



Oral Immunization with YncE-Encapsulated Chitosan Nanoparticles Confers Protective Immunity against Enterotoxigenic Escherichia coli (ETEC) in a BALB/c Murine Model

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ABSTRACT

Background: Every year, a large number of people worldwide suffer from gastrointestinal diseases such as diarrhea. Among this population, children under five are particularly vulnerable and are at increased risk of death or malnutrition. Enterotoxigenic Escherichia coli (ETEC) is a major cause of this severe diarrhea. The bacteria first adhere to intestinal cells using colonization factors (CFs), then produce heat-stable (ST) and heat-labile (LT) toxins, which together are responsible for causing the severe diarrheal illness.

Objective: to investigate the potential of the recombinant periplasmic protein YncE, encapsulated within chitosan nanoparticles, as a novel vaccine candidate, and to evaluate its immunogenicity.

Methods: The recombinant YncE gene, encoding the periplasmic protein YncE, was cloned, expressed, purified. Successful expression and purification were confirmed via Western blot analysis. The purified recombinant protein was subsequently encapsulated within chitosan nanoparticles, and the particle size was measured. BALB/c mice were divided into five groups: recombinant protein injection, nanoparticle injection, oral nanoparticle administration, combined oral-injection nanoparticle regimen, and control. Immunogenicity was evaluated by measuring antibody titers using an enzyme-linked immunosorbent assay (ELISA). Finally, a bacterial challenge assay was conducted on all immunized groups to evaluate protective efficacy.

Results: Following expression and purification, the size of the YncE-containing nanoparticles was measured. These nanoparticles successfully induced both humoral and mucosal immune responses. Furthermore, immunized mice challenged with the YncE-containing chitosan nanovaccine demonstrated protective immunity against ETEC.

Conclusion: Taken together, our results show that the YncE protein when packaged in chitosan nanoparticles protects mice against ETEC infection. This makes YncE a strong candidate for future vaccine development.

Keywords: Diarrheal disease, Enterotoxigenic Escherichia coli, YncE gene, Chitosan nanoparticles

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INTRODUCTION

Diarrhoeal disease remains a major global public health problem and one of the leading causes of death among children under five years of age, ranking among the top infectious causes of under-five mortality (1). According to the Global Burden of Disease Study 2021, although the global burden of diarrhoeal disease in children under five has steadily declined from 1990 to 2021, it remains a significant health threat (1, 2). In 2021, diarrhoeal diseases caused approximately 444,000 deaths among children under five worldwide, with rotavirus and *Shigella* remaining the leading causes of mortality (2). The heaviest burden of diarrhoeal diseases remains concentrated in sub-Saharan Africa and South Asia, where mortality rates in children under five reach approximately 152 per 100,000 in males and 132 per 100,000 in females, compared with less than 1 per 100,000 in high-income regions (3). Various pathogens have been identified as causes of infectious diarrhoea, with rotavirus, *Shigella*, and diarrhoeagenic *Escherichia coli* being among the most frequently implicated globally. In 2021, rotavirus remained the leading pathogen attributed to diarrhoeal deaths in children under five, accounting for approximately 35% of deaths, followed by *Shigella* (approximately 24%) (3). Many strains of bacteria, including Enterotoxigenic *Escherichia coli* (ETEC), cause diarrhea. According to the Centers for Disease Control and Prevention, travelers' diarrhea (TD) is the most common travel-related illness (4), and ETEC is the most common bacterial cause of TD (5). Colonization factors (CFs), together with heat-labile (LT) and heat-stable (ST) toxins, constitute the main pathogenic factors of ETEC. The genes encoding these two enterotoxins are located on plasmids, and the enterotoxins can cause diarrheal disease independently or simultaneously. CFs, which are major virulence factors, attach to the surface of small intestinal cells and trigger toxin production. Binding of CFs to host

cell receptors is the initial step required for ETEC infection. More than 25 different CFs, including CFA/I, CFA/II, CS1, CS2, CS4, and CS14, have been identified and characterized (6). Although antibiotics remain an important treatment for bacterial infections worldwide, resistance has emerged among many bacterial pathogens. Given the increasing multidrug resistance among these organisms, new and effective interventions, including vaccines, are urgently needed (7).

The *YncE* gene of *E. coli* encodes a predicted periplasmic protein of unknown function. The protein is localized within the periplasmic space of *E. coli*. When a truncated form of *YncE* is overexpressed in *E. coli*, the bacterial cell envelope is damaged, leading to release of cytoplasmic contents (8). Preliminary crystallization studies indicate that the protein contains a single beta-propeller domain composed of seven four-stranded beta-sheets. Iron limitation strongly induces *YncE* expression, and a Fur box has been identified in its promoter region (9). The *YncE* gene is 1062 base pairs in length and encodes a protein of 353 amino acids. The first 30 amino acids constitute a signal peptide, while amino acids 31 to 353 form the mature protein. The *YncE* protein, characterized as a conserved immunogenic antigen, is highly immunogenic, capable of inducing a strong IgG response, and can protect against acute systemic infection (10). The *YncE* gene is conserved across *E. coli* phylogenetic groups, and its expression has been demonstrated in diverse clinical isolates (10). Studies suggest that *YncE*, as a novel antigen, is a promising vaccine component against pathogenic *E. coli* strains, though its use may require combination with pathotype-specific antigens. Given the high prevalence of infections caused by antibiotic-resistant strains, there are limited effective treatments available for these infectious diseases (10).

The activation of local immunogenic responses is a rational approach to preventing enteric infections. To enhance vaccine efficacy, antigen-specific antibody titers on

the mucosal surface should be increased, as this reduces bacterial colonization. Oral vaccination induces antigen-specific secretory IgA (SIgA) responses at mucosal surfaces (11), and in an ETEC murine model, oral immunization with the conserved ETEC adhesin EtpA afforded significant protection against small intestinal colonization, with protection correlating with fecal antibody responses (12). Nanoparticles play a crucial role in improving the effectiveness of mucosal vaccines because these vaccines are poorly absorbed following mucosal administration. Chitosan, a polymer composed of β -(1 $\rightarrow$ 4)-linked D-glucosamine and N-acetyl-D-glucosamine units, is derived by deacetylation of chitin. The primary advantage of chitosan over its precursor chitin lies in its pH-dependent solubility and cationic nature, which enable interactions with negatively charged mucosal surfaces. Chitin, the precursor of chitosan, is a structural component of fungal cell walls and crustacean exoskeletons. Chitosan is produced industrially by deacetylation of chitin extracted primarily from crustacean exoskeletons (13, 14). One of the key advantages of chitosan nanoparticles is their biodegradability, which reduces environmental concerns associated with persistent synthetic materials. They are widely utilized across various scientific fields, including pharmaceutical sciences (15, 16). In addition to its biocompatibility, chitosan can enhance the paracellular permeability of hydrophilic macromolecular drugs, including peptides, across mucosal epithelia by transiently opening tight junctions (17). This absorption-enhancing property, combined with its ability to form antigen-loaded nano- and microparticles, has made chitosan highly valuable in mucosal vaccine development (18).

The current study aimed to develop an YncE nanovaccine targeting enterotoxigenic *Escherichia coli* (ETEC), a major causative agent of travelers' diarrhea. A 323-amino-acid segment of the YncE protein was utilized. The recombinant YncE protein was expressed in *E. coli* BL21 (DE3) and subsequently encapsulated within chitosan

nanoparticles. Finally, mice were immunized with the chitosan nanoparticles containing the recombinant YncE protein. Antibody titers were measured, and a bacterial challenge assay was conducted using ETEC.

MATERIALS AND METHODS

T4 DNA ligase, Pfu DNA polymerase, Taq polymerase, RNase A, *Hind*III, and *Eco*RI were obtained from Thermo Scientific (Waltham, MA, USA). Medium molecular weight chitosan was purchased from Sigma-Aldrich (St. Louis, MO, USA). A PCR extraction kit and a plasmid extraction kit (Roche Diagnostics, Mannheim, Germany), as well as a protein extraction solution (iNtRON Biotechnology, Seongnam, Korea), were also acquired. DNA molecular weight marker (SM0333, GeneRuler DNA Ladder Mix) and protein molecular weight marker (SM0671, PageRuler Prestained Protein Ladder) were obtained from Thermo Scientific. All other chemicals were supplied by Merck KGaA (Darmstadt, Germany) and were of molecular biology grade purity.

Construction of Prokaryotic Expression Vectors

A 972 bp DNA fragment encoding the mature YncE protein (residues 31–353, excluding the N-terminal 30-amino-acid signal peptide) was amplified, digested with *Eco*RI and *Hind*III, and subcloned into the corresponding sites under the control of the T7 promoter in the pET28a vector. The insertion of the YncE coding sequence into the recombinant pET28a plasmids was confirmed by PCR, restriction digestion, and sequencing. Subsequently, pET28a-YncE was introduced into *E. coli* BL21 (DE3) using the heat-shock transformation method (19).

Confirmation of pET28a-YncE in Host Cells

The gene of interest was ligated into the pET28a vector and then transferred into host cells for cloning. The plasmid containing

Table 1: PCR thermal cycling parameters employed for amplification of the yncE gene

PCR steps	Temperature	Time	Number of cycles
Initial denaturation	95°C	5 min	1 cycle
Denaturation	95°C	45 sec	30 cycles
Annealing	62°C	45 sec	
Extension	72 °C	45 sec	
Final incubation	72°C	5 min	1 cycle

the YncE gene was extracted from DH5 α competent cells. PCR was performed using T7 primers to confirm the presence of the gene, with pET28a serving as the negative control. PCR-amplified DNA fragments (1300 bp) were separated on a 1% agarose gel and visualized under ultraviolet light. PCR was performed under the following conditions (see Table 1).

Expression of the YncE Recombinant Protein in E. coli BL21(DE3) Cells

For recombinant YncE protein expression, 100 μ L of an overnight culture of *E. coli* BL21 (DE3) harboring the recombinant pET28a-YncE plasmid were inoculated into 5mL of LB medium supplemented with 50 μ g/mL kanamycin. The culture was incubated at 37°C with shaking at 180 rpm until the optical density at 600 nm (OD_{600}) reached 0.7. An uninduced sample was collected immediately before induction. Recombinant protein expression was then induced by adding IPTG to a final concentration of 1 mM, and the cultures were further incubated at 37°C with shaking at 180 rpm. Samples were collected at 2, 4, and 16 h after IPTG induction. At each indicated time point, the cells were harvested by centrifugation at $8,000 \times g$ for 10 min at 4°C. The resulting cell pellets were resuspended in 100 μ L of 1 $\times$ sample buffer. The samples were heated at 95°C for 5 min to denature the proteins before electrophoresis. Equal volumes of the prepared samples were loaded onto a 12% SDS-PAGE gel.

Purification of Recombinant YncE Protein

The recombinant YncE protein, containing an N-terminal His-tag, was purified by Ni-NTA affinity chromatography under denaturing conditions. Briefly, bacterial

cells were lysed in denaturing lysis buffer containing 50 mM sodium phosphate, 300 mM NaCl, and 8 M urea at pH 8.0. Following clarification by centrifugation, the soluble fraction was loaded onto a pre-equilibrated Ni-NTA column. The column was washed with washing buffer containing 50 mM sodium phosphate, 300 mM NaCl, 8 M urea, and 20 mM imidazole (pH 6.3). The His-tagged YncE was eluted with elution buffer containing 50 mM sodium phosphate, 300 mM NaCl, and 8 M urea (pH 4.5). Eluted fractions were analyzed by SDS-PAGE (12%), and YncE-containing fractions were pooled. The purified protein was subsequently dialyzed and quantified using the Bradford assay with BSA as the standard (20, 21).

Western Blot Analysis

To perform electrophoresis on a 12% SDS-PAGE gel, three samples were used: *E. coli* BL21 (DE3) containing pET28a-YncE before and after induction, and a positive control. The YncE protein band was electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Germany) using transfer buffer composed of 40 mM glycine, 50 mM Tris base, 20% methanol, 0.04% SDS, at pH 8.3, in a tank containing blot buffer (Bio-Rad, USA). The membrane was blocked with 5% nonfat milk powder in TBST buffer for 2 hours, then washed three times with TBST (TBS buffer plus 0.05% Tween-20) for 5 minutes each to prevent nonspecific antibody binding. To detect the YncE protein, the membrane was incubated with an anti-His tag antibody at a 1:1000 dilution for 1 hour at 37 °C. After three washes of 10 minutes each, the blot was visualized using DAB (3, 3'-diaminobenzidine). The substrate

solution was prepared by dissolving DAB in 5 mL of deionized water (Sigma, Germany), and the reaction was stopped by rinsing the membrane twice with distilled water (22, 23).

Encapsulation of YncE Recombinant Protein in Chitosan Nanoparticles

Chitosan (50 mg) was dissolved in 25 mL of 2% acetic acid, and 50 mL of distilled water was added. The mixture was stirred for 1 h to ensure complete dissolution. The YncE recombinant protein solution (1 mg/mL) was added dropwise to 7.5 mL of the chitosan solution over 12 min with continuous stirring. Subsequently, 5 mL of 1 mg/mL sodium tripolyphosphate (TPP) solution was added dropwise over 5 min. The mixture was then sonicated for five cycles of 21 s each. Nanoparticles were collected by centrifugation at 13,000 rpm for 30 min, and the supernatant was retained for quantification of free YncE protein (24). Encapsulation efficiency was determined by measuring the residual YncE protein concentration in 100 μ L of the supernatant using the Bradford assay with BSA as the standard (25). The particle size (Z-average), polydispersity index (PDI), and zeta potential of the YncE-loaded chitosan nanoparticles were determined by dynamic light scattering (DLS) and laser Doppler electrophoresis using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Samples were diluted in double-distilled water for size and PDI measurements, and in 10 mM NaCl for zeta potential measurements, with three measurements per sample.

Oral and Injectable Immunization in Animal Models

Twenty-five BALB/c mice were divided into five groups of five animals each: 1) recombinant YncE protein injection group, 2) nanoparticle injection group, 3) oral nanoparticle group, 4) combined oral-injection nanoparticle group, and 5) control group (PBS).

Group 1 received a subcutaneous injection of 15 μ g of purified recombinant protein

with complete Freund's adjuvant (CFA) for the first injection, while the second and third injections used incomplete Freund's adjuvant (IFA), all administered at 14-day intervals. The lower injectable dose was sufficient due to the potent adjuvant effect of CFA, which enhances antigen immunogenicity and reduces the required antigen quantity.

Mice in Group 2 were immunized via injection with chitosan nanoparticles encapsulating 15 μ g of recombinant YncE protein, administered in three doses at 14-day intervals.

Group 3 received oral chitosan nanoparticles containing 100 μ g of encapsulated YncE protein by gavage at 7-day intervals over 5 weeks.

Group 4 received a combined regimen of oral nanoparticle administration by gavage at 7-day intervals followed by a subcutaneous nanoparticle injection at week 1. The control group received an injection and oral dose of PBS.

Titration of IgG and IgA Antibodies by ELISA

Blood samples were collected to determine IgG and IgA antibody titers in serum and feces after each administration. Anti-YncE antibody titers were measured using the specific mouse IgG and IgA ELISA kit (BioCheck, USA) with anti-mouse horseradish peroxidase (HRP)-conjugated IgG at a 1:5000 dilution. Serum and fecal IgA titers were determined using an indirect ELISA kit (BioCheck, USA) and HRP-conjugated anti-IgA at a 1:8000 dilution. For fecal samples 0.5g of fresh feces was suspended in 1 mL of PBS. The optical density at 492 nm of experimental and control samples was measured using an ELISA reader. Endpoint titers were defined as the highest serum dilution yielding an OD value above the cutoff calculated as the mean OD of the negative controls plus three standard deviations (mean + 3 SD). Area under the curve (AUC) values were calculated for quantitative comparison of antibody responses among the samples.

Immunized Mice Challenge Assay

Two weeks after the final immunization, mice were challenged by intraperitoneal (IP) injection of 0.5 mL of PBS containing 1×10^8 CFU of ETEC (strain C83902) (K88ac: LT⁺, STa⁺). The (IP) challenge model was selected to establish a standardized lethal challenge, as oral ETEC challenge in adult mice does not reliably produce consistent mortality (12). The IP route enabled evaluation of systemic protective immunity as an initial screening step.

Statistical Analysis

Three independent experiments were performed three times, and results are presented as means $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple group comparisons. A p-value of less than 0.05 was considered statistically significant. For survival analysis, the log-rank (Mantel-Cox) test was used to compare survival distributions between groups.

RESULTS

Confirmation of Recombinant Constructs Using PCR, Extraction, and Digestion of Recombinant pET28a-YncE

The pET28a-YncE plasmid was extracted

from *E. coli* DH5 α . PCR was performed using T7 vector-specific primers to confirm the presence of the YncE insert (Fig. 1A). Subsequently, pET28a-YncE was transformed into *E. coli* BL21 (DE3) for protein expression. To verify successful transfer of the plasmid into the expression host, colony PCR was conducted using T7 vector-specific primers, followed by restriction digestion with EcoRI and HindIII (Fig. 1B, C). Amplified products were visualized on a 1% agarose gel. The PCR product migrated at approximately 1300 bp, and restriction digestion released the expected 972 bp insert, confirming successful construction of pET28a-YncE.

Expression and Purification of the YncE Recombinant Protein

The recombinant YncE protein expression was analyzed using 12% SDS-PAGE. A band of approximately 41 kDa was observed in IPTG-induced *E. coli* BL21(DE3) harboring pET28a-YncE, and its intensity increased with induction time (2, 4, and 16 h), while the band was absent in the uninduced sample (Fig. 2A). This band is consistent with the expected size of the His-tagged YncE construct (calculated molecular weight: 41 kDa). Purification was performed using Ni-NTA column chromatography, and the presence of the YncE protein was confirmed by SDS-PAGE on a 12% gel (Fig. 2B).

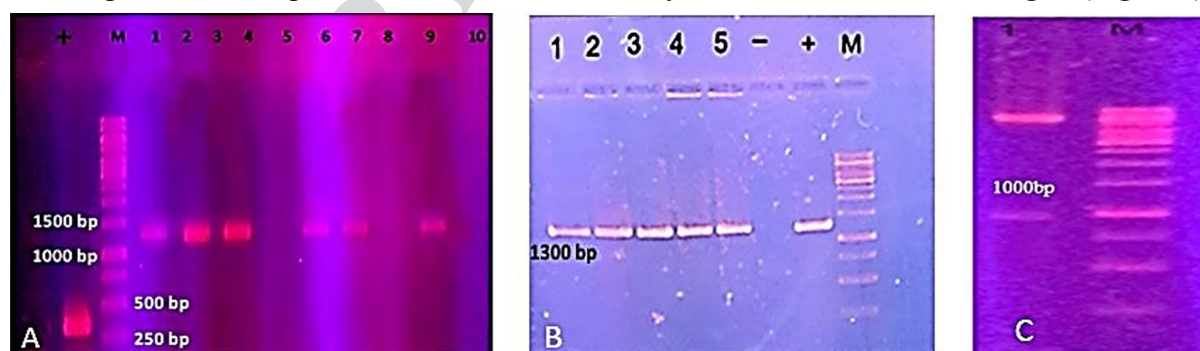


Fig. 1. Molecular analysis of the pET28a-YncE plasmid in host bacteria (a: *E. coli* DH5 α and c: *E. coli* BL21(DE3)). A, B) PCR amplification of the pET28a-YncE construct using T7 promoter and terminator primers. The expected product size for pET28a-YncE is approximately 1300bp. A) Lane M, DNA ladder; Lanes 1-10, pET28a-YncE plasmid extracted from *E. coli* DH5 α ; negative control, empty pET28a plasmid. B) Lanes 1-5, pET28a-YncE plasmid from *E. coli* BL21(DE3); negative control, *E. coli* BL21(DE3) without pET28a; Lane M, DNA ladder. C) Restriction digestion of pET28a-YncE with EcoRI and HindIII, Lane 1, the released YncE insert (approximately 972 bp) and the linearized pET28a vector (approximately 5.4 kb); Lane M, DNA ladder.

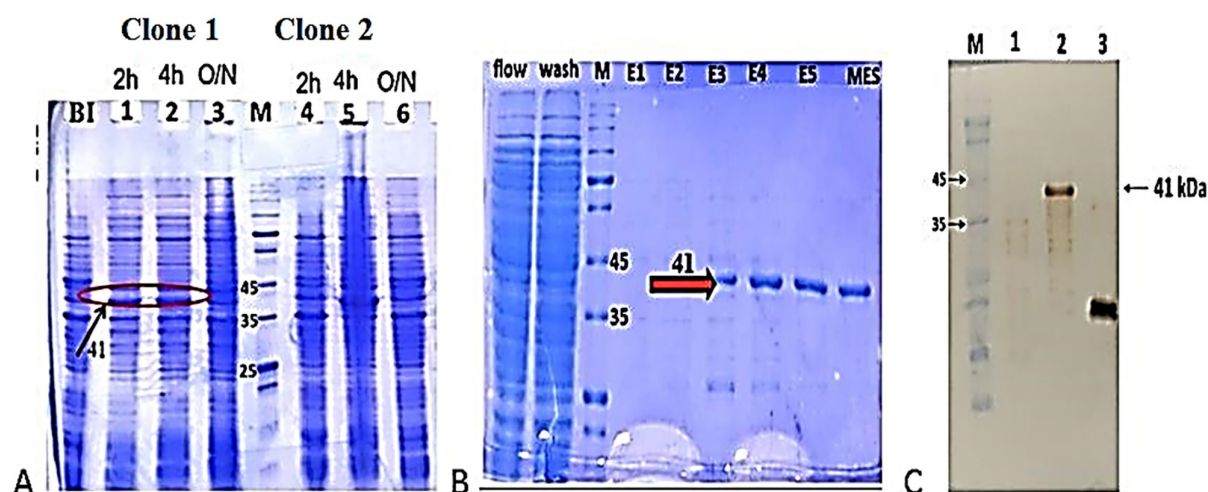


Fig. 2. Expression and purification of recombinant YncE protein analyzed by 12% SDS-PAGE. A) Lane BI, before induction; Lane M, protein ladder; Lanes 1 and 2, two independent colonies of *E. coli* BL21(DE3) containing the recombinant YncE gene; lane 1, sample induced with IPTG for 2 h; lane 2, sample induced with IPTG for 4 h; lane 3, sample induced with IPTG for 16 h. The expected YncE band is indicated at approximately 41 kDa. B) Purification of YncE protein by Ni-NTA column chromatography. Lanes 1-5 elution fractions showing a band of approximately 41 kDa. C) Western blot validation of YncE expression. Lane M, protein ladder; lane 1, before induction sample; lane 2, *E. coli* BL21(DE3) containing pET28a-YncE induced with 1 mM IPTG; lane 3, positive control (a recombinant protein with His tag).

Results

		Diam. (nm)	% Volume	Width (nm)
Z-Average (r.nm): 146.2	Peak 1:	91.55	100.0	16.28
Pdl: 0.399	Peak 2:	0.000	0.0	0.000
Intercept: 0.906	Peak 3:	0.000	0.0	0.000
Result quality Good				

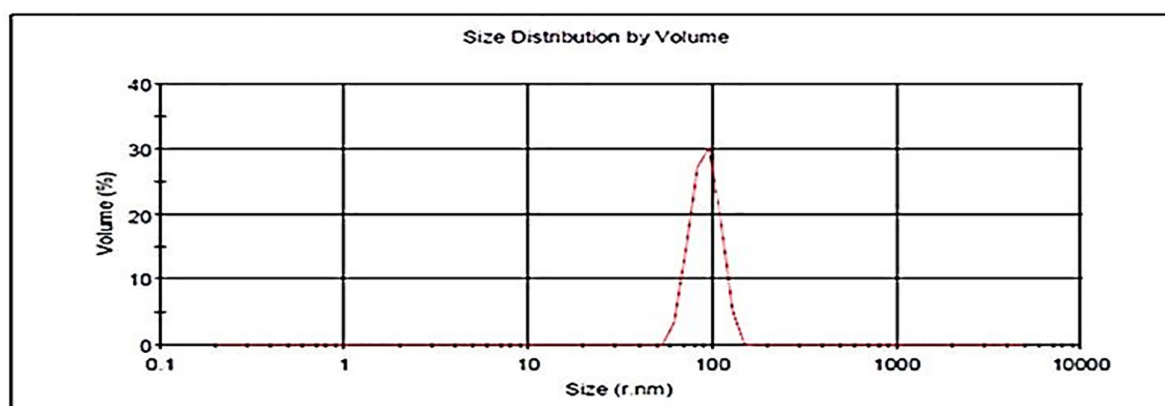


Fig. 3. Particle size characterization of chitosan nanoparticles encapsulating recombinant YncE protein determined by dynamic light scattering (DLS). The formulated nanoparticles displayed an average hydrodynamic diameter (Z-average) of 146.2 nm and a polydispersity index (PDI) of 0.399. The zeta potential was $+32.5 \pm 4.2$ mV. The PDI indicates a moderately polydisperse distribution, which is within the acceptable range for mucosal vaccine delivery systems. Measurements were performed in double-distilled water at 25°C for size and PDI, and in 10 mM NaCl for zeta potential.

Expression of the YncE protein was further validated by Western blot analysis, in which the induced sample and positive control showed the expected band, while the uninduced sample did not (Fig. 2C).

Evaluation of the Size of Chitosan Nanoparticles Containing the YncE Recombinant Protein

Zeta potential is an important parameter for assessing the stability of nanoparticle

surface charge. Chitosan is a suitable material for protein encapsulation due to its biocompatibility and cationic nature. In this study, the encapsulation efficiency of the YncE recombinant protein was 80%, and the Z-average size of the chitosan nanoparticles was 146.2 nm with a polydispersity index (PDI) of 0.399 (Fig. 3). The PDI indicates a

moderately polydisperse distribution, which is within the acceptable range for mucosal vaccine delivery systems. The zeta potential of the nanoparticles was determined to be $+32.5 \pm 4.2$ mV, indicating a positively charged surface that contributes to colloidal stability and facilitates interaction with negatively charged mucosal surfaces.

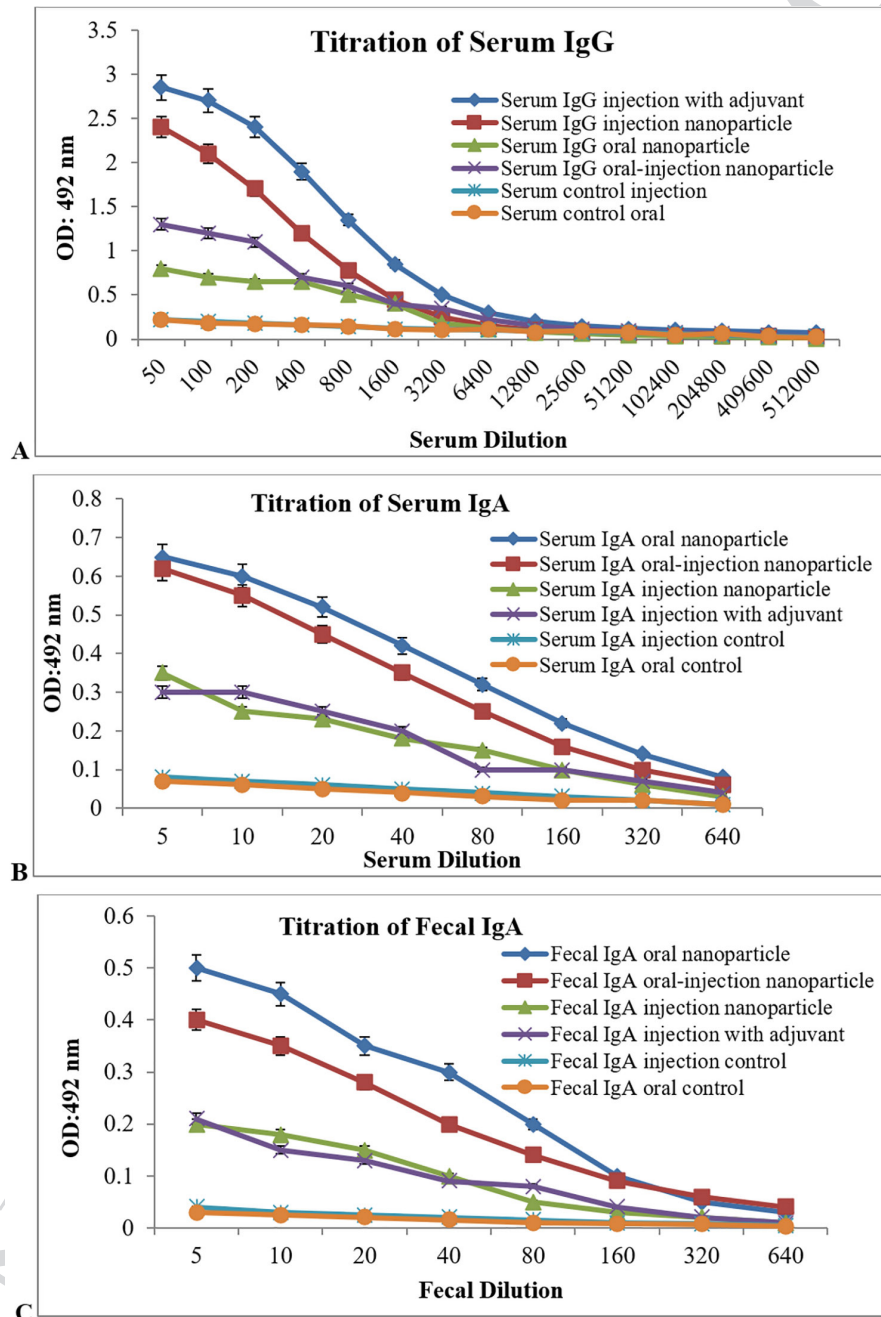


Fig. 4. Antibody titration in mice immunized with the YncE nanovaccine. A) Serum IgG antibody titers, with the highest titer in the group injected with recombinant YncE protein. B) Serum IgA titers, with the highest titer in the oral nanoparticle group. C) Fecal IgA titers, with the highest titer in the oral nanoparticle group. Data are presented as means $\pm$ SD ($n = 5$ per group). Statistical significance was determined by one-way ANOVA with Tukey's post hoc test. Asterisks indicate significant differences compared with the control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Immunogenicity of the YncE Recombinant Protein in Mice

To evaluate the stimulation of humoral and mucosal immunity by the YncE recombinant protein, five groups of BALB/c mice were immunized using different strategies: recombinant protein injection, nanoparticle injection, oral nanoparticle administration, combined oral and injection nanoparticle administration, and a control group. Serum ELISA results showed that the IgG antibody titer was highest in the YncE recombinant protein injection group compared to all other groups. Additionally, serum and fecal IgA antibody titers were highest in the oral nanoparticle group (Figs. 4A-C).

The endpoint titers for serum IgG were as follows: injection group (1:12,800), nanoparticle injection group (1:6,400), oral nanoparticle group (1:3,200), combined oral-injection group (1:6,400), and control group (<1:100). Serum IgA endpoint titers were:

oral nanoparticle group (1:3,200), combined oral-injection group (1:1,600), nanoparticle injection group (1:800), injection group (1:400), and control group (<1:100). Fecal IgA endpoint titers were: oral nanoparticle group (1:800), combined oral-injection group (1:400), nanoparticle injection group (1:200), injection group (1:100), and control group (<1:50).

One-way ANOVA with Tukey's post hoc test confirmed that the injection group had significantly higher IgG titers compared to all other groups ($p < 0.001$), while the oral nanoparticle group had significantly higher serum and fecal IgA titers compared to all other groups ($p < 0.01$).

Challenge Assay in Immunized Mice

Fourteen days after the final immunization, mice were challenged by intraperitoneal injection with 0.5 mL of PBS containing 1×10^8 CFU of ETEC (strain C83902) (K88ac:

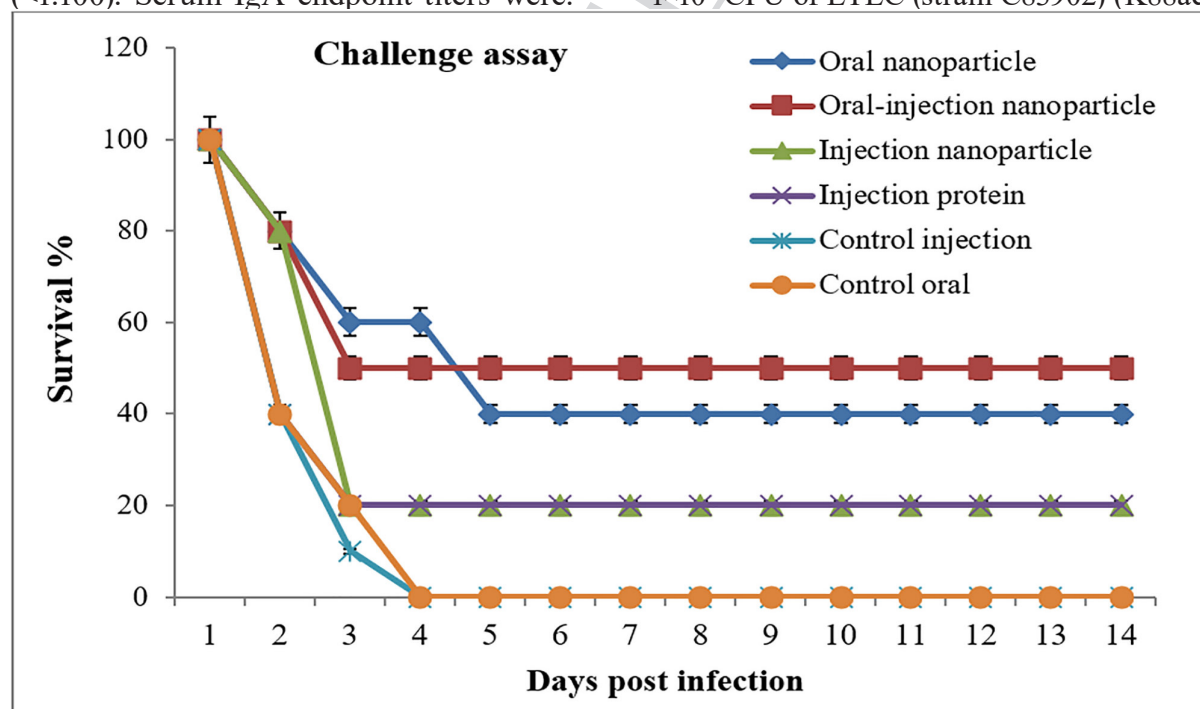


Fig. 5. Survival curves of BALB/c mice immunized with recombinant YncE protein or YncE-loaded chitosan nanoparticles following challenge with Enterotoxigenic *Escherichia coli* (ETEC) strain C83902 (K88ac: LT⁺, STa⁺). Mice ($n = 5$ per group) were challenged intraperitoneally with 1×10^8 CFU of ETEC 14 days after the final immunization and monitored for 14 days. Complete mortality was observed in the control group by day 4 post-challenge, whereas survival rates of 60%, 50%, 20% and 20% were observed in oral nanoparticle; combined oral-injection; recombinant protein and nanoparticle injection groups, respectively, indicating that YncE-based vaccination conferred partial protection against ETEC infection. Survival curves were compared using the log-rank (Mantel-Cox) test. $p < 0.05$ compared to the control group.

LT⁺, STa⁺). The groups were monitored for two weeks to assess their survival. Four days after challenge, 100% of the control mice had died. Survival rates were 50% in the combined YncE-oral-injection group, 60% in the oral nanoparticle group, and 20% in both the recombinant protein injection and nanoparticle injection groups (Fig. 5). Log-rank test analysis revealed statistically significant differences in survival between the control group and each immunized group ($p < 0.05$), indicating that YncE-based immunization conferred partial protection. However, no significant differences were observed between the immunized groups ($p > 0.05$).

DISCUSSION

In developing countries, mortality rates and gastrointestinal infections remain alarmingly high due to poverty, lack of access to safe drinking water, and inadequate sanitation infrastructure. Diarrhoeal disease was the third leading cause of death among children aged 1–59 months worldwide in 2019 (26, 27). In 2019, diarrhoea accounted for 9.1% of all under-5 deaths globally (26). To prevent the pathogenicity of enterotoxigenic *Escherichia coli* (ETEC), two key pathogenic factors must be controlled: first, the prevention of colonization factor binding to host cell surfaces, and second, the inhibition of toxin activity. Considerable attention has been directed toward developing subunit vaccines based on colonization factors (CFs) and toxins to combat ETEC-induced diarrhoeal disease (28–30). The YncE protein, conserved immunogenic antigen from the core *E. coli* genome, has emerged as a promising recombinant antigen candidate for inclusion in vaccines targeting multiple *E. coli* strains. Currently, no licensed recombinant subunit vaccine for ETEC-induced diarrhoeal disease has been commercialized, and the World Health Organization prioritizes the prevention and development of effective treatments for this condition (31–33). Subunit vaccines,

particularly oral formulations, hold significant promise for preventing gastrointestinal and intestinal infections (34).

Our results demonstrate that chitosan nanoparticles effectively deliver the YncE recombinant protein and induce both humoral and mucosal immune responses. The finding that IgG antibody titers were highest in the injection group was expected, as parenteral immunization with adjuvant typically elicits strong systemic antibody responses. Conversely, the highest IgA titers observed in the oral nanoparticle group confirm the capacity of oral chitosan nanovaccines to stimulate mucosal immunity, consistent with the role of IgA as the primary immunoglobulin isotype at mucosal surfaces (35, 36). This differential antibody profile is significant because it suggests that the route of immunization can be tailored to achieve specific immune outcomes: systemic immunity via injection, or mucosal immunity via oral administration.

The protection rates observed in the challenge assay indicate partial but significant protection against ETEC challenge. The combined oral-injection group showed the highest survival (50%), followed by the oral nanoparticle group (40%) and the recombinant protein and nanoparticle injection groups (20% each). The lower survival in the nanoparticle injection group suggests that while injectable nanovaccines elicit systemic immune responses, they may be less effective than oral formulations in generating protective immunity, possibly due to the absence of strong mucosal IgA induction, although other factors such as antigen release kinetics and limited sample size ($n = 5$ per group) cannot be excluded. The complete mortality observed in the control group confirms the lethality of the IP challenge model and supports the protective effect of YncE-based immunization. The complete mortality observed in the control group confirms the lethality of the IP challenge model and supports the protective effect of YncE-based immunization. Compared to previous studies,

our 40% survival rate is in line with findings by Ghaffari Marandi et al. (2019), who reported partial protection using an LSC chimeric protein vaccine encapsulated in chitosan nanoparticles (37). This similarity suggests that YncE is a promising antigen candidate comparable to other known protective antigens. However, the partial protection also indicates that combination strategies such as incorporating additional CF antigens or toxins may be necessary to achieve higher efficacy. For example, Svennerholm (2011) developed an oral ETEC vaccine comprising recombinant cholera toxin B subunit (rCTB) and formalin-inactivated *E. coli* bacteria expressing major CFs. This vaccine demonstrated immunogenicity in both adults and children (38). In a 2012 study, Tobias & Svennerholm investigated the production of specific monoclonal antibodies against CF subunits. Oral immunization with inactivated recombinant bacteria overexpressing key CFs of the enterotoxigenic strain, under the control of the strong Tac promoter, elicited significantly higher antibody responses compared to immunization with previously used vaccine strains (39). Furthermore, Lundgren et al. (2014) designed a vaccine targeting major ETEC CFs-CFA/I, CS3, CS5, and CS6-combined with an LTB-related toxoid (LCTBA). Vaccine recipients exhibited significant mucosal IgA responses, with approximately 90% of all vaccinees responding to at least four antigens (40). Ramakrishnan et al. (2021) utilized a CS6+ ETEC strain, B7A, in an *Aotus nancymae* non-human primate model to evaluate the immunogenicity and protective efficacy of a recombinant CS6-based subunit vaccine. Vaccination with the dscC_{ss}BA + dmLT induced robust serum antibody responses, and following challenge, none of the immunized animals developed diarrhea (41).

In the present study, the YncE recombinant antigen was designed. Our results demonstrated enhanced stimulation of both humoral and mucosal immune responses following oral and injectable administration

of the YncE recombinant protein and chitosan nanoparticles containing the YncE protein in an animal model. Chitosan nanovaccines effectively enhance mucosal immune responses and serve as efficient carrier systems (42, 43). Additionally, a study by Carroll (2016) showed that chitosan nanoparticles activate dendritic cells and elicit a strong immune response (44). Three critical characteristics of chitosan nanoparticles are their size, the amount of protein loaded, and their overall quality. In this study, the loading efficiency of the YncE recombinant protein was 90.3%, and the nanoparticle size was 146.2 nm with a zeta potential of $+32.5 \pm 4.2$ mV. Both the loading efficiency and nanoparticle size demonstrated good quality compared to other studies. For instance, protein loading efficiencies reported in experiments by Mirzaei Tabrizi et al. (2018) (45) and Ghaffari Marandi et al. (2019) (37) were 88% and 90%, respectively. Our ELISA results specifically measured anti-YncE antibodies, confirming the specificity of the immune response to the YncE antigen. Chitosan itself is known to possess immunostimulatory properties (42, 44); therefore, it is possible that chitosan contributed to the overall magnitude of the immune response. The primary objective of the current study, after producing, confirming, purifying, and encapsulating the YncE recombinant protein in chitosan nanoparticles, was to evaluate its immunogenicity following injection, oral, and combined oral-injection administration in mice. ELISA results showed that IgG and IgA antibody titers were generated in all immunized groups. The highest IgG antibody levels were observed in the group receiving the YncE recombinant protein via injection. Conversely, the highest IgA levels were detected in the oral administration group, consistent with the fact that IgA is the predominant immunoglobulin isotype in the intestinal mucosa (35, 36). Consistent with previous studies, our results revealed that IgG antibody titers were highest in the injection group, while IgA antibody titers were highest

in the oral groups (46).

In the present research, immunized mice were challenged with enterotoxigenic *E. coli* (ETEC) 14 days post-immunization. After the challenge, survival rates were 40% in the oral nanoparticle group, 20% in the recombinant protein injection group, and nanoparticle injection group. All mice in the control group succumbed. Another study immunizing mice with a chimeric protein containing multiple toxin subunits and various colonization factors (CFs) reported approximately 70% survival rate in immunized mice against bacterial challenge (47). The bacterial challenge was performed via intraperitoneal (IP) injection rather than the natural oral route. This approach was chosen because oral ETEC challenge in adult mice does not reliably produce consistent mortality, as mice are not the natural host for ETEC and the bacterium does not efficiently colonize the murine intestine. The IP model enabled us to evaluate systemic protective immunity induced by the vaccine as an initial screening step. However, we acknowledge that this model does not fully replicate the natural gastrointestinal pathophysiology of ETEC infection. Therefore, the observed protection likely reflects systemic immune-mediated defense against bacterial dissemination rather than intestinal mucosal protection.

CONCLUSION

In conclusion, the YncE protein was encapsulated in chitosan nanoparticles as a vaccine candidate. This protein not only stimulated humoral and mucosal immune responses, producing anti-YncE antibodies of the IgG and IgA immunoglobulin classes, but also demonstrated partial protective efficacy in bacterial challenge studies. Therefore, the YncE protein appears to be a promising candidate for the design and development of vaccines against intestinal diseases caused by this bacterium and other *E. coli* strains that harbor the conserved YncE gene.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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