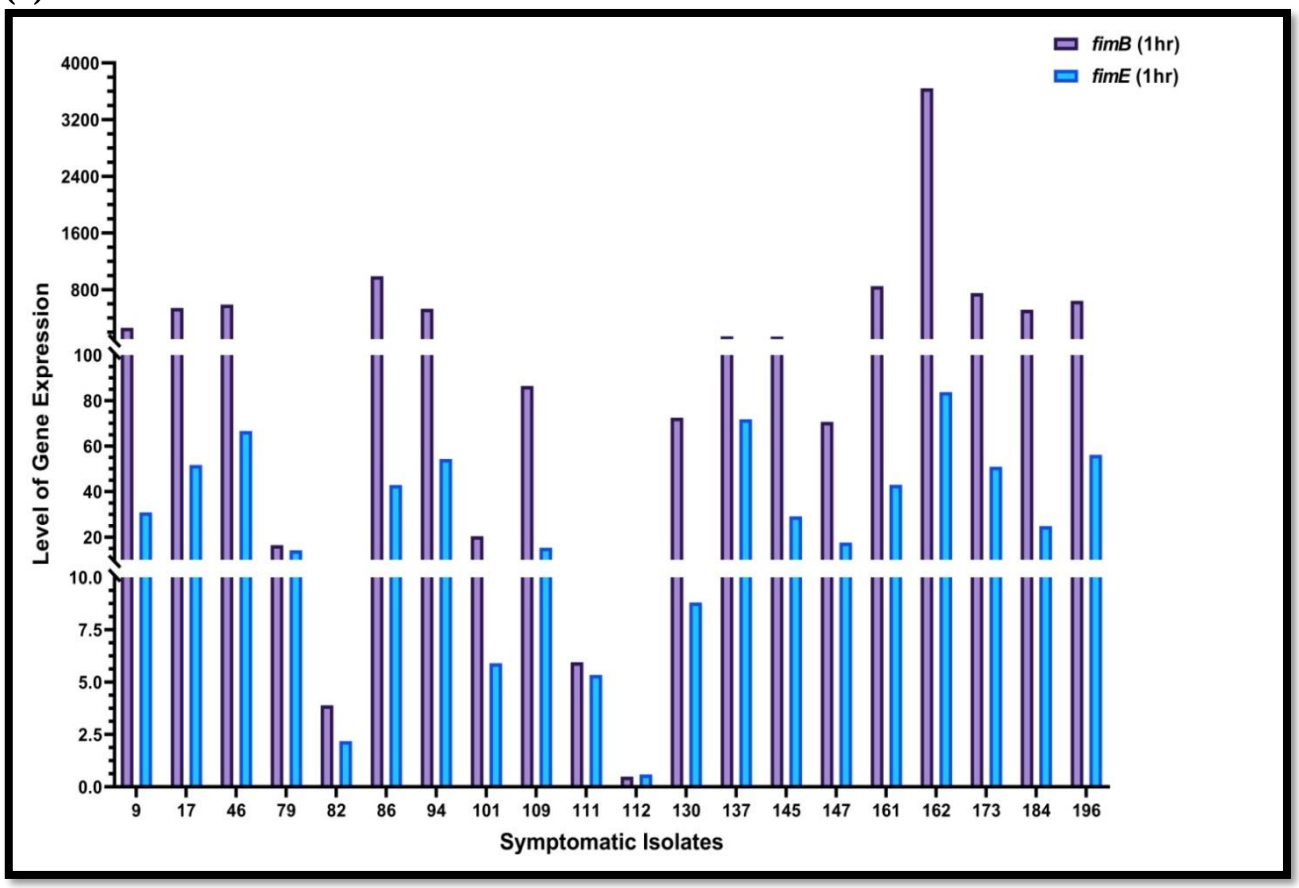

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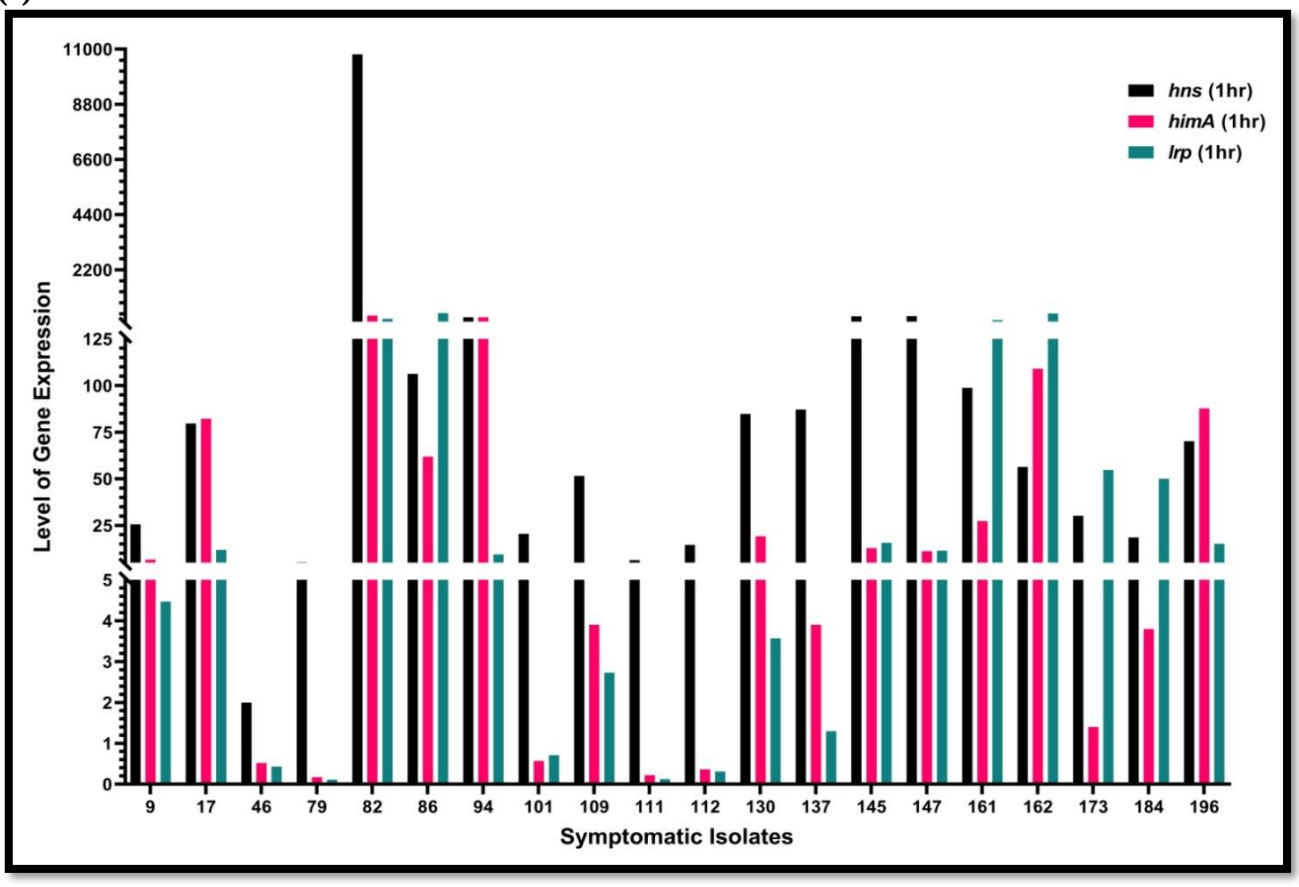

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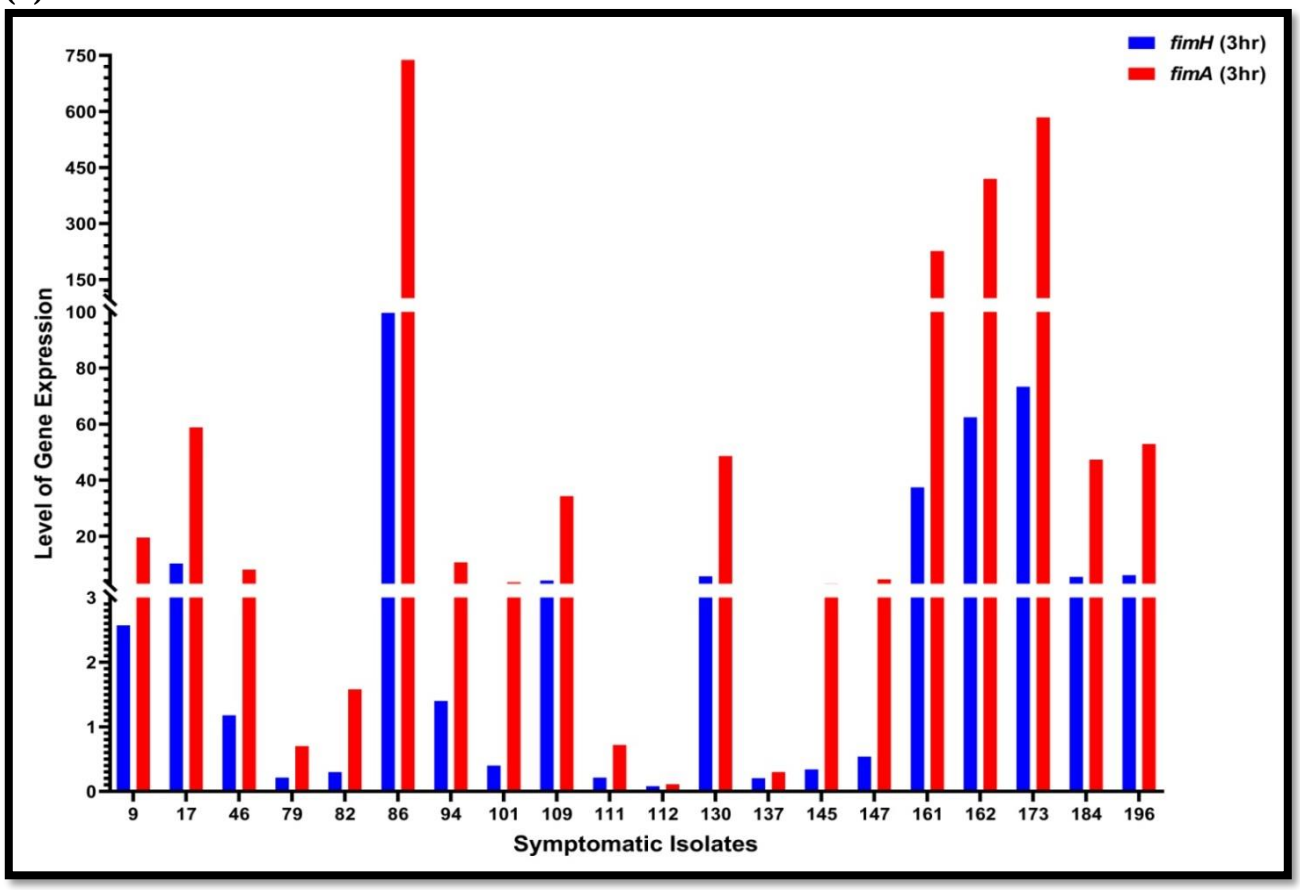
(d)



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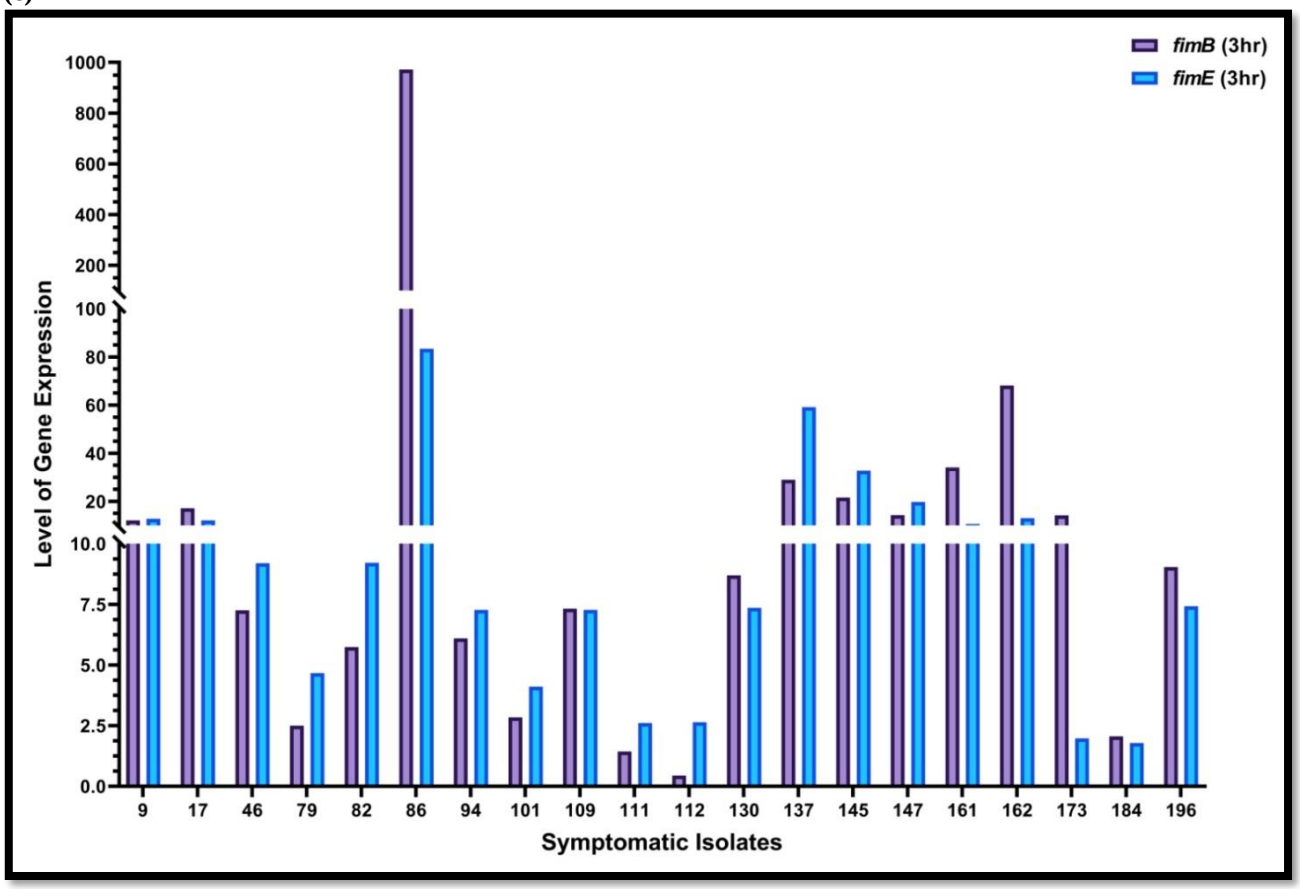

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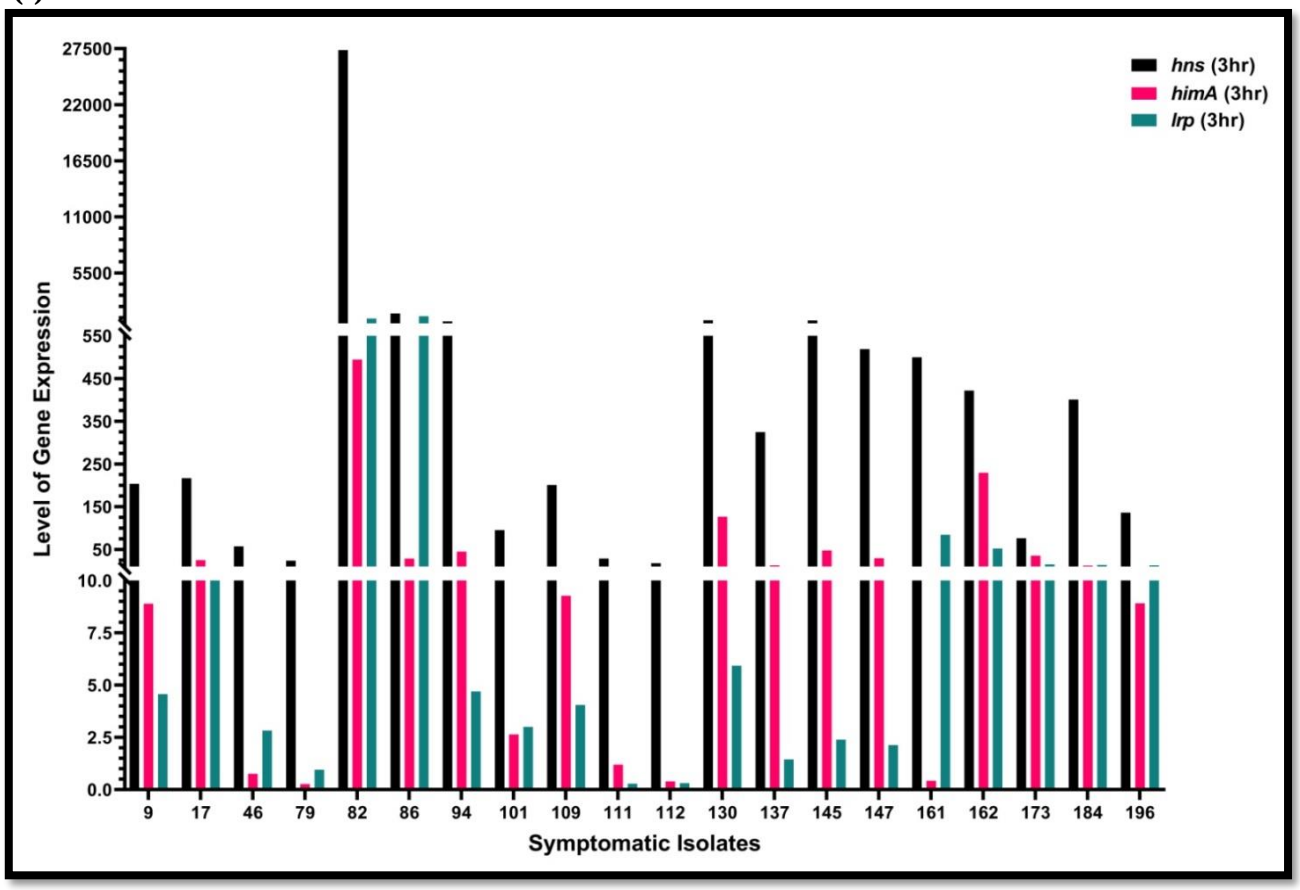

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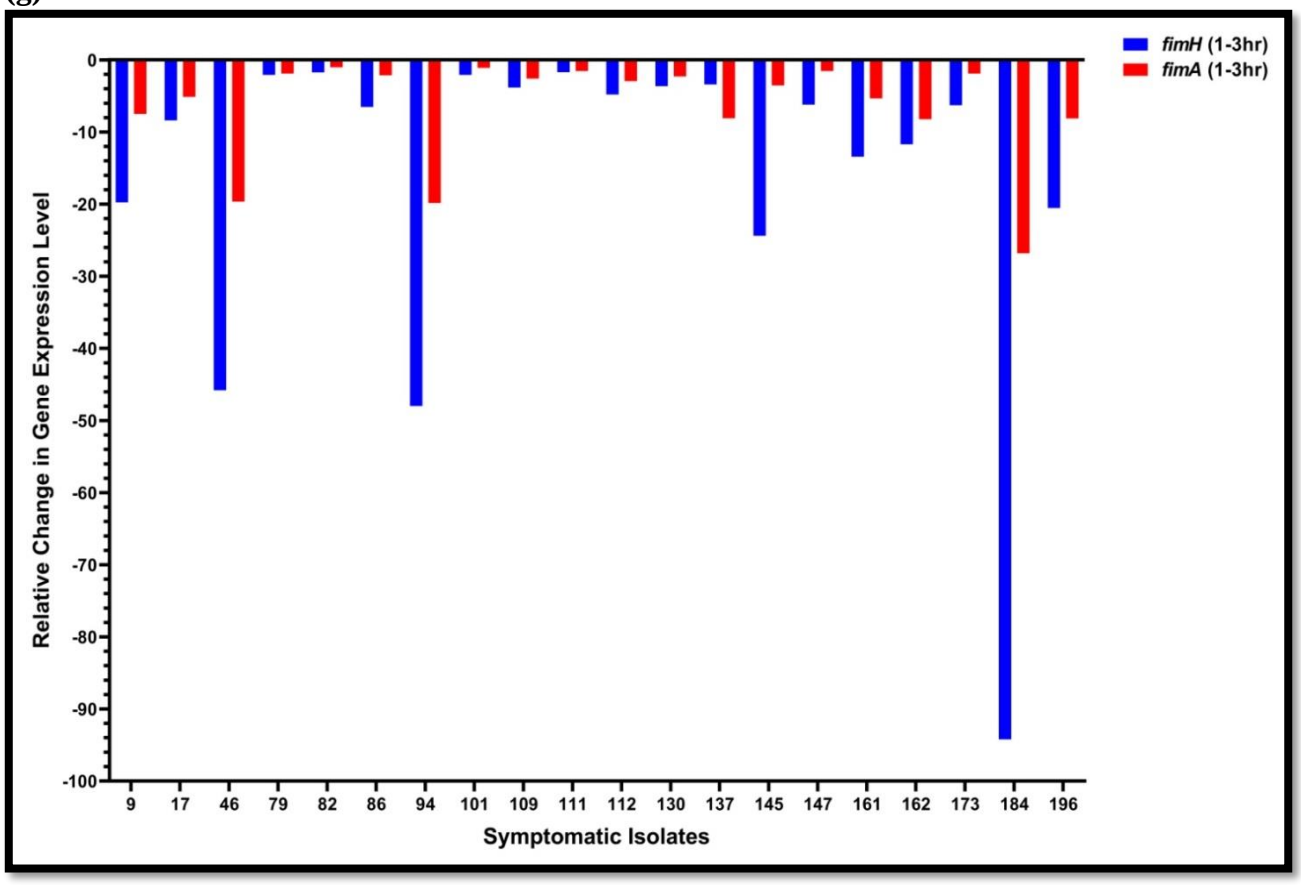
(g)



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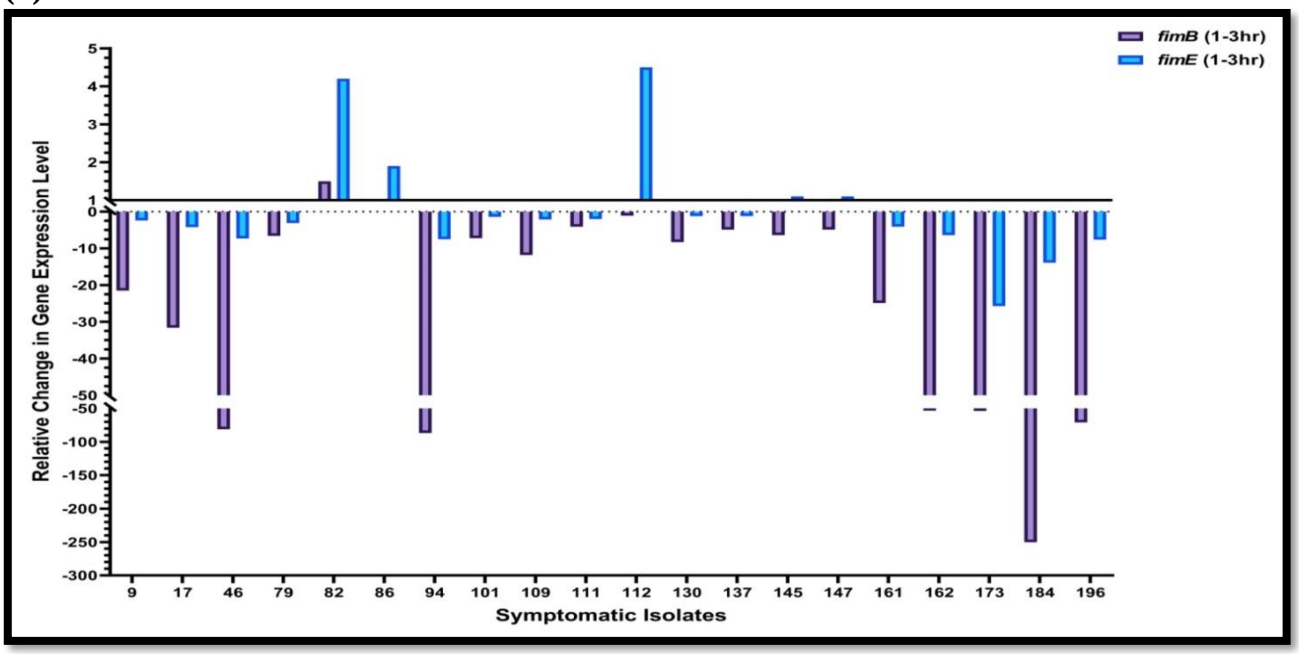

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(i)

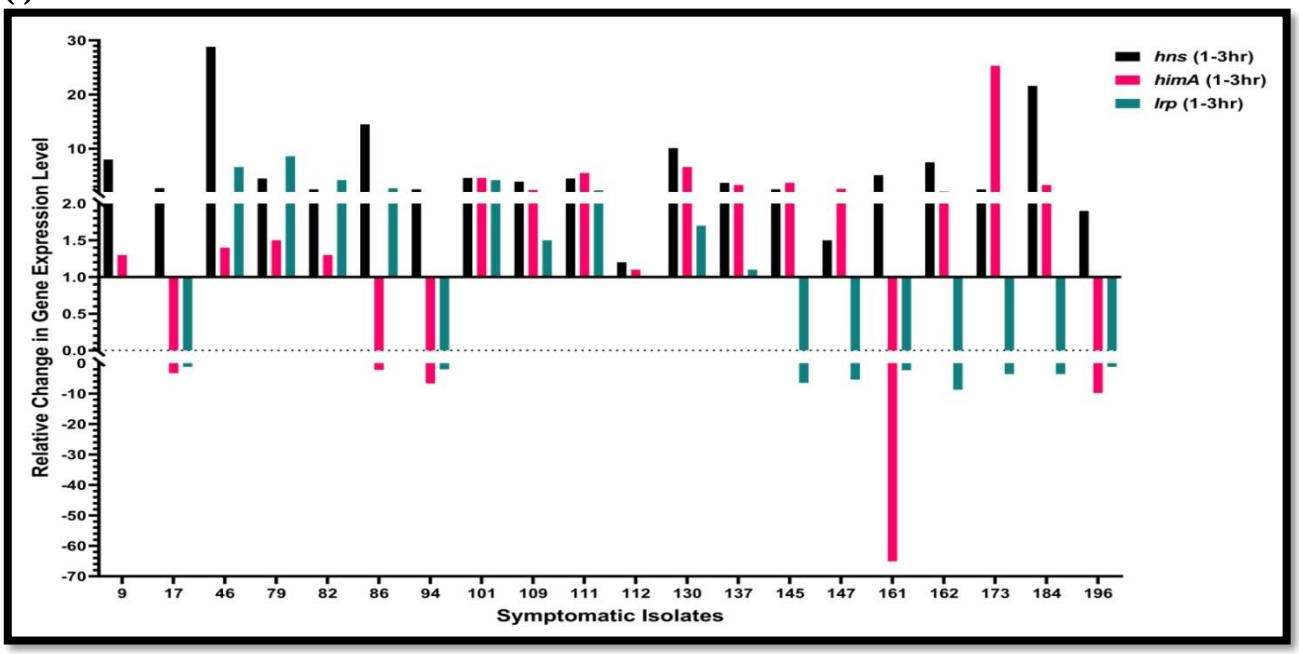

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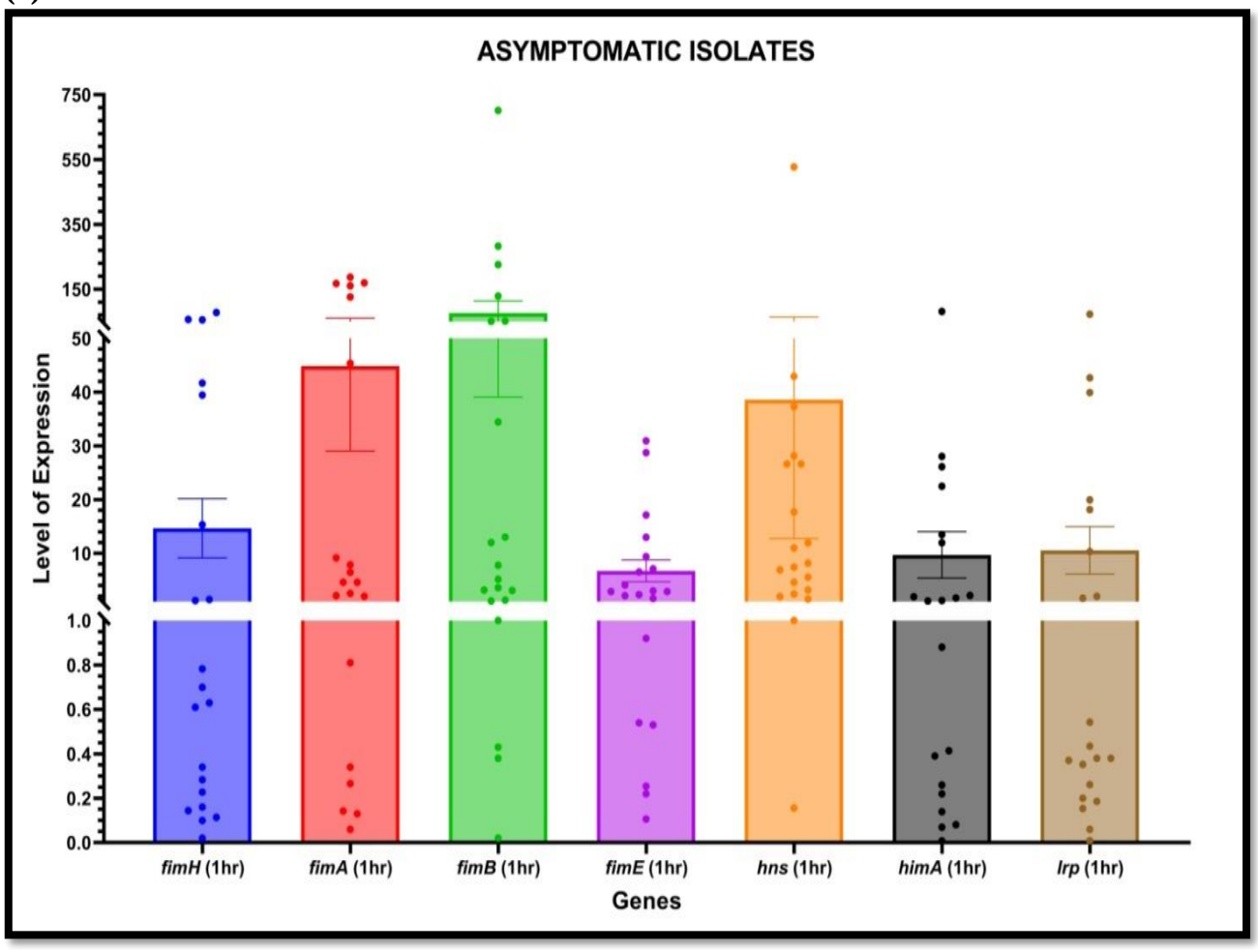
Fig. 5.9: Graphical representation of the level of quantitative expression of different target genes at different studied infection durations and also their relative changes (a) *fimH* (1hr); *fimA* (1hr) (b) *fimB* (1hr); *fimE* (1hr) (c) *hns* (1hr); *himA* (1hr); *lrp* (1hr) (d) *fimH* (3hr); *fimA* (3hr) (e) *fimB* (3hr); *fimE* (3hr) (f) *hns* (3hr); *himA* (3hr); *lrp* (3hr) (g) *fimH* (1 to 3hrs relative changes); *fimA* (1 to 3hrs relative changes) (h) *fimB* (1 to 3hrs relative changes); *fimE* (1 to 3hrs relative changes) and (i) *hns* (1 to 3hrs relative changes); *himA* (1 to 3hrs relative changes); *lrp* (1 to 3hrs relative changes) in adherent the symptomatic UPECs (n=20), computed with the $2^{-\Delta\Delta Ct}$ method using 16srRNA gene as reference and *E. coli* ATCC 25922 as a calibrator generated using GraphPad Prism version 9 (Prism software package). Different genes were represented by bar graphs with varied colours.

Moreover, the inferential statistics demonstrated the sampling distribution of the population mean of ABU **(Fig. 5.10a-b)** and symptomatic **(Fig. 5.10c-d)** UPECs relating to the echelon of expression of the *fimH*, *fimA*, *fimB*, *fimE*, *hns*, *himA*, and *lrp* genes at 1 and 3hrs of infections respectively. This analysis revealed that 6; 6 **(Fig. 5.10a)**, 6; 6 **(Fig. 5.10b)**, 4; 6 **(Fig. 5.10a)**, 6; 3 **(Fig. 5.10b)**, and 2; 6; 5 **(Fig. 5.10a)**, 3; 3; 4 **(Fig. 5.10b)** isolates respectively fell outside the population mean of ABU UPECs relating to the echelon of expression of the *fimH*; *fimA*, *fimB*; *fimE* and *hns; himA*; *lrp* genes at 1 and 3hrs of infections respectively. Nonetheless, in the case of symptomatic UPECs, 5; 5 (**Fig. 5.10c)**, 4; 4 **(Fig. 5.10d)**, 9; 9 (**Fig. 5.10b)**, 2; 4 **(Fig. 5.10d)** and 1; 6; 4 (**Fig. 5.10b)**, 1; 3; 2 **(Fig. 5.10d)** isolates respectively contravened from the population mean with regard to the *fimH*; *fimA*, *fimB*; *fimE* and *hns; himA*; *lrp* gene expression levels at 1 and 3hrs respectively. Furthermore, the difference in the expression levels of *fimH*, *fimA*, *fimB*, *fimE*, *hns*, and *lrp* genes between 1and 3hrs of expression was found to be highly statistically significant (p-value ≤ 0.01) in the case of both ABU (**Fig. 5.10e)** and symptomatic **(Fig. 5.10f)** UPECs except *lrp in* case of symptomatic **(Fig. 5.10f)** UPECs. To boot, the difference in expressions of the *himA* gene between 1and 3hrs of expression was found to be statistically non-significant (p-value ≥ 0.05) among both ABU (**Fig. 5.10e)** and symptomatic **(Fig. 5.10f)** UPECs.

(a)



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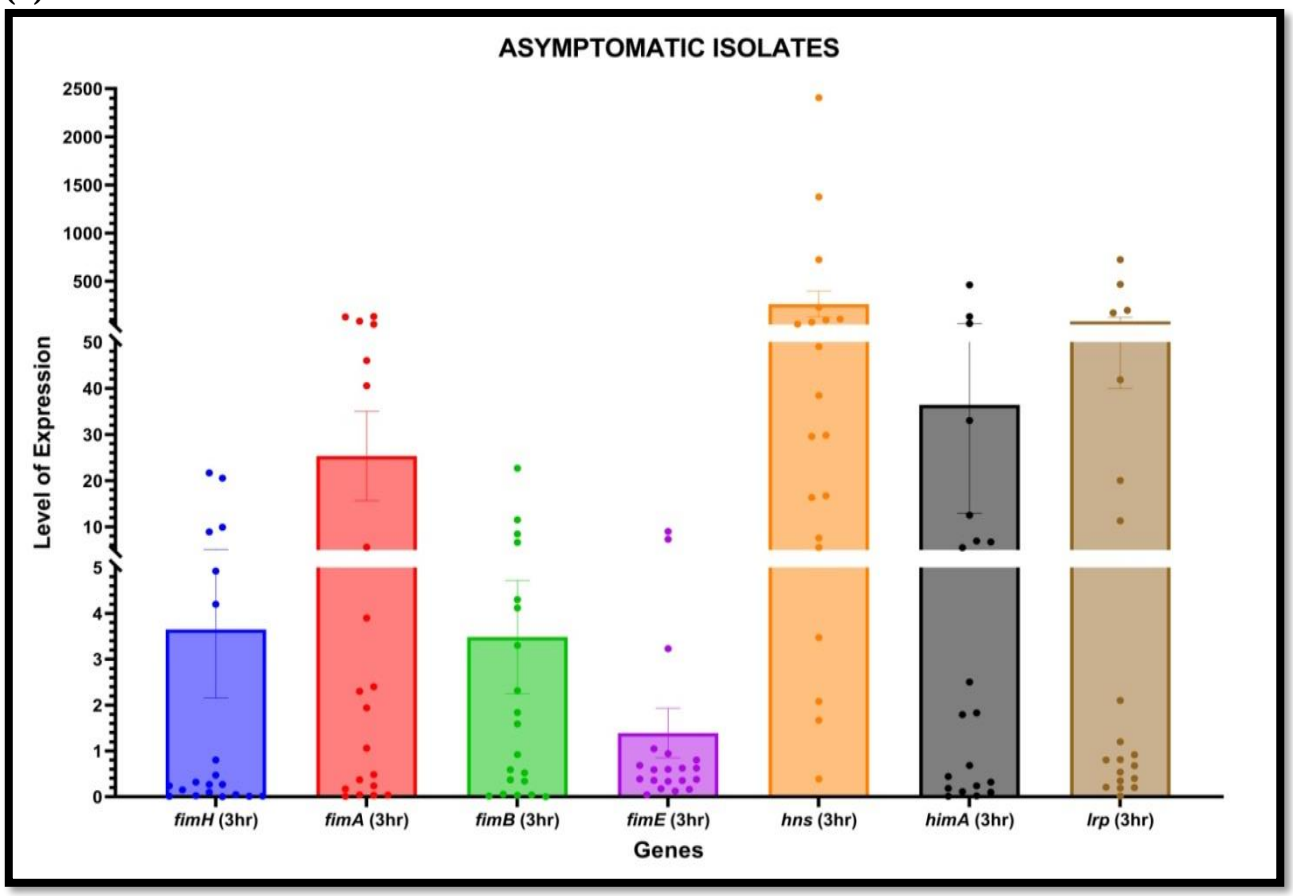
(b)



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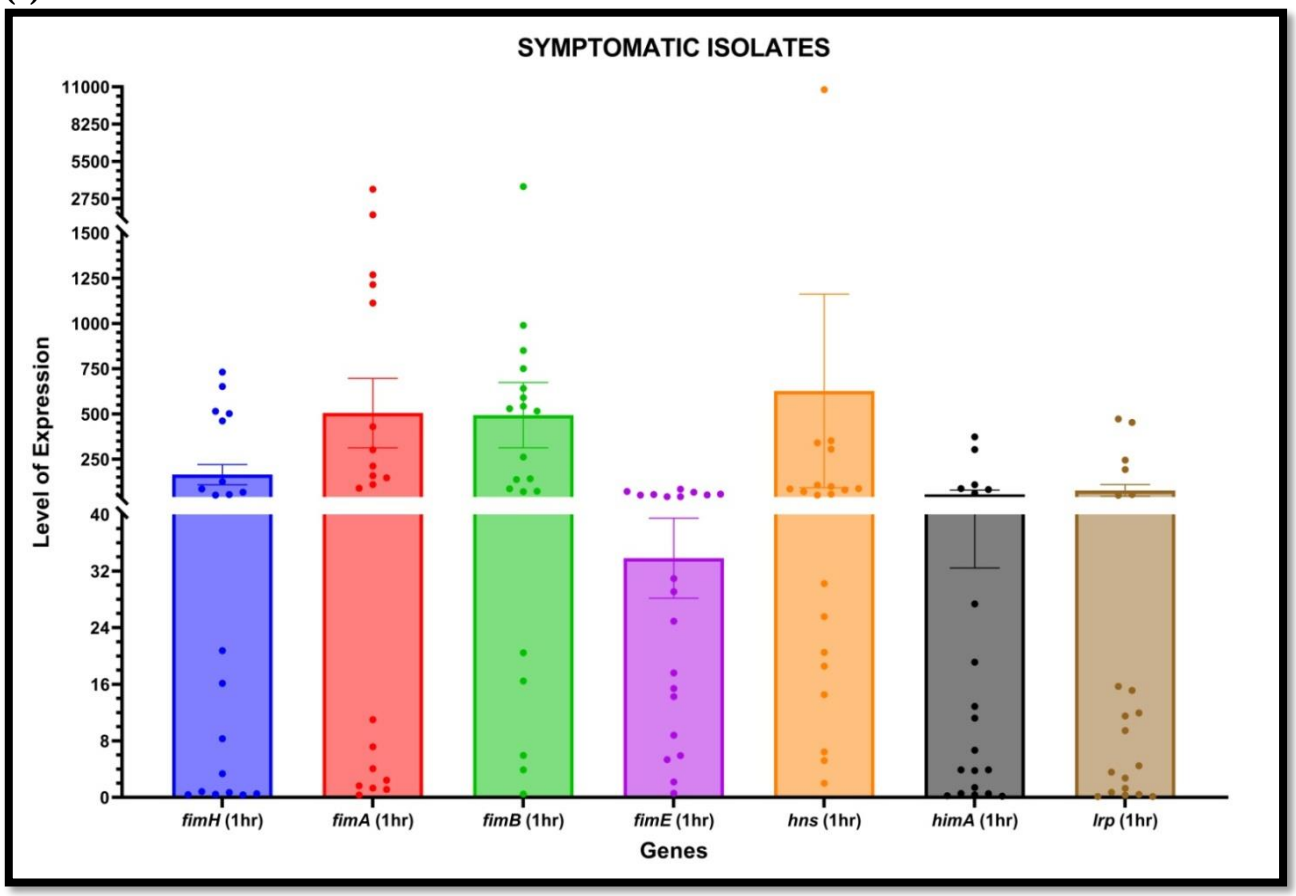

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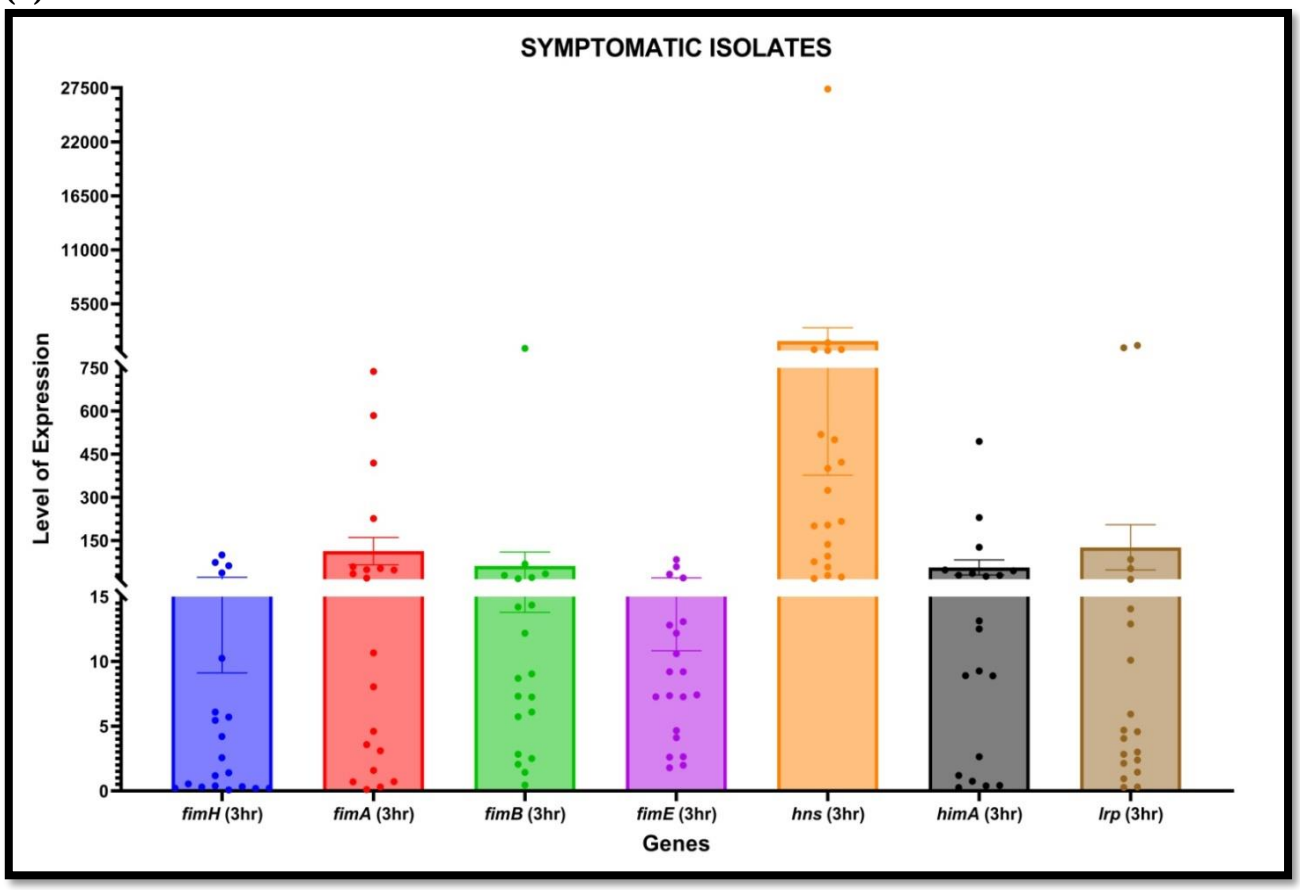
(d)



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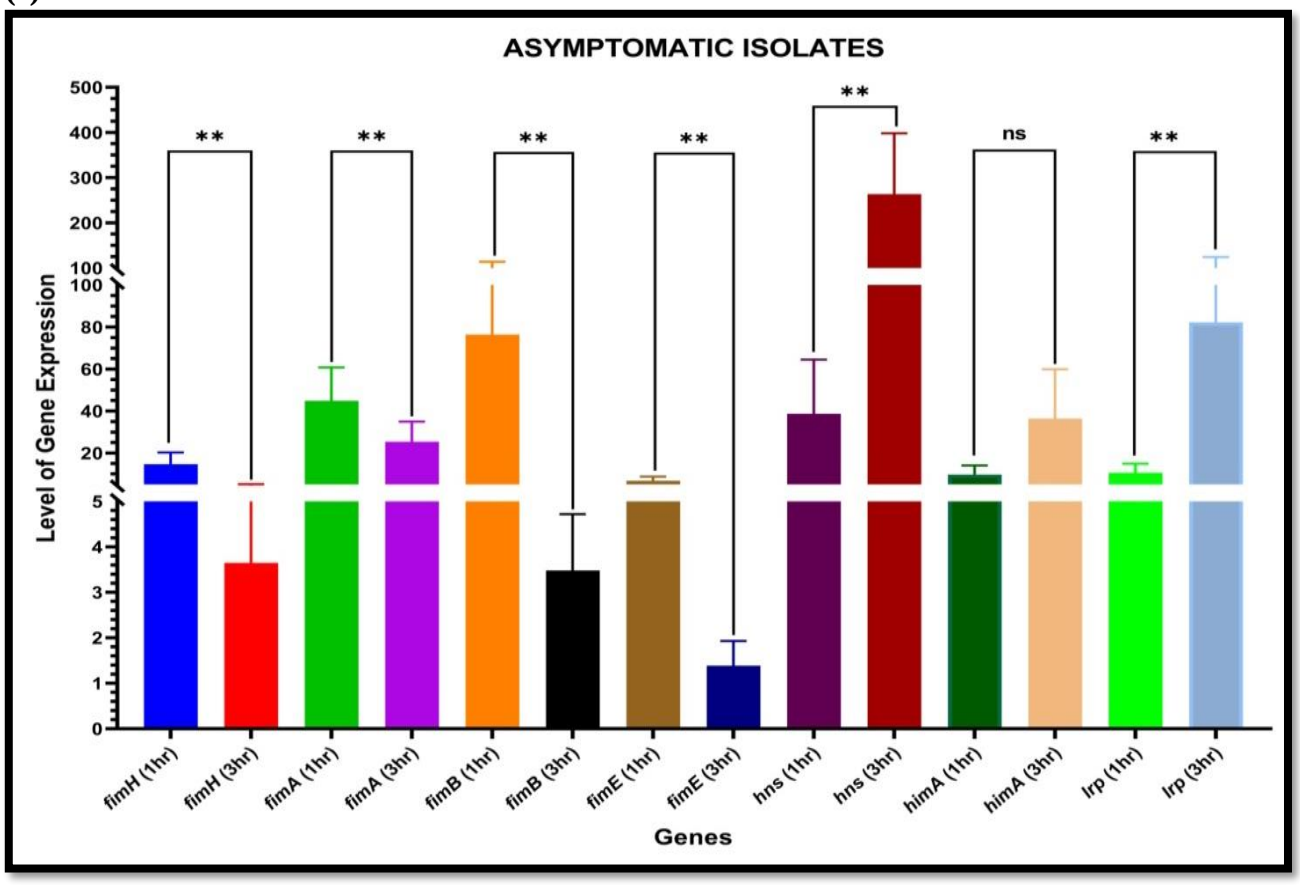

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(f)

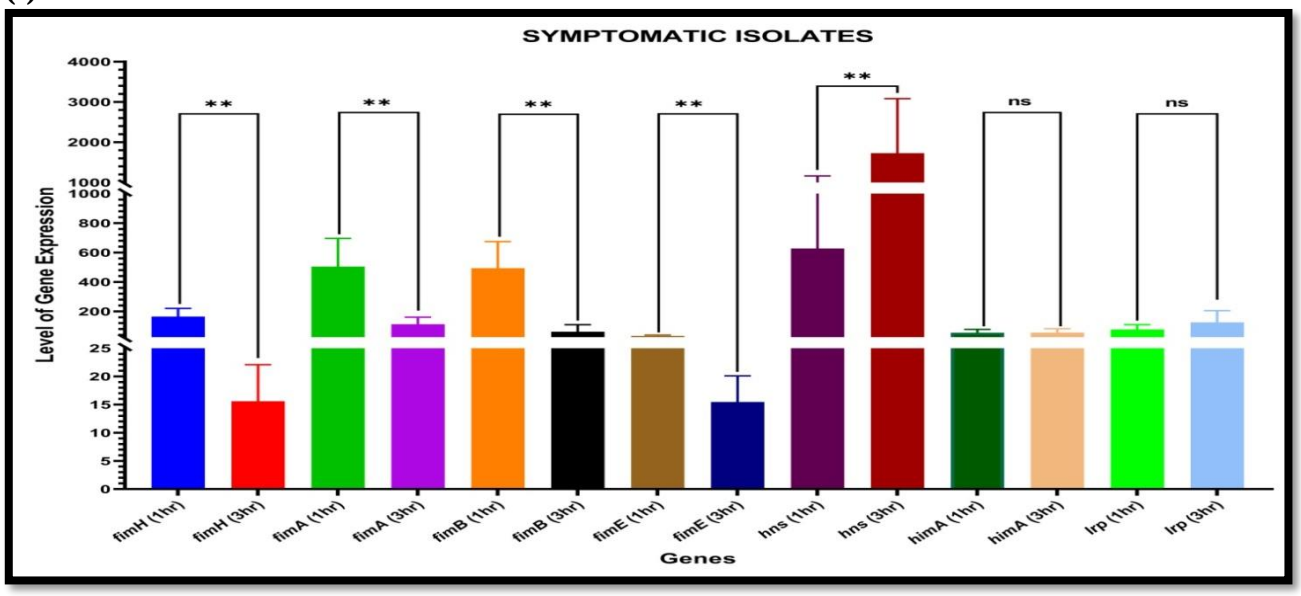

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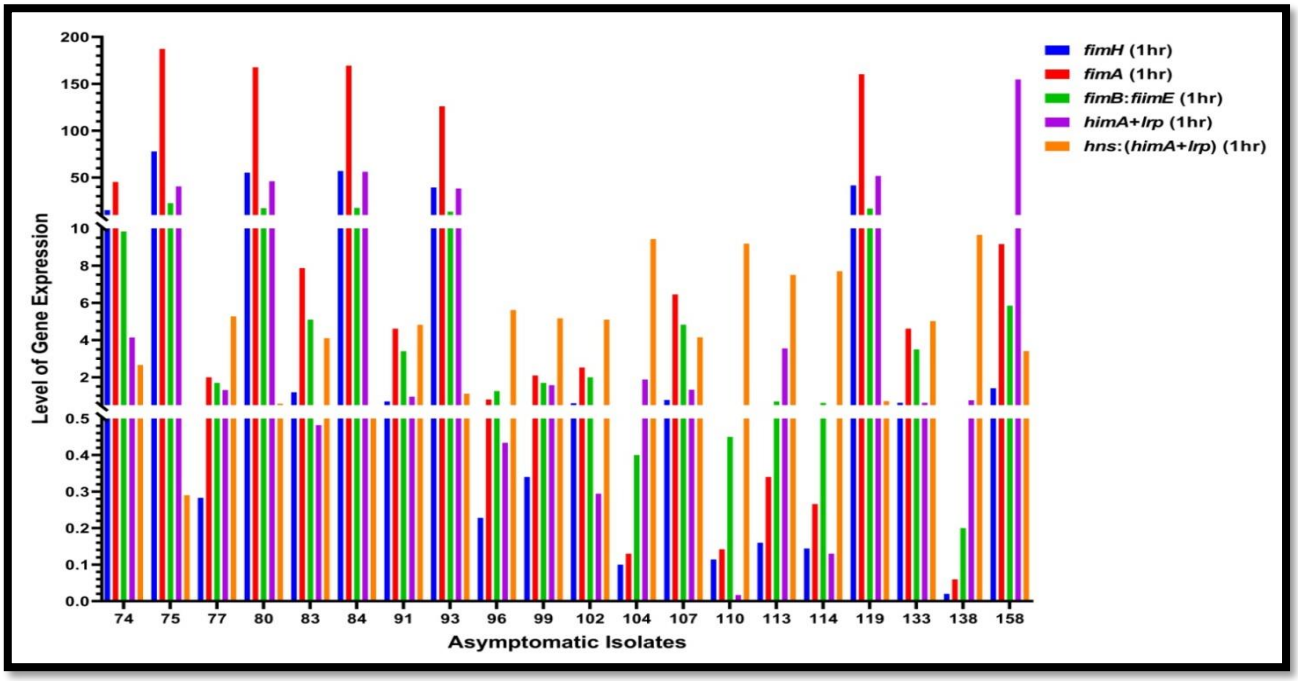
Fig. 5.10: 1 Graphical representation of the mean with standard error of mean (SEM) values of *fimH, fimA, fimB, fimE, hns, himA, lrp* at (a) 1hr of infection duration in asymptomatic UPECs (b) 3hrs of infection duration in asymptomatic UPECs (c) 1hr of infection duration in symptomatic UPECs (d) 3hrs of infection duration in symptomatic UPECs; and paired t- test values displaying difference in quantitative expression of the aforementioned genes between 1 and 3hrs of infection durations in (e) asymptomatic UPECs (f) symptomatic UPECs, based on their level of quantitative expression among asymptomatic and symptomatic UPECs generated using GraphPad Prism version 9 (Prism software package). Different genes were represented by scatter dot plots (a-d) and bar graphs (e-f) with varied colours. Error bars indicated the spread of data in case of each of the individual genes at the studied infection durations (a-d). Double stars (**) indicated significant difference (p value ≤ 0.01) of each of the individual gene between 1 and 3hrs of infection duration (e-f).

Over and above that, varied *fimB: fimE* and [*hns:* (*himA+lrp*)] ratios could be perceived together with the heterogeneous expressions of the *fimH* and *fimA* genes among both the asymptomatic (**Fig. 5.11a-b)** and symptomatic group **(Fig. 5.11c-d)** at 1 and 3hrs of infection respectively. However, the highest and lowest *fimB: fimE* ratio, deduced from the expression level of the said recombinases was found in asymptomatic (75; 138 and 84; 138) **(Fig. 5.11a-b)** and symptomatic (162; 112, and 86; 112) **(Fig. 5.11c-d)** isolates after 1 and 3 hrs of infections respectively. Nonetheless, the highest and lowest [*hns*: (*himA+lrp*)] ratio deduced from the expression level of the mentioned regulatory factors was found in asymptomatic (138; 75 and 138; 84) **(Fig. 5.11a-b)** and symptomatic (112; 162, and 112; 86) **(Fig. 5.11c-d)** isolates after 1 and 3 hrs of infections respectively. Furthermore, a decrease in the expression of *fimH* and *fimA* was perceived among the entire studied UPECs irrespective of their asymptomatic **(Fig. 5.8g)** and symptomatic (**Fig. 5.9g)** nature from 1 to 3hrs of infection**.** A decrease in expression of *fimB* and *fimE* genes was observed in the bulk of the adherent UPECs studied

irrespective of their asymptomatic **(Fig. 5.8h)** and symptomatic **(Fig. 5.9h)** nature from 1 to 3hr of infection**.** Withal, an increase in expression of the aforementioned regulatory factors could be noticed in the majority of the adherent ABU **(Fig. 5.8i)** and symptomatic UPECs **(Fig. 5.9i)** studied from 1 to 3hrs of infection. Likewise, an increase in *hns*: (*himA+lrp*) ratio together with the decrease in *fimB: fimE* ratio and expression of *fimH* and *fimA* genes was perceived in the adherent UPECs regardless of their asymptomatic **(Fig. 5.11e)** or symptomatic **(Fig. 5.11f)** nature from 1 to 3hrs of infections.

(a)



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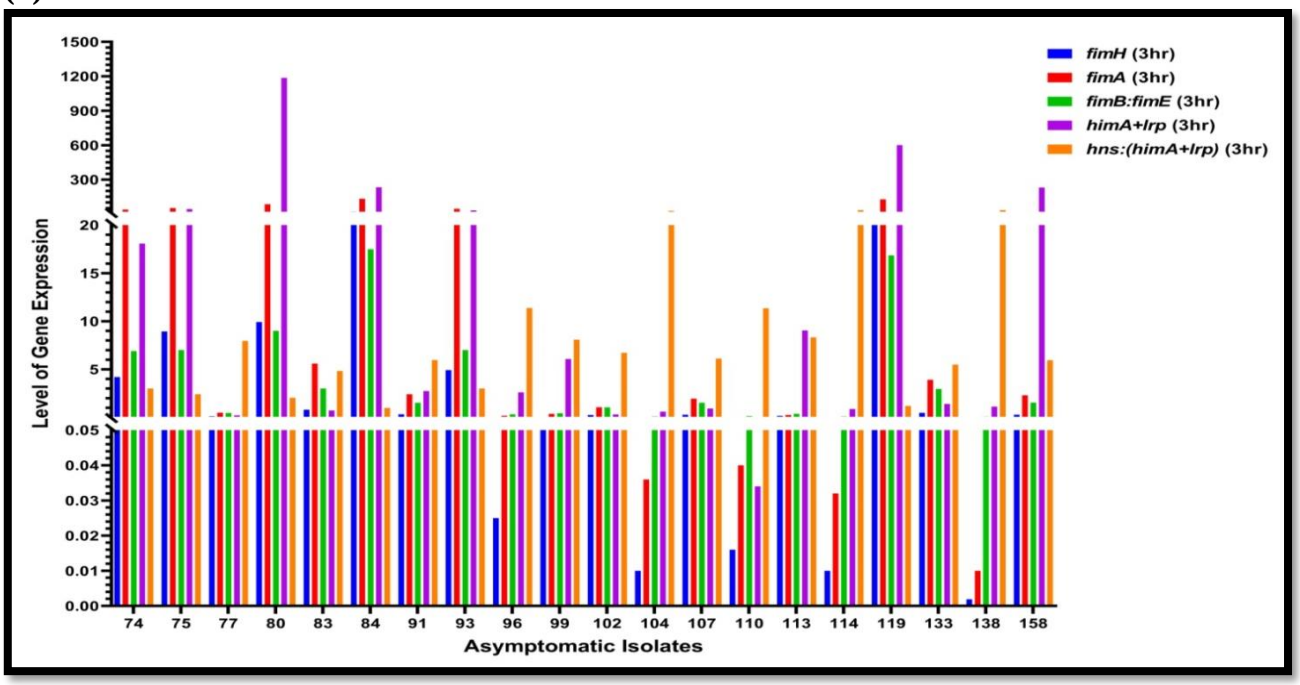
(b)



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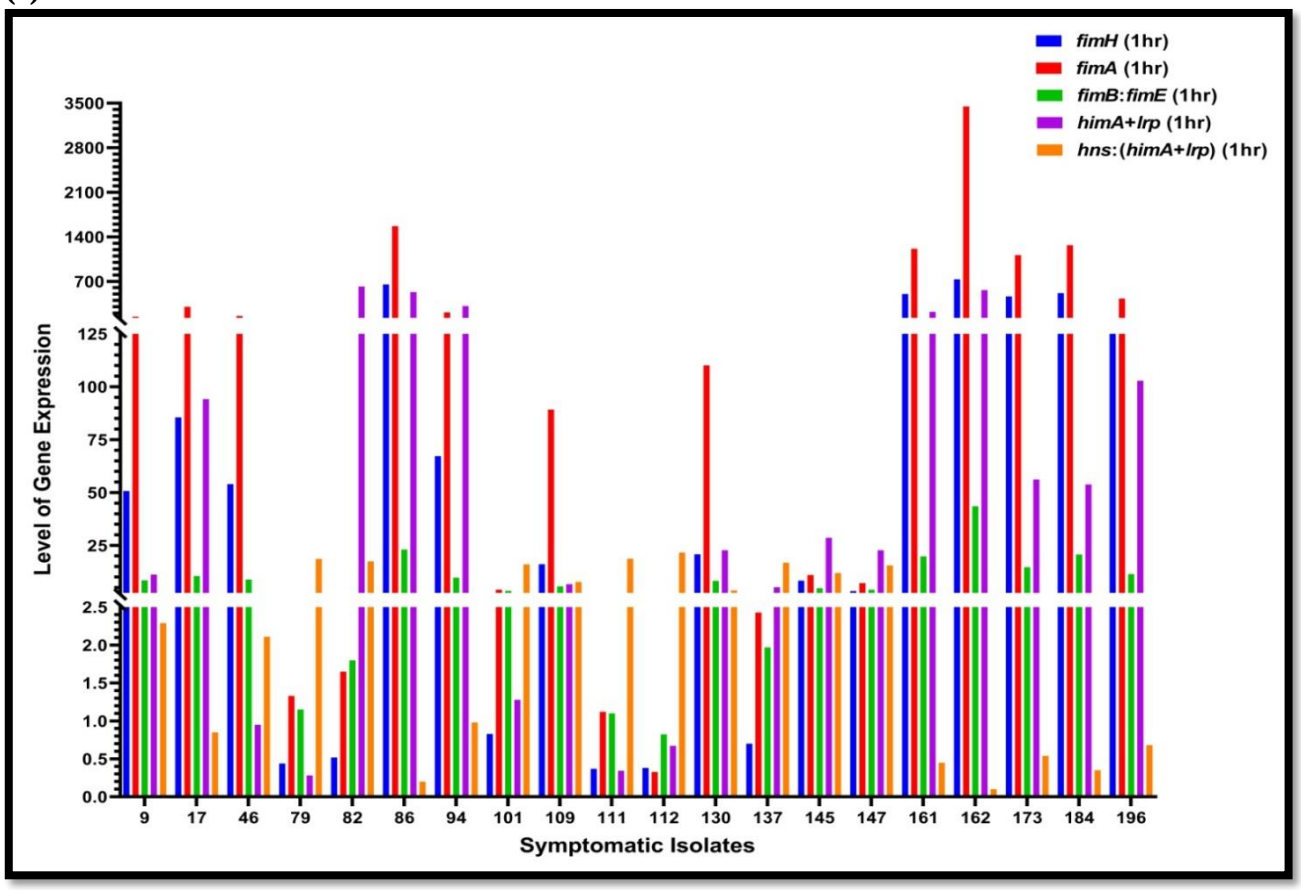

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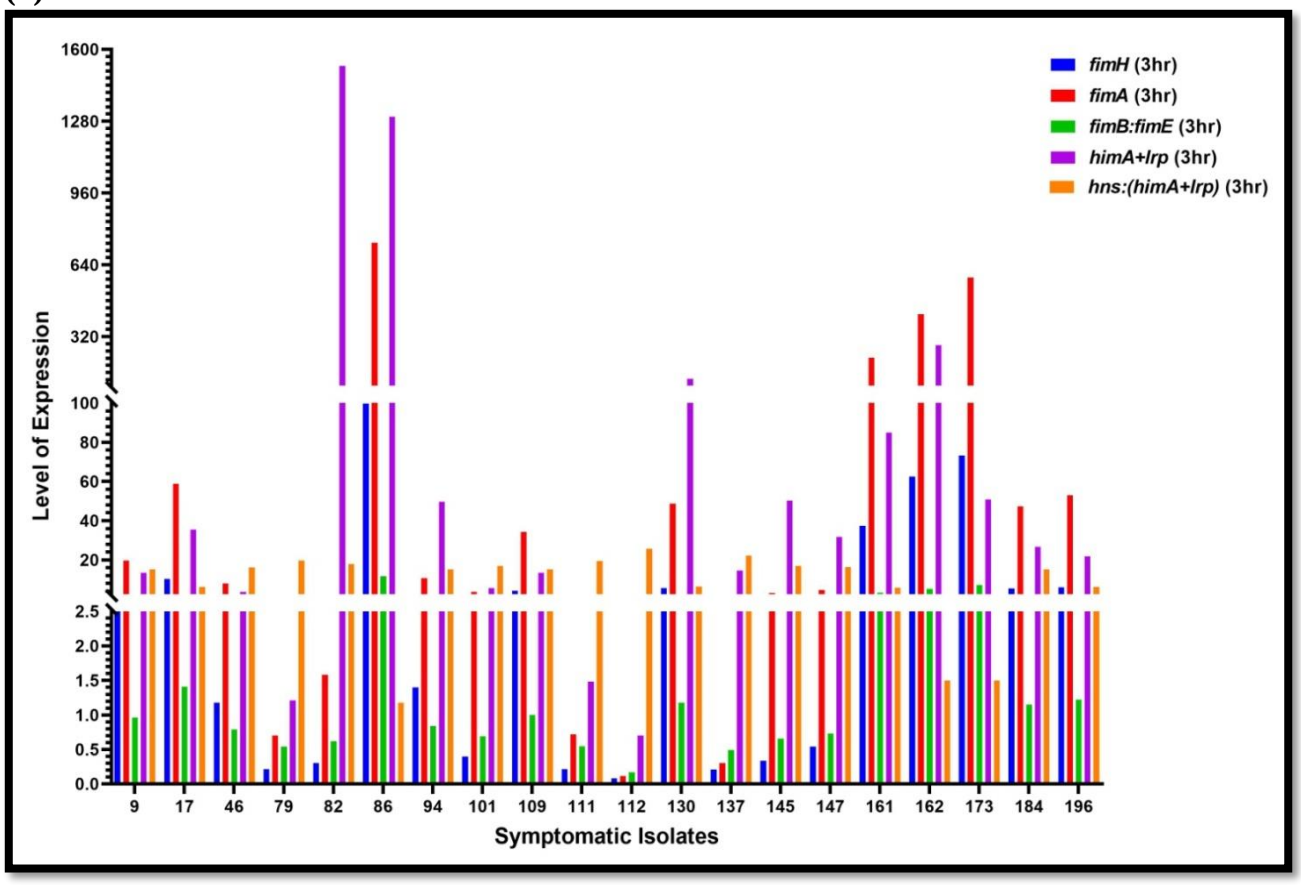

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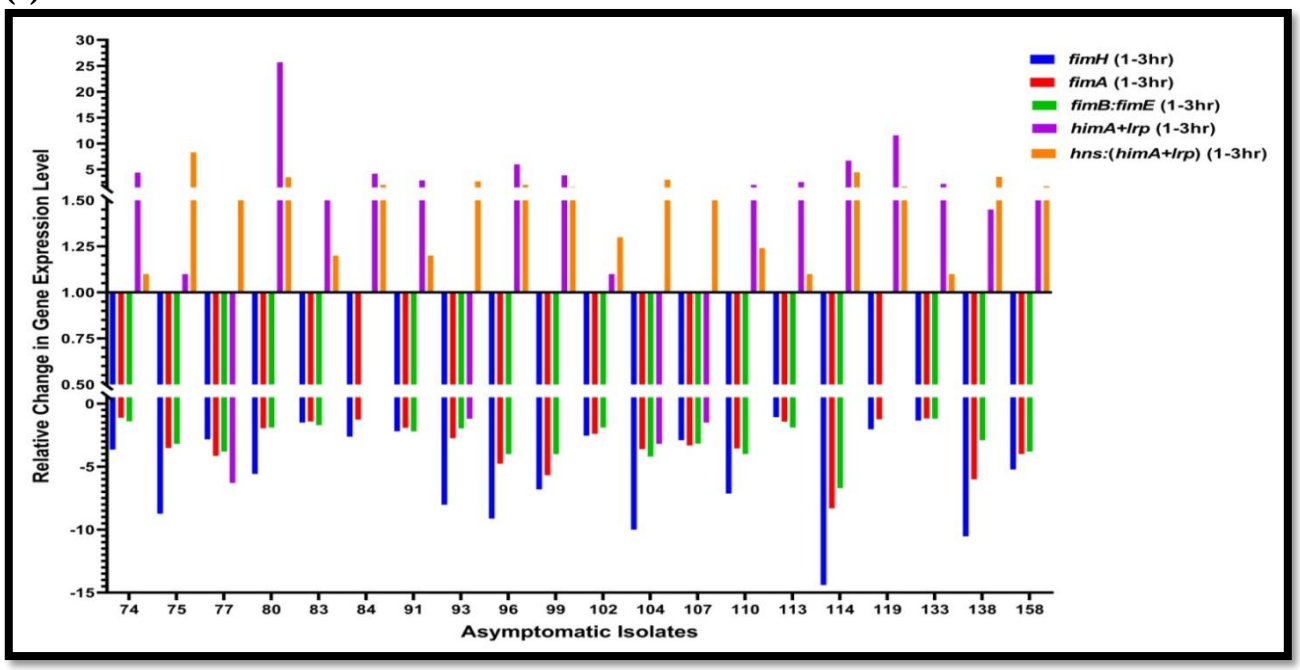

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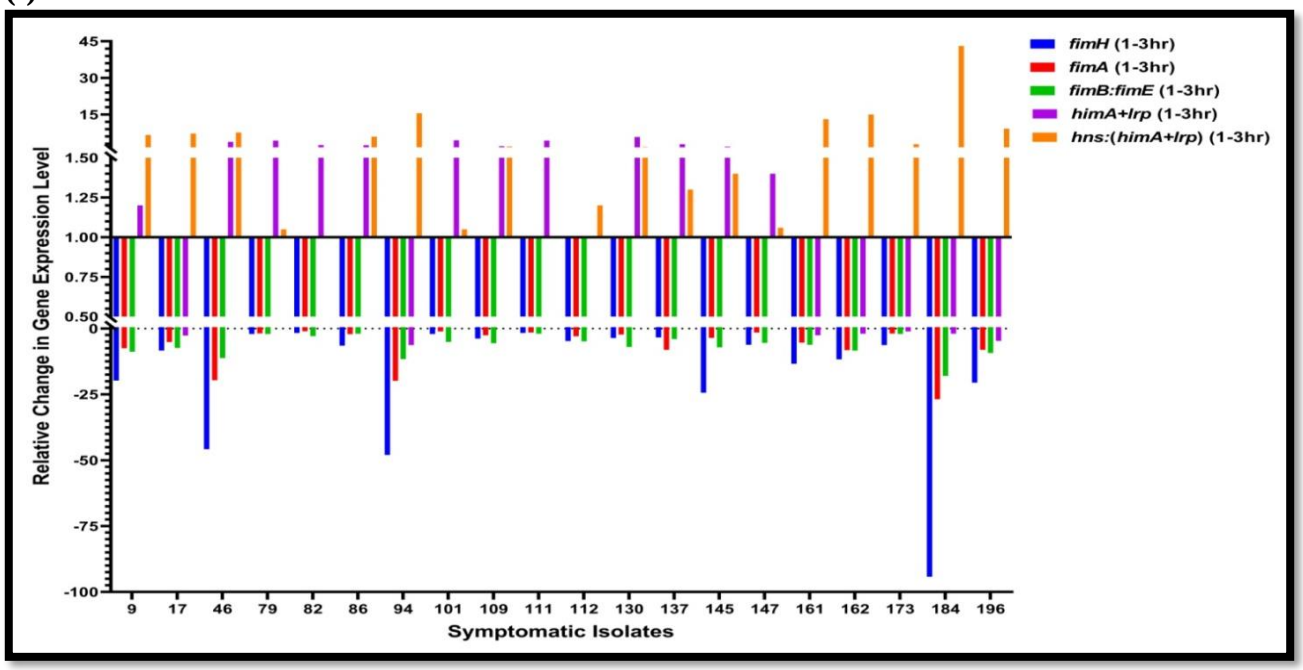


(This study)

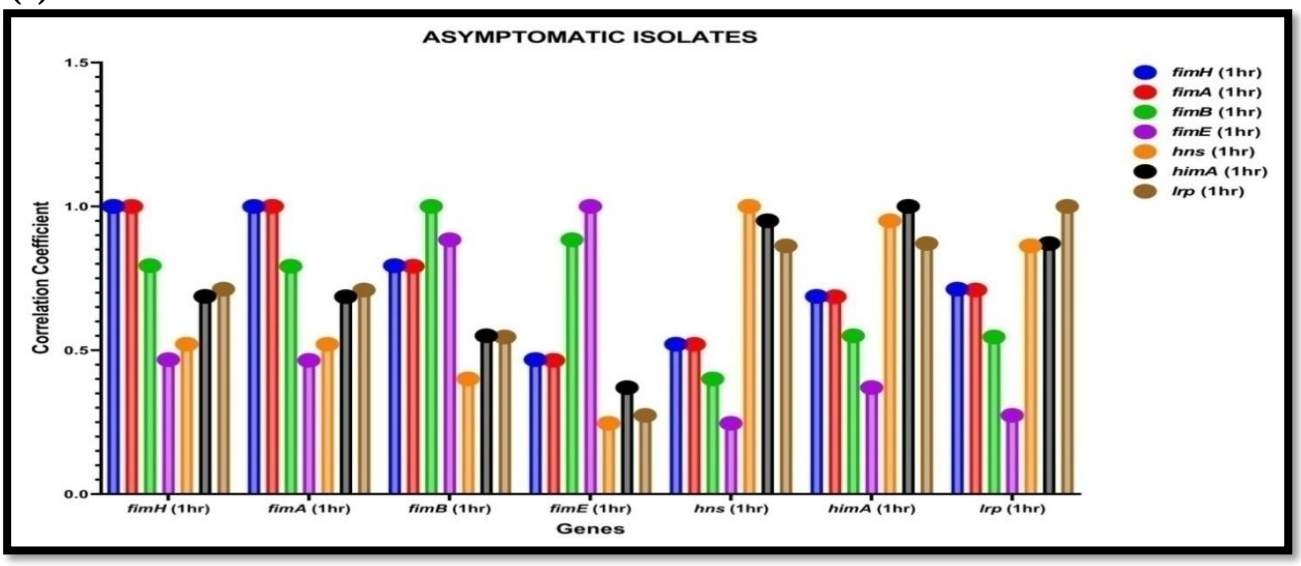
Fig. 5.11: Graphical representation of the level of quantitative expression of two type 1 fimbrial genes *fimH*, *fimA*, ratio of their recombinases (*fimB*: *fimE*), regulator combinations (*himA*+*lrp*) and ratio of regulator combinations [*hns*: (*himA*+*lrp*)] at different studied infection durations and also their relative changes in (a) asymptomatic UPECs [1hr] (b) asymptomatic UPECs [3hr] (c) symptomatic UPECs [1hr] (d) symptomatic UPECs [3hr] (e) asymptomatic UPECs [1to 3hr] (f) symptomatic UPECs [1to 3hr] computed with the $2^{-\Delta\Delta Ct}$ method using 16srRNA gene as reference and *E. coli* ATCC 25922 as a calibrator generated using GraphPad Prism version 9 (Prism software package). Different genes were represented by bar graphs with varied colours.

5.5.8 Correlation among the type 1 fimbrial genes, recombinase genes, and regulatory factor genes

The significant (p-value ≤ 0.05) positive and/or negative correlations that ranged from low to high was observed in the extent of expressions of the aforesaid fimbrial, recombinase, and regulatory factor genes **(Fig. 5.12a-c; Fig. 5.12d-f)** and their combinations **(Fig. 5.13a-c; Fig. 5.13d-f)** among both the ABU and symptomatic UPECs at 1, 3 and 1 to 3hrs of infections respectively. *fimH* and *fimA* expressions level were found to be strongly correlated (positive correlation) among isolates of both the asymptomatic (**Fig. 5.12a-c)** and symptomatic (**Fig. 5.12d-f)** groups with regard to the two aforementioned studied time durations and their relative changes from 1 to 3hrs of infections respectively. Withal, the *fimB* expression was found to be highly (positive correlation) correlated to *fimH* and *fimA* expressions among isolates of both the asymptomatic **(Fig. 5.12a-b)** and symptomatic **(Fig. 5.12d-e)** groups post 1 and 3hrs of infection respectively. However, low; low, and moderate; moderate positive correlations could be perceived in the level of *fimE with fimH; fimE with fimA* expressions among adherent ABU **(Fig. 5.12a)** and symptomatic **(Fig. 5.12d)** UPECs respectively, after 1 hr of infection. Nevertheless, post 3hrs, a significant positive correlation (low) relating to the expression of *fimE* could only be observed in the ABU UPECs when associated with the expressions of *fimH; fimA* (**Fig. 5.12b)**. High and low positive correlations were observed relating to the relative changes in expressions of the *fimB* and *fimE* recombinases respectively from 1 to 3hrs of infection with that the two other type 1 fimbrial genes in case of adherent symptomatic (**Fig. 5.12f)** UPECs, unlike the asymptomatic (**Fig. 5.12c)** ones. A moderate, low, moderate, strong, and strong positive correlations were perceived in the level of expressions of *hns* with *fimH*; *fimA, hns* with *fimB*; *fimE, himA* with *fimH*; *fimA*; *fimB, lrp* with *fimH*; *fimA* and *hns* with *himA; lrp* respectively among isolates of the asymptomatic (**Fig. 5.12a)** group, post 1hr of infection. However, post 3hrs of infection, moderate, moderate, strong, and strong positive correlations could be observed in the level of *hns* with *fimH*; *fimA, himA* with *fimH*; *fimA*; *fimB, lrp* with *fimH*; *fimA*; *fimB* and *hns* with *himA; lrp* expressions respectively among adherent ABU **(Fig. 5.12b)** UPECs. However, very low to moderate positive correlations (*hns* with *fimH*; *fimA*; *fimB*; *fimE* and *himA* with *fimH*; *fimA*; *fimB*; *fimE*) and high positive correlations (lrp with *fimH*; *fimA, hns* with *himA* and *himA* with *lrp*) were observed in the level of expressions of the aforementioned genes among symptomatic UPECs after 1(**Fig. 5.12d)** hr and/or 3 (**Fig. 5.12e)** hrs of infection respectively. However, relating to the relative change in *hns* expression from 1 to 3hrs of infection, moderate negative correlations were perceived with the two type 1 fimbrial genes and the recombinase *fimB* in the case of adherent symptomatic (**Fig. 5.12f)** UPECs, unlike the asymptomatic (**Fig. 5.12c)** ones. High positive correlations could be observed between *fimH;* (*fimB: fimE)* and *fimA;* (*fimB: fimE)*; respectively among all isolates irrespective of their asymptomatic **(Fig. 5.13a-c)** or symptomatic **(Fig. 5.13d-f)** nature at 1, 3 and 1to 3hrs (relative changes in the expression)

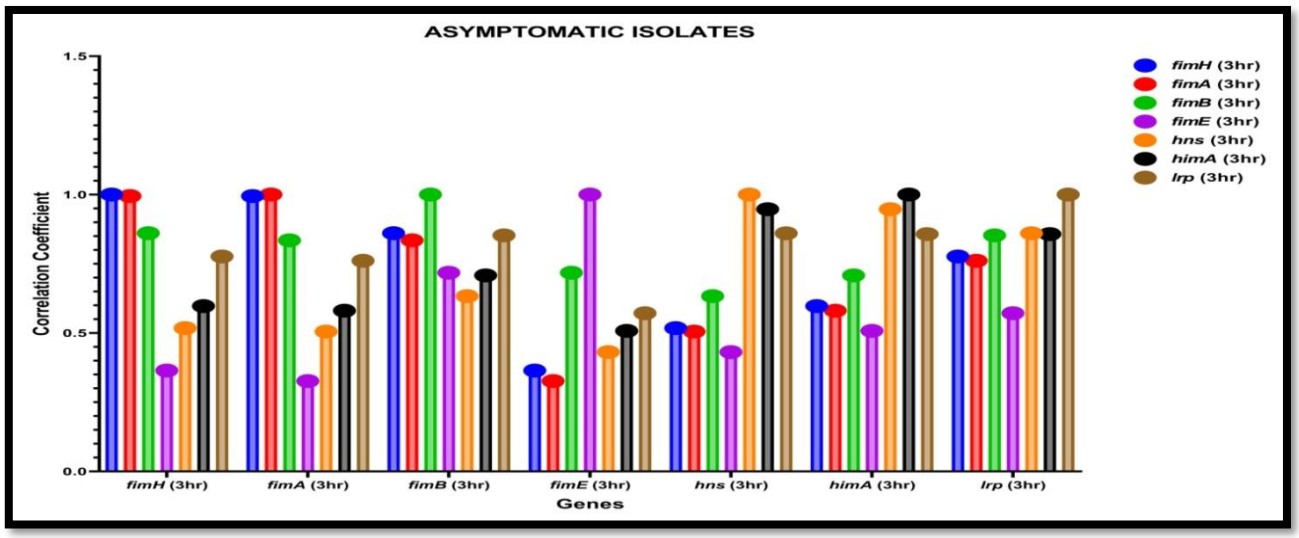
of infections respectively. A low to high correlation was observed between the combination of expressions of *himA* and *lrp* (*himA+lrp*) with that of two type 1 fimbrial genes and the ratio of their recombinases (*fimB: fimE*) among ABU **(Fig. 5.13a-c)** and symptomatic **(Fig. 5.13d-f)** UPECs, with regard to both 1 and 3hrs of infections and also their relative changes. Negative correlations that varied from moderate to high could be observed between various combination [{*hns*: (*himA+lrp*); *fimH}*, {*hns*: (*himA+lrp*); *fimA*}, {*hns*: (*himA+lrp*)]; (*fimB: fimE*)}, and {*hns*: (*himA+lrp*); (*himA+lrp*)}] of the aforementioned genes among all adherent isolates irrespective of their asymptomatic **(Fig. 5.13a-b)** or symptomatic **(Fig. 5.13d-e)** nature at both the studied time points. Nonetheless, strong negative correlations were perceived between the combinations *hns*: (*himA+lrp*); *fimH*, *hns*: (*himA+lrp*); *fimA*, *hns*: (*himA+lrp*); *fimB: fimE* among both ABU **(Fig. 5.13a-c)** and symptomatic UPECs **(Fig. 5.13d-f)** except ABU UPECs (1 to 3hrs relative changes) **(Fig. 5.13c)**.

(a)



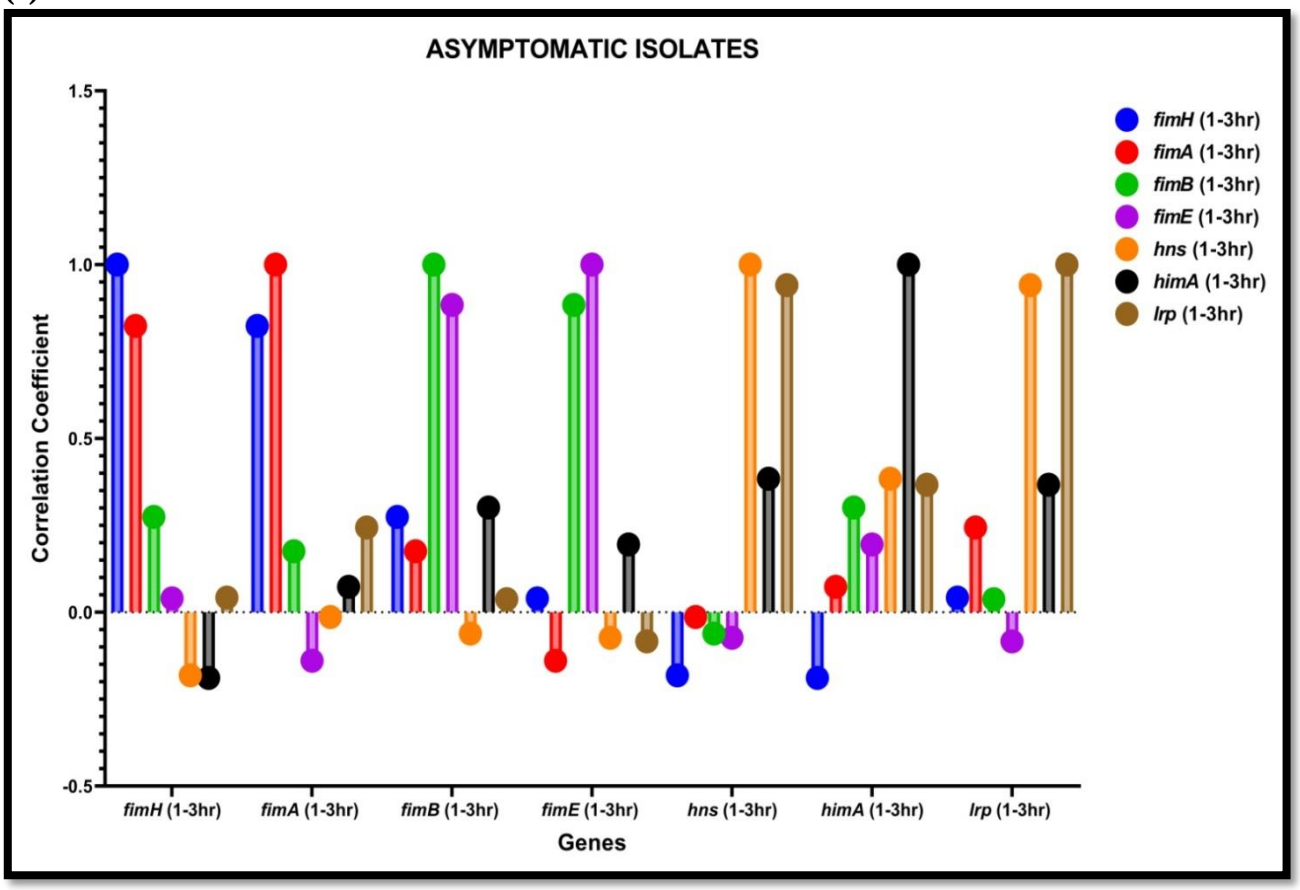
(This study)

(b)



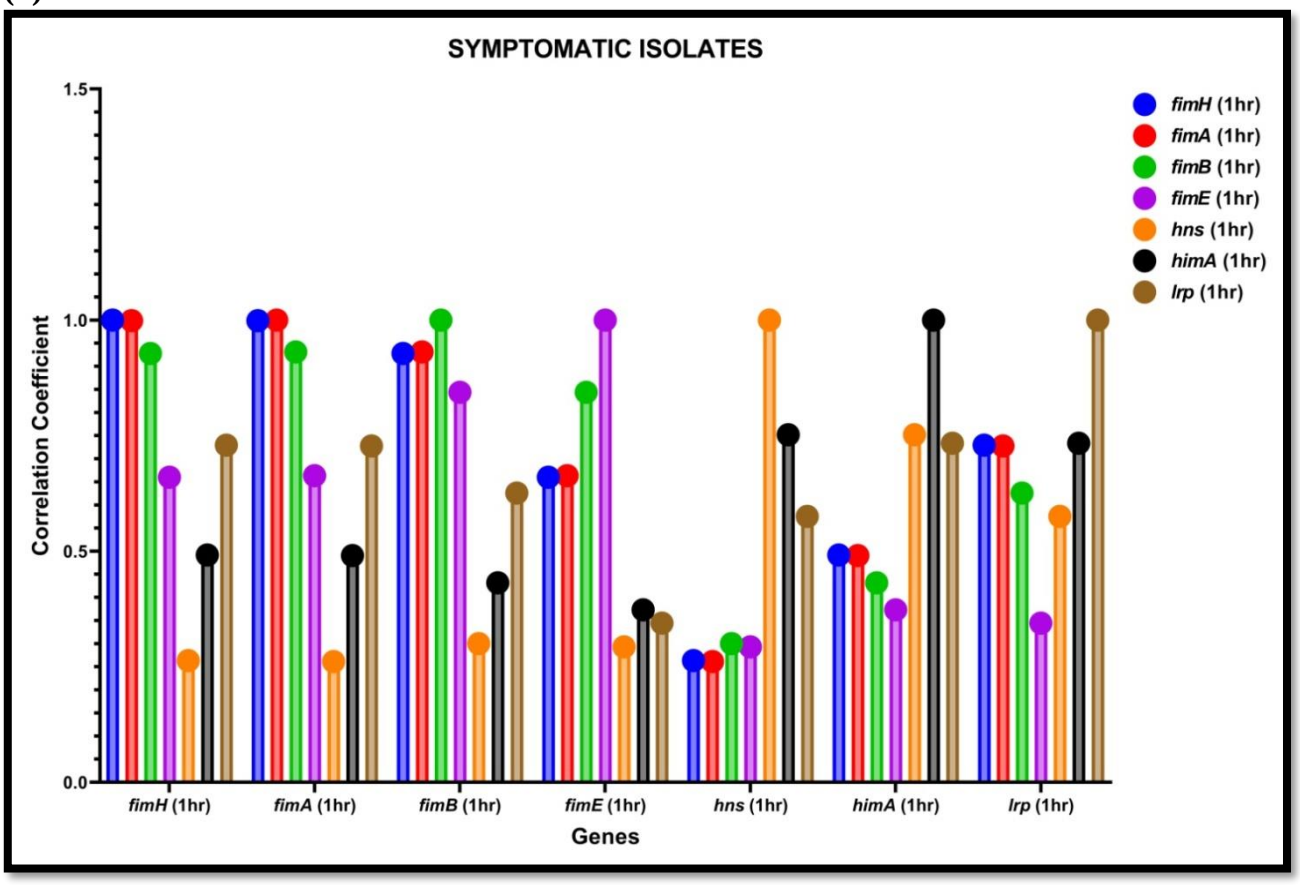
(This study)

(c)



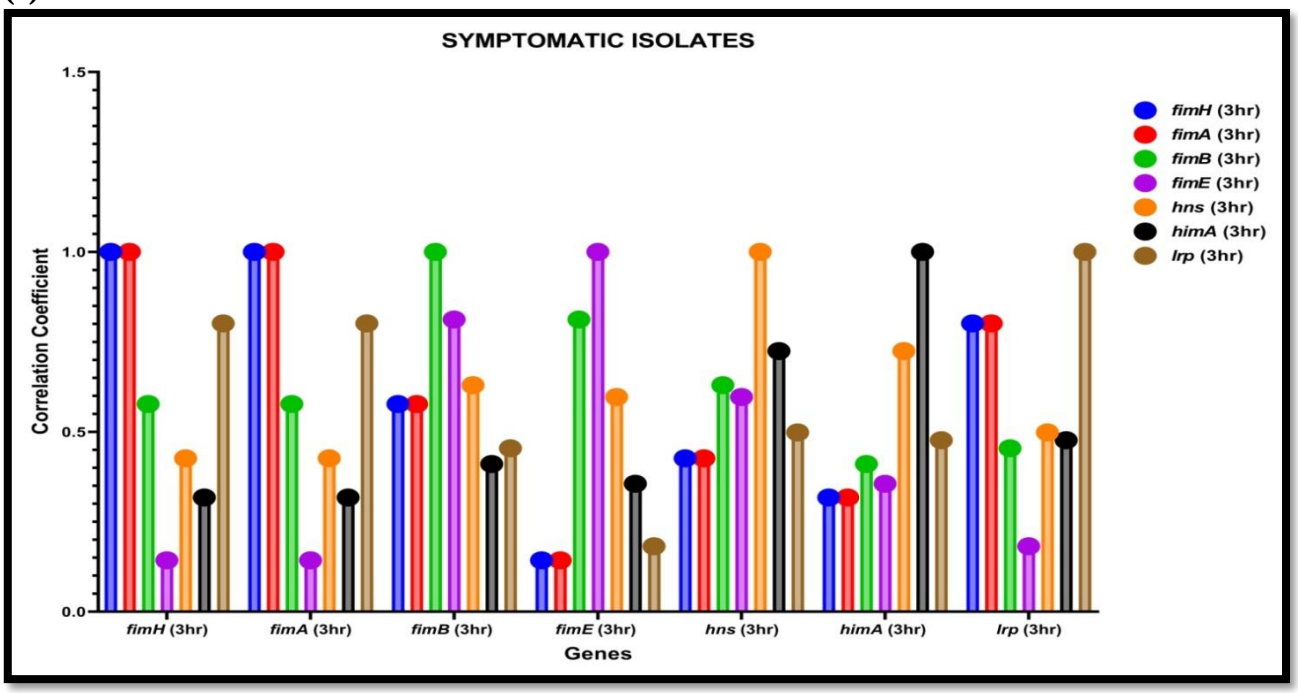
(This study)

(d)



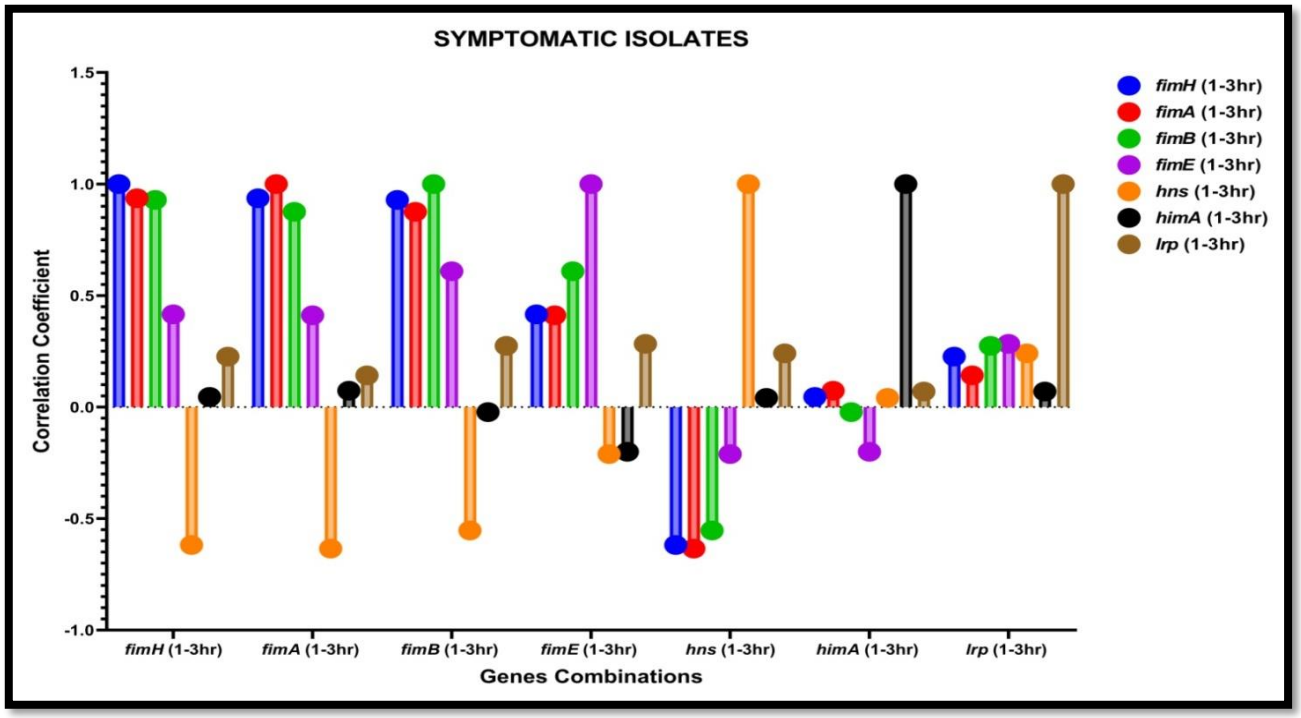
(This study)

(e)



(This study)

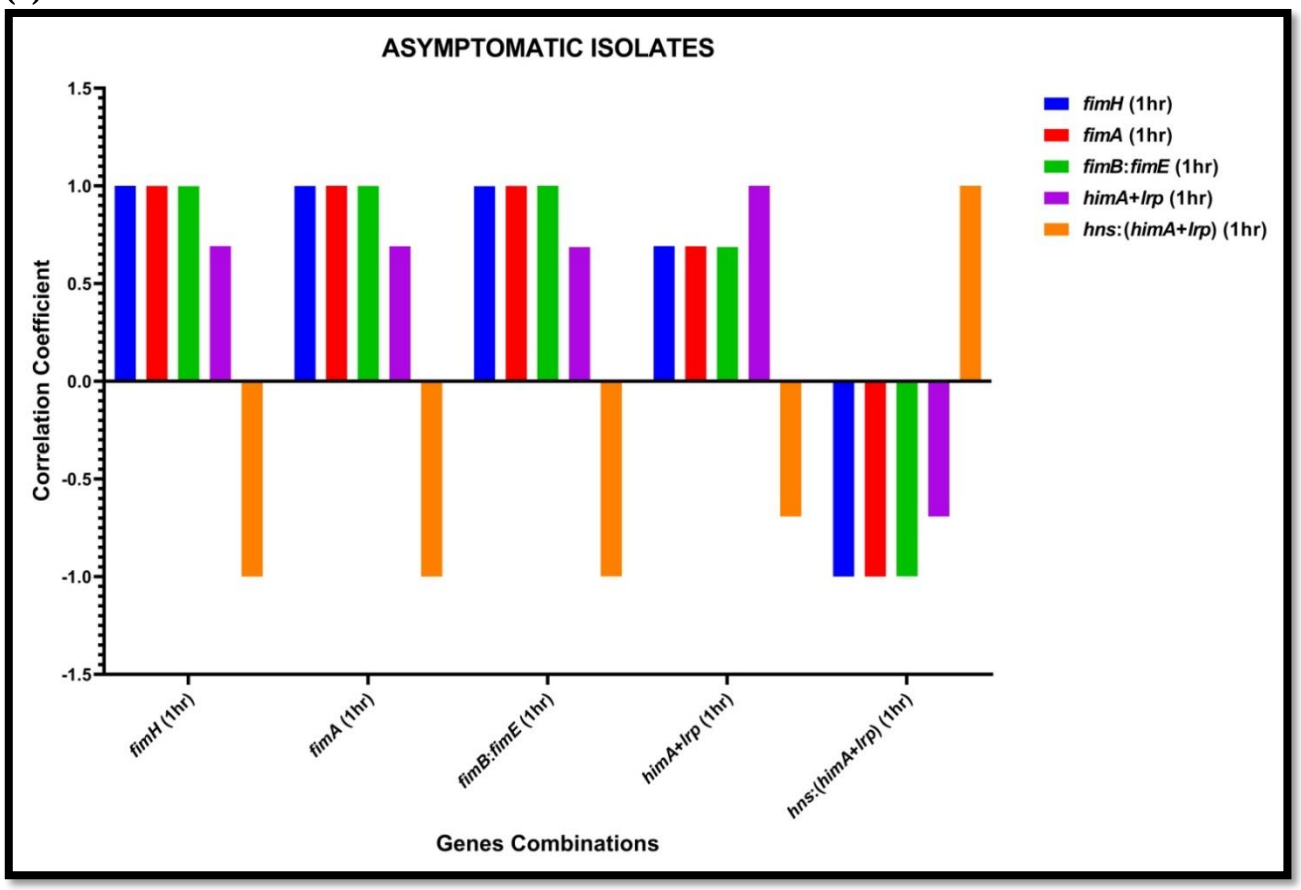
(f)



(This study)

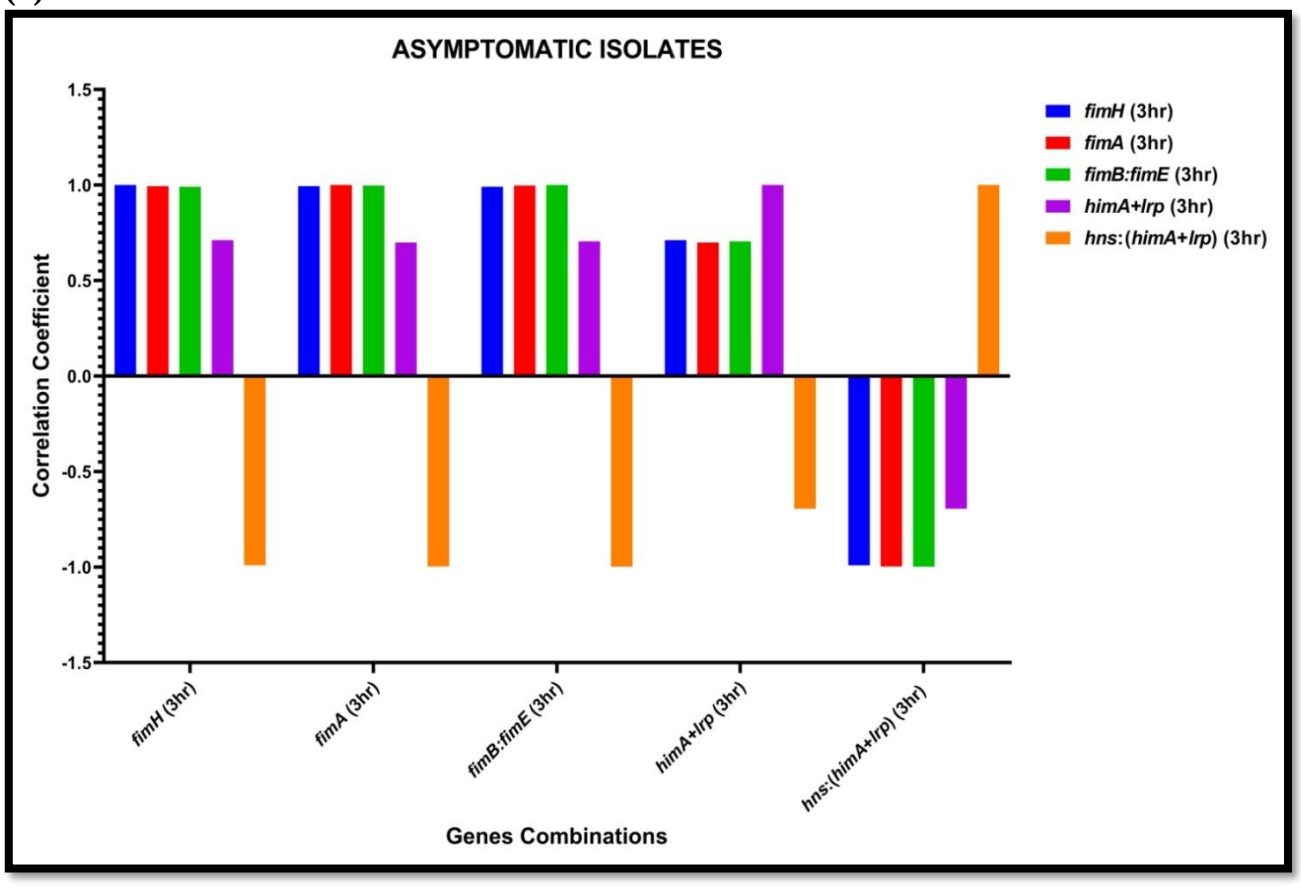
Fig. 5.12: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of quantitative expression of seven different genes (*fimH*, *fimA*, *fimB*, *fimE*, *hns*, *himA*, *lrp*) in adherent asymptomatic UPECs at (a) 1hr of infection duration (b) 3hrs of infection duration (c) 1to 3hrs (relative changes) and symptomatic UPECs at (d) 1hr of infection duration (e) 3hrs of infection duration (f) 1to 3hrs (relative changes) UPECs. Different genes were represented by scatter dot plots with varied colours.

(a)



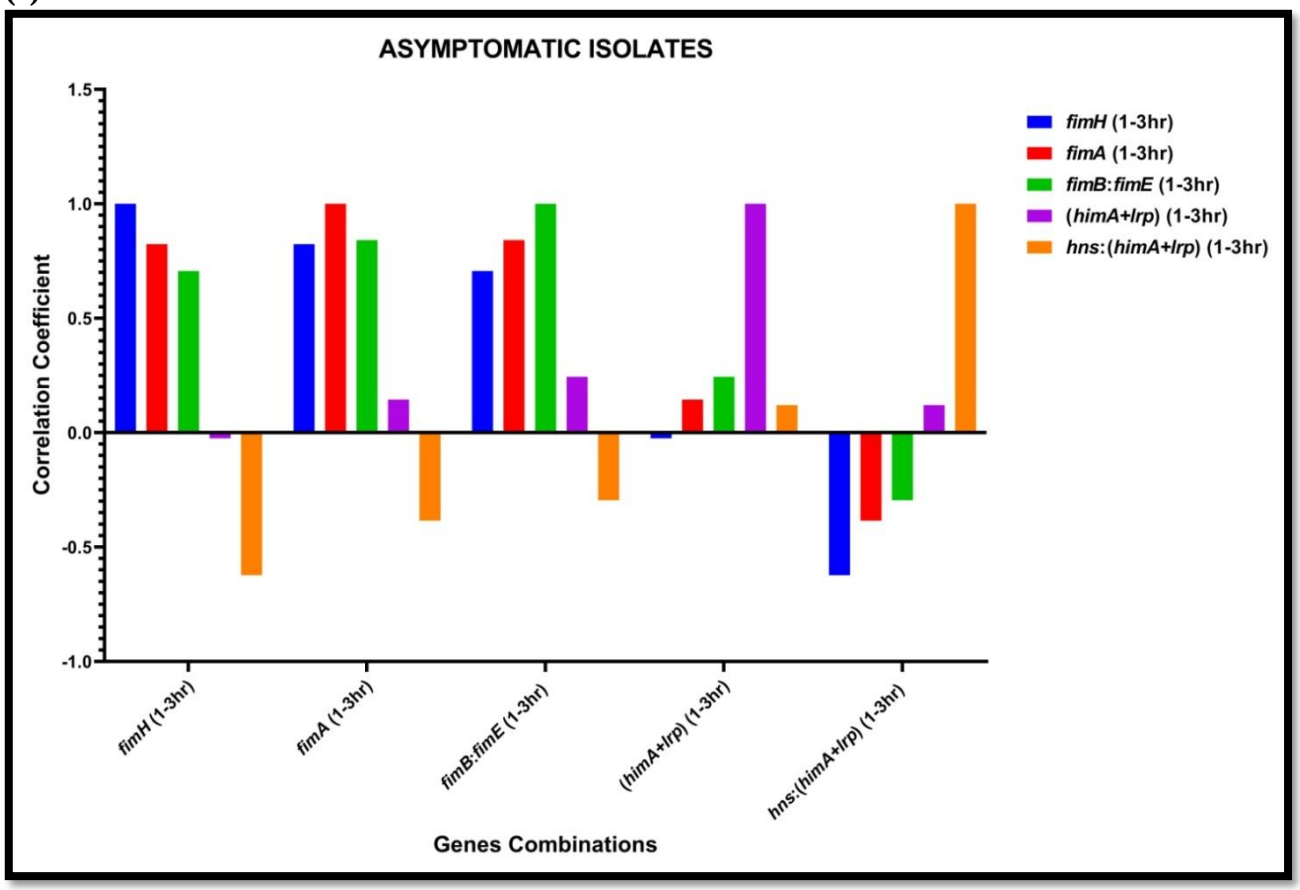
(This study)

(b)



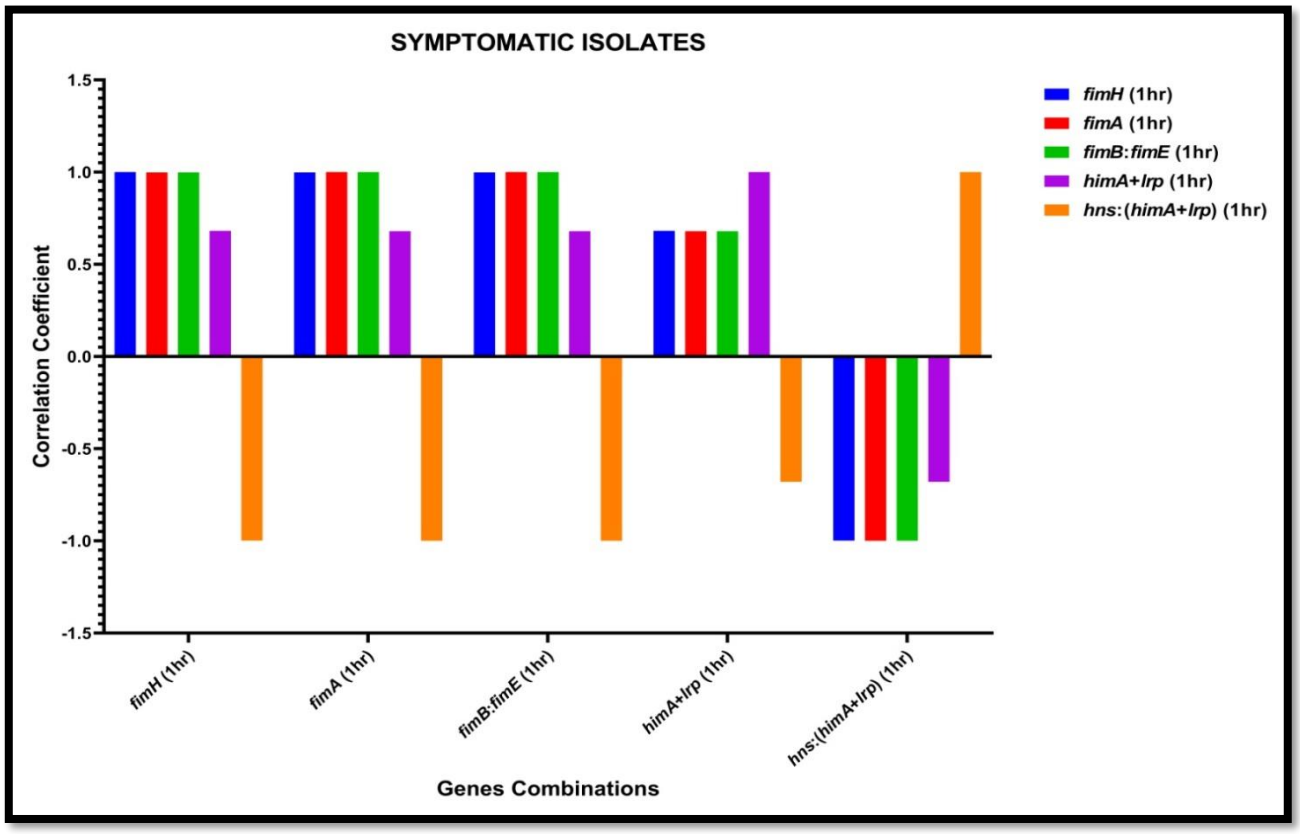
(This study)

(c)



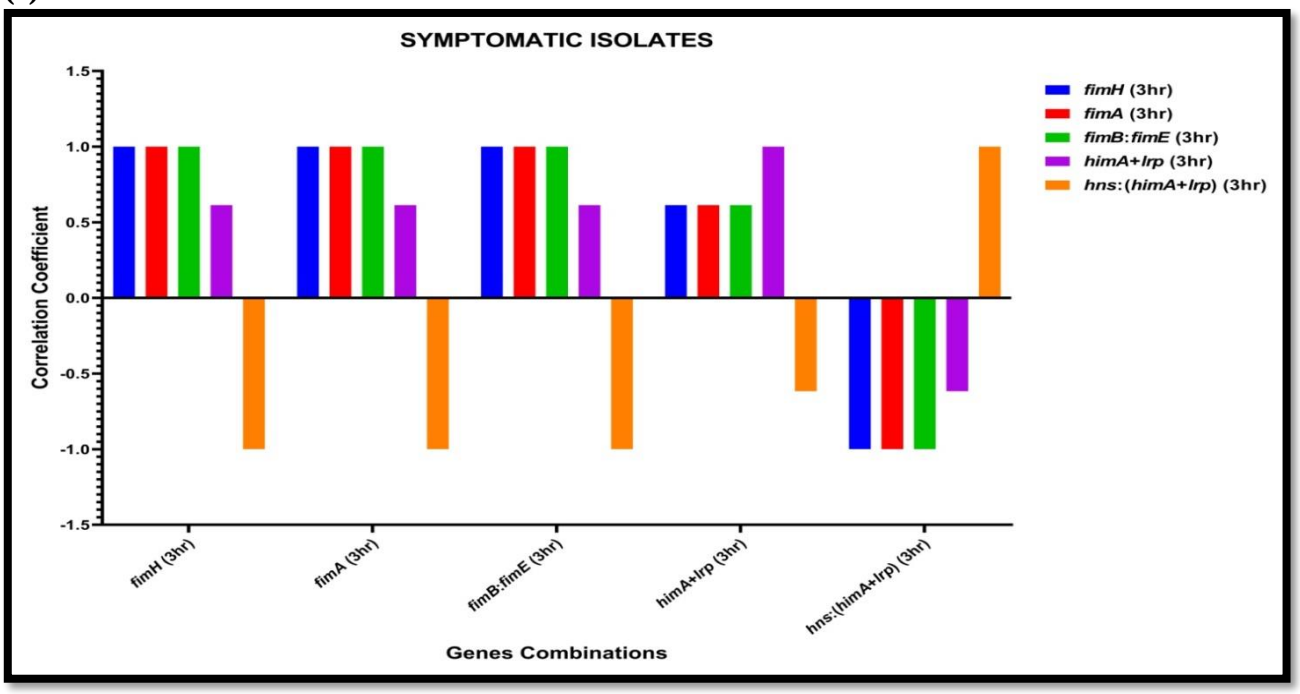
(This study)

(d)



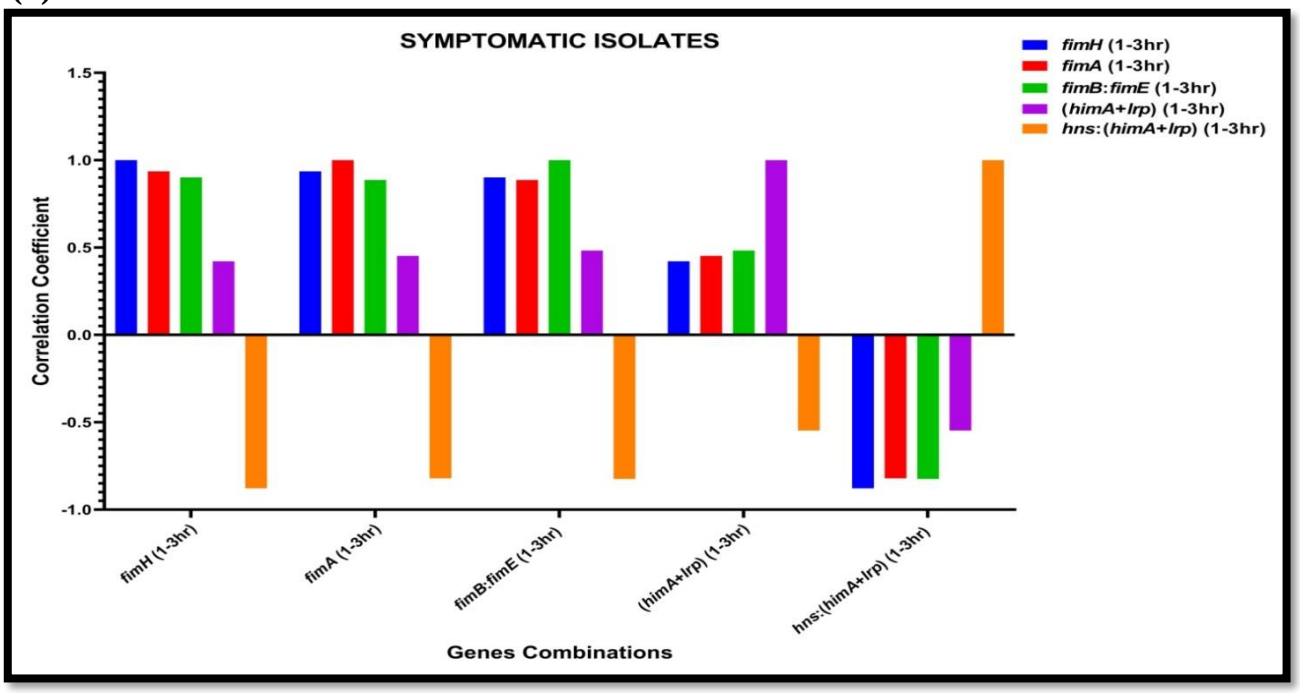
(This study)

(e)



(This study)

(f)



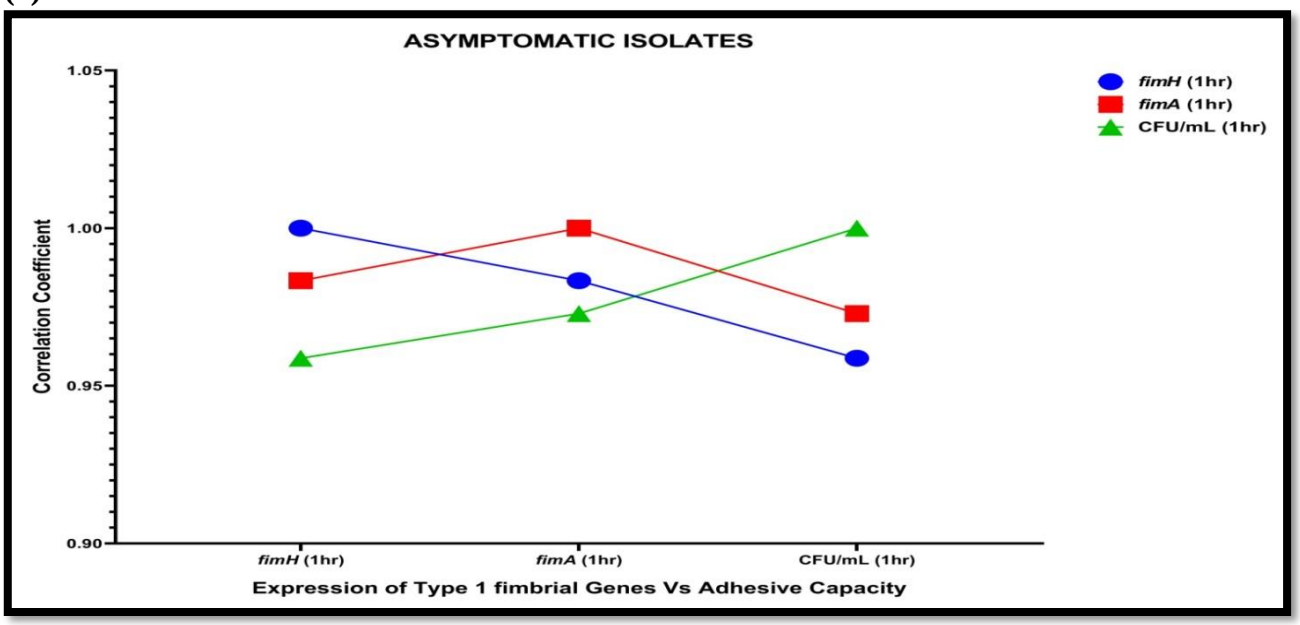
(This study)

Fig. 5.13: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of quantitative expression of two type 1 fimbrial genes *fimH*, *fimA*, ratio of their recombinases (*fimB*: *fimE*), regulator combinations (*himA+lrp*) and ratio of regulator combinations [*hns*: (*himA+lrp*)] at different studied infection durations and also their relative changes in asymptomatic (a) 1hr (b) 3hr (c) 1 to 3hrs relative changes and symptomatic UPECs (d) 1hr (e) 3hr (f) 1 to 3hrs relative changes. Different genes were represented by bar graphs with varied colours.

5.5.9 Interrelationships between the Type 1 fimbrial genes (*fimH*, *fimA*) and adhesive capacity of UPECs

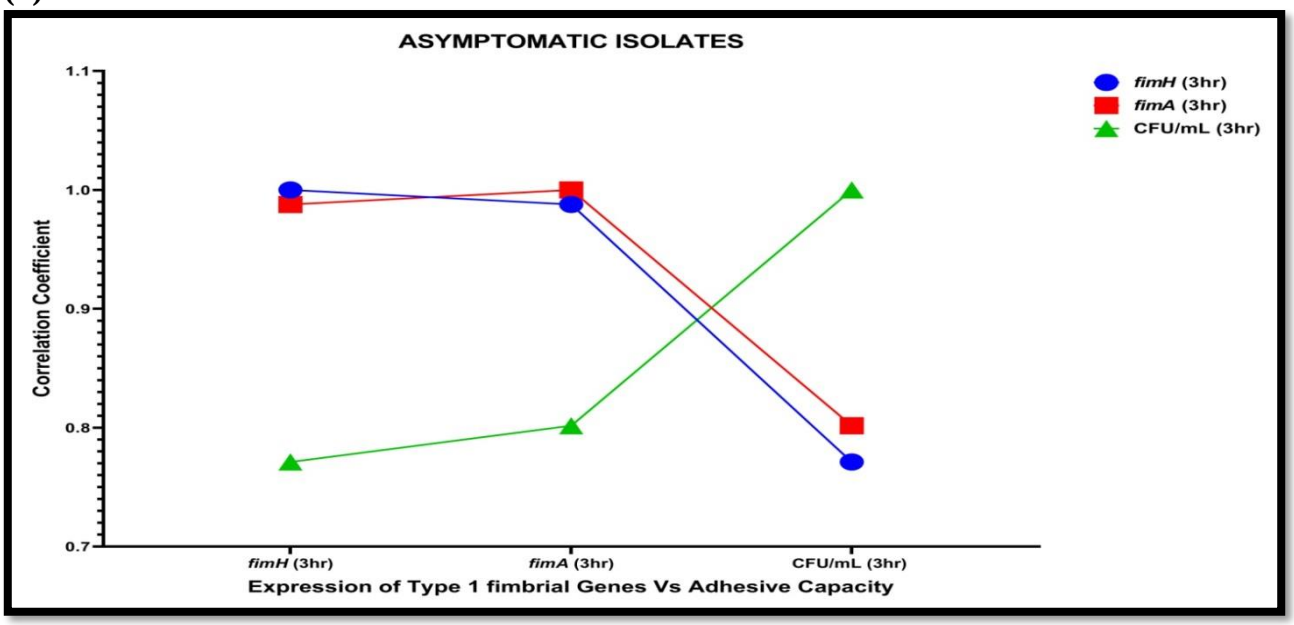
A strong positive correlation was discerned in the expression level of *fimH*, *fimA*, and infection potential (adhesive capacity; CFU/mL) among both ABU **(Fig. 5.14a-b)** and symptomatic UPECs **(Fig. 5.14c-d)** at both the time durations. However, the extent of correlation was lower at 3hrs duration compared to the 1hr. Nonetheless, a significant but moderate level of negative correlation **(Fig. 5.14f)** between relative changes in the echelon of expression of *fimH*, *fimA* with that of the adhesive capacity was perceived among the symptomatic UPECs from 1 to 3hrs of infection unlike the asymptomatic ones **(Fig. 5.14e)**.

(a)



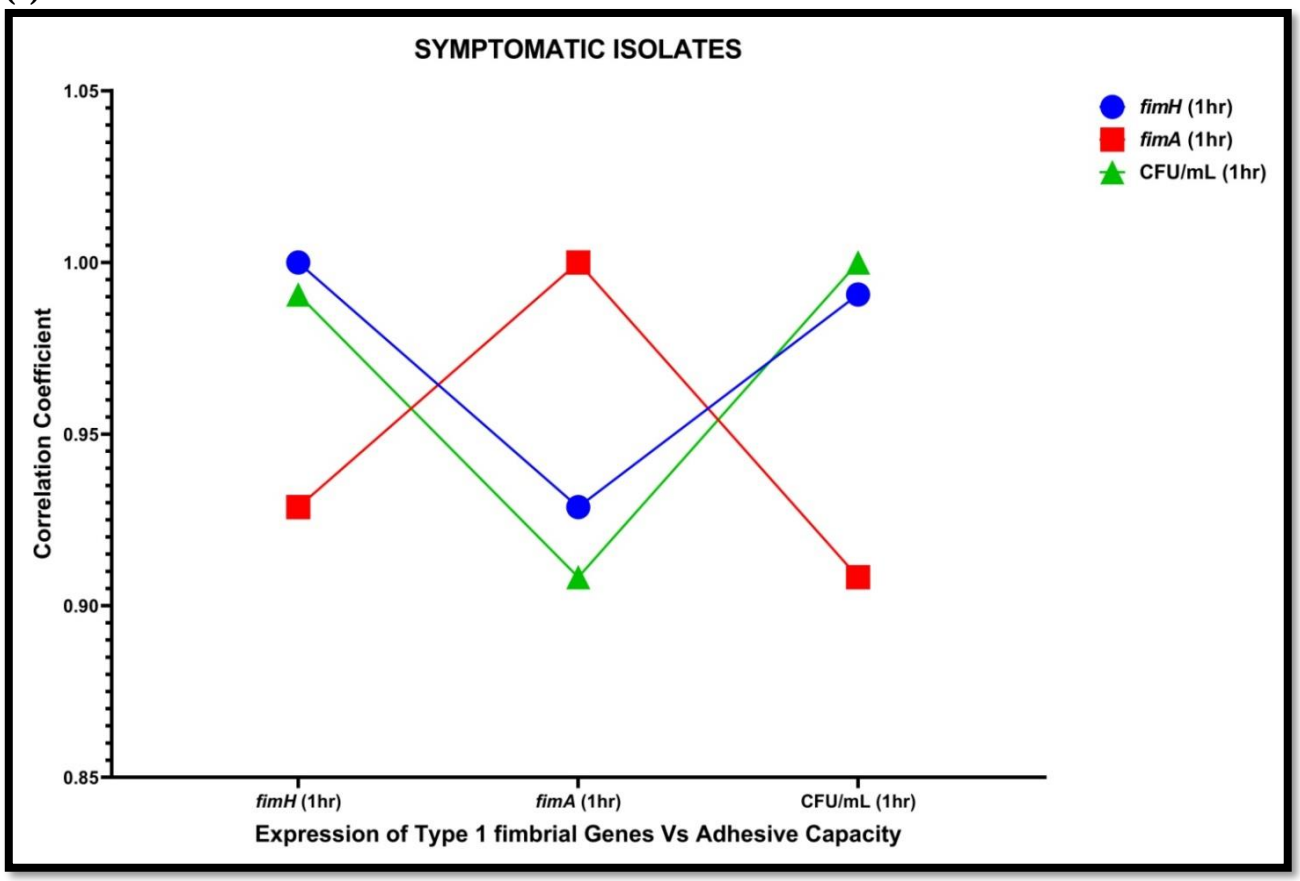
(This study)

(b)



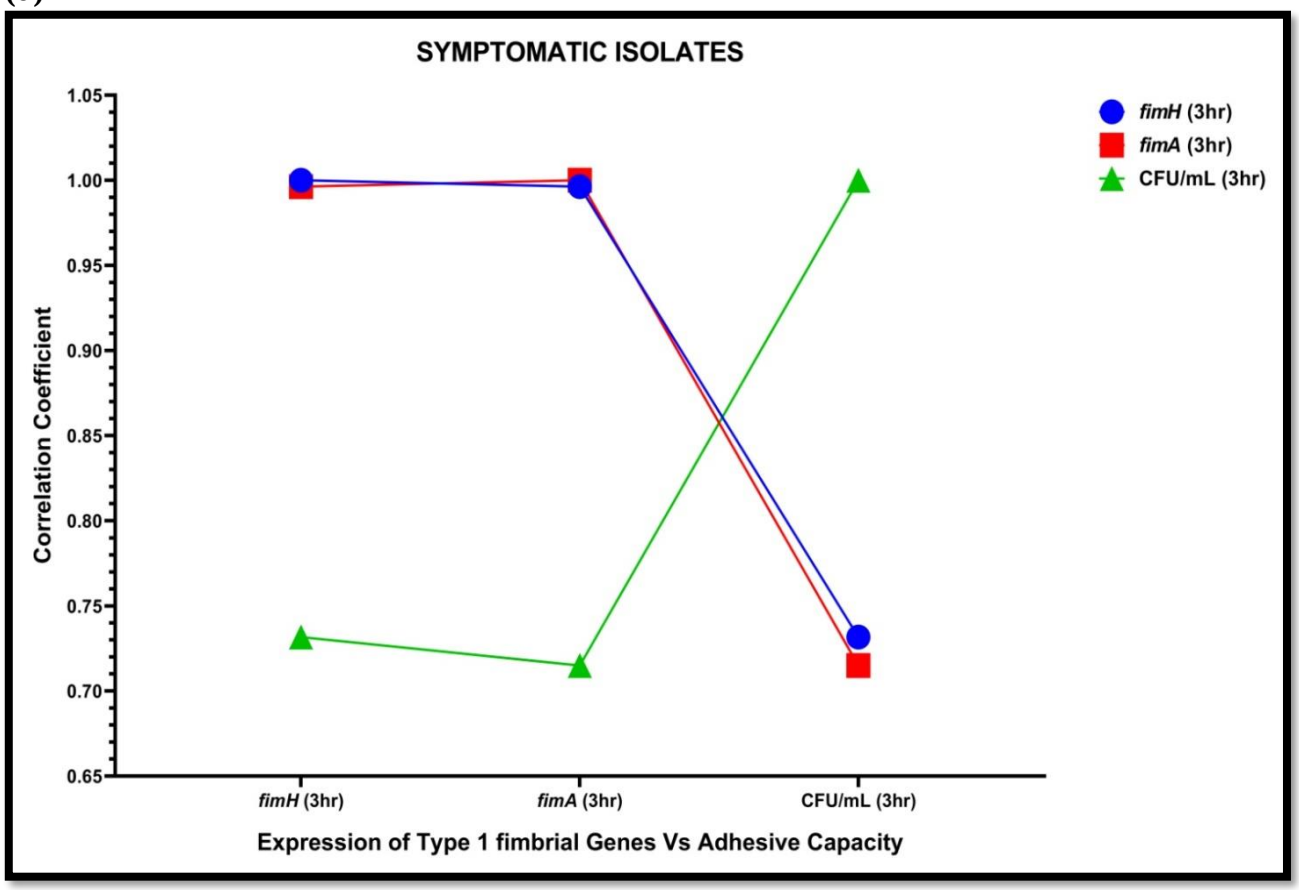
(This study)

(c)



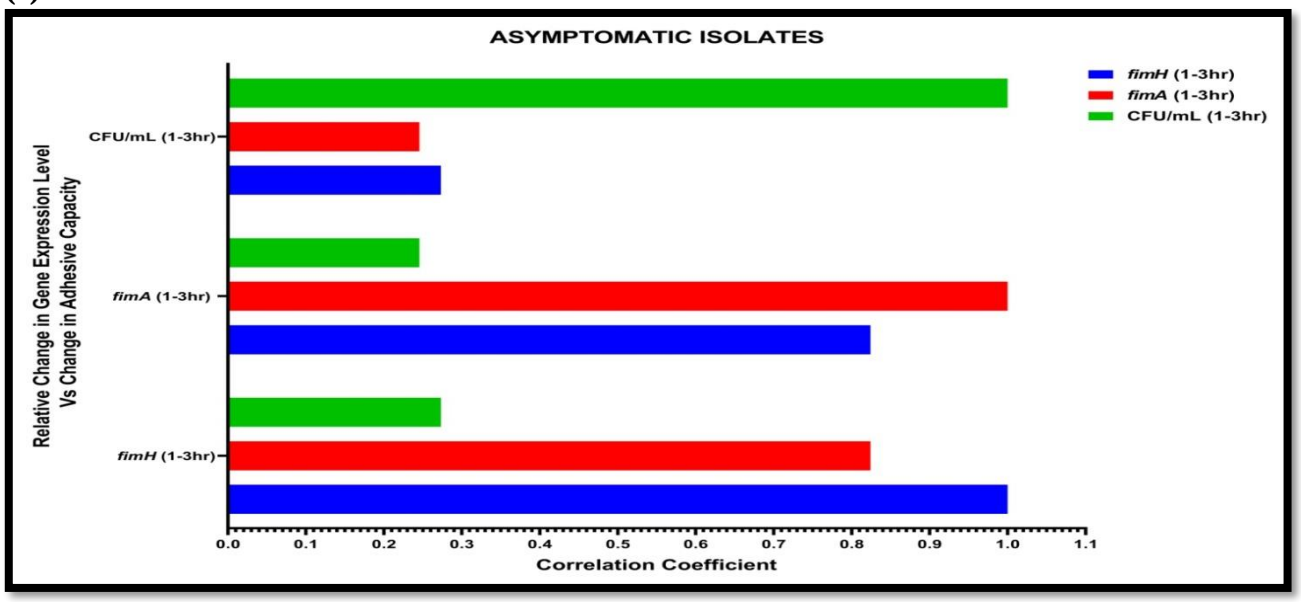
(This study)

(d)



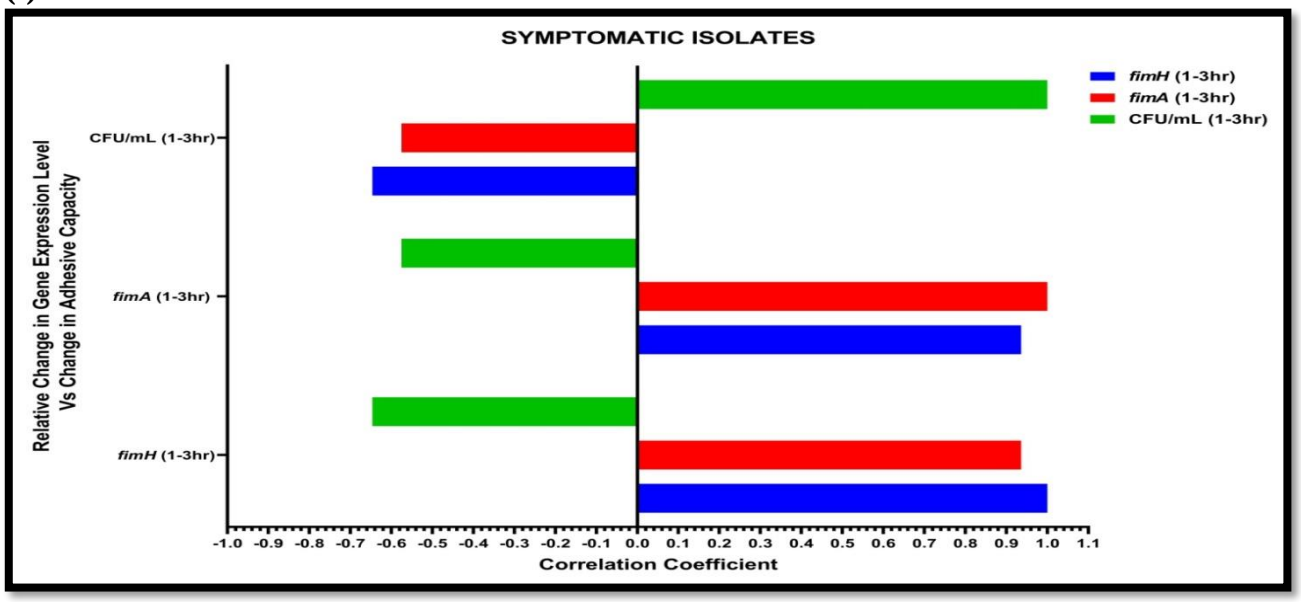
(This study)

(e)



(This study)

(f)



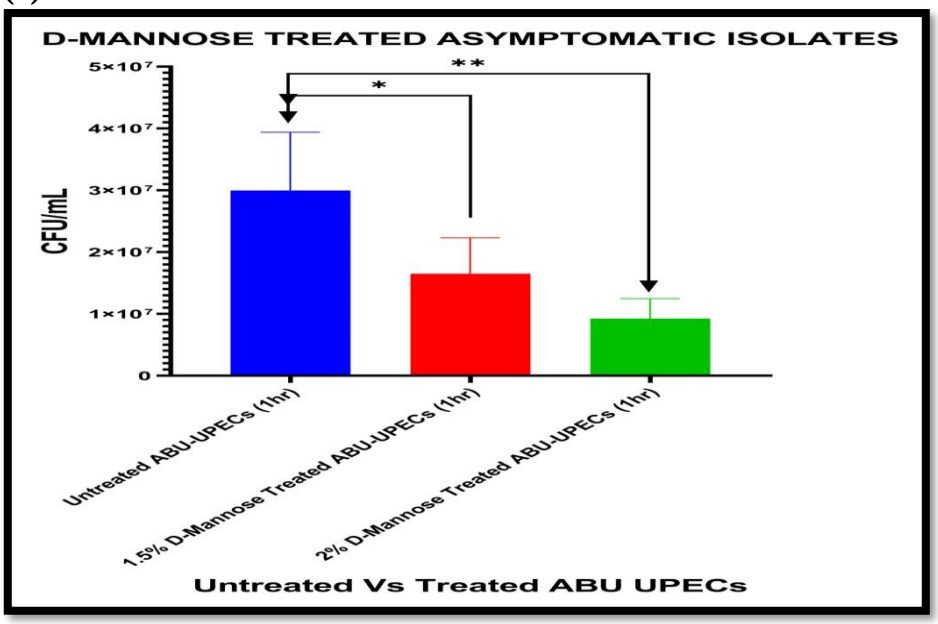
(This study)

Fig. 5.14: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of quantitative expressions of two different type 1 fimbrial genes (*fimH*; *fimA*) and adhesive capacity of asymptomatic and symptomatic UPECs to T24 uroepithelial cells (a) *fimH* (1hr); *fimA* (1hr); CFU/mL (1hr) in asymptomatic UPECs (b) *fimH* (3hr); *fimA* (3hr); CFU/mL (3hr) in asymptomatic UPECs (c) *fimH* (1hr); *fimA* (1hr); CFU/mL (1hr) in symptomatic UPECs (d) *fimH* (3hr); *fimA* (3hr); CFU/mL (3hr) in symptomatic UPECs (e) *fimH* (1 to 3hrs relative changes); *fimA* (1 to 3hrs relative changes); CFU/mL (1to 3hrs relative changes) in asymptomatic UPECs (f) *fimH* (1 to 3hrs relative changes); *fimA* (1 to 3hrs relative changes); CFU/mL (1to 3hrs relative changes) in symptomatic UPECs. Different genes and adhesive capacity were represented by one symbol per row with varied colours.

5.5.10 D-Mannose as Inhibitor of UPECs Attachment to T24 Uroepithelial Cells

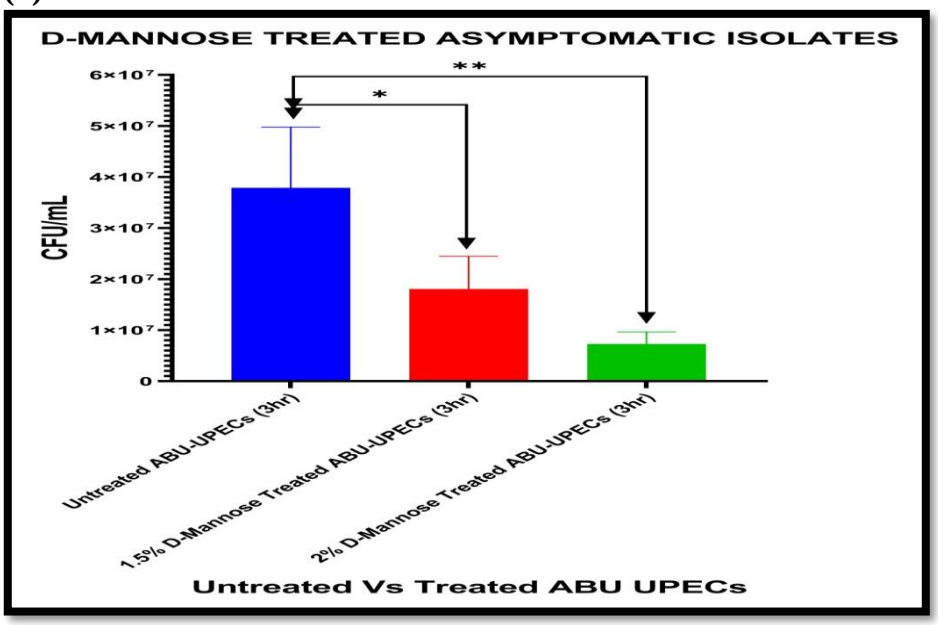
No microscopic differences could be observed between T24 cell monolayers incubated with 1.5% and 2% D-mannose with that observed before D-mannose exposure concerning their shape, integrity, adhesiveness and cytoplasmic proliferation. The significantly lower extent of adhesion to T24 uroepithelial cells was observed in the case of both the 1.5% and 2% D-mannose treated ABU **(Fig. 5.15a-b)** and symptomatic **(Fig. 5.15c-d)** UPECs after both 1 and 3 hrs of infection relative to their untreated controls, except in the case of 1.5% D-mannose treated symptomatic UPECs. Moreover, significant number (p value $\leq$ 0.05) of adherent isolates regardless of their asymptomatic and symptomatic nature showed OFF orientation of *fim* switch after 2% D-mannose treatment.

(a)



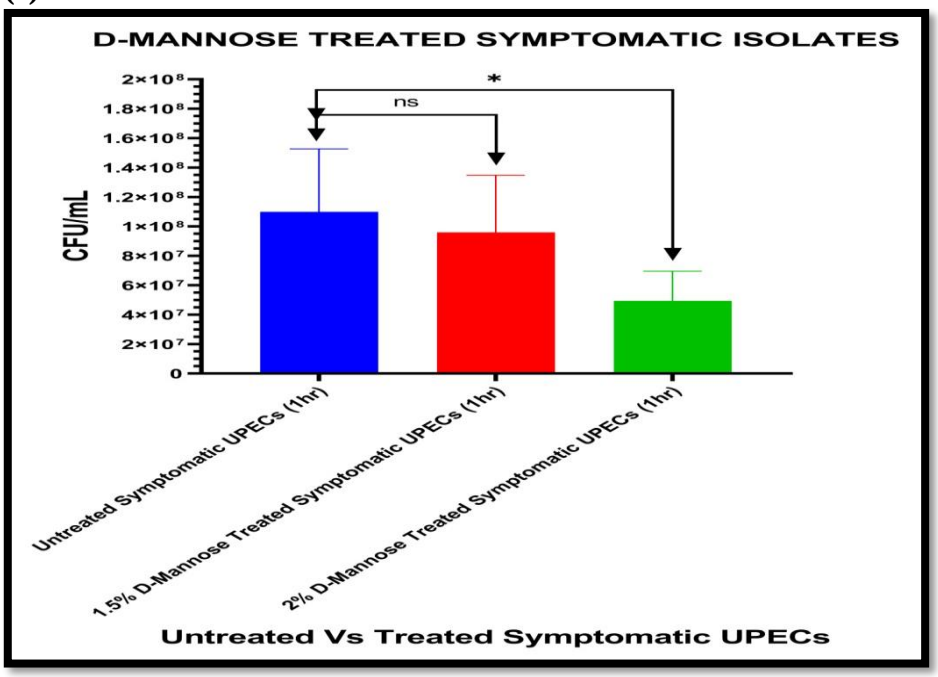
(This study)

(b)



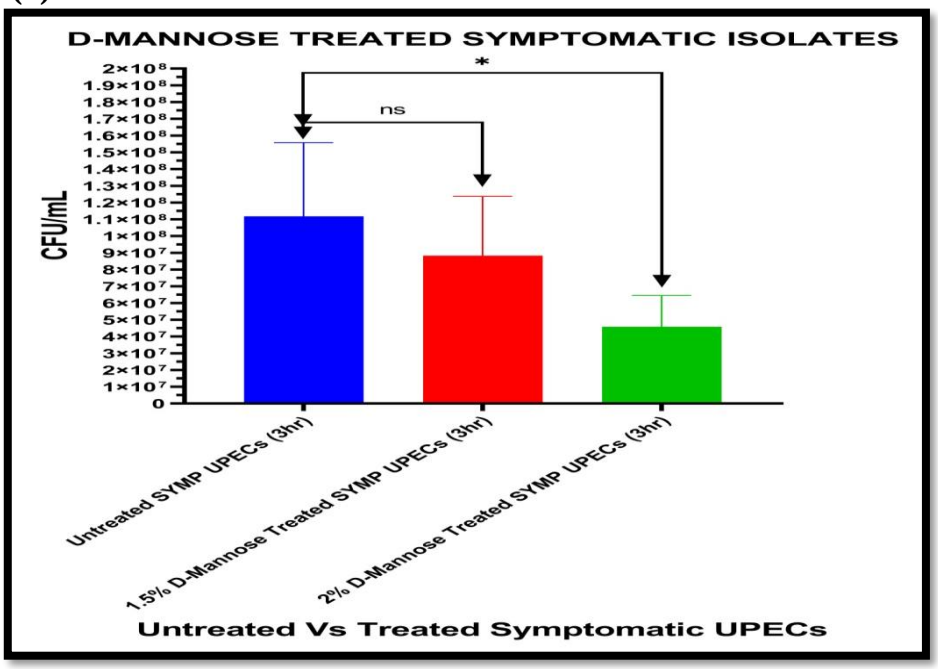
(This study)

(c)



(This study)

(d)



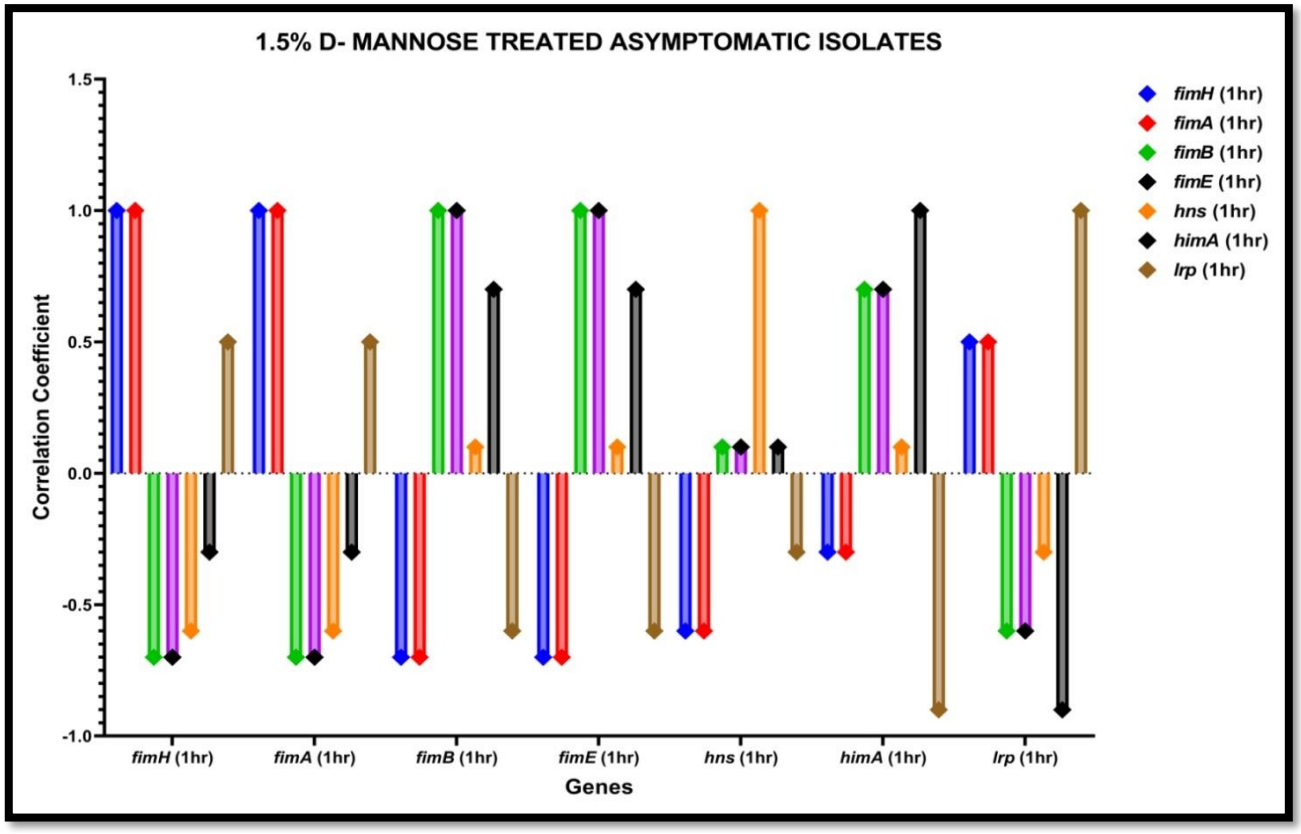
(This study)

Fig. 5.15: Graphical representation of statistical differences computed with confidence level of 95% (p values ≤ 0.05) using one- way analysis of variance (one-way ANOVA) in GraphPad Prism version 9 (Prism software package) based on the differences in adhesive capacity (CFU/mL) of untreated, 1.5% and 2% D-mannose treated asymptomatic (a) 1hr post infection incubation (b) 3hrs post infection incubation and symptomatic (c) 1hr post infection incubation (d) 3hrs post infection incubation, UPECs to T24 uroepithelial cells. Treated and untreated UPECs were represented by bar graphs with varied colours. Single (*) (p value ≤ 0.05) and double stars (**) (p value ≤ 0.01) indicated significant differences between treated and untreated adherent UPECs. "ns" indicated non-significant differences.

5.5.11 Correlation among the type 1 fimbrial genes, recombinase genes and regulatory factor genes, post D-Mannose treatment

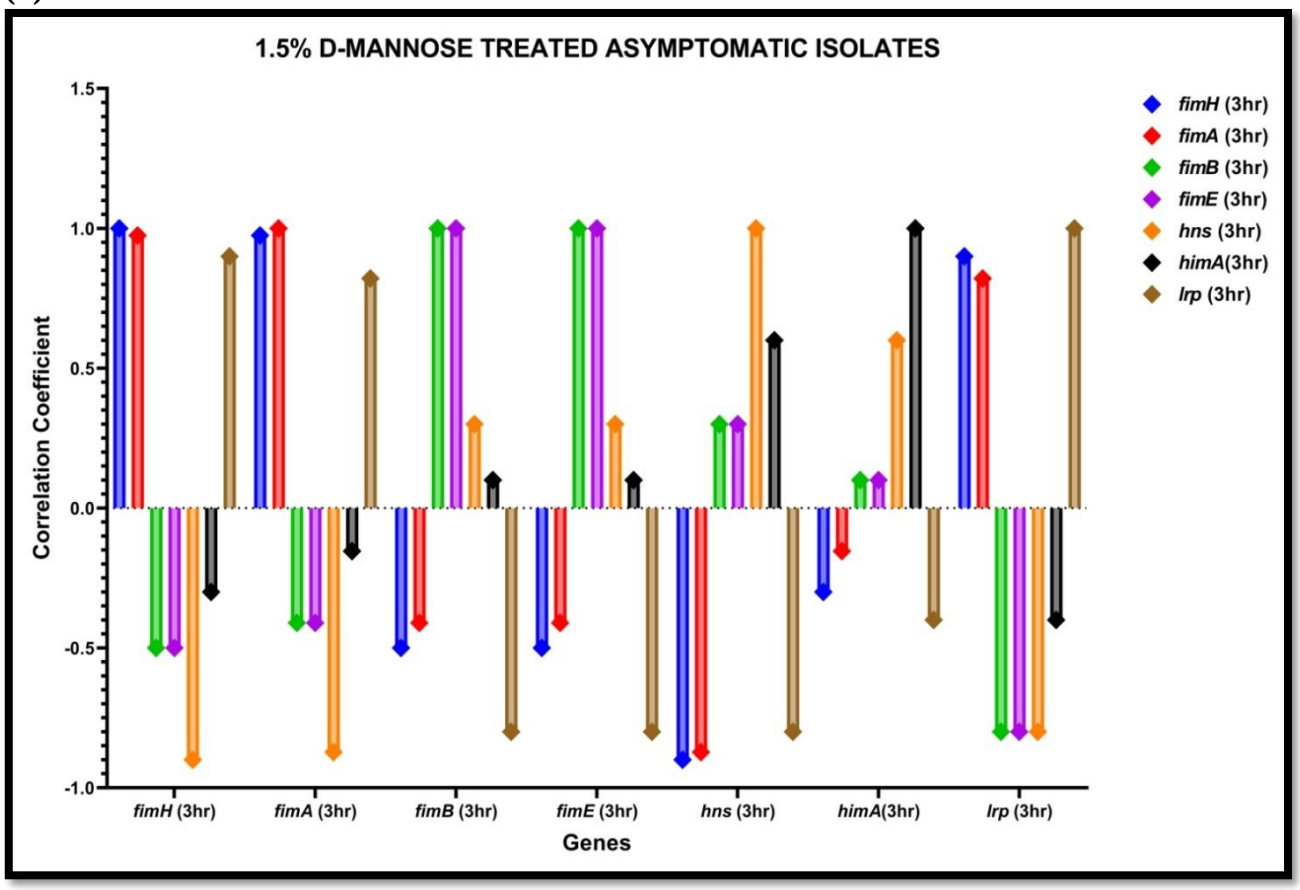
The significant (p-value ≤ 0.05) positive and/or negative correlations that ranged from low to high was observed in the level of expressions of the *fimH*, *fimA*, *fimB*, *fimE*, *hns*, *himA* and *lrp* [**(Fig. 5.16a-c; Fig. 5.16d-f), (Fig. 5.17a-c; Fig. 5.17d-f)]** and their combinations [(**Fig. 5.18a-c; Fig. 5.18d-f**), **(Fig. 5.19a-c; Fig. 5.19d-f**)] among both the 1.5% and 2% D-mannose treated ABU and symptomatic UPECs at 1, 3 and 1to 3hrs of infections respectively, however the extent of correlations differed between treated and untreated isolates of both the asymptomatic and symptomatic groups. Strong positive correlations could be perceived between *fimH;* (*fimB: fimE)* and *fimA;* (*fimB: fimE)*; respectively among all 1.5% (**Fig. 5.18a-c; Fig. 5.18d-f**) and 2% **(Fig. 5.19a-c; Fig. 5.19d-f)** D-mannose treated adherent isolates irrespective of their asymptomatic or symptomatic nature at 1, 3 and 1to 3hrs (relative changes in expression) of infections respectively. Moreover, high positive correlation was also observed between the combination of expressions of *himA* and *lrp* (*himA+lrp*) with that of two type 1 fimbrial genes and the ratio of their recombinases (*fimB: fimE*) among both the 1.5% **(Fig. 5.18a-c; Fig. 5.18d-f)** and 2% **(Fig. 5.19a-c; Fig. 5.19d-f)** D-mannose treated adherent ABU and symptomatic UPECs, relating to both 1 and 3hrs of infections and also their relative changes respectively, except 1.5% D-mannose treated ABU (1-3hrs), symptomatic (3hrs), symptomatic (1-3hrs) and 2% D-mannose treated symptomatic (1-3hrs) UPECs respectively.

(a)



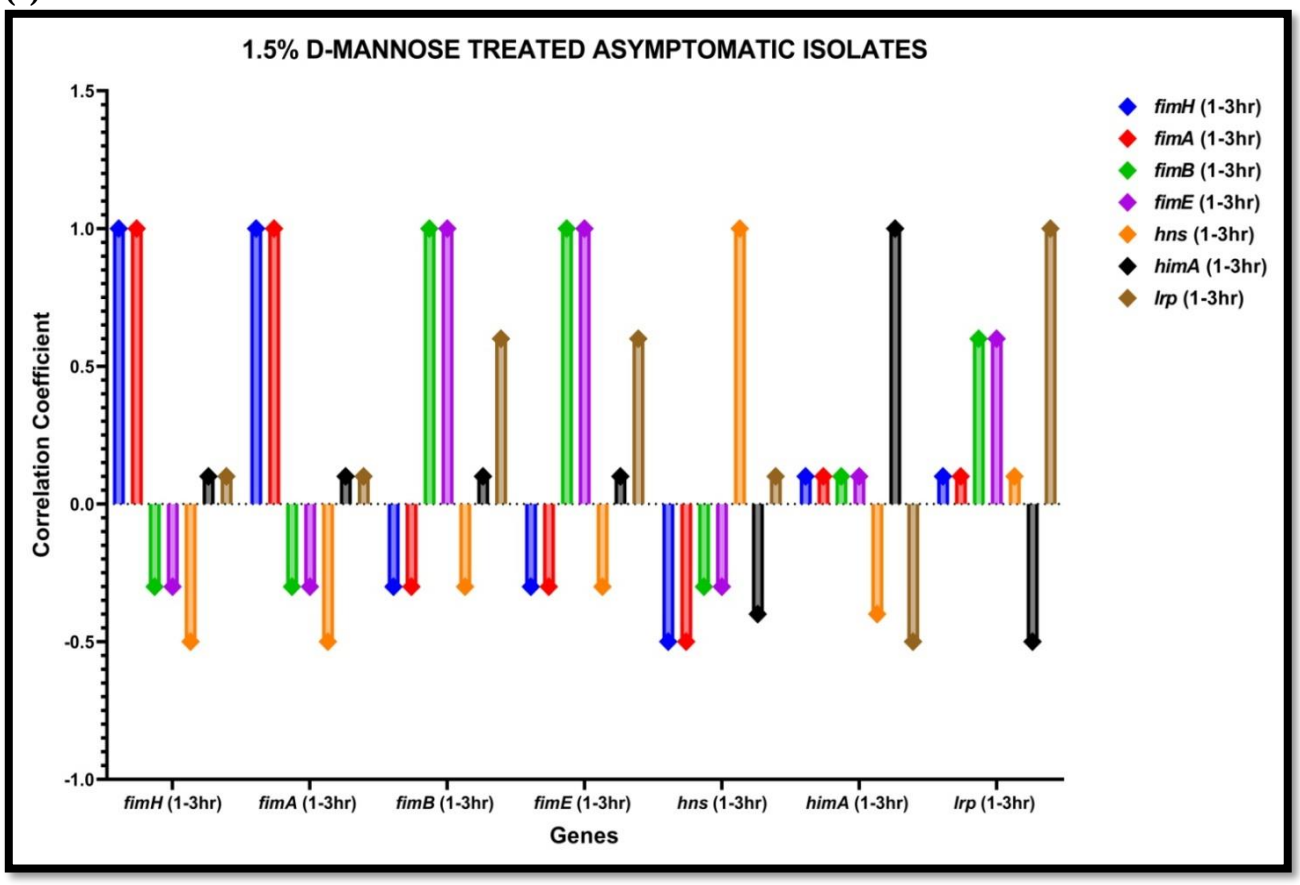
(This study)

(b)



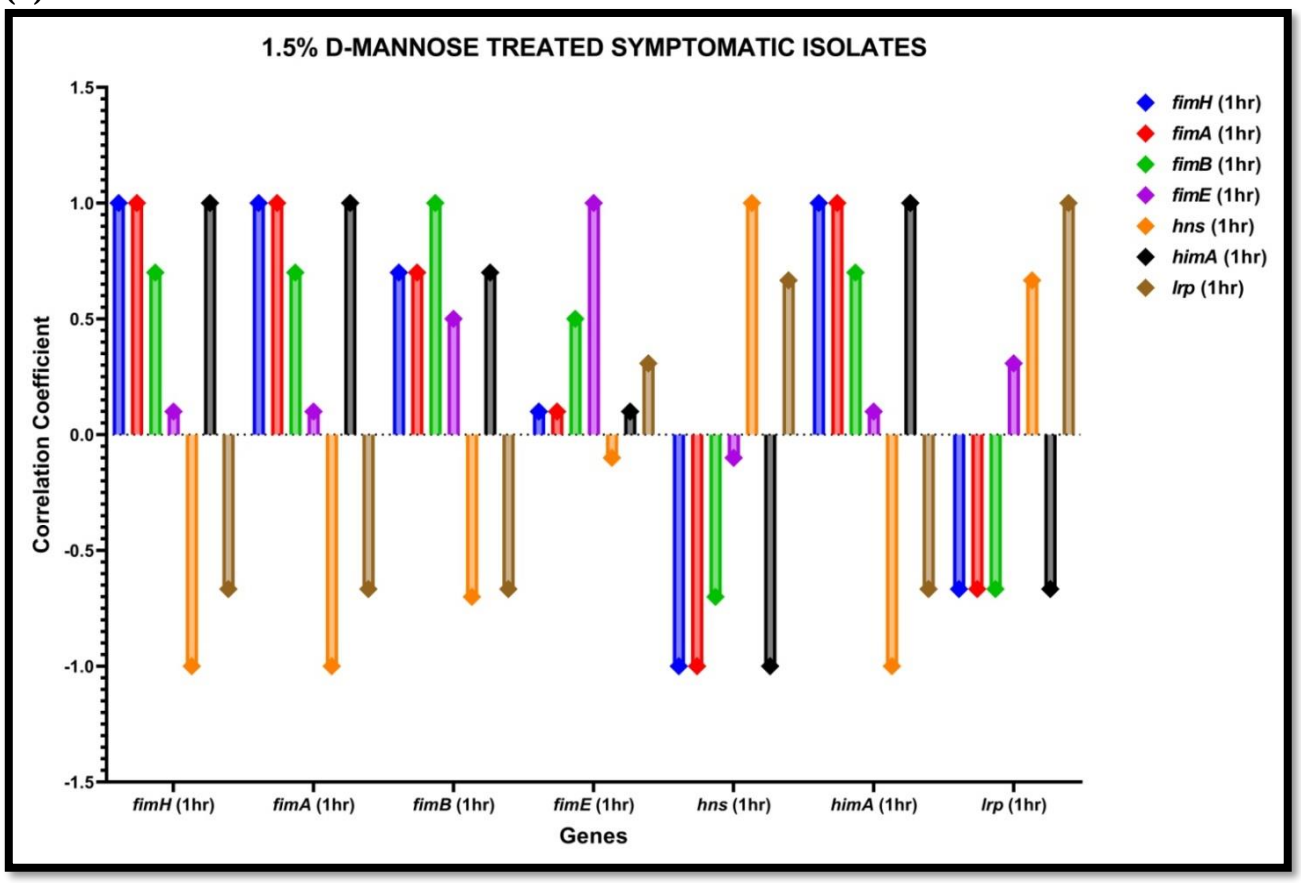
(This study)

(c)



(This study)

(d)



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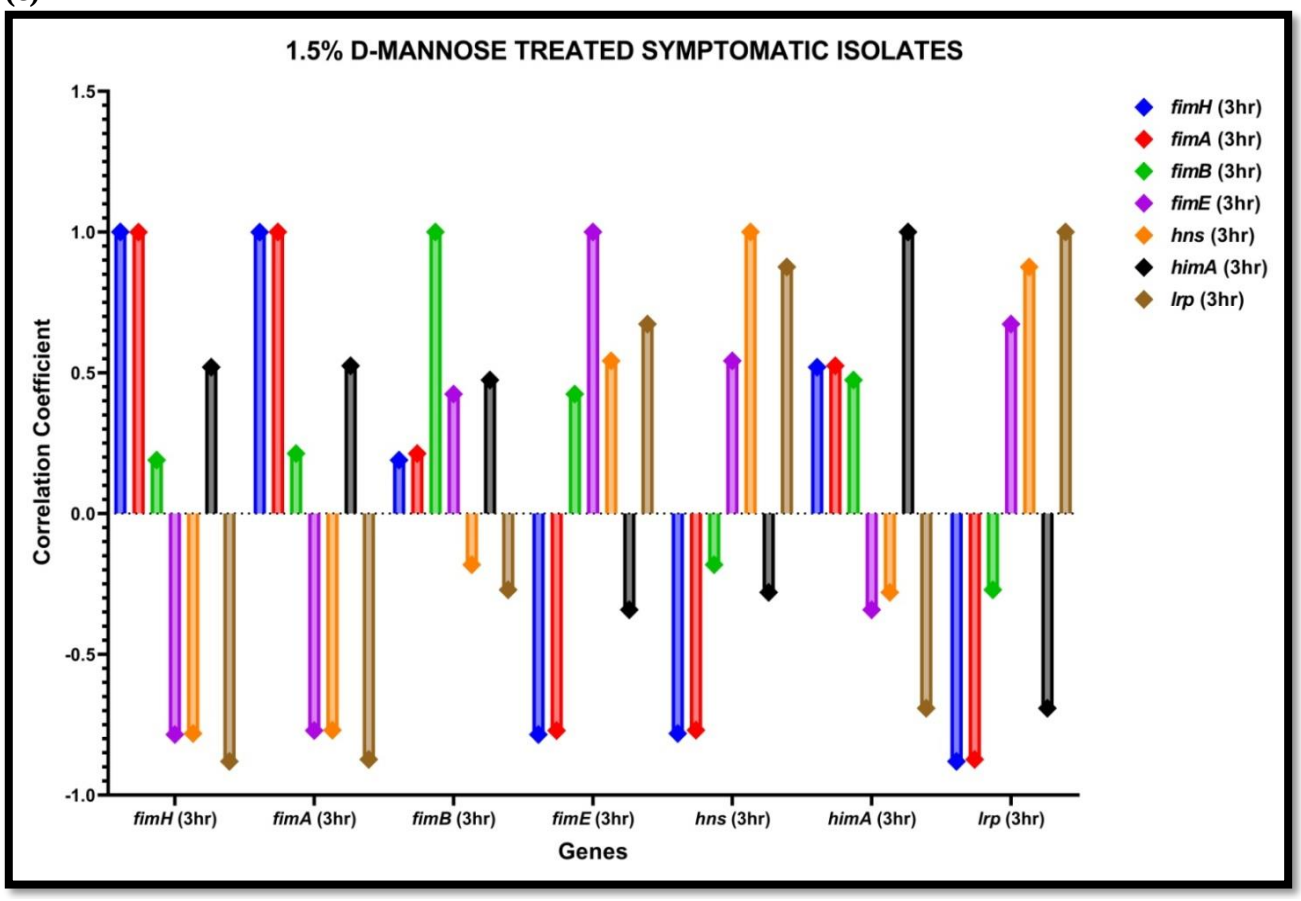
(e)



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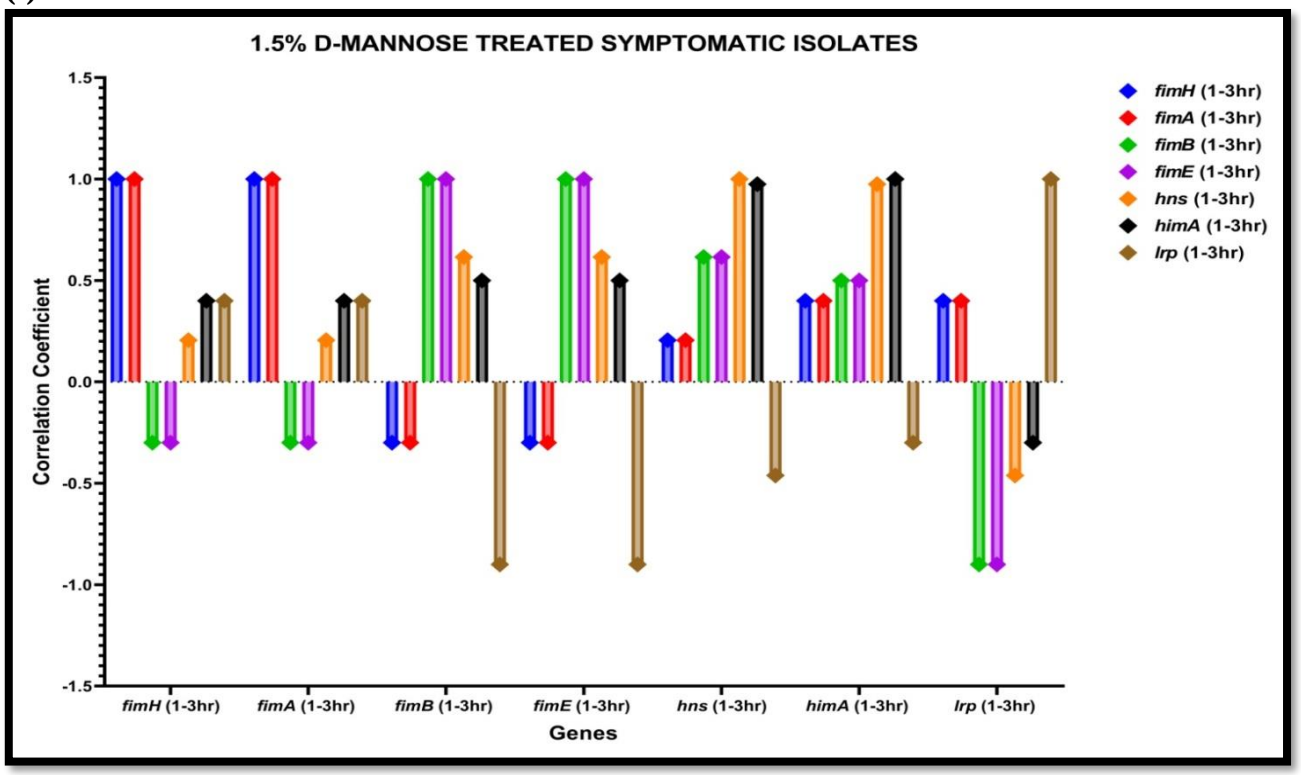
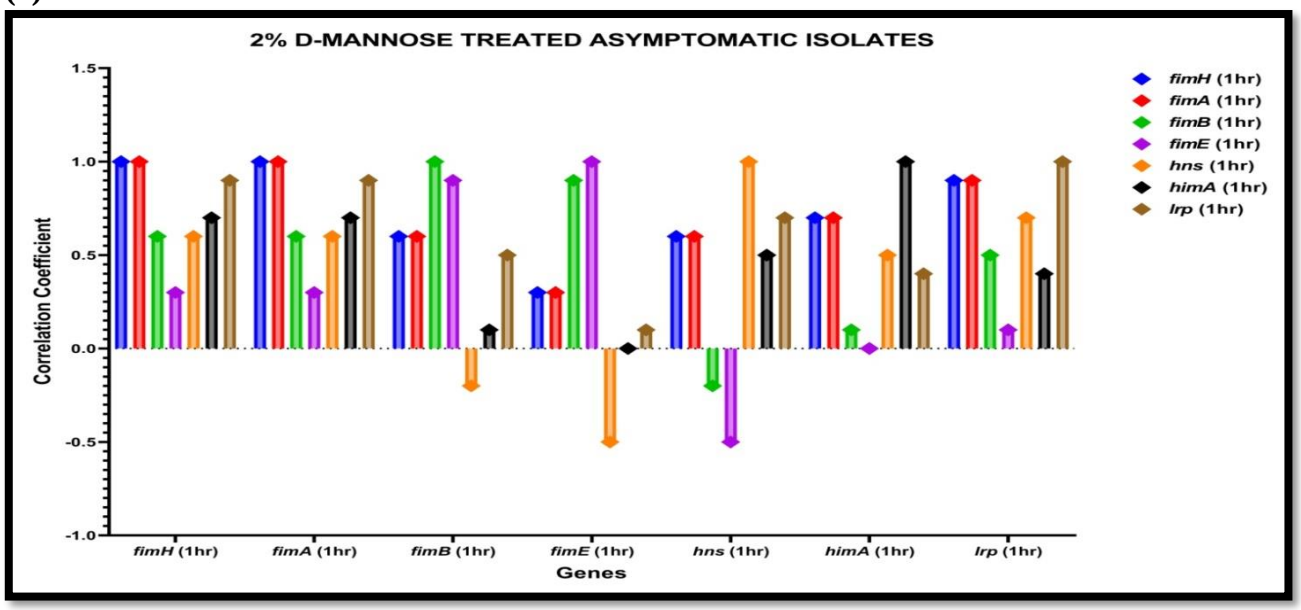
(f)



(This study)

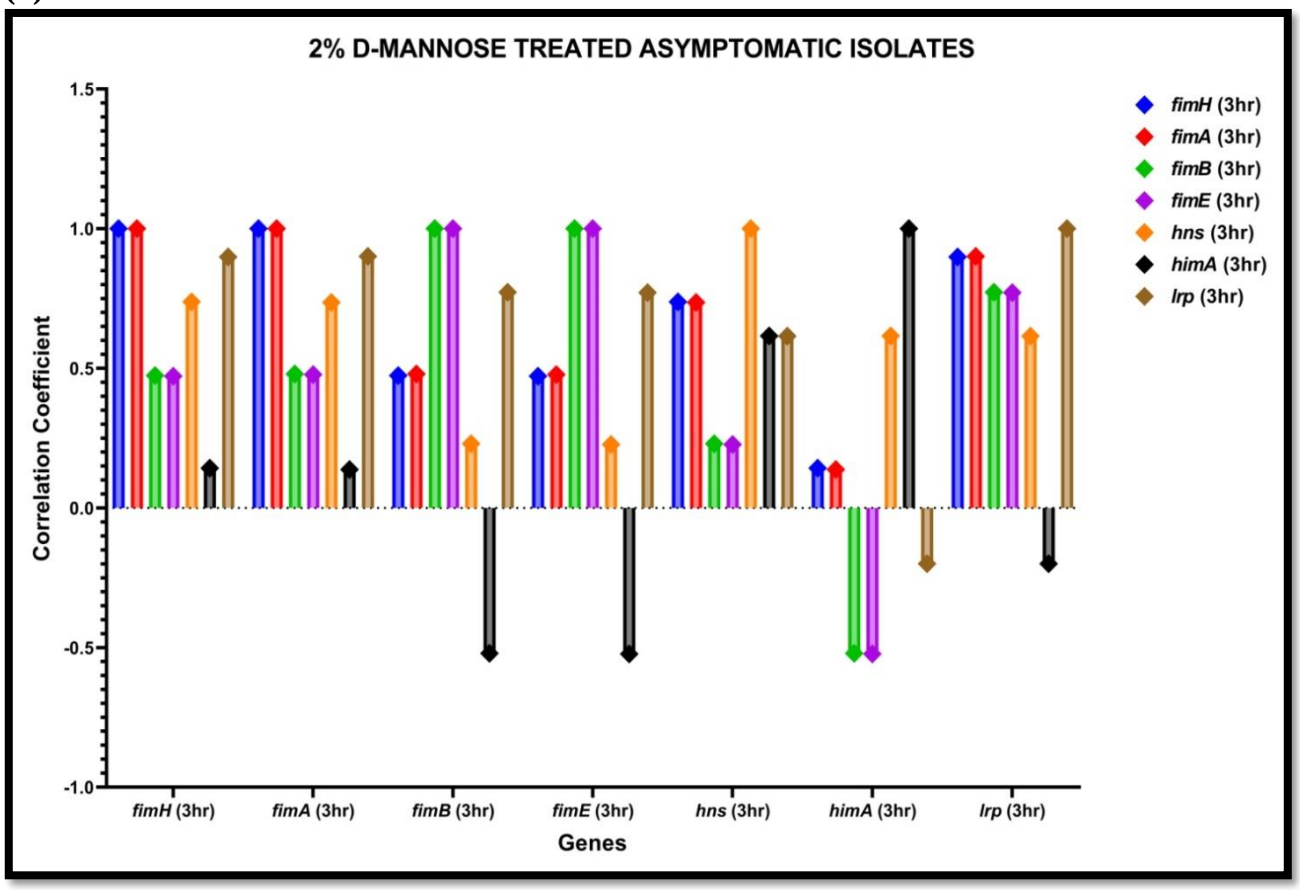
Fig. 5.16: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of quantitative expression of seven different genes (*fimH*, *fimA*, *fimB*, *fimE*, *hns*, *himA*, *lrp*) among 1.5% D-mannose treated adherent asymptomatic (a) 1hr of infection duration (b) 3hrs of infection duration (c) 1to 3hrs (relative changes) and symptomatic (d) 1hr of infection duration (e) 3hrs of infection duration (f) 1to 3hrs (relative changes) UPECs. Different genes were represented by scatter dot plots with varied colours.

(a)



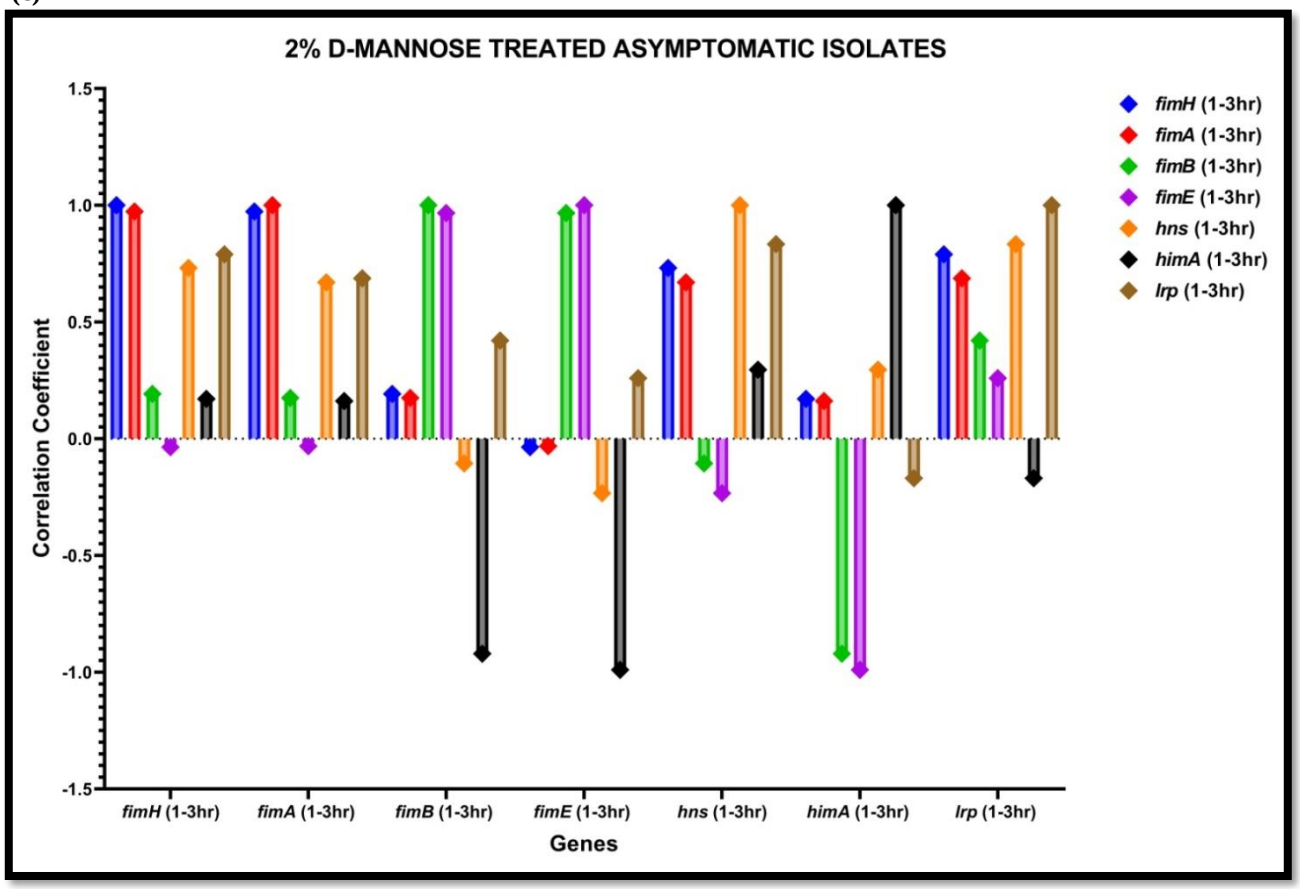
(This study)

(b)



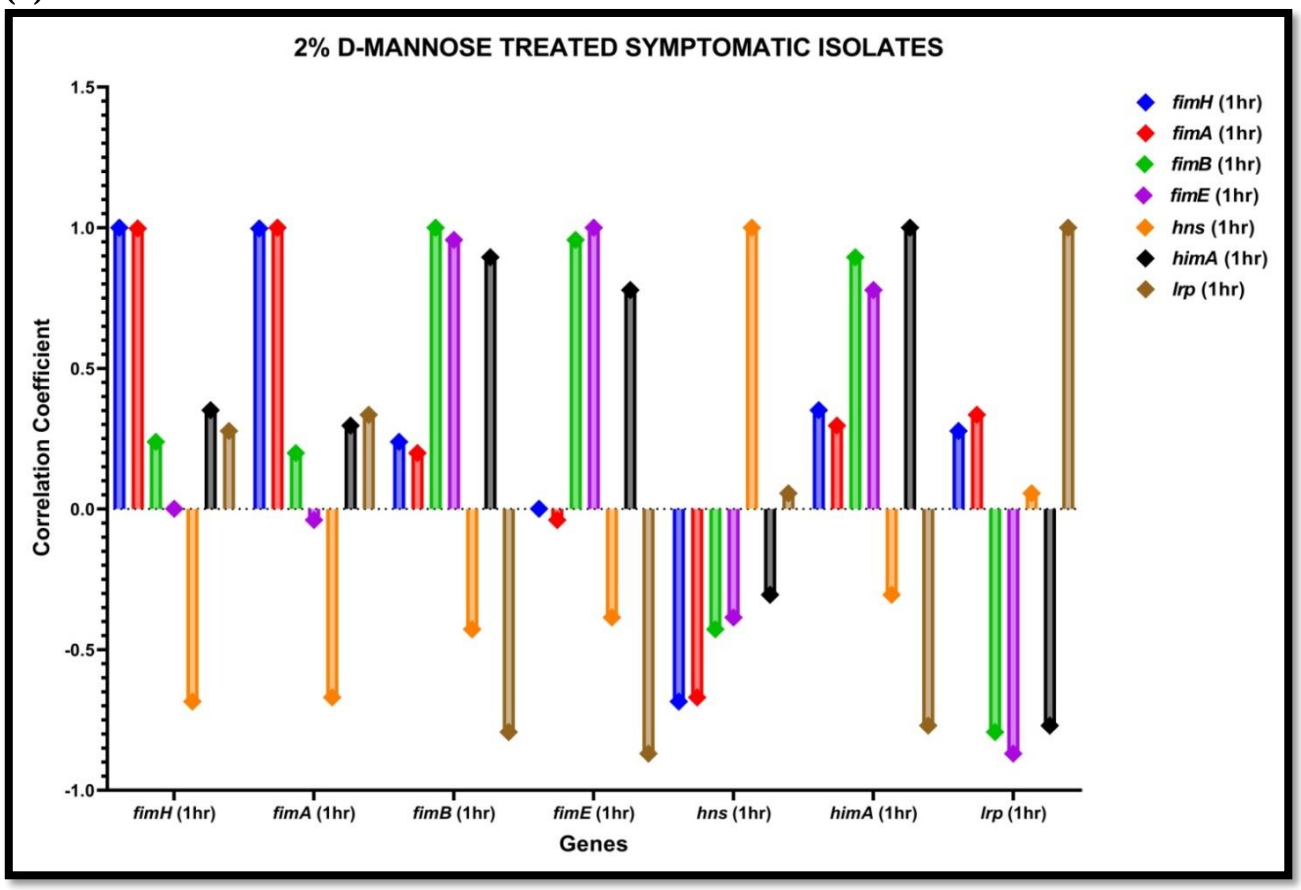
(This study)

(c)



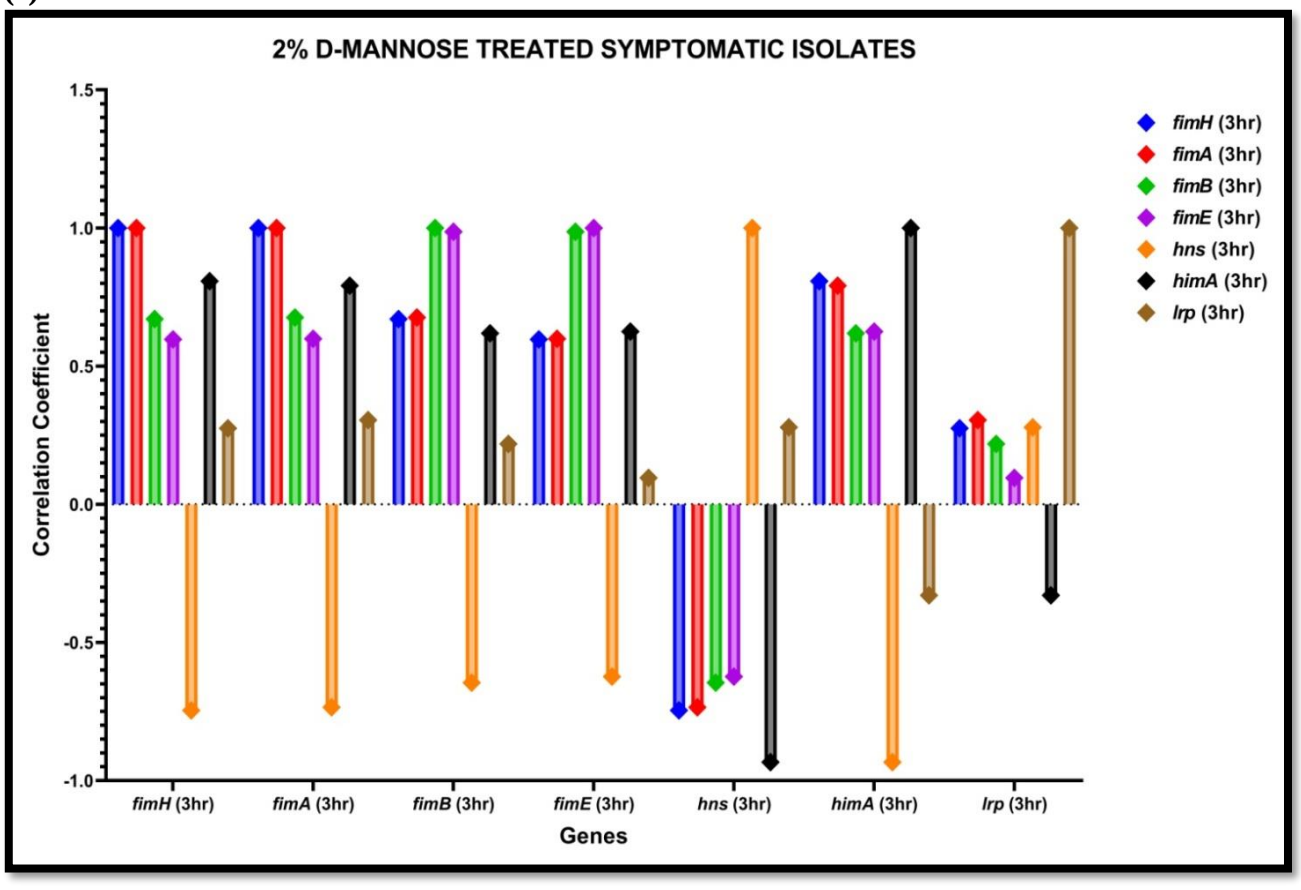
(This study)

(d)



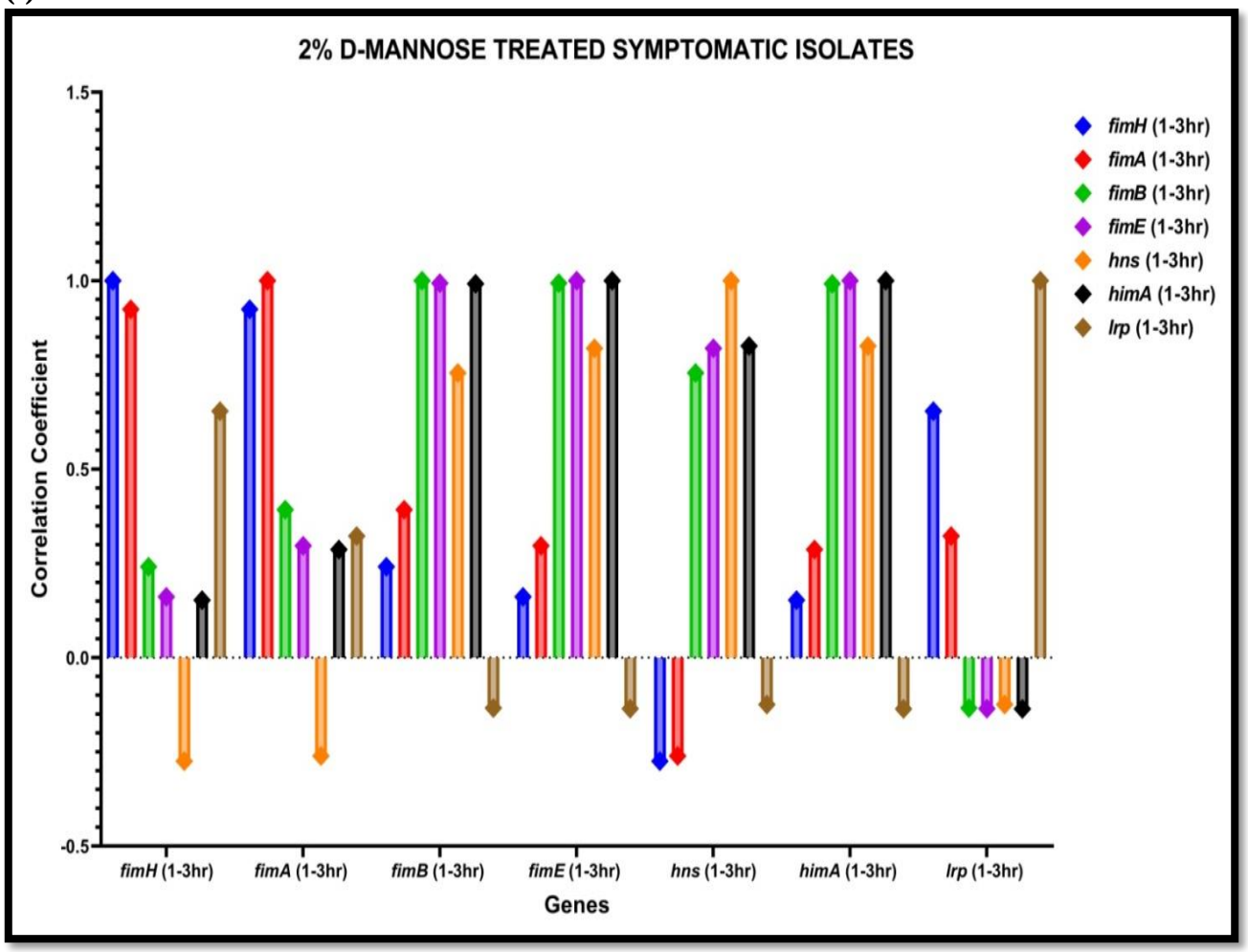
(This study)

(e)



(This study)

(f)

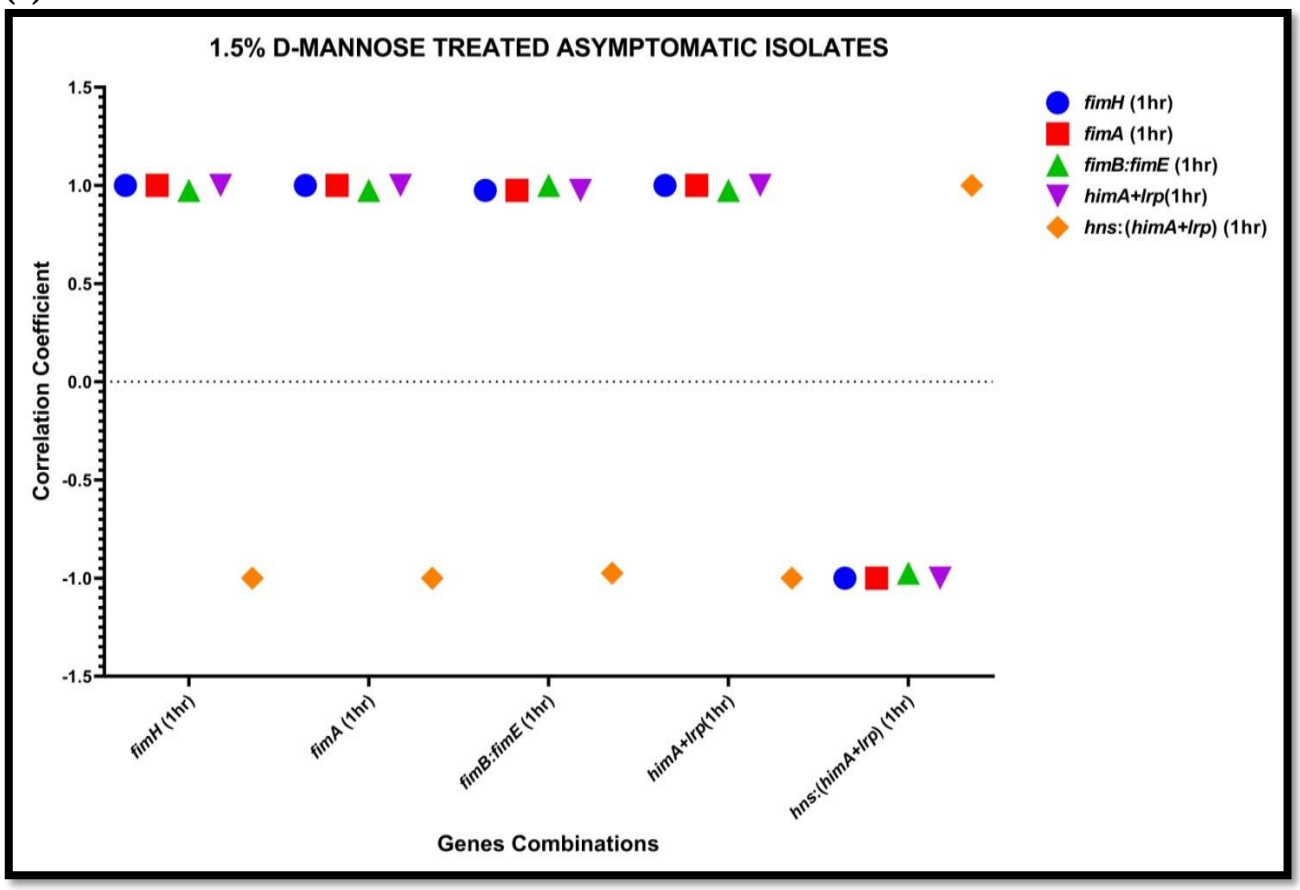


(This study)

Fig. 5.17: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of quantitative expression of seven different genes (*fimH, fimA, fimB, fimE, hns, himA, lrp*) among 2% D-mannose treated adherent asymptomatic (a) at 1hr of infection duration (b) at 3hrs of infection duration (c) at 1to 3hrs (relative changes) and symptomatic (d) at 1hr of infection duration (e) at 3hrs of infection duration (f) at 1to 3hrs (relative changes) UPECs. Different genes were represented by scatter dot plots with varied colours.

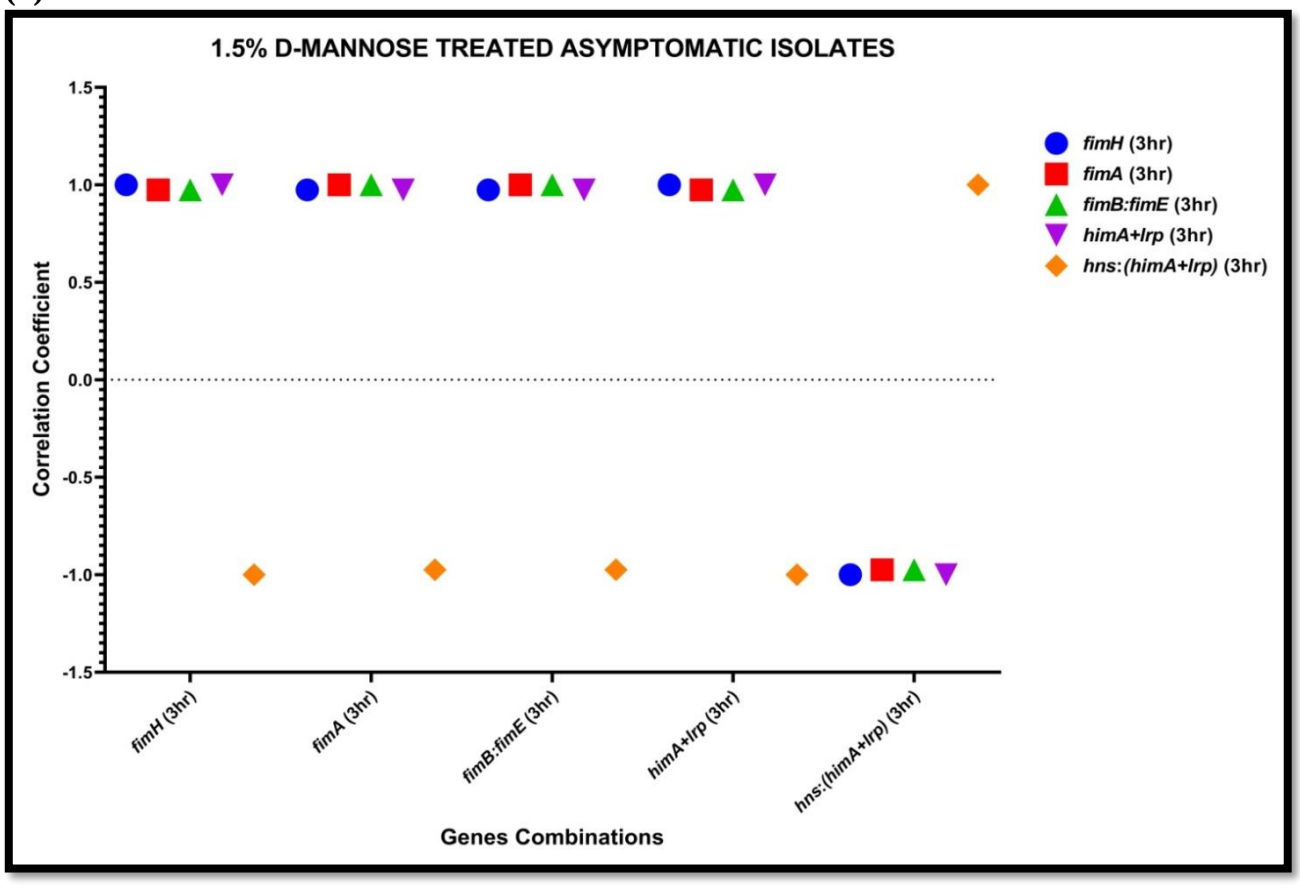
Furthermore, high negative correlations could be discerned between various combination [{*hns*: (*himA*+*lrp*); *fimH}*, {*hns*: (*himA*+*lrp*); *fimA*}, {*hns*: (*himA*+*lrp*)]; (*fimB: fimE*)}, and {*hns*: (*himA*+*lrp*); (*himA*+*lrp*)}] of the aforementioned genes among both the 1.5% **(Fig. 5.18a-c; Fig. 5.18d-f)** and 2% **(Fig. 5.19a-c; Fig. 5.19d-f)** D-mannose treated adherent ABU and symptomatic UPECs, relating to both 1hr, 3hrs of infections and also their relative changes respectively, except 1.5% D-mannose treated ABU [{*hns*: (*himA*+*lrp*); (*himA*+*lrp*)} (1-3hrs)], symptomatic [{*hns*: (*himA*+*lrp*); (*himA*+*lrp*)} (3hrs)], symptomatic [{*hns*: (*himA*+*lrp*); (*himA*+*lrp*)} (1-3hrs)] and 2% D-mannose treated symptomatic [{*hns*: (*himA*+*lrp*); (*himA*+*lrp*)} (1-3hrs)] UPECs respectively.

(a)



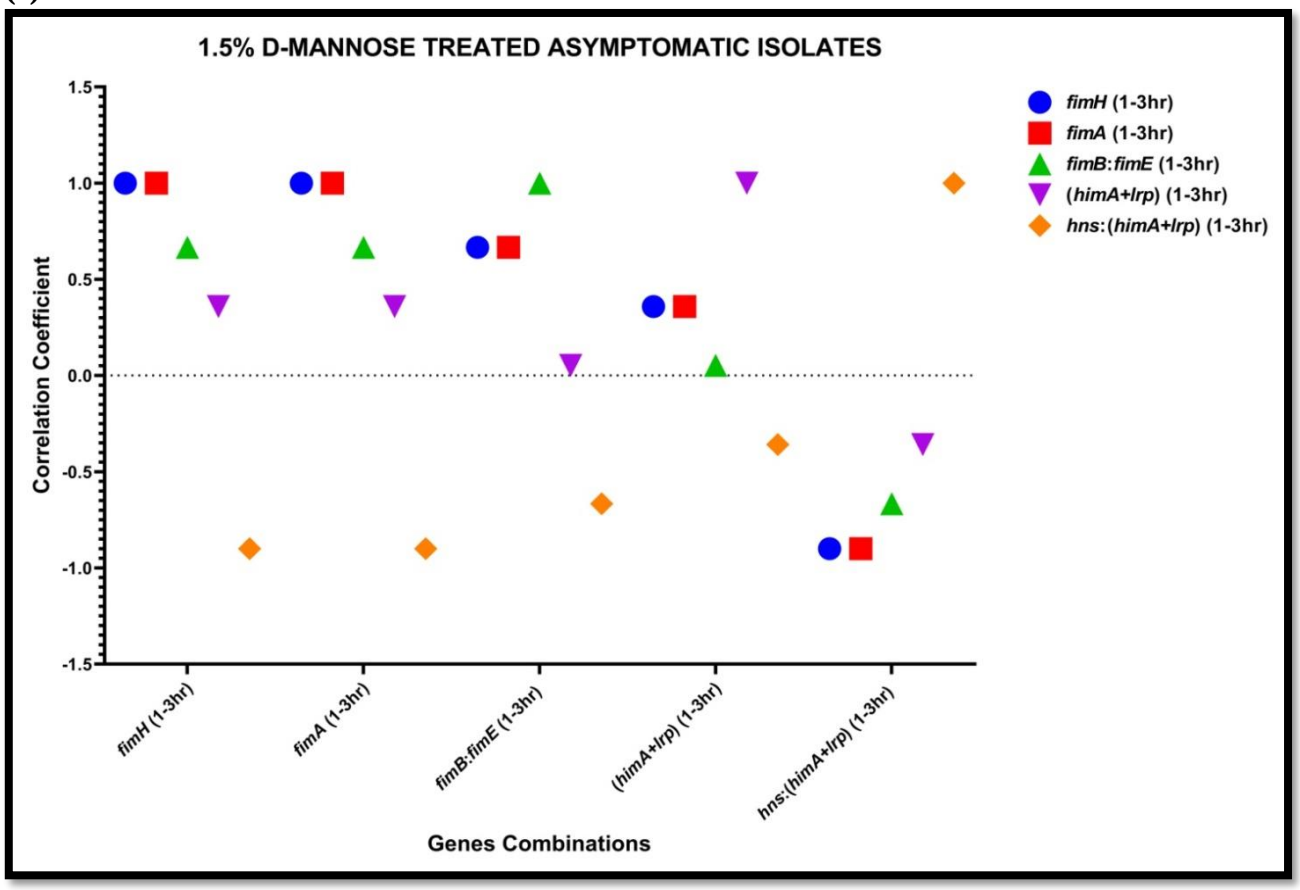
(This study)

(b)



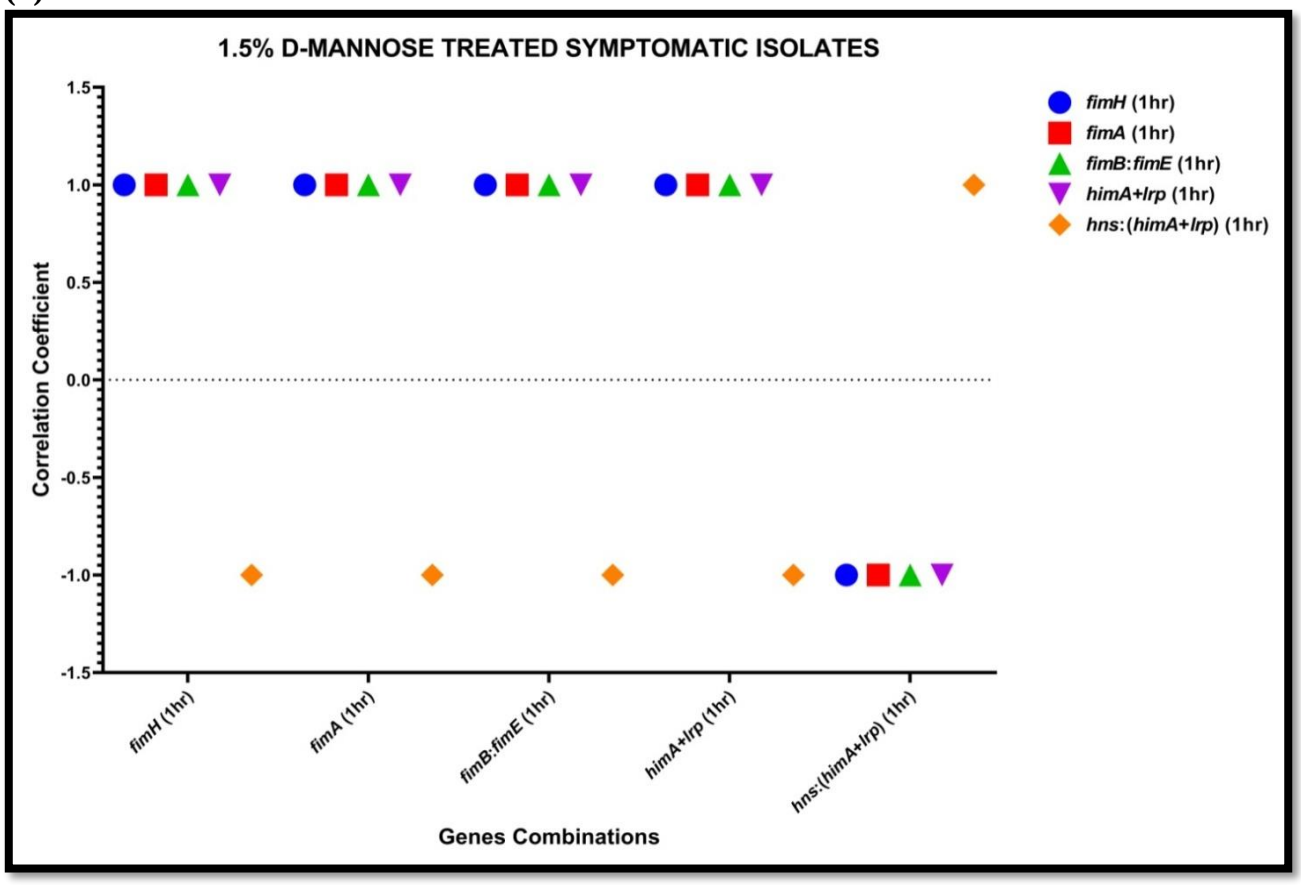
(This study)

(c)



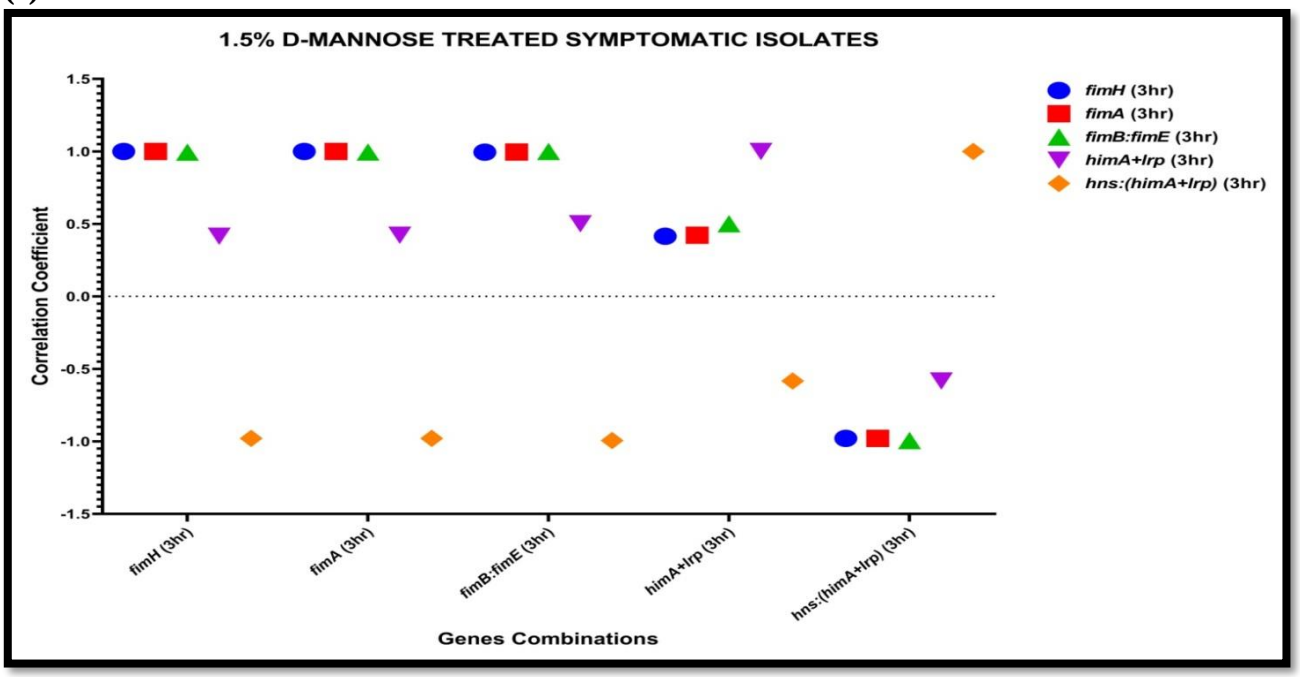
(This study)

(d)



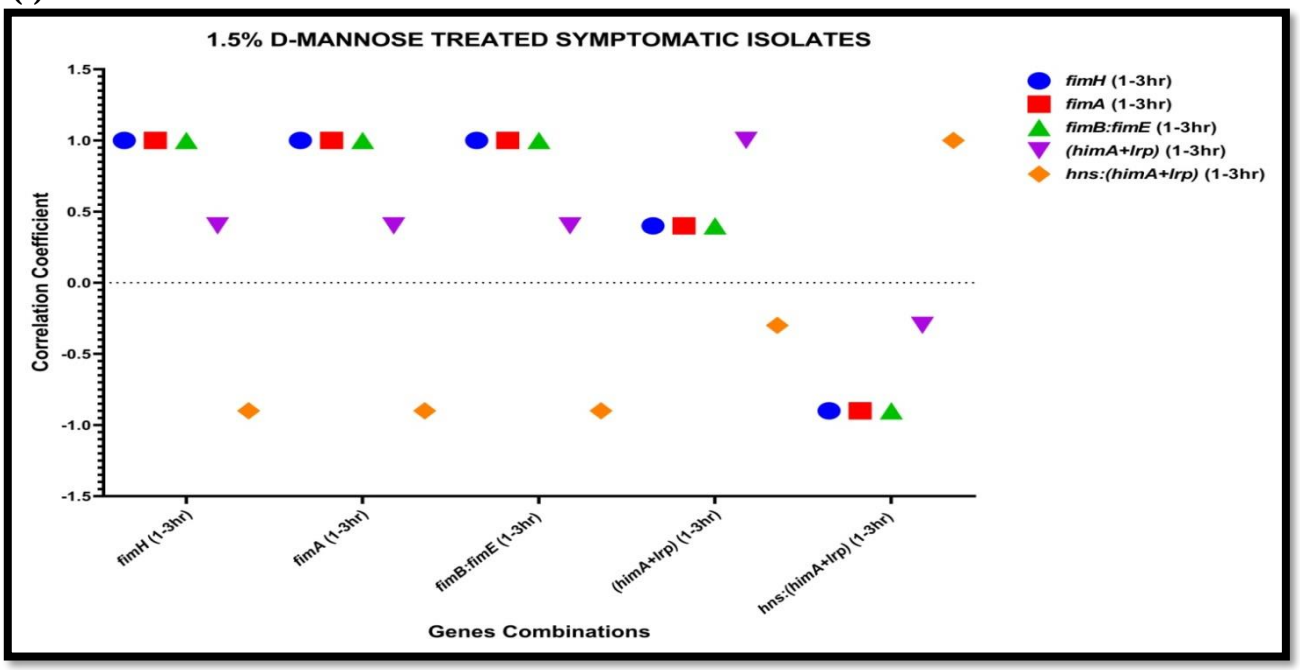
(This study)

(e)



(This study)

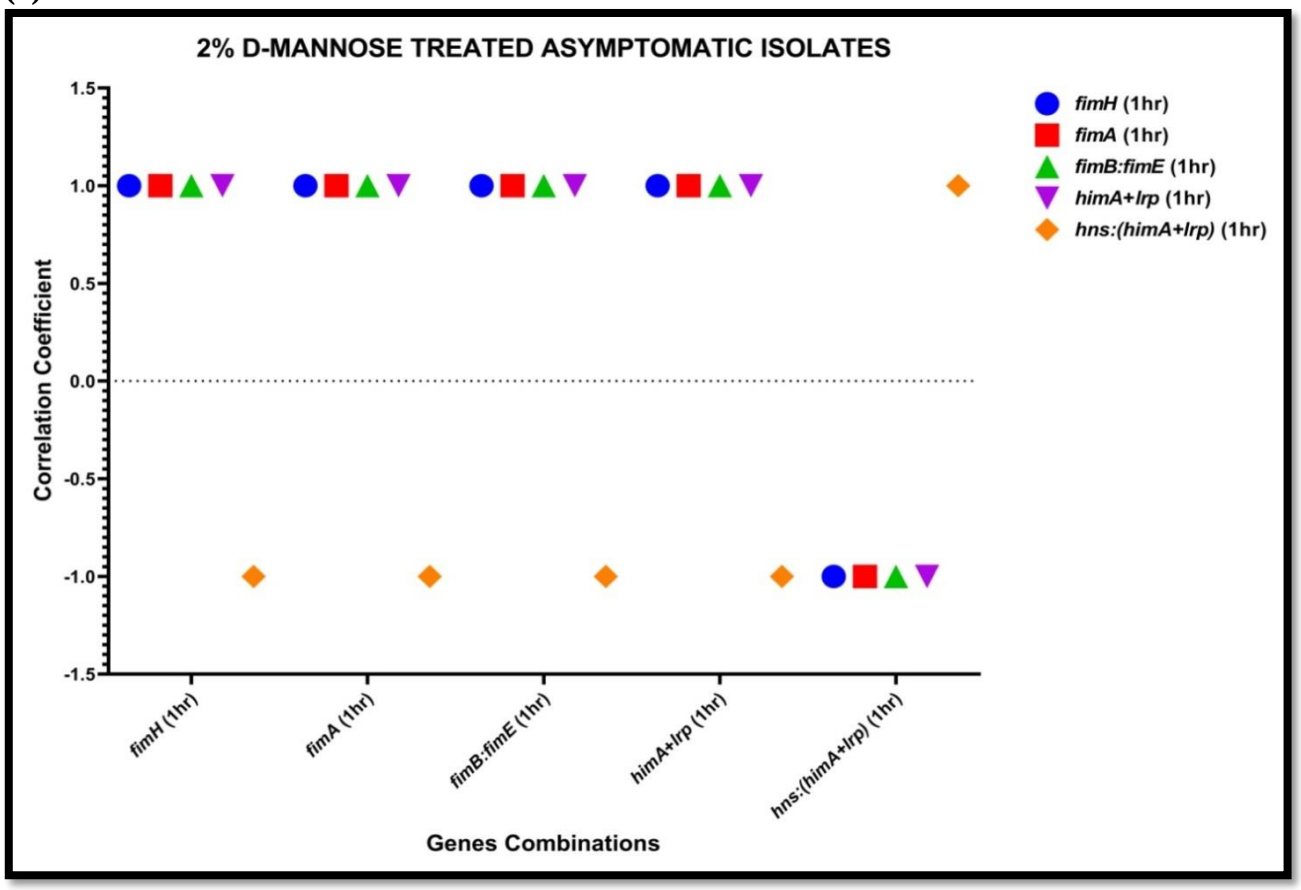
(f)



(This study)

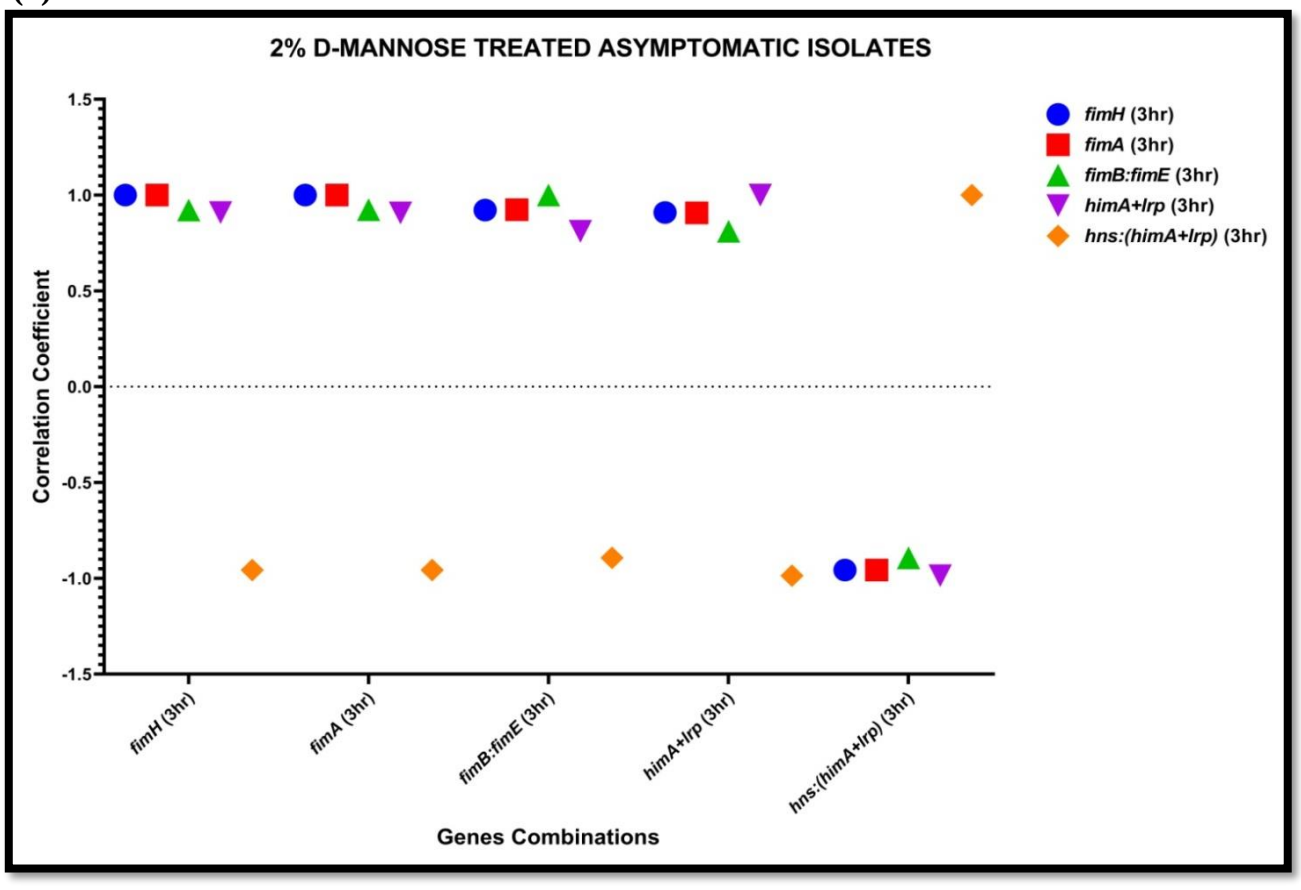
Fig. 5.18: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of quantitative expression of two type 1 fimbrial genes (*fimH* and *fimA*), ratio of their recombinases (*fimB*: *fimE*), regulator combinations (*himA+lrp*) and ratio of regulator combinations [*hns*: (*himA+lrp*)] at different studied infection durations and also their relative changes in among 1.5% D-mannose treated asymptomatic (a) 1hr (b) 3hr (c) 1 to 3hrs relative changes and symptomatic UPECs (d) 1hr (e) 3hr (f) 1 to 3hrs relative changes. Different genes were represented by one symbol per row with varied colours.

(a)



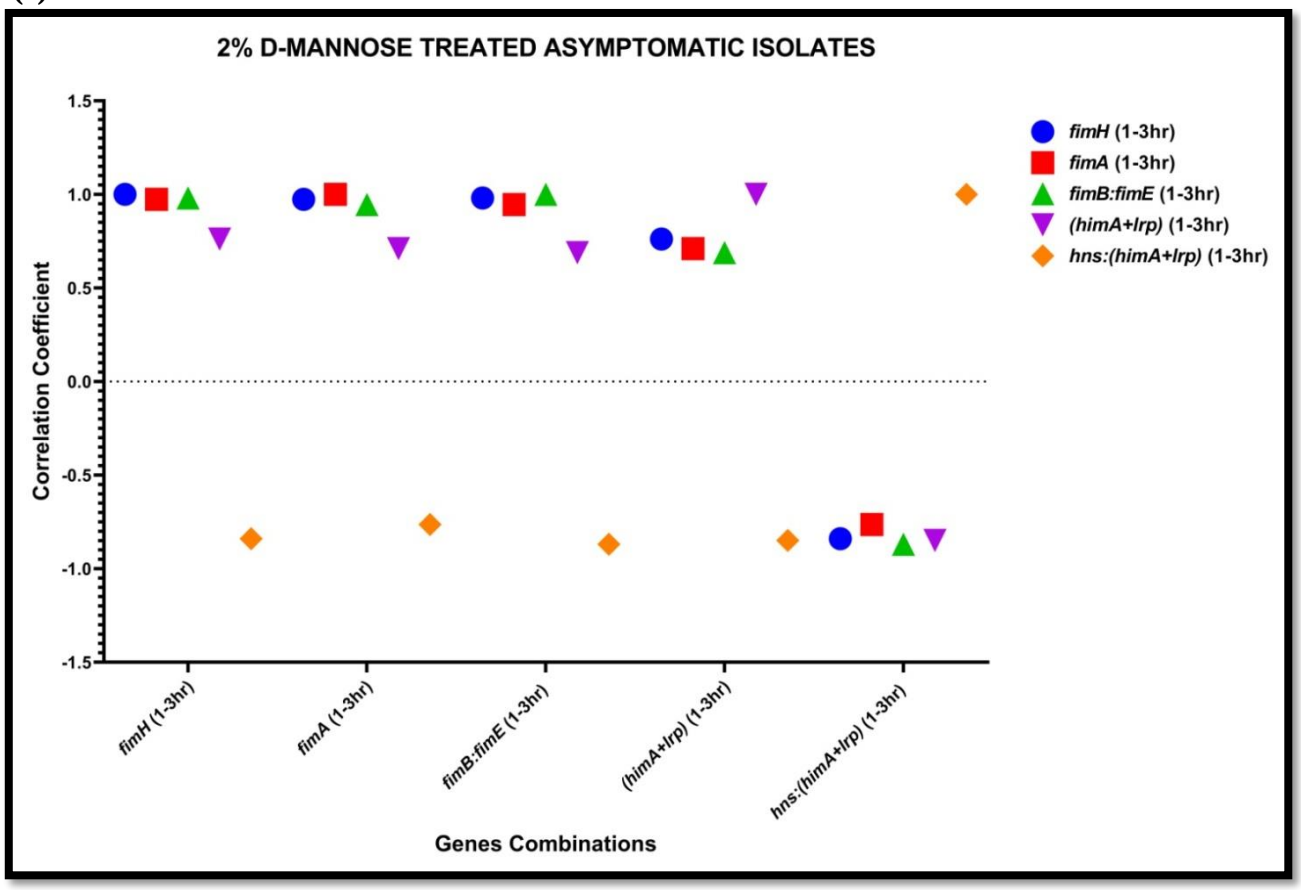
(This study)

(b)



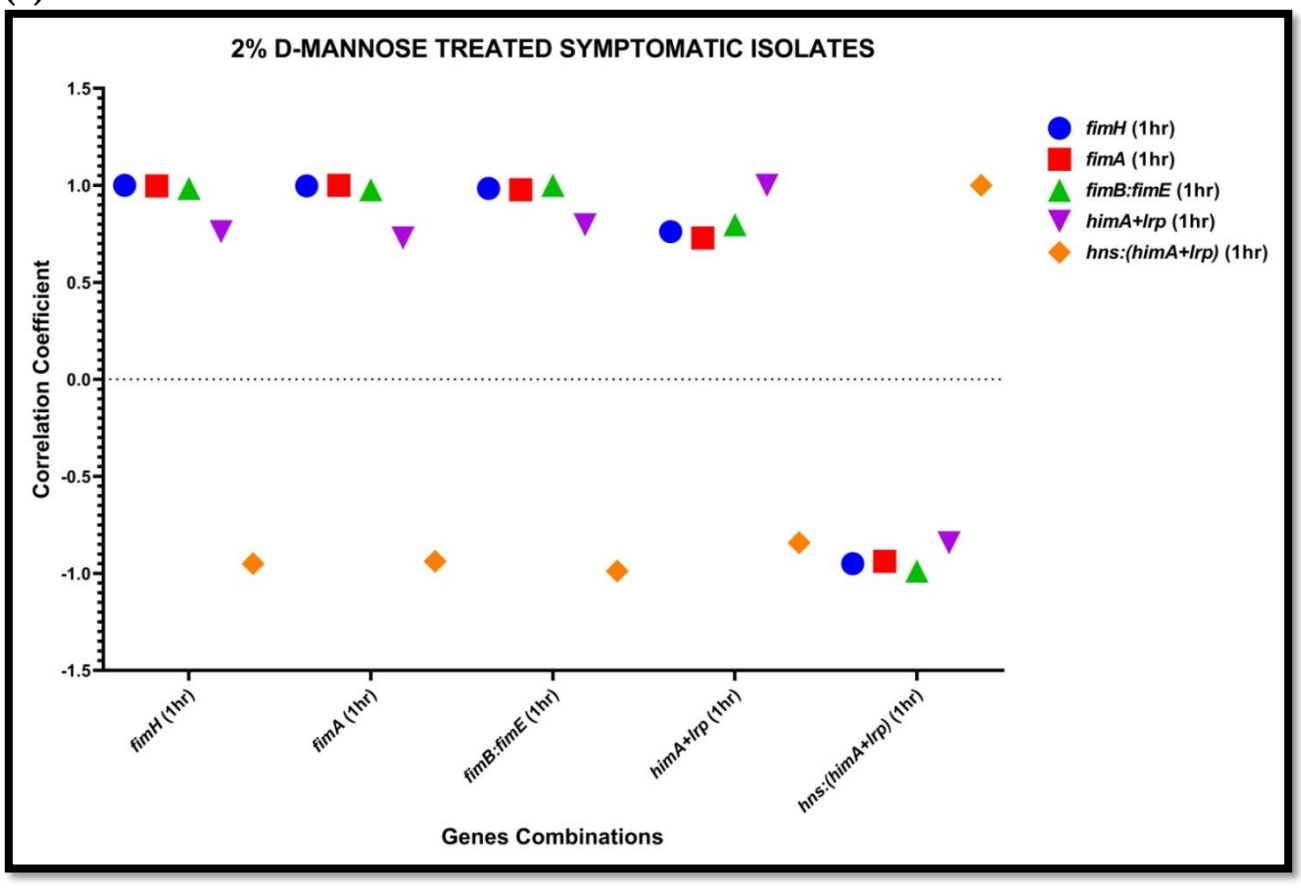
(This study)

(c)



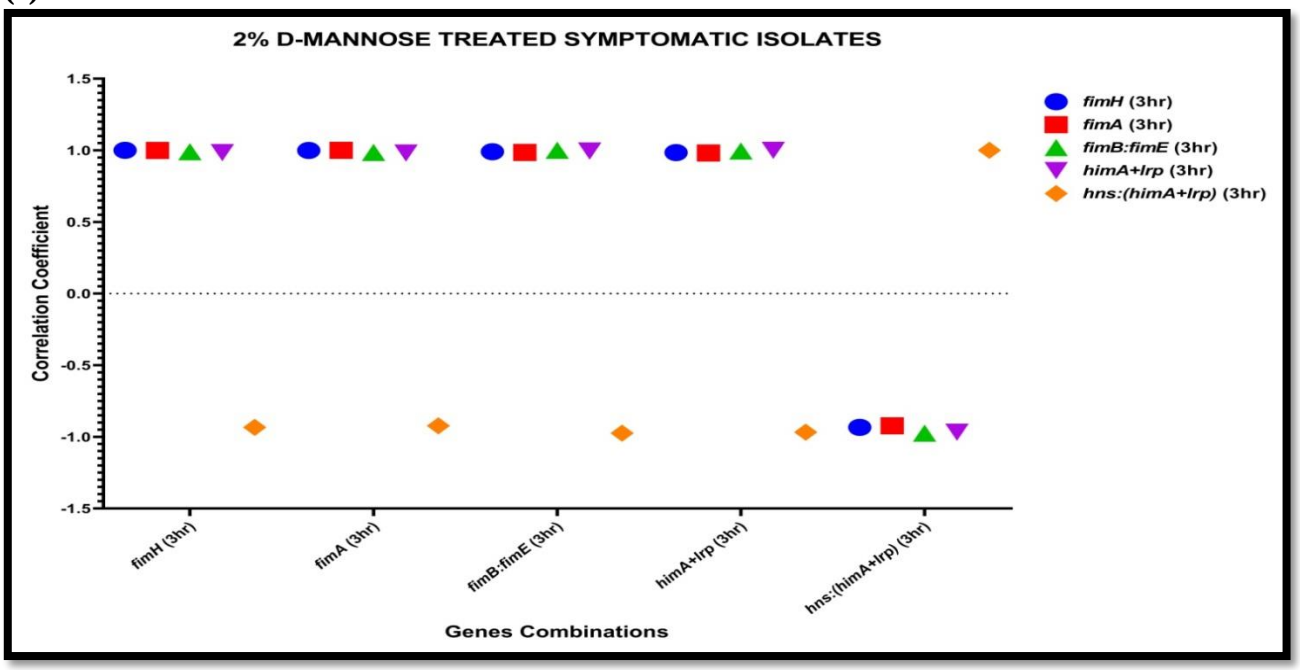
(This study)

(d)



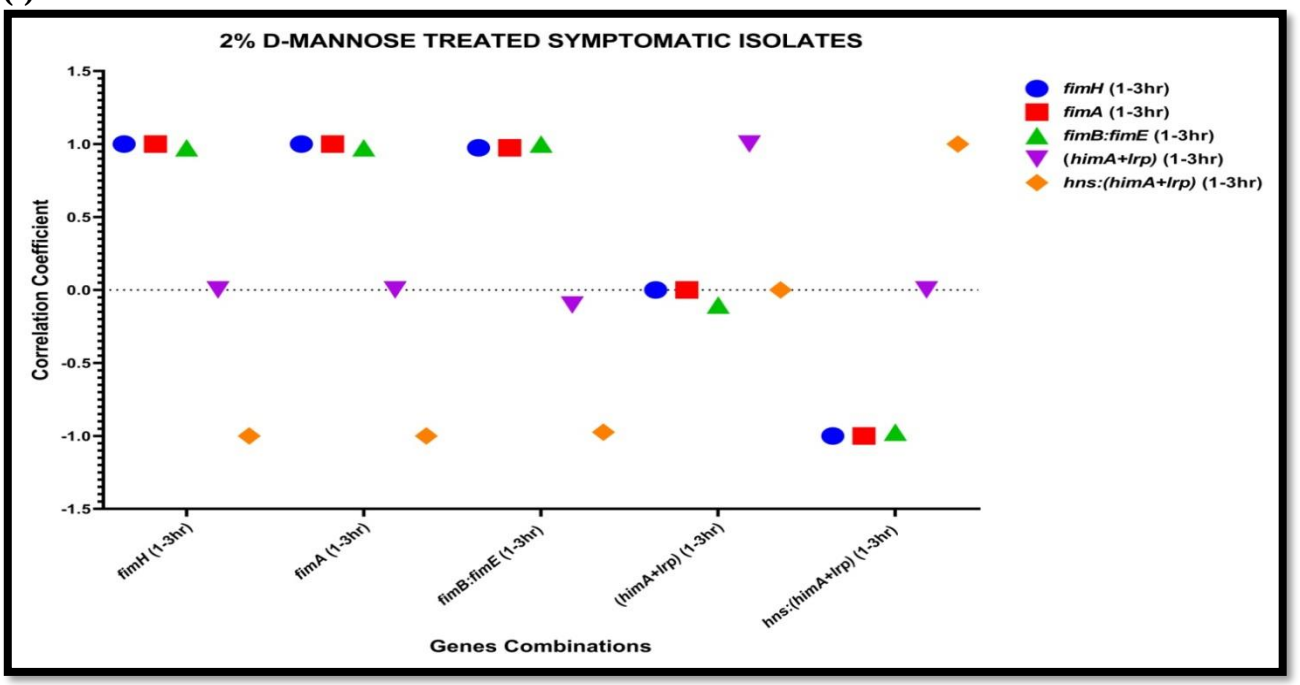
(This study)

(e)



(This study)

(f)



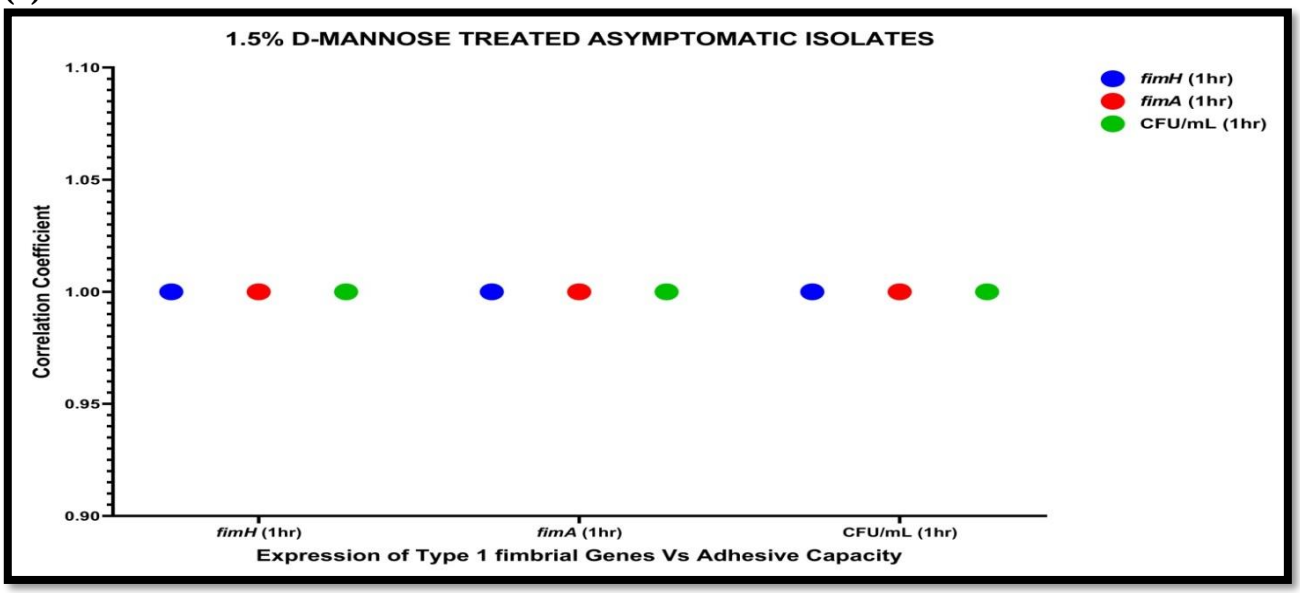
(This study)

Fig. 5.19: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of quantitative expression of two type 1 fimbrial genes (*fimH* and *fimA*), ratio of their recombinases (*fimB*: *fimE*), regulator combinations (*himA+lrp*) and ratio of regulator combinations [*hns*: (*himA+lrp*)] at different studied infection durations and also their relative changes in among 2% D-mannose treated asymptomatic (a) 1hr (b) 3hr (c) 1 to 3hrs relative changes and symptomatic UPECs (d) 1hr (e) 3hr (f) 1 to 3hrs relative changes. Different genes were represented by one symbol per row with varied colours

5.5.12 Interrelationships between the Type 1 fimbrial genes (*fimH*, *fimA*) and adhesive capacity of UPECs post D-Mannose treatment

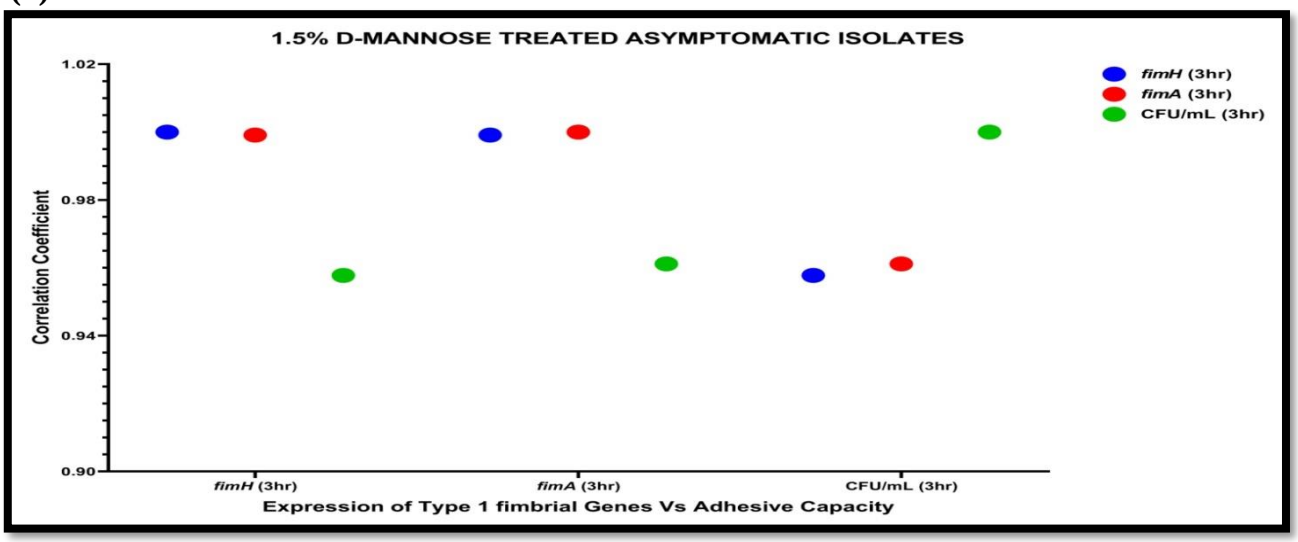
Strong positive correlations were perceived in the expression level of *fimH*, *fimA*, and adhesive capacity (CFU/mL) among both the 1.5% **(Fig. 5.20a-b; Fig. 5.20d-e)** and 2% **(Fig. 5.21a-b; Fig. 5.21d-e)** D-mannose treated adherent ABU and symptomatic UPECs, relating to both 1hr and 3hrs of infections respectively. However, the extent of correlation was slightly diminished at 3hrs duration compared to the 1hr. Nevertheless, a significant but low to moderate level of positive correlations between relative changes in the level of expression of *fimH*, *fimA* with that of the adhesive capacity was perceived among 1.5% and 2% D-mannose treated adherent ABU **(Fig. 5.20c; Fig. 5.21c)** unlike the symptomatic UPECs **(Fig. 5.20f**; **Fig. 5.21f)** respectively from 1 to 3hrs of infection.

(a)



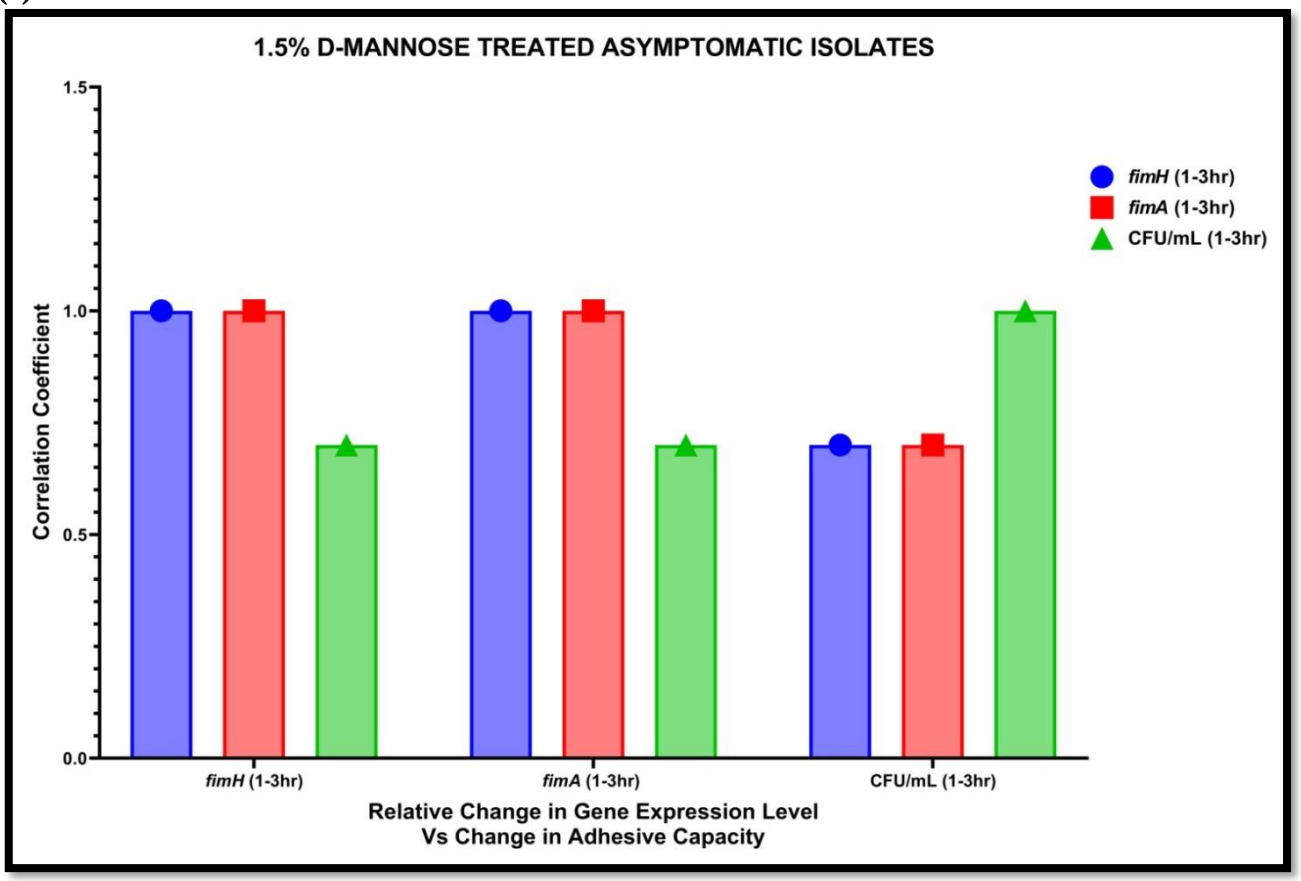
(This study)

(b)



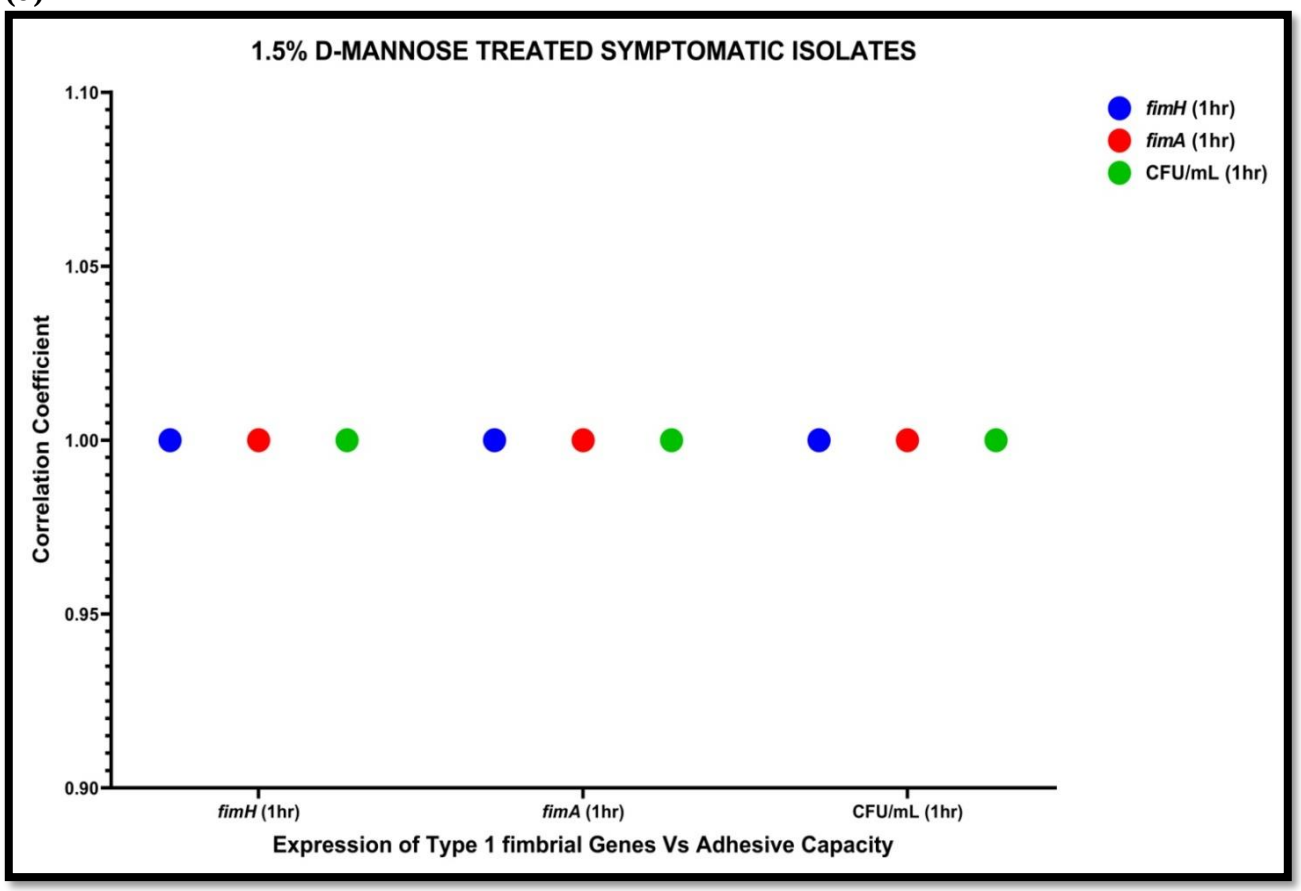
(This study)

(c)



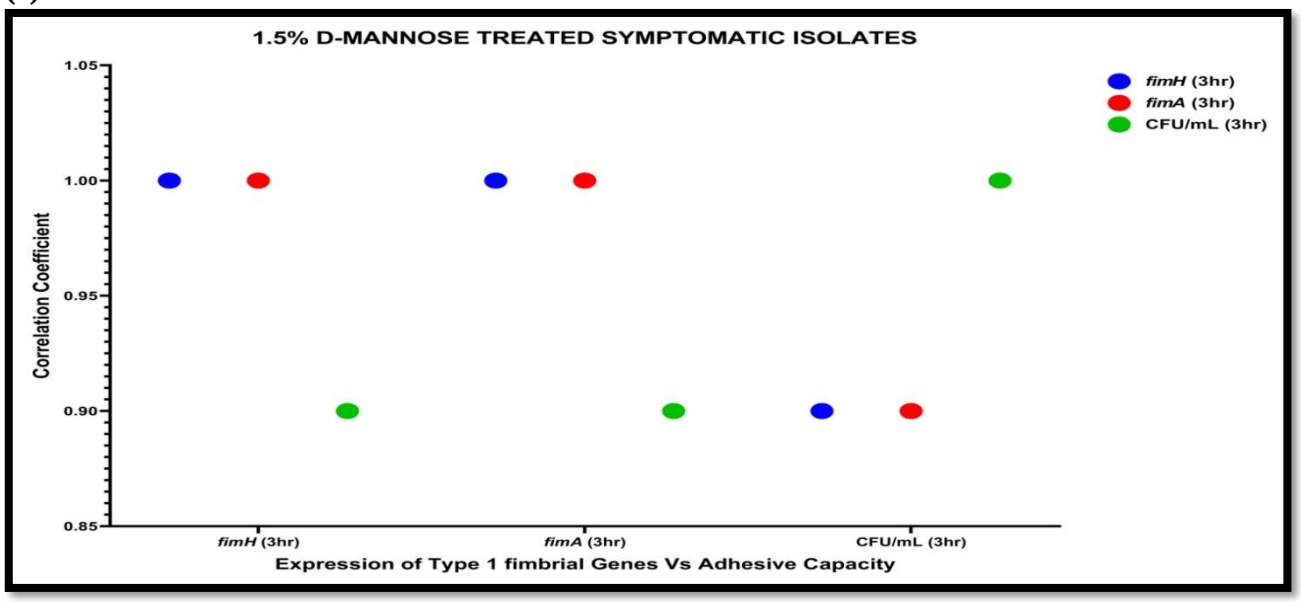
(This study)

(d)



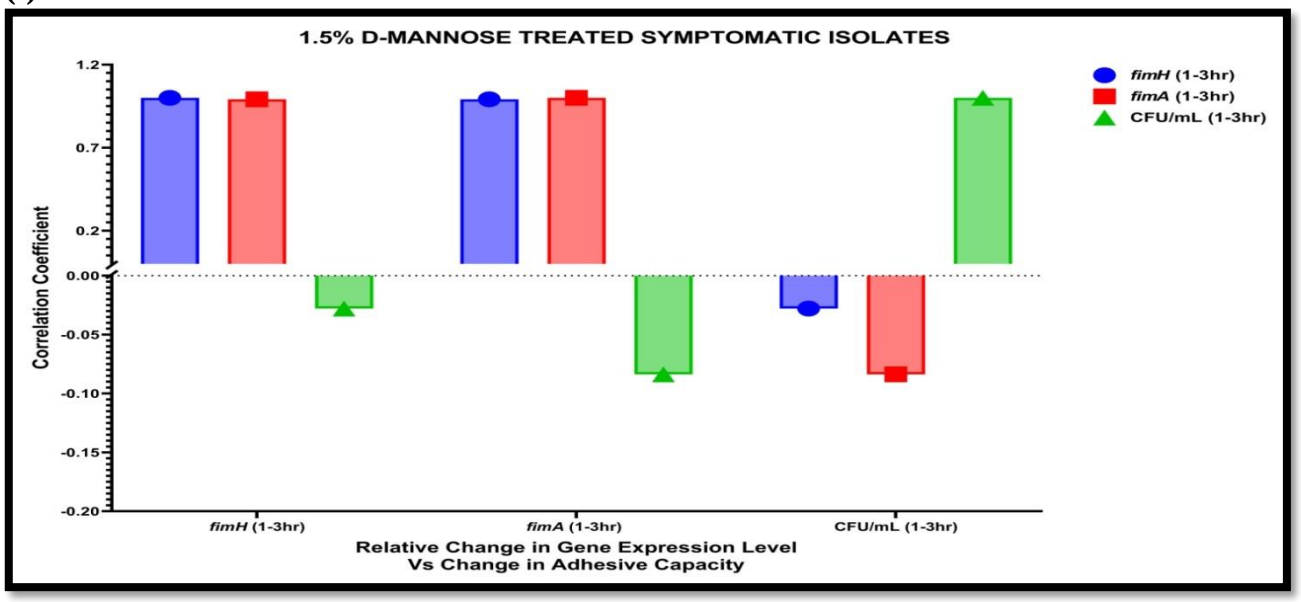
(This study)

(e)



(This study)

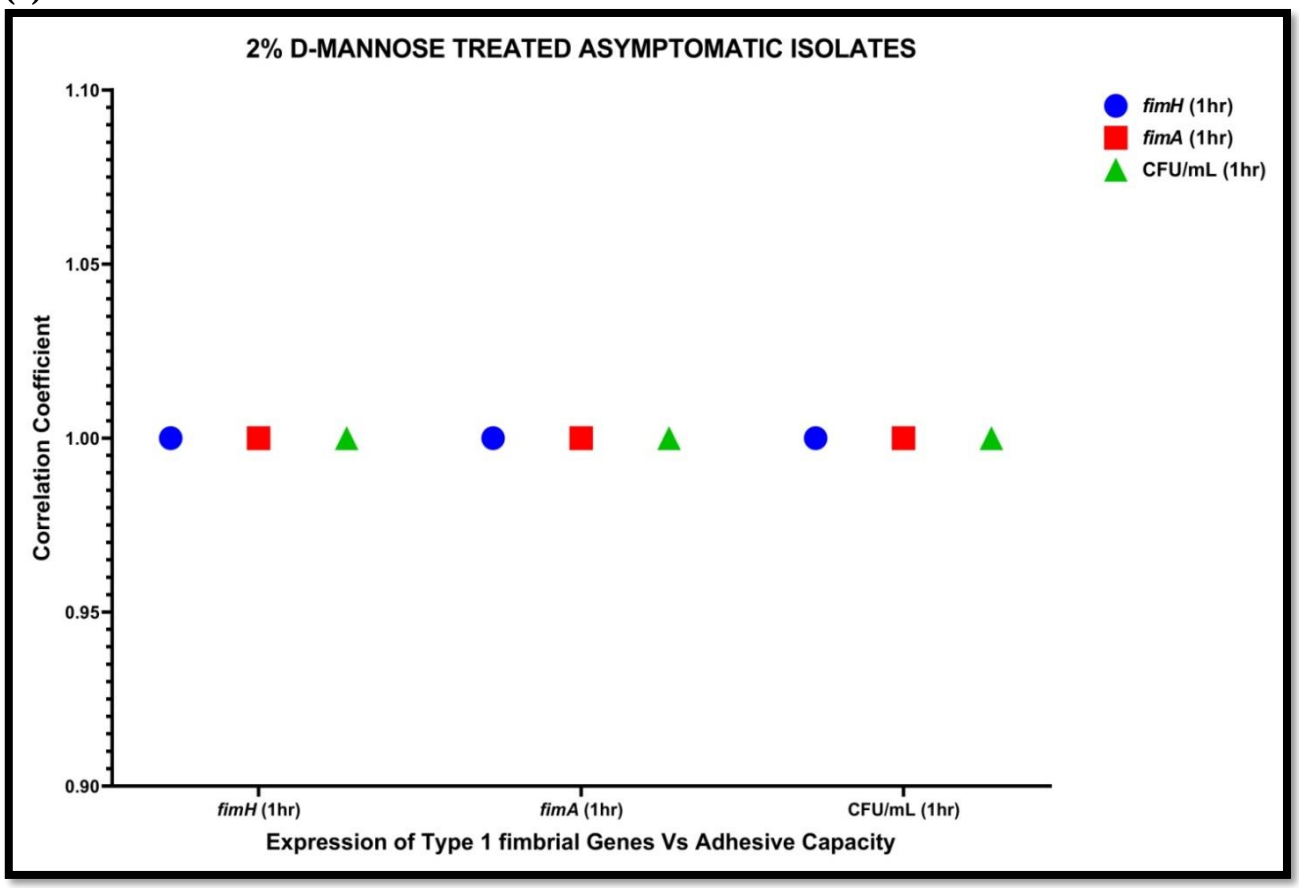
(f)



(This study)

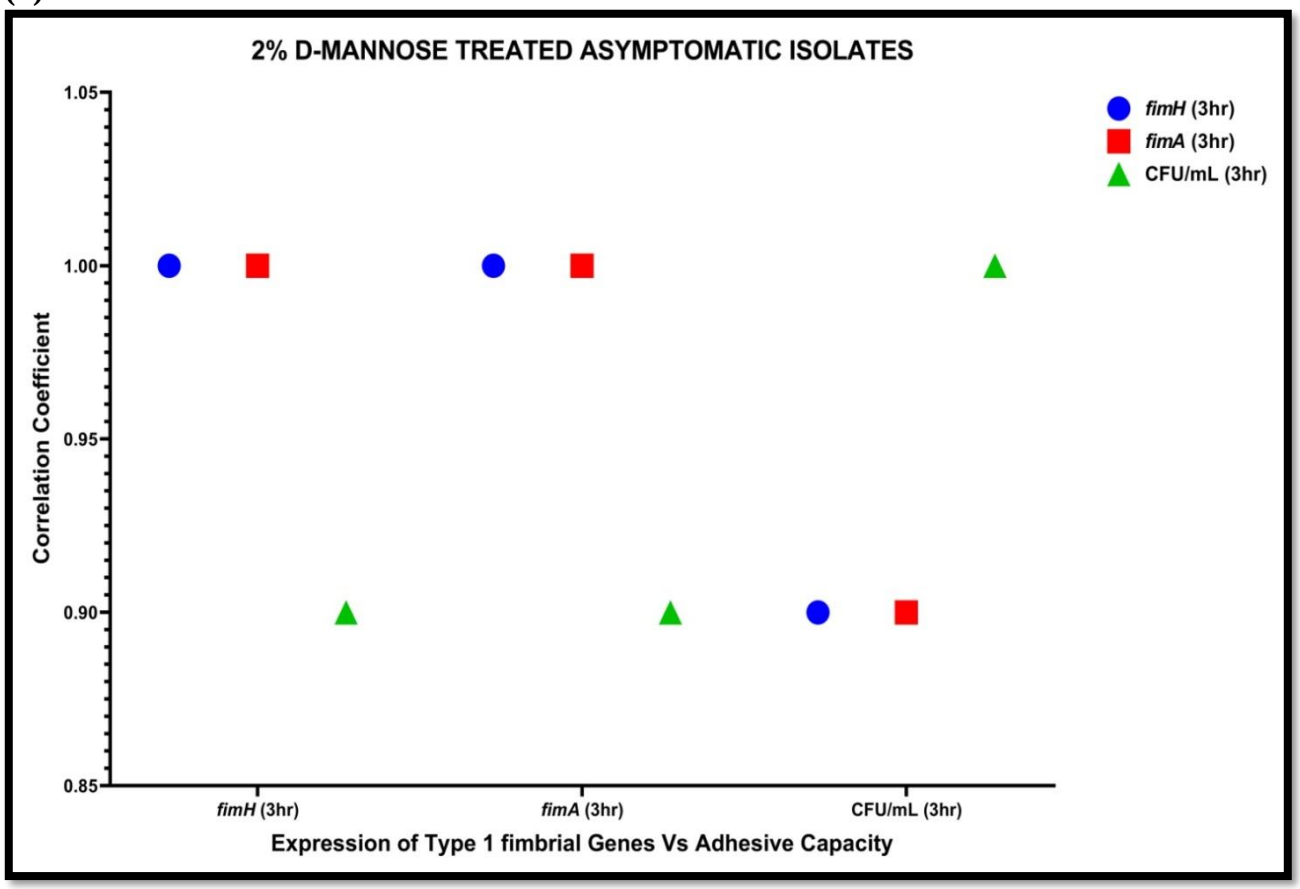
Fig. 5.20: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of quantitative expressions of two different type 1 fimbrial genes (*fimH*; *fimA*) and adhesive capacity of asymptomatic and symptomatic UPECs to T24 uroepithelial cells post 1.5% D-mannose treatment respectively: (a) *fimH* (1hr); *fimA* (1hr); CFU/mL (1hr) in asymptomatic UPECs (b) *fimH* (3hr); *fimA* (3hr); CFU/mL (3hr) in asymptomatic UPECs (c) *fimH* (1 to 3hrs relative changes); *fimA* (1 to 3hrs relative changes); CFU/mL (1to 3hrs relative changes) in asymptomatic UPECs (d) *fimH* (1hr); *fimA* (1hr); CFU/mL (1hr) in symptomatic UPECs (e) *fimH* (3hr); *fimA* (3hr); CFU/mL (3hr) in symptomatic UPECs (f)) *fimH* (1 to 3hrs relative changes); *fimA* (1 to 3hrs relative changes); CFU/mL (1to 3hrs relative changes) in symptomatic UPECs. Different genes and adhesive capacity were represented by bar graphs associated with one symbol per row with varied colours.

(a)



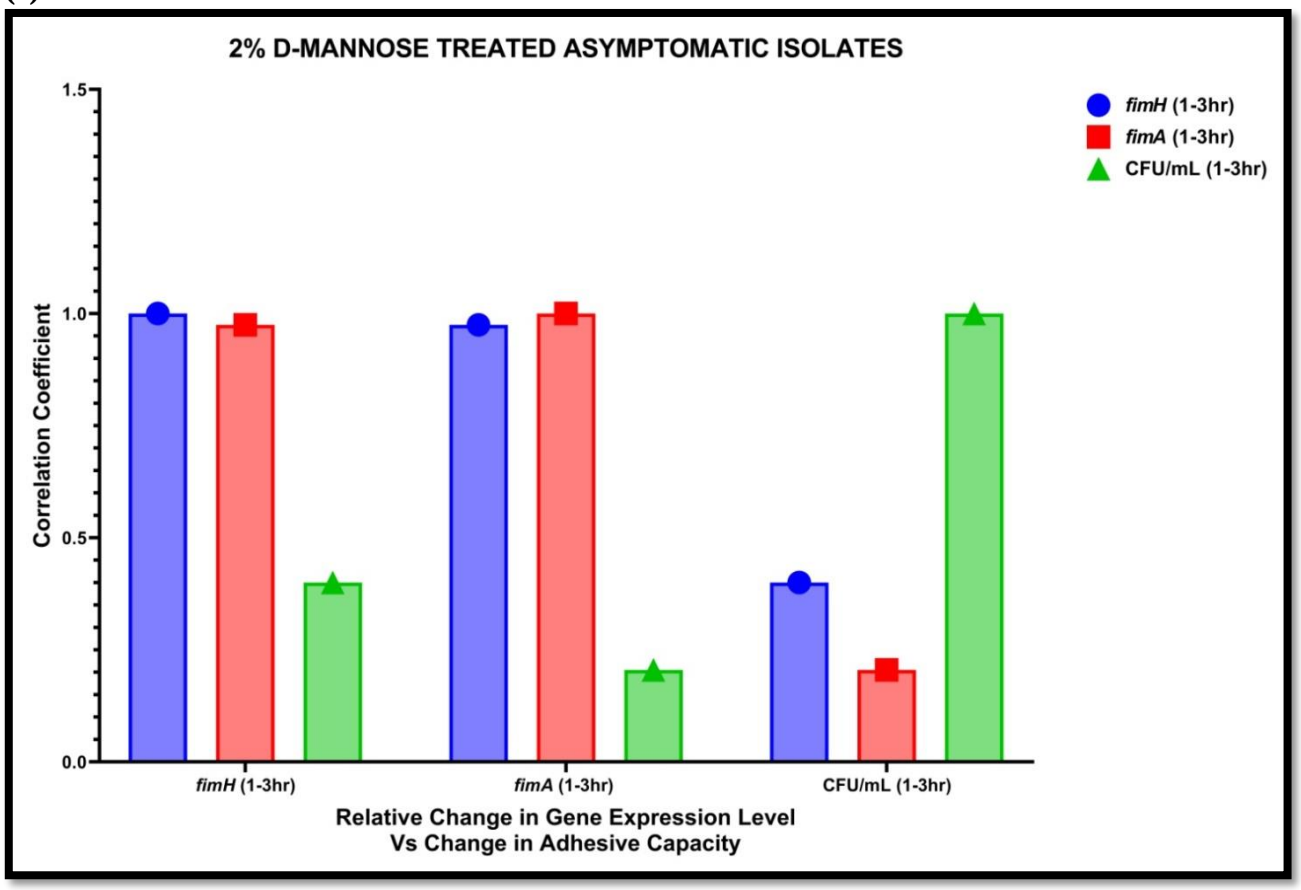
(This study)

(b)



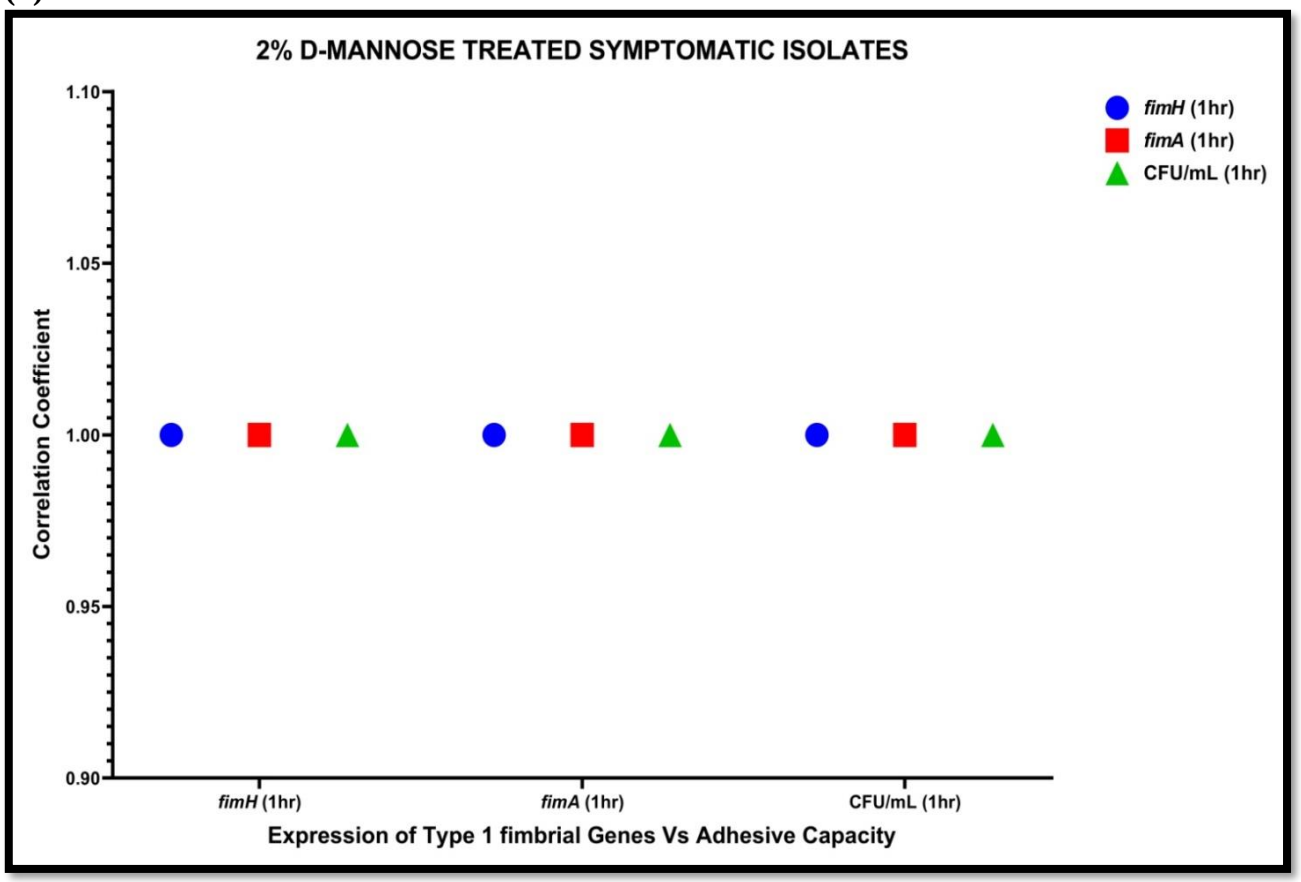
(This study)

(c)



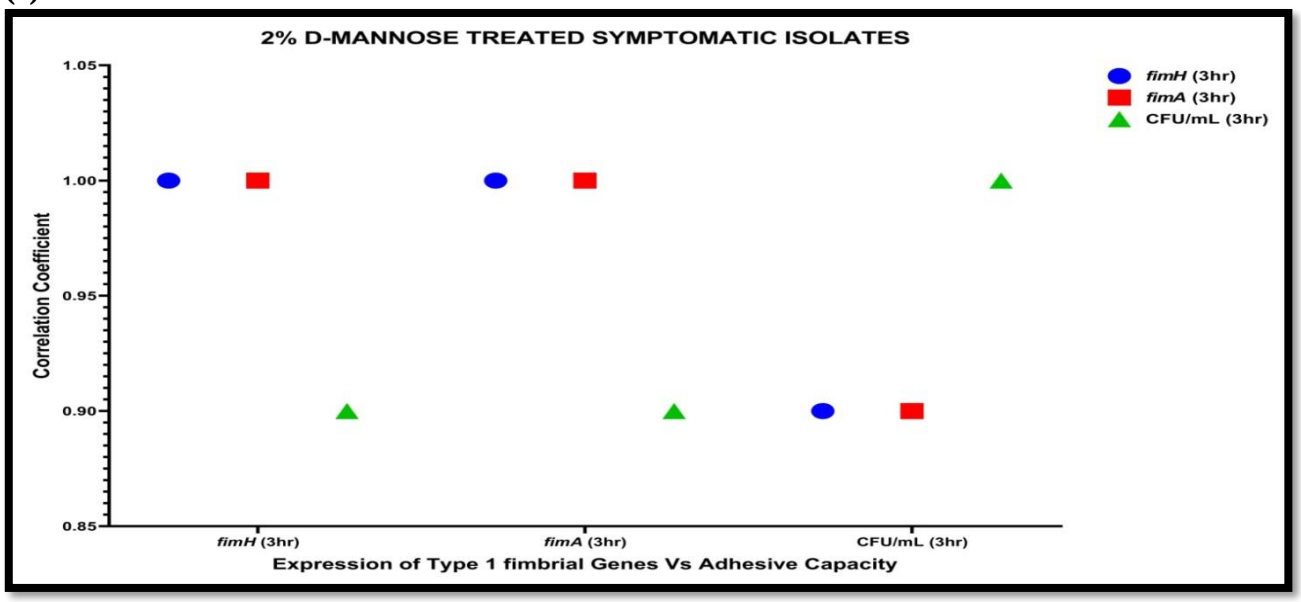
(This study)

(d)



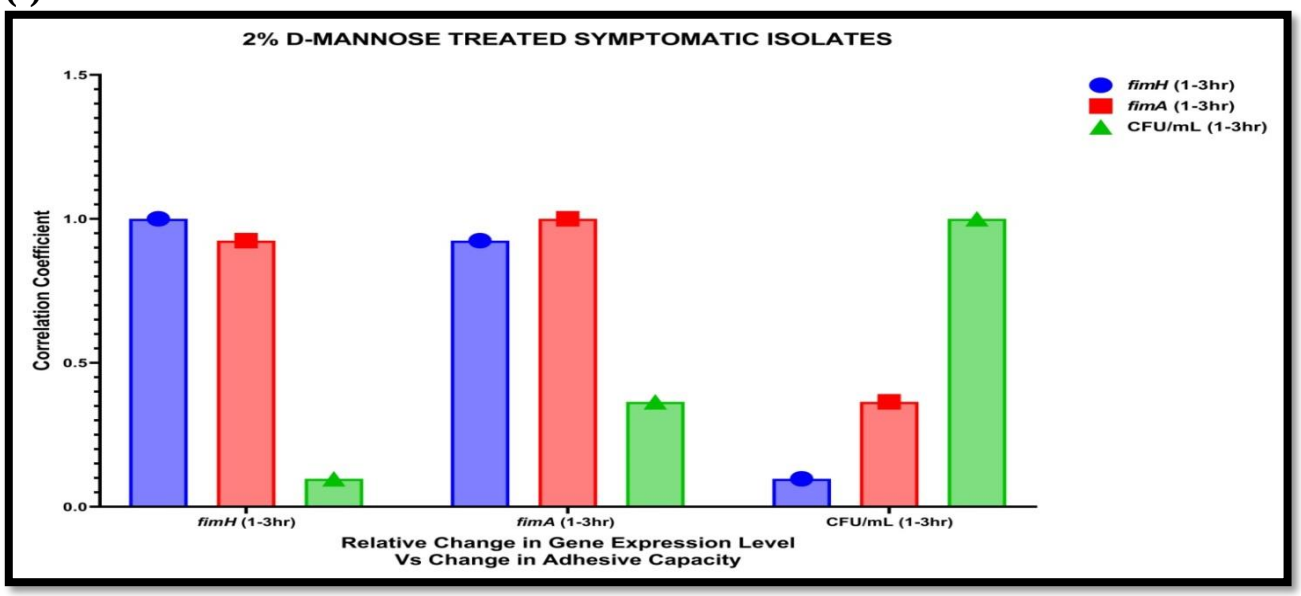
(This study)

(e)



(This study)

(f)



(This study)

Fig. 5.21: Graphical representation of correlation coefficient values computed with confidence level of 95% (p values ≤ 0.05) using GraphPad Prism version 9 (Prism software package) based on the correlation of quantitative expressions of two different type 1 fimbrial genes (*fimH*; *fimA*) and adhesive capacity of asymptomatic and symptomatic UPECs to T24 uroepithelial cells post 2% D-mannose treatment respectively: (a) *fimH* (1hr); *fimA* (1hr); CFU/mL (1hr) in asymptomatic UPECs (b) *fimH* (3hr); *fimA* (3hr); CFU/mL (3hr) in asymptomatic UPECs (c) *fimH* (1 to 3hrs relative changes); *fimA* (1 to 3hrs relative changes); CFU/mL (1to 3hrs relative changes) in asymptomatic UPECs (d) *fimH* (1hr); *fimA* (1hr); CFU/mL (1hr) in symptomatic UPECs (e) *fimH* (3hr); *fimA* (3hr); CFU/mL (3hr) in symptomatic UPECs (f)) *fimH* (1 to 3hrs relative changes); *fimA* (1 to 3hrs relative changes); CFU/mL (1to 3hrs relative changes) in symptomatic UPECs. Different genes and adhesive capacity were represented by bar graphs associated with one symbol per row with varied colours.

5.6 Discussion

The current study exhibited the universal incidence of MSHA, evocative of type 1 fimbrial expression, among all the studied clinical ABU UPECs, similar to symptomatic ones, quite contrary to the previous reports from Australia and India **(Mabbett et al. 2009; Srivastava et al. 2016).** However, unlike the clinical ABU UPECs, prototype ABU strain *E. coli* 83972 did not express functional type 1 fimbriae *in vitro*, akin to earlier reports **(Hull et al. 1999; Roos et al. 2006)** from the United States of America and Denmark**.** The functional type 1 fimbriae *in vitro* in the entire set of clinical UPECs irrespective of their asymptomatic and symptomatic nature suggested their plausible cogent adhesive capacity and colonization potential relating to the uroepithelial cells, discordant to the prototype ABU strain. However, although *in vitro* phenotypic study (MSHA) indicated the presence of type 1 fimbrial expression in all UPECs regardless of their asymptomatic or symptomatic nature, genotypic analysis concerning the orientation of *fim* switch mostly showed phase OFF orientation at the various studied durations, growth and temperature conditions suggestive of the absence (**Schwan 2011; Schwan and Ding 2017**) of the type 1 fimbrial expression, however the results observed were inconsistent (5.5.2). This contrariety between the *in vitro* genotypic and phenotypic studies might be due to the high inversion rates of the existent *fim* switch in the studied UPECs. The aforementioned observations might also be due to the fact that UPECs found in the infected urine are mainly in OFF orientation when cultured *in vitro* unlike to that observed in the case of the UPECs attached to the uroepithelium as reported previously **(Graham et al. 2001)** from the United Kingdom. However, the aforesaid inconsistency in the *in vitro* genotypic study together with its discordance to the phenotypic study at log phase of culture demanded the further evaluation of these strains after attachment to human uroepithelial cells.

Earlier reports from different parts of the world like the United States of America **(Kariyawasam and Nolan 2009; Tchesnokova et al. 2011)** and the United Kingdom **(Li et al. 2009)** indicated that FimH adhesin of type 1 fimbriae is necessary for the MSHA (phenotypic) property of the UPECs. Moreover, Bien et al. **(Bien et al. 2012)** from Poland specified that the piliated cells were known to bind to the urothelial mannosylated glycoproteins uroplakin Ia and IIIa (UPIIIa) via the adhesin subunit FimH, located at the fimbrial tip. Additionally, the previous part of this study (Chapter 3) **(Ghosh and Mukherjee 2019)** displayed that universal incidence of the *fimH* adhesin gene among all the ABU and symptomatic UPECs, mostly in accordance with the previous reports from Denmark **(Roos et al. 2006)** and India **(Srivastava et al. 2016)** respectively. Likewise, all isolates included in this study showed MSHA property connotative of the functional FimH adhesin. Therefore, the aforementioned reports along with the results obtained in this study demanded further analysis of the isolated UPECs regardless of their asymptomatic and symptomatic behaviour with regard to the FimH

adhesin. Additionally, an earlier report from the United States of America (**Weissman et al. 2007**) stated that mutations occur in this adhesin very frequently and other from France **(Hommais et al. 2003)** and the United States of America **(Sokurenko 2016)** indicated that certain mutations in FimH adhesin are sometimes known to increase the pathogenicity of *E. coli* and also known to offer significant advantage upon bacteria during bladder colonization. Withal, in this study several mutations identified in the lectin (V27A, G66S, N70S, S78N, P102S, A119V) and pilin (R166H, A202V, Q269K) domain of FimH adhesins present in the ABU UPECs **(Table 5.4a; 5.5a)**, were also common to those identified in the symptomatic UPECs **(Table 5.4b; 5.5b)**, except for A119V and Q269K which were only observed in the ABU UPECs and Q41K, G66V, V118G, V128M, V163A that were unique to the symptomatic isolates respectively. Moreover, earlier reports from France and Italy showed that some random point mutations in FimH lectin domain (V27A, G66S, N70S, S78N, A119V) and pillin domain (V163A) enhanced pathogen's fitness during an infection that is they were potentially pathoadaptive **(Hommais et al. 2003; Iebba et al. 2012)**. The degree of naive mucosal inflammation was related to specific FimH alleles that harbor V27A and was reported as pathoadaptive in Crohn's disease **(Iebba et al. 2012)**. This study revealed that 80% of the ABU UPECs harboured the hotspot mutation V27A in lectin domain of FimH adhesin, either alone or in combination with the other NSMs exactly similar to that found in the symptomatic UPECs. Furthermore, this study also showed that overall prevalence of the other aforesaid previously reported pathoadaptive FimH mutations in isolated ABU were almost similar to that of the symptomatic ones. This high incidence of the pathogenic variants of FimH adhesin in our ABU isolates might be considered to facilitate the first step in the initiation of infection **(Ghosh and Mukherjee 2019)**.

Moreover, previous reports from the United States of America **(Hull et al. 1999)** and Denmark **(Roos et al. 2006**) stated limited uroepithelial cell adherence of the prototype ABU strain *E. coli* 83972, but they were reported to be capable of deliberate long-term bladder colonization (**Roos et al. 2006; Stork et al. 2018).** Additionally, several studies conducted various parts of the world like Denmark **(Roos et al. 2006)**, Australia **(Mabbett et al. 2009)** and Poland **(Bien et al. 2012)** stated the nonadherent nature of different ABU strains. This study for the first time reported 100% successful adhesion of clinical ABU UPECs to the human epithelial cells, akin to the symptomatic ones. This observation was in contrary to the previous studies conducted on the clinical ABU UPECs from Australia (**Mabbett et al. 2009)** and Hungary (**Stork et al. 2018)** that intimated the nonadherent properties of the majority of the studied ABU UPECs. Nonetheless, limited uroepithelial cell adherence was perceived in the case of the prototype ABU strain *E. coli* 83972 both post 1 and 3hrs of infection which was in accordance with previous reports from the United States of America **(Hull et al. 1999)**, Denmark **(Roos et al. 2006)**

and Stork et al. 2018) Moreover, statistically significant **(Table 5.7)** incidence of remarkable adhesive capacity among ABU UPECs, greater than the control strain, with efficiency greater than 30% of the symptomatic UPECs, post 1hr of infection **(Table 5.6a)**, was highly distressing. This highlighted the fact that bladder epithelial cell adherence of ABU UPECs inside the human urinary tract without manifestation of symptoms might be due to the inhibition of NFkB-dependent transcription factors and hence the pro-inflammatory cytokine response as suggested in a study conducted by Mabbett et al. **(Mabbett et al. 2009)** from Australia. Alternately**,** this also adduced the possibility that ABU in our population might have persisted in the bladder cells without eliciting antibacterial defence by the host due to evasion of normal immune surveillance by binding to a class of receptors that do not contribute to the signalling and host cell activation.

This study for the first time investigated the *fim* switch of ABU UPECs isolated from males and non-pregnant females, and also compared them to symptomatic UPECs. The significant predominance of "both OFF and ON" orientation of *fim* switch among both ABU and symptomatic UPECs after both 1 and 3hrs of infection **(Table 5.7)** was mostly in congruence with a previous study **(Struve and Krogfelt, 1999)** from Denmark that examined the orientation of *fim* switch in symptomatic UPEC *in vivo* during a mouse model infection. This was indicative of an active and an alternating *fim* switch that might have interspersed between the piliated and non-piliated phase states due to their high inversion rates. Moreover, the presence of invertible *fim* switch was identified among all the clinical ABU UPECs, unlike the prototype, ABU strain *E. coli* 83972 which delineated the fact *fimS* region in the former isolates was not deleted as in the case of the prototype strain **(Roos et al. 2006; Ambite et al. 2019).** This study reported that isolates with 75% and lesser adhesive capacity than the control strain, showed OFF orientation of *fim* switch, evocative of a non-functional type 1 fimbriae **(Schwan, 2011; Schwan and Ding 2017)** irrespective of their asymptomatic **(Table 5.6a; Table 5.6b; Table 5.8a)** or symptomatic **(Table 5.6a; Table 5.6b; Table 5.8b)** nature especially after 3hrs of infection durations. Furthermore, 25% of symptomatic UPECs with only ON orientation of *fim* switch, connotative of the functional type 1 fimbriae **(Schwan, 2011; Schwan and Ding 2017)** exhibited strikingly high adhesive capacity compared to the control strain. The aforementioned observations betokened a plausible relationship between adherence and phase switch of UPECs, further evincive of an efficacious role of type 1 fimbriae in mediating UPECs attachment to uroepithelial cells, a process governed by inversion of phase switch in type 1 fimbriae operon as suggested in previous studies from the United States of America **(Schwan, 2011; Schwan and Ding 2017)**. Furthermore, a significant number of ABU UPECs switched to only OFF orientation of *fim* switch, an inactive phase state post 3hrs of infection, unlike 1hr which indicated a gradual loss of type 1 fimbrial expression post 3hrs of infection. Therefore, a significant incidence of the only OFF orientation of *fim* switch together with marked but overall low

adherence potential among ABU UPECs in comparison to symptomatic ones adumbrated the lower expression of type1 fimbrial genes in the case of the former group. This highlighted the fact that procuration of these chromosomal genes on different pathogenic islands (PAIs) by mobile genetic elements (MGEs) and their consequent horizontal gene transfer (HGT) might have occurred differently in the case of ABU and symptomatic UPECs that led to the acquisition of different adhesin variants in the case of the former group, owing to unbridled drug usage.

The present study reported the significant number of UPECs from both ABU **(Fig. 5.8a-b; Fig. 5.8d-e; Fig. 5.11a-b)** and symptomatic **(Fig. 5.9a-b; Fig. 5.9d-e; Fig. 5.11c-d)** group to demonstrate higher expression of recombinase gene *fimE* compared to the *fimB* recombinase, a lower *fimB: fimE* ratio post 3hrs of infection**,** together with lowly expressive type1 fimbrial adhesin *fimH* and major structural gene *fimA*, compared to their expression post 1hr. These observations were further validated by the significant difference in the echelon of expressions of *fimH*, *fimA*, *fimB*, and *fimE* genes between 1 and 3hrs of infection in the case of both ABU **(Fig. 5.10e)** and symptomatic **(Fig. 5.10f)** UPECs. The aforementioned observations, along with the high positive correlations between the *fimB: fimE* ratio and the two other type 1 fimbrial genes (*fimH* and *fimA*) among all isolates irrespective of their asymptomatic **(Fig. 5.13a-c)** or symptomatic **(Fig. 5.13d-f)** nature at 1, 3 and 1to 3hrs (relative changes in the expression) of infections respectively indicated a potential positive role of *fimB* recombinase in activation of type 1 fimbrial genes towards a piliated state by inducing their expression **(Schwan, 2011; Schwan and Ding 2017)** and a negative role of *fimE* recombinase in actuating their expression thereby silencing the transcription of the structural gene *fimA* and adhesin *fimH* **(Abraham et al. 1985; Schwan, 2011; Schwan and Ding 2017)**, paving the way of the *E. coli* isolates towards a non-piliated type.

Furthermore, the previous studies conducted from different countries of the world like the United States of America **(Blomfield et al. 1993)**, Ireland **(O'Gara and Dorman 2000; Corcoran and Dorman, 2009)** and the United States of America **(Schwan, 2011)** reported the regulatory role of Lrp; IHF, and H-NS proteins in maintaining the phase ON (piliated state) and phase OFF (non-piliated state) orientation respectively of *E. coli* isolates. Nonetheless, this study displayed an increase in *hns*: (*himA+lrp*) ratio together with the decrease in *fimB: fimE* ratio and expression of *fimH* and *fimA* genes UPECs regardless of their asymptomatic **(Fig. 5.11e)** or symptomatic **(Fig. 5.11f)** nature from 1 to 3hrs of infections, although the level of individual regulatory factors differed among isolates at all the studied infection durations irrespective of their asymptomatic **(Fig. 5.8c; Fig. 5.8f; Fig. 5.8i)** and symptomatic **(Fig. 5.9c; Fig. 5.9f; Fig. 5.9i)** nature, quite contrary to an earlier study **(Olsen et al. 1998)** from Denmark that demonstrated the individual role of H-NS in the modulation of type1 fimbrial expression towards a fimbriate state. This indicated the predominant effect of the combination of all regulatory factors rather than the individual ones on the activation of type 1 fimbrial genes and their recombinases.

This was further substantiated by the observations of inconsistent levels of positive or negative correlations between individual regulatory factor genes *hns*, *himA* encoding IHF, *lrp*, the type 1 fimbrial genes, and their recombinases among both ABU and symptomatic UPECs at 1, 3, and 1 to 3hr (relative changes) durations respectively in the absence **(Fig. 5.12a-f)** and presence of 1.5 % **(Fig. 5.16a-f)**; 2% **(Fig. 5.17a-f)** D-mannose exposure respectively. However, a consistent negative relationship of [*hns:* (*himA*+*lrp*)] ratio with that of *fimB: fimE* ratio and individual expressions of *fimH* and *fimA* genes among both ABU and symptomatic UPECs was espied at the studied infection durations and their relative changes respectively in the absence **(Fig. 5.13a-f)** and presence of 1.5 % **(Fig. 5.18a-f)**; 2% **(Fig. 5.19a-f)** D-mannose. Additionally, in the present study *fimH* expression of isolates was found to be highly correlated to *fimA* which was further positively correlated to their adherence potential **(Fig. 5.14a-d)** and was also mostly associated with their *fim* switch orientation **(Table 5.9a-b)** regardless of their asymptomatic or symptomatic nature and the studied infection durations. This observation was mostly in compliance with previous reports on symptomatic UPECs from Poland and China respectively (**Bien et al. 2012; Duan et al. 2017).** Comparable observations were also perceived after the D-mannose exposure **(Fig. 5.20a-f; Fig. 5.21a-f)**. Therefore, the aforesaid observations delineated a concerted action of three studied regulatory factors on the regulation of the *fimB* and *fimE* recombinases which further governed the expression of type 1 fimbrial genes among UPECs irrespective of their asymptomatic and symptomatic nature by controlling the inversion of *fim* switch and adhesive capacity. To boot, this study is the first of its kind that threw spotlight on the role of regulatory factors that control the type 1 fimbrial expression in ABU UPECs.

Studies conducted from Italy (**Scribano et al. 2020; Scaglione et al. 2021**) reported the momentous role of different concentrations of D-mannose in the inhibition of bacterial adhesion to uroepithelial cells and others from countries like the United States of America (**Kariyawasam and Nolan 2009)**, United Kingdom **(Li K et al. 2009)**, Iran **(Tabasi et al. 2015)**, India **(Desai et al. 2013)** promulgated their remarkable role in blockage of type 1 fimbrial expression phenotypically. The present study exhibited a significantly lower extent of adhesion to T24 uroepithelial cells in the case both the 1.5% and 2% D-mannose treated ABU **(Fig. 5.15a-b)** and symptomatic **(Fig. 5.15c-d)** UPECs after both 1 and 3 hrs of infection relative to their untreated controls, except in the case 1.5% D-mannose treated symptomatic UPECs. This observation was mostly in agreement with a previous study (**Scribano et al. 2020)** from Italy performed mainly on the symptomatic prototype strain. This inhibition of adhesion on D-mannose treatment was highly significant in the case of ABU UPECs **(Fig. 5.15a-b)** compared to the symptomatic **(Fig. 5.15c-d)** ones which implied a plausible connection between the extent of adhesion of UPECs and the efficaciousness of the D-mannose treatment. However, a significant increase in the inhibition of adhesion at 2% concentration of exogenous D-mannose compared to 1.5% was discordant

to a previous report from **(Duan et al. 2017)** China that reported a high inhibition using 0.5% D-mannose which did not significantly differ from the 8% concentration. The aforementioned observations, in line with previous reports from Croatia **(Kranjcec et al. 2014)**, Italy **(Domenici et al. 2016; Scaglione et al. 2021)**, further delineated the cogent role of D-mannose as a suitable alternative to prevalent antibiotic regimens in managing UTI.

The current study demonstrated enhanced and strikingly high adhesive capacity **(Table 5.7a-b)** and type1 fimbrial expression **(Fig. 5.8a-c; Fig. 5.9a-c)** in the case of one isolate (75) and three (86,161 and 162) isolates of the asymptomatic and symptomatic group respectively compared to the others of each group, especially post 1 hr of infection. However, this study also identified pathoadaptive FimH mutations (V27A, N70S, and S78N) among all of the aforementioned isolates **(Table 5.5a-b)**. This betokened an association of these amino acid mutations with the higher adhesive capability of UPECs and type 1 fimbrial expression, regardless of their asymptomatic or symptomatic nature, quite similar to an earlier report **(Iebba et al. 2012)** conducted from Italy on *E. coli* isolated from patients suffering from Crohn's disease and ulcerative colitis that stated high adhesiveness of strains with the aforesaid mutations. Moreover, the ABU (74, 80, 84, 93, and 119) and symptomatic (173, 184, 196) isolates that showed marked adhesive capacity **(Table 5.7a-b)** and strong type 1 fimbrial expression **(Fig. 5.8a-c; Fig. 5.9a-c)**, although at extent lower than those mentioned above, harboured either the mutation A119V or G66S in addition V27A except symptomatic isolate 184 **(Table 5.5a-b) (Ghosh and Mukherjee 2019).** These observations were mostly in congruence with the previous reports from Italy and France respectively that related A119V **(Iebba et al. 2012)** and G66S **(Hommais et al. 2003)** to highly adhesive and potentially pathoadaptive *E. coli* strains.

To boot, in this study, most highly adherent UPECs with the marked but varied echelon of type 1 fimbrial expression, irrespective of their asymptomatic (74, 75, 80, 84, 93 and 119) or symptomatic nature (86, 161, 162, 173, 184, 196) were found to be ones with NPP (novel phylotype property) or unknown phylotype as reported in earlier part of this study (Chapter 4) **(Ghosh et al. 2022).** The majority of these isolates were ESBL producers and harboured MGEs *ISEcp1* and/or *IS26* (Chapter 4) **(Ghosh et al. 2022)** reported to be predominantly associated with the dissemination of resistance determinants **(Cattoir et al. 2008; Harmer et al. 2019)**. Nevertheless, no specific relationship could be drawn between the potentially adherent and type 1 fimbriae expressive nature of isolates (this study) with their strain types as described earlier **(Ghosh et al. 2022).** The aforesaid observations professed our earlier observations **(Ghosh and Mukherjee 2019; Ghosh et al. 2022)** thereby stating the fact that the emergence of the strain types with interminable genetic diversity, varied pathogenic and adhesive properties especially among ABU UPECs besides symptomatic ones might be accredited to acquisition and dissemination of resistance genes that led to the selection of random mutations through the

movement of MGEs or chromosomal rearrangements (genome instability) likely in response to indiscriminate drug usage.

5.7 Conclusion

The present study for the first time displayed the universal presence of MSHA among MDR ABU UPECs along with the significant incidence of pathoadaptive FimH mutations *in vitro*. This study also displayed the successful adhesion of all clinical ABU UPECs to human epithelial cells, unlike the prototype ABU strain *E. coli* 83972 but similar to symptomatic ones. Moreover, the significant incidence of remarkable adhesive capacity among ABU UPECs, greater than the control strain, quite similar to symptomatic ones was highly disconcerting. This threw the spotlight on the incidence of the human bladder epithelial cell adherence and persistence of ABU UPECs inside the human urinary tract without manifestation of symptoms. Furthermore, this is the first study that betokened statistically significant incidence of "both OFF and ON" orientation of *fim* switch among the adherent ABU UPECs akin to symptomatic ones which was suggestive of an operative and an alternating fim switch that might have commutated between the piliated and non-piliated phase states. Withal, this is the first study that delineated a concerted action of three studied regulatory factors (H-NS, IHF, and Lrp) on the regulation of the *fimB* and *fimE r*ecombinases which further controlled the functioning of the two major type 1 fimbrial genes, *fimH* adhesin gene, and the structural gene *fimA* among ABU UPECs alike symptomatic UPECs thereby domineering the inversion of fim switch and adhesive capacity. However, despite the incidence of remarkable adhesive capacity and type 1 fimbrial expression in the case of both the MDR ABU and symptomatic UPECs, an overall distinct expression profile of the two type1 fimbrial genes, their recombinases, and regulatory factor genes among isolates of former and the latter groups could be perceived. Therefore, the aforementioned analysis advocated the fact that amassment of these chromosomal genes might have occurred on diverse PAIs by the MGEs and their subsequent HGT that occurred differently in the case of ABU and symptomatic UPECs that led to the procuration of disparate adhesin variants in case of both the groups, due to the rampant drug usage. This study showed a potential relationship between incidence of pathoadaptive FimH mutations, high adhesive capability and type 1 fimbrial expression among MDR ABU UPECs, similar to symptomatic UPECs. Nonetheless, this study also exhibited the exigency for ABU screening, their enhanced cognizance, felicitous understanding, characterization, and inception of antibiotic regimen not only in pregnant females, geriatric patients with unbalanced minds, and in individuals undergoing invasive urologic procedures but also in general individuals with the evidence of acute and/or chronic life-threatening medical conditions, which otherwise might lead to severe complications in the individuals at risk.

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THESIS CONCLUSION

This study provided a detailed insight into the clinical characteristics; antibiogram profile, ESBL/BLIR phenotypic property, distribution of PAIs; virulence factor gene repertoire, and phylogenetic background of the ABU UPECs circulated in Kolkata, an eastern region of the resource-poor country like India, and also further compared them to the symptomatic ones. Moreover, the current study also presented a comprehensive view of the aforementioned isolated ABU UPECs, besides the symptomatic ones with regard to their acquisition of the β-lactamase genes; mobile genetic elements, intricate phylotype property, genetic heterogeneity, STs, CCs, evolutionary/phylogenetic and quantitative relationships to understand their epidemiology and evolutionary origin. Withal, the present study also gave an extensive perspicacity of the type 1 fimbrial phase variation and the regulatory interplay of cellular factors governing this phase variation among adherent ABU UPECs and compared them to the symptomatic ones. Furthermore, this study also subjected ABU and symptomatic UPECs to more intricate analysis to establish a prospective association between their adhesive capacity, the ascertained pathoadaptive FimH mutations, phylotype property, ESBL phenotype, and acquisition of MGEs, in order to get an insight into their interdependence.

This is the first study that identified the significant prevalence of PAI markers (PAI IV536, PAI I CFT073), MGEs (*intI1, intI2, ISEcp1, IS5* and *IS26*), NPPs, MSHA, pathoadaptive FimH mutations among ABU UPECs isolated from hospitalized patients with the predominance of MDR and ESBL production which exhibited the detrimental effect of MDR among this pathogen. The gratuitous use of newer and potent antibiotics in the last few years might have facilitated the selection of novel β-lactamase variants that had further developed complications in health care management. Moreover, this study for the first time attempted to introduce a new approach to determine the phylotype property of the unassigned UPECs. The present study also displayed the clonal heterogeneity and predominance of ST940 (CC448) among ABU UPECs akin to the symptomatic ones along with the evidence of zoonotic transmissions. Moreover, the significantly striking incidence of remarkable adhesive capacity among the ABU UPECs, greater than the control strain, quite akin to symptomatic ones highlighted the incidence of the human bladder epithelial cell adherence of ABU UPECs inside the human urinary tract without manifestation of symptoms. Withal, this is the first study that specified the statistically significant incidence of "both OFF and ON" orientation of type 1 fimbrial phase switch among the adherent ABU UPECs similar to symptomatic ones, indicative of an operative and an alternating *fim* switch that might have commutated between the piliated and non-piliated phase states. Besides this, the current study is the first study of its kind that delineated a concerted action of three studied regulatory factors (H-NS, IHF, and Lrp) on the regulation of the *fimB* and *fimE* recombinases which

further controlled the functioning of the two major type 1 fimbrial genes, *fimH* adhesin gene, and the structural gene *fimA* among ABU UPECs alike symptomatic UPECs thereby dictating the inversion of *fim* switch and adhesive capacity. Furthermore, the incidence of the highly adherent UPECs with remarkably expressive type 1 fimbrial genes that were MDR, mostly ESBL producers, harboured pathoadaptive FimH mutations, MGEs *ISEcp1* and/or *IS26*, known to be associated with dissemination of resistant determinants and possessed NPP or unknown phylotype property, but mostly unrelated to their sequence and clonal types, among ABU UPECs akin to symptomatic ones was highly appalling. This could be attributed to a likely response to indiscriminate drug usage that led to the emergence of strain types with colossal genetic diversity and divergent adhesive properties, especially among ABU UPECs in addition to symptomatic ones. This might be have occurred, likely owing to genome instability caused due to acquisition and dissemination of resistance genes that guided the selection of random mutations through the movement of MGEs or chromosomal rearrangements.

CONTRIBUTION FROM THIS STUDY

- UTIs, one of the most prevalent bacterial infections, are the major public health predicament in terms of morbidity and mortality, thereby affecting millions of people worldwide. UPECs are among the most common ExPEC encountered in the vast majority of ABU and symptomatic UTIs, including cystitis and pyelonephritis. Having appropriate knowledge of the ABU and symptomatic UPECs relating to their PAIs and virulence factor genes distribution, phylogenetic background, genetic diversity, and adhesive properties in association with drug resistance and clonality, keeping geographical locations into consideration is a prerequisite to decipher a strain's proclivity towards infection. The current guidelines from the IDSA suggest screening for ABU and treating it in explicit circumstances such as during pregnancy or before invasive urologic procedures, however, antibiotic overuse for ABU seems to be overpowering in clinical practice. However, recent reports also enunciated deleterious consequences of undiagnosed ABU in the individuals at risk. Therefore, this study is expected to recuperate the society by providing detailed knowledge about the asymptomatic UPECs in comparison to the symptomatic ones regarding their resistance profile various phenotypic, genotypic, epidemiologic, and molecular characteristics with special reference to type 1 fimbriae (key mediator of UPECs attachment to uroepithelial cells). Moreover, this study's essentiality also lies in the fact that it is the first of its kind that threw the spotlight on the regulatory interplay of different cellular factors controlling the expression of different type 1 fimbrial genes, thereby guiding and modulating the inversion of phase switch among adherent ABU UPECs and further compared them to the symptomatic UPECs. This can be exploited for increased efficacy of therapeutics in future.
- This study displayed the high occurrence of MGEs among the MDR and ESBL producing ABU UPECs similar to symptomatic ones which further indicated the strong association between rampant use of antibiotics, dissemination, and the emergence of antimicrobial resistance through the acquisition of MGEs.
- ERIC-PCR typing, MLST, MEGA, and MST analysis betokened a high degree of genetic heterogeneity among the asymptomatic and symptomatic UPECs circulating in Kolkata, an eastern region of resource-poor country, India, which further gave an insight into their epidemiology and evolutionary origin.
- The comparable characteristics of ABU and symptomatic UPECs indicated to the probable incidence of the genome plasticity caused due to acquisition of resistance genes that led to the

selection of random mutations through the movement of MGEs or chromosomal rearrangements due to indiscriminate antibiotic usage. Therefore, this study exhibited the detrimental consequences of MDR among these pathogenic microbes that accentuated the importunateness for the proper establishment of all three types (broad; pharmacy driven; infection and syndrome specific) of antimicrobial stewardship globally to provide a reasonable touchstone for empiric antibiotic de-escalation. This also implied the need to intervene in the alternative therapeutic strategies.

- This study also displayed the fact that ABU, although generally not considered as a clinical condition, their increased recognition, proper understanding, and characterization together with appropriate therapeutic measures when necessary is the need of the era which otherwise might lead to serious complications in the vulnerable population and also, in turn, might increase the overall pathogenic microbial pool.

LIST OF PUBLICATIONS

- **Ghosh A**, Mukherjee M. Incidence of multidrug resistance, pathogenicity island markers, and pathoadaptive FimH mutations in uropathogenic *Escherichia coli* isolated from asymptomatic hospitalized patients. Folia Microbiol (Praha). 2019 Jul;64(4):587-600. doi: 10.1007/s12223-019-00685-4. Epub 2019 Mar 5. PMID: 30835050.

- **Ghosh A**, Bandyopadhyay D, Koley S, Mukherjee M. Uropathogenic *Escherichia coli* in India-an Overview on Recent Research Advancements and Trends. Appl Biochem Biotechnol. 2021 Jul;193(7):2267-2296. doi: 10.1007/s12010-021-03521-z. Epub 2021 Feb 17. PMID: 33595784.

- **Ghosh A**, Ghosh B, Mukherjee M. Epidemiologic and molecular characterization of β-lactamase-producing multidrug-resistant uropathogenic *Escherichia coli* isolated from asymptomatic hospitalized patients. Int Microbiol. 2022 Jan;25(1):27-45. doi: 10.1007/s10123-021-00187-9. Epub 2021 Jun 30. PMID: 34191193.

- **Ghosh A**, Mukherjee M. Regulatory interplay of cellular factors in phase variation of type 1 fimbriae operon in adherent uropathogenic *Escherichia coli* isolated from asymptomatic hospitalized patients. Under review.

SEMINARS AND WORKSHOPS PRESENTED/ATTENDED

Seminars:

- Poster presentation at the **International Conference** (5th India Biodiversity Meet 2018), held at Agricultural & Ecological Research Unit, Indian Statistical Institute, Kolkata. **Ghosh A**. and Mukherjee M. (2018). Raising public awareness against asymptomatic uropathogenic *Escherichia coli* in males and non-pregnant females -a study on urine samples, isolated from hospitalized patients of Kolkata, an eastern region of India.
- Oral presentation at the **International Conference** on Biotechnology and Biological Sciences (Biospectrum 2020) held at the department of Biotechnology, of University of Engineering and Management, Kolkata, in association with Indian Ecological Society, Microbiologists society, India, Smart Society, USA. **Ghosh A**, Bandyopadhyay D, Koley S, Mukherjee M. (2020). Uropathogenic *Escherichia coli* in India-an Overview on Recent Research Advancements and Trends.
- Participated in **CME7 on Tropical and Infectious Diseases** organized by Society of Tropical Medicine and Infectious Diseases in India, held on 11th June, 2017.
- Participated in **CME9 on Tropical and Infectious Diseases** organized by Society of Tropical Medicine and Infectious Diseases in India, held on 18th August, 2019.

Workshop:

- Participated in the **"Workshop and hands on training on Biomedical Techniques"** organised by Department of Biochemistry and Medical Biotechnology, School of Tropical Medicine, Kolkata from 13th-15th February, 2017.