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Review

Nucleic Acid Amplification Testing for *Neisseria gonorrhoeae*

An Ongoing Challenge

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Nucleic acid amplification tests (NAATs) for the detection of *Neisseria gonorrhoeae* became available in the early 1990s. Although offering several advantages over traditional detection methods, *N. gonorrhoeae* NAATs do have some limitations. These include cost, risk of carryover contamination, inhibition, and inability to provide antibiotic resistance data. In addition, there are sequence-related limitations that are unique to *N. gonorrhoeae* NAATs. In particular, false-positive results are a major consideration. These primarily stem from the frequent horizontal genetic exchange occurring within the *Neisseria* genus, leading to commensal *Neisseria* species acquiring *N. gonorrhoeae* genes. Furthermore, some *N. gonorrhoeae* subtypes may lack specific sequences targeted by a particular NAAT. Therefore, NAAT false-negative results because of sequence variation may occur in some gonococcal populations. Overall, the *N. gonorrhoeae* species continues to present a considerable challenge for molecular diagnostics. The need to evaluate *N. gonorrhoeae* NAATs before their use in any new patient population and to educate physicians on the limitations of these tests is emphasized in this review. ***(J Mol Diagn 2006, 8:3–15; DOI: 10.2353/jmoldx.2006.050045)***

Gonorrhea Epidemiology and Management

Neisseria gonorrhoeae is the etiological agent of the sexually transmitted disease (STD) gonorrhea, which globally causes an estimated 60 million new cases of gonococcal disease annually.[1] In 2003, it was second to *Chlamydia trachomatis* as the most reported notifiable sexually transmitted disease in the United States, with 335,104 cases of gonorrhea reported.[2] Infections with *N. gonorrhoeae* are primarily restricted to the mucus membranes of the endocervix, urethra, rectum, and pharynx. In females, gonorrhea is a major cause of pelvic inflammatory disease and may lead to tubal infertility, ectopic pregnancy, and chronic pelvic pain, whereas in males, it primarily causes urethritis. Importantly, these infections may often be asymptomatic, thereby contributing to further transmission and maintenance of the disease within populations.[1–3]

Control of gonorrhea, with a consequent reduction in morbidity due to its complications, is difficult and involves, among other factors, the need for complex social and behavioral change.[4] Laboratory contributions to the control of this disease include enhanced diagnosis and surveillance of antimicrobial resistance in the gonococcus to ensure that disease is both recognized and treated optimally.[4] However, the gonococcus is a readily transmissible, highly transformable, strictly human pathogen that is highly adapted to a particular biological niche where it adapts rapidly to host influences.[5,6] Notably, it has the capacity to alter its phenotypic and genotypic characteristics by numerous mechanisms, some of which are unique to the pathogenic *Neisseria*.[7] When coupled with its fastidious growth requirements, this capacity has led to difficulties in laboratory diagnosis and confirmation of this diagnosis by traditional culture-based methods and molecular-based approaches alike.

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An ideal diagnostic test for gonococcus is one in which sample collection is noninvasive and in which testing is cheap and can be performed simply and rapidly while the patient waits to obtain a result that is both sensitive and specific and that provides guidance regarding optimal treatment. Such a test does not exist, of course, despite concerted attempts for over a decade boosted by the added incentive of a substantial "reward" offered by the Rockefeller Foundation.[8] However, once developed and applied, testing that results in enhanced diagnosis can, if combined with appropriate treatment, effect significant reductions in disease incidence.[9] Thus, the applications for diagnostic tests for gonococci are many, and there is a need for improved laboratory tests that can be used both to screen for disease on a population basis and to establish an etiological diagnosis in the individual case.

Laboratory Diagnosis

Traditional Methods

Until the late 1980s, laboratory diagnosis of gonorrhea was limited to gram stain and bacterial isolation. The gram stain is a rapid tool and has comparable sensitivity to bacterial culture for symptomatic urethral gonorrhea in men. However, it is relatively insensitive for specimens collected from women and for specimens from extragenital sites where the specificity of gram stain may also be affected by the presence of commensal *Neisseria* species. Bacterial culture is generally regarded as sensitive and specific for the detection of gonorrhea, and to date, it remains the gold standard for definitive diagnosis. In addition to its relatively low cost, it is suitable for a broad range of specimen types and provides a viable organism for both antibiotic susceptibility testing and epidemiological investigation. Disadvantages of bacterial culture include the need to collect invasive specimens, which must be transported under appropriate conditions to maintain organism viability.[10,11] During the 1980s, an enzyme immunoassay (Gonozyme; Abbott Diagnostics, Abbott Park, IL) was also available but was withdrawn because of poor sensitivity.[12]

Nucleic Acid Tests

In the early 1990s, nucleic acid tests first became available for routine use. These include both nucleic acid hybridization assays and nucleic acid amplification tests (NAATs). The hybridization assays include the Gen-Probe PACE II (Gen-Probe, San Diego, CA)[13] and the Digene Hybrid Capture II assays (Digene Corp., Beltsville, MD).[14] These assays use a specific oligonucleotide probe to hybridize directly to *N. gonorrhoeae* nucleic acid present within a specimen. Reported sensitivity and specificity values of the hybridization assays showed that these may be below that of bacterial culture.[11,15,16]

To date, there have been four main commercial *N. gonorrhoeae* NAAT assays, including the Roche Cobas Amplicor (Roche Molecular Systems, Branchburg, NJ), the Gen-Probe APTIMA Combo 2 (AC2; Gen-Probe), the Becton Dickinson ProbeTec assay (Becton Dickinson, Sparks, MD), and the Abbott Ligase Chain Reaction (LCx) (Abbott Laboratories). All of these use multiplex NAAT assays, targeting both *C. trachomatis* and *N. gonorrhoeae*. In addition, each of these multiplex assays has used a unique *N. gonorrhoeae* gene target and amplification technology (Table 1). The Abbott LCx has previously been recalled because of manufacturing issues.[17] In addition to the commercial assays, numerous in-house *N. gonorrhoeae* NAAT assays have also been described. These have primarily used polymerase chain reaction (PCR), have targeted various *N. gonorrhoeae* genes, and for the most part, have not been multiplexed with other assays.

There are several advantages of *N. gonorrhoeae* NAATs. First, they offer improved sensitivity compared with bacterial culture. When compared with *N. gonorrhoeae* NAATs, gonoccocal culture ranges in sensitivity from 85 to 95% for acute infections and may fall as low as 50% for females with chronic infection.[11,18–20] The increased sensitivity of NAATs makes them particularly suitable for screening, enabling accurate diagnosis of both symptomatic and asymptomatic gonococcal infections, which is critical to control of the disease.[10] Second, specimens collected for NAAT assays do not require the

TABLE 1. Overview of Commercial *N. gonorrhoeae* NAATs

	Roche Amplicor	ProbeTec SDA	Abbott LCx	Gen-probe APTIMA
Gene target	Cytosine DNA methyltransferase gene	Multicopy pilin gene-inverting protein homologue	Opacity protein genes	16S ribosomal RNA gene
Amplification technology	PCR	SDA	LCR	TMA
Sensitivity	64.8 to 100%	84.9 to 100%	88.2 to 97.3%	91.3 to 98.5%
Specificity	93.9 to 100%	98.4 to 100%	98.5 to 100%	98.7 to 99.3%
Positive predictive value	31.3 to 100%	54.8 to 100%	59.3 to 100%	88.1 to 97.4%
Negative predictive value	99.5 to 100%	95.2 to 100%	98.5 to 100%	99.2 to 99.9%
Cross-reactivity with other *Neisseria* species	*N. cinerea, N. flavescens, N. lactamica, N. sicca, N. subflava*	*N. flavescens*, *N. lactamica*, *N. subflava*, *N. cinerea*	None identified	None identified
References	23, 49, 55, 56, 64, 72, 75, 88, 89, 104	23, 56, 57, 105	54, 56, 57, 76, 88, 89, 94–98	27, 76, 99

organism to be viable for detection and so require less stringent transport conditions compared with those collected for bacterial culture. Finally, NAATs can be used effectively on noninvasive specimens such as urine and self-collected specimens. This is particularly useful for patients in remote regions where sexual health services may not be available and for special populations where religious or cultural restrictions otherwise restrict opportunities for specimen collection.[10,21]

N. gonorrhoeae NAATs do have some limitations. These include the typical problems associated with the use of NAAT protocols, such as high cost, carryover contamination, inhibition of the reaction, high quality control requirements, and the absence of antibiotic resistance data. More importantly, there are sequence-related limitations that are unique to *N. gonorrhoeae* NAATs and include the generation of both false-negative and false-positive results. This is because target sequences may either be absent in some *N. gonorrheoae* subtypes or otherwise present in some commensal *Neisseria* strains. Overall, there is a broad range of technical challenges associated with the successful implementation of *N. gonorrhoeae* NAATs.

Common Challenges Associated with the Use of NAAT Technology

NAAT Inhibition

Inhibitory Substances

A disadvantage of NAAT methods is that they are susceptible to inhibition by substances that may be present in patient samples. These include β-human chorionic gonadotropin, crystals, hemoglobin, and nitrites.[22] Unless controlled for, these inhibitory substances may lead to the false-negative results in the assay. Inhibitors are common in urine and thus are particularly relevant to the sensitivity of *N. gonorrhoeae* NAATs.[22–25] The problem has been addressed by most commercial *N. gonorrhoeae* NAAT assays by the incorporation of an internal control reaction, which enables inhibition to be identified. However, the use of internal control reactions remains an issue for many diagnostic laboratories developing or using in-house NAAT assays, because these in-house assays do not often incorporate tests for inhibition.

Hesitance to use internal controls in in-house tests is usually associated with the additional costs involved; essentially the reagent costs and workload may double if the internal control reaction is run separately from the target organism. Although multiplexed reactions (incorporating both the internal control and target organism assays) are a cost-efficient alternative, they can reduce the sensitivity of the target organism NAAT and so may not be a suitable solution. Nevertheless, laboratories failing to use internal control reactions run the risk of issuing false-negative results. Recently, there has been a call to make internal control reactions mandatory.[26] In any event, with increasing government regulation of *in vitro* diagnostics, laboratories using NAATs may soon be required to use all appropriate controls, including an internal control.

Competitive Inhibition

One of the benefits of commercial *N. gonorrhoeae* NAAT assays is that most are multiplexed with assays to detect *C. trachomatis* and inhibitory substances (internal control reactions). Thus, these multiplex NAATs are in theory able to simultaneously detect and distinguish the presence of both common STDs or otherwise identify inhibition. Unfortunately, in multiplex NAATs, competitive amplification may occur between the different reactions. Such competition is most pronounced where the nucleic acid concentration of one target greatly exceeds the other and may lead to the target of lesser concentration failing to amplify. Thus, co-infections of *N. gonorrhoeae* and *C. trachomatis* may be missed by multiplex assays. Gaydos et al[27] found that of 56 known *N. gonorrhoeae* and *C. trachomatis* co-infections, six were missed by the Gen-Probe AC2 transcription-mediated amplification (TMA) assay, with *C. trachomatis* identified in two specimens only and *N. gonorrhoeae* identified in four specimens only. Competitive inhibition was also reported in the Roche Cobas Amplicor multiplex system.[28] Overall, there has been little recognition of this phenomenon in the literature, suggesting that this problem is not common. However, there may be two other reasons for this. First, co-infections of *N. gonorrhoeae* and *C. trachomatis* are only common in areas of high incidence of both diseases and may often represent only a small proportion of positive samples in many studies. Second, co-infections could be missed by the methodology used by such studies. This is because many studies merely compare one multiplex NAAT with another multiplex NAAT, and so both assays may be subject to the same problem. To determine whether co-infections are indeed being missed, it may be more prudent to include individual *N. gonorrhoeae* and *C. trachomatis* NAATs in the evaluations of multiplex assays.

NAAT Cost and Specimen Pooling

NAATs require careful cost analysis before implementation because they are generally more expensive to perform than traditional methods such as bacterial culture. In addition, NAATs can be considerably more laborious and time consuming. To reduce NAAT costs, some laboratories have investigated pooling specimens.[10] The overall impact of pooling specimens for *N. gonorrhoeae* NAATs is still unclear, although at least two studies have shown some benefit using such an approach. Kacena et al[29] found that the costs of using an *N. gonorrhoeae* ligase chain reaction assay could be decreased as much as 60% with a minimal loss of sensitivity (95.8% compared with nonpooled specimens) when testing pools of up to 10 urine specimens.[29] Similar findings were reported by Kissin et al.[30] A number of other studies have found similar results when pooling specimens for *C. trachomatis* NAATs.[31–35] Nevertheless, some concerns have been

raised over this practice. Pooling may decrease the sensitivity of *N. gonorrhoeae* NAATs, with the decrease in sensitivity of an assay being relative to the number of specimens pooled. For example, the pooling of 10 specimens has the potential to decrease the detection limit of the NAAT by a factor of 10. Whether this impacts on clinical sensitivity will depend on the detection limit of the assay being used. The results of Kacena et al[29] showed that the pooling of 10 urine specimens had little effect on the clinical sensitivity of the Abbott LCx assay. When investigating pooling of three to four specimens for *C. trachomatis*, Bang et al[36] showed that the sensitivity of the Roche Cobas Amplicor PCR was unaffected, whereas the sensitivity of the BD ProbTec strand displacement amplification (SDA) assay decreased to 86.5%. This demonstrates that the success of pooling is assay dependent and may be detrimental to some assays, even if a small number of samples are pooled.

Other factors to consider include the rate of inhibition. Potentially, the pooling of specimens with a high inhibition rate could lead to even higher rates of inhibition within the pools, necessitating the retesting of all of the individual specimens. Conversely, pooling may decrease the inhibition rate for some specimens.[31] Positivity rates are also important. Arguably, pooling may only be suitable for low incidence populations. This is because the benefits of pooling stem from the ability to simultaneously issue negative results for all specimens within a pool. Positive pools require the individual samples to be retested, and so high positivity rates may hinder the use of pooling. Furthermore, although pooling may generate substantial savings in reagent costs, it may save little in hands on time because there is additional work required to physically pool the samples and then analyze the results. These steps may also create further avenues for the introduction of human error into the testing procedure.[10] Overall, the success of pooling is dependent on the particular assay being used, the incidence of positive results, the incidence of NAAT inhibition, and the numbers of specimens being pooled. It should also be noted that such procedures may deviate from the manufacturer's instructions of FDA-approved tests. Therefore, stringent validation is an intrinsic requirement for any laboratory considering adopting this approach.

Assay Complexity and Quality Control

A further disadvantage of NAATs is that they are more complex than traditional methods. NAATs generally involve the performance of three intrinsic steps: nucleic acid extraction, amplification, and detection. Each one of these steps has the potential to introduce error, including human error, reagent failure, and carryover contamination. Thus, effective internal quality control and monitoring as well as the participation in proficiency panel testing are imperative to ensure efficiency of all of these processes. A detailed analysis of NAAT quality control issues is described by Burkardt[37] and Johnson et al.[10] Overall, such complexities highlight the need for adequate staff training and the need to allocate resources to ensure effective quality control, which again increases the cost of performing *N. gonorrhoeae* NAATs.

It should be noted that as NAAT technology has evolved, the complexity of performing commercial assays has significantly decreased. In fact, some commercial assays are now fully automated. For example, the TIGRIS version of the APTIMA Combo 2 test offers full automation from sample processing to detection. In addition, most commercial assays now incorporate the necessary controls for internal laboratory quality control. However, such quality control measures remain an issue for laboratories running "in-house" NAAT methods. Furthermore, the performance of any *N. gonorrhoeae* NAAT needs to be validated for any new patient population and should continually be monitored using external proficiency panels. In contrast, the limited complexity of bacterial culture isolation and identification requires few quality control measures, and validation of bacterial culture is not required for new patient populations.

Antibiotic Resistance

A notable limitation of *N. gonorrhoeae* NAATs is the inability to provide antibiotic resistance data, which is an emerging health concern given the increased reports of *N. gonorrhoeae* antibiotic resistance.[38] There have been several studies investigating the use of molecular techniques for identifying *N. gonorrhoeae* antibiotic resistance, and they have examined genes including the *penicillin-binding protein* and *gyrase A* genes[39,40] Nonetheless, such an approach is not yet a plausible alternative to phenotypic methods of sensitivity testing, because many genetic mechanisms of resistance have not as yet been identified. However, in our opinion, the inability of NAATs to provide antibiotic resistance data should not be a reason to exclude them as screening methods for *N. gonorrhoeae* detection. On the contrary, a testing algorithm would be enhanced if specimens providing positive results in the NAAT screen were then subjected to appropriate bacterial culture to provide antimicrobial data by phenotypic methods.

The aim of gonococcal susceptibility testing is to define a suitable treatment, defined as that which cures a minimum of 95% of infections when given as a single dose, preferably on diagnosis or first presentation.[41] This means that susceptibility testing is used to establish effective standardized or programmatic treatments and differs from practices in other conditions in which a suitable treatment is determined by examination of individual isolates tested on an emerging basis.[42] This further means that a sufficient and representative sample of gonococcal isolates, rather than all available gonococci, needs to be tested for antibiotic susceptibility. Thus, such a sample can be obtained by means of targeted culture of selected samples. For example, patients with a positive NAAT for *N. gonorrhoeae* are likely to return for follow-up treatment, and a swab may then be collected for bacterial culture and sensitivity testing.[43] Alternatively, if NAAT is performed in a timely manner, then *N. gonorrhoeae* may still be isolated from the specimen without need for further specimen collection.[44]

It should be noted that concerns have also been raised over the use of NAATs for monitoring responses to antibiotic therapy. The high sensitivity of NAATs allows them to detect nucleic acid from nonviable organisms after effective antibiotic treatment. Therefore, positive results can arise if patients are tested too soon after treatment, possibly resulting in unnecessary additional antibiotic administration. It was shown that after successful therapy for gonorrhea, NAATs continued to give positive results for several days, but gonococcal DNA was cleared from urogenital specimens within 2 weeks.[45] Certainly, support for such concerns has been found in studies investigating NAAT testing for chlamydia. In fact, the Centers for Disease Control (CDC) recommend that a follow-up sample for NAAT testing should not be taken for at least 3 weeks after antibiotic treatment for chlamydia.[10]

Sequence-Related Challenges for Gonorrhea NAAT Assays

Sequence Variation between N. gonorrhoeae *Subtypes*

The variation between *N. gonorrhoeae* subtypes and the continually changing prevalence of these subtypes in a patient population can have a significant impact on the success of *N. gonorrhoeae* NAATs. The *N. gonorrhoeae* species comprises a broad range of subtypes that exhibit considerable genetic variation, and these are not randomly distributed. That is, their distribution varies geographically, temporally, and between patient groups.[46–48] This has considerable implications for *N. gonorrhoeae* NAATs because their performance can vary between different patient populations, and potentially in the same patient population, or over time. A good example was recently provided by studies investigating the use of *N. gonorrhoeae* NAATs targeting the *cppB* gene. Some *N. gonorrhoeae* types lack the *cppB* gene, and there is a potential for false-negative results using this target. For instance, Palmer et al[49] showed that the *cppB* gene was absent in 18 of 20 specially selected *N. gonorrhoeae* isolates of the PAU$^-$ auxotype. Recently, two independent studies investigated the use of *N. gonorrhoeae cppB* PCR assays on populations in Australia and the Netherlands.[50,51] Tabrizi et al[51] found no false-negative results and considered the *cppB* gene assay to be suitable for use in a population in Victoria, Australia, whereas Bruisten et al[50] found a high incidence (5.8%) of false-negative results and concluded that the *cppB* gene target was not suitable for use in the Dutch population. Interestingly, other Australian studies have revealed a high incidence of *cppB*-related false-negative PCR results in a patient population in the Northern Territory in Australia, due to the recent appearance and expansion of a non-PAU$^-$ gonococcal subtype.[52,53] The disparity between these results clearly indicates that the incidence of *cppB*-negative *N. gonorrhoeae* strains varies between patient groups, presumably because of differences in the predominant *N. gonorrhoeae* subtypes in these populations. Furthermore, this highlights that successful evaluation of a *N. gonorrhoeae* NAAT on one patient population at one time point may not necessarily reflect the assay's suitability for use on another patient population or even in the same patient population over an extended period.

It should be noted that false-negative results arising from sequence variation have not as yet been reported for other *N. gonorrhoeae* NAAT assays. However, specimens that are culture positive and negative by NAAT methods are commonly reported.[54–57] For instance, Van Dyck et al[57] found specimens that were positive by culture but provided negative results by one or more of the NAATs used, including the Amplicor PCR, Abbott LCx, and ProbeTec SDA assays. Inhibitory substances were not identified in these specimens. Sequence variation could have been responsible for these negative results, but the isolates from these specimens were not further investigated. Therefore, other sources of NAAT failure cannot be excluded.

Cross-Reaction with Other Neisseria *Species*

Sources of Cross-Reaction

A further sequence-related problem with *N. gonorrhoeae* NAAT assays is cross-reaction with related *Neisseria* species resulting in false-positive results. The problem stems from the high degree of sequence homology between *N. gonorrhoeae* and these other *Neisseria* species. There are two sources of this homology. First, the genomes of *N. gonorrhoeae* and *N. meningitidis* are very similar and are thought to have evolved from a common ancestor. In fact, it has been suggested that the *N. gonorrhoeae* species may have arisen from a clone of *N. meningitidis* that was able to colonize the genital tract.[58,59] Second, the *Neisseria* species are unusual in that they are fully competent for DNA uptake throughout their entire life cycle. This enables frequent interspecific horizontal genetic exchange within the *Neisseria* genus.[60–63] These processes are well illustrated by the fact that phylogenetic grouping of *Neisseria* species using housekeeping genes, including the 16S rRNA gene, can be distorted by interspecies recombination events.[63] Crucially, for molecular diagnostics, this genetic exchange can lead to commensal *Neisseria* species acquiring gonoccocal genes. In combination, the above factors create a considerable dilemma for the molecular detection of *N. gonorrhoeae*. The high homology with *N. meningitidis* makes much of the genome unsuitable for NAAT targeting because of cross-reactions of the assay with *N. meningitidis* DNA, whereas genetic exchange can lead to the commensal *Neisseria* species acquiring regions of sequence that may otherwise be distinct from *N. meningitidis*.

Factors Limiting the Rate of Cross-Reaction

Neisseria strain variation: There are some factors that limit the potential for cross-reaction. Although a particular commensal *Neisseria* species may be known to cross-react with a particular *N. gonorrhoeae* NAAT, not all

strains of that species will necessarily produce a cross-reaction. Palmer et al[49] used 10 *N. meningitidis*, 23 *Neisseria cinerea*, 6 *Neisseria flavescens*, 11 *Neisseria subflava*, 7 *Neisseria mucosa*, 13 *Neisseria lactamica*, 8 *Neisseria sicca*, and 2 *Neisseria elongata* isolates to test the intragenus specificities of five different *N. gonorrhoeae* NAATs. Of these, 1 *N. flavescens*, 1 *N. lactamica*, and 1 *N. sicca* isolate cross-reacted with the Amplicor PCR test; 1 *N. flavescens*, 1 *N. lactamica*, 1 *N. subflava* and 2 *N. cinerea* isolates cross-reacted with the ProbeTec SDA assay; and 1 *N. cinerea* isolate cross-reacted with an in-house PCR targeting the *cppB* gene. Farrell[64] also investigated the specificity of the Amplicor PCR assay by testing an Australian panel of commensal *Neisseria* species comprising 2 *N. cinerea*, 5 *N. lactamica*, 4 *N. meningitidis,* 2 *N. mucosa*, 1 *N. sicca*, and 14 *N. subflava* strains and found cross-reactions with 6 *N. subflava* isolates only. Therefore, the production of false-positive results is dependent on the particular *Neisseria* strains present within a sample.

Neisseria strain distribution: There is some suggestion that the extent of cross-reaction of any given *N. gonorrhoeae* NAAT may vary depending on the patient population. For example, Farrell[64] postulated that the low positive predictive values of Amplicor PCR assay reported by his laboratory may be attributed to differences in the normal flora of the local Australian population, particularly the Australian indigenous population. It was contended that patients carrying commensal species that cross-react with the Amplicor PCR target may be at a higher incidence in this Australian population. It is interesting to note that Farrell[64] also investigated the intragenus specificity of an in-house *cppB* gene using Australian commensal *Neisseria* species and found no cross-reaction. Yet, a later Australian study found cross-reactions with *N. meningitidis* and *N. subflava* strains using a similar *cppB* PCR when testing *Neisseria* strains derived from a different Australian patient population.[65] This suggests that commensal *Neisseria* strains carrying certain gonococcal genes may also be nonrandomly distributed. Alternatively, the differences in these studies may just reflect sampling error and the possibility that larger panels of *Neisseria* strains may be required to adequately assess intragenus specificity. Importantly, this would suggest that testing small panels of commensal *Neisseria* strains may give little indication as to the actual positive predictive value of a test. Nevertheless, these studies highlight the importance of evaluating *N. gonorrhoeae* NAATs before use on any new patient population.

NAAT gene target: The above also shows that the incidence of cross-reactions varies depending on the test. In fact, studies have shown that the rate of *Neisseria* genetic exchange varies between genes, presumably because of differences in immune pressure.[62] This may explain why some assays suffer a higher rate of false positivity. Of two *N. gonorrhoeae* NAATs investigated by Palmer et al,[49] an in-house PCR targeting the OMPIII gene and the Abbott LCx targeting the *opa* genes, no cross-reactions were observed with the commensal *Neisseria* isolates tested. It was hypothesized that this was due to genetic exchange occurring less frequently at these loci.[49]

Specimen site restrictions: Finally, many commercial NAATs are restricted, being accredited only for testing urogenital specimens from adults.[10] Interestingly, this restriction highlights the cross-reactivity problems of *N. gonorrhoeae* NAATs. The reasoning for this restriction is that commensal *Neisseria* species are uncommon in the urogenital sites of adults and so there is less potential for obtaining false-positive results, in contrast to throat and rectal specimens (which are discussed below). In any event, the false-positive results that are obtained from urogenital specimens are most likely due to transient carriage of commensal *Neisseria* species in the genital tract, which occurs infrequently in adult populations. In fact, nongonoccocal species account for less than 1% of gram-negative diplococci isolated from the genital sites in heterosexuals.[66,67]

It should be noted that the current CDC recommendation is that bacterial culture should be used for urogenital, pharyngeal, or rectal specimens obtained from children who are suspected victims of child abuse. This is so that an isolate can be retained for additional testing. In addition, this prevents false-positive NAAT results potentially leading to erroneous reports of child abuse and unjustified prosecution.[10,68] Likewise, bacterial culture is the preferred method for diagnosis of gonococcal ophthalmia in neonates, given that neonatal conjunctivitis can be caused by several other *Neisseria* species.[69] Where bacterial culture is not available, some specialists support the use of *N. gonorrhoeae* NAATs in the above circumstances if positive results are verified by a supplementary test targeting a different *N. gonorrhoeae* sequence.[10]

Overall Impact of Cross-Reaction on NAAT Performance

Because of the above limitations on the potential for cross-reaction, the reported specificity rates of most *N. gonorrhoeae* NAATs are generally high, often exceeding 98% (Table 1). However, the positive predictive values vary depending on the patient population. In particular, positive predictive values can be unacceptably low in populations in which the prevalence of *N. gonorrhoeae* is low.[10,70] A simplified explanation for this is provided by Klausner[71]: if the specificity of a *N. gonorrhoeae* NAAT is 99.5%, then 0.5% of positive results will be false-positive results. Therefore, if the rate of test positivity in the population is 1.0%, then one-half of the observed positive results may be false-positive results, providing a positive predictive value of only 50%. When testing urogenital specimens in a low prevalence (0.5%) Canadian population, Diemert et al[72] found that the specificity of the Amplicor *N. gonorrhoeae* PCR assay was 98.7%, whereas the positive predictive value was only 31.3%. Thus, if positive results were issued for this Canadian population without supplementary testing, then close to 70% of positive results issued would in fact be false-positive results.

To improve positive predictive values, the CDC recently issued guidelines requiring supplementary testing for *N. gonorrhoeae* NAATs in which the positive predictive value of the screening assay is less than 90%.[10] Of course, this again impacts on the costs of performing *N.*

gonorrhoeae NAATs, and practical problems of patient recall for repeat sampling and testing also arise. However, in the absence of supplementary testing, repeat testing is still an issue for the commercial assays. This is because all of the commercial *N. gonorrhoeae* NAATs have an equivocal result zone that ultimately requires a small proportion of samples to be repeated. Furthermore, concerns have been raised over repeat testing of samples using the same test, given that such an approach is more likely to reproduce a false-positive result.[10] We recommend that a second *N. gonorrhoeae* NAAT, using an alternative gene target, be used for both repeat and supplementary testing. The need to determine the positive predictive value of an assay further emphasizes the requirement for validation of *N. gonorrhoeae* NAATs before using such tests in a diagnostic setting.

There has been some suggestion of overuse and overinterpretation of *N. gonorrhoeae* NAAT results. The criticism of overuse is aimed at the fact that *N. gonorrhoeae* NAATs are often used to screen patients who are at low risk of having the infection and that such testing may further decrease the positive predictive values of the NAAT. It was suggested that screening should be more selective and should be aimed at high risk patients only.[10,71] In addition, there are concerns that clinicians may rely too heavily on the NAAT for diagnosis of gonorrhea, and Klausner[71] suggested that clinicians should be aware of assay specificity when assessing test results.

Issues for Assay Validation

The sequence-related problems described above have also created considerable difficulties for *N. gonorrhoeae* NAAT validation. Because of the potential for both false-positive and false-negative results, samples providing discrepant results between the new and reference tests may often remain unresolved. This problem is further compounded by that fact that NAATs are generally more sensitive than traditional reference methods, including bacterial culture, so additional NAAT-positive results are common in such studies.[10]

Various approaches have been adopted to solve these problems. A common approach has been the use of discrepant analysis. Essentially, any specimen providing discrepant results between the new test and the reference test is retested using one or more different assays. For an evaluation of a new NAAT assay, this will usually involve retesting the specimen using one or more NAAT assays targeting different genes. The results of the additional NAAT assays are then used in the calculation of the sensitivity and specificity of the new test. There are some advantages to this approach, including that it is cheap and quick and involves little workload. However, discrepant analysis has been heavily criticized for the potential to introduce data bias in the calculations of test sensitivity and specificity, particularly where an NAAT is being compared with a less sensitive traditional method.[73,74]

To avoid such criticisms, other studies have adopted a consensus criterion for defining *N. gonorrhoeae* true positive results. Essentially, these algorithms act as an expanded gold standard. Rather than just retesting discrepant specimens, all specimens are tested by all assays. For example, a new NAAT test may be compared with the combined results of bacterial culture and two other NAAT assays; Van Dyck et al[57] defined an *N. gonorrhoeae* true positive result as either positive by culture or positive by two NAAT assays. Such algorithms may also use an "infected patient" standard, whereby a patient's infectivity status is determined on the basis of results obtained from both urine and genital swab specimens.[27,56,75] Importantly, these expanded gold standards accommodate the increased sensitivity of NAAT tests while also providing statistical validity. However, these algorithms operate under the assumption that both gonococcal NAAT targets are unlikely to be present in commensal *Neisseria* strains occurring in these specimens. This assumption may be valid for urogenital specimens where commensal species are uncommon but would not hold for extragenital sites such as the pharynx or rectum (discussed below). Furthermore, the assumption will also only hold if the NAAT assays target different gene sequences, rather than just different sequences on the same gene.

Other disadvantages to this approach include the substantial costs and workload involved in such a study. In addition, these expanded gold standards may also introduce other forms of bias. For instance, "infected patient" standards that depend on positive results being obtained from both urine and genital swab specimens may underestimate the sensitivity of tests. This is because some infected patients may have one specimen positive only.[76] Differences in the analytical sensitivities of NAAT assays may also create complications for such evaluations. By using TMA technology to amplify rRNA, the Gen-Probe AC2 assay has in theory the potential to detect lower concentrations of *N. gonorrhoeae* because the copies of 16S rRNA will usually exceed that of DNA targets used by other commercial *N. gonorrhoeae* NAATs.[27] Gaydos et al[27] found that the positive predictive values of the AC2 were 88.1 and 92.1% for swab and urine specimens, respectively. However, these figures were calculated using the Abbott LCx as the reference standard. When the false positives were retested by a supplementary TMA method (AGC; Gen-Probe), the majority of specimens produced positive results, suggesting that the additional AC2-positive specimens actually represented true positive results. It should also be recognized that bacterial culture identification systems are not infallible and that commonly used biochemical tests can fail to confirm the identity of some *N. gonorrhoeae* isolates.[44,77] This will also impact on evaluations of NAAT assays in which culture is used as the standard. Nonetheless, the expanded gold standard remains the preferred approach for *N. gonorrhoeae* NAAT evaluations.

In our opinion, evaluations of an *N. gonorrhoeae* NAAT should use at least two other *N. gonorrhoeae* NAATs targeting different gene targets as the standard. Preferably, these evaluations would include bacterial culture. Specimens providing positive results by bacterial culture or by both NAATs used as the standard are considered to represent true positive results. All other specimens are

considered to be negative. However, consideration should also be given to the type of specimens being tested. We would consider that a standard comprising two NAATs would be sufficient for urogenital specimens but would recommend a standard comprising at least three NAATs for extragenital sites, including pharyngeal and rectal specimens (discussed below).

Diagnosis of Pharyngeal and Rectal Gonorrhea

The current recommendation from the CDC is that only bacterial culture should be used to test for pharyngeal or rectal gonorrhea.[10] This recognizes both the specificity problems of *N. gonorrhoeae* NAATs and the high incidence of commensal *Neisseria* species in these sites. However, this creates somewhat of a quandary for clinical laboratories because testing for both urogenital and extragenital specimens requires both bacterial and NAAT protocols to be used. Interestingly, there is some suggestion that bacterial culture may be an insensitive tool for detecting pharyngeal and rectal gonorrhea in men who have sex with men (MSM). Ligase chain reaction (LCR) testing has indicated that the true incidence of pharyngeal and rectal gonorrhea in MSM may be double that detected by bacterial culture, suggesting that the sensitivity of bacterial culture may be as low as 50% for these specimen sites.[43,78,79] These results are supported by a more recent study using an *N. gonorrhoeae porA* pseudogene PCR assay.[80] The lower sensitivity of bacterial culture in the extragenital sites may be attributed to the heavy colonization of these sites by a broad range of other organisms, including other *Neisseria* species, which may interfere with *N. gonorrhoeae* isolation.[43] In general, pharyngeal and rectal gonorrhea are considered to be higher in MSM, and STDs are generally prevalent in these populations.[81,82] Many of these infections may be asymptomatic and may make a significant contribution to transmission of gonorrhea in MSM. Consequently, implementation of routine NAAT screening for pharyngeal or rectal gonorrhea could improve the control of gonorrhea in MSM.[43]

Unfortunately, commercial *N. gonorrhoeae* NAAT assays currently available appear to be unsuitable for screening these specimens. In fact, no commercial *N. gonorrhoeae* NAAT is licensed for use on pharyngeal or rectal specimens. Even the Abbott LCx assay, which was shown to be highly specific by Palmer et al,[49] has provided false-positive results in these samples. Young et al[43] found that the positive predictive value of the LCR was 94.1% for rectal swabs and 88.9% for pharyngeal swabs in a population of MSM, and similar results were reported by Page-Shafer et al.[78] In both of these studies, the incidence of *N. gonorrhoeae* was approximately 10%. Consequently, the positive predictive value of the LCR may be considerably less if a low incidence population were tested. More recently, Leslie et al[55] showed that the positive predictive value for the Roche Cobas Amplicor *N. gonorrhoeae* assay was only 20% for rectal swabs and as low as 5.6% for pharyngeal swabs in a Australian population comprising a high proportion of MSM.

Given these problems, it has been suggested that any *N. gonorrhoeae* NAAT-positive result from an extragenital specimen should be confirmed using a NAAT assay targeting a different genetic sequence.[55] This would improve the performance of *N. gonorrhoeae* NAAT assays on specimens from these sites. However, supplementary testing by only one NAAT may be insufficient to raise the positive predictive values of some *N. gonorrhoeae* NAAT assays to an acceptable level because the broad range of *Neisseria* species or strains in such specimens may enable one strain to cross-react with the screening assay while another could cross-react with the supplementary assay. This would give a false-positive result from the algorithm. Overall, it would appear that attempts to use any *N. gonorrhoeae* NAAT assay on pharyngeal or rectal specimens would require thorough validation for any prospective patient population. As discussed above, such validations would need to incorporate multiple *N. gonorrhoeae* NAATs targeting different genes as well as bacterial culture. Also, it should be noted that debate is ongoing over the clinical significance of pharyngeal gonorrhea, with suggestions that pharyngeal gonorrhea may spontaneously clear or otherwise not make a significant contribution to transmission of disease.[83–87] However, it is also recognized that pharyngeal carriage predisposes to disseminated gonococcal infection.[87]

Overview of Gonorrhea NAA Tests

Overall, it is clear from published evaluations that the performance of different *N. gonorrhoeae* NAATs varies widely depending on the patient population and reference standard used (Table 1). Certainly, some assays appear to have a greater requirement for supplementary testing, whereas the sensitivities published for some assays are notably higher than others.

Roche Cobas Amplicor

It is generally accepted that the Amplicor *N. gonorrhoeae* assay cross-reacts with strains of several commensal *Neisseria* species (Table 1). Notably, numerous studies conducted worldwide have shown positive predictive values for the Amplicor PCR assay on urogenital specimens below the 90% CDC threshold.[23,55,72,88,89] Increasing the positive result cutoff of the Amplicor assay will increase the positive predictive value of the test. This is because commensal *Neisseria* strains cross-reacting with the Amplicor assay may generally be at a low concentration or may contain minor base variations with the Amplicor PCR oligonucleotides. However, increases in the positive result cutoff can significantly reduce the sensitivity of the assay. Thus, supplementary testing is the only appropriate approach.[57,72]

Supplementary tests for the Roche Amplicor assay have included a 16S PCR assay provided by Roche as well as various in-house PCR methods targeting the *cppB* gene.[51,64,90] Roche later withdrew the 16S assay from the marketplace, so *cppB* gene-based PCR assays were widely adopted for confirmation. However, *cppB* gene-

based NAAT assays are now no longer considered adequate for some patient populations because of the limitations outlined previously.[50] More recently, a PCR assay targeting the *N. gonorrhoeae porA* pseudogene showed considerable promise as a suitable supplementary test for the Amplicor assay.[91] So far, this assay has only been validated in an Australian population and will need further validation in other populations. It should also be recognized that considerable sequence homology exists between the *porA* sequences of *N. gonorrhoeae* and *N. meningitides*, so there is a potential for cross-reaction with *N. meningitidis* using this gene as a NAAT target. However, the *porA* pseudogene PCR assay described above uses carefully selected *porA* target sequences that are specific to *N. gonorrhoeae* and do not cross-react with *N. meningitidis* DNA.[91] The porA protein is not expressed in *N. gonorrhoeae*, and therefore the *porA* pseudogene is not under immune pressure.[58] This makes the sequence a particularly stable target for NAAT development, a fact supported by a recent Swedish study that showed the *porA* pseudogene to be highly conserved across *N. gonorrhoeae* subtypes.[92] One question remaining is whether the target sequences used by the assay are likely to be found in other *Neisseria* species. Arguably, the lack of immune pressure on the *porA* pseudogene may make the sequence less likely to be transferred to other *Neisseria* via genetic exchange events. Intragenus specificity testing of the *porA* pseudogene assay provides some support for this.[80]

The Becton Dickinson ProbeTec SDA Assay

There have also been some reports of positive predictive values for the BD ProbeTec SDA assay falling below the 90% threshold. Chan et al[23] determined the positive predictive value for this assay to be 88.2% for 1224 urine specimens collected in a Canadian population. Also, Van Der Pol et al[56] conducted a multicenter evaluation of the SDA assay on 4131 urogenital specimens in the United States and found that the positive predictive value varied broadly depending on the center location, specimen type, and patient sex; the positive predictive values across the different locations ranged from 54.8 to 100% and 75.2 to 100% for female urine and swab specimens, respectively, and from 83.1 to 100% and 81.8 to 100% for male urine and swab specimens, respectively. The lowest values were recorded in areas of lowest *N. gonorrhoeae* incidence (1.2%). It should be noted that separate analyses based on specimen type and patient sex may underestimate the overall positive predictive value of the assay in each location. Nevertheless, these values highlight the difference in performance of the assay across these populations. Interestingly, Van Dyck et al[57] found that the BD ProbeTec SDA assay was the only assay of three *N. gonorrhoeae* NAATs, including the Roche PCR and Abbott LCx, to provide a specificity of 100% for 733 endocervical swab specimens collected in a Belgian population. Irrespective of this, a more recent German study has highlighted the need for supplementary testing of specimens positive in the ProbeTec SDA *N. gonorrhoeae* assay.[93] Clearly, the ProbeTec SDA assay cross-reacts with some commensal *Neisseria* strains,[49] thus suggesting that supplementary testing is required in some patient populations to satisfy the CDC recommendations.

The Abbott LCx Assay

In contrast, the Abbott LCx appeared to be suitable for use in most populations without the need for supplementary testing. Notably, the reported positive predictive values for the LCx usually fell within the range of 91.7 to 100%.[54,57,76,89,94–98] However, in the same multicenter evaluation described by Van Der Pol et al[56] above, it was shown that the positive predictive values obtained of the Abbott LCx fell below 90% for some specimen types; two laboratories provided positive predictive values of 59.3 and 85.6% for urine collected from female patients, whereas another laboratory reported a positive predictive value of 81.2% for urine samples collected from males. Again, it should be emphasized that separate analyses of each specimen type and patient sex may underestimate the overall positive predictive values of the assay. Overall, the positive predictive values for the LCx did exceed 90% for most specimen types in most locations used in the study.[56] In any event, the LCx has been withdrawn from the market because of issues related to poor quality assurance.[17]

Gen-Probe APTIMA Combo 2

Initial results for the Gen-Probe APTIMA Combo 2 (AC2) *N. gonorrhoeae* assay are favorable. Notably, Golden et al[99] found the positive predictive value for the AC2 assay to be 97.4% for 59,664 urogenital specimens collected from a female patient population in Washington state for whom the *N. gonorrhoeae* incidence was only 0.5%. This suggests that this assay may satisfy CDC recommendations in low *N. gonorrhoeae* incidence populations without the need for supplementary testing.[99] To date, there have been no reports of cross-reactions by the APTIMA Combo 2 with other commensal *Neisseria* species.[10] However, the literature suggests that this has not been thoroughly investigated as yet. Gen-Probe does offer a supplementary test (AGC), which also uses TMA technology. The AGC assay has similar sensitivity and specificity to the AC2 and thus is a suitable supplementary test for the AC2, or other *N. gonorrhoeae* NAATs, where required.[100] Perhaps the only disadvantage of the AGC supplementary assay is that it also targets the *N. gonorrhoeae* 16S rRNA molecule rather than using a different gene to that of the AC2. The assay design at least prevents the AGC from being susceptible to false-positive results arising from carryover contamination from the AC2 assay. However, by targeting the same gene, both assays may be affected by the same genetic exchange events.

In-House Assays

Gene targets for in-house PCR assays have included the ORF1, *cppB*, OMPIII, and *opa* genes as well as the *porA* pseudogene.[80,88,101–103] With the exception of *cppB* gene-based assays, limited performance data are available for these in-house assays, with most only investigated in a single study. Briefly, Chaudhry et al[101] reported sensitivity, specificity, and positive predictive values of 100, 98.7, and 99.7%, respectively, using an ORF1-based PCR method on 489 urogenital specimens collected from an STD clinic in India. In addition, this ORF1 assay provided positive results for 40 *N. gonorrhoeae* strains and negative results for 17 nongonococcal *Neisseria* strains, suggesting the ORF1 targets are conserved and specific to *N. gonorrhoeae*.[101] In contrast, our laboratory has investigated the intragenus specificity of an in-house ORF1 real-time PCR using Australian commensal *Neisseria* strains and found cross-reactions with these bacteria. In particular, 4 of 12 local *N. subflava* isolates produced positive results in the assay. Similar cross-reactions were found using an in-house real-time PCR assay targeting the *N. gonorrhoeae gyrA* gene (D.M. Whiley and T.P. Sloots, unpublished data). In the United Kingdom, Palmer et al[49] investigated the intragenus specificity of an OMPIII assay and concluded that it was specific for *N. gonorrhoeae*. However, it was noted that amplification products were produced by commensal *Neisseria* strains and that these products only differed slightly in size from *N. gonorrhoeae* amplification products using gel electrophoresis.[49] This suggests that the amplification step was not specific but rather that the specificity was derived from the detection method. In other Australian studies, Farrell et al[88] reported a *cppB* gene-based PCR assay with sensitivity, specificity, and positive predictive values of 97.9, 100, and 100%, respectively, for 260 urine specimens collected in an Australian population. Yet as previously discussed, the *cppB* gene has serious limitations as a NAAT target in many patient populations.[50] More recently, a PCR assay targeting the multicopy *opa* genes was described previously.[103] This assay was evaluated using 135 clinical samples and a panel of 173 microorganisms, including 73 nongonococcal *Neisseria* strains, and reported 100% sensitivity and specificity. Also, Whiley et al[80] investigated the use of the *N. gonorrhoeae porA* pseudogene as a target for a real-time PCR assay and reported 100% sensitivity and 100% specificity for 636 genital and extragenital specimens. This assay did not cross-react with the 102 commensal *Neisseria* strains tested.

Conclusions

The numerous factors that may diminish the performance of NAAT include carryover contamination, human error, inhibition, reagent or instrument failure, cross-reaction, and absence of the target sequences in local *N. gonorrhoeae* strains. The first four of these factors can be controlled by good laboratory practices and effective quality control. However, sequence-related problems, which may vary considerably with the patient population studied and over time, cannot be dealt with easily and may require the use of supplementary or even alternative assays. These issues may be of lesser importance if the NAATs are used for epidemiological purposes, eg, to establish indicative prevalence rates for gonorrhea or to define the major components of individual STI syndromes. However, they assume a greater relevance when NAATs are used to establish a precise etiological diagnosis of gonococcal infection.

Overall, it would appear that any *N. gonorrhoeae* NAATs may be subject to cross-reaction with commensal *Neisseria* strains unless the sequence targets used by the assay are protected from genetic recombination.[49] Therefore, false-positive results may be an ongoing issue for *N. gonorrhoeae* NAATs and may continue to preclude *N. gonorrhoeae* NAAT testing of the extragenital sites; testing of the pharynx and rectum may only be possible with the use of numerous supplementary assays, which may make such testing cost prohibitive. It should also be recognized that a false-positive diagnosis can be detrimental to the patient, with the potential for medical, legal, social, and psychological consequences.[10] Thus, appropriate measures should be taken by laboratories to avoid such incidents. This includes thorough evaluation and validation of *N. gonorrhoeae* NAATs and the use of supplementary tests if required. The need for NAAT validation is further emphasized by the fact that some *N. gonorrhoeae* may lack particular NAAT targets, creating the potential for false-negative results in some populations. Overall, the *N. gonorrhoeae* species presents some considerable challenges for molecular diagnostics. These challenges have not yet been adequately overcome and continue to cause problems for diagnosis of the disease even a decade after *N. gonorrhoeae* NAATs were first implemented. Recognition of these issues does not devalue the overall utility of NAAT in enhancing diagnosis of gonorrhea, which in any event is problematic. Rather, their resolution, to the extent that this is possible, allows a proper appreciation of the value as well as the limitations of NAAT so that a valid and reliable laboratory result can be produced and properly interpreted within a relevant clinical context.

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