Seroprevalence and comparison between methods of Syphilis detection among Blood Donors in Khartoum State/Sudan

الأنتشار والمقارنة بين طرق كشف مرض الزهرى لدى المتبرعين بالدم في ولاية الخرطوم-السودان

A dissertation Submitted in Partial Fulfillment for the Requirements of M.Sc. in Medical Laboratory Sciences (Microbiology)

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Dedication

To my parents

To my brothers and sisters

To my friends

To my teachers
Acknowledgements

My deep thanks to AllAH, my gratitude to my supervisor Prof. Yousif Fadlallah Hamed Elnil who mad this study possible by his advices and effort. My appreciations to all working staff of Central Blood Bank of Khartoum State for giving me the permission and their cooperation to do this study. My deep thanks to all blood donors specially those whose samples were collected, also special thanks to all my teachers. Finally my great thanks to anyone who helped me in this study.
Abstract

This study was conducted among blood donors in Central Blood Bank of Khartoum. On a total of ninety tow samples were collected, during the period from March to May 2018, to demonstrate the frequency of syphilis and to compare between different diagnostic methods for detection of syphilis. All samples were tested from ELISA, ICT and RPR. The seroprevalence of syphilis revealed from ELISA method was 7 (7.6 %), which was equal to ICT but was slightly increase in RPR 8 (8.7%). The high frequency of syphilis 3 (42.9%) was found in both age group of (26-35 and 36-45). There was no positive cases between the ages of (46-55) years. 5 (71.4%) of positive samples were seen among single blood donors, 2 (28.6%) among married donors and no positive cases among divorced blood donors. ICT method showed 100% sensitivity and 100% specificity. RPR showed 100% sensitivity and 98.8% specificity. Results showed that the ICT used in this study was the same when compared with ELISA but the RPR showed less specificity than ELISA.

The study concluded that ICT with ELISA were the best methods for screening blood donors.
ملخص الدراسة

اجريت هذه الدراسة على م平坦عي الدم بولاية الخرطوم (بنك الدم المركزي). في الفترة ما بين مارس الي مايو 2018 في 92 عينة دم, وذلك لتحديد مدى انتشار مرض الزهري وسط هذه الفئة وللمقارنة بين عدة اختبارات لفحص مرض الزهري. كل العينات تم فحصها بواسطة تقنية الكروماتوقرفي الممنع وتقنية الاليزا وتقنية الكشف عن المستضد رياجين .

كانت النتائج كالتالي. (7.6%) كان لديهم المستضد بواسطة فحص الاليزا وقد أعطت تقنية الكروماتوقرفي الممنع نفس النتائج بينما كانت هناك زيادة قليلة في نسبة وجود المرض عند الفحص بواسطة تقنية الكشف عن المستضد رياجين(8.7%). وكان انتشار المرض اكبر في الفئتين العمرية (35-26)/(36-45) بنسبة (42.9%) في كل فئة. بينما نجد انعدام تواجد المرض وسط الفئة العمرية (46-55) ، وأيضا كانت نسبة انتشار المرض بين الغير متزوجين(71.4%) تفوق نسبة انتشاره بين المتزوجين (28.6%) , ولا توجد حالات وسط المطلقين.

أعطت تقنية الكروماتوقرفي الممنع حساسية وخصوصية بنسبة 100% بينما أعطت تقنية الكشف عن المستضد الرياجين حساسية 100% وخصوصية 98.8% .

أثبتت هذه الدراسة ان تقنية الكروماتوقرفي الممنع المستخدمة في هذه الدراسة لها نفس النتائج مقارنة بتقنية الاليزا بينما أعطت تقنية الكشف عن المستضد خصوصية اقل مقارنة بتقنية الاليزا. اثبتت هذه الدراسة ان تقنية الكروماتوقرفي الممنع والأليزا هي الطرق الأفضل لفحص المتبرعين بالدم.
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CHAPTER ONE
INTRODUCTION
1. Introduction

1.1. Background

*Treponema pallidum* is the causative agent of syphilis, the most common sexually transmitted disease (Parij, 2012). Syphilis, or lues, is a chronic venereal infection caused by the spirochete *Treponema pallidum*. First recognized in epidemic form in sixteenth-century Europe as the great pox, syphilis has remained an endemic infection in all parts of the world. Although penicillin and public health programs resulted in a gratifying reduction in cases of syphilis from the late 1940 until the late 1970, a significant resurgence of cases of both primary and secondary syphilis has been documented over the past 2 decades. There is a strong racial disparity, with African Americans affected 30 times more than whites in United States (Kumar et al; 2003).

World health organization recommends that all blood donations be screened for evidence of infection prior to the release of the blood and its components for clinical or manufacturing use. Screening of all blood donations should be mandatory for human immune deficiency virus HIV, hepatitis B and C and syphilis (WHO, 2017).

It is the responsibility of governments to assure a safe and sufficient supply of blood and blood products for all patients requiring transfusion. Each country should formulate a national blood policy and plan, as part of the national health policy, to define how safe blood and blood products will be made available and accessible to address the transfusion needs of its population, including how blood transfusion services will be organized and managed (WHO, 2009).

The provision of safe and efficacious blood and blood components for transfusion or manufacturing use involves a number of processes, from the selection of blood donors and the collection, processing and testing of blood donations to the testing
of patient samples, the issue of compatible blood and its administration to the patient. There is a risk of error in each process in this “transfusion chain” and a failure at any of these stages can have serious implications for the recipients of blood and blood products. Thus, while blood transfusion can be life-saving, there are associated risks, particularly the transmission of blood borne infections (WHO, 2009).

*T. pallidum* may be found in the blood stream, but levels are variable, and bacteremia is often short lived even in recent contamination. Moreover, the treponemes are relatively fragile and sensitive to cold, storage below +20°C for more than 72 hours destroys the organism and reduces dramatically the infectious risk. Although clearly potentially infectious, the risk of transmission through the transfusion of blood and blood components stored below +20°C is very low (Tagny, 2011).

Serological tests for syphilis contributed greatly to the detection of *T. pallidum* infection in blood donors and especially in those who were not identified during the medical selection. (Tagny, 2011).

Although it had some false positive results, it was a major advancement in the prevention of syphilis because it helped to diagnose the disease before the clinical manifestation and thus prevent its spread (Tagny, 2011).

Several labs tests, treponemic or not treponemic exist, among which rapid tests, immunological tests, and genomic. The laboratory assessment of syphilis is generally based on the detection of antibodies against *T. pallidum* antigens in blood by the use of either specific or nonspecific reagents. The detection of genomic particle are more specific but not affordable for most of laboratories (Tagny, 2011).
1.2. Rationale

Syphilis is a transfusion transmitted infection (TTI) due to a *spirocheta* called *Treponema pallidum*. The germ is present in the blood of a contaminated blood donor and infects the recipient. The transmissibility of syphilis by blood transfusion has been frequently reported, chiefly based on animal experiments. Cases of syphilis transmitted by blood have been described in literature, with more than a hundred cases since the first description. The main cases reported were shown to occur when donors were in the primary or secondary stage of the disease (Tagny, 2011).

Developing countries are characterized by a difficult epidemiologic, sociological and economic environment which limits the implementation of a high quality of blood safety. Thus, this context requires that tests and algorithms should be selected so that they correspond with the high prevalence of the disease, limited technical know how of the personnel and limited availability of reagents and equipments. The selection criteria of screening strategy must include simple techniques, reliability, sustainability and cost effectiveness. Several blood banks use rapid test technique as it does not required sophisticated lab materials (Tagny, 2011).

Sudan is one of the developing countries, data from this study could be helpful in detection of the syphilis among healthy blood donors in Central Blood Bank in Sudan in addition to compare between different methods (Enzyme Linked immunosorbent assay ELISA, Immunochromatographic test ICT and Rapid plasma regain RPR for the diagnosis of syphilis.
1.3. Objectives

1.3.1. General objective

To study Seroprevalence and comparison between methods of Syphilis detection among Blood Donors in Khartoum State/Sudan.

1.3.2. Specific objectives

A- To determine the frequency of syphilis (*Treponema pallidum*) among blood donors in Central Blood Bank (Khartoum State).

B- To compare between different methods for detection of syphilis (*Treponema pallidum*) in the study area.
CHAPTER TWO
LITERATURE REVIEW
2. LITERATURE REVIEW

2.1. Historical background

In 1831 Ricord has designed a larger study on syphilis and gonorrhea and succeeded to show that the last occurs only after contact with gonorrhea 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 Previous Study in Sudan and other different countries

Study about prevalence of syphilis in pregnancy in Addis Ababa was conducted. A total of 410 pregnant women were included in the study, twelve tested positive for syphilis using VDRL giving a seroprevalence of 2.9% (Kebede E and Chamiso B, 2000).

Across-sectional study about syphilis among pregnant women in antenatal clinic of Juba Teaching Hospital, Malakaia National Health Insurance Center and Munuki Primary Health Care Centre in 2010 in Juba Southern Sudan, 231 pregnant women participants, 51 (22.1%) were positive for syphilis with the rapid
plasma regain test and 79(34.2%) were positive with the *Treponema pallidum* heamagglutination assay. In conclusion there is a high prevalence of syphilis in pregnant women attending the selected health facilities. *Treponema pallidum* haemagglutination assay can be used as a field test for syphilis due to its high sensitivity and specificity (Emmanuel, 2010).

In Africa, syphilis prevalence rates amongst pregnant women varies from 2.5% in Burkina Faso to 17.4% in Cameroon (WHO 2001).

A study about safety of blood transfusion was conducted in Egypt covering the period between 2006 and 2012, of 308762 donors, the overall prevalence of HCV antibodies HBs Ag, human immunodeficiency virus (HIV) and syphilis antibodies were 4.3%, 1.22%, 0.07%, and 0.13%, respectively (Omram *et al*; 2012).

The rate of primary and secondary syphilis reported in the United States decreased during the 1990, in 2000 the rate was the lowest since reporting began in 1941, although the rate of primary and secondary syphilis in the United States declined 89.7% during 1990-2000, the rate increased annually during 2001-2009 before decreasing in 2010 and remaining unchanged during 2011. During 2012, rates again increased (Braxton *et al*; 2012).

### 2.3. *Treponema pallidum*

The spirochetes are a large, heterogeneous group of spiral motile bacteria. One family (*Spirochaetaceae*) of the order *Spirochaetales* consists of two genera whose members are human pathogens, *Borrelia* and *Treponema*. The other family (*Leptospiraceae*) include one genus of medical importance (*Leptospiraceae*) (Carroll *et al*; 2016).
2.3.1. Typical organisms

*T. Pallidum* are slender spirals measuring about 0.2 um in width and 5-15 um in length. The spiral coils are regularly spaced at a distance of 1 um from one another. The organisms are actively motile, rotating steadily around their endoflagella even after attaching to cells by their tapered ends. The long axis of the spiral is ordinarily straight but may sometimes bend so that the organism forms a complete circle for moments at a time, returning then to its normal straight position (Carroll *et al*; 2016). The spirals are so thin that they are not readily seen unless immunofluorescent stain or dark-field illumination is used. They do not stain well with aniline dyes, but they can be seen in tissues when stained by silver impregnation method (Carroll *et al*, 2016).

Pathogenic *T. pallidum* has never been cultured continuously on artificial media, infertile eggs, or in the presence of reducing substances, *T. pallidum* may remain motile for 3-6 days at 25°C. In whole blood or plasma stored at 4°C, organisms remain viable for at least 24 hours, which is of potential importance in blood transfusion (Carroll *et al*; 2016).

2.3.2. Reaction to physical and chemical agents

Drying kills the spirochete rapidly, as dose elevation of the temperature. Treponemes are rapidly immobilized and killed by trivalent arsenical, mercury, and bismuth (contained in drugs of historical interest in the treatment of syphilis). Penicillin is treponemicidal in minute concentrations, but the rate of killing is slow, presumably because of the metabolic inactivity and slow multiplication rate of *T. pallidum* (estimated division time is 30 hours). Resistance to penicillin has not been demonstrated in syphilis (Carrol *et al*; 2016).
*T. pallidum* is a very delicate bacterium. It is readily killed by drying or heating at 41–42°C for 60 minutes, at 0–4°C for 1–3 days. They are also readily killed on contact with distilled water, soap, arsenic compounds, and common antiseptics. *T. pallidum* strains can be preserved for laboratory use by many methods. It can be stored frozen in a medium containing 5% glycerol at 70°C or in liquid nitrogen (Parija, 2012).

### 2.3.3. Genome

The *T. pallidum* genome is a circular chromosome of approximately 1,138,000 base pairs, which is small for bacteria. Most pathogenic bacteria have transposable elements, but *T. pallidum* does not, which suggests that the genome is highly conserved and may explain its continued susceptibility to penicillin. There are few genes involved in energy production and synthesis of nutrients, indicating that *T. pallidum* obtains these from the host (Carrol et al; 2016).

### 2.3.4. Antigenic structure

The fact that *T. pallidum* cannot be cultured in vitro has markedly limited the characterization of its antigens. The outer membrane surrounds the periplasmic space and the peptidoglycan-cytoplasmic membrane complex. Membrane proteins are present that contain covalently bound lipids at their amino terminals. The lipids appear to anchor the proteins to the cytoplasmic or outer membranes and keep the proteins inaccessible to antibodies. The endoflagella are in the periplasmic space. *T. pallidum* has hyaluronidase that breaks down the hyaluronic acid in the ground substance of tissue and presumably enhances the invasiveness of the organism. The endoflagella are composed of three core proteins that are homologous to other bacterial flagellin proteins plus an unrelated sheath protein.
Cardiolipin is an important component of the treponemal antigens (Carrol et al; 2016).

2.3.5. Transmission and epidemiology

*Treponema pallidum* is transmitted from spirochete containing lesions of skin or mucous membranes (e.g. genitalia, mouth, and rectum) of an infected person to other persons by intimate contact. It can also be transmitted from pregnant women to their fetuses. Rarely, blood for transfusions collected during early syphilis is also infectious. *T. pallidum* is a human organism only. There is no animal reservoir (Levinson, 2014).

Unprotected sex, promiscuous sex, and intravenous drug use are the major risk factors for syphilis. Doctors, nursing staff, and other healthcare workers are at occupational risk. (Parija, 2012).

Syphilis occurs worldwide, and its incidence is increasing. It is one of the leading notifiable diseases 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).

2.3.6. Pathogenesis and Clinical Findings

*T. pallidum* causes venereal (transmitted through sexual contact) syphilis. The clinical presentation of venereal syphilis is varied and complex, often mimicking many other diseases (Forbes *et al*; 2007).

*T. pallidum* produces no important toxins or enzymes. The organism often infects the endothelium of small blood vessels, causing endarteritis. This occurs during all stages of syphilis but is particularly important in the pathogenesis of the brain and cardiovascular lesions seen in tertiary syphilis. In primary syphilis, the spirochetes multiply at the site of inoculation, and a local, nontender ulcer (chancre) usually
forms in 2 to 10 weeks. The ulcer heals spontaneously, but spirochetes spread widely via the bloodstream (bacteremia) to many organs. One to 3 month later, the lesions of secondary syphilis may occur. These often appear as a maculopapular rash, notably on the palms and soles, or as moist papules are called condylomata lata. These lesions are rich in spirochetes and are highly infectious, but they also heal spontaneously. Patchy alopecia also occurs. Constitutional symptoms of secondary syphilis include low-lymph-adenopathy. Pharyngitis, meningitis, nephritis, and secondary stages may not occur, and yet the disease may progress. (Levinson, 2014).

About one –third of these early (primary and secondary) syphilis cases will cure themselves, without treatment. Another third remain latent (on lesions appear, but positive serologic tests indicate continuing infection). The latent period can be divided into early and late stages, the symptoms of secondary syphilis can last for many years, no symptoms occur and patients are not infectious. In the remaining one-third of people, the disease progresses to the tertiary stag. Tertiary syphilis may show granulomas, especially of skin and bones, central nervous system involvement, also known as neurosyphilis, or cardiovascular lesions (e.g, aortitis, aneurysm of the ascending aorta). In tertiary lesions, treponemes are rarely seen. Also T.pallidum causes congenital syphilis (Levinson, 2014).

2.4. Nonvenereal syphilis

Congenital syphilis and occupational syphilis are example of nonvenereal syphilis (Parija, 2012 ).
2.5. Congenital syphilis

It is the most severe outcome of syphilis in humans. The infection occurs by vertical transmission from mother to fetus during pregnancy. If the mother is suffering from secondary syphilis and not treated for the same, a higher proportion of infants are affected compared to untreated early latent syphilis. In more than 40% of untreated maternal infection, *T. pallidum* causes late abortion, still birth, and death. Deaths in neonates may occur due to secondary bacterial infection, fulminant hepatitis, or pulmonary hemorrhage. The infants suffering from congenital syphilis are born usually without any overt clinical manifestation of the disease. Poor feeding and rhinitis may be the earliest signs of congenital syphilis. Manifestations of the late onset congenital syphilis include neurosyphilis and involvement of the eighth cranial nerve, teeth, and bones (Parija, 2012).

2.6. Occupational syphilis

It is a condition that may occur in medical and paramedical workers handling a case of secondary syphilis. The lesion develops usually on the palm of infected health workers and may also occur on other exposed body part (Parija, 2012).

2.7. Syphilitic gumma

It is a solitary, localised, rubbery lesion with central necrosis, seen in organs like liver, testis, bone and brain. In liver, the gumma is associated with scarring of hepatic parenchyma (hepar lobatum). Histologically, the structure of gumma shows the following features, Central coagulative necrosis resembling caseation but is less destructive so that outlines of necrozed cells can still be faintly seen,
Surrounding zone of palisaded macrophages with many plasma cells, some lymphocytes, giant cells and fibroblasts (Mohan, 2010).

2.8. Habitat

*T. pallidum* inhabits the genital tract of infected males and females. Reservoir, source, and transmission of infection *T. pallidum* is a strict human pathogen and does not naturally occur in any animal species. Humans are the only natural hosts. Infected human hosts secreting *T. pallidum* in serous transudates from moist lesions, such as primary chancre, condyloma latum and mucous patch, are the sources of infection (Parija, 2012).

2.9. Laboratory Diagnosis

2.9.1. Direct detection of *T. pallidum*

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 results were non-specific. More recently described monoclonal antibodies are more specific (Gillesspie and Hawkey, 2006).
2.9.2. Serology

2.9.2.1. Non-Treponemal or Nonspecific Serologic Tests

These tests involve the use of nontreponemal antigens. Extracts of normal mammalian tissues (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 reagin antibodies. Flocculation tests are Venereal Disease Research Laboratory VDRL and rapid plasma reagin RPR tests detect the presence of these antibodies. These tests are positive in most cases of primary syphilis and are almost always positive in secondary syphilis. The titer of these nonspecific antibodies decreases 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. Therefore, positive results have to be confirmed by specific tests (Levinson, 2014). Results of nonspecific tests usually become negative after treatment and should be used to determine the response to treatment. These tests can also be falsely negative as a result of the prozone phenomenon. In the prozone phenomenon, the titer of antibody is too high (antibody excess), and no flocculation will occur. On dilution of the serum, however, the test result becomes positive. These tests are inexpensive and easy to perform and therefore are used as a method of screening the population for infection (Levinson, 2014).

The laboratory diagnosis of congenital syphilis is based on the finding that the infant has a higher titer of antibody in the VDRL test than has the mother. Furthermore, if a positive VDRL test result in the infant is a false-positive one because maternal antibody has crossed the placenta, the titer will decline with time. If the infant is truly infected, the titer will remain high. However, irrespective of the VDRL test results, any infant whose mother has syphilis should be treated (Levinson, 2014).
2.9.2.2. *Treponema*-specific tests

The *Treponema* specific tests measure antibodies specific for *T. pallidum*. These tests use live *T. pallidum* strains (*T. pallidum* immobilization test), or killed *T. pallidum* (*T. pallidum* agglutination test, *T. pallidum* immune adherence test, and fluorescent treponemal antibody test), also *T. pallidum* extracts as antigens (TPHA test and EIA enzyme immunoassa] (Levinson, 2014).

**T. pallidum immobilization test:** TPI test was the first specific treponemal test, which was introduced in 1949. This test detects the treponemal antibodies in patient’s serum, which immobilize motile virulent *T. pallidum*. The test is performed by incubating live *T. pallidum* strains with test serum in the presence of complement. If the serum contains treponemal antibodies, the treponemes become immobilized, which can be demonstrated under dark ground microscope. The test when introduced was the most specific serological test for diagnosis of syphilis. But because of its complexity and difficulty in maintaining live treponemal strains, this test is no longer used and is replaced by newer tests, such as TPHA and fluorescent treponemal antibody absorption (FTA-Abs) tests(Levinson, 2014).

**T. pallidum agglutination test:** *T. pallidum* agglutination test uses killed *T. pallidum* suspension inactivated by formalin. The test is performed by mixing the formalin inactivated suspension of *T. pallidum* with patient’s serum. If antibodies are present in the serum, it leads to agglutination of treponemal antigen, which can be demonstrated by dark ground microscopy. However, the test is no longer used, because it is non-specific and is associated with false positive reactions(Levinson, 2014).

**T. pallidum immune adherence test:** In this test, a suspension of inactivated treponemes is incubated with test serum, complement, and fresh heparinized whole blood from normal individuals. If antibodies are present, treponemes are found to adhere to the erythrocytes. If antibodies are absent, the treponemes do not adhere to the erythrocytes. This test is also not used now adays(Levinson, 2014).
**Fluorescent treponemal antibody test:** FTA test is the most specific and popular test used for diagnosis of syphilis. FTA is an indirect immunofluorescence (IIF) test, which uses acetone fixed smears of *T. pallidum* on the slides. The test is performed by adding a drop of test serum to the smear on the slide followed by washing and re incubating the smear with fluorescent labeled antihuman immunoglobulin. The slide is examined under a fluorescence microscope (Levinson, 2014). Demonstration of fluorescent treponemes is suggestive of a positive FTA test. FTA absorption (FTA-Abs) is a modification of FTA test, which shows high sensitivity and specificity. In this test, patient’s serum is first absorbed with a sonicated extract of non-pathogenic *T. phagedenis* to remove group specific antibodies. The test is almost as specific as the TPI test and is considered as a standard reference test in syphilis serology. The FTA-Abs test is positive in 80% primary syphilis, 100% secondary syphilis, and 95% tertiary syphilis. The test is highly specific (92–99%). The test shows occasional false positive reactions in patients with rheumatoid arthritis, systemic lupus erythematosus, cirrhosis, and hypergammaglobulinemia. The IgM FTA-Abs test is another modification of FTA-Abs used to detect serum IgM antibodies in congenital syphilis. This test is used to differentiate seropositivity due to passively transferred IgG maternal antibodies to the fetus from IgM antibodies found in utero in congenital syphilis (Levinson, 2014).

**TPHA test:** TPHA test uses erythrocytes sensitized with asonicated extract of *T. pallidum* as antigen. TPHA is now been modified to perform in microtiter plates and is referred to as microtiter hemagglutination *T. pallidum* (MHA-TP) test. This test is performed by incubating the serial dilution of the patient’s serum with erythrocytes sensitized with *T. pallidum* antigen in a microtiter plate. If antibodies are present, hemagglutination of RBCs occur . The serum samples before testing for TPHA are reabsorbed with a diluent containing Reiter’s treponeme, rabbit testes, and sheep erythrocytes. TPHA is the most widely used treponemal test
available commercially. It is simple, economical, and does not require any expensive sophisticated equipment (Levinson, 2014).

**Enzyme immunoassay:** EIA uses ultrasonicated *T. pallidum* antigen coated on tubes or ferrous metal beads as solid-phase carrier for antigen. The serum antibodies are detected by an enzymatic reaction. The test is available commercially. All the treponemal tests are not completely specific for syphilis. They also show false positive reactions in patients with other spirochetal diseases, such as leptospirosis, relapsing fever, Lyme disease, pinta, yaws, and rat-bite fever (Parija, 2012).

### 2.9.3. Rapid test

several simple rapid tests for treponemal antibody are now commercially available. most are in the format of an immunochromatographic strip. 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).

### 2.9.4. Culture

*T. pallidum* does not grow in artificial culture media. *T. pallidum* had been maintained for long time by subculture in animals. **Niche’s strain of T. pallidum:** It is a pathogenic strain, which has been maintained for several decades by serial passage in rabbit testes. This strain was isolated in 1913, originally from the brain of a fatal case of a patient with general paralysis of insane. This strain is most commonly used for diagnostic and research purposes in the laboratory. Strains of *T. pallidum* have been maintained for a short period in cell lines, such as Eagle
and McCoy cell lines supplemented with fetal bovine serum and reducing agent (Parija, 2012).

2.9.5. Molecular techniques

Clinical specimens often contain very low numbers of an infecting pathogenic microbe, amplification methods can be used to increase the sensitivity of the test and the likelihood of detection. Such methods remove the need to grow the organism in vitro, a slow and variable process. Polymerase chain reaction PCR is an example of molecular techniques using a variety of targets and test formats with conventional or real time PCR (De La Maza et al; 1997).

2.10. Treatment of syphilis

Primary, secondary, or early latent use penicillin G or tetracycline (2 weeks), late, latent or benign tertiary use penicillin G benzathine or tetracycline (4 weeks), neurosyphilis or pregnancy or HIV use aqueous crystalline or procaine penicillin G or desensitization and penicillin. (Sastry and Bhatk, 2015).

2.11. Prevention

Prevention depends on early diagnosis and adequate treatment, use of condoms, administration of antibiotic after suspected exposure, and serologic follow-up of infected individuals and their contacts. The presence of any sexually transmitted disease makes testing for syphilis mandatory, because several different infections are often transmitted simultaneously. There is no vaccine against syphilis. (Levinson, 2014). Avoiding transmission of T.Pallidum in blood The transmission of T.pallidum by blood transfusion can be avoided by storing all donor blood at 2-
6 C for 3 – 5 days and collecting blood from low risk donors or preferably from donors previously serologically screened for syphilis (Cheesbrough 2003).

2.12. Other treponemal infections

Three geographically localized treponemal diseases closely mimic syphilis. They include bejel (found in hot, arid areas of Africa, Southeast Asia, and the Middle East), yaws (found in humid, tropical countries), and pinta (found in South and Central America, Mexico, and the Phillipines). Unlike syphilis, direct skin contact, crowded living conditions, and poor hygiene contribute to the spread of these diseases. Sexual contact is usually not the mode of transmission, and congenital infections occur rarely if at all. All three diseases are curable with penicillin. (Harvey et al; 2007).

2.13. Syphilis and Blood Safety

Syphilis is a transfusion-transmitted infection (TTI). The germ is present in the blood of a contaminated blood donor and infects the recipient. The transmissibility of syphilis by blood transfusion has been frequently reported, chiefly based on animal experiments. Cases of syphilis transmitted by blood have been described in literature, with more than a hundred cases since the first description (Tangy, 2011).

The main cases reported were shown to occur when donors were in the primary or secondary stage of the disease. (Syphilis) detection of specific Treponema antigens is possible using methods as passive agglutination, as T. pallidum hemagglutination (TPHA) assay or the T. pallidum particle agglutination (TPPA) assay, indirect immunofluorescence as the fluorescent treponemal antibody absorbed (FTA-ABS) assay or enzyme immunoassay (EIA) for the detection of specific IgG and IgM or total Ig. Non-treponemal methods are based on non-
treponemal lipid antigens (cardiolipin), using frequently the flocculation technique. Of these, the Venereal Disease Research Laboratory (VDRL) and rapid plasma reagin (RPR) tests are the most commonly used. These tests are cheap, fast and more sensitive. They are able to identify the contaminated blood donors few days before the treponemal test and thus useful for acute infection. However, VDRL and RPR cannot be automated and are time-consuming if used for large scale testing. Moreover, they produce more false positive results. These tests are routinely used to screen blood donors. False positives on the rapid tests can be seen in viral infections such as hepatitis, tuberculosis, malaria, or varicella. Thus, non-treponemal tests should be followed up when possible by treponemal test. The treponemal tests are based on monoclonal antibodies and immunofluorescence they are more specific and more expensive. The tests based on enzyme-linked immunoassays are the more specific and are usually used to confirm the results of simpler screening tests for syphilis (Tangy, 2011).
CHAPTER THREE
MATERIALS AND METHODS
3. Materials and Methods

3.1. Study design

Cross sectional study.

3.2. Study area

This study was conducted in Central Blood Bank / Khartoum State.

3.3. Study population


3.4. Study duration

Study was carried out during March to May, 2018.

3.5. Sample size

A total of ninety two subjects (n=92) were enrolled in this study.

3.6. Data collection

Personal and clinical data were collected from blood donors by direct interviewing questionnaire from each subject.
3.7. Ethical considerations

Permission to carry out the study was obtained from the College of Graduate Studies, Sudan University of Science and Technology.

3.8. Sample collection

Under aseptic condition, venous blood (3 mls) were obtained from blood donors, samples were collected by vein puncture using tubes did not contain anticoagulant, serum was collected from whole blood and left to settle for 30 minutes in the rack for blood coagulation and then was centrifuged to get serum specimen supernatant with avoidance of hemolysis. If specimen were not immediately tested they were kept at –20 °C.

3.9. Laboratory Methods

The serological techniques Immunochromatographic test ICT, Rapid plasma regains RPR and Enzyme linked immunosorbent assay ELISA were used for detection of syphilis among blood donors.

3.9.1. Immunochromatography test

3.9.1.1. Principle of the test

The syphilis rapid test cassette (whole blood/serum/plasma) is a qualitative membrane based immunoassay for the detection of treponemal antibodies (IgG and IgM) in whole blood, serum or plasma. In this test procedure, recombinant syphilis antigen is immobilized in the test line region of the test. After specimen is added to the specimen well of the cassette, it reacts with syphilis antigen coated
particles in the test. This mixture migrates chromatographically along the length of the test and interacts with the immobilized syphilis antigen. The double antigen test format can detect both IgG and IgM in specimens. If the specimen contains treponemal antibodies, a colored line will appear in the test line region, indicating a positive result. If the specimen does not contain treponemal antibodies, a colored line will not always appear in the control line region, indicating that proper volume of specimen has been added and membrane wicking has occurred.

3.9.1.2. Storage and stability

**Kits:** Stored as packaged in the sealed pouch at room temperature 25 °C. They could be kept at (2-30°C). The kits were used before the expiration date printed on the sealed pouch. The test remained in the sealed pouch until use.

**Specimens:** Serum or plasma stable for 72 hours at 2-8°C. Samples free from contamination and haemolysis. For long term storage, specimens should be kept below -20 °C.

3.9.1.3. Procedure of the test

The test cassette was removed from the sealed pouch and placed on a clean and level surface, the dropper was hold (which found with the cassette) vertically and 1 drop of serum was transferred (approximately 40ul) to the specimen area, then 1 drop of buffer was added (approximately 40ul), the result was read at 5 minutes and not more than 20 minutes.
3.9.1.4. Interpretation of the results

According to manufacture instructions as follows:

**Positive:** Two lines appear. One colored line should be in the control line region (C) and another apparent colored line should be in the test line region (T). The intensity of the color in the test line region (T) will vary depending on the concentration of treponemal antibodies present in the specimen. Therefore, any shade of color in the test line region (T) considered positive.

**Negative:** One colored line appears in the control line region (C). No line appears in the test line region (T).

**Invalid:** Control line fails to appear. Insufficient specimen volume or incorrect procedural techniques are the most likely reason for control line failure.

3.9.1.5. Quality control

A procedural control is included in the test. A colored line appearing in the control line region (C) is considered an internal procedural control. It confirms sufficient specimen volume, adequate membrane wicking and correct procedural technique. Control standards are not supplied with this kit however, it is recommended that positive and negative controls be tested as a good laboratory practice to confirm the test procedure and to verify proper test performance.

3.9.2. Rapid Plasma Regain RPR

3.9.2.1. Principle of the test

The Rapid Plasma Reagin or RPR card test is a non treponemal method for the serological detection of syphilis. The antigen a particulate carbon suspension coated with lipid complexes agglutinates in the sera of syphilitic patients. Visible
agglutination in the form of black clumps which can be viewed macroscopically, indicate the presence of such antibodies in the sample tested.

3.9.2.2. Storage and stability

**Reagents:** Components stored at 2-8°C. Cards and pipettes may be kept at room temperature.

**Specimens:** Serum or plasma stable for 48 hours at 2-8°C. Samples should be free from contamination and haemolysis. Lipemic will not affect the test results unless it is severe enough to obscure the state of the antigen particles.

3.9.2.3. Reagent preparation

According to manufacture instructions as follows:

The carbon antigen was resuspended gently to ensure thorough mixing. The required volume of carbon antigen was transferred to the dispensing bottle. The dispensing bottle was labeled with the antigen lot number, expiry and transfer dates. Once the antigen has been transferred to the dispensing bottle; it is stable for 3 months.

3.9.2.4. Procedure of the test

According to manufacture instructions as follows:

The reagents and samples were brought to room temperature. 50ul of sample and 1 drop of control were placed into separate circles on the card. The antigen gently resuspended. One drop of free falling antigen were added to each test circle and mixed with the disposable pipette/stirrer and spread over the entire area enclosed by the ring. A new stirrer was used for each sample. The cards rotated at 100 r.p.m for 8 minutes.
3.9.2.5. Interpretation of the results

According to manufacture instructions as follow:

**Reactive:** Medium and Large clumps.

**Weakly Reactive:** Small clumps

**Non Reactive:** No clumping or very slight roughness.

3.9.2.6. Quality control

Each run of tests should be validated with a positive and negative control.

3.9.3. Inzyme Linked Immunosorbent assay ELISA

3.9.3.1. Principle of the assay

The detection of anti treponemal 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 micro wells together with recombinant treponemal antigens conjugated to HRP. The pre coated antigens, but are expressed in different hosts. In case of presence of anti treponemal in the sampl, during incubation the pre coated and conjugated antigens will be bound to the two variable domains of antibody and the specific antigen antibody immunocomplex 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 is hydrolyzed by the bound HRP conjugate to a blue coloured product, which turns 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 treponemal antibodies remain colorless.

Assay principle scheme: Double antigen sandwich ELISA
Ag(p) + Ab(s) + (Ag)ENZ→ [Ag(p)-Ab(s)-(Ag)ENZ] →blue→yellow (positive) .
Ag(p)+(Ag)ENZ→[Ag(p)]→no color (negative).

Ag(p) pre coated recombinant anti treponemal antibodies.
Ab(s) treponemal antibodies in sample.
(Ag) ENZ HRP conjugated recombinant treponemal antigens.

3.9.3.2. Storage and stability

**Kits:** Components stored at 2-8°C.

**Specimens:** Serum or plasma stable for 72 hours at 2-8°C if delay occur stored frozen at -20°C or less. Multiple freeze – thaw cycles should be avoided. Samples should be free from contamination and haemolysis.

3.9.3.3. Reagent preparation

According to manufacture instructions as follow:
The reagents and samples allowed to reach room temperature (18-30°C) for at least 15 – 30 minutes. The wash buffer concentrate was checked for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37°C until crystals dissolve. The stock wash Buffer was diluted 1 to 20 with distilled or deionized water. Useing clean vessels to dilute the buffer. For one plate, 50ml of the concentrate was mixed with 950ml of water for a final volume of 1000ml diluted wash Buffer.
3.9.3.4. procedure of the test

**Numbering wells:** The strips needed was set in strip holder and numbered sufficient number of wells including three negative control, two positive control.

**Adding HRP conjugate:** 100 HRP conjugate were added in to each well.

**Adding sample:** 20ul of positive control, negative control, and specimen were added into their respective wells. Upon addition of the sample the HRP conjugate sample mixture will appear blue. A separate disposable tip was used for each specimen.

**Incubating:** The plate was mixed by tapping gently. And covered over the plate with the plate cover and incubated for 60 minutes at 37°C.

**Washing:** At the end of the incubation, the plate cover was removed and discarded. Each well was washed 6 times with diluted wash buffer. Each time, the microwells allowed soaking for 30-60 seconds. After the final washing cycle, the plate was turned down onto blotting paper or clean towel, and tapped to remove any remaining solution.

**Colouring:** 50ul of chromogen A and 50ul chromogen B solution were dispensed into each well, and mixed by tapping the plate gently. The plate was incubated at 37°C for 15 minutes avoided light. The enzymatic reaction between the chromogen solutions and the HRP conjugate produced blue color in positive control and anti treponemal positive sample wells.

**Stopping reaction:** 50ul stop solution were added into each well and mixed gently. Intense yellow color developed in positive control and anti treponemal antibodies positive sample wells.

**Measuring the absorbance:** The reference wavelength were set at 630nm. The cut-off value were calculated and the results evaluated (the absorbance was read within 5 minutes after stopping the reaction.)
3.9.3.5. Interpretation of the results

According to manufacture instructions as follow:
Each micro plate considered separately when calculating and interpreting results of the assay. The results were calculated by relating each samples optical density (OD) value to the cut – off value (C.O) of the plate.

**Calculation of cut-off value:** Cut-off value (C.O) = *Nc + 0.18
*Nc = the mean absorbance value for three negative controls.

=0.011+0.009 =0.02/2=0.01
0.01+0.18=0.19
C.O = 0.19

The mean value is calculated using the two values of negative control.

**Negative Results (S/C.O < 1):** samples giving an absorbance less than the cut-off value were considered negative, which indicates that no anti treponemal antibodies have been detected with this anti treponemal ELISA kit, and there are no serological indications for past infection with Treponema pallidum.

**Positive Results (S/C.O. > or =1):** Samples giving an absorbance greater than or equal to the cut-off value were considered initially reactive, which indicates that anti treponemal antibodies have been detected with this anti treponemal ELISA kit.

**Borderline (S/C.O = 0.9-1.1):** Samples with absorbance to cut – off ratio between 0.9 and 1.1 are considered. Repeatedly positive samples can be considered positive for ant treponemal antibodies.
3.9.3.6. Quality control

The test results are valid if the quality control criteria are verified. The OD value of the positive control must be equal to or greater than 0.800 at 450/630nm. The OD value of the negative control must be less than 0.100 at 450/630nm.

3.10. The evaluation of the test results

In order to determine how useful the test is to detect a disease the sensitivity and specificity were calculated. ELISA method was used as gold standard test to determine the prevalence of disease, RPR and ICT tests were interested in determining their sensitivity and specificity.

The sensitivity and specificity were calculated as follow: For people that have the characteristic (as determined by the gold standard which we used ELISA method), the number of people who tested positive and negative were recorded. And same was done with the people that do not have the characteristic (as determined by the gold standard). And ended up with four numbers. People with the characteristic and tested positive were true positive (TP). People with the characteristic and tested negative were the false negatives (FN). People without the characteristic and tested positive were the false positive (FP). People without the characteristic and tested negative were true negatives (TN). To calculate the sensitivity, TP was divided by (TP+FN) and to calculate the specificity, TN was divided by (FP+TN) (wikihow, 2018).
3.11. Data analysis

Statistical Package of Social Sciences (SPSS version 18). Computer software was used for data analysis.
CHAPTER FOUR
RESULTS
4. Results

A total of ninety two blood donors were tested for syphilis. The age range was 18 to 50 years, all of them were males. The seroprevalence of syphilis revealed from ELISA method 7(7.6 %), were it was equal in ICT and increase to 8(8.7 %) in RPR.

4.1. Distribution of syphilis among blood donors according to age (result given by ELISA)

The higher frequency of syphilis was found in age group [26-35] 3(42.9%) and age group [36-45] 3(42.9%), and there was no positive cases among the ages of [46-55] years as shown in fig 4.2.

4.2. Distribution of syphilis among blood donors according to marital status (result given by EILISA)

As shown in fig.4.3, out of 92 examined samples 5 (71.4%) of positive samples were single blood donors, 2 (28.6)% were married and there were no positive cases among divorced blood donors.

4.3. ELISA results

Out of 92 examined blood samples 7 (7.6%) were found positive and 85 (92.4) were found negative for syphilis by ELISA method as shown in fig 4.1.
4.4. ICT results

Out of 92 examined blood samples 7 (7.6%) were found positive and 85 (92.4) were found negative for syphilis by ICT method. The same result was found by ELISA.

4.5. RPR results

Out of 92 examined blood samples 8 (8.7%) were found positive and 84 (91.3) were found negative for syphilis by RPR method.

4.6. Sensitivity and specificity of ICT

ELISA was the gold method
True positive = 7
False negative = 0
False positive = 0
True negative = 85
Sensitivity = TP/ (TP+FN).
Sensitivity = 7/ (7+0) = 1x100 = 100%
Specificity = TN/ (FP+TN)
Specificity = 85/(0+85) = 1x100=100%
ICT method shows 100% sensitivity and 100% specificity.
4.7. Sensitivity and specificity of RPR

ELISA was the gold method

True positive = 7
False negative = 0
False positive = 1
True negative = 84

Sensitivity = TP/ (TP+FN).
Sensitivity = 7/ (7+0) = 1x100 = 100%

Specificity = TN/ (FP+TN)
Specificity = 84/ (1+84) = 0.988 x100 = 98.8%

RPR show 100% sensitivity and 98.8% specificity.
CHAPTER FIVE

DISCUSSION
5. Discussion

This study was conducted to detect the frequency of syphilis among blood donors in Central Blood Bank of Khartoum State in Sudan from March to May 2018. Ninety two samples were tested for syphilis, the age range was 18 to 50 years, all of them were males. The seroprevalence of syphilis revealed 7 (7.6 %) by ELISA which was the gold standard method in this study compare to other study we can find different results. A retrospective analysis study about sero-prevalence of HIV and syphilis infections among blood donors at Kosti Teaching Hospital –White Nile State Sudan was conducted between January 2014 and May 2014, a total number of donors were 1204 all were males, the sero-prevalence for antibodies against HIV and Treponema pallidum was positive in 8 (0.7%) and 82 (6.8%) donors, respectively (Bazie et al; ,2014).

Another study about prevalence of syphilis infection among adult rural residents of Hassai and Bir Agam area in Red Sea State in Sudan,27 of the 125 samples tested in this study (21.6) were positive for anti Treponema pallidum antibodies and 98 of them (78.4%) were negative (Abdalla and Ismail, 2014).

In vitro comparative study, conducted in augus 2015 at Central Blood Bank, Gezira State Sudan. 200 samples were collected from blood donors in Central Blood Bank 187 were negative by enzyme linked immuno sorpant assay (ELISA),while they were 191 by immunochromatography test(ICT) ,the positive cases were 13 for ELISA test ,while they were 9 cases for ICT (Abdalla et al; 2017). The Gezira State results disagree with this study not only in the prevalence of the disease but also in the specificity and sensitivity of ICT method which show sensitivity 69.2%, specificity 97.9% but in this study the specificity and sensitivity of ICT were equal to ELISA which show 100% sensitivity and 100% specificity, and it may be due to the difference in quality in these test which can differ according to the manufacture. A study about prevalence of syphilis among blood and stem cell donors in Sudi Arabia was conducted. About 240,000 blood donors
were screened, most of the blood donors were males (98.3%), approximately 0.044% of all the blood donors were syphilis positive. No cases were detected as positive among stem cell donors (Elyamamy et al; 2016). In this study there was different prevalence but all showed that the hazard was found if using blood for transfusion before testing for this disease.

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).
5.1. Conclusion

ICT and ELISA were the best methods for screening blood donors. High prevalence was observed among the age of [26-45] years and also among single cases.

5.2. Recommendations

1. More studies are needed with large sample size to support the result which was obtained in this work.
2. Blood donor should be tested for syphilis before donation.
3. ELISA, ICT and RPR can be used for the screening of blood donors, those tests showed high sensitivity according to this study. In the area of Sudan which have poor health service and there is no ability for doing ELISA, ICT and RPR can be the substitution.
4. ICT and RPR can use just for screening but to confirm the diagnosis for the patient it is better to use other more advanced techniques like ELISA or more better Polymerase chain reaction PCR.
References
References


Appendices
Appendix (1)

Sudan University of Science and Technology
College of Graduate Studies
Microbiology Department

Questionnaire

Prevalence of syphilis among blood donors in Kartoum state-Sudan

Date…………….                             Questionnaire NO…..

1/Age
A/15-25          B/26-35          C/36-45          D/46-55

2/Job
A/Employee   B/Labor   C/Free work   D/Other…………..

3/Social State
A/Married     B/Single     C/Divorce     D/Widow

4/Suffering from other STD diseases
A/AIDS         B/Hepatitis B   C/Hepatitis C
Fig 4.2 Distribution of syphilis among blood donors according to age by ELISA method.

Fig 4.3 Distribution of syphilis among blood donors according to material status by ELISA method.
Fig 4.4. RPR card sample NO 1 was control negative and sample NO 2 was control positive, the other samples were all non reactive.

Fig 4.5. RPR card in this card all was non reactive except NO 10 was reactive.
Fig 4.6. RPR reactive reaction.

Fig 4.7. ELISA microtiter plate yellow wells indicate positive results the tow upper left was control positive.
Fig 4.8 ICT rapid test device the upper was positive and the lower was negative.
**Assay Procedure:**

**Step 1: Reagents preparation**
- Allow the reagents and samples to reach room temperature (18-30°C) for at least 1-2 hours before use.
- Check the wash buffer concentrate for the presence of solid crystals. If crystals have formed in the solution, redissolve by warming at 37°C until crystals dissolve. Dilute the stock Wash Buffer 1:10 with distilled or sterile water. Use only the top half of the bottle.

**Step 2: Numbering Wells**
- Set the strips needed in triplicate, and number sufficient number of wells including three negative control (e.g., A1, C1, D1), two Positive Control (e.g., F1, I1), and one blank (e.g., A1). Neither samples nor HRP-Conjugate should be added to the blank well.

**Step 3: Adding HRP Conjugate**
- Add 100 µl HRP conjugate into each well except the blank well.

**Step 4: Adding Sample**
- Add 20 µl of Positive control, Negative control, and samples into their respective wells. Upon addition of the sample, the HRP conjugate - sample mixture will appear blue, like a separate disposable tip for each specimen. Negative control and Positive control are added to avoid cross-contamination.

**Step 5: Incubating**
- Mix the plate by tapping gently. Cover the plate with the cover plate and incubate for 30 minutes at 37°C. It's recommended to use thermostat-controlled water bath to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

**Step 6: Washing**
- At the end of the incubation, remove and discard the plate cover. Wash each well 2 times with diluted Wash Buffer. Each time, add 100 µl of diluted Wash Buffer to each well to look 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.

**Step 7: Coloring**
- Dispense 50 µl of Chromogen A and 50 µl Chromogen B solution into each well including the blank and mix by tapping gently. Incubate the plate at 37°C for 10 minutes avoiding light. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in positive control and anti-IP positive sample wells.

**Step 8: Stopping Reaction**
- Use a multichannel pipette or manually, add 100 µl Stop Solution into each well and mix gently; intense yellow color develops in Positive control and anti-IP positive sample wells.

**Step 9: Measuring the Absorbance**
- Calibrate the plate reader with the Blank well and read the absorbance of 405 nm if a dual wave instrument is used. Set the reference wavelength of 630 nm. Calculate the Cutoff value and evaluate the result (take the absorbance within 6 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 Cutoff value (CV) of the plate. If the OD reading is based on single filter plate reader, the results should be calculated by subtracting the blank well OD value from the plate OD. If the reading is based on dual filter plate reader, do not subtract the blank well OD from the plate report values of samples and controls.

1. **Calculation of Cutoff value:**
   - Cutoff value (CV) = OD at 0.18
   - OD = 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 this mean value is calculated again using the remaining two values. If this results in 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 is 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.
   - The OD value of the Blank well, which contains only Chromogen and Stop solution, is less than 0.000 at 405 nm.
   - The OD value of the Positive control must be equal to or greater than 0.000 at 405-600 nm, or at 405 nm after 60 minutes. If the OD value of the Negative control must be less than 0.100 at 405-600 nm or at 405 nm after blanking.
   - There are no serological indications for past infection with HPV.

3. **Interpretations of the results:**
   - S = Individual absorbance (OD) of each specimen.
   - Negative Results (S/C/O > 1): Samples giving an absorbance less than the Cutoff value are considered negative, which indicates that no anti-IP antibodies have been detected with the anti-IP ELISA kit. There are no serological indications for past infection with HPV.
   - Positive Results (S/C/O < 1): Samples giving an absorbance greater than or equal to the Cutoff value are considered positive, which indicates that anti-IP antibodies have been detected with the anti-IP ELISA kit. Any blood containing antibodies to human papillomavirus should be immediately discarded.
   - Borderline (S/C/O = 1-1.5): Samples with absorbance to Cutoff ratio between 0.5 and 1.5 are considered borderline samples and retesting is recommended. Repeatedly positive samples can be considered positive for anti-IP antibodies.

**Fortress anti-IP ELISA Performance:**

**Clinical Performance:**
- The clinical performance of this assay has been evaluated by a panel of samples obtained from 340 healthy donors. The results were compared to 170 blood donors and a panel of samples from 192 Syphilis positive patients. The evaluation results are given below:

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>False Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>3392</td>
<td>99.91%</td>
<td>99.91%</td>
<td>0.1%</td>
</tr>
<tr>
<td>400</td>
<td>99.91%</td>
<td>99.91%</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

**Analytical Specificity:**
- 1. No cross-reactivity observed with samples from patients infected with HIV, HCV, HBV, CMV, HAV and HUS.
- 2. No interference from rheumatoid factors up to 2000 U/ml observed.
- 3. No high dose hook effect during clinical testing.
- 4. The assay performance characteristics are unaffected by elevated concentrations of microgram, hemoglobin, and protein.

**Quality Control System**

**Reagents:**
- Western USA, Western USA, Western USA, Western USA, Western USA, Western USA, Western USA, Western USA.
- Western USA, Western USA, Western USA, Western USA, Western USA, Western USA, Western USA, Western USA.

**References:**