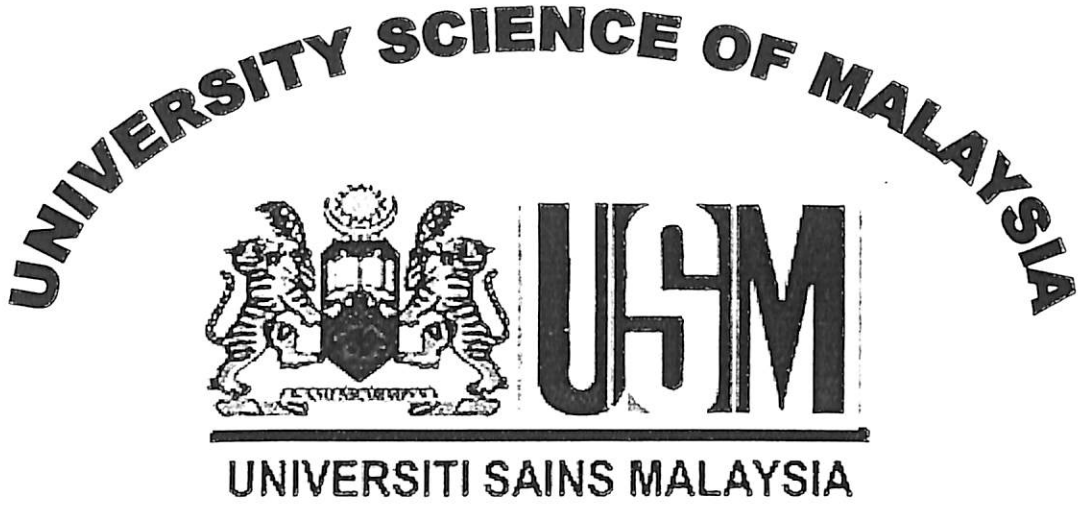

Development of an ELISA Using a Native *Salmonella typhi* 50kDa Antigen for Detection of IgA Anti-typhoid Antibodies in Human Sera.

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

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2004

CERTIFICATE

This is to certify that the dissertation entitled,

"Development of an ELISA Using a Native *Salmonella typhi* 50kDa Antigen for Detection of IgA Anti-typhoid Antibodies in Human Sera."

is the bonafide record of research work done by

Ms Kiu Siik Hung

during the period from August, 2003 to March, 2004

under my supervision.

.....

Prof. Madya Dr. Phua Kia Kien
School of Medical Sciences,
Universiti Sains Malaysia.

Date: 27/3/2004

ACKNOWLEDGMENTS

With God's help and His amazing grace, I have successfully completed this project. I have learned much during this period as I am given the opportunity to step foot into the realm of the true research work. I am thankful and glad that at last I managed to finish my laboratory work and dissertation as well.

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ABSTRACT

Typhoid fever remains an unsolved public health problem especially in the Third World countries like Philippines, Vietnam and Indonesia. Hence, a study on the reliability of an enzyme-linked immunosorbent assay (ELISA) for the detection of typhoid fever was conducted. This ELISA test measures the concentration of IgA against *Salmonella typhi* in human serum. This test is an indirect ELISA, based on a method that makes use of a 50kDa outer membrane protein specific for *S. typhi* as the solid-phase antigen. Peroxidase conjugated rabbit anti-human IgA and *ortho*-phenylenediamine as chromogen for the substrate were used to detect the presence of serum IgA bound to the solid-phase antigen. The amount of IgA was determined by reading the OD of the chromogen substrate using an ELISA reader at a wavelength of 492nm and 620nm as reference wavelength.

The optimal antigen coating concentration of the 50kDa antigen was 20μg/ml, determined by checker-board titration. IgA anti-typhoid antibodies were expressed as arbitrary units (Au) based on an IgA positive acute typhoid fever subject which was designated as 100 Au. The results of this study confirmed that the ELISA has a high reliability for the detection of typhoid fever in human sera, based on the finding of a high degree of diagnostic sensitivity (83.3%) and specificity (96%). The cut-off value, defined as the mean plus 2SD of normal human serum was 5.1 Au (n=50). Intra-assay CV was 4.8% (n =54) and inter-assay CV was 7.1% (n = 5). This assay offers advantages of speed, simplicity, and sensitivity over conventional cell-culture methods for detection of typhoid fever.

INTRODUCTION

Today, typhoid fever still is one of the major health problems in most developing countries such as Asia, Africa and Latin America. Globally, there are more than 16 million cases of typhoid each year, with more than 600,000 deaths (WHO, 1996). It is an important cause of morbidity in many regions of the world, with an estimated 13 million cases occurring annually in Asia alone (Ivanhof, 1997). In Malaysia, an estimated 4,153 cases of typhoid fever occurred between 1995 and 1999. Kelantan is the state which has the highest number of cases during those periods (Ministry of Health Malaysia, 1999).

Transmission

Typhoid fever is transmitted by food and water contaminated by the faeces and urine of patients and carriers. The infection is spread by the faecal-oral route and closely associated with poor food hygiene and inadequate sanitation. School children and young adults are most frequently affected. Besides polluted water being the most common source of typhoid, shellfish taken from sewage contaminated beds, vegetables fertilized by human faeces and contaminated milk and milk products, have also been shown to be important sources of infection.

Clinical features

Typhoid fever is a serious multi-systemic illness, which is caused by the bacterium *Salmonella typhi.* It is an enteric fever, which starts with infection of the gastrointestinal tract and can develop into fatal illness. Typhoid fever is characterized by the sudden onset of sustained fever, severe headache, nausea, severe loss of appetite, constipation or sometimes diarrhoea. Severe forms have been described with mental dullness and meningitis. The clinical features of typhoid fever are often non-specific and sometimes confusing.

Pathogenesis of *S. typhi*

Natural infection begins by ingestion of *S. typhi.* This is followed by either direct invasion of the mucosa of the small intestine, or multiplication of the bacterium for several days in the gut lumen before invasion. Stool culture findings are positive for several days after *S. typhi* ingestion and do not become negative until after the onset of clinical illness.

The M cells, which are epithelial cells that overlie the Peyer patches, are the potential sites where *S. typhi* is internalized and transported into the underlying lymphoid tissues. From the submucosa, the organism travels to the mesenteric lymph nodes, where they multiply before entering the blood stream via the thoracic duct (transient primary bacteremia). This marks the end of the incubation period, which may last from 3 to 60 days but is usually between 7 to 14 days.

During this bacteremic phase, the organisms may invade any organ but most commonly

are found in reticuloendothelial tissues of the liver, spleen, bone marrow, gall bladder, and Peyer patches in the terminal ileum. The gall bladder probably is infected via the liver, and the resultant cholecystitis is usually subclinical. The infected bile renders stool cultures positive. Preexisting gall bladder disease predisposes to chronic biliary infection, leading to long-term fecal carriage of *S. typhi.*

Invasion of Peyer patches occurs during either the primary intestinal infection or secondary bacteremia, and further seeding occurs through infected bile. The Peyer patches become hyperplastic with infiltration of chronically inflamed cells, which may lead to necrosis of the superficial layer and ulcer formation, with potential hemorrhage from blood vessel erosion or peritonitis from transmural perforation. The pathogenesis of the prolonged fever and toxemia of the enteric fever syndrome is not as well understood. Pyrogens and mediators produced at the sites of inflammation have been postulated as factors responsible for the prolonged fever.

Diagnostic methods

There are several methods that are used by general hospitals and clinics to diagnosis *S. typhi*. They are growth and non-growth methods. Growth methods are conventional methods that utilize the culture enrichment and plating procedures. They include blood culture, stool culture and bone marrow culture. Non-growth methods of detection include Widal test, Dot blot, TyphiDot™, TyphiDot-M™, DNA probes, PCR, counter-immunoelectrophoresis and haemagglutination assays. Coagglutination tests have also been used for the detection of antigens in urine and serum (Rockhill *et al.*, 1997).

Prevention

Prevention is always better than cure, so a good plan to eradicate typhoid fever must include prevention efforts. Vaccination of high-risk populations is considered the most promising strategy for the control of typhoid fever (Ivanoff, 1995). However, with large numbers of asymptomatic typhoid carriers in endemic region, identification of these person become a major logistic issue. WHO defines a 'chronic carrier' as one who is asymptomatic and still showed positive stool or rectal swab after 1 year of having had the disease. Approximately 1–4% of patients continue to harbour *S. typhi* in their intestinal tract and gall bladder for months or years, thus becoming "chronic carriers" (Caygill *et al.*, 1994).

Bacteriological confirmation of the chronic carrier state requires either multiple stool cultures or cultures of bile or bile-stained duodenal fluid. These procedures are not amenable to large-scale screening. In addition, because chronic biliary carriers are often intermittent or light fecal *S. typhi* excreters, multiple bacteriological examinations are usually required to make a reliable diagnosis (Feemster *et al.*, 1945). It is therefore crucial to develop a serological diagnostic test to detect the carriers.

The serological diagnostic test must be able to determine the specific antibody immunoglobulin class that would indicate carrier status. Losonsky *et al.* (1987) who had studied Vi antigen had shown that IgG is the primary indicator for carriers. IgM is an acute typhoid indicator and IgA can be found in both the acute and carrier states. When further tested, IgA and secretory IgA were found most frequently in the sera of dysentery and typhoid carriers. Lashin *et al.* (1976) reported in their studies that the carrier state showed high IgA and IgG

content which began as early as the acute period.

Ciznar *et al.* (1975) had also shown that IgA among carriers seemed to be elevated 2.4 times compared to non carriers (but with a previous typhoid history) while IgM is only elevated among acute typhoid cases. The high IgA content among carriers may reflect prolonged mucosa immunological stimulation since IgA when formed does not last long in the body (in cases of acute typhoid). It has been suggested that the continuous presence of IgA among typhoid carriers may be due to *S. typhi* being the primary occupant of the biliary system during chronic infection. Hence, developing an ELISA to detect specific IgA may help in detection of carriers.

Native 50kDa antigen

The sensitivity of antibody detection depends on the use of diagnostically relevant epitopes on the solid-phase. Hence, the antigen used for coating microtiter plates should be as pure as possible. Previous studies were undertaken to determine the presence of a specific antigenic proteins on the outer membrane of *S. typhi*. Immunoblot analysis using sera from patients with typhoid fever revealed that a 50kDa OMP was specifically recognized by typhoid sera. The 50kDa OMP is highly antigenic and is specific to *S. typhi* only. This native antigen has been used to detect IgM and IgG anti-50kDa antibodies and has been available as a commercial kit since 1991 (Ismail *et al.*, 1991).

Enzyme-linked Immunosorbent Assay (ELISA).

Enzyme-linked immunosorbent assay (ELISA) is similar in principle to Radioimmunoassay but depends on an enzyme rather than a radioactive label. An enzyme conjugated to an antibody reacts with a colourless substrate to generate a coloured reaction product. Three main methods form the basis of ELISAs. They are referred to as direct, indirect and sandwich ELISAs. The sandwich ELISA is used to detect or quantitate antigen, whereas the indirect ELISA is widely used for antibody measurements.

ELISA is also objective and can be automated. In addition, its use is free from restriction legislation and the reagents used have a long shelf life. The advantages of this method include high specificity and sensitivity for detection of either antibody or antigens. It is an accurate test and the interpretation of results is also easier by the use of a spectrometer to obtain a cut-off value to discriminate between positive and negative results. It is also rapid, requiring 2-3 hours for diagnosis, so that treatment can be given as soon as possible. The low cost of the ELISA is also another advantage, which is currently less than RM3.00 per test.

The growth in demand for laboratory diagnostic tests by clinical laboratories have prompted the search for low-cost, rapid methods, which are sensitive and specific. The ELISA has fulfilled all those demands.

REVIEW OF LITERATURE

Since the signs and symptoms of typhoid fever are common to many other conditions, such as malaria, dengue and hepatitis, it is therefore difficult to diagnose typhoid fever from the clinical features of the disease alone. Early and definitive diagnosis of the disease is not only important in relieving patients' suffering, but also critical in avoiding fatal complications, such as perforation of the intestines. The pathogen can be eliminated rapidly if the specific treatment is given at an early stage. Otherwise, the patient's excreta, especially stool, becomes a constant source of spread of the disease. Hence, results obtained from the laboratory are important in confirming the clinical diagnosis of typhoid and will contribute to the effective management and treatment of the disease.

There are two categories of detection of *S. typhi,* including growth method and non-growth method. The growth methods include blood culture, stool culture and bone marrow culture. Widal test, Dot blot, TyphiDot™, TyphiDot-M™, DNA probes, PCR and ELISA tests are the recent non-growth methods.

Farooqui *et al.* (1991) indicated that blood culture method has a high specificity result especially at the first week, but its sensitivity is poor. The main reason for the low sensitivity of blood culture is due to the fact that only a few bacteria is sufficient to cause severe disease. False negative results of blood culture are common for the patients who have received prior antibiotic therapy.

Brodie *et al.* (1977) also reported that bone marrow culture has a higher sensitivity than blood culture. They found that bone marrow samples may remain positive for up to 5 days longer after starting effective treatment with fluoroquinolones. The viable organism counts in the bone marrow were considerably less affected by antibiotic treatment than the quantitative blood counts, and this contributed to the superiority of bone marrow isolation over blood culture for diagnosis. However, the procedure is invasive and painful and, therefore, limits in practicality.

Stool culture could not differentiate between acute infection and carrier status. Isolation of *S. typhi* from the stool only confirms the diagnosis if characteristic clinical features are also present since the patient may be a chronic carrier. Stool culture is not only tedious and costly but also has a low sensitivity. Christie *et al.* (1987) doing studies on typhoid carriers have reported that positive fecal culture results only after 196 negative culture results. It can be concluded that the growth methods are still not good methods for detection of *S. typhi*. The culture method is time consuming, needs 2-7 days to produce the result and it is well recognized that facilities for cell cultures are not readily available or are limited in many endemic areas. Vallenas *et al.* (1985) also reported that culture method is less sensitive for diagnosis of infection especially among children compared to adults. Besides that, these techniques are laborious and cumbersome.

Non-growth methods include Widal test, TyphiDot™ , TyphiDot-M™, ELISA, DNA probes, PCR, counter-immunoelectrophoresis, haemagglutination assay and coagglutination. Widal test, TyphiDot™ and TyphiDot-M™ are serological tests, which are applied mostly in established laboratories.

The Widal test was introduced over a century ago and is still widely used for the serological diagnosis of this disease. It detects agglutinating antibodies to lipopolysaccharide (LPS) (TO test) and flagella (TH test) and required acute- and convalescent-phase serum samples taken approximately 10 days apart. Because patient management cannot wait until results for the convalescent-phase, hence, more recently, the test has been adapted for use with a single, acute-phase serum sample. However, numerous studies including Schroeder *et al.* (1969), Sen *et al.* (1969), Reynolds *et al.* (1970) and Wicks *et al.* (1971), have produced data which cast serious doubts on the value of the Widal test in the diagnosis of typhoid fever.

Several factors have contributed to this uncertainty. These include poorly standardized antigens, the sharing of antigenic determinants with other salmonellae species, and the effects of treatment with antibiotic and previous immunization with TAB vaccine. The false-positive diagnosis might occur due to the raised antibodies which is the result of typhoid immunization or from earlier infection with salmonellae or other Gram-negative bacteria sharing common antigens. The antibody titres of normal population are often not known in the endemic area, it causes problem in the interpretation of the Widal test result. In the clinical settings, an interpretation based on a single serum specimen does not reflect the diagnostic value of the test. More often even when paired sera are obtained, a decrease in titer is commonly observed when comparing the convalescent titer to the acute titer. This could be due to the fact that most patients attended the hospital during the convalescent phase, after initial pretreatment by the general practioners failed (Pang *et al.*, 1983).

Ismail *et al.* (1998) had developed a laboratory test based on the Enzyme Immunoassay (EIA) Dot Blot method using a 50kDa outer membrane protein (OMP) of *S. typhi.* This test is commercially available as the TyphiDot™ and TyphiDot-M™ test kits. The test used the 50kDa OMP as the specific antigen for *S. typhi.* The 50kDa antigen could detect the presence of specific IgM and IgG antibodies found in the sera of patients with acute typhoid using the EIA Dot Blot method. Evaluating the test kits in clinical settings, K.E *et al.* (1994) showed that the TyphiDot™ offered simplicity, speed (1-3 hours), specificity (75%), economy, early diagnosis, sensitivity (95%) and with high negative and positive predictive values.

The detection of IgM alone or with IgG would suggest acute typhoid while the detection of IgG alone in test samples posed several interpretations. 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 the more accurate diagnosis. This modified test called TyphiDot-M™ have shown that inactivation of IgG would remove competitive binding and allow accessibility of the antigen to the specific IgM, when present.

The detection of specific IgM was reported to be useful in areas of high endemicity since it could differentiate new from convalescence typhoid cases. Ismail *et al.* (1998) and Choo *et al.* (1999) reported that TyphiDot™ and TyphiDot-M™ were superior than Widal test and culture method. TyphiDot™ has high sensitivity (>95%) as well as high negative predictive value (>96%) whereas TyphiDot-M™ shows a better results than TyphiDot™ with >95% sensitivity and >97% specificity. A variant of the TyphiDot-M™ test is now available as TyphiRapid.

Tynes *et al.* (1962) have shown that asymptomatic carriers play a crucial role in the transmission of typhoid fever. These asymptomatic carriers represent an important reservoir that helps to perpetuate the disease. Approximately, 1-4% of typhoid cases become chronic biliary carriers and hence perpetuates the endemicity of the disease. Ames *et al.* (1943) also showed that the chances of becoming a carrier increases with age and is evidently greater amongst women. The detection of these carriers, thus, is an important aspect of disease control. Stool culture is the current gold standard to detect for carriers which presents a tedious, costly and low sensitivity test (Braddick *et al.*, 1991). Hence, developing a new serological diagnostic test specifically for typhoid carriers, which is specific, sensitive and cost-effective, is an important strategy in the management and control of typhoid fever.

Lanata *et al.* (1983) developed a passive agglutination assay which was reported to be highly specific and sensitive especially for highly endemic areas. The assay uses Vi antigen which is an indicator for carriers status but they found that it was not suitable for screening large population since the sera needed to be preabsorbed with sheep erythrocytes before being

used.

Losonsky *et al.* (1987) had shown that IgG is the primary indicator for carriers and IgM does not play a role. They developed and evaluated an ELISA assay using tyraminated Vi antigen to detect Vi antibodies for detection of chronic *S. typhi* carriers. The ELISA method was recommended for carrier diagnosis with IgG as the indicator at equal to or greater than the cut-off titer of 1:200. The test has a sensitivity of 86% and a specificity of 95%. The anti-Vi test is still used for the lack of a better test for carrier diagnosis.

Losonsky *et al.* (1987) also had reported that IgA can be found in both the acute and carrier state. Continuous presence of IgA among typhoid carriers may be due to *S. typhi* being the primary occupant of the biliary system during chronic infection. Gol *et al.* (1978) reported that IgA and secretory IgA were found most frequently in the sera of dysentery and typhoid carriers. Lashin *et al.* (1976) had shown that the carrier state showed high IgA and IgG content which began as early as the acute period. Other studies have also shown that IgA among carriers seemed to be elevated 2.4 times compared to non carriers (but with a previous typhoid history) while IgM is only elevated among acute typhoid cases (Ciznar *et al.*, 1975). The high IgA content among carriers may reflect prolonged immunological stimulation since IgA when formed does not last long in the body (in cases of acute typhoid). Hence IgA detection among healthy individuals may also indicate typhoid carrier state.

Currently, molecular biology-based techniques including Polymerase Chain Reaction (PCR) assays and DNA hybridization assays have been reported for the rapid, specific, and

sensitive detection of microorganisms in blood samples. Rubin *et al.* (1989) had reported that hybridization by using DNA probes was the first molecular biology technique used for the diagnosis of typhoid fever. Although detection of *S. typhi* by using labeled probes is 99% specific, but its sensitivity is poor because it cannot detect less than 500 bacteria/ml. Hashimoto *et al.* (1995) introduced PCR techniques which offers highly specific, sensitive and reasonably quick diagnostic modality. Even 1-5 bacteria/ml can be detected with absolute specificity within 1-2 days. For PCR, they preferred to target the flagellin gene because its hypervariable region Vi is unique for *S. typhi,* and its amplification provides 100% specificity.

Recently, development of DNA tests for carriers is carried at INFORMM, USM, using the ST50kDa gene sequence. The PCR test was reported to be 100% sensitive and specific against DNA of pure cultures. Currently they are working to combine specific DNA probes from various bacteria to create a DNA multi-test system using nanotechnology. Due to the need for extensive infrastructure and specialized skills, the PCR facility cannot be made available everywhere, especially in developing countries. Another factor to be considered is the relatively high cost, which is almost twice that of blood culture cost.

Hence, developing better test with rapid, higher specificity and sensitivity for the diagnosis of typhoid fever is important. In my research study, a new test has been developed to detect IgA antibodies by applying the Indirect ELISA technique and using a 50kDa native antigen. It is theorized that the presence of IgA antibodies in test sera, may indicate acute and chronic carrier states. ELISA is a useful and powerful method in estimating 10^{-9} to 10^{-12} gram order of materials in solution, such as serum, urine and culture supernatant (ELISA

homepage, 1999). ELISA is a versatile, simple, sensitive and quantifiable test. It promises a high specific and sensitive method for detection of IgM, IgG and IgA in human sera, even though the level is low. The interpretation of result is also easier using an ELISA reader to remove subjectivity.

LACUNA

So far, there has not been any study to develop an ELISA test using the 50kDa native antigen of *S. typhi* to detect specific IgA antibodies in typhoid sample.

OBJECTIVE OF STUDY

To develop an Indirect ELISA using a native 50kDa OMP antigen for the detection specific IgA antibodies in human sera.

MATERIALS AND METHODS

Antigen

50kDa native antigen was provided by MBDr, Kuala Lumpur.

Serum samples

a. 14 typhoid fever patients tested positives by TyphiDot™ were obtained from hospital KB.

   159 serum samples were from food handlers.

   From the 159 food handler serum samples,

   i. 6 samples were stool culture positive. All were TyphiDot™ IgM negative and 1 sample was TyphiDot™ IgG positive.

   ii. 19 samples were TyphiDot™ IgG positive, 2 samples were TyphiRapid–M IgM positive.

   iii. 4 samples were TyphiDot™ IgA positive, 1 sample was TyphiRapid–M IgM positive.

   iv. 19 samples were TyphiRapid–M IgM positive.

   v. 115 samples were negative for all tests.

b. 50 serum samples from normal healthy humans were obtained from Blood bank HUSM.

c. High positive control serum was selected from the 14 serum samples, which showed high OD readings in a preliminary ELISA screen.

d. Low positive control serum was obtained from the 159 serum samples, which showed low OD reading units during a preliminary ELISA screen.

e. Negative control serum was selected from the 159 serum samples, which showed the lowest OD reading units during a preliminary ELISA screen.

All serum samples were provided by INFORMM, USM, serum bank.

Reagents, chemicals and test kits

All the reagents, chemicals and test kits used are tabulated below (Table 1).

Table 1: Reagents, chemicals and test kits

No	Reagents, chemicals and kits
1.	Coating buffer
2.	3% skimmed milk (Anlene)
4.	Peroxidase-conjugated rabbit anti-human IgA (Dako, Denmark)
5.	Substrate buffer
6	PBS buffer (Biosystem, Barcelona Spain)
7	OPD (Fluka, Switzerland)
8	4M H_2SO_4

Equipment

Equipment used are tabulated below (Table 2).

Table 2: Equipment

No.	Equipment
1.	Microtiter plates (NUNC maxisorp, Denmark)
2.	Eight-channel pipettor
3.	20,100 and 1000 microliter pipetters
4.	UV microtiter plate reader (U Quant)
5	ELISA machine washer (Fluido)
6	37^0C dry Incubator
7	Vortex mixer
8	pH meter

Methods

Preparation of reagents and media

1. Coating buffer

Carbonate-bicarbonate buffer (pH 9.6) consisting of 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$ and 0.2 g of NaN_3, made up to 1 liter with distilled water. Adjusted to pH 9.6 with sodium hydroxide.

2. Washing and dilution buffer

1 package of PBS (Biosystem, Barcelona Spain) diluted in 1 liter distilled water.

3. Blocking Reagent

3% skimmed milk, consisting of 0.9 g of skimmed milk made up to 30ml with PBS buffer, made up fresh for each usage.

4. Enzyme labeled conjugates

1/1000 dilution of peroxidase-conjugated rabbit anti-human IgA (Dako, Denmark)

5. Chromogen substrate

Ortho-phenylenediamine(OPD) (Fluka, Switzerland)

1 tablet OPD was dissolved in 10 ml distilled water with 5µl 20 volumes hydrogen peroxide. The substrate solution was prepared immediately before used.

7. Stopping solution

4M H_2SO_4 was prepared by adding 219ml of 18.2N stock sulphuric acid in 781 ml of distilled water and made up to 1 liter.

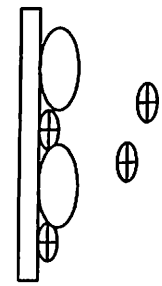
Indirect ELISA Procedure:

Enzyme immunoassays make use of enzymes attached to one of the reactants in an immunoassay to allow quantification of the analyte through the development of color after the addition of a suitable substrate.

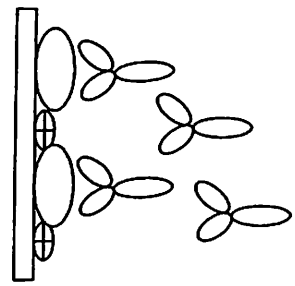
100µl of antigen, at varying concentrations was pipetted into each well of a 96-well flat-bottom microtiter plate. The microtiter plate was incubated overnight at 4^0C. On the next day, the microtiter plate was washed 4 times with PBS using an ELISA washing machine and the washed plates inverted to dry. Next, 300µl of 3% skimmed milk as blocking reagent was added to each well, incubated for 1 hour in room temperature and then washed 4 times in PBS. 100 µl of appropriately diluted test sera, control positive and control negative sera were dispensed into each well and incubated at 37^0C for one hour. After washing 4 times to remove excess antibodies, 100µl of 1:1000 diluted HRP conjugated rabbit anti-human IgA reagent was added into each well and incubated at 37^0C for one hour. After washing 4 times in PBS, 100µl chromogenic substrate (OPD) was added and incubated at room temperature for 4 minutes. 100 µl of stopping solution is added to stop the enzyme reaction. The OD readings of the substrate product in each well was obtained using an ELISA reader set at 492nm and 620nm wavelength as reference.

Figure 1: Diagrammatic representation of indirect ELISA.

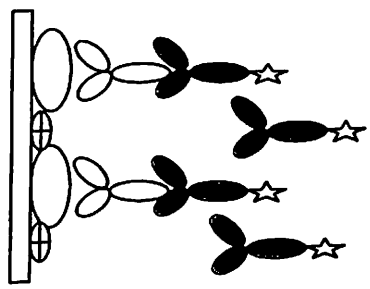
Step 1: Adsorption of antigen



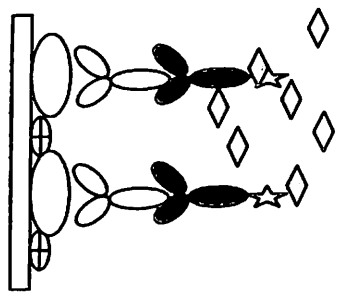
Step 2: Blocking the non-specific binding sites with skimmed milk.



Step 3: Addition of test serum

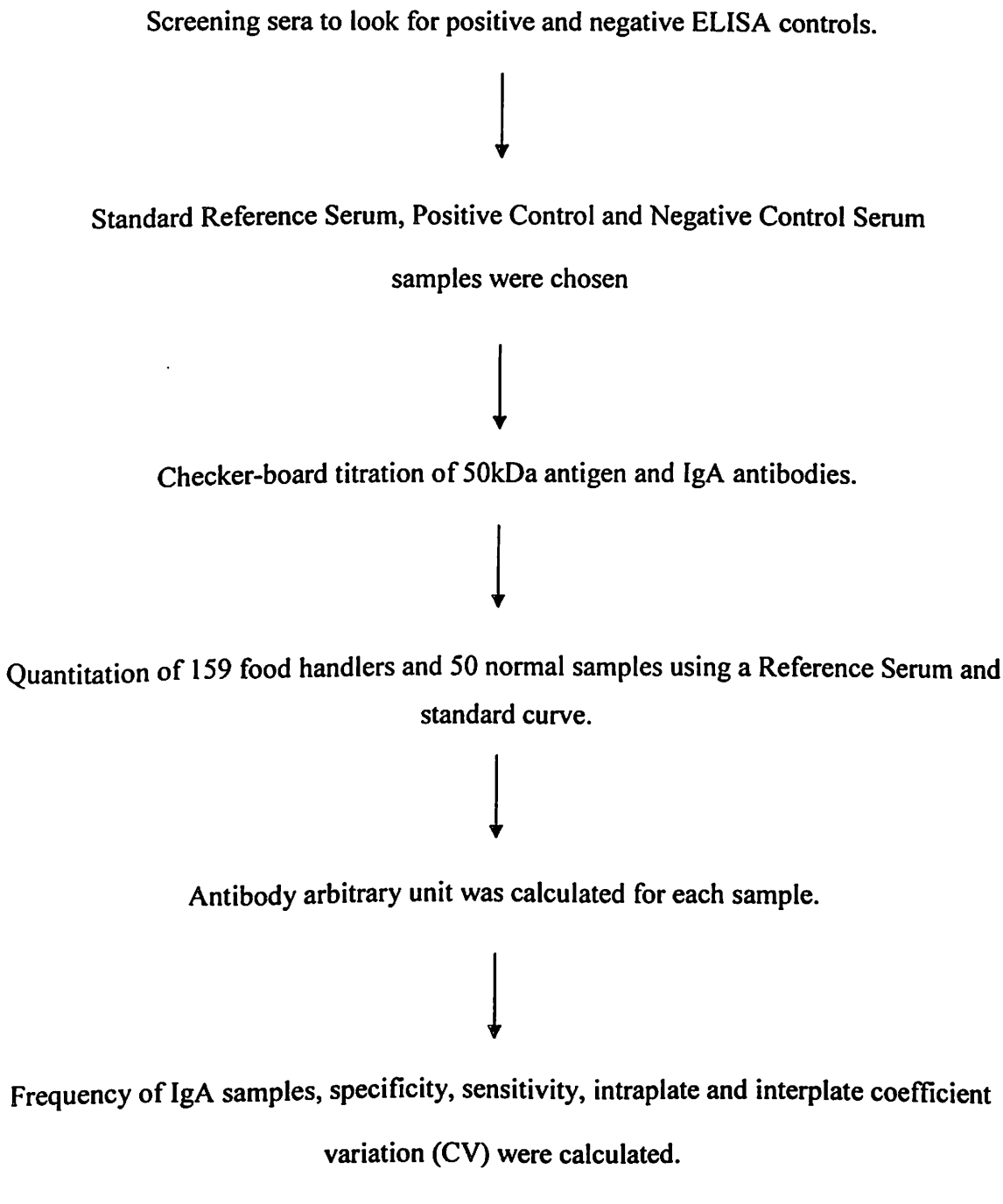


Step 4: Addition of label, HRP-Conjugated rabbit anti-human IgA reagent.



Step 5: Addition of Substrate and colour development.

Figure 2: Flow chart for Research Methodology



Anti-50kDa IgA antibody screening using 50kDa native antigen.

The 50kDa OMP was used as the solid-phase antigen to look for the anti-50kDa IgA antibody in test serum. 174 serum samples were tested to determine their IgA levels relative to the reference Standard Serum.

Titration of coating antigen (50kDa native antigen) and primary IgA antibody

The main purpose of the checker-board titration was to find out the optimal amount of antigen needed to coat microtiter plate wells. This is to avoid coating the wells with excessive antigen to avoid wastage. The optimal dilution of primary antibody was also determined through the checker-board titration.

Table 3: Checker-board titration using native antigen for IgA anti-typhoid antibody detection.

	Column 1	2	3	4	5	6	7	8	9	10	11	12
Antigen Concentration	100	50	25	12.5	10	5	2.5	1.25	1	0.5	0.25	0.125µg/ml
Row A 1/50												
A/B DIL B 1/100												
C 1/200												
D 1/400												
E 1/800												
F 1/1600												
G 1/3200												
H NC (1/100)												

A/B DIL= Antibody dilution, NC= Negative Control

Screening 159 food handler samples and 50 normal serum samples.

The aim was to screen those samples to ascertain whether the ELISA method can detect IgA antibody in human sera. Based on the checker-board titration, 20μg/ml of the 50kDa native antigen was found to be sufficient. Each sample was screened in duplicate and its antibody concentration was calculated relative to the reference standard serum, which was arbitrary assigned, 100 antibody units.

Table 4: Screening.

Row A/B DIL	Column 1	2	3	4	5	6	7	8	9	10	11	12
A 1/100			C1		6		14		22		30	
B 1/125			C2		7		15		23		31	
C 1/167			C3		8		16		24		32	
D 1/250			1		9		17		25		33	
E 1/500			2		10		18		26		34	
F 1/1000			3		11		19		27		35	
G 1/2000			4		12		20		28		36	
H 0			5		13		21		29		37	
	Reference Serum		Test sera in duplicate.									

A/B DIL= Antibody dilution

All 209 serum samples were screened using 6 microtiter plates, each plate containing the positive standard serum and control positive and negative sera. Each test serum was assayed in duplicate. All test samples and sera were diluted 1/100 dilution in PBS.

Example of Dilution Method

Concentration of stock antigen=1mg/ml. Antigen is diluted in coating buffer

Figure 3: Antigen dilution