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RESEARCH ARTICLE

Shigella Flexneri Ghost Cells as a Vaccine with Correct Surface Antigens

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Abstract

Increasing interest was shown in the last five years concerning preparation of the microbial ghosts including the bacterial cells as a vaccination method by using the newly-introduced protocol, the sponge-like protocol. The current study is concerned with preparing *Shigella flexneri* cell ghosts (SFGs) for generating vaccine against the intestinal infection caused by *S. flexneri* to minimize the incidence of watery and dysentery diarrhea among people. SFGs were successfully produced in *vitro* with and their surface antigens were evaluated with specific antibodies. The oral vaccination with SFGs through a challenge test proved that both of (O) and (H) antigens in rat sera were confirmed after treatment with SFGs. The agglutination test showed correct *in vitro* and *in vivo* antigen-antibody binding. That demonstrated the validity of SFGs in rat immunization against *viable S. flexneri*. This study is the first in showing the feasibility of SFGs as a vaccine and in different immunological applications such as the preparation of the adjuvants and the antibodies, the production of the diagnostic kits and experiments aimed to study the surface antigens of *S. flexneri*. This study is an important process for establishing proper SFGs for different immunological directed applications.

Keywords: *Ghost cells, Shigella flexneri.*

Introduction

S. flexneri is responsible for most diarrheal conditions in both children [1, 4] and adults [5, 6]. Several *Shigella spp*. invade the epithelial lining of the colon and rectum causing severs inflammation and cell death in the infected areas [7]. *S. flexneri* infection results in loose diarrhea and dysentery that could weaken the immune system creating other reported complications [1].Therefore, there is an urgent need for an effective way to control such illnesses including other species of antibiotics resistance *Shigella*.

Numerous assays were developed world-widely that utilized different parts of the bacteria to trigger the immune response against the infection [8, 14]. Bacterial ghosts (BGs) are dead empty bacterial cells with the same 3-D structure and the surface antigens. *S. flexneri* as gram-negative bacterial cells, their BGs are envelopes each represent the fused inner and outer bacterial cell wall [15, 20]. Although, BGs lack of inner bacterial contents and genetic material [21], BGs keep the bacterial antigenic properties and especially regarding stimulating the humeral and cellular immunities. Hou, Li [22] developed bacterial ghosts by expressing phage-derived holin. Studies demonstrated that BGs are applied as adjuvants and able to stimulate prion inflammatory interleukins [23].

In addition, they revealed strong defense against different enterobacteriaceae [24]. *S. flexneri* ghosts (SFGs) were made following the *Sponge like Protocol* according to [25], where the procedure was described extensively. In this study, the cell wall surface antigens were investigated via

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common commercial antibodies, thereby the specific SFGs were positive following the agglutination test. At the same time, immunized rats were subjected to a challenge test for further verification of the results.

Material and Methods

Bacterial Strains

S. flexneri used in this study was obtained from the Department of Microbiology, Al-Meri-Hospital, Faculty of Medicine, Alexandria University, Egypt. Incubation at 37˚C for 24hrs in MacConkey Lactose Agar (MLA) and Xylose Lysine Deoxycholate Agar (XLD) (Oxiod) were introduced to the study for understanding the morphology of *Shigella flexneri*. API 20E kit-based test (Biomerieux, France) was utilized for bacterial identity confirmation. Bergys Manual of Determinative Bacteriology (Bergey and Holt, 1994) was followed in this section. DNA isolation kit (Primer Design-UK) and its protocol were used to extract the bacterial DNA.

Polymerase Chain Reaction and Partial Gene Sequencing

Polymerase chain reaction (PCR) and partial gene sequencing (PGS) were followed to better prove the identity of the bacterial isolate using the *rpoB* gene as DNA target. The primer set was used (F: 5-AAC CAG TTC CGC GTT GGC CTG G-3 and R: 5- CCT GAA CAA CAC GCT CGG A-3). The PCR reaction volume was 25µl (30ng DNA, 15pmol for each primer, master mix at 2X 12.5µL (Kappa Biosystem), and 6.5µl molecular biology directed H_2O. The PCR conditions in the thermocycler (Primus 25 peQLab) were 94°C for 5min initial denaturation, 35 cycles of (94°C for 2min denaturation, 60°C for 1min annealing, and 72°C for 1min extension), and 72°C for 10mins of the final extension.

One-percent-agarose-gel electrophoresis was used (J. Sambrook, E.F. Fritsch, 1989). A UV-based imager was employed to read the band lengths. For the PGS, the *rpoB* gene piece was gel-purified using PCR Clean UP-kit and its protocol (Promega, Co.). Then, ABA applied Biosystem was employed for run the sequencing process. BLASTN at http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi was inserted to process-analyze the raw data of the sequencing.

Preparation of *S. flexneri* Inactivated Vaccine

Sponge like Reduced Protocol was used to prepare SFGs and different experiments were conducted to validate the quality of the SFGs generated as recommended by Amara et al. (2013) and Amro et al (2014).

SFGs Quality Inspection

Crystal violet stain (for 5mins) was used in both experiments 1 and 2, Table 1. Light microscopy was employed for understanding the quality of the cells relying on the preserved 3D structure. The cells were imaged using MICROS Camera analyzed by MICROVISIBLE v 1.11.10.

Table 1: Shows DNA and protein concentrations during each step in *S. flexneri* preparation.

Basic experiment			H_2O_2 step		Ethanol step		
Experiment	Protein	DNA	Protein	DNA	Protein	DNA	(SFGs %)
No.	Mg ml^{-1}	Mg ml^{-1}	Mg ml^{-1}	Mg ml^{-1}	Mg ml^{-1}	Mg ml^{-1}	
1	2955.80	166.3	2910.13	87.65	2635.16	143	95
2	5422.32	187.4	5098.11	108.74	3936.11	122	100

Bacterial Envelope Assessment

The 3D cell structure was examined using electron microscopy (EM). Glutaraldehyde standard method for fixation was recruited to stain bacterial smears coated after dryness with ~15nm gold (JEC-1100 E Sputter Coater). An electron microscope (JEOL JSM-5300) was utilized to scan the ghost cells. Then, images were collected.

Immunization and Challenge Test

Thirteen males of Sprague-Dawley rats (60±10g) purchased from the animal house, the College of Veterinary Medicine, University of Al-Qadisiyah, Iraq. Animal care and use of national and international criteria were followed. The animals were placed in standard cages in which food and water were provided ad libitum. Housing environment at 22±2°C, 40-60%, and 12hrs/12hrs of temperature, humidity, and a light/dark cycle, respectively, was provided. After two-week-acclimation period, the animals were

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randomly divided into two groups (15 rats/each), intraperitoneally vaccinated group with SFGs (SFG group) at 70µg in 100µl PBS with 100µl complete Freund's adjuvant and the second group is control group (CG) received sterilized. Initially, SFG primary vaccinations were given at week nought followed by three booster doses at week 2, 4, and 8, then the rats were challenged with (3.8×10^9 cells/ml) of *S. flexneri*. Animals were sacrificed after seven days of this challenge, and blood samples were processed for obtaining sera which were stored at -20C for later biochemical analysis. At the same time, livers were collected and homogenized under aseptic conditions. The samples were test-bacterial-cultivated on the Maconky agar medium at 37°C for one week to ensure the absence of viable cells.

SDS-PAGE Analysis and Western Blot Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on 12% polyacrylamide gels according to method described by Dehghani et al [27]. Samples for were centrifuged at 10000×g for 10mins to collect the cells and extract SFGs by using the lysis buffer.

Supernatant was decanted and pellets of the whole cells were resuspended in PBS at 20µl and boiled up for 5mins at 100˚C in 5×SDS loading buffer at 5µl. Then, an electrophoresis process for 10mins at 110V was performed using a 3% stacking gel and a 12.5% running gel in vertical Bio-Rad mini-Protean II cell. Coomassie blue (CB) staining (for 24hrs) was recruited for visualizing protein bands. For the western blot (WB), 20µg of SFG lysates were exposed to a 12%-SDS-PAGE. WBs and the vaccine-generated sera at (1:1000), a primary antibody, were incubated together in non-fat dry milk. Then, goat anti-rat IgG conjugated alkaline phosphatase (1:7,000; Sigma-Aldrich) acted as a secondary antibody. Nitrobluetetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrate (MBI Fermentas, Canada) were used to soak the membranes for 30s which were exposed to G-box or X-ray film for scanning the gel bands.

Results

Morphological and Biochemical Identification

SS agar showed 1-3mm measured diameter colonies with pale-yellow to no-color appearance and negative lactose fermentation. Moreover, circular colonies with the presence of gas-bubbles and black spots of H_2S were recognized. In addition, small black-centered red convex-smooth-circular colonies were noticed on the XLD agar. Those findings provided proofs of the correctness of the bacterial family identity. The numerical profile (6704752) of the API 20 kit confirmed the identity of the genus *Salmonella*.

Molecular Detection of *S. flexneri*

The *rpoB* gene at 1090bp, Figure 1, was detected. The *rpoB* PGS, 897bp, was one-hundred-percent-identified with a zero e-value recognizing the isolate as *S. flexneri* 100% matching with *Shigella flexneri* of NCBI accession no. CP002201055.1. this confirmed the present strain identity.

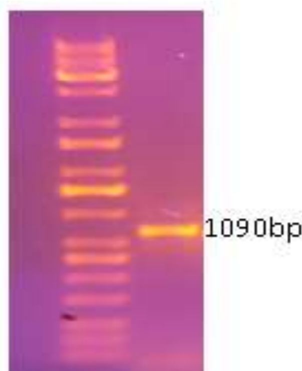

Figure 1: Agarose gel electrophoresis of the rpoB gene. Lane M: the ladder. Lane 2: PCR product at 1090bp of the Shigella flexneri

Electron Microscope Findings

Nanogold labeled SFGs showed great similarity to the control naïve *S. flexneri,* signifying the validly of this approach in preserving the 3-D structural integrity of the pathogen, Figure 2.

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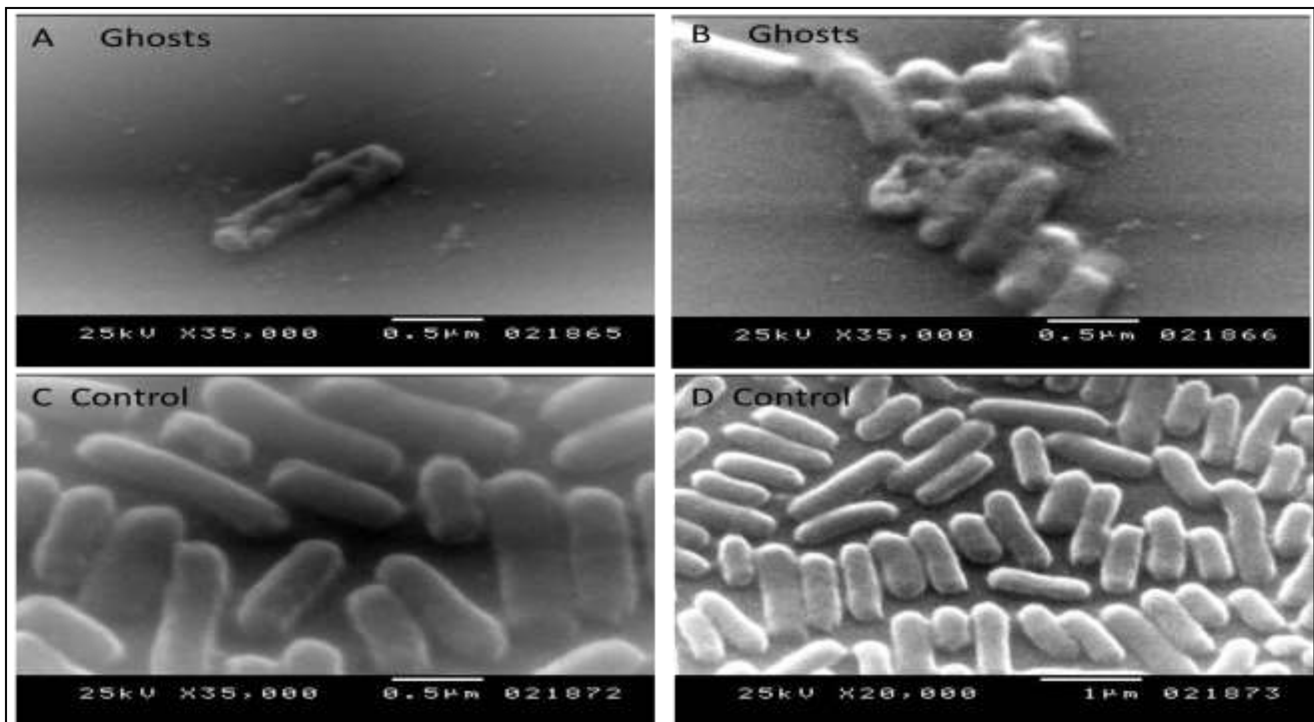
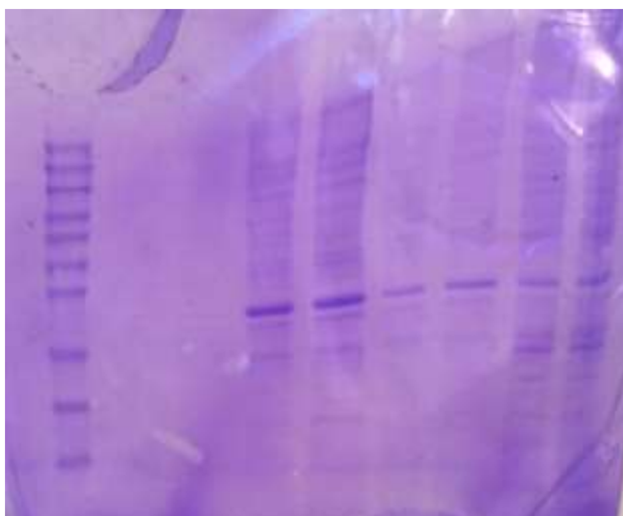


Figure 2: Electron microscope images. They reveal *S. flexneri* bacterial 3D profiles. A and B are SFGs while C and D normal *S. flexneri* bacterial

Immune Responses to *S. flexneri* Ghost Vaccine

The challenge test in Rats with *S. flexneri* showed significant reductions in the total colony count formation in SS agar. The CB-stained 12%-SDS-PAGE inoculated with the proteins of the *S. flexneri* envelope mixed with the serum antibodies generated from the challenged rats with either the SFGs or the PBS. Figure 3 provides the challenge results from the collected organs. SFG group revealed a significant ($p< 0.01$) lower bacterial count in those organs than that in those from PBS group. The WB showed stronger responses against proteins and LPS appeared by the resulted bands, 82, 62, 59, 57, 50, 40, 38,37, 36, 27, 25, 21, 19, 17, 15, 12, 11 and, 10 kDa respectively.



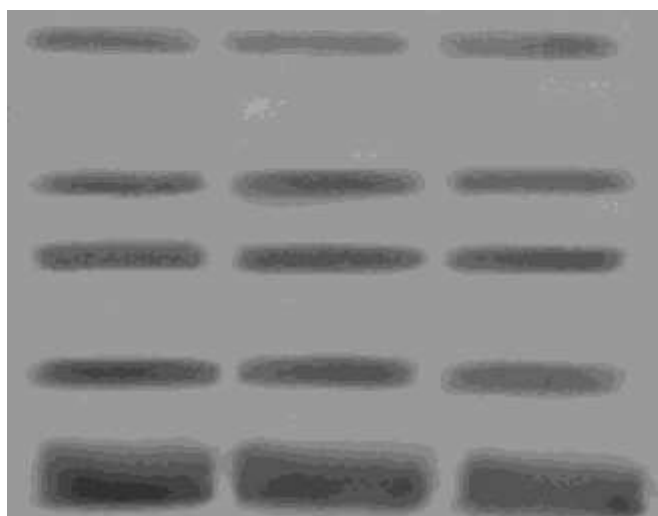


Figure 3: Western blot images of the proteins of the bacterial envelopes (a) *S. flexneri* and (b) SFG sera

Discussion

Although the mortality associated with Shigella has decreased considerably, shigellosis persists to be a serious disease spread around the world, especially in Asia and Africa. Shigella is an extremely virulent pathogens consisting of four main species and a humongous number subtypes. The limited resources in certain health environments are common for infections induced by Shigella flexneri. Mild watery diarrhea is one presentation of the disease that can be intensified to a serious dysentery accompanied with infection-based ystemic reactions such as losing of electrolytes, seizure attacks, and hemolytic uremic syndrome [28].

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Bacterial ghost (BG) is a revolutionary technique for the supply of vaccines, pharmaceuticals and active substances. Cell envelopes from Gram-negative bacteria form the BGs. BGs are free of any cytoplasmic materials, but their cellular morphological properties are conserved. In addition, BGs show adjuvant characteristics that help in better humoral and cellular immune response [29].

The current experiment was successfully able to generate SFGs which were identified having preserved 3D structure as shown by the EM results. EM based studies showed that the production of the tunnel is linked to the fusion of the internal and external membranes resulting in the periplasmic space sealing. The cytoplasmic cellular materials like DNA are dispelled via the tunnel forming the BGs because of high osmotic pressure. It has been shown that the tunnel development on the bacterial surface is not assigned to randomness but happens on possible sites of cellular division [30].

BGs consist of envelope antigens for triggering Immune responses. BG is an easy-to-construct vaccine that has great safety features and is room temperature stable. Traditional bacterial deactivation methods can lead to the remove of sufficient immunogenic antigens required to stimulate the immune function effectively. In addition, entirely killed bacteria may lead to the insertion of virulence factor genes into host bacteria. Interestingly, BG keeps the antigenic epitopes untouched for better immune responses [30].This completely agrees with present study results that showed significant immune responses generated in SFG-immunized rats against active *S. flexneri*.

Conclusion

BG formation of *S. flexneri*, here, is considered as for-the-first-time study employing NaOH and H_2O_2. The study explains the ability of the envelope antigens to work as a successful candidate vaccine. After the oral immunization of the rats with prepared SFGs, the rats show more effective immune function following active-*S. flexneri* challenge as revealed by agglutination of immunized rat serum antibodies to both (H) and (O) antigens.

This study is the first in showing the feasibility of SFGs as a vaccine and in different immunological applications such as the preparation of the adjuvants and the antibodies, the production of the diagnostic kits and experiments aimed to study the surface antigens of *S. flexneri*. This study is an important process for establishing proper SFGs for different immunological directed applications.

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