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New typhoid vaccine using sponge-like reduced protocol: development and evaluation

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Purpose: Typhoid remains a major health problem, especially in the developing world. Furthermore, the emergence of multidrug-resistant and extensively drug-resistant strains of *Salmonella typhi* added a sense of urgency to develop more effective typhoid vaccines, one of which is bacterial ghosts (BGs), prepared by both genetic and chemical means. The chemical method includes incubation with numerous agents for a short time at their minimum inhibitory or minimum growth concentrations. This study included the preparation of BGs by a sponge-like reduced protocol (SLRP).

Materials and Methods: Critical concentrations of sodium dodecyl sulfate, NaOH, and H_2O_2 were used. Moreover, high-quality BGs were visualized by scanning electron microscope (SEM). Subculturing was used to confirm the absence of vital cells. Besides, the concentrations of the released DNA and protein were estimated spectrophotometrically. In addition, the integrity of cells was proved by visualizing Gram-stained cells using a light microscope. Furthermore, a comparison between the immunogenicity and safety of the prepared vaccine and the available whole-cell killed vaccine was established.

Results: Improved preparation of high-quality BGs of *S. typhi*, visualized by SEM, revealed punctured cells with intact outer shells. Moreover, the absence of vital cells was confirmed by subculturing. At the same time, the release of respective amounts of proteins and DNA is another evidence of BGs' production. Additionally, the challenge test provided evidence that the prepared BGs are immunogenic and have the same efficacy as the whole cell vaccine.

Conclusion: The SLRP provided a simple, economical, and feasible method for BGs preparation.

Keywords: Bacterial ghost, *Salmonella typhi*, Sponge-like reduced protocol, Vaccines

Introduction

Typhoid fever is an acute generalized infection caused by a highly virulent and invasive enteric bacterium, *Salmonella typhi*. Typhoid fever is an important public health problem in many low- and middle-income countries with low socioeconomic levels and inadequate hygiene. Global estimates of the typhoid fever burden range between 11 and 21 million cases and approximately 128,000 to 161,000 deaths annually. As a result, it is regarded as one of the most serious infectious disease threats to global public health, with particular concern over the rapid and widespread emergence of resistance to multiple antibiotics [1]. In 2000, the estimated incidence of typhoid fever in Egypt was 15 per 100,000 persons per year (Egyptian national syndrome-based sur-

veillance, unpublished data). However, this estimate may not reflect the true disease incidence, as less than 1% of these cases were culture-confirmed. The disease is either waterborne, foodborne, or spread through significant person-to-person contact [2].

S. typhi is Gram-negative bacilli, anaerobic bacteria that are non-capsulated, non-sporulating, and have distinct flagellar, somatic, and outer coat antigens. It has a long, oval tube appearance when arranged individually or in pairs. Virulence factors of *S. typhi* play a significant role in the pathogenicity of typhoid fever. They are involved in the various stages of infection, including the production of toxins, colonization, adhesion and invasion, and survival inside the host cells [3].

Antibiotics are typically used to treat typhoid fever, where the early start of effective antimicrobial treatment has been demonstrated to lessen the duration of the illness and the risk of complications and death, as there is a massive risk of morbidity and mortality if the disease is not treated [4]. Since the discovery of chloramphenicol's efficacy in treating typhoid in 1948, there has been a pattern of antibiotic use and the resulting development of antibiotic resistance. As a result, antimicrobial resistance has emerged as a major threat to typhoid treatment, elevating treatment failure rates [5]. The multidrug-resistant *S. typhi* is now regarded as endemic in several developing countries, particularly in South and Southeast Asia, mediated by the spread of the specific H58 lineage across Asian and African countries [6].

For the prevention of typhoid, a variety of vaccines are available. Inactivated vaccines were developed using various inactivation techniques for *S. typhi* cells, including acetone inactivation, alcohol inactivation, heat inactivation, and phenol preservation. However, in clinical trials, it has been linked to fever and systemic reactions in 9% to 34% of recipients. As a result, the inactivated whole-cell typhoid vaccine is regarded as inappropriate for use as a public health vaccine, and it is no longer available for use, despite being licensed [7]. Ty21a is a live oral vaccine prepared from an attenuated strain of *S. typhi* Vi polysaccharide vaccine based on *S. typhi* Vi antigen, a purified capsular polysaccharide. This vaccine is licensed for people aged 2 years and older. It does not induce immunity in younger children. It is advised to take a vaccine every 3 years [8]. This was overcome by covalently conjugating ViPS to carrier proteins by transforming the immunological response from T cell-independent to T cell-dependent, allowing vaccination even in young children [9].

Bacterial ghosts (BGs) are a biotechnology platform that can help generate safe vaccines, biological carriers, and effective drug delivery. The BG is a Gram-negative bacteria's empty cellular envelope that retains its surface and antigenic characteristics but lacks interior components [10]. They are dead, empty cells with a proper three-dimensional (3D) structure. The cells will no longer be able to proliferate after their cytoplasm has been removed. As a result, when they enter the bodies of humans or animals, they can safely trigger the immune system to produce the required antibodies. The definition was restricted to Gram-negative bacteria because they have the unique ability to translate the highly specific protein E of phage/X174, which was the only way to generate BGs until recently [11]. Chemical agents, on the other hand, can be utilized in critical concentrations and periods to generate Gram-positive BGs [12], Gram-negative BGs [13,14], yeasts ghosts [15], and even viral ghosts [16]. The same principles used to make Gram-negative BGs can be employed to make Gram-positive BGs by using the appropriate chemicals and controlling their concentrations, temperature, and shaking speed, resulting in high-quality ghosts [12,17].

Materials and Methods

S. typhi strain was obtained from the Clinical Microbiology Unit, Central Health Laboratories, Ministry of Health and Population of Egypt, where it was identified by serotyping with Kauffmann-White-LeMinor Scheme using *Salmonella* antisera (*Salmonella* O [9-O, group D], Vi, and *Salmonella* H [d-H]) [18].

Bacterial isolates were cultured on XLD, Hektoen enteric agar (Himedia, Thane, India), and other selective media for *Salmonella* and incubated at 35°C–37°C for 24 hours. The morphological characteristics were detected, and pure colonies were maintained on nutrient agar slants at 4°C. They were then sub-cultured on nutrient agar plates and incubated at 37°C overnight. A frozen stock was prepared from the overnight culture in Luria–Bertani (LB) broth (Oxoid, Basingstoke, UK) containing 20% glycerol and stored at -80°C [19].

The inoculum of *S. typhi* was prepared by the direct colony suspension method. The bacterial isolate was cultivated on nutrient agar media and incubated at 37°C for 24 hours. The inoculum was prepared by suspension of the appropriate number of separated colonies picked up from overnight nutrient agar plate with sterile saline solution (0.9%, weight by volume) and adjusted to 0.5 MacFarland standard solution

with turbidity (absorption) of 0.08–0.13 at a wavelength of 625 nm. It was used for the determination of the minimum inhibitory concentration (MIC) and minimum growth concentration (MGC) of chemicals used for the preparation of BGs [20].

The MIC and MGC (the concentration which shows the first growth after the MIC) of NaOH, sodium dodecyl sulfate (SDS), and H_2O_2 were determined using standard criteria [20]. NaOH and SDS were prepared as 10% sterile stock solutions. H_2O_2 was a 30% sterile solution. The standard serial dilution method was used to determine MIC and MGC values, where 1 mL of each of the above solutions was added to the first tube, which contained 9 mL of sterile nutrient broth (NB) medium to gain the final volume of 10 mL, and the 1:10 dilution. And 1 mL was then transferred from the first test tube to the second one and so on until reaching eight tubes for each compound. For the last tube, 1 mL was discharged, then the tubes were mixed gently. Each tube was inoculated with 100 μL of 0.5 MacFarland of overnight *S. typhi* culture and incubated at 37°C for 18–24 hours under static cultivation conditions.

Two experiments with different parameters were conducted (as in Table 1). The two experiments were selected from the results for the best conditions for BGs preparation. They are mainly experiment numbers one and 11 in the original sponge-like protocol [21] and as described in the sponge-like reduced protocol (SLRP) [17,21] with some modifications. The different variables were four chemical compounds representing SDS (X_1), H_2O_2 (X_2), $CaCO_3$ (X_3), and NaOH (X_4), and two physical parameters were represented as one variable, temperature-shaking rate (X_5). Each variable was represented at two levels (high and low), which were given by +1 and –1 as in Table 1. For each SDS, H_2O_2, and NaOH, the +1 value represents the MIC while the –1 value represents the determined MGC. The +1 value of the fifth variable represents 37°C and 100 rpm. In the case of $CaCO_3$, the used amount of the +1 (MGC) value was 1.05 μg/mL, while the –1 (MIC) value was 0.35 μg/mL [21].

S. typhi was cultivated in 500 mL of NB in a 1-liter flask and incubated at 37°C under static conditions for 72 hours. The biomass was collected using centrifugation at 3,500 rpm for 15 minutes. The biomass was washed gently with 0.5% saline and re-centrifuged at 3,500 rpm for 15 minutes. The supernatant was then discarded, and the cells were collected as cell pellets and washed again. The cells were then collected and resuspended in water for injection.

Table 1. Experiments one and two for bacterial ghosts preparation

Experiment no.	Experiment variable				
	SDS (X_1)	H_2O_2 (X_2)	$CaCO_3$ (X_3)	NaOH (X_4)	Temperature-shaking rate (X_5)
1	1	1	1	-1	1
2	1	-1	-1	-1	1

SDS, sodium dodecyl sulfate.

Twenty-five-time concentrations (25×) for NaOH, SDS, and H_2O_2 were prepared from the –1 and +1 values, determined from the MIC and MGC above. A 25-time concentration for $CaCO_3$ has been prepared from the quantities described above.

The two experiments were conducted in three steps. The first step contains all the variables except H_2O_2. After saline washing, the second step contains only H_2O_2. In the first step, 5 mL of each of the 25× (+1 or –1) NaOH, SDS, and $CaCO_3$ was added to 10 mL of the bacterial suspension to have a final volume equal to 25 mL and to give a final concentration equal to 1× for each. The BG first mixture was incubated at 37°C and 100 rpm for 1 hour in the presence of SDS, NaOH, and $CaCO_3$. The cells are then collected using centrifugation at 3,500 rpm for 15 minutes. The supernatant was then transferred to clean, sterile Falcon tubes to determine released DNA and protein concentrations.

The cell pellets were then washed with 0.5% sterile saline and re-centrifuged. The supernatant was discarded, and the cells were suspended in 5 mL of water for injection to readjust the bacterial volume to its original volume. The second step contained only H_2O_2, where 5 mL of the bacterial suspension had been diluted by adding 15 mL of water for injection, followed by adding 5 mL of +1 or –1 value of H_2O_2 according to the number of the experiment to have a final volume of 25 mL. Then the mixture was incubated at 37°C and 100 rpm for 30 minutes. The cells and the supernatant were collected separately as above. The cell pellets were washed with a saline solution followed by centrifugation (as above). Finally, in the third step, the cell pellets were resuspended in 60% ethanol and left at room temperature for 30 minutes with a gentle vortex every 5 minutes for 30 seconds. The cell pellet collection and washing were repeated as above. The wet cells were then suspended in 5 mL of water for injection and used for either light or electron microscope examination.

An Olympus light microscope (Olympus, Tokyo, Japan) was used to investigate cellular external surface integrity [22]. The cells' quality was determined for each preparation based on the quality of the 3D structure as either being correct or

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deformed. The overall bacterial ghost quality (BGQ) for each was given as a %. BGQ had given one of 10. Ten means that all 10 tested cells are corrected BGs [21]. The images were collected using the camera scanning of bacterial cells; an electron microscope was an additional step for studying the BGQ. The centrifuged pellets of bacterial cells were investigated by scanning electron microscope (SEM) (JEOL, Tokyo, Japan) at the Agriculture Research Center, Giza, Egypt [23].

The Biophotometer Plus-Eppendorf spectrophotometer (Eppendorf, Hamburg, Germany) was used for the determination of released DNA and protein concentrations in (μg/mL) in the supernatant at 260 nm and 280 nm, respectively [24]. The DNA concentration was calculated using the following equation: as each 1 OD_{260} unit for double-strand DNA=50 μg/mL.

The prepared *S. typhi* ghost was tested for the presence of any viable cells, where samples (about 25 μL) from each experiment (1 and 2) were cultured on XLD agar plates. The plates were incubated at 37°C for 2 days [17].

Thirty female BALB/c mice (Bagg and Albino Laboratory-bred strain of the house mice), with an average age of 8–12 weeks (25±5 g), were supplied from the Central Administration of Drug Control, Egyptian Drug Authority, Giza, Egypt. All mice were maintained and cared for according to the US National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011. They were divided randomly into three groups, each containing 10 mice.

The prepared *S. typhi* ghost was diluted using phosphate-buffered saline (PBS) and adjusted to 1 MacFarland standard solution (3×10^8 colony-forming unit [CFU]/mL) with an absorbance of 0.257 at a 600 nm wavelength; for every 1 mL of vaccine, 1 mg of alum adjuvant was added. The parenteral heat-inactivated whole-cell typhoid vaccine (provided by VACSERA, Giza, Egypt) was used at a concentration of 21% by diluting 1 mL of vaccine to 5 mL with PBS. Crude mucin-type II from the porcine stomach was prepared at a concentration of 5% using PBS.

Three groups of mice were randomly assigned; each one contained 10 mice as follows: (1) The first group was used as a control and was first injected intraperitoneally with 0.2 mL of 5% mucin-type II and then with 0.5 mL of a mixture of PBS and alum adjuvant (1:1). (2) The second group was first injected intra-peritoneally with 0.2 mL of 5% mucin-type II and then with 0.5 mL of a mixture of *S. typhi* ghost with alum adjuvant. (3) The third group was first injected intra-peritoneally with 0.2 mL of 5% mucin-type II and then injected with 0.5 mL of the old parenteral heat-inactivated whole-cell typhoid vaccine.

After 2 weeks, all groups received a booster dose for the first time. A challenge solution was prepared from cells of *S. typhi* grown in LB broth and incubated at 37°C for 4 hours with agitation. Then, the inoculum was adjusted to 0.5 MacFarland standard solution using PBS, and from this suspension, 1×10^7 CFU/mL suspension was prepared; this suspension was used for the challenge test.

All groups were first injected intra-peritoneally with 0.2 mL of 5% mucin-type II and then injected with 0.2 mL of the prepared bacterial suspension [25].

Animals were handled strictly with good animal practice as defined by the relevant local animal welfare bodies. All animal procedures were supervised and approved by the Faculty of Pharmacy, Cairo University ethics committee for animal experimentation (no., MI 1664; April 28, 2016). The care and handling of the animals were conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no., 85-23 revised 1996) and the European Agreement on Vertebrate Animal Protection for Experimental Use (86/609).

Results

In order to prepare BG from *S. typhi*, the SLR protocol was used. First, the MIC (+1 value) and MGC (–1 value) of NaOH, SDS, and H_2O_2 were determined; the results are summarized in Table 2. In the case of $CaCO_3$, the +1 value was 1.05 μg/mL, while the –1 value was 0.35 μg/mL. The tubes, which were incubated overnight, were observed, and both the MGC and the MIC for each treatment are shown in Fig. 1.

The cells from experiments one and two were examined for their integrity (correct or deformed 3D structure) compared with viable cells of *S. typhi* using the light microscope. BGs prepared by experiment number 1 had the correct 3D structure of cells, while those prepared by experiment num-

Table 2. The MIC and MGC of 10% NaOH, 10% SDS, and 30% H_2O_2

Chemicals	MIC (+1 value)	MGC (–1 value)
10% NaOH	1 mg/mL	0.1 mg/mL
10% SDS	1 mg/mL	0.1 mg/mL
30% H_2O_2	0.03% v/v	0.003% v/v

MIC, minimum inhibitory concentration; MGC, minimum growth concentration; SDS, sodium dodecyl sulfate; v/v, volume per volume.

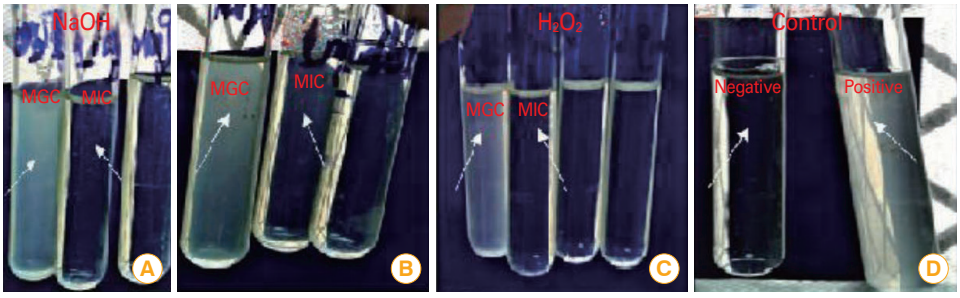

Fig. 1. Determination of minimum inhibitory concentration (MIC) and minimum growth concentration (MGC). (A) MIC & MGC of 10% NaOH. (B) MIC & MGC of 10% sodium dodecyl sulfate (SDS). (C) MIC & MGC of 30% H_2O_2. (D) Positive and negative control. Turbid test tube in (A), (B), and (C) represents MGC, clear test tube represents MIC while turbid tube in (D) represents positive control and clear one indicates negative control.

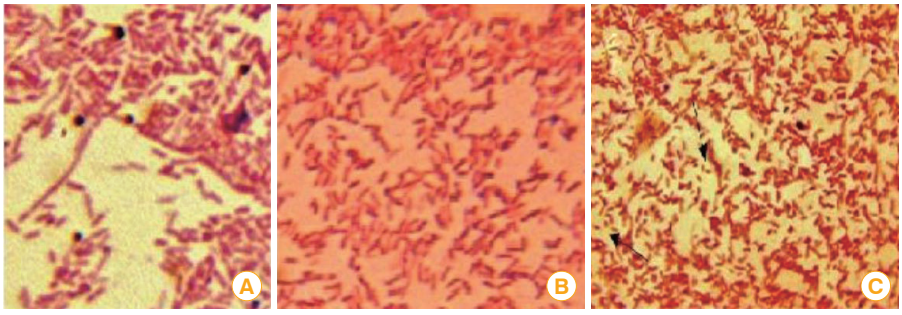

Fig. 2. Visualization of three-dimensional (3D) structures of bacterial ghosts using the light microscope. (A) Viable cells of *Salmonella typhi* show correct 3D structure. (B) *S. typhi* ghosts of experiment number 1 show correct 3D structure. (C) *S. typhi* ghosts of experiment number 2 show cells with deformed 3D structure (arrows).

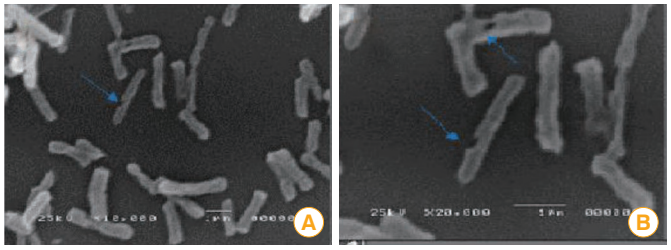

Fig. 3. Scanning electron microscope (SEM) image for bacterial ghosts (BGs) of *Salmonella typhi* prepared by experiment number 1. SEM image for BGs of *S. typhi* prepared by experiment number 1 showing intact cells with surface pores. (A) SEM image for BGs at ×10,000 (arrows). (B) SEM image for BGs at ×20,000 (arrows).

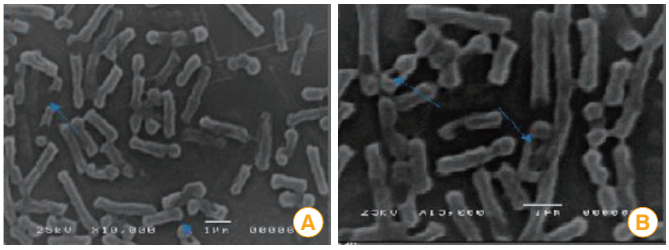

Fig. 4. Scanning electron microscope (SEM) image for bacterial ghosts (BGs) of *Salmonella typhi* prepared by experiment number 2. SEM image for BGs of *S. typhi* prepared by experiment number 2 showing formation of surface pores in intact cells while others were with deformed outer shell. (A) SEM image for BGs at ×10,000 (arrows). (B) SEM image for BGs at ×15,000 (arrows).

Table 3. BG quality as % for BGs prepared from experiments one and two

Experiment no.	Experiment variable					
	SDS (X_1)	H_2O_2 (X_2)	$CaCO_3$ (X_3)	NaOH (X_4)	Temperature-shaking rate (X_5)	BGs quality %
1	1	1	1	-1	1	100
2	1	-1	-1	-1	1	90

BG, bacterial ghost; SDS, sodium dodecyl sulfate.

ber 2 had some cells with a deformed 3D structure (Fig. 2), which was the cause for lowering the BGQ. The overall BGQ for each was demonstrated in Table 3.

The SEM was used to investigate the integrity of BGs, which was considered an additional step for studying the BGQ after the light microscope. It revealed punctured cells with intact outer shells and at least one intramembranous tunnel (Fig. 3), while BGs prepared by experiment number

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Table 4. Determination of released DNA and protein from (bacterial ghosts) prepared by experiments numbers 1 and 2

Experiment no.	Basic experiment		H_2O_2 step		Ethanol step	
	Protein (mg/mL)	DNA (μg/mL)	Protein (mg/mL)	DNA (μg/mL)	Protein (mg/mL)	DNA (μg/mL)
1	2,721	415	444	43.1	318	267.8
2	1,337	219.8	334	62.3	244	206

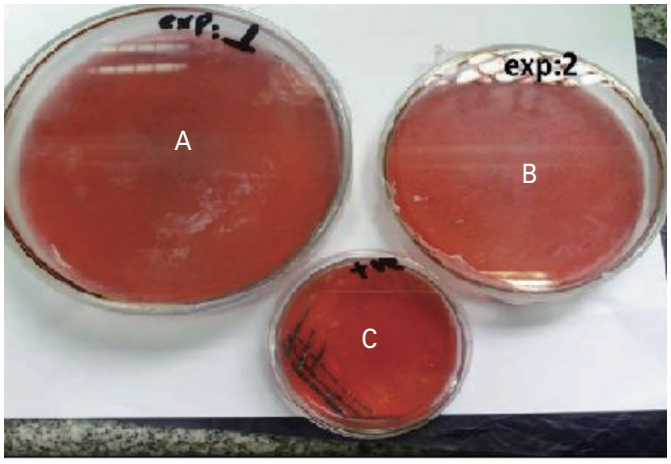

Fig. 5. Bacterial ghost (BGs) of *Salmonella typhi* prepared from experiments numbers 1 and 2 versus positive control. (A) BGs prepared by experiment number 1 (no viable cells). (B) BGs prepared by experiment number 2 (no viable cells). (C) *S. typhi* cells (positive control) (red colonies with black centers).

two showed some cells with deformed 3D structures and others with intact cell wall surface pores formed in them (Fig. 4).

Quantifying released DNA and protein from the two experiments was carried out to prove the release of cytoplasmic content of bacterial cells through pore formation, which is considered an essential step in the formation of BGs to confirm the absence of viable cells. The results are illustrated in Table 4.

A viability test was conducted to confirm the absence of viable cells to guarantee loss of their ability to replicate and, subsequently, their pathogenicity while they are still immunogenic due to the presence of surface antigens. As shown in Fig. 5, no viable cells were found after 2 days of incubation from BGs prepared from experiments one and two, while the positive control showed growth (red colonies with black centers).

A challenge test was carried out to evaluate the immunogenic effect of the prepared BG vaccine and compare this effect with the traditional parenteral heat-inactivated whole-cell typhoid vaccine. It was found that the challenge test demonstrated that the prepared BG vaccine was immunogenic; where all tested mice stayed alive for more than 15 days of observation after the challenge dose of *S. typhi*, while the control group died after 4 days.

Discussion

The demand for a simple, cost-effective, and viable process for preparing BGs is growing at the same time as the number of applications for BGs grows. Traditionally, BGs were made using the E-lysis gene, only found in Gram-negative bacteria, responsible for forming a well-formed transmembrane tunnel [26]. Multiple processes were used to produce a 100% of non-living lysed cells. The high cost and sophistication of genetic procedures for creating BGs are limiting constraints for the generation of Gram-positive BGs [12] and Gram-negative BGs [13,14].

The preparation of BGs according to SLRP has been previously established [17,21]. The current study used the SLR protocol to prepare BG from *S. typhi*. It was found that the efficacy of the prepared BG vaccine was similar to that of the traditional parenteral heat-inactivated whole-cell typhoid vaccine. These findings agree with another study's findings, in which the immunization of rats by subcutaneous injection of *Listeria monocytogenes* BGs generated via sponge-like technique, vaccinated rats were protected by 100%, in contrast to 0% survival in non-immunized animals [17]. In another study in 2022, the survival rate in the *Salmonella typhimurium* BG subcutaneously vaccinated rats' group was 100%, while in the BG-adjuvant subcutaneously vaccinated group (75%), but the lowest survival rate was in the orally vaccinated group (25%) [27].

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