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# Optimization of an ELISA Using a 50 kDa OMP Antigen *Salmonella typhi* for Detection of Anti-Typhoid Antibodies in Human Sera

Dissertation submitted in partial fulfillment for the
Degree of Bachelor of Science (Health) in Biomedicine

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# CERTIFICATE

This is to certify that the dissertation entitled

"Optimization of an ELISA Using a 50 kDa OMP Antigen of *Salmonella typhi* for Detection of Anti-typhoid Antibodies in Human Sera"

is the bonafide record of research work done by

Ms. Lau Wai Hoe

During the period August 2003 to April 2004

under my supervision

.....

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# ABSTRACT

In most tropical parts of the world particularly the developing countries, such as Malaysia, Thailand and Indonesia, typhoid fever has remained a major public health problem. The currently available laboratory methods for diagnosis of typhoid fever in endemic areas including blood culture, Widal test or other serodiagnostic methods are still unsatisfactory. This is because they failed to meet the ideal criteria of rapidity, simplicity, sensitivity, specificity, cost-effectiveness and practicality.

It is well recognized that the indirect enzyme-linked immunosorbent assay (ELISA) has high reproducibility and sensitivity as compared to the currently used methods. In this study, the ELISA was developed and optimized for the detection of serum specific IgG and IgM antibodies to against a 50 kDa Outer Membrane Protein (OMP) antigen preparation of *Salmonella typhi*. The optimal antigen coating concentration for the 50 kDa OMP was 10 μg/ml for both IgG and IgM ELISA. The ideal dilution of test serum for both IgG and IgM detection was 1:200 dilution, whilst the horseradish peroxidase-conjugated rabbit anti-human IgG and IgM reagent were each 1:1000 dilution. Both the test antibody and horseradish peroxidase-conjugate reagent were each incubated at 37°C for 1 hour. Enzyme-substrate reaction was 1, 2-Ortho-phenylenediamine dihydrochloride (OPD) in 30% (W/V) hydrogen peroxide ($H_2O_2$) was used as the enzyme-substrate and the reaction was carried out at room temperature for 5 minutes before the optical density (OD) reading was taken.

This study evaluated three groups of sera: typhoid fever sera with positive blood culture for *Salmonella typhi*, non-typhoid sera patients with negative blood culture for *Salmonella typhi*, and lastly healthy blood donors. Blood Culture method was used as a gold standard for diagnosis of typhoid fever.

Antibody concentration in test sera was expressed as Arbitrary Antibody Units (AAU) using a reference standard serum which was assigned 100 Arbitrary units. Quantitation of antibody concentration was carried by superimposing OD readings on the standard curve with dilutions of the reference serum between 1:200 and 20,000 to represented 100 and 1 AAU.

For the IgG ELISA, it was found that the coefficient of variation (CV) for both intra-assay and inter-assay was 19.0% and 45.0% respectively. The cut-off value was 47 AAU. The sensitivity and specificity of the ELISA were 42.9% and 100%, respectively, whereas, the Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were 100% and 76.2%, respectively.

In contrast, the IgM ELISA had an intra-assay and inter-assay Coefficient of variation (CV) of 20.0% and 27.4%, respectively. The cut-off value for antibody arbitrary unit was 40 AAU. The sensitivity and specificity were 71.4% and 87.5% respectively, whereas, the Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were 62.5 % and 89.4%, respectively.

These results indicate that both the IgG and IgM ELISA test were not successfully optimized. Amongst the reasons for failure to optimize the assay include a lack of a high titer positive control serum for investigation, and pipetting error in the test.

# INTRODUCTION

## The organism and transmission

Typhoid fever is caused by a Gram negative bacterium known as *Salmonella typhi* (Figure 1). The incubation period for *Salmonella typhi* until the appearance of the initial symptoms is about 1-2 weeks. It can, however, be as short as three days or as long as two months depending on the size of the ingested dose. The infective dose of *Salmonella typhi* is $10^5$-$10^8$ organisms (Haque A *et al.*, 2002).

Humans are the only natural host and reservoir for the disease. The infection is transmitted via fecal-oral route. Typhoid fever primarily involves person-to-person transmission because it lacks of an animal reservoir. Food and water contaminated by human feces and the shedding of *Salmonella typhi* by human carriers during food preparation are the main routes of transmission.

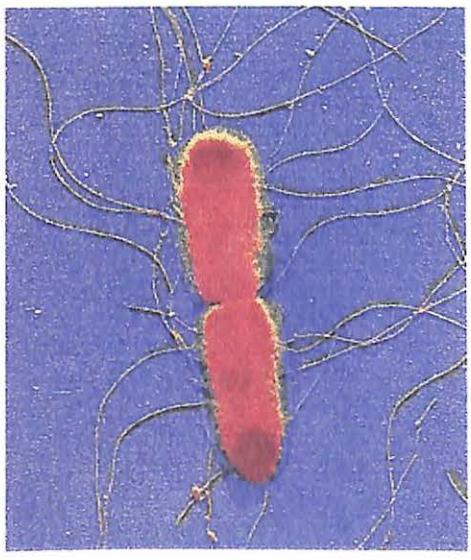

Figure 1: Scanning electro-microcopy morphological structure of *Salmonella typhi*.
Image source: GlaxoSmithKline Biologicals, Belgium.

**Symptoms of typhoid fever**

Typhoid fever is often presented as a clinical dilemma because of its highly variable, non-pathognomonic and relatively non-specific clinical manifestation. It is a systemic and prolonged febrile illness which begins with mounting fever, headache, vague abdominal pain and constipation, followed by the appearance of rashes. During the third week, the patient reaches a state of prolonged apathy, toxemia, delirium, disorientation and/or coma followed by diarrhoea. If untreated, it can lead to complications affecting various organ and systems, such as gastrointestinal bleeding, intestinal perforation, gall-bladder carcinoma and dysfunction. This systemic manifestation is due to invasion of the infective organisms into the bloodstream of the host via the Peyer's patches in the intestine, causing an acute systemic disease (Christopher MP *et al.*, 2002).

**Epidemiology of typhoid fever**

Typhoid fever continues to be a significant global problem. It is known as one of the most prevalent acute infectious diseases in the developing world with a high mortality and morbidity rate (Figure 2). Worldwide, there are 16.6 million cases of typhoid fever each year worldwide and this resulting in approximately 600,000 deaths (Ivanoff B *et al.*, 1997). The majority of those affected are 3-19 years of age, with case fatality rates ranging from 0-5% (Pang T *et al.*, 1998).

According to the 'Malaysia Annual Report', 2000, Malaysia had an incidence rate of at least 3.28 per 100,000 populations, with a fatality rate 0.52%. Since it takes several months for a typhoid patient to recover and be able to work again, typhoid fever also has important socio-economic impact due to its high morbidity rate. At current, typhoid fever continues to exist as a hyper-endemic disease due to improper sanitation and low socio-economic status. Hence the control and prevention of typhoid fever is important.

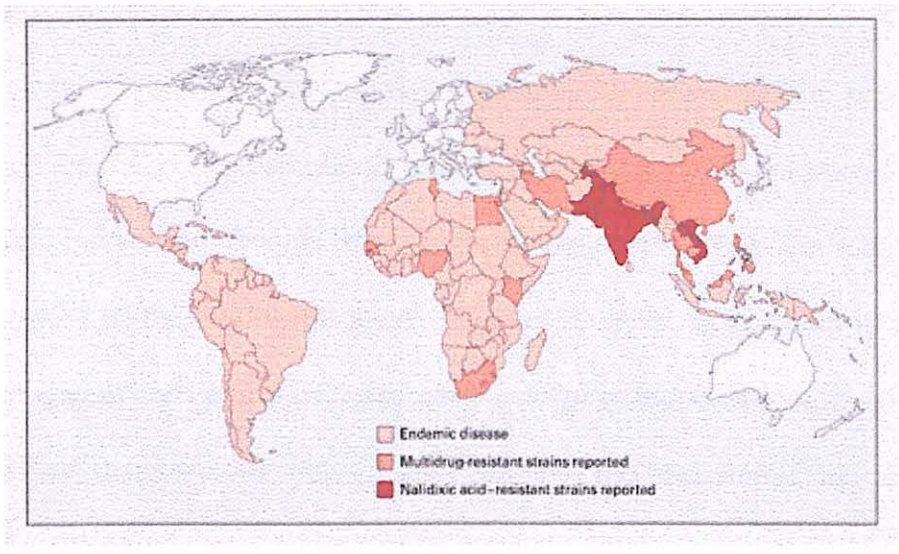


Figure 2: Global distribution of *Salmonella typhi*, 1990 through 2002. All shaded areas are areas of endemic disease (Christopher MP *et al.*, 2002).

**Case definition of typhoid fever based on World Health Organization (WHO)**

i. Confirmed case of typhoid fever

A patient with fever (38°C and above) that has lasted for at least three days, with a laboratory-confirmed positive culture (blood, bone marrow or bowel fluid) for *Salmonella typhi.*

ii. Probable case of typhoid fever

A patient with fever (38°C and above) that has lasted for at least three days, with a positive serodiagnosis or antigen detection test but without *Salmonella typhi* isolation.

iii. Chronic carrier

Excretion of *Salmonella typhi* in stool or urine (or repeated positive bile or duodenal string cultures) for more than one year after the onset of acute typhoid fever. Short-term carriers also exist but their epidemiological role is not as important as chronic carriers. Some patients excreting *Salmonella typhi* have no history of typhoid fever.

**Diagnosis of typhoid fever**

Diagnosis of typhoid fever on clinical grounds is difficult as the presenting signs and symptoms are diverse and similar to those observed with other common febrile illness such as malaria, non-severe dengue fever, hepatitis and scrub typhus. Early rapid detection and identification of the etiological agent, *Salmonella typhi*, is essential in diagnosis as well as for treatment and control of the disease in order to reduce morbidity and mortality. The conventional diagnosis for typhoid fever includes the bacteriological culture method and antibody detection tests. The diagnosis of the disease requires the isolation of *Salmonella typhi* from the blood, feces, urine or other body fluids. The Widal test and blood culture still remain the only universally practiced diagnostic procedures.

**Gold standard for detection of typhoid fever and typhoid carriers**

Blood culture is generally recognized as the gold standard of bacteriological confirmation of typhoid fever and the most practiced procedure for definitive diagnosis of early typhoid fever. However, culture negative typhoid fever is also commonly encountered especially in endemic areas, where over-the-counter drug availability increases the proportion of under treated patients.

Although blood culture is very specific, its sensitivity is poor in the first week due to various factors. Its detection limit is 10 bacteria/ml (Hoffman SL *et al.*, 1978). Hence, positive culture yields are low and this can elude definitive diagnosis. Other limiting factors include the bacteriostatic effect of antibiotics, nature of culture medium used, the time of blood collection, the host's immune response system, and the intracellular characteristic of *Salmonella typhi* (Hoffman SL *et al.*, 1978). Besides, blood culture is a time consuming process, cumbersome, and require microbiological culturing facilities. On

the other hand, bone marrow culture has a higher degree of sensitivity than blood culture. It is less affected by antibiotic drug usage but is a more invasive procedure.

The current gold standard for detection of typhoid carriers is by means of stool culture and it plays an important role in the disease control. However, stool culture is tedious, costly and less sensitive (Braddick MR *et al.*, 1987). The sensitivity is dependent on the amount of feces obtained for culture. For instance, stool cultures for carriers need several samples for examination because of the irregular nature of shedding of bacteria.

**Serological Test for Detection of Typhoid Fever**

Serological tests based on antibody detection provide a convenient alternative for diagnosis of typhoid fever. The traditional Widal agglutination test measures antibodies against flagellar (H) and somatic (O) antigens of *Salmonella typhi*. Rising or high O antibody titers thus generally indicate acute infection, while a raised H antibody helps to identify the type of enteric fever. The Widal test, however, may often be time consuming and required paired serum for interpretation. For instance, raised antibodies can result from typhoid immunization or earlier infection with salmonellae or other Gram-negative bacteria sharing common antigens that cross reacts with other salmonella strains. High Vi capsular antibody is suggestive of a carrier state, but there are high rates of false-positives and false-negatives as well (Genevieve AL *et al.*, 1987). In low endemicity areas, the frequency of weak and delayed H or O antibody responses had severely limited the usefulness of the Widal test in these populations.

Because of these problems, a number of newer tests which are antigen-based detections such as passive haemagglutination, countercurrent immuno-electrophoresis (CIEP), coagglutination (COAG), latex agglutination, fluorescent antibody test, and the more recent enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction

(PCR) have been tried for the rapid diagnosis of typhoid fever. Detection of soluble microbial antigens in body fluids have been used in rapid tests for the diagnosis of typhoid fever. These tests have been evaluated with promising results, but are not widely used in routine diagnostics.

Since the incubation period of typhoid fever is usually sufficient for a humoral response to be elicited by the time clinical symptoms develop, serological assays with adequate specificity are able to identify most cases of infection by the time of hospital admission. The remarkable specificity of the antigen-antibody reaction has been exploited in identifying and serotyping the bacteria. As a bonus, detection of specific antibodies against the somatic, flagellar or OMP antigen on *Salmonella typhi* have made it possible to diagnose the disease by immunological tests. The ELISA was one such technique that was developed by many studies for diagnosis of typhoid fever (refer to review of literature).

Recently, a rapid test known as the TyphiDot® was developed to detect the antibodies against the OMP of *Salmonella typhi.* An improved version of the TyphiDot® test, known as TyphiDot-M® is able to inactivate IgG before detecting the presence IgM in patient's serum. Technically, it was reported to be a simpler and easy-to-perform test. However, the subjectivity in the interpretation of results was a common problem due to the variability in visualization of the colorimetric reaction. This resulted in the need for repeated testing which inadvertently increased the cost of the test and prolonged the reporting time.

**The importance of 50 kDa OMP antigen preparation as the candidate antigen**

The 50 kDa OMP antigen had undergone a full-scale multi-national clinical trial to evaluate its diagnostic value (Figure 3). The 50 kDa OMP is antigenic as well as specific for *Salmonella typhi* since it only reacted immunologically with typhoid sera (Asma I *et al.*, 1991). In addition, the OMP antigen is obviously superior to the virulent (Vi) antigen. Even though the Vi antigen is present in *Salmonella typhi* and can provoke anti-Vi antibodies which have bactericidal action in serum of infected patients, it is also present in some other bacteria *Citrobacter freundii*, *Salmonella paratyphi C* and *Salmonella Dublin* which render is non-specific.

The OMP antigens (especially the porins) are more immunogenic than the lipopolysaccaride (LPS) antigen. The OMP antigen has greater potency in stimulating B-cells production and the release of high concentration of immunoglobulin. This is because the OMP antigen is more stable to the effects of proteolysis by proteolytic enzymes. The stable state of the OMP antigen may permit it to be maintained in blood for a longer period of time and thus elicit the immune response. Furthermore, the OMP antigens have the capacity to enhance cell-mediated immunity, thereby inducing long lasting immune responses against *Salmonella typhi* (Gam, 1992). There is no cross-reaction encountered between the OMP antigens of *Salmonella typhi* and *Escherichia coli* and other gram negative bacteria (Indro H and Rita D, 2000). Therefore, the 50 kDa OMP of *Salmonella typhi* has been selected as the best candidate antigen in our study.

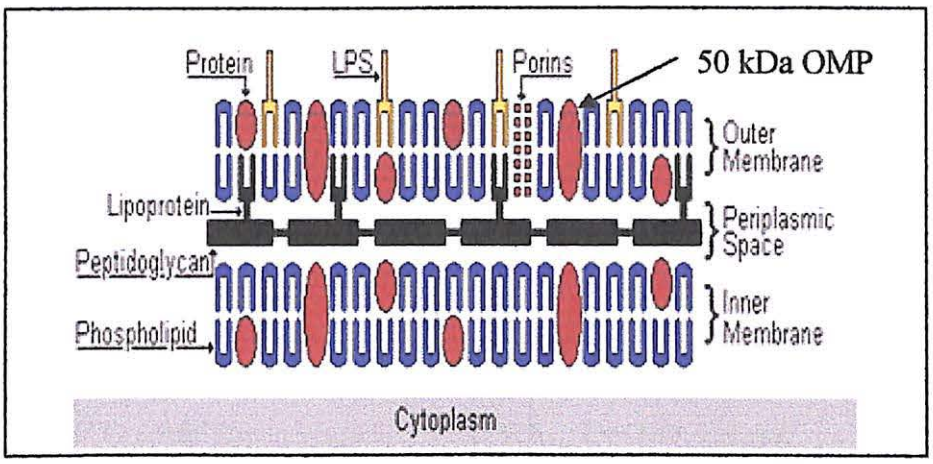


Figure 3: Location and structure of OMP on *Salmonella typhi.*

**The importance of indirect ELISA for detection of typhoid fever**

In our study, the indirect ELISA using the 50 kDa OMP on *Salmonella typhi* was used for detection of specific antibodies for typhoid fever. The ELISA is a simple and common immuno-enzymatic technique which is based on antigen-antibody reaction. Firstly, the 50 kDa OMP antigen is adsorbed onto the surface of the solid phase. Then the addition of antibodies from patient's serum, if present will bind specifically against to the antigen. Next, the complex is targeted by the enzyme-conjugated antibody and substrate is added to detect the bound conjugate complex. Lastly, the color developed as a result of conversion of colourless substitute to a colour product is stopped and read with an ELISA reader. The amount of test antibody measured is directly proportional to the optical density (OD) of the coloured end-product.

The indirect ELISA is a versatile technique for passive attachment of antigen to solid phase and the easy separation of bound and unbound reactants by a washing step. It is also simple to perform by using microtiter plates and allied equipment. Besides, the ELISA is technically more sensitive and precise than Dot-blot assay.

**Summary**

The aim of the study is to develop an ELISA to determine objectively the amount of IgM and IgG antibodies to *Salmonella typhi* 50 kDa OMP antigen in patients' sera. This study also evaluates the diagnostic value of the assay for typhoid fever by determining the cut-off value for delineating the antibody positive serum from antibody negative serum. The quantitative value for determination of IgM and IgG antibody levels obtained in this study is important to avoid the subjectivity inherent in Dot blot assays.

In this study, an indirect ELISA using the 50 kDa OMP of *Salmonella typhi* developed by optimizing antigen coating concentration, serum dilution and HRP-conjugated rabbit anti-human conjugated IgM and IgG dilution. The reproducibility, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of this assay were calculated.

In conclusion, an ELISA test for *Salmonella typhi* assist the clinician to make a definitive diagnosis and hence treatment at an early stage. Otherwise, the patients' excreta would become a constant source of spread for the disease. Moreover, the early and accurate diagnosis is important to relieve patients' suffering and avoid fatal systemic complications. Therefore, the effective management of acute and chronic typhoid fever is dependent on the speed and accuracy of diagnosis of the disease.

# REVIEW OF LITERATURE

In endemic areas where cell culture facilities are lacking or limited, the Widal test remains among the few tests available to differentiate enteric infection from other illnesses due to bacteria, viruses or animal parasites. The traditional Widal agglutination test based on antibody detection which is used to measure titer for both flaggellar (H) and somatic (O) antibodies in acute typhoid infection based on base-line cut-off titer in healthy population. However, it is also recognized this agglutination test has serious shortcomings.

Gopalakrishnan V *et al.*, (2002) summarized that the Widal agglutination test could give false positive results due to previous vaccination, infection or autoimmune diseases, whereas false negative results due to early treatment, hidden organisms in bone and joints and with relapse of typhoid fever. Furthermore, the Widal test needed paired sera for interpretation which can result in a long reporting time. Besides, they also found out that H and O antigen cross-react with other *Salmonella* strains.

Yupin M *et al.* (1990) developed an indirect ELISA for detection of serum IgG and IgM antibodies to lipopolysaccharide (LPS) and protein antigens of *Salmonella typhi* found that the indirect ELISAs were more sensitive and specific for the diagnosis of typhoid fever compare to the Widal test, which used O and H antigen.

In 1987, Genevieve A *et al.* (1987) reported that the capsular polysaccharide antigen (Vi) had poor binding on microtiter plates. In order to solve this problem, the Vi capsular polysaccharide antigen was tyraminated to increase its binding efficiency to microtiter plates. Unfortunately, the resultant IgG Vi-tyr ELISA was found to be sensitive for detection of typhoid carriers whereas the IgM Vi-tyr EliSA was found to lack sensitivity for detection of acute typhoid subjects. Hence, they concluded that the IgM Vi-tyr ELISA was unable to discriminate between patient with acute typhoid fever and

chronic carriers. This may be due to the fact that Vi is non-specific since it is also found in *Citrobacter* sp.

At the same year, Hatsadde A *et al.* (1987) reported that sandwich ELISA for detection of *Salmonella* protein antigen in patient's serum. Even though, it was able to detect *Salmonella typhi* protein antigen as low as 0.5 μg/ml and superior to the detection of Vi antigen. However, false positive results were found in normal healthy individuals and pyrexia patients. Cross-reaction of protein antigens from other related enteric bacteria were reported.

Tassanee S *et al.* (1994) reported the production of a monoclonal antibody to a 52 kDa protein from *Salmonella typhi* which showed no cross-reactivity with proteins from other bacteria causing enteric fever and enteric-like illness. The 52 kDa protein of *Salmonella typhi* was a strong immunogen and could be used to detect specific antibodies in typhoid fever serum.

ELISA-Ty using OMPs antigen has been developed by Indro H and Rita D (2000), they reported that ELISA-Ty had high sensitivity and specificity for detection of antibodies to antigens in typhoid fever.

According to the analyses of Deborah H, *et al.* 2001, ELISAs for detection of specific immunoglobulin classes of anti-LPS and anti-flagellum antibodies were compared with other serological tests such as Widal somatic (O) test and Widal flagellum (H) test. They found that a higher degree of sensitivity and specificity of the LPS IgM, IgG, IgA ELISAs as compared to Widal test. In contrast, flagellum IgG ELISA showed a lowest specificity and sensitivity as compared to other test as mention above.

Currently, molecular biology-based techniques including Polymerase Chain Reaction (PCR) assays has been reported for the rapid, specific, and sensitive detection of microorganisms in blood samples. PCR is much superior to both blood culture and Widal

test. It has great discriminating value due to its very high sensitivity and specificity. However, since PCR needs extensive infrastructure, specialized skills and high cost, PCR facility cannot be made easily available in developing countries (Abdul *et al.*, 1999)

An ideal diagnostic test for typhoid and typhoid carriers should be low cost, rapid, specific as well as sensitive. To overcome the limitations of the existing tests, new specific antigens and new diagnostic techniques have been employed. Khan E *et al.*, (1994) showed that the dot-blot Enzyme Immunoassay (EIA) test, TyphiDot® offers simplicity, speed (1-3 hours), specificity (75%), economy, early diagnosis, sensitivity (95%) and has high negative and positive predictive values. This EIA test was based on a using a 50 kDa outer membrane protein (OMP) of *Salmonella typhi* was developed by Ismail *et al.* (1998). The 50 kDa OMP was reported to be immunogenic as well as specific for *Salmonella typhi* since it only reacted immunologically with typhoid sera (Ismail *et al.*, 1991).

Since IgG could persist for more than 2 years after typhoid infection, the detection of specific IgG sometimes could not differentiate between acute and convalescent cases. Due to the lack of immunity to typhoid fever, patients in highly endemic areas often have re-infections. In the event of current re-infection, there will be a secondary immune response with a significant 'boosting' effect of IgG over IgM such that the latter is masked and could not be detected (Ismail *et al.*, 1998). Hence, a modification to the original TyphiDot® test was done by Ismail *et al.* (1998) to inactivate total IgG in the serum sample in order to achieve a more accurate diagnosis. This modified test called TyphiDot-M®, have been available commercially since 1998 and have been shown to be superior than Widal test and culture methods. (Ismail *et al.*, 1998, Choo *et al.*, 1999).

However, the TyphiDot®-M was not able to discriminate between dengue, paratyphoid, typhoid fever. This is cause by a cross-reaction of 50 kDa antigen dengue virus and *Salmonella paratyphi* A and B antigens. Furthermore, TyphiDot-M® and

TyphiDot® EIA present problems with the subjective interpretation of results. In contrast, ELISA uses OD reading for interpretation of antibody concentration, thus giving quantitative rather than subjective quantitive results.

ELISA using antigens for diagnosis of typhoid fever offers a high degree of sensitivity and specificity as well as a moderate degree of practicability as indicated by Gam (1992). The other advantage of the indirect ELISA is its ability to detect the class of antibody (IgG and IgM) against *Salmonella typhi* (Verdugo-rodrigue *et al.*, 1993 and Gam, 1992).

From the theoretical point of view, developing an ELISA using the 50 kDa OMP antigen, gives a good balance between practicability of assay as well as sensitivity and specificity much needed in the diagnosis of typhoid fever.

# LACUNA OF STUDY

Many studies using the indirect ELISA for detection of antibodies to the OMP of *Salmonella typhi* have been described by researchers world wide in an attempt to provide a plausible laboratory diagnosis of typhoid fever (Verdugo-Rodrigues *et al.*, 1993 and Indro I and Rita D, 2000). The elevated level of IgG and IgM antibodies to OMP antigen can give a comparable diagnostic value for the detection typhoid fever.

In this study, a 50 kDa OMP antigen was used to develop an indirect ELISA and for quantitation of specific amount of IgG and IgM antibodies in patient's serum. By using a reference serum assigned 100 Arbitrary units, OD reading of the ELISA could be converted to arbitrary antibody unit to compensate for plate-plate variation.

# OBJECTIVES OF STUDY

1. To developed an indirect ELISA using a 50 kDa *Salmonella typhi*-specific OMP antigen to detect IgM and IgG antibodies in sera of typhoid patients.
2. To evaluated the reproducibility of the ELISA, by using typhoid patient sera and normal sera.
3. To determine the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the ELISA for diagnosis of typhoid fever.

# MATERIALS AND METHODS

## 1. Materials

### 1.1 Antigens

50 kDa OMP antigen preparations were gifts from Malaysian Biodiagnostic Research (MBDr) Sdn. Bhd., Kuala Lumpur, Malaysia. 100 µl aliquots of the 50 kDa OMP antigen preparation at 1.0 mg/ml concentration were stored at -20°C until ready for used.

### 1.2 Serum samples

Serum samples with blood culture confirmed positive for typhoid fever were collected and stored at -20°C until ready for assay. The serum samples were gifts from Malaysian Biodiagnostic Research (MBDr) Sdn Bhd, Kuala Lumpur, Malaysia. All serum samples were tested using the TyphiDot® or TyphiDot-M® test kits before evaluation using the indirect ELISA technique.

Serum samples were used for evaluation of reproducibility, sensitivity and specificity studies are as follows:

**(A) Reproducibility of ELISA**

**Control serum**

1. Positive control: Pooled serum samples confirmed by Blood Culture and TyphiDot® positive for *Salmonella typhi* with known typhoid fever.
2. Negative control: Pooled serum samples confirmed by Blood Culture and TyphiDot® negative for *Salmonella typhi.*

**(B) Sensitivity and Specificity Evaluation**

**1. Reference standard serum**

Serum sample confirmed by blood culture and TyphiDot® positive for *Salmonella typhi* with known typhoid fever. These sera were also tested positive by double radial immunodiffusion.

**2. Test panel serum**

1. Typhoid fever sera: 7 serum samples confirmed with Blood Culture positive for *Salmonella typhi.*
2. Non-typhoid fever sera: 7 serum samples confirmed with Blood Culture negative for *Salmonella typhi.*
3. Healthy blood donors: 13 healthy blood donors 'serum confirmed with Blood Culture negative for *Salmonella typhi.*

**1.3 Reagents and buffers**

1. Hydrogen peroxide (30% w/v, stored at 4°C until ready for use)
2. 1, 2-Ortho-Phenylenediamine dihydrochloride (OPD), (Fluka Biochemica, Switzerland). Store at 4°C.
3. Enzyme horseradish peroxidase-conjugated rabbit anti-human IgG and IgM reagent (Dakopatts, Glostrup, Denmark).
4. Coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6)
5. Stopping solution (4M sulphuric acid).
6. Skimmed milk (household grade)
7. Tween 20. Store at room temperature

## 1.4 Special equipments for ELISA

1. 96-well flat bottom microtiter plates (NUNC immunoplate, MaxiSorp™, Denmark.) with total volume of 400 µl/well.
2. Microtiter plate washing device

   Fluido microtiter plate washer (Anthos Labtec Instrument, Austria) which is a fully automated, programmable 96-well microtiter plate washer using 8 needles to aspirate 400 µl/well and programmed to perform four washings with PBS (pH 7.2). Duration of time for four washing cycle are 4 minutes.
3. ELISA reader

   ELISA reader (Bio-Tek Instruments, Inc, USA) which is configured to work with Bio Tek's PC-based KC junior software. The absorbance color development of OPD substrate was read at 490 nm wavelength. The reference wavelength was 620 nm.
4. Corning™ double immunodiffusion plates were used for immunodiffusion assay and viewed using a light box.

## 1.5 Common materials and equipments

Single channel Eppendorf micropipettes (1000 µl, 200 µl, 100 µl and 10 µl), 8-channel Eppendorf micropipette (100 µl), micropipette tips (blue, crystal and yellow), 10 mm x 100 mm plastic or glass tubes, test tube racks, substrate and stopping solution troughs, volumetric flasks (500 ml and 1000 ml), 50 ml universal tubes, glass cylinder (10 ml, 50 ml and 100 ml), beakers (100 ml and 1000 ml), pH meter, electronic balance, incubator (37°C), refrigerator (4°C), absorbent paper, timer, vortex device, Eppendorf tubes (0.5 ml, 1 ml and 1.5 ml), laboratory sealing film (100 mm x 40 mm), moist chamber, stirring device, gloves, discard

jar containing Clorox, aluminium foil, light box, and plastic syringe (1 ml) and distilled water were standard laboratory supplies.

## 2. Methods

### 2.1 Control serum immunodiffusion

Immunodiffusion assay was carried out using Corning™ double immunodiffusion plates according to manufacture's instructions 20 µg/ml 50 kDa antigen preparation was added to the central well and the 20 µl test serum added to each of the peripheral holes. The plate stored in a humidified chamber for 24 hour until ready for reading.

### 2.2 Preparation of buffers and reagents

a) Coating buffer

0.05M Carbonate-Bicarbonate buffer, pH 9.6, (1.59 g $Na_2CO_3$, 2.93 g $NaHCO_3$, $NaN_3$) in 500 ml distilled water and the pH was adjusted to 9.6 using sodium hydrochride (3M NaOH). The solution was then made up to one liter by adding distilled water. Coating buffer was stored at 4°C (cold room). This carbonate-bicarbonate buffer is stable for 3 months. When using coating buffer, it was pre-warmed to room temperature for at least 30 minute before use.

b) Washing and diluting buffer PBS

Phosphate-buffered saline (PBS) powder Packet (Scimedx, Denvile, USA), (0.01M, pH 7.2 ± 0.1). Each packet contains sufficient powder to make one liter and stored at room temperature. This was freshly prepared and used as washing and diluting buffer.

c) PBS with 0.05% Tween 20 (PBST)

Phosphate-buffered saline powder (Scimedx, Denvile, USA), (0.01M, pH 7.2 ±0.1). One packet of washing buffer PBS dissolved in 1000 ml distilled water dissolved in 0.5 ml of Tween 20 to give a 0.05% solution. It stored at 4°C (cold room) until ready for use.

f) 1, 2-Ortho-phenylenediamine dihydrochloride (OPD) tablet (Fluka Biochemica, Switzerland)

Each tablet contains 30 mg of 1, 2-Ortho-Phenylenediamine dihydrochlorid (OPD), (Fluka Biochemica, Switzerland) was dissolved in 11 ml of distilled water and then was added 5 µl of hydrogen peroxide (30% W/V). This substrate solution was freshly prepared immediately before use.

g) Enzyme conjugate

10 µl of enzyme horseradish peroxidase-conjugated rabbit anti-human IgG or IgM reagent (Dakopatts, Glostrup, Denmark) was diluted in 10 ml of PBS. (1: 1000).

h) Stopping solution (4M sulfuric acid, $H_2SO_4$)

To preparing 4M sulphuric acid, 219 ml of stock 18.2N sulphuric acid was diluted in distilled water and make up to 1 liter with distilled water.

Calculation for 4M sulphuric acid preparation:

Sulphuric acid ($H_2SO_4$), (SG = 1.84; % Purity = 97; molecular weight = 98.00 g)

Normality of concentrated sulfuric acid

$$= \frac{\text{Specific gravity x \% purity x 10g}}{\text{Molecular Weight}}$$

$$= \frac{1.84 \times 97\% \times 10g}{98.00}$$

$$= 18.2N$$

Volume of 18.2N sulphuric acid required to make up 1000 ml of 4M sulphuric acid, V

$$18.2N \times V = 4M \times 1000 \text{ ml}$$

$$V = \frac{4M \times 1000 \text{ ml}}{18.2N}$$

$$= 219 \text{ ml}$$

Therefore, 219 ml of stock 18.2N sulphuric acid was required and diluted in distilled water make up to 1 liter 4M $H_2SO_4$.

## 2.3 Preparation of 10 µg/ml antigen from stock 1 mg/ml of 50 kDa OMP antigen

Calculation of the amount of coating buffer and antigen concentration needed to coat 96 wells. An excess volume of antigen was prepared to compensate for solution lost during preparation and pipetting.

Calculation of the total volume of antigen at 10 µg/ml required to add 100 µl of antigen to the wells of microtiter plate:

Total volume of coating buffer

(96 wells/plate) x (100 µl/well) = 9600 ml

Prepare 10 ml of coating buffer to dilute 10 µg/ml of antigen

Total volume of antigen at 10 mg/ml or 1000 µg/ml, V

$$V \times (1000\ \mu g/ml) = 10{,}000 \times (10\ \mu g/ml)$$

$$V = \frac{10{,}000 \times (10\ \mu g/ml)}{1000\ \mu g/ml}$$

$$V = 100\ \mu l$$

Therefore, 100 µl of stock 1 mg/ml 50 kDa OMP antigen preparation was diluted in 10 ml of coating buffer.

## 2.4 Storage of 50 kDa OMP antigen preparation

100 µl of 1 mg/ml 50 kDa OMP antigen preparation needed and diluted in 10 ml of coating buffer in order to prepare a 10 µg/ml antigen for one ELISA plate coating. Therefore, dispense 0.1 ml aliquots from 1 mg/ml stock 50 kDa OMP antigen preparation into individual Eppendorf tubes for storage at -20°C. One aliquot of antigen is thawed to room temperature and used for each 96-wells microtiter plate.

## 2.5 Storage of standard, control positive and control negative serum

Dispense 20 µl aliquots of reference standard serum, control positive and control negative serum into each individual Eppendorf tubes for storage at or below -20°C. One aliquot of each serum is thawed to room temperature and used for experiments.