Development and evaluation of modern enzyme immunoassays for comprehensive syphilis serology

Ontwikkeling en evaluatie van moderne enzym immunoassays voor toepassing in de verschillende aspecten van syfilisserologie

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"Ma dimmi prima, come hai indovinato che si trattava del secondo libro di Aristotele?"
"Non mi sarebbero bastati certo i tuoi anatemi contro il riso, né il poco che ho saputo sulla discussione che avesti con gli altri. Sono stato aiutato da alcuni appunti lasciati da Venanzio. Non capivo a tutta prima cosa volessero dire. Ma c'erano alcuni riferimenti a una pietra svergognata che rotola per la pianura, alle cicale che canteranno da sotto la terra, ai venerandi fichi. Avevo già letto qualcosa del genere: ho controllato in questi giorni. Sono esempi che Aristotele faceva già nel primo libro della Poetica, e nella Retorica. Poi mi sono ricordato che Isidoro da Siviglia definisce la commedia come qualcosa che racconta stupra virginum et amores meretricum... Piano piano mi si è disegnato nella mente questo secondo libro come avrebbe dovuto essere. Te lo potrei raccontare quasi tutto, senza leggere le pagine che dovrebbero infettarmi. La commedia nasce nelle komai ovvero nei villaggi dei contadini, come celebrazione giocosa dopo un pasto o una festa. Non racconta degli uomini famosi e potenti, ma di esseri vili e ridicoli, non malvagi, e non termina con la morte dei protagonisti. Raggiunge l'effetto di ridicolo mostrando, degli uomini comuni, i difetti e i vizi. Qui Aristotele vede la disposizione al riso come una forza buona, che può avere anche un valore conoscitivo, quando attraverso enigmi arguti e metafore inattese, pur dicendoci le cose diverse da ciò che sono, come se mentisse, di fatto ci obbliga a guardarle meglio, e ci fa dire: ecco le cose stavano proprio così, e io non lo sapevo. La verità raggiunta attraverso la rappresentazione degli uomini, e del mondo, peggiori di quello che sono o di quello che li crediamo, peggiori in ogni caso di come i poemi eroici, le tragedie, le vite dei santi ce li hanno mostrati.
E' così?"

From: Eco, Umberto. Il nome della rosa, 10 th ed.
Gruppo Editoriale Fabbri, Bompiani, Sonzogno, Etas S.p.A., Milano, 1983;475.

To Isabella, Rocco and Elisa
To my parents and parents in law

This dissertation includes the following articles:

1. IJsselmuiden OE, Schouls LM, Stolz E, Aelbers GNM, Agterberg CM, Top J, Van Embden JDA. Sensitivity and specificity of an enzyme-linked immunosorbent assay using the recombinant DNA-derived Treponema pallidum protein TmpA for serodiagnosis of syphilis and the potential use of TmpA for assessing the effect of antibiotic therapy. J. Clin. Microbiol. 1989;27:152-157. Presented at the seventh congress of the international society for sexually transmitted diseases research, Atlanta, 2-5 august, 1987.
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3. IJsselmuiden OE, Beelaert G, Schouls LM, Tank B, Stolz E, van der Groen G. Line Immunoassay and Enzyme Linked Line Immunofiltration Assay using the purified Reiter Treponeme Axial Filament and the recombinant Treponema pallidum protein TmpA for simultaneous detection of antibody to different treponemal antigens. (Submitted for publication)
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6. IJsselmuiden OE, van der Sluis JJ, Mulder A, Stolz E, Bolton KD, van Eijk RVW. IgM antibodies to treponemes in patients with syphilis detected by an IgM capture enzyme-linked immunosorbent assay. Genitourin. Med. 1989;in press. Presented at the Spring meeting on sexually transmitted diseases, Antwerp, 14-16 may, 1987.

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Chapter 1

1 Introduction

Syphilis is a venereal treponemal infection with a world-wide distribution and a high morbidity if left untreated. In 1987, in The Netherlands 388 new cases of infectious (primary and secondary) syphilis were reported to the chief public health inspector at the Ministry of Health (1). The annual incidence in 1987 was 2.8/100,000 inhabitants. In the U.S.A., in 1987 approximately 35,000 new cases were reported to the Center for Disease Control in Atlanta, Georgia (U.S.A.) (2). An incidence of 14.7/100,000 inhabitants was projected for 1987. In The Netherlands, most of the cases were from the cities of Amsterdam and Rotterdam. In Amsterdam, the majority of the patients were homosexuals or bisexuals, but in 1987 an increase in heterosexual syphilis patients was reported (3). Both in The Netherlands and in the U.S.A., the incidence of infectious syphilis has begun to increase again, especially in heterosexuals (2, 3). If the disease is left untreated, serious complications may arise either soon after the infection or may rise after many years. Therefore, early detection and treatment of syphilitic infection is imperative for the benefit of the individual patient and for the interruption of the infection chain.

The non-venereal treponemal infections consist of yaws, endemic syphilis and pinta. Together there are about 2.5 million cases, of which 75 % are children, mainly in developing countries (4). The organisms causing human treponematoses belong to the spirochaetaceae family that include the Treponema pallidum subspp. and the Treponema carateum (causes pinta). The T. pallidum subspp. consist of T. pallidum subsp. pallidum (causes venereal syphilis), T. pallidum subsp. endemicum (causes endemic syphilis or Bejel) and T. pallidum subsp. pertenue (causes yaws) (5). The pathogenic treponematoses can be differentiated by inoculation into golden hamsters and rabbits (6). In vitro differentiation of the subspp. via serological identification (7), protein analysis (6) or DNA-DNA hybridization is not feasible as yet (8).

The presence of a treponemal infection can be established by the detection of antitreponemal and anticardiolipin antibodies. The currently available tests for the detection of antitreponemal antibody are the fluorescent treponemal antibody-absorbed (FTA-ABS) test and the T. pallidum hemagglutination assay (TPHA). After treatment, the results of these tests remain positive for a long period depending on the length of infection before treatment. As a consequence, during this period, a reinfection or relapse after treatment may be not recognized. An additional test, the Venereal Disease Research Laboratory (VDRL) test, that detects antibodies to cardiolipin, is currently used for monitoring the therapeutic effect of the treatment. In contrast to antitreponemal antibodies, anticardiolipin antibodies are usually present during an active infection but disappear gradually upon successful treatment. Therefore, a reinfection or relapse after therapy can be detected using the VDRL test. Disadvantages of the VDRL test are its limited specificity and sensitivity and the occurrence of serofast patients, i.e. patients with persisting positive VDRL test reactions for years after adequate treatment. For the diagnosis of congenital syphilis the detection of antitreponemal IgM is required. This can be accomplished by the 19S(IgM)FTA-ABS test. Although the current serological tests for syphilis are satisfactory, their complexity of operation, their costs, the diversity of test systems, the antigens

used and inability to differentiate between the various treponematoses, necessitates the development of a comprehensive test system for diagnosis, screening, confirmation and monitoring the effectiveness of treatment that can distinguish venereal syphilis from other treponematoses. Recently, new test systems based on enzyme immunoassay techniques and purified treponemal proteins have become available. The technical advance during the past 5 years concerning immunochemical analysis, recombinant DNA technology and the development and the application of specific monoclonal antibodies has attracted new ideas for the development of improved serological tests for the diagnosis of syphilis (5). The first application of ELISA technology in serodiagnosis of syphilis by Veldkamp and Visser in 1975 created the basis for additional intensive investigations in syphilis ELISAs (9). These new approaches and acquisitions in the fundamental syphilis research provided the impetus for the investigations reported in this dissertation.

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Chapter 2

2 Syphilis

2.1 Introduction

The venereal syphilitic infection in man is usually transmitted via sexual contact to moist mucosal or cutaneous lesions. Syphilis can also be acquired by blood transfusion, intravenous drug abuse, tattooing and placental transmission. The classical course of an active syphilitic infection is characterized by three different stages namely the primary, the secondary and the tertiary stage. The periods of active disease may alternate with the stages of latency. The untreated primary, the secondary and the early latent stages of syphilis together are referred to as early infectious syphilis (duration of shorter than 2 years).
In congenital syphilis, the unborn child acquires a syphilitic infection by transplacental transmission of T. pallidum. It is especially in early infectious syphilis that a mother may transmit the infection to her fetus. The transmission of a syphilitic infection to the unborn child is reported to occur as early as the ninth week of pregnancy (1). The risk of transmission gradually declines during the course of the untreated infection.
Information on the natural progress of the untreated syphilitic infection was described in the Oslo study conducted between 1890 and 1951 and reported by Boeck, Bruusgaard and Gjestland (2, 3). The number of patients entered into this study, was 1978. In 1949, this number was reduced by Gjestland to 1404 patients who were Norwegian residents of Oslo. Up to 7 relapses were observed in 25 % of the patients. Gummas (granulomatous benign syphilitic lesions) were observed in 14.4 % of the males and 16.7 % of the females. Late neurosyphilis developed in 9.4 % of the males and 5.0 % of the females. Cardiovascular syphilitic lesions were observed in 13.6 % of the males and 7.6 % of the females. An underestimation of these figures was confirmed by the higher prevalence of cardiovascular syphilis in autopsied patients (4).
Secondary lesions may recur in untreated syphilis, usually within the first two years after primary infection. These lesions are often nodular and larger than those in secondary syphilis. Chancre redux may be a relapse of the original primary lesion. Lesions occurring after an inadequate therapy could be due to a recurrence or a reinfection.

2.2 Primary syphilis

Clinical picture
A painless indurated ulcus with a firm base, the chancre, develops about 3 weeks after sexual contact with an infected individual. The minimum and maximum incubation periods are 10 and 90 days respectively. Atypical herpes-like or chancroid-like ulcers may also occur. A painless regional adenopathy develops within a week after the appearance of the primary

lesion. The chancre generally disappears after 2 to 8 weeks, but may remain during the secondary stage. The demonstration of treponemes in the chancre by darkfield microscopy confirms the diagnosis of primary syphilis.

Histopathology

The typical chancre may be surrounded by a wall of acanthosis. The epidermis is thin or may be absent in the center of the lesion. At the base of the chancre, an obliterative endarteritis with perivascular mononuclear cuffing, characteristic of syphilis, is observed. The infiltrate mainly consists of mononuclear cells and edema is also present. In the biopsies from patients with primary and secondary skin lesions treponemes are demonstrated by silver impregnation methods (5, 6). Treponemes are also observed usually around the capillaries and at the dermal-epidermal junction. The Steiner staining (5) facilitates the distinction between spirochetes and reticulin fibers which are difficult in the Whartin-Starry silver staining technique (6). However, immunofluorescence is a more sensitive method to detect treponemes in tissues (7). The main disadvantage of immunofluorescence technique is that it cannot be performed using formalin-fixed tissues.

2.3 Secondary syphilis

Clinical picture

About 6 weeks after the primary lesion has appeared, the generalized character of the disease is demonstrated by the presence of a macular, papular, pustular, psoriasiform, erythemato-papulosquamous or nodular rash including the palms and soles, generalized painless lymphadenopathy and frequently hepatitis and a transitory proteinuria. Sometimes "moth-eaten" alopecia, arthritis, myositis or a meningitis may also occur.

Not only a rash, but other mucocutaneous lesions may also appear. Condylomata lata may be the result of confluent moist elevated papules which are covered by a white mucoid exudate. Sometimes, split papules occur in intertriginous regions. Smallpox-like pustular syphilids may resemble impetigo. Erythematopapulosquamous lesions may resemble psoriasis vulgaris or seborrhoeic dermatitis. On mucous membranes, erosive patches can be seen. Within 2 to 10 weeks, the cutaneous lesions may disappear but may relapse after a period of latency. Hyperpigmentation or hypopigmentation (leukoderma) may occur especially in the neck ("Collier de Vénus").

Histopathology

In macular lesions, the endothelium of superficial capillaries are swollen, surrounded by an infiltrate of lymphoid cells and plasma cells. However, the number of plasma cells is limited and therefore is of no diagnostic value. In condylomata lata, papular, pustular, psoriasiform, erythemato-papulosquamous and nodular lesions the deeper vessels are also involved with endothelial swelling and pronounced lymphoid and plasma cell infiltrate (vasculitis) which may occasionally lead to the diagnosis of syphilis. In addition, sometimes, small granulomas consisting of epitheloid and giant cells resembling tertiary syphilis lesions may occur. Ulcerating lesions may occur in the very rare lues maligna with endothelial swelling and plugging of the vessels with fibrinoid causing infarction and necrosis.

Sometimes silver staining or immunofluorescence examination may also be helpful in establishing the diagnosis of secondary syphilis. Treponemes can be demonstrated in chancres and in secondary syphilis skin eruptions and even leukoderma by electron microscopy (8, 9). In these studies, it was observed that there was a degeneration of the vessel wall and the nerve fiber. The nerve degeneration may be responsible for the painless skin lesions in primary and secondary syphilis. Treponemes were also demonstrated within phagosomes of the endothelial cells, macrophages, neutrophils, plasma cells and epidermal cells (6).

2.4 Latency

After a remission of the cutaneous lesions in secondary syphilis, if the infection is left untreated, it becomes latent but may relapse several times. Any individual without clinical symptoms of syphilis but with serological evidence of an untreated syphilitic infection is classified as a patient with latent syphilis. If upon patient history, the impression is formed that the infection already exists for less than two years, it is arbitrarily classified as early latent syphilis. Otherwise, the patient is classified as being in the late latent stage. If the patient is untreated, early lesions may relapse or tertiary syphilis may develop.

2.5 Tertiary syphilis

Benign and severe syphilitic lesions may develop in the 3 decades following an untreated syphilitic infection. These lesions include neurosyphilis, the formation of gummas and cardiovascular syphilis.

Neurosyphilis may already occur within 1 year after syphilitic infection. The neurological lesions may be located anywhere in the central nervous system and may remain asymptomatic. Special attention must be given to clinically asymptomatic neurosyphilis. Although, no neurological symptoms may be observed, the central nervous system may be affected as reflected by pathological changes in the cerebrospinal fluid. Symptomatic neurosyphilis may occur rapidly (syphilitic meningitis), after a few years (meningovascular syphilis) or after some decades (parenchymatous neurosyphilis).

Late benign syphilis consists of a proliferative or a destructive gummatous inflammation that does not involve tissues essential for life. Gummas are commonly observed in the skin and bones but may also appear in mucosae, viscera, muscles and eyes. They are benign syphilitic lesions, possibly induced by a hypersensitive reaction which induces scar formation that may impair the function of the structure involved.

In untreated syphilis, cardiovascular symptoms could develop after a period of 30 years. Owing to the modern antisyphilitic treatment, only few syphilis patients ever suffer for such a long time from active syphilis. As a result, nowadays, cardiovascular syphilis is a rare event.

2.6 Neurosyphilis

In the tertiary stage of the syphilitic infection, the central nervous system (CNS) is often involved. In fact, the cerebrospinal fluid is invaded by treponemes in the primary and the secondary stages of the disease in 15 to 40 % of the cases (10), reflecting in changes of the cerebrospinal fluids.
Asymptomatic neurosyphilis is the most common form of neurosyphilis. It is defined by the absence of neurological symptoms but with pathologic changes in the cerebrospinal fluid; TPHA and/or FTA reactive liquor adjusted for leakage of the blood-CSF barrier in combination with either VDRL-reactivity or mononuclear cell count $> 5/\mu l$, or total protein > 0.5 g/l or IgG index > 0.7, or oligoclonal immunoglobulins (11, 12). Asymptomatic neurosyphilis can occur in up to 20 % of the untreated patients (13). In patients with asymptomatic neurosyphilis the disease seldom progresses to neurosyphilis.
In symptomatic neurosyphilis, the lesions can occur anywhere in the CNS and may cause meningeal, meningovascular, parenchymatous and gummatous lesions.

2.7 Symptomatic neurosyphilis

Syphilitic meningitis
Clinical picture
Syphilitic meningitis may develop within 1 year after the infection with T. pallidum.
Symptoms resembling a viral meningitis (fever, stiff neck, lymphocytosis and normal glucose) may be observed. More severe symptoms indicative of an acute syphilitic hydrocephalus (vomiting, headache, papilledema), focal cerebral involvement (seizures, hemiplegia), and cranial nerve palsies may also appear. In addition, in 20 % of the cases sensorineural deafness may be observed and which can be corrected by proper penicillin therapy.

Spinal pachymeningitis
Clinical picture
Spinal pachymeningitis is caused by a thick fibrous sheet embracing the spinal cord resulting from a hypertrophic inflammatory reaction or a gumma. This rare condition commonly affects the cervical region. A slowly developing radicular syndrome may be observed.

Meningovascular syphilis
Clinical picture
Pathologic changes in the medium and larger-sized arteries of the brain and the spinal cord leads to meningovascular syphilis. In cerebrovascular syphilis the middle cerebral arteries are usually involved resulting in a large cerebrovascular accident frequently accompanied by seizures.
Meningovascular syphilis of the spinal cord is a rare condition that appears as syphilitic meningomyelitis or spinal vascular syphilis. In syphilitic meningomyelitis the spinal cord may be affected either by cord infarction or by parenchymatous degeneration due to chronic

spinal meningitis. This may cause weakness of the legs and paresthesias sometimes progressing to paraparesis. With the development of spinal vascular syphilis, transection or hemisection, usually at the thoracic level occurs resulting in a spinal shock syndrome.

Histopathology

The pathologic changes in meningovascular syphilis consist of obliterative endarteritis (endothelial and fibroblastic thickening of small arteries) with perivascular cuffing (infiltration by lymphocytes and plasma cells) of the vasa vasorum and adventitia. Subsequently, the smooth muscles and elastic tissue of the media are destroyed and concentric narrowing of the vessel due to the proliferation of subintimal fibroblasts occurs until it is totally occluded by thrombus formation.

Parenchymatous neurosyphilis

Parenchymatous neurosyphilis may be represented by dementia paralytica or tabes dorsalis. Meningoencephalitis caused by invasion of the cerebrum by T. pallidum occurs and will result in a dementia paralytica after a few decades, and results finally in death. This condition may resemble almost any psychiatric or neurological disorder. Frequent symptoms include early memory loss, diminishing intellectual functions, personality changes and dementia. Seizures may be observed and transient ischemic attacks and strokes resulting in hemiparesis, aphasia or persisting psychotic behaviour can occur. Argyll Robertson pupils may occur but are more frequently observed in tabes dorsalis. Tabes dorsalis is very rare since the introduction of treatment with penicillin. Early symptoms are; lightening pains, paresthesias, diminishing deep tendon reflexes and Argyll Robertson pupils. Late symptoms include ataxia, paresthesias, optic atrophy, incontinence of urine and visceral crises.

Histopathology

Pathologic changes in parenchymatous neurosyphilis consist mainly of fibrosis of the meninges, frontal and temporal lobes atrophy and demyelinization of white matter.

2.8 Cardiovascular syphilis

The cardiovascular system may be involved in the tertiary stage of syphilis with clinical symptoms in 10% of the cases. Pathological changes, however, may be observed in 80% of these syphilitic patients. In early stages of syphilis, the cardiovascular system is not affected and therefore, adequate therapy of early syphilis prevents syphilitic heart disease.

Clinical picture

Three major cardiovascular syndromes may occur: aneurysm formation in the ascending aorta, aortic valve incompetence and coronary ostial stenosis.

The syphilitic aneurysm is the most common manifestation of tertiary syphilis and is usually located at the proximal end of the ascending aorta. The clinical symptoms are minor and may consist of pain in the chest or hoarseness from pressure on the recurrent laryngeal nerve. The compliance of the aorta is usually reduced resulting in tambouric heart sounds. The chest radiograph may reveal a typical "egg-shell" calcification but the diagnosis of aortic aneurysm is established by aortic root angiography.

Aortic valve disease, commonly present in cardiovascular syphilis, consists of aortic regurgitation without stenosis. This is caused by a widening of the aortic valve commissures due to aortic root dilatation with stretching and thickening of the aortic valve leaflets. Diastolic blowing and prominent second heart sound result. Subsequent hypertrophy and dilation of the left ventricle may occur.
Syphilitic disease of the coronary arteries usually only affect the ostia and the most proximal few millimeters, and results in narrowing of the coronary arteries causing angina pectoris and sudden death due to extensive infarction. Limited myocardium infarct seldom occurs because an occlusion of a proximal coronary artery will lead to an extensive infarct resulting in death.
Histopathology
T. pallidum spread in the early stages of syphilis probably via the lymphatics to the vasa vasorum of the proximal aorta. An obliterative end-arteritis occurs which includes the proximal portions of the coronary arteries. The inflammatory infiltrate consists of lymphocytes and plasma cells. This process may last for years indicating the possibility of an immunological process.
The aortic media becomes necrotic with focal scarring and destruction of elastic fibers which leads to aortic aneurysm formation. The adventitia undergoes fibrous thickening and the intima wall contains atherosclerotic changes with extensive plaque formation and calcification.

2.9 Late benign syphilis

A proliferative or destructive inflammatory process called gumma formation provides the basis for late benign syphilis. Gummata mainly affect the skin and bones and sometimes the mucosae brain, myocardium, spinal cord, trachea, the viscera and muscles.
Clinical picture
Skin
Gummata of the skin can be divided into nodular and solitary types. A nodular gumma is a deep indurated papule with a brownish-red colour. Predilection areas are the face, the scapular and interscapular regions, and the extremities. The gumma may remain for weeks or months and may heal without severe scarring or develop into noduloulcerative lesions. Healing of these lesions may result in atrophic non-contractile scars. Without treatment, a serpiginous relapse at the border of this lesion may occur. The solitary nodule is a subcutaneous process that secondarily involves the skin. It may become necrotic and may drain through one or more areas.
Skeleton
The tibia, fibula, clavicle and skull (nose and palate) are the most affected bones of the skeleton. Gummatous bone lesions can be classified as follows:
1. periostitis, i.e. periosteal thickening.
2. osteomyelitic osteitis, sometimes destructive and with

   sclerosis of the surrounding bone.

3. sclerosing osteitis

Clinical symptoms include pain, especially during the night, and swollen bones.

Respiratory tract and mouth

Osteitis of the hard palate often leads to ulceration with possible perforation. Due to perichondritis of the nose septum, a saddle-shaped nose may develop. A chronic hoarseness is caused by the destruction of the epiglottis or the larynx. Occasionally gummata in the trachea and bronchi may occur with narrowing of the lumen.

Digestive system

Gummata in the esophagus appear as ulcers, tumors or strictures. Gastric gummata may mimic gastric ulcer. Gumma of the liver is the most frequent type of gastrointestinal late benign syphilis often accompanied with splenomegaly.

Ocular late benign syphilis

Iritis may occur in late syphilis. Occasionally the more serious syphilitic chorioretinitis occurs.

Histopathology

A gumma consists of a granuloma with central coagulative necrosis (gummy like) surrounded by epitheloid cells and infiltrated by mononuclear leukocytes (principally plasma cells) and enclosed by a fibroblastic wall. The small vessels enclosing the lesion may reveal obliterative endarteritis and a perivascular mononuclear infiltrate. Gummata closely resemble the granulomas observed in tuberculosis and sarcoidosis. Multinucleated giant cells are seldom observed. The gumma is probably an allergic reaction to the few treponemes present at this site.

2.10 Congenital syphilis

The unborn child may acquire the syphilitic infection via the transmission of T. pallidum from the mother through the placental wall. It has been demonstrated that this transmission may occur very early in gestation; spirochetes were observed in 9 and 10 weeks old fetuses (1). A syphilitic infection in the fetus at this stage of the gestation is difficult to demonstrate due to the absence of inflammation in the affected fetal tissues. This may explain why formerly it was assumed that infection of the fetus did not occur before the 18^{th} week of gestation (14).

Congenital syphilis may be overt directly at birth or develop within the first two years of life (early congenital syphilis) or it may develop at an older age (late congenital syphilis). The diagnosis of congenital syphilis may be difficult since the symptoms may be nonspecific.

Clinical picture

Early congenital syphilis occurs within the first two years of life. There are usually no visible symptoms at birth. No primary stage can be observed. In 60 % of the cases, the first symptoms are recognized 3 to 8 weeks after birth. After 3 months, new cases are rare. The clinical symptoms of early congenital syphilis are usually nonspecific. They include low birth weight, hepatosplenomegaly, purpura, pneumonia, eye and skin lesions. In Table 1, the typical symptoms of early congenital syphilis are summarized. The most important early congenital syphilitic lesions include snuffles, pseudoparalysis, secondary syphilis-like lesions,

palmo-plantar bullae, hepato-splenomegaly and generalized lymphadenopathy. Epitrochlar lymphadenopathy is considered to be pathognomonic of congenital syphilis. Jaundice and petechiae may appear, cutaneous extramedullary hematopoiesis may produce a "blue berry muffin" rash. Typical bone lesions due to osteochondritis and periostitis may lead to the diagnosis of congenital syphilis. Neurosyphilis may develop after 3 months with features comparable to those involved in adult neurosyphilis patients. First meningitis appears leading to meningovascular disease. If untreated, it will lead to dementia paralytica or tabes dorsalis after 6 or more years.

Late congenital syphilis may appear two years or later after birth. The lesions of late congenital syphilis are comparable to those of late syphilis in adults. Late congenital syphilis is recognized by malformations which developed during the initial period of growth, called stigmata. The children usually have bone deformities. Chondritis and osteitis of the skull may cause craniofacial malformations. The nose may be affected leading to a saddle nose. The teeth are small, widely spaced with conical incisors and molars with mulberry-like surface. The enamelization may be defective. The first molars and the incisors mainly of the permanent set are affected. The long bones are predominantly involved, especially at the metaphyseal-epiphyseal junction. If this junction is destroyed, pseudo-Charcot's joints may develop. Saber shins may occur due to periostitis of the long bones. The eyes and ears may be involved resulting in interstitial keratitis and neural deafness due to osteochondritis with cochlear degeneration. Local gummata may affect the palate, throat and the brain. Neurosyphilis, dementia paralytica and tabes dorsalis may be found after puberty.

Histopathology

Basically, the histopathology of neonatal and adult syphilis is very similar. A vasculitis predominantly of the small vessels, resulting in obliterative endarteritis with necrosis and fibrosis. Microscopic gummas with granulomatous histologic changes are commonly observed.

2.11 Essentials of antisyphilitic therapy

Since its introduction in 1944 (15) the penicillin treatment still remains the treatment of choice. Although, T. pallidum containing plasmids have been reported (16), resistance to penicillin, due to plasmid factors, has never been observed. In vitro experiments have shown that penicillin at a concentration of 0.17 U/ml medium could immobilize T. pallidum (10^6 - 10^7 treponemes/ml) completely within 12 hours (17). Higher concentrations of penicillin did not reduce the immobilization time. However, lower doses have been recommended for in vivo treatment. Dose of penicillin of 0.03 U/ml serum has been effective in all forms of syphilis (18). Since penicillin inhibits cell wall construction, only growing bacteria are destroyed. The replication time of T. pallidum is about 30 hours in early syphilis (19) but may be much longer in late syphilis (20). Therefore, effective blood levels of penicillin for longer period is required in late syphilis.

In The Netherlands, the generally accepted treatment for early syphilis of shorter duration than 2 years without CNS involvement is weekly intramuscular administration of 2.4 MU

Table 1. Clinical symptoms of early congenital syphilis.

liver and spleen	hepatosplenomegaly
lymphatics	generalized lymphadenopathy
skin and mucous membranes	rhinitis ("snuffles")
	macular, maculopapular or papular lesions
	furuncle-like lesions
	vesiculobullous eruptions on the palms and soles
	desquamation especially around the finger nails
	paronychia, especially 4^{th} and 5^{th} finger
	ezematous, impetiginous or gangrenous lesions in the midface, perineum and intertriginous areas
skeleton	epiphysitis of the radius, femur, humerus and fibula
	osteitis of the occipital and parietal bones
	frontoparietal tumefactions of Parrot
blood	anemia, normochrome, normo- or macrocytosis with autoimmune hemolysis
	pleocytosis of neutrophils, thrombocytopenia
kidneys	proliferative or membranous glomerulonephritis
eyes	chorioretinitis
	glaucoma
	uveitis
CNS	CSF abnormalities
	meningovascular neurosyphilis
	after third to sixth month postpartum
	CVA caused by arteritis and thrombocytopenia

benzathine penicillin for three weeks (21). In Rotterdam, patients with syphilis of longer than 2 years duration are treated daily with 1.2 MU procaine penicillin G injections intramuscularly for 10 days and with 1.2 MU benzathine penicillin G injections intramuscularly at weekends. In case the patient has penicillin-allergy, tetracycline or erythromycin is used. A patient with a syphilitic infection of shorter than two years duration is treated 4 times a day with 500 mg tetracycline orally or erythromycin orally for 15 days. Patients with infections of longer than 2 years duration receive the treatment for 30 days.

The current treatment of neurosyphilis in the Netherlands consists of therapy during 15 days with 0.15 MU penicillin G/kg body weight/24 h divided into 6 doses administered intravenously. To avoid Jarisch-Herxheimer reactions, prednisolon is administered intravenously during the first 3 days of treatment in tapering amounts (75, 50 and 25 mg). Benzathine penicillin G does not reach the cerebrospinal fluids. Therefore, benzathine penicillin G is unsuitable for treating neurosyphilis (22).

Patients suffering from congenital syphilis with CSF deviations are treated with 50,000 U penicillin G/kg body weight divided in two doses and administered intramuscularly or intravenously for 10 days (in Rotterdam: 100,000 U penicillin G/kg body weight divided in eight doses for 14 days). In case of penicillin-allergy: erythromycin 20 mg/kg divided in four doses administered intravenously or 30 mg/kg orally for 14 days. Without CSF deviations, one dose of 50,000 U penicillin G/kg body weight is sufficient (in Rotterdam: 100,000 U penicillin G/kg in eight doses).

2.12 Side effects in antibiotic treatment of the syphilitic infection.

The Jarisch-Herxheimer reaction may appear after treating a syphilitic infection with penicillin or another antibiotic therapy. Side effects are mild fever, sometimes high fever, worsening of the syphilitic lesions (swelling, increased rash, tender lymphnodes), hyperventilation, nausea and increased blood pressure with peripheral vasoconstriction. Leukocytosis and lymphopenia usually develop. Finally the blood pressure drops due to peripheral vasodilatation. The reaction starts at 4 hours posttreatment and may last for 24 hours. A severe Jarisch-Herxheimer reaction is rare. During the treatment of neurosyphilis the neurological disorders may increase or seizures may occur. The treatment of the Jarisch-Herxheimer reaction is symptomatic. Several possible mechanisms of this reaction have been proposed. Among them are the response to an endotoxin possibly released by the gut (23), an activation of the immune system by release of treponemal antigens (24) and destabilization of mast cells through treponemal antigen-IgE binding (25).
Hoigné's syndrome is an acute reaction to the treatment with procaine penicillin G treatment. This reaction may be caused by microembolism of procaine penicillin crystals. Psychotic reactions may appear including depersonalization and hallucinations and somatic reactions consisting of bradycardia, increased blood pressure and dilation of the pupils (26). Adequate therapy of this syndrome consists of treatment with a single dose of 10 mg diazepam rectioles or administered intravenously.

2.13 Alterations of syphilitic disease due to a concurrent infection with human immunodeficiency virus (HIV)

The reaction in man against T. pallidum infection is characterized by the partial failure of the immune system to eliminate the pathogenic treponemes. In keeping with non-specific immunosuppression due to syphilis as has been suggested by some authors, the disease has been observed to be associated with a higher risk of HIV infection and may induce progressive immunodeficiency in HIV-seropositive individuals (27, 28, 29). Recently, in HIV-seropositive patients with concurrent syphilitic infections alterations in the natural course of syphilis have been observed (28).
Due to immunosuppression in HIV-seropositive patients, a syphilitic infection may be more severe and cause more serious damage than in patients without HIV infection. Serious complications reported in early syphilitic patients with concurrent HIV infections are listed in Table 2.
Blurred vision may be the only complaint (30) possibly caused by necrotizing retinitis which is usually a benign complication in secondary syphilis. With concurrent HIV infection however, the prognosis may be poor unless treated adequately. Neurosyphilis appears seldom ($< 6.5\%$) in early syphilis. However, four cases of neurosyphilis were reported by Johns et al. (28) in patients who were seropositive for HIV and with concurrent active syphilis. In one of the patients with meningovascular syphilis, the neurological involvement developed within four months. In the uncompromized host, meningovascular syphilis usually occurs 5 to 12 years after the primary infection but seldom appears as early as a few months

Table 2. Manifestation of severe syphilitic symptoms in patients with early syphilis and concurrent HIV infection.

Diagnosis	symptoms	literature cited
active syphilis	necrotizing retinitis	30
secondary syphilis	chorioretinitis, uveitis, vitreous haze	31
secondary syphilis	relapse after treatment with CVA	32
primary syphilis	meningovascular neurosyphilis	28
active syphilis	acute syphilitic meningitis	28
secondary syphilis	asymptomatic neurosyphilis	28

after infection (33). In the syphilitic patients mentioned, the concurrent HIV infection may have led to syphilitic symptoms that occurred earlier or more severe than usual. In addition, syphilitic infection in HIV-infected patients may result in non-reactive tests for syphilis (28), although others reported no difference in treponemal antibody response in patients infected with HIV (34). Therefore, careful examination is recommended for syphilitic patients with neurological disorders and who are also HIV-seropositive since serious complications can be prevented by adequate antitreponemal treatment.

Syphilis may also have consequences in the acquisition of HIV. Several studies have demonstrated an association between syphilis and HIV infection, suggesting that syphilis may facilitate HIV acquisition (29, 35, 36, 37). In the industrialized countries, especially homosexual man are involved. In Africa, a positive relationship between syphilis and HIV-infection has been observed in heterosexuals. It may be that the syphilitic chancre facilitates the entry of HIV. Indeed, increased risk of HIV transmission due to genital ulcers was reported by Greenblatt et al. (38) which seems to confirm the influence of syphilitic ulcers on HIV transmission. Another explanation may be that oozing syphilitic chancre release HIV more readily, thus promoting concomitant infection. In women T-helper cells in the vaginal discharge may be responsible for the transmission of HIV (37).

In conclusion, concurrent HIV and syphilitic infections create new features in diagnosis, treatment, severity and progression of syphilitic disease. In addition, the transmission and progression of HIV infection to AIDS seem to be associated with syphilis. Impairment of cell-mediated immunity, helper cells for humoral immunity and macrophage function in HIV infected patients may diminish the host's defense against syphilis and facilitate the progression of syphilitic disease. Transient immunosuppression suggested during the early stages of syphilis may impair the host's defense against HIV. Therefore, synergistic immunodeficiency may develop permitting the progression of both diseases. Rapid progression of syphilis to severe neurologic or other organic syphilis require the clinician to be vigilant since

postponement of the appropriate treatment may lead to serious complications. Distinction between syphilitic and HIV associated neurologic disorders is necessary but not always easy. Therefore, it has been recommended that patients seropositive for both syphilis and HIV have their cerebrospinal fluid examined (30). In addition, treatment regimens should be reevaluated for the early stages of syphilis in HIV-seropositive patients. Since treponemes are often present in the CSF during the early stages of syphilis and within the CSF the concentration of benzathine benzylpenicillin after intramuscular administration is therapeutically insufficient (39), a regimen with penicillin G administered intravenously or procaine penicillin G administered intramuscularly may be more effective. In 1988, Lukehart et al. reported on the ineffectiveness of a single dose of 2.4 MU of benzathine penicillin administered intramuscularly to HIV-infected patients suffering from secondary syphilis (40). In 2 out of 3 of these patients, viable T. pallidum was isolated from the CSF and was cultured by inoculation of rabbit testes. The third patient had a positive CSF-VDRL 8 months after the initial therapy. A patient not infected with HIV but with viable T. pallidum in the CSF had a nonreactive serum VDRL 10 months after treatment. These data suggest that the usual treatment for early syphilis in the U.S.A. (a single dose of 2.4 MU benzathine penicillin administered intramuscularly) is not sufficient if the patient is HIV-seropositive. Therefore, in patients with early syphilis, screening for the presence or absence of a concurrent HIV infection has to be considered (41). In fact, Quinn et al. suggested that attendants at clinics for sexually transmitted diseases with presumptive diagnosis of syphilis should be examined for a possible HIV infection (29). It should be stressed that this form of additional diagnosis should be accompanied by counseling and intensive education on HIV infection (29). However, it should be realized that screening for HIV infection with or without informed consent is still widely discussed in view of the lack of resolving therapy and victimization (42, 43, 44). Therefore, further research is needed to consider the testing for HIV-antibodies in patients with early syphilis if concomitant HIV infection necessitates different treatment regimens for syphilis. This question will not further be discussed in the context of the present study.

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Chapter 3

4 Serodiagnosis of syphilis

4.1 Introduction

The diagnosis of treponematoses, can be established by the detection of serum antibodies against treponemes or to cardiolipin. However, it must be realized that antibodies to treponemes not only detect syphilis but also point to the presence of an endemic treponematosis. Antibodies to cardiolipin can also be detected in patients with diseases other than syphilis. Therefore, in the serodiagnosis of syphilis, the detection of antibodies to treponemes is more relevant than the detection of anticardiolipin antibodies. However, anticardiolipin antibodies tend to disappear gradually from the serum after the syphilitic infection has been treated effectively. This is only partially observed with antitreponemal antibodies. Current tests for the detection of antitreponemal antibodies are the fluorescent treponemal antibody-absorption (FTA-ABS) test and the T. pallidum hemagglutination assay (TPHA).

Table 1. Characteristics of tests currently used for syphilis.

Attribute	FTA-ABS	TPHA	VDRL	RPR	19S(IgM)FTA-ABS
Antigen used	Tp[a]	Tp	CL[b]	CL	Tp
Antibody type	IgG	IgG/M	IgG/M	IgG/M	IgM
Sensitivity	+++++	++++	+++	+++	++++[c]
Specificity	+++	++++	++	++	+++[c]
Screening	++	++++	+++	+++	+
Confirmation	++++	++++	+	+	++
Treatment monitoring	+	+	+++	++	++
Simplicity	++	++++	+++	++++	+
Costs	++++	+	+++	++	+++++
Automation	+	+++	+	+++	+
Simultaneous sample testing[d]	++	++++	++	+++	-

[a] Tp = T. pallidum.
[b] CL = cardiolipin.
[c] for congenital syphilis and early infectious syphilis.
[d] more than one sample can be tested at the same time.
The results are shown using an arbitrary scale.

Anticardiolipin antibodies are detected in the Venereal Disease Research Laboratory (VDRL) test and the rapid plasma reagin (RPR) card test. Antitreponemal IgM antibodies are currently detected by the 19S(IgM)FTA-ABS test. Relevant characteristics of these tests are summarized in Table 1.

4.2 Evolution of the serological test for syphilis

Tests detecting antibodies to cardiolipin

The first serological test for syphilis was introduced by Wassermann in 1906. He described a complement fixation test using antigens from liver extracts of fetuses that had died from congenital syphilis (1). Using this test it was demonstrated that a mother could transmit syphilis to her unborn child since antibodies reactive in the Wassermann test could always be demonstrated in the mother. This event emphasizes the importance of serological tests in the pathogenesis of the disease and in discarding the belief in hereditary syphilis before that time (2). The test was improved further with respect to sensitivity and specificity by using purified antigens and the addition of cholesterol and lecithin (3). These tests are now referred to as lipoidal tests.

A problem in complement fixation tests was the presence of complement-consuming complexes in patients' serum that could result in false-positive reactions. This problem was circumvented by the development of flocculation tests like the VDRL in 1946 and the RPR card test in 1962 (4, 5). Although, improved considerably, the tests with lipoidal antigens are not specific for syphilis since antilipoidal antibodies can also be found in individuals not suffering from syphilis. These so called Biologically False Positive reactions (BFPs) may be observed in any test using lipoidal antigens. Transient BFPs can be observed in pregnancy, after acute febrile disease (e.g. mycoplasma pneumonia) and immunizations (e.g. smallpox). Chronic BFPs may occur in narcotic addicts, systemic lupus erythematodes, lepromatous leprosy or autoimmune disease (6).

The possible differences between positive reactions in lipoidal tests in syphilis patients and those in other individuals were explored by Harris et al. (7). It appeared that antibodies to phospholipids from syphilis patients were mainly against cardiolipin and those from patients with autoimmune disorders reacted not only with cardiolipin but also with a variety of negatively charged phospholipids. In the VDRL test, the largest part of the latter antibodies are absorbed by the present cholesterol and lecithin in the test system (7).

Tests detecting antibodies to treponemes

The first widely used test for the detection of antitreponemal antibodies, the T. Pallidum Immobilization (TPI) test, was introduced in 1949 by Nelson and Mayer (8). In this test pathogenic treponemes were immobilized by antitreponemal antibodies and complement. The TPI test required freshly harvested treponemes from rabbit testes and was rather complicated to perform. In The Netherlands, the TPI was carried out at the Dutch National Institute of Public Health and Environmental Protection (Bilthoven) until 1987, but due to its low sensitivity, use of complement, complexity, high costs, need for live treponemes cultured in rabbits and the availability of alternative tests, the TPI program was discontinued. It was argued that the TPI was not very sensitive and not 100 % specific (9).

In 1957, a second treponemal test, the fluorescent treponemal antibody (FTA) test was developed by Deacon et al. (10). Patient's serum was used at a 1:5 dilution. Unfortunately, too many false-positive reactions were observed. Therefore in the later versions of the

test Deacon used a serum dilution of 1:200. The resulting FTA-200 test, however, was insensitive for the detection of primary syphilis. It was suggested that the aspecific reactions were due to the occurrence of common or group antigens shared by both pathogenic and saprophytic treponemes. In 1964, Hunter described the fluorescent treponemal antibody absorption (FTA-ABS) test using a sorbens prepared from sonicated Reiter treponemes to absorb non-specific antibodies from the patient's serum (11). Serum was used at a 1:5 dilution in sorbens which resulted in a highly sensitive test with a specificity which was at least equal to that of the FTA-200 test.
The main drawback of the fluorescent test was that it was a technically complicated test which could only be performed by a skilled technician. Each test result had to be read by the technician and automation was therefore difficult. False-positive results can be observed in the FTA-ABS test. Therefore, it should be used for confirmation. Increased immunoglobulin levels in the serum may occasionally result in a false-positive FTA-ABS test (12). A beaded pattern of immunofluorescence due to the attachment of antinuclear serum antibodies to extruding treponemal DNA may be observed with sera from SLE patients (13). The conjugate used in the FTA-ABS test should detect only IgG since the detection of IgM may lead to non-specific results (12).
The exact nature of the absorbing compound in the FTA-ABS test remained obscure. Other compounds such as the culture filtrate of a Reiter culture, choline, VDRL antigen, yeast, thioglycolate with absorbing activity were found effective (12, 14). It was observed that the high osmolarity was at least partially responsible for the absorbing effect (14).

In 1965 Rathlev first described the T. pallidum hemagglutination assay (TPHA) (15). This test used tanned sheep erythrocytes which had been sensitized with an ultrasonicate of T. pallidum. Initially, normal rabbit serum was used as absorbens to prevent non-specific reactions. These days commercially available TPHA-kits contain fairly complicated absorbing diluents. Erythrocyte membrane lysates, rabbit testicular extract, Reiter treponeme lysate and Tween 80 may all be included (16). The sensitivity of the TPHA differs between European and American studies (9, 17).
Despite these efforts to eliminate non-specific reactions, false-positive results may still be observed due to known (heterophile antibodies) or unknown causes (18). The Hemagglutination Treponemal Test for syphilis (HATTS) developed in the U.S.A. utilized glutaraldehyde stabilized turkey erythrocytes instead of formalinized tanned sheep erythrocytes as used in the TPHA (19). A higher specificity may be expected from this test due to a lack of reactivity with heterophile antibodies.

4.3 Detection of syphilitic IgM antibodies

The primary antibody response to a syphilitic infection mainly consists of antitreponemal IgM class immunoglobulins as was demonstrated by Shannon and Booth (20). A serological test that detects antitreponemal IgM may trace a primary syphilitic infection very early. It was also shown that antitreponemal IgM is the first class of immunoglobulins that disappears after treatment. Furthermore, in contrast to IgG, IgM cannot pass through the placental wall. Therefore, demonstration of antitreponemal IgM in the serum of a neonate from a mother with syphilis may confirm a congenital syphilitic infection. Finally, the demonstration of antitreponemal IgM in cerebrospinal fluid may provide evidence for the diagnosis neurosyphilis. In 1968, a FTA-ABS IgM test to detect congenital syphilis

was developed by Scotti and Logan (21). The test was not sensitive due to competition of antitreponemal IgM with antitreponemal IgG and the production of IgM directed against maternal IgG in large quantities with concomitant low antitreponemal IgM (22). The specificity was limited by the presence of rheumatoid factors (IgM anti-IgG) causing false-positive reactions, since these antibodies can bind to antitreponemal IgG and are subsequently detected by anti-IgM conjugate (23).

An attempt to circumvent these problems was undertaken by Müller and Loa in 1974 who introduced the 19S(IgM)-FTA-ABS test (24). Before the sera were tested in the FTA-ABS test using μ-chain specific anti-IgM conjugate, the IgM containing fraction (antitreponemal and non-antitreponemal) was separated from the IgG by a Sephadex G 200 column. This did not always result in IgG free IgM fractions and the Sephadex G 200 column was therefore replaced by the ultragel AcA 34 column (25).

In 1982, Schmidt described the high performance size exclusion chromatography using TSK G 3000 SW columns for isolation of IgM from the serum (26). Not only a better separation was obtained, but the IgM was also less diluted after the separation. The time required for separation was only 10 minutes instead of hours and the amount of serum needed (0.05 ml) was much less than that required for normal gel filtration (0.7 ml). The latter feature was in greatly favoured for testing neonatal sera which were only available in small amounts.

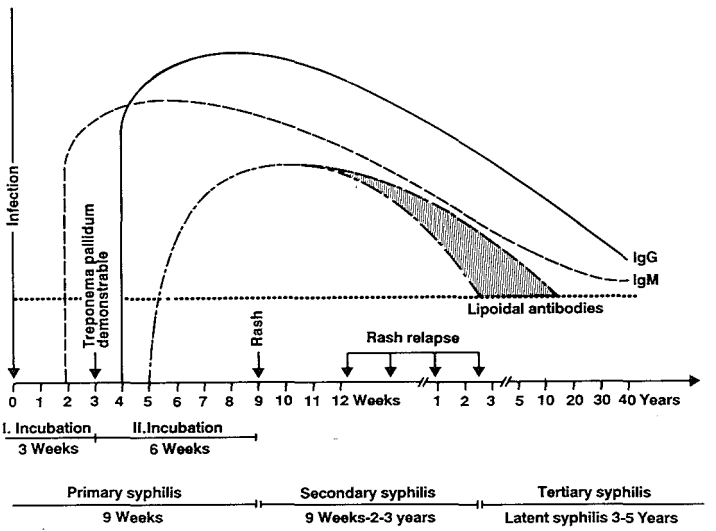
The value of the 19S(IgM)-FTA-ABS test in the serodiagnosis of congenital syphilis is undeniable and may be the only meaningful application of this test. In other syphilitic conditions, the role of antitreponemal IgM is doubtful. Although, the 19S(IgM)-FTA-ABS test is the most sensitive and specific test for the detection of antitreponemal IgM at present, a negative test result does not exclude an active infection nor does a positive test more than one year after treatment indicate that the infection is not over. If IgG is produced in large amounts, IgM may be not detected. The production of 19S(IgM) is hampered by high titres of antitreponemal 7S(IgG) by means of a negative feedback mechanism (20). High IgG titres may be found in secondary, latent and tertiary syphilis. Müller observed that a TPHA-titre of less than 1:40,000 excluded the feedback phenomenon (27). Therefore, in late active syphilis, when often high antibody titres are present, the 19S(IgM)-FTA-ABS test may be non-reactive. In addition, reinfection of a patient treated for late syphilis may not result in detectable levels of 19S IgM. This may be due to the boost in the production of 7S(IgG) rather than 19S(IgM) (27). An active infection in such patients is detected better by tracing antitreponemal or lipoidal IgG.

Finally, the separation of 19S(IgM) from 7S or 8S(IgM) is an additional important feature of the 19S(IgM)-FTA-ABS test. Müller and Schmidt observed antitreponemal 7S and 8S(IgM) in the sera of syphilis patients (27, 28). These antibodies may not be related to an active infection and therefore should not be detected in an antitreponemal IgM test.

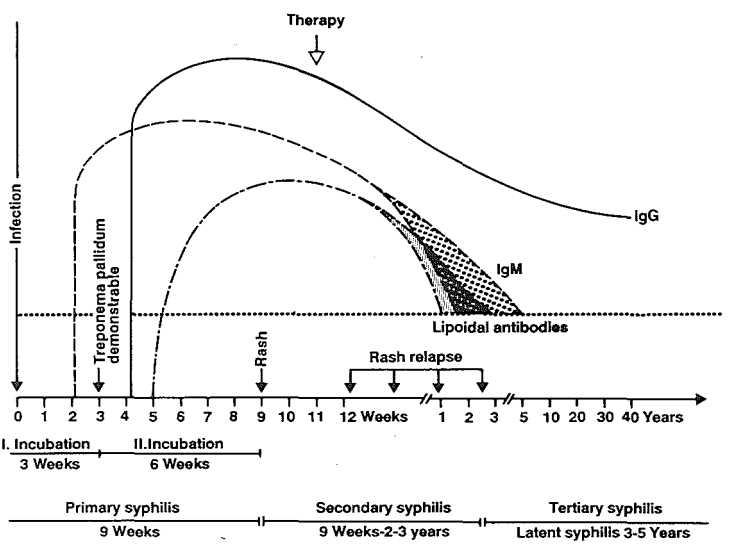
The 19S(IgM)-FTA-ABS test is laborious and requires expensive equipment. In 1980, Schmidt developed the Solid-phase hemadsorption (SPHA) assay which was simpler to perform. This assay eliminated the inhibition of 19S(IgM) by 7S(IgG) and did not detect 7S(IgM) (28). This was achieved by the selective adsorption of IgM from the patient's serum to the wells of a microtitration plate coated with μ-chain specific antihuman IgM. In doing so the competitive antitreponemal IgG and monomeric rheumatoid factor was removed. Only 19S(IgM) anti-IgG still caused false-positive results in the SPHA assay (28). The SPHA test appeared to be less sensitive for low IgM titres than the 19S(IgM)-FTA-ABS test (29). Therefore, further investigations should establish the usefulness of this test.

In conclusion, effective screening for syphilis can be achieved using the TPHA. The specificity of the screening can be enhanced by confirmation of positive TPHA test results using the FTA-ABS test. Treatment monitoring can be done with the VDRL. Antitreponemal IgM can be effectively detected in the 19S(IgM)FTA-ABS test. The 19S(IgM)FTA-ABS test is most useful in the detection of congenital syphilis. However, this test is not suitable for routine use due to its complexity, the expense and the lack for testing samples simultaneously. In the figure the different tests for syphilis and their relationship to the period of infection in treated and untreated syphilis patients are summarized. From this figure it can be concluded that cardiolipin tests become negative after a few years with or without therapy. Tests that detect IgG against treponemes remain positive for many years in adequately treated and untreated syphilis patients.

Figure. Antibody concentration in different stages of untreated syphilis.



Antibody concentrations in different stages of treated syphilis.



Courtesy Professor Dr.med.Dr.h.c.mult. O. Braun-Falco. From: Braun-Falco O, Plewig G, Wolff HH. Dermatologie und Venerologie, 3^{d} ed. Berlin, Heidelberg, New York, Springer-Verlag 1984;98-99.

4.4 Management of the cerebrospinal fluid abnormalities in syphilis

Involvement of the central nervous system (CNS) is a common feature in a syphilitic infection. T. pallidum isolated from the cerebrospinal fluid (CSF) can be cultivated in rabbits from up to 30 % of the secondary syphilis patients (30). This emphasizes the need for careful examination of the CNS in any syphilitic infection. Therefore, in Rotterdam, each patient is examined one year after the treatment for CNS involvement by examining the CSF. A proven syphilitic infection of the CNS requires special treatment since the usual therapy for early syphilis of 3 times 2,4 MU benzathine penicillin will not result in treponemacidal penicillin levels in the CSF (31).
The diagnosis of symptomatic and asymptomatic neurosyphilis is mainly based on the examination of the CSF. The examination of the CSF consists of 2 parts: The demonstration of antitreponemal antibody synthesis within the CNS and the detection of non-specific inflammation. Both are influenced by a possible dysfunction of the blood-CSF barrier which should be investigated separately.
In many infectious diseases of the CNS the blood-CSF barrier is damaged due to an acute or chronic meningitis. The function of the blood-CSF barrier is commonly measured by the albumin quotient (32) and is a better indicator of impaired blood-CSF barrier than CSF-protein.

$$\text{Albumin quotient} = \frac{\text{CSF-albumin}}{\text{serum-albumin}} \times 10^3$$

The albumin quotient normally ranges from 3 to 8 depending on the age of the patient (33).
Inflammation of the CNS leads to increased total protein levels and lymphocytic pleocytosis in the CSF. Total protein levels and mononuclear cell count in the CSF of normal individuals usually do not exceed 0.5 gr/l (32) (0.6 gr/l in Rotterdam) or 5/mm^3 respectively (34). Unfortunately, these changes are not specific for neurosyphilis and normal levels do not exclude CNS involvement (32).
Demonstration of IgG-production in the CSF and the detection of oligoclonal CSF immunoglobulins may indicate a local infection of the CNS. The CSF is assumed not to be contaminated with serum antibodies if no blood is visible in the CSF. More precisely, the erythrocyte concentration in the CSF should not exceed 0.1 % (35). It was observed in experiments that addition of 0.3 % (v/v) whole blood with a 1/256 positive VDRL test to 1 ml CSF did not result in a reactive CSF-VDRL. Higher amounts of whole blood added to the CSF was visible. The IgG levels in the CSF should be determined with respect to the function of the blood-CSF barrier. Normally, the concentration of IgG in the serum is about 400 times higher than in CSF. Intrathecal production of IgG will raise the IgG concentration in CSF but this increase may also be due to an impairment of the blood-CSF barrier. Therefore, the concentration of IgG in the CSF should be adjusted for the leakage of the blood-CSF barrier. This may be achieved by the correction of the CSF-IgG concentration by the albumin quotient as was done for the IgG-index (33):

$$\text{IgG-index} = \frac{\text{CSF-IgG} \quad \text{x } 10^3}{\text{serum-IgG}} \quad \div \quad \text{Albumin quotient}$$

Index values of > 0.7 may be considered abnormal (33).
However, the IgG index is frequently normal in up to 40 % or more symptomatic neurosyphilis patients (32, 34). Similarly, IgM levels in the CSF adjusted for an assumed blood-CSF barrier dysfunction can be estimated by means of the IgM-index with abnormal values > 1.0 (33). An abnormal IgM-index is seldom observed in neurosyphilis (34).
Oligoclonal immunoglobulins as a result of the proliferation of a limited subset of B lymphocytes may occur in the CSF due to an intrathecal infection. Oligoclonal immunoglobulins can be demonstrated by isoelectric focussing using thin-layer polyacrylamide gels or by cellulose acetate electrophoresis (36, 37). The prevalence of oligoclonal immunoglobulins in neurosyphilis is high varying from 40 % to 90 % in different studies (34, 36).
If the CNS is affected by the syphilitic infection, antitreponemal antibodies will be synthesized in the CSF. Again, demonstration of antitreponemal IgG or IgM in the CSF may be the result of intrathecal production or leakage through the blood-CSF barrier or both. Determination of antitreponemal IgM may seem an attractive method to detect an active syphilitic infection of the CNS. Unfortunately, in late syphilis when the CNS is involved, antitreponemal IgM is often not detectable (34, 38). Antitreponemal IgM in the CSF with intact blood-CSF barrier can be determined by the 19S(IgM)-FTA-ABS test. An intact blood-CSF barrier is simply established if anti-Adenovirus immunoglobulins are absent in CSF and present in serum (39). If the blood-CSF barrier is damaged, antitreponemal IgM in the CSF, adjusted for impaired blood-CSF barrier function can be calculated from the IgM intrathecal T. pallidum antibody (ITPA) index (40).

$$\text{IgM-ITPA-index} = \frac{\text{CSF-IgM-TPHA titre}}{\text{serum-IgM-TPHA titre}} \quad \div \quad \frac{\text{CSF-IgM}}{\text{serum-IgM}}$$

Unfortunately, this index may be normal in neurosyphilis. Determination of antitreponemal 19S(IgM) in serum of untreated syphilitic patients with neurological disorders may be indicative for an active infection but 19S(IgM) is often absent in neurosyphilis as stated before. A reactive VDRL in the CSF also indicates an active syphilitic infection. However, the VDRL test is negative in 30 % to 85 % of the neurosyphilis patients (32, 34). Antitreponemal IgG titres in the CSF can be measured by the TPHA or FTA-ABS test, but must be adjusted for blood-CSF barrier dysfunction. This calculation requires quantification of the antitreponemal IgG concentration. This concentration is approximated by titration which

can be easily performed in the TPHA. Different formulas to adjust the CSF-TPHA titre for blood-CSF barrier leakage have been proposed. In 1983 Müller described the ITPA-index.

$$\text{ITPA-index} \quad \frac{\text{CSF-TPHA titre}}{\text{serum-TPHA titre}} \div \frac{\text{CSF-IgG}}{\text{serum-IgG}}$$

In this index, the quotient CSF-TPHA titre/serum-TPHA titre was corrected for blood-CSF barrier dysfunction by dividing it by the IgG-quotient (40). Müller defined an ITPA-index > 2.0 as indicative for local production of antitreponemal IgG. This implies a 100 % specificity of the ITPA-index. Other authors observed a much lower specificity ranging from 55 % to 73 % (34, 39). The sensitivity among neurosyphilis patients was high ranging from 82 % to 97 % (36, 39). In 1981 Luger used the TPHA titre in CSF only to develop a analogous formula (32). In this TPHA-index the TPHA titre in CSF was divided by the albumin quotient.

$$\text{TPHA-index} = \frac{\text{CSF-TPHA titre}}{\text{albumin quotient}}$$

In 1988, Luger compared the ITPA-index and the TPHA-index using the sera and CSFs of 45 patients with active neurosyphilis and 67 patients with early or latent syphilis without CNS involvement as controls (39). With the new limit of 70 for the TPHA-index, he observed the sensitivity and the specificity of 100 %. In this study the many false-positive (45 %) and some false-negative (3 %) results in the ITPA-index were observed to be due to the extreme variations in the serum TPHA titre (39). The serum TPHA titre was not a part of the TPHA-index formula and therefore, these extreme variations of the serum TPHA titre could not affect the TPHA-index.

In general practice, the diagnosis of the CSF disorders is performed on a population with an extremely low prevalence of neurosyphilitic disease. This condition requires a test of high specificity. Obviously, such a test is not provided by the ITPA-index, but according to Luger, the TPHA-index may be a promising candidate (39).

In summary, neurosyphilis can be excluded by a negative TPHA in the CSF. Demonstration of antitreponemal 19S(IgM) can almost prove neurosyphilis only if the blood-CSF barrier is intact, but the sensitivity is low. In addition, a TPHA-index > 500 also strongly suggests neurosyphilis.

If a TPHA-index between 70 and 500 is observed, the diagnosis of neurosyphilis must be supported by one or more of the following laboratory test results; CSF mononuclear cell count > 5/mm^3, CSF total protein > 0.5, IgG-index > 0.7, reactive VDRL in CSF or presence of oligoclonal immunoglobulins.

4.5 New approaches in syphilis serology

Serology in syphilis has always been of great importance for the diagnosis of the disease since the causative agent cannot be cultivated in the laboratory. Therefore, much effort has been directed into developing good tests. Almost any era in the new generation of tests is represented in the history of the syphilis serology. Each method had its drawbacks that provided the basis for the improvement of the next generation of tests.
The first serological tests for syphilis exploited the complement fixation principle (Wassermann, Kolmer and RPCF test). A severe disadvantage of these methods was the presence of anticomplementary activity often found in the test sera. In 1974 a complement independent counterimmunoelectrophoresis (CIE) assay using sonicated Reiter treponemes was introduced by Bänffer (41). This method was improved by Nell using purified Axial Filament of the same treponemes (42). Other methods that were developed required experimental animals (TPI) or needed expensive equipment (FTA-ABS test). Large-scale testing became available with the introduction of the TPHA (15).
This hemagglutination assay combined high specificity and sensitivity with simplicity in the testing of many samples simultaneously. This feature made the TPHA extremely useful as a screening test. Although, the TPHA could be automated to a large extent, the reading of the test results still needed the human eye.
In 1971, Engvall and Perlmann described the Enzyme-linked immunosorbent assay (ELISA) for the detection of pathogen-specific antibody (43). This assay used chromogenic substrates that, initially colourless, were changed into a coloured product by an enzymatic reaction. The enzyme was attached to antibodies that could bind to human immunoglobulins (the conjugate). Such a conjugate could detect antigen-specific human antibodies in the patients sera that were bound to the antigens which were precoated onto a solid phase. Since a single enzyme molecule could interact with many substrate molecules, the colour reaction was magnified markedly. As a consequence a very sensitive assay was available with test results that could easily be read by eye and could be quantified. The test could be carried out in small plastic vials that allowed clustering of samples in low space consuming trays. Application of the ELISA technique in serodiagnosis of syphilis was done for the first time by Veldkamp and Visser in 1975 (44). Although, the sensitivity of this ELISA for serodiagnosis of syphilis was high (90 % - 100 %), the specificity was not evaluated in an extensive number of control sera from non-infected individuals. Other authors have reported no improvement in the sensitivity or the specificity of an ELISA using T. pallidum antigens. Even lower sensitivities than the FTA-ABS test, or TPHA in primary syphilis were reported (45, 46, 47, 48).
It was shown that the preincubation of the sera with a FTA-ABS sorbens-like substance could enhance the specificity in such a test (45) or that purification of treponemal antigens could improve the sensitivity of the ELISA (49). These studies showed that the use of T. pallidum as antigen in a serological test led to aspecific results possibly due to cross-reacting antibodies raised by presumed commensal organisms. It was concluded that these cross-reacting antibodies were effectively removed by absorption with the Reiter treponeme used for preabsorption in the FTA-ABS test. Therefore any serological test that uses T. pallidum must contain a procedure for the removal of non-specific antibodies from the serum.
Another approach for avoiding the non-specific reactivity of such antibodies in a syphilitic test is the purification of distinct treponemal antigens. Isolation and purification of such antigens from T. pallidum is difficult because these organisms are available in small amounts only. Purification of the endoflagellum from the cultivable T. phagedenis may circumvent

this difficulty. Strandberg Pedersen et al. used this endoflagellum as antigen in an ELISA (50). This test was valuable since an antigen that could be easily obtained by cultivation of non-pathogenic treponemes was used. Although high sensitivity using syphilitic sera and high specificity using donor sera was observed, the reactivity in this test of sera that reacted falsely in other tests for syphilis was higher than that in the FTA-ABS test. Another method to obtain purified treponemal antigens is the expression of distinct treponemal antigens in an easily cultivable host via recombinant-DNA technology. Treponemal recombinant-DNA derived antigens are well-defined and can be highly purified as compared to the natural treponemal antigens because recombinant-DNA derived proteins are available in large quantities. In addition, easily cultivable hosts containing recombinant plasmids produce treponemal antigens cheaper than by cultivating T. pallidum in rabbit testes. Recently, several recombinant-DNA derived treponemal antigens have become available (51-57). However, the application of these antigens in serological tests remains to be established. Single treponemal epitopes can be detected in a test using monoclonal antibodies. It remains to be determined whether these determinants, involved in small numbers only and therefore highly specific, are suitable for use in such a test.

ELISA for the serodetection of antitreponemal IgM

The detection of antitreponemal IgM antibodies may be impaired by the presence of the rheumatoid factor or the excess of antitreponemal IgG. Therefore, before the detection of antitreponemal IgM in ELISA, this IgM should be separated from the serum before it can be detected. Duermeyer and Van der Veen have described an ELISA using microtitration plates with anti-human IgM immunoglobulins coated into the wells for binding of serum IgM (58). In this method, IgG is easily washed away but the rheumatoid factor still may cause false-positive results due to its binding to anti-IgM antibodies coated onto the wells of the microtitration plate. An ELISA for serodiagnosis of syphilis using this IgM-capture principle was described by Strandberg Pedersen et al. (59). Lindenschmidt et al. absorbed the rheumatoid factor from the serum by preincubation with heat-aggregated human IgG. Competitive inhibition by antitreponemal specific IgG was not impaired (60). A complete solution for these problems was given by Müller et al. (61). Preincubation of the sera with sheep anti-human IgG not only inhibited the interference by the rheumatoid factor but also the competition by antitreponemal IgG. However, the immunoprecipitation of total IgG was very critical and therefore the test remained laborious.

In conclusion, application of purified or recombinant-DNA derived antigens in a test for serodiagnosis of syphilis may result in the higher specificity and circumvent the need for a sorbens. A test using such antigens is not yet widely available and for which further development and evaluation is necessary. For the detection of antitreponemal IgM a simple test with a performance equal to the 19S(IgM)FTA-ABS is required. Such a test is not yet available.

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Chapter 4

4 Requirements for a new laboratory test for the serological diagnosis of syphilis

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4.1 General requirements for a new laboratory test

Diagnostic tests are used either for the diagnosis of a disease in individuals, or for the diagnosis of a disease in groups of individuals, or both (1, 2, 3).
The diagnosis of a disease in an individual with signs (perceived by the clinician) and symptoms (perceived by the patient) has the purpose of categorizing these signs and symptoms into a known disease entity, and is called "clinical diagnosis" (1). In this case, a diagnostic test may also provide additional clues to the severity and prognosis of the condition, and to the expected response to treatment, and may also be helpful in evaluating the response to treatment.
The use of a diagnostic test in groups of apparently healthy individuals is used to detect the disease prior to the development of signs and symptoms. This is called "screening" (1, 2, 3). "Case finding" as defined by Griffiths and Ruitenberg (2) is a variation on screening in that a diagnostic test is used in individuals with signs and symptoms which are unrelated to the disease being tested.
The diagnosis of a disease in groups of individuals is done in epidemiological surveys (2). However, even in the case of a "community diagnosis" (4) it is the total of individual test results that form the basis for such diagnosis. No specific laboratory tests have been developed which measure group characteristics only.
In summary, the main two uses of diagnostic laboratory tests are clinical diagnosis and screening. The essential difference between these two possible applications of a diagnostic test is that, by definition, the prevalence of the target disease is higher in the case of clinical diagnosis than in screening. The relevance of this is discussed below.

4.2 General requirements for a test aimed at improving clinical diagnosis of a disease

The most comprehensive set of requirements for a new diagnostic test for a disease was compiled by Sackett et al. (1) and is presented in Table 1.

Table 1. Evaluation of a diagnostic test.

1. The new test must be compared blindly and independently with the current "gold standard" of diagnosis.
2. The study population in which the new test is to be evaluated must comprise an appropriate[a] spectrum of mild and severe, treated and untreated disease, as well as of disease commonly confused with the disease under study.
3. The setting of the evaluation, and the in- and exclusion criteria for entering subjects into the study, must be clearly and unambiguously defined.
4. Variability in test results and interpretation must be clearly determined.
5. There must be a sensible definition of the a "normal" test result.
6. If the test is to be used as a part of a cluster or sequence of tests, its individual contribution to the overall validity of the cluster or sequence must be established.
7. The tactics for carrying out the test must be described in adequate detail to allow exact replication.
8. The utility of the test must be determined[b].

[a] "appropriate spectrum" indicates that the study population must as far as possible resemble the population in which the test is intended for use.
[b] "utility of the test" refers to the benefits of the new test in terms of patient's health and comfort, of costs, and of resources needed to perform the test.

4.3 General requirements for a test aimed at improving screening for disease

The application of a new diagnostic test for the purposes of screening differs from its use in the clinical diagnosis in that the test is applied to a population with low prevalence, and to individuals who are apparently healthy. For this reason there are specific requirements for screening tests. These requirements are summarized in Table 2 (2, 3).

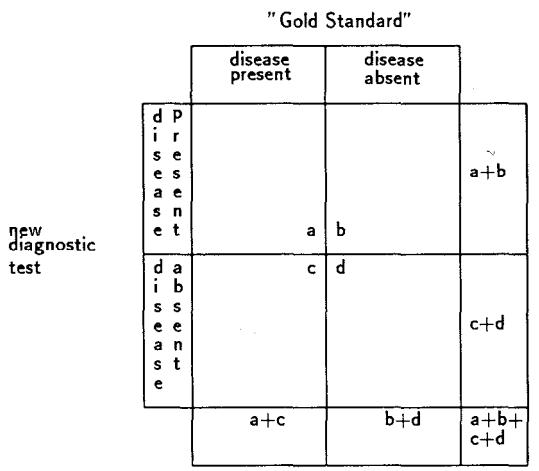
Table 2. Evaluation of a screening test.

1. The test must be safe.
2. The test must be simple to execute and apply.
3. The test must be acceptable to the population to be screened.
4. The test must be relatively inexpensive.
5. The test must be valid and reproducible.
6. The test must have a high sensitivity.
7. The test should preferably increase the examinee's understanding of the disease tested.

4.4 Comparison of a new diagnostic test with a "gold standard"

The basic format for the comparison of a new test with the current "gold standard" of diagnosis is the 2x2 table. This method assesses the agreement between the results of the new test and those of the "gold standard". The validity of this comparison is, therefore, only as good as the validity of the "gold standard" itself. In Figure 1 the basic table of comparison is presented.

Figure 1.



Using this format of comparison, the following parameters can be calculated:

- Sensitivity of the test: i.e. the proportion of patients with the disease who are correctly identified by the new test as having the disease. This can be expressed as a/a+c.
- Specificity of the test: i.e. the proportion of patients without the disease who are correctly identified by the new test as not having the disease, or d/b+d.
- Positive Predictive Value (PPV) of the test: i.e. the proportion of patients, diagnosed by the new test as having the disease, who actually have this disease, or a/a+b.
- Negative Predictive Value (NPV) of the test: i.e. the proportion of patients, diagnosed by the new test as not having the disease, who actually do not have the disease, or d/c+d.
- False Positive Rate (FPR) of the test: i.e. the proportion of patients without the disease who are diagnosed by the new test as having the disease, or b/b+d, or (100 % - specificity).

- False Negative Rate (FNR) of the test: i.e. the proportion of patients with the disease who are diagnosed by the new test as not having the disease, or c/a+c, or (100 % - sensitivity).
- Prevalence of the disease: i.e. the proportion of the study population actually having the disease, or a+c/a+b+c+d.

These parameters allow an adequate description of the validity of the new test in cases where a dichotomised test result i.e. the disease is either absent or present, is sufficient. More sophisticated mathematical calculations such as odds ratios may be needed to determine the appropriate cut-off points in continuous and non-dichotomous test results. Since the tests under consideration in this study are principally aimed at providing a dichotomous answer, these calculations are not further considered here.
The parameters most commonly used in the evaluation of new diagnostic tests are the sensitivity, the specificity, the PPV and the NPV. The sensitivity and the specificity indicate the performance of the test when applied to a population. The PPV and the NPV, however, indicate to the clinician in the typical doctor patient setting the likelihood that the test result accurately reflects the patient's condition. For this reason clinicians may tend to consider only the reported PPV and NPV of a new diagnostic test. However, there is a crucial difference between the sensitivity and the specificity of a test on the one hand, and the PPV and the NPV of the same test on the other. Using the 2x2 table presented above it can be easily shown that sensitivity and specificity are independent of the prevalence of the condition. The PPV and the NPV on the other hand do vary with varying prevalence: the PPV increases with increasing prevalence while the NPV decreases, and vice versa. Any report on a new diagnostic test must, therefore, provide sufficient information to allow calculation of all these four parameters.
These parameters apply to tests whether they are intended for clinical diagnosis or for screening. However, the emphasis on each parameter differs between the two applications of a test. In Table 3 the relative emphasis on each parameter is assessed for the application of a test in clinical diagnosis as compared to its application in screening.

Table 3. Relative emphasis on test evaluation criteria in clinical diagnosis and screening.

Evaluation Criterion	Clinical Diagnosis	Screening
Sensitivity	Low	High
Specificity	High	Low
PPV	High	Low
NPV	Low	High
FPR	Low	High
FNR	High	Low

4.5 Specific requirements for a new laboratory test for the serodiagnosis of syphilis

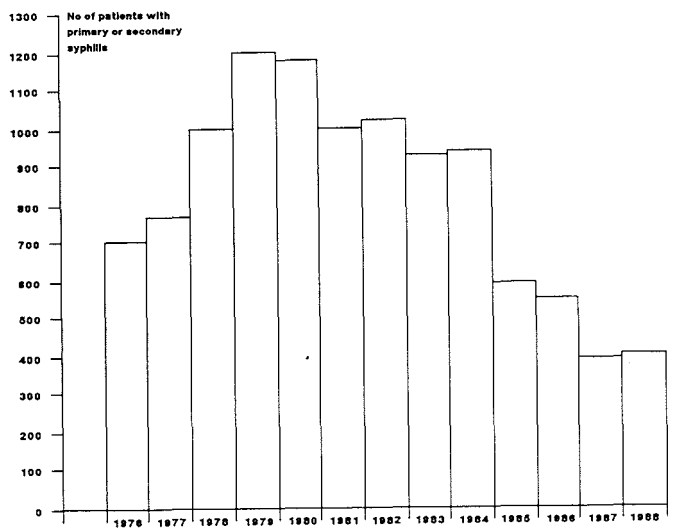
The Centers for Disease Control (CDC) in Atlanta, Georgia, (U.S.A.) has provided detailed guidelines for the evaluation of new serological tests for the diagnosis of syphilis (5). These guidelines are essentially adapted from Sackett's general test criteria (see Table 1) to syphilis.
The current "gold standard" for the serodiagnosis of syphilis (Sackett's first criterion) are the FTA-ABS and VDRL tests. Comparison with the FTA-ABS test provides information about the performance of the new test in terms of clinical diagnosis, while comparison with the VDRL test demonstrates the new test's performance as a screening tool. Sackett's second criterion is fulfilled by the CDC's requirement that the new test should be evaluated in 100 cases of untreated primary syphilis, 50 cases of untreated secondary syphilis, 100 cases of untreated latent syphilis, in 300 cases of other diseases including diseases causing false-positive reactions in FTA-ABS and VDRL tests, and in 300 apparently healthy individuals. Sackett's third criterion is implicit. The fourth criterion is covered by the requirement to have the test evaluated at least by two different laboratories, using at least 500 specimens, and to determine the variability of the test results over time period. Criteria 5, 6 and 7 are also implicit in the CDC requirements. Criterion 8 does not seem to be covered by the CDC guidelines.
The evaluation of the new test which is the subject of this study will therefore have to satisfy the CDC requirements for a new test, Sackett's criteria for application of the test in clinical diagnosis, and the requirements relating to the application of this test for the purposes of screening.

4.6 Specific requirements for a new test for the serodiagnosis of syphilis in the Netherlands

Primary and secondary syphilis became notifiable on an anonymous basis in The Netherlands in 1976. The cases reported since then are shown in Figure 2. (6, 7, 8).

The increase in the reported incidence of syphilis in the first four years is probably an artefact, and probably related to an increasing awareness among health workers that syphilis had become notifiable. The sharp decrease since 1985 may be related to a more prudent sexual lifestyle consequential upon the advent of the Acquired Immune Deficiency Syndrome (AIDS). This is supported by the finding that primary syphilis has continued to decrease among homosexuals and bisexuals in the city of Amsterdam (9).
Congenital syphilis is relatively rare. Between 1982 and 1985, IgM antibodies to treponemes were demonstrated in 19 children. (10).
In summary, between 500 and 1100 cases of primary and secondary syphilis were reported annually in The Netherlands over the past 13 years. Taking into consideration that the reported incidence is probably an underestimate of the true incidence, it can be concluded that syphilis was relatively common in The Netherlands but that its incidence declines.

Figure 2.



However, the continued screening of the approximately 525.000 blood donors and 170.000 pregnant women annually can be discussed (11).

For the clinical diagnosis of primary and secondary syphilis in The Netherlands, the most common protocol is to apply the TPHA test first, and to confirm the positive TPHA test results using the FTA-ABS test. For monitoring the response to treatment, the VDRL test is used. To diagnose congenital syphilis and neurosyphilis, the 19S(IgM)FTA-ABS test is used which is only available at the National Institute of Public Health and Environmental Protection in Bilthoven.
Sensitivities and specificities of these tests are depicted in Table 4 (12, 13).

These tests routinely used in The Netherlands, and probably also in many other developed countries, make the accurate clinical diagnosis of and the screening for syphilis a costly and laborious undertaking. The FTA-ABS and 19S(IgM)FTA-ABS tests are labour intensive, expensive and require a high level of quality control to ensure reliable results. However, the tests have high specificity. The TPHA and VDRL tests on the other hand, have high sensitivity and are relatively cheap, but both have relatively low specificity and therefore lead to a high false-positive rate.

A new serological test for the diagnosis of syphilis in The Netherlands should address particularly the disadvantages of the routinely used tests.

Table 4. Sensitivity and specificity of syphilitic tests in The Netherlands.

Diagnosis	No. of samples	percentage of samples positive in: VDRL	TPHA	FTA-ABS	19S(IgM)FTA-ABS
Primary syphilis	61	66	90	95	95
Secondary syphilis	96	99	100	100	100
Early latency	62	89	100	100	24
Neurosyphilis	25	68	100	100	32
No syphilis	500	0.4	0.6	0.4	not done

The new test should therefore:

- have high sensitivity and specificity to reduce the need for multistage testing.
- have potential for automation in view of the large numbers of tests performed annually.
- take no more time for execution than the average time required for consultation, in order to eliminate the need for reconsultation with the consequent risk of non-compliance and loss of follow up.
- be simple to perform, require no sophisticated equipment and be cost-competitive.

In the following Chapters of this dissertation, the new tests described were assessed according to the specific situation in The Netherlands. These conditions, however, are likely to be relevant to most other developed and developing countries.

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Chapter 5

5 Objectives of the study

In the previous Chapters of this dissertation the major shortcomings of the current serological tests for syphilis were investigated. These include an insufficient sensitivity and specificity, reactivity of a diagnostic test long after the syphilitic infection had been cured, complexity of operation, high costs, long test duration, unsuitability for automation, no quantifiable test results available and the inability to distinguish between the distinct treponematoses. The aim of this study was to eliminate these shortcomings and to develop and evaluate enzyme immunoassays for serodiagnosis of syphilis. To achieve this, four different performance aspects of the enzyme immunoassays were investigated. These are as follows:

1. A sensitive and a specific treponemal screening test that also permitted treatment monitoring without the phenomenon of serofast test results after adequate treatment and biological false- positive reactions.
2. To reduce the operation time to around 10 to 20 minutes in order to facilitate its use in an outpatient clinic to provide the test results within the course of one consultation.
3. To develop a simple automated confirmation test for syphilis to replace the current confirmation tests that are complex and laborious.
4. To devise a simple, sensitive and specific test for the detection of antitreponemal IgM. Such a test should permit the simultaneous testing of several serum samples and provide automated test results.

Integration of tests that meet these objectives will permit simple, sensitive, specific, objective, rapid and automated testing for human antitreponemal IgG and IgM antibodies to detect untreated syphilis, congenital syphilis and to monitor the effect of treatment. Since there are no known antigenic differences between the treponematoses, the distinction between the various treponematoses by serological means was not studied. Therefore, in the experimental chapters of this dissertation the terms "antitreponemal" or "antitreponemal antibody" are used only in relation to syphilis.
Enzyme immunoassay methods were investigated in this study in order to exploit their simple operation and extensive possibilities for automation. New acquisitions obtained from modern biochemistry like recombinant-DNA derived antigens, monoclonal antibodies and the newly developed immunofiltration technique for enzyme immunoassays were evaluated to improve syphilis serology.

Chapter 6

6 Sensitivity and specificity of an enzyme-linked immunosorbent assay using the recombinant DNA-derived Treponema pallidum protein TmpA for serodiagnosis of syphilis and the potential use of TmpA for assessing the effect of antibiotic therapy

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6.1 Abstract

The recombinant DNA-derived Treponema pallidum membrane protein TmpA, purified from Escherichia coli K-12, was used in an enzyme-linked immunosorbent assay (ELISA) to evaluate its suitability in a screening test for syphilis and to monitor the effect of antibiotic treatment. The sensitivity of the TmpA ELISA was 76 % for primary syphilis, 100 % for secondary syphilis and 98 % for early latent syphilis. All except 1 of 15 serum samples positive for yaws were positive in this test. A specificity of 99.6 % was found by testing more than 938 donor samples. The sensitivity and the specificity of the TmpA ELISA are comparable to that of the T. pallidum hemagglutination assay, and therefore the test may be useful for the diagnosis of untreated syphilis. After antibiotic treatment, the level of anti-TmpA antibodies in sera of syphilis patients dropped sharply within 1 year. Thus, TmpA might be a useful antigen for monitoring successful treatment of syphilis.

6.2 Introduction

The serodiagnosis of syphilis is commonly based on the detection of antibodies against the causative organism, Treponema pallidum, or against cardiolipin (19).

In Europe, the most frequently used screening test for syphilis is the T. pallidum hemagglutination assay (TPHA; Japan Lyophilization Co.) (5,7,15). A positive TPHA is commonly confirmed by the fluorescent treponemal antibody absorption test (FTA-ABS) (19). Although it is more sensitive than the TPHA, the FTA-ABS is laborious and unsuitable for screening of large numbers of serum samples.
Cardiolipin-based flocculation tests such as the Venereal Disease Research Laboratory (VDRL) test and the Rapid Plasma Reagin (RPR) card test are also used in screening for syphilis. However, the sensitivities and specificities of such tests have been reported to be less than that of the TPHA (4,9,15). Treatment monitoring is currently done by determination of the decline of anticardiolipin or antitreponemal immunoglobulin M antibodies (5).
Recently, individual T. pallidum antigens became available as a consequence of the use of recombinant DNA techniques (8,10-13,17,18). One of the recombinant DNA-derived antigens investigated for use in syphilis serology is the T. pallidum membrane protein TmpA (2). This 42-kilodalton antigen has been obtained from Escherichia coli K-12 containing plasmid pRIT4661, which could be induced to express high levels of TmpA (L.M. Schouls, O.E. IJsselmuiden and J.D.A. van Embden, submitted for publication). In recent studies, TmpA was purified from E. coli K-12 membranes by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis. TmpA purified in this way was used to develop an enzyme-linked immunosorbent assay (ELISA) to detect antibodies in sera from syphilis patients. The preliminary results of that study showed that the sensitivity of the TmpA ELISA to detect primary, secondary, and early latent syphilis was comparable to that of the TPHA. However, as only a limited number of syphilitic and control serum samples was studied, no conclusions on the general applicability of the TmpA ELISA could be drawn.
The aim of this study was to evaluate the sensitivity and specificity of the TmpA ELISA in comparison with those of the TPHA, FTA-ABS and VDRL test for serodiagnosis of untreated syphilitic disease. Furthermore, in the TmpA ELISA, the reactivity of serum samples from patients treated for syphilis was investigated.
To evaluate the TmpA ELISA for suitability in monitoring the effect of treatment, we studied the levels of anti-TmpA antibodies in sera of syphilitic patients at various intervals after antibiotic treatment. The changes in the anti-TmpA antibody levels in these sera as a consequence of treatment were compared with changes in levels of antibodies against cardiolipin and against endoflagellum purified from cultivable Treponema phagedenis. The axial-filament (AF) ELISA (AF-ELISA) using this endoflagellum was included in this study because this ELISA appeared to be about as sensitive as the TmpA ELISA for diagnosis of syphilis (Schouls et al., submitted)

6.3 Materials and Methods

Sera
Serum samples from patients with syphilis, including 148 untreated and 167 treated patients, and from 190 patients without any evidence of syphilis were obtained from the Sexually Transmitted Disease (STD) Clinic of the University Hospital Dijkzigt in Rotterdam. The diagnosis and classification of syphilis were based on criteria described previously (3). To investigate the reactivity in serological tests of posttreatment sera, another 27 untreated patients were included in this study. These patients were monitored serologically after treatment. The group of 167 serum samples from treated patients were predominantly

from drug-treated individuals with early syphilis of less than 2-years duration. Furthermore, 86 samples obtained from individuals without a history of syphilis that were reactive in only one of the syphilis tests (TPHA, FTA-ABS or VDRL) were tested. All samples from the last group were obtained from the University Hospital of Rotterdam, except for 24 samples which were reactive in the VDRL test and which were kindly provided by A. Paris-Hamelin (Institut Alfred Fournier, Paris, France). The 15 yaws-positive serum samples were collected by S. Sadal (Paramaribo, Suriname). The sera were from untreated children aged 12 years or younger. Finally, 938 serum samples were obtained from donors at the blood banks in Rotterdam and The Hague. All sera were stored at -20 °C. All sera, except the blood donor sera, were tested by the TPHA, the FTA-ABS (14) and the VDRL test (14). The blood donor serum samples were screened by TPHA only.

Purification of TmpA

Isolation and purification of TmpA were performed by the method of Schouls et al. (Schouls et al., submitted), with modifications. E. coli K-12 cells carrying plasmid pRIT4661, which encodes for temperature-dependent hyperexpression of TmpA, were cultured at 28 °C for 6 hours and subsequently induced to hyperexpression by raising the temperature to 42 °C for 1 h. The culture was centrifuged, and the pellet was suspended in 50 mM Tris hydrochloride-5 mM EDTA (pH 8.0). After ultrasonic treatment of the cell suspension, the membrane fraction was solubilized with 1 % N-tetradecyl-N,N,-dimethylammonio-1-propanesulfonate (SB 3-14) for 1 h at 37 °C. Unsolubilized material was removed by centrifugation at 10,000 x g. The supernatant was purified by DEAE column chromatography using high performance liquid chromatography equipment with a TSK 545 column (7.5 x 150 mm; LKB-Producter, Bromma, Sweden). Therefore, the supernatant was diluted five times in TE buffer (0.01 M Tris hydrochloride, 1 mM EDTA (pH 8.0)). About 10 mg of TmpA in 100 ml of TE buffer containing 0.1 % SB 3-14 was loaded into the column. To remove the detergent, the column was washed with 10 ml of a 0.1 to 0 % SB 3-14 gradient in TE-buffer. Subsequently, TmpA was eluted by 50 ml of a 0 to 0.5 M NaCl gradient in TE buffer at a flow rate of 0.5 ml/min. The fractions containing the TmpA were subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the TmpA-containing polyacrylamide was cut out and TmpA was extracted from the gel by electroelution. TmpA was tested for stability by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and for equal reactivity in the TmpA ELISA during 10 freeze-thaw cycles. No change of characteristics was found. TmpA was preserved either at -20 °C or as dry protein. Before use, the latter product was solubilized in TS buffer (0.01 M Tris hydrochloride (pH 7.4), 0.15 M NaCl) containing 0.05 % sodium dodecyl sulfate.

ELISAs

A 0.1-ml solution of TS buffer containing TmpA (1 μg/ml) was added to each well of a 96-well microdilution plate (Dynatech Deutschland GmbH, Plochingen, Federal Republic of Germany) and incubated for 1 h at 37 °C. The plate was washed for 30 s by a continuous low-pressure flow of wash buffer (TS buffer containing 0.05 % Tween 20). Serum was diluted 1:100 in TS buffer, containing 0.5 % Tween 20. A 0.1-ml portion of the diluted serum was incubated for 1 h at 37 °C. Positive and negative control sera were included in each experiment. After being washed as described above, the wells were incubated for 1 h at 37 °C with a 0.1-ml solution of sheep anti-human immunoglobulin horse radish peroxidase conjugate (obtained from the RIVM, The Netherlands) diluted 1:25.000 in TS buffer with 0.05 % Tween 20. After the final wash, 0.1 ml of substrate solution containing

0.11 M sodium acetate (pH 5.5), 0.1 mg of 3,3',5,5'-tetramethylbenzidine per ml, and 0.009 % hydrogen peroxide was added to each well. After 5 min, the reaction was stopped by adding 50 μl of 3 M sulfuric acid to each well and the optical density (OD) was read at 450 nm (Titertek Multiscan). OD values below 0.2 were regarded as negative.
The AF-ELISA was carried out as described previously (16).

6.4 Results

Sensitivity and specificity of the TmpA ELISA

The sensitivity of the TmpA ELISA was compared to that of the TPHA, FTA-ABS, and VDRL test (Table 1). The sensitivity of the TmpA ELISA was virtually identical to those of the TPHA and the FTA-ABS in cases of untreated secondary and early latent syphilis and in the 15 cases of untreated yaws. The reactivities of the sera from untreated primary syphilitics was 76, 75, 84, and 75 % for TmpA ELISA, TPHA, FTA-ABS and VDRL test, respectively.

Table 1. TmpA reactivity of serum samples from individuals with a history of syphilis or yaws.

Diagnosis	No. of serum samples tested	No. (%) of serum samples reactive by			
		TmpA-ELISA	TPHA	FTA-ABS	VDRL test
Untreated syphilis					
Primary	55	42(76)[a]	41(75)	46(84)	41(75)
Secondary	39	39(100)	39(100)	39(100)	39(100)
Latent	54	53(98)	54(100)	54(100)	51(94)
Yaws, untreated	15	14(93)	14(93)	14(93)	14(93)
Treated syphilis					
VDRL test reactive	53	45(85)	50(94)	52(98)	53(100)
VDRL test nonreactive	114	42(37)	102(89)	95(83)	0(0)

[a] Of the 13 TmpA ELISA-nonreactive serum samples, 9 were positive only by darkfield microscopy; 2 were reactive in TPHA and VDRL test; 1 was positive by TPHA, FTA-ABS and VDRL test; and 1 was positive by TPHA and FTA-ABS.

To compare the specificity of the TmpA ELISA with those of the classical syphilis tests, we analyzed 938 serum samples from blood donors and 190 samples from individuals who visited an STD clinic but did not have any clinical or serological evidence for syphilis. The specificity of the TmpA ELISA among the 938 blood donors was 99.6 %, which is comparable to those of the TPHA (99.8 %), FTA-ABS (99.6 %) and the VDRL (99.6 %) test (Table 2). Among the 190 samples from visitors of the STD clinic, the specificity of the TmpA ELISA was 98.4 %. All positive blood donor sera were positive in TmpA

ELISA, TPHA, FTA-ABS, or VDRL test only. Furthermore, we tested 86 serum samples which were from individuals without a history of syphilis and which were positive by TPHA, FTA-ABS or VDRL test only. In none of these sera were TmpA-reactive antibodies found.

Table 2. TmpA reactivities of the serum samples from nonsyphilitic individuals in TPHA, FTA-ABS and the VDRL test.

Source of sera	No. of serum samples	No. (%) of serum samples reactive by			
		TmpA ELISA	TPHA	FTA-ABS	VDRL test
Blood donors	938	4(0.4)[a,b]	2(0.2)	4(0.4)	4(0.4)
Visitors to STD clinic	190	3(1.6)[b,c]	0(0)	0(0)	2(1.1)
TPHA (only)-positive patients	18	0	18	0	0
FTA-ABS (only)-positive patients	21	0	0	21	0
VDRL test (only)-positive patients	47	0	0	0	47

[a] ODs were: 0.58, 0.30, 0.26, and 0.23.
[b] None of the samples was positive in the TPHA, FTA-ABS or the VDRL test.
[c] OD of each was 0.22.

Decline of anti-TmpA antibodies as a result of treatment

Fresh sera obtained from patients treated for syphilis were divided into VDRL-test-reactive and VDRL-test-non-reactive sera (Table 1). Each serum sample was reactive in at least the TPHA or the FTA-ABS test. The reactivities of the sera of both groups were lower in the TmpA ELISA than in the TPHA and FTA-ABS. In particular, the VDRL-test-non-reactive sera were remarkably less reactive in the TmpA ELISA (37 %) than in the FTA-ABS (83 %) and the TPHA (89 %).

This suggested that the level of anti-TmpA antibodies, like that of anticardiolipin antibodies, dropped after adequate treatment. This prompted us to investigate the kinetics of the decrease of anti-TmpA antibodies in more detail. The sera of 27 syphilis patients from whom serum samples had been collected before treatment and at various intervals after treatment were compared in the TmpA ELISA, AF-ELISA and the VDRL test (Table 3). The VDRL test titers of all except two sera dropped at least two steps or became nonreactive within 9 months. The results show that the level of anti-TmpA antibodies in the majority of the individuals dropped sharply within a period of 9 months after treatment. Furthermore, a close resemblance between the drop in OD values obtained by TmpA ELISA and that of VDRL titers was observed. To illustrate this resemblance, the OD values of serum samples from 27 individuals in different stages of syphilis are represented graphically in Fig. 1.

In the majority of the cases studied, the anti-AF levels also dropped after treatment, but the drop was generally less steep than that of the anti-TmpA antibody levels. It should be noted that all sera remained positive in the TPHA and the FTA-ABS. The serum samples of only 2 patients (numbers 17 and 26) showed an OD drop of less than 0.3 and remained highly reactive in the TmpA ELISA. Both were cases of latent syphilis (Table 3). With the serum samples of 25 of 27 patients, either the reactivity in the VDRL test declined at least two steps within 9 months after the start of treatment or the samples were not reactive in the VDRL test at all at the start of treatment. A negative VDRL test result 9 months after treatment was observed in 13 patients. One patient remained VDRL test positive after treatment with undiluted serum. The mean levels of anti-TmpA, anti-AF and anti-cardiolipin antibodies for the different stages of syphilis are shown in Fig. 2. These mean values confirm that anti-TmpA antibodies decline more rapidly than anti-AF antibodies. However, the kinetics of anticardiolipin and anti-TmpA antibodies show a close resemblance in their response to therapy in all stages of early syphilis.

6.5 Discussion

In this study, we used recombinant DNA-derived, extensively purified TmpA as antigen in an ELISA procedure to evaluate the usefulness of this protein in the serodiagnosis of syphilis.

Among serum samples from untreated syphilis patients, the sensitivity of the TmpA ELISA was high: 76, 100, and 98 % for primary, secondary and early latent syphilis, respectively. These values are comparable to those obtained with the TPHA and the FTA-ABS. When FTA-ABS-reactive sera were chosen from primary-syphilis sera, as was done by Radolf et al. (11) for evaluation of an ELISA using the 4D recombinant protein, the sensitivity of the TmpA ELISA was found to be 91 %. The sera not reactive in the FTA-ABS test were from patients from whom T. pallidum organisms were observed by dark-field microscopy, without reactivity in any serological test for syphilis. It should be noted that 11 of the 13 TmpA-nonreactive primary-syphilis serum samples tested were also nonreactive in the TPHA and the VDRL test. Nine of these were also negative by FTA-ABS (Table 1). Since the TmpA-nonreactive samples showed a positive reaction in the FTA-ABS test only or were obtained from patients found positive by dark-field microscopy without any serological evidence of syphilis, these samples were probably taken very soon after these individuals were infected by T. pallidum.

Among the 938 donor serum samples, 4 showed OD values above the cutoff value of 0.2 in the TmpA ELISA. This resulted in a TmpA-ELISA specificity 99.6 %, which is comparable to the specificities of the TPHA and FTA-ABS obtained in this study. For the 190 STD clinic visitors without syphilis, the specificity of the TmpA ELISA was 98.4 %. The false-positive sera of both groups have been investigated by immunoblotting with T. pallidum antigen and purified TmpA. No reaction with T. pallidum antigens or the TmpA antigen was observed, which suggests that the antibodies in these sera probably did not react with TmpA itself but with E. coli contaminants (L.M. Schouls, unpublished data). Further purification of TmpA by acid precipitation before DEAE chromatography consistently resulted in nonreactivity of three of the seven false-positive sera from donors and nonsyphilitic STD visitors (L.M. Schouls, manuscript in preparation). These results suggest that further purification could improve the specificity of the TmpA ELISA.

Moreover, in the analysis of the 86 serum samples from patients with positive reactions

in only the VDRL tests, TPHA or FTA-ABS, no samples showed a positive reaction in the TmpA ELISA. This demonstrates the specificity of the TmpA ELISA for a treponemal infection. Thus, it seems that the TmpA ELISA is a useful alternative screening test for syphilis. It has the advantage over the TPHA in that mechanized or automated test systems based on the ELISA can be applied.

An unexpected finding in this study was the resemblance between reactivity in the TmpA ELISA and that in the VDRL test after treatment of syphilis patients (Table 3). This observation suggested that anti-TmpA antibodies disappear concomitantly with anticardiolipin antibodies after treatment of an active T. pallidum infection. This idea was confirmed by the study of the well-monitored serum samples from 27 syphilis patients, taken at various intervals up to 12 months after treatment. In all patients except 2, the OD values of the TmpA ELISA dropped at least 0.3 U or became negative. All these samples remained TPHA and FTA-ABS positive. Interestingly, the 2 patients who did not show a significant drop in OD were the only ones suffering from late syphilis. It seems that the TmpA antigen is not unique with respect to decline of anti-TmpA antibodies, since a similar drop of antibody titers of other protein antigens was observed in samples from treated patients by using immunoblotting techniques (1,6). In contrast to this, the decline in OD determined by the AF-ELISA was much smaller or absent.

The conclusion is that TmpA is a useful antigen in syphilis serology for mass screening, detection of active syphilis and yaws, and monitoring of the success of antibiotic treatment of syphilis. Since no data are available yet about the presence and the level of anti-TmpA antibodies in patients with late treponematoses, we are in the process of collecting serum samples from such patients to test in the TmpA ELISA.

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Table 3. Comparison of OD values from TmpA ELISA and AF-ELISA with VDRL test titers of samples from 27 patients at various intervals after treatment.

	TmpA ELISA			VDRL test			AF-ELISA		
Type of syphilis and patient no.	OD at mo[a]: 0	1-5	6-9	titer at mo: 0	1-5	6-9	OD at mo: 0	1-5	6-9
Primary									
1	1.12	0.65	0.40	32	4	1	0.73	0.83	0.83
2	0.98	0.50	0.42	8	1	0	0.78	0.81	0.69
3	0.62	0.15	0.13	4	0	0	0.53	0.26	0.17
4	0.57	0.18	0.18	4	1	0	0.77	0.43	0.30
5	0.44	0.15	0.12	16	4	0	0.28	0.20	0.20
6	0.22	0.11	0.12[b]	4	1	1[b]	0.53	0.21	0.19[b]
Secondary									
7	1.32	1.24	0.94	64	1	4	1.00	0.77	0.81
8	1.29	1.05	0.62	32	8	1	0.85	0.79	0.63
9	1.15	0.68	0.45	64	4	1	0.74	0.71	0.52
10	1.07	0.63	0.42	64	2	0	0.72	0.83	0.77
11	1.07	0.75	0.43	16	2	0	0.67	0.80	0.78
12	1.05	1.12	0.46	32	32	1	0.56	0.72	0.37
13	0.98	0.63	0.33	8	0	0	0.89	0.63	0.51
14	0.87	0.61	0.22	16	4	0	0.70	0.67	0.41
15	0.82	0.41	0.26	8	0	0	0.75	0.45	0.40
Early latent									
16	1.24	1.08	0.48	128	16	1	0.80	0.77	0.76
17	1.16	1.24	0.93	16	16	4	0.80	0.78	0.77
18	1.08	0.98	0.77[b]	32	8	4[b]	0.79	0.88	0.81[b]
19	1.00	0.55	0.25	8	2	1	0.78	0.78	0.58
20	0.98	0.66	0.31	0	0	0	0.73	0.80	0.67
21	0.91	0.30	0.16[c]	16	1	0[c]	0.76	0.70	0.48[c]
22	0.87	0.83	0.61	16	8	4	0.65	0.54	0.52
23	0.86	0.57	0.39	8	1	1	0.64	0.41	0.29
24	0.80	0.56	0.10	16	2	0	0.62	0.34	0.59
25	0.25	0.25	0.17	0	1	1	0.71	0.69	0.67
Late latent									
26	1.21	1.14	1.12	16	8	8	0.78	0.75	0.75
Asymptomatic neurosyphilis									
27	0.25	0.23	0.19	0	0	0	0.62	0.56	0.53

[a] Posttreatment.
[b] Taken 12 months posttreatment.
[c] Taken 10 months posttreatment.

Fig. 1. Decline of anticardiolipin (VDRL), anti-TmpA, and anti-AF antibodies of 27 syphilitic patients up to 12 months after treatment. The serum samples from the patients are divided according to primary (LI), secondary (LII), and early latent (LL) stages of syphilis. DIL, Highest dilution of serum resulting in a positive VDRL test.

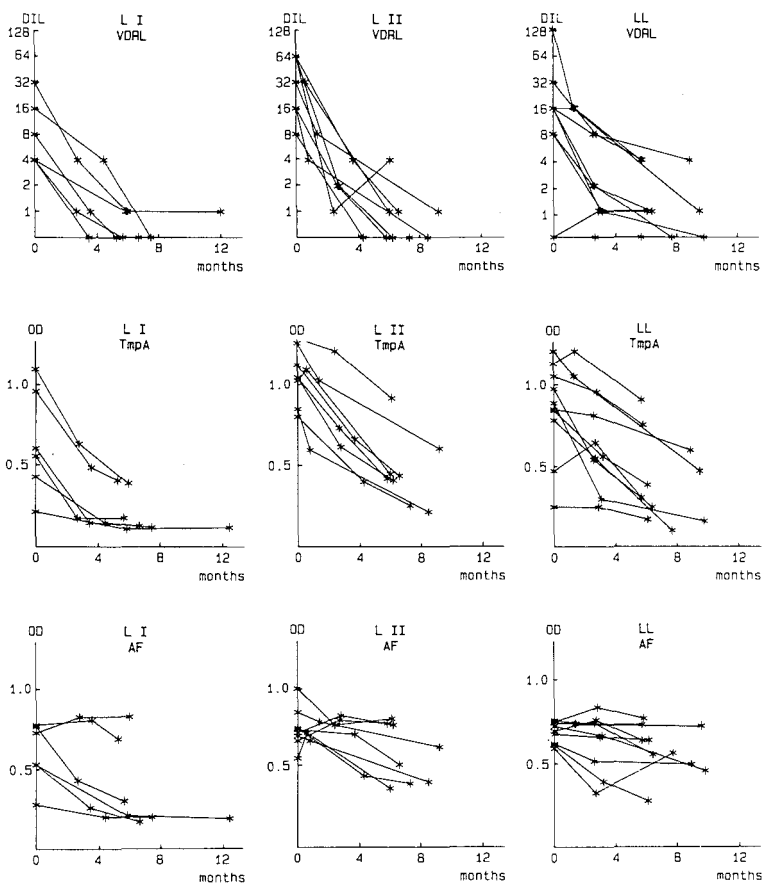
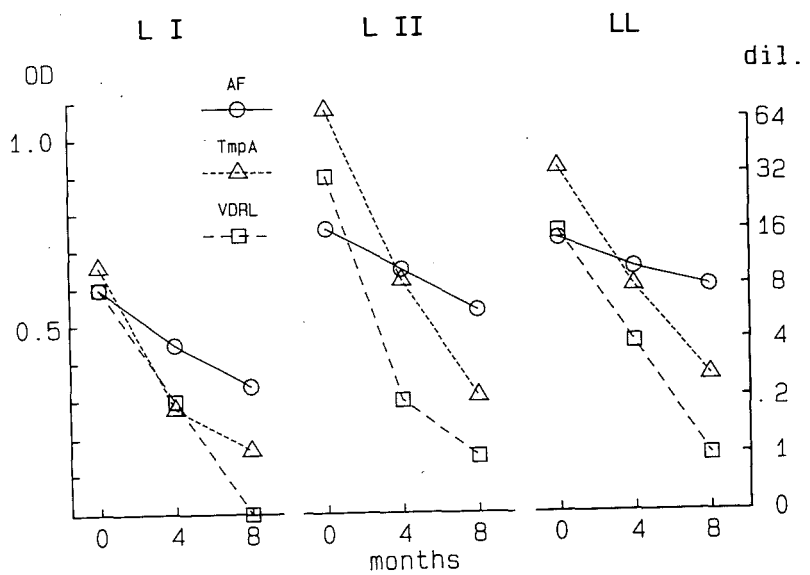


Fig. 2. Mean values of anti-TmpA, anti-AF, and anticardiolipin antibodies of 27 syphilitic patients up to 8 months after treatment. Abbreviations are as defined in the legend to Fig. 1.



Chapter 7

7 Enzyme-linked immunofiltration assay for rapid serodiagnosis of syphilis

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7.1 Summary

A new rapid technique for detection of serum treponemal antibodies is described which is based on an enzyme-linked immunoassay using nitrocellulose as solid phase. With this technique antigen-antibody binding is accelerated by the filtration of the antibody solution through the antigen-coated nitrocellulose filter instead of its remaining over the solid phase for incubation. Test results are available in less than 15 min. Serum specimen from 255 syphilitics and 829 non-infected subjects were investigated. The sensitivity and specificity of the Treponema pallidum enzyme-linked immunofiltration assay were comparable to those of the Treponema pallidum haemagglutination assay and the fluorescent treponemal antibody absorption test.

7.2 Introduction

Serological testing is an important tool in the diagnosis of syphilis. Assays must be rapid, sensitive and specific to be of value in the management of the disease. Currently, the World Health Organization recommends using a combination of the Venereal Disease Research Laboratory (VDRL) or the rapid plasma reagin card (RPR) test and the Treponema

pallidum haemagglutination assay (TPHA) for screening and the fluorescent treponemal antibody absorption (FTA-ABS) test for sera giving discrepant results in initial testing (1). Enzyme-linked immuno-assays (ELISA) are being increasingly applied as convenient procedures for detection of treponemal antibody (2-5). Recently, nitrocellulose replacing polystyrene or other materials as solid phase for antigen binding was applied in an ELISA for detection of antibody. This procedure, called dot immuno-binding (6) or antigen spot test (7), offers a number of advantages over the commonly used ELISA, including superior antigen binding capacity, as well as equal or greater sensitivity, easier discrimination between specific and non-specific background reactivity and simpler operation. Unfortunately, the procedure does not result in a substantial reduction of time needed to perform the ELISA, which still requires 2 to 3 h.
By modifying the ELISA and using nitrocellulose as solid phase, we developed a new, very rapid technique, the enzyme-linked immunofiltration assay (ELIFA), which reduces the time to obtain results to 15 min. Using Treponema pallidum antigens spotted on nitrocellulose sheets, we applied ELIFA for rapid serological screening of syphilis and compared the sensitivity and specificity of this assay (TP-ELIFA) with those of the TPHA, and the FTA-ABS test.

7.3 Materials and Methods

Sera.
A total of 202 serum samples from syphilitics, 51 of them treated, 504 serum samples from healthy blood donors and 104 serum samples from patients with clinical and serological evidence of (infectious) diseases other then syphilis were used from a collection of sera stored at -20 °C. Fresh sera in this study consisted of 274 serum samples collected from visitors attending a sexually transmitted disease clinic at the University Hospital in Rotterdam. All serum samples were tested with the TPHA (Japan Lyophilization Company), the FTA-ABS test (8), and the VDRL test (8). Before testing, all sera were heat-inactivated at 56 °C for 30 min.

The diagnosis and classification of syphilis were made on the basis of clinical and laboratory data according to the following criteria:

a. non-syphilitic: history and results of clinical and laboratory investigation gave no conclusive evidence of syphilitic infection.
b. primary syphilis: localized skin and/or mucosal lesions alone or in combination with regional lymphadenopathy and demonstration of Treponema pallidum in the lesions or in the lymph node and/or reactive serological tests.
c. secondary syphilis: generalized lesions of skin and/or mucosa alone or in combination with generalized lymphadenopathy and demonstration of Treponema pallidum in the lesions and reactive serological tests.

d. latent syphilis: no clinical signs of syphilis, history of past infection, reactive serological tests.

e. neurosyphilis: neurological disorders in combination with changes in the cerebrospinal fluid; TPHA and/or FTA reactive in combination with either VDRL-positive or mononuclear cell count > 5.0 μl, or IgG index ≥ 0.7, or IgM index ≥ 0.1 or oligoclonal immunoglobulins.

f. treated syphilis: diagnosed syphilis, treated according to current treatment plans, with no evidence of reinfection; serum samples were collected six months to three years after treatment.

Preparation of Antigen.

Treponema pallidum (Nichols strain) was maintained by serial passage in rabbit testes; organisms were extracted as described previously (9). Treponemes were purified from residual tissue components by Hypaque density centrifugation (10). Preparations adjusted to 2 x 10^{10} treponemes in 1 ml phosphate buffered saline (PBS), pH 7.2, were stored at -70 °C. For use in the ELIFA treponemes (5 x 10^9/ml) were solubilized for 30 min at 37 °C in PBS, containing 0.5 % (w/v) N-tetradecyl-N,N,-dimethylammonio-1-propanesulfonate and protease inhibitors (2 mM phenyl-methylsulfonyl fluoride, 0.028 mM N-p-tosyl-L-lysine chloromethyl ketone HCl, 2 mM 4-aminobenzaminidine, and 10 μg/ml soybean trypsin inhibitor). Finally, the preparation was diluted in PBS as indicated, maintaining the detergent concentration at 0.005 % (w/v).

Preparation of Sorbent.

A procedure different from the one described earlier (11) was employed to prepare a sorbent from Treponema phagedenis, biotype Reiter, for use as a serum diluent in the ELIFA. Treponemes were cultured in Brewer thioglycolate medium with 10 % (v/v) heat-inactivated rabbit serum as previously described (12). Suspensions of treponemes containing 50 g wet weight sediment in 1 l PBS were sonicated using a Branson sonifier at 20 kHz, 100 W, for 10 min at 0 °C. Thereafter, proteinase K was added to yield a final concentration of 100 μg/ml and the suspension was incubated for 1 h at 37 °C. The latter treatment was repeated once and residual proteinase K activity destroyed by boiling the suspension for 30 min. Particulate matter was removed by centrifugation of the suspension at 100,000 x g for 1 h and filtration of the supernatants through a nitrocellulose membrane (pore size 0.2 μm). Aliquots of sorbent were stored at -70 °C. For use as serum diluent, sorbent was diluted 1:2 in PBS containing 0.5 % v/v Tween 20 (PBS-T).

Enzyme-Linked Immunofiltration Assay.

The assay was performed in a specially designed multi-hole filtration device, which allows spotting of antigen on a nitrocellulose membrane sheet and simultaneous handling of up to 32 serum samples. The device is constructed of three blocks of perspex, which are clamped together during assay. The bottom section has an external outlet and a valve,

and constitutes a reservoir attached to the upper sections. The middle and top sections, designed to accommodate a nitrocellulose sheet in between, contain 32 corresponding holes with a diameter of 5 mm and neoprene "O" rings facing the nitrocellulose sheet to prevent lateral flow.

All assay steps were carried out at room temperature (22-24 °C). PBS was added to each hole and the fluid removed by vacuum. Five microliters of Treponema pallidum antigen were applied under vacuum as a small tight dot on the nitrocellulose sheet in a single well. Thereafter the nitrocellulose sheet was washed once with PBS-T, 0.2 ml per hole, which was then removed by vacuum. Two hundred microliters of serum, diluted in serum diluent, were added to each hole and filtered through the nitrocellulose sheet by calibrated suction using a peristaltic pump at the indicated flow rate. Positive and negative control sera were included in each experiment. The nitrocellulose sheet was washed as before. Two hundred microliters of sheep anti-human immunoglobulin (IgA, IgG, IgM, Fc and Fab) conjugated to horse radish peroxidase (HRP) (13), diluted in PBS-T, were then added to each hole and filtered through the membrane sheet using the same conditions as for serum. The fluid was removed and the membrane sheet washed three times with PBS-T removed by vacuum. To prepare the substrate, 3 ml of buffer containing 0.005 M citrate- 0.01 M phosphate, pH 5.0, was mixed with 1 ml of an ethanol solution containing 2.4 mg tetramethylbenzidine and 8 mg dioctylsodium-sulfosuccinate. Hydrogen peroxide was added to yield a 0.015 % v/v final concentration, and 200 μl of this solution was poured in each hole. After 3 min the substrate was removed and the membrane sheet washed with tap water removed by vacuum. The results were compared visually with the positive and negative control dots run on the same sheet.

The following individual serum samples were used as positive controls: strong positive control; secondary syphilis, TPHA titer 1:81920, VDRL titer 1:64, positive control; secondary syphilis, TPHA titer 1:20480, VDRL titer 1:16, and weak positive control; primary syphilis, TPHA titer 1:1280, VDRL negative. The development of color with equal or greater intensity than that of the weak positive control sample was read as positive.

Statistical Analysis.
Sensitivity and specificity were expressed as point estimated with 95 % confidence limits (14).

7.4 Results

Determination of Optimal Conditions
Figure 1A shows how the intensity of the final ELIFA reaction depends on the amount of solubilized Treponema pallidum organisms spotted on the nitrocellulose sheet. The fewest organisms still giving a detectable spot with the positive control serum amounted to 1 x 10^4. More organisms gave a more distinct positive reaction. The generation of a non-specific reaction following incubation with the negative control serum or serum diluent was

not observed. For subsequent experiments an optimal amount of 5 x 10^5 organisms per spot was chosen.

To optimize the serum concentration, increasing dilutions of the positive and the negative control serum were tested in the ELIFA (compare Figure 1B). Reactivity was still detectable at a dilution of 1:1250 of the positive control, but not at higher dilutions. Compared to a 1:50 dilution, a 1:10 dilution sometimes gave a slightly darker background, reducing the discriminatory power of the reading. A 1:50 serum dilution was considered optimal.

The relation between the final ELIFA result and the dilution of the anti-human immunoglobulin HRP conjugate is illustrated in Figure 1C. A 1:20,000 diluted HRP-conjugate still yielded maximal reactivity. Therefore, subsequent experiments were carried out with this dilution.

Figure 1: Effect of varying antigen, serum or HRP-conjugate concentration on TP-ELIFA test results. The TP-ELIFA had a filtration rate of 40 μl per min. A: 1:50 dilution of serum and 1:20,000 dilution of HRP-conjugate; B: 5x10^5 detergent-solubilized organisms per spot and 1:20,000 diluted HRP-conjugate; C: 5x10^5 detergent-solubilized organisms per spot and 1:50 diluted serum. Row one: negative control serum; row two: positive control serum.

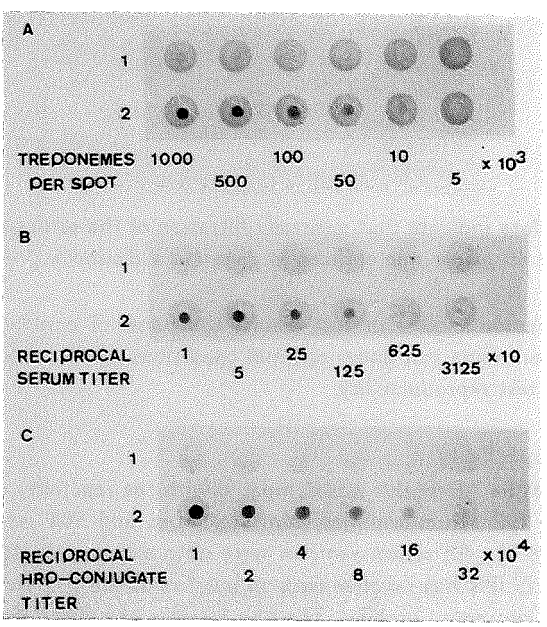
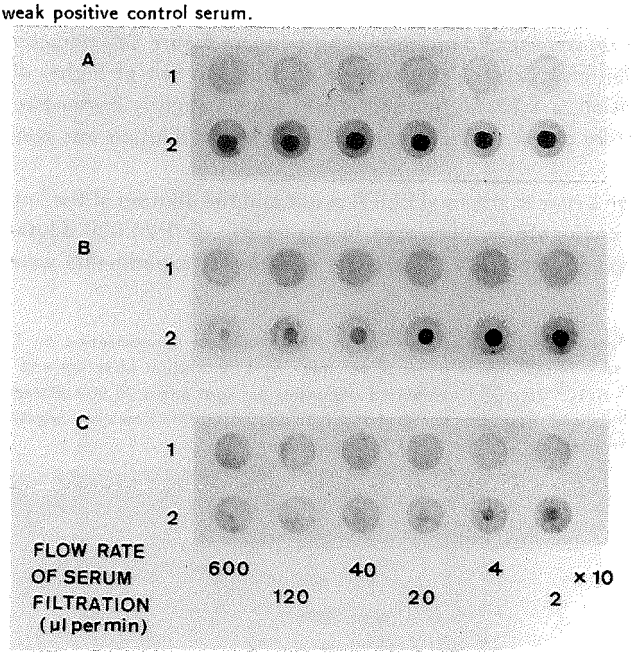


Figure 2 shows the effects of varying the flow rate during filtration of serum through the nitrocellulose membrane. With the 1:50 diluted strong positive control serum, a maximal reaction occurred at a flow rate of 6 ml per min (Figure 2A). A lower flow rate of 40 μl per min, however, was required to achieve a maximal reaction with the 1:50 diluted positive and weak positive control serum (Figures 2B and C). A flow rate of 40 μl per min was needed for the HRP-conjugate to be detectable with the positive control sera (results not

Figure 2: Effect of varying the filtration rate of serum on TP-ELIFA test results. The TP-ELIFA used 5 x 10^5 detergent-solubilized organisms per spot, 1:50 diluted serum, and 1:20,000 diluted HRP-conjugate. Filtration rate of the HRP-conjugate was 40 μl per min. A: row one: negative control serum, row two: strong positive control serum; B: row one: negative control serum, row two: positive control serum; C: row one: negative control serum, row two: weak positive control serum.



shown). These data indicate that generally filtration of the serum and the HRP-conjugate at a flow rate of 40 μl per min suffices to generate a distinctly positive result.

To investigate reproducibility of test results the panel of control sera was examined in 20 separate experiments run under optimal conditions. The test readings were identical, indicating good test reproducibility.

Sensitivity and Specificity

The reactivity of the TP-ELIFA was compared with the reactivity of the TPHA and FTA-ABS test using 202 serum samples from syphilitics and 504 serum samples from non-syphilitics (Table 1). All serum samples were obtained from a stock collection frozen at -20 °C. The TP-ELIFA was reactive with all but five of the 151 serum samples from documented cases of untreated syphilis. The non-reactive samples were obtained from primary syphilis patients, four of them darkfield-positive and seronegative, and the remaining one FTA-ABS positive. In treated syphilis five of 51 patients were TP-ELIFA-negative. The sensitivity of the TP-ELIFA (95 $\pm$ 3 %) was comparable to that of the TPHA (95 $\pm$ 3 %) and the FTA-ABS test (97.5 $\pm$ 2.1 %)

Of 504 sera from non-syphilitics three, one and two serum sample(s) were found to react

positively in the TP-ELIFA, TPHA and the FTA-ABS test, respectively (Table 1). The specificity of the TP-ELIFA (99.4 $\pm$ 0.7 %) was comparable to that of the TPHA (99.8 $\pm$ 0.3 %) and the FTA-ABS test (99.6 $\pm$ 0.5 %). Treatment of serum with sorbent in the TP-ELIFA proved to be essential for a high specificity.

To determine the false positive rates in conditions other than syphilis a total of 104 serum samples from patients with various infectious diseases and rheumatoid arthritis were investigated. No positive results with either the TP-ELIFA or TPHA were observed. The FTA-ABS test gave positive results with one serum sample obtained from a patient with measles and a second one from an individual with rheumatoid arthritis.

Table 1: Results of serological tests for syphilis on serum samples from patients with syphilis and non-infected subjects (percentage given in parenthesis).

Diagnosis	Serum samples (n)	Samples positive by		
		TP-ELIFA	TPHA	FTA-ABS
Primary syphilis	50	45(90)	40(80)	45(90)
Secondary syphilis	43	43(100)	43(100)	43(100)
Latent syphilis	47	47(100)	47(100)	47(100)
Neurosyphilis	11	11(100)	11(100)	11(100)
Treated syphilis	51	46(90.2)	51(100)	51(100)
No syphilis	504	3(0.6)	1(0.2)	2(0.4)

Table 2: Results of serological tests for syphilis in relation to clinical data on 58 positive serum samples.

Serological reactions by			No.(%) of serum samples	Patients with known syphilis		Subjects without clinical evidence or history of
TP-ELIFA	TPHA	FTA-ABS		treated	untreated	syphilis
+	+	+	46(79.3)	43	3	
-	+	+	4(6.9)	4		
+	-	-	3(5.2)			3
-	+	-	5(8.6)	3		2

Evaluation of the Assay with Fresh Sera

A total of 274 serum samples submitted for syphilis serology were investigated with the TP-ELIFA and the results compared with those of the TPHA and FTA-ABS test. Fifty-eight of the examined sera were reactive in one or more of the tests (Table 2). Seven of 53 (13.2 %) cases diagnosed as syphilitics were TP-ELIFA-negative. The FTA-ABS tests failed to score three (5.7 %) sera. The sera showing these negative results were from patients treated for syphilis. The remaining five reactive serum samples were from individuals with neither clinical evidence nor reliable history of syphilis. The three serum samples showing an isolated positive reaction with the TP-ELIFA were obtained respectively from a female suffering from chronic adnexitis, a male with gonorrhoea and a male with chancroid. Two serum samples reactive only with the TPHA were obtained from a patient with non-venereal treponematosis and an individual born in Indonesia.

7.5 Discussion

A modification of the ELISA using nitrocellulose as solid phase for detection of antibody (6,7) was described and evaluated as a treponemal test for the serodiagnosis of syphilis. In this new and very rapid method (TP-ELIFA) the antibody solution is filtered through an antigen-coated nitrocellulose sheet instead of remaining over the solid phase for incubation. Since the translational motion rate of antibody toward the immobilized antigen is increased, the time needed for appreciable generation of antigen-antibody complex is substantially reduced from 30-60 min to only 5 min. Thus test results are available in less than 15 min; this is especially useful for rapid serological screening.

The results of this study indicate that the TP-ELIFA is suitable for serodiagnosis of syphilis. Investigation of frozen and fresh serum samples from patients with untreated syphilis and non-infected subjects showed that the sensitivity and specificity of the TP-ELIFA were comparable with those of the TPHA and the FTA-ABS tests.

In contrast to the FTA-ABS test, positive TP-ELIFA reactions were not found in the 104 serum samples from patients with conditions other than syphilis. From the five fresh serum samples reactive in the TP-ELIFA or TPHA in patients without reliable evidence for syphilis, three were from patients suffering from a sexually transmitted disease, one with non-venereal treponematosis and one from an immigrant born in a tropical country. Antitreponemal reactivity in this group could have been due to antibody formation against commensal treponemes sharing antigens with Treponema pallidum. It could, however, also reflect earlier exposure to Treponema pallidum. The overall sensitivity (93.3 $\pm$ 3 %) and specificity (99.3 $\pm$ 0.7 %) of the TP-ELIFA are in general agreement with sensitivity and specificity values that have been reported for ELISA using Treponema pallidum as antigen (2,4,15,16). Test reproducibility proved to be good.

Although interpretation of the TP-ELIFA was visual and therefore subjective, positive and negative reactions were easily differentiated. Positive results appear as a distinct spot against an almost white background, which also serves as an internal control for non-

specific antibody binding. Endpoint titrations can be performed, but they are not generally necessary. The reading of test results can be automated (17), but for small clinical laboratory settings visual interpretation with a yes-no type answer is preferred.

It was clearly more advantageous to use N-tetradecyl-N,N,-dimethylammonio-1-propanesulfonate-solubilized Treponema pallidum organisms as antigen than sonicated or whole organisms, since higher sensitivity and reproducibility of test results were possible. Since binding of antigen to nitrocellulose was slightly depressed by the presence of detergent at the indicated concentrations, under assay conditions (results not shown) specific antibody binding might be increased. Inadequate antigen presentation due to clumping of antigens, masking of epitopes and unfavourable interaction with the solid phase, can probably be avoided by adding a detergent. The association of antigens to nitrocellulose in the presence of detergents has been shown to be affected both by the kind of detergent used and its concentrations (18,19). In conclusion, the TP-ELIFA using detergent-solubilized Treponema pallidum organisms as antigen is an efficient, relatively simple and inexpensive assay. Its main advantage over standard treponemal tests is the short operation time. The TP-ELIFA appears to be as sensitive and specific as the TPHA and the FTA-ABS test and seems suitable for rapid detection of serum treponemal antibody. Finally, ELIFA is not limited to detection of antibody; the method can also be applied for rapid antigen detection, for example antigen of Treponema pallidum. Such studies are currently in progress in our laboratory.

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Chapter 8

8 Line Immuno Assay and Enzyme Linked Line Immunofiltration Assay using the purified Reiter Treponeme Axial Filament and the recombinant Treponema pallidum protein TmpA for simultaneous detection of antibody to different treponemal antigens

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8.1 Summary

Two enzyme immunoassays, the Line Immuno Assay (LIA) and the Enzyme Linked Line Immunofiltration Assay (ELLIFA) were studied for suitability in serodiagnosis of syphilis. In both assays, antibody to treponemes was detected by treponemal antigens coated in parallel lines on nitrocellulose membranes.
In the LIA procedure, nitrocellulose strips were coated with treponemal antigens in parallel lines. For detection of treponemal antibody, the strips were soaked in the incubation solutions. In the ELLIFA procedure nitrocellulose sheets coated with treponemal antigens in parallel lines were used. Incubation was performed in a filtration device and incubation solutions were sucked through the nitrocellulose filter, thereby reducing the assay operation time considerably.
The sensitivities and specificities of both assays for the serodiagnosis of syphilis were determined using the recombinant DNA derived treponemal protein TmpA and the purified Axial Filament (AF) derived from the Reiter treponeme. The sensitivities and specificities

were compared with those of the Treponema pallidum haemagglutination assay (TPHA), the fluorescent treponemal antibody absorption test, the Axial Filament and TmpA enzyme linked immunosorbent assays (ELISAs).
Serum samples from 65 untreated syphilitics, 95 patients treated for syphilis and 60 blood donors were investigated. In untreated early syphilis the sensitivities and specificities of LIA and ELLIFA were found to be comparable to those of the TPHA, except for the AF LIA.
The LIA procedure could detect simultaneously serum antibodies directed to 2 antigens within 2 hours, but 10 or more antigens can be easily used in this procedure. This assay may be useful in fieldwork. The ELLIFA procedure could detect simultaneously antibodies directed to two antigens within 15 minutes. Combination of these assays may provide a multiple antigen test in which the assay operation time is reduced.

8.2 Introduction

The World Health Organization (WHO) has recommended a two stage procedure for the routine serological diagnosis of a syphilitic infection (1). Sera, reactive in a standard screening test for syphilis like the Treponema pallidum haemagglutination assay (TPHA) or the Venereal Disease Research Laboratory (VDRL) test, must be confirmed by the fluorescent treponemal antibody absorption (FTA-ABS) test.
Recently, new methods based on enzyme immunoassay techniques have been developed for serological identification of a syphilitic infection (2, 3). These methods not only have the advantage in that the results can be easily read, but they are also simple to execute and speedy in operation. The recently developed Treponema pallidum Enzyme Linked Immunofiltration Assay (TP-ELIFA) combines the high sensitivity and specificity of a test using Treponema pallidum as antigen with a short test duration of only 15 minutes (3).
The specificity and sensitivity of enzyme immunoassays have been enhanced by the use of purified treponemal proteins obtained either from the cultivable Treponema phagedenis or E. coli recombinants producing Treponema pallidum proteins (4, 5, 6, 7).
Simultaneous detection of antibodies directed to antigens of different pathogenic microorganisms in one test procedure may simplify the screening of individuals at risk for multiple infectious diseases. This may be useful in cases where a particular Sexually Transmitted Disease (STD) has been diagnosed: the patient may concurrently be suffering from other sexually transmitted infections that need treatment. The recently described Line Immuno Assay in which multiple antigens were applied in parallel lines on nitrocellulose strips permitted the simultaneous detection of serum antibodies to multiple antigens (8).
In the TP-ELIFA procedure, the simultaneous use of different antigens in combination with Treponema pallidum itself to detect more than one infectious disease is not recommended since the test sera must first be absorbed with a Treponema phagedenis derived absorbens to reduce false positive reactions (3). This absorbens may interfere with the detection of antibodies to other antigens. However, the application of purified or recombinant DNA derived antigens in the TP-ELIFA procedure circumvents the need for an absorbens and also permits simultaneous testing for infections other than syphilis (4, 7).

In this study, the suitability of the purified Treponema phagedenis biotype Reiter derived Axial Filament (AF) and of the recombinant DNA derived Treponema pallidum membrane protein A (TmpA) were assessed for use in LIA and ELIFA procedures. In addition, the coating of antigens in parallel lines was evaluated for its suitability to deliver multiple treponemal antigens to one well in an ELIFA procedure. This new assay is designated Enzyme Linked Line Immunofiltration Assay (ELLIFA). The sensitivities and specificities of the LIA and ELLIFA procedures using either antigen were evaluated for serodiagnosis of syphilis. Furthermore, the LIA and ELLIFA procedures may offer the opportunity to detect simultaneously antibodies induced by different pathogenic microorganisms. Purified treponemal antigens like AF and TmpA could be incorporated in such a multiple antigen test.

8.3 Materials and Methods

Sera

A total of 160 syphilitic sera, obtained from 65 untreated syphilis patients and from 95 patients treated for syphilis, were collected from individuals attending the Sexually Transmitted Disease (STD) clinic at the University Hospital in Rotterdam. The untreated syphilitic sera were obtained from a collection described previously (3). The sera from treated syphilis patients were samples submitted for syphilis serology to the National Institute of Public Health, laboratory of Bacteriology. All samples were tested in the TPHA (Japan Lyophilization Company), the FTA-ABS test (9) and the VDRL test (9). All syphilis sera were classified according to previously stated criteria (3).
The 60 sera obtained from blood donors were tested by TPHA only. None of these sera were reactive in the TPHA.

Antigens

The isolation and purification of the recombinant DNA derived TmpA was done by Schouls et al. (10) with some modifications as described previously (7). In preliminary experiments it was shown that concentrations of Sodium Dodecyl Sulfate (SDS) higher than 0.05 % in the antigen preparation may hamper the non-electrophoretic attachment of proteins to nitrocellulose (O.E. IJsselmuiden, unpublished results). To remove the SDS (0.1 %) and the Coomassie Brillant Blue from these preparations, the TmpA was precipitated with a solution containing 5 % triethylamine, 5 % acetic acid and 10 % acetone for 10 minutes and subsequently washed twice with acetone using the micromethod for removal of SDS from proteins (11). After the final centrifugation, the acetone was removed from the TmpA pellet by evaporation under vacuum. The protein was redissolved in Tris-buffer (0.01 M Tris.HCl pH 7.4 and 0.15 M NaCl) containing 0.05 % SDS and it was stored at -20 °C until use. The 0.05 % SDS stabilized TmpA in solution and did not interfere with the binding of TmpA to nitrocellulose.
T. phagedenis biotype Reiter was cultured and the Axial Filament was prepared and purified as described previously (12).

Coating of antigen onto nitrocellulose sheets

The TmpA and AF antigens were diluted in phosphate-buffered saline pH 7.0 (PBS) and coated on nitrocellulose sheets (Schleicher and Schuell, BA 85, 0.45 μm) in lines of 180 mm length ($\pm$ 0.3 mm width) as shown in Figure 1. For use in the LIA procedure, coated nitrocellulose sheets were cut into 3 mm wide strips perpendicular to the antigen lines. Sheets to be used in ELLIFA were cut into rectangles (70 x 40 mm).

To determine the optimal concentration of TmpA and AF in both assays, various amounts of both antigens were applied to nitrocellulose sheets. Twenty, 40, 80 and 160 fold dilutions of the TmpA stock solution (1 mg/ml) were coated onto the nitrocellulose sheets in lines giving the final concentrations of TmpA antigen of 5.0, 2.5, 1.3 and 0.6 ng/mm. The AF stock solution (5 mg/ml) was diluted 100, 200, 400 and 800 fold and coated to the nitrocellulose sheets in the same manner, giving the final concentrations of 5, 2.5, 1.3 and 0.6 ng AF/mm. In addition, 1.3 ng human IgG/mm was coated onto the nitrocellulose sheets as an internal control on the quality of the conjugates used in the test and to provide a reference control for positive test results.

Line Immuno Assay

Nitrocellulose strips were incubated for 2 hours at room temperature with serum diluted 1:200 in a buffer consisting of 10 mM Tris-HCl pH 7.4, 0.1 M $MgCl_2$, 0.5 % Tween 20, 1 % bovine serum albumin and 5 % fetal calf serum (TMTB buffer). After rinsing three times (10 minutes each) in TMTB buffer, the strips were incubated for 2 hours with 1 ml of Horse Radish Peroxidase conjugated goat anti-human IgG (Tago, Denmark) which had been diluted 1:1000 in TMTB buffer. After two rinses of 10 minutes each with TMTB buffer and 3 rinses in Tris-buffer, the substrate solution containing 4-chloro-1-naphtol and hydrogen peroxide was added and incubated at room temperature in the dark for 30 minutes. After 3 rinses with distilled water, the strips were dried and examined visually. Reactions with a lower colour intensity than that observed using the negative control serum were regarded as negative.

Enzyme Linked Line Immunofiltration Assay

The assay was performed in a specially designed multi-hole filtration device as described previously (3). Precoated nitrocellulose sheets were inserted into the filtration device in such a way that two antigen lines were enclosed by each well of the filtration device. 0.2 ml phosphate-buffered saline containing 0.5 % Tween (PBS-T) was added to each well and subsequently removed by vacuum (0.5 kg/cm^2). Two hundred microliters of serum which had been diluted 1:50 in PBS-T were added to each well and filtered through the nitrocellulose sheet at a consistent flow rate obtained by calibrated suction for 5 minutes as described previously (3). After rinsing the wells with 0.2 ml PBS-T, 0.2 ml of Horse Radish Peroxidase conjugated sheep anti-human IgG (Nordic, The Netherlands) which had been diluted 1:20,000 in PBS-T, was incubated for another 5 minutes by calibrated suction. The wells were rinsed and incubated with 0.2 ml of a substrate solution (0.6 mg/ml tetramethylbenzidine, 2 mg/ml dioctylsodiumsulfosuccinate and 0.015 % hydrogen peroxide in 25 % ethanol and citrate/phosphate buffer containing 5 mM citric acid, 10 mM

Na2HPO4, pH 5.0) for 3 minutes. The colour intensity was examined visually.

Enzyme Linked Immunosorbent Assay

The ELISA was performed with TmpA or AF antigen as has been described previously (7, 12).

Statistical expression

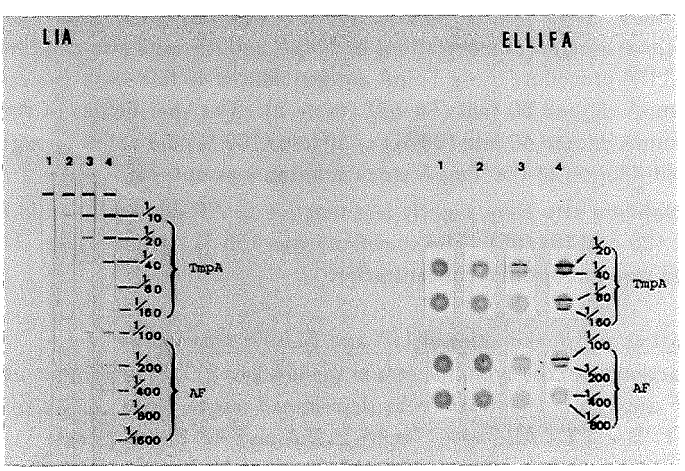
Sensitivity and specificity were calculated within their 95 % confidence limits (CL). The McNemar test was used for statistical evaluation.

8.4 Results

Determination of optimal antigen concentration in LIA and ELLIFA

Figure 1 illustrates the test results of a checkerboard titration of dilution buffer, a non-syphilitic serum and of two syphilitic sera in the LIA and ELLIFA. To determine the optimum antigen concentration in the LIA procedure, the sensitivity and specificity of the LIA were measured at various concentrations of TmpA and AF antigens.

Figure 1: LIA and ELLIFA test strips showing various concentrations of TmpA and AF. Sera used were: a control (no serum) (1), a non-syphilitic serum (2), a weakly reactive serum (3), and a strongly reactive syphilitic serum (4). The upper line on the LIA strips represents the IgG control that was directly coated to the nitrocellulose membrane.



The results depicted in table 1 show that the reactivity of primary, secondary and early latent syphilitic sera dropped substantially at higher dilutions of TmpA. When TmpA was diluted 40 fold, 63 out of 65 sera from untreated patients with various stages of syphilis reacted positively representing a sensitivity of 97 %, whereas only 30 sera reacted positively when TmpA was diluted 80 fold. The reactivity of the 60 blood donor sera declined substantially when TmpA was diluted more than 20 fold. Only 1 donor serum reacted positively when

TmpA was diluted 40 fold giving a specificity of 98 %. Therefore, TmpA diluted 40 fold appeared to be optimal with respect to the sensitivity and specificity in the TmpA LIA.

Table 1: Optimalization of antigen concentration in LIA.

		No of sera reactive to							
		TmpA diluted				AF diluted			
diagnosis	No of sera tested	20	40	80	160	100	200	400	800
primary syphilis	16	15	14	6	5	15	15	12	7
secondary syphilis	21	21	21	5	11	21	21	21	14
early latent syphilis	28	28	28	19	16	28	28	24	17
no syphilis	60	9	1	0	0	31	9	0	0

In table 1 it can be seen that the reactivity of the sera from untreated patients with various stages of syphilis to AF diluted 800 fold was significantly less (38/65) than to AF diluted 400 fold (57/65). The blood donor sera appeared not to react at all with AF diluted 400 fold or more. Therefore, AF diluted 400 fold was optimal for use in the AF LIA.
The optimal antigen concentrations of TmpA and AF were also determined for the ELLIFA. The ELLIFA procedure using TmpA antigen diluted 40 fold was more sensitive (61/65) than with TmpA diluted 80 fold (56/65) (table 2). The specificities of the ELLIFA procedure using TmpA diluted 40 fold (98 %) or 80 fold (100 %) did not differ significantly. Therefore a 40 fold dilution of the TmpA stock solution was optimal.

Early syphilitic sera were slightly less reactive to AF diluted 800 fold (58/65) than to AF diluted 400 fold (64/65). The specificity was 100 % in both cases. Therefore, AF diluted 400 fold was the optimal concentration.

Sensitivities and specificities of LIA and ELLIFA procedures

The reactivities of the syphilitic sera in the LIA and ELLIFA procedure using optimum TmpA and AF antigen concentrations as determined above, were compared to their reactivities in the TPHA, FTA-ABS test, TmpA ELISA and AF ELISA (Table 3). In primary syphilis, the sensitivities of the TmpA LIA (88 %, with 95 % confidence limits (CL): 72-100 %) and the TmpA ELLIFA (81 %, CL: 62-100 %) were comparable to those of the TPHA (88 %, CL: 72-100 %) and the TmpA ELISA (88 %, CL: 72-100 %). The reactivity of primary syphilitic sera in the AF ELLIFA (94 %, CL: 82-100 %) was slightly higher than in the AF LIA (75 %, CL: 54-96 %) or AF ELISA (75 %, CL: 54-96 %). Consequently, the sensitivity of the ELLIFA procedure using AF was comparable to that of the TPHA in primary syphilis. In secondary syphilis the sensitivities of all tests were 100 %. In early

Table 2: Optimalization of antigen concentration in ELLIFA.

diagnosis	No of sera tested	No of sera reactive to TmpA diluted 20	40	80	160	AF diluted 100	200	400	800
primary syphilis	16	16	13	10	6	16	15	15	14
secondary syphilis	21	21	21	19	19	21	21	21	20
early latent syphilis	28	28	27	27	25	28	28	28	24
no syphilis	60	12	1	0	0	11	2	0	0

latent syphilis, the sensitivities of the LIA and ELLIFA procedure using either TmpA or AF were comparable to that of the TPHA and ELISA except for the AF LIA which sensitivity for early latent syphilis was lower.
The reactivity of treated syphilis sera was determined for 95 sera using the TmpA antigen (table 3). The reactivity of TmpA in LIA (87 %) and ELLIFA (95 %) was comparable to its reactivity in ELISA (84 %). The specificities of both LIA and ELLIFA using TmpA or AF were comparable to those of the TPHA and the FTA-ABS test (table 3).

Comparison of titration of serum antibodies in the LIA and ELLIFA procedures

The difference in sensitivity between the LIA and ELLIFA procedure was studied by titration of a strongly, a moderately, and a weakly reactive syphilitic serum in both assays (figure 2). The three sera were titrated against 3 different TmpA dilutions and 2 different AF dilutions. All three sera were reactive at a 2 to 4 fold higher titre in ELLIFA than in the LIA when TmpA was used at the lowest dilution in both the assays. At the other two TmpA dilutions (80 and 160 fold) the reactivity of the three sera in ELLIFA was equal to or higher than in LIA, except that the weakly reactive serum reacted to TmpA diluted 80 times. In this particular case, the LIA was slightly more sensitive than the ELLIFA procedure. The reactivities of both the strongly and the moderately reactive sera to AF were higher at dilutions of 400 fold and 800 fold respectively in ELLIFA than in LIA.

Reduction of aspecific reactivity of sera with high lipid content by treatment with organic solvent

Sera with a high lipid content may cause severe non-specific reactivity in the ELLIFA procedure. The high amount of lipids in the serum may cause delayed and incomplete filtration of the diluted serum during its incubation and aspecific attachment of serum particles to the surface of the nitrocellulose membrane. The organic solvent 1,1,2-trichloro-trifluoroethane (arcton) was used to remove serum contaminants from a serum rich in lipids and from a

Table 3: Results of serological tests for syphilis on serum samples from patients with untreated and treated syphilis and from blood donors.

diagnosis	No of sera tested	No (%) of sera reactive by: TmpA LIA	TmpA ELLIFA	TmpA ELISA	Axial Filament LIA	Axial Filament ELLIFA	Axial Filament ELISA	TPHA	FTA-ABS
untreated syphilis,									
primary	16	14(88)	13(81)[b]	14(88)	12(75)[a]	15(94)	12(75)[b]	14(88)[b]	16(100)
secondary	21	21(100)	21(100)	21(100)	21(100)	21(100)	21(100)	21(100)	21(100)
early latent	28	28(100)	27(96)	28(100)	24(86)	28(100)	27(96)	28(100)	28(100)
treated syphilis	95	83(87)	90(95)	80(84)	n.d.	n.d.	n.d.	92(97)	91(96)
no syphilis	60	1(2)	1(2)	n.d.	0(0)	0(0)	n.d.	0(0)	n.d.

n.d. = not done

[a] Of the 4 non-reactive sera:
one serum was not reactive in TmpA and AF LIA, TmpA and AF ELLIFA, TmpA and AF ELISA
second serum was not reactive in TmpA LIA, AF ELISA and TPHA
third serum was not reactive in TmpA ELLIFA, TmpA and AF ELISA
fourth serum was not reactive in AF LIA only.

[b] The remaining non-reactive sera were not reactive in one test only.

low lipid containing serum. This solvent was chosen because of its low denaturing capacity (R. van den Akker, personal communication). Therefore, treatment of a syphilitic serum rich in lipids with arcton reduces the non-specific binding to the nitrocellulose membrane without a reduction in specific binding to the antigens. To determine a quantitative estimation of the serum reactivity before and after arcton treatment, a syphilitic serum with high lipid content and a syphilitic serum with low lipid content without arcton treatment, after centrifugation and after arcton treatment and centrifugation, were titrated against 4 different dilutions of AF (figure 3). The serum with low lipid content showed at all antigen dilutions a decrease in reactivity after arcton treatment and centrifugation. This reduction in reactivity was not observed by centrifugation alone. Virtually no difference in reactivity was found using the low lipid containing serum with or without arcton treatment.

8.5 Discussion

In this study two new enzyme immuno assays using nitrocellulose as solid phase to which treponemal antigens were coated in parallel lines, were studied for the serodiagnosis of syphilis. The LIA procedure could accommodate many different antigens on one single nitrocellulose strip so as to allow the detection of antibodies to several antigens simultaneously. The rapid ELLIFA procedure could detect antibodies to one or two antigens

Figure 2: Comparison of the sensitivity of LIA and ELLIFA using 3 sera. (A) a strongly reactive serum, (B) a moderately reactive serum and (C) a weakly reactive serum. ■ represents LIA test results and □ represents ELLIFA test results.

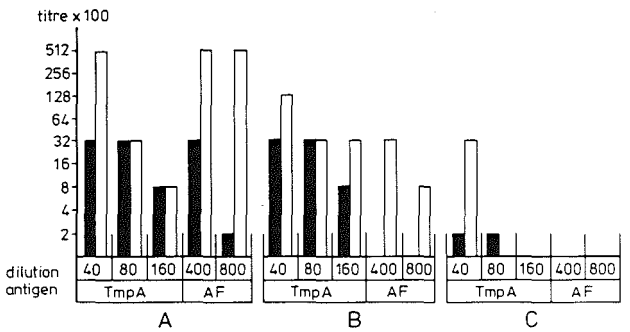
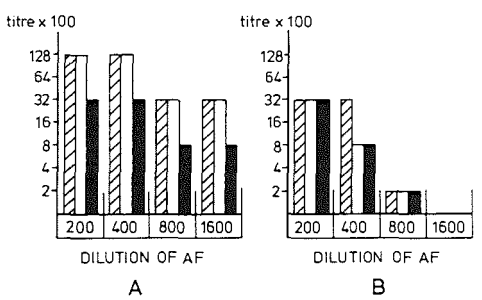


Figure 3: Comparison of arcton treatment of a serum with high lipid content (A) and a serum with low lipid content (B). ▨ represents the untreated serum, □ represents the serum after centrifugation and ■ represents the serum after arcton treatment with subsequent centrifugation.



simultaneously within 15 minutes. The results were visible as coloured lines and could be read by eye.

The specificities of LIA and ELLIFA using TmpA or AF antigens did not differ significantly from that of the TPHA. However, in both LIA and ELLIFA, a single serum from a blood donor reacted to TmpA antigen. A more extensive purification of TmpA reduced the non-specific reactivity to TmpA of this serum in ELISA (10). Therefore, these aspecific reactions to TmpA may be due to contaminants derived from E. coli in which TmpA was expressed (10).

The sensitivities of the TmpA LIA, the TmpA ELLIFA and AF LIA in all early stages of untreated syphilis were comparable to those of the TPHA. In contrast to this, the sensitivity of the AF LIA for the diagnosis of primary syphilis and early latent syphilis appeared to be less than that of the TPHA. However, as was shown in Table 1, AF diluted less than 400 fold increased the sensitivity substantially, but at the same time there was a reduction of the specificity. This indicates that a slight decrease in dilution of the antigen may enhance

the sensitivity and emphasizes that this dilution is critical.

The ELLIFA procedure proved to be more sensitive than the LIA procedure in titration experiments using sera of different titres from patients with different stages of syphilis. This may be due to the filtration in ELLIFA. The reactants not only react at the surface of the membrane but also with the antigens impregnated below the surface of the filter sheet due to the filtration of the reactants through the membrane. The higher sensitivity of the ELLIFA may permit the use of this assay to detect antibodies to multiple antigens that require different optimal serum dilutions for the respective antigens.

In preliminary experiments it was observed that a transmission thin-layer-chromatography (TLC) scanner could be easily used to detect positive reactions in the LIA procedure. If the absorption of light transmitted through nitrocellulose membranes in ELLIFA tests was measured with a TLC scanner, maximum absorption was not only observed at the dark coloured lines of a positive reaction but also at the non-specific weak background that surrounded the coloured line. Therefore, light transmission could not be used to read ELLIFA reactions. In contrast, a reflection TLC-scanner was found to be suitable for both ELLIFA and LIA tests. This method would also allow quantitative evaluation of LIA and ELLIFA tests.

The filtration through a nitrocellulose membrane is efficient provided the reactants have a minimal lipid content (3, 13). Arcton treatment followed by centrifugation was effective in reducing the lipid content of the sera rich in lipids. Therefore, the arcton treatment may be useful when a substantial number of sera of which some sera have high lipid content must be screened. The LIA procedure offers a simple multi-antigen test with easily to preserve nitrocellulose strips. Preliminary results indicated that the LIA procedure may be useful to detect antibodies to human immunodeficiency virus, herpes simplex virus, cytomegalovirus, varicella-zoster virus and mumps antibodies simultaneously (8). Detection of Hepatitis B surface antigen was demonstrated in a separate study (van der Groen, unpublished).

The 15 minutes duration time of the ELLIFA procedure may be useful when rapid test results are required. The use of ELLIFA in an outpatient clinic would have the advantage that the test results are available at one and the same consultation. The assay is easy to perform and only requires the basic laboratory equipment. The development of a new filtration device is currently in progress. The ELLIFA filtration device can be adapted to test for up to 14 antigens simultaneously. Preliminary results indicate that up to 14 antigen lines in a single reaction field can be accommodated in this new filtration device. Testing for multiple antigens has already been demonstrated using the LIA procedure (8). Both the LIA and the ELLIFA assays not only allow to test for multiple antigens in infectious diseases, but may also be useful to screen for allergic antigens, serotyping or screening for monoclonal antibody producing hybridoma cell cultures.

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Chapter 9

9 Optimizing the solid-phase immunofiltration assay A rapid alternative to immunoassays

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9.1 Summary

The technical variables of the solid phase immunofiltration assay (SPIA) for the detection of antibodies bound to antigens on a solid-phase filter have been investigated.
The binding to solid-phase filters of ^{125}I-labelled axial filament proteins derived from Treponema phagedenis and the optimal conditions for blocking non-specific protein binding were analysed. Axial filament was applied to nitrocellulose, Hybond Nylon and Zeta Probe. After extensive rinsing, the highest amount (68 %) of axial filament was observed bound to Zeta Probe. However, blocking non-specific protein binding by pre-wetting the filter with rinsing buffer containing 0.5 % Tween-20, prevented the binding of protein to the filter only when nitrocellulose was used as solid-phase. Tween 20 (0.5 %) in rinsing and incubation solutions was found to be necessary for the reduction of non-specific binding of contaminants in turbid sera. However, the use of such solutions resulted in a substantial leakage of antigen (47 %) during rinsing procedures.
Binding of antigen specific antibody was analysed using ^{125}I-labelled protein A. The maximal possible binding of the antibody occurred within 5 min when the antibody solution was filtered. For optimal binding of ^{125}I-labelled protein A an incubation time of 1 h was needed.

It is suggested that solid-phase immunofiltration may provide a rapid alternative for radioimmunoassays or enzyme immunoassays for the detection of specific antibodies.

9.2 Introduction

The diagnosis of infectious diseases often relies on the results of serological assays. The enzyme immunoassays are widely used for serodiagnosis of infectious diseases due to their high sensitivity, simple operation and possibilities for automation (Engvall, 1971; Yolken, 1982).
Recently, solid-phase immunoassays using nitrocellulose or diazobenzyloxymethyl membranes as alternative carriers for antigens in enzyme-linked immunosorbent assays (ELISA) or radioimmunoassays (RIA) have been developed to permit easier application of antigen to the solid-phase and to enhance their sensitivity (Hawkes, 1982; Herbrink, 1982; Towbin, 1984).
However, despite these improvements, the enzyme immuno assays are still laborious. The binding of the primary antibodies and the conjugate immunoglobulins to their fixed targets are time consuming. Shorter rinsing procedures in immunoassays may accelerate their performance. The ^{125}I-staphylococcal protein A immunoassay (SPA) described by Cleveland et al. (1979), has the advantage of very rapid rinsing procedures involving the suction of rinsing liquids through a filter at the bottom of the incubation wells. This method, however, does not accelerate the rate at which the antibody binds to the antigen. In the spot test described by Lin et al. (1986), hard polystyrene cards are used as the solid-phase providing a simple and rapid test procedure of 30 min. However, this method still remains to be confirmed by others.
Recently, we developed a very rapid immunoassay which involved the filtration of the antibody and rinsing solutions through a nitrocellulose filter which has been pre-coated with the antigen. In the specific procedure developed (the Treponema pallidum enzyme-linked immunofiltration assay (TP-ELIFA) for use in the serodiagnosis of syphilis) the assay time was reduced to 15 min (IJsselmuiden et al., 1987). A further advantage of the procedure is the possibility of testing multiple antigens in a single run without affecting the time required for the execution of the assay.
In the present study, the possibility of increasing the efficiency of the ELIFA procedure was investigated. The effect of various types of solid-phase material, their antigen binding capacity and the retention of antigen during various blocking and incubation steps were investigated with respect to maintaining a short assay time.

9.3 Materials and methods

Antigen
The axial filament (AF) of Treponema phagedenis, biotype Reiter was used as antigen. Treponema phagedenis, biotype Reiter, was cultured in Brewer thioglycolate medium (Difco laboratories) with 10 % heat-inactivated rabbit serum, as described previously (De Bruin,

1960). The axial filament was prepared as described by Hardy et al. (1975). The AF was radioiodinated using the chloramine-T method (Hunter, 1962). The radioactivity of the labelled AF was 5.3 x 10^6 Bq/μg. Both the unlabelled and ^{125}I-labelled AF were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to Laemmli (1970). After staining with Coomassie brilliant blue or autoradiography, a pattern of four distinct bands with molecular weights of 39,000, 35,000, 34,000 and 32,000 was obtained in both cases. The antigenic properties of the labelled AF were compared to those of the unlabelled AF in ELIFA (IJsselmuiden et al., 1987) using rabbit anti-AF hyperimmune serum and normal rabbit serum as a control. No change was observed in the capacity to bind antibodies after labelling the AF. Protein concentrations were measured by the Lowry procedure (Lowry, 1951). The ^{125}I-labelled AF preparation was stored at -70 °C in aliquotes and used within 1 month.

Sera

Hyperimmune anti-axial filament serum and normal serum (not reactive in any serological test for syphilis) were obtained from rabbits. To produce hyperimmune anti-axial filament serum, a rabbit was injected intravenously with 0.1, 0.2, 0.3, 0.4 or 0.5 mg AF in 1 ml Tris buffer (0.01 M Tris-HCl, pH 7.4 and 0.15 M NaCl) on days 0, 7, 9, 14 and 19 respectively. On day 28, the rabbit was bled and the serum harvested. To remove serum components that may interfere in the experiments, IgG-enriched fractions from both the sera were prepared by two successive precipitations in 50 % saturated ammonium sulfate and subsequent centrifugation at 10,000 x g for 30 min. The immunoglobulin fractions were resuspended in PBS. The rabbit anti-axial filament derived immunoglobulin preparation (RAS) and normal rabbit serum derived immunoglobulin preparation (NRS) were stored at -20 °C. The titre of the RAS was determined in the axial filament ELISA and was 1/25,000 (Van Eijk, 1986).

To investigate the possible interference by serum components other than immunoglobulins, a turbid serum rich in lipid was used. This serum was obtained from a patient with diabetes mellitus (DM) without clinical signs or serological evidence indicative for syphilis.

^{125}I-labelled protein A

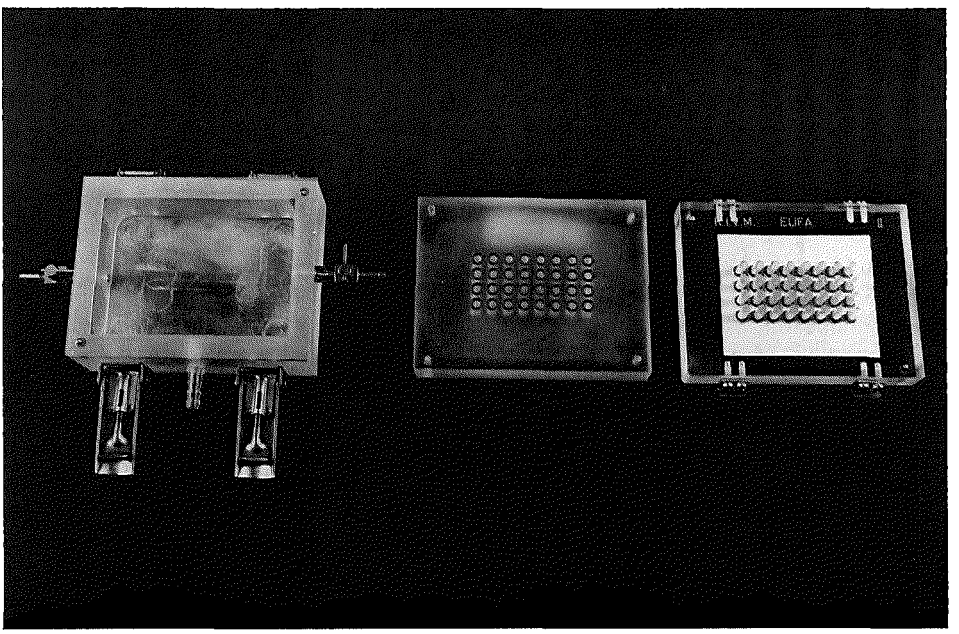
^{125}I-protein A was obtained from Amersham (UK). The specific activity was 3.5 x 10^8 Bq/μg protein A (0.031 μg/ml).

Filtration device

A specially designed 32 well filtration device (patent registered, RIVM, The Netherlands) was used and is shown in Fig. 1. The device consisted of three blocks of perspex. A sheet of a solid-phase filter was placed between the top and the middle part containing 32 corresponding holes (4 x 8 holes in a rectangle, 4 mm apart). In both the upper and the middle part, neoprene O-rings of 5 mm diameter, were fitted to support the filter. Filtration was achieved by applying vacuum to the lower part of the device. This construction successfully prevented lateral flow of the various reactants to the adjacent wells. None of the commercially available dot-blot devices (Bio-Rad, Schleicher and Schuell, BRL) met

this criterion since in these devices a single layer of gasket material (at most) was used instead of a double layer.

Fig. 1. The 32 well immunofiltration device. The device consists of three blocks of perspex. The filter is inserted between the two upper parts, which each contain 32 holes with neoprene O-rings.



Solid-phase immunofiltration assay

The ELIFA procedure (IJsselmuiden et al., 1987) was modified to permit quantitation of the bound ligands. The enzyme-labelled antibody used in the ELIFA procedure was replaced by ^{125}I-labelled protein A. Therefore, in this study the assay is designated solid phase immunofiltration assay (SPIA).

Briefly, the SPIA procedure at optimum conditions was performed as follows: 15 μg/cm^2 AF in 50 μl phosphate-buffered saline (PBS) was coated to the filter over 10 min. After removal of the remaining solution by vacuum (0.5 kg/cm^2), the filter was rinsed with 0.2 ml PBS containing 0.5 % Tween 20 (PBS-T) per well under vacuum. 200 μl of serum diluted 1/50 in PBS-T were added to the wells and slowly sucked through the filter over 5 min using a peristaltic pump. Subsequently, the filter was rinsed as stated previously. 100 μl solution containing 3.7 ng/cm^2 ^{125}I-labelled protein A was sucked through the filter within 10 min. After rinsing, the filter was cut into square pieces according to the dimensions of the wells and radioactivity was counted in a gamma counter.

Binding capacity of the solid-phase for axial filament

Blocking of unoccupied binding sites to inhibit non-specific binding in the solid phase immunofiltration assay was achieved using incubation and rinsing media containing 0.5 %

Tween 20 (IJsselmuiden et al., 1987).
The binding capacity of the different solid-phases such as nitrocellulose (NC) (BA 85, Schleicher and Schuell, F.R.G.), Hybond Nylon (Amersham, U.K.) and Zeta Probe (Bio-Rad, U.S.A.) for AF was determined by adding 50 μl of a solution containing 3 μg AF mixed with approximately 10,000 cpm of ^{125}I-labelled AF, to each well (15 μg/cm^2 solid-phase). Immobilization was allowed to occur on the sheets for 10 min after which they were rinsed with PBS or PBS-T. Finally, radioactivity was measured in each well.
In preliminary experiments using NC membranes the amount of AF and the amount of ^{125}I-protein A were varied to determine the optimum binding. After immobilization of the antigen, the NC sheet was rinsed with 0.2 ml PBS-T per well under vacuum. Further rinsing and incubations with serum immunoglobulins and protein A were carried out as described above.

Reduction of non-specific binding
The detachment of protein from the solid-phase in relation to the reduction of non-specific protein binding by a blocking agent was investigated. Initially, for the determination of non-specific protein binding to the solid-phase, ^{125}I-labelled AF was used after wetting the various membranes with PBS containing 0.5 % Tween 20. The influence of various concentrations of Tween 20 on the non-specific binding of NRS and serum components of the turbid serum was investigated. After immobilization of antigen, 0.2 ml of diluted RAS, NRS or the turbid serum was added, followed by a slow controlled filtration using a peristaltic pump. After rinsing, 100 μl of a solution containing 3.7 ng/cm^2 ^{125}I-protein A were added. After filtration for 10 min the NC sheet was rinsed.
The incubation times for both serum immunoglobulins and protein A were altered by varying the rate of filtration using a peristaltic pump. Finally the radioactivity was determined.

Expression of test results
The test results were expressed as the percentage mean binding of four test samples within the range of the standard deviation. The binding percentage was calculated according to Cleveland (1979) as follows:

$$\text{Percent binding} = \frac{\text{cpm } ^{125}\text{I-labelled protein bound}}{\text{cpm } ^{125}\text{I-labelled protein added to the well}}$$

9.4 Results

The results of the preliminary experiments showed that 15 μg/cm^2 of AF produced the best sensitivity with economical use of the antigen and 3.7 ng/cm^2 ^{125}I-protein A appeared to be sufficient for the detection of bound antibody.

Binding of protein to different solid-phase materials
Binding of AF to three different solid-phase materials: NC, Hybond Nylon and Zeta Probe was investigated for their suitability for use in SPIA. First, the percentage of antigen binding

to the solid-phase materials was determined. The binding percentages of AF coated at 15 $\mu g/cm^2$ to NC, Hybond Nylon and Zeta Probe as determined in four experiments were 41 $\pm$ 4 %, 44 $\pm$ 4 % and 68 $\pm$ 4 %, respectively. Further experiments concerning protein binding to solid-phase materials, were limited to the use of NC. An analysis was made of the binding of AF to NC in large amounts approaching the maximum binding capacity of NC (Gershoni, 1982). The application of 100 $\mu g/cm^2$ AF to NC resulted in a binding percentage of 11 $\pm$ 1.1 %. Application of higher amounts of AF resulted in a lower percentage being bound.

Inhibition of protein binding to the solid-phase materials

Since in the immunofiltration procedure several incubations with protein containing solutions were necessary, it was of importance to establish in which way the binding of protein to the membranes could be prevented in order to avoid non-specific reactions. For this purpose the ^{125}I-labelled AF preparation was used after the solid-phase had been blocked. Blocking agents such as bovine serum albumin and fetal calf serum were unsuitable because the membranes became clogged during the filtration steps. Blocking of the membranes with Tween 20 was successful only in combination with nitrocellulose membranes. Only 3.6 $\pm$ 0.8 % AF bound to PBS-T wetted NC. The reduction of protein binding to Hybond Nylon was 0 $\pm$ 4 % and to Zeta Probe was 6 $\pm$ 4 %. In all further studies nitrocellulose was chosen as the solid-phase.

Optimizing the time of antigen binding to NC

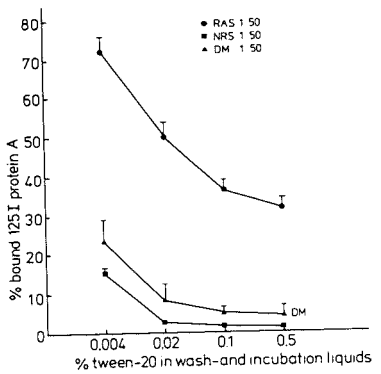
An amount of 15 $\mu g/cm^2$ ^{125}I-labelled AF was applied to each well and left to attach for various times. Antigen binding time was varied from a minimum of a few seconds up to 30 min. The amounts of protein that became bound did not differ significantly from that after 10 min binding time. Binding times longer than 1 h increased the percentage of AF bound by approximately 20 % (results not shown). For practical reasons, 10 min was chosen as the antigen immobilization time in further experiments.

Reduction of non-specific binding of protein to nitrocellulose and retention of bound antigens

The results of using various concentrations of Tween 20 in the rinsing and the incubation solutions are shown in Fig. 2. The largest decline in non-specific binding of NRS was obtained between 0.004 and 0.02 % Tween 20. Concomitantly with the declining non-specific activity, the binding of RAS also decreased, but the signal/noise ratio was improved considerably. In this study immunoglobulin fractions of sera were used. To investigate the non-specific binding of serum components, serum rich in lipids from a patient suffering from diabetes mellitus (DM) was analysed. A much higher background activity was obtained with this serum than with NRS. This high non-specific binding could be reduced by increasing the concentration of Tween 20 in the rinsing and incubation buffers to at least 0.1 %. The use of a Tween 20 concentration between 0.1 % and 0.5 % resulted in an optimum signal/noise ratio. For further experiments a Tween 20 concentration of 0.5 % was used.

To investigate possible leakage in SPIA due to the use of PBS containing 0.5 % Tween

Fig. 2. Influence of Tween 20 concentration in the incubation and rinsing buffers on the amount of bound ^{125}I-protein A. The binding of immunoglobulins derived from hyperimmune rabbit anti-axial filament serum (RAS), from normal rabbit serum (NRS) and the serum of a patient with diabetes mellitus (DM) to AF-coated nitrocellulose (15 $\mu g/cm^2$) as measured by ^{125}I-protein A was studied at different Tween 20 concentrations.



20, the detachment of antigen after rinsing with PBS was compared to that observed after rinsing with PBS-T. Two sheets of nitrocellulose were coated with 15 $\mu g/cm^2$ of AF (mixed with about 100,000 cpm ^{125}I-AF/cm^2) and rinsed with PBS or PBS-T. Rinsing with PBS resulted in 88 $\pm$ 6 % bound AF antigen whereas rinsing with PBS-T resulted in only 41 $\pm$ 4 % bound antigen. Although this suggested that a substantial leakage of antigen was caused by rinsing with PBS containing 0.5 % Tween 20, an appreciable amount of RAS still remained bound.

Influence of incubation time with serum immunoglobulins on antibody detection

The effect of incubating with serum Ig for 0, 1, 5, 10 min or 1 h on the percentage of protein A that was bound is shown in Fig. 3. Using 1/200 diluted RAS, maximal antibody binding was obtained after 5 min. Longer incubation times did not significantly increase the percentage of protein A that was bound. When 1/1000 diluted RAS was used, a 5 min incubation time resulted in maximum antibody binding. When NRS was used, the binding of protein A was low and not influenced by different periods of incubation with the NRS. An incubation time of 5 min was subsequently taken to be optimal in SPIA procedures.

The effect of the amount of bound serum antibody and the incubation time with protein A on the percentage of ^{125}I-protein A bound

The percentage of protein A binding at various dilutions of RAS and NRS is shown in Fig. 4. Using RAS, a maximum of protein A binding was observed at dilutions between 1/50 and 1/200. Higher and lower dilutions of these Ig preparations resulted in lower percentages of protein A binding. Using a RAS dilution of less than 1/100 resulted in a reduced binding of protein A. Although the maximum amount of bound protein A was achieved at the optimal serum Ig dilution there was no risk of false negative test results if the serum Ig was diluted to a lesser extent.

The effect of different incubation times of ^{125}I-protein A on the amount of bound protein

Fig. 3. Influence of duration of serum immunoglobulin incubation. Serum antibody was detected by ^{125}I-protein A. Incubation time ranged from nil to 10 min according to the speed of the peristaltic pump.

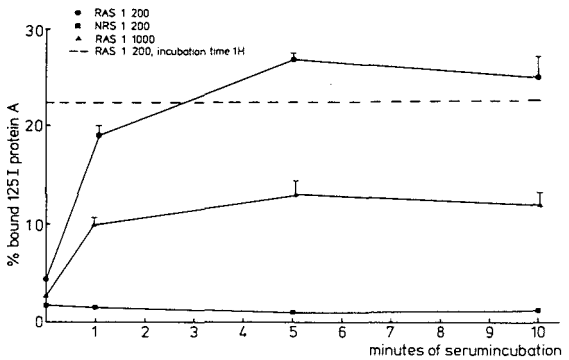
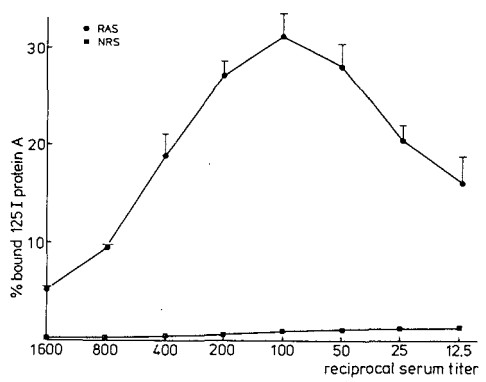


Fig. 4. Effect of dilution of immunoglobulins derived from hyperimmune rabbit anti-axial filament serum (RAS) and rabbit control serum (NRS). Dilution of serum immunoglobulins ranged from 1/1600 to 1/12.5.

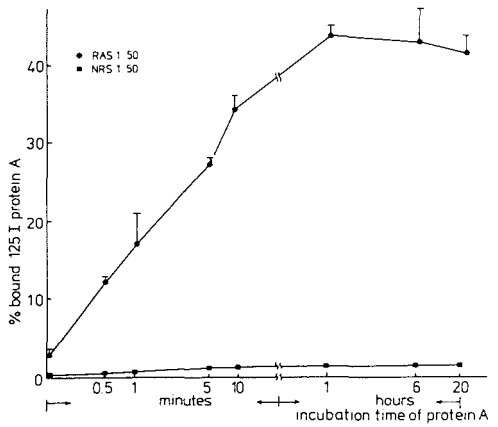


A is illustrated in Fig. 5. The incubation time was varied from a few seconds to 20 h by changing the speed of the peristaltic pump. After 1 hour maximum binding of ^{125}I-protein A to RAS was reached. Furthermore, non-specific binding of protein A after incubation of the control serum Ig increased slightly with longer incubation times. Since after an incubation time of 10 minutes 80 % of the maximum amount of protein A was bound, we selected this incubation time as the most practical in a rapid assay.

9.5 Discussion

In this study the conditions for the optimum performance of the solid phase immunofiltration assay were investigated. A specially designed immunofiltration device was constructed to permit the filtration of the incubation and rinsing solutions without any cross-over leakage to the adjacent wells. A rapid and almost plateau level binding of filtered antibody to the fixed antigen was obtained within 5 min (IJsselmuiden et al., 1987). The binding con-

Fig. 5. Influence of incubation time of protein A on the binding of ^{125}I-protein A. 3.7 ng/cm^2 of ^{125}I-protein A were added to the wells. Incubation time ranged from nil to 20 h.



ditions of protein to the different solid-phase materials, the detachment of proteins during rinsing and the limitation of non-specific protein binding using a blocking agent were investigated. The binding of AF was only slightly affected by the variation in the immobilization time. Immobilization of AF for 10 min resulted in sufficient binding. An appreciable binding percentage of AF was obtained with 15 μg/cm^2 of AF. However, coating with higher concentrations (15 - 150 μg/cm^2) resulted in a decrease of the binding percentages. This decline in the binding percentage could indicate that the maximum binding of AF to NC had been reached (Gershoni, 1982).

The loss of antigen bound to NC by rinsing with PBS containing 0.5 % Tween 20, reduced the bound antigen. A similar phenomenon was described by Lehtonen (1980). Leakage of bound antigen during rinsing and incubation procedures was described for polystyrene cuvettes and we assume therefore, that Tween 20 in the rinsing and incubation solutions was responsible for a substantial detachment of bound antigen. However, this did not substantially influence the detection of antibody. An advantage may be that only firmly bound antigen was retained on the membrane.

A reduction in the non-specific binding of serum components including immunoglobulins was accomplished by the addition of blocking agents to the rinsing and incubation solutions. Our previous experiments demonstrated that fetal calf serum, gelatin or bovine serum albumin could not be used in the immunofiltration procedure (IJsselmuiden et al., 1987) since the addition of such compounds hampered the filtration at a calibrated rate due to the high viscosity of the solutions. Batteiger (1982) has claimed that Tween 20 is an efficient blocking agent. Accordingly, the capacity of Tween 20 to reduce the non-specific protein binding in the SPIA procedure was investigated by comparing the binding of ^{125}I-labelled AF to sheets through which PBS-containing 0.5 % Tween 20 and sheets through which PBS only had been filtered. It was shown that the use of PBS containing 0.5 % Tween 20 resulted in a substantial reduction in the amount of protein that was bound compared to the sheets treated with PBS only. A substantial reduction in the background activity due

to non-specific binding of NRS in the SPIA procedure was accomplished by the addition of at least 0.1 % Tween 20 to the rinsing and the incubation solutions. Non-specific binding of components from a highly turbid serum was reduced to an acceptable level by using PBS containing 0.5 % Tween 20. Although the binding of RAS was reduced, higher dilutions of RAS still resulted in positive reactions suggesting that even weakly reactive sera could be detected. The ability of Tween 20 to prevent non-specific protein binding to NC is in contrast to the results of Cleveland et al. (1979) who used cellulose filters. These authors reported that Tween 20 greatly increased the non-specific binding of antibodies in their filtration assay. No reduction of protein binding was observed when Tween 20 was used as a blocking agent with Zeta Probe and Hybond Nylon membranes.

Immunofiltration of antibody was found to be optimal using an incubation time of 5 min. Using a more concentrated antibody solution resulted in sufficient binding over shorter incubation times. Incubation times longer than 5 min only slightly increased the non-specific binding of the control serum. The rate of antigen-antibody interaction mainly depends on the diffusion rate of the ligands (Velick, 1960; Yolken, 1981). In immunofiltration procedures, the mean diffusion time for antibodies encountering antigen is considerably reduced, thereby resulting in short incubation times.

RAS, at a dilution of less than 1/100, decreased the binding of ^{125}I-protein A. This may be explained by a phenomenon similar to the prozone effect that is observed in certain flocculation tests. However, test results remained positive and we concluded that, although in SPIA procedures a prozone-like effect may be observed, it will usually not affect the outcome of the results.

The optimal incubation time of ^{125}I-protein A was found to be 1 h, but at shorter incubation times, sufficient binding was still observed. In the immunofiltration procedure, the incubation times for enzyme-labelled antibody were appreciably shorter (IJsselmuiden, 1987). This difference may be explained by the known binding characteristics of protein A: the affinity constant of protein A binding to rabbit IgG is about 10^{-8} M (Goding, 1978), whereas that for hyperimmune anti-IgG immunoglobulins is usually lower (Yolken, 1982). Increasing the amount of protein A used for antibody detection by mixing the ^{125}I-labelled protein A with unlabelled protein A up to 20 times the original protein A concentration did not significantly alter the percentage of protein A that was bound (results not shown).

In conclusion, nitrocellulose in combination with Tween 20 permitted the optimum detection of specific serum antibody. The optimum filtration time for antibody was 5 minutes. However, for each pair of ligands the optimum incubation conditions for immunofiltration assays should be determined experimentally. Enzyme-labelled conjugate to detect specific antibody was more rapid than protein A resulting in an assay duration of only 20 min (IJsselmuiden, 1987). Immunofiltration may therefore, be generally useful in the rapid detection of antibodies.

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Chapter 10

10 Development and evaluation of a monoclonal antibody inhibition Enzyme-linked Immunosorbent Assay for serodiagnosis of syphilis

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10.1 Summary

A highly specific inhibition Enzyme-linked Immunosorbent Assay (ELISA) for the serodiagnosis of syphilis using murine anti-treponemal monoclonal antibodies was developed.
The monoclonal antibodies used in this study were reactive to antigens of both Treponema pallidum and Treponema pertenue and not to antigens of non-pathogenic treponemes. Inhibition of the binding of monoclonal antibody to the treponemal antigens was successful with serum antibodies of syphilitic patients in an inhibition ELISA using monoclonal antibodies raised against T. pallidum antigens of 42 kDa and 47 kDa. In contrast, the binding of monoclonal antibodies obtained by immunization of mice with recombinant DNA derived treponemal protein TmpB, was not inhibited by serum antibodies from syphilis patients.
The sensitivity of the inhibition ELISA using the monoclonal antibody against the 47 kDa T. pallidum antigen was 93 % among 58 sera of untreated syphilis patients. The sensitivity was 79 % if the monoclonal antibody against the 42 kDa T. pallidum antigen was used. By a combination of the test results obtained by these two inhibition assays a sensitivity of 97 % among the 58 sera of untreated syphilitics and 64 % among 64 treated syphilis sera was obtained. The specificity of the inhibition ELISA performed with either monoclonal antibody was 100 % among 500 sera from non-infected individuals. The specificity among 432 non-syphilitic patients attending a sexually transmitted disease clinic was 98.8 % for

the monoclonal antibody against the 42 kDa antigen, 99.5 % for the monoclonal antibody against the 47 kDa antigen and 98.4 % for the combined antibodies.
The sensitivity and specificity of the inhibition ELISA using the combination of test results obtained by the application of the monoclonal antibodies against the 42 kDa treponemal protein TmpA and against the 47 kDa T. pallidum antigen, were comparable to those of the Treponema pallidum haemagglutination assay (TPHA) and the fluorescent treponemal antibody absorption (FTA-ABS) test for the diagnosis of early untreated disease. The inhibition ELISA offers the potential for additional confirmation of early untreated syphilis. Its use for confirmation of late untreated syphilis is still under investigation. The test is highly specific for pathogenic treponemes and lacks the need of a sorbens.

10.2 Introduction

Most infectious diseases lead to an immune response that elicits antibodies against different antigenic determinants of the causative organism. The range of determinants to which antibodies are raised is large and will include pathogen specific epitopes as well as determinants that are common to (un)related bacteria. The immune response in syphilis to common antigens leads to aspecific reactions in diagnostic tests using Treponema pallidum spp. pallidum (T. pallidum) as antigen. Therefore, in these serological tests a sorbens is often used to remove serum antibodies that are not specific for T. pallidum (1, 2, 3). The effectivity of such a sorbens is not well defined. This stresses the need for serological tests using well defined, specific antigens. Immunoassays using purified antigens (4) or recombinant proteins (5, 6) and immunoblotting techniques (7) have been proposed as serological tests for syphilis without the need for a sorbens.
Application of monoclonal antibodies (MoAbs) in the detection of treponemal antibody may provide a highly specific tool for the diagnosis of treponematoses. It can be anticipated that such a test, that takes advantage of the high specificity of MoAbs, will show the high specificity required of a confirmatory test.
Studies on the reactivity of MoAbs raised against T. pallidum or T. pallidum components have resulted in MoAbs that were specific for pathogenic treponemes and those that were reactive with non-pathogenic and pathogenic spirochetes (8-14). These studies also showed that thusfar the pathogen-specific MoAbs cannot distinguish between different pathogenic treponema subspecies, e.g. T. pallidum spp. pallidum, spp. pertenue and spp. carateum. This is in agreement with the observation that the T. pallidum spp. pallidum and spp. pertenue (causative organisms of syphilis and yaws respectively) showed only minor differences in their protein patterns in two-dimensional gels, Western blots and in studies on the genetic relationship (15, 16).
Binding of pathogen-specific MoAbs to T. pallidum antigens can be inhibited by sera of patients suffering from syphilis (13) indicating that a competitive inhibition assay using pathogen-specific MoAbs may be suitable for the serodiagnosis of syphilis.
In this study eight monoclonal antibodies, raised to three different T. pallidum antigens of molecular weights 47, 42 and 34 kDa were investigated for their suitability for use in an inhibition ELISA. We developed an inhibition ELISA using MoAbs directed to 42 kDa and

47 kDa proteins and compared this assay for sensitivity and specificity with the Treponema pallidum haemagglutination assay (TPHA), fluorescent treponemal antibody absorption (FTA-ABS) test and the Venereal Disease Research Laboratory (VDRL) test for diagnosis of untreated and treated syphilis.

10.3 Materials and Methods

Antigens

T. Pallidum spp. pallidum (T. pallidum), T. pallidum spp. pertenue (T. pertenue , T. phagedenis, biotype Reiter, were used for the evaluation of specificity of the different monoclonal antibodies used in this study. T. pallidum (Nichols strain) was cultured by serial passage in rabbit testes. Extraction from the rabbit testicular tissue and subsequent purification of treponemes by urografin density centrifugation was performed as described previously (17, 18). Preparations of 10^{10} treponemes/ml in phosphate buffered saline (PBS) were stored at -70 °C.

T. pertenue, strain Gauthier kindly provided by P. Hindersson, Kopenhagen, was essentially prepared in the same manner as T. pallidum. T. phagedenis was cultured in Brewer thioglycolate medium with 10 % (v/v) heat-inactivated rabbit serum as described earlier (19).

Prior to use in ELISA or immunoblotting experiments, the treponemes were suspended in Tris buffer (0.01 M Tris HCl, pH 7.4, 0.15 M NaCl) and submitted to ultrasonic treatment with three bursts of 15 seconds using a Branson sonifier at 20 kHz, 50W. The sonicates were stored at -20 °C until use.

Sera

Sera from 58 untreated syphilitic patients that had been stored at -20 °C, and fresh sera submitted for syphilis serology from 496 syphilitic and non-syphilitic patients were obtained from patients attending the sexually transmitted disease clinic at the University Hospital in Rotterdam. Sixty-four samples from the fresh sera submitted for syphilis serology were obtained from patients who had been treated for syphilis. These sera were reactive in at least one treponemal test. Five hundred sera from non-infected individuals were obtained from blood donors at the Rotterdam bloodbank. Clinical and serological classification of the syphilitic patients was made as described before (3). All sera were tested in the TPHA (Japan lyophilization company), the FTA-ABS and VDRL test (20). To investigate the possible cross-reactivity of the sera from patients with other spirochaetoses in the inhibition ELISA, 6 sera, reactive in an immunofluorescence test to Borrelia burgdorferi (21) and 7 sera, reactive in an ELISA to Leptospira, strain Wijnberg, serotype icterohaemorragiae (22) were also tested in the inhibition ELISA (courtesy dr. W.J. Terpstra, WHO/FHO collaborating centre for serology and research on Leptospirosis, Amsterdam).

Preparation and characterization of Monoclonal antibodies (MoAbs)

Details of the production and selection of MoAbs have been described (11). Selection of anti T. pallidum hybridoma cultures was done by T. pallidum ELISA as reported by

Van Eijk et al. (23). MoAbs C3E5, D6F7C6 and 1-14M1 were prepared from mice that had been immunized with T. pallidum and MoAbs 15, 21, 29, 30 and 34 were prepared from mice that had been injected with the 34 kDa recombinant DNA derived treponemal protein-TmpB (24).

The MoAbs were purified by the addition of a saturated ammonium sulfate solution to the ascites fluids to a final 50 % saturation and mixing for 1 h at 4 °C under slow end over end rotation. The insoluble proteins were pelleted by centrifugation for 10 minutes at 10,000 x g. The pellet was rinsed with 50% saturated ammonium sulfate and recentrifugated. The final pellet was dissolved in 10 mM sodium phosphate buffer (Na_2HPO_4/NaH_2PO_4, 0.01 mM $CaCl_2$ pH 6.8) and dialyzed overnight against the same buffer. Final purification was accomplished by High Performance Liquid Chromatography using a hydroxylapatite column (100 x 7.8 mm, LKB, Sweden). Upto 2.5 mg of monoclonal antibody dissolved in 2 ml of a 10 mM sodium phosphate buffer was loaded onto the column. A 10 to 350 mM sodium phosphate buffer gradient was run in 30 minutes at a flow rate of 1 ml/minute. The protein containing fractions as determined by absorption at 280 nm were collected and used for further study.

Labelling of monoclonal antibody with horse radish peroxidase (HRP) was performed according to the method of Nakane (25).

MoAbs were characterized with the FTA, TPHA and ELISA using T. pallidum and T. phagedenis biotype Reiter antigens. The titre of each undiluted MoAb in the T. pallidum ELISA was defined as the reciprocal of that MoAb dilution that showed an extinction half of that of the undiluted MoAb solution.

The suitability of the MoAbs was studied for possible use in the inhibition ELISA by inhibiting the binding of the conjugated MoAb by its homologous unlabelled counterpart that was previously bound to the immobilized antigen. Binding of conjugated MoAb was inhibited by the unlabelled homologous MoAb in all cases except for D6F7C6. Therefore MoAb D6F7C6 was excluded from further experiments.

SDS-PAGE and Western Blotting

Sonicated T. pallidum, T. pertenue and T. phagedenis were solubilized in 3 % Sodium Dodecyl Sulfate (SDS) by heating in a boiling waterbath for 3 min. under reducing conditions using 2 % 2-mercaptoethanol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 13 % slab gels (17 x 12 cm) according to Laemmli (26). Immunoblotting was performed to identify the components of pathogenic and non-pathogenic treponemes to which the monoclonal antibodies were directed. Electrophoretic transfer of proteins to nitrocellulose was performed with a Trans-blot apparatus (Biorad, U.S.A.) using the method of Burnette (27). After the transfer of the proteins, the nitrocellulose was cut into separate strips. Each strip was soaked in Phosphate Buffered Saline containing 0.05 % Tween-20 (PBS-T) for 15 min. and incubated for 1 hour with MoAb diluted 1:100 in PBS-T. After rinsing with PBS-T for 10 minutes, the nitrocellulose strips were incubated with 1:1000 dilution of horse radish peroxidase labelled anti-mouse (RIVM, The Netherlands) or anti-human (Nordic, The Netherlands) immunoglobulin for one hour. The nitrocellulose strips were rinsed again and the substrate solution containing

0.6 mg/ml tetramethyl-benzidine, 2 mg/ml dioctylsodiumsulfosuccinate and 0.015 % (v/v) hydrogenperoxide in citrate/phosphate buffer (5 mM citric acid, 10 mM Na_2HPO_4 pH 5.0 and 25 % (v/v) ethanol) were added and incubated for 10 minutes.

Titration of monoclonal antibodies in T. pallidum ELISA

The optimal dilution of each HRP-labelled monoclonal antibody was determined by titration in the T. pallidum ELISA. For this purpose a microtitration plate pre-coated with T. pallidum ultrasonicate was incubated for 1 hour with labelled MoAb in increasing dilutions. Ten minutes after the addition of 0.1 ml substrate solution (0.1 mg/ml tetramethylbenzidine, 0.009% H2O2, 0.11 M sodium acetate, adjusted with citric acid to pH 5.5), the reaction was stopped by addition of 3M sulfuric acid. The extinctions were measured and plotted against the appropriate dilution of the labelled monoclonal antibody.

Inhibition ELISA

Preliminary experiments had demonstrated that maximum inhibition of monoclonal antibody binding to the antigens by serum antibodies was achieved by incubation of low dilution of the patient's serum before the incubation of MoAb. In addition, the patient's serum diluted to the same extent had to be added to the MoAb solution during its incubation. To incorporate these conditions, the inhibition ELISA was performed as follows: 0.1 ml of T. pallidum sonicate (2.7×10^7 treponemes/ml PBS) was coated onto the wells of a microtitration plate (96 wells, Dynatech, Germany) by incubating the plates for 1 h at 37 °C. After rinsing for 30 seconds in a continuous low pressure flow of rinsing buffer (TS-buffer containing 0.05 % Tween-20), patient's serum diluted 1:5, 1:50 and 1:100 in PBS-T was added to the wells and incubated for 1 h at 37 °C. The wells were rinsed with PBS-T and subsequently a mixture of appropriately diluted test serum and HRP-labelled monoclonal antibodies at the indicated working dilution were incubated for 1 h at 37 °C. After rinsing with PBS-T, 0.1 ml substrate solution was added to the wells and incubated (30). The reaction was stopped by adding 50 μl of 3 M sulfuric acid. The extinction was measured with a Multiscan (Titertek) at 450 nm. Appropriate control samples were included in each assay. Reactivity of each MoAb was checked by incubating the appropriate HRP-labelled MoAb without serum. The specificity of the reaction was checked using HRP-labelled alfa-fetoprotein as control on each plate.

10.4 Results

Specificity of monoclonal antibodies

To define the reactivity and specificity of MoAbs reactive to T. pallidum antigens, eight MoAbs, directed to treponemal antigens of molecular weights 34 kDa, 42 kDa and 47 kDa were investigated in ELISA, TPHA, FTA test and Western Blot using T. pallidum, T. pertenue and T. phagedenis antigens.

The results of the evaluation of the eight monoclonal antibodies in ELISA using T. pallidum or T. phagedenis antigens and in the FTA test and TPHA using T. pallidum antigens are shown in Table 1. None of the investigated MoAbs reacted with T. phagedenis antigens in

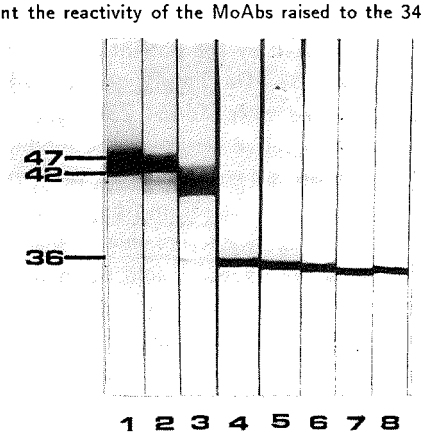
ELISA. All but one MoAb (1-14M1) reacted in the T. pallidum ELISA, FTA and TPHA. MoAb 1-14M1 reacted in the T. pallidum ELISA and TPHA but not in the FTA.

Table 1. Comparison of the reactivity of monoclonal antibodies in various tests using T. pallidum or T. phagedenis, biotype Reiter antigens.

MoAb	Mw(kDa) of reactive antigen	reciprocal ELISA titre		FTA	reciprocal TPHA titre
		T.pall.	T.phag.	T.pall.	T.pall.
15	34	25600	-	+	128
21	34	800	-	+	128
29	34	800	-	+	128
30	34	1600	-	+	128
34	34	1600	-	+	128
C3E5	47	12800	-	+	4
D6F7C6	47	12800	-	+	128
1-14M1	42	51200	-	-	128

The reactivity of the MoAbs to T. pallidum, T. pertenue and T. phagedenis was analyzed by immunoblotting technique. The results of the immunoblotting with T. pallidum antigens are shown in Fig. 1. MoAbs C3E5 and D6F7C6 reacted with both T. pallidum and T. pertenue antigens of 47 kDa and MoAb 1-14M1 with 42 kDa antigens. MoAbs 15, 21, 29, 30, 34 reacted with 34 kDa antigens of T. pallidum and T. pertenue. None of the MoAbs were reactive to T. phagedenis antigens in immunoblotting (results not shown).

Figure 1. Monoclonal antibody reactivity to T. pallidum on Western blots (2.5 x 10^6 treponemes per lane). Lane 1 and 2 show the reactivity of MoAbs C3E5 and D6F7C6. In lane 3 the reactivity of MoAb 1-14M1 is shown. Lane 4 through 8 represent the reactivity of the MoAbs raised to the 34 kDa recombinant DNA derived treponemal protein-TmpB.



Titration of monoclonal antibodies

A titration curve for each MoAb was constructed using the T. pallidum ELISA. For MoAbs 15, 21, 29, 30 and 34 the maximal extinction plateau was maintained until a dilution of 1:800, for MoAbs C3E5, D6F7C6 and 1-14 M1 a plateau was maintained until dilutions of 1:3200, 1:1600 and 1:1600 respectively. At higher dilutions a sharp decline in extinction was noticed. The steepest part of this slope represented the optimal dilution area for inhibition experiments. Therefore, the working dilution for each MoAb was chosen at the beginning of the slope of their respective titration curve. These titration curves were highly reproducible. The correct working dilution was confirmed by testing several syphilitic sera in the inhibition ELISA using MoAbs at different dilutions. At dilutions other than stated, no improved inhibition was obtained.

Discrimination between positive and negative results in inhibition ELISA

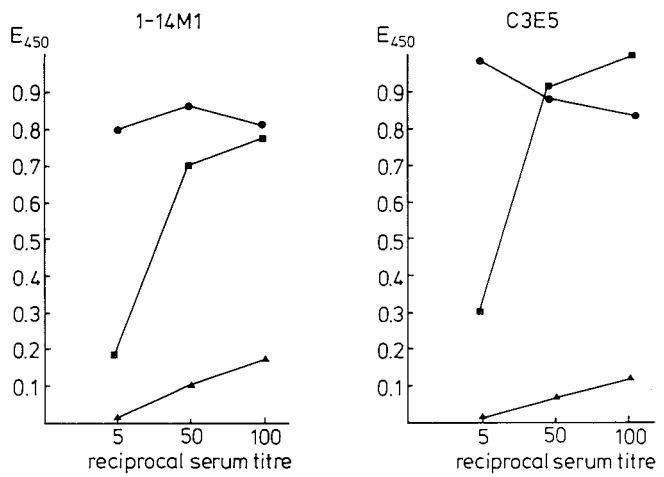
The criterion for discrimination between the inhibitory effect of syphilitic serum samples and that of non-syphilitic samples on monoclonal antibody binding was determined by evaluating the test results of 20 sera from untreated syphilis patients and 100 control sera. All sera were tested at 3 different dilutions (1:5, 1:50 and 1:100).

High variation in extinctions of sera with little or no inhibitory capacity made it impossible to correlate the test results to the extinction that was obtained with the labelled MoAb alone. However, it was initially observed that an inhibition of extinction of at least 0.5 occurred between the lowest and highest dilution of each syphilis serum sample that was tested. Such an inhibition was not observed in any control serum that was tested. Therefore, sera showing an inhibition of extinction of at least 0.5 when diluted as stated, were considered positive. In further experiments some of the sera from syphilis patients showed an inhibition of extinction of less than 0.5. These sera also showed an extinction of less than 0.3 when diluted at the 3 different dilutions (Fig. 2). Such sera were retested at higher dilutions. Upon retesting an inhibition of 0.5 was always observed. Such sera were considered strongly positive in the inhibition ELISA.

Inhibition of monoclonal antibody binding by sera from syphilis patients

All MoAbs were evaluated in the inhibition ELISA. None of the MoAbs directed against the 34 kDa TmpB could be inhibited by the patient's serum (results not shown). Therefore all MoAbs raised against the 34 kDa TmpB were excluded from further investigations. Marked inhibition was observed with MoAbs C3E5 and 1-14M1. The results of typical experiments with a strongly reactive serum in the inhibition ELISA (secondary syphilis, TPHA 2+, FTA-ABS 3+, VDRL 1:4), a moderately reactive serum (treated syphilis, TPHA 2+, FTA-ABS 3+) and a non-reactive serum using HRP-labelled MoAb 1-14M1 or C3E5 are shown in Fig. 2. The non-reactive serum showed high extinctions at all dilutions. The moderately reactive serum when diluted 5 fold significantly inhibited the binding of the labelled conjugate resulting in low extinctions. This serum, when diluted 100 fold did not substantially inhibit the binding of labelled MoAb. A strongly reactive serum inhibited the binding of the labelled MoAb to a high extent. Low extinctions were observed for all three dilutions.

Figure 2. The reactivity of syphilitic and non-syphilitic sera in the inhibition ELISA. (■–■) represents a moderately positive syphilitic serum, (▲–▲) a strongly positive serum and (•–•) a negative serum.



Sensitivity and specificity of the inhibition ELISA

Fifty-eight sera from patients with early syphilis and 500 sera from non-infected individuals were tested in the inhibition ELISA, TPHA and FTA-ABS test to determine the sensitivity and specificity, respectively (Table 2). All 58 sera from untreated early syphilis patients were reactive in at least the FTA-ABS test. A larger number of sera from patients with secondary and latent syphilis reacted in the inhibition ELISA using labelled MoAb C3E5 than when MoAb 1-14M1 was used and resulted in sensitivities of 98 % (mean 100 % and 96 %) and 77 % (mean 83 % and 72 %) respectively. For primary syphilis, the inhibition ELISA using MoAb C3E5 was less sensitive (80 %) than with MoAb 1-14M1 (87 %). In the group of untreated early syphilis as a whole the sensitivity of the inhibition ELISA using labelled MoAb C3E5 only (93 %) was comparable to that of the TPHA (98 %) and the FTA-ABS test (100 %). The combined results of both C3E5 and MoAb 1-14M1 inhibition ELISAs (97 %) also showed results comparable to that of the TPHA and FTA-ABS test.

The specificity of the inhibition ELISA using either MoAb (C3E5, 1-14M1) after testing of 500 sera of healthy individuals was 100 %. This is comparable to the specificities of the TPHA (99.8 %) and FTA-ABS test (99.6 %). To detect possible cross-reactivity of the sera from subjects with other spirochaetoses in the inhibition ELISA, the 6 sera reactive in the Borrelia burgdorferi immunofluorescence test and the 7 sera reactive in the Leptospira icterohaemorrhagiae ELISA were tested. These 13 sera were negative in syphilis serology (results not shown). The six sera reactive in the Borrelia burgdorferi immunofluorescence test did not react in the inhibition ELISA using labelled MoAbs C3E5 and 1-14M1, but 1 out of 7 sera reactive in the Leptospira ELISA also reacted in the inhibition ELISA using both labelled MoAbs.

Reactivity of the fresh sera in the inhibition ELISA

Table 2. Results of the monoclonal inhibition ELISA with serum samples from patients with syphilis and non-infected individuals.

Diagnosis	No. of sera	No (%) of samples positive in inhibition ELISA MoAbs: C3E5 and 1-14M1	1-14M1	C3E5	TPHA	FTA-ABS
primary syphilis	15	14(93)	13(87)	12(80)	14(93)	15(100)
secondary syphilis	18	18(100)[a]	15(83)[b]	18(100)[c]	18(100)	18(100)
early latent syphilis	25	24(96)	18(72)	24(96)[d]	25(100)	25(100)
blood donors	500	0(0)	0(0)	0(0)	1(0.2)	2(0.4)

[a,b,d] Three sera in these groups did not show 0.5 extinction decrease but had an extinction $\leq$ 0.3.
[c] One serum did not show 0.5 extinction decrease but had an extinction $\leq$ 0.3.

The 496 sera which were submitted for syphilis serology consisted of 432 sera from non-syphilis patients and 64 sera from patients treated for syphilis. None of these 64 sera were positive in the VDRL test. These sera were investigated in the inhibition ELISA using labelled MoAb C3E5 or 1-14M1. The results are shown in Table 3. These results were compared with those of the TPHA and FTA-ABS test. The specificity of the inhibition ELISA in the high risk group of the 432 non-syphilis patients was dependent on the MoAb that was used and was 98.8 % and 99.5 % for MoAb 1-14M1 and C3E5 respectively. After combining the results of both inhibition ELISAs, the specificity was 98.4 %. Of the 432 sera not reactive in either treponemal test, 5 reacted in the inhibition ELISA using labelled MoAb 1-14M1, whereas 2 other sera were positive in inhibition ELISA when labelled MoAb C3E5 was used. None of these patients who were positive in the inhibition ELISA had a history of syphilis infection.

Of the 64 sera from treated patients, 39 sera (61 %) reacted in the inhibition ELISA using MoAb C3E5, and 24 sera (38 %) when MoAb 1-14M1 was used. Forty-one sera (64 %) reacted positively when the results using both MoAbs were combined. The sensitivity of the inhibition ELISA for the diagnosis of treated syphilis after combining the test results of the two MoAbs (64 %) was less than the sensitivity of the TPHA (92 %) and the sensitivity of the FTA-ABS (86 %) test.

Table 3. Results of serological tests on serum samples submitted for syphilis serology.

reaction pattern of			No. of serum samples	patients with known treated syphilis	individuals without evidence of syphilis
TPHA or FTA-ABS	inhibition ELISA 1-14M1	C3E5			
+	+	+	22	22	
+	-	+	17	17	
+	+	-	2	2	
+	-	-	23	23	
-	+	-	5		5
-	-	+	2		2
-	-	-	425		425

10.5 Discussion

In the present study an inhibition ELISA for the serodiagnosis of syphilis using antitreponemal monoclonal antibodies is reported. Eight MoAbs were investigated for their suitability in the inhibition ELISA. Three MoAbs had been raised against T. pallidum organisms and 5 were raised against the 34 kDa recombinant protein TmpB. All 8 MoAbs were reactive in the T. pallidum ELISA, indicating that the T. pallidum antigens used in the inhibition ELISA, contained the epitopes to which the MoAbs were reactive. Furthermore, the MoAbs reacted in the TPHA and the FTA test with the exception of MoAb 1-14M1 in the FTA test. Although MoAb 1-14M1 was raised against the treponemal membrane associated 42 kDa protein TmpA (24), it did not react with presumed exposed treponemal surface antigens in the FTA test. Since MoAbs directed to the 34 kDa TmpB were not inhibited by sera from syphilis patients, they were not suitable for use in the inhibition ELISA. Homologous inhibition of labelled MoAb by its unlabelled counterpart was demonstrated for all MoAbs except MoAb D6F7C6. This indicated that the labelling of the latter MoAb may have altered the structure of its antigen binding sites. With respect to its specificity among presumed normal individuals, the inhibition ELISA using MoAbs C3E5 and 1-14M1 was comparable to the specificity to the TPHA and FTA-ABS test. Among the 432 sera from patients attending the STD clinic, the specificity of the inhibition ELISA was lower. This may be due to incomplete disease histories obtained from these patients with high risk for suffering from STDs. In untreated syphilis the sensitivities of the inhibition ELISA using either MoAb 1-14M1 or C3E5 or both was 79 %, 93 % and 97 % respectively. Therefore, the sensitivity of the inhibition ELISA using both MoAbs was comparable to that of the TPHA and the FTA-ABS test. The use of MoAb C3E5 alone provided a test with 93 % sensitivity. This in combination with the test results obtained with MoAb 1-14M1 slightly enhanced the sensitivity for primary syphilis patients, but this has to be confirmed

by testing a larger number of sera. The ideal inhibition assay takes advantage of a MoAb that is able to react in all stages of syphilis. Such a MoAb was not found in this study. There are several possible explanations for the differences in reactivity between the MoAbs in the inhibition ELISA. The avidity of the MoAb may be higher than that of the polyclonal serum directed to the common determinant which results in less inhibition. Serum antibodies reactive to the common determinant may be present in small amounts. Serum antibodies directed to the appropriate determinant may not be present depending on the stage of the syphilitic infection. It is possible that the epitope to which the MoAb was reactive, may not be presented to the host immune system during natural infection. This possibility may apply to the MoAbs raised against the recombinant protein TmpB. The use of these MoAbs in the inhibition ELISA indicated that they could not be inhibited by sera from syphilis patients. This could be due to an antibody response of mice to epitopes of the recombinant treponemal protein TmpB that differ from the epitopes of the naturally occurring TmpB from treponemes. In a direct ELISA using TmpB as antigen only a limited number of sera of untreated syphilitic patients, positive in other tests for syphilis, were reactive, indicating that certain patients do not produce antibodies to the 34 kDa protein TmpB (Schouls et al., submitted for publication). This confirms the poor antibody response to TmpB in man. The use of more than one MoAb in the inhibition ELISA may overcome the disadvantages of using a single MoAb. However, as can be expected from this theoretical viewpoint, the specificity of the test would decline for each additional MoAb that is used. This was shown by combining the results of the inhibition ELISA using MoAbs C3E5 and 1-14M1 to screen a high risk population. In the diagnosis of treated syphilis, the sensitivity of the inhibition ELISA using MoAb 1-14M1 in 64 sera of treated syphilis patients that were not reactive in the VDRL but reactive in at least one treponemal test was significantly less than the sensitivity using MoAb C3E5. The fact that the MoAb 1-14M1 was less effective in the serodiagnosis of treated syphilis than the MoAb C3E5 is in agreement with the observation that the serum antibodies reactive to the 42 kDa recombinant DNA derived treponemal membrane protein A (TmpA) show a rapid decline in reactivity soon after the patients were treated (28). Technically, non-reactive sera showed high variation of extinction in the inhibition ELISA . This was circumvented by testing each serum sample at three different dilutions. The dilutions that were chosen were based on a limited number of sera tested. An improvement in future experiments may be the inclusion of dilutions higher than 1:100 of each serum specimen. The application of MoAbs in an inhibition assay offers the opportunity to demonstrate antibody response to distinct epitopes. Although there is a considerable similarity between the antigenic structures of pathogenic treponemes, epitopes of different pathogenic treponemal species may differ. In future, monoclonal antibodies directed to such epitopes may provide an inhibition ELISA to differentiate between various pathogenic treponemes. In conclusion the inhibition ELISA using the combination of test results of two MoAbs directed to 42 kDa and 47 kDa treponemal proteins may be a highly specific and sensitive test for the diagnosis of early untreated syphilis. Due to its high specificity, the inhibition ELISA may have potential for confirmation of early untreated syphilis. Furthermore, the inhibition ELISA forms a model for the study of a wider panel of monoclonal antibodies whose inhibition pattern

may possibly give guidance to the stage of syphilis and treatment status.

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Chapter 11

11 IgM antibodies to treponemes in patients with syphilis detected by an IgM capture enzyme-linked immunosorbent assay

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11.1 Summary

A new IgM capture enzyme-linked immunosorbent assay (ELISA) was compared with the 19S(IgM) fluorescent treponemal antibody absorbed (19S(IgM)FTA-ABS) test for detecting IgM antibodies to treponemes. Serum samples from 180 people, 109 with various stages of untreated syphilis, 45 patients with treated syphilis, and 26 non-infected, were investigated.

In all diagnostic groups of syphilis the reactivity of the IgM capture ELISA was similar to that of the 19S(IgM)FTA-ABS test except in untreated neurosyphilis, for which the IgM capture ELISA was significantly less sensitive. The IgM capture ELISA was very sensitive in congenital (100 %, 5/5), and primary syphilis (82 %, 18/22), but less sensitive in secondary (60 %, 12/20), latent (53 %, 16/30), neurosyphilis (34 %, 11/32) and treated (11 %, 5/45) syphilis.

False positive IgM capture ELISA results were not found in five people who gave false positive Venereal Disease Research Laboratory (VDRL) reactions or in 21 neonates born to mothers adequately treated for syphilis before or during pregnancy. This indicated that the IgM capture ELISA was very specific.

The course of antitreponemal IgM reactivity after treatment of early infectious syphilis was followed up in six patients. The quantity of IgM antibody declined in nearly all patients after treatment, but still remained detectable in five patients up to six months after treatment. In contrast, non-treponemal antibodies measured by the VDRL test disappeared in four out of six patients within five months from starting treatment.
In conclusion, the IgM capture ELISA may be useful for easy and sensitive detection of IgM antibodies to treponemes in patients with congenital and primary syphilis. A positive test result in these cases indicates that patients should receive treatment if they have not been treated recently. The test is not, however, recommended to replace the VDRL test to monitor patients treated for syphilis.

11.2 Introduction

IgM class antibodies to treponemes are usually the first to be produced during treponemal infection. IgG class antibodies appear rapidly thereafter, and soon become predominant (1). Testing for antitreponemal IgM has been thought to be useful for detecting active disease early (2), evaluating treatment (13), and screening for congenital syphilis in neonates (4). Some investigators (2,5) have indicated a need to separate the IgM fraction from serum before testing, to avoid false negative results caused by IgG masking IgM and to avoid false positive results caused by rheumatoid factor. Several methods to accomplish this have been described. They include ultracentrifugation (1), ion exchange (6), size exclusion (5), and affinity chromatography (6). These techniques are used to separate antitreponemal IgM before testing by immunofluorescence or haemagglutination. In The Netherlands the 19S(IgM) fluorescent treponemal antibody absorption (FTA-ABS) test, using size exclusion chromatography to isolate IgM antibodies, is generally accepted as the standard procedure for detecting antitreponemal IgM. Application of this test is, however, limited by the need for special equipment and by technical complexity. Technically simpler methods suitable for detecting antitreponemal IgM routinely have been proposed. They include the IgM solid phase haemadsorption assay (SPHA) (7), the enzyme linked immunosorbent assay (ELISA) to detect antiaxial filament IgM (IgM-ELISA) (2), and the Treponema pallidum specific IgM haemagglutination assay (TP-IgM-HA) (8). Because of doubts raised about the sensitivity of the SPHA (9) and the lack of commercially available reagents for the IgM-ELISA and the TP-IgM-HA, these tests have never been generally accepted as suitable for detecting IgM antibodies to treponemes. In the present study we compared a recently introduced IgM capture ELISA, Captia Syphilis-M (Mercia Diagnostics, Guildford, England), with the 19S(IgM)FTA-ABS test to detect IgM antibodies to treponemes in patients with treated or untreated syphilis.

11.3 Patients, materials, and methods

Serum samples

Serum samples from 154 patients with syphilis were obtained from the sexually transmitted disease clinic at the University Hospital, Rotterdam, and the Department of Neurology, the Free University, Amsterdam. One hundred and nine of these patients were untreated. The samples from the 45 patients treated for syphilis were collected at various intervals after the start of treatment. The diagnosis and classification of syphilis was made according to criteria described previously (10). All samples were reactive in at least the FTA-ABS test. Consecutive samples from five patients with primary syphilis and one patient with early latent syphilis, which were collected at the start of treatment and at various intervals after treatment, were used to evaluate IgM antibody to treponemes after treatment. The samples were tested in the same run by the IgM capture ELISA and the Venereal Disease Research Laboratory (VDRL) test, and in the same day by the 19S(IgM)FTA-ABS test. The serum samples of all patients were reactive in the FTA-ABS and VDRL tests at the start of treatment. In addition samples from five patients with solitary false positive VDRL test reactions were used. Samples from five neonates with clinical signs indicative of congenital syphilis were obtained from the Baragwanath Hospital in Johannesburg. Samples giving positive results in the T pallidum haemagglutination assay (TPHA) and the FTA-ABS test were obtained from 21 neonates without clinical signs indicative of syphilis who attended various clinics in The Netherlands. All serum samples were tested using the TPHA (Japan Lyophilisation Company, Japan), the FTA-ABS test (11), the 19S(IgM)FTA-ABS test, the VDRL test (11), and the antitreponemal IgM capture ELISA. Rheumatoid factor was detected by a latex agglutination test (Rapitex, Behring Werke, West Germany). All samples were stored at -20 °C.

19S(IgM)FTA-ABS test

IgM was separated by gel filtration chromatography using high performance liquid chromatography (HPLC; LKB, Sweden). A 50 μl volume of serum, prefiltered through a 0.2 μm filter (Millex G-V, Millipore), was passed through a 7.5 x 75 mm column (TSK GSWP; Toya Soda, Japan) that had been equilibrated with 0.25 mmol/l phosphate buffered saline (PBS), pH 7.2, at a flow rate of 1 ml/minute. The eluate was monitored at 280 nm, and fractions of 50 μl were collected. The IgM fraction was partially excluded by the gel. The mean IgG contamination in the fraction containing IgM was less than 25 mg/l and the molar IgM/IgG ratio was 200-fold greater in favour of IgM than before fractionation. The IgM was diluted seven times. Concentrations of IgM and IgG were measured by a fluoroimmunoassay (12). The IgM fractions were investigated with an indirect immunofluorescent assay according to the protocol of the FTA-ABS test (11). To minimise the risk of false positive and negative test results because of contaminating IgG, IgG was investigated separately using the FTA test with the appropriate anti-IgG conjugate. In all cases except one, as indicated, no fluorescence was observed.

Antitreponemal IgM capture ELISA

IgM antibodies to treponemes were detected by a capture ELISA procedure using Captia Syphilis-M test kits, which included microtitration plates coated with antihuman IgM, positive and negative control serum samples, dilution buffer, wash buffer, tracer complex, and

substrate. The test was performed according to the manufacturer's instructions. Briefly, microtitre plate wells coated with rabbit antihuman IgM were incubated for one hour at 37 °C with 100 μl serum diluted 1/50 in dilution buffer. Control serum samples (positive at high and low titres and negative) were included in every experiment. After the wells had been emptied and washed five times with 300 μl wash buffer, the plates were incubated for one hour with 100 μl tracer complex diluted 1/20. The tracer complex consisted of T pallidum antigens, a biotinylated antiaxial filament monoclonal IgM antibody, and streptavidin conjugated with horseradish peroxidase (HRP). The monoclonal antibody appeared to be of the IgM class by radial immunodiffusion. The wells were washed again as mentioned above, and 100 μl substrate was added. The reaction was stopped after 30 minutes by adding 25 μl of 2 mol/l sulphuric acid. The plates were read on a multiscan reader (Titertek) at 450 nm. The cut off point between positive and negative was set at the absorbance of the low titre positive control according to the manual supplied by the manufacturer. Serum samples showing an absorbance within about 10 % of the low titre positive control were tested again.

Statistical analysis
The sign test was used to compare the results of the different tests.

11.4 Results

IgM antibodies to treponemes in patients with untreated syphilis
Serum samples from 109 patients with untreated syphilis were investigated with the IgM capture ELISA and the results compared with those of the 19S(IgM)FTA-ABS test (Table 1). Both tests showed comparable sensitivity at all stages of untreated syphilis except for neurosyphilis. The sensitivity of the IgM capture ELISA was high in congenital (100 %) and primary (82 %) syphilis, but less in secondary (60 %) and latent syphilis (53 %). In patients with neurosyphilis the reactivity of the IgM capture ELISA was significantly lower (34 %) than that of the 19S(IgM)FTA-ABS test (72 %). Of the 12 samples from patients with neurosyphilis that were reactive in the 19S(IgM)FTA-ABS test but not the IgM capture ELISA, two had absorbance values within 10 % below those of the low titre positive control. Retesting those samples did not alter the results. Fluorescence in the 19S(IgM)FTA-ABS test was strong in seven out of these 12 samples and weak in the remaining five. All 32 samples from patients with neurosyphilis gave negative results when tested for the presence of rheumatoid factor.
IgM antibodies to treponemes in patients with treated syphilis and non-infected people
Table 2 shows the incidence of IgM antibodies to treponemes in 71 serum samples from patients with adequately treated syphilis, people showing false positive VDRL reactions, and non-infected neonates who had positive treponemal reactions. The patients with treated syphilis were divided into two categories; those with positive treponemal test results and a positive VDRL reaction, and those with only positive treponemal test results. Of the 23 samples from patients with treated syphilis who showed positive VDRL reactions, three were positive in both the IgM capture ELISA and the 19S(IgM)FTA-ABS test and two were

Table 1. IgM antibodies to Treponema pallidum in serum samples from 109 patients with untreated syphilis comparing results in IgM capture ELISA with those in 19S(IgM)FTA-ABS test.

Diagnostic category of syphilis	No	No. positive in: ELISA	FTA-ABS	Percentage agreement
primary	22	18	18	100
secondary	20	12	14	80
latency	30	16	18	87
congenital	5	5	5	100
neuro-syphilis	32	11	23	63

ELISA = enzyme linked immunosorbent assay. FTA-ABS = fluorescent treponemal antibody absorption.

each reactive in one test only. Of the 22 samples from treated patients with a negative VDRL reaction, only one gave a positive result in the IgM capture ELISA. No evidence of rheumatoid factor was found in any of these serum samples. IgM antibodies to treponemes were not found in the five people who had false positive VDRL reactions. The 21 serum samples from neonates of mothers adequately treated for syphilis before or during pregnancy also gave negative results in both IgM tests. All neonates, however, gave positive results in the TPHA and the FTA-ABS test.

Course of antitreponemal IgM reactivity after treatment

To assess the influence of treatment on reactivity in the IgM capture ELISA, consecutive serum samples from six patients with early infectious syphilis were investigated for up to 10 months after the start of treatment. The figure compares the results of the IgM capture ELISA with those of the 19S(IgM)FTA-ABS and VDRL tests at various intervals after the start of treatment.

In all patients with primary syphilis the titre of IgM in both tests for IgM antibodies to treponemes declined as a result of treatment. After six months, however, three out of the six patients still gave positive IgM capture ELISA results and four gave positive 19S(IgM)FTA-ABS test results. All five patients with primary syphilis showed a positive VDRL reaction at the start of treatment, but five months after treatment only one still showed a positive result. A patient suffering from early latent syphilis was followed up for three months after treatment and was found to give negative results in the IgM capture ELISA and positive results in both the 19S(IgM)FTA-ABS and VDRL tests.

11.5 Discussion

Treponeme specific IgM can most reliably be detected in the 19S(IgM) fraction after its separation from serum (2,5,7-9). The 19S(IgM)FTA-ABS test is widely used to show treponeme specific IgM, but the time consuming operation of this method makes a more simple procedure desirable.

Table 2. IgM antibodies to Treponema pallidum in serum samples from 45 patients with treated syphilis and 26 non-infected people comparing results in IgM capture ELISA with those in 19S(IgM)FTA-ABS test.

Diagnostic category and results of other tests	No	No. positive in: ELISA	FTA-ABS
Treated syphilis: TPHA, FTA-ABS, and VDRL reactive	23	4	4
Treated syphilis: TPHA and FTA-ABS positive, VDRL negative	22	1	0
False-positive VDRL	5	0	0
Non-infected neonates: TPHA and FTA-ABS positive	21	0	0

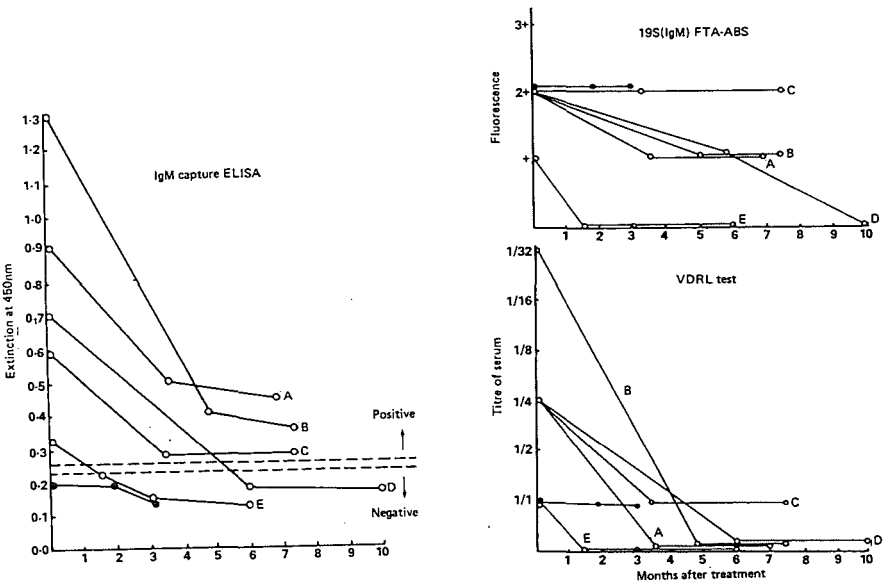
ELISA = enzyme linked immunosorbent assay.
FTA-ABS = fluorescent treponemal antibody absorption.
TPHA = T pallidum haemagglutination assay.
VDRL = Venereal Disease Research Laboratory.

The IgM capture ELISA shares with other recently developed tests the specific procedure of IgM immune adsorption (the binding of IgM antibodies to a solid phase that has been coated previously with antibodies to IgM raised in animals) (13). However, detecting immune adsorbed IgM antibodies specific to treponemes using red blood cells sensitised to T pallidum antigens (7) or by ELISA (2) may be insensitive (13). Although detecting adsorbed IgM by sensitised red blood cells is very specific, this technique is insensitive because the blood cells adhere insufficiently in the haemadsorption step (9,13). In contrast to the SPHA, high sensitivity can be expected in the IgM capture ELISA because of the separation of IgM from IgG and the use of a biotin-avidin complex, which amplifies the reaction of antigen with antibody.

Regarding the specificity of a capture ELISA used to detect IgM, rheumatoid factor can cause false positive results because, bound to the anti-IgM antibodies coated to the solid phase, rheumatoid factor can directly bind the crystallisable fragment of the IgG class conjugate (2,14). In the IgM capture ELISA, false positive results are not likely to occur in this way because a tracer complex is used instead of an IgG class conjugate.

In early stages of syphilis the sensitivity of the IgM capture ELISA was 93 % compared

Figure. Course of IgM reactivity in the IgM capture enzyme linked immunosorbent assay (ELISA), the 19S(IgM) fluorescent treponemal antibody absorption (FTA-ABS) test, and the Venereal Disease Research Laboratory (VDRL) test after treating five patients (A-E) with primary and one with early latent syphilis.



with the 19S(IgM)FTA-ABS test. In the case of neurosyphilis, however, the sensitivity of the IgM capture ELISA only reached 47 % compared with that of the 19S(IgM)FTA-ABS test. In addition, the consecutive serum samples from one patient with latent syphilis, which were collected at the onset of treatment and thereafter, did not react in the IgM capture ELISA although they showed strong fluorescence in the 19S(IgM)FTA-ABS test. Several explanations may be relevant for the differences in the sensitivity of the two tests in the patient with latent syphilis and those with neurosyphilis. Firstly, the immune response in these patients could be directed against a limited range of T pallidum antigens. If this range did not include the T pallidum axial filament (32-39 kilodalton) components, to which the monoclonal antibody used in the IgM capture ELISA was directed, negative results could be expected. However, western blot analysis of IgM fractions from these patients did not show a different reaction pattern in the 32-39 kilodalton antigen range from that of a patient suffering from primary syphilis (results not shown). Performing the IgM capture ELISA on these serum samples using a monoclonal IgG class antibody directed to the 47 kilodalton antigen, which had been labelled by the manufacturer under the same conditions as the monoclonal antibody to axial filament, did not give positive reactions either. Secondly, the difference in sensitivity of the IgM capture ELISA and the 19S(IgM)FTA-ABS test could be due to the fact that some epitopes of T pallidum antigens first become immunogenic in late syphilis and that IgM antibodies raised to these epitopes may be detected in a immunofluorescence test and not in an ELISA, as has been pointed

out by Strandberg Pedersen et al. (2).
In our hands the IgM capture ELISA was less successful than the currently used VDRL test in monitoring the effect of treatment. We do not know the reason for this, but the serum of treated patients with syphilis may contain 8S(IgM) treponemal antibodies long after 19S(IgM) antibodies have disappeared (13). Detecting 8S(IgM) antibodies by an IgM detection test may therefore mask the success of treatment as indicated by the absence of 19S(IgM) antibodies. The IgM capture ELISA probably cannot distinguish between 19S(IgM) and 8S(IgM) antibodies (13), and therefore serum samples from which 19S(IgM) antibodies are absent but that still contain 8S(IgM) antibodies may react in the IgM capture ELISA.
In conclusion, the IgM capture ELISA in its present form can be a useful test in early stages of syphilis and for screening for congenital syphilis. It might, however, be possible to improve the test for late syphilis, especially neurosyphilis, by setting the cut off point between positive and negative results to a lower value. Treatment monitoring with the IgM capture ELISA is not very useful because IgM antibodies to treponemes can be detected for longer after treatment than non-treponemal antibodies reactive in the VDRL test. The VDRL test is therefore able to detect the response to treatment more rapidly than a test detecting treponemal IgM. Finally, the absence of IgM antibodies to treponemes does not imply that syphilis is absent (2).

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Chapter 12

12 General discussion

12.1 Current status of syphilis serology

The definite diagnosis of syphilis still greatly depends on the detection of antitreponemal antibody in the serum and the cerebrospinal fluid of the patient. Since the first symptom of syphilis, the syphilitic ulcer, may mimic other genital ulcers, the clinical diagnosis of syphilis can be difficult. In addition, the syphilitic infection may progress slowly and destroy the vital organs in the absence of external symptoms and sometimes, neurosyphilis cannot be distinguished from other neurological disorders without serological confirmation of syphilis. In Europe, currently, a syphilitic infection is detected using the TPHA, and is confirmed using the FTA-ABS test. The effect of antibiotic treatment is monitored by the VDRL. The diagnosis of congenital syphilis is established by the detection of antitreponemal IgM using the 19S(IgM) FTA-ABS test.

The TPHA is a sensitive and specific test, but requires at least three hours to execute. The test results cannot be reliably read automatically, and for quantitation of the results, the samples must be titrated.

The FTA-ABS is more sensitive than the TPHA but of equal specificity. This is a disadvantage for its use as a confirmatory test. However, the combination of positive outcomes of the TPHA and the FTA-ABS test, is highly specific. The FTA-ABS test is complicated. The test results can be correctly read only by a skilled technician, and the quantitation of the results is also difficult in the FTA-ABS test. Crude T. pallidum antigens and a sorbens to exclude the false-positive reactions are used in both the TPHA and the FTA-ABS tests. Neither the antigens nor the sorbenses are well-defined. Therefore, investigations into the reasons for the false-positive results are not clear. Monitoring the effect of treatment is difficult since positive reactions are observed in both these tests long after treatment has been terminated. The treponemal antigens which are used in both these tests do not allow their use for distinguishing the various treponematoses from each other.

Although, the VDRL test can be used to monitor the effect of treatment, the sensitivity and specificity of the test are less than that of the FTA-ABS test. Quantitation of treatment-monitoring results can only be obtained after titration studies. The use of the VDRL is limited since positive reactions (biological false-positive reactions, BFPs) are observed in diseases other than syphilis.

The detection of antitreponemal IgM is useful in case of suspected congenital syphilis. The 19S(IgM)FTA-ABS test for the detection of antitreponemal IgM is highly sensitive and specific but it is very laborious and samples cannot be tested simultaneously. Therefore, the detection of antitreponemal IgM is limited to only a few laboratories. This test has not yet been automated and cannot be used to distinguish between the different human treponematoses. Quantitation of the results is also not feasible as yet.

12.2 Application of recombinant-DNA derived treponemal antigen in ELISAs for screening syphilis

In Chapter 6, the recombinant-DNA derived treponemal antigen TmpA was investigated for its possible use in an ELISA test for screening syphilis. The sensitivity of the TmpA ELISA in early syphilis was comparable to that of the TPHA test but was somewhat less than that of the FTA-ABS test in primary syphilis. The specificity of 99.6 % was comparable to that of the TPHA. The availability of punitive TmpA in large quantities enabled attempts to increase the specificity of the test. These investigations showed that the non-specific reactions in the TmpA ELISA were probably due to contaminants derived from E. coli. Therefore, investigations on the further purification of TmpA are necessary. Recently, TmpA has been purified further. This could permit the use of the TmpA ELISA to screen populations with a low prevalence of syphilis (e.g. at blood banks) and may even allow the development of a new screening test for syphilis eliminating the need for confirmation using the FTA-ABS test. Quantitation and automatic reading of test results were easily achieved using an ELISA microtitration plate reader. In addition, it was observed that the level of anti-TmpA antibodies after treatment dropped concomitantly with that of the VDRL. Therefore, the TmpA ELISA may represent the first treponemal test with potential to monitor the effect of therapy. However, this potential has yet to be confirmed in the clinical setting. In conclusion, the TmpA ELISA appeared to be a simple, sensitive and specific test for screening syphilis. It has also the potential for use as a confirmatory test and for monitoring the effect of treatment. The antigen, TmpA can be produced cheaply in large quantities. The antigen, TmpA appeared to recognize the antibodies to pathogenic treponemes without the need of a sorbens, but the test could not be used to differentiate between the human treponematoses. Several recombinant-DNA derived treponemal antigens for use in diagnostic tests for syphilis have been described. Their value in syphilis serology remains to be confirmed.

12.3 Rapid serodiagnosis of syphilis

In Chapters 7, 8 and 9 an attempt was made to develop a rapid immunoassay that could be used to detect antitreponemal antibodies within a period of 15 minutes. First, a new immunofiltration assay was developed using a mixture of antigens derived from T. pallidum. The sensitivity and specificity of this new assay, designated TP-ELIFA, were observed to be comparable to those of the TPHA indicating that the immunofiltration assay could be used successfully in the serodiagnosis of syphilis. However, the use of T. pallidum antigens was cumbersome and sorbens had to be used to remove non-specific antibodies from the serum. Moreover, coating of antigen manually in the dot form onto nitrocellulose membranes was laborious. In Chapter 8, the LIA procedure, involving the mechanical coating of antigens onto nitrocellulose membranes was adopted for use in the serodiagnosis of syphilis (1). In this assay multiple antigen lines were coated onto 3 mm wide nitrocellulose strips permitting the detection of antibodies to multiple antigens simultaneously. Purified or recombinant-DNA derived antigens had to be used since the use of T. pallidum required

for a sorbens which hampered the simultaneous detection of antitreponemal antibodies and antibodies to other infectious agents. Although, the limited number of sera tested provided only preliminary results, it was shown that the LIA procedure using TmpA or AF antigens could efficiently detect antitreponemal antibodies. Since air-dried antigen coated nitrocellulose strips could be stored easily in small boxes, the LIA may be useful in fieldwork. The mechanical application of antigen in parallel lines in the LIA procedure was also incorporated into the immunofiltration procedure. A preliminary study on this newly developed enzyme-linked line immunofiltration assay (ELLIFA) using TmpA and AF as antigens and a limited number of sera showed a sensitivity and specificity comparable to those of the TPHA. In this procedure TmpA and AF antigens were applied to nitrocellulose in lines and permitted a rapid detection of antibodies directed to two different treponemal antigens simultaneously. One of these antigens can be combined with antigens derived from other infectious agents in the ELLIFA procedure. However, extensive evaluation of the ELLIFA using multiple antigens is required before final evaluation.
In Chapter 9, various factors that influence the performance of the immunofiltration assay were investigated. Five minutes incubation was observed to be optimal for immunofiltration of antibody. In addition, evidence was obtained that immunofiltration could only be performed if the dissociation affinity constant of the ligands was low enough for the rapid binding during the filtration. This is valid in antigen-antibody binding which confirmed that serum antibodies and enzyme-labelled immunoglobulins could be effectively incubated during immunofiltration. Furthermore, it was shown that the choice of solid phase and blocking-agent was critical. From all solid-phases that were investigated, only nitrocellulose in combination with Tween-20 was found suitable for immunofiltration. In conclusion, the ELLIFA may offer a rapid test for screening syphilis. It is cheap, easy to perform, and can be automated. Characteristics of TmpA as antigen in the ELLIFA requires further investigations. Rapid immunoassays have been described by others but did not involve filtration during incubation. Consequently, these assays required substantially more time for execution.

12.4 Development of an ELISA for the confirmation of syphilis

A confirmation test should have the highest specificity and a high sensitivity. Therefore, in the development of a confirmation test, attention must be focused on improving the specificity of the test. In general, high specificity of a test is limited by the range of epitopes present on the antigen which is used in the test. Cross-reacting antibodies raised to epitopes that resemble epitopes found on antigens from other organisms can be observed. Such non-specific antibodies can be observed in individuals who do not have the disease. The specificity of a test can be enhanced by limiting the number of non-specific antigens that occur in the test or by limiting the non-specific reacting serum antibodies in every sample tested by means of an absorbing compound. The latter manner is not well-defined and some antigens will always be missed. The use of recombinant-DNA derived antigens could eliminate non-specific reactions. However, the complete removal of contaminants of the host origin in which the recombinant-DNA derived antigen was expressed is difficult.

In addition, the recombinant antigen itself may contain epitopes that react non-specifically and which cannot be eliminated. A third approach is the use of monoclonal antibodies in the confirmation test. Since monoclonal antibodies that are reactive to one epitope only can be selected, a highly specific test can be developed. To investigate this approach, the specificity of two monoclonal antibodies was investigated (Chapter 10). It was observed that the use of these monoclonal antibodies resulted in a very specific and sensitive test but that the non-specific reactions were not completely eliminated. In addition, it was shown that combining the results obtained using the two monoclonal antibodies directed to different antigens may enhance the sensitivity of the test without compromising the specificity. A general disadvantage of the inhibition ELISA is the fact that the discriminative extinction between positive and negative test results has to be set at a high level. High extinctions in ELISA vary considerably which limits their value for discriminative use. It was shown that this disadvantage could be overcome by testing different dilutions of the serum samples. A difference in the extinction between the different serum dilutions was found to be a better discriminator between a positive and a negative sample than the inhibition measured in one single sample. Furthermore, it is recommended that a dilution of 1/1000 be included in the range of standard dilutions of each sample tested. In conclusion, the inhibition ELISA can be used as confirmation test for the diagnosis of untreated syphilis. In treated syphilis, the sensitivity was low as that observed in the TPI. Monitoring the effect of treatment can be an additional benefit, but further investigations on this subject are needed. The inhibition ELISA is cheaper and more simple than the FTA-ABS test and can be automated. However, despite its high specificity, yaws and syphilis cannot be distinguished from eachother by the monoclonal antibodies used in this test. Other inhibition ELISAs using monoclonal antibodies have not yet been described.

12.5 Detection of antitreponemal IgM

An automated test for the detection of antitreponemal IgM would be of great value in syphilis serology, since the current test for detection of antitreponemal IgM, the 19S(IgM)FTA-ABS test is complicated and does not allow testing of more than one sample simultaneously. In Chapter 11, it was shown that the recently introduced capture ELISA (Captia Syphilis-M test), for the detection of antitreponemal IgM, is sensitive and specific in early syphilis. However, these results should be considered preliminary because of the small number of samples that were tested. The five sera from congenital syphilis patients all showed a positive reaction in the capture ELISA. Therefore, this test may be an effective tool in the diagnosis of congenital syphilis. In neurosyphilis, the sensitivity of the capture ELISA appeared to be less than that of the 19S(IgM)FTA-ABS test. It may be argued, that the difference in sensitivity of the two tests could be due to the non-specific reactions in the 19S(IgM)FTA-ABS test. In addition, testing for IgM is of limited use in the diagnosis of neurosyphilis. A better diagnostic aid (the TPHA-index) was formulated by Luger (2). In conclusion, the specificity of the capture ELISA for the detection of antitreponemal IgM was comparable to the 19S(IgM)FTA-ABS test. The very rare 19S IgM-anti-IgG autoantibodies may cause false-positive reactions in the presence of antitreponemal IgG

similar to that observed in the SPHA test (3). The sensitivity of the capture ELISA for early syphilis is also comparable to that of the 19S(IgM)FTA-ABS test. The capture ELISA is simple to perform, more than one sample simultaneously can be tested, and it can be automated. However, since the results obtained in the capture ELISA are still preliminary, it is recommended to confirm each positive sample in the 19S(IgM)FTA-ABS test.

In this study the application of ELISA methodology in syphilis serology was investigated. An ELISA test using the recombinant-DNA derived TmpA, the TmpA ELISA for monitoring the effect of treatment, a confirmatory inhibition ELISA using monoclonal antibodies and a capture ELISA in which antitreponemal IgM could be detected were shown to be of value as compared to the current corresponding tests for syphilis. However, the use of these new ELISAs in syphilis serology requires further investigations in clinical setting. Future experiments should show that screening, diagnosis and monitoring the effect of treatment can be incorporated in one single test using TmpA or other suitable recombinant DNA-derived antigens. In addition, the rapid enzyme-linked immunofiltration assay was developed for syphilis using T. pallidum, Axial Filament or TmpA as antigen. This test can be a suitable complementary test to the rapid RPR-card test eliminating the biological false-positive reactions. In combination with the RPR-card test, a useful tool for the rapid serodiagnosis of syphilis was developed and in which antitreponemal and anticardiolipin antibodies could be detected within 20 minutes. Rapid serodiagnosis of syphilis using T. pallidum antigens was shown to be possible by immunofiltration. Whether the RPR-card test could be replaced by an immunofiltration assay also remains to be investigated in the clinical setting. The performance of an immunofiltration assay using recombinant DNA-derived antigens eliminates the need for a sorbens. Therefore, such test could be incorporated in multiple antigen tests.
Although, the tests which were modified and evaluated in this dissertation did not strictly conform to the criteria that new tests are required to fulfil in terms of the minimum number of sera that must be examined at different centers, the results did establish a definite value of these new tests. However, these tests must satisfy the strict requirements for new tests described in Chapter 4 prior to their final acceptance into the already existing battery of tests for the diagnosis of syphilis.

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13 Summary

Syphilis is a sexually transmitted disease (STD) which is spread world-wide (Chapter 1). It can be cured easily by antibiotic treatment. If left untreated, syphilis may cause severe damage even after many years. Diagnosis during the first consultation of the patient is important since many patients with STD fail to return. Therefore, much effort has been made towards to the early and rapid diagnosis of syphilis by both individual health services and public health care.

In Chapters 2 and 3, the clinical aspects of syphilis infection and the current outlook on syphilis serology were discussed, respectively. Tests for syphilis in Europe to date consist of a treponemal screening test, the TPHA, a confirmation test, the FTA-ABS test, a test for treatment monitoring, the VDRL or RPR-card test, and the 19S(IgM)FTA-ABS test for detection of antitreponemal IgM in congenital syphilis. All these tests have several disadvantages. The antigens used in these tests consist of a blend of treponemal antigens (TPHA, FTA-ABS and 19S(IgM)FTA-ABS) or consist of non-treponemal antigens like cardiolipin (VDRL and RPR card test). Therefore, the elimination of false-positive results in these tests is laborious if not impossible. Furthermore, the VDRL and RPR card test are not only reactive in syphilis but also in other systemic diseases. Therefore, a treponemal test that can be used to evaluate the effect of treatment is desired.

Rapid testing within 20 - 30 minutes cannot be performed and automation is difficult using the current tests for syphilis except for the VDRL and RPR card test. Finally, the 19S(IgM)FTA-ABS test is complex to execute and therefore, limited only to a small number of laboratories with special equipment.

In Chapter 4, the requirements of tests for screening, diagnosis and confirmation of syphilis and other infectious diseases were described.

A candidate test system that can potentially meat the requirements for syphilis serology is the enzyme-linked immunosorbent assay (ELISA), because of its many applications, its simple performance and the possibilities for its automation (Chapter 5). In the present study various modifications of ELISA were investigated in an attempt to develop efficient assays for a comprehensive syphilis serology with respect to diagnosis, screening and monitoring the response to treatment.

In Chapter 6, an ELISA using the recombinant DNA derived treponemal protein TmpA was discussed as screening test for syphilis. A sensitivity of 76 % for primary syphilis, 100 % for secondary syphilis, and 98 % for early latent syphilis was observed. The specificity was 99.6 % among 938 sera from blood donors. The sensitivity and specificity of the TmpA ELISA were found to be comparable to those of the TPHA. Therefore, the TmpA ELISA was observed to be suitable as a screening test for syphilis. The TmpA-ELISA could be easily automated and produced quantifiable test results. In addition, it was found that the level of anti-TmpA antibodies declined after treatment concomitantly with that of the VDRL. Therefore, the TmpA ELISA may be a treponemal test with a potential for monitoring the effect of antibiotic treatment. Furthermore, the previously described AF-ELISA using the purified Axial Filament derived from the T. phagedenis, was investigated for its potential for treatment monitoring. It was demonstrated that the AF ELISA was not a suitable

alternative for treatment monitoring.

In Chapters 7, 8 and 9, a rapid immunoassay, the enzyme-linked immunofiltration assay (ELIFA) for serodiagnosis of syphilis was discussed. In the T. pallidum ELIFA (TP-ELIFA), T. pallidum antigens were coated onto a filtrable nitrocellulose membrane through which diluted serum was filtered (Chapter 7). A test duration of 15 - 20 minutes was obtained. The non-specific reactions were inhibited by the absorption of the non-specific reacting antibodies by a T. phagedenis derived sorbens. The specificity and the sensitivity of the TP-ELIFA obtained were comparable to those of the TPHA. Recently, a new technique for application of antigen in lines, the Line Immunoassay (LIA) became available. The potential use of the LIA in serodiagnosis of syphilis was investigated using TmpA and AF antigens (Chapter 8). The test results of the LIA were found to be comparable to those of the TPHA for virtually all sera tested. In addition, AF and TmpA were applied in parallel lines onto nitrocellulose membranes that were subsequently used in the immunofiltration procedure (Chapter 8). The results of this newly developed enzyme-linked line immunofiltration assay (ELLIFA) using AF and TmpA antigens were comparable to those of the TPHA for the limited number of sera tested. The AF and TmpA LIA could be used for the serodiagnosis of syphilis in the field since the antigen precoated nitrocellulose strips used in this assay could be preserved easily in a little space consuming dry box. The TmpA and AF ELLIFA eliminated the need for a sorbens and therefore permitted these antigens to be used in combination with other antigens for simultaneous detection of antibodies to multiple antigens.

The coating, rinsing and incubation steps in immunofiltration were optimised by means of ^{125}I labelled AF and protein A (Chapter 9). Briefly, it was observed that a 5 minutes incubation time was optimal if antibody-antigen binding was involved, but this was longer for another pair of ligands as was demonstrated with protein A. Blocking the non-specific binding to nitrocellulose in the incubation steps was only possible if Tween-20 (0.5 %) [Bwas used. Zeta Probe and Hybond Nylon could not be blocked effectively for non-specific protein binding in immunofiltration.

A confirmation test with high specificity that could be automated was developed to replace of the FTA-ABS test (Chapter 10). Monoclonal antibodies raised against three different treponemal antigens were investigated for application in an inhibition ELISA. Two of them, C3E5, directed to the 47,000 dalton treponemal protein and 1-14M1, directed to the 42,000 dalton treponemal protein TmpA were found to be promising in preliminary studies. The sensitivity of the inhibition ELISA using C3E5 for early untreated syphilis was 93 % and was 79 % if 1-14M1 was used. If the positive results of the ELISAs using both monoclonal antibodies were combined, a sensitivity of 97 % was obtained. The specificity of the inhibition ELISA using either monoclonal antibody was 100 % among 500 sera from blood donors.

Furthermore, the specificity of the inhibition ELISA was investigated using sera reactive with other spirochetes. Six sera reactive with Borrelia burgdorferi did not react in the inhibition ELISA but 1 serum out of 7 sera reactive to Leptospira icterohaemmorhagiae was reactive in the inhibition ELISA. In 432 non-syphilitic sera from individuals attending

an STD outpatient clinic, 7 sera were found to be reactive in the inhibition ELISA using either monoclonal antibody. These results suggests that although a very small number of epitopes to which cross-reactivity could occur were involved in the inhibition ELISA, non-specific reactions did occur. High variance in the extinction among the non-reactive sera was observed in the inhibition ELISA. Therefore, all sera were tested at 3 different dilutions. A serum was defined positive in the inhibition ELISA if a difference of at least 0.5 was found between the 3 dilutions or if the extinction at all dilutions was less than 0.3. In Chapter 11, the recently introduced capture ELISA (Captia Syphilis-M test), a highly sensitive ELISA for the detection of antitreponemal IgM was discussed as alternative to the complicated 19S(IgM)FTA-ABS test. The capture ELISA test was sensitive in congenital syphilis, but only 5 specimen were tested. The sensitivity of the capture ELISA in early untreated syphilis was comparable to that of the 19S(IgM)FTA-ABS test but it was less sensitive in neurosyphilis. The specificity was comparable to that of the 19S(IgM)FTA-ABS test for samples false-reactive in the VDRL and for samples from non-infected neonates with antitreponemal IgG. These results indicated that the capture ELISA is a sensitive and specific alternative for diagnosis of congenital syphilis.

14 Samenvatting

Venerische syfilis is een sexueel overdraagbare aandoening (SOA) die wereldwijd voorkomt (hoofdstuk 1). De ziekte kan eenvoudig worden behandeld met antibiotica. Wordt syfilis niet behandeld, dan kunnen, soms pas na vele jaren, ernstige afwijkingen ontstaan. De diagnose syfilis kan het beste tijdens het eerste consult geheel worden afgerond omdat vele patienten met SOA's niet voor een tweede consult komen. Daarom wordt via de individuele en maatschappelijke gezondheidszorg veel moeite gedaan om tot een vroege en snelle diagnose van syfilis te komen.

In de hoofdstukken 2 en 3 werden respectievelijk de klinische symptomen en huidige serodiagnostiek van syfilis besproken. De huidige testen voor syfilis welke doorgaans in Europa worden gebruikt, bestaan uit een treponemale screeningstest, de TPHA, een confirmatietest, de FTA-ABS test, een test voor meting van het effect van behandeling, de VDRL of RPR-card test en de 19S(IgM)FTA-ABS test voor het aantonen van antitreponemaal IgM bij verdenking op congenitale syfilis. Al deze testen hebben bepaalde nadelen. De antigenen die in deze testen worden gebruikt, bestaan uit een mengsel van treponemale antigenen (TPHA, FTA-ABS en 19S(IgM)FTA-ABS) of zelfs uit niet-treponemale antigenen als cardiolipine (VDRL en RPR-cardtest). Daarom is de eliminatie van fout positieve reacties uit deze testen moeilijk of misschien zelfs onmogelijk. Daarbij komt nog dat de VDRL en de RPR-card test niet alleen positief kunnen reageren bij syfilis maar ook bij andere systemische ziekten. Daarom zou een treponemale test die het effect van behandeling kan meten, een aanwinst zijn. Een snel testresultaat is met de huidige serologische syfilistesten niet binnen 20 - 30 minuten te verkrijgen en automatisering van deze testen is problematisch uitgezonderd de VDRL en RPR-cardtest. De 19S(IgM)FTA-ABS test is complex om uit te voeren en het gebruik ervan is derhalve beperkt tot slechts enkele laboratoria met speciale voorzieningen.

In hoofdstuk 4 werden de eisen voor screening-, diagnose- en confirmatietesten beschreven voor infectieuze ziekten en voor syfilis in het bijzonder.

Vanwege zijn vele toepassingsmogelijkheden, zijn simpele verwerking en goede mogelijkheden voor automatisering, is de enzyme-linked immunosorbent assay (ELISA) een testsysteem dat potentieel aan de eisen voor moderne syfilisdiagnostiek kan voldoen (hoofdstuk 5). In de onderhavige studie zijn enkele modificaties van de ELISA onderzocht en nieuwe antigenen toegepast in een poging om een geintegreerd pakket van efficiente testen voor de verschillende toepassingsgebieden van syfilisserologie met betrekking tot diagnose, screening en meting van het effect van behandeling te ontwikkelen.

In hoofdstuk 6 werden de eigenschappen en resultaten van een ELISA screeningstest voor syfilis besproken die gebruik maakt van antigeen dat beschikbaar was gekomen via recombinant-DNA technieken. Er werd een gevoeligheid van 76 % voor primaire syfilis, 100 % voor secundaire syfilis en 98 % voor vroege latente syfilis gemeten. De specificiteit was 99,6 % onder 938 sera van bloeddonors. De gevoeligheid en specificiteit van de TmpA-ELISA bleek vergelijkbaar met die van de TPHA. De TmpA-ELISA kan eenvoudig worden geautomatiseerd en de testresultaten zijn kwantificeerbaar. Tevens werd gevonden dat de concentratie van antiTmpA antistoffen na behandeling daalde overeenkomstig de concentratiedaling van anticardiolipine antistoffen gemeten in de VDRL. Daarom kan de TmpA-ELISA de eerste treponemale test worden genoemd die potentieel het effect van behandeling kan meten. Een andere, reeds eerder beschreven ELISA die gebruik maakt van het gezuiverde Axiaal Filament van de T. phagedenis (AF-ELISA), werd onderzocht

of deze dezelfde karakteristieken vertoonde bij het meten van het effect van behandeling. Er werd aangetoond dat de AF-ELISA het effect van behandeling onvoldoende kan meten.

In de hoofdstukken 7, 8 en 9 werd een nieuwe snelle immunoassay, de enzyme-linked immunofiltration assay (ELIFA) besproken voor serodiagnose van syfilis. In de T. pallidum ELIFA (TP-ELIFA) werden T. pallidum antigenen aangebracht op een nitrocellulose membraan waardoor vervolgens verdund serum werd gezogen (hoofdstuk 7). Op deze wijze werd de totale testduur beperkt tot 15 - 20 minuten. Niet-specifieke reacties werden beperkt door absorptie van de niet-specifiek reagerende antistoffen met een sorbens verkregen van bewerkte T. phagedenis antigenen. De gevoeligheid en specificiteit van de TP-ELIFA waren vergelijkbaar met die van de TPHA. Kort geleden kwam een nieuwe techniek voor applicatie van antigenen op dragermateriaal, the Line Immuno Assay (LIA), beschikbaar. De toepasbaarheid van de LIA in serodiagnose van syfilis werd onderzocht met behulp van de antigenen TmpA en AF (hoofdstuk 8). De testresultaten van de LIA waren vergelijkbaar met die van de TPHA voor bijna alle sera die werden getest. Verder werden AF en TmpA in parallelle lijnen gecoat op nitrocellulose membranen welke vervolgens werden gebruikt in de immunofiltratieprocedure (hoofdstuk 8). De testresultaten van deze nieuw ontworpen enzyme-linked line immunofiltration assay (ELLIFA) waren met gebruik van AF en TmpA antigenen vergelijkbaar met die van de TPHA voor het beperkte aantal sera dat was getest. De AF en TmpA-LIA kunnen worden gebruikt in een eenvoudige omgeving zonder koeling omdat de nitrocellulose strips gecoat met antigeen gemakkelijk droog kunnen worden bewaard in een kleine, weinig ruimte innemende doos. Met toepassing van TmpA en AF in de ELLIFA procedure werd het gebruik van een sorbens overbodig waardoor deze antigenen gebruikt kunnen worden in combinatie met andere antigenen voor simultane detectie van antistoffen tegen verscheidene antigenen.

De coating, was- en incubatiestappen in de immunofiltratie-procedure werden geoptimaliseerd door middel van ^{125}I gelabeld AF en proteine A (hoofdstuk 9). In deze studie werd aangetoond dat 5 minuten incubatietijd optimaal was voor antistof-antigeen binding, maar dat een veel langere incubatietijd nodig was indien een ander ligandenpaar werd gebruikt als werd aangetoond met proteine A. Blokkering van niet-specifieke binding aan nitrocellulose tijdens de incubatiestappen bleek alleen mogelijk met 0.5 % Tween-20. Andere membranen als Zeta Probe en Hybond Nylon konden niet effectief worden geblokkeerd voor niet-specifieke proteinebinding in de immunofiltratieprocedure.

In hoofdstuk 10 werden de ontwikkeling en resultaten van een bevestigingstest met hoge specificiteit die ook kan worden geautomatiseerd besproken voor eventuele vervanging van de FTA-ABS test. Monoclonale antistoffen tegen drie verschillende treponemale antigenen werden onderzocht op hun bruikbaarheid voor toepassing in een inhibitie-ELISA. Twee van deze, C3E5 gericht tegen het 47.000 dalton wegend treponemale eiwit en 1-14M1 gericht tegen het 42.000 dalton wegend treponemale eiwit TmpA leken veelbelovend te zijn in vooronderzoekingen. De gevoeligheid van de inhibitie-ELISA met C3E5 voor vroege onbehandelde syfilis was 93 % en met 1-14M1 79 %. Als de positieve resultaten van de ELISA's met beide monoclonale antistoffen werden gecombineerd, werd een gevoeligheid van 97 % bereikt. De specificiteit van de inhibitie-ELISA met beide monoclonale antistoffen was 100 % onder 500 sera van bloeddonors.

Vervolgens werd de specificiteit van de inhibitie-ELISA onderzocht onder sera die positief waren voor andere spirochetosen. Zes sera die reageerden met Borrelia burgdorferi waren negatief in de inhibitie-ELISA maar 1 van de 7 sera die reageerden met Leptospira icterohaemorrhagiae was positief in de inhibitie-ELISA. In 432 niet-syfilitische sera van patienten die een SOA-polikliniek bezochten werden 7 sera positief bevonden in de inhibitie-

ELISA wanneer beide monoclonale antistoffen werden gebruikt. Deze resultaten suggereren dat hoewel slechts een zeer klein aantal epitopen betrokken is in de inhibitie-ELISA, toch niet-specifieke reacties kunnen optreden. Ook werd een grote variatie in extinctie gezien in de inhibitie-ELISA wanneer niet-reactieve sera werden getest. Om dit probleem te omzeilen, werden alle sera in drie verschillende verdunningen getest. Een serum werd als positief beschouwd in de inhibitie-ELISA wanneer een verschil van tenminste 0,5 werd gevonden tussen de hoogste en de laatste verdunning of als de extincties van alle verdunningen minder waren dan 0,3.
In hoofdstuk 11 werd de recent geintroduceerde capture-ELISA (Captia Syphilis-M test) beschreven, een heel gevoelige ELISA voor het aantonen van antitreponemale IgM antistoffen. Deze test werd geevalueerd als alternatief voor de gecompliceerde 19S(IgM)FTA-ABS test. De capture-ELISA was gevoelig in congenitale syfilis, maar slechts 5 sera konden worden getest. De gevoeligheid van de capture-ELISA in vroege onbehandelde syfilis was vergelijkbaar met die van de 19S(IgM)FTA-ABS test maar de test was minder gevoelig in neurosyfilis. De specificiteit was vergelijkbaar met die van de 19S(IgM)FTA-ABS test in sera die fout positief waren in de VDRL en in sera van niet-geinfecteerde pasgeborenen met antitreponemaal IgG. Deze bevindingen geven aan dat de capture-ELISA een gevoelig en specifiek alternatief is voor de diagnose van congenitale syfilis.

15 Abbreviations

AF = Axial Filament
AIDS = Acquired Immunedeficiency Syndrome
Albumin-quotient = $\frac{\text{CSF-albumin}}{\text{serum-albumin}} \times 10^3$
BFP = Biological False Positive reaction
CIE = Counter Immunoelectrophoresis
CL = Confidence Limits
CNS = Central Nervous System
CSF = Cerebrospinal Fluid
CVA = Cerebro Vascular Accident
DM = serum of a patient with diabetes mellitus
EDTA = ethylenediamine-tetraacetic acid
ELIFA = Enzyme-linked Immunofiltration Assay
ELISA = Enzyme-linked Immunosorbent Assay
ELLIFA = Enzyme-linked Line Immunofiltration Assay
FTA-ABS = Fluorescent Treponemal Antibody-Absorption
HATTS = Hemagglutination Treponemal Test for Syphilis
HIV = Human Immunodeficiency Virus
HPLC = High Performance Liquid Chromatography
HRP = horse radish peroxidase
IgG-index = $\frac{\text{CSF-IgG}}{\text{serum-IgG}} \times 10^3 \div$ Albumin quotient
IgM-index = $\frac{\text{CSF-IgM}}{\text{serum-IgM}} \times 10^3 \div$ Albumin quotient
ITPA = Intrathecal Treponema pallidum antibody
IgM-ITPA-index = $\frac{\text{CSF-IgM-TPHA titre}}{\text{serum-IgM-TPHA titre}} \div \frac{\text{CSF-IgM}}{\text{serum-IgM}}$
ITPA-index = $\frac{\text{CSF-TPHA titre}}{\text{serum-TPHA titre}} \div \frac{\text{CSF-IgG}}{\text{serum-IgG}}$
LIA = Line Immuno Assay
MoAb = Monoclonal Antibody
MU = Million Units
NC = Nitrocellulose
NRS = immunoglobulins derived from normal rabbit serum
PBS = Phosphate-Buffered Saline
PBS-T = Phosphate-Buffered Saline containing 0.5 % Tween-20
RAS = immunoglobulins derived from rabbit anti-axial filament hyperimmune serum
RIA = Radio Immunoassay
RPR = Rapid Plasma Reagin
SB 3-14 = Sulfobetain 3-14 (N-tetradecyl-N,N,-dimethylammonio-1-propanesulfonate)
SDS-PAGE = Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
19S(IgM)FTA-ABS = 19S(IgM) Fluorescent Treponemal Antibody-Absorption
SOA = Sexueel Overdraagbare Aandoening
SPA = ^{125}I-staphylococcal protein A immunoassay
SPHA = Solid-phase Hemadsorption Assay
SPIA = Solid-phase Immunofiltration Assay
STD = Sexually Transmitted Disease
TLC = Thin-layer-chromatography
TE-buffer = 0.01 M Tris.HCl, 1 mM EDTA pH 8.0
TmpA = Treponema pallidum membrane protein A (42 kDa)

TmpB	= Treponema pallidum membrane protein B (34 kDa)
TMTB-buffer	= 10 mM Tris-HCl pH 7.4, 0.1 M $MgCl_2$, 0.5 % Tween 20, 1 % bovine serum albumin and 5 % fetal calf serum
TP-ELIFA	= Treponema pallidum-Enzyme-linked Immunofiltration Assay
TP-IgM-HA	= Treponema pallidum specific IgM haemagglutination assay
TPHA	= Treponema pallidum Hemagglutination Assay
TPHA-index	= $\frac{\text{CSF-TPHA titre}}{\text{albumin quotient}}$
TPI	= Treponema pallidum Immobilization
Tris-buffer	= 0.01 M Tris-HCl, pH 7.4 and 0.15 M NaCl
TS-buffer	= 0.01 M Tris.HCl, pH 7.4 and 0.15 M NaCl
VDRL	= Venereal Disease Research Laboratory

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Curriculum vitae

De schrijver van dit proefschrift werd op 29 december 1956 geboren te Haarlem. In 1976 werd het diploma Atheneum B behaald aan het J.F. Kennedy Atheneum te Dongen. In 1976 werd met de studie Electrotechniek begonnen aan de Technische Universiteit te Eindhoven. In 1977 werd de studie Geneeskunde begonnen aan het Rijks Universitair Centrum te Antwerpen. Na het behalen van het kandidaatsdiploma aldaar in 1980, werd de studie voortgezet aan de Erasmus Universiteit te Rotterdam. In 1984 werd het artsexamen afgelegd.
Van februari 1985 tot december 1987 was de schrijver werkzaam in het Laboratorium voor Bacteriologie, Rijksinstituut voor Volksgezondheid en Milieuhygiene te Bilthoven onder leiding van prof.dr. E.J. Ruitenberg en dr. P.A.M. Guinée en onder begeleiding van dr. R.V.W. van Eijk en later dr. J.D.A. van Embden. Binnen deze periode werd een vier maanden durend deelonderzoek verricht in het Laboratorium voor Klinische Immunologie van de Stichting Samenwerkende Delftse Ziekenhuizen onder leiding van dr. P. Herbrink. Een ander deelonderzoek werd uitgevoerd aan het Instituut voor Tropische Geneeskunde te Antwerpen onder leiding van dr. G. van der Groen. Van december 1987 tot juni 1988 werd een opleiding tot programmeur Cobol en systeemanalist gevolgd bij Volmac Toptraining (TT) te Utrecht in het kader van het Promotie Informatica Omscholing in Nederland (PION) project. Sinds juli 1988 is de schrijver werkzaam als arts-assistent in opleiding aan de afdeling Dermato-venereologie, Academisch Ziekenhuis Dijkzigt te Rotterdam (opleider: prof.dr. E. Stolz).