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by kunza latif

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ABSTRACT

Objectives: This study was design to explore the immunogenic behavior of LamB and SpaO outer membrane protein of *Salmonella* serovar Typhi against enteric fever in BALB/c mice.

Methods: SpaO or LamB genes of locally isolated strain of *S.Typhi* was amplified, cloned and expressed. Recombinant protein SpaO and LamB were purified and lyophilized to prepare aluminum hydroxide Gel precipitated vaccine. Clinical trials were performed in BALB/c mice. The Immunoprophylactic potential of recombinant SpaO or LamB alone and in combination at different concentrations were determined using Complement fixation test along with whole culture attenuated and commercial vaccine.

Results: The immune response of rSpaO and rLamB proteins in combination at 500 μ g/ml was significantly high at 48th day and maintained until 60thday. Protective immune potential of recombinant monovalent and multivalent vaccine assessed upon challenge in mice with 10^9 cells of *S.Typhi* virulent culture. Out of five no mice could survive in control group although rSpaO and rLamB in combination at 500 μ g/ml conferred 80% protection. Whereas the monovalent rSpaO and rLamB vaccine elucidate protection 60% or 40% respectively.

Conclusion: From this study, it is concluded that combine vaccine rSpaO and rLamB effectively enhanced the immunological response and withstand the challenge of the causative agent more effectively as compared to the monovalent vaccines.

Keywords: Outer membrane protein Immunological response Recombinant vaccine *Salmonella* Typhi Complement fixation test

1. Introduction

Enteric fever is one of common infectious diseases of South East Asian countries like Pakistan (Yasin et al., 2018) and cause high rate of mortality among children and adults. For several years, the phenotypic trait of multidrug resistance (MDR) extensively distributed among *S.Typhi* (Eng et al., 2015). Multi drug resistance strains are present all over the world particularly in India, Vietnam or Pakistan instead of European countries (Ochiai et al., 2008). In 2015 approximately 17 million cases of paratyphoid and typhoid fever reported globally in Southeast Asia, South Asia or sub-Saharan African region and cause 2,50000 deaths reported each year in Pakistan (Mogasale et al., 2014). Vaccine is effective way to control the occurrence of diseases, *Salmonella* whole cell, live attenuated and recombinant vaccine are effective approaches to control the disease (Klemm et al., 2018). *Salmonella* outer membrane proteins are good candidate for vaccine preparation as it act as potential Immunoprophylactic agent and it induce good humoral and cellular response (Saxenan et al., 2017). First line for the treatment of enteric fever is ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol have been used from many years (Crump et al., 2015). Currently limited vaccines for enteric fever are commercially available, nevertheless some limitations including short-term immunity and high cost have been reported (Singh et al., 2017).

OMPs: *OmpF*, *OmpC*, *OmpL* and *OmpA* that exhibit high immune response against typhoid (Yang, 2013). However, when in combination one or more OMP used for immunization a strong protein immunity can be achieved (Muthiadin et al., 2018). OMP 28 could be use in animal study for better protection (Saxena et al., 2012). OMP 28 comprises eight variable regions, which are found on the membrane outer side or having highest probability presented for recognition of B-cell and provoke immune response. For eliciting immune response, these variable regions highly predicted to act as possible B-cell epitopes (Arockiasamy et al., 2004). In human response, it was observe that after vaccination, activation of B or T cells were observed which showed, significantly, circulatory antibody- secreting cells or highest Omp-

specific serum IgG titers. Likewise highest expansion of CD4⁺ T cells of OMP- specific in peripheral blood along substantial increase in production of TNF α or IFN- γ were also observe (Carreno et al., 2017). Immunization with 30 μ g of OMPs showed 100% protection when mice were challenge with two strains of *S.Typhi* (9,12,d,Vi and Ty2) upto 1,000 50% lethal doses (LD50). Moreover, protection rate of 30 % have been attain when challenged with *Salmonella* Typhimurium up to 500 LD50 (Isibasi et al., 1988).

SpaO is a surface presentation antigen or performed invasion or adhesion of pathogen such as *Salmonella* Typhi, Paratyphi or Typhimurium. Foremost components of the sorting platform for Typhimurium SPI-1 injectosome: the AAA+ ATPase InvC, its regulator SpaO, OrgB or OrgA protein identified by proteomic analyses (Hueck, 1998). Although for formation of the sorting platform SpaO have been shown to be essential about its molecular structure (Bzymek et al., 2012). Wharse outer membrane protein such as maltoporin β -barrel play a vigorous role in adhesion, virulence or act as enzymes responsible for the uptake and metabolism of glucose polymers (maltodextrins) (Gromiha and Suwa, 2007). One of a porin named LamB that encodes an outer membrane protein had play their vital role in transport of maltodextrins maltose also in phage adsorption. It usually active in trimer form (Boulain et al., 1986). A huge assortment of LamB mutants have been utilize in order to probe roles of different parts of protein. It has shown that outer membrane protein of similar size of LamB (49kDa) successfully expressed and purified (Kaur and Jain, 2013).

The current study was undertaken to determine the immune response of rLamB or rSpaO of outer membrane protein alone or in combination from local *S.Typhi* were tested in vivo at different concentration to draw a clear picture of their immunopotential so that data can be used in future for the clinical .

2. Materials and Methods

2.1 Ethical approval

Animal Right Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan rendered the ethical approval for this work.

2.2 Collection of samples

Total fifty blood samples of patient affected with enteric fever were collected from tertiary care hospital of Sialkot, Punjab, Pakistan. Thirty samples isolated as positive for *S. Typhi* were confirmed by ¹ commercial available kit Analytical Profile Index (API 20-E kit, BiOMerieux, France). For further confirmation of the strains 16S rRNA ribotyping were performed. The sequence of primer listed in Table 1. After the amplification of the genes DNA extracted by using ² GeneJET Gel Extraction Kit (Thermo Fisher Scientific). Afterwards Gel electrophoresis excised the bands. In equal amount GB buffer was added in gel slice. About 5-10 min gel slice was melt at 55°C. After that, samples were transferred to extraction ² column or at 10,000 rpm centrifuged for 1 min. Discarded the flow throw. Wash buffer 750µl was used for washing the column. Transfer the column to new centrifuge tube or in 50 µl distilled water eluted the DNA.

Hundred male mice BALB/c (weight 18-20 gram) recruited from National Institute of Health (NIH), Islamabad Pakistan. The mice were kept at the animal house of University of Veterinary and Animal Sciences, Lahore, Pakistan (UVAS) and provided the recommended feed or water under control conditions.

2.3 Amplification, expression and purification of outer membrane protein

Target genes SpaO and LamB (APV96323, AYT88671) amplified and cloned into pTZ57R/T cloning vector (Thermo Fisher USA) and expressed in pET28a expression vector (Takara/Clontech). Prokaryotic expression system pET28a-SpaO-*E.coli* BL21 (DE3) and pET28a-LamB-*E.coli* BL21 (DE3) were induced with 0.5mM IPTG in LB medium to express the SpaO and LamB proteins. Prior to immunization, prokaryotic expression system pET28a-SpaO-*E.coli* BL21 (DE3) and pET28a-LamB-*E.coli*BL21 (DE3) were induced with 0.5mM IPTG in LB medium to express the SpaO and LamB proteins.

Resuspended BL21 (DE3) with recombinant protein for lysis of bacteria using binding buffer (20mM Tris-Cl, 20mM Imidazole, 250mM NaCl) of pH 8.0 or exposed to sonication for 3 min with 5 sec on and 8 sec pulse off. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) 12% was use for visualization of samples. The cell pellets were washed at 4°C for overnight. Resuspend the IB pellet of SpaO using 30ml of buffer with 8 M urea pH 8.0 (10mM Tris-Hcl, 100mM NaH₂PO₄, 100mM NaCl, 8M urea) on the other hand for LamB used solubilization buffer with 6M, 8M or 10 M urea after that stirred for overnight at 37°C on magnetic stirrer and next day centrifuged at 4°C at 15000 rpm for 30 min. Supernatant was collected. 3M urea was used for dialysis the samples for overnight. Ni-NTA chelating Sapharose column were used for purification of rLamB and rSpaO (Hochuli, 1990). After that

equilibrated the column for 2-3 hour with 20 ml of an IB solubilization buffer. The supernatant were ¹bind with the column. Then using the wash buffer (20mM Tris-Hcl, 250mM NaCl, and 50mM imidazole) the column was washed. SpaO and LamB was eluted by the use of ¹elution buffer (20mM Tris-Hcl, 250mM NaCl, 250mM imidazole), (20mM Tris-Hcl, 250mM NaCl, 150mM imidazole). Collected 1ml fractions or examined with 12% SDS-PAGE. Ni-NTA affinity chromatography was used for purification of recombinant proteins. Purified protein was lyophilized for vaccine formulation. Protein concentration was measurement by taking absorbance at 280nm on spectrophotometer and OD at 280 considered 1mg/ml. Spectrum was very clear and only one peak at 280nm that shows the purity of protein.

2.4 Formulation of Aluminum Hydroxide gel recombinant vaccine

Recombinant vaccines based of Aluminum Hydroxide Gel (AHG) were prepared (Peng, 2015). The adjuvant used for human that approved worldwide was alum (Harandi et al., 2010). To separate the water from the gel 15ml of alum gel were centrifuged for 5 minute then mixed with recombinant protein. The Aluminum Hydroxide Gel (AHG) was prepared with the addition of Aluminum Ammonium Sulphate 800 grams in 1000 ml carbonated water comprising Sodium Carbonate ($\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$) of 1000 grams. Using daily manual of 2 hours stirring all gases produced were remove. The suspension was washed using cold distilled water 6 time by centrifugation. Mixed the collected pellet with vaccine suspension with rate of 2%. In order to obtain homogenized suspension stirred the suspension for 15 minutes at 300 rpm.

2.5. Commercial vaccine formulation

The commercial vaccine (Vi Capsular Polysaccharide Typhoid Vaccine, AMSON PHARMA (PVT LTD) injected in the mice at the rate of 0.1ml per mice. Mixed with phosphate buffer saline of 0.5 ml Tybbar vaccine or 0.1 ml to each five mice were injected.

2.6 Preparation of whole cell culture vaccine

Total viable bacterial count estimated in the purified suspension as described by Miles and Misra, 1938. Briefly, sterilized 10ml nutrient broth in test tubes. Prepare tenfold dilutions

of the stock culture (10^{-1} to 10^{-10}). Finally, poured one ml of each concentration on Salmonella Shigella agar on petri plates. Then incubated the plates at 37°C for 24 hours.

Determined the bacterial count ¹ in the culture with the following formula.

$$\text{Total viable bacterial count: } \frac{\text{dilution factor} \times \text{volume of the culture transferred to the Agar plate} \times \text{Number of colonies}}{\text{Agar plate} \times \text{Number of colonies}}$$

Added 2.5 ml of formaldehyde (0.5 %) in culture flask for inactivation of bacteria or incubate ⁹ at 37°C for 24 hours on shaking. After 24 hours inactivation of cells confirmed on SS agar plates.

2.7 Vaccination trial in BALB/c mice

Five different groups were designed to investigate the immunogenic potential of the vaccine. Each experimental group comprises of five mice. ² The groups were; Group 1 (rSpaO), Group 2 (rLamB), Group 3 (Combine rSpaO + rLamB), Group 4 (Typbar Commercial vaccine) and Group5 (Whole culture vaccine *S.Typhi*). Five different concentration of recombinant outer membrane protein for each group were used (500, 250, 125, 60 and $30 \mu\text{g/ml}$) respectively as shown in table below (Table 1). One group was taken as control and adjuvant phosphate buffer saline (PBS) was injected. While group 1,2 and 3 subcutaneously injected with recombinant proteins of different concentrations (Table1) along with adjuvant aluminum hydroxide gel (AHG) (final concentration of 2%) followed by booster at 14th day after the primary ¹⁰ dose.

2.8 Collection of blood samples

Blood samples was collected and serum was obtained by centrifugation on 0, 14, 28, 48, 60 and 90 days post primary dose and stored at -80°C for further analysis. Evaluation of antibody titer performed using Complement fixation test (CFT) (Boulain et al., 1986).

2.9 Challenge study

For challenge study, used 24 hours broth bacterial culture. The viable bacterial were count

by dilution pour plate. Bacterial suspension (*S.Typhi*) with 2×10^9 CFU/ml was used (Adone et al., 2016). 2.5 ml of formaldehyde (0.5%) was use for the inactivation of bacteria and incubated on shaking at 37°C for 24 hours. 1 ml inactivation culture poured on plates and incubated at 37°C for 24 hours. The control was inject with 0.2 ml of culture as well as in the experimental groups of mice. For seven days, post challenge mice observed.

2.10 Complement fixation test (CFT)

Briefly, poured 50ml of PBS in all wells of the round bottom 96 well plate. 50 μ l of the respective sample added in the first wells of first column and sample were diluted upto 10th wells. 50 μ l of 96 well plate (row wise 1-12) serum samples was poured in first well and diluted in 2-4 pattern upto 9th well. Next 50 μ l of the antigen added in all wells 1-10th. Incubated the plate incubated for 15 min at 37°C for antigen antibody reaction. After incubation, 50 μ l of Guinea pig complement added from 1-12th well. Lastly 50 μ l sensitized RBCs (RBCs react with amboceptor's) was poured in each well plate and incubated for 2 hours at 37°C. The formation of RBCs in the bottom of the well was representative of positive sample (positive for antibody). Whereas the hemolysis was the indication of negative result.

2.11 Statistical Analysis

The statistical significance of data was find out using One- way and two way analysis of variance (ANOVA) by means of SPSS program version 20 (International Business Machines Corporation) and at $p \leq 0.05$ significance was tested. Presented the results as mean values \pm standard error, and selected for each condition three independent replicates.

3. Results

The sample streaked on Salmonella-shigella agar (SSagar) which was positive for *Salmonella*. Blackish center colonies were observe on SSagar specifying that the sample was positive for *Salmonella*. Bacilli gram negative appearance was indicate the confirmation of *Salmonella*. Further confirmation of suspected *Salmonella enterica* were carried out with biochemical test using API (E-20) Kit. Positive samples were further confirmed by 16SrRNA

PCR for molecular characterization.

3.1 *In vivo* Immune response assay

Complement fixation test (CFT) used to determine the serum antibody titer. Different vaccine groups antibody titer rSpaO, rLamB, combine (rSpaO and rLamB), commercial vaccine or whole culture at all concentrations shown in fig 1. After booster dose on 14th day immune response was highly significant ($P < 0.01$). Whereas it declined on 90th day post vaccination. At concentration 500 $\mu\text{g/ml}$, the serological comparison of commercial Typbar and combine (rSpaO and rLamB) vaccine concluded that vaccine in combination (rSpaO and rLamB) is relatively effective same as commercial Typbar vaccine. When the result was statistically analyzed particularly with in the groups it was concluded that antibody titer of whole culture vaccine was significantly higher ($P < 0.01$) at all concentrations as compared to monovalent rSpaO, rLamB, combine (rSpaO and rLamB) or commercial Typbar vaccine on 48th and 90th day post priming. Whereas, immune response of combine vaccine with respect to monovalent vaccine is significantly better. Antigens when used in combination synergize each other by producing better immune response on 48th and 60th day (Fig 1). Moreover, when statistically analyzed vaccine in combination of 500 and 250 $\mu\text{g/ml}$ concentration it was concluded that both were immunogenically producing same antibody titer with no significant difference. However, upon 125, 60 and 30 $\mu\text{g/ml}$ concentrations, the combine vaccine (rSpaO and rLamB) produced better efficacy on 48th and 60th day than respective monovalent rSpaO and rLamB vaccine.

Fig. 1. Immune response of different vaccines at concentration 500, 250, 125, 60 and 30 $\mu\text{g/ml}$: a) rSpaO, b) rLamB, c) combine (rSpaO and rLamB), d) whole culture vaccine and e) commercial vaccine against *S.Typhi*.

3.2 Challenged study against *S. Typhi*

Protective immune potential of different recombinant vaccine in BALB/c mice evaluated by challenge study for which challenged the vaccinated groups of mice with pathogenic field isolate of *Salmonella Typhi* (2×10^9) subcutaneously. Immune response of combine (rSpaO and rLamB) and monovalent vaccine assessed against challenge of *S. Typhi*. Results shown in Table 2. In all vaccinated group no mice could survive at 14th day. At 500 $\mu\text{g/ml}$ percentage of protection on 60th day was 60 %, 60%, 100%, 100% and 80% of rSpaO, rLamB, combine (rSpaO and rLamB), for whole culture vaccine or commercial Typbar vaccine. Whereas the protection rate was 60%, 40%, 80%, 100% and 80% on 90th day at rSpaO, rLamB, combine (rSpaO and rLamB), for whole culture vaccine and commercial vaccine Typbar. Although the percentage protection of recombinant combine, vaccine at 500 $\mu\text{g/ml}$ was higher(80%) with respect to others (Table 2)

3.3 ¹ Immuno comparison of different vaccine between the groups

To access the appropriate concentration of rSpaO, rLamB, combine (rSpaO and rLamB). They were compared with each other in kill cross manner means rSpaO x rLamB, rSpaO x combine and rLamB x combine. Selected the concentration in order to comparison at which percentage protection was above 50 % and there were 500 and 250 ug/ml per dose. Vaccine in combination at 250 ug/ml each showed protection as compared to rSpaO ² 250 ug/ml or LamB 250 ug/ml per dose vaccine. In challenged study percentage, protection was 80 % and 60%. Vaccine at concentration of 500 ug rSpaO is highly protected than 250 rSpaO that showed rSpaO or rLamB incombination at ¹ 250 ug/ml it result is equal or better to the rSpaO vaccine 500 ug/ml per dose.

4. Discussion

Enteric fever, caused by *Salmonella enterica* serovars, is a foremost public health challenge for the developing nations (Das et al., 2015). Although in Pakistan with respect of *Salmonella* multidrug-resistant strains occurrence its management become more complex due to increased survivability, communicability as well as virulence as a result there is an increased ratio of mortality and morbidity (Qamar et al., 2014). Presently there is no local vaccine available, which could protect against *S. Typhi* infection in Pakistan. Therefore, it is essential that from the local isolate the effective vaccine formulated. In current study, two recombinant outer membrane proteins of *Salmonella* expressed and formulated to check their immune response *in vivo*.

In the present study, it were concluded that antibody titer at concentration of 500 and 250 $\mu\text{g/ml}$ of rSpaO reached to peak on 48th day and remained significantly higher until 60th day, was decline on 90th day. It is revealed that vaccine antibody titer at different phases because of the reason that an extra follicular response had been observed with the stimulation of initial exposure of vaccine antigen. Due this response, there is a quick appearance of immunoglobulins or formerly immune response starts to build up (Mendes et al., 2018). When there is a proliferation of B cells occurs and differentiate into plasma cells, as a result there is an increase of immunoglobulins titers up to a peak value. Due to these plasma cells there are fast decline in antibody titers. Though, when secondary immune response happen by means of booster exposure to antigen, reactivation of immune cells had been observed, so there is a rapidly raise in immunoglobulins level (Clem, 2011). During a few weeks, peak antibody levels maintain by means of short-lived plasma cells. Subsequently, initially there is a decline in antibody titer with an equal quick kinetics as following primary immunization (Margolick et al., 2006). With long-lived plasma cells antigen specific antibodies produced continuously which extended survival niches in the bone marrow that eventually decline by slower kinetics. These explanations are in line with Ruan et al. (2008) they used rSpaO vaccine doses of 500 or 250 $\mu\text{g/ml}$ and injected into rats subcutaneously. While compared the antibody titer of monovalent vaccine it was conclude that rLamB vaccine showed less efficacy as compared to rSpaO at same concentration. Through, in zebra fish vaccination or challenge with heterogeneous virulent *Vibrio* strain Lun et al. (2014)

evaluated cross protective property of rLamB. Afterwards, immunization with rLamB protein, it revealed that from *Vibrio* infection zebra fish showed protected. From this study it was concluded that LamB was a conserved antigen between the tested *Vibrio* species and it might be used as a better potentially vaccine candidate for *Vibrio*'s. These results are in line with Hamid and Jain, (2008) 49kDa OMP of *S.Typhimurium* is highly immunogenic and evoked both humoral and cell mediated response while upon challenge with high doses (upto 100 times the 50 % lethal dose) to immunized rates (upto 100 times the 50 % lethal dose) then the protection percentage was 100%. In combination, (rSpaO and rLamB) antibody titer was high as related with particular monovalent vaccines. Our findings are in line with Jiang et al. (2012) when mice were challenge with *S. Paratyphi A*, 66.7% and 83.3% percentage protection have been showed by 100 µg or 200 µg of rSpaO-OmpA. Immune protective rates produced were suggestively higher as compared of equal rSpaO or rOmpA ($P < 0.05$). Agglutination titers 1:5-1:40 presented from sera of rSpaO-OmpA immunized mice to the different *S. paratyphi* species H antigens while for proteins rSpaO, rOmpA or rSpaO-OmpA immunodiffusion titers were 1:1-1:16 respectively.

Immune stimulatory is *Salmonella* OmpA as exposed with IFN-g production stimulation or in Salmonellosis have a vital role in modulation of the immune response on the other hand MHC expression enhanced as well as co- stimulatory molecules in T cells or dendritic cells (Jang et al., 2013). Protection level enhanced when the mice were immunized with two antigens as compared to monovalent rSpaO or rHIa against subsequent infection (Ruan et al., 2008). rOmp and rOmF of *S.Typhi* are more immunogenic protein in murine models and enhanced the cell-mediated immunity (Verma et al., 2010). On the basis of findings of earlier workers we can conclude that recombinant combine outer membrane protein are conserved and it can be used as a strong candidate for vaccine development against *S.Typhi*.

Subsequently, this study suggests that against typhoid fever recombinant combine vaccine (rSpaO and rLamB) formulation with alum based as an adjuvant provoked long lasting immunity. Live vaccines induced strong immune protection attributed to the probability that natural infection may imitate by live vaccine with secreted proteins or therefore naturally evoke the immune response of the host (Jang et al., 2013). *Salmonella* is widely use in live attenuated vaccine and its protection can be improved by using

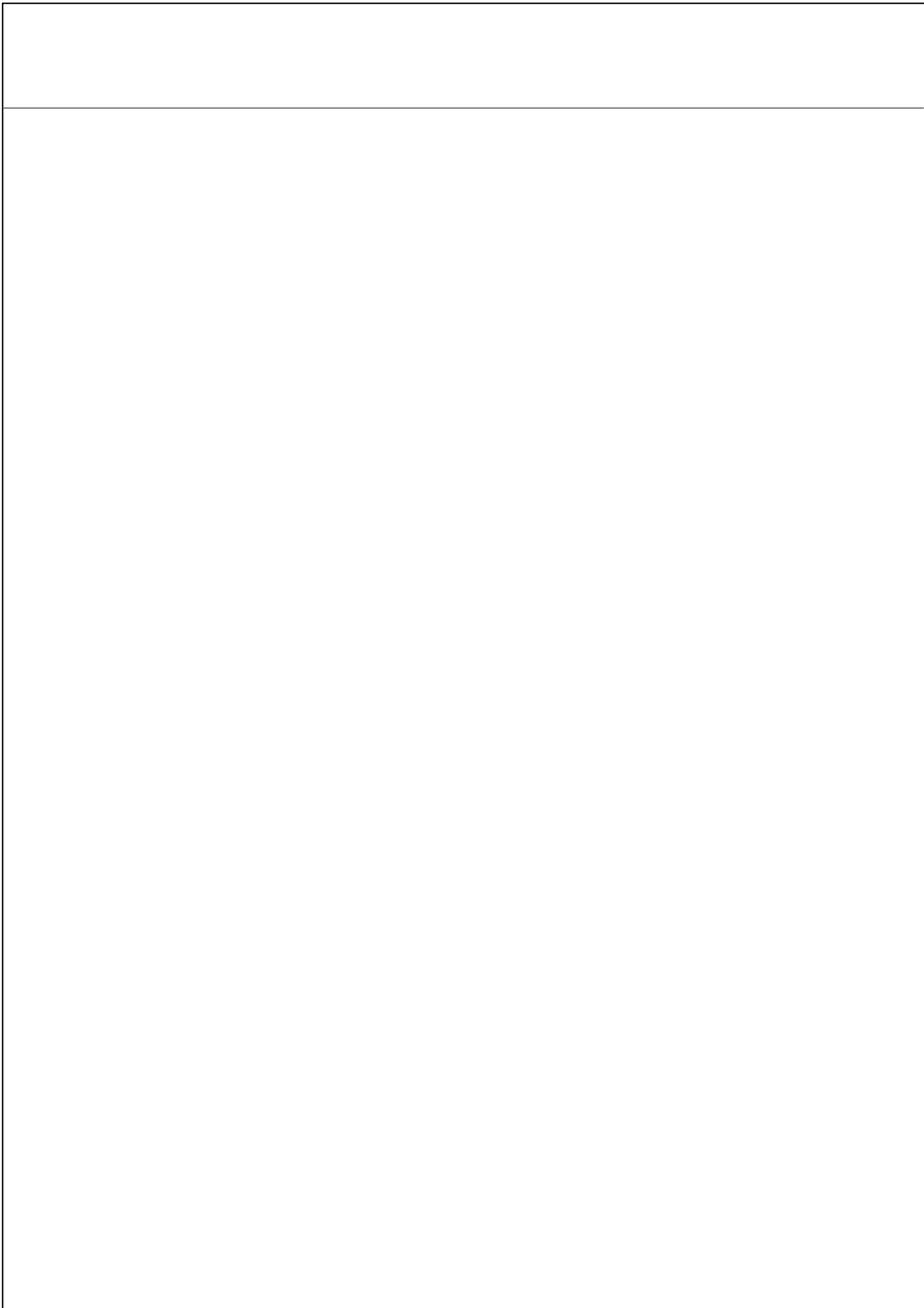
engineered strains of *Salmonella* (Periaswamy, et al., 2012). Nevertheless, numerous live attenuated vaccines are limited by their essential ability to access systemic organs in many of the vaccinated hosts, particularly those which are immunocompromised (Vishwakarma et al., 2012). Whole cell vaccine found to be short lived usually of two or three years. In small percentage of infants the encephalitis-type reaction occurred receiving the whole-organism pertussis vaccine, these vaccines for pertussis have led to development of a new cellular vaccine (Zaitsev, 2013). Furthermore, certain vaccinated individuals developed a hypersensitivity reaction (Taylor and Francis, 2008). To overcome these disadvantages, as an alternative a non- toxic recombinant vaccine has developed.

Most Whole culture vaccines attenuated which are using today with adapting the wild type virus to a new environment (e.g. replication in a novel cell line or low temperature) attained their reduced virulence along with replication rate reduced in humans. For pathogens such as HTV the usage of attenuated vaccines is hazardous so there is a need to develop a recombinant vaccine with better efficacy where pathogen genes are successfully expressed from a benign virus vector along with immunogenic activity (Bull et al., 2019).

It is suggested from this study that whole cell vaccine is LPS based and short lived and need repeated booster doses, to overcome this problem recombinant combine vaccine containing rSpaO and rLamB can be produced an ideal solution for a long-term immunity.

6.5. Conclusion

The findings from the study suggested that outer membrane protein could be protective vaccine candidate when used in combination. In order to explore different aspects of enteric fever. This study can be broaden with different assessments that may comprise exploration of immunological behavior of combine and conjugated vaccine of outer membrane protein. A new effective vaccine with better efficacy could be launched if the study extended to the phase 2, 3 and 4 in biological developments.



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