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Advances in diagnostic tests for bacterial STDs

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Abstract

Because of their asymptomatic nature and nonspecific symptoms, laboratory tests are often required to diagnose a sexually transmitted infection. Over the past few years, there have been advances in technology, such as the development of nucleic acid amplification assays, which have improved our ability to diagnose infections caused by *Chlamydia trachomatis*. The finding that nucleic acid amplification tests can detect more infected individuals and are useful in screening low prevalence populations, has led to the development of strategies designed to reduce the cost of these assays without significantly impacting their sensitivity. The development of new tests for the diagnosis of syphilis has gained momentum from the report of a synthetic VDRL antigen, which will result in better nontreponemal antibody tests for syphilis. In spite of the completion of the genome sequence of *Treponema pallidum* and its annotation, we are still unable to cultivate this microorganism *in vitro*. However, the molecular revolution has resulted in the development of PCR assays for detecting *Treponema pallidum* in various types of clinical specimens, and to the production of recombinant antigens for use in tests that detect treponemal-specific antibodies. Further research will improve the availability of low cost, sensitive tests for the diagnosis of sexually transmitted infections. The English version of this paper is available too at: http://www.insp.mx/salud/index.html

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Resumen

Las pruebas de laboratorio son necesarias a menudo para el diagnóstico de las infecciones transmitidas sexualmente, debido a la naturaleza asintomática o a la presencia de síntomas inespecíficos de esas infecciones. En este sentido, durante los años relativamente recientes se han registrado importantes avances tecnológicos, como por ejemplo los ensayos de amplificación de ácidos nucleicos que han permitido una mejora en la posibilidad de diagnosticar las infecciones causadas por *Chlamydia trachomatis*. El descubrimiento de que las pruebas de amplificación de ácidos nucleicos permiten diagnosticar a un mayor número de individuos infectados y de que son útiles para tamizar poblaciones con bajas prevalencias de infección, han conducido al desarrollo de estrategias diseñadas para reducir el costo de los ensayos de laboratorio sin que ello impacte significativamente en la sensibilidad de las pruebas diagnósticas. Por otra parte, el desarrollo de nuevas pruebas para el diagnóstico de la sífilis ha ganado momento a partir de la factibilidad de producir un antígeno de VDRL sintético, que deberá resultar en mejores pruebas de anticuerpos no-treponémicos para el tamiz de la sífilis. Ahora bien, aún cuando se ha completado el conocimiento de la secuencia genética del *Treponema pallidum*, este microrganismo todavía no es susceptible de cultivarse *in vitro*. Sin embargo, la revolución de la biología molecular ha facilitado la implantación de ensayos de la reacción en cadena de la polimerasa para detectar al *Treponema pallidum* en varios tipos de muestras clínicas, así mismo ahora es posible la producción de antígenos recombinantes de esa bacteria para utilizarse en pruebas serológicas de anti-

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cuerpos treponémicos específicos. En conclusión, es de esperarse que la investigación futura favorecerá la disponibilidad de pruebas de laboratorio sensibles y de bajo costo para el diagnóstico de las infecciones transmitidas sexualmente. El texto completo en inglés de este artículo también está disponible en: http://www.insp.mx/salud/index.html

Key words: laboratory diagnosis; bacterial STD; syphilis; gonorrhea; chlamydial infection

Palabras clave: diagnóstico de laboratorio; ETS bacterianas; sífilis; gonorrea; clamidiasis

Sexually transmitted diseases (STDs), are among the most common causes of illness in the world, STDs disproportionally affect the health of women and their infants. STDs have also been implicated as a cofactor in the transmission of HIV.[1] Global estimates of the STD prevalence suggest that there are more than 162 million new cases of the three major bacterial STDs, i.e., syphilis, gonorrhea, and chlamydia, each year.[2] Many of these infections are asymptomatic and when symptoms are present they are often nonspecific. Thus, laboratory tests are required to make a definitive diagnosis, These laboratory tests can be technically difficult and expensive. During the past decade, there have been major advances in the application of nucleic acid amplification (NAA) technologies for the development of assays for the diagnosis of gonorrhea, chlamydial infections, and other sexually transmitted infections.[3] While NAA tests are more sensitive, and in many cases more specific, than conventional laboratory methods they have not achieved the widespread acceptance of older, less sensitive technologies, such as serology and antigen detection. It is hoped that the recent completion of the genome sequences of Treponema pallidum, *Chlamydia trachomatis*, and *Mycoplasma genitalium*[4-6] will provide the impetus for the development of a new generation of diagnostic tests. The purpose of this article, which is to review the recent advances in laboratory tests for STDs, will focus on tests for bacterial STDs. For a more extensive review on NAA assays, the reader is referred to other recent review.[3]

Syphilis

The diagnosis of syphilis depends on a combination of clinical findings, microscopic examination of lesions for treponemes, and/or serologic tests. Serological tests can be divided into nontreponemal and treponemal tests. In the United States, nontreponemal tests such as the Rapid Plama Reagin (RPR) tests or the Venereal Disease Research Laboratory (VDRL) test are used for screening, while treponemal tests such as the fluorescent treponemal antibody absorption (FTA-ABS) assay or the microhemagglutination (MHA-TP) assay are used as confirmatory tests. VDRL antigen is the basic ingredient in the preparation of antigens used in all nontreponemal tests including the VDRL, RPR, and toluidine red unheated serum test (TRUST). For more than 60 years, the isolation and purification of cardiolipin and lecithin from beef hearts by the method described by Pangborn[7] has been used for the preparation of natural VDRL antigen. This process was tedious and time-consuming and resulted in VDRL antigen with a variable purity range. Recently, a synthetic VDRL antigen has been developed using tetramyristoyl cardiolipin and synthetic 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine and was shown to be as specific in detecting syphilis as a VDRL antigen made with natural components.[8] Moreover, this synthetic antigen had a higher level of reactivity with 85% (169/200) of the positive specimens tested than the natural VDRL antigen. The purity of the synthetic VDRL antigen was greater than 99%, which should eliminate this variabile as well as problems with stability that have been encountered with reagents prepared with natural VDRL antigen.

Screening large numbers of serum specimens using the RPR or VDRL tests is labor intensive. Enzyme immunoassays (EIAs) are ideally suited for processing large numbers of specimens because they can be automated, results are read objectively, and reports are generated electronically, reducing transcription errors. SpiroTek Reagin II (Organon Teknika, Durham, NC), a nontreponemal test in an EIA format, may eventually replace the RPR test for screening large numbers of serum specimens.[9] When compared with RPR, the SpiroTek Reagin II test detected more cases of syphilis based on the results of the treponemal test; however,

the number of false-positive results appeared to be higher than with the RPR test. The decreased specificity of the SpiroTek Reagin II test may be offset by its increased sensitivity when it is used in certain settings. It should be pointed out that the SpiroTek Reagin II test is not a quantitative assay and therefore can not be used to monitor treatment efficacy, since that depends on a fourfold decrease in titer.

Recombinant DNA technology involving the cloning, expression, and purification of treponemal antigens such as TpN15, TpN17, TpN47, and TmpA has led to the development of a new generation of treponemal antibody tests. In some European countries, syphilis screening is primarily based on the use of treponemal antibody test such as the MHA-TP (10); however, this test is being phased out by its manufacturer.[9] Schmidt *et al*[10] assessed the performance characteristics of nine EIAs for treponemal antibodies that are commercially available in Europe (Trepanostika, Organon; ICE Syphilis, Murex; Enzygnost Syphilis, Behring; Pathozyme Syphilis Competition, Omega; Bioelisa Syphilis, Biokit; Trep-Check, Phoenix; TmpA-ELISA, Eurodiagnostic; Captia Syphilis G, Trinity; Captia Syphilis M, Mercia). The authors evaluated the sensitivity of these assays with a panel of 52 MHA-TP negative sera from patients with early primary syphilis because sera from patients with secondary or early latent syphilis (titers of ≥1:640 by MHA-TP and ≥1:4 by VDRL) were reactive in all of these tests. Eight of the EIAs exhibited greater sensitivity (48.5% to 86.5%) than the commonly used VDRL test (44.2%). The results of this evaluation demonstrated that: 1)IgM assays were more sensitive than those that measured both antitreponemal IgG and IgM, or IgG only; 2) assays that used a greater volume of serum or serum at a lower dilution were more sensitive; 3) design-related differences were important as capture or competition assays were more sensitive than sandwich type assays; 4) the use of recombinant antigens did not necessarily result in better performance than tests using purified native antigens; and 5) tests employing multiple antigens performed better than those that used a single antigen. Routine testing of 2 053 unselected serum specimens showed that, when compared with the MHA-TP test, the specificity of the ICE Syphilis and Enzygnost Syphilis tests was 99.5 and 99.8%, respectively. However, additional data on the specificity of the other tests are needed as well as on the performance of these tests on specimens from patients with late syphilis.

In syphilis screening programs in the US and much of Europe, latent infection now predominates. Therefore, a screening test should be able to detect all stages of disease. The IgM-specific Captia Syphilis M test had the highest sensitivity (86.5 %) of the EIAs compared in this study.[10] Unfortunately, treponemal-specific IgM is not often detected in late syphilis and therefore, IgM-specific assays should not be used for screening. However, for early syphilis, tests that detect *T pallidum*-specific IgM are the most sensitive.

The Captia Syphilis G test is an EIA that detects treponemal-specific IgG antibodies. Three recent studies have evaluated the performance of this test using different panels of sera and different standard tests for comparison. Schmidt *et al*[10] observed an 80.6% agreement with results using the Captia Syphilis G test on 31 MHA-TP negative serum specimens from patients with early syphilis. In another evaluation, Pope *et al*[9] found that there was an overall agreement of 97.7% between Captia Syphilis G and MHA-TP results on a panel of 390 unselected serum specimens submitted to a state public health laboratory for syphilis testing. In the last study, Halling *et al*[11] observed that there was an 85% agreement between the Captia Syphilis G and FTA-ABS IgG test results with a panel of 89 stored sera from individual patients previously tested by the FTA-ABS IgG test (41 negative sera and 48 positive sera). However, the FTA-ABS IgG test requires a subjective interpretation and thus may be biased if the reader has knowledge of the RPR results. This was illustrated by evaluating the 13 discrepant results after repeating the FTA-ABS IgG test in a blinded fashion, patient chart reviews, and testing with the MHA-TP test. When this was done, the agreement between the two tests increased to 97.8%. The Captia Syphilis G test, like other EIAs, is prone to equivocal results. Thus, it is important to repeat tests with equivocal results, and if the results are still equivocal, a second serum specimen should be obtained at least 7 days after the first specimen was collected.

Pope *et al*[9] compared the Serodia *Treponema pallidum* particle agglutination (TP-PA) test (Fujirebio) with the MHA-TP test on a panel of 390 unselected serum specimens that had been previously submitted to a state public health laboratory for syphilis testing. Because there was a 97.4% agreement between the results of these two assays, the authors concluded that the TP-PA tests was an appropriate substitute for the MHA-TP test.

Young *et al*[12] compared the performance of the Enzywell TP (Diesse, Sienna, Italy), a new rapid enzyme immunoassay that uses 2 recombinant T *pallidum* antigens, with the Syphilis ICE EIA (Murex Biotech Ltd., Dartford, UK), a test that uses 3 recombinant antigens (TpN15, TpN17 and TpN47). Both tests detect treponemal-specific IgG and IgM. The specificity of the Enzywell TP on initial and repeat testing (99.6% and

99.7%, respectively) was similar to that of the Syphilis ICE test (99.8% and 99.9%, respectively). The sensitivities of the Enzywell TP (100%) and Syphilis ICE (99.4%) were similar and were significantly greater than that of the FTA-ABS test (94.5%). A potential advantage of the Enzywell TP was the ability to complete the assay in approximately 1 hour as compared with the 2.5 to 3 hours required to run the Syphilis ICE assay.

The treponemal Western blot (WB) has been studied as a possible alternative to either the FTA-ABS or MHA-TP tests for the confirmation of the serological diagnosis of syphilis. The WB can be used to detect either *T pallidum*-especific IgG or IgM.[13] Marangoni *et al*[14] determined the sensitivity of the WB using srum specimens obtained from patients with a clinical diagnosis of primary (N=15) or secondary (N=6) syphilis. Clinical diagnosis was the reference method used to compare the performance of the WB, FTA-ABS, and TPHA tests. The person reading the WB strips was blinded to the identity of the serum samples. A WB was considered to be positive when at least 3 of the 4 bands at 15.5, 17, 44.5, and 47 kDa were present; a test was considered negative when no band or less than 3 of the above mentioned bands were present. If one assumes that the clinical diagnosis was accurate, the sensitivities of the WB, FTA-ABS, and TPHA were 100%, 88.5%, and 86%, respectively. Using a panel of sera from blood donors, patients with Lyme disease and patients with leptospirosis, the specificities of these tests was 100%, 98%, and 100%, repectively.

The sensitivity and specificity of serologic tests for syphilis is based on standard confirmatory testing using the FTA-ABS as the gold standard.[15] However, in autoimmune diseases, the false positive rate for syphilis testing is significantly higher than in the general population. In order to improve the diagnostic accuracy of syphilis testing in patients with autoimmune disease, Murphy *et al*[16] determined the sensitivity and specificity of standard tests such as RPR, VDRL, and FTA-ABS using the WB as the reference standard. Using specimens obtained from a prospective cohort study of 107 patients with autoimmune disease (50 with at least one positive serologic test for syphilis and 57 disease matched controls with negative serologic tests for syphilis), the sensitivities and specificities of the RPR and VDRL tests were determined to be 62.5% and 91.9% and 37.5% and 89.9%, respectively. The sensitivity and specificity of the FTA-ABS test was 100% and 67.7%, respectively. Thus, the variability in the immunologic response in patients with rheumatic disease was especially apparent in confirmatory tests that require subjective interpretation. The significant number of false-positive FTA-ABS test results indicate that it should not be used to confirm syphilis in patients with autoimmune diseases and that it may be better to use a less subjective method such as the WB.

A line immunoassay (INNO-LIA) Syphilis kit, (Innogenetics NV, Ghent, Belgium) has been developed as a confirmatory test for syphilis.[17] This assay is similar to the WB but instead employs three recombinant immunodominant treponemal proteins (TpN15, TpN17, TpN47) and a synthetic peptide derived from the N-terminal region of TmpA that are immobilized on a nylon strip in discrete lines. The strip also has control lines that are used for semiquantitative evaluation (+/-, 1+, 3+) of the results as well as for the verification of sample addition and reagents. Serum or plasma sample are diluted 1:100 and incubated with the strip at room temperature overnight followed by three washing steps before the addition of a goat anti-human IgG conjugated to alkaline phosphatase. Bound IgG is detected following the addition of a chromogen. The assay has color-coded reagents for ease of performance. Interpretation of the results is facilitated by comparison of the intensity of the TpN15, TpN17, TpN47 and TmpA lines to that of the control lines. A positive specimen must have ≥ 2 reactive bands with an intensity equal to or greater than the +/- control. An evaluation of this new test using 840 serum specimens found a sensitivity and specificity of 99.6% and 99.5%, respectively, when compared to standard tests such as VDRL, TPHA, FTA-ABS, TPI, and ICE Syphilis EIA. There was no reactivity of the INNO-LIA with ninety serum specimens from patients with diseases other than syphilis (eg, Lyme disease) or autoimmune diseases. Based on a limited evaluation using CSF, it appears that the INNO-LIA may also be useful in the diagnosis of neurosyphilis. Unfortunately, the required overnight incubation period and cost may preclude widespread use of this test. A simple, rapid (15 minute), 1-step immunochromatographic test for syphilis (Determine Syphilis TP, Abbott Laboratories, Abbott Park, IL) has recently been evaluated.[18] This test detects antibodies to *T pallidum* through their binding to an antigen-selenium colloid that is subsequently captured by immobilized antigen forming a red line on the test strip. A positive control is also present. No specialized equipment is required because the results are interpreted visually. Either serum, plasma, or whole blood can serve as specimens. This test may be ideal for use in resource limited settings or for rapid screening. Unfortunately, the specificity was not determined in this evaluation as the gold standard FTA-ABS test was not performed.

Nevertheless, there was good agreement (99.3%, 289/291) between the Determine Syphilis TP, Serodia

TP (Fujirebio) and test results among the patient sample examined. One whole blood specimen from a high-risk patient showed a negative result, although the serum, plasma, and confirmatory test results were positive and a second whole blood specimen from another high-risk patient showed a negative result, but exhibited a weakly positive result with the serum and plasma and was classified as negative by a confirmatory test (OTC Trepanostika Microelisa, Organon Teknika). Thus, it is possible that the sensitivity and specificity may be somewhat less when using whole blood as the specimen; however, this needs to be further evaluated.

Marangoni *et al*[19] used a surface immunofluorescence assay (SIFA) to detect treponemal antibodies in serum samples from patients with syphilis. This assay requires a source of viable *T pallidum*. Serum samples are heated to inactivate complement, diluted 1:20 and incubated for 1 hour at 37 ºC under an atmosphere of 95% N_2 and 5% CO_2 with viable treponemes. At least 99% of the treponemes must be motile after the incubation period to have a valid test. After several washes, the treponemes are diluted, spotted onto microscope slides and fixed with acetone. Bound antibody is detected by using fluoroscein-conjugated rabbit anti-human IgG. The sensitivity of this assay using samples from patients with primary, secondary, early latent, and late syphilis was equivalent to that of the WB or VDRL. This assay is complex, technically difficult, expensive (rabbit colony for maintaining viable *T pallidum*), and does not appear to be more sensitive than other confirmatory tests such as the WB or INNO-LIA. Because of the lack of a culture system for *T pallidum*, serology and microscopy (eg. darkfield microscopy) have been the primary laboratory methods that have been used for the diagnosis of syphilis. Several NAA assays have been developed for *T pallidum*;[3] however, none of these are commercially available. Pietravalle *at al*[20] recently described a commercially available, nested PCR kit for *T pallidum* (BIOLINE Diagnostici s.r.l., Turin, Italy), DNA extraction and amplification instructions are provided with the kit. The authors were able to detect the presence of treponemal DNA in ulcers from 6/6 IgM-positive patients with darkfield-positive lesions, 5/6 IgM-positive patients with darkfield-negative lesions, and 2/2 IgG-positive patients with reinfection. Perhaps the most exciting finding in this report was that they were able to detect treponemal DNA in serum specimens from these patients (5/6 IgM positive patients with darkfield-positive lesions, 2/6 IgM-positive patients with darkfield-negative lesions, 5/5 IgM-and IgG-positive patients with latent syphilis, 1/4 IgM-negative IgG-positive patients with latent syphilis, and 2/2 patients with reinfection) as well. No treponemal DNA was detected in the serum of healthy subjects or in those previously affected with syphilis and recovered for at least three years. Further work need to be done to expand on these findings. Nevertheless, the ability to detect treponemal DNA in serum (or other specimens) by PCR may prove to be useful in the diagnosis of syphilis in HIV-infected patients as well as in establishing that the patient had been adequately treated.

Chancroid

EIAs employing undefined antigens that have been used in many studies to determine the seroprevalence of chancroid the disease caused by Haenophilus ducreyi require adsorption of the serum to remove cross-reacting antibodies. These tests are difficult to standardize and their results difficult to interpret. Elkins *et al*[21] developed and evaluated a new EIA employing purified recombinant *H ducreyi* outer membrane proteins (rHgbA, rTdhA, rD15). Serum specimens from patients with and without PCR-confirmed chancroid were used to optimize the sensitivity and specificity and to establish cutoff values. The presence of antibodies to these proteins was strongly correlated with current infection with *H ducreyi* In addition, a significant proportion of patients from a chancroid hyperendemic area presenting with either urethritis or with ulcers due to herpes or syphilis also had antibodies to these proteins. A significantly lower proportion of high-risk and medium-risk patients from STD clinics in a chancroid endemic area of the United States were also seropositive. Further studies are needed to determine the persistence of these antibodies and the effect of reinfection on antibody level.

Transport medium for *Neisseria gonorrhoeae*

Despite declining numbers of reported cases of gonorrhea in the US and many European countries and the development of nucleic acid-based diagnostic tests, culture and Gram stain remains the only tests used in many clinics for the diagnosis of gonorrhea. Currently, culture is the only way to determine the antimicrobial susceptibility of this organism. However, direct plating is not always feasible. Thus, specialized transport systems have been developed and evaluated for their ability to maintain gonococcal viability during transport. Olsen *et al*[22] compared Copan Amies gel agar with and without charcoal (Copan Diagnostics, Corona, CA) to direct inoculation of specimens onto modi-

fied Thayer-Martin medium for detection of *N gonorrhoeae* in 1 490 endocervical specimens obtained from women attending a STD clinic (12% prevalence of *N gonorrhoeae* by culture). Historically, charcoal is added to transport systems to neutralize bactericidal fatty acids present in agar; however, the presence of charcoal makes Gram stains more difficult to interpret. Copan transport systems have a plastic-laminated film pouch that is flushed with nitrogen gas to expel atmospheric air. Swabs are inserted into an agar gel in a polypropylene tube that contains compounds to neutralize oxygen, superoxide, and free radicals. Swabs maintained in the Copan transport system without charcoal performed as well as direct inoculation when specimens were inoculated within 6 hours of collection; recovery of viable gonococci was <95%, if the transport time exceeded 24 hours. Specimens containing low numbers of *N gonorrhoeae* were most likely to be negative or overgrown with commensal bacteria or yeast if held for 24 hours before plating.

Enzyme immunoassay for *Chlamydia trachomatis* infections

EIAs are widely used to screen for *C trachomatis*[23] in spite of data indicating that they are less sensitive than NAA assays.[3,24] Okadome *et al*[25] determined the analytical sensitivity of a new dual amplified immunoassay (IDEIA PCE *Chlamydia*, Dako Diagnostics, Denmark) for detecting chlamydial LPS. This is a new version of the IDEIA Chlamydia EIA whereby the signal amplification system has been modified by using a polymer conjugate incorporating multiple copies of antibody and enzyme molecules on a dextran backbone. The use of polymer conjugates has been reported to increase the analytical sensitivity of EIAs by 10-40-fold. However, an examination of the data indicates that the use of a polymer conjugate resulted in only a 2-4 fold increase in analytical sensitivity based on number of IFUs/ml when compared to the earlier version of the test. This is likely to be an underestimation of the number of elementary bodies (EBs) required since only viable EBs form IFUs and are counted. Furthermore, the implication that IDEIA PCE *Chlamydia* test has the same analytical sensitivity as a NAA assay is unfounded. Taylor-Robinson and Thomas[26] expressed similar criticisms of this assay. The IDEIA PCE *Chlamydia* test has also been used to assay pooled first void and midstream urine specimens from female high school students (overall prevalence of *C trachomatis* was 5.1%).[27] However, only 49% of the *Chlamydia* positive females were identified, with a significantly lower number of positives identified if they were in the second and third week following menstruation.

The ACCESS immunoassay for *C trachomatis* (Beckman/Sanofi Diagnostics, Brea, CA) uses a monoclonal antibody coupled to paramagnetic particles to detect chlamydial LPS antigens in a chemiluminescent immunoassay. Waites *et al*[28] compared the ACCESS *Chlamydia* antigen assay with the ligase chain reaction (LCR) using the Abbott LCx System (Abbott Laboratories, Abbott Park, IL) on 356 endocervical specimens that were obtained from women presenting to a hospital emergency department. The prevalence of *chlamydial* infection was 8.7% in this population. Using confirmed positive results by LCR as a reference, ACCESS had a sensitivity of 83.9%, a specificity of 99.7%, a PPV of 96.3%, and a NPV of 98.5%. However, using the manufacturer's definition of a positive test, ACCESS would have only correctly identified 25/31 infected women (80.6% sensitivity). In addition, these authors reported that the total cost of the ACCESS immunoassay, calculated on the basis of the cost for labor, reagents, quality control and standards, was significantly greater than that of the Abbott LCx.

The CHLAMYDIA OIA (BioStar, Boulder, CO) is a rapid antigen detection assay based on the principle of optical interference that has recently become commercially available for office-or clinic-based detection of *C trachomatis*. Widjaja *et al*[29] conducted a multicenter cross-sectional survey in three hospital outpatient clinics in South Kalimantan, Indonesia comparing this assay to the LCx *C trachomatis* assay (Abbott Laboratories). Endocervical samples from 415 outpatients visiting dermatovenerology and nondermatovenerology clinics were evaluated. The overall prevalence of *C trachomatis* by LCR was 9.2%. Relative to the LCR, the overall sensitivity and specificity of the OIA were 31.6 and 98.9%, respectively. The sensitivity varied among the three hospital laboratories, ranging from 20 to 50%. The authors concluded that the educational level and experience of the personnel in the three hospital laboratories were not major factors in determining how well the assay performed, but felt that the light source may be crucial in reading the results of the OIA test. Unfortunately, the authors did not consider that adequate specimen collection during the routine gynecological examination might be responsible for the poor performance of the OIA. For the diagnosis of chlamydial infections, an adequate specimen is more important for the performance of immunoassays than for the performance of NAA tests such as LCR.[30]

Nucleic acid amplification assays for *Chlamydia trachomatis and/or Neisseria gonorrhoeae*

Several nucleic acid amplification assays for the detection of ch1amydial or gonococcal DNA have been developed and evaluated.[3,24] This review will focus on new assays as well as the applications and problems that have been encountered with some of the older assays.

Madicio *et al*[31] developed a touchdown enzyme time release (TETR)-PCR assay using primer sequences for specific regions of the 16S and 16S-23S spacer rRNA genes to detect and identify *C trachomatis, C pneumoniae,* and *C psittaci.* The three amplified products were designed to be sufficiently different in size so that they could be easily discriminated by agarose gel electrophoresis. The primers of the TETR-PCR assay could be used either individually or multiplexed in a single assay. The 96.7% sensitivity and 99.6% specificity of the TETR-PCR for detection of *C trachomatis* in vaginal specimens were comparable to the sensitivity and specificity of the commercially available AMPLICOR *C trachomatis* test. The distinguishing features of the TETR-PCR are: a hot start polymerase to avoid artifacts before amplification, a touchdown protocol for annealing temperatures to improve the specificity of primer binding, and an enzyme time release protocol to allow 60 cycles of amplification. The latter improved the analytical sensitivity of the test, allowing detection of 0.004 to 0.063 IFU per PCR reaction compared to 0.1 to 4 IFU when a conventional PCR protocol with 35 cycles was used. This is not a commercially available assay.

A commercially available, FDA approved, second-generation nucleic acid amplification assay has been developed by Becton Dickinson Microbiology Systems[32] for the detection of *C trachomatis* and *N gonorrhoeae.* This assay uses strand displacement amplification (SDA) and real-time detection of the amplification products. The mechanism of SDA is described in detail in.[33] Amplification and detection occur simultaneously during the SDA process. The detection process is distinguished from Taqman (another real-time nucleic acid amplification technology) in several ways, including the fact that Taqman uses PCR, thus exploiting the 5'-3' exonuclease activity of the Taq polymerase, whereas SDA uses an exonuclease-free polymerase *(Bst* DNApolymerase), and that the Taqman method requires thermocycling for the formation of the fluorescent product while SDA is an isothermal process. The BDProbeTEC^{TM}ET system uses sealed microwells to minimize amplicon contamination. This method is simpler, has a higher throughput, and is more user friendly than other commercially available nucleic acid amplification assays. The authors report that the BDProbeTEC^{TM}ET will reliably detect as few as 10-15 EBs of *C trachomatis* or cells of *N gonorrhoeae.* The system configuration (96 well format) and workflow permits a throughput of 564 patient results per shift. The assay steps and times include: sample lysis (30min), sample cooling(15 min), priming incubation at room temperature (20 min), priming and prewarm incubation (10 min), and amplification and detection (60 min). However, 96 specimens and controls can be lysed at one time; lysed specimens can be held for up to 6 hours. Thus, a sufficient number of specimens can be prepared for multiple runs. The system can be configured to detect either *C trachomatis* or both *C trachomatis* and *N gonorrhoeae.* For each 96-well plate, one positive control and one negative control are included in the microwell set up and are tested like specimens. A separate microwell for each control and specimen is used for an amplification control (AC). The AC well contains an amplifiable DNA sequence added to the specimen, which serves to flag inhibitory specimens. Thus, for a *C trachomatis* test, a 96-well plate will contain one positive control, one negative control and up to 46 samples; each of the48 wells has a corresponding AC well. For specimens being tested for both *C trachomatis* and *N gonorrhoeae,* one plate contains one positive control, one negative control and up to 30 samples. Each of the 30 samples and 2 controls has a corresponding AC well. The algorithm resident in the instrument reports results as positive, negative, indeterminate (inhibited AC), or equivocal on the basis of preselected cutoff values. Internal studies of interfering substances showed that the presence of up to 2% blood (swab specimens) did not affect results. In a limited evaluation (57 specimens), reportable results using the BDProbeTEC^{TM}ET were 100% sensitive and specific relative to results obtained by the LCx. Further clinical evaluations of this assay are needed; nevertheless, this assay has several attractive features. For example, with urine specimens, a urine processing pouch (Becton Dickinson Microbiology Systems) is added to the sample. The pouch contains a proprietary material capable of removing amplification inhibitors and stabilizing the urine specimens. Stabilized specimens containing *C. trachomatis* can be stored up to 6 days or *N gonorrhoeae* up to 4 days at 18 - 30 °C, or 6 days at 2-8°C for specimens containing *N gonorrhoeae* or *C trachomatis.* This urine processing pouch may algo be useful for screening programs that require self-collected urine specimens that are sent by mail to a central laboratory.[27,34,35]

The Hybrid Capture II (HCII CT/GC) test (Digene Corporation, Silver Spring, MD) is an investigational

nucleic acid probe-based chemiluminescent assay that will detect chlamydial or gonococcal DNA in cervical specimens. The target DNA is hybridized with RNA probes and the DNA/RNA hybrids are immobilized in an antibody capture system on microtiter plates. Rather than amplifying the target or probe, as is done in PCR and LCR, respectively, the HCII system uses a signal amplification method. The format of the HCII CT/GC test allows simultaneous detection of *C trachomatis* and *N gonorrhoeae* in a single specimen within 4 hours; an initial positive result is followed by repeat tests with probes to specifically identify these microorganisms. This identification step requires an additional 4 hours. A proprietary endocervical brush called a cervical sampler is used to collect specimens from nonpregnant women, while a Dacron swab is used to collect specimens from pregnant women. In a multicenter validation study, Schachter *et al*[36] found that the HCII CT/GC test was both sensitive and specific in detecting these organisms. The overall prevalence of chlamydia infection among symptomatic patients was 17.9% (range 7.9-21.9) and among asymptomatic patients it was 8.6% (range 4.9-25). The overall prevalence of gonorrhea was 6.9%. Compared to gonococcal culture, the HCII had a sensitivity of 93% (87/94) and a specificity of 98.5% (1 244/1 263). Compared to *C trachomatis* culture, the sensitivity was 97.7% and the specificity was 98.2%. Testing of discrepant specimens by PCR suggested that the test would actually prove to be even more specific. It is likely that this test could be used for screening low- prevalence populations.

Roche Molecular Systems has developed a multiplex PCR assay for *C trachomatis* and *N gonorrhoeae* that is available in two formats. In the semiautomated AMPLICOR CT/NG test, amplified products are detected by an EIA on microwell plates while the fully automated COBAS AMPLICOR CT/NG test is performed on the COBAS AMPLICOR analyzer, an integrated unit that automatically amplifies nucleic acid targets and detects the tesulting amplicons. Both of these tests use a master mix containing oligonucleotide primers to simultaneously amplify *C trachomatis* and *N gonorrhoeae* in a single specimen. The master mix also contains internal control (IC) DNA to check for the presence of inhibitors in the clinical specimen. The IC consists of primer binding regions identical to those of the *C trachomatis* target sequence and a unique probe binding region that differentiates the amplified IC from amplified *C trachomatis* target DNA. The *C trachomatis, N gonorrhoeae,* and IC amplicons are detected separately using specific, oligonucleotide capture probes. Van Der Pool *et al*[37] published a multicenter comparison of the AMPLICOR and COBAS AMPLICOR tests for *C trachomatis.* Six sites were involved with the prevalence of *C trachomatis* by culture ranging from 2.1-15.1% for women and 1.7-18.9% for men. Patients were recruited from STD clinics and family planning centers. Test performance was compared to culture for 2 236 matched endocervical swab and urine specimens obtained from women and 1 940 matched urethral swab and urine specimens obtained from men. Culture-negative, PCR-positive specimens were resolved as true positives if they tested positive in either a DFA test, or in a confirmatory PCR test for an alternative target sequence. The two assay formats yielded concordant results for 98.1% of the specimens. With the infected patient as the reference standard, the resolved sensitivities of COBAS AMPLICOR were 89.7% for endocervical swab specimens, 89.2% for female urine specimens, 88.6% for male urethral specimens, and 90.3% for male urine specimens. However, in most clinical situations, only a single patient specimen is collected and run. Thus, when results were analyzed on this basis, the resolved sensitivities were always higher (92.1%-98.8%). The resolved specificities of COBAS AMPLICOR were 99.4% for endocervical swab specimens, 99.0% for female urine specimens, 98.7% for male urethral swab specimens, and 98.4% for male urine specimens. The IC revealed that 2.4% (203/8,618) of specimens were inhibitory when initially tested, with urine specimens being more inhibitory than swab specimens (152/203 versus 51/203). The only data on *N gonorrhoeae* presented in this paper was that the coinfection rate was 3.7% (159/4,315) among the individuals tested; however, among those infected with *C trachomatis,* 27% were coinfected with *N gonorrhoeae.* Overall, this evaluation demonstrated that both formats exhibited excellent sensitivity and specificity for *C trachomatis,* with essentially the same sensitivity for urine and urogenital swab specimens. The sensitivity of culture varied markedly between sites. Thus, PCR performed on any one sample detected approximately l0 to 20% more chlamydial infections than culture. Pallidino *et al*[38] reported that the sensitivity and specificity of the Amplicor CT/NG on urine specimens from 73 men (prevalence of *N gonorrhoeae was* 52.1%) were 100%; the sensitivity and specificity for C *trachomatis* was 80.0% and 95.2%, respectively. The addition of 2% boric acid to the urine specimen may prevent the growth of bacteria during mailing and perhaps prevent the degradation of nucleic acid in the sample.[39]

Morre *et al*[40] compared the performance of COBAS Amplicor with LCx using 2 906 mailed, first void urine specimens (1,138 from men and 1,717 from women) from an asymptomatic population (overall prevalence of *C trachomatis* was 2.9%). The IC results indicated that

4.0% of the urine specimens from men and 7.9% of the urine specimens from women were inhibitory. Samples positive in only one assay were subjected to discrepant analysis using an in-house plasmid PCR assay. After discrepant analysis, the sensitivities and specificities of the COBAS and LCx were: 98.8% versus 78.6% and 99.9% versus 99.7%, respectively. In this asymptomatic population, the COBAS Amplicor test performed better than LCx with mailed urine specimens.

de Barbeyrac *et al*[41] evaluated the sensitivity and specificity of the AMP CT (Gen-Probe, Incorporated, San Diego, CA) assay on urogenital specimens taken from symptomatic patients and on first void urine specimens from asymptomatic patients. A clinical specimen was considered to be truly positive if either cell culture was positive or both AMP CT and Amplicor *C. trachomatis* PCR was positive. A total of four amplification methods (AMP CT, Amplicor, COBAS, and LCx) were used to identify those asymptomatic patients who were infected; a subject was considered to be infected when two or more amplification methods were positive. After analysis of discordant results, the sensitivity and specificity of AMP CT was 100% and 98.3%, respectively. The authors concluded that AMP CT was as sensitive as other amplification methods for the identification of chlamydial infection in symptomatic and asymptomatic men and women on genital and urine specimens.

The high analytic sensitivity of NAA assays enables them to be used on specimens that would be less than optimal with other technologies. Carder *et al*[39] found that self-collected vaginal-introital specimens were an acceptable alternative to clinician-obtained cervical swab specimens. Vaginal-introital specimens had a higher sensitivity compared with first void urine specimens for the detection of *C. trachomatis* and were acceptable to the patient. Domeika *et al*[42] observed that self-obtained vaginal swabs were more sensitive that a clinician-obtained cervical swab or a first catch urine specimen for the detection of *C. trachomatis* among women with genital symptoms. However, cervical swab specimens were more sensitive than vaginal swabs for detecting *C. trachomatis* in asymptomatic women. It was interesting to note that C. *trachomatis* was less often detected in urine of women who routinely practiced genital washing. Patient-administered tampon specimens are another easy and sensitive method for obtaining a specimen for the diagnosis of chlamydial and gonococcal infections by PCR.[43]

NAA tests have generally been considered to perform adequately with specimens containing low numbers of organisms. Nevertheless, a proportion of specimens collected for PCR testing for *C. trachomatis* may be inadequate as measured by b-globin gene amplification.[44] *C. trachomatis* was detected in a significantly greater proportion of specimens that were β-globin positive than in those that were β-globin negative. A linear relationship between chlamydia positivity rates and the number of endocervical columnar or metaplastic cells observed using a semiquantitative cytologic staining method was also observed using a less-sensitive, non-amplified probe assay.[45]

NAA tests are generally more expensive than tests such as EIA or non-amplified probe tests. Because most urine specimens collected for screening purposes will test negative, pooling algorithms have been developed to take advantage of the high analytic sensitivity of these technologies and to reduce the cost of these tests.[46,47] Pooling cervical swab specimens can also save considerable time and cost.[48] Wilcox *et al*[49] found that combining a cervical swab with a urine specimen did not affect the sensitivity of PCR testing for *C. trachomatis* and may detect more infected patients with a concomitant increase in the cost effectiveness of DNA based screening.

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