

# Technology Matters: Next-Generation RNA-Based Diagnostics Improve Disease Detection

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## Introduction

Molecular assays are rapidly becoming the standard of care in the diagnosis of many infectious gynecologic conditions as providers seek objective tools for guiding clinical practice. However, misconceptions surrounding these essential tools, such as understanding the attributes of assays that detect DNA versus RNA sequences, currently exist among clinicians. The capability to detect nucleic acid sequences, made possible by recent advancements in nucleic acid amplification tests (NAATs), has powerful clinical ramifications for the detection of infectious organisms as well as the identification of cervical cancer precursors. Clinicians can now diagnose patients with improved accuracy for numerous pathogens, including *Mycoplasma genitalium*, the organisms that are associated with bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC), *Trichomonas vaginalis* (TC), and human papillomavirus (HPV). Aptima® assays, RNA-based molecular diagnostics, will be reviewed in this report.

## Background and Benefits of NAATs

Stemming from their superior sensitivity and specificity compared to traditional microscopic techniques and quick turnaround time compared to culture, NAATs are currently recommended by the Centers for Disease Control and Prevention (CDC) to detect *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, herpes simplex

virus, and other gynecologic pathogens.<sup>1-3</sup> NAATs can detect low-titer infections by amplifying DNA or RNA molecules by a million-fold within hours, improving diagnostic sensitivity and accuracy.<sup>4</sup>

The first NAAT was a DNA amplification method known as polymerase chain reaction (PCR) that was developed in the 1980s.<sup>4</sup> PCR denatures a segment of DNA into two single-stranded DNA molecules and then rebuilds the double-stranded DNA via DNA polymerase.<sup>5</sup> This process, resulting in a duplication of the original DNA, can be repeated, creating numerous copies of the target DNA. After amplification, nucleic acid hybridization is performed using a fluorescently-labeled probe that pairs with a sequence of interest, such as one unique to an infectious organism.<sup>5</sup> An instrument then measures the amount of fluorescent signal emitted, which increases relative to the amount of the target pathogen present. Other types of NAATs are based on RNA amplification. Transcription-mediated amplification (TMA), one type of RNA amplification, follows a process similar to that of PCR but uses RNA sequences as the initial target.

## Differentiating NAAT Technologies: The Aptima® System

Unlike other RNA-based NAAT methods, the Aptima® assays use magnetic microparticles to capture the hybridized target.<sup>6</sup> The captured oligonucleotides, which are hybridized to the target RNA sequence, are drawn to

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the side of the tube by magnets and separated from the rest of the specimen.<sup>6</sup> After multiple washes to remove unbound material, including potentially interfering substances, just the hybridized target remains.<sup>6</sup>

The next step, target amplification, is performed using a reverse transcriptase and RNA polymerase.<sup>6-10</sup> Finally, using a chemiluminescent marker, nucleic acid hybridization is performed, allowing for the detection of the specific RNA transcript.<sup>6</sup> RNA-based NAAT assays can be used to detect ribosomal RNA (rRNA), a structural component of the ribosome of which there can be thousands of copies per cell. NAATs can also detect messenger RNA (mRNA), which is the precursor of proteins in the cell and can indicate not only the presence of a specific nucleotide sequence, but also that it is functional and may be contributing to a disease process.

## **M. genitalium: Prevalence and Detection Challenges**

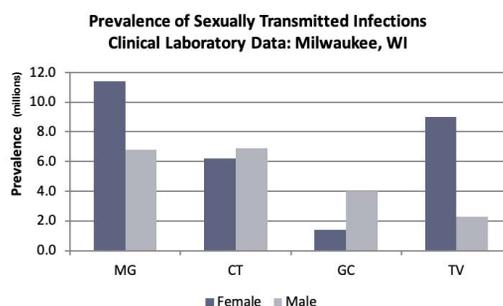
*M. genitalium*, an underdiagnosed and undertreated genitourinary pathogen, has traditionally been difficult to diagnose. Culture of the organism can take up to 6 months.<sup>11,12</sup> Gram stains, used to screen for other bacterial pathogens, are not useful given that *M. genitalium* lacks a cell wall.<sup>12</sup> Although the CDC recommends diagnosis by NAAT, no US Food and Drug Administration (FDA)-cleared tests have been available until recently, leading to missed opportunities for diagnosis and treatment.<sup>11,12</sup>

Data over the last decade have demonstrated that *M. genitalium* is not only more prevalent than previously thought, but a significant healthcare concern with long-term sequelae, similar to *C. trachomatis* or *N. gonorrhoeae*.<sup>11,13-17</sup> Although prevalence is estimated at 0.8% to 4.1% among low-risk women, higher rates have been reported among high-risk patients presenting to sexually transmitted infection (STI) clinics.<sup>11,13-17</sup>

Found in the epithelial cells of the genital and urinary tracts, *M. genitalium* is more prevalent than *N. gonorrhoeae* and just as common, if not more, than *C. trachomatis*.<sup>11,18,19</sup> Figure 1 illustrates the prevalence of *M. genitalium* when compared to other common STIs. The referenced studies were performed in both symptomatic and asymptomatic men and women in diverse clinical sites, including family medicine, obstetrics and gynecology, family planning, public health, and STI clinics.<sup>17-19</sup>

As most women infected with *M. genitalium* are asymptomatic, a sensitive test is essential.<sup>17,19</sup> Without early diagnosis and treatment, these women are at risk for cervicitis, pelvic inflammatory disease (PID), infertility, preterm birth, and other adverse pregnancy outcomes.<sup>20,21</sup> *M. genitalium* has been identified in up to 30% of women with clinical cervicitis, and up to 22% of women with pelvic inflammatory disease.<sup>12</sup> As *M. genitalium* infections have similar symptoms as chlamydia or gonorrhea, empiric treatment with azithromycin frequently occurs, but suboptimal treatment can contribute to macrolide resistance and difficult-to-treat organisms.<sup>22</sup>

Figure 1: *M. genitalium* Prevalence in the United States<sup>17,19</sup>  
MG = *Mycoplasma genitalium*; CT = *Chlamydia trachomatis*, GC = *Neisseria gonorrhoeae*, TV = *Trichomonas vaginalis*



## **The Aptima® M. genitalium Assay**

In January 2019, the FDA approved the first in-vitro diagnostic assay for *M. genitalium*, the Aptima® *M. genitalium* assay, which detects rRNA.<sup>22</sup>

The Aptima® *M. genitalium* assay was shown to be both sensitive and specific for the detection of *M. genitalium* in a multicenter prospective trial of over 3,000 sexually active men and women.<sup>22</sup> Up to four specimens were collected from each female (including urine, patient-collected vaginal, clinician-collected vaginal, and endocervical samples), and up to three specimens collected from each male (including urethral, penile meatal, and urine samples). All specimens were tested using the Aptima® *M. genitalium* assay as well as three alternate TMA assays to ensure validity. Patients were considered to be infected with *M. genitalium* if at least two alternate TMA assays resulted positive. Among females, vaginal swabs achieved a specificity of approximately 98% and sensitivity of 92% to 98%. Urine testing and endocervical swabs, while slightly less sensitive and specific, still resulted in acceptable accuracy.<sup>22</sup> Another study, recently published in the *Journal of Clinical Microbiology*, revealed that the Aptima® *M. genitalium* assay achieved 100% sensitivity and 99.9% specificity in the detection of *M. genitalium*.<sup>24</sup>

DNA testing methods have also been studied for the detection of *M. genitalium*, rRNA testing is preferable, as it is more sensitive at detecting infections with a low bacterial load. When compared to DNA, rRNA is much more abundant in the cell. Given that many *M. genitalium* infections are low titer and require a highly sensitive detection method, rRNA-based assays are preferable to DNA-based assays. A 2017 study compared the Aptima® *M. genitalium* assay to both PCR testing and the SpeDx DNA assay.<sup>25</sup> While both RNA and DNA methods showed similar specificities, Aptima® testing achieved higher sensitivity over DNA PCR (100% versus 59.74% respectively), indicating that the use of DNA-based assays are more likely to result in missed diagnoses.<sup>25</sup>

## **Vaginitis Diagnosis via rRNA**

Just as the diagnostic accuracy of *M. genitalium* can be improved using rRNA detection, the diagnosis of infectious vaginitis can also be improved via rRNA testing methods. Using NAATs, rRNA from bacteria associated with BV and fungal organisms associated with yeast infections can be detected and quantified, ensuring an accurate diagnosis.

Vaginitis, defined as vaginal inflammation associated with vulvovaginal itching, burning, irritation, and discharge, remains one of the most frequent reasons women visit their gynecologists.<sup>26</sup> These symptoms can lead to significant pain and sexual dysfunction, resulting in poor self-image.<sup>26</sup> Common infectious causes of vaginitis, representing over 90% of cases, include BV, vulvovaginal candidiasis, and *T. vaginalis*.<sup>26,29</sup> Among women with symptomatic vaginitis, 22% to 50% are diagnosed with BV, 17% to 39% with vulvovaginal candidiasis, and 4% to 35% with *Trichomonas*.<sup>27</sup> As symptoms can be nonspecific and overlapping, clinical diagnosis alone is often insufficient. The CDC currently recommends NAAT for *Trichomonas*, given that the traditional wet mount and visualization of organisms is only about 50% sensitive.<sup>28</sup> BV and vulvovaginal candidiasis are traditionally diagnosed clinically.

Similar to BV, the diagnosis of vulvovaginal candidiasis is typically performed in the office setting using microscopy via direct visualization of blastospheres or pseudohyphae on saline or 10% KOH microscopy.<sup>26</sup> Up to 89% of cases are caused by *Candida albicans*.<sup>31</sup> *Candida glabrata*, representing the majority of non-albicans infections, causes similar symptoms but is less likely to respond to azole treatment.<sup>26</sup> As traditional methods of *Candida vaginitis* diagnosis do not discriminate between species, these women may experience persistent or recurrent symptoms. Although species evaluation can be performed using a yeast culture, this can take up to 7 days.<sup>26</sup>

However, NAAT technology using rRNA has recently become available, promising to improve the sensitivity and specificity in diagnosing these infections.

### The Aptima® BV and CV/TV Assays

The Aptima® BV assay is a NAAT that detects rRNA from anaerobic bacteria commonly implicated in BV, such as *G. vaginalis* and *A. vaginae*, as well as three species of *Lactobacillus* (*L. crispatus*, *L. jensenii*, and *L. gasseri*).<sup>7</sup> Using the assay software, an algorithm then calculates the relative abundance of healthy lactobacillus to pathogenic bacteria in order to diagnose BV.<sup>7</sup> Test results are reported as positive, negative, or invalid for BV.<sup>7</sup> The Aptima CV/TV assay detects a *Candida spp* group (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. dubliniensis*), *C. glabrata*, and *Trichomonas vaginalis*.

Both the Aptima® BV and CV/TV assays were evaluated in prospective, multicenter studies of over 1,000 women with symptomatic vaginitis, using traditional diagnostic methods for reference testing.<sup>7,8</sup> As shown in Table 1, both tests were found to be highly sensitive and specific for the detection of both BV and vaginal *Candida spp*. Looking at *C. glabrata*, specificity was even further improved (Table 1).

In addition to the detection of *Candida*, the Aptima® CV/TV assay also detects *T. vaginalis*, another common cause of vaginitis.<sup>8</sup> *T. vaginalis* is a sexually transmitted pathogen that leads to similar symptoms as BV and vulvovaginal candidiasis. In addition, infection has been associated with an increased risk of HIV acquisition, prolonged HPV infection, and increased risk of

Table 1: Sensitivities and Specificities for the Aptima® Vaginitis Assays<sup>7,8</sup>

Aptima® Assay	Sensitivity	Specificity
Bacterial Vaginosis		
Clinician-Collected Swab	95%	89.6%
Patient-Collected Swab	97.3%	85.8%
<i>Candida</i> species (general)		
Clinician-Collected Swab	94.5%	94.2%
Patient-Collected Swab	94.4%	90.9%
<i>Candida glabrata</i>		
Clinician-Collected Swab	94.3%	99.1%
Patient-Collected Swab	88.9%	98.6%

concurrent STIs.<sup>8</sup> Traditional microscopic wet mount is only 50% sensitive for the detection of *Trichomonas*.<sup>28</sup> Therefore, the CDC currently recommends NAAT for the diagnosis of this pathogen.<sup>28</sup> The Aptima® CV/TV assay uses the same CDC-recommended assay as is currently offered as a standalone *Trichomonas* assay. Using the Aptima® assays, the three main infectious causes of vaginitis can be identified with improved specificity and sensitivity over traditional microscopic diagnostic methods.

### Cervical Cancer Screening via mRNA

Improved cervical cancer screening has also been made possible via the detection of HPV with RNA-based technology. Given that high-risk HPV subtypes are responsible for most cervical cancer cases, expert organizations, such as the American College of Obstetricians and Gynecologists (ACOG) and the Society of Gynecologic Oncologists (SGO), now recommend HPV screening for all women over 30.<sup>32,33</sup> Screening can be performed using DNA or RNA detection methods. Although DNA testing methods are acceptable, the presence of HPV DNA only indicates that the virus is present. It does not distinguish between active disease with oncogenic potential verses an infection which is likely to be cleared by the immune system.<sup>34–36</sup> Alternatively, an RNA-based assay detects messenger RNA, which is produced after the HPV DNA integrates into the host genome, indicating an active infection with true oncogenic potential.<sup>34–36</sup>

### The Aptima® HPV Assay

Currently, six HPV tests are approved for cervical cancer screening by the FDA.<sup>37,38</sup> Five of these detect DNA; one, the Aptima® HPV assay, detects HPV mRNA, including 14 high-risk types of HPV.<sup>37</sup> While all of the available HPV detection assays are highly sensitive and minimize false negatives, the HPV RNA detection assay exhibits a higher specificity, indicating that when a positive infection is detected, it is more likely to correlate with clinically-relevant cervical disease.<sup>39–58</sup>

The largest trial to date performed on the Aptima® HPV mRNA test was the CLEAR trial, which included over 11,000 women.<sup>39</sup> The study consisted of two arms: women with atypical squamous cells of undetermined

Table 2: Long-Term Equivalency of HPV mRNA Detection Compared to DNA-Based Assays<sup>67-70</sup>

Study	Screening Population	# Years of Follow-Up	Risk of CIN2+ Following Baseline HPV mRNA-	Risk of CIN2+ Following Baseline HPV DNA-	Statistically Significant Difference?
Reid et al. CLEAR study <sup>62</sup>	n=10,509	3	0.23%	0.26%	No
Cook et al. FOCAL study <sup>63</sup>	n=3,476	4	0.53%	0.56%	No
Iftner et al. GAST study <sup>66</sup>	n=10,040	6	0.62%	0.47%	No
Forslund et al. <sup>65</sup>	n=65,911	7	0.16% for CIN3+	0.12% for CIN3+	No

significance (ASCUS) and women negative for intraepithelial malignancy (NILM) on routine cytologic examination. Women with an ASCUS result were referred for colposcopy while those with negative cytology underwent colposcopy, but only if Aptima<sup>®</sup> HPV mRNA testing was positive. Researchers concluded that, when compared to HPV DNA testing methods, the Aptima<sup>®</sup> HPV assay had similar sensitivity and superior specificity for the detection of CIN2 and CIN3.<sup>39</sup> Since the CLEAR trial, numerous studies have confirmed these findings.

Trials have also demonstrated that results from HPV mRNA detection are equivalent to the DNA-based assays for detecting moderate to severe cervical dysplasia up to 7 years after baseline testing (summarized in Table 2).<sup>62-65</sup> By reducing false-positive screening tests, clinicians can reduce unnecessary colposcopies, decrease patient anxiety, and improve patient quality of life.

## Conclusion

RNA-based testing modalities represent an improvement over clinical and microscopic assessments as well as DNA assays, both in the detection of infectious organisms and the identification of cervical cancer precursors. The high sensitivity of RNA detection methods ensures that infections are diagnosed and treated, while the high specificity of these methods prevents patients from being subjected to unnecessary treatment.

Emerging NAAT technologies have enabled numerous RNA amplification techniques to be utilized in women's health, including those used for the detection of *M. genitalium*, BV, vulvovaginal candidiasis, *T. vaginalis*, and HPV. With advanced RNA-based diagnostics, such as the Aptima<sup>®</sup> assays reviewed above, infectious organisms can be identified and treated earlier, preventing long-term sequelae and patient suffering while minimizing false-positive results. Using these tools, clinicians can feel confident they are achieving accurate diagnoses and providing optimal patient care.

## Authors' Biographies

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Erik Munson is affiliated with the College of Health Sciences at Marquette University in Milwaukee, Wisconsin. Dr. Munson completed postdoctoral training at the Wisconsin State Laboratory of Hygiene and the University of Iowa Hospitals and Clinics. He has attained diplomate status with the American Board of Medical Microbiology. Dr. Munson and his staff have published peer-reviewed manuscripts with respect to molecular diagnostics of STI pathogens, optimization of FDA-cleared assays, and local surveys of antimicrobial resistance. Dr. Munson has served as past Chair of American Society for Microbiology Division V and currently is associate editor for the *Journal of Clinical Microbiology*.

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