Review Article

Efforts in blood safety: Integrated approach for serological diagnosis of syphilis

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Abstract:

Recent efforts in transfusion medicine are focused on improving blood safety as well as establishing effective and efficient diagnostic algorithms for donor screening. To date, syphilis is a transfusion-transmitted infection re-emerged in many countries as a public health threat especially among populations at specific risk. This task requires new diagnostic tools and hemovigilance programs. The current diagnostic methodologies are debated, since presenting limitations and unresolved issues with special regard to the clinical interpretation of serological patterns, especially in asymptomatic patients and in blood donors. Furthermore, the switch from the traditional to alternative diagnostic algorithms underlines the lack of a gold standard, which has not been supported by shared guidelines. Besides, a lot of ongoing clinical trials on the performance of diagnostic assays, on the serological response associated with different pharmacological treatments, as well as on the prevention programs are currently under investigation. Here, we review the recent literature about the diagnosis of syphilis especially for low-risk populations proposing the adoption of an algorithm for blood donor screening that should satisfy the need of increasing safety for transfusion-transmitted infections in the modern blood transfusion centers.

Key words:

Blood donors, confirmatory testing, diagnostic tests syphilis, serology

Introduction

Syphilis is a re-emerging disease caused by the spirochete *Treponema pallidum*. The clinical manifestations of syphilis have been recognized for centuries and classified into four infectious stages: Primary syphilis, secondary syphilis (subdivided into early latent syphilis and late latent syphilis), and the noninfective tertiary syphilis.[1,2] The first stage is characterized by painless sores called chancre that disappear within 6 weeks. If the disease is untreated, the secondary stage appears within 10 weeks from the onset of the first chancre and includes fever, malaise, lymphadenopathy, loss of appetite, and maculopapular rash. The tertiary stage results in the spread of the spirochete to the nervous system, heart, and bone. Historically, the disease was imported in Europe from the New World at the end of the 15th century and reached an epidemic proportion after a few decades, spreading also to the rest of the world and becoming ubiquitous by the beginning of the 19th century [Figure 1].[3] During the last decades of the 20th century, a radical decline in its prevalence was obtained through the use of penicillin.[4] In spite of this, at the start of the 1990s, an increase in the incidence of primary and secondary syphilis was observed all over the world.[5] This new epidemic peaked first among men having sex with men and bisexuals, but subsequently spread to the heterosexual population as well.[6] In Europe, surveillance data are available from most countries and periodical updates are issued: Recently, the European Centers for Disease Control reported a remarkable increase of syphilis cases.[7] According to this trend, in Italy, data by the National Institute of Health showed an eightfold increase of primary and secondary syphilis cases from 1996 to 2008.[8] Furthermore, the reappearance of syphilis has been ascribed to HIV and a new emphasis has been put on the need to promote syphilis awareness and screening in those patients.[9]

Both control and surveillance of syphilis require an accurate sexual risk anamnesis, a correct interpretation of clinical manifestations as well as the application of serological tests, based on reliable methods. Since laboratory tests are not equally sensitive and specific, their rational choice plays a crucial role for a correct diagnosis

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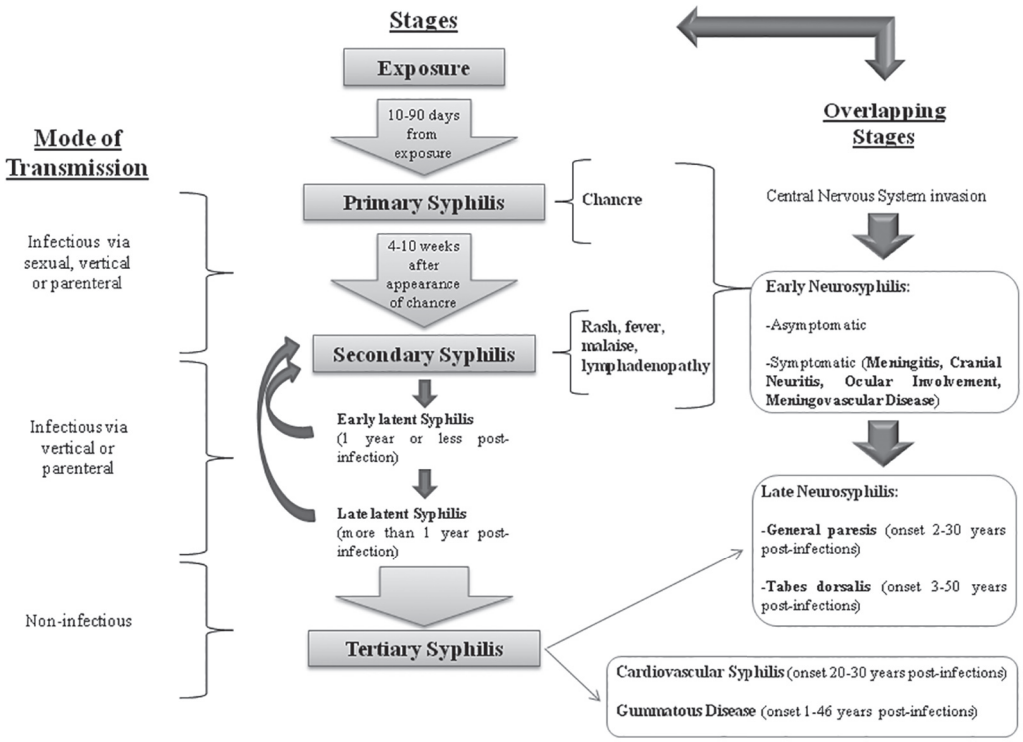


Figure 1: The natural history of untreated syphilis in immunocompetent individuals (based on data from Golden *et al*., 2003, ref#1)

and a proper management of the patient. To date, there is not a generally recognized diagnostic algorithm. Since 1982, the World Health Organization (WHO) has been recommending both nontreponemal and treponemal tests for syphilis serological screening and diagnosis.[4]

Here, we provide a critical update of syphilis diagnosis through an overview of current available serological algorithms applicable especially in a low-prevalence population (blood donors) as well as in emerging categories (HIV and immunocompromised patients). Besides, the reported ongoing clinical trials emphasize the need of recognized diagnostic protocols and novel prevention programs highlighting the renovated interest in this topic.

Diagnostic Approaches

There is no an uniform screening method for syphilis. The diagnostic processes are based on direct examination in the early stage of syphilis when a lesion is present coupled to indirect treponemal and nontreponemal serological tests.

Direct identification tests: An early detection of syphilis is still a major clinical challenge. Since *T. pallidum* is a noncultivable bacterium, the diagnosis of syphilis is based on direct identification of the pathogen in the lesion and the identification of a specific immunological response. Dark-field microscopy and/or polymerase chain reaction (PCR)[10,11] are useful in acute primary infection when spirochete can be detected directly. In particular, dark-field microscopy allows an immediate diagnosis of syphilis with a prompt start and a follow-up of the therapy. A principal limitation of this technique consists of the requirement for a great experience of each operators; moreover, the presence of nonpathogenic spirochetes can limit its use. Recently, PCR appears quite promising, but its routine use cannot yet be proposed.[10,11] It is known that molecular tests for syphilis are too expensive for many clinical laboratories and cannot replace the serology. Besides, they are not suitable for blood donors.[12,13]

Serological tests: Serological tests are still considered the most useful approach for the diagnosis.[12,14,15] The serological diagnosis is based on the detection of two distinct antibodies, the nontreponemal antibody (reagin), which binds to cardiolipin released from damaged host cells and the treponemal antibody directed against specific antigens.

The nontreponemal tests are rapid plasma reagin (RPR) and venereal disease research laboratory test (VDRL), derived from the first available laboratory test, the Wassermann reaction for cardiolipin. These tests are cheap and simple to perform and have a sensitivity of approximately 70-85%, which approaches 100% only in the secondary stage when, the infection is still active. Since RPR/VDRL takes 30 min, it can be performed in emergency departments and it is particularly suited for patients with a strong clinical suspicion of syphilis.[16] Nontreponemal antibodies become detectable in the early infection (7-10 days after the appearance of the primary lesion) or a few weeks after the infection. They are indicative of active infection and important for monitoring treatment; indeed, a reduction of their titer shows the efficacy of the antibiotic treatment while an increase shows a relapse or re-infection.[17]

When the incidence and prevalence of syphilis in blood donors appear elevated, it might be necessary to consider the use of a nontreponemal assay to identify those donors with the evidence of recent infections. However, one of the major disadvantages of

nontreponemal tests are the biological false-positive reactions since nontreponemal antibodies can also be present in other diseases such as other spirochetal infections, mononucleosis, varicella, measles, malaria, leprosy, connective tissue diseases such as systemic lupus erythematosus and malignancy.[1,17,18]

Since nontreponemal tests are not-specific, treponemal specific assays have been developed and improved. Treponemal tests use native or recombinant *T. pallidum* antigens and allow the detection of specific anti-treponemal antibodies; anti-treponemal IgM are detectable within approximately 2 weeks postinfection, while anti-treponemal IgG appear at about 4 weeks after the postinfection.[19] Anti-treponemal IgM and nontreponemal antibodies decline following treatment of early syphilis, while anti-treponemal IgG antibodies persist longer and are usually detectable for many years after the disease has been thought to be eradicated.[19,20] The treponemal tests evaluate the antibody reactivity against specific *T. pallidum* antigens and are based on different agglutination reactions: *Treponema pallidum* hemagglutination assay (TPHA) uses red blood cells, and the *Treponema pallidum* particle agglutination assay (TPPA), or the microhemagglutination assay for *T. pallidum* use gelatin particles. Higher titers of these tests are correlated to an active infection while they decrease in the latent phase. Clinically, TPHA reactivity may be detectable around the 4th week of infection with an overall sensitivity in the untreated primary stage in the 70-80% range by increasing to about 100% in the secondary stage. TPPA is generally superior to TPHA for the detection of primary syphilis.[21]

Treponemal assays meet the requirements for use in blood center and contribute importantly to optimizing workflow and efficiency; on the other hand, they are technically difficult to perform and more expensive than nontreponemal tests and false positive reactions can occur.[16,18]

Automated immunoassay: In the last decades, a number of highly sensitive and specific enzyme immunoassays (EIAs) for syphilis testing have become available as appropriate alternative to the combined RPR/VDRL and TPHA. EIAs have been often chosen for syphilis screening because they are particularly well-suited for automation.[22-28] The first treponemal EIA was initially approved for blood bank screening in the USA during the 1980s, and later on, it was approved for clinical diagnostic use by the Food and Drug Administration in 2001. A survey conducted in the USA showed that treponemal EIAs or chemiluminescent immunoassays (CIAs) tests increased from 0 in 2001 to over 3,90,000 performed in 2007, with a concomitant decrease in total RPRs and VDRLs performed from approximately 2.9-1.9 million. Furthermore, the most recent generation of automated immunoassays appear to be more sensitive (95-99%) and specific (98-99%) than the first generations of these assays.[29] Some of these recent assays can simultaneously detect syphilis IgG and IgM,[24,30] thus shortening the seronegative window phase following infection.

In the search for a possible confirmation strategy for an initial positive screening result, the fluorescent antibody absorption test (FTA-Abs) has also been employed for several years as a confirmatory assay.[31,32] FTA-Abs is technically more complex than the agglutination assays. This treponemal test performs quite well when used for sera found to be positive on screening, less well to confirm the presence of negative sera; indeed, false negative results have been reported in HIV infection.[24] Due to all these reasons and after reevaluation of assay performances,[14] FTA-Abs is not recommended anymore for syphilis confirmation[7,29] although some laboratories decided to continue its use. In the United States, Pope *et al*.,[27] reported that TPPA was an appropriate substitute for the TPHA as a confirmatory assay. Another report found that TPPA was significantly more sensitive than FTA-Abs and TPHA[21] making TPPA very suitable as a confirmatory test.

Recently, immunoblots for specific treponemal antibodies, as well as for reagin-directed antibodies, have gained importance.[33] Treponemal Western blot assays have proved a valid alternative choice to the FTA-Abs because of their high sensitivity and specificity together with their simplicity.[34] In addition, the LIA syphilis score immunoblot assay, which uses recombinant and synthetic polypeptide *T. pallidum* antigens (Fujirebio), has been evaluated satisfactory as a confirmatory testing for syphilis.[35-37]

A major breakthrough in syphilis serology has been allowed by the sequencing of the complete genome of *T. pallidum* and the subsequent recognition of some major protein antigens *Treponema. pallidum* proteins-15 (TpN15), (TpN17, TpN47, TpN44) which have strong immunogenicity and thus, considered important candidate targets for the serological diagnosis of syphilis.[17,30,38-42] The serological response to these antigens might be related to the different phases of the infection. Antibodies directed against TpN47 are usually present in phases of the disease, anti-TpN17 is more frequently observed in patients with early syphilis (primary, secondary, and early latent), while patients with other clinical forms of disease show reactivity against TpN15. Furthermore, in tertiary syphilis the reactivity of TpN15 is reported to be stronger than that of TpN47.[43,44] Recently, new specific chimeric antigens have been described that may enhance the diagnostic accuracy of syphilis.[45] Fully automated treponemal assays employ a combination of recombinant antigens on the solid phase and this feature has contributed to their enhanced sensitivity due to the selection of immune-dominant epitopes coupled with a higher specificity in comparison with previous EIAs employing spirochetal lysates.

The use of one treponemal test for screening purpose is not without limitations which include the potential risk for false-negative and false-positive results.[23,27,28] The use of a nontreponemal assay for routine screening is not suitable for high-volume testing and brings the risk of an elevated number of false negative due to its low sensitivity compared to specific treponemal tests even when the infection is recent[28,46] or due to the prozone phenomenon.[47,48] Besides, false-positive reactions, which almost certainly occur with nontreponemal tests and with treponemal EIAs, create clinical management dilemmas (e.g., other infections)[1,17,18] that prompt either to repeat the test or to an unnecessary treatment. This problem will be greatest in routine screening of low-risk populations, such as blood donors.[25,46,49] In addition, it should also be considered that the interpretation of manual method results such as the RPR/VDRL and TPHA/TPPA assays can vary significantly among different laboratories and operators. However, all these methods require confirmatory testing with a different treponemal assay almost with a similar sensitivity and with a greater specificity compared to that used for the first screening.[15]

Many syphilis rapid point-of-care (POC) tests have been extended in the last 20 years. Their use is fundamental in the WHO strategy (syphilis control programs) for reducing syphilis especially in countries where high rates of syphilis and HIV co-infections are observed.[9,50] At first, POC tests detected only the presence of treponemal antibodies presenting low sensitivity compared to traditional methods, even if most recent, they have improved the sensitivity.[51,52] Moreover, dual rapid tests for treponemal antibodies and reagin have been developed[53] although they cannot distinguish among active, historical, or treated cases.[54] Syphilis POC rapid tests have also been coupled with tests for other sexually transmitted infections as HIV.[55] These multi-infection tests have showed a good sensitivity and specificity for HIV and for treponemal antibodies with a poor sensitivity for nontreponemal antibodies making it difficult to discriminate between an active case and a treated case of syphilis. For this reason, rapid POC tests are suitable in resource-limited settings and are not useful for the screening.

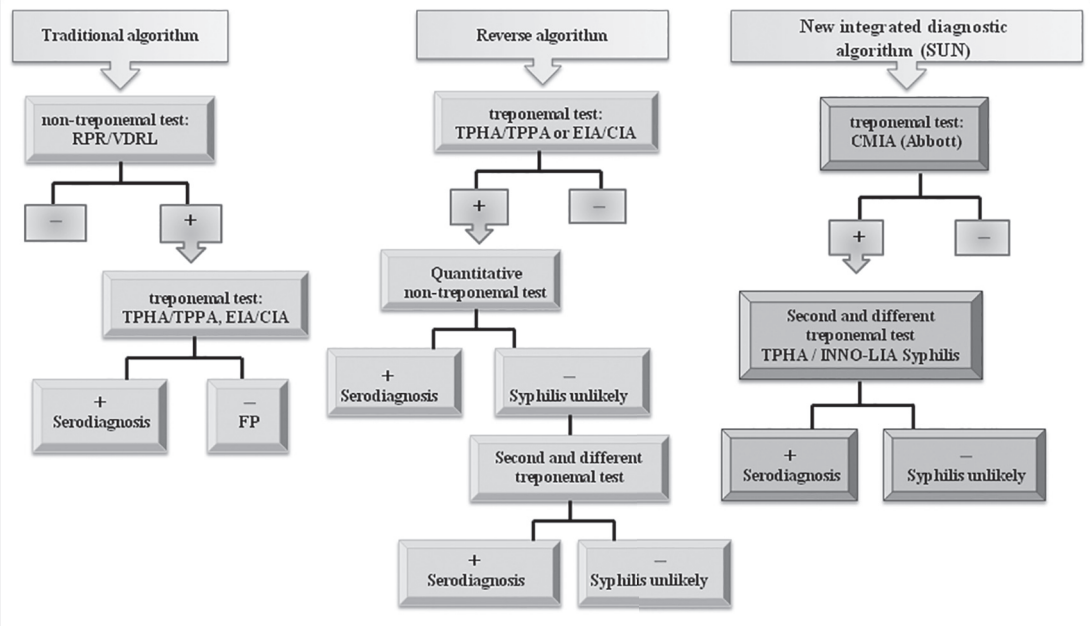
Testing Guidelines and Algorithms

The different guidelines recognize that there are a number of available tests with different performance characteristics.[21,28,46,44,49,56] The Centers for Diseases Control (CDC) recommend serologic screening with an inexpensive nontreponemal test to identify subjects with an untreated active infection followed by a more specific treponemal test to confirm syphilis infection in positive patients.[57] Syphilis guidelines in the United Kingdom recommend screening with either EIAs when early primary syphilis is suspected or a combination of VDRL and TPHA tests in other cases.[15,21] Other European guidelines for syphilis recommend either EIA or TPPA as a screening test.[58] Furthermore, in Italy and in many other countries, blood transfusion services are forced by law to screen for syphilis.

Due to the high variability of syphilis antibodies, the results of a single test are not always sufficient for an accurate screening, and there is no evidence on which algorithm should be preferred. No standardized protocols and approved screening or confirmatory testing of an initial reactivity have been established yet for this setting.[59] This contributes to generate high discrepancies both in clinical laboratories and in blood transfusion centers stressing the need to generate an efficient algorithm.[60]

Two possible algorithms for serological screening have been proposed: The traditional algorithm and the reverse algorithm, as reported in Figure 2. The traditional algorithm recommends the use of nontreponemal tests such as RPR/VDRL followed by treponemal agglutination assays or EIA/CIA as confirmatory treponemal testing. While this algorithm is suitable for the diagnosis of active syphilis, it does not allow identifying past infections and thus, is not useful for donor screening and for specialized settings such as blood centers where high sensitivity for all stages of *T. pallidum* infection is required. The reverse algorithm starts with treponemal tests such as TPHA/TPPA or EIA/CIA followed by a quantitative nontreponemal assay on positive samples.[23,25,61] If the latter results are negative or discordant (e.g., EIA reactive, RPR nonreactive) it would be necessary to perform a third test with a different treponemal assay [Figure 2].[56,62] The reverse algorithm detects active, latent, and past syphilis, and then it could be more useful for donor screening and population surveys.

As recommended by CDC,[5] the protocol for syphilis screening should include not only the utilization of two different treponemal assays with the same sensitivity, but also a successive step with another sensitive treponemal test in case of discordant results. Taking this into account, our Immunohematology Laboratory at the Second University of Naples has adopted an European Centre for Disease Prevention and Control (ECDC) modified algorithm,[63] considering a treponemal test, such as the chemiluminescent



RPR, rapid plasma reagin; VDRL, Veneral Disease Research Laboratory; TPHA, Treponema pallidum Haemoagglutination Assay; TPPA, Treponema pallidum particle agglutination; EIA, enzyme immunoassay; CIA, chemiluminescenze immunoassay; CMIA, Chemiluminescent Microparticle Immunoassay; FP, false positive., SUN: Second University of Naples.

Figure 2: The actual testing algorithms for diagnosis of syphilis (modified from Tong *et al*., 2014, ref #63)

Table 1: Ongoing clinical studies on syphilis from ClinicalTrials.gov.

Trial Registration Number	Study Type	Enrollment	Partecipant Group	Condition	Purpose	Study Phase	Recruitment Status
NCT00300534	Interventional	1000 Patients and Controls	Both sex 18 years and older	Syphilis	Diagnosis of syphilis by an investigational immune-chromatographic strip (ICS) test compared with the Abbott Laboratories Determine ICS test.	Not Provided	Completed
NCT01317784	Interventional Randomized	1200 Volunteers	Both sex 18 years and older	HIV Hepatitis C Hepatitis B Syphilis	Diagnosis of syphilis by rapid tests.	Not Provided	Currently recruiting
NCT01530672	Interventional	2900 Healthy Volunteers	Female	HIV Syphilis	Diagnostic validation study for a combined Syphilis/HIV test by MBio Diagnostics, Inc.	Not Provided	Active not recruiting
NCT00732355	Observational Prospective	600 Patients and Controls	Both sex 18 years and older	Syphilis	Diagnosis of syphilis to evaluate the performance of a rapid membrane test comparing the results to currently licensed laboratory tests.	//	Unknown
NCT01543295	Observational Prospective	915 Participants	Both sex 13 years and older	Syphilis	Diagnosis of syphilis to evaluate the performance of the Chembio Diagnostics Systems, Inc. Syphilis Screen and confirm rapid test.	//	Suspended
NCT02191527	Interventional	600 Participants	Female 18 years and older	HIV Syphilis	To evaluate the cost-effectiveness of Point-of-care Diagnostic Technologies for syphilis.	Not Provided	Completed
NCT02262390	Interventional	1752 Patients	Both sex 14 years and older	Syphilis	This project will recruit pregnant women and partner with a sexually transmitted infection with minimal risk and with the potential to prevent future re-infection.	Not Provided	Not yet recruiting
NCT02019043	Interventional Randomized	3131 Patients	Male 16 years and older	Syphilis	Diagnosis of syphilis to enhance syphilis testing among HIV-positive men.	Not Provided	Not yet recruiting
NCT02257658	Interventional Randomized	30 Patients	Male 18 years and older	Syphilis	To investigate the feasibility of conducting a large, randomized trial comparing a structural intervention to contingency management to reduce incident syphilis infections by doxycycline in an especially high risk group: HIV+ men who have sex with men who have had syphilis twice or more since their HIV diagnosis.	Not Provided	Completed
NCT02059525	Observational Prospective	150 Patients	Both sex 18 years and older	Syphilis	To study the proteomic, immunological, serological and clinical changes associated with pre-and post-treatment syphilis.	//	Currently recruiting
NCT01944358	Observational Prospective	1128 Patients	Both sex 20 years and older	Syphilis Early Syphilis HIV	To compare serologic response of syphilis to penicillin treatment between HIV-infected and HIV-uninfected patients and serologic response of early syphilis to benzathine penicillin G among HIV-infected patients.	//	Completed
NCT01540227	Observational Prospective	120 Patients	Both sex 18 years and older	Primary, Secondary, Early-Latent Syphilis	To determine if current dose of penicillin recommended to treat syphilis is sufficient and if treatment failures differ between HIV-positive and HIV-negative individuals.	//	Enrolling participants by invitation only
NCT00031499	Interventional Randomized	593 Patients	Both sex 18 to 55 years old	Syphilis	To determine if azithromycin is as effective for syphilis.	Phase 3	Completed
NCT00000648	Interventional	100 Patients	Both sex 13 years and older	HIV Neurosyphilis	To determinate if penicillin G and ceftriaxone are effective for presumed neurosyphilis in HIV.	Not Provided	Completed
NCT01382004	Interventional Randomized	255 Patients	Both sex 6 months to 15 years	Yaws Treponema Infection Neglected Tropical Disease	To determinate if azithromycin treatment is effective with a decline in the VDRL titer of at least two dilutions by six months after treatment and in primary yaws also by epithelialization of ulcers within two weeks.	Phase 3	Completed

(Continued)

Table 1: ***(Continued)***

Trial Registration Number	Study Type	Enrollment	Partecipant Group	Condition	Purpose	Study Phase	Recruitment Status
NCT00207506	Interventional Randomized	354 Participants	Male 18 years and older	Syphilis Gonorrhea Chlamydia HIV	Prevention on sexually transmitted disease and model health behavior.	Phase 1 Phase 2	Completed
NCT00553111	Interventional Randomized	168 Healthy Volunteers	Male Age: not provided	Syphilis	Prevention by promoting knowledge of syphilis and in encouraging syphilis and HIV diagnosis testing.	Not Provided	Completed
NCT00552539	Interventional Randomized	220 Healthy Volunteers	Male 18 to 55 years old	Syphilis	Prevention by promoting knowledge of syphilis.	Not Provided	Completed
NCT01465607	Interventional Randomized	1080 Participants	Female 18 years and older	HIV Syphilis Gonorrhea Chlamydia	Implementation of effective programs for reducing the spread of HIV and other sexually transmitted infections.	Not Provided	Currently recruiting
NCT01315054	Interventional Randomized	7700 Participants	Both sex 18 to 55 years old	HIV Hepatitis C Syphilis Herpes Simplex Type II	To evaluate the effectiveness of a tailored education program for methadone maintenance treatment service providers using subsequent methadone dose prescribed to new patients.	Not Provided	Active, not recruiting
NCT02122094	Interventional	1500 Participants	Males 16 to 29 years old	HIV Chlamydia Gonorrhea Syphilis HCV	To implement a sexual health promotion intervention.	Not Provided	Currently recruiting
NCT00362791	Interventional Randomized	6000 Participants	Both sex 14 years and older	HIV Sexually Transmitted Diseases	Prevention by self-reported condom use and new diagnoses of Sexually Transmitted Diseases (gonorrhea, Chlamydia, syphilis, HIV) defined by laboratory tests.	Not Provided	Completed
NCT01465607	Interventional Randomized	1080 Participants	Female 18 years and older	HIV Syphilis Gonorrhea Chlamydia	To reduce the spread of HIV and other sexually transmitted infections.	Not Provided	Currently recruiting

microparticle immunoassay, for screening followed directly by two different treponemal assays (TPHA and INNO-LIA immunoblot assay) as confirmatory test [Figure 2]. (Sommese *et al*.,[64] syphilis detection: Evaluation of serological screening and confirmatory assays in blood donors, submitted). This procedure has resulted to be more suitable and more sensitive than TPHA. A potential drawback of the use of an immunoblot for confirmation of a positive screening result and the resolution of discrepancies among different treponemal assays is the occurrence of "indeterminate" results (i.e., reactivity patterns that do not fulfill the criteria for a confirmed positivity, according to the manufacturers' recommendations) as also reported in literature.[51,65-70] Nevertheless, the reverse screening algorithm presents several significant benefits. It allows to achieve a higher sensitivity for all stages of *T. pallidum* infection including resolved cases with a consequent reduction of false positive results among blood donors; furthermore, it provides a more objective interpretation of screening results.[6,43]

It is impossible to conclude if the reverse algorithm causes a higher number of false results than traditional since RPR screening is not provided in the reverse algorithm. Different evidence and specific clinical settings that may favor the traditional or the reverse algorithm have been nicely summarized in a recent point-counterpoint discussion.[71] Some factors support the persistent use of a traditional algorithm, particularly in small clinical laboratories, to consent a more rapid screening assay without expensive instrumentations and clinicians should be vigilant that alternative testing algorithms exist. Nevertheless, since the screening tests of syphilis cannot distinguish between treated and untreated disease, the anamnesis of the patient will continue to be crucial for a correct diagnosis and for a blood safety.[25]

Ongoing Clinical Trials

The relevance of continuous interest on syphilis is well documented by several ongoing clinical trials, as reported in Table 1. We searched on the web for studies and clinical trials selected from the USA National Institutes of Health using the following keywords (also combining them): Syphilis, diagnosis, and sexually transmitted diseases. We got back 18 interventional and five observational studies. Some of these studies considered patients with also other sexually transmitted infections as HIV [Table 1]. We found eight studies on the performance and relevance of diagnostic tests; seven investigations on the serological response associated to different pharmacological treatments also in the presence of co-infections, and eight clinical trials on the implementation of prevention programs. All these ongoing projects are aimed to control the real impact of this ancient disease.

Conclusions

While a consolidated practice and clinical guidelines are available for symptomatic cases, the screening for asymptomatic infections is still a challenge. Many issues remain to be investigated to definitively establish the gold standard for diagnostic algorithm. Nevertheless, serologic testing remains the principal tool for syphilis diagnosis. Indeed, the most recent evidence suggests that an algorithm based on the ECDC model, as performed in our center, could guarantee an adequate sensitivity and a good overall accuracy and may then be adopted for blood donor screening.

By considering the most recent studies, the diagnosis and the monitoring of this infection are still intensely examined especially in patients with HIV considering their increased risk of severe complications.[72,73] Therefore, further researches need to be performed and the ongoing clinical trials could shed light on unresolved issues.

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Conflicts of interest

There are no conflicts of interest.

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