Original Article

Immunogenic Potential of Chitosan Nanoparticles Encapsulating Recombinant SlyB Antigen of Enterotoxigenic Escherichia coli (ETEC) in an Animal Model

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ABSTRACT

Intestinal bacterial infections are a significant cause of mortality in developing countries, with Enterotoxigenic Escherichia coli (ETEC) being a leading cause of severe diarrheal diseases. These infections are characterized by the production of enterotoxins and colonization factors that disrupt the small intestine, leading to diarrhea. While antibiotic treatments face limitations, vaccination has emerged as a critical tool for prevention. This study evaluates the immunogenicity of chitosan nanoparticles (NPs) that encapsulates the recombinant surface antigen SlyB of ETEC in an animal model. The SlyB antigen was expressed in an expression vector, purified, and encapsulated into chitosan nanoparticles using the ionic gelation method. Rabbits were immunized using three different administration methods: oral, oral-injection, and injection. Antibody levels in serum and feces were measured via ELISA, and the neutralization ability of immune sera was assessed using an ileal loop assay. The study's findings revealed that the oral administration of chitosan nanoparticles led to the highest titers of serum IgG and fecal IgA antibodies, suggesting a potential for enhanced mucosal immune responses.The encapsulation of the recombinant SlyB protein within the chitosan nanoparticles not only maintained antigen stability but also promoted controlled release, thereby stimulating robust cellular and humoral immunity.The efficacy of immune sera in neutralizing ETEC toxins was confirmed through a challenge test, with the oral vaccination group demonstrating the most significant neutralizing activity. This study underscores the potential of chitosan nanoparticles as an effective delivery platform for mucosal vaccines against ETEC.By encapsulating recombinant antigens, this method not only enhances immunogenicity but also offers a promising alternative to conventional vaccination strategies for diarrheal diseases.Further research is recommended to explore scalability and efficacy in broader populations.

Keywords: Enterotoxigenic *E. coli*, *SlyB*, Subunit Vaccine, Bacterial Toxin, Intestinal Infections.

1. Introduction

Infectious diseases account for more than 54% of deaths in developing countries (1).Enterotoxigenic Escherichia coli (ETEC) is a leading cause of acute diarrhea, responsible for significant mortality among children under five in low-income regions (2,3).ETEC pathogenesis involves host cell attachment, colonization, and toxin production, with the PhoP/PhoQ two-component signaling system in ETEC regulating resistance to antimicrobial peptides (AMPs). AMPs disrupt the membranes of pathogens, thereby aiding host defences. One such system in Escherichia coli is the PhoQ/PhoP system, which controls the expression of genes such as the Mg2+ transporter, as well as genes necessary for LPS changes (4). AMP (Antimicrobial Peptide) compounds are cationic and react with the membrane lipopolysaccharide of pathogens, thereby disrupting the outer membrane and penetrating the periplasmic space. This process contributes to the host's ability to thwart bacterial invasion. The PhoPQ signal transduction system plays a regulatory role in the resistance of AMPs in the Enterobacteriaceae family. It is evident that bacteria have developed diverse strategies to evade or resist the antimicrobial effects of AMPs (5). In Enterobacteriaceae, the PhoP and PhoQ genes are under the control of the PhoPQ system, which contributes to antimicrobial peptide resistance (6). Research has shown that the high expression of SlyB inhibits the activity of PhoP protein, and that this gene modulates the activity of PhoP protein. By targeting this system in bacteria, it is possible to affect the pathogen's ability to resist and promote the bactericidal activity of AMPs in the host body (6,7).SlyB, a gene under PhoPQ regulation, has been found to inhibit PhoP protein activity, modulating bacterial resistance. Targeting this system can reduce bacterial resistance and enhance AMP activity, offering a promising alternative to antibiotics. Diarrheal diseases continue to represent a significant health challenge, particularly in developing regions, with the development of effective vaccines being of critical importance due to the prevalence of ineffective treatments and the risk of resistance.Studies have indicated that plant-based edible vaccines offer a novel approach to disease prevention and have demonstrated potential in enhancing immune responses (8).Furthermore, the diverse pathotypes exhibited by Escherichia coli necessitate vaccination strategies that are tailored to its versatility (9). Research has demonstrated that nanoparticle-based vaccine delivery systems can enhance mucosal immunity, thereby addressing the limitations of conventional vaccine delivery methods (10,11). Previous studies have also indicated that antibodies, such as anti-K99, offer limited protection against ETEC (12). It is vital to acknowledge the significance of systemic and mucosal immune responses, including secretory IgA, in defense mechanisms. The utilization of platforms for delivering pathogens to mucosal surfaces, such as chitosan nanoparticles, has the potential to enhance vaccine efficacy. Chitosan, a natural polymer derived from chitin, possesses several advantageous properties including low toxicity, biodegradability, and immune-stimulating capabilities, rendering it particularly well-suited for antigen delivery (13,14). A study of the design of a multivalent ETEC vaccine containing different colonization factors and ETEC toxin may provide protection against a broad spectrum of bacterial strains. In this paper, the importance and pathogenesis of ETEC and the latest results of ETEC vaccine research are discussed (15). A significant corpus of research has been dedicated to the utilization of nanoparticles in the domains of medicine and veterinary science. For instance, Anvar et al. conducted a comprehensive review of the extant literature concerning the application of nanoparticles biosynthesized from cyanobacteria and microalgae in these disciplines (16).Chitosan, a deacetylated form of chitin, is a nano polymer that possesses inherent and natural adjuvant properties, thereby capable of stimulating cytokines. Its low toxicity, biodegradability, biocompatibility, antimicrobial activity, and mucoadhesive properties make it a suitable carrier for protecting antigens against proteolytic enzymes during mucosal administration (17-19).The encapsulation of recombinant antigens with chitosan has been shown to induce high levels of IgG and IgA antibodies in several studies. However, this study encapsulates the recombinant protein SlyB in chitosan nanoparticles to investigate its immunogenic potential.

2. Materials and Methods

2.1. Materials, Strains, and Plasmids

Chemical materials were procured from Merck, Qiagen, Roche, Fermentas and Sigma companies. Plasmid extraction and Gel Extraction kits were prepared from GeNet Bio (Korea), and molecular markers were purchased from Thermo Fisher Scientific. Low molecular weight CS was supplied by Sigma. The enterotoxigenic E. coli strain was used in the present study to isolate the SlyB gene, and the challenge test and the host bacterial strains were procured from the National Research Institute of Genetic Engineering and Biotechnology (NIGEB, Tehran).

2.2. Bioinformatics Analysis of *SlyB* Gene Sequence

The protein and nucleotide sequences of SlyB were retrieved from the UniProt protein and GeneBank databases, respectively. To ascertain the conserved sequence of the gene, BLASTn analysis was performed. To facilitate the amplification and sequencing of the gene, specific primers, designated SlyBF and SlyBR, were meticulously designed. These primers were engineered to incorporate sequence-specific sites for the EcoRI and HindIII enzymes. The forward primer, SlyBF, was designed to include the sequence ACTAGAGAATTCTGTGTTAATAACGACACCCTGT CAG, while the reverse primer, SlyBR, was designed to include the sequence ATATATAAGCTTGCGCGGAGAAACGGTCAC.

2.3. Screening of Recombinant Clones and Confirmation of *SlyB* Gene

The SlyB gene was extracted from the bacterial genome and amplified by Polymerase Chain Reaction (PCR). The PCR reaction conditions comprised an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 63.9°C for 20 seconds, and extension at 72°C for 30 seconds. This was followed by a final extension step at 72°C for 5 minutes. The restriction enzymes EcoRI and HindIII were utilized for the enzymatic digestion of the PCR product and the pET28a expression vector. Subsequently, a ligation reaction was performed using T4 ligase for a duration of 2 hours at 22°C.Following the transformation of the pET28a-SlyB vector into E. coli BL21 as the expression host, the recombinant colonies were screened by the colony PCR method (19). Furthermore, one of the colonies in which the presence of the SlyB gene was confirmed was analysed by enzymatic digestion.

2.4. Expression of Recombinant Protein

The recombinant E. coli BL21 (DE3) strain containing the pET28a-SlyB plasmid was selected for the expression of the SlyB protein.A volume of 50 µl of the selected colony overnight culture was added to 5 ml of fresh LB medium containing 50 µg/ml kanamycin. For the induction of gene expression, the bacterial cell density (OD 600 nm) was allowed to reach 0.6-0.8. At this point, isopropyl β-D-1-thiogalactopyranoside (IPTG) with a final concentration of 1 mM was added to the culture media. The culture was then incubated at 37 °C with shaking at 150 rpm. In addition, a control medium was prepared without adding IPTG, according to the above conditions. Subsequently, the culture media was subjected to a centrifugal process at a speed of 5000 rpm for a duration of 5 minutes. Thereafter, the cell lysate-containing protein was subjected to analysis using SDS-PAGE on a 12% polyacrylamide gel (20).

2.5. Purification of Recombinant Protein SlyB

The 6His-SlyB recombinant protein was purified by means of an affinity chromatography process, with Ni-NTA resin according to the manufacturer's protocol. In summary, following induction, 50 ml of culture media was subjected to centrifugation at 5000 rpm for 5 min. The resultant pellet was then suspended in 5 ml of lysis buffer solution, to which lysozyme (1 mg/ml) was added. The mixture was then subjected to shaking at room temperature for 1 following this, the lysate obtained from the previous step was subjected to centrifugation at 12,000 rpm for 10 min. At this stage, a 5 ml lysis buffer solution containing 8M Urea and a pH of 8 was added to the pellet, which was then shaken at room temperature for 1 h. The mixture was then subjected to centrifugation at 12,000 rpm for 10 min, after which the resultant pellet was washed with a solution of denatured washing buffer (pH=5.9). This was followed by elution with 2 ml of elution buffer (pH=4.5), which was repeated four times to ensure complete elution of the recombinant protein. To remove urea from the samples, the dialysis method was performed using phosphate buffer saline (PBS).The collected proteins were analyzed by SDS-PAGE (12%) and the purified protein concentration was determined using the Bradford assay (21).

2.6. Western Blotting Analysis

Following purification, the protein samples were transferred to a nitrocellulose membrane using a transfer buffer composed of 39 mM glycine, 48 mM Tris-base, 0.037% SDS and 20% methanol.The membrane was then treated with a blocking buffer (3% skim milk) for a period of 2 hours. Following a thorough washing step, the membrane was then subjected to an incubation with an anti-His-tag antibody (diluted 1:2000) in TPBS (0.05% Tween-20 dissolved in PBS). This was followed by a 1-hour incubation at 37°C.The membrane was then washed once more and subsequently incubated with an anti-mouse IgG antibody (HRP-conjugated, diluted 1:50,000), as described in the previous step. Finally, the protein band was detected using 3,3'-diaminobenzidine (DAB) (22).

2.7. Preparation of Nanoparticles Containing Recombinant Protein

The synthesis of the chitosan nanoparticles was achieved through the process of ionic gelation, whereby the chitosan was amalgamated with sodium tripolyphosphate (TPP) (23).The preparation of the chitosan solution involved the dissolution of 50 mg of the compound in 25 mL of acetic acid (2%).Subsequently, the nanoparticles containing the recombinant protein were prepared by the drop-by-drop addition of the purified protein SlyB to the prepared chitosan solution, utilizing a magnetic stirrer, for a duration of 10 minutes. The pH of the mixture was then adjusted to 5.5 using a NaOH solution, and 5 ml of TPP solution was added. The sonication process was then initiated. In order to prepare the control sample, PBS was added to the chitosan instead of the recombinant protein. The solutions were then subjected to centrifugation at 13,000 rpm for 45 minutes at 4°C. The amount of protein loading was evaluated using the resulting pellet.

2.8. Determination of nanoparticle properties

Loading capacity (LC) values were calculated as follows (24):

$$\text{Loading efficiency (\%)} = \frac{[(\text{Total antigen}) - (\text{antigen in the supernatant})]}{(\text{Total antigen})} \times 100$$

The size of the nanoparticles was measured using a Malvern Zeta Sizer device at Baqiyatallah University. The CS-SlyB was suspended in a Phosphate Buffered Saline (PBS) solution (pH 7.4) with constant stirring at 100 rpm at 37 °C. Sampling was performed at time intervals of 0, 3, 12, 24, 48, 72, 96, and 120 hours. The amounts of the protein released into the supernate were measured by Bradford protein assay (25).

2.9. Animal Immunization Assay

Eighteen 7-10 week-old female New Zealand rabbits from Razi Vaccine and Serum Research Institute were divided into six groups. In all groups, the interval between the administration of one dose of protein and the subsequent

dose was 14 days. The first blood sample was collected one week after the third dose was administered, and the second blood sample was collected after the fourth dose. The immunization groups and the method of administration are as follows:

Group 1) Injection of recombinant protein: first injection, 150 µg of recombinant protein with complete Freund's complete adjuvant (FCA); second to fourth injections, 150 µg of recombinant protein with Freund's incomplete adjuvant (FIA), administered subcutaneously.

Group 2) Injection of nanoparticle: in all four doses, chitosan nanoparticles containing 150 µg of recombinant protein, administered subcutaneously.

Group 3) The administration of oral nanoparticle solution: in all four doses, chitosan nanoparticles containing 300 µg of recombinant protein were administered to subjects via gavage.

Group 4) The administration of oral injection nanoparticle solution: three doses, nanoparticles containing 300 µg of recombinant protein were administered to subjects via gavage (oral administration) and a booster dose, a single dose of 50 µg of recombinant protein, was injected subcutaneously.

Group 5) Injection control: four doses of PBS solution were injected subcutaneously.Group 6: oral control that was received four doses of nanoparticles without recombinant protein through gavage.The ELISA method was used to determine the level of IgG and IgA antibodies in serum and faeces.

2.10. Neutralizing Test in the Immunized Animal

In order to evaluate the ability of serum from immunized animals to neutralize the toxin, the ileal loop assay was performed. The rings, with a length of approximately 2 cm, were created in the ileum of the intestine through surgery in immunized animals. The serum from immunized groups was treated with an overnight culture mixture of ETEC bacteria (LT+) and was injected into separate rings. The negative control involved the injection of PBS, while the positive control involved the injection of bacteria.18 hours later, the amount of fluid accumulation in each ring was measured.

2.11. Statistical Analysis

The statistical analysis in all experiments was carried out by SPSS software.

3. Results

3.1. Bioinformatics Analysis of *SlyB* Gene Sequence

GC content of the *SlyB* gene is equal to 52% and the codon adaptation index (CAI) is 0.8 which indicates the high efficiency of gene translation. The codon frequency distribution (CFD) suggests an optimum translation (Figure 1). The GC content of the SlyB gene is 52%, and the codon adaptation index (CAI) is 0.8, indicating high efficiency of gene translation. The codon frequency distribution (CFD) suggests an optimum translation (Figure 1).

3.2. Transformation of Recombinant Construct pET28a-SlyB

The colony-PCR technique, utilizing specific primers for the SlyB gene, was employed to verify the presence of the recombinant construct, pET28a-slyB, within bacterial colonies. The products of the amplification reaction are depicted in Figure 2. The targeted product was visualized as a 750 bp band on a 1% agarose gel. Moreover, enzymatic digestion provided additional evidence that these colonies harbor the recombinant construct.

3.3. Expression and Purification of Recombinant Protein

Following confirmation of recombinant colonies, the expression of the construct was induced by using 1 mM IPTG (Figure 3A). The appearance of a 20-kDa band in the sample after induction (Figure 3A) suggests that this band is related to the recombinant SlyB protein. The best expression was observed at 16 hours after induction in colony number 2. Therefore, this colony was selected for protein expression and further analysis. It was further established that the predominant form of the SlyB protein is expressed as insoluble inclusion bodies. The purification of the SlyB protein was achieved through the use of Ni-NTA chromatography, and the expression of the recombinant protein was confirmed through Western Blotting (Figure 3B). The concentration of the purified protein post-dialysis was determined to be 362 µg/ml.

3.4. Physical Properties of Chitosan Nanoparticles

The analysis indicates that the nanoparticles containing SlyB have an average size of 116.6 nm and an average zeta potential of 19.6 mV, suggesting that these values are within an optimal range. The loading efficiency was determined to be 88%, and the analysis of antigen release indicates that approximately 76% of SlyB was released from the chitosan nanoparticles over a period of 120 hours (Figure 4).

3.5. Determining the Serum IgG and Fecal IgA Antibodies

The ELISA results indicate an increase in IgG and IgA levels, suggesting that the administration of Cs-SlyB nanoparticles has led to the stimulation of the immune system in immunised rabbits (Figure 5A).The titration of IgG in the nanoparticle injection group is almost equivalent to that in the protein injection group. However, in the Cs-SlyB group, a notable difference in the amount of IgG antibody is observed between the two blood samples. Furthermore, the oral administration of Cs-SlyB nanoparticles resulted in a significant increase in IgG antibody titers when compared to the other groups (Figure 5B).The presence of high titers of IgA antibody in the feces of rabbits immunized orally indicates that the Cs-SlyB nanoparticles effectively stimulated the mucosal surfaces (Figure 5C).

3.6. Neutralization Assay

The ileal loop assay was utilised to assess the neutralization capacity of Lt toxin by the serum of immunized animals. The extent of liquid accumulation in each ring was

quantitated in relation to ring length and reported as an index of toxicity (Figure 6).The serum of immunized animals exhibited a substantial reduction in fluid accumulation in comparison to the serum of non-immune control animals ($p<0.001$). The magnitude of reduction in fluid accumulation in the oral nanoparticle group was found to be more significant in comparison to the other groups. Consequently, it can be concluded that the antibodies present in the immune serum were capable of neutralizing the effects of Lt toxin.

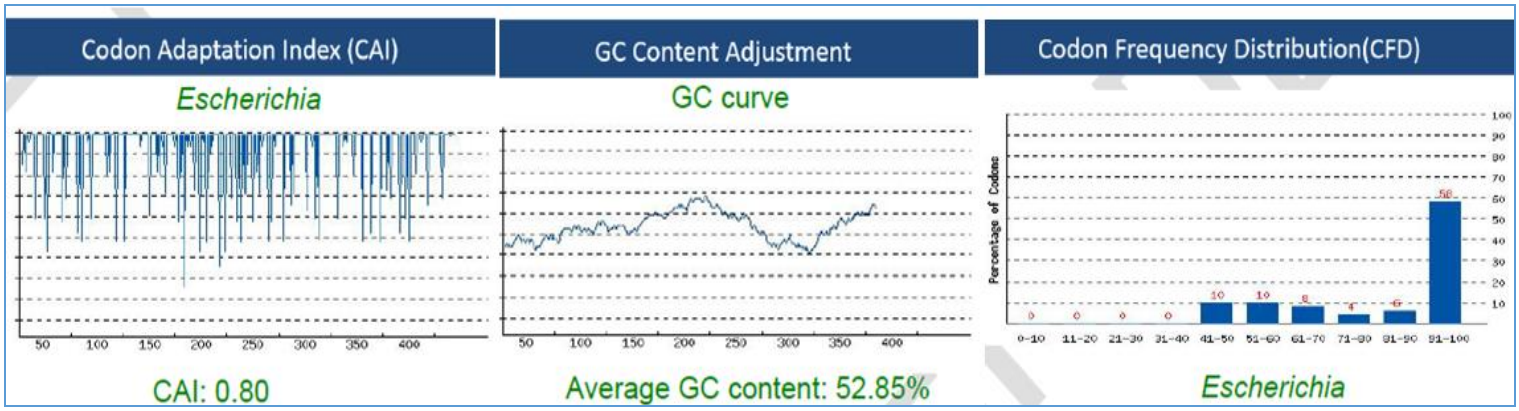

Figure 1. Codon adaptation index (CAI), GC content, and codon frequency Distribution (CFD) of the SlyB gene.

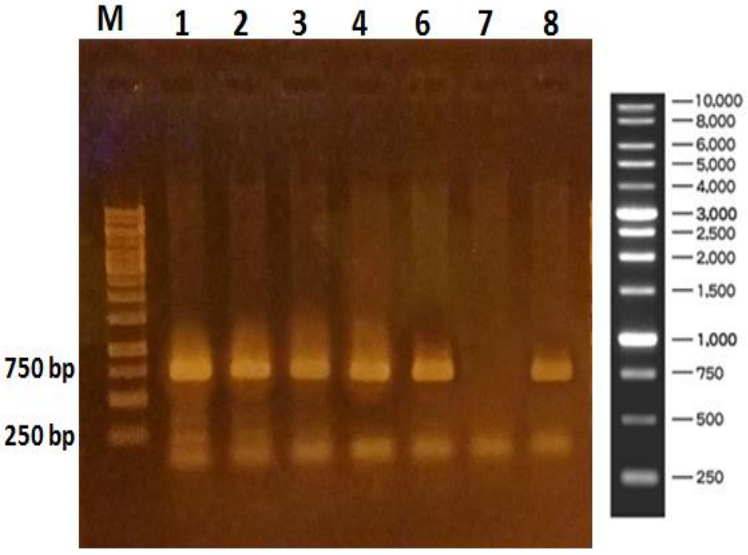

Figure 2. PCR product obtained from different colonies: lanes 1–6 recombinant colonies containing pET28a-*slyb*, lane 7 negative control, lane 8 positive control, lane M DNA ladder.

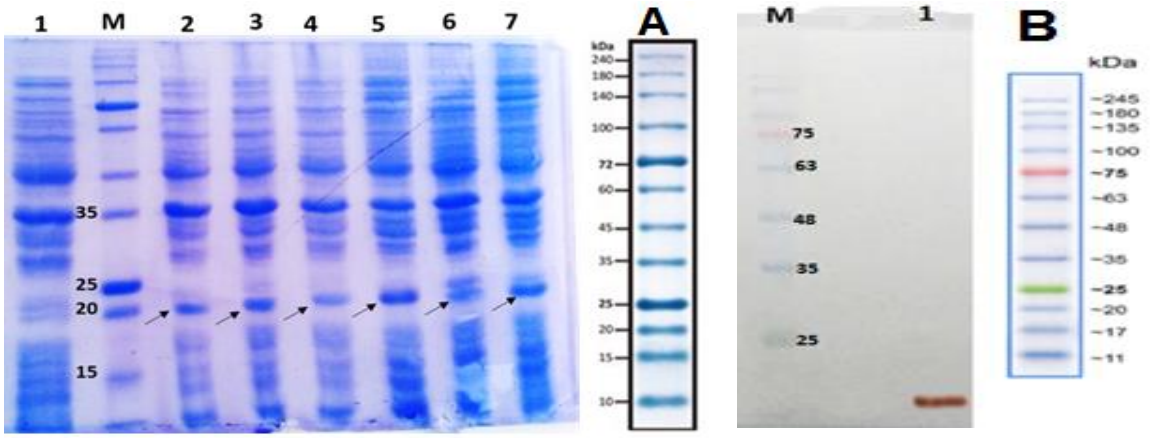

Figure 3. Expression and western blot evaluation. A) The profile expression of recombinant SlyB protein on 12% SDS-PAGE gel. Lane1 sample before induction, lane 2 to 7 samples after 16 inductions.

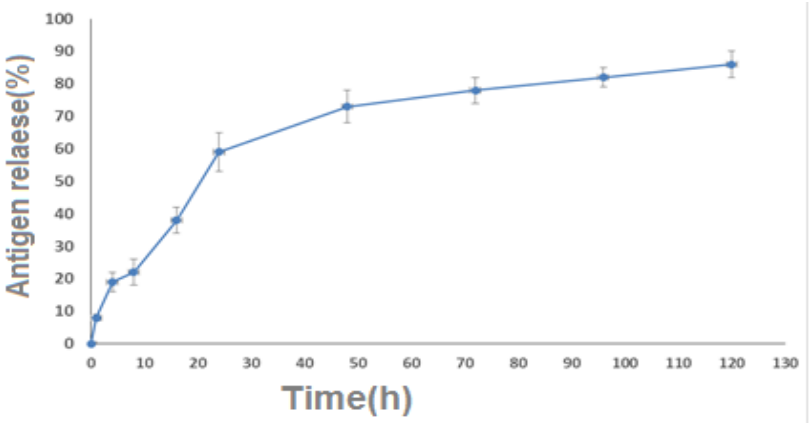

Figure 4. The profile of SlyB release from Cs-SlyB at pH 7.4 at 37 °C.

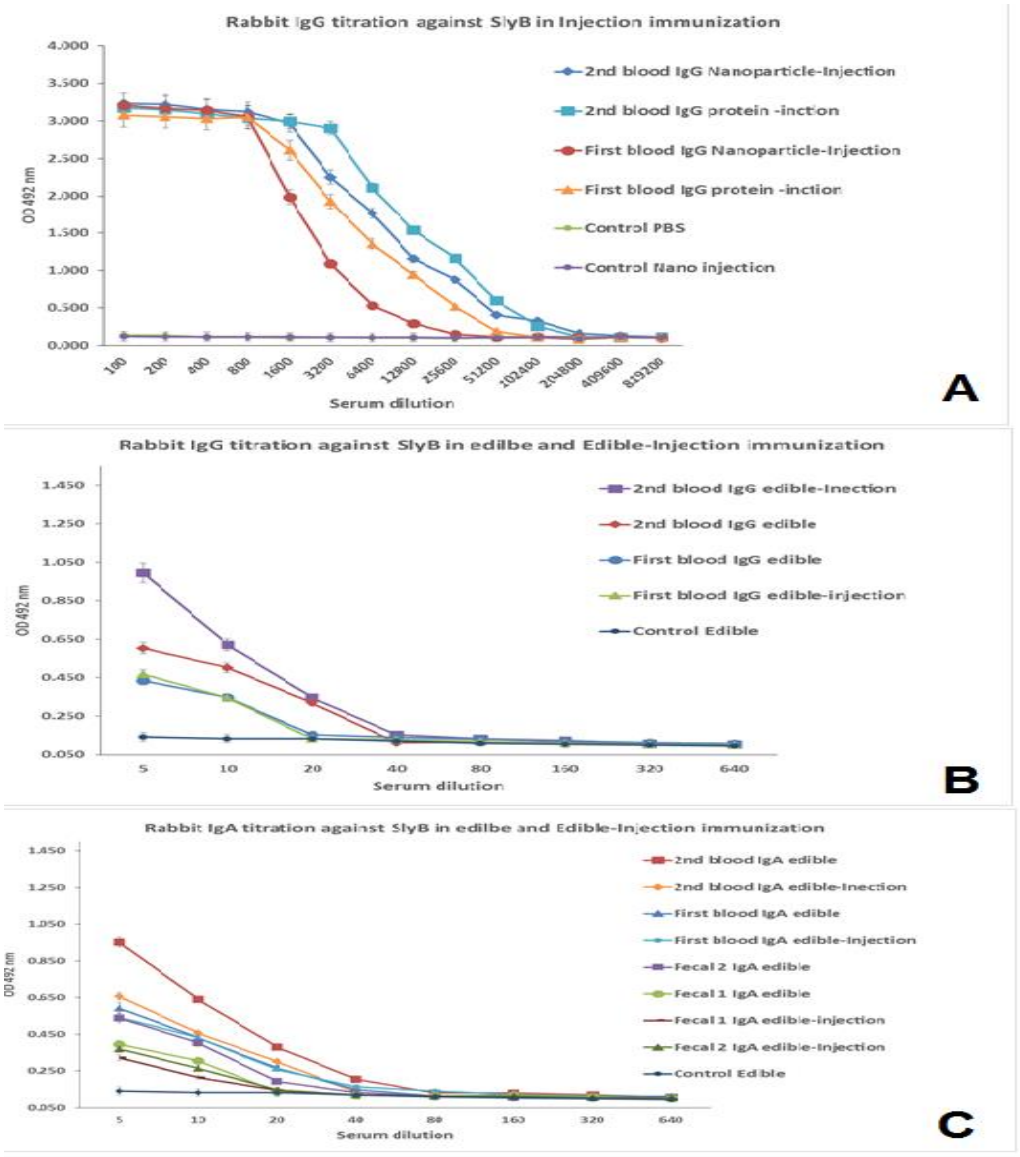

Figure 5. The Serum antibody levels in the immunized groups; A) IgG titration in the injection group, B) IgG titration in edible formulation, C) IgA titration.

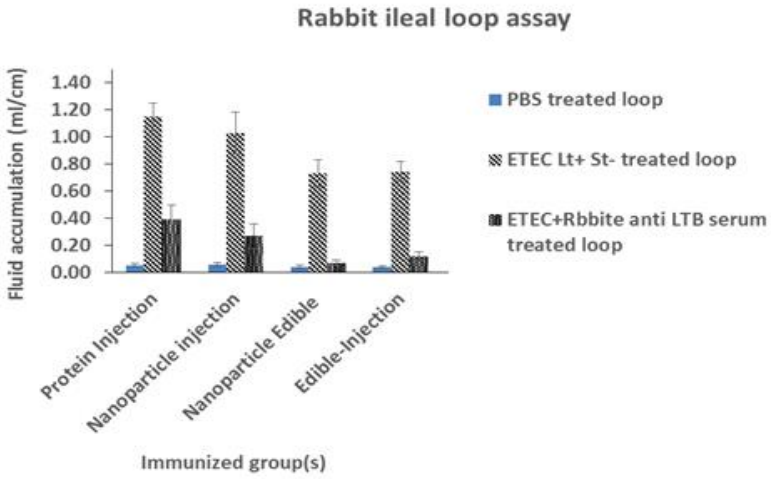

Figure 6. The effect of immunized serum on fluid accumulation in the ileal loop assay.

4. Discussion

Intestinal pathogenic bacteria, such as enterotoxigenic Escherichia coli (ETEC), are a primary cause of diarrheal diseases, with vaccination proving effective in reducing the mortality associated with ETEC infections (3). In developing countries, this bacterium is responsible for 280-400 million cases of diarrhea in children under five years of age, and accounts for over 50,000 human deaths annually (25). However, it has been observed that exposure to these infections can result in the development of immunity in the host, thereby underscoring the significant role of vaccination in immunity and the prevention of these infections (26). The choice of adjuvant and the route of vaccine administration can have a substantial impact on the intensity of the immune response. The predominant route of infection for these bacterial agents is the oral-fecal route; therefore, the most effective solution to combat them is the induction of a high amount of secretory IgA in the mucosal surfaces. The oral vaccine has a significant effect in preventing these diseases (27). Peyer's patches are defined as organized structures located within the intestinal wall, which are recognized as primary sites for initiating immune responses within the gastrointestinal tract. The M cells that are present within these patches are specialized in the uptake and transport of antigens through mucous epithelial cells. Subsequent to this transfer, the antigen and microorganisms are processed by antigen-presenting cells, thereby initiating immune responses and IgA production (28). The encapsulation of antigens within the particles of chitosan has been shown to enhance the effectiveness of stimulating the immune system. Chitosan, a natural polymer of D-glucosamine and N-acetyl D-glucosamine, has demonstrated enhanced antibacterial properties in Gram-negative bacteria through its binding to lipopolysaccharide and disruption of cell wall dynamics. The interaction between the positive charge of glucosamine in chitosan and the negative charge of the bacterial cell membrane can result in a change in membrane permeability, leading to osmotic imbalance and cell death. These nanoparticles are absorbed by the M cells. Chitosan's role as an adjuvant has been demonstrated to promote cellular immunity by producing cytokines and activating dendritic cells. The mucoadhesive property of chitosan results in prolonged retention at the absorption site and the release of antigens in a controlled manner. Chitosan nanoparticles have been shown to enhance antigen stability, prolong retention at mucosal surfaces, and stimulate immune responses (29). The interaction of chitosan nanoparticles with bacteria makes them very effective against intestinal infections (18). Antimicrobial peptides (AMPs) are cationic peptides that are classified into two groups, Defensin and Cathelicidin, based on their structure and function (Smith et al., 2022). The positive charge of these compounds promotes their attachment to the bacterial membrane and, by disrupting the outer membrane, they access the periplasmic space (Jones et al., 2021).Studies have shown that transgenic mice that expressed these human compounds were resistant to salmonella infections (Brown et al., 2020). To evade the bactericidal action of AMPs, bacteria must detect the presence of AMPs and regulate the expression of genes involved in AMP resistance (5).In the Enterobacteriaceae family, the PhoPQ transport system controls genes that modulate AMP resistance, including the three genes SlyB, PhoP, and PhoQ. Bacteria have developed several mechanisms to counteract the AMPs and facilitate their penetration into host cells (7). Studies have shown that the high expression of the SlyB gene, which encodes a lipoprotein in the outer membrane of bacteria, inhibits the activity of the PhoP protein; in fact, it negatively regulates the activity of this protein.Based on this, by inhibiting the activity of this transmission system, the host can have an effective defence against the invading bacteria and reduce the resistance of bacteria.Also, the bactericidal activity of the host's AMP can be improved. This anti-virulence method has the potential to serve as a solution for the treatment of bacterial infections and the problem of resistance to antibiotics (6–8). In this study, we expressed the SlyB gene from ETEC bacteria in an expression vector and encapsulated the

purified protein in chitosan nanoparticles to investigate its immunogenicity in the animal model. The presence of a 20-kDa band in western blotting analysis can suggest the correct translation in the expression system (30).After dialysis, the concentration of purified protein was estimated to be 362 µg/ml. Research has indicated that a concentration ranging from 1 to 3 mg/ml of chitosan promotes the desired formation of chitosan nanoparticles (30).The results of our study revealed that the concentration of 2 mg/ml chitosan and 1 mg/ml TPP leads to the highest loading efficiency and slow-release rate. A higher percentage of encapsulation efficiency indicates that a larger proportion of the recombinant protein is incorporated into the nanoparticles. It has been demonstrated that increasing the amount of vaccine available for target cells can enhance the immune response. The results of the protein loading percentage in nanoparticles showed that 88% of SlyB recombinant protein was encapsulated within the chitosan nanoparticles, which is consistent with the findings of other researchers (31). It has been hypothesized that the antimicrobial activity of chitosan with a lower molecular weight is enhanced, and that this activity increases in gram-negative bacteria with decreasing nanoparticle size (18).In the present study, the average size of the SlyB-containing nanoparticles was 116.6 nm, and the average zeta potential was 19.6 Mv. It is hypothesized that the favorable electrostatic charge of the nanoparticles contributes to their stability and improved interactions with cells. It has been demonstrated that enhancing the zeta potential and reducing the size of nanoparticles in chitosan nanoparticles can augment their antibacterial activity (22).The loading capacity of nanoparticles is a pivotal factor in determining the immunogenicity of recombinant protein. In Hosseini's study, the recombinant protein containing CfaB, ST, CfaE, and LtB was encapsulated within chitosan nanoparticles, with a size of 112 nm and a loading efficiency estimated to be 98.8% (29). In a separate study, Nazarian reported that the chimeric protein encapsulated in PLGA nanoparticles had an average size of 252 nm and a loading efficiency of 91.96% (32).In the study of Noroozi, the loading efficiency of chitosan nanoparticles was 90% and the size of the nanoparticles was 532 nm (33).Chitosan has been shown to release the active substance in a controlled manner, and the release profile shows this controlled release at different times. As illustrated in Figure 4, the initial 24-hour release phase accounts for approximately 60% of the total release, followed by a gradual decline. This observation aligns with the findings reported by other researchers, suggesting that this sustained release mechanism enhances the uptake of antigens by mucosal surfaces and improves immunogenicity (32). In this study, the gavage method was employed for the purpose of oral immunization, with the objective of increasing antigen protection against proteolytic enzymes by using chitosan. The titration results of IgG and IgA in rabbits immunized with recombinant protein indicate that this protein is capable of effectively stimulating the immune system in both oral and intravenous administration. The results indicate similar titration between nanoparticle injection and protein-adjuvant injection, and a high level of IgG antibody is observed in the serum of rabbits that received the nanoparticle orally. In the oral method, the group that received a booster dose by injection had a higher antibody titer compared to the group that received all four doses orally. In terms of IgA antibody production, the group receiving nanoparticles containing the recombinant protein orally exhibited the highest titer of IgA antibodies in the serum, and this titer continued to higher dilutions in the oral group compared to the oral injection group. Furthermore, the oral group exhibited significantly higher levels of IgA antibodies in stool samples when compared to the injected oral group. This outcome suggests that the delivery of the antigen to the desired site in the digestive system and its protection from protease degradation may be facilitated by encapsulating the recombinant protein in chitosan nanoparticles. The encapsulation of CFA/I, CS3, and CS6 in PLGA has been found to stimulate the local and systemic immune response, resulting in high titers of IgA and IgG antibodies (32). The investigation of the immunogenicity of chitosan nanoparticles containing OMV has revealed that the group receiving the antigen in the form of nano capsules exhibited a significantly higher fecal IgA antibody titer. In the early 1950s, researchers discovered that injecting enterotoxins into closed segments of the intestine (closed intestinal loops) in rabbits and later in other animals caused fluid accumulation (Jones et al., 1957). This test was subsequently employed extensively to identify enterotoxins (Smith et al., 1964).In the ileal loop assay, it was found that the immunized serum was able to significantly reduce the amount of fluid accumulation in the rings (Brown et al., 1966). The oral group that received nanoparticles containing recombinant protein orally demonstrated a highly significant difference in the reduction of fluid accumulation compared to the other groups. Therefore, mucosal immunogenicity induced by the oral administration of nanoparticles containing recombinant protein has been effective in neutralizing the toxin. In the Noroozi study, fluid accumulation was not observed in loops containing ETEC that were treated with serum immunized with chitosan nanoparticles containing antigen, while fluid accumulation occurred in the control group (33). The investigation focused on the encapsulation of the important pathogenic factor LTB from ETEC in PLGA, and the subsequent investigation of its immunogenicity. The study demonstrated that the IgG antibody titer was significantly elevated, and that fluid accumulation in the ETEC group treated with serum from immunized mice was significantly reduced. This indicated that the antibody against this recombinant LTB was able to recognize and neutralize the toxin effectively (34). In a separate study, Hosseini et al. encapsulated intimin from E. coli O157:H7 bacteria in chitosan nanoparticles and observed higher IgA titers in the serum and feces of the oral group compared to

other groups (10). In a separate study by Khanifer et al., the immunogenicity of encapsulated EIT and Stx2B proteins in an organism model was investigated. The results demonstrated that orally injected vaccinated groups exhibited a more favorable immune response to encapsulated antigens. This method was found to be effective in reducing bacterial attachment and neutralizing the resulting toxin (35). Conclusive findings from related studies have shown increased mucosal IgA production when using nanoparticles as delivery platforms, thereby validating the findings of this study (36). Furthermore, this study aligns with previous research indicating that encapsulated proteins provide enhanced antigen protection and slow-release benefits, which are critical for effective immune response activation (37). Previous studies have highlighted the challenges and strategies in developing vaccines against ETEC, including the importance of enhancing mucosal immune responses and targeting bacterial toxins effectively (38). Comparatively, studies on other polymer-based nanoparticles also indicate similar immunogenicity outcomes, suggesting a generalizable advantage of nanotechnology in vaccine development (39). The conclusion drawn from this study indicates that the incorporation of recombinant SlyB protein into chitosan nanoparticles results in the effective stimulation of immune responses. This finding suggests that the utilization of such nanoparticles as a vaccine platform for ETEC infections holds considerable promise. The encapsulation of antigens within nanoparticles has been demonstrated to promote mucosal immunity and to serve as an effective vaccination method for the neutralization of ETEC infections. Further research is recommended to investigate the scalability and efficacy of this approach in broader populations, as well as to examine the optimal delivery of the protein to the immune system in other organism models and the persistence of the vaccine.

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Authors' Contribution
Writing paper, Doing experiment: S. KA.
Supervisor, Project manager, Concept design: J. A.
Supervisor, Editing paper: E.T.
Advisor, editing paper: S. ZK.

Ethics
It is hereby stated that all ethical standards have been adhered to in the preparation of the submitted article.

Conflict of Interest
The authors declare that they have no conflict of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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