EVALUATION OF TEST KITS FOR SYPHILIS SCREENING IN BLOOD DONORS

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EVALUATION OF TEST KITS FOR SYPHILIS SCREENING IN BLOOD DONORS

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ABSTRACT

The purpose of this research is to evaluate the sensitivity and specificity of three different Enzyme Link Immunosorbent Assay (ELISA) test kits compared with Rapid Plasma Reagin (RPR) assay which currently is used for syphilis screening in blood donors and to compare sensitivity and specificity between different ELISA test kits. One thousand two hundred and fifty unselected donor samples and 206 Treponema pallidum Heamagglutination (TPHA) positive donor samples from Thai National Red Cross were included in this research. TPHA was used as gold standard.

The sensitivity of ELISA test kits: Bioelisa, Ice Syphilis and New Market were 92.72%, 98.06%, 98.06% respectively while the sensitivity of RPR was 75.24%. The specificity of Bioelisa, Ice Syphilis, New Market and RPR were 99.18%, 99.51 %, 99.92 %, 99.75 % respectively. A discordant result between TPHA and ELISA test kits was observed. RPR assay missed 13 TPHA positive cases and Bioelisa missed 15 TPHA positive cases. All cases were detected by New Market and Ice Syphilis ELISA.

Data from this research showed that ELISA had comparable specificity and higher sensitivity than RPR. Different ELISA tests kit for Syphilis screening have different sensitivity and specificity. New Market and Ice Syphilis are more suitable because they have slightly higher sensitivity, higher specificity and significantly greater accuracy than Bioelisa.

KEY WORDS: SYPHILIS / T.PALLIDUM / ELISA / EVALUATION

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การศึกษาความแม่นยำของน้ำยาที่ใช้ตรวจกรองซิฟิลิสในผู้บริจาคโลหิต (EVALUATION OF TEST KITS FOR SYPHILIS SCREENING IN BLOOD DONORS)

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คณะกรรมการควบคุมวิทยานิพนธ์: ปาริชาต เพิ่มพิกุล, พ.บ. , วิโรจน์ จงกลวัฒนา,พ.บ., สมศรี รัตนวิจิตราศิลป์, พ.บ.

บทคัดย่อ

วัตถุประสงค์ของการวิจัยนี้คือ การตรวจเพื่อประเมินหาความไวและความจำเพาะของชุดตรวจ Enzyme Link Immunosorbent Assay (ELISA) 3 ชุดตรวจ โดยเปรียบเทียบกับวิธี Rapid Plasma Reagin (RPR) ซึ่งปัจจุบันใช้สำหรับตรวจคัดกรองหาซิฟิลิสในผู้บริจาคโลหิต นอกจากนี้ยังเปรียบเทียบความไวและความจำเพาะระหว่างชุดตรวจ ELISA ทั้ง 3 ชุดตรวจ การวิจัยนี้ได้ใช้วิธี Treponema Pallidum Heamagglutination (TPHA) เป็นวิธีมาตรฐานโดยใช้ตัวอย่างโลหิตของผู้บริจาคโลหิตปกติจำนวน 1,250 ราย และ ตัวอย่างโลหิตของผู้บริจาคที่ให้ผลบวกต่อซิฟิลิส จำนวน 206 ราย ซึ่งได้มาจากศูนย์บริการโลหิตแห่งชาติ สภากาชาดไทย

ความไวของชุดตรวจ ELISA ซึ่งประกอบด้วยชุดตรวจ Bioelisa, Ice Syphilis และ New Market คือ 92.72%, 98.06%, 98.06% ตามลำดับ ขณะที่ความไวของวิธี RPR คือ 75.24 % ส่วนความจำเพาะของชุดตรวจ Bioelisa, Ice Syphilis, New Market และ RPR คือ 99.18%, 99.51 %, 99.92 %, 99.75 % ตามลำดับ จากการสังเกตผลการตรวจที่ไม่สอดคล้องกันระหว่างผลของชุดตรวจ TPHA, ELISA และ RPR พบว่า วิธี RPR ให้ผลการตรวจเป็นลบกับกลุ่มตัวอย่างที่ TPHA มีผลเป็นบวก จำนวน 13 ราย และ Bioelisa ให้ผลการตรวจเป็นลบกับกลุ่มตัวอย่างที่ TPHA มีผลเป็นบวกจำนวน 15 ราย ซึ่งชุดตรวจ New Market และ Ice Syphilis สามารถให้ผลเป็นบวกกับตัวอย่างเหล่านี้ได้

ข้อมูลจากการวิจัยครั้งนี้แสดงให้เห็นว่า ชุดตรวจ ELISA มีความจำเพาะเทียบเท่าวิธี RPR และ มีความไวสูงกว่าวิธี RPR ซึ่งชุดตรวจ ELISA ที่ใช้ตรวจคัดกรองซิฟิลิสที่ต่างชุดกัน จะมีค่าความไวและความจำเพาะต่างกัน ชุดตรวจ New Market และ Ice Syphilis ซึ่งมีความไวและความจำเพาะสูงจึงมีความเหมาะสมมากกว่าวิธีอื่นที่จะนำมาใช้ตรวจคัดกรองหาซิฟิลิสในโลหิตของผู้บริจาคโลหิตในงานประจำวัน

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CONTENTS

		PAGE
ACKNOWLEDGEMENTS		iii
ABSTRACT		iv
LIST OF TABLES		viii
LIST OF FIGURES		ix
LIST OF ABBREVIATION		x
CHAPTER		
I	INTRODUCTION	1
II	OBJECTIVES	4
III	LITERATURE REVIEW	5
	1. Origin of Syphilis	5
	2. Character of Treponema pallidum	7
	3. Stages of Disease	10
	4. Diagnosis of Syphilis	13
	5. Direct Detection Methods	14
	6. Non-Treponemal Tests: History	19
	7. Treponemal Tests: History	25
	8. Major Antigens	27
	9. The last Reported Case	32
IV	MATERIALS AND METHODS	33
	1. Study Design, Study Population, Samples Size	33
	2. Character of Population and Samples	36
	3. Research Design	36
	4. Materials and Research Instruments	37
	5. Method	37
	6. Procedure of ICE SYPHILIS	39
	7. Procedure of Bioelisa Syphilis	45

CONTENTS (CONT.)

CHAPTER		Page
	8. Procedure of New Market Syphilis EIA	50
	9. Procedure of New Market TPHA Screening	52
	10. Data processing and analysis	55
V	RESULTS	58
VI	DISCUSSION	68
VII	CONCLUSION	75
BIBLIOGRAPHY		77
BIOGRAPHY		88

LIST OF TABLES

Tables		Page
1	Characters for four pathogenic sub-species of *T.pallidum*	8
2	History of tests for syphilis: non-treponemal tests	19
3	Non-syphilitic condition giving biological false positive results (BFPs) using VDRL and RPR tests	23
4	History of tests for syphilis: treponemal tests	25
5	Major antigens of Treponema pallidum	27
6	Preparation of substrate solution for Ice Syphilis	41
7	Preparation of conjugate solution for Bioelisa Syphilis	47
8	Preparation of substrate solution for Bioelisa Syphilis	48
9	Statistical formula, for analyses data between TPHA and RPR	55
10	Statistical formula, for analyses data between TPHA and ELISA	55
11	Test results of unselected samples (N=1,250)	61
12	Specificity, PPV and NPV of three ELISA and RPR assays	61
13	Concordant results of unselected samples (n = 1,250)	62
14	Discrepancy results of unselected samples (all samples, TPHA positive)	62
15	Test results of positive samples	63
16	Sensitivity, PPV and NPV of three ELISA and RPR assays	63
17	Discrepancy results for three ELISA in positive samples testing	64
18	Mean of samples OD. , Cut-off and S/CO for three ELISA assays when tested with unselected and positive samples	64
19	A total of initial and repeated reactive in unselected samples	65
20	Characteristic of three ELISA assays for detection of *T. pallidum* Antibodies	66
21	Summarise data of each assay	67

LIST OF FIGURES

Figure		Page
1	Machines were used to running the ELISA processing	38
2	Principle of Indirect ELISA for positive and negative result	38
3	distribution of S/CO for three ELISA in unselected samples	65

LIST OF ABBREVIATIONS

IR	Initial Reactive
RR	Repeat Reactive
NC	Negative Control
PC	Positive Control
%CV	Percentage of Coefficient Variation
S/CO	Sample/Cut off
OD	Optical Density
RPR	Rapid Plasma Reagin
TPHA	Treponema pallidum Hemagglutination
FTA-ABS	Fluorescent treponemal antibody absorption test
ELISA	Enzyme Linked Immunosorbent Assay
PPV	Positive Predictive Value
NPV	Negative Predictive Value
TP	True Positive
TN	True Negative
FP	False Positive
FN	False Negative
VDRL	Venereal Disease Research Laboratory
BFP	Biological false positive
STD	Sexually transmitted disease
CSF	Cerebrospinalfluid
MHA-TP	Microhemagglutination assay for T. pallidum antibodies
kDa	Kilo Dalton
TmpA	Treponemal membrane protein A
TmpB	Treponemal membrane protein B
CNS	Central nervous system
Rec	Recombinant

CHAPTER I
INTRODUCTION

Syphilis is a chronic disease, usually sexually transmitted, caused by the spirochete: *Treponema pallidum*. Most of transmissions occur via direct contact with lesions or blood transfusion (the organism enters via mucous membranes or microscopic skin abrasions). Infection may also occur by the transplacental route and cause congenitally syphilis.

Definitive diagnosis of syphilis is complicated by the inability to cultivate *T pallidum* subspecies pallidum in vitro. Clinical manifestations, demonstration of treponemes in lesion material, and serologic reactions are used for diagnosis. For primary stage syphilis a direct microscopic examination should be performed, since the *T. pallidum* can not be cultured "in vitro".

Serologic tests are mainstays of syphilis diagnosis. They are the only means of identifying asymptomatically infected individuals. Serological tests have been developed over the past years and fall into two general categories: “Non-treponemal tests” and “Treponemal test”. Both treponemal and nontreponemal serological tests have been highly standardized by the Centers for Disease Control and Prevention (CDC) of USA.

Non-treponemal tests are non-specific tests, which test for a common mammalian protein (cardiolipin) on the organism. This protein is also present in other diseases and non-diseased states and can result in positive tests in a patient without syphilis. These tests are used to screen for the presence of nonspecific reagin antibodies that appear and rise in titer following infection. They are quantitatively performed and used to monitor response to therapy. Tests in current use include Venereal Disease Research Laboratory (VDRL) and Rapid Plasma Reagin (RPR).

These tests are good indicators of active disease, but they are susceptible to nonspecific reactions. RPR or VDRL may be negative in primary syphilis if the lesion is seen early. One third of patients may reverse to a negative non-treponemal test after adequate treatment for syphilis.

Treponemal tests remain reactive in the patients with previous, treated infection. The positive predictive value of treponemal tests is high, and reactive results are likely to represent true infection with syphilis. Treponemal tests may also be useful in patients with suspected late syphilis and nonreactive nontreponemal tests, since declining antibody titers may produce false-negative nontreponemal tests.

Two terms relevant for syphilis serodiagnostic testing are sensitivity and specificity. The perfect test, not yet developed, would detect 100 percent of the treponemal infections and would be non-reactive in all other diseases. Sensitivity refers to the ability to detect the tested variable, in this case syphilis. A false negative occurs when serum from a syphilitic patient fails to react. Specificity refers to the ability to recognize when the variable is not present. A false positive occurs when serum from a nonsyphilitic patient reacts positively.

The sensitivity of the nontreponemal and treponemal tests varies with the stage of the disease. The results of nontreponemal tests usually parallel with the extent of infection; titers tend to be highest during secondary syphilis and subside during subclinical infection (latency) or following antibiotic therapy. While, the treponemal tests often remain reactive for life.

TPHA, FTA-ABS, these are treponemal tests or specific tests for *T. pallidum* and measure surface antibodies to a laboratory synthesized (non-virulent) form of *T. pallidum*. FTA-ABS is a fluorescent labeled antigen-antibody complex identification method. *T. pallidum* - specific IgM is commonly detected by the fluorescent treponemal antibody test with absorption in combination with removal of IgG (19S IgM FTA-ABS). This test is sensitive, but very laborious. It cannot be automated and only a limited number of samples can be processed at one time. It requires highly skilled technologists in order to obtain reproducible "readings". TPHA is a hemagglutination test, which detects presence of antibodies by the principle of agglutination of red cells enabling macroscopic reading of a result. Thus, it is simple to use and is both sensitive and specific. TPHA is an easier and less expensive test than FTA-ABS

RPR or VDRL and TPHA are traditionally use for screening routine sera. If either of these is positive, a quantitative RPR or VDRL test is carried out and FTA-Abs done to check that the reactions of the screening tests are specific and not biological false positive reactions.

The Enzyme-linked immunosorbent assay (ELISA) is a very specific, sensitive and reliable method for the detection of antibodies to syphilis allowing an objective determination of antibody status to be made on a single dilution of the test specimen. The genes, that coding for a number of antigens have now been isolated and recombinant proteins produced. These proteins have been incorporated ELISA tests and promise a new generation of highly specific and sensitive tests for syphilis. The ELISA tests, gives a quantitative colorimetric result and can be automated. The color intensity is proportional to the amount of antibodies present in the patient's serum.

For this research, I want to evaluate the sensitivity and specificity of ELISA assays when we use them for syphilis screening in routine laboratory of blood bank compare with TPHA. Thus, ELISA tests are perform in parallel with TPHA and RPR test which use as routine screening for blood donor. I use three different commercial kits for ELISA reagents. In order to ascertain the diagnostic value of each new serological method, it is necessary to determine its specificity and sensitivity. Therefore, the results of ELISA tests are compared with RPR test and compare between of three ELISA assay. TPHA is used to confirm samples that ELISA or/and RPR repeat reactive. TPHA is regard to gold standard test for this research, then discrepancy results determine with TPHA results, for true positive or true negative.

CHAPTER II
OBJECTIVE

1. To evaluate the sensitivity and specificity of three different Enzyme Linked Immunosorbent Assays (ELISA) and Rapid Plasma Reagin (RPR) assay, which commonly use in routine screening for syphilis (using TPHA as gold standard).

2. To compare the sensitivity and specificity between of three different ELISA assay.

CHAPTER III
LITERATURE REVIEW

Origin of Syphilis

The origins of syphilis have been discussed for many centuries (Dennie 1962, Oriel 1994, Quetel 1990, U.S. Department of Health.1968). Two main theories have been proposed the New World or Columbian theory and the Old World or pre-Columbian theory. The former holds that syphilis was endemic in the part of the world now known as Haiti and was then acquired and carried to Europe by Columbus in the 1400s. The pre-Columbian theory purports that syphilis originated in central Africa and was introduced to Europe prior to the voyage by Columbus. A third theory, the Unitarian theory, could be made to fit the pre-Columbian theory (Oriel 1994). This theory proposed that syphilis and the non-venereal treponematoses were all manifestations of the same infection, with the observed clinical differences being due mainly to environmental factors, especially temperature. However, recent bacteriological work has demonstrated genetic differences between these organisms (Centurion et al. 1998). Regardless of the origins, however, it remains clear that by 1495 a widespread syphilis epidemic had spread throughout Europe (Oriel 1994). From there the disease spread to India in 1498 and China in 1505 (Oriel 1994).

Many different names for syphilis, which into the following:

- Scientific publications: The Great Pox
- The Scandinavians called it the German Pox
- The Germans called it Polish Illness
- The Spanish called it Portuguese disease
- The French called it Castilian infection

- Throughout Europe it was called the French Disease
- The Turks called it the disease of Christians

  Eventually, the medical community gave it a Latin name: Morbus Gallicus
- Girolamo Fracastoro coined the name Syphilis in 1530

Early names for syphilis included the Great Pox, lues venereum (venereal disease), morbus gallicus (French disease), and the Italian, Spanish, German, or Polish disease, but the name that was to become part of the everyday language was syphilis (Oriel 1994). Hieronymus Fracastorius in 1530 is believed to be the first to coin the term "syphilis" derived from a mythical shepherd, Syphilus, described in his poem Syphilis Sive Morbus Gallicus, which means "Syphilis or the French Disease"(Oriel 1994).

Many early investigators contributed to our knowledge of syphilis, and the following highlights but a few. John Hunter (1728 to 1793) believed the Unity or Monist Theory, which holds that syphilis and gonorrhea were the same disease (Dennie1962, Oriel 1994). This theory was supported by his well-known experiment of 1767 in which he inoculated matter from a patient whom he believed to have gonorrhea onto the prepuce and glans of a recipient, who traditionally is believed to be himself. However, since the experiment was reported in the third person, it is now believed that the recipient was someone else (Dempster 1978). Ten days after the inoculation, a chancre appeared, followed by signs of secondary syphilis "(Oriel 1994). It is now believed that the donor had both syphilis and gonorrhea, but Hunter was convinced that he had induced syphilis by inoculation of gonorrheal pus. It was not until 1838 that Philippe Ricord demonstrated conclusively that syphilis and gonorrhea were separate diseases based on over 2,500 human inoculations "(Oriel 1994, U.S. Department of Health.1968). Ricord was also the first to propose a scheme for the categorization of syphilis into primary, secondary, and tertiary stages, which is still used today (Oriel 1994). Giovanni Lancisi (1654 to 1720) and Herman Boerhaave (1668 to 1738) implicated syphilis as a cause of cardiovascular disease. Alfred Fournier (1832 to 1914) confirmed the syphilitic origins of neurosyphilis (Sartin, Perry 1995). Paul Diday (1812 to 1894) and Jonathan Hutchinson (1828 to 1913) contributed greatly to our knowledge of congenital infections (Oriel 1994). Syphilis rarely diagnosed by isolation and characterization of the causative organism. Instead, less precise method of diagnosis was used: direct darkfield microscopy, immunofluorescence, immunoperoxidase or silver staining, and epidemiological, serologic, and clinical finding.

In 1905, The organism responsible for the disease was first recognition, when Metchnikoff and Roux infected monkeys with extracts of infected tissues from persons with syphilis and the association of Treponema pallidum with syphilis was described by Schaudinn and Hoffman, who demonstrated spirochetes in Giemsa-stained smears of fluid from secondary syphilitic lesions (Schaudinn and Hoffman. 1905). In 1906, Landsteiner introduced darkfield microscopy as a means to visualize this organism and August von Wassermann devised a serum reaction test for syphilis, then serologic tests for syphilis were born (Wassermann et al. 1906).

Character of Treponema pallidum

Syphilis is a complex sexually transmitted, infectious disease (STD) caused by the bacterium called Treponema Pallidum.

Family;	Spirochaete
Genus;	Treponema
Species;	pallidum, divide into four sub-species, which cause different diseases

Sub-species	**Disease**
pallidum	Syphilis
pertenue	Yaws
endemicum	Bejel
carateum	Pinta

Table.1 Characters for four pathogenic sub-species of *T.pallidum*.

	T. pallidum **ssp. pallidum**	***T. pallidum*** **ssp. endemicum**	***T. pallidum*** **ssp. pertenue**	***T. pallidum*** **ssp.carateum**
Transmission	Venereal, blood transfusion	Direct mucosol contact	Direct skin contact	Direct skin contact
Disease	Venereal Syphilis	Bejel (Endemic, nonvenereal syphilis)	Yaws	Pinta
Distribution	Worldwide	Warm climates: India, North Africa, Southeast Asia, Middle East, Yugoslavia	Humid,warm climates: South and central America, tropical Asia, Pacific Island	Warm climates: Central and South America, Mexico, Philippines
Symptoms	Chancre, rash, gummas (1^{st}, 2^{nd}, 3^{rd} stages)	Like syphilis, but non-venereal	Red papule surrounded by erythema: framboise	Dischromic (blue, grey, brown) cutaneous lesions
Age of onset	Adolescents, adults	Children to adults	Children	Children, adolescents
Congenital Infection	yes	no	rarely	no

T. pallidum is a member of the order Spirochaetales, family Spirochaetaceae, and genus Treponema**,** which includes four human pathogens and at least six human nonpathogens (Norris and Larsen. 1995). The pathogenic species are

T. pallidum subsp. pallidum which causes venereal syphilis, *T. pallidum* subsp. endemicum, which causes endemic syphilis (bejel), *T. pallidum* subsp. pertenue, which causes yaws, and T. carateum, which is the etiologic agent of pinta. Initial studies indicated that these four agents were morphologically indistinguishable, with >95% DNA homology (Miao and Fieldsteel 1980, Noordhoek et. al.1989). Recently, however, a genetic signature was defined in the 5'-flanking region of the 15-kDa-lipoprotein gene (tpp15) that distinguishes *T. pallidum* subsp. pallidum from *T. pallidum* subsp. pertenue and endemicum (Centurion-Lara et al. 1998). Syphilis is a systemic disease, caused by a bacterium called Treponema pallidum. The syphilis bacterium is very fragile, and the infection is almost always spread by sexual contact or direct contact with lesions. The bacterium spreads from the initial ulcer of an infected person to the skin or mucous membranes of the genital area, the mouth, or the anus of a sexual partner. It also can pass through broken skin on other parts of the body. Within hours to days after *T. pallidum* penetrates the intact mucous membrane or gains access through abraded skin, it enters the lymphatics or the blood stream, where it adheres to the surface proteins of cells, especially endothelial cells and disseminates throughout the body. Endarteritis, the main lesion of syphilis, can lead to endothelial scarring and over the long term, can cause an intense inflammatory reaction that may be followed by extensive tissue necrosis. The infectious dose varies from patient to patient, but in rabbits an inoculum contains as few as four spirochetes can establish an infection. The organism divides every 30 to 33 hours (Edmund 2000). The incubation period is directly proportion to the size of the inoculum (Magnuson et al. 1956). The median incubation period is 3 weeks, but it may vary from 3 to 90 days. Infection may also occur by the transplacental route to her unborn child, who may be born with serious mental and physical problems as a result of this infection or give congenital syphilis. Syphilis is a chronic disease. The bacteria move throughout the body, damaging many organs over time, especially the central nervous system (CNS). Medical experts describe the course of the disease by dividing it into four stages.

Stages of Disease

Primary Stage: After exposure to an infected source (someone with primary or secondary syphilis), a painless, papule appears at the site of inoculation. The papule grows into an indurate painless ulcer (chancre) with clear base and without exudate. The size of the chancre varies from 0.3 to 3.0 cm. and occasionally there are multiple lesions (Chapel 1978 and DiCarlo, Martin 1997). Multiple chancres can occur, especially in persons infected with HIV. Clinical lesions appear when a concentration is reached of approximately 10^7 organism per gram of tissue (Edmund 2000). Because the chancre may be painless and may occur inside the body, it may go unnoticed. It usually is found on the part of the body exposed to the partner's ulcer, such as the penis, the vulva, or the vagina. A chancre also can develop on the cervix, tongue, lips, or other parts of the body. The chancre disappears within a few weeks (2 to 8 weeks) whether or not a person is treated and may persist for longer periods, especially in immunocompromised hosts (e.g. HIV infected persons). If not treated during the primary stage, about one third of people will progress to chronic stages.

Secondary Stage: There may be no sharp demarcation between primary and secondary syphilis. This is the disseminated stage of disease and can occur at the same time as the primary stage or up to 6 months following healing of the chancre. A primary chancre is still present in as many as one-third of patients with secondary syphilis (Chapel 1980., Mindel et al. 1989). Secondary syphilis is often marked by a skin rash that is characterized by brown sores about the size of a penny. The rash appears anywhere from three to six weeks after the chancre appears. While the rash may cover the whole body or appear only in a few areas, the palms of the hands and soles of the feet are almost always involved. Because active bacteria are present in these sores, any physical contact – sexual or nonsexual – with the broken skin of an infected person may spread the infection at this stage. The rash usually heals within several weeks or months. Secondary syphilis is often a subtle disease; the skin lesions may be easily overlooked and may mimic other dermatological diseases (Chapel, 1980 , Stokes, Beerman and Ingraham. 1944). Other symptoms also may occur, such as mucous patches in the oral or genital regions that occurring in 5 to 22% of patients

(Chapel 1980, Mindel, Tovey, Timmins, and Williams 1989, Stokes, Beerman and Ingraham 1944). Wart like growths in moist intertringinous regions (condyloma lata), malaise, fatigue, headache is present in up to one-third of patients. Fever is usually low-grade, seldom exceeding 100°F, sore throat, as well as patchy hair loss;the classic 1989, Stokes, Beerman and Ingraham 1944) , and swollen lymph glands throughout of primary syphilis, will disappear without treatment. This stage resolves in 3 – 12 weeks. There may be recurrence of the secondary stage in 25% of untreated patients. The signs of secondary syphilis may come and go over the next one to two years.

Latent Stage: The latent or asymptomatic stage of syphilis is defined as the period from disappearance of the secondary manifestations until therapeutic cure occurs or tertiary manifestations develop (Howles 1943). Latent syphilis is the period of infection when patients are seroreactive for syphilis but are without clinical symptoms or signs. Many people who are not treated will suffer no further consequences of the disease. Approximately one-third of those who have secondary syphilis. Patients who have acquired syphilis within one year are classified as having early latent syphilis. In general, these patients are not infectious, however, the 25% of untreated patients, who have a recurrence of secondary syphilis, will have it occur within one year of acquisition of primary disease. Patients who acquired primary syphilis greater than one year prior are classified as having late latent syphilis. While patients in latency are asymptomatic and non-infectious, treatment of this stage is required to prevent long-term sequel.

Tertiary Stage: This stage occurs in up to 30% of untreated patients and can occur from months to decades after the primary infection. Tertiary syphilis can involve multiple organ systems:

(i) Cardiovascular syphilis. Although in the prepenicillin era, it was estimated that cardiovascular syphilis accounted for 10 to 15% of all clinical cardiovascular disease (Stokes, Beerman and Ingraham 1944) and was demonstrable in 55 to 86% of all patients with syphilis at autopsy (Howles 1943), it is now considered rare (Heggtveit 1964, Jackman and Radolf 1989). Concomitant involvement of the nervous system has been reported in up to 43% of patients (Cole 1937). Cardiovascular involvement usually occurs between 10 and 30 years after the initial infection (Cole 1937). Syphilitic aortitis is the most common manifestation and typically involves the ascending aorta (Cole 1937, Howles 1943, Kampmeier 1964). Uncomplicated aortitis is usually asymptomatic, but symptoms include substernal dull, aching pain in about 20% of patients with aortitis and heart failure in 25% (Kampmeier and Morgan 1952, Stokes, Beerman and Ingraham 1944). The most common complication of untreated syphilitic aortitis is aortic regurgitation (Howles 1943, Kampmeier and Morgan 1952). Clinical presentation usually occurs in the second or third decade of infection.

(ii) Neurosyphilis (asymptomatic, meningeal, meningovascular, parenchymatous, and gumma). Syphilis bacteria frequently invade the nervous system during the early stages of infection, and approximately 3 to 7 percent of persons with untreated syphilis develop neurosyphilis. In some instances, the time from infection to developing neurosyphilis may be up to 20 years. Neurosyphilis may be more difficult to treat and its course may be different in people with HIV infection. Although many patients with syphilis undergo cerebrospinalfluid (CSF) invasion by spirochetes, not all will develop CSF abnormalities or neurosyphilis (Chesney and Kemp 1924, Lukehart et. al. 1988, Wile and Stokes 1915). After initial invasion of the CSF by spirochetes, untreated or inadequately treated infection may follow one of several courses: spontaneous resolution, asymptomatic meningitis, or acute syphilitic meningitis (Merritt, Adams and Solomon 1946). After this, the disease may either remain asymptomatic or progress to meningovascular syphilis, tabes dorsalis, or paresis (Merritt, Adams and Solomon 1946). Although neurosyphilis has been divided into five major categories, i.e., asymptomatic, meningeal, meningovascular, parenchymatous, and gummatous, these entities represent a continuum and frequently overlap (Merritt, Adams and Solomon 1946). Asymptomatic neurosyphilis is defined as the presence of CSF abnormalities in the absence of neurologic symptoms or signs (Hahn and Clark 1946). Asymptomatic, ill-defined syndromes have become more common in the antibiotic era and comprises one-third of clinically diagnosed cases of neurosyphilis (Hooshmand Escobar and Kopf 1972, Hotson 1981, Lukehart 1988, Simon 1985). The incidence of CSF abnormalities, including an elevated leukocyte count and protein or reactive CSF VDRL test, peaks 12 to 18 months following infection (Moorthy, T. T., C. Lee, K. Lim, and T. Tan. 1987). Early in the disease, symptoms of aseptic meningitis are common, usually occurring within the first 6 months of infection or at the time of the secondary rash (Merritt 1940, Merritt and Moore 1935, Simon 1985). Clinical findings include severe headache, confusion, nausea, vomiting, and stiff neck without fever. Meningovascular syphilis represents about 10% of all cases of neurosyphilis, with peak occurrence at 4 to 7 years after primary infection (Hooshmand, Escobar and Kopf 1972, Hotson 1981, Simon 1985).

(iii) Late benign syphilis**.** The essential lesion of late benign syphilis is the gumma (Olansky 1964). The term "benign" implies that these lesions rarely cause total physical incapacity or death, but when the lesions occur in organs such as the brain or heart, serious complications may occur (Howles 1943, Kampmeier and Morgan 1952, Merritt 1940, Sohval 1935, Stokes, Beerman and Ingraham. 1944). The most common sites of involvement are the skin, bone, and liver (Kampmeier 1964). The gumma develops from 1 to 46 years after healing of secondary lesions, with the majority developing by the end of the 15th year (Clark and Danbolt. 1964). Skin lesions may appear as nodular, noduloulcerative, or ulcerative lesions (Chung, G., G. R. Kantor, and S. Whipple. 1991, Olansky 1964). Bone lesions are marked by periostitis involving the cranial bones, tibia, and clavicle, with the cardinal features of nocturnal pain and local swelling (Kampmeier 1964).

An infected person who has not been treated may infect others during the first two stages, which usually last one to two years.

Diagnosis of Syphilis

Syphilis has sometimes been called "the great imitator" because its early symptoms are similar to those of many other diseases. Sexually active people should consult a doctor about any suspicious rash or sore in the genital area. Those who have been treated for another STD, such as gonorrhea, should be tested to be sure they have not also acquired syphilis.

There are three ways to diagnose syphilis: a doctor's recognition of its signs and symptoms; microscopic identification of syphilis bacteria; and blood tests. The doctor usually uses these approaches together to detect syphilis and decide upon the stage of infection.

To diagnose syphilis by identifying the bacteria, the doctor takes a scraping from the surface of the ulcer or chancre, and examines it under a special "darkfield" microscope to detect the organism itself. Blood tests also provide evidence of infection, although they may give false- negative results (not show signs of infection despite its presence) for up to three months after infection. False-positive tests also can occur; therefore, two blood tests are usually used. Interpretation of blood tests for syphilis can be difficult, and repeated tests are sometimes necessary to confirm the diagnosis.

Various test methods for the diagnosis of syphilis have been developed. These tests for syphilis fall into 4 categories:

1. Direct microscopic examination, used when lesions are present.
2. Non-treponemal tests used for screening.
3. Treponemal tests that are confirmation.
4. Direct antigen detection tests currently used in research setting and as gold standards for test evaluation.

Direct Detection Methods

The oldest, and also the newest, methods for the diagnosis of syphilis are direct antigen detection procedures. Before the etiologic agent for syphilis was isolated, nonsyphilitic humans were inoculated with specimens from infected persons, and their reactions were examined to differentiate chancroid from syphilis and to study the nature of the disease (Magnuson et. al. 1956). The various sources of inoculum ranged from lesion exudate to blood. The uninfected recipient was inoculated by single or multiple injection or by implanting biopsy material into the forearm or thigh (Magnuson et. al. 1956). Although lesions developed, precise interpretation of results was difficult because of the diverse clinical symptoms of syphilis and the lack of serologic or microscopic proof of infection (Catterall 1972). The first transfer of *T. pallidum* from a human to the testicle of a rabbit was reported in 1907 (Parodi 1907). Later, rabbit infectivity test (RIT) studies (Magnuson, Eagle and Fleischman 1948) determined that one or two treponemes were infectious for 47% of rabbits inoculated intratesticularly with *T. pallidum*. The oldest technique is still the most sensitive of the methods. The newest technology, PCR, has not reached the sensitivity level of the RIT (Grimprel et al. 1991). The first association of Spirochaeta pallida, as *T. pallidum* was then known, with the disease syphilis was made in 1905 by Schaudinn and Hoffmann (Schaudinn and Hoffmann 1904–1905), who used a modified Giemsa stain to examine lesion material from individuals with chancres. Coles (Coles 1909) in 1909 described the use of dark-field illumination for the examination of S. pallida, noting especially the motility of the organism. Today, dark-field examination is still a viable method for the diagnosis of syphilis. In the mid-1960s, the direct fluorescent-antibody (DFA) test for *T. pallidum* was developed (Kellogg 1970, Kellogg and Mothershed. 1969, Yobs, Brown, and Hunter 1964). Later, it was modified for use with monoclonal antibodies (Hook et al.1985, Ito, George, Hunter, Larsen, and Pope 1992) and tissue sections in the direct fluorescent-antibody tissue test for *T. pallidum* (DFAT-TP) (Hunter 1990, Hunter et al. 1984, Ito, George, Hunter, Larsen, and Pope 1992, Ito et al. 1991).

For primary stage syphilis a direct microscopic examination should be performed, since the *T.pallidum* cannot be cultured "in vitro". The most common method for diagnosis of syphilis is serological test. These tests detect either antibodies to lipoidal antigens indicative of an infection (non-treponemal methods) or antibodies against specific treponemal antigens (treponemal methods). Serum is the specimen of choice for all serological tests. However, plasma samples also may be used in the microscopic non-treponemal card tests. The non-treponemal (reagin) tests measure

IgM and IgG antibodies to lipoidal material released from damaged host cells; to lipoprotein-like material; and possibly to cardiolipin released from the treponemes (Mathews, Yang and Jenkin 1979, Belisle et al. 1994). The antilipoidal antibodies are not only produced as a consequence of syphilis and other treponemal disease, but also may be produced in response to non-treponemal disease of an acute and chronic nature in which tissue damage occurs (Catterall 1972). Without some other evidence for diagnosis of syphilis, a reactive non-treponemal test does not confirm *T. pallidum* infection.

Dark-field Microscopy

When lesions are present, the most specific and easiest means of diagnosing syphilis is by direct detection of the organism. Currently, dark-field microscopy (Creighton, E. T. 1990) and the direct fluorescent-antibody (DFA) tests are commonly used to detect *T. pallidum*. A positive result on microscopic examination is definitive evidence of syphilis if infection with other pathogenic treponemes can be excluded. When collecting, preparing, and examining specimens, observes universal safety precautions (Centers for Disease Control. 1987, Centers for Disease Control. 1988). Because viability of the Treponema is necessary to distinguish *T. pallidum* from morphologically similar saprophytic spirochetes within and near the genitalia, dark-field examination must be accomplished immediately after the specimen is obtained. Equipment and personnel for dark-field examination must be readily available, or the patient must be sent to a facility where the procedure can be carried out. Dark-field examination is most productive during primary, secondary, infectious relapsing, and early congenital syphilis when moist lesions containing large numbers of treponemes (e.g., chancres, condylomata latum, or mucous patches) are present.

Enlarged regional lymph nodes can also serve as a specimen source if the involved node is aspirated and the material obtained is examined. Dark-field examination of lesions of the cervix and vagina are possible if special techniques are used for collection of the specimen. Briefly, a specimen for dark-field microscopy consists of serous fluid that contains *T. pallidum*, but it should be free of erythrocytes, other organisms, and tissue debris. The lesion should be cleansed only if encrusted or obviously contaminated and only tap water or physiologic saline (without antibacterial additives) should be used. A minimum amount of liquid should be used for cleaning because large amounts may dilute organisms and hinder the ability to recover treponemes. Antiseptics or soaps should not be used because they may kill the treponemes and invalidate interpretation of the dark-field examination. After cleansing the lesion, gently abrade it and apply gentle pressure until only clear serum exudes. Place a drop of serum on the surface of a coverslip or slide. Then place the coverslip on a microscope slide, and examine the specimen while the organism is still motile. For cervical and vaginal lesions, after the lesion is identified by speculum examination, it should be cleansed with physiologic saline and then abraded by being rubbed with a gauze pad held in suitable forceps. When serous fluid appears, the specimen should be collected with a bacteriologic loop or Pasteur pipette and transferred to a glass slide for examination. Even the experienced observer may find it difficult or impossible to differentiate *T. pallidum* from saprophytic spirochetes in the mouth; thus, dark-field microscopy should not be used for the examination of samples from oral lesions. Because of their narrow width, treponemes cannot be observed with the ordinary light microscope.

Microscopes equipped with a double-reflecting or single-reflecting dark-field condenser, a 340 to 345 objective, and a 390 to 3100 objective with a funnel stop are needed to perform the dark-field examination. Illumination for dark-field microscopy is obtained when light rays strike the object in the field at an oblique angle so that no direct light rays enter the microscope, but only the rays that are reflected from the object. Therefore, the object itself appears to be illuminated against a dark background (Creighton, E. T. 1990). Details for the test are found in A Manual of Tests for Syphilis (Creighton, E. T. 1990). Because the specimen must be read immediately, the microscope adjustment should always be completed, and the microscope should be in satisfactory working condition before the specimen is collected.

Usually stock strains of a nonpathogenic Treponema or gingival scrapings in a drop of saline are used for adjustment. *T. pallidum* is distinguished from other spiral organisms by the tightness of the spirals and characteristic corkscrew movement. However, *T. pallidum* subsp. pallidum cannot be distinguished from the other pathogenic organisms, *T. pallidum* subsp. pertenue, *T. pallidum* subsp. endemicum, and T. carateum. *T. pallidum* is a delicate, corkscrew-shaped organism with rigid, uniform, tightly wound, deep spirals. The length of the organism is 6 to 20 mm; the width is 0.10 to 0.18 mm. The length of the spiral wave is 1.0 to 1.5 mm, and the spiral depth is 0.5 to 0.7 mm. The characteristic motion of *T. pallidum* is a deliberate forward and backward movement with rotation about the longitudinal axis. A soft bending, twisting, or undulation of the organism may accompany rotation from side to side. When attached to or obstructed by heavier particles, the organism may contort, convolute, or bend and thereby distort the coils, but the organism will snap back to its original form in a coil-like manner. Organisms easily confused with *T. pallidum* are T. refringens and T. denticola (Creighton, E. T. 1990). Positive findings on dark-field examination permit a specific and immediate diagnosis of syphilis. Also, primary syphilis can be diagnosed by dark-field examination several days to several weeks before the appearance of reactive serologic tests.

However, a negative dark-field finding does not exclude the diagnosis of syphilis. Too few organisms may be present to be observed, because the lesion may be in the healing stage, or the spirochete may have been altered by systemic or topical treatment. In addition, the dark-field sample may be reported as unsatisfactory as a result of the presence of too many blood cells, air bubbles, or tissue fragments for an accurate reading. The sensitivity of the dark-field examination approaches 80% (Daniels and Ferneyhough. 1977. , Romanowski, et al. 1987). Adequate training and experience are necessary to make an accurate diagnosis by dark-field microscopy. Artifacts such as cotton fibers and Brownian motion may deceive the untrained observer. Because lesions also characterize other sexually transmitted diseases, when the direct microscopic results are negative, other diseases, such as herpes and chancroid, should be considered.

Non-Treponemal Tests: History

Table. 2 History of tests for syphilis: non-treponemal tests

Date	Author	Accomplishment
1906	Wassermann, Neisser and Bruck	Developed complement-fixation test
1907	Michaelis	Developed first precipitation test without need for complement
1922	Kahn	Introduced a flocculation test that required no complement
1941	Pangborn	Isolated and purified cardiolipin
1946	Harris, Rosenberg and Riedel	Developed Venereal Disease Research Laboratory (VDRL) test
1957	Portnoy, Carson and Smith	Modified the VDRL to create the unheated serum reagin (USR)
1961	Portnoy et al.	Modified USR to create the rapid plasma reagin (RPR)
1980	March and Stiles	Developed reagin screen test (RST)
1983	Pettit et al.	Modified USR to create tolidine red unheated serum test (TRUST)
1984	Koike and Norberg et al.	Developed ELISAs to identify and quantitate anti-cardiolipin antibodies
1987	Pedersen, Orum and Mouritsen	Developed non-treponemal enzyme-linked immunosorbent (ELISA)

*From. S.A. Larsen et al., Microbiology and Microbial infection; edition9; vol.3;1998 .

In 1906, Wessermann, Neisser, and Bruck adapted the complement fixation test, previously introduced by Bordet and Gengou in 1901, to serological testing for syphilis. The Wassermann test was the first serologic test for identification of syphilis.

(Douglas A. Triplett, 2000). The antigen used in the Wassermann test for syphilis was an extract of liver from newborns that had died of congenital syphilis. Initially, the Wassermann antigen was thought to be specific, but later Landsteiner demonstrated that other tissues, particularly beef heart extracted in alcohol, could be used equally well as antigens (Eagle 1937). Cholesterol and lecithin were added to increase the sensitivity of the antigens (Eagle 1937). Although the complement fixation tests contributed immensely to the diagnosis of syphilis, they were complicated to perform and required many reagents and 24 h to complete. Subsequent work by Michaelis in 1907, using watery extracts of syphilitic liver and Meinicke in 1917, using distilled water or sodium chloride extracts of liver, resulted in the first precipitation tests that did not require complement (Eagle 1937). In 1922, Kahn introduced a flocculation test without complement that could be read macroscopically in a few hours. Many modification of the Kahn test was appeared (Kampmeier 1983). However, because of the crudely derived extracts of tissue serving as antigens, the test varied in quality, sensitivity and specificity.

A major breakthrough in antigen standardization occurred in 1941 when Margaret Pangborn successfully isolated from beef heart the active antigenic component, cardiolipin. Cardiolipin, when combined with lecithin and cholesterol, forms a serologically active antigen for the detection of syphilitic antibodies. In contrast to the crude tissue extract antigens, the pure cardiolipin-cholesterol-lecithin antigens could be standardized chemically as well as serologically, thus ensuring greater reproducibility of test results both with in and between laboratories. With the advent of these new purified antigens, microflocculation tests, such as Venereal Disease Research Laboratory (VDRL) test (Haris, Rosenberg and Riedel 1946), were developed. The addition of choline chloride and EDTA to the VDRL antigen enhanced the reactivity of the test and stabilized the antigen suspension (Portnoy et al. 1961). The VDRL test requires fresh and accurate preparation of antigen and microscopic visualization of agglutination.

In the resulting unheated serum reagin (USR) test, as the name implies, the need for heating serum was eliminated and plasma was also found to be an acceptable test sample source. The next modification in the late 1950 was the incorporation of charcoal particles into the URS antigen to aid in reading the reaction (Portnoy, Carson and Smith 1957). The resulting test was the rapid plasma reagin (RPR) test performed on the plastic coated card. Additional modifications of the (RST) (March and Stiles 1980) and the toluidine red unheated serum test (TRUST) (Pettit et al. 1983) and numerous varying on the RPR test. Recently a radioimmunosorbent assay and an enzyme linked immunosorbent assay (ELISA) have been described for the detection of anti-cardiolipin antibodies (ACA). Subsequently, ELISAs to identify and quantitative anti-cardiolipin antibodies were developed by Koike et al. and Norberg et al. in 1984 (Douglas A. Triplett, 2000). These tests have not been evaluated as syphilis diagnostic tests but have been used to detect anti-cardiolipin antibodies in the patients with autoimmune disease, i.e., systemic lupus erythematosus (SLE). Frequently used non-Treponemal antibody tests are the reagin tests such as RPR and VDRL: these tests are good indicators of active disease, but they are susceptible to non-specific reactions.

Problem of Non-Treponemal Test

Three major problems are encountered with the non-treponemal tests: the prozone phenomenon, false positive reactions and test interpretation. Serum samples containing large amounts of non-treponemal antibody occasionally demonstrate a prozone reaction in the non-treponemal serological tests. Prozone reactions occur in 1-2 % of patients with secondary syphilis (Spangler et al. 1964, Jurado, Campbell and Martin 1993). A prozone occured when an antibody is in excess, incomplete, or blocks the normal antigen-antibody reaction. Initially, these strongly reactive serum samples may show a weakly reactive, atypical, or on rare occasion, a negative rough reaction or grainy appearance in undiluted serum. Dilution of the antibody to 1:16 is usually adequate to obtain the proper optimal concentration and a readily detectable reaction. All tests with a rough appearance should be quantitated. A serologist may not detect a prozone reaction because of a lack of reading experience. When fresh samples are tested, some exhibit innate roughness. Many serologists compensate for this roughness in their reading and fail to quantitative all serum samples exhibiting a grainy appearance (negative roughs). A second reason that the prozone reaction may not be detected is that the antigen is added to the serum before sample is spread to fill the circle.

Following World War II, the phenomena of biological false positive (BFP) was studied by Joseph Moore and Charles Mohr (Johns Hopkins University) in 1952. These investigators divided biological false positive (BFP) into acute and chronic categories. Patients with an acute biological false positive (BFP) had positive laboratory results which persisted for less than six months. Chronic biological false positive (BFP) by definition were those individuals who had positive results of greater than six months duration. Acute biological false positive (BFP) were usually identified in the context of infections (viral, bacterial, spirochetal, etc.). In contrast, patients with chronic biological false positive (BFP) typically had no history of infection. In their landmark study, Moore and Mohr found a high incidence of autoimmune disease (41.9%) in patients with chronic biological false positive (BFP). In addition, during their studies, they observed a high incidence of biological false positive (BFP) in young women with clinical thrombocytopenia as well as thrombosis.

The incidence of false positive reactions depends on the test used and the population studied. Acute false positive of non-treponemal reactions have been associated with hepatitis, infectious mononucleosis, viral pneumonia, chicken pox, measles, other viral infections, malaria, immunizations, pregnancy and laboratory or technical error. Chronic false positive reactions have been associated with connective tissue diseases such as systemic lupus erythematosus or diseases associated with immunoglobulin abnormalities which are more common in women; thus, chronic false positive reactions are more common in women than in men. Other conditions associated with chronic false positive reactions are necrotic addition, aging (Tuffanelli 1966), leprosy and malignancy (Jaffe and Musher 1990). HIV infection has not been associated with an increase in false positive non-treponemal test results, in individuals with a low risk of drug addiction (Rusnak et al 1994). At one time, titer that was used to distinguish between false and true positive results, titer of >8 consider as true positive and those <8 as possible false positives. The titre of false positive reactions is usually low, but on rare occasion can be extremely high: therefore, the quantitative titre cannot be used to differentiate between a false positive reaction and syphilis. This is especially true for persons who inject illegal drugs. More than 10% of intravenous drug users have false positive test results with titers >8 (Larsen 1983).

Table.3 Non-syphilitic condition giving biological false positive results (BFPs) using VDRL and RPR tests.

Disease	**Approximate Percentage BFPs**
Malaria	100
Leprosy	60
Relapsing fever	30
Active immunization in children	20
Lupus erythematosus	20
Lymphogranuloma venereum	20
Rat-bite fever	20
Vaccinia	20
Infectious mononucleosis	20
Typhus fever	20
Pneumonia (atypical)	20
Infectious hepatitis	10
Leptospirosis (Weil's desease)	10
Periarteritis nodosa	10
Trypanosomiasis	10
Chancroid	5
Chickenpox	5
Measles	5
Rheumatoid arthritis	5-7
Rheumatoid fever	5-6
Scarlet fever	5

Table.3 Non-syphilitic condition giving biological false positive results (BFPs) using VDRL and RPR tests (continued)

Disease	Approximate Percentage BFPs
Subacute bacterial endocarditis	5
Pneumonia, pneumococcal	3-5
Tuberculosis, advanced pulmonary	3-5
Blood loss, repeated	? (low)
Common cold	? (low)
Pregnancy	? (low)

* From. Frances Fischbach, a manual of Laboratory & Diagnostic test; edition 6; 2000

The interpretation of non-treponemal test results depends on the population being tested. The predictive value of the non-treponemal tests is increased when combined with a reactive treponemal test. Therefore, when the non-treponemal tests are used as screening tests in a low risk population, all reactive results should be confirmed with a treponemal test. In some low risk populations, every reactive result may be a false positive result. Non-treponemal test results also must be interpreted according to the stage of syphilis suspected. A reactive or weakly reactive result can be seen in all stages of syphilis. These results can also indicate a person who is serofast or could represent a false positive reaction. A non-reactive result generally excludes active infection. However, serological tests may be non-reactive during incubating disease. Otherwise, the rapid plasma reagin (RPR) tests are easier to perform, but interpretation of weak positive results may often be difficult.

Treponemal Tests: History

Table.4 History of tests for syphilis: treponemal tests

Date	Author	Accomplishment
1949	Nelson and Mayer	Developed the first treponemal antibody test, the Treponema pallidum immobilization (TPI) test
1953	D'Allesandro and Dardanoni	Developed the Reiter complement fixation test
1957	Deacon, Falcone and Harris	Developed the fluorescent treponemal antibody (FTA) test
1964	Hunter, Deacon and Meyer	Modifted the FTA by the addition of sorbent, FTA-ABS test
1965	Rathlev	Developed the haemagglutination test for syphilis (TPHA)
1969	Cox, Logan and Norins	Modified TPHA to a micromethod (MHA-TP)
1975	Veldekamp and Visser	Developed treponemal enzyme-linked immunosorbent test (ELISA)
1982	Hanff et al.	Applied the western blot technique to the diagnosis of syphilis
1989	Schouls et al.	First to use a cloned antigen in a serological test for syphilis

*From. S.A. Larsen et al., Microbiology and Microbial infection; edition9; vol.3;1998 .

The serological detection of specific antibodies to Treponema pallidum is of particular importance in the diagnosis of syphilis, since the natural course of the infection is characterized by periods without clinical manifestation (Bruno, Marzieh and Anton 2000). The serological diagnosis of syphilis is performed to demonstrate the presence or absence of significant levels of specific *T. pallidum* antibodies in the serum sample. All treponemal tests use *T. pallidum* or the components thereof as the antigen and are base on the detection of antibodies directed against treponemal components. Treponemal tests are used primarily to verify reactivity in the non-treponemal tests. The treponemal tests may also be used to confirm a clinical impression of syphilis in which the non-treponemal test is non-reactive, but there is evidence of syphilis, such as might occur in late syphilis.

When treponemal tests are for screening purposes, about 1% of the general population will have false positive results (Goldman and Lanzt 1971). However, a reactive treponemal test result on a sample that is also reactive in a non-treponemal test is highly specific. Since treponemal tests remain reactive despite therapy, they cannot be used to monitor response to treatment. Initial attempts to develop a test using an antigen derived from the treponeme were unsuccessful until 1949 when Nelson and Mayer developed the first treponemal antibody test, the Treponemal pallidum immobilization (TPI) test. The TPI test used *T. pallidum* (Nichols strain) grown in rabbit testes as the antigen and was base on the ability of patients' antibody and complement to immobilize living treponemes, as observed by darkfield microscopy. The TPI test was rapidly accepted as a specific test for syphilis. However, because the TPI test was complicated, technically difficult, time-consuming and expensive to perform, a simpler procedure was sought. In addition, studies in the 1970 found that the TPI test was less sensitive and specific than the treponemal tests developed in the 1960 (Hedersted 1976, Rein et al 1980).

As with the non-treponemal tests, an array of treponemal tests was later developed, some of which enjoyed short periods of popularity. In Europe, screening is based mainly on treponemal antigen tests such as the microhemagglutination assay for *T. pallidum* antibodies (MHA-TP), whereas in the United States the Rapid Plasma Cardiolipin antigen test (RPR) or the Venereal Disease Research Laboratory test (VDRL) is recommended as a screening test. *T. pallidum* specific tests such as the MHA-TP also lack sensitivity in the very early stage of the disease; however, they offer the highest sensitivity for late stages of the disease.

A doctor will administer a confirmatory blood test when the screening test is positive. These tests include the fluorescent treponemal antibody-absorption (FTA-ABS) test and the *T. pallidum* hemagglutination assay (TPHA). These tests detect syphilis antibodies (proteins made by a person's immune system to fight infection). They are not useful for diagnosing a new case of syphilis in-patients who have had the disease previously because once antibodies are formed, they remain in the body for many years. These antibodies, however, do not protect against a new syphilis infection. In some patients with syphilis (especially in the latent or late stages), a lumbar puncture (spinal tap) must be done to check for infection of the nervous system.

Major Antigens

Table.5 Major antigens of Treponema pallidum

Designation[a]	**MW (kDa)**	**Description and Comments**
TpN83	82	Major component of cytoplasmic filaments of fibronectin binding protein
TpN60	59	The homologue of Hsp60 or GroEL. Heat shock protein. Cross reactive antigen
TpN47	45	Lipoprotein, the most abundant polypeptide and dominant antigen
TpN44.5 (TmpA)	42	Lipoprotein, that also abundant. Recombinant from purified and tested successfully as a diagnostic reagent
TpN41	39.5	Lipoprotein.Homologue of the Mg1B periplasmic sugar binding protein of Escherichia coli

Table.5 Major antigens of Treponema pallidum (continued)

Designation[a]	**MW (kDa)**	**Description and Comments**
TpN37	37	The FlaA flagellin abundant of dominant antigen. Member of the class of unique spirochaetal flagellar sheath proteins
Tpn35	35.5	Lipoprotein.Less abundant and antigenically dominant than TpN47, 44.5
TpN34.5	34.5	The FlaB flagellins, homologues of other bacterial flagellins from flagellar core
TpN33	33	The FlaB flagellins, homologues of other bacterial flagellins from flagellar core
TpN30	32	The FlaB flagellins, homologues of other bacterial flagellins from flagellar core
TpN29-35 (TpD)	30-38	The Lipoprotein, moderately antigenic. Diffuse molecular weight-forms smear on electrophoresis
TpN24-28 (TpE)	24-30	Lipoprotein, that similar properties to TpN29-35
TpN19 (TpF1,4D*)	19	Subunit of a large, heat labile complex which in recombinant E. coli is a ring structure. In yaws strains, homologue TyF1 usually differs in one base of the sequence
TpN17	17	Lipoprotein, strongly antigenic
TpN15	15	Lipoprotein, strongly antigenic

* From C W Penn, Microbiology and Microbial infection; edition9; vol.2; 1998

[a] TpN designations are those of norris (1993). Names in parentheses were mainly given by van Embden et al. (1983)

* Designation of Walfield, Hanff and Lovett (1982).

TmpA (Transmembrane protein A)

The Enzyme Linked Immunosorbent Assay (ELISA) was developed independently by Engvall, Perlman, van Weeman and Schuurs in 1971. In 1975, Ruitenberg et al. and Voller et al. was Published microtechniques that improved test methodology. In addition, in 1975, Veldkamp and Visser was published a method for detecting treponemal antibody by using the ELISA. The ELISA was first applied to the area of syphilis serology in 1975. Since, that time many other tests using the ELISA format have been developed and evaluated as treponemal tests (Pedersen et al 1982, Pope, Hunter and Feeley 1982, Farshy et al. 1985, Burdash et al. 1987, Moyer, Hudson and Hausler 1987, Young et al. 1992, Naryar and Campos 1993). The ELISA using as antigen the axial filament of Treponema phagedenis biotype reiteri and cardiolipin, cholesterol, and lecithin, as well as sonicate of purified *T. pallidum* organisms, had been developed. Serum immunoglobulin responses to individual *T. pallidum* polypeptides have been studied by Western blotting.

During primary syphilis, the earliest responses are against TpN47 and some of the flagellar proteins, followed by TpN15 and TpN17. Antibodies against TpN15. TpN17, TpN44.5 (TmpA), and TpN47 appear to be diagnostic for acquired syphilis. With the availability of individual *T.pallidum* antigens produced with recombinant DNA techniques, new test was developed. The test used of recombinant *T.pallidum* antigens in place of poorly defined mixtures of antigens from the Nichols strains of *T.pallidum*, which may be contaminates with rabbit testicular components, has the potential for improving the specificity of serological assays. Test based on antigens produced with recombinant DNA techniques from single genes like TmpA; the 4D antigen, a ring-forming decamer on the outer membrane; or a combination of different recombinant proteins have become available. Most of the new tests have been evaluated in comparison with a standard test, e.g., the MHA-TP, the VDRL, or theCapital G test, an ELISA-based test commercially available for more than 10 years. However, the commercial ELISA tests designed to replace the FTA-ABS and haemagglutination tests as confirmatory tests for syphilis, initial evaluations have found all to have sensitivities and specificities similar to those of the other treponemal tests (Naryar and Campos 1993), but more extensive evaluation is necessary (Norgard 1993).

Many of the ELISA tests are based on the use of cloned antigens. The first cloned antigen to be used in the development of a serological test for the diagnosis of syphilis was the TmpA protein (Schouls et al. 1989). This protein has since been show to be a membrane-localized lipoprotein. It is found closely associated with another membrane protein (TmpB), and the 2 genes may be transcriptionally coupled since the start ATG of one overlap with the termination codon of the other. The genes yield a mature protein of approximately 42 kDa. When used for an ELISA, this protein proved to be both sensitive and specific: results were almost exactly the same as those seen with the MHA-TP and FTA-ABS tests. Several recombinant proteins have been used to develop ELISA since, the most prominent being the TmpB and TmpC proteins. The protein on which the greater amount of work has been bine is the 47kDa protein (Jones et al. 1984), which is now thought to be a penicillin-binding protein. This protein has been shown to be immunodominant, produced in large quantities by the treponemes and do not cross-react to any extent with similar proteins from the commensal treponemes. The ELISA system that uses this protein is the visuwell syphilis test. The main advantages of ELISA are the capacity to process large numbers of samples and the automated readout.

The presence of IgM antibodies is a feature of current infection in primary, secondary and congenital syphilis. After successful treatment the level of IgM antibody falls, eventually to negative levels. IgM antibodies may persist for a long time in patients with untreated, partially treated or inadequately treated syphilis.A variation on the ELISA test is the Captia syphilis M test for the detection of congenital syphilis in the newborn (Ijsselmuiden et al. 1989, Lefevre Bertrand and Bauriaud 1990, Stoll et al. 1993). Because infected infants can produce IgM in utero after 3 months of gestation and the fetus can be infected with *T.pallidum* at any time during gestation, an IgM ELISA test has been developed. The test is based on using anti-human IgM antibody to capture IgM in the patient's serum, followed by the addition of a purified *T. pallidum* antigen to detect those IgM antibodies in the patient's serum directed toward *T. pallidum* (Ijsselmuiden et al. 1989). One study (Stoll et al. 1993) found that the IgM capture ELISA was more sensitive than the FTA-ABS 19s IgM test in detecting probable cases of congenital syphilis. However, another study (Bromberg, Rawstron and Tannis 1993) found the test to be equal in sensitivity to the IgM western immunoblot in neonatal congenital syphilis, but less sensitive than the Western blot in detecting delayed onset congenital syphilis. The interpretation of the test result is linked to the treatment status of the mother and her stage of syphilis.

Although false positive results in the treponemal tests are often transient and their cause is unknown. A definite association has been made between false positive FTA-ABS test results and the diagnosis of systemic discoid (Shore and Faricelli 1977, Anderson and Stillman 1978) and drug-induced varieties of lupus erythematosus (Kraus, Haserick and Lanzt 1970, Kraus et al. 1971, McKenna, Schroeter and Kierland 1973, Monson 1973). Patients with systemic lupus erythematosus can have false positive FTA-ABS tests that exhibit an atypical beading fluorescence pattern. Unexplained reactive serological results may also occur, in elderly patients. Further, some false positive reactions may be due to the failure of the sorbent used in the test to remove all cross- reacting group, genus, or family antibodies (Hunter et al. 1986, Magnarelli et al. 1990). The two standard treponemal test systems (fluorescent antibody and heamagglutination) currently in used, the heamagglutination methods give fewer false positive test results (Jaffe et al. 1978, Wentworth et al. 1978, Larsen et al. 1981). In general, the occurrence of false positive heamagglutination tests is rare in healthy persons (<1%). Presumably, false positive heamagglutination tests also occur in samples from drug addicts, patients with collagen disease, patients with leprosy and patients with other miscellaneous conditions (Wentworth et al. 1978, Rein et al. 1980, and Larsen et al. 1981). In some cases, the results are difficult to assess because syphilis may coexist with these other conditions. If both the FTA-ABS and heamagglutination test were reactive, the sample is most likely (95%) from a person who has or has had syphilis (Rein et al. 1980). However, the diagnosis rests with clinical judgement. Treponemal tests vary in their reactivity in early primary syphilis: the varied sensitivities of the treponemal tests in primary syphilis are related to the time of serum collection after lesion development. For 85% of successfully treated, test results can remain reactive for years, for some people a lifetime (Schroeter et al. 1972). In addition, for the diagnosis of late untreated syphilis, reactivity in the treponemal test may be the only indication of a previous treponemal infection.

The last Reported Case

In 1966, a patient was seen at the clinical center, the hospital of the NIH USA, with a diagnosis of lymphoma (Chambers et al 1969). His VDRL test was negative on admission. He received 5 units of RBCs and 25 units of fresh platelets, none of which reacted in the VDRL test. Two months later, he had a maculopapular rash. One month after that, the rash was generalized and was most pronounced on the extremities. His VDRL, Kolmer complement fixation, and fluorescent treponema antibody tests were all positive. He was treated with penicillin and the rash cleared, although his VDRL was still positive 12 months later.

Of his 30 donors, 27 repeatedly serologically negative, but 2 could not be traced and 1 refused to be retest. The presumption is that 1 of those latter 3 donors, a platelet donor, was infectious for syphilis. That case 35 years ago was the last reported case of posttransfusion syphilis in the United States.

CHAPTER IV
MATERIALS AND METHOD

Study Design:

- Cross sectional

Study Population:

1. Unselected sample (non-disease group) collected from blood donors at National Blood Centre, Thai Red Cross Society, which use RPR assay for screening syphilis infection (collected from 20th OCT.2002 – 4th DEC 2002).

2. TPHA positive sample (disease group) collected from blood donors at Regional Blood Centre, Thai Red Cross Society, which use TPHA assay for screening syphilis infection (collected from 1st JAN 2002 – 31st AUG 2002).

Samples Size

- Prevalence of syphilis in blood donor which screening by RPR assay, at NBC,TRC is 0.17% (from annual report of NBC,TRC, 2002)
- This research need sensitivity 98.0% and specificity 98.0% (from literature reviews), then the formula for calculate the samples size, following below:

Sensitivity (for Positive Samples)

$$n = \frac{Z^2_{\alpha/2} P(1-P)}{d^2}$$

n = samples size

$Z^2\alpha/_2$ = Z value at alpha error two-tailed (0.05) = 1.96

P = probability of expected sensitivity = 0.98

1-P = 0.02

d = probability of error, that accept = 0.02 (samples size have sensitivity error less than 2%)

$$n = \frac{(1.96)^2 \times 0.98 \times 0.02}{(0.02)^2}$$

The minimum of sample size = 188.24 = 188 samples

Thus, if this research need 188 samples, the population size for these samples can calculate with prevalence rate, following below

Prevalence of syphilis by RPR assay = 0.17%

0.17 samples use population size = 100

$$188 \text{ samples use population size} = \frac{100 \times 188}{0.17}$$

The minimum of population size = 110,588

Specificity (for Unselected Samples)

$$n = \frac{Z^2\alpha/_2\, P(1-P)}{d^2}$$

n = samples size

$Z^2\alpha/_2$ = Z value at alpha error two-tailed (0.05) = 1.96

P = probability of expected specificity = 0.98

1-P = 0.02

d = probability of error, that accept = 0.02 (samples size have specificity error less than 2%)

$$n = \frac{(1.96)^2 \times 0.98 \times 0.02}{(0.02)^2}$$

The minimum of sample size = 188.24 = 188 samples

Thus, if this research need 188 samples, the population size for these samples can calculate with non-disease rate, following below

Non-syphilis disease rate = 99.83%

99.83 samples use population size = 100

$$188 \text{ samples use population size} = \frac{100 \times 188}{99.83}$$

The minimum of population size = 188

Character of Population and Samples

The number of sample sized if we want 188 positive samples was too high and we didn't have enough resource to test so we decided to collect only 188 positive samples from donor population in regional blood center which use TPHA as Syphilis screening test.

For specificity the calculation gave number only 188 cases of non-disease was too small if we want to see character of evaluated test so we decide to increase number to 1250 cases so we could study character of result like initial reactive rate, compare with repeated reactive, and % CV of test which have benefit to decision making for kit selection.

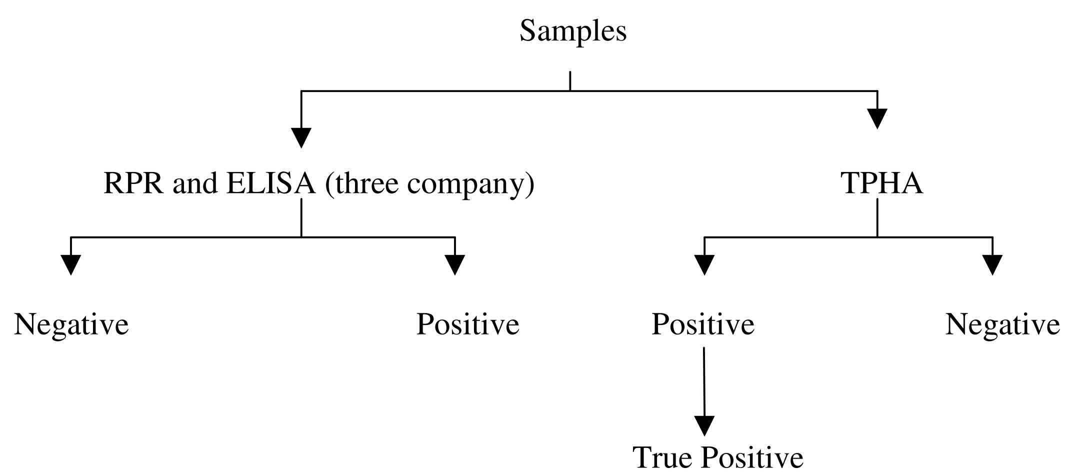
- Two groups of sera were used, which divided into the following.

1. One thousand, two hundred and fifty sera of regular donors (unselected) were collected from National Blood Centre, Thai Red Cross Society, which screening with Rapid Plasma Reagin (RPR)

2. Two hundred and six positive sera of donors, who positive for TPHA assay, were collected from Regional Blood Centre, Thai Red Cross Society, that screening with TPHA. Samples were collected to micro-tube and keep at less than -20 C ^{0}.

Research Design

Two group of samples (1,250 unselected and 206 positive samples), serum of donors: were performed ELISA, TPHA and RPR. TPHA is regarding to gold standard test for syphilis in this study. Then, discrepancy results determine with TPHA results, if TPHA positive that sample is determining to true positive.



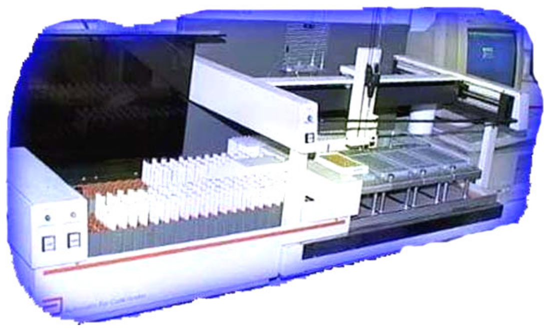
Materials and Research Instruments:

1. Flexible Pipetting Center (FPC) (Abbott Co. LTD.) for put samples and controls into each well of microplate.
2. Behring ELISA Processing III (BEPIII) (Dade Behring Co. LTD.) for perform the ELISA processing.
3. Glass slide, Reagent Dispensing and Rotator for perform the RPR test.
4. ELISA reagents support by Abbott Laboratory Co.LTD., Blood Transfusion Service Co. LTD. and Zest-Med Co. LTD.
5. RPR reagent that use in routine screening from Zest-Med Co. LTD.

Method:

1. Collect samples an optimal amount and number, which need to use.
2. Samples were putting into the wells of microplate by FPC machine. After that, unselected samples, ELISA, TPHA and RPR tests are performing following the procedure recommended in the reagent manual. Each assay was processing by BEP II machine.
3. In the same way, positive samples, ELISA, RPR and TPHA tests are performing after the unselected samples were tested already.
4. Samples that initial reactive are repeat in duplicate with same reagent (include RPR test). Data handling and statistical analysis: The comparison between RPR and TPHA assay, ELISA and TPHA assays are calculates with two-by-two table. Also, data is compare between three ELISA assay.

Figure1: Machines were used to running the ELISA processing

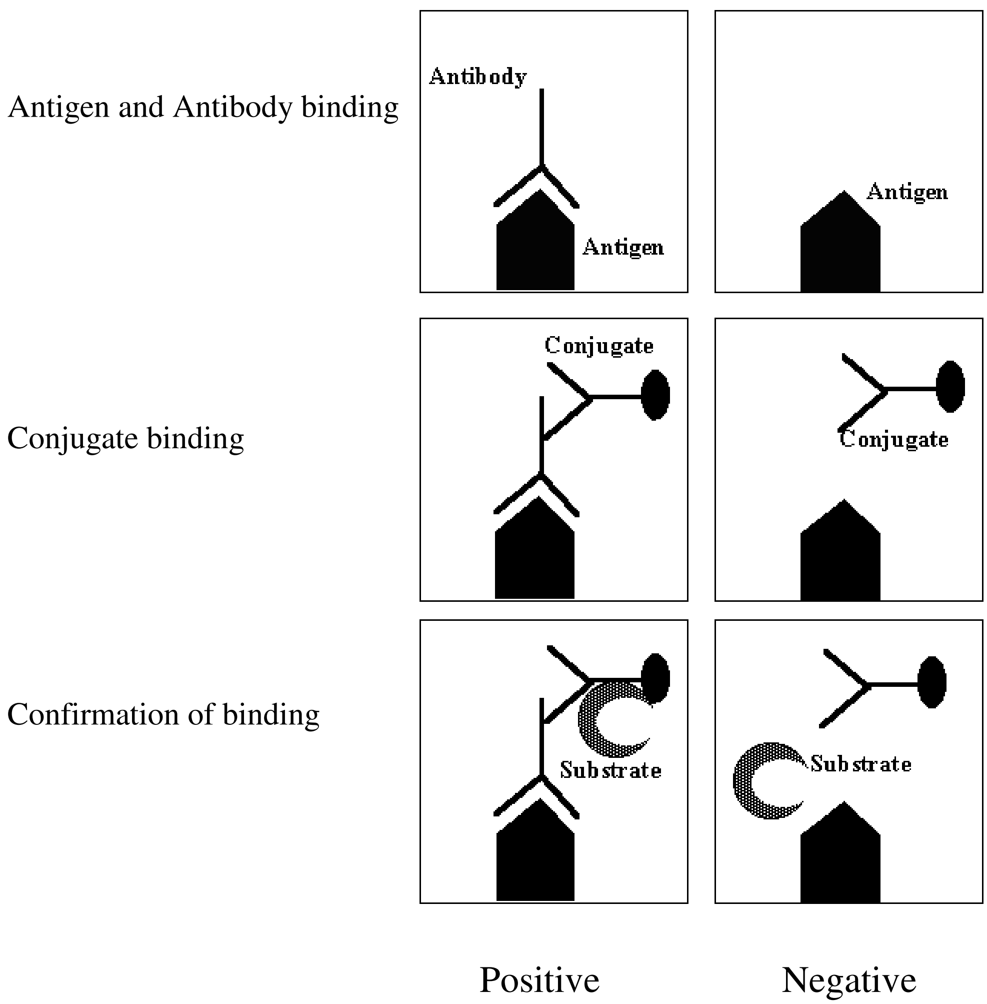


FPC Machine

BEPIII Machine

Principle of Indirect ELISA

Figure2: Principle of Indirect ELISA for positive and negative result



Procedure of Each Assay: follow the list below

1. ICE SYPHILIS Lot. 8E04-01 Exp.11-2002
2. BIOELISA SYPHILIS Lot. F-0802 Exp. 24 MAR 03
3. NEW MARKET SYPHILIS EIA Lot. 217201 Exp. 01/2004
4. NEW MARKET TPHA SCREENING Lot. 221201 Exp. 01/2004

1. Ice Syphilis

1.1 Intended Use

Ice Syphilis is designed to detect IgM and IgG antibodies directed against specific *T.pallidum* antigens, responses to that are present throughout all stages of the disease.

1.2 Principle of the Procedure

Ice Syphilis is based on the recombinant proteins TpN15, TpN17, and TpN47 both labeled and unlabelled which carry the immunodominant epitopes from *T.pallidum*. The unlabelled antigens are coated on the microwells with the anti-human-IgG and anti-human IgM. Serum or plasma samples are incubate in the wells and the specific antibodies against *T.pallidum*, if present, are capture by their related antigens on the plate. In addition a proportion of the total IgG and IgM present in the sample is capture by the anti-human antibodies. The sample, including any unbound antibody, is then removed by washing. In a subsequent step Conjugate is added, which is captured by any specific antibody already bound to the plate. Unbound Conjugate is washed away and a solution containing 3,3,5,5'-tetramethylbenzidine(TMB) and hydrogen peroxide is added to the wells. Wells with bound Conjugate develop a purple color, which is converted to an orange color when the reaction is stopped with sulphuric acid.

1.3 Components

All components must be store at 2 to 8 ^{0}C, unless otherwise stated, under which condition they will retain activity until the expiry date of the kit.

1. Antibody Coated Wells
2. Sample Diluent
3. Negative Control
4. Positive Control
5. Conjugate Diluent
6. Conjugate
7. Substrate Diluent
8. Substrate Concentrate
9. Wash Fluid

Reconstitution of Conjugate

Tap the bottle of conjugate gently on the bench to remove any material adhering to the robber stopper. Pipette about 2 ml. or about 5 ml. of Conjugate Diluent into the Conjugate bottle. Re-cap and allow to dehydrate for 5 to 10 minutes with occasional swirling and inversion. Before use, transfer the entire volume of dehydrated conjugate into the bottle of conjugate diluent using a transfer pipette and mix thoroughly. Label the bottle as containing syphilis conjugate. After reconstitution: The syphilis Conjugate may be stored at 2 to 8 ^{0}C for up to 10 days or frozen (-15 ^{0}C or colder) for up to three months. Do not freeze thaw more than 11 times.

Substrate Solution

To prepare the substrate solutions add a volume of colorless substrate diluent to an equal volume of pink substrate concentrate in either a clean glass container or a new polystyrene vessel. It is extremely important that this order of addition is followed and that any pipettes and glassware used to prepare substrate solution are clean. Alternatively, the substrate solution may be prepared by pouring the entire contents of the bottle of substrate diluent into the bottle of substrate concentrate. One bottle of substrate solution provides sufficient for at least five plates – see Table below:

Table 6. Preparation of substrate solution for Ice Syphilis

Number of wells											Number of plates			
8	16	24	32	40	48	56	64	72	80	88	1	2	3	4
Substrate Concentrate (ml)														
1.0	1.5	2.0	2.5	2.5	3.0	3.5	4.0	4.5	4.5	5.0	6	12	18	22
Substrate Diluent (ml)														
1.0	1.5	2.0	2.5	2.5	3.0	3.5	4.0	4.5	4.5	5.0	6	12	18	22

Addition reagent may be required for use with automated systems. Keep away from direct sunlight. The Substrate Solution should be pink; if it is purple before being used it should be discarded and fresh Substrate Solution prepared. The prepared Substrate Solution is stable refrigerated (2 to 8 ^{0}C) or at 15 to 25 ^{0}C for up to two days but must be discarded if crystals have formed.

Reconstitution of Wash Fluid

Dilute the wash fluid 1 in 20 with distilled or demonized water to give the require volume or dilute the entire contents of one bottle of wash fluid to a final volume of 2500 ml. When diluted the wash fluid contains 0.01% bronidox preservative. Store the working wash fluid at 18 to 30 ^{0}C in a closed vessel under which conditions it will retain activity for one month.

1.4 Test Procedure

Addition of the various components of the assay to the wells may be confirmed visually by examining the plate for the following colors. Sample diluent is green/brown in color. On addition of the sample or control the color will change to blue/green. The color change will vary from sample to sample but some change should always be visible. Reconstituted conjugate is red in color. Substrate solution is initially pink with any reactive wells becoming purple. On addition of stop solution the purple color of the reactive will change to orange, whilst the negatives remain pink.

Step 1 Reconstitute and mix the conjugate, prepare the substrate solution and wash fluid.

Step 2 Use only the number of wells required for the test.

Step 3 Add 50 µl of sample diluent to each well.

Step 4 Add 50 µl of sample or controls to the wells. For each plate use the first column of wells for the assay controls. Add the controls to the designated wells after dispensing the samples. Pipette 50 µl of the negative control into each of three wells [A1 to C1] and 50 µl of syphilis positive control into well D1. Use of a white background will aid visualization of Sample addition.

Step5 Cover the wells with the lid and incubate for 30 minutes at 37^0C

Step6 At the end of the incubation time wash the plate as described under wash procedures.

Step7 Immediately after washing the plate, add 50 µl of Conjugate to each well.

Step8 Cover the wells with the lid and incubate for 60 minutes at 37^0C

Step9 At the end of the incubation time wash the plate as described under wash procedures.

Step10 Immediately after washing the plate, add 100 µl of Substrate Solution to each well.

Step11 Cover the wells with a lid and incubate for 30 minutes at 37 ^{0}C.

Step12 Add 50 μl of stop solution (0.5M to 2M Sulphuric Acid) to each well.

Step13 Within 15 minutes read the absorbance at 450 nm. Wave length if available. Blank the instrument on air (no plate in the carriage). Wells containing either sample diluent or sample diluent and sample may be left at room temperature for up to one hour before starting Step5.

Protocol for Automated Stripwasher

Perform 5 wash cycles using working strength Wash Fluid. Ensure, where possible, that:

(i) The fill volume is 500 μl/well with instrumentation, ensure that the well is completely filled.

(ii) The dispenser height is set to completely fill the well without causing an overflow.

(iii) The time taken to complete one aspirate/wash/soak cycle is approximately 30 seconds.

(iv) Ensure that no liquid is left in the well (by use of a double aspirate step in the final cycle where possible).

1.5 Results: **Calculation**

Each plate must be considered separately when calculating and interpreting results of the assay, regardless of the number of plates processed at the same time.

Negative control. Calculate the mean absorbance of the negative controls.

Example:

Well 1 = 0.084, well 2 = 0.086, Well 3 = 0.070

Total = 0.240

Mean negative control = 0.240/3 = 0.080

If one of the negative control wells has an absorbance more than 0.15 above the mean of all three discards that value and calculate the new negative control mean from two remaining replicates.

Cut-off Value. Calculate the cut-off value by adding 0.2 to the mean of the negative control replicates.

Example:

Mean Negative control = 0.080

Cut-off Value = 0.080 + 0.200 = 0.280

1.6 Quality Control

Results of an assay are valid if the following criteria for the controls are met:

Negative control: The mean absorbance is less than 0.15

Positive control: The absorbance of the positive control is more than 0.8 above the mean absorbance of the negative control.

1.7 Interpretation of Results

Negative results: Samples giving an absorbance less than the cut-off values are considered negative in Ice Syphilis.

Reactive Results: Samples giving an absorbance equal to or greater than the cut-off values are considered initially reactive in the assay. Such samples should be retest in duplicate using the original source, Sample that are reactive in at least one of the duplicate retest are considered repeatedly reactive in Ice Syphilis and are presumed to contain antibodies against *T. pallidum*. Such samples should be further investigated. Samples that are non-reactive in both wells on retest should be considered non-reactive for antibodies against *T. pallidum*.

No sample addition: Absorbance values significantly higher than the negative control may be obtained in wells where the sample has been omitted but all the regents have been added.

1.8 Limitations of the Procedure

1. The test procedure and interpretation of results must be followed.
2. This test is to be used only with individual (unpooled) serum or plasma samples. Ice Syphilis has not been evaluated for any other purpose.
3. A negative result with an antibody detection test does not preclude the possibility of infection.
4. Non-repeatable reactive results may be obtained with and EIA procedure.
5. The most common sources of error are:
    a) Imprecise delivery of sample, conjugate or substrate into the wells.
    b) Contamination of substrate with conjugate.
    c) Contamination with conjugated from other assays.
    d) Blocked or partially blocked washer probes.
    e) Insufficient aspiration leaving a small volume of wash fluid in the wells.
    f) Failure to ensure that the bottom surface of the wells is clean and dry, and that no air bubbles are present on the surface of the liquid in the wells before a plate is read.
    g) Failure to read at the correct wavelength (450 nm.) or use of an incorrect reference wavelength.
6. Use of highly hemolysed samples, incompletely clotted sera, plasma samples containing fibrin or samples with microbial contamination may be obtained with samples from cadavers.

2. Bioelisa Syphilis

2.1 Intended Use

Bioelisa Syphilis is designed for detection of antibodies to *T.pallidum* in human serum or plasma.

2.2 Principle of the Procedure

Bioelisa syphilis is an immunoenzymatic method in with the well of a microtiter plate. Plates were coated with antigens representing epitopes of Treponema pallidum. Serum or plasma samples are added to these wells. If antibodies specific for *T.pallidum* are present in the sample, they will form stable complexes with the antigens on the well. After washing to remove the unbound material, a rabbit conjugate anti-human IgG and anti-human IgM labeled with horseradish peroxidase is added and, if the antigen/antibody complex is present, the conjugate will bind to the complex. After second wash, an enzyme substrate solution containing a chromogen is added. This solution will develop a blue color if the sample is positive. The blue color change to yellow after blocking the reaction with sulphuric acid. The intensity of color is proportional to the anti-*T.pallidum* antibody concentration in the sample. Wells containing negative samples remain colorless.

2.3 Components

1. Microtiter plate: Antibody Coated Wells
2. Sample Diluent
3. Negative Control [contains yellow dye]
4. Positive Control [contains green dye]
5. Conjugate Diluent [contains yellow dye]
6. Concentrated conjugate [contains green dye]
7. Substrate solution

8. Chromogen [TMB]
9. Washing solution [concentrated 10x]
10. Stopping solution [1N sulphuric acid]
11. Adhesive seals [cover plate during incubation]

2.4 Test Procedure

Previous Operations

Allow all the reagents to reach room temperature (20-25 ^{0}C) before running the assay. Gently mix all liquid reagents before use. Dilute the concentrated washing solution 1/10 with distilled or deionized water. Dilute the concentrated conjugate 1/51 with the conjugate diluent according to table below. Mix thoroughly. The diluted conjugate is stable for 15 days at 2-8 ^{0}C.

Table 7. Preparation of conjugate solution for Bioelisa Syphilis

Strips used	1	2	4	6	8	10	12
Conjugate Diluent ml	1.0	2.0	4.0	6.0	8.0	10.0	12.0
Conc. conjugate µl	20	40	80	120	160	200	240

Assay Procedure:

1. Use only number of strips required for the test. Reserve 6 well for blank and controls. Pipette to the rest of the well 200 µl. of sample diluent and 10 µl. of each sample to the designated wells.

2. Transfer 200 µl. of negative control to 3 wells and 200 µl. of positive control to 2 wells. Do not dilute controls. They are ready to use. Leave a well empty for the blank.

3. Cover the plate with adhesive seal mix gentry and incubate for 1 hour at 37 ^{0}C

4. Remove and discard the adhesive plate cover. Aspirate the content of the wells and fill them completely [approximately 300 µl.] with the diluted washing solution. Repeat the process of aspiration and washing 3 more times. After the last washing blot the microplate on absorbent tissue to remove any excess liquid from the wells.

5. Transfer 100 µl. of diluted conjugate into each wells of microplate, except the blank.

6. Cover the with the adhesive seal and incubate for 30 minutes at 37 ^{0}C

7. During the last 5-10 minutes of this incubation prepare the substrate chromogen solution. If the entire plate is used add 280 µl. of chromogen [TMB] to the bottle containing the substrate buffer [14 ml.] and mix wells. If the entire is not used, follow table below. The final solution should be colorless: discard if it become blue.

Table 8. Preparation of substrate solution for Bioelisa Syphilis

Strips required		1	2	4	6	8	10	12
Substrate buffer	ml	1.0	2.0	4.0	6.0	8.0	10.0	12.0
Chromogen solution	µl	20	40	80	120	160	200	240

Note: As the melting point of the DMSO is 18 ^{0}C, the chromogen solution should be to reach a temperature of 20-25 ^{0}C, and be well mix before use. A yellowish color is normal for the chromogen solution.

8. Remove and discard the adhesive plate cover. Aspirate and wash the plate as in step.4

9. Add 100 µl. of substrate solution to each well.

10. Incubate uncover for 30 minutes at room temperature.

11. Stop reaction by adding 100 µl. of stopping solution in the same sequence and time intervals as for the substrate solution.

12. Blank the spectrophotometer at 450 nm, use reference filter at 620 nm. with the blank well and read the absorbance of each well within 30 minutes.

2.5 Quality Control

The validity of an assay requires the accomplishment of the following points:

Negative control mean: Absorbance of individual of negative control values must be less than or equal to 0.200. If one value is outside this range, discard this value and recalculate the mean. If two values are outside range, the run should be repeated.

Positive control mean: The positive control mean must be equal to or greater than 0.600. If the mean is less than 0.600 the run should be repeated.

2.6 Results

The present or absence of anti-*T.pallidum* antibodies in the samples analyzed is determined by relating the absorbance value of each sample to cut-off value of the technique. This value is the mean value obtained for the negative control plus 0.300

Cut-off = NCx + 0.300

- If the initial test result absorbance value is less than the calculated cut-off value the sample is considered non-reactive.
- If the screening value of a sample is equal to or greater than the cut-off, retest it in duplicate. If both retest values are lower than the cut-off, the interpretation of the total testing is non-reactive for *T.pallidum* antibodies. If both retest values are equal to or greater than the cut-off or if one of the duplicate is equal to or greater than the cut-off and one is lower than the cut-off, the interpretation of the testing result is

repeated reactive. The sample should be considered reactive or positive for *T.pallidum* antibodies by the criteria of this test.

- A negative result does not exclude the possibility of exposure or infection with *T.pallidum*.

3. New Market Syphilis EIA

3.1 Intended Use

New market syphilis EIA is designed for detection of antibodies to *T.pallidum* in human serum or plasma.

3.2 Principle of the Procedure

New Market Syphilis EIA use three recombinant antigens in a sandwich test to produce a test that is both highly specific and sensitive. The antigens will detect *T. pallidum*-specific IgG, IgM, and IgA, enabling the test to detect antibodies during all stages of infection.

All reagents except the conjugate and wash solution are supplied ready to use and color coded, and the procedure uses undiluted samples and standard volumes for ease of both manual and automated use. The assay can be used with both serum and plasma.

3.3 Components

3.3.1 Plate (96 wells, coated with antigens)

3.3.2 Positive control (Red)

3.3.3 Negative control (Yellow)

3.3.4 Conjugate (Blue)

3.3.5 Conjugate dilution buffer (Green)

3.3.6 Substrate (TMB/Peroxide) (Pink)

3.3.7 Wash (10 x Conc.) (clear)

3.3.8 Stop (0.5 M Sulphuric acid)(clear)

3.3.9 Bag for storing unused wells

Storage

All reagents are stable at 4^0C until stated expiry date.

Do not freeze.

Do not expose substrate to direct sunlight.

3.4 Test Procedure

Bring all reagents to room temperature prior to use. Use fresh serum or plasma samples. Dilute wash buffer 1/10 in distilled water prior to use.

2.4.1 Add 50 µl. undiluted sample to each well. Incubate at 37 ^{0}C for 30 minutes.

2.4.2 Wash 5 times with working strength wash buffer. A short soak time of about 30 seconds is recommended between each wash cycle. Tap out excess liquid.

2.4.3 Dilute conjugates 1 + 10 in conjugate buffer [50 µl. +500µl. per 10 wells]. Add 50 µl. diluted conjugate to each well. Incubate at 37 ^{0}C for 30 minutes.

2.4.4 Wash 5 times with working strength wash buffer. A short soak time of about 30 seconds is recommended between each wash cycle. Tap out excess liquid.

2.4.5 Add 50 µl. substrate to each well. Incubate at room temperature for 30 minutes. As the substrate is light sensitive it is recommended that the plate be protected from light during this incubation.

2.4.6 Add 50 µl. sulphuric acid to each well. Blue color change to yellow.

2.4.7 Read at 450 nm. and use reference filter at 620-690 nm.

3.5 Quality Control

Positive and negative controls provide should be run with each batch of test. Use diluted conjugate within 8 hours. Read assay within 30 minutes of adding the stop reagent.

3.6 Results

$OD \leq 0.15$ = Negative

$OD > 0.15$ = Positive

The assay is valid if the kit negative OD is ≤ 0.15 and the kit positive OD is > 1.0.

4. New Market TPHA Screening

4.1 Intended Use

Will detect antibodies to *T. pallidum* in human sera & plasma using micro haemagglutination.

4.2 Principle of the Procedure

New market TPHA screening uses preserved avian erythrocytes coated with antigens of *T. pallidum* (Nichol's strain) to bind with specific antibody present in patient sera or plasma. The cells are suspended in diluent containing components to eliminate non-specific reactions. Positive reactions are characterized by haemagglutination. Eye or plate reader may interpret test patterns.

4.3 Components

4.3.1 Test cells [Preserved chicken erythrocytes coated with antigens of *T.pallidum*]

4.3.2 Control cells [Preserved chicken erythrocytes]

4.3.3 Positive control [Human sera or defibrinated plasma]

4.3.4 Negative control [Human sera or defibrinated plasma]

4.3.5 Diluent saline solution containing absorbents

All reagents contain less than 0.1% sodium azide.

Storage

All reagents are stable at 4^0C until shelf life stated on reagent labels.

Store bottles in an upright position. Do not freeze.

2.4 Test Procedure

2.4.1 Bring all reagents to room temperature prior to use.

2.4.2 Use fresh sera or plasma.

2.4.3 Use U well plates.

2.4.4 Sample Dilution (1/20)

Add 190 µl of TPHA Diluent to a well. Add 10 µl of sample to the same well. Ensure thorough mixing. Positive & negative controls provided should be treated as samples (Diluted 1/20)

2.4.5 Assay

Add 25 µl of diluted sample from 2.4.4 to test well. Gently mix test cells to ensure thorough resuspension.

Add 75 µl of test cells to test well. Ensure thorough mixing. Final sample dilution after addition of cells is 1/80.

Incubate at room temperature on a vibration free surface for a minimum of 45 minutes.

Read & interpret the settling pattern.

2.4.6 Interpretation

	Test cells	Control cells
Positive: Strong	Full cell pattern covering the bottom of the well.	Negative
: Weak	Cell pattern covers approximately 1/3 of the well bottom.	Negative
Indeterminate	Cell pattern shows a distinctly open center	Negative
Negative	Cells settled to a compact, typically with a small clear center.	Negative
Non-specific*	Positive	Positive

***Absorption of Non-specific Reactions**

1. Add 10 µl of sample to 190 µl. of resuspended control cells, mix & incubate for 30 minutes.
2. Centrifuge for compact the cells at 2000 rpm for 3 minutes.
3. Add 25 µl of supernatant from step 2 to each of 2 wells.
4. Gently mix the test & control cells to ensure thorough resuspension.
5. Add 75 µl of test cells to the 1st well.
6. Add 75 µl of control cells to the 2nd well.

7. Ensure thorough mixing & incubate at room temperature for a minimum of 45 minutes.
8. Read & interpret the settling pattern.

Data processing and analysis

Table 9. Statistical formula, for analyses data between TPHA and RPR.

	TPHA		Total
RPR	**Positive**	**Negative**	
Positive	**a**	**b**	
Negative	**c**	**d**	
Total			

a = True positive (TP), when samples were positive in both TPHA, RPR and confirmed to have syphilis

b = False positive (FP), when samples were positive with RPR but negative with TPHA

c = False negative (FN), when samples were negative with RPR but positive with TPHA

d = True negative (TN), when samples were negative in both TPHA and RPR

Table 10. Statistical formula, for analyses data between TPHA and ELISA.

	TPHA		Total
ELISA	**Positive**	**Negative**	
Positive	**a**	**b**	
Negative	**c**	**d**	
Total			

a = True positive (TP), when samples were positive in both TPHA, ELISA and confirmed to have syphilis

b = False positive (FP), when samples were positive with ELISA but negative with TPHA

c = False negative (FN), when samples were negative with ELISA but positive with TPHA

d = True negative (TN), when samples were negative in both TPHA and ELISA

Definition and calculated formula for sensitivity, specificity, positive predictive value and negative predictive value

Sensitivity = Proportion of positive test result among diseased persons

$$= \frac{a}{a+c} \qquad = \frac{TP}{TP+FN}$$

Specificity = Proportion of negative test result among non-diseased persons

$$= \frac{d}{b+d} \qquad = \frac{TN}{FP+TN}$$

Positive Predictive Value = Proportion of diseased among those with positive test result

$$= \frac{a}{a+b} \qquad = \frac{TP}{TP+FP}$$

Negative Predictive Value = Proportion of non-diseased among those with negative test result

$$= \frac{d}{c+d} \qquad = \frac{TN}{FN+TN}$$

Calculation of percentage the sensitivity, specificity, and predictive value of a diagnostic test, I used the formula below.

$$\text{\% Sensitivity} = \frac{\text{true positives}}{\text{true positives + false negatives}} \times 100$$

$$\text{\% Specificity} = \frac{\text{true negatives}}{\text{false positives + true negatives}} \times 100$$

$$\text{\% Positive predictive value} = \frac{\text{true positives}}{\text{true positives + false positives}} \times 100$$

$$\text{\% Negative predictive value} = \frac{\text{true negatives}}{\text{true negatives + false negatives}} \times 100$$

CHAPTER V
RESULTS

The result of 1,250 unselected donor samples which were tested by Bioelisa , Ice Syphilis [Murex] , New market and RPR was shown in table 11 . For Bioelisa when compared the result with result of TPHA which we used as gold standard, true positive [Bioelisa and TPHA positive] was observed in 13 samples (1.04 %) while true negative [Bioelisa and TPHA negative] were observed in 1,219 (97.52%) samples. Bioelisa gave 8(34.78%) false negative result and 10 (43.48%) false positive results.

Comparing data between Ice Syphilis [Murex] and TPHA (table-11), it had 20(1.60 %) total positive samples and 14(1.12 %) samples were true positive results, but 6(30.00%) samples were false positive results. Ice Syphilis [Murex] had negative results in 1,230 samples and 1,223 were true negative, while 7 were false negative .

New market gave positives result in 16 samples (1.28%), only one (06.25%), sample was false positive. There were 1234 negative results, in which 1,228 were true negative . RPR which was currently screening test for blood donor gave 13 (1.04 %) positive, 10 (0.80 %) were true positive and 3(23.07%) were false positive. In 1,237 RPR negative samples , 11(0.89%) were false negative and 1,226 samples were true negative results.

The Initial Reactive (IR) and Repeated Reactive (RR) was shown in table table15 . Reductive of reactive rate for Ice Syphilis Bioelisa, New Market, and RPR [shield] were 6 (30.00%) and 10(43.48%), 1(06.25%), and 3(23.07%) respectively. Then, if we need to choose the assay for syphilis screening, these reductive numbers should to consider for expense and unit cost. The distribution of ratio of optical density /cut-off when unselected 1250 samples were tested with various assay was shown in figure 3.

Specificity calculation , which answers the question “Among patients who do not have the disease, what proportion or percentage have a negative test” was shown in table 12 . In this research, when tested each assays with 1,250 unselected samples. All assays had specificity around 99%. New market had highest specificity (99.92%) and Bioelisa had lowest specificity (99.18%) which was less than RPR .

In clinical practice, however, a clinician's question is “If the patient has a positive test, how likely is he or she to have the disease?” (What is the predictive value of a positive test, or PPV?) Alternatively, “If the patient has a negative test, how likely is he or she not to have the disease?” (What is the predictive value of a negative test, or NPV?). In unselected donor samples group, New Market gave highest PPV (93.75%) and Bioelisa had lowest PPV (56.50%). PPV for Ice Syphilis, RPR were 70.00%, 76.92% respectively. All assays had NPV around 99% which New Market had highest value (99.51%) and RPR had lowest value (99.11%).

In unselected donor samples group, there were twenty-one positive result with TPHA, which could separated three groups. Seven samples gave positive result with all assays and 14 samples had discrepancy result between various assay . The result and detail of ratio of optical density /cut-off was shown in table 13 and 14.. In seven samples that gave positive with all assays, the New market had ratio values (S/CO) higher than Bioelisa and Ice Syphilis, while Bioelisa had less value than New market and Ice Syphilis. In 14 discrepancy samples between TPHA and any one of ELISA kit, TPHA had highest agreeable result with TPHA while RPR had lowest agreeable result with TPHA.

After following these 21 donors, which positive with gold standard (table13 and 14), 5 donors were first time donors (4 of 5 disappear after donated blood and another one was negative at 20 month later). The following results showed that, 4 donors were positive at three months later, 3 donors were positive at six months later and 2 donors were positive at one year later. Otherwise, 5 donors were negative at more one year later.

The results of positive samples (N = 206), 202 (98.06%) were reactive by the Ice Syphilis, 191 (92.72%) by Bioelisa, 202 (98.06%) by New Market and 155 (75.24%) by RPR.

Sensitivity which answers the question " Among patients who have the disease, what proportion or percentage will have a positive test? ". In this study, sensitivity of New market equal with Ice Syphilis was 98.06 % (highest sensitivity). Incontrast, RPR had lowest sensitivity (75.24%). Also, sensitivity of Bioelisa was 92.72 %. In positive samples group, 15 samples had discrepancy results for three ELISA. Fifteen samples were negative with Bioelisa (ratio less than 1.000), but only 4 samples for New market and Ice syphilis, also 13 samples for RPR. Eleven samples in these 15 samples group, were positive for New Market and Ice Syphilis with high ratio, and also 2 samples were RPR reactive with titer 1:1 or prediluted. There were four samples negative with three ELISA and RPR, these samples positive with TPHA (positive with Omega reagent, which used for screening at RBC, TRC and New Market in this study).

Coefficients of agreement between assays are shown in Table 21. Overall assay, were shown around 99 % coefficients of agreement with TPHA(TP+TN). New market was shown highest agreement (99.44%) and lowest for Bioelisa (98.56%), also 98.96%, 98.88% for Ice syphilis and RPR respectively.

Table11. Test results of unselected samples (N=1,250)

		TPHA		Total
		Pos	Neg	
BIOELISA	Pos	13	10	23
	Neg	8	1219	1227
	Total	21	1229	1250
MUREX ICE SYPHILIS	Pos	14	6	20
	Neg	7	1223	1230
	Total	21	1229	1250
NEW MARKET	Pos	15	1	16
	Neg	6	1228	1234
	Total	21	1229	1250
RPR [Shield]	Pos	10	3	13
	Neg	11	1226	1237
	Total	21	1229	1250

Table 12. Specificity, PPV and NPV of three ELISA and RPR assays

Trade Name	**Specificity**	**PPV**	**NPV**
New Market	99.92 %	93.75 %	99.51 %
RPR [shield]	99.75 %	76.92 %	99.11 %
Ice Syphilis	99.51 %	70.00 %	99.43 %
Bioelisa	99.18 %	56.50 %	99.35 %

Table 13. Concordant results of unselected samples (n = 1,250)

Samples ID	New market	Ice syphilis	Bioelisa	RPR	T*	Sex	Age	Results in routine testing (following results)
4-12461	22.364	9.55	2.523	+	12	M	43	Positive (3 Months later)
7-16863	21.169	12.368	1.282	+	1	W	38	Disappear***
7-16862	20.2	11.505	1.524	+	1	M	38	Disappear***
3-14089	16.15	9.692	2.657	+	2	M	60	Disappear***
3-14137	9.77	10.569	3.383	+	23	M	39	Positive (6 Months later)
3-14172	6.636	11.375	6.809	+	21	M	42	Disappear***
3-14100	1.508	2.106	1.164	+	15	M	43	Negative (9 Months later)

Table 14. Discrepancy results of unselected samples (all samples, TPHA positive)

Samples ID	New market	Ice syphilis	Bioelisa	RPR	T*	Sex	Age	Results in routine testing (following results)
4-12507	17.763	11.418	2.74	-	17	M	44	Positive (3 Months later)
3-14175	16.343	11.214	5.6	-	17	W	47	Positive (6 Months later)
G-18013	7.862	2.271	1.23	-	4	W	37	Disappear***
J-12475	7.759	0.21	2.393	-	34	M	47	Positive (12 Months later)
H-13357	7.582	10.04	0.467	-	1	M	35	Disappear***
A-08356	5.98	5.062	1.211	-	62	M	53	Positive (3 Months later)
N-04728	4.4	5.357	0.882	+	13	W	50	Positive (3 Months later)
4-12523	1.139	6.605	0.333	-	23	M	41	Negative (18 Months later)
A-01720	0.293	0.634	2.865	+	42	M	52	Negative (19 Months later)
5-11179	0.197	0.238	0.133	+	1	M	22	Negative (20 Months later)
6-11016	0.182	0.246	0.142	-	1	M	32	Disappear***
5-11201	0.146	0.238	0.089	-	3	W	21	Positive (12 Months later)
3-14154	0.136	0.796	0.428	-	3	W	41	Positive (6 Months later)
1-11037	0.133	0.268	0.483	-	4	M	21	Negative (6 Months later)

T* = Time of donations *** donor no comeback

Table.15. Test results of positive samples

		TPHA	Total
		Pos	
BIOELISA	Pos	191	191
	Neg	15	15
	Total	206	206
MUREX ICE SYPHILIS	Pos	202	202
	Neg	4	4
	Total	206	206
NEW MARKET	Pos	202	202
	Neg	4	4
	Total	206	206
RPR [Shield]	Pos	155	155
	Neg	51	51
	Total	206	206

Table16.Sensitivity, PPV and NPV of three ELISA and RPR assays.

Trade Name	**Sensitivity**
Bioelisa	92.72 %
Ice Syphilis	98.06 %
New market	98.06 %
RPR	75.24 %

Table 17. Discrepancy results for three ELISA in positive samples testing

No	Samples ID	New Market	Ice Syphilis	Bioelisa	TPHA	RPR
1	10145-J-01045	17.732	10.540	0.722	P	N
2	10145-M-03543	16.693	12.620	0.574	P	N
3	16545-1-09373	9.273	13.518	0.726	P	R
4	11245-0-00059	6.709	13.033	0.486	P	N
5	11245-1-05542	6.492	13.029	0.145	P	N
6	35045-1-07685	6.195	12.066	0.758	P	N
7	35045-4-01109	4.836	5.761	0.676	P	N
8	10145-J-01044	3.488	11.208	0.890	P	W
9	10145-M-3242	2.578	5.607	0.381	P	N
10	10145-M-02945	2.078	10.849	0.623	P	N
11	10145-M-03280	1.250	3.798	0.132	P	N
12	14945-1-24782	0.933	0.474	0.258	P	N
13	10145-J-00681	0.455	0.599	0.382	P	N
14	10145-M-03263	0.303	0.313	0.097	P	N
15	35045-1-07855	0.297	0.265	0.409	P	N

Table 18. Mean of samples OD. , Cutoff and O.D. / Cut off for three ELISA assays when tested with unselected and positive samples

Trade Name	Tested with unselected samples			Tested with Positive samples		
	OD.	Cutoff	S/CO	OD.	Cutoff	S/CO
Bioelisa	0.077	0.330	0.234	0.747	0.317	2.356
New Market	0.044	0.141	0.315	2.601	0.131	20.057
Ice Syphilis	0.171	0.295	0.584	3.166	0.274	11.561

Table19. A total of initial and repeated reactive in unselected samples

Trade Name	Initial Reactive	Repeated Reactive	Total Reduction
Ice Syphilis	20 [1.60%]	14 [1.12%]	6[30.00%]
Bioelisa	23 [1.84%]	13 [1.04%]	10[43.48%]
New Market	16 [1.28%]	15 [1.20%]	1[06.25%]
RPR [shield]	13 [1.04%]	10 [0.80%]	3[23.07%]

Figure 3: distribution of S/CO for three ELISA in unselected samples

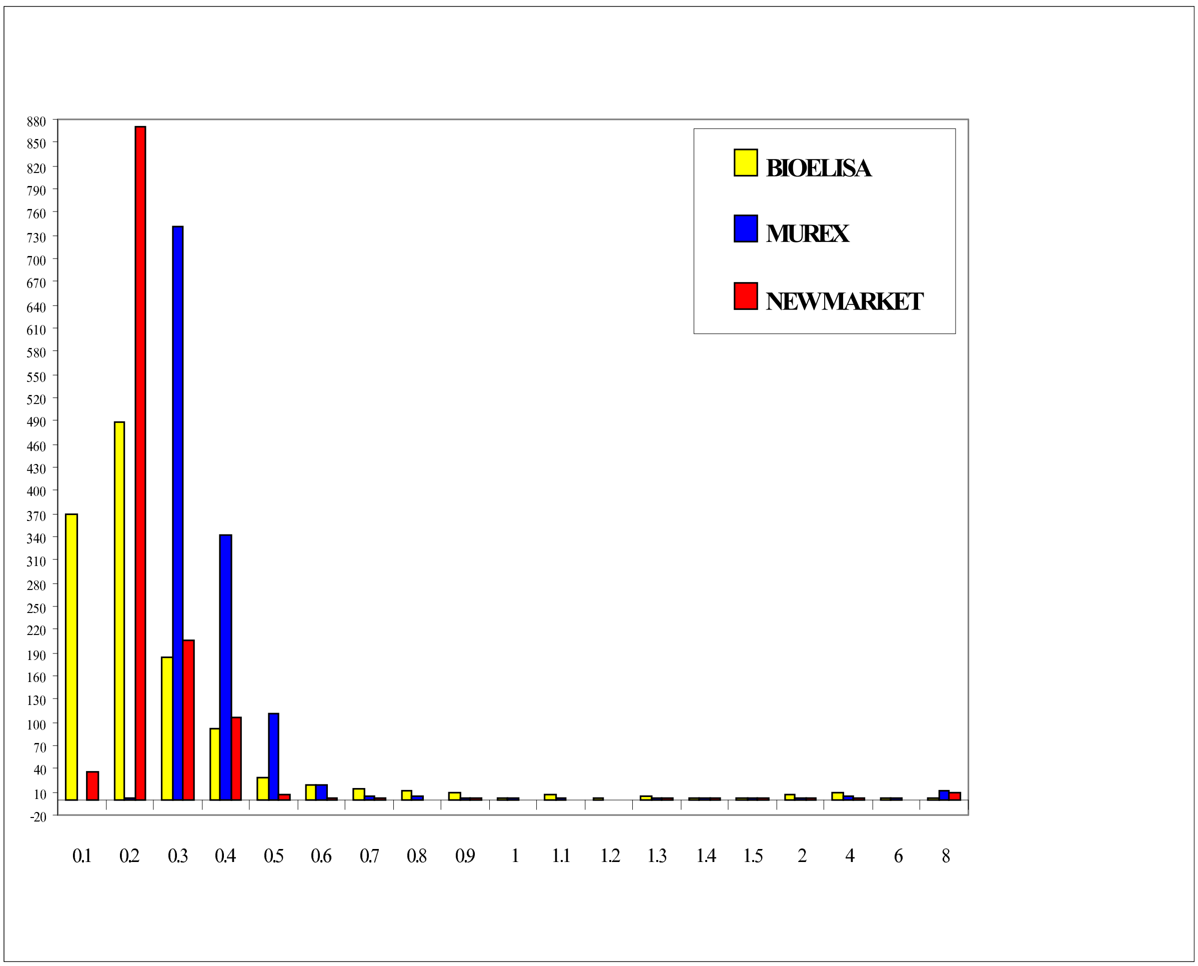


Table 20. Characteristic of three ELISA assays for detection of *T. pallidum* antibodies

Trade Name	**Murex**	**Bioelisa**	**New Market**
Predilution (serum+diluent)	No	Yes (10+200)	No
Serum (µl)	50	100	50
Diluent (µl)	50	0	0
Dilution	1:2	1:21	0
Antigen	Rec.TpN15	Rec.TpN15	Rec.TpN15
	Rec.TpN17	Rec.TpN17	Rec.TpN17
	Rec.TpN47		Rec.TpN47
Method	Capture	Sandwich	Sandwich
No.of incubations (min)	120	120	90
Cutoff	NC+0.200	NC+0.300	NC+0.100
Detection of Ig	IgM+IgG	IgM+IgG	IgM+IgG+IgA

Table 21. Summarize data of each assay.

	BIOELISA	MUREX ICE	NEW MARKET	RPR [Shield]
Control				
Mean of PC OD.	1.29	2.87	1.88	-
Mean of NC OD.	0.03	0.10	0.04	-
%CV of NC	56.56	23.83	44.08	-
%CV of PC	43.59	8.51	19.38	-
Unselected Samples n = 1,250				
IR	30 (2.4%)	23 (1.84%)	18 (1.44%)	13 (1.04%)
RR	23 (76.66%)	20 (86.95%)	16 (88.88%)	13 (100%)
FP (TPHA= Neg.)	10	6	1	3
FN (TPHA= Pos.)	8	7	6	11
% Agreement	98.56%	98.96%	99.44%	98.88%
(TP+TN)	(1232/1250)	(1237/1250)	(1243/1250)	(1236/1250)
PPV	56.50	70.00	93.75	76.92
NPV	99.35	99.43	99.51	99.11
Mean of OD	0.077	0.171	0.044	-
Mean of Cut off	0.330	0.295	0.141	-
Mean of S/CO ratio	0.234	0.584	0.315	-
Specificity	99.18	99.51	99.92	99.75
Positive Samples n = 206				
TP	191	202	202	155
FN (TPHA= Pos.)	15	4	4	51
Mean of OD	0.747	3.166	2.601	-
Mean of Cut off	0.317	0.274	0.131	-
Mean of S/CO ratio	2.356	11.561	20.057	-
Sensitivity	92.72	98.06	98.06	75.24

CHAPTER VI
DISCUSSION

All samples were tested with ELISA, RPR and TPHA within 24 hours including the repeated tests after initial reactive. The titer or diluted method is unnecessary for this study, because I need to know which tests have reactive or non-reactive results when standard assay (TPHA) were reactive or non-reactive. Otherwise, RPR may have titer lower than 1:8 if samples obtain from the active syphilis persons and they were syphilis in late stage, which normally low titer. The color code which were color change after adding samples or reagent were useful to monitor adding samples or reagent and I can checked or observed again before I put the microplate into the automatic processing machine (BEP III).

Data in this research came from using only the kit and lot that were specified in materials and methods and in donor population. Now, each reagent may develop or upgrade to new version (e.g. Bioelisa) and sometime may have lot variation. Then, these data could not use for another kit lot. Also, other factors were considers (e.g. population, equipment) when decide to use these reagent in laboratory.

The Ice Syphilis and New Market have clear background, so they have little doubtful results and interpretation more simple than Bioelisa.

TPHA (New Market) was easy to done, but the precaution of this assay was to avoid the vibration or shake area and heat source. Because this assay used created red cell of chicken as test cell and control cell, then the vibration may move the cell to the central of well and false negative can occur in this event.

Overall, data from this research indicate that New market is the best assay, because it had highest both specificity and sensitivity. While, RPR had lowest sensitivity this assay was common used in routine blood bank and can not detected the late syphilis persons because these person may lack of non specific antibodies (Anti-lipoidal) or were treated already. Then, RPR assay had many false negative results and had shown only 75.24 % of sensitivity. Also, only RPR not recommend for blood screening. Many countries in Europe, they used combine assays for blood screening in blood bank e.g. RPR combine with TPHA or ELISA with VDRL.

Specificity is the ratio of subjects with a negative test result to all uninfected subjects (true negative fraction). Test specificity is of maximum concern when testing patient populations having a low prevalence of disease. A high specificity suggests that test is good for “ruling in” disease. High specificity is also important when the consequences of a false positive test are serious.

Specificity of three ELISA and RPR were very close value, same as NPV value which both specificity and NPV more than 99 %. The value of NPV and specificity were differed less than 1 %, but were too differed the PPV value. New market had shown highest PPV value (93.75%) indicate that it had false positive results less than other assays or had true positive results more than other assays. Opposite for Bioelisa, it had lowest PPV value indicate that it had false positive results more than other assays.

PPV and NPV depend not only on the test sensitivity and specificity (stable properties of the test), but also on the prevalence of the disease in the population examined. As prevalence of disease decrease, the proportion of individuals with a positive test result who actually are infected falls, and the proportion of uninfected persons who are falsely identified as being infected rise. Tests with high NPV are desirable when it is essential not to miss any infection.

It is the ratio of subjects with a positive test result to all infected subjects. Test sensitivity is of maximum concern in patient populations having a high prevalence of disease. A high sensitivity suggests that the test is good for ruling out disease. High sensitivity is also important when the treatment available is very cost-beneficial and when the consequences of false-negative test are seriousness.

The mean O.D. of positive control for Ice Syphilis, Bioelisa and New Market are 2.87, 1.29, 1.88 respectively. Also the mean O.D. of negative controls for Ice Syphilis is 0.10 which higher than Bioelisa (0.03) and New Market (0.04), same as the mean O.D. of positive control. In this research, Bioelisa show the mean O.D. of negative controls and positive control lowers than New Market and Ice Syphilis. Otherwise, the O.D. of negative and positive control for each plate of three ELISA assays was in range.

The mean S/CO ratio value for Ice Syphilis was 0.584, Bioelisa 0.234 and 0.315 for New Market, when tested with negative samples and 2.356, 11.561, 20.057 for Bioelisa, Ice Syphilis, and New Market respectively when tested with positive samples. Base on same samples group, in negative samples group the mean S/CO ratio value for Ice Syphilis was highest but in positive samples group the mean S/CO ratio value for New Market was highest. Also, the mean S/CO ratio value for Bioelisa were lowest in two samples group.

The main influence on sensitivity can be attributed to the design of the assay: Sandwich-based tests, where the solid phase is coated with the antigen and after incubation with serum the bound immunocomplex is detected by an anti-human IgG or IgM conjugate. Competitive ELISAs also have a surface-bound antigen, but antibodies present in serum have to compete with added, labeled *T. pallidum* antibodies, resulting in low optical density (OD) values, if specific antibodies are present in tested serum. Capture ELISAs have anti-human IgG or IgM molecules bound to the microtiter well. After incubation with serum, a part of the human immunoglobulins is bound to the solid phase. In the second incubation, specificity is achieved with a complex of antigen and labeled anti-*T. pallidum* antibodies.

Principle of Method for Bioelisa Syphilis and New Market are used sandwich method [Plates were coated with antigens representing epitopes of Treponema pallidum. If antibodies specific for *T.pallidum* are present in the sample, they will form stable complexes with the antigens on the well. After washing to remove the unbound material, a rabbit conjugate anti-human IgG and anti-human IgM labeled with horseradish peroxidase is added and, if the antigen/antibody complex is present, the conjugate will bind to the complex]. While Ice Syphilis is used capture method [The unlabelled antigens are coated on the microwells with the anti-human-IgG and anti-human IgM. Serum or plasma samples are incubate in the wells and the specific antibodies against *T.pallidum*, if present, are capture by their related antigens on the plate. In addition a proportion of the total IgG and IgM present in the sample is capture by the anti-human antibodies]. Except IgM and IgG, only New Market can detected IgA antibodies.

The immune response involves production of non-specific antibodies as well as specific treponemal antibodies. The first demonstrable response to infection is the production of specific treponemal IgM, which may be detected at the end of the second week of infection. Specific treponemal IgM can be used to assess the stage of the infection. In untreated patients, its presence implies active disease. IgM becomes undetectable within 3-9 months after adequate treatment of early syphilis, but it may persist for 12-18 months after treatment of late disease. IgG appears at about 4 weeks. Non-specific antibodies and specific IgM decline rapidly after adequate treatment of early syphilis but specific IgG generally persists. HIV infection may reduce or delay immune response in primary syphilis but in most cases, the response is normal or exaggerated.

Laboratorians and clinicians should remember that use of a treponemal test, as a screening procedure will detect old case as well as active untreated case of syphilis. The syphilis IgM test kit that base on the use of anti-human IgM antibody to capture IgM in the specimen, followed by the use of a purified *T.pallidum* antigen to detect IgM antibodies in the patient's serum directed toward *T.pallidum*. This test most useful in the diagnosis of congenital syphilis. One study (Stoll, et al 1993) found that the IgM capture EIA was more sensitive than FTA-ABS 19S IgM test in detecting probable case of congenital syphilis. However, another study found the test to be equal in sensitivity to the IgM Western Blot for the detection of neonatal congenital syphilis but less sensitive than the Western Blot for detection of delay onset congenital syphilis. The usefulness of the Syphilis M test in adult onset syphilis has not been determined fully.

With the availability of individual *T.pallidum* antigens produced with recombinant DNA techniques. The test used of recombinant *T.pallidum* antigens in place of poorly defined mixtures of antigens from the Nichols strains of *T.pallidum*, which may be contaminates with rabbit testicular components, has the potential for improving the specificity of serological assays.

The coated antigens in the bottom of wells of microplate for Ice Syphilis are Rec.TpN15, Rec.TpN17 and Rec.TpN47, which same as New Market, but Bioelisa Syphilis coated only Rec.TpN15, Rec.TpN17. These antigens are membrane proteins of *T. pallidum*. TpN15 and TpN17 are lipoprotein and strongly antigenic. TpN47 is lipoprotein and the most abundant polypeptide, which as dominant antigen.

During primary syphilis, the earliest serum immunoglobulin (Antibodies), those responses to individual *T. pallidum* polypeptides are against TpN47 and some of the flagellar proteins, followed by TpN15 and TpN17. These antibodies appear to be diagnostic for acquired syphilis. Then, test coated with TpN47 antigen may be more sensitive than test did not coated with TpN47 antigen.

To date many investigators agree that the detection of antibodies to the immunodeterminants with molecular masses of 15.5, 17, 44.5 and 47 kDa appears to be diagnostic for acquired syphilis. Tests utilizing recombinant T.pallidum antigens are currently under evaluation. These include a rapid test utilizing 47, 17, 15.5 kDa recombinant proteins, an EIA and a western blot format recombinant assay. It is anticipated that serologic assays involving recombinant antigens will become more widely used in the future, given the difficulties in preparing T.pallidum antigen preparation from infected rabbit tissue.

Only Bioelisa Syphilis has to diluted serum (predilution) before put into the microwells. It used 10 µl. of samples include with 200 µl. of diluent, it has dilution 1:21. New Market do not used diluent and put samples only 50 µl., so it don't has dilution, but Ice Syphilis used diluent 50 µl. dilution include with 50 µl. of samples, so it has dilution 1:2. Then, antigens-antibodies reaction in New Market testing may occur, more often than Bioelisa Syphilis and Ice Syphilis.

Incubation times throughout processing, Ice Syphilis and Bioelisa Syphilis have 120 minutes, while New Market has 90 minutes. Then, New Market can completed running the process shorter than Bioelisa Syphilis and Ice Syphilis.

An important principle of syphilis serology is the detection of treponemal antibody by a screening test, followed by confirmation of a reactive screening test. The confirmatory test should ideally have equivalent sensitivity, greater specificity and be independent methodologically. Serology, however, cannot differentiate between the different treponematoses.

The testing strategy varies and depends on several factors, including whether the aim is to detect all stages of syphilis or only infectious syphilis. In the United States, France and Belgium, non-treponemal tests are used for screening. This does not detect most adequately treated cases, thus simplifying patient assessment. There are, however, several disadvantages. It may yield false negative reactions in early infection (prozone phenomenon) and with concomitant HIV infection. It also lacks sensitivity in late stage infections. In Germany and the Netherlands, the TPHA is used for screening. This provides a good screen for all stages beyond the early primary stage, but because more primary infections are detected by the VDRL/TPHA combination, the latter is the preferred strategy in the UK (Chan Yuin Chew. 1995). However the VDRL/TPHA combination is labor intensive requires subjective interpretation and cannot be easily automated.

FTA-abs is still generally regarded as the "gold standard". However, the TPHA is more sensitive, except in the third and fourth weeks of infection. The TPHA is also more specific. In addition, false negative FTA-abs results have been described in HIV infection. Overall, TPHA is the most appropriate test for confirming reactive non-treponemal tests or EIA results at present. FTA-abs is probably best reserved for discrepant results. Follow-up of seronegative patients at recent risk of STD is essential due to seronegative window in early primary syphilis

EIA tests can detect treponemal IgG and/or IgM. Published data show comparable results to the VDRL/TPHA combination and they are useful in HIV infection (Young I-I et al. 1992). A recent report suggested that a new recombinant antigen-based treponemal IgG and IgM EIA is the most sensitive treponemal test; it is also highly specific (Young I-I et al. 1992). The EIA produces objective results and allows easy automation. As a screening test, the EIA is replacing VDRL/TPHA in the UK. Laboratories with large workloads (>20 000 tests yearly) should use EIA (Chan Yuin Chew. 1995).

CHAPTER VII
CONCLUSION

The objective of this research, is to evaluate the sensitivity and specificity of three different Enzyme Linked Immunosorbent Assays (ELISA) and Rapid Plasma Reagin (RPR) assay, which common use in routine screening for syphilis. Also to compare the sensitivity and specificity between of three different ELISA assay. Sensitivity of New market is 98.06 %, while Ice Syphilis, Bioelisa, RPR are 98.06 %, 92.72 %, and 75.24 % respectively. When, I reviewed many literatures they show sensitivity and specificity of ELISA assay for syphilis detection less than 99.0 % or around 98.0 %. Then, I used this point (98%) as criteria for this research. All assays could pass these criteria for specificity, but only New market and Ice Syphilis could pass criteria for sensitivity. Overall, in two sample groups New market had show highest both sensitivity and specificity. ELISA may suitable for syphilis screening more than RPR. Because, if we use RPR assay for syphilis screening in routine laboratory, false negative results may occur more than other assay and also patient may receive blood, which contain syphilis disease.

From overall results, I can answer my research question (Are sensitivity and specificity of ELISA assay different from RPR assay for screening and detection of syphilis?), when I use these assays to test with blood donors.

Hypothesis of this research is sensitivity and specificity of ELISA assay different from RPR assay. The results could show sensitivity and specificity of RPR assays lower than three ELISA, so agreement with hypothesis.

EIA has practical advantages as a screening test for all stages of syphilis and it is recommended that laboratories with large workloads (eg. above 20,000 tests pa.) use this approach; depending on local circumstances it may also be appropriate for lower workloads. Details of several EIAs with acceptable performance characteristics have been published, but it is important to note that there is variation in treponemal EIA performance, as with other EIA tests.

However, when using any one of the ELISAs tested as a screening assay, one has to consider the facts that: (I) Special precautions should be taken in handling (e.g., washing of plates). (II) More data for specificity should be evaluated and (III) insufficient data are available at present to verify proper reactivity in late syphilis.

Compared to standard screening tests, more handling steps [(I) Preparation of serum dilution. (II) Dispensing the appropriate volume of diluted serum into the wells of the microtiter plate. (III) Dispensing of negative, positive, and cutoff controls. (IV) Addition of conjugate, substrate, and stop solution (V) Washings; incubation at elevated temperatures; and optical readings of the plates] are necessary for performing ELISAs.

If one has to make decisions on which EIA to use, one will also need to take account of other factors, including cost (all ELISAs are more expensive than the hemagglutination tests, VDRL or RPR), easy of use, availability of suitable automated processing equipment and compatibility with other EIA format tests already in use in the laboratory.

I hope the usefulness of this research are to known sensitivity and specificity of ELISA and RPR assay, when test with sera donor and for blood banking, this data can provide information when blood screening by ELISA is better than RPR assay. Finally, public health benefits: active syphilis cases sometimes more detect and treat, because sensitivity and specificity of ELISAs are better than RPR.

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