CHAPTER ONE

1. INTRODUCTON

1.1. Introduction

Treponema pallidum is the causative agent of syphilis, the most common sexually transmitted disease (Parij, 2012). Syphilis is transmitted primarily by sexual intercourse(venereal syphilis) including genital, oral and /or anal contact with or without penetration or during pregnancy from mother to fetus, the spirochete is able to pass through intact mucous membrane or compromised skin (Kent and Eomanelli 2008, Stamm2010). The disease can also be transmitted in vitro (via placenta) blood transfusion accidental inoculation from infectious materials.

Since the syphilis is almost always transmitted bysexual intercourse, it is thus classified as one of the Sexual Transmitted Diseases (STDs) (Abdelbagi *et al*., 2008). Syphilis has acquired a higher potential of morbidity and mortality with the increasing prevalence of HIV infection. If syphilis is rare in developed countries, it is much more common in developing countries where prevalence can reach 25% amongst blood donor, (Tagny *et al*., 2009; Tagny*et al*., 2010). Syphilis is believed to have infected 12 million people in 1999, with greater than 90% of cases in the developing world. It affects between 700,000 and 1.6 million pregnant women a year, resulting in spontaneous abortions, stillbirths and congenital syphilis. InSub-SaharanAfrica, syphilis contributes to approximately 20% of prenatal deaths. Rates are proportionally higher among intravenous drug users, those who are infected with HIV and men who have sex with men. In the United States, rates of syphilis in 2007 were six times greater in men than women, while they were nearly equal in 1997. 2 African Americans accounted for almost half of all cases in 2010, (Tagny *et al*., 2010).

Approximately 10.6 million new cases reported in 2005 and 2008, according to the World Health Organization (WHO, 2012). Primary, secondary, early latent syphilis: benzathine penicillin is first-line and oral azithromycin single dose is second-line (British Association for Sexual Health and HIV, 2008). Azithromycin has been shown to be as effective as benzathine penicillin in treating early syphilis. In case of late latent syphilis: benzathine penicillin is first-line,and in neurosyphilis: procaine penicillin with oral probenecid (Hook *et al*., 2010).

1.2. Rationale

Many previous studies of syphilis were done in sudan, the prevalence of syphilis obtained in study among pregnant women in Tricapital Khartoum state in sudan was 9% reported by Abdelbagi et al (2008) and 2.4% according to WHO(1999).

It is important to screen for syphilis among pregnant women in first trimester, because primary syphilis may be asymptomatic. Because primary syphilis may be asymptomatic; the disease may pass unnoticed. Moreover, there is a risk of disease transmission from mother to her unborn child. Screening and treating mothers for syphilis during pregnancy can prevent adverse pregnancy outcomes associated with maternal infection. This study sought to determine the prevalence of syphilis among pregnant women in Berber Teaching Hospital.

1.3 Objectives

1.3.1. General Objectives:

To detect the anti-*Treponemal* antibodies among pregnant women in Berber Teaching Hospital.

1.3.2. Specific Objectives:

-To detect the anti-*Treponemal* antibodies using Rapid Plasma Reagin test as screening test.

-To confirm the result of rapid syphilis test by using Enzyme Linked Immune Sorbent Assay (ELISA).

-To compare between Enzyme Linked Immune Sorbent Assay (ELISA) and Rapid Plasma Reagin (RPR) in the diagnosis of syphilis among pregnant women.

-To determine risk factors associated with syphilis among pregnant women.

CHAPTER TWO

2.Literature Review

2.1 History of syphilis

In 1831 Ricord has designed a larger study on syphilis and gonorrhea and succeeded to show that the last occurs only after contact with gonorrohea patients, whilst the former only after contact with syphilis patients (Tampa *et al*; 2014). It was not earlier than 1905 that Schaudinn (1871-1906) and Hoffman (1868- 1959) have discovered the etiologic agent of syphilis, whom they have named Spirochaeta pallid, on various syphilis lesions, proving its existence in both fresh and Giemsa coloured specimens. It was them who changed the name of the bacterium subsequently to *Treponema pallidum*. (Tampa *et al*; 2014) In 1906 Landsteiner introduced the use of the dark-field microscopy method for the detection of the spirochete of syphilis. In 1910 the German bacteriologist August Wasserman (1866-1925) came with the first serologic test for syphilis and in 1949 Nelson and Mayer have conceived *Treponema pallidum* immobilization test (TPI) the first specific test for *T.pallidum* (Tampa *et al*; 2014).

2.2 *Treponema pallidum*

2.2.1Structure and Biology *Treponema pallidum*

TheSpirochaeate are large group of spiral, motile bacteria.One family (Spirochaetaceae) of the order Spirochaetales consist of three genera of free-living, large spiral organism. The other family (Treponemataceae) include three genera whose members are human pathogen, Treponema ,Borrelia andLeptospira (*Pope etal;2007*).

The spirochaeate have many structural characteristics, they are long, slender, helically, coiled, and spiral or corkscrew shaped, Gram negative bacilli. *T.pallidum* has an outer sheath or glycosaminoglycan coating inside the sheath is the outer membrane, which contain peptidoglycan and maintain the structural integrity of the organism. End flagella (axial filaments) are the flagella –like organelles in the periplasmic space encased by outer membrane. The end flagella begin at each end of the organisms and wind around it, extending to and overlapping at the midpoint .Inside the endo flagella the inner membrane (cytoplasmic membrane) that provide osmatic stability and covers the

proplasmic cylinder. A series of cytoplasmic tubules (body fibrils) are inside the cell near the inner membrane. Treponemes reproduced by transverse fission. (*Popeetal*; 2007).

These organisms are slender bacteria, 0.2 wide and 5–15 lm long; they feature 10–20 primary windings and move by rotating around their lengthwise axis. The organisms are actively motile, rotating steadily around their end flagella even after attaching to cells by their tapering end. The long axis of the spiral is ordinarily slight but may sometimes bend , so that organisms forms a complete circle for moments as the time , returning then to normal straight position , The spirals are so thin that they are not readily seen unless immune fluorescent stain or dark field illumination is employed (*Jawetezet al* ;2010).

Their small width makes it difficult to render them visible by staining. They can be observed in vivo using dark field microscopy. In-vitro culturing has not yet been achieved (*Kayser*, 2005).

T.pallidum subspecies pallidum hashyaluronic acid in the ground substance of tissue and presumably enhanced the invasiveness of the organism. The protein profiles of *T.pallidum* (all subspecies) are indistinguishable; more than 100 protein antigens have been noted. The end flagella are composed of three core proteins, plus an unrelated sheath protein. Cardiolipin is an important component of treponemal antigen (*Jawetezet al*;2010).

2.3.Transmisstion

Venereal syphilis is a worldwide disease of only humans; there is no animal reservoir (LaFond and Lukehart, 2006). Syphilis is acquired by direct contact, usually sexual, with active primary or secondary lesions. Studies have shown that 16 to 30% of individuals who have had sexual contact with a syphilis-infected person in the preceding 30 days become infected (Peeling *et al.*, 2004). Actual transmission rates may be much higher. Infection also occurs when organisms cross the placenta to infect the foetus in a pregnant woman causing congenital syphilis and this happens particularly during the first two years of infection (LaFond and Lukehart, 2006). *T.pallidum* may also occasionally be transmitted as a blood-borne infection (Peeling and Hook, 2006).

2.4 Epidemiology

Syphilis occurs world wide, and its incidence is increasing .It is one of the leading notifiable disease in the United States .Many cases are believed to go unreported , which limits public health efforts . There has been a marked increase in incidence of the disease in homosexual men in recent years.(*Levinson*, 2014). The wide spread epidemics of syphilis that occurred in Russia in 1990 and more recently in China mostly involved heterosexuals , smaller outbreaks in the United States , Canada and England predominately involved men who have sex with men (MSM)(*Martin et al* ,2009)

2.5 Pathogenesis

T.pallidum initially enters from mucosal or cutaneous lesions of an infected through skin or mucous membranes abraded during sexual activity. The organisms reach local lymph nodes within 30 minutes after skin inoculation.

Viable treponemes attach to cells, after which polymorph nuclear leukocytes accumulate in the area of infection (Musher *et al.*, 1983). Both B and T lymphocytes accumulate in the area. Activated macrophages ingest treponemes. By the time a chancre appears, most patients have evidence of antibody formation to the organism. Although both humeral and cell- mediated immunity to *T.pallidum* have been demonstrated. The treponemes may actually suppress host immunity to facilitate their own survival (Lukehart et*al.*, 1980; Soltain *et al.*, 1978).

2.6 Signs and symptoms

Syphilis can present in one of four different stages: primary, secondary, latent, and tertiary and may also occur congenitally (Stamm, 2010).

2.6.1. Primary syphilis

Primary syphilis is typically acquired by direct sexual contact with the infectious lesions of another person.Approximately 3 to 90 days after the initial exposure (average 21 days) a skin lesion, called a chancre, appears at the point of contact (Kent and Eomanelli, 2008).This is classically (40% of the time) a single, firm, painless, non-itchy skin ulceration with a clean base and sharp borders between 0.3 and 3.0 cm in size. The lesion, however, may take on almost any form (Eccleston *et al.*, 2008). In the classic form, it evolves from a macule to a papule and finally to an erosion or ulcer (Eccleston *et al.*, 2008). Occasionally, multiple lesions may be present (~40%) with multiple lesions more common when coinfected with HIV. Lesions may be painful or tender (30%), and they may occur outside of the genitals (2– 7%). The most common location in women is the cervix (44%), the penis in heterosexual men (99%), and anally and rectally relatively commonly in men who have sex with men (34%) (Eccleston *et al.*, 2008), Lymph node enlargement frequently (80%) occurs around the area of infection, Occurring seven to 10 days after 10 chancre formation (Eccleston *et al.*, 2008). The lesion may persist for three to six weeks without treatment (Kent and Eomanelli, 2008).

2.6.2. SecondarySyphilis

Typical presentation of secondary syphilis with a rash on the palms of the handsReddish papules and nodules over much of the body due to secondary syphilis. Secondary syphilis occurs approximately four to ten weeks after the primary infection (Kent and Eomanelli, 2008). While secondary disease is known for the many different ways it can manifest, symptoms most commonly involve the skin, mucous membranes, and lymph nodes. (Mullooly and Higgins, 2010). There may be a symmetrical, reddish-pink, nonitchy rash on the trunk and extremities, including the palms and soles (Kent and Eomanelli, 2008; Dylewski and Duong, 2007). The rash may become maculopapular or pustular. It may form flat, broad, whitish, wart-like lesions known as condyloma latum on mucous membranes. All of these lesions harbor bacteria and are infectious. Other symptoms may include fever, sore throat, malaise, weight loss, hair loss, and headache, (Kent and Eomanelli, 2008). Rare manifestations include hepatitis, kidney disease, arthritis, periostitis, optic neuritis, uveitis, and interstitial keratitis (Kent and Eomanelli, 2008; Bhatti, 2007). The acute symptoms usually resolve after three to six weeks, however, about 25% of

people may present with a recurrence of secondary symptoms. Many people who present with secondary syphilis (40–85% of women, 20–65% of men) do not report previously having had the classic chancre of primary syphilis (Mullooly and Higgins, 2010).

2.6.3. Latent syphilis

Latent syphilis is defined as having serologic proof of infection without symptoms of disease (Larry and Pickering, 2008). It is further described as either early (less than 1 year after secondary syphilis) or late (more than 1 year after secondary syphilis) in the United States (Bhatti, 2007). The United Kingdom uses a cut-off of two years for early and late latent syphilis (Eccleston *et al.*, 2008). Early latent syphilis may have a relapse of 11 symptoms. Late latent syphilis is asymptomatic, and not as contagious as early latent syphilis, (Bhatti, 2007).

2.6.4. Tertiary syphilis

Tertiary syphilis may occur approximately 3 to 15 years after the initial infection, without treatment, a third of infected people develop tertiary disease(Bhatti,2007) People with tertiary syphilis are not infectious (Kent and Eomanelli, 2008).

2.6.5. Congenital Syphilis

Congenital syphilis is a rare disease in most developed countries; but it remains a severe pregnancy outcome in developing countries (Walker and Walker, 2002). *T. pallidum* can be transmitted from the bloodstream of the infected woman to her developing fetus at any time during pregnancy, although risk of fetal infection is much higher during early maternal syphilis (the first year of infection) than during later stages (Sheffield, *et al.*, 2002). Transmission of syphilis to a fetus depends largely on the duration of the disease in the mother. A long interval between infection and pregnancy results in a benign outcome in the infant (Lindstrand *et al*, 1993). The risk of transmission is 70%– 100% in women with primary or secondary syphilis, 40% with early latent syphilis 12 and 10% in late latent cases. About 40% of pregnancies in women with infectious syphilis result in the death of the fetus (Finelli, *et al.*, 1998). Antibiotic treatment of the mother during the first two trimesters is usually sufficient to prevent negative outcomes, but later treatment or lack of treatment may result in foetal death, foetal damage, or birth of an infected infant

(Mascola, *et al*., 1985). Destructive effects are thought to depend upon the immune response of the foetus and include spontaneous abortion, stillbirth, and premature delivery. Affected infants typically have low weight at birth, and infants with congenital syphilis may be underweight even relative to other infants of the same gestational age (Mascola, *et al*., 1985). Pulmonary haemorrhage, secondary bacterial infection, and severe hepatitis cause death of approximately 4% of *T. pallidum*-infected neonates soon after delivery (LaFond and Lukehart, 2006).

2.7. Laboratory diagnosis

In laboratory diagnosis always there are three important approaches. Specimens can be pus or tissue fluid from lesions to look for spirochetes and blood serum for serological tests (Emerson, 2009).

2.7.1. Direct diagnostic methods include

Darkground microscopy is the traditional method for direct detection of *T.pallidum* in lesion exudates;it can provide rapid results, with identification of the organism by its characteristic morphology and motility. Immunofluorescence using fixed smears of lesion material, or tissue specimens, has several advantages and is of comparable sensitivity, neither technique differentiates between the pathogenic treponemes, the resluts were non-specific.More recently described monoclonal antibodies are more specific (Gillesspie and Hawkey, 2006).

2.7.2. Indirect diagnosis

Is based on serological tests for the detection of antibodies.

2.7.3. Serological tests

Fall into two categories: non Treponemal tests for screening, and Treponemal tests for confirmation (Fears and Pope, 2001).

2.7.4. Non-treponemal tests

These tests involved the use of non treponemal antigens. Extracts of normal mammalian tissue (e.g cardiolipin from beef heart) react with antibodies in serum samples from patients with syphilis. These antibodies, which are a mixture of IgG and IgM, are called regain antibodies. These tests are positive in

most cases of primary syphilis and are almost always positive in secondary syphilis.

The titer of this nonspecific antibodies decrease with effective treatment, in contrast to the specific antibodies, which are positive for life. False positive reactions occur in infections such as leprosy, hepatitis B, and Infectious mononucleosis and in various autoimmune diseases (Edwards, 2000).

2.7.5. Treponemal tests

These tests involve the use of treponemal antigens and therefore are more specific than those described earlier. In these tests, *Treponema pallidum* reacts in immunofluorescence or hem agglutination assays (TPHA) with specific treponemal antibodies in the patient serum. These antibodies arise within 2 to 3 weeks of infection; therefore, the test results are positive in most patients with primary syphilis.

The tests remain positive for life after effective treatment and cannot be used to determine the response to treatment or reinfection. They are more expensive and more difficult to perform than the nonspecific tests and therefore are not used as screening procedure.

Treponema pallidum Particle Agglutination (TPPA) these are very valuable and simple tests using an indirect haemagglutination method with red cells or by gelatin particle together with Venereal Disease Research Laboratory (VDRL), it is probably the best combination for routine use. False positive reaction occurs in up to 2% (Wright and Jones, 2003)

2.7.6. Immunochromatographic based strips (ICT)

The simplest rapid tests for treponemal antibody commercially available. An evaluation of these assays was recently published by the WHO sexually Transmitted Diseases Diagnostics Initiative, the rapid assays are potentially suitable for non-laboratory use in the developing world, and they may also have a role as point of care tests elsewhere (Gillesspie and Hawkey 2006).

2.7.7. Molecular methods

Molecular methods are not commonly used in the detection of *T.pallidum* in a clinical setting but can be considered a complimentary technique to be used in combination with conventional dark – field microscopy or serology . Some of

these molecular methods used for detection of the pathogen include the use of PCR and Real time PCR assays the application of PCR in the detection of *T.pallidum* DNA has the advantage of being a diagnostic method with the ability to characterize strains susceptible to macrolide antibiotics.

The sensitivity of PCR detection assay has been found depending on the specimen types and the stages of the disease (Morshed *et al*., 2007).

2.8. Prevention and Control of Syphilis in Pregnancy

To prevent congenital syphilis, it is imperative to screen for syphilis early in pregnancy; but this remains a challenge. A study found that pregnant women who came for a first antenatal care visit had a median gestational period of 20 weeks (Saloojee, *et al*., 2004). Promotion of earlier attendance at antenatal care is a simple prevention strategy which should be advocated (Romoren and Rahman, 2006). Another study also determined that antenatal screening and treating women during pregnancy can prevent maternal syphilis (French, 2007). Interventional program launched in Nairobi by the Nairobi City Council department of public health in early 1990s, has been subjected to monitoring and evaluation; and shows that the effort lead to the downward trend of syphilis during pregnancy from prevalence of 7.2% in 1993 to 3.4% in 1998, and also changes in health seeking behaviour and improved health care (Temmerman, *et al*., 2000). Likewise, in Shenzhen (China) a screening and interventional program led to high increase in coverage for antennal care clinics; which lead to a cost effective decrease in incidence of congenital cases by half from 43.3 per 100,000 to 22.0 per 100,000 pregnant woman screened in 2002 and 2003 (Cheng et al., 2007).

2.9. Risk Factors for Syphilis

There are several factors that can be associated with syphilis in pregnancy. A Study in Mwanza, Tanzania has shown that the age of 15 years to 34 years is associated with high incidence of syphilis especially in women; also women who had lived away from their community during the past 2 years were significantly at higher risk of syphilis incident and that there was no association between syphilis and polygamy (Todd *et al*., 2001). Another study in Nairobi showed that lower education, being single, having multiple sex partners, a history of STD, a history of preterm delivery, inadequate antenatal care, and HIV infection were risk factors for syphilis infection (Temmerman *et al*., 2000).

Syphilis seropositive women were also more likely to have a history of alcohol use, to be clinically wasted, to have clinical anaemia, and pruritic dermatitis (Temmerman *et al.*, 2000). Another study in Shenzhen (China) found a significant association between unmarried status and lower education attainment as risk factors for syphilis infection (Zhou *et al.*, 2007); and also found a significant association of syphilis infection in pregnant women with the overnight travel of a sex partner during the past year (Zhou *et al.*, 2007). An interventional study in the Shenzhen (China) showed syphilis positive pregnant women to have the adverse pregnancy outcomes like miscarriages, stillbirth and perinatal death (Cheng *et al.*, 2007). A study in Nairobi (Kenya) confirmed risk factors for maternal syphilis infection at delivery and the predictable fact that syphilis positive women more often showed a risky sexual behaviour, had a lower level of education and inadequate antenatal care (Temmerman *et al.*, 2000)

2.10. Treatment

Penicillin is effective in the treatment of all stages of syphilis. A single injection of benzathine penicillin G (2.4 million units) can eradicate *T.pallidum* and cure early (primary and secondary) syphilis .Benzathine penicillin is used because the penicillin is released very slowly, from this depot preparation. *T.pallidum* grows 15 very slowly, which required that the penicillin be present in bactericidal concentration .If the patient is allergic to penicillin; doxycycline can be used but Must be given for prolonged periods to effect a cure. In neurosyphilis, high doses of aqueous penicillin G are administered because benzathine penicillin penetrates poorly into the central nervous system. No resistance to penicillin has been observed. However, strains resistant to azithromycin have emerged. Pregnant women with syphilis should be treated promptly with the type of penicillin used for the stage of their disease. (Workokowski and Berman, 2010)

CHAPTER THREE

3. Materials and Methods

3.1. Study design

This is study a cross-sectional, analytical study

3.2. Study area

The study was conducted in Berber Teaching Hospital in River Nile State

3.3. Study duration

The study was conducted during the 2019, in the period from April to October.

3.4. Study Population

The study population included the pregnant women, attending Berber Teaching Hospital.

3.5. Sample size

Atotal 100 serum samples were collected from Berber Teaching Hospital.

3.6. Inclusion criteria

Pregnant women were included in this study.

3.7. Exclusion criteria

Nonpregnantwomen were excluded in this study.

3.8.Data collection

After explaining the purpose of the study, data were collected from each subject by interviewing questionnaire with informed consent. The data include the demographic information (age and trimester).

3.9. Methods

Hundered serum samples were collected from pregnancy women has different age range from 16-43 year in the department of gynecology and obstetrics of Berber Teaching Hospital to detect seroprevelence of syphilis among pregnant women. All specimens were tested by Rapid Plasma Reagin test (RPR) and then confirmed by enzyme linked immune sorbent assay (ELISA).

3.10. Ethical consideration

Approval to conduct this study was taken from the college research ethical commitlee and them from the Hospital Authority

3.11. Specimen collection and preparation

Using sterile disposable syringes, about 3ml of blood were withdrawn from the anticubital vein under aseptic conditions. The site of collection was disinfected using alcohol. The blood samples were collected in sterileplain containers without any additives, and left to clot at room temperature. Each blood sample was then centrifuged at 1500 rpm for 5 minutes, and each serum was separated in another sterile plain container. Samples were labeled by giving laboratory numbers. Serum samples were collected in another plain sterile container with corresponding laboratory number,and then the samples were transported in an ice bag, from the site of collection to the lab, where they stored, foranalysis. The samples were kept frozen at -20 °Cwithout addition of preservatives, until the time of analysis (not more than 3 months).

3.12. Laboratory examination

All the specimens were tested for syphilis;Rapid Plasma Reagin Test (RPR) and enzyme immune sorbent assay (ELISA).

3.12.1. Rapid Plasma Reagin test (RPR)

The Rapid plasma Reagin (RPR) test is a macroscopic, nontreponemal flocculation card test used to screen for syphilis. The kits used were manufactured by Spin React (Spain). Rapid: Its rapid test that can be done within few minutes.Plasma: The sample is plasma (or serum),Reagin:

The term "reagin" means that this test does not look for antibodies against the actual bacterium, but rather for antibodies against substances released by cells when they are damaged by *Treponema pallidum*.

Principle of the method

The RPR-carbon is non- treponemal slide agglutination test for the qualitative and semiquantitative detection of plasma regains in human serum. Carbon particles coated with a lipid complex are agglutinated when mixed with samples containing regains of the patients affected by syphilis.

Reagents

Antigen used in RPR Test: RPR Antigen contains cardiolipin, lecithin, cholesterol, 10% choline chloride, EDTA, charcoal etc in Buffer. The antigen is prepared from a modified Venereal Disease Research Laboratory (VDRL) antigen suspension containing choline chloride to eliminate the need to heat inactivate serum, ethylenediaminetetraacetic acid (EDTA) to enhance the stability of the suspension, and finely divided charcoal particles as a visualizing agent. Positive Control: Prepared from human serum samples containing antibodies to *Treponema pallidum*(regain). Serum is ready to use(artificial). Bring to room temp before use. (titer more or equal ¼). Control negative: Animal serum preservative. Basic Test

Procedure

The Rapid plasma Reagin (RPR) antigen is mixed with unheated or heated (to inactivate complement) serum or with unheated plasma on a plastic-coated card. If antibodies are present, they combine with the lipid particles of the antigen, causing them to agglutinate. The charcoal particles coagglutinate with the antibodies and show up as black clumps against the white card (macroscopically visible flocculation-type precipitation if the patient's sera contain reagin). Preparation for the RPR assay: Rapid plasma Reagin Antigen Suspension, Positive Control, Negative control and samples were left at room temperature (20–30oC) before use to worm. The reagentswere gently mixed before used.

1. The Rapid plasma Reagin card was labeled according to sample number, being careful not to interfere with the test areas of the card.

2. Disposable serum dispensers or droppers was used, one free-falling drop (0.05 ml) of serum sample was dispending onto a circle on the test card.

3. One free-falling drop of positive control and negative control were also dispending in situ, new dispenser for each sample were used.

4. The sample was smoothly across the circle area using the paddle side of the dispenser as shown by instructor, the same for positive and negative control.

5. After mixing the antigen solution by swirling, one drop of the antigen suspension was added to each sample / control testing area.

6. The card was placed on an automatic rotator and then covered to maintain humidity, then rotated at 100 ± 5 rpm for 8 minutes (7 minutes 50 seconds to 8 minutes 30 seconds). Following rotation, a brief hand rotation and tilting of the card (3–4 times) was performed to aid in differentiating nonreactive from minimally reactive results.

7. Results were immediately read macroscopically in the "wet" state under a high intensity light source.

Interpretation of RPR Test

1. Non-reactive (NR) – smooth suspension, no clumping or slight roughness

2. Reactive (R) – any degree of clumping

3.12.2. Enzyme linked immunosorbent assay ElISA

Determination of ELISA

The reagent was supplied by Biorex, united kingdom, ELISA gen, fourth generation ELISA for in vitro determination of specific antibodies to syphilis in human plasma and serum. Fortress Syphilis ELISA is an in vitro diagnostic kit for the detection of antibodies to T.pallidum in human serum or plasma.

Principle of the Assay

The detection of *anti-TP* antibodies is achieved by antigen sandwich enzyme linked immune sorbent assay, where the micro wells are coated with recombinant *Treponema pallidum* antigens expressed in E. coli. The sample is incubated in the micro wells together with recombinant TP antigens conjugated to HRP. The pre-coated antigens express the same epitopes as hosts. In case of presence of anti-TP in the sample, during incubation the pre-coated and conjugated antigens will be bound to the two variable domains of the antibody and the specific antigen-antibody immune complex is captured on the solid phase. After washing to remove sample and unbound conjugates, chromogen solution containing TMB and urea peroxidase are added into the wells. In presence of the antigen-antibody sandwich complex, the colorless chromogen hydrolyzed by the bound HRP conjugate to a blue colored product, which turn yellow upon addition of the stop solution. This color is then read photo metrically and is directly proportional to the amount of the antibody in the sample. Wells containing samples negative for anti-TP remain colorless.

Procedure

Control

3x negative control and 2x positive controls included with each batch of samples to be tested .and the controls were treated as though they are patient samples.

Conjugate and samples incubation

Twenty microliterof negative control (x3) and positive control (x2) and sample were added into their respective wells. Fifty microliter of conjugateHRPwas added to each well.Then Incubated (covered) at 37oCfor 60 minutes.

Wash The plate was washed 6 times with diluted washing buffer,soaked for about 30 seconds between each cycle. Excess liquid was tapped out.

Substrate incubation

Fifty microliter of the TMBchromogen solution was added to each well then mixed by tapping the plate gently and incubated at 37 for 15 minutes avoiding light. the in the presence of positive sample, the color turned to blue in positive control and anti-TP positive sample wells, due to enzymatic reaction between the chromogen solution and the HRP-conjugate.

Stop color development

Fifty microliter stop solution (H2SO4-0.5M) were added to each well, mixed gently blue colorchanged to yellow in positive control and anti-TP positive sample wells.

Reading:

The plate bottom was carefully wiped and OD was read at 450nm using a plate readerwithin5 minutes after stopping the reaction.

Calculation and Interpretation of results

Assay validation

The OD value of the positive control must be equal to or greater than 0.0800 at 450nm. The OD value of the negative control must be less than 0.100 at 450nm.

Calculation of cut-off value

Cut-off value (C.O.) = MNC+0.18. The mean absorbance value for three negative controls+0.18

Interpretation:

(S= the individual absorbance (OD) of each specimens).

Negative Results (S/C.O.</=1)

samples giving on absorbance greater than or equal to the cut-off value are considered initially reactive, which indicates that anti-TP antibodies have been detected with this anti-TP ELISA kit and there are no serological indications for past infection with TP.

Positive Results (S/C.O.>/=1)

samples giving on absorbance greater than or equal to the cut-off value are considered initially reactive, which indicates that anti-TP antibodies have been detected with this anti-TP ELISA kit.

Borderline (S/C. O=0.9-1.1)

Samples with absorbance to cut-off ratio between 0.9 and 1.10 are considered borderline samples and retesting is recommended.

CHAPTER FOUE

4. RESULTS

Hundred serum blood samples were collected from pregnant women have different age in the department of gynecology and obstetrics of BerberTeaching hospital to detect seroprevalence of syphilis among pregnant women. All specimens were tested by two different methods first by Rapid Plasma Reagin test (RPR) and then confirmed by Enzyme Linked Immune Sorbent Assay (ELISA). The result9% of positive cases with syphilis was detected by ELISA where 10% was detected by RPR.

The age of the patients range: 16-43with amean of amost of participant,were in the age group23-36(table 1):-

Table 1: Distribution of the population according to the age group (n=100)

Age	Frequency	Percent%
16-22	15	15%
23-29	41	41%
30-36	33	34%
37-43	11	10%
Total	100	100%

The majority of the patient, were in the third trimester(Table 2)

Table2: show distribution of population according to trimester:

Trimester	Number	Percent (%)
First	12	12%
Second	26	26%
Third	62	62%

Ten out of hundred women were positive for syphiliswhen tested by RPR(Table3)

Table3: Detection of anti-*Treponema pallidum* antibodies using RPR.

RPR	Frequency	Percent (%)
Positive	10	10%
Negative	90	90%
Total	100	100%

Nine out of hundred patients were positive for syphilis when using ELISA (Table4)

Table4: Detection of anti-*Treponema pallidum* antibodies using ELISA.

ELISA	Frequency	Percent (%)
Positive	9	9%
Negative	91	91%
Total	100	100%

Syphilis was detected in all age group with exception of age 37-43 (Table5) when using ELISA

Table 5: Positive syphilis cases detected by ELISA in different age group(n=100)

Age	Positive	Negative	Total	(%)
16-22	3	12	15	15
23-29	4	37	41	41
30-36	2	31	33	33
37-43	0	11	11	11
Total	9	91	100	100

The diseasewas found in all age when tested by RPR(table6)

Table 6: Positive syphilis casesdetected by RPR in different age group (n=100).

Age	Positive	Negative	Total	(%)
16-22	3	12	15	15
23-29	4	37	41	41
30-36	2	31	33	33
37-43	1	10	11	11
Total	10	90	100	100

The pregnant woman in third trimester showed the higher frequency of syphilis(5/9)

Table 7:Detection of anti-*Treponema pallidum* antibodies in pregnant women according to trimester

Trimester	Positive	Negative	Total	(%)
First	1	11	12	1/12
Second	3	23	26	3/26
Third	5	57	62	5/62
Total	9	91	100	9

Show no significant difference between the two techniques

Table (8): Comparison between detection of u anti-*Treponema pallidum* sings RPR and ELISA

Technique	Positive	Negative	Total
RPR	10/100	90/100	100%
ELISA	9/10	91/100	100%

5 DISCUSSION

5.1 Discussion

This study was conducted to detect the frequency of syphilis among pregnant women in Berber Teaching Hospital of River Nile State in Sudan from April to October 2019. Hundred samples `were tested for syphilis; the age range was 16 to 43.The seroprevalence of syphilis obtained in this study among pregnant woman in Berber Teaching Hospital was (9%)by ELISA which was the gold standard method in this study compare to other study we can find different results. This results, high in comparison to the previous reports by the WHO (1.6%) (WHO, 1999) however, this prevalence was considered nearly similar to the result reported by Kadiga who reported seroprevelence of syphilis among pregnant women in Khartoum Teaching Hospital (7%) (Kadiga, 2012). Percentage of syphilis with in age group 23-29 years (4%) and 16-22 years (3%) respectively was higher more than those in the other ages. These findings were similar to result reported by, (Abdelbagi*etal*; 2008). By employing the two serological techinques in this study, (10%) was positive by (RPR)test, (9%) was positive when confirmed by enzyme linked immunosorbant Assay, (1%) positive by (RPR) test and negative when confirmed byELISA Showed no significant difference between the two techniques.

The RPR result in this study show specificity 98.8%, one of the negative samples by ELISA show positive by RPR and this may be due to different causes. The false positive result by the RPR is related to anti-cardiolipin antibodies produced in other infections eg, narcotic drug abuse, chickenpox, acute malaria and HIV (Cheesbrough, 2003).

Syphilis prevalence data from the rest of Africa has been reported to range from a low of 2.5% in Burkina Faso, 13.7% in North West Ethiopia (Azize *et al*., 1995), 17.4% in Cameroon (WHO, 1999) and a high of 42% in Mozambique (Folgosa *et al*., 1996). It is nearly similar to study done in Khartoum the capital of Sudan by Abdel-bagi *et al*., (2008), reported maternal syphilis to be 9% among the antenatal care attendees.

5.2. Conclusion

The results of this study conclude that:

1. The seroprevalence of syphilis obtained in this study among pregnant women in BerberTeaching Hospital was (9%) by ELISA and (10%) by RPR.
2. For diagnosis of syphilis all results must be confirmed by more specific and sensitive methods like PCR.
3. ELISA is better than RPR in diagnosis of syphilis.
4. Large sample size using advance Technique.

5.3. Recommendations:

1. All pregnant women should be screened to detect syphilis during pregnancy.

2. All the cases should be treated to prevent further complication in mother and transmission of these diseases to new born infants.

3. Health education in rural area is necessary for women in all ages.

4. Provision of many centers for voluntary screening for sexual transmitted disease to detect sexually transmitted diseases before marriage for both male and female.

Referance:

Abdelbagi M. Nagi,Hager A. Wedaat Allah and Omer . M. Khalil (2008) Seroprevalence of syphilis among pregnant women in the Tri-capital, Khartoum, SudanResearch.*Journal of Medical sciences*,**3**(1):48-52.

Azize,B., Fantahun, M., Kidan, K and, K and Haile, T (1995). seroprevallence of syphilis amongst women attending antenatal clinics in a rural hospital in North West Ethiopia. BMC Inf.Dis. ;8:119.

Bhatti M.T (2007). Optic neuropathy from viruses and spirochetes. *Int Ophthalmol Clin***47** (4): 37–66.

Brillman, J.C and R.W. Quenzer, (1998). Infectious Disease in Emergency Medicine, 2 ed. Lippincott-and Raven, 686-92.

British Association for Sexual Health and HIV (2008), Management of syphilis.

Cheesbrough M (2003). District Laboratory Practice in Tropical countries.part 2,Cambridge university press, UK, Pp 218-221.

Cheng J,Zhou H., F C Hong F., Zhang D., Y J Zhang J, P Pan and Y M Cai (2007). Syphilis screening and intervention in 500 000 pregnant women in Shenzhen, the People's Republic of China. *J Sex Transm Inf*, **83**: 347-350

Chiu M.J, Cockerell CJ, Houpt KR et al (1990): Spirochetal infections of the skin. In: Atlas of Infectious Diseases. Mandell GE and Stevens DL (eds) Churchill Livingstone, Philadelphia.

Dylewski Jand Duong M (2007). "The rash of secondary syphilis". Canadian Medical Association Journal176 (1): 33–5

Eccleston, K, Collins, L and Higgins, SP (2008). "Primary syphilis". International journal of STD &AIDS,**19** (3): 145–51

Edwards R (2000). Syphilis in women. Prim Care Update Ob /Gyns; 7 :186-91.

Emerson C.R (2009). Syphilis: A review of diagnosis and treatment. Op Infect Dis,**3**:143-147.

Fairley C.K and Read TR (2012). Vaccination against sexually transmitted infections. *Current Opinion in Infectious Diseases*,**25** (1): 66–72.

Fears M. and Pope V. (2001). Syphilis fast latex agglutination test, a Rapid confirmatory test. Clinical & diag, lab, *immuno* p 841-842 vol 8 No 4

Finelli L., Berman SM., Koumans E., Levine W., (1998). Congenital syphilis.Bull World Health Organ; *sex transm Dis*76 (suppl 2): 126–28.

Folgosa E., Bique O., Gonzalezt C., Hagerstrand I., Bergstrom S., Ljungh A., (1996). Syphilis Seroprevalence among pregnant women and its role as a risk factor for stillbirth in Maputo, Mozambique. Genitourin Med; 72:339-342

French P., (2007) Syphilis. *Biomed J.* 334; 143-147

Gillesspie. S and Hawkey P (2006). Clinical Bacteriology .2nd edition, John Wiley& Sons Ltd,UK, 503.

Hook EW 3rd, Behets F and Van Damme K (2010). A phase III equivalence trial of azithromycin versus benzathine penicillin for.*J Infect Dis*. **201**(11):1729-35.

Jawetesz ,Melnick and adelbergs, (2010) medical microbiology, twentyfifth editions, ch2, pp301.

Kayser,(2005) Medical Microbiology 2005 Thieme All right reserved. Usage subject to terms and condition of license.

Kent, M, and Eomanelli, F. (2008), Reexiaming syphilis: an update on epidemiology clinical manifestation, and management, *Ann pharmacother*, **42**(2):226-36.

Khadiga, A. (2012) Screening of Syphilis Antibodies among pregnant women Using ELISA and ICT, A Dissertation in partial fulfillment for the requirement of post graduate Diploma in Medical Laboratory Science (Microbiology).

LaFond R., and Lukehart S., (2006), Biological Basis for Syphilis. *Clinical Microbiology Reviews.***19**(1): 29–49.

Larry K. and Pickering. ED. (2006), Committee on Infectious Diseases. Red book 2006 Report of the committee on Infectious Diseases (27th ed). Elk Grove Village, IL:American Academy of Pediatrics.pp.631-ch44.

Levinson W (2014). Review of Medical Microbiology and Immunology.13th edition, McGraw Hill education, USA, Pp 444-450.

Lindstrand A, Bergstrom S., Bugalho A., Zanconato G., Helgesson A. M., Hederstedt B (1993). Prevalence of syphilis infection in Mozambican women with second trimester miscarriage and women attending antenatal care in secondtrimester. *Genitouri Med*,**69**:431-3.

LukehartS.A, Baker-zander SA, Seu S (1980): characterization of lymphocyte responsiveness in early experimental syphilis: II Nature of cellular infiltration and Treponema pallidum distribution in testicular lesions. *J Immunol*,**124**:454

Martin IE, W, Yang Y and Tsang RSW (2009). Macrolide resistance and molecular types of Treponema pallidum causing primary syphilis in Shangai, China. Clinical Infectious Disease .; 49: 184-191.

MascolaL., Pelosi R., Blount J. H., Alexander C. E., and W. Cates C. E. Jr., (1985). Congenital Syphilis Revisited. Am. *J. Dis. Child*. **139**:575– 580.P. 29–49 Vol. 19, No. 1.

Morshed M.G, Lee MK, Jorgensen D and Isaac-Renton JL (2007). Molecular methods used in clinical laboratory: prospects and pitfalls.*FEMS Immun.Med.Microb* , **49**:184-191.

Mullooly, **C** and Higgins, SP (2010). "Secondary syphilis: the classical triad of skin rash, mucosal ulceration and lymphadenopathy". International.*journal of STD &AIDS*,**21** (8): 537–45.

Murray, P. R., Baron, E. J., Jorgensen, J. H., Landry, M. L., Pfaller, M. A., & Yolken, R. H. (Eds.). (2003). Manual of Clinical Microbiology (8th ed.). Herdon, VA, United States of America: American Society for Microbiology.

MusherD.M (1990). Biology of Treponema pallidum. In: Sexually Transmitted Diseases (Holmes KK et al, eds.), Second Edition, McGraw-Hill Book Company, New York,

Parija S (2012). Text Book of Microbiology and Immunology. 2nd edition, Elsevier India private limited, India, Pp 371-377.

Peeling R.W and Hook EW (2006). The pathogenesis of syphilis: The great mimicker,revisited. Journal *of pathology*, 208:224-232.

Peeling R.W., Mabey D., Fitzgerald D.W., and Watson-Jones D. (2004) Avoiding HIV and dying of Syphilis. *Lancet***364**: 1561–1563.

Pope, Norris Johnson RE (2007): treponema and other human host associated apirochaeata. In manual of chinical microbiology, 9th ed. Murray RR et al (editors). Asm press,

Romoren M. and Rahman M. (2006). Syphilis screening in the antenatal care: a cross-sectional study from Botswana. *BMC International Health and Human Rights,***6**:8 1186/1472-698X-6-8

Saloojee H., Velaphi S., Goga Y., Afadapa N., Steen R. and Lincetto O. (2004). The prevention and management of congenital syphilis: an overview and recommendations. *Bull World Health Organ*, 82:424-30.

Scheffield S., Sanchez P. J., Morris G., Maberry M., Zeray F., McIntire D. D., and G. D. Wendel, Jr. (2002). Congenital Syphilis After 38 Maternal Treatment for syphilis during pregnancy. Am. *J. Obstet. Gynecol*. **186**:569– 573

Stamm LV(2010). "Global Challenge of Antibiotic-Resistant Treponema pallidum". Antimicrob. *Agents Chemother* ,**54** (2): 583–9

Tagny, CT., Diarra, A., Yahaya, R., Hakizimana, M., Ngussanm A., Mbensa, G., et al.,(2009). Characteristics of blood donors and donated blood in Sub-Saharan Francophone Africa. *Transfusion*, **49** :1592-9. 42.

Tagny, CT., Owusu-Ofori, S., Mbanya, D. and Deneys, V. (2010). The blood donors in Sub –Saharan Africa: *a review*. *Transf* . *Med*. **20**:1-10.

Tampa M, Sarbu I, Matei C, Beuea V and Georgescu S (2014). Brief Historyof Syphilis. Journal of medicine and life. 15(1):4-10.

Temmerman M., Gichangi P., Fonck K., Apers L., Claeys P., Van Renterghem L., Kiragu D., Karanja G., Ndinya-Achola J., Bwayo J. (2000). Effect of a syphilis control programme on pregnancy outcome in Nairobi, Kenya. *J Sex Transm Infect* , **76**:117–21.

Todd J., Munguti K., Grosskurth H., Mngara J., Changalucha J., Mayaud P., Mosha F., Gavyole A., Mabey D. and Hayes R. (2001); Risk factors for active syphilis and TPHA Seroconversion in a rural African population . *J. Sex TransmInf*, **77**:37–45

Walker D., and Walker J., (2002). Forgotten but not gone: the continuing scourge of congenital syphilis . *Lancet Infect Dis* , **2**: 432–36.

WHO.(1999). Treponemal Infection : Technical Reports series 674, Geneva, WHO.

Workowski K. A and Berman S (2010). "Sexually transmitted disease treatment guildelines ," Morbidity and Mortality Weekly Report, **59,**:1-113.

World Health Organization (2012): baseline report on global sexually transmitted infection surveillance

World Health Organization (WHO),(1999) Treponemal Infections: Technical Report series 674, Geneva , WHO

Writght D and Jones (2003). Syphilis . In :Benz E, ed .Oxford University Press ,; 1607-18

Zhou H., Chen X. S., Fu-C. H., Peng P., Fan Y., Yu M.C., Yue-Ping Y., Peeling R. W. and David M. (2007) Risk factors for syphilis infection among pregnant women: results of a case-control study in Shenzhen, China. *J Sex Transm Infect* ,**83**:476–480.

APPENDICES

APENDIX 1

fortress diagnostics

ISO 13485 accredited company

8X6095A

96 TESTS

STORE AT 2-8ºC

FOR IN-VITRO DIAGNOSTICS USE ONLY

Syphilis Treponema pallidum Antibody Elisa

Fortress Syphilis Elisa is an in vitro diagnostic kit for the detection of antibodies to Treponema pallidum in human serum or plasma.

Intended Use:

1. For screening of blood donors.
2. Aid in diagnosis and management of clinical conditions of syphilis.

Principle of the Assay:

The detection of anti-TP antibodies is achieved by antigen sandwich enzyme linked immunosorbent assay, where the microwells are coated with recombinant Treponema pallidum antigens expressed in E.coli. The sample is incubated in the microwells together with recombinant TP antigens conjugated to HRP. The pre coated antigens detect the same epitopes on the HRP conjugate antigens, but are expressed in different hosts. In case of presence of anti-TP in the sample, during incubation the pre-coated and conjugated antigens will be bound to the two variable domains of the antibody and the specific antigen-antibody immunocomplex is captured on the solid phase.

After washing to remove sample and unbound conjugate, chromogen solution containing H2O2 and urea peroxide are added into the wells. In presence of the antigen-antibody sandwich complex the colourless chromogen is hydrolysed by the bound HRP conjugate to a blue coloured product, which turns yellow upon addition of the stop solution. The colour is then read photometrically and is directly proportional to the amount of the antibody in the sample. Wells containing samples negative for anti-TP remain colourless.

Assay principle scheme: Double antigen sandwich ELISA

Ag(s) + Ab(t) + (Ag)tN2 →(Ag)s-Ab(t)-(Ag)tN1

→blue →yellow (+)

Ag(s) +(Ag)tN2 → (Ag)s }– no colo) (-)

Incubation Immobilized Complex Coloring Results

40 min. 15min.

Ag(s)=pre-coated/reconcilant anti-treponema pallidum antibodies

Ab(t)=TP antibodies in sample

(Ag)tN2=HRP conjugated recombinant TP antigens.

Fortress Diagnostics Limited, Unit 2C Antrim Technology Park, Antrim, BT41 1QS (United Kingdom)
Tel: +44 (0) 2894 487676 | Fax: +44 (0) 2894 469933 | Website: www.fortressdiagnostics.com

Kit Contents:

Kit Contents:	Volume
Microwell Plate 96 Tests	1 plate (12x8/8x12 well strips per plate)
Negative Control	1x0.5ml
Positive Control	1x0.6ml
HRP-Conjugate Reagent	1x13 ml
Stock Wash Buffer	1x60ml (Dilute 1 to 20 with distilled water before use. Once diluted, stable for two weeks at 2-8ºC).
Chromogen Solution A	1x7ml (Ready to use and once open, stable for one month at 2-8ºC)
Chromogen Solution B	1x7ml (Ready to use and once open, stable for one month at 2-8ºC)
Stop Solution	1x7ml
Plastic Sealable Bag	1 Unit
Plate Cover	1 Sheet
Package Insert	1 Copy

Additional Materials And Instruments Required But Not Provided:

1. Distilled/ distilled or deionized water.
2. Disposable gloves and timer.
3. Appropriate waste containers for potentially contaminated materials.
4. Disposable V-shaped troughs.
5. Dispensing system and/or pipette single or multichannel).
6. disposable pipette tips.
7. Absorbent tissue or clean towel.
8. Dry incubator or water bath, 37±0.5ºC.
9. Microshaker for dissolving and mixing conjugate with samples.
10. Microwell plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm.
11. Microwell washer/washing system.

Specimen Collection and transportation:

1. **Sample Collection:** Either fresh serum or plasma samples can be used for this assay. Blood collected by venipuncture should be allowed to clot naturally and completely – the serum/plasma must be separated from the cells as early as possible so to avoid hemolysis of the RBC. Care should be taken to ensure that the serum samples are clear and not contaminated by microorganisms. Any visible particulate matters in the samples should be removed by centrifugation at 3000 RPM for at least 20 minutes at room temperature, or by filtration on 0.22µ filters. Plasma samples collected into EDTA, sodium citrate or heparin may be tested, but high lipidemic, icteric, or hemolized samples should not be used as they could give erroneous results in the assay. Do not heat inactivate samples, this can cause sample deterioration.
2. **Transportation and Storage:** Store samples at 2-8ºC. Samples not required for assaying within 3 days should be stored frozen (-20ºC or lower).Multiple freeze-thaw cycles should be avoided. For shipment, samples should be packaged and labelled in accordance with the existing local and international regulations for transport of clinical samples and etiological agents.
4. Do not touch the bottom, exterior of the wells/ fingerprints or scratches may interfere with microwell reading.
7. When reading the results, ensure that the plate bottom is dry and there are no air-bubbles inside the well.
8. Never allow the microplate wells to dry after the washing step, immediately proceed to the next step. Avoid the formation of air-bubbles when adding the reagents.
9. Avoid assay steps interspersed with time interruptions.
10. Assure same working conditions for all wells.
11. Calibrate the pipette frequently to ensure the accuracy of samples/reagents dispensing. Always use different disposal pipette tips for each specimen and reagents, so to avoid cross-contamination. Never pipette solutions by mouth.
12. The use of calibrated pipettes is recommended.
13. Assure that the incubation temperature is 37º inside the incubator.
14. When adding samples, avoid touching the well's bottom with the pipette tip.
15. When reading the results with a plate reader, it is recommended to determine the absorbance at 450nm or at 450nm with reference at 630nm.
16. All specimens from human origin should be considered as potentially infectious.
17. Materials from human origin may have been used in the kit. These materials have been tested with kits with accepted performance and found negative for antibodies to HIV 1&2, HCV, TP and HBsAg. However there is no analytical method that can assure that infectious agents in the specimens or reagents are completely absent, therefore, handle reagents and specimens with extreme caution as if capable of transmitting infectious disease. Strict adherence to OLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.
18. Bovine derived sera may have been used in this kit. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE free-geographical areas.
19. The plate lids, vials, strips and sample containers should be collected and autoclaved for 1hour at 121ºC or treated with 10% sodium hypochlorite for 30minutes to decontaminate before any further steps for disposal.
20. The Stop solution (2M H_2SO_4) is a strong acid. Corrosive. Use it with appropriate care. Wipe up with immediately or wash with water if come into contact with the skin or eyes. Acidic 300 used as a preservative can cause irritation of the skin.
21. The enzymatic activity of the HRP-conjugate might be affected by: dust, reductive chemical and substances like sodium hypochlorite, acids, alkalis etc. Do not perform the assay in the presence of such substances.
22. If using fully automated microplate processing system, during incubation, do not cover the plates with the plate cover. Use the sealing foil of the nanosheets inside the plate after washing, can also be omitted.

Additional Notes:

3. Make sure that all reagents are within the validity indicated on the kit box and are of the same lot. Never use reagents beyond the expiry date stated on reagents labels or on the kit box.
3. **CAUTION - CRITICAL STEP:** Allow the reagents and samples to stabilize at room temperature (18-30ºC) before use. Shake reagents gently before, and return to 2-8ºC immediately after use.
4. Shake the reagent gently before use, and return to the storage temperature immediately after use.
5. Use only sufficient volume of sample as indicated in the procedure steps, failure to do so may cause low sensitivity of the assay.

QUALITY MANAGEMENT SYSTEM
ISO13485:2003 CERTIFIED COMPANY

SYPHILIS ELISA | Revision No : 11 SEPT/16| Page 1 of 2

Assay Procedure:

Step1 Reagents preparation: Allow the reagents and samples to reach room temperature (18-30°C) for at least 15-30minutes. Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37°C until crystals dissolve. Dilute the stock Wash buffer **1 to 20** with distilled or deionized water. Use only clean vessels to dilute the buffer.

Step2 Numbering Wells: Set the strips needed in strip-holder, and number sufficient number of wells including three Negative control **(e.g. B1, C1, D1)**, two Positive control **(e.g. E1, F1)** and one blank **(e.g. A1**, neither samples nor HRP-Conjugate should be added into the Blank well.
If the results are determined by using dual wavelength plate reader, the requirement for use of blank well could be omitted. Use only number of strips required for the test.

Step 3 Adding HRP Conjugate: Add 100ul HRP Conjugate into each well except the Blank well.

Step3 Adding Sample: Add **20µl** of Positive control, Negative control, and specimen into their respective wells. **Upon addition of the sample the HRP conjugate – sample mixture will appear Blue. Use a separate disposable tip for each specimen. Negative control and Positive control to avoid cross-contamination.**

Step4 Incubating: Mix the plate by tapping gently. Cover the plate with the plate cover and incubate for **60 minutes at 37°C.** It is recommended to use thermostat-controlled water bath to assure the temperature stability and humidity during the incubation. If any incubator is used, do not open the door frequently.

Step5 Washing: At the end of the incubation, remove and discard the plate cover. Wash each well **6** times with diluted Wash buffer. Each time, allow the microwells to soak for 30-60 seconds. After the final washing cycle, turn the plate down onto blotting paper or clean towel, and tap it to remove any remaining solution.

Step6 Colouring: Dispense **50µl** of Chromogen A and **50µl** Chromogen B solution into each well including the **Blank**, and mix by tapping the plate gently. Incubate the plate at **37°C for 15minutes avoiding light.** The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in Positive control and anti-TP positive sample wells.

Step7 Stopping Reaction: Using a multichannel pipette or manually, add **50µl** Stop Solution into each well and mix gently. Intense yellow colour develops in Positive control and anti-TP positive sample wells.

Step8 Measuring the Absorbance: Calibrate the plate reader with the Blank well and read the absorbance at **450nm.**If a dual filter instrument is used, set the reference wavelength at **630nm.** Calculate the Cut-off value and evaluate the results **(Note:** read the absorbance within **5** minutes after stopping the reaction).

Interpretation of Results:
Each microplate should be considered separately when calculating and interpreting results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each sample's optical density (OD) value to the Cut-off value (C.O.) of the plate. If the Cut-off reading is based on single filter plate reader, the results should be calculated by subtracting the Blank well OD value from the print report values of samples and controls. In case the reading is based on dual filter plate reader, do not subtract the Blank well OD from the print report values of samples and controls.

1. Calculation of Cut-off Value
Cut-off value (C.O.) = *Nc +0.18

*Nc = the mean absorbance value for three negative controls.
If one of the Negative control values does not meet the Quality control range specifications, it should be discarded and the mean value is calculated again using the remaining two values. If more than one negative control OD value does not meet the Quality control range specifications, the test is invalid and must be repeated.

2. Quality control range:
The test results are valid if the Quality Control criteria are verified. It is recommended that each laboratory must establish appropriate quality control system with quality control material similar to or identical with the patient sample being analyzed

1. The OD value of the Blank well, which contains only Chromogens and Stop solution, is less than 0.080 at 450 nm.
2. The OD value of the Positive control must be equal to or greater than 0.800 at 450/630nm, or at 450nm after blanking.
3. The OD value of the Negative control must be less than 0.100 at 450/630nm or at 450nm after blanking.

3. Interpretations of the results:
(I = the individual absorbance (OD) of each specimen)
Negative Results (I/C.O. <1): samples giving an absorbance less than the Cut-off value are considered negative, which indicates that no anti-TP antibodies have been detected with this anti-TP ELISA kit, and there are no serological indications for past infection with TP.

Positive Results (I/C.O.≥1): samples giving an absorbance greater than or equal to the Cut-off value are considered initially reactive, which indicates that anti-TP antibodies have been detected with this anti-TP ELISA kit. Any initially reactive samples should be retested in duplicates. Repeatedly reactive samples could be considered positive for antibodies to anti-TP , therefore there are serological indications for current or past infection with TP. Any blood unit containing antibodies to Treponema pallidum should be immediately discarded.
Borderline (I/C.O=0.9-1.1): Samples with absorbance to Cut-off ratio between 0.9 and 1.10 are considered borderline samples and retesting is recommended. Repeatedly positive samples can be considered positive for anti-TP antibodies.

Fortress anti-TP Elisa Performance:

Clinical Performance: The clinical performance of this assay have been evaluated by a panel of samples obtained from 3400 healthy blood donors from 3 blood banks and by a panel of samples from 192 Syphilis positive patients. The evaluation results are given below.

	*	Confirmed positive	Specificity	False Positives
Donors	3392	8	3	99.91%	3
3400					

Analytical Specificity:
1. No cross reactivity observed with samples from patients infected with HAV, HCV, HIV, CMV, HBV and HTLV.
2. No interference from rheumatoid factors up to 2000IU/ml observed.
3. No high dose hook effect during clinical testing.
4. The assay performance characteristics are unaffected from elevated concentrations of bilirubin, haemoglobin and triglycerides.

REPRODUCIBILITY		WITHIN RUN		BETWEEN RUN	
SPECIMEN	N	MEAN S/CO	CV	MEAN S/CO	CV
WEAK +VE	10	3.35	8.4%	3.20	9.0%
MODERATE +VE	10	6.75	7.0%	6.40	7.5%
STRONG +VE	10	10.90	4.2%	10.30	4.4%

Limitations:
1. Non-repeatable positive result may occur due to the general biological and biochemical characteristics of ELISA assays. The test is design to achieve very high performance characteristics of sensitivity and specificity. However, in very rare cases some HBV mutants or subtypes can remain undetectable. Antigens may be undetectable during the early stages of the disease and in some immunosuppressed individuals.
2. If, after retesting of the initially reactive samples, the assay results are negative , these samples should be considered as non-repeatable (false positive) and interpreted as negative. As with many very sensitive ELISA assays, false positive results can occur due to the several reasons, most of which are related but not limited to inadequate washing step.
3. Any positive results must be interpreted in conjunction with patient clinical information and other laboratory testing results.
4. Common sources for mistakes: kits beyond the expiry date, bad washing procedures, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
5. The prevalence of the marker will affect the assay's predictive values.
6. This kit is intended ONLY for testing of individual serum or plasma samples. Do not use it for testing of cadaver samples, saliva, urine or other body fluids, or pooled (mixed) blood.
7. This is a qualitative assay and the results cannot be use to measure antigens concentrations.

Indications of Instability or deterioration of Reagents:
1. Values of the Positive or Negative controls ,which are out of the indicated Quality control range, are indicator of possible deterioration of the reagents and/or operator or equipment errors. In such case, the results should be considered as invalid and the samples must be retested. In case of constant erroneous results classified as due to deterioration or instability of the reagents, immediately substitute the reagents with new ones.
2. If after mixing of the Chromogen A and B solutions into the wells, the, the color of the mixture turns blue within few minutes, do not continue carrying out the testing and replace the reagents with fresh ones.

Reference:

1. Fraser C.M, et al. Complete genome sequence of Treponema pallidum, the syphilis spirochete. Science 1998; 281:375.
2. Holmes KK, Iemon SM, Mardh P, Piot P, Sparling PF, Stamm WE, Wasserheit JM, Weisner PF, Chapters 33-36. In Sexually transmitted diseases, 2nd ed. New York: McGraw-Hill, 1999.

Appendix 2

A-ELISA reading

B- ELISA washer