For Drug-Resistant STIs, Detection Is Not Enough

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A new method of detecting *Mycoplasma genitalium* holds promise for broader use

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Mycoplasma genitalium is a sexually transmitted infection (STI) that can bring about a wide range of clinical conditions, including cervicitis, endometritis, urethritis, and pelvic inflammatory disease.[1,2] In the general population, the prevalence of *M. genitalium* ranges from 1% to 3%, and in higher risk populations it ranges from 10% to 40%, often exceeding the prevalence of *Neisseria gonorrhoeae* infection.[1,3–7]

M. genitalium infection has been largely underreported due to the difficulty of isolating and culturing the organism. In practice, such difficulties mean that molecular testing is the only practical method that can be used to reliably identify *M. genitalium*. Although a small number of CE-marked molecular diagnostics are available to detect the organism, there is currently no FDA-approved equivalent available to the US market.

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Further, identification of the bacterium alone may no longer be sufficient to guide effective treatment. Treatment failure by azithromycin, the recommended antimicrobial treatment, is rapidly emerging. The median cure rate for both men and women has been approximately 85%, but was only 40% in the most recent trial.[8,9] Recent advances in molecular diagnostics have allowed for rapid detection of not only the organism, but also antimicrobial resistance markers in *M. genitalium* that can be used to guide clinical treatment.

UNDERSTANDING *M. GENITALIUM*

M. genitalium was first identified in 1980 in two patients with urethritis. [10] The bacteria are extremely slow growing and fastidious, which makes them difficult to culture in the lab and difficult to follow up by performing phenotypic studies. It is only with the recent availability and acceptance of molecular testing in diagnostic labs that the relationships between *M. genitalium* and genital infections have been confirmed.

M. genitalium is now recognized as an STI that can cause serious morbidities. [1,2] Studies have shown that it is present in 10% to 35% of men diagnosed with non-chlamydial non-gonococcal urethritis (NCNGU).[1] Similarly, associations have also been established between *M. genitalium* and cervicitis and pelvic inflammatory disease in women.[11]

Treatment and Resistance. Early in vitro studies showed that *M. genitalium* was highly susceptible to tetracyclines and macrolides.[12] In the clinic, however, doxycycline (a tetracycline) showed poor efficacy for eradicating *M. genitalium* (<50%), while azithromycin (a macrolide) was shown to be potent. [12,13] Consequently, azithromycin is now the recommended treatment for patients infected with *M. genitalium*, although various countries have adopted different dosing guidelines and there is continuing controversy about the optimal dosing schedule for eradication.[14] The regimens followed typically provide either a single 1 g dose or an extended azithromycin schedule of 500 mg on day 1 followed by 250 mg on days 2 through 5.

Recent studies have shown azithromycin resistance developing among patients treated with a single 1 g dose. [14] Mutations conferring resistance are now detected in 30% to 100% of cases of *M. genitalium*-infected patients in countries where 1 g azithromycin is used for treating genital infections and where limited testing for *M. genitalium* is available to guide treatment.[12–15]

The most disturbing observation in the evolving resistance to this antimicrobial is that cure rates for *M. genitalium* infections appear to be declining globally. There now appears to be an accelerated rate of increase in resistance to azithromycin as well as in the number of patients who have failed treatment and do not respond to higher doses of the drug.[8]

MOLECULAR DETECTION

Resistant strains of *M. genitalium* had been shown to develop point mutations in the 23S rRNA gene.[19] Mycoplasmas naturally have a high mutation rate, so it is reasonable to expect that point mutations conferring high-level resistance would likely be randomly present in a population of *M. genitalium* organisms.[20] The random presence of such minority strains among infected patients before they have been treated may be contributing to the rapid emergence of resistant mutants.[6]

Mutations at two loci in the 23S rRNA gene have been detected in clinical samples from azithromycin-resistant patients. They may have one or more mutations at positions A2058G, A2059G, A2058C, A2059C, or A2058T (*E. coli* numbering). Studies have shown there is 100% agreement between in vitro macrolide resistance, failure of azithromycin therapy, and the presence of one or more of these 23S mutations.[21,22]

For *N. gonorrhoeae*, another STI that is renowned for its antimicrobial resistance, the US Centers for Disease Control and Prevention recommends identification through the use of molecular diagnostics, followed by antimicrobial susceptibility culture to identify the resistance profile of the organism. Indeed, culture is the gold standard for resistance characterization in most bacterial investigations. From a clinical point of view, however, culture is impractical for the slow-growing *M. genitalium,* as it can take months to culture this bacterium.

The discovery of resistance-associated mutations for *M. genitalium* opened the possibility of using molecular methods to detect resistance in addition to identification. Sequencing technology allowed researchers to characterize the 23S rRNA mutations in their patient specimens, and studies from Australia, France, Japan, the Netherlands, and Norway, showed that these mutations were present in 10% to 40% of *M. genitalium*-positive specimens.[1–4] Despite this advance in sequencing technology, obtaining timely resistance information that can guide the initial course of treatment has remained a challenge.

ADVANCES IN MOLECULAR DIAGNOSTICS

Molecular diagnostics based on polymerase chain reaction (PCR) and transcription mediated amplification (TMA) can overcome turnaround time and throughput challenges. Several companies have already developed commercial tests for rapid identification of *M. genitalium* (see Table 1). In a typical assay based on PCR, complimentary primers are used to selectively amplify the genetic sequence of interest (the target). However, in order to utilize PCR to discriminate antibiotic-resistant mutations, the amplification needs to selectively distinguish the one or two bases where mutations are known to occur.

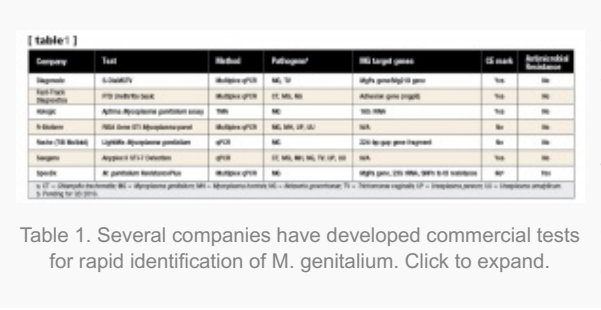

Table 1. Several companies have developed commercial tests for rapid identification of M. genitalium. Click to expand.

Recent advances in molecular techniques are improving the sensitivity and selectivity of real-time PCR techniques (qPCR) so that multiple resistant mutations at very close positions can be detected at the same time as the wild-type RNA. *M. genitalium* ResistancePlus is a new assay developed by SpeeDx, Eveleigh, NSW, Australia, and is the only test currently available to simultaneously identify *M. genitalium* and detect the five mutations associated with azithromycin resistance in the 23S rRNA gene.

The SpeeDx assay enhances qPCR using two novel tools called PlexPrime and PlexZyme technology. PlexPrimers selectively amplify mutants over wild-type RNA, and PlexZymes allow for efficient multiplexed detection and signaling. This unique combination allows “stacking” of the five mutation assays for a single readout.

PLEXPRIME AND PLEXZYME TECHNOLOGY

PlexPrime is a novel priming technology designed to selectively amplify mutations. Each PlexPrime primer works by combining three functional regions: a 5′ target recognition sequence, a 3′ target-specific sequence, and an intervening insert sequence (INS) region which is mismatched with respect to the target (see Figure 1). The 5′ target recognition sequence anchors the primer to a particular location on the target near the site of the mutation. This target sequence will be common to mutant and wild-type organisms. The 3′ target-specific sequence selectively recognizes a mutant sequence of interest by matching the terminal base to the mutation. This sequence will be present only in the mutant. The mismatched INS region inserts a target-independent sequence into the resulting amplicon. These mutant-derived amplicons now vary from wild type by 12–15 additional bases, so they are amplified more efficiently during subsequent amplification cycles.

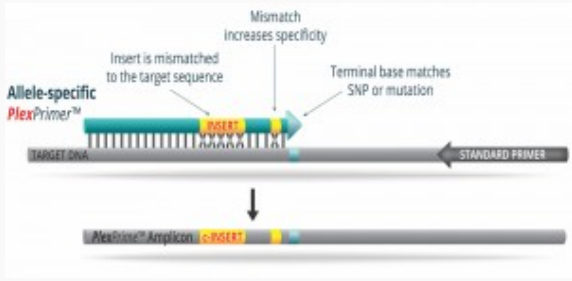

Figure 1. PlexPrime technology selectively amplifies mutations. Click to expand.

Molecular diagnostics typically employ one of two reporter methods to detect amplicon products: nonspecific fluorescent dyes that intercalate with the amplicon double-stranded DNA, or sequence-specific probes labeled with a fluorescent reporter that is quenched until the probe hybridizes with its complementary sequence. PlexZyme uses the second of these methods, but instead of a simple 1:1 hybridization between probe and amplicon that generates a signal, PlexZyme cleaves the probe and releases it, making it available to hybridize and cleave repeatedly, and creating a larger signal from a single amplicon.

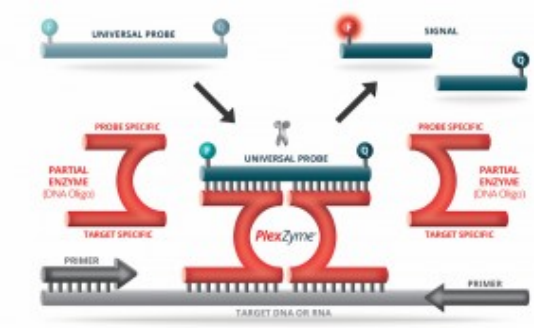

Figure 2. The mechanism of action of the PlexZyme technology. Click to expand.

Specifically, PlexZymes are catalytic DNA-based enzymes that assemble only in the presence of a target sequence and are capable of cutting the probes, releasing a fluorescent signal that can be monitored in real time.[23] PlexZymes are made up of two DNA oligonucleotides (called partial enzymes or “partzymes”). These partzymes are initially inactive. When bound adjacently on an amplicon, however, they form active, catalytic PlexZymes (see Figure 2).

PlexPrimers containing a mismatched INS region create amplicons that are distinctly different from their parent sequence. Unique INS regions in the primers can be matched with each individual mutation. The INS region can also be matched with a corresponding PlexZyme that cleaves a uniquely labeled probe (see Figure 3). Using different mutation-specific PlexPrime and PlexZyme combinations, the five *M. genitalium* mutations associated with azithromycin resistance can be detected in a single well, using standard qPCR instruments (see Figure 3).

Impact on Clinical Outcomes

The *M. genitalium* ResistancePlus test has been evaluated on retrospective patient samples in a clinical setting, and was found to provide high sensitivity and specificity compared to both the reference assay and sequencing method for identifying mutations.[23] Unlike sequencing, which is too slow to guide treatment, this molecular assay can provide significant time benefits in a clinical setting.

Figure 3. Together PlexPrime and PlexZyme facilitate multiplexing. Click to expand.

Using the SpeeDx test, patients with detectable mutations at A2058 or A2059, who may fail azithromycin treatment, could be more quickly directed to a second-line treatment. This practice could also reduce the

transmission period for macrolide-resistant strains. Timely detection of antibiotic resistance could enable the development of better algorithms for the treatment of *M. genitalium* infection and promote responsible stewardship of antibiotics.

These developments constitute a model for personalized treatment for *M. genitalium* that can be expanded to include any STIs, or indeed any infectious organism with recognizable molecular biomarkers for antibiotic resistance. Multiplex detection of different antibiotic markers could generate resistance profiles to multiple antibiotics, which will be increasingly useful for the emerging problem of multidrug resistance.

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