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RESEARCH ARTICLE

APPLICATION OF *Salmonella typhi's* OUTER MEMBRANE (OMP) IN DIAGNOSIS OF TYPHOID

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ABSTRACT

In this study, a modern technique for the diagnosis of *Salmonella typhi* using the outer membrane protein was established. To reduce the identification time of the infection by typhoid fever; *Salmonella typhi* outer membrane protein was extracted from *Salmonella typhi* bacteria ATCC 19430 growing on optimized growth conditions. Partially purified outer membrane protein was immobilized on a nitrocellulose membrane and tested against serum of typhoid patients to detect the antigenicity of the protein isolated using immunoblotting technique. The molecular weight of the highly antigenic protein was measured and it was about 50KDa. These antigens showed high antigenisity against the sera of Typhoid fever patients using Western blotting technique. So, this can be an initial step towards the rapid immunochromatographic strips and ELISA diagnostic kits for the efficient detection of antibodies against *Salmonella typhi* in serum specimens.

INTRODUCTION

Salmonella typhi (*S. typhi*) was observed by Eberth (1880) in the mesenteric lymph nodes & spleen from a patient who died from typhoid (Todar, 2005) The bacterium *S. typhi* causes typhoid fever is a gram-negative, motile, non-sporing, non-capsulated bacillus (Doughari and Okafor, 2008). *Salmonella* are chemoorganotrophic, with the ability to metabolize nutrients by both respiratory and fermentative pathways. Also *Salmonella* grow at temperature of between 2 – 47 °C, with rapid growth occurring at 25 to 43 °C. The minimum temperature for growth prevails at neutral pH and increases sharply with increasing acidity or alkalinity of the suspending medium and the optimum pH for growing is between 6.5 and 7.5 (Sangvatanakul, 2007). It belongs to proteobacterial family (National standard methods, 2008).There are two distinct syndromes caused by *Salmonella*; Typhoid and paratyphoid fevers (enteric fevers) and Gastroenteritis/salmonellosis. Typhoid fever is endemic throughout Africa and Asia and persists in the Middle East, a few southern and eastern European countries and central and South America. In the US and most of Europe, apart from occasional point source epidemics, typhoid is predominantly a disease of the returning traveller (Preechakasedkit *et al*., 2012). Typhoid fever is endemic in the Mediterranean North African countries (Morocco, Algeria, Tunisia, Libya, and Egypt) with an estimated incidence of 10-100 cases per 100,000 persons (Ghenghesh *et al*., 2009). The absence of specific symptoms or signs makes the clinical diagnosis of typhoid difficult (Parry *et al*., 2002). Enteric fever is a very common diagnosis for any fever coming to a general practitioner in the developing countries (Neopane *et al*., 2006) because of a fever without evident cause that lasts more than one week should be considered typhoid until proved otherwise (Parry *et al*., 2002). The 'gold standard' for identifying the cause of an infection is the isolation and identification of the causative agent of disease (Chart *et al.,* 2007) so the definitive diagnosis of typhoid fever depends on the isolation of *S.typhi* from blood, bone marrow or a specific anatomical lesion. The presence of clinical symptoms characteristic of typhoid fever or the detection of a specific antibody response is suggestive of typhoid fever but not definitive. Blood culture is the mainstay of the diagnosis of this disease (WHO, 2003). In the absence of a viable bacterium, antibody tests can give evidence of infection provided that suitable immunoassays, based on well-characterized antigens, are used (Chart *et al.,* 2007). Widal test is the most widely used immunological tests for the detection of antibodies against *S.typhi* antibodies developed for the diagnosis of typhoid fever, the test is simple and inexpensive but of limited value, however, because of false positive and negative results (Appassakij *et al*., 1987). Therefore, we considered the establishment of a biotechnological technique for the detection of a *S. typhi* protein antigen, with the hope that it might be useful for rapid diagnosis of typhoid fever in endemic areas**.**

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MATERIALS AND METHODS

Bacterial strains and media

S.typhi (ATCC 19430) (Provided kindly by Prof. Houda Mansour, CEO, Egyblood, VACSERA, Egypt). *Salmonella* enteric subspecies enterica (extracted by Kauffmann and Edwards) Le Minor and Popoff serovar *Typhi* deposited as *Salmonella typhi* (Warren and Scott). Tryptic soya broth (TSB) medium and Tryptic soya agar (TSA) medium were prepared. The pH was adjusted at pH 7.5 for both media.

Optimization of *Salmonella typhi* culture

Bacterial cells were activated by growing them over night (O/N) on TSA plate at 37°C. Fresh TSB medium was inoculated by single colonies and incubated O/N at 37°C. Two ml O/N culture was inoculated into 100 ml fresh TSB medium at 37°C and 150 RPM for 8 hrs. The bacterial growth curve was monitored spectrophotometrically at 405 nm. Viable bacterial count (colony forming units, CFU) was carried out to obtain the CFU per ml culture according to (Zwietering, 1990).

Effect of temperature on the growth

S.typhi cells were activated as described before. Activated cells were used to inoculate 3x100 ml TSB medium. Cultures were grown at different temperatures (25°C, 37°C and 45°C), with shaking at 150 RPM. The growth was monitored as described before (Ratkowsky *et al*., 1983).

Effect of pH on the growth

To study the effect of pH; the activated *S.typhi* cells were used to inoculate TSB fresh medium with different pH (3, 7.3, and 9). Cultures were grown at 37°C with shaking at 150 RPM as above and the growth was determined as mentioned before (Sampathkumar *et al*., 2004).

Effect of inoculums size on the growth

Activated *S. typhi* cells with different inoculum size (10µl, 100µl, 1ml, and 10ml) were used to inoculate TSB fresh medium. The growth was determined as mentioned before (Butler, 2001).

Effect of addition of nitrogen and carbon sources on the growth

Yeast extract was used as nitrogen supplement in TSB medium at the level of (0.1%, 0.5% and 1%) (Kalil *et al*., 2008). Also, glucose was used as a carbon source at concentrations (0.5%, 1% and 1.25%) in TSB medium (Kiran *et al*., 2005). In another experiment; the effect of TSB medium supplemented with yeast extract at the level (1%) combined with glucose at concentration (1.25%) was mentioned. In all cases; the bacterial growth was determined as mentioned before.

Proteins production

Optimized growth culture for preparative purification (4 liters TSB medium supplemented with 1% yeast extract and 1.25% glucose, pH 7.3) was done. Four hundreds ml of TSB medium was inoculated with single colony and incubated O/N at 37°C with shaking (150 RPM). A 400 ml O/N culture was added to the TSB medium. The bacteria were grown at 37°C with shaking for 8 hour. Two ml of growth medium were collected every 2 hours, 1 ml for protein determination and the other one for performing optimized bacterial growth curve. Cells were harvested by centrifugation at 7000 RPM for 30 min at 4°C then wash twice with 0.9% Normal Saline then centrifugation at 7000 RPM for 30 min at 4°C. The pellet was collected and resuspended in Tris HCl pH (7.2) at 4°C. After which the suspension was sonicated for 3 minutes on ice using sonicator (Sonics and Materials INC., USA).After centrifugation at 12000 RPM for 10 min at 4°C, the supernatant was collected, crude membrane supernatant was dialyzed O/N against the PBS buffer (Ortiz *et al*., 1989).

Protein content determination

The protein content was determined according to Bradford method (Bradford, 1976). BSA stock (1mg/1ml) [sigma, USA] and 1X phosphate buffer saline (PBS) were prepared. Bio-Rad protein assay (Dye) was used. Serial dilutions from BSA were prepared as follow; 0.0, 0.1, 0.25, 0.5, 0.75 and 1(µg/µl). In maxisorp plate (Nunc, USA); 145µl of diluted Bio-Rad protein assay were distributed. Three µl of BSA standard or protein were added according the standard and samples plate map. The plate was shacked on the orbital shaker for 15 minutes then the absorbance were read at 650 nm using UV-max (Molecular Devices, USA).

Protein electrophoresis (SDS – PAGE):

Proteins were used for electrophoresis. For protein elctrophoresis; 100µg from each sample were boiled in the presence of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol then separated in a 12% gel according to Laemmli method, 1970. The proteins were stained with coomassie brilliant blue R-250.

Immunostaining tests

Fourty blood samples [30 are positive typhoid (+ve widal test) and 10 are negative typhoid (-ve widal test)] were collected from the Abasia fever hospital and Medpark laboratories (Cairo, Egypt). The Sera were separated. One hundred µg of protein sample was electrophoresed on a polyacrylamide gel as mentioned before. The gel was transferred to a positively charged membrane in a buffer tank blotting apparatus. The membranes were cut into strips using Immuno - stripTM bladeless 2MM membrane cutter (Novex, USA). Strips containing antigens were immersed in diluted sera (in PBS containing Tween and non fat milk) and incubated over the shaker platform O/N. Wash with hot PBS / Tween was done. An enough amount of Goat antihuman IgG labeled peroxidase enzyme conjugate (GAHU G-POD, KPL, USA) covered the strips for 1 hour at room temperature then washed with PBS - Tween at room temperature, followed with PBS only. Addition of [5% Diaminobenzidine; DAB, Sigma) in solution of PBS and H_2O_2 on the strips was done. After colour developing the reaction was stopped by washing the filter with distilled H_2O (Bolt *et al*., 1997).

RESULTS AND DISCUSSION

Typhoid fever is a multisystemic disease which still remains a global public health concern particularly in developing countries as its occurrence is fuelled by unhygienic and poor sanitary conditions (Jain *et al*., 2012). It is a life-threatening illness caused by the bacterium *S.typhi*, a gram-negative, rod-shaped, motile bacterium. It is only known to infect humans, and has an estimated global incidence of 22 million cases and 200,000 deaths per year (Preechakasedkit *et al*., 2012).

Optimization of bacterial growth:

The current study was undertaken to provide an efficient and simple system for diagnosis of typhoid infection. The antigen production was carried out under several conditions in an attempt to optimize the fermentation process. Temperature is probably the most important environmental factor affecting growth. If temperature is too hot or too cold microorganisms will not grow.The minimum and maximum temperatures for microbial growth vary widely among microorganisms and are usually a reflection of the temperature range and average temperature of their habitat. So, it was found that the most suitable temperature was 37ºC which was compatible with the results of (Leclerc *et al*., 1998) and (Chart *et al*., 2007) while (Thayer, *et al*., 1987) proved that *Salmonella typhimurium* can grow at 19 ºC. Bronikowski *et al*., 2001, stated that; *S. enterica* can grow at (27.7–39.8°C). (Fig. 1).

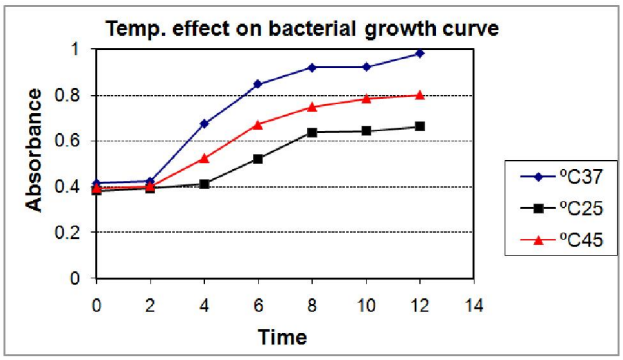

Fig. 1. Monitoring the effect of temp. on the growth curve of *S. typhi*, symbols ♦ , ■ and ▲ represent the growth of *S. typhi* at 37°C, 25°C and 45°C respectively

Most natural environments have pH values between 5 – 9 and most organisms have pH optima in this range. In this study, the most yields were obtained at pH 7.3(Fig.2) which is complied with (Thayer, *et al*., 1987), (Mcdermid *et al*., 1996) and (Compliance Guideline for Controlling Salmonella and Campylobacter). Mcdermid *et al*., 1996 stated that; the steady-state of *Salmonella* growth was possible over the pH range 4.35 - 9.45. On the other hand (Idziak and Suvanmongkol, 1972) proved that; *Salmonella sp*. increased in virulence during growth in an acid environment whereas in a near

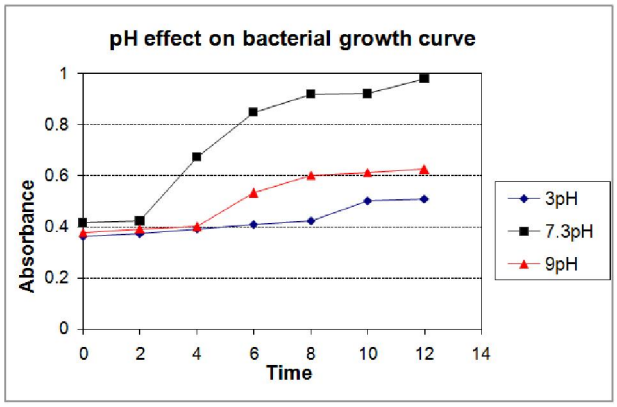

Fig. 2. Monitoring the effect of pH on the growth curve of *S. typhi*, symbols ♦, ■ and ▲ represent the growth of *S. typhi* at pH 3, 7.3 and 9 respectively.

neutral environment, the reverse was true. The growth of *Salmonella*e was observed to occur at pH values as low as 4.05 × 0.05. The growth-limiting pH was dependent on several factors, most important the acid molecule itself, the effect of temperature, relative oxygen supply and level of inoculum (Chung and Goepfert, 1970). In order to optimize the antigen production process, the bacterial medium was inoculated with different sizes from inoculums (mother culture). Cultures were grown as described earlier at 37ºC for 8 hours. The maximum yield was obtained at the highest inoculums size (10 ml) 100 ml media as shown in Fig.(3).

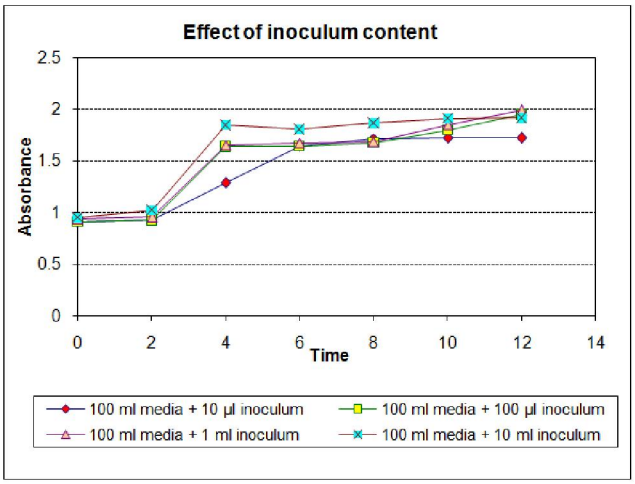

Fig. 3. Monitoring the effect of inoculum size on the growth curve of S.*typhi*, symbols ♦ , ■, ▲, x represent the growth of *S. typhi* with starting inoculum size 10µl, 100µl, 1ml, and 10ml respectively

It was proved that; there was an exponentially relationship between Salmonella growth and inoculums size (Cogan *et al*., 2001, Iturriaga, *et al*; 2003). (Butler, 2001) studied the effect of increased inoculums of S. typhi on azithromycin and resultant growth characteristics. Turbidity developed only with large inocula. While (Mackey and Kerridge; 1988) found that; Maximum growth rates and lag times of *Salmonella*e were unaffected by inoculums size. The present work deals with selecting and optimization of carbon and nitrogen sources for producing biomass from *S. typhi*. Yeast extract was evaluated as a nitrogen source (Fig 4) and glucose was as a carbon source (Fig.5). As shown in Fig.6, the biomass maximum yield from bacterial culture was higher in the cultures containing 1% yeast extract and 1.25% glucose. The bacterial growth was raised up by supplementing the culture by a combination of 1% yeast extract and 1.25% glucose (Tracy *et al*., 2002). In an attempt to integrate more than one factor studied above, the growth curve of S.typhi was monitored. The optimized bacterial growth was higher than that is of control culture

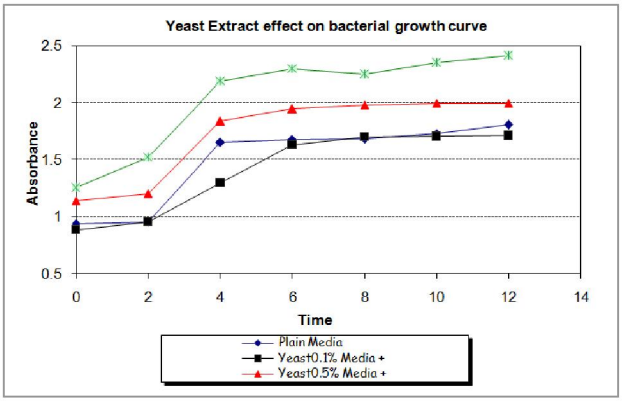

Fig. 4. Monitoring the effect of yeast extract growth curve of S.*typhi*, symbols ■, ▲, x represent the bacterial growth on TSB medium supplemented with 0.1%, 0.5% and 1% Yeast Extract respectively against plain media(♦).

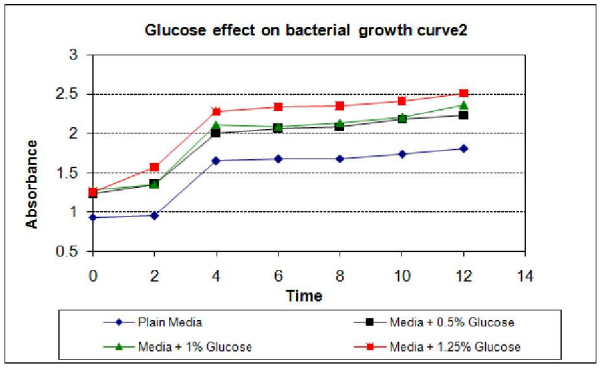

Fig. 5. Monitoring the effect of glucose on the growth curve of S.*typhi*, symbols ■, ▲, ■ represent the bacterial growth on TSB medium supplemented with 0.5%, 1% and 1.25% Glucose respectively against plain media (♦).

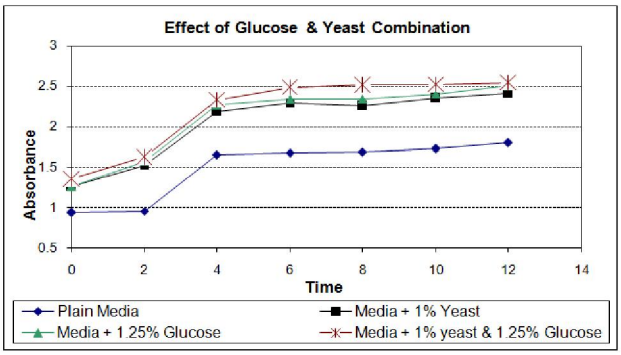

Fig. 6. Monitoring the growth curve of S.*typhi*, symbols ■, ▲, x represent the bacterial growth on TSB medium supplemented with 1% Yeast Extract, 1.25 % Glucose and Combination of 1% Yeast & 1.25% Glucose respectively against plain media (♦).

considerable the maximum bacterial growth was found in the culture by hour 8(Fig.7). As it was expected SDS-PAGE assured the same result where the level of antigen produced was started with low level then it was produced gradually parallel with its growth curve until reached to the maximum after 8 hours growth (Fig.8).

S.typhi Antigen extraction

In the current study *S. typhi*'s antigen had been extracted and partial purified in a simple method from optimized large scale *Salmonella* culture using sonication and dialysis. SDS-PAGE was carried out to check the presence of target antigen and effect of dialysis. Figure (9) shows that there are some expected undesired proteins were gotten

ride of after dialysis. In previous studies; (Blais and Yamazaki, 1989); extracted the antigen by heating *Salmonella typhi*murium in ethylenediaminetetraacetate. In another study; the 52 kDa specific protein antigen of *S. typhi* has been studied with respect to its physicochemical stability, purification by affinity chromatography and immunochemical specificity. It was found that the 52 kDa protein was degraded into smaller antigenic fragments of MW 30-51 kDa when treated with acetone, ethanol, sodium thiocyanate, 0.3M sodium chloride and Veronal and Tris buffers (Anuntagool *et al*., 1991). Extraction of the outer-membrane protein, OmpC, from *S. typhi* was done by using a modified salt-extraction procedure. It was possible to extract only the OMP from the crude membrane using this method. The purity of the preparation was confirmed by denaturing urea SDS-PAGE and N-terminal sequencing. The major OMP extracts had LPS of both bound and free forms. The free form of LPS could be removed by gel filtration and the bound form, largely, was removed using ion-exchange chromatography and by passing through ultrafiltration devices (Arockiasamy and Krishnaswamy, 2000).

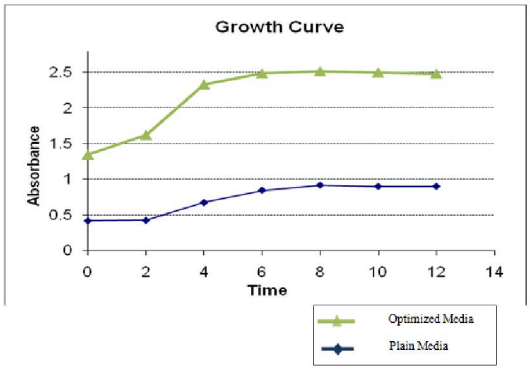

Fig. 7. demonstrate that *S. typhi* growth curve throughout growth in optimized condition; Symbols (▲, ♦) represent cultures growth in optimized conditions and growth in normal condition respectively.

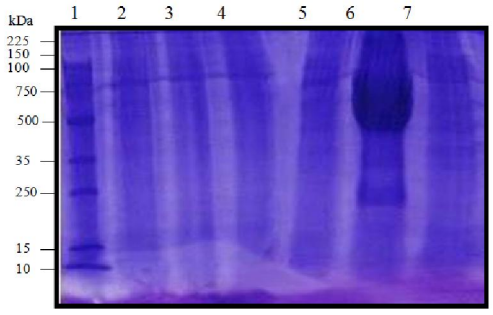

Fig. 8. 10% SDS-PAGE shows the antigen production of *S. typhi* throughout (0 – 10 hours) Lane (2-6) where lane (1) is standard protein marker (10 – 225 KDa), lane (2 to 7) represent 100 µg of cultivation production from hour (0) to hour (10).

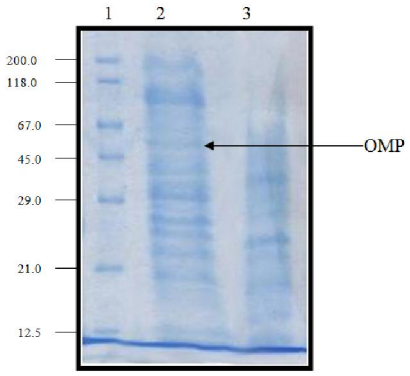

Fig. 9. 10% SDS-PAGE shows the *S. typhi* protein production and the effect of dialysis. Where Lane (1) where lane (1) is standard protein marker (12.5– 200 KDa), lane (2, 3) are the *S. typhi* proteins before dialysis and after dialysis respectively.

Immunoblotting

The diagnosis of typhoid fever in endemic areas mainly depends on the isolation of *S. typhi* from hemoculture. It takes several days before the results can be obtained however. In addition, hemoculture can be falsely negative due to prior antibiotic treatment and inappropriate timing of specimen collection, there are many immunological tests for the detection of antibody to *S. typhi* that have been developed for the diagnosis of typhoid fever, the most widely used is the Widal test, which is an inexpensive and simple method (Appassakij *et al*., 1987). But there are many limitation lead to difficulties in the interpretation of Widal test results (Choo *et al*., 1994). Several variations of standard PCR, such as multiplex PCR and real-time PCR, have recently been employed for Salmonella detection, and these methods have provided high sensitivity with some assays being able to detect as few as 30 cells per sample. The important criteria in the development of a nucleic acid based detection assay for Salmonella is the ability to detect all the diverse serotypes of the organism and PCR has been employed to replace conventional serotyping methods. PCR-based serotypings depend on specific virulence genes, and have provided high specificity. (Hatta and Simts, 2007) and (Jarquin *et al*., 2009). However there is a limitation on the number of target Salmonella serovars which can be detected in single PCR reaction. Even in multiplex PCR, it is difficult to incorporate more than five to six primer sets (correlating to five or six serovars) in one reaction due to cross-reactivity. Also the primary difficulty with routine application of molecular assays is the problem of extracting and recovering representative samples for molecular analyses. Molecular techniques also may be hindered due to chemicals present in feed samples that can inhibit PCR reactions (Jarquin *et al*., 2009).

Because hemoculture and the Widal test, which are currently used as standard methods for the diagnosis of typhoid fever, still possess some disadvantages, many alternative methods have been developed to achieve quick, sensitive and reliable results (Appassakij *et al*., 1987). Recent attempts to improve the diagnosis of enteric fever have focused on two approaches: the first is an attempt to improve the serological diagnosis for enteric fever by using new ELISA assays and the second relies on nucleic acid testing for S.typhi and paratyphi. (Olsen *et al*., 2004) and (Wain and Hosoglu, 2008). In previous study in UK to evaluate an immunoassay for the detection of human serum antibodies to the LPS and flagellar antigens of Salmonella Typhi and Salmonella Paratyphi A, B and C, and to the Vi capsular polysaccharide of S. typhi and S. Paratyphi C. A total of 330 sera were used; these originated from 15 patients who were culture-positive for S. typhi and 15 healthy controls, together with 300 sera submitted to the Laboratory of Enteric Pathogens for Salmonella serodiagnosis. The immunoassays described here provide a sensitive means of detecting serum antibodies to the LPS, flagellar and Vi antigens of S. typhi and S. Paratyphi, and constitute a viable replacement for the Widal assay for the screening of sera (chart *et al*., 2007). Furthermore, none of them has been validated in travelers. A recently developed DOT enzyme immunoassay known as "Typhidot" for detecting IgM antibody against 50 KDa OMP antigen of Salmonella typhi, was evaluated on 100 clinically suspected typhoid fever cases and 40 age-sex matched controls, in the Department of Microbiology, Mymensingh Medical College during, the period from June 2006 to July 2007. Blood culture, Widal test, and DOT EIA for IgM test were performed in all patients. Among 100 clinically suspected typhoid fever cases, 35 were subsequently confirmed on the basis of positive blood culture for S. typhi and/or significant rising titre of Widal test. The DOT EIA IgM test could produce results within 1 hour. The result of the DOT EIA IgM test showed a good diagnostic value for typhoid fever. The sensitivity, specificity, positive and negative predictive value of the test was found as 91.42%, 90.00%, 88.88% and 92.30% respectively. On the other hand corresponding values for Widal test were of 42.85%, 85.00%, 71.42% and 62.96% respectively. Thus, The DOT EIA IgM seems to be a practical alternative to Widal test for early diagnosis of typhoid fever (Begum, *et al*., 2009). (Nalbantsoy, 2012); proving that Salmonella enteritidis reaction with

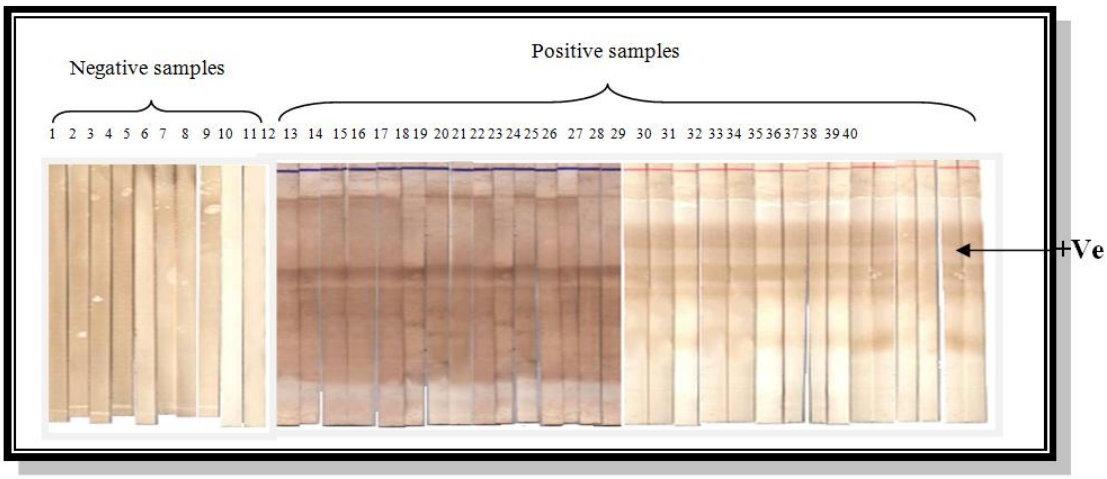

Fig. 10. shows the Antiginicty of *S. typhi* antigens where the samples (1 -10) are negative samples and (11-40) are positive samples

specific antisera and stained antigen helps to reduce the identification time of the infection and offers clinical and epidemiological advantages for the control of salmonellosis. In another study; A rapid and sensitive gold-nanobioprobe based immunoassay format has been presented for the detection of capsular Vi polysaccharide of Salmonella enterica serovar Typhi (surface antigen) using anti-Vi antibodies. The Vi antigen was extracted from serovar Typhi cells, under the optimised growth conditions for its over-expression (Pandey *et al*., 2012). In the current study, immunochromatography was developed under the optimized conditions for the rapid detection of S. typhi using the principle of a sandwich immunoblotting assay to examine the antigenisity of the bacterial antigens using positive S.typhi sera and compared with negative ones. By analyzing the result of the sandwich immunochromatographic strip test which showed that one brown line appeared for a positive test (positive sera), while no line appear for the negative test (negative sera) on the nitrocellulose membrane. It was found that the most antigenic S.typhi protein is 50 KD OMP which is compatible with (Choo *et al*., 1994) and (Hayat *et al*., 2011) which can be used for developing a rapid test for detection of S.typhi (Fig.10).

Conclusion

The current study indicates that the Immunoblotting assay is comparable to the Widal test for the serodiagnosis of typhoid fever and that it also offers the advantages of specificity, speed, early diagnosis, simplicity, and economy. In addition, it offers flexibility as other specific antigen dots can be added to the test strips, enabling other diagnostic tests to be con-ducted simultaneously. These results indicate that the Immunoblotting assay might replace the Widal test as a routine technique for early and accurate diagnosis of typhoid fever in busy hospitals in areas in which the disease is endemic. *S.typhi* 50 KD OMP was successfully isolated and used as a rapid detector for typhoid fever by detection of antibody against *S.typhi* using immunoblotting assay. This work needs a further study to optimize the 50KD outer membrane antigen production in high yield and to purify it completely. Also these antigens showed high antigenisity against the sera of Typhoid fever patients using Western blotting technique. So, this can be an initial step towards the rapid immunochromatographic strips and ELISA diagnostic kits for the efficient detection of antibodies against *S.typhi* in serum specimens.

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