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Research paper

First characterization of immunogenic conjugates of Vi negative *Salmonella* Typhi O-specific polysaccharides with rEPA protein for vaccine development

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ABSTRACT

Efficacious typhoid vaccines for young children will significantly reduce the disease burden in developing world. The Vi polysaccharide based conjugate vaccines (Vi-rEPA) against *Salmonella* Typhi Vi positive strains has shown high efficacy but may be ineffective against Vi negative *S.* Typhi. In this study, for the first time, we report the synthesis and evaluation of polysaccharide-protein conjugates of Vi negative *S.* Typhi as potential vaccine candidates. Four different conjugates were synthesized using recombinant exoprotein A of *Pseudomonas aeruginosa* (rEPA) and human serum albumin (HSA) as the carrier proteins, using either direct reductive amination or an intermediate linker molecule, adipic acid dihydrazide (ADH). Upon injection into mice, a significantly higher antibody titer was observed in mice administrated with conjugate-1 (OSP-HSA) ($P = 0.0001$) and conjugate 2 (OSP-rEPA) ($P \leq 0.0001$) as compared to OSP alone. In contrast, the antibody titer elicited by conjugate 3 (OSP_{ADH}-HSA) and conjugate 4 (OSP_{ADH}-rEPA) were insignificant ($P = 0.1684$ and $P = 0.3794$, respectively). We conclude that reductive amination is the superior method to prepare the *S.* Typhi OSP glycoconjugate. Moreover, rEPA was a better carrier protein than HSA. Thus OSP-rEPA conjugate seems to be efficacious typhoid vaccines candidate, it may be evaluated further and recommended for the clinical trials.

1. Introduction

Typhoid fever is one of the major public health problems worldwide (Crump and Mintz, 2010). It enormously contributes to morbidity and mortality in the developing world because of overcrowding, with inadequate clean water and unhygienic conditions (Crump et al., 2004; Waddington et al., 2014). The reported global burden of typhoid in 2010 was 26.9 million cases per annum (Buckle et al., 2012), however the true burden of this disease is likely to be higher (Crump and Mintz, 2010; Buckle et al., 2012). The greatest typhoid incidences have been reported in South-Central and South-Eastern Asia (Crump et al., 2004; Akter et al., 2017; Javed et al., 2017). A retrospective study found that among five countries (China, Pakistan, India, Indonesia and Vietnam), South Asia was the most affected region, especially Pakistan (Ochiai et al., 2008; Munir et al., 2016).

In developing countries, *S.* Typhi has developed multidrug resistance (MDR) against most of the available first-line antibiotic drugs (Munir et al., 2016). Moreover, the growing rate of fluoroquinolones and cephalosporin resistant isolates has seriously outpaced the development of new drugs (Koirala et al., 2012; Afzal et al., 2013; Kaur, 2013). Because of high antibiotic resistance, it is critical to adopt comprehensive vaccination strategies in high-risk localities.

Currently, available typhoid vaccines are based on Vi capsular polysaccharides. The polysaccharide vaccine was found to be safe and well tolerated (Sur et al., 2009) but Vi is not immunogenic in children younger than 2 years of age (Wain et al., 2005). The Updated reports from endemic areas showed a substantial typhoid fever burden in children below 2 years (Cui et al., 2010), supporting the need for effective conjugate vaccines against *S.* Typhi infection in this age group. Vi capsular polysaccharide was coupled with recombinant exoprotein A (rEPA) of *Pseudomonas aeruginosa* to produce Vi-rEPA conjugates, followed by testing in infants in Vietnam. These testing results from Vietnam suggested that the Vi-rEPA vaccine was safe, provoked protective levels of anti-Vi antibodies in about 95% of vaccinees and was compatible with Extended Program on Immunization (EPI) vaccines (Thiem et al.,

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2011; Szu, 2013). Hence the rEPA is widely used suitable carrier protein for vaccine research (Pan et al., 2016).

The Vi (capsular) polysaccharide of *S.* Typhi has been purified, conjugated with a carrier protein and evaluated in mice by different researchers (Thiem et al., 2011; Fiorino et al., 2012). However, no such study has been performed using Vi negative strains of *S.* Typhi. These strains have been isolated from Asian countries (Baker et al., 2005; Wain et al., 2005; Ali et al., 2012; Ali et al., 2014). Recently, a study from Kathmandu, Nepal has shown that naturally occurring Vi negative *S.* Typhi may cause typhoid fever similar to Vi positive *S.* Typhi (Pulickal et al., 2013). This scenario highlights the need to explore conjugate vaccine candidates based on polysaccharides *S.* Typhi that are present in both Vi negative and Vi positive *S.* Typhi strains. O-specific polysaccharide (OSP) antigens are important component of lipopolysaccharide (LPS), present on outer membrane of *Salmonella* as like other Gram negative bacteria. Composition of OSP in various gram negative bacteria is extremely variable, it acts as a fingerprint for bacteria. OSP of the *S.* Typhi is comprised of five monosaccharide sugars; glucose, galactose, rhamnose, mannose and tyvelose (Rahman et al., 1997).

The OPS plays a major role in protection from immune system of the host (Raetz and Whitfield, 2002; Baliban et al., 2017) and against antibiotics (Banemann et al., 1998). Hence, it induces the immunoglobulins such as IgM and IgG. The OSP of *S.* Typhi has been used as potential candidate for conjugate vaccine preparation against this bacterium (Robbins et al., 1999). In this study, we have synthesized four glycoconjugate vaccine candidates of Vi negative *S.* Typhi OSP with human serum albumin (HSA) and recombinant exoprotein A (rEPA) of *Pseudomonas aeruginosa* using reductive amination and cyanylation methods. The IgG immune response was determined in mice against OSP alone and the prepared conjugates. We anticipate that the current study will contribute toward development of a vaccine to eradicate infections caused by *S.* Typhi.

2. Materials and methods

2.1. Fermentation of Vi negative S. Typhi

Already confirmed *S.* Typhi Vi negative (AS-8) isolate taken from National Institute for Biotechnology and Genetic Engineering (NIBGE) stock cultures was revived overnight on tryptic soy broth (TSB). Bacterial cells were grown in a 20 L fermenter as described (Ali et al., 2012; Salman et al., 2015) and the bacterial pellet was harvested by centrifugation at 7000 ×*g* at 4 °C for 40 min.

2.2. Purification of OSP from LPS of S. Typhi

LPS were extracted from the bacterial cell pellet by the hot phenol-water method (Westphal, 1965). Impurities were eliminated by enzyme treatment followed by dialysis and ultracentrifugation at 96,000 ×*g* for 5 h at 4 °C. The pellet was lyophilized as purified LPS. Core hydrolysis of the purified LPS was done for OSP extraction as described earlier (Chu et al., 1991; Ali et al., 2012; Salman et al., 2015). Briefly, the purified 10 mg/ml LPS of *S.* Typhi was dissolved in 1% glacial acetic acid and heated at 100 °C in reaction-block for 90 min. The mixture was cooled, transferred to Corex tubes and centrifuged for 20 min at 8000 x g. The supernatant was passed through chromatography column (1.5 × 34 cm) using G-25 Sephadex (Amersham Biosciences, USA). The void volume fractions were pooled in a round bottom flask, frozen with dry ice and lyophilized (Chu et al., 1991). The purified OSP was stored at −20 °C. Alditol acetate assay was performed to determine the sugars components of *S.* Typhi OSP, using GLC-MS as described earlier (Cox et al., 2010).

2.3. Conjugation strategy 1: reductive amination

Five mg of protein (HSA or rEPA) along with 200 × molar excess of OSP were resuspended in 5 ml of 200 mM of sodium phosphate (Na_2HPO_4) buffer pH 8.0 and sonicated for 2–5 min to solubilize them completely. Meanwhile, 25 mg of sodium cyanoborohydride ($NaBH_3CN$) was dissolved in 500 μl of sodium phosphate buffer and added to the protein-OSP mixture without pipetting. The protein-OSP mixture was then passed through a 0.22 μm sterile filter unit (Part No SLGV033RK, Millipore, Ireland) using a 10 ml disposable syringe inside a laminar air flow cabinet. The mixture was kept at 37 °C for 72 h.

The prepared conjugates were purified by passing through Sepharose CL 6B column (column size 96 cm) equipped with a refractive index detector using PBS citrate buffer (pH 8.0), with the flow rate 5 cm/h. High molecular weight fractions were pooled and concentrated using a Millipore Ultra 30 kDa MW 30,000 MW cut off spin device, and then subjected to PBS buffer exchange at 5000 rpm for 20 min, three times.

2.4. Conjugation strategy 2: CDAP activation method

S. Typhi OSP was derivatized using the 1-cyano-4-dimethylaminopyridinium tetrafloroborate (CDAP) method (Lees et al., 1996) under the conditions described earlier (Konadu et al., 1996; Ali et al., 2012). For conjugation of OSP_{ADH} with protein, 5 mg of protein (HSA or rEPA) was dissolved in 0.5 ml of 4-morpholine ethane sulfonic acid (MES) and 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was dissolved in 0.5 ml of Milli-Q water. Derivatized OSP (OSP_{ADH}) was added to the stirred protein solution. The pH of the mixture was maintained at 5.2 for 20 min and the mixture was placed at 4 °C overnight. The prepared conjugates were purified and concentrated as mentioned in strategy 1.

To determine adipic dihydrazide (ADH) group in conjugation reactions Trinitrobenzene sulfonic acid (TNBS) assay was performed using ADH as standard (Chu et al., 1983). The ^{1}HNMR spectroscopy was performed to analyze the OSP_{ADH} as described previously (Cox et al., 2002). Briefly, 5 mg of each OSP_{ADH} as sample and OSP as standard were dissolved in D_2O and solutions were transferred to NMR tubes and examined with NMR parameters: frequency 400 mHz, temperature 25 °C, acquired time 2.5 s, relax 0.05 s, pulse 45°, repetitions 8 total, time 36 s NMR active and nuclei was ^{1}H.

2.5. Determination of carbohydrates and proteins in prepared conjugates

The DuBois assay was used to determine the carbohydrate concentrations in conjugates (DuBois et al., 1956). Briefly, 200 μl of 5% phenol was added to each tube containing 100 μl of sample (column fractions) or control (column effluent), vortexed followed by addition of 2 ml of concentrated sulphuric acid. Reaction mixtures were cooled for 30 min, negative control was set to zero absorbance and OD was taken at 490 nm.

Bicinchoninic acid assay (BCA) was used for determination of protein concentrations in conjugates (Smith et al., 1985). Briefly, 200 μl of BCA reagent were put in each well of NuncMaxiSorp plate starting from standard BSA protein followed by unknown protein dilutions. The plate was incubated at 37 °C for 30 min. Samples were cooled to room temperature and reading was taken using Dynatech lab MR 500 at 562 nm.

2.6. SDS-PAGE and Western blotting for conjugates

One mg/ml HSA and 1 mg/ml *S.* Typhi OSP were taken as standards and separated on 10% Tris–HCl pre-cast gels under reducing conditions with the buffer system of Laemmli (Cox et al., 2010). An unstained duplicate gel was trans blotted (Biorad trans blot semi-dry SD cell) to polyvinylidene fluoride (PVDF) membrane at 12 V for 1 h. The membrane was blocked with 1% BSA-TBST (Tris Buffer Saline with Tween) and immuno-stained with in-house produced monoclonal antibodies developed to *S.* Typhi D1 at a suitable dilution (1:20,000) followed by anti-mouse IgG (Bio-Rad, USA) conjugated to alkaline phosphatase and developed with alkaline phosphatase substrate kit (Bio-Rad, USA).

2.7. Mice immunization

Female Balb/C mice 6–8 weeks old were purchased from Charles River Laboratories, Canada and acclimatized for one week prior to initiating animal experiments. All protocols of animal experiments were approved by the NRC Animal Care and Use Committee. Groups of five mice were immunized intra-peritoneally with four *S.* Typhi OSP – protein conjugates. Three mice were immunized with OSP and protein alone or mixture (with adjuvant) in each group. Each mouse received 2 μg of carbohydrate in 0.2 ml Sigma adjuvant system (Sigma, USA). An equal amount of adjuvant was added to the prepared conjugate in 1:1 ratio per injection. Mice were boosted on day 21 and 42 with same amount. Sera were recovered on day 0 and 35 from facial jugular vein and by terminal heart puncture on day 56.

2.8. ELISA

A 96 well NuncMaxiSorp plate was coated with purified *S.* Typhi LPS (0.5 μg/well). Solid-phase indirect ELISA was performed to analyze the antibody titer elevated by the mice sera as mentioned previously (Cox et al., 2005). The result was obtained from plotted reader and prepared graph using Graph pad prism 4.0 software.

3. Results

3.1. Extraction of LPS and purification of OSP from Vi negative S. Typhi

Core hydrolysis of the purified LPS followed by size exclusion chromatography and lyophilization resulted in lipid A removal and yielded the purified OSP as 54 mg per 100 mg of purified LPS. By treatment of DNase and RNase the nucleic acid impurities were reduced to 0.06% and proteinase K decreased protein impurities to 0.07%. The alditol acetate assay showed that OSP sample of Vi negative *S.* Typhi contained galactose, glucose, mannose, rhamnose and tyvelose sugars (Rahman et al., 1997) (Fig. 1).

3.2. The OSP-HSA and rEPA-OSP conjugates prepared by reductive amination

Two conjugates were prepared using reductive amination denoted as OSP-HSA [Conjugate 1] and OSP-rEPA [Conjugate 2]. Fractions of size exclusion chromatography Sepharose 6B column resulted in a clear baseline separation of high molecular weight (HMW) conjugated fractions tubes 35 (105 ml) to 41 (123 ml) from un-reacted proteins and OSP column volume (figure not shown). Fractions of both conjugates were pooled separately and concentrated in MW 30 kDa cut off spin columns.

3.3. The OSP_{ADH}-rEPA and OSP_{ADH}-HSA conjugates prepared by CDAP chemistry

Two conjugate constructs of activated OSP were produced using CDAP chemistry (Lees et al., 1996) to functionalize the OSP with ADH. The ADH-OSP was subsequently linked via the spacer to HSA and rEPA using carbodiimide chemistry. The prepared conjugates were designated as OSP_{ADH}-HSA [Conjugate 3] and OSP_{ADH}-rEPA [Conjugate 4]. Both conjugates were passed through Sepharose 6B column using PBS citrate as eluent to remove free OSP. Void volume fractions tubes 51 (153 ml) to 75 (225 ml) were collected and concentrated using a MW 30 kDa cut off spin device. The concentrations of protein and carbohydrates in each conjugate are summarized in Table1.

3.4. Analytical methods for OSP and conjugates

The ^{1}H NMR spectra of OSP showed five signals: for anomeric protons, the peak was between 4.5 and 5 ppm, ring protons signals were located at 3.0–4.5 and two signals were at 1.0–2.0 which indicated the presence of methyl protons of rhamnose and tyvelose. After derivatization of OSP; new peaks were found between 2 and 3 ppm because of OSP_{ADH} linkage (Fig. 2). Initially the concentration of OSP was 1 mg/ml and after the derivatization the concentration of OSP_{ADH} linkage analyzed with trinitrobenzene sulfonic acid (TNBS) assay was 22.1 μg/ml.

3.5. SDS-PAGE and Western blot of conjugates

An increase in molecular weight was observed following conjugation of the OSP and protein. Protein load of conjugate was determined on 7.5% SDS-PAGE in comparison to relevant protein (HSA and rEPA) controls (Fig. 3a) whereas, carbohydrate load was determined by comparing conjugates with OSP alone using carbohydrate specific antibody in Western blot (Fig. 3b).

3.6. Immunogenicity of OSP conjugates in mice

The serum IgG antibody titers (on day 56) following the second booster dose were analyzed. Each of the conjugates showed an increased ELISA titer against the OSP as compared to control group (OSP alone). The geometric mean titer of conjugate 2 was the best (3.306), conjugate 1 (2.197) showed good immune response, while immune response against conjugate 3 (1.342) and conjugate 4 (1.089) were not significantly higher than the OSP alone control (Table 2).

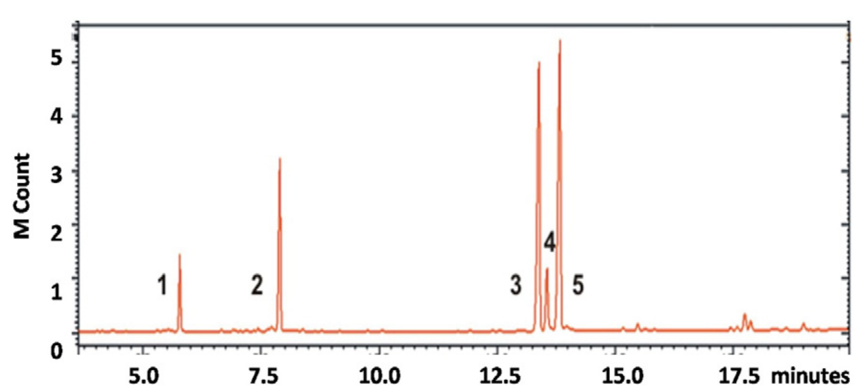

Fig. 1. Alditol acetate assay for Sugar determination in *S.* Typhi OSP. Vi negative *S.* Typhi LSP mixture was partially methylated with alditol acetates. Total ion chromatogram was examined by GC–MS for separation and quantification of LPS. Resulted sugar contents were 1. Tyvelose, 2. Rhamnose, 3. Mannose, 4. Glucose, and 5. Galactose.

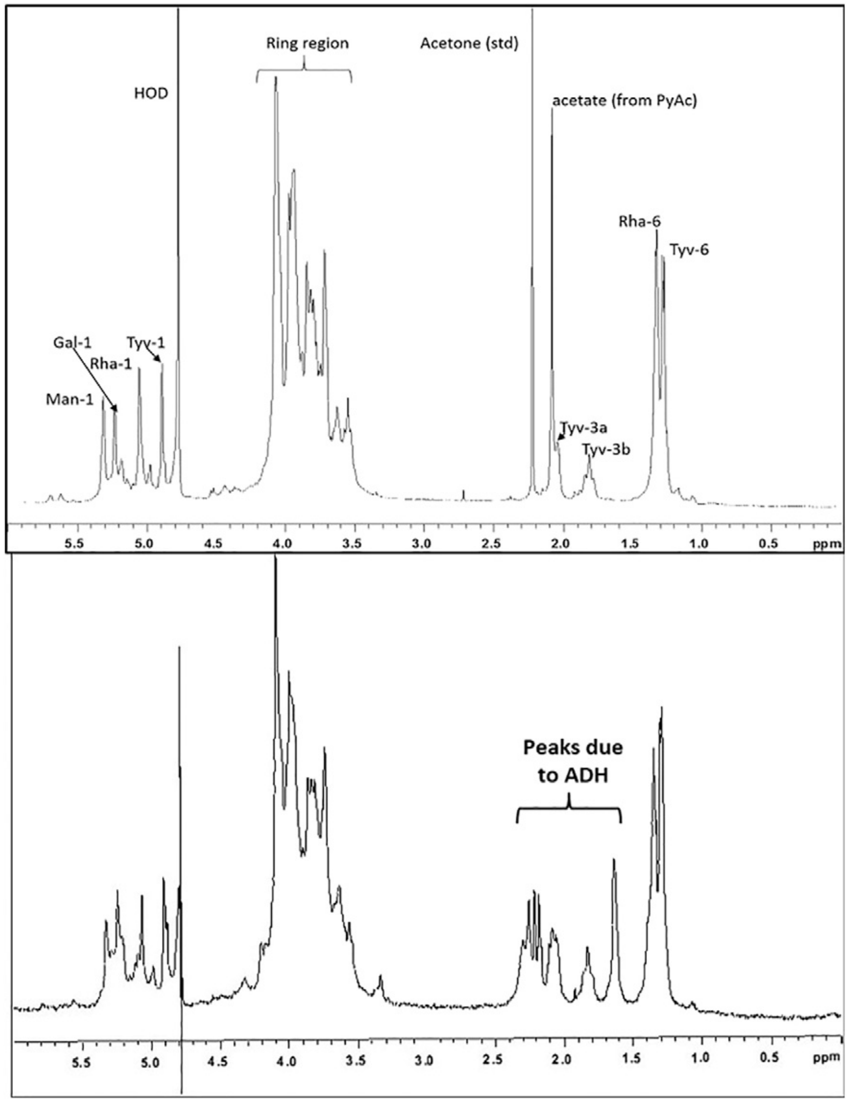

Fig. 2. ^{1}H Spectrum of the NMR for OSP (with and without ADH linker) of *S.* Typhi Vi negative. 400 MHz ^{1}H NMR spectra of two NMR traces, A); contained 1 mg of OSP only in 0.5 ml of D_2O and B); contained 1 mg of OSP-ADH in 0.5 ml of D_2O. Spectra were obtained at an indicated probe temperature of 25 °C. Acetone was used as standard.

3.7. Statistical analysis

Overall analysis of all conjugates along with control using one way ANOVA showed significant difference for these groups. This was further probed with a post hoc analysis using the Tukey-Kramer test, which showed significant differences ($P < 0.05$) for IgG for two of the conjugates. Conjugate 1 ($P = 0.0001$) and conjugate 2 ($P \leq 0.0001$) were significant as compared to OSP control group. Conjugate 3 ($P = 0.1684$) and conjugate 4 ($P = 0.3794$) induced antibody titers that were not significant relative to the control group (Table 2).

The immune response to the OSP with rEPA as the carrier protein was better than with HSA. Furthermore, the ELISA titer was higher for conjugates made using reductive amination method than CDAP method. Therefore, the conjugate 2 (OSP-rEPA; made using reductive amination) was the most immunogenic of the four conjugates.

4. Discussion

S. Typhi strains lacking the Vi antigen have been reported from various regions of the world. These strains have been detected in patients from India, China, Pakistan and Nepal (Arya, 2002; Baker et al., 2005; Wain et al., 2005; Pulickal et al., 2013). In Pakistan, 60 fresh isolates from typhoid patients' blood were examined and 9 (15%) were found negative for both tviA and tviB genes (Baker et al., 2005). In India, 10% of freshly isolated strains were also found to be negative for Vi agglutination (Maurya et al., 2010). Recently, the prevalence of Vi negative *S.* Typhi isolates were confirmed in children from Kathmandu, Nepal. Sixty eight isolates were tested, out of which 5.9% were found negative for capsular expression through slide agglutination test (Pulickal et al., 2013). Conjugate vaccines are among the most efficacious and safest vaccines (Pace, 2013; MacLennan et al., 2014). Various typhoid conjugate vaccines using Vi capsular polysaccharide have been developed using different carrier proteins and conjugation chemistry, e.g., Vi-rEPA, Vi-DT (Diphtheria toxoid) and Vi-CRM_{197} (cross reacting material) (Konadu et al., 2000; Cui et al., 2010; Micoli et al., 2012b). However, these Vi based glycoconjugates are not likely to be effective against Vi negative *S.* Typhi strains. In previous study we have purified and checked the antigenicity of the OSP of Vi negative *S.* Typhi (Salman et al., 2015). Identical OSP antigens of bacterial LPS are found both in Vi positive and Vi negative *S.* Typhi isolates, which cannot be differentiated by morphological or biochemical tests (Baker et al., 2005), however comparative growth of these isolates can be analyzed in-vitro (Liaquat et al., 2015). It implies that vaccines based on these OSP will be universally effective against all *S.* Typhi strains. In this study, LPS of Vi negative *S.* Typhi were isolated in high yield. We obtained 50% greater yield of

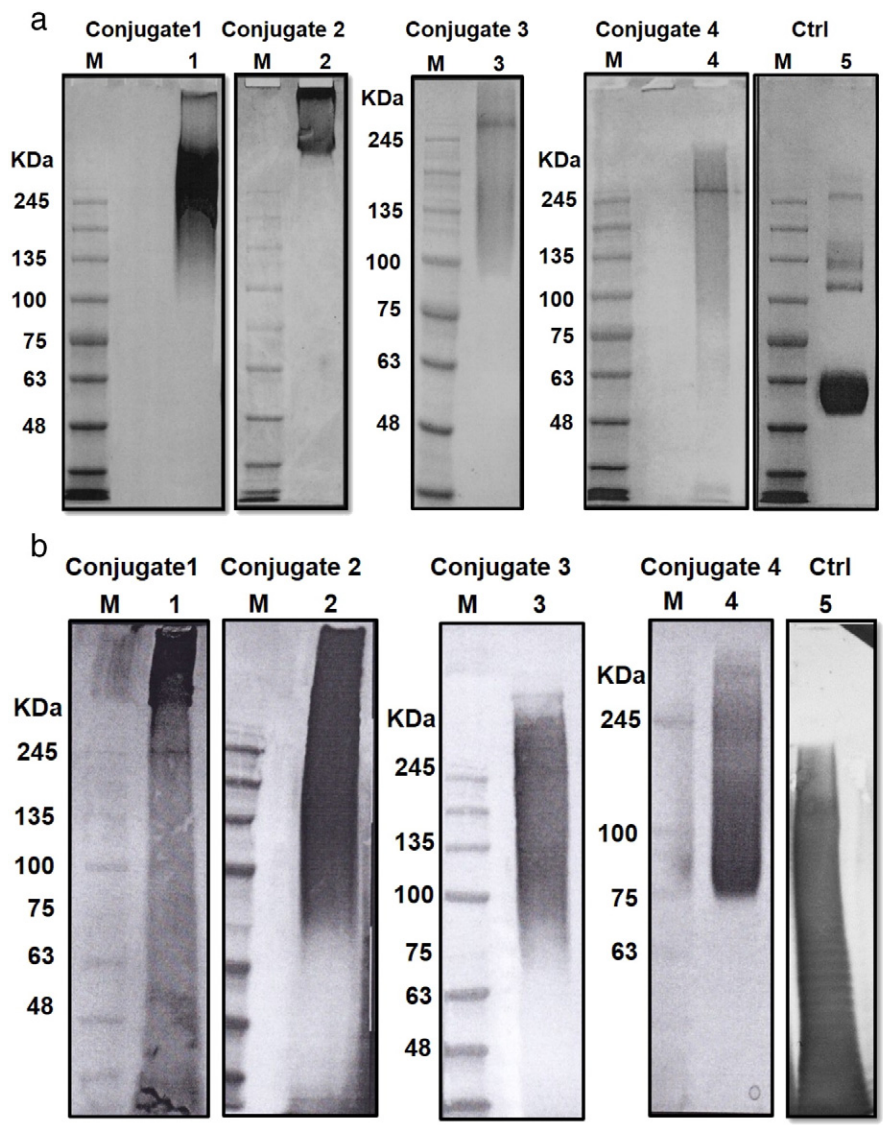

Fig. 3. PAGE and Western blot for conjugates. Coomassie staining and Western blot analysis of carbohydrates and protein conjugates a) Coomassie staining of samples after 15 μl conjugates loading lane 1); conjugate 1 (OSP – HSA) and lane 2); conjugate 2 (OSP-rEPA) were prepared with reductive amination, while lane 3); conjugate 3 (OSP_{ADH}-HSA) and lane 4) conjugate 4 (OSP_{ADH}-rEPA) were prepared with CDAP chemistry; lane 5 shows 1 mg/ml HSA protein. b) Western blot analysis of conjugates 1–4 (lane 1–4) using in-house produced monoclonal antibody developed to *Salmonella* Typhi D1 at a dilution of 1:20,000, lane 5 shows OSP only control. Lane M shows protein ladder of known molecular mass.

OSP as compared with a previous study (Ali et al., 2012). Using GLC-MS we found five types of monosaccharide (glucose, galactose, rhamnose, mannose and tyvelose), as reported in an earlier study (Rahman et al., 1997).

The OSP of *S.* Typhi and *S.* Paratyphi A have previously been conjugated with diphtheria toxoid and CRM_{197} followed by evaluation of immunogenicity in mice (Ali et al., 2012; Micoli et al., 2012a; Ali et al., 2014; Baliban et al., 2017). However, we have found difference in chemical composition of OSPs of Vi positive and Vi negative *S.* Typhi isolates (Jabbar, 2014). To date, no information is available regarding the conjugation of Vi negative *S.* Typhi OSP with proteins. In preparation of conjugate vaccines, parameters such as conjugation chemistry, carbohydrate modification, use of protein-carbohydrate linkers can have a big role (Baliban et al., 2017). In present study we prepared four different conjugates using Vi negative *S.* Typhi OSP and HSA or rEPA as carrier protein. Two of these conjugates were based on reductive amination using cyanoborohydride. In reductive amination, usually the lysine of the protein reacts with reducing end of the sugars. In rEPA protein there were 17 possible attachment sites for binding with OSP, whereas HSA has 61 possible sites to bind with OSP. Two other conjugates of *S.* Typhi were prepared with CDAP chemistry using derivatized OSP (OSP_{ADH}). Although reductive amination was slower as compared to CDAP, we observed better activated yield of OSP. We also linked ADH to OSP as it is more reactive linker (Micoli et al., 2012a). It helped in reduction of the reaction time and increased the conjugation yield.

All four constructed conjugates were analyzed under denaturing conditions of SDS-PAGE to disaggregate the conjugates. We assumed the molecular weight of OSP as 5 kDa, while molecular weights of HSA and rEPA were 66.5 kDa and 69 kDa, respectively. The resulted conjugates were about 120 kDa. Furthermore, we confirmed higher carbohydrate loading OSP-HSA and OSP-rEPA using anti LPS monoclonal antibody in Western Blot pattern of the conjugates; 1 mg/ml of OSP and protein were run as controls.

Immunogenicity of the conjugates was evaluated in mice. IgG titer was determined by indirect ELISA on day 56. The antibody titer was sufficient and recognized target well as previous study (Cox et al., 2013). Although our protein and carbohydrate levels varied considerably from conjugate to conjugate for reasons that we cannot adequately explain, we did follow the same work up for each conjugate and the immunogenicity's as determined by ELISA were not drastically different, possibly suggesting that the quantifications of protein and carbohydrate were somewhat limited. Antibody titres generated by conjugates prepared with reductive amination (OSP-HSA), (OSP-rEPA) were significantly higher ($P < 0.05$) than the OSP control, whereas conjugates synthesized with CDAP chemistry (OSP_{ADH}-HSA), (OSP_{ADH}-rEPA) failed to do so. The relative poor performance of the conjugates with the ADH linkers compared to the direct reductive amination technique, could also partially be due to an immune response to the ADH linkers, diminishing the response to the target carbohydrate epitopes. This distraction would not be possible with the direct reductive amination methodology, since no linkers were involved. Thus higher activities of conjugates, produced through reductive amination over ADH linker, seems to be correlated with the higher modification ratio (ratio of the amount of OSP carbohydrate loading to each protein), as exhibited by conjugates (Table 1, Conjugate No. 1 & 2 versus 3 & 4). These results demonstrate that conjugation ratio may be critical for efficient immunization in this case at least. Between the two tested techniques, the reductive amination yielded the efficient conjugation ratio and consequently gave higher immunization activity (Tables 1 & 2). In addition, reductive amination chemistry was simpler and easier to perform as compared with CDAP chemistry. Nevertheless, direct conjugation (sugars and proteins through reductive amination) without linker demonstrated consistency in immunization, whereas with linker (sugar, ADH and protein) the conjugates yielded highly variable immunization results and lost their consistency (Table 2). This may be due to the facts that linkers have departed away the immunogenic (epitopic) carbohydrates components from the protein through five highly rotatable sp^3 hybridized C—C bonds (present in ADH linker), which may give random, delocalized and variable position to the carbohydrate on the protein surface, generating several kinds of isomeric structures, thereby declining the proper recognition by the immune response. On contrary, the reductive amination process may fix the carbohydrates on protein surface, consequently generating the more signatured, repeatedly recognisable and relatively defined regioizainsomeric glycoconjugate structure better sensed by the immune response (Table 2, lower – higher ELISA readings, Conjugates 1, 2 vs 3, 4). Sugar-protein connectivity may have significant effect on the immunogenicity of conjugates (Stefanetti et al., 2015). However, this area is debatable and needs more investigation to clarify the exact mechanism (Stefanetti et al., 2015). Between the two tested proteins, HSA was found to be the less immunogenic as compared to rEPA protein (Szu, 2013). The higher antibody titers were raised against conjugates containing rEPA, even having less number/amount of carbohydrates as compared to conjugates containing HSA.

Overall, the conjugate 2 (OSP-rEPA) produced the best antibody titres among all of the tested immunogens and produced the most significant results ($P = 0.0001$) as compared to the control group. This work has elucidated that conjugation, specifically by using reductive amination can be easily used to develop conjugate vaccines candidates against Vi negative *S.* Typhi. The OSP-rEPA conjugate vaccine candidate has demonstrated the potential to be a clinically useful vaccine. We would suggest that a bivalent conjugate vaccine based on Vi and OSP or OSP of Vi positive and Vi negative *S.* Typhi could be a very good option for prevention of typhoid in developing countries. Furthermore, in the future, the efficiency of this vaccine could be tested against OSP of *S.* Paratyphi (Rahmat et al., 2016) along with that of *S.* Typhi (Vi positive and negative) as a new vaccine candidate to cover all these strains for its attractive use.

Table 1
Carbohydrate and proteins ratios in conjugates.

Conjugate no.	Conjugate name	Carbohydrate conc. (mg/ml)	Protein conc. (mg/ml)
1	*S.* Typhi OSP-HSA	2.91	0.148
2	*S.* Typhi OSP-rEPA	0.12	0.06
3	*S.* Typhi OSP_{ADH}-HSA	0.25	1.5
4	*S.* Typhi OSP $_{ADH}$-rEPA	0.99	1.0

Table 2
Determination of OSP concentration in Vi negative *S.* Typhi OSP conjugates.

Conjugate name	Mouse name	ELISA reading IgG dilution (1:40)	Geometric means	95% confidence limit		*P* value when compared to OSP alone
				Lower	Higher	
Conjugate 1 (OSP-HSA)	MSTV1	2.304	2.197	2.050	2.350	0.0001
	MSTV2	2.160				
	MSTV3	2.221				
	MSTV4	2.302				
	MSTV5	2.013				
Conjugate 2 (OSP-rEPA)	MSAV1	3.231	3.306	2.926	3.710	<0.0001
	MSAV2	3.703				
	MSAV3	2.949				
	MSAV4	3.583				
	MSAV5	3.124				
Conjugate 3 (OSP_{ADH}-HSA)	MTHV1	2.404	1.342	0.519	2.590	0.1684
	MTHV2	0.693				
	MTHV3	2.221				
	MTHV4	1.802				
	MTHV5	0.653				
Conjugate 4 (OSP $_{ADH}$-rEPA)	MTXV1	3.304	1.089	−0.066	2.954	0.3794
	MTXV2	2.062				
	MTXV3	0.721				
	MTXV4	0.648				
	MTXV5	0.483				
OSP alone as control group	MSTC1	1.334	0.840	0.408	2.954	N/A
	MSTC2	0.862				
	MSTC3	0.521				

Conflict of interest

The authors declare that they have no conflict of interest.

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