Original Article

Immune response to Vi polysaccharide, heat-killed whole cells, and outer membrane protein of *Salmonella typhi*

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Abstract

Introduction: *Salmonella typhi* Vi capsular polysaccharide (ViCPS) is a licensed vaccine against typhoid fever in many countries; in Egypt, the killed whole-cell vaccine is still used. In this study, mice were used as an animal model to evaluate the immune response to ViCPS and other *S. typhi* antigens such as heat-killed whole cells and outer membrane protein (OMP).
Methodology: The three antigens were laboratory prepared, injected into mice groups, and the humoral response was evaluated using the indirect whole-cell enzyme-linked immunosorbent assay (ELISA). The sensitivity of this assay was investigated using *in situ* or pre-heated whole cells as coating antigens. In addition, the effect of the immunization route for ViCPS was examined.
Results: Immunizing doses of heat-killed whole cells as well as ViCPS, 2 and 4 µg given subcutaneously (SC) and 4 µg given intraperitoneally (IP), showed significant immune response compared to controls. However, the responses to these doses were not significantly different from each other. The OMP showed a higher significant response. The sensitivity of indirect whole-cell ELISA was enhanced significantly by *in situ* heat treatment of the coating antigen rather than the pre-heated coating antigen.
Conclusions: The three antigens showed significant immune response. The immune response to OMP was higher. Though heat-killed whole cells and ViCPS are almost similar in immunizing level, ViCPS is recommended. The SC route was more immunizing than the IP one. Furthermore, the sensitivity of the indirect whole-cell ELISA technique could be enhanced by *in situ* heat inactivation of the coating cells.

Key words: *Salmonella typhi*; Vi capsular polysaccharide; outer membrane protein; ELISA; immune response.

Introduction

Salmonella typhi (*S. typhi*) is the causative agent of typhoid fever, a serious health problem in many regions of the world, especially in developing countries such as Egypt. The global annual incidence was reported to be 21 million cases with an annual fatality rate of 200,000 deaths (1%–4% deaths worldwide) [1]. In Egypt, population-based surveillance indicated a moderate incidence of typhoid fever, where the annual incidence rate was estimated to be about 59/100,000 persons [2]. The earliest available vaccine was the TAB vaccine (combining typhoid, paratyphi A,-and paratyphi B), but due to its increased reactogenecity and increased incidence of systemic side effects, its use was undermined and it is no longer commercially available [3]. Currently, the available vaccines for *S. typhi* are the oral living attenuated vaccine of Ty21a strain (VIVOTIF), as well as the Vi capsular polysaccharide (ViCPS) vaccine (TYPHIM Vi,TYPHERIX). The attenuated vaccines are formulated as coated capsules, and three doses are given every other day to individuals older than five years of age. However, Ty21a, being a live attenuated vaccine, is not recommended for use in immune-suppressed individuals, while in the case of Vi capsular polysaccharide vaccine, only a single intramuscular (IM) dose is given to all individuals older than two years of age [4-6].

The vaccine of Vi capsular polysaccharide is recommended by the World Health Organization (WHO) as the vaccine of choice for typhoid fever, as it has many advantages over other vaccines, including that it is free from endotoxin (LPS), is without systemic side effects, requires only a single dose to yield consistent immunogenicity and efficacy, and can be given to immune-compromised individuals over two years of age and to individuals receiving antibiotic therapy [7-11].

Accordingly, the present study aimed to evaluate the serological responses of different antigens prepared from ViCPS of a standard strain, outer membrane protein (OMP) fraction, another candidate

vaccine against *S. typhi* that is still under investigation, to that of heat-killed whole cells.

Methodology

Bacterial strains

A standard strain of *Salmonella typhi* Ty2 was used for preparing ViCPS, OMP, and heat-killed whole cells. This strain is recommended by the WHO for the preparation of ViCPS [12].

Culturing conditions

Salmonella typhi Ty2 was grown in 100 mM phosphate-buffered media containing 10 g l^{-1} glucose, 10 g l^{-1} yeast extract dialysate, and 5 g l^{-1} casamino acid at 32°C and pH 7.2 [13].

Fractionation and purification of ViCPS antigen

A modified method described by Gotschlich *et al.* [14] was applied. Briefly, the bacterial liquid culture was centrifuged at 4,000 rpm for 20 minutes and the ViCPS was obtained by precipitation, from the supernatant, using hexadecyl-trimethyl ammonium bromide (cetavlon). The precipitate (crude Vi -CPS) was purified by dissolving in 1 M $CaCl_2$, followed by alcohol fractionation, phenol extraction on cold, then alcohol precipitation of crude polysaccharide and finally ultracentrifugation (Beckman L8-80M ultracentrifuge, Beckman Coulter Inc., Brea, USA). The extracted sample containing ViCPS was lyophilized (Lyph-Lock 4.5 lyophilizer, Labconco, Kansas City, USA) and was stored at -20°C until further use.

The *O*-acetyl content of the purified ViCPS was determined according to the method of Hestrin [15], using acetylcholine chloride as a reference.

Isolation and purification of OMP fractions

This was carried out using two different extraction buffers; the first one followed the method of Foulaki *et al.* [16] modified by Hamid and Jain [17], using Tris extraction buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, and 6 M urea). In brief, the harvested bacterial cells were washed using 10 mM Tris-HCl (pH 7.5), then extracted with the extraction buffer (20 mL/1 g wet bacteria shaken using a Bellco roller drum, Bellco Glass Inc., Vineland, USA) for one hour at 4°C. The extract was dialyzed against distilled water, centrifuged, and the supernatant containing surface proteins was collected. It was then sterilized by filtration, lyophilized, and stored at -20°C until further use. In the second method of OMP preparation, lithium chloride was used as an extraction buffer

following a modified Frasch method [18]. The harvested bacterial pellet was washed with 0.1 M phosphate buffered saline (PBS) (pH 7.2), and was extracted with 0.2 M $LiCl_2$ in 0.1 M acetate buffer (pH 5.8) with rapid shaking, for two hours in a water bath at 45°C. The extract was then centrifuged, dialyzed against sterile 0.1 M PBS, sterilized by filtration, and kept at -20°C until use. The protein content in the samples was estimated using Lowry *et al*'s. method [19].

Preparation of heat-killed whole cells

The method of Cronlly-Dillon [20] was used. The bacterial suspension was heated at 56°C for one hour in a water bath, and complete killing was confirmed by the absence of viable bacteria after treatment.

Ethics statement

Animals were housed in accordance with the principles of laboratory animal care etsbalished by National Institutes of Health (NIH) [21]. The experimental protocol was approved as well by the Faculty of Pharmacy, Cairo University/VACSERA ethical committees.

Laboratory animals

Outbred Swiss albino mice weighing approximately 20–22 g were obtained from Helwan farm for laboratory animals. They were housed at 25°C in the animal house of the research center of the Egyptian Holding Company for Biological Products and Vaccines (VACSERA, Agouza, Egypt). They were fed a *Salmonella*-free pellet diet and supplied with clean drinking water. Existing *Salmonella* infection of the used mice was ruled out by culturing tissues from randomly selected test mice.

Immunization schedule

Mice were divided into 11 groups of 10 mice each, including the control groups. Four groups were injected with Vi antigen, two of which were subcutaneously (SC) injected, one with 2 µg and one with 4 µg ViCPS. The other two groups were intraperitoneally (IP) injected, one with 2 µg and one with 4 µg ViCPS at days 0, 14, and 28.

In case of Tris-extracted OMP, 50 µg of the antigens, determined by the method of Lowry *et al.* [19], was SC injected at days 0, 7, 21, and 28, and a booster dose was administered at day 40. The test was repeated in another group but using lithium-extracted OMP [17]

The test group for the heat-killed whole cells was IP injected with two doses of 10^8 heat-killed cells (in 200 μL PBS) one week apart. For the different tested antigens, a control group was included for comparison under similar experimental conditions with PBS. Antisera obtained from immunized mice one week after the final immunization were pooled, and 20 μL aliquots of each were stored at -70°C until use.

Indirect whole-cell ELISA assay

The assay was done using the method of Tsang and Zollinger [22] with some modifications. In brief, bacterial suspension of an overnight *S. typhi* Ty2 cultured on a brain heart solid agar was resuspended in sterile PBS (pH 7.4) inactivated in a water bath at 56°C for 60 minutes. The absorbance of bacterial suspension was adjusted to an absorbance of 0.1 at 620 nm by dilution in sterile PBS. From this suspension, 100 μL/well was used as an ELISA antigen for coating. Capsular polysaccharide-binding microtiter plates were used (Nunc Covalink NH plate well; Nunc, Wiesbaden, Germany). The plates were overnight incubated at 37°C to allow drying and adherence of bacterial cells onto the wells. Another plate was coated with live bacterial suspension where 50 mL of a fresh overnight culture, cultured on a brain heart solid agar, was suspended in PBS; 10 mL was heat inactivated at 56°C for 60 minutes, and its absorbance was adjusted to be 0.1 at 620 nm. By knowing the dilution factor, the living bacterial suspension was adjusted to the same absorbance and was used for coating another plate (100 μL/well). The microorganism was *in situ* heat inactivated by exposing the plate to 56°C for 60 minutes and incubating it at 37°C for one hour. The two plates were washed five times by 0.01 M PBS with 0.05% Tween 20. The plates were blocked by 1% bovine serum albumin in PBS (1% BSA in PBS) for one hour at room temperature. After washing, 100 μL PBS-diluted sera were added (per well), incubated at room

temperature for one hour, and then washed. Effective dilution factors were applied (1:250, 1:500, and 1:1,000), as pre-determined by titration.

Horseradish peroxidase goat anti-mouse IgG conjugate, 1:1,000 whole molecule in PBS, was used to detect the bounded immunoglobulins. The reactants were incubated at 37°C for one hour, washed, and then *ortho*-phenylenediamine dihydrochloride (OPD) substrate was added. The reaction was left at room temperature, for color development, before it was terminated by 4N H_2SO_4 (50 μL/well). A BIO-TECK ELx800 ELISA reader (Biotek, Winooski, USA) was used to read the ELISA plates at 492 nm. In general, a volume of 200 μL per well were used in blocking and washing steps. The optical density was then plotted against sera dilution.

Statistical analyses

The data were expressed as the arithmetic mean ± the standard deviation (SD). The differences between mean antibody responses, to different test antigens was statistically analyzed with SPSS version 22 using ANOVA one-way Duncan multiple comparison test, with $p \leq 0.05$ accepted as significant.

Results

As shown in Table 1, the response to the lower dose (2 μg), injected via IP route, was not significantly different from that of controls, but significant difference was recorded to 4 μg ViCPS when compared to lower dose (2 μg) and controls. In contrast, the results obtained in Table 2 revealed that the response to lower dose (2 μg), injected via SC route, was significantly different from controls, but no significant difference was found in response to both dose levels (4 μg and 2 μg ViCPS). These results could be summarized by saying the low dose (2 μg) was immunizing when administered SC rather than IP. In both tables, the response to 4 μg ViCPS, injected either IP or SC, was significantly higher in cases of

Table 1. Antibody responses to intraperitoneal injection of 2 and 4 μg *S. typhi*ViCPS, using indirect ELISA assay, for *in situ* heated and pre-heat-treated (56°C/60 minutes) whole cell as a coating antigen

Sera dilution	Mean antibody response * ± SD (p ≤ 0.05)					
	In situ heated coating antigen			Pre-heat-treated coating antigen		
	Control	2 μg ViCPS	4 μg ViCPS	Control	2 μg ViCPS	4 μg ViCPS
1:250	1.405 ± 0.032	1.472 ± 0.007	1.952^{S*} ± 0.117	1.375 ± 0.015	1.38± 0.064	1.801^{SS} ± 0.02
1:500	0.96 ± 0.046	1.036 ± 0.055	1.741^{S*} ± 0.181	0.911 ± 0.031	0.951 ± 0.062	1.396^{SS} ± 0.013
1:1,000	0.588 ± 0.059	0.603 ± 0.024	1.369^{S*} ± 0.152	0.505 ± 0.016	0.554 ± 0.053	1.04^{SS} ± 0.010

ViCPS: Vi capsular polysaccharide antigen; SD: standard deviation; *: Mean of duplicate determinations of absorbance measured at 492 nm; S*: Significant responses compared to their control, 2 μg ViCPS and the corresponding response of 4 μg using pre-heated coating antigen; SS: Significant antibody responses compared to their control and the response of 2 μg ViCPS

using *in situ* heated whole cells as coating antigens when compared to their response in cases of pre-heated coating antigen.

The above findings are supported by results illustrated in Table 3; upon comparing the responses of both doses given by both routes, when using *in situ* heated coating antigens, the antibody responses to low dose (2 µg SC injected) of ViCPS antigen as well as to high dose (4 µg) of ViCPS antigen injected either SC or IP were significantly higher than the response to IP injected 2 µg ViCPS. However, the responses to 2 µg SC and to 4 µg IP or SC, at all dilution levels, showed no significant differences when compared to each other.

The results obtained in Tables 4 and 5 show that the responses after four immunizing doses of both Tris-extracted and lithium-extracted OMP antigen, within four weeks, were significantly higher than in controls. However, this response significantly increased after a booster dose, given at day 40, and the response was observed to be significantly higher when using the *in situ* heated whole cell as a coating antigen.

In Table 6, the antibody response to heat-killed whole cell antigen immunization in mice was significantly higher than that in controls, and this response was significantly enhanced using the *in situ* heated whole cell as a coating antigen in the indirect ELISA assay, which reflects the reliability of the assay in the determination of the serological response.

The response to vaccination through different routes, antigens, and doses (Table 7) could be summarized by saying that no significant difference in

Table 2. Antibody responses to subcutaneous injection of 2 and 4 µg *S. typhi*ViCPS, using indirect ELISA assay, for *in situ* heated and pre-heat-treated (56°C/60 minutes) whole cell as a coating antigen

Sera Dilution	Mean antibody response * ± SD (p ≤ 0.05)					
	In situ heated coating antigen			Pre-heat treated coating antigen		
	Control	2 µg ViCPS	4 µg ViCPS	Control	2 µg ViCPS	4 µg ViCPS
1:250	1.4 ± 0.077	1.790$^{S°}$ ± 0.003	2.142^{SS*} ± 0.113	1.398 ± 0.045	1.819$^{S°}$ ± 0.002	1.777$^{S°}$ ± 0.045
1:500	1.104 ± 0.015	1.772$^{S°*}$ ± 0.108	1.902$^{S°*}$ ± 0.069	1.052 ± 0.014	1.609$^{S°}$ ± 0.035	1.560$^{S°}$ ± 0.025
1:1,000	0.706 ± 0.038	1.482$^{S°}$ ± 0.124	1.587$^{S°*}$ ± 0.152	0.714 ± 0.016	1.326$^{S°}$ ± 0.033	1.227$^{S°}$ ± 0.05

ViCPS: Vi capsular polysaccharide antigen; SD: standard deviation; *: Mean of duplicate determinations of absorbance measured at 492 nm; S°: Significant antibody responses to their control only; S°*: Significant responses compared to their control and the corresponding response of pre-heated coating antigen; SS*: Significant response compared to their control, 2 µg ViCPS, and the corresponding response of 4 µg using pre-heated coating antigen.

Table 3. Comparison between the antibody responses to ViCPS injected subcutaneously and intraperitoneally, using indirect ELISA assay, for *in situ* heated (56°C/60 minutes) whole cell as a coating antigen

Sera dilution	Mean antibody response* ± SD (p ≤ 0.05)			
	2 µg ViCPS (IP injected)	2 µg ViCPS (SC injected)	4 µg ViCPS (IP injected)	4 µg ViCPS (SC injected)
1:250	1.472 ± 0.007	1.790^{S} ± 0.003	1.952^{S-} ± 0.117	2.142^{SS} ± 0.113
1:500	1.036 ± 0.055	1.772^{S} ± 0.108	1.741^{S} ± 0.181	1.902^{S} ± 0.069
1:1,000	0.603 ± 0.024	1.482^{S} ± 0.124	1.369^{S} ± 0.152	1.587^{S} ± 0.152

ViCPS: Vi capsular polysaccharide antigen; SD: standard deviation; SC: subcutaneously; IP: intraperitoneally; *: Mean of duplicate determinations of absorbance measured at 492 nm; S: Significant antibody responses compared to IP-injected 2 µg ViCPS only; S⁻: Significant antibody response compared to IP-injected 2 µg ViCPS but not significantly different from SC-injected 2 or 4 µg ViCPS; SS: Significant antibody response compared to 2 µg ViCPS injected IP and SC.

Table 4. Antibody responses to subcutaneous injections of Tris Buffer extracted *S. typhi* outer membrane protein antigen, using indirect ELISA assay, for *in situ* heated and pre-heat-treated (56°C/60 minutes) whole cell as a coating antigen

Sera dilution	Mean antibody response* ± SD (p ≤ 0.05)					
	For *in situ* heated coating antigen			For pre-heat-treated coating antigen		
	Control	4 doses (50 µg) within 4 weeks	50 µg booster dose at day 40	Control	4 doses (50 µg) within 4 weeks	50 µg booster dose at day 40
1:250	1.409 ± 0.035	2.029^{S} ± 0.014	2.495^{S*} ± 0.085	1.383 ± 0.01	2.112^{S} ± 0.029	2.28^{SS} ± 0.051
1:500	1.078 ± 0.036	1.779^{S} ± 0.015	2.394^{S*} ± 0.103	0.778 ± 0.04	2.008^{S} ± 0.002	2.164^{SS} ± 0.013
1:1000	0.710 ± 0.005	1.581^{S} ± 0.409	2.319^{SS} ± 0.354	0.58 ± 0.18	1.838^{S} ± 0.052	1.983$^{S°}$ ± 0.06

OMP: outer membrane protein antigen; SD: standard deviation; SC: subcutaneously; *: Mean of duplicate determinations of absorbance measured at 492 nm; S: Immunizing doses showed significant responses compared to controls; S°: Significant antibody response to control only; S*: Significant responses compared to control, immunizing doses, and the corresponding response of pre-heated coating antigen; SS: Significant response, after a booster dose, compared to controls and to the responses of immunizing doses.

response using the heat-killed whole cells, 4 µg ViCPS IP injected or SC injected. In addition, in spite of being slightly higher, the response to lithium buffer-extracted OMP was not significantly different than the Tris buffer-extracted OMP. However, both lithium buffer-extracted and Tris buffer-extracted OMP antigens resulted in a more significant response than the other three mentioned antigens.

Discussion

Because of unsuitable level of efficacy and some unacceptable side effects of the currently available typhoid fever vaccines, research into new immunogens suitable for vaccination has become pertinent. Several typhoid vaccines are licensed for use including Vi polysaccharide vaccine (ViCPS) and Ty21a live oral vaccine. However, other subunit antigens, such as *Salmonella* OMPs, have been considered to be possible candidates for conferring protection against typhoid. This study was conducted to compare the humoral immune response of laboratory-prepared *S. tyhpi* antigens, including ViCPS, OMP (porins) and heat-killed whole cells, traditionally used for immunization against *S. typhi*,in Egypt. In addition, the effect of vaccination route of ViCPS was studied. This Vi antigen enabled *S. typhi* to survive in the blood by preventing the binding to anti-O antigen, inhibiting complement activation and complement-mediated lysis as well as phagocytosis [23,24]. Therefore, the development of serum-specific anti-Vi

Table 5. Antibody responses to subcutaneous injections of lithium buffer-extracted *S. typhi* outer membrane protein, using indirect ELISA assay, for *in situ* heated and pre-heat-treated (56°C/60 minutes) whole-cell as a coating antigen

Sera dilution	Mean antibody response* ± SD (p ≤ 0.05)					
	For *in situ* heated coating antigen			For pre-heat-treated coating antigen		
	Control	4 doses (50 µg) within 4 weeks	50 µg booster dose at day 40	Control	4 doses (50 µg) within 4 weeks	50 µg booster dose at day 40
1:250	1.409 ± 0.035	2.065[S] ± 0.011	2.539[S*] ± 0.113	1.383 ± 0.015	2.112[S] ± 0.055	2.333[SS] ± 0.024
1:500	1.078 ± 0.036	2.243[S] ± 0.011	2.495[S*] ± 0.095	0.778 ± 0.042	2.122[S] ± 0.015	2.243[SS] ± 0.021
1:1000	0.71 ± 0.005	2.254[S] ± 0.002	2.47[S*] ± 0.056	0.580 ± 0.183	2.227[S] ± 0.077	2.235[S°] ± 0.03

OMP: outer membrane protein antigen; SD: standard deviation; *: Mean of duplicate determinations of absorbance measured at 492 nm; S: Immunizing doses showed significant responses compared to controls; S°: Significant antibody response to control only; S*: Significant responses compared to control, immunizing doses, and the corresponding response of pre-heated coating antigen; SS: Significant response, after a booster dose, compared to controls and to the responses of immunizing doses.

Table 6. Antibody responses to intraperitoneal injection of *S. typhi* heat-killed whole cells, using indirect ELISA assay, for *in situ* heated and pre-heat treated (56°C/60 minutes), whole-cell coating antigen

Sera dilution	Mean antibody response* ± SD (p ≤ 0.05)			
	For in situ heated coating antigen		For pre-heat treated coating antigen	
	Control	HK whole cells	Control	HK whole cells
1:250	1.396 ± 0.018	2.109[S*] ± 0.087	1.282 ± 0.056	1.778[S] ± 0.001
1:500	1.09 ± 0.024	1.903[S*] ± 0.007	0.922 ± 0.012	1.502[S] ± 0.065
1:1000	0.75 ± 0.007	1.692[S*] ± 0.007	0.622 ± 0.014	1.265[S] ± 0.033

HK: heat killed; SD: standard deviation; *: Mean of duplicate determinations of absorbance measured at 492 nm; S*: Significant antibody responses using *in situ* heated coating antigen compared to control and the corresponding response using pre-heated coating antigen; S: Significant antibody responses than their control.

Table 7. Comparison between antibody responses of *S. typhi* antigens using indirect ELISA assay for *in situ* heated (56°C/60 minutes) whole-cell as a coating antigen

Sera dilution	Mean antibody response* ± SD (p ≤ 0.05)				
	IP injected			SC injected	
	2 doses heat-killed whole cells (10⁸ cells) 1 week apart	4 µg ViCPS doses at days 0, 15, and 42	4 µg ViCPS doses at days 0, 15, and 42	50 µg booster dose of Tris buffer extracted OMP at day 40	50 µg booster dose of lithium buffer extracted OMP at day 40
1:250	2.109 ± 0.087	1.952 ± 0.117	2.142 ± 0.113	2.4955[S] ± 0.08556	2.5395[S] ± 0.113
1:500	1.903 ± 0.007	1.741 ± 0.181	1.902 ± 0.069	2.394[S] ± 0.103238	2.4955[S] ± 0.095459
1:1000	1.692 ± 0.007	1.369 ± 0.152	1.587 ± 0.152	2.3195[S] ± 0.35426	2.603[S] ± 0.250316

ViCPS: Vi capsular polysaccharide antigen; OMP: outer membrane protein antigen; SD: standard deviation; SC: subcutaneously; IP: intraperitoneally; *: Mean of duplicate determinations of absorbance measured at 492 nm; S: Significant antibody responses to booster dose of Tris- and lithium-extracted OMP.

antigen is necessary for clearance of *S. typhi* through complement activation. In addition, it was reported to induce rapid and dominant humoral immune responses [25] as well as eliciting a cross-reactive plasmablast response to *S*. paratyphi A, B, and C)[26].

The immune response to ViCPS, OMP, and heat-killed whole cell antigens were evaluated using mice, the results of which are well correlated with efficacy in humans [27]. The humoral immune response was estimated using indirect whole-cell ELISA, a useful technique for determination of surface bacterial antigens [28]. The immune response was evaluated for all of the three studied antigens by applying the indirect whole-cell ELISA technique in which the whole cell coating antigen was heat inactivated either *in situ* in the microtiter plate or before its plating.

Immunizing doses of ViCPS, 2 and 4 µg administered SC and 4 µg administered IP, showed significant immune response compared to controls. However, the response to these doses was nearly similar without significant difference, in agreement with the findings of Szu *et al.*[29], who reported a non-significant difference in responses using different dose levels of Vi antigen. In general, the responses to 4 µg ViCPS, injected via SC or IP, were relatively higher when compared to the lower doses justified by immunogenicity studies which linked the *O*-acetylation to the antibody response, as *O*-acetyls are important in the binding of Vi to antibodies [30]. The response obtained from three consecutive doses of 2 µg ViCPS injected SC showed significant immune response. This finding is inconsistent with that of Micoli *et al*. [31], who reported that Vi antigen failed to generate a detectable antibody response after three subcutaneous injections of 2.5 µg, two weeks apart; this may be related to the use of whole-cell ELISA in determination of the immune response in our study.

Regarding the effect of injection route, results obtained revealed the superiority of SC over IP route; 2 µg SC injected showed matching response to that of 4 µg IP supported by the current use of deep SC injection of ViCPS for human vaccination[8]. However, the immunization route was reported to have little effect on immune response [32,33], which could be attributed to the difference in the mice strain used, the use of different serological technique, hemagglutination, or to the use of a single high dose of Vi antigen.

Regarding the use of lithium or Tris buffer-extracted OMP as an immunizing antigen at 50 µg dose levels, the use of four consecutive doses within four weeks resulted in a significant immune response,

which was also significantly enhanced after a booster dose was given at day 40. This may be due to the relatively short surface of OMP loops exposed from the surface of the whole cell [34]. The relatively high response obtained was explained by MacLennan [35] to be due to the high immunogenic character of protein antigen; the antigen is T-cell dependent, inducing several antibody isotypes, long-lasting memory, and affinity maturation. On the other hand, a significant humoral immune response was obtained using whole cells, heat killed at 56°C for 60 minutes, after injection of two IP doses (10^8 cells/200 µL PBS) two weeks apart. The ELISA readings of the control groups reflect the intensive cross-reaction phenomena of Enterobacteriaceae antigens with the *S. typhi* antigens [36].

Comparing the immune response to ViCPS, OMP, and heat-killed whole cells, a similar immune response was recorded using heat-killed *S. typhi* whole cell, 4 µg SC or IP injected ViCPS. The response to OMP was relatively higher than the previously mentioned antigens. Nevertheless, the heat-killed whole cell vaccine has higher systemic and local reactions [10], while the OMP protective capacity was shown to be lower against Vi expressing *Salmonella* strains [33].

The relationships and the overall results mentioned before did not change when similar numbers of coating cells were used. The sensitivity of the indirect ELISA results was, however, significantly increased, in general, when the *in situ* inactivated whole cell coating technique was used. A potential explanation of such result is that the *in situ* heat treatment of bacterial whole cell followed by one hour incubation may prevent denaturing of antigens on their surface and may prevent the loss of polysaccharide antigen, thus allowing antigens to absorb more anti-*S. typhi* Vi and OMP IgG from hyperimmune sera than could the pre-heat treatment of bacterial whole cells followed by an overnight incubation.

Conclusions

Though ViCPS and heat-killed whole cells are similar in immunizing level, ViCPS is usually recommended. The subcutaneous route, in general, was more immunizing than the intraperitoneal route. In addition, the immune response to OMP seems to be higher, but its protective capacity against Vi expressing *Salmonella* is low. Furthermore, the sensitivity of indirect ELISA technique, used for measurement of antibody responses, could be enhanced by *in situ* heat inactivation of the coating cells.

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Conflict of interests: No conflict of interests is declared.