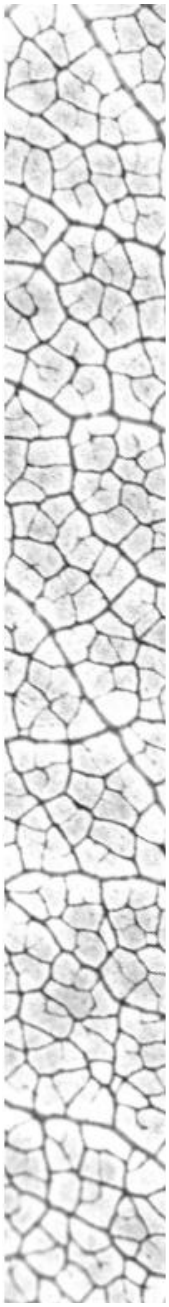

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Evaluation of S*almonella enterica* serotype *typhi* Lipopolysaccharide immunization induction in rats

Ibtisam Habeeb Alazawi [1*]

[1] Department of Microbiology, College of Medicine, University of Al-Qadisiyah, IRAQ
*Corresponding author: Ibtisam.alazzawi@qu.edu.iq

Abstract

Background: Salmonella enterica serotype typhi (SeT) is a bacterium with a gram-negative staining characteristic. This pathogen is globally known for its responsibility of causing typhoid fever. Controlling the disease burden is a great deal especially in the developing countries. According to this, finding a sufficient vaccine against the bacterium may provide a successful tool to prevent detrimental outcomes induced by the disease especially in the health and economic sectors. **Objectives:** This study was conducted to test if a purified lipopolysaccharide (PLPS) from SeT was able to induce neutralizing antibodies (NAb) against SeT O and H antigens in rats. **Materials and methods:** First, traditional cultivation and biochemical assays, Api-E20 test, and a 16S rRNA based polymerase chain reaction (PCR) and partial gene sequencing (PGS) analyses were employed to confirm the identity of the bacterium. Then, PLPS was obtained using a modified hot phenol-water extraction method and fractionated by SDS-PAGE electrophoresis. After that, bacterial-protein- and nucleic-acid-based contaminations of the PLPS were tested using silver and coomassie blue staining (SCBS) and ethidium bromide electrophoresis (EBE), respectively. Later, 10 rats were subcutaneously injected with PLPS at week 0 (wk-0) followed by booster doses at wk-2, wk-4, and wk-8. Then, a challenge test was applied to those rats using (3.8×10^9cells/ml) of SeT. This was followed by slide-agglutination screening of NAb-O and NAb-H complexes. Those complexes also were visualized using SDS-PAGE. **Results:** The identity of SeT was positively recognized using the confirming tests. The purity tests showed clearly that there were no protein and nucleic acid contaminations accompanied PLPS. The PLPS based immunization revealed successful NAb-O and NAb-H slide agglutination which later was successfully confirmed by SDS-PAGE. **Conclusions:** LPS induces successful neutralizing antibodies in the serum of the tested rats that show high agglutination activity against the O and H antigens of the S. Typhi. Collectively, LPS may be considered as a vital candidate vaccine against the bacterium.

Keywords: Lipopolysaccharide, Salmonella Typhi, typhoid, vaccine

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INTRODUCTION

Salmonella enterica serotype typhi (SeT) is a gram-negative bacterium which causes typhoid fever across decades and has been a concern for developing countries. The term “typhoid fever” popularized in 1829, after recognizing abnormalities of the abdominal lymph nodes of patients with “gastric fever”, was used by Pierre Louis to define the delirium that was shown by the illness, as an inferred from the Greek word “typhus” as “smoky”. While the agent for typhoid fever was first identified in the early 1800s, it was not until 1880 (Barnett, 2016). SeT is a flagellated with a -ve Gram staining rod-shaped bacterial pathogen that the human body is the only reservoir. LPS O9 and O12 antigens as well as the separate Vi capsular polysaccharide are serologically positive in this bacterium. The Vi-negative strains tend to be less degrees of causing infections and less virulent than the Vi-positive strains (Parry et al. 2002; Crump et al. 2015).

In children and young adults, typhoid fever is more frequent and linked to areas with low incomes where inadequate sanitation is widespread. It was stated that typhoid fever caused 21,7 million infections and 216,000 fatalities worldwide in 2000, and that 11,9 million infections and 129,000 fatalities in low- to medium-income nations were reported by the International Vaccine Institute in 2010. Such statistics are, nevertheless, more than likely to not reflect the true burden of disease, considering that a significant number of cases are treated privately or do not undergo any care. About 200 to 300 cases of SeT are recorded every

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year in the US; about 80% of these cases come from endemic area travelers (Crump et al. 2004; Lynch et al. 2009; Imanishi et al. 2014; Mogasale et al. 2014; Wain et al. 2015).

The intake of food or drink, which is affected by excrements of people who have the bacterium, normally leads to contracting SeT and has to overcome the gastric pH barrier in the stomach until adhering to the small intestine. An infectious SeT dose of 1000 to 1 million bacteria may induce the disease but be compared to healthy people and the status of their immune mechanisms. SeT enters the submucosal zone of the intestine by either a direct penetration into the epithelial tissue regulated by the cystic fibrosis transmembrane conductance regulator (CFTR) or through a specific lymphoid epithelial cells, M-cells. The pathogen, once inside the submucosa, induces Peyer patches to have hypertrophy (Ashurst et al. 2008).

Due to the existing health issue of the disease, vaccination is a better way to prevent the illness. Some of these vaccinations contain LPS, an outer-membrane virulence factor, characteristics in their function. The use of some vaccines was employed to have expression of the LPS component such as in the case of the S. Typhi Ty21 vaccine (Kintz et al. 2017). According to that, this study was conducted to test if a purified lipopolysaccharide (PLPS) from SeT was able to induce neutralizing antibodies (NAb) against SeT O and H antigens in rats.

MATERIALS AND METHODS

S. Typhi identification

The strain of the SeT was confirmed for its identity a multiple-test protocol. First, Gram staining was performed. Later, a cultivation method utilizing SS and XLD agars (Oxoid) was conducted followed by visualizing the colony morphological features. After that, the Api-E20 system (BioMerieux, France) was used as one of the confirming methods. Finally, a 16S rRNA based PCR and PGS analyses were employed to confirm the identity of the bacterium.

DNA Extraction and PCR

Genomic DNA Mini Bacteria Kit and its protocol were used. In brief, 1ml of the bacterial culture was placed in a 1.5-ml tube which was followed by a centrifugation step at 10,000rpm for 1min. Then, the supernatant was removed. To the solid phase, a 200µl lysozyme buffer was added. Finally, the extraction steps were completed according to the kit protocol. The DNA was evaluated for its purity and concentration (ng/µl) using a NanoDrop spectrophotometer.

PCR that targeted the 16S rDNA gene at its full length was performed depending on the primer set; F:5'-ATGCTTAGTGCTGGTTTAGG-3' and R:5'-GCCTTCATCATTTCGCTTTC-3'). The total reaction volume was 25µl that included 3µl (30ng) DNA, 1.5µl (15pmol) of each primer, 12.5µl Master Mix (Kapa Biosystem CO., South Africa), and 6.5µl water for molecular biology. Then, a thermocycler (Primus 25 peQLab, Germany) was used at 5mins-95^{o}C (initial denaturation), 30 cycles of; 1min-94^{o}C (denaturation), 1min-58^{o}C (annealing), and 30s-72^{o}C (extension), and 72^{o}C-10min (final extension). Finally, a 2% agarose was used and visualized under a UV-transilluminator.

Sequencing of PCR product

Excision of the amplified piece from the PCR agarose gel was performed using PCR Clean UP-kit (Promega Co., USA) and relying on its accompanied protocol. The above mentioned primer set was employed for the sequencing process with a model 373A automated fluorescent- DNA sequencer (Applied Biosystem Co.,Ltd., USA).

Analysis of the sequencing data

The BioEdit software was handled to analyze the sequencing data. BLAST N (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) was utilized to perform alignment and identify the current isolate. The isolate was deposited in the NCBI GeneBank under the accession No. MT726224.

Immunization of rats

Extraction and purification of lipopolysaccharide

The extraction and purification processes of the lipopolysaccharide were conducted using a method described by Rezania et al. (2011). Simply, SeT isolates were shake-overnight-cultivated in Luria-Bertani broth (usb, Cleveland, USA) at 37°C. The bacterial sediments resulted from a centrifugation step to the tubes of the growth were collected and utilized for the extraction and purification of the LPS. Then, pellets were collected after centrifuging 10^{8} colony-forming units/ml at 10,000×g for 5mins. These pellets were double-washed with PBS (pH=7.2) (0.15M) of 0.15mM $CaCl_2$ and 0.5mM $MgCl_2$. Later, 10ml PBS and sonicated for 10mins on ice were recruited to resuspend the pellets. Then, proteinase K (100µg/mL at 65°C for 1hr) (Roche, Mannheim, Germany) was used to remove any protein presence. For the removal of nucleic acids, RNase (40µg/ml) (Roche, Mannheim, Germany) and DNase (20µg/ml) (Roche, Mannheim, Germany) were added subsequently to the suspension with 1µL/ml 20% $MgSO_4$ and 4µl/ml chloroform at 37°C overnight. The extraction of the lipopolysaccharide was performed by using a hot phenol-water method and according to Rezania et al. (2011).

Silver, Coomassie Blue and Ethidium Bromide Staining

According to Rezania et al. (2011), solubilizing of the PLPS was performed in a sample buffer to 1mg/ml and boiled for 5mins. In each well, 16µl was used in a 15%-SDS gel with a 4%-stacking gel at 100mA for 2hrs using mini-PROTEAN electrophoresis (Bio-Rad Laboratories, California, USA). For the confirmation of no protein

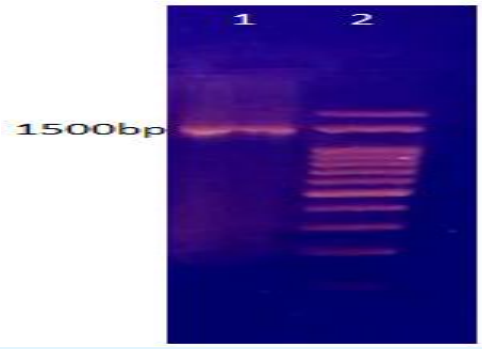

Fig. 1. Agarose gel image of the 16S rRNA gene that belongs to S. Typhi. Lane 1: positive amplification at 1500bp. Lane 2: Ladder

presence, SCBS of the gels was conducted. For assuring no nucleic acid presence, EBE was performed using 10µl/well of PLPS and 10µl/well 10^8 colony-forming units/mL of the bacterial suspension, as a positive control, in an agarose gel stained with ethidium bromide.

Rat PLPS injection and S. Typhi challenge

In accordance with international criteria of handling Lab animals, 20 male Sprague-Dawley rats (80±10g) obtained from the Animal Facility, College of Veterinary Medicine, University of Al-Qadisiyah, Diwaniyah, Iraq were housed (22±2°C, 40-60% humidity, and 12hr/12hr light/dark cycle) in standard cages and given food and water ad libitum. Acclimatization of the rats was performed for two weeks. Then, randomly, these animals were separated into two groups; PLPS-given group (PLPSG) and PBS-given group (PBSG). PLPS 50µg/200µl PBS complete Freund's adjuvant was used. Both PLPS or PBS were administered subcutaneously at week 0 (wk-0) followed by booster doses at wk-2, wk-4, and wk-8. Then, a challenge test was applied to those rats using (3.8×10^9cells/ml) of SeT. This was followed by slide-agglutination screening of NAb-O and NAb-H complexes. Those complexes also were visualized using SDS-PAGE

RESULTS

Cultivation and biochemical tests

Pale-yellow or nearly colorless colonies (1-3mm diameter) were recorded. The bacterium did not show lactose fermentation. In addition, SS-agar colorless colonies were circular (1-2mm diameter) with the presence of gas bubbles and H2S-based black spots. The small colonies on XLD agar displayed the characteristics of family enterobacteriaceae specifically Salmonella sp. These colonies were rounded, smooth, convex with red color and black-centered. The chromogenic agar colonies were size-different, convex, and mauve in color. The Api-E20 test confirmed the identity of the bacterium with the numerical profile (6704752).

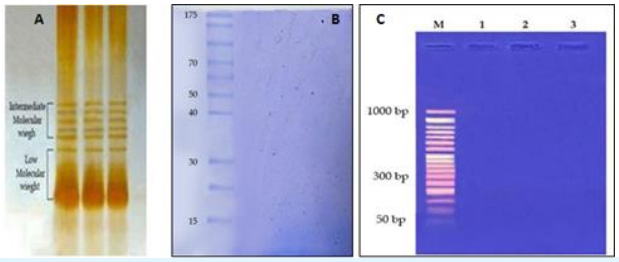

Fig. 2. LPS fractionation and protein and nucleic acid removal confirming assays. A. SDS-PAGE fractionation. B. Silver and coomassie blue staining (SCBS), no staining (no protein presence). C. Ethidium bromide electrophoresis (EBE) (no bands): no nucleic acid presence in the PLPS

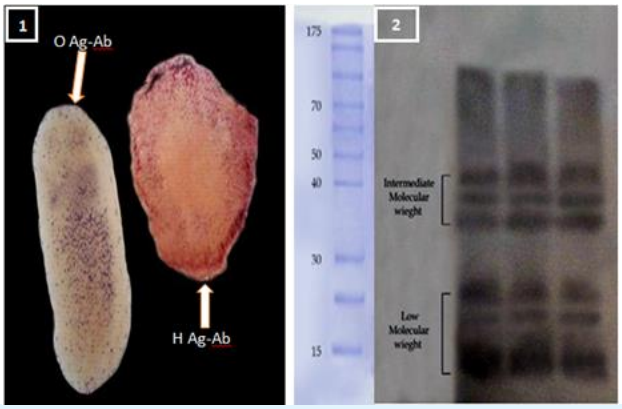

Fig. 3. Antibody evaluation due to PLPS immunization in rats. 1. Slide agglutination of vaccinated rat sera with O and H antigens of S. Typhi. 2. X-ray film of PLPS with 1/500 diluted vaccinated rat sera

Polymerase chain reaction and sequencing

The PCR and sequencing revealed positive results of the SeT identity. The amplification band was at 1500bp (**Fig. 1**).

The purity tests showed clearly that there were no protein and nucleic acid contaminations accompanied PLPS (**Fig. 2**). No staining by the SCBS was seen indicating that was no protein contamination present in the PLPS (**Fig. 2B**). The EBE showed no nucleic-acid separation (no bands) indicating that was no presence of any nucleic acids in the PLPS (**Fig. 2C**).

The PLPS based immunization revealed successful NAb-O and NAb-H slide agglutination (**Fig. 3-1**) which later was successfully confirmed by SDS-PAGE (**Fig. 3-2**).

The PLPS based immunization revealed successful NAb-O and NAb-H slide agglutination (**Fig. 3-1**) which later was successfully confirmed by SDS-PAGE (**Fig. 3-2**).

DISCUSSION

Typhoid fever management is one of the significantly challenging health issues around the world. Antibiotics ensure limited successful treatment of the disease; however, this success rate is affected by the bacterial

behaviors inside the host systems and cells. According to that, vaccination is the best choice to prevent the occurrence of the illness especially in endemic areas or people travel to endemic regions (Yang, Chong and Song, 2018).

The current study revealed successful usage of the LPS in inducing the production of NAb against the bacterial O and H antigens as it was confirmed by the slide agglutination test and the SDS-PAGE analysis. These results came in agreements with the current global aim of using LPS as a potential component of vaccines against the illness. The Vi LPS is a signaling triggering factor for inflammation that activates a pathway named as Toll-like receptor 4 (TLR4) and the subsequent activation of TNF-α and IL-6 cytokines that recruit inflammatory cells such as neutrophils to the infection site. S. Typhi has developed a suppressing mutation in fepE gene that controls the repeating O-antigen length to even more reduce the activation of the TLR4 (Seth-Smith, 2008; Tsolis et al. 2008; Wilson et al. 2008; Tran et al. 2010; Crawford et al. 2013).

The idea of using PLPS as a main component of these vaccines is supported by studies performed around the world that targeted liposaccharide and the immune responses against it in affected patients. In a study from Bangladesh, where the disease is endemic, patients have revealed lipopolysaccharide-based serum IgA and IgG antibodies and antibodies against whole-cell extract and membrane component preparations. However, the preparations of the membrane unveiled frequent IgA antibodies more than IgG. These immune inductions are significantly high in adults with IgA antibody formation in children against the membrane antigens; however, they get a reduction at the late stages of convalescence (Khanam et al. 2015).

Currently, Vaccines against SeT are categorized into two candidates. First, the live Ty21a vaccine (orally administered) that has mutations in the galE and ViCPS that encourage no survival of the bacterium inside the human cells. The other candidate is the Vi polysaccharide (ViCPS or Vi) vaccine, The ViCPS is a purified capsular polysaccharide of the SeT. The galE gene generates codes for the synthesis of the uridine-diphosphate-galactose-4-epimerase (UDP-Gal-4-epimerase). UDP-Gal-4-epimerase absence can lead to galactose accumulation in the bacterial cell. This eventually makes Ty21a an attenuated vaccine. The vaccine is used for people of five-year-old and older. The vaccine generates both cell-mediated and humoral immunity against the bacterial pathogen (Xu et al. 2013; Galen et al. 2016; Chong and Song, 2018).

CONCLUSION

LPS induces successful neutralizing antibodies in the serum of the tested rats that show high agglutination activity against the O and H antigens of the S. Typhi. Collectively, LPS may be considered as a vital candidate vaccine against the bacterium.

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