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Original Work

Screening Assays to find out Late Latent Syphilis Cases – Which is the best one?

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ABSTRACT: The serological methods for diagnosis of syphilis are classified into non-specific (non-treponemal) such as Rapid Plasma Reagin (RPR), and specific (treponemal) such as Treponema pallidum hemagglutination test (TPHA) and Enzyme-linked immunosorbent assay IgG, IgM (ELISA IgG, IgM) tests. The aim of this retrospective study was to estimate and compare the sensitivity and specificity of RPR, TPHA and Syphilis ELISA IgG, IgM. The study was conducted on 18 799 clinically healthy persons who had visited the Outpatient clinic of Ministry of the Interior Clinical Centre, Latvia during 2 years period from August 2006 to November 2007. Patients were screened to find out possible late latent syphilis cases by using RPR, TPHA and ELISA IgG, IgM assays. The results showed the highest sensitivity indices of ELISA IgG, IgM and TPHA methods, and the lowest for the RPR test. Highest specificity indices were observed by using ELISA IgG, IgM method followed by TPHA method with lower values and RPR method showing the lowest specificity. To conclude, ELISA IgG, IgM and TPHA methods should be used for screening late latent syphilis cases and ELISA IgG, IgM for diagnosis confirmation. RPR is not recommended for screening purposes.

KEY WORDS: Late latent syphilis; Serological diagnostics; Screening

INTRODUCTION

Syphilis is the disease caused by *Treponema pallidum* subspecies (ssp.) *pallidum*. The term "serological activity" is considered in describing syphilis, to indicate the level of antibodies against *Treponema pallidum* ssp. *pallidum* in human blood. False positive serologic reactions are not rare because of cross-reactions with a concomitant disease or technical errors[1-3].

Since 1990, there has been an upward trend in the incidence of both acquired and congenital syphilis in European countries, especially in Eastern Europe. Indeed, the incidence of syphilis has increased worldwide, and this phenomenon is clearly associated with sexual promiscuity and sexual tourism destinations[4]. The syphilis incidence in Latvia has been steadily decreasing in the past few years, but remains still high in comparison with more developed countries[5]. As a syphilitic infection can produce a variable range of symptoms in humans, laboratory tests are often required to definitively diagnose an infection[6].

Infection is initiated when *Treponema pallidum* ssp. *pallidum* enters tissues through dermal microabrasions or by penetration of intact mucosa, typically resulting in a single chancre at the site of inoculation. The primary chancre develops after an average incubation period of 3 weeks. The chancre usually heals

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spontaneously within 4-6 weeks, but it may still be discernible in about 15% of patients at the onset of secondary syphilis[7]. Within hours after inoculation, and during evolution of the primary stage, *Treponema pallidum* ssp. *pallidum* disseminates widely and may lodge in any organ. The manifestations of secondary syphilis usually develop within 12 weeks of initial infection. The most common manifestation of secondary syphilis is disseminated mucocutaneous lesions. The lesions of secondary syphilis gradually resolve spontaneously within 12 weeks of appearance. Untreated individuals enter a variable period of latent infection in, which no clinical manifestations are evident. Latent syphilis is divided into two stages : early (high likelihood of relapse) or late (recurrence unlikely), based on an approximate duration of infection. For the first year after infection, patients are considered to have early latent syphilis and up to 25% of patients may have recurrent secondary manifestations[8]. Late latent syphilis is defined as asymptomatic infection for a period more than one year or unknown duration. Serologic tests during the late latent stage are positive, but sexual transmission is unlikely. Organisms may seed the bloodstream intermittently during latent syphilis and can infect the fetus during pregnancy. A schematic diagram of untreated syphilis is shown in **Figure 1**.

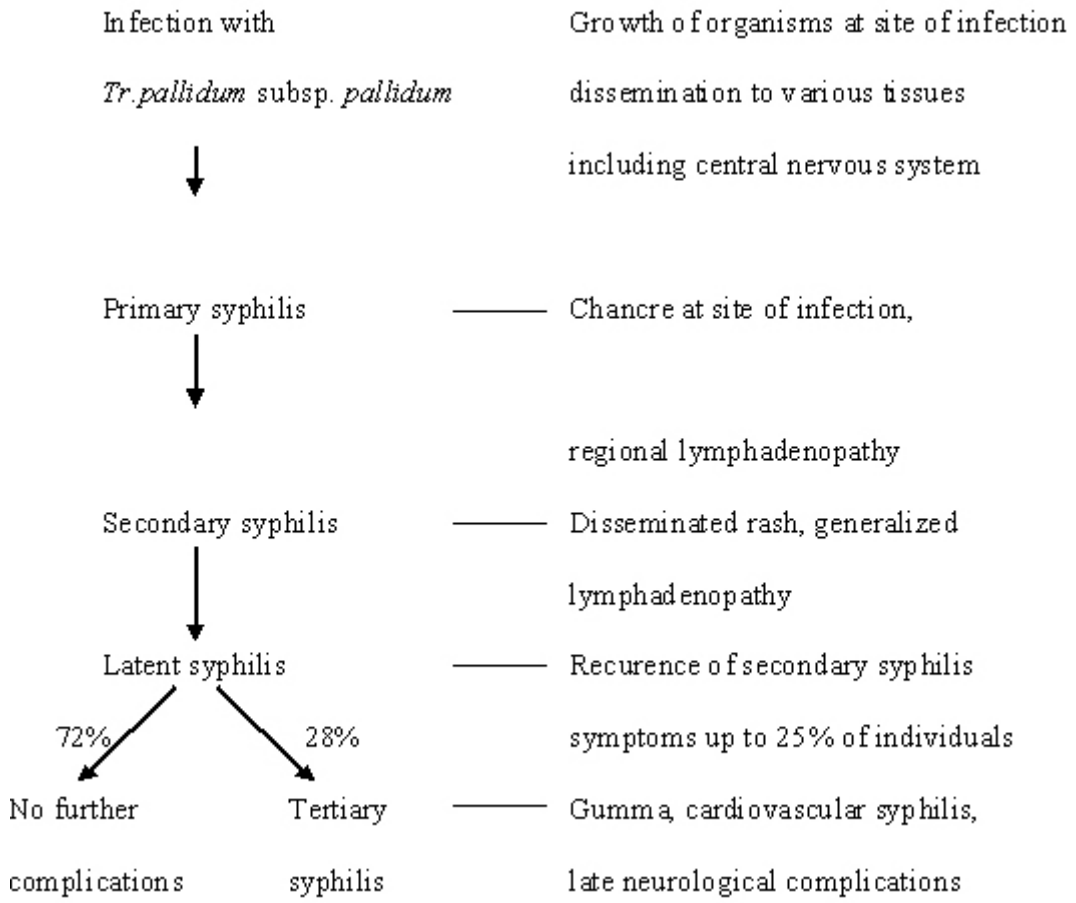

Figure 1: Natural history of untreated syphilis (Gjestland 1995)

The determination of the stage of disease is important, because the sensitivity and specificity values of the applied diagnostic methods vary according to the different stages of the disease and the prognosis and outcome of treatment depends on the stage of disease[9, 10].

The *Treponema pallidum* ssp. *pallidum* genome, which is known to be small[11, 12], was confirmed by the Genome Sequencing Project as 1.14 Mb and encodes 1041 putative proteins[13]. The *Treponema pallidum* genome sequence does not reveal any obvious classical virulence factors that could account for syphilis signs and symptoms[14]. Unlike the related spirochetes *Treponema denticola*[15] and *Borrelia burgdorferi*[16], no system for genetic manipulation of *Treponema pallidum* yet exists. Because of the fragility of its outer membrane, genetic manipulation of *Treponema pallidum* may prove impossible. Heterologous expression in related organisms

such as *Treponema denticola* may be the most practical way to study *Treponema pallidum* genes and advance our understanding of this enigmatic organism[14].

Spirochaete *Treponema pallidum* ssp. *pallidum* belongs to a family of spiral-shaped bacteria- the Spirochaetaceae (spirochaetes), and is related to other pathogenic treponemes that cause non-venereal diseases. The *Treponema pallidum* subspecies are virtually identical based on their morphology, antigenic properties, and DNA homology; although more recent evidence suggests that there may be molecular signatures than can be used to differentiate the subspecies[17]. *Treponema pallidum* lacks lipopolysaccharide- the endotoxin, which is found in the outer membranes of many gram-negative bacteria that causes fever and inflammation. However, *Treponema pallidum* does produce a number of lipoproteins which may induce expression of inflammatory mediators via toll-like receptor 2 (TLR2) recognition[18]. Antibodies are usually produced either against *Treponema pallidum* ssp. *pallidum* or part of its component. Basic antigens, the determinants of *Treponema pallidum* ssp. *pallidum* are components of its triple layer outside wall and in separate cases the capsule-shaped mucopolysaccharides coupler. The most investigated protein antigens of *Treponema pallidum* ssp. *pallidum*, which contain a fraction are known to be common in both – pathogenic and saprophytic treponemas, and the antibodies are raised against these antigens. They also contain a fraction that is specific only to pathogenic treponemas. Components of *Treponema pallidum* ssp. *pallidum* proteins have high immunogenicity. Virulent *Treponema pallidum* ssp. *pallidum* induces cultured endothelial cells to express the adhesion molecules ICAM-1, VCAM-1 and E-selection. These are also activated by 47-kDa *T. pallidum* lipoprotein TpN47[19]. Compared to the wealth of information about the disease-causing mechanisms of many bacterial pathogens, little is known about how *T. pallidum* causes the protean manifestations of syphilis. In the absence of cytotoxins and other known virulence factors, the inflammation and ensuing adaptive immune response to *Treponema pallidum* ssp. *pallidum* probably causes the tissue destruction characteristic of syphilis infection. Specific *Treponema pallidum* ssp. *pallidum* molecules that have been shown to stimulate dendritic cells, the lipoproteins TpN17 and TnN47, are not located on the surface. The initiation of lipoprotein signalling of dendritic cells is not likely to occur until the organisms are being depredated, exposing the lipoproteins to the TLR2 receptors. This theory supports the observation that longer time is required for *Treponema pallidum* ssp. *pallidum* to stimulate dendritic cells[1]. A delay in dendritic cells maturation, resulting in slower inflammatory response, could allow the early dissemination of *Treponema pallidum* ssp. *pallidum,* which gives opportunity to the organism to penetrate organs and tissues before an active inflammatory response has been mounted by the host.

Bacteriological investigation in a case of syphilis is not possible because *Treponema pallidum* ssp. *pallidum* does not grow on artificial media. Two basic methods are applied in the routine diagnosis of syphilis – microscopy (exudates from an ulcer, erosion or punctuate obtained from a lymph node) using dark visual field and serology. Microscopy shows *Treponema pallidum* ssp. *pallidum* in all lesions of early and late, but not in latent syphilis.

In the human, infection with *Treponema pallidum* ssp. *pallidum* results in rapid production of two types of antibodies (non-specific and specific) at the end of the incubation period or during the first week after onset of *ulcus durum*. The serological methods for the diagnosis of syphilis are classified as non-specific (non-treponemal) and specific (treponemal) tests. In non-specific tests, a non-treponemal antigen called cardiolipin, which is extracted from bovine heart muscle is used. These are mostly flocculation tests, where the formed "antigen + antibody" complexes comprise of flakes. In Latvia the non-specific tests, mostly Rapid Plasma Reagin (RPR) test are used. Non-treponemal tests are widely used for screening, although they are not reliable alone to confirm the diagnosis of syphilis. According to the European guidelines, the non-treponemal tests are used for the monitoring of serologic activity and the treatment of syphilis[9].

Antibodies to specific antigens of *Treponema pallidum* ssp. *pallidum* in blood serum and/or plasma are detected with the specific tests. Commercially available test systems use Nichol's strain TpN15, TpN17 and TpN47 recombinant antigens and synthetic peptide TmpA. Other recombinant antigens like Tp0453, Tp92 and Gpd can also be used[20], although such test systems are not commercially available. In Latvia, TPHA (*Treponema pallidum* hemagglutination test) and ELISA (Enzyme-linked immunosorbent assay) are used recently. The above-mentioned specific treponemal tests are used to confirm

the diagnosis of syphilis and as differential diagnostics[9, 21].

Historically, to confirm the cure of syphilis, in addition to non-treponemal tests, the reaction of complement binding (Wasserman) reaction and *Treponema pallidum* immobilisation reaction (TPIR) or Nelson test, both being specific tests, were used. Nowadays these tests are not used routinely, although the TPIR is applied in specialised laboratories in Latvia. The diagnostic value of non-specific antibodies is limited: **firstly**, in early primary disease, antilipoidal antibodies may not be developed and in late syphilis (late latent and tertiary), up to 30 % of individuals there may be lack of antilipoidal antibodies[20] and secondly, non-treponemal tests are highly sensitive in secondary syphilis, but in other forms their sensitivity is not sufficient (**Table 1**)[10]. Therefore, it is essential to find out highly sensitive tests in monitoring the serological activity and the efficacy of treatment in cases of latent syphilis.

Table 1. **Sensitivities of serological tests for syphilis in different stages of disease ***

Test	Stage of syphilis			
	Primary	Secondary	Latent	Late
VDRL	78 (74-87)	100	95 (88-100)	71 (37-94)
RPR	86 (77-100)	100	98 (95-100)	73

*Values are percent sensitivities. Numbers in paranthesis represent ranges of sensitivities in studies at the Center for Disease Control and Prevention.
VDRL – Venereal disease research laboratory
RPR – Rapid plasma reagin

Studies of this type have several problems, the major one being lack of a "gold standard" for the direct detection of *Treponema pallidum* ssp. *pallidum*. Serological confirmation may be delayed, absent, or difficult to interpret the cases of potential reinfection or reactivation of disease[22]. This study was aimed to estimate and compare the sensitivity and specificity of RPR, TPHA and Syphilis ELISA IgG, IgM. A retrospective analysis of medical documentation was used as the reference.

METHODOLOGY

Study population

The retrospective study was conducted by analyzing the medical records of 18,799 patients (10,220 males and 8,579 females) of age ranges from 17 to 67 years (mean age 40.7 years) during a period of approximately 2 years from August 2006 to November 2007. These patients came to Outpatient Clinic of Ministry of Interior Clinical Centre of Sexually transmitted Diseases without any clinical symptoms and/or epidemiological data related to syphilis. They were screened to find out possible late latent syphilis cases using RPR, TPHA and ELISA IgG, IgM assays. The choice of the assay used for the screening was decided by clinician dermatovenerologist. The final late syphilis diagnosis was confirmed for 17 persons (8 males and 9 females). Demographic data of late latent syphilis patients are shown in **Figure 2**.

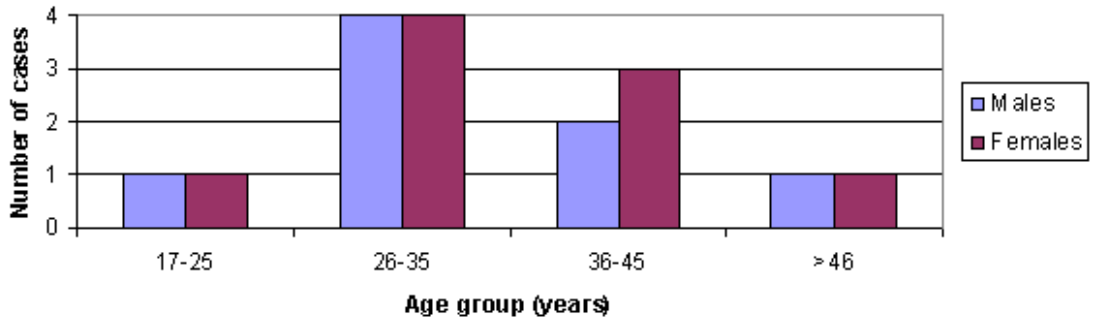

Figure 2: Demographic data of late latent syphilis patients

Statistical calculations

Diagnostic sensitivity is defined as:
SE = TP/(TP+FN) x 100 (where, SE – diagnostic sensitivity; TP – number of true positive test results; FN – number of false negative test results)
Test specificity is the percentage of negative results in the non-infected patients:

S = AN/(AN+FP) x 100 (where, S – test specificity; AN – number of actual negative test results; FP – number of false positive test results)

Results were identified as true positive and false negative in relation to the retrospective study of the participants' clinical records.

Techniques

The assay was performed strictly following manufacturer's instructions:

***RPR*:** This assay was performed in 15,829 patients. The commercially available cardiolipin antigen produced by "Biokit" (Spain) was used[21]. The assay was performed according to manufacturer's instructions.

***TPHA*:** It was performed in 1,474 patients. The test system "Cellognost Syphilis H" produced by "Dade Behring" (Germany) was used. Absorbed serum was added to sheep red blood cells sensitised with *Treponema pallidum* (Nichols strain) sonicate in a microtiter tray. Agglutination of the red cells was produced by antibody containing serum[21]. Test was considered as negative, if the agglutination was not stated. Test was positive, if hemagglutination occured, that was expressed semi-quantitatively in the interval of 1+ till 4+.

***SYPHILIS ELISA IgG, IgM*:** It was performed in 1,496 patients. The testsystem "Enzygnost" produced by "Dade Behring" (Germany) was used. The test was performed by incubating test specimens in microplate well coated with p15, p17 and p47 *Treponema pallidum* recombinant antigens. The specific antibodies IgG and IgM presented in sample bound to the solid-phase antigens. Subsequently, the wells were washed to remove residual test sample and p15, p17 and p47 *Treponema pallidum* recombinant antigens conjugated with the enzyme peroxidase were added. The conjugate bound to the captured specific antibodies. After another washing to eliminate unbound material, a solution of enzyme substrate and chromogen was added. This solution developed blue colour if the sample contained anti- *Treponema pallidum* antibodies. The blue colour changed to yellow after blocking the reaction with sulphuric acid. The intensity of colour was proportional to the anti- *Treponema pallidum* antibodies concentration in the sample[21].

RESULTS

The sensitivity of TPHA and ELISA IgG, IgM was much higher i.e. 100.0 % as compared to the sensitivity of RPR (58.8 %). As far as specificity is concerned, it was found to highest by using ELISA IgG, IgM method (100%). The specificity of TPHA and RPR was 66.7% and 33.3 % respectively.
The obtained results of sensitivity and specificity with RPR, TPHA and syphilis ELISA IgG, IgM methods are shown in **Figure 3**.

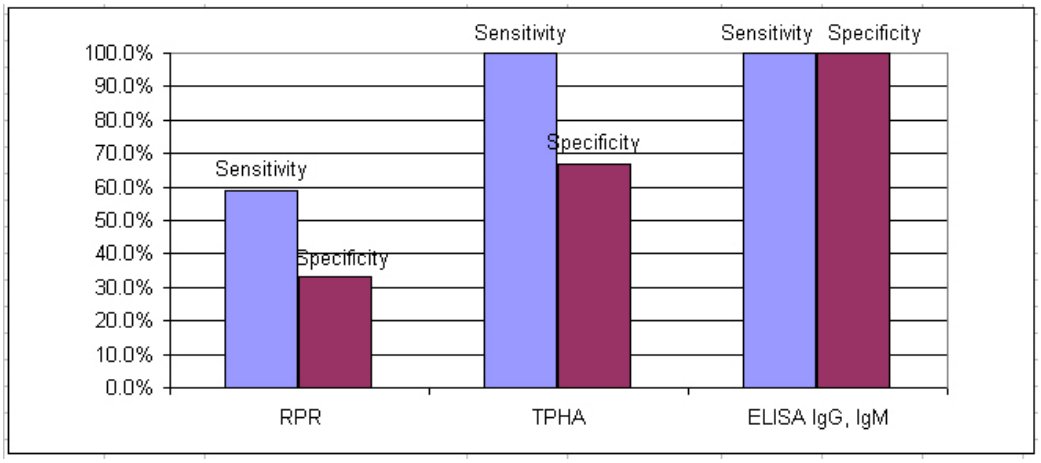

Figure 3: The obtained results of sensitivity and specificity with RPR, TPHA and Syphilis ELISA IgG, IgM methods

DISCUSSION

The sensitivity of non-treponemal tests to find out late latent syphilis patients in the population without clinical and epidemiological signs of syphilis is not high enough. Our result of sensitivity of RPR (58.8 %) agrees with the data of RPR (73%) presented in **Table 1**[10]. Non-treponemal tests used for screening have the advantage of being widely available, inexpensive, convenient to perform on large numbers of specimens, and necessary for determining the efficacy of treatment. Limitations of the nontreponemal serological tests include their lack of sensitivity in early dark-field-positive primary cases and in late syphilis due their possibility of a prozone reaction or false-positive results[10]. Therefore, RPR assay is not recommended for screening purposes to find out late latent syphilis cases due to its limitations and because of the availability of other more reliable syphilis screening tests.

TPHA and ELISA IgG, IgM methods could be recommended for screening diagnostics because they possess high sensitivity and ELISA IgG, IgM is reasonably used to confirm the diagnosis due its highest specificity (100%). Limitations of the ELISAs are time and cost when small numbers of samples are to be processed[10]. TPHS alone could not be used to confirm the diagnosis because of having low specificity (66.7%).

Despite the dramatic advances in other biomedical fields, the tools for the management and control of syphilis have changed little in the past sixty years[23]. New molecular tests for syphilis are unlikely to replace serology in the short term because they are fairly expensive and require sophisticated equipment[24]. Nevertheless, there are data concerning the importance of treating syphilis during pregnancy with penicillin, and the endorsement of the use of molecular techniques to identify *Treponema pallidum* subsp. *pallidum* in clinical samples to diagnose congenital early syphilis[25]. Also, a one-step sandwich chemoluminescence immunoassay (CLIA) is reported as a screening test and as a confirmatory test for the diagnosis of syphilis[26].

The use of recombinant *Treponema pallidum* ssp. *pallidum* antigens TpN47 (Tp0574), TpN17, TpN15 (Tp0171), TmpA, TpN44.5 (Tp0768), and TpN17 (Tp0435) to demonstrate seroreactivity has advantages over lipoidal antigen-based and crude *Treponema pallidum* ssp. *pallidum* tests, which may have up to 30 % of false negative results in individuals with early and late syphilis[20]. Regarding the results of the INNO-LIA™ Syphilis obtained at the Institut Alfred Fournier[27] and at OKCON[28] where 681 out of 683 syphilis-positive samples tested positive while 2 samples gave an indeterminate result, resulting in 100% sensitivity, including the indeterminate results. So, Syphilis Immunoblot IgG might be very effective for screening purposes to find late latent syphilis patients. However, this test is relatively expensive.

CONCLUSION

The sensitivity of non-treponemal tests for late latent syphilis patients is not high enough to monitor the treatment of syphilis. TPHA and ELISA IgG, IgM methods are useful for syphilis screening diagnostics because of having high sensitivity and ELISA IgG, IgM is recommended to confirm the diagnosis due to its highest specificity (100%).

REFERENCES

1. Bouis DA, Popova TG, Takashima A, et al. Dendritic cells phagocytose and are activated by Treponema pallidum. *Infect Immun*. 2001 Jan;69(1):518-28.
2. Haake DA. Spirochetal lipoproteins and pathogenesis. *Microbiology.* 2000 Jul;146(Pt7);1491-504.
3. Porcella SF, Schwan, TG. Borrelia burgdorferi and Treponema pallidum: a comparison of functional genomics, environmental adoptions and pathogenic mechanisms. *J Clin Invest*. 2001;107(6):651-6.
4. Woznicova V, Valisova Z. Performance of CAPTIA SelectSyph-G enzyme-linked immunosorbent assay in syphilis testing of a high-risk population: analysis of discordant results. *J Clin Microbiol.* 2007 Jun;45(6):1794-7.
5. Ozoliņš D, Hartmane I, Dērveniece A, et al. Diagnostics of syphilis: Evaluation of the enzyme-linked immunosorbent assay of IgG and IgM in comparison with other methods of serological diagnostics. *Proceedings of the Latvian Academy of Sciences.* 2006;60(4):127-32.
6. Brinkman MB, McKevitt M, McLoughlin M, et al. Reactivity of antibodies from syphilis patients to a protein array representing the *Treponema pallidum* proteome. *J Clin Microbiol.* 2006 Mar;44(3);888-91.
7. Rompalo AM, Joesoef M R, O'Donnell JA, et al. Clinical manifestations of early syphilis by HIV status and gender: results

of the syphilis and HIV study. *Sex Transm Dis*. 2001 Mar;28(3):158-65.
8. Gjestland T. The Oslo study of untreated syphilis; an epidemiologic investigation of the natural course of the syphilitic infection based upon a re-study of the Boeck-Bruusgaard material. *Acta Derm Venereol Suppl (Stockh)*. 1995;35(Suppl 34):3–368.
9. Goh BT, van Voorst Vader PC. European guideline for the management of syphilis. *Int J STD AIDS*. 2001 Oct;12 Suppl 3;14-26.
10. Larson SA, Steiner BM**,** Rudolph AH. Laboratory diagnosis and interpretation of tests for syphilis. *Clin Microbiol Rev*. 1995 Jan;8(1):1-21.
11. Walker EM, Arnett JK, Heath JD, et al. Treponema pallidum subsp. pallidum has a single, circular chromosome with a size of approximately 900 kilobase pairs. *Infect Immun*. 1991 Jul;59(7):2476-9.
12. Walker EM, Howell JK, You Y, et al. Physical map of the genome of Treponema pallidum subsp. pallidum (Nichols). *J Bacteriol*. 1995 Apr;177(7):1797-804.
13. Fraser CM, Norris SJ, Weinstock GM, et al. Complete genome sequence of Treponema pallidum, the syphilis spirochete. *Science.* 1998 Jul;281(5375):375-88.
14. Lafond RE, Lukehart SA. Biological Basis for Syphilis. *Clin Microbiol Rev.* 2006 Jan;19(1):29-49.
15. Girons IS, Chi B, Kuramitsu H. Development of shuttle vectors for spirochetes. *J Mol Microbiol Biotechnol*. 2000 Oct;2(4):443-5.
16. Eggers CH, Caimano MJ, Clawson ML, et al. Identification of loci critical for replication and compatibility of a Borrelia burgdorferi cp32 plasmid and use of a cp32-based shuttle vector for the expression of fluorescent reporters in the Lyme disease spirochete. *Mol Microbiol.* 2002 Jan;43(2):281-95.
17. Fenton K, A, Breban R, Vardavas R, et al. Infectious syphilis in high-income settings in the 21st century. *Lancet Infect Dis.* 2008 Apr;8(4):244-53.
18. Lien E, Sellati TJ, Yoshimura A, et al. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J Biol Chem*. 1999 Nov;274(47):33419-25.
19. Lee KH, Choi HJ, Lee MG, et al. Virulent Treponema pallidum 47 kDa antigen regulates the expression of cell adhesion molecules and binding of T-lymphocytes to cultured human dermal microvascular endothelial cells. *Yonsei Med J*. 2000 Oct;41(5):623-33.
20. Wesley CV, Barrett LK, Lukehart SA, et al. Serodiagnosis of syphilis: Antibodies to recombinant Tp0453, Tp92, and Gpd proteins are sensitive and specific indicators of infection by Treponema pallidum. *J Clin Microbiol*. 2003 Aug;41(8):3668-74.
21. Smith MB, Hayden RT, Persing DH, et al. Spirochete infections. In: *Clinical diagnosis and management by laboratory methods*. Henry J. B. Saunders company, Philadelphia, London, New York, St. Louis, Sydney, Toronto. 2001;1131-44.
22. Leslie DE, Azzato F, Karapanagiotidis T, et al. Development of a real-time PCR assay to detect Treponema pallidum in clinical specimens and assessment of assay's performance by comparison with serological testing. *J Clin Microbiol*. 2007 Jan;45(1):93-6.
23. Hook EW 3rd, Peeling RW. Syphilis control -- a continuing challenge. *N Engl J Med*. 2004 Jul;351(2)**;**122-4.
24. Muller I, Brade V, Hagedorn H. Is serological testing a reliable tool in laboratory diagnosis of syphilis? Meta-analysis of eight external quality control surveys performed by the german infection serology proficiency testing program. *J Clinl Microbiol*. 2006 Apr;44(4):1335-41.
25. Woznicova V, Valisova Z. Performance of CAPTIA SelectSyph-G enzyme-linked immunosorbent assay in syphilis testing of a high-risk population: analysis of discordant results. *J Clin Microbiol.* 2007 Jan;45 (6);1794-7.
26. Knight CS, Crum MA, Hardy RW. Evaluation of the LIAISON chemiluminescence immunoassay for diagnosis of syphilis. *Clin Vaccine Immunol.* 2007 Jun;14(6):710-3.
27. Ebel A, Vanneste L, Cardinaels M, et al. Validation of the INNO-LIA syphilis as a confirmation assay for Treponema pallidum antibodies. *Scand J Immunol*. 2000 Jan;38(1);215-9.
28. Hagedorn J, Vanneste L, De Bosshere K, et al. Validation of the interpretation algorithm of INNO-LIA™ Syphilis using well-characterized sera. *Innogenetics NV*, Gent, Belgium, Study Report code DR-SYP.