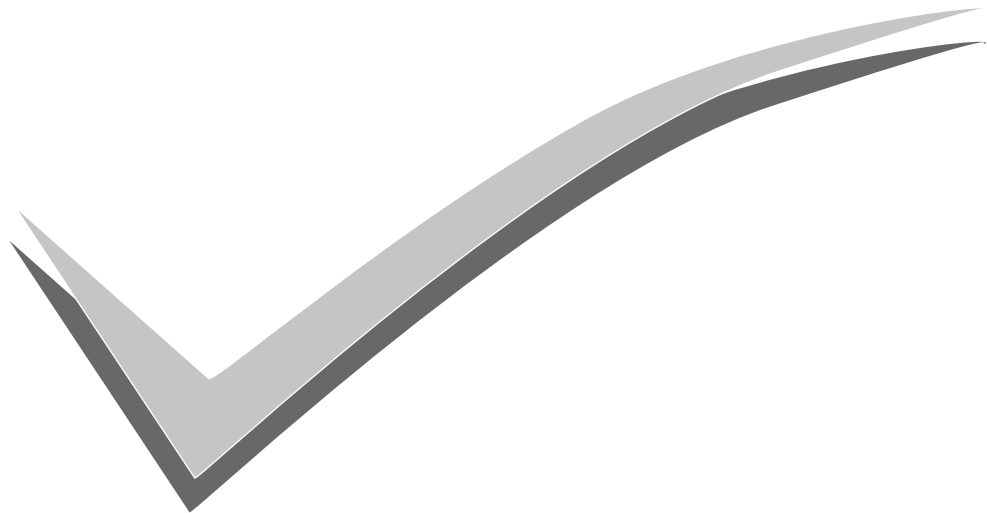




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INDEX		
S. No.	Name of the Publication	Pg Nos
1.	Thesis submitted at Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University	1-46

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Widal antigens for slide and tube tests

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Addis Ababa University
College of Health Sciences
School of Allied Health Sciences



**Diagnostic Performance of Commercially Available Widal Test Kits for the
Diagnosis of Typhoid Fever: The Case of One Public Hospital and a Health
Center in Addis Ababa, Ethiopia**

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A thesis submitted to the Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Clinical Laboratory Science

(Diagnostic and Public health Microbiology specialty track)

November, 2015

Addis Ababa, Ethiopia

Addis Ababa University
College of Health Sciences
School of Allied Health Sciences

Department of Medical Laboratory Sciences
(Diagnostic and Public Health Microbiology Specialty Track)

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Table of Contents

Contents	Page
<i>Approval sheet</i>	II
<i>Acknowledgement</i>	III
<i>List of Tables</i>	VI
<i>List of Abbreviations</i>	VII
<i>Abstract</i>	VIII
1. Introduction.....	1
1.1. Background	1
1.2. Statement of the Problem.....	3
1.3. Literature Review.....	5
1.4. Significance of the Study.....	11
2. Objective of the Study.....	12
2.1.General Objective	12
2.2.Specific Objective	12
3. Materials and Methods.....	13
3.1.Study Design	13
3.2. Study Area	13
3.3. Study Period	14
3.4. Study Population.....	14
3.5. Source Population	14
3.6. Selection Criteria	14
3.6.1. Sampling procedure.....	14
3.6.2. Sample size consideration	14
3.7. Study Variables.....	15
3.7.1. Dependent variables.....	15
3.7.2. Independent variables.....	15
3.8. Measurement and Data Collection	15
3.8.1. Data collection.....	15
3.8.2. Specimen collection and laboratory testing.....	15
3.8.3. Quality control and quality assurance.....	17
3.9. Statistical Analysis.....	18
3.10. Operational Terms	18
3.11. Ethical Clearance	18
4. Result	19
4.1. Study Participants.....	19
4.2. Blood Culture and Widal Test Results.....	20
4.2.1. Blood culture results.....	20
4.2.2. Widal test results	22

5. Discussion	28
6. Conclusions and Recommendations.....	31
6.1. Conclusions	31
6.2. Recommendations.....	32
7. Limitations of the Study.....	33
8. References	34
9. Annexes.....	37

List of Tables

Table 1: Characteristics of study participants from Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015	18
Table 2: Blood culture results of febrile patients suspected of having typhoid fever at Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015.....	19
Table 3: Qualitative slide agglutination reaction results of febrile patients suspected of typhoid fever as tested by the four brands of Widal tests, Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015.....	21
Table 4: Distribution of anti-O & anti-H antibody titers of reactive and weakly reactive samples of febrile patients, Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015.....	22
Table 5.1: Association between quantitative tube titration results and slide agglutination results of Kit 1, Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015.....	23
Table 5.2: Association between quantitative tube titration results and slide agglutination results of Kit 2, Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015.....	23
Table 5.3: Association between quantitative tube titration results and slide agglutination results of Kit 3, Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015.....	24
Table 5.4: Association between quantitative tube titration results and slide agglutination results of Kit 4 Yekatit 12 Hospital and Jan Meda Health center, Addis Ababa, Ethiopia, 2015.....	24
Table 6: Sensitivity and Specificity results of the four Widal test brands, Yekatit 12 Hospital and Jan Meda Health center, Addis Ababa, Ethiopia, 2015.....	24

List of Abbreviations

CDC: Center for Disease Control

DNA: Deoxyribonucleic Acid

EIA: enzyme immunoassay

MDR: Multi Drug Resistant

OMP: outer membrane protein

PCR: polymerase chain reaction

SGOT: serum glutamic oxaloacetic transaminase

SGPT: serum glutamic pyruvic transaminase

SOP: Standard Operating Procedure

QC: Quality Control

WHO: World Health Organization

ABSTRACT

Background: The causative agent of typhoid fever was first isolated in the late 19th century. The Widal agglutination test, utilizes a suspension of killed *Salmonella Typhi* as an antigen, to detect anti-H and anti-O agglutinins in serum from suspected *Salmonella Typhi*-infected patients. Information concerning the utility of Widal test in Ethiopia is limited and studies using multiple Widal test kits are even far less common.

Objective: The objective of this study was to investigate the diagnostic performance of commercially available Widal test kits for the diagnosis of typhoid fever in Ethiopia.

Methods: A cross-sectional study was conducted in two governmental health facilities in Addis Ababa, from October 2014 to February 2015 and a total of 192 typhoid fever suspected patients participated. Descriptive statistical analysis was conducted on the collected dataset for the purpose of data exploratory analysis. Different measures of agreements, between the Widal test kit brands as well as between slide agglutinations and tube titrations were carried out.

Results: Results of the four commercially available Widal test kits under investigation showed plausibly strong test agreement with each other for slide agglutination methods (kappa statistics was $k = 0.967$ for anti-H and $k = 0.786$ for anti-O antibodies). Also in tube titration method, the four brands showed strong agreement ($k = 0.875$ for anti-H and $k = 0.850$ for anti-O antibodies). In contrast, slide agglutination and tube titration agreement results of each brands showed a relatively lower agreement in anti-H ($k < 0.40$) and a slightly better agreement in anti-O agglutinins, ($k: 0.40–0.68$). Among the 190 patient blood samples collected, 4 bacterial isolates were obtained in which, two of them were *S. Typhi*.

Conclusion: In this study, the slide agglutination results of the four brands were generally good and comparable but the tube titration result agreements were lower than the agglutination agreement results. Also, a significance difference between slide agglutination and tube titration results of the four brands was found. The current study indicates that although the Widal test can be used as a good screening method treatment decisions should not be made solely based on results of slide agglutination.

Key words: *Typhoid fever, Salmonella, Widal test kits*

1. INTRODUCTION

1.1. Background

Advances in hygiene and public health issues has led to the reduction of cases of enteric fever, or more commonly known as typhoid fever, in most of the developed countries. And yet, the disease is still a burden and remains endemic in most of these countries where such problems as unsafe drinking water, poor hygienic handling of food and inadequate sanitation exist widely. Typhoid fever is a disease caused by the bacteria *Salmonella enterica* subspecies I serotype *Typhi* (the nomenclature of these organisms is somehow not uniformly agreed on; but the widely accepted and often used short form nomenclature is *Salmonella typhi*). A very similar but often less severe disease is caused by *Salmonella enterica* subspecies I serotype *Paratyphi* A and B or in short form *Salmonella Paratyphi* A and B [1].

Typhoid or enteric fever was first successfully defined by Sir William Jenner in the mid-19th century although most scholars agree that known outbreaks due to typhoid fever can be dated back to the 17th century[2]. Some scholars even claim outbreaks of typhoid fever in Athens happened from 478-460 BC and this speculation might have given an insight to possible ancestral strain of *Salmonella enterica* serovar *Typhi*[3].

Crowded and impoverished populations with inadequate sanitation that are exposed to unsafe water and food with high probability of contamination with feces are at risk of developing the infection. Humans are the natural reservoirs for the *Salmonella typhi*. Studies show that *Salmonella* bacteria can survive for days in groundwater or seawater and for months in contaminated eggs and the infectious dose may vary between 10^3 - 10^6 organisms[4]. Besides the above mentioned risk factors, climatic variables such as, rainfall, vapor pressure and temperature have been shown to have important effects on the transmission/distribution of typhoid infections in human populations[5].

From the first to the third week of illness symptoms may range from dry cough, dull frontal headache, fever that rises to 39-40°C to intestinal perforation and peritonitis[6].

By the end of the third week, and the fourth week, the individual may descend into the typhoid state, which is characterized by apathy, confusion, and even psychosis. Hepatosplenomegaly worsens and intestinal bleeding with secondary bacteremia may develop[7]. Typhoid hepatitis

(increased bilirubin, SGOT and SGPT) and pneumonia has been reported in some complicated infections. Some survivors become asymptomatic *S typhi* carriers and have the potential to transmit the bacteria for a long time[6-8].

According to some studies, the clinical course of a given individual with typhoid fever may deviate from the above description of classic disease. Atypical manifestations of typhoid fever include isolated severe headaches that may mimic meningitis, acute lobar pneumonia, isolated arthralgia (joint pain) urinary symptoms, severe jaundice, or fever alone. Some patients, especially in India and Africa, present primarily with neurologic manifestations such as delirium[9].

Globally, an estimated 12–33 million typhoid fever cases occur and, even if the estimates vary from time to time, 190,000–600,000 deaths per year occur due to typhoid fever [10-13]. According to WHO report and other studies, south-central and south-east Asia are the main regions of the world where typhoid fever is highly prevalent[14]. A recent study in Ethiopia indicated that, the prevalence of typhoid fever cases is high, although coordinated epidemiological surveillance is necessary to know the true burden of the disease[12, 15].

The causative agent of typhoid fever was first isolated in the late 19th century, and the first serological test for the diagnosis of the disease was introduced in 1896 by Fernand Widal[16]. The test is based on the principle of agglutination reaction between *Salmonella enterica* serotype Typhi somatic lipopolysaccharide O and flagellar H antigens and agglutinins produced against them [17]. Even though more than 100 years have passed since its introduction, Widal test is still widely used in developing countries, while its use in most industrialized countries has been terminated[17].

Besides the serological markers, bacterial culture and DNA amplification are among the options that exist for the diagnosis of typhoid fever. The isolation of *S. typhi* from blood or bone marrow is diagnostic of typhoid fever; whereas stool culture is important for monitoring the carriage of *S. typhi* and may also help in the diagnosis. Although culture of bone marrow is considered gold standard, studies show blood culture can give a comparable result if enough blood is cultured and hence, culture of blood is performed more frequently[12, 18].

As mentioned above, several years have passed since the introduction of Widal test and still, the assay is surrounded with controversies and the sensitivity and specificity issues associated with the assay are not still resolved. In developing countries like Ethiopia, Widal test seems to be the most convenient and preferred way to diagnose typhoid fever since culture and molecular techniques are expensive and not widely available. Although the question of cost and ease of performing the test has led to the wide use of the assay in Ethiopia, and other developing countries in general, problems associated with the test started being mentioned in the developed world starting from 1936, and unfortunately, the question of insensitivity and non-specificity of Widal antigens continues until today[18].

1.2. Statement of the Problem

Typhoid fever was a major cause of morbidity and mortality in developed countries, such as the United States and Europe, in the 19th century. However, the incidence of the disease is not at a worrying stage in these regions anymore mainly due to the provision of clean water and good sewage systems, but it remains an important public health problem in developing countries[12]. In Ethiopia, although population-based surveys, established on good microbiological diagnosis are absent, the few existing evidences suggest that typhoid fever is still a pressing public health problem. Thus, knowing the true burden of the disease can ultimately lead to better control measures that can improve the public health[15].

The clinical diagnosis of typhoid fever is often difficult because the presenting signs and symptoms are diverse and similar to those of other common febrile illnesses, such as malaria and dengue fever. One study in Cameroon describes that malaria is the most common cause of fever in patients with symptoms clinically compatible with typhoid fever [19]. A specific diagnosis of typhoid fever requires access to a competent laboratory that can process different specimens. Even though blood culture, bone marrow and stool are the most reliable diagnostic methods, these methods are expensive techniques and are not routinely used in resource-poor countries. Hence, with all its controversies, Widal test is the most commonly and widely used diagnostic assay in most developing and enteric fever endemic areas as it is an easy, inexpensive and relatively noninvasive diagnostic assay.

The Widal agglutination test is commonly performed in two ways; the rapid slide Widal agglutination testing and conventional tube titration Widal testing. Nowadays, rapid semi-quantitative slide test has replaced conventional tube Widal test in many laboratories[20].

Despite its extensive use, the Widal test unfortunately suffers from serious cross-reactivity with other infectious agents, and may produce false-positive results, leading to an over-diagnosis of typhoid fever. Several other diseases caused by non-*Salmonella* organisms (malaria, dengue fever, Miliary tuberculosis, endocarditis, chronic liver disease, brucellosis, etc.) have been shown to exhibit this cross-reactivity in typhoid fever endemic regions, and these cross-reactions increase the error rate of the result of the Widal test[16]. In one study in Riyadh, Saudi Arabia, which was conducted to determine the prevalence of *Salmonella* agglutinins in patients with

other febrile illnesses and healthy blood donors, showed that a significant number of patients suffering from other febrile illnesses, and normal individuals with no history of prior exposure to *Salmonella* either by vaccination or actual *Salmonella* infection had positive Widal tests[21].

Besides the known limitations of the Widal test, other challenges may arise from the lack of proper validation and evaluation of the different Widal test kits available in the market. In typhoid fever endemic countries like Indonesia, comparison of the diagnostic value of the different test brands has shown a significant difference between the different brands available in the country[22]. Results from another study, which was done in Kenya where the burden of the disease is also high, indicated that the test performances of the different serological kits available in the country vary with respect to their sensitivity and specificity[23].

Different brands of Widal test kits are commercially available in Ethiopia but there is lack of records that show whether the diagnostic accuracy of any of the available test kits is properly evaluated and whether the results of different Widal test kit brands agree with each other with respect to their diagnosis results. On top of that, nowadays, rapid slide agglutination technique of Widal testing is becoming the common way of performing the test and tube titration technique of the test is not utilized in most local laboratories. The need to confirm slide agglutination results by tube titration method seemed to be ignored by most laboratories.

In view of providing an insight to the variation of test results of some of the commercially available Widal test kits in our country, and in an intention to compare the results of the semi-quantitative slide agglutination results and tube titration results, this study was undertaken using the different Widal test kits available in Ethiopia.

1.3. Literature Review

Reports that estimate the global burden of typhoid fever indicate the disease is still a public health issue. WHO reported, in its 2000 global typhoid and paratyphoid fever estimate, that there were around 22 million typhoid fever cases and 5 million paratyphoid fever cases[11, 12]. A recent publication that revised typhoid fever incidence data indicated that, in 2010, there were an estimated 27 million typhoid fever episodes globally; and a systematic analysis for Global Burden of Disease Study confirms that, in 2010, there were about 190,000 deaths due to typhoid fever[13, 14].

Asia is the top ranking part of the world where the prevalence of typhoid fever is high compared to the other parts. A prospective population-based surveillance conducted by WHO in five Asian countries result was consistent with previous reviews and showed higher incidences of typhoid fever in India, Indonesia and Pakistan, and relatively lower incidences in Vietnam and China[24]. On the other hand, in Europe, North America, Australia and New Zealand, the incidence of new enteric fever cases has significantly decreased mostly due to the improved water quality and sanitation[4, 11, 25].

Typhoid fever is endemic in most parts of Africa but the continent is considered to have a medium prevalence of the disease even though the burden of typhoid fever is poorly characterized, especially sub-Saharan Africa. Surveillance (2000-2002) supported by WHO ranked *Salmonella Typhi* as the third (8%) most prominent *Salmonella* species isolated in Africa[26]. A study in five Mediterranean North African countries (Morocco, Algeria, Tunisia, Libya, and Egypt) indicated that typhoid fever is indeed endemic in Mediterranean North Africa[27]. Another study which was conducted in Malawi and South Africa also confirmed a similar prevalence of the disease in these two countries [28].

In Sub-Sahara Africa, the epidemiology of typhoid fever is still not clearly understood. A recent review on the issue of typhoid fever in Sub-Sahara Africa stated that epidemiological study is important to have a clear image on the real prevalence of enteric fever[29].

Ethiopia is among the countries in Sub-Saharan Africa where the disease burden is not clearly known. A recent review on this issue has indicated that, even if the epidemiology of

salmonellosis has not been well investigated in the country, there is widespread distribution of *Salmonella* isolates in the community[15].

Several options exist for diagnosing typhoid fever. Some of them are clinical signs and symptoms, serological markers, bacterial culture, antigen detection, and DNA amplification. When monitored using such measures of performance as sensitivity and specificity, none is completely satisfactory and even some of them lack at least moderate performance measure[30, 31].

Bacterial culture is one of the frequently used diagnostic methods and remains the most effective diagnostic procedure in suspected typhoid fever. Blood has been the mainstay of culture for *Salmonella Typhi* since 1900 although, recently, bone marrow culture has been shown to have a more satisfactory result[32]. One study showed that although bone marrow culture is considered gold standard for the diagnosis of enteric fever by some scholars, blood culture can give a similar reliable result if enough blood is cultured and sample is taken prior to the administration of any antibiotic[30]. The other reliable diagnostic tool developed recently is an advanced polymerase chain reaction called nested PCR. A nested PCR makes the detection more sensitive and is able to detect the presence of even 3-5 bacilli[33-35]. Recent studies on this diagnostic tool stated that nested PCR can be of great use in the diagnosis of culture negative typhoid fever cases and can be the next gold standard for the diagnosis of the disease[33, 36].

The availability facilities for microbiological culturing or PCR are often limited in regions in which typhoid is endemic and clinicians are often forced to count on serological results. The recently developed enzyme immunoassay test, i.e., Typhidot test, and the century old Widal test are among this mentioned group[11]. Although further evaluation of the assay in typhoid endemic areas is important, the available studies show that Typhidot test has a promising performance and showed a better sensitivity and specificity than the Widal test[37].

The Widal test has been used very extensively in the serodiagnosis of typhoid fever and, in developing countries, remains the only practical test available. In the original format, the test required acute- and convalescent-phase serum samples taken approximately 10 days apart with a four-fold rise in antibody titer to be considered diagnostic of typhoid fever. More recently, the test has been adapted for use with a single, acute-phase serum sample[38, 39].

One study conducted about a decade ago[40] and another recent study[41] in which both investigated the usefulness of a single Widal test reported the convenience of using a single Widal test in typhoid endemic and resource poor countries. These two studies and others suggest that, although an 'O' and an 'H' agglutinin titer of 1:80 are indicative of typhoid fever, a titer of 1:160 (a fourfold rise) should be considered of greater significance, because of its greater ability to rule out those who do not have the disease (specificity) and higher yield of true positive cases or PPV[40-42].

Recently, tube Widal test is being largely replaced by a quantitative slide test for its rapidity and convenience. However, the semi-quantitative slide agglutination as an alternative to tube Widal test needs to be further evaluated in view of the statistically significant differences with respect to measures of performance (i.e., sensitivity and specificity) observed in the results obtained by the two methods in some studies[43].

Along the years, several studies have evaluated the diagnostic accuracy of the Widal test. In one study that was conducted in typhoid fever endemic regions of India and Vietnam, different serological test assays including Widal test, for the diagnosis of typhoid fever were evaluated and showed relatively varying results[44].

Another study in Iraq that compared Widal agglutination test with ELISA typhi test on 120 patients showed that the specificity and sensitivity of ELISA typhi test were significantly higher than those of the Widal test and the ELISA test is more accurate method for detection *Salmonella Typhi* antibody than the Widal test[45].

In Turkey, one study evaluated the significance of Widal testing in the diagnosis of typhoid fever and indicated that although Widal test is an easy, inexpensive and relatively non-invasive way to diagnose typhoid fever, care must be taken in interpretation of its results as it quite large results of false negatives and false positives[46].

A study was conducted by WHO in two sub-Saharan African sites: South Africa and United Republic of Tanzania which were selected to represent patients from southern and eastern Africa, respectively. The study evaluated three diagnostic kits that are commercially available internationally using four rapid methods for detecting antibodies to *Salmonella Typhi* (typhoid

rapid antibody tests) and used blood culture as the standard for comparison. The result of the study showed that both ways of Widal testing (Semi-quantitative slide agglutination and single-tube Widal tests) performed poorly[47].

Currently several brands of Widal test kit are commercially available. These different brands have varying test performances which, in turn, bring the need of evaluating each of these several brands by carrying out a thorough investigation on each of them from clinical and statistical point of views.

One recent study compared the diagnostic accuracy, in terms of sensitivity and specificity, of four different commercial kits used to perform Widal test (Remel, BioSystems, Dialab and Biotec). The result of the study showed marked discrepancies among the different Widal brands. Regarding anti-O antibodies, Remel brand showed the highest sensitivity and accuracy of 89.50% and 87.91%, respectively, while Biosystem and Dialab brands showed the highest specificity (95.38%, each). Regarding anti-H antibodies, Remel brand also showed the highest sensitivity of 83.58% but with decreased accuracy of 67.03%, while Dialab brand still showing the highest specificity of 91.66%[48].

In Indonesia, one study compared the diagnostic value of local Widal test kit with imported test kit and found a significant difference in sensitivity and specificity between the two[22].

Another study in Kenya that evaluated five Widal test kits available in the country using antisera that is experimentally produced in rabbits, showed varying sensitivity and specificity results in the five test kits. The study also showed antibody titers detected against 'O' and 'H' antigens were different in the five test assays[23].

In Ethiopia, the test performance of the Widal test is not well investigated and for antibody tube titration method, cutoff values are not clearly defined. One study performed in Jimma Town, Southwestern Ethiopia, to determine the baseline antibody tube titration and slide agglutination pattern to Widal antigen and the usefulness of rapid slide agglutination test for diagnostic purposes indicated a cutoff value highly reactive (+4) for rapid slide agglutination and a titer of 1:320 and above for tube titration test be used. It also indicated that a well-defined cutoff value is important if the test continues to be used for clinical purposes[49].

A cross-sectional descriptive type of study was also carried out in Oromia Region using six years surveillance data reported to the zonal health division. The surveillance data from 2007–2012 showed an increasing incidence of typhoid fever cases, the highest being in 2012 (incidence rate of 46 cases per 10,000 residents per year) and the lowest being in 2007 (with the incidence rate of 11 cases per 10,000 residents per year)[50].

Another research conducted in St. Paul's General Specialized Referral Hospital in Addis Ababa compared the Widal test with blood culture in the diagnosis of typhoid fever. The Widal test brand used was Linear Cromotest® (Linear Chemicals, Barcelona, Spain) and the sensitivity, specificity, PPV and NPV of Widal test were found to be 71.4 %, 68.44%, 5.7%, and 98.9% respectively[51].

1.4. Significance of the Study

Widal test has been the main diagnostic tool for Typhoid fever for several decades. In many countries, the test is most widely used because it is relatively affordable, easy to perform and requires minimal training and equipment. Although other serological tests like Typhidot are becoming available in other countries recently, Widal test is still extensively used in many clinical settings here in Ethiopia [11].

Several brands of the Widal test are marketed in our country. It is not entirely known if these brands are properly transported from where they are manufactured and if their test performance is evaluated prior to marketing. There is also lack of records that show whether the test results of different brands agree with each other. This study is performed to see the test performance and agreement of four Widal brands in hope of initiating other researchers and stake holders to do further study on the matter. The results of this study will serve as a baseline to perform a wider range study to compare the different Widal test brands with blood culture and with each other so that the provision of quality health care can be strengthened.

Qualitative Slide agglutination technique of the Widal testing is becoming the most common way of performing the test. This may pose a problem when the slide agglutination result is positive and reported without further processing using tube titration[43]. The management and treatment of Typhoid fever is highly influenced by antibody titration levels[49] and hence, clinicians should be provided with titration results when slide agglutination results are positive. The present study provides information on the agreement of slide agglutination and tube titration methods so that it can help to provide a quality test result for patients and by extension help in preventing unnecessary antimicrobial treatment of patients.

2. OBJECTIVE OF THE STUDY

2.1. General Objective

The objective of this study was to investigate the diagnostic performance of commercially available Widal test kits for the diagnosis of typhoid fever in Ethiopia, with the following specific objectives.

2.2. Specific Objectives

Specific objectives of this study were:

- To investigate diagnostic performance of both slide agglutination and quantitative tube titration results of four of commercially available Widal test kits denoted as
 - Kit 1, Kit 2, Kit 3 and Kit 4
- To compare the results of slide agglutination and tube titration
- To determine the yield of blood culture for the diagnosis of Typhoid fever

Hypothesis

- The different Widal test kits used in this study are expected to show a varying test agreement and slide agglutination and tube titration results of each brand are expected to show a low agreement.

3. MATERIALS AND METHODS

3.1. Study Design

A descriptive type of cross-sectional study was conducted to investigate the diagnostic performance of commercially available Widal test kits for the diagnosis of typhoid fever in Ethiopia by collecting test results from patients recruited in a public hospital and a health center in Addis Ababa city.

3.2. Study Area

The study was conducted in two health facilities found under the Health Bureau of Addis Ababa City Administration. The health facilities were Yekatit 12 Hospital Medical College and JanMeda Health Center.

Yekatit 12 Hospital Medical College is one of the six hospitals under the Addis Ababa City Administration Health Bureau and is located at Arada Sub-city. The Hospital is established by Emperor Haile Selassie I in 1915 making it the second governmental hospital in Addis Ababa's history and, formerly, it was known by the name 'Bete-Sayida'. A medical college was established under the Hospital in 2012. Currently, Yekatit 12 has 522 clinical, 412 supportive and 61 academic staffs providing health and medical services with 272 beds. More than 19 types of services are provided at the facility besides being a referral hospital for burn, ENT (ear, nose, and throat), and neonatal services for cases from all parts of Addis Ababa. Currently, an estimated 600 visits are recorded per day from outpatient and emergency units.

Jan Meda Health Center is a community-based health facility under the Arada Sub-city Health Office and was established in 2012. The Health Center provides primary health services to more than 25,000 residents of the area and, in 2014-2015 fiscal budget year, approximately 17,500 patients visited the health facility. More than a hundred clinical and non-clinical staffs provide service at the Health Center. The nearby Yekatit 12 Hospital laboratory serves as a back-up laboratory service provider for the Health Center. Minilik the II Hospital and the Addis Ababa City Health Bureau regional lab also serve as the immediate referral sites for the Health Center.

3.3. Study Period

The study was conducted from October 2014 to February 2015.

3.4. Source Population

The source population was all patients attending the two health facilities during the time of the study.

3.5. Study Population

Febrile patients with symptoms clinically similar to typhoid fever that have visited one of the two health facilities during the time of the study and who were willing to give consent and blood sample.

3.6. Selection Criteria

Inclusion criteria

- Febrile patients at all ages that were willing to participate in the study
- Febrile patients that had no recent antibiotic treatment for the Typhoid fever or for any other bacterial infections
- Patients or guardians who were willing to give informed consent for the study,

Exclusion criteria

- Individuals that had any obvious signs and/or symptoms of infectious disease other than Typhoid fever

3.6.1. Sampling procedure

All individuals with febrile condition and with symptoms clinically similar to typhoid fever visiting one of the two health facilities during the study period were recruited and convenient sampling method was used.

3.6.2. Sample size consideration

In a period of four months, 120 febrile patients from Yekatit 12 Hospital Medical College and 85 febrile patients from Jan Meda Health Center were included.

3.7. Study Variables

3.7.1. Dependent variables

- Widal test results
- Positive blood culture results

3.7.2. Independent variables

- Age
- Sex
- Duration of fever
- Typhoid fever symptoms

3.8. Measurement and Data Collection

3.8.1. Data collection

Valid and reliable standard questionnaire and consent form was designed and instituted to obtain relevant information and clinical information from participating patients and clinicians. The data was collected by nurses and laboratory technicians/technologists.

The data for the study was derived from culture results, serological testing and questionnaires. Socio demographic data, clinical sign and symptoms, and other relevant data were collected using a standard structured questionnaire prepared for this purpose.

3.8.2. Specimen collection and laboratory testing

Blood sample collection and culturing: The specimens for this study were also collected by nurses and laboratory technicians/technologists. Using a sterile syringe and needle, about 8-10ml of blood from an adult and 2-5 ml from a child was collected. Then 5–8ml of the blood was inoculated into the automated Bactec Plus Aerobic/F culture medium bottle for an adult and the 1-3ml into Bactec Ped Plus/F blood culture medium bottle for a child. Without delay, the blood was mixed with the broth and then transported to the microbiology department at Yekaktit 12 hospital and incubated at 37⁰C in the Bactec automated incubator. Vials entered into the instrument are automatically tested every ten minutes for the duration of the testing period. The Bactec identifies positive blood cultures and signals in red light. The positive samples were taken out and gram staining and subsequent sub culturing was performed. When the gram staining showed gram negative short rod, the sample was sub-cultured on blood agar and MacConky

Agar. Negative cultures were taken out when the Bactec signals negative after 5 days of incubation. Gram staining was performed on both the negative and positive culture bottles as a QC. The remaining 2–3 ml blood from the total collected blood was used for Widal testing.

Sub-culturing and Identification of bacterial isolates: Those culture bottles which showed growth were further sub-cultured on MacConky Agar (Mac) and blood agar media. On Mac, *S. typhi* is non-lactose fermenter and the media shows a characteristic of transparent colorless colonies. On blood agar media *S. typhi* shows smooth, non-hemolytic colonies. Suspected colonies obtained on the above sub-cultured blood agar media were screened by means of biochemical tests. Indole test, Citrate utilization test, motility test, Urease test, KIA, lysine decarboxylation test were among the biochemical tests performed.

Serotype *Typhi* strains were identified as Indole negative, Citrate negative, motile, urease negative, Lysine decarboxylase positive, H₂S positive, non-gas forming, and coagulase positive [46].

Antimicrobial sensitivity tests: The antimicrobial sensitivity pattern of the blood culture isolates was determined using CLSI guideline 2012 version as a guide. For the sensitivity testing, Muller-Hinton media was used. A max of 12 discs on 150mm plate and an inoculum size of 0.5 McFarland were used and the inoculated discs were incubated at 35±2°C in ambient air for 16-18hrs [52].

Widal tests: A single acute phase sample was used to perform the Widal test in this study. All serum samples were first screened by slide agglutination technique and, the reactive samples were further tested by tube agglutination technique using each of the four Widal test kit brands (Chromatest, Fortress, Atlas and Tydal brands) under study. All the tests were carried out according to the manufacturer's instruction.

Slide agglutination technique: Generally, one drop of positive control from the respective Widal test kits was added onto a reaction circle of the glass slide. Physiological saline, 50µl measure, was added onto the next reaction circle of the glass slide. One drop of patient's serum sample to be tested was added onto each of the required number of reaction circles. One drop of appropriate Widal[®] antigen suspension brand to be tested was added to the reaction circles

containing positive control & physiological saline. One drop of appropriate Widal® antigen suspensions was added to the reaction circles containing the patient's serum sample. The contents of each circle were mixed uniformly over the entire circle with separate mixing sticks and rotated gently for one minute. The observation of clumping macroscopically at one minute was considered as positive.

Quantitative tube titration technique: In the tube agglutination test (titration), patient serum sample was diluted from 1:40 to 1:640 for anti-TO and anti-TH separately in 10 test tubes for each Widal test brand. The patient's sera were serially diluted in normal saline, starting by 1/40 to obtain dilutions of 1:40, 1:80, 1:160, 1:320, and 1:640. One ml of each dilution was dispensed in each of 10 tubes making two rows each of 5 tubes. For the first row of tubes a drop of anti-O antibodies (of each of the four used Widal brands) was added, while to the second row of tubes a drop of anti-H antibodies (of each of the four used Widal brands) was added. A tube containing 1 ml saline was included as a negative control. All tubes were mixed, and were incubated in a water bath at 37°C for 16–18hrs. In a positive O reaction, there was a granular agglutination; while H agglutination has a characteristic floccular appearance. In a negative reaction and in the saline the appearance of the suspension should be unchanged, and show a typical swirl when the tube is flicked. The tubes were not shaken.

Again in each test brand, manufacturer's instruction and SOP was followed. A cutoff value of 1:80 for anti-TO and 1:160 for anti-TH was used to consider the titer results as positive or indicative of recent Typhoid fever infection.

3.8.3. Quality control and quality assurance

The questionnaire were prepared in English and Amharic and first revised by two internal medicine specialists and one microbiologist to ensure the necessary screening questions relevant to the study were included. Then the questionnaire was Pre-tested before the main study on patients. The data was collected by trained professionals (nurses and laboratory technicians). No personal identifier was included; patients were given a unique code numbers that were attached on the questionnaire and their laboratory specimens. Daily all the collected data was checked for completeness by the principal investigator.

The laboratory tests were done by strictly following standard operating procedures. Control samples were run before and together with patient samples when performing the Widal test and ATCC strains of *E.coli* and *S. typhi* were cultured prior all the new batches of blood culture bottles opened.

3.9. Statistical Analysis

Statistical analysis software (SAS, version 9.3; SAS Institute Inc., Cary, NC) was used to analyze the data obtained. The Fleiss' kappa statistic was used to calculate result agreements between each brand and Cohen's Kappa statistics was used to see associations between the results of the slide agglutinations and the titers results in both anti-H and anti-O antibodies for each brand.

3.10. Operational Terms

- **Widal Test:** is an agglutination test which detects the presence of serum agglutinins (H and O) in patients serum with typhoid and paratyphoid fever.
- **Febrile patient:** A patient with a temperature on admission of $>37.5^{\circ}\text{C}$, or history of fever of >2 days duration, and no identified cause of fever.
- **Confirmed case of typhoid fever:** A febrile patient with a laboratory confirmed positive blood culture for *Salmonella typhi*.
- **Titer Cut-off value:** The significant titer taken as indicative for the diagnosis of Typhoid fever. The titers of 1:80 (O agglutinins) and 1:160 (H agglutinins) were taken as minimum cut-offs.

3.11. Ethical Clearance

This study was done after getting the necessary ethical clearance from the Ethical Clearance Committee of Addis Ababa University, College of Health Sciences, Department of Medical Laboratory Sciences, research committee and from Addis Ababa City Administration Regional Health Bureau. Information about the study was given to all the study participants and assured about the human subjects' protection, confidentiality and anonymity of data and are only for research proposes. Written informed consent was obtained from voluntary study participants. Clinicians were notified about the results of the tests and any abnormal findings were immediately reported.

4. RESULTS

4.1. Study Participants

A total of 205 patients (from Yekatit 12 Hospital Medical College and Jan Meda Health Center) were recruited at the beginning of the study. Among them, 13 patients were excluded for not qualifying the exclusion-inclusion criteria (nine patients were taking antibiotic during or few days prior to sample collection and four patients had incomplete demographic and other relevant information). A total of 192 patients finally included in the study. Of these patients, 67.7% (130) were female patients. The average age of the patients were about 32 years (mean= 31.7 years, SD= 15.9 years) and their ages range from 6 months to 80 years. Detailed demographic information about the study participants is presented in Table 1. It should be noted that the average ages of female and male patients in the study were closely comparable (mean ages: for females= 32.2 years and for males= 30.7 years). In general, it was observed that 52.6% (101) of the patients were aged between 15 and 35 years and only 7.8% (15) of patients are older than 55 years.

None of the participated patients have taken antibiotic at least before two weeks and above. Demographic results and duration of fever are summarized in the table below.

Table 1: *Characteristics of the study participants from Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015*

Characteristic	Statistics
Age in years: mean (SD)	31.59 (15.89)
Sex	
Female	130 (67.71%)
Male	62 (32.29%)
Fever	
Present	192 (100%)
Duration of Fever	
≤3 days	56 (26.74%)
1 week	92 (47.67%)
2 weeks	29 (16.86%)
≥ 3 weeks	15 (8.72%)
Antibiotic in the last 2 wks	
No	192.00%)

Although the duration of the fever varies among the study participants, all the 192 of them complained of having fever when they came to the health facility. It was observed that 29.2% of the total patients have had fever and symptoms similar to typhoid fever for three days and 7.8% of the total patients have had fever and symptoms similar to typhoid fever for three or more weeks. The remaining 63% of the total patients have had such symptoms for more than three days but less than three weeks.

4.2. Blood Culture and Widal Test Results

4.2.1. Blood culture

A total of 192 patient blood samples were tested by blood culturing for the presence of *S. typhi*. Only two patient samples yielded *S. typhi* and two other patient samples yielded *S. pneumoniae* and *K. pneumoniae*.

Table 2: Blood culture results of febrile patients suspected of having Typhoid fever at Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015

Blood culture results	Frequency (%)
Negative culture results	188 (97.92%)
<i>K. pneumoniae</i>	1 (0.52%)
<i>S. pneumoniae</i>	1 (0.52%)
<i>S. typhi</i>	2 (1.04%)

Antimicrobial sensitivity test results: Antimicrobial sensitivity and resistance pattern of the *salmonella enterica* isolates were performed against 10 antimicrobial agents. Both the two isolates of *S. Typhi* were resistant to Ceftriaxone, Cefotaxime, Trimethoprim- Sulfamethoxazole, Ceftazidime and Tobramycin. Ciprofloxacin was the only antimicrobial the two isolates have responded and were sensitive for.

The other non-salmonella isolates were also tested for antimicrobial sensitivity. The *S. pneumoniae* was tested against Oxacillin, Erythromycin, Clindamycin and Trimethoprim-Sulfamethoxazole. The isolate was resistant to only Oxacillin and was sensitive to the other three drugs. The *Klebsiella Spp.* was tested against six drugs and was resistant to Ampicillin and Trimethoprim- Sulfamethoxazole and was sensitive to the rest four drugs.

4.2.2. Widal test results

Widal test using four different brands, namely (Chromatest (Spain), Fortres (United Kingdom), Atlas (Jordan) and Tydal (India) brands) was performed on all the 192 patient samples. Qualitative slide agglutination test was performed on each patient sample using each of the four brands as a screening test. All the reactive and weakly reactive samples were further tested by tube titration method.

Qualitative slide agglutination Widal test results on the four brands of the Widal test kits for both anti-H and anti-O antigens are presented in Table 3. As it can be seen, most of the tests were detected with non-reactive results in both anti-H and anti-O antigens; and a few tests were detected with weakly reactive tests in both anti-H and anti-O antigens. Most of the patient samples were found to be non-reactive by all the four brands for anti-H antibodies (which were 72.9% for each of the four) and the lowest percentage weakly reactive result was found for Kit 4, anti-H antigen (4.69%).

Four (2.08%) patient samples were reactive only for anti-H agglutinins when tested by kit 1, 2 and 3 and three (1.5%) were reactive when using kit 4. Reactive results only for anti-O agglutinins were found for six (3.12%) patient samples using kit 1, 2 and 4 and for five samples when using kit 3.

We assessed whether the four brands agree each other in their slide agglutination test result in the two antibodies using the Fleiss' Kappa. It was found that there were strong agreements between the results of the four brands in each of the two antibody (for anti-H antibodies, $k= 0.967$ with $p < 0.001$ and for anti-O antibodies, $k= 0.786$ with $p < 0.001$).

Table 3: *Qualitative slide agglutination reaction results of febrile patients suspected of typhoid fever as tested by the four brands of Widal tests, Yekatit 12 Hospital Medical College and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015*

Kit 1(H)	Freq.	%	Kit 1 (O)	Freq.	%
NR¹	140	72.92	NR	123	64.06
WR²	10	5.21	WR	25	13.02
R³	42	21.88	R	44	22.92
Kit 2 (H)	Freq.	%	Kit 2(O)	Freq.	%
NR	140	72.92	NR	126	65.63
WR	10	5.21	WR	22	11.46
R	42	21.88	R	44	22.92
Kit 3 (H)	Freq.	%	Kit 3 (O)	Freq.	%
NR	140	72.92	NR	128	66.67
WR	13	6.77	WR	24	12.5
R	39	20.31	R	40	20.83
Kit 4 (H)	Freq.	%	Kit 4 (O)	Freq.	%
NR	140	72.92	NR	96	50
WR	9	4.69	WR	44	22.92
R	43	22.4	R	52	27.08
¹ NR = Non-reactive, ² WR = Weakly reactive; and ³ R = Reactive					

Next, we evaluated the blood titer results of the reactive and weakly reactive samples in the four brands of the Widal test for the anti-H and anti-O antibodies for the study participants. As next procedure, 52 samples for H and 69 samples for O antibodies using kit 1, 52 samples for H and 66 samples for O antibodies using kit 2, 52 samples for H and 64 samples for O antibodies using kit 3 and 52 samples for H and 96 samples for O antibodies using kit 4 were further processed by tube titration method because they were either weakly reactive or reactive during the slide agglutination technique.

In general, less than 5% of the patients had blood titer results 1:320 in all brands for both anti-H and anti-O antibodies; and for anti-H and anti-O antibodies of kit 3, there wasn't any participant with result 1:320. No kit had a 1:160 and above titer result for anti-H agglutinin only. The patient samples that had 1:80 and above titer for anti-O agglutinin only were four when tested by kit 1, 2 and 4 and three when tested by kit 3. All the titer results can be referred in Table 4 given below.

Table 4: *Distribution of anti-O & anti-H antibody titers of Reactive and weakly reactive samples, Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015*

Kit 1 (H)	Freq.	%	Kit 1 (O)	Freq.	%
1:40	13	25	1:40	31	44.93
1:80	23	44.23	1:80	20	28.98
1:160	15	28.85	1:160	17	24.64
1:320	1	1.92	1:320	1	1.45
Kit 2 (H)	Freq.	%	Kit 2(O)	Freq.	%
1:40	12	23.1	1:40	27	40.91
1:80	25	48.06	1:80	22	33.33
1:160	14	26.92	1:160	14	21.21
1:320	1	1.92	1:320	3	4.55
Kit 3 (H)	Freq.	%	Kit 3 (O)	Freq.	%
1:40	16	30.76	1:20	6	9.37
1:80	24	46.15	1:40	24	37.5
1:160	12	23.1	1:80	23	35.94
			1:160	11	17.2
Kit 4 (H)	Freq.	%	Kit 4 (O)	Freq.	%
1:40	9	17.3	1:40	43	44.81
1:80	25	48.1	1:80	27	28.12
1:160	16	30.76	1:160	23	23.95
1:320	2	3.84	1:320	3	3.12

In all brands, greater than 20% of the slide agglutination positive samples had anti-O titer result of 1:80 and above and more than 12% of anti-H agglutinins had a titer of 1:160 and above. A titer result greater than 1:320 was not obtained using any of the four brands.

Agreements between titer results

We used the Fleiss' kappa measure of agreement statistic to investigate whether the four Widal brands provided us blood test results which are agreeable to each other in both anti-H and anti-O antibodies titers. Without labeling the titer results as 'positive' or 'negative' using the earlier mentioned cutoff values, for the anti-H antibodies; the Fleiss' kappa statistic was estimated to be 0.904 ($p < 0.001$) and for the anti-O antibodies, it was found to be 0.685 ($p < 0.001$). On the other hand, after categorizing the original results into 'positive' and 'negative' using commonly used cutoff values, (i.e., using 1:80 cutoff value for anti-O titer results and 1:160 for anti-H titer results), the Fleiss' kappa statistic was found to be 0.875 ($p < 0.001$) for anti-H antibodies and for

the anti-O antibodies, this statistic was found to be 0.850 ($p < 0.001$). All these results of the Fleiss' kappa measure of agreement give us the same conclusion that, in either of anti-H or anti-O antibodies, there is significantly strong agreement between the titer results of the four brands.

Agreements between slide agglutination and tube titration results

Finally, we investigated whether there are associations between the results of the slide agglutinations and the titers results in both anti-H and anti-O antibodies for each brand using the Cohen's Kappa statistic. The results are presented in Tables 6.1–6.4 below and in each of the eight cases the p-values were found to be less than 0.001. As the table shows, the kappa results of the anti-H antibody tests of all the four brands are less than 0.40 suggesting there is only a poor and fair agreement between slide agglutination and tube titration results. On the other hand, the anti-O antibody test results of all the brands range between 0.40 and 0.68 suggesting some of the brands showed moderate agreement (kit 4 showing the lowest agreement) in their slide agglutination and tube titration results and some of the brands showed a good agreement in their slide agglutination and tube titration results (kit 1 showing the highest agreement). As a result, we concluded that, in each of the brands in either of the H or O antibodies, there was a test agreement that ranged from poor to good when positive slide agglutination results were further processed by tube titration.

Table 5.1: Association between quantitative tube titration results slide agglutination results of Kit 1, Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015

Anti-H antibody (Kit 1)			Anti-O antibody (Kit 1)		
Tube Titration	Slide Agglutination		Tube Titration	Slide Agglutination	
	Positive	Negative		Positive	Negative
Positive	16	0	Positive	33	5
Negative	35	141	Negative	19	135
$k = 0.3933$			$k = 0.6543$		

Table 5.2: Association between quantitative tube titration results and slide agglutination results of Kit 2 Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015

Anti-H antibody(Kit 2)			Anti-O antibody(Kit 2)		
Tube Titration	Slide Agglutination		Tube Titration	Slide Agglutination	
	Positive	Negative		Positive	Negative
Positive	15	0	Positive	39	0
Negative	54	123	Negative	25	128
$k = 0.2625$			$k = 0.6753$		

Table 5.3: Association between quantitative tube titration results and slide agglutination results of Kit 3 Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015

Anti-H antibody (Kit 3)			Anti-O antibody (Kit 3)		
Tube Titration	Slide Agglutination		Tube Titration	Slide Agglutination	
	Positive	Negative		Positive	Negative
Positive	12	0	Positive	34	0
Negative	40	140	Negative	21	137
$k = 0.3043$			$k = 0.6449$		

Table 5.4: Association between quantitative tube titration results and slide agglutination results of Kit 4 Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015

Anti-H antibody (Kit 4)			Anti-O antibody (Kit 4)		
Tube Titration	Slide Agglutination		Tube Titration	Slide Agglutination	
	Positive	Negative		Positive	Negative
Positive	18	0	Positive	53	0
Negative	48	126	Negative	43	96
$k = 0.3298$			$k = 0.5521$		

Although the sensitivity, specificity, positive predictive value and negative predictive value of all the four kits were calculated, the results were inconclusive and inadequate to draw any sort of conclusion since the *S. typhi* isolates and the true positives were only two.

The following table shows the sensitivity and specificity results of the four titers (in the two categories). In all cases, the sensitivity results equal 1.00 for the H-agglutinins and O-agglutinins. On the other hand the specificity results range from 0.73 to 0.93. It can be observed that the specificity results for H-agglutinins are greater than those for O-agglutinins.

Table 6: *Sensitivity and Specificity results of the four Widal Test brands, Yekatit 12 Hospital and Jan Meda Health Center, Addis Ababa, Ethiopia, 2015*

		Kit 1	Kit 2	Kit 3	Kit 4
Anti--H agglutinin	Sensitivity	1.00	1.00	1.00	1.00
	Specificity	0.93	0.93	0.95	0.92
Anti--O agglutinin	Sensitivity	1.00	1.00	1.00	1.00
	Specificity	0.81	0.81	0.83	0.73

5. DISCUSSION

Widal test has been the main diagnostic tool for Typhoid fever for more than a century now. Although the diagnostic reliability of the test kit has been questioned for a long time, it is still widely used in Typhoid fever endemic developing countries [16]. Ethiopia is no different in this regard and the wide use of Widal test for the diagnosis of typhoid fever is a testimony for this.

The present study has used a single Widal testing for practical purposes and treatment decisions. This is supported by Shaidul et al [41] and Kulkarni [40] who evaluated the use of a single Widal testing. It should be noted that both studies have emphasized the need to interpret results cautiously and have a defined cut-off value in the region based on the epidemiology of typhoid fever the specific countries of study.

In this study, each patient serum was tested by four different Widal test brands. The slide agglutination results vary in some degrees and Fleiss's kappa statistics was used to see the diagnosis result agreements between these brands. Overall all the four brands show a significant number of positive results. This finding is strongly comparable with a study done in Sudan by Elseed [53] and a study in Northern Ethiopia, Mekelle by Wasihun et al [54], suggesting the need to explore the reasons for the high positivity rates in the Widal test.

Based on the suggestion by Landis and Koch [55] and later by Sim and Wright [56], The Fleiss's kappa results in our study showed strong agreements of slide agglutination tests results among the four brands in both anti-H and anti-O antibodies. These result are in line with the proposition by Sood et al, that slide agglutination can be used as a good screening method for rapid testing of Typhoid fever [8, 57]. On the other hand several studies argue that there is no question to the need to further perform quantitative titration test for positive slide agglutination results [17, 58, 59].

Quantitative tube titration test was performed on reactive and weakly reactive serum samples. The agreement of titer result of each brand was then compared by Fleiss's kappa. The results were comparable with the results of the study by Elseed and the study concluded that the Widal test has a good negative predictive value which indicates that negative result have a good indication for the absence of the disease [53].

In this study, using Landis and Koch proposal, the kappa agreement can be considered strong especially in anti-H agglutinin and the overall result showed an increased test agreement between brands. When compared to a study performed in Egypt by Baker WM et al, which compared four Widal test brands, the later study showed that marked discrepancies were obtained when comparing four Widal brands at different cut-off values [48]. This difference might be accounted to the larger scale of sample size and method of comparisons they used. Also in this regard, Olopoenia and King stated that the value of Widal test depends upon the standardisation as well as maintenance of the antigens (produced by different manufacturers) to produce consistent results[16].

In many laboratories, the rapid slide agglutination seems to be the routine way of performing the Widal test [51]. Slide agglutination and quantitative tube titration of each reactive sample was also compared in this study. Each reactive serum sample was tested by the four different brands and the results showed an agreement that ranged from poor to moderate. Similar comparison of results obtained by semi-quantitative slide agglutination and quantitative tube titration was done by Lavanya V et al in India [43]. The study concluded that the slide agglutination as an alternative to tube Widal test needs to be further evaluated in view of the statistically significant difference in the results obtained by the two methods in the study.

Further, according to Olopoenia and King, studies show that *S. typhi* O antigen obtained from different manufacturers tested against the same serum, which had previously been shown to be positive by the slide agglutination test, revealed marked variability with the tube agglutination titer[16]. Another retrospective study using five years data from International Clinical Laboratories, Addis Ababa, Ethiopia, indicated that Widal slide test should be used in combination with tube titration test [60].

Similar to the findings of our study, Andualem also reported that there was a moderate agreement between slide agglutination test and tube agglutination titer for O antigen and a fair agreement for H antigen slide agglutination and tube agglutination titer [51].

On the other hand, in contrast to our study, a decreased and a very poor agreement between slide agglutination and tube titration was reported by Wasihun et al in which the agreement was found to be ($\kappa=0.02$ for O) and ($\kappa=0.06$ for H) for both antigens [54].

The variation in agglutination pattern in the four different brands in this study is supported by a study in Jimma Town, Southwest Ethiopia. The study was performed on apparently healthy individuals and it indicated that, almost all the blood tested showed some titer of the antibody reactivity of agglutination slide tests and it recommended that if Widal test is to be used for the clinical use to aid the diagnosis of typhoid fever in adult population, a titer of 1:320 and above for tube titration test be used [49]. Also this and other studies indicate that local base line titer based on the epidemiology of Typhoid fever should be established [61].

A significant number of positive slide agglutination results in each brand were shown to have a tube titer results less than or equal to 1:40 in this study. This result is similar with a study by Gaikwad and Rajrukar which found 209 samples to be negative by tube titration method from 294 slide agglutination positive samples [62]. The increased number of slide agglutination positive results may be attributed to cross reactions with other febrile illnesses like Malaria as seen in studies in India and Bahir Dar, Ethiopia [63].

Although blood culture was performed as a gold standard test in this study, the numbers of *S. typhi* isolates were very low. Based on the two isolates, the sensitivity of all the four brands was found to be 100% with a specificity ranging from 73% to 93% in both anti-H and anti-O agglutinins. A very similar finding was reported by Gaikwad and Rajrukar [62]. But our results are in contrast with several other studies which reported a decreased sensitivity and specificity results of the Widal test [17, 47, 48, 51, 64]. The reason for this could most likely be due to the larger sample size these studies used and the greater yield of blood culture they had and in one of the study, the type of gold standard test it used. It should be noted that the sensitivity and specificity results in the current study cannot be taken to evaluate the test performances of the four brands.

The two blood culture isolates in this study were multi drug resistant. Most of the isolates in Wasihun G. et al study in Mekelle Town were also MDR [54] which may suggests the emergence of MDR species of *S. typhi* and the over prescription of antimicrobials which may be the result of the over diagnosis of Typhoid fever.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

It is known that the definitive diagnosis of typhoid fever relies on the isolation of the causative agent from body fluids like blood and bone marrow and from stool and urine although the sensitivity of these tests may vary with time. Being one of the developing and resource poor countries in the world, the Widal test is the primary means used to diagnose typhoid fever in Ethiopia. The current study indicates that although the Widal test can be used as a good screening method treatment decisions should not be made solely based on results of slide agglutination.

This study also found good agreements between the four different Widal brands. The slide agglutination results of the four brands were generally good and comparable but the tube titration result agreements were lower than the agglutination agreement results. Although this study primarily intended to compare each of the four brands with blood culture, the limited number of isolates was an obstacle. Based on the two isolates the four brands showed 100% sensitivity but the specificities were as low as 73% indicating the need for further study. Hence, more investigation with bigger sample size should be strongly encouraged.

The current study has found a significance difference between slide agglutination and tube titration results of the four brands. The use of tube titration method is mostly neglected in many labs in our country. Although this and several other studies emphasized the need to perform tube titration for slide agglutination positive results to increase the sensitivity and specificity of the Widal test, during this study, it was observed that only slide agglutination is performed routinely at both study areas (health facilities).

6.2. Recommendations

- It is recommended to use blood, stool or urine culture besides using Widal test as a screening test if the facility has the necessary laboratory set up for microbiological testing.
- It is strongly recommended to use tube titration for slide agglutination positive results before reporting results.
- Authorized governmental bodies should perform a coordinated surveillance to know the true burden of Typhoid fever and the use of Widal testing to control the over diagnosis of Typhoid fever and over usage of antimicrobial treatment.
- A defined and standardized local cut-off values for both anti-H and anti-O agglutinins are vital and should be endorsed according to the epidemiology of typhoid fever in our country.

7. LIMITATIONS OF THE STUDY

There are some limitations in this study:

- The primary intention of this study was to compare four of the commercially available Widal test kits with blood culture and evaluate their test performance. Only two isolates of *S. typhi* were obtained by the blood culture which limited our ability to evaluate the test accuracy of the Widal test kits.
- A single acute phase sample was used for Widal testing for practical purposes and treatment decisions.
- The sample size was affected by the availability of blood culture bottles and other resources.
- Tube titration was performed for only reactive and weakly reactive slide agglutination results for practical purposes.
- During clinical selection some recruiting conditions were based solely on the response of the study participants which may have had an effect on the yield of blood culture.
- Blood film was not performed for all patients to rule out malaria and other febrile illness.

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9. ANNEXES

Annex 1: Participant Information Sheet and Consent Forms

Information Sheet

My name is Mastawesha Temtem and I am a postgraduate (M.Sc. program) student in Medical Laboratory Science at Addis Ababa University, College of Health Sciences. I am conducting a research to evaluate the test performance one of the diagnostic tools of typhoid fever called the Widal test and I am evaluating the different brands available in Ethiopia.

If you agree to participate in the study, with your permission, your card number, age, sex and medical history will be obtained from the history that you gave to the clinician. About 5-10 ml of blood will be collected from you or you will allow us to use the sample that you will give for your medical examination. During collection of blood, you may feel some discomfort, but this does not produce serious pain. All the data obtained will be kept strictly confidential by using only code numbers and locking the data, only study personnel will have access to the files. Anonymous testing will be undertaken, that is sample will be coded and positive result will not be identified by names. There will be no costs to you as a result of taking part in this study and you are not asked to pay for the laboratory examination. I will give you the result and if your result is clinically significant, I will refer you to the physician for further diagnosis and treatment. Your participation is purely voluntary, and you cannot participate or you can withdraw any time after you get involved in the study. Participating and not participating has no influence on the service you seek to get.

Consent Form (English Version)

Study Participant's unique code: *(to be filled by data collector)* _____

Study Participant's card number: *(to be filled by data collector)* _____

Participant's response: I am free to decline to be in this study, or to withdraw from it at any point. She promised to give the result without cost. My decision as to whether or not to participate in this study will have no influence on my present or future medical service. My signature below indicates that I agree to participate in this study.

Study participant's signature

date of signature

Signature of Person Obtaining Consent

date of signature

Person to contact

Please direct any questions or problems you may encounter during this study to the principal investigator:

Mastawesha Temtem

Department of Medical Laboratory Sciences, College of Health Sciences

Addis Ababa University

Cell phone: +251- 09 11 83 53 44

Email: masttem@gmail.com

Socio-demographic and clinical information of the patient

1. Card number of the patient_____
2. Age (in years) _____
3. Sex : Male_____ Female_____
4. Does the patient complain of having a fever?
 - A. Yes
 - B. No
5. If the patient has a fever, for how long does He / She had it?
 - A. Three days
 - B. One week
 - C. Two weeks
 - D. More than two weeks
6. Is the preliminary clinical diagnosis of the patient is AFI?
 - A. Yes
 - B. No
7. Have the patient had any recent antibiotic treatment for typhoid fever or any other infectious disease either prescribed by physicians or taken over the counter?
 - A. Yes
 - B. No

በአዲስ አበባ ዩኒቨርሲቲ የላቦራቶሪ ት/ቤት

በጥናቱ ለሚሳተፉ ግለሰቦች የፈቃድ መጠየቂያና መቀበያ ፎርም፡፡

ማስታወሻ ተምትም እባላለሁ፡፡

በአዲስ አበባ ዩኒቨርሲቲ የህክምና ፋኩልቲ የላቦራቶሪ ሳይንስ የማስተርስ ዲግሪ ተማሪ ነኝ፡፡ በአሁኑ ሰዓት ለታይፎይድ በሽታ መመርመሪያነት የሚውለውን እና ዋይዳል ቴስት የሚባለውን የምርመራ ዘዴ ለመገምገም ጥናት እያካሄድኩ ነው፡፡

የጥናቱ ዓላማ

በጥናቱ በኢትዮጵያ ውስጥ በአሁኑ ወቅት በጥቅም ላይ እየዋሉ ያሉ የተለያዩ አይነቶች የዋይዳል ቴስት የምርት አይነቶች ውስጥ አራቱ ይገመገማሉ፡፡ እርስዎ በጥናቱ ለመሳተፍ ፍቃደኛ ከሆኑ በኋላ እድሜዎትን፣ የካርድ ቁጥሮን፣ ጾታዎን ፣ የህመም ምልክቶችን እና ሌሎች መረጃዎችን ከሃኪሞ እንድንወስድ በመፍቀድ ይተባበሩናል፡፡ ከ5 እስከ 10 ሚሊት ደም ይሰጡናል ወይም ለሌላ ምርመራ የሰጡትን ደም እንድንጠቀም ይፈቅዱልናል፤ ደም በሚሰጡበት ሰዓት የተወሰነ የህመም ስሜት ይኖራል፡፡ ሆኖም ግን ምንም ዓይነት የከፋ ጉዳት አይደርስብዎትም፡፡ የሰጡት ማንኛውም መረጃ ሁሉ ሚስጥራዊነቱ የተጠበቀ ነው፡፡ በስም አይገለፅም፤ በጥናቱ ለመሳተፍ ምንም ዓይነት ክፍያ አይከፍሉም፤ ምንም ዓይነት የሚያገኙት ገንዘብም አይኖርም፡፡ ነገር ግን የምርመራ ውጤትዎ አሳሳቢ ውጤት ካለው ወይም የምርመራ ውጤቱ ህክምና የሚያስፈልገው ከሆነ ተጨማሪ ምርመራ እና ህክምና እንዲያገኙ ከባለሙያ ጋር አገናኝታለሁ፡፡ በጥናቱ የሚሳተፉት ፈቃደኛ ከሆኑ ብቻ ነው፡፡ ስለዚህ መሳተፍ ከጀመሩ በኋላ ማቋረጥ ሙሉ መብትዎ ነው፡፡ በጥናቱ መሳተፍ ወይም አለመሳተፍ በሚያገኙት አገልግሎት ላይ ምንም ዓይነት ጥቅምም ሆነ ጉዳት አይኖረውም፡፡

የጥናቱ ተሳታፊ ልዩ የኮድ ቁጥር: (በመረጃ ሰብሳቢ የሚሞላ) _____

የጥናቱ ተሳታፊ የካርድ ቁጥር: (በመረጃ ሰብሳቢ የሚሞላ) _____

የተሳታፊውን ፈቃድ መግለጫ

መሳተፍ፣ አለመሳተፍ፣ ከጀመሩ በኋላ ማቋረጥ እንደሚቻል፣ በጥናቱ መሳተፍ አለመሳተፍ በማግኘቱ አገልግሎት ላይ ምንም አይነት ጥቅምም ሆነ ጉዳት እንደሌለው እና በውጤቱ መሰረት ሀኪም ጋር እንደሚያገናኙኝ በመሳተፊም ምንም አይነት ክፍያ እንደማይሰጠኝ እና እኔም እንደማልከፍል ተገልጾልኛል፡፡

የጥናቱ አላማ ግልፅ ስለሆነልኝ ለመሳተፍ ተስማምቻለሁ፡፡ ፊርማዬንም እንደሚከተለው አስቀምጫለሁ፡፡

የጥናቱ ተሳታፊ ፊርማ _____ ቀን _____

ፍቃደኛነትን የጠየቀ ሰው ፊርማ _____ ቀን _____

ማንኛውም ጥያቄ ካሎዎት

ይህን ጥናት በተመለከተ ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥሙ ድንገተኛ ችግሮች ጥያቄ ወይም አስተያየት ካሎት በሚከተለው አድራሻ ይጠቀሙ፡፡

ማስታወሻ ተምቶም

የሕክምና ላብራቶሪ ሳይንስ ት/ክፍል፣ የጤና ሳይንስ ኮሌጅ፣ አዲስ አበባ ዩኒቨርሲቲ

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የታካሚው የግል መረጃዎች እና የህመም ምልክቶች (በመረጃ ሰብሳቢ ወይም በህኪም የሚሞላ)

የጤና ማዕከሉ ስም _____

1. የታካሚው የካርድ ቁጥር _____

2. የታካሚው እድሜ _____

3. የታካሚው ጾታ፡- ወንድ _____ ሴት _____

4. ታካሚው ትኩሳት አለው?

ሀ) አለው ለ) የለውም

5. ትኩሳት ካለው፡ ከጀመረው ምን ያህል ጊዜ ሆነው?

ሀ) ሶስት ቀናት ለ) አንድ ሳምንት ሐ) ሁለት ሳምንት መ) ከሁለት ሳምንት በላይ

6. ታካሚው ከታይፎይድ በሽታ ጋር የሚመሳሰሉ ወይም የሚጣጣሙ የህመም ምልክቶችን

እንደሚያሳይ በሃኪሙ የመጀመሪያ ምርመራ ተገልጧል?

ሀ) አዎ ተገልጧል ለ) አልተገለጠም

7. ታካሚው ለታይፎይድ ህመምም ሆነ ለሌላ ህመም በሃኪም የታዘዘ ወይም ያልታዘዘ የጸረ

ባክቴርያ መድሃኒት ወስደዋል?

ሀ) አዎ ወስደዋል

ለ) አልወሰዱም

Annex 2: Laboratory Testing Procedures

Materials that will be used for slide test method

- Stop watch, Variable Micropipettes.

Materials that will be used for quantitative tube method

- Timer, test tubes, Pipettes, Physiological saline, Incubator (37°C), Test tube rack.

Materials that will be used for culturing and biochemical tests

- Blood culture Medias like Trypticase soy broth, MacConkey or XLD.
- Wire loop, Pasture pipettes, Incubator
- The necessary biochemical tests, medias for drug sensitivity tests, Gram's stain

Slide agglutination method

Procedure:

- Place one drop of positive control from the respective Widal test kits onto a reaction circle of the glass slide.
- Place 50µl of physiological saline onto the next reaction circle of the glass slide. Add one drop of patient's serum to be tested onto each of the required number of reaction circles.
- Add one drop of appropriate Widal ® antigen suspension to the reaction circles containing Positive control & physiological saline.
- Add one drop of appropriate Widal® antigen suspensions to the reaction circles containing the patient's serum.
- Mix contents of each circle uniformly over the entire circle with separate mixing sticks. Rock the slide gently back and forth, and observe for agglutination macroscopically at one minute.

Quantitative test-tube procedure

Procedure:

- Take appropriate number of sets (as required; one set for each antigen suspension) of 6 test tubes and label them 1 to 6.
- Pipette into tube No. 1 of all sets 1.9 ml of physiological saline. To each of the remaining tubes (2 to 6) add 1 ml of physiological saline.
- To tube No. 1 of all sets add 0.1 ml of serum sample to be tested and mix well. Transfer 1 ml of the diluted serum sample from tube No. 1 to tube No. 2 and mix well. Transfer 1 ml of the diluted serum sample from tube No. 2 to tube No. 3 and mix well. Continue this serial dilution till tube No. 5 in each set.
- Discard 1.0 ml of the diluted serum from tube No.5 of each set. Now the dilutions of the serum sample achieved from tube No. 1 to 5 respectively in each set is as follows: 1:20, 1:40, 1:80, 1:160 and 1: 320.
- Tube No. 6 in all the sets, serves as a saline control. To all the tubes (1 to 6) of each set add one drop of the respective well-mixed WIDAL® antigen suspensions from the reagent vials and mix well.
- Cover and incubate at 37⁰C overnight (approximately 18 hours). Dislodge the sediment button gently and observe for agglutination

Blood culturing

Specimen collection

- Specimens must be collected aseptically following the laboratory's venipuncture protocol.
- It is recommended to collect the blood just prior to administration of antibiotics.
- Blood drawn from an intravenous or intra-arterial catheter is acceptable only if the blood cannot be drawn from a peripheral venipuncture.

Volume:

- The volume of blood is dependent upon the type of blood culture system used. Generally 5 to 10 ml of venous blood will be drawn.

➤ **Collection of specimen:**

1. Remove flip caps from blood culture vials and clean vial tops with an alcohol swab
2. Select the vein and prepare the arm using double application of 70% alcohol starting at the center of the site and swabbing concentrically for 1 min.
3. Allow the venipuncture to dry.
4. Do not touch the venipuncture site after preparation or prior to phlebotomy.
5. Perform venipuncture.
6. Transport directly to laboratory

In the laboratory:

Proceed with incubation and subsequent Subcultures of the bottles as per the manufacturer's instructions for the system involved.

Detection and work-up of positive cultures:

Negative: no growth after 5 to 7 days of incubation. Discard if it fails to yield any growth even after seven days.

Positive: Report organism with corresponding antimicrobial susceptibility. In order to optimize the success of blood cultures each laboratory should establish a standard procedure for performing the test

Identification of bacterial isolates: On MacConkey, *S. typhi* is non-lactose fermenter and the media shows a characteristic of transparent colorless colonies. On blood agar media *S. typhi* shows smooth, non-hemolytic colonies. Suspected colonies obtained on the above sub-cultured blood agar media were screened by means of biochemical tests. Indole test, Citrate utilization test, motility test, Urease test, KIA, lysine decarboxylation test were among the biochemical tests performed. Serotype Typhi strains were identified as Indole negative, Citrate negative, motile, urease negative, Lysine decarboxylase positive, H₂S positive, non-gas forming, and coagulase positive

Antimicrobial sensitivity tests: The antimicrobial sensitivity pattern of the blood culture isolates was determined using Muller-Hinton media. A max of 12 discs on 150 mm plate and an

inoculum size of 0.5 McFarland were used. The inoculated discs were incubated at $35 \pm 2^{\circ}\text{C}$ in ambient air for 16-18 hrs

Annex 3: Declaration

I, the undersigned, declare that this MSc thesis is my original work, has not been presented for a degree in Addis Ababa University or any other universities. I also declare that all sources of materials used for the thesis have been duly acknowledged.

Name of the candidate: Mastawesha Temtem (BSc)

Signature_____

Place: Addis Ababa University School of Medical Laboratory Sciences, Ethiopia

Date of submission_____/_____/_____

This thesis has been submitted with my approval as university advisor.

Name of advisor: Kasu Desta (MSc, PhD)

Signature_____

Place: Addis Ababa University, Department of Medical Laboratory Sciences, Ethiopia

Date of submission_____/_____/_____

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