

Isolation of Vi Antigen of *Salmonella typhi* by a Simple Method and its Immunization Evaluation in Animal Model

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Typhoid is a widespread disease. The most common cause of this disease is *Salmonella typhi*. Emergence of multidrug resistant strains has complicated the treatment of typhoid so that it is considered as a threat to future epidemics. Vaccination is accounted as the most successful approach for dealing with this disease. Polysaccharide capsule of *S. typhi* is used as a safe and facile tool for preventing typhoid. The aim of this study was to isolate *S. typhi* Vi antigen and perform animal tests in order to examine its potential for human vaccination. *S. typhi* Ty2 strain was cultivated in Muller-Hinton agar medium. The polysaccharide capsule was extracted by cetavlon organic solvent after collecting the cells and suspending them in physiological serum. The nucleic acid and protein content of the samples were measured by spectrophotometry at 260 nm and Bradford's test, respectively. SDS-PAGE and immunodiffusion were used to analyze Vi antigen. This antigen was injected to mice and rabbit in order to examine immunization and pyrogenicity, respectively. The smear of the test sample was completely similar to that of the standard. Occurrence of the precipitation zone confirmed the presence of Vi antigen. It produced 80% immunization in mice and its injection to rabbit demonstrated that LPS was eliminated to a great extent. The proposed method can be a facile and cheap procedure with sufficient efficiency for Vi antigen purification.

Key words: Vi antigen, *Salmonella typhi*, Vaccine, Purification.

Enteric fever (typhoid) is a systemic disease caused by *Salmonella typhi*. This disease is globally widespread in a way that, estimatedly, 16 million cases occur annually in the world from which 600000 cases result in the patient's death¹⁻³. In developed countries the prevalence of this disease has been reduced significantly due to the existence of efficient water and sewage systems, however it remains a prevalent disease in developing countries^{2,4,5}. It is identified by bacteremia and the involvement of several organs. *Salmonella typhi* is consumed with water and food. The bacteria then grow, pass the intestinal wall,

colonize the mesenteric lymph nodes, and finally spread out in the blood which in turn results in septicemia and transmission to other organs⁶⁻⁸. *Salmonella typhi* has three antigens; O antigen (somatic), H antigen (flagellar), and capsular antigen (Vi) which is an acidic polysaccharide layer outside the cell wall. Due to some features such as resistance to a variety of antibiotics, *Salmonella typhi* has been considered as a threat to the future epidemics. Vaccination is accounted as a necessary tool in efficient management of the disease. Common typhoid vaccines include injectable inactivated vaccines, Ty21a live edible vaccine, and non-edible vaccine which is made of purified Vi polysaccharide¹. The latter has been commercialized since 1989⁶. Vi antigen, being on the surface of the typhoid bacilli, has a antiphagocytosis characteristic and help the bacterium resist the phagocytosis together with the complement system.

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A new type of vaccine made of *S. typhi* Vi antigen covalently conjugated to exotoxin A from the non-pathogenic *Pseudomonas aeruginosa* (rEPA) has been evaluated in Vietnam. This new vaccine induces much more IgG in comparison with the purified Vi polysaccharide. The increased immunization was observed not only in adults but also in 5-14-year old and preschool (2-4 years old) children. This vaccine stimulates the booster responses in 2-4-year old children. According to the examinations, this conjugated vaccine provides a 92% protection in children with the age of 2-5 years old¹. Ty2 cells are cultivated in large scales in order to produce the vaccine. The polysaccharide capsule is precipitated by treating the supernatant of the culture with Cetrimonium bromide and dried in vacuum before it is resuspended in the buffer. This also contains phenol as the preservative. In Vi purification it is important to keep the polysaccharide from denaturation in order to maintain its immunization properties.

More facile and low-cost procedures should be used in order to obtain high amounts of Vi antigen. The aim of this study was to isolate and purify *S. typhi* Ty2 Vi antigen by optimizing the present methods in order to facilitate the isolation procedures and to evaluate the quality of the purified antigen in mice.

MATERIAL AND METHODS

Cultivation and identification of the bacteria

S. typhi Ty2 was identified and confirmed through several biochemical tests such as cultivation on blood agar, Salmonella-Shigella agar, MacConkey agar, and EMB media, gram staining, oxidase and urease tests, ONPG, MRVP, and sugar and carbohydrates fermentation.

Vi antigen isolation

A colony of *S. typhi* Ty2 was picked and cultivated on Müller-Hinton agar media and incubated at 37 °C. After 24 hours a single colony was picked and subcultivated on Müller-Hinton agar media for another 24 hours. The grown bacteria were collected from the plates and suspended in sterile saline. The amounts of saline was dependent on the number of the well-grown plates. Four milliliter saline was implemented per each plate.

The resulted suspension was incubated

at 35 °C and 180 rpm shaking for an hour. The suspension was then aliquoted in microtubes and centrifuged for 5 minutes at 10000 rpm. The supernatant was collected and 2% cetavlon was added to reach the final concentration of 0.1%.

Vi polysaccharide antigen is precipitated in this solvent. The transparent solution is expected to become opaque.

The turbid solution was transferred into microtubes and centrifuged for 5 minutes at 13000 rpm. The precipitant was dissolved in 1 ml of 1 M calcium chloride and placed on ice before the addition of absolute ice-cold alcohol. The mixture was kept in -20 °C freezer for 10-15 minutes until the precipitants were observed sporadically. The samples were then centrifuged at 13000 rpm for 5 minutes.

The supernatant was removed completely and the precipitants were allowed to dry in laboratory temperature. The dried precipitant (Vi polysaccharide) was dissolved in 500 µl distilled water.

Adding 5 µl RNase (500 µg/ml) and 5 µl DNase (50 mg/ml) the remaining RNA and DNA were removed, respectively and kept in room temperature for an hour. Proteinase K was then added with a concentration of 0.5 mg/ml in order to eliminate the protein remnants. The optical density of samples were subsequently measured at 260 and 280 nm wavelengths. .

Protein concentration was calculated using Bradford's procedure. (Reference). SDS-PAGE with a 15% gel was implemented in order to visualize the purity of the protein.

Confirmation of the purified Vi through immunodiffusion

Anti-Vi polyclonal antibody was used in this method. Twenty milliliter of 1% agarose with sodium azide was poured in a sterile plate and three wells were made by a sterile pipet tip. Anti-Vi polyclonal antibody was placed in the middle one and the others were filled with distilled water and the antigen obtained through precipitation by cetavlon. The plate was then kept in refrigerator for 24-48 hours. Finally, it was analyzed for the existence of the precipitation zone around the wells.

Immunization evaluation in mice

Male mice with an age of 20-25 grams were selected. Forty male mice were categorized in the subject group and 10 in the control. An amount of

800 μ l of Vi antigen was diluted with 8 ml physiological serum from which 200 μ l was subcutaneously injected to each subject mouse. Control mice received 200 μ l physiological serum. Second injection was carried out 14 days after the first round and 10^9 bacteria were injected to both groups Intraperitoneally at the 17th day.

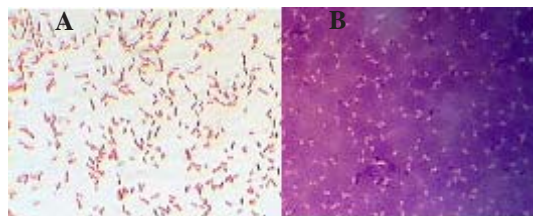
Examination of LPS contamination by pyrogenicity test on rabbits

Two milliliter diluted Vi antigen was intravenously injected to a 1.5-kg male rabbit in order to examine LPS contamination. The body temperature was measured and recorded every half an hours for two hours after the injection.

RESULTS

Biochemical confirmation of the bacterium

Red colonies with a black center were observed on XLD medium and colorless transparent colonies grew on EMB and MacConkey media. Gram staining revealed gram



1A: Gram staining showed that this is a gram negative bacterium.

1B: Capsule staining; the halos indicate the capsule in *S. typhi*.

Fig. 1. Confirmation of *S. typhi* by staining:

negative single bacilli under the light microscope. (figure 1A). KOH (3%) test was implemented to confirm the gram staining results. The result (gram negative) was confirmed since the bacterium was able to make the potash sticky. Capsule staining revealed the structure in the form of a halo around the purple bacteria in the dark field. (Figure 1B)

The results of the analytical biochemical tests are summarized in table 1. The data confirmed the bacterium to be *S. typhi*.

Agglutination test was used to confirm the Vi antigen. The formation of the agglutinated particles in a suspension of *S. typhi* Ty2 cells in a drop of anti-Vi polyclonal antibody on a slide showed that there is a reaction between Vi antigen and anti-Vi antibody; i.e. the presence of these particles confirmed the existence of Vi antigen in *S. typhi* Ty2. (figure 2)

Purification of Vi antigen

Precipitation was carried out by cetavlon organic solvent after the purification procedures. The concentration of the antigen was calculated to be approximately 10 μ g through Bradford's analysis. SDS-PAGE was used to visualize the purified antigen. Figure 3 represents the polyacrylamide gel after the electrophoresis. Standard Vi antigen was used as the positive control. Results demonstrated the complete similarity of the purified Vi to the standard Vi. (figure 3)

Immunodiffusion is a standard method for antigen conformation. Anti-antigen Polyclonal antibodies are implemented in the procedures. The presence of the precipitation zone between the middle well (containing anti-Vi polyclonal antibody) and the one filled with the purified

Table 1. Biochemical analysis of *S. typhi*

Results	Tests	Results	Tests
-	oxidase	F	OF
-	urease	-	ONPG
-	simmons citrate	-	KCN
-	Gelatin liquidification at 22 °C	-	malonate
+	Methyl red	+	sorbitol
-	vogesproskauer	+	arabinose
-	indole	+	xylose
+	Motility	-	lactose
+	H ₂ S	-	sucrose
+	Acidification in the presence of glucose	-	Gas from glucose

antigen in cetavlon organic solvent confirmed the reaction between the antibody and Vi antigen. (figure 4).

Evaluating the immunization of the purified antigen in mice

After the injection of antigen and physiological serum to the subject and control groups, respectively, at the first and 14th day, both groups were challenged by an amount of 109 *S. typhi* cells at the the day 17. All the control mice died in less than 24 hours while the number of deaths was 8 in the subject group where 32 mice survived the challenge. The conditions of the subject mice were monitored for ten days. At the end all 32 subjects were still alive. Thus, this method resulted in up to 80% immunization.

Examination of LPS contamination in the purified antigen

Pyrogenicity test was performed in rabbit in order to confirm the absence of LPS in the purified antigen. The rabbit's body temperature was measured before and after the injection. It



Fig. 2. The agglutinated particle resulted from the reaction between Vi antigen and anti-Vi polyclonal antibody

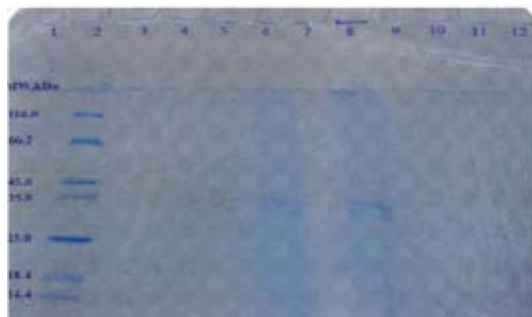


Fig. 3. SDS-PAGE analysis for visualization of the purified antigen: the smears of the 6th and 8th lanes represent the purified and standard Vi antigen, respectively.

remained at 39.7 °C from the first measurement (before injection) to the fourth half an hour after the injection.

DISCUSSION

Due to the emergence of antibiotic resistant *S. typhi* strains and the complication of typhoid fever, vaccination has been considered as the best known and most successful approach for dealing with this disease. The polysaccharide layer outside the cell wall, which is called Vi antigen, is implemented as a safe and facile material to produce vaccines in order to prevent typhoid fever. Several methods have been developed by researchers to day to isolate *S. typhi* Vi antigen. The resulted Vi antigens in these methods were different in physicochemical and immunological properties. These variations have been related to the alteration in the antigen nature during the purification procedures⁹. performed the isolation of Vi antigen by ethanol in the presence of sodium chloride and acetic acid hydrolysis which resulted in large amounts of high pure Vi antigen. However, due to the usage of hot acetic acid in hydrolysis the antigen was depolymerized and its immunization properties were affected⁹

Baker *et al.* extracted the antigen using saline-ethanol in various temperatures¹⁰ Jarvis *et al.* used 1 M warm acetic acid treatment of *Citrobacter freundii* 5396/38 for hydrolysis and electrophoresis for the purification of Vi antigen. Their purified antigen had a more immunization effect in comparison with Webster *et al.*¹¹. Wong *et al.* obtained Vi antigen from salty extract of



Well 1: test sample; Well 2: anti-Vi polyclonal antibody; Well 3: distilled water

Fig. 4. Immunodiffusion Test

Citrobacter freundii and concentrated it with ethanol which was then precipitated by hexadecyltrimethyl ammonium bromide (cetavlon). The resulted Vi antigen in their experiments produced a 18-23 fold higher immunization in mice in comparison with the previous two studies in which the extraction was based on acid hydrolysis¹². In other study, Wong *et al.* used *S. typhi* Ty2 acetone-dried cells and precipitated Vi antigen by cetavlon and evaluated its immunological properties in tissue culture and animals¹³

Tesheva *et al.* isolated Vi antigen through cetavlon precipitation (ct-Vi), purification by chromatography (cp-Vi), and triple precipitation by cetavlon (3-ct-Vi)¹⁴. Chibber *et al.* carried out the purification by seed culture preparation and precipitation by cetavlon and ethanol¹⁵

In this study, we used Chibber's method with some modifications and elimination of the seed culture step in order to invent a simple and cheap procedure which can be carried out in a minimal laboratory. On the other hand, this method does not need intensive chemical treatments which affect the immunization properties of the antigen.

The results were consistent with the outcomes of Tesheva's scp-Vi method. Currently this chromatographically purified Vi antigen (cp-Vi) is used in the production of typhoid vaccine¹⁴. The visualized smear in the 15% polyacrylamide gel electrophoresis of the purified Vi antigen and the observed band in the immunodiffusion assay in 15% agarose gel was exactly similar to the results of a study by Shousunchenszu *et al.* which was published in the journal of experimental medicine¹⁶. Our purified antigen produced 80% immunization in mice while according to WHO reports the efficiency of Vi polysaccharide-containing vaccine has been estimated to be 72-77%. Stone and Szu also suggested that Vi-containing vaccines produce an immunization against typhoid fever with an efficiency of approximately 70%¹⁷.

Since in a 2-hour period after the injection the rabbit's body temperature increased less than a degree, it can be concluded that the purified *S. typhi* Ty2 Vi antigen in our method was not pyrogenic and the LPS was removed to a great extent. In addition, the increase in body temperature was not a fever, being a hyperthermia. Thus, the purified antigen does not induce the fever in rabbit

CONCLUSION

Finally we conclude that the purification of Vi antigen through the proposed method in this study results in high purity and immunization. Furthermore, due to its simplicity and low cost, this procedure can be carried out in minimal laboratories with limited equipment.

REFERENCES

1. Guzman CA, Borsutzky S, Griot-Wenk M, Metcalfe IC, Pearman J, Collioud A, *et al.* Vaccines against typhoid fever. *Vaccine*. 2006; **24**(18):3804-11. Epub 2005/11/10.
2. Shima Shaigan Nia, Fateme Rostami, Farhad Safarpour Dehkordi, Ebrahim Rahimi, Emad Yahaghi, Ebrahim Khodaverdi Darian, Mahrokh Bagheri Moghadam . Isolation and Evaluation Virulence Factors of *Salmonella typhimurium* and *Salmonella enteritidis* in Milk and Dairy Products . *Iran J Med Microbiol* 2014, **8**(1): 54-61
3. Andrews JR, Ryan ET. Diagnostics for invasive *Salmonella* infections: Current challenges and future directions. *Vaccine*. 2015; **33** Suppl 3:C8-C15. Epub 2015/05/06.
4. Khan MI, Pach A, 3rd, Khan GM, Bajracharya D, Sahastrabudde S, Bhutta W, *et al.* Typhoid vaccine introduction: An evidence-based pilot implementation project in Nepal and Pakistan. *Vaccine*. 2015; **33** Suppl 3:C62-C7. Epub 2015/05/06.
5. Agtini MD, Ochiai RL, Soeharno R, Lee HJ, Sundoro J, Hadinegoro SR, *et al.* Introducing Vi polysaccharide typhoid fever vaccine to primary school children in North Jakarta, Indonesia, via an existent school-based vaccination platform. *Public health*. 2006; **120**(11):1081-7. Epub 2006/09/29.
6. Hessel L, Debois H, Fletcher M, Dumas R. Experience with *Salmonella typhi* Vi capsular polysaccharide vaccine. *European journal of clinical microbiology & infectious diseases* : official publication of the *European Society of Clinical Microbiology*. 1999; **18**(9):609-20. Epub 1999/10/26.
7. Khan MI, Ochiai RL, Clemens JD. Population impact of Vi capsular polysaccharide vaccine. *Expert review of vaccines*. 2010; **9**(5):485-96. Epub 2010/05/11.
8. MacLennan CA, Martin LB, Micoli F. Vaccines against invasive *Salmonella* disease: current status and future directions. *Human vaccines &*

- immunotherapeutics*. 2014; **10**(6):1478-93. Epub 2014/05/09.
9. Webster ME, Landy M, Freeman ME. Studies on Vi antigen. II. Purification of Vi antigen from *Escherichia coli* 5395/38. *Journal of immunology* (Baltimore, Md : 1950). 1952; **69**(2):135-42. Epub 1952/08/01.
 10. Baker EE, Whiteside RE, Basch R, Derow MA. The VI antigens of the Enterobacteriaceae. I. Purification and chemical properties. *Journal of immunology* (Baltimore, Md : 1950). 1959; **83**: 680-96. Epub 1959/12/01.
 11. Jarvis FG, Mesenko MT, Kyle JE. Electrophoretic purification of the Vi antigen. *Journal of bacteriology*. 1960; **80**:677-82. Epub 1960/11/01.
 12. Wong KH, Feeley JC. Isolation of Vi antigen and a simple method for its measurement. *Applied microbiology*. 1972; **24**(4):628-33. Epub 1972/10/01.
 13. Wong KH, Feeley JC, Northrup RS, Forlines ME. Vi antigen from *Salmonella typhosa* and immunity against typhoid fever. I. Isolation and immunologic properties in animals. *Infection and immunity*. 1974; **9**(2):348-53. Epub 1974/02/01.
 14. Tesheva AM, Aparin PG, L'Vov V L. [Isolation of a highly purified capsular polysaccharide from *Salmonella enterica* serovar Typhi (*Salmonella typhi*)—Vi-antigen and its use in serological diagnosis of typhoid fever]. *Bioorganicheskaya khimiya*. 2002; **28**(1):64-8. Epub 2002/03/06. Kapsul'nyi polisakharid *Salmonella enterica* serovara Typhi (*Salmonella typhi*)—Vi-antigen vysokoi stepeni ochistki dlia serologicheskoi diagnostiki briushnogo tifa.
 15. Chibber S, Bhardwaj SB. Protection in a mouse peritonitis model mediated by iron-regulated outer-membrane protein of *Salmonella typhi* coupled to its Vi antigen. *Journal of medical microbiology*. 2004; **53**(Pt 7):705-9. Epub 2004/06/09.
 16. Szu SC, Stone AL, Robbins JD, Schneerson R, Robbins JB. Vi capsular polysaccharide-protein conjugates for prevention of typhoid fever. Preparation, characterization, and immunogenicity in laboratory animals. *The Journal of experimental medicine*. 1987; **166**(5):1510-24. Epub 1987/11/01.
 17. Stone AL, Szu SC. Application of optical properties of the Vi capsular polysaccharide for quantitation of the Vi antigen in vaccines for typhoid fever. *Journal of clinical microbiology*. 1988; **26**(4):719-25. Epub 1988/04/01.