Neisseria gonorrhoeae NAAT – a problem down under

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Nucleic acid amplification tests (NAATs) are used worldwide for the detection of *Neisseria gonorrhoeae,* either in conjunction with or in place of traditional bacterial culture techniques. There are numerous advantages of gonococcal NAATs, including increased sensitivity, that a viable organism is not needed for detection, and they can be used effectively on non-invasive specimens such as urine and self-collected specimens [1]. For these reasons, NAATs have been particularly useful for patients in remote regions of Australia where sexual health services may not be available and where religious or cultural restrictions otherwise restrict opportunities for specimen collection. Australian studies have been at the forefront of investigating the use of self-collected NAAT specimens and particularly successful at introducing the use of tampon self-collected specimens in remote populations of Indigenous Australians [2].

Limitations of *N. gonorrhoeae* NAATs, including cost, risk of carryover contamination, NAAT inhibition, and the inability to provide antibiotic resistance data, are well recognised and are, for the most part, easily circumvented by good laboratory practice. However, there are also sequence-related limitations of *N. gonorrhoeae* NAATs that make them susceptible to both false-positive and false-negative results. It is the latter that have been particularly detrimental to the reliability of gonococcal NAATs in Australia and have effectively curbed their use in certain testing situations. In recent years, there has been an extensive body of research and assay development conducted in Australia aimed at identifying, further characterising and resolving the sequence-related issues associated with gonococcal nucleic acid testing. This culminated in March 2005 when the Public Health Laboratory Network (PHLN) convened a workshop of Australian experts to formulate guidelines for use of gonococcal NAATs in Australia. These national consensus guidelines are now published in *Communicable Diseases Intelligence* [3].

False-positive results

Problems with gonococcal NAAT false-positive results were first reported in Toowoomba, Queensland in 1999 when David Farrell observed a particularly low positive predictive value (PPV = 84%) using the Amplicor *N. gonorrhoeae* NAAT assay [4]. Previous evaluations of the assay in the United States and Canada had reported PPVs in excess of 90% and so Farrell's findings were a significant deviation leading to the conclusion that confirmatory testing should be adopted for all specimens testing positive in the Amplicor *N. gonorrhoeae* assay. Farrell hypothesised that the poor performance of the Amplicor assay reported by his laboratory may be due to differences in normal flora, suggesting that patients carrying commensal bacterial strains which could cross-react with the Amplicor NAAT target were present at a higher incidence in the Australian population. Similar low PPVs for the Amplicor assay have subsequently been reported by other Australian laboratories [5,6].

False-positive results are now recognised as a significant limitation of gonococcal NAATs worldwide. They primarily stem from the frequent horizontal genetic exchange occurring within the *Neisseria* genus, leading to commensal *Neisseria* species acquiring *N. gonorrhoeae* sequences. We now know that the incidence of cross-reaction depends on the choice of nucleotide sequence targets used in the NAAT, and the particular commensal *Neisseria* strains (rather than species) present within any given sample or patient population [1]. Thus, simply validating an assay by testing a few *Neisseria* species in a gonococcal NAAT and finding no cross-reaction is meaningless. Many *N. gonorrhoeae* NAATs have been found to cross-react with commensal *Neisseria* strains, including the Roche Cobas Amplicor and Becton Dickenson ProbeTec SDA assays as well as in-house NAAT assays targeting the *N. gonorrhoeae cppB* and OMPIII genes [4,7]. Many other

gonococcal NAATs simply have not been extensively tested for cross-reaction.

In 2002, the Centers for Disease Control and Prevention (CDC; United States) issued guidelines suggesting the use of additional testing for *N. gonorrhoeae* NAATs where the positive predictive value of the screening assay is <90% [8]. The use of supplementary testing is now advocated by the Australian PHLN [3].

Supplementary testing

The need for supplementary testing in itself has created a further problem; which test should be used for confirmation? Roche originally marketed a confirmatory test for the Amplicor assay targeting the *N. gonorrhoeae* 16S gene, but the assay was later withdrawn. This created a testing void which needed to be filled. It is in this context that Australian laboratories have been both innovative and reactive.

Following the withdrawal of the Roche 16S confirmatory assay, laboratories moved towards in-house NAAT assays for confirmation of results. The first of these was a conventional polymerase chain reaction (PCR) assay, described by Farrell, and targeted the *N. gonorrhoeae cppB* gene [4]. Real-time PCR assays targeting the *cppB* gene were subsequently described by two other Australian laboratories [9,10]. The original appeal of the *cppB* gene target was its high copy number. However, the popularity of *cppB* based assays was later unravelled by additional Australian research showing that some gonococcal strains lack this target (discussed below). Hence, new targets were sought. In response, PCR assays targeting the *N. gonorrhoeae porA* pseudogene and multicopy opa genes were developed and described by laboratories in Brisbane and Melbourne respectively [6,11]. Initial evaluations of these assays are very favourable and the assays are gaining broad acceptance both locally and internationally.

The current view of the PHLN is that all specimens providing positive results in a gonococcal NAAT screening assay should also be positive on a reliable supplemental assay (preferably a different gene target to the screening assay) before a positive result is reported. Acceptable targets for supplementary assays include the *N. gonorrhoeae* CMT gene, pilin gene, 16S RNA gene, *opa* gene and the *porA* pseudogene. However, the PHLN emphasises that laboratories need to continue to assess the suitability of their targets based their own or published data given the propensity for genetic exchange to impact upon the performance of gonococcal assays [3].

Extragenital specimens and validations of gonococcal NAATs?

NAAT validations and the use of extragenital specimens are both contentious issues when testing for gonorrhoea by NAAT. For instance, a 'true-positive' result in a gonococcal NAAT evaluation can be difficult to determine given the potential for cross-reaction with commensal *Neisseria* strains. Also, there is no consensus in the literature as to what comprises a suitable standard. Further, the incidence of commensal *Neisseria* strains in pharyngeal and rectal specimens is significantly higher than in urogenital specimens and so these specimens are more likely to produce false-positive results in gonococcal NAATs. It is for this reason the CDC only recommends using bacterial culture for extragenital specimens [8]. The latter is of particular relevance to Australia given that many laboratories receive specimens from remote communities and so observe poor sensitivity using bacterial culture because of sample degradation during prolonged transport, and hence are heavily reliant on molecular methods.

The Australian PHLN guidelines now provide clear guidance on the issues.

The guidelines state that a true-positive result *for the purposes of an evaluation* is defined as a culture positive, or positive by three gonococcal NAATs targeting separate genes, or by sequencing a gene known to distinguish gonococcal from non-gonococcal species.

For the extragenital sites, the PHLN still recommends cultures as the preferred test. However, if tested by NAAT then it should meet the 'test evaluation' criteria above to be considered a 'true positive' [3].

False-negative results

As discussed above, the genetic composition of commensal *Neisseria* flora can vary between patient populations. However, it is important to note that this also applies to gonococcal strains. The *N. gonorrhoeae* species comprises numerous subtypes that exhibit considerable sequence diversity and are not randomly distributed. That is, the distribution of gonococcal subtypes can vary geographically, temporally and between patient groups. Thus, the performance of *N. gonorrhoeae* NAATs may similarly vary between patient populations due to the presence of different subtypes.

Recent experiences with *cppB* gene based PCR assays have provided a good example of these problems. When originally evaluated in South East Queensland and Victoria, *cppB* gene based PCR assays proved to be highly sensitive and specific for the detection of *N. gonorrhoeae* [4, 9, 10]. However, a high number of false-negative results were observed when a *cppB* gene based PCR method was adopted at the Royal Darwin Hospital. Lum *et al* conducted a study of 143 gonococcal isolates from the Northern Territory and found that 14 (9.8%) were non-reactive in the *cppB* gene based PCR [12]. The high false-negative rate was due to the recent appearance and expansion of a particular (non-PAU) gonococcal subtype, which lacked the cryptic plasmid carrying the *cppB* gene.

The above studies clearly indicate that the incidence of *cppB* gene negative *N. gonorrhoeae* strains varies between patient groups. In fact, a recent multi-centre study conducted in the Netherlands determined that the *cppB* gene was missing in 5.8% of *N. gonorrhoeae* strains [13]. Subsequent studies by our laboratory have indicated that *cppB*-negative gonococcal strains are rare in Queensland. Overall, this highlights that successful evaluation of a *N. gonorrhoeae* NAAT on one patient population at one point in time may not necessarily reflect the assay's suitability for use on another patient population or even on the same patient population over an extended period.

It should be noted that false-negative results arising from sequence variation have not been reported for other *N. gonorrhoeae* NAAT assays. However, specimens that are culture-positive and negative by NAAT methods are commonly reported in published evaluations (but not further investigated). Further, the possibility needs to be taken seriously given the propensity of the organism to undergo frequent transformation and recombination.

The PHLN guidelines state that the *cppB* gene should not be used for either screening or supplemental assays. Again, laboratories should continue to assess the suitability of their assay targets based on their own or published data [3].

Conclusion

Since their inception, gonococcal NAATs have been plagued by sequence related problems affecting sensitivity and specificity. Australians researchers have maintained a pivotal role in characterising these issues and have responded quickly to solve emerging problems, including those related to the *cppB* target. As a result, many of the issues have now been identified and publicised. However, there are still challenges ahead. These include determining the long-term stability and specificity of current NAAT genetic targets, further examining the suitability of NAATs on extragenital specimens as well as in child abuse situations, and the detection of antimicrobial resistance by molecular methods. Identifying norms that are acceptable to all is a difficult process for gonococcal nucleic acid testing. In fact, the PHLN guidelines are not likely to be welcomed by all Australian laboratories. Nonetheless, we feel such guidelines are warranted given the medical, legal, social and psychological consequences that may arise from issuing incorrect *N. gonorrhoeae* NAAT results.

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MICRO-FACT

The most prevalent STD is Chlamydia trachomatis; *in Australia 0.2% of the population is infected annually. Reported diagnoses of chlamydial disease are highest in and continue to increase in the 15 - 29 year age group, reaching 0.7% of the population.*