STANDARDIZATION OF ELISA ASSAY FOR DETECTION OF Salmonella enterica

Bagul C. D., Bodake N. L.¹ and Pethkar A. V.²

1. Department of Biotechnology, KTHM College, Gangapur Road, Nashik, Maharashtra (INDIA)
2. Department of Microbiology, Govt. Institute of Science, Aurangabad, Maharashtra (INDIA)

Received February 29, 2018 Accepted September 09, 2018

ABSTRACT
Salmonella enterica, an organism belonging to family enterobactericeae is responsible for foodborne infection, viz. salmonellosis, enteric fever or typhoid. Monitoring the food samples, raw materials and process equipment for presence or absence of the pathogen is necessary in order to avoid the threat to public health. It is also necessary to develop reliable assays for diagnosis of enteric fever in humans. Conventional detection methods based on physiological and biochemical analyses are time consuming; whereas, newer molecular-based methods are expensive. An indigenously developed immunological ELISA based method could provide a rapid, high sensitivity diagnosis in addition to improved specificity and affordability. Research was undertaken in our laboratory to test the possibility of developing an indirect ELISA assay for infections caused by Salmonella enterica serovar typhi using a heat inactivated strain isolated from pathological sample. Further efforts will be targeted at the direct detection of the pathogen from food samples and food processing equipment.

Key Words : Foodborne infection, Pathogen Non lactose fermenting, Typhoid

INTRODUCTION
Salmonella, a non-lactose fermenting, Gram negative rod, motile with peritrichous flagella causes salmonellosis (enteric fever) and is transmitted to man via faeco-oral route¹. Foods of animal origin, fruits and vegetables contaminated by infected manure are the major cause of food borne illness due to Salmonella, while birds and fleas play an important role in spreading the disease. The disease is transmitted mainly through water or food contaminated with human feces. The risk for infection by typhoidal Salmonella (Salmonella enterica serovars Typhi, Paratyphi A, Paratyphi B, and Paratyphi C) is high in low- and middle-income countries with poor sanitation². Post-ingestion, the bacteria penetrate gastrointestinal mucosa, enter mononuclear phagocytes where they multiply and by 10-14 days of infection, they are released in large numbers into the blood stream resulting in symptoms such as high fever, nausea, vomiting, constipation, fatigue, headache, rose-spots across the abdomen and joint pain. The bacteria further invade the gallbladder and lymph tissue of the intestine causing inflammation, intestinal bleeding leading to systemic infection with fatal peritonitis, pneumonia, hepatitis, enlargement of spleen, kidney injury, myocarditis, etc.³.³ In view of the potential threat posed by Salmonella, it is essential to develop indigenous sensitive and affordable assays for diagnosis of enteric fever or typhoid. The assays may be suitably modified in order to monitor presence of the pathogen in food as well as raw material and processing equipment. Conventional methods are based on pre-enrichment, routine microbiology, cultural characteristics, growth on selective media such as bismuth sulfite (BS) agar, xylose lysine deoxycholate (XLD) agar, Hektoen enteric (HE) agar, brilliant green sulfa agar (BGS), double modified lysine iron agar (DMLIA), triple sugar iron agar (TSI) and biochemical reactions such as sugar fermentation, oxidase and catalase tests, IMViC test, H₂S production, etc. However,
these are laborious and time consuming, typically requiring 5 to 6 days and forbid selling of the food during this procedure. A number of immunological assays for the detection of *Salmonella* spp. are available commercially, viz. Widal agglutination test, enzyme linked immunosorbent assay (ELISA), latex agglutination (LA), immuno-precipitation (Ab-ppt), etc. in addition to the recently developed DNA-based and polymerase chain reaction (PCR) based assays for *Salmonella* detection. Diagnostic methods based on molecular, immunological and spectroscopic analyses are costly and may have sensitivity and specificity issues. In the case of food testing, the major challenge is the difficulty in the detection of a small number of *Salmonella* from amongst a large number of indigenous microorganisms in the food samples; while affordability of diagnostic tests is the major concern for patients.

**MATERIAL AND METHODS**

**Culture and culture conditions:** Pathogenic strains of *Salmonella typhi* (isolated from fecal and blood samples) were kindly gifted by Bactest Laboratories, Nashik and The Department of Microbiology, KTHM College, Nashik. The isolates grown on MacConkey’s agar containing brilliant green and Wilson Blair’s medium and showing typical morphology were characterized by routine microbiological and biochemical tests.

**Inactivation of the pathogen:** Enumeration of bacteria was carried out using a log phase culture of *S. typhi* grown in nutrient broth by Total Viable Count (TVC) method. Cells were then recovered by centrifugation at RT and 5000 rpm. Cell pellet was dispersed in 10ml phosphate buffered saline (pH 6.8) containing 0.3% formalin. The suspension was heated at 100°C for one hour in boiling water bath to inactivate the organisms. The suspension (0.1ml) was then spread on an NA plate and incubated at 37°C overnight. The plate was observed for the number of colonies that appeared subsequently. A control plate consisting in non-inactivated organisms (diluted appropriately) was included to ascertain level of inactivation.

**Immunization of animals and determination of antibody titer:** For the purpose of developing polyclonal antibodies against *S. typhi*, a total of 10 balb/c mice (5 males and 5 females weighing approximately 20-25 g each) were obtained from Bharat Serum Ltd., Thane. The animals were housed in an animal house and allowed an adaptation time of about 5-6 days during which no experimental work on animals was carried out. The temperature in the animal house was maintained at 25-30°C with proper light, air circulation and humidity levels. The animals were fed with standard diet consisting of dried pellets. Day-to-day monitoring of the animal health and behaviour as well as cleanliness was carried out and utmost precautions were taken in order to ensure that the animals were not put into any discomfort by following the norms of the Institutional Animal Ethics Committee. Prior to immunization blood was collected from the animals and sera were obtained after blood coagulation. These served as negative controls (normal animal sera). Suspension of inactivated bacteria (0.2 ml) containing 2X10⁶ cells/ml in phosphate buffer was mixed with Freund’s incomplete adjuvant (0.2 ml) and administered in mice through the intraperitoneal (I.P.) route. On the 15th day post immunization, blood was collected from the animals by retro-orbital vein puncture for preparation of sera and determination of antibody titer. Second immunization was carried out on the 16th day using Freund’s complete adjuvant and subsequently booster dose (antigen without adjuvant) was injected on the 31st day. Antibody titers of sera were determined prior to each immunization dose by the antigen-down ELISA assay. Briefly, antigen suspension (100µl inactivated bacteria, 2.2x10⁸ cells/ml) in coating buffer (bicarbonate buffer, 0.2M, pH 9.4) was added to the wells of a high binding microtiter plate (Tarsons, India). The plate was incubated at 2-8°C overnight after which excess antigen was discarded by blotting the plates dry. The wells were blocked with 5% skimmed milk powder followed by addition of test antisera and control antisera (1:500 dilution in PBS). After incubation at 37°C for 2 hr, the excess antisera were discarded and wells washed with PBS-tween 3-4 times. Goat anti-mouse IgG-HRP (1:5000) was then added to the wells and
incubated at 37°C for 1 hr. Subsequently, soluble TMB substrate (Bangalore Genei, India; 100 µl) was added to the wells and reactions were stopped after 10 minute by 2M sulphuric acid (100 µl). The plate was read at 450nm with an ELISA reader (Transasia, India).

Development of antigen-down (indirect) enzyme linked immunosorbent assay: Various coating parameters such as coating buffer (phosphate buffer; PB and bicarbonate buffer; BB), antigen concentration, coating time (3 hr and 16 hr) and temperature (2-8°C and 37°C) were optimized by varying one parameter at a time and keeping the others constant. Briefly, cell suspensions (2.2x10^{12} cells/ml) were prepared in either 0.2M sodium bicarbonate buffer (pH 9.4) or phosphate buffer (pH 7.4). The suspensions (100 µl) were added to into the wells of a microtiter plate (Tarsons, India, high binding). The plates were incubated at 37°C for 3 hr or 2-8°C overnight (16 hr). After the incubation, excess antigen was discarded and blocking agent was added. Different blocking agents (100 µl) such as bovine serum albumin (1% w/v in PB), gelatin (1% w/v in PB), skimmed milk powder (5% w/v in PB), etc. were used in order to block nonspecifically interacting sites in the microtiter plate. Excess blocking agent was discarded and the plate was washed thrice with washing buffer (phosphate buffered saline with 0.02% tween-20 (PBST). This was followed by addition (100 µl) of primary antibody, i.e. antisera from mice that were diluted (1:1000 to 1:5000) using PB. After incubation at 37°C for 2 hr, the excess primary antibodies were discarded and plate was washed thrice with PBST. Secondary labelled antibodies (goat anti-mouse IgG-HRP) were then added to the wells at dilutions of 1:5000 and 1:10000. The wells were again washed thoroughly with PBST followed by addition of the soluble TMB substrate (100 µl, Bangalore Genei, India). The reactions were stopped by addition of 2M H_{2}SO_{4} (100 µl) that resulted in change of colour from blue to yellow and the samples were read at 540 nm using an ELISA reader. In another set of experiments, the coating antigen concentration was reduced to 2.2x10^{5} cells/ml in order to test the sensitivity of the assay.

RESULTS AND DISCUSSION

Culture and culture conditions: Prior to beginning of any experimentation, the as-obtained cultures of S. typhi were initially tested for growth and biochemical characteristics. For this, the isolates were grown on nutrient agar and isolated colonies were tested for Gram staining and biochemical characters. The cultures were plated on selective media (MacConkey’s agar containing Brilliant Green and Wilson-Blair medium). The identity of the test culture was confirmed on the basis of Gram negative nature, motile cells, black centered colonies on MacConkey’s agar, IMViC test and evolution of gas with H_{2}S production on TSI agar slopes.

Inactivation of the pathogen: It was observed that there was no growth of S. typhi on the NA plate after inactivation, while the non-inactivated culture showed presence of more than 300 colonies. This indicated that the inactivation procedure was adequate for the subsequent use of the culture for animal immunization.

Immunization of animals and determination of antibody titer: During the entire course of immunization, the health of the animals was normal with respect to body weight and the general behaviour. Antibody titres of the sera of animals (five males and five females) after the primary immunization increased only marginally over the control (average OD_{540}= 0.07) and were not significant. The booster dose however resulted in significant increase in the antibody titer as evidenced from the increase in absorbance to 0.717 at 540nm (data not shown). This indicated that the desired types of immunoglobulins were produced and could be isolated for further experimentation.

Development of antigen-down (indirect) enzyme linked immunosorbent assay: Experiments were targeted initially at the development of indigenous diagnostic assay that would be affordable, sensitive and specific for detection of enteric fever. The assay would be further modified suitably in
order to detect the presence of *Salmonella* in food/pathological samples. It was observed that at the coating antigen concentration of 2.2x10^{12} cells/ml that was used in preliminary optimization, overnight coating at 4°C and phosphate buffer as the coating buffer resulted in consistently higher detection levels of the antigen (Table 1). The detection levels with carbonate buffer and ambient coating temperature were comparatively lower. Among the different blocking agents, BSA (1% w/v in PB) was found to be the most effective blocking agent for all combinations of the primary and secondary antibodies, followed by skimmed milk powder (SMP, 5% w/v in PB). Primary antibody (antisera from immunized mice) diluted to 1:1000 and secondary labelled antibody (goat anti-mouse IgG-HRP) diluted to 1:5000 were found to yield highest level of detection of the antigen (Table 1). Lower dilutions of the corresponding antibodies did not improve the sensitivity of detection (data not shown), while higher dilutions showed negligible absorbance values. In order to test the sensitivity for disease diagnosis, the coating antigen concentration was reduced from 2.2x10^{12} cells/ml to 2.2x10^{8} cells/ml. It was found that lowering the antigen concentration still resulted in significant absorbance value of 0.215. A change in format of the assay to sandwich ELISA will be required for detection of pathogen in food samples. The use of monoclonal antibodies will ensure a further enhancement in the sensitivity of the assay.

Although the results are preliminary in nature, the experiments point to the possibility of developing indigenous reagents (polyclonal and monoclonal antibodies) for the development of ELISA kits to detect enteric fever. Gelinski et al., (2002) demonstrated the effectiveness of a novel 3-component (SPRINT, MSRV and Salmonella Latex Test system) for rapid detection of *Salmonella* in foods^5_. However, the authors conclude that the latex agglutination test did not perform well for naturally infected food samples^5_. In this context, it might be worthwhile to use a sandwich ELISA format for sensitive detection of the pathogen in the food samples, whereby, polyclonal or monoclonal antibodies against selected *Salmonella* antigen can be used for detection of antigen (bacteria) in food samples. The use of well characterized monoclonal antibodies with monoeptitopic specificity for detection and diagnostic applications has been highlighted by Rementeria et. al. (2009) and points to the necessity of further investigations in this area^6_. Schneid et. al. (2005) showed the effectiveness of monoclonal antibodies for detection of *Salmonella enterica* in chicken meat using antigen capture ELISA assay^7_. Combination of mono- and polyclonal antibodies have also been investigated for possible use in the development of biosensors for the detection of *Salmonella enterica*^8_. The use of nanotechnology platform could offer the prospects of high sensitivity with multiparameter analysis for simultaneous detection of various food borne pathogens^9_.

The presence of various gram positive psychrophilic bacteria in milk and fermented milk products has been demonstrated by Patil and Gandhi^10_ by routine culturing and 16s rRNA sequencing. Kamble et al. used PCR technique to detect aerolysin gene that codes for an important virulence factor in Aeromonas sp. in order to detect presence of the pathogenic strain in edible fish^11_. The authors concluded that the PCR-based method is rapid, sensitive and specific for detection of virulence factors. It needs to be mentioned that the ELISA-based method requires less time for detection compared to the DNA extraction, PCR amplification and agarose gel electrophoresis put together in the PCR-based method. Development of ELISA based methods for potential pathogens would proffer the advantage of a rapid and reliable screening method for foods and for the development of effective food preservation strategies. In view of the fact that the pathogen may be present in municipal sewage treatment plants^12_, the assay may also be applicable for monitoring the effectiveness of treatment processes.
Table 1: Optimization of parameters for ELISA-based detection of *Salmonella typhi*. Absorbance values represent corrected values after subtraction of absorbance of the controls (normal sera). Each value is mean of the 10 observations corresponding to number of animals used in the experiments.

<table>
<thead>
<tr>
<th>Coating: overnight at 4°C</th>
<th>Coating: 3 hr at ambient temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating- PB</td>
<td>Coating- CB</td>
</tr>
<tr>
<td>SMP</td>
<td>Gelatin</td>
</tr>
<tr>
<td>2.2x10^12 cells/ml</td>
<td></td>
</tr>
<tr>
<td>PAb1:1000</td>
<td>0.347</td>
</tr>
<tr>
<td>SAb1:5000</td>
<td>0.312</td>
</tr>
<tr>
<td>PAb1:1000</td>
<td>0.276</td>
</tr>
<tr>
<td>SAb1:5000</td>
<td>0.212</td>
</tr>
<tr>
<td>PAb1:3000</td>
<td>0.153</td>
</tr>
<tr>
<td>SAb1:10000</td>
<td>0.114</td>
</tr>
<tr>
<td>2.2x10^8 cells/ml</td>
<td></td>
</tr>
<tr>
<td>PAb1:1000</td>
<td>0.197</td>
</tr>
<tr>
<td>SAb1:5000</td>
<td>0.146</td>
</tr>
<tr>
<td>PAb1:1000</td>
<td>0.135</td>
</tr>
<tr>
<td>SAb1:5000</td>
<td>0.124</td>
</tr>
<tr>
<td>PAb1:3000</td>
<td>0.092</td>
</tr>
<tr>
<td>SAb1:10000</td>
<td>0.077</td>
</tr>
</tbody>
</table>

**CONCLUSION**

The experiments point to the feasibility of using ELISA-based approach for the detection of a food-borne pathogen such as *Salmonella typhi*. Although at the preliminary level due to the use of polyclonal antibodies, the sensitivity of the assay may be increased to significantly high levels with the use of monoclonal antibodies against specific antigenic determinants of the pathogenic bacterium. Further, the use of sandwich ELISA format would allow the sensitive and specific detection of the pathogen from food samples.

**ACKNOWLEDGEMENT**

The authors thank The Sarchitnis, MVPS, Nashik; Principal, MVPS’s KTHM College, Nashik and Director, Govt. Institute of Science, Auranganaad, Maharashtra, India for laboratory infrastructure and experimental facilities. The authors also thank Dr. V. S. Nade and Dr. L.A. Kavale, Maratha Vidya Prasarak Samaj’s (MVPS) College of Pharmacy, Nashik, India for animal house facilities and technical assistance.

**REFERENCES**


2. Crump J. A. Sjölund-Karlsson M., Gordon M. A., Parry C. M., Epidemiology, clinical


