Immunological evaluation of Vi capsular polysaccharide of *Salmonella enterica* subsp. Typhi vaccine by serum bactericidal assay

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*Salmonella enterica* subsp. Typhi (S. Typhi) Vi antigen capsular polysaccharide (Vi-CPS) is a licensed vaccine against typhoid fever. As there is no animal model for S. Typhi fever to evaluate the protective efficacy of the Vi-CPS vaccine, a serum bactericidal assay (SBA) is the recommended 'gold standard' to evaluate its potency. Vi-CPS was extracted from S. Typhi Ty6S (CSBPI-B191) using a modified Gotschlich method. Purified Vi-CPS (50 μg) was injected intramuscularly into three groups of five rabbits; group 2 received an additional booster dose of 50 μg Vi-CPS on day 15 and group 3 received two additional boosters on days 15 and 30. The sera obtained from each group were tested by SBA on days 0, 15, 30 and 45. The anti-Vi-CPS titres for groups 1, 2 and 3 on days 15, 30 and 45 were 4, 16 and 16; 4, 32 and 32; and 16, 64 and 64, respectively. Thus, Vi-CPS was shown to be a potent immunogen, as even one dose could induce an efficient bactericidal effect against S. Typhi. Although Vi-CPS is a reliable vaccine, sometimes depolymerization during purification can affect its potency, which can be resolved through a potency test. As the passive haemagglutination test recommended by the World Health Organization does not indicate vaccine potency, we recommend using an SBA to evaluate the bactericidal ability of Vi-CPS.

INTRODUCTION

Typhoid fever remains a serious health threat in developing countries, with 12.5 million reported cases annually. The final goal is control of this infection through vaccination. The classical typhoid + paratyphoid A + paratyphoid B (TAB) vaccine and oral *Salmonella enterica* subsp. Typhi (S. Typhi) Ty21a vaccine offer good immunity production in humans, but neither is a complete and ideal vaccine, resulting in the necessity for further studies (Mastroeni & Ménager, 2003; Tang *et al.*, 2003). It has been shown that the S. Typhi Vi capsular polysaccharide (Vi-CPS) subunit vaccine can prevent typhoid fever. A single intramuscular injection of this vaccine conferred about 65 and 72% protection in South Africa and Nepal, respectively (Acharya *et al.*, 1987; Klagman *et al.*, 1987; Szu *et al.*, 1994; Tang *et al.*, 2003; Chibber & Bhardwaj, 2004). The Vi-CPS vaccine has now been licensed in >93 countries worldwide, although it is not commonly used as part of vaccination programmes in developing countries. The antibody response to the Vi-CPS vaccine is measured using various serological methods. Serum bactericidal antibody activity has shown that there is a direct correlation between application of the Vi-CPS vaccine and the induction of both natural and vaccine-induced immunity to typhoid fever (Yang *et al.*, 2001; Tang *et al.*, 2003; Canh *et al.*, 2004; Fadeel *et al.*, 2004; Wain *et al.*, 2005). The induction of complement-dependent bactericidal antibodies after vaccination with Vi-CPS has been widely accepted as evidence of the potential efficacy of this vaccine (WHO, 1981). However, as the animal models used for Vi-CPS vaccine efficacy testing do not cause typhoid fever with human pathogenic strains, in order to evaluate the protective efficacy of the Vi-CPS vaccine, we recommend the serum bactericidal assay (SBA) as a 'gold standard' method.

METHODS

Bacterial strains. Vi-CPS vaccine was produced from a standard rough strain of S. Typhi Ty6S (CSBPI-B191), which is rich in CPS. Hyperimmune Vi antiserum was prepared by injection of *Citrobacter bullerup* (CSBPI-A124) in rabbit. *C. bullerup* and S. Typhi share some antigenic structures in their CPS, resulting in cross-immunity.
between these two bacteria (Nolan et al., 1980). These strains were obtained from the Collection of Standard Bacteria of the Pasteur Institute of Iran (CSBPI).

**Culture.** S. Typh Ty6s was grown in 90 l modified Frantz medium in a Novo-Paljas unit bioreactor (Frantz, 1942). The fermentation parameters were adjusted to 36 ± 1 °C, pH 7.6, 450 r.p.m. final speed of rotation and air flow of 5 l h⁻¹ (Ahmadi et al., 1999).

**Purification of Vi-CPS.** At the late exponential growth phase (after 12 h), the cells were harvested by centrifugation at 3500 g at 4 °C. The supernatant was treated with 1 g cetyltrimethylammonium bromide (Fluka Chemical) L⁻¹ with continuous shaking at 4 °C for 30 min, and after centrifugation at 3500 g at 4 °C for 30 min, the precipitate (crude Vi polysaccharide) was dissolved in 1 M CaCl₂, and further purification was performed by alcohol fractionation, cold phenol extraction, alcohol precipitation and ultracentrifuge differentiation, using a Beckman Instruments LM 80 ultracentrifuge (Gotschlich, 1975; WHO, 1981). Purified Vi-CPS was washed twice with alcohol, acetone and diethyl ether. The extracted sample was then filter sterilized and dispensed in 50 ml vials, dried in a P₂O₅ desiccator at 37 °C and kept at -20 °C until used.

**Moisture content.** The moisture content of the purified Vi-CPS was determined using the Karl Fischer method.

**O-Acetyl content.** The O-acetyl content of the purified Vi-CPS was determined according to the method of Hestrin (1949), using acetyltoluidine chloride as a reference.

**Nucleic acid and protein contamination.** Nucleic acid content was determined by UV spectroscopy (WHO, 1974) and protein content was determined using the method of Lowry et al. (1951) using BSA as a reference.

**Chromatography.** Size exclusion analysis of Vi-CPS was performed by gel filtration chromatography, using a Sepharose 4B-CL column (1.5 × 85 cm). The polysaccharide was eluted with 0.2 M sodium chloride containing 2 mM sodium azide (WHO, 1976, 1981).

**Sterility test.** The purified Vi-CPS was tested for bacterial and mycotic sterility according to WHO (1973).

**Abnormal toxicity test.** The purified Vi-CPS was tested for abnormal toxicity by intraperitoneal injection of 50 µg purified Vi-CPS into five mice (weighing 17–22 g each) and 250 µg purified Vi-CPS into two guinea pigs (weighing 250–350 g each). The test was considered satisfactory if the animals survived for at least 7 days without weight loss (WHO, 1994).

**Pyrogenicity studies.** The purified Vi-CPS was tested for pyrogenic activity by intravenous injection into New Zealand white rabbits (weighing 2–2.5 kg each). Three healthy rabbits were used in each test. The polysaccharide was reconstituted in special diluents. Further dilution was carried out in pyrogen-free physiological saline. Each rabbit received 0.0025 µg purified Vi-CPS kg⁻¹ (WHO, 1979).

**Immunogenicity studies.** Purified Vi-CPS (50 µg) in 0.5 ml was injected intramuscularly into three groups of five rabbits each. Group 2 received a booster dose of 50 µg Vi-CPS on day 15, whilst group 3 received two boosters of 50 µg Vi-CPS on days 15 and 30. Each group of pooled serum was tested by SBA on days 0, 15, 30 and 45.

**ELISA.** The cross-titration table (plate checkerboard) method was used to determine the optimum serum titres. Vi-CPS (2 mg) was dissolved in 100 ml 0.05 M carbonate buffer (pH 9.6) and 100 µl was added to each well of the plate. The plate was incubated overnight at 4 °C. The following day, the plate was washed three times with buffer [0.05% Tween 20 and 3% sodium azide in 0.01 M PBS (pH 7.2)] and the diluted sera were added to each well. The plate was incubated for 6 min at room temperature. After rewashing the wells with buffer, goat anti-mouse antibodies were diluted 1:1000 in PBS and 100 µl was added to each well and incubated for 1 h at room temperature. After washing, horseradish peroxidase-conjugated goat anti-IgG (diluted 1:3000) was added to the wells and incubated, and substrate was then added. After 15 min, 50 µl stopping buffer (1 M sulfuric acid) was added, and absorbance at 450 nm (A₄₅₀) was read using an ELISA reader (Sunrise Microplate Reader, Tecan).

**SBA.** S. Typhi Ty2 (CSBPI-B190; Collection Standard Bacterial Pasteur Institute, Tehran, Iran) was used in the SBA for this study. The strain was cultured in brain–heart infusion (BHI) agar for 18 h. The culture was diluted in PBS (pH 7.2) to give ~10⁵ c.f.u. ml⁻¹. Sera collected from two S. Typhi-negative 3-week-old baby rabbits were used as the complement source, and were filter sterilized with a 0.45 µm filter and kept at −20 °C until use. The serum samples were treated at 56 °C for 30 min prior to use to inactivate the complement activity (Holst et al., 2003). The SBA was performed in sterile 96-well polystyrene microplates. The heat-inactivated
A

Table 1. Antibody titres determined by SBA

Rabbits were injected intramuscularly with 50 μg Vi-CPS in 0.5 ml. Rabbits in group 2 received a booster on day 15 and those in group 3 received boosters on days 15 and 30.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of injections</th>
<th>Titre Day 0</th>
<th>Titre Day 15</th>
<th>Titre Day 30</th>
<th>Titre Day 45</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>&lt;2</td>
<td>4</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>&lt;2</td>
<td>4</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>&lt;2</td>
<td>16</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

serum samples (50 μl) were serially diluted in PBS (pH 7.2), and 25 μl bacterial suspension and 25 μl baby rabbit complement were then added to each well. Control wells contained: (i) bacteria plus complement; (ii) bacteria plus sample serum; and (iii) bacteria plus PBS. The microplates were incubated at 37 °C for 1 h. Before and after incubation, 10 μl from each well was plated onto BHI agar plates, which were incubated for 24 h at 37 °C. The number of colonies before (T0) and 1 h (T1) after incubation were counted. The actual number of c.f.u. per well added before incubation was obtained by multiplying by three the mean colony count after overnight incubation. The serum bactericidal titre is reported as the reciprocal of the highest serum dilution yielding >50% bacterial killing compared with the number of c.f.u. present before incubation with serum and complement at T0.

Identity test. The identity of the Vi-CPS was estimated using the Ouchterlony gel (Hudson & Hay, 1989) double diffusion technique using hyperimmune serum prepared from multiple injections of a standard culture of C. ballerup Vi+ (CSBP1-A124). The Ig was purified and concentrated against 45% saturated ammonium sulphate.

RESULTS

The biochemical and some of the immunological properties of purified Vi-CPS prepared on a semi-industrial scale in a 90 l bioreactor were studied by different methods. S. Typhi Ty6s was grown under controlled submerged cultural conditions and Vi-CPS was extracted from the fermentation broth. The product was highly purified, containing >4.7 mM O-acetyl and <10 mg protein and <20 mg nucleic acid (g polysaccharide)−1 as contaminants. The moisture content was <2.5% of the dry weight of the polysaccharide.

Table 2. Serum IgG titres against Vi-CPS determined by ELISA

Results are shown as A450 units (means ± sd). *P<0.01 compared with the control group.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Serum titre Two weeks after first injection</th>
<th>Serum titre Two weeks after second injection</th>
<th>Serum titre Two weeks after third injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vi-CPS</td>
<td>131.2 ± 7*</td>
<td>400.0 ± 9*</td>
<td>550.4 ± 11*</td>
</tr>
<tr>
<td>None (negative control)</td>
<td>0.2 ± 0.03</td>
<td>0.1 ± 0.03</td>
<td>0.1 ± 0.05</td>
</tr>
</tbody>
</table>

Chromatography

As shown in Fig. 1, Vi-CPS was eluted as a high-molecular-mass aggregate with two major adjacent peaks in tube numbers 25–29 and 30–40, respectively.

Gel precipitation reaction

Gel precipitation was used to determine the identity of the product. Fig. 2 shows that there was a single precipitation line of identity exhibited by the purified monospecific polyclonal anti-Vi antibodies in the central well and wells containing highly purified Vi-CPS. The well containing CPS of N. meningitidis type A showed no cross-reaction with Vi antiserum. In addition, anti-LPS antibodies of S. Typhi Ty2 did not exhibit any precipitation line of reaction with Vi-CPS.

Determination of anti-Vi-CPS antiserum titre

The bactericidal antibody titres of immunized rabbits were determined by SBA and the anti-IgG titre of the sera evaluated by ELISA. As shown in Table 1, the final bactericidal antibody titres of Vi-CPS for groups 1, 2 and 3 were 1:16, 1:32 and 1:64, respectively. Table 2 shows that the titres of anti-IgG against Vi-CPS were 131.2, 400.0 and 550.4, respectively, after the first, second and third injections.

DISCUSSION

The role of Vi-CPS as a protective antigen against infection caused by S. Typhi is well known (Kim et al., 1995; Lim et al., 2007), but production of Vi-CPS in its native configuration is problematic. Being a sensitive molecule in nature, depolymerization sometimes takes place during steps in product recovery, which in turn results in a loss of potency (Szu et al., 1991). To overcome this problem, standard potency tests should be performed on the final products. However, as S. Typhi is a human parasite, there is no reliable animal model for such a potency test. Klugman et al. (1996) and Lim et al. (2007) recommended a passive haemagglutination test to evaluate the level of anti-Vi-CPS antibodies in sera of the vaccinees. In addition, the World Health Organization recommended a challenge potency test using mice sensitized with mucin to evaluate the potency of both classical whole-killed cell and Vi-CPS vaccines (Kasi, 1964). As both of these tests have drawbacks, Maslanka et al. (1997) and Rodriguez et al.
(2002) performed SBAs to assess the rate of antibacterial activity of people vaccinated with \textit{N. meningitidis} CPS. As shown in the present investigation, live pathogenic \textit{S. Typhi} was also killed when challenged with the pooled sera of mice vaccinated with Vi-CPS, and these data matched the results obtained by ELISA. Therefore, SBA can be regarded as a standard and reliable method to evaluate the potency of the Vi-CPS vaccine.

**REFERENCES**


Immunogenicity, efficacy and serological correlate of protection of \textit{Salmonella typhi} Vi capsular polysaccharide vaccine three years after immunization. \textit{Vaccine} 14, 435–438.


