JOURNAL OF APPLIED BIOANALYSIS, December 2025, p. 112-121
http://doi.org/10.53555/jab.v11si4.933 (ISSN 2405-710X)
Vol. 11, No. 4s

OPEN ACCESS

Development and Immunological Evaluation of O Specific Antigen from Salmonella enterica Serovar Paratyphi B: A Novel Candidate for Vaccine Development

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Abstract

Enteric fever, resulting from different Salmonella species such as S. typhi and S. paratyphi A and B, continues to pose a major public health challenge in developing countries. Although Vi polysaccharide-based vaccines offer effective protection against S. typhi, they fail to safeguard against S. paratyphi B because these serovars lack the Vi antigen. Currently, there is no licensed vaccine available for Salmonella Paratyphi B, highlighting a critical gap in preventive measures against this pathogen. To address this issue, researchers have focused on the O antigen, a crucial component of Gram-negative bacteria, as a potential vaccine candidate. The investigation outlined in the abstract focuses on the extraction, refinement, and evaluation of the immunogenic properties of the O antigen derived from Salmonella paratyphi B using a murine model. The research encompasses large-scale production, purification, and analytical characterization of the S. paratyphi B O-antigen. The purified antigen demonstrated high immunogenicity in the mouse model, suggesting its potential as a novel vaccine candidate. This research contributes to the development of a future paratyphoid fever vaccine, potentially addressing the unmet need for protection against S. paratyphi B infections.

Keywords: O-Specific Ag, Salmonella Typhi, S. paratyphi B, lipopolysaccharide, vaccine candidate

Introduction

Enteric fever, which includes typhoid and paratyphoid fever, continues to be a major global health issue, especially in low- and middle-income nations. The illnesses are attributed to Salmonella enterica serovars Typhi and Paratyphi A, B, and C, respectively. [2] The transmission of these pathogens primarily occurs via the fecal-oral route, frequently as a result of contaminated water and food sources. The World Health Organization (WHO) estimates that typhoid fever impacts 11-20 million individuals each year, leading to around 128,000-161,000 fatalities. Every year, typhoid disease affects 11 to 20 million individuals and causes death in about 128,000 to 161,000 of them Paratyphoid fever, while less prevalent, still contributes significantly for overall impact of enteric fever [3,6,10]. The epidemiology of enteric fever is closely linked to socioeconomic factors and public health infrastructure. Regions lack of proper sanitation, poor availability of clean and pure water, and lack of cleanliness are particularly vulnerable. South Asia, Southeast Asia, and sub-Saharan Africa experience the highest impact from disease. In these endemic areas, children and young adults are disproportionately affected, with long-term consequences on health, education, and economic productivity. The clinical presentation of enteric fever can vary from mild to severe, making diagnosis challenging. Common symptoms include sever fever, headache, stomach pain, and problem with the gastrointestinal system. In acute cases, complications such as intestinal perforation, encephalopathy, and myocarditis can occur. The gold standard for diagnosis remains blood culture, but its sensitivity is limited, particularly at the beginning of an infection or after taking antibiotics. This has led to the development of alternative diagnostic methods, including serological tests and molecular techniques, to improve detection rates.

The treatment of enteric fever is increasingly complicated due to the rise of antimicrobial resistance. Instances of multidrug-resistant and extensively drug-resistant strains of S. Typhi have been recorded in various regions around the world, especially in South Asia. The presence of these resistant strains complicates effective treatment for individuals and highlights the critical necessity for innovative therapeutic strategies and preventive actions. Vaccination is essential for preventing and

managing enteric fever. The creation of vaccines for these illnesses has a lengthy and intricate background. The earliest vaccines, such as the whole-cell killed typhoid vaccine, provided some protection but were associated with significant side effects. The advent of Vi polysaccharide vaccines in the 1990s marked a significant improvement, offering better tolerability and efficacy against S. Typhi infections. However, these vaccines protect against S. Paratyphi strains, particularly S. Paratyphi B, which lacks the Vi antigen. The limitations of current vaccines have spurred research into new vaccine candidates. One promising approach focuses on the O-specific polysaccharide (OSP) of the bacterial lipopolysaccharide (LPS). The OSP is a key virulence factor and a significant focus of the host immune system response. Vaccines based on OSP conjugates have shown potential in preclinical studies, offering the possibility of broader protection against multiple Salmonella serovars, including S. Paratyphi B. The development of OSP-based vaccines involves several critical steps. First, the cultivation of Salmonella strains under controlled conditions to optimize OSP production. This is followed by extraction and purification processes, which often involve acid hydrolysis to separate the OSP from the lipid A component of LPS. Advanced chromatographic techniques are then employed to further purify the OSP and ensure its quality and consistency.

Characterization of the purified OSP is a crucial step in vaccine development. Additionally, immunological assays were performed to assess the antigenicity and specificity of the purified OSP. The next phase in vaccine development involves conjugation of the OSP to a carrier protein. This step is crucial for enhancing the immunogenicity of the polysaccharide, especially in young children with compromised immune systems might not react properly to polysaccharide antigens solely. Common carrier proteins include tetanus toxoid, diphtheria toxoid, and CRM197, a non-toxic mutant of diphtheria toxin. Preclinical evaluation of OSP-based vaccines typically begins with immunogenicity studies in animal models, most commonly mice. These studies assess the ability of the vaccine to induce specific antibodies against the OSP. Enzyme-linked immunosorbent assays (ELISA) are used to measure antibody titers. [11,12] Challenge studies in animal models are a critical component of vaccine evaluation. These studies involve immunizing animals with the candidate vaccine and subsequently exposing them to virulent Salmonella strains. The level of protection conferred by the vaccine is assessed by monitoring survival rates, bacterial loads in various organs, and other parameters. The development of OSP-based vaccines for enteric fever, particularly those targeting S. Paratyphi B, represents a promising avenue for addressing the current gaps in prevention. By focusing on a conserved and immunologically relevant antigen, these vaccines have the potential to provide broader protection against multiple Salmonella serovars. However, significant challenges remain, including the need for large-scale production methods, optimization of vaccine formulations, and comprehensive clinical trials to establish safety and efficacy in human populations. In conclusion, the ongoing research into OSP-based vaccines for enteric fever, including S. Paratyphi B, reflects the continued commitment of the scientific community to addressing this significant global health challenge. As these efforts progress, they hold the promise of developing more effective and comprehensive vaccines that could significantly reduce the burden of enteric fever worldwide.

Materials and Methods

Cultivation of strain & media preparation

Salmonella Paratyphi B strain NCTC 10412 was used in this study. The Soybean Casein Digest Medium (SCDM) (Himedia,#M011) was used for growth as well as production of the polysaccharide. The solution was produced by dissolving 30 g of SCDM in 1 L purified water and autoclaved for 15 min at 121 °C, the sterilized medium was used for further studies. To start the culture revival, 1.0 mL of WCB was added onto 50 mL of sterile SCDM and then incubation was done for 5 h at 35 °C and 200 rpm in an orbital shaker. By determined the optical density of the culture at 600 nm (OD600nm) in an UV–visible spectrophotometer (Shimadzu UV-1900), the growth of S. paratyphi B was measured over a predetermined period of time. The 50 mL of pre-seed culture was transferred to 800 mL of SCDM in 2 L flask. Fermentation was carried out up to 18 hours, samples were withdrawn in specific time intervals and growth patterns were recorded. After fermentation the culture was inactivated, purified and kept at 2-8 °C till needed for subsequent applications.

The fermentation process was scaled up by transferring 50 mL of the pre-seed culture to 800 mL of SCDM in a 2 L flask. The fermentation was carried out for 18 hours, with samples collected at specific time intervals to record growth patterns. Following fermentation, the culture underwent inactivation and purification processes. The resulting product was stored at 2-8 °C for future use. This detailed approach to strain cultivation and media preparation ensures optimal growth conditions and polysaccharide production for further studies involving Salmonella Paratyphi B.

Culture inactivation and O-antigen purification

The harvested culture was hydrolysed with acetic acid (1 % v/v) and incubated at 95 °C for 3h. To

obtain a pH of 6.5 ± 0.2, 25% of NH_4OH was added. For the extraction of supernatant, centrifuged of the mixture carried out at 11,000 g for 30 minutes at 4°C. A 30 kDa Sartorius Hydrosart 0.1 m² cassette was used to concentrate the supernatant eight times in order to remove impurities. The concentrate was diafiltered 10 times against 1 M NaCl, followed by 10 diafiltration cycles with water for injection (WFI).

The diafiltered material was mixed constantly at room temperature with a citrate buffer at pH 2.8 at a concentration of 20 mM until a precipitate developed, which produced a final pH of 3.0 ± 0.2. The solution was then centrifuged with 10,000 rpm speed at 10°C temperature for ten minutes after being allowed to settle at ambient temperature for 30 ± 5 minutes. Filtration of supernatant was done with 0.2 μm filter.

Cation exchange chromatography was used to further purify the collected supernatant using SP Sepharose (Cytiva) resin that was equilibrated previously using sodium citrate buffer (20 mM, pH3.0). The O-antigen-containing fraction was collected in the flow-through. To this fraction, 18 mM Na_2HPO_4, 20% (v/v) absolute ethanol, and 200 mM $CaCl_2$ were added, stirring of the mixture was carried out continuously at ambient temperature for about 30 ± 5 min. After centrifugation, the mixture for 30 minutes at 10 °C at 10,000 rpm, the supernatant was collected. The collected supernatant was tenfold concentrated by using 30 kDa Sartorius Hydrosart 0.1 m² cassette and diafiltered 20 times with 0.02 M phosphate buffer having pH 7.4. A 0.22 μm filter was used to filter the final purified O-antigen solution, which was subsequently stored at 4°C. The protein concentration of the purified samples was quantified via Bicinchoninic acid assay method employing Bovine serum albumin as the reference standard and measured the OD at 562 nm and run the sample in duplicate.

Estimation of Nucleic acid

Nucleic acid analysis used UV spectroscopy at 260 nm is a widely employed method for quantifying DNA and RNA concentrations in biological samples. This technique exploits the characteristic absorption of nucleic acids at this wavelength, primarily due to the presence of purine and pyrimidine bases. The Shimadzu UV-1900 spectrophotometer, utilized in this analysis, offers high precision and reliability for such measurements.

The principle behind this method relies on the Beer-Lambert law, which establishes a linear relationship between absorbance and concentration. For double-stranded DNA, an optical density (OD) of 1.0 at 260 nm corresponds to a concentration of approximately 50 μg/mL. This relationship serves as a standard for estimating nucleic acid concentrations in unknown samples. To ensure accuracy and reproducibility, measurements were typically performed in duplicate. This practice allows for the detection of potential errors or inconsistencies in sample preparation or instrument performance, thereby enhancing the reliability of the results obtained from the nucleic acid analysis.

Molecular size estimation by HPLC-SEC method

HPLC-SEC with UV detection is a powerful analytical technique for determining molecular size distribution and assessing the purity of polysaccharide samples. This method uses the size-based fractionation principle of size exclusion chromatography and the separation power of high-performance liquid chromatography. In the described setup, a TSK-GEL G3000PWXL column from Tosoh Bioscience was employed to separate the purified O-antigen samples based on their molecular size. The mobile phase, consisting of 100 mM NaCl in 0.5 M phosphate buffer, was used to elute the samples under isocratic conditions with 0.5 mL/min rate of flow for 35 minutes.

The use of a UV detector set at 214 nm allows for the detection and quantification of eluted polysaccharide molecules. This wavelength is particularly suitable for detecting peptide bonds and other chemical groups commonly found in polysaccharides. By analyzing the elution profile of chromatogram can determined the molecular size distribution of the purified O-antigen and confirm the absence of degradation products. This information is crucial for ensuring the quality and integrity of the polysaccharide samples, which is important for many uses in areas like glycobiology, immunology, and vaccine development.

Estimation of polysaccharide purity by SDS-PAGE method:

The SDS-PAGE method is a widely used technique for estimating polysaccharide purity and assessing the effectiveness of various purification steps prior to immunogenicity studies. This method involved the separation of proteins based on their molecular weight using polyacrylamide gel electrophoresis. The process begun with sample preparation, where the polysaccharide samples were mixed with β-mercaptoethanol and a sample buffer containing SDS, glycerol, and other components. The samples were then heated to denature the proteins, ensuring they were in their linear form for accurate separation.

The protein separation was carried out using 4-12% Tris-Bis gels, which provide a gradient for optimal resolution of proteins with different molecular

weights. The electrophoresis was performed using a Tris-Bis SDS running buffer at a constant voltage for a specified duration. After that, the gel was stained with the dye Coomassie Brilliant Blue (CBB) R-250, a dye that bound to proteins, allowed for visualization of the separated protein bands. The gel was then destained for removing extra unnecessary dye and increase the clarity of the protein bands. This method allowed to assess the purity of the O-antigen by examined the presence or absence of contaminated proteins, provided valuable information about the success of the purification process.

Dosage strength optimization study:

The dosage strength optimization study aimed to assess the immunogenic potential of the finished product using female BALB/c mice as the animal model. The experiment involved six mice per group, ensuring a sufficient sample size for statistical analysis. The intramuscular injection route was chosen for administering the immunizations, likely due to its effectiveness in eliciting a robust immune response. The study design incorporated a two-dose immunization schedule, with injections given on days 0 and 7, allowing for a prime-boost strategy to enhance the immune response.

Three different dosage levels of pure O-antigen were evaluated: 5, 10, and 25 µg. This range of doses was likely selected to determine the optimal concentration that would elicit the strongest immune response while minimizing potential side effects or waste of material. Blood samples were collected from the mice on days 28 and 42 post-immunization, providing two time points to assess the development and persistence of the immune response. The serum extracted from these blood samples was preserved at −20 °C to maintain the integrity of the antibodies. Subsequently, an indirect ELISA (enzyme-linked immunosorbent assay) was employed to analyze the serum samples for specific antibody reactions, allowing for quantification of the immune response generated by each dosage level of the O-antigen.

Screening for Antigenicity of Isolated O ag. Protein by Indirect ELISA

The expanded text describes a detailed protocol for an Indirect Enzyme-Linked Immunosorbent Assay (ELISA) to screen for the antigenicity of isolated O-antigen. This method is used to measure anti-O-antigen antibody levels in mice serum collected on days 28 and 42. The process begins with antigen coating, where purified O-antigen is applied to a 96-well plate using an antigen coating carbonate-bicarbonate buffer. After overnight incubation, the plates undergo a series of washing and blocking steps to prevent non-specific binding. Serum samples at various dilutions done, then added to the wells and incubated, followed by the addition of a secondary antibody conjugated with horseradish peroxidase (HRP).

The final steps of the ELISA involve the addition of a substrate (TMB) that reacts with the HRP enzyme to produce a colored product. The reaction is stopped using sulfuric acid, and the absorbance is measured using a plate reader at 450 nm, with a reference wavelength of 620 nm. The intensity of the color and the corresponding absorbance value directly correlate with the amount of specific antibodies present in the sample, allowed for quantification of the immune response to the O-antigen. This method provides a sensitive and specific way to evaluate the antigenicity of the isolated O-antigen and assess the effectiveness of potential vaccine candidates or immunological responses in experimental settings.

Results

Bacteria cultivation in Soybean Casein Digest Medium (SCDM) was optimized to minimize external biological contamination. The culture incubated at 35 °C and 200 rpm for 18 hours yielded the highest optical density (OD) at 600 nm, reaching 0.773 nm, which surpassed all other studied conditions. This optimized cultivation process ensured the production of a robust bacterial population for subsequent purification steps.

The harvested culture supernatant underwent a series of purification steps as outlined in the flow chart **(Figures 1 and 2).** This systematic purification process was crucial for isolating the O-antigen (O-ag) from other cellular components. The final purified O-ag was subjected to various analytical tests to assess its quality and composition.

Protein content in the purified O-ag was determined using the bicinchoninic acid (BCA) method, revealing a concentration of 0.68 mg/mL. This relatively low protein content indicates effective removal of cellular proteins during the purification process. Nucleic acid content was measured at 38.04 µg/mL, suggesting minimal DNA/RNA contamination. The O-acetyl content, an important structural feature of many O-antigens, was quantified at 0.115 µmol/mL **(Table 1).** These biochemical analyses provide a comprehensive profile of the purified O-ag, confirming its purity and structural integrity.

SDS-PAGE analysis of the purified O-ag obtained through different purification strategies revealed a heterogeneous population of molecules. The gel electrophoresis showed distinct bands at approximately 75 kDa and 37 kDa (Figure 3A),

indicating the presence of both high- and low-molecular-weight species. This molecular weight distribution is typical for O-antigens, which often exist as polymers of varying lengths.

To further characterize the molecular weight distribution and assess the purity of the O-ag preparation, high-performance liquid chromatography–size exclusion chromatography (HPLC–SEC) was performed using a TSK-GEL G3000PWXL column (Tosoh Bioscience). The chromatogram showed two main peaks with retention times of approximately 11 minutes and 14 minutes, corresponding to the high- and low-molecular-weight species, respectively (Figure 3B). Importantly, the absence of additional peaks or shoulders in the chromatogram confirmed the lack of degradation products or contaminating saccharide fragments. This result validates the effectiveness of the purification process in yielding a homogeneous O-ag preparation suitable for immunological studies. The purified and well-characterized O-ag was subsequently used in immunogenicity studies to evaluate its potential as a vaccine candidate. Doses of 5 µg, 10 µg and 25 µg were administered to test subjects, with a control group receiving a placebo. The immune response was monitored over time, with significant results observed by day 28. Both the 10 µg and 25 µg doses induced higher antibody titers compared to the control group, demonstrating the immunogenicity of the purified O-ag.

Interestingly, by day 42, the antibody titers showed a gradual decline (Figure 4). This observation suggests that while the O-ag is capable of eliciting an immune response, the durability of this response may be limited. This finding has important implications for potential vaccine development, indicating that multiple doses or the inclusion of adjuvants might be necessary to maintain long-term immunity.

In conclusion, the optimized cultivation conditions, coupled with the rigorous purification and characterization processes, yielded a high-quality O-ag preparation. The immunogenicity studies provide promising initial results, demonstrating the potential of this O-ag as a vaccine candidate. However, the observed decline in antibody titers over time highlights the need for further research to enhance the longevity of the immune response. These findings lay the groundwork for future studies aimed at developing an effective vaccine against the target pathogen.

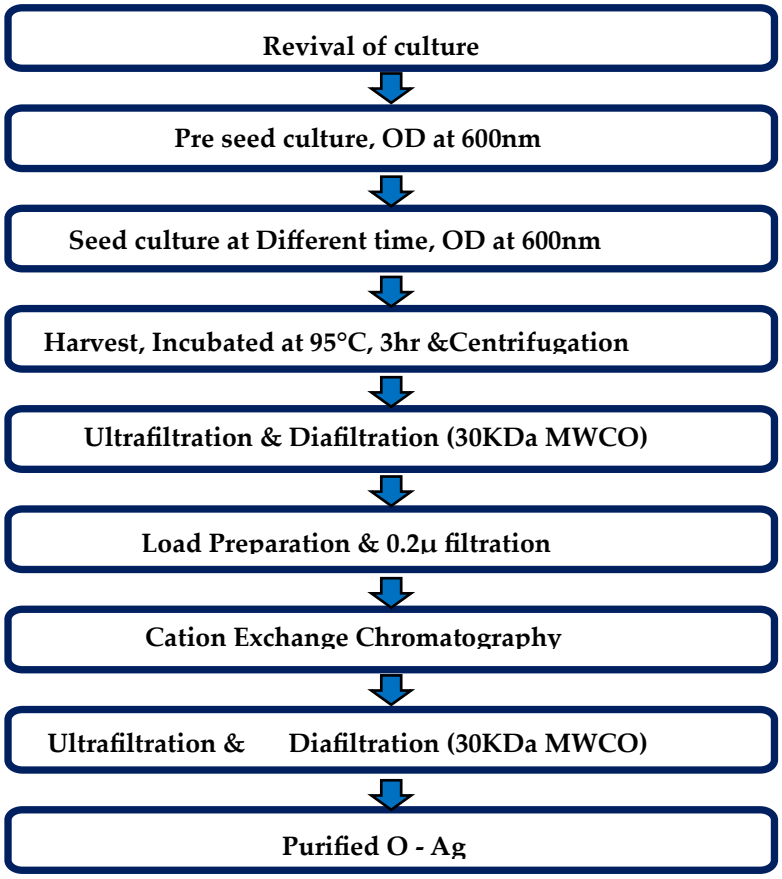

Figure 1. Schematic representation of the O-antigen O-Ag purification process from Salmonella Paratyphi B.

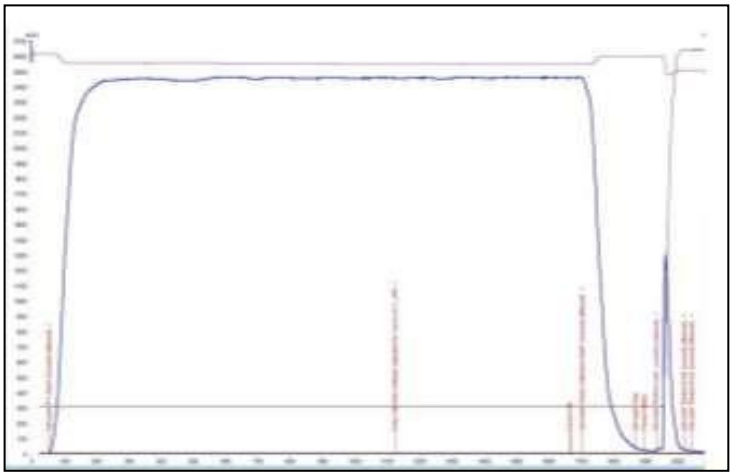

Figure 2. Chromatogram of SP sepharose chromatography, Elution buffer contains 20mM sodium citrate contain 1 M NaCl and pH 3.0, Column Type- XK 26/30 cytiva, Column volume-100ml, Packing Flow Rate- 13ml/min, Running Flow rate – 10ml/min

Table 1: characteristics of purified O-Ag sample by different test methods; Protein content by BCA assay, Nucleic acid content, O acetyl content, Purity by SDS-PAGE and SEC-HPLC and Immunogenicity determination by Indirect ELISA method

	Purified O- Ag Sample
Tests	**Results**
Protein Content	0.68 mg/ml
Nucleic acid content	38.04 μg/mL
O acetyl content	0.115 μmol/mL
Purity	target protein was present
SEC-HPLC	Peak was detectable at 11 min and 14 min, no Impurities observed
ELISA	Antibody titre was four fold higher than control group

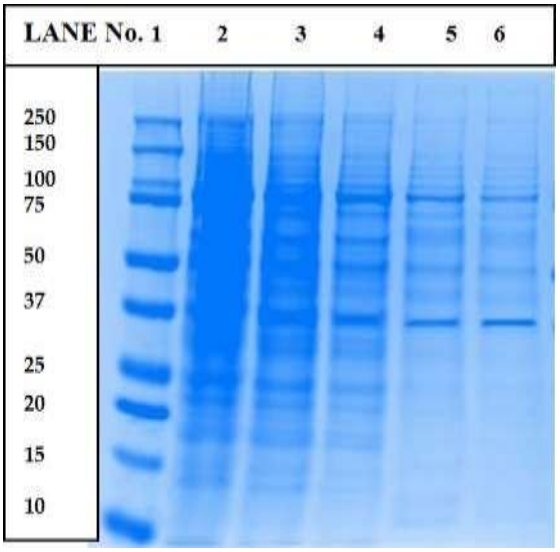

Figure 3 (A). SDS-PAGE image of O Ag. protein obtained in various purification strategies, Lane 1 represents marker, Lane 2 is Hydrolysed sample, Lane 3 is Retentate sample, Lane 4 is FT sample, Lane 5 is Purified Sample -1, and Lane 6 is Purifed Sample -2

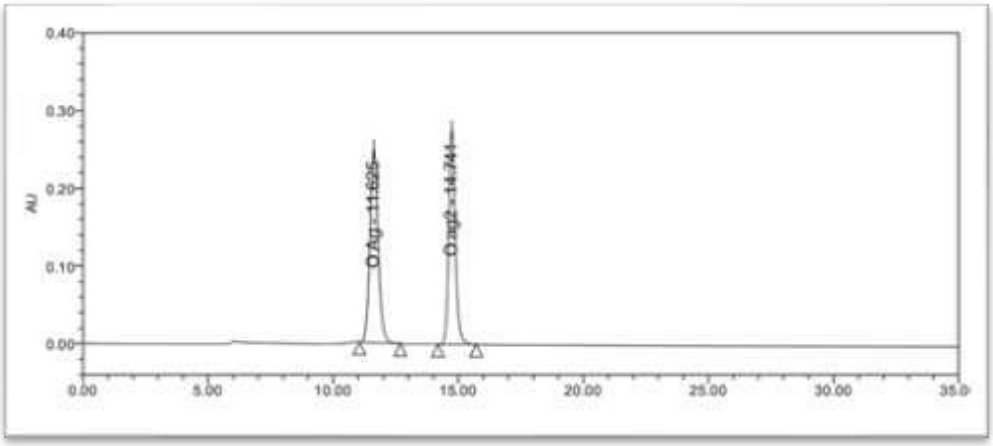

Figure 3 (B) Purified O Ag. from Salmonella Paratyphi B analysed by SEC-HPLC, to assess its molecular size distribution and homogeneity. The analysis was performed using TSK GEL 3000PWXL, (Tosoh Bioscience) column, according to molecular weight of high and low, peak were observed at the retention time 11 min and 14min.the absence of additional peak observed.

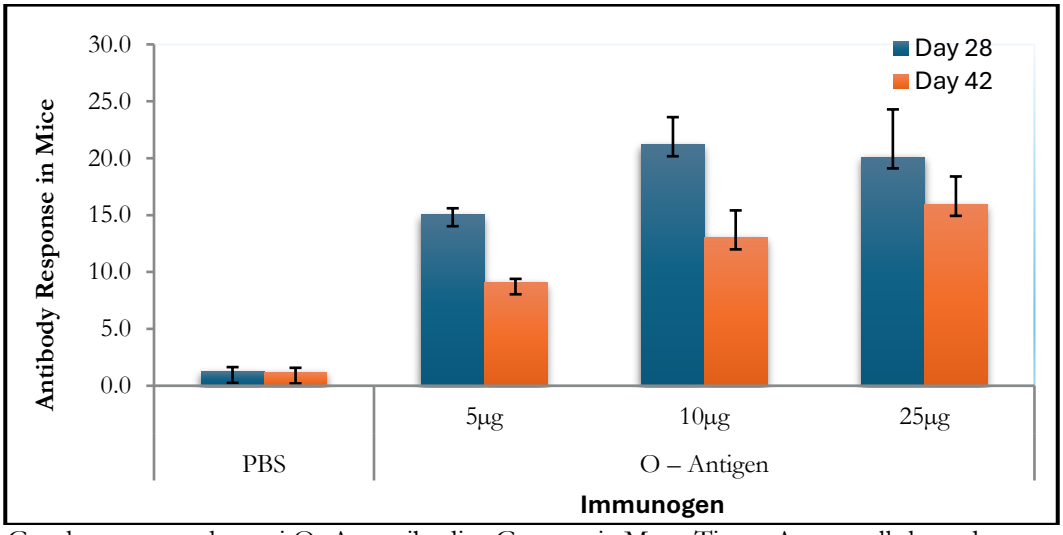

Figure 4: Graph represent the anti O–Ag antibodies Geometric Mean Titres, Among all three dose strengths, the 10ug and 25 ug dose were giving similar immune response which was higher than the immune response generated by 5ug dose. Geometric Mean and Standard Deviations from 6 number of animals & Run the triplicate each of the sera samples, Statistical analyses for immunogenicity outcomes (Day 28 vs Day 42 within each dose group) were performed using t-tests under the 5 % significant level to assess equivalence or difference in antibody levels. control group showed no significance change in antibody generation between Day 28 and Day 42. the O–Antigen groups, all dose strengths showed a decrease in antibody levels from Day 28 to Day 42. While the 5 µg and 10 µg doses showed a significant decline, the 25µg dose did not, indicating that the change over time is not strictly dose-dependent. P Value < 0.0001 for all test group.

Discussion

The extraction and purification of the O-antigen core component from Salmonella Enteric Serovar strains have been substantially enhanced through the development of a scalable and efficient method. This innovative approach marks a significant departure from conventional techniques that traditionally relied on bacterial sedimentation and hot phenol extraction of lipopolysaccharide (LPS) [8]. The newly developed method employs direct acetic acid hydrolysis on bacterial culture supernatants, effectively eliminating the need for toxic chemicals previously utilized in LPS hydrolysis [5].

This streamlined process has demonstrated considerable promise for larger batch productions and potential application to other Gram-negative bacteria, as evidenced by successful extractions from Shigella sonnei [4] and various other Salmonella strains [7,8]. The versatility of this method across different bacterial species highlights its potential as a universal tool in vaccine development, potentially revolutionizing the field of bacterial vaccine production.

A key advantage of this optimized extraction method is its ability to preserve crucial O-antigen characteristics, particularly the O-acetylation content, which plays a vital role in maintaining

immunogenicity [1]. The preservation of these essential properties is clearly demonstrated by the higher antibody titers observed in immunized groups compared to control groups (Figure 4). This preservation of immunogenic properties is critical for developing effective vaccines, as it ensures that the extracted antigens closely mimic those found on the surface of live bacteria, thereby eliciting a robust and specific immune response.

Furthermore, the stability of the purified O-antigen population has been rigorously confirmed through comprehensive analyses using SDS-PAGE and SEC-HPLC techniques, which conclusively show no signs of degradation. This stability is crucial for vaccine development, as it ensures the consistency and reliability of the antigen preparation, which is essential for both regulatory approval and long-term vaccine efficacy.

The process has proven to be highly effective, reliable, and reproducible for both pilot-scale and larger-scale batches of Salmonella Enteric Serovar strains. By employing this mild extraction protocol in conjunction with simplified tangential flow filtration (TFF) parameters, the method offers significant advantages for vaccine development. These benefits extend beyond mere efficiency, potentially leading to substantial cost reductions and enhanced productivity in the fields of immunology and vaccinology. The implications of this improved method are far-reaching, as it addresses several key challenges in the production of O-antigen-based vaccines. By simplifying the extraction process and eliminating the use of toxic chemicals, this approach not only enhances safety but also increases the feasibility of large-scale production. This could potentially accelerate the development and availability of vaccines against Salmonella and other Gram-negative bacteria, addressing critical public health needs globally.

The scalability of this method is particularly noteworthy, as it allows for the production of large quantities of purified O-antigen without compromising quality or yield. This is crucial for meeting the global demand for vaccines, especially in the context of emerging infectious diseases or widespread outbreaks. The ability to rapidly scale up production could significantly reduce response times to new threats and ensure a steady supply of vaccines for ongoing immunization programs.

Moreover, the method's demonstrated effectiveness across different bacterial strains suggests its potential as a versatile tool in vaccine research and development. This versatility could prove invaluable in responding to emerging pathogens or in developing multivalent vaccines that target multiple bacterial species or strains simultaneously. Such multivalent vaccines could provide broader protection against a range of related pathogens, potentially reducing the number of individual vaccinations required and improving overall public health outcomes.

The elimination of toxic chemicals from the extraction process not only improves safety for laboratory personnel but also reduces environmental impact. This aligns with growing concerns about sustainable and environmentally friendly manufacturing processes in the pharmaceutical industry. Additionally, the simplified process may lead to reduced production costs, potentially making vaccines more affordable and accessible, particularly in resource-limited settings where the burden of infectious diseases is often highest.

As research in this area continues, further refinements and applications of this method may emerge. For instance, the technique could be adapted for the extraction of other bacterial components relevant to vaccine development, such as protein antigens or capsular polysaccharides. Additionally, the principles underlying this method could inspire new approaches to antigen extraction and purification in other areas of biotechnology and pharmaceutical production.

In conclusion, this innovative O-antigen extraction method represents a significant advancement in the field of vaccine development. Its efficiency, scalability, and versatility address many of the challenges associated with traditional extraction methods, paving the way for more rapid and cost-effective vaccine production. As this technology continues to evolve and find new applications, it has the potential to significantly impact global health by accelerating the development and distribution of vaccines against a wide range of bacterial pathogens.

Conclusion

The successful development of a rapid, safe, and cost-effective method for extracting and purifying O-antigen from Salmonella Paratyphi B represents a significant advancement in vaccine research. This innovative approach not only preserves the structural integrity and O-acetylation of the antigen, crucial for maintaining its immunogenic properties, but also demonstrates promising results in immunization studies. The strong antigen-specific antibody responses observed in mice provide compelling evidence for the potential of this purified O-Ag as a viable vaccine candidate against S. Paratyphi B infections.

The scalability and environmental sustainability of this method offer substantial advantages for large-scale vaccine production. By reducing production time and costs while maintaining high-quality standards, this platform has the potential to accelerate the development and distribution of O-antigen-based vaccines. This breakthrough could have far-reaching implications for public health,

particularly in regions where S. Paratyphi B infections are prevalent.

Furthermore, the success of this approach may pave the way for similar methodologies to be applied to other pathogens, potentially revolutionizing the field of vaccine development and contributing to global efforts in combating infectious diseases. The adaptability of this extraction and purification technique could lead to the development of a new generation of vaccines against various bacterial infections, enhancing our ability to respond rapidly to emerging health threats.

Future aspects of this research may include:

1. Clinical trials to evaluate the safety and efficacy of the purified O-antigen vaccine in humans.
2. Investigation of potential adjuvants to enhance the immune response and provide long-lasting protection.
3. Exploration of combination vaccines incorporating multiple antigens to broaden protection against various Salmonella serovars.
4. Development of novel delivery systems to improve vaccine stability and efficacy, such as nanoparticle-based formulations or oral delivery methods.
5. Application of this extraction and purification method to other bacterial pathogens, expanding the range of potential vaccine candidates.
6. Integration of this technology with other emerging vaccine platforms, such as mRNA-based vaccines, to create hybrid approaches that maximize protection
7. Optimization of large-scale production processes to further reduce costs and increase accessibility in resource-limited settings.
8. Long-term studies to assess the duration of immunity and the need for booster doses.

These future directions hold promise for advancing the field of vaccinology and improving global health outcomes in the fight against infectious diseases.

Abbreviations

The following abbreviations are used in this manuscript:

SCDM	Soyabean Casein Digest Medium
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC-HPLC	Size Exclusion Chromatography
OD	Optical Density
ELISA	Enzyme Link Immunosorbent assay

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