Research Article

# Extraction of VI Antigen from *Salmonella typhi* for Establishing its Antigenic Evaluation by Elisa in Comparison to Commercial VI Antigen

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**ABSTRACT**

Typhoid is a disease caused by bacteria *Salmonella typhi* leading to severe fever, headache, nausea and weakness etc. The patient suffering from the disease needs the treatment of antibiotics which put a check on further systemic infection. In current study the *Salmonella typhi* bacteria was characterized by biotyping and serotyping using reference anti-serum. The Vi-antigen was targeted in this study and was extracted by cultivation of *Salmonella typhi.* The Vi-antigen was used for performing the ELISA to verify its antigenicity in comparison to the commercially available antigen. The Vi-antigen exhibits the ELISA titers with reference rabbit antisera implicating the retention of antigenic nature along with the O-acetyl units. This methodology can probably be used in human typhoid diagnosis. The role of Vi-antigen in case of natural infection of *S.typhi* is a matter of concern and studies for exploring behavior of this antigen should be emphasized.

**Keywords:** *Salmonella typhi,* Biotyping, Serotyping, Vi antigen, ELISA.

## INTRODUCTION

Typhoid fever is an infectious disease which is an acute generalized infection of the reticulo-endothelial system, gallbladder and intestinal lymphoid tissues. *Salmonella typhi* is the causal organism for enteric fever along with *Salmonella paratyphi* A and B which are the most prevalent species related to the fever[2]. The consumption of *Salmonella* through contaminated food items or drinks is the common mode of typhoid spread. The source of infection is variable and may include the contamination by person to person as well as poor hygiene standards, sewage contamination and poor water supply[3].

The developing countries of Asia like India, Pakistan, Bangladesh, Nepal having common factors like poverty, over-population and lower living standards, are still combating with the endemic zones of enteric fever. In India typhoid diseases is endemic and have significant mortality and morbidity in adult and pediatric population[1].

Investigators from the US Centres for Disease Control and Prevention estimate that there are 21.6 million typhoid cases annually, with the annual incidence varying from 100 to 1000 cases per 100000 population. Population based studies from Asia suggest that higher rates of complications and hospitalisation occurs in children aged less than 5 years[2-6].

Treatment of typhoid includes antibiotic treatment given in appropriate dose and duration for the treatment of complications of typhoid. The usage of chloramphenicol or amoxicillin is allied with a relapse rate of 5-15% or 4-8% respectively, whereas the newer quinolones and third generation cephalosporins are associated with higher cure rates[7].

The *S. enterica* serotype t*yphi* is assigned the antigenic formula I 9,12[Vi]:d:_ [8]. *Salmonella typhi* comprises of three basic antigens; the Somatic O-antigen, the flagellar H-antigen and the capsular Vi-antigen which lead to the virulence of bacteria. Vi antigen was first discovered by Felix and Pitt[9]. It is present mainly in *S.typhi* and *Salmonella paratyphi* C (*S. paratyphi* C), as well as *Salmonella dublin* (*S. Dublin*) and *Citrobacter freundii* (*C. freundii*)[10, 11]. The Vi-antigen is a polysaccharide which is linear homo polymer of α-D-(1-4)-linked *N*-acetyl-galactosaminuronate (GalNAcA) with 60 to 70% of the monomeric units O-acetylated at the C-3 position and has a molecular mass typically over 200 kDa[12, 13, 21]. It is an antigen that enhances *S.typhi* virulence in mice and has been shown to induce immune response in rabbits[14, 15].

Vi antigen is found to be a highly immunogenic antigen and is used as a vaccine candidate with the majority of the antibody response directed toward the O-acetyl groups. The *in vitro* studies have shown that Vi is antiopsonic and antiphagocytic, reduces the level of secretion of *S.typhi* induced tumor necrosis factor α by human macrophages[16] and increases the level of resistance of the organism to oxidative killing . The process of phagocytosis and complement mediated killing are inhibited in Vi+ strains of *S. typhi*[17].

*Salmonella* antigens are used in various diagnostic kits for the diagnosis of typhoid infection. For the diagnosis of typhoid, three commercially rapid diagnostic kits are available which are Tubex (Sweden), Multi-Test Dip-S-Ticks (Baltimore) and Typhi-Dot (Malaysia). In TUBEX, polystyrene particle agglutination principle is used to detect IgM antibodies to the O9 antigen semi-quantitatively[18]. The Multi-Test Dip-S-Ticks tests for five

pathogens, including *Salmonella* serotype *typhi*. The test is qualitative in nature and available in a dipstick format that detects anti-O, anti-H, anti-Vi, IgM, or IgG antibodies in patient serum, plasma, or heparinized whole blood[19]. Typhi-Dot is a qualitative test that works on principle of dotblot enzyme immunoassay using the outer membrane protein as antigen of serotype Typhi that detects either IgM or IgG antibodies. For specimens that are indeterminate (IgM negative and IgG positive), a confirmatory test, TyphiDot-M, is recommended by the manufacturer[18,19]. In these tests the H and O antigen of *S. typhi* are used profoundly however Vi-antigen is used only by Multi-Test Dip-S-Ticks tests.

The diagnosis of antibodies against Vi antigen in acute typhoid infection is of clinical importance. Vi polysaccharide is a T-independent antigen, the IgM antibody responses will develop at the time of bacterial infection. Therefore, IgM anti-Vi antibody should disappear from the sera of previously *S. typhi* infected individuals and even in the late convalescent stage of the disease. The usefulness of detecting specific IgM to this type of antigen in acute sera of typhoid patients is recognized[20]. The detection of IgM anti Vi antigen in acute sera of typhoid patients should be the most benefit for accurate diagnosis of this disease, since no salmonella which have phase-l d flagellin has Vi polysaccharide, except *S. typhi*.

Diagnosis of typhoid fever has been progressed from the conventional methods like blood culture and Widal test to modern techniques like Enzyme-linked immunosorbent assays (ELISA), dipstick assays and dotblot ELISAs. Among these techniques, ELISA is used because of stringent data providing results with clarity of antibody production against *Salmonella antigens thus* making diagnosis more accurate among typhoid cases. Considering the need for rapid screening of patients with suspected typhoid salmonellosis, the ELISA technique was an optimal candidate for serological diagnosis of Salmonella infections. Attempts to use the outer-membrane antigens and LPS in ELISAs showed promising results for detecting antibodies in human sera[22]. The current study had been designed for the extraction of Vi-antigen (Vi-Ag) from characterized *S. typhi* for usage in ELISA. These ELISA results were compared with the ELISA results Vi-antigen and anti Vi- antibody available commercially.

In the present study we have tried to assess the efficacy of in house extracted Vi-antigen as a diagnostic antigen that can be used for diagnosis of typhoid fever by ELISA.

## MATERIALS AND METHODS

### Characterization

The *Salmonella typhi* was grown on Makconkey agar and cultivated in Soyabean Casein Digest media (SCDM). The bacterium was characterized by the biotyping as well as by serotyping. The media and biochemical from Hi Media were used in the biotyping. *S. typhi* biotyping was performed by the biochemical testing[23,24] and confirmed by serotyping on the basis of somatic O and phase 1 and phase 2 flagellar antigens by agglutination test using standard *Salmonella* agglutinating factor sera in accordance to the scheme of Kauffmann-White[25,26]. The bacterium was cultured on nutrient agar and Mac-conkey agar plates. For serotyping purpose 15 hour broth culture of *S. typhi* was formalinized (0.25 %) and kept at room temperature for 60 minutes. Centrifuged the culture at 2,500 rpm for 10 minutes and collected the deposit. The deposit was mixed with drops of polyvalent O, H-factor and Vi-antisera. The tube agglutination test was also performed respectively.

### Vi antigen

*Salmonella typhi* was grown by fermentation in SCDM containing 5% glucose, 2% yeast extract at 37°C and pH 7.2[27]. The method described by Gotschlich et al.[28] was applied for purification of Vi antigen. Briefly, the bacterial liquid culture was centrifuged at 4,000 rpm for 20 minutes and the Vi was obtained by precipitation, from the supernatant, using hexadecyl-trimethyl ammonium bromide (Cetavlon). The precipitate (crude Vi-antigen) was purified by dissolving in 1M $CaCl_2$, followed by alcohol fractionation, cold phenol extraction, alcohol precipitation of crude polysaccharide and centrifugation[29]. The O-acetyl content test (Hestrin Assay) was performed for the quantification of the Vi-antigen in the study samples. The reference rabbit Vi-antiserum was obtained from N.S.E.C, Kasauli. Vi-polysaccharide vaccine (Typbar, Bharat Biotech, India) was purchased and used as reference Vi-antigen.

### ELISA

Standard checkerboard titrations[30] were performed to determine the optimum concentration for loading *S. typhi* Vi-antigen onto ELISA plates. ELISA was performed in flat bottom polystyrene plates (Tarsons). The plates were coated by Vi-antigen (2µg/200µl) diluted in the phosphate buffer saline. Overnight incubation at 4°C was provided to the plate. Commercial Vi-polysaccharide (Typbar, Bharat Biotech, India) was also added in similar quantity as a reference comparative antigen in a separate plate. After incubation, decanted the antigen dilutions and washed the plates three times with PBST (phosphate buffer saline containing 0.05% Tween 20). The coated plates were then blocked by adding 200µl/well of blocking buffer with 3% w/v bovine serum albumin, BSA (Sigma-Aldrich) and incubating for one hour at room temperature. After decanting the blocking solution, the plates were washed three times with PBST as before. The reference rabbit Vi-antiserum was diluted in PBST with 1% w/v BSA and 200 µl was added to each well (in duplicate). For each plate blank and negative controls diluted in PBST buffer were also added. After incubation for one hour at room temperature, plates were washed as described above and added 200 µl/well peroxidase-labeled goat anti-rabbit (IgG) conjugate (Sigma), diluted 1:15,000 in PBST with 1% w/v BSA. Plates were incubated for 60 minutes at room temperature, then washed three times again. O-

phenylene diamine (OPD) substrate solution (Sigma) was prepared according to the manufacturer's instructions, 200 µl was added to each well, and the plates were incubated for 30 minutes at room temperature in the dark. The reaction was stopped by adding 50 µl of stopping solution (1M $H_2SO_4$) to each well. Color intensities were read using ELISA microplate reader (BioRad) at 490 nm.

## RESULTS AND DISCUSSION

*S. typhi* was grown on nutrient agar and Mac-conkey agar. After overnight incubation at 37°C the colonies grown were non lactose fermenting, pale, opaque, non-hemolytic, moist, circular with a smooth convex surface and the entire edge. Colonies were catalase positive and oxidase negative. Gram stained smear from the growth revealed Gram-negative bacilli.

*Salmonella typhi* gave alkaline slant and acid butt, with production of $H_2S$ in Triple sugar Iron agar. It was positive for methyl red and glucose fermentation. Voges-Proskauer test was negative with citrate utilization, oxidase, indole and urease negative tests. The results were positive for catalase test and motility.

The agglutination was positive, the *Salmonella* serotype was identified. The slide and tube agglutination results are shown in figure1,2 and 3. These confirmatory tests were performed by using standard diagnostic polyvalent antisera for *Salmonella typhi*.

**Figure 1:** Negative slide aglutination

**Figure 2:** Positive slide aglutination

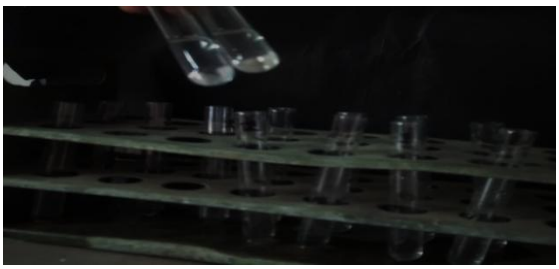

**Figure 3:** Tube aglutination

### ELISA

The ELISA was performed using the Vi-antigen (Vi-Ag) extracted from *S. typhi* during the study and the reference rabbit Vi-antiserum. This serum had braced the specificity of the Vi-Ag under study along with the comparison of antigenicity to the commercial Vi-antigen (Typbar, Bharat Biotech, India). The Vi-Ag content was quantified by the Hestrin assay for O-acetyl content. The Vi polysaccharide was diluted in PBS to have the Vi polysaccharide content equivalence to the commercial Vi-Ag for coating on ELISA plates. Similar concentrations of both antigens were used for the coating of the ELISA plates. The test had been repeated with various Vi-Ag concentrations starting from 0.5, 1.0, 1.5, 2.0, 2.5 µg/well. The best results were obtained at 1.5, 2.0 µg/well so the Vi-Ag concentration finalized for the performing ELSIA was 2.0 µg/well for coating of ELISA plate. The final results of the ELISA test are summarized in Table-01.

**Table 1:** Results of ELISA performed on Vi-antigen and commercial Vi-antigen

| S.No. | Reference Sera dilution | Mean antibody response (OD at 490 nm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | (Coating of 1.5µg) | | (Coating of 2µg) | | (Coating of 2.5µg) | |
| | | Vi-Ag | Commercial Vi-Ag | Vi-Ag | Commercial Vi-Ag | Vi-Ag | Commercial Vi-Ag |
| 1. | 1:5 | 0.173 | 0.449 | 0.235 | 0.598 | 0.253 | 0.598 |
| 2. | 1:10 | 0.204 | 0.493 | 0.285 | 0.520 | 0.29 | 0.534 |
| 3. | 1:25 | 0.286 | 0.602 | 0.368 | 0.653 | 0.376 | 0.654 |
| 4. | 1:50 | 0.377 | 0.581 | 0.437 | 0.670 | 0.45 | 0.691 |
| 5. | 1:100 | 0.105 | 0.380 | 0.208 | 0.461 | 0.220 | 0.483 |
| 6. | 1:200 | 0.065 | 0.169 | 0.081 | 0.246 | 0.091 | 0.261 |
| 7. | 1:400 | 0.052 | 0.060 | 0.053 | 0.097 | 0.053 | 0.109 |
| 8. | 1:500 | 0.052 | 0.055 | 0.051 | 0.054 | 0.052 | 0.053 |
| 9. | Control | 0.054 | 0.055 | 0.054 | 0.058 | 0.053 | 0.056 |

The dilutions for reference rabbit Vi-antisera used for the test were 1:5, 1:10, 1:25, 1:50, 1:100, 1:200, 1:400 and 1:500. The ELISA test results were affirmative up to 1:100 and 1:200 dilutions of reference rabbit Vi antisera with the study Vi-Ag and commercial Vi antigen respectively.

The trend line as well as coefficient of determination ($R^{2)}$ value for various concentrations of the Vi-Ag and commercial Vi antigen were calculated. The comparison was made in between the Vi-sample as well as commercial Vi antigen. The maximum value of $R^2$ was at 2µg concentration of both antigens which was 0.8211 and 0.8182 respectively (Figure-04, Figure-05). The $R^2$ value of the both Vi antigen at 2µg concentration was found comparatively equivalent to each other.

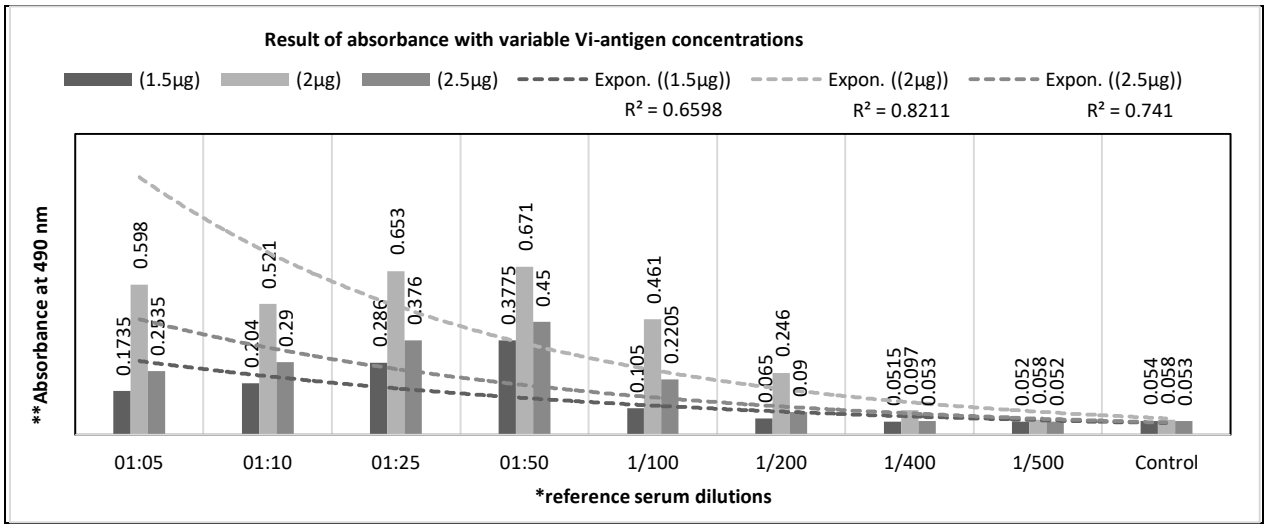


**Figure 4:** Results of ELISA for Vi-antigen * Dilutions of rabbit Vi-antiserum ** Absorbance at 490nm

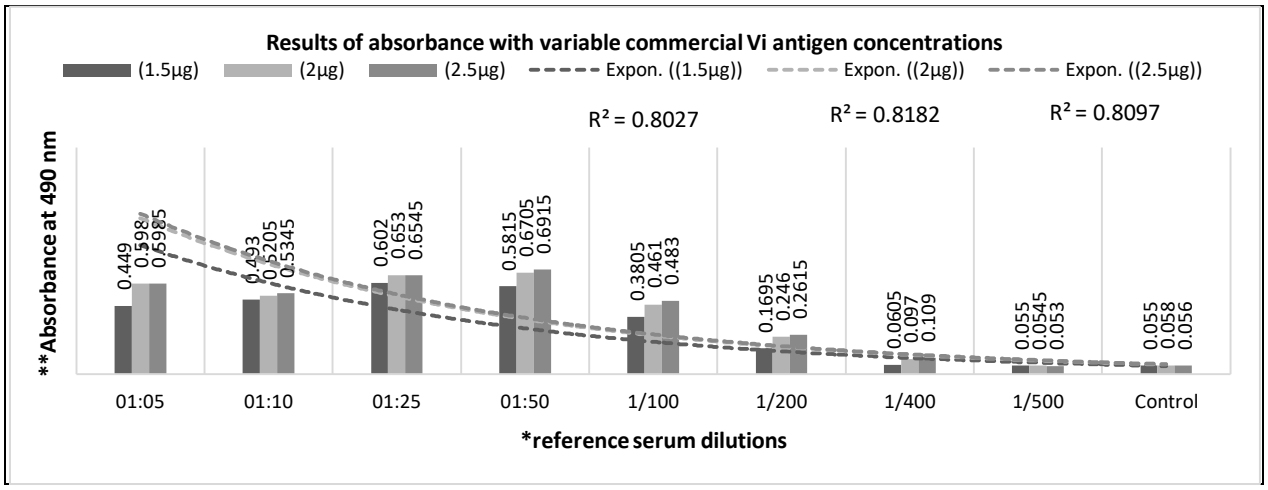


**Figure 5:** Results of ELISA for commercial Vi-antigen * Dilutions of rabbit Vi-antiserum **Absorbance at 490 nm

The blocking buffers used for test standardization were 2%, 3% and 5% w/v of BSA in PBST. The final blocking concentration used for the test was 3% w/v of BSA in PBST. The results of test were studied for temperature variation also. The tests were repeated comparatively at room temperature and $37^0$C. The results were found to be satisfactory at room temperature and the correlation of determination ($R^2$) was equivalent to 1 for both Vi-antigens at temperatures studied (Figure 06). The incubation time for primary and secondary antibody treatment was studied for 30, 60 and 90 minutes. It was found that 60 minutes incubation was giving equivalent results to 90 minutes. However reducing time to 30 minutes decreases the values of absorbance at higher dilutions of the Vi-antiserum wells. The value of correlation of determination ($R^2$) is equivalent to 1 for both Vi-antigens at variable incubation time periods (Fig-07).

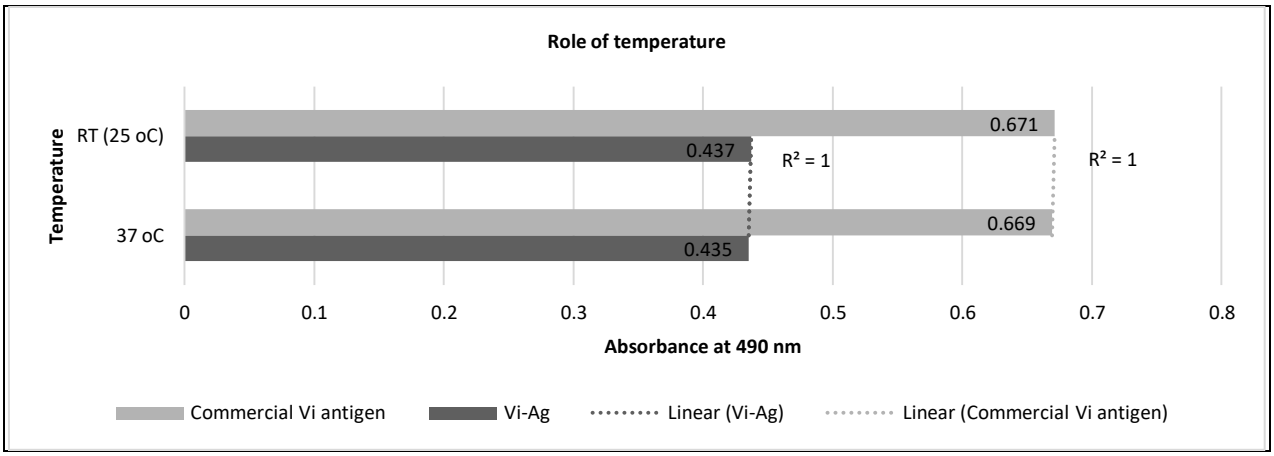


**Figure: 6** ELISA results at room temperature (RT) and 37$^0$C

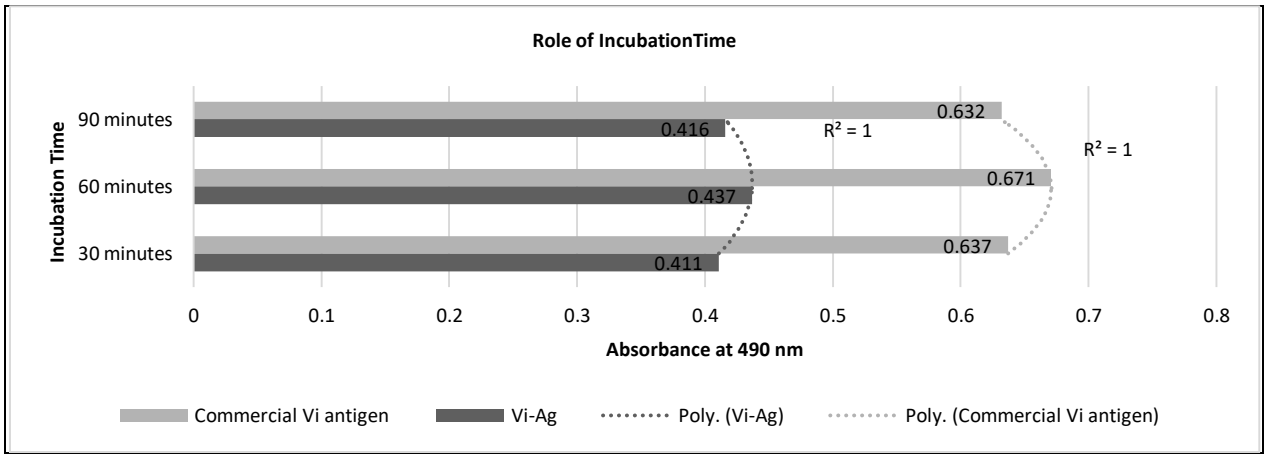


**Figure 7:** ELISA results at variable incubation time (90, 60 and 30 minutes)

The HRP labeled anti-rabbit secondary antibody was used at variable dilutions of 1:10,000, 1:15,000, 1:20,000 and 1:25,000 for repeating the tests. The best absorbance results were observed at 1:15,000 dilution of the secondary antibody.

## CONCLUSION

Vi-antigen is a virulent polysaccharide which is present in *S. typhi* bacteria. The lower molecular weight and higher molecular weight compounds when conjugated to various proteins had led to variable immunological behavior[31]. Similarly the immunogenic characteristics of Vi-antigen had been found to be enhanced when conjugated to tetanus or diphtheria toxoid[31]. Typhoid Vi-polysaccharide conjugate vaccine is currently available in market and is found to induce immunity in population. The vaccine is found to be safe and immunogenic in children too[13, 32]. These characteristics of Vi-antigen had enthused this study.

Vi- antigen had been profusely found and isolated from the patients suffering from typhoid. The antigen had been isolated from the urine of the patients and significant presence of the Vi-antigen in urine had been used in the diagnosis of typhoid[33, 34, 35]. The role of Vi-antigen in case of natural infection of *S. typhi* is a matter of concern.

The current study comprised Vi-antigen extraction from the *S. typhi* and establishing ELISA to check its antigenic role in comparison to the commercially available Vi-antigen. The ELISA was standardized in the study using various dilutions of reference Vi-antiserum. The results were positive for the Vi- antiserum raised in rabbit and found to be comparable to commercially available Vi-antigen. In ELISA, the parameters like antigen content, antibody content, conjugate content, blocking agent, incubation temperature and time used were varied to various levels to finalize the optimum values for imparting the most acceptable range of results.

The ELISA results indicated that qualitatively the Vi-Ag was found to be immuno-reactive with commercial rabbit raised Vi-antiserum. The Vi-Ag under study was identified by the reference Vi-antiserum as well have the antigenic similarity to the commercial Vi-antigen used for this comparative study. Hence the extraction of Vi antigen from *S. typhi* was appropriate and the antigen quality is comparable to standard Vi antigen available commercially. The ELISA was repeated three times at similar concentrations so the relativity and contingency of both Vi antigens were found acceptable. This study emphasized that the ELISA can be performed at room temperature and this test need no incubation at higher temperature of 37$^o$C. The incubation time during various steps of the test were finalized to be 60 minutes for

performing the test. The final dilution of HRP labelled anti rabbit dilution was standardized to be used in dilution of 1:15,000 for the final testing based on the absorbance results, color intensities and reaction completion timings.

The study indicates the limited use of Vi-antigen of *S. typhi* can be expanded so the charge of this antigen in diagnostic ELISA can be standardized. An approach that the diagnostic values of Vi-antigen need to be considered at clinical level. The Vi-antigen can be used in combination to somatic antigens, lipopolysaccharides and flagellar antigens of Salmonella in the diagnostic assays for typhoid detection using human sera[20]. The emphasis should be brought upon the Vi-antigen and studies for exploring behavior of Vi-antigen need to be improved.

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