Multidimensional Approaches in Developing a Vaccine against Circulating Strains of *Helicobacter pylori*

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By

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "**Multidimensional Approaches in Developing a Vaccine Against Circulating Strains of *Helicobacter pylori*"** submitted by **Mr. Sanjib Das** who got his name registered on **3rd December, 2021 (Index number: 134/21/Life. Sc./27, Registration number: SLSBT1213421**) for the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Hemanta Koley** and that neither this nor any part of it has been submitted for either any degree/ diploma or any other academic award anywhere before.

Hemanta Koley 25/09/2024

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I dedicate my thesis to everyone who has positively influenced my life…

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Declaration

This PhD thesis was prepared at the Division of Bacteriology, ICMR-National Institute for Research in Bacterial Infections, in fulfillment of the requirements for acquiring the PhD degree. The research work embodied in the thesis entitled "**Multidimensional Approaches in Developing a Vaccine Against Circulating Strains of *Helicobacter pylori*"** was carried out at **ICMR-National Institute of Research in Bacterial Infections, Kolkata, India under the supervision of Dr. Hemanta Koley, Scientist-F, Division of Bacteriology.**

The thesis has been composed in its entirely by the candidate except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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Contents

Abstract

Title of the thesis:

"Multidimensional Approaches in Developing a Vaccine against Circulating Strains of *Helicobacter pylori*"

Submitted by: Sanjib Das
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Helicobacter pylori is a major health concern worldwide, particularly in developing nations, affecting nearly half of the global population. Classified as a class I pathogen by the WHO, it is a significant contributor to various gastric diseases, including chronic gastritis, ulcers and gastric cancer. Current diagnostic methods are costly and invasive, making them inaccessible to many individuals. Additionally, the lack of an effective vaccine means that antimicrobial therapies are the primary treatment option, leading to the development of antimicrobial resistance (AMR). In this present study, initially we screened strains isolated from patients suffering from various gastric diseases. Phenotypic and genotypic features of these indigenous strains with putative virulence features allowed selection of the immunogen strains. Selected strains were then evaluated for two types of vaccine platforms: (i) Outer membrane vesicles (OMVs) based and (ii) Nanocurcumin-induced Bacterial Ghosts (CurBGs) based against circulating strains of *H. pylori*. Outer membrane vesicles (OMVs) are protein-rich microvesicles secreted by gram-negative bacteria, with the potential to stimulate an immune response. On the other hand, nanoformulated curcumin disrupts bacterial cell membranes, resulting in the formation of empty bacterial envelopes known as Bacterial Ghosts (CurBGs). Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) were used to characterize both OMVs and CurBGs. Proteomic analysis revealed the OMVs laden with a wide range of somatic antigens. Conversely, nanocurcumin formulation led to the development of curcumin nanoparticles less than 100nm in size and subsequent treatment to bacteria induced bacterial ghost cells devoid of any inclusion bodies. In-vitro cytotoxicity assay with both the immunogens using murine macrophage cells (RAW 264.7) resulted in significantly low toxicity. Three doses of successive oral immunization using C57BL/6 mice on the 0th, 14th, and 28th day showed a significant increase in serum and secretory antibody titers against outer membrane proteins (OMPs) and whole cell lysate (WCL) of the virulent wild-type strain. Serum antibodies from vaccinated animals showed excellent bactericidal activity and mitigate motility and mucin penetration ability. Ex vivo analysis of harvested spleen cells re-stimulated with respective antigens showed a steep spike in the inflammatory cytokine profile in immunized animals compared to non-immunized animals. In addition, we established an intra-gastric surgical model whereby the stomach was exposed surgically to inject the bacterial inoculums directly into the gastric environment leading to the development of an active infection in less time. In terms of protective efficacy study, histopathological observation showed a significant improvement in gastric architecture in immunized animals, which was further supported by the reduction of colonization. Therefore, this model can be extremely useful in prophylactic, therapeutic, or pathogenesis study. In conclusion, both CurBGs and OMVs have shown to be better immunogens, eliciting a more pronounced immune response in mice compared to the non-immunized, indicating the prospects as potential vaccine candidates.

Sanjib Das.

Ph.D Thesis submitted by Sanjib Das

Hemanta Koley 25/09/2024

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Background

Helicobacter pylori (H. pylori) is a spiral-shaped bacterium that colonizes the human stomach and duodenum. It is a major cause of gastritis, peptic ulcers, and gastric cancer. Despite decades of research and the availability of antibiotics, the eradication of *H. pylori* remains challenging due to its ability to evade the immune system and develop antibiotic resistance. As a result, the development of a vaccine against *H. pylori* has become a significant research priority.

A multidimensional approach is essential for developing a successful *H. pylori* vaccine due to the bacterium's complex biology and the diverse immune responses it elicits. This approach encompasses several key aspects: (i) Antigen selection: The choice of antigens is crucial for a vaccine's efficacy. *H. pylori* produces numerous proteins, and identifying the most immunogenic ones is essential. Previous researches focused on outer membrane proteins, flagella, and urease, which are key virulence factors. Additionally, the genetic diversity of *H. pylori* strains necessitates the inclusion of multiple antigens to broaden the vaccine's coverage. (ii) Delivery platform: The delivery system plays a vital role in presenting antigens to the immune system. Traditional approaches include subunit vaccines, which consist of purified antigens, and live attenuated vaccines, which use weakened *H. pylori* strains. However, these methods have limitations, such as the potential for adverse reactions and the risk of reversion to virulence. Newer approaches, such as recombinant protein vaccines, DNA vaccines, and viral vector vaccines, offer promise in overcoming these challenges. (iii) Adjuvants: Adjuvants are substances that enhance the immune response to a vaccine. They can increase antigen uptake by immune cells, stimulate the production of cytokines, and promote the development of long-lasting immunity. Alum is a commonly used adjuvant, but researchers are exploring other options, such as liposomes, microparticles, and Toll-like receptor agonists, to improve vaccine efficacy. The desired immune response for a *H. pylori* vaccine is a strong, durable, and

protective antibody response, as well as a robust cellular immune response. Antibodies can neutralize the bacterium and prevent colonization, while cellular immunity can eliminate infected cells. Understanding the specific immune mechanisms involved in *H. pylori* infection is essential for designing vaccines that elicit the appropriate responses. (iv) Clinical research: Clinical trials are necessary to evaluate the safety and efficacy of *H. pylori* vaccines in humans. Phase I trials assess safety and immunogenicity, while phase II trials evaluate efficacy in preventing infection or reducing the severity of disease. Phase III trials are large-scale studies that confirm efficacy in a broader population. Despite significant progress, several challenges remain in developing a *H. pylori* vaccine. These include the bacterium's ability to evade the immune system, the genetic diversity of *H. pylori* strains, and the potential for antibiotic resistance. Future research should focus on identifying novel antigens, improving vaccine delivery systems, and developing adjuvants that enhance immune responses. Additionally, studies on the immune mechanisms underlying *H. pylori* infection and the factors that contribute to vaccine failure are essential. *H. pylori c*olonizes the mucosal lining of the stomach and duodenum, making mucosal immunity a critical factor in vaccine development. Mucosal vaccines, which are designed to induce immunity at mucosal surfaces, may be particularly effective against *H. pylori*. Researchers are exploring various mucosal vaccine strategies, including intranasal, oral, and rectal delivery. The emergence of antibiotic resistance is a major challenge in *H. pylori* eradication. Vaccines that can prevent infection or reduce the severity of disease can help reduce the reliance on antibiotics, thereby limiting the development of antibiotic resistance. Additionally, researchers are investigating combination therapies that combine vaccines with antibiotics to improve eradication rates and reduce the risk of resistance. The genetic diversity of *H. pylori* strains can make it difficult to develop a vaccine that is effective against all strains. Personalized vaccines,

which are tailored to an individual's specific *H. pylori* strain, may offer a solution to this challenge. By sequencing the genome of an individual's *H. pylori* strain, researchers can identify the most relevant antigens for inclusion in a vaccine. The development of a successful *H. pylori* vaccine could have a significant economic impact, as it could reduce the burden of *H. pylori*-related diseases and healthcare costs. Vaccines can also be more cost-effective than long-term antibiotic therapy, particularly in developing countries with limited access to healthcare.

In conclusion, a multidimensional approach is necessary to address the complexities of *H. pylori* infection and develop a vaccine that can effectively protect against this prevalent pathogen. By combining advances in antigen selection, delivery systems, adjuvants, immune response engineering, and clinical trials, researchers are making significant strides towards achieving this goal. Future research should focus on mucosal immunity, addressing antibiotic resistance, personalized vaccines, and the economic impact of vaccine development.

Chapter 1

Review of Literature

1. An account on discovery of *Helicobacter pylori*

Barry James Marshall, one of four siblings originally from Kalgoorlie, near Perth, Australia, moved to Carnarvon because his mother wanted her children to receive a university education. A mediocre student, Marshall had immense curiosity and interests in many things. However, when he realized that mathematics was not his strong suit, he quickly gave up his initial preference of being an electrical engineer and joined the University of Western Australia as a medical student.

After finishing his internship and residency in internal medicine at Queen Elizabeth II Medical Centre in Perth, he was appointed at Royal Perth Hospital and started learning about cardiology and gastroenterology. During his rotation in the gastroenterology division, he met a young professional, J. Robin Warren, who was working as a pathologist. While looking for a research topic, Marshall learned that a "curved" bacterium was found in many patients diagnosed with gastric illnesses. Warren, being an enthusiast, wanted a clinician to diagnose these patients as he was keen to study post-treatment changes. While Marshall was practicing, he encountered a woman complaining of severe abdominal pain. After a careful examination, he found nothing but erythema and "curved" bacteria in gastric biopsies. Unaware of her diagnosis, Marshall referred the patient to a psychiatrist who then prescribed antidepressants to the patient.

To find the real culprit, both Warren and Marshall began their investigation. In their first meeting, Warren showed Marshall the bacterium along with histological abnormalities of the gastric mucosa. By that time, Marshall was aware of *Campylobacter jejuni* and its ability to cause gastroenteritis. These newly discovered pathogens had a very similar appearance to that of *C. jejuni*. While going through the literature, he found that these curved bacteria in gastric biopsies were never given importance and were considered as artifacts. He finished his rotation in gastroenterology and became a hematology registrar at Port Hedland Hospital, 1200 miles

north of Perth. However, he continued his study and followed up with 100 patients that he and Warren had collected. At Port Hedland Hospital, he presented his work at the local College of Physicians Meeting and received a mixed response. They were not yet ready to move away from their dogma on the effect of stress on gastritis and ulcers.

Due to a technical glitch, he ended up at Fremantle Hospital, the smallest teaching hospital in Perth. There, he found a group of enthusiasts and co-workers who supported his work. Moreover, he continued to receive more cases of patients with peptic ulcers infected by the curved bacteria, which in 1982, became known as *Campylobacter pylori*. He then contacted Dr. Martin Skirrow, a renowned gastroenterologist at Worcester Infirmary, England, who invited him to present his work at the European Campylobacter meeting in Brussels. Here, he was surprised to see that Cliodna McNulty, a registrar at Worcester, had successfully isolated the same curved organism from gastric biopsies, just as he had seen in Australia.

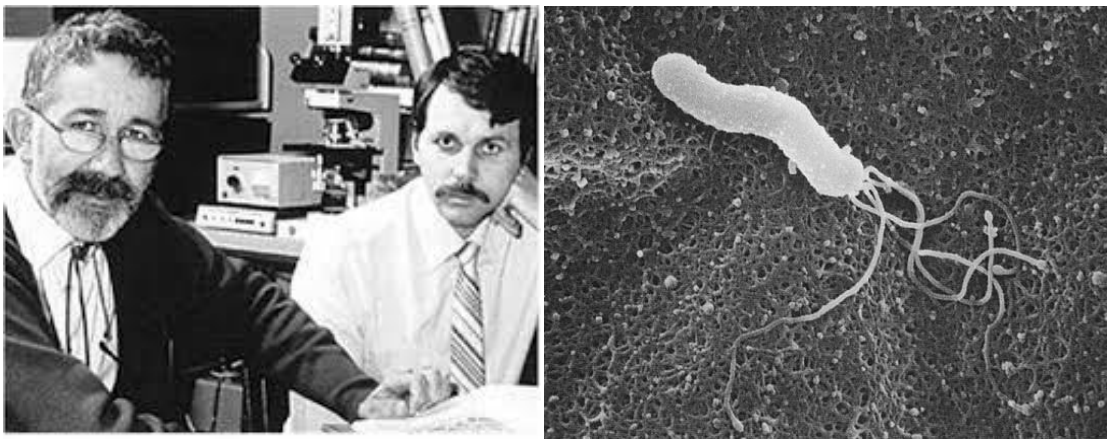


Figure 1: Warren (left) and Marshall (right), EM image of *H. pylori*

After the meeting, a lot more cases started to surface from different countries in Europe along with Australia. In 1984, Marshall and Warren published their work in "The Lancet," where they reported that 77% of 22 gastric ulcer patients and 100% of 13 patients with duodenal ulcers had the curved bacteria. However, they failed to culture the bacteria in 34 cases as they were

discarded too soon. Finally, in 1982, on an Easter weekend, a prolonged culture showed these organisms, which were renamed in 1989 as Helicobacter pylori after ribosomal sequencing revealed a clear distinction from Campylobacter.

Research on *Helicobacter pylori* took off after its discovery. However, Marshall was unable to develop an animal model and took matters into his own hands. He volunteered himself for the sake of science and initiated an endoscopy, which showed normal gastric mucosa. He then drank a bacterial culture and developed nausea and chlorhydria. His condition worsened on day 8 with vomiting and a positive endoscopy result for *H. pylori*. By day 14, he developed severe gastric problems and began treatment with antibiotics and bismuth. He recovered successfully and proved his point on the involvement of gastric illnesses due to *Helicobacter pylori* infection. Their efforts were not only recognized by scientific communities worldwide but also by the Nobel committee. They received the Nobel Prize for Medicine or Physiology in 2005 [1, 2].

Epidemiological Trends:

Gastro-duodenal diseases refer to a broad range of medical conditions affecting stomach and duodenum, encompassing issues like gastritis, peptic ulcers, gastric cancer, gastroenteritis, and various functional gastro-duodenal disorders. According to the World Health Organization (WHO) *Helicobacter pylori* is the major infectious agent causing stomach cancer of approximately 850,000 individuals only in 2020 [3]. It has been estimated almost 50% of world population has been affected by the pathogen at least once in their life time and left untreated, remains for life [4].

Gastric cancer (GC) ranks third in terms of mortality rates for both sexes and is the fifth fastest growing kind of cancer worldwide. The worldwide projections for GC in 2020 were 1.09 million new cases and 0.77 million deaths [5]. Globally, the annual incidence rates for males and women

are 15.6 to 18.1 and 6.7 to 7.8 per 100,000 individuals, respectively [6]. Anatomical subsites allow for the classification of GC into two groups: non-cardia GC and cardia GC. Due to their unique pathogenesis and disparate epidemiological traits, cardia and non-cardia GC are treated as two distinct disorders. Compared to cardia GC, non-cardia GC is more prevalent. Up to 82% of all GC cases worldwide in 2018 were non-cardia GC cases [7]. High prevalence of GC is not usually linked to high incidence of *H. pylori* infection. Holcombe originally referred to this mystery surrounding *H. pylori* infection and GC as the "African Enigma" in 1992 [8]. It is characterized by a relatively high incidence of infection yet a low rate of GC. Therefore, the African enigma is a change in the inflammatory response that the infection caused, which is why there are no neoplastic signs. Similar mysteries have been reported from China, Colombia, India, Costa Rica, and Malaysia, among other nations. A number of prior research studies have indicated that certain lifestyle choices, like heavy alcohol use, smoking cigarettes, eating a lot of salt, consuming processed meat, and eating little fruit, may raise the risk of gastric cancer [9]**.**

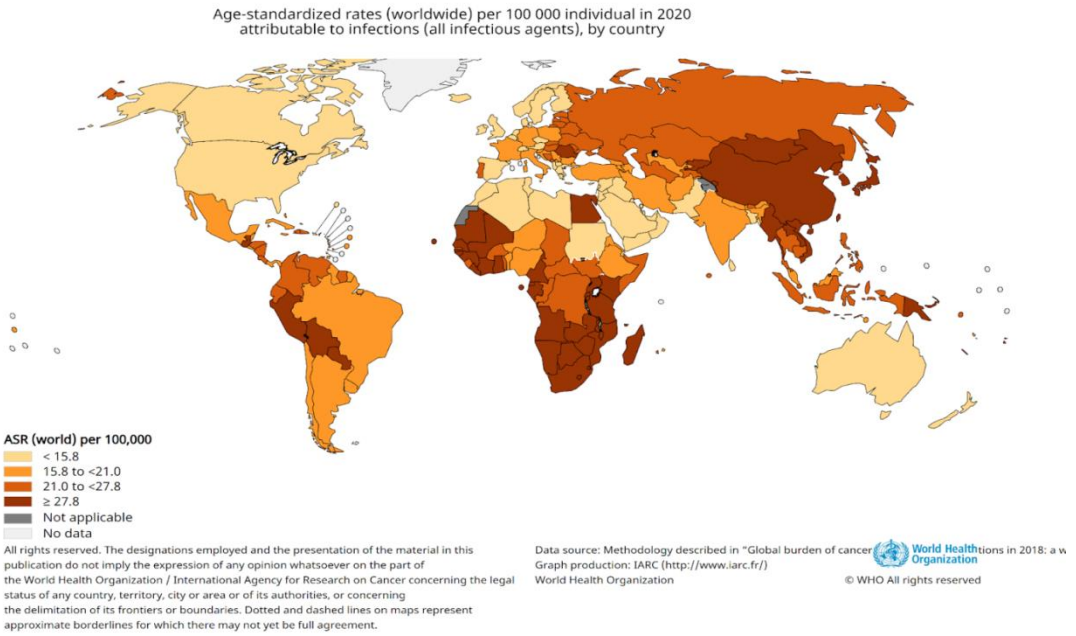


Figure2: Global occurrence of *H. pylori* mediated stomach cancer cases (International Agency for Research on Cancer, WHO)

Research indicates that the prevalence of *H. pylori* in European populations ranges from 20% to 40% [10]. In the USA, a cross-sectional survey found that the infection was present in 25.4% of people older than three. It has been reported that in Japan, the prevalence was close to 90% among those born before the year 1950, but then declined, with people born after the year 2000 having a prevalence of fewer than 2%]11]. Because stomach cancer in the Japanese population is strongly associated with *H. pylori*, this low prevalence is pretty significant. Conversely, there have been reports of up to 80% *H. pylori* prevalence in the Eastern Mediterranean region [12].

A little over 1.2 billion people call India home. It is estimated that over 726 million people worldwide would have *H. pylori* infection if the prevalence of the infection was 60%. At least 18 million people in India may require anti-*H. pylori* therapy, as the estimated prevalence of duodenal ulcers is 3%. If treated over a year, this translates to almost 50,000 cases every day [13]. It is possible that the government and some doctors would be discouraged from taking a strong approach to treating *H. pylori* infection due to the immense difficulty of the undertaking. That said, the problem is considerably easier to solve if one approaches it one patient at a time. Numerous epidemiological studies carried out in South India have revealed a greater incidence of stomach cancer compared to North India. The results of the relationship between *H. pylori* and stomach cancer are conflicting, as evidenced by the high frequency of *H. pylori* infection (49.94% - 83.30%) in India and the relatively low incidence of gastric cancer. *H. pylori* and stomach cancer have a clear correlation in about 50% of patients, while the remaining patients have a negative correlation, according to human epidemiological research [14].

The prevalence of *H. pylori* infection is strongly influenced by the local antibiotic profile. According to Thyagarajan*et al*. (2003), isolates from various areas had resistance to amoxicillin

in 32.8% of cases, metronidazole in 77.9% of cases, and clarithromycin in 44.7% of cases. The highest rates of metronidazole resistance were found in Lucknow (68%), Chennai (88.2%), and Hyderabad (10%), whereas moderate rates were seen in Chandigarh (38.2%) and Delhi (37.5%). On the other hand, among the isolates, ciprofloxacin and tetracycline resistance was lowest, ranging from 1.0 to 4.0% [15]. A further study conducted in 2005 under the direction of Datta *et al.* found that 85% of the isolated strains from Kolkata exhibited resistance to metronidazole, and that the percentage of bacteria that were resistant to tetracycline (7.5%) varied considerably [16]. Among the isolates from Northern India, Gehlot et al. (2015) revealed that 70.6% of the isolates had drug resistance, including resistance to metronidazole (48.5%), furzolidone (22.1%), amoxicillin (17.6%), tetracycline (16.2%), and clarithromycin (11.8%) [17]. Since antibiotics have a significant impact on the development of antimicrobial resistance, all of these investigations have demonstrated the significance of extensive antibiotic profiling.

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Epsilonproteobacteria

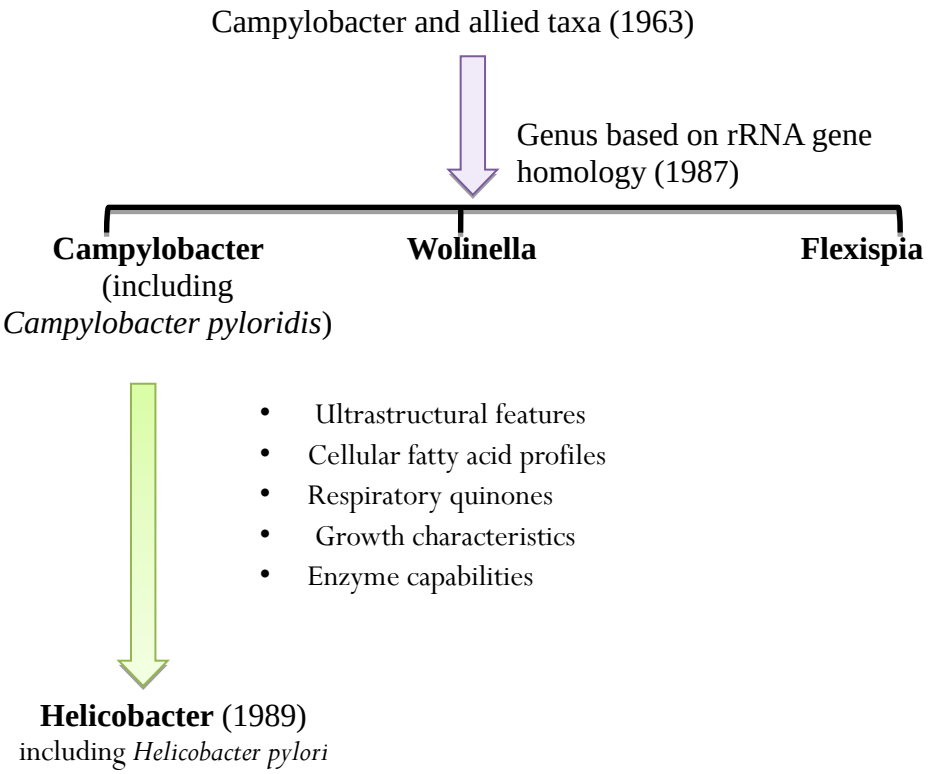
Order: Campylobacterales

Family: Helicobacteraceae

Genus: Helicobacter

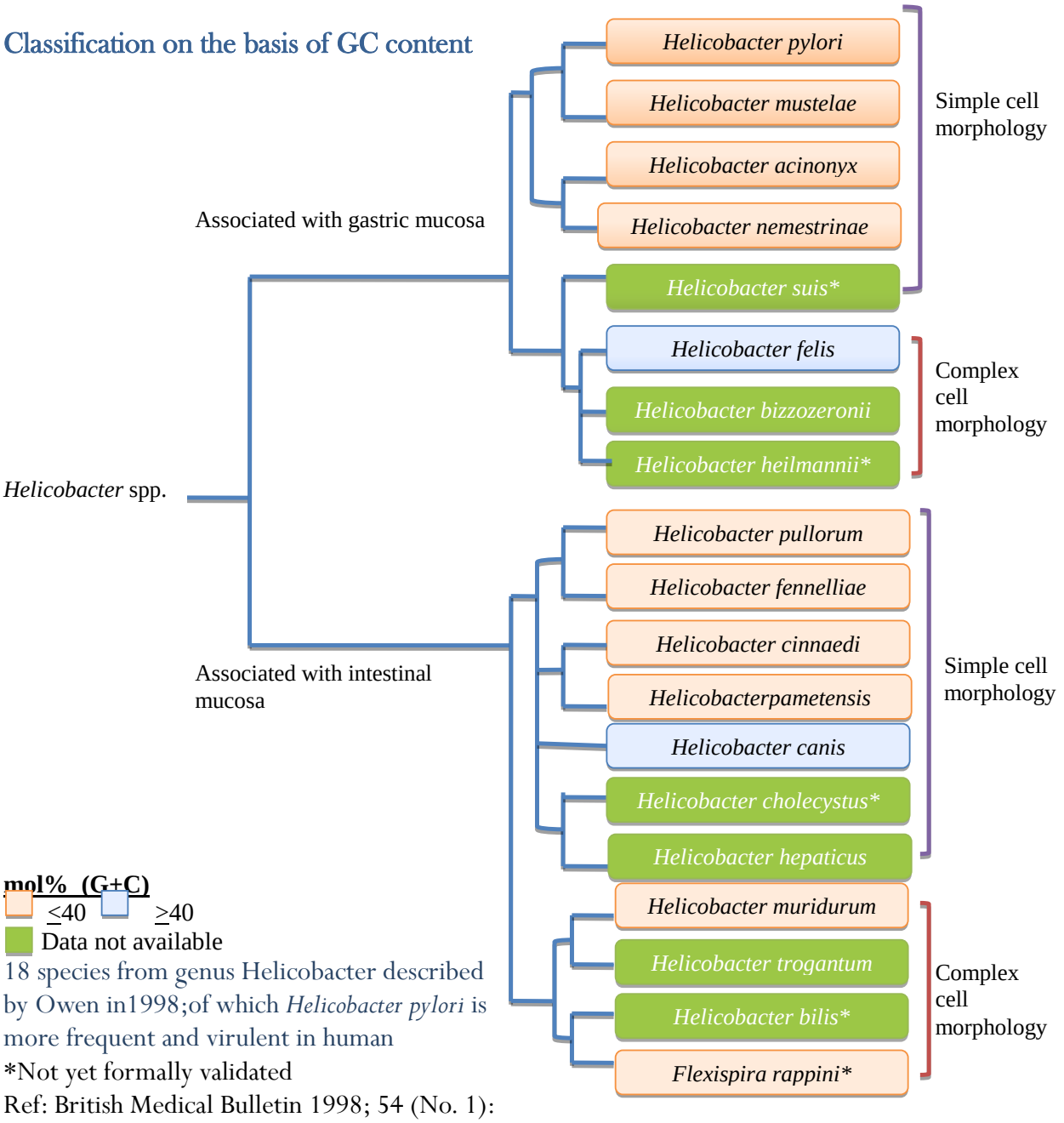
Species: Pylori

Binomial nomenclature: *Helicobacter pylori* **(Ref: Marshall et al., 1985, Goodwin et al, 1989)** [18, 19]



(Ref: Goodwin et al.,1989)

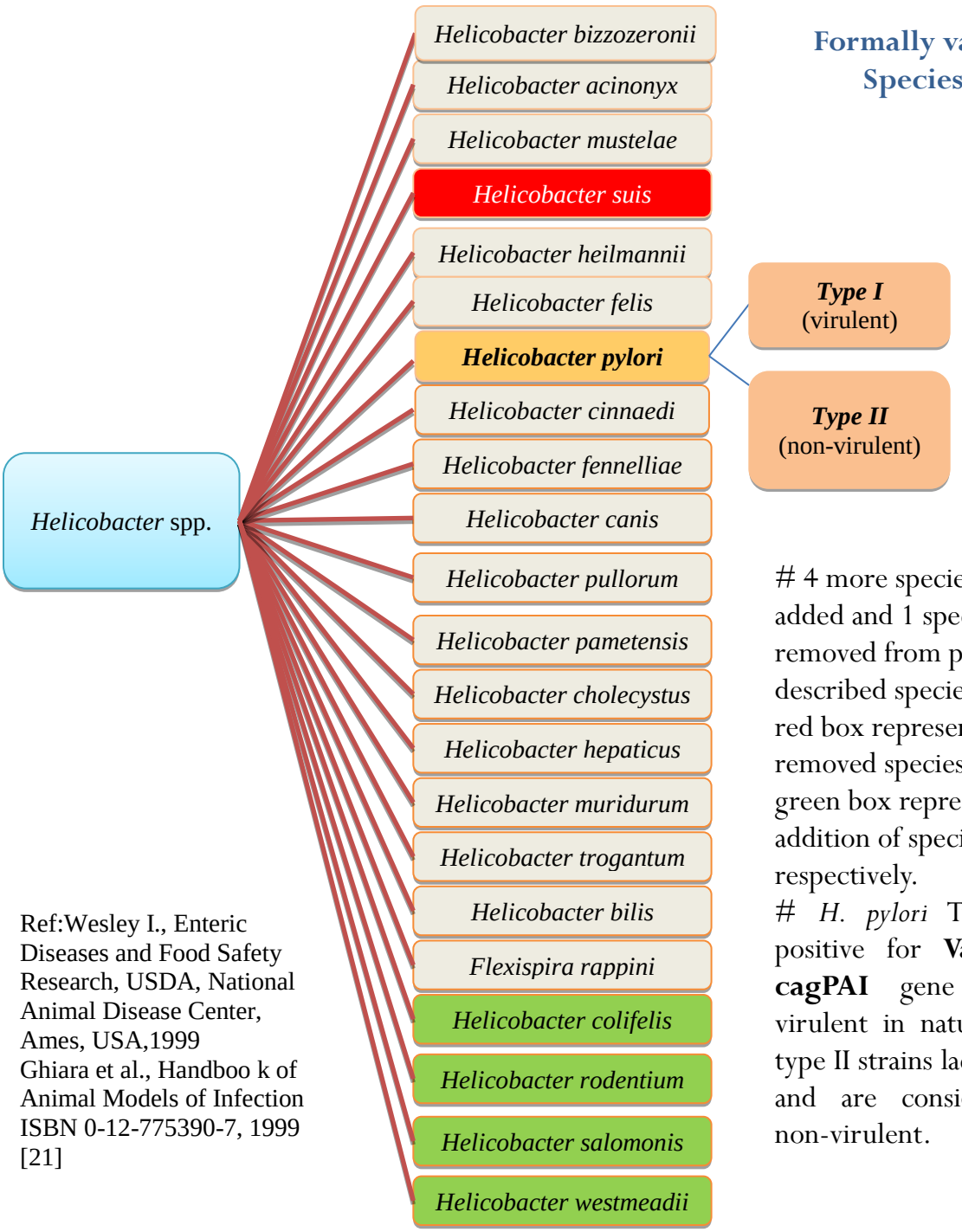
Classification on the basis of GC content



18 species from genus Helicobacter described by Owen in1998;of which *Helicobacter pylori* is more frequent and virulent in human

*Not yet formally validated

Ref: British Medical Bulletin 1998; 54 (No. 1): 17-30 [20]



Ref:Wesley I., Enteric Diseases and Food Safety Research, USDA, National Animal Disease Center, Ames, USA,1999
Ghiara et al., Handboo k of Animal Models of Infection ISBN 0-12-775390-7, 1999 [21]

4 more species were added and 1 species was removed from previously described species; here red box represents removed species while green box represents addition of species respectively.

H. pylori Type I is positive for **VacA** and **cagPAI** gene and is virulent in nature while type II strains lack cagPAI and are considered as non-virulent.

The pathogenic mechanism of *Helicobacter pylori (H. pylori)* is a result of a complex interaction between human responses and bacterial virulence factors. When *H. pylori* colonizes the stomach mucosa, it uses adhesins to adhere to epithelial cells securely, avoiding removal processes and creating a chronic infection. By neutralizing stomach acid, its urease synthesis helps it survive in the acidic environment of the stomach. Meanwhile, virulence factors such as the VacA toxin and the cag pathogenicity island (cagPAI) of *H. pylori* induce a strong inflammatory response that leads to tissue destruction. Gastritis, ulcers, and even gastric cancer are made more likely by persistent infections that disturb the physiology of the stomach, changing acid secretion and barrier function. Despite being triggered, host immune responses frequently fall short of eliminating the infection because of *H. pylori* 's evasion techniques. Recognizing this complex pathophysiology is crucial for devising effective therapeutic interventions against *H. pylori* -associated diseases.

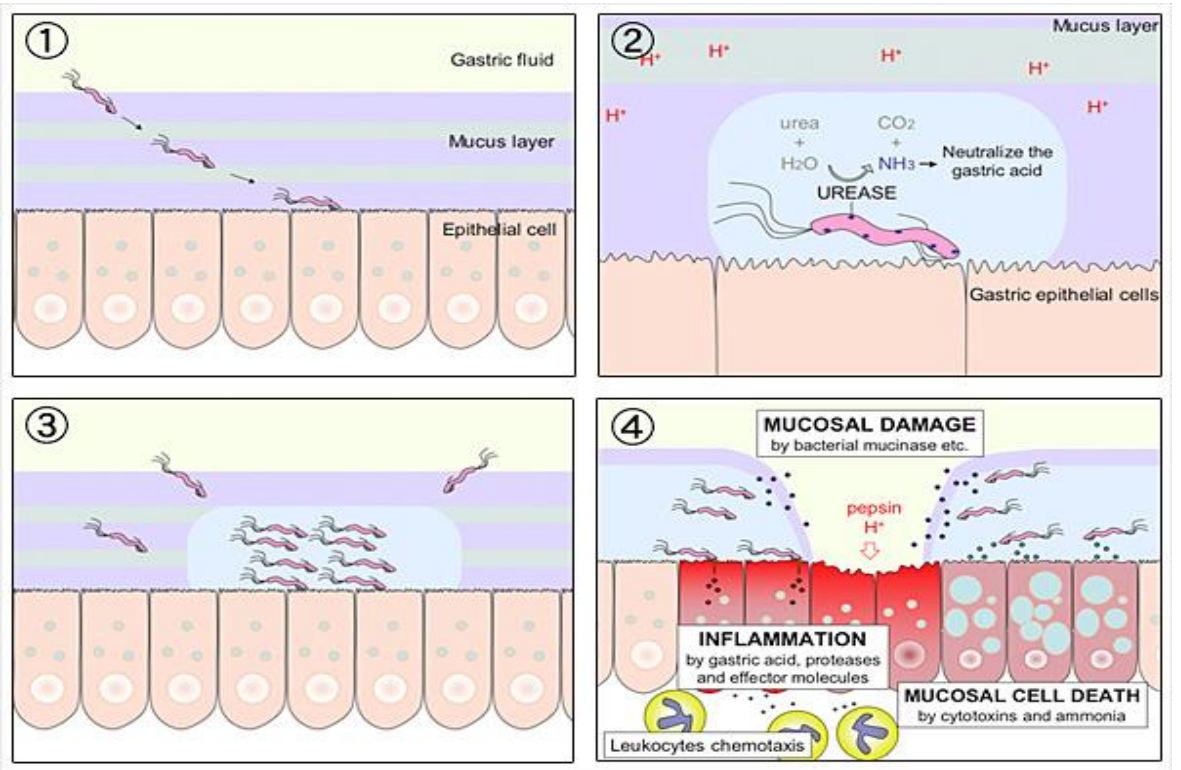


Figure 3: Pathogenesis of *Helicobacter pylori*

Major factors behind pathogenesis
a. Biochemical factors
i. Urease:

A vital pathogenic component of *Helicobacter pylori (H. pylori)* infection is urease. This enzyme is essential because it catalyzes the conversion of urea into carbon dioxide and ammonia. The stomach's acidic environment is successfully neutralized by this process, making a more favorable habitat for bacterial survival. Urease helps *H. pylori* survive in the gastric mucosa by increasing the local pH, protecting the bacteria from the harsh acidic environment of the stomach [22].

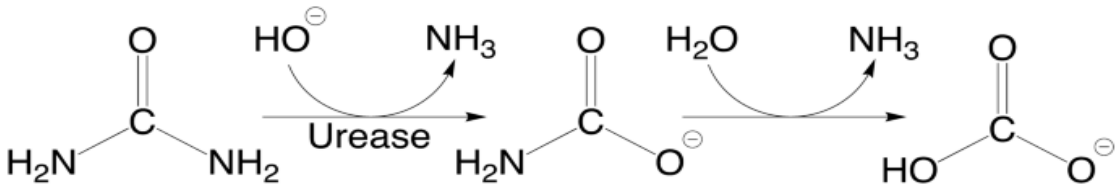


Figure 4.a: Neutralization of gastric acidity by elevating the pH by urease production

Furthermore, the production of ammonia through urease activity has the potential to cause harm to stomach epithelial cells and compromise the mucosal barrier, so promoting bacterial colonization and causing inflammation. Ammonia can also provide *H. pylori* with a source of nitrogen, which will aid in the bacteria's growth and multiplication. Therefore, urease modulates the gastric milieu, enhances bacterial survival, and contributes to inflammation and tissue damage in the host, all of which are important aspects of the pathophysiology of *H. pylori* infection.

ii. Catalase and Oxidase:

Chronic stomach infections are caused by the bacterium *Helicobacter pylori*, which depends on both oxidase and catalase to survive. The bacterial cell is shielded from oxidative damage by

catalase, which aids in the breakdown of hydrogen peroxide, a hazardous consequence of bacterial metabolism and host immunological responses. Oxidase, on the other hand, helps the bacteria produce energy and is essential to its survival in the stomach's acidic and oxygen-rich environment. When these enzymes work together, *H. pylori* is better able to withstand oxidative stress and preserve its cellular activities, which increases its ability to survive and grow pathogenically in the harsh environment of the stomach. The bacterium's ability to persist and produce chronic illnesses depends on their combined activity.

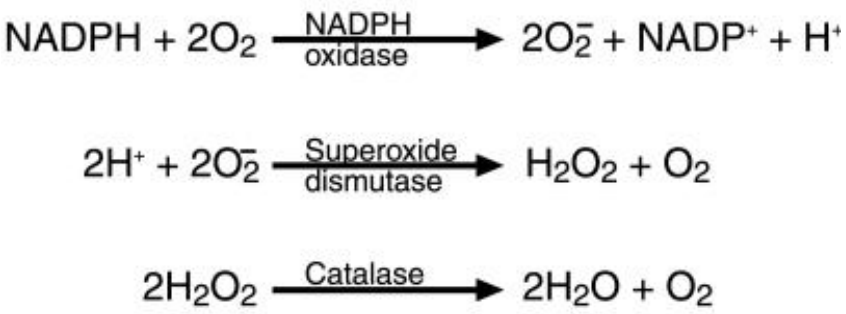


Figure 4.b: Catalase and Oxidase prevent oxidative stress and maintain cellular functions

b. Genotypic factors

i. cagPAI:

In *Helicobacter pylori (H. pylori)* infections, the cag pathogenicity island (cagPAI) is an essential virulence component that is critical to the pathogenesis of the bacteria. This is a section of the genome that contains about 40 genes, one of which is the cagA gene, which produces the oncoprotein CagA. Upon contact with gastric epithelial cells, *H. pylori* utilizes a type IV secretion system encoded by cagPAI to inject CagA into host cells. Inside the host cell, CagA undergoes phosphorylation and subsequently interacts with various host signaling proteins, leading to dysregulation of cellular processes such as cell proliferation, apoptosis, and inflammation. This dysregulation contributes to the development of gastric diseases, including

gastritis, peptic ulcers, and gastric cancer.Furthermore, other virulence factors, like as heat shock protein and peptidoglycan, can be delivered into host cells by cagPAI's type IV secretion system, aggravating tissue damage and the inflammatory response.

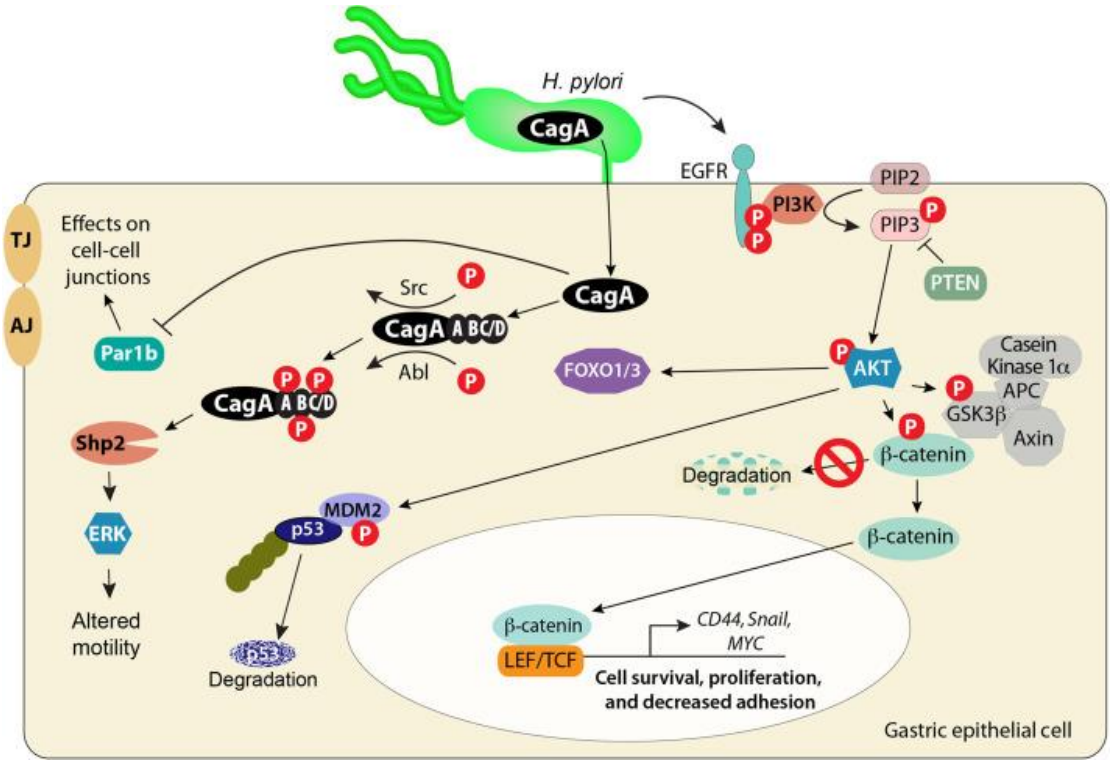


Figure 5: Role of cagA in pathogenesis

Protein CagA has the ability to influence cell activity through its effects on host signaling, which are mostly linked to the EPIYA motifs found in the protein's C-terminus. Tyrosine residues act as the site of phosphorylation in the five amino acid sequence that makes up the EPIYA motifs: Glu-Pro-Ile-Tyr-Ala. The cagA genes, which include EPIYA-A, -B, -C, and -D, have distinct counts and kinds of motif repeat. In every positive cagA, the EPIYA-A and -B motifs are consistently present. But because of the pathogenicity power and bacterial strains, the number and order of the other two segments varies. According to reports, there is a significant incidence of stomach cancer cases in East Asian strains found in Central Asia, East Asia, and North America. These strains are categorized as EPIYA-A, -B, and -D in the segments. Western strains

with EPIYA-A, -B, and -C patterns, on the other hand, are found in countries in the West and the Middle East; the C motif, in particular, can vary geographically and repeat itself several times. According to research by Yamaoka et al., the bacteria become more sensitive to the pH of the surrounding environment and more effective in triggering the release of interleukin 8 as the segment C repetition count rises [23, 24].

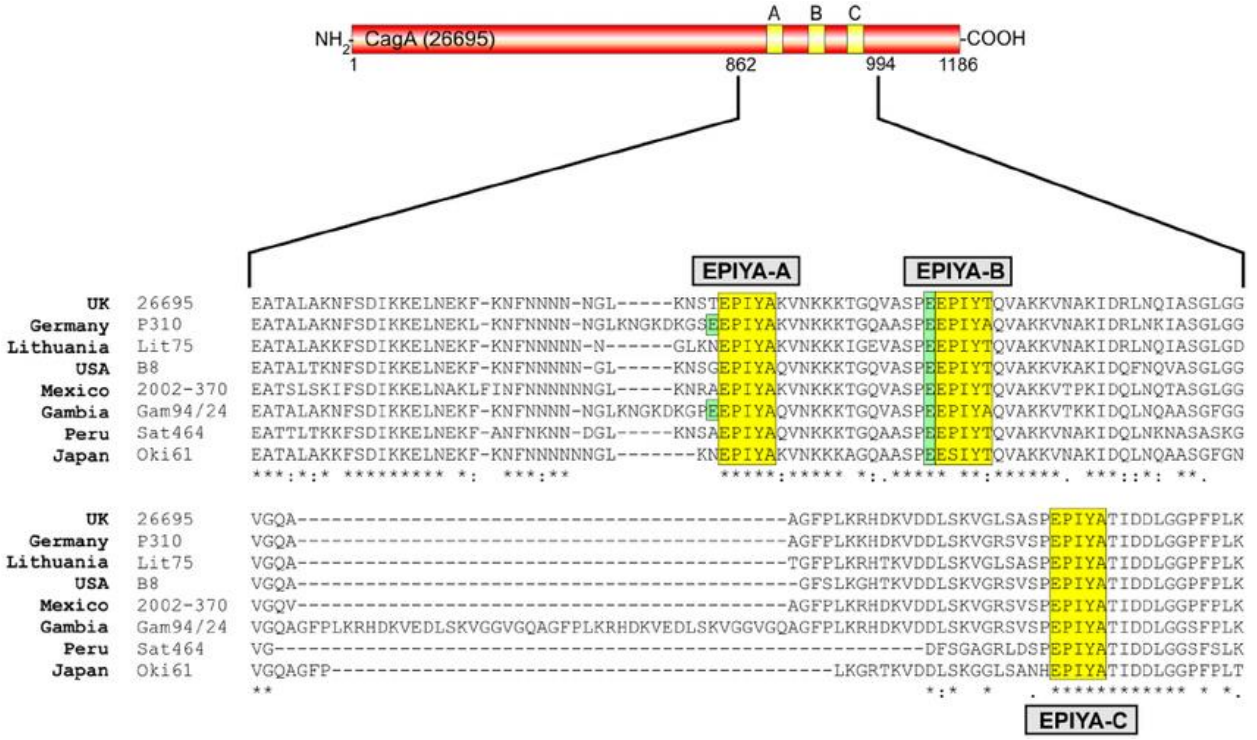


Figure 6: EPIYA motif and its relevance pertaining to cagA induced infection

ii. vacA:

One of the main virulence factors of *Helicobacter pylori (H. pylori)* is the Vacuolating Cytotoxin A (VacA) gene, which plays a complex function in the pathogenesis of the bacteria. VacA targets the epithelial cells of the stomach when it secretes, causing vacuolation and damaging cellular integrity, which leads to tissue damage and the development of ulcers. Additionally, VacA suppresses T-cell activation and proliferation and triggers lymphocyte death, which helps

H. pylori evade host immunological responses. The s1/m1 allelic mutation, in particular, is associated with a higher incidence of stomach cancer and peptic ulcers, as well as increased virulence and disease severity. VacA is known to exhibit activity through pathways other than vacuolating effects that are dependent on toxins, such as interfering with intracellular trafficking.

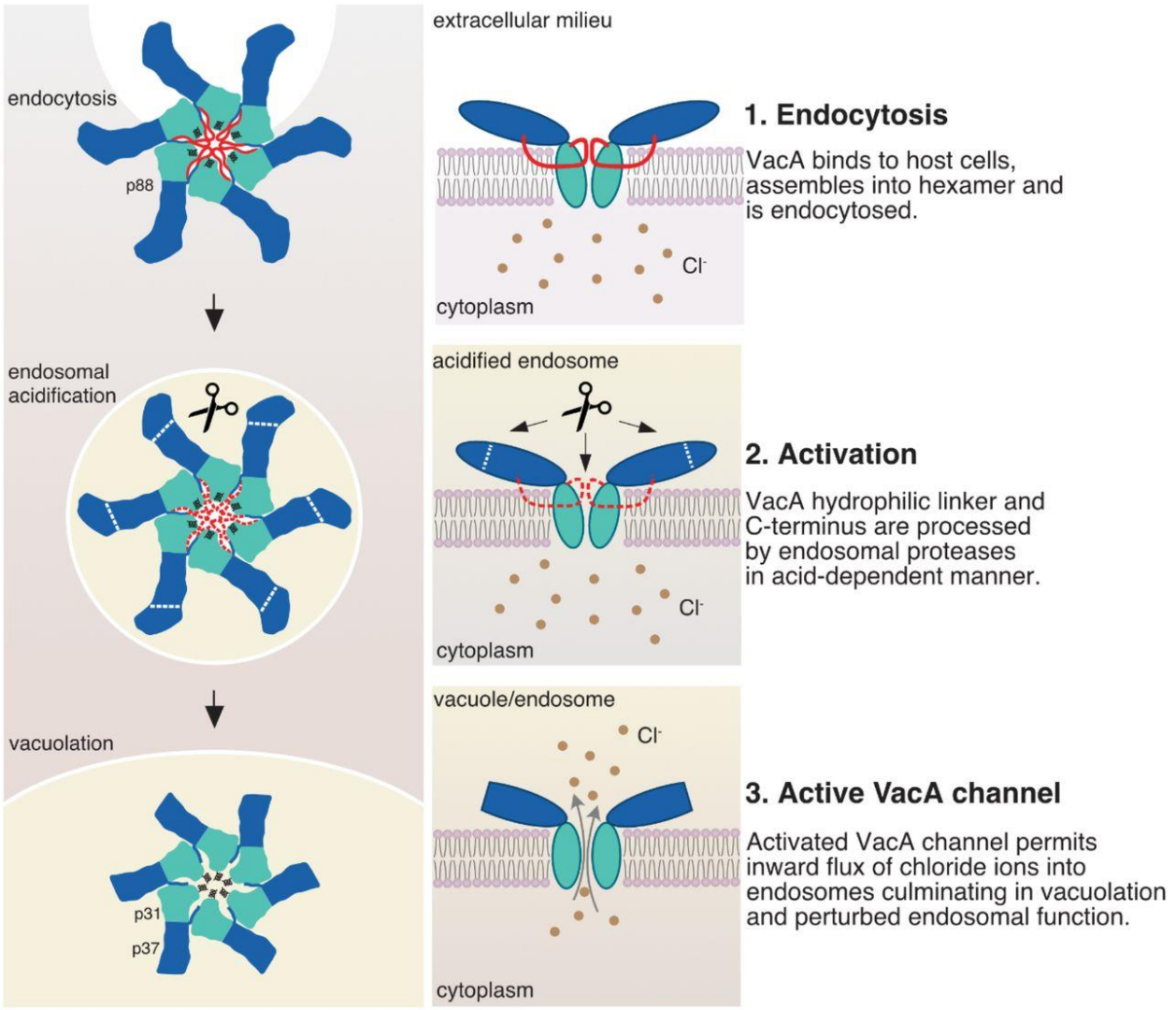


Figure 7: Mode of action of vacA toxin

Moreover, VacA's correlation with the severity of the disease goes beyond its immediate cellular consequences. Research has demonstrated that it plays a part in impairing the integrity of the gastric barrier, which makes it easier for other bacterial virulence factors and toxins to move

across the epithelium and exacerbate tissue damage and inflammation. Furthermore, the immunomodulatory characteristics of VacA aid in the development of a chronic inflammatory state in the stomach mucosa, which further encourages the persistence of *H. pylori* and the advancement of the disease. It's interesting to note that VacA demonstrates a remarkable variability in structure and function between strains of *H. pylori*, with differences in its domain organization and amino acid sequence affecting its pathogenic potential and activity. Its significance as a major factor influencing *H. pylori* virulence and disease prognosis is shown by its diversity [24].

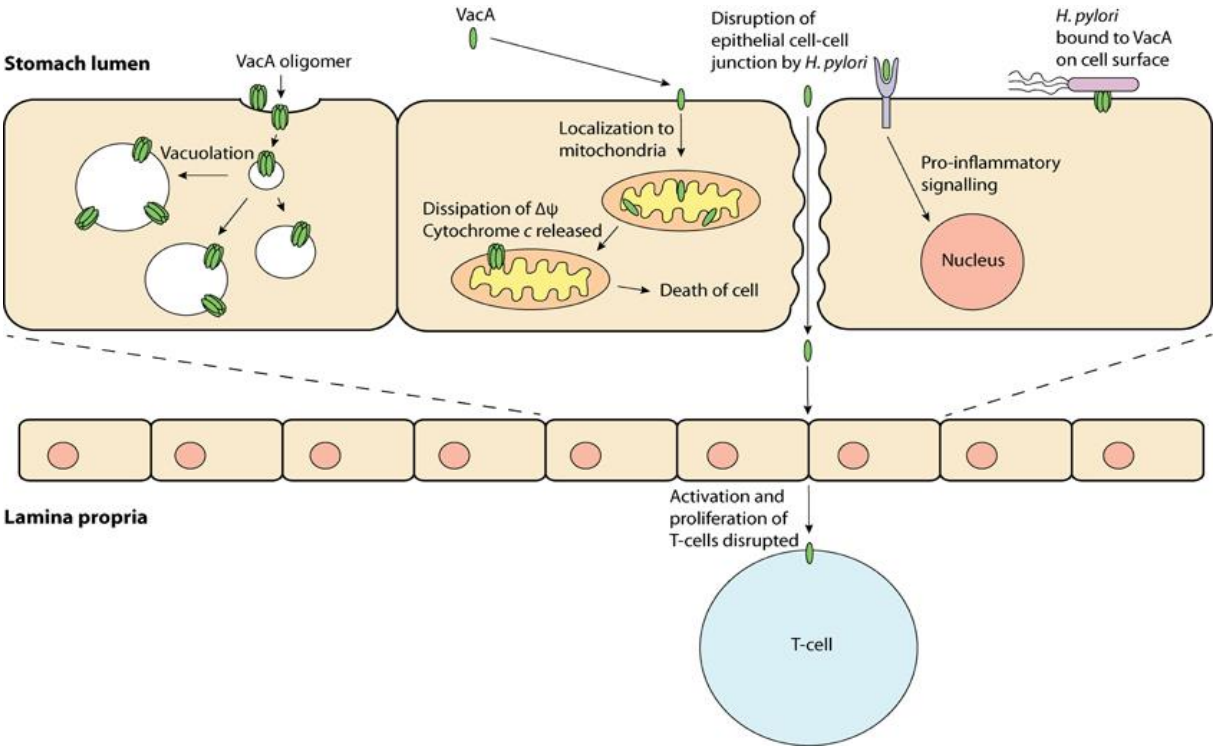


Figure 8: Effect of vacA toxin on pathogenesis

iii. *babA2:*

BabA2, an essential adhesin protein that *Helicobacter pylori(H. pylori)* expresses, is essential to the pathophysiology of *H. pylori* infection. Many gastrointestinal conditions, such as gastritis, peptic ulcers, and potentially stomach cancer, have this bacterium as a primary causative agent.

BabA2 binds particularly to the Lewis b (Leb) antigen on the surface of the gastric mucosa, which helps *H. pylori* attach itself to the gastric epithelial cells. This attachment is essential to *H. pylori*'s colonization and survival in the stomach, as it allows the bacteria to elude the host's immune system and cause a chronic infection. Furthermore, BabA2-mediated binding contributes to the pathophysiology of gastritis and the development of ulcers by causing inflammation and damaging the integrity of the stomach epithelial barrier.

The Lewis b (Leb) antigen on the surface of the gastric mucosa is recognized and selectively bound to by BabA2, a well-known adhesin protein of *Helicobacter pylori (H. pylori)*, which helps the bacteria connect to gastric epithelial cells. A β-barrel structure inserted in the bacterial outer membrane distinguishes BabA2, a member of the outer membrane protein family. Certain amino acid residues in BabA2's binding pocket operate as a mediator between the ligand, which is the glycan structure known as the Lew^b antigen, and the protein.

BabA2 triggers an array of actions that culminate in the development of *H. pylori* infection upon attaching to Le^b antigen. A portion of this involves the bacterial adhesion, colonization, and immune response evasion caused by the activation of downstream signaling pathways within the host cells. In addition, pro-inflammatory reactions triggered by BabA2-mediated binding include the production of cytokines and chemokines, which draw immune cells to the infection site and cause persistent inflammation [25].

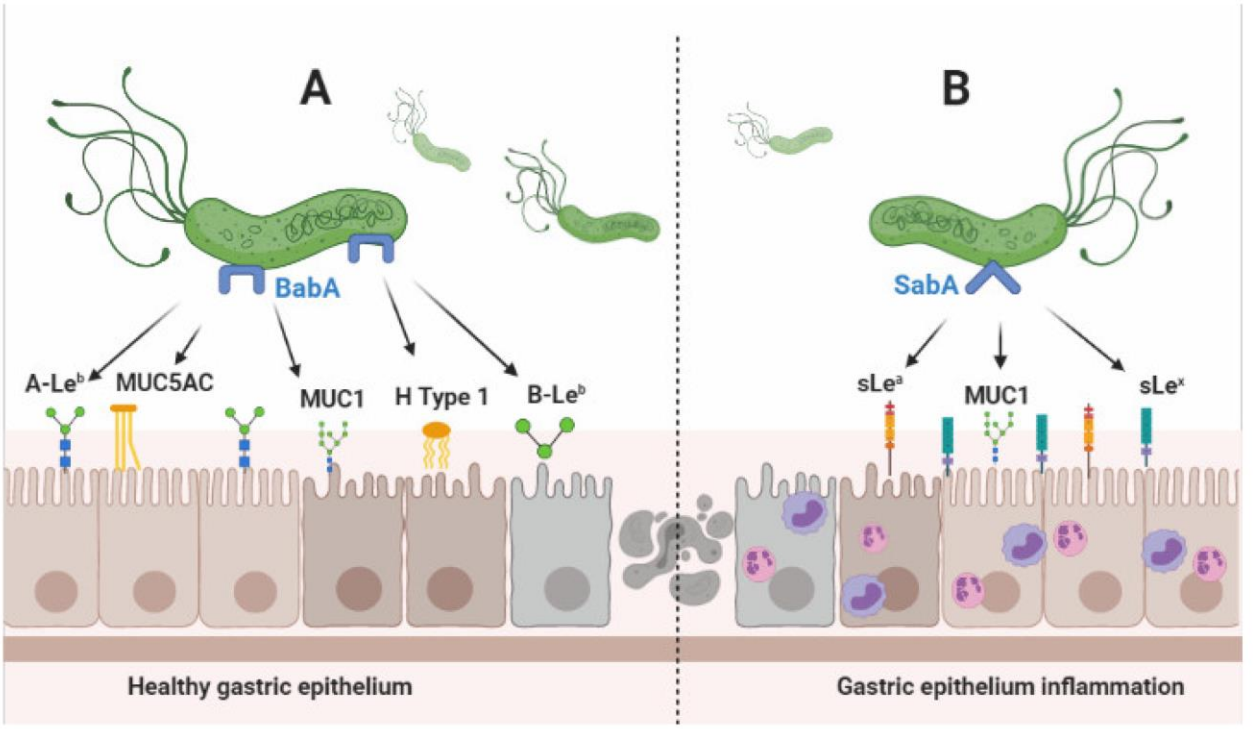


Figure 9: Pathological role of BabA2 and SabA

iv. dupA:

Within the plasticity zone of the *Helicobacter pylori (H. pylori)* genome, the Duodenal Ulcer Promoting Gene (dupA) is a virulence factor linked to the pathogenesis of gastroduodenal disorders. Its existence may have a preventive effect on duodenal ulcers, but it may also raise the risk of other gastroduodenal conditions, such as stomach cancer. It is believed that dupA affects other virulence factors, changes the adhesion characteristics of bacteria, or modulates host immune responses, albeit the exact processes underpinning its effects are still unknown. A better knowledge of disease progression and the development of specific therapeutic approaches for *H. pylori* -related gastroduodenal diseases are possible with continued research into dupA's function in *H. pylori* pathogenesis, despite some contradictory findings [26].

c. Role of Gut microbiome in pathogenesis:

Particularly in relation to the human stomach, *Helicobacter pylori (H. pylori)* is well-known for its complex interactions with the gut microbiome. This microbe's effects on stomach disorders like gastritis, peptic ulcers, and even stomach cancer have prompted a great deal of research. There are several facets and a complex interaction between *H. pylori* and the gut microflora. When *H. pylori* first enters the stomach mucosa, it carves out a home where it might remain unchecked for decades. The immunological response sparked by this colonization results in persistent inflammation. The composition of the gut microbiota can be indirectly affected by this inflammatory response, which is interesting since it can change the gastric environment. According to studies, variations in the variety and number of other stomach microorganisms are correlated with the presence of *H. pylori*. It may affect the general health of the gut, for instance, reducing the diversity of microbes in the stomach. In addition, *H. pylori* has developed defense mechanisms to control the immune system of its host, preventing it from being identified and eliminated. This alteration may affect the balance of gut microbes and systemic immunity more broadly. Changes in gastric acid secretion are also linked to the presence of the bacteria, and this could have an impact on the microbial communities in the stomach and possibly further down the digestive tract. However, the diversity and composition of the gut microbiome can affect the pathogenicity of *H. pylori* infection. *H. pylori* growth may be stimulated or inhibited by specific microbial communities, which can affect how the disease progresses. Comprehending these interplays is essential for formulating focused treatments that may eliminate *H. pylori* or alter the gut microbiota to lessen its deleterious consequences. In summary, there are important health consequences for humans arising from the dynamic connection between *H. pylori* and the gut flora. To precisely understand how *H. pylori* affects gut microbial ecosystems and vice versa, more research is required. This information may open the door to new treatment approaches

meant to correct the microbial balance and stop gastrointestinal disorders brought on by *H. pylori* [27].

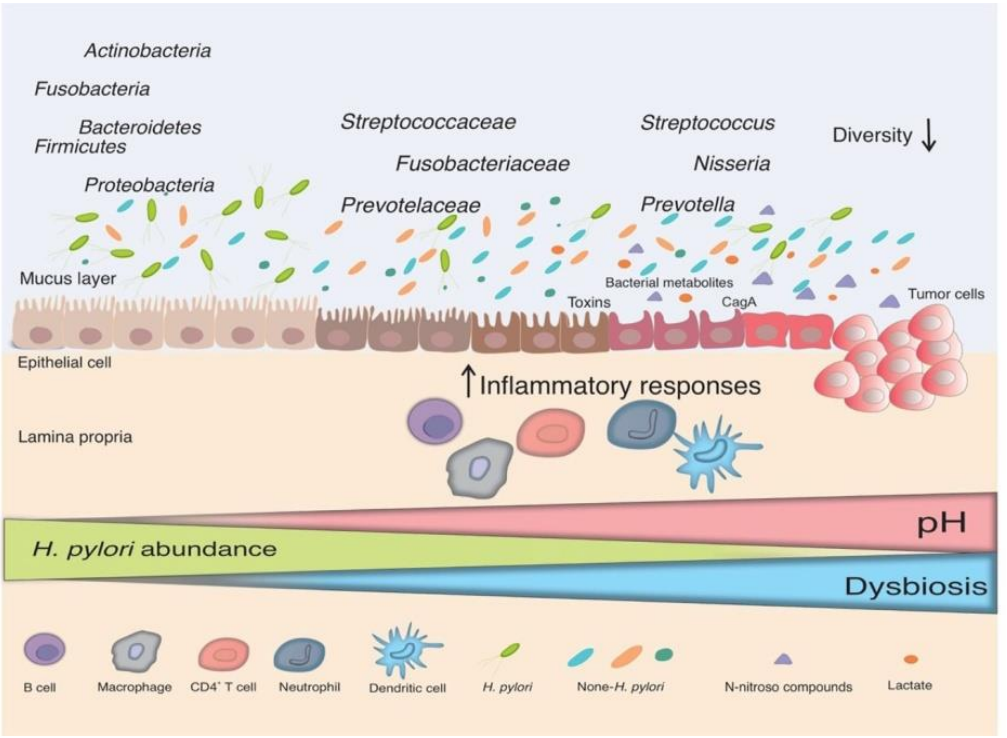


Figure 10: Interaction between *H. pylori* and gut microbiome

d. Role of mucus layer in pathogenesis:

As a dynamic barrier separating the bacteria from the gastric epithelium, the stomach's mucus layer is vital in the setting of *Helicobacter pylori (H. pylori)* infection. Mucins, glycoproteins released by epithelial cells, make up this layer. They cover the epithelial surface in a thick matrix that resembles gel. First, *H. pylori* is physically prevented from coming into direct touch with and attaching itself to stomach epithelial cells by the mucus layer. Bacterial penetration into the stomach mucosa is restricted by the physical barrier that is created by the dense and viscous nature of mucins. Second, the mucus layer contains antimicrobial components that support innate immune response, including as immunoglobulins and antimicrobial peptides (AMPs). These

elements may interfere with the growth of *H. pylori* directly or damage bacterial membranes, preventing colonization. In order to avoid immune recognition, *H. pylori* has also developed strategies to break through and modify the mucus layer. These strategies include the development of adhesins, which aid in adhesion to mucins, and changing the glycosylation patterns of mucins. Developing methods to stop or treat disorders linked to *H. pylori* requires an understanding of how the stomach mucus layer and *H. pylori* interact. Potential treatment interventions to address *H. pylori* infection and related gastric diseases may be made possible by disrupting this barrier or improving its protective properties [28, 29].

Transmission:

The most frequent means of person-to-person transmission for *Helicobacter pylori (H. pylori)* is fecal-oral or oral-oral transmission. Although the precise modes of transmission are still unclear, a number of factors influence the spread of *H. pylori*. These include sharing utensils, inadequate sanitation standards, and close contact inside families, especially among family members. Furthermore, research indicates that *H. pylori* may persist in food and water sources, which may aid in transmission. Other possible pathways of infection include iatrogenic transmission through infected endoscopes or medical equipment, and gastroesophageal reflux, in which *H. pylori* from the stomach may reflux into the mouth. Furthermore, although the importance of this route is still up for question, oral-to-oral transmission can happen through actions like kissing. Although *H. pylori* infection is usually acquired in childhood, there are significant individual and community differences in the precise timing and circumstances of transmission. Environmental factors, hygiene habits, and socioeconomic considerations all affect the chance of *H. pylori* transmission. Implementing strategies to enhance hygiene and sanitation, such as frequent hand-washing, appropriate sanitation techniques, and refraining from sharing utensils and food, is the main

strategy for preventing the spread of *H. pylori*. Furthermore, treating and screening infected people can help lower the prevalence of *H. pylori* -related illnesses and stop future transmission within communities, especially in those with a history of stomach cancer or peptic ulcers [30, 31].

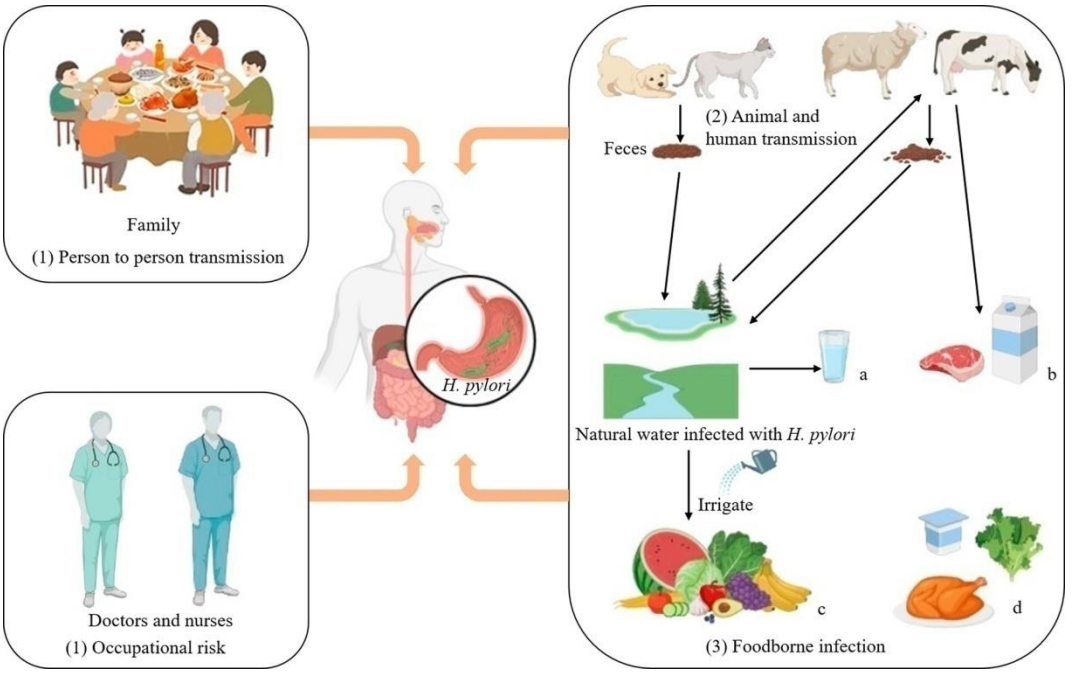


Figure 11: Transmission of *H. pylori*

Detection of *H. pylori*

i. Rapid Urease Test (RUT):

A popular diagnostic technique for identifying *Helicobacter pylori (H. pylori)* infections, which are linked to a number of gastrointestinal conditions such as gastritis and peptic ulcers, is the rapid urease test (RUT). The efficacy of this test depends on *H. pylori's* production of the urease enzyme, which quickly hydrolyzes urea to create carbon dioxide and ammonia.

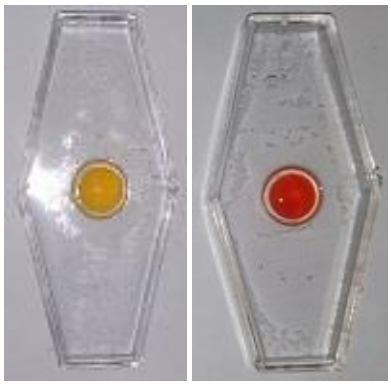


Figure 12: Rapid urease Test Kit

During an endoscopy, a biopsy specimen from the stomach mucosa is obtained as part of the procedure. A tiny bit of tissue is inserted into a particular culture medium that has pH indicator and urea in it. The urease that *H. pylori* produces will hydrolyze the urea in the tissue sample, raising the pH if the bacteria is present. The indicator changes color as a result of the pH shift, signifying a positive result for *H. pylori* infection. The test is a somewhat quick way to diagnose, usually producing results in 24 to 48 hours.The fast urease test has a high sensitivity and specificity; reported values exceed 90%. This is one of its main advantages. In comparison to alternative diagnostic techniques like bacterial culture or histological investigation, the test is also rather easy to do, non-invasive, and affordable. However, because proton pump inhibitors and antibiotics can suppress *H. pylori* activity, individuals who have recently taken these drugs may receive false-negative findings from the fast urease test. Because of this, it's critical to consider the patient's medication history and, in the event that a negative RUT result doesn't lower clinical suspicion, to run additional confirmatory tests [32].

ii. Urea Breath Test (UBT):

An effective and non-invasive diagnostic technique for identifying *Helicobacter pylori (H. pylori*) infections, which are frequently linked to gastrointestinal conditions such as gastritis and

peptic ulcers, is the urea breath test (UBT). This test is dependent on *H. pylori's* capacity to produce the urease enzyme, which is capable of catalyzing the hydrolysis of urea to produce carbon dioxide and ammonia.During the UBT process, the patient swallows a liquid, pill, or granule that contains a particular type of tagged urea, like nitrogen-15 or carbon-13 urea. Labeled carbon dioxide will be released from the stomach if *H. pylori* is present because the bacteria's urease enzyme will break down the labeled urea. Following the patient's exhalation into a collecting device, a breath sample is collected and subjected to mass spectrometry or infrared spectrophotometry to detect the presence of tagged carbon dioxide [33].

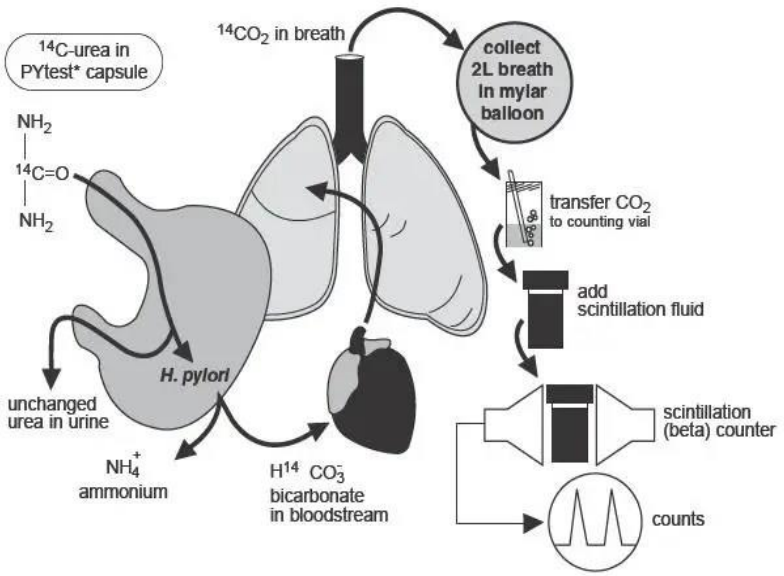


Figure 13: Steps involving Urea Breath Test

When compared to alternative *H. pylori* infection diagnosis techniques, the UBT has a number of benefits. The procedure is safe, non-invasive, and convenient for patients, merely requiring one clinic visit. Furthermore, regardless of the patient's usage of antibiotics or proton pump inhibitors, the UBT may detect an active *H. pylori* infection due to its excellent sensitivity and specificity, which typically surpass 95%. Nonetheless, a few things like recently taking

antibiotics, substances containing bismuth, or proton pump inhibitors may affect how accurate the UBT readings are. Consequently, in order to guarantee the validity of the test, it is crucial that patients adhere to certain pre-test guidelines. In summary, the urea breath test, which offers great accuracy, non-invasiveness, and convenience, is a useful and popular diagnostic method for identifying *H. pylori* infection.

Treatment: Therapeutic and Prophylactic

Therapeutic Treatment

i. Standard Antibiotic Therapy (Triple therapy):

A combination of proton pump inhibitors (PPIs) and antibiotics is the standard treatment for *Helicobacter pylori (H. pylori)* infections. The most often given combination is called triple therapy, which includes two antibiotics (typically clarithromycin and amoxicillin or metronidazole) and a PPI (omeprazole or lansoprazole). Eliminating the *H. pylori* bacteria and treating related symptoms including gastritis and peptic ulcers are the goals of this regimen. But typical triple therapy's effectiveness has been reduced in some areas due to rising antibiotic resistance, especially to metronidazole and clarithromycin. As such, other treatment plans, including bismuth subsalicylate triple therapy, might be advised, particularly in regions where the prevalence of antibiotic resistance is high. Even though it's the first line of treatment, treatment failure for *Helicobacter pylori (H. pylori)* infections can still happen, mostly as a result of patient non-compliance and antibiotic resistance. The increasing prevalence of antibiotic resistance, especially to metronidazole and clarithromycin, might severely compromise the effectiveness of routine triple therapy. Treatment failure can also be caused by insufficient dosage, interrupted treatment regimens, and noncompliance with the recommended regimen by

the patient. In addition, variables such bacterial virulence, age of the patient, and underlying co-morbidities may also affect how well a treatment works. Antimicrobial susceptibility testing-guided alternate antibiotic regimens, such as levofloxacin-based therapy or bismuth-containing quadruple therapy, may be used by clinicians in cases of treatment failure in order to maximize eradication rates. Additionally, probiotics and mucosal protecting agents are examples of adjuvant therapy that can be taken into consideration to improve treatment efficacy and lessen side effects. For successful eradication and to avoid disease recurrence, management of *H. pylori* treatment failure requires a customized strategy based on unique patient variables and antibiotic sensitivity patterns [34].

ii. Bismuth containing quadruple therapy:

In areas where antibiotic resistance rates are high, bismuth-containing triple treatment is a viable alternative for managing *Helicobacter pylori (H. pylori)* infections. Proton pump inhibitors (PPIs), bismuth salts, tetracycline, and nitroimidazole antibiotics like metronidazole or tinidazole usually make up this regimen. The addition of bismuth compounds, which are well-known for their antibacterial and anti-inflammatory qualities, improves the treatment's effectiveness by preventing urease activity, rupturing bacterial cell membranes, and decreasing *H. pylori* colonization. Additionally, bismuth aids in shielding the stomach mucosa from the harmful effects of bacterial toxins and acid. To help further eradicate bacteria, tetracycline and the antibiotic nitroimidazole target the production of bacterial proteins and the replication of bacterial DNA, respectively.

When compared to traditional triple therapy, bismuth-containing quadruple therapy has shown better eradication rates, particularly in cases of clarithromycin resistance. On the other hand, negative outcomes include a metallic taste, blackened stools or tongue, and upset stomach are

possible. Bismuth-containing triple therapy is still a useful treatment option for *H. pylori* infection, with high efficacy and the possibility of successful eradication, despite severe side effects [35].

Prophylactic Treatment

i. Vaccine development:

Due to the intricate biology of the bacteria and the host immune response, developing a vaccine against *Helicobacter pylori (H. pylori)* presents substantial obstacles. The capacity of Helicobacter pylori to establish chronic colonization in the stomach mucosa and to subdue the host immune system by a variety of strategies, including immunological regulation and antigenic diversity, is one of the main challenges. Furthermore, *H. pylori* infection presents a challenge in identifying appropriate vaccine targets that might generate protective immunity without inducing deleterious inflammatory responses because it evokes a complex immune response combining both innate and adaptive immunity [36]. Preclinical vaccine evaluation is further hampered by the absence of trustworthy animal models that faithfully mimic the course of a human *H. pylori* infection. The great genetic diversity of *H. pylori* strains also presents a major obstacle to the development of vaccines, since a vaccine has to be broadly protective against a variety of strains in order to be considered therapeutically relevant. In spite of these difficulties, current research is concentrated on finding conserved antigenic targets and cutting-edge vaccine delivery methods in order to get beyond these barriers and create a vaccine that is effective against *H. pylori* infection [37].

In the last several years, research on the *H. pylori* vaccination has mostly focused on identifying and using particular antigens that are essential for triggering immune responses that protect against *H. pylori* infection. In order to efficiently target *H. pylori* while reducing the possibility

of immune evasion, researchers have concentrated on finding conserved and immunogenic antigens. The FlaA and FlaB proteins, which are crucial to bacterial motility and host colonization, are examples of key antigens that are now being studied. Furthermore, because of their role in *H. pylori* adhesion, virulence, and host interactions, outer membrane proteins (OMPs), including as BabA, VacA, and CagA, have attracted a lot of interest. Moreover, antigens such as urease an enzyme essential to *H. pylori* survival in the acidic stomach environment have been investigated as possible targets for vaccinations. With continuous work targeted at refining antigen selection and formulation to boost vaccination efficacy and produce robust and lasting immune responses against *H. pylori* infection, these antigens provide interesting paths for vaccine development [38, 39].

ii. Vaccines at different stages of development:

A vaccine is one of the most outstanding achievements of modern science and medicine. In the past, it has helped to eliminate many fatal, infectious diseases from our society. Various types of vaccines are available that use live attenuated or inactivated pathogens (bacteria and viruses) or conjugated polysaccharides to develop immunity. To be precise, these foreign objects or antigens stimulate the immune system to produce antibodies. Antibodies are produced by the plasma cells (primarily from B lymphocytes) and directly encounter the antigens or their toxins by their epitopes. A cell-mediated immune response is mediated by T-lymphocytes that differentiate into cytotoxic T lymphocytes or CTLs. At the same time, helper T lymphocytes recognize and produce cytokines, ultimately initiating a cascade of death-associated pathways that kill the pathogen and induce antigen-specific memory T cells. Activation and sustenance of these helper T cells control the degree of response toward an infection. In case of *H. pylori*, another type of T

lymphocyte, were found called Treg or T-regulator cells that mediate immune tolerance by regulating the actions of effector-T cells [40].

Candidate	Vaccine (s)/ components	Preclinical	Phase I	Phase II	Phase III
Wuhu Kangwei Biological technology	UreB/LTB fusion vaccine	√	√	√	Discontinued
Imax	IMX101	√			
Helicovaxor®	Engineered Vibrio cholerae vaccine, Inactivated H. pylori	√			
Sichuan University	Urease epitope vaccine	√			
Southern Medical University	Lp220 vaccine	√			
China Pharmaceutical University	Probiotic vaccine delivery	√			

Table 1: Status of vaccine under pipeline

In 2004-2005 Wuhu Kangwei Biological Technology Co., Ltd conducted a vaccination program that involved oral administration of a recombinant *H. pylori* vaccine. The preliminary report published in 2015 revealed, of 99% participants who completed a 3year trial, reported 64 events of *H. pylori* re-infection within first year,7% of both vaccine group and placebo group participants with adverse reaction and <1% reported serious adverse effects when exposed to circulating strains of *H. pylori* . In spite of achieving a vaccine efficacy of 71.8% with a significant reduction in *H. pylori* infection and good immunogenic response among naive

children, the study was terminated after phase III clinical trial [41]. This was the first reported vaccination program of its kind but certainly left a huge grey area to ponder on, like 10 years delay in publishing the preliminary report or why the complete report has never been disclosed in public.

Vaccines/Adjuvants	**Pros**	**Cons**
Live attenuated	• A non-virulent strain of a pathogen is used to induce an immune response. • One or two doses are enough to produce life-long immunity.	• Not suitable for immuno-compromised people. For example, people with HIV infection or under chemotherapy.
Bacterial ghost	• Cell envelopescontaining outer membrane particles are devoid of any cytoplasmic components.	• Possibility to develop a reaction like inflammation or become hypersensitive due to its reactogenicity.
Heat kill	• Heat-kill pathogens lack active functional genetic material or proteins.	• Requires booster shots to train the body to produce immunity.
Synthetic nanoformulation	• Synthetic nanoparticles have easy uptake via DCs depending upon the particle size. • Highly modifiable.	• The relationship between efficacy and the size of the particle still needs to be discovered. • Less complexity.
OMVs	• It can be modified to express recombinant proteins. • Self-adjuvancy	• Endotoxic content.

Table 2: Pros and Cons of different vaccine candidates

IMX101, developed by ImeVax, essentially targets a key survival process of *H. pylori* in the human stomach that isto reduce the effect of low gastric pH. One of the most potent *H. pylori* antigens, γ-glutamyltranspeptidase (GGT) is responsible for inducing immunosuppressive activity by neutralizing highly acidic environment of the stomach disabling the inflammatory response in the host. While the result of phase I clinical trial of the vaccine is yet to be published, the approach certainly is novel and thus intriguing to see whether targeting a single antigenic component is efficient enough or not in order to generate immunity given the fact that the bacteria employ a number of other defensive mechanisms related to adhesion, colonization and destruction of the mucosal lining of the stomach [42].

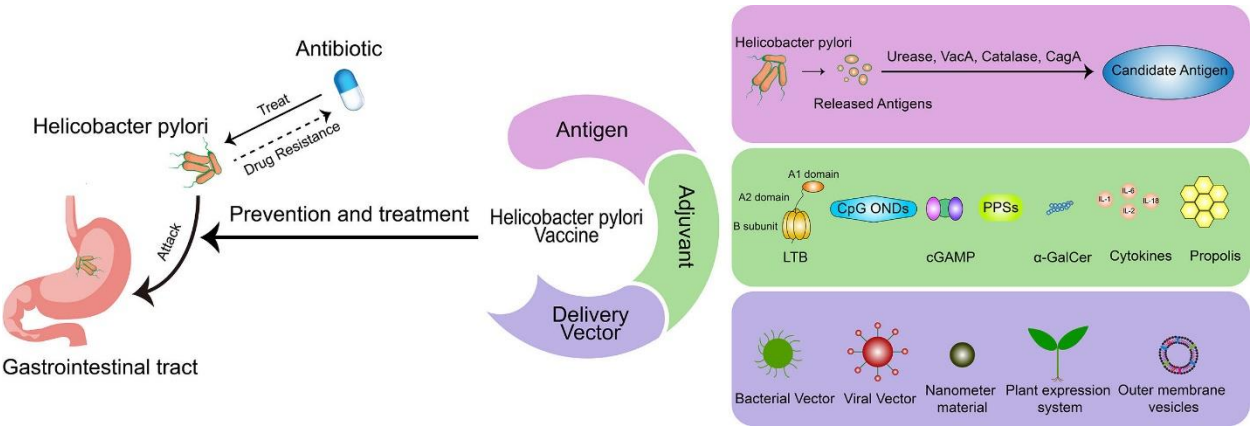


Figure 14: Prophylactic approaches to reduce *H. pylori* mediated health burden

Recently many small companies and academic institutions started showing interest and took initiatives towards *H. pylori* vaccine development. All these approaches are at the preclinical stages and involve mainly purified or recombinant antigen components accompanied by an adjuvant. Though the preliminary results of these studies are promising but they lack consistency and clarity. For example, (i) an epitope-based vaccine EpiVax, involves a DNA vaccine initially followed by a peptide-liposome. The vaccine showed some therapeutic protection but is

restricted only in mice,(ii) Dual approaches taken by Helicovaxor® -first one involving genetically engineered non-virulent *Vibrio cholerae* strain expressing *H. pylori* antigens (HpaA, UreB, and FlaA) while other one involves inactivated *H. pylori* strain engineered to induce serum antibody response both of which are at research level, (iii)Two recent studies, one on urease subunits and another on Lp220 (lipopolysaccharide220), conducted among BALB/c mice have shown minimal effect in generating protection, (iv) Probiotic as a vehicle for vaccine delivery involved microbes like *Lactococcus lactis* recombinantly expressing cholera toxin B subunit in addition to *H. pylori* urease epitope, has also not been found effective in developing immunity [42].

Preclinical vaccine research against prevalent strains of *H. pylori*

i. Animal model and its implications

Animal models are essential to *Helicobacter pylori (H. pylori)* research, especially when it comes to the creation and assessment of vaccinations. The pathophysiology of *H. pylori* infection, the host immune response, and the effectiveness of possible vaccine candidates are all greatly enhanced by these models. Researchers can examine the dynamics of *H. pylori* colonization, persistence, and disease progression in a controlled environment by using animal models, which replicate important elements of human infection. Animal models are crucial instruments used in vaccine research to evaluate the safety, immunogenicity, and protective effectiveness of vaccines. Mice especially genetically modified or strains devoid of specific pathogens are frequently employed because of their well-studied immune responses and genetic malleability. To simulate human *H. pylori* infection and evaluate vaccine-induced immune responses, several animal models are being used, including non-human primates and Mongolian gerbils [43]. For instance, researchers used a mouse model to show the effectiveness of a

recombinant protein-based vaccine against *H. pylori* in a paper published in the Vaccine journal. When *H. pylori* was challenged, the vaccination produced strong humoral and cellular immune responses that resulted in marked decreases in bacterial colonization and gastritis. Similar to this,

a study evaluated the protective effectiveness of a DNA vaccine encoding *H. pylori* antigens using Mongolian gerbils, showing a decrease in stomach inflammation and bacterial colonization after immunization.All things considered, animal models are a great resource for research on *H. pylori*, offering vital insights into the pathophysiology of the disease and aiding in the development and refinement of vaccinations meant to prevent *H. pylori* infection and related stomach disorders.

Sl. No.	Animals	Advantages	Disadvantages
1.	Mouse	• Well documented immune response. • Knockout mice lack specific components of the immune system. • Easily available. • Cost effective and requires less maintenance.	• Infection often leads to lymphocytic gastritis and not peptic ulcers or gastric cancer lead by H. pylori. • Differences in the architecture between human and mouse stomach thereby affecting gastric pathology. • Murine stomach isn't sterile and contamination is a big issue when it comes to testing colonization of H. pylori.
2.	Mongolian gerbil	• Convenient size and ease in terms of husbandry. • Long term infection has proven to cause gastritis similar to that of human.	• They are out-breeding animals. • Lack of defined knowledge of the immune system. • Proper genetic tools are lacking for genetic mutations.
3.	Guinea pig	• Nutritional requirement for vitamin C just like humans gives an advantage as low vit C level has been found to be directly associated with H. pylori infection.	• Limited only for testing H. pylori immunization and vaccination and also studies like vitamin supplementation or mutation of adhesions. • Long-term colonization leads to only gastritis and not peptic ulcers or adenocarcinoma.

4.	Gnotobiotic pig	• Monogastric mammal. • Dietary habits similar to human. • Similarity in anatomical and physiological characters of both the stomach.	• Only gastric ulcers and MALT lymphoma has been found upon infection. • Lack of availability as they're currently not in use.
5.	Non human primates	• Used for testing efficacy of therapeutic intervention by antimicrobials. • Study of efficacy of vaccine and antiadhesion compounds. • Permitted study of the role of mucins and Lewis antigens in adhesion of H. pylori.	• Expensive and requires a lot of maintenance. • Though cancer has been reported but not gastric cancer.

Table 2: Advantages and disadvantages of existing animal models

ii. Clinical potential of OMVs as vaccine:

An OMV is an outer membrane vesicle released from the outer membrane of gram-negative bacteria as a survival response. The size of these vesicles typically ranges from 20 to 400 nm and usually contains electron-dense material encased within a single lipid bilayer. Depending upon the size of these vesicles, internalization of OMVs into the epithelial cells takes place. The internalization process involves micropinocytosis, clarithin, or caveolin-mediated endocytosis [44, 45]. It has been observed that the degree of antigenicity is higher for larger OMVs as they contain more outer membrane proteins, channel-forming proteins, lipoproteins, and lipopolysaccharides. Interestingly these features allowed considering OMVs as a vaccine candidate with tremendous potential.

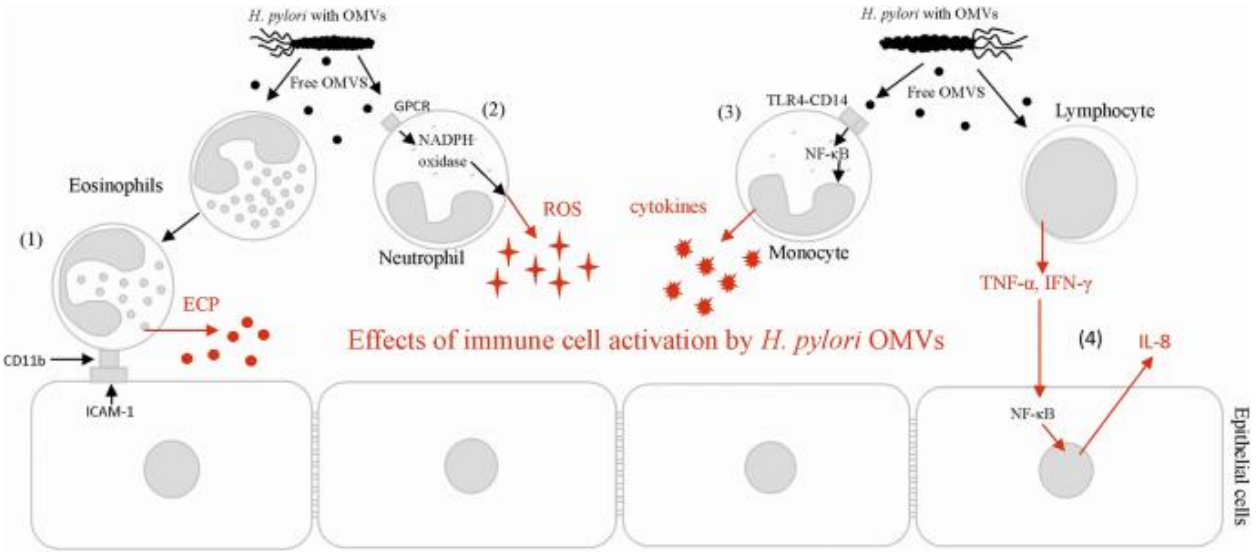


Figure 15: Immune cells activation by *H. pylori* OMVs

Chronic gastrointestinal disorders are associated with *Helicobacter pylori*, a gram-negative bacterium that is common in human stomachs worldwide. It is vital to identify an appropriate adjuvant to increase antigen efficiency because to the rising medication resistance of *H. pylori* and the limited protective performance of certain anti-pylori vaccinations. Earlier researchers found that *H. pylori's* multi-component secretion, known as outer membrane vesicles (OMVs), was harmless and could elicit strong, long-lasting immune responses in mice against the gram-negative bacterium. The adjuvanticity of OMVs in mice was evaluated in this work using two widely used vaccines: whole cell vaccine (WCV) and outer membrane proteins (OMPs). Cholera toxin (CT), a common adjuvant, was employed as a control.

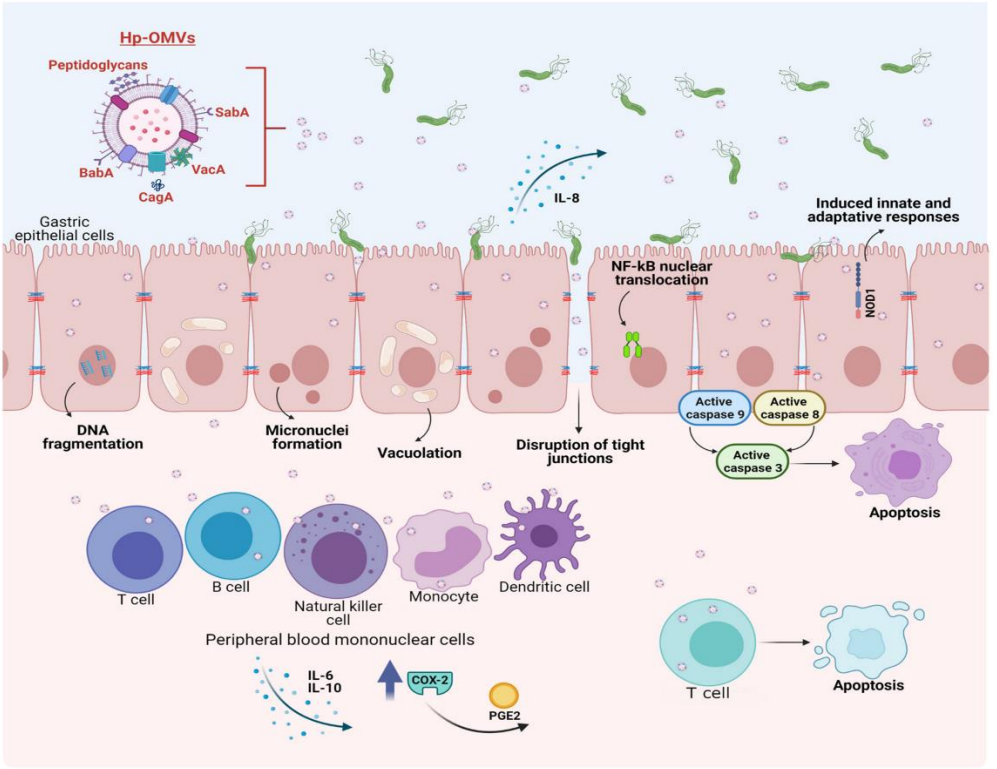


Figure 16: Activation of different immune cells due to OMVs

In general, OMVs show the following characters- • They are acellular. Therefore, they cannot replicate within the host. • They do not alter the antigen's structural conformations, which helps in antibody production more effectively. • They remain stable after long-term storage at 50 C, thereby increasing their shelf life [46]. Immune response has been found to be directly associated with the amount of OMVs used as an adjuvant. In case of H. pylori, administration of a low dose of OMVs from cag PAI+ toxicogenic and cag PAI- nontoxigenic strains have shown increased proliferation in AGS cells [47]. Growth arrests, increased toxicity, and production of IL-8 are some key effects observed at higher doses. A consistent administration of OMVs has been found effective in developing low-grade gastritis, indicating the importance of determining a safe and controlled dose. *H. pylori* release OMVs both in vitro and in vivo, containing proteins, lipopolysaccharide (LPS), and lipoproteins which ultimately lead to the generation of an immune

response by stimulating Toll-like receptors [48]. While the efficacy or route of administration of OMVs requires further research but they certainly provide an ideal platform to generate desired immune response in animals.

iii. Clinical potential of Bacterial Ghost

The Bacterial Ghost (BG) platform technology is an innovative system for vaccine, drug or active substance delivery and for technical applications in white biotechnology. BGs are cell envelopes derived from Gram-negative bacteria. BGs are devoid of all cytoplasmic content but have a preserved cellular morphology including all cell surface structures. Using BGs as delivery vehicles for subunit or DNA-vaccines the particle structure and surface properties of BGs are targeting the carrier itself to primary antigen-presenting cells. Furthermore, BGs exhibit intrinsic adjuvant properties and trigger an enhanced humoral and cellular immune response to the target antigen. Multiple antigens of the native BG envelope and recombinant protein or DNA antigens can be combined in a single type of BG. Antigens can be presented on the inner or outer membrane of the BG as well as in the periplasm that is sealed during BG formation. Drugs or supplements can also be loaded to the internal lumen or periplasmic space of the carrier. BGs are produced by batch fermentation with subsequent product recovery and purification via tangential flow filtration. For safety reasons all residual bacterial DNA is inactivated during the BG production process by the use of staphylococcal nuclease A and/or the treatment with β-propiolactone. After purification BGs can be stored long-term at ambient room temperature as lyophilized product. The production cycle from the inoculation of the pre-culture to the purified BG concentrate ready for lyophilization does not take longer than a day and thus meets modern criteria of rapid vaccine production rather than keeping large stocks of vaccines. The broad spectrum of possible applications in combination with the comparably low production costs

make the BG platform technology a safe and sophisticated product for the targeted delivery of vaccines and active agents as well as carrier of immobilized enzymes for applications in white biotechnology [49, 50].

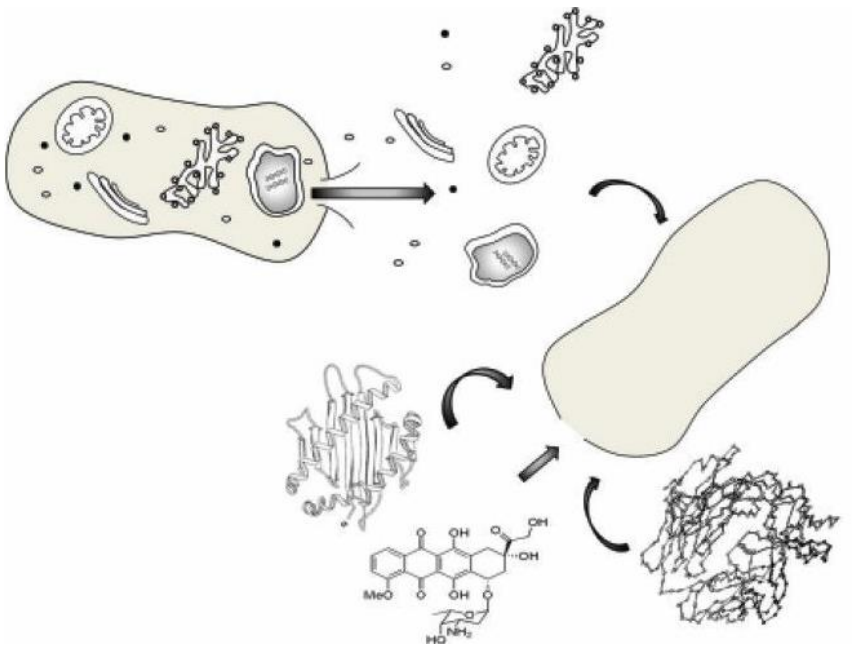


Figure 17: Bacterial Ghost, novel vaccine platform

The empty bacterial envelopes known as "bacterial ghosts" (BGs) are created when cloned gene E is expressed under control, creating a lysis tunnel structure inside the active bacteria's envelope. BGs do not carry any infectious potential and are free of cytoplasm. They nevertheless have all of the original bacterial bio-adhesive surface characteristics. As an advanced drug delivery system (ADDS) for hazardous chemicals in tumor therapy, BGs are perfectly appropriate. The ability to load individual components or mixtures of peptides, medications, or DNA into the interior of BGs presents a chance to create novel (polyvalent) drug delivery systems. The leukemia cells showed the same result. Additionally, melanoma cells demonstrated a strong capacity for BG internalization. These findings suggest that BGs have the ability to target many cancer types. Moreover, BGs have been studied as DNA delivery vehicles.

According to studies, professional APCs and tumor cells may effectively phagocytose and internalize DNA-loaded BGs, resulting in up to 82% of the cells expressing the reporter gene encoded in the plasmid [51].

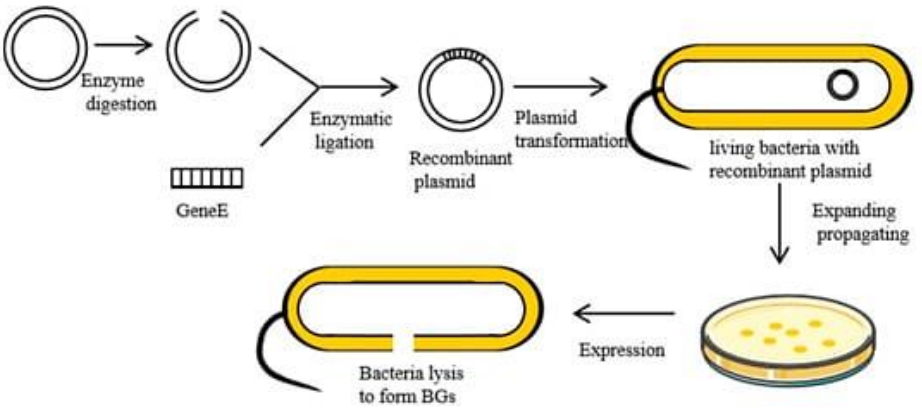


Figure 18: Bacterial Ghost production using genetic manipulation

BGs have an inherent immunogenicity due to intact reservation of bacterial cell membranes, which allows for targeted drug delivery and controlled release. As carrier vehicles, BGs shield drugs from interference from outside sources. In recent years, BG-based delivery systems have gained traction against tumors, inflammation, and infection, among other conditions. They are strong activators of a wide variety of cell types involved in both innate and adaptive immunity and comprise a large number of innate immunostimulatory agonists. Numerous noteworthy investigations have exhibited the efficacy of BG as adjuvants and their capacity to stimulate the production of proinflammatory cytokines by a variety of immunological and non-immune cell types. In order to increase the likelihood that T and B lymphocytes will come into contact with their specific antigen and subsequently evoke strong immune responses, these proinflammatory cytokines cause a widespread recruitment of these cells to lymph nodes. Because of BG's flexibility, immunologically active envelope-bound foreign antigens have been produced, and these vaccines have shown promise in animal models. BGs have been used as vaccine adjuvants

and delivery systems worldwide, and there is growing interest in the development of novel delivery systems based on BGs for biomedical applications [52].

Chapter 2

Objectives of the study

Grounds:

Vaccine research against *Helicobacter pylori (H. pylori*) represents a promising frontier in combating the bacterium responsible for a range of gastrointestinal disorders, including peptic ulcers and gastric cancer. *H. pylori* is a prevalent pathogen with the ability to chronically infect the stomach lining, leading to persistent inflammation and significant health complications. Current treatment regimens, involving antibiotics and acid-reducing agents, often suffer from limitations such as antibiotic resistance and patient compliance issues. A vaccination could provide a more effective and long-lasting therapy by eliciting a strong immune response that avoids initial infection or allows for faster elimination of the pathogen. Researchers are working to develop antigens that can elicit a powerful and protective immune response, as well as to improve vaccine delivery systems. The successful development of an *H. pylori* vaccine could significantly lower the incidence of related disorders, enhance quality of life, and alleviate the global healthcare burden posed by this ubiquitous bacterium. The objectives of present study are as follows-

1. **To select and characterize prevalent *H. pylori* strains isolated from different parts of India on the basis of different virulence factors.**

2. **To formulate immunogens from selected wild type virulent *Helicobacter pylori* strains**

3. **To study reactogenicity, immunogenicity and protective efficacy of formulated immunogens**

4. **To study the duration of protective efficacy of formulated immunogen after subsequent challenge with currently circulating wild type *Helicobacter pylori***

Chapter 3

Objective 1

To select and characterize prevalent H. pylori strains isolated from different parts of India on the basis of different virulence factors.

Background

Gastroduodenal disorders are the cumulative effect of carefully orchestrated molecular interactions between host and pathogen factors belonging to the genus *Helicobacter* [53]. With almost 50% of the population worldwide infected by the pathogen, it is one of the major health burdens in developing nations [54]. Although *H. pylori* has been recognized as a class I carcinogen by the WHO, very little has been explored thus far. This is primarily due to the asymptomatic nature of infected individuals, expensive clinical detection (e.g., endoscopy, urea breath test, etc.) and diagnosis with considerable information scarcity [55]. In addition to this, the global antimicrobial resistance (AMR) pattern of *H. pylori* is changing alarmingly, resulting in a paradigm shift in "treatment of choice" by clinical practitioner [56]. Research in in vitro and in vivo systems of *H. pylori* is continuously enriching our understanding of pathophysiology and genetic predisposition related to adaptation, survival and co-evolution of the pathogen [57, 58]. For instance, *H. pylori* has the inherent ability to modulate the gastric microenvironment, such as increasing gastric pH by means of urease upregulation, employing different adhesion proteins or simply dislodging itself when the pH becomes overwhelmingly acidic [59,60]. Such responses, along with others, act as the precursor to a chronic infection that largely depends on the gastric acid neutralizing capacity unique to each strain. The pathogen is known to recruit different adhesins depending upon the stages of disease progression, such as BabA during early infection or SabA during ongoing inflammation [61]. In addition, host antigens present on the surface of host cells, mucins and other gastric cells, such as A/B-Leb , MUC5AC, MUC1 and H type 1, play important roles in bacterial adhesion, further promoting the severity of different gastric maladies [62, 63]. To date, a combination of antibiotics with a proton pump inhibitor (PPI) is the only mode of treatment available due to the lack of a potent vaccine [64]. Moreover, an efficient

animal model is crucial to understanding the immunological attributes of different immunogen(s) for vaccine development, which existing models fail to satisfy. To date, considerable efforts have been made to establish a reliable murine (gerbil or mouse) model to serve this purpose, including extensive application of transgenic animals with single or double mutations, but unfortunately, no significant efforts have been made toward the route of administration to induce an infection [65]. The preexisting method relies on the oral administration of multiple doses of inoculums along with antibiotic pretreatment to induce an infection [66]. Moreover, it takes a minimum of two weeks to develop an infection using the traditional approach, which is significantly higher than any other enteric pathogens, such as E. coli or Salmonella, while using an animal model [67–69].

Therefore, in this study, we introduced an infection by surgically exposing the stomach of C57BL/6 mice and directly injecting *H. pylori* inoculums. We assessed different pathological and immunological markers for active infection and applied the same to study the vaccine efficacy of OMVs and CurBGs-based immunogens isolated from a prevalent strain.

1.Methods

1.a.i. Bacterial strains and culture conditions

Bacterial strains were revived from glycerol stock using brain heart infusion agar (BD Difco, USA) supplemented with 7% horse blood, 0.4% IsoVitaleX with antibiotics such as amphotericin B, trimethoprim, and vancomycin (Sigma Aldrich, USA) at concentrations as described previously [70]. Inoculated plates were then kept under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2 at 37°C) for 48 hrs and sub-cultured before conducting any experiment. Broth culture was prepared using Brucella Broth (BD, Difco, USA) supplemented

with 10% horse serum and vancomycin (Sigma, USA). The inoculated flask was then kept in shaking conditions (100 rpm) overnight while maintaining the microaerophilic environment [71].

Sl no.	Strains	Demography	Disease status
1	BHU 8A	Bhubaneswar	Duodenal ulcer (DU)
2	KO 8A	Kochi	Duodenal ulcer(DU)
3	AS 2	Assam	Duodenal ulcer (DU)
4	OT-10 (A)	Kolkata	Gastric cancer (GC)
5	B34	Bangalore	Non ulcer Dyspepsia (NUD)
6	D383	Delhi	Non ulcer Dyspepsia (NUD)
7	SD13	Kolkata	Non ulcer Dyspepsia (NUD)
8	B6	Bangalore	Duodenal Ulcer (DU)
9	M28	Mizorum	Gastric cancer (GC)
10	L7	Lucknow	Duodenal ulcer (DU)
11	A61C(1)	Kolkata	Gastric cancer (GC)

Table 3: Demography of Pan-Indian strains as per diagnosis

1.a.ii. Characterization and selection of strains

All strains were checked for oxidase, catalase and urease as mentioned elsewhere [72]. Next, an antibiogram was performed using the agar dilution method following CLSI guidelines. PCR-based detection was applied for genotypic characterization. Some major virulence factors, such as *cagA*, *vacA*, *babA* and *dupA*, were checked using either simplex or multiplex PCR [73, 74].

Sl. no	Strains		Urease (RUT)	Catalase (H_2O_2)	Oxidase (Cytochrome C)	Antibiotics					
						Clarithromycin		Amoxicillin		Metronidazole	
						S	R	S	R	S	R
1.	Ref. strains	26695	+	+	+	+	-	+	-	-	+
		AM1	+	+	+	+	-	+	-	-	+
		SS1	+	+	+	+	-	+	-	-	+
2.	BHU 8A		+	+	+	-	+	+	-	-	+
3.	KO 8A		+	+	+	+	-	+	-	-	+
4	AS 2		+	+	+	+	-	+	-	-	+
5	OT-10 (A)		+	+	+	+	-	+	-	-	+
6	B34		+	+	+	+	-	+	-	-	+
7	SD13		+	+	+	+	-	+	-	-	+
8	D383		+	+	+	+	-	+	-	-	+
9	B6		+	+	+	+	-	+	-	-	+
10	M28		+	+	+	+	-	+	-	-	+
11	L7		+	+	+	+	-	+	-	-	+
12	A61C(1)		+	+	+	+	-	+	-	-	+

Table 4: Phenotypic characterization of strains

1.a.iii. PCR confirmation, Genome Library preparation, Genome sequencing and assembly of immunogen strain

DNA isolated from pure culture was initially subjected to PCR confirmation for *ure*B, *cag*A and *vac*A genes. Simplex PCR was employed for *ure*B while a multiplex PCR was used to determine the *cag*A and *vac*A status of the immunogen strain. A *cag*+ and a *cag*- strains were also used as controls. The primers used for the PCR amplification are enlisted in Supplementary table 1.

For library preparation, gDNA was extracted using Quiagen mini DNA isolation kit following the manufacturer's protocol and quantified using NanoDrop (ThermoFisher). Next, raw fastq reads were acquired using Illumina based platform and processed using Fastp v.0.23.4. The processed reads were de novo assembled using Unicycler v.0.4.4 with default parameters. Reference based scaffolding of the draft assemblies was done using RagTag v2.1.0 based on the corresponding reference genomes. The 16s rRNA gene fragment of the draft assemblies were extracted using the ContEST16S tool of EzBioCloud and taxonomy identification based on 16s rRNA was done using EzBioCloud. Genome-based classification and identification of prokaryotic strains and generation of phylogenetic tree was performed using Type Strain Genome Server (TYGS) (https://tygs.dsmz.de/) and plotted using Interactive Tree Of Life (iTOL). Circular genome plot was plotted using GenoVi v.0.4.3.

2. Results

2.a.i. Characterization and selection of *H. pylori* strains used in the study

A total of 11 strains including 3 reference strains and 8 clinical strains were checked for the presence or absence of major virulence genes i.e. cytotoxin-associated gene or *cagA* representing cag pathogenicity island (*cag*PAI), vacuolating toxin A or *vacA,* blood group antigen binding adhesin 2 or *babA2* and duodenal ulcer promoting gene or *dupA.* A type I or type II strain is defined by the presence of *cag* and allelic variations of *vacA* with signal region (s1 or s2) and middle region (m1 or m2). A *cag+s1m1* is considered to be more virulent, thereby influencing diseases development than *cag+s2m2* or any combination of s1, s2, m1 and m2.

Abbreviations	Strains
M	Marker
L1	SS1
L3	J99
L4	AM1
L5	BHU8A
L6	KO8A
L7	AS2
L8	OT10A
L9	B34
L10	B6
L11	M28
L12	L7
L13	A61C(1)
L14	D383
L15	Blank

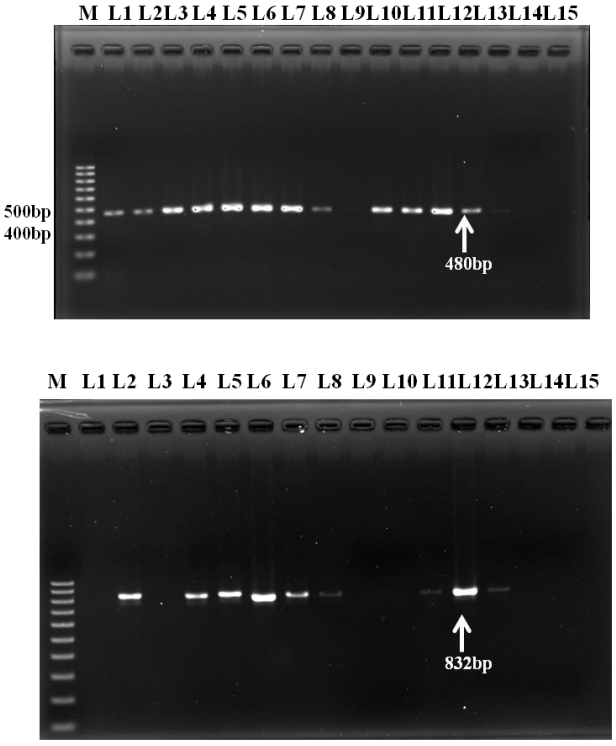


Figure 19: Simplex PCR for (a)***ureB*** subunit of Urease enzyme, a typical confirmatory PCR based method for *H. pylori* strains,(b)***babA2***, an adhesion

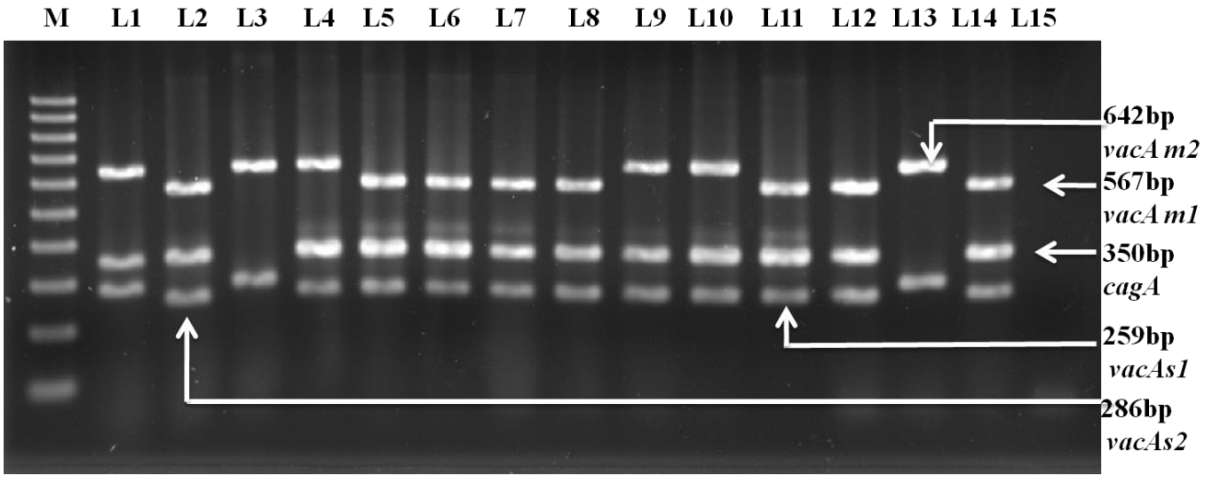


Figure 20.: Genotypyic characterization using Multiplex PCR method (Chattopadhyay et al,2004), ***L1****-s1m1cag+26695,* ***L2****-s2m2cag-D383,* ***L3****-s1m2cag+B6,* ***L4****-s1m2cag+M28,* ***L5****-s1m1cag+KO8A,* ***L6****-s1m1cag+AS2,* ***L7****-s1m1cag+OT10A,* ***L8****-s1m1cag+B34,* ***L9****-s1m2cag+BHU8A,* ***L10****-s1m1cag+L7,* ***L11****-s1m1cag+A61C(1)*

Additionally, allelic variations of babA i.e babA2, plays a key role in adhesion to the Lewis B (Leb) antigen of blood as babA1 is known to be non-functional [73]. dupA belongs to a plasticity region (jhp0917- jhp0918) and found to be responsible in developing ulcers in *H. pylori* infected individuals.

Immunogen strains	**Purpose**
A61C (1)	OMVs preparation
SD13	CurBGs preparation
SS1	Challenge study

Table 5: Strains used in this study

Therefore, any strain positive for all these genes can be considered to be more virulent than others. A61C (1) is positive for all these virulence genes and therefore is selected for immunogen preparation. However, for model establishment and challenge study purpose, SS1 is considered to be more suitable than others as it is a mouse adapted strain. The result of genetic characterizations of all strains is listed in a table.

Genetic Features			**Virulence Marker**					**Adhesion**	**Duodenal Ulcer Promoting Gene**		**Plasticity Region (Presumed to promote apoptosis)**	
Strains			*cagA*	*vacA*				*babA2*	*dupA*		*jhp 0947*	*jhp 0950*
				s1	*s2*	*m1*	*m2*		0917	0918		
1	Ref strains	26695	+	+	-	+	-	-	-	-	-	-
		AM1	-	-	+	-	+	-	-	-	-	-
		SS1	+	-	+	-	+	-	-	-	-	-
2	BHU 8A		+	+	-	-	+	+	-	-	+	+

3	KO 8A	+	+	-	+	-	+	-	-	+	+
4	AS 2	+	+	-	+	-	-	-	-	-	+
5	OT-10 (A)	+	+	-	+	-	-	-	-	-	-
6	B34	+	+	-	+	-	+	-	-	-	-
7	SD13	**+**	**+**	**-**	**+**	**-**	**+**	**+**	**+**	**+**	**-**
8	D383	-	-	+	-	+	-	-	-	+	+
9	B6	+	+	-	-	+	+	-	-	+	+
10	M28	+	+	-	-	+	-	-	-	-	+
11	L7	+	+	-	+	-	+	-	-	-	-
12	**A61C(1)**	**+**	**+**	**-**	**+**	**-**	**+**	**+**	**+**	**-**	**-**

Table 6: Genotypic makeup of different virulence genes

2.a.ii. Genomic features of immunogen strain

PCR results of immunogen strain revealed positive for the virulence associated genes. Genomic characteristics of *H. pylori* strain SD13 comprised a genome size of 1.66Mbp with 13 contigs of DNA sequence. The strain encodes 1,572 coding genes, 36 tRNA and 3 rRNA and 38.77 GC% content with a single chromosome. The reference genome sequence of *H. pylori* strain, ATCC 43504 was used to compare the sequence with 99.51% similarity based on 16s rRNA taxonomic identification.

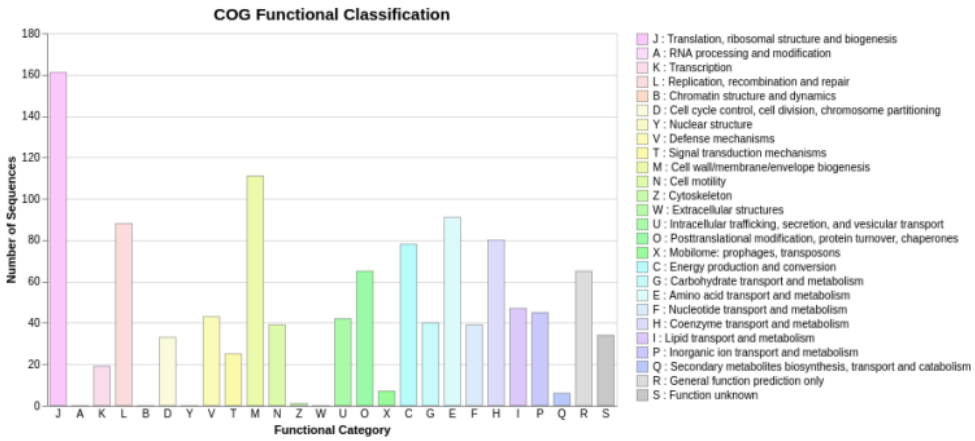


Figure 21: Cluster of gene orthologs,

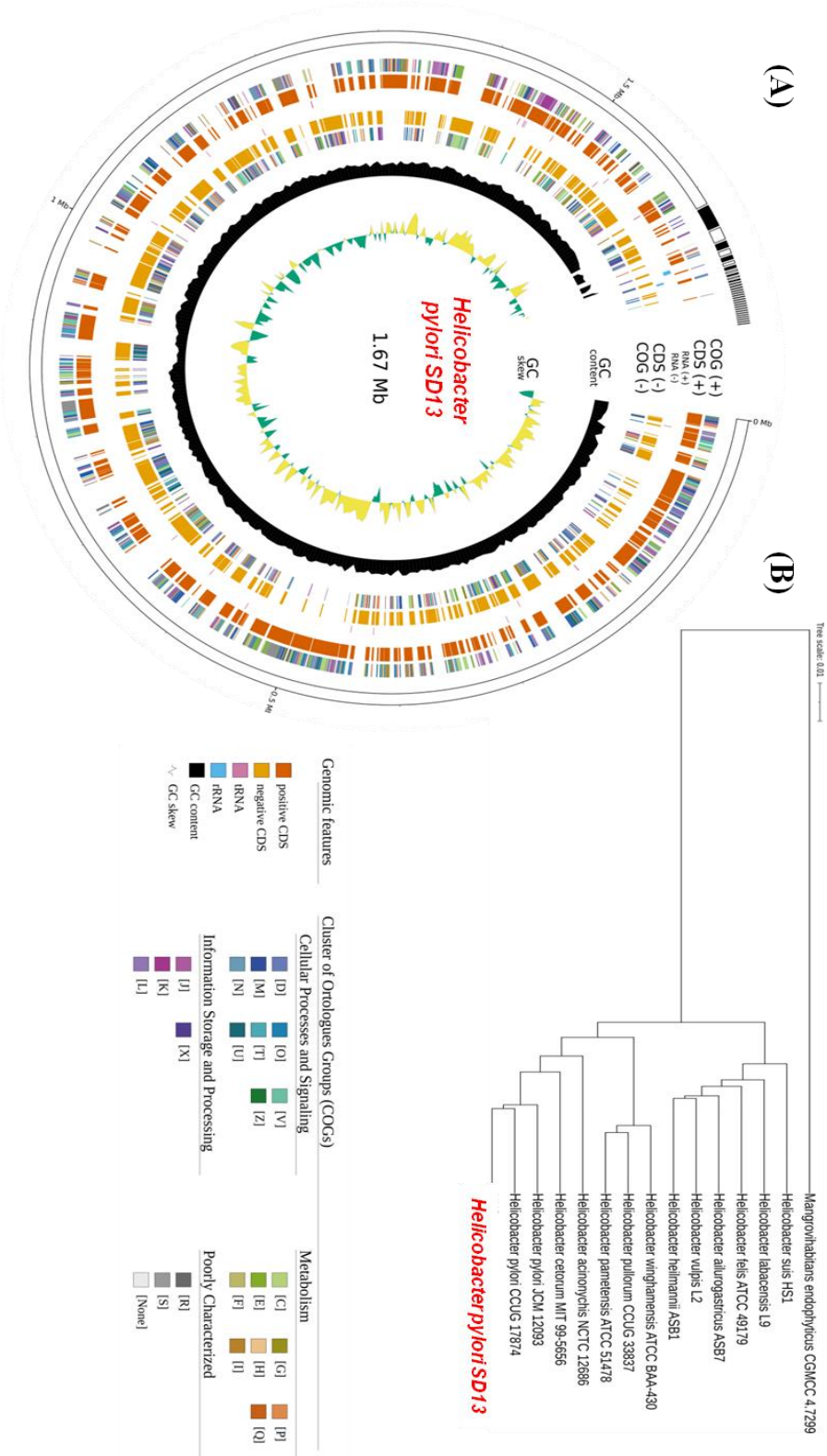


Figure 22: A, Genetic features of immunogen strain. **B.** Phylogenetic tree on the basis of 16s rRNA

Figure 23: Classification based on genotype

3. Discussion

H. pylori, is a gram negative bacterium that is closely related to human health, especially when it comes to stomach disorders. Researchers have found the diversity of *H. pylori* strains to be an interesting field since the traits unique to each strain has a major impact on disease outcomes and subsequently treatment approaches. Understanding the pathogenic processes, antibiotic

resistance patterns, and host interactions of *H. pylori* requires careful strain characterization and effective selection. In order to further our understanding of *H. pylori* biology and enhance clinical management techniques, this study involves the methods, difficulties, and consequences of strain selection and characterization. The degree of illnesses including gastritis, peptic ulcer disease, and stomach cancer that are linked to *H. pylori* is determined by virulence factors. Determining the presence of these characteristics and their role in pathogenesis facilitates the prediction of illness outcomes and the development of effective treatment plans. The ability of *H. pylori* to interact and modify host cells, tissues, and immunological responses is facilitated by virulence factors. Investigating these elements sheds light on the methods the bacteria uses to develop a persistent infection and elude the host's defenses. Antibiotics and other treatment approaches are not as effective when certain virulence factors are present. Having an understanding of these variables can help choose the right treatment plans based on the patient's unique *H. pylori* strain. Geographical locations and distinct *H. pylori* strains can have varying virulence factors. Understanding the epidemiology of *H. pylori* infections and detecting newly emerging strains with higher pathogenic potential are made possible by the monitoring and characterization of these parameters.

Our study involves strain A61C(1) for OMVs production and SD13 for nanocurcumin induced bacterial ghost (CurBGs) formulation. Both of these strains were isolated from patients seeking medical attention for acute/chronic gastroduodenal problems. The phenotypic features, PCR results as well as whole genome sequencing report revealed the unique feature that enabled us to select these strains for immunogen preparation while SS1 is used for challenge as the strain is a reference strain particularly adapted to colonize murine stomach.

4. Conclusion

To sum up, the meticulous identification and selection *H. pylori* strains are critical to the advancement of vaccine research against this enduring infection. One can more effectively target the processes by which *H. pylori* evades the immune system and produces chronic infection by selecting and concentrating on strains with important virulence factors and immunogenic profiles. This methodology not only advances our comprehension of pathogenicity differences particular to individual strains but also contributes to the development of vaccines that effectively elicit strong protective immune responses. Furthermore, precise strain selection guarantees that prospective vaccine candidates are optimized for potential efficacy and application in a variety of populations by addressing the heterogeneous worldwide landscape of *H. pylori* strains. The critical role that accurate strain selection plays in opening the door to successful disease prevention and better public health outcomes globally cannot be emphasized as attempts to develop workable *H. pylori* vaccination continues to be a matter of deep scientific endeavors.

Chapter 4

Objective 2

To formulate immunogens from selected wild type virulent Helicobacter pylori strains

Background:

A vaccine is one of the most outstanding achievements of modern science and medicine. In the past, it has helped to eliminate many fatal, infectious diseases from our society. Various types of vaccines are available that use live attenuated or inactivated pathogens (bacteria and viruses) or conjugated polysaccharides to develop immunity. To be precise, these foreign objects or antigens stimulate the immune system to produce antibodies. Antibodies are produced by the plasma cells (primarily from B lymphocytes) and directly encounter the antigens or their toxins by their epitopes. A cell-mediated immune response is mediated by T-lymphocytes that differentiate into cytotoxic T lymphocytes or CTLs. At the same time, helper T lymphocytes recognize and produce cytokines, ultimately initiating a cascade of death-associated pathways that kill the pathogen and induce antigen-specific memory T cells. Activation and sustenance of these helper T cells control the degree of response toward an infection. In case of *H. pylori*, another type of T lymphocyte, were found called Treg or T-regulator cells that mediate immune tolerance by regulating the actions of effector-T cells [75]

In 2004–2005 Wuhu Kangwei Biological Technology Co., Ltd conducted a vaccination program that involved oral administration of a recombinant *H. pylori* vaccine. The preliminary report published in 2015 revealed, of 99 % participants who completed a 3year trial, reported 64 events of *H. pylori* re-infection within first year,7 % of both vaccine group and placebo group participants with adverse reaction and <1 % reported serious adverse effects when exposed to circulating strains of *H. pylori*. In spite of achieving a vaccine efficacy of 71.8 % with a significant reduction in *H. pylori* infection and good immunogenic response among naive children, the study was terminated after phase III clinical trial [76]. This was the first reported vaccination program of its kind but certainly left a huge grey area to ponder on, like 10 years

delay in publishing the preliminary report or why the complete report has never been disclosed in public.

IMX101, developed by ImeVax, essentially targets a key survival process of *H. pylori* in the human stomach that isto reduce the effect of low gastric pH. One of the most potent *H. pylori* antigens, γ-glutamyltranspeptidase (GGT) is responsible for inducing immunosuppressive activity by neutralizing highly acidic environment of the stomach disabling the inflammatory response in the host. While the result of phase I clinical trial of the vaccine is yet to be published, the approach certainly is novel and thus intriguing to see whether targeting a single antigenic component is efficient enough or not in order to generate immunity given the fact that the bacteria employ a number of other defensive mechanisms related to adhesion, colonization and destruction of the mucosal lining of the stomach [77].

Recently many small companies and academic institutions started showing interest and took initiatives towards *H. pylori* vaccine development. All these approaches are at the preclinical stages and involve mainly purified or recombinant antigen components accompanied by an adjuvant. Though the preliminary results of these studies are promising but they lack consistency and clarity. For example, (i) an epitope-based vaccine EpiVax, involves a DNA vaccine initially followed by a peptide-liposome. The vaccine showed some therapeutic protection but is restricted only in mice,(ii) Dual approaches taken by Helicovaxor® -first one involving genetically engineered non-virulent *Vibrio cholerae* strain expressing *H. pylori* antigens (HpaA, UreB, and FlaA) while other one involves inactivated *H. pylori* strain engineered to induce serum antibody response both of which are at research level, (iii)Two recent studies, one on urease subunits and another on Lp220 (lipopolysaccharide220), conducted among BALB/c mice have shown minimal effect in generating protection, (iv) Probiotic as a vehicle for vaccine

delivery involved microbes like *Lactococcus lactis* recombinantly expressing cholera toxin B subunit in addition to *H. pylori* urease epitope, has also not been found effective in developing immunity [76].

Based on extensive literature review selected strains were used to formulate immunogen i.e. outer membrane vesicles and nanocurcumin induced bacterial ghost.

Methodology:

1. Outer Membrane Vesicles (OMVs)

1.1. OMVs preparation

Outer membrane vesicles (OMVs) were isolated from the *Helicobacter* strain [A61C(1), *cagA*+, *vacAs1m1*] following the methods described previously with slight modification [78]. In brief, BB broth (BD, Difco, USA) was inoculated with log phase (OD_{600} ~0.6) pre-culture of the respective strains and kept overnight in microaerophilic conditions under constant shaking (100 rpm) at 37°C. On the next day, centrifugation was performed consecutively first at 8000xg for 15 minst 4°C, followed by 30 min with same conditions. The supernatants were then filtered twice with 0.45 µm and 0.22 µm syringe filters (Millipore, USA). To prevent protein degradation, a protease inhibitor cocktail was incorporated into the filtrate and ultra-centrifuged at 140,000 x g at 4°C for 4 hrs using a P27A-1004 rotor (Hitachi). A density gradient centrifugation allowed obtaining the purified OMVs. Protein content was measured using a Lowry protein estimation kit (Pierce, USA) and stored at -20°C until further use.

1.2. Characterization of the Outer Membrane Vesicles (OMVs)

1.2.1. Dynamic Light Scattering

Concentrated OMVs were diluted 10-fold to reach a concentration of 0.1 mg/mL. The hydrodynamic size of OMVs was measured using a Malvern Zetasizer ZS90 (Malvern Instruments, Germany) and analyzed using ZS Xplorer version 3.1.0.64 [79].

1.2.2. Transmission electron microscopy

Diluted OMVs were placed on a carbon-coated grid and left for 10-20 min for absorption. The samples were then washed twice with drops of Tris buffer solution. Excess fluid was soaked using blotting paper, followed by staining with 2% uranyl acetate and air drying. Finally, the OMV-coated grids were observed under a JEOL JEM 2100 HR (JEOL, Tokyo, Japan) [80].

1.2.3. LC/MS of OMVs and analyses

Proteins present in OMVs were used for digestion and reduced with 5 mM TCEP and further alkylated with 50 mM iodoacetamide and again digested with trypsin (1:50, trypsin/lysate ratio) for 16 h at 37°C. Digests were cleaned using a C18 silica cartridge to remove the salt and dried using a speed vac. The dried pellet was resuspended in buffer A (2% acetonitrile, 0.1% formic acid). Experiments were performed on an Easy-nlc-1000 system coupled with an OrbitrapExploris mass spectrometer. One microgram of peptide sample was loaded on a C18 column (15 cm, 3.0 μm Acclaim PepMap, Thermo Fisher Scientific), separated with a 0–40% gradient of buffer B (80% acetonitrile, 0.1% formic acid at a flow rate of 500nl/min) and injected for MS analysis. LC gradients were run for 60 minutes. MS1 spectra were acquired in the Orbitrap (Max IT = 25 ms, AGQ target=300%; RF lens = 70%; R=60 K, mass range = 375−1500; profile data). Dynamic exclusion was employed for 30s, excluding all charge states for a given precursor. MS2 spectra were collected for the top 12 peptides. MS2 (Max IT= 22 ms,

R= 15 K, AGC target 200%). All samples were processed, and the generated RAW files were analyzed with Proteome Discoverer (v2.5) against the UniProt organism database. For dual Sequest and Amanda searches, the precursor and fragment mass tolerances were set at 10 ppm and 0.02 Da, respectively. The protease used to generate peptides, i.e., Enzyme specificity was set for trypsin/P (cleavage at the C-terminus of "K/R: unless followed by "P"). Carbamidomethyl on cysteine as a fixed modification and oxidation of methionine and N-terminal acetylation were considered variable modifications for the database search.

2. Results:

2.1. Isolation and characterization of OMVs from *H. pylori* strain A61C (1)

The OMVs isolated from the broth culture of A61C (1) were purified and assessed using dynamic light scattering (DLS), transmission electron microscopy (TEM) and proteomics analyses using LC/MS. The data revealed uniformity in OMVs structure with a diameter of 50nm. TEM image showed the OMVs to be circular in shape with distinct bilayers. The protein components present in OMVs isolated from the immunogen strain [A61C (1)] revealed 18 major proteins including UreB, UreA, FtnA, GroEL, UbiX, Tuf, SecA, RplI, LpxK, RimO, AroB along with some other proteins with unknown localization. Presence of proteins like UreA, UreB and GroEL on OMVs indicate the potential to generate a strong immune response as these proteins are known for their immunomodulatory activities. The sub-cellular localization of proteins indicated by the software includes cytoplasm, membrane, periplasmic space, plasma membrane of the bacteria. *H. pylori* LPS is known to have no cytotoxicity which is also evident from cytotoxicity assay.

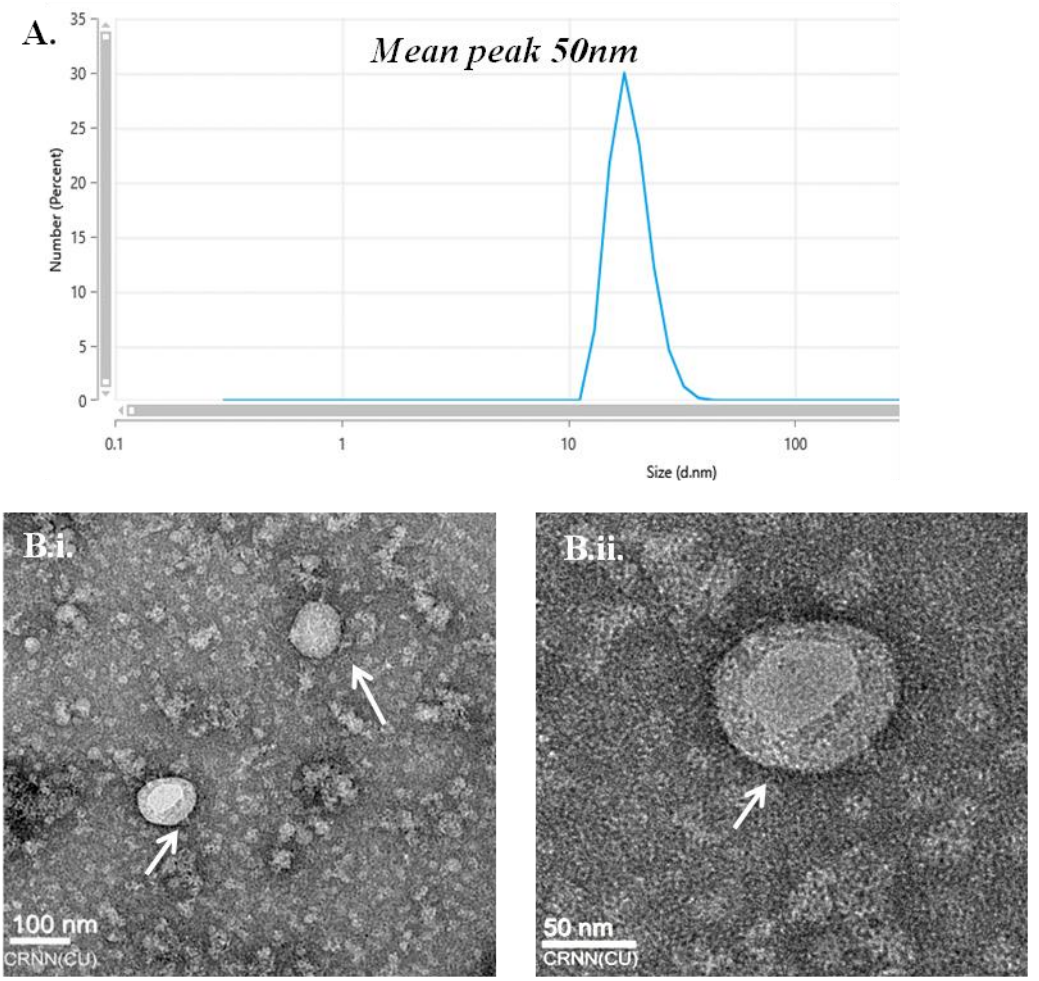
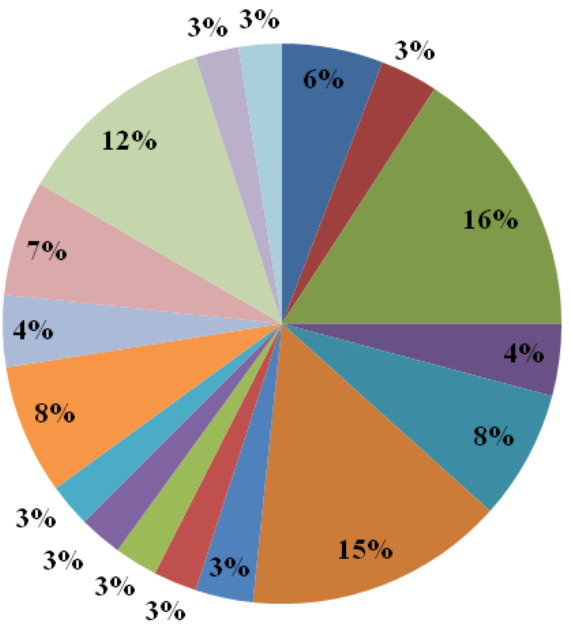


Figure 24: Characterization of *Helicobacter pylori* OMVs isolated from strain A61C(1); (A) Dynamic Light Scattering showing a uniformity in OMVs population with mean peak at 50nm**,** (B) Transmission Electron microscopy images revealing the circular morphology of *Helicobacter pylori* OMVs of A61C(1) strain; (B.i) Image taken at 100nm scale, (B.ii) Image taken at 50nm scale. Both TEM images revealed the thick bilayer structure with hollow center of the OMVs. (C) Percentage of major proteins present on OMVs.

C.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Bacterial non-heme ferritin
Chaperonin GroEL
Alpha-ketoglutarate permease (KgtP)
Flavin prenyltransferase UbiX
Elongation factor Tu
Protein translocase subunit SecA
LUD domain-containing protein
Flagella basal body P-ring formation protein FlgA
VirB4 homolog (VirB4)
Large ribosomal subunit protein bL9
Tetraacyldisaccharide 4'-kinase
Ribosomal protein uS12 methylthiotransferase RimO
ABC transporter, ATP-binding protein (HI1087)
Outer membrane beta-barrel protein
3-dehydroquinate synthase

Figure 25: Characterization of *Helicobacter pylori* OMVs isolated from strain A61C(1); (A) Dynamic Light Scattering showing a uniformity in OMVs population with mean peak at 50nm**,** (B) Transmission Electron microscopy images revealing the circular morphology of *Helicobacter pylori* OMVs of A61C(1) strain; (B.i) Image taken at 100nm scale, (B.ii) Image taken at 50nm scale. Both TEM images revealed the thick bilayer structure with hollow center of the OMVs. (C) Percentage of major proteins present on OMVs.

Accession	**Protein assigned**	**Molecular mass (kDa)**	**No. of peptides**	**Sequence coverage (%)**
P69996	nickel cation binding, urease activity	61.6	3	7
P14916	nickel cation binding, urease activity	26.5	1	4
P52093	ferric iron binding, ferrous iron binding, ferroxidase activity	19.3	2	19
P42383	ATP binding, ATP-dependent protein folding chaperone, isomerase activity, unfolded protein binding	58.2	3	5
O25723	transmembrane transporter activity	47.5	1	9
O26011	carboxy-lyase activity, flavin prenyltransferase activity	20.6	1	18

P56003	GTP binding, GTPase activity, guanosine tetraphosphate binding, translation elongation factor activity	43.6	2	4
O25475	ABC-type protein transporter activity, ATP binding, metal ion binding, protein-exporting ATPase activity	99	1	3
O24949	Hypothetical protein	23.6	1	3
O26012	Hypothetical protein	24.3	1	3
O25206	ATP binding, DNA binding	98.4	1	3
P56035	rRNA binding, structural constituent of ribosome	16.5	1	9
O25095	ATP binding, tetraacyldisaccharide 4'-kinase activity	35.5	1	5
O25434	4 iron, 4 sulfur cluster binding, aspartic acid methylthiotransferase activity, metal ion binding,proteinmethylthiotransferase activity	49.6	1	8
O26001	ATP binding,ATP hydrolysis activity	29.2	1	14

O25992	Hypothetical protein	82.3	1	3
P56081	3-dehydroquinate synthase activity, metal ion binding, nucleotide binding	39.1	1	3

Table 7: Proteomic analysis of OMVs along with their molecular weight

3. Discussion:

An OMV is an outer membrane vesicle released from the outer membrane of gram-negative bacteria as a survival response. The sizes of these vesicles typically range from 20 to 400 nm and usually contain electron-dense material encased within a single lipid bilayer. Depending upon the size of these vesicles, internalization of OMVs into the epithelial cells takes place. The internalization process involves micropinocytosis, clarithin, or caveolin-mediated endocytosis [81, 82]. It has been observed that the degree of antigenicity is higher for larger OMVs as they contain more outer membrane proteins, channel-forming proteins, lipoproteins, and lipopolysaccharides. Interestingly these features allowed considering OMVs as a vaccine candidate with tremendous potential. In general, OMVs show the following characters- • They are acellular. Therefore, they cannot replicate within the host. • They do not alter the antigen's structural conformations, which helps in antibody production more effectively. • They remain stable after long-term storage at 50 C, thereby increasing their shelf life [83]. Immune response has been found to be directly associated with the amount of OMVs used as an adjuvant. In case of H. pylori, administration of a low dose of OMVs from cag PAI+ toxicogenic and cag PAI- nontoxigenic strains have shown increased proliferation in AGS cells (Ismail et al., 2003).

Growth arrests, increased toxicity, and production of IL-8 are some key effects observed at higher doses. A consistent administration of OMVs has been found effective in developing low-grade gastritis, indicating the importance of determining a safe and controlled dose. H. pylori release OMVs both in vitro and in vivo, containing proteins, lipopolysaccharide (LPS), and lipoproteins which ultimately lead to the generation of an immune response by stimulating Toll-like receptors [84]. While the efficacy or route of administration of OMVs requires further research but they certainly provide an ideal platform to generate desired immune response in animals.

4. Conclusion

As a highly efficient immunogen in mice, outer membrane vesicles, or OMVs, have gained popularity. Packed with a wide range of bacterial components that elicit both innate and adaptive immune responses, these nanostructures are naturally produced by Gram-negative bacteria. OMVs stimulate T cell activation and antibody production by causing dendritic cells to become activated. In order to provide long-lasting immunity, they stimulate the development of memory T and B cells. In comparison to other vaccine formulations, OMVs have a better safety profile that reduces the possibility of adverse effects. OMVs are a promising tool for improving immunization techniques since they can be used to generate vaccines against bacterial infections in mice.

2. Curcumin nanoparticle induced Bacterial Ghost (CurBGs)

2.1. Preparation and characterization of nanocurcumin

Nanocurcumin (CurNPs) were synthesized using the previously described process with a few modifications [85]. In summary, 5 mg/ml of a stock solution was made using dichloromethane

and pure curcumin (≥80% curcumin, ≥94% curcumioid content Sigma Aldrich, USA). Next, using ultrasonic conditions (100W, frequency 30 kHz, 10min), the solution was sonicated and subsequently sprayed dropwise (0.2 ml/min) into deionized boiling water. Next, at room temperature, the suspension was agitated until the yellow tint changed to orange. Ultimately, the suspension underwent concentration, dehydration, and storage at 4◦C till its next utilization. The nanoparticles were examined using Zetasizer (Malvern Panalytical) to assess size distribution, mean particle size, polydispersity index, and shape, as well as scanning electron microscopy (Quanta 200 SEM; FEI, Netherlands) and dynamic light scattering (DLS) for further biophysical characterization.

2.2. Bacterial culture and conditions

Brain Heart Infusion agar (BD Difco) supplemented with 7% horse serum, antibiotics such as vancomycin (6µg/ml), trimethoprim (5µg/ml), and polymixin B (10µg/ml) coupled with 0.04% IsoVitaleX was used to revive bacterial strains from glycerol stock. For 24 to 48 hours, inoculated plates were maintained in a microaerophilic environment (10% CO2, 5% O2, and 85% N2). In order to prepare the broth culture, 10% horse serum was added to Brain Heart Infusion broth (BD, Difco), which was then shaken at 120 revolutions per minute for the whole night. At a wavelength of 600 nm, optical density was measured, and serial dilution was used to calculate CFU [86].

2.3. Determination of MIC

The minimum inhibitory concentration (MIC) of nanocurcumin on *H. pylori* strains was determined as described previously [87]. To do this, BHIA plates comprising ascending concentrations of CurNPs were prepared. Bacteria that were growing exponentially were

centrifuged, resuspended and adjusted to 1x 10^8CFU/ml in PBS. Next, 10µl of progressively increasing concentrations of CurNPs were put onto BHIA. Final concentrations comprised 5, 10, 15, 20, 25 and 30µg/ml of CurNPs respectively. Before typical *H. pylori* colonies could be seen, the inoculation plates were maintained under microaerophilic conditions for three to four days.

2.4. Preparation of nanocurcumin induced bacterial ghosts

Using a procedure previously published, bacterial ghosts were generated from *H. pylori* strain SD13 [88]. In short, the cells were cultured in microaerobic conditions for 24 hours using Brain-Heart Infusion broth that contained 10% horse serum and CurNPs at a 2xMIC concentration. The next day, the cells were extracted using centrifugation and carefully resuspended in sterile PBS (pH 7.4). A viability assay was performed to check the presence of any viable bacteria.

2.5. Characterization of bacterial ghosts

Scanning electron microscopy was used to visualize high-quality images of freshly produced bacterial ghosts. After fixing the bacterial pellets with a 2.5% gluteraldehyde solution, the pellets were dehydrated using ethanol gradation in sequence. After drying, coating, and SEM visualization at various magnifications, the samples were ready for analysis. Using the spread plate method, the cells' vitality was evaluated. To find any viable colonies, 100µl of each serially diluted batch of CurBG cells was plated [89].

3. Results

3.1 Synthesis and evaluation of nanocurcumin

Wet-milling technique resolved one of the major drawbacks of curcumin by transforming it into small particles that increased their solubility without affecting their integrity. The crude solution

of curcumin when sprayed on hot water under ultrasonification produced nanoparticles that exhibited effortless dispersion without any surfactants. A change in color of nanocurcumin indicated a more soluble form in water than unprocessed curcumin. Further, synthesis of nanocurcumin was confirmed using scanning electron microscopy. DLS results revealed the average size approximately 125nm with a polydispersity index of 1.

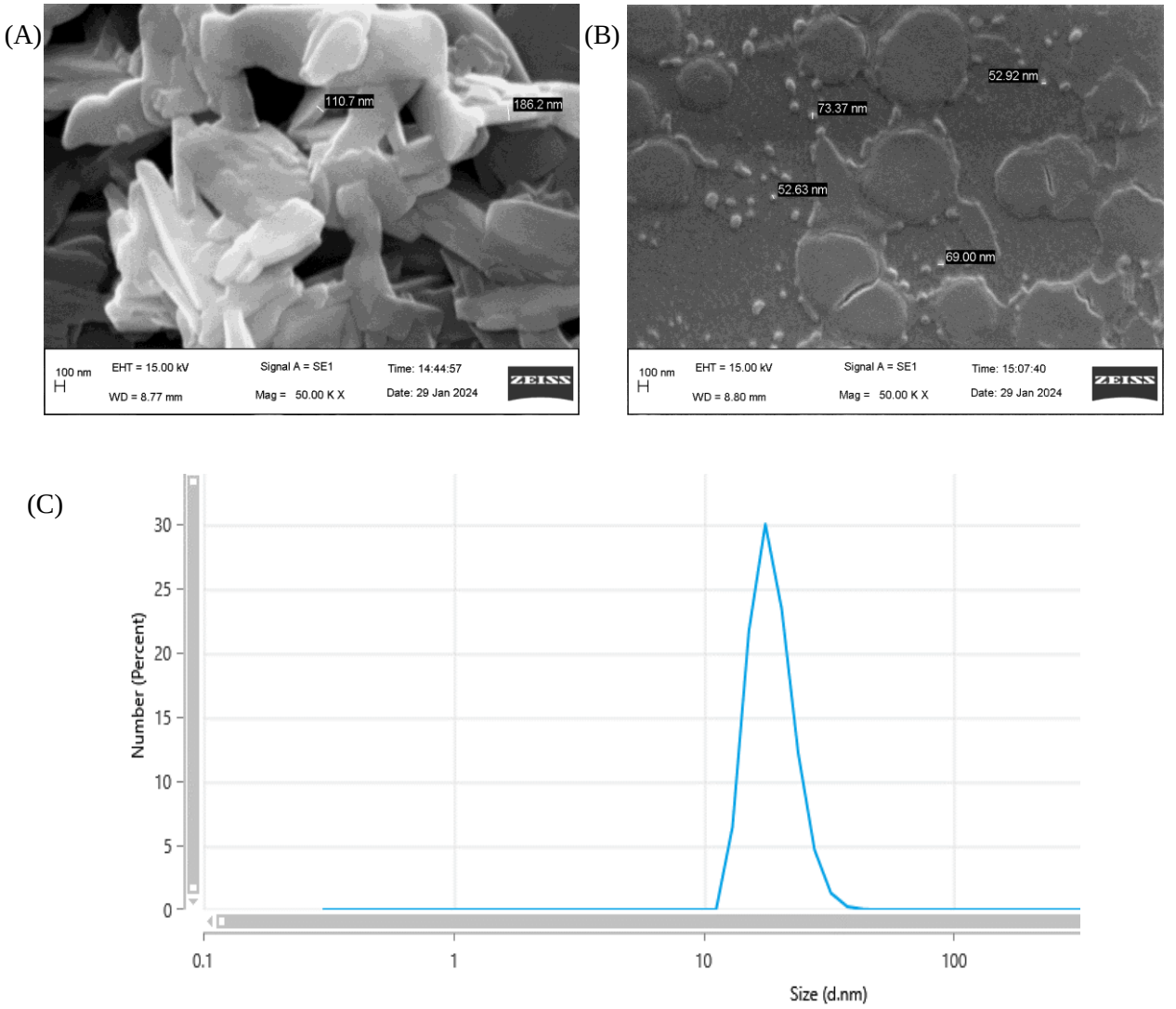
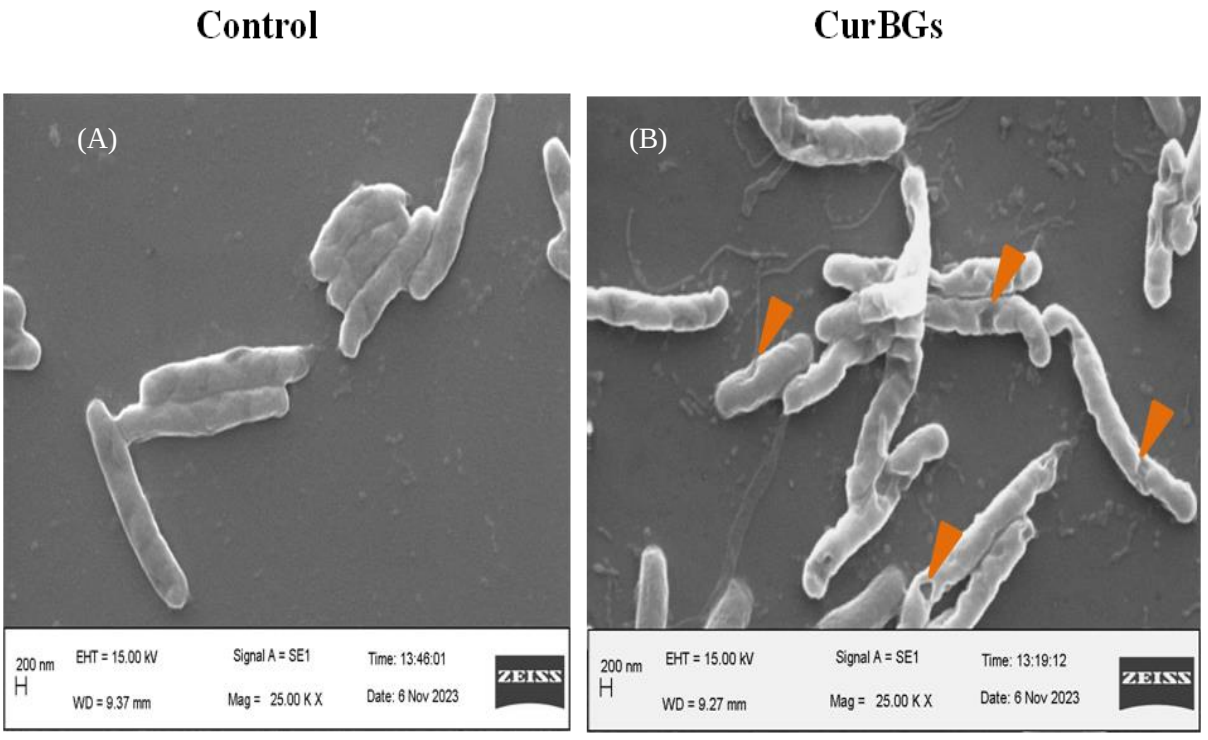


Figure 26: Biophysical characterization of nanocurcumin. SEM image of (A) raw curcumin and (B)nanocurcumin at 100nm scale. (C)Dynamic Light Scattering (DLS) analysis of nanocurcumin indicating a size distribution ranging from 50 nm to 90nm

3.2 Production and characterization of nanocurcumin induced bacterial ghost

Bacterial ghost cells were prepared by exposing overnight grown *H. pylori* with MIC concentration of nanocurcumin (10µg/ml). The culture was kept under shaking condition for 24hrs. This treatment allowed to inhibit the growth of the cells which when plated for viability showed no growth. Moreover, scanning electron microscopic images revealed nanocurcumin treated *H. pylori* containing several trans-membrane tunnel-like structures indicating the passages through which inclusion bodies were removed. Compared to the untreated bacterial cells, such structures confirmed the formation of bacterial ghost cells.



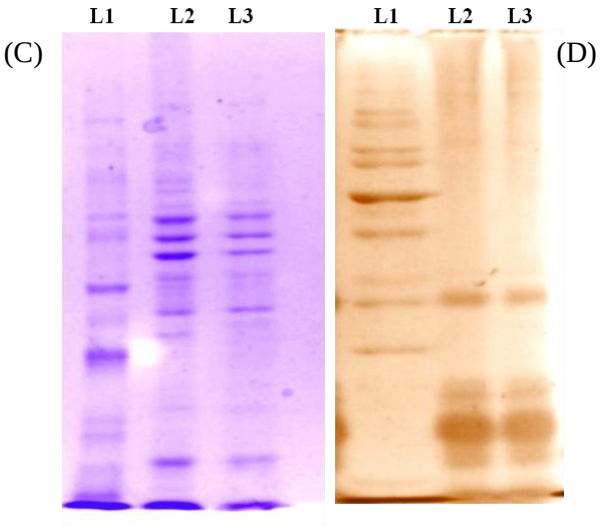


Figure 27: Development of nanocurcumin induced bacterial ghost (CurBGs). SEM images of **(A)** Untreated control, **(B)** Nanocurcumin treated bacterial ghosts. Orange arrowhead indicate formation of transmembrane holes to expel out the inclusion bodies. **(C)** SDS-PAGE of untreated and CurBGs comparing the surface protein profile; **L1: M**arker, **L2:** OMPs of untreated control bacterial cells, **L3:** OMPs of CurBGs ; **(D)** Comparative lipopolysaccharide (LPS) profile, **L1:** Marker, **L2:** LPS of untreated control bacterial cells, **L3:** LPS of nanocurcumin induced bacterial ghosts.

4. Discussion

The rising prevalence of antimicrobial resistance (AMR) in *H. pylori* presents serious threats to develop an effective treatment. Currently, no licensed vaccine is available and formulation of a successful vaccine requires extensive research on antigens capable of eliciting a robust immune response. So far commendable efforts have been made; however, several studies indicated *H. pylori* antigens to be less immunogenic, sometimes requiring additional adjuvant to evoke better immunogenicity. Bacterial ghost (BGs) is an empty cell envelope and an emerging platform in vaccine research. Despite its tremendous potential as a vaccine candidate, research in this area

has been limited. Genetically manipulated bacteria expressing pore forming genes like Lysis E, is one of the most widely used technique besides chemical modification. Using chemical agents for BGs preparation has a number of advantages over genetic manipulation, including feasibility of production time and cost while maintaining surface integrity and efficacy intact. Exposure to chemicals for certain duration allows formation of transmembrane tunnel-like structures on bacteria that promote removal of inclusion bodies, leaving behind the empty envelops. Inorganic chemical agents like sodium hydroxide (NaOH), hydrochloric acid (HCl), sulfuric acid (H_2SO_4), Tween-80 are some of the chemicals used to produce BGs. However, harnessing the potential of a phytochemical based agent to produce BGs is yet to be explored extensively thereby eliminating any possibilities of side effects due to residual chemicals.

The major problem using a phytochemical agent is their solubility which is very much dependent on the nature of solvent along with parameters like temperature and pH. Crude extracts of any plant derived product are usually non-polar in nature. For example, bioreactive component of turmeric, curcumin, is completely water-insoluble in its raw form and appears as "shards of glasses" under scanning electron microscope. Despite that, several techniques are available that breaks down raw curcumin into particles <250nm in size, thereby improving solubility in water and reducing hydrophobicity. Additionally previous studies with curcumin confirmed that formulation of nanocurcumin changes the size without altering their chemical integrity. *In-vivo* studies of *H. pylori* infection and treatment revealed significant improvement in gastric architecture when curcumin is employed therapeutically due to its antimicrobial effect. However, translating such results to treat human infection is challenging because of bioavailability issues subsequently affecting the determination of effective dosing of curcumin in human. When bacteria come in contact with nanocurcumin, the lipid bilayer interacts with the curcumin

particles and changes membrane integrity. Disruption in membrane integrity facilitates pore-mediated leakage which irreversibly damages the bacteria. Scanning electron microscopic images of nanocurcumin induced BGs confirming formation of pores.

5. Conclusion

The difficulty in creating vaccines against *H. pylori* stems from the bacteria's capacity to withstand host immune responses and endure in the stomach mucosa. On the other hand, novel developments employing curcumin-induced bacterial ghosts and outer membrane vesicles (OMVs) have demonstrated encouraging potential as vaccine candidates. OMVs are produced spontaneously by Gram-negative bacteria and include immunogenic substances such lipopolysaccharides and outer membrane proteins that can elicit both innate and adaptive immune responses. Conversely, curcumin-induced bacterial ghosts function as empty shells that allow the immune system to encounter bacterial antigens without running the danger of infection. These ghosts are produced by eliminating the cellular contents of bacteria while maintaining the structure of their outer membrane. When combined, these creative strategies open up new possibilities for the development of *H. pylori* vaccines, offering promise for the future of efficient *H. pylori*-associated disease prevention and treatment.

Chapter 5

Objective 3

To study reactogenicity, immunogenicity and protective efficacy of formulated immunogens

Background

The careful process of developing vaccines depends critically on research into the immunogenicity, reactogenicity, and protective effectiveness of prepared immunogens. When an immunogen is immunogenic, it means that it can stimulate strong immunological responses, which are necessary for producing antibodies and triggering T cells to provide long-term protection against infections. Aiming to maintain safety without sacrificing effectiveness, reactogenicity evaluation concurrently concentrates on detecting and controlling any possible negative reactions following vaccination.

The gold standard for evaluating a vaccine's effectiveness in preventing illness or disease in vaccinated persons as opposed to their unvaccinated counterparts is its protective efficacy. Careful measurement of these factors through extensive preclinical and clinical assessments, tailoring vaccination formulations with adjuvants and stabilizers to maximize immunogenicity and reduce reactogenicity hazards. This all-encompassing strategy protects public health more broadly by supporting international efforts to combat infectious illnesses and improving vaccination safety and efficacy.

1. Methodology

1.a Reactogenicity Assay

1.a.i. Quaantification of cytotoxicity of OMVs on murine macrophage cell line (RAW264.7)

Using a cell proliferation kit, the cytotoxicity assay of outer membrane vesicles (OMVs) evaluates the effects of the particles on grown cells through a systematic process. Multiwell plates are first used to seed cells, which are then left to adhere and proliferate. Then, at different concentrations—usually ranging from low to high doses—OMVs are introduced to the cells. Additionally supplied for comparison are control wells devoid of OMVs. A cell proliferation kit is used to test the viability and proliferation of cells after incubation. Colorimetric assays, which

measure cellular metabolic activity and are suggestive of alive cells, are frequently used in the kit. The assay offers quantifiable information on the long-term effects of OMVs on cell health, enabling researchers to identify cytotoxic thresholds and evaluate biocompatibility. Decisions about OMVs' possible usage in biological applications, such as vaccinations, are informed by this methodical methodology, which guarantees a full review of their safety profile.

1.a.ii. Evaluation of macrophage-mediated cytotoxicity of CurBGs using MTT assay

Cytotoxicity of the curcumin treated bacteria (CurBGs) was assessed using MTT assay kit (Roche, Merck). Murine macrophage cells (RAW 264.7) were grown using a 96 well plate and seeded with $1x10^5$cell/well and kept at 37°C for 24 hrs under 5% CO_2. The cells were then incubated with $1.0x10^8$CFU/ml of CurBGs along with *Salmonella* LPS (positive control) and PBS (negative control) overnight while maintaining the previous conditions. Next, day 10µl of substrate was added to each well and incubated for 4 hours before adding the solubilizing solution. Finally the optical density was measured using a microtiter reader (BioRad) [100].

1.b Immunogenicity against OMVs

1.b.i. ELISA

Serum immunoglobulin (IgG, IgM, IgA, IgG2c) levels were measured against OMPs or LPS following the method described previously [90]. Twofold serial dilutions were prepared from serum isolated from both immunized and non-immunized groups. HRP-conjugated secondary anti-mouse IgG, anti-IgA, anti-IgG2c and anti-IgM antibodies (Sigma Aldrich, USA) were used to detect the antibody titer. Each experiment was replicated thrice with pooled sera from different groups.

1.b.ii. Serum Bactericidal Assay (SBA) and Scanning Electron Microscopy

The effect of immunized mouse sera on bacterial morphology was measured and visualized using scanning electron microscopy (SEM) following a previously described protocol [91]. Bacteria along with heat-inactivated mouse sera and 25% guinea pig complement (with/without) were incubated for 1 hr under microaerophilic conditions followed by plating for viable colonies or fixation with 3% glutaraldehyde overnight followed by a gradual dehydration step initially with alcohol and then substitution later with a mixture of alcohol and hexamethyldisilazane (HMDS) at ratios of 2:1, 1:1 and 1:2. Finally, the samples were mounted on specimen stubs, sputter-coated with gold and analyzed on a Quanta 200 SEM (FEI, Netherlands).

1.b.iii. Cytokine assay

Both immunized and non-immunized mice were sacrificed, and the spleens were harvested. After isolating spleen cells; ~105 cells were cultured for two hours in RPMI1640 containing 10 % FBS incubated with 50 μg of OMVs and incubated overnight at 37°C (with 5 % CO2) for 24 h. IL-10, IFN-γ, IL-1β, IL-6, IL-4, TNF-α and IL-17 were measured in the culture supernatant using a cytokine measuring kit (Invitrogen, USA) [92].

1.b.iv. Fluorescence-activated cell sorting (FACS) analysis

Spleen cells were harvested, cultured for two hours in RPMI1640 containing 10 % FBS and restimulated using isolated OMVs (50 μg) and incubated overnight at 37°C (with 5 % CO2) for 24 h. The next day, the cells were scraped, washed thoroughly, blocked and then incubated with mouse anti-CD4+, CD8+ or CD19+ antibodies. Splenocytes were stained with anti-Mabs: CD4-phycoerithrin (PE), CD8 PE, CD19 PE or an isotype control PE (Miltenyi Biotec, USA). Unbound antibodies were washed, and a specific epitope of the immune cell population was observed using FACS Aria II [93].

1.c. Immunogenicity against CurBGs

1.c.i. Determination of antibody levels using ELISA

A 96 well flat bottom plates were coated with outer membrane proteins (OMPs) and whole cell lysates (WCLs) separately as described earlier[94]. The plates were then kept at 4°C overnight. Next the plates were washed with PBS and blocked using skim-milk. The wells were washed thoroughly using PBS-T (PBS and 0.5% Tween-20) and incubated with diluted sample serum for 1hr. The wells were washed again with PBST and incubated with HRP-tagged secondary antibody (IgG, IgG1, IgG2c, IgA). Finally, the wells were washed and substrate was added, incubated for ten minutes at room temperature and stopped using 2(N) sulphuric acid. The measurement was taken at 492nm using a microplate reader.

1.c.ii. Western Blot analysis

Outer membrane proteins were isolated using previously described protocol [95]. 20µg of proteins were run using a 12% SDS-PAGE with 100V using the AE-6530 SDS-PAGE apparatus from ATTO Corporation (Japan). The proteins were then transferred onto a nitrocellulose membrane using ATTO AE-6687 (Japan). After blocking with 5% non fat skimmed milk overnight, the membrane was washed several times and incubated with primary serum isolated from the immunized animals. After discarding the primary serum the membrane was washed thoroughly and incubated with anti-mouse secondary IgG conjugated with alkaline phosphatase. Finally, the secondary antibody was removed, washed and incubated with substrate (BCIP/NBT) until the bands were developed.

1.c.iii. Serum bactericidal assay and Scanning Electron Microscopy

In line with previously detailed method, serum bactericidal assay was performed [96]. Initially, the sera isolated from both immunized and non-immunized cohorts were heat inactivated at 56°C

for 20 minutes and serially diluted from 1:50 to 1:6400 in PBS. 50µl of this diluted serum was mixed with 12.5 µl of baby rabbit complement (12.5% of total volume), 10 µl of diluted bacteria (500 CFU, T_{0h}) and adjusted to 100 µl using PBS. The concoction was then incubated for 1hr at microaerophilic condition under shaking. Next, viable colonies were determined by spreading the mixture onto the plate and incubated for 48hrs. Negative control contained only bacteria and complement. A 50% reduction in CFU numbers in T_{1h} than T_{0h} was considered to be significant bactericidal activity. Bacteria incubated with lowest dilutions of immunized and non-immunized serum were fixed using gluteraldehyde and kept at 4°C. The samples were then processed and visualized under scanning electron microscope.

1.c.iv. Motility and mucin penetration assay

The motility assay was performed following a previously described protocol [97, 98]. 0.3% soft agar was mixed with immunized and non-immunized serum separately and poured over BHIA plate. A shallow puncture was done at the center of the plate and log-phase bacteria (0.6,OD_{600}) was added. The inoculated plates were kept for 48hrs under microaerophilic conditions. For mucin penetration assay, 1% (w/v) mucin (Sigma chemicals, USA) was mixed with 0.3% BHIA and allowed to solidify into columns. 100µl of log phase bacteria incubated with either immunized or non-immunized sera was then added to each of the column and incubated for 1hr. 500µl of the sample from lower portion of the columns were collected, diluted and plated to determine the bacterial count.

1.c.v. Ex-vivo splenocyte re-stimulation assay

Spleens were harvested from immunized and non-immunized animals and splenocytes were isolated. The cells were then cultured using RPMI containing 10% FBS along with 1x 10^6CFU/ml CurBG cells and kept at 37°C for 24hrs. The supernatants were then collected and

used to measure TNFα, IFNγ, IL-1β, IL-6, IL-17, IL-12p40/70 using individual ELISA kits (Invitrogen, USA**).**

1.c.vi. FACS analysis

35th day post-immunization spleens of both immunized and non-immunized animals were harvested and homogenized [99]. The homogenate was then strained through a cell strainer. Freshly isolated spleenocytes were then incubated with anti-Mabs: CD4-phycoeerithrin (PE), CD8a PE, CD19 PE along with isotype control PE (Miltenyi Biotec, USA). The populations of different immune cells were then quantified with BD FACS ARYA III flow cytometer and analyzed using FACS DIVA software

2. Results

2.a. Both OMVs and CurBGs showed low toxicity *in-vitro* using RAW 264.7

In contrast to lipopolysaccharide (LPS), a powerful component of Gram-negative bacterial cell walls that is well-known for its pro-inflammatory qualities, outer membrane vesicles (OMVs) demonstrated noticeably less cytotoxicity in our investigation. OMVs continuously maintained greater cell viability and lower levels of cytotoxicity indicators than LPS-treated cells when cultured cells were exposed to both substances at similar concentrations. According to this result, OMVs have lower toxicity profiles than LPS-derived bacterial membranes, which may be helpful for their possible use as drug delivery systems or vaccine candidates. The findings emphasize that OMVs are attractive candidates for more research and development, with the ability to maximize their immunogenic benefits and minimize their deleterious effects on host cells.

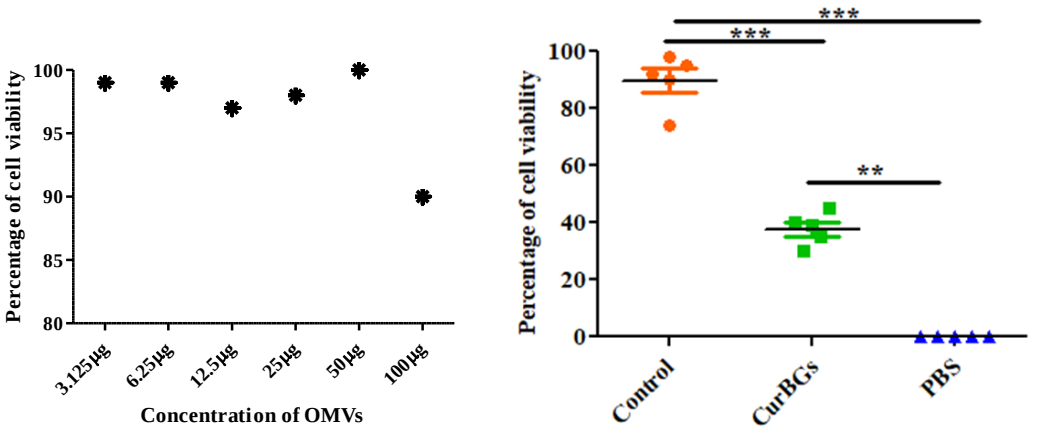


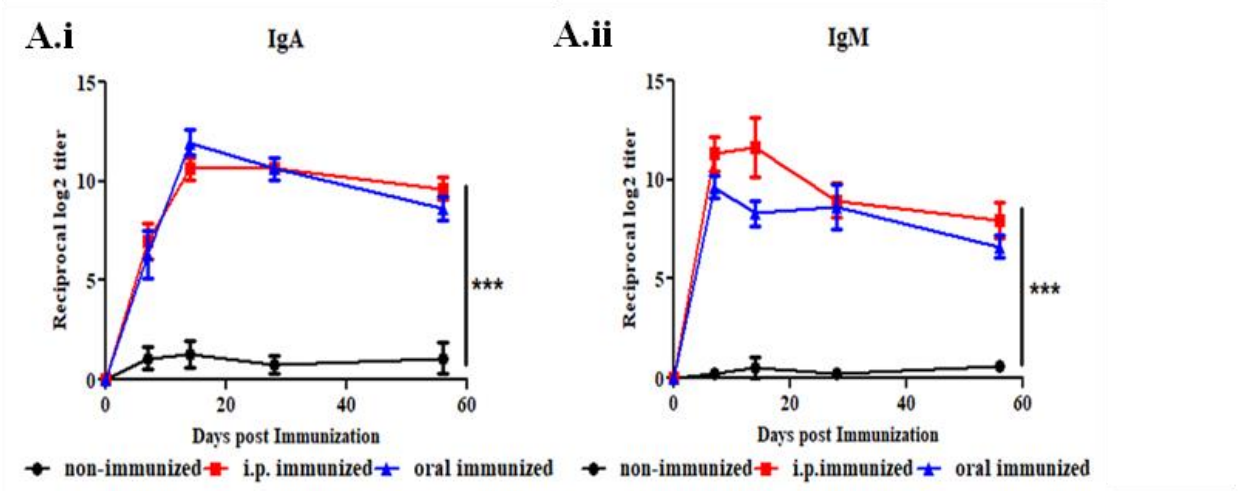
Figure 28: *in-vitro* cytotoxicity test with different concentrations of OMVs and CurBGs. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. (*** p value < 0.001, ns-Non-significant).Each bar represents median and error values of five ± SE of three independent experiments

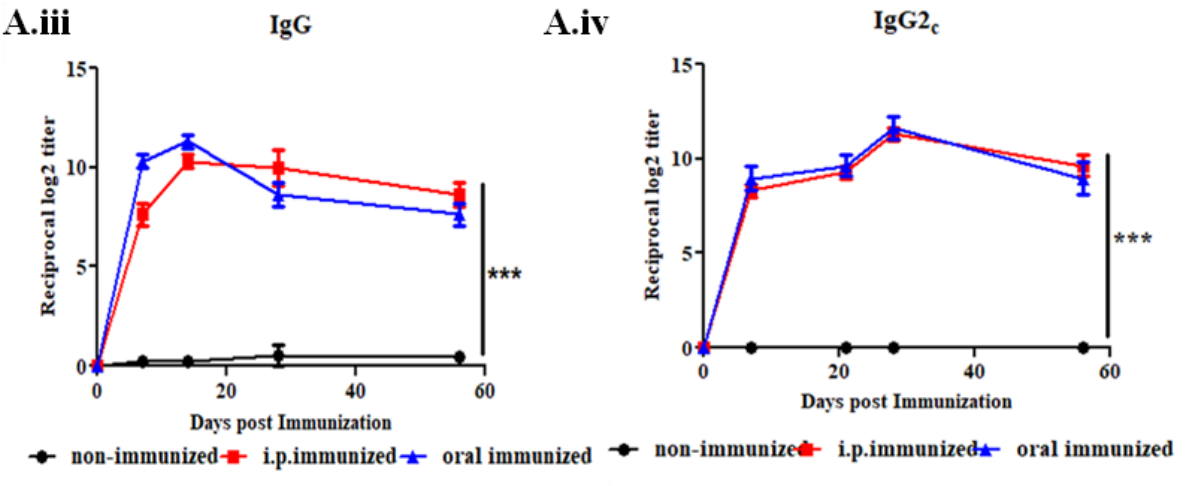
Our study revealed that, in comparison to LPS, curcumin-induced bacterial ghosts exhibited significantly reduced cytotoxicity. Bacterial ghosts are possible candidates for a variety of biomedical applications since they are produced by removing the cellular contents of bacteria while maintaining the structure of their outer membrane. Cultured cells were subjected to both LPS and curcumin-induced bacterial ghosts at comparable doses; the bacterial ghosts showed consistently lower levels of cytotoxicity indicators and higher cell survival than the intact bacterial cells. This suggests that bacterial ghosts created by curcumin maintain the immunogenic qualities required for the creation of vaccines while greatly reducing their propensity to cause harm to host cells.

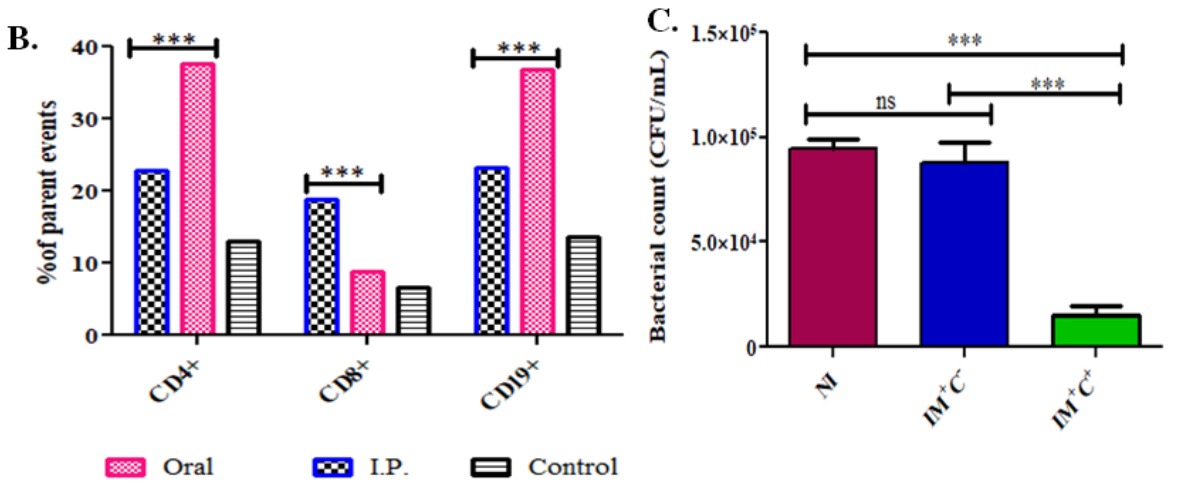
2.a.i. Immunization of *H. pylori* OMVs elicited higher adaptive immune response

Previous studies on bacterial extracellular vesicles revealed OMVs to be an excellent vaccine candidate against bacterial pathogens [102]. OMVs are known to induce both humoral and

cellular arms of immune responses usually mediated by outer membrane proteins (OMPs) and lipopolysaccharides (LPS). We investigated serum immunoglobulin levels of oral and intraperitoneally immunized mice and found significant difference compared to the control. However, we did not find significant differences between oral and intraperitoneal immunization. Next, we evaluated the bactericidal activity of the immunized serum. The data showed significant reduction in bacterial number when immunized serum is incubated with 25% guinea pig serum as compared to non-immunized mice serum. This confers activation of complement mediated pathway, along with sufficient antibody titer in immunized C57BL/6 mice that effectively kill the bacteria by damaging the bacterial surface as viewed under SEM. Comparative analyses of the splenic cell population of immunized and non-immunized mice were done using a flow cytometer. Immunization with OMVs showed significantly higher population of CD4+, CD8a+ and CD19+ cells indicating a strong immune response in immunized mice. In all, immunization with *H. pylori* OMVs generated adaptive immune responses in C57BL/6 mice and significantly activated adaptive immune responses which could in turn help to provide long term protective immune response against infections caused by *H. pylori*.




A.iii
IgG
Reciprocal log2 titer
Days post Immunization
non-immunized
i.p.immunized
oral immunized
***
A.iv
IgG2c
Reciprocal log2 titer
Days post Immunization
non-immunized
i.p.immunized
oral immunized
***


B.
%of parent events
CD4+
CD8+
CD19+
Oral
I.P.
Control
C.
Bacterial count (CFU/mL)
NI
IM+C-
IM+C+
ns
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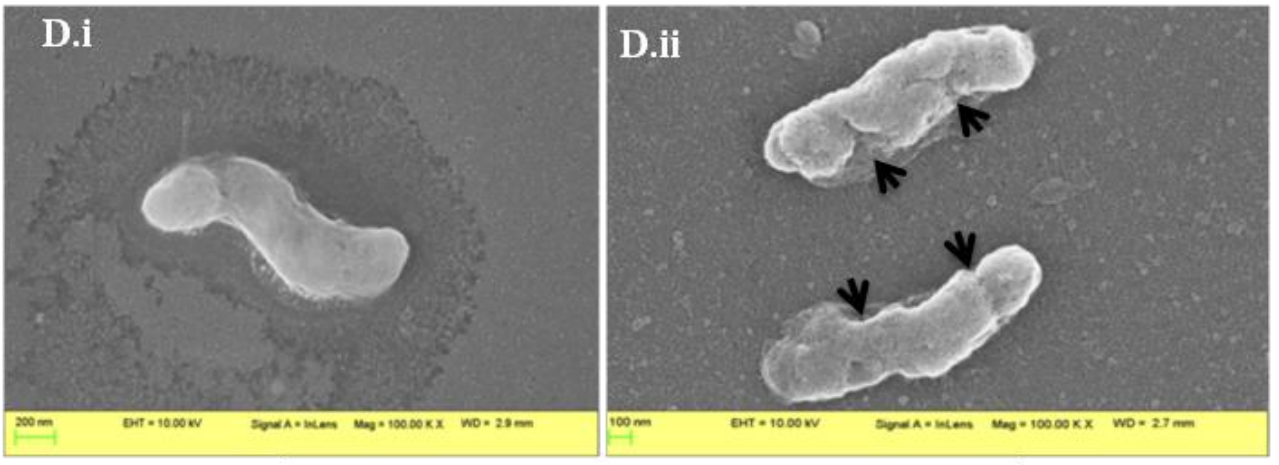

D.i
D.ii

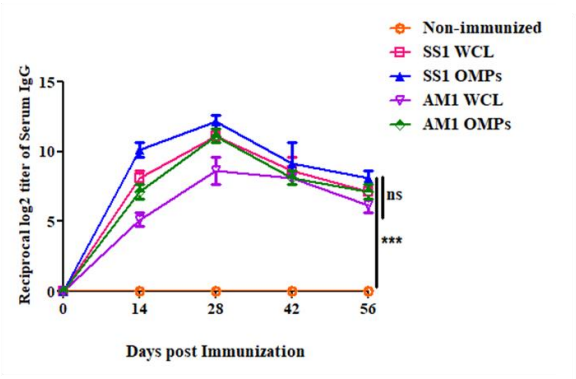
Figure 29: Reciprocal log2 titer of serum IgA, serum IgM, serum IgG, and serum IgG2c immunoglobulins from *Helicobacter pylori* OMVs immunized and non-immunized (PBS immunized) group against OMPs. Immunization induces the population of CD4+, CD8+, and CD19+ splenic cells of immunized over non-immunized (PBS immunized) mice and the microscopic image of serum bactericidal activity of immunized and non-immunized. Mouse serum IgA (**A.i**), serum IgM (**A.ii**), serum IgG (**A.iii**), serum IgG2c (**A.iv**) was measured separately after three doses of intraperitoneal or oral immunization against Outer membrane protein (OMP) of *Helicobacter pylori.* **(B)** Bar diagram represents the percentage of CD 19+, CD 4+, and CD 8+ spleen cells from immunized and non-immunized mice using FACS analyses. Significant statistical difference was found between OMVs immunized and non-immunized spleen cell population (*** p value< 0.001). **(C)** OMVs immunized mouse serum is effective in complement mediated lysis of *H. pylori* (SS1). *H. pylori* (SS1) was separately incubated with OMVs immunized serum or non-immunized serum with or without guinea pig complement for 1 h at 37 ◦C. Viable bacterial count were determined by spread-plate method. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. (*** p value < 0.001, ns-Non-significant.). NI, non-immunized serum; IM+C-, OMVs immunized serum without complement; IM+C+, OMVs immunized serum with complement. (**D**)Scanning electron microscopic images after Serum Bactericidal Assay using non-immunized serum with complement (i) and immunized serum with complement (ii) (black arrowheads indicate immunized antibody-mediated lysis in presence complement).

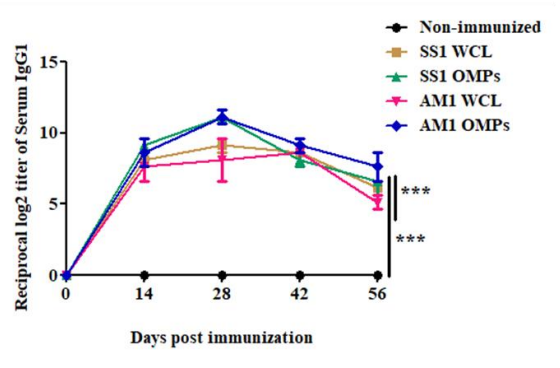
2.b.i. CurBGs induce humoral immunity in mice

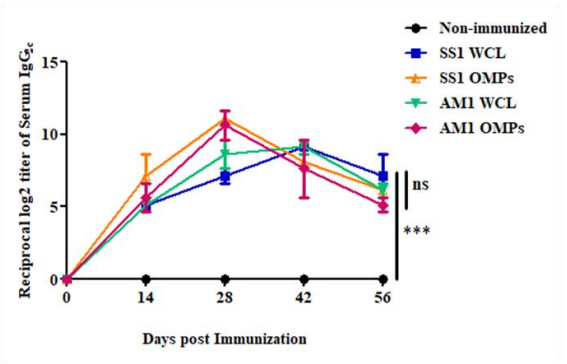
An ELISA was used to evaluate the induction of humoral immunity up to 90 days following the first immunization during and after the immunization with nanocurcumin induced bacterial ghost cells. Serum immunoglobulins such as IgG, IgA were found to be significantly induced in immunized animals than in non-immunized animals. Furthermore, we assessed the IgG subtypes IgG1, IgG2c, IgG3 to confirm the induction of long lasting memory response for an effective

adaptive immune response. All the results indicated a successful specific antibody-mediated immune response in immunized animals. Antibody titers began to increase 7days post immunization till 35 days. The booster immunizations on day 28 and day 35 significantly induced serum IgG and IgA titers. A prominent mucosal immunity (sIgA) was also observed from gastric lavage. Antibody profile was done against outer membrane protein (OMP) and lipopolysaccharides (LPS) of both *cagA*+ (SS1) and *cagA*- (AM1) strains. These results indicate CurBGs can be an excellent vaccine candidate against *H. pylori.*

Western blot analyses against both OMPs and LPS showed induction of specific antibodies which was initially visualized using SDS-PAGE. An array of proteins were recognized which were indicated by the presence of bands on western blot. However, the western blot for LPS was not prominent compared to the OMPs depicting their role in generation of an immune response.







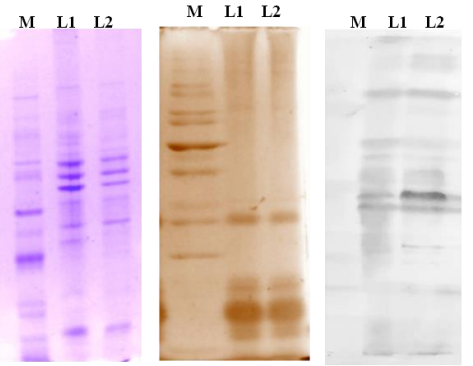


Figure 30: Induction of CurBGs mediated immune response in C57BL/6. (A) Schematic diagram of immunization and challenge study. Serum immunoglobulin levels **(B)** IgG and its subtypes **(C)** IgG1 and **(D)** IgG2C against whole cell lysate (WCL) and outer membrane proteins (OMPs) of cag+ (SS1) and cag -(AM1) strains. **(E)** Elevation of secretary IgA (sIgA) level post immunization. All immunoglobulis are measured by ELISA (n = 6). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non significant). Each bar represents median and error values of six ± SE of three independent experiments.

2.b.ii. CurBGs immunized serum mitigates bacterial motility and mucin penetration ability

Flagella play very important role not only in bacterial motility but also in overall pathogenesis. *H. pylori* is known to dislodge and move from one part of the stomach to another when stomach becomes overwhelmingly acidic. Incubation with immunized and non-immunized serum separately along with bacterial inoculums stunted bacterial motility in immunized groups than the PBS immunized groups. This clearly indicates that our immunogen induce antibodies might impede the bacterial motility via agglutination. Gastro-intestinal mucosa releases a thick mucus gel that entraps any possible pathogen. Mucin, is an essential component of this mucus and crosslinks in order to produce a mucin layer that contain secretary IgA (sIgA). Secretary IgA is essential for mucosal immunity. Thus, we evaluated the mucin penetration ability of bacterial cells in presence of immunized and non-immunized serum which was found to be significantly lower in CurBGs immunized mice than non-immunized serum treated bacteria.

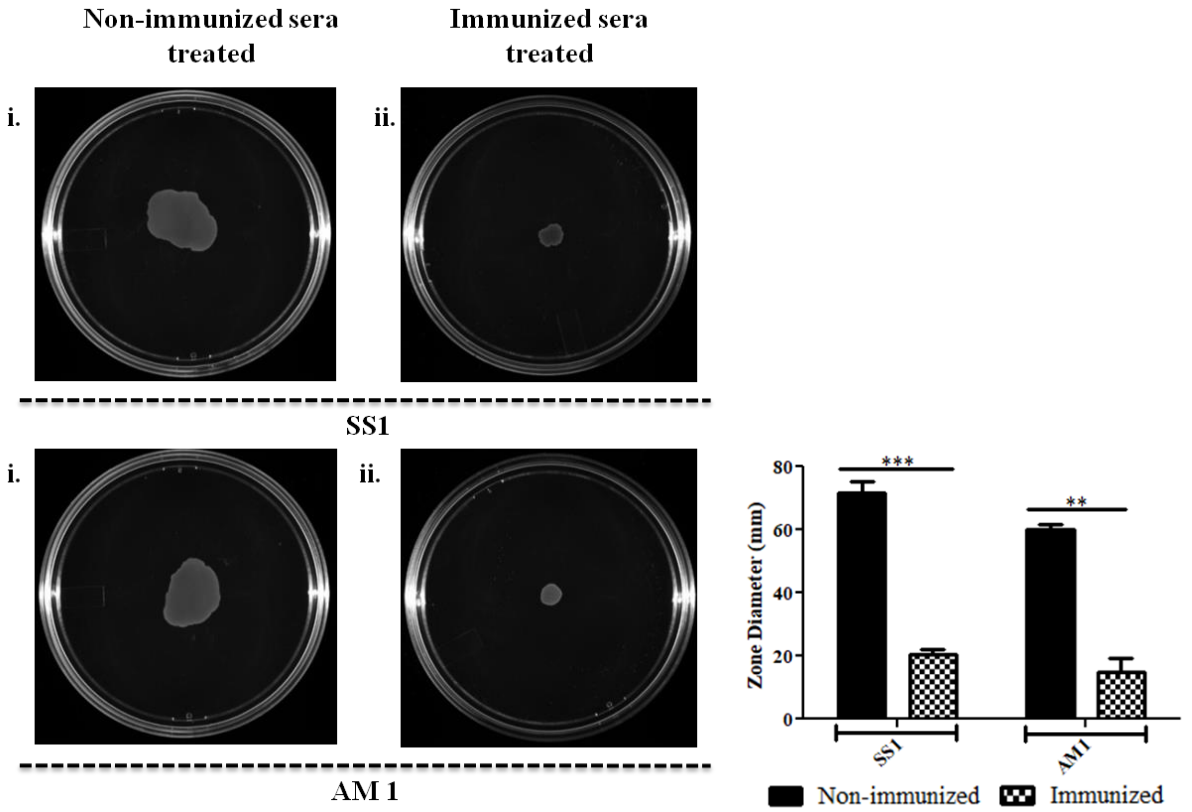


Figure 31: Mucin penetration ability of *H. pylori* strains SS1(cag+) and AM1(cag-). Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. (*** p value < 0.001, ns-Non-significant).Each bar represents median and error values of six ± SE of three independent experiments

We further wanted to check that CurBGs induced antibodies show any bactericidal activity or not as indicated by previous studies. Complement-mediated bactericidal activities were remarkably higher in case of immunized mice serum when mixed with externally supplemented complement. A gradient dilution of both heat inactivated immunized and non-immunized mice serum and complement system when exposed to bacterial cells resulted in transmembrane pores which were readily observable under scanning electron microscope. Therefore, this result indicates the generation of CurBGs-specific antibodies due to priming of immunized cells.

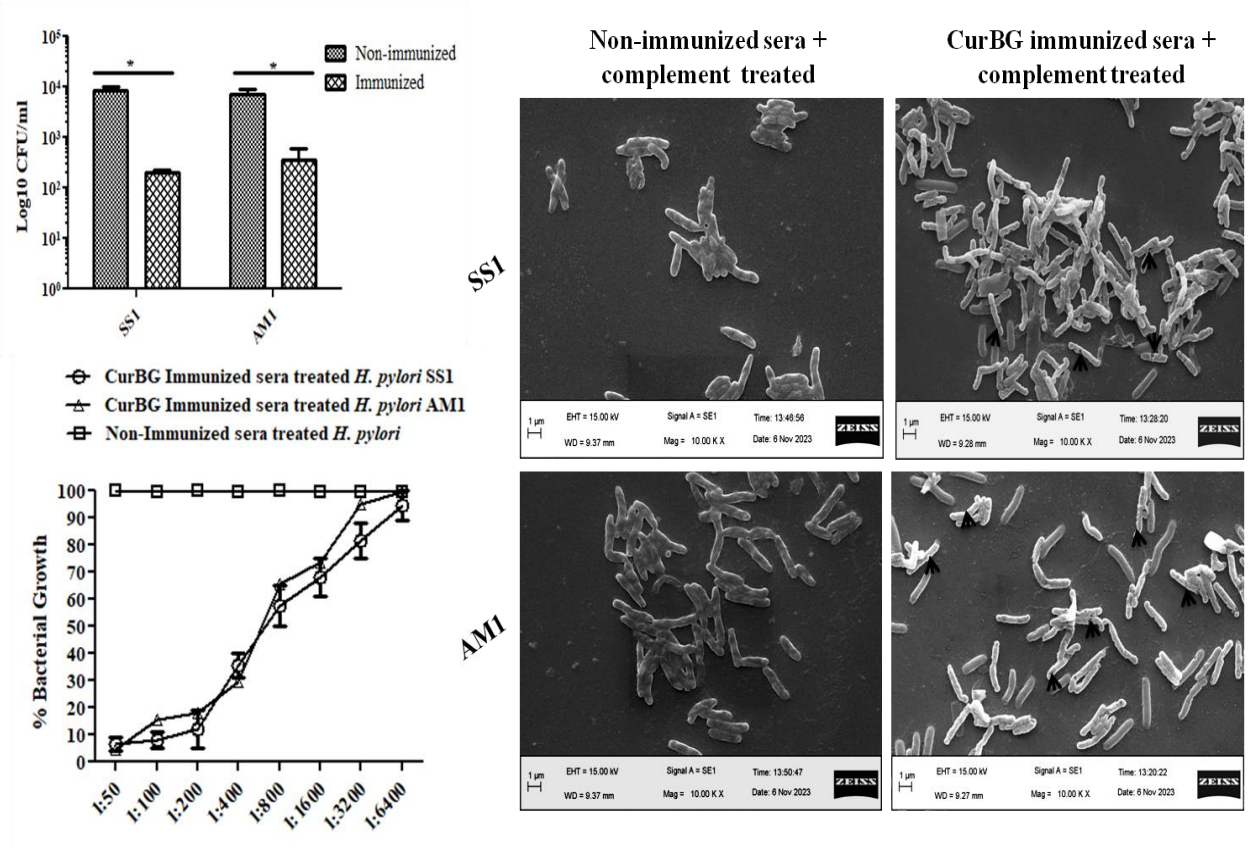


Figure 32: Serum Bactericidal assay of *H. pylori* strains SS1(cag+) and AM1(cag-). Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. (*** p value < 0.001, ns-Non-significant).Each bar represents median and error values of six ± SE of three independent experiments

2.b.iii. Inflammatory cytokine response triggered by curcumin-induced bacterial ghost cells

On the 35th day following the initial immunization, mice immunized with CurBGs and PBS was sacrificed, their spleens were harvested, and a single cell suspension had been produced and cultured. Next, the cells were re-stimulated with CurBGs and incubated overnight. The supernatant collected revealed significant up-regulation in TNFα, IFNγ, IL-1β, IL-6, IL-17, IL-12p40/70. These results indicate a robust Th1/Th2/Th17 mediated inflammatory response post immunization in CurBGs immunized mice. Additionally, FACS analysis of different T-cell

populations measured by their cell surface markers such as CD4+, CD8a+ and CD19+ were found to be elevated significantly in immunized animals than PBS immunized groups.

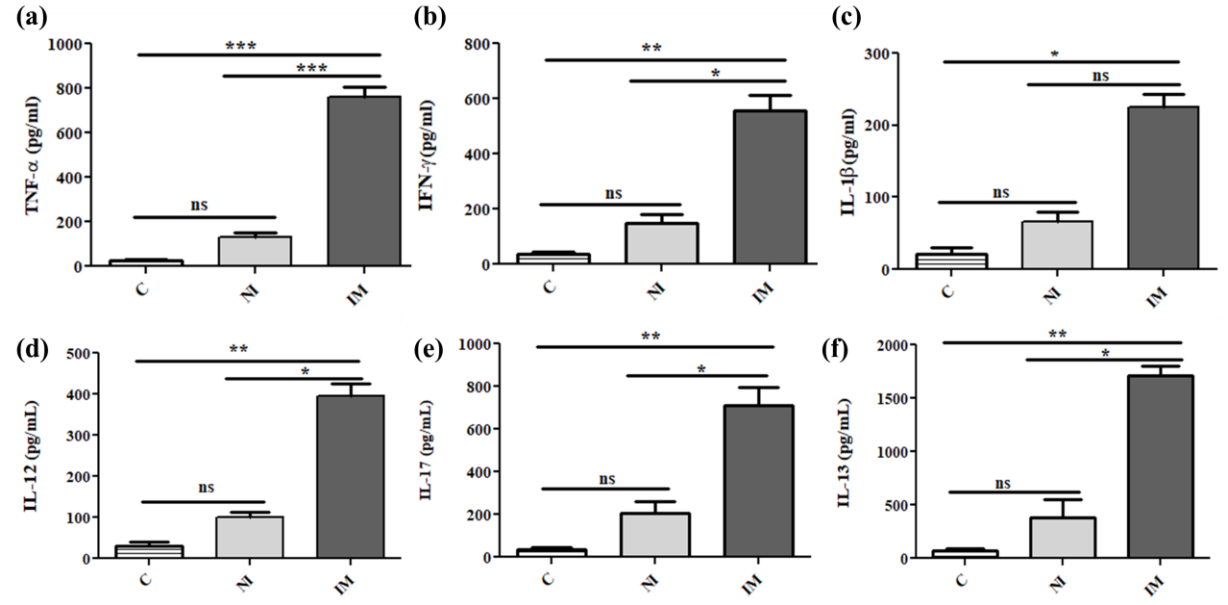


Figure 33: *Helicobacter pylori* CurBGs induce the production of cell mediated cytokines responses. (a) TNF-α, **(b)** IFN-γ, **(c)** IL-1β, **(d)** IL-12, **(e)** IL-17, **(f)** IL-13, cytokines in culture supernatant of ex-vivo cultured splenic cells of immunized and non-immunized (PBS immunized) mice after 24 h of restimulation with OMVs. The differences in immunized mice serum response of each of the studied cytokines were highly significant than nonimmunized. All cytokines are measured by ELISA (n = 6). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non significant). Each bar represents median and error values of six ± SE of three independent experiments. **C**-control**, NI**-non-immunized**, IM**-immunized

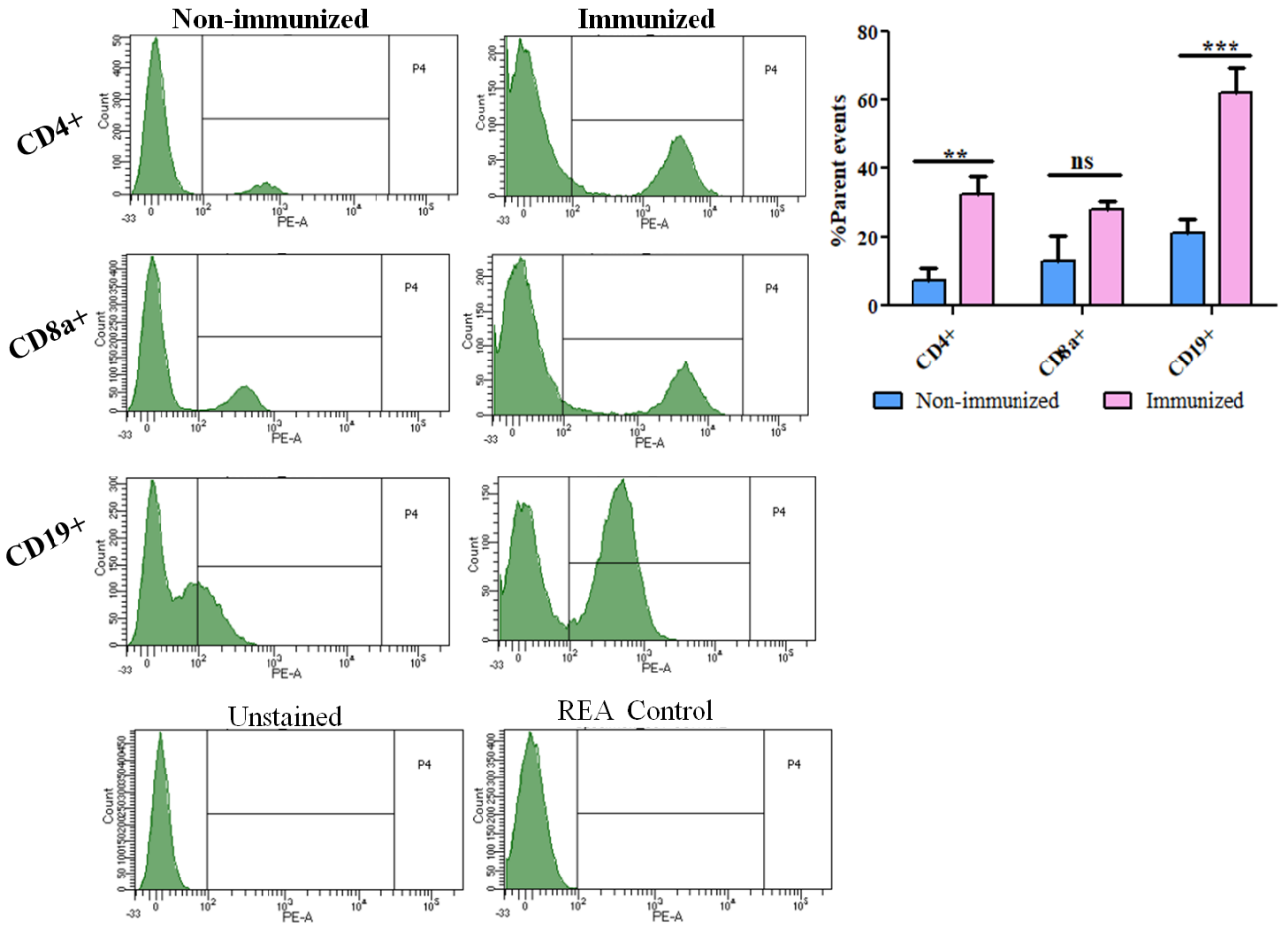


Figure 34: Cytokine response and FACS analysis of immunized and non-immunized spleen cells). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non significant). Each bar represents median and error values of six ± SE of three independent experiments.

3. Discussion

Two different immunization routes were assessed to observe any alterations in the immune response due to changes in the route of administration. Immunization was performed both orally and intraperitoneally (i.p.) on days 0, 14 and 28. Initially, an elevation of serum IgG, IgM and IgA levels was observed against OMPs but not LPS of H. pylori. This can be due to the structural similarity between H. pylori LPS and blood antigens of the host [103-104]. Furthermore, we

evaluated IgG2c (IgG subtype) and found it to be increased in immunized rather than non-immunized groups [102]. Our study found oral immunization to be better responsive than i.p. route, which can be due to the presence of different surface proteins on OMVs that are more readily absorbed and reactive to gastric epithelial cells than peritoneal immune cells. A splenic cell re-stimulation (ex vivo) assay revealed enhanced Th2-based cytokine responses, such as IL-4, IL-13, IL-10 and IL-12, coinciding with previous studies with H. pylori-derived OMVs used as immunogens [101]. Interestingly, our study did not find any biased immune response against OMVs, indicating that the immune response to OMVs is not general but rather unique to each strain. CD4+, CD8a+ and CD19+ cell populations were increased due to OMV immunization independent of the route of administration. OMV immunization ultimately leads to a reduction in bacterial colonization in immunized animals but not in non-immunized animals. Serum bactericidal assay (SBA) typically denotes the functional aspect of immunogen-invoked antibody response in killing the bacterial population via complement mediated pathway. This in-vitro method involves incubation of bacteria in presence of heat-inactivated serum isolated from both OMVs immunized and PBS immunized mice. Antibodies generated in host due to immunization are sufficient enough to reduce the bacterial CFU by means of agglutination as demonstrated in OMVs induced immune response against S. Typhi and Paratyphi A [91]. However, agglutination doesn't directly imply a bacteriostatic or bactericidal activity of the antibodies. Therefore, addition of purified baby rabbit [105] or guinea-pig [106] complement externally to these antibodies ensured the lysis of the bacteria via complement-mediated pathway. In the present study, incubation of bacteria treated with OMVs immunized or PBS immunized sera in presence of guinea-pig complement lead to significant reduction in viable colony numbers in immunized compared to the PBS immunized mice groups.

Curcumin has no effect on the outer membrane proteins or LPS of bacteria. The comparison between surface protein and LPS profile of the CurBGs to that of whole cell lysate of the same strain found no significant variations indicating conservation of surface antigens. LPS is known to be highly toxic due to their inflammatory nature due to their interaction with TLR-4 or toll like receptor 4. Cytotoxicity assay confirmed the CurBGs to be significantly less toxic than positive control, allowing it to be used as an immunogen to evoke a robust immune response in C57BL/6 mice. Oral administration of CurBGs thrice (day 0, 14 and 28) at an interval of 14 days significantly elevated serum immunoglobulin levels of IgG and its subtypes IgG1 and IgG2c against OMPs and WCL isolated from *cagA+/cagA*- strains. This depicts generation of long term immunity in immunized mice compared to the non-immunized groups. Moreover, rise in secretary IgA (sIgA) level in gastric lavage indicated development a prominent mucosal immune response in immunized animals. Next, we assessed the effect of generated antibodies on motility and mucin penetration ability of *cagA+/cagA*-strains and found observable differences in both. Compared to the control, serum treated bacteria were unable to move through the soft agar plate and mucin validating generation of antigen-primed antibodies against these antigens. Additionally, when immunized and non-immunized heat-inactivated sera was supplemented with complement system externally and incubated with these strains, their growth reduced noticeably. Further, SEM images confirmed lysis of bacterial cells in immunized sera treatment. Western blot analyses showed multiple bands indicating the development of antibodies against OMPs of *cagA+/cagA*-strains explaining the role of surface antigens in developing immunogenicity. To evaluate whether the immune response is Th1/Th2/Th17 mediated, ex-vivo spleen cell re-stimulation was performed. TNFα, IFNγ, IL-1β, IL-6, IL-17, IL-12p40/70 was measured from the culture supernatant of both immunized and non-immunized spleenocytes re-stimulated with

CurBGs. Pronounced expressions of these cytokines indicate both cell mediated and humoral immune response is responsible to reduce the bacterial burden in immunized animals compared to the non-immunized.

4. Conclusion

To sum up, a promising approach to fighting the enduring infection *H. pylori* is the production of outer membrane vesicles (OMVs) and curcumin-induced bacterial ghosts as prospective vaccine candidates. OMVs have the benefit of having several immunogenic components from the outer membrane of the bacteria, which increases their capacity to elicit a strong immune response. The immunogenicity of the remaining surface antigens combined with the safety of non-viable bacterial cells is what makes curcumin-induced bacterial ghosts, on the other hand, a balanced approach to vaccine production. When compared to conventional vaccination methods, both systems exhibit significant promise in terms of safety, effectiveness, and ease of production. In order to completely investigate their safety and efficacy characteristics and pave the road for a potentially successful *H. pylori* vaccination, more investigation and clinical studies are necessary.

Chapter 6

Objective 4

To study the duration of protective efficacy of formulated immunogen after subsequent challenge with currently circulating wild type Helicobacter pylori

Background

Expanding our knowledge of long-term immunity in relation to *Helicobacter pylori* requires examining how long formulated immunogens defend against this pathogen. Because of its genetic variety and propensity to linger in the human stomach, the bacterium *H. pylori* is a major obstacle when it comes to treating chronic gastritis, peptic ulcers, and potentially gastric cancer. One possible method of treating *H. pylori* infections is the use of formulated immunogens, which are intended to boost immunity and offer protection. The durability of this defense, however, continues to be a crucial consideration when assessing these immunogens' efficacy. The purpose of this work is to examine the duration of these immunogens' protective effects following successive challenges with wild-type strains of *H. pylori* that are currently in circulation. We can evaluate the vaccines' long-term efficacy and capacity to offer enduring defense against this persistent infection more accurately if we are aware of the length of immune protection.

Due to the absence of any natural model of *H. pylori,* often existing animal models are being manipulated physiologically, immunologically or genetically to be more susceptible for the infection. Several studies implicated the need of a model for studying the pathogenesis and immunization of *H. pylori.* However, selecting a suitable model has proven to be difficult as it may prevent exact translation to the human condition due to its own defense mechanisms. Though black mice (C57BL/6) are commonly used model for *H. pylori* study, studies are also done in animals including Sprague-Dawley rats, Mongolian gerbil, guinea pig, gnotobiotic piglets and non-human primates.

1. Methodology

1.a.i. Development of an Intragastric surgical model for vaccine efficacy study

Experimental animals were kept in fasting conditions overnight with sterile water. Initially, animals were sedated by an intraperitoneal injection of a mixture of ketamine (87.5 mg/kg) and xylazine (12.5 mg/kg) [107]. The stomach was exposed through a 2-3 cm midline incision without compromising any major blood supply. A disposable syringe with a 26G needle containing 200 µl (~2x108 CFU) of the inoculums in PBS was directly injected into the stomach. Hydration was maintained in the exposed stomach using sterile normal saline throughout the surgery. The stomach was placed back inside the abdominal cavity, and the incision was sutured back. The incision site was monitored for any infection and occasionally washed with 5% povidone-iodine (betadine) soaked in a sterile gauge for 72 hours [108]. Sterile food and water were provided to the animals once they regained consciousness (**Figure: 35**).

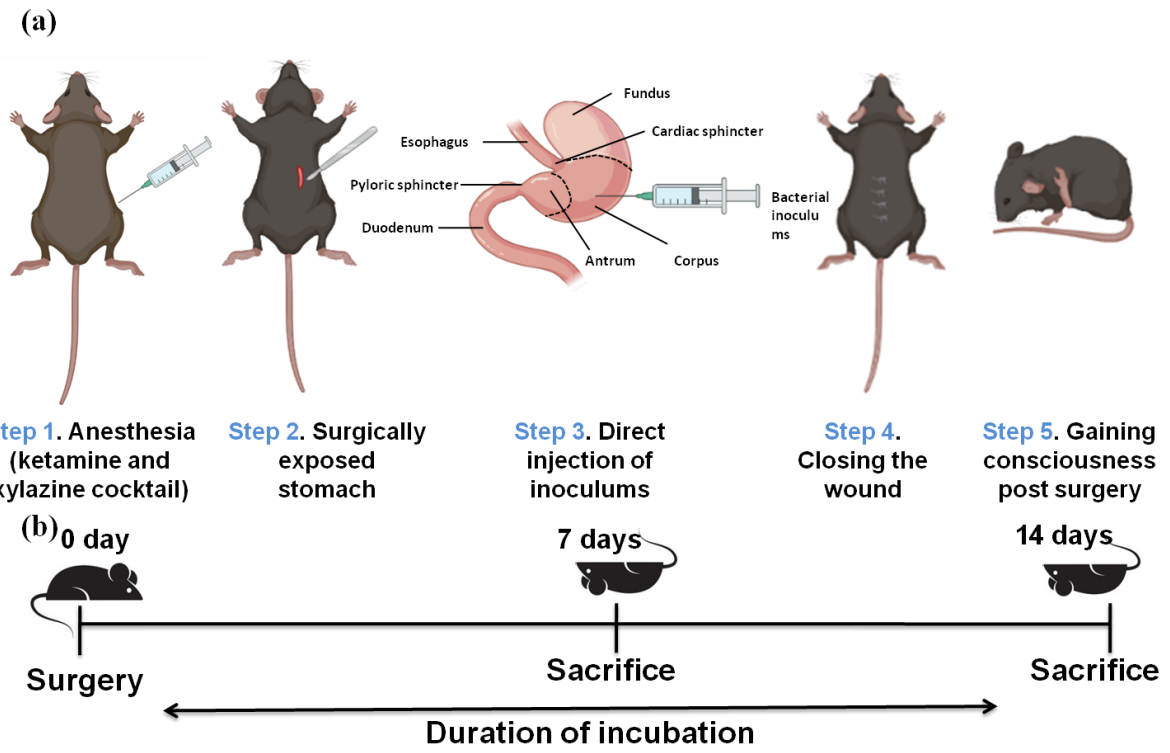


Figure 35: Schematic diagram of the intragastrically infection model in C57BL/6 mice. (a) Graphical Representation of the "Surgical Model" using C57BL/6.Bacterial inoculation (~$2x10^8$CFU/mL) is directly injected into the stomach, **(b)** Schematic schedule from infection to sacrifice.

1.a.ii. Post Surgery Observation

All infected mice were observed twice a day for 7 days. Physical parameters were checked along with stool consistency and the nature of mucus or blood (if any) present in the feces. Rectal swabs were taken daily and were subjected to RUT solution and spread-plate to observe the shedding of the organism. Isolated colonies (if any) were confirmed using a PCR-based technique. *H. pylori* infection augments the modulation of both pro- and anti-inflammatory cytokines in the host [109]. Therefore, IL-1β, TNFα, IFNγ, IL-6, IL-10 and IL-17 were tested using cytokine measuring kits (Invitrogen, USA) following the manufacturer's protocol. 50µl of serum samples from 0-day, 7-day- and 14-day-infected mice were used to quantify the inflammatory response after post-operational (OP) observation due to infection.

1.a.iii. Protective efficacy Study of OMVs immunized animals

Seven days after the last immunization, both the immunized and non-immunized groups were challenged with the wild-type SS1 strain using a newly developed surgical procedure and housed for 7 days before being sacrificed. The antrum of stomach of both immunized and nonimmunized groups was isolated and separated into two parts. Half of each part was immediate transferred to BHI kept on ice, and the other half was transferred to neutral buffered formalin (NBF, 10%) solution to fix the tissue and left at room temperature. Harvested tissue in BHI was weighed, homogenized and serially diluted using PBS. The diluted samples were then spread onto BHIA and kept under microaerophilic conditions for 3-5 days. Any visible colonies

were then counted and confirmed using RUT and PCR. Histopathological assays were performed as described elsewhere [110, 111]. Briefly, samples kept in 10% formalin were washed and gradually dehydrated using the alcohol gradation method followed by preparing a paraffin block. A thin section (approximately 5 μm) was prepared using a microtome. The slides were then de-waxed, rehydrated, and stained. Hematoxylin-Eosin was used for the study because they enhance tissue or bacterial contrast. Finally, the slides were mounted and observed under a microscope. Histological scoring was assigned for each sample based on their morphological changes. The gastric tissue observed under a microscope revealed various degrees of gastritis, which was then categorized according to the Houston-updated Sydney System based on the infiltration of inflammatory cells within the lamina propria [112].

1.a.iv. Protective efficacy studies of CurBGs immunized animals

Both immunized and non-immunized animals were challenged with SS1 strain with a dose of $2x10^8$ CFU/mouse 7 days post last immunization. Animals from each of immunized and non-immunized groups were infected with SS1 intragastrically using surgical intervention, while two received nothing but 100μl of PBS only. Infected animals were euthanized after 7days to observe the histopathological changes.

The stomach tissues were harvested from animals 14 days post surgery-induced infection and fixed with 10% normal buffered formalin (NBF) followed by paraffin fixation. 5μm sections were then stained with haematoxylin and eosin (H&E) and viewed at different magnifications using a light microscope and were scored accordingly.

2. Results

2.a.i. Clinical response caused by surgical intervention

In the present study, $2x10^8$ CFU of bacteria were used to induce an active infection. Oral inoculation with the aforementioned dose revealed inconsistent results. Moreover, in the majority of cases, very little or no recovery of the bacterial population was observed using available detection techniques. Mice receiving WTSS1 directly to their stomachs by surgical means developed various degrees of gastric changes. Recovery of bacterial colonies from stool was insignificant and erroneous compared to gastric tissues, which were considerably higher (~2-3 times) and were confirmed to be positive upon RUT, spread-plate and PCR. The recovery rate of *H. pylori* from the 7-day infected mice was comparatively higher than that at 14 days post infection (**Figure: 36**).

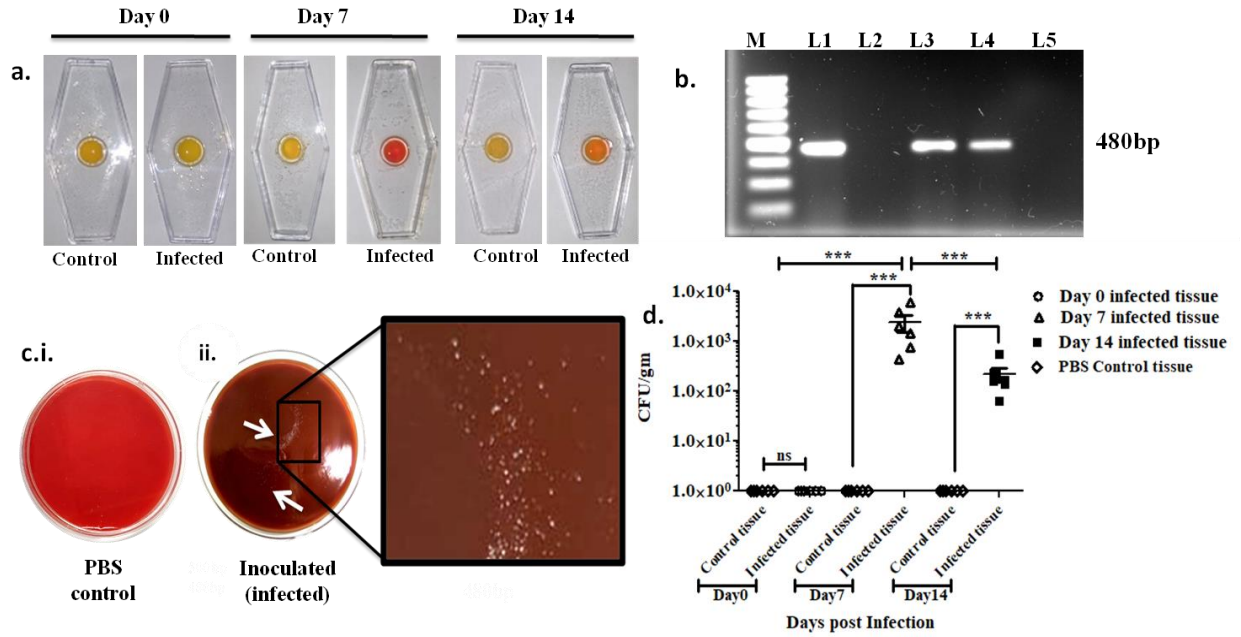


Figure 36: Intragastric infection induced by wild type (WT) SS1 observed through urease test, *ureB* PCR, colonization in gastric tissue. (a) RUT of infected gastric tissue; Day 0, Day7 and Day 14 with respective PBS control**, (b)** Confirmatory *ureB* PCR for the presence of *H.*

pylori recovered from the gastric tissue of infected mice**, M**-100bp marker,**L1-**positive control, **L2**-day 0 post infection, **L3**-day7 post infection, **L4**-14 day post infection, **L5**-PBS control.(**c**) Blood agar plates showing **(c.i.))**plate containing no *H. pylori* colonies recovered from gastric tissue of 7days post PBS inoculated mice **(c.ii)** plate containing *H. pylori* colonies recovered from gastric tissue of 7days post SS1 infected mice. **(d)** Colonies recovered from mice of 0, 7 and 14 days post infection. Data represented here are the mean values +/-Standard Deviation (SD) of three independent experiments. The differences in day wise response of each colonization assay were highly significant with respect to PBS control tissue. Statistical significance was found between 0day, 7day and 14day infected mice tissue (***$p<0.001$, ns-non significant).

2.a.ii. Intragastric surgical evoked inflammatory response

Cytokine analysis of intragastrically infected mice of different time points i.e. 0 day, 7 day, and 14 days, showed drastic differences in serum cytokine levels. IFN-γ, IL-1β, TNF-α, IL-10, and IL-17 are increasing more on day 7 after infection and also reducing progressively on day 14.In case of IL-6, which is responsible for sustaining inflammation is increasing on day 14. The majority of the pro-inflammatory cytokines were upregulated after 7 days post infection, except IL-6, which was found to be more pronounced at 14 days than at 7 days post infection (**Figure:37**) indicating active *H. pylori* infection.

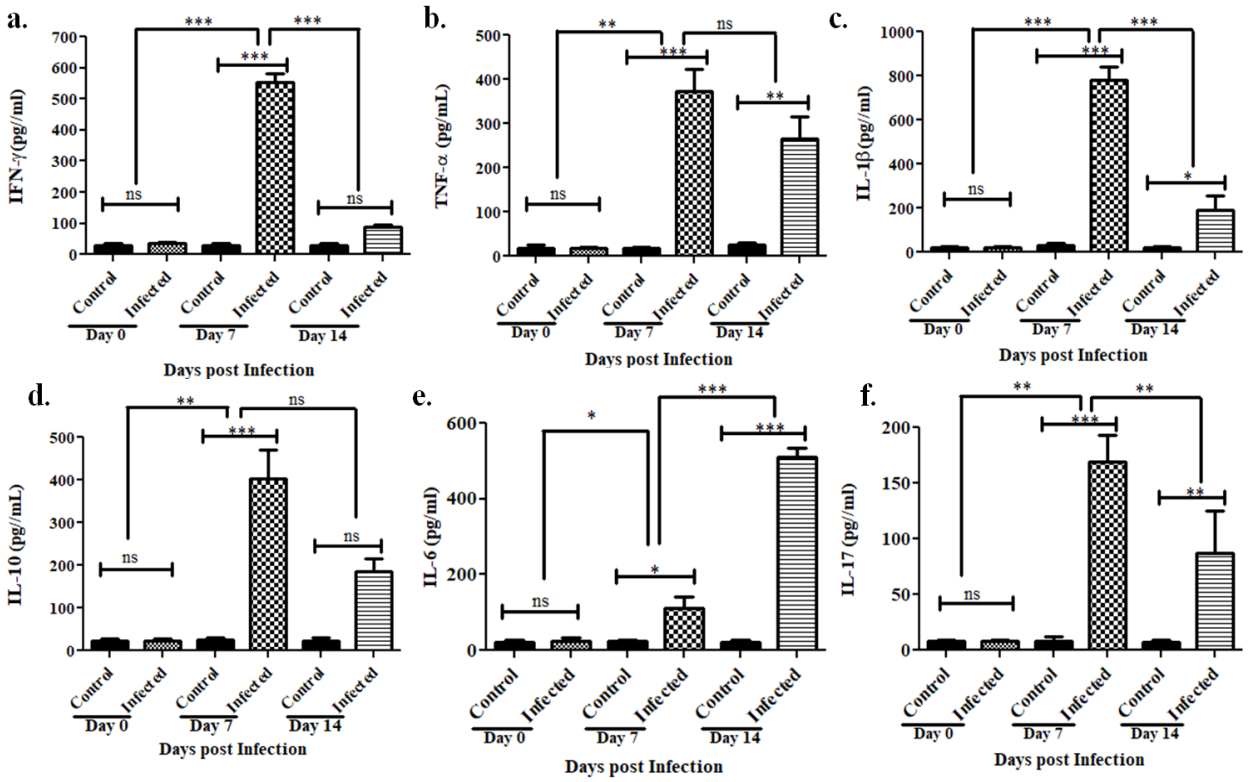


Figure 37: *Helicobacter pylori;* wild type (WT) SS1 induces the production of cell mediated cytokines responses post surgical intervention. (a) IFN-γ, **(b)** TNF-α, **(c)** IL-1β, **(d)** IL-10, **(e)** IL-6, **(f)** IL-17 cytokines in serum isolated on Day 0, Day 7 and Day 14 post surgical infection with respective PBS controls. All cytokines are measured by ELISA (n = 6). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non significant), Each bar represents median and error values of Six ± SE of three independent experiments.

2.a.iii. Histopathological changes due to intragastric infection

Histopathological observation plays crucial role in *H. pylori* diagnosis. *H. pylori* infection causes local inflammation in gastric tissue marked by various degrees of inflammatory infiltration with substantial damage in gastric epithelium leading to the survival of the bacteria to the gastric microenvironment. Moreover, previous study has already showed a pronounced effect

on gastric tissue of C57BL/6 mice upon *H. pylori* infection. Therefore, in order to establish a successful infection mediated by surgical intervention, stomach samples were taken at different time points and the topographical changes were compared. Negative control mice, receiving only PBS and Day0 had no inflammation (**Figure 38.i: a, b**) whereas inflammatory infiltration was significantly higher on day7 (**Figure 38.i: c**) compared to day 14(**Figure 38.i: d**). In addition to this, mucosal epithelium was severely damaged with exposed gastric pits in both cases. In contrast, metaplasia due to infection was more prominent on day 14 than day 7, indicating a successful infection. Histopathological scoring was assigned based on Sydney system (**Figure 38. ii**)

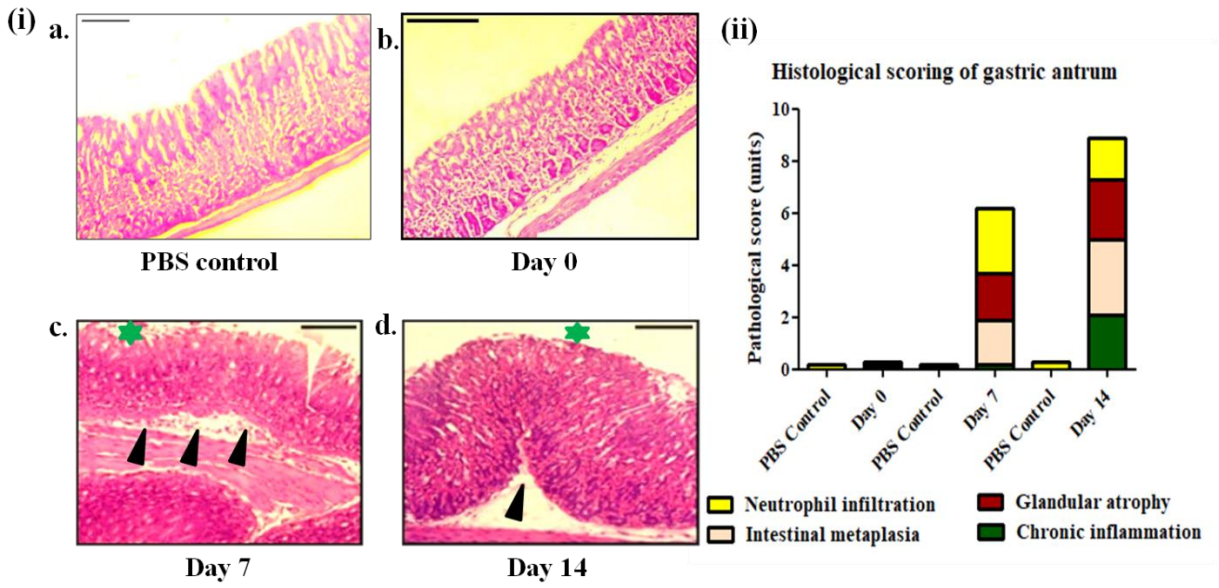


Figure 38: Histological (H&E staining) observation and histological scoring of gastric epithelium after intragastric surgical infection with *Helicobacter pylori* (SS1). (i) a, b, c, d; all are the antrum part of the stomachs harvested from C57BL/6 mice**. (i.a)** no distinct changes observed in PBS control, mice receiving PBS only, **(i.b)** zero inflammation in Day 0 post surgical infection of C57BL/6 mice, **(i.c)** severe inflammatory cell infiltration, glandular atrophy, intestinal metaplasia of 7 days post surgical infection of C57BL/6 mice, **(i.d)** mild to moderate

inflammatory cell infiltration, disruption in epithelial lining, glandular atrophy, chronic inflammation in 14 days post surgical infection of C57BL/6 mice. Images were captured at 20× magnification. Scale bar represents 100 μm. Inflammatory cell infiltration indicated by (black arrowhead), gastric epithelial damage (green star); **(ii)** Histopathological scoring is done according to Huston updated Sydney classification system. Colored histogram represents the mean scores of histological scoring of experimental animals (n=6) and PBS controls (n=6). All experiments were performed in triplicate.

2.b.i. *H. pylori* OMVs induce pro-inflammatory cytokine response

35th day post immunization splenic cells were harvested from both immunized and nonimmunized mice and re-stimulated with 50μg of OMVs. A significant induction in IFN-γ, TNFα, IL-1β, IL-4, IL-10, IL-17, IL-6and IL-12, IL-13 levels were observed (**Figure 39: a-i**). Moreover, our data revealed oral immunization to be a better route for immunization as most of proinflammatory cytokines. Contrary to previous studies [101], our study did not find any Th1 or Th2 biased response indicating the immune response against OMVs is independent of routes of administration, compared to the control, both oral and i.p. immunized animals revealed elevation in cytokines. Altogether, immunization invoked an array of cytokines than non-immunized implying the potential of OMVs for a vaccine candidate.

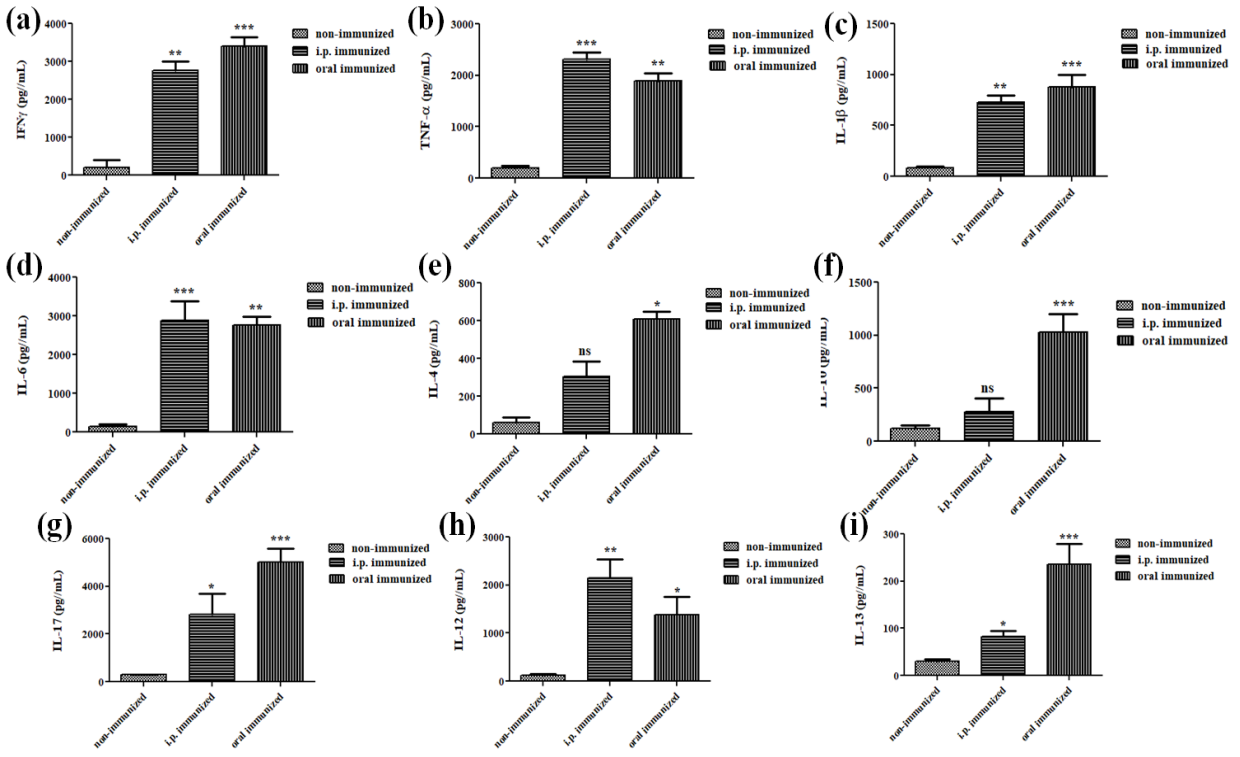


Figure 39: *Helicobacter pylori* OMVs induces the production of cell mediated cytokines responses. (a) IFN-γ, **(b)** TNF-α, **(c)** IL-1β, **(d)** IL-6, **(e)** IL-4, **(f)** IL-10, **(g)** IL-17,(**h**) IL-12, (**i)** IL-13, cytokines in culture supernatant of ex-vivo cultured splenic cells of immunized and nonimmunized (PBS immunized) mice after 24 h of restimulation with OMVs. The differences in immunized (i.p. and oral immunization) mice serum response of each of the studied cytokines were highly significant than nonimmunized. All cytokines are measured by ELISA (n = 6). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non significant). Each bar represents median and error values of six ± SE of three independent experiments.

2.b.ii. Protective efficacy study post OMVs immunization

After immunization with OMVs, the immunized and non-immunized animals were challenged with wild type SS1 with a dose mentioned before (i.e. 2x108 CFU) and the colonization were

analyzed 7 days post infection. A significant decrease in colonization was observed in the stomach tissue of immunized than non-immunized animals indicating a substantial reduction in bacterial load (**Figure 40:.D**). To confirm further, DNA was extracted from the gastric tissue and subjected to ureB PCR for the presence of *H. pylori*. All non-immunized mice were found to be positive whereas immunized animals were found insignificant presence - of *H. pylori* DNA among OMVs immunized animals (**Figure 40:C**). Histopathological changes of both immunized and non-immunized mice stomachs were analyzed 7 days post infection using surgical intervention (**Figure 40:A.i-ii**). OMVs immunized mice showed a significant reduction in gastric epithelial damage, altered gastric mucosa, inflammatory infiltration, exposed gastric pit, and metaplasia. Pathological scores were also less in immunized mice than non-immunized mice (Fig. 8.B). Overall, reductions in bacterial numbers were observed upon immunization.

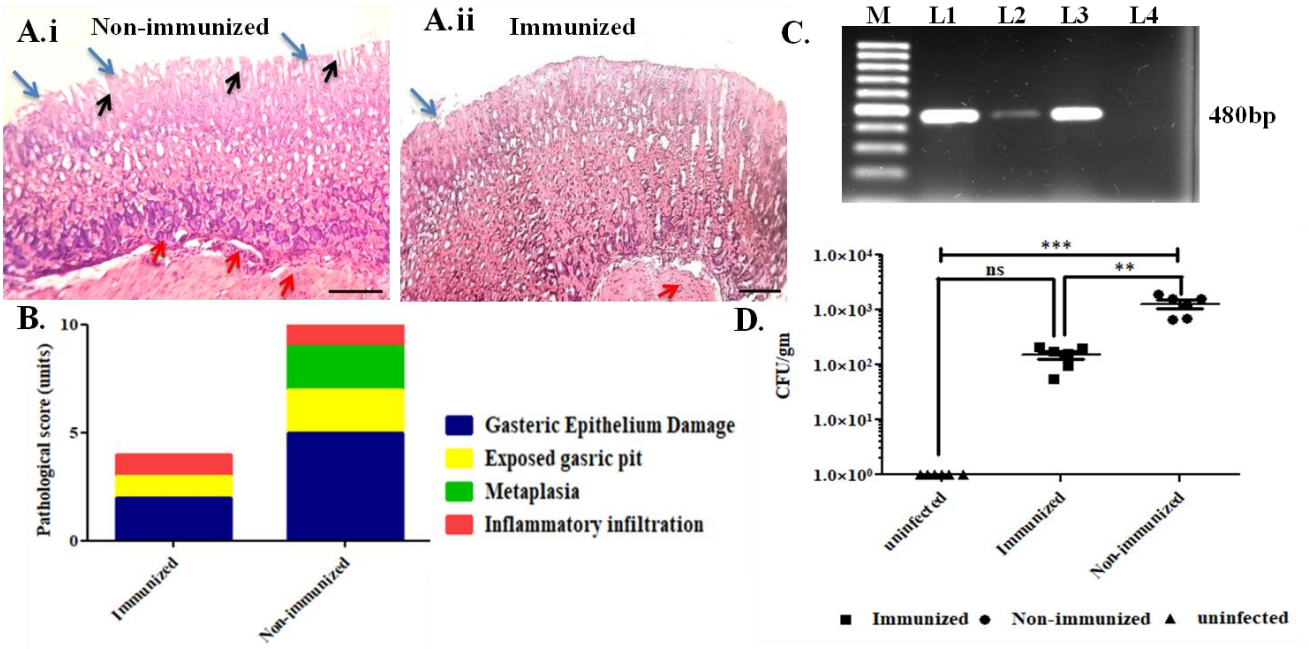


Figure 40: OMVs immunized mice shows reduced gastric tissue damage, inflammation after infection with SS1 (2 x 10^8 CFU) and reduce bacterial colonization in gastric tissue. Histological images represent both **(A.i)** non-immunized (PBS immunized) antrum of stomach

and **(A.ii)** immunized antrum of stomachs. OMVs immunized mice showed mild epithelial layer damage, less altered gastric mucosa and inflammatory infiltration, whereas non-immunized mice displayed marked epithelial damage, inflammatory infiltration, exposed gastric pit and early signs of gastric metaplasia. (Blue arrow: gastric epithelium; Black arrow: exposed gastric pit; Red arrow: Inflammatory infiltration) **(B)** Pathological scores of immunized or non-immunized mice post *Helicobacter pylori* SS1 challenge. **(C)** ***u****reB* PCR shows significant changes in bacterial DNA yield harvested from gastric tissues of both non-immunized and immunized mice post intragastric surgical challenge; **M:** 100bp ladder, **L1:** Positive control, **L2**: Immunized mice gastric tissue, **L3**: Non-immunized mice gastric tissue, **L4**: Negative control **(D)** *Helicobacter pylori* colonization in gastric tissue of immunized and non-immunized mice 7 days post challenge.

2.c.i Inflammatory cytokine response triggered by CurBGs

On the 35th day following the initial immunization, mice immunized with CurBGs and PBS was sacrificed, their spleens were harvested, and a single cell suspension had been produced and cultured. Next, the cells were re-stimulated with CurBGs and incubated overnight. The supernatant collected revealed significant up-regulation in TNFα, IFNγ, IL-1β, IL-13, IL-17, IL-12. These results indicate a robust Th1/Th2/Th17 mediated inflammatory response post immunization in CurBGs immunized mice (**Figure 41: a-f)**. Additionally, FACS analysis of different T-cell populations measured by their cell surface markers such as CD4+, CD8a+ and CD19+ were found to be elevated significantly in immunized animals than PBS immunized groups

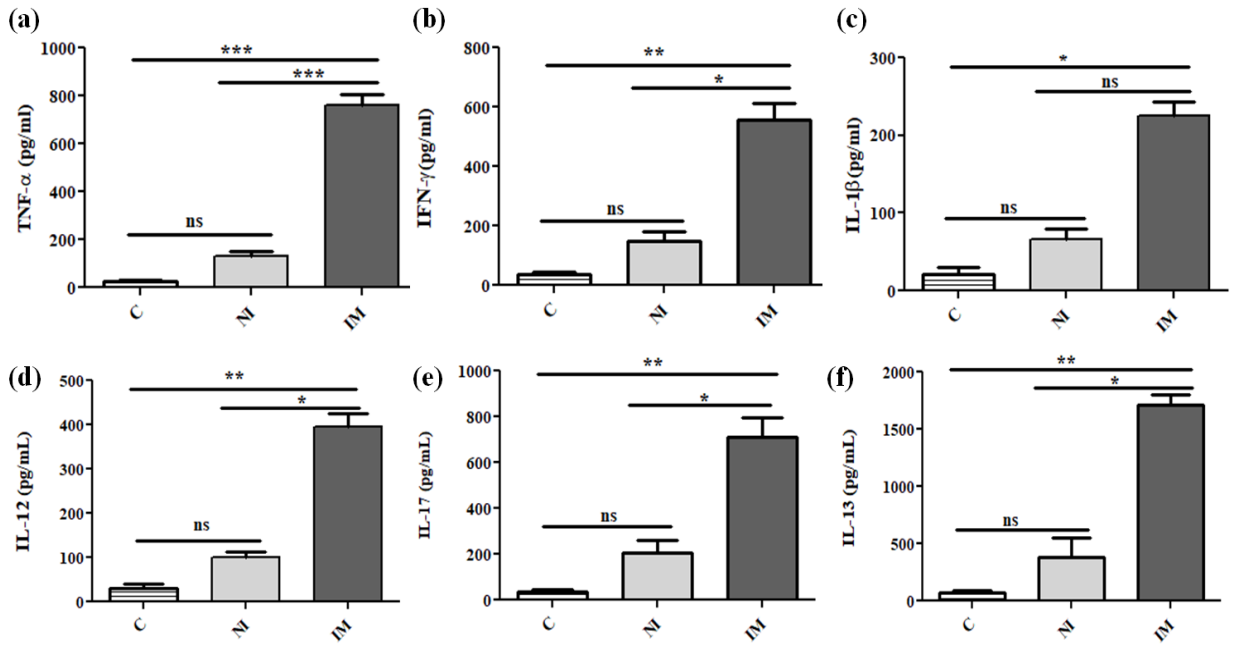


Figure 41: *Helicobacter pylori* CurBGs induce the production of cell mediated cytokines responses. (a) TNF-α, (b) IFN-γ, (c) IL-1β, (d) IL-12, (e) IL-17, (f) IL-13, cytokines in culture supernatant of ex-vivo cultured splenic cells of immunized and non-immunized (PBS immunized) mice after 24 h of restimulation with OMVs. The differences in immunized mice serum response of each of the studied cytokines were highly significant than nonimmunized. All cytokines are measured by ELISA (n = 6). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non significant). Each bar represents median and error values of six ± SE of three independent experiments.

2.c.ii. Bacterial colonization and histopathological analysis of CurBGs immunized animals

7 days post last immunization both immunized and non-immunized (or PBS immunized) animals were challenged with $2x10^8$CFU/mice with SS1 and bacterial colonization were determined from gastric tissues. A notable reduction in bacterial colonization was observed in immunized and non-immunized groups. Histopathological evaluation of gastric tissue was ascertained based on the degree of inflammation. Inflammatory response caused by *H. pylori* infection largely

depends on the inflammatory cell infiltration in the lamina propria of the stomach. Exposed gastric glands, gastric pits and damage in gastric epithelial lining are some of the changes in gastric topology directly associated with an active infection (**Figure 41: A-B**).

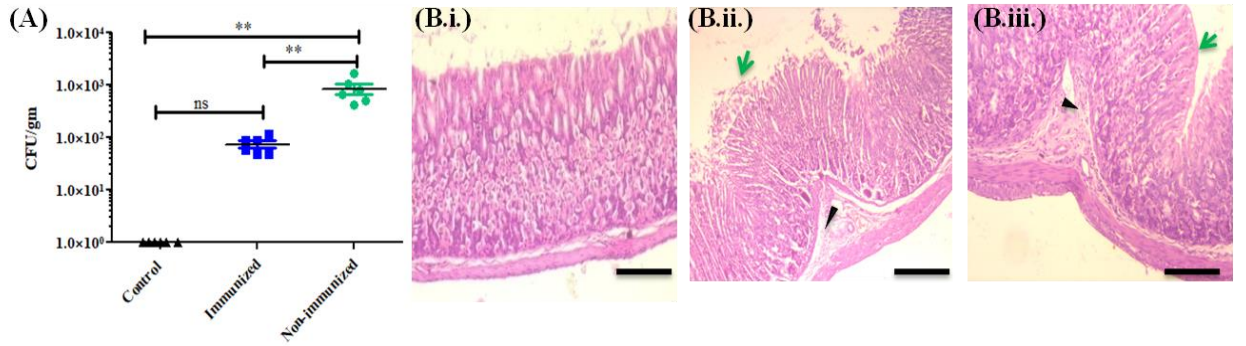


Figure 42: (A) *Helicobacter pylori* colonization in gastric tissue of immunized and non-immunized mice 7 days post challenge. **(B) Histopathological observation of antrum challenged with virulent wild type strain SS1**; **B.i.** PBS control, **B.ii.** Non-immunized, **B.iii.** CurBGs immunized, green arrows indicate damage due to infection, and black arrowheads indicate inflammatory cell infiltration. Statistical analyses were performed using the two tailed Student's t test (***p value <0.001, **p value <0.01).

3. Discussion

Over the years, a number of animal models have been evaluated for pathophysiology or treatment against *H. pylori*, including gnotobiotic pigs, dogs, cats, Mongolian gerbils, guineapigs, rhesus monkeys, and mice [113-123]. In most cases, C57BL/6 or black mice were explored extensively because of their substantial contribution in *H. pylori* -related studies. The proper route for administering the pathogen and/or immunization also has a key impact on the development of an infection and assessment of immune response, which is another important consideration when selecting an animal model for in vivo studies. Therefore, cytokine alterations along with histological changes described in the present study represent a successful infection

achieved through our newly developed surgical model. Conventional approaches for studying *H. pylori* infection in animals usually involve multiple oral inoculations using an oral gavage [124–125]. However, relying solely on the oral route to induce an infection and expecting the bacterium to outcompete the existing microflora and successfully colonize the stomach may not always yield a consistent result in any given experimental setting. This can cost significant time and resources being invested while still fostering uncertainty about an actual infective status in experimental animals. Therefore, it is important to consider the limitations and variability of the in vivo systems and look for alternative approaches that could provide a more reliable method of *H. pylori* -mediated pathogenesis in animal models. Clinical detection of *H. pylori* infection generally involves histology and PCR apart from the Rapid Urease Test (RUT) [126–129]. Serological tests are often avoided, as previously invoked antibodies fail to recognize the actual infective status of recent manifestations [130, 131]. As a consequence, this increases the chances of false positive results. In addition, histology allows visualization of pathogen-induced changes in gastric tissues, such as the intensity of inflammatory cell infiltration or aberrations in gastric topology, while PCR detects the presence of genomic DNA of *H. pylori* in gastric tissue samples [132]. However, it should be noted that neither histological observations nor negative PCR results rule out the presence of an infection [133]. Thus, a number of different techniques must be employed simultaneously to achieve a more accurate diagnosis of *H. pylori* infection [134]. Our study comprised a combination of histological observation, PCR detection and quantification of serum cytokine levels to confirm active *H. pylori* infection.

. To evaluate whether the immune response is Th1/Th2/Th17 specific, an *ex-vivo* spleen cell re-stimulation was performed. Inflammatory cytokines like TNFα, IFNγ, IL-1β, IL-13, IL-17, IL-12 was measured from the culture supernatant of both immunized and non-immunized spleenocytes

re-stimulated with CurBGs . Pronounced expressions of these cytokines highlighted the evolution of both innate and adaptive immune responses responsible to reduce the bacterial load in immunized animals compared to the non-immunized. Fluorescence activated cell sorting (FACS) analysis of both immunized and non-immunized animal derived spleen cells indicated generation of a substantial CD4+ and CD19+ population compared to CD8a+. Cytotoxic T cells expressing CD8a+ on their surface are generally responsible for initiating an innate immune response upon infection onset. This is accompanied by elevation in pro-inflammatory cytokines like TNFα, IFNγ, IL-1β. However, FACS result of CD8a+ population didn't coincide with the pro-inflammatory cytokine levels indicating a rise in Th1 cells, NK cells or macrophage cells derived cytokine response other than CD8a+ epithet. Further studies are needed to confirm the involvement of different immune cells in generating a robust immune response.

Histopathological analysis of gastric tissues harvested from both immunized and non-immunized mice post challenge shows some key differences. Epithelial lining of gastric mucosa was severely damaged along with surplus amount of inflammatory cell infiltration which is typical to *H. pylori* infection. In addition to this, glandular atrophy was prominent in non-immunized mice apart from early signs of metaplasia.

4. Conclusion:

Our model showed an initial elevation in these cytokines, which decreased over time, indicating progression toward a chronic infection. However, as 7 days were not sufficient to develop a chronic infection, our model showed promising results in a time-dependent manner. Consistent with the cytokine analysis, histopathological observations also validate such changes to some extent. Intense inflammatory cell infiltration was observed on day 7 than on day 14, and the

gastric lining was found to be more damaged with exposed gastric pits, indicating destruction caused by bacteria. Nevertheless, we did not find any striking structural abnormalities in gastric tissue 7 days post infection. PCR results from the same samples confirmed the presence of bacterial genomic DNA in experimental animals.

Histopathological analysis of gastric tissues harvested from both immunized and non-immunized mice post challenge shows some key differences. Epithelial lining of gastric mucosa was severely damaged along with surplus amount of inflammatory cell infiltration which is typical to *H. pylori* infection. In addition to this, glandular atrophy was prominent in non-immunized mice apart from early signs of metaplasia.

Chapter 7

General Discussion

Helicobacter pylori(*H. pylori*) is a gram-negative bacterium that colonizes the human stomach and causes a variety of gastrointestinal problems, including chronic gastritis, peptic ulcers, and even gastric cancer [135]. The development of a vaccination against *H. pylori* is an important public health goal due to the pathogen's broad prevalence and the difficulties associated with eradicating it using traditional treatments [136]. Vaccination could be used as a preventive technique to minimize the number of *H. pylori* infections and associated consequences. However, producing an effective vaccine is difficult due to the bacterium's ability to resist the immune system and its many strain variants. Research efforts are needed on discovering important antigens that elicit a powerful and long-lasting immune response, as well as understanding *H. pylori*'s immune evasion mechanisms. A successful vaccine development program could transform the management of *H. pylori*-related disorders and enhance global health outcomes.

Helicobacter pylori (H. pylori) infection affects over half of the global population, with considerable geographical differences. The highest prevalence is found in poorer countries, where it can surpass 80% due to factors such as inadequate sanitation and overcrowded living circumstances [137]. In wealthy countries, prevalence is often between 30 and 50%, indicating better living conditions and healthcare infrastructure. Despite these variations, *H. pylori* remains a serious public health concern, causing chronic gastritis, peptic ulcers, and stomach cancer. Addressing this widespread infection necessitates improved diagnostic, therapeutic, and preventive efforts, especially in high-prevalence areas where the impact is most severe [138].

Antimicrobial resistance in *Helicobacter pylori* has emerged as a rising public health concern, with more cases of resistance to medicines such as clarithromycin, metronidazole, and levofloxacin [139]. This resistance complicates treatment regimens and forces the employment of more expensive or ineffective alternative medicines. The growth in resistance is frequently caused by inadequate or improper antibiotic usage, underlining the need for better adherence to treatment guidelines and more precise diagnostic methods to customize medication to individual resistance profiles [140]. Addressing this issue is critical for increasing eradication rates and lowering the prevalence of *H. pylori*-related illnesses. The developing problem of antibiotic resistance in *Helicobacter pylori* has a considerable impact on treatment success rates. Resistance to first-line antibiotics like clarithromycin and metronidazole has resulted in more treatment failures, necessitating the adoption of other, often more expensive or complex regimens. This resistance is commonly related to antibiotic overuse and ineffective treatment regimens [141]. To address this tendency, it is critical to apply rational antibiotic usage methods, create novel therapeutic options, and expand surveillance systems that monitor resistance patterns and advise successful treatment decisions. *H. pylori* poses significant hurdles for effective treatment, with growing resistance rates weakening the efficacy of conventional medicines. Antibiotic resistance, such as that seen with clarithromycin and metronidazole, is particularly troubling since it increases the likelihood of treatment failure and infection persistence. Addressing this issue involves a multimodal approach that includes optimizing antibiotic stewardship, monitoring local resistance, and investigating new treatment alternatives. Improved diagnostic approaches for quickly identifying resistant strains can assist customize treatment tactics and enhance patient outcomes.

The treatment of *Helicobacter pylori (H. pylori)* infection has progressed dramatically over time, with several regimens aimed to efficiently eliminate the pathogen. The choice of therapy has a significant impact on therapeutic success and failure. A proton pump inhibitor (PPI) combined with clarithromycin and either amoxicillin or metronidazole is frequently used as first-line treatment for *H. pylori*. This regimen, known as triple therapy, works in a large proportion of instances but has limits. PPIs lower stomach acid, which improves antibiotic efficacy by boosting stability and penetration into bacterial cells. Clarithromycin and amoxicillin or metronidazole work together to destroy the bacterium [142]. Despite its early success, triple therapy confronts challenges with antibiotic resistance. Clarithromycin resistance has become a big issue, particularly in countries with high resistance rates. Metronidazole resistance is also frequent, which can reduce the regimen's efficacy. In addition, noncompliance owing to the regimen's intricacy or side effects might have a major impact on treatment outcome. The standard triple therapy for *H. pylori* consists of a PPI, clarithromycin, and amoxicillin. This strategy usually lasts 7 to 14 days, depending on the regimen and local requirements. It has been effective in many situations, although success rates are declining due to rising clarithromycin resistance. According to studies, resistance rates can surpass 20% in some places, reducing the efficiency of this regimen [143].

To address the limitations of triple therapy, quadruple therapy was devised. This treatment regimen contains a PPI, bismuth subsalicylate, doxycycline, and metronidazole. The inclusion of bismuth subsalicylate protects the stomach mucosa and has antibacterial effects. Tetracycline and metronidazole are used to combat the resistance difficulties related with clarithromycin and amoxicillin. Quadruple therapy has a greater eradication rate and is frequently employed when triple therapy fails or resistance is suspected. Quadruple therapy is more effective than single

therapy because it can combat resistance to many antibiotics at the same time [144]. However, therapy failure is still possible. Resistance to tetracycline and metronidazole, while uncommon, can emerge, and patient noncompliance can still affect success rates. Furthermore, the complexity of triple therapy may impair adherence, since patients must follow a more sophisticated regimen and may face greater adverse effects. The ultimate challenge in treating H. pylori is to overcome resistance while ensuring patient adherence. Antibiotic resistance is commonly the cause of treatment failure, rendering normal regimens ineffective. Local differences in resistance patterns need a personalized strategy to therapy that takes into account both local resistance data and patient-specific characteristics. Adherence is another essential element, as complex regimens can be difficult for patients to follow, resulting in partial treatment rounds and diminished efficacy. To summarize, while first-line and routine triple therapies for H. pylori have historically been effective, developing antibiotic resistance and patient adherence concerns have resulted in the creation and widespread usage of quadruple therapy. This more sophisticated regimen frequently results in a better eradication rate, but it also has its own set of obstacles. Finally, the efficacy of H. pylori treatment is dependent on a combination of effective regimens, knowing local resistance patterns, and patient compliance. The development of a vaccination against Helicobacter pylori (H. pylori) has been a focus of significant research, owing to the bacterium's role in peptic ulcers and gastric cancer. H. pylori affects more than half of the world's population, frequently asymptomatic, although it can cause significant gastrointestinal problems. The reason for vaccine research is clear: a successful vaccination might reduce the occurrence of these illnesses while reducing reliance on antibiotics, lowering the danger of antibiotic resistance. Early vaccination studies aimed to target major H. pylori

antigens such as the CagA protein, urease, and VacA, which are essential to the bacterium's pathogenic processes [145].

Preclinical trials have showed promise for both subunit vaccines, which employ pure proteins or peptides, and whole-cell vaccines, which use killed or attenuated bacteria. Subunit vaccines targeting urease have shown promise in triggering immune responses in animal models, but translating these findings into human efficacy has been difficult. Whole-cell vaccines, while comprehensive in antigen presentation, raise safety and adverse reaction issues, especially in live attenuated forms. Conjugate vaccines, which combine H. pylori antigens with carrier proteins, have demonstrated enhanced immunogenicity in tests, although there are still challenges in optimizing formulation and achieving long-term effectiveness. Clinical trials of vaccine candidates, such as those targeting the urease enzyme, have produced conflicting findings, with some showing encouraging immune responses and others revealing limitations in efficacy and safety. The bacterium's genetic variety hinders the identification of universally effective antigens, and vaccines must stimulate powerful, long-lasting immune responses. Despite these challenges, researchers are still looking at new ways, such as using adjuvants to boost immune responses, optimizing antigen delivery systems, and improving vaccine formulations. Advances in genetic engineering and a better knowledge of H. pylori's molecular mechanics provide hope for addressing these hurdles. In conclusion, while great progress has been made, the creation of an effective H. pylori vaccination remains a complicated and changing area, with future research aimed at overcoming current limits and achieving a successful preventive approach. Preclinical research is critical in the development of Helicobacter pylori (H. pylori) vaccines because it provides key data on safety, effectiveness, and immunogenicity prior to human trials. Vaccines are preclinically studied in vitro and in animal models to determine their ability to produce a

protective immune response. This phase is critical for identifying promising vaccine candidates and optimizing their formulations. Preclinical research with H. pylori aid in the evaluation of different antigens and adjuvants, exploration of various vaccine platforms (e.g. subunit, whole-cell, conjugate), and the determination of acceptable dosages and administration routes [146].

However, preclinical research has its limits. While animal models are useful, they do not always correctly mirror human reactions to H. pylori, which can lead to disparities in preclinical and clinical outcomes. Furthermore, preclinical studies may fail to capture the complexities of human immune responses or the genetic variety of H. pylori strains, reducing vaccination efficacy. Because of these differences, translating data from animal models to human trials can be difficult, and impressive preclinical results do not always ensure success in the clinical phases. Despite these limitations, preclinical research remains critical for directing the development of H. pylori vaccines and minimizing hazards prior to human trials [147].

Over the years, a number of animal models have been evaluated for pathophysiology or treatment against H. pylori, including gnotobiotic pigs, dogs, cats, Mongolian gerbils, guineapigs, rhesus monkeys, and mice [148]. In most cases, C57BL/6 or black mice were explored extensively because of their substantial contribution in H. pylori-related studies. The proper route for administering the pathogen and/or immunization also has a key impact on the development of an infection and assessment of immune response, which is another important consideration when selecting an animal model for in vivo studies. Therefore, cytokine alterations along with histological changes described in the present study represent a successful infection achieved through our newly developed surgical model. Conventional approaches for studying H. pylori infection in animals usually involve multiple oral inoculations using an oral gavage [149]. However, relying solely on the oral route to induce an infection and expecting the bacterium to

outcompete the existing microflora and successfully colonize the stomach may not always yield a consistent result in any given experimental setting. This can cost significant time and resources being invested while still fostering uncertainty about an actual infective status in experimental animals. Therefore, it is important to consider the limitations and variability of the in vivo systems and look for alternative approaches that could provide a more reliable method of H. pylori-mediated pathogenesis in animal models. Clinical detection of H. pylori infection generally involves histology and PCR apart from the Rapid Urease Test (RUT). Serological tests are often avoided, as previously invoked antibodies fail to recognize the actual infective status of recent manifestations [150]. As a consequence, this increases the chances of false positive results. In addition, histology allows visualization of pathogen-induced changes in gastric tissues, such as the intensity of inflammatory cell infiltration or aberrations in gastric topology, while PCR detects the presence of genomic DNA of H. pylori in gastric tissue samples. However, it should be noted that neither histological observations nor negative PCR results rule out the presence of an infection. Thus, a number of different techniques must be employed simultaneously to achieve a more accurate diagnosis of H. pylori infection [151]. Our study comprised a combination of histological observation, PCR detection and quantification of serum cytokine levels to confirm active H. pylori infection. Surgical intervention initially spiked pro-inflammatory cytokines such as IFN-γ and IL-1β along with IL-17 significantly more on day7 than on day14. However, as the infection progressed, these cytokines were lowered and finally balanced, except for IL-6, which was found to be elevated more on day 14 than on day 7. A pronounced IL-6 level at later stages might indicate ongoing inflammation in the gastric lining with potentially developing chronic gastritis. Such responses were further supported by the induction of other cytokines. IFN-γ is an early effector molecule responsible for generating a

Th1-mediated response by initiating different signaling cascades. However, up-regulation of IFN-γ transiently down-regulates IL-1β production. In addition, IL-17, a cytokine regulating the Th-17-based response, plays important roles in both pathogenesis and host immunity. Studies with chronic diseases have revealed that well-balanced IL-1β and IL-17 levels are constitutively produced to sustain inflammation due to infection in the long term [152]. In the case of H. pylori infection, both IL-1β and IL-17 play crucial roles in pathogenesis; in particular, IL-17 influences the disease outcome upon infection. Our model showed an initial elevation in these cytokines, which decreased over time, indicating progression toward a chronic infection. However, as 7 days were not sufficient to develop a chronic infection, our model showed promising results in a time-dependent manner. Consistent with the cytokine analysis, histopathological observations also validate such changes to some extent. Intense inflammatory cell infiltration was observed on day 7 than on day 14, and the gastric lining was found to be more damaged with exposed gastric pits, indicating destruction caused by bacteria. Nevertheless, we did not find any striking structural abnormalities in gastric tissue 7 days post infection. PCR results from the same samples confirmed the presence of bacterial genomic DNA in experimental animals. Next, we evaluated the surgical model for vaccine efficacy studies. Two different immunization routes were assessed to observe any alterations in the immune response due to changes in the route of administration. Immunization was performed both orally and intraperitoneally (i.p.) on days 0, 14 and 28. Initially, an elevation of serum IgG, IgM and IgA levels was observed against OMPs but not LPS of H. pylori. This can be due to the structural similarity between H. pylori LPS and blood antigens of the host [153]. Furthermore, we evaluated IgG2c (IgG subtype) and found it to be increased in immunized rather than non-immunized groups [154]. Our study found oral immunization to be better responsive than i.p. route, which can be due to the presence of

different surface proteins on OMVs that are more readily absorbed and reactive to gastric epithelial cells than peritoneal immune cells. A splenic cell re-stimulation (ex vivo) assay revealed enhanced Th2-based cytokine responses, such as IL-4, IL-13, IL-10 and IL-12, coinciding with previous studies with H. pylori-derived OMVs used as immunogens [156]. Interestingly, our study did not find any biased immune response against OMVs, indicating that the immune response to OMVs is not general but rather unique to each strain. CD4+, CD8a+ and CD19+ cell populations were increased due to OMV immunization independent of the route of administration. OMV immunization ultimately leads to a reduction in bacterial colonization in immunized animals but not in non-immunized animals. Serum bactericidal assay (SBA) typically denotes the functional aspect of immunogen-invoked antibody response in killing the bacterial population via complement mediated pathway. This in-vitro method involves incubation of bacteria in presence of heat-inactivated serum isolated from both OMVs immunized and PBS immunized mice. Antibodies generated in host due to immunization are sufficient enough to reduce the bacterial CFU by means of agglutination as demonstrated in OMVs induced immune response against S. Typhi and Paratyphi A [156]. However, agglutination doesn't directly imply a bacteriostatic or bactericidal activity of the antibodies. Therefore, addition of purified baby rabbit or guinea-pig complement externally to these antibodies ensured the lysis of the bacteria via complement-mediated pathway. In the present study, incubation of bacteria treated with OMVs immunized or PBS immunized sera in presence of guinea-pig complement lead to significant reduction in viable colony numbers in immunized compared to the PBS immunized mice groups.

The rising prevalence of antimicrobial resistance (AMR) in *H. pylori* presents serious threats to develop an effective treatment. Currently, no licensed vaccine is available and formulation of a

successful vaccine requires extensive research on antigens capable of eliciting a robust immune response. So far commendable efforts have been made; however, several studies indicated *H. pylori* antigens to be less immunogenic, sometimes requiring additional adjuvant to evoke better immunogenicity. Bacterial ghost (BGs) is an empty cell envelope and an emerging platform in vaccine research [157]. Despite its tremendous potential as a vaccine candidate, research in this area has been limited. Genetically manipulated bacteria over-expressing pore forming genes like Lysis E, is one of the most widely used technique besides chemical modification. Using chemical agents for BGs preparation has a number of advantages over genetic manipulation, including feasibility of production time and cost while maintaining surface integrity and efficacy intact. Exposure to chemicals for certain duration allows formation of transmembrane tunnel-like structures on bacteria that promote removal of inclusion bodies, leaving behind the empty envelops. Inorganic chemical agents like sodium hydroxide (NaOH), hydrochloric acid (HCl), sulfuric acid (H_2SO_4), Tween-80 are some of the chemicals used to produce BGs [158]. However, harnessing the potential of a phytochemical based agent to produce BGs is yet to be explored extensively thereby eliminating any possibilities of side effects due to chemical residue.

The major problem using a phytochemical agent is their solubility which is very much dependent on the nature of solvent along with parameters like temperature and pH. Crude extracts of any plant derived product are usually non-polar in nature. For example, bioactive component of turmeric, curcumin, is completely water-insoluble in its raw form and appears as “shards of glasses” under scanning electron microscope. Despite that, several techniques are available that breaks down raw curcumin into particles <100nm in size, thereby improving solubility in water and reducing hydrophobicity. Additionally previous studies with curcumin confirmed that formulation of nanocurcumin changes the size without affecting their chemical integrity [159].

In-vivo studies of *H. pylori* infection and treatment revealed significant improvement in gastric architecture when curcumin is employed therapeutically due to its antimicrobial effect. However, translating such results to treat human infection is challenging because of bioavailability issues subsequently affecting the determination of effective dosing of curcumin in human. When bacteria come in contact with nanocurcumin, the lipid bilayer interacts with the curcumin particles and changes membrane integrity. Disruption in membrane integrity facilitates pore-mediated leakage which irreversibly damages the bacteria. Scanning electron microscopic images of nanocurcumin induced BGs confirmed formation of such pores on bacterial surfaces [160].

Curcumin has no effect on the outer membrane proteins or LPS of bacteria. The comparison between surface protein and LPS profile of the CurBGs to that of whole cell lysate of the same strains found no significant variations indicating conservation of surface antigens. LPS is known to be highly toxic due to their inflammatory nature prompted by their interaction with TLR-4 [161]. Cytotoxicity assay confirmed the CurBGs to be significantly less toxic than positive control, allowing it to be used as an immunogen to evoke a robust immune response in C57BL/6 mice. Oral administration of CurBGs thrice (day 0, 14 and 28) at an interval of 14 days significantly elevated serum immunoglobulin levels of IgG and its subtypes IgG1 and IgG2c against OMPs and WCL isolated from *cagA*+(SS1) and *cagA*-(AM1) strains [162]. This depicts generation of long term immunity in immunized mice compared to the non-immunized groups. Moreover, rise in secretary IgA (sIgA) level in gastric lavage indicated development a prominent mucosal immune response in immunized animals. Next, we assessed the effect of generated antibodies on motility and mucin penetration ability of *cagA+/cagA*-strains and found observable differences in both. Compared to the control, immunized serum treated bacteria were unable to

move through the soft agar plate and mucin validating generation of antigen-primed antibodies against these antigens [163]. Additionally, when immunized and non-immunized heat-inactivated sera was supplemented with complement system externally and incubated with these strains, their growth reduced noticeably. Further, SEM images confirmed lysis of bacterial cells due to immunized sera treatment [164]. Western blot analyses showed multiple bands indicating the development of antibodies against OMPs of *cagA+/cagA*-strains explaining the role of surface antigens in developing immunogenicity. To evaluate whether the immune response is Th1/Th2/Th17 mediated, ex-vivo spleen cell re-stimulation was performed. TNFα, IFNγ, IL-1β, IL-13, IL-17, IL-12p40/70 was measured from the culture supernatant of both immunized and non-immunized spleenocytes re-stimulated with CurBGs [165]. Pronounced expressions of these cytokines indicate both cell mediated and humoral immune response is responsible to reduce the bacterial burden in immunized animals compared to the non-immunized.

Histopathological analysis of gastric tissues harvested from both immunized and non-immunized mice post challenge shows some key differences. Epithelial lining of gastric mucosa was severely damaged along with surplus amount of inflammatory cell infiltration which is typical to *H. pylori* infection [166]. In addition to this, glandular atrophy was prominent in non-immunized mice apart from early signs of metaplasia [167].

Chapter 8

Pivotal findings of the Thesis work in nutshell

- Biochemical tests of the strains revealed positive for oxidase, catalase and urease. Antimicrobial susceptibility test (AST) with antibiotics such as metronidazole, clarithromycin and amoxicillin revealed majority of the strains resistant to metronidazole and sensitive towards clarithromycin, amoxicillin.

- PCR based genotypic characterization revealed presence of virulence genes e.g. *ure*B, *cag*A, *vac*A with its allelic orthologs, *dup*A, *bab*A2 along with plasticity associated genes like *jhp0947, jhp0950* at various degrees among the strains.

- Whole genome sequencing was performed for extensive molecular characterization based on genome content analysis. A 1.67Mb genome with 38.77% GC content along with 36 tRNA and 3 rRNA annotations coincided with that of reference strains deposited in NCBI. Moreover, 99.51% similarity was observed with ATCC43504 strain in respect to their 16s rRNA sequence.

- Transmission electron microscopy (TEM) revealed OMVs to be spherical in shape with distinct lipid bilayers while dynamic light scattering (DLS) showed homogeneity in OMVs population with a diameter of 50nm.

- Proteomic analysis of the OMVs revealed presence of a number of antigenic proteins with putative functions such as nickel cation binding activity, urease activity, transmembrane transporter activity etc responsible for developing an immune response.

- Nanocurcumin was formulated from pure raw curcumin using wet-milling technique.

- Scanning electron microscopy (SEM) revealed the particles with a size less than 100nm which was further backed up by the dynamic light scattering (DLS) observation confirming presence of heterogenous population of nanoparticles less than 100nm in size.

- Bacterial culture treated with nanocurcumin gave rise to bacterial ghosts (CurBGs).

- Scanning electron microscopy (SEM) revealed CurBGs having multiple transmembrane tunnels on their surface to expel out the inclusion bodies from the cells.

- Viability was checked by plating the CurBGs on enriched media and no colonies were recovered.

- Cytotoxicity assay was performed using murine macrophage cells revealed each of the immunogens, OMVs and CurBGsto be significantly less toxic.

- In-vivo administration of either OMVs (50µg) or CurBGs (10^8cells) in animals (black mice) revealed no changes in physiological parameters indicating they are safe to be administered to the animals.

- Immunization was done either orally and intraperitoneally (OMVs) or orally (CurBGs) on day 0, 14 and 28 while blood was drawn out 7 days post each successive immunization.

- Serum immunoglobulin levels of OMVs immunized animals showed significant elevation in IgG, IgA and IgM levels against outer membrane proteins (OMPs) and whole cell lysates (WCL) but not lipopolysaccharides (LPS) due to their structural resemblance with host blood antigens (Lewis $^{a/b}$).

- Serum bactericidal assay (SBA) and SEM image revealed bactericidal activity of OMVs immunized serum in presence of externally supplied complement implying generation of specific immunogen-primed antibodies.

- Serum cytokine levels showed induction in inflammatory cytokines IFN-γ,TNF-α, IL-1β, IL-6, IL-4, IL-10, IL-17, IL-12, IL-13 indicating a prominent Th1/Th2/th17 based immune response with a significant CD4+, CD8a+ and CD19+ population in OMVs immunized animals.

- Similar to OMVs, CurBGs evoked long term immunity which was evident in heightened serum IgG level and its subtypes IgG1 and IgG2c against outer membrane proteins (OMPs) and whole cell lysates (WCL) but not lipopolysaccharides (LPS) due to their structural resemblance with host blood antigens (Lewis $^{a/b}$).

- Serum bactericidal assay (SBA) and SEM image revealed bactericidal activity of CurBGs immunized serum in presence of externally supplied complement implying generation of specific immunogen-primed antibodies.

- Mucin penetration ability and motility of the bacterium were greatly mitigated in presence CurBGs immunized sera implying the evolution of antibodies against surface antigens including flagella.

- Serum cytokine levels showed induction in inflammatory cytokines IFN-γ,TNF-α, IL-1β, IL-17, IL-12, IL-13 indicating a prominent Th1/Th2/th17 based immune response with a significant CD4+, CD8a+ and CD19+ population in CurBGs immunized animals.

- An animal model was developed to study the protective efficacy of the immunogens.

- Intragastric surgical model involved infection directly to the stomach of an animal without receiving any preparatory treatment.

- The process induced infection within 7 days and a single dose of inoculum was sufficient to serve the purpose thereby minimizing any experimental errors.

- Infection with wild type SS1 (WTSS1) strain of *H. pylori* evoked an array of cytokines including IFN-γ,TNF-α,IL-1β, IL-10, IL-6, IL-17. All of these cytokines except IL6 was found to be more on day 14 than day 7 post-surgical infection, indicating a transition from an acute phase to chronic phase of infection.

- Histopathological observation of intragastric surgical model significantly affected gastric architecture indicated by heightened expression of neutrophil infiltration, glandular atrophy, intestinal metaplasia and chronic infection.

- Protective efficacy of both OMVs and CurBGs immunized animals was assessed by challenging both OMVs and CurBGs immunized animals with WT SS1.

- Seven days post challenge revealed a significant reduction in bacterial colonization in immunized animals than non-immunized animals. Moreover, gastric topology was much more ameliorated in immunized animals than non-immunized cohorts indicating the potential of these immunogens as a vaccine candidate.

Chapter 9

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Chapter 10

Patents applied

The following patents have been applied through IPR Unit of ICMR:

1. "A Novel Formulation of Sodium Alginate Encapsulated Green-Synthesized Titanium Nanoparticle Coated OMVs based vaccine (SGTiOMVs) against prevalent strains of *Helicobacter pylori*" the complete patent application of this invention has been filed on 11th April, 2024 and the patent application number is 202411029436

2. "A novel Trivalent Iron Nanoparticles Electroporated Outer Membrane vesiclesbased antigen (TINEOMVs) as a vaccine candidate against *Campylobacter Jejuni, Salmonella Typhimurium* and *Salmonella Enteritidis*" the complete patent application of this invention has been filed on 30th May, 2023 and the patent application number is 202311037297

Chapter 11

Original Publications

1.**Das S**, Halder P, Banerjee S, Mukhopadhyay AK, Dutta S, Koley H. Establishment of an intragastric surgical model using C57BL/6 mice to study the vaccine efficacy of OMV-based immunogens against Helicobacter pylori. *Biol Open*. Published online April 11, 2024. doi:10.1242/bio.060282

2. **Das, S**., Banerjee, S., Halder, P., Dutta, S., Mukhopadhyay, A., & Koley, H. (2024). A review for the Prevention and management of helicobacter pylori induced gastritis through development of novel vaccine candidates. *The Microbe*, 100114. https://doi.org/10.1016/j.microb.2024.100114

3.Halder, P., Maiti, S., Banerjee, S., **Das, S**., Dutta, M., Dutta, S., & Koley, H. (2023). Bacterial ghost cell based bivalent candidate vaccine against salmonella typhi and salmonella paratyphi A: A prophylactic study in BALB/C Mice. *Vaccine*, *41*(41), 5994–6007. https://doi.org/10.1016/j.vaccine.2023.08.049

4.Banerjee S, Halder P, **Das S**, Maiti S, Bhaumik U, Dutta M, Chowdhury G, Kitahara K, Miyoshi SI, Mukhopadhyay AK, Dutta S, Koley H. Pentavalent outer membrane vesicles immunized mice sera confers passive protection against five prevalent pathotypes of diarrhoeagenic Escherichia coli in neonatal mice. Immunol Lett. 2023 Nov;263:33-45. doi: 10.1016/j.imlet.2023.09.009. Epub 2023 Sep 19. PMID: 37734682.

Chapter 12

Published abstracts for presentation in scientific meetings

1. Outer Membrane Vesicles based livestock Non-typhoidal Salmonella Candidate Vaccine help to reduce Clinical Health Burden Hemanta Koley*1 , Suhrid Maiti1 , Prolay Halder1 , Soumalya Banerjee1 , **Sanjib Das1** , Moumita Dutt2 , Asish Kumar Mukhopadhyay1 , Shanta Dutta1, 16th Asian Conference on Diarrhoeal Disease and Nutrition,2022, WestInn, Kolkata.

2. Development and Evaluation of a simple PCR assay and nested PCR for rapid detection of clarithromycin resistant Helicobacter pylori from culture and directly from biopsy samples Bipul Chandra Karmakar1 , Sangita Paul1 , Puja Bose1 , **Sanjib Das1** , Sujit Chaudhuri2 , Shanta Dutta1 , and Asish Kumar Mukhopadhyay1*, 16th Asian Conference on Diarrhoeal Disease and Nutrition,2022.

3. "Santalol"- A corridor to alternative therapeutic approach against Helicobacter pylori clinical isolates Sangita Paul1 , Bipul Chandra Karmakar1 , Puja Bose1 , **Sanjib Das1** , Sujit Chaudhuri2 , Shanta Dutta1 and Asish Kumar Mukhopadhyay1*, 16th Asian Conference on Diarrhoeal Disease and Nutrition,2022.

4. Selection and characterization of Helicobacter pylori to formulate a next generation OMVs-based immunogen **Sanjib Das1** , Prolay Halder1 , Soumalya Banerjee1 , Sangita Paul1 , Shanta Dutta1 , Asish Kumar Mukhopadhyay1 , Hemanta Koley1 *, 16th Asian Conference on Diarrhoeal Disease and Nutrition,2022.

5. The prospective therapeutic actions of natural agent Curcumin for Shigellosis Puja Bose1 , Tanmoy Kumar Dey1 , Bipul Chandra Karmakar1 , Sangita Paul1 , **Sanjib Das1** , Shanta Dutta1 , Asish Kumar Mukhopadhyay1*, 16th Asian Conference on Diarrhoeal Disease and Nutrition,2022.

6. Bivalent typhoidal bacterial ghost acts as a protective immunogen against Salmonella Typhi and Salmonella Paratyphi A in mice model. Prolay Halder*, Soumalya Banerjee, **Sanjib Das**, ShantaDutta, Hemanta Koley**, 16th Asian Conference on Diarrhoeal Disease and Nutrition,2022.

7. Development of a pentavalent next generation Outer Membrane Vesicles based immunogen to ameliorateprevalent multi drug resistant strains of diarrhoeagenic Escherichia coli Soumalya Banerjee* , Prolay Halder, **Sanjib Das**, Shanta Dutta, Hemanta Koley**, 16th Asian Conference on Diarrhoeal Disease and Nutrition,2022.

8. Preparation and immunogenic characterization of Helicobacter pylori to formulate a next generation OMVs-based vaccine, **Sanjib Das*,** Prolay Halder, Soumalya Banerjee, Sangita Paul, Asish Kumar Mukhopadhyay, Shanta Dutta, Hemanta Koley**, 108th Indian Science Congress Association, Nagpur, Maharashtra.

9. Studies on passive protection in suckling mice model conferred by pentavalent outer-membrane vesicles immunized mice sera against infection caused by five prevalent strains of diarrhoeagenic Escherichia coli Soumalya Banerjee * , Prolay Halder, **Sanjib Das**, Shanta Dutta, Hemanta Koley ** 108th Indian Science Congress Association, Nagpur, Maharashtra.

10. Bivalent typhoidal bacterial ghost based immunogen of Salmonella Typhi and Salmonella Paratyphi A induce adaptive immunity and protective efficacy in mice. Prolay Halder*,

Soumalya Banerjee, **Sanjib Das**, Shanta Dutta, Hemanta Koley**,108th Indian Science Congress Association, Nagpur, Maharashtra.

11. Establishment of an intra-gastric surgical model in C57BL/6 mice to study the vaccine efficacy against Helicobacter pylori. **Sanjib Das*,** Prolay Halder, Soumalya Banerjee, Shanta Dutta, Asish Kumar Mukhopadhyay, Hemanta Koley**, Frontiers in Modern Biology, 2023, IISER Kolkata, Mohanpur,West Bengal, India.

12. Unravelling immunological insights using an intra-gastric surgical model in C57BL/6 mice for assessing vaccine efficacy against Helicobacter pylori. **Sanjib Das***, Prolay Halder, Soumalya Banerjee, Shanta Dutta, Asish Kumar Mukhopadhyay, Hemanta Koley**, Immunocon 50, AIIMS New-Delhi.

13. A prophylactic study on curcumin nanoparticle induced bacterial ghosts against *Helicobacter pylori*. **Sanjib Das**, Prolay Halder, Soumalya Banerjee, Samima Sultana, Asish Kumar Mukhopadhyay, Shanta Dutta, Hemanta Koley*, Frontiers in Disease Biology, Kalimpong, West Bengal.

14."Hypocholesterolemic effect of chemically modified Mango Kernel Starch on diet induced dyslipidemia in animal model". Samima Sultana*, **Sanjib Das**, Prolay Halder, Anusree Giri, Ananya Roy, Hemanta Koley, Samadrita Sengupta**. One day National Symposium on Human Origin, Health and Diseases-New Dimension, Sister Nivedita University, Kolkata.

Establishment of an intragastric surgical model using C57BL/6 mice to study the vaccine efficacy of OMV-based immunogens against *Helicobacter pylori*

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Abstract

Chronic gastritis is one of the major symptoms of gastro-duodenal disorders typically induced by *Helicobacter pylori* (*H. pylori).* To date, no suitable model is available to study pathophysiology and therapeutic measures accurately. Here, we have presented a successful surgical infection model of *H. pylori*-induced gastritis in C57BL/6 mice that resembles features similar to human infection. The proposed model does not require any preparatory treatment other than surgical intervention. C57BL/6 mice were injected with wild-type SS1 (Sydney strain 1, reference strain) directly into the stomach. Seven days post infection, infected animals showed alterations in cytokine responses along with inflammatory cell infiltration in the lamina propria, depicting a prominent inflammatory response due to infection. To understand the immunogenicity and protective efficacy, the mice were immunized with outer membrane vesicles (OMVs) isolated from an indigenous strain with putative virulence factors of *H. pylori* [A61C (1), *cag+/vacA s1m1*]. In contrast to the non-immunized cohort, the OMV-immunized cohort showed a gradual increase in serum immunoglobulin(s) levels on the 35th day after the first immunization. This conferred protective immunity against subsequent challenge with the reference strain (SS1). Direct inoculation of *H. pylori* into the stomach influenced infection in a short time and, more

importantly, in a dose-dependent manner, indicating the usefulness of the developed model for pathophysiology, therapeutic and prophylactic studies.

Keywords: Animal model, Surgical intervention, Gastric illness, Outer membrane vesicles, Vaccine efficacy, *Helicobacter pylori*.

Background

Gastroduodenal disorders are the cumulative effect of carefully orchestrated molecular interactions between host and pathogen factors belonging to the genus *Helicobacter* [1]. With almost 50% of the population worldwide infected by the pathogen, it is one of the major health burdens in developing nations [2]. Although *H. pylori* has been recognized as a class I carcinogen by the WHO, very little has been explored thus far. This is primarily due to the asymptomatic nature of infected individuals, expensive clinical detection (e.g., endoscopy, urea breath test, etc.) and diagnosis with considerable information scarcity [3]. In addition to this, the global antimicrobial resistance (AMR) pattern of *H. pylori* is changing alarmingly, resulting in a paradigm shift in "treatment of choice" by clinical practitioner [4].

Research in *in vitro* and *in vivo* systems of *H. pylori* is continuously enriching our understanding of pathophysiology and genetic predisposition related to adaptation, survival and coevolution of the pathogen [5, 6]. For instance, *H. pylori* has the inherent ability to modulate the gastric microenvironment, such as increasing gastric pH by means of urease upregulation, employing different adhesion proteins or simply dislodging itself when the pH becomes overwhelmingly acidic [7,8]. Such responses, along with others, act as the precursor to a chronic infection that largely depends on the gastric acid neutralizing capacity unique to each strain. The pathogen is known to recruit different adhesins depending upon the stages of disease progression, such as BabA during early infection or SabA during ongoing inflammation [9]. In addition, host antigens present on the surface of host cells, mucins and other gastric cells, such as A/B-Le^b, MUC5AC, MUC1 and H type 1, play important roles in bacterial adhesion, further promoting the severity of different gastric maladies [10,11].

To date, a combination of antibiotics with a proton pump inhibitor (PPI) is the only mode of treatment available due to the lack of a potent vaccine [12]. Moreover, an efficient animal model is crucial to understanding the immunological attributes of different immunogen(s) for vaccine development, which existing models fail to satisfy. To date, considerable efforts have been made to establish a reliable murine (gerbil or mouse) model to serve this purpose, including extensive application of transgenic animals with single or double mutations, but unfortunately, no significant efforts have been made toward the route of administration to induce an infection [13]. The preexisting method relies on the oral administration of multiple doses of inoculums along with antibiotic pretreatment to induce an infection [14]. Moreover, it takes a minimum of two weeks to develop an infection using the traditional approach, which is significantly higher than any other enteric pathogens, such as *E. coli* or *Salmonella*, while using an animal model [15–17].

Therefore, in this study, we introduced an infection by surgically exposing the stomach of C57BL/6 mice and directly injecting *H. pylori* inoculums. We assessed different pathological and immunological markers for active infection and applied the same to study the vaccine efficacy of OMV-based immunogens isolated from a prevalent strain.

Methods

Bacterial strains and culture conditions

Bacterial strains were revived from glycerol stock using brain heart infusion agar (BD Difco, USA) supplemented with 7% horse blood, 0.4% IsoVitaleX with antibiotics such as amphotericin B, trimethoprim, and vancomycin (Sigma Aldrich, USA) at concentrations as described previously [18]. Inoculated plates were then kept under microaerophilic conditions (5% O2, 10% CO2, and 85% N2 at 37°C) for 48 hrs and sub-cultured before conducting any experiment.

Broth culture was prepared using Brucella Broth (BD, Difco, USA) supplemented with 10% horse serum and vancomycin (Sigma, USA). The inoculated flask was then kept in shaking conditions (100 rpm) overnight while maintaining the microaerophilic environment [19].

Characterization and selection of strains

All strains were checked for oxidase, catalase and urease as mentioned elsewhere [20]. Next, an antibiogram was performed using the agar dilution method following CLSI guidelines (**Supplementary table:1**). PCR-based detection was applied for genotypic characterization. Some major virulence factors, such as *cagA, vacA, babA* and *dupA,* were checked using either simplex or multiplex PCR [21,22]. The primers used in the present study are listed in a table **(Supplementary table: 2)**.

Animals

Six- to eight-week-old female C57BL/6 mice were received from the NICED-Animal house facility. The animals were kept in a condition maintained at 25±2°C with 65 ± 2% humidity and a 12/12-hour light/dark cycle. Animals weighing ~22 grams were selected for the study and provided with sterile food and water *ad libitum*. All experiments were performed following the standard operating procedure outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (CPCSEA Registration no. 68/GO/ReBi/S/1999/CPCSEA valid 17.07.2024) and Institutional Animal Ethics Committee (IAEC) of NICED was approved (Approval No. PRO/194/June 2022-25) and supervised experimental design and protocols from time to time.

Animal experimental design

Thirty-six C57BL/6 mice were randomly assigned into two major groups, each comprising 18 animals. To determine an infectious dose for the surgical model, the first set of mice was further divided into three subgroups and infected with a dose of either $1x10^8$ (n=6) or $2x10^8$ CFU/mL (n=6, each) along with PBS control (n=6). All groups were housed for one or two weeks under sterile conditions.

For immunological studies, the remaining 18 mice were separated into two groups as: non-immunized (NI) (n=6) and oral or i.p. immunized (IM) (n=6 in each group). An oral or

intraperitoneal immunization with 50 µg of OMVs dissolved in PBS was administered on days 0, 14, and 28. Blood was collected at different time points, and the serum was isolated and stored at -20°C for use in different immunological assays. For the protective efficacy study, both groups (IM and NI) were infected surgically on the 35th day post-first immunization and sacrificed 7 days post-infection **(Supplementary figure:1)**.

Intragastric surgical model development

Experimental animals were kept in fasting conditions overnight with sterile water. Initially, animals were sedated by an intraperitoneal injection of a mixture of ketamine (87.5 mg/kg) and xylazine (12.5 mg/kg) [23]. The stomach was exposed through a 2-3 cm midline incision without compromising any major blood supply. A disposable syringe with a 26G needle containing 200 µl (~2x10^{8} CFU) of the inoculums in PBS was directly injected into the stomach. Hydration was maintained in the exposed stomach using sterile normal saline throughout the surgery. The stomach was placed back inside the abdominal cavity, and the incision was sutured back. The incision site was monitored for any infection and occasionally washed with 5% povidone-iodine (betadine) soaked in a sterile gauge for 72 hours [24]. Sterile food and water were provided to the animals once they regained consciousness (**Fig. 1**).

Post-Surgery observation

All infected mice were observed twice a day for 7 days. Physical parameters were checked along with stool consistency and the nature of mucus or blood (if any) present in the feces. Rectal swabs were taken daily and were subjected to RUT solution and spread-plate to observe the shedding of the organism. Isolated colonies (if any) were confirmed using a PCR-based technique. *H. pylori* infection augments the modulation of both pro- and anti-inflammatory cytokines in the host [25]. Therefore, IL-1β, TNFα, IFNγ, IL-6, IL-10 and IL-17 were tested using cytokine measuring kits (Invitrogen, USA) following the manufacturer's protocol. 50µl of serum samples from 0-day, 7-day- and 14-day-infected mice were used to quantify the inflammatory response after post-operational (OP) observation due to infection.

Immunogen preparation

Outer membrane vesicles (OMVs) were isolated from the *Helicobacter* strain [A61C (1), *cagA*+, *vacA s1m1*] following the methods described previously with slight modification [26]. In brief, BB broth (BD, Difco, USA) was inoculated with log phase (OD_{600} ~0.6) pre-culture of the respective strains and kept overnight in microaerophilic conditions under constant shaking (100 rpm) at 37°C. On the next day, centrifugation was performed consecutively first at 8000xg for 15 min st 4°C, followed by 30 min with same conditions. The supernatants were then filtered twice with 0.45 µm and 0.22 µm syringe filters (Millipore, USA). To prevent protein degradation, a protease inhibitor cocktail was incorporated into the filtrate and ultracentrifuged at 140,000 x g at 4°C for 4 hrs using a P27A-1004 rotor (Hitachi). A density gradient centrifugation allowed obtaining the purified OMVs. Protein content was measured using a Lowry protein estimation kit (Pierce, USA) and stored at -20°C until further use.

Characterization of the Outer Membrane Vesicles (OMVs)

Dynamic Light Scattering

Concentrated OMVs were diluted 10-fold to reach a concentration of 0.1 mg/mL. The hydrodynamic size of OMVs was measured using a Malvern Zetasizer ZS90 (Malvern Instruments, Germany) and analyzed using ZS Xplorer version 3.1.0.64 [27].

Transmission electron microscopy

Diluted OMVs were placed on a carbon-coated grid and left for 10-20 min for absorption. The samples were then washed twice with drops of Tris buffer solution. Excess fluid was soaked using blotting paper, followed by staining with 2% uranyl acetate and air drying. Finally, the OMV-coated grids were observed under a JEOL JEM 2100 HR (JEOL, Tokyo, Japan) [28].

LC/MS of OMVs and analyses

Proteins present in OMVs were used for digestion and reduced with 5 mM TCEP and further alkylated with 50 mM iodoacetamide and again digested with trypsin (1:50, trypsin/lysate ratio) for 16 h at 37°C. Digests were cleaned using a C18 silica cartridge to remove the salt and dried using a speed vac. The dried pellet was resuspended in buffer A (2% acetonitrile, 0.1% formic acid). Experiments were performed on an Easy-nlc-1000 system coupled with an Orbitrap

Exploris mass spectrometer. One microgram of peptide sample was loaded on a C18 column (15 cm, 3.0 μm Acclaim PepMap, Thermo Fisher Scientific), separated with a 0–40% gradient of buffer B (80% acetonitrile, 0.1% formic acid at a flow rate of 500nl/min) and injected for MS analysis.LC gradients were run for 60 minutes. MS1 spectra were acquired in the Orbitrap (MaxIT = 25 ms, AGQ target=300%; RF lens = 70%; R=60 K, mass range = 375−1500; profile data). Dynamic exclusion was employed for 30s, excluding all charge states for a given precursor. MS2 spectra were collected for the top 12 peptides. MS2 (Max IT= 22 ms, R= 15 K, AGC target 200%). All samples were processed, and the generated RAW files were analyzed with Proteome Discoverer (v2.5) against the UniProt organism database. For dual Sequest and Amanda searches, the precursor and fragment mass tolerances were set at 10 ppm and 0.02 Da, respectively. The protease used to generate peptides, i.e., Enzyme specificity was set for trypsin/P (cleavage at the C-terminus of "K/R: unless followed by "P"). Carbamidomethyl on cysteine as a fixed modification and oxidation of methionine and N-terminal acetylation were considered variable modifications for the database search.

Extraction of lipopolysaccharide (LPS) and outer membrane proteins (OMPs)

LPS and OMPs were extracted following the methods described earlier [29]. LPS was then treated with proteinase-K to ensure the absence of any protein residue. The carbohydrate content of LPS was then quantified using the phenol–sulfuric acid method and measured at a wavelength of 492 nm [30]. Isolated proteins were quantified for their concentration using a Modified Lowry's Kit (Pierce, USA) and measured at 660 nm using a spectrophotometer.

ELISA

Serum immunoglobulin (IgG, IgM, IgA, IgG2c) levels were measured against OMPs or LPS following the method described previously [31]. Twofold serial dilutions were prepared from serum isolated from both immunized and non-immunized groups. HRP-conjugated secondary anti-mouse IgG, anti-IgA, anti-IgG2c and anti-IgM antibodies (Sigma Aldrich, USA) were used to detect the antibody titer. Each experiment was replicated thrice with pooled sera from different groups.

Serum Bactericidal Assay (SBA) and Scanning Electron Microscopy

The effect of immunized mouse sera on bacterial morphology was measured and visualized using scanning electron microscopy (SEM) following a previously described protocol [32]. Bacteria along with heat-inactivated mouse sera and 25% guinea pig complement (with/without) were incubated for 1 hr under microaerophilic conditions followed by plating for viable colonies or fixation with 3% glutaraldehyde overnight followed by a gradual dehydration step initially with alcohol and then substitution later with a mixture of alcohol and hexamethyldisilazane (HMDS) at ratios of 2:1, 1:1 and 1:2. Finally, the samples were mounted on specimen stubs, sputter-coated with gold and analyzed on a Quanta 200 SEM (FEI, Netherlands).

Cytokine assay

Both immunized and non-immunized mice were sacrificed, and the spleens were harvested. After isolating spleen cells; ~10^5 cells were cultured for two hours in RPMI1640 containing 10 % FBS incubated with 50 µg of OMVs and incubated overnight at 37°C (with 5 % CO2) for 24 h. IL-10, IFN-γ, IL-1β, IL-6, IL-4, TNF-α and IL-17 were measured in the culture supernatant using a cytokine measuring kit (Invitrogen, USA) [33].

Fluorescence-activated cell sorting (FACS) analysis

Spleen cells were harvested, cultured for two hours in RPMI1640 containing 10 % FBS and re-stimulated using isolated OMVs (50 µg) and incubated overnight at 37°C (with 5 % CO2) for 24 h. The next day, the cells were scraped, washed thoroughly, blocked and then incubated with mouse anti-CD4+, CD8+ or CD19+ antibodies. Splenocytes were stained with anti-Mabs: CD4-phycoerithrin (PE), CD8 PE, CD19 PE or an isotype control PE (Miltenyi Biotec, USA). Unbound antibodies were washed, and a specific epitope of the immune cell population was observed using FACS Aria II [32].

Protective efficacy Study

Seven days after the last immunization, both the immunized and non-immunized groups were challenged with the wild-type SS1 strain using a newly developed surgical procedure and housed for 7 days before being sacrificed. The antrum of stomach of both immunized and non-immunized groups was isolated and separated into two parts. Half of each part was immediately

transferred to BHI kept on ice, and the other half was transferred to neutral buffered formalin (NBF, 10%) solution to fix the tissue and left at room temperature. Harvested tissue in BHI was weighed, homogenized and serially diluted using PBS. The diluted samples were then spread onto BHIA and kept under microaerophilic conditions for 3-5 days. Any visible colonies were then counted and confirmed using RUT and PCR. Histopathological assays were performed as described elsewhere [34,35]. Briefly, samples kept in 10% formalin were washed and gradually dehydrated using the alcohol gradation method followed by preparing a paraffin block. A thin section (approximately 5 μm) was prepared using a microtome. The slides were then de-waxed, rehydrated, and stained. Hematoxylin-Eosin was used for the study because they enhance tissue or bacterial contrast. Finally, the slides were mounted and observed under a microscope. Histological scoring was assigned for each sample based on their morphological changes. The gastric tissue observed under a microscope revealed various degrees of gastritis, which was then categorized according to the Houston-updated Sydney System based on the infiltration of inflammatory cells within the lamina propria [36].

Statistical analysis

The presented data do not follow a normal distribution due to biological variations. Nonparametric tests were adopted for all data analyses. Triplicate data were expressed as the mean ± SD (standard deviation) using GraphPad Prism version 5.02. Two-way analysis of variance (ANOVA) or the Mann–Whitney test (for animal data) was performed as per the requirements, and statistical significance was determined from the *P* values mentioned in the figure legends.

Results

Characterization and selection of *H. pylori* strains used in the study

A total of 12 strains including 3 reference strains and 9 clinical strains were checked for the presence or absence of major virulence genes i.e. cytotoxin-associated gene or *cagA* representing cag pathogenicity island (cagPAI), vacuolating toxin A or *vacA*, blood group antigen binding

adhesin 2 or *babA2* and duodenal ulcer promoting gene or *dupA*. A type I or type II strain is defined by the presence of *cag* and allelic variations of *vacA* with signal region (*s1* or *s2*) and middle region (*m1* or *m2*). A *cag+s1m1* is considered to be more virulent, thereby influencing diseases development than *cag+s2m2* or any combination of *s1*, *s2*, *m1* and *m2*. Additionally, allelic variations of *babA* i.e *babA2*, plays a key role in adhesion to the Lewis B (Le^b) antigen of blood as *babA1* is known to be non-functional [21]. *dupA* belongs to a plasticity region (*jhp0917-jhp0918)* and found to be responsible in developing ulcers in *H. pylori* infected individuals [66]. Therefore, any strain positive for all these genes can be considered to be more virulent than others. A61C (1) is positive for all these virulence genes and therefore is selected for immunogen preparation. However, for model establishment and challenge study purpose, SS1 is considered to be more suitable than others as it is a mouse adapted strain. The result of genetic characterizations of all strains is listed in a table (**Supplementary table: 3)**.

Clinical response caused by surgical intervention

In the present study, $2x10^8$ CFU of bacteria were used to induce an active infection. Oral inoculation with the aforementioned dose revealed inconsistent results. Moreover, in the majority of cases, very little or no recovery of the bacterial population was observed using available detection techniques. Mice receiving WTSS1 directly to their stomachs by surgical means developed various degrees of gastric changes. Recovery of bacterial colonies from stool was insignificant and erroneous compared to gastric tissues, which were considerably higher (~2-3 times) and were confirmed to be positive upon RUT, spread-plate and PCR. The recovery rate of *H. pylori* from the 7-day infected mice was comparatively higher than that at 14 days post infection (**Fig.2**).

Intragastric surgical evoked inflammatory response Cytokine analysis of intragastrically infected mice of different time points i.e. 0-day, 7 day, and 14 days, showed drastic differences in serum cytokine levels. IFN-γ, IL-1β, TNF-α, IL-10, and IL-17 are increasing more on day 7 after infection and also reducing progressively on day 14. In case of IL-6, which is responsible

for sustaining inflammation is increasing on day 14. The majority of the pro-inflammatory cytokines were upregulated after 7 days post infection, except IL-6, which was found to be more pronounced at 14 days than at 7 days post infection (**Fig.3**) indicating active *H. pylori* infection.

Histopathological changes due to intragastric infection

Histopathological observation plays crucial role in *H. pylori* diagnosis. *H. pylori* infection causes local inflammation in gastric tissue marked by various degrees of inflammatory infiltration with substantial damage in gastric epithelium leading to the survival of the bacteria to the gastric microenvironment. Moreover, previous study has already showed a pronounced effect on gastric tissue of C57BL/6 mice upon *H. pylori* infection. Therefore, in order to establish a successful infection mediated by surgical intervention, stomach samples were taken at different time points and the topographical changes were compared. Negative control mice, receiving only PBS and Day0 had no inflammation (**Fig 4.i: a, b**) whereas inflammatory infiltration was significantly higher on day7 (**Fig 4.i:c**) compared to day 14(**Fig 4.i: d**). In addition to this, mucosal epithelium was severely damaged with exposed gastric pits in both cases. In contrast, metaplasia due to infection was more prominent on day 14 than day 7, indicating a successful infection. Histopathological scoring was assigned based on Sydney system (**Fig.4. ii**).

Isolation and characterization of OMVs from *H. pylori* strain A61C (1)

The OMVs isolated from the broth culture of A61C (1) were purified and assessed using dynamic light scattering (DLS), transmission electron microscopy (TEM) and proteomics analyses using LC/MS (**Fig. 5.A**). The data revealed uniformity in OMVs structure with a diameter of 50nm (**Fig. 5.B.i, ii**). TEM image showed the OMVs to be circular in shape with distinct bilayers. The protein components present in OMVs isolated from the immunogen strain [A61C (1)] revealed 18 major proteins including UreB, UreA, FtnA, GroEL, UbiX, Tuf, SecA, RplI, LpxK, RimO, AroB along with some other proteins with unknown localization (Fig. 5.C, **Supplementary table 4**). Presence of proteins like UreA, UreB and GroEL on OMVs indicate the potential to generate a strong immune response as these proteins are known for their

immunomodulatory activities. The sub-cellular localization of proteins indicated by the software includes cytoplasm, membrane, periplasmic space, plasma membrane of the bacteria. *H. pylori* LPS is known to have no cytotoxicity which is also evident from cytotoxicity assay (**Supplementary figure 2**).

H. pylori OMVs induce pro-inflammatory cytokine response

35th day post immunization splenic cells were harvested from both immunized and non-immunized mice and re-stimulated with 50µg of OMVs. A significant induction in IFN-γ, TNF-α, IL-1β, IL-4, IL-10, IL-17, IL-6and IL-12, IL-13 levels were observed (**Fig. 6.a-i)**. Moreover, our data revealed oral immunization to be a better route for immunization as most of pro-inflammatory cytokines. Contrary to previous studies [63], our study did not find any Th1 or Th2 biased response indicating the immune response against OMVs is independent of routes of administration, compared to the control, both oral and i.p. immunized animals revealed elevation in cytokines. Altogether, immunization invoked an array of cytokines than non-immunized implying the potential of OMVs for a vaccine candidate.

Immunization of *H. pylori* OMVs elicited higher adaptive immune response

Previous studies on bacterial extracellular vesicles revealed OMVs to be an excellent vaccine candidate against bacterial pathogens [62]. OMVs are known to induce both humoral and cellular arms of immune responses usually mediated by outer membrane proteins (OMPs) and lipopolysaccharides (LPS). We investigated serum immunoglobulin levels (**Fig 7.A.i-iv**) of oral and intraperitoneally immunized mice and found significant difference compared to the control. However, we did not find significant differences between oral and intraperitoneal immunization.

Next, we evaluated the bactericidal activity of the immunized serum. The data showed significant reduction in bacterial number when immunized serum is incubated with 25% guinea pig serum as compared to non-immunized mice serum (**Supplementary figure 3**). This confers activation of complement mediated pathway, along with sufficient antibody titer in immunized

C57BL/6 mice that effectively kill the bacteria by damaging the bacterial surface as viewed under SEM (**Fig 7. C, D.i-ii**). Comparative analyses of the splenic cell population of immunized and non-immunized mice were done using a flow cytometer. Immunization with OMVs showed significantly higher population of CD4+, CD8a+ and CD19+ (**Fig 7. B**) cells indicating a strong immune response in immunized mice.

In all, immunization with *H. pylori* OMVs generated adaptive immune responses in C57BL/6 mice and significantly activated adaptive immune responses which could in turn help to provide long term protective immune response against infections caused by *H. pylori* (**Figure 7.A.iii-iv**).

Protective efficacy study post immunization

After immunization with OMVs, the immunized and non-immunized animals were challenged with wild type SS1 with a dose mentioned before (i.e. $2x10^8$ CFU) and the colonization were analyzed 7 days post infection. A significant decrease in colonization was observed in the stomach tissue of immunized than non-immunized animals indicating a substantial reduction in bacterial load (**Fig. 8.D)**. To confirm further, DNA was extracted from the gastric tissue and subjected to *ureB* PCR for the presence of *H. pylori.* All non-immunized mice were found to be positive whereas immunized animals were found insignificant presence - of *H. pylori* DNA among OMVs immunized animals (**Fig. 8.C)**. Histopathological changes of both immunized and non-immunized mice stomachs were analyzed 7 days post infection using surgical intervention (**Fig. 8.A.i-ii)**. OMVs immunized mice showed a significant reduction in gastric epithelial damage, altered gastric mucosa, inflammatory infiltration, exposed gastric pit, and metaplasia. Pathological scores were also less in immunized mice than non-immunized mice (**Fig. 8.B)**. Overall, reductions in bacterial numbers were observed upon immunization.

Discussion:

Over the years, a number of animal models have been evaluated for pathophysiology or treatment against *H. pylori,* including gnotobiotic pigs, dogs, cats, Mongolian gerbils, guinea

pigs, rhesus monkeys, and mice [37–48]. In most cases, C57BL/6 or black mice were explored extensively because of their substantial contribution in *H. pylori*-related studies. The proper route for administering the pathogen and/or immunization also has a key impact on the development of an infection and assessment of immune response, which is another important consideration when selecting an animal model for *in vivo* studies. Therefore, cytokine alterations along with histological changes described in the present study represent a successful infection achieved through our newly developed surgical model. Conventional approaches for studying *H. pylori* infection in animals usually involve multiple oral inoculations using an oral gavage [49–51]. However, relying solely on the oral route to induce an infection and expecting the bacterium to outcompete the existing microflora and successfully colonize the stomach may not always yield a consistent result in any given experimental setting. This can cost significant time and resources being invested while still fostering uncertainty about an actual infective status in experimental animals. Therefore, it is important to consider the limitations and variability of the *in vivo* systems and look for alternative approaches that could provide a more reliable method of *H. pylori*-mediated pathogenesis in animal models. Clinical detection of *H. pylori* infection generally involves histology and PCR apart from the Rapid Urease Test (RUT) [52–54]. Serological tests are often avoided, as previously invoked antibodies fail to recognize the actual infective status of recent manifestations [55, 56]. As a consequence, this increases the chances of false positive results. In addition, histology allows visualization of pathogen-induced changes in gastric tissues, such as the intensity of inflammatory cell infiltration or aberrations in gastric topology, while PCR detects the presence of genomic DNA of *H. pylori* in gastric tissue samples [57]. However, it should be noted that neither histological observations nor negative PCR results rule out the presence of an infection [58]. Thus, a number of different techniques must be employed simultaneously to achieve a more accurate diagnosis of *H. pylori* infection [59]. Our study comprised a combination of histological observation, PCR detection and quantification of serum cytokine levels to confirm active *H. pylori* infection.

Surgical intervention initially spiked pro-inflammatory cytokines such as IFN-γ and IL-1β along with IL-17 significantly more on day7 than on day14. However, as the infection progressed, these cytokines were lowered and finally balanced, except for IL-6, which was found to be elevated more on day 14 than on day 7. A pronounced IL-6 level at later stages might indicate ongoing inflammation in the gastric lining with potentially developing chronic gastritis. Such

responses were further supported by the induction of other cytokines. IFN-γ is an early effector molecule responsible for generating a Th1-mediated response by initiating different signaling cascades. However, up-regulation of IFN-γ transiently down-regulates IL-1β production. In addition, IL-17, a cytokine regulating the Th-17-based response, plays important roles in both pathogenesis and host immunity. Studies with chronic diseases have revealed that well-balanced IL-1β and IL-17 levels are constitutively produced to sustain inflammation due to infection in the long term [67]. In the case of *H. pylori* infection, both IL-1β and IL-17 play crucial roles in pathogenesis; in particular, IL-17 influences the disease outcome upon infection. Our model showed an initial elevation in these cytokines, which decreased over time, indicating progression toward a chronic infection. However, as 7 days were not sufficient to develop a chronic infection, our model showed promising results in a time-dependent manner. Consistent with the cytokine analysis, histopathological observations also validate such changes to some extent. Intense inflammatory cell infiltration was observed on day 7 than on day 14, and the gastric lining was found to be more damaged with exposed gastric pits, indicating destruction caused by bacteria. Nevertheless, we did not find any striking structural abnormalities in gastric tissue 7 days post infection. PCR results from the same samples confirmed the presence of bacterial genomic DNA in experimental animals.

Next, we evaluated the surgical model for vaccine efficacy studies. Two different immunization routes were assessed to observe any alterations in the immune response due to changes in the route of administration. Immunization was performed both orally and intraperitoneally (i.p.) on days 0, 14 and 28. Initially, an elevation of serum IgG, IgM and IgA levels was observed against OMPs but not LPS of *H. pylori*. This can be due to the structural similarity between *H. pylori* LPS and blood antigens of the host [60-61]. Furthermore, we evaluated IgG2c (IgG subtype) and found it to be increased in immunized rather than non-immunized groups [62]. Our study found oral immunization to be better responsive than i.p. route, which can be due to the presence of different surface proteins on OMVs that are more readily absorbed and reactive to gastric epithelial cells than peritoneal immune cells. A splenic cell re-stimulation (ex vivo) assay revealed enhanced Th2-based cytokine responses, such as IL-4, IL-13, IL-10 and IL-12, coinciding with previous studies with *H.* pylori-derived OMVs used as immunogens [63]. Interestingly, our study did not find any biased immune response against OMVs, indicating that the immune response to OMVs is not general but rather unique to each strain. CD4+, CD8a+ and

CD19+ cell populations were increased due to OMV immunization independent of the route of administration. OMV immunization ultimately leads to a reduction in bacterial colonization in immunized animals but not in non-immunized animals. Serum bactericidal assay (SBA) typically denotes the functional aspect of immunogen-invoked antibody response in killing the bacterial population via complement mediated pathway. This in-vitro method involves incubation of bacteria in presence of heat-inactivated serum isolated from both OMVs immunized and PBS immunized mice. Antibodies generated in host due to immunization are sufficient enough to reduce the bacterial CFU by means of agglutination as demonstrated in OMVs induced immune response against *S. Typhi* and *Paratyphi A* [31]. However, agglutination doesn't directly imply a bacteriostatic or bactericidal activity of the antibodies. Therefore, addition of purified baby rabbit [64] or guinea-pig [65] complement externally to these antibodies ensured the lysis of the bacteria via complement-mediated pathway. In the present study, incubation of bacteria treated with OMVs immunized or PBS immunized sera in presence of guinea-pig complement lead to significant reduction in viable colony numbers in immunized compared to the PBS immunized mice groups.

Conclusion

In conclusion, the intragastric surgical model of *H. pylori* infection can be used to study the pathophysiology, immune response, and potential therapies for *H. pylori* infection. Our study indicates that a minimum of 7 days is enough to develop an infection in this model. All experimental results showed that tissue samples collected at 7days post infection can provide better results for diagnosing *H. pylori* infection than samples obtained at 14 days post infection, as histological changes and inflammatory cell infiltration are typically more pronounced at earlier time points post infection. Moreover, the cytokine response and antibody generation further support this model for vaccine efficacy studies. The immunization of mice with *H. pylori* OMVs has been shown to reduce the bacterial load with elevated antibody titers and protect gastric tissue from destruction. Therefore, the intragastric surgical model can become a valuable tool for understanding the pathophysiology of *H. pylori* infection, formulation and evaluation of potent vaccine candidates and development of potential therapeutics.

Author contributions

Sanjib Das: Conceptualization, experimental design and performance, data analysis and interpretation, manuscript preparation, **Prolay Halder:** Experiment performed, data analysis and interpretation, review and edit manuscript, **Soumalya Banerjee:** Experiment performed, data analysis and interpretation, review and edit manuscript, **Asish Kumar Mukhopadhyay:** Review and edit manuscript**, Shanta Dutta:** Review and edit manuscript, **Hemanta Koley**: Conceptualization, experimental design and supervision, data analysis and interpretation, manuscript preparation, funding acquisition.

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Conflict of interest

The authors declare no conflicts of interest.

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Figures

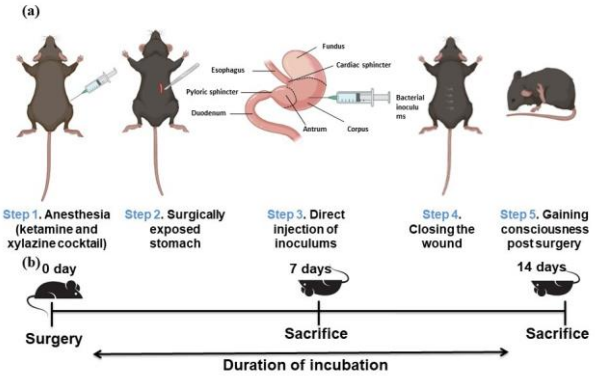

Fig. 1. Schematic diagram of the intragastrically infection model in C57BL/6 mice. (a) Graphical Representation of the "Surgical Model" using C57BL/6.Bacterial inoculation (~2x10^{8}CFU/mL) is directly injected into the stomach, **(b)** Schematic schedule from infection to sacrifice.

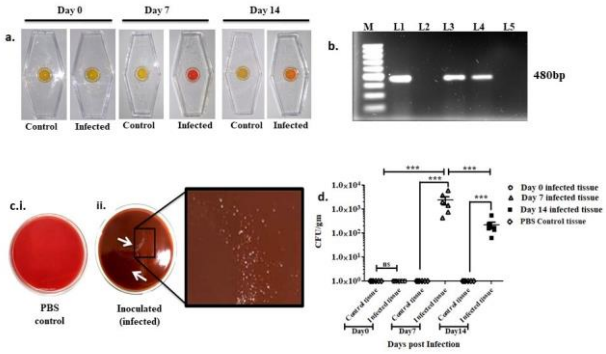

Fig. 2. Intragastric infection induced by wild type (WT) SS1 observed through urease test, *ureB* PCR, colonization in gastric tissue. (a) RUT of infected gastric tissue; Day 0, Day7 and Day 14 with respective PBS control**, (b)** Confirmatory *ureB* PCR for the presence of *H. pylori* recovered from the gastric tissue of infected mice**. M**-100bp marker, **L1**-positive control, **L2**-day 0 post infection, **L3**-day7 post infection, **L4**-14-day post infection, **L5**-PBS control. (**c**) Blood agar plates showing **(c.i.)** plate containing no *H. pylori* colonies recovered from gastric tissue of 7days post PBS inoculated mice **(c.ii)** plate containing *H. pylori* colonies recovered from gastric tissue of 7days post SS1 infected mice. **(d)** Colonies recovered from mice of 0, 7- and 14-days post infection. Data represented here are the mean values +/-Standard Deviation (SD) of three independent experiments. The differences in day wise response of each colonization assay were highly significant with respect to PBS control tissue. Statistical significance was found between 0day, 7day and 14day infected mice tissue (***p<0.001, ns-non-significant).

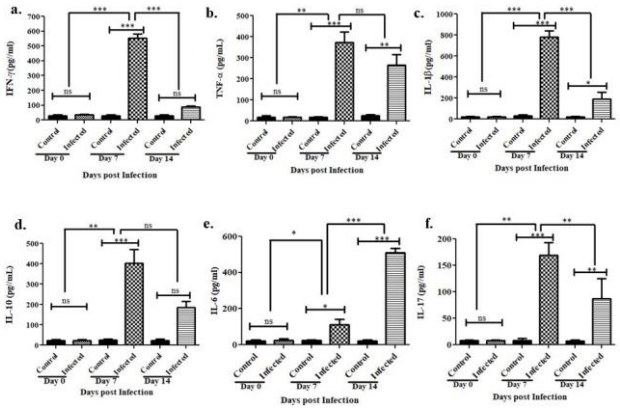

Fig. 3. *Helicobacter pylori;* wild type (WT) SS1 induces the production of cell mediated cytokines responses post-surgical intervention. (a) IFN-γ, **(b)** TNF-α, **(c)** IL-1β, **(d)** IL-10, **(e)** IL-6, **(f)** IL-17 cytokines in serum isolated on Day 0, Day 7 and Day 14 post-surgical infection with respective PBS controls. All cytokines are measured by ELISA (n = 6). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non-significant), Each bar represents median and error values of Six ± SE of three independent experiments.

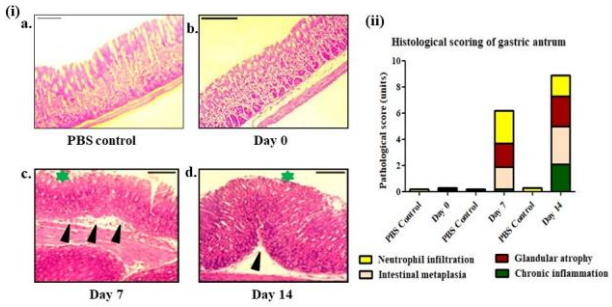

Fig. 4. Histological (H&E staining) observation and histological scoring of gastric epithelium after intragastric surgical infection with *Helicobacter pylori* (SS1). (i) a, b, c, d; all are the antrum part of the stomachs harvested from C57BL/6 mice**. (i.a)** no distinct changes observed in PBS control, mice receiving PBS only, **(i.b)** zero inflammation in Day 0 post-surgical infection of C57BL/6 mice, **(i.c)**severe inflammatory cell infiltration, glandular atrophy, intestinal metaplasia of 7 days post-surgical infection of C57BL/6 mice, **(i.d)** mild to moderate inflammatory cell infiltration, disruption in epithelial lining, glandular atrophy, chronic inflammation in 14 days post-surgical infection of C57BL/6 mice. Images were captured at 20× magnification. Scale bar represents 100 μm. Inflammatory cell infiltration indicated by (black arrowhead), gastric epithelial damage (green star); **(ii)** Histopathological scoring is done according to Huston updated Sydney classification system. Colored histogram represents the mean scores of histological scoring of experimental animals (n=6) and PBS controls (n=6). All experiments were performed in triplicate.

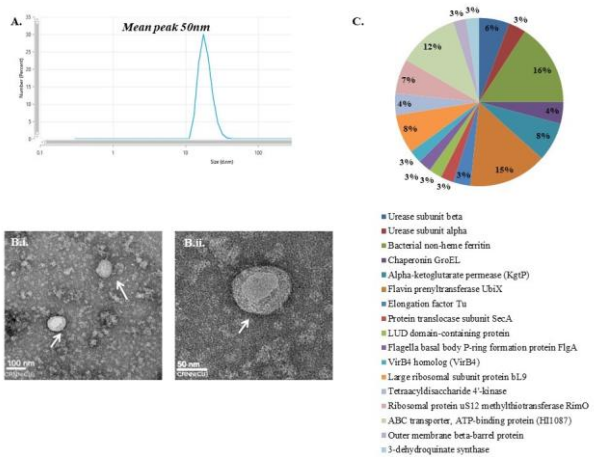

Fig. 5. Characterization of *Helicobacter pylori* OMVs isolated from strain A61C (1); (A) Dynamic Light Scattering showing a uniformity in OMVs population with mean peak at 50nm**,** (B) Transmission Electron microscopy images revealing the circular morphology of *Helicobacter pylori* OMVs of A61C (1) strain; (B.i) Image taken at 100nm scale, (B.ii) Image taken at 50nm scale. Both TEM images revealed the thick bilayer structure with hollow center of the OMVs. (C) Percentage of major proteins present on OMVs.

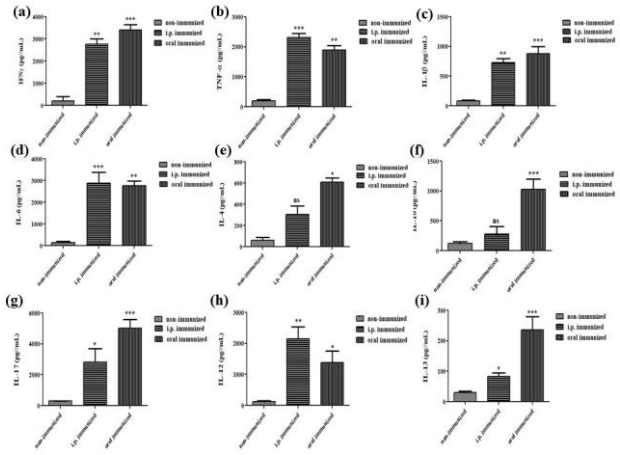

Fig. 6. Helicobacter pylori OMVs induces the production of cell mediated cytokines responses. (a) IFN-γ, **(b)** TNF-α, **(c)** IL-1β, **(d)** IL-6, **(e)** IL-4, **(f)** IL-10, **(g**) IL-17, (**h**) IL-12, (**i)** IL-13, cytokines in culture supernatant of ex-vivo cultured splenic cells of immunized and non-immunized (PBS immunized) mice after 24 h of re-stimulation with OMVs. The differences in immunized (i.p. and oral immunization) mice serum response of each of the studied cytokines were highly significant than nonimmunized. All cytokines are measured by ELISA (n = 6). Statistical analyses were performed using the non-parametric Student's *t* test (Mann-Whitney tests) to evaluate data; (***p value <0.001, **p value <0.01, *p value <0.05, ns=Non-significant). Each bar represents median and error values of six ± SE of three independent experiments.

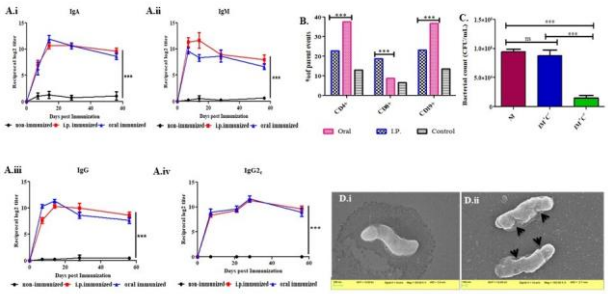

Fig. 7. Reciprocal log2 titer of serum IgA, serum IgM, serum IgG, and serum IgG2c immunoglobulins from *Helicobacter pylori* OMVs immunized and non-immunized (PBS immunized) group against OMPs. Immunization induces the population of CD4+, CD8+, and CD19+ splenic cells of immunized over non-immunized (PBS immunized) mice and the microscopic image of serum bactericidal activity of immunized and non-immunized. Mouse serum IgA (**A.i**), serum IgM (**A.ii**), serum IgG (**A.iii**), serum IgG2c (**A.iv**) was measured separately after three doses of intraperitoneal or oral immunization against Outer membrane protein (OMP) of *Helicobacter pylori.* **(B)** Bar diagram represents the percentage of CD 19+, CD 4+, and CD 8+ spleen cells from immunized and non-immunized mice using FACS analyses. Significant statistical difference was found between OMVs immunized and non-immunized spleen cell population (*** p value< 0.001). **(C)** OMVs immunized mouse serum is effective in complement mediated lysis of *H. pylori* (SS1). *H. pylori* (SS1) was separately incubated with OMVs immunized serum or non-immunized serum with or without guinea pig complement for 1 h at 37 ◦C. Viable bacterial count was determined by spread-plate method. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. (*** p value < 0.001, ns-Non-significant.). NI, non-immunized serum; IM+C-, OMVs immunized serum without complement; IM+C+, OMVs immunized serum with complement. (**D**)Scanning electron microscopic images after Serum Bactericidal Assay using non-immunized serum with complement (i) and immunized serum with complement (ii) (black arrowheads indicate immunized antibody-mediated lysis in presence of complement).

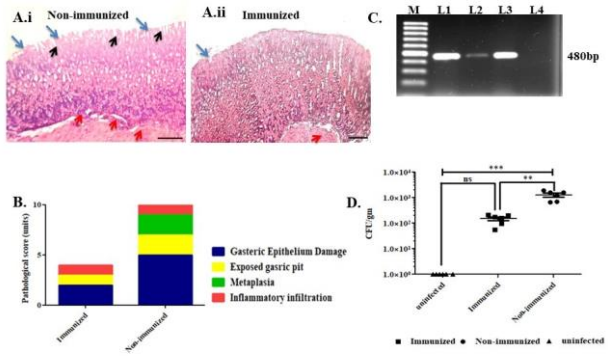

Fig. 8. OMVs immunized mice shows reduced gastric tissue damage, inflammation after infection with SS1 (2 x 10^8 CFU) and reduce bacterial colonization in gastric tissue. Histological images represent both **(A.i)** non-immunized (PBS immunized) antrum of stomach and **(A.ii)** immunized antrum of stomachs. OMVs immunized mice showed mild epithelial layer damage, less altered gastric mucosa and inflammatory infiltration, whereas non-immunized mice displayed marked epithelial damage, inflammatory infiltration, exposed gastric pit and early signs of gastric metaplasia. (Blue arrow: gastric epithelium; Black arrow: exposed gastric pit; Red arrow: Inflammatory infiltration) **(B)** Pathological scores of immunized or non-immunized mice post *Helicobacter pylori* SS1 challenge. **(C)** ***u****reB* PCR shows significant changes in bacterial DNA yield harvested from gastric tissues of both non-immunized and immunized mice post intragastric surgical challenge; **M:** 100bp ladder, **L1:** Positive control, **L2**: Immunized mice gastric tissue, **L3**: Non-immunized mice gastric tissue, **L4**: Negative control **(D)** *Helicobacter pylori* colonization in gastric tissue of immunized and non-immunized mice 7 days post challenge.

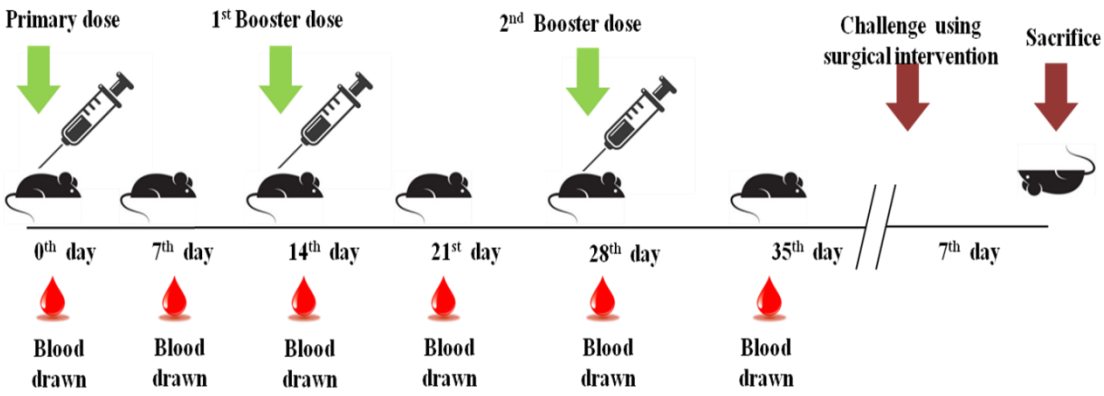

Fig. S1. Immunization and blood collection schedule Oral or Intraperitoneal (i.p.) route of immunization

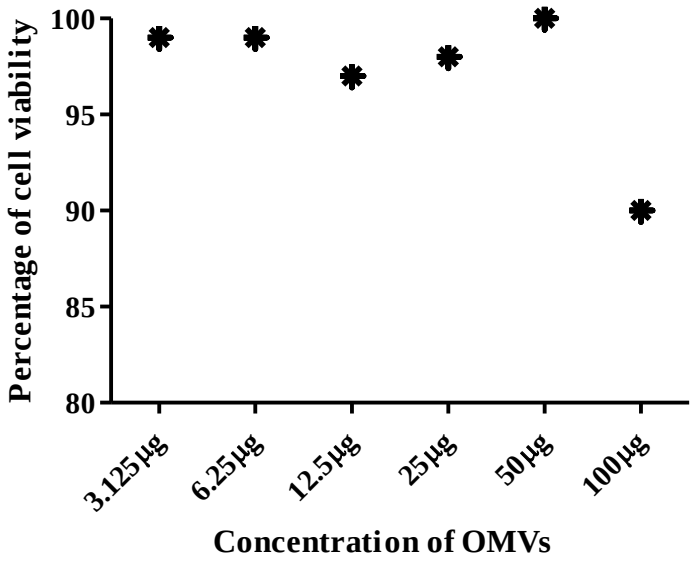

Fig. S2. *in-vitro* cytotoxicity test with different concentrations of OMVs

Table S1. The Antibiotic profile of *Helicobacter pylori*used for immunogen strain selection.

Strains			Antibiotics					
			Clarithromycin		Amoxicillin		Metronidazole	
			S	R	S	R	S	R
1	Ref. strains	26695	+	-	+	-	-	+
		J99	+	-	+	-	-	+
		SS1	+	-	+	-	-	+
2	BHU 8A		-	+	+	-	-	+
3	KO 8A		+	-	+	-	-	+
4	AS 2		+	-	+	-	-	+
5	OT-10 (A)		+	-	+	-	-	+
6	B34		+	-	+	-	-	+
7	D383		+	-	+	-	-	+
8	B6		+	-	+	-	-	+
9	M28		+	-	+	-	-	+
10	L7		+	-	+	-	-	+
11	A61C(1)		+	-	+	-	-	+
12	AM1		+	-	+	-	-	+
S - sensitive					R - resistant			

Table S2. Primers of specific genes of *Helicobacter pylori*used in this study.

Gene Assigned	Primer	Sequence	Amplicon (bp)	Reference
cagA	cag5c-F cag3c-R	5′-GTTGATAACGCTGTCGCTTCA-3′ 5′-GGGTTGTATGATATTTTCCATAA-3′	350	Chattopadhyay et al, 2004 [20]
vacA s1/s2	VA1-F VAI-R	5′-ATGGAAATACAACAAACACAC-3′ 5′-CTGCTTGAATGCGCCAAAC-3′	259/286	
vacA m1/m2	VAG-F VAG-R	5′-CAATCTGTCCAATCAAGCGAG-3′ 5′-GCGTCAAAATAATTCCAAGG-3′	567/642	
babA2	babA2R babA2F	5′-AATCCAAAAAGGAGAAAAAGTATGAAA-3′ 5′-GTTTTCTTTGAGCGCGGGTAAGC-3′	607	Ghosh et al, 2016 [21]
ureB	ureBF ureBR	5′-CGTCCGGCAATAGCTGCCATAGT-3′ 5′-GTAGGTCCTGCTACTGAAGCCTTA-3′	480	Ghosh et al, 2016 [21]
dupA	jhp0917F jhp0917R	5′-TGGTTTCTACTGACAGAGCGC-3′ 5′-AACACGCTGACAGGACAATCTCCC-3′	307	Lu et al, 2005 [66]
	jhp0918F jhp0918R	5′-CCTATATCGCTAACGCGCGCTC-3′ 5′-AAGCTGAAGCGTTTGTAACG-3′	276	
16SrRNA	16SF 16SR	5′-CTGGAGAGACTAAGCCCTCC-3′ 5′-ATTACTGACGCTGATTGCGC-3′	110	Kashyap et al., 2020 [67]

Table S3. Result of major virulence genes of *Helicobacter pylori*screened for immunogen strain selection

Genetic Features			Virulence Marker					Adhesion	Duodenal Ulcer Promoting Gene	
Strains			*cagA*	*vacA*				*babA2*	*dupA*	
				s1	*s2*	*m1*	*m2*		*jhp0917*	*jhp0918*
1	Ref. strains	26695	+	+	-	+	-	-	-	-
		J99	+	+	-	+	-	+	+	+
		SS1	+	-	+	-	+	-	-	-
2	BHU 8A		+	+	-	-	+	+	-	-
3	KO 8A		+	+	-	+	-	+	-	-
4	AS 2		+	+	-	+	-	-	-	-
5	OT-10 (A)		+	+	-	+	-	-	-	-
6	B34		+	+	-	+	-	+	-	-
7	D383		-	-	+	-	+	-	-	-
8	B6		+	+	-	-	+	+	-	-
9	M28		+	+	-	-	+	-	-	-
10	L7		+	+	-	+	-	+	-	-
11	A61C(1)		+	+	-	+	-	+	+	+
12	AM1		-	-	+	-	+	-	-	-

Table S4. Proteomic analyses of OMVs isolated from*Helicobacter pylori*strain A61C(1).

Accession	Protein assigned	Molecular mass (kDa)	No. of peptides	Sequence coverage (%)
P69996	nickel cation binding, urease activity	61.6	3	7
P14916	nickel cation binding, urease activity	26.5	1	4
P52093	ferric iron binding, ferrous iron binding, ferroxidase activity	19.3	2	19
P42383	ATP binding, ATP-dependent protein folding chaperone, isomerase activity, unfolded protein binding	58.2	3	5
O25723	transmembrane transporter activity	47.5	1	9
O26011	carboxy-lyase activity, flavin prenyltransferase activity	20.6	1	18
P56003	GTP binding, GTPase activity, guanosine tetraphosphate binding, translation elongation factor activity	43.6	2	4
O25475	ABC-type protein transporter activity, ATP binding, metal ion binding, protein-exporting ATPase	99	1	3

	activity			
O24949	Hypothetical protein	23.6	1	3
O26012	Hypothetical protein	24.3	1	3
O25206	ATP binding, DNA binding	98.4	1	3
P56035	rRNA binding, structural constituent of ribosome	16.5	1	9
O25095	ATP binding, tetraacyldisaccharide 4'-kinase activity	35.5	1	5
O25434	4 iron, 4 sulfur cluster binding, aspartic acid methylthiotransferase activity, metal ion binding,proteinmethylthiotransferase activity	49.6	1	8
O26001	ATP binding,ATP hydrolysis activity	29.2	1	14
O25992	Hypothetical protein	82.3	1	3
P56081	3-dehydroquinate synthase activity, metal ion binding, nucleotide binding	39.1	1	3

Table S5. Sydney classification of histopathological scoring used in the study

	Neutrophil infiltration	**Glandular atrophy**	**Intestinal metaplasia**	**Chronic inflammation**
Grade I	<1/3 of surface infiltrated	Mild	<1/3 of surface infiltrated	5-10 cells x 40
Grade II	1/3- 2/3 of surface infiltrated	Moderate	1/3- 2/3 of surface infiltrated	11-20 cells x 40
Grade III	>2/3 of surface infiltrated	Severe	>2/3 of surface involved	>21cells x 40

Scores assigned: mild=1, moderate=2, severe=3

Table S6. List of Antibodies used in the study

Sl no.	Antibody	Catalog no	Company
1.	Anti mouse-IgA	AB97235	Abcam
2.	Anti mouse-IgG2c	AB97255	Abcam
3.	Anti mouse-IgG	AB97023	Abcam
4.	Anti mouse-IgM	A8786	Sigma aldrich

Table S7.

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A review for the prevention and management of *Helicobacter pylori* induced gastritis through development of novel vaccine candidates

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ABSTRACT

The circulating strains of *Helicobacter pylori (H.pylori)* cause chronic gastritis, leading to diseases like gastric ulcers, duodenal ulcers, dyspepsia, gastric adenocarcinoma or gastric cancer, mucosal-associated lymphoid tissue lymphoma globally. In 2012, a report estimated around 723,000 deaths, i.e., 8.8 % of total 14.1 million cancer cases due to gastric cancer only, making it the third significant cause of deaths worldwide due to its malignancy. Nonetheless, this estimate fails to represent the actual status of the disease due to the lack of a unified diagnostic approach and proper reporting system prevailing in the endemic as well as other regions of low and middle-income countries. Current control strategies include antimicrobial therapies that are losing their efficacy due to rapid introduction of genetic mutations among the circulating strains promoting antimicrobial resistance. Moreover, therapies can only provide temporary fixation and no permanent solution or risk of re-infection. However, recognition of *H. pylori* as a significant contributor to a broad spectrum of gastric illnesses by WHO, demands a prophylactic measure than a therapeutic measure. To this date, no vaccine is available against *H. pylori*, which calls for a comprehensive approach where a potent vaccine will be effective enough against all the gastric diseases leading to a healthy future. In this review, we focused on aspects of *H. pylori* infection, present status of vaccine development including animal models and its prospective future.

1. Introduction

1.1. Pathogenesis and transmission

The onset of *Helicobacter pylori* mediated infection in gastric mucosa typically starts in childhood and if left untreated, remains for life (Aguilera Matos et al., 2020).The pathogen is considered to be an etiological agent of chronic gastric ulcers and cancers, causing global morbidity and mortality (Abadi et al., 2015). Even though half the population gets infected at least once in their lifetime, surprisingly, a tiny percentage develops extreme outcomes like cancer (Kumar et al., 2020). Asymptomatic nature of the infected individuals, lack of suitable diagnostic tests and poor surveillance systems aggravates the situation manifold. Although prevalence in some developed and developing nations have been declining over the past two decades, it remains to be a major burden in many developing countries, particularly that of Asian, African, and South American continents [Fig. 1].

Heterogeneity in the prevalence trend within a region is a unique feature of *H. pylori* and depends upon its geo-social diversities, food habits, lifestyle of the inhabitants (Vinagre et al., 2013).Besides this, factors like population density play a crucial role in prevalence which can be justified by the lower financial status, infrastructural deficit, and inability to maintain personal hygiene and sanitation (Salih, 2009). For instance, in China, the prevalence rate is higher (66 %) in rural areas compared to urban (47 %) regions (Nagy et al., 2016).

While the exact mode of transmission is a matter of extensive research, many rooted for a possible fecal-oral or oral-oral routes for the transmission of the bacteria (Mladenova et al., 2006).This belief is quite popular and supported by the ability to cultivate *H. pylori* in laboratory condition, isolated from infected stool, vomit or dental plaque (Al Asqah et al., 2009). Interestingly, intra-familial bacterial analyses indicate a possible transmission from mothers (not fathers) to their children, indicating a vertical transmission (Konno et al., 2008). Nevertheless, contaminated food and water remains the primary infection source

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(Zamani et al., 2017), while possibility of zoonotic transmission is still a matter of investigation.

Travelers returning from endemic regions (Nguyen et al., 2021) are more susceptible in developing acute or chronic gastritis induced by *H. pylori* and are likely to become carriers for the pathogen. Thus precautions must be taken while traveling in rural areas as chances of contamination via food or water is higher in the countryside due to a lack of awareness and cleanliness (Wang et al., 2022). This is extremely concerning as primary suspects are nothing but a threat to an uninfected population (Blanchard and Czinn, 2001). Increase in cases of infection reported from non-endemic regions puts foreign travelers under radar as primary suspects for the transmission of the pathogen (Lindkvist et al., 1995). Such incidences can further promote genetic alterations among the circulating non-virulent strains; thereby increasing resistances while the host acting as a permanent reservoir, utterly unaware of the infection (Payão and Rasmussen, 2016).

1.2. Why do we need H. pylori vaccine research?

Till date, there is no vaccine available against *H. pylori* (Sutton and Boag, 2019). Antimicrobial therapies are the only option available to the mass. A combination of antibiotics is prescribed to eradicate the pathogen. However, practical results could be more consistent as prolonged exposure to these antibiotics had shown to induce antibiotic resistance at an alarming level (Mehrotra et al., 2021).Thus a search for an alternative treatment led to the development of a therapy that incorporates metals, like bismuth containing Quadruple therapy as bismuth has already proven to have anti-*H. pylori* effect (Alkim et al., 2017). Treatments like these can certainly buy some time, but developing a permanent solution like a vaccine must be given priority. However small it is, some steps have been taken toward vaccine development in recent times. For instance, UreB/LTB fusion vaccine primarily showed efficacy in developing immune responses in naïve children (6–14years old) but the trial stopped after the third phase because ofsome unknown reasons (Xie et al., 2021). IMX101, which used *H. pylori* antigen γ-glutamyltranspeptidase (GGT), an outer membrane protein and a mucosal adjuvant, completed phase I clinical trial but is yet to publish any result (de Brito et al., 2019). With such opacity in vaccine research, the question arises as to why we don'tgo for a population-wide eradication program using antibiotics.

To answer the question, we must consider the potential drawbacks of a population-wide antibiotic eradication program. A recent trial in China, a large population (>184,000) with a *H. pylori* prevalence rate of 57 % has been able to achieve an eradication rate of about 73 % using antimicrobial therapies. However, the withdrawal of one in four (25 %) volunteers from the study raised concerns about the challenges for an entire population-based eradication program. Even if this mountainous goal is achieved, such a drive isn't economically feasible, labor intensive and increase re-infection rates (Pan et al., 2016). This was confirmed by a hospital-based study in China, where re-infection rate of 1.75 % in one year and 4.61 % in three year follow-up after receiving the treatment has been reported previously (Ding et al., 2022).

Epidemiological reports across the world indicate a significantly higher prevalence rate of *H. pylori* infection in countries like Russia,

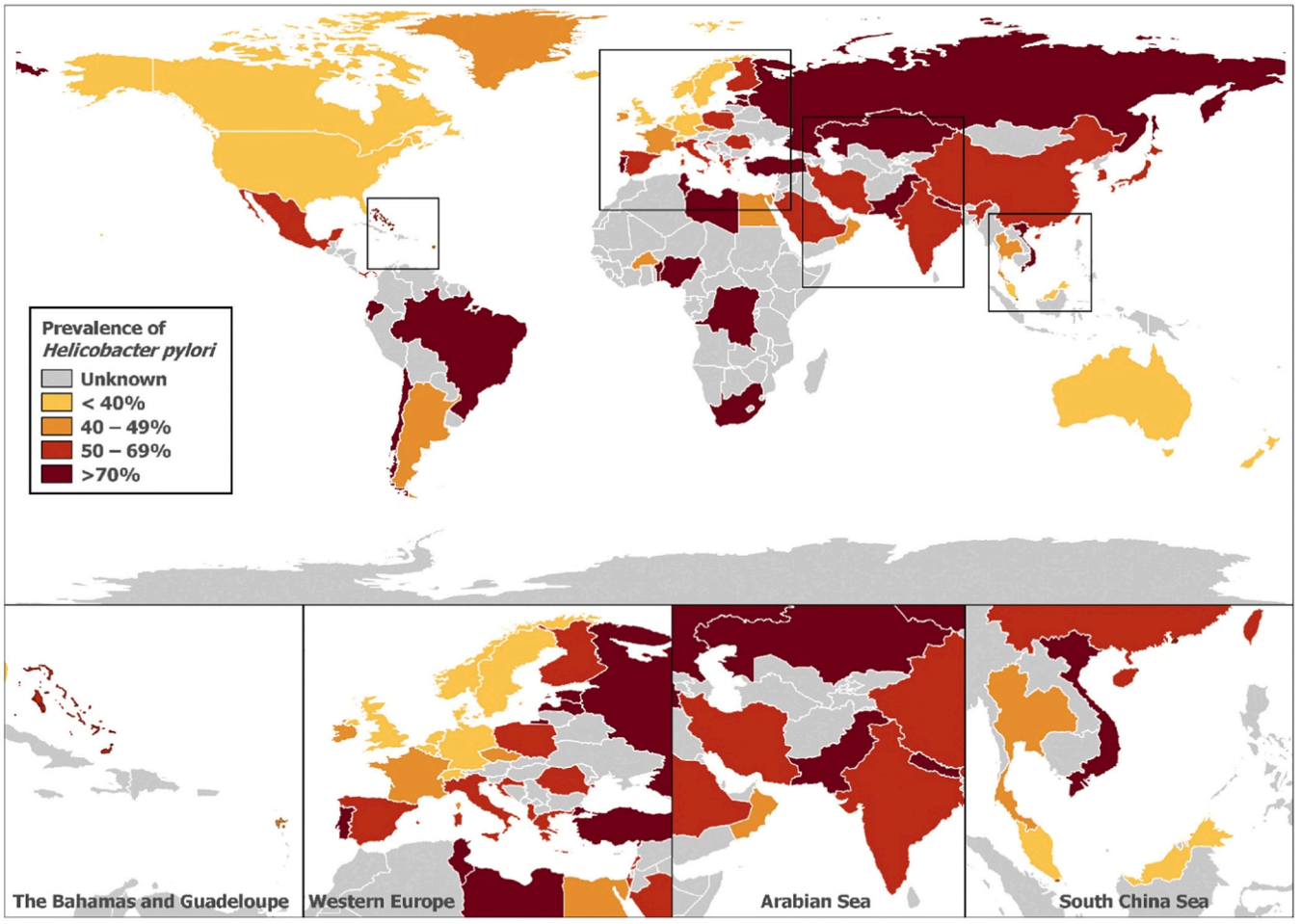

Fig. 1. Current global prevalence status (Different colors represent the percentage). Adopted from: Hooi, J. K., Lai, W. Y., Ng, W. K., Suen, M., Underwood, F. E., Tanyingoh, D., Malfertheiner, P., Graham, D. Y., Wong, V. W., Wu, J. C., Chan, F. K., Sung, J. J., Kaplan, G., & Ng, S. C. (2017). Global Prevalence of Helicobacter pylori Infection: Systematic Review and Meta-Analysis. *Gastroenterology*, *153*(2), 420–429. https://doi.org/10.1053/j.gastro.2017.04.022.

Jordan, Iran, China, and Latin America, along with parts of Canada (Mezmale et al., 2020). In Canada, *H. pylori* positive cases are higher among indigenous Arctic population than non-indigenous population (Fagan-Garcia et al., 2019). An interrelation between ethnicity and prevalence has already been established before. A significantly higher prevalence of *H. pylori* sero-positive in ethnic minority groups of Amsterdam and the Netherlands compared to the Dutch population validates its ethnic connection (Alberts et al., 2020). In recent times, Armenia published a report for the first time indicating a 41.5 % *H. pylori* prevalence (Gemilyan et al., 2019)while Chile raised concerns about a higher infection rate in newborns within the first month after their birth (Solnick et al., 2003). Although Hungary and Taiwan published evidence of declination in *H. pylori* infection simply by improving the socioeconomic status, the pathogen continues to be endemic, with a re-infection rate of 1.5 % in China to 3.1 % in Korea (Li et al., 2021; Kim et al., 2013).

2. Co-infection: A major impediment in *H. pylori* vaccine research

Seroprevalence data is an essential measure and a source of knowledge about infection, its spread into the population, and the potential for an outbreak in the future.It plays a significant role in epidemiological studies depicting the current infection status or having immunity after being exposed to a specific infectious agent across the population in a spatiotemporal manner. Therefore seroprevalence data can be an excellent tool in vaccine research (Wilson et al., 2012).

Another vital aspect of sero-prevalence study is co-infection. In recent times, Italy conducted a study on gut micro-flora among migrants and found co-infection of *Blastocystis* spp and *H. pylori* and found a higher prevalence of the *Blastocystis* co-infection in first-generation migrants and a steady elevation in infection among second-generation migrants coinciding with the Dutch findings on *H. pylori* and co-infection (Alberts et al., 2020; Pomari et al., 2020).In a different scenario, 27 % of Egyptian patients with Irritable Bowel Syndrome (IBS) and 67 % of Pakistani patients diagnosed with chronic diarrhea were found to be positive for *Blastocystis* co-infection along with *H. pylori* (El-Badry et al., 2018; Yakoob et al., 2018).However, despite the previously described association between *H. pylori* and gut parasites, Moreira *et al.* found no significant association between *E. histolytica* and *H. pylori* (Moreira et al., 2005), suggesting the importance of extensive screening of gut microbiota and sero-prevalence prior to administering any therapy. In past, misinterpretation of serological data resulted in exclusion of such observations prior to therapeutic intervention (Lindsetmo et al., 2008).

Undoubtedly seroprevalence studies can provide directions in many cases but posses unique limitations in case of *H. pylori.* According to several studies, serological tests for *H. pylori* are inconsistent and have wide variations in sensitivity and specificity (Best et al., 2018). This could be due to the presence of detectable anti-*H. pylori* antibodies post infection or treatment, making it hard to consider seroprevalence as an essential parameter to distinguish between the present or past encounter.

3. Limitations of clinical detection

Generally, when patients fail to clear an infection within one year of recovery, it is called chronic carriage (Gunn et al., 2014). Chronic carriage occurs due to either prolonged or incomplete antibiotic exposure following an infection. Inside the human body, *H. pylori* alter the gastric microenvironment and the foundation of a chronic infection is being laid silently. As children usually develop symptoms at a much later age and stage, consequently early detection and identification of a chronic carriage become a herculean task.

Several diagnostic tests are frequently advised for the detection of *H. pylori* infection. Urea breath test, rapid urease test, stool antigen test, histology, and culture, along with endoscopic tests, are routinely used in diagnosis and research practice. Most of these high-end assays are expensive and have limitations in determining the actual pathophysiology of the organism *in vivo (*Best et al., 2018*)*.With intermittent shedding of the bacteria through human excreta; a chronic carriage can trigger a ripple effect in spreading the infection from a family to a community in a larger sense. This cries for tests that are cost effective, time saving and accurate (De Korwin, 2003).While PCR-based methods showed promising results for determining presence of *H. pylori* in laboratories with proper setup, they might not always be accessible by the common (Rimbara et al., 2013).

4. Existing Control measures

The pathogen's survival adaptations in the natural environment are poorly understood, and its transmission method is dubious. However, a lot of people believed that the sickness originated primarily from contaminated food and water (Quaglia and Dambrosio, 2018). It has also been demonstrated that upholding good sanitation practices, such as pasteurizing milk and managing wastewater better significantly lowers infection (Fujimura et al., 2002).

4.1. Therapeutic intervention

Triple therapy, a standard antibiotic regimen comprising of a proton pump inhibitor and a combination of clarithromycin, amoxicillin or metronidazole was considered to be the gold standard in *H. pylori* treatment for a long time (Wang et al., 2015). Prolonged exposure to these antibiotics induced AMR so much so that 90 % of African (Kouitcheu Mabeku et al., 2019) developed resistance against metronidazole whereas 49 % of Spanish population developed resistance against clarithromycin (Agudo et al., 2010). As a result, global trend of treatment moved from conventional to alternatives such as incorporation of bismuth or probiotics with existing treatment (Goderska et al., 2018). These treatments function in a time and dose dependent manner with limitations such as bismuth-toxicity (Hafeez et al., n.d.).

4.2. Control measures by vaccine

A vaccine is one of the most outstanding achievements of modern science and medicine. In the past, it has helped to eliminate many fatal, infectious diseases from our society. Various types of vaccines are available that use live attenuated or inactivated pathogens (bacteria and viruses) or conjugated polysaccharides to develop immunity. To be precise, these foreign objects or antigens stimulate the immune system to produce antibodies. Antibodies are produced by the plasma cells (primarily from B lymphocytes) and directly encounter the antigens or their toxins by their epitopes. A cell-mediated immune response is mediated by T-lymphocytes that differentiate into cytotoxic T lymphocytes or CTLs. At the same time, helper T lymphocytes recognize and produce cytokines, ultimately initiating a cascade of death-associated pathways that kill the pathogen and induce antigen-specific memory T cells. Activation and sustenance of these helper T cells control the degree of response toward an infection. In case of *H. pylori*, another type of T lymphocyte, were found called Treg or T-regulator cells that mediate immune tolerance by regulating the actions of effector-T cells (Janeway et al., 2001).

In 2004–2005 Wuhu Kangwei Biological Technology Co., Ltd conducted a vaccination program that involved oral administration of a recombinant *H. pylori* vaccine. The preliminary report published in 2015 revealed, of 99 % participants who completed a 3year trial, reported 64 events of *H. pylori* re-infection within first year,7 % of both vaccine group and placebo group participants with adverse reaction and <1 % reported serious adverse effects when exposed to circulating strains of *H. pylori*. In spite of achieving a vaccine efficacy of 71.8 % with a significant reduction in *H. pylori* infection and good immunogenic response

among naive children, the study was terminated after phase III clinical trial (Sutton and Boag, 2019). This was the first reported vaccination program of its kind but certainly left a huge grey area to ponder on, like 10 years delay in publishing the preliminary report or why the complete report has never been disclosed in public.

IMX101, developed by ImeVax, essentially targets a key survival process of *H. pylori* in the human stomach that isto reduce the effect of low gastric pH. One of the most potent *H. pylori* antigens, γ-glutamyl-transpeptidase (GGT) is responsible for inducing immunosuppressive activity by neutralizing highly acidic environment of the stomach disabling the inflammatory response in the host. While the result of phase I clinical trial of the vaccine is yet to be published, the approach certainly is novel and thus intriguing to see whether targeting a single antigenic component is efficient enough or not in order to generate immunity given the fact that the bacteria employ a number of other defensive mechanisms related to adhesion, colonization and destruction of the mucosal lining of the stomach (Formichella et al., 2013).

Recently many small companies and academic institutions started showing interest and took initiatives towards *H. pylori* vaccine development. All these approaches are at the preclinical stages and involve mainly purified or recombinant antigen components accompanied by an adjuvant.Though the preliminary results of these studies are promising but they lack consistency and clarity. For example, (i) an epitope-based vaccine EpiVax, involves a DNA vaccine initially followed by a peptide-liposome. The vaccine showed some therapeutic protection but is restricted only in mice,(ii) Dual approaches taken by Helicovaxor® -first one involving genetically engineered non-virulent *Vibrio cholerae* strain expressing *H. pylori* antigens (HpaA, UreB, and FlaA) while other one involves inactivated *H. pylori* strain engineered to induce serum antibody response both of which are at research level, (iii)Two recent studies, one on urease subunits and another on Lp220 (lipopolysaccharide220), conducted among BALB/c mice have shown minimal effect in generating protection, (iv) Probiotic as a vehicle for vaccine delivery involved microbes like *Lactococcus lactis* recombinantly expressing cholera toxin B subunit in addition to *H. pylori* urease epitope, has also not been found effective in developing immunity (Sutton and Boag, 2019)[Table 1] .

5. Advantages and disadvantages of existing animal models in *H. pylori* vaccine research

Due to the absence of any natural model of *H. pylori*, often existing animal models are being manipulated physiologically, immunologically or genetically to be more susceptible for the infection. Several studies implicated the need of a model for studying the pathogenesis and immunization of *H. pylori* (Amalia et al., 2023). However, selecting a suitable model has proven to be difficult as it may prevent exact translation to the human condition due to its own defense mechanisms. Though black mice (C57BL/6) are commonly used model for *H. pylori* study, studies are also done in animals including Sprague-Dawley rats, Mongolian gerbil, guinea pig, gnotobiotic piglets and non-human primates (Ansari and Yamaoka, 2022; Nedrud, 1999).A brief account on these animals is described in Table 2.

From the perspective of scientific experimentation, black mice have a number of advantages over others which allow them to be considered as a model of choice; one such is immunological manipulation. To understand how immunological manipulation works in vivo, experiments like the effect of *H. pylori* in allergic asthma can serve the purpose. The experiment revealed, when both neonate and adult mice were infected with *H. pylori* antigens and induced with OVA as an allergen; stimulated asthma like symptoms and showed considerable increase in the pulmonary CD4+FoxP3+ Treg level. The effect was more profound in neonatal mice compared to the adults. This indicates a long-lived

Table 2
Advantages and disadvantages of existing animal models.

Sl. No.	Animals	Advantages	Disadvantages
1.	Mouse	• Well documented immune response. • Knockout mice lack specific components of the immune system. • Easily available. • Cost effective and requires less maintenance.	• Infection often leads to lymphocytic gastritis and not peptic ulcers or gastric cancer lead by H. pylori. • Differences in the architecture between human and mouse stomach thereby affecting gastric pathology. • Murine stomach isn't sterile and contamination is a big issue when it comes to testing colonization of H. pylori.
2.	Mongolian gerbil	• Convenient size and ease in terms of husbandry. • Long term infection has proven to cause gastritis similar to that of human.	• They are out-breeding animals. • Lack of defined knowledge of the immune system. • Proper genetic tools are lacking for genetic mutations.
3.	Guinea pig	• Nutritional requirement for vitamin C just like humans gives an advantage as low vit C level has been found to be directly associated with H. pylori infection.	• Limited only for testing H. pylori immunization and vaccination and also studies like vitamin supplementation or mutation of adhesions. • Long-term colonization leads to only gastritis and not peptic ulcers or adenocarcinoma.
4.	Gnotobiotic pig	• Monogastric mammal. • Dietary habits similar to human. • Similarity in anatomical and physiological characters of both the stomach.	• Only gastric ulcers and MALT lymphoma has been found upon infection. • Lack of availability as they're currently not in use.
5.	Non human primates	• Used for testing efficacy of therapeutic intervention by antimicrobials. • Study of efficacy of vaccine and antiadhesion compounds. • Permitted study of the role of mucins and Lewis antigens in adhesion of H. pylori.	• Expensive and requires a lot of maintenance. • Though cancer has been reported but not gastric cancer.

Table 1
Status of different vaccine researches.

Candidate	Vaccine (s)/ components	Preclinical	Phase I	Phase II	Phase III
Wuhu Kangwei Biological technology	UreB/LTB fusion vaccine	√	√	√	Discontinued
Imax	IMX101	√			
Helicovaxor®	Engineered Vibrio cholerae vaccine, Inactivated H. pylori	√			
Sichuan University	Urease epitope vaccine	√			
Southern Medical University	Lp220 vaccine	√			
China Pharmaceutical University	Probiotic vaccine delivery	√			

immunological tolerance by Tregs through infiltration of semi mature DCs in alveoli at an early stage (Zuo et al., 2021). In another study, Mukhupadhaya *et al.* showed that black mice infected with a non-virulent strain AM1 when exposed to a virulent and mice adapted strain SS1, had elevated TH1, cytokine IFN-γ level with significantly reduced TH17 response.Thus studies like these emphasize the role of introducing non-virulent primed Treg cells to reduce the severity of gastric pathology in black mice (Ghosh et al., 2017).Therefore, it is evident that *H. pylori* infection and its degree of immunological manipulation determine the severity of the gastritis, which is more readily observable and measurable in C57BL/6 than others.

6. Routes of administration affecting the efficacy of *H. pylori* vaccine

The hostile acidic environment inside a stomach acts as a physiological barrier discouraging adhesion of any intruder. Due to selective permeability of gastric epithelial lining, only certain essential micro and macro nutrients are allowed to pass through them. On arrival, *H. pylori* enters into the stomach where it employs sophisticated mechanisms that not only allow the pathogen to adhere but also thrive in the microaerophilic condition. There it begins to neutralize the acidic gastric juice by producing urease and other metabolic products further suppressing the host's immune response by restricting immune cells proliferation.

The route of administration of a vaccine determines the immune response at cellular level. Several cell-signaling pathways such as EGFR, MAPK and JAK/STAT, are found to be involved in the inflammatory response induced by *H. pylori (*Sierra et al., 2018; Khanna et al., 2015). Early activation of these cell-signaling pathways leads to the production, expression and secretion of various chemokines (CXCL8, CXCL1, and CXCL5), resulting in an enhanced inflammatory response (Mustapha et al., 2014). Interestingly JNK signaling pathway has not been found accountable in early chemokine production conversely TOLL-like receptors are found directly involved in the inflammatory response in Primary Human Gastric Epithelial Cells (PGEC) (Sierra et al., 2018). Therefore, a novel vaccine strategy is necessary to generate and sustain immunity by activating different signaling pathways against these mucosal pathogens regulated by Antigen-primed immune cells.

The major hurdle in developing an effective *H. pylori* vaccine is the need for a proven vaccine-effective antigen and safe mucosal adjuvant. The presence of high genetic variations among the circulating strains and their role in disease status is largely unknown. Thus, developing a safe and effective route to generate an immune response has limitations. So far, attempts at oral immunization in vaccine development have shown promising results in mice. For instance, oral administration of a non-toxic adjuvant α-galactosylceramide (α-GalCer) along with prophylactic intragastric immunization with a whole-cell killed *H. pylori* antigen showed a substantial elevation in the systemic and intestinal Th1 response including significant antigen-specific mucosal and antibody response. TH1 response due to the adjuvant is dependent on IL-based signaling (CD1d, IL-1R, and IL-17R). Moreover, CD1d activates DCs and selectively promotes TH1 responses while adjuvant-induced IFN-γ, which may progress into local pro-inflammation mediated by different chemokines, ultimately leading to the activation of M1 macrophage population (Longet et al., 2019).

The numbers of DCs are generally higher in well-vascularized muscles and, thus a preferable route of injection for most vaccines. Adipose tissues in the subcutaneous layer contain fewer DCs than the intramuscular region. Therefore, subcutaneous injection may be less effective while administering the vaccine. On encountering a pathogen, immune cells proliferate rapidly and then move toward different organs for maturation. The mature cells with specific surface receptors migrate to secondary lymph nodes where T and B cell responses are induced (Nurgalieva et al., 2005). As *H. pylori* is non-invasive, an intra-venous administration of immunogen holds no logic.

7. Approaches to stimulate host immune system: antigen presentation

An imunogen is a pathogen derived component used in vaccine design that has the potential to induce an immune response with or without any additional help. Advancement in biotechnologies, especially nanotechnology, has contributed to developing particles that mimic bio-particles such as a Pathogen-Like-Particle or PLPs that initiates an immune response triggering both innate and adaptive immune systems, similar to an infection-mediated response. These particles are highly versatile and can range from simple surface proteins to different secretary proteins and most importantly, they are susceptible to modification according to their requirement. Nowadays, a number of different PLPs are not only used as vaccine but also as adjuvants. Table 3 shows a contrasting picture of the pros and cons of different immune-stimulatory agents that are frequently used in vaccine development.

7.1. Clinical potential of Outer membrane vesicles

An OMV is an outer membrane vesicle released from the outer membrane of gram-negative bacteria as a survival response. The size of these vesicles typically ranges from 20 to 400 nm and usually contain electron-dense material encased within a single lipid bilayer. Depending upon the size of these vesicles, internalization of OMVs into the epithelial cells takes place. The internalization process involves micropinocytosis, clarithin, or caveolin-mediated endocytosis (Parker et al., 2010; Parker and Keenan, 2012). It has been observed that the degree of antigenicity is higher for larger OMVs as they contain more outer membrane proteins, channel-forming proteins, lipoproteins, and lipopolysaccharides. Interestingly these features allowed considering OMVs as a vaccine candidate with tremendous potential. In general, OMVs show the following characters-

- They are acellular. Therefore, they cannot replicate within the host.
- They do not alter the antigen's structural conformations, which helps in antibody production more effectively.
- They remain stable after long-term storage at 5^0 C, thereby increasing their shelf life (Carvalho et al., 2019).

Immune response has been found to be directly associated with the amount of OMVs used as an adjuvant. In case of *H. pylori,* administration of a low dose of OMVs from cag PAI^+ toxicogenic and cag PAI^- non-toxigenic strains have shown increased proliferation in AGS cells (Ismail et al., 2003). Growth arrests, increased toxicity, and production of IL-8 are some key effects observed at higher doses. A consistent administration of OMVs has been found effective in developing low-grade gastritis, indicating the importance of determining a safe and controlled dose. *H. pylori* release OMVs both *in vitro* and *in vivo*, containing proteins, lipopolysaccharide (LPS), and lipoproteins which ultimately lead to the generation of an immune response by stimulating Toll-like receptors (Pachathundikandi et al., 2015). While the efficacy or route of administration of OMVs requires further research but they certainly provide an ideal platform to generate desired immune response in animals.

7.2. Clinical potential of Recombinant-based vaccine candidate

Recombinant technology has revolutionized many of the traditional approaches in modern medicine by manipulating the expression of a specific gene isolated from an organism expressed in different organism. This allowed the expression of isolated proteins in desired quantity which doesn't affect the host and thus can further be used for different studies, including vaccine. For example, studies on recombinant attenuated *Salmonella typhimurium* modified to express *H. pylori* antigen UreA and UreB, subunits of Urease, have shown promising results as a vaccine delivery system. The efficacy of this methodology can be interpreted in

Table 3
Pros and Cons of conventional and alternative approaches to stimulate an immune response.

Vaccines/Adjuvants	Pros	Cons
Live attenuated	• A non-virulent strain of a pathogen is used to induce an immune response. • One or two doses are enough to produce life-long immunity.	• Not suitable for immuno-compromised people. For example, people with HIV infection or under chemotherapy.
Bacterial ghost	• Cell envelopescontaining outer membrane particles are devoid of any cytoplasmic components.	• Possibility to develop a reaction like inflammation or become hypersensitive due to its reactogenicity.
Heat kill	• Heat-kill pathogens lack active functional genetic material or proteins.	• Requires booster shots to train the body to produce immunity.
Synthetic nanoformulation	• Synthetic nanoparticles have easy uptake via DCs depending upon the particle size. • Highly modifiable.	• The relationship between efficacy and the size of the particle still needs to be discovered. • Less complexity.
OMVs	• It can be modified to express recombinant proteins. • Self-adjuvancy	• Endotoxic content.

terms of induction of an immune response where a single nasal dose of the vaccine has been shown to induce both Th1 and Th2 responses in BALB/c mice as much as 60 % reported resistance to *H. pylori* infection after receiving live attenuated recombinant *Salmonella* (Corthésy-Theulaz et al., 1998 Feb 1). Study with *Helicobacter pylori* heat shock protein (HspA) recombinantly expressed in measles virus (MV) and infected in measles-permissive transgenic mice not only developed a long-time anti-Hsp antibody response but also remained protected against MV. These results are encouraging, but their reactogenicity assessment requires more in-depth investigation (Iankov et al., 2020).

7.3. Clinical potential of Bacterial Ghost

Bacterial ghost (BGs) is a relatively new approach that employs an empty, non-denatured envelope of gram-negative bacteria. This is achieved by recombinantly expressing lysis mediated by protein-E, which helps retain all structural and morphological features of the standard cell until they are induced. Any changes in the physical parameters caninduce lysis in the cell wall, thereby releasing the cellular content. The surface molecules present on these empty envelopes or ghosts can induce an immune response similar to the actual infection, making them an excellent platform for a vaccine delivery system (Langemann et al., 2010).

H. pylori ghost generation mediated by PhiX protein E, when administered orally in BALB/c mouse, showed a significant reduction in the bacterial load in the immunized group.Further co-administration with cholera toxin (which acts as a mucosal adjuvant) showed complete protection against *H. pylori* (Panthel et al., 2003). When attenuated *Salmonella* ghosts are used to deliver *H. pylori* specific outer inflammatory protein DNA (oipA) and administered orally in black mice, resulted in significantly higher levels of IgG2a/IgG1 antibodies and IFN-γ/IL-4 cytokines. The oipA DNA-BG vaccine, when challenged with *H. pylori* virulence strain SS1, showed a reduction in the colonization density along with a mixed Th1/Th2 response, thus indicating the potential of BG as an immune stimulator (Chen et al., 2014).

8. Emergence of an mRNA-Based Vaccine platform

The Discovery of mRNA-based vaccines revolutionized the face of vaccine development. Conventional approaches involving the whole cell, subunit, synthetic peptides, or even DNA have pros and cons. Table 4 shows some of the advantages of an mRNA vaccine. Increasing efficacy, reducing reactogenicity, and generating anti-inflammatory responses or antibodies are some primary goals that play a crucial role in approaches, subsequently influencing the outcome. A relatively new approach in this arena, m-RNA vaccine provides an excellent alternative against conventional approaches and has shown encouraging outcomes in diagnosing complex diseases like cancer and other infectious diseases due to their high potency, capacity for rapid development, low-cost manufacture, and safe administration (Pardi et al., 2018). Recent application of mRNA vaccine shows their tremendous versatility and ability to adapt to both infectious and non-infectious diseases (Gu et al., 2022). The problem with the traditional approach is not the effectiveness but their large-scale production in less time.

Table 4
Advantages of mRNA as a vaccine.

Features	Parameters	Advantages of mRNA-based vaccine
1.	Safety	• mRNA is non-infectious. • Non-integrating platform. • Less risk of insertional mutagenesis. • They are naturally degraded. • Half-life can be regulated by modification. • Inherent immunogenicity can be downregulated.
2.	Efficacy	• Stability and translatability can be modified. • In vivo delivery by formulating mRNA into carrier molecules thereby allowing rapid uptake and expression in the cytoplasm. • Anti-vector immunity is avoided as mRNA is considered a minimal genetic vector. • Repeated administration is possible.
3.	Production	• Rapid and inexpensive. • High yield due to transcription reaction in vivo.

An mRNA-based vaccine generally contains desired antigen coding region along with both 5′ and 3′ untranslated regions (UTRs). However, a self-amplifying RNA not only encodes the antigen but also employs replication machinery that enables intracellular RNA amplification and abundance in protein expression. Many cell surface, endosomal, and cytosolic immune receptors recognize exogenous mRNA. They are inherently immune-stimulatory, and thus therapeutic application determines the aftermath of such vaccines (Wang et al., 2021). It can either be beneficial or detrimental for the host as it can stimulate DC maturation giving rise to robust T and B cell immune response. This simple yet highly sophisticated immune system machinery also increases the risk of reactogenicity (Powell et al., 2021), leading to inflammation.

So far, a handful of genes have been explored from *H. pylori*, while the rests are yet to be explored. Based on facts, preliminary research of vaccine development using existing molecular techniques has shown to be effective in generating an immune response. Therefore, it would be interesting to see the efficiency of an m-RNA based vaccine both *in vitro* and *in vivo* systems leading to develop a more safe and potent vaccine candidate that will be safe to use in larger animals and, hopefully, humans.

9. Lacunae of present *H. pylori* vaccine research

Although initial steps have been taken, getting a vaccine seems far from reality. Some of the major loopholes in *H. pylori* vaccine research can be summed up into the following-

i. Lack of information on this highly genetically variable organism. Thus, it becomes tough to pinpoint any particular gene (or genes) solely responsible for the disease development.
ii. World Health Organization declared *H. pylori* a class I carcinogen. Due to the asymptotic nature of the infected individuals, detection and diagnosis are essential, which requires proper tools and techniques, whichare only possible in laboratories equipped with advanced testing pieces of equipment to conduct a higher level of research.Thus, infrastructural barriers must be resolved.
iii. Despite having the potential to cause an outbreak, most pharmaceutical companies have not shown any interest yet, and thus, research is only restricted the research institutes and university laboratories.
iv. Infection of *H. pylori* and its circulating strains is restricted only in humans. There are no other natural models available. In laboratories, animals are being modified physiologically, immunologically, and surgically for research. Thus, unavailability of a proper model hampers the research.

10. Conclusion in *H. pylori* vaccine research

In conclusion, the review emphasizes the critical need for novel vaccine candidates in the prevention and treatment of *Helicobacter pylori*-induced gastritis. *H. pylori* infection is a major global health concern, contributing to a variety of gastrointestinal ailments. While antibiotic therapy has generally been the major treatment technique, the emergence of antibiotic-resistant bacteria and the difficulties associated with eradication underscore the need for other strategies. Vaccination offers a promising approach to controlling *H. pylori* infection by preparing the immune system to recognize and kill the bacterium. However, producing an effective *H. pylori* vaccine has been difficult due to the bacterium's ability to avoid host immune responses and establish persistent colonization in the stomach mucosa. Optimal vaccine design, formulation, and delivery strategies necessitate ongoing research efforts going ahead. Moreover, extensive clinical trials are required to evaluate the safety, effectiveness, and long-term preventive benefits of potential vaccinations in a variety of groups. In order to overcome the remaining obstacles and develop effective *H. pylori* vaccines, cooperation between academic institutions, private sector companies and public health agencies will be essential. The development of next-generation *H. pylori* vaccines appears to be promising in light of developments in molecular biology, vaccine technology, and immunological knowledge. The investigation of innovative vaccination platforms that may improve vaccine efficacy and immunogenicity is a part of this, including recombinant vector-based vaccines, nucleic acid vaccines, and mucosal delivery systems. Using cutting-edge technology like artificial intelligence and systems biology could also speed up vaccination.

Authors contribution

SD and HK wrote the manuscript. SB, PH, AKM, S Dutta provided valuable input and edited from time to time. HK and SD made the final corrections and edits.

CRediT authorship contribution statement

Sanjib Das: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Soumalya Banerjee:** Conceptualization. **Prolay Halder:** Writing – review & editing, Writing – original draft. **Asish Kumar Mulhopadhyay:** Writing – review & editing, Conceptualization. **Shanta Dutta:** Writing – review & editing. **Hemanta Koley:** Writing – review & editing, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Pentavalent outer membrane vesicles immunized mice sera confers passive protection against five prevalent pathotypes of diarrhoeagenic *Escherichia coli* in neonatal mice

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ABSTRACT

Diarrhoeagenic *Escherichia coli* (DEC) pathotypes are one of the major causative agents of diarrhoea induced childhood morbidity and mortality in developing countries. Licensed vaccines providing broad spectrum protection against DEC mediated infections are not available. Outer membrane vesicles (OMVs) are microvesicles released by gram-negative bacteria during the growth phase and contain multiple immunogenic proteins. Based on prevalence of infections, we have formulated a pentavalent outer-membrane vesicles (POMVs) based immunogen targeting five main pathotypes of DEC responsible for diarrhoeal diseases. Following isolation, OMVs from five DEC pathotypes were mixed in equal proportions to formulate POMVs and 10 μg of the immunogen was intraperitoneally administered to adult BALB/c mice. Three doses of POMVs induced significant humoral immune response against whole cell lysates (WCLs), outer membrane proteins (OMPs) and lipopolysaccharides (LPS) isolated from DEC pathotypes along with significant induction of cellular immune response in adult mice. Passive transfer of POMVs immunized adult mice sera protected neonatal mice significantly against DEC infections. Overall, this study finds POMVs to be immunogenic in conferring broad-spectrum passive protection to neonatal mice against five main DEC pathotypes. Altogether, these findings suggest that POMVs can be used as a potent vaccine candidate to ameliorate the DEC-mediated health burden.

1. Introduction

Diarrhoeal diseases are the second major cause of mortality in children less than five years of age and one of the main reasons people seek medical care globally [1,2]. *Escherichia coli* (*E. coli*) is a part of the normal microbiota of human intestine and is among the first bacteria to colonize the neonatal gut after birth [3]. Certain subgroups of *E. coli* are known to cause a broad range of diseases in humans and are considered pathogenic. On the basis of type of infections, pathogenic *E. coli* are divided into intestinal or diarrhoeagenic *E. coli* (DEC) and extra-intestinal *E. coli* (ExPEC) [4]. DEC causes more than 300 million infections each year with nearly 200,000 deaths globally [5]. 30–40% of

List of abbreviations: CFU, colony forming unit; *E.coli*, *Escherichia coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; EAEC, enteroaggregative *E. coli*; EIEC, enteroinvasive *E. coli*; EHEC, enterohemorrhagic *E. coli*; OMV, outer-membrane vesicle; IP, intraperitoneal; OMP, outer-membrane protein; LPS, lipopolysaccharide; WCL, whole cell lysate; TSB, tryptic soy broth; TSA, tryptic soy agar; PBS, phosphate buffered saline; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; sIgA, secretory IgA; TNF-α, tumor necrosis factor- alpha; IL, interleukin; IFN-γ, interferon gamma.

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diarrhoeal episodes are caused by DEC in developing countries and in 2011, DEC caused 120,000 deaths in children worldwide [6]. DEC are classified into five main pathological types, namely, enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC) [7]. ETEC is known to cause watery diarrhoea in infants and travellers, EPEC causes acute and chronic watery diarrhoea in the paediatric population, EIEC causes dysentery characterized by bloody mucoid diarrhoea, identical to that caused by *Shigella* sp., EHEC is associated with haemorrhagic colitis and haemorrhagic uremic syndrome (HUS) and EAEC causes acute and persistent watery diarrhoea in low to middle income countries and in immunocompromised patients [8].

Multi-drug resistant *E. coli* strains are emerging due to inappropriate use of antibiotics [9]. In the post-antibiotic era, prophylactic approaches like vaccines can work as a major asset for reduction of DEC mediated health burden [10]. Currently, researchers are trying to develop potential vaccines against DEC but are facing a major setback due to the presence of vast number of DEC serotypes and lack of production of an effective gut immune response [11]. As of now, no single licensed vaccine against DEC is available for public health use [12].

Outer membrane vesicles (OMVs) are nanosized proteoliposomes released from the outer membrane of gram-negative bacteria [13] and can serve as potential acellular vaccine candidates as they are readily taken up by mammalian cells and are strongly immunogenic in nature. OMVs contain natural antigens like phospholipids, peptidoglycan, lipopolysaccharides (LPSs), outer membrane proteins (OMPs) and components of membrane lipoproteins which mimic the bacterial outer surface. An OMV-based vaccine against *Neisseria meningitides* has been approved by the European Medicine Agency (EMA) for active human immunization [14–16]. Previous studies revealed that intraperitoneal (i.p.) immunization with *E. coli* OMVs protected mice against subsequent infections [17]. OMVs have been shown to elicit good mucosal immune responses [18], but currently no combination OMVs based vaccine has been developed against the pathotypes of DEC. Recently, a formalin-killed whole-cell combined vaccine candidate co-administered with either alum or Cholera toxin B (CTB) was developed and found to be effective against five main pathotypes of DEC in BALB/c mice [11]. OMVs possess intrinsic adjuvant properties due to the presence of LPS, flagellin, peptidoglycans, lipoproteins, and other outer membrane proteins (OMPs) which activate various Toll-like receptors (TLRs) [19], eliminating the need to add external adjuvants to enhance its immunogenicity.

Previous studies have reported that neonatal mice are more susceptible to *E. coli* mediated infections than adult mice [20,21]. Passive protection studies are conducted using a neonatal mice model to evaluate the potency and efficacy of a vaccine candidate [22–25]. Due to an immature immune system, protection of neonatal mice through vaccination is difficult to achieve as they lack sufficient time to generate an immune response against infection due to a short susceptibility period [26,27]. In a previous study, it was reported that passive transfer of immunized adult mice sera was able to protect neonatal mice against group B streptococcal infection [28]. Recently, our group demonstrated that transfer of OMVs immunized adult mouse sera conferred passive protection to suckling mice against non-typhoidal *Salmonella* mediated gastroenteritis [29].

In this study, we have developed a combined pentavalent OMVs-based immunogen (POMVs) from five main subgroups of DEC (EPEC, ETEC, EAEC, EIEC and EHEC) and determined the adaptive immune response induced by the immunogen. This study also aims to assess the passive protection conferred by POMVs immunized adult mouse sera in neonatal mice. From this study, we have found that POMVs induce significant mucosal and systemic immune responses in adult mice. The study depicts the role of POMVs-immunized adult mouse sera to passively protect neonatal mice against the five pathotypes of DEC. Our study indicates that POMVs can act as a vaccine candidate to reduce infections mediated by the five pathotypes of DEC.

2. Methods and materials

2.1. Bacterial strains and culture conditions

Pentavalent outer membrane vesicles-based immunogen (POMVs) were prepared from five circulating strains of DEC subgroups H10407 (ETEC), PD124 (EHEC), DSM411 (EAEC), BCH8865 (EPEC) and IDH10106 (EIEC). For challenge studies, another five strains of DEC subgroups were used 4266 (ETEC), VT3 (EHEC), BCH04060 (EAEC), BCH9327 (EPEC) and BCH10790 (EIEC). All the strains were obtained from ICMR- National Institute of Cholera and Enteric Diseases (NICED), Kolkata strain repository. All the strains were preserved in 20% glycerol in Brain Heart Infusion Broth (BHIB, Difco, USA) at −80 °C. For experimentation, strains were grown in Tryptic Soy Agar (TSA, Difco, USA) plates or in Tryptic Soy Broth (TSB, Difco, USA) at 37 °C under constant shaking conditions (100 rpm). For further studies, MacConkey media and antibiotic supplemented plates were used [30]. All the strains used in this study are listed in Table 1.

2.2. Preparation of POMVs

OMVs were isolated from five subgroups of DEC as previously described with slight modifications [29]. Briefly, 1 litre of TSB was inoculated with 10 mL of overnight culture of DEC subgroups and grown to log phase (8 h) under constant shaking at 37 °C. Bacteria were removed by centrifugation (8000 X g for 15 min and 30 min consecutively) and the supernatants were collected. This was followed by filtration of the supernatants consecutively with 0.45 μm and 0.22 μm pore size filters (Millipore, USA). To confirm the presence of viable bacteria in the supernatant, 100 μL of the collected filtrate was plated and incubated overnight at 37 °C on TSA plates. Protein degradation in the filtrate was prevented by addition of protease cocktail inhibitor (Roche, Switzerland). The supernatant was then ultracentrifuged using a Hitachi P27A-1004 rotor at 140,000 X g for 4 h at 4 °C. The OMV pellets obtained by ultracentrifugation were resuspended in phosphate buffered saline (PBS, 7.4). Further purification of OMVs were achieved using density gradient centrifugation. The resuspended OMVs were layered with care in a centrifuge tube containing a gradient of sucrose ranging from 10%−50%. Tubes were then centrifuged using a swinging bucket rotor (Beckman-Coulter; SW 32.1 Ti) at 150,000 X g for 5 h at 4 °C. The OMV fractions found between 20% and 30% of sucrose were collected and centrifuged at 150,000 X g for 2 h. The pellet obtained was resuspended in PBS and was again filtered with 0.22 μm pore size filters [31]. Purified OMVs of DEC subgroups obtained were either stored separately or mixed in a 1:1:1:1:1 ratio on the basis of protein content to make a final concentration of POMVs of 10 μg/100 μL PBS and stored at −80 °C for further experiments.

2.3. Transmission electron microscopy (TEM) analysis by negative staining of OMVs

On carbon-coated grids, a 5 μL aliquot of OMVs was placed for 1 min, after which it was washed with 2 drops of Tris-HCL buffer. After blotting of the excess buffer, OMVs were negatively stained using 2% aqueous solution of uranyl acetate for 30 s. Excess stain was blotted off and the grids were air dried. Finally, the negatively stained OMVs were analysed using a JEOL JEM-2100 transmission electron microscope operating at 100 kV [32] (Fig. 1a).

2.4. Particle size analysis of OMVs by dynamic light scattering (DLS)

Size distribution of OMVs was measured by DLS. Briefly, OMVs were diluted to a concentration of 0.1 mg/mL in milli-Q water and subsequently the sizes of different OMVs were measured using Zetasizer Nano

Table 1
List of strains used in this study.

Strain name	Purpose of use	DEC subtype	Abbreviation	Reference
H10407	Immunogen preparation	Enterotoxigenic *E. coli*	ETEC	[30] (Reference strain)
PD124	Immunogen preparation	Enterohaemorrhagic *E. coli*	EHEC	[30]
DSM411	Immunogen preparation	Enteroaggregative *E. coli*	EAEC	[30]
BCH8865	Immunogen preparation	Enteropathogenic *E. coli*	EPEC	Clinical isolate (This study)
IDH10106	Immunogen preparation	Enteroinvasive *E. coli*	EIEC	Clinical isolate (This study)
4266	Challenge study	Enterotoxigenic *E. coli*	ETEC	[30]
VT3	Challenge study	Enterohaemorrhagic *E. coli*	EHEC	[30]
BCH04060	Challenge study	Enteroaggregative *E. coli*	EAEC	[30]
BCH9327	Challenge study	Enteropathogenic *E. coli*	EPEC	Clinical isolate (This study)
BCH10790	Challenge study	Enteroinvasive *E. coli*	EIEC	Clinical isolate (This study)

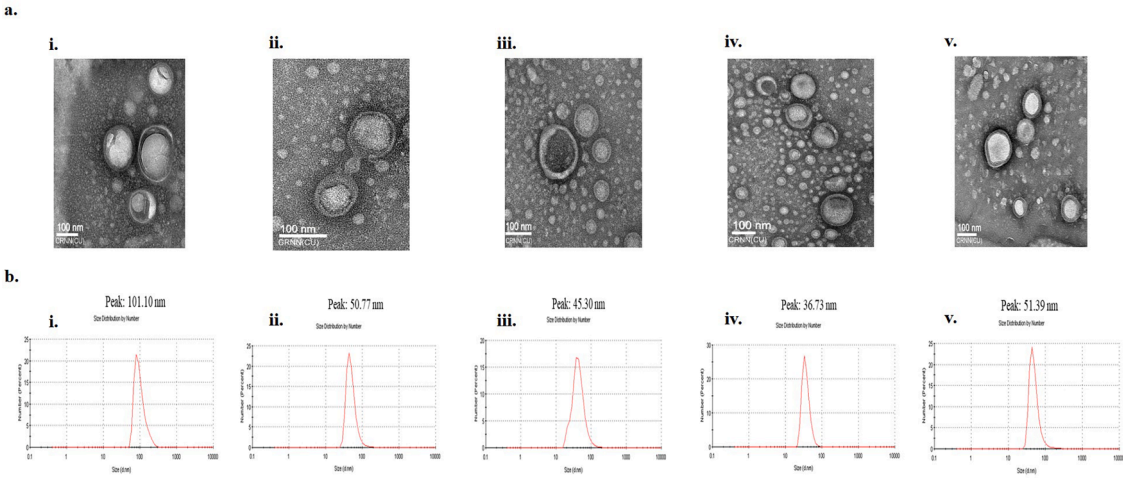

Fig. 1. (a) Transmission electron microscopic images of OMVs isolated from DEC subtypes: (i) EPEC OMVs, (ii) ETEC OMVs, (iii) EAEC OMVs, (iv) EIEC OMVs, (v) EHEC OMVs. (b) Dynamic light scattering analysis of OMVs from DEC subtypes with peaks ranging from 36.73 nm to 101.10 nm: (i) EPEC OMVs, (ii) ETEC OMVs, (iii) EAEC OMVs, (iv) EIEC OMVs, (v) EHEC OMVs.

series (Malvern, Worchestershire, UK) [33] (Fig. 1b).

2.5. Animals and housing

6–8 week old BALB/c mice were acquired from the ICMR-NICED animal house facility. Two female mice were housed with one male mouse of the same age for mating. Following conception, female mice were separated from the male and observed until birth. Other female adult mice were grouped ($n = 10$) in separate cages and maintained at 25 ± 2 °C temperature with 65 ± 2% humidity. Mice were provided with sterile food and water *ad libitum*. All the animal experiments were conducted following the standard operating procedure as outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The animal experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of NICED with Registration No. 68//Rebi/S/1999/CPCSEA valid17/7/2024, approval number PRO/183/-Jan 2021–24.

2.6. Mouse immunizations

Female adult BALB/c mice ($n = 6$) were immunized intraperitoneally (i.p.) on day 0, followed by boosters on the 14th and 28th days, with POMVs and the control group was administered with PBS on the same days. A dose of 10 μg of OMVs per mouse was used for i.p. immunizations. Immunization schedule and animal experiments are described schematically in supplementary figure 1.

2.7. Collection of serum and gut lavages

From the lateral tail vein of mice, blood was collected on the following time intervals: 0th, 7th, 21st, 35th and 56th days. The blood collected was taken in BD microtainer tubes (BD, NJ, USA) for separation of sera by centrifugation at 3000 rpm for 10 min at 4 °C. One week after the last immunization, 3 mice each from POMVs immunized and non-immunized group were sacrificed and the small intestines were dissected. The intestinal lumens were then flushed with PBS containing protease inhibitor (Roche, Sigma, USA). Then the collected lavage fluid was centrifuged at 1000 X *g* for 10 min at 4 °C, after which the supernatant was collected. Both the sera and intestinal supernatant were stored at −80 °C until further use.

2.8. Preparation of whole cell lysates (WCLs) from DEC subgroups

WCLs were prepared from the DEC pathotypes according to the previously mentioned protocol [32]. Briefly, bacteria were cultured overnight followed by centrifugation at 8000 rpm for 10 min. This was followed by a PBS wash and sonication (Heilcher UP100H). After checking the cells for membrane lysis, they were centrifuged at 10,000 rpm for 10 min and the supernatant was collected. WCLs were stored at

−80 °C until further use.

2.9. Extraction of outer membrane proteins (OMPs) from DEC subgroups

OMPs from DEC pathotypes were extracted according to the previous mentioned protocol [29] with slight modifications. Briefly, 50 mL of overnight culture was centrifuged at 8000 rpm for 10 min and cells were isolated. The isolated cells were washed with HEPES buffer (10 mM, pH 7.5) once, centrifuged and re-suspended in HEPES containing protease inhibitor. For lysis, cells were then sonicated using a Heilcher UP100H sonicator. The cells that failed to lyse were removed by centrifugation at 13,000 X *g* for 10 min and the supernatant was collected. The supernatant was then ultracentrifuged at 100,000 X *g* for 1 h at 4 °C. The pellets obtained were dissolved in 10 mM HEPES containing 1% sarcosyl and incubated for 30 min at 37 °C, followed by ultracentrifugation at 100,000 X *g* for 1 h at 4 °C. Finally, the pellets containing OMPs were washed twice with 10 mM HEPES and ultimately re-suspended in 10 mM HEPES. Protein content of the OMPs was measured using a Lowry protein estimation kit (Pierce, USA). The OMPs were stored at −80 °C until further use.

2.10. Extraction of lipopolysaccharide (LPS) from DEC subgroups

From a 50 mL overnight bacterial culture, cells were harvested by centrifugation at 8500 X *g* for 20 min, washed with normal saline and re-suspended in 150 mM NaCl containing Phenol-saturated 3-[N-*morpholino*]propen sulfonic acid (MOPS), followed by incubation with shaking at 65 °C in a water bath for 30 min. This was followed by incubation on ice for 10 min and centrifugation at 8500 X *g* for 30 min, following which the upper aqueous phase was collected. The collected aqueous phase was mixed with 95% chilled ethanol (−20 °C), 4 times the volume of aqueous phase, and kept at −20 °C overnight. LPS was ultimately isolated by centrifugation at 8500 X *g* for 20 min at 4 °C on the next day and re-suspended in milliQ water and stored at −20 °C for further use [29].

2.11. SDS-PAGE and western blot

SDS-PAGE analysis of WCLs, OMPs and OMVs of both the immunization and challenge strains was done using an AE-6503 SDS-PAGE apparatus (ATTO corporation, Japan). After estimation of protein concentration of the samples by the Lowry method, samples were denatured at 100 °C for 10 min. Aliquots of equal amount of proteins were separated by 12% SDS-PAGE and stained with Coomassie blue (Supplementary figure 2).

For western blot, the samples mentioned were first electrophoresed in 12% SDS-PAGE and transferred onto nitrocellulose membrane (Biorad, USA) by semi-dry transfer (AE 6687, ATTO corporation, Japan). Presence of immunogenic components in samples was determined using 35th day POMVs immunized mouse sera post 1st immunization. Alkaline phosphatase substrate BCIP/ NBT (MP, USA, Cat# 980,621) was used for developing the blots (Fig. 3a).

2.12. ELISA

Immunoglobulins from serum (IgG along with its subtypes IgG1, IgG2a, IgG2b, IgG3 and IgA) as well as intestinal lavage (sIgA) were measured by ELISA as described by Keren [34]. Briefly, disposable polystyrene micro-titre wells (Nunc, Denmark) were separately coated with 5 μg/well of bacterial WCLs, 5 μg/well of OMPs and 5 μg/well of LPS of each strain from which immunogens were prepared and incubated for 18 h at 4° C. Wells were washed and blocked with 5% Bovine Serum Albumin (BSA; Sigma Chemical, USA) for 2 h at 37 °C. After washing the wells with PBS-T (PBS with 0.5% Tween-20, Sigma Chemicals, USA), plates were incubated with serially diluted serum samples for 1 h at 37 °C and 100 μL of HRP-conjugated goat anti-mouse immunoglobulin was added, followed by incubation for 1 h at 37 °C. After washing with PBS, the substrate o-phenyl-Di-amine (OPD) was added to each well, followed by stopping the reaction after 10 min by addition of 100 μL of 2 N sulphuric acid. Measurement was taken at OD_{492} in a microplate reader. The experiment was repeated three times for each immunoglobulin, with the immunized and non-immunized serum, collected from individual mice, before, during and after immunization [29].

2.13. Flow cytometry analysis of splenic cells

Spleens were isolated from both non-immunized and immunized mice on the 35th day post three immunization doses for flow cytometry analysis. Mice were sacrificed under proper aseptic conditions and spleens were isolated, followed by disassociation using a sterile plunger of a 5 mL syringe and a cell strainer. RBCs were lysed using RBC lysis buffer (Sigma, USA) followed by re-suspension of the splenocytes in RPMI 1640 containing 10% FBS, 2-ME (50 mM) and antibiotics (5 U/mL penicillin G, 5 μg/mL streptomycin, and 0.1% gentamycin) (Gibco, USA). CD4+, CD8a+ and CD19+ splenic cell populations were determined by staining splenocytes with CD4-phycoerythrin (PE), CD8a-PE, CD19-PE and isotype control (PE) anti-mouse mAbs. BD FACS ARYA III flow cytometer was used for assessment of the cells and FACS DIVA software was used for analysis of data [29].

2.14. Assessment of serum bactericidal properties of POMVs immunogen and scanning electron microscopy (SEM)

Serum bactericidal assay was conducted according to the protocol mentioned previously with slight modifications [29]. Briefly, sera were collected from POMVs immunized and non-immunized mice on the 35th day post primary immunization. Collected sera was heat inactivated for 30 min at 56 °C. Each challenge strain of DEC was cultured on TSB. All the strains were grown for 2 h ($O.D._{600} = 0.4$) and bacterial pellet was collected by centrifugation at 1100 X g for 5 min and resuspended in PBS. 1:50 dilution of heat-inactivated sera with or without 25% guinea pig complement was added to the bacterial solution to make a total 50 μL reaction mixture and incubated 1 h at 37 °C. The reaction was subsequently stopped by addition of 950 μL TSB and ultimately plated on TSA plates by serial dilution for determination of CFU count. Bacteria incubated with non-immunized serum were used as control.

Bactericidal properties of POMVs immunized serum was also assessed by scanning electron microscopy (SEM) analysis. The strains were cultured as mentioned above, followed by incubation with POMVs immunized and non-immunized serum for 1 h at 37 °C and subsequently processed for SEM analysis. In short, samples were fixed using 3% glutaraldehyde and left overnight. The next day, samples were dehydrated in ascending grades of alcohol (30, 50, 70, 90, 100%), followed by chemical drying of the samples with hexamethyldisilazane (HMDS) by increasing the HMDS ratio gradually (2-part ethanol: 1-part HMDS; 1 part ethanol: 1-part HMDS; 1 part ethanol: 2-part HMDS). Finally, the samples were treated with 100% HMDS and left overnight in a fume hood for evaporation of HMDS, followed by mounting the samples on specimen stubs, sputter-coating with gold and analysis of images on Quanta 200 SEM (FEI, Netherlands).

2.15. Passive protection study in neonatal mice

One day old BALB/c neonatal mice were divided into 11 groups (A, B, C, D, E, F, G, H, I, J and K, $n = 6$ per group). Challenge study on neonatal mice was conducted as previously described with slight modifications [21,22,35]. Neonatal mice were orally inoculated with 1×10^5 CFU/neonate of challenge strains of DEC. Groups A and B were orally inoculated with EPEC BCH9327, C and D with ETEC 4266, E and F with EAEC BCH04060, G and H with EIEC BCH10790, I and J with EHEC VT3 and group K served as the control and was inoculated with PBS (uninfected). 2 h after challenge with DEC strains, groups B, D, F, H and J

were administered orally with pooled 20 μL of 35th day POMVs immunized adult mice sera (with a 1:10 dilution in PBS) while groups A, C, E, G and I received non-immunized adult mice sera of the same dilution. The neonates were returned to their dams and observed for 7 days for survival analysis.

In another set of experiments, neonatal mice were again divided into 11 groups ($n = 6$ per group) and inoculated with the same dose of challenge strains of DEC as stated above. Mice were passively immunized as described above and sacrificed after 24 h. Small intestines were collected for determination of bacterial load, homogenized, serially diluted, and plated on selective antibiotic supplemented agar plates as described previously [24,29,30]. The same set of experiments was conducted but with an inoculation dose of 1×10^3 CFU/neonate for determination of change in body weight up to 3 days post infection [29].

24 h post bacterial challenge with a dose of 1×10^5 CFU/neonate, small intestinal tissues from mice of the above mentioned groups ($n = 6$ per group), were homogenized in cytokine buffer and supernatants were stored at −80 °C as described previously for cytokine measurement [29]. For histological analysis, small intestinal samples from all the groups were collected 24 h post bacterial challenge and fixed using 10% buffered formalin solution.

2.16. Histological analysis

Following fixation using 10% buffered formalin, small intestinal samples were paraffin embedded, sectioned, double stained with haematoxylin and eosin (H & E) and visualized using an Olympus IX51 light microscope. Pathological scoring of samples (Table 2) was done as described previously with slight modifications [29,36].

2.17. Quantification of cytokines

IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70 and IL-23 were quantified from small intestinal supernatants stored in cytokine buffer using cytokine ELISA kits according to manufacturer's protocols (Invitrogen, USA).

2.18. Statistical analysis

Data are expressed as mean ± S.D. Comparison between three or more groups of data was analysed using two-way analysis of variance (ANOVA) and one tailed Student's t-test was used to analyse two grouped data sets. Mean values of triplicate experiments were taken for final calculation. A probability of $p < 0.05$ or $p < 0.01$ or $p < 0.001$ was considered as significant level. GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA) and Microsoft Excel was used for statistical analyses.

3. Results

3.1. Extraction of OMVs from DEC subgroups and preparation of POMVs immunogen

OMVs of all DEC subgroups secreted during the growth phase were found to be spherical in shape, as was revealed by TEM images (Fig. 1a i–v). The peaks of the OMVs of the DEC subgroups ranged from approximately 36 nm to 101 nm as analysed by dynamic light scattering (Fig. 1b i-v). Protein content of the OMVs was estimated and OMVs from five DEC subgroups were mixed in equal proportions (1:1:1:1:1 ratio) to formulate POMVs immunogen with a total concentration of 10 μg POMVs/100 μL of PBS.

3.2. POMVs elicited adaptive immune response in adult BALB/c mice

The magnitude of immunogenicity triggered by POMVs was determined after three doses of intraperitoneal immunization of adult BALB/c mice (10 μg of OMVs/mice) with 14 days intervening period between immunizations. The control group was administered with PBS intraperitoneally for comparative measurements. Immunogenicity of serum immunoglobulins was observed for 56 days after 1st immunization. The results demonstrated a boost in the production of serum antibodies, namely, IgG, IgG1, IgG2a, IgG2b, IgG3 and IgA against LPS, OMPs and WCLs isolated from homologous DEC subtypes with highest immunogenicity observed at 35th day post primary immunization and reached plateau afterwards (Fig. 2, a–c). Significantly higher levels of secretory IgA (sIgA) were found against LPS, OMPs and WCLs of each of the five DEC subtypes in the intestinal lavage of immunized mice as compared to non-immunized control groups, showing a significant activation of mucosal immune response (Fig 2d).

Western blot analysis was performed using 35th day polyvalent immunized adult mice sera against components of both the immunization and challenge strains. Analysis revealed immunogenic bands ranging from 200 KDa to 20 KDa against WCLs and OMPs with distinct bands in the range of 70 KDa to 20 KDa (Fig. 3a i & ii). In case of OMVs, distinct immunogenic bands ranged in the region of 40 KDa – 20 KDa (Fig. 3a iii).This signifies that POMVs contain antigenic components present in WCLs and OMPs due to which significant immunogenicity against a range of proteins present in these components can be seen in

Table 2
Pathological score reference table for histological images.

Inflammatory cell infiltrate			Mucosal architecture			
Severity	Extent	Score 1	Severity	Extent	Mucosal and epithelial changes	Score 2
Minimal	Mucosa	1	**Minimal**	Mucosa	Minimal hyperplasia	1
					Minimal villous blunting	1
Mild	Mucosa and sometimes submucosa	2	**Mild**	Mucosa, sometimes submucosa	Mild hyperplasia	2
					Mild to moderate villous blunting	2
					Mild goblet cell loss	2
Moderate	Mucosa and submucosa, sometimes transmural	3	**Moderate**	Mucosa, submucosa	Moderate hyperplasia	3
					Moderate villous blunting and broadening, sometimes villous atrophy	3
					Goblet cell loss	3
					Cryptits	
Marked	Transmural	4	**Marked**	Mucosa, submucosa, often transmural	Marked hyperplasia	4
					Villous atrophy	4
					Marked goblet cell loss	4
					Multiple crypt absences	4
Sum of Score 1 and 2: 0–20						

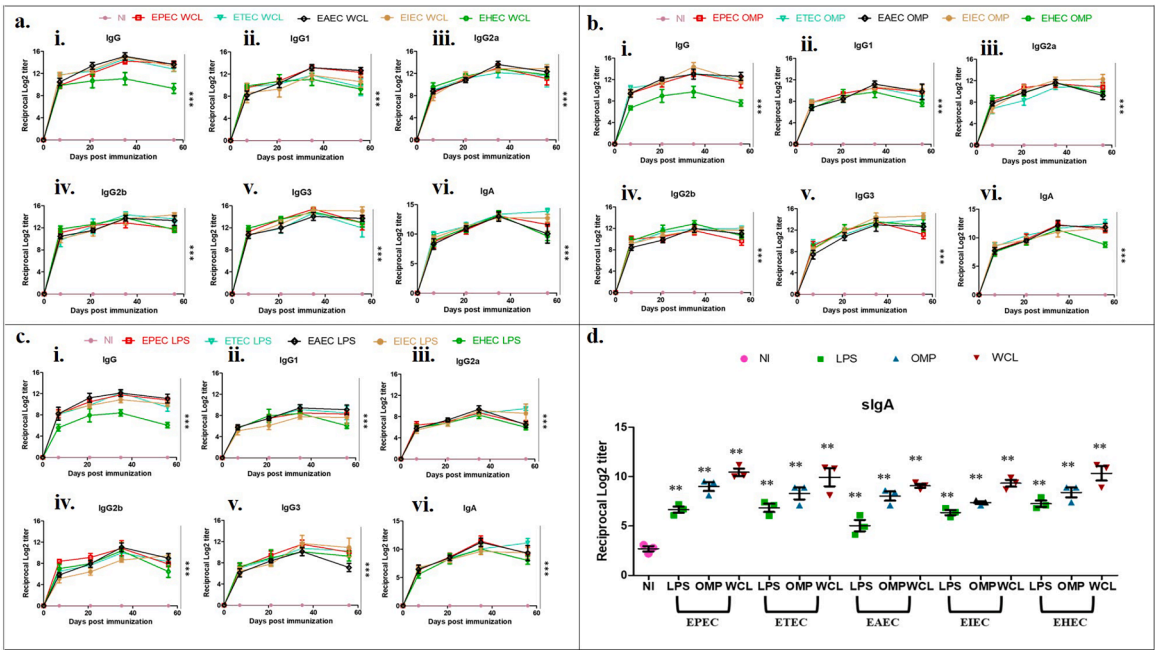

Fig. 2. Reciprocal Log2 titer of serum IgG, IgG subtypes and IgA immunoglobulin from POMVs immunized and non-immunized mouse serum were measured separately after three doses of intraperitoneal immunization against WCLs (a, i-vi) OMPs (b, i-vi) and LPS (c, i-vi) of each subtype specific five DEC strains. The horizontal axis represents pre-immunization, immunization and post-immunization periods during which antibody titers were measured. Values are represented as mean ± S.D. of three independent experiments. Significant statistical difference was found between POMVs immunized and non-immunized serum (*** p value < 0.001). (d) Reciprocal Log2 titer of mucosal IgA (sIgA) from POMVs immunized mice intestinal lavage obtained at 35th day after primary immunization. Significant statistical difference was found between POMVs immunized and non-immunized groups. Results are presented as one out of three experiments using six mice per group. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Data represented as mean ± S.D., $n = 6$. **p value < 0.01. WCL- whole cell lysate, OMP- outer-membrane protein, LPS- lipopolysaccharide.

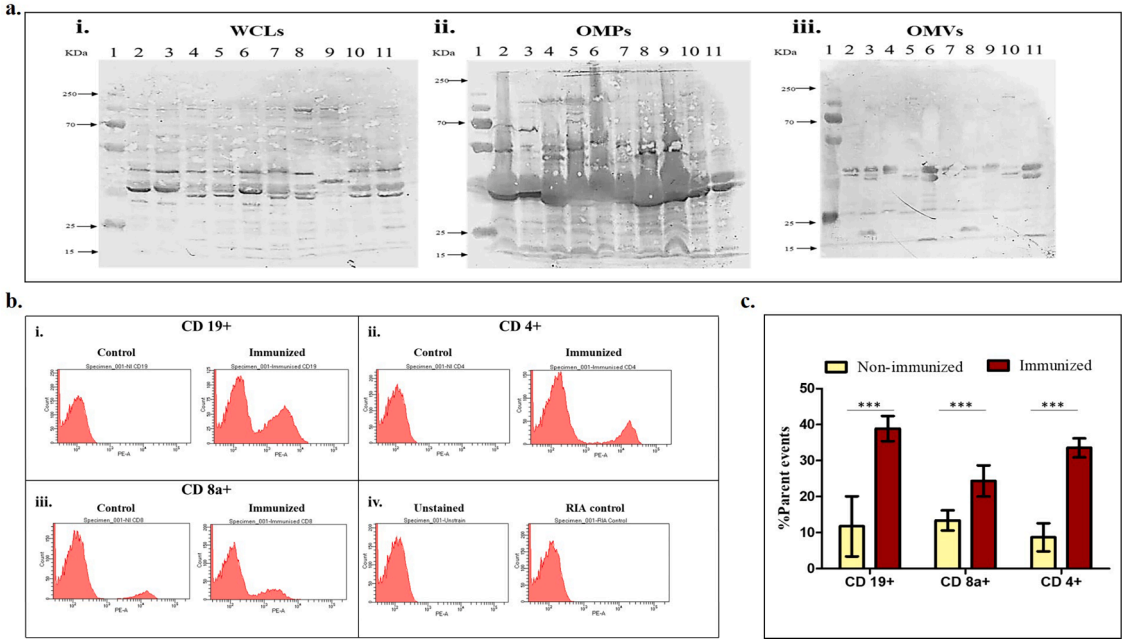

Fig. 3. (a) Immunoblot using 35th day POMVs immunized mice sera against WCLs (i), OMPs (ii) and OMVs (iii), isolated from both the immunization and challenge strains used in this study. For (i) WCLs and (ii) OMPs, the lanes numbered represent samples from the following: 1- Marker, 2- EPEC BCH8865, 3- EPEC BCH9327, 4- ETEC H10407, 5- ETEC 4266, 6- EAEC DSM411, 7- EAEC BCH04060, 8- EIEC IDH10106, 9- EIEC BCH10790, 10- EHEC PD124, 11- EHEC VT3. For (iii) OMVs, the following samples were loaded in the respective lanes: 1- Marker, 2- EPEC BCH8865, 3- ETEC H10407, 4- EAEC DSM411, 5- EIEC IDH10106 6- EHEC PD124, 7- EPEC BCH9327, 8- ETEC 4266, 9- EAEC BCH04060, 10- EIEC BCH10790, 11- EHEC VT3. (b) Representative histograms of FACS analysis of POMVs immunized and non-immunized CD 19+ (i), CD 4+ (ii), CD 8a+ splenic cells (iii) stained using CD 19-PE, CD 4-PE,CD 8a-PE and counted in FACS Aria III flow-cytometer (BD Bioscience, USA) . (iv) Represents unstained and RIA control. (c) Bar diagram comparing the percentage of CD 19+, CD 4+, CD 8a+ splenic cells from immunized and non-immunized mice. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. Significant statistical difference was found between POMVs immunized and non-immunized splenic cell population (*** p value < 0.001).

western blot.

Comparative analysis of the splenic cell populations of immunized and non-immunized mice were done using a flow cytometer. POMVs immunized mice showed a significantly greater population of CD4+, CD8a+ and CD19+ splenic cells as compared with non-immunized mice 35 days post primary immunization (Fig. 3b and c). This result suggests that antigenic components of OMVs help to elicit heightened immune responses due to activation of CD4+, CD8a+ and CD19+ splenic cells.

Altogether, i.p. immunization with POMVs generated adaptive immune responses in adult BALB/c mice and significantly activated both the cellular and humoral arms of the immune system, which could subsequently help to provide long term protective immune response against infections caused by DEC pathotypes.

3.3. POMVs immunized sera from adult BALB/c mice conferred bactericidal effect

Previous results in this study reported that POMVs immunization significantly induces high titres of anti-WCL, anti-OMPs and anti-LPS antibodies in adult BALB/c mouse sera for all the five subtypes of DEC. Bactericidal effect was analysed by serum bactericidal assay (SBA). Here, we found that immunized sera pooled from BALB/c mice, containing 25% guinea pig complement, significantly lowered the bacterial count when incubated with heterologous challenge strains of DEC subtypes, as compared to heat-inactivated (not supplemented with guinea pig complement) and non-immunized mouse serum. This signifies activation of complement pathway, along with significant antibody titres in POMVs immunized BALB/c mouse serum sufficient to effectively kill bacterial cells and subsequently lower the bacterial count (Fig. 4a). The data were further supported by SEM analysis of SBA. Pore formation, lysis and agglutination of bacterial cells were seen in DEC subtypes incubated with immunized mouse serum, whereas non-immunized mouse serum failed to effectively lyse the bacterial cells (Fig. 4b, i-v).

3.4. POMVs immunized adult mouse serum conferred passive protection to suckling mice against DEC subtypes mediated infections

Survival analysis was conducted for up to 7 days for suckling mice receiving POMVs immunized and non-immunized adult mouse sera. After challenge of suckling mice with 1×10^5 CFU/neonate of respective heterologous challenge strains of DEC subtypes, immunized and non-immunized pooled adult mouse sera (diluted in a 1:10 ratio) was administered orally 2 h post infection. Groups receiving immunized mouse sera represented significant better survival than the groups receiving non-immunized mouse sera (Fig. 5, a-e). Immunized sera recipient groups after challenge with EPEC, ETEC, EAEC, EIEC and EHEC strains represented a survival rate of 81%, 82%, 80%, 75% and 78% respectively. Suckling mice receiving non-immunized mice sera died within 2 – 4 days post infection. This result suggests that POMVs immunized adult mouse serum can significantly protect suckling mice against DEC mediated infections.

After administering a similar dose of DEC challenge strains mentioned above, assessment of changes in small intestinal colonization was analysed in immunized and non-immunized sera treated groups. 24 h post infection, a significant decrease in colonization of the five DEC pathotypes was noted in small intestine of immunized sera treated neonates as compared to non-immunized neonates (Fig. 5f).

For assessment of changes in body weight, suckling mice were administered a low dose of 1×10^3 CFU/neonate of challenge strains of DEC subtypes. Non-immunized sera treated groups displayed significant decrease in body weight observed over a 3 day period, as compared to immunized sera treated mice whose body weight remained approximately similar with that of uninfected groups (Fig. 6).

Histopathological changes of neonatal small intestine were analysed 24 h post infection with DEC challenge strains (1×10^5 CFU/neonatal mice). Recipient suckling mice administered with immunized mice sera showed a significant reduction in epithelial layer damage, altered intestinal mucosa, polymorphic nuclear neutrophilic (PMN) infiltration,

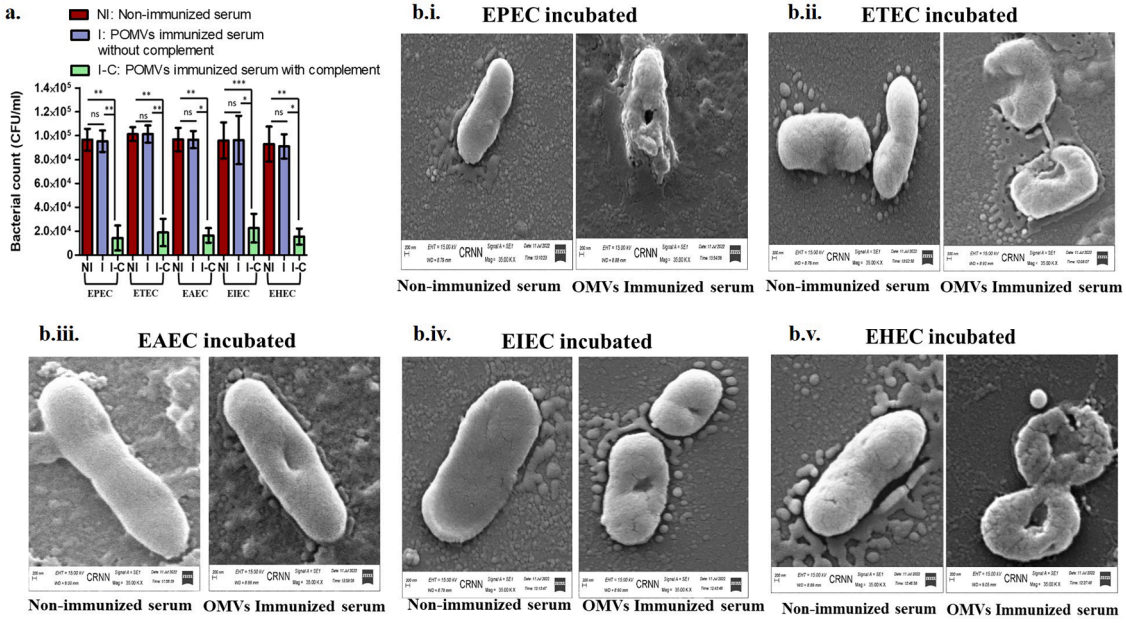

Fig. 4. (a) POMVs immunized mouse serum is effective in complement mediated lysis of DEC pathotypes. 1×10^5CFU/mL each challenge strains of EPEC, ETEC, EAEC, EIEC, EHEC were separately incubated with denoted dilution of either POMVs immunized serum or non-immunized serum with or without guinea pig complement for 1 h at 37 °C. Viable bacterial count were determined by plating on TSA plates. Two-way analysis of variance (ANOVA) test was used for statistical analysis. Bars represent mean ± S.E. of three individual experiments. *** p value < 0.001, ** p value < 0.01, * p value < 0.5. NI, non-immunized serum; I, POMVs immunized serum without complement; I-C, POMVs immunized serum with complement added. (b) Scanning electron microscopic images of DEC challenge strains incubated with either 35th day POMVs immunized mice serum or non-immunized mice serum. (i) EPEC incubated either with or without POMVs immunized serum, (ii) ETEC incubated either with or without POMVs immunized serum, (iii) EAEC incubated either with or without POMVs immunized serum, (iv) EIEC incubated either with or without POMVs immunized serum, (v) EHEC incubated either with or without POMVs immunized serum. Scale bar 200 μm.

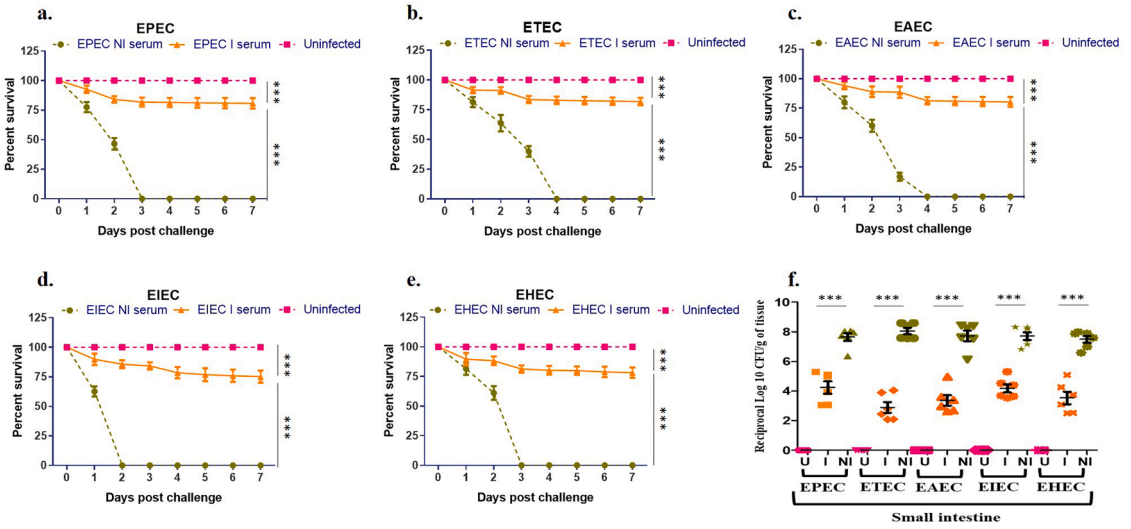

Fig. 5. One day old neonatal mice were challenged with 1×10^5CFU of DEC heterologous challenge strains separately. After two hours of challenge, neonatal mice were administered with pooled serum from POMVs immunized and non-immunized adult mice. Neonatal mice were observed for survival up to 7 days post infection. Mortality was determined based on humane endpoints. POMVs immunized sera recipient groups after challenge represented survival rate of 81% for EPEC (a), 82% for ETEC (b), 80% for EAEC (c), 75% for EIEC (d) and 78% for EHEC (e). Data are represented as mean ± S.E. of three independent experiments ($n = 6$). Protective efficacy was calculated using the equation {[(percent deaths of non-immunized neonates) - (percent deaths of immunized neonates)] / [percent deaths of non-immunized neonates]} X 100. (f) Comparison of protection determined from colonization data in small intestine of neonatal mice ($n = 6$), exerted by POMVs immunized and non-immunized adult mouse serum, after challenge with 1×10^5CFU/ neonatal mice of DEC challenge strains. Each dot represent colonization data obtained from a single neonatal mouse. On the vertical axis, data are represented as Log10 of recovered colony forming unit per gram of small intestine of each neonatal mouse, 24 h post challenge. The difference in colonization between the immunized and non-immunized sera recipient neonatal mice were highly significant (p value < 0.001). Two-way analysis of variance (ANOVA) test was used for statistical analysis. Data represented as mean value of six neonatal mice ± S.E. of three independent experiments. *** p value < 0.001. UI, uninfected; I, immunized sera recipient; NI, non-immunized sera recipient.

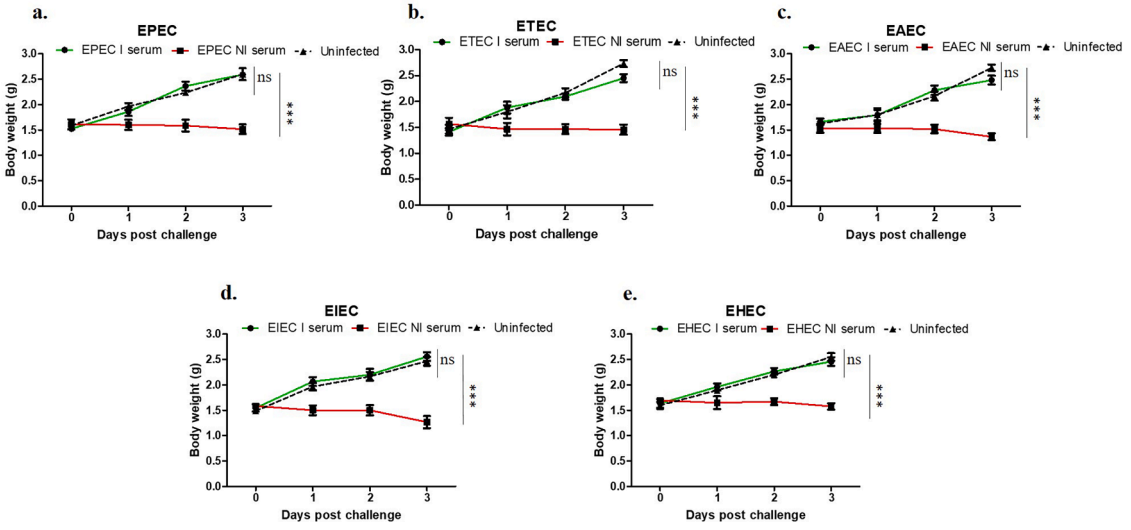

Fig. 6. One day old neonatal mice were challenged with 1×10^3CFU of DEC heterologous challenge strains separately ($n = 6$). 2 h post challenge, neonatal mice were administered pooled serum from POMVs immunized and non-immunized adult mice. Changes in body weight were monitored for 3 days (a-e). Every single dot represents mean and error value of six neonatal mice ± S.D. of three independent experiments. *** p value < 0.001. ns, non-significant. I, immunized; NI, non-immunized.

goblet cell loss, hyperplasia and villous atrophy as compared to non-immunized sera recipient suckling mice (Fig. 7a). Pathological scores of immunized sera treated suckling mice were also lower than non-immunized sera treated suckling mice (Fig. 7b). Overall, passively transferred POMVs immunized adult mouse sera, exerted a significant level of passive protective efficacy against five DEC pathotypes in neonatal mice.

3.5. POMVs immunized mouse serum lowers pro-inflammatory cytokine levels in neonatal mouse small intestine after infection

Significant differences in the amount of pro-inflammatory cytokines were noted in small intestinal tissue of immunized sera treated and non-treated neonates after infection with DEC subtypes (1×10^5 CFU/ neonate). Immunized sera recipient neonatal mice had substantially reduced levels of IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70 and IL-23 in small intestinal tissues as compared to non-immunized sera recipient neonatal mice after infection (Fig. 8). These pro-inflammatory cytokines

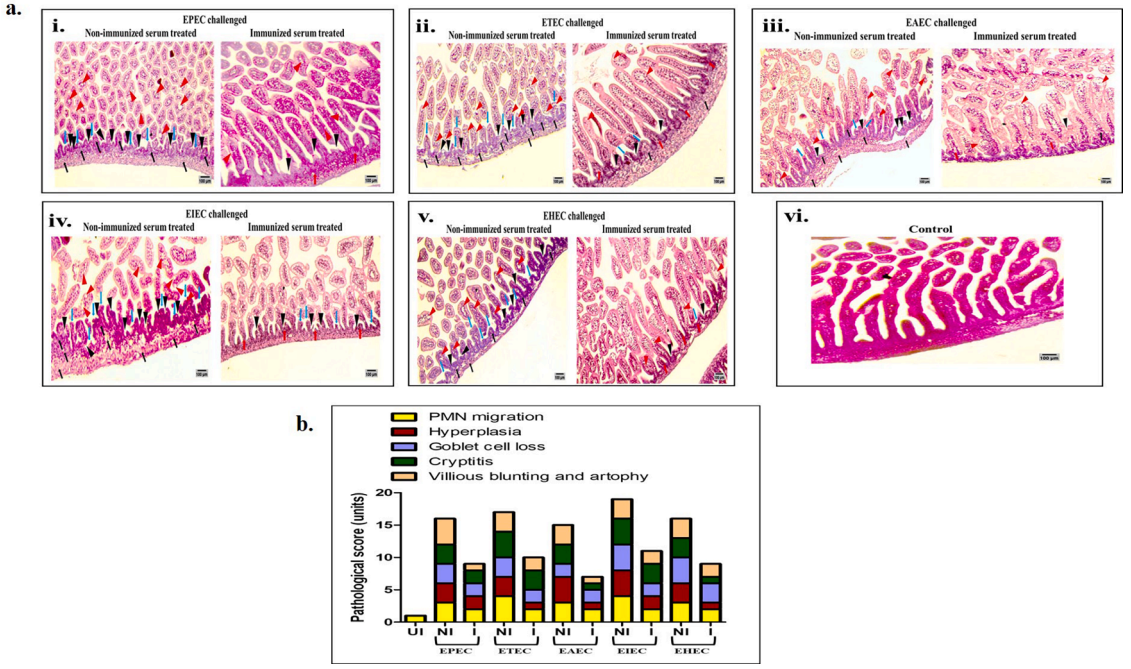

Fig. 7. Pentavalent OMVs immunized mouse serum decreases small intestinal tissue damage and inflammation in neonatal mice after infection with DEC pathotypes separately (1×10^5CFU). (a) Histological images of small intestine of neonatal mice administered with either 35th day POMVs immunized mouse serum or non-immunized mouse serum. (i) EPEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (ii) ETEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (iii) EAEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (iv) EIEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (v) EHEC challenged neonatal mice treated with either POMVs immunized or non-immunized serum, (vi) uninfected control neonatal mice small intestine. Small intestine of neonatal mice treated with POMVs immunized mouse serum showed mild epithelial layer damage, altered intestinal mucosa and polymorph nuclear neutrophilic (PMN) infiltration, whereas non-immunized sera treated neonatal mice displayed marked epithelial layer damage, altered intestinal mucosa, polymorph nuclear neutrophilic (PMN) infiltration, goblet cell loss, hyperplasia and villous atrophy. (Blue arrow– Villous blunting and atrophy; Black arrow – PMN migration marked level; Red arrow- PMN migration mild level; Black arrowhead – Crypt alteration with mucus or inflammatory cell; Red arrowhead – Goblet cell loss. Images were taken at 200X magnification, 100 μm scale bar. (b) Pathological scores of immunized or non-immunized sera treated neonatal mice small intestine post challenge. Immunized sera treated neonatal mice small intestine displayed less inflammation as compared to that of non-immunized sera treated neonatal mice. UI, uninfected; I, immunized sera recipient; NI, non-immunized sera recipient.

are responsible for epithelial cell damage and villous atrophy. Therefore, administration of POMVs immunized adult mouse sera significantly lowered intestinal inflammation in neonatal mice after infection, which ultimately lowers the level of intestinal destruction as is evident from previous results.

4. Discussion

Childhood mortality due to diarrhoeal diseases are a major concern in developing nations with children less than five years of age most affected [37]. DEC pathotypes are among the main agents responsible for diarrhoea-related illnesses. Due to the emergence of multi-drug resistant (MDR) bacteria, vaccination is an advantageous approach to mitigate infections caused by DEC pathotypes [38,39]. Polysaccharide conjugate vaccine efficacy was previously studied against O111 *E. coli* [40]. ETVAX, an inactivated whole-cell vaccine, which is comprised of four recombinant *E. coli* strains overproducing adhesins, along with recombinant hybrid B subunit of heat labile toxin and cholera toxin, was found to be safe and immunogenic in phase 1/2 trials [41]. But none of these vaccines were effective in providing broad spectrum immunogenicity and protection against multiple DEC subtypes. Combination-based vaccines are considered a better approach to prevent diarrhoea associated infections [42]. Recently, a study reported formalin killed whole-cell vaccine, when administered subcutaneously in BALB/c mice along with cholera toxin B subunit as an adjuvant, was effective in eliciting immunogenicity and provided protection against five main subtypes of diarrhoeagenic *E. coli* [11]. The intrinsic adjuvant properties of OMVs, due to the presence of LPS, peptidoglycan, flagellin and lipoproteins [19,43,44], eliminates the need to supplement OMVs with external adjuvants to enhance immunogenic properties. Therefore, we have formulated OMVs-based immunogen isolated from EPEC, ETEC, EAEC, EIEC and EHEC so as to provide broad spectrum protection against DEC pathotypes. OMVs-based vaccines have already been shown to be effective against *Shigella, Salmonella* and *Neisseria* species [45–48]. Previous reports stated that three doses of 25 μg intramuscular immunizations of *Neisseria meningitis* OMVs-based vaccine (MenBvac and MeNZB) was effective in eliciting a protective immune response [16,49,50]. Using a similar approach, we found that i.p. immunization with three doses of 10 μg of POMVs at 14 day intervals activated both the cellular and humoral arms of the immune response in BALB/c mice. Similar types of immune responses were found in our previous studies, both of which were bivalent OMVs-based vaccines, one against *S.* Typhi and *S.* Paratyphi, and another against *S.* Typhimurium and *S.* Enteritidis in an adult BALB/c mouse model [29,32]. In this study, it was noticed that a minimum of 10 μg of OMVs per mice was sufficient to effectively stimulate secretion of systemic antibodies. Levels of serum IgA, IgG and its subtypes were found to significantly increase after immunization. IgG2a and IgG2b lead to activation of the complement pathway, which ultimately leads to opsonisation of the antigen of interest. Bacterial capsular polysaccharides activates IgG3 subclass in mice [51] and IgG1 subclass mediates effective uptake of bacteria by phagocytes [52]. The elevated levels of IgG subclass in immunized mice might be due to the presence of a variety of antigens on POMVs, which may cause activation of cellular arm of immune response required for passive protection of neonatal mice.

Secretory IgA is associated with prevention of host tissue invasion

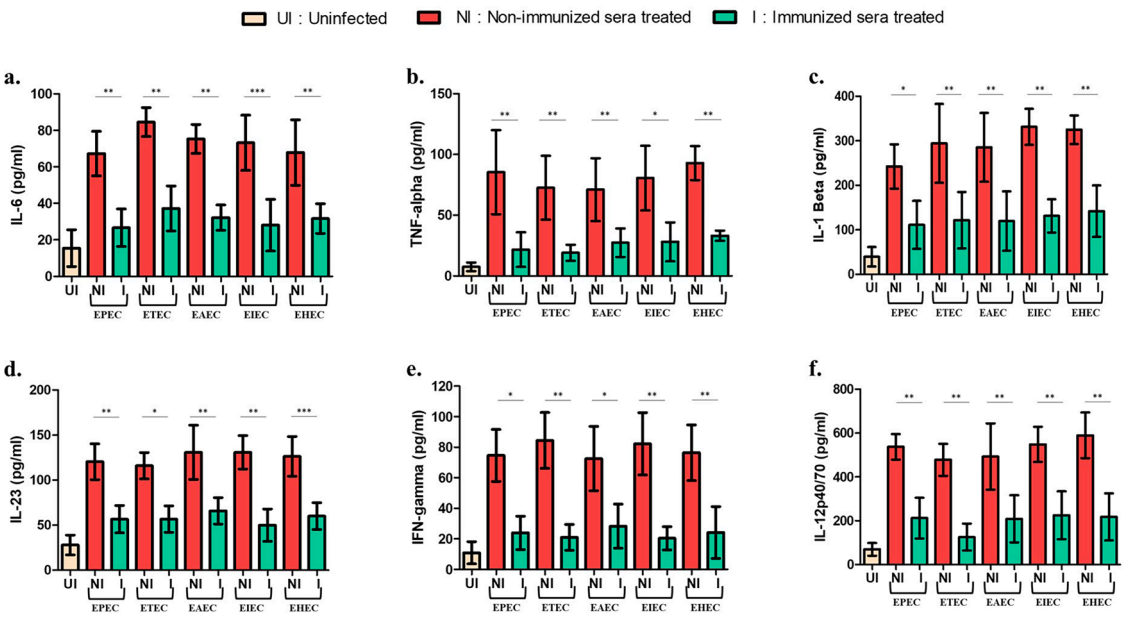

Fig. 8. Passive immunization with POMVs immunized adult mouse sera significantly lowers expression of inflammatory markers in small intestinal tissues of neonatal mice. Small intestines of immunized or non-immunized sera treated neonatal mice were isolated 24 h post challenge with respective DEC pathotypes (1×10^5CFU) and processed as described in materials and methods. Pro-inflammatory cytokine levels were measured in small intestinal tissue extracts. Cytokine levels of (a) IL-6, (b) TNF-α, (c) IL-1β, (d) IL-23, (e) IFN-γ and (f) IL-12p40/70. ($n = 6$). Two-way analysis of variance (ANOVA) test was used for statistical analysis. Significant statistical difference was found between uninfected, immunized sera treated and non-immunized sera treated neonatal mice small intestinal tissue inflammatory markers (*** p value < 0.001, ** p value < 0.01, * p value < 0.5). Bars represent mean of six neonatal mice ± S.E. of three individual experiments. UI, uninfected; I, immunized sera recipient; NI, non-immunized sera recipient.

and faster clearance of bacteria from the gut lumen. The clearance of bacteria by sIgA from the intestinal lumen is mediated either by the classical agglutination or the enchained growth pathway. In classical agglutination, sIgA prevents bacterial interaction with the mucosal layer, which results in elimination of the bacteria from the intestinal lumen. In the case of the enchained growth pathway, sIgA causes formation of a coat outside bacteria, which prevents bacterial sister cells from dividing, ultimately leading to clearance of undissociated cells [29,53,54]. Elevated titres of anti-LPS, anti-OMPs and anti-WCLs sIgA was also found in this study in the gut lumen of mice after i.p. immunization with POMVs. This indicates that POMVs immunization can mediate clearance of DEC subtypes and can eventually protect against subsequent infections.

Recently, studies have shown that different outer membrane proteins of *E.coli* like OmpA, OmpC, BamA and OmpF have immunogenic properties and can therefore act as potential immunogens [55,56]. In our study, western blot analysis revealed polyvalent POMVs immunized mouse anti-sera are able to recognize a range of immunogenic proteins present in WCLs, OMPs and OMVs. Moreover, polyvalent immunized anti-sera could recognize immunogenic proteins of heterologous challenge strains, along with those of homologous immunization strains. This indicates that these conserved immunogenic proteins may be present in POMVs and might play a pivotal role in modulating host immune responses. Previously, it was reported that i.p. immunization with *E. coli* OMVs increases the population of CD4+ and CD8+ splenic T-cells [17], necessary for a long-term humoral immune response. Other studies have also reported increases in splenic T-cell populations after subsequent immunization with OMVs isolated from enteric disease causing bacteria [25,29,32]. In the current study, a hike in the CD4+ and CD8a+ splenic cell populations was seen after i.p. immunization of mice with POMVs, which is similar to those observed previously. Therefore, POMVs potently activate both the cellular and humoral arms of the adaptive immune response, which is necessary for providing a long-term protective immune response against DEC-mediated infections.

In a previous study, it was reported OmpC in *E. coli* is required for activation of the complement pathway by Cq1 and loss of OmpC function renders serum inactive to effectively kill *E. coli*, allowing it to escape the immune response [57]. In this study, we observed bactericidal effect of POMVs immunized serum by SBA and scanning electron microscopy analysis and found that immunized mouse sera can efficiently bind and kill the DEC subtypes by causing lysis of the bacterial cells. SBA analyses the functionality of antibodies so as to assess the immunogenicity and potential protective capacity of the immunogen in preclinical studies [58]. POMVs contain various components of outer membrane proteins, as was evident from the results of immunoblot, which can play a vital role in the activation of classical complement pathway thereby leading to lysis of the bacterial cells.

Due to an immature immune system, neonates are not adequately protected in early life from infectious diseases [27]. Serum therapy or passive antibody therapy has long been used against infectious organisms responsible for causing disease in humans [59]. Neonatal mice, having an immature immune system, are a suitable animal model for studying passive protection conferred by immunized mouse sera [29,60]. In our study, passive transfer of POMVs immunized adult mouse sera significantly protected the neonatal mice from infections by DEC pathotypes. Neonatal mice administered orally with immunized adult mouse sera demonstrated better survivability, insignificant or no loss in body weight and a several fold decrease in small intestinal colonization against five DEC subtypes. Our previous studies also showed passive protection exerted by OMVs isolated from *Shigella* sp. [24], *Vibrio cholerae* [25], *Salmonella* Typhimurium and *Salmonella* Enteritidis [29]. Small intestinal histopathological scores of immunized sera treated neonatal mice were found to be lower than those treated with non-immunized sera. Neonatal mice administered with immunized sera displayed less epithelial layer damage, altered intestinal mucosa, polymorph nuclear neutrophilic (PMN) infiltration, goblet cell loss, hyperplasia and villous atrophy than those treated with non-immunized sera after infection. Previously, a study reported that anti-LPS IgG in serum protects against *E. coli* infections [61]. In this study, we hypothesize that anti-LPS IgG in immunized mice serum might trap the bacteria and

prevent infection in neonatal mice.

IL-6 was found to promote inflammation by *E. coli* in the intestine and acted as a major pro-inflammatory cytokine, expressed differentially based on disease severity [62]. IL-1β increases epithelial cell permeability by affecting tight junctions. IL-1β causes upregulation of NF-κβ, which in turn enhances permeability by affecting the tight junctions [63]. LPS binding to toll-like receptors (TLRs) leads to TNF-α production [64] and pathogenesis of inflammatory bowel disease has been found to be associated with the overproduction of TNF-α and IFN-γ [65]. IFN-γ has been found to cause micropinocytosis of tight junction proteins into early endosomes, thereby increasing permeability of the intestinal epithelial cell barrier [65]. Bacterial infection induces monocytes, macrophages and dendritic cells to secrete IL-12 and IL-23, which further stimulates T-lymphocytes to secrete inflammatory cytokines, especially IFN-γ. IL-12 and IL-23 also stimulate T-cells to produce pro-inflammatory markers such as IL-1, IL-6, TNF-*α* and chemokines causing inflammation [66]. This study suggests that administration of POMVs immunized adult mice sera downregulated the production of IL-6, IL-1β, TNF-α, IFN-γ, IL-12p40/70 and IL-23 pro-inflammatory cytokines in neonatal mice small intestines after challenge with DEC subtypes, leading to attenuation of inflammation. This correlates with the histopathological observations in which immunized sera treated neonatal mice had lower pathological changes when compared to those treated with non-immunized sera.

5. Conclusion

This study demonstrates POMVs can be used as a potent immunogen to elicit broad spectrum immunogenicity against five DEC pathotypes after intraperitoneal administration in adult BALB/c mice. Immunized mouse sera displayed characteristic bactericidal properties thereby effectively killing the DEC pathotypes. Moreover, passive transfer of POMVs immunized adult mouse sera provided broad spectrum protection to neonatal mice against infections caused by five pathotypes of DEC.

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Declaration of Competing Interest

The authors declare no financial or personal conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2023.09.009.

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Bacterial ghost cell based bivalent candidate vaccine against *Salmonella* Typhi and *Salmonella* Paratyphi A: A prophylactic study in BALB/c mice

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ABSTRACT

Typhoid and emerging paratyphoid fever are a severe enteric disease worldwide with high morbidity and mortality. Licensed typhoid vaccines are in the market, but no paratyphoid vaccine is currently available. In the present study we developed a bivalent vaccine against *Salmonella* Typhi and Paratyphi A using a bacterial ghost platform. Bacterial ghost cells (BGs) are bacteria-derived cell membranes without cytoplasmic contents that retain their cellular morphology, including all cell surface features. Furthermore, BGs have inherent adjuvant properties that promote an enhanced humoral and cellular immune reaction to the target antigen. Sodium hydroxide was used to prepare ghost cells of *Salmonella* Typhi and Paratyphi A. The bacterial ghost cells were characterised using electron microscopy. Then BALB/c mice were immunized three times (0^{th}, 14^{th} and 28^{th} day) with the bivalent typhoidal bacterial ghost cells. Haematological study of adult mice throughout immunization period reflected that the immunogen was safe to administer and does not affect the animals' health. After complete immunization, we found significant serum antibody titter against whole cell lysate, outer membrane protein and lipopolysaccharide of both bacteria, and cell-mediated immunity was observed in an ex-vivo experiment. CD4+, CD8a+ and CD19+ splenic cell populations were increased in immunized animals. Bivalent Typhoidal ghost cell immunized mice showed better survival, less bacterial colonization in systemic organs, and less inflammation and/or destruction of tissue in histopathological analysis than non-immunized control mice. Serum antibodies of immunized animals can significantly inhibit bacterial motility and mucin penetration ability with better killing properties against *Salmonella* Typhi and Paratyphi A. Furthermore, significant passive protection was observed through the adoptive transfer of serum antibody and lymphocytes of immunized animals to naïve animals after bacterial infection. In summary, Bivalent Typhoidal Bacterial Ghost cells (BTBGs) enhances immunogenic properties and serves as a safe and effective prevention strategy against *Salmonella* Typhi and Paratyphi A.

1. Introduction

Enteric fever (including Typhoid fever caused by *Salmonella* Typhi and Paratyphoid fever caused by *Salmonella* Paratyphi A), is a life threating bacterial infection which poses one of the most important health problem of developing and under developed countries. *S*. Typhi is more endemic and various modelling studies have assessed the worldwide estimated risk burden was 20.6 million (17.5–24.2) cases and 223,000 (131,000–344,000) deaths [1]. A pooled assessment of local area-based reports in India proposed a paratyphoid event of 105 for every 100,000 people yearly, signifying the importance of *S*. Paratyphi (A, B and C) [2]. Enteric fever mainly effects areas with very low sanitation, poor hygiene, and congested rural areas without clean water, in addition to in travellers returning from these endemic regions. In the near future it is estimated that the major agent causing enteric fever will be *Salmonella* Paratyphi [3]. Currently, the only treatment available for typhoidal fever patients are antibiotics, but this treatment is facing a global challenge, which is increasing antibiotic resistance among enteric bacteria (both *S*. Typhi and *S*. Paratyphi A). Resistance to various antimicrobial treatments of enteric fever has been consistent for decades, as evidenced by the first reports of chloramphenicol resistance in *S*. Typhi in the 1970s [4]. *S*. Typhi's resistance to chloramphenicol, amoxicillin,

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and co-trimoxazole was linked with many outbreaks in the late 1980s and early 1990s [5], and is now majorly found in South Asia. This multi-drug resistant issue of *S.* Typhi has been complicated the therapeutic management of enteric fever. The only way to reduce this health burden is by vaccination [6]. Though there are several vaccines available against *S.* Typhi infection for global use, there is no vaccine which can protect people simultaneously from both *S.* Typhi and *S.* Paratyphi A. An important virulence factor of *S.* Typhi i.e., Vi polysaccharide (Vi-PS) has been targeted as an antigen for protective immunity against *S.* Typhi [7,8]. Vaccine efficacy report from various clinical data showed live attenuated vaccine without expressing Vi-PS has a protective efficacy of ~60 % but due to safety concerns, this is not recommended for children under the age of six [9]. The immune responses prompted by Vi-PS vaccines were short-lived and efficacy ranges-between 55 and 60 % in older children (>6years) and adults [10]. Although Subunit vaccines are safe for all ages, Vi-PS vaccines fail to make optimal immunity in children lower than 2 years of age. When Vi-PS is conjugated with a protein, it can induce anti-Vi-PS antibody in infants and young children up to 90 % efficacy [11]. In current *Salmonella* Paratyphi A vaccine research, there are a few potential subunit vaccine candidates like GMMA of *S.* Typhi A and protein capsular matrix [12]. The problem with these vaccine candidates is high production cost, and as the endemic regions of enteric fever are mainly in lower middle-income countries, the economic burden always comes before the health burden. From this point of view, it is most essential to formulate a bivalent typhoidal vaccine candidate [13] which is cost effective and also easy to make at an industrial scale.

In this study, we have developed a novel bivalent bacterial ghost based typhoidal immunogen using *S.* Typhi and *S.* Paratyphi A ghost cells. After three doses of intra-peritoneal immunization with this bivalent ghost immunogen, we checked protective efficacy in an adult mouse model. We also evaluated the humoral and cell-mediated immune response after immunization by quantifying outer membrane protein (OMP) and lipopolysaccharide (LPS) specific serum immunoglobulin and Th1/Th17 specific cytokine response from splenic cells and bone marrow derived dendritic cells. We have also aimed to induce common mucosal immunity to kill the administered bacteria through oral transmission. Low systemic circulation of bacteria after infection in fully immunized animals compared to placebo also proved the mode of protection. This preclinical study suggests that a Bivalent Typhoidal Bacterial Ghost (BTBG) cell-based vaccine might be one of the best choices for a human vaccine to reduce the typhoidal *Salmonella* mediated health burden, especially in lower middle-income countries.

2. Material and methods

2.1. Bacteria and culture method

Salmonella Typhi (C-6.946) and *Salmonella* Paratyphi A (BCR148) were used for bacterial ghost cell preparation and *Salmonella* Typhi (K554) and *Salmonella* Paratyphi A (K580) were used for challenge studies. All strains were obtained from National Institute of Cholera and Enteric Diseases (NICED) strain culture repository. All strains were kept in 20 % glycerol, maintained at-80 °C in Tryptic soy broth (Difco, USA). Prior to use, strains were cultured in Tryptic Soy Broth (TSB; Difco, USA) at 37 °C with shaking or on Tryptic Soy Agar (TSA; Difco, USA) plates. Table 1 lists the strains used in this study.

Table 1
List the strains used in this study.

Strain Name	Serotype	Abbreviation	Reference
C-6.946	*S.* Typhi	ST	Clinical Isolate [13]
BCR148	*S.* Paratyphi A	SPA	Clinical Isolate [13]
K554	*S.* Typhi	ST	Clinical Isolate [This study]
K580	*S.* Paratyphi A	SPA	Clinical Isolate [This study]

2.2. Bacterial ghost cell preparation

Determination of the minimum inhibitory concentration (MIC) of NaOH for *S.* Typhi and *S.* Paratyphi A was performed by the 2-fold broth dilution method as described previously with some modifications [14]. The biomass of overnight *S.* Typhi and *S.* Paratyphi A culture cells was centrifuged (8,000×g, 10 min, 4 °C) before being resuspended in sterile phosphate buffered saline (PBS, pH-7.4). The bacterial cells were then adjusted to 1×10^8 CFU/ml. 1 ml (5×) of MIC of sodium hydroxide concentration was added to 2 ml of cell suspension with 2 ml of sterile solvent and incubated for 90 min at 37 °C. After centrifugation (8,000×g, 10 min, 4 °C) and three PBS washes, ghost cells were collected. In ice-cold PBS, the final cell pellets were resuspended. Based on bacterial cell count (1×10^8 CFU/ml), recovered bacterial ghost cells from the two strains were combined in a 1:1 ratio and the final mixture was kept at 4 °C until further use [14,15]. 100 µl of each strain of bacterial ghost cells were spread on agar plates to check for complete lysis of bacterial cells.

2.3. Scanning electron microscopy (SEM) imaging of bacterial ghost cells

Ghost bacteria and non-treated control bacteria were fixed using buffered 2.5 % glutaraldehyde (pH 7.0) for 2 h at 4 °C, washed with buffer and then post fixed in 1 % osmium tetroxide for 1.5 h at 4 °C. Again, washed samples are dehydrated through a series of ethanol concentrations. After samples reached the critical dried point, they were mounted on SEM stubs, coated with gold–palladium, and observed under a scanning electron microscope, Tecnai12 Bio Twin Transmission Electron Microscope (FEI, Netherlands) [14].

2.4. Macrophage-mediated cytotoxicity assay (LDH assay)

To check the cytotoxicity of NaOH treated Bacteria, we used Pierce LDH Kit (Invitrogen, USA). Murine macrophage cell line (RAW 264.7) was cultured in 96-well plates (Nunc, USA) for 24 h in a 37 °C incubator (with 5 % CO2). 1.0×10^5 cells/well was then incubated for 24 h with 1.0×10^8 CFU/ml of ghost cells and heat-killed cells in culture medium. LDH-positive samples are used for positive controls and PBS was used for negative control [15].

2.5. Animals

Six weeks old female BALB/c mice were obtained from the animal house facility of NICED, Kolkata. Mice were separated into different groups with sterile food and water. The Institutional Animal Ethical Committee of NICED (CPCSEA registered, Registration No. 68//Rebi/S/1999/CPCSEA valid17/7/2024), approved the animal experimental protocol with the project approval number. PRO/167/January 2020-November 2022.

2.6. Immunization of animals

Mice were injected intra-peritoneally on the 0^{th}, 14^{th} and 28^{th} day with 100 µl of bacterial Ghost Immunogen (1:1) containing 1×10^8 CFU/ml cell. Non-immunized control group mice received 100 µl of sterile PBS. The vaccination schedule for animals is shown schematically in Supplementary Fig. 1.

2.7. Collection of serum and intestinal lavage

On the 0^{th}, 7^{th}, 21^{st}, 35^{th}, 49^{th}, 70^{th}, 90^{th} and 120^{th} days following the initial vaccination, at time intervals, blood was taken from tail veins. Blood was kept in microtainer tube (BD, USA) and serum isolated by centrifugation (1000×g, 10 min, 4 °C). Intestinal lavage from immunized and control mice was collected in a sterile microcentrifuge tube by PBS wash of small intestine after dissection. Lavage was centrifuged at

6000×g, 10 min; supernatant was collected and stored at −20 °C. The animal blood and intestinal lavage collection schedule for animals is shown in Supplementary Fig. 1.

2.8. Haematological parameter analysis

Animals of immunized and control groups were monitored for the signs of acute toxicity. After 7 days of every immunization, animals were bled and blood samples were collected to study haematological parameters, such as the Haemoglobin (Hb), Erythrocyte Sedimentation Rate (ESR), Total Leucocyte Count (TLC), Differential Leucocyte Count (DLC), and Packed Cell Volume (PCV) [16]. All parameters were analysed within few hours of sample collection.

2.9. Purification of whole cell lysates (WCL)

WCL were isolated according to the previously described protocol [13] as follows; Bacteria were cultured 18 h in Tryptic Soy Broth (TSB; Difco, USA), centrifuged to obtain cell pellets, washed with PBS, and sonicated. Again, samples were centrifuged and supernatant was collected and stored.

2.10. Purification of outer membrane proteins (OMPs)

Outer membrane proteins were isolated based on a previously described protocol [17] as follows; Bacteria were cultured for 18 h in Tryptic Soy Broth (TSB; Difco, USA), centrifuged, and pellets were washed with HEPES buffer (pH 7.5) and protease inhibitor cocktail (Roche, Sigma, USA). After that, they were sonicated for ten minutes using a Hielscher (UP100H) sonicator in an ice bath. After centrifugation, the supernatants were collected and centrifuged at 100,000×g. The pellet was re-suspended with same buffer containing 1 % N-lauryl Sarcosine and placed at 37 °C for 30 min. Again, it was centrifuged and the final collection was re-suspended in same buffer. Then the OMP concentration was determined, which was then adjusted to 1 μg/1 μl with HEPES buffer and stored at −20 °C.

2.11. Purification of lipopolysaccharide (LPS)

LPS was isolated based on a previously described protocol [17] as follows; *S.* Typhi and *S.* Paratyphi A were cultured overnight in Tryptic Soy Broth (TSB; Difco, USA), centrifuged, and the cell pellet was re-suspended with 0.15 M NaCl containing Phenol-saturated 3-[N-morpholino] propen sulfonic acid [0.02 M MOPS]. The mixture was incubated for 30 min at 65 °C with random shaking, and then for 10 min on ice. After a second centrifugation, the upper aqueous layer was collected, mixed with four times the volume of the sample with chilled ethanol, and left overnight at −20 °C. LPS was purified and collected on the following day by centrifuging at 8500×g for 20 min, resuspending in distilled water, and storing at −20 °C.

2.12. Determination of antibody levels by ELISA

96 well flat bottom ELISA plate (Tarson, India) was separately coated with whole cell lysate (WCL), outer membrane proteins (OMPs), lipopolysaccharide (LPS), and Vi-polysaccharide (Bharat biotech, India) as previously described [13,17]. Then each plate was kept at 4 °C for 18 h. The wells were washed with PBS and blocked with skim milk (BD, USA). After that wells were washed with PBS-T (PBS having 0.5 % Tween-20, Sigma, USA) and incubated for 1hr with consecutively diluted serum samples and intestinal lavage for mucosal antibody. Again, wells were washed with PBS-T and incubated after adding HRP-tagged secondary antibody (IgG & IgA, Abcam & Sigma, USA). After a PBS wash, substrate was added to all wells and kept for ten mins. The reaction was stopped using sulphuric acid (2 N). Finally, the OD_{492} nm was measured using a microplate reader. A table of antibodies used in this study are listed in Supplementary Table with RRID designations (Supplementary Table 1).

2.13. SDS-PAGE, immunoblot and dot blot

LPS and total protein content of the OMPs from *Salmonella* strains were determined by SDS-PAGE. 20 μg of OMPs, were boiled in SDS-PAGE buffer and LPS samples were boiled in LPS sample buffer. The samples were then loaded onto a 12 % SDS-PAGE gel separately depending on their staining reagent. 100 V was then applied for running the gel in an AE-6530 SDS-PAGE apparatus from ATTO Corporation (Japan). The gel was then stained by either coomassie or silver stain. For immunoblot, proteins were boiled in 5× loading buffer and separated on a 12 % SDS gel. Using ATTO AE-6687 (Japan) blot apparatus, proteins were transferred onto a nitrocellulose membrane for immunoblotting. Antisera from immunized mice and the ALP-conjugated goat anti-mouse secondary IgG was used for immunoblotting [13]. We measured the concentration of extracted LPS, with 5 μg, 10 μg and 15 μg of LPS being used for a dot blot assay of either strain. First, LPS was absorbed by nitrocellulose membrane at room temperature. 5 % BSA solution was used for blocking and then the membrane was washed with TBS-T following a standard protocol. Antisera from immunized mice and the ALP-conjugated anti-mouse secondary IgG was used in the dot blot [13]. A table of antibodies used in this study are listed in Supplementary Table with RRID designations (Supplementary Table 1).

2.14. Serum bactericidal assay

Serum bactericidal assay was performed according to an earlier described method [18]. Sera collected from the immunized mice on day 35 of first immunization was heat inactivated at 56 °C for 20 min. Serum samples were diluted from 1:50 to 1:128,000 in PBS. A master mix composed of 12.5 μl of baby rabbit complement (12.5 % final concentration) with 27.5 μl of PBS, 50 μl of diluted mouse serum, and 10 μl of diluted bacteria (320 CFU, T_{0h}) was prepared. The mixture was then incubated for 1 h (T_{1h}) with shaking at 115 rpm at 37 °C. Viable bacterial colonies were counted after spreading the mixture onto the plate followed by overnight incubation at 37 °C. The negative control contained bacteria and complement only. Bactericidal activity was determined as serum dilutions necessary to obtain a 50 % reduction in CFU counts at T_{1h} compared with T_{0h}. For SEM imaging, samples (bacteria and complement with heat inactivated immunized serum, nonimmunized serum respectively) with lowest serum dilution (1:50) were fixed by adding 900 μl glutaraldehyde and incubated at 4 °C overnight. Cells were then prepared using the previously described method for scanning electron microscopic analysis and observed [17].

2.15. Motility and mucin penetration assay

A motility assay performed based on a published method, with modifications [19]. Vaccinated and control serum were mixed with PBS in 1:400 dilutions, separately, and dispensed on soft agar (0.3 %) plates. Log-phase bacteria with an OD_{600} value of 0.4 were punctured in the middle of the plates once the serum mixture had dried. After that, the plates were incubated for 24-hours at 37 °C. Then the bacterial spreading across the surface was measured. The mucin penetration assay was performed according to a published method [20]. Briefly, a solution containing 1 % (w/v) mucin (MP Biomedicals, USA) and soft agar (0.3 %) media (TSB) was allowed to form a soft gel at room temperature in a 1 ml tuberculin syringe fitted with a stopcock. Log-phase bacteria were incubated for one hour at 37 °C with heat-inactivated vaccinated and control serum. 100 μl aliquot (containing 1×10^7 CFU/ml organisms) of incubated bacterial suspension were placed in the top of the columns and kept at 37 °C for 30 min in a vertical position. To determine the bacterial count, a 500 μl sample from the column's lower portion was collected, serially diluted, and plated.

2.16. Cytokine measurement:

2.16.1. Splenocytes re-stimulation assay

Splenocytes from vaccinated mice were cultured for two hours in RPMI containing 10 % FBS one week after the last vaccination. 1×10^6 CFU/ml bivalent typhoidal bacterial ghost cells were used to treat the splenocytes at 37 °C (with 5 % CO2) for 24 h. From the culture supernatants, TNF-α, IFN-γ, IL-6 and IL-17 were estimated by individual ELISA kits (Invitrogen, USA) [13].

2.16.2. Separation of bone marrow-derived dendritic cells (BMDCs) and re-stimulation assay

From naive BALB/c mice, bone marrow was isolated and cultured in RPMI containing 10 % FBS and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). 1×10^6 CFU/ml bivalent typhoidal bacterial ghost cells were used to treat the mature BMDCs at 37 °C (with 5 % CO2) for 24 h [13]. From the culture supernatants, IL-1β, IL-6, IL-23, and IL-12p40/70 were estimated by individual ELISA kits (Invitrogen, USA).

2.16.3. BMDC-Splenic CD4+ T cell co-culture

BMDCs (previously stimulated) and splenic CD4+ T cells were isolated (BD IMag™ anti-mouse CD4 Magnetic Particles, Cat. No. 551539, USA) and cultured at a 1:1 ratio for 24 h [21]. From the culture supernatants, IFN-γ, IL-2, IL-6 and IL-17 were estimated by individual ELISA kits (Invitrogen, USA).

2.17. Fluorescence-activated cell sorter (FACS) analysis

The spleens of both the immunized and control mice were removed and sterilely homogenized on the 35th day after vaccination to determine the CD4+, CD8a+, and CD19+ cell populations. Using Cell Strainer (Corning, USA) and a sterile syringe, the spleen was homogenized. [17]. Splenocytes were stained with anti- Mabs: CD4-phycoerithrin (PE), CD8a PE, CD19 PE or an isotype control PE (Miltenyi Biotec, USA). Expression was measured on a BD FACS ARYA III flow cytometer and data was assessed with FACS DIVA software.

2.18. Histopathology analysis

The liver and spleen tissues that were obtained at 48 h and 72 h after infection were fixed in a solution of 10 % buffered formalin, and 5 μM paraffin-embedded sections were stained with haematoxylin and eosin (H&E). The slides were viewed at 20× and 40× magnification using an Olympus IX51 light microscope, followed by observer-blind histopathological analysis.

2.19. Protective efficacy studies

2.19.1. Bacterial challenge study

The immunized and non-immunized animals were infected intra-peritoneally with 5×10^8 CFU/mouse of heterologous strain of bacteria (*S.* Typhi; K554 and *S.* Paratyphi A; K580) on the 35th day after first vaccination. Total of 30 mice were divided into five groups. 6 mice per group were challenged intra-peritoneally. Two immunized group were infected with *S.* Typhi and *S.* Paratyphi A. Two nonimmunized groups were infected with *S.* Typhi and *S.* Paratyphi A. Another group received only 100 μl PBS; as the non-immunized, non-infected negative control. The infected mice were observed for 10 days to assess survival. To examine bacterial colonization in different systemic organs, animals were infected intra-peritoneally at the 35th and 180th day post first immunization with 5×10^5 CFU/mouse of heterologous typhoidal strains (*S.* Typhi; K554 and *S.* Paratyphi A; K580). Infected animals (both vaccinated and control mice) were sacrificed at different times post infection to determine the bacterial count.

2.19.2. Passive protection study

35th day post first immunization serum and splenocytes (spleen was processed as before) were isolated from vaccinated and a control group of mouse then transferred to a naïve mouse via tail vein (100 μl of serum). RBCs were lysed and splenocytes were re-suspended in phosphate saline buffer and 100 μl (1×10^6 splenic cell) was injected via tail vein. Afterwards, animals were infected intra-peritoneally with 1×10^5 CFU/mouse with heterologous strains of *Salmonella* to observe bacterial colonization after 72 h. One group of mice was infected on the day of the adoptive transfer (0th day) and another group was infected at the 7th day post adoptive transfer.

2.20. Statistical analysis

All data are presented as the median ± SE. A Mann-Whitney test was used to compare the experimental data of immunized group versus control group. An analysis of variance (ANOVA) with Kruskal-Wallis for multiple comparisons was used when three or more groups were compared. Kaplan-Meier analysis was performed to compare survival curves in the challenge study. The remaining data were analysed using Student's *t* test. Two-tailed P values of $P < 0.05$ were considered statistically significant. All experiments were triplicated for statistical significance. All the data were evaluated using GraphPad Prism 8.0.2 (GraphPad Software, Inc.) and MS Excel software.

3. Results:

3.1. Preparation and characterization of bacterial ghost cells

For preparation of bacterial ghost cells from *Salmonella* Typhi and Paratyphi A, we used a 2-fold broth dilution method, with the MIC of NaOH found to be 3.125 mg/ml. At the end of the lysis there was no bacterial growth up to 48 h incubation at 37 °C. The determined MIC completely lysed the bacterial cells and stopped further growth (Supplementary Fig. 2). Scanning electron microscopic structure revealed that the NaOH treated *S.* Typhi and *S.* Paratyphi A had some transmembrane tunnel like structures that differentiated bacterial ghost cells from untreated live cells (Fig. 1.A, 1.B, 1.C, 1.D). Through this rupture in bacterial cell, the cytoplasmic contents leave the cell and the ability to grow was lost. The bacterial ghost cell surface structure seemed to retain normal cell morphology.

3.2. In-vitro cytotoxicity tests in murine macrophages exposed to bacterial ghost cells and in-vivo effect of bacterial ghost cells on blood parameter

In our experiments, NaOH-treated bacterial ghost cells showed much less cytotoxicity in an in-vitro murine macrophage (RAW 264.7) cell line than the LDH positive control sample provided by the manufacturer (Supplementary Fig. 3.A). This suggests that the NaOH treated bacterial cells are less reactogenic in nature. No major alteration in any blood parameters was observed in between the immunized and PBS control mice groups [16]. All the parameters studied were within the normal range (Supplementary Fig. 3.B).

3.3. Bivalent typhoidal Salmonella ghost cells immunization induces humoral and mucosal immune response in adult mice

Induction of humoral immunity during and after the immunization with bivalent typhoidal bacterial ghost cells was measured using an ELISA up to 120th days after 1st immunization. In this experiment, we observed significant induction of serum immunoglobulin IgG and IgA antibodies along with a mucosal antibody (sIgA) response in intestinal lavages of immunized mice than in non-immunized animals. The level of different antibodies in immunized animals increases from day 7 to 35 days post immunization, and then maintain a steady level of antibody titre up to 120 days (P value < 0.0001). A significant peak of IgG and IgA

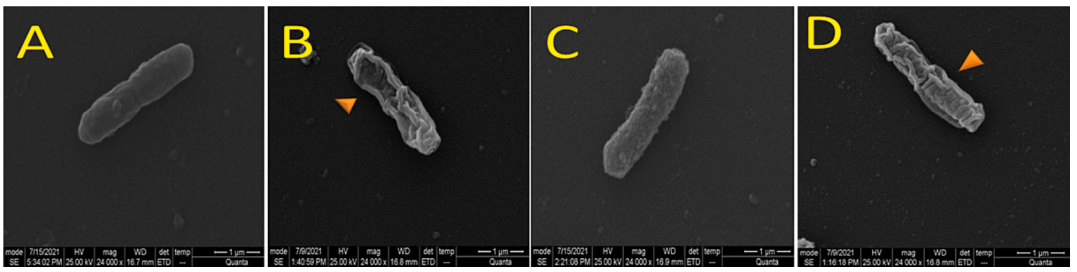

Fig. 1. Scanning electron microscopic analysis (SEM); (**A**) Untreated control S. Paratyphi A (**B**) NaOH-treated S. Paratyphi A (**C**) Untreated control S. Typhi (**D**) NaOH-treated S. Typhi. Arrows shows *trans*-membrane lysis tunnels.

titre were seen after the second dose of immunization, between day 28 and 35 post immunization. Three doses of immunization with the bivalent typhoidal bacterial ghost cells are sufficient to get a prominent mucosal antibody (sIgA) titre from the intestinal lavage. The entire antibody titre was measured against the whole cell lysate (WCL), outer membrane protein (OMP), and lipopolysaccharide (LPS) of *Salmonella* Typhi and Paratyphi A (Fig. 2; A.i,A.ii,B.i,B.ii). Mucosal antibody titres were also significant increased against these components i.e. OMP and LPS of both bacterial ghost cells individually (Fig. 2.C). As well as serum IgG antibody titre was measured against Vi-polysaccharide (Vi-PS) of *Salmonella* Typhi (Fig. 2.D). From the ELISA results it is clear that our bivalent typhoidal bacterial ghost cells are immunogenic against the antigenic components of *Salmonella* Typhi and Paratyphi A separately compared to non-immunized animals. Bacterial cells of *Salmonella* Typhi and Paratyphi A can induce good humoral and mucosal immunity in animals up to 120th day post immunization. This is very much needed to control and prevent infections from *Salmonella* Typhi and Paratyphi A.

Isolated purified OMP and LPS of *S.* Typhi and *S.* Partyphi A was used in ELISA, immunoblot and dotblot were visualised through SDS-PAGE gel separation and coomassie and silver staining respectively (Supplementary Fig. 4.A, 4.B). Serum antibodies were capable of recognizing the WCL, bacterial ghost cells, and OMP of both bacteria in immunoblot and LPS in dotblot assays. Proteins from 20 KD to 80 KD size were viewed as immunogenic (Supplementary Fig. 4.C). A dot blot assay was performed for three different concentrations of LPS (5 μg, 10 μg, and 15 μg) from either strain with 35th day sera of bivalent typhoidal ghost cell immunized mice. We observed a prominent band against every concentration of LPS (Supplementary Fig. 4.D, 4.E). That shows that bacterial ghost cells are immunogenic against lipopolysaccharide of both strains. It's confirmed that whole outer membranes of NaOH-induced bacterial ghost cells contain preserved epitopes i.e. outer membrane proteins and LPS.

3.4. *Bivalent typhoidal ghost-cells (BTBGs) induce a pro-inflammatory (Th1/Th17) cytokine response:*

35th day post 1st immunization both BTBG and PBS immunized mice were sacrificed, and their spleens were collected, and a single cell suspension were prepared and cultured for 24 h. Cells were treated with respective immunogens and checked for their cytokine response post treatment. From the culture supernatants we found BTBGs treatment significantly upregulate levels of IFN-γ, TNF-α, IL-6 and IL-17 (Fig. 3.A, B, C, D) than PBS treated group. To further illustrate the generation of innate immune response by BTBGs; we investigated dendritic cells (DCs) response that controls T cell variation. For that isolated mature bone marrow derived dendritic cells (BMDC) and were stimulated with BTBGs. We observed a significant induction of IL-1β, IL-6, IL-23, and IL-12p40/70 cytokines levels from the culture supernatants. Pro-inflammatory cytokines secreted by DCs regulate Th1/Th17 cell differentiation (Fig. 3.E, F, G, H). To check this reaction, CD4+ T cells separated or isolated from immunized mice and PBS control mice were then co-cultured for 24 h with BTBGs pulsed BMDCs. Cytokine ELISA results had significantly higher levels of Th1/Th17 cytokines (IFN-γ, IL-2, IL-6 and IL-17) in the culture supernatants (Fig. 3.I, J, K, L). All these outcomes direct a robust Th1/Th17 media immunity in BTBGs immunized mice. To check the upregulation of adoptive immune responses post immunization with BTBGs in mice, we check the different surface markers of T cells (CD4+, CD8a+) and B cells (CD19+) 35th day post immunization. From the result we observed that BTBGs immunization significantly increases the both CD4+, CD8a+ and CD19+ cells populations than PBS immunized mice (Fig. 3.M.i,M.ii,M.iii, Supplementary Fig. 5).

3.5. *Bacterial colonization, survivality check after heterologous bacterial challenge*

After one week of last immunization of BTBG immunized group and nonimmunized group (PBS group) were challenged with 5 × 10^5 CFU/mice of *S.* Typhi and *S.* Paratyphi A and bacterial colonization were enumerated from different systemic organs of mice. The results showed bacterial colonization was 2–3 fold less in immunized group than non-immunized group (Fig. 4.A, B). At different time point post challenge, we found gradual increase of bacterial colonization in nonimmunized group but in case of immunized groups colonization was decreased. Then we checked long term protection after challenge with sublethal dose of *S.* Typhi and *S.* Paratyphi A after 180 days post final immunization. We found at least 2-fold less bacterial load on organs of immunized animals against control (nonimmunized group) animals (Fig. 4.C). This may be due to good antibody titter present for a long period after completion of immunization. To further support our result, we challenge both group of mice with lethal dose of *S.* Typhi and *S.* Paratyphi A (5 × 10^8 CFU/mice) via intraperitoneal route and their survival was recorded. Mice were observed for 10 days post challenge and from the result we found that all mice immunized with BTBGs were survived till 10 days, but all PBS immunized mice challenged with S. Typhi died within 4 days post challenge and all PBS immunized *S.* Paratyphi A challenged mice died 9 days post challenge. Immunized mice showed better survival, with 100 % survival for *S.* Typhi and ≥80 % for *S.* Paratyphi A. All of the non-immunized control mice died during this observation period after infection (Fig. 4.D). The body weights of immunized mice very less decreased throughout the observation period, but dramatically decreased in non-immunized control mice (Fig. 4.E.i, E.ii). This result indicates that our immunogen can protect mice from *Salmonella* infection. Some other clinical signs of disease like diarrhea, lethargy and all the symptoms including weight loss were assessed to measure not only mortality, but also the morbidity signs. Non-immunized, *S.* Typhi and *S.* Paratyphi A infected mice showed major clinical signs of morbidity i.e. severe fur ruffling was noticed over time, severe weight loss over time, severe diarrhea started overnight of infection, all animals showed severe lethargy over time, and ultimately all animals are died within 4–9 days of infection in nonimmunized group. Whereas the number of morality was very less in BTBG immunized mice including the clinical sign of morbidity like very mild fur ruffling for short period and weight loss was mild but recovered shortly, in case of S. Paratyphi A challenged group.

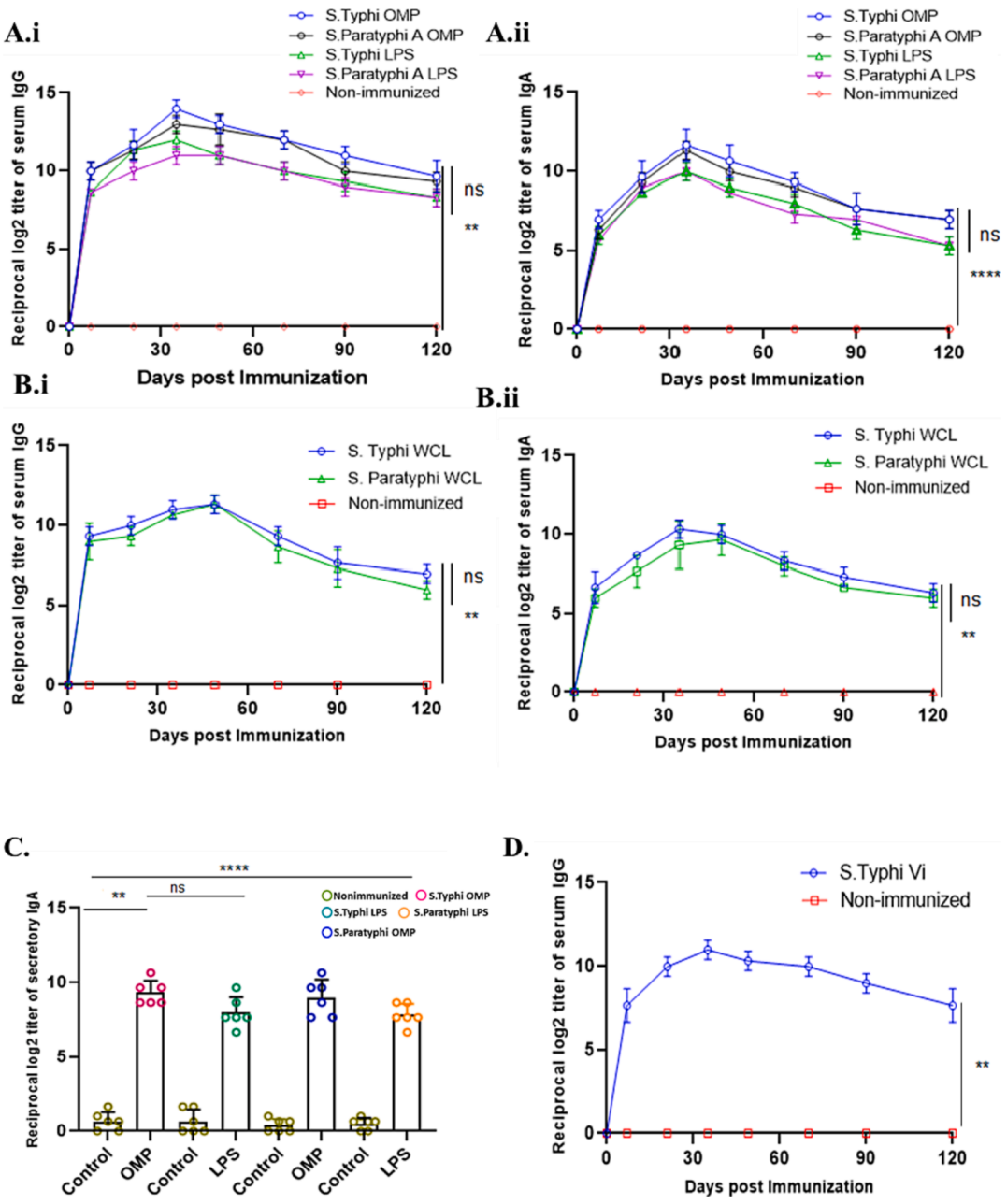

Fig. 2. Reciprocal log2 titer of serum IgG, serum IgA and secretory IgA immunoglobulin from Bivalent Typhoidal Bacterial Ghost cells immunized and non-immunized group. Mouse serum IgG (**A.i**), serum IgA (**A.ii**) was measured separately after three doses of intraperitoneal immunization against Outer membrane protein (OMP) and LPS of S. Typhi and S. Paratyphi A. Mouse serum IgG (**B.i**), serum IgA (**B.ii**) were measured separately after three doses of intraperitoneal immunization against whole cell lysate (WCL) of S. Typhi and S. Paratyphi A. **C.** Reciprocal Log2 titter of Secretory IgA was measured after 35th day of primary immunization against OMP and LPS of *S.* Typhi and *S.* Paratyphi A. **D.** Mouse serum IgG was measured separately after three doses of intraperitoneal immunization against Vi-polysaccharide of S. Typhi. Difference between immunized and non-immunized group was statistically significant. Statistical analyses were performed non-parametric two tailed Student's *t* test (Mann-Whitney tests) and one-way analysis of variance (ANOVA) test (Kruskal-Wallis test) to evaluate data; (****P value < 0.0001,**p value < 0.001). Each bar represents median and error values of six sample ± SE of three independent experiments.

All the observed protective and good survival results in case of immunized animals were due to a *Salmonella* Typhi and Paratyphi A specific strong antibody mediated immunity with other cellular and humoral immune responses as was generated through BTBG immunization.

3.6. *Histopathlogical analysis of BTBGs from immunized and non-immunized animal tissue*

The degree of inflammation and tissue damage were determined by histopathological examination of the respective organs (Fig. 5). Normal uninfected immunized and non-immunized mice showed no abnormalities upon histopathological examination in the spleen or liver of both groups (Fig. 5; A, D, G, J). However, the infected mice displayed extensive inflammation indicative of bacterial spread. Spleens of infected non-immunized mice showed augmented states of inflammation, infiltrating lymphocytes and granulomas (Fig. 5; Ei, E.ii, F.i, F.ii) when compared to BTBG immunized mice (Fig. 5; K.i, K.ii, L.i, L.ii). Liver and spleen tissues had similar types of histopathlogical findings. Granulomas were detected in the livers of infected control mice, which were not present in the livers of *S.* Typhi and/or very reduced in *S.* Paratyphi A

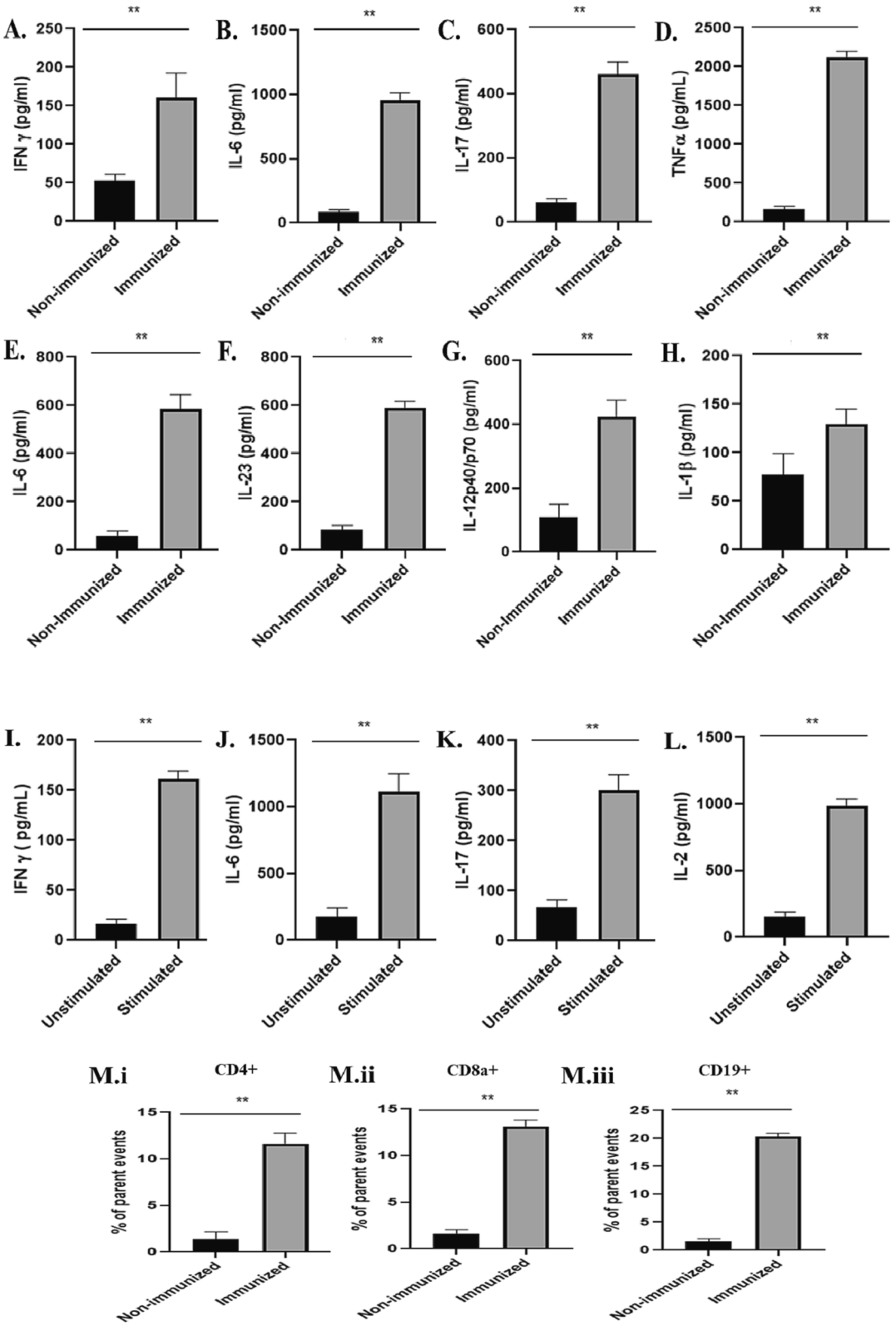

Fig. 3. Bivalent Typhoidal Bacterial Ghost cells (BTBGs) induces the production of cell mediated cytokines responses and increases the population of CD4+, CD8a+ and CD19+ splenic cells. **A.B.C.D.** IFN-γ, TNF-α, IL-6 and IL-17 cytokines in culture supernatant of ex-vivo cultured splenic cells of immunized and nonimmunized mice after 24 h of restimulation with BTBGs; **E.F.G.H.** IL-23, IL-1β, IL-6 and IL-12p40/70 cytokines in culture supernatant of ex-vivo bone marrow derived dendritic cells (BMDC) from naïve mice with and without BTBGs stimulation for 24 h, **I.J.K.L.** IFN-γ, IL-6, IL-17 and IL-2 cytokines in culture supernatant of ex-vivo co-culture of bone marrow derived dendritic cells (BMDC) of naïve mice and CD4+ splenic cell of immunized and nonimmunized mice after 24 h. All cytokines are measured by ELISA (n = 6). **M.i., M.ii., M.iii.** CD4+, CD8a+ and CD19+ cell population in splenocytes of immunized and nonimmunized mice by FACS analysis. Statistical analyses were performed using the non-parametric two tailed Student's *t* test (Mann-Whitney tests) to evaluate data; (**p value < 0.001). Each bar represents median and error values of six sample ± SE of three independent experiments.

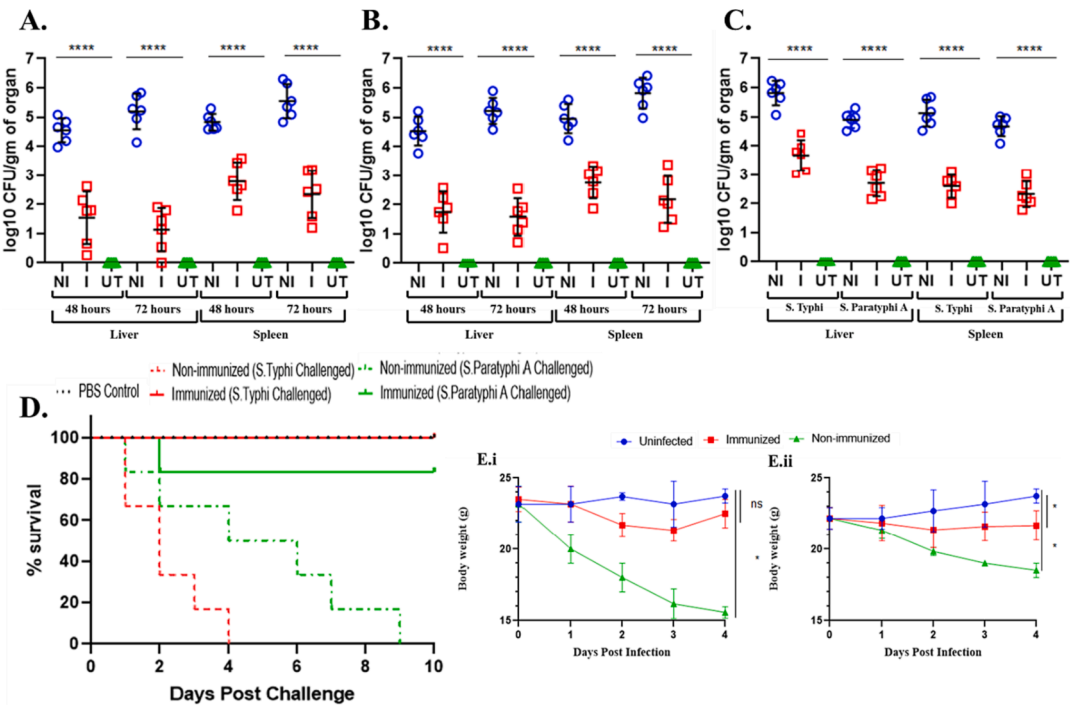

Fig. 4. Colonization and protective efficacy analysis of Ghost cells against *Salmonella* Typhi and *Salmonella* Paratyphi A infection. **A.** Colonization of *S.* Typhi **B.** Colonization of *S.* Paratyphi A, respectively after 35th day post immunization, **C.** Colonization (post 72 h post infection) to systemic organs after 180th day post first immunization. In every case immunized and nonimmunized groups were challenged with 5×10^5 CFU/mice of S. Typhi and S. Paratyphi A. NI: non-immunized, I: immunized, UT: untreated. **D.** Survival graph (Kaplan-Meier Curves) depicts percentage (%) of survival of the immunized and nonimmunized BALB/c mice after Intra-peritoneal challenge with 5×10^8 CFU/mice of S. Typhi and S. Paratyphi A. **E.i, E.ii.** Comparative data of change in body weight between non-immunized and immunized adult mice, after challenge with *S.* Typhi and *S.* Paratyphi A respectively. Data were expressed as median value of six mice ± S.E of three separate experiments. Statistical analyses were performed using non-parametric two tailed Student's *t* test (Mann-Whitney tests) and one-way analysis of variance (ANOVA) test (Kruskal-Wallis test) (****P value < 0.0001, **p value < 0.001, *p value < 0.01).

infected immunized mice. Although *S.* Typhi infected immunized mice presented lobular inflammation in the liver (Fig. 5; H.i, H.ii, I.i, I.ii), and lobular inflammation was witnessed to be higher in control mice. In addition, *S.* Typhi-infected PBS control mice had more portal inflammation and leukocyte infiltration in their livers (Fig. 5; B.i, B.ii, C.i, C.ii) than in the livers of animals that had been immunized. Immunized and unvaccinated *S.* Typhi-infected mice also had acidophil bodies.

3.7. Adoptive transfer of serum and splenocytes followed by bacterial challenge

Adoptive transfer of immunized and non-immunized mice serum and splenocytes intravenously (via tail vein) to naïve mice was done to check the humoral and cell mediated passive protection. We found that immunized blood serum and splenocytes confers protection to naïve mice similarly. We observed bacterial colonization differences in systemic organs of animals after adoptive transfer. Non-immunized sera and splenocytes transferred mice had a very high rate of colonization in the liver and spleen. However, immunized sera and splenocyte-treated mice showed considerably lower bacterial load at day 0 (Fig. 6.A.i, ii, iii, iv) and day 7 (Fig. 6.B.i, ii, iii, iv) in adoptively transferred mice.

3.8. Serum bactericidal activity, bacterial motility and mucin penetration assay

tWe observed that bivalent typhoidal bacterial ghost cells were able to generate protein and LPS specific serum antibodies against *S.* Typhi and *S.* Paratyphi A. Previous studies showed that both protein and LPS-specific anti *Salmonella* antibodies have complement-dependent bactericidal activity [22,23]. So, we decided to check the bactericidal activity of our immunized mice serum. To do this, immunized mice serum was heat inactivated and then incubated in different serum dilution with bacteria and complement. Whenever we incubated the bacteria with heat inactivated immunized serum supplemented with baby rabbit complement, the bacterial cells were killed significantly. The bactericidal effect of BTBG immunized serum dilution on *Salmonella* Typhi is 1:12,800 and for *Salmonella* Paratyphi A is 1:6400 (Fig. 7.A). Therefore, from this experiment we conclude that our typhoidal *Salmonella* ghost immunized mice serum could eliminate Typhoidal *Salmonella* by activating complement pathways. To further support our result scanning electron microscopic analysis was performed. Scanning electron microscopy (SEM) images revealed clearly visible lysis of bacterial cell when treated with immunized heat inactivated serum treated group (Fig. 7B.i, B.ii, C.i, C.ii).

Bacterial motility is very important for typhoidal *Salmonella's* pathogenesis as motility help them to move from epithelial lumen to epithelial surface. Thus, to find our BTBG immunogens' effect on typhoidal *Salmonella's* motility, we performed bacterial motility assay. We found that immunized serum, compared to non-immunized showed a major inhibition of *Salmonella* motility. This indicates that our bivalent formulation does indeed agglutinate bacteria, which may contribute to the immunogens' protective nature (Fig. 7.D.i, D.ii,E.i,E.ii,F) [14]. Intestinal epithelial wall also covered with a thick layer of viscoelastic mucus gel which is formed with a dense network of entangled and cross linked mucin. This mucin layer is always abundant with various antibodies specifically secretory IgA (sIgA) and sometimes IgG. To find the effect of the BTBGs immunization on both typhoidal *Salmonella* strains, we performed mucin penetration assay. Mucin penetration by immunized serum-treated bacteria was found to be lower whereas, that of non-agglutinated bacteria were much higher (Fig. 7.G). According to the findings, bacterial ghost cell-specific sera considerably hinder motility, which can prevent mucin penetration from reaching the epithelial cells and thereby prevents colonization or invasion.

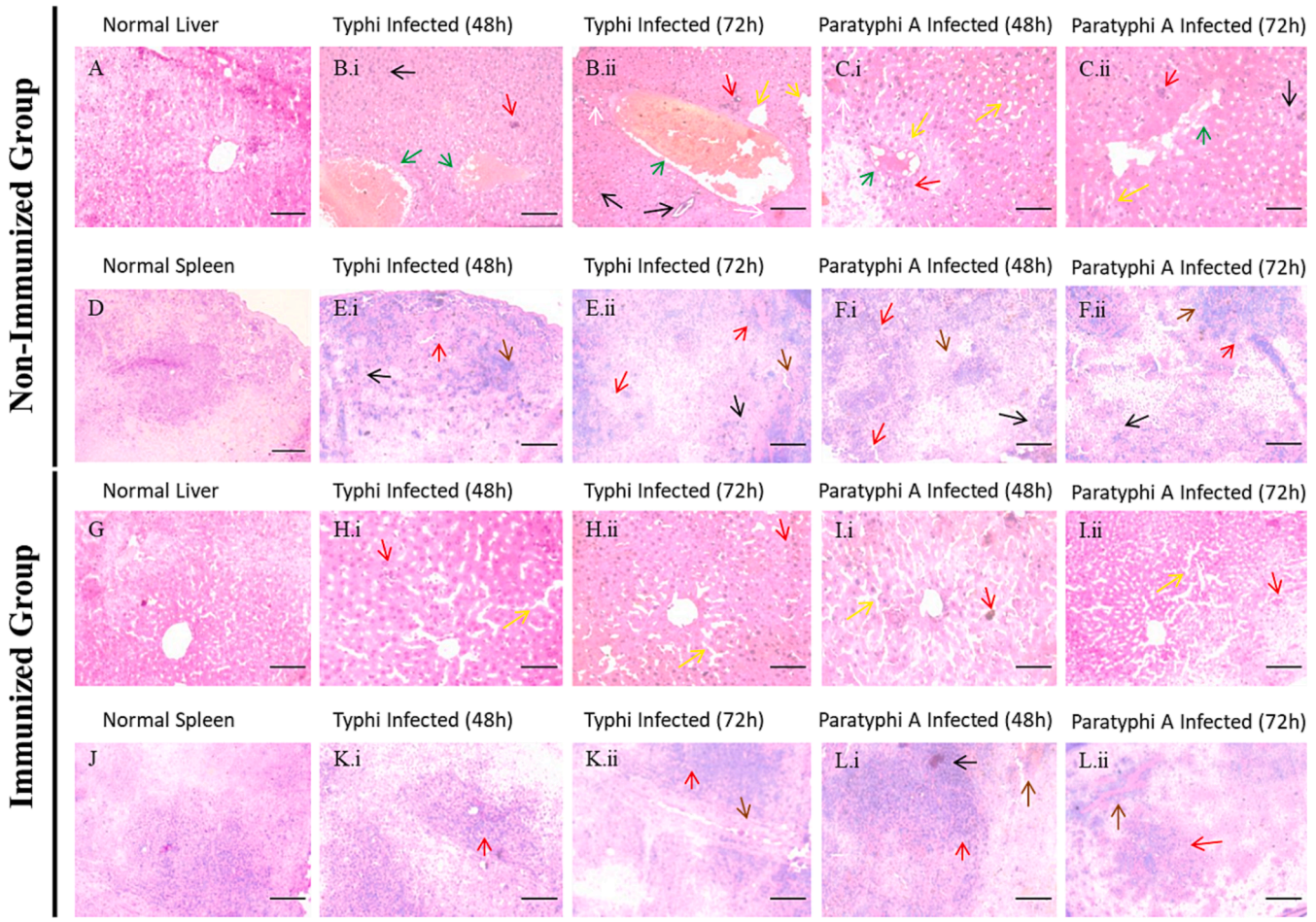

Fig. 5. Histopathology (H&E staining) of the liver and spleen of non-immunized and immunized mice after 48 h and 72 h of intra-peritoneal challenge with *Salmonella* Typhi and *Salmonella* Paratyphi A. **A.** & **D.** Liver and spleen of normal non-immunized mice. Liver and spleen of nonimmunized mice after 48 h and 72 h post challenged with *S.* Typhi (**B, i** & **ii**; **E, i** & **ii**) and *S.* Paratyphi A (**C, i** & **ii**; **F, i** & **ii**). Histological images show extensive portal inflammation, acidophilic bodies, infiltrating leukocytes, lobular inflammation and extensive granulomas. **G.** & **J.** Spleen and liver of normal immunized mice. Liver and spleen of immunized mice after 48 h and 72 h post challenged with *S.* Typhi (**H, i** & **ii**; **K, i** & **ii**) and *S.* Paratyphi A (**I, i** & **ii**; **L, i** & **ii**). Histological images represent mild leukocyte infiltrates, mild lobular inflammation, no portal inflammation and acidophil bodies in spleen and liver respectively. Images were captured at 20× and 40× magnification. Scale bar represents 100 μm. Inflammation (brown arrow), Extensive granulomas (black arrow), Portal inflammation (green arrow), Lobular inflammation (yellow arrow), Acidophil bodies (white arrow), Infiltrating leukocytes (red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Due to the global rise in multi-drug resistance, *Salmonella* Typhi and *Salmonella* Paratyphi are reaching an extremely concerning level with regards to human wellbeing. The advancement of vaccine development against enteric diseases should include a combination vaccine against *Salmonella* Typhi and *Salmonella* Paratyphi A [12]. Currently, no licensed bivalent vaccine or paratyphoid-only vaccines are available. In recent times, various techniques including bacterial ghost cells have been used to develop a vaccine against the cellular surface antigens of bacteria [24]. As an immunogen, both innate and adoptive immune responses can be elicited by the BGs preserved surface structures and components [25]. Vaccine formulations often include surface associated conserved motifs (i.e. pathogen associated molecular patterns (PAMP)) such as the lipopolysaccharide of gram-negative bacteria, which serve as ligands for host pattern recognition receptor to induce host immune responses. Previous work reported that surface antigens may induce strong humoral and cell-mediated immune responses against intracellular bacteria like *Salmonella* [26]. The use of bacterial ghosts (BGs) as a platform is reasonable, cost-effective, easy to produce, and at the same time intensified with expanded applications [25].

Lysis gene E-based recombination has been used in gram-negative bacteria to produce ghost cells with regular transmembrane tunnels [27]. Expense and intricacy are significant limitations for using genetic strategies to prepare bacterial ghosts. To avoid this, chemical agents are now widely used to prepare gram-positive [28], gram-negative [14,15], yeast-based fungal ghosts [29], and even viral ghosts [30]. In this study, we prepared bivalent bacterial ghost cells of *Salmonella* Typhi and *Salmonella* Paratyphi A by treatment with sodium hydroxide and characterized them. Scanning electron microscopic images show hollow structures on the surface of our treated bacterial cells. That confirms the formation of bacterial ghost cells. We observed no bacterial growth in these treated *Salmonella* suggesting that they are non-living lysed cells. An equal ratio of sodium hydroxide treated *Salmonella* Typhi ghost cells and *Salmonella* Paratyphi A ghost cells were mixed to formulate our bivalent typhoidal bacterial ghost cell mixture. After immunization of mice with BTBGs in three doses with two-week intervals, we observed an anti-LPS, anti-OMP, anti-Vi-PS and anti-WCL antibody response. Throughout BTBGs immunization, we compared the blood parameters of immunized mice with non-immunized mice to screen for any abnormalities. There are no significant changes in the blood profile between BTBG immunized and non-immunized animals. This suggests that our

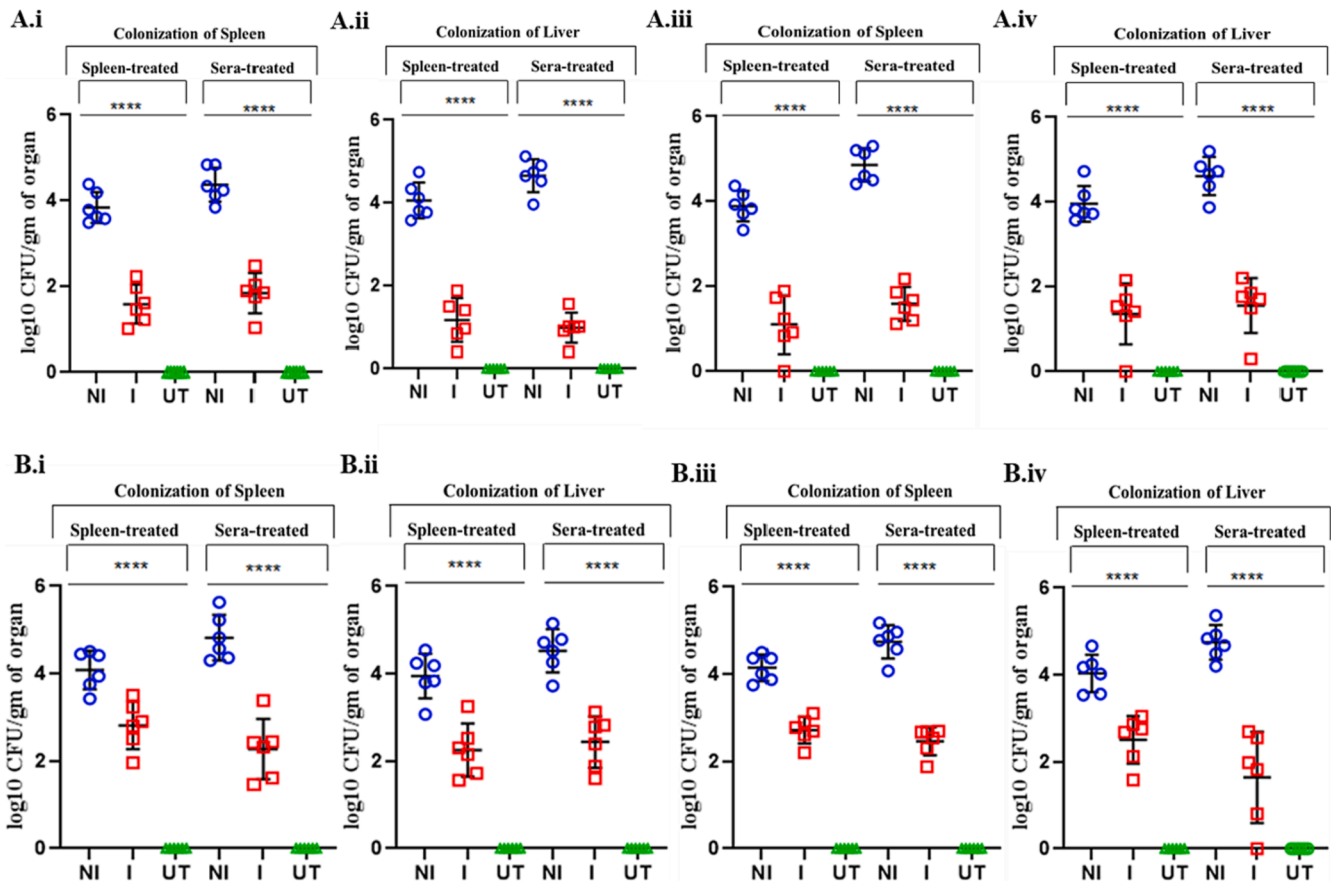

Fig. 6. Adoptive transfer (of immunized serum and splenocytes) reduces bacterial number in naïve mice after infection. **A.i, A.ii,** Bacterial number (72 h post infection) of *S.* Typhi in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 0^{th} day. **A.iii, A.iv.** Bacterial number (72 h post infection) of *S.* Paratyphi A in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 0^{th} day. **B.i, B.ii,** Bacterial number (72 h post infection) of *S.* Typhi in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 7^{th} day **B.iii, B.iv.** Bacterial number (72 h post infection) of *S.* Paratyphi A in spleen and liver after adoptive transfer (serum and splenocytes) in mice on 7^{th} day. Data were expressed as median value of six mice ± S.E of three separate experiments. Statistical analyses were performed using non-parametric one-way analysis of variance (ANOVA) test (Kruskal-Wallis test) (****P value < 0.0001, **p value < 0.001, *p value < 0.01). NI: non-immunized, I: immunized, UT: untreated.

BTBGs are not reactogenic or toxic in an in-vivo model [16].

Flow cytometry analysis of immunized mouse splenic cells showed significant increases in CD4+, CD8a+, and CD19+ cell populations. This suggests that B-cells and T-cells are activated by our immunogen [13,17]. Long-term protective immunity relies heavily on both humoral and cell-mediated immune responses to reduce infection [31,32,33]. In the vaccine against *Salmonella* Typhi, the lipopolysaccharides (LPS) and Vi-polysaccharides (Vi-PS) very important to produce protective immunity [34,35], but both are working in a T cell-independent manner [36]. That is the reason why the Vi-PS based Typhoid vaccine produces a very short-term immune response [37]. However, when Vi-PS is conjugated with a protein (tetanus toxoid), it works in a T-cell dependent manner [38,39]. In our study, we have found that adequate amount of Vi-polysaccharide specific anti-Vi-polysaccharide serum IgG was generated after bacterial ghost cell immunization. The anti-Vi-polysaccharide IgG antibody has a significant role in the protection against *Salmonella* Typhi in this study. Protection might be achieved due to typhoid bacterial ghost cells immunization could elicit anti-Vi antibody in addition to the cell-mediated responses and other immunologic responses after bivalent typhoidal bacterial ghost cells immunization [40]. Different surface proteins present in the outer membrane of *Salmonella* can act as an adjuvant and produce T-cell dependent immunity in animals [41]. Our ELISA, immunoblot, and dotblot studies revealed that immunized mice produce both anti-LPS and anti-OMPs specific antibodies, which may be due to BTBGs inducing the T-cell facilitated immune response in immunized animals. Previous studies reported that purified porins containing OmpC and OmpF of *S.* Typhi act as strong immunogen, responsible for conferring specific bactericidal antibody responses [23,42,43]. In our study also, range of immunogenic proteins were observed in immunoblot with distinct immunogenic bands in the region of 25 KDa–55 KDa, indicating the possibility of presence of porin specific antibodies in immunized serum. BTBGs also induce an anti-LPS and anti-OMP secretory IgA response in immunized animals. A mucosal antibody response is also necessary for protection against enteric pathogens like *Salmonella* [17]. A steady antibody titre up to the 120^{th} day post immunization suggests a long-term immune response is induced by BTBGs. Ex-vivo restimulation of immunized mouse splenic cells produces a Th1 and Th17 based cytokine response. In this study, a substantial amount of IFN-γ, TNF-α, IL-6 and IL-17 were produced, which helps to activate the innate and cell-mediated responses [44,45]. As dendritic cells (DCs) are essential for bacterial-specific T-cell priming in the event of *Salmonella* infection, we examined the response of bone marrow derived mature DCs to BTBGs. This led to the induction of pro-inflammatory cytokines IL-1β, IL-6, IL-12p40/70 and IL-23. This is necessary for differentiation between the Th1 and Th17 response in T cell receptor (TCR) stimulation [46]. The BMDCs-splenic CD4 T-cell co-culture experiment suggested antigen presentation and activation of different paths for immune coordination. In this case, induction of IFN-γ and IL-12 cytokines may activate cytotoxic T-cell and Th1-responses. IL-6 has a standing for the gaps concerning innate as well as adoptive immune response, and helps T cells to secrete IFN-γ and IL-2 [47,48]. IFN-γ, TNF-α also activates macrophages for antibacterial activity. To

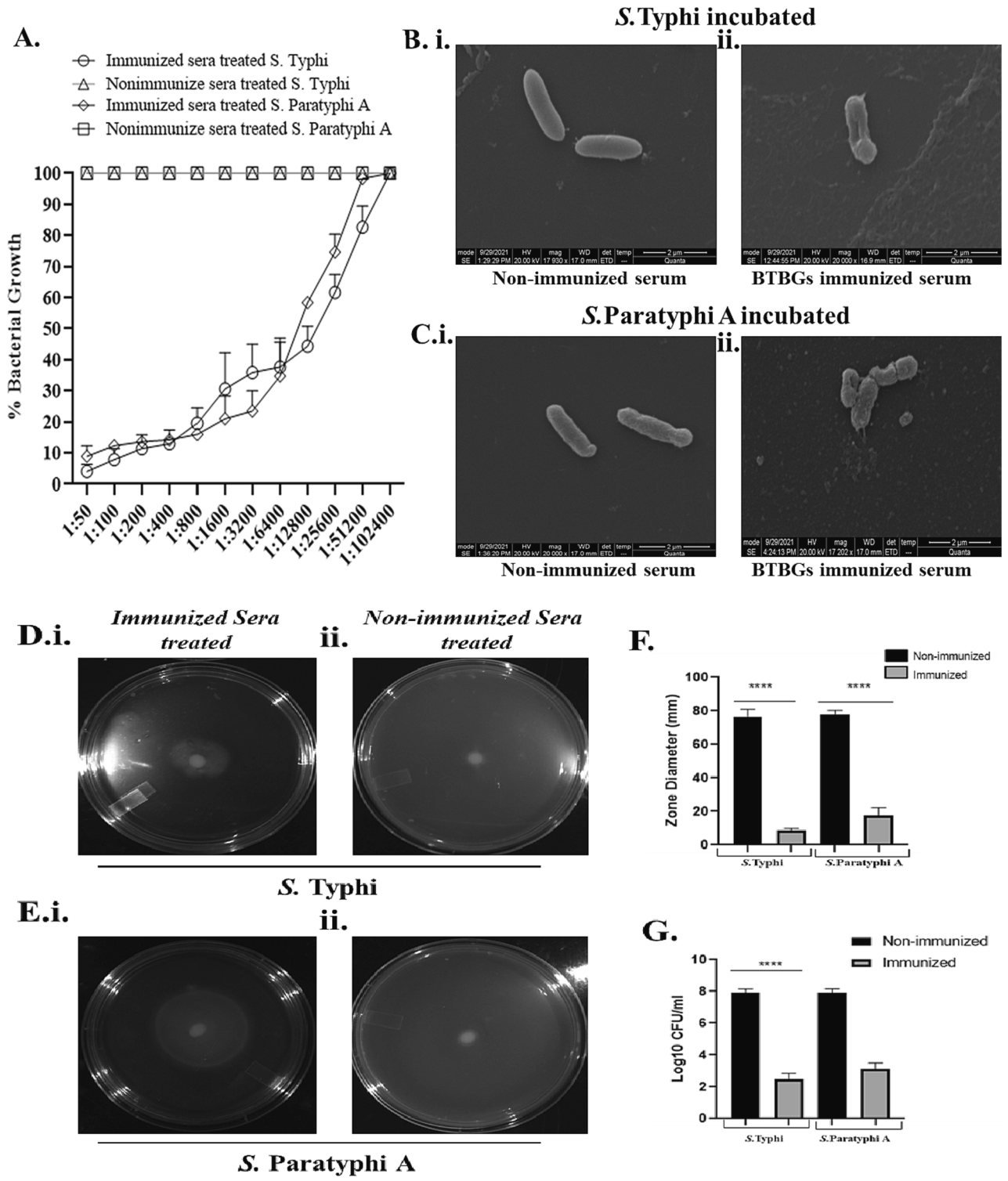

Fig. 7. Serum bactericidal activity, bacterial motility and mucin penetration assay in presence of immunized and non-immunized serum. **A.** Immunized serum is capable of complement mediated lysis of *Salmonella* Typhi (K554) and *Salmonella* Paratyphi A (K580). X-axis represents the serum dilution and Y-axis represent the percentage of bacterial growth occur after overnight incubation. **B–C**. Scanning electron microscopic (SEM) image of non-immunized and immunized sera treated bacteria; **B.i.** Nonimmunized sera treated *S.* Typhi **B.ii**. Immunized sera-treated *S.* Typhi **C.i**. Nonimmunized sera treated *S.* Paratyphi A **C.ii.** Immunized sera treated *S.* Paratyphi A. Scale bar represent 2 μm length. **D-F.** Bacterial motility test on soft agar (0.3 % agar) plates; **D.i. E.i**. The motility of *S*. Typhi and *S*. Paratyphi A was significantly reduced in case of immunized serum spreaded plate. **D.ii. E.ii.** The motility of *S*. Typhi and *S*. Paratyphi A in case of nonimmunized serum spreaded plate. **F.** Bar diagram of zone diameter of bacterial motility in immunized and nonimmunized serum spreaded plate after overnight growth. **G**. Mucin penetration assay; Bacteria treated with non-immunized or immunized serum were loaded on top of the 1 ml mucin column and allowed to penetrate. Immunized serum agglutinated bacteria showed reduced ability to penetrate mucin. Statistical analyses were performed using the two tailed Student's *t* test (****$p < 0.0001$).

prevent intracellular infection of *Salmonella* and its spread (to infect adjacent cells), cell-mediated immune responses, particularly Th1, are required [49,50]. Previous research showed that IFN-$\gamma^{-/-}$ mice are more prone to *Salmonella* infections than wild-type mice. It also showed that IFN-γ is necessary to activate macrophages to clear the invasive bacteria [51,52]. Th17 responses are enhancing the infiltrating leukocytes to the infection site to kill the bacteria [53] and also maintain the mucosal barrier thereby limiting the bacterial spread from the intestines [54]. All of these results indicate that BTBGs can significantly elicit both the cellular and humoral arms of the immune response that can protect against *Salmonella* infection. In our protective efficacy study, all of the animals were infected with a lethal dose of heterologous clinical isolates of *Salmonella* Typhi and Paratyphi A. Due to its reproducibility, we used the intra-peritoneum challenge model instead of the iron overload model for our vaccine efficacy study of *Salmonella* Typhi [55] and Paratyphi A [13]. BTBG immunized mice showed 100 % and 80 % protective efficacy or survivability against *Salmonella* Typhi and Paratyphi A respectively. The bacterial loads in the liver and spleen after 48 h and 72 h of infection was significantly lower in immunized mice than in non-immunized mice. The same results were obtained (i.e. lower bacterial colonization in immunized animals than nonimmunized animals after 48 h of infection) post 180th day of primary immunization. The histopathological study of spleen and liver tissue of both immunized and nonimmunized mice showed distinct differences in bacterial infection, dissemination, and inflammation. The cell mediated immune response plays a crucial role in reducing inflammation, granulomas, portal inflammation, lobular inflammation, acidophil bodies, and infiltrating leukocytes in liver and spleen tissues of immunized mice. We observed marked inflammation, extensive granulomas, significant portal inflammation, high lobular inflammation, the presence of acidophil bodies, and a large count of infiltrating leukocytes in nonimmunized mice [56]. Adoptive transfer of serum and splenocytes from immunized mice significantly lowers bacterial count in tissues of naïve mice, which also establishes the protective efficacy of our vaccine candidate. This adds to the evidence that humoral and cell-mediated immune responses are necessary to prevent typhoidal *Salmonella* infection [57,58]. In our study, immunized mice sera exhibited characteristic bactericidal properties as was evident from serum bactericidal assay. Functional capabilities of antibodies to elicit immunogenicity and potential protective capacities are measured by serum bactericidal assay [56]. SEM images from the bactericidal assay showed that our immunized serum can bind to *Salmonella* and rupture the cell, thereby aiding in complement-mediated killing. The BTBG immunized serum also inhibits bacterial motility and lowers the penetration ability of mucin layer, which reduces bacterial adherence and invasion through the epithelial cell membrane [59]. Thus, antibodies in BTBGs immunized mice serum can effectively opsonize and kill the bacteria. All of this strongly suggests that our bivalent typhoidal bacterial ghost cells (BTBGs) have the potential to be used as a vaccine candidate against *Salmonella* Typhi and *Salmonella* Paratyphi A.

5. Conclusion

A sodium hydroxide induced BTBG formulation can stimulate humoral and cellular immunity in mice. This antigen-specific long-term immune response provides protection against heterologous strains of *Salmonella* Typhi and Paratyphi A. Therefore, BTBGs can be used as vaccine candidate to prevent the typhoid and paratyphoid fever-mediated heath burden.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Authors contributions

PH: Conceptualization, design, methodology, investigation, analysis and writing; SM: methodology, analysis, review and editing. SB: methodology, review and editing; SD: methodology, review and editing. MD: methodology; SD: funding, review and editing; HK: conceptualization, design, funding, supervision and writing. All authors read and approved the final manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2023.08.049.

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