EVALUATION OF WIDAL TUBE DILUTION AND ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DIAGNOSING TYPHOID FEVER AT A REFERRAL HOSPITAL IN KENYA

BY

BUIGUT, JEMUTAI ABIGAEL

BSc. Env. Health

A Thesis Submitted in Partial Fulfillment for the Requirements of the Degree of Master of Science in Immunology of Department of Immunology, School of Medicine, Moi University Kenya.

MARCH 2015

DECLARATION

DECLARATION BY THE CANDIDATE

This thesis is my original work and has not been presented in any university for a degree. No part of this thesis may be reproduced without the prior written permission of the author and/ or Moi University.

Buigut J. Abigael SM/PGI/01/10…….…….. Date……..……

DECLARATION BY SUPERVISORS

This thesis has been submitted for examination with our approval as university supervisors.

Dr. Kirtika Patel, BSc, MSc, PhD.

Lecturer Department of Immunology,

School of Medicine,

Moi University. ……..……..…… Date……...…….….

Dr. Nathan G. Buziba, MBChB, MMed.

Lecturer Department of Hematology and Blood Transfusion,

School of Medicine,

Moi University. …….…….……. Date…….

DEDICATION

This thesis is dedicated to those who have been diagnosed with typhoid fever, researchers in the field of health sciences, and my family for the sacrifices they made to see me succeed.

ABSTRACT

Background: Typhoid fever is a disease caused by the pathogen *Salmonella enterica* serotype *typhi*. It is a water and food-borne disease of public health importance. To diagnose typhoid fever, Kenyan health facilities have relied on the Widal slide test, a serological technique. The documented Sensitivity and Specificity values of the Widal slide test are inconsistent ranging from 34-86% and 42-99% respectively. In 2011 in Kenya, the Ministry of Health banned the use of the Widal slide test for diagnosing typhoid fever in health facilities because of its poor performance. This was in accordance with the set World Health Organization guidelines, which stipulate that the sensitivity and specificity values of a test should approach 100% and should be consistent. To replace the Widal slide test in routine diagnosis of typhoid fever, WHO recommends the Widal tube dilution test, which is a more accurate version of the Widal slide test. Apart from that, use of culture to diagnose typhoid fever is also recommended. However, these tests present challenges in terms of speed and cost. Thus, there was need to evaluate other existing tests, which are cheaper and faster.

Objective: To analyze the value of the Widal tube dilution test vis-à-vis the Enzyme-Linked Immunosorbent Assay (IgG) in diagnosis of typhoid fever.

Setting Design and Methodology: The study was cross-sectional, done at the Moi Teaching and Referral Hospital outpatient clinic and the wards. Calculation of sample size arrived at a minimum of 118 clinically suspected cases using a formula derived from Fischer's formula for use in Sensitivity and Specificity studies. Convenience sampling was used to select the participants. Only willing cases were enrolled in the study. Participants were both males and females of all ages. Blood cultures were done using venous blood of all the participants. The Widal tube dilution test and Enzyme-Linked Immunosorbent Assay (IgG) were performed using prepared serum of all the participants. Culture, Widal tube dilution, and ELISA (IgG) test results were analyzed to determine any variation in the tests.

Ethical Considerations: Permission to conduct the study was sought and granted from the Institutional Research and Ethics Committee and Moi Teaching and Referral Hospital.

Results: Widal tube dilution test had a sensitivity of 76.92%, specificity of 77.36%, Positive Predictive Value of 80.65%, and Negative Predictive Value of 73.21%. The test was easy to perform and cost-effective when compared to culture. However, it lacked in speed and in Negative Predictive Value. The ELISA (IgG) test had higher sensitivity (87.69%), specificity (92.45%), Positive Predictive Value (93.44%) and Negative Predictive Value (85.96%). It was fast, easy to perform, and cheap when compared to culture.

Conclusion and Recommendations: The study showed ELISA (IgG) to be a practical alternative to the Widal slide test. It had higher specificity and sensitivity values than the Widal tube dilution test. It is cost effective, fast, and easy to perform as compared to culture. The cost per patient for ELISA (IgG) amounted approximately $5. It should be considered for use in routine diagnosis of typhoid fever in health facilities.

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LIST OF ABBREVIATIONS

CIE- Counter-current Immuno-Electrophoresis

CFTR- Cystic Fibrosis Trans-membrane conductance Regulator

AIDS- Acquired Immune Deficiency Syndrome

COAG- Co-agglutination

DNA- Deoxyribo Nucleic Acid

EIA- Enzyme Immunoassay

ELISA- Enzyme-Linked Immunosorbent Assay

FPR- False Positive Rate

GM-CSF- Granulocyte-Macrophage Colony Stimulating Factor

HIV- Human Immunodeficiency Virus

HLA- Human Leukocyte Antigens

IDL- Intermediate Density Lipoprotein

Ig- Immunoglobulin

IL- Interleukin

Ipaf- Ice Protease Activating Factor

IREC- Institutional Research and Ethics Committee

LPS- Lipopolysaccharide

MTRH- Moi Teaching and Referral Hospital

NPV- Negative Predictive Value

NTS- Non-Typhoidal Salmonellosis

OD- Optical Density

PBS- Phosphate Buffered Saline

PMNL- Polymorphonuclear Leukocytes

PPV- Positive Predictive Value

SPI-2- *Salmonella* Pathogenicity Island-2

TMB- Tetramethylbenzidine

TNF- Tumour Necrosis Factor

TPR- True Positive Rate

WHO-World Health Organization

ACKNOWLEDGEMENT

I am particularly grateful to my supervisors, Dr. Kirtika Patel and Dr. Nathan G. Buziba for their insightful comments, guidance, and support through all stages of writing of this thesis and carrying out of the research. I would also like to sincerely thank Mr. Richard Biegon, Dr. Wilfred I. Emonyi, and the MTRH and AMPATH lab technicians for their help with the laboratory procedures. I thank biostaticians, Mr. Alfred Keter of AMPATH and Dr Ann Mwangi of School of Medicine, who assisted me in the calculation of the sample size and in the analysis of the data. I also thank the, Department of Immunology, Professors Simeon K. Mining, Alex K. Chemtai, and Eleanor Fish (Visiting professor - University of Toronto), for their contributions to my work and their dedication towards my training.

Finally, I want to thank my family, for their understanding, support, and inspiration. Above all, I thank Jehovah God for enabling me to accomplish this task.

CHAPTER 1: INTRODUCTION

1.1 Typhoid Fever and *Salmonella enterica typhi*

There are over 2500 serotypes of pathogenic bacteria in the genus *Salmonella*, of which 200 are known to cause human infections [(1)]. The infections/diseases caused by pathogens of the *Salmonella* genus are collectively known as salmonellosis. Salmonellosis can present as typhoid fever or NTS (non-typhoidal salmonellosis) [(1)]. Salmonellosis is among the most widely distributed water and food-borne disease. Millions of cases of human salmonellosis have been reported worldwide [(2)]. Every year the incidence and severity of these diseases continues to increase especially in developing countries resulting in high mortality rates [(2)]. This has led to a major concern for those in the field of public health. For the purposes of this study, this paper does not discuss NTS but focuses on typhoid fever.

Typhoid fever or enteric fever are terms that have been used for the disease that is caused by several serovars of *Salmonella enterica* including *Salmonella typhi* and *Salmonella paratyphi A, B (Salmonella schottmuelleri),* and *C (Salmonella hirschfeldii)* [(3)]. Typhoid fever is found only in humans. Globally, *Salmonella enterica* serotype *typhi* is the most common cause of typhoid fever [(4)]. Typhoid fever resulting from *Salmonella paratyphi A* infections occurs in some parts of the world and is mostly associated with travelers, thus is of significant importance in travel medicine [(5)]. *Salmonella enterica typhi*, a Gram-negative bacterium, is very similar to *Salmonella paratyphi A*, but often causes a less severe disease than the latter. *Salmonella paratyphi A, B,* and *C* are rare [(6)]. With respect to other Salmonella serovars, *Salmonella enterica typhi* causes a greater

disease burden and there is a better understanding of this organism [1]. WHO (World Health Organization) estimates 16-33 million cases of typhoid fever annually resulting in 216, 000 deaths [2] [4] [7]. The global distribution of typhoid fever is shown in Figure 1.

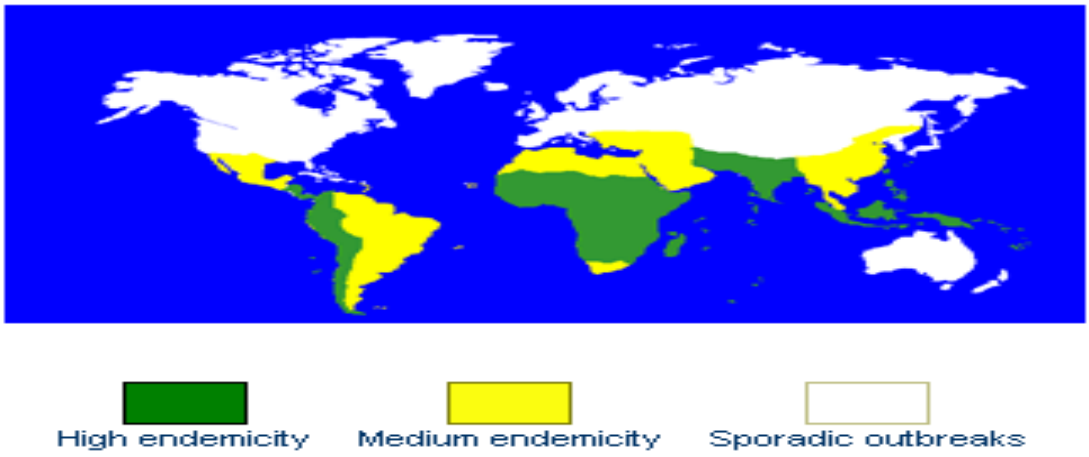

Figure 1: Global distribution of typhoid fever (risk areas).
Source: World Health Organization Department of Vaccines and Biologicals. Background document: The diagnosis, treatment and prevention of typhoid fever. *Communicable Disease Surveillance and Response, Vaccines and Biologicals (WHO/V&B/03.07)*. 2003, pp. 19-23.

A study done in an urban informal settlement in Nairobi, Kenya found the incidence rates of typhoid fever to be 247 cases per 100,000 person years of observation (pyo) [8]. The Moi Teaching and Referral Hospital, where this study was carried out, lies in the western part of Kenya in Uasin-Gishu County. It also serves Keiyo-Marakwet, Tranzoia, Turkana, Nandi, Baringo, Nakuru, Kericho, Bomet, Kisii, Nyamira, Homabay,

Migori, Kisumu, Siaya, Busia and Bungoma Counties. The western part of Kenya is in an endemic zone with estimated prevalence of occurrence typhoid fever of 50% from previous cross-sectional studies [4] [8].

This study focused on the bacteria *Salmonella enterica* serotype *typhi*, which is the commonest cause of typhoid fever [3]. From here henceforth, the bacteria will be referred to as *S. typhi* in the thesis.

1.2 How *S. typhi* Causes Infection

S. typhi is transmitted through faeco-oral or urino-oral routes. It is ingested in either food or water, and finally reaches the small intestine. It penetrates the mucosal epithelium and arrives in the lamina propria, where it rapidly elicits an influx of macrophages that ingest the bacilli but do not kill them. *S. typhi* has been known to persist within macrophages [9]. Macrophages may be the main cell type that supports bacterial growth in vivo. Both the host and the *S. typhi* regulate this growth. The ability of *S. typhi* to survive within macrophages contributes to the dissemination of the microorganism from the sub-mucosa to the circulation. Intracellular bacterial growth within phagocytes is limited by mechanisms requiring reactive oxygen intermediates, reactive nitrogen intermediates, lysosomal enzymes, and defensins [9].

Some bacilli remain within macrophages of the small intestinal lymphoid tissue [10]. Other bacilli are drained into mesenteric lymph nodes where there is further multiplication and ingestion by macrophages. During an acute infection, *S. typhi* multiplies in mononuclear phagocytic cells before being released through lymph drainage from the mesenteric nodes, after which they enter the thoracic duct and then the general circulation (into the bloodstream). Because of this silent primary bacteraemia, the

pathogen reaches the intracellular sanctuary of the spleen, liver, and bone marrow within 24 hours of ingestion [11]. They reside here during the incubation period, usually of 8 to14 days. However, incubation periods ranging from 3 to more than 60 days have been reported. The incubation period in a particular individual depends on the quantity of the inocula and other host factors. The larger the quantity of inocula, the shorter the incubation period [12].

Factors that influence the severity and overall clinical outcome of infection by *S. typhi* can be grouped into; host factors and bacterial factors. The host factors include: impaired cell-mediated immunity [13], impaired phagocytic function, extremes of ages (the neonates and the elderly), previous exposure, or vaccination history, decreased gastric acidity [14], and altered intestinal function. Bacterial factors include: host specificity, virulence of the bacterial strain, quantity of inocula ingested, survival within macrophages, acid resistance, mucosal invasion, and intraluminal factors such as mucous layer penetration and bile salts resistance. Blaser et al explain all of these factors in detail in their book, *Infections of the Gastrointestinal Tract* [14].

Studies have shown that Cystic Fibrosis can offer protection against typhoid fever. The CFTR (Cystic Fibrosis Trans-membrane Conductance Regulator) protein is present in lungs and the intestinal epithelium and is responsible for the transport of chloride ions across the membranes. The mutant cystic fibrosis form of CFTR protein prevents entry of the typhoid bacterium into the body through the intestinal epithelium [2]. It has been suggested that the Cystic Fibrosis mutation may be one of the several mutations that has spread in the European populations because this population has shown

increased resistance to infectious diseases [3]. However, cellular and molecular evidence is currently not available for this hypothesis.

1.3 Immunological Aspects of *S. typhi* Infection

S. typhi carry a variety of virulence plasmids. A highly conserved 8-kilobase region of DNA contained within these plasmids has been associated with the ability of strains to induce bacteremia and persist within macrophages. The virulence plasmid stimulates IL-12 production in a mouse model, which may lead to attenuated T cell proliferation. In addition, both the phoP/phoQ virulence regulatory locus and the SPI-2 in the bacteria contain genes that are important for its survival within macrophages. The importance of this process is illustrated by studies that showed that phoP/phoQ-deleted *Salmonella typhimurium*, when used as a vaccine vector, caused no bacteremia or late sequelae in a limited number of subjects who received a large oral inoculum [15].

Important antigens of the *S. typhi* are the LPS, the capsular antigens and the flagellar antigens. Immunogenically, the LPS for instance the (O9) antigen is immunodominant and robust. The capsular (Vi) and flagellar antigens are thymus-independent type II in nature are poorly immunogenic in infants. On the other hand, the O9 antigen (or LPS in general) is thymus-independent type I, immunogenic in infants, and a potent B cell mitogen [16]. It can stimulate B cells without the help of T cells (unlike protein antigens) and, consequently, this makes the anti-LPS responses rapid [17].

The innate immune system plays a critical role in the initial response to *S. typhi* infection. It may be the determining factor in whether the infection is subclinical or more aggressive. Macrophages and polymorphonuclear (PMN) leukocyte response are important in *S. typhi* infection. It has been shown that depressed PMN function for

example, as seen in sickle cell anemia, malaria, schistosomiasis, and histoplasmosis increases the incidence of *S. typhi* infection in humans [18] [19]. Toll-like receptor agonists such as LPS, lipoprotein, and flagellin stimulate proinflammatory cytokine production, resulting in TNF-alpha and interleukin responses such as IL-8. In addition, there is GM-CSF, and Monocyte Chemotactic Protein-1 production in colonic epithelial cells, which in turn mediate further immune responses. Activation of Toll-like receptor 5 and Ipaf (Ice Protease Activating Factor) by *S. typhi* flagellin has been a significant finding. Ipaf activates caspase-1 leading to the secretion of proinflammatory cytokines, such as interleukin IL-1beta and IL-18, and is important in host defense against *S. typhi*. Caspase-1-deficient mice are more susceptible to several pathogens of the Sallmonella genus including *S. typhi* [20].

Cell-mediated immunity plays an important role in clearing infection and protecting against subsequent *S. typhi* infection. Thus, clinical vigilance in the diagnosis and management of typhoid fever should be increased in settings associated with cellular immunosuppression since infection is more severe and prolonged in patients with depressed cellular immunity due to glucocorticoid therapy, those infected with HIV, those who have developed AIDS, and those with malignancies. Even though this was not evident before, recent studies have shown that immunocompromised patients fare poorly with typhoidal infections. One study of four individuals with HIV infection in Peru described atypically severe diarrhea or colitis in those patients [13]. Other case reports document unusual manifestations of *S. typhi* infection such as arteritis and chorioamnionitis in HIV-infected patients.

Nude mice and mice deficient in alpha-beta T cells are more susceptible to *S. typhi* infections. Murine models suggest that CD4+ T cells are of more importance than CD8+ T cells in clearing the bacteria [20]. Humoral immune responses are also important in containing *S. typhi* infection as illustrated by the protective immunity induced in vaccination studies with *S. typhi*. Mice deficient in B cells due to a targeted deletion of the Ig-mu gene show increased susceptibility to *S. typhi* infection and are unable to mount a significant convalescent immune response [21]. In addition, mucosal humoral immune responses may play a contributory role. Murine studies have shown that secretion of large amounts of a single monoclonal IgA directed against *Salmonella typhimurium* LPS into the intestinal lumen provides significant protection against systemic disease. Furthermore, mucosal antibodies to *S. typhi* appear to inhibit the "take" of the oral live attenuated typhoid fever vaccine Ty21a [22].

1.4 Clinical Presentation of Typhoid Fever

Clinical illness is accompanied by a fairly sustained but low level of secondary bacteraemia (approx 1-10 bacteria per ml of blood). The clinical presentation of typhoid fever ranges from a mild illness to a severe clinical picture with multiple complications [23].

Acute typhoid fever without complications is characterized by prolonged fever, slight dry cough, disturbances of bowel function (constipation in adults and diarrhoea in children), headache, malaise, and anorexia. Bronchitic cough is common in the early stage of the illness. During the period of fever, up to 25% of patients show exanthem (rose spots) on the chest, abdomen, and back [24].

On the other hand, up to 10% of typhoid patients may develop serious complications. Since the gut-associated lymphoid tissue exhibits prominent pathology, the presence of occult blood is a common finding in the stool of 10-20% of patients, and up to 3% may have melena (dark coloured and tarry stools). Abdominal discomfort is present in these patients and increases in intensity with time. Intestinal perforation and peritonitis has also been reported in up to 3% of hospitalized cases [25]. The symptoms and signs of intestinal perforation and peritonitis are accompanied by a sudden rise in pulse rate, hypotension, marked abdominal tenderness, rebound tenderness and guarding, and subsequent abdominal rigidity. A rising white blood cell count with a left shift and free air on abdominal radiographs confirms the diagnosis [26].

1.5 Chronic Carriage of *S. typhi*

Some patients exhibit chronic carriage of *S. typhi* bacteria. These patients continue to excrete the bacteria in stool or urine for more than 12 months after acute infection [27]. Rates of chronic carriage after *S. typhi* infection range from 1 to 6 percent [28]. Chronic carriage occurs more frequently in women and in patients with cholelithiasis or other biliary tract abnormalities. Chronic excretion of the bacteria in the urine is usually associated with a defect in the urinary tract such as, urolithiasis and prostatic hyperplasia or concurrent bladder infection with *Schistosoma* [29]. In animal and in vitro models, *S. typhi* exposure to bile appears to induce formation of specific bacterial polysaccharide components that contribute to biofilm formation, and flagellar proteins that may enhance bacterial adherence [30]. In rare cases, chronic carriage has been observed to persist even after antibiotic therapy and cholecystectomy, suggesting that factors other than biliary abnormalities also may contribute to the carrier state [31].

In general, chronic carriers do not develop recurrent symptomatic disease. They appear to reach an immunologic equilibrium in which they are chronically colonized and may excrete large numbers of the bacteria, but have a high level of immunity and do not develop clinical disease [28]. Chronic carriers frequently have high serum antibody titers against the Vi antigen, which is a clinically useful test for rapid identification of such patients [32] [33]. Chronic carriers represent an infectious risk to others, particularly in the setting of food preparation [34]. The story of "Typhoid Mary," a cook in early 20th century, living in New York who infected approximately 50 people and three fatally, highlights the role of asymptomatic carriers in maintaining the cycle of person-to-person spread [35]. For this reason, eradication of carriage when identified should be attempted.

1.6 Diagnosis of Typhoid Fever

The definitive diagnosis of salmonellosis depends on the isolation of *S. typhi* from blood, stool, urine, bone marrow, or a specific anatomical lesion like rose spots (exanthem). Culture, biochemical, or molecular testing techniques are used to identify the *S. typhi* bacteria. This is considered the gold standard diagnostic test for the confirmation of typhoid fever. This procedure of isolating *S. typhi* takes about one week to get the conclusive results. Bone marrow aspirate culture is very reliable for the diagnosis of typhoid fever and is particularly valuable for patients who have been previously treated [36], those who have a long history of illness, and for those whom there has been a negative blood culture with the recommended volume of blood. Duodenal aspirate culture has also proved satisfactory as a diagnostic test but has not found widespread acceptance because of poor tolerance of duodenal aspiration, particularly in children [24].

It has been reported that more than 80% of patients with typhoid fever have the causative organism in their blood [37]. Thus, blood culture is also a satisfactory technique for diagnosis. The recommended media for culturing enteric fever pathogens (*S. typhi* and *S. paratyphi*) is Ox bile medium (Ox gall). However, only these pathogens can be grown on it [38]. Therefore, in a general diagnostic laboratory where other pathogens are suspected, a general blood culture medium should be used. A failure to isolate the organism may be caused by several factors including: limitations of laboratory media, the presence of antibiotics, the volume of the specimen cultured, and the time of collection since patients with a history of fever for 7 to 10 days are more likely than others to have a positive blood culture [39]. Blood culturing of *S. typhi*, whilst considered "routine," is expensive and requires special facilities and expertise. The development of cheap and reliable diagnostics would undoubtedly benefit long-term disease control and treatment [4] [40].

To diagnose typhoid fever, many countries rely on serological techniques because of their speed, simplicity, and economy. In Kenya, the serological test that has commonly been used for several years now, is the Widal rapid slide test. The principle behind this test is to demonstrate the presence of somatic (O) and flagellar (H) agglutinins to *S. typhi* in the patient's serum [41]. Prepared suspensions of O and H antigens are used. A Widal test that is considered positive is based on a 4-fold rise in O agglutinins in repeated tests for a titer of 1:80, over 2-3 weeks or greater in a single test [42]. The emphasis on the O agglutinins is because of the fact that the IgM somatic (O) antibody appears first and represents the initial serologic response in acute typhoid fever, while the IgG flagella (H) antibody usually develops more slowly but persists for longer [41].

Clinicians generally accept that, toward the end of the first week of illness, titers of either O or H antibody may rise to as high as 1:160 [43] [44]. However, the presence of clinical symptoms that are characteristic of typhoid fever, or the detection of a specific antibody response against *S. typhi* antigens is suggestive of typhoid fever but not definitive [45].

The Widal test has two types of agglutination techniques that are available: the slide test and the tube dilution test. The slide test is rapid and is meant to be used as a screening procedure. Using commercially available antigens of *S. typhi*, a drop of the suspended antigen is added to an equal amount of previously prepared serum [46]. An initial positive screening test requires the determination of the strength of the antibody. This is done by adding together equal amounts of antigen suspension and serially diluted serum from the suspected patient [47]. Agglutinations are visualized as clumps. Weakly reactive agglutinations may require an adequate light source for proper visualization, while strongly reactive agglutinations are easily seen. The result of the tests are scored from 0 to 4+, that is, 0 (no agglutination), 1+ (25% agglutination), 2+ (50% agglutination), 3+ (75% agglutination) or 4+ (100% agglutination) [48]. The smallest quantity of serum that exhibits 2+ or 50% agglutination is considered the end-point of serum activity or titer [49]. This test takes as little as five minutes to obtain results from the patient's serum.

The second type of agglutination technique of the Widal test is commonly referred to as tube dilution and it serves as a means of confirming the results of the slide test. A mixture of suspended antigen and antibody is incubated for up to 24 hrs at 37°C in a water bath. Agglutinations are visualized in the form of pellets, clumped together at the

bottom of the test tube. Results are scored from 0 to 4+ positive agglutination as described above for the slide test. The tube test is useful to clarify erratic or equivocal agglutination reactions obtained by the more rapid slide test [50]. If used in routine diagnosis, the patients will have to come back for the results after 24 hrs.

Apart from these diagnostic tests, other tests for the diagnosis of typhoid fever also exist in the market. For example;

1. The Immuno-Enzymatic Dipstick test (detects *S. typhi* directly from stool and takes about 30 minutes to obtain the results) [51].
2. Enzyme-Linked Immunosorbent Assay (detects either IgG or IgM antibodies specific to *S. typhi* from serum and takes about 1.5 hours to obtain the results).
3. Enzyme Immunoassay – Typhi Dot test (detects IgG and IgM antibodies specific to *S. typhi* from serum and takes about three hours to perform). Typhidot is an ELISA-based method, miniaturized into an immunodot test format [52].
4. IDL-TUBEX® (detects IgM antibodies specific to *S. typhi*, soluble antigens of *S. typhi*, and even whole bacteria from serum and urine and takes about 5 minutes to obtain the results) [53].
5. Diazo urine test. It is known that the putrefaction of a protein in the intestine of patients with typhoid fever leads to a breakdown product being excreted in the urine as a phenol-ring compound. This is detected by Diazo test [54].
6. Microarray technology (detects nucleic acid targets and base mismatches) [55].
7. COAG (Co-agglutination) and CIE (Counter-current Immuno Electrophoresis) [56].

1.7 Problem Statement and Justification

Several developing countries including Kenya have relied on the Widal slide test to diagnose typhoid fever. The problem is that studies of *S. typhi* have indicated that there exists persistent antibody titers specific to *S. typhi* antigens in individuals that have previously been exposed to *S. typhi* [57]. Thus, a positive Widal test titer may be seen in a healthy individual. The results of a study done in Vietnam indicated that up to 55% of persons without *S. typhi* infection exhibit detectable O and H agglutinins [58].

The situation is similar in Kenya, especially the western part where MTRH (Moi Teaching and Referral Hospital) lies, which is an area endemic to typhoid fever [2]. Hence, this has given rise to situations where clinicians give too much significance to positive Widal slide test results leading to over-diagnosis and misdiagnosis of typhoid fever. This often happens even when there is absence of compatible clinical symptoms and the individual is given antibiotic therapy [59]. To add on this, the follow-up Widal tests have been used inappropriately to determine the efficacy of the treatment given. As a result, clinicians have put some patients on prolonged courses of antibiotic agents because of a "persistent positive" Widal test, which in real sense is persistent false positive results [60]. This has led to the emergence of drug resistant *S. typhi* bacteria, further affecting patient care significantly [61] [62].

Second, studies have reported cross-reactivity of the Widal test reagents with other antibodies that are not specific to *S. typhi* in areas where febrile diseases are common leading to a false positive Widal test result [63]. The Moi Teaching and Referral Hospital lies in an area with high occurrences of other febrile illnesses most common being malaria [64]. This may undermine the performance of the Widal slide test.

Third, other studies have reported that some culture proven cases of typhoid fever are associated with a negative Widal test, that is, insignificant titers. This outcome particularly occurs if the test is done early in the course of illness. This limits the predictive value of a negative Widal test [65].

The Widal slide test has been reported to have low sensitivity and specificity values that range from 34-86% and 42-99% respectively. The huge differences in these values may be because of the use of un-standardized antigens, the level of expertise of the technician performing the test, and whether the patient is from an endemic zone or not [66]. Thus, these reports indicate that the test cannot give reliable diagnostic results. According to the international standards set by WHO (World Health Organization), it is recommended to only use diagnostics whose sensitivity and specificity values are consistent and approaching 100%. In the case of the Widal slide test, values as low as 34% for sensitivity and 42% for specificity have been reported, hence it is not recommended for use by WHO.

In Kenya, in 2011, the Ministry of Health issued a ban on the use of Widal slide test in all health facilities. A Newsletter released in 2011 from the KMLTTB (Kenya Medical Laboratory Technicians and Technologists Board), said, *"Poor diagnostics lead to reduction of quality of care given to patients and create a breeding ground for drug resistance. The ministry of health is enforcing international standards for the health care industry, and is therefore banning the use of Widal test for diagnosis of typhoid fever after studies demonstrated its inefficiency* [64]*."*

Since the use of cultures in routine diagnosis presents many challenges in terms of speed, simplicity, and economy, several health facilities in Kenya continue to use Widal

slide test in routine diagnosis of typhoid fever. It is important to identify a new diagnostic that offers speed, economy, simplicity, and gives accurate and reproducible results. Hence, there was need to evaluate Widal tube dilution and ELISA (Enzyme-Linked Immunosorbent Assay) to determine if they can overcome the mentioned challenges and be used in point-of-care diagnosis at the MTRH (Moi Teaching and Referral Hospital) and other Kenyan health facilities to replace the banned Widal slide test.

1.8 Significance of the Study

It is not possible to provide good healthcare services to patients without reliable diagnostic tests for typhoid fever. A good diagnostic for typhoid fever enables definitive typhoid fever therapy and thus better patient care and management. In addition, it would limit the emergence of multiple drug resistant strains of *S. typhi*, which will in turn lead to a reduction in the morbidity, and mortality rates associated with typhoid fever [(67)]. Therefore, there is need for an accurate diagnostic for use at MTRH and all other facilities in Kenya to facilitate definitive therapy and limit emergence of antimicrobial resistance [(68)]. An evaluation of alternatives for use in the diagnosis of typhoid fever will provide results that can be used in the selection of a proper diagnostic tool.

1.9 Research Questions

The following research questions were answered:

1. Does the Widal tube dilution test show high specificity and sensitivity?
2. Does the ELISA (IgG) test show high specificity and sensitivity?
3. Does the ELISA (IgG) kit manufactured by *MyBiosource.com* show high precision/ similar results from a single serum sample analyzed in different wells?

4. Which of the three techniques (Widal tube dilution, culture, and ELISA) is cheapest, fastest, and simple to perform?

1.10 Study Objectives

The broad objective of this study was to analyze the value of Widal tube dilution test vis-à-vis ELISA using blood culture as the gold standard at the Moi Teaching and Referral Hospital.

The specific objectives were as follows:

1. To evaluate the performance of Widal tube dilution using blood culture as the gold standard.
2. To evaluate the performance of ELISA (IgG) test using blood culture as the gold standard.
3. To determine the precision of the ELISA (IgG) kit manufactured by *MyBiosource.com*. (That is, to determine if similar results can be obtained from a single serum sample analyzed in different wells).
4. Compare the protocols, cost, and speed of culture, Widal tube dilution and ELISA tests in diagnosis of typhoid fever.

1.11 Hypotheses

The following hypotheses were tested:

1. The Widal tube dilution test is highly specific and sensitive.
2. The ELISA (IgG) antibody-detection test is highly specific and sensitive.
3. The ELISA (IgG) kit manufactured by *MyBiosource.com* shows high precision/ similar results from a single serum sample analyzed in different wells.

4. ELISA (IgG) test is the cheapest, fastest, and easiest to perform when compared to culture and Widal tube dilution.

CHAPTER 2: LITERATURE REVIEW

In developing countries, the Widal rapid slide test is the common laboratory test used in the diagnosis of typhoid fever in clinically suspected patients [69]. Research has shown that the test suffers from serious cross-reactivity with other antibodies that are not specific to *S. typhi*. Since the ultimate goal of the test is antigen–antibody complex reaction, cross-reactions are encountered in infections like malaria, dengue, miliary tuberculosis, endocarditis, and brucellosis. Cross-reaction is also encountered in chronic liver disease [70] [71]. Thus, it may produce false-positive results [72].

In addition, in endemic areas, there is persistence of *S. typhi* antibodies in individuals with previous exposure to *S. typhi*, thus, leading to an over-diagnosis of typhoid fever [73] [74] [75]. The use of typhoid vaccine also tends to lower the specificity of the Widal test because it leads to the development of antibodies that persist for a long time [76]. Thus, diagnosis of typhoid fever based on Widal rapid test alone is frequently inaccurate [77].

The value of the Widal test also depends upon the standardization of the antigens to produce consistent results. It has become evident from work done in recent years on standardization of the Widal test and interpretation of the results, that both the O and H antigens are necessary for proper serologic analysis of the suspected serum [78]. However, other evidence has indicated that no Widal test, regardless of the composition and standardization of the antigens used, is infallible, and thus it is unlikely that any will be developed that will lower the validity of the isolation of *S. typhi* [79] [80].

Clinically, a single Widal test in an unvaccinated or unexposed patient may have some diagnostic relevance. However, the result of a single test has limited diagnostic significance in an endemic region, in part due to difficulty in establishing a baseline (cut-off) titer of Widal agglutination [81]. The baseline titer that is currently used is based on 50% agglutination and over to indicate positive results.

While bacteriological culture remains the gold standard for definitive diagnosis of typhoid fever, lack of its immediate availability during the acute febrile illness may limit its use. Clinicians usually elect to treat, rather than wait for blood or stool culture results, which usually take about a week.

Advances in molecular immunology have led to the identification of sensitive and specific markers for typhoid fever and the technology needed to manufacture practical and inexpensive kits for their rapid detection [82].

Khan et al described a rapid immuno-enzymatic dipstick test for detection of *S.typhi* directly from the stool. The test, which is non-invasive, involves homogenization of stool sample in a buffer solution and immersion of a dipstick (previously coated with antibodies) in a tube containing the supernatant from the homogenized stool samples [51]. The contents of the tube (dipstick and supernatant) are incubated at room temperature for fifteen minutes and a second tube is incubated for an additional five minutes for full development of color. The dipstick is air-dried and the result is visualized as a horizontal mark on the dipstick. A sensitivity of 94%, specificity of 98%, negative predictive value of 99.5%, and positive predictive value of 74% were reported [51]. Such a direct stool testing can be a useful discriminating test that can be used with confidence in areas where both malaria and typhoid may have similar clinical presentations [83].

Rajesh Bansal carried out a study to determine the efficacy of diazo urine test in the diagnosis of typhoid fever in children. One hundred twelve children suffering from enteric fever (based on suggestive clinical picture and either positive blood culture, Typhidot M or Widal) in study group and 60 children with fever of confirmed aetiology other than enteric fever in control group were included in the study. Results of diazo test were compared with blood culture positive, Typhidot M positive or Widal. Of the 112 blood cultures, only 18 (16.1%) were positive for Salmonella typhi. In the first week of illness, Typhidot M was positive in 35 (89.7%) out of 39 cases whereas it was positive in all the 112 cases in the second week of illness. Diazo test showed sensitivity, specificity, positive and negative predictive value, and likelihood ratio of positive and negative test of 88.4%, 93.3%, 96.1%, 81.2%, 13.2% and 0.12% respectively as compared to Typhidot M and 90%, 93.5%, 96.1%, 81.2%, 13.9% and 0.11% as compared to Widal and 88.9%, 11.7%, 16.1%, 84.6%, 1% and 0.95% as compared to blood culture. Diazo test is a simple bedside, quick and cheap test with significant amount of reliability for the diagnosis of enteric fever in children [(54)].

Another test is the IDL TUBEX® test marketed by a Swedish company. It is able to detect IgM O (somatic) antibodies from patients' serum within a few minutes. The TUBEX® test detects not only typhoid-specific antibodies but also soluble antigens and whole bacteria. TUBEX® (IDL Biotech) is a five-minute semi-quantitative colorimetric test [(84)]. TUBEX® detects anti *S. typhi* O (somatic) antibodies from a patient's serum by the ability of these antibodies to inhibit the binding between an indicator antibody-bound particle and a magnetic antigen-bound particle [(84)]. TUBEX® could also be used to

specifically detect soluble O (somatic) lipopolysaccharide in antigen-spiked buffer by the ability of the antigen to inhibit the same binding between the particles [53].

One study used a modified protocol of the TUBEX® test, where the test sample was mixed with the indicator particles first, rather than with the magnetic particles as in antibody detection. It showed that sensitivity was improved. The antigen was also detectable both in spiked serum and in urine samples, albeit less well than in buffer generally [85]. In addition, whole organisms of *S. typhi* (15 strains) and *Salmonella enteritidis* (6 strains) (both O+ Salmonella), grown in simulated blood broths or on Mac Conkey agar, were also detectable by TUBEX®. Expectedly, *Salmonella paratyphi* A (seven strains), *Salmonella typhimurium* (1 strain) and *Escherichia coli* (2 strains) were negative in the test. Thus, the same TUBEX® kit may be used both serologically and microbiologically for the rapid diagnosis of typhoid fever [86]. The TUBEX® test pack includes:

1. Sets of specially designed V-shaped tubes that allow six samples per set to be examined simultaneously
2. Reagent A, comprising magnetic particles coated with *S. typhi* LPS (Lipopolysaccharide)
3. Reagent B, comprising blue-colored latex particles coated with a monoclonal antibody specific for the O antigen.

These reagents are stable for over a year at 4°C, and for at least some weeks at ambient temperature [86]. For reasons yet to be elucidated TUBEX® detects IgM antibodies but not IgG. This makes it invaluable as an aid in the diagnosis of current

infections. However, validation of these applications will require the systematic examination of real patient and laboratory materials [84].

Another serological test is the Typhidot®, (an Enzyme Immunoassay test), which takes three hours to perform. It was developed in Malaysia for the detection of specific IgM and IgG antibodies from serum against a 50 kD antigen of *S. typhi* [87]. A newer version of the test, Typhidot-M®, was recently developed to detect specific IgM antibodies only [88]. It has undergone full-scale multinational clinical evaluation of its diagnostic value [89]. This dot EIA (Enzyme Immunoassay) test offers simplicity, speed, early diagnosis and is economical. The study reported a specificity of 75%, sensitivity of 95%, and high negative and positive predictive values [52] [90].

The detection of IgM reveals acute typhoid in the early phase of infection, while the detection of both IgG and IgM suggests acute typhoid in the middle phase of infection. In areas of high endemicity where the rate of typhoid transmission is high, the detection of specific IgG increases. Since IgG can persist for more than two years after typhoid infection, the detection of specific IgG cannot differentiate between acute and convalescent cases. False-positive results attributable to previous infection may occur. On the other hand, IgG positivity may also occur in the event of current re-infection. In cases of re-infection, there is a secondary immune response with a significant boosting of IgG over IgM, such that the latter cannot be detected and its effect is masked [91]. A possible strategy for solving these problems is to enable the detection of IgM by ensuring that it is unmasked. In order to increase diagnostic accuracy in these situations the original Typhidot® test was modified by inactivating total IgG in the serum sample. Studies using the modified test, Typhidot-M®, have shown that inactivation of IgG removes

competitive binding and allows antigen to be accessed by specific IgM when it is present. The detection of specific IgM within three hours suggests acute typhoid infection. Evaluations of Typhidot® and Typhidot-M® in clinical settings showed that they performed better than the Widal test and the culture method [91] [92].

Microarray technology is an important tool for detection and analysis of nucleic acid targets. It can be used in the diagnosis of typhoid fever. Immobilization of modified and unmodified oligonucleotides on epoxy-functionalized glass surfaces is done in microarray fabrication. A protocol that employs coating of SU-8 (glycidyl ether of bisphenol A) onto glass micro slides to obtain high density of epoxy functions for efficient immobilization of aminoalkyl-, thiophosphoryl-, and phosphorylated oligonucleotides with uniform spot morphology is followed [93]. The resulting microarrays exhibit high immobilization (approximately 65%) and hybridization efficiency of (30-36%) and are sufficiently stable over a range of temperature and pH conditions. The prominent feature of the protocol is that spots can be visualized distinctly at 0.05 μM probe (a 20-mer oligonucleotide) concentration. The constructed microarrays are subsequently used for detection of base mismatches and bacterial diseases like typhoid fever and meningitis [55].

Other methods are COAG (co-agglutination) and CIE (counter-current immuno-electrophoresis). A study of rapid diagnosis of typhoid fever looked at these methods in parallel, using clot and blood cultures on 114 clinically suspected cases. Sera from 50 controls were subjected to both tests [56]. Analysis of data with reference to retrospectively confirmed typhoid cases show that *S. typhi* was isolated in 58.6% and 58.3% of blood and clot cultures. Antigen detection by CIE in their supernates was

81.1% and 79.2%, respectively. This correlated closely with serum COAG (81.0%) in contrast to serum CIE (5.7%). Thus, COAG was superior to CIE for serology [94] [95].

Sonja et al did a large comparative study in a typhoid endemic zone with limited laboratory capability. Eighty individuals participated in the study, (59 patients, and 21 controls) [96]. Cases were patients with *S. typhi* isolated from blood samples, and controls were patients with other laboratory-confirmed illnesses. Patients presenting with less than 4 days of fever were enrolled at two hospitals in Southern Vietnam. Isolation and identification of *S. typhi* was done. Three commercial kits for serologic diagnosis of typhoid fever were evaluated. *S. typhi* isolates were confirmed and tested for antimicrobial susceptibility at the Pasteur Institute in Ho Chi Minh City. The Widal test was run at the hospitals and the Pasteur Institute. Frozen Sera were shipped to the Centers for Disease Control and Prevention and tested by using Multi-Test Dip-Sticks, Enzyme immunoassay (Typhi Dot®), and IDL TUBEX to detect IgG and IgM, and IgM, respectively.

The sensitivity and specificity findings were as follows: 89% and 53% for Multi-Test Dip-Sticks, 79% and 89% for Typhi Dot, 78% and 89% for TUBEX, and 64% and 76% for Widal testing in hospitals, 61%, and 100% for Widal testing at the Pasteur Institute respectively. For all assays, the sensitivity was highest in the second week of illness. The Widal test was insensitive and displayed inter-operator variability. Two rapid kits, Typhi Dot and TUBEX, demonstrated promising results [96].

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Design and Setting

The study was a cross-sectional carried out at the MTRH routine labs. Patients were from both the outpatient clinics and the wards. MTRH is situated in Uasin-Gishu County, Eldoret Town, which is 320km Northwest of Nairobi city, the capital of Kenya. MTRH was established as the second National Hospital in Kenya after Kenyatta National Hospital in Nairobi. It has specialized facilities for teaching, research and provision of health services. It serves the population in the western part of Kenya, which constitutes 50% of the Kenyan population. This region includes the Uasin-Gishu, Keiyo-Marakwet, Tranzoia, Turkana, Nandi, Baringo, Nakuru, Kericho, Bomet, Kisii, Nyamira, Homabay, Migori, Kisumu, Siaya, Busia and Bungoma Counties with a cumulative population of about 19 million people [97]. Residents of Eastern Uganda, South Sudan and from as far as Burundi, Rwanda, DR Congo, also benefit from health services at MTRH courtesy of the highway traversing these countries.

3.2 Study Population and Sampling Procedure

Study population was formed by patients of MTRH of all ages both male and female who complained of abdominal pain, diarrhea, and fever during the months of January to December in 2012. Convenience sampling method was used in the selection of the participants. The participants would be selected in the clinic as they came, whilst adhering to the inclusion and exclusion criteria as elaborated under topic 3.3.

3.2.1 Sample Size Determination

There was a need to calculate a sample size that would give an appropriate precision for estimating sensitivity and specificity. Calculation of sample size was done using the formulas designed by Dr Lin Naing, of School of Dental Sciences of the *Universiti Sains Malaysia* in 2004 as an improvement of the Fischer's formula to be used in sensitivity and specificity studies [98].

The sample size that was to be calculated for this study is =*N* (total sample size). From the 2x2 diagnostic table, sensitivity is calculated by a/ (a+c) and specificity by b/ (b+d) as shown in table 1.

Table 1: 2x2 Diagnostic Table for Calculating Sensitivity and Specificity

	Disease +ve	**Disease -ve**	
Test +ve	(a)	(b)	(a+b)
Test –ve	(c)	(d)	(c+d)
	(a+c)	(b+d)	***N***

By using the usual single proportion sample size formula, labeled as formula 1 below, the sample size for (a+c) and (b+d) can be calculated. *P* can be used as either specificity or sensitivity.

$n = \{Z^2 \times P (1-P)\} / \Delta^2$…… (1)

In formula 1, n = (a+c) if *P* is used as sensitivity. On the other hand, n= (b+d) if *P* is used as specificity. Documented specificity and sensitivity values of Widal tube dilution test were used. Documented Sensitivity and Specificity in a certain hospital in an endemic zone of Tanzania was 76% and 96% respectively for the Widal tube dilution test [99]. By using the single proportion sample size formula, (formula 1), the sample size of

(a+b) and (b+d) can be calculated. In the formula 1, the Z variable modulated the errors and 1.96 was used while Δ was the precision and 0.05 was used. By using the documented prevalence of the disease as shown in formulas 2 and 3 the sample size was calculated.

For the purposes of this study, sample size was calculated for (b+d) by using specificity as *P* in formula 1. After obtaining the value of (b+d), the total sample size was calculated using formula 3 and 50% was used as the prevalence of typhoid fever as obtained from previous cross-sectional studies [4] [8].

N = (a+c) /Prevalence…… (2)

N = (b+d) /1-Prevalence…… (3)

The calculation is shown below.

$(b+d) = 1.96^2 \times \{0.96 (1-0.96)\} \div 0.05^2$.....….. (1)

(b+d) = 59

N= 59/0.5……..……...……..... (3)

N= **118**

A minimum of 118 clinically suspected cases was sufficient to answer the objectives. However, a hundred and thirty six clinically suspected cases were recruited to the study. This helped to maintain the power to answer the objectives in case there arose any problems that led to discarding of participant samples, for example haemolyzed and contaminated samples.

3.3 Subject Inclusion and Exclusion Criteria

Only willing cases of abdominal pain, diarrhea, and fever were enrolled in the study. Unwilling cases and those not reporting the above symptoms were excluded. Only

patients who had experienced the above symptoms for four or more days were recruited because it is difficult to culture *S. typhi* early in the course of illness. Patients who have had these symptoms for four or more days are more likely to have a blood culture positive for *S. typhi* than those who have had the symptoms for less than four days [36]. Patients already on antibiotic therapy by the time of their visit were excluded, since this would interfere with the culturing of *S. typhi* [36].

3.4 Ethical Considerations

Permission to conduct the study was sought and granted from the hospitals' administration, and the Institutional Research and Ethics Committee (Appendix 4). The purpose of the study was explained to the participants, after which consent/assent was sought from the participants or their legal guardians prior to enrollment into the study. The consent form is in Appendix 1. Codes were used to identify participants to ensure confidentiality. The study did not expose participants to unusual risks, as trained hospital staff using standard approved methods collected the specimens.

3.5 Specimen Collection and Analysis/ Processing

3.5.1 Administering Questionnaires/ Participant Information Forms

Each participant was allocated a code number and their details; Age, Sex, Education level, Occupation, and Previous exposure/Vaccination history were recorded (Form in Appendix 2). The information was later entered together with the test results into MS Access database for analysis.

3.5.2 Blood Cultures

1. Preparation of MAC Media: The dehydrated media (manufactured by *HiMedia Laboratories*) was dissolved in water and heated until it boiled. It was then transferred to a bottle and autoclaved at 121ºC for 15 minutes. Next, it was allowed to cool to 44ºC -47ºC and stored at 1-5ºC for up to 14 days (that is, when it was not possible to inoculate all of the prepared media sooner).
2. Venous blood was collected from the all participants and aseptically transferred to Castaneda biphasic medium bottles, leaving behind about 2ml of blood for the Widal tube dilution and ELISA tests.
3. For children between 5 years and 12 years, 2-5ml of blood was used for the cultures while for those participants above 12 years of age; 10-15ml was used as recommended by WHO [(2)].
4. Incubation of cultures in the Castaneda biphasic media bottles was done at 37ºC in 5% CO_2 for 18 hours.
5. When signs of bacterial growth were observed, (air bubbles, turbidity or both), they were sub-cultured onto the prepared MAC media and incubated for a further 18-24 hours.
6. Inoculation of the set MAC blood cultures was done using sterile loops and was evaluated for growth every 24 hours.
7. The remaining blood cultures in the Castaneda biphasic media bottles were re-incubated for a further 3 weeks or until positive.
8. Observation for the colony characteristics of *S. typhi* was done on the sub-cultures. On MAC, they form non-lactose fermenting colonies that are white (or

colourless), smooth, and circular. Sometimes they can be golden brown with dark centers. (*S. typhi* uses up peptone in addition, forms ammonia. This raises the pH of the agar. They also clear bile precipitates that are brought about by other organisms). The following three tests were done for confirmation of *S.typhi.*

I. TSI (Triple Sugar Iron)

1. Suspected colonies were picked using an inoculating loop (1µl). (A single well-isolated colony was used in each case).
2. Inoculation of the TSI bottle (agar containing glucose, lactose, and sucrose) was done by stabbing the butt and streaking the surface of the slant.
3. They were incubated for 18-24 hours at 35ºC- 37ºC. (Caps were loosened before incubation).
4. To identify *S. typhi,* alkali (red)/acid (yellow) + H_2S + no gas was noted. (These results meant that glucose had been fermented). When positive *S. typhi* was suspected and *Pseudomonas aeruginosa* and *Shigella* were eliminated.

II. Urease test (Stuart's urea broth)

1. The tubes of the urea broth were inoculated with growth from each presumed positive TSI slant culture using a sterile loop.
2. An uninoculated tube of urea broth was included as control, since occasionally uninoculated tubes of this broth turn purple/red (positive test) on standing.
3. The tubes were incubated for 22-26 hours at 35ºC. {Positives turned purple red while negatives had no colour change (remained pale yellowish)}.
4. *S. typhi* is urease negative. Therefore, positives were discarded. The positives were suspected to be *Proteus* or *Klebsiella.*

III. Indole test

1. The reagents are tryptone or tryptophane broth and Kovac's indole reagent.
2. The broth was inoculated with a small growth from positive TSI agar culture and incubated at 35ºC for 22-26 hours.
3. 5ml of the tryptophane broth culture was transferred to an empty test tube and 0.2-0.3ml of Kovac's reagent was added.
4. Salmonella cultures gave negative test, that is, lack of deep red/violet colour at the surface of the broth. Instead, they remained yellow at the surface.
5. Positive reactions with violet/deep red colour at the surface were discarded.
6. The results were then entered into the MS Access database.

3.5.3 Widal Tube Dilution Test

1. The remaining blood from all the participants was centrifuged to get serum samples, which were used to perform the tube dilution test as follows:
2. A working PBS solution was prepared by adding 1ml of concentrated PBS (made by *Dade Behring Marburg*) to 20ml of normal saline.
3. 10 µl of the prepared patient serum was added to 190 µl of the prepared working PBS solution making a total of 200µl in this test tube. Thus, the first tube had 10µl: 200µl (1:20). This was the starting point.
4. 100µl of working PBS was transferred to a second tube. Next, 100µl of the contents of the first tube (1:20) was transferred to the second tube and mixed thoroughly. The mixture in the second tube was 1:40.

5. 100µl of working PBS was transferred to another tube (third tube). Then, 100µl of the contents of the second tube (1:40) was transferred to the third tube and mixed thoroughly. The mixture in the third tube was 1:80.
6. 100µl of working PBS was transferred to another tube (fourth tube). Then, 100µl of the contents of the third tube (1:80) was transferred to the fourth tube and mixed thoroughly. The mixture in the fourth tube was 1:160.
7. 100µl of working PBS was transferred to another tube (fifth tube). Then, 100µl of the contents of the fourth tube (1:160) was transferred to the fifth tube and mixed thoroughly. 100µl of the contents in the fifth (and last) tube was discarded so that all the tubes had equal volume i.e. 100µl. The mixture in the fifth tube was 1:320.
8. A drop of the reagents manufactured by *Dade Behring Marburg*, (prepared suspensions of *Salmonella* O and H antigens) was added to each of the tubes.
9. All the tubes were incubated overnight at 37ºC and then checked for signs of agglutination.
10. A cut-off value (baseline titre) of 1:80 was used. This meant those that exhibited 50% agglutination or more. Those with 1:80 and above were considered positive.
11. The results from the cultures were compared to the results from the Widal tube dilution test to determine the accuracy of the tube dilution test.
12. Queries were created in the MS Access database for comparison of the two tests.
13. A 2x2 diagnostic table was used to calculate Sensitivity, Specificity, Negative, and Positive predictive values of the Widal tube dilution test.

3.5.4 ELISA (IgG) Test

1. ELISA (IgG) plates with wells coated with *Salmonella typhi* antigens were used. (Manufactured by *MyBiosource.com*). The insert/protocol of the ELISA (IgG) kit is shown in Appendix 3.
2. 100µl sample diluent was added to the first two wells (blanks), 100µl of diluted negative control was added to the third and fourth wells and 100µl of diluted positive control to the fifth and sixth wells, and 100µl of diluted calibrator was added to the seventh and eighth wells.
3. 100µl of the diluted patients' serum was added to the rest of the wells. (NB: all the dilutions were done 1:101)
4. The plates were then incubated at room temperature for 20 minutes then washed three times with a micro-well washer with a volume that filled the wells. A prepared wash solution was used, as directed by the insert (Appendix 3).
5. 100µl of enzyme conjugate was added to each of the wells.
6. Next, they were again incubated at room temperature for 20 minutes then washed.
7. The wells were washed three times with the micro-well washer as in step 4.
8. 100µl of TMB was added to each well and incubated at room temperature for 10 minutes.
9. Immediately after the 10 minutes, 100µl of the stop solution was added to each well and the OD (Optical Density) was read on a spectrophotometer (ELISA reader).
10. The results were then classified as positive or negative using the cut-off values of each kit calculated from the calibrator, positive and the negative controls.

14. The results from the cultures were compared to the results from ELISA (IgG) tests to determine accuracy of ELISA (IgG) kit.
15. Queries were created in the MS Access database for comparison of the two tests.
16. A 2x2 diagnostic table was used to calculate Sensitivity, Specificity, Negative, and Positive predictive values of the ELISA (IgG) test.

3.6 Limitations and Assumptions of the Study

1. The study relied on self-report when recording information on whether the participants had been diagnosed with typhoid fever before. Those who reported previous exposure were 86%. This number could be lower or higher since self-report can be erroneous. This did not affect the study in any way. However, if this figure is to be quoted in other studies then it should be mentioned that it was obtained from self-reporting by participants.

CHAPTER 4: DATA ANALYSIS AND RESULTS

The sample was made up of 118 participants who were clinically suspected cases. The collected data was analyzed using MS Access database. Queries were created to get the number of participants with a certain desired characteristic.

4.1 Age Distribution of Participants with *S. typhi* from Blood Culture

The youngest participant was 4 years old while the oldest was 83 years old. Of the 118 participants, 55.08% (that is 65 out of 118) had culture-proven *S. typhi* infection. The most affected age group in the MTRH catchment area was determined by calculation of age ratios. The age group that was most affected was (10-19) years as shown in Table 2. The modal class/ age group therefore was the (10-19) years. Of the 23 participants in the age group (10-19) years, 21 (91.30%) of them were school going children and teenagers. The age with the highest frequency (mode) was 22 years. The mean age of all the participants positive for *S. typhi* was 27.61 years. The variance was 188.22 while standard deviation was 13.719.

Table 2: Age Ratios of Participants with *S. typhi* from Blood Culture

Age Group	**Number of Culture +ve Participants**	**Total Number of Culture +ve and Culture - ve Participants**	**Percentage of Culture Positive Participants**	**Age Ratio**
0-9 years	5	9	7.69%	**1.01**
10-19 years	23	28	35.38%	**1.76**
20-29 years	15	27	23.08%	**1.01**
30-39 years	11	31	16.92%	**0.57**
40-49years	5	12	7.69%	**0.74**
50-59 years	4	8	6.15%	**0.90**
Above 60 years	2	3	3.08%	**1.22**

Total Number	**65**	**118**	**100%**	

4.2 Determination of Sex Ratios for Participants with *S. typhi* from Blood Culture

The number of males and females with culture-proven *S. typhi* infections was used to calculate the sex ratio. *N* (which is the sample size) was 118. Of these participants, 46 males and 72 were females.

Sex Ratio = [Number of males (culture positives)/ Total number of males] ÷ [Number of females (culture positives)/ Total number of females]

Sex Ratio = 26/46 ÷ 39/72

Sex Ratio = **1.043**

4.3 Education Level and Occupation of Participants with *S. typhi* from Blood Culture

Tables 3 and 4 below indicate a summary of education level and occupation of those who were culture positive.

Table 3: Education level of participants with *S. typhi* from Blood Culture

Education level	**Percentage of culture positive participants**
None	5.47%*
Primary	36.71%*
Secondary	28.52%*
Tertiary	21.15%*

Note:* These figures include those that are still in school at the respective level

Table 4: Occupation of participants with *S. typhi* from Blood Culture

Occupation	**Percentage of culture positive participants**
Student	55.56%
Employed	22.22%
Unemployed	9.52%

House –Wife	12.7%

4.4 Previous Exposure to *S. Typhi* / Vaccination History

One hundred and two (102) out of the 118 clinically suspected cases reported that they had been previously exposed to *S. typhi.* They reported that they had been diagnosed with typhoid fever at least once in their lifetime. Those who had received the *S. typhi* vaccine were also to be included in this number. However, none of the 118 participants reported receiving vaccination against typhoid fever. Those with previous exposure made up 86% of the 118 participants. Only a small percentage (14%), reported no previous exposure to *S. typhi.*

4.5 Widal Tube Dilution Test as Compared to Blood Culture

Widal tube dilution test was easier to perform as compared to blood cultures. Culturing of blood was a very complicated procedure that required care in handling of the materials to avoid contamination of the samples. To obtain results from the blood cultures, it took approximately a week, whereas for the Widal tube dilution test it took approximately 24 hours. The cost per patient for blood cultures was approximated at 700 Ksh, while for the Widal tube dilution the cost per patient was approximated at 350 Ksh. Therefore, in terms of cost, speed, and simplicity, Widal tube dilution was better than blood culture. The results from the Widal tube dilution test as compared to the results from blood culture are shown in Table 5.

Table 5: Outcomes of Widal tube dilution test in Comparison to Blood Culture

Tube Dilution	**Disease (Culture)**		**TOTAL**
	Present	***Absent***	
Positive	50 ***(TP)***	12 ***(FP)***	62
Negative	15 ***(FN)***	41 ***(TN)***	56
TOTAL	65	53	**118**

Blood cultures were positive in 65 (55.08%) of the clinically suspected cases. On the other hand, tube dilution was positive in 62 (52.54%) of the suspected cases. To calculate the Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and Accuracy of the tube dilution, the formulae below were used:

i. Sensitivity = True Positives/ (True Positives+ False Negatives).

Sensitivity (of Widal tube dilution) = 50/ (50+15) = 0.7692 = **76.92%.**

ii. Specificity = True Negatives/ (True Negatives+ False Positives).

Specificity (of Widal tube dilution) = 41/ (41+12) = 0.7736 = **77.36%.**

iii. PPV (Positive Predictive Value) = TP (True Positives) / {TP (True Positives) FP (False Positives)}

Positive Predictive Value (of Widal tube dilution) = 50/ (50+12) = 0.8065 = **80.65%.**

iv. NPV (Negative Predictive Value) = TN (True Positives) / {TN(True Negative +FN (False Negatives)}

Negative Predictive Value (of Widal tube dilution) = 41/ (41+15) = 0.7321 = **73.21%.**

v. Accuracy = TP (True Positives) +TN (True Negatives) / *N* (which is the samp size).

Accuracy (of Widal tube dilution test) = (50+41) / 118 =0.7712 =**77.12%.**

vi. TP (True Prevalence) = {TP (True Positives) + FN (False Negatives)}/ *N* (whi is the sample size).

True Prevalence (as given by culture) = 65/118 = **0.5508.**

vii. AP (Apparent Prevalence) = {TP (True Positives) + FP (False Positives)} / (which is the sample size).

Apparent Prevalence (as given by Widal tube dilution) = 62/118 = **0.5254.**

4.6 ELISA (IgG) Test as Compared to Blood Culture

Like the Widal tube dilution, ELISA (IgG) test was easier to perform as compared to blood cultures. To obtain results from the blood cultures, it took approximately a week, whereas for the ELISA (IgG) test it took approximately 1.5 hours. The cost per patient for blood cultures was approximated at 700 Ksh while for the ELISA (IgG) test the cost per patient was approximated at 425 Ksh or $5 (The exchange rate as at May 2013 was used). Therefore, in terms of cost, speed, and simplicity, ELISA (IgG) test was better than blood culture. The results from ELISA (IgG) test as compared to the results from blood culture are shown in Table 6.

Table 6: Outcomes of ELISA (IgG) test in Comparison with Blood Culture

ELISA (IgG)	**Disease (Culture)**		**TOTAL**
	Present	***Absent***	
Positive	57 ***(TP)***	4 ***(FP)***	61
Negative	8 ***(FN)***	49 ***(TN)***	57
TOTAL	65	53	**118**

ELISA (IgG) was positive in 61 (51.69%) of the suspected cases. To calculate the Sensitivity, Specificity, PPV (Positive Predictive Value), NPV (Negative Predictive Value), and Accuracy of ELISA (IgG), the formulae below were used:

i. Sensitivity = True Positives / (True Positives + False Negatives}

Sensitivity (of ELISA IgG) = 57/ (57+8) = 0.8769 = **87.69%.**

ii. Specificity = True Negatives/ (True Negatives+ False Positives).

Specificity (of ELISA IgG) = 49/ (49+4) = 0.9245 = **92.45%.**

iii. PPV (Positive Predictive Value) = TP (True Positives) / {TP (True Positives)+ (False Positives)}

Positive Predictive Value (of ELISA IgG) = 57/ (57+4) = 0.9344 = **93.44%.**

iv. NPV (Negative Predictive Value) = TN (True Negatives) / {TN (True Negativ +FN (False Negatives)}

Negative Predictive Value (of ELISA IgG) = 49/ (49+8) = 0.8596 = **85.96%.**

v. Accuracy = TP (True Positives) + TN (True Negatives) / *N* (which is the samp size)

Accuracy (of ELISA IgG) = 57+49/118 = 0.8983 = **89.83%.**

vi. AP (Apparent Prevalence) = {TP (True Positives) + FP (False Positives)} / *N* (which is the sample size)

Apparent Prevalence (as given by ELISA IgG) = 61/118 = **0.5169.**

vii. TP (True Prevalence) as calculated earlier (as given by culture) was **0.5508.**

ELISA (IgG) test was compared to Widal tube dilution in terms of cost, speed, and simplicity. The two tests were equally easy to perform since both of them had to do with pipetting serum samples and their diluents. Though ELISA (IgG) test would cost 125 kshs more for the patients, it was fast as compared to the Widal tube dilution. To obtain results from the ELISA (IgG) test, it took approximately 1.5 hours, whereas for the Widal tube dilution test it took approximately 24 hours.

To compare the results from the two methods, that is, the Widal tube dilution test and the ELISA (IgG), false positive rate of both methods was calculated, then a ratio was calculated.

i. FPR (False Positive Rate) = 1- Specificity.

   a. False Positive Rate (of ELISA IgG) = 1-0.9245= **0.0755.**

   b. False positive rate (of Widal tube dilution test) = 1-0.7736= **0.2264.**

ii. Ratio of False Positive Rate of ELISA (IgG) vs. Widal tube dilution

   a. Ratio = FPR_{ELISA}/ FPR_{tube}

   b. Ratio = 0.0755/0.2264 = **0.3335.**

iii. TPR (True Positive Rate), which is the same as sensitivity was also used:

   a. TPR of ELISA (IgG) = Sensitivity of ELISA (IgG) = **0.8769.**

   b. TPR of Widal tube dilution = Sensitivity of Widal tube dilution = **0.7692 .**

iv. Ratio of TPR of ELISA (IgG) vs. Widal tube dilution test

a. Ratio = TPR_{ELISA}/ TPR_{tube}

b. Ratio = 0.8769/0.7692 = **1.1400.**

4.7 Precision of the ELISA (IgG) Kits Manufactured by MyBiosource.com

Two ELISA (IgG) kits for detection of *S. typhi* specific antibodies were used in this study in order to accommodate the 118 serum samples. These kits, each with 90 wells were manufactured by *MyBiosource.com.* No two wells are the same. There is usually a difference in the outcomes when the same serum sample is used in several wells. However, the difference should not be too much as that would mean that the ELISA kit would give unreliable results. A coefficient of variation of no more than 15% is acceptable for a kit to pass the precision test [(100)].

To ensure the consistency of the results, the kits from *MyBiosource.com* were tested by running multiple replicates of sera from participants in several wells. This was done for both the kits. Replicates of sera were also run in the two kits at the same time. Results from these wells with the same serum sample of a particular participant were recorded. Standard deviation was calculated then the coefficient of variation was determined. Tables 7, 8 and 9 show the coefficient of variation within each kit and between kit A and B.

Table 7: Coefficient of Variation of Results Obtained from Multiple Sera Replicates within Kit A

Serum Sample	Number of Replicates	Mean	Standard Deviation	Coefficient of Variation (%)
1	3	0.467	0.014	3.00
2	3	1.59	0.035	2.20
3	3	0.317	0.008	2.52
4	3	0.394	0.035	8.88

5	3	1.226	0.069	5.63

Table 8: Coefficient of Variation of Results Obtained from Multiple Sera Replicates within Kit B

Serum Sample	**Number of Replicates**	**Mean**	**Standard Deviation**	**Coefficient of Variation (%)**
6	3	1.113	0.033	2.96
7	3	0.443	0.012	2.71
8	3	0.390	0.011	2.82
9	3	0.875	0.107	12.23
10	3	0.321	0.019	5.92

Table 9: Coefficient of Variation of Results Obtained from Multiple Sera Replicates across Kits A and B

Serum	**No of Replicates**	**Mean**	**Standard Deviation**	**Coefficient of Variation %**
11	2	1.459	0.059	4.05
12	2	0.297	0.014	4.72
13	2	0.266	0.182	3.01
14	2	0.306	0.016	5.24
15	2	1.032	0.115	11.15
16	2	0.299	0.020	6.70

CHAPTER 5: DISCUSSION

The participants of this study were clinically suspected cases of typhoid fever. Blood cultures were then used to determine if the participants had *S. typhi* (true disease). The age group of 10-19 years had the highest age ratio of the number of participants testing positive for *S. typhi* from blood cultures (Table 2). From the study findings, as well as reports from other studies, it can be said that this group shows the highest susceptibility making typhoid fever a disease of children and teenagers [101] [102]. However, it is possible for people in other age groups to contract typhoid fever as seen in the column showing the distribution of the culture positive participants (Table 2). The reasons behind the age group of 10-19 years having the highest age ratio of culture positive participants may be related to personal hygiene, state of public health facilities like contaminated water sources, and physiology of the participants [103] [104].

Poor sanitation and lack of potable water have been reported in many areas in Kenya [105]. To add on this, children and teenagers may not understand the importance of good personal hygiene and may therefore not pay keen attention to their surroundings. Since 91.30% of the participants in the most affected age group were enrolled in school, then measures such as campaigns that are focused on improving personal hygiene and intensive school health education programs if introduced, will help to prevent and control the spread of typhoid. Others measures include improving the provision of clean water and adequate sanitation, the identification of carriers, and sustained and targeted vaccination programs [106].

For governments to make rational decisions about committing resources for this purpose, information concerning the burden of disease in the human population in defined areas is essential. The required information must include detecting the majority of cases and incorporate those individuals that have been exposed but experience an asymptomatic or mild infection that does not require medical attention and those that are transiently or chronically carrying the organism. Granted, this was not a 'burden of disease study', but the results give a rough picture on disease burden. Thus, any efforts to prevent and control typhoid fever should make use of this age distribution information.

In this study, 36.71% were either primary school children or participants who dropped out at primary school level (Table 3). The level of education may be a contributing factor in the spread of typhoid fever. We expect those with a higher level of education to have a less chance of contracting *S. typhi* because it is assumed that they have better knowledge on prevention of the disease through measures like good hygiene and sanitation [7]. However, this is not always the case as other factors may be of

importance. More studies should be done on this subject to ascertain if the hypothesis is true. If the hypothesis is true, then community health programs should be strengthened in order to reach those who dropped out of school. There are professions where individuals may have an increased risk in contracting the infection than others, for example, those handling food, infectious material or live cultures [107]. However, this study did not go into the details of the kind of work the culture positive participants did. From the reports of other studies, food handlers and contaminated water sources are considered important in maintaining enteric fever in endemic areas [103].

The sex ratio for the participants who were positive for *S. typhi* (blood culture) was 1.043. This means that both sexes were almost equally susceptible to typhoid fever. The males were slightly more at risk than the females in the MTRH catchment area. Therefore, gender is not considered a significant/special risk factor concerning contracting typhoid fever. Possible reasons could be that poor sanitation and inadequate public health facilities cuts across gender. It does not affect one gender more than the other does [107].

A large number of the participants (86%) reported previous exposure to *S. typhi*. They reported being diagnosed with typhoid fever at least once in the past. Some reported having been diagnosed with the disease more than once. However, none of the participants reported ever receiving the typhoid fever vaccine. Using these findings, though self reported, we could conclude that the MTRH catchment area indeed lies in an endemic zone. This presents a challenge when it comes to selection of a diagnostic test because of the persistent antibodies in individuals that were previously exposed to *S. typhi*. It is the major reason behind the failure of the Widal rapid kits, because the

antigens used in the Widal rapid kits react with the persistent antibodies and give false positive results [68].

A good diagnostic should not only be cheap and fast but also produce accurate and reproducible results. This study evaluated two commercial rapid diagnostic kits for *Salmonella* serotype *typhi* with sera collected from participants with acute febrile illness with a duration of four or more days at MTRH, Eldoret, Kenya. Blood culture was used as the gold standard and was a very complicated, time consuming and expensive procedure. The Widal tube dilution has been reported to have higher accuracy than that of the Widal rapid kits in previous studies [81]. Thus, if Widal agglutination is to be used, then the tube dilution is the best method. In this study, the sensitivity, specificity, PPV and NPV for the Widal tube dilution were above 70%. Twelve cases were false positives. This could mean that the antigens could have reacted with existing antibodies [74] or cross reacted with other antibodies produced as a result of non-salmonella infections [71] [72]. Even though this method was the cheapest of the three, it took 24 hours for the results to be available.

On the other hand, ELISA (IgG) demonstrated a higher accuracy than the Widal tube dilution test. ELISA has been used to more precisely define the normal antibody response and its relevance for diagnosis [57] [108]. The findings from this study showed that ELISA (IgG) was better than the Widal-tube dilution as supported by the FPR and TPR ratios calculated in section 4.6. Sensitivity, Specificity, NPV, and PPV of ELISA (IgG) were all above 80%, and approaching 100%. Four cases were found false positives, meaning that the antigens could have reacted with existing antibodies [74] or cross-reacted with other antibodies produced as a result of non-salmonella infections like malaria [71]

[72]. This number was lower as compared to the false positives in tube dilution. This method was simple to perform, cheap, took a short time, and gave reliable results. Small co-efficient of variations (less than 15%) within and across the kits was indicative of high precision of the MyBiosource ELISA (IgG) salmonella kits [100].

CHAPTER 6: CONCLUSIONS, RECOMMENDATIONS, AND FURTHER WORK

6.1 Conclusions

ELISA (IgG) had the highest accuracy, sensitivity, specificity, PPV and NPV. This means that it is able to deliver reliable and reproducible results. The test was easy to perform, cost effective and fast.

6.2 Recommendations

Following the above conclusion, the use of ELISA to diagnose typhoid fever as a replacement to the Widal rapid kits is recommended. Because of it is affordable, fast, and able to give reliable results, it should be considered for use in routine diagnosis in Kenyan hospitals.

6.3 Further Work

Regarding this area more work should be done to:

1. Evaluate other tests available for the diagnosis of typhoid fever, for example, the IDL-TUBEX®, and the Dot enzyme test (Typhidot®).
2. Investigate the existence of inter-operator variability and antigen variability in Widal tube dilution and the ELISA tests; that is their ability to produce consistent results.
3. Develop new titres or cut-off points (other than the 1:80 ≤ that was used in this study) that can be used in diagnosis of typhoid fever using the Widal tube dilution test. This will be able to answer the question about whether the Widal tube dilution test can perform better that it did in this particular study.

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APPENDICES

Appendix 1: Consent Form

Information Sheet

Purpose of the Study. As part of the requirements for Master of Science at Moi University School of Medicine, I have to carry out a research study. The aim of the study is evaluate how the tube dilution test and a new ELISA (IgG) test in the diagnosis of typhoid perform.

What the study involves. The study will involve drawing of venous blood. Trained personnel (phlebotomist) will do it. It will only take a few minutes of your time and cause minimal pain. After the blood is drawn, culture, ELISA, and tube dilution tests will be done.

Why have you been asked to take part in the study? You have been asked because many people in this region are diagnosed with typhoid. There have been questions raised about the performance of the old Widal rapid testing method, thus it is important to evaluate new tests. Participation is voluntary. If you do not want to take part, your decision will be respected. For those who participate, I will ensure that no clues to your identity appear in the thesis.

What will happen to the results? The results from the study will be presented in the thesis. My supervisor, a second marker, and the external examiner will see them. Future students on the course may read the thesis. The study may be published in a research journal. In addition to that, the results may lead to introduction of new highly performing diagnostic tests.

Who has reviewed this study? Approval to conduct this study was given by the Institutional Research and Ethics Committee, the MTRH management and the Department of Immunology, Moi University, School of Medicine. .

Further questions. If you need any further information, you can contact me:

Name:……..…… Phone:…… Email:…….

If you agree to take part in the study, please sign the consent form below.

Consent Form

I……agree to participate in "evaluation of Widal (tube dilution) and ELISA tests for diagnosis of salmonellosis at Moi Teaching and Referral Hospital, Kenya research study.

The purpose and nature of the study has been explained to me in writing. I am participating voluntarily. I understand that anonymity will be ensured in the write-up by disguising my identity. I understand that disguised extracts from my interview may be quoted in the thesis and any subsequent publications if I give permission below: (Please tick one box)

I agree to participate in the study ☐

I do not agree to participate in the study ☐

Signed……. Date…….

Appendix 2: Questionnaire/ Participant Information Form

Section 1: Demographic Data

1. Identification/ Lab Number …….
2. Age (years) ……..
3. Gender : ☐ Male ☐ Female
4. Level of Education: ☐ None ☐ Elementary
   ☐ High School ☐ Tertiary
5. Occupation: ☐ Employed ☐ Student
   ☐ House Wife ☐ Unemployed

Section 2 Clinical Data

1. Previous exposure/Vaccine ☐ Yes ☐ No

Section 3: Lab Results from Tests Performed

1. Culture: ☐ Positive ☐ Negative
2. Widal Tube Dilution Test

"O" Titre	**"H" Titre**

   Conclusion: ☐ Positive ☐ Negative
3. ELISA (IgG) Test: ☐ Positive ☐ Negative

Interview conducted by…….

Appendix 3: MyBiosource.com ELISA (IgG) Kit Protocol

The *Salmonella typhi* ELISA (IgG) kit was obtained from MyBiosource.com and the insert/ protocol that was followed is attached after this page.

Appendix 4: Approval Letters to Carry Out the Research

The proposal was reviewed by the MTRH-Moi University School of Medicine Institutional Research and Ethics Committee (IREC) and approval was given before the study was done. Approval was also sought from MTRH for conducting the research in the hospital's labs. The documents are attached following this page.