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Review

Molecular Methods for the Detection of *Mycoplasma* and *Ureaplasma* Infections in Humans

A Paper from the 2011 William Beaumont Hospital Symposium on Molecular Pathology

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Mycoplasma* and *Ureaplasma* species are well-known human pathogens responsible for a broad array of inflammatory conditions involving the respiratory and urogenital tracts of neonates, children, and adults. Greater attention is being given to these organisms in diagnostic microbiology, largely as a result of improved methods for their laboratory detection, made possible by powerful molecular-based techniques that can be used for primary detection in clinical specimens. For slow-growing species, such as *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, molecular-based detection is the only practical means for rapid microbiological diagnosis. Most molecular-based methods used for detection and characterization of conventional bacteria have been applied to these organisms. A complete genome sequence is available for one or more strains of all of the important human pathogens in the *Mycoplasma* and *Ureaplasma* genera. Information gained from genome analyses and improvements in efficiency of DNA sequencing are expected to significantly advance the field of molecular detection and genotyping during the next few years. This review provides a summary and critical review of methods suitable for detection and characterization of mycoplasmas and ureaplasmas of humans, with emphasis on molecular genotypic techniques. *(J Mol Diagn 2012, 14:437–450; http://dx.doi.org/10.1016/j.jmoldx.2012.06.001)

The bacteria commonly referred to as mycoplasmas are included within the phylum Tenericutes, class Mollicutes, which is composed of four orders, five families, eight genera, and approximately 200 known species distributed among humans, animals, insects, and plants.[1] Mollicutes are smaller than conventional bacteria, in cellular dimensions and genome size, making them the smallest free-living organisms known. Lack of a cell wall, coupled with their extremely small genome and limited biosynthetic capabilities, explains the parasitic or saprophytic existence of these organisms, their sensitivity to environmental conditions, resistance to β-lactam antibiotics, and fastidious growth requirements. Among the mollicutes that are the most important human pathogens, there are one or more type strains for which the genome has been completely sequenced and annotated. Genome sizes range from 580 to 2200 kbp, with *Mycoplasma genitalium* being the smallest.[2] Mollicutes require enriched growth medium supplemented with nucleic acid precursors, fatty acids, and amino acids. Most mollicutes require sterols in growth media, supplied by the addition of horse or bovine serum.

There are 16 mollicute species that have been isolated from humans, excluding those of animal origin that have been detected occasionally in humans, usually in immuno-

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suppressed hosts, but which are generally considered transient colonizers. There are at least six species considered to be of pathological significance, either as primary pathogens or opportunists: *Mycoplasma pneumoniae, Mycoplasma hominis, M. genitalium, Mycoplasma fermentans, Ureaplasma urealyticum,* and *Ureaplasma parvum.* A newly described species, *Mycoplasma amphoriforme*, has been detected in the lower respiratory tracts of several immunodeficient people with respiratory disease, but there is no conclusive evidence that this mycoplasma is a significant pathogen of humans.[1] Other species, such as *Mycoplasma penetrans* and *Mycoplasma pirum*, have been studied because of a possible association with HIV and AIDS, but there is no evidence that either species is independently pathogenic in humans.[3] Although *M. fermentans* can be considered an uncommon opportunistic pathogen of humans,[3] it is rarely sought; methods for detection by culture are not well described, and few laboratories offer molecular-based tests for its detection. This article focuses on molecular methods for detection and identification of *M. pneumoniae*, *M. genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum*, because these are the mollicute species that are most important clinically and for which diagnostic testing is most commonly performed.

Major Pathogenic Mollicutes of Humans

A brief summary describing important biological characteristics of each species and salient clinical features of the infections they cause is provided. Readers are referred to other reference texts and reviews for more detailed descriptions.[3–9]

M. pneumoniae

This mycoplasma is a common cause of upper and lower respiratory tract infections in children and adults. The organism is easily spread through respiratory droplets and can cause a variety of clinical manifestations, including pharyngitis, tracheobronchitis, and pneumonia. Extrapulmonary manifestations sometimes occur after primary respiratory tract infection, either by direct spread or autoimmune effects.[4,8] Attachment of *M. pneumoniae* to host cells in the respiratory tract is required for colonization and infection. Cytadherence is mediated by the P1 adhesin and other accessory proteins, followed by induction of chronic inflammation, and cytotoxicity is mediated by hydrogen peroxide, which also acts as a hemolysin. *M. pneumoniae* stimulates B and T lymphocytes and induces formation of autoantibodies that react with a variety of host tissues and the I antigen on erythrocytes, which is responsible for production of cold agglutinins.[4] An ADP-ribosylating toxin, known as the community-acquired respiratory distress syndrome (CARDS) toxin, causes vacuolation and ciliostasis in cultured host cells and is becoming appreciated as a significant virulence factor.[10] Although mycoplasmas are generally considered to be extracellular organisms, intracellular localization is appreciated for *M. pneumoniae* and other species, including *M. genitalium*.[11,12] Intracellular localization may be responsible for protecting the organisms from antibodies and antibiotics, as well as contributing to disease chronicity and difficulty in cultivation. The genome of *M. pneumoniae* M129 (type strain for subtype 1) consists of 816 kbp with 687 protein-coding genes.[13] The 811-kbp genome sequence of the FH strain (type strain for subtype 2) was published in 2010,[14] and the third complete genome of *M. pneumoniae* strain 309 from Japan, classified as subtype 2a variant, consists of 817 kbp.[15] A brief comparison of the three genomes indicated that they are similar, with variations in a region involving insertion changes in the putative lipoprotein genes.[15] Comparative analysis of genomic differences among the type strains and the inclusion of clinical strains representing all of the major P1 subtypes could provide useful information in developing diagnostic tests and treatment strategies.

M. genitalium

This mycoplasma was initially isolated from men with urethritis and is a significant cause of this condition and female cervicitis and pelvic inflammatory disease.[9] Unlike other genital mycoplasmas that are rather common as commensals in the lower urogenital tract of many healthy adults, *M. genitalium* is less commonly detected in the absence of clinical infection. *M. genitalium* possesses a terminal structure, the MgPa adhesin, that facilitates its attachment to epithelial cells,[16] attaches to spermatozoa and erythrocytes, and invades epithelial cells with evidence of nuclear localization.[9] A family of repetitive DNA elements homologous to the MgPa adhesin gene is believed to contribute to variation in the protein of the MgPa adhesin gene. Sequence divergence among strains of *M. genitalium* and antigenic variation may help avoid the host immune response and optimize adhesion.[17] The 580-kbp genome of *M. genitalium* contains only 485 protein-coding genes, making it the smallest known free-living microorganism.

M. hominis

Approximately 21% to 53% of asymptomatic sexually active women are colonized with this mycoplasma in the cervix or vagina, but the occurrence is somewhat lower in the male urethra.[3] *M. hominis* is often present concurrently with *Ureaplasma* species and is transmissible venereally and vertically. *M. hominis* is associated with a variety of conditions, including pyelonephritis, pelvic inflammatory diseases, chorioamnionitis, postpartum endometritis bacterial vaginosis, arthritis, osteoarthritis, wound infections, and several conditions in neonates (eg, congenital pneumonia, meningitis, bacteremia, and abscesses). Systemic infections sometimes occur in neonates, older children, and adults. Such extragenital infections outside of the neonatal period are usually, but not always, associated with an immunocompromised host status.[3] Henrich et al[18] demonstrated the presence of the variable adherence-associated antigen, which displays high-frequency phase and size variation that is believed to be a major adhesin of *M. hominis* and may also assist in evasion of host immune responses. Additional surface proteins, such as OppA, which is an oligopeptide per-

mease substrate-binding protein, are also believed to be involved in cytadherence and may also induce ATP release from cells, resulting in apoptosis.[19] The *M. hominis* genome contains 665 kbp and 527 protein-coding genes, placing it second, behind *M. genitalium,* as the smallest known self-replicating free-living organism.[19] Genome analysis indicates that *M. hominis* has undergone horizontal gene transfer with *Ureaplasma* species.[19]

U. parvum *and* U. urealyticum

Members of the genus *Ureaplasma* hydrolyze urea and use it as a metabolic substrate for generation of ATP. This genus has seven recognized species, with *U. parvum* and *U. urealyticum* being the two species found in humans. As many as 40% to 80% of healthy adult women may harbor ureaplasmas in their cervix or vagina. Their occurrence is somewhat less in the lower urogenital tract of healthy men.[3] The organisms are readily transmitted venereally and vertically, either *in utero* or at delivery of the neonate.[3] *U. parvum* is more common than *U. urealyticum* as a colonizer of the male and female urogenital tracts and in the neonatal respiratory tract.[3] Although detected less frequently than *U. parvum* in most patient populations, *U. urealyticum* may be more pathogenic in male urethritis.[3,20] Ureaplasmas reside primarily on the mucosal surfaces of the urogenital tracts of adults or the respiratory tracts in infants. Despite their frequent occurrence in healthy people, *Ureaplasma* species may cause or be associated with a variety of clinical conditions in adults, including urethritis, arthritis, chorioamnionitis, postpartum endometritis as well as preterm birth, pneumonia, bacteremia, abscesses, meningitis, and chronic lung disease in neonates.[3] Systemic spread is uncommon beyond the neonatal period unless there is an immunosuppressed condition, such as hypogammaglobulinemia.[3,21] Ureaplasmas are capable of attaching to a variety of cell types, such as urethral epithelial cells, spermatozoa, and erythrocytes.[3] The adhesins of ureaplasmas have not been characterized completely, but evidence suggests that the receptors are sialyl residues and/or sulfated compounds.[3] A major family of surface proteins, the multiple-banded antigens, is immunogenic during ureaplasmal infections. Ureaplasmas produce an IgA protease and release ammonia through urea hydrolysis, both of which are considered possible virulence factors.[3] Variation in surface antigens may be related to persistence of these organisms at invasive sites. Details of the pathogenic mechanisms through which ureaplasmas mediate human disease have been described elsewhere.[3] *Ureaplasma* genomes range from 750 kbp (594 genes) for *U. parvum* to 947 kbp (711 genes) for *U. urealyticum.*

Limitations of Culture and Serological Analysis as Diagnostic Tests for Mycoplasmas and Ureaplasmas

Culture

Ureaplasma species grow rapidly in media containing urea, such as 10 B broth and A 8 agar, producing colonies visible with a stereomicroscope within 1 to 3 days.[1] The appearance of brown granular colonies on A 8 agar is sufficient for the diagnosis of *Ureaplasma* species, but culture alone cannot distinguish between the two species. *M. hominis* grows well in SP 4 broth or SP 4 agar supplemented with arginine, but it will also grow on A 8 agar and in 10 B broth.[1] Colonies appear on agar within 2 to 3 days, visible with a stereomicroscope. To confirm species identity for mycoplasmas growing on agar, additional procedures (eg, a PCR assay) must be performed because there are no phenotypic tests that can distinguish them.

PCR is more sensitive than culture for diagnostic purposes, even for organisms such as *M. hominis* and *Ureaplasma* species, which are relatively easily and quickly cultivated. Data from the University of Alabama at Birmingham Diagnostic *Mycoplasma* Laboratory showed that real-time PCR detected ureaplasmal DNA in 52 (39.4%) of 132 specimens versus 32 (24.2%) detected by culture.[22] Even though culture was considered the reference method, PCR is theoretically able to detect fewer organisms; therefore, PCR-positive, culture-negative specimens likely represent true positives.

Despite PCR being more sensitive for detection of *M. hominis* and *Ureaplasma* species, culture remains the most economical and practical means for detection of these organisms for most laboratories with a low to moderate test volume. Cultures can be set up one at a time, whereas the most efficient and cost-effective use of PCR is to run the assays in batches of several specimens. However, batching lengthens the turnaround time for reporting results. Culture also has additional advantages in that it provides an isolate on which antimicrobial susceptibility testing can be performed. Highly sensitive PCR assays are useful for research studies when turnaround time is not so critical and when samples can be batch tested for cost-effectiveness. Although various modalities have been developed to enhance the ability to detect *M. genitalium* in culture, the high failure rate and extremely slow growth make culture impractical and rarely attempted now that molecular-based assays have been developed.

M. pneumoniae can be cultivated in SP 4 glucose broth, although its slow growth, requiring several days to weeks, and low sensitivity make culture unattractive for diagnostic purposes.[1] Colonies growing on agar must undergo additional testing, typically by PCR, to confirm their identity because several commensal mycoplasmal species often inhabit the human oropharynx. Isolation of the organism from the respiratory tract or detection of its DNA is clinically significant in most instances. This should be correlated with the presence of clinical respiratory tract disease, because occasional asymptomatic carriers may exist.

Detailed methods for obtaining specimens, culturing, and identifying mycoplasmas and ureaplasmas of humans *in vitro* using culture-based methods have been described in other reference texts.[1,23] Whether culture- or non–culture-based detection methods should be used for diagnostic purposes depends on the resources and facilities available in individual laboratories and the species being sought.

Serological Analysis

Serological testing was the first method developed for detection of *M. pneumoniae* infections. Complement fixation assays were used for many years until the development of alternative serological methods sold commercially, such as enzyme immunoassays, immunofluorescence, and particle agglutination assays. More recently, molecular-based nucleic acid amplification tests (NAATs) have further reduced the need for serological diagnosis. Despite its widespread use, at least partly because of the expense and limited availability of NAATs, serological analysis has far more limitations than advantages for detection of acute *M. pneumoniae* infections. The main disadvantages of serological analysis are the requirement for acute and convalescent serum samples that are tested simultaneously for IgM and IgG to confirm seroconversion, difficulty in distinguishing current or recent infection from past infection, and need to wait 1 to 2 weeks from onset of the infection until detectable antibody develops.[24] Adults may never develop a measurable IgM response, presumably because of reinfections.[4] Moreover, IgM antibodies can persist for weeks to months, making it risky to base diagnosis of acute infection on a single assay for IgM.[4] In addition, as a result of repeated infections, many healthy adults can also have a high background seropositivity for IgG[25]; thus, there is a need to measure antibody increase and decrease in acute and convalescent specimens. Antibody production may also be delayed in some infections or even absent if the patient is immunosuppressed.

Problems with sensitivity and/or specificity have been reported for practically all serological assay formats and commercial products when rigorously compared with other detection methods, such as PCR.[24] However, many serological tests have never undergone extensive comparative evaluations and comparison with other diagnostic methods, so their accuracy is unknown. Serological testing of *M. pneumoniae* and the various commercial methods available for antibody measurement have been described in other reference texts and reviews.[1,4,23,24]

Serological test methods for *M. hominis*, *Ureaplasma* species, and *M. genitalium* include microimmunofluorescence, metabolism inhibition, and enzyme immunoassay,[1] but the ubiquity of ureaplasmas and *M. hominis* in healthy people makes interpretation of antibody titers against these organisms difficult. No serological assays for the genital mycoplasmas have been standardized, and they are not available in the United States for diagnostic purposes.

Overview of Molecular-Based Tests for Detection of Mollicutes

Interest in non–culture-based detection of mycoplasmas dates back to the 1980s when assays for *M. pneumoniae* antigen detection and nonamplified DNA probes were developed, driven by the drawbacks of culture and serological analysis. Once NAATs began to be developed, interest in nonamplified DNA probes and antigen detection assays waned, and there are no products of this nature sold commercially in the United States for detection of *M. pneumoniae*.

Since 1989, hundreds of publications have described various NAATs and their applications to detect mycoplasmas and ureaplasmas in clinical specimens. These assays enable detection of extremely fastidious species, such as *M. genitalium*, that might never be detected otherwise. NAATs are also useful for the identification of organisms grown in culture to the species level, replacing older and cumbersome technologies.

PCR is the most widely applied NAAT for detection of mycoplasmas and ureaplasmas. PCR has also been adapted to detect antimicrobial resistance determinants and to analyze genetic relatedness of clinical isolates. Conventional PCR measures the end-stage PCR products using gels or other methods, whereas real-time PCR detects and quantifies the products simultaneously with amplification. Nested PCR can increase sensitivity through re-amplification of a PCR product with a second set of primers.[26] However, the nested PCR method may also enhance the risk of contamination.[27] Publications describing real-time PCR for detection and characterization of mycoplasmas and ureaplasmas have used the ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA), the iCycler iQ (Bio-Rad, Hercules, CA), and the LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN). Detection systems include agarose gel electrophoresis, SYBR Green, TaqMan probes, hybridization probes, molecular beacons, and microchip electrophoresis (Foster City, CA).[28] The UAB Diagnostic *Mycoplasma* Laboratory has eliminated conventional PCR in favor of real-time PCR using the Roche LightCycler for detection and identification of all of the major mollicute pathogens of humans because of its advantages in accuracy, quantitation, and turnaround time. The improved specificity of real-time PCR compared with conventional PCR is mainly because of the use of a third oligonucleotide probe that binds to the target sequence. The use of a labeled probe minimizes the probability of cross-reaction and detection of undesired amplicons. Another feature of real-time PCR that contributes to the specificity of the assay is that the amplicon melting temperature is determined at the end of the assay, so it can be used to verify whether the desired PCR product is being detected.

Because organism viability does not have to be maintained for NAAT-based detection, specimen collection, handling, and transport are somewhat simpler than for culture, in that a specialized nutritive transport medium is not required. Although conventional PCR methods can take 2 to 3 days, real-time PCR can potentially provide results the same day a specimen is received and provide quantitative data to determine the bacterial load. This can be important for interpretation of results for organisms that are known to colonize asymptomatic people. Two studies have reported that the bacterial load of *M. pneumoniae* in throat swabs was significantly greater in patients requiring hospitalization than in those who were not hospitalized, indicating the potential importance of such quantitative data.[29,30] The pros and cons of NAATs, culture, and serological analysis, as they apply to detection

Table 1. Advantages of Molecular-Based Methods Compared with Culture and Serological Analysis for Detection of Mycoplasmas and Ureaplasmas in Humans

Criteria	Molecular-based assays	Culture	Serological analysis
Availability	Not commercially available in the United States, a few PCR kits are available in Europe and Asia. Nonproprietary PCR assays are available in a few US reference laboratories.	Commercially prepared SP 4, 10 B, and A 8 media and test kits for *M. hominis* and *Ureaplasma* species are available. Culture is available in many large hospital and reference laboratories. Additional immunoserological or genotypic tests are required to confirm species identity of large-colony mycoplasmas.	Commercial qualitative and quantitative antibody assays are available for *M. pneumoniae*. No such assays are available for other *Mycoplasma* or *Ureaplasma* species in the United States.
Cost	Cost of equipment and reagents is significant, and personnel trained in molecular diagnosis are required. Costs are less if equipment, facilities, and personnel can be used for other molecular diagnostic testing.	Media are somewhat expensive to obtain or prepare. Equipment used for general microbiology purposes is usually sufficient. Length of time cultures have to be held for slow-growing mycoplasmas adds to personnel costs.	Commercial serological kits for *M. pneumoniae* vary in cost. Some assay formats are suitable for testing single specimens, whereas others are more practical for batches. Cost per test depends on the volume of specimens and equipment requirements.
Turnaround time	Real-time PCR can be completed in a few hours. Batching specimens and running the assays once or twice each week decrease the costs but delay turnaround time.	*M. hominis* and *Ureaplasma* species can be grown in culture within 1–3 days, whereas *M. pneumoniae* requires from 5 days up to several weeks. *M. genitalium* cannot be reliably grown in culture.	Hands-on time varies from a few minutes to a few hours. Acute and convalescent serum sample collection time spans 2–3 weeks.
Analytical sensitivity	High: most assays detect <100 CFU/mL organisms or 100 genome copies.	May detect 100–1000 viable organisms per test.	Serological tests do not measure the presence of the microorganism, but instead measure the host immune response. Compared with PCR, serological analysis may miss many infected individuals.
Specificity	PCR assays that are carefully validated with targets chosen for diagnostic accuracy and lack of cross-reactivity are specific.	Culture is 100% specific when positive.	Older complement fixation tests had problems distinguishing *M. pneumoniae* from *M. genitalium.* Newer commercial ELISAs do not have this problem.
Specimen requirements	Organisms do not have to be viable. The same specimen types used for culture can be submitted for PCR assays. Specimens require frozen storage until processing. Formalin-fixed tissue can also be processed by PCR.	Properly collected specimens require appropriate transport media, frozen storage, and shipment to maintain viability.	Serum is the only required specimen type. No special handling or storage, other than refrigeration, is needed.

ELISA, enzyme-linked immunosorbent assay.

of mycoplasmas and ureaplasmas in clinical specimens, are summarized in Table 1.

Use of Molecular-Based Tests for Mollicute Detection

M. pneumoniae

Gene targets for PCR assays have included 16S ribosomal RNA (rRNA), *P1*, *tuf*, *parE*, *dnak*, *pdhA*, ATPase operon, CARDS toxin gene (*mpn372*), and the noncoding repetitive element *repMp1*.[6,26,28,31–33] Analytical sensitivity is generally high, with some assays being capable of detecting a single organism when purified DNA is used.

The UAB Diagnostic *Mycoplasma* Laboratory adapted the real-time PCR assay published by Dumke et al,[32] targeting the *repMp1* noncoding DNA sequence for routine diagnostic use. Its theoretical advantage is the fact that sensitivity may be improved by amplification of a gene present in multiple copies. This assay provided acceptable sensitivity and specificity when tested against several *M. pneumoniae* reference strains, detecting as few as seven DNA molecules per microliter, including both major P1 subtypes. The assay also detected *M. pneumoniae* DNA in a large group of clinical specimens obtained from patients with radiologically proved pneumonias who were positive for *M. pneumoniae* by serological analysis, culture, and/or conventional PCR. No other mollicute species or other respiratory pathogens tested positive by this method.[32,34]

Use of PCR for detection of *M. pneumoniae* infection in extrapulmonary sites can also be helpful because cultures from nonpulmonary sites are rarely positive as a result of low organism load. PCR has also been advan-

tageous when insufficient time has elapsed since onset of illness for an antibody response to develop and for testing fixed lung tissue obtained at biopsy.[6] The advantage of real-time PCR over conventional PCR in detection of systemic infection was demonstrated in a study that found 15 (52%) of 29 patients with serological evidence of *M. pneumoniae* infection had a positive assay result when serum samples were tested by real-time PCR. Conventional PCR assay results for those specimens were entirely negative.[35]

Although PCR may theoretically be more sensitive than culture, some studies have yielded contradictory results.[6] For PCR-positive, culture-negative patients, it is important to ascertain whether clinically significant respiratory disease is actually present, because the positive PCR assay result may reflect asymptomatic carriage with a low bacterial load, prior antibiotic therapy, persistence of mycoplasmal DNA after resolution of infection, organisms residing in an intracellular location not amenable to culture, or the possibility that the PCR target was nonspecific. A positive PCR assay result in a patient who is serologically negative may indicate that the specimen was obtained too early in the infection for measurable antibody to have developed, antimicrobial therapy that may have blunted the immune response, or an inadequate immune response because of immunosuppression. Negative PCR results in patients who are culturally and/or serologically positive could indicate technical problems with the PCR assay or inhibitors. Using a PCR assay with a different gene target may resolve the problem. One issue that can be problematic with PCR assays for *M. pneumoniae* is that they may not have been tested adequately for specificity for this organism by making certain there is no reactivity with the numerous commensal mycoplasmal species of the human respiratory tract. The use of a highly specific PCR for *M. pneumoniae* has been particularly useful in our experience when mycoplasma species are detected in respiratory tract cultures because they frequently turn out to be one of the common commensal species and not *M. pneumoniae*. Performing PCR assays with two different gene targets is theoretically the best diagnostic approach for *M. pneumoniae* infections, but using the second target increases costs and takes more time. Combining PCR with serological analysis has also been advocated as a possible means to distinguish colonization from active disease, but this also adds to the cost of testing and will not overcome the problem common in older people who often fail to mount an acute-phase IgM response, necessitating testing paired serum samples and prolonging the time until diagnosis can be confirmed.

There is no universal consensus regarding what constitutes the best respiratory specimen to be tested by PCR. Combining nasopharyngeal and oropharyngeal specimens may provide the greatest diagnostic yield.[36] One study reported that sputum was superior to oropharyngeal or nasopharyngeal specimens in young adults with serologically proved *M. pneumoniae* infection.[37] However, young children and many adults with mild illness often do not produce sputum, so nasopharyngeal or oropharyngeal samples may be the only specimen types available. In our experience, many children with severe pneumonia requiring hospitalization will have positive PCR results for *M. pneumoniae* on bronchoalveolar lavage fluid obtained by bronchoscopy.[34]

There have been few side-by-side comparisons to determine whether one assay format or gene target is better than another. Many of the comparisons that have been done compared PCR using culture or serological analysis as a reference method, and predictably yielded disparate results in some cases. The Centers for Disease Control and Prevention compared three real-time PCR assays to detect *M. pneumoniae*.[31] They performed triplicate PCR assays using the Applied Biosystems ABI 7500 system using two different TaqMan primer-probe sets targeting the ATPase gene and a new assay targeting the CARDS toxin gene on 54 respiratory samples from an outbreak in a college setting. Eighteen cases had positive results with all three assays. When dilutions of *M. pneumoniae* reference strains were tested, the CARDS toxin PCR assay consistently detected 1 to 5 colony-forming units (CFUs), whereas the other two assays targeting the ATPase genes detected 5 to 50 CFUs. Two multicenter comparisons of various NAATs for *M. pneumoniae* detection[38,39] reported significant variations in test performance among participating laboratories, making a strong case for an organized proficiency test program, which is available in some European countries.[40] In the United States, where there are no commercially sold Food and Drug Administration–approved PCR assays for any *Mycoplasma* or *Ureaplasma* species, reference laboratories that offer their own internally developed NAAT should participate in alternative proficiency testing, in which clinical specimens are exchanged in a blinded manner, or use the *M. pneumoniae* proficiency testing specimens that are available through the College of American Pathologists.

Both conventional and real-time nucleic acid sequence-based amplification (NASBA) have been used to detect *M. pneumoniae* RNA.[41] NASBA can provide rapid results with sensitivity as good as or better than PCR, with a detection threshold as low as 5 to 50 CFUs.[41,42] This assay has been described in monoplex and multiplex format and has been developed as a commercial kit (NucliSENS; bioMérieux, Marcy l'Etoile, France) sold in various European countries. Multiplex PCR and NASBA assays have been developed in a variety of formats for detection of *M. pneumoniae*, along with *Chlamydophila pneumoniae* and *Legionella pneumophila*.[43–46] Generally speaking, some loss in analytical sensitivity occurs in multiplex assays when compared with monoplex assays that may be related to incompatible amplification conditions for multiple targets and the high concentration of primers that can cause elevated background readings and reduced efficiency. Although the multiplex NASBA has potential to detect *M. pneumoniae* and *C. pneumoniae* and *Legionella* species, it had a slightly lower sensitivity than monoplex NASBA when applied to dilutions of wild-type RNA generated *in vitro*.[47] Other techniques, including reverse line blot assays and microarrays, have been used alone or in combination with multiplex PCR assays for detection of *M. pneumoniae* and other respiratory pathogens.[33,45,46,48,49]

Table 2. Examples of Real-Time PCR Assays for Detecting *Mycoplasma* and *Ureaplasma* Species in Clinical Samples

Specificity	Target gene/region	Analytical sensitivity (genome copies or CFU/reaction)	Ref. no.
Up and Uu	*16S rRNA*	10 copies	Yoshida et al[52]
Up	Urease gene subunits and adjacent regions	5 copies	Mallard et al[53]
Up	*UU063*	0.6 CFU	Xiao et al[22]
Up	Urease gene subunits and adjacent regions	10 copies	Cao et al[54]
Uu	Urease gene subunits and adjacent regions	10 copies	Cao et al[54]
Uu	Urease gene subunits and adjacent regions	5 copies	Mallard et al[53]
Uu	*UUR10_0680*	0.8 CFU	Xiao et al[22]
Mp	P1	10 copies	Pitcher et al[55]
Mp	*repMp1* in P1	0.2 CFU	Dumke et al[32]
Mp	CARDS toxin (*mpn372*)	1–5 CFU	Winchell et al[31]
Mp	ATPase operon	5–50 CFU	Winchell et al[31]
Mp	16S rRNA	1 copy	Khanna et al[45]
Mp	16S rRNA	5 CFU	Raggam et al[44]
Mg	*gap*	5 copies	Svenstrup et al[56]
Mg	16S rRNA	10 copies	Yoshida et al[57]
Mh	*gap*	10 copies	Baczynska et al[58]
Mh	*yidC*	7 copies	Ferandon et al[59]

Mg, *M. genitalium*; Mh, *M. hominis*; Mp, *M. pneumoniae*; Up, *U. parvum*; Uu, *U. urealyticum*.

The loop-mediated isothermal amplification assay has been applied to detection of *M. pneumoniae* in clinical specimens using *P1* sequences for primers in direct comparison to real-time PCR.[50] The assay was specific, with a detection limit of 200 copies, and had 100% concordance with PCR when applied to 95 nasopharyngeal specimens. The development of commercial assays using this type of technology for *M. pneumoniae* is anticipated because instrumentation and reagents are undergoing clinical trials.

Commercial PCR assays have been available in Europe for several years, and additional products are still in development. Limited evaluations have shown they work in a comparable manner to noncommercial assays.[45,46,51] These assays include the Artus RepMp1 (Qiagen, Valencia, CA), Venor Mp-QP (Minerva Biolabs, Berlin, Germany), Chamylege (Argene Inc., Shirley, NY), and Pneumoplex (Prodesse Inc., Waukesha, WI).

A comprehensive review of diagnostic methods for *M. pneumoniae* respiratory tract infection, published by Loens et al,[28] indicated that, as of 2010, there were at least 61 published in-house PCR assays for *M. pneumoniae*, many of which have been validated only for their analytical sensitivity and not tested against many clinical samples, or against one another. Some of the more recently described real-time PCR assays for detection of *M. pneumoniae* and other species are listed in Table 2.

Macrolide resistance in *M. pneumoniae* is a major problem in Asia and is spreading to Europe and North America.[6] PCR assays have been developed to detect three major mutations in domain V of 23S rRNA that confer high-level macrolide resistance in isolates of *M. pneumoniae* or directly in clinical specimens.[34,60,61] A rapid and inexpensive method that combines nested PCR, single-stranded conformation polymorphisms, and capillary electrophoresis can also detect macrolide-resistant mutants directly from throat swabs.[62] Finally, pyrosequencing has also been applied for detection of macrolide resistance in *M. pneumoniae* and for strain typing.[63]

The UAB Diagnostic *Mycoplasma* Laboratory performs a multiplex real-time PCR assay to detect point mutations in all three positions of the 23S rRNA gene (2063, 2064, and 2617) that are related to the macrolide resistance on all clinical samples that are positive for *M. pneumoniae* in the *repMp1* real-time PCR assay. This assay uses fluorescence resonant energy transfer hybridization probes and the Roche LightCycler 2.0 instrument. This method is based on the fact that nucleic acid will melt at a precise temperature that is related to the nucleotide base composition. The presence of one or more point mutations in 23S rRNA that impair drug attachment to the bacterial ribosome will be detected by this extremely sensitive method, which can be completed in just a few hours. The detection limit is as low as seven mutant molecules per microliter in the PCR mixture.[34] Figure 1 illustrates how this assay detects and distinguishes the mutants.

Given the relatively mild clinical course of most mycoplasmal respiratory tract infections, lack of widespread availability of rapid diagnostic tests, and their associated costs, many clinicians choose to treat empirically when infection with this organism is strongly suspected. Testing for *M. pneumoniae* would almost certainly become more widespread in the United States if rapid real-time PCR assays become more readily available so that test results can play a greater role in guiding initial patient management. Even if specimens have to be sent to a reference laboratory with a longer turnaround time, it is reasonable to pursue a comprehensive microbiological diagnosis for patients with lower respiratory tract infections, including testing for *M. pneumoniae* by PCR, when respiratory illness is of sufficient severity to warrant hospitalization and no other causative microorganism is readily apparent. Testing for *M. pneumoniae* should also be considered if there is an unsatisfactory clinical response to empirical treatment with macrolides because this may indicate the presence of a macrolide-resistant strain, in patients with an underlying comorbid condition or immunodeficiency that would make severe and disseminated disease more likely, and when there are significant extrapulmonary symptoms present.

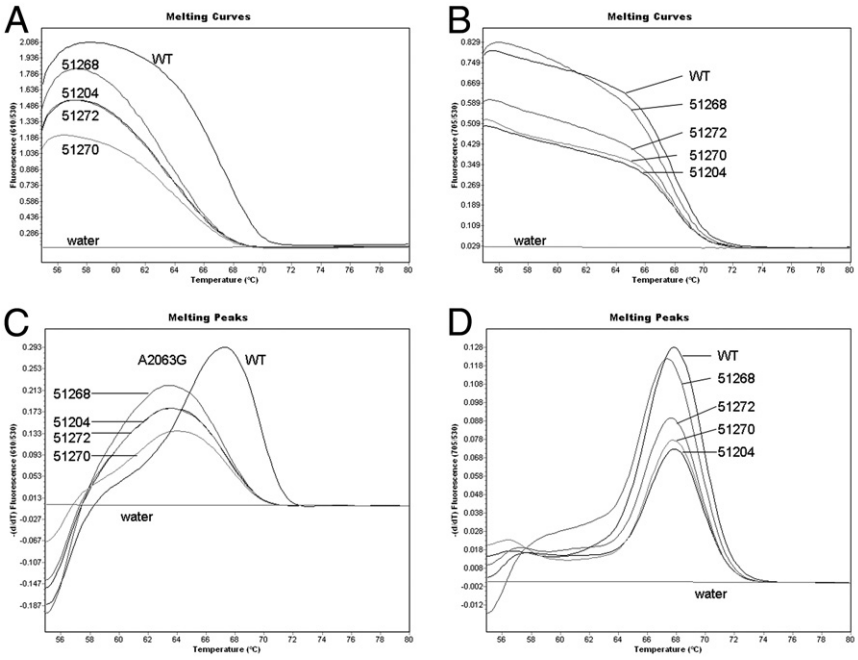

Figure 1. Real-time PCR detection of macrolide-resistant *M. pneumoniae* in clinical samples. Genomic DNA samples of two patients containing the A2063G mutation, verified by sequencing, are purified and tested together with a wild-type (WT) control (*M. pneumoniae* strain M129, ATCC number 29342). Melting curves (**A** and **B**) and corresponding melting peaks (**C** and **D**) are shown. A2063/A2064 mutations are analyzed in channel 610 (**A** and **C**). The WT melting peak is 67.31°C, whereas the melting temperature (Tm) of A2063G mutants is 63.25°C ± 0.04°C. Thus, a 4°C difference between WT and mutant is observed. The C2617 assay is shown in channel 705 (**B** and **D**). Because all samples do not have mutations at this position, they show similar WT Tm values of approximately 68°C, as predicted. Data are reproduced from Xiao et al,[34] with permission of Walters Kluwer Health (copyright 2009).

M. genitalium

Although *M. pneumoniae* and *M. genitalium* are structurally and antigenically related, they are genomically different. Most of the early PCR assays targeted various regions of the MgPa operon, but some have used the 16S rRNA gene.[64,65] Additional molecular-based assays, including transcription-mediated amplification, have also been used for epidemiological purposes.[66–68] Quantitative, rapid, real-time PCR assays have used targets such as MgPa operon, 16S rRNA, the 115-kDa gene, and *gap* encoding glyceraldehyde-3-phosphate dehydrogenase.[56,57,68–72] It has become apparent that not all primers used in various studies react with all *M. genitalium* strains, especially those using the MgPa target. Ma et al[73] examined the three genes of the *M. genitalium* MgPa operon (*mgpA*, *mgpB*, and *mgpC*) and nine repetitive sequences (termed MgPars) dispersed throughout the genome in 15 geographically diverse strains. They discovered that all operon sequences and all MgPars differed from each other more than from the published G37 operon sequence at both the nucleotide and deduced amino acid levels. They determined that one of 19 primers contained up to 19 variable nucleotides and that the target for one of two typing systems was located in a hypervariable region, indicating the likelihood of erroneous results with some assays. This has been demonstrated in studies using primers MGS-2 and MgPa-903.[73] These data suggest that there is an efficient recombination system enabling generation of numerous variants that may facilitate evasion of host defenses and that additional research and development must be undertaken to identify and validate the most appropriate PCR primers for diagnostic testing.

In view of concerns for use of MgPa, and 98% identity of the 16S rRNA gene for *M. pneumoniae* and *M. genitalium*, the UAB Diagnostic *Mycoplasma* Laboratory has adapted the real-time PCR assay described by Svenstrup et al[56] for detection of *M. genitalium* in clinical specimens. This protocol targets the conserved housekeeping gene *gap* (National Center for Biotechnology Information accession number U39710) in a primer and probe system. This target is different from other species, including the *gap* homologue in *M. pneumoniae* (72.3% identity) and is present in the genome as a single copy.[56] PCR-based assays have also been developed to detect mutations in DNA gyrase and/or topoisomerase IV, mediating fluoroquinolone resistance in *M. genitalium*, thereby circumventing the need for culture *in vitro* to determine antimicrobial susceptibilities to these agents.[74]

Gen-Probe (San Diego, CA) has developed a transcription-mediated amplification real-time PCR assay that performs well compared with other methods, but it is available in the United States only for research purposes.[68,75,76] Multiplex PCR-based systems for detection of *M. genitalium*, along with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and other urogenital mycoplasmas and ureaplasmas, are sold as kits in several European countries by multiple companies, including Bio-Rad (Hercules, CA), Amplex Biosystems (Giessen, Germany), PCR Diagnostics.eu (Bratislava, Slovak Republic), and Seegene, Inc. (Rockville, MD), using various formats and instrument platforms.

Because the requirements for marketing diagnostic products in Europe are not as stringent as those in the United States, mandated by the Food and Drug Administration, many products sold there have not been subjected to large and rigorous clinical trials or comparisons with existing assays.

Lack of commercial NAATs for detection of *M. genitalium* in the United States has greatly limited interest among physicians in seeking this mycoplasma in clinical specimens. Given the importance of this mycoplasma in

conditions such as urethritis, cervicitis, and pelvic inflammatory disease, and the likelihood of venereal transmission of disease to other individuals, use of accurate molecular-based assays with a rapid turnaround time would undoubtedly increase if they become available. If *M. genitalium* is detected in the lower urogenital tract of a symptomatic patient, it should be considered medically significant.

M. hominis

Conventional PCR assays for *M. hominis* have mainly used 16S rRNA as a gene target.[77,78] Because some heterogeneity has been reported in the 16S rRNA gene of *M. hominis*,[79] other targets, including *gap*, *fstY*, and *yidC*, have been developed.[58,59,80] The UAB Diagnostic *Mycoplasma* Laboratory uses a real-time PCR assay to detect *M. hominis* in clinical specimens targeting *gap* (National Center for Biotechnology Information accession number AJ243692) using the Roche LightCycler 2.0, as adapted from Baczynska et al.[58]

Because *M. hominis* is a common commensal in the female cervix and vagina and the male urethra, a positive PCR result on specimens from such sites may not be meaningful in the absence of clinical manifestations known to be associated with this organism. However, if the *M. hominis* load reaches $\geq 10^4$ CFU/mL, this can be a crucial criterion for urogenital infections in women.[81] In the case of extragenital specimens in adults or neonates, a positive *M. hominis* PCR assay or culture result should be considered clinically significant. There are many reports of systemic infections that were caused by *M. hominis* and for which diagnosis was delayed simply as a result of delays in appropriate testing because no one attempted to test.

Ureaplasma *Species*

PCR assays are important to detect and identify the individual *Ureaplasma* species for research purposes. Gel-based conventional PCR assays targeted sequences of 16S rRNA and 16S rRNA to 23S rRNA intergenic spacer regions, the urease gene, and *mba,*[82–90] whereas real-time PCR assays have mainly targeted the urease genes and their subunits or *mba*.[22,53,54,57,91,92] Yoshida et al[78] described a conventional PCR assay applied to urine specimens of patients with urethritis targeting 16S rRNA genes of *M. genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum.* After amplification, PCR products were then subjected to hybridization assays using four species-specific capture probes to detect the targets. When compared with direct sequencing, this technique produced similar results and no cross-reactivity.

The UAB Diagnostic *Mycoplasma* Laboratory performs a real-time PCR assay for detection and differentiation of *Ureaplasma* species based on *UU063* (NP_077893), which encodes a conserved hypothetical protein that is identical in all four *U. parvum* serovars and a 15,072-bp open reading frame, *UUR10_0680* (NC_011374.1), that is conserved (>99.97%) in all 10 *U. urealyticum* serovars.[22] This assay detected more positive clinical specimens than a conventional PCR assay based on a urease gene target in intralaboratory method evaluations.

Some molecular-based assays that include detection of *Ureaplasma* species are commercially available in various European countries, but not in the United States. Seegene, Inc. markets their products STD6 and STD6B ACE Detection, which simultaneously detect *Trichomonas vaginalis*, *M. hominis*, *M. genitalium*, *C. trachomatis*, *N. gonorrhoeae*, and *Ureaplasma* species in endocervical/urethral swabs. The novel feature of the Seegene, Inc., technology is a dual-priming oligonucleotide system that contains two separate priming regions linked by a polydeoxyinosine spacer.[93] The Seegene, Inc., STD6 ACE kit works with any thermocycler, and the post-PCR assay is designed for either manual or automated gel electrophoresis. The STD6 *Ureaplasma* assay amplifies a 130-bp region of the *ureD* cassette.[94] Their new version, STD6B, differentiates *U. urealyticum* from *U. parvum ureC* genes. Other companies, including PCR Diagnostics.eu and Amplex Biosystems, have PCR-based diagnostic products to detect *Ureaplasma* species in clinical specimens. PCR Diagnostics.eu has an assay in traditional PCR format with detection of PCR products on agarose gels that will differentiate the two *Ureaplasma* species. The multiplex PCR/reverse line blotting assay method has been used to detect numerous respiratory pathogens, including *M. pneumoniae* and urogenital pathogens, including both *Ureaplasma* species, *M. genitalium*, and *M. hominis*.[95–97] Examples of real-time PCR assays used for detection and differentiation of *Ureaplasma* species are shown in Table 2.

Such as *M. hominis*, *Ureaplasma* species are also common commensals in the lower urogenital tracts of healthy people. Therefore, a positive PCR assay or culture result from specimens from these sites is usually expected. It is the increased bacterial load determined by real-time PCR or quantitative culture that can sometimes be valuable as a clinical indication of infection.[52] Positive PCR or culture results for *Ureaplasma* species from the urethra in men with urethritis, from tracheal aspirates of neonates with respiratory distress, from the bloodstream or cerebrospinal fluid in neonates with pleocytosis, and from normally sterile extragenital sites should be considered diagnostic of clinically significant infection. *Ureaplasma* species can be relatively common opportunistic pathogens causing systemic infections involving various body sites in immunosuppressed people; many of them are missed initially because they are not considered, and diagnostic tests are performed only after treatment with antimicrobials that are not active against these organisms has failed.

Xiao et al[98] evaluated 1061 clinical isolates of *Ureaplasma* species from various patient populations and found that *U. urealyticum*, but not *U. parvum*, was associated with male urethritis, as noted by others.[20,52] Even though there have been conflicting reports regarding differential pathogenicity of the two *Ureaplasma* species, there is no compelling necessity to identify ureaplasmas to the species level in most circumstances because antimicrobial treatment would not differ. The only possible exception would be cases of male urethritis, in which

ureaplasmas were detected, because one could argue that *U. parvum*, if detected alone by PCR, might not warrant treatment. However, because the real-time PCR assay we use for diagnostic purposes uses gene targets that enable species distinction, the specific organisms that are detected are reported whenever there is a positive result. As mentioned earlier, our laboratory still provides culture as the primary means for *Ureaplasma* detection for clinical purposes, and we perform PCR only by special request or for research studies.

Important Technical Aspects of Molecular-Based Assays Used for Mollicute Detection

Specimen Collection

All clinical specimens suitable for culture are acceptable for diagnostic testing for mycoplasmas and ureaplasmas by molecular methods if they are collected, stored, and processed correctly. Blood should be collected in a tube containing acid citrate dextrose. Other specimens can be inoculated into transport media, culture media, or PBS at collection or as soon as possible thereafter. For swab specimens, use of only Dacron or polyester swabs as calcium alginate and cotton swabs can be inhibitory. The 10 B or SP 4 broths used for culture do not have any deleterious effect on performance of the real-time PCR assay using the Roche LightCycler 2.0 (Roche Applied Science, Penzberg, Germany), but it is possible that culture broth could be inhibitory when using other primers or reaction conditions, or thermocyclers. Thus, it is mandatory to verify that culture broth or any liquid other than a designated PCR transport buffer is not inhibitory before using it for PCR transport.

DNA Extraction

Lysis and proteinase K treatment usually yield PCR-detectable DNA, unless the specimen is inhibitory.[99] Suitable specimens for this procedure include body fluids (other than blood) and transport systems containing material obtained from swabs. Potentially inhibitory specimens, including blood, tissue samples, lower respiratory tract secretions, and subcultures, may be purified by the QIAamp DNA Blood Mini Kit (Qiagen) or other commercial genomic DNA purification kits. Automated or semi-automated nucleic acid isolation methods, such as Qiagen BioRobot EZ1 (Qiagen), NucliSENS (bioMérieux), easyMAG (bioMérieux), or MagNaPure LC (Roche Applied Science), can also be used to prepare samples. Some automated extraction systems appear to work as well as manual systems. The easyMAG nucleic acid extractor actually enabled superior amplification results for *M. pneumoniae* when applied retrospectively to clinical specimens when compared with the QIAgen blood mini kit and the NucliSENS miniMAG systems.[100,101]

PCR Programs and Operating Conditions

Most aspects of the real-time PCR procedures are instrument and protocol specific, such that analytic sensitivity, specificity, primer selection, and all aspects of the operating program must be validated separately for each method and instrument. Each laboratory must perform an evaluation of every assay component, from sample type, transport media, and extraction method, to final PCR amplification and detection procedures using the specific primers and probes, reaction conditions, and controls applicable to the assay to ensure that the techniques are valid and that there is no PCR inhibition at any step.

Quality Control

Careful attention to quality control procedures should limit the risk of false-positive and false-negative results. False-positive results from contamination can be a major problem for conventional PCR, but are less problematic with real-time PCR. In addition to human errors, reasons for false-negative results include the presence of PCR inhibitors in the specimen, suboptimal reagent preparation and reaction conditions, and inefficient extraction of the target DNA. Inhibitory factors and suboptimal PCR conditions can be detected by adding a positive control DNA after purification. However, this external control strategy cannot reveal inefficient DNA extraction. Use of an internal control added directly to the crude sample and coprocessed for purification and amplification is the most accurate method to monitor the important steps of diagnostic PCR protocols.

Determination of Analytical Sensitivity and Specificity

The PCR analytical sensitivity test should be performed against serial dilutions of template DNA, either bacterial genomic DNA from a defined inoculum titer or a plasmid containing the target sequence, and can be expressed in terms of amount of DNA detected or numbers of organisms (CFUs). Published or commercially sold PCR assays used for diagnostic purposes can reasonably be expected to detect $<10^2$ CFUs of the targeted organism.

The analytical specificity of PCR assays should be tested against all other human mollicutes, including commensal mycoplasmal species and other bacteria that may appear in the same body locations or show sequence similarities to the targets. An assay should be validated against various type strains and low-passage clinical isolates. For *M. pneumoniae*, strains representing both of the main P1 subtypes should be included. For *U. parvum*, the four recognized serovars should be included; and for *U. urealyticum*, the 10 known serovars should be tested. Human genomic DNA should always be included in the evaluation because of its presence in clinical specimens and possible inhibitory effects. The assay reproducibility should be verified by testing the same samples multiple times.

Additional PCR Assay Validation

It is important to perform PCR assays on well-characterized clinical specimens, with or without the organism of interest, that have been tested by other acceptable methods, such as culture and other PCR assays using different gene targets. Before a laboratory can begin routine PCR-based diagnostic work, it must be able to demonstrate that its molecular results compare favorably or exceed the detection ability of conventional culture-based techniques to establish a clinical sensitivity for the assay. The lack of comprehensive published comparative data for validation of the wide variety of PCR assays used for detection of human mycoplasmas and ureaplasmas remains a significant shortcoming.

Summary and Conclusions

Development and application of molecular-based methods during the past two decades has significantly improved the ability to detect and identify mycoplasmas and ureaplasmas in clinical specimens, enabled expansion of knowledge about the diseases they may cause, and provided more rapid and accurate diagnosis. It has also heightened interest in obtaining diagnostic testing for these organisms among many clinicians. This has been especially true for *M. pneumoniae* and *M. genitalium*, for which real-time PCR methods are clearly the diagnostic methods of choice. NAAT-based detection methods have lessened the reliance on the problematic serological detection systems. The enthusiasm for development of NAAT-based systems for application in mycoplasmology has resulted in dozens of published assays using a broad array of gene targets and methods. When used for diagnostic or epidemiological purposes in a clinical setting, there is justifiable concern over accuracy because most assays have never been sufficiently validated against other molecular- or culture-based methods to ensure their accuracy. Because none of these assays has thus far been evaluated or approved by the Food and Drug Administration, much is still unknown about their sensitivity and specificity. The few comparative clinical studies of various NAATs and preliminary studies of interlaboratory proficiency testing have indicated that there are considerable differences with these assays for detection of mycoplasmal infections, as well as the capabilities of the individual testing laboratories. The future of diagnostic mycoplasmology and epidemiological research rests with molecular-based technology, although culture, phenotypic methods, and traditional antimicrobial susceptibility testing will still have an important role, especially for *M. hominis* and *Ureaplasma* species. Therefore, it is important that large-scale comparisons must be performed to compare reproducibility and accuracy of NAATs. This must include side-by-side comparisons of new assay formats and gene targets with existing assays using the same, and different, targets and with other established methods, including culture. Such comparisons should ideally include a broad selection of specimen sources from different geographic areas. Eventually, it seems likely that commercial development of NAATs for all of the important pathogenic mycoplasmal and ureaplasmal species that infect humans will come to the United States, as they have to Europe. Standardization of reagents and rigorous quality control would then be more realistic.

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