Syphilis, caused by the bacterium Treponema pallidum subsp. pallidum, is an infection recognized since antiquity. It was first reported at the end of the 15th century in Europe. Infections may be sexually transmitted as well as spread from an infected mother to her fetus or through blood transfusions. The laboratory diagnosis of syphilis infection is complex. Because this organism cannot be cultured, serology is used as the principal diagnostic method. Some of the issues related to serological diagnoses are that antibodies take time to appear after infection, and serology screening tests require several secondary confirmatory tests that can produce complex results needing interpretation by experts in the field. Traditionally, syphilis screening was performed using either rapid plasma reagin or Venereal Disease Research Laboratory tests, and confirmed by treponemal tests such as MHA-TP, TPPA or FTA-Abs. Currently, that trend is reversed, ie, most of the laboratories in Canada now screen for syphilis using treponemal enzyme immunoassays and confirm the status of infection using rapid plasma reagin or Venereal Disease Research Laboratory tests; this approach is often referred to as the reverse algorithm. This chapter reviews guidelines for specimen types and sample collection, treponemal and non-treponemal tests utilized in Canada, the current status of serological tests for syphilis in Canada, the complexity of serological diagnosis of syphilis infection and serological testing algorithms. Both traditional and reverse sequence algorithms are recommended and the algorithm used should be based on a combination of local disease epidemiology, test volumes, performance of the proposed assays and available resources.

**Key Words**: Enzyme immunoassay; Reverse algorithm; RPR; Serological diagnosis; Syphilis; Treponema pallidum

**INTRODUCTION**

There is no single serological test that can be performed on a single blood specimen that can tell you if that patient has active syphilis or not. Serological tests are divided into those which detect non-treponemal antibodies (or reags) and those which detect treponemal antibodies. Currently only two non-treponemal antibody assays have updated their licensing status for diagnostic testing by Health Canada. These include one Venereal Disease Research Laboratory (VDRL) test and one rapid plasma reagin (RPR) product. A larger number of treponemal antibody assays have been licensed. Several EIA/CIA assays were licensed, some capable of detecting immunoglobulin (Ig) G antibodies only and others capable of detecting both IgG and IgM antibodies. In addition, there are four immunoassays licensed for...
use on automated analyzers. A single fluorescent treponemal antibody-absorption (FTA-ABS) assay and a single Treponema pallidum particle agglutination (TP-PA) assay have been licensed for use in Canada.

The confirmation of infection with syphilis is often based on the presence of syphilis-specific antibody in a serum or plasma sample detected by treponemal assays. However, a positive treponemal test result does not differentiate between active and past infections. A person with active infection may have both non-treponemal reagents and treponemal antibodies in the blood, while patients with adequately treated past infection may still have a positive treponemal test result but their non-treponemal test findings will be negative or low titre (1:1 or 1:2). Traditionally, patients with suspected syphilis are screened with a non-treponemal test and any positive results are confirmed by a treponemal test. However, with the advent of newer automated treponemal tests, some laboratories have modified their diagnostic algorithm to a reverse algorithm of screening with a treponemal test (1). This change in diagnostic algorithms for the serological diagnosis of syphilis has created challenges that will be discussed in the present review, together with an overview of the types of treponemal and non-treponemal tests commonly employed in the diagnostic laboratories in Canada. Based on a literature review of the common current practice and the experience of the Canadian Public Health Laboratory Network’s (CPHLN) national task group on laboratory diagnosis of syphilis, suggested guidelines on how to follow the two diagnostic algorithms have been developed and the pros and cons of each algorithm will be discussed in this section.

GUIDELINES FOR SPECIMEN

TYPES AND SAMPLE COLLECTION

In the early 1960s, plasma was the sample of choice as screening tests for syphilis were designed to be performed in the field, without access to laboratory equipment (2). However, the poor specificity of this assay and development of better screening assays have resulted in serum being used almost exclusively. Venous blood should be allowed to clot adequately in a clean tube without anti-coagulant before the serum is being used almost exclusively. Venous blood should be allowed to clot adequately in a clean tube without anti-coagulant before the serum is separated and removed because samples not completely clotted may contain fibrogen or fibrin strands that may interfere with the assays, especially those based on flocculation (3).

Cord blood, once regarded as a useful sample for the detection of congenital syphilis, is now considered a suboptimal specimen type. Whatton’s jelly, comprised of mucopolysaccharides, is found in the umbilical cord and can cause false-positive non-treponemal results (4). The issue associated with cord blood, which applies to all serological tests, is the possibility of contamination with the maternal blood, which is, by far, a more probable source of false positivity.

TYPES OF SEROLOGICAL TESTS

Non-treponemal tests

Non-treponemal tests detect antibodies (reagins) that react with lipoidal particles containing cardiolipin (5). Historically, the antigen was obtained by Wasserman from the liver of an infant who had died of congenital syphilis, and used in the complement fixation test. However, it was quickly realized that the antibodies were cross-reactive and an alcohol extract from bovine heart was equally suitable for this purpose (6). The identification of the phospholipid cardiolipin as the active antigenic component led to the development of standardized antigens containing cardiolipin, cholesterol and lecithin (7,8).

The VDRL test (8) is a flocculation test developed using the standardized antigen preparation, and remains in use today. The antigen was further modified by the addition of chloride and EDTA to produce the unheated serum regain test (USR), in which either plasma or unheated serum was an acceptable sample matrix (9).

Other non-treponemal tests were developed, including the RPR test and the toluidine red unheated serum test (TRUST). In the RPR test, the antigen suspension incorporates charcoal particles to enhance flocculation (10), while in the TRUST the carbon particles were replaced with toluidine red particles (11).

All non-treponemal tests have similar performance characteristics. They detect both IgM and IgG antibodies (Figure 1). The sensitivity of non-treponemal tests during primary syphilis is approximately 75% (12). All non-treponemal tests in current use are flocculation tests, in which the reaction between the antigen and reagin is evidenced by clumping of particles. Interpretation of flocculation tests is subjective and, therefore, depends on staff experience, with a minimum of ±1 dilution margin of error associated with these types of tests.

The non-treponemal tests currently licensed and available in Canada are the RPR and the VDRL. The VDRL is now used primarily for testing CSF (6).

Non-treponemal tests are used as qualitative assays for screening in the traditional algorithm or as quantitative assays to assess the response to treatment. Screening tests are performed using undiluted serum. Results are reported as non-reactive or reactive. Some specimens give a granular or ‘rough’ appearance. A prozone phenomenon occurs in high-titre specimens, which appear as non-reactive unless the samples are diluted (13). Reactive specimens are re-tested in a two-fold dilution series for quantitative results. Titres usually decline significantly after successful therapy (14). Treated patients should be followed for up to 24 months (15); this prolonged follow-up is necessary to ensure titres decline to the expected extent.

False-positive reactions occur with non-treponemal tests (16). These are categorized as either acute (occurring for <6 months) or chronic. The recognized causes of acute false-positive reactions include other febrile illnesses, immunizations and pregnancy (17). Patients with acute false-positive reactions should be re-tested in three to six months. Chronic false-positive reactions are associated with hepatitis C infection (1), connective tissue diseases, intravenous drug use, malignancy, older age, malaria, Chagas disease, tuberculosis and leprosy (6).

Treponemal tests

The first treponemal tests were developed >60 years ago, using either live or killed cells of the Nichols strain of T. pallidum (6,18). Treponemal tests detect both IgM and IgG antibodies. The antibodies detected by treponemal assays appear up to a few weeks earlier than those detected by non-treponemal tests.

The tests that remain available today include the T. pallidum hemagglutination assay (TPHA), the T. pallidum particle agglutination assay (TPPA), and the FTA-ABS (19,20). These early treponemal assays utilized whole cells, or antigens derived from cells of T. pallidum.

The TPHA is an indirect immunofluorescent staining assay, in which fixed cells of the Nichols strain of T. pallidum are exposed to the test serum after they have been absorbed with a sorbent, which is an extract from a non-pathogenic T. phagedenis strain sometimes referred to as the Reiter strain. After washing to remove unbound antibody, the reaction mixtures are incubated with fluorescein-conjugated anti-human globulin. In a positive test, the presence of antibodies to T. pallidum in the serum specimen is indicated by the appearance of fluorescent spirochetes when viewed with a fluorescence microscope. The FTA-ABS is slightly more sensitive than either the TPHA or the TPPA, and is usually the first serological test to become reactive, during the primary stage of the disease (6). However, the FTA-ABS is subjective and occasionally gives false-positive (non-specific fluorescence) readings.

The TPHA and TPPA are indirect agglutination tests in which surface antigens extracted from T. pallidum cells are coated onto red cells (TPHA) or gelatin particles (TPPA) and mixed with the test serum. Serum containing specific T. pallidum antibodies will react with the antigen-sensitized red cells or particles, causing agglutination.

In recent years, new treponemal tests have been developed that utilize recombinant antigens derived from T. pallidum. This approach may allow greater specificity and sensitivity and also promotes standardization (see Table 1 for a list of recombinant treponemal tests available in Canada). Recombinant treponemal assays suitable for screening are available in EIA/CIA format (for IgG, for IgM, and for total immunoglobulins), in chemiluminescence assay format and in a multiplex bead immunoassay format. Table 1 shows the available assays.
The chemiluminescence and multiplex bead immunoassays are capable of high throughput and even random-access testing, but each is only capable of running on the manufacturer's platform. Limited data suggest that the performance of these assays for screening is very similar (21-23).

Accumulating anecdotal experience with treponemal assays as screening tests suggests that these assays generate a small proportion of positive results, which cannot be explained by previous infections. Because these tests have not been applied as tests for screening populations before, it should not be a surprise that apparently false-positive results occur in a small proportion of the population. Among the causes of false-positive treponemal tests are other spirochetal infections, such as borrelial infections, in addition to the causes of false-positive results in non-treponemal tests. Further studies will be necessary to quantify the rate of false-positive results, although considering their higher analytical sensitivity and also in the absence of a gold standard, it is difficult to prove that the results obtained using these new platforms are necessarily false positive, hence demanding a thorough search into the patient's medical and social history, and to perform risk assessment by the health care provider.

A number of immunoblot assays are available, but none are currently licensed by Health Canada. A potential application of these assays is to reconcile discordant results because they allow the users to visualize the reacting antigens (24-26).

ALGORITHMS FOR SEROLOGICAL DIAGNOSIS

Currently there are two commonly used approaches to the serological diagnosis of syphilis. In the traditional algorithm (Figure 2), a reactive non-treponemal screening test is followed by a treponemal confirmatory assay.

The second algorithm – commonly referred to as a reverse sequence algorithm (Figure 3) – utilizes a treponemal test for screening (27) and, in most cases, is followed by a quantitative non-treponemal test. This algorithm presents several potential advantages, which include the elimination of biological false-positive results in non-treponemal tests and the possibility of detecting antibodies earlier in the course of primary syphilis than would be the case if a non-treponemal screening test were used as well as in some RPR-negative latent syphilis patients. Both algorithms require the titration of reactive sera using RPR and VDRL in order to help with disease staging, assess response to treatment and to help determine if re-infection has occurred (28).

A variation of the reverse sequence algorithm is recommended by the European Centre for Disease Prevention and Control (ECDC): a reactive treponemal screening test is followed by a second treponemal test but is not accompanied by a non-treponemal test (29) This latter version of the reverse sequence algorithm is not currently in use in Canada.

Adoption of the second algorithm will produce some cases with positive treponemal and negative RPR tests (30,31), which will need to be managed appropriately. A small proportion (<1%) of normal sera may be reactive in treponemal tests (6,16). While this proportion is very small, it may translate to a large number of individual patients when treponemal tests are used for screening instead of being used to confirm a reactive non-treponemal test. A recent report suggested that sera with discordant results should be tested with a second treponemal test (32), preferably with equal or higher sensitivity than the primary treponemal test (33). Some have expressed the opinion that samples that have been screened positive by a treponemal test but are negative by non-treponemal test and a second treponemal test likely represent false positives and syphilis is unlikely (34). However, more recent studies have argued that caution should be taken before discarding these isolated EIA or CLA results as false positives because they may occur in high-risk individuals with early or late syphilis, and some have been shown to seroconvert upon follow-up examination (35,36). In such situations, it is prudent to repeat the serological tests two to four weeks after the initial test to ensure that the patient does not have early primary syphilis.

A recent analysis suggested that whether the algorithm used employed a treponemal assay followed by a non-treponemal assay, or alternatively a non-treponemal assay followed by a treponemal assay, the outcome in case numbers detected or adverse outcomes prevented would be the same (37). In addition, there were similar outcomes in terms of overtreatment rates. A recent Canadian study, however, demonstrated an increase in late latent cases of syphilis that required additional public health follow-up (38). The use of treponemal tests for screening, followed by non-treponemal tests, also resulted in higher overall testing costs (37). Tong et al (39) conducted a cross-sectional study of three syphilis testing algorithms: traditional algorithm, reverse algorithm, and the European Centre for Disease Prevention and Control (ECDC) algorithm and reported that the traditional algorithm had the highest negative likelihood ratio (0.24), a missed diagnosis rate of 24.2%, and only 75.81% sensitivity. However, both the reverse and ECDC algorithms had higher diagnostic efficacy than the traditional algorithm with reported sensitivity, specificity, and accuracy of 99.38% to 99.85%, 99.98% to 100.00% and 99.93% to 99.96%, respectively. While their study supported the use of the ECDC algorithm, in which syphilis screening begins with a treponemal immunoassay that is followed by a second, different treponemal assay as a confirmatory test in high-prevalence populations, they acknowledged that a non-treponemal assay is recommended for determining serological activity and the effect of syphilis treatment.

The decision to use a treponemal or non-treponemal assay as the first screening test in the diagnostic algorithm should be based on local syphilis epidemiology, the expected workload, the requirement for automation and the available budget for labour and consumables (40).

CURRENT STATUS OF SEROLOGICAL TESTS FOR SYPHILIS IN CANADA

Serological testing for syphilis diagnosis in Canada was surveyed recently (41). Serological testing is performed in all public health laboratories in each of the provinces, and in many regional, hospital and/or private laboratories in New Brunswick, Nova Scotia, Newfoundland and Labrador, Prince Edward Island, Quebec and Alberta. Diagnostic testing in the territories varies, with most of the territories relying on used other provincial laboratories for either primary and/or confirmatory testing, except Nunavut, where RPR screening is performed. In some provinces, syphilis serology testing is centralized at the provincial public health laboratory, while in other provinces, eg, Quebec, there were >80 laboratories providing the initial screening test.

Syphilis test algorithms varied significantly in provinces and territories. Both non-treponemal and treponemal tests are performed for screening tests (Figures 1 and 2) (41) and a wide range of commercial products are used for non-treponemal and treponemal testing. Although line immunoassay (LIA) has not been approved for use as medical device by the Therapeutic Products Directorate of Health Canada, a few laboratories are using this test for the discrepant samples, under special access licenses from the Therapeutic Products Directorate (42).

RECOMMENDED ALGORITHMS: TREPONEMAL AND NON-TREPONEMAL

After reviewing the current data on syphilis serological testing and group discussion, the CPHLN Syphilis Laboratory Task Group came to the conclusion that both the traditional algorithm (screening by a non-treponemal assay followed by a treponemal assay) and the reverse algorithm (screening by a treponemal assay followed by a non-treponemal assay) are appropriate, and that decisions to implement a diagnostic algorithm should be based on local syphilis epidemiology and other considerations, including expected workload, need for automation and budget considerations.

The traditional algorithm performs well in identifying persons with active infection, while minimizing false-positive results in low-prevalence populations. If the reverse sequence algorithm is used, CPHLN Syphilis Laboratory Task Group recommends that a specimen with reactive EIA/CLIA results be tested reflexively with a quantitative non-treponemal test (eg, RPR or VDRL) as well as TPPA to reduce false-positive cases (Figure 3). In high-test-volume laboratories, discordant
Figure 1) 2009 Canadian syphilis survey serological testing summary for non-treponemal screening (41).

Figure 2) 2009 Canadian syphilis survey serological testing summary for treponemal screening (41)
Figure 3) Traditional algorithm (non-treponemal screening)

Figure 4) Reverse algorithm (treponemal screening)
results are more likely to occur and a third treponemal test, such as INNO-LIA (Innogenetics NV, Belgium), may be needed to resolve those few cases. In addition, based on preliminary data (unpublished) from in-house evaluations presented by some Canadian laboratories, testing algorithms may be modified using a RPR cutoff (eg, 1:8 dilutions), above which a second treponemal test may not be necessary to confirm infection with syphilis; such modifications may be made by individual laboratories based on their local evaluations until published data are available to guide recommendations for further modifications to the algorithms.

Results from all reactive serological testing should be reported promptly and concurrently to the clinician and public health department. Patients with discordant EIA/CIA and RPR/VDRL serological results and whose sera are reactive by TPPA testing are considered to have past or present syphilis; if TPPA is nonreactive, syphilis is unlikely; however, every effort should be taken to consider potential early primary or latent syphilis, especially if such result is from an individual at high risk for syphilis (Figure 4).

It is important to note that clinicians should always assess for evidence of syphilis, especially primary disease (eg, ulcerative genital, oral or anal lesions), and consider additional factors such as sexual behaviour, medical history, previous treatment history of syphilis, etc. If the clinical assessment suggests possible or probable syphilis, and the initial nontreponemal test is nonreactive (traditional algorithm), the patient should still be treated and the laboratory test should be repeated after two to four weeks. Previously untreated patients whose sera are tested with discordant EIA/CIA and RPR/VDRL results and a reactive TPPA should be treated according to Health Canada’s 2010 STD Treatment Guidelines (15).

### RECOMMENDATIONS/SUMMARY

Based on the current information, both the traditional or reverse algorithms are acceptable for the serological diagnosis of syphilis infection.

Each laboratory should choose the algorithm best suited to its needs, taking into consideration the laboratory’s test volumes, local disease prevalence, sensitivity and specificity of the proposed assay, and available resources.

If the traditional algorithm is followed, positive non-treponemal screening test results should be confirmed by a treponemal test. If syphilis is suspected even in the absence of serological confirmation, treatment should be offered and the laboratory tests repeated after two to four weeks. If lesions are present (eg, suspect chancres), a direct test should be collected.

If the reverse algorithm is followed, and the treponemal screening test is positive, a quantitative non-treponemal test is essential to differentiate between active and past treated infection, and a second treponemal test is recommended in most situations to reduce false positivity. If the initial treponemal screening test is positive but the non-treponemal test and the second treponemal test are negative, every effort should be taken to exclude early primary or latent syphilis by repeating the test after two to four weeks before concluding that this represents a false-positive reaction.

### CONCLUSION

Both traditional and reverse sequence algorithms are appropriate for the diagnosis of syphilis and decisions to use either algorithm should be based on a combination of local disease epidemiology, test volumes, performance of the proposed assays and available resources.

### DISCLOSURES
The authors have no conflicts of interest to declare.

### TABLE 1

**Recombinant treponemal tests available in Canada, September 2014**

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Test name or platform</th>
<th>Test format</th>
<th>Specimen</th>
<th>Treponemal antigens included</th>
<th>Antibodies detected</th>
<th>Health Canada Reference(s) approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbott, USA</td>
<td>Architect Syphilis TP</td>
<td>Two-step sandwich chemiluminescent microparticle assay</td>
<td>30 µL serum or plasma</td>
<td>TpN15, TpN17, TpN47</td>
<td>IgG/IgM</td>
<td>4 Yes</td>
</tr>
<tr>
<td>Biokit, Spain</td>
<td>Bioelisa Syphilis 3.0</td>
<td>Microplate EIA</td>
<td>50 µL serum</td>
<td>P15, p17, p47</td>
<td>IgG/IgM</td>
<td>Yes</td>
</tr>
<tr>
<td>Bio-Rad, USA</td>
<td>BioPlex</td>
<td>Multiplex flow immunoassay</td>
<td>5 µL serum</td>
<td>r15kd, r17kd, r47kd</td>
<td>IgG</td>
<td>2 Yes</td>
</tr>
<tr>
<td>Bio-Rad, USA</td>
<td>INNO-LIA</td>
<td>Line immunoassay</td>
<td>10 µL serum or plasma</td>
<td>TpN15, TpN17, TpN47, TmpA</td>
<td>IgG</td>
<td>1 No</td>
</tr>
<tr>
<td>Diasorin, Italy</td>
<td>Liaison</td>
<td>One-step sandwich chemiluminescent assay</td>
<td>70 µL serum or citrated plasma</td>
<td>TpN17</td>
<td>IgG/IgM</td>
<td>3,4 Yes</td>
</tr>
<tr>
<td>Dieess</td>
<td>Enzy-Well Syphilis IgG</td>
<td>Microplate EIA</td>
<td>20 µL serum or plasma</td>
<td>TpN15, TpN17, TpN47</td>
<td>IgG</td>
<td>Yes</td>
</tr>
<tr>
<td>Euroimmun, Germany</td>
<td>Anti-Treponema pallidum Screen ELISA (IgG/IgM)</td>
<td>Western blot</td>
<td>100 µL serum or plasma</td>
<td>TpN (not specified)</td>
<td>IgG/IgM</td>
<td>5 Yes</td>
</tr>
<tr>
<td>Euroimmun, Germany</td>
<td>Anti-Treponema pallidum ELISA (IgG)</td>
<td>Western blot</td>
<td>100 µL serum or plasma</td>
<td>TpN15 and Tp17</td>
<td>IgG/IgM</td>
<td>Yes</td>
</tr>
<tr>
<td>Euroimmun, Germany</td>
<td>Anti-Treponema pallidum Euroline- WB</td>
<td>Western blot</td>
<td>100 µL serum or plasma</td>
<td>TpN17</td>
<td>IgG/IgM</td>
<td>Yes</td>
</tr>
<tr>
<td>Phoenix Airmid, Canada</td>
<td>TrepSure Anti-Treponema EIA Screen</td>
<td>Microplate EIA</td>
<td>100 µL serum or plasma</td>
<td>TpN (not specified)</td>
<td>IgG/IgM</td>
<td>Yes</td>
</tr>
<tr>
<td>Siemens, Germany</td>
<td>ADVIA Centaur</td>
<td>One-step chemiluminescent assay</td>
<td>100 µL serum or plasma (EDTA, heparinized, citrate)</td>
<td>TpN15 and Tp17</td>
<td>IgG/IgM</td>
<td>Yes</td>
</tr>
<tr>
<td>Siemens, Ireland</td>
<td>Immulite 2000</td>
<td>One-step chemiluminescent assay</td>
<td>100 µL serum or plasma (EDTA, heparinized, citrate)</td>
<td>TpN15, TpN17, TpN47</td>
<td>IgG, IgM and IgA</td>
<td>Yes</td>
</tr>
<tr>
<td>Trinity Biotech, Ireland</td>
<td>Captia Syphilis TA</td>
<td>Microplate EIA</td>
<td>50 µL Serum or plasma</td>
<td>TpN15, TpN17, TpN47</td>
<td>IgG, IgM and IgA</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table references: 22, 23, 43-45. EIA Enzyme immunoassay; Ig Immunoglobulin
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