A comparative study of virulence factors and antibiotic susceptibility pattern of enteric *Escherichia coli* isolated from diarrheal patients

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by

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CERTIFICATE FROM THE SUPERVISOR(S)

This is to certify that the thesis entitled "**A comparative study of virulence factors and antibiotic susceptibility pattern of enteric *Escherichia coli* isolated from diarrheal patients**" submitted by Smt. **Debjani Ghosh** who got her name registered on 12th **October, 2020** for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Asish Kumar Mukhopadhyay** 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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Declaration

I do, hereby, declare that the work embodied in this thesis entitled "**A comparative study of virulence factors and antibiotic susceptibility pattern of enteric *Escherichia coli* isolated from diarrheal patients**" submitted for the award of Doctor of Philosophy (Science) in Life Sciences and Biotechnology, is the completion of work carried out under the supervision of **Dr. Asish Kumar Mukhopadhyay**, Scientist-F (Sr. Deputy Director), at the Division of Bacteriology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata. Neither this thesis nor any part of it has been submitted for either any equivalent degree/diploma or anyother academic award elsewhere.

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Dedicated to

my parents

and

husband

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A comparative study of virulence factors and antibiotic susceptibility pattern of enteric *Escherichia coli* isolated from diarrheal patients.

Abstract: Diarrhoea is mostly prevailing in developing countries, especially in Asia and Africa, where people suffer due to poor hygiene and limited healthcare facilities. According to the WHO report, diarrheal disease is the second leading cause of death in children under 5 years of age. One of the major causative agents of diarrhoea is *E. coli* which is prevalent in the human gastrointestinal tract as harmless commensals but can also turn to the pathogenic form on acquiring certain features by mobile genetic elements. Besides their prevalence, a major public health concern in diarrhoea endemic regions is the spread of antibiotic resistance (AMR) and the treatment has become more and more challenging due to the emergence of resistance against the first-line antimicrobial agents. Therefore, in this study, we targeted to analyze the prevalence and antimicrobial resistance of *E. coli* in diarrheal patients of Kolkata, India. We reported the presence of a large number (n=1153/14,613) of Diarrheagenic *E. coli* (DEC) such as EAEC, EPEC and ETEC along with EIEC during 2012-2022. Prevalence of EAEC was found more followed by ETEC, EPEC and EIEC in Kolkata among children which are interestingly different from the prevalence pattern of past decade. Antimicrobial susceptibility assay showed that the isolates have acquired increased resistance to fluoroquinolone group of antibiotics followed by β-lactams and tetracyclines. EIEC is found to be prevalent in Kolkata, India with two distinct forms LF and NLF. Most interestingly, the LF isolates which is a rare type of EIEC were found to coexist almost equally with the mostly studied NLF-EIEC strains. There is no previous study reporting the virulence of LF-EIEC isolates. Therefore, we extensively characterized the LF-EIEC and compared the pathogenicity between the two groups of EIEC showing that LF strains were more invasive and expressed the virulence associated genes at increased levels compared to the NLF-EIEC strains when tested on cultured mammalian cell-line. The LF strains were found to colonize more in the rabbit intestine and caused severe keratoconjunctivitis in guinea pigs. Plasmid profiling showed that the EIEC strains harboured multiple plasmids irrespective of NLF or LF forms which correlates with virulence. In another part, we isolated an EAEC strain that harbors $bla_{\text{NDM-1}}$ gene conferring resistance to the carbapenem antibiotics, used as a last line of drug treating microbial infections. Therefore, this study also reports the first isolation of $bla_{\text{NDM-1}}$ carrying EAEC in India and the $bla_{\text{NDM-1}}$ carrying plasmid harbored multiple antibiotic resistance markers which can be threatening to the human population. We also investigated a bunch of fluoroquinolone resistant DEC strains for the presence of plasmid mediated quinolone resistance genes and nucleotide mutations in the chromosomal genes that are reported to contribute to quinolone resistance. However, no such novel mutation has been observed. Collectively, the findings of this study highlight the high prevalence of multidrug resistance in major DEC pathotypes that could be considered as the leading etiological bacterial agent responsible for diarrhea suggesting a significant public health threat and needs special attention. Also, monitoring the AMR surveillance needs special attention because the DEC isolates were highly resistant to commonly used antimicrobials in the treatment of diarrhea.

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CONTENTS

Part 1

Introduction

1. Introduction

Infectious diarrheal diseases are a great problem worldwide and are severe cause of morbidity and mortality in developing countries (Takahashi *et al*., 2008; Dutta *et al*., 2013). Diarrhea remains the major cause of mortality among children of low and mid-income countries of Africa, South Asia and Latin America due to poor living conditions (Croxen *et al.,* 2013; Gomes *et al.,* 2016). It was estimated that 3 to 4 million children died annually from diarrhea. In 2010 out of the 4.879 million children below 5 years of age all over the world were affected with infectious diseases, 0.801 million children died solely due to diarrhea (Takahashi *et al*., 2008; Dutta *et al*., 2013). A wide range of viruses, bacteria and parasites can be regarded as etiological agents of diarrhea among which diarrheaogenic *Escherichia coli* (DEC) is the prominent cause of diarrhea in developing countries (Dutta *et al*., 2013; Zhou *et al*., 2018). According to 2019 CDC reports, an outbreak of *E. coli* strain O157:H7 was observed where 102 people from about 23 states were infected. Although diarrhoea can be caused by various different types of pathogens, in this study we have focused only on the diarrhoea caused by the bacterial pathogen *E. coli* as it remains as one of the major causes of infant diarrheal cases in India.

Within a few hours of birth, the gastrointestinal tract of the newborn is colonized by *E. coli* where they maintain a good healthy beneficial mutual relationship with its host (Kaper *et al.,* 2004). These harmless commensal *E. coli* remain as the most abundant facultative anaerobe in the mucus layer of the mammalian colon (Kaper *et al.,* 2004; Jafari *et al.,* 2012) but in immune compromised individuals it can have several pathogenic effects like causing diarrhea and other extra-intestinal diseases like urinary tract infections (UTI), meningitis and septicemia (J.P Natoro *et al.,* 1998; Croxen *et al.,* 2013; Kaper *et al.,* 2004; Jafari *et.al*., 2012). *E. coli* was considered as a major avirulent commensal flora of the gut for over a century. This view has changed progressively over the years with an accumulation of several evidences, indicating that *E. coli* can cause a variety of diseases. In some cases, commensal *E. coli* turns to pathogenic forms by acquiring a combination of mobile genetic elements through horizontal gene transfers and can cause a wide range of diseases (Croxen *et al*., 2013). A recent case study on pediatric diarrheal disease conducted by Global Enteric Multi-Centre Study (GEMS) has illustrated that ETEC and *Shigella* as two of the major agents of pediatric diarrhea in South Asia and sub-Saharan Africa (Levine *et al.,* 2012). High frequency of diarrheagenic *E. coli* (DEC) has been reported to cause acute diarrhea in children in Kenya, Indonesia, Andaman Islands, Southeast China, South Africa, South-eastern Brazil and

Japan (Iijima *et al*., 2016; Osawa *et al*., 2013; Raghavan *et al*., 2017; Chen *et al*., 2019; Aijuka *et al*., 2018; Spano *et al*., 2017; Wang *et al*., 2003).

Pathogenic *E. coli* strains differ from the predominately existing enteric flora present in healthy individuals and are more likely to express virulence factors that are directly connected to pathogenesis. The virulence factor expression causes disruption of the normal host physiology resulting in acute disease processes. Along with their role in disease development, virulence factors also enable the pathogens to exploit their hosts in ways that are unavailable to commensal strains, resulting in the spread and persistence of these pathogenic forms in the bacterial community (Chowdhury *et al,* 2022). Several *E. coli* pathotypes have been involved with diarrheal illness thus making diarrhea a major global public health problem (Bhan *et al*., 1989; Giron *et al*., 1991; Levine *et al*., 1993). Pathogenic, diarrhea causing i.e., diarrheagenic *E. coli* or DEC strains are categorized into 6 pathotypes depending on the presence of virulence factors – Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Shiga toxin producing *E. coli* (STEC) and Diffusely adherent *E. coli* (DAEC) (Jafari *et.al*., 2012).

Before the development of appropriate molecular techniques, DEC pathotypes were detected by serotyping (J.P Natoro *et al.,* 1998; Croxen *et al.,* 2013). Serogroups and serotypes are chromosomal markers which are used to identify the different virulent clones of the DEC pathotypes. Nowadays, both the phenotypic properties and genetic marker genes involved in the pathogenesis of DEC are considered for identification of different DEC pathotypes (Nataro and Kaper, 1998; Suzart *et al*., 1999; Vieira *et al*., 2007; Ghosh *et al.,* 2022). Characterization of DEC strains may include various detection arrays of different chromosomal or plasmid-encoded virulence genes as well as their pattern of adherence in cultured cell lines and the identification of specific surface antigens. These virulence markers are useful in distinguishing between potentially pathogenic and non-pathogenic strains. Among the various virulence factors, one or more forms of heat stable (*stI* and *stII*) and heat labial (*ltI* and *ltII*) enterotoxins are associated with ETEC which is known to cause traveler's diarrhea. The two structural genes *bfpA* and *eae* which code for bundle forming pilus and intimin respectively (required for attachment as well as effacement on intestinal epithelial cells) are considered as virulence factors for EPEC (Jerse *et al*., 1990; Rajendran *et al*., 2010; Dutta *et al*., 2013). The gene *stx* is associated with the Shiga toxin producing *E. coli* (Lima *et al*., 2017). It has been reported that EAEC is generally the most common among DEC

pathotypes in causing acute diarrhea in both adults and children (Jafari *et al*., 2012) where the virulence factors involved are *aaiC* and *cvd*.

Apart from the 3 major prevailing pathotypes of DEC, we have focused on enteroinvasive *E. coli* which are considered as a rare DEC pathotype. Enteroinvasive *E. coli* (EIEC) causes bacillary dysentery like *Shigella spp*. and is found in two virulent forms-lactose fermenting (LF) and non-lactose fermenting (NLF). Previous studies investigated the prevalence and pathogenicity of NLF-EIEC but the presence of LF-EIEC has been rarely documented, and their virulence property is largely unknown. Therefore, we determined the prevalence and pathogenicity of these rarely focused LF-EIEC isolates and compared them with the NLF-EIEC isolated from diarrheal patients in Kolkata, India. The pathogenicity of EIEC is due to the presence of large F-type invasion plasmid (pINV) which encodes the genes required for invasion, survival, and cell to cell dissemination of bacteria. Our study also focuses on the molecular determinants of invasiveness, intracellular growth and spreading of EIEC isolates in both in-vivo and in-vitro model environments.

Bacterial resistance to antibiotics has become one of the biggest challenges of modern society. Antibiotics play an important role in public health all over the world. Although, they help in limiting infectious diseases, their misuse results in the emergence and spread of multidrug resistant and pan-resistant bacterial strains at an alarming rate. Recent surveillance data from the 2000s show that *E. coli* strains have developed resistance to all the major classes of antibiotics including the modern fluroquinolones and third generation cephalosporins which result in extended β-lactamase (ESBL) production (Paitan *et al*., 2018; Pons *et al*., 2014). Thus, antibiotic resistance profile of the DEC isolates from Kolkata would also give us some valuable information about their innate strategy which they use to overcome our treatment barriers.

Generally, bacteria use two major strategies to develop antimicrobial resistance- spontaneous genetic mutations and obtaining resistance genes by horizontal gene transfer (HGT). Mutations in the genes can modify the mode of action of the antibiotics by modifying the drug targets consequently diminishing the drug affinity, increasing the number of target sites, increase in the number of efflux pumps resulting in faster and easy removal of the drugs or reducing the drug uptake by the cells and by modifying the vital metabolic pathways (Kumar *et al*., 2017; Munita *et al*., 2016). The main vehicles of HGT are the mobile genetic elements (MGEs) which include plasmids, transposons, integrons and integrative conjugative elements

(ICEs) that help in acquisition and spread of the resistant genes among the different species of bacteria (Rajpara *et al*., 2018; Munita *et al*., 2016; Kumar *et al*., 2017; Amin *et al.*, 2021). Thus our study includes the characterization of a bla_{NDM-1} containing plasmid in one of the meropenem resistant EAEC strain obtained from a diarrheal patient in Kolkata. We have also focused on finding unique point mutations in the chromosomal genes responsible for imparting fluroquinolone resistance along with searching for some plasmid mediated fluoroquinolone resistance genes.

ESBLs are broad spectrum enzymes of the β-lactamase group produced by bacterial strains which make them resistant to penicillins, cephalosporins as well as to other groups of antibiotics such as aminoglycosides, fluoroquinolones, trimethoprim, sulfonamides and tetracyclines (Colodner *et al*., 2005; Nepal *et al*., 2017; Rawat *et al*., 2010). ESBL enzyme producing bacterial strains inactivate the antibiotics by hydrolyzing the β-lactum ring present in the antibiotics (Colodner *et al*., 2005; Nepal *et al*., 2017). The genes coding ESBL are mostly found on large plasmids and integrons which can easily be transferred among different species, thus posing a threat among the hospitalized patients (Colodner *et al*., 2005; Nepal *et al*., 2017; Rawat *et al*., 2010). The major ESBL producing genes found in gram negative bacteria are bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX-M} (Colodner *et al*., 2005; Peymani *et al*., 2017). Nowadays, more than 90 types of TEM and more than 25 types of bla_{SHV} enzymes have been deciphered depending upon the mutations found at particular loci within the genes of these enzymes (Bradford *et al*., 2001). Thus, another aim of my study is to find the different AMR genes that are responsible for conferring resistance phenotypes to our isolates obtained from two diarrhea treating hospitals in Kolkata. Altogether, this study will provide us with the information regarding the prevalence of major DEC isolates in India, antimicrobial resistance, pathogenicity in cell culture and animal models with the help of molecular biology and cell biology techniques along with bioinformatics.

Part 2

Importance of study

2. Importance of the study

Almost 525,000 deaths primarily in children below 5 years occur due to gastroenteritis all over the world (Liu *et al.,* 2016). Diarrheagenic *E. coli* (DEC) is the significant causative agent of gastroenteritis and diarrhoea in children and thus poses a serious worldwide public health challenge (Canizalez-Roman *et al.,* 2016). A recent survey indicated that diarrhoea is more prone in underage children in India and is responsible for 37% of the total diarrheal deaths globally. Thus, diarrheal disease remains the commonest cause of illness and death among the developing countries where *Escherichia coli* remains one of the major causative agents of diarrhoea and is responsible for 30 to 40% of all diarrheal episodes in developing countries (Salleh *et al.,* 2022).

E. coli is generally known as non-pathogenic facultative flora of the human intestine. However, in the immune-suppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause severe diseases. Non-pathogenic forms of *E. coli* can turn to the pathogenic form by acquiring a combination of mobile genetic elements. DEC strains are one of the main reasons for diarrhea and based on the presence of virulence factors they can be categorized into 6 pathotypes. High frequency of DEC has been reported to cause acute diarrhoea in children in most of the developing countries (Zhang *et al.,* 2018; Zhou *et al.,* 2021). Another report suggests that EAEC and ETEC remain the common cause of diarrhoea among the high and low income counties of Southeast China. Thus the purpose of the study is to find out the prevalence of DEC (EAEC, EPEC and ETEC) in the diarrhoea endemic region of Kolkata, India along with their mode of antimicrobial resistance and also to find its impact on the population of Kolkata.

Another rare DEC pathotype known as enteroinvasive *E. coli* (EIEC) causes bacillary dysentery quite similar to that of *Shigella* pathotypes in children as well as in adults. EIEC isolates are closely related to *Shigella* species with respect to their phenotypic, biochemical and genetic characteristics making it difficult to differentiate between the two genera. Despite several worldwide reports on the prevalence of EIEC, very less is known about them and there is no study regarding their virulence from India. Thus, the aim of our study is to characterize the virulence profile of both the lactose fermenting as well as non-lactose fermenting EIEC isolates from India and also to focus on the molecular determinants of invasiveness, intracellular growth and spreading of EIEC isolates in both *in-vivo* and *in-vitro* model environments.

Although, antibiotics help in limiting infectious diseases, but the inappropriate use of third generation antibiotics has resulted in the increase of antimicrobial resistance phenotype among the bacterial species. The increase in the global incidence of antimicrobial resistance in *E. coli* has raised a crucial situation and demands for the advent of proper medical interventions in order to control its transmission. Moreover, the spread of multidrug-resistant (MDR) and extended beta- lactamase (ESBL) producing DEC pathotypes have made treatment against DEC infections more difficult and challenging as the first-line antimicrobial agents have become less effective against the different *E. coli* pathotypes (Salleh *et al.,* 2022). Recent surveillance data from the 2000s show that *E. coli* strains have developed resistance to all the major classes of antibiotics including the modern fluroquinolones and third generation cephalosporins such as ceftriaxone, cefotaxime, cefepime and cefuroxime (Kayastha *et al.,* 2020). Thus, in this study we targeted to monitor the antimicrobial resistance of *E. coli* in diarrheal patients from two remote hospitals in Kolkata, India in order to understand the pattern of spread of antibiotic resistance so that the treatment of diarrhoea becomes easier for the doctors. We have also aimed to find the prevalence of the different AMR genes responsible for the spread of resistance among the different DEC pathotypes.

Among these newly emerging antibiotic-resistant *E. coli* pathotypes, the dramatic increase in the dissemination of NDM isolates has created a worrying AMR situation. Thus, we have investigated the prevalence of such NDM resistant strains among the hospitalized patients of Kolkata. We have found one EAEC isolate (BCH 7846) from a diarrheal patient in Kolkata that exhibited meropenem resistance phenotype due to the presence of a carbapenem resistant plasmid. As the strains are gaining antibiotic resistance genes through horizontal gene transfer and rising as highly pathogenic forms, it is necessary to monitor their changing susceptibility pattern towards the latest generation antibiotics and find their mode of action resulting in their spread in the human population.

Before 1990, the resistance to fluroquinolone was rarely found in *E. coli* but as the use of fluroquinolones increased in the treatment of several different infectious diseases, there is a rise of fluroquinolone resistant bacteria species in India. Thus, the spread of fluroquinolone resistance has motivated us to decipher the mechanism of fluroquinolone resistance by searching for a point mutation in the quinolone-resistance determining regions (QRDR) of chromosomal genes such as *gyrA*, *gyrB*, *parC* and *parE* as well as to find the prevalence of plasmid mediated quinolone resistance genes (PMQR). The purpose of our study is to correlate the abundance of chromosomal mutations as well as the presence of different PMQR genes

with the increase of fluroquinolone resistance in order to help in finding ways to control the spread of this resistance.

Therefore, the results of this study can help to improve the understanding of the epidemiology of DEC infections in patients with diarrhea. Monitoring of AMR surveillance needs special attention because the DEC isolates are becoming highly resistant to commonly used antimicrobials in the treatment of diarrhea, therefore calling for an urgent need of new treatment facilities.

Part 3

Review of Literature

3. Review of literature

3.1. Diarrhea: a common threat in developing countries

Diarrhea can be defined as the passage of two or more times loose stools accompanied by abdominal cramps and sometimes fever. Diarrheal disorder has emerged as the second major cause of childhood mortality and morbidity in the entire world. Almost 1.7 million childhood diarrheal cases are reported globally out of which death has been reported in 5,25,000 cases annually (Shine *et al.*, 2020; Ghosh *et al.*, 2021). According to the latest World Health Organisation reports, almost 8% of children less than 5 years of age die due to diarrheal disease which has aroused a severe alarming situation worldwide (WHO, 2020). The recent reports from the United Nations International Children's Emergency Fund (UNICEF) also state that diarrhea is the major cause of malnutrition among children less than 5 years of age and has accounted for about 8% of the diarrheal cases in developing countries (WHO, 2017; UNICEF, 2019).

Diarrhea has been reported in low income countries such as Kenya, Indonesia, Andaman Islands, Southeast China, South Africa, South-eastern Brazil and Japan (Osawa *et al*., 2013; Iijima *et al*., 2016; Raghavan *et al*., 2017). In 2008, in 5 countries such as India, Pakistan, Nigeria, China and Congo 4.2 million pediatric diarrheal deaths occurred which has contributed to almost half of the deaths caused by diarrhea (Black *et al.*,2008; Ghosh *et al.*, 2021).

Studies have revealed that diarrheal disorders resulted in deaths among children less than 5 years of age in India. Reports from The National Family Health Survey highlights that there has been an increase in childhood diarrheal episodes from 9% to 9.2% from the year 2016 to 2020 in India thus making it one of the major public health issues in India (Lakshminarayanan *et al.,* 2015; Ghosh *et al.,* 2022). States such as Uttar Pradesh and Assam are more affected by this disease than the rest of the country (Kamath *et al.*, 2018). The diarrheal cases in India are influenced by the mother's age, social class, child's age, religion, and wealth index (Ghosh *et al.*, 2021).

Thus, it is clear from the above facts that diarrhea poses a crucial threat to the Indian children population making it evident to decipher new preventive strategies in order to reduce child deaths in different parts of India (Ghosh *et al.,* 2021; Ghosh *et al.,* 2022).

3.2. *Escherichia coli*: the gut-friendly microbiota turning into a global pathogen

E. coli is the predominant facultative anaerobe that colonizes the gastrointestinal tract of human infants within a few hours after birth where *E. coli* and its human host coexist with mutual benefit. This commensal *E. coli* rarely causes disease except in immune-compromised hosts where it can have several pathogenic effects in the intestines causing diarrhea and other wide range of diseases like urinary tract infections (UTI), meningitis and septicemia (Nataro *et al.,* 1998; Kaper *et al.,* 2004; Croxen MA *et al.,* 2010; Jafari *et.al*., 2012; Croxen MA *et al.,* 2013).After the initial description of *E. coli* by a German paediatric named Theodar Escherich in 1885, it became apparent that certain distinctive *E. coli* strains (by appearance and smell) were frequently found in clinical specimens of patients with specific clinical syndromes. It is now known that most *E. coli* isolates are harmless intestinal commensals, even when the human is susceptible to infection by *E. coli* clones which have evolved the ability to cause a spectrum of human diseases. Pathogenic strains of *E. coli* that causes diarrhea have evolved through the acquisition of genetic characteristics through horizontal gene transfers (Natoro *et al.,* 1998; Croxen MA *et al.,* 2013; Gomes *et al*., 2016).

3.3. Historical background: recognition of *E. coli* as a gastrointestinal pathogen

In 1884, *E. coli* was discovered as fast-growing bacteria by a German paediatrician Theodor Escherich while struggling against neonatal mortality due to dysentery in his laboratory of Otto von Bollinger in Munich (Shulman *et al.,* 2007). These fast growing bacteria were named *Bacterium coli commune* but later came to be known as *E. coli* (Escherich, 1988; Shulman *et al.,* 2007; Blount, 2015). Due to its nonpathogenic and fast growing nature in chemically defined media *E. coli* has been referred to the “workhorse” in molecular biology (Blount, 2015; Idalia *et al.,* 2017). Many groundbreaking studies on bacterial physiology and genetics were developed on *E. coli* by eminent scientists such as Wollman; Werkman; Wollman and Wollman and others (Blount, 2015). The spontaneity of bacterial mutations was discovered in 1940 by Luria and Delbrück while genetic recombination and bacteria is being infected by DNA has been discovered by Hershey and Chase. Simultaneously, structure of genetic unit was discovered by Benzer and bacterial sexual recombination by J. Lederberg and E. Tatum. Some years later, Lederberg's group discovered plasmids as the extrachromosomal unit and the phenomenon of transduction. After A. Lwoff discovered lysogeny at the Pasteur Institute in Paris F. Jacob and E. Wollman revealed the mechanism of the sexual process in *E. coli* and defined that bacterial chromosomes are

circular in nature (Ullman, 2011; Blount, 2015). In the 1960s, J. Monod and F. Jacob analyzed the lactose system of *E. coli* which resulted in the establishment of the operon model for gene regulation. Not only the operon model, but they also highlighted messenger RNA for the first time during their study (Ullman, 2011; Blount, 2015). The revelation of the DNA double helix structure in 1953 by F. Crick and J. Watson formulated major discoveries such as the establishment of the copying mechanism by Meselson and Stahl, the discovery of the nature of the genetic code by S. Brenner leading to the development of recombinant DNA technology based on the discovery of the restriction-modification system by W. Arber (Ullman, 2011; Blount, 2015).

After the establishment of *E. coli* as the model organism, several reports by Roberts-Browne incriminated *E. coli* as the cause of severe infantile gastroenteritis known as 'cholera infantum' in the early 20th century. Reports by Bray and Beaven (Bray, 1945; Bray and Beaven, 1948) led to the acknowledgment of *E. coli* as the most important pathogen of the gut. In the 20th century, an *E. coli* strain named Bacterium coli neopolitanum was found to be significantly associated with neonatal diarrhea. Robins-Browne discovered several pathogenic strains of *E. coli* that were involved in various nosocomial and community diarrhea outbreaks in the 20th century. All these pathogenic strains were given unique epithet by Robins-Browne and resulted in a confusion which was later resolved by a Danish bacteriologist named Fritz Kauffman who discovered a serotyping scheme for *E. coli* (Kauffmann, 1947). The importance of this scheme was highlighted when various strains of *E. coli* were involved in several diarrhea outbreaks in the 1920s and 1940s belonged to O serogroups, O111 and O55 (Robins-Browne, 1987).

According to this scheme, *E. coli* isolates were distinguished based on the presence of O (somatic), H (flagellar) and K (capsular) antigens. 186 O antigens and 53 H antigens have being identified (Fratamico *et al.,* 2016). A few of the O group strains are more frequently associated with diarrhea. K antigens were further subdivided into thermo-labile antigens (L and B antigens) and thermo-stable antigens (A antigens). The strains containing the K antigens are more pathogenic than those lacking the K antigen. These serogroups have more than 10,000 possible combinations in which some isolates termed as 'rough' (OR) auto agglutinate in serum while some isolates were unable to be typed using existing antisera (O non-typable or 'ONT') (Kauffmann, 1947; Fratamico *et al.,* 2016). Due to the difficulty in standardizing this serotyping scheme involving the different antisera in various laboratories, genetic methods such as polymerase chain reaction (PCR) and multilocus sequence typing

(MLST) were considered standard and easy methods to identify the *E. coli* pathotypes (Panchalingam *et al*., 2012).

The pathogenicity of different serogroups of *E. coli* strains such as O111, O55 and O127 for humans was established by Robins-Browne (Robins-Browne, 1987). Enteropathogenic *E. coli* (EPEC) was discovered by Neter in 1955 as the primary intestinal pathogen that causes infections when introduced into extra-intestinal tissues. Some varieties of *E. coli* were found to cause cholera-like diarrhea while others can cause *Shigella*-like dysentery (Dupont *et al*., 2016). Enterotoxigenic *E. coli* (ETEC) was identified in the lab of Bradley Sack in Kolkata in 1968. ETEC is the major cause of traveler's as well as childhood diarrheal cases globally (Adachi *et al.,* 2002; Zhang *et al.,* 2022). In 1999, PCR amplification of probe cvd432 resulted in the identification of EAEC in children's stool samples where they produced watery diarrhea stool instead of mucoid diarrheal stool. In 1993, a massive childhood diarrheal outbreak was observed in which more than 2000 children were affected (Gomes *et al.,* 2016). In 2004, EAEC with *aggR* gene was found among children in Mongolia (Sarantuya *et al.,* 2004). A multiplex PCR survey of certain EAEC genes such as *astA, aagR* and *aafII* genes in south India showed the existence of EAEC among children below 5 years (Pai *et al.,* 1997). A massive *E. coli* outbreak was reported in 2011 in Europe, which involved a hybrid strain of EAEC and STEC. The hybrid strain was identified as EAEC O104:H4 that contained the marker EAEC virulence factors such as *aggA*, *aggR*, *set1*, *pic*, and *aap* along with *stx2* gene of STEC (Rasko *et al.,* 2011; Philipson *et al.,* 2013). The strain affected 4,321 of healthy Europeans out of which hemolytic uremia syndrome (HUS) was developed in 900 patients and 50 individuals died (Rasko *et al.,* 2011). EAEC became the major cause of acute diarrheal disorders in children in the United States and thus EAEC was included on the National Institutes of Health category B list containing infectious organisms which has the potential to be used as bio-weapons in 2002 (Huang *et al.,* 2004). Recent Centers for Disease Control and Prevention (CDC) reports indicated 4 hospitalization cases due to EPEC (CDC reports, 2013). Annually, ETEC infections cause a huge number of diarrheal episodes, resulting in approximately 75,000 numbers of neonatal deaths in tropical areas with poor sanitary conditions (Gomes *et al.,* 2016). According to CDC 2012 report, 203,000 individuals in the USA were affected by DEC infections causing 21 deaths from the year 2000 to 2008. *E. coli* became the third most abundant pathogen in different food-borne outbreaks in Brazil after *Salmonella spp.* and *Staphylococcus aureus* (Lima *et al.,* 2017). Several *E. coli* outbreaks have been reported in 2006-2021 by CDC which involved different food sources as

the cause of the outbreak. Recent *E. coli* outbreaks of serogroups O121, O157:H7 in 2022 have been reported by CDC due to different food sources such as ground beef and frozen falafel (CDC reports, 2020). Thus all these reports indicated the chances of future outbreaks which revived great interest in DEC and stimulated to investigate their various pathogenic properties.

3.4. Structure

E. coli are gram negative facultative anaerobic rods (Nataro *et al.,* 1998) following the basic infection strategy like most of the other mucosal pathogens such as the colonization of a mucosal site, evasion of host defence followed by multiplication and damage to the host.

3.4.1. Physical structure

E. coli is gram negative, oxidase negative anaerobic rods with peritrichous flagella belonging to the family Enterobacteriaceae (Ewing *et al.,* 1986; Gomes *et al.,* 2016). Most of the diarrheagenic *E. coli* (DEC) are motile while enteroinvasive *E. coli* (EIEC) are non motile (Nicolas-Chanoine *et al.,* 2014). The most conserved feature of DEC is its ability to colonize intestinal mucosa and compete with other gut microflora in order to get established in the gut as the major pathogenic bacteria. DEC consists of specific fimbrial antigens and colonizing factors that enhance their intestinal colonizing ability. Rigid rods, flexible fibrillae, bundle-forming fimbriae and non-fimbrial (afimbrial) adhesions are the 4 major different types of adhesions found in DEC (Nataro *et al.,* 1998). 4 different aggregative adherence fimbriae (AAF) help in the colonization of intestinal mucosa in the case of EAEC (Jafari *et al*., 2012). Bundle forming pilus (BFP) help in initial attachment of typical EPEC strains to the host intestinal epithelium (Scaletsky *et al.,* 1984; Jerse and Kaper, 1991). More than 22 serologically different colonizing factors are present in ETEC which differ in their composition, structure as well as function. The CFs are mostly fibrous in structure while other adhesions are simple outer membrane proteins (Croxen *et al.,* 2013; Madhavan *et al.,* 2015).

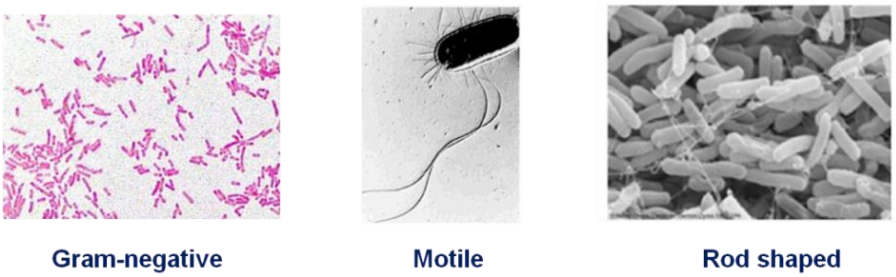


Figure I1: *General features of E. coli. E. coli is a gram-negative, motile, and rod-shaped bacterium (Source: google images).*

3.4.2. Genetic structure

The genomic plasticity of *E. coli* is due to the presence of mainly two genetic configurations - virulence-related plasmids and chromosomal pathogenicity island. DEC strains are categorized into 7 pathotypes depending on the presence of virulence factors – Enteroaggregative*E. Coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), *Enteroinvasive E. coli* (EIEC), Shiga toxin producing *E. coli* (STEC), Diffusely adherent *E. coli* (DAEC) and adherent-invasive *E. coli* (AIEC) (Jafari *et.al*., 2012; Santos *et al.,* 2020). All these categories of DEC carry at least one virulence character which is encoded by plasmids. Pathotypes such as EAEC, EIEC and EPEC contain low copy numbers and conjugative plasmids which are greater than 50MDa in size (Wood *et al*., 1986; Nataro *et al*., 1987; Hales *et al*., 1992). Each of these pathotypes represents a group of specific clone on the basis of virulence factors that are acquired by horizontal gene transfer. Some of the pathogenic *E. coli* isolates harbour virulence factors of 2 or more than 2 DEC pathotypes and thus are regarded as hyper-virulent "hybrid" or "hetero-pathogenic" *E. coli* (Croxen MA *et al.,* 2013; Gomes *et al.,* 2016; Santos *et al.,* 2020).

'Pathogenicity islands' (PAI) are clusters of chromosomal virulence genes found in EPEC and EHEC pathotypes (McDaniel *et al*., 1995). PAI represents a common way in which the genomes of pathogenic and non-pathogenic *E. coli* diverge genetically. Plasmids and pathogenicity islands carry clusters of virulence traits that may be encoded by transposons, such as ST in ETEC or phage, such as Stx in EHEC (O'Brien *et al*., 1992).

3.5. Evolution of diarrheagenic *E. coli* (DEC)

Phylogenetically *E. coli* can be categorized into 5 major groups such as A, B1, B2, D and E. Commensal *E. coli* isolates are placed in phylogroup A, while other pathogenic DEC such as

ETEC is in phylogenetic group A and B1 and EAEC is in A, B1, B2 and D thus signifying the incongruent nature of *E. coli* pathotypes. Commensal and pathogenic *E. coli* genome sizes differ by a million base pairs and the genes for virulence and fitness are contained in the extra genetic material. Comparative analysis of the *E. coli* genome has highlighted the presence of a conserved set of genes forming the core genome and a flexible gene pool that is formed by the gain or loss of genes at several hot spots throughout the entire genome. Horizontal gene transfer (HGT) by mobile genetic elements (transposons, bacteriophages, insertion sequences, and plasmids) occurs through the process of conjugation, transformation, and transduction in prokaryotes. These mobile genetic elements can either remain integrated into the genome or can self-replicate in order to provide new traits and fitness advantages to the host. Most of the ETEC toxin genes and colonization factors (CFs) are found exclusively on plasmids, while a chromosomal pathogenicity island (PAI) called the locus of enterocyte effacement (LEE) is present in EPEC and some STEC strains. PAI expresses a 35kb virulence genes cluster which is required for the attaching and effacing (A/E) phenotype of EPEC. The Afa/Dr adherence fimbriae in DAEC isolates and many EAEC virulence factors are found on the pAA plasmid. Moreover, the O104:H4 serotype of EAEC, which was involved in the 2011 German outbreak, acquired the *stx*$_2$ gene of STEC by HGT. The invasive property of EIEC/*Shigella* is due to the presence of the invasive plasmid pINV. Genome analysis of STEC that are LEE negative have revealed homologs and subunits of ETEC toxins in some of the isolates, indicating the potential for the emergence of novel hyper-virulent pathogenic *E. coli* hybrids in future.

Genetic analysis of pathogenic *E. coli* shows the vast diversity of this organism. HGT plays a pivotal role in *E. coli* pathogenicity and it becomes challenging to define a particular pathotype based on a small set of features as these defining genes may not be restricted to a particular pathotype (Croxen MA *et al.*, 2013).

3.6. Major DEC pathotypes

DEC strains are categorized into six pathogenic groups based on the distinct virulence factors, phenotypic traits, epidemiological features as well as clinical features (**Table I1**). The six pathotypes of DEC are- Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Shiga toxin producing *E. coli* (STEC) and Diffusely adherent *E. coli* (DAEC) (Takahashi *et al.*,2008; Jafari *et.al*., 2012). Among all these DEC pathotypes, almost 30-40% of diarrheal cases in

developing countries including India are due to EAEC, EPEC and ETEC (Dutta *et al*., 2013; Ghosh *et al.*, 2022).

Table I1: *Clinical features and virulence factors of Diarrheagenic E. coli (DEC).*

DEC Pathotypes	Virulence Factors	Clinical features
Enteroaggregative *E. coli* (EAEC)	Pili, cytotoxins	Diarrhea with mucus
Enteropathogenic *E. coli*(EPEC)	Bundle forming pilus	Attaching and effacing lesions, watery diarrhea and vomiting
Enterotoxigenic *E. coli*(ETEC)	Colonizing factors and toxins	Watery diarrhea
Enteroinvasive *E. coli* (EIEC)	Invasive plasmid	Bloody diarrhea
Shiga toxin producing *E. coli* (STEC)	Shiga toxins	Watery diarrhea, haemolytic ureamic syndrome(HUS)
Diffusely adherent *E. coli* (DAEC)	Fimbriae	Diarrhea

3.7. Typical characteristics of DEC to be considered for isolation

Although there are a number of assays available in order to identify all categories of DEC, in many situations it is not necessary to identify the specific *E. coli* pathotype. Based on the type of strain and the objective of isolation, different isolation techniques are used. In cases of persistent diarrhea, especially in travelers, children and immune-compromised individuals as well as in outbreaks culturing the stool sample for DEC isolation is important. *E. coli* can be recovered easily from clinical specimens on differential or selective media at 37°C under aerobic conditions. *E. coli* is a lactose fermenter can be easily distinguished from other non-lactose fermenting (NLF) bacteria of stool, food, water and other sources by simply plating them on Mac Conkey which selectively grow members of Enterobacteriaceae and permit differentiation of enteric organism on the basis of lactose fermentation (Lupindu *et al.*, 2017).

Stool samples are streaked onto Mac Conkey agar plates followed by incubation of 18–24 hours at 37°C. *E. coli* colonies appear as pink (due to lactose fermentation), round medium-sized colonies. Some *E. coli* pathotypes are non-lactose fermenters, which may give pale yellow colonies on Mac Conkey agar. All *E. coli* strains can be captured on Mac Conkey agar but sometimes certain variations of Mac Conkey agar such as sorbitol MacConkey (SMAC) agar are used for the serotype O157:H7 as they cannot ferment sorbitol, unlike most *E. coli*. Thus, these sorbital negative colonies are selected from SMAC plates and are further

processed as O157:H7 *E. coli* (March *et al.,* 1986). Thermophilic *E. coli* strains grow at 45°C on MacConkey agar.

For epidemiological and clinical purposes, *E. coli* is often selected from agar plates after presumptive visual identification of lactose positive, non-spreading colonies and is usually identified using different biochemical reactions.

3.7.1. Phenotypic Assays for Identification

a) Biochemical Tests for *E. coli* identification

IMViC is the traditional biochemical test for *E. coli* which comprises of 4 tests that are used to discriminate the members of *Enterobacteriaceae*. IMViC stands for the Indole, Methyl red, Voges-Proskauer, and Citrate utilization tests.

i. Indole Test: In this test, the bacteria's decomposition ability of tryptophan is verified. It is tested whether the bacteria can degrade tryptophan in order to produce indole by using the enzyme tryptophanase. The enzyme is required for the deamination of tryptophan to indole. A test tube containing peptone water (which contains tryptophan) is inoculated with testing bacteria which is incubated overnight at 37°C. Then, a few drops of Kovac's reagent (contains hydrochloric acid and p-dimethylaminobenzaldehyde in amyl alcohol) are added to the mixture. When indole reacts with Kovac's reagent, the color of the solution turns yellow or cherry red. As amyl alcohol is non soluble, a pink or red colored ring formation occurs at the top indicating the bacteria to be indole positive (Lupindu *et al.,* 2017).

ii. Methyl Red: This test is used to determine the capacity of glucose fermentation of the bacteria. It detects the ability of bacteria to ferment glucose which results in the formation of stable acids such as lactic acid, formic acid or acetic acid as the end products. Acid formation brings down the pH to less than 4.5 which is indicated by a color change of methyl red from yellow to red. The type of acid formation depends on the bacteria type and the pathway that it follows for lactose fermentation.

Glucose phosphate (MRVP) broth (containing glucose and a phosphate buffer) is inoculated with the testing bacteria and incubated at 37°C for 48 h. When three to five drops of MR reagent are added to the tube, color formation occurs. The development of red color indicates a positive reaction while yellow discoloration occurs in the case of MR-negative bacteria. *E. coli* is MR-positive bacteria.

iii. Voges-Proskauer: Voges-Proskauer test is used to determine whether the bacteria can produce acetylmethyl carbinol from glucose fermentation. Acetylmethyl carbinol is oxidized to diacetyl in the presence of alpha-naphthol to produce a red color.

VP test is performed by incubating the bacteria into glucose phosphate (MRVP) for 72 h followed by the addition of 15 drops of alpha-naphthol to the test broth which is followed by the addition of five drops of 40% potassium hydroxide (KOH) to the broth and shake well. The tube is allowed to stand for 15 min to see a positive red color. *E. coli* is VP negative (Lupindu *et al.,* 2017).

iv. Citrate utilization test: This test determines the ability of the bacteria to use sodium citrate as the sole source of carbon and energy. The color of the medium changes from green to blue at an alkaline pH in the presence of pH indicator bromothymol blue. A loopful of bacteria is streaked onto a citrate agar slant which is incubated at 37°C for 24 h with a loose cap. Citrate in the media breaks down to oxaloacetate by the enzyme citritase which is again broken down to pyruvate and CO_2. Na_2CO_3 is produced from sodium citrate that causes the media to turn alkaline resulting in a color change from green to blue. A positive test is indicated by the slant becoming blue in color whereas the slant remaining green in color is an indicator of a negative citrate test. *E. coli* is citrate negative (Lupindu *et al.,* 2017) (**Table I2**).

Table I2: *Biochemical tests and their results for typical E. coli*

Name of the tests	Result of the tests
Indole production	+
Methyl red reaction	+
Voges Proskauer test	-
Citrate utilization test	-
H_2S gas formation	-

b) Serotyping

Serotyping of *E. coli* was very important in the history of these pathogens prior to the discovery of specific virulent factors in DEC strains. According to the modified Kauffman scheme, *E. coli* is serotyped on the basis of their antigenic properties of lipopolysaccharide (O), flagellar (H) and capsular (K) within determinants of the organism. In the present scenario, there are almost 186 O-antigens, 53 H-flagellar and 80 K antigens (Orskov *et al.,*

1992; Fratamico *et al.,* 2016). A huge number of O, H and K combinations are found in nature. Serotypes of DEC are mostly species-specific and are used as epidemiological markers for specific bacterial clones along with their virulence gene markers. Their O-antigen lipopolysaccharides may be regarded as virulence factors. O antigens are formed by 10 to 25 repeating units of oligosaccharides (the O unit) and are the hypervariable part of the bacterial cell. These O units are further translocated and polymerized by the O-antigen processing proteins, which are encoded by *wzx* (O-antigen flippase) and *wzy* (O-antigen polymerase) (Joensen *et al.,* 2015). Each serotype of pathogenic *E. coli* is associated with a different set of characteristic O:H (capsular) antigens. This serotyping system was an important weapon for defining pathogenic categories of DEC but nowadays, due to lack of sensitivity and specificity of serotyping method, other molecular based techniques are taken into consideration (Joensen *et al.,* 2015; Fratamico *et al.,* 2016).

c) Adherence property of DEC

One of the most useful phenotypic assays for the diagnosis of DEC is the HEp-2 adherence assay. This assay has recently been reviewed in detail (Donnenberg and Nataro, 1995). Cravioto *et al.* in 1979 were the first to describe an adherence assay which remains the "gold standard" for the diagnosis of EAEC and DAEC pathotypes of DEC. EAEC is defined by its “stacked brick” “honeycomb” and “aggregative” pattern of adherence to the epithelial cells (Nataro *et al.,* 1987). EPEC forms distinctive lesions and thus known as attaching and effacing (A/E) lesions on the intestinal epithelial cells surfaces (Croxen *et al*., 2013). A diffused adherence pattern is seen in the case of DAEC.

In HEp-2 adherence assay the test strain is incubated onto a semiconfluent HEp-2 monolayer for 3-6 hrs at 37°C under 5% CO. After incubation, the monolayer is washed, fixed and stained followed by examination under a light microscope. Localized adherence (LA), aggregative adherence (AA) and diffused adherence (DA) are the three patterns of HEp-2 adherence that could be seen. However, some strains may yield equivocal results reproducibly in the assay (Nataro *et al.,* 1987).

3.7.2. Molecular methods of DEC identification

Molecular methods are the most renowned and reliable technique for differentiating DEC strains from other non-pathogenic strains present in stool and help in distinguishing one DEC pathotype from another. Progress has been made in the field of polymerase chain reaction

(PCR) assay as well as nucleic acid-based probe methods. Other molecular techniques such as restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MLEE) and randomly amplified polymorphic DNA-PCR (RAPD-PCR), are currently being used to distinguish among the diverse variety of DEC pathotypes.

a) By polymerase chain reaction (PCR)

Multiplex PCR was performed with 6 sets of primers (*aaiC, aatA, bfpA, eae, st* and *lt*) to identify the 3 pathogenic types of *E. coli*(EAEC, EPEC, and ETEC) (Vidal *et al.,* 2005; Dutta *et al.,* 2013; Panchalingum *et al.,*2012). Simplex PCRs were also performed to confirm the multiplex PCR results with each of the single primer sets.

b) Using nucleic acid probes

Among the various virulence factors, one or more forms of heat stable (*stI* and *stII*) and heat labial (*LTI* and *LTII*) enterotoxins are associated with ETEC which is known to cause traveler's diarrhea. The two structural genes *bfpA* and *eae* which code for bundle forming pilus and intimin (required for attachment as well as effacement on intestinal epithelial cells) are considered as virulence factors for EPEC (Dutta *et al*., 2013). The gene *stx* is associated with the Shiga toxin producing *E. coli* (Lima *et al*., 2017). EAEC is the most common among DEC in causing acute diarrhea in both adults and children (Jafari *et al*., 2012) where the virulence factors involved are *aaiC* and *cvd*. The *ipaH* gene which is present in the chromosomes as well as virulence plasmids of EIEC is considered as marker for the EIEC strains.

c) By multi locus sequence typing (MLST)

MLST is the most common method of distinguishing and finding the relatedness among the pathogenic *E. coli* isolates. A small number of housekeeping genes are sequenced and assigned a unique allele, and the allelic profile of the housekeeping genes can be used to give an isolate a sequence type (e.g., *E. coli* O104:H4 is ST678 and many STEC O157:H7 isolates are ST11, based on the MLST databases). Sequence types can be further grouped into clonal complexes based on their similarity. However, genetic diversity can be found within strains of a similar sequence type, so higher-resolution typing may be required to understand evolutionary relationships (Hao *et al.,* 2016).

3.8. Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* (EAEC) is one of the diarrheagenic *E. coli* which are defined by their ability to form characteristic "stacked-brick" like aggregative adherence (A) phenotype to cultured cell monolayer Hep2 cells (Nataro *et al.*, 1998; Kaur *et al.*, 2010) (**Figure I1**). The adherence pattern of EAEC was different from those of DAEC and EPEC (Harrington *et al.*, 2006). This adherence phenotype is due to its ability to attach to other EAEC, to the epithelial cells as well as to the surface of tissue culture plates (Nataro *et al.*, 1998; Estrada-Garcia *et al.*, 2012). Epidemiological investigations have revealed the fact that EAEC is a significant cause of infantile diarrhea in developing countries and a major cause of persistent diarrhea which may last more than 14 days resulting in severe illness or death (Nataro *et al.*, 1998; Nataro and Kaper, 1998; Estrada-Garcia *et al.*, 2012). EAEC has been recently shown to induce growth impairment and is significantly associated with malnutrition among children even in cases without diarrhea (Steiner *et al.*, 1998). Thus, this implies that the long-term effects of this pathogen in developing countries are even more threatening and devastating than the short-term self-limiting diarrhea caused by other pathogens.

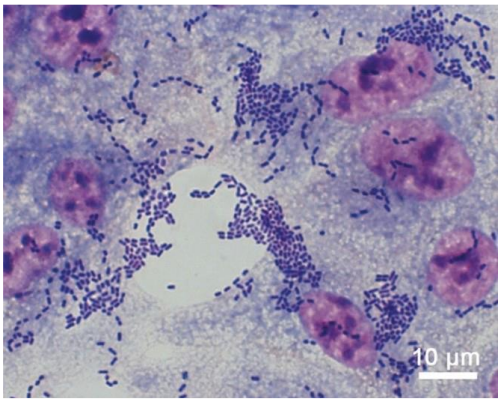


Figure I2: *Stacked brick like appearance of typical EAEC isolates (Karch et al., 2012).*

3.8.1. Historical Background

E. coli have been recognized as a major etiologic agent for diarrheal illnesses in 1920s. In 1979, Cravioto while studying the adherence factors of the isolates from different diarrheal outbreaks observed that 51 of the isolates belonged to enteropathogenic *E. coli* pathotype (EPEC) while other 17 isolates belonged to other serotypes other than ETEC and EPEC (Cravioto *et al.*, 1991). The discovery of EAEC came into existence from the studies that showed the distinctive pattern of EPEC adherence to HEp-2 cells in tissue culture when a

collection of diarrheal *E.coli* strains were found to adhere to HEp-2 cells and the phenotype was totally different from that of EPEC strains (Cravioto *et al*., 1991). The investigators further reported a non-EPEC and AF probe negative strain, *E. coli* 042 (044: H18) harboring a plasmid of 65 MDa that was nonhomologous to the EAF plasmid found in EPEC. *E. coli* 042 was confirmed to be enteroaggregative *E. coli* by Vial *et al.* in 1988 (Vial *et al*., 1988). The ability of EAEC strains to form its characteristic aggregative adherence (AA) to Hep2 cells is attributed to the presence of this large plasmid that contains several virulence genes regulated by the master regulator AggR (Kaur *et al*., 2010; Estrada-Garcia *et al.,* 2012). The stacked-brick like pattern of adherence was further subdivided into aggregative and diffuse adherence by Nataro *et al* in 1985. Vial and his colleagues replaced the term "enteroadherent-enteroaggregative" with enteroaggregative in order to discriminate between aggregative and diffusely adherent *E.coli* (Vial *et al*., 1988). The pathogenicity of this group of DEC was confirmed by several outbreaks occurring all over the world. The aggregative adherent groups detected among infantile diarrhea cases in Santiago, Chile led to proposal of two independent pathotypes: EAEC and diffusely adherent *E.coli* (DAEC) (Nataro *et al*., 1987). The report derived from traveler's diarrhea in Mexico (Mathewson *et al*., 1987) stated the strains that were capable of adhering to Hep2 cells belonged to enteroadherent *E. coli* (EAEC) pathotype. The EAEC strain 042 came into prominence as a prototypic EAEC for studying the various characteristics of EAEC such as its pathogenicity, virulence factors and genetic framework after it elicited diarrhea in Peruvian children (Nataro *et al.,* 1985; Estrada-Garcia *et al.,* 2012). EAEC obtained from diarrheal patients with a frequency similar to that of asymptomatic healthy patients, casted doubt on the virulence of EAEC (Gomes *et al*., 1989; Cravioto *et al*., 1991; Echeverria *et al*., 1992). Over the past 15 years, several case control and cohort studies have indicated that the EAEC has now become a prominent and emergent cause of persistent and acute diarrhea worldwide (Okeke *et al*., 2001).

3.8.2. Epidemiology

Previously EAEC was known as an opportunistic pathogen causing diarrhea among malnourished children and HIV patients but the recent outbreaks of EAEC have highlighted their significant role in causing diarrhea among travelers and healthy individuals of various industrialized countries (Hebbelstrup *et al.,* 2014; Hebbelstrup *et al.,* 2016). The etiology of EAEC was first described to be associated with diarrheal diseases among children in a case control study conducted in Santiago, Chile (Nataro *et al*., 1987). *E. coli* strains with

aggregative and diffuse adherence patterns were differentiated. Of 253 *E. coli* strains that were negative for the EAF probe isolated from Chilean patients suffering from diarrhea, 84 strains (33%) showed the AA adherence pattern. However, the strains showing diffused adherence were not associated with significant diarrhea (Nataro *et al*., 1987). Studies conducted by Bhan *et al*. (1989), have established the significant association of EAEC with persistent diarrhea. In a longitudinal study conducted in Anapurpalla in northern India (Bhan *et al*., 1989), EAEC was isolated from 30% of children with acute diarrhea, 12.7% with persistent and 9.8% of healthy controls. Children with EAEC diarrhea had a mean duration of illness of 17 days with fever and grossly bloody stools. Subsequent case-control studies reports from other developing countries (Fang *et al*., 1995; Geyid *et al*., 1998) have established EAEC as a major cause of pediatric persistent diarrhea but very few reports have correlated EAEC with the cause of acute diarrhea (Paul *et al*., 1994; Okeke *et al*., 2000). The inconsistent association of EAEC with acute diarrhea might be a function of the large numbers of healthy people from whom EAEC is often recovered during controlled studies (Oberhelman *et al.*, 1998). In some studies, significant association was detected in diarrheal cases than in controls (Okeke *et al*., 2000). The etiologic role of EAEC in diarrhea in developed countries had implicated in a Scandinavian case-control investigation, which showed EAEC was more prevalent than EPEC among diarrhea patients (Bhatnagar *et al*., 1993). Other reports from east London revealed that EAEC was the most frequently detected pathogen among infants with acute diarrhea (Chan *et al*., 1994). Studies conducted in Germany (Huppertz*et al*., 1997) and Switzerland (Pabst *et al*., 2003) have further underscored the etiologic role of EAEC. In more recent study among infants in Cincinnati, USA, EAEC was shown to be an important cause of diarrhea (Cohen *et al*., 2005).

In 1999, EAEC was identified in the stool samples of children by performing Hela cell assay and PCR amplification of the gene probe complementary to cvd432. Here the children were recorded to produce watery diarrhea instead of mucoid diarrheal stool. A massive outbreak of EAEC was observed in 1993 affecting 2,697 children in Japan. Further in 1998, Steiner *et al* discovered the association of growth impairment and fecal inflammatory markers associated with EAEC among Brazilian children. EAEC in these children was identified by PCR amplification of AA probe and Hela cell assay. EAEC was reported among children in Dhaka in 1993-1994. In 2004, EAEC with *aggR* gene was found among children in Mongolia. A survey conducted in 2003-2006 in south India showed the presence of EAEC among children less than 5 years of age. Here multiplex PCR of *astA*, *aggR* and *aafII* genes was done. In

2008, EAEC was found among the food handlers indicating it to be the cause of travelers' diarrhea. Single nucleotide polymorphisms (SNPs) in IL-8 promoter regions have been seen to be prone to EAEC diarrheal infections and individuals with lactoferin SNPs are involved in increased susceptibility to EAEC in traveler's diarrhea (Jiang *et al.,* 2003). Recent studies have highlighted the prevalence of EAEC in Iran and Spain (Cabal *et al.,* 2016; Darbandi *et al.,* 2016).

During the 2011 European outbreak, hybrid strains of EAEC and STEC were isolated which affected 4,321 healthy individuals out of which 900 patients developed hemolytic uremia syndrome (HUS) and 50 died. The gene *stx* was identified as EAEC O104:H4 which contained both the virulence genes of typical EAEC such as *aggA*, *aggR*, *set1*, *pic*, and *aap* as well as *stx2* gene of STEC (Ellis *et al.,* 2020). Various reports from Japan, Northern Ireland, France, and Central African Republic state the isolation of *stx* positive EAEC resulting in HUS formation in patients. Occurrence of large outbreaks due to EAEC infection has been reported in Japan (Itoh *et al*., 1997), England (Dallman *et al*., 2012) and South Korea (Shin *et al*., 2015). Thus, it is an indication of such outbreaks in the future.

3.8.3. Clinical features

EAEC has been known to be associated with watery, mucoid (Huang *et al.,* 2004) and often prolonged diarrhea which may last for more than 14 days depending on the patient's immunity, nutritional status as well as genetic susceptibility. Diarrhea caused by EAEC is often associated with abdominal pain, fever and vomiting (Lima *et al.,* 1992; Hebbelstrup *et al.,* 2014). Bloody stool has been detected in rare cases of infantile EAEC infections (Cravioto *et al*., 1991; Denno *et al.,* 2012). Both persistent and acute diarrheal cases have been reported where persistent diarrhea was observed in case of children below 1 years of age (Pereira *et al.,* 2007; Shazberg *et al.,* 2003). Steiner in his lab found that a large percentage of patients excreting EAEC have detectable fecal lactoferrin (a sensitive indicator of fecal leukocytes) and high levels of IL-8 in their stool. They also deciphered that EAEC infections resulted in growth retardations in children (Steiner *et al.,* 1998). Boukenooghe *et al*. (2000) have reported the presence of similar inflammation marked by raised fecal lactoferrin in adults with EAEC infection. These observations suggest that EAEC infection may be accompanied by a subtle form of mucosal inflammation.

Not only diarrhea EAEC is also involved in urinary tract infections (Abe *et al.,* 2008; Olesen *et al.,* 2012). In 1991, a Danished study reported an UTI outbreak due to EAEC infection where virulence genes of EAEC such as *sat, aggR, pic, aaiC, aatA, aap* and *aggA* were detected (Olesen *et al.,* 2012; Hebbelstrup *et al.,*2014).

3.8.4. Animal models of EAEC infection

The pathogenicity of EAEC has been investigated using various different animal models. EAEC shows significant histopathological changes, characterized by villi shortening, hemorrhagic necrosis of villous tips and a mild inflammatory response followed by edema and mononuclear infiltration in both rat and rabbit ileal loop assays (Vial *et al.,* 1988). In the 1988 study, NZB rabbits and Fisher 344 rats were used where EAEC infection has led to intestinal lesions followed by limb paralysis and sometimes death in severe cases of infections **(**Philipson *et al*., 2013). Studies using the reversible ileal-tie in adult rabbit diarrhea (RITARD) model have been used effectively to show the secretogenicity and colonization of some EAEC strains and to point out strain heterogeneity (Tickoo *et al*., 1992; Kang *et al*., 2001; Kaur *et al*., 2010). Rat ileal loops and rat tissue mounted in using chambers have been used to assess a plasmid-encoded toxin (Pet) (Eslava *et al*., 1998).

The pathogenicity of EAEC can be tested in the gnotobiotic pig model which shows a high resemblance with the pathogenic effects of EAEC in humans. Pigs show gastrointestinal, metabolic and immunological similarities with humans and thus were chosen for the study (Saif *et al.,* 1996; Rothkötter *et al.,* 2002; Philipson *et al*., 2013). Examination of the mucosa showed moderate stunting of villi and nuclear fragmentation (Tickoo *et al*., 1992). Tzipori and his colleagues also investigated the effect of EAEC infection in gnotobiotic piglets (Tzipori *et al.,* 1992) where two strains of EAEC were fed to gnotobiotic piglets which resulted in severe diarrhea or death in most of the piglets. Histology of the gastrointestinal tract revealed moderate hyperemia of the distal small intestine and caecum, swelling of small intestinal villi and “stacked brick” appearance of bacteria (Tzipori *et al.,* 1992). Six-day-old C57BL/6 mice with or without malnutrition were used as a model to test infantile diarrhea caused by EAEC. Antibiotic treated adult mice are also used for different pathogenicity studies of EAEC (Philipson *et al*., 2013). All these studies confirm the formation of distinctive intestinal lesions by EAEC infections and indicate the role of EAEC as a potent pathogen in humans.

3.8.5. Transmission and reservoir

Transmission of EAEC infections generally occurs through contaminated food and water in travelers as well as children in developing countries. In Mexico, food items such as salads, salsa and deserts remain the major mode of transmission of EAEC infections (Koo *et al.*, 2008; Vigil *et al.*, 2009). In one of the studies, the growth of EAEC was analyzed in different drinking water samples where the survival capacity of EAEC was found to be up to 60 days under normal temperature in mineral water than in normal water (Hebbelstrup *et al.*,2014). The survival capacity of EAEC infections in mineral water was higher as compared to normal spring water due to the presence of Ca^{2+} and Mg^{2+} ions which enhanced its genetic competence. The presence of EAEC was also surveyed in surface waters from rivers and creeks in Australia and Bangladesh (Akter *et al.*, 2013). Another study performed in Ouagadougou, Burkina Faso, showed that EAEC could also be transmitted by food handlers, emphasizing good food handling practices (Hebbelstrup *et al.*, 2014). EAEC was detected in different meat and beef by PCR targeting the genes such as *pic* and *aggR* prevalent in EAEC (Kagambega *et al.*, 2012). EAEC was detected in 3 out of 100 milk samples from infant feeding bottles in the low socioeconomic areas of São Paolo, Brazil (Hebbelstrup *et al.*, 2014).

Some British and Brazilian studies have indicated the presence of EAEC in sheep, pigs, cats and dogs. The presence of atypical EAEC has been seen in calves, piglets and horses, but it is not yet conclusive whether animals are a true source of EAEC infection (Uber *et al.*, 2006). Thus, from the above-mentioned facts, it is clear that contaminated food and drinks are a major source of EAEC infections in humans.

3.8.6. Pathogenicity and virulence factors

There are several virulence conferring factors which help to discriminate between virulent strains of *E. coli* from non-virulent strains of *E. coli*. Based upon these virulence factors various models of pathogenesis have been developed to distinguish the various pathogenic strains. Strains of *E. coli* may express a wide range of pathogenic mechanisms such as the production of enterotoxins and cytotoxins, adhesive structures and proteins that induce enterocyte cytoskeletal changes that lead to cell invasion. Present studies suggest that EAEC initially adheres to intestinal mucosa followed by the formation of mucoid biofilm. They induce toxic effects on the intestinal mucosa that result in diarrhea. There are three stages of

EAEC pathogenesis, the first stage involves the adherence of the bacteria to the intestinal mucosa with the help of aggregative adherence fimbriae (AAF) and other such adherence factors followed by an increase in mucus production which propels EAEC to the surface of enterocytes and lastly, the release of toxins which elucidates inflammatory response along with mucosal toxicity and secretions from the intestines (Harrington *et al.,* 2005; Nataro *et al.,* 2005). EAEC can be distinguished from other pathogenic *E. coli* by its distinct "stacked-brick" adherence pattern which has received considerable attention as adhesion to the gut epithelium forms the first major step in the pathogenesis of *E. coli* pathotypes (EPEC and ETEC). Epidemiological studies suggest certain plasmid-borne and chromosomal virulence factors are regulated by the master regulator, *AggR* (Aggregative adherence regulator) (Kaur *et al*., 2010; Meza-Segura *et al.,* 2020).

a) Histopathology and biofilm formation

A number of virulence factors and characteristic histopathological lesions have been described along with the formation of a thick layer of auto-aggregating bacteria that remained loosely attached to the mucosal surface of the infected animals. The most important feature of the EAEC strains is the enhancement of mucous secretion from the mucosa resulting in the formation of bacterium-mucous biofilm (Kaur *et al*., 2010). When an EAEC strain was used to infect the gnotobiotic pigs an unusual mucoid gel packed with a huge number of dense aggregating bacteria was observed adhering to the small intestinal mucosa of the pigs (Tzipori *et al*. 1992). In addition to hypersecretion of mucus, pitting of goblet cells was also observed. This goblet cells pitting and attachment of aggregating bacteria within a periodic acid- Schiff (PAS)-staining blanket (Vial *et al.,* 1988) has been recorded in ligated rabbit ileal loop models infected with EAEC. In vitro organ cultures have shown the EAEC adhered to small bowel mucosa in children (Hicks *et al.,* 1996) where they formed a thick mucus biofilm. The volunteers of EAEC study have developed mucoid diarrhea due to the survival of EAEC within the thick aggregating mucus layer (Wanke *et al*., 1990; Nataro *et al*., 1995; Wakimoto *et al.,* 2004; Kaur *et al*., 2010). Although the purpose of excess mucus formation remains unclear, heavy biofilm formation is directly related to its diarrhoeagenicity and its capacity to cause persistent diarrhea and colonization of mucosa (Kaur *et al*., 2010; Wakimoto *et al.,* 2004). EAEC biofilm formation is under the regulation of *AggR* and requires the involvement of several other genes such as *fis*, *yafF, eilA* regulator genes and *air* (Sheikh *et al.,* 2006). A recent discovery has suggested the involvement of another gene

encoding a 32.8 kDa protein named shf that is located in one of the three open reading frames between *aafC* and *aatA* (Fujiyama *et al.,* 2008).

Various cytotoxic effects on the mucosa of the intestines have been reported due to EAEC infections. Vial and his colleagues were the first to demonstrate the formation of destructive lesions in rabbit as well as rat ileal loop models which are characterized by shortening and hemorrhagic necrosis of the villous tips, with mild inflammatory response followed by mononuclear infiltration of the submucosa. Ileum specimens from patients who died of EAEC infection have shown damage to mucosa (Eslava *et al*., 1998).

b) Adhesins

The pathogenesis of EAEC involves the formation of characteristic aggregative adherence (AA) pattern on the surface of Hela or Hep2 cells where the bacteria adhere together to form a "stacked brick wall" with the help of aggregative adherence fimbriae (AAF) (Moreira *et al.*,2003; Kaper *et al*.,2004). These AAF belong to the Dr family of adhesions comprising of fimbrial as well as non fimbrial adherence factors that recognize the antigens of Dr blood group (Savarino *et al.,* 1994). Bacterial interactions with the cellular monolayer cause cellular detachment and cytotoxic activity within the cells (Savarino *et al.,* 1993).

Aggregative adherence (AA) pattern of EAEC is due to the presence of both fimrial [aggregative adhesin fimbria (AAF)] and afimbrial adhesins. According to the reviews of Estrada- Garcia and Navarro-Garcia in 2012, afimbrial adhesins otherwise known as outer membrane proteins help in adhesion. 3 different types of AAF are found to be encoded by the pAA plasmid of the EAEC strains which are: *aggA* (AAF/I), *aafA* (AAF/II), and *agg-3* (AAF/III) although only a single type of AAF is present in each EAEC isolate (Nataro *et al.,* 1993; Kaur *et al*., 2010). Other 2 fimbriae variants such as AAF/IV and AAF/V have also been identified (Jonsson *et al.,* 2015; Jonsson *et al.,* 2017; Ellis *et al.,* 2020). Along with conferring the aggregative phenotype, AAF/I adhesins are involved in erythrocyte hemagglutination. AAF/II helps in intestinal adhesion (Kaur *et al*., 2010).

AAF/I is a flexible bundle forming fimbriae of 2-3 nm in diameter that confers the aggregative phenotype and the genes required for the expression of AAf/I are located in 2 separate gene clusters (region 1 and 2) on a 60 MDa plasmid separated by a 9kb intervening DNA region (Nataro *et al.*,1993). Sequencing of region 1, revealed the presence of four open reading frames (ORFs) known *Agg D, C, B, A* respectively each having specific functions.

AggA is involved in encoding a major fimbrial subunit of 15.6 kDa molecular weight, while *AggC* encodes outer membrane usher and *AggD* encodes a periplasmic chaperone (Savarino *et al*., 1994). Region 2 comprises of 5 ORFs, 3 of which share homology with insertion elements of other enterobacteria. ORF3 encodes a 29.4 kDa protein, known as AggR, which is highly homologous to *CfaR* and other genes that positively regulate fimbrial expression in *E. coli*. AggR is a major transcriptional regular belonging to a AraC family, which plays a vital role in fimbrial biogenesis of AAF/I as well as AAF/II fimbriae. The gene *aggR* promotes the expression of both chromosomal and plasmid encoded virulence factors such as AAF and dispersin (Dudley *et al.,* 2006). The effect of AggR was most remarkable at pH 5.5 (Morin *et al.,* 2010). The term 'typical EAEC' was coined by Nataro in 2005 where it was used for those strains that harbored *AggR* and at least a subset of *AggR*-regulated genes and the term 'atypical EAEC' was used for strains lacking the *AggR* regulon. EAEC prototype strain 042, which has been shown to be pathogenic in volunteers (Nataro *et al*., 1995), does not express AAF/I fimbriae and produces a honeycomb pattern of adherence (Nataro *et al*., 1995). 25% of identity has been shown by AAF/I and AAF/II fimbriae (Gonyar *et al.,* 2020). The AAF/II fimbriae are 5 nm in diameter and are antigenically distinct from AAF/I (Czeczulin *et al*., 1997). AAF/I is expressed in the majority of EAEC (31%) while AAF/II is expressed by only 12% of EAEC strains (Gonyar *et al.,* 2020). AAF fimbriae have been shown to mediate aggregative adherence to epithelial cells, hemagglutination and are necessary for biofilm formation. In contrast to bundle forming structure of AAF/I and AAF/II fimbriae, AAF/III shows peritrichous, long flexible filaments that aid in bacterial aggregation. The genetic organization of *agg-3* shows similarity to *aag* and *aaf* operons found in EAEC. AAF/V was found to be a chimeric form of AAF/III comprising of all the accessory genes as well as the minor pilin subunit gene (*agg3B*) (Jonsson *et al.,* 2017).

The fimbria can be thrust out of the bacteria due to the presence of Dispersin protein encoded by the gene *aap* which is present in the upstream region of *aggR* gene (Harrington *et al.,* 2005). Dispersin, when secreted out of the EAEC strains, binds non-covalently to lipopolysaccharide (LPS) thus neutralizing the electrostatic surface of the LPS layer of the bacteria, making it possible for the positively charged fimbriae to thrust out and bite to appropriate sites (Harrington *et al.,* 2005). The secretion of dispersin is dependent on the ABC transporter complex encoded by the pAA plasmid.

c) Toxins

Once EAEC has successfully adhered to the intestinal mucosa, it starts secreting a number of cytotoxins and enterotoxins that promote cytotoxic damage to the epithelium and help in intestinal colonization (Ellis *et al.,* 2020; Kaur *et al*., 2010). The toxins secreted by EAEC include the plasmid encoded toxin (Pet), the enteroaggregative *E. coli* heat-stable enterotoxin (EAST-1) which is encoded by the gene *astA*, the protein involved in colonization (Pic), autotransporter proteases such as *sepA* and *sigA*, which were first found in *Shigella flexneri* and hemolysin E (*hlyE*) (Kaur *et al*., 2010; Dias *et al.,* 2020; Ellis *et al.,* 2020). Pic and the oligomeric enterotoxin Shigella enterotoxin 1 (ShET1) is encoded by the opposite strands of the same chromosomal locus by the genes *setBA.* (Noriega *et al*.,1995; Henderson *et al*., 1999; Harrington *et al.,* 2006). ShET1 results in secretary diarrhea found in EAEC and *Shigella* infections and reported to cause fluid accumulation in rabbit ileal loops. The mechanism underlying the enterotoxic activity of ShET1 is not dependent on the activity of cyclic guanosine monophosphate, cyclic adenosine monophosphate and Ca^{++}, and may involve nitric oxide signaling (Harrington *et al.,* 2006).

EAST1 is a plasmid borne enterotoxin found in most of the EAEC strains which is a 38-amino-acid protein homologous to the STa toxin present in ETEC (Savarino *et al*., 1993). The *astA* gene which codes for the EAST1 enterotoxin is abundant among the various pathogenic and non-pathogenic *E. coli* isolates that not only 41% of EAEC isolates harbors *astA* gene but it is also present in 100% of 0157: H7 EHEC, 6-41% of other DEC, as well as in 38% of commensal *E. coli* (Harrington *et al.,* 2006). Several EAST-1 positive strains were found to be associated with diarrhea in Spanish students. Although EAST1 contributes to watery diarrhea in EAST1-positive strains; the gene for EAST1 production is also present in the commensal *E. coli* strains thus making the role of EAST1 in diarrhea a mystery (Menard and Dubreuil, 2002).

Pet is a toxin of molecular weight of 108 kDa which is encoded by the 60 MDa plasmid present in the EAEC isolates that cause exfoliation of epithelial cells of the colon and mucosal damage (Eslava *et al*., 1998). The EAEC strain 042 expressing Pet has demonstrated the bulging of intestinal enterocytes as well as the distention of crypt openings and intercrypt crevices. Reports suggest that the toxicity of Pet is due to its cleaving of the apha-III spectrin within the epithelial cytoskeleton. Pet is a serine protease which was first identified as a protein whose secretion reduces resistance and increases short-circuit current across rat

jejunal tissue (Eslava *et al*., 1998; Navarro-Garcia *et al*., 1998). The *pet* gene is found in the pathogen-specific cluster of pAA plasmid and is found in almost 44% of EAEC isolates (Eslava *et al*., 1998).

d) Intestinal inflammation

The first clinical inflammatory response to EAEC infection varies from individual to individual depending upon the host's innate immune response and its genetic composition. Diarrhea induced by EAEC is associated with markers of intestinal inflammation such as fecal lactoferrin, a stable neutrophil product, fecal neutrophils and occult blood as well as with a series of pro-inflammatory markers and cytokines, such as interleukin-8 (IL-8), interleukin-1ra (IL)-1ra, interferon-γ and interleukin-1ß (IL-1ß) (Steiner *et al*., 1998; Greenberg *et al*., 2002). IL-8 plays an important role as a pro-inflammatory chemokine in EAEC pathogenesis. It is involved in neutrophil recursion to the epithelial mucosa without damaging the mucosal surface, and results in intestinal fluid secretion. Results of infant diarrhea from Brazil have shown the presence of inflammatory markers such as IL-8, IL-1β in the stool samples of the patients (Lima *et al*., 1992; Fang *et al*., 1995; Steiner *et al*., 1998). It has been reported that Caco-2 cells when infected by EAEC, result in upregulation and early release of IL-8 and other pro-inflammatory factors such as IL-1β, IL-6, IL-10, IL-12 and TNF (Kaur *et al*., 2010; Estrada-Garcia *et al.,* 2014). The increase in IL-8 production is due to the presence of AA genotype at -251 position of the IL-8 promoter. Apart from IL-8, intercellular adhesion molecule (ICAM)-1, a growth-related gene product (GRO)-*α*, GRO-*γ* and granulocyte macrophage colony-stimulating factor (GM-CSF) are also elevated in vitro studies which are mediated by the EAEC surface protein flagellin (*fliC*) (Greenberg *et al.*,2002; Harrington *et al.*, 2005). EAEC induces mitogen-activated protein kinases (MAPK) activation on intestinal cells, which in turn activates the transcriptional factor NF-κB, resulting in the hyper secretion of IL-8 and other cytokines involved in pro-inflammation of the intestinal mucosa (Khan *et al.*,2010; Goyal *et al.,* 2010; Estrada-Garcia *et al.,* 2014) (**Figure I3**).

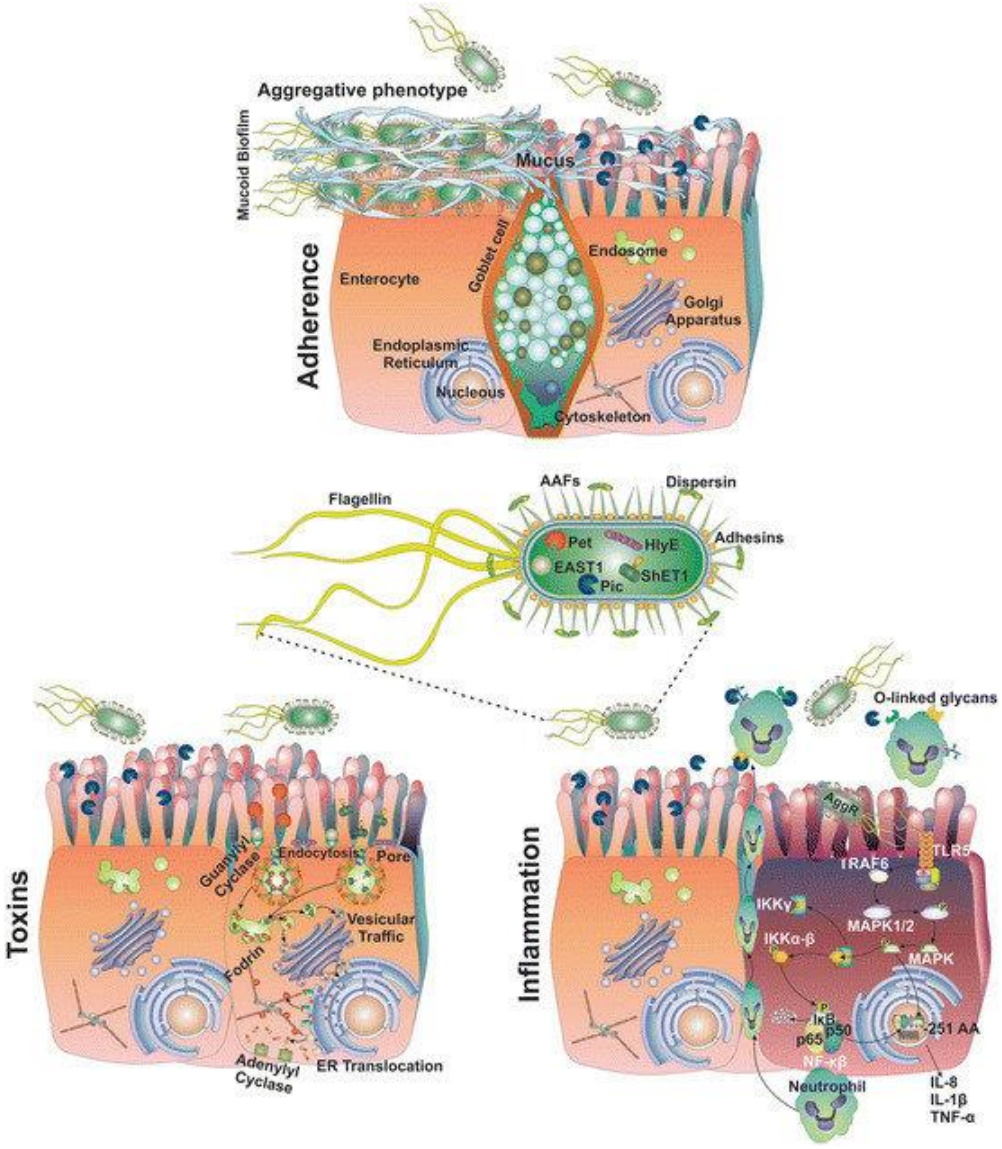


Figure I3: *The pathogenesis model of EAEC infections: a) EAEC containing the virulence factors adhering to the intestinal mucosa b) biofilm formation and secretion of toxins c) intestinal inflammation (Estrada-Garcia et al., 2014).*

3.8.7. Strain heterogeneity

A devastating outbreak of diarrhea and HUS occurred in Germany in the year 2011 due to a heterogeneous EAEC strain O104:H4 that harbored the virulence features of STEC. The O104:H4 strain harbored the pAA plasmid and expressed the AAF/I fimbrae which helps it to adhere to the intestinal epithelial cells with stacked brick appearance. The gene for dispersin protein that is known to be involved in intestinal colonization and the *Shigella* enterotoxin (*set1*) was detected in this strain. Phage-mediated Shiga toxins 2a, resistance to all penicillins, cotrimoxazole and cephalosporin, tellurite and mercury resistance colicin were some of the characteristics acquired by this strain due to horizontal gene transfer. Genomic plasticity and

the fact that any isolate has the potential to acquire novel virulence factors were illustrated by this strain.

3.8.8. Diagnosis

Considering the fact that EAEC is often recovered from symptom-free cases, the isolation of EAEC in a case of diarrhea does not assure that it is the causative agent. However, in the absence of other known pathogens, or if EAEC is isolated repeatedly, particularly if the diarrhea is persistent, EAEC should be presumed to be the offending pathogen. The gold standard for EAEC identification remains the HEp-2 adherence test. Other enteric pathogens have shown the ability to exhibit aggregative phenotypes and therefore confirmation of *E. coli* speciation is necessary to conclusively identify EAEC. Most strains of EAEC carry one of a family of large virulence plasmids, which encode the AA phenotype. A 1.0-kb Sau3AI fragment of the AA plasmid from strain 17-2, which hybridizes with a fragment of similar size from the 65-MDa plasmid of strain 042, was selected by Baudry *et al*. (1990) and named CVD432. In its original evaluation, the CVD432 probe was found to be 89% sensitive and 90% specific for EAEC (Baudry *et al*., 1990). Later studies have confirmed the high specificity of this probe but have shown that sensitivity varies between 15% and 90% for EAEC from different locations. This finding has important consequences for the conclusions that can be drawn from studies that use the probe alone to identify EAEC. The epidemiological significance of probe-positive as against probe-negative strains has not so far been determined. Although *aggR* is a sensitive target, it is much less specific than the CVD432 locus (Okeke *et al*., 2000). Although, several studies have attempted to find methods for providing a simple test for identifying EAEC, including erythrocytes agglutination, serotyping, pellicle formation in broth culture and outer membrane protein analysis (Wai *et al*., 1996; Bangar *et al.*, 2007; Ellis *et al.,* 2020) the HEp-2 adherence assay and/or the CVD432 standard probe represent the present means for detecting EAEC (Hebbelstrup *et al.*, 2014); however, it is clear that more sensitive, specific and practical methods are needed to improve diagnosis of infected cases and understanding of the disease.

3.8.9. Treatment

EAEC can cause prolonged diarrhea with long term effects such as malnutrition and growth retardation in children, thus proper measures should be taken to prevent EAEC infections. The decision to provide antibiotic therapy depends on the individual and also on the susceptibility pattern of the infecting bacteria. The susceptibility pattern of EAEC is different

in different locations. EAEC isolated from travels of Jamaica and the US are found to be susceptible to fluoroquinolone, rifampicin, nalidixic acid, azithromycin and amoxicillin/clavulanic acid. Haider and his colleges have reported the prevalence of 71% of multidrug resistant EAEC in children. Oral rehydration therapy (ORT) should be preferred in case of mild diarrhea while antibiotic therapy is recommended in case of severe infections to decrease the prolonged period of persistent diarrheal episodes in children. ORT alone is not amenable in case of severe infections, thus the development of vaccinations and antibiotic therapy has arisen. In severe cases of vomiting and dehydration, intravenous hydration therapy is used. Several reports have suggested the use of lactoferrin to protect infants from gastrointestinal infections, including EAEC, and may be an alternative treatment for antibiotic-resistant EAEC strains.

3.9. Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) colonizes the surface of the small-bowel mucosa and elaborates their enterotoxins, giving rise to a net secretary state of dehydration. ETEC causes watery diarrhea, which can range from mild, self-limiting disease to severe purging disease. ETEC is one of the major causes of childhood diarrhea in developing countries (Barry *et al.,* 2006; Deng *et al.,* 2013). Almost 15-20% of children under 5 years of age are suffering from diarrhea caused by ETEC (Byrd *et al.,* 2006; Isidean *et al.,* 2011). In the year 2013, approximately 89,000 children below 5 years of age died due to diarrhea caused by ETEC in Africa and South Asia (Ziethlow *et al.,* 2008; Mansouri *et al.,* 2013). It is also a major causative organism of travelers' diarrhea in Asia, Africa and Latin America. Approximately 10 million of diarrheal cases in travelers are caused due to ETEC (Ziethlow *et al.,* 2008; Mansouri *et al.,* 2013; Mirhoseini *et al.,* 2018).

3.9.1. Historical background

ETEC came into eminence in the late 1960s and early 1970s, due to the work carried out in Calcutta by De and Chatterjee (1953); Gorbach *et al*., (1971) and others (DN *et al.,* 1953; Gorbach *et al.,* 1971). The injection of sterile culture filtrates of *Vibrio cholerae* O1 gave rise to a secretary response in the rabbit ileum in the gut of anesthetized animals, thus indicating the diarrhoeagenic effect of the exogenous product of *Vibrio cholerae* O1 (Sack *et al.,* 2011). The etiology of severe cholera-like diarrhea in cases where *Vibrio*was not demonstrated puzzled clinicians and microbiologists and prompted closer investigations of the dominant *E.*

coli flora from the stool. S. N. De used single *E. coli* colonies from these diarrheal cases where *Vibrio* was not recorded to test in rabbit ileal loops (RIL). The RIL yielded positive results which led to the discovery of enterotoxin production by certain human strains of *E. coli* (Sack *et al.,* 2011). In the late 1960's it was found that *E. coli,* which caused diarrhea in piglets, also produced enterotoxins (Smith and Gyles, 1970). In the early 1970s; it had become clear that the ETEC strains were of global significance as an agent of diarrheal disease in both human and domestic animals. ETEC is a major cause of infantile diarrhea in less developed countries (Black *et al.,* 1982) and also serves as the main cause of traveler's diarrhea. The RIL model developed by S. N. De was used to demonstrate the pathogenic serogroups of *E. coli* causing diarrhea in children less than 5 years of age (Taylor *et al.,* 1966).

3.9.2. Epidemiology

Annually, a surprising number of diarrheal cases are reported due to infections with different ETEC strains. The ETEC infections are caused due to intake of contaminated food or water and are transmitted through fecal-oral route. Fecal contamination of food and water remains the major cause of ETEC diarrhea in developing countries. The endemic pattern of ETEC spread is due to a number of factors such as: a) exposed individuals develop mucosal immunity to ETEC infections, b) discharge of virulent pathogenic ETEC in stools of asymptotic individuals (DuPont *et al.,* 1971; Nataro *et al.,* 1998).

ETEC infections mainly occur among children below 5 years and travelers in developing countries. 10% to 30% of the sporadic infant diarrheal cases are due to ETEC (Nataro *et al.,* 1998). ETEC infections are characterized by the discharge of profuse watery stool caused by the disruption of intestinal functions by two ETEC enterotoxins- heat-labile (LT) and heat stable (ST) enterotoxins (Heymann *et al.,* 2008; Buuck *et al.,* 2020). There are reports greatly exceeding 200 million cases, causing approximately 75,000 deaths mainly among babies and young children in tropical areas due to poor sanitary conditions (Gomes *et al.,* 2016). The peak incidence of ETEC infections in combination with Rotavirus and *Shigella* occurs among children of 6 to 36 months of age. Infant studies conducted in Egypt, West Africa and Bangladesh reported that the rate of ETEC infections increases from 3 to 6 months of age (Steinsland *et al.,* 2002; Rao *et al.,* 2003; Medus *et al.,* 2016). In the study conducted in Guinea Bissau, children of 3 months of age were seen to be infected by ETEC strains producing STh and LT enterotoxins, whereas 6 to 7 months children were infected by ETEC

strains producing STp, and STh (Steinsland *et al.,* 2002; Qadri *et al.,* 2005). ETEC has been reported as the second and fourth major pathogenic diarrhea causing bacteria in a surveillance study conducted in 2 different clinical laboratories of Minnesota during the time span 2000-2008. The incidence of ETEC infections decreases in the age group between 5 to 15 years of age while it increases above 15 years of age. The majority of ETEC endemic cases are due to ST producing ETEC strains (Nataro *et al.,* 1998). ETEC has been reported to be the primary cause of travelers' diarrhea affecting almost 20% to 60% of travelers all over the world (Fleckenstein *et al.,* 2019). 40,000 ETEC cases have been reported in the USA annually in which 55% of the cases are associated with international travel (Scallan *et al.,* 2011). Travelers' diarrhea due to ETEC is mostly recorded in warm and wet months when the rate of multiplication of ETEC is high in food and water. Travelers who are traveling to developing countries for the first time are mostly affected by ETEC (Nataro *et al.,* 1998).

As it was not possible to detect ETEC by conventional stool culture methods, diagnostic laboratories have started using culture-independent diagnostic tests (CIDTs), such as multiplex polymerase chain reaction (PCR) for the routine detection of ETEC positive samples (Murphy *et al.,* 2017; Cybulski *et al.,* 2018). Film Array gastrointestinal panel (GIP) has been adopted in Minnesota since 2015 which can detect 22 different enteric pathogens including ETEC and other pathotypes of diarrheagenic *E. coli* (Buuck *et al.,* 2020).

3.9.3. Clinical features

Diarrhea caused due to ETEC infections was first reported among adults as well as children in Calcutta (Sack *et al.,* 1971; Qadri *et al.,* 2005). ETEC diarrhea is very similar to diarrhea caused by *Vibrio cholerae* (Sack *et al.,* 1971). After the first report from Calcutta, several other reports from around the world indicated that ETEC diarrhea may range from very mild to very severe cases (Black *et al.,* 1981).

ETEC causes a secretary type of diarrhea with a sudden onset of watery stool usually without blood, mucus or pus followed by vomiting which results in dehydration due to loss of fluids and essential electrolytes such as sodium, potassium and chloride from the body (Sack *et al.,* 1975; Black *et al.,* 1981; Qadri *et al.,* 2005; Wang *et al.,* 2019). This abrupt fluid loss causes drying of mouth, lethargy, decrease in blood pressure, rapid pulse, decreased skin turgor, muscle cramps and shock in severe cases. The degree of dehydration ranges from mild to severe and on the basis of this clinical feature appropriate therapies are given to the patients

(Wang *et al.,* 2019). The diarrhea caused by ETEC lasts for about 3 to 4 days and is often self-limiting. The patients survive if proper hydration is maintained. The mortality rate can be decreased to less than 1% by means of adequate treatment (Qadri *et al.,* 2005). Travelers' should be counseled to maintain proper dehydration status in case of severe diarrhea episodes. For decreasing the severity of diarrhea loperamide or bismuth subsalicylate should be administered. However, loperamide should not be given to patients suffering from fever or dysentery if antibiotics are not given (Nataro *et al.,* 1998). Administration of antibiotics results in shortening the diarrheal episodes but the rise of resistance to antibiotics is a major concern in the modern world. Fluoroquinolones such as ciprofloxacin, ofloxacin and norfloxacin are commonly used against ETEC diarrhea as resistance to other antibiotics has been documented in many different areas (DuPont *et al.,* 1993; Nataro *et al.,* 1998).

The pathophysiology of the infection is quite a similar to that of caused by *Vibrio cholerae* documenting similar clinical picture in adults (Khan *et al.,* 1988). Studies have shown a high dose of infection in both cases of infection. In the case of ETEC, the dose ranges from 10^6 to 10^{10} CFU (Levine *et al.,* 1979). In both cases, a high dose of infection results in bacterial proliferation in the small intestine with the help of colonizing factors followed by the secretion of enterotoxins which causes secretary watery diarrhea followed by dehydration (Croxen *et al.,* 2010). The attack rates of ETEC are higher in children and decrease in higher age groups (Black *et al.,* 1981).

3.9.4. Transmission and reservoir

Contaminated food and water supply is the main cause of ETEC infection and transmission among individuals (Gilligan *et al.,* 1999; Daniels *et al.,* 2000; Beatty *et al.,* 2004). For severe infections, a high dose of about 10^8 CFU of bacteria is required which implies that the food and water sources have to be highly infected for outbreaks to occur (Nataro *et al.,* 1995). Food sources that are reported to be involved in ETEC infections include parsley, scallops, basil, fresh fruits and vegetables (especially lettuce), shrimp, tuna paste, crab meat prepared salads, and soft cheeses. Various types of contaminated salads such as salads of potato, macaroni and eggs and contaminated seafood are the major vehicles of ETEC infections. Waterborne outbreaks pose a serious threat, particularly in cruise ships. One such outbreak reported on cruise ship was due to failure to properly chlorinate water after bunkering which resulted in the spread of ETEC outbreaks in thousands of individuals (Daniels *et al.,* 2000).

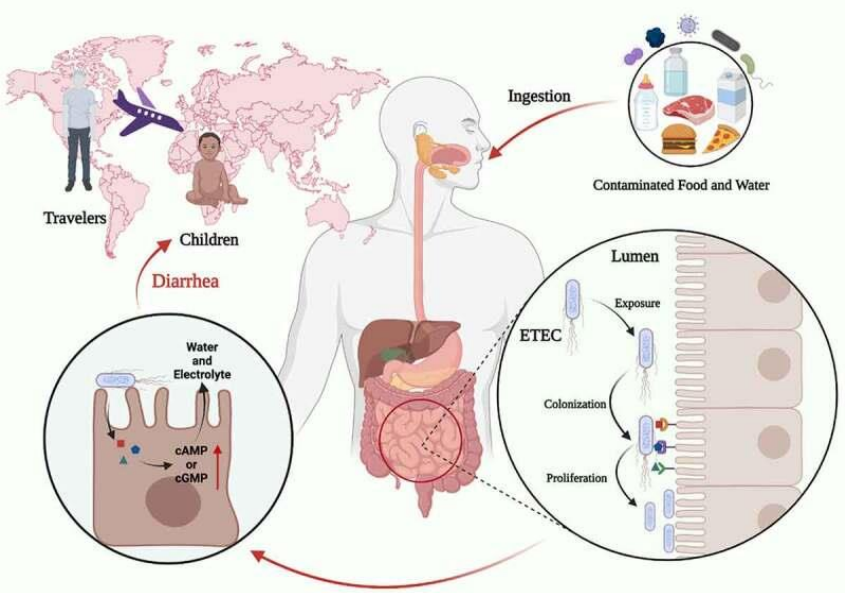


Figure I4: *Mode of transmission and reservoirs of ETEC infections. ETEC is the major enteric pathogen in travelers and children of developing countries. ETEC infection is caused by ingestion of contaminated food and water and the ETEC pathogens colonize the small intestine (Zhang et al., 2022).*

3.9.5. Pathogenesis

Two major virulence determinants have been identified in ETEC: enterotoxins and colonization factor antigens (CFA) which are often encoded by plasmids. The plasmid borne enterotoxins are heat-stable (ST) and/or heat-labile (LT) enterotoxins (Nataro and Kaper, 1998). ETEC infections are caused by the intake of contaminated food and water which results in the colonization of the small intestine followed by the secretion of enterotoxins causing severe diarrhea (Nataro *et al.*, 1998; Mirhoseini *et al.*, 2018). Distinct types of CFAs have been described in human ETEC strains and they are mainly constituted by protein fimbriae. A considerable proportion of ETEC strains do not appear to express known CFA. Once attached to the enterocytes, the bacteria produce either LT or ST or both, leading to diarrhea (Nataro and Kaper, 1998; Mirhoseini *et al.*, 2018; Zhang *et al.*, 2022).

a) Enterotoxins

ETEC strains evoke a non-invasive, highly secretory kind of diarrhea with the help of two enterotoxins- heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) either singly or together. It was in 1967 that the first ETEC enterotoxin was discovered from a cell-free heat-stable solution from a porcine *E. coli* strain which was demonstrated to cause fluid accumulation in a ligated pig intestine. Shortly, thereafter a distinct heat-labile toxin which showed similarity to cholera toxin (CT) was discovered. Two types of ST (ST-I and ST-II)

have been described based on solubility in methanol as well as their activity in infant mice (Moseley *et al*., 1983). There are two types of LT toxins based on genetic sequence and antigenic capacity which are designated as LT-I and LT-II (Mirhoseini *et al.,* 2018; Zhang *et al.,* 2022). LT-Ih and LT-Ip are isolated from humans and porcine respectively while LT-II is isolated from animals (Zhang *et al.,* 2022). ETEC strains are capable of producing either toxin class alone or in combination. Human ETEC may produce LT and ST-I but not ST-II, which is solely produced in animal ETEC strains.

i. Heat-labile toxins (LTs)

The heat-labile enterotoxin (LT) produced by ETEC is a higher molecular weight oligomeric class of toxin. It is encoded by the *eltAB* operon present on a virulence plasmid and is closely related functionally, structurally as well as biologically to the cholera enterotoxin (CT), the major virulence determinant of *V. cholera* (Zhang *et al.,* 2022). The LT found predominantly in human strains, called LT-I shows ~75% similarity with CT at the nucleotide level and shares several phenotypes, including in its primary receptor and mechanism of action (Zhang *et al.,* 2022). The LT-II toxin is found primarily in animal *E. coli* strains and rarely in human strains, but is not associated with disease.

LT-I enterotoxin

LT enterotoxin is a hetero-hexamer comprising of a single A subunit and a pentameric B subunit. The LT toxin is 86kDa which possess one 28 kDa subunit and five identical 11.5 kDa B subunits (Hajishengallis *et al.,* 2013). The B subunit is arranged in the form doughnut or ring of five subunits and binds strongly to the GM1 ganglioside present at the intestinal epithelium (Henrique *et al.,* 2021). The A subunit is made of 2 domains (A1 & A2) which are linked together by disulfide bonds (Henrique *et al.,* 2021). The A1 domain shows the major catalytic activity while the A2 domain acts as a bridge anchoring the A1 domain to the B subunit (Beddoe *et al.,* 2010; Zhang *et al.,* 2022). The A2 domain is also involved in the transportation of proteins into the intercellular region (Zhang *et al.,* 2022).

The genes encoding the LT toxin reside on virulence plasmids and are flanked by inverted DNA repeats. The expression of the plasmid genes is controlled by both the chromosomal genes as well as the genes in the flanking region. Both the A and B subunits are synthesized individually and are secreted through the inner membrane. Apart from its secretary activity, LT also acts as a mucosal adjuvant (Hajishengallis *et al.,* 2013).

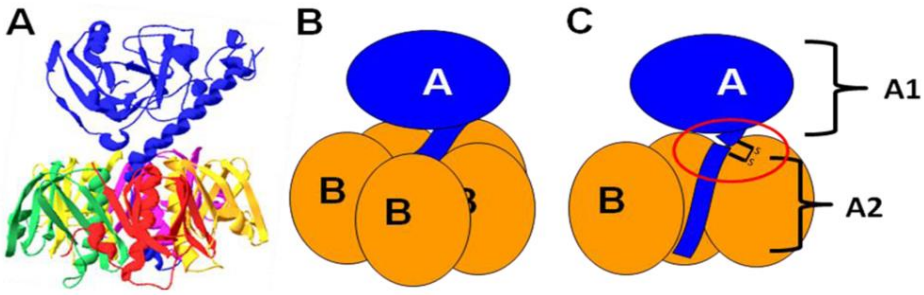


Figure I5: *Diagrammatic representation of LT enterotoxin. Ribbon model (A), Holotoxin (B) and Cleavage domain (C) (Hajishengallis et al., 2013).*

LT-II enterotoxin

The second group in the LT family, LT II, has a protein structure and mechanism of action similar to that of LT-I but is different with respect to its immune-reactivity and ganglioside binding capacity (Pickett *et al*., 1989). The gene encoding LT-II is chromosomal (Pickett *et al*., 1989). LT-IIa has been purified from an *E. coli* strain isolated from a water buffalo in Korat, Thailand (Moseley *et al*., 1983). The other toxin, LT-IIb, which has also been purified, has several properties that distinguish it from LT-IIa, including partial antigenic identity, differences in isoelectric point (LT-IIa pI=6.8; LT-IIb pI=5.4) (Guth *et al*., 1986).

Receptors of LT family

The membrane receptor of the LT-I family has been identified as GM ganglioside, as in the case of CT. LT can also bind to GM2 (sialo-GM,) and to glycoprotein (Jobling *et al.*, 2016). Chemical modification has revealed that the tryptophan-88 (Mullin *et al*., 1976) and the glycine-33 from the amino-terminus of the B subunit (Tsuji *et al*., 1982) are important for binding the B subunit to its ganglioside receptor. LT-IIa binds with the greatest affinity to ganglioside GD1b, while LT-Ib binds preferentially to ganglioside GD1a (Fukuta *et al*., 1988).

Mode of action of LT toxins

After binding to the host G1 ganglioside receptor, the toxin A1 is internalized through the process of endocytosis. The toxin A1 subunit translocates through the cell via trans-Golgi vesicular transport. The active A1 toxin results in ribosylation of α subunit of G_S protein which in turn activates adenylate cyclase. The activated adenylate cyclase causes an increase in the intercellular cAMP level which in turn elicits the activation of cAMP dependent

protein kinase A (PKA). The active PKA causes phosphorylation of multiple serine residues present within the R-domain of CFTR (cystic fibrosis transmembrane regulator) which in turn causes secretion of Cl^- and HCO_3^- anions and decrease in absorption rates of Na^+ and Cl^- ions (Viswanathan *et al.,* 2009; Duan *et al.,* 2019). This change in water and electrolyte balance causes severe watery diarrhea (Henrique *et al.,* 2021).

ii. Heat-stable toxins (STs)

In contrast to the large, oligomeric LTs, the STs are small, monomeric toxins that contain multiple cysteine residues, whose disulfide bonds account for the heat stability of these toxins (Wang *et al.,* 2019; Kiefer *et al.,* 2020). Heat-stable enterotoxins produced by ETEC are subdivided into 2 types based on their protease sensitivity and methanol solubility. These types are STa and STb. STa is methanol soluble and is associated with the onset of diarrhea in humans and animals whereas STb is methanol insoluble and is virulent in animals (Weiglmeier *et al.,* 2010; Wang *et al.,* 2019). The term ST-I and ST-Il are also commonly used as synonyms for STa and STb, respectively which differ in both structure and mechanism of action. The enterotoxins are produced by plasmid borne genes named *estA* (ST-I) and *estB* (ST-II) (Wang *et al.,* 2019). Only toxins of the STa class have been associated with human disease (Nataro and Kaper, 1998).

STa enterotoxin

Based on the host in which the toxin is identified, STa is subdivided into 2 classes- STh isolated from humans and STp isolated from swines. The STa gene encodes a 72 amino acids pre-pro-peptide precursor. Based on the difference in their pro region, six different variants of alleles were discovered:*estA1*, *estA5* and *estA6* from the porcine origin (STp) and *estA2*, *estA3/4* and *estA7* from the human origin (STh) (Von Mentzer *et al.,* 2014; Joffre *et al.,* 2016). Among STp+, the *estA5*gene is the most common variant causing diarrhea in adults and children while *estA¾* is the prevalent variant in the case of STh^+. Apart from the pro region variation, each STa allele variant is translated into a pro-peptide, consisting of a 19 amino acids (aa) signal peptide, followed by a 34 aa pro-sequence, and the mature STa peptide. The pro-peptide is cleaved to form the mature STa peptide after being translocated from the inner membrane into the periplasm (STh: 19 aa; STp: 18 aa) (Weiglmeier *et al.,* 2010). In the periplasm, the three intramolecular disulfide bonds between cysteine residues Cys6-Cys11, Cys7-Cys15, and Cys10-Cys18 in STh or Cys5-Cys10, Cys6-Cys14, and Cys9-

Cys17 in STp are connected by the help of disulfide oxidoreductase DsbA (Yamanaka *et al.,* 1994; Wang *et al.,* 2019). These disulfide bridges are essential for the correct folding and proper functioning of the mature STa peptide. The mature STa peptide is similar to guanylin and uroguanylin peptides found in human (Yamanaka *et al.,* 1994). The efflux protein TolC mediates the secretion and delivery of mature STh and STp into the extracellular space (Zhu *et al.,* 2018).

STb enterotoxin

STb enterotoxin is highly conserved in ETEC isolates all over the world. Until now only one variant (a His_{12}→Asn change) of STb has been reported which is generally found among the STa and STx positive ETEC isolates (Fekete *et al.,* 2003; Taillon *et al.,* 2008). STb is synthesized as a 71 amino acid pre-peptide consisting of a signal sequence and a 48 amino acid mature STb enterotoxin (Turner *et al.,* 2006). The signal sequence is cleaved in the periplasm releasing the mature peptide. Correct folding is mediated by the formation of 2 disulfide bonds at Cys21-Cys36andCys10-Cys48 by DsbA (Turner *et al.,* 2006). STb has been identified as the major diarrhea causing factor in animals, particularly in weaning piglets (Loos *et al.,* 2012; Wang *et al.,* 2019).

Receptors of STs

The first step in the biological action of STa is its interaction with specific high-affinity receptors. The main receptor for STa is a membrane-spanning guanylate cyclase (GC-C). The binding of STa to GC-C stimulates guanylate cyclase activity, leading to increased intracellular cyclic GMP levels (Pig. 2), which in turn produces stimulation of chloride absorption. Guanylin is a 15-amino-acid peptide, which contains four cysteines and is less potent than STa in activating GC-C (Weiglmeier *et al.,* 2010). Guanylin is presumed to play a role in normal gut homeostasis, and GC-C is apparently used opportunistically by STa to cause diarrhea. Few studies were done on the first step of STb pathogeny; the binding of the toxin to the intestinal epithelial cells via a specific attachment molecule.

Mode of action of ST toxins

STa binds to the receptor and activates the intracellular domain of guanylate cyclase C resulting in hydrolysis of guanosine triphosphate (GTP) and accumulation of intracellular cyclic GMP (cGMP) levels (Vaandrager *et al.,* 1997; Kaper *et al.,* 2004). The increase in cGMP level in turn activates cGMP-dependent protein kinase II (PKGII) as well as cAMP-

dependent protein kinase A (PKA) (Chao *et al.*, 1994; Vaandrager *et al.*, 1997) Activated PKGII and PKA phosphorylation causes the activation of CFTR, inducing release of Cl^- and HCO_3^- into the intestinal lumen of the host (Chao *et al.*,1994; Vaandrager *et al.*, 1997; Vaandrager *et al.*, 1998; Wang *et al.*, 2019). Protein kinase A also phosphorylates the sodium/hydrogen exchanger 3 (NHE3) that inhibits Na^+ reabsorption (Ostedgaard *et al.*, 2001; Wang *et al.*, 2019) (**Figure I6**).

Table I3: *Different enterotoxins secreted by ETEC.*

Enterotoxins	Variants	Encoding Gene	Location of Genes	Host Specificity	Receptor
Heat-labile enterotoxin (LT)	LTIh	eltAB	plasmid	humans	GM1a
	LTIp	eltAB	plasmid	piglets	GM1a
	LTIIa	eltAB	chromosome, prophages	water-buffalo, humans	GD1b
	LTIIb	eltAB	chromosome, prophages	unknown	GD1a
	LTIIc	eltAB	chromosome, prophages	humans, calves	GM1a
Heat-stable enterotoxin (STa)	STp	estA1, estA5, estA6	plasmids	piglets, calves, humans	GC-C
	STh	estA2, estA3/4, estA7	plasmids	humans	GC-C
Heat-stable enterotoxin (STb)	STb	estB	plasmids	post-weaning pigs	sulfatide
	STb_{H12N}	$estB_{C34A}$	plasmids	post-weaning pigs	sulfatide

GM = Monosialotetrahexosylganglioside; GD = Disialoganglioside; GC-C = guanylate cyclase.

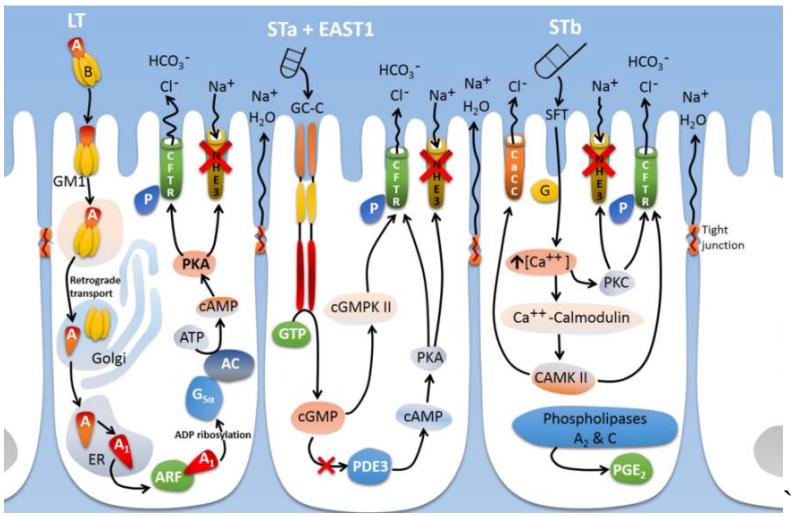


Figure I6: *Mode of action of the ETEC enterotoxins (Wang et al., 2019).*

STb interacts with the sulfatide on the intestinal epithelial cell surface of porcine jejunum which activates pertussis toxin-sensitive GTP-binding regulatory protein (Gαi3). The activated Gαi3 stimulates Ca^{2+} ion influx through the Ca^{2+} gated channels. The increase in Ca^{2+} ions in the intracellular space results in activation of calmodulin-dependent protein

kinase II (CaMKII) as well as activation of CFTR resulting in fluid accumulation in the host intestine (**Figure I6**).

b) Colonization factors

Colonization of the host is a crucial step in ETEC pathogenesis and the onset of infections (Madhavan *et al.,* 2015). The major aspect of ETEC virulence lies in the attachment of bacteria to the enterocytes of the small intestine followed by expression of the enterotoxins (Vidal *et al.,* 2019). These enterotoxins decrease absorption by the villus tip and elicit secretion of both water and electrolytes by the crypt cells (Levine *et al.,* 1987; Vidal *et al.,* 2019). ETEC colonization is mediated by one or more proteinaceous fimbrial or fibrillar colonization factors (CF), which are designated by CFA (colonization factor antigen), CS (coli surface antigen) which anchor the bacteria to intestinal mucosal receptors. Based on antigenic and genetic relationships, there are 30 antigenically distinct CFs that are associated with human disease (Gaastra and Svennerholm, 1996; Vidal *et al.,* 2019). The sequences of the structural and assembly proteins of several of these pili are closely related. CFA/I, CFA/II and CFA/IV are the three major families of CFAs which promote diarrhea in humans. The first family of CFA contains only CFA/I. CFA/II strains encode coli surface (CS) antigen 3 (CS3) alone or a combination of CS1 or CS2 (Levine *et al.,* 1987), while CFA/IV strains encode CS6 alone or in combination with CS4 or CS5 (Levine *et al.,* 1987). CFA/I, CS1, CS2, CS4 and CS5 are rigid fimbriae of about ~6–7 nm in diameter, the diameter of CS3 is 2-3 nm in diameter and are thin flexible fibrillae (Levine *et al.,* 1984). A large number of other, less common adhesins have also been described in ETEC, yet epidemiological studies suggest that together CFA/I is expressed by approximately 30% of human ETEC worldwide. Combination of CFs and toxins that are commonly found in humans ETEC isolates of temporal and other geographic locations are CS2 + CS3(±CS21) LT + STh, CS1 + CS3(±CS21) LT + STh, CS6 STp, CS5 + CS6 LT + STh, CFA/I(±CS21) STh and CS7 LT (von Mentzer *et al.,* 2017). CFA/I, CS1-CS6 and CS21 are prevalently found among the ETEC isolates while other CFs such as CS7, CS14 and CS17 are prevalent in specific geographic areas (von Mentzer *et al.,* 2017).

i. Nomenclature of CFs

The nomenclature of CFs of human ETEC is rather confusing. Terms like colonization factor antigen (CFA), coli surface antigen (CS) and putative colonization factor (PCF) are used to

describe different ETEC CFs. Historically, the term CFA was used for the first CF (CFA/1) for which it was demonstrated that in its absence the same ETEC strain caused no disease in a volunteer. The second CFA described in the literature, CFA/ID had similar properties to CFA/I but was clearly misnamed, as it was later shown that CFA/II consisted of three antigenically distinct components. At that time the part played by each component in colonization was not known and the different subcomponents were not called colonization factors but were coli surface antigens (CS1, CS2 and CS3). The same was true for CFA/IV, which consists of CS4, CS5 and CS6 (Turner *et al.,* 2006). Subsequently, the term PCF was used for factors that had similar properties to CFA/I but had not been tested in animal or human models for their role as colonization factors. Furthermore, some CFs was named after the serogroup or serotype or simply after the strain in which they were first identified. For the CFs of human ETEC was proposed the designation CS was followed by a number, which indicates the order in time they were discovered (Gaastra and Svennerholm, 1996). This would probably be the most logical system and cause the least confusion. Gaastra and Svennerholm proposed the use of the genetic designation followed by the letter A for genes encoding the major subunit protein, followed by C for genes encoding the molecular usher, followed by D for chaperone-encoding genes and followed by G for genes encoding the adhesin protein. Genes encoding regulatory proteins should be followed by R V (Gaastra and Svennerholm, 1996).

ii. Identification and classification of CFs

In the beginning, the capacity of ETEC to exhibit haemagglutination was used as a simple, readily available system to identify and discriminate between potential CF. The specific methods like slide agglutination, immunodiffusion, immunoblotting and enzyme-linked immunosorbent assays (ELISA) were developed (McConnell *et al*., 1985). Recently, monoclonal antibodies (mAbs) against most i.e., 12 of the known CFs, have been produced. These mAbs are highly specific in identifying the -CF in slide agglutination, dot-blot or ELISA inhibition tests. Probes and PCR methods (Gaastra and Svennerholm, 1996) remain a major useful tool in epidemiological studies of CFs or in the screening of large numbers of ETEC from clinical samples (Puiprom *et al.,* 2010).

iii. CF receptors

There has been a great deal of interest regarding the exact nature of the receptors for CAs. The molecules to which ETEC fimbriae bind are glycol-conjugates including mannosides, N-

acetylgalactosamine, and N-acetyl-D-glucosamine (Moseley *et al*., 1986). Tip-localised adhesions are present in most of ETEC isolates which bind to carbohydrate receptors present in the host and result in colonisation. A number of glyco-sphingolipid receptors are present in both humans (Jansson *et al.,* 2006; Jansson *et al.,* 2009) and pigs (Teneberg *et al.,* 1990; Von Mentzer *et al.,* 2020). One of the commonly found CFs, CFA/1 mediates colonization of the host by binding to various glycosphingolipids such as lactosylceramide with phytosphingosine and/or hydroxy fatty acids, glucosylceramide, gangliotriaosylceramide , the Lea-5 glycosphingolipid, neolactotetraosylceramide, gangliotetraosylceramide, the Ley-6 glycosphingolipid, the Lex-5 glycosphingolipid, and the H5 type 2 pentaglycosylceramide (Jansson *et al.,* 2006; Von Mentzer *et al.,* 2020). The non-fimbrial adhesin coli surface antigen 6 (CS6) binds to sulfatide receptors present on the host (Jansson *et al.,* 2009). The glycol-conjugates on eukaryotic cell membranes exhibit an enormous diversity in oligosaccharide sequences. The oligosaccharides are not distributed uniformly among species, tissues and cells, hence the preference of pathogens for specific host tissues and the relative specificity of haemagglutination. Most CFs agglutinate erythrocytes from one or more species and several mono or oligosaccharides may inhibit haemagglutination.

iv. Genetics involving CFs

Most CF operons contain eight to eleven genes on a continuous stretch of DNA, which encode proteins involved in the regulation of expression, the structural subunit or major sub-unit protein, two assembly proteins (a periplasmic chaperone and an outer-membrane molecular usher) and proteins that specify the adhesive properties of the CF (the minor subunits). Though the genes for CFs of ETEC are located on plasmids but the interplay between chromosomal and plasmid genes is important for successful virulence and transmission of the ETEC strains. CFA/I, the most common CFs of ETEC has a fimbrial structure that comprises of repetitive units of CfaB and CfaE as a single tip subunit (Jansson *et al.,* 2006). The genes required for the production and assembly of CFA/1 fimbriae are located on a plasmid of approximately 60 MDa in a *cfaABCE* operon (Gaastra and de Graaf, 1982). Two regions, CFA/1 region 1 and CFA/1 region 2 on this plasmid are important for fimbrial expression (Jordi *et al.,* 1992). Region 1 encodes the structural genes while region 2 contains a positive regulator. The genes *cfa A, cfa B, cfa C, cfa D* and *cfa E* are located in region 1 (Hamers *et al.,* 1989). The upstream of *cfa B* contains the gene *cfa*A while the *cfa C* is present at the downstream of *cfa B* (Hamers *et al.,* 1989). *Cfa A* is required for exporting

the pilus across the periplasmic membrane while *cfaB* helps in binding to the different glycosphingolipids, such as asialo-GM1 (Jansson *et al.,* 2006) and Lewis A (Le^A) glycans present in young children and adults containing non-functional alleles of *FUT2* (Mottram *et al.,* 2017; Mottram *et al.,* 2018). Region 2, separated from region 1 by approximately 40 kb, codes for a positive regulator called CfaR protein that acts on CFA/I promoter (Jordi *et al.,* 1992). The expression of the CFA/I-like operons is temperature regulated, but this temperature- dependent regulation is absent in *E. coli* mutated in the histone-like protein H-NS. Furthermore, the positive regulator, CfaR, is not needed in H-NS-negative mutants, suggesting that H-NS acts as a repressor of the CFA/I operon (Jordi *et al.,* 1992). Reports have shown that CFA/III is also encoded by a 55kb plasmid (Taniguchi *et al.,* 2001).

3.9.6. Diagnosis and vaccine development

Recent field studies have indicated that it is feasible to develope vaccines against ETEC. WHO the Public health stakeholders have encouraged as well as supported the development of the ETEC vaccine over the past 20 years (Khalil *et al.,* 2021). At this point, ETVAX is a recent cellular ETEC vaccine at Phase 2b trial administered in Finnish travelers to Benin indicating a reduction in the use of antibiotics among vaccinated subjects when compared to placebo recipients (Lundgren *et al.,* 2014). The vaccines target at inducing immune responses against one or both ETEC toxins and the CFs which are important in protective immunity. As ETEC comprises of a diverse number of CFs/CS the vaccines are developed against most of those CF/CS antigens and toxin components that are epidemiologically prevalent (Bourgeois *et al.,* 2016; Barry *et al.,* 2019). A vaccine formulated to include four to five of these factors would cover for 70–80% of the most common strains associated with illness in infants and international travelers (Isidean *et al.,* 2011; von Mentzer *et al.,* 2017; Khalil *et al.,* 2021). This CFs/CS in some of the candidates cross-reacts with the other related CF/Cs antigens thus increasing the coverage of protection. There are other 2 cellular approaches for the development of ETEC vaccines such as the development of attenuated, hybrid *Shigella* vaccine vectors expressing the antigens of ETEC (Barry *et al.,* 2019, Harutyunyan *et al.,* 2020). Subunit candidates also focus on the induction of anti-CF/CS and anti-toxin immunity by the parenteral route of administration. The most advanced subunit vaccine is a fusion of representative adhesin-pilin subunits namely class 5a (CfaEB), class 5b (CsbDA-CooA), class 5c (CotDA), and CS6 (CssBA). It is anticipated that these components,

along with an LT-based adjuvant, like dmLT, could provide over 75% coverage against circulating ETEC strains (Isidean *et al.,* 2011; Khalil *et al.,* 2021).

3.9.7. Treatment

The treatment for diarrhea caused due to ETEC is the same as that cholera or any other diarrheal disease caused by other organisms. Proper hydration is very important and administration of antibiotics is required in severe cases.

a) Rehydration

Patients with severe diarrhea should be treated with intravenous fluids such as Ringer's lactate in order to restore blood pressure and other major symptoms of dehydration. After the initial treatment to restore the main signs of dehydration, the patients can be treated with oral rehydration solutions for the rest of the treatment period. Less degree of dehydration can be treated with rehydration solutions only until the diarrhea stops.

b) Antimicrobials

The rise and spread of resistance to several different antibiotics has been designated as a global burden in medical sciences. Extensive use of antibiotics and poor prescription practices are the major causes of drug resistance in developing countries (Qadri *et al.,* 2005; Chellapandi *et al.,* 2017). Thus the antimicrobials used for treating traveler's diarrhea have changed over time due to the rise in antibiotic resistance. Antimicrobials that have been used in effective treatment include trimethoprim-sulfamethoxazole, doxycycline, norfloxacin, ofloxacin, ciprofloxacin, erythromycin, azithromycin, and rifamycin (Qadri *et al.,* 2005; Ericsson *et al.,* 2013).

3.10. Enteropathogenic *E. coli* (EPEC)

Of the diarrheagenic *E. coli*, EPEC was the first pathotype to be identified. The term "EPEC" was first utilized in 1955 (Neter *et al.,* 1955) for describing a number of *E. coli* strains epidemiologically related to a series of outbreaks of infantile diarrhea in the 1940s and 1950s (Bray *et al.,* 1945). These *E. coli* outbreaks were regarded as "summer diarrhea" that were reported to cause frequent outbreaks in developing countries (Levine *et al.,* 1984; Robins-Browne RM, 1987; Deborah *et al.,* 2005). EPEC remains as the major cause of infant mortality in developing countries. Recent studies in Mexico, Brazil, Bangladesh and South

Africa have shown 30-40% of infant diarrheal episodes are due to EPEC infections (Deborah *et al.,* 2005).

3.10.1. Historical background

EPEC was the first pathotype of *E. coli* to be described in the 1950s. The identification of *E. coli* as the causative factor of summer diarrhea in infants during 1940-1950 was at first hampered as the organism is also a component of the normal intestinal flora. After performing several bacteriological examinations little or no difference between patients with gastroenteritis and healthy individuals was seen. The distinction between strains causing the disease and those that were part of the normal flora of the human gut was only possible after the rising of antibodies against an *E. coli* strain isolated during an outbreak from a child with severe protracted diarrhea, who later died. Although the intestinal pathogenicity of *E. coli* had already been described in 1889 and 1897, Bray's work (1945) helped to describe a group of *E. coli* strains that were serologically distinct (Bray *et al.,* 1945). Kauffman's serotyping scheme allowed systemic characterization of the *E. coli* strains (Kauffman, 1947). A study of Bray's strains showed that they belonged to serogroup O55, while later analysis of stored samples of stool indicated that strains of serogroup O111 were similarly associated with hospital outbreaks of infantile diarrhea. The virulence of *E. coli* was confirmed in healthy adult volunteers (Feeguson and June, 1952).

The term "enteropathogenic" was used to describe *E. coli* strains incriminated in infantile diarrhea and was coined by Neter *et al*. (1955). The main basis of the classification of EPEC lies in the O (somatic), H (flagellar) and K (capsular) antigens. The serogroups O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 were recommended to be considered as EPEC serogroups by the World Health Organization in 1987(Nataro and Kaper, 1998; Campos *et al.,* 2004; Mare *et al.,* 2021). Recent studies have included serogroups such as O39, O88, O103, O145, O157, and O158 in EPEC pathotype (Mare *et al.,* 2021). Among the flagellar antigens H2 and H6 are the most common followed by certain less common H types such as H7, H8, H9, H12, H21, H27, H25, and H34 (Nataro and Kaper, 1998; Trabulsi *et al.,* 2002; Croxen *et al.,* 2013). Strains that are H negative are also considered as EPEC (Mare *et al.,* 2021). Because of the high diversity of these antigens, serotyping is no longer considered a rapid diagnostic tool. As the understanding of EPEC pathogenicity improved it become clear that EPEC strains exhibit specific virulence characteristics (Sánchez *et al.,* 2015; Joensen *et al.,* 2015).

3.10.2. Epidemiology

EPEC remains the major cause of infantile diarrhea in both community settings as well as hospitals in developing countries including India (Snehaa *et al.*, 2021; **Figure I7**). EPEC infection is primarily a disease of infants younger than 2 years (Nataro *et al.*, 1998; Zhou *et al*., 2021). Most of the infantile diarrheal cases go under-diagnosed due to the lack of improved tools for diagnosis (Snehaa *et al.*, 2021).

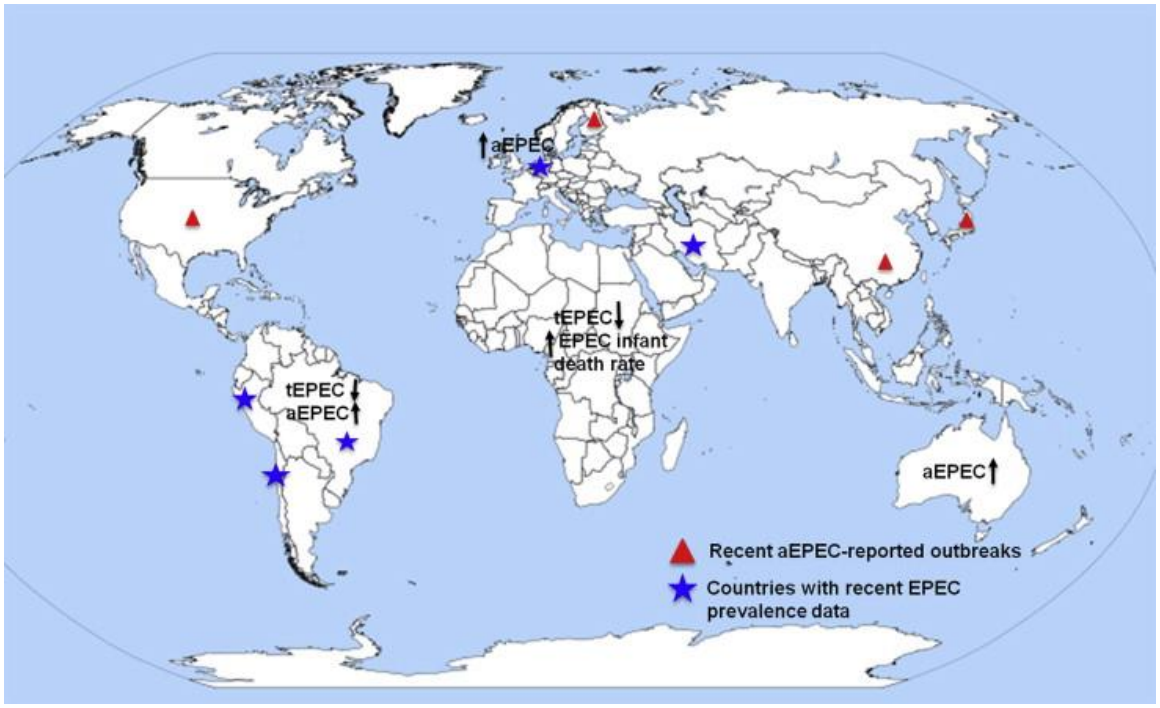


Figure I7: *Distribution of recent worldwide prevalence of Enteropathogenic Escherichia coli (EPEC). The arrows represent the increase or decrease of typical EPEC (tEPEC) and atypical EPEC (aEPEC) incidence per geographical region. The blue stars depict those countries with increased EPEC prevalence reported in recent years. The red triangles represent countries with recently reported aEPEC outbreaks (Snehaa et al., 2021).*

Levine and Edelman (Dupont *et al.*, 2016) reviewed numerous case-control studies in many countries that have shown a strong correlation of isolation of EPEC from infants with diarrhea compared to healthy infants. The correlation is strongest with infants younger than 6 months. Based on recent GEMS data, tEPEC was significantly associated with moderate to severe diarrhea in children under 2 years of age in Kenya, whereas aEPEC was not associated with this type of diarrhea at any of the GEMS sites (Kotloff *et al.*, 2013). Overall, tEPEC was not strongly associated with cases of moderate to severe diarrhea, but when present, it was associated with an increased risk of death in patients aged 0 to 11 months (Kotloff *et al.*, 2013). Recent estimates from the Centers for Disease Control and Prevention (CDC) on food-

related illness in the United States listed only 4 hospitalizations as a result of EPEC infection (CDC reports, 2013); however, this pathogen continues to persist in other parts of the world and continues to be regarded as a serious threat to children under the age of 2.

3.10.3. Clinical features

Clinical features of EPEC infections include watery diarrhea accompanied by mild fever and vomiting. In case of severe EPEC infections, vomiting may hamper the oral hydration therapy making it life threatening in some of the cases. Further, the disease caused by EPEC can last for a longer time resulting in malnutrition and sometimes death in severe cases. Thus EPEC infections can be sometimes life-threatening. Watery diarrhea, nausea, abdominal pain, vomiting, and fever are the major symptoms of EPEC infections. In addition to humans, EPEC infections are also documented in animals such as cattle, dogs, cats, and rabbits (Singh *et al*., 2015). The infectious dose of EPEC in healthy adults has been estimated to be 10^8 organisms (Croxen *et al*., 2013). EPEC-induced diarrhea is self-limiting in most of the cases and can be effectively treated by rehydration therapy (Yang *et al.*, 2017). Antibiotics should be used if the infection persists (Yang *et al.*, 2017).

3.10.4. Transmission and reservoir

Like other *E. coli* transmission of EPEC is fecal-oral, with contaminated hands, contaminated weaning foods or formula, or contaminated objects serving as vehicles. Unless strict decontamination procedures are followed, admission of an infant to a pediatric ward can result in contamination of crib linen, toys, tabletops, hand towels, scales, carriages, rubber nipples, etc. In one study, EPEC was isolated from dust and aerosols, suggesting potential airborne transmission, either directly through inhalation followed by ingestion or indirectly via contamination of other fomites (Nataro *et al.*, 1998).

3.10.5. Pathogenesis

The pathogenesis of EPEC infection has been proposed to occur in four distinct stages (Donnenberg and Kaper, 1992) (**Figure I8**). The formation of attaching and effacing lesions is the hallmark of EPEC infections which is characterized by bacterial attachment to the epithelial cells and microvilli effacement at the site of attachment. EPEC pathogenesis is common to both typical and atypical EPEC strains.

a) Expression of *bfpA* and *espA* by the bacteria

The first stage of EPEC pathogenesis is the expression of *bfpA* and *espA* by the bacteria which helps in attachment to the enterocytes (Nataro *et al.,* 1998). The expression of *bfp* and e*spA* is both plasmid and chromosomal genes dependent (Nataro *et al.,* 1998; Mare *et al.,* 2021). In the 2nd stage, EPEC cells adhere to the epithelial cell by using bfp and EspA filaments and result in the formation of dense microcolonies on the cell surface. After the attachment, the type III secretion system injects the translocated intimin receptor (Tr) and other effector molecules which facilitate bacterial colonization, immune evasion, and regulation of inflammatory response resulting in host cell death. Tir results in the formation of actin pedestral like structures which extend from the epithelial surface to the lumen. Various cell signaling pathways are activated by these effecter molecules causing cytoskeleton alterations and microvilli loss (Donnenberg *et al.,* 1992; Vallance *et al.,* 2000; Clarke *et al.,* 2003; Scholz *et al.,* 2015; Pearson *et al.,* 2013; Ugalde-Silva *et al.,* 2016; Shenoy *et al.,* 2018; Mare *et al.,* 2021). In the third stage, loss of the EspA filaments from the bacterial cell surface occurs followed by binding of *eae* encoded bacterial intimin to the modified Tir, ensuing intimate bacterial attachment to the host cells (Vallance *et al.,* 2000; Clarke *et al.,* 2003; Mare *et al.,* 2021). In this phase secretion of EspC protein via type V secretion system (T5SS) (Guignot *et al.,* 2015; Serapio-Palacios *et al.,* 2016; Sanchez-Villamil *et al.,* 2019) mediates epithelial cell cytotoxicity as well as bacterial biofilm formation and replication (Navarro-Garcia *et al.,* 2014; Serapio-Palacios *et al.,* 2016; Govindarajan *et al.,* 2020). The fourth step is marked by a massive accumulation of cytoskeletal elements at the point of bacterial attachment leading to pedestal structure formation which is the characteristic feature of EPEC infection (Clarke *et al.,* 2003; Kaper *et al.,* 2004; Croxen *et al.,* 2013). The translocated effecter molecules disrupt host cell processes, causing loss of tight-junction integrity and mitochondrial function, leading to both electrolyte loss and eventual cell death (Mare *et al.,* 2021).

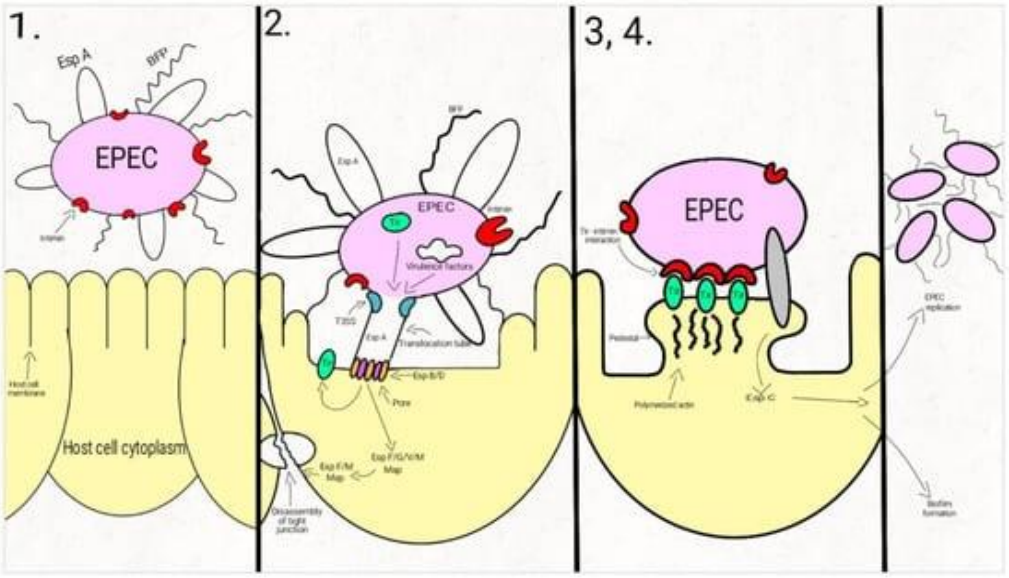


Figure I8: *This represents the mechanism of EPEC adherence (Clarke et al., 2003; Mare et al., 2021).*

b) Adherence pattern of EPEC

tEPEC initially attaches to the epithelial surface with the help of bundle forming pilus (BFP). BFP mediates tethering of bacteria to one another resulting in the formation of localized adherence (LA) pattern which is visualized as three-dimensional micro colonies on HeLa/HEp-2 cell lines within 3 hours of infection (**Figure I9**). The LA pattern of adherence is found in strains containing large EAF plasmids such as pMAR2 and pB171. Both these plasmids consist of *bfp* operon as well as another operon named *perABC* which is also associated with bacterial virulence. *bfp* and *per* loci show 99% sequence similarity. Plasmid encoded regular PerABC governs the expression of *bfp* and *perABC* as well as LEE encoded global regulator Ler thus linking the expression of *bfp* to LEE expression in tEPEC strains. aEPEC donor harbor pEAF and thus do not contain BFP. Due to the lack of bfp in aEPEC, loose clusters of bacteria are formed on the cell surface. This pattern of adherence is known as "localized adherence-like" (LAL) which takes more than 6hrs to establish. Apart from LAL which is the most common pattern of adherence in aEPEC strains; alternate adherence patterns such as aggregative adherence (AA) and diffuse adherence (DA) are also seen in some strains. Lymphocyte inhibitory factor (LifA) is another surface protein known which mediates both epithelial cell adherence *in vitro* (as well as intestinal colonization of mice by the related A/E pathogen *C. rodentium* (Klapproth *et al.*, 2005). *lifA* gene is more common among tEPEC than aEPEC strains; however, aEPEC strains harboring *lifA* gene are seen to

cause diarrhea in children less than 5 years (Ochoa *et al.*, 2011). Another adherence factor called *E. coli* common pilus (ECP), commonly found in commensal and pathogenic *E. coli* has been found to play an accessory role in adherence and bacterial interactions in EPEC. While BFP has been shown to be the predominant factor required for initial attachment of tEPEC, less efficient attachment is done by EspA filaments and could mediate adherence of strains lacking BFP (Cepeda-Molero *et al.*, 2017).

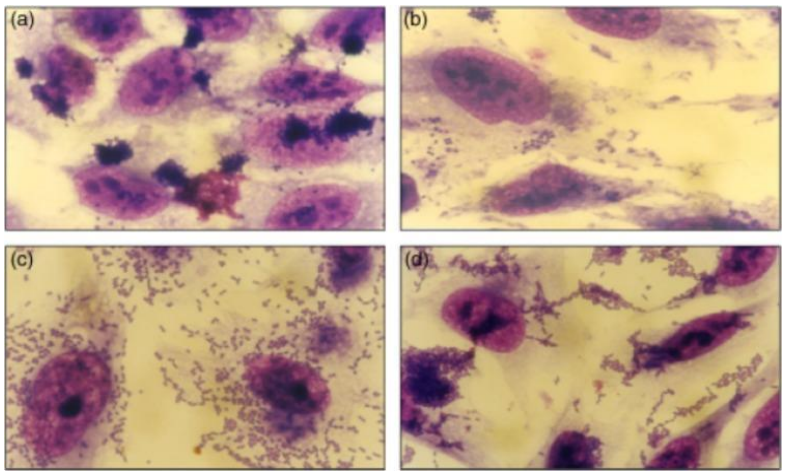


Figure I9: *Adherence patterns of enteropathogenic E. coli (EPEC) on tissue culture cells. (a) Localized adherence (LA); (b) localized adherence like (LAL); (c) diffuse adherence (DA); (d) aggregative adherence (AA). (Source: google images)*

c) Characteristic attaching and effacing phenotype of EPEC and the signaling pathways involved in EPEC infections

Attaching and effacing (A/E) lesions are treated as the hallmark of EPEC infection (Cepeda-Molero *et al.*, 2017). A/E lesion is characterized by localized destruction of brush border microvilli and intimate bacterial adherence to the membrane of enterocyte in the form of cuplike pedestal structures followed by the formation of dense plaque of action and other cytoskeletal filaments underneath the adhered bacteria (Lai *et al.*, 2013).

The epithelial membrane below the adherent bacteria is raised in the form of pedestral structure which extends up to 10μm outside the cell in the form of pseudopod-like structures. These pedestral structures formed by EPEC are dynamic in nature and can bend as well as undulate and become longer or shorter while remaining attached to the cell surface (Lai *et al.*, 2013). Intimate attachment of EPEC to intestinal cells induces diverse signal transduction pathways within the host, which helps the bacteria to bypass many cellular processes that

have an adverse effect on the bacteria (Cepeda-Molero *et al.,* 2017). The mediators of A/E lesions and initialization of cellular signaling pathways are the genes situated on the pathogenicity island named locus of enterocyte effacement (LEE). LEE encodes the adhesin intimin, a type III secretion system (T3SS) comprising the translocator proteins such as EspA, EspB and EspD, the six effectors (EspF, EspG, EspH, Map and EspZ) (Gaytán *et al.,* 2016), including the essential translocated intimin receptor (Tir) (Frankel *et al.,* 2008; Cepeda-Molero *et al.,* 2017). Apart from these seventeen additional effectors are encoded by genes that are not located in LEE. Various studies have shown that strains that lack the non-LEE effectors have a marginal capacity to produce these A/E lesions (Santos *et al.,* 2015). Certain proteins such as Efa1/LifA help in A/E lesion formation. The interaction of intimin with its receptor Tir causes actin polymerization leading to pedestral structure formation. Thus the interaction between intimin and Tir plays a vital role in EPEC infection (Frankel *et al.,* 2008).

The activity of NF-κB via tumor necrosis factor alpha (TNF-α) receptor-associated factors has been inhibited by Tir. The other six LEE-encoded effectors such as Map, EspF, EspG, EspZ, EspH, and EspB play significant roles in A/E pathogen infection. Map activates the formation of membrane filopodia and mitochondrial dysfunction along with disruption of the epithelial barrier. EspF and EspG have been reported to affect aquaporin localization, leading to diarrhea (Guttman *et al.,* 2006). Like Map, EspF localizes to mitochondria (Nougayrède *et al.,* 2004) and disrupts tight junctions (Guttman *et al.,* 2006), while EspG interacts with tubulin causing alteration of host cytoskeletal components (Hardwidge *et al.,* 2005). EspZ stimulates the survival of host cells (Mare *et al.,* 2021), EspH affects filopodium formation, engages in actin signaling during the formation of pedestal structures (Mare *et al.,* 2021), and acts as a RhoGEF inhibitor (Wong *et al.,* 2012). Both EspH and EspB can inhibit phagocytosis of EPEC by macrophages (Dong *et al.,* 2010). There is considerable variation in the number and type of Nle-encoded effectors among A/E pathogens. NleB, NleC, NleD, NleE, and NleH have been reported to inhibit NF-κB activation by various different mechanisms (Gao *et al.,* 2013; Yen *et al.,* 2010; Nadler *et al.,* 2010; Newton *et al.,* 2010; Sham *et al.,* 2011). In addition to immune-modulatory functions, Nle effectors such as EspJ exhibit anti-phagocytic properties (Marchès *et al.,* 2008), while NleA alters host protein secretion (Kim *et al.,* 2007) and tight junction integrity (Thanabalasuriar *et al.,* 2010) and inhibits vesicle trafficking (Thanabalasuriar *et al.,* 2012). NleH is capable of modulating apoptotic response (Hemrajani *et al.,* 2010).

In addition to T3S effectors, some EPEC strains also encode the type V-secreted virulence-associated protein EspC. EspC is a Per-activated serine protease autotransporter that acts as an enterotoxin, causing cytopathic effects on tissue culture cells. EspC confers enhanced lysozyme resistance to EPEC (Salinger *et al.,* 2009), and purified EspC promotes the degradation of hemoglobin (Elisa *et al.,* 2006) and other proteins such as pepsin, factor V, and spectrin (Dutta *et al.,* 2002). Additionally, oligomerization of EspC gives rise to rope-like structures that serve as a substratum for adherence and biofilm formation as well as to protect bacteria from antimicrobial compounds and thus play a significant role in bacterial survival (Mare *et al.,* 2021).

d) Mechanism of diarrhea induced by EPEC

The microvilli effacement at the bacterial attachment site causes a decrease in absorptive surfaces, thus contributing to diarrhea (Mare *et al.,* 2021). A number of T3S effectors are known to have an impact on the different water and ion channels of the intestinal epithelia and are considered to be involved in EPEC-induced diarrhea but the exact contribution of each mechanism is unknown. The fluid uptake transporter from the intestine called sodium–d-glucose transporter (SGLT1) is inhibited by Tir, Map, EspF, and EspH (Dean *et al.*,2006; Dean *et al.,* 2009), while EspF and EspG cause alteration in localization of aquaporins, which also play a role in water transport (Guttman *et al.,* 2007). EspG and EspG2 disrupt the chloride transport across the apical membrane resulting in decreased Cl^-/OH^- exchange activity (Gill *et al.,* 2007). Additionally, disruption of tight junctions by EspF (Viswanathan *et al.,* 2004), EspG (Matsuzawa *et al.,* 2005), and Map (Dean *et al.*,2004) leads to increased intestinal permeability, which could also contribute to EPEC-induced diarrhea.

3.10.6. Typical and atypical EPEC

It is a matter of debate whether EPEC strains that lack the EAF plasmid encode the BFP adhesion and Per regulators are true pathogens or not. Although the EAF plasmid does not play a vital role in the formation of A/E lesions, its presence enhances the efficiency of its formation, probably through the help of a cluster of plasmid-borne regulatory genes (per A, B, C) which results in increasing the chromosomal LEE genes expression (Nataro and Kaper, 1998). In 1995, during the Second International Symposium on EPEC in Sao Paulo, EPEC was defined as DEC result in the formation of the characteristic pathological condition known as attaching and effacing (AE) on the intestinal cell surface and that does not produce

Shiga, Shiga-like, or verocytotoxins. Recently EPEC have been subdivided into 2 types based on the presence of EPEC adherence factor plasmid (pEAF) namely typical and atypical EPEC (Trabulsi *et al.,* 2002). The two important genes encoded by the plasmid are *bfp* and *per* where *bfp* is involved in encoding type IV bundle forming pilus that helps in bacterial microcolony formation (Bieber *et al.,* 1998). The *per* operon is involved in encoding transcriptional activator known as plasmid-encoded regulator of the Locus for Enterocyte Effacement (LEE) pathogenicity island (Gómez-Duarte *et al.,*1995; Frankel *et al.,*1998). Typical EPEC of human origin possess a virulence plasmid known as the EAF (EMBC adherence factor) plasmid that ensures localized adherence on cultured epithelial cells mediated by the *bfp*, while atypical EPEC do not harbor this plasmid. The typical EPEC strains are more homogenous than those of the aEPEC strains. The virulence factors produced by EPEC are encoded by the LEE region and EAF plasmid (Trabulsi *et al.,* 2002). Atypical EPEC might possess enteroaggregative heat-stable toxin (EAST1) and other potential virulence factors such as hemolysis which is not encoded in the LEE (Trabulsi *et al.,* 2002).

Typical EPEC that is known to cause infantile diarrhea is more prevalent in developing countries than that of industrialized countries (Trabulsi *et al.,* 2002). In the case of typical EPEC, human is the only reservoir while in the case of atypical EPEC it is found in both human and animal reservoirs. Atypical EPEC are observed to be closely related to Shiga toxin producing *E. coli* (STEC) (Trabulsi *et al.,* 2002).

The majority of typical EPEC strains fall into certain well-recognized O: H serotypes (Trabulsi *et al.,* 2002). As shown in the table below, the typical and atypical EPEC strains belong to two different sets of serotypes.

Table I4: *Frequently isolated EPEC serotypes, including both typical and atypical EPEC strains.*

Strains	Serotypes
Typical EPEC	O55:H6, O86:H34, O111:[H2],O114:H2, O119:[H6], O127:H6, O142:H6, O142:H34
Atypical EPEC	O26:H[11], O55:H[7], O55:H34, O86:H8, O111ac:H[8], O111:H9, O111:H25, O119:H2, O125ac:H6, O128:H2

3.10.7. Treatment

Most of the EPEC infections are self-limiting and the patients recover from diarrheal conditions by taking rest and without any other treatment but sometimes patients need oral rehydration therapy so that they do not lose too much water from their bodies (Denham *et al.,* 2018). Patients with a severe form of EPEC-induced diarrhea are given antimicrobials such as ciprofloxacin, azithromycin and in some cases loperamide to treat residual diarrhea (Denham *et al.,* 2018).

3.11. Enteroinvasive *E. coli (EIEC)*

Enteroinvasive *Escherichia coli* (EIEC) causes intestinal illness indistinguishable from that caused by *Shigella*, mainly in developing countries. EIEC are responsible for shigellosis in humans which is characterized by the destruction of the colonic epithelium provoked by the inflammatory response that is induced upon invasion of the mucosa by bacteria (Michelacci *et al*., 2016). EIEC are strains that possess some of the biochemical properties of *E. coli* and are capable of causing bacillary dysentery using the same invasive method found in *Shigella.* Sequencing results of multiple housekeeping genes prove that EIEC is more closely related to *Shigella* than to any other non-invasive *E. coli*. The characteristic features of EIEC are due to the presence of a large virulence plasmid of approximately 220 kb in size which helps in invasion of the bacteria to the human mucosa. After penetrating the colonic epithelium, EIEC replicates within the cells and spreads to the adjacent cells resulting in the destruction of epithelial barrier of the intestines (Harris *et al.,* 1982; Taylor *et al.,* 1988; Pasqua *et al.,* 2017).

3.11.1. Epidemiology

EIEC has been commonly reported in countries of Asia and Latin America such as India (Chellapandi *et al.,* 2017), Bejing (Kain *et al.,* 1991), Chile (Faundez *et al.,* 1988) and Thailand (Taylor *et al.,* 1986; Taylor *et al.,* 1988). In Europe and the United States, EIEC is prevalent among travelers and migrant workers returning from high incidence countries (Niyogi *et al.,* 2005; Michelacci *et al.,* 2016). Sporadic cases of EIEC infections are seen but EIEC has also been reported to cause major outbreaks in United States and Europe in 1971 and 2012 respectively (Newitt *et al.,* 2016; Herzig *et al.,* 2019). In 2012, a severe food-born EIEC outbreak was documented in Italy where 32 individuals were hospitalized out of 109 affected cases (Escher *et al.,* 2014). In 2014, 2 severe EIEC outbreaks were reported in

Nottingham city of the United Kingdom within 2 days interval (Newitt *et al.,* 2014; Lagerqvist *et al.,* 2020). A recent outbreak was reported in 2017 with 83 cases in a conference venue of Halland, Sweden (Michelacci *et al.,* 2016; Lagerqvist *et al.,* 2020). EIEC is widely distributed in Middle East, Europe, western Africa, Central and South America and southeastern Asia (Vieira *et al.,* 2007). It is evident from these outbreaks that EIEC has the capacity to cause major diarrheal outbreaks and hence should be considered as a potent diarrheal pathogen. EIEC has been reported in travelers returning from countries having a higher incidence of EIEC infections (Michelacci *et al.,* 2016). Contaminated food and water remain the major source of EIEC infections, however, sometimes certain secondary infections are also due to contaminations caused by human sources (Escher *et al.,* 2014). Apart from sporadic cases, EIEC has been involved in some major outbreaks. In the 1970s there was a huge EIEC outbreak caused by cheese contaminated with O124 *E. coli* strain. In 2012, an increase in a recent emerging EIEC strain was reported to cause a severe bloody diarrhea outbreak affecting more than 100 individuals in Europe (Escher *et al.,* 2014). The O96:H14 EIEC clone was isolated from cooked vegetables as well as asymptomatic food handlers working in the canteen thus supporting the hypothesis that secondary contamination of foods occurs due to improper handling of food by contaminated food handlers (Escher *et al.,* 2014). In 2014, there were 2 outbreaks in the US ione of which was caused by consumption of salad infected with O96:H19 (Newitt *et al.,* 2016). This serotype was further isolated from travelers' diarrhea occurring during the year 2013 in Spain.

3.11.2. Clinical features

EIEC belong to one of the diarrheagenic *E. coli* (DEC) pathotypes which cause bacillary dysentery quite similar to that of *Shigella* pathotypes in children as well as in adults. The EIEC strains resemble *Shigella* in their ability to invade the gut epithelium and formation of dysentery. The invasive process involves the entry of EIEC into the host followed by multiplication and intestinal spread causing cellular destruction and an inflammatory response similar to that found in *Shigella spp.*

Bacillary dysentery results in nausea, fever, abdominal cramps and bloody diarrhea with mucus. The disease caused by EIEC is quite similar to shigellosis where it causes colonic lesions accompanied with ulcers, mucosal and sub-mucosal edema, hemorrhage and infiltration due to polymorphonuclear leukocytes (PMNs).

3.11.3. Transmission and reservoir

Consumption of contaminated food and water along with poor sanitation remains the major cause of high incidence of EIEC infections in rural and low income regions. Since no animal reservoir has been identified, the EIEC infected humans remain the main source of infections. Travelers returning from high incidence countries also remain a vital source of the spread of EIEC infections.

3.11.4. Pathogenesis

The pathogenicity of EIEC is due to the presence of a large F-type invasion plasmid (pINV) which encodes the genes of the type III secretion system required for the invasion, survival and cell to cell dissemination of bacteria (Mohammadzaden *et al.,* 2015). The pINV plasmid which is 220kb large consists of a large conserved region of 31kb called the entry region encoding the two divergent gene clusters of type III secretion system (T3SS), namely *Mxi* (membrane excretion of Ipa) and *Spa* (surface presentation of invasion plasmid antigen). The 31kb entry region is called the *ipa-spa-mxi* region which encodes 50-60 genes that are associated with virulence such as *IpaB*, *IpaC*, and *IpaD* with their chaperons *IpgA*, *IpgE*, *IpgC* and *Spa15*, and also two transcriptional regulators *VirB* and *MxiE*, both of which are associated with activation of various virulence genes which are also present in the plasmid. T3SS plays an important role in bacterial entry into the host and is composed of several proteins (*IpaB*, *IpaC*, and *IpaD)* forming a needle shaped oligomer which helps in connecting the bacterial and host cytoplasm. Outside the entry region, the plasmid contains *virG* that encodes an outer membrane protein, VirG which is involved in bacterial movement within the infected cell cytoplasm, a transcriptional activator gene *virB* that controls the expression of *virB* and *icsA* and a serine protease *sepA* that belongs to the autotransporter family which is involved in inflammation of the intestines. The plasmid carries the gene called invasion associated locus (*ial*) that is responsible for cell penetration by EIEC. Invasion plasmid antigen H (*ipaH*) involved in the dissemination process of the bacteria in the epithelial cells is present in multiple copies on both pINV plasmid and chromosomes. Another plasmid encoded gene named *sen* is involved in the production of *Shigella enterotoxin 2* (*ShET*-2) that has a role in both toxicity as well as plays vital role in the transport of water and electrolytes in the intestine. Thus, the plasmid encoded genes are responsible for bacterial entry into the host cell epithelium. *Shigella* and EIEC require a combined expression of both pINV plasmid

as well as chromosomal genes for invasion and maintenance in the host (Mohammadzaden *et al.*, 2015; Pasqua *et al.*, 2017).

Apart from the entry region, a small islet contains the genes encoding IcsA, VirA and RnaG. There are other genes that encode proteins such as OspG, OspF, PhoN2 and ipaH required for the invasive process. OspG and OspF proteins interfere with the host immune system, thus enabling the bacteria to invade the host while PhoN2 protein is important for localization of IcsA. IpaH protein interferes with the host protein degradation. The primary transcriptional factor virF is located far away from the virulence genes such as *virB* and *icsA* which are under its direct control in a "deserted island" surrounded by several IS elements. The position of *virF* indicates that it has been acquired independently in order to govern the expression of other virulence genes. Along with the activation of virulence genes present on the plasmid, *virF* is involved in the expression of other chromosomal genes (**Figure I10**).

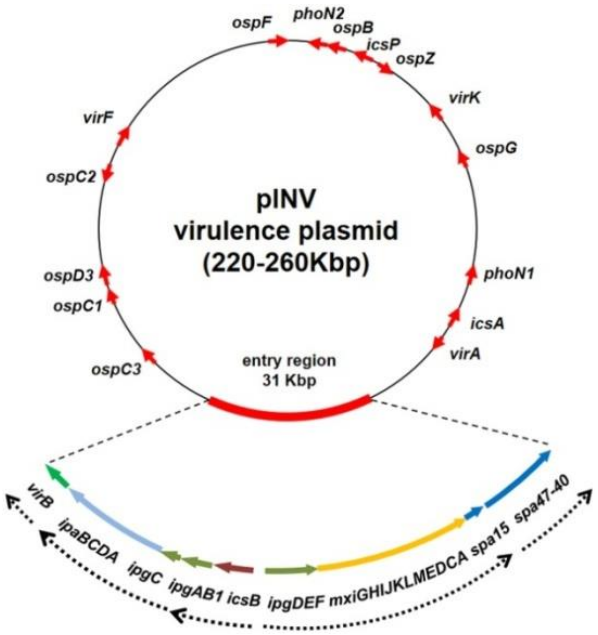


Figure I10: *The genetic map of the pINV plasmid present in enteroinvasive E. coli. The red arrows indicate major virulence determinants. Due to the variability in position and number, the ipaH genes are not shown. The genetic organization of the entry region is shown in detail, with dashed arrow lines indicating known transcriptional units (Pasqua et al., 2017).*

Outside the human host, the key regulators of the pINV are repressed by the H-NS repressors. Within the human intestines, the environmental conditions in the intestines result in an increase of the VirF which counteracts the H-NS repression at the promoter sites of *icsA* and *virB*. virB then activates several other invasive operons present at the entry site including the gene for *mxiE* and other genes scattered in the pINV plasmid except *icsA*. Finally, MxiE,

along with IpgC, activates the transcription of genes resulting in the encoding of the late effectors (Mohammadzaden *et al.,* 2015).

3.11.5. Invasive property

In order to enter the host epithelium, the bacteria first migrate from the lumen to the submucosa by entering the M-cells found in the Payer's patches. From the M-cells they are phagocytosed by macrophage which is accompanied by the release of T3SS effectors and PAMPs by NLRs resulting in pyroptosis with the release of proinflammatory cytokines such as IL-1β and IL-18. Within the epithelial cells, PRR detects the PAMPs and DAMPs of bacteria which in turn stimulate the defense signal pathways of the host involving MAPKs and NF-kB causing the release of IL-8 and TNF-α. The cytokines cause the recruitment of phagocytosis cells to the site of infection, thus clearing the bacteria. Thus, in order to establish infection bacteria must overcome the innate defense system of the host which is achieved by the release of T3SS effectors that inhibit the MAPK and NF-kB pathways thus decreasing inflammatory cytokines (Pasque *et al.,* 2017).

3.11.6. Treatment

The treatment is the same as that of other *E. coli* pathotypes where the patients are given rehydration therapy. In severe cases, antibiotics of the fluoroquinolone group are used to combat the severity of the situation.

3.12. Emergence of MDR in DEC strains as a potent threat to the society

Antimicrobials are used to reduce the severity of the disease and shorten the span of infection. It also prevents the transmission of disease from one person to another which is important for slowing outbreaks because the organisms present in an infected person may be more virulent than those acquired in the wild (Oeke *et al.,* 2007). Indiscriminate use of antimicrobials in irrigation and agricultural farms with untreated sewage water has resulted in the increase of the potential risk to a growing number of antibiotic-resistant organisms in developing countries like Pakistan (Shah *et al.,* 2015). Antimicrobial agents may also be life-saving at certain situations but the unscrupulous use of antimicrobial result in the emergence and dissemination of multidrug resistant (MDR) diarrheagenic *E. coli* (Prasad *et al*., 2022; Okeke *et al.,* 2007; Shah *et al.,* 2015). Most of the diarrheal diseases are self limiting and does not need treatment with antibiotics or other agents such as chemotherapy. Recent studies from Kenya (Senerwa *et al.,* 1989), Gabon (Prester l*et al.,* 2003), Tanzania (Vila *et al.,* 1999),

Senegal (Okeke *et al.,* 2007) and Nigeria (Okeke *et al.,* 2000) suggest that there is rise in MDR EPEC, ETEC and EAEC. Reports from Kenya and Tanzania have shown the occurrence of notable outbreaks and sporadic cases of drug resistant EPEC (Senerwa *et al.,* 1989; Vila *et al.,* 1999; Okeke *et al.,* 2000). The most common cause of persistent childhood diarrhea is recently identified multidrug-resistant enteroaggregative *E. coli* (Okeke *et al.,* 2001), thus indicating the rise of threatening antimicrobial drug resistant diarrheagenic *E. coli* pathotypes (Okeke *et al.,* 2007).

Surveillance studies have demonstrated the fact that commensal *E. coli* are a rich source of genetic material which is passed on to other organisms via mobile genetic materials. A study in Nigeria has shown that the commensal *E. coli* is becoming resistant to an extensive number of microbial over a short span of time (Okeke *et al.,* 2000). Moreover, it has also been documented that the urban population of Nigeria is displaying more resistance than the rural and provincial population (Okeke *et al.,* 2000; Nys *et al.,* 2004). Thus, this finding highlighted the adverse effect of urbanization in these countries and other parts of the continent where travel networks have become a more efficient and extensively used mode of rapid spread of this resistance in the population. Therefore, just as Africa has had to deal with imported resistant organisms, resistant strains that emerge or are amplified in Africa will be exported (Okeke *et al.,* 2001; Roper *et al.,* 2004).

Generally, bacteria use four different types of antimicrobial resistance mechanisms which are – limiting drug uptake, modifying the target of the drug, inactivating the drug and active efflux of the drug. These mechanisms are present either within the bacterial chromosomes or are acquired from other bacteria through mobile genetic elements or plasmids. *E. coli* is a common microflora has become resistant to a number of antibiotics such as beta lactam antibiotics, tetracycline, fluoroquinolone, gentamicin, trimethoprim and sulfamethoxazole.

3.12.1. Emergence of Carbapenem resistance

Carbapenems are broad spectrum antibiotics that are generally used as the last line of defence against multidrug resistant bacteria for severe cases of infection (Murugan *et al.,* 2019). Carbapenems belong to the β-lactum family of antibiotics which are structurally related to penicillins. The mode of carbapenem action begins by entering the bacterial cell wall and attaching to the enzyme called penicillin binding proteins (PBPs) followed by inhibition of transpeptidase reaction thus hindering bacterial cell wall formation (Codjoe *et al.,* 2017). A covalent bond comprising of carboxypeptidase is formed by PBPs through transpeptidation

and transpeptidase enzymes which in effect prevent their peptide cross-linking activities during peptidoglycan biosynthesis. The lethal effects result in bacterial cell death by autolytic action within the bacterial cell and destruction of the cell by osmotic pressure (Van Dam *et al.,* 2009; Papp-Wallace *et al.,* 2011).

A few examples of carbapenem drugs are meropenem, imipenem, ertapenem, doripenem, biapenem and panipenem which are in use all over the world against severe infections due to the rising resistance to cephalosporin antimicrobials in the Enterobacteriaceae group (Codjoe *et al.,* 2017). Carbapenem resistance may develop due to various factors such as (a) due to the presence of the enzyme, carbapenemase, a plasmid borne lactamase which hydrolyses carbapenems, (b) loss of membrane porins and (c) over expression of efflux pumps (Murugan *et al.,* 2019).

Carbapenemases are enzymes that belong to β-lactamases, which are capable of hydrolyzing penicillins, carbapenems, mono-bactams and cephalosporins. There are two types of carbapenem hydrolyzing enzymes based on the reactive site of the enzymes: metallo-β-lactamases and serine carbapenemases both of which are induced by exposure of bacteria to β-lactams (Bhardwaj *et al.,* 2015; Aslam *et al.,* 2020). Carbapenems are classified into 3 different classes (class A, B and D) by scientist "Frere" and his colleagues (Aslam *et al.,* 2020). Class A carbapenemases require a serine active site and are capable of hydrolyzing penicillins, carbapenems, aztreonam, and cephalosporins (Ambler *et al.,* 1991). Class B is metallo-β-lactamases which require a zinc ion at their active sites in order to hydrolyze carbapenems, penicillins, and cephalosporins but do not hydrolyze aztreonam (Walsh *et al.,* 2005). Class D carbapenemases are OXA β-lactamases with the capacity to hydrolyze penicillin, oxacillin, ceftazidime and cloxacillin except imipenem (Queenan *et al.,* 2007).

Table I5: *Molecular classification of carbapenems* (Codjoe *et al.,* 2017, Aslam *et al.,* 2020).

Classification	Enzymes
Class A	SME, KPC, NMC, GES, IMI
Class B	GIM,VIM, IMP, SPM
Subclass B1	VIM-2, SPM-1, IMP-1, BcII, CcrA
Subclass B2	Sfh-1, CphA
Subclass B3	FEZ-1, Gob-1, L1, CAU-1
Class D	OXA

Most of the carbapenemases that are acquired by Enterobacteriaceae are plasmid-mediated which can spread to other species by several different mechanisms (Bedenić *et al.,* 2014). Moreover, there are many other important mechanisms that can confer carbapenem-resistance that have been observed recently. First, the carbapenems that are resistant to hydrolysis can be destroyed in the presence of plasmid AmpCs in combination with ESBL enzymes, making Gram-negative bacteria insusceptible to carbapenem agents (Bedenić *et al.,* 2014).

Since carbapenem is the last line of defence against multidrug resistant bacteria, the spread of resistance of this drug among different bacterial species is an emerging problem in modern society.

3.12.2. Fluoroquinolone resistance

The majority of infections acquired in hospitals and epidemics related to the gastrointestinal tract are due to multidrug resistant bacteria that have gained resistance to fluoroquinolone drugs. Fluoroquinolone antibiotics are generally broad spectrum antibiotics that are used to treat gram negative bacterial infections. These have become frequently used medicines against urinary, gastrointestinal, respiratory and other infections. Thus, gastrointestinal and urinary tract E. coli infections are also addressed with the commonly prescribed fluoroquinolone drugs which have resulted in the emergence of fluoroquinolone-resistant *E. coli* strains (Rath *et al.,* 2015).

Fluoroquinolone drugs impact their bactericidal effect by disrupting bacterial DNA replication. Fluoroquinolone resistance is mediated by mutations in the chromosomal genes such as gyrase and topoisomerase genes. The most common mutation in gram negative is *gyrA* mutations however several plasmid mediated quinolone resistance (PMQR) genes have been reported. These PMQR genes are more frequent nowadays and can spread the quinolone resistance with the help of horizontal gene transfer. The known PMQR genes are *qnr, qepA, aac(6')-Ib-cr* and *oqxAB* where there are several qnr genes such as *qnrA-qnrE, qnrS* and *qnrVC* (Ruiz *et al.,* 2019). *qnrA, qnrB,* and *qnrS* are mostly seen in *Salmonella enterica* and *Escherichia coli* found in the United States (Tyson *et al.,* 2019).

Transmission of fluoroquinolone is dangerous since it results in the spread of fluoroquinolone resistance in the bacterial species creating a serious public health issue. The transfer of resistance among different species and genera is also possible as *qnrB* genes were thought to

have originated in *Citrobacter* spp. (Ribeiro et al., 2015), but have now been found in many pathogens, including *E. coli*, *S. enterica*, and *Klebsiella pneumoniae*, among others (Tyson *et al.*, 2019). Fluoroquinolone drugs such as ciprofloxacin are very important medicines in the treatment of severe gram negative infections. Thus this spread of fluoroquinolone resistance is a serious public health issue and may result in treatment failures if proper preventive measures are not taken.

Part 4

Objectives of study

4. Objectives of the study

Diarrhoeal illness constitutes one of the major infections causing a great deal of morbidity and mortality in various developing countries. It is the second leading cause of child death in the world and the third leading cause of childhood mortality in India. It kills almost 2.5 million people each year globally and 70% of them are under 5 years of age. Acute diarrhea caused by diarrheagenic *Escherichia coli* (DEC) is one of the major public health problems in developing countries mainly in Asia and Africa. Among the 6 pathotypes (ETEC, EAEC, EPEC, EIEC, EHEC and DAEC) ETEC has been regarded as a prime cause of infant diarrhea and has also been implicated as an aetiological agent of traveler's diarrhea. EAEC has also been reported to cause persistent diarrhea among children less than 5 years of age. Since diarrheal infections caused by ETEC, EPEC and EAEC are frequently been identified all over the world our study deals with the prevalence and its impact on the Indian population. Moreover, the indiscriminate use of antibiotics has resulted in the arousal of MDR *E. coli* making treatment difficult for the doctors. Thus, in the present scenario, our study deals with the prevalence of DEC and its antibiotic resistance profile as well as the mechanism of resistance of the presently circulating strains of DEC in Kolkata. In order to achieve the diarrheal disease study, the following objectives have been undertaken:

- Isolation and identification of enteric *E. coli* (Diarrheagenic and commensal) isolated from diarrheal stool samples
- To access the magnitude of antimicrobial resistance in enteric *E. coli* isolated from diarrhea patients
- To identify the genetic determinants and mechanism of resistance to fluroquinolone and of associated multidrug resistance in *E. coli* isolates
- Characterization of carbapenem resistance (*bla*-NDM) *E. coli* strains isolated from diarrhea patients

Part 5

Materials and methods

5. Materials and methods

5.1. Study design

5.1.1. Sample history

The present diarrheal study was conducted in the National Institute of Cholera and enteric diseases, Beliaghata, eastern part of Kolkata, India. From Infectious Diseases Hospital (ID&BG) a total of 9501 diarrheal stool samples were collected during 2012-2022 and 5112 diarrheal stool samples were collected from B. C. Roy Hospital (BCH), Kolkata from the year 2018-2022. ID&BG and BCH are the 2 main hospitals that admit diarrhea patients in Kolkata and its suburbs for years. ID&BG hospital is located within the NICED campus that deals with critical patients who are given special care by admitting them in the hospital while BCH is located away from the campus which discharges the patients after providing clinical aids. In the active surveillance program, systematic stool specimens were collected from every 5th patient irrespective of who was receiving treatment. Passage of three or more loose stools in 24 hr duration with one or more clinical symptoms of enteric disorder (nausea, vomiting, abdominal pain or cramps, fecal urgency or dysentery) has been regarded as diarrhea.

5.1.2. Sampling

The patients admitted to ID&BG hospital were generally of all age groups whereas BCH treats patients less than 5 years of age. The presence of different DEC pathotypes was correlated with the patient's age.

5.1.3. Clinical parameters of diarrheal patients

After admission, clinical parameters of patients suffering from acute diarrhea were recorded in a standard proforma. The features recorded were a) duration of diarrhea a) nature of diarrhea i.e., watery, mucoid, bloody, bloody-mucoid b) frequency of vomiting c) abdominal pain d) presence or absence of fever and e) dehydration status. The degree of dehydration was assessed by the criteria recommended by WHO and was classified as some, none or severe (WHO, 1987).

5.1.4. Transport of clinical samples

Stool specimens were collected in sterile McCartney bottles and examined for certain characteristics of stool such as stool consistency (Watery, loose, formed) and its type (bloody or mucoid) which were noted. Rectal swabs were taken with sterile cotton tipped swab sticks which were introduced in Cary-Blair (Difco, Mich.).The stool samples were processed in the laboratory for the identification of enteric organisms within 2 hours of collection following the standard protocols (Panchalingam*et al.,* 2012). The clinical, demographic and laboratory data were entered in a specific data entry proforma supported with the SPSS.17.0 software (SPSS Inc, USA).

5.2. Isolation and identification

5.2.1. Preparation of selective medium

E. coli being gram negative lactose fermenting in nature are generally identified by streaking on Mac Conkey media as it is a selective medium which permits the growth of gram negative bacteria and further helps in the detection of lactose fermenting from non lactose fermenting bacteria. MacConkey agar (MAC) (Difco) in dehydrated form was used for primary isolation of *E. coli*. Medium was prepared according to the manufacturer's direction, autoclaved at 121°C for 15 minutes, cooled to 50-55°C and then poured (20 ml/plate) aseptically in sterile disposable plastic petri plates (15 mm. × 90 mm. Hi media, Mumbai, India).

5.2.2. Isolation procedure of *E. coli*

A loopful of stool specimens and fecal swabs were streaked on the Mac Conkey agar medium for colony isolation and incubated aerobically at 37°C overnight. The Mac Conkey agar contains disaccharide lactose, bile salts, crystal violet and neutral red as the pH indicator. The lactose in this medium is fermented by lactose fermenting bacteria such as *E. coli* resulting in the formation of lactic acid which lowers the pH. This decreased pH causes neutral red to change its color from off while to pink or red. Thus *E. coli* colonies appear as pink colonies in Mac Conkey medium. After overnight incubation, at least 3 pink (lactose-fermenting) colonies were picked up from MacConkey agar plates and were streaked in normal Luria Berteni (LB) plates for further confirmation by PCR before they are stored as stabs in nutrient agar (Difco) supplemented with 0.5% NaCl for further characterization of different categories of DEC.

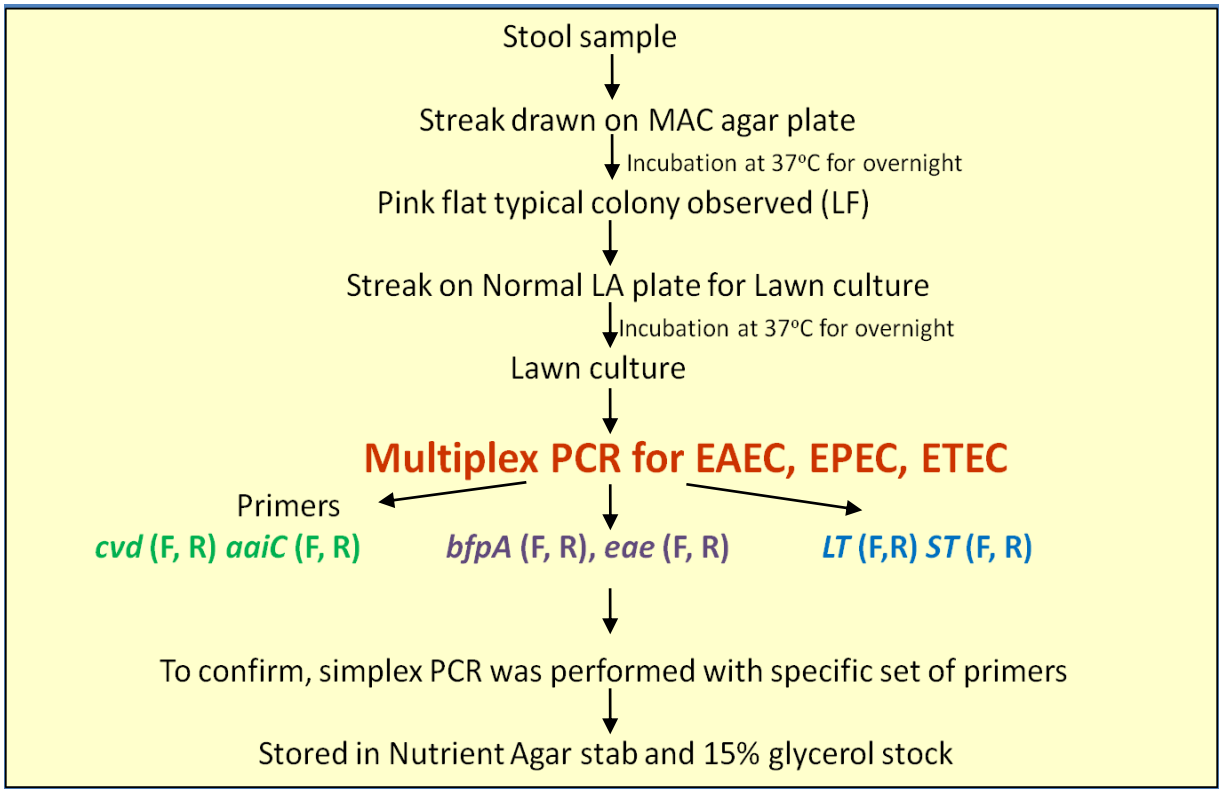


Figure M1: *Flowchart of isolation and identification of pathogenic E. coli from diarrheal stool samples*

5.2.3. Biochemical tests

E. coli belongs to the group of gram-negative Enterobacteriaceae which can be differentiated based on certain biochemical tests such as TSI (Triple sugar iron), catalase test, oxidase test and IMViC test (indole test, methyl red, Voges-Proskauer and citrate test).

a) Indole test: It is a biochemical test which is used to determine the bacteria's capacity for the formation of indole from tryptophan by the help of an enzyme tryptophanase. The result of this test is indicated by a color change on addition of a reagent (Kovac's reagent).

In this test, the bacteria are grown in sterile peptone water broth for 24hrs followed by addition of 5 drops of Kovac's reagent (isoamyl alcohol, para-dimethylaminobenzaldehyde and concentrated hydrochloric acid). A positive result is indicated by the formation of pink/red color on the top of the liquid whereas a yellow color in case of negative result.

b) Methyl red and Voges-Proskauer test: Both these tests utilize the same media for the growth of bacteria known as MRVP broth. After the bacterial growth, the broth is separated into 2 different test tubes for methyl red and VP test respectively.

MR test is used to determine the ability of the bacteria to convert glucose to other stable acids such as acetic acid, lactic acid or formic acid. In this test, the bacteria is grown for about 24 hrs in a broth containing glucose and the reagent methyl red is added to it. In case of a positive test, the color of the media changes from yellow to red due to the lowering of pH below 4.5.

In VP test 2 reagents such as alpha-naphthol and potassium hydroxide are added to the bacterial culture for the detection of acetoin formation during the 2,3-butanediol fermentation pathway. The tube is vigorously shaken after the addition of the reagents followed by an incubation of 10 minutes in static condition. The yielding of pinkish-red color indicates a positive result.

c) Citrate test: This test is used to determine a bacteria's ability to utilize citrate and the test is performed on a slant for better growth of bacteria. Simmon's citrate agar is used which contains citrate as the only source of carbon along with ammonium ions and bromophenol blue as the pH indicator. The slant remains green before inoculation, but a positive result causes the change of color of the slant from green to blue.

d) TSI test: This test is used to determine whether the organism can ferment lactose, sucrose and glucose and determines its hydrogen sulfide gas production. When one of the carbohydrates is fermented the color of the TSI media changes from reddish orange to yellow due to lowering of pH of the medium. Alkalinization of the peptones is indicated by the dark red color of the media. The reagent sodium thiosulfate present in the medium is reduced to hydrogen sulfide by some of the bacterial species which react with ferric ions to yield iron sulfide, turning the slant black.

e) Oxidase test: This test is used to determine whether an organism possesses cytochrome c oxidase, an enzyme that is involved in transportation of electrons between the bacteria and a redox dye- tetramethyl-*p*-phenylene-diamine. Organisms containing cytochrome c are known as oxidase positive which reduces the dye to purple color whereas the organisms lacking cytochrome c (oxidase negative) do not change the color of the dye.

f) Catalase test: This test is performed to determine the presence of the enzyme catalase. The test is performed by the addition of a few drops of hydrogen peroxide to the bacterial culture. Hydrogen peroxide dissociates in the presence of catalase to peroxide and water resulting in foam formation.

5.2.4. Molecular analysis

a) Different types of PCR used in this study

PCR is a method which is used for the detection of the presence of a known genetic fragment within an unknown genetic content of an organism. The materials required for running a PCR are the following: a DNA template, two oligonucleotide primers of length from 15-25 bases, deoxyribonucleotides (dNTPs), buffer and the enzyme Taq polymerase. A thermal cycler that can shuffle the temperature of its blocks shortly (ramp rate) was used for the amplification (making copies) of the DNA fragment of our interest. This PCR product was run in an agarose gel which was further stained and viewed for the presence of amplified product with the help of a gel doc (Mullis et al., 1986). 2 different types of PCRs were used in our study:

i. Multiplex PCR

Multiplex PCR allows the amplification of multiple target genes simultaneously within a single reaction tube using specific primer sets. The primers for different sets were designed such a way that the difference among products was at least 50bp apart. Also, the melting temperature (tm) should coincide with another set of primers that will be amplified within the same reaction tube. In this study, a multiplex has been used to amplify six different virulence genes of three different pathotypes of *E. coli* (described in the later section).

ii. Simplex PCR

In this PCR method, only a single gene is amplified within a PCR reaction tube using only one set of primer (one forward and one reverse). This is the simplest method of optimization, amplification and detection of a gene from an unknown species. The primers designed require little optimization and there is no competition for the targets between the primers (**Table M1**).

Table M1. *List of primers used in this study.*

Primer	Target gene	Primer sequences (5'-3')	Tm (ºC)	Amplicon size (bp)	Reference
CVD432F	*aatA*	CTGGCGAAAGACTGTATCAT	57	630	Panchalingam *et al*., 2012
CVD432R		CAATGTATAGAAATCCGCTGTT			
aaiCF	*aaiC*	ATTGTCCTCAGGCATTTCAC		215	
aaiCR		ACGACACCCCTGATAAACAA			
bfpA-F	*bfpA*	GGAAGTCAAATTCATGGGGG	57	367	
bfpA-R		GGAATCAGACGCAGACTGGT			
eae-F	*eae*	CCCGAATTCGGCACAAGCATAAGC		881	
eae-R		CCCGGATCCGTCTCGCCAGTATTCG			
ST-F	*est*	GCTAAACCAGTAG/AGGTCTTCAAAA	57	147	
ST-R		CCCGGTACAG/AGCAGGATTACAACA			
LT-F	*elt*	CACACGGAGCTCCTCAGTC		508	
LT-R		CCCCCAGCCTAGCTTAGTTT			
EIEC-F	*ipaH*	GTTCCTTGACCGCCTTTCGGATACCGTC	60	620	Casabonne *et al*., 2015
EIEC-R		GCCGGTCAGCCACCCTCTGAGAGTAC			
bla$_{OXA-1}$F	*bla$_{OXA-1}$*	GCAGCGCCAGTGCATCAAC	50	198	Maynard *et al.*, 2003
bla$_{OXA-1}$R		CCGCATCAAATGCCATAAGTG			
bla$_{TEM}$F	*bla$_{TEM}$*	GAGTATTCAACATTTTCGT	57	857	
bla$_{TEM}$R		ACCAATGCTTAATCAGTGA			
bla$_{CTX-M-3}$F	*bla$_{CTX-M}$*	AATCACTGCGTCAGTTCAC	60	701	
bla$_{CTX-M-3}$R		TTTATCCCCCACAACCCAG			
bla$_{SHV}$F	*bla$_{SHV}$*	TCGCCTGTGTATTATCTCCC	55	768	
bla$_{SHV}$R		CGCAGATAAATCACCACAATG			
Sul2-F	*sul2*	TTCGGCATTCTGAATCTCAC	50	822	
Sul2-R		ATGATCTAACCCTCGGTCTC			
StrA-F	*strA*	CCA ATCGCAGATAGAAGGCAAG	65	580	
StrA-R		ATCAACTGGCAGGAGGAACAGG			
tet(B)-F	*tetB*	CCTTATCATGCCAGTCTTGC	50	774	Sarkar *et al*., 2015
tet(B)-R		ACTGCCGTTTTTTCGCC			
qnrBF	*qnrB*	GATCGTGAAAGCCAGAAAGG	64	476	
qnrBR2		ATGAGCAACGATGCCTGGTA			
qnrS(F)	*qnrS*	GCAAGTTCATTGAACAGGGT	64	428	
qnrS(R)		TCTAAACCGTCGAGTTGCGCG			
faa-F	*aac6'-Ib-cr*	GCAACGCAAAAACAAAGTTAGG	55	561	
faa-R		GTGTTTGAACCATGTACA			
dfrIa F	*dfrA1*	GTGAAACTATCACTAATGG	55	474	Marbou *et al.*, 2020
dfrIa R		TTAACCCTTTTGCCAGATTT			
catI F	*catI*	AGTTGCTCAATGTACCTATAACC	55	547	
catI R		TTGTAATTCATTAAGCATTCTGCC			
bla$_{NDM-1}$F	*bla*NDM	GGGCAGTCGCTTCCAACGGT	55	475	Shanthi *et al.*, 2016
bla$_{NDM-1}$R		GTAGTGCTCAGTGTCGGCAT			

(continued)

virF_RT_F virF_RT_R	*virF*	CAGCTGTTTCTGATGAGGAAGC CAGAAAGACGCCATCTCTTCTC	55	120	This study
virB_RT_F virB_RT_R	*virB*	CATCCGAGAACTTGGTATCGG CCTGTGGAACGCTTGCTG	55	131	
ipaA_RT_F ipaA_RT_R	*ipaA*	CAGTAGCCGTGTTTCCTGAG GTCCGCCATCTGGCATATC	55	123	
ipaB_RT_F ipaB_RT_R	*ipaB*	CCCTGAATCCGATCATGAAAGC CCCCCAGAATAGAGCCAATC	55	129	
ipaC_RT_F ipaC_RT_R	*ipaC*	TGCTACAAAATCAGCTGCAGAG GTCGCTAATCCCTGAATGCG	55	133	
ipaD_RT_F ipaD_RT_R	*ipaD*	GTGGAACAATCGGCAAGGTATC CATTATCTAGCACAACCTCGCC	55	129	This study
icsA_RT_F icsA_RT_R	*icsA*	CCCAATGTCCACCATTACCG CGTTCGCTGGAGGCAAC	55	132	
icsB_RT_F icsB_RT_R	*icsB*	GTATCCCGGCGGGAATG TCTTTCGGCTGTTTGGTCTG	55	127	
RecA_RT_F RecA_RT_R	*recA*	ATGTGAGAGTCGCCGATTTCG AGCAGGCACTGGAAATCTGTG	55	124	
gyrA(F) gyrA(R)	*gyrA*	ACGTACTAGGCAATGACTGG AGAAGTCGCCGTCGATAGAAC	55	190	Pazani *et al.*, 2010
gyrB(F) gyrB(R)	*gyrB*	CTCCTCCCAGACCAAAGACA TCACGACCGATACCACAGCC	55	447	Sorlozano*et al.*, 2007
parC(F) parC(R)	*parC*	TGTATGCGATGTCTGAACTG CTCAATAGCAGCTCGGAATA	55	265	Pazani *et al.*, 2010
parE(F) parE(R)	*parE*	TACCGAGCTGTTCCTTGTGG GGCAATGTGCAGACCATCAG	55	266	Sorlozano *et al.*, 2007

5.3. DEC identification

The virulence mechanisms that characterize *E. coli* are genetically coded in the chromosomal, and plasmid DNAs. The most common virulence factors include heat-labile (LT) and heat-stable (ST) toxins for ETEC; *eae* and *bfpA* for EPEC, *aaiC and cvd* for EAEC. With the advent of PCR, it has become possible to identify these genes, offering the possibility of diagnosis of the virulence mechanisms existing in the specific *E. coli* strains.

For identification of the specific type of *E. coli* strain a multiplex PCR containing the 6 different sets of primers of *LT, ST, eae, bfpA, aaiC* and *cvd* genes was employed followed by a simplex PCR in order to identify the colony containing the specific *E. coli* type (**Table M2**).

Table M2: *The E. coli pathotypes with their virulence genes functions.*

***E. coli* type**	**Genes present**	**Gene function**	**Reference**
Enteroaggregative *E. coli* (EAEC)	*aaiC*	Encodes type IV secretion system	Hebbelstrup *et al.*, 2017
	cvd	Helps in adhesion	
Enteropathogenic *E. coli* (EPEC)	*bfpA*	Virulence, localized adhesion to epithelial cell	Black *et al.*, 2008
	eae	Attachment to epithelial cells	Donnenberg *et al.*, 1995
Enterotoxigenic *E. coli* (ETEC)	*ST*	Enterotoxin	Zhang *et al.*, 2022
	LT	Enterotoxin	Kaper *et al.*, 2004
Enteroinvasive *E. coli* (EIEC)	*ipaH*	Helps in invasion	Michelacci *et al.*, 2016

5.3.1. Preparation of template DNA

Genomic DNA can be used as template in the PCR reaction. There are various methods of isolating genomic DNA. A few methods that have been used to isolate DNA from the bacterial cell are as follows:

a) Boiled template method: Bacterial culture was taken in small amount form the Luria Bertani agar (LA) plate with the help of autoclaved toothpick and suspended in 200 µl of autoclaved water or phosphate buffer saline (PBS) in a 1.5ml micro-centrifuge tube. The culture was mixed well by vortexing and placed in boiling water bath for 10 minutes followed by snap chilling on ice for 5 minutes and centrifugation at 10000 rpm for 10 minutes. The supernatant was collected and directly used as template for PCR.

b) Phenol-chloroform method: In this technique of DNA isolation, the DNA remains in the aqueous phase while hydrophobic lipids fall into the lower organic phase and protein remains at the interphase in between the upper aqueous layer and lower organic layer. In phenol-chloroform method, one loopful of an overnight culture from LA plate was suspended in 200 µl of Tris-EDTA buffer (pH 8.0) and then lysed by vigorous vortexing with mixture of phenol: chloroform: isoamyl alcohol (25:24:1) saturated with 10 mM Tris and 1mM EDTA. (Sigma-Aldrich, St Louis, MO, USA) As the phenol: chloroform mixture is immiscible in water, it results in the formation of 2 distinct layers after centrifugation- the upper aqueous

layer that contains the DNA and the lower organic layer containing the hydrophobic lipids along with the phenol chloroform mixture. Supernatant was collected carefully following centrifugation at 12,000 rpm for 15 min and was mixed once again with 100µl of mixture of chloroform: isoamyl alcohol (24:1) and centrifuged for 15 min at 12,000 rpm. The supernatant containing the DNA was collected and was diluted 30times in water in order to be used as template for PCR analysis. All the centrifugation steps were carried out at room temperature.

c) Cetyltrimethyl ammonium bromide (CTAB) method: A modification of the method of Murray and Thompson, 1980 was used for DNA extraction. Cells were harvested from 16-18 h grown culture in LA by centrifugation at 3000 rpm for 1 min at 4^{o}C suspended in 1ml PBS. The bacterial pellet was resuspended in 540ul of TE buffer (pH 8), followed by sequential addition of 60ul 10% SDS solution and 9ul freshly prepared proteinase K solution (20 mg/ml). The suspension was then incubated at 50^{o}C for 1 h for the digestion of proteinaceous material. After incubation, 6ul of RNase A (final 100 ug/ml) was added and incubated at 37^{o}C for ½ h. Then 100ul of 5M NaCl solution was added followed by the addition of 80ul CTAB/NaCl and incubated at 65^{o}C for 10 minutes. The DNA was extracted once with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1) by collecting the aqueous phase at each of the extraction step followed by the centrifugation at 12,000 rpm for 10 min, into fresh microfuge tubes. The aqueous phase was transferred to a fresh micro-centrifuge tube and the DNA was precipitated with 0.6 volume of isopropanol and recovered as an insoluble pellet by centrifugation at 10,000 rpm at 4^{o}C for 10 min. The DNA pellet was washed with 70% ethanol, dried and then suspended in 80ul of TE buffer (pH 8.0). The concentration of the DNA was determined spectrophotometrically. Then the DNA was diluted to 20-100ng/ul and used as template for PCR (Murray and Thompson, 1980).

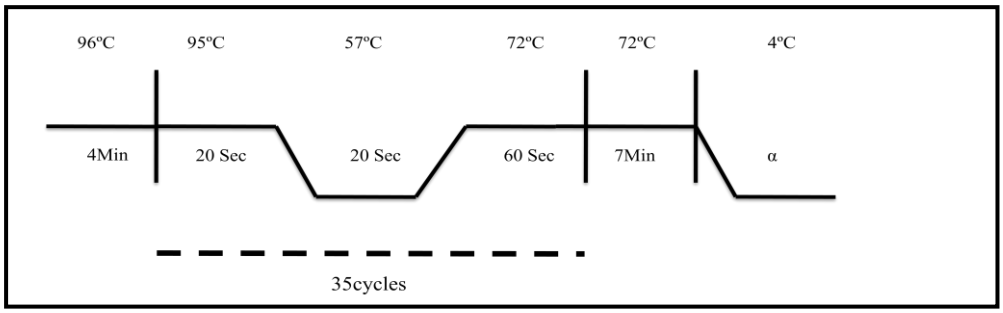
5.3.2. Preparation of PCR reaction mixture

The components that were mixed in appropriate proportions in order to form a proper PCR mixture (20ul of total reaction volume) were as follows: 2ul of a 1.25 mM mixture of deoxyribonucleoside triphosphates (dNTPs), 2.5 ml of 10XPCR buffer, 20 mM of each primer (Sigma), 0.25 ml of 5 U of Taq-polymerase per ml (New England Biolabs, USA) and 3ul of DNA template. The crude cell lysate prepared by boiled template method mentioned before was used as DNA template for the PCR reactions.

In case of multiplex PCR 6 different sets of primers containing the *LT, ST, eae, bfpA, aaiC* and *cvd* genes were used to identify the specific *E. coli* type. Simplex PCR for DEC utilized a combination of *LT* and *ST* to identify ETEC, *eae* and *bfpA* for EPEC and *aaiC* and *cvd* genes for EAEC. The PCR mixture remained the same with a variation of the primer sets for the different gene amplification.

5.3.3. PCR cycling conditions

The cycling conditions were set in a GeneAmp PCR system 9700 (AB Applied Biosystem). The samples were amplified for 35 cycles where each cycle was as follows: PCR reaction mixture was preheated to 96ºCfor 4 mins, followed by denaturation at 94ºC for 20 secs, annealing at 57ºC for 20 secs, and strand elongation at 72ºC for 1 minute with a final extension at 72ºC for 7 minutes. PCR cycle, annealing temperature and time, and extension time were same in both multiplex and simplex PCR (**Figure M2**)



FigureM2: *A representative figure for PCR temperature cycling conditions.*

5.3.4. Agarose gel electrophoresis and documentation

Gel electrophoresis has been used to separate DNA molecules (PCR amplified) depending on the size of nucleic acid molecules and the size is estimated by comparing it with the migration of molecules of known length (molecular size marker or DNA ladder) in agarose gels. The fluorescent dye ethidium bromide (EtBr) (Sigma) solution is used to stain the agarose gel where EtBr binds to DNA by intercalating between the base pairs. This intercalation concentrates ethidium in the DNA and also increases its intrinsic fluorescence. As a result, when the gel is illuminated with ultraviolet light, bright fluorescence is seen in the regions of the gel containing DNA than the regions of the gel without DNA.

4µl of the product were mixed with 1µl of bromophenol blue loading dye (gel loading dye in Appendix) and loaded on agarose (SRL, India) gels (concentration of agarose is varied according to the size of DNA molecule). Electrophoresis was carried out in 1X Tris-acetate

EDTA (TAE) buffer (Appendix) in a horizontal gel electrophoresis apparatus (BioRad, USA) at 100 volts for 1h using a power supply (BioRad). A 100 bp or 1 kb ladder (New England Biolabs, NEB, UK) was run along with the PCR products for size determination of the PCR amplified products. After electrophoresis, the gels were stained in a freshly prepared EtBr solution (0.5mg/ml) for 15 min. The gels were then viewed and documented with Gel-Doc 2000 system (BioRad).

5.3.5. Statistical analysis

The age of the patients was classified into 6 age groups comprising of ≤ 2, >2-5, >5-14, >14-30, >30-50 and ≥50 years. The prevalence of the categorized DEC was compared with each age group along with other pathogens detected in this study. Multinomial logistic regression analysis was performed. *P* values of <0.05 were considered as statistically significant and odds ratio (OR) and the 95% confidence interval (CI) was also calculated to determine the significant risk age group.

5.4. EIEC isolation and identification

5.4.1. Colony selection

A loopful of stool samples was streaked on MacConkey agar plates which were incubated at 37°C for 18-20hrs. Three pink lactose fermenting colonies along with three pale non-lactose fermenting colonies were selected from MacConkey plates for each sample which were sub-cultured on Luria agar (LA, Difco) plate. The lactose fermenting cultures from the LA plate were further confirmed by oxidase, indole production and acid/gas production in triple sugar iron agar for presumptive *E. coli* detection (Dutta *et al*., 2013; Panchalingam *et al*., 2012). The non lactose fermenting colonies were confirmed by acid/gas production in TSI agar followed by serological testing. The colonies were further confirmed by Vitek 2 compact system (bioMérieux). The cultures confirmed by the above tests were then suspended in 200 µl of phosphate buffered saline (PBS) or sterile water and were subjected to PCR for the detection of virulence marker gene *ipaH* which is encoded in multiple copies by both chromosomal DNA and the virulence plasmid pINV.

5.4.2. Serotyping

Fresh overnight grown cultures were used for performing the agglutination tests for determining the O and H antigens present with O (O1–O181) and H (H1–H56) following the

manufacturer's protocol (Denka-Seiken Co. Ltd., Japan) (Chowdhury *et al.,* 2022).8 polyvalent and 43 monospecific antisera were found in the serogrouping kit.

The small amount of culture was taken by a toothpick from the LA plate and tested in 8 sets of O antisera such as polyvalent 1, polyvalent 2, polyvalent 3, polyvalent 4, polyvalent 5, polyvalent 6, polyvalent 7 and polyvalent 8. If the culture agglutinizes in any of the polyvalent antiseras then it was further checked in the respective monovalent antisera present under that polyvalent antiserum (**Table M3**).

Table M3: The various antisera used for *E. coli* identification.

Sl. No	Polyvalent antisera	Monovalent antisera
1	Polyvalent 1	O1, O26, O86, O111, O119, O127a, and O128
2	Polyvalent 2	O44, O55, O125, O126, O146, and O166
3	Polyvalent 3	O18, O114, O142, O151, O157, and O158
4	Polyvalent 4	O6, O27, O78, O148, O159, and O168
5	Polyvalent 5	O20, O25, O63, O153, and O167
6	Polyvalent 6	O8, O15, O115, and O169
7	Polyvalent 7	O28ac, O112ac, O124, O136, and O144
8	Polyvalent 8	O29, O143, O152, and O164

5.4.3. VITEK-2

This is an identification system which is used to identify organisms based on the different biochemical tests and their nutrient utilization. The bacterial cultures are adjusted to a Mac Ferland OD. The VITEK-2 cards contain 64 wells of different nutrients and biochemical tests required for identification of the microorganism. The card was inoculated with the specific organism solution that reacts with the different biochemical reagents to yield positive or negative results. This pattern of positive and negative results was compared to a library in order to provide the name of the organism.

5.4.4. Pulse Field Gel Electrophoresis (PFGE)

In regular electrophoresis, all linear double-stranded DNA molecules that are larger than a certain size migrate through agarose gels at the equal time and cannot be separated as distinct bands. To solve this issue, Schwartz and Cantor, 1984 first reported the development of

PFGE capable of resolving DNA of up to 10 mega base (Mb) pairs in length. Large DNA molecules, ranging from $2x10^4$ to 10^7 base pairs [20 Kb to 10Mb] in length, can be separated by size with PFGE. This technique depends on the unique behavior of large DNAs in an electric field that is turned on and off (pulsed) sequentially with the alternating, orthogonal electric fields. When an electric field is applied in the gel, the DNA molecules migrate in the direction of the field and stretch out lengthwise. If the current is stopped, the molecules begin to "relax" into random coils. The time required for relaxation is directly proportional to the length of a molecule. The electric field then is reapplied at 90^o or 180^o to the first direction. Longer molecules relax less than shorter ones during the time the current is turned off. Since the molecules must relax into a random coil before moving off in a new direction, longer molecules start moving in the direction imposed by the new field more slowly than the shorter ones. Repeated alternation of the field direction gradually forces large DNA molecules of different sizes further and further apart.

a) Media, buffers and other reagents- The media required are LB broth and agar plate and listed in the Appendix. The composition of the buffers i.e., cell suspension buffer, lysis buffer washing buffers, 0.5X Tris-borate EDTA (TBE), 0.5X Tris-EDTA (TE)and proteinase K solution are listed in the Appendix.

b) Preparation of DNA plugs- To perform PFGE, genomic DNA was brought out from the bacterial cells in the agarose embedded form following the methodology described by Kurazono et al., 1994 with certain modifications. The strains of *E. coli* were revived on LA plates and a single colony was inoculated into 3ml of LB. Following incubation at 37^oC for 16-18 h with shaking (150rpm), 30ul of the culture was used as seed inoculum to generate fresh 3ml culture, which was allowed to grow at room temperature for 3 hr till it reached 1.2 OD measured at 600 nm. 1 ml of normalized bacterial culture was taken and harvested by centrifugation at 1,000xg for 5 minutes at 4^oC, washed and resuspended in 400ul of cell suspension buffer. To this mixture 20ul of proteinase K solution (20mg/ml stock) was added. A 1% low melting molecular grade agarose (SeaKem Gold) in water was also equilibrated at 55^oC-60^oC. The cell suspension was then mixed with an equal volume of the SeaKem Gold agarose (Bio-Rad) at 50^oC and the mixture was poured into plug moulds (Bio-Rad). The solidified gel plugs were cut into two small sizes and then transferred into 2 ml tubes and soaked in 1 ml of lysisbuffer with 25 µl proteinase K solution (20 mg/ml stock) at 55^oC in shaking condition for overnight. Following incubation, the gel plugs then washed twice with distilled water for about 20 minutes each followed 3 times washing with 1X washing buffer

for 20 minutes each at room temperature with gentle agitation. After removing the washing buffer, the gel plugs were equilibrated in 30ul of CutSmart buffer (NEB) diluted in 270 µl of distilled water for the desired enzyme (*XbaI*) for 1 hr at room temperature. The 1X CutSmart buffer was then replaced with 0.6 ml of the fresh 1X restriction enzyme buffer and 60U of the *XbaI* restriction enzyme was added and mixed gently. The plugs were then incubated at 37ºC overnight without shaking. Following the incubation, the digestion mixture was removed from the gel plugs and kept in 0.5X TBE buffer until run. A similar set was prepared with the strain *Salmonella* serotype Braenderup (H9812) which has been used as the universal control for PFGE and digested with the restriction enzyme *XbaI* (60U).

c) Electrophoresis- A 100 ml 1% gel was made using SeaKem Gold (Bio-Rad) in 0.5 X TBE. PFGE chamber of the CHEF Mapper (Bio-Rad) was filled with 2 liters of 0.5 XTBE and cooled to 14ºC using the cooler model 1000 minichiller (Bio-Rad). The gel plugs were kept on the comb heads and the comb was placed on the casting tray and then 1% low melting agarose was slowly poured. After 1 hr of solidification, the gel was carefully placed into the electrophoresis chamber. PFGE of bacterial strains were performed by using contour-clamped homogeneous electric field method on a CHEF-mapper (Bio-Rad) using 0.5X TBE buffer for 18 hrs. The temperature of buffer during the run was maintained at 14ºC. Run conditions were generated by auto-algorithm mode of CHEF Mapper Pulsed Field Gel Electrophoresis system using a size range of 20-300 Kb. The calibration factor was kept fixed at 1.5 and all other parameters including initial and final switch times were generated by default [electrophoresis buffer: 0.5X TBE pH 8.0; run temperature 14ºC; electrical parameters: 6 V/cm; run time 18 hrs; included angle 120ºC; initial switch time 6.76 secs and final switch time 35.38 secs with linear ramping factor].

d) Documentation of the result- After the completion of electrophoresis the gel was stained with EtBr solution (0.5mg/ml; Sigma) and visualized and documented using Gel Doc 2000 system (Bio-Rad). The banding patterns were then analyzed and the dendrogram was constructed with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

5.4.5. PCR for detection of EIEC virulence genes

The colonies confirmed for the presence of *ipaH* gene were further subjected to characterization for the presence of different virulence marker genes. The EIEC isolates were subjected for the detection of 8 virulence genes such as *ipaH*, *ipaBCD*, *ial*, *sat*, *virF*, *sig*, *sepA* and *pic* by PCR. 20ug of reaction mixture was made containing 4ul of template DNA,

1X of standard Taq buffer, 0.33 mM of dNTP with 0.33 mM of forward and reverse primers of each gene and 1U of Taq polymerase (NEB) (**Table M1**).

5.4.6. Determination of pathogenicity of EIEC strains

a) Tissue culture assays

Tissue culture assay generally represents the experiments involved in infection of bacteria to the mammalian cultured cell-lines. In this study, Int407 (intestine 407, Human intestinal epithelial cell line) cell line was used to analyze the invasive property of the different strains of EIEC.

i. Culturing methods

Revival of the stored cell line includes the following steps. The stored cell line was taken out from liquid nitrogen (-196^{o}C) and placed in a floater kept in a beaker containing pre warmed (37^{o}C) water. 1ml of 20% fetal bovine serum (FBS, Gibco) containing Dulbecco's modified eagles medium (DMEM, Gibco) and pen-strep-glutamine (Gibco) was added to the vial, mixed and emptied in to a T-25 flask (Tarsons, India) containing 5ml of 20% DMEM (20%FBS + DMEM + pen-strep-glutamine). The flask was then shaken briefly and incubated at 37^{o}C with 5% CO_2 till it reached confluence. Sub-culturing was performed in T-75 flask containing the same media composition except the FBS concentration (10% in this case). After the cells of the T-25 flask reached confluence, the media was discarded, washed the cells with 1X PBS and 1ml of 1X Trypsin-EDTA solution (0.025% trypsin and 0.01% EDTA in PBS, Gibco) was added. The flask was then incubated at 37^{o}C for 3-5 mins. After the cells were dislocated from the flask bottom surface, 2 ml of 10% DMEM was added, mixed and taken in a 15ml centrifuge tube. The tube was centrifuged to 1500g for 2mins. Supernatant was discarded and 1ml of fresh 10% DMEM was added and mixed thoroughly by pipetting. A T-75 flask previously filled with 14ml of 10% DMEM was used to inoculate 250 µl of above cell suspension for sub-culturing and incubated at 37^{o}C with 5% CO_2 till it reached confluence.

ii. Plaque assay

Shigella has the ability to form plaques in cellular monolayer of tissue culture and the size of the plaques is a function of its replication capacity within the host cell cytosol as well as its dissemination capacity to the adjacent cells. Thus, the intercellular spreading of EIEC and *Shigella* could be evaluated by the help of plaque assay.

For performing this plaque assay, int 407 cells were seeded in 6 wells plates with a cell density of $1X10^5$ cells per well and were incubated until they become confluent. Subcultures were given from overnight bacterial cultures and were kept in shaker until the cultures reach 1OD. 10^4 bacteria were used for inoculating the confluent cellular monolayer followed by an incubation of 2 hrs at 37ºC with 5% CO_2. After 2 hrs of incubation, the supernatant containing the bacteria was replaced and incubated for 30 mins with DMEM containing 10% FBS and 50 µg/ml gentamycin. The monolayer was washed and further incubated with fresh DMEM containing 10% FBS, 50 µg/ml gentamycin along with 1% agar for 24 hrs and 48 hrs. A contrast solution of 0.033% neutral red in PBS was used to incubate the cellular monolayer for 2hrs at 37ºC in order to visualize the plaques.

iii. Adhesion assay

Int 407 cells were cultured in 6 well plates and exponential phase bacterial cultures of EIEC and *S. flexneri* were used to perform triplicate sets of infection. The cellular monolayer was infected with 10^7 bacteria and incubated for 2 hrs at 37ºC with a constant supply of 5% CO_2. The cellular monolayer was washed with phosphate buffered saline (PBS) after 2hrs of incubation followed by an incubation of 1 hr with 50ug/ml of gentamycin to eliminate the extracellular bacterial growth. After this the cells were trypsinized and plated on LA/TSA plates for quantification of the bacterial growth in CFU/ml.

b) Expression of virulence genes by reverse transcriptase PCR

i. Infection protocol of Int 407

The transcriptional profile of the genes expressed during the intracellular bacterial spread were analysed by infecting the cellular monolayer with 10^7 bacteria and incubated for 2 hrs at 37ºC with a constant supply of 5% CO_2 followed by 50ug/ml gentamycin treatment for 3 hrs. The cells were then strapped and RNA was isolated from them.

ii. Quantitative Real-time (qRT) PCR for gene expression

Relative mRNA expression among the LF EIEC and NLF EIEC strains of the genes *ipaH, virF, virB, ipaABCD, icsA* and *icsB* were studied to find out the virulence capacity of the different EIEC strains. For this, the strains were grown and used for the extraction of total cellular RNA for qRT PCR. For the expression analysis of bacterial mRNA a similar method was followed unless otherwise mentioned.

Isolation of total bacterial RNA

After infection the bacteria along with the cellular monolayer was scraped from the wells and was collected in a 1.5ml centrifuge tube which was centrifuged at 10,000 rpm to get a pellet of bacterial cell. The pellet was suspended in 1ml of TRIZOL reagent (Ambion, Life Technologies), homogenized by pipetting vigorously and kept at room temperature for 5mins. 200ul of chloroform (Merck) was added and mixed by inverting the capped tube several times and kept again at room temperature for 10mins. After 10mins, the tube was centrifuged at 13,000rpm for 15 mins at 4^{o}C. Clear supernatant (~300ul) was collected carefully without disturbing the bottom layer and an equal volume of isopropanol was added and mixed by inverting the tube. The tube was then incubated at room temperature for 10mins and centrifuged at 13,000rpm for 10 mins at 4^{o}C. The supernatant was discarded carefully and the nucleic acid material was seen to precipitate as white to transparent mass in the bottom of the tube. The tube was then soaked by keeping the tube inverted over a tissue and 75% chilled ethanol solution was added. The tube was vortexed and centrifuged at 13,000rpm for 8 mins at 4^{o}C. Supernatant was decanted carefully; pellet was air dried for 15-20mins and dissolved with 30ul RNase free autoclaved water by pipetting well. The extracted RNA can be stored at -80^{o}C for 2 weeks.

Removal of contaminant DNA from RNA

The concentration of the extracted RNA was measured using a NanoDrop spectrophotometer and normalized to 100ng/µl. A 20ul 100ng/µl concentration of RNA was made such a way that it can accommodate the 3 µl 10X DNaseI buffer and 2ul (40U) within the 20 µl total volume. For example, if the RNA concentration comes to 363.0 ng/µl, then the RNA needed to make 20 µl of 100 ng/µl concentration is 5.5 µl, 10X buffer 2 µl, DNaseI enzyme 2.5 µl and 10ul RNase free water. The reaction set up was subjected to incubation in a thermal cycler using the condition as follows: 37^{o}C for 3.5 hrs, 75^{o}C for 10 mins and 4^{o}C for 10 mins. After the reaction was over, a PCR for *recA* gene was run using 3ul of the DNaseI treated RNA as template for checking of any remaining traces of contaminated DNA. If no bands were found with the DNaseI treated sample in the PCR, then it was considered as DNA contamination free RNA and were used to the next step of cDNA preparation.

cDNA synthesis

A total of 2-3 µg of RNA was used for synthesis of total cDNA according to the manufacturers' protocol (Revert Aid first strand cDNA synthesis kit, Thermo Scientific, USA). A 20ul reaction volume was prepared. First, 11.5 µl of RNA, 1ul of random hexamer (for eukaryotic sample 1µl of oligodT primer was used instead of random hexamer) and 0ul of autoclaved double distilled water were added and the reaction mixture was heated at 65^{o}C for 5 mins and snap chilling on ice. Then, 4 µl of 5X reaction buffer, 2 µl of dNTP mix, 0.75 µl of ribolock and 0.75 µl of reverse transcriptase enzyme were added into the snap chilled mix. The final reaction was subjected to thermal cycling using the condition 42^{o}C for 90 mins, 70^{o}C for 10 mins and 4^{o}C on hold. After the process was done, concentration of the DNA was measured using a NanoDrop spectrophotometer and finally normalized to 1000 ng/µl (for eukaryotic cDNA 1500 ng/µl) and used for qRT PCR.

Reaction mixture and temperature cycling for qRT PCR

qRT PCR was performed using the fluorescent SyBr green dye (Thermo Scientific, USA) which fluoresces when intercalated in double stranded DNA. To quantify the relative mRNA expression for a particular gene, three types of reaction were set up (each well contains 20 ul). The reaction mix for standard was set up using 10ul of 2X SyBr green mix, 0.6ul of each forward and reverse primers (usually *recA* for bacterial and GAPDH for eukaryotic samples were used), 7.8 ul of water and 1ul of template of known concentration (prepared previously using PCR amplification of the standard gene such as *recA* followed by gel run, gel purification and concentration determination). Usually, six reactions for standard were prepared using different known template concentration. Four for sample concentration of 1 ng/ul, 0.2 ng/ul (diluting 1ng/ul 5-times), 0.04 ng/ul, 0.08 ng/ul, positive and negative controls. The reaction mix for unknown was prepared for two genes, one is the calibrator gene *recA* (or GAPDH) and other is the gene of our interest. The reaction mixture for each was prepared as follows: 2X SyBr green mix 10 ul, 0.6 ul of each forward and reverse primer, 3.8ul of water and 5ul of total cDNA (~1000 ng/ul). All the primers have been designed in such a way that the GC% was 50-55 and the tm was about 56^{o}C (**Table M1**). The reaction was prepared in a 96-well plate, sealed, centrifuged (2000 rpm-2 mins) and run in LightCycler 480 Instrument II (Roche diagnostics, UK) using the following program: pre-incubation at 95^{o}C for 3 mins, 40cycles of denaturation at 94^{o}C for 20 secs, annealing 56^{o}C

for 20 secs and extension 72^{o}C for 20 secs (as the product length was 110-150bp) with a single acquisition at the extension step (bandwidth channel 465-510) followed by a cooling step at 40^{o}C for 30 secs.

Analysis of result

After the run has ended the standard curve and the Ct values were generated using the Abs Quant/2nd Derivative Max program. The Ct values were then compared between the calibrator and the experimental gene using the $2^{-\Delta\Delta Ct}$ method to know the relative expression of a particular gene among different strains (Pfaffl et al. 2002). Fold increase and error bars were then calculated using the Graphpad prism software (Ver. 6.0).

c) In vivo estimation of virulence and colonizing capacity of the EIEC isolates

i. Animals used

BALB/c mice of either sex was obtained from the animal resource of National Institute of Cholera and Enteric Diseases. Mice of either sex were kept in a group of 10 in two separate cages which were maintained at 25$\pm$2^{o}C temperature with 65$\pm$2% humidity. Water ad libitum and sterile food were fed to the mice. An antibiotic cocktail containing ciprofloxacin (500 mg), metronidazole (400 mg), albendazole (400 mg) and erythromycin (500 mg) was used for gut sterilization of the mice. This antibiotic treatment was carried out for a period of 5 days (Mitra *et al.,* 2012; Koley *et al.,* 1995). Non-albino "old English colored" guinea pigs of either sex was used for studying the development of keratoconjunctivitis (Sereny test) in order to test the virulence capacity of the EIEC strains. Standard operational procedure as stated Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India was used to conduct the animal experiment. Institutional Animal Ethical Committee (IAEC) of NICED with registration Number No. 68/Rebi//S/1999/CPCSEA valid 17/7/2024 approved all the animal experiment protocol.

ii. Bacterial strains used

2 LF EIEC (BCH 12902 and BCH 10711), 2 NLF EIEC (IDH 9427 and BCH 8159) and a *Shigella sp.* strain were used for the challenging the mice in order to find their invasion and colonization property in mice gut. In the case of the sereny test similar strains were used to estimate their virulence property.

iii. Colonization and invasion assay in mice model of the EIEC isolates

Both LF and NLF EIEC strains were grown overnight at 37^{o}C. Next day fresh LB were re-inoculated with these overnight grown cultures and were kept in shaking at 37^{o}C until the cultures reach 0.8-1 OD. Before the oral infection, the mice were fed 5% solution of sodium bicarbonate and were orally fed with 10^{7} colony forming units (CFU) (Mitra *et al.,* 2012; Yang *et al.,* 2014). The actual bacterial dose that was given to the mice was determined by platting serial dilutions on LA or TSB agar plates (Yang *et al.,* 2014). All the immunized mice were returned to their respective cages and were provided with limited amount of food and water (Mitra *et al.,* 2012). Mice were sacrificed at respective hours (6 hrs, 24 hrs, 48 hrs and 96 hrs) and the parts of large intestine (colon and caecum), ileum, liver, spleen and stoolwere smashed to make a homogeneous mixture which was diluted to different dilutions for plating on selected medium containing specific antibiotics. Colonies were counted the next day. The tissues from intestines, liver and spleen were also sent for histological analysis.

iv. Sereny test

Keratoconjunctivitis of the guinea pigs were induced by using 1 LF EIEC (BCH 12902), 1 NLF EIEC (IDH 9427) and 1 Shigella strain. Keratoconjunctivitis were sereny test helps to estimate the invasive potential of the EIEC strains. The bacterial strains were allowed to grow until the culture reached 1OD. 1 OD 1ml culture of bacterial strains were pelleted down and washed with PBS. Bacterial strains (5.0×10^{8}CFU/eye) were dropped onto the conjunctival sac on the right eyes of the guinea pigand the eyelids were gently massaged after the treatment. The animals were then returned to their respective cages. This treatment with the same bacterial dose (5.0×10^{8}CFU/eye) was repeated the next day for better results. Keratoconjunctivitis was observed after the after dose of bacterial culture in the conjunctival sac of the guinea pig.

v. Histological analysis

The mouse tissues (liver, spleen, colon, ileum and caecum) that were obtained from 6hrs, 24 hrs 48 hrs, 72 hrs and 96 hrs of infection were fixed in a solution of 10% buffered formalin and 5 μM paraffin-embedded sections were stained with haematoxylin and eosin (H&E). During the staining procedure, the embedded tissue sections were deparaffinized in xylene for about 10-20 minutes followed by rehydration in 100% and 95% of alcohol for 1-2

minutes. The tissue sections were then rinsed in tap water followed by rinsing in distilled water before staining them in hematoxylin stain for about 3-5 minutes. After staining, the tissue sections were dipped in a solution of 1% HCL in 70% alcohol for differentiation of the sections. The tissue section slides were further washed in running tap water followed by staining in eosin stain for 1-4 minutes. The tissue slides were again rehydrated in 100% and 90% alcohol before dipping in xylene solution for 2 minutes. The slides mounted in mounting media and were viewed at 20x magnification using light microscope followed by histopathological analysis.

5.5. Antibiotic resistance profile and prevalence of antibiotic resistance genes (AMR) in the *E. coli* isolates

5.5.1. Antibiotic susceptibility tests

Antibiotic susceptibility test has been performed to get the idea about the current trend of antimicrobial resistance of recently circulating *E. coli* strain in India. Generally, one third proportion of the isolated strains representing the whole population were selected and tested for their pattern of antimicrobial susceptibility. A number of recent generation antibiotics were chosen and the susceptibility was analyzed with the following methods:

a) Kirby-Bauer disc diffusion test

Antimicrobial susceptibility testing was performed by disk diffusion method (Bauer *et al.*, 1966) using commercially available discs (Difco, B.D, USA and Himedia, India). Single colony of the strain that need to be tested was picked up from overnight culture in LA agar (Difco) plate and inoculated into 3 ml of Luria Bertani broth (LB) (Difco). The broth was incubated at 37°C for about 2-3 hrs until the cells reached McFerland OD. A swab stick dipped in this culture was then spread onto the entire surface of a MH Agar (Difco) plate. Antibiotic discs were placed on the agar plate sufficiently separated from each other to avoid the overlap of the zones of inhibition by using sterile fine forceps. The plates were then incubated overnight at 37°C.

Representative isolates were tested for their susceptibility to the antibiotics ampicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), sulfamethoxazole-trimethoprim(23.75/1.25 μg), ciprofloxacin (5 μg), ofloxacin (5 μg), gentamycin 42 (10 μg), streptomycin (10 μg), ceftazidime (30 μg), ceftriaxone (30 μg), azithromycin (10 μg) and doxycycline (30 μg) by Kirby-Bauer disk diffusion method using commercially available

antibiotic disks (Difco, B.D, USA and Himedia, India. The *E. coli* strain ATCC 25922 was used as the control to check disc accuracy for this assay (Bauer *et al.*, 1966). Breakpoints were interpreted following the guideline mentioned by CLSI 2019 (**Table M4**).

Table M4: *Different breakpoints of antibiotics tested by disc diffusion method.*

Antibiotics	Abbreviations	Strength(ug/disc)	Interpretation of the zone diameter		
			Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Ampicillin	AM	10	<13	13-17	>17
Ceftriaxone	CRO	30	<19	19-23	>23
Ceftazidime	CAZ	30	<17	17-21	>21
Meropenem	MEM	10	<19	19-23	>23
Tetracycline	Te	30	<14	14-19	>19
Doxycycline	D	30	<10	10-14	>14
Streptomycin	S	10	<11	11-15	>15
Ciprofloxacin	CIP	5	<21	21-26	>26
Ofloxacin	OFX	5	<12	12-16	>16
Gentamycin	GM	10	<12	12-15	>15
Chloramphenicol	C	30	<12	12-18	>18
Sulfamethoxazole Trimethoprim	SXT	23.75/1.25	<10	10-16	>16

b) Minimal inhibitory concentration (MIC) test with E-strips

MIC is necessary to determine the extent of antimicrobial resistance property of a particular organism. In this study MICs have been performed with the representative strains that showed high level of resistance to the latest generation antibiotics. The procedure was the same as described in the above section (**Table M5**).

Table M5: *MIC values of different antibiotics tested.*

Sl no.	Antibiotic strips used	Range of E-strip (µg/ml)
1	Imipenem (IP)	4-256
2	Ofloxacin (OF)	0.002-32
3	Ampicillin/Sulbactam (AB)	0.016-256
4	Streptomycin (SM)	0.016-256
5	Ciprofloxacin (CI)	0.002-32
6	Tetracycline (TC)	0.016-256
7	Azithromycin (AZ)	0.016-256
8	Ceftriaxone (TX)	0.002-32
9	Trimethoprim/Sulfamethoxazole (TS) (1/9)	0.002-32
10	Imipenem/Imipenem+EDTA (MBL) (IP/IPI)	1-64
11	Cefepime/Cefepime+Cla (ESBL) (PM/PML)	0.25-16/0.064-4
12	Cefotetan/Cefotetan+ Cloxacillin	0.016-256

5.6. PCR for AMR genes

MDR strains were subjected to PCR for the presence of the following AMR determining genes: *bla*$_{\text{TEM-9}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{CTX-M3}}$, *bla*$_{\text{SHV}}$for β-lactams (Maynard et al. 2003); *qnrB, qnrS*and *aac6'-Ib-cr* for plasmid-mediated quinolone resistance, *sul2* and *dfrA1* for sulfamethoxazole-trimethoprim (SXT) element; *strA*and *aadA* for aminoglycosides; *catI*for chloramphenicol and *tetB* for tetracycline group of antibiotics using the primer sets listed in Table S1 (Sarkar et al. 2015; Marbou et al. 2020). 30µl PCR reaction mixture was prepared containing 2 µl of a 1.25 mM dNTP mixture, 3 µl of 10XPCR buffer, 20 mM of each forward and reverse primer (Sigma), 0.25µl of 5U/ml of Taq polymerase (NEB, USA) and 3 µl of template DNA. Amplicon size and the annealing temperatures of the primers for each target gene are furnished in **Table M1.**

5.7. Determination of mechanism of fluroquinolone resistance in the *E. coli* isolates

The resistance to fluroquinolone drugs such as nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin depends on the presence of certain plasmid mediated fluroquinolone resistance (PMQR) genes and also on the presence of mutations in the chromosomal genes such gyrase A, gyrase B, *parC* and *pare*. Therefore, both the PCR identification of PMQR genes such as *qnrB, qnrS* and *aac6'-Ib-cr* as well as determination of novel mutations at the chromosomal genes were performed in the study.

5.7.1. PCR for PMQR genes determination

qnrB, qnrS and *aac6'-Ib-cr* genes for plasmid-mediated quinolone resistance were checked in the *E. coli* isolates in order to find the cause of resistance of the quinolone drugs.

5.7.2. Nucleotide sequencing analysis of the chromosomal genes

Nucleotide sequencing is one of the most valuable tools in the field of molecular biology. It is generally used to read short and partially unknown genome sequences. In this study, nucleotide sequencing technique has been used to monitor the presence of novel genetic mutations in the chromosomal genes such as *gyrA, gyrB, parC* and *parE* in order to decipher the cause of high resistance of fluroquinolone drugs.

a) **Primer designing for sequencing-** Sequencing has been performed for many purposes in this study. To know the sequence of a complete gene of length <1 Kb primers have been designed from the immediate flanking regions of the gene. If the gene length is >1 Kb, the primers have been designed in such a way that it covers

only the portion of our interest or the mutational hot spots of the gene within the range of ~1Kb. No such specific criteria for primer designing have been implemented for sequencing. Primers used were 17-25 bp in length and the tm was kept at ~55^{o}C.

b) **Reaction set up and product purification-** The reaction was set up for 50 µl which consists of 5 µl of 10X reaction buffer with $MgCl_2$, 4ul of 2.5 mM dNTPs, 1 µl of 10 µM each primer, 10 µl of appropriately diluted DNA template and 1ul of high fidelity Taq polymerase (TaKaRa, Japan). The cycling conditions were mentioned in **Table M1**. After the PCR was done, the product was purified with GeneJet PCR purification kit (Thermo Scientific, USA) following the manufacturers' protocol. 1-2 µl of the purified product was run in a 1% agarose gel to estimate the concentration of the product and to use in cycle sequencing reaction.

c) **Cycle sequencing-** For the cycle sequencing reaction, specific amount of DNA present in purified PCR product was added as template depending on the size of the product to be sequenced. The PCR primer pair used to generate the amplicon was generally used for sequencing of both the strands of the amplicon. The sequencing mix was obtained from Big Dye Terminator v3.1 kit (Applied Biosystems, USA) and consisted of Big Dye® Terminator 3.1 Sequencing Buffer (5X) and ready to use reaction mixture (2.5X). The terminators used were di-deoxynucleotides labeled with fluorescent dyes. For cycle sequencing, the following components were added in a 0.2 ml thin-walled PCR tube: Sample DNA amounts to 100 ng/kb of template to be sequenced, Ready reaction mixture (2.5X)- 2ul, Big Dye Sequencing Buffer (5X)-1ul, Primer (forward or reverse)- 1ul (from 3.3 pmoles/µl) and Sterile triple-distilled water to a final volume of 10ul.

Cycle sequencing was carried out in a thermal cycler (Applied Biosystems, USA). The tubes were allowed to stand at 96^{O}C for 2 min followed by 25 complete cycles comprising of denaturation at 96^{O}C for 10 seconds, annealing at 50^{O}C for 5 seconds and extension at 60^{O}C for 4 minutes and finally allowed to reach to 4^{o}C before the tubes were taken out from the thermal cycler. The cycle sequencing products were kept on ice for further purification.

d) **Cycle sequencing product purification-** After cycle sequencing, the products of each reaction (10 µl) were pipetted into a 1.5 ml tube and diluted by adding 30ul

sterile deionized water. In the same tube 5 µl of 3M sodium acetate (pH 4.8) and 50ul of 95% ethanol (Sigma) were added. The contents were thoroughly mixed by vortexing and placed on ice for 15 mins. Tubes were centrifuged at 13,000 rpm for 15 min at room temperature and the supernatant were carefully aspirated with a separate fine tipped pipette tip for each sample and discarded. Next, the pellets were rinsed in 70% ethanol and centrifuged at 13,000 rpm for 10 mins. The residual fluid was removed by aspiration and were re-suspended in 20 µl of Hi-Di™ Formamide (Applied Biosystems, USA) by vortexing vigorously for 1 min and heating at 95^{o}C for 2 min in a thermal cycler. Samples were snap chilled on ice for 5 min and vortexed thoroughly and loaded in the automated DNA sequencer (ABI PRISM 3100, Applied Biosystems).

e) **Capillary electrophoresis-** The purified extension products from cycle sequencing reactions were loaded onto sample tubes, sealed with septa, and arranged on a 96-well tray. The tray was loaded to an automated sequencer (PE Applied Biosystems) and samples were subjected to electrophoresis through 47cm x 50cm capillary (PE Applied Biosystems) coated with POP-6 (Performance Optimized Polymer-6), (PE Applied Biosystems). Based on the nature of end-labeled bases four different types of fluorescence could be obtained. The machine recorded the different types of fluorescence obtained from end labeled molecules and analyzed the data to generate the output file with nucleotide sequence for the sample. The raw sequencing data was analyzed using the ABI PRISM DNA Sequencing Analysis Software (PE Applied Biosystems).

f) **Assembly and analysis of sequencing data-** The nucleotide sequence data was assembled to generate sequence contig for each gene from respective forward and reverse sequence read using software (DNASTAR, USA). Then searches for identical sequences were performed using the Basic Local Alignment Search Tool (BLAST) program available on the National Centre for Biotechnology Information (NCBI).

5.8. Characterization of *bla*$_{\text{NDM-1}}$ positive plasmid containing EAEC

Since carbapenems are the last line of defense against bacterial infections we focused on finding the association of carbapenems with diarrheagenic e. coli. By antibiogram screen we obtained an EAEC strain that was highly resistant to carbapenem antibiotic meropenem. The

meropenem resistance was verified by Kirby Baurer disc diffusion method followed by E-strip tests (both the methods are described in the above section).

5.8.1. Minimal inhibitory concentration (MIC) test with E-strips

MIC is necessary to determine the extent of antimicrobial resistance property of a particular organism. In this study MICs have been performed with the representative strains that showed high level of resistance to the latest generation antibiotics. The procedure was same as described in the above section (**Table M5**)

5.8.2. Plasmid Isolation

As the*bla*$_{\text{NDM-1}}$ genes are generally located in large plasmids conferring resistance to carbapenem antibiotics we went for isolation for plasmid. Plasmid was isolated by the help of two protocols which were:

a) Kado & Liu method

The plasmid was extracted from the *bla*$_{\text{NDM-1}}$ positive strain by Kado and Liu's method (1981) with slight modifications. For plasmid isolation, the bacterial culture was grown overnight and was sub-cultured the next day until the culture reached 1OD. The culture was centrifuged at 12,000rpm for 2 mins and was washed with TE buffer. The culture was then lysed with 100 µl of lysis buffer for about 45 mins at 55^{o}C which was followed by equal volumes of phenol chloroform treatment for about 10 minutes. This treated culture was centrifuged for about 30 minutes at 8000rpm. The clear supernatant at the top contained the plasmid which was collected and analyzed by gel electrophoresis using 0.8% Agarose.

b) Plasmid isolation by Qiagen maxi prep kit

An overnight grown bacterial culture was centrifuged at 6000 x g for 15 min at 4°C followed by resuspension of the pellet in 5 ml of P1 buffer (containing RNase A). To this, 5 ml of P2 buffer was added and was gently mixed by inverting until the lysate appeared viscous and incubated at room temperature (15‑25°C) for 3 mins. After this, 10 ml of pre chilled P3 buffer was added which was mixed by inverting 4-5 times followed by incubation on ice for about 20 mins. The mixture was then centrifuged at 20,000g for 30 mins at 4^{o}C. The supernatant was again recentrifuged at 20,000g for 15 mins at 4^{o}C. The Qiagen tip was equilibrated by 10 ml of buffer QBT, the column was allowed to empty by gravity flow. The supernatant was applied to the Qiagen tip and was allowed to enter the resin by gravity flow.

The Qiagen tip was washed twice with 30 ml of buffer QC. The DNA was eluted with 15 ml of QF buffer. The DNA was precipitated with 10.5 ml of isopropanol and the mixture was centrifuged at 15000g for 30 minutes at 4^{o}C. The supernatant was decanted and 70% ethanol was added to the DNA pellet that was again centrifuged at 15000g for 15 minutes. The supernatant was decanted and the DNA pellet was air dried for about 5-10 mins followed by dissolving the pellet in TE buffer (pH 8).

5.8.3. Purification of Plasmid

If multiple bands of plasmid appear, then we need to cut and purify the desired band of the plasmid.

a) Purification of plasmid isolated by Kado & Liu method

The plasmid DNA obtained was run in a 0.8% low melting agarose gel and was visualized by the help of gel doc machine. After visualizing the plasmid band, the band was cut and stored in 4^{o}C until a sufficient amount of gel is collected which can purify to yield a required concentration of plasmid. Band cuts from two gels were collected in one eppendrof tube followed by addition of 500 µl of TE buffer and water saturated phenol and kept at 55^{o}C for melting the gel. Equal volumes of Phenol: Chloroform: Isoamylalcohol (P:C:I) was added and centrifuged at 8,000rpm for 20 mins in room temperature until a clear solution was formed at the top layer. After centrifugation, 2 distinct layers were seen containing an upper clean layer with the DNA and a lower organic layer. The upper layer containing the DNA was collected and equal volumes of C:I was added to it followed by a centrifugation of 8000rpm for 20 mins. Ethanol precipitation was done with 2.5X volume chilled absolute ethanol+ 1/10th volume of 0.3M sodium acetate that was kept in 4^{o}C overnight. The next day, the mixture was centrifuged at 13,000rpm for 10 mins at 4^{o}C and the supernatant was discarded, washed twice in 70% Ethanol and was resuspended in TE buffer.

b) Verification of the presence of *bla*$_{\text{NDM-1}}$gene in the plasmid

The band of the plasmid obtained is cut from the gel and is melted to perform the PCR specific for *bla*$_{\text{NDM-1}}$ gene. In order to prove that it belongs to a plasmid and not genomic DNA, the gene (*aaiC*) specific for chromosomal DNA were checked by PCR.

5.8.4. Transfer of Plasmid

a) Conjugation

Conjugation assay was used in order to check the transfer of *bla*$_{NDM-1}$ gene to other bacterial species where pNDM positive EAEC was taken as donor with two recipients such as sodium azide resistant E. coli J53 and Salmonella serovar Bareilly. Overnight grown bacterial cultures were used for giving inoculum for late exponential phase cultures whose OD was adjusted to $1.5X10^8$ cells/ml. in a 5ml LB, the bacterial cultures of donor and recipients were mixed in the ratio of 1:2 and was kept overnight at 37^{o}C. The next day 100ul of the culture from the 5ml tube was spread on several selective mediums in order to recover the plasmid containing transconjugants. Mac Conkey with sodium azide and meropenem were used to select the J53 transconjugant. Similarly, xylose lysine deoxycholate (XLD, Difco) containing meropenem (5 µg/ml) was used to recover the transconjugant of *Salmonella*. PCR specific for *bla*$_{NDM-1}$ was used to select the putative transconjugant colonies and conjugation frequency was calculated using the number of transconjugants received per cell.

b) Transformation of plasmid DNA in *E. coli* J53

i. Preparation of competent cells

Inoculate a single colony in 5 ml LB and incubate at 37^{o}C overnight. Subculture in 50 ml LB broth (1:100) and incubate at 37^{o}C until it reaches ~0.45 OD followed by a 45-60 mins incubation in ice. Centrifuged at 1500g for about 10 mins at 4^{o}C and the supernatant was discarded. The pellet was washed with 5 ml TSS, suspended and centrifuged for 10 mins at 4^{o}C. The supernatant was discarded, and the pellet was resuspended in 1 ml TSS, kept in ice for 45-60 minutes. After the incubation, the cells were aliquoted in vials and stored at -80^{o}C.

ii. Reagents used for transformation

Competent cells, DNA to be transformed, TCM (11mM Tris-Cl, 11mM $CaCl_2.2H_2O$, 11mM $MgCl_2$, Filter sterilized), LB solution.

iii. Transformation procedure by TSS

10-20 µl DNA (conc 0.1 ng) was taken in an eppendrof tube in which 50 µl of TCM was added to incubate the mixture for 10-30 mins. 50 µl of thawed competent cells were added to the DNA mix in order to incubate it in ice for 20-30 mins. The mixture was given heat shock

at 42ºC for 1 min and half minutes followed by immediate putting the mixture in ice for 3 mins. 200 µl of LB media was added to revive the shock and was incubated at 37ºC for 1 hr at 200rpm. Then the cells were plated on plates containing markers and were incubated at 37ºC overnight for the appearance of transformed colonies.

c) Electroporation

i. Competent cell preparation

A single colony was incubated overnight at 37ºC which was subcultured in 100 ml LB the next day till 0.5 OD reached. The cells were harvested by chilling it on ice and centrifugating at 4,000g for 1 minute at 4ºC. Cells from 100 ml culture were resuspended in 100 ml cold autoclaved water and centrifuged at 4,000g for 15 minutes at 4ºC. The pellet was resuspended in 50 ml cold water, centrifuged, and again resuspended in 20 ml 10% glycerol again centrifuged and resuspended in 1 ml 10% glycerol and stored at -80ºC.

ii. Electroporation process

70 µl of thawed competent cells were mixed with 10 µl of plasmid DNA and placed on ice for about 1 min. The cell mixture was transferred to electroporation cuvette and pulse was given followed by addition of 800 µl of LB solution and incubation at 37ºC for 1 hr in shaking condition. The cell solution plasmid DNA+ DH5α was plated on LA plate containing 30 mg of ceftriaxone, the cell solution pCVD+DH5α was plated on ampicillin plate which was taken as positive control and another cell solution plasmid DNA+ DH5α was plated on normal LA plates which was used for cell viability check.

5.8.5. Plasmid sequencing and analysis

Amplicons were purified with the help phenol chloroform method described above which were sequenced by ABI Big Dye terminator cycle sequencing ready reaction kit of version 3.1 (Amplified Biosystems, Foster City, CA, USA) in an automated DNA sequencer (ABI 3730; Applied Biosystems).

5.9. Tissue culture

5.9.1. Stacked brick like appearance of EAEC

$5x10^5$ cells were seeded in a T-25 flask for infection and incubated for 24 hrs for doubling. Pure bacterial cultures were prepared by inoculating 2 ml LB which was kept at 37ºC

overnight. The next day bacterial subculture was given in a 5ml LB in order to get a log phase culture used for giving infection. 1.0 OD_{600nm} cells in 1ml PBS ($1x10^9$ cells/ml), from which 10^8 cells were used for infection to cell line (MOI 1:100) in tissue culture. After 2 hrs of infection, the flask was observed for the typical stacked brick appearance of the EAEC strains.

5.9.2. Il-8 production

The cell content of a confluent T-75 flask was trypsinized following the above-mentioned procedure and re-suspended in 2 ml of 10% DMEM. $5x10^5$ cells were seeded in a T-25 flask for infection and incubated for 24 hrs for doubling. For preparation of bacteria, pure cultures were inoculated into LB broth and incubated overnight at 37ºC. On the next day, bacterial subculture was made in 5 ml of fresh LB broth (1:100) and grown for an OD_{600nm} of 0.5-0.8. The culture was then normalized to 1.0 OD_{600nm} cells in 1ml PBS ($1x10^9$ cells/ml), from which 10^8 cells were used for infection to cell line (MOI 1:100). Prior to infection, the media of the T25 flask was removed, washed the cells with PBS and sub-merged the cells with 2 ml of DMEM without FBS and pen-strep. Bacteria in 1ml PBS was centrifuge, the PBS was discarded and 1 ml of DMEM without FBS and pen-strep was added, 100 µl (10^8 cells) of which was added to the T-25 flask and incubated for 3.5 hrs at 37ºC with 5% CO_2. After incubation, the media was decanted, cells were washed twice with 1 ml PBS and 1ml trypsin-EDTA was added. Incubated for 3 mins and the trypsin was inactivated by adding 2 ml of 10% DMEM. Cells were taken in a 15 ml centrifuge tube, washed with PBS and suspended in 1 ml of Trizol reagent (Ambion) for RNA isolation to perform qRT PCT for the housekeeping gene Glyceraldehyde-3phosphate dehydrogenase (GAPDH) of the cell line and the gene of our interest interlukin-8 (IL-8) and interlukin-1β (IL-1β) to analyze the level of induction of the pro-inflammatory cytokines. The Ct values were converted to relative expression difference using the formula $2^{-\Delta\Delta Ct}$ mentioned by Pffafl et al. The experiment was performed in triplicate for a single strain (Bandyopadhaya *et al*., 2007; Woida *et al*., 2020).

5.10. Appendix for materials and methods

- **Bromophenol Blue Loading Dye (10ml)**

- 1M Tris-HCl (pH-7.5): 100ul
- Glycerol: 6ml
- Bromophenol blue: 0.003gm
- 500mM EDTA: 1.2ml
- Water: 2.7ml

- **50X Tris-acetate EDTA (TAE) (1 lt)**

- Tris Base: 242gm
- Glacial acetic acid: 55ml
- 0.5M EDTA (pH-8.0): 18.6 gm

- **Transformation & storage solution (TSS) (50ml):**

- PEG8000: 5gm
- 1M $MgCl_2$: 1.5ml
- DMSO: 2.5ml
- Luria Bertani broth: 50ml

- **TCM:**

- 11mM Tris Cl
- 11mM $CaCl_2$, $2H_2O$
- 11mM $MgCl_2$

- **Luria Bertani Broth (1lt)**

- Tryptone: 10g
- Yeast Extract: 5gm
- NaCl: 10gm

- **Luria Bertani Agar**

- Tryptone: 10g
- Yeast Extract: 5gm
- NaCl: 10gm

- Bacto Agar: 20gm

- **Cell Suspension Buffer (10ml)**

- 0.1ml of 1.0M Tris-HCl, pH 7.2
- 0.04ml of 5.0M NaCl
- 1.0ml of 0.5M EDTA, pH 8.0

- **10X restriction digestion buffer (buffer H) (5ml)**

- 500mM Tris HCl (pH 7.2): 2.5ml
- 100mM $MgCl_2$: 0.5ml
- 10mM Dithiothreitol: 0.5ml
- 1000mM NaCl: 1ml
- Distilled water: 0.5ml

- **1X Washing Buffer (20ml)**

- 1M Tris-HCl, pH 7.2: 400ul
- 0.5M EDTA, pH 8.0: 2.0ml
- 100mM PMSF: 200ul

Adjust the volume with sterile reagent grade water to 20ml

- **Proteinase K Solution (50ml)**

- Proteinase K: 50mg
- 0.5M EDTA, pH 8.0: 10ml
- Na-deoxycholate: 0.1gm
- Na-laurylsarcosine: 0.5gm

- **Plasmid lysis buffer**

- SDS: 1500mg
- Tris: 304mg
- NaOH: 1 and half tablet

Adjust the volume with sterile water to 50

Part 6

Results

6. Results

Diarrheal episodes caused by DEC are a major public concern as they are associated with high rates of mortality and morbidity in children less than 5 years of age particularly in developing countries including India (Gomes *et al.,* 2016; Lakshminarayanan *et al.,* 2015). In 2008, 4.2 million pediatric diarrheal deaths which is equivalent to half of the diarrheal deaths were reported in 5 countries such as India, Pakistan, Nigeria, China and Congo (Black *et al.,* 2008; Ghosh *et al.,* 2021). The rise of pediatric diarrheal cases from 9% to 9.2% from the year 2016 to 2020 in India has been indicated in reports from The National Family Health Survey, thus making it as one of the major public health issues in India (Lakshminarayanan *et al.,* 2015; Ghosh *et al.,* 2022).

Moreover, the rise of multidrug resistant DEC is a major public concern since it is very difficult and challenging to deal with such infections due to the spread of antibiotic resistance against the first-line antimicrobial drugs among different *E. coli* pathotypes. Although antibiotics help in curing severe infections, their excessive use has resulted in the spread of antibiotic resistance all over the world. Antimicrobial susceptibility profile suggests that the isolates have acquired greater resistance towards fluoroquinolone group of antibiotics followed by ampicillin and tetracycline. As the strains are gaining antibiotic resistance genes through horizontal gene transfer and emerging as highly pathogenic forms, it is necessary to monitor their changing susceptibility pattern to the latest generation antibiotics and their prevalence in diarrheal patients.

DEC remains the most common cause of illness and death among children in developing countries (Takahashi *et al*., 2008; Dutta *et al*., 2013). ETEC and *Shigella* have been identified as the two major pathogenic agents of pediatric diarrhea in African and Asian countries (Levine *et al.,* 2012). *E. coli* is generally known as non-pathogenic facultative flora of the human intestine. However, in the immune-suppressed host, or when gastrointestinal barriers are violated, even normal “nonpathogenic” strains of *E. coli* can cause infection by acquiring the virulence factors necessary for infection (Nataro *et al*., 1998; Croxen *et al*., 2010). DEC pathotypes have resulted in several outbreaks worldwide. Thus in order to check DEC epidemics it is very important to monitor the characteristics and prevalence of the circulating strains. DEC associated diarrhea is a crucial threat to the Indian children population making it evident to decipher new preventive strategies in order to reduce child deaths in different parts of India (Ghosh *et al.,* 2021; Ghosh *et al.,* 2022).

Objective 1:

Isolation and identification of enteric *E. coli* (Diarrheagenic and commensal) isolated from diarrheal stool samples.

6.1. Prevalence of DEC in Kolkata, India

Since Kolkata is one of the diarrhea endemic regions, we have focused on analyzing the trends in prevalence of the different pathotypes of DEC among the hospitalised diarrheal patients in Kolkata. In our study, 3 pathogenic types of DEC (Enteroaggregative *E. coli*, Enteropathoigenic *E. coli* and Enterotoxigenic *E. coli*) were considered as the major categories of DEC due to their high prevalence in Kolkata. Another rare form of DEC known as enteroinvasive *E. coli* (EIEC) was also isolated in our study. These 4 varieties of DEC were collected from 2 different hospitals: 1) ID&BG Hospital (IDH) that is located on the campus where critical diarrheal patients are admitted and 2) B. C Roy Hospital which is located outside the campus and where only children from 0- 5 years of age are treated as out-patient (OPD).

6.1.1. ETEC was most prevalent in patients of ID&BG hospital

The diarrheal surveillance data from ID&BG hospital during 2008-2011 has revealed the predominance of EAEC pathotype among the diarrheal patients. Thus, we were further interested in finding the trends in the population of DEC pathotypes during the recent years. During an 11-year diarrheal disease surveillance period from 2012 to 2022, a total of 9501 patients were admitted to the ID&BG hospital for this study. Based on the selection strategy, this number represents almost one-third of the total number of patients who were treated in the hospital. The patients admitted to ID&BG hospital include both children as well as the adult population of different age groups.

DEC was identified in 7.5% (710/9501) of the patients among which ETEC (47.5%) was most prevalent followed by EAEC and EPEC pathotypes (38.8% and 13.7%, respectively) (**Table R1**). The prevalence of DEC was higher in the years 2015 and 2016. Stool samples were collected for only 3 months (Jan-Mar) in the year 2020 due to COVID-19 outbreak. As ID&BG hospital was one of the main centers of treatment of COVID-19 infected patients in Kolkata, no stool samples were collected in the year 2021 and the collection resumed only after August 2022, thus there were no samples during 2021 and fewer isolates in 2022.

Table R1: *Year-wise prevalence pattern of different DEC pathotypes isolated from patients during 2012-2022 from ID&BG Hospital.*

Year	No. of DEC	No. of ETEC	No. of EPEC	No. of EAEC
2012	93	37 (39.8)	14 (15.0)	42 (45.2)
2013	57	25 (43.9)	8 (14.0)	24 (42.1)
2014	90	39 (43.3)	17 (18.9)	34 (37.8)
2015	152	77 (50.7)	26 (17.1)	49 (32.2)
2016	109	56 (51.4)	5 (4.6)	48 (44.0)
2017	53	37 (69.8)	4 (7.6)	12 (22.6)
2018	83	34 (41.0)	12 (14.4)	37 (44.6)
2019	53	24 (45.3)	10 (18.9)	19 (35.8)
2020	11	2 (18.2)	1 (9.1)	8 (72.7)
2021	0	0	0	0
2022	9	6 (66.7)	0	3 (33.3)
Total (%)	**710**	**337 (47.5)**	**97 (13.7)**	**276 (38.8)**

Our study (2012-2022) result differed from the previous (2008-2011) study obtained from diarrheal patients from ID&BG hospital (Dutta *et al*., 2013). In the previous study, the rate of EAEC infected patients was higher in number followed by ETEC and EPEC infected patients. In our present study, the number of ETEC infected cases was higher followed by EAEC and EPEC infected cases. Therefore, it is evident from this study that ETEC is emerging as the most significant DEC pathotype and affecting the human population in Kolkata, India during the current decade.

DEC was identified as the sole pathogen in about 72.9% (518/710) of the patients while 27.1% (192/710) of the patients had mixed pathogens. Other enteric bacteria such as *Vibrios, Shigellae, Salmonellae, Campylobacters* and *Aeromonads* were identified in the mixed infection category. *Vibrio spp.* was more prevalent in the mixed infection category followed by *Campylobacter spp.* and *Shigella spp.* The association of *Aeromonas sp.* was rare with DEC infection (**Tables R2-R4**). Among the ETEC isolates, 73.29% of the infections were caused by sole ETEC pathogen i.e., the samples consisted of only ETEC isolates while 26.70% of ETEC infection was found in association with other enteric bacteria like *Shigella, Salmonella, Vibrio, Campylobacter* and *Aeromonas*. *Vibrio spp.* remained as the most prevalent enteric pathogen in association with the ETEC isolates (**Table R2**). In the case of

EPEC isolates, 75.3% of the stool samples consisted of solely EPEC pathogens while 24.7% of the isolates comprised of EPEC which were associated with other enteric bacteria. Similarly, like the ETEC isolates, *Vibrio sp.* remained dominant among all the enteric pathogens (**Table R3**). Among the EAEC isolates, 71.74% of the stool samples consisted of EAEC as the sole pathogen whereas 28.26% of the samples consisted of EAEC along with other enteric bacteria. Here also, *Vibrio spp.* remained the major pathogen among all other enteric pathogens in association with EAEC infections (**Table R4**).

When the rate of sole (individuals affected with DEC as the only pathogen) and mixed (individuals affected with DEC along with other enteric bacteria) DEC infections among the 3 categories of DEC was compared, it was found that sole infection rate among the 3 categories of DEC were higher than the mixed infection rate among these 3 categories (**Figure R1**).

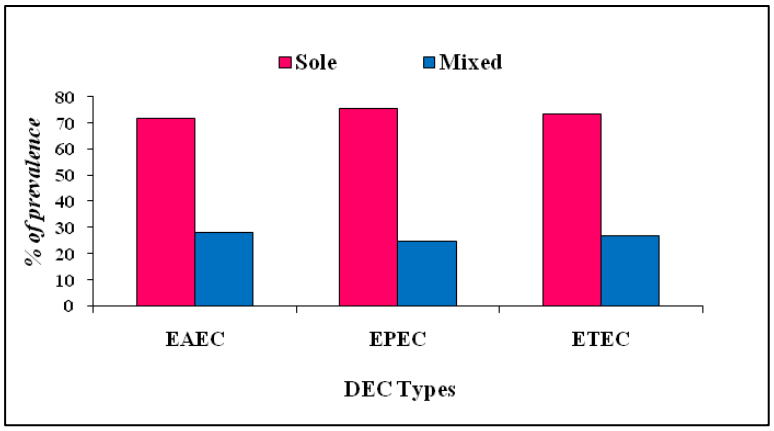


Figure R1: *Prevalence of sole and mixed infection rate of the DEC pathotypes from ID&BG Hospital. Sole infection rate was higher than mixed infection rate among all the DEC pathotypes.*

Table R2: *Prevalence of ETEC and other pathogens (enteric bacteria) as co-pathogens in patients with diarrhea.*

Year	No of DEC positive samples	No of ETEC	Sole pathogen	Mixed Pathogens	Mixed pathogens (other bacteria) in combination with ETEC
2012	93	37	25	12	ETEC+*Vibrio*= 9, ETEC + *Shigella*= 3
2013	57	25	15	10	ETEC+Vibrio=6, ETEC + *Campylobacter*=2 ETEC+*Vibrio* (NAG)+*Campylobacter*=1 ETEC+*Vibrio*+*Campylobacter*=1
2014	90	39	26	13	ETEC+*Vibrio*=9, ETEC + *Campylobacter*=2 ETEC+*Shigella*=1 ETEC+*Vibrio*(NAG)+*Campylobacter*=1
2015	152	77	60	17	ETEC+*Vibrio*=10, ETEC + *Campylobacter*=3 ETEC+*Shigella*=2 ETEC+*Vibrio*(O1)+*Campylobacter*=1 ETEC+*Vibrio* (O1)+*Shigella*=1
2016	109	56	44	12	ETEC+*Vibrio*=6, ETEC+*Shigella*=3,ETEC+*Vibrio*(O1)+ *Campylobacte*r=1 ETEC+*Vibrio*(O1)+ NAG+*Campylobacter*=1 ETEC+*Vibrio*(O1)+*Campylobacter*=1
2017	53	37	24	13	ETEC+*Vibrio*=7, ETEC+*Campylobacter*=3 ETEC+*Shigella*=1 ETEC+*Vibrio*(O1)+*V. fluvialis*=1, ETEC+*Campylobacter*=1
2018	83	34	26	8	ETEC+*Vibrio*=3 ETEC+*Aeromonas*=1 ETEC + *Campylobacter*= 2 ETEC+*Vibrio*(O1)+*Vibrio*(NAG)+ *Campylobacter*=1 ETEC+*Vibrio*(O1)+*Campylobacter*=1
2019	53	24	20	4	ETEC+*Vibrio*=2, ETEC+*Campylobacter*=2
2020	11	2	2	0	
2021	0	0	0	0	
2022	9	6	5	1	ETEC+*Salmonella*=1
Total	**710**	**337**	**247**	**90**	

Table R3: *Prevalence of EPEC and other pathogens (enteric bacteria) as co-pathogens in patients with diarrhea.*

Year	No of DEC positive samples	No of EPEC	Sole pathogen	Mixed Pathogens	Mixed pathogens in combination with EPEC
2012	93	14	12	2	EPEC+*Vibrio*=2
2013	57	8	7	1	EPEC+*Vibrio*=1
2014	90	17	12	5	EPEC+*Vibrio*=4, EPEC+*Campylobacter*=1
2015	152	26	17	9	EPEC+*Vibrio*=6, EPEC+*Campylobacter*=1 EPEC+*Shigella*=1, EPEC+*Aeromonas*+*Salmonella*=1
2016	109	5	4	1	EPEC+*Campylobacter*=1
2017	53	4	2	2	EPEC+*Vibrio*=1, EPEC+*Campylobacter*=1
2018	83	12	9	3	EPEC+*Vibrio*=1, EPEC+*Aeromonas*=1 EPEC+*Campylobacte*r=1
2019	53	10	9	1	EPEC+*Vibrio*=1
2020	11	1	1	0	
2021	0	0	0	0	
2022	9	0	0	0	
Total	710	97	73	24	

Table R4: *Prevalence of EAEC and other pathogens (enteric bacteria) as co-pathogens in patients with diarrhea.*

Year	No of DEC positive samples	No of EAEC	Sole Pathogen	Mixed Pathogens	Mixed pathogens in combination with EAEC
2012	93	42	30	12	EAEC+*Vibrio*=7, EAEC+*Aeromonas*=1 EAEC+*Shigella*=2,EAEC+*Vibrio*(O1)+*Shigella*=1 EAEC+*Vibrio*(O1)+*Campylobacter*=1
2013	57	24	21	3	EAEC+*Vibrio*=3
2014	90	34	13	21	EAEC+*Vibrio*=1, EAEC+*Campylobacter*=2, EAEC+*Shigella*=2, EAEC+Rotavirus=14, EAEC+*Campylobacter*+*Shigella*=1, EAEC+*Vibrio* (O1)+ *Campylobacter*=1
2015	152	49	37	12	EAEC+*Vibrio*=7, EAEC+*Shigella*=4, EAEC+*Vibrio*(O1)+*Vibrio*(NAG)=1,
2016	109	48	33	15	EAEC+*Vibrio*=10, EAEC+*Campylobacter*=2 EAEC+*Shigella*=2, EAEC+*Vibrio*(O1)+*Campylobacter*=1
2017	53	12	10	2	EAEC+ *Shigella*=1, EAEC+*Vibrio*(O1)+*Vibrio*(NAG)=1
2018	83	37	29	8	EAEC+*Vibrio*= 3, EAEC+*Aeromonas*=2, EAEC+*Campylobacter*=1, EAEC+*Vibrio*(O1)+*Campylobacter*=2
2019	53	19	16	3	EAEC+*Vibrio*= 1, EAEC+*Shigella*=1, EAEC+*Salmonnella*=1
2020	11	8	7	1	EAEC+Vibrio=1
2021	0	0	0	0	
2022	9	3	2	1	EAEC+*Salmonella*=1
Total	710	276	198	78	

When the presence of typical isolates was monitored among the ETEC infected cases, it was found that 54% (183/337) of the isolates contained *elt* as well as *est*. Remaining 46% of the ETEC carried either *est* or *elt*, respectively. Among the EAEC infected cases, 15% (41/276) of the isolates harbored both *aaiC* as well as *aat* gene while 85% (235/276) of the isolates contained only *aaiC* or *aat* respectively. 23.7% (23/97) of the EPEC isolates contained both *bfpA* and *eae* gene while 76.3% of the EPEC isolates contained any of the genes among *bfpA*

and *eae*. The DEC isolates that contained both the virulence genes were regarded as typical isolates while those that contained any one of the virulence genes were regarded as atypical isolates (**Figure R2**).

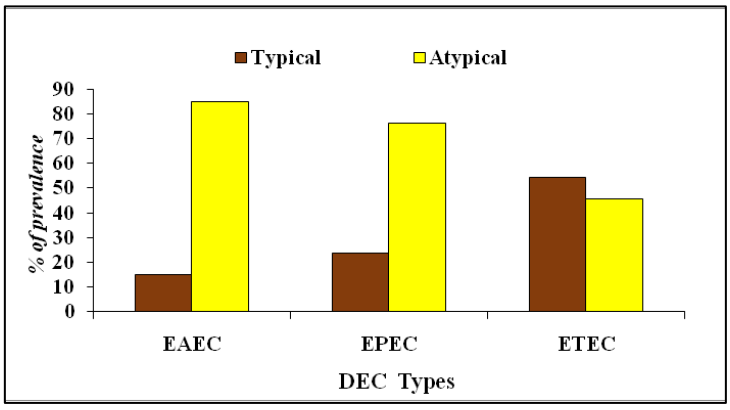


Figure R2: *Graphical representation of typical and atypical isolates among the 3 different DEC isolates (EAEC, EPEC & ETEC). Most of the EAEC and EPEC isolates belong to the atypical category while most of the ETEC isolates belong to the typical category harbouring both the virulence genes.*

Month-wise isolation rate of DEC from the year 2012-2022 showed a seasonal variability of DEC isolates with the highest number of isolates in monsoon season (July and August). The isolation rate was relatively low during the winter season, i.e., December, January (**Figure R3**).

Due to the onset of COVID-19 outbreak, sample collection was discontinued from April 2020 to July 2022. Therefore, the number of samples represented in the graph was relatively lower than in the previous years.

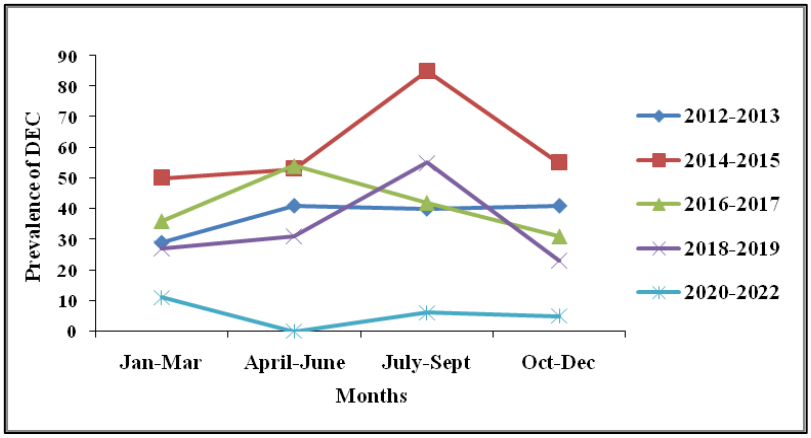


Figure R3: *Month-wise distribution pattern of DEC isolates from the year 2012 to 2022. A higher number of DEC isolates were found during the monsoon season while the number decreased during the winter season.*

6.1.2. EAEC strains were found with a higher rate among patients of B. C. Roy Hospital

Since DEC is one of the potential causes of child death all over the world, we have also focused on finding the prevalence and impact of the DEC pathotypes in the children population of India. During a 5-year study from the year 2018-2022 a total of 5112 stool samples were collected from B. C. Roy hospital where children from the age 0-5 years were treated. Among 5112 stool samples, 443 were found to be DEC positive in which EAEC (67%) remained as the dominant DEC pathotype followed by EPEC (17.2%) and ETEC (15.8%) pathotypes (**Tables R5**). More number of DEC was isolated during the years 2018 and 2019. The prevalence of DEC was very low in 2020 as stool samples were collected for only 3 months (January-March) due to COVID-19 outbreak. The normal flow of sample collection resumed from January 2021 onwards.

From this B. C. Roy hospital prevalence data, it is clear that the children population was mostly affected by EAEC pathotype of DEC. The ID&BG hospital data on the contrary highlighted the dominance of ETEC pathotype among the higher age groups. Thus our study highlighted that in the population of Kolkata, EAEC predominates among the children while ETEC dominates in the higher age groups. A regular surveillance study should be carried out to get an idea of the changing patterns of prevalence of DEC pathotypes in the population of Kolkata.

Table R5: *Year-wise prevalence pattern of different DEC pathotypes isolated from patients during 2018-2022 from B.C. Roy Hospital.*

Year of isolation	No of DEC	No of ETEC	No of EPEC	No of EAEC
2018	140	20(14.3)	26 (18.7)	94(67.2)
2019	146	22(15.1)	29 (19.8)	95 (65.1)
2020	30	1 (3.3)	8 (26.7)	21 (70)
2021	83	20 (24.1)	10 (12)	53(63.9)
2022	44	7 (11.9)	3 (6.8)	34 (77.27)
Total	**443**	**70 (15.8)**	**76 (17.2)**	**297 (67.0)**

Among the total DEC isolated during 2018-2022, DEC as sole pathogen was identified in about 86% (381/443) of the patients while 14% (62/443) of the patients had mixed DEC

infections. Other enteric bacteria such as *Vibrios, Shigellae, Salmonellae, Campylobacters* and *Aeromonads* were identified in the mixed infection category. *Vibrio spp.* was the most prevalent pathogen in the mixed infection category followed by *Campylobacter spp* and *Shigella spp*. Here also the association of *Aeromonas sp.* was rare with DEC infections (**Tables R6-R8**).

Among the patients infected with ETEC, 77.14% of the infections were caused due to sole ETEC infections whereas 22.86% of the infections were due to mixed infections caused by ETEC along with other enteric pathogens such as *Vibrios, Shigellae, Salmonellae, Campylobacters* and *Aeromonads.* Among the other enteric pathogens that were found in association with ETEC, *Vibrio spp* remained the major bacteria followed by *Shigella spp.* The detailed list of pathogens in association with ETEC is provided below in **Table R6**. A higher association of *Vibrio spp* with ETEC was also seen in the case of the ID&BG isolates where almost 57% of the mixed ETEC infections showed an association with *Vibrio spp.*

Table R6: *Prevalence of ETEC and other pathogens (enteric bacteria) as co-pathogens in patients with diarrhea from B.C. Roy Hospital.*

Year	No of DEC positive samples	No of ETEC	Sole pathogen	Mixed Pathogens	Mixed pathogens (other bacteria) incombination with ETEC
2018	140	20	16	4	ETEC+*Shigella*=1 ETEC + *Campylobacter*= 1 ETEC+*Vibrio*(NAG) =1 ETEC+*Vibrio*(O1)=1
2019	146	22	18	4	ETEC+*Vibrio*=1, ETEC+*Campylobacter*=2 ETEC+*Shigella*=1
2020	30	1	1	0	
2021	83	20	14	6	ETEC+*Vibrio*=3 ETEC+*Shigella*=3
2022	44	7	5	2	ETEC+*Vibrio*(O1)=2
Total	443	70	54	16	

Among the EPEC infected cases, 79% of the infections were caused due to sole EPEC pathogen while 21% of the cases were caused due to mixed infections of EPEC along with other bacteria such as *Shigella, Salmonella, Vibrio, Campylobacter* and *Aeromonas.* Among the different pathogens in association with EPEC *Campylobacter sp* served as the major enteric bacteria in association with EPEC followed by *Shigella sp.* (**Table R7**).

In this study, EAEC infections were mostly caused by the sole EAEC infections. Almost 90% of the infected cases were due to sole EAEC infections while only 10% of the cases were due to mixed DEC infections. Among the various enteric bacteria, *Campylobacter* was predominant followed by *Shigella* and *Vibrio sp* which remained associated with EAEC pathotype (**Table R8**). Thus, from the above result, it can be stated that *Campylobacter sp.* was the major enteric pathogen that remained associated with the EAEC and EPEC pathotype isolated from B. C. Roy hospital.

Table R7: *Prevalence of EPEC and other pathogens (enteric bacteria) as co-pathogens in patients with diarrhea from B.C. Roy Hospital*.

Year	No of DEC positive samples	No of EPEC	Sole pathogen	Mixed Pathogens	Mixed pathogens (other bacteria) in combination with EPEC
2018	140	26	17	9	EPEC+*Shigella*=3 EPEC + *Campylobacter*= 5 EPEC+A*eromonas* =1
2019	146	29	26	3	EPEC+ A*eromonas* =1, EPEC+*Campylobacter*=2
2020	30	8	8	0	
2021	83	10	8	2	EPEC+ *Campylobacter*=1 EPEC+ A*eromonas* =1
2022	44	3	1	2	EPEC+*Shigella*=2
Total	443	76	60	16	

Table R8: *Prevalence of EAEC and other pathogens (enteric bacteria) as co-pathogens in patients with diarrhea from B.C. Roy Hospital.*

Year	No of DEC positive samples	No of EAEC	Sole pathogen	Mixed Pathogens	Mixed pathogens (other bacteria) in combination with EAEC
2018	140	94	80	14	EAEC+*Shigella*=3 EAEC + *Campylobacter*= 6 EAEC+A*eromonas* =1 EAEC+NAG=3 EAEC+*Campylobacter*+*Vibrio*=1
2019	146	95	89	6	EAEC+*Shigella* =1, EAEC+*Campylobacter*=5
2020	30	21	20	1	EAEC+ *Campylobacter*=1
2021	83	53	45	8	EAEC+ *Shigella*=3 EAEC+ A*eromonas* =3 EAEC+*Vibrio*=2
2022	44	34	33	1	EAEC+*Shigella*=1
Total	443	297	267	30	

When the rate of sole and mixed DEC infections among the 3 categories of DEC were compared, it was found that sole and mixed infection rate with EAEC was higher compared to that of ETEC and EPEC (**Figure R4**). Among the EAEC infected cases it was seen that the rate of sole infections was greater (90%) as compared to that of mixed infection rate (10%). In the case of ETEC, only 22.86% of the isolates remained in association with other enteric bacteria while the rest 77.14% of the ETEC infected cases were due to sole ETEC infections. Similarly, 21% of the EPEC infected cases were in association with other enteric pathogens while 79% of EPEC cases were due to sole EPEC infections.

Among the ETEC infected cases, almost 44% (31/70) of the isolates contained *elt* as well as *est*. The remaining 56% of the ETEC isolates carried either *est* or *elt*, respectively. Among the EAEC infected cases, almost 20% (62/297) of the isolates harbored both *aaiC* as well as *aat* gene while 80% (235/297) of the isolates contained only *aaiC* or *aat* respectively. 33% (25/76) of the EPEC isolates contained both *bfpA* and *eae* genes while 67% of the EPEC isolates contained any of the genes among *bfpA* and *eae*. The DEC isolates that contained both the virulence genes were regarded as typical isolates while those that contained any one of the virulence genes were regarded as atypical isolates. The detailed data is represented in graphical format below (**Figure R5**).

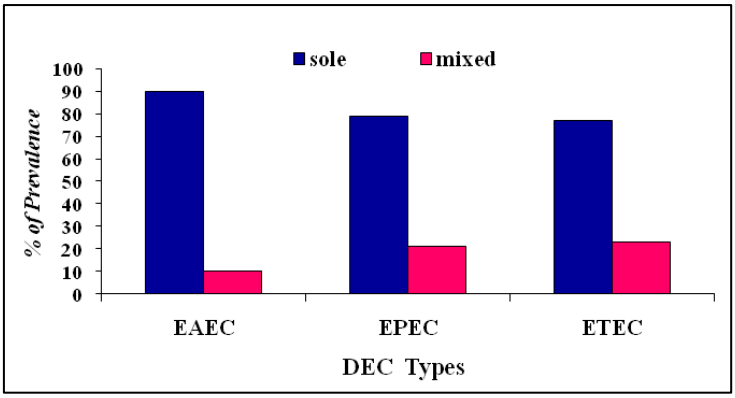


Figure R4: *Prevalence of sole and mixed infection rate of the DEC pathotypes. Sole and mixed infection rate with EAEC is higher compared to that of ETEC and EPEC.*

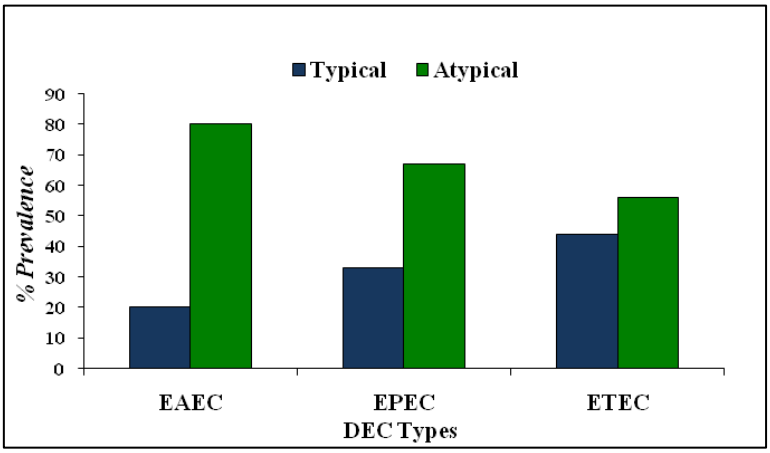


Figure R5: *Graphical representation of typical and atypical isolates among the 3 different DEC isolates (EAEC, EPEC & ETEC). Most of the 3 categories of DEC isolates belonged to the atypical type i.e. harbouring only one of the virulence genes.*

When the month-wise isolation rate of DEC was analyzed for the years 2018-2022 it showed a seasonal variability of DEC isolates with the highest number of isolates in July and August during 2018 and 2019. During the years 2020-2022, the isolation rate showed a gradual decrease from July month onwards and reached almost zero during the winter months. The isolation rate was relatively low during the winter season, i.e., during December-January (**Figure R6**).

Due to COVID-19 outbreak, samples were collected only for January, February and March 2020 and resumed in January 2021. Thus, the sample number was low during 2020-2022.

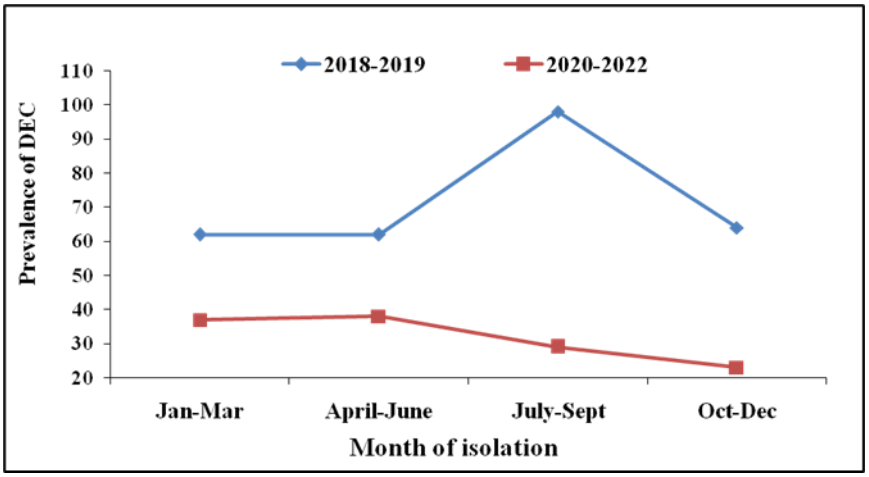


Figure R6: *Month-wise distribution pattern of DEC isolates obtained from B. C. Roy hospital during the year 2018 to 2022. The monsoon months (July and August) consist of a higher number of DEC isolates.*

The seasonality pattern was similar to the seasonality pattern seen in case of ID&BG isolates showing a higher prevalence of DEC isolates during the monsoon months and low in the winter months. This result further highlighted the fact that the poor hygienic condition and unsafe drinking water during the monsoon season has resulted in DEC predominance.

6.1.3 Commensal *E. coli* isolates co-existed with DEC

Since *E. coli* is one of the common colonizers of the gastrointestinal tract of both children and adults we have focused on the isolation of these commensal bacteria as they have potential role in the transfer of genetic material and spread of antibiotic resistance. The study for commensal screening was performed during the period of April 2018 to October 2018 as the facilities were limited. A total of 556 commensals were isolated within that period (**Figure R7**). It had also been found that the prevalence of commensal *E. coli* was more than the DEC isolates in hospitalized diarrheal patients as commensal *E. coli* belongs to the normal flora of the gut.

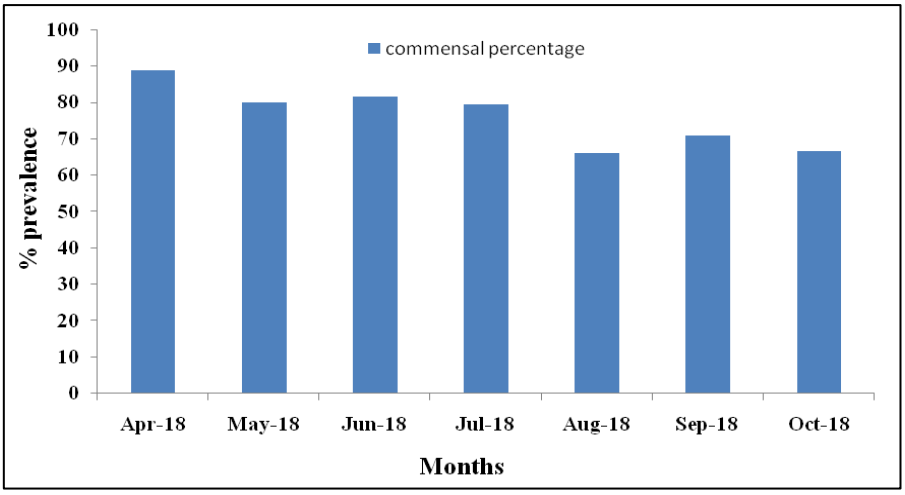


Figure R7: *Percentage prevalence of commensal E. coli from April2018 to October2018. The percentage of isolation of commensal E. coli remained high during the summer and monsoon months and gradually started decreasing after the monsoon season.*

6.1.4 Both the LF and NLF forms of EIEC were prevalent in Kolkata, India

Like *Shigella*, EIEC is also known to cause bacillary dysentery, but the clinical symptom caused by EIEC is less severe when compared to *Shigella*. The pathogenicity of EIEC is due to the presence of pINV plasmid (F plasmid) which is similar with that found in *Shigella*. Since EIEC is a very rare type of DEC isolated from India, we have focused on finding the prevalence and characterization of EIEC isolated from ID&BG and B. C. Roy hospital, Kolkata. EIEC isolates are mainly non lactose fermenting in nature but we have isolated some of lactose fermenting isolates harboring the *ipaH* gene which is the main gene for EIEC identification (**Figure R8**).

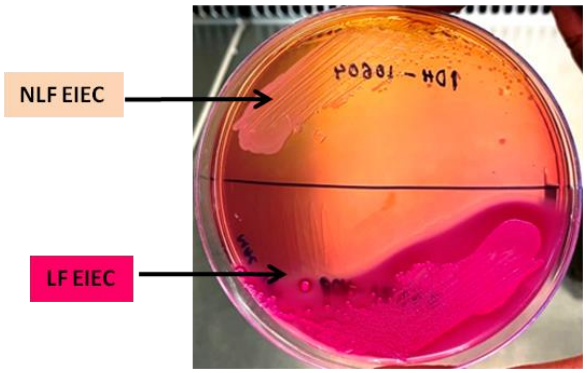


Figure R8: *MAC plate showing both the NLF and LF EIEC isolates. The pale-yellow colonies formed on the MAC plate indicate NLF E. coli while the pink coloured colonies indicate the presence of LF E. coli isolates.*

In case of the EIEC isolates, the prevalence was found during the year 2016-2022. A total of 13,682 patients were admitted in ID&BG and B.C. Roy Hospital from the year 2016-2022 among which 7.45% (1020/13682) were DEC positive in which 1.5% (15/1020) were EIEC positive. Out of the 15 EIEC positive strains, 40% (6/15) of the strains were lactose fermenting while 60% (9/15) of the strains were non lactose fermenting (**Table R9**).

Table R9: *Prevalence of EIEC isolates from ID&BG Hospital and B. C. Roy Hospital.*

Year	Total stool collected	Total no. of DEC isolated	No of EIEC isolated	LF EIEC	NLF EIEC
2016	2565	261	4	0	3
2017	2922	153	5	0	5
2018	2583	222	1	1	0
2019	2631	198	3	3	0
2020	1111	44	1	0	1
2021	572	82	1	1	0
2022	1298	60	1	1	0
Total	13,682	1020 (7.45%)	15 (1.5%)	6 (40%)	9 (60%)

Despite several worldwide reports on the prevalence of EIEC, there is no study regarding their virulence from India. Thus, the aim of our study is to characterize the virulence profile of both the LF as well as the NLF-EIEC isolates from India. Our study focused on the molecular determinants of invasiveness, intracellular growth and spreading of EIEC isolates in both *in-vivo* and *in-vitro* model environments.

EIEC and *Shigella* isolates are very difficult to differentiate based on the different biochemical methods that are available. However, in TSI, the LF EIEC isolates produced an acidic slant and an acidic butt (A/A reaction) with gas production and the NLF isolates were found to show alkaline slant and acidic butt (K/A reaction) without gas production (**Figure R9**). Other biochemical tests such mucate, salicin, esculin and acetate fermentation were negative for all the EIEC isolates (**Table R10**).

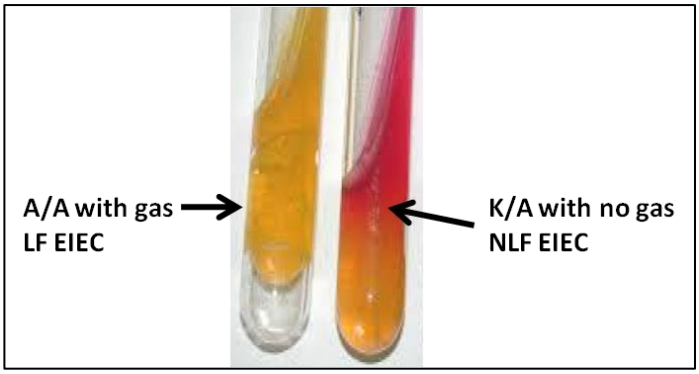


Figure R9: *TSI result of LF and NLF EIEC isolates. LF EIEC showed A/A reaction with gas formation while NLF EIEC yielded K/A reaction without gas formation. In case of LF EIEC we find yellow slant and yellow butt with gas production and NLF EIEC resulted in the formation of red slant and yellow butt without gas production.*

EIEC and *Shigella* are very difficult to distinguish due to their similar phenotypic, genotypic and invasive characteristics. Thus, we have tried to identify the strains based on serology and VITEK 2. EIEC generally have 14 major *E. coli* O-antigenic serogroups that include O28ac, O29, O112ac, O121, O124, O135, O136, O143, O144, O152, O159, O164, O167 and O173 and all are devoid of the flagellar (H) antigen. None of the EIEC isolated in this study cross-reacted with the *Shigella* antisera. Of the nine NLF EIEC strains 44.44% (n=4) of the strains belonged to O125 and 55.56% (n=5) were untypable. Out of the six LF EIEC strains, 33.33% (n=2) were untypable while other 66.67% of the strains belonged to O125 (n=1), O136 (n=1), O152 (n=1) and O28ac (n=1) serogroups (**Table R10**).

VITEK 2 machine performs bacterial identification based on certain biochemical reactions such as the variable capacity of the organism to ferment different salts (glucose, maltose and lactose) and other reactions. In case of the NLF isolates, the VITEK 2 showed them to be ~97% similar with *Shigella spp.* while the LF isolates it showed that they were 99% similarity to *E. coli* species based on the chemical reactions.

Table R10: *Detailed methods of differentiation of EIEC isolates*

Strain	Serogroup	TSI result	VITEK 2 result	Virulence genes	Mucate/ Acetate/ Salicin/ Esculin
BCH-13316 (LF)	ONT	A/A no gas	99% probability *E. coli*	*ipaH, lacY, ipaBCD, ial, virF*	Negative
BCH-10711 (LF)	O136	A/A no gas	99% probability *E. coli*	*ipaH, lacY, ipaBCD, ial, virF, sig*	Negative
BCH-12902 (LF)	O28ac	A/A no gas	99% probability *E. coli*	*ipaH, lacY, ipaBCD, ial, virF, sig, sepA*	Negative
PT1 (LF)	O125	K/A no gas	50% probability *E. coli*	*ipaH, lacY, ipaBCD, ial, virF, sig, sepA*	Negative
BCH-10678 (LF)	O152	A/A no gas	99% probability *E. coli*	*ipaH, lacY, ipaBCD, ial, virF, sen, sig*	Negative
BCH-12109 (LF)	ONT	A/A with gas	99% probability *E. coli*	*ipaH, lacY, ipaBCD, ial, virF*	Negative
IDH-10604 (NLF)	ONT	K/A no gas	96% probability *Shigella* group	*ipaH, ipaBCD, ial, sen, sig*	Negative
IDH-9427 (NLF)	ONT	K/A no gas	98% probability *Shigella* group	*ipaH,ipaBCD, ial, virF, sen, sig, sepA*	Negative
BCH-8165 (NLF)	ONT	K/A no gas	99% probability *Shigella* group	*ipaH, virF, sen*	Negative
BCH-12034 (NLF)	O125	K/A no gas	97% probability *Shigella*	*ipaH, ipaBCD, sig, sepA*	Negative
IDH-10106 (NLF)	O125	K/A no gas	97% probability *Shigella*	*ipaH, sig*	Negative
IDH-10275 (NLF)	O125	K/A no gas	97% probability *Shigella* group	*ipaH,ipaBCD, ial, virF, sen, sig*	Negative
BCH-8159 (NLF)	O125	K/A no gas	99% probability *Shigella* group	*ipaH, ipaBCD, ial, virF, sen, sig*	Negative
IDH-9625 (NLF)	ONT	K/A no gas	96% probability *Shigella* group	*ipaH, ipaBCD, ial, sen, sig*	Negative
BCH-7181 (NLF)	ONT	K/A no gas	96% probability *Shigella* group	*ipaH,ipaBCD, ial, virF, sen, sig, sepA*	Negative

a) Clinical symptoms were more prominent in the patients infected with NLF EIEC

66.67% (10/15) of the patients infected with EIEC showed the presence of watery diarrhea while bloody mucoid diarrhea and loose diarrheal stool was reported in 13.33% (2/15) of the individuals and only 6.67% (1/15) individuals showed the presence of only mucoid stool without blood. 100% of the LF EIEC strains resulted in the formation of watery diarrhoeal stool in the individuals while only 44.44% of the NLF strains showed watery diarrhea and 44.44% and 11.12% of bloody mucoid and loose diarrheal stools respectively. Bloody mucoid stool was one of the vital symptoms observed solely in the case of NLF strains.

Fever, vomiting (4 times) and abdominal pain were seen only in 16.66% (1/6) of the LF strains. Fever was recorded in 55.56% (5/9) of individuals affected with NLF strains. Vomiting was reported in 77.78% (7/9) of the individuals infected with NLF strains while only 44.44% (4/9) of individuals affected with NLF strains suffered from abdominal pain. Severe dehydration status was documented in only 11.12% (1/9) of the patients infected with a NLF strain. 33.33% (2/6) of the LF strains and 55.55% (5/9) of the NLF strains showed to some extent dehydration. 33.33% (2/6) of the NLF strains showed no dehydration status while 66.67% of LF strains had "no dehydration" status (**Table R11**).

Table R11: *Clinical symptoms of patients suffering from EIEC infection. Symptoms include watery diarrhea along with fever, vomiting and abdominal pain.*

Attributes	**EIEC**	
	LF	**NLF**
Duration of diarrhea (before admission) [hrs.]	29.5 ± 1.9	34.2 ± 2.1
Duration of hospital stay [hrs.]	34.9 ± 1.8	41.2 ± 2.1
Dehydration status: [%]		
No	66.67%	33.33%
Some	33.33%	55.55%
Severe	0%	11.12%
Type of diarrhea: [%]		
Watery	100%	44.44%
Loose	0%	11.12%
Blood / Mucus	0%	44.44%
Presence of clinical symptoms: [%]		
Fever	16.67%	55.56%
Vomiting	16.67%	77.78%
Abdominal pain	16.67%	44.44%

b) Close genetic relatedness observed between LF and NLF-EIEC isolates

PFGE profiles were analyzed to find out the clonal relationship among the EIEC isolates. We found close genetic relatedness among the LF and NLF isolates. Almost 75% similarity was observed among the LF isolates and 70% among the NLF isolates (**Figure R10**). A few of the NLF strains showed 90% homology. Also, there is about 60% identity between the LF and NLF isolates which suggested that both the types share common genetic features.

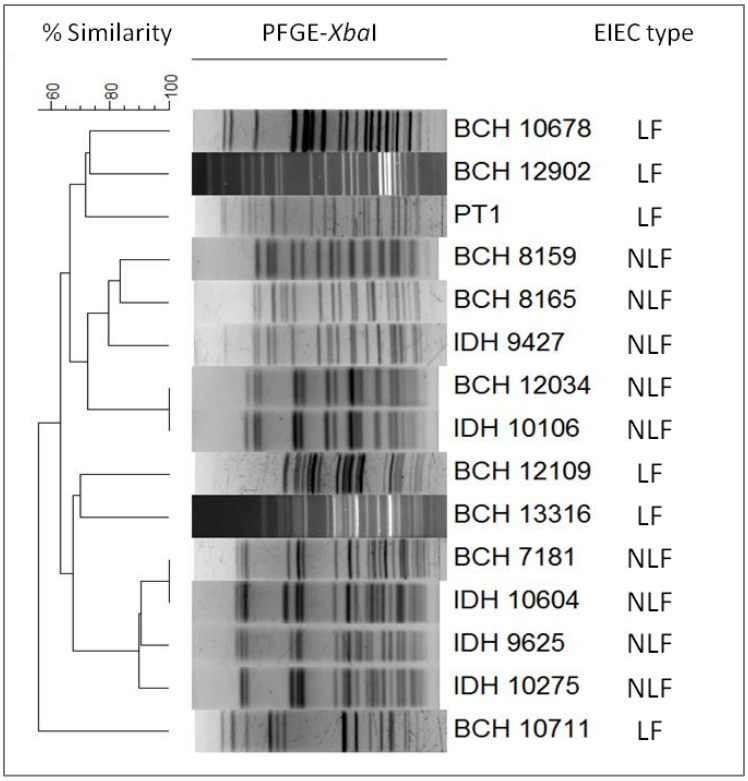


Figure R10: *Restriction fragment length polymorphism (RFLP) pattern of XbaI digested clinical LF and NLF EIEC isolates. Dendrogram showed about 60% similarity between the LF and NLF EIEC isolates indicating close clonal relationship among the isolates.*

c) LF isolates carried most of the virulence genes

Since the virulence genes are found among the *Shigella* sp. and both *Shigella* and EIEC share common genetic features, we investigated the presence of the virulence genes among the EIEC isolates. The detection of virulence genes revealed that 100% (n=6) of the LF EIEC isolates harbored the *ipaH*, *ial*, *virF* and *ipaBCD* genes while *sen*, *sig* and *sepA* were prevalent among 16.67% (n=1), 66.67% (n=4) and 33.33% (n=2) of the LF isolates, respectively. None of the LF EIEC isolates harbored the *pic* gene. Among the NLF isolates, all the 9 NLF strains harbored *ipaH* gene while *virF*, *ipaBCD*, *sen*, *sig* and *sepA* were positive among 55.56% (n=5), 77.78% (n=7), 77.78% (n=7), 66.66% (n=6) and 33.33% (n=3) of the NLF isolates respectively. Similar to the LF stains, the NLF strains also did not harbor the *pic* gene (**Figure R11**).

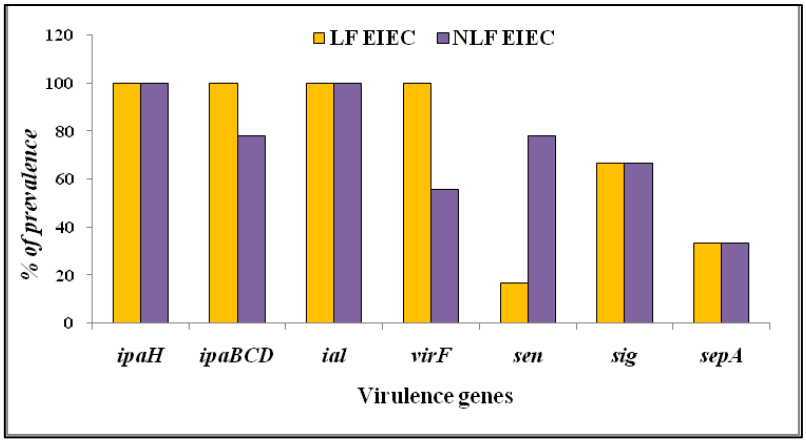


Figure R11: *Prevalence of virulence genes among the EIEC isolates. All the LF and NLF EIEC isolates harboured ipaH and ial genes while ipaBCD and virF were prevalent among the LF EIEC isolates.*

d) All the EIEC strains were non-motile

Since EIEC are generally non-motile like the *Shigella sp.* we have checked whether these rare LF EIEC isolates were motile. The motility was analyzed in 0.3% motility agar plates.

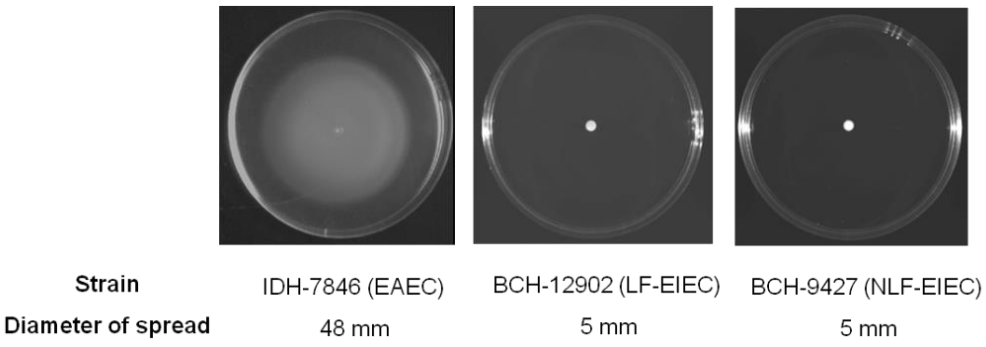


Figure R12: *Motility assay of the LF and NLF EIEC strains showed that all the LF and the NLF EIEC strains were non motile in nature. A clinical* enteroaggregative *E. coli (BCH-7846) isolate was taken as positive control.*

Motility analysis confirmed that all the EIEC strains irrespective of NLF and LF were non motile in nature (**Figure R12**). The diameter of spread of the LF and NLF isolates on the 0.3% motility agar plates were 5 mm for both which confirmed that the EIEC strains were non motile in nature.

e) All the EIEC stains harbored multiple plasmids

The invasive property of EIEC is due to the presence of a large pINV plasmid (~220 kb) which is present in all the *Shigella spp.* and carries all the genes required for invasion.

Therefore, we have checked the plasmid profile in order to see the presence of the pINV plasmid in the isolates. The plasmid profile of the EIEC strains revealed the presence of multiple plasmids in all the strains. The Image Lab software (Bio-Rad) predicted the sizes of the plasmids between 3.8 kb to 254.5 kb. The largest plasmid of ~220 kb was found in almost all the EIEC strains. Most of the LF isolates harbored multiple numbers of plasmids and between 2 to 7 in numbers whereas NLF contained 2 to 5 plasmids in a strain and were 3.8 kb to 223.3 kb in length (**Table R12**). The strains containing at least 2 plasmids were PT1 (LF), BCH-10678 (LF) and IDH-9427 (NLF). 3 plasmids were carried in IDH-10604 (NLF) and IDH-10275 (NLF). At least 4 plasmids were carried in strains 4 strains which were BCH-12109 (LF), BCH-12902 (LF), BCH-8165 (NLF) and BCH-12304 (NLF). Another 4 strains such as IDH-8159 (NLF), BCH-7181 (NLF), BCH-10711(LF) and IDH-10106 (NLF) harbored 5 plasmids. More than 6 plasmids were present in only BCH-13316 (**Figure R13**).

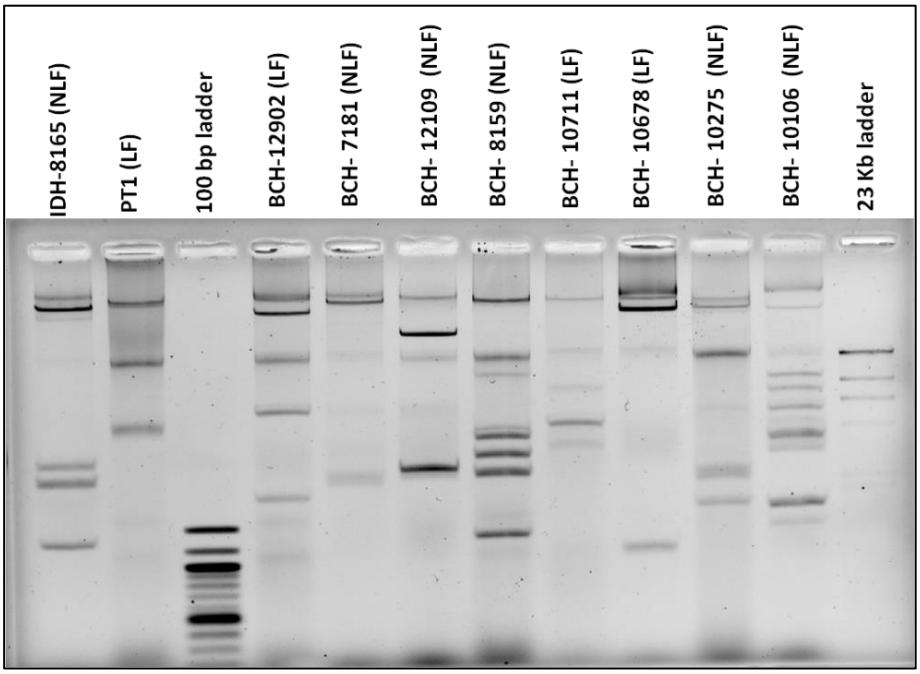


Figure R13: *The plasmid profiles of the LF and NLF EIEC strains. The Figure shows a representative number of EIEC strains whose plasmid profiles were generated. All the EIEC strains contained large plasmids required for virulence along with several other small plasmids.*

The multiple bands present in the EIEC strains are represented in the form of a table giving the detailed size and number of plasmids contained in each of the strains (**Table R12**).

Table R12: *Length of the plasmids carried by EIEC isolates and determined using the Image Lab software (BioRad).*

Strain	Size of the plasmids in kb						
	1st	2nd	3rd	4th	5th	6th	7th
BCH-13316 (LF)	236.1	114.3	35.3	25.0	15.6	9.6	4.2
BCH-10711 (LF)	239.3	93.2	24.3	13.3	7.2		
BCH-12902 (LF)	254.0	175.9	18.7	2.3			
PT1 (LF)	254.5	14.1					
BCH-10678 (LF)	180.8	17.9					
BCH-12109 (LF)	252.5	110.8	15.8	4.3			
IDH-10604 (NLF)	223.3	137.8	3.7				
IDH-9427 (NLF)	204.9	3.8					
BCH-8165 (NLF)	207.7	162.0	5.8	3.8			
BCH-12034 (NLF)	29.9	21.9	8.7	4.0			
IDH-10106 (NLF)	170.5	29.8	21.3	16.0	8.3		
IDH-10275 (NLF)	179.4	13.5	3.7				
BCH-8159 (NLF)	203.5	35.8	9.6	6.0	3.8		
IDH-9625 (NLF)	216.9						
BCH-7181 (NLF)	225.6	133.4	113.7	14.7	4.0		

f) Most of the virulence genes were present in the large plasmids

As the EIEC strains harbored several plasmids of varying lengths we were interested in finding the location of the different virulence genes in the different plasmids present in the strains. A representative number of strains were checked for the distribution of the virulence genes in the different plasmids and it was found that most of the virulence factors such as *icsA, icsB, ipaA, ipaC, ipaD, ipaH, virF* were located on the largest size plasmid of approximately 220 kb. The gene *ipaH* was found to be situated in the other plasmids as well in some of the strains such as BCH 10678 (LF) and BCH 8159 (NLF) (**Figure R14**).

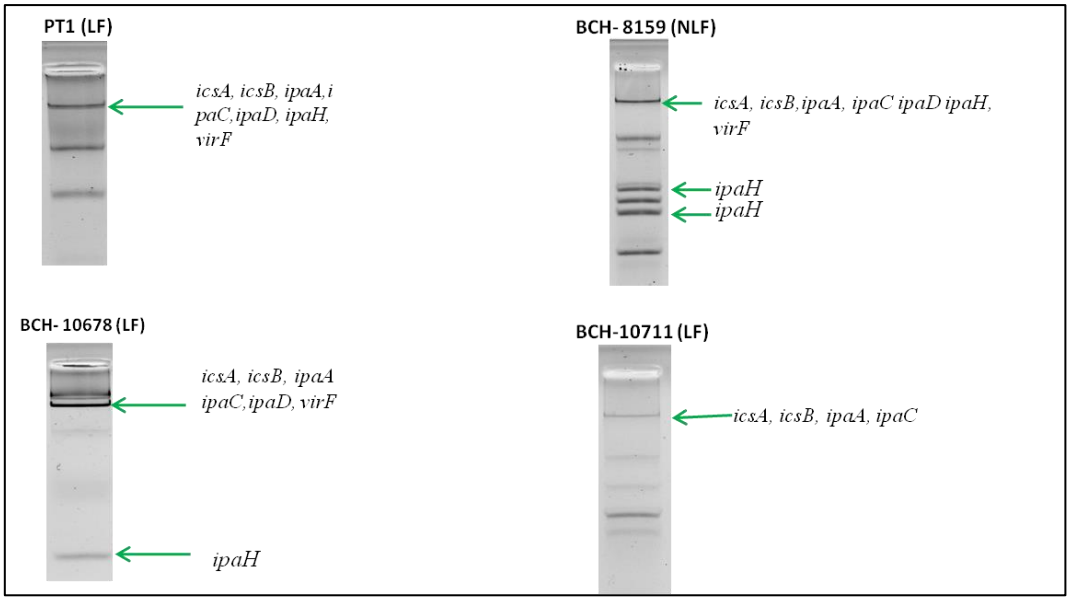


Figure R14: *Arrangements of virulence genes in the different plasmids present in the EIEC isolates. Most of the markers were found in the large plasmid sized >200kb and ipaH was found to be present in multiple plasmids for a few of the strains.*

g) Plaque assay: Cell to cell dissemination was poorly observed in both types of EIEC

Since the dissemination potential of *Shigella* spp. is determined by the help of plaque formation, we have used plaque formation assay to estimate the dissemination potential of the EIEC isolates. After 24 hrs of incubation, the Int 407 cells were stained the next day to visualize the plaque formation. There was no plaque formation in case of both the LF and NLF isolates. A *Shigella* strain was taken as a positive control where there was formation of plaques (**Figure R15**).

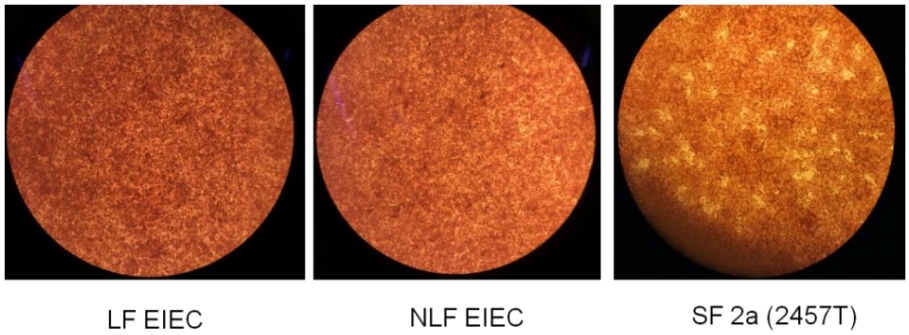


Figure R15: *Plaque assay showing extent of cell to cell spreading of EIEC and Shigella isolates. No particular plaque formation was observed in both the LF and NLF EIEC types. A Shigella flexneri 2a (2457T) strain was taken as positive control.*

h) LF EIEC strains were more invasive than the NLF EIEC strains

As the EIEC and *Shigella* strains are reported to be invasive in nature, we focused on determining the invasive potential of these EIEC isolates. During bacterial invasion, it was seen that the invasive properties of the LF strains were more than the NLF EIEC strains. After 2 hours of infection, the Int 407 cellular layer was lysed to recover the number of intercellular bacteria. The CFU (colony forming units) count of the LF EIEC strains was much higher than the NLF EIEC strains. After 2 hrs we observed $1.56x10^6$ CFU/well, $1.89x10^6$ CFU/well, $4.4x10^2$ CFU/well, $0.9x10^6$ CFU/well, $7.45x10^6$ CFU/well, $1.55x10^5$ CFU/well in case of the 6 LF EIEC strains whereas $4.5x10^2$ CFU/well, $0.35x10^2$ CFU/well, $3.3x10^2$ CFU/well, $1.85x10^6$ CFU/well, $2x10^2$ CFU/well, $5.5x10^3$ CFU/well, $7.2x10^3$ CFU/well, $3.9x10^3$ CFU/well, $3.17x10^3$ CFU/well for the 9 NLF EIEC strains. Among the LF EIEC strains, PT1 shows less CFU/well may be due to its slow growing capacity.

The log values of the CFU/well were plotted on the Y axis and from the graph we can conclude that the log values of CFU/well of all the LF strains were more than the NLF strains. Statistical analysis was performed using an unpaired t-test which showed that the invasive rate of all the LF strains was significantly higher ($p<0.0001$) than the NLF strains. Each of the LF EIEC strains showed a significant difference ($p<0.0001$) among each of the NLF strains. A *S. flexneri 2a* strain was taken as a positive control in the experiment. It was further seen that the invasive property of the LF EIEC was higher than *S. flexneri 2a* while the invasive property of the NLF EIEC strains were significantly lower than *S. flexneri 2a* (**Figure R16**).

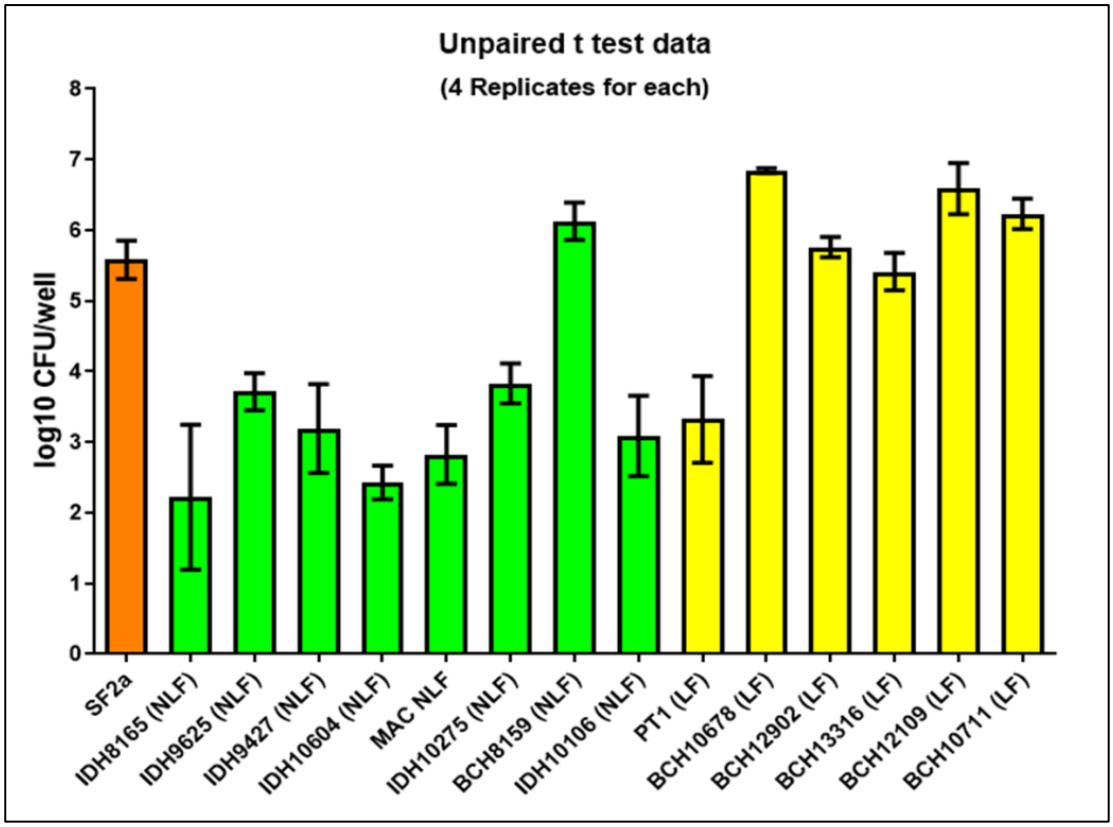


Figure R16: *Invasion assay showing the invasive property of the EIEC isolates. Results indicated that LF EIEC was more invasive than NLF EIEC strains.*

i) LF EIEC expressed the virulence genes at a higher level than the NLF EIEC

The invasive properties of the LF-EIEC isolates were further compared to the NLF-EIEC isolates by analyzing the expression level of the virulence regulatory genes such as *virF* and *virB* as well as the other genes required for invasion such as *ipaABCD, icsA, icsB* and *ipaH* to understand the potential cause behind their greater invasiveness (**Figure R17**).

A representative number of LF (2 among 6 LF strains) and NLF EIEC (2 among 9 NLF strains) strains were taken for the comparative study. When we compared the expression of bacterial genes of both LF and NLF strains it was revealed that LF EIEC expresses virulence genes at a significantly higher level than NLF EIEC (**Figure R17**). In the case of *icsA* gene which is involved in actin-based motility and adhesion (Qin *et al.,* 2020) it was found that *icsA* was expressed more by the LF EIEC strains than the NLF EIEC strains. The expression of *icsA* gene by the LF EIEC strains was significantly higher than the *S. flexneri 2a* which was taken as control strain ($p<0.0001$) while the expression level of the NLF EIEC strains was significantly lower ($p<0.01$) than *S. flexneri 2a*.

A similar pattern of expression was found in the case of *icsB*, a gene that is involved in invasion. When the expression of *icsB* of both LF and NLF were compared with *S. flexneri 2a* and it was found that expression levels of both the LF EIEC strains were significantly more ($p<0.0001$) than *S. flexneri 2a* and the expression levels of both the NLF EIEC strains were significantly less than ($p<0.001$) than *S. flexneri 2a*.

The expression levels of other genes involved in invasion such as *ipaA*, *ipaB*, *ipaC* and *ipaD* showed similar patterns of expression like *icsA* and *icsB* genes. Expression level of *ipaA* gene was higher in case of the LF EIEC than the NLF EIEC strains. When the expression levels were compared with *S. flexneri 2a*, it was seen that the LF strains expressed more *ipaA* gene than *S. flexneri 2a*. Among the 2 LF strains, it was further observed that the expression levels of *ipaA* gene in case of BCH12902 and BCH 10711 were higher than *S. flexneri 2a* with p values greater than 0.0001 and 0.001 respectively. The expression levels of both the NLF EIEC strains were significantly lower than *S. flexneri 2a*. Similar results were observed for *ipaB* gene which is involved in adhesion during entry and secretion of virulence factors by *Shigella*, destruction of phagosome and phagocytosis inductuion in macrophages (Yang *et al.*, 2015).

ipaC gene which is involved in similar functions like *ipaB* (Yang *et al.*, 2015) was also expressed more in case of the LF isolates and less in case of the NLF isolates when compared to *S. flexneri 2a*. Among the 2 LF EIEC strains, it was further observed that BCH 12902 showed higher expression of *ipaC*($p<0.0001$) in comparison to *S. flexneri 2a* than the other LF strain BCH 10711. The expression levels of the NLF strains were less than *S. flexneri 2a*.

Similarly, the gene *ipaD* which is also involved in controlling T3SS was expressed more in LF EIEC than the NLF EIEC strains in comparison to *S. flexneri 2a*. The LF strains showed a higher expression of *ipaD* ($p<0.001$) when compared to *S. flexneri 2a* whereas the NLF strains showed a lower expression of *ipaD* compared to *S. flexneri 2a*. Among the LF strains, the expression level was higher in BCH 12902 than BCH 10711 when compared to *S. flexneri 2a*.

The gene *virF*, a major transcriptional activator of *virB* and *icsA*, regulates the transcription of other invasion genes (Di Martino *et al.*, 2016) was also expressed higher in LF strains than the NLF strains when compared to *S. flexneri 2a*. The expression pattern of *virF* was similar to the expression pattern of the other invasion genes such as *ipaA, ipaB, icsA* and others.

The expression pattern of *virB*, the gene that is activated by *virF* was similar to*virF*. The LF strains expressed a greater amount of *virB* protein as compared to *S. flexneri 2a* while the NLF strains expressed less amount of *virB* protein as compared to *S. flexneri 2a*. Among the LF strains, BCH 12902 expressed higher *virB* ($p<0.0001$) than BCH 10711 ($p<0.001$) as compared to *S. flexneri 2a*. The expression pattern of the NLF strains was lower than *S. flexneri 2a* similar to the other genes involved in invasion.

Similarly, like the other genes involved in invasion, the LF EIEC strains expressed higher *ipaH* than *S. flexneri 2a* while the expression of NLF EIEC is less compared to*S. flexneri 2a.* The expression level of both the LF strains was significantly higher ($p<0.0001$) than *S. flexneri 2a* while the expression level of *ipaH* by the NLF strains was less compared to *S. flexneri 2a*.

Thus, from the results of the expression level of the various genes involved in invasion, it can be concluded that the LF strains may be more virulent than the NLF strains since the expression levels of all the genes required for invasion (*icsA, icsB, ipaABCD, virF, virB and ipaH*) were higher in LF than the NLF EIEC strains when compared to *S. flexneri 2a* (**Figure R17**). The higher expression of *virF* might have resulted in the higher activation *virB* which has resulted in more expression of other invasion genes and causing more invasions.

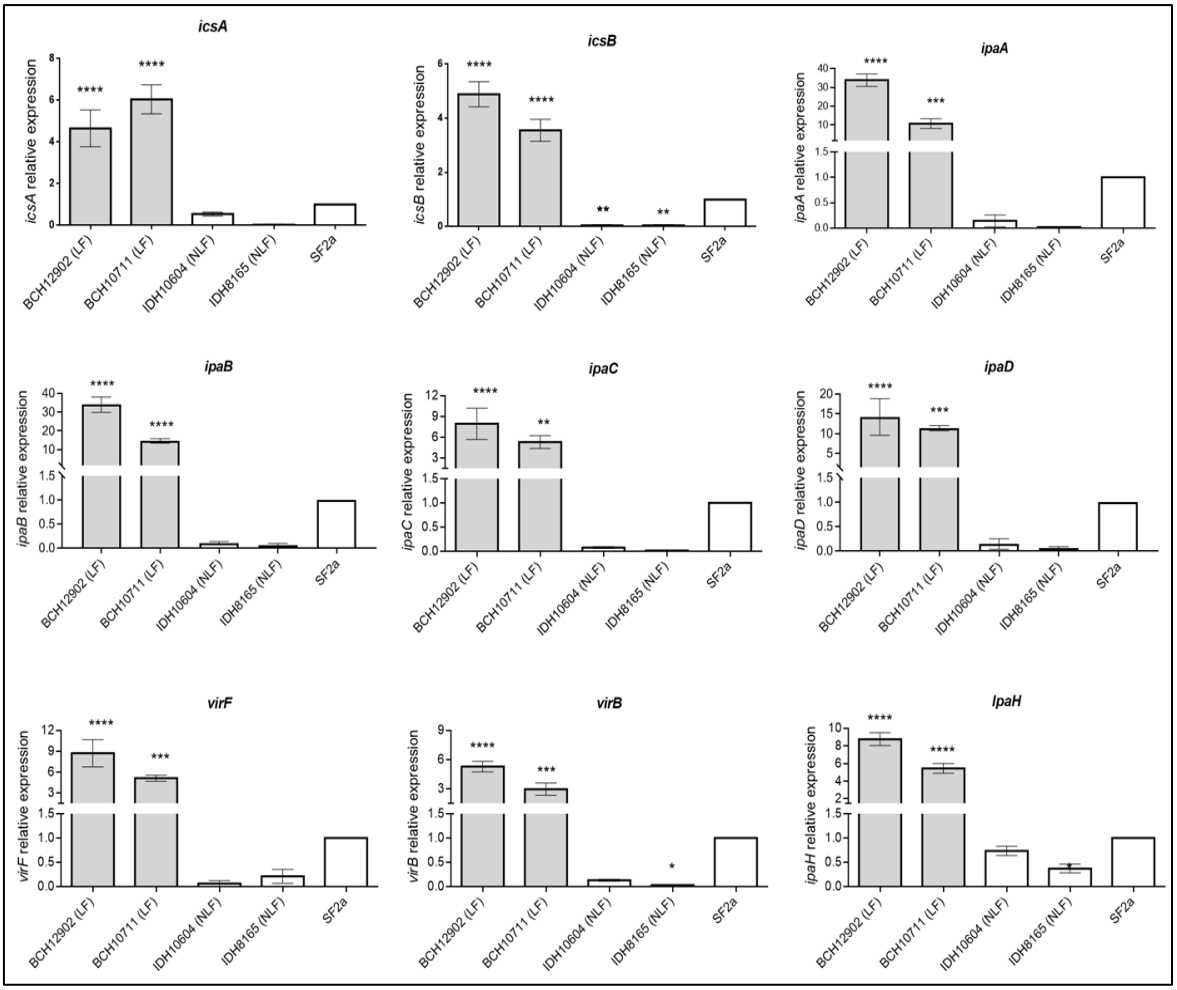


Figure R17: *Transcription profiles of common virulence associated genes of LF and NLF-EIEC strains expressed during spreading into Int407 cell monolayer after 2h of infection. Real-time PCR was performed, and C_t values were normalized to recA expression using the $2^{-\Delta\Delta Ct}$ method, with expression of a Shigella flexneri2a set to 1.0. The graphs represent the mean expression levels from three independent experiments performed in duplicate. One-way analysis of variance (ANOVA) and Dunnett's multiple-comparison tests were used to determine the statistical significance. The error bars represent standard deviations from the mean. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.*

j) LF strains colonized more in the gut epithelium

In order to decipher whether the colonization capacity as well as the invasive capacity of the LF EIEC strains were more than the NLF EIEC strains and *S. flexneri* 10^7 CFU of bacteria were fed to antibiotic treated mice and was observed for 6 different time points (6 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs). From this protocol, it was observed that the colonization of the LF strains was more than SF2a and NLF EIEC strains and it was measured in CFU/gm weight of respective tissues (**Figure R18**).

In the ileum, it was seen that LF EIEC colonized more than NLF EIEC strains. In case of BCH 12902, a bell shaped graph was observed where the colonization rate increased from 6 hrs onwards and reached a peak at 48 hrs followed by a gradual decrease of colonization at 72 hrs, 96 hrs and 120 hrs. Another LF strain, BCH 10711, showed high colonization at 6 hrs with a sudden drop down at 24 hrs which again increased at 48 hrs followed by a gradual decrease of colonization at 72 hrs and 96 hrs with zero colonization at 120 hrs. The colonization pattern of NLF strains also showed a bell-shaped graph like LF strain 12902 but the level of colonization of the NLF strains was lower than both the LF strains.

In the large intestine, the degree of colonization of LF EIEC was more than NLF EIEC strains. In caecum, a more or less bell-shaped pattern was observed in case of the LF strains where the rate of colonization of both the strains increased from 6 hrs with a decrease at 24 hrs and a sudden increase at 48 hrs with the highest colonization at 72 hrs followed by a gradual decrease in colonization at 96 hrs and 120 hrs. In the colon, colonization rate of 2 LF strains were not similar. The LF strain, BCH 12902, showed a gradual increase of colonization reaching a peak at 48 hrs followed by a gradual decrease at rest of the hrs. Another LF strain BCH 10711 colonized more at 6 hrs followed by a steady decline of colonization in 24 hrs, 48 hrs, 72 hrs and 96 hrs with zero colonization at 120 hrs. The colonization level of NLF strains was lower than the LF strains in colon. When compared, it was seen that the average colonization level of LF strains was high while it was zero in the case of both the NLF strains (**Figure R18**).

The LF strains were found in the liver and spleen of the mice tissue. When the liver tissues were checked, a high CFU/gm of BCH 12902 was recovered from the liver at 6 hrs which increased at 24 hrs and lower colonization at 48 hrs which again increased at 72 hrs followed by a decrease at 96 hrs and 120 hrs. LF strain BCH 10711, showed high stable colonization at 6 hrs, 24 hrs and 48 hrs with a gradual decrease in 72 hrs, 96 hrs and 120 hrs respectively. In comparison to the LF strains, very less NLF strains were recovered from liver. The colonization in the liver of LF strains was higher than that of NLF strains (**Figure R18**).

In the spleen LF strains colonized more than NLF strains. The LF strain BCH 12902, showed a high colonization in 6 hrs, 24 hrs, 48 hrs with a gradual decrease after 72 hrs, 96 hrs and 120 hrs respectively. Similarly, the other LF strain BCH 10711, showed a highest colonization at 24 hrs which decreased and reached zero in the rest of the hrs. The NLF strain IDH 10604, showed zero colonization in spleen. Other NLF IDH 9427 showed very less at 24

and 48 hrs with nil colonization at 72 hrs, 96 hrs and 120 hrs respectively. From the data of the liver and spleen the invasive properties of LF strains may be estimated to be more than NLF strains (**Figure R18**).

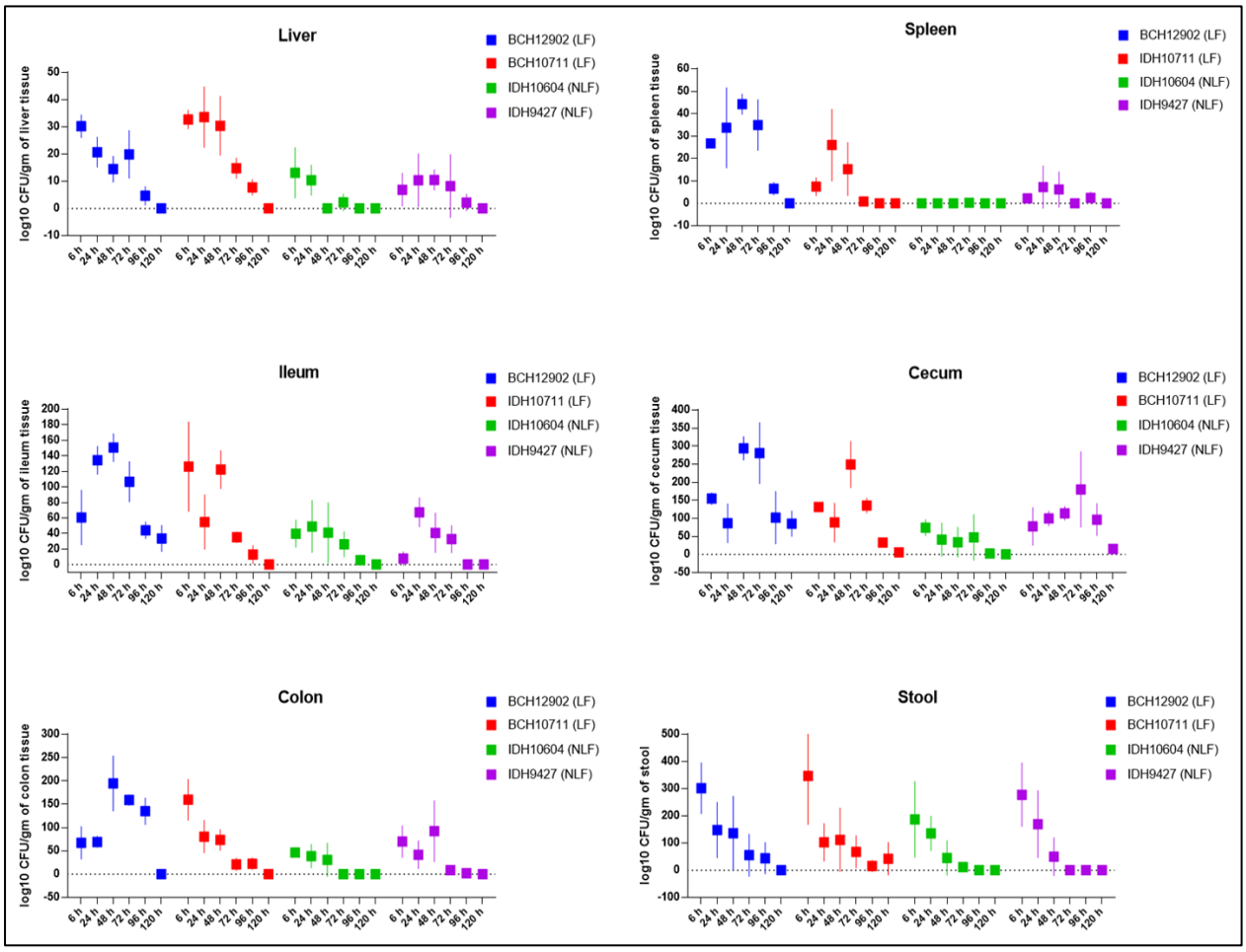


Figure R18: *The colonization and shedding pattern of the EIEC isolates. LF EIEC strains showed a high colonization pattern in all the organs of the mice gut than NLF EIEC strains. The shedding pattern was almost similar in both LF and NLF strains.*

k) LF strains showed higher tissue disruption in mice

In order to check the damage provoked by oral enteroinvasive *E. coli* (EIEC) challenge, we assessed the presence of morphological changes at different time points such as 6 hrs, 24 hrs, 48 hrs and 72 hrs after infection in the large intestine, small intestine, liver and spleen. Extensive morphological changes in crypts and villi, epithelial shedding, extensive granulomas, and inflammatory cell infiltration were seen during 24 hrs, 48 hrs and 72 hrs while mild symptoms were noted in 6 hrs after infection (Erben *et al.*, 2014).

After 24 hrs of LF and NLF-EIEC infection, the cross section of the colon showed infiltration of mixed leukocytes, blunting of villi, crypt abscess, and mucosal infiltration of inflammatory cells. Transmural inflammatory cells, loss of goblet cells, distorted crypts and infiltration of leukocytes were observed after 48 hrs of infection (Erben *et al.*, 2014). During 72 hrs post infection, loss of surface epithelium was observed, highlighting erosion with extensive granuloma and infiltration of inflammatory cells (**Figure R19**).

In the case of ileum, the histopathology of the control strain showed intact villi containing simpler columnar epithelium, intact crypts, submucosa, and muscle layers. 6 hours post infection, showed destruction of villi along with infiltration of mixed leukocytes and other granuloma formation (Erben *et al.,* 2014). These morphological changes were prominently observed in the case of the LF-EIEC infected ileum, while the NLF-EIEC infected ileum showed mild changes (**Figure R19**).

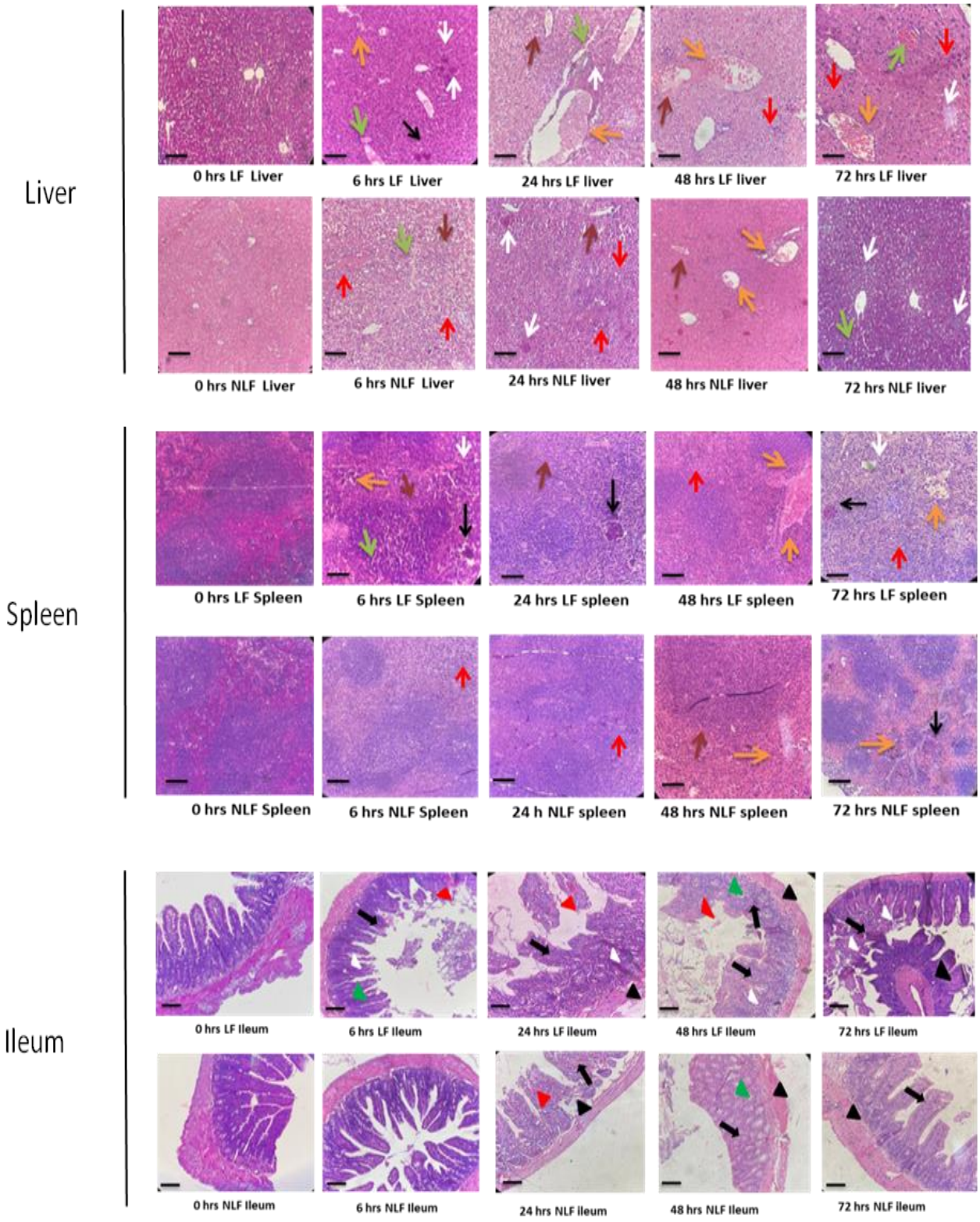
The affected caecum showed infiltration of eosinophilic/heterophilic cells during 24 hrs after infection, erosion of the epithelial cells along with severe infiltration that extend to the submucosa was observed during 48 hrs and 72 hrs after the onset of infection. Extensive necrosis and the appearance of transmural infiltration of the various inflammatory cells were observed during these later hours (Abdelhamid *et al.,* 2021). All these changes were prominent in the LF-EIEC isolates and less in the NLF-EIEC isolates (**Figure R19**).

For finding the systemic spreading of EIEC we examined the extent of damage done to the liver and spleen by bacterial infection and determined the histopathology at different hours (6 hrs, 24 hrs, 48 hrs and 72 hrs) post infection. Very few histological changes were observed in the liver and spleen infected with NLF-EIECs whereas both the liver and spleen infected by LF-EIEC showed marked pathological changes during respective hours post infection. Hepatic steatosis of liver tissue had been observed with infiltrating cells (Nur-Amalina *et al.,* 2023). Granulomas were observed in the liver with both types of EIEC challenged mice. The onset of mild histopathological changes such as leukocyte infiltration, portal inflammation along with lobular inflammation was observed at 6 hrs. In addition, more instances of portal inflammation and leukocyte infiltration were observed in the liver of the LF-EIEC challenged mice than NLF counterparts (**Figure R19**).

The spleen infected by LF EIECs showed an increase of apoptotic as well as necrotic activity with infiltration of inflammatory cells. The black arrowheads represent apoptotic and necrotic activities such as the presence of apoptotic bodies and/or cellular debris. The white arrows

indicate infiltration of inflammatory cells (Jiménez *et al.*, 2011). The extent of impairment found in the spleen of the NLF-EIEC infected mice was comparatively less than the LF-EIEC (**Figure R19**).

Thus, it can be concluded that the degree of damage done to the intestinal parts was more or less the same for both the LF and NLF EIECs whereas the spread and damage were more in the systemic organs in case of the LF EIECs.


Liver
0 hrs LF Liver
6 hrs LF Liver
24 hrs LF liver
48 hrs LF liver
72 hrs LF liver
0 hrs NLF Liver
6 hrs NLF Liver
24 hrs NLF liver
48 hrs NLF liver
72 hrs NLF liver
Spleen
0 hrs LF Spleen
6 hrs LF Spleen
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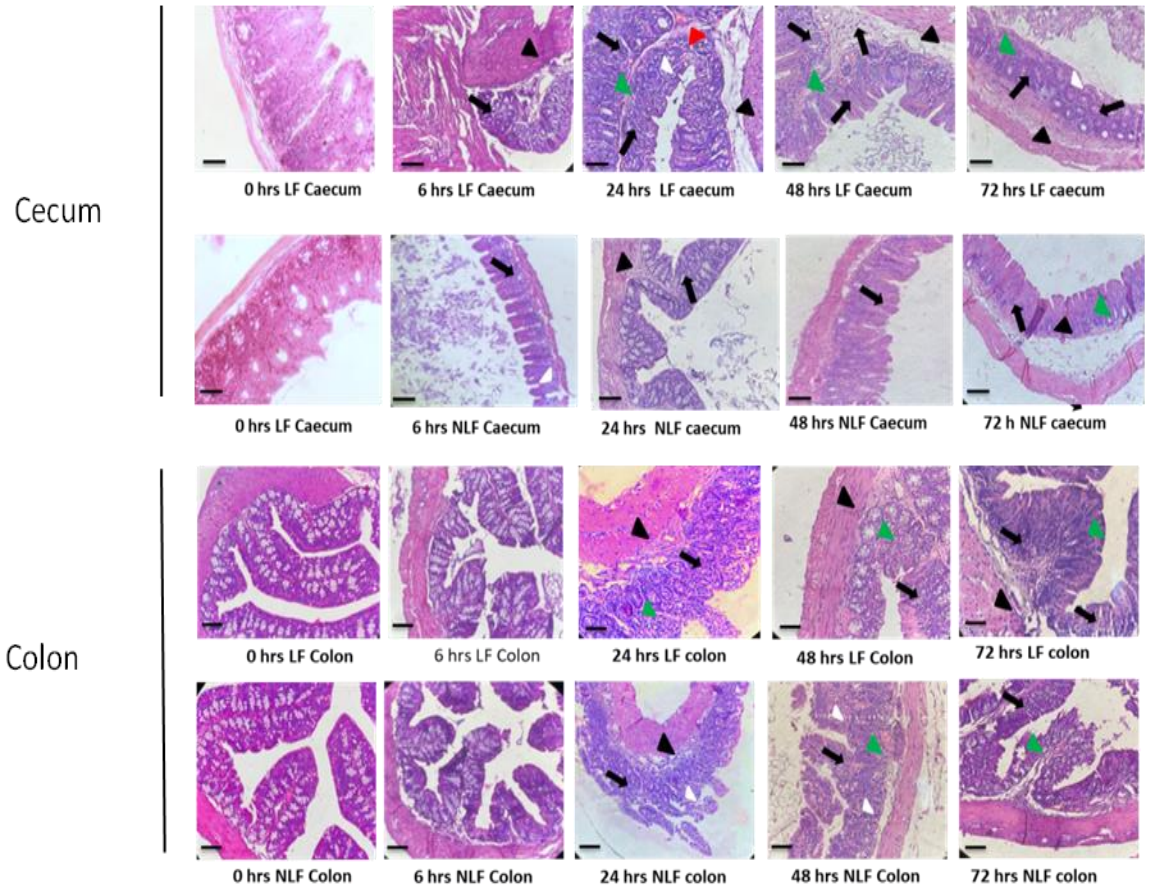

Figure R19 (a): *Hematoxylin and Eosin-stained tissue sections of different parts of mice (liver spleen and ileum) orally infected with LF and NLF EIEC strains and challenged for 6h, 24h, 48h, 72h. Damages in liver and spleen were almost equally observed in both the types.* ***(b)****tissue sections of cecum and colon at different time points were shown. Shapes with different colors represent: Inflammatory cell infiltrates within mucosa (arrows) and submucosa (arrowheads); goblet cell loss (green arrowhead); crypt loss (White arrowhead); hemorrhage (red arrowhead); Inflammation (brown arrow); Extensive granulomas (black arrow); Portal inflammation (green arrow); Lobular inflammation (yellow arrow); Acidophil bodies (white arrow); Infiltrating leukocytes (red arrow).*

l) LF-EIEC instigates severe keratoconjunctivitis

Sereny test was done in order to understand and estimate the invasive property of the EIEC isolates by observing the degree of severity of disease symptoms. For the test, the conjunctival sac of one eye of each guinea pig was inoculated with bacteria and the eyelids were gently massaged and monitored daily for 4 consecutive days for the development of keratoconjuctivitis. For the EIEC isolates keratoconjunctivitis occurred on the next day whereas pathologenicity induced by *S. flexneri* took 48 hours for development after infection. In case of the LF EIEC strain, the degree of infection was severe but mild or very less infection was observed in NLF strains and *Shigella* spp. Moreover, the keratoconjunctivitis

developed by LF EIEC took several days to heal while that of the NLF EIECs was healed in just 2 days (**Figure R20**).

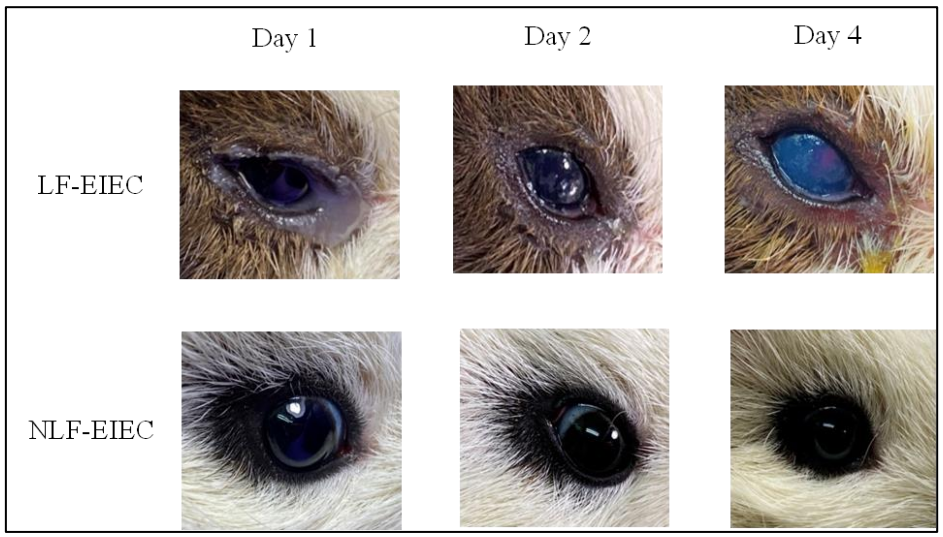

Figure R20: *Guinea pig keratoconjunctivitis induced by infection with LF and NLF-EIEC isolates and monitored for a 4-days period. Severity of the illness was found with the LF-EIEC isolates whereas NLF-EIEC caused mild infection. Each picture is representative of three independent experiments.*

Objective 2:

To access the magnitude of antimicrobial resistance in enteric *E. coli* isolated from diarrheal patients

6.2. Antimicrobial profile of ID&BG Hospital DEC isolates

Due to the increasing worldwide prevalence of antibiotic resistant *E. coli* isolates, we determined the prevalence of antibiotic resistance among the acute diarrheal patients admitted to ID&BG hospital. Multidrug resistance has become one of the challenges in the modern society, thus we screened for the prevalence of MDR *E. coli* among the ID&BG isolates to get an idea about the drug resistance pattern prevailing among the diarrheal patients in Kolkata. We have also screened for the presence of different antimicrobial resistance genes in find the potential cause of antibiotic resistance.

6.2.1. EAEC strains were highly resistant to the different group of antibiotics

A total of 710 DEC positive stool samples were obtained during 2012-2022 from ID&BG Hospital out of which 276 were EAEC, 337 ETEC and 97 EPEC isolates. Among the 276 EAEC, 337 ETEC and 97 EPEC isolates, 111 EAEC, 104 ETEC and 48 EPEC isolates were

considered for checking the antibiotic resistance profile. In our study, the EAEC isolates were seen to be more resistant to most of the antibiotics than EPEC and ETEC strains studied.

The study showed almost 83% of the EAEC strains were resistant to ampicillin followed by the 3rd generation β-lactam antibiotics called ceftriaxone (55%). Only 22% of the EAEC strains showed resistance to ceftazidime and all the EAEC strains were susceptible to meropenem. The resistance to β-lactam group of antibiotics was closely followed by the resistance to fluoroquinolone group of antibiotics where almost 68% of the EAEC strains showed resistance which was again followed by tetracycline group of antibiotics where the EAEC strains showed 60% resistance to tetracycline and almost 42% of the EAEC were resistant to another tetracycline antibiotic called doxycycline. Lower resistance was seen in case of gentamycin and chloramphenicol group where only 14% and 21% of the EAEC strains were resistant. Only 32% of the EAEC were resistant to sulfamethoxazole-trimethoprim antibiotic.

Among the β-lactam antibiotics, 84% of the EPEC strains were resistant to ampicillin followed by ceftriaxone (34%) and ceftazidime (17%). Similar to the EAEC strains, EPEC strains were 100% susceptible to meropenem. More than 50% of the EPEC strains were fluoroquinolone group resistant followed by streptomycin (46%) and sulfamethoxazole-trimethoprim (44%). Almost 70% of the EPEC strains were resistant to tetracycline and 47% of the EPEC strains were doxycycline resistant. Similar to the EAEC strains, the resistance of EPEC strains towards gentamycin and chloramphenicol was low (13%) and (15%) respectively.

The ETEC strains showed the highest resistance of 67% to ampicillin followed by 55% resistance towards the fluoroquinolone drugs namely ciprofloxacin and ofloxacin. 49% of the ETEC strains were resistant to tetracycline and 37% of the ETEC strains were resistant to doxycycline. Among the other β-lactam antibiotics, 33% and 16% of the strains were resistant to ceftriaxone and ceftazidime respectively. None of the ETEC strains were resistant to meropenem. Lower resistance was observed for gentamycin (7%), chloramphenicol (10%), streptomycin (12%) and sulfamethoxazole-trimethoprim (24%) (**Figure R21**).

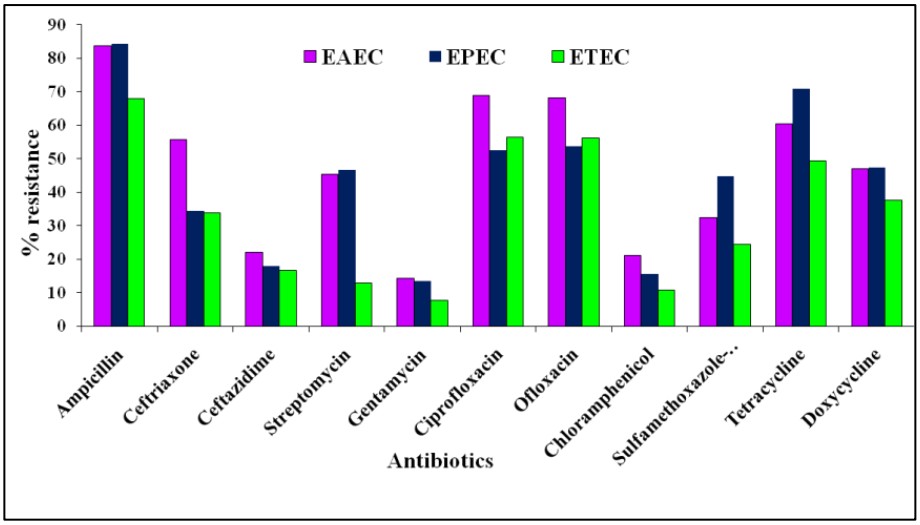

Figure R21: *Antibiotic resistance profile of the DEC isolates obtained from ID&BG Hospital during 2012-2022. EAEC strains showed more resistance to ampicillin, ceftriaxone and fluroquinolone drugs followed by EPEC and ETEC strains.*

6.2.2. Multidrug resistance was mostly found in EPEC among ID&BG hospital isolates

MDR analysis of the DEC isolates obtained from ID&BG Hospital during 2012-2022, showed the presence of many patterns. Thus, the MDR isolates were grouped as MDR1, MDR2, MDR3 and MDR4 that represent the isolates resistant to at least 3, 4, 5 and 6 different classes of antimicrobials (β-lactams, sulphonamides/trimethoprims, aminoglycosides, tetracyclines, quinolones and chloramphenicol), respectively. About 56% (147/263) of isolates were found resistant to at least three different classes of antimicrobials, while 16% (42/263) of the isolates were resistant to either one or two classes. Prevalence of MDR was higher in EPEC (72.9%, 35/48) followed by EAEC (64.86%, 72/111) and ETEC (38.4%, 40/104). Prevalence of EPEC pathotype was higher in MDR1 group (resistant to at least 3 groups of antimicrobials) while EAEC was found to be prevalent in MDR2 (resistant to 4 groups of antimicrobials) and MDR3 group (resistant to 5 groups of antimicrobials). Result showed the prevalence of MDR1 (34.5%) was more among the total MDR isolates followed by MDR2 (29%), MDR3 (27.5%) and MDR4 (9%) groups (**Figure R22).**

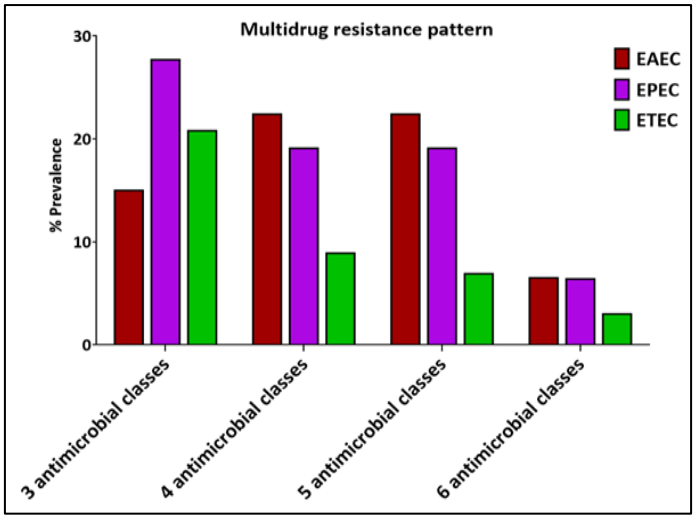

Figure R22: *MDR profile of DEC isolates of ID&BG Hospital during 2012-2022. EPEC strains were mostly resistant to 3 different antimicrobial classes whereas EAEC was resistant to 4-5 antimicrobial classes of antibiotics.*

6.2.3. Existence of different AMR encoding genes among DEC isolates

Of the 263 DEC isolates, 151 isolates that showed resistance towards β-lactamases were tested in PCR assay. Among these, 46.3% (70/151), 51% (77/151), 15.2% (23/151), 2% (3/151) isolates harboured *bla*$_{\text{TEM-9}}$, *bla*$_{\text{CTX-M3}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{SHV}}$, respectively. In 77 sulfamethoxazole-trimethoprim resistant isolates, 41.5% (32/77) harboured *sul2* gene and 5.2% (4/77) were positive for *dfrA1*. Sixty-one isolates were resistant to streptomycin, of which 49.2% (30/61) were positive for *strA* and 41% (25/61) contained *aadA* gene. Tetracycline resistance was noticed in 104 isolates in which 44.2% (46/104) contained *tetB* gene. Quinolone resistance was shown by 131 of the isolates in which 6.9% (9/131) were positive for *qnrB* gene, 27.5% (36/131) for *qnrS* and 9.2% (12/131) for *aac6'-Ib-cr*. About 27% (11/41) of the chloramphenicol resistant isolates harboured *cat1A* gene (**Figure R23**). Further, among the MDR isolates, *bla*$_{\text{CTX-M3}}$ and *bla*$_{\text{TEM-9}}$ were more prevalent, followed by *bla*$_{\text{OXA-1}}$, *sul2*, *strA*, *tetB*, *qnrS* and *qnrB*. Our analysis also showed that the presence of *bla*$_{\text{CTX-M3}}$, *bla*$_{\text{OXA-1}}$, *aadA* and *aad6'-Ib-cr* were more in EAEC. s*ul2*, *dfrA1*, *strA*, *tetB*, *qnrB* and *catI* were mostly detected in EPEC, whereas, *bla*$_{\text{TEM-9,}}$ *bla*$_{\text{SHV}}$ and *qnrS* were more common among the ETEC isolates (**Figure R23**).

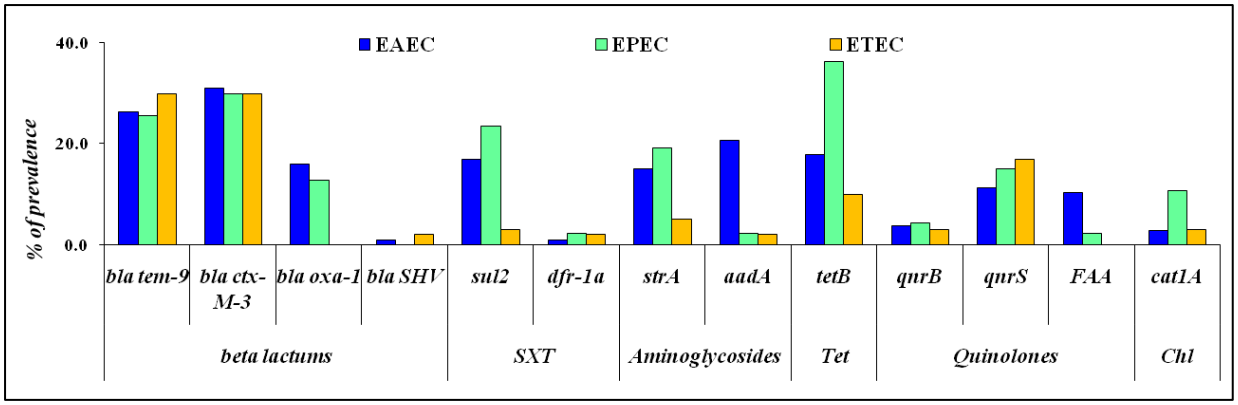

Figure R23: *Antibiotic resistance genes profile of DEC isolates of ID&BG Hospital during 2012-2022. Prevalence of* $bla_{CTX\text{-}M3}$, $bla_{OXA\text{-}1}$, *aadA and aad6'-Ib-cr were more in EAEC. sul2, dfrA1, strA, tetB, qnrB and catI were more in EPEC, whereas,* $bla_{TEM\text{-}9}$, bla_{SHV} *and qnrS were more commonly harboured among the ETEC isolates. SXT, Sulfamethoxazole-Trimethoprim; Tet, Tetracycline; Chl: Chloramphenicol.*

6.3. Antimicrobial profile of B. C. Roy Hospital DEC isolates

Since antibiotic resistance has spread to the children population due to the indiscriminate use of antibiotics we have focused on finding the prevalence of antibiotic resistant *E. coli* isolates from the children population admitted to the B. C. Roy hospital. Multidrug resistant *E. coli* was also screened in order to estimate the resistance pattern among the children population so that better treatment options can be available to the doctors. AMR genes were also screened to find the cause behind the spread of resistance.

6.3.1. Antimicrobial resistance pattern of the DEC isolates from 2018-2022

During 2018-2022, a total of 297 EAEC, 76 EPEC and 70 ETEC were isolated from which 60 EAEC, 32 EPEC and 25 ETEC strains were tested for antibiotic resistance. Similar to the ID&BG Hospital isolates, EAEC was more resistant followed by EPEC and ETEC isolates of B.C. Roy Hospital.

EAEC isolates showed greater resistance towards β-lactam group of antibiotics followed by fluoroquinolone, tetracycline, and streptomycin group of antibiotics. In the β-lactam group, the highest resistance was seen in case of ampicillin (86%) followed ceftriaxone (41%) and ceftazidime (8%). The resistance towards fluoroquinolone group of antibiotics such as ciprofloxacin and ofloxacin were 41% and 38% respectively. EAEC strains were 36% resistant to tetracycline and 21% were resistant to doxycycline. 25% of the EAEC strains showed resistance to streptomycin and sulfamethoxazole-trimethoprim. Lowest resistance

was seen in case of gentamycin where only 2% of the EAEC strains showed resistance. All the EAEC strains were susceptible to meropenem (**Figure R24**).

Similarly, the EPEC isolates showed higher resistance to the β-lactam group followed by fluoroquinolone group of antibiotics. β-lactam antibiotics such as ampicillin, ceftriaxone and ceftazidime showed 65%, 37% and 15% resistance respectively. Ofloxacin and ciprofloxacin showed 28% and 25% resistance respectively while 18% of the strains showed resistance to doxycycline and sulfamethoxazole-trimethoprim. Tetracycline resistance was shown by 25% of the strains while the lowest resistance was seen in case of chloramphenicol (6%) and no resistance was seen for meropenem and gentamycin group of antibiotics (**Figure R24**).

A relatively lower resistance pattern was seen among the ETEC isolates where 64% of the strains were resistant to ampicillin, 32% to ceftriaxone and no resistance to ceftazidime in case of the β-lactam group of antibiotics. 24% of the strains showed resistance to streptomycin and sulfamethoxazole-trimethoprim. Only 16% of the strains were resistant to the fluroquinolone group of antibiotics (ciprofloxacin and ofloxacin). Lower resistance was seen for gentamycin (8%) and the ETEC strains were susceptible to meropenem, ceftazidime and chloramphenicol antibiotics (**Figure R24**).

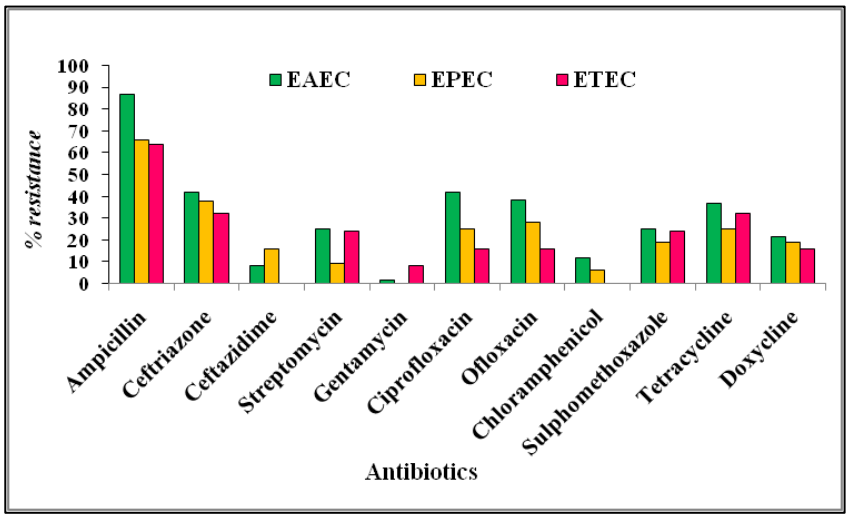

Figure R24: *Antibiotic resistance profile of the DEC isolates obtained from B.C. Roy Hospital during 2018-2022. EAEC strains were more resistant to most of the antibiotics used followed by ETEC and EPEC strains.*

6.3.2. Prevalence of MDR was more among EAEC isolates of the B. C. Roy Hospital

MDR analysis of the DEC isolates obtained from B. C. Roy Hospital showed the presence of atleast 4 distinct patterns. MDR isolates were grouped as MDR1, MDR2, MDR3 and MD4

which signified the isolates were resistant to at least 3, 4, 5 and 6 different classes of antimicrobials (β-lactams, sulphonamides/trimethoprims, aminoglycosides, tetracyclines, chloramphenicol and quinolones), respectively. About 10% (12/117) isolates were found resistant to at least three different classes of antimicrobials. Prevalence of MDR was more in EAEC (30%, 18/60) followed by ETEC (24%, 6/25) and EPEC (18.75%, 6/32). Results showed the prevalence ETEC was higher in the MDR-3 group than in any of the other groups while EAEC prevailed in both the MDR-1 and MDR-2 groups. EAEC strains were also resistant to 6 antimicrobial classes i.e. they belonged to MDR-4 groups (**Figure R25**).

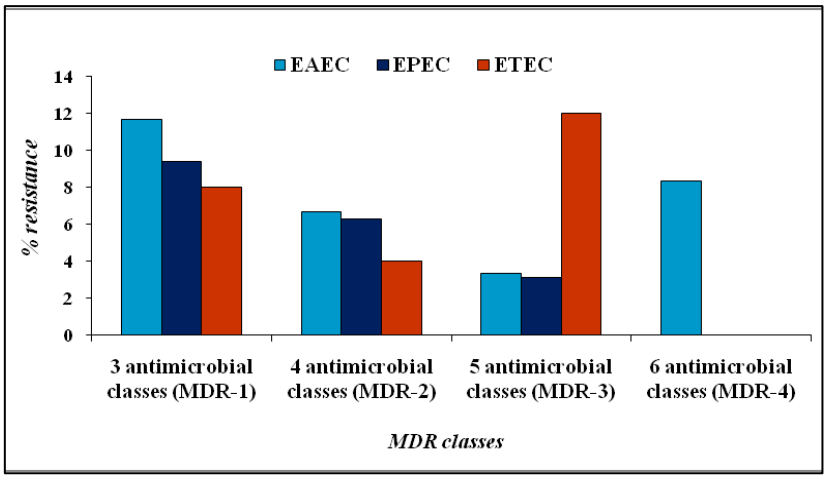

Figure R25: *Prevalence of MDR phenotype among the DEC isolates of B. C. Roy isolated during 2018-2022. EAEC strains were highly resistant to 6 antimicrobial classes while most of the ETEC isolates were resistant to 5 different antimicrobial classes.*

6.3.3. ETEC contained most of the antimicrobial resistance genes

Among the B. C. Roy isolates, EAEC strains showed maximum accumulation of resistance genes that result in conferring resistance to the different antimicrobial classes such as β-lactams, sulphonamides/trimethoprims, aminoglycosides, tetracyclines, chloramphenicol and quinolones. Almost 36%, 57% of EAEC strains harboured $bla_{\text{TEM-9}}$, $bla_{\text{CTXM-3}}$, while 44% of EAEC were harboured *tetB* followed by *sul2* (40%) and *strA* (40%). The EAEC isolates harboured only *qnrB* among the fluoroquinolone resistant genes. 66% of ETEC isolates carried $bla_{\text{CTXM-3}}$ and *sul2* while 33% of the ETEC isolates contained *strA*, *aadA1* and *dfrA1*. The highest number of ETEC (80%) harboured qnrS gene. Almost 62% of EPEC possessed $bla_{\text{CTXM-3}}$ followed by *tetB* (50%). Thus, it can be concluded that $bla_{\text{CTXM-3}}$ was most prevalent among the 3 DEC pathotypes while FAA was very less among the isolates from B. C. Roy hospital (**Figure R26**).

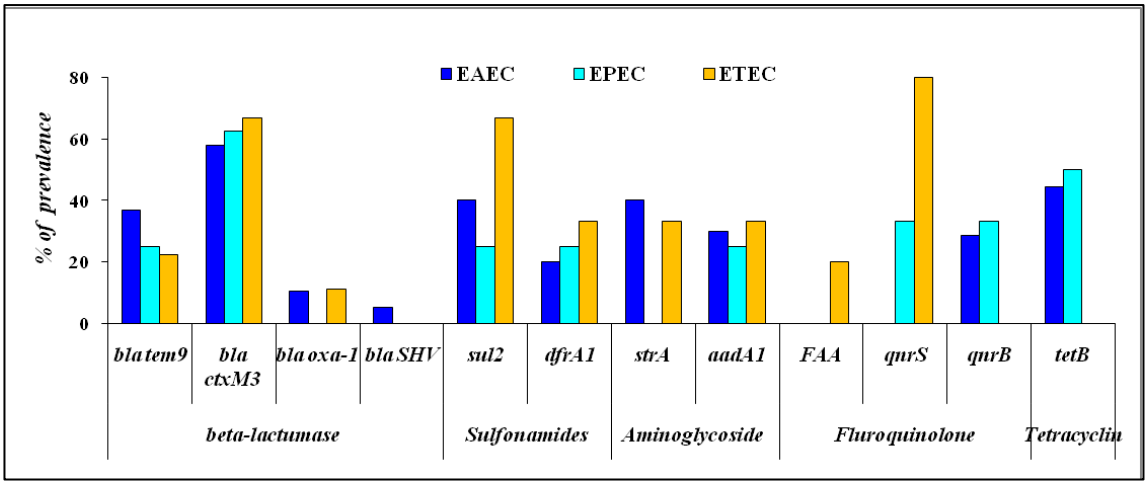

Figure R26: *Antibiotic resistance genes profile of DEC isolates of B. C. Roy Hospital during 2018-2022. Prevalence of* bla_{TEM-9}, bla_{OXA-1}, bla_{SHV} *and strA were more in EAEC. tetB and qnrB were mostly detected in EPEC, whereas* bla_{CTX-M3}, *sul2, dfrA1, aadA1, aad6'-Ib-cr and qnrS were more common among the ETEC isolates.*

6.4. MDR3 group was prevalent in 2018-2019 in both hospital isolates in age group 0-5 years

Due to the high prevalence of multidrug resistant bacteria in our study, we have further focused on comparing the prevalence of MDR *E. coli* isolates among the two hospitals in different years. This comparison was done in order to estimate the rise of MDR phenotype in the similar age group (0-5 years) in both hospitals.

In ID&BG hospital, *E. coli* strains resistant to 4 different antimicrobial classes (MDR 2) were prevalent during the years 2012- 2013 while strains resistant to 3 different antibiotic groups (MDR 1) were prevalent during the years 2014-2017. Among the B. C. Roy hospital isolates, there was a rise in *E. coli* resistant to at least 5 antibiotic groups (MDR-3) during 2018-2019 and there was an increase of *E. coli* isolates resistant to 3 antibiotic groups (MDR-1) during 2020-2022.

From the data, we can see that during the year 2018-2019, there was an increase of *E. coli* pathotypes that were resistant to 5 different antibiotic classes such as β-lactam, fluoroquinolone, sulfonamides, tetracycline, and aminoglycosides in both the hospitals. Again during 2020-2022, there was a rise of *E. coli* resistant 3 antimicrobial groups in both the hospitals (**Figure R27**).

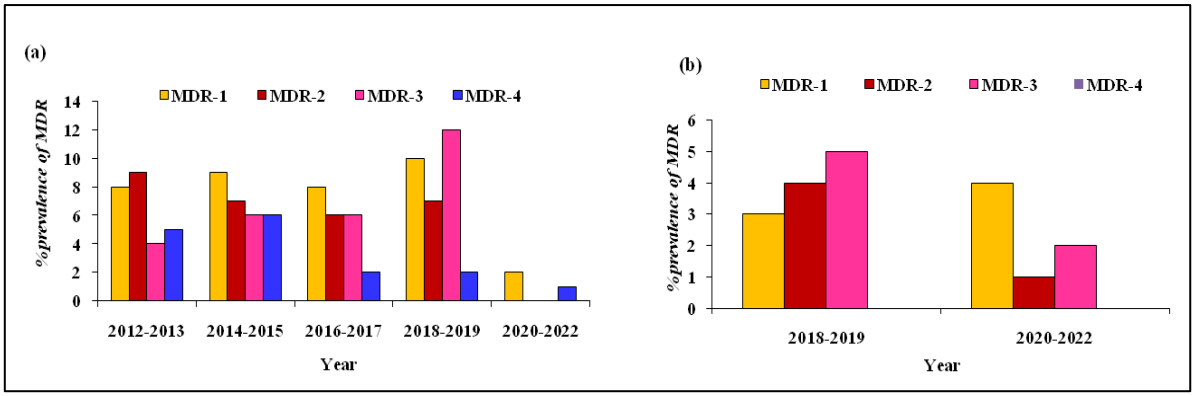

Figure R27: *MDR status of the DEC isolates from both the hospitals (a) Represents the MDR status of the ID&BG hospital isolates. (b) Represents the MDR status of the B. C. Roy hospital isolates. The MDR profile showed the prevalence of MDR-3 group during the years 2018-2019.*

6.5. Antimicrobial resistance profile of ID&BG and B. C. Roy EIEC isolates

DEC and *Shigella* both are generally found to confer a higher degree of resistance to most of the antimicrobials used in the treatment of acute diarrhea. Therefore, we aimed to understand the antimicrobial resistance profile of the EIEC isolates.

6.5.1. EIEC isolates were mostly susceptible to latest generation of antibiotics

LF strains showed resistance to ampicillin, ceftriaxone, tetracycline, doxycycline, sulfamethoxazole-trimethoprim and streptomycin (**Figure R28a**). NLF-EIEC was resistant to almost all the antimicrobials tested except ceftazidime, ceftriaxone, meropenem, gentamycin, and azithromycin. The NLF strains showed greater resistance to the antimicrobials used than that of the LF strains. Resistance to fluoroquinolones drugs such as ciprofloxacin and ofloxacin and chloramphenicol drugs was specially seen with the NLF isolates (**Figure R28a**).

As the emergence of multidrug resistance in pathogenic bacteria has become a serious public health threat, we checked the spread of multi-drug resistance phenotype in this rare group and found that 4 of the NLF and 1 of the LF-EIEC isolates were resistant to about 5 different antimicrobial classes including β-lactam, tetracycline, streptomycin, SXT and fluoroquinolone group of antibiotics (**Figure R28b**).

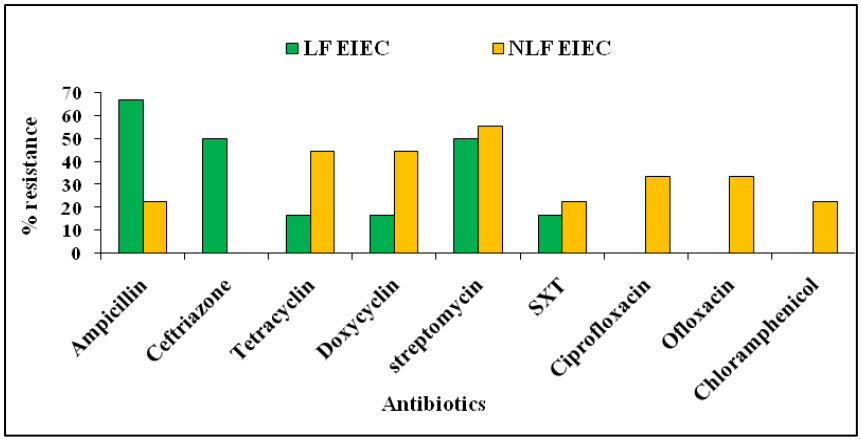

Figure R28 a: *Antimicrobial resistance pattern of the EIEC isolates from B. C. Roy and ID&BG Hospital during 2016-2022. The NLF EIEC strains showed higher resistance to the antibiotics used in the study.*

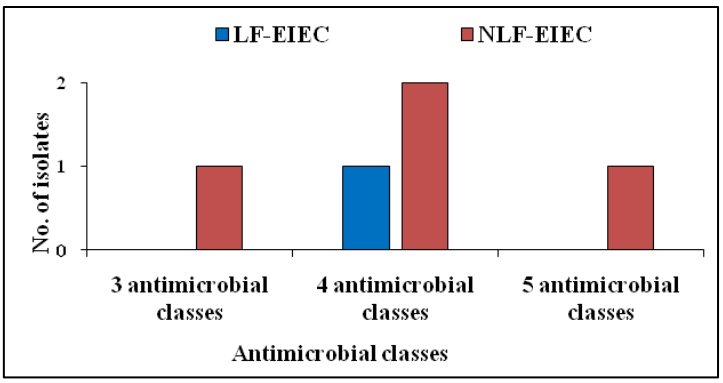

Figure R28 b: *Strains were categorized into 3 different antimicrobial classes to determine multidrug resistant (MDR) phenotype. Very few of the isolates were found show MDR phenotype and mostly confined to the NLF strains.*

6.5.2. Prevalence of AMR genes among the EIEC resistant isolates

The presence of antimicrobial resistance conferring genes was also determined. Among the LF isolates, 2 (33.3%) harboured *bla*$_{\text{CTX-M3}}$ while only one (16.7%) of them harbored *bla*$_{\text{OXA-1}}$ and *bla*$_{\text{TEM-9}}$. The *strA* gene responsible for conferring streptomycin resistance was present among the 2 (33.3%) of the LF-EIEC. The NLF isolates showed a greater frequency of resistance encoding genes than LF-EIEC isolates. In addition to the genes found in LF isolates, NLF strains were found to carry SXT and fluoroquinolone encoding resistance genes such as *sul2*, *dfrA1* and *qnrS*. Two (22.2%) NLF isolates harbored the *sul2* gene while *dfrA1* (11.1%) was present in 1 of the NLF-EIEC isolates. Two (22.2%) NLF isolates contained *strA* gene, which was responsible for streptomycin resistance (**Figure R29**).

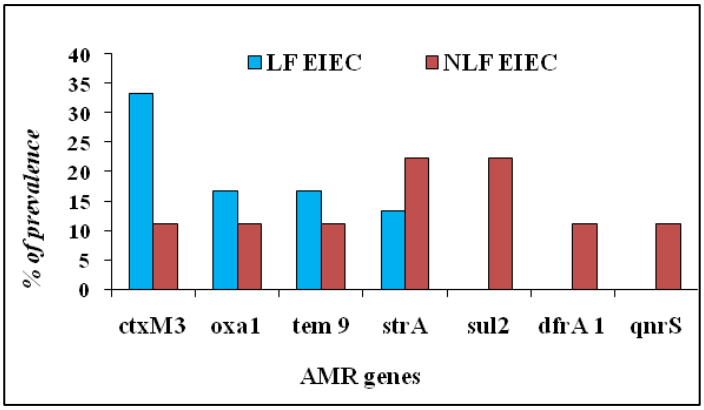

Figure R29: *Prevalence of AMR genes among the EIEC isolates.sul2, dfrA1 and qnrS were present in NLF EIEC while these are absent in LF EIEC strains.*

6.6. Commensal *E. coli* strains were highly resistant to the antibiotics used

The spread of antibiotic resistance is a growing public health threat and commensal *E. coli* being one of the major carriers of resistance. Thus we have analyzed the antimicrobial resistance pattern of the commensal *E. coli* isolates. In case of commensal *E. coli*, strains showed high levels of resistance to fluoroquinolone group of antibiotics such as ciprofloxacin (98%), ofloxacin (98%) followed by ampicillin (83%), tetracycline (72%), sulfamethaxzole (60%), azithromycin (52%) and ceftriaxone (40%). Almost 40%, 30%, 12% and 10% of commensal *E. coli* strains were doxycycline, streptomycin, gentamycin and chloramphenicol antibiotics respectively. The strains were highly susceptible to meropenem (**Figure R30**).

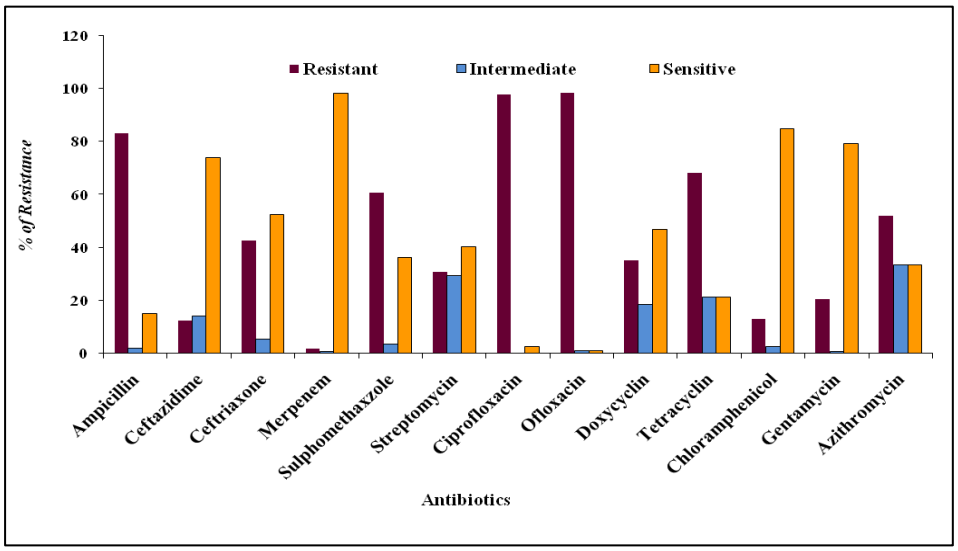

Figure R30: *The antimicrobial resistance pattern of commensal E. coli isolated during the period Apr'2018 to Oct'2018. The E. coli strains showed high resistance to ampicillin, tetracycline and fluroquinolone antibiotics.*

Objective 3:

To identify the genetic determinants and mechanism of resistance to fluoroquinolone and of associated multidrug resistance in *E. coli* isolates

6.7. Role of plasmid mediated fluroquinolone resistant genes (PMQR) conferring fluroquinolone resistance in the *E. coli* isolates

6.7.1. PMQR gene *qnrS* was prevalent among the ETEC strains isolated from ID&BG hospital

PMQR genes result in the protection of DNA gyrase and topoisomerase IV from quinolone activity mediated by *qnr* genes. These genes provide some degree of reduced susceptibility to fluoroquinolone drugs resulting in survival of the bacterial species. Thus we have screened for the presence of such PMQR genes in order to understand the mechanism of reduced susceptibility of our *E. coli* isolates.

Among the EAEC isolated from ID&BG hospital, the prevalence of PMQR gene *qnrS* (20.68%) was highest among the other two PMQR genes *qnrB* (6.89%) and *aac(6')-Ib-cr* (FAA) (17.24%). In the EPEC as well as ETEC isolates *qnrS* gene was prevalent than the other two PMQR genes. 37.5% of the ETEC strains harbored the *qnrS* gene whereas only 20% of the EPEC isolates harbored the *qnrS*. Almost 6% of ETEC and EPEC strains harbored *qnrB*. *aac(6')-Ib-cr* was present in only 6% of EPEC isolates (**Figure R31**).

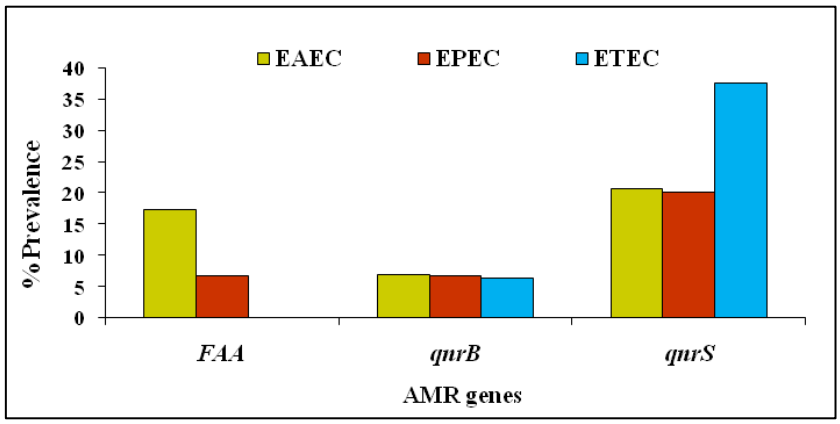

Figure R31: *Prevalence of PMQR genes from ID&BG isolates. qnrS was high in case of ETEC for ID&BG isolates and FAA remained high among the EAEC isolates.*

6.7.2. PMQR gene *qnrB* was prevalent among the ETEC strains isolated from B. C. Roy hospital

Among the B.C. Roy isolates, 80% of the ETEC strains harbored *qnrB* and only 20% of them harbored *aac(6')-Ib-cr* (FAA). *qnrB* and *qnrS* was found in 33.3% of EPEC strains while only 28.5% of EAEC strains harbored *qnrS* (**Figure R32**).

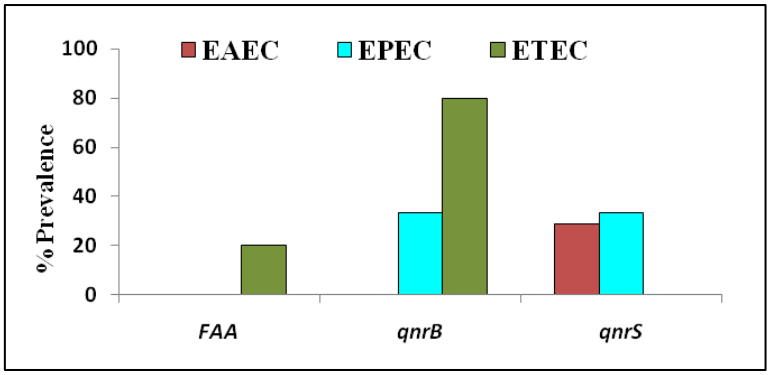

Figure R32: *Prevalence of PMQR genes from B. C. Roy isolates. qnrB and FAA were high in case of ETEC for B. C. Roy isolates and qnrS remained high among the EPEC isolates.*

6.8. Mutations in the quinolone resistance determining region (QRDR) of chromosomal genes

The main mechanism of quinolone resistance is the accumulation of mutations in the bacterial enzymes targeted by fluoroquinolones: DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*).

6.8.1. Presence of 2 point mutations in *gyrA* and several synonymous mutations in *gyrB*

Multiple mutations in *gyrA* and *gyrB* genes are reported in the clinical isolates, thus a representative number of EAEC, EPEC and ETEC strains from both hospitals were checked for the presence of mutations in the QRDR region of *gyrA* and *gyrB* genes.

In case of *gyrA* gene, all the EAEC, EPEC and ETEC strains harbored a change in nucleotide from C to T at 248th position which resulted in the amino acid change at codon 83 from Serine to Leucine. Another point mutation has been observed resulting in nucleotide change from G to A at 259th position resulting in the change of amino acid from Aspartate to Asparagine at codon 87 (**Figure R33**).

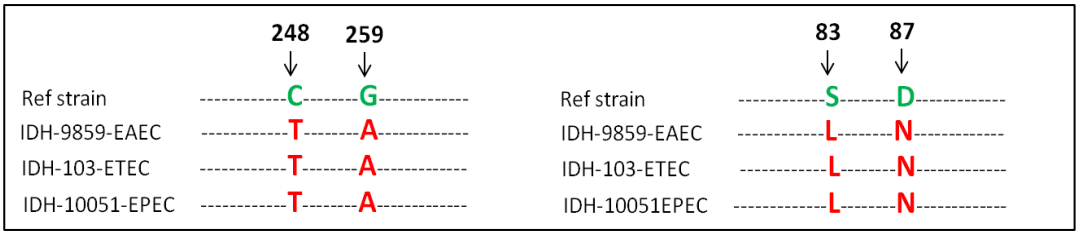

Figure R33: *Mutations present in gyrA resulting in change of amino acids at 83 and 87 positions. Serine to Leucine at codon 83 and Aspartate to Asparagine at codon 87 were the 2 mutations found in our E. coli strains.*

gyrB gene had a large number of mutations at several positions of the genome in case of all the EAEC, EPEC and ETEC strains. In case of the EAEC strains 6 different mutations (G→A, T→G, G→C, T→C, T→C and G→A) have been observed at 1074, 1152, 1194, 1196, 1203 and 1266 positions respectively. The ETEC strain showed 6 mutations such as G→A, G→C, T→C, T→G, T→G and C→T at1164, 1194, 1196, 1203, 1248 and 1359 positions of the genome, respectively. The EPEC strain had no mutations. All the mutations found in *gyrB* were synonymous (coding similar amino acids) (**Figure R34**)

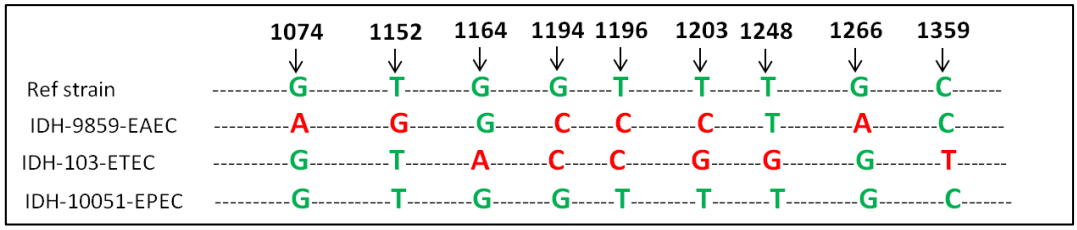

Figure R34: *Figure shows mutations in gyrB gene of representative EAEC, ETEC and EPEC isolates aligned with a reference wild type gyrB gene. All the mutations were synonymous.*

6.8.2. Presence of 3 point mutations in *parC* and several synonymous mutations in *parE*

In the *parC* gene, both the EAEC and EPEC strains harbored change of nucleotides at 239 and 240 positions where there is a change from G→T and T→C respectively resulting in amino acid change from Serine to Isoleucine at codon 80. There was no change in the ETEC strain. Another mutation was found at nucleotide position 273 resulting in a nucleotide change of G→A, but there was no change in amino acid due to this mutation (**Figure R35**)

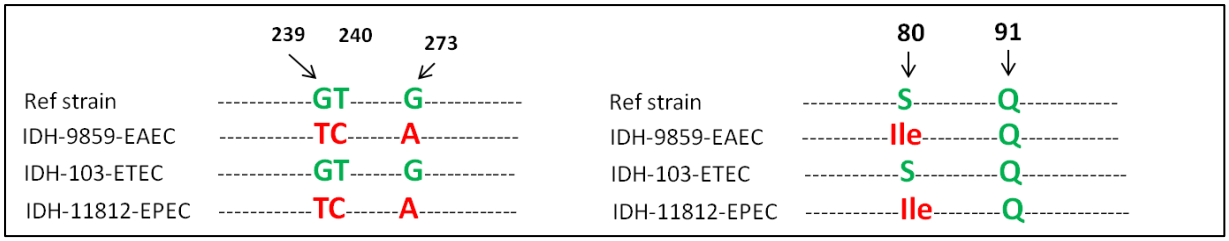

Figure R35: *Mutations in parC gene along with amino acid changes of representative EAEC, ETEC and EPEC isolates of Kolkata and aligned with a reference wild type parC gene. Serine to Isoleucine at codon 80 was the only amino acid change found among the isolates.*

For the *parE* gene, all the EAEC, EPEC and ETEC strains harbored a number of mutations at different positions but all these mutations are synonymous in nature, resulting in coding of similar amino acids. EAEC, EPEC and ETEC strains harbored 4 common mutations at positions which were T→C, C→T alternatively at positions 1338, 1350, 1371 and 1386 (**Figure R36**).

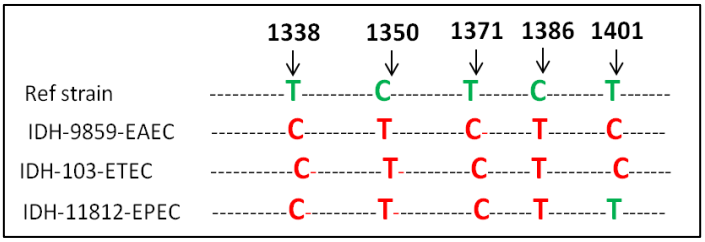

Figure R36: *Mutations in parE gene along with amino acid changes of representative EAEC, ETEC and EPEC isolates of Kolkata and aligned with a reference wild type parE gene. All the mutations were synonymous in nature.*

Objective 4:

6.9. Characterization of carbapenem resistance (*bla*$_{NDM-1}$) *E. coli* strains isolated from diarrheal patients

Carbapenems are the last line of defence against diarrheal infections and the spread of its resistance is a growing global health problem. Therefore, we concentrated at finding carbapenem resistant strains in the population of Kolkata.

6.9.1. Confirmation of meropenem resistance by disc diffusion method

Kirby-Bauer disc diffusion method was performed in order to decipher the meropenem resistance among the *E. coli* isolates and one such EAEC isolate was found to be meropenem resistant. Antibiotic susceptibility comparison of meropenem (30U) between BCH-7846

(resistant) and BCH-10245 (sensitive) by disc diffusion method was performed (**Figure R37**).

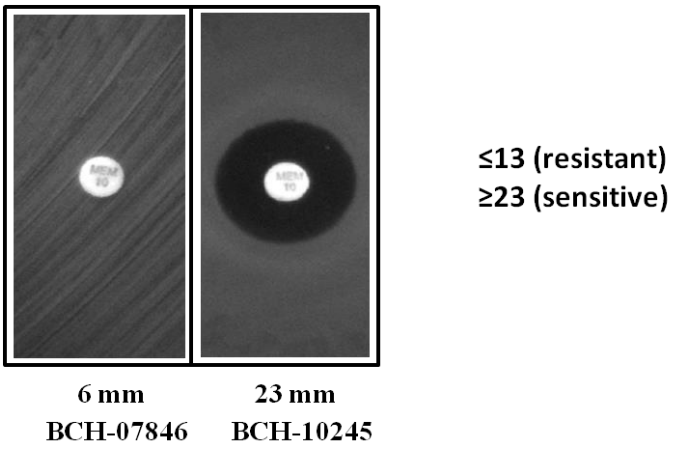

Figure R37: *Disc diffusion assay to compare the susceptibility of a meropenem resistant EAEC (BCH- 7846) and a meropenem susceptible EAEC strain (BCH- 10245).*

6.9.2. EAEC was confirmed by PCR and typical stacked brick appearance in tissue culture

The meropenem resistant strain was checked by PCR to confirm the presence of typical virulence genes of DEC. The strain was found to be positive for the *cvd* gene only and hence categorized as atypical EAEC (**Figure R38**). It was further confirmed by the appearance of stacked brick-like phenotype in cell line upon infection which is the typical characteristic of EAEC pathotype. The stacked brick like appearance was shown in (**Figure R39**).

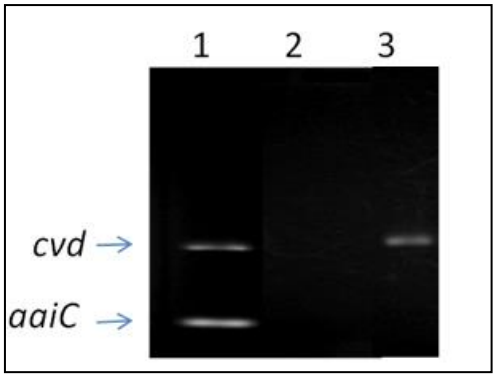

Figure R38: *PCR for identification of EAEC. Lane 1: EAEC positive control, Lane 2: Negative control, Lane 3: BCH 7846. The meropenem resistant strain showed the presence of cvd* gene which is a typical virulence gene of EAEC isolates.

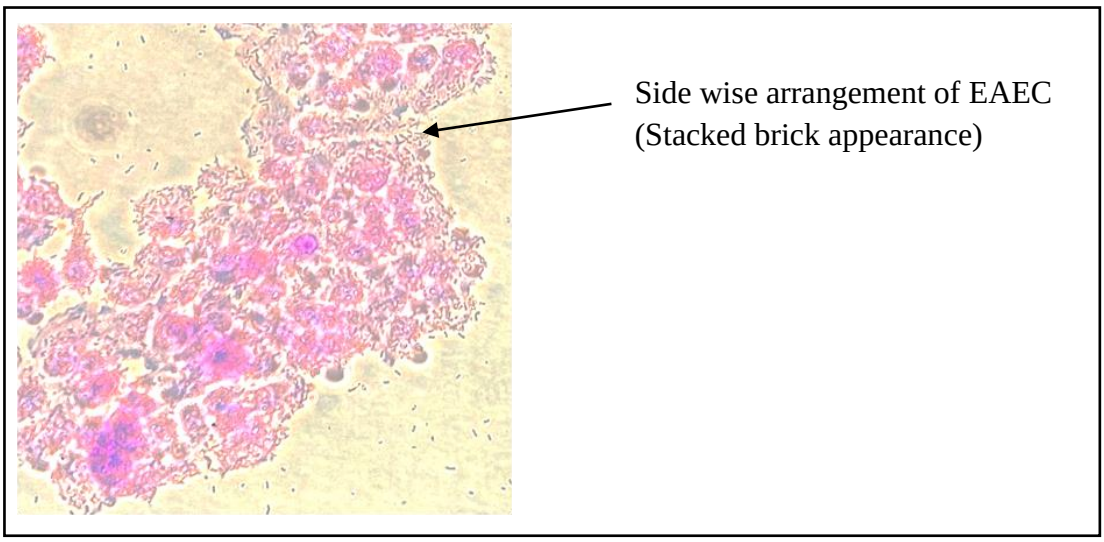

Figure R39: *Stacked brick-like structure formed when infected to cultured cell-line (Int407) which is a typical characteristic of EAEC.*

6.9.3. Detection of *bla*$_{NDM-1}$ gene

The meropenem resistant EAEC (BCH 7846) was subjected for amplification of carbapenem resistance genes and was found to be *bla*$_{NDM-1}$ positive (**Figure R40**). Since the *bla*$_{NDM-1}$ gene is generally present in plasmid, thus we focused on the isolation of plasmid harboring *bla*$_{NDM-1}$ gene (**Figure R41**).

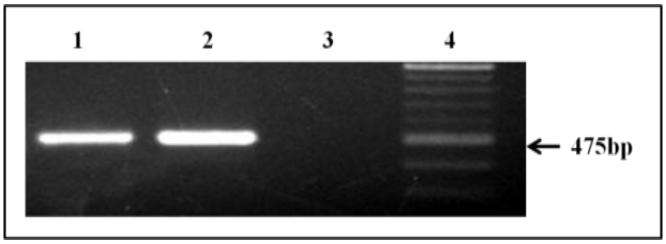

Figure R40: *Gel picture showed the meropenem resistant strain positive for bla*$_{NDM.-1}$ *Lane 1: meropenem resistant strain (BCH 7846), Lane 2: Positive control, Lane 3: Negative control, Lane 4: 100 bp ladder.*

6.9.4. Isolation of meropenem resistant conferring plasmid

The strain was further seen for the presence of plasmids conferring meropenem resistance. The plasmid was isolated by Kado and Liu method and was separated in a 0.8% agarose gel. The lamda Hind III marker was used in Lane 3 in order to distinguish the chromosomal DNA fragment (**Figure R41**).

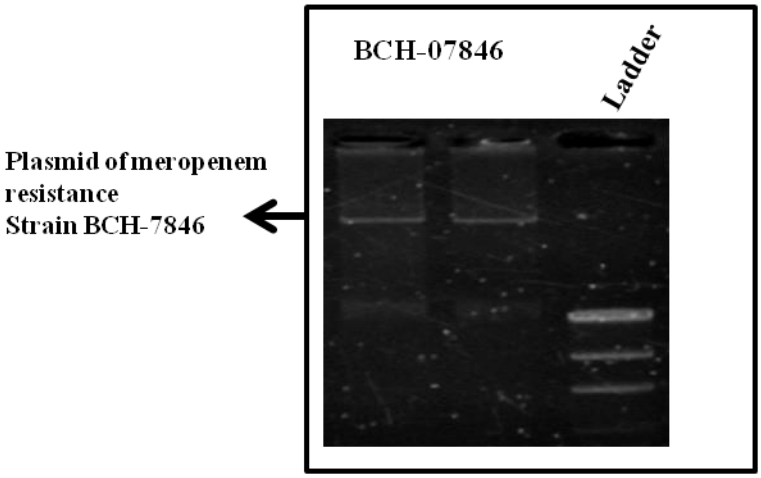

Figure R41: *Plasmid isolated from meropenem resistant strain (BCH 7846). Lane 1 and Lane 2: plasmid isolated from meropenem resistant strain (BCH 7846), Lane 3: 23kb λ Hind III ladder.*

6.9.5. In silico analysis of the plasmid revealed the presence of multiple AMR genes

Only a single plasmid of size ~180 kb was detected in the meropenem resistant EAEC strain by Image Lab software. Plasmid typing stated that the pNDM-7846 plasmid belonged to Inc H1 replicon. The plasmid was analysed by ANOVA software and the different AMR genes and virulence genes (VF) were annotated by red and yellow boxes respectively (**Figure R42**).

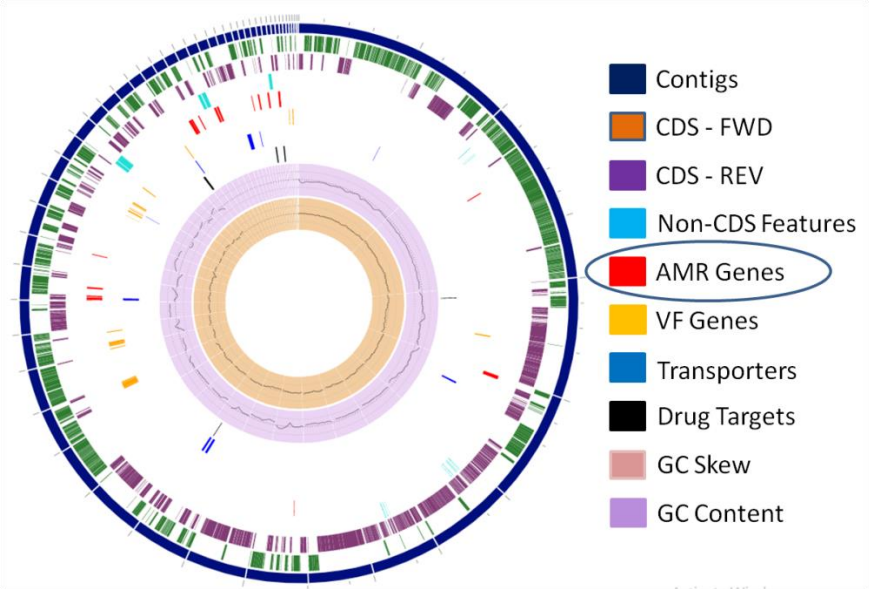

Figure R42: *The circular map of the pNDM-7846 meropenem resistant plasmid. The different contigs and coding sequences in forward and reverse directions are represented by dark blue, orange and purple colours respectively. The various AMR genes, virulence genes, transporters, drug targets are represented by red, yellow, blue, and black colours. The GC content of the plasmid is highlighted light purple colour.*

6.9.6. Sequence analysis of the *bla*$_{NDM-1}$ resistant plasmid

The sequence analysis of the plasmid revealed that the bla$_{NDM-1}$ gene located on the plasmid pNDM-7846 in a complex integron connected by two insertion sequences, IS26 in the upstream and IS110 in the downstream of the gene. The presence of these insertion sequences may help in the mobility of the resistance genes to other species and result in the dissemination of resistance among other bacteria. A truncated class 1 integron intI1 was also present in the upstream of the bla$_{NDM-1}$ gene. The flanking genetic structure of the bla$_{NDM-1}$ of pNDM-7846 plasmid further showed the presence of the genes *ble*$_{MBL}$ (bleomycin resistance gene), *trpF* (phosphor ribosyl anthranilate isomerase), *bla*$_{DHA-1}$(AmpC β-lactamase), *ampR* (transcriptional regulator), *hypA* (putative hydrogenase nickel incorporation protein), *qacEΔ1* (ethidium bromide resistance protein) and *sul1* (dihydropteroate synthase) in downstream. The upstream region of pNDM-7846 consisted of genes such as *pcoS* (Sensor protein), *pcoE* (Probable copper binding protein), *rcnA*, *rcnR*, *DUF* and some other genes marked as B (ATP/GTP binding protein), A (Duf 1173 family protein), Y (DNA cystein methyl transferase) and 2 hypothetical genes marked as X and Z (**Figure R43**).The presence of bleomycin resistant protein *ble*$_{MBL}$ aided in providing an advantage to the *bla*$_{NDM-1}$ positive *E. coli* since both genes *ble*$_{MBL}$ and *bla*$_{NDM-1}$ were controlled by a single promoter; therefore, the presence of *ble*$_{MBL}$ might help the *bla*$_{NDM-1}$ bearing plasmids to disseminate in other bacterial species.

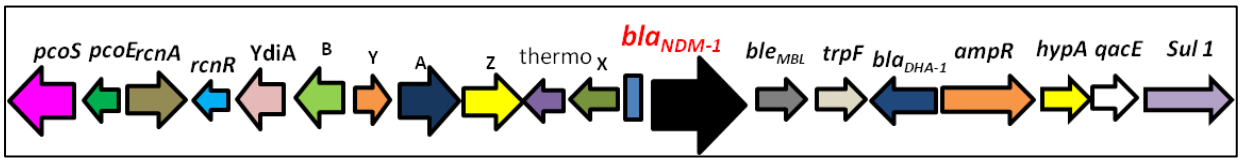

Figure R43: *Schematic representation bla*$_{NDM-1}$ *flanking region of our plasmid pNDM-7846 showing the different antimicrobial resistance encoding genes. Arrow lengths are proportionate to the lengths of the genes or open reading frames. Genetic structure of pNDM-7846 consists of ble*$_{MBL}$ *(bleomycin resistance gene), trpF (phosphor ribosyl anthranilate isomerase), bla*$_{DHA-1}$*(AmpC β-lactamase), ampR (transcriptional regulator), hypA (putative hydrogenase nickel incorporation protein), qacEΔ1 (ethidium bromide resistance protein) and sul1 (dihydropteroate synthase) in downstream while pcoS (Sensor protein), pcoE (Probable copper binding protein), rcnA (, rcnR, DUF, some other genes marked as B (ATP/GTP binding protein), A (Duf 1173 family protein), Z (DNA cysteine methyl transferase) and 2 hypothetical genes marked as X and Z in the upstream.*

6.9.7. pNDM-7846 sequence showed homology to *bla*$_{NDM}$ plasmids from other organisms

Our plasmid pNDM-7846 was compared with the other organisms harbouring the *bla*$_{NDM-1}$ and it was found that our plasmid pNDM-1 showed homology to the plasmid sequences of various bacteria such as *E. coli*, *Klebsiella pneumonia*, *Citrobacter freundii* and *Salmonella enterica*. When the *bla*$_{NDM-1}$ flanking region was observed the genes in the downstream of *bla*$_{NDM-1}$ showed homology to the genes carried by plasmids in *E. coli* (HQQ451074), *Klebsiella pneumonia* (JX988621), *Citrobacter freundii* (MH722216.1) while the upstream region was highly similar to plasmids of *Salmonella enterica* (CP048299.1) and another *Citrobacter freundii* harbouring the *bla*$_{NDM-1}$ of accession no. CP038659.1 (**Figure R44**)

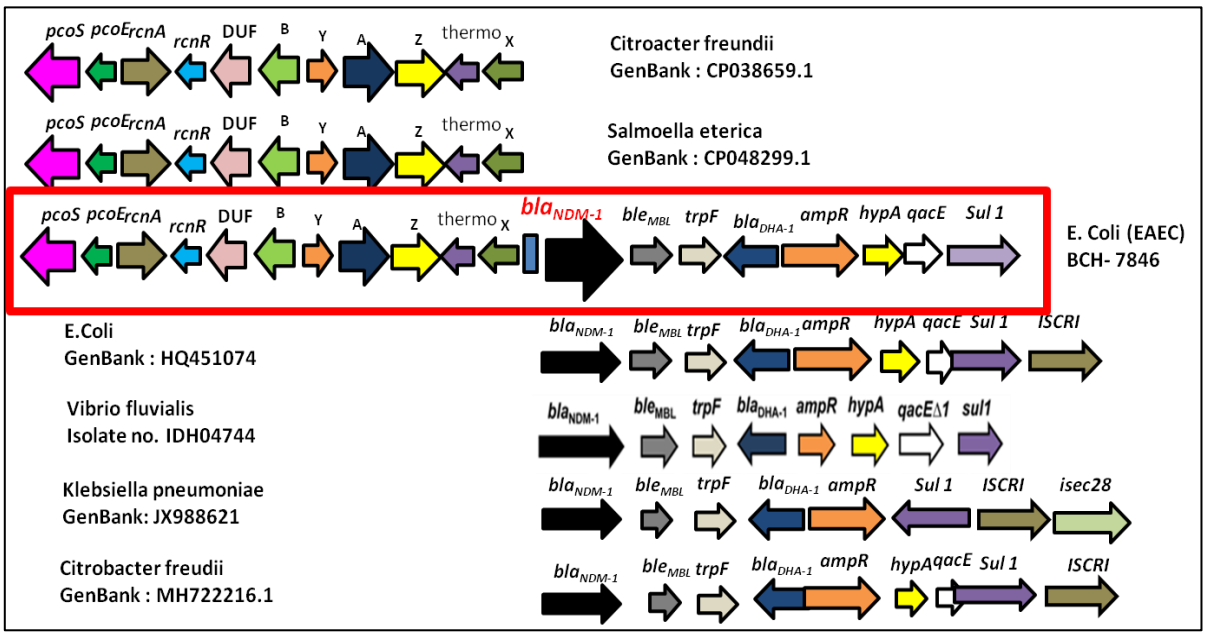

Figure R44: *Structural comparison of bla*$_{NDM-1}$ *flanking regions of pNDM-7846 (EAEC, BCH-7846) with other bacterial species. The plasmid sequence shows a high homology with bacterial species such as Klebsiella pneumonia, Citrobacter freundii, Salmonella enterica and other E. coli harboring the bla*$_{NDM-1}$ *plasmid.*

6.9.8. pNDM-7846 harboured several AMR encoding genes

Since several AMR genes are present in plasmids, we were interested in finding the AMR genes in our pNDM-784 plasmid. The plasmid showed resistance to β-lactam group of antibiotics such as meropenem, imipenem, ampicillin, cefotaxime, ceftazidime, ceftriaxone due to the presence of *bla*$_{TEM-29}$, *bla*$_{DHA-1}$, *bla*$_{NDM-1}$ resistance genes. Resistance to the tetracycline group of antibiotics was conferred by *tetC*. Genes *such as dfrA23, sul1* conferred resistance to trimethoprim and sulfamethoxazole. Resistance to macrolides such as erythromycin and azithromycin was provided by *mphE* and *msrE* genes. *rmtC* and *qacEΔ1*

conferred resistance towards aminoglycosides (amikacin, gentamycin, kanamycin, tobramycin) and quaternary ammonium compounds (ethidium bromide, chlorhexidine) while the gene *cmlA1* provided resistance to chloramphenicol (**Table R13**).

Table R13: *The list of resistance genes presents in the plasmid that confers resistance to beta lactam, tetracycline, folate pathway antagonist, macrolide, aminoglycoside, quaternary ammonium compound and amphenicol class of antibiotics.*

Antibiotics Class	Resistant Phenotype	Resistance genes
Beta-lactums	Meropenem, imipenem, ampicillin, cefotaxime, ceftazidime, ceftriaxone	*bla-TEM29, bla-DHA1, bla-NDM1*
Tetracycline	Doxycline, Tetracycline	*tetC*
Folate pathway antagonist	Trimethoprim, sulphomethoxazole	*dfrA23, sul1*
Macrolide	Erythromycin, azithromycin	*mph(E), msr(E)*
Aminoglycoside	Amikacin, Gentamycin, Kanamycin, Tobramycin	*rmt(C)*
Quaternery ammonium compound	ethidium bromide, chlorhexidine	*qacE*
Amphenicol	Chloramphenicol	*cmlA1*

6.9.9. MIC of the pNDM-7846 carrying EAEC

As the meropenem resistant strain (BCH 7846) was resistant to most of the antibiotics used in the study, thus we focused on finding the minimal inhibitory concentration range of the strains. E-strips were used to finds the different MIC values for the different antibiotics used such as imipenem, fluroquinolone drugs like ofloxacin and ciprofloxacin, tetracycline, streptomycin and others. The strain was resistant to all the antibiotic E-strips used (**Figure R45**).

The result of the E-strip test demonstrated the resistant phenotype of the isolate as there was no zone of inhibition formation in case of plates containing E-strips of imipenem, ciprofloxacin, ofloxacin, Trimethoprim/Sulfamethoxazole (TS) and ceftriaxone. The MIC values obtained in the plates containing E-strips of streptomycin, tetracycline and azithromycin indicated that the strain to be resistant to these antibiotics. Further in order to check the production of metallo beta-lactamase (MBL), Imipenem/Imipenem+EDTA (MBL) (IP/IPI) strip was used. The MIC result of the strip was ≥ 8, thus indicated MBL production

by the strain. The ESBL production was further checked by Cefotetan/Cefotetan+ Cloxacillin E-strip. The MIC value was 0, indicating that the strain produced no ESBL.

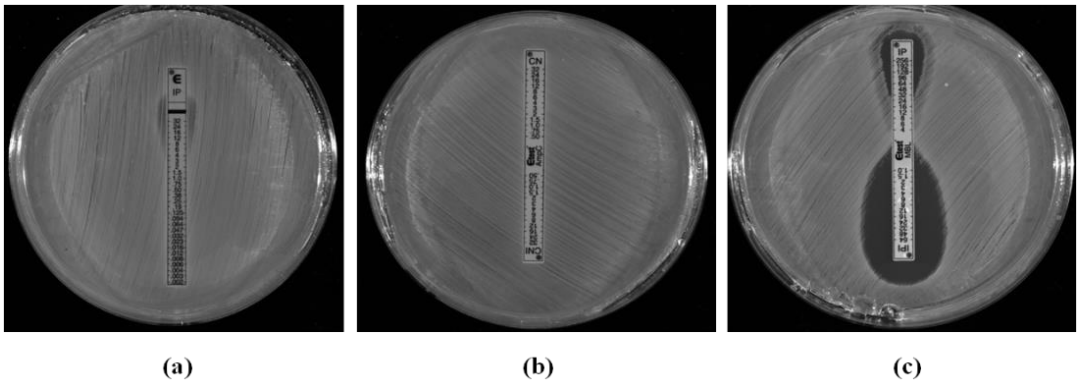

Figure R45: *The different E-strip values of the strain. (a) Imipenem strip showing imipenem resistance (b)E-strip for Amp-C beta lactamase production indicating a negative result i.e, no AmpC production by the strain (c) E-strip for MBL production, showing that the strain is positive for MBL production.*

Table R14: *MIC values of the strain pNDM-1 EAEC*

Antibiotic strips used	Zone of resistance (Diameter)	Resiatant/Sensitive
Imipenem (IP)	0	R
Oflaxacin(OF)	0	R
Ampicillin/Sulbactam (AB)	0	R
Streptomycin (SM)	12	S
Ciprofloxacin (CI)	0	R
Tertacycline (TC)	24	R
Azithromycin (AZ)	25	R
Ceftriaxone (TX)	0	R
Trimethoprim/Sulphomethoxazole (TS) (1/9)	0	R
Imipenem/Imipenem+EDTA (MBL) (IP/IPI)	32/<1	MBL production
Cefepime/Cefepime+Cla (ESBL) (PM/PML)	0/0	No ESBL production
Cefotetan/Cefotetan+ Cloxacillin	0/0	R
Cefotaxime/Cefotaxime+Cla (CT/CTL)	0/0	R

6.9.10. pNDM-7846 was nontransferable to other bacteria

Since plasmids can easily get transferred to other species resulting in the spread of antibiotic resistance, various experiments such as conjugation, transformation and electroporation were performed to check whether the plasmid was able to be transferred to the other species. For

transferring the plasmid pNDM-1, we used gene transfer techniques such as transformation, conjugation and electroporation to the other laboratory strains.

a) Conjugation

The plasmid pNDM-1 was not transferable to the laboratory strain *E. coli* J53 as well as to the other clinical strains of *E. coli*, *Shigella*, *Salmonella* and *Vibrio spp.*

b) Transformation

The transformation of the plasmid to other strains such as *E. coli*, *Shigella*, *Salmonella* and *Vibrio spp.* yielded negative results.

c) Electroporation

The electroporation experiment was not successful as pNDM-1 plasmid was not transferred to any other strains.

Thus, from the above experiments it has been concluded that the plasmid pNDM-1 was nontransferable to other organisms might have been due to the absence of transfer regulating genes and a complete functional *traJ* operon.

Part 7

Discussion

7. Discussion

Acute secretary diarrhea caused by the different classes of diarrheagenic *E. coli*, mainly ETEC, EPEC and EAEC, is one of the serious health problems in many parts of the world. DEC is associated with early child deaths in developing countries and has been identified as an important evolving pathogen. It has emerged as one of the major public health risks in children next to Rotavirus and Cholera which result in hospitalization of the patients especially in developing countries (Nair *et al.*, 2010; Raghavan *et al.*, 2017). Among the hospitalized diarrheal children aged up to 5 years of age, the prevalence of DEC was next to that of rotavirus while in children above 5 years of age, DEC mediated infection was placed next to cholera (Nair *et al.*, 2010; Dutta *et al.*, 2013).

Among the different categories of DEC, ETEC results in 75 million annual episodes of diarrhea in children that are less than 5 years and accounts for about ~4.2% of diarrheal deaths (Silwamba *et al.,* 2022). Moreover, ETEC is also responsible for acute diarrhoea in developing countries with an estimated number of 400 million cases per year among patients aged more than 15 years. Diarrhea caused due to ETEC is also known to be associated with chronic nutritional faltering (Black *et al.,* 1981). This disease burden is concentrated primarily in young children in less-developed countries of Asia, Africa, Middle East and Latin America (Dutta *et al.,* 2013; Bagamian *et al.,* 2020). ETEC has been a major cause of diarrhoea in children less than 5 years of age that has resulted in about 60,000 deaths in 2015 (Anderson *et al.,* 2019; Bagamian *et al.,* 2020). It is the major enteric bacteria known to cause tens of millions of diarrheal episodes in the developing world per year (Anderson *et al.,* 2019). It is the major etiological factor causing traveller's diarrhea in low income countries and almost one-third of the travellers suffering due to diarrhea was diagnosed with disturbances in their gastrointestinal tract. ETEC infections are seen to be formed due to the uptake of contaminated food and drinking water and since developing countries lack the infrastructure to provide clean water and disposal of waste materials the risk of ETEC diarrhoea is more in these countries (Ahmed *et al.,* 2013; Zhang *et al.,* 2022).

Recent patho-physiological studies on EPEC infection support the molecular basis of disease mediated by EPEC. Although during 1940-1950, outbreaks were frequent in developing countries, the incidence of EPEC infection was reported during the 1960s in the United States and the United Kingdom. One of several categories of DEC, EPEC is considered to be a well-established pathogen causing diarrhea among children due to its high prevalence in both

hospital settings as well as in communities. A recent hospital based study conducted in India has reported 3.2% of 648 diarrheal stool samples collected from children below 5 years of age were positive for EPEC (Nair *et al.,* 2010; Ochoa *et al.,* 2011). Thus EPEC infection is estimated to cause the deaths of several hundred thousand children per year making EPEC a significant health threat to children worldwide (Ochoa *et al.,* 2011).

EAEC is being recognized as an important cause of persistent diarrhea (14 days) in the entire world, especially in developing countries (Nataro and Kaper, 1998; Nataro *et al.,* 1998). In several studies, the detection of EAEC from the stool of patients in the first few days of diarrhea indicated the continuation of the illness for a longer period (Donnenberg *et al.,* 1992; Estrada-Garcia *et al.,* 2014). The presence of EAEC has been documented in sporadic diarrheal cases and also in gastroenteritis outbreaks in different parts of the world (Hebbelstrup *et al.,* 2018). In recent times, EAEC has been identified as a major etiological cause of acute diarrhea which requires hospitalization in children and travelers worldwide (Estrada-Garcia *et al.,* 2014). EAEC is characterized as a heterogeneous pathogen but shares a common phenotypic character of forming an aggregative adherence (AA) pattern on epithelial cells such as HEp-2 or HeLa cells (Nataro *et al.,* 1987; Estrada-Garcia *et al.,* 2014). EAEC comprises several genetic variations and consists of both pathogenic and non pathogenic strains (Jenkins *et al.,* 2006). A huge number of children who were asymptotic carriers have been reported in several different studies (Nüesch-Inderbinen *et al.,* 2013). However, their association with several outbreaks and in volunteer studies has confirmed their role in being an important etiologic agent (Mathewson *et al.,* 1987; Nataro *et al.,* 1985; Okeke *et al.,* 2007). The heterogeneity of EAEC is due to the presence of virulence factors and their regulating mechanism (Estrada-Garcia *et al.,* 2014; Hebbelstrup *et al.,* 2014; Hebbelstrup *et al.,* 2018).

Enteroinvasive *Escherichia coli* (EIEC) belong to one of the diarrheagenic *E. coli* (DEC) pathotypes that cause bacillary dysentery quite similar to that of *Shigella* pathotypes in children as well as in adults (Michelacci *et al.,* 2016; Nataro *et al.,* 1998; Farajzadeh-Sheikh *et al.,* 2020). Despite several worldwide reports on the prevalence of EIEC, very little is known about them and there is no study regarding their virulence from India. In the present study, an attempt was made to document the prevalence of 3 major pathotypes of DEC (EAEC, EPEC, ETEC) along with the rare DEC pathotype EIEC in Kolkata and also provide information on the phenotypic and genotypic traits, antibiotic resistance and antibiotic resistance genes profile of DEC strains. Our study also focuses on the molecular determinants

of invasiveness, intracellular growth and spreading of EIEC isolates in both *in vivo* and *in vitro* model environments.

7.1. Prevalence of DEC in Kolkata, India and its distribution to various age groups

Thus based on the above scenario of DEC infections and its harmful impact on the population we have concentrated on finding the impact of DEC pathotypes in the population of Kolkata. Our clinical observational study was conducted to find out the preponderance of DEC pathotypes to any specific age group, drug resistance pattern and genetic markers for virulence among the patients admitted to ID&BG hospital and B.C Roy Hospital, Kolkata, India. In ID&BG hospital, stool samples were collected from acute diarrheal patients (all age groups) from 2012 to 2022 and in B. C. Roy hospital, the stool samples were collected from children under 5 years of age from the year 2018 to 2022.

Prevalence of DEC from ID&BG Hospital in our study was 7.47% which is comparable to the reports in other developing countries (Schroeder *et al.,* 2002; Takahashi *et al.,* 2008; Lima *et al.,* 2017). Of all DEC infected diarrheal cases, infections caused solely by the DEC was 73% followed by 27% of mixed infections caused by DEC in association with other pathogens such as *Vibrio*, *Campylobacter*, *Shigella* and *Salmonella*. However, this sole infection pattern is quite higher in contrast to other previous reports (Dutta *et al.,* 2013). ETEC was found to be mostly associated with diarrhea followed by EAEC and EPEC. Among all the 3 DEC pathotypes, 73% of the diarrheal cases were due to sole ETEC infections. This scenario is quite different from the previous studies in Kolkata from 2008 to 2011 where EAEC was prevalent. Moreover, our present study also revealed that the infections caused by ETEC strains containing both the ST and LT toxins were significantly higher compared to infections caused by ETEC strains containing either LT or ST alone. Although EAEC and EPEC has been reported as the major diarrhea causing agent in developing countries like Brazil, Tanzania and several parts of India including Northern India, Manipal and Karnataka, our study from ID&BG Hospital suggested ETEC as the major diarrhea causing pathotype followed by EAEC and EPEC among the hospitalized patients suffering from diarrhea (Okeke *et al.,,* 2000; Takahashi *et al.,* 2008; Shetty *et al.,* 2012; Dutta *et al.,* 2013; Canizalez-Roman *et al.,* 2013; Canizalez-Roman *et al.,* 2016; Singh *et al.,* 2019).

The study from B. C. Roy hospital highlighted contrasting results to the ID&BG hospital study. Among the B. C. Roy isolates, the prevalence of DEC was 8.66% (443/5112) among 5112 stool samples collected during the year 2018-2022 which is in accordance with a

previous study from Kolkata (Dutta *et al.,* 2013). 86% of the diarrheal cases were due to sole DEC infections while only 14% of the cases were due to DEC infections along with other bacterial infections (*Vibrio*, *Campylobacter*, *Shigella* and *Salmonella*). This result was contradictory to the result documented by Dutta *et al* in 2013 where the percentage of sole infection was much lower than that of mixed infections comprising DEC along with other enteric pathogens (Dutta *et al.,* 2013). In the B. C. Roy isolates, the prevalence of EAEC was highest followed by EPEC and ETEC. Our study result was similar to the study conducted in the four provinces of Mozambique during 2014-2017 where we observed a similar pattern of prevalence among the DEC isolates (Manhique-Coutinho *et al.,* 2022). From B. C. Roy Hospital, the number of typical ETEC (harboring both LT and ST genes) was less compared to atypical ETEC infections (containing only ST or LT genes). Our observation from both these hospitals showed a greater incidence rate of DEC infection in children ≤2 years of age than in older age groups which was reported from Kolkata and other locations during past years (Rajendran *et al.,* 2010; Dutta *et al.,* 2013). Moreover, significant association of EAEC was seen in case of children ≤2 years from both the hospitals but the prevalence of ETEC was higher in age group >30-50 years which is very similar to the previous reports of Pondicherry and Vietnam (Nguyen *et al.,* 2006; Natarajan *et al.,* 2018; Ghosh *et al.,* 2022). Thus, these observations demand a continuous study of DEC surveillance in order to understand the distribution pattern in the upcoming years to develop strategies that can prevent the spread of these pathogenic strains. Further, Zhou *et al.,* showed the seasonal variations of DEC prevalence in China where it was found that a higher frequency of isolates obtained during late summer and monsoon months indicated a specific dependence of DEC infection to certain environmental conditions such as moisture and temperature (Zhou *et al.,* 2018). In our study, it has been found that the rate of DEC isolation from both the hospitals was higher in July and August during the monsoon season which entails a similar seasonality pattern of DEC infection.

7.2. Significance of newly variant lactose fermenting EIEC strains in Kolkata

Enteroinvasive *E. coli* forms part of the distinctively important clinical *E. coli* pathotype which causes watery diarrhea and shows pathogenic similarity to the four species of *Shigella* (Mohammadzadeh *et al.,* 2015). Although EIEC has been recognized as a human pathogen but very little research has been conducted on EIEC due to the low incidence rate of this diarrheal pathogen in comparison to the other diarrheal pathogens (Rajendran *et al.,* 2010; Chellapandi *et al.,* 2017; Garrine *et al.,* 2020). Our study deals with the prevalence,

characterization and pathogenic comparison of the EIEC isolates obtained from the diarrheal patients in Kolkata.

Prevalence of the EIEC isolates obtained from diarrheal patients is only about 1.5% which is similar to the other previous reports of EIEC isolation from southeastern Iran (Alizade *et al.,* 2014) Vietnam (Hien *et al.,* 2008) and south India (Rajendran *et al.,* 2010). A very low prevalence of EIEC isolates was found among adults as well as children below 5 years of age in other parts of Iran (Eybpoosh *et al.,* 2021). Similar to the previous reports, 60% of the EIEC isolated from Kolkata were from children below 5 years of age (Michelacci *et al.,* 2016; Mohammadzadeh *et al.,* 2015; Garrine *et al.,* 2020). Most of the EIEC isolates obtained from children in our study were lactose fermenting in nature. EIEC shows the variable ability to ferment lactose whereas most of the *Shigella spp* are lactose negative due to the absence of lactose permease gene (*lacY*) required for lactose fermentation (Løbersli *et al.,* 2016). The LF EIEC found in our study showed higher virulence properties and invasiveness than the NLF EIEC isolates found in the Indian population. In fact, their virulence property was higher than that of *Shigella sp.* From this study, it is seen that EIEC can cause acute diarrheal disorders in all age groups and can be dangerous in children below 5 years. Moreover, the indiscriminate use of antibiotics has resulted in the rise of multidrug resistance to these new EIEC isolates making it a challenge to treat these pathotypes which may emerge as a potent enteric pathogen having an adverse effect on the worldwide population.

7.3. Virulence profile of the LF EIEC in comparison with the NLF EIEC strains

Our study deals with the distribution of several virulence genes in the EIEC isolates that were mostly studied in the *Shigella spp*. in the various previous reports (Nave *et al.,* 2016). The *ial* gene which is required for invasion was reported to be absent in some of the *Shigella sp* (Thong *et al.,* 2005; Sousa *et al.,* 2013; Nave *et al.,* 2016) but all the EIEC isolates in our study showed 100% prevalence of *ial* gene which is in agreement to the various other studies carried in Iran and Shiraz (Abbasi *et al.,* 2014; Nave *et al.,* 2016). In our study, the major transcriptional regulator *virF* was found in almost 73% of the isolates which is slightly lower than the study conducted by Farajzadeh *et al* in Iran (Farajzadeh-Sheikh *et al.,* 2020). Transcription of *virF* results in the activation of other regulatory genes involved in conferring virulence nature to the EIEC isolates. The *sen* gene that is involved in intestinal inflammation and diarrhea was found in only 46% of the EIEC isolates which was contradictory to the high

prevalence of *sen* gene found in different studies carried out in Iran (Nave *et al.,* 2016; Farajzadeh-Sheikh *et al.,* 2020). Similar to our study, Boisen *et al.* and Hosseini Nave *et al.* found a high prevalence of the SPATE gene *sigA* among the EIEC isolates (Boisen *et al.,* 2009; Nave *et al.,* 2016). However, the *sepA* gene was found in almost 33% of the EIEC isolates quite contradictory to the observations of Boisen *et al* and Hosseini Nave *et al.,* (Boisen *et al.,* 2009; Nave *et al.,* 2016). Consistent with previous reports, our EIEC isolates also harbored a large plasmid of 220kb that contained the genes required for invasion such as *ipaA, ipaB, ipaC, ipaD, ipaH, virF, virB, icsA* and *icsB* (Sansonetti *et al.,* 1982; Parsot *et al.,* 2005; Pasqua *et al.,* 2017). NLF-EIEC isolates have previously been reported to contain multiple plasmids and we found LF-EIEC isolates also harbored multiple plasmids with high abundance similar to the study demonstrating the different plasmids in *Shigella* spp. by Nandy and his colleagues in Kolkata (Nandy *et al.,* 2010). The presence of these virulence genes on the virulence plasmid was further validated by PCR where it was observed that apart from *ipaH* all the other genes were present on the large size virulence plasmid. *ipaH* was present in the large plasmid as well as in the other small plasmids of varying lengths thus proving the presence of multiple copies of *ipaH* genes as reported before (Farajzadeh-Sheikh *et al.,* 2020).

Apart from the prevalence and characterization, we determined virulence induction in cell culture and animal models with NLF and LF isolates. However to our knowledge, this is the first study of estimating the virulence pattern of these rarely documented LF-EIEC isolates and interestingly we found the virulence expression in terms of invasion, or the virulence gene expression was significantly higher than the NLF-EIEC as well as the *Shigella sp*. Since the expression of the virulence regulator, *virF* was higher in LF-EIEC isolates, other effector genes like *virB* were also higher correlating with increased virulence. The invasive capacity of the bacteria was further confirmed with the conventional sereny test where the direct evidence of higher invasiveness was found in the form of keratoconjunctivitis development in guinea pig eye within 24 hrs of LF-EIEC infection and this infection was self-curing over time. Our result with the NLF-EIEC was in accordance with a previous study where the pathogenicity of the NLF-EIEC strains was lower than *Shigella spp*. The *in-vitro* results were further supported by the *in-vivo* evidence where the rate of colonization of the LF-EIEC strains was higher than NLF-EIEC strains in the mice intestines. Apart from higher colonization, LF-EIEC strains disseminated to the systemic organs such as liver and spleen thus depicting the more invasiveness of LF-EIEC than that of NLF-EIEC isolates. Higher

colonization and dissemination of these LF isolates may be directly attributed to the higher expression of virulence genes by LF-EIEC strains. However, the major genetic factor(s) driving the higher pathogenic potency of the hypervirulent LF-EIEC strains is yet to be determined and is under investigation. The disease triggered by LF-EIEC would have been more fatal than the NLF-EIEC and *Shigella sp.* as the systemic spread to the liver and spleen was highly observed. Therefore, it is very important to understand the changing patterns of EIEC infections and their mechanism of invasion which would help us in designing better medical strategies to combat the hyper-virulent EIEC infections.

7.4. Antibiotic resistance profile of the recently circulating *E. coli* strains in Kolkata

In Kolkata, patients with acute diarrhea are treated with oral rehydration solution as well as antibiotic intervention. Since many patients suffering from gastroenteritis are treated with antibiotics; it is very important to explore the prevailing antibiotic resistance patterns of the common etiologic agents. As ETEC, EPEC and EAEC are associated with diarrhoea in this region, attention was paid to their role in antibiotic treatment therapy as this information on the resistance patterns of DEC in this region is important for the incorporation of appropriate control strategies.

ID&BG hospital:

Our results indicated more than 75% of *E. coli* isolates were resistant to ampicillin followed by fluoroquinolone drugs such as ciprofloxacin, ofloxacin along with sulfamethoxazole-trimethoprim and tetracycline. Studies carried out in China and Mexico, have shown a similar pattern of higher resistance to the first line of antibiotics ampicillin, sulfamethoxazole-trimethoprim and tetracycline (Canizalez-Roman *et al.,* 2016; Zhu *et al.,* 2016; Zhou *et al.,* 2018). Most of the DEC strains showed resistance to fluoroquinolone drugs demonstrating resistance by horizontal gene transfer similar to the studies carried out by Ciesielczuk et al (Ciesielczuk *et al.,* 2013). Another example of resistance by horizontal gene transfer explains more resistance to sulfamethoxazole-trimethoprim in EPEC strains than that in EAEC accounting for almost 60% of EPEC resistant phenotypes. The beta-lactam resistance showed by the isolates in our study was due to the greater prevalence of $bla_{CTX\text{-}M}$, bla_{TEM} followed by $bla_{OXA\text{-}1}$ and bla_{SHV} similar to the study carried out in the past. The presence of plasmid mediated quinolone resistance (PMQR) genes such as *qnrA*, *qnrS* and *aac6'-Ib-cr* have been reported in studies carried out in Spain and India (Rozwandowicz *et al.,* 2018). Similarly, our

study also reflects the presence of *qnrS, qnrB* and *aac6'-Ib-cr* in the isolates in high frequencies.

B. C. Roy isolates:

Similar to the studies in Vietnam, the diarrheagenic *E. coli* strains found here were resistant to ampicillin, streptomycin and sulfamethoxazole (Nguyen *et al.,* 2006). The study results in Vietnam showed higher prevalence of ampicillin resistant EAEC strains quite similar to our study. Our study results depicted a higher resistance to β-lactums antibiotics followed by fluoroquinolone, tetracycline and streptomycin group of antibiotics which were regarded as the common antibiotics in diarrheal treatment. Similar to the studies by Dela *et al.,* more than 30% of our EAEC isolates were resistant to fluoroquinolone drugs (Talan *et al.,* 2016). However, 25% and 18% of EPEC and ETEC strains were resistant to fluroquinolone drugs. The gradual increase of resistance to co-trimoxazole, β-lactam, tetracycline and fluoroquinolone groups of antibiotics reflects well-documented global trends. The β-lactum resistance is conferred by genes such as $bla_{\text{TEM-9}}$, $bla_{\text{CTXM-3}}$ similar to the study reports of Bajpai *et al* (Bajpai *et al.,* 2017). 66% of ETEC isolates harboured $bla_{\text{CTXM-3}}$ and *sul2* while 33% of the ETEC isolates harboured *strA, aadA1* and *dfrA1* for streptomycin and co-trimoxazole resistance respectively. Almost 80% of ETEC isolates harboured the fluroquinolone resistant gene *qnrS* whereas *qnrB* was only found among the EAEC isolates. Thus, monitoring of antimicrobial resistance data should be continued as recent reports documented the emergence of quinolone-resistant diarrheagenic *E. coli* strains (Vila *et al.,* 1999). The low prevalence of chloramphenicol and gentamycin resistance among DEC strains of B. C. Roy hospital suggests the validity of using these drugs as the treatment of choice for diarrhoea in children less than 5 years. 41.27% of *E. coli* strains in our study isolated from both the hospitals belonged to the MDR phenotype which is similar to the study carried out in paediatric patients from Iran (Abbasi *et al.,* 2020). There are other studies where the percentage of MDR *E. coli* is as high as 66.7% (Zhou *et al.,* 2018). Thus the spreading of MDR *E. coli* possesses a serious menace to the proper management of diarrheal diseases worldwide (Abbasi *et al.,* 2020). The high rate of MDR *E. coli* strains in children may lead to treatment failures. Moreover, these resistant *E. coli* requires to be treated with the more recent generation antibiotics thus increasing the cost of treatment.

The global rate of incidence of antimicrobial resistance has raised an alarming condition and requires the need for the development of proper interventions which can prevent the

transmission of this resistance. This study suggests the emergence of LF variant EIEC strains with increasing antimicrobial resistance and high pathogenic properties that can result in large diarrheal outbreaks across the globe and an upcoming threat to humans if proper healthcare strategies are not taken. This rare group of *E. coli* has developed resistance to cephalosporin and quinolone group of antibiotics (Chellapandi *et al.,* 2017). The EIEC isolates in our study showed resistance to β-lactums, tetracycline, and streptomycin as well as fluoroquinolone group of antibiotics where the NLF-EIEC strains demonstrated a high resistance pattern towards most of the antibiotics than that of the LF-EIEC strains. Much like the previous study conducted on diarrheagenic *E. coli* in Kolkata (Ghosh *et al.,* 2022), all the EIEC strains were susceptible to meropenem. The resistance showed by the EIEC isolates was similar to the *Shigella spp* and *E. coli* isolates reported in various reports all over the world (Nandy *et al.,* 2010; Qin *et al.,* 2020; Salleh *et al.,* 2022). 4 (44.44%) of the NLF strains showed MDR phenotype by being resistant to 5 different classes of antibiotics namely β-lactum, tetracycline, streptomycin, sulphomethaxazole and fluoroquinolone class of antibiotics much like the MDR *Shigella* isolates found in Japan in 2006 (Ahmed *et al.,* 2013).

Similar to the study conducted on *Shigella spp*. by Pazhani and his colleagues in India, the EIEC isolates were resistant to β-lactum, streptomycin and chloramphenicol group of antibiotics due to the presence of bla_{OXA-1}, *strA* and *cat1* genes (Pazhani *et al.,* 2008). Moreover, *dfrA1* gene responsible for conferring resistance to trimethoprim was also found in these EIEC isolates similar like the *Shigella* isolates of the study conducted in India (Pazhani *et al.,* 2008). Only the plasmid mediated *qnrS* conferring fluoroquinolone resistance was found among the EIEC isolates in contrast to reports that highlighted the presence of various plasmid mediated genes such as *qnrS, qnrB* and *aac6'-Ib-cr* in diarrheagenic *E. coli* (Ghosh *et al.,* 2022) and *Shigella* isolates (Qin *et al.,* 2020). Therefore, the information on the resistance patterns of the aetiological agents is important for therapeutic aspects and for implementing appropriate control strategies (Vila *et al.,* 1999).

7.5. Documentation of a carbapenem resistant EAEC strain for the first time in Kolkata, India

The increase in resistance to carbapenems is a major public health concern since carbapenems are considered as the last line of defence antibiotics for the treatment of infectious disease caused by multidrug resistant (MDR) bacteria (Peirano *et al.,* 2011). Carbapenems are β-lactum antibiotics which hinder the cell wall synthesis by binding to the

penicillin binding proteins. The most commonly used carbapenem antibiotics are meropenem, imipenem, doripenem and ertapenem (Potter *et al.,* 2016). Carbapenemases are enzymes secreted by bacteria which inactivate the carbapenems by hydrolyzing the beta lactum ring (Potter *et al.,* 2016). In addition to the commonly found carbapenemases in Enterobactericea such as IMP, VIM, KPC and OXA-48, a novel class B metallo β-lactamase called New Delhi beta lactumases-1 (NDM 1) has been reported in 2008 (Yong *et al.,* 2009; Solé *et al.,* 2011; Flerlage *et al.,* 2020). NDM enzymes were first identified in *Klebsiella pneumonia* and *Escherichia coli* clinical isolates recovered in Sweden from a traveller returning from India which exhibited broad spectrum activity against penicillins, cephalosporins and carbapenems except aztronem (Solé *et al.,* 2011; Marchetti *et al.,* 2020). Since the discovery in 2008, 24 variants of NDM have been identified in association with other resistance genes which are rapidly distributed all over the world (Pitart *et al.,* 2015; Marchetti *et al.,* 2020). The spread of NDM producing Enterobacteriaceae (mostly *K. pneumonia* and *E. coli*) in India, Pakistan and United Kingdom was reported by Kumarasamy and his colleagues in 2010 (Peirano *et al.,* 2011; Bora *et al.,* 2013). Further reports of the presence of NDM producing Enterobacteriaceae have been obtained from patients in the United States, Netherlands, Australia, Canada, France and Sultanate of Oman (Poirel *et al.,* 2010; Poirel *et al.,* 2010; Pitout *et al.,* 2010; Poirel *et al.,* 201; Peirano *et al.,* 2011). Thus NDM enzymes have received special attention due to their rapid global dissemination and association with other resistance genes (Pitart *et al.,* 2015). Thus it became important to find the presence of such $bla_{\text{NDM-1}}$ harboring strains in the population of Kolkata.

Carbapenem resistance was found among EPEC pathotype isolated from children less than 5 years of age (Zhou *et al.,* 2018), from *E. coli* present in urine samples (Arzanlou *et al.,* 2022) and also from commensal *E. coli* found in the gut of normal individuals (Chowdhury *et al.,* 2022). However, the presence of carbapenem resistance in EAEC was first documented in our study where the $bla_{\text{NDM-1}}$ flanking environment of the plasmid showed high similarities with the $bla_{\text{NDM-1}}$ plasmid present in *E. coli* strain HK451074 which is the first *E. coli* strain harboring $bla_{\text{NDM-1}}$ plasmid found in China (Liu *et al.,* 2013). Much like the HK451074 strain and the plasmid containing commensal *E. coli* strains, our plasmid harboring strain also showed multidrug resistance phenotypes towards other antibiotic groups such as β-lactums, fluoroquinolones, tetracycline, aminoglycoside groups. The antimicrobial resistant genes such as bla_{TEM29}, bla_{DHA1}, bla_{NDM-1}, *tetC, sul1, mphE, rmtC, qacE* and *cmlA1* were responsible for conferring resistance to the various antibiotics. The plasmid showed complex structure

where the upstream region of *bla*$_{NDM-1}$ showed similarities with *bla*$_{NDM-1}$ plasmid present in *Citrobacter freundii* (GenBank: CP038659.1) and *Salmonella enterica* (GenBank: CP048299.1) while the downstream region showed similarities with *bla*$_{NDM-1}$ plasmids of *E. coli* (GenBank: HQ451074), *Vibrio fluvialis* and *Klebsiella pneumonia* (GenBank: JX988621). However, the multidrug resistant plasmid is non transferable to other species thus lowering the threat of global spread of this particular plasmid isolated from our lab to the world population.

Part 8

Highlights of the study

8. Highlights of the study

8.1. Isolation and identification of enteric *E. coli* (Diarrheagenic and commensal) isolated from diarrheal stool samples

1) Kolkata has been considered an endemic region where diarrhea is common among children as well as in adults and DEC as a causative agent accounts for 40% of the total reported diarrheal cases. Therefore, considering the DEC-infected diarrhea in India, we studied the prevalence of different DEC pathotypes in the population of local endemic regions and focused on the difference of infection patterns in children below five years of age and in adults. Two study groups were prepared to compare the geographical location-based as well as age-group based infection patterns. Stool samples were collected from two remote hospitals in Kolkata-

    i. **ID&BG Hospital** that treats patients of all age groups suffering from diarrhea and admitted to the hospital to receive treatment.

    ii. **B. C. Roy Hospital** which treats children who are below 5 years of age and are treated in the outpatient department (OPD).

2) In this 11-year (2012-2022) study period we found patients with different susceptibility to DEC infections in these two hospitals. ETEC pathotype was prevalent followed by EAEC and EPEC among the ID&BG hospital isolates while EAEC was prevalent followed by EPEC and ETEC pathotypes in case of B. C. Roy hospital isolates. From this result, it was indicated that EAEC and ETEC served as the major DEC pathotypes in the population of Kolkata and thus, provided base line data of the current situation of DEC prevalence in Kolkata.

3) Mixed pathogenic infections such as DEC co-infection with other pathogens, the presence of other diarrhea causing bacteria showed that *Vibrio spp*. was prevalently associated with ETEC pathotype isolated from both the hospitals while *Shigella sp*. was found to be prevalent with EAEC isolates of ID&BG Hospital. *Campylobacter* was highly associated with EAEC isolates of B. C. Roy Hospital. Thus, it indicates other pathogens may also remain associated with DEC pathotypes and may induce severe damage to human health.

4) The distribution of the sole and mixed DEC infections among different age groups showed that EAEC and EPEC infection as both sole and mixed form was highest among children ≤2 years but sole and mixed infections of ETEC was highest in older age groups.

5) The seasonality pattern of the DEC isolates was highest in the monsoon season (July-Sept) in both the hospitals thus focusing that there is an increase of diarrheal infections during the rainy season due to unhygienic conditions and unsafe drinking water during that time of the year.

6) Along with the 3 major pathotypes of DEC, this study focused on the comparison of phenotypic and virulence traits of the rarely reported lactose-fermenting EIEC with the non-lactose-fermenting EIEC isolates, highlighting the hyper-virulent nature of the lactose-fermenting EIEC which is emerging as a significant threat to human health.

7) 0.15% of the patients from both hospitals were found to be affected by EIEC. Among the positive isolates, 40% were lactose fermenting (LF) in nature. Apart from the presence of the major virulence gene *ipaH*, other virulence associated genes such as *ial* and *virF* were found to be more common in LF whereas NLF strains mostly contained *ipaBCD* virulence genes.

8) The strains were checked for the presence of multiple copies of plasmids, motility, clonality and antibiotic resistance. All the strains were non-motile and harboured multiple plasmids. The strains harbored the large pINV (~220 kb) plasmid that is responsible for conferring the virulence phenotype as it carries all the genes required for virulence and invasion. Greater antimicrobial resistance was found in the NLF strains with the presence of beta-lactamase, tetracycline and fluoroquinolone resistance genes.

9) Virulence induction assays were performed in cell culture with these NLF and LF isolates showing higher virulence expression in terms of invasion, or the virulence gene expression in the case of the LF EIEC strains.

10) This data was further confirmed with *in-vivo* studies which showed higher colonization by LF-EIEC strains in mouse intestine and development of severe keratoconjunctivitis in the eyes of guinea pigs. Along with higher colonization, LF strains disseminated to the systemic organs such as the liver and spleen thus depicting the invasiveness of LF-EIEC than that of NLF-EIEC isolates.

11) Our study demonstrated the estimation of the prevalence of DEC pathotypes which improves the understanding of the epidemiological aspects of DEC infections in the population of Kolkata, India. The study results call for increased vigilance for such etiological agents in the Indian population to combat any emergency that may arise due to negligence.

8.2. To access the magnitude of antimicrobial resistance in enteric *E. coli* isolated from diarrhoea patients

1) The antibiotic resistance profile of the isolates showed a high prevalence of MDR *E. coli* strains that were isolated from the diarrheal patients from both hospitals. The rise of MDR *E. coli* can be regarded as a serious threat to critical patients in hospitals and nursing homes. Studies with *E. coli* strains are particularly important as they can efficiently accept and transfer genetic materials among various enteric pathogens.

2) From both hospitals, a higher percentage of EAEC strains were resistant towards β-lactum groups of antibiotics such as ampicillin, ceftriaxone as well as fluoroquinolone antibiotics followed by EPEC and ETEC strains.

3) EIEC strains were mostly susceptible to the antibiotics used but showed resistance to tetracycline, streptomycin and fluoroquinolone group of antibiotics. Greater antimicrobial resistance was found in the NLF strains with the presence of β-lactamase, tetracycline and fluoroquinolone resistance genes.

4) In the ID&BG hospital isolates, most of the EPEC strains showed MDR phenotype followed by EAEC and ETEC. Most of the EPEC strains were resistant to at least 3 groups of antimicrobials while EAEC was found to be resistant to 4 and 5 groups of antimicrobials. In the B. C. Roy isolates, EPEC was resistant to at least 4 groups of antimicrobials and ETEC showed resistance to at least 3 antimicrobial classes.

5) Thus, from our study we find a progressive increase in the MDR status of the *E. coli* isolates among the diarrheal patients in Kolkata which has even spread to the children population. This high prevalence of multidrug-resistant DEC strains circulating in the Indian population is a major risk factor and urges for fast and better treatment facilities against multidrug resistant strains.

8.3. Characterization of carbapenem resistance (*bla*NDM) *E. coli* strains isolated from diarrheal patients

1) Carbapenems are broad spectrum antibiotics which are generally used as the last line of defence against multidrug resistant bacteria. Since carbapenem are the last line of defence against multidrug resistant bacteria and the spread of resistance of this drug among different bacterial species are an emerging problem thus we have taken one archived meropenem resistant strain (BCH-7846) for further studies.

2) $bla_{\text{NDM-1}}$ positive enteroaggregative *E. coli* strain isolated for the first time in the Indian population

3) The size of the plasmid was determined as ~180kb and was found to be non-transferable.

4) The pNDM-7846 plasmid belongs to Inc HI 1 replicon.

5) The plasmid conferred resistance to antibiotic groups such as β-lactums, tetracycline, macrolides and aminoglycosides.

6) The flanking region of bla_{NDM-1} showed similarity with bla_{NDM-1} flanking region of plasmid of other species such as *V. fluvialis, K. pneumoniae, C. freundii.*

8.4. To identify the genetic determinants and mechanism of resistance to fluoroquinolone and of associated multidrug resistance in *E. coli* isolates

1) Fluoroquinolone resistance is mainly caused by point mutations in the quinolone resistance-determining regions (QRDRs) of the DNA gyrase (encoded by *gyrA* and *gyrB*) and topoisomerase IV (encoded by *parC* and *parE*) subunits. Thus, we have sequenced the QRDR of the chromosomal genes (*gyrA* and *gyrB, parC* and *parE)* to find unique point mutations resulting in fluoroquinolone resistance.

2) Among the chromosomal genes, *gyrA* and *parC* harboured mutations that are already reported while *gyrB* and *parE* genes harboured synonymous mutations.

3) Mutations found: *gyrA* S83 → L and D87 → N, *parC* S80 → I

4) Among the plasmid mediated quinolone resistance (PMQR) genes, *qnrS* and *qnrB* were highly prevalent among the isolates from both the hospitals. The presence of *qnrS* was

higher in case of ETEC for ID&BG isolates and *qnrB* was higher in ETEC for B. C. Roy isolates.

5) Therefore, fluoroquinolone resistance in DEC isolates in India outlined the presence of plasmid-mediated resistance-conferring genes along with mutations in DNA replication genes.

Part 9

References

9. References

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Part 10

List of publications

10. List of publications

Publications in peer reviewed journals

1) **Ghosh D,** Chowdhury G, Samanta P, Shaw S, Deb AK, Bardhan M, Manna A, Miyoshi SI, Ramamurthy T, Dutta S, Mukhopadhyay AK**.** Characterization of diarrhoeagenic *Escherichia coli* with special reference to antimicrobial resistance isolated from hospitalized diarrhoeal patients in Kolkata (2012-2019), India. **J Appl Microbiol. 2022**; 132:4544-4554. doi: 10.1111/jam.15548.

2) Chowdhury G, Ramamurthy T, Das B, **Ghosh D,** Okamoto K, Miyoshi SI, Dutta S, Mukhopadhyay AK. Characterization of NDM-5 Carbapenemase-Encoding Gene (*bla*$_{\text{NDM-5}}$) - Positive Multidrug Resistant Commensal *Escherichia coli* from Diarrheal Patients. **Infect Drug Resist**. **2022**; 15:3631-3642. doi: 10.2147/IDR.S364526.

3) Shaw S, Samanta P, Chowdhury G, **Ghosh D,** Dey TK, Deb AK, Ramamurthy T, Miyoshi SI, Ghosh A, Dutta S, Mukhopadhyay AK**.** Altered molecular attributes and antimicrobial resistance patterns of Vibrio cholerae O1 El Tor strains isolated from the cholera endemic regions of India. **J Appl Microbiol. 2022**; 133:3605-3616. doi: 10.1111/jam.15794.

4) Chowdhury G, Das B, Kumar S, Pant A, Mukherjee P, **Ghosh D,** Koley H, Miyoshi SI, Okamoto K, Paul A, Dutta S, Ramamurthy T, Mukhopadhyay AK. Extensively Drug Resistant *Pseudomonas aeruginosa* Associated with Hospitalized Acute Diarrhea Case. **Future Microbiol. 2023**; 18:173-186. doi: 10.2217/fmb-2022-0140.

Manuscripts in communication

1) Bose P; Chowdhury G; Halder G; **Ghosh D**; Deb AK; Kitahara K; Miyoshi S; Masatomo M; Ramamurthy T; Dutta S; Mukhopadhyay AK. Prevalence and changing antimicrobial resistance profiles of *Shigella* spp. isolated from diarrheal patients in Kolkata during 2011-2019. Submitted to **PLOS Neglected Tropical Disease** in September 2023.

2) Chowdhury G; **Ghosh D**; Zhou Y; Deb AK; Mukhopadhyay AK; Dutta S; Chakraborty S. Field evaluation of a simple and rapid diagnostic test, RLDT to detect *Shigella* and enterotoxigenic *E. coli* and determine epidemiology in Indian children. Submitted to **Emerging Infectious Diseases** in August 2023.

Manuscripts under preparation

1) **Ghosh D;** Halder P; Samanta P; Chowdhury G; Shaw S; Roy D; Ramamurthy T; Koley H; Dutta S; Mukhopadhyay AK. High virulence pattern of lactose fermenting enteroinvasive *Escherichia coli* isolated from diarrheal patients in India.

2) **Ghosh D**; Sarkar A; Chowdhury G; Samanta P; Ramamurthy T; Dutta S; Mukhopadhyay AK. Carbapenem resistant enteroaggregative *Escherichia coli* isolated from a diarrheal patient in Kolkata, India.

Published abstract of papers presented at different scientific meetings

1) **Ghosh D**; Chowdhury G; Samanta P; Shaw S; Deb AK; Bardhan M; Manna A; Miyoshi S; Ramamurthy T; Dutta S; Mukhopadhyay AK. Rising trend in the prevalence of multidrug resistant diarrheagenic *Escherichia coli* among hospitalized diarrheal patients in Kolkata (2012-2019), India. [Poster presented at 16th Asian Conference on Diarrheal Disease and Nutrition (ASCODD) held in Kolkata, India on 11th – 13th November 2022]

2) **Ghosh D**; Chowdhury G; Samanta P; Shaw S; Deb AK; Bardhan M; Manna A; Miyoshi S; Ramamurthy T; Dutta S; Mukhopadhyay AK. High prevalence of multi-drug resistant diarrheagenic *Escherichia coli* among hospitalized diarrheal patients in Kolkata, India. [Oral presentation (online) in the BioSangam 2022 An International Conference held at Department of Biotechnology, MNNIT Allahabad, Prayagraj, India on 10th – 12th March 2022].

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Journal of Applied Microbiology

ORIGINAL ARTICLE

Characterization of diarrhoeagenic *Escherichia coli* with special reference to antimicrobial resistance isolated from hospitalized diarrhoeal patients in Kolkata (2012–2019), India

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Abstract

Aims: This study analyses the prevalence and antimicrobial resistance (AMR) of major diarrhoeagenic *Escherichia coli* (DEC) pathotypes detected in hospitalized diarrhoeal patients in Kolkata, India, during 2012–2019.

Methods and Results: A total of 8891 stool samples were collected from the Infectious Diseases Hospital, Kolkata and screened for the presence of enteric pathogens. Multiplex PCR identified the presence of DEC in 7.8% of the samples, in which ETEC was most common (47.7%) followed by EAEC (38.4%) and EPEC (13.9%). About 54% cases were due to sole DEC infections. Majority of the mixed DEC infections were caused by the *Vibrio* spp. (19.1%) followed by Rotavirus (14.1%) and *Campylobacter* spp. (8.4%). ETEC and EAEC were associated significantly with diarrhoea in children <5 years of age, whereas EPEC and also ETEC were prevalent in patients aged between 5 and 14 years. AMR profile showed high prevalence of multidrug resistance (MDR) among DEC (56.9%) in which 9% were resistant to antibiotics of six different antimicrobial classes. Screening of the AMR conferring genes of DEC showed the presence of $bla_{\text{CTX-M3}}$ (30.2%) in highest number followed by bla_{TEM} (27.5%), *tetB* (18%), *sul2* (12.6%), *strA* (11.8%), *aadA1* (9.8%), $bla_{\text{OXA-1}}$ (9%), *dfrA1* (1.6%) and bla_{SHV} (1.2%).

Conclusions: These findings highlighted the high prevalence of MDR in major DEC pathotypes that could be considered as the leading aetiological bacterial agent responsible for diarrhoea and suggests a significant public health threat.

Significance and Impact of the Study: The results of this study can help to improve the understanding of the epidemiology of DEC infections in patients with diarrhoea. Monitoring of AMR surveillance needs special attention because the DEC isolates were highly resistant to commonly used antimicrobials in the treatment of diarrhoea.

KEYWORDS

antimicrobial resistance, diarrhoeagenic *Escherichia coli*, EAEC, EPEC, ETEC

INTRODUCTION

Diarrhoea is prevailing in the developing countries, especially in Asia and Africa, where people suffer due to poor availability of safe water and lack of proper hygiene and healthcare facilities (Kotloff et al., 2013; Mills, 2014; Troeger et al., 2018). It is the second leading cause of child death at the global level and third leading cause of child mortality in India (Fenta et al., 2020; Laxminarayan & Chaudhury, 2016). Overall, this disease kills almost 2.5 million people each year globally and 70% of them are under 5 years of age (Diarrheal disease, WHO, 2017; Fenta et al., 2020). The causative agents belong to the broad group of intestinal pathogens, which includes bacteria, viruses and parasites. Among them rotavirus, calcivirus and diarrhoeagenic *Escherichia coli* (DEC) accounted for more than half of the diarrhoeal deaths worldwide (Raghavan et al., 2017; Saka et al., 2019). A recent outbreak was also reported due to DEC in the United States where 167 people from 27 states were infected (CDC, 2019). In India, 30–40% of all diarrhoeal episodes are generally found to be associated with DEC infection (Dutta et al., 2013). Therefore, DEC has been considered as the leading diarrhoeal pathogen and needs a constant surveillance to understand the disease causing property and their antimicrobial resistance.

DEC consist of six pathotypes, among them enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC) and enteropathogenic *E. coli* (EPEC) are the most common and are characterized by the presence of unique virulence factors for colonization and pathogenesis in humans (Phillips et al., 2000; Takahashi et al., 2008; Zhou et al., 2018). ETEC infections are associated with heat-stable (ST) and/or heat-labile (LT) enterotoxins encoded in the *est* and *elt* genes and fimbriae colonizing factors (CFs) (Dutta et al., 2013). In EPEC, *bfpA* and *eae* genes encode for bundle forming pilus and intimin are required for the attachment on intestinal epithelial cells (Dutta et al., 2013; Jerse et al., 1990; Rajendran et al., 2010). EAEC encode a transcriptional factor, aggregative regulator (AggR, part of the AraC family of transcription activators). AggR activates the expression of the genes *aat* and *aaiC* encoding the dispersin translocator and the chromosomal type 6 secretion system, which are present in typical EAEC (Baudry et al., 1990; Dudley et al., 2006). These virulence marker genes are unique to each pathotype and have been used in the detection of DEC (Dutta et al., 2013; Panchalingam et al., 2012).

Besides their prevalence, a major public health concern in diarrhoea endemic regions is the spread of antibiotic resistance (AMR). Antibiotics are important in treating bacterial infections and have been used for the treatment of severe diarrhoeal diseases and traveller's diarrhoea to reduce the duration of illness (Nguyen et al., 2005). Extensive use of chloramphenicol and tetracycline in the treatment for diarrhoea has increased the resistance in DEC. Hence, the use of fluoroquinolones is recommended for DEC-associated infections due to their lesser resistance (Heidary et al., 2014; Omolajaiye et al., 2020). Other *E. coli* types, such as extraintestinal pathogenic *E. coli* (ExPEC) that are related to bacteraemia and urinary tract infections, have also found to develop resistance to several antibiotics (Alhashash et al., 2013). Although antibiotics help in limiting infectious diseases, their excessive use results in the development and spread of antibiotic resistance at an alarming rate. Persistence of DEC infection is mostly due to the development of multidrug resistance (MDR) with repeated exposures of antibiotics during the course of treatment (Baym et al., 2016; Omolajaiye et al., 2020). MDR *E. coli* belongs to the critical category of pathogens, as it possesses serious threat to the patients in daily care hospitals in many countries (Rajpara et al., 2018; WHO reports, 2017). Resistance to extended spectrum β-lactamases (ESBLs), aminoglycosides, fluoroquinolones, macrolides, sulphonamides and tetracyclines are reported in clinical *E. coli* isolates (Colodner, 2005; Nepal et al., 2017; Paitan, 2018; Pons et al., 2014; Rawat & Nair, 2010). Therefore, it is very important to understand the AMR pattern so that proper medication and treatment can be provided to the patients suffering with the DEC infection (Heidary et al., 2014). The enhanced AMR is generally conferred by the acquirement of several AMR encoding genes through the mobile genetic elements (MGEs) (Natarajan et al., 2018). Hence, investigation of such AMR genes could help in understanding the role of MGEs in the spread of resistance among the DEC isolates.

Considering the importance of DEC in high rates of childhood morbidity, we conducted an 8-year study from 2012 to 2019 to investigate its prevalence in different age group of patients suffering from diarrhoea who are admitted in the Infectious Diseases and Beliaghata General Hospital (IDH) in Kolkata, India. All the DEC isolates were also tested for AMR encoding genes based on their antimicrobial resistance patterns. We have also studied the co-infection of DEC with other enteric pathogens to understand the age-specific infection and disease severity.

MATERIALS AND METHODS

Collection of clinical samples and patient history

This diarrhoeal disease surveillance study was conducted at the Infectious Diseases and Beliaghata General Hospital (IDH), in Kolkata, India. The Institutional

Ethics Committee (IEC) of the National Institute of Cholera and Enteric Diseases (NICED) approved this study (Approval Number: A-1/2015-IEC). Informed consent was obtained from each patient or parents in the case of children and enrolled in this study. Confidentiality of all the information was maintained as per the clinical ethics. Stool specimens were collected during 2012–2019 from diarrhoeal patients. The samples were collected from every 5th diarrhoea patient who may or may not receive a treatment before admission. This fraction of patients represents a total of almost 40,000 diarrhoeal cases received treatment after hospitalization. Discharge of three or more loose stools in 24 h, with or without symptoms such as abdominal pain or cramps, vomiting, faecal urgency, dehydration or dysentery, was considered as diarrhoea. The stool samples were processed in the laboratory for the identification of enteric organisms within 2 h of collection following the standard protocols (Panchalingam et al., 2012). The clinical, demographic and laboratory data were entered in a specific data entry proforma supported with the SPSS.17.0 software (SPSS Inc, USA).

Microbial culture techniques for the isolation of DEC and other pathogens

A loopful of stool sample was streaked on MacConkey agar (Difco, BD, USA) plate for the isolation of major DEC pathotypes and incubated at 37°C for 18–20 h. Three lactose fermenting pink colonies from each sample were selected and sub-cultured on Luria agar (LA, Difco) plate. For the presumptive identification of *E. coli*, cultures from the LA plate were checked for oxidase, acid/gas production in triple sugar iron agar and indole production (Dutta et al., 2013; Panchalingam et al., 2012). Further confirmation was performed using multiplex PCR for the detection of virulence marker genes for each pathotype. Other enteric pathogens were isolated and identified following the published procedures (Panchalingam et al., 2012).

Multiplex PCR assay for DEC identification

To get the DNA template for multiplex PCR, colonies were picked up from LA plate, suspended in 200 µl of phosphate-buffer saline (PBS) and boiled for 10 min followed by centrifugation at 8000*g* during 10 min and supernatant containing DNA was used in PCR experiment. The multiplex PCR was carried out for the presence of specific virulence genetic markers: *est* and/or *elt* for ETEC, *eae* and/or *bfpA* for EPEC, and *aatA* and/or *aaiC* for EAEC. A total of six primer pairs were used to detect the different virulence-associated genes (Table S1). PCR was performed following published procedures (Panchalingam et al., 2012). Presence of at least one of the virulence genes was considered as positive for the respective DEC pathotype (Dutta et al., 2013; Panchalingam et al., 2012).

Antimicrobial susceptibility testing

A total of 255 DEC isolates were selected for antimicrobial susceptibility testing. These isolates have covered each DEC pathotypes (107 EAEC, 47 EPEC and 101 ETEC) and represented almost one-third of the isolates from each year. Kirby-Bauer disc diffusion method was followed using 12 different antimicrobials, including ceftazidime (30 µg), ampicillin (10 µg), ceftriaxone (30 µg), meropenem (10 µg), gentamicin (10 µg), sulphamethoxazole–trimethoprim (23.75 and 1.25 µg, respectively), streptomycin (10 µg), tetracycline (30 µg), doxycycline (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg) and chloramphenicol (30 µg). Fresh cultures were grown at 37°C in Luria-Berteni (LB) broth until they reach 0.5 McFarland optical density. The culture was then spread onto Muller-Hinton agar (MHA, Difco) plates before placing the antimicrobial discs (BD, BBL, USA). Zone diameters were measured after an incubation of 18–20 h at 37°C as per the Clinical and Laboratory Standards Institute guideline (CLSI, 2019). The *E. coli* strain ATCC 25922 was taken as a control for checking the disc accuracy (CLSI, 2019).

PCR for AMR encoding genes

MDR strains were subjected to PCR for the presence of the following AMR determining genes: bla_{TEM}, $bla_{\text{OXA-1}}$, $bla_{\text{CTX-M3}}$, bla_{SHV} for β-lactams (Maynard et al., 2003); *qnrB*, *qnrS* and *aac6′-Ib-cr* for plasmid-mediated quinolone resistance, *sul2* and *dfrA1* for sulfamethoxazole–trimethoprim (SXT) resistance; *strA* and *aadA* for streptomycin (aminoglycoside); *catI* for chloramphenicol and *tetB* for tetracycline group of antibiotics using the primer sets listed in Table S1 (Marbou et al., 2020; Sarkar et al., 2015; Sunde & Norström, 2005). The PCR mixture (total volume, 30 µl) consists of a final concentration of 1X Standard Taq Reaction Buffer (NEB, USA), 0.33 mM dNTP mixture, 0.33 µM of each forward and reverse primer (Sigma), 1 U of Taq DNA Polymerase (NEB, USA) and 50–80 ng of template DNA. Amplicon size and the annealing temperatures of the primers for each target gene are furnished in Table S1.

Statistical analysis

The patient's age was classified into six age groups comprising of ≤2, >2–5, >5–14, >14–30, >30–50 and ≥50 years (Dutta et al., 2013). The DEC prevalence was aligned with the age groups along with the pathogens. Multinomial logistic regression analysis was performed. The differences marked as statistically significant that yield *p*-values of <0.05. Odds ratio (OR) along with the 95% confidence interval (CI) was also calculated to determine the age group at significant risk.

RESULTS

Detection of DEC and other pathogens

During an 8-year diarrhoeal disease surveillance period from 2012 to 2019, 8891 patients admitted in the IDH were enrolled for this study. Based on the selection strategy, this number represents almost one-fifth of the total patients who received treatment in the hospital. DEC was identified in 7.8% (690/8891) of the patients. Among DEC, ETEC and EAEC remained predominant pathotypes (47.7% and 38.4%, respectively) (Table 1). Among the ETEC, 55% (182/329) of the isolates harboured *elt* as well as *est*. Remaining 32% (105/329) and 13% (42/329) of the ETEC carried *est* and *elt*, respectively. In case of EPEC, 26% (25/96) of the isolates were typical carrying both the genes *bfpA* and *eae*. Among the EAEC, 38.5%, 47.2% and 14.3% of the isolates harboured *aat*, *aaiC* and *aat* and *aaiC* genes, respectively. About 54% (374/690) of the patients were identified to have DEC as a sole pathogen and 46% (316/690) of the patients had mixed pathogens, that is, the presence of the other common diarrhoea causing enteric pathogen(s) concurrently with DEC. Other enteric pathogens such as shigellae, vibrios, campylobacters, salmonellae, aeromonads, viruses and parasites were identified in the mixed infection category. *Vibrio* spp. was the most prevalent in the mixed infection category, followed by Rotavirus, *Campylobacter* spp., *Giardia* and *Shigella* spp. Other pathogens such as aeromonads, *Cryptosporidium*, *Entamoeba* and *Salmonella* spp. were rarely associated with DEC infections (Tables S1).

Seasonal isolation rate was highest during the monsoon season (June–September) for all the years followed by pre-monsoon (March–May) and post-monsoon seasons (October–December). The isolation rate was relatively low during the winter season, that is, during January and February (Indian Meteorological Department, 2022) (Figure 1).

Susceptibility of different age groups for DEC infection

Sole infection frequency of DEC was predominately higher in most of the age groups than the mixed infection. The detection rate of DEC in age group ≤2 years was the highest (31.4%) followed by the age group >30–50 years (22.5%) and >14–30 years (18.6%). Further observation showed that the frequency of EAEC was higher in children ≤2 years of age (20.5%) than in adults. Similarly, the prevalence of EPEC was higher in children ≤2 years of age (5.2%) than in other age groups. Interestingly, ETEC was mostly prevalent in adults viz., >30–50 years (14.4%), >14–30 years (11.4%) and >50 years (11.5%) (Figure 2). The sole infection rate with EAEC and EPEC was observed highest in the age group of ≤2 years. However, the ETEC sole infection rate was higher in older age groups. When the mixed pathogenic infection was considered, the scenario was unchanged, that is, EAEC and EPEC showed a high

TABLE 1 Year-wise prevalence pattern of different DEC pathotypes isolated from patients during 2012–2019

Year	No. of DEC	No. of ETEC	No. of EPEC	No. of EAEC
2012	93	37 (39.8)[a]	14 (15.0)	42 (45.2)
2013	57	25 (43.9)	8 (14.0)	24 (42.1)
2014	90	39 (43.3)	17 (18.9)	34 (37.8)
2015	152	77 (50.7)	26 (17.1)	49 (32.2)
2016	109	56 (51.4)	5 (4.6)	48 (44.0)
2017	53	37 (69.8)	4 (7.6)	12 (22.6)
2018	83	34 (41.0)	12 (14.4)	37 (44.6)
2019	53	24 (45.3)	10 (18.9)	19 (35.8)
Total (%)	690	329 (47.7)	96 (13.9)	265 (38.4)

[a]Figures in parentheses represent percentage of total against each year.

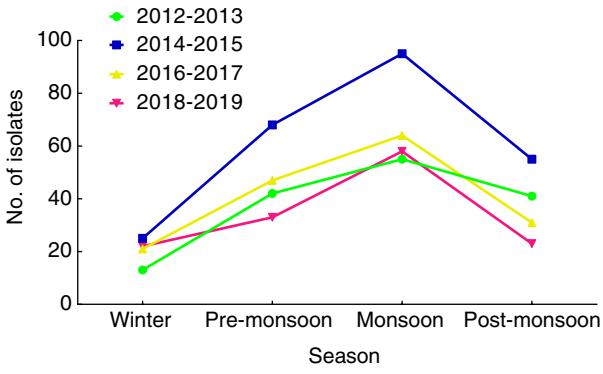

FIGURE 1 Season-wise distribution pattern of DEC isolates from the years 2012 to 2019. Monsoon season presents a higher number of DEC isolates

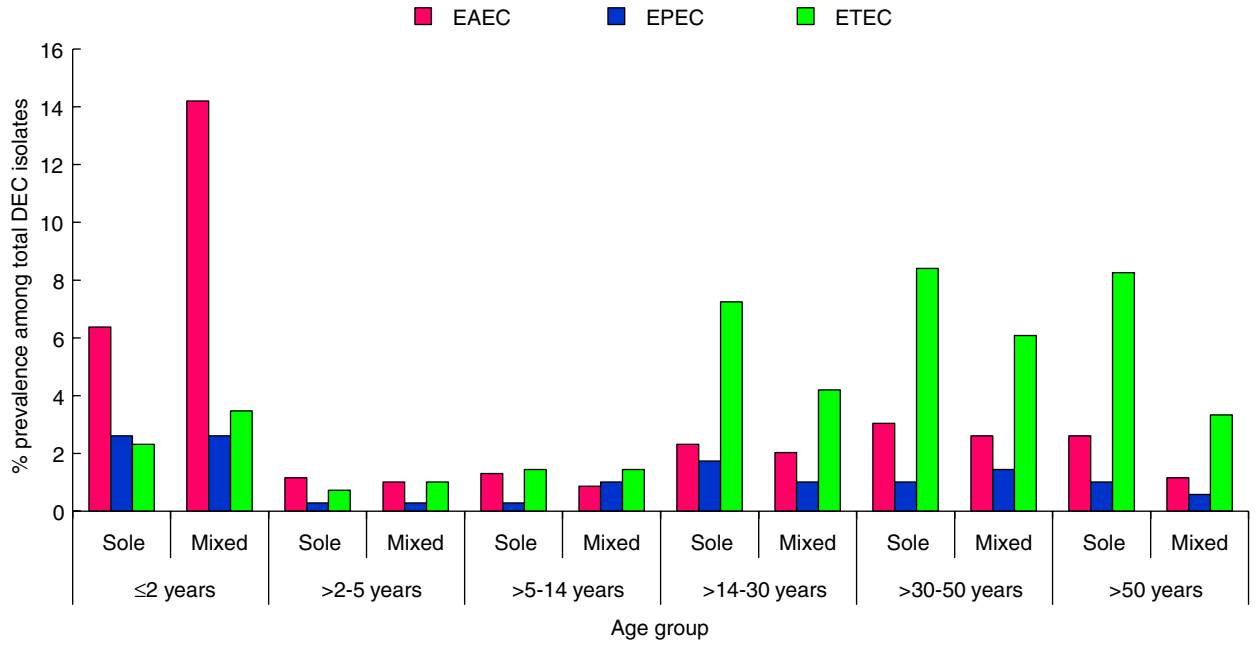

FIGURE 2 Sole and mixed pathogenic distribution of different DEC pathotypes according to the age groups of the infected patients. Figure shows high prevalence of EAEC sole and mixed infections in age group ≤2 years. ETEC shows a higher prevalence in older age groups

frequency in ≤2 than other age groups, but ETEC mixed infection was more in older age groups (Figure 2).

The most significant risk groups for DEC infection

To find out the high-risk age groups to specific DEC pathotypes, a multinomial logistic regression model was made and in this, >50 years age group was considered as the reference category. According to this model, ETEC and EAEC were significantly detected more in children ≤2 years of age ($p = 0.000$) (Table 2). It has also been found that the age group >2–5 years as a significant risk age group for ETEC and EAEC infections ($p = 0.003$ and $p = 0.005$, respectively). The age group >5–14 years is the only age group where EPEC infection was found significantly ($p = 0.049$). In this same age group, ETEC infection was also significant ($p = 0.018$) (Table 2).

Clinical symptoms of different pathotypes of DEC

Watery diarrhoea was seen in more than 60% of the DEC-infected individuals and the rate was higher (>70%) in patients who were detected with ETEC, sole EPEC and EAEC mixed with other enteric pathogens (Table 4). Bloody diarrhoea was recorded (3.4%) among EAEC-infected cases. Comparing the other clinical symptoms and disease severity caused by the different pathotypes of DEC, it was observed that vomiting is a predominant symptom (>80%) among EAEC and EPEC sole infection. Abdominal pain was mainly found among solely infected patients with ETEC and EPEC (>50%). Severe dehydration was observed in patients suffering from sole ETEC and EAEC infections (>9%). Fever was seen in >18% of the DEC-positive cases. Patients with EAEC infections suffered more and stayed about 37 h on average at the hospital for treatment (Table 3).

Antimicrobial resistance in DEC

An interesting pattern of resistance was observed for EAEC pathotype which showed resistance towards most of the antibiotics followed by EPEC and ETEC. The rate of β-lactam resistance is high in EAEC. Almost 86% of the EAEC isolates were found resistant to ampicillin, whereas 61.7% and 22.4% of the isolates showed resistance to the third-generation β-lactam antibiotics, ceftriaxone and ceftazidime, respectively. Similarly, the resistance to fluoroquinolones such as ciprofloxacin (72%) and ofloxacin (71%) has been high in EAEC. Resistance to gentamicin (15%, 8% and 5% for EAEC, EPEC and ETEC, respectively) and chloramphenicol (20%, 19% and 11% for EAEC, EPEC and ETEC, respectively) was rarely found in the DEC, but all the isolates remained susceptible to meropenem. EPEC showed highest resistance to sulphomethoxazole–trimethoprim (61.7%), tetracycline (63.8%) and doxycycline (59.6%) compared to other pathotypes. Among the ETEC, high resistance rates were observed to ampicillin

TABLE 2 Multinomial logistic regression models exploring significant risk age group of predominant DEC infection at IDH, Kolkata

Age group in years	DEC pathotypes	B (regression coefficient)	Odds ratio (95% CI)	*p*-value
≤2	ETEC	2.275	9.72 (5.79–16.34)	0.000[a]
	EPEC	−0.63	0.53 (0.25–1.08)	0.083
	EAEC	−1.89	0.15(0.09–0.26)	0.000[a]
>2–5	ETEC	1.23	3.42 (1.50–7.78)	0.003[a]
	EPEC	−0.35	0.7 (0.20–2.37)	0.567
	EAEC	−1.2	0.3(0.13–0.69)	0.005[a]
>5–14	ETEC	0.86	2.37 (1.15–4.88)	0.018[a]
	EPEC	−0.96	0.38 (0.14–0.99)	0.049[a]
	EAEC	−0.56	0.57 (0.26–1.24)	0.157
>14–30	ETEC	0.31	1.36 (0.81–2.32)	0.243
	EPEC	−0.51	0.6 (0.27–1.32)	0.206
	EAEC	−0.105	0.9 (0.49–1.63)	0.737
>30–50	ETEC	0.15	1.16 (0.70–1.95)	0.553
	EPEC	−0.17	0.83 (0.37–1.86)	0.661
	EAEC	−0.17	0.84 (0.47–1.48)	0.554
>50	Reference category			

[a]Statistically significant.

TABLE 3 Clinical attributes of patients identified with sole and mixed infections with different pathotypes of DEC

	EAEC		EPEC		ETEC	
Attributes	**Sole**	**Mixed**	**Sole**	**Mixed**	**Sole**	**Mixed**
Duration of diarrhoea (before admission) (hours)	29.5 ± 1.9	34.2 ± 2.1	28.7 ± 2.9	31.7 ± 3.0	30.2 ± 1.6	29.3 ± 2.7
Duration of hospital stay (hours)	34.9 ± 1.8	41.2 ± 2.1	39.1 ± 2.7	33.8 ± 3.1	33.2 ± 1.4	34.9 ± 1.8
Dehydration status: (%)						
No	0.90	0.00	0.00	2.20	1.00	0.00
Some	89.70	95.90	91.80	91.30	89.10	91.10
Severe	9.50	4.10	8.20	6.50	9.90	8.90
Type of diarrhoea: (%)						
Watery	68.10	78.40	77.60	69.60	73.40	77.00
Loose	28.50	18.20	20.40	28.30	24.50	21.50
Blood/Mucus	3.40	3.40	2.00	2.20	2.10	1.50
Presence of clinical symptoms: (%)						
Fever	24.10	29.10	28.60	21.70	22.40	18.50
Vomiting	82.80	79.70	83.70	82.60	78.10	82.20
Abdominal pain	40.50	32.40	51.00	43.50	56.20	52.60

(71.3%), ciprofloxacin (53.5%) and ofloxacin (53.5%). Greater resistance against aminoglycosides such as streptomycin and gentamicin were noticed in EAEC followed EPEC and ETEC (Figure 3).

MDR analysis of the DEC isolates showed the presence of many patterns. About 57% (145/255) isolates were found resistant to at least three different classes of antimicrobials, while 14.9% (38/255) of the isolates were resistant to either one or two classes. Prevalence of MDR was more in EPEC (72.3%, 34/47) followed by EAEC (66.3%, 71/107) and ETEC (39.6%, 40/101). MDR isolates were grouped as MDR1, MDR2, MDR3 and MDR4 that represent the isolates resistant to at least 3, 4, 5 and 6 different classes of antimicrobials (β-lactums, sulphonamides/trimethoprims, aminoglycosides, tetracyclines, quinolones and chloramphenicols), respectively. Among the total MDR

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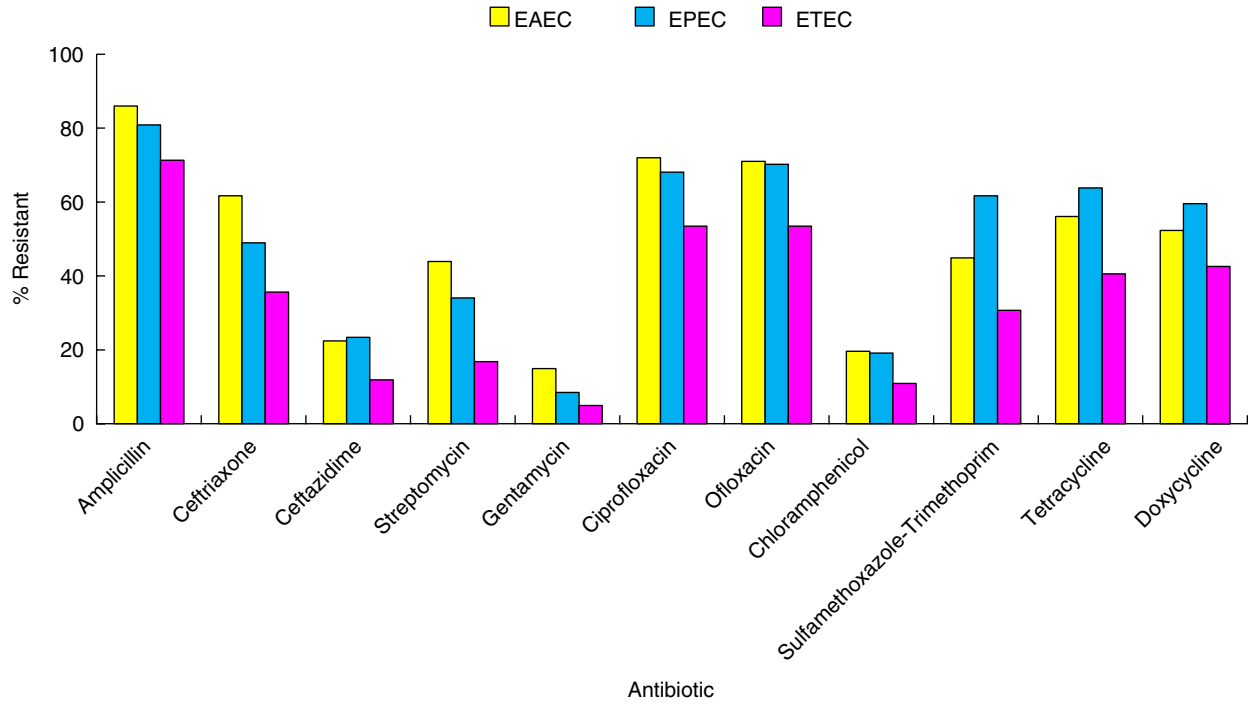

FIGURE 3 Antimicrobial resistance pattern of different DEC pathotypes isolated in Kolkata during 2012–2019

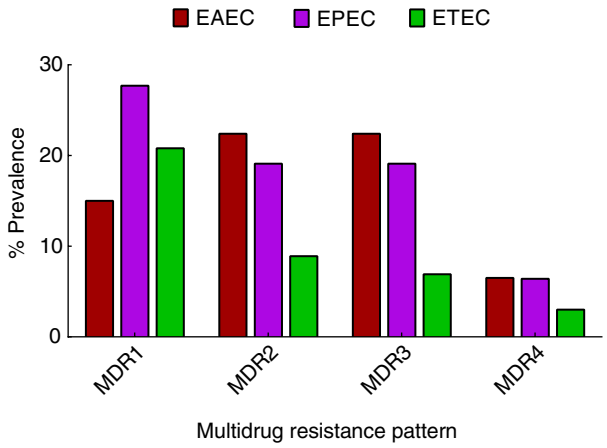

FIGURE 4 Multidrug resistance profile of different DEC isolates of Kolkata. MDR1, MDR2, MDR3 and MDR4 represent the strains those are resistant to at least 3, 4, 5 and 6 different classes of antimicrobials, respectively. Higher prevalence of MDR phenotype was found in EPEC isolates compared to other DEC pathotypes

isolates, those exhibiting the MDR1 phenotype were the most prevalent (34.5%), followed by MDR2 (29%), MDR3 (27.5%) and MDR4 (9%) (Figure 4).

Existence of different genes encoding AMR among DEC isolates

Of the 255 DEC isolates, 151 isolates that showed resistance towards β-lactam antibiotics were tested in PCR assay. Among these, 46.3% (70/151), 51% (77/151), 15.2% (23/151), 2% (3/151) isolates harboured bla_{TEM}, $bla_{\text{CTX-M3}}$, $bla_{\text{OXA-1}}$, bla_{SHV}, respectively. In 77 sulphamethoxazole–trimethoprim-resistant isolates, 41.5% (32/77) harboured *sul2* gene and 5.2% (4/77) were positive for *dfrA1*. In all, 61 isolates were resistant to streptomycin, of which 49.2% (30/61) were positive for *strA* and 41% (25/61) contained *aadA* gene. Tetracycline resistance was noticed in 104 isolates in which 44.2% (46/104) contained *tetB* gene. Quinolone resistance was shown by 131 of the isolates in which 6.9% (9/131) were positive for *qnrB* gene, 27.5% (36/131) for *qnrS* and 9.2% (12/131) for *aac6′-Ib-cr*. About 27% (11/41) of the cholramphenicol-resistant isolates harboured *cat1A* gene (Table 4). Furthermore, among the MDR isolates, $bla_{\text{CTX-M3}}$ and bla_{TEM} were more prevalent, followed by $bla_{\text{OXA-1}}$, *sul2*, *strA*, *tetB*, *qnrS* and *qnrB*. Our analysis also showed that the presence of $bla_{\text{CTX-M3}}$, $bla_{\text{OXA-1}}$, *aadA* and *aad6′-Ib-cr* were more in EAEC. *sul2*, *dfrA1*, *strA*, *tetB*, *qnrB* and *catI* were mostly detected in EPEC, whereas bla_{TEM}, bla_{SHV} and *qnrS* were more common among the ETEC isolates (Table 4).

DISCUSSION

DEC has emerged as one of the major public health risks in children after rotavirus and cholera (Dutta et al., 2013; Konaté et al., 2017; Takahashi et al., 2008). Due to the clinical complexity of the infection, these patients need hospitalization and administration of antimicrobials along with treatment for severe dehydration.

TABLE 4 Prevalence of antimicrobial resistance-conferring genes among the resistant EAEC ($n = 107$), EPEC ($n = 47$) and ETEC ($n = 101$) isolates

Confers resistance to	Genes	No. of EAEC (%)	No. of EPEC (%)	No. of ETEC (%)
Beta-lactams	bla_{TEM}	28 (26.2)	12 (25.5)	30 (29.7)
	bla_{OXA-1}	17 (15.9)	6 (12.8)	0 (0.0)
	bla_{CTX-M}	33 (30.8)	14 (29.8)	30 (29.7)
	bla_{SHV}	1 (0.9)	0 (0.0)	2 (2.0)
Sulphonamides and trimethoprims	*sul 2*	18 (16.8)	11 (23.4)	3 (3.0)
	dfrA1	1 (0.9)	1 (2.1)	2 (2.0)
Streptomycin (Aminoglycoside)	*strA*	16 (15.0)	9 (19.1)	5 (5.0)
	aadA	22 (20.6)	1 (2.1)	2 (2.0)
Tetracyclines	*tetB*	19 (17.8)	17 (36.2)	10 (9.9)
Quinolones	*qnrB*	4 (3.7)	2 (4.3)	3 (3.0)
	qnrS	12 (11.2)	7 (14.9)	17 (16.8)
	aad6′-Ib-cr	11 (10.3)	1 (2.1)	0 (0.0)
Chloramphenicols	*catI*	3 (2.8)	5 (10.6)	3 (3.0)

Prevalence of DEC in this study is 7.8%, which is comparable to the reports from other affected countries (Lima et al., 2017; Schroeder et al., 2002; Takahashi et al., 2008). Diarrhoea caused by the DEC as sole pathogen was 54%. Mixed infections caused by DEC with other pathogens (*Vibrio*, *Campylobacter*, *Shigella*, *Salmonella*, Rotavirus and parasites) were high (46%), which is more than the previous investigations (28%) (Farfán-García et al., 2020; Nair et al., 2010). In addition, several aspects of present observation differed from a previous study conducted in Kolkata (Dutta et al., 2013), which includes (i) higher incident rate (54%) of DEC sole infection, (ii) ETEC was found to be mostly associated with diarrhoea as against EAEC and (iii) infections caused by ETEC containing both the ST and LT toxins were higher compared to the other ETEC with LT or ST alone. Although EAEC and EPEC are more associated with diarrhoea in countries like Brazil, Tanzania and several parts of India including Northern India and Karnataka (Canizalez-Roman et al., 2013, 2016; Dutta et al., 2013; Okeke et al., 2000; Shetty et al., 2012; Singh et al., 2019; Takahashi et al., 2008), our study shows that ETEC as the major DEC pathotype.

DEC-associated diarrhoea was found to vary among different age groups of the patients. Similar to the previous finding, ETEC and EAEC infections were significantly higher in younger age groups (Dutta et al., 2013; Rajendran et al., 2010). However, compared to the other reports from Vietnam and India, ETEC was higher in age group >30–50 years (Natarajan et al., 2018; Nguyen et al., 2006). Therefore, it is important to consider older age groups for monitoring DEC infections.

Similar to our previous studies, DEC-associated clinical symptoms are much less compared to cholera and Rotavirus gastroenteritis (Dutta et al., 2013; Nair et al., 2010). A recent study in Cameroon suggested that among DEC-associated diarrhoeal patients, only fever and vomiting were seen in EPEC-infected cases (Marbou et al., 2020). However, in Kolkata, these symptoms remain prominent in all the DEC cases regardless of their pathotypes. Globally, the seasonal prevalence of DEC was more during summer and rainy seasons (Ahmed et al., 2013; Behiry et al., 2011; Estrada-Garcia et al., 2009; Zhou et al., 2018). We have seen a higher incidence of DEC during 2015, and this might be associated with heavy Southwest monsoon and floods in several Districts of West Bengal, contaminating the water resources.

Widespread and arbitrary use of antimicrobials has increased the resistance ability of many bacteria, which also leads to the emergence of highly pathogenic MDR strains. In this study, majority of the DEC isolates have been identified as MDR, which was relatively higher than that of the other reports (Abbasi et al., 2020; Ghorbani-Dalini et al., 2015; Ramirez Castillo et al., 2013; Shah et al., 2015; Zhou et al., 2018). Furthermore, our findings showed a high prevalence of fluoroquinolone resistance in DEC isolates, which is one of the drugs advocated for the treatment of diarrhoea (Alikhani et al., 2013; Heidary et al., 2014; Omolajaiye et al., 2020). Studies conducted in China and Mexico also have shown higher resistance of DEC to ampicillin, sulfamethoxazole–trimethoprim and tetracycline (Canizalez-Roman et al., 2016; Zhou et al., 2018; Zhu et al., 2016). Most of the DEC isolates showed resistance to fluoroquinolone drugs demonstrating resistance may be acquired by horizontal gene transfer similar to the observation made by Ciesielczuk et al. (2013). Similar to a study conducted in France, we found that DEC from Kolkata has also

shown β-lactam resistance mostly with isolates harbouring $bla_{CTX\text{-}M3}$, bla_{TEM}, $bla_{OXA\text{-}1}$ and bla_{SHV} (Blanc et al., 2014). The presence of several plasmid-mediated quinolone resistance genes such as *qnrS*, *qnrB* and *aac6′-Ib-cr* has been encountered in this study. This trend has been reported in other investigations also (Herrera-León et al., 2016; Pazhani et al., 2011). However, the prevalence of *aadA*, *catA1* and *tetB* gene was relatively low as reported from Iran and South Africa (Heidary et al., 2014; Omolajaiye et al., 2020). EPEC isolates were found to harbour most of the AMR conferring genes followed by EAEC and ETEC.

This study highlights the important contribution of DEC in the prevalence of acute diarrhoea and also reveals existence of MDR among several pathotypes challenging the success of clinical management of diarrhoea.

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CONFLICT OF INTEREST

No conflict of interest declared.

AUTHORS' CONTRIBUTION

DG, PS, TR and AKM conceptualized the study. DG, GC, PS and SS performed the experiments. DG, PS and AKM prepared the original draft of the manuscript. AD performed the statistical analysis. MB, AM, SM, SD and AKM collaborated in this study. All the authors did data analysis, draft review, editing and approval.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Journal of Applied Microbiology

ORIGINAL ARTICLE

Altered molecular attributes and antimicrobial resistance patterns of *Vibrio cholerae* O1 El Tor strains isolated from the cholera endemic regions of India

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Abstract

Aims: The present study aimed to document the comparative analysis of differential hypervirulent features of *Vibrio cholerae* O1 strains isolated during 2018 from cholera endemic regions in Gujarat and Maharashtra (Western India) and West Bengal (Eastern India).

Methods and Results: A total of 87 *V. cholerae* O1 clinical strains from Western India and 48 from Eastern India were analysed for a number of biotypic and genotypic features followed by antimicrobial resistance (AMR) profile. A novel polymerase chain reaction was designed to detect a large fragment deletion in the Vibrio seventh pandemic island II (VSP-II) genomic region, which is a significant genetic feature of the *V. cholerae* strains that have caused Yemen cholera outbreak. All the strains from Western India belong to the Ogawa serotype, polymyxin B-sensitive, hemolytic, had a deletion in VSP-II (VSP-IIC) region and carried Haitian genetic alleles of *ctxB*, *tcpA* and *rtxA*. Conversely, 14.6% (7/48) of the strains from Eastern India belonged to the Inaba serotype, polymyxin B-resistant, nonhemolytic, harboured VSP-II other than VSP-IIC type, classical *ctxB*, Haitian *tcpA* and El Tor *rtxA* alleles. Resistance to tetracycline and chloramphenicol has been observed in strains from both regions.

Conclusions: This study showed hypervirulent, polymyxin B-sensitive epidemic causing strains in India along with the strains with polymyxin B-resistant and nonhemolytic traits that may spread and cause serious disease outcomes in future.

Significance and impact of the study: The outcomes of this study can help to improve the understanding of the hyperpathogenic property of recently circulating pandemic *Vibrio cholerae* strains in India. Special attention is also needed for the monitoring of AMR surveillance because *V. cholerae* strains are losing susceptibility to many antibiotics used as a second line of defence in the treatment of cholera.

KEYWORDS

cholera, El Tor biotype, nonhemolytic, polymyxin B, *Vibrio cholerae*, vibrio seventh pandemic island II

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ORIGINAL RESEARCH

Characterization of NDM-5 Carbapenemase-Encoding Gene (bla_{NDM-5}) – Positive Multidrug Resistant Commensal *Escherichia coli* from Diarrheal Patients

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Purpose: The multidrug resistance Enterobacteriaceae cause many serious infections resulting in prolonged hospitalization, increased treatment charges and mortality rate. In this study, we characterized bla_{NDM-5}-positive multidrug resistance commensal *Escherichia coli* (CE) isolated from diarrheal patients in Kolkata, India.

Methods: Three CE strains were isolated from diarrheal stools, which were negative for different pathogroups of diarrheagenic *E. coli* (DEC). The presence of carbapenemases encoding genes and other antimicrobial resistance genes (ARGs) was detected using PCR. The genetic arrangement adjoining bla_{NDM-5} was investigated by plasmid genome sequencing. The genetic relatedness of the strains was determined by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) methods.

Results: In addition to colistin, the bla_{NDM-5}-positive CE strains showed resistance to most of the antibiotics. Higher MICs were detected for ciprofloxacin (>32 mg/L) and imipenem (8 mg/L). Molecular typing revealed that three CE strains belonged to two different STs (ST 101 and ST 648) but they were 95% similar in the PFGE analysis. Screening for ARGs revealed that CE strains harbored *Int-1,* bla_{TEM}, bla_{CTX-M3}, bla_{OXA-1}, bla_{OXA-7}, bla_{OXA-9}, *tetA, strA, aadA1, aadB, sul2, floR, mph(A)*, and *aac(6′)-Ib-cr*. In conjugation experiment, transfer frequencies ranged from 2.5×10^{-3} to $8.4x10^{-5}$. The bla_{NDM-5} gene was located on a 94-kb pNDM-TC-CE-89 type plasmid, which is highly similar to the IncFII plasmid harboring an IS26-IS30-bla_{NDM-5}-ble_{MBL}-*trpF-dsbd*-IS*91-dhps* structure.

Conclusion: To the best of our knowledge, this is the first report on carbapenem resistance involving the bla_{NDM-5} gene in CE from diarrheal patients. The circulation of bla_{NDM-5} gene in CE is worrisome, since it has the potential to transfer bla_{NDM-5} gene to other enteric pathogens.

Keywords: antimicrobial resistance, carbapenem-resistance, commensal *E. coli*, plasmid; bla_{NDM-5}

Introduction

The emergence and rapid spread of carbapenemases-producing Enterobacteriaceae is a serious public-health concern because carbapenems are the last resort antibiotics to treat extensively multidrug-resistant (MDR) bacterial infections.[1] Amongst the newly emerging carbapenemases-producers, NDM is very important due to its increased MDR phenotype and rapid global dissemination with frequent allelic variations.[2] Since its first discovery in 2008, more than 20 variants of NDM have been identified in different bacterial species spread across different countries.[3–5] In 2011, the NDM-5 was first identified from an MDR *E. coli* isolated in the United Kingdom from a patient who had been previously treated in India.[6] The NDM-5 varied from NDM-1 by only two amino acid replacements at positions 88 (Val → Leu) and 54 (Met

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Future MICROBIOLOGY

Genomic insights into extensively drug-resistant *Pseudomonas aeruginosa* isolated from a diarrhea case in Kolkata, India

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Aim: To characterize extensively drug-resistant *Pseudomonas aeruginosa* from a patient with diarrhea. **Materials & methods:** Antimicrobial susceptibility was tested by the disk diffusion method. The *P. aeruginosa* genome was sequenced to identify virulence, antibiotic resistance and prophages encoding genes. **Results:** *P. aeruginosa* had a wide spectrum of resistance to antibiotics. Genomic analysis of *P. aeruginosa* revealed 76 genes associated with antimicrobial resistance, xenobiotic degradation and the type three secretion system. **Conclusion:** This is the first report on diarrhea associated with *P. aeruginosa*. Since no other organism was identified, the authors assume that the patient had dysbiosis due to antibiotic exposure, leading to antibiotic-associated diarrhea. The *in vivo* toxicity expressed by the pathogen may be associated with T3SS.

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Pseudomonas aeruginosa is a nonfermenting, aerobic, Gram-negative pathogen often reported in hospital-acquired infections with an attribute of antimicrobial resistance (AMR) [1]. *P. aeruginosa* is part of the normal human intestinal flora of healthy individuals and one of the most important opportunistic human pathogens associated with clinical infections, including wound infections, pneumonia, urinary tract infections, endocarditis, meningitis, brain abscesses and bacteremia [2–4]. The mechanisms of causing the disease are attributed mainly to cell-associated and extracellular virulence factors [5,6]. *P. aeruginosa* is a major cause of infection-related mortality among critically ill patients and has the highest fatality rate of all Gram-negative pathogens, even among patients who receive appropriate antimicrobial treatment. Infections caused by multidrug-resistant or extensively drug-resistant (XDR) *P. aeruginosa* are emerging, which has drastically reduced the efficacy of several clinically important antibiotics [7]. The increasing use of broad-spectrum antibiotics in health and food animal sectors has facilitated the emergence and spread of multidrug-resistant *P. aeruginosa*, which complicates therapy and limits treatment options [8,9]. Infections caused by this organism are hard to treat because of the presence of several resistance mechanisms to multiple classes of antibiotics, including β-lactams, aminoglycosides and fluoroquinolones [10,11]. Generally, bacterial resistance can evolve by either the accumulation of mutations in specific genes or horizontally acquired genetic elements such as plasmids, transposons and integrons [12,13]. Even though *P. aeruginosa* is not considered a