PERFORMANCE CHARACTERISTICS OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY FOR RAPID DIAGNOSIS OF TYPHOID INFECTIONS AT CITY CLINICS IN NAIROBI

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ABSTRACT

Background

Enteric fever continues to be a major public health burden in Kenya as it is in other developing countries. The current diagnostic options are limited with the recent phasing out of the Widal serological test that was unreliable. The gold standard is blood culturing which is also beset with cost and logistical challenges which are common in the typhoid endemic and holo-endemic areas. Due to these diagnosis setbacks, it has been impossible to correctly evaluate the exact burden of typhoid and inform specific mitigation measures at policy and practice levels. Additionally, this has contributed to the prevailing mis-diagnosis and over-diagnosis which are potentially fatal and costly respectively.

Objectives

Loop isothermal amplification assay (LAMP) is a recently developed technology that is both cost effective, easy to perform, sensitive and specific as shown in previous studies. Furthermore, because using the LAMP method a large amount of DNA is synthesized, simple turbidity can be used to detect the products thus expensive equipment is not necessary in order to give high level of precision. The aim of this study is to test this new technology against the existing options in the diagnosis of typhoid.

Methods

A cross sectional study will be carried out at city clinics in Nairobi which has a population of approximately 4 million residents with stretched social amenities. Simple random sampling will be done and 160 subjects will be included in the study. Samples will be taken from the subjects and subjected to both blood culture and Loop isothermal amplification assay (LAMP).

Laboratory data will be transcript generated by the turbid meter and then entered manually into access database. Data analysis will be done using STATA.

Results

The LAMP tests had a reasonable agreement with gold standard test. Therefore, laboratories should perform the standard laboratory procedure of LAMP test and follow the standard reporting instead of in ‘reactive’ and ‘non reactive’ terms. The sensitivity, specificity, PPV and NPV of LAMP test were 92.64%, 84.14, 82.89% and 93.24% respectively.

Conclusion

As typhoid is a highly infectious disease it requires accurate diagnosis so as to enable prompt initiation if therapy and also to reduce the rate of over diagnosis and misdiagnosis. With this novel technology (LAMP), diagnosis of typhoid will be faster and more accurate and can be adopted as the gold standard in detection of typhoid. This study will potentially be the preliminary of a major study on national ‘Typhoid watch’.

DEDICATION

This dissertation is dedicated to all clinicians and researchers. I hope this information will be useful.

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To Almighty God, for his grace, love and faithfulness

I would like to sincerely thank my supervisors Dr. Julius Oyugi who amidst his busy work and research schedules spared time to go through my work, provided the apparatus to make the work implementable and tirelessly guided and advised me throughout the entire research.

To my family and friends who have been with me through this entire journey.

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

A	Adenine
ACMA	Annals of Clinical Microbiology and Antimicrobials
APCs	Antigen Presenting Cells
BA	Blood Agar
BIP	Reverse Inner Primer
BMJ	British Medical Journal
C	Cytosine
CBA	Chocolate Blood Agar
DC	Dendritic Cells
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetra acetic Acid
EQA	External Quality Assurance
FEMS	Federation of European Microbiological Societies
FIP	Forward Inner Primer
G	Guanine
ICAM1	Inter-cellular Adhesion Molecules 1
IFN	Interferon
IL	Interleukin
JAMA	The Journal of the American Medical Association
KEMRI	Kenya Medical Research Institute
KIA	Kliger Iron Agar

KNH/UON-ERC Kenyatta National Hospital/ University Of Nairobi Ethics and Research Committee

LAMP Loop Mediated Isothermal Amplification

LPS Lipopolysaccharide

MAb Monoclonal Antibody

MAC Maconkey Agar

NPV Negative Predictive Value

OMP Outer Membrane Protein

PCR Polymerase Chain Reaction

PPV Positive Predictive Value

RUQ Right Upper Quadrant

T Thymine

TNF Tumor Necrosis Factor

UNITID University Of Nairobi institute of Tropical and Infectious Diseases

VCAM-1 Vascular Cell Adhesion Molecule1

WHO World Health Organization

CHAPTER ONE
1.0 INTRODUCTION

Typhoid fever continues to be an important medical burden in Kenya and other developing countries in general due to unhygienic food handling procedures and contamination of water ways and water sources (Crump *et al.*, 2004, Crump & Mintz, 2004). It is caused by the Gram Negative Bacterium *Salmonella enteric* serovar *typhi*. The transmission is feco-oral though chronic carriage in the gall bladder can result in new infection (Gali *et al.*, 2004). Once sufficient inoculum is ingested, the bacteria invade the Peyer's patches at the terminal ileum where it resides inside the macrophage and is then translocated to the mesenteric lymph nodes. Together with absorbed lipids, it passes through the thoracic duct into the subclavian vein after which it goes into the general circulation and then to the liver where it multiplies exponentially and is secreted together with bile into the gall bladder and duodenum. It then re-invades the Peyer's patches and is systemically disseminated to other tissues (House *et al.*, 2001).

Symptoms of typhoid fever include persistent high fever with low pulse rate, severe headache, toxaemia, enlargement of the spleen, nausea, and apathy or mental confusion. The organisms multiply in reticuloendothelial cells. Invasion of the intestine causes inflammation and ulceration, epistaxis, intestinal hemorrhage and perforation, toxaemia and renal failure may occur in untreated late typhoid (often fatal). A rash (rose spots) on the trunk may be seen on light coloured skin. In uncomplicated typhoid, the total white cell count is normal or low with a relative lymphocytosis. There may also be anaemia. A sudden increase in white cell count may occur with intestinal perforation. Infection with *S.* Typhi can also cause osteomyelitis and typhoid arthritis particularly in those with sickle cell disease and thalassaemia.

Presently, the diagnosis of typhoid is a major challenge for the clinician due to several differential diagnoses in the tropics and the poor sensitivity and specificity of the previously widely used Widal test and the dot-enzyme linked immunoassay (Typhidot).In Kenya for example, 40% of all widal tests are declared positive although on blood culture testing only 1-2% of these are truly positive. Caution has been called in interpreting the Widal test (Parry *et al*., 1999) pointing to its ambivalence in usefulness. Consequently this test has lately been phased out of practice in Kenya as a diagnostic test. In a study to evaluate the effectiveness of the dot-enzyme linked immunoassay, (Bhutta *et al*.,1999)noted a sensitivity and specificity of 85-94% and 77-89% respectively. Of significance, the varying structure of the Outer Membrane Protein (OMP) of the salmonella which is the target antigen in the Typhidot assay is a potential Achilles heel in attaining test performance uniformity across different geographical locations (Bhutta & Mansurali, 1999). The gold standard has been blood culture and this has many limitations ranging from cost of agar, requirements for electricity and contamination. Blood culture is the recommended diagnostic method, but it is reported to be positive in only 40-80% of cases (Bhutta*et al*.,1999). Culture of the bone marrow is more sensitive than blood but not feasible in routine practice (Parry C.M*etal*, 1999). These diagnostic challenges have undermined proper evaluation of the magnitude of the burden leading to paucity of data needed by policy makers in making decisions on deployment of enteric fever prevention measures and vaccines (Crump*et al.*,2010).

Consequently, this has resulted in over diagnosis of typhoid in patients presenting with fever and unnecessary use of antibiotics which not only pose risk of resistance developing but is also costly (Poulos *et al.* 2004). Emergence of multi-drug resistance typhoid has been reported in outbreaks in Pakistan (Shanahan *et al.,* 2000), India, Kenya (Kariuki *et al.,* 2004) and other countries (Holt *et al.,* 2004). The present main stay of treatment is fluoroquinolones especially ciprofloxacin and third generation cephalosporins e.g. ceftriaxone. To maintain the efficacy of

these drugs, it is critical that unnecessary usage should be avoided (Rowe *et al.*,1997). Even so, under diagnosis of typhoid may result in complications such as intestinal perforation (Osifo & Ogiemwonyi, 2004), gall bladder perforation (Gali et al.,2004) brain abscesses and death.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology

An estimated 16-33 million cases of typhoid fever and 216,000 – 600,000 typhoid-related deaths occur annually worldwide (WHO). It is more common in children and young adults than in older patients. Worldwide typhoid fever is most prevalent in impoverished areas that are overcrowded with poor access to sanitation.

Typhoid fever remains an important cause of enteric disease in Africa, Latin America and particularly in developing areas of Asia with incidence of *Salmonella typhi* infection (more than 100 cases per 100,000 person years) (Crump *et al*,2004).However, it has become rare in industrialized countries with improved water and sanitation systems and hygiene education. The sporadic cases of typhoid fever reported in developed countries are mostly imported from endemic areas. However outbreaks are still occasional. Nonetheless, the true burden of typhoid infections in developing countries is difficult to estimate due to lack of inexpensive rapid diagnostic tools, infrequency of laboratory testing, poor disease reporting systems and the fact that the clinical presentation of the disease is often confused with other febrile illnesses. The true burden is also limited by lack of consistent reporting from different parts of the world and is based on extrapolation of data across regions and age groups. As an example, the incidence estimates within Africa are based upon reports from Egypt and South Africa only and thus may not be accurately defined.

Since humans are the only reservoir *of Salmonella enterica* serotype *typhi,* a history of travel to a setting in which sanitation is poor or contact with a known typhoid case or carrier is useful for identifying people at risk of infection outside of endemic areas although a specific source or contact is identified in a minority of cases. Approximately 200-300 cases of *Salmonella*

typhi are reported in the United States each year (Lynch *et al*, 2009). About 80% of these occur among travellers to countries where typhoid is endemic.

It is estimated that a total of 400,000 cases occur annually in Africa, an incidence of 50 per 100,000 persons per year (Kariuki S. *et al*, 2004).

The global concern over typhoid is reflected in perceptions that typhoid is a common and serious disease among children and adults in Kenya. One consequence is the common use of the Widal test for screening in both inpatient and outpatient settings as few centers have the capacity to perform blood or bone marrow cultures, the accepted gold standard diagnostic tests (Chart *et al*. 2000; Willke *et al*., 2002)

In Kenya there have been reports of sporadic outbreaks of typhoid but these cases have not always been confirmed, leading to lack of reliable data on the prevalence of typhoid in many areas reporting outbreaks. Some of the areas that have reported outbreaks previously include three districts in Central Kenya, Malindi and Kwale in coast region, and parts of the counties around Lake Victoria.

2.2 Pathogenesis

The infectious dose of S. Typhi in volunteers varies between1,000 and 1 million organisms (Hornick et al.,1970). The low gastric pH is an important defense mechanism as the bacteria must survive the gastric acid barrier to reach the small intestine. In the small intestine, bacteria move across the intestinal epithelial cell and reach the M cells, thus penetrating in the Peyer's patches. The M cells are specialized epithelial cells overlying Peyer's patches that have probably originated from intestinal epithelial cells and small pockets in the mucosal surface. After contact with M cells, the infectious bacteria are rapidly internalized and they reach a group of antigen-presenting cells (APCs), being partially phagocytized and neutralized. The

infected phagocytes are organized indiscrete foci that become pathological lesions, surrounded by normal tissue. Lesion formation is a dynamic process that requires the presence of adhesion molecules such as ICAM1 (Inter-Cellular Adhesion Molecule 1), VCAM-1 (Vascular Cell Adhesion Molecule 1) and the balanced action of cytokines tumor necrosis factor (TNF), interleukin (IL)-12, IL-18, IL-14, IL-15 and interferon (IFN).Failure to form pathological lesions results in abnormal growth and dissemination of the bacteria in the infected tissue. Some bacteria escape this barrier, and reach the developed lymphoid follicles (Peyer's patches); formed mainly by mononuclear cells as T lymphocytes, as well as dendritic cells (DC). DC presents the bacterial antigens to immune cells that provoke activation of T and B lymphocytes.

The T and B lymphocytes come from the lymphatic nodules, reaching the liver and spleen via the reticuloendothelial system. In these organs the bacteria are killed mainly by phagocytosis through the macrophage system. However, Salmonella are able to survive and multiply within the mononuclear phagocytic cells (House et al.,2001). At a threshold level determined by the number of bacteria, the bacterial virulence and the host immune response, the bacteria are released from their sequestered intracellular habitat into the bloodstream. This bacteremic phase of disease is characterized by dissemination of the organisms. The most common sites of secondary infection are the liver, spleen, bone marrow, gallbladder and Peyer's patches in the terminal ileum. In liver, S. Typhi provokes Kupffer cell activation. Kupffer cells have high microbicidal power and neutralize the bacteria with oxidative free radicals, nitric oxide as well as enzymes, active in acid pH. The survived bacteria invade hepatocytes and cause cellular death, mainly by apoptosis.

2.3 Diagnosis

The diagnosis of typhoid fever is made by any blood, bone marrow or stool cultures and with Widal test(demonstration of salmonella antibodies against O- somatic and H – flagellar). The widal test is what is routinely used in the diagnosis of typhoid fever with the gold standard being blood cultures. The widal test is a tube agglutination test employed in the serological diagnosis of enteric fever whereby bacteria causing typhoid fever are mixed with serum containing specific antibodies obtained from an infected individual. It has been used very extensively in the serodiagnosis of typhoid fever and, in developing countries particularly, remains the only practical test available. Widal testing done on an acute phase serum of a patient suspected to have typhoid fever had limited diagnostic capability given its low sensitivity of 26% (Omuse *et al*,2010).A rise in titre over time or a single high test rest is diagnostically significant. Classically, a four-fold rise of antibodies in paired sera Widal test is considered diagnostic of typhoid fever. However, paired sera are often difficult to obtain and specific chemotherapy has to be instituted on the basis of a single widal test. This may lead to false negative results if the blood is collected too early at the onset of the disease; therefore negative results do not rule out typhoid fever. False positive results may be associated with a past history of immunization for typhoid fever, cross reacting antibodies or a host of infections and conditions.

Tubex is a 5 minute semi-quantitative colorimetric test that uses polystyrene particle agglutination to detect IgM antibodies to the salmonella 09 antigen. It detects anti-Salmonella O9 by inhibiting the binding between an anti-O9 IgM monoclonal antibody (MAb) conjugated to colored latex particles and S. typhi lipopolysaccharide (LPS) conjugated to magnetic latex particles. The reactants are mixed in a specially designed microtube for 2 minutes, and the result is read based on the resultant colour of the supernatant following forced sedimentation

of the magnetic beads. In the absence of inhibitory antibodies, there is a color change (from blue to red) due to cosedimentation of the indicator particles with the magnetic particles, whereas if these antibodies are present, they prevent such a change to a degree dependent on their concentration. Tubex has shortcomings regarding scoring of its results as sharp scores are obtained only in the two extremities (strong positive and strong negative cases). In cases where a sharp score is not obtained it is difficult to interpret. This test is rarely used since the Widal test is thought to be better as it has subjective interpretation of colour results and hemolysis may result in difficulty in interpretation. It has a sensitivity of 73% and specificity of 69% (Bulletin of the WHO vol.89, 2011).

Typhidot is a dot enzyme linked immunoassay that detects the presence of IgM and IgG antibodies against the 50kd Outer Membrane Protein (OMP) of the *Salmonella typhi* which is impregnated on nitrocellulose strips. IgM shows recent infection whereas IgG signifies remote infection. This test becomes positive within 2-3 days of infection. The most important limitation of this test is that it is not quantitative and the result is either positive or negative whereas a detailed widal test can tell the titres of the specific antibodies. Typhidot lacks both sensitivity and specificity. For both sensitivity of 75% and 69% and specificity of 60.7% and 70.4% for IgM and IgG respectively (Bulletin of the WHO vol.89, 2011).

Blood culture is the gold standard in the diagnosis of typhoid fever. Since blood is normally sterile, the isolation and identification of an organism has great diagnostic significance. Blood is collected from the patient (preferably before antibiotic therapy is initiated) by vein puncture with a needle and syringe (or blood collecting set) and immediately transferred aseptically to the blood culture bottle containing the desired growth medium. The bottle is then incubated and can be observed for turbidity, color change, hemolysis, gas formation or other evidence of

microbial growth. Appropriate conventional subculturing methods should be used. Subculture on blood agar (BA),chocolate blood agar (CBA), and maconkey agar (MAC). The BA and MAC plates are incubated aerobically and the CBA plate in carbon dioxide atmosphere (candle jar). On blood agar Salmonella produce grey white colonies 2-3mm in diameter also non hemolytic colonies. On MAC agar it produces non-lactose fermenting pale coloured colonies. Biochemical test are also carried out to confirm the presence of Salmonella. The Kliger Iron Agar (KIA) is used to presumptively identify Salmonella which produces a pink-red (alkaline) slope and a yellow butt indication fermentation of glucose and not lactose. Other biochemical tests include a negative urease, indole and citrate tests. There are limitations to this method as it requires skilled personnel. It may lead to contamination of the blood culture media giving false positive results and it is also labour intensive as one needs to visibly inspect for growth.

There are a greater number of bacteria found in the bone marrow, ten-fold more per volume than in blood and they may be protected from the presence of systemic antibiotics. Bone marrow cultures are the gold standard diagnostic test although it is rarely used due to its invasive nature hence cannot be routinely performed as it requires skilled personnel who may not be found in resource poor settings.

Molecular based studies have also been used in the diagnosis of typhoid fever. Polymerase Chain Reaction (PCR) is a sensitive and specific method used in the diagnosis of a number of infectious diseases. It is effective since it can be used even in cases where antibiotic therapy has been started or the pathogen load is too low. A nested PCR makes the detection more sensitive and is able to detect the presence of even 3-5 bacilli. Due to its expensive nature and requirement of skilled personnel, it’s difficult to be used as routine screening test.

Loop isothermal amplification assay (LAMP) is a nucleic acid amplification based technique that unlike the polymerase chain reaction does not require thermocycling. This is attained by the novel design of looping primers at the 3' and 5' ends of the gene segment under amplification. It is characterized by the use of four different primers specifically designed to recognize six distinct regions on the target gene and the reaction process proceeds at a constant temperature using strand displacement reaction. Amplification and detection of the gene can be completed in a single step by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 65^{o}C). It provides high amplification efficiency with DNA being amplified $10^9 – 10^{10}$ times in 15 – 60 minutes. The amplified products can be detected by gel electophoresis, turbidimetry or can be visualized by the naked eye as either turbidity or in form of a colour change when SYBR® green is used.

LAMP has been tested for microbiological diagnosis of diverse agents with high sensitivity and specificity. Importantly, testing for *Salmonella spp*. in chicken eggs demonstrated higher sensitivity than PCR and comparable specificity (Ohtsuka *et al.*,2005, Hara-Kudo *et al.*2005).

2.4 Significance of the study

This study will be useful in rapid and accurate diagnosis of typhoid fever to reduce number of patients who are over-diagnosed or misdiagnosed. It will also help reduce the level of drug resistance which is associated with overuse of antibiotics.

2.4.1 Study Problem

The diagnosis of typhoid remains a big challenge in the tropics due to the myriad of differential diagnoses and inadequacy of the available laboratory tests. The aim of this study is to test a novel technology, LAMP, in the diagnosis of typhoid.

2.5 Rationale and justification

Although advances in public health and hygiene have lead to the virtual disappearance of enteric fever from the developed world, the disease remains endemic in many developing countries (Bhutta *et al*.,2006). It is therefore difficult to estimate the true burden of typhoid infections as few established surveillance systems exist in the developing world.

Currently the diagnosis of typhoid is restricted to clinical suspicion which poses problems since typhoid fever may mimic many common febrile illnesses. There is an atypical presentation of typhoid in areas where malaria is endemic and where schistosomiasis is common.

Although the mainstay of diagnosing typhoid fever is a positive blood culture, the test is only positive in 40-60% of cases usually early in the course of the disease (WHO 2003) and the specimen cultures take several days to yield results. Stool and urine cultures become positive after the first week of infections, but their sensitivity is much lower. In much of the developing world, widespread antibiotic availability and prescribing is another reason for the low sensitivity of blood cultures. Although bone marrow cultures are more sensitive they are difficult to obtain, relatively invasive and of little use in public health setting.

The Widal test which provided a rapid serological diagnosis is not reliable as it lacks both sensitivity and specificity and reliance on it alone in areas where typhoid is endemic will lead to over diagnosis. Newer diagnostic test have been developed such as typhidot and tubex which directly detect IgM antibodies against the host specific *S. typhi* antigens but these have not proved to be sufficiently robust in large scale evaluations in community setting. A nested PCR has been used to amplify specific genes of *S.typhi* in the blood of patients. It has a high sensitivity and specificity rate but is expensive to be used on a large scale (Bhutta et al.,2006).

LAMP is a new technology that is easy to use, takes only 2 hours, can test up to 96 samples at a time, and is potentially cost effective, with high sensitivity and specificity.

To be able to correctly diagnose typhoid fever, there is need to develop and apply new technologies in testing both active cases and carriers.

2.6 Hypothesis

There is no difference in test diagnostic performance between blood culturing and LAMP in diagnosing *salmonella enterica* serovar *typhi.*

2.7 Broad objective

To evaluate the loop mediated isothermal amplification (LAMP) for rapid diagnosis of typhoid infections.

2.8 Specific objectives

1. To determine the sensitivity and specificity of loop mediated isothermal amplification assay in diagnosis of typhoid fever
2. To determine the predictive value of loop mediated isothermal amplification assay in diagnosis of typhoid fever

CHAPTER THREE

3.0 PROJECT METHODOLOGY

3.1 Study design

A cross sectional study will be carried out among patient attending outpatient clinics in Nairobi.

3.2 Study Area

The study will be carried out in Nairobi County. With a bursting population of over 3 million residents, stretched social amenities ranging from water supply to sewerage system, and informal settlements, anecdotal reports indicate that typhoid is among the top 3 infectious diseases at the hospital.

3.3 Sample Size

3.3.1 Sample size calculation

The prevalence of Salmonella in patients with fever and a request for a widal test is 25% (Kariuki et al.,2004).

With estimate prevalence rate of 25% of patients with typhoid, the sample size at 95% confidence level was calculated as

$$n = \frac{Z_{\propto}{}^{2}\, p(1-p)}{\sigma^{2}}$$

Where n = sample size, Z = Z statistic for a level of confidence, P = expected prevalence and d = standard deviation

$$n = \frac{1.96 * 0.25(1 - 0.25)}{0.05^2}$$

=147 patients rounded off to 150 patients.

3.3.2 Study population

An estimated 150 subjects will take part in the study and this will include both voluntary adults and children above 5 years who assent while their parents or guardian give consent.

3.3.3 Inclusion criteria

Patients having the symptoms of interest (fever, abdominal discomfort, lethargy and headache).

i. Adults and children over 5 years will be recruited.
ii. Informed consent for participation in the study.

3.3.4 Exclusion criteria

i. Patients who do not give consent for blood collection.
ii. Children
iii. Not suspected to be having disease.
iv. Patients who appear clinically pale.

3.3.5 Definition of terms

Sensitivity is the ability of a test to correctly classify an individual as diseased also known as true positive rate

Specificity is the ability of a test to correctly classify an individual as disease free also known as true negative rate.

Gold standard test

Novel test	Positive	Negative
Positive	True Positives	False Positive
Negative	False Negative	True Negatives

Sensitivity = $\frac{\text{True positives}}{\text{True positives + False negatives.}}$

Specificity = $\frac{\text{True Negatives}}{\text{True negatives + False positives}}$

3.4 Specimen collection

Blood will be collected using aseptic technique using a sterile needle gauge 16 (G16) and 20 millimetre (ml) syringe. The blood (11ml) will be obtained from a peripheral vein after thoroughly cleaning the area with spirit and betadine solution. 10mls of the blood will be put in a blood culture bottle and the other 1mls will be used in the LAMP procedure.

3.4.1 Blood culturing procedure

A minimum of 10 ml of venous blood from an adult patient will be added to each of 50 ml of sodium taurocholate broth and glucose broth. From children at least 5 ml blood shall be inoculated in each of the bottles. The inoculated media will then be incubated at 37°C overnight and sub-cultured onto MacConkey agar and blood agar. If subcultures fail to yield any bacterial growth, the incubation of blood culture bottle will be extended upto 7 days. Subcultures will be performed again on day 7 and if no growth is obtained blood culture will be declared as negative for enteric fever bacilli.

3.4.2 LAMP procedure

Several primers will be designed and optimized for the local environment for all the three genes of Salmonella using a bioinformatics tool, Primer Explorer v3 software. The three genes are

16s rRna gene, The primers will be selected based on criteria described previously (Hara-Kudo et al.,2005, Ohtsuka et al.,2005). The two outer primers will be described as being forward outer primer (F3) and reverse outer primer (B3). The inner primers will be described as being forward inner primer (FIP) and reverse inner primer (BIP). FIP will consist of a complementary sequence of F1 and a sense sequence of F2 while BIP will consist of a complementary sequence of B1 and a sense sequence of B2. FIP and BIP being the nested primers and tool for enhancing specificity will need to be prepared under highly purified conditions to eliminate issues of contamination that may compromise validity of results obtained.

Primer	**Sequence**
FIP	GACGACTGGTACTGATCGAGTTTTTCAACGTTTCCTGCGG
BIP	CCGGTGAAATTATCGCCACACAAAACCCACCGCCAGG
F3	GGCGATATTGGTGTTTATGGGG
B3	AACGATAAACTGGACCACGG
Loop F	GACGAAAGAGCGTGGTAATTAAC
Loop B	GGGCAATTCGTTATTGGCGATAG

One ml of blood sample collected containing Potassium EDTA as anticoagulant will be centrifuged at 1,000 rpm for 5 minutes. The supernatant will be discarded. One ml of lysis buffer is added to the pellet. The mixture is gently aspirated to effect hemolysis. The tube is then centrifuged at 12,000rpm for 6 minutes. The supernatant is discarded. The tube is sealed, kept in boiling water for 20 minutes and brought back to room temperature before being used as a sample for LAMP.

To amplify the DNA a mixture of the DNA sample, the 4 primers (FIP, F3, BIP, and B3), DNA polymerase with strand displacement activity, substrates (deoxynucleotides triphosphates) and

a reaction buffer are incubated in isothermal conditions, between 60-65ºC for 1 hour. The amplification can be detected through presence of amplified products by visual detection of white turbidity. A positive result will be seen as white turbidity.

2.4.3 Quality Control/Assurance

The blood samples collected will be delivered to the laboratory as soon as possible. Quality control samples used in the study will be from patients who are confirmed to have typhoid infections and also from those without the infection as positive and negative controls respectively and these control samples will be run concurrently for each of the samples. This will be able to help in validating the results obtained.

An external quality assurance (EQA) program will not be included in this study but the UNITID laboratory has EQA programs for other studies. In this study internal quality assurance will be used as stated above.

3.4.4 Referral system for positive patients

Participants who turn out positive will be asked to return to the facility as soon as possible so as to be able to receive the appropriate treatment. The results will also be communicated to the clinician found at the facility. If the treatment is not available at that particular facility, the participant will be referred to the nearest health facility which can offer the appropriate treatment.

CHAPTER FOUR

4.0 DATA MANAGEMENT AND ANALYSIS

Socio-demographic and health variables will be collected for each sample. The data will be entered into an access database. Laboratory data will be transcript generated by the turbidimeter and then entered manually into access database and counter checked for consistency. Data analysis will be done using STATA.

4.1 Impact of project

The project will provide prompt diagnosis of enteric fever and contribute to patient management at various hospitals in Nairobi.

4.2 Anticipated outputs

The diagnostic test data derived from this study will inform on the sensitivity and specificity of LAMP and thus usability. This study may then preclude a major “typhoid watch study” that will be rolled out at sites across Kenya to ascertain the ‘hidden’ epidemiology of the disease, consequently informing policy on control and treatment strategies.

4.3 Ethical considerations

Permission to carry out the study will be sought from the Kenyatta National Hospital/ University of Nairobi Ethics and Research Committees (KNH/UON-ERC). The adult or the parent/guardian of the children enrolled in the study will not incur any cost in the transport nor processing of the samples, neither will they receive any monetary inducements to participate in the study. An informed written consent will be sought from the parent/guardian of the children enrolled in the study. The patients will be given an appointment when the results will be made available to them. If evidence of recent infection is found, the primary clinician will

be informed of the result in order to institute the necessary supportive management in line with the prevailing clinical condition of the patient.

4.3.1 Consenting process

Eligible participants will be approached by a research assistant or the principal investigator, given information about the study and asked if they will be willing to participate. All participants will be informed that participation is completely voluntary; refusal to participate will in not affect services provided. Those who participate will not be identifiable from any data as numbers will be used instead of names. The study will be fully explained as well as the rights and obligations as research participants, including the right to withdraw at any point in the study without negative consequences. Any questions will be answered and signed informed consent will be obtained from all women willing to participate. Thus all participants will be assured that confidentiality, privacy and anonymity will be strictly maintained throughout all aspects of the study. A copy of a signed and dated consent form will then be given to the partcipants.

4.4 Budget

Items	**Unit cost**	**Total cost**
Blood cultures x 150	400	60,000
LAMP assay x 150	500	75,000
Transport (to UNITID lab) x 15	1,000	15,000
Lab assistants x2	15,000	30,000
Miscellaneous	11,000	10,000
Total		**190,000**

4.5 Project Time Plan: Gannt chart

ACTIVITY	**2014**								**2015**					
	M	J	J	A	S	O	N	D	J	F	M	A	M	J
Protocol development														
Ethical approval														
Data collection														
Laboratory analysis														
Study findings/ dissemination														

CHAPTER FIVE

5.0 RESULTS

5.1 Profile of Patients in the study

One hundred and fifty (150) eligible patients from age cohorts of 5 - 17 years and 18 years and above presenting at the four out patients hospitals in Nairobi, namely Kayole hospital, KNH Out Patient, Victory Hospital and Patanisho Hospital with suspected typhoid disease underwent the standard typhoid pack of investigations and were tested for typhoid disease. All the patients granted consent, and the children consent was provided by their parents or guardians and was enrolled in the study.

The study had two age cohorts of children and adults aged between 5 -17 years (37.5%) and over 18 years (62.5%) respectively. Of this 36.25% were drawn from Kayole Hospital, 9.36% from KNH Out Patient, 27.5 % from Victory Hospital and 26.86% from Patanisho Hospital.

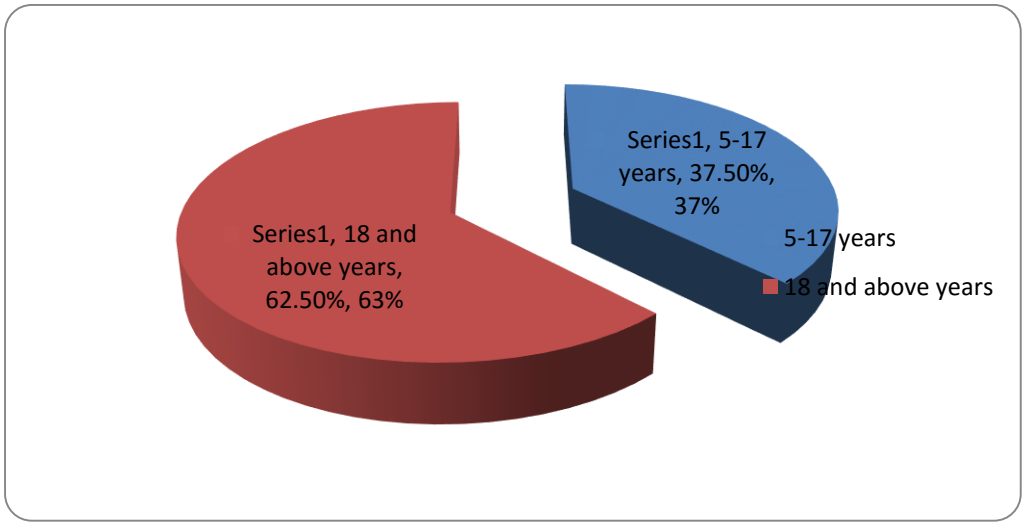

Figure1: Age cohorts of Patient in the study

5.2 Primer sequences

Salmonella spp. 18S ribosomal gene

Forward primer	5¢- AATAAATCATAAGTATTCAGATGTCAGAGGTG -3¢
Reverse primer	5¢- AATAAATCATAAGRCAAATGCTTTCGCAGTTG -3¢
Probe	5¢- MGB-FAM- TTCTGGAGACG*A*G*CAA*CT -Quencher -3¢

PHPRT1 human housekeeping gene

Forward primer	5¢-CCGCAGCCCTGGCGTCGTGATTAGTGA -3¢
Reverse primer	5¢- CCCTTTCCAAATCCTCAGCATAATGATTAGGT -3¢
Probe	5¢- MGB-Yellow Dye- G*ATTTATTTTGCA*TACCTQ -3¢

Underlined in primer sequences are twelve-mer AT-rich non-complementary ‘flaps’. Modified bases are indicated by * in the sequence.

Table 1 Primers and probes for Salmonella spp real-time LAMP assays targeting the 18S ribosomal gene sequence.

5.3 Design and performance of universal Salmonella spp. LAMP reagents

Pleiades probe-based LAMP tests were developed targeting the 18S ribosomal RNA gene for Salmonella spp. All assays were performed biplexed with LAMP reagents targeting the Salmonella spp 18S sRNA gene and PHPRT1 human housekeeping gene as an endogenous control as described in the materials and methods. In the LAMPs, 2 ll of template nucleic acid was used. The Pleiades probe-based reagents were tested for specificity against a negative pooled human genomic DNA, which were obtained from patient who were correctly identified as disease negative.

5.4 Performance characteristics of LAMP and RT-LAMP on clinical specimens

Total nucleic acid was extracted from whole blood specimens using the QIAmp DNA mini kits. Two microliters of whole blood equivalents was used per LAMP; 51% (76/150) specimens were positive by LAMP.

Table 1: Summary of positive results obtained from the study

PATIENTS (n)	INSTITUTIONS							
	KAYOLE HOSPITAL		KNH OUTPATIENT		VICTORY HOSPITAL		PATANISHO HOSPITAL	
	GOLD STD	LAMP	GOLD STD	LAMP	GOLD STD	LAMP	GOLD STD	LAMP
5-17	45.4% (10/22)	50% (11/22)	40% (2/5)	60% (3/5)	52.9% (9/17)	52.9% (9/17)	46.7% (7/15)	53.3% 8/15
>18	45.2% (14/31)	48.4% (15/31)	44.4% (4/9)	55.6% (5/9)	40.7% (11/27)	48.1 (13/27)	45.8% (11/24)	50% (12/24)
TOTAL	45.3% (24/53)	49.1% (26/53)	42.8% (6/14)	57.1% (8/14)	45.4% (20/44)	50% (22/44)	46.2% (18/39)	51.3% (20/39)

The results as shown in the table above show that the LAMP assay performance is better compared to the blood culture. The LAMP result was highest at 60% compared to the blood culture at 40%. This shows that the LAMP was able to correctly diagnose the patients with disease who would have been missed out if the Lamp test was not done.

5.5 Sensitivity specificity, PPV and NPV

Gold standard test

Novel test	Positive	Negative
Positive	True Positives	False Positive
Negative	False Negative	True Negatives

Sensitivity = $\frac{\text{True positives}}{\text{True positives + False negatives}}$

Specificity = $\frac{\text{True Negatives}}{\text{True negatives + False positives}}$

Positive Predictive Value = $\frac{\text{True Positives}}{\text{True positives + False Positives}}$

Negative predictive value = $\frac{\text{True Negatives}}{\text{True Negatives + False Negatives}}$

Table 2: Results of the two tests performed on the patients

	BLOOD CULTURE (GOLD STANDARD)		
LAMP TEST	POSITVE	NEGATIVE	**TOTAL**
POSITIVE	63	13	**76**
NEGATIVE	5	69	**74**
TOTAL	**68**	**82**	**150**

Statistic	Value	95% CI
Sensitivity	92.64%	86.90% to 98.53%
Specificity	84.14%	76.11% to 92.30%
Positive Predictive Value	82.89%	73.72% to 91.45%
Negative Predictive Value	93.24%	86.90% to 98.53%

The results shown above show that the test has a very high sensitivity rate (92.64%) meaning that it is able to correctly identify patients with disease. It also has a high Negative Predictive Value (93.24%) which shows that it is correctly identify patients without disease.

CHAPTER SIX

6.0 DISCUSSION

High incidences of typhoid fever have been noted and outbreaks are not uncommon. Due to the similarities with other febrile illness it's difficult to differentiate typhoid in early phase of disease. Many patients with typhoid do not have the opportunity to obtain a rapid and reliable diagnosis because of lack of diagnosis and technical personnel in economically poor areas.

Therefore the general lack of etiological diagnosis of typhoid results in the persistence and accumulation of patients and carriers which to some extent attributes to the outbreak and high incidence of typhoid fever demanding the necessity of developing a rapid accurate and cost effective method applied in developing countries with high incidence of typhoid.

Simple, specific and sensitive diagnostic tests are needed for early detection of pathogens especially in low resource setting. Although the mainstay of diagnosing typhoid fever is a positive blood culture, the test is only positive in 40-60% of cases usually early in the course of the disease. Stool and urine cultures become positive after 1st week of infection but their sensitivity is much lower. In much of the developing world, widespread antibiotic availability and prescribing is also another reason for the low sensitivity of blood cultures.

Current diagnosis is via culturing and molecular methods such as PCR and RT-PCR. However, the conventional culture method is time consuming and can take more than three days to obtain results. Despite the rapidity and sensitivity provided by PCR based detection methods, they are not widely used due to sophisticated equipment and well trained staff to conduct the testing.

It should be noted that the cost of LAMP assay is higher than the available rapid diagnostic techniques but its sensitivity is much higher. Its use in clinical diagnosis may allow detection

of the causative organism and facilitate initiation of prompt treatment among patients with typhoid fever.

The sensitivity and specificity of LAMP and Gold standard in this study were about 94.6% and 74.1% respectively. This is similar with the study conducted in the endemic area of Vietnam by Olsen *et al*. for the evaluation of serodiagnostic assay of acute enteric fever. Another study done in Kenya has shown that Widal testing done on acute phase serum of patients suspected to have typhoid fever had limited diagnostic capability given its low sensitivity in which among all typhoid cases only 26% had diagnostic titer. LAMP use in this study had relatively good NPV (94.67%) and a satisfactory PPV of (84.4%). Positive predictive value is more important than other measure of clinical diagnostic methods because it gives the proportion of patients with positive test results that are correctly diagnosed but it is highly affected by a prevalence of the disease. A Negative LAMP test result has a good predictive value for the absence of the disease as well as a positive result would have a satisfactory predictive value for the presence of typhoid fever.

CHAPTER SEVEN

7.0 CONCLUSION

The LAMP tests had a reasonable agreement with gold standard test. Therefore, laboratories should perform the standard laboratory procedure of LAMP test and follow the standard reporting instead of in ‘reactive’ and ‘non reactive’ terms. The sensitivity, specificity, PPV and NPV of LAMP test were 92.64%, 84.14%, 82.89% and 93.24% respectively.

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APPENDICES

INFORMATION AND CONSENT FORM

Performance characteristics of loop mediated isothermal amplification (lamp) assay for diagnosis of typhoid infections

***Introduction*:** Hello. My name is Dr. Miriam Anyango Etole from the University of Nairobi. I am conducting a research to test a new diagnostic kit for typhoid infections which is common in this country. I am going to give you information and invite you to be part of this research. However, no one is obligated to participate and individuals can refuse to answer any questions or withdraw from the interview at any time.

***Purpose of the research*:** Typhoid infections are common and may lead to sickness. There are many tests which are available for the diagnosis of typhoid but most are not accurate. There is a new test which may work better. The reason for this research is to find out if the new test (LAMP) is better than blood cultures which are currently being used.

***Type of Research Intervention*:** This research will involve a single injection in your arm as well as one follow up visit at the clinic.

***Participant selection*:** We are inviting all adults and children over five years of age to participate in this research for the new test for typhoid.

***Voluntary participation*:** Your participation in this research is entirely voluntary. It is your choice whether to participate or not. You will still be able to receive the services if you do not want to participate.

***Procedure*:** Since we do not know if the new test for typhoid is better than what is currently available we need to compare the two. We will take blood from your arm using a needle and a syringe. Part of this blood will be put directly into a bottle for blood culture and the rest will

be carried to the UNITID laboratory for LAMP assay. You will receivetreatment for your condition according to the national guidelines.

***Risks*:** There are no risks involved by participating in this research.

***Benefits*:** There may not be any direct benefit for you but your participation is likely to help us find a more accurate diagnostic kit for typhoid.

***Confidentiality*:** The information that we collect from this research project will be kept private. Any information you give will be reported anonymously and no names will be used. Numbers will be use instead. Information about you that will be collected during the research will be put away and no one but the researcher will be able to access it.

You can ask me any questions about any part of the research study, if you wish to. Do you have any questions?

Whom do we call if we have questions or problems?

For questions about the study or a research-related injury, call or contact Dr. Miriam Etole, the Principal Investigator, Mobile No: 0727795993.

For questions about your rights as a research participant, contact Professor Chindia, who is the chairperson of the Kenyatta National Hospital/ University of Nairobi-Ethics and Review Committee, by calling Tel: 726300-9

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and these have been answered to my satisfaction. I consent voluntarily to be a participant in this study.

FOMU YA IDHINI

Performance characteristics of loop mediated isothermal amplification (LAMP) assay for diagnosis of typhoid.

Utambulisho: Habari. Jina langu ni Daktari Miriam Anyango Etole kutoka chuo kikuu cha Nairobi. Ninafanya utafiti wa kupimo kipya cha maambukizi homa ya matumbo ambayo ni ya kawaida nchi hii. Nita kupa habari na kuwakaribisha kuwa sehemu ya utafiti huu. Hata hivyo , hakuna mtu ana wajibu wa kushiriki na mtu binafsi anaweza kukataa kujibu maswali yoyote au kujiondoa katika mahojiano wakati wowote.

Madhumuni ya utafiti: Maambukizi homa ya matumbo ni ya kawaida na inaweza kusababisha ugonjwa. Kuna vipimo vingi ambavyo vinapatikana kwa ajili ya utambuzi wa homa ya matumbo lakini vingi si sahihi. Kuna kipimo kipya ambacho kinaweza kufanya kazi bora kushinda hizo zingine. Sababu ya utafiti huu ni ili kujua kama kipimo hiki kipya ni bora kuliko zingine ambazo zinatumika kwa sasa.

Aina ya utafiti**:** Utafiti huu utahusisha kudungwa sindano moja katika mkono wako na kufuatiliwa kwa kliniki mara moja tu.

Mshiriki: Ninawakaribisha watu wazima na watoto juu ya umri wa miaka mitano kushiriki katika utafiti huu kwa ajili ya kipimo kipya cha homa ya matumbo

Ushiriki kwa hiari**:** Ushiriki wako katika utafiti huu ni kwa hiari yako kabisa. Ni hiari yako kama kushiriki au la. Bado utakuwa na uwezo wa kupokea huduma kama hutaki kushiriki.

Utaratibu: Kwa vile hatujui kama kipimo hiki kipya ya homa ya matumbo ni bora kuliko yale yanapatikana tunahitaji kulinganisha mbili. Tutachukua damu kutoka mkono wako kwa kutumia sindano na sirinji. Gawanyiko ya damu hii itawekwa moja kwa moja kwenye chupa na ingine itafikishwa kwa maabara UNITID kwa LAMP assay. Utaweza kupokea matibabu kwa ajili ya hali yako kulingana na miongozo ya kitaifa.

Hatari: Hakuna hatari ya kushiriki katika utafiti huu

Faida: Kunaweza kuwa hakuna faida moja kwa moja yoyote kwa ajili ya ushiriki wako lakini kuna uwezekano wa wewe kutusaidia kupata sahihi zaidi ya kipimo kipya ya homa ya matumbo.

Siri : Habari ambayo tutakusanya katika utafiti huu yatawekwa binafsi. Taarifa yoyote utakayo toa itawekwa siri na hakuna majina zitatumika nambari zitatumika badala yake. Habari ya kukuhusu ambayo itakuwa ilikusanywa wakati wa utafiti itawekwa siri na hakuna mtu isipokua mtafiti atakuwa na uwezo wa kupata huduma hiyo.

Unaweza kuniuliza swali lolote kuhusu sehemu yoyote ya utafiti, kama unataka . Je, una maswali yoyote?

Ni nani tutapigia kama tuko na maswali au shida?

Kwa maswali kuhusu utafiti, pigia Dakari Miriam Etole , kwa nambari ya simu 0727795993

Kwa maswali kuhusu haki za mshiriki kwa utafiti huu, pigia Professor Chindia ambaye ni mwenyekiti wa KNH/UON-ERC kwa nambari ya simu 726300-9

Nimesoma habari hii, ama nimesomewa habari hii na nimekua na fursa ua kuuliza maswali kuhusu utafiti huu na nimejibiwa na kuridhika. Nimekubali kibinafsi kushiriki kwa utafiti huu.

INFORMED CONSENT DOCUMENT

I, (name of volunteer).....

Of (address)..... agree to take part in the research project entitled: "**Performance characteristics of loop mediated isothermal amplification (LAMP) assay for rapid diagnosis of typhoid infections at city clinics in Nairobi**". I have been told in detail about the study and know what is required of me. I understand and accept the requirements. I understand that my consent is entirely voluntary and I may withdraw from the research study for any reason, and this will not affect the legal rights I may otherwise have.

Participant: Print name: .....

Signature/ Mark or Thumbprint: .....

Date: .....

Person obtaining consent

I have explained the nature, demands and foreseeable risks of the above study to the volunteer and answered his/her questions

Print name: .....

Signature: .....

Date: .....

CHETI CHA MAKUBALIANO

Mimi, (Jina la muhusika).....

Anwani ya muhusika……..... ninatoa idhini kuwa; nitashiriki kwenye mradi wa udadisi uitwao: "**Performance characteristics of loop mediated isothermal amplification (LAMP) assay for rapid diagnosis of typhoid infections at city clinics in Nairobi**". Nimepata maelezo kamili na nina elewa kwa ufasaha yatako jiri. Hata hivyo, nina elewa kuwa naweza kutoka kwenye mradi bila haki zangu kuadhirika kwa wakati wowote bila dhamira wala kupeana sababu zozote.

Jina la mshiriki: .....

Sahihi/amaalama ya gumba: .....

Tarehe : .....

Anaye omba idhini

Nimeeleza taswira, matarajio na vikwazo vya huumradi kwa muhusika; na pia nimejibu maswali yote aliyo omba kuuliza kwa ufasaha:

Jina: .....

Sahihi: .....

Tarehe : .....

ASSENT FORM

Project title: Performance characteristics of LAMP assay for rapid diagnosis of typhoid infections at city clinics in Nairobi

Investigator: Dr. Miriam AnyangoEtole

I am carrying out a research study about a new test for typhoid. A research study is a way to learn more about something. There are many tests which are available for the diagnosis of typhoid but most are not accurate. There is a new test which may work better than the tests which are currently available.

If you allow your child to be part of this study you will be asked to answer a few questions about your child's illness and a little blood (the size of a table spoon) will be taken from the child's arm.

The child does not have to join this study. It is up to you. You can say okay now and change your mind later. All you have to do is tell us you want to stop. No one will be mad at you if you don't want your child to be in the study or if you join the study and change your mind later and stop.

Before you say **yes or no** to being in this study, we will answer any questions you have. If your child joins the study, you can ask questions at any time. Just tell the researcher that you have a question.

If you have any questions about this study please feel free to contact the researcher on 0727795993.

□ Yes, I will be in this research study. □ No, I don't want to do this.

_____	_____	_____
Child's name	Signature of the parent/ guardian	Date
_____	_____	_____
Person obtaining Assent	Signature	Date

QUESTIONNAIRE

Thank you for agreeing to fill in this questionnaire. The purpose of this study is to assess performance of a new diagnostic kit for typhoid infections.

All medical information is strictly confidential

Respondent #: _____

Date of data collection: ____/____/_____ (dd/mm/yyyy)

Interviewer Initials: _____

Section A: Socio-Demographic

1. Date of birth : ____/____/_____ (dd/mm/yyyy)
2. Sex : [ ] Male [ ] Female
3. Place of residence: _____
4. Occupation : _____

Section B: Medical History

1. Do you have any of the following symptoms?(*please tick appropriate box*)
    - Fever
    - Chills
    - Anorexia
    - Diaphoresis
    - Malaise
    - Insomnia
    - Confusion/ delirium
    - Frontal headache
    - Dry cough
    - Constipation
    - Diarrhoea
    - Bloating
    - Abdominal pain
    - Myalgia

- Arthritis
- Arthralgia

2. What is the source of water in your household?
   - Borehole
   - City council
   - River

3. On examination
   - Febrile
   - Tachypnoea
   - Rose spots
   - Abdominal distension
   - Abdominal pains(RUQ)
   - Crackles over lung bases

Section C: Laboratory results

1. Widal test titres
   [ ] nil
   [ ] 1: 80
   [ ] 1:160
   [ ] 1:320
2. Malaria blood slide
   [ ] Positive
   [ ] Negative
   [ ] Not done
3. Stool microscopy for amoebiasis
   [ ] Positive
   [ ] Negative

ORODHA YA MASWALI YA UCHUNGUZI

Asante kwa kukubali kujaza orodha ya maswali ya uchunguzi hili. Lengo la utafiti huu ni kuthamini utendaji wa kipimo kipya kwa homa ya matumbo.

Habari zote za utafitini madhubuti za siri

Mshiriki #: _____

Tarehe ya ukusanyaji wa takwima: ____/____/_____ (dd/mm/yy)

Herufi za mhojaji: _____

Section A:

1. Siku ya kuzaliwa: ___/___/_____(dd/mm/yy)
2. Jinsia: [ ]mwanaume []mwanamke
3. Makazi: _____
4. Kazi: _____

Section B:

1. Je, una yeyote ya dalili zifuatazo? (*tafadhali jibu sanduku sahihi*)
    - Joto ya mwili
    - Baridi
    - Upungufu wa hamu ya kula
    - Jasho jingi
    - Kuchoka kwa mwili
    - Kukaukwa na usingizi
    - Mchafuko /kuweweseka
    - Maumivu ya kichwa
    - Kikohozi kavu
    - Kifunga choo
    - Kuhara
    - Maumivu ya tumbo
    - Ugongwa wa baridi ya bisi
2. Ni wapi unapata maji yakutumia nyumbani kwako?
    - Kisima

- Halmashauriyajiji
- Mtoni

3. Kwa kupima
    - Joto mwilini
    - Kupumua haraka
    - Kufura tumbo
    - Maumivu ya tumbo sana sana kwa ini
    - Kelele kwa mapafu
    - Upele mwilini

Section c: majibuyamahabara

1. Kipimo cha Widal
    - Hakuna
    - 1:80
    - 1:160
    - 1:320
    - Haijapimwa
2. Kipimo cha malaria
    - Iko
    - Hakuna
    - Haijapimwa
3. Kipimo cha choo kuangalia amoeba
    - Iko
    - Hakuna
    - Haijapimwa