REVIEW

CURRENT OPINION

Molecular tests for the detection of antimicrobial resistant *Neisseria gonorrhoeae*: when, where, and how to use?

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Purpose of review
Molecular methods for the diagnosis of *Neisseria gonorrhoeae* are replacing bacterial culture in many settings. This review focuses on recent progress in the development of molecular tests to detect resistant *N. gonorrhoeae* both to enhance surveillance and to guide decisions about individual patient management.

Recent findings
Assays to enhance surveillance have been developed to detect determinants of resistance for all antibiotics used as first-line gonorrhoea treatment, or to detect specific 'superbug' strains, but few have been applied in clinical practice. The most advanced strategy relevant to individual case management is to identify ciprofloxacin-sensitive strains so that unnecessary use of ceftriaxone can be avoided. Cross-reactivity with pharyngeal commensal *Neisseria* species reduces specificity and is a challenge for many assays.

Summary
Progress with laboratory-based molecular tests to detect gonococcal resistance is being made but substantial challenges remain. No laboratory-based assay has been subjected to a field evaluation and no assay so far can be used as a point-of-care test. Given the threat of antimicrobial resistance, now is the time to exploit the molecular technologies used for diagnosis and to invest in the development of molecular gonococcal resistance tests that can be implemented for public health good.

Keywords
antimicrobial resistance, antimicrobial surveillance, *Neisseria gonorrhoeae*, point-of-care tests

INTRODUCTION

Antimicrobial resistant *Neisseria gonorrhoeae* is a global public health challenge [1]. New diagnostic strategies and novel antimicrobials are urgently needed to conserve ceftriaxone [2], the last antimicrobial for empirical first-line monotherapy for gonorrhoea in many countries [3,4▪]. Gonococcal strains with resistance to extended-spectrum cephalosporins have caused treatment failure with ceftriaxone and/or cefixime in Europe, North America, Asia, and Africa [5–9]. In this evolving situation, dual antimicrobial therapy (ceftriaxone plus azithromycin) has been introduced for first-line treatment in Europe, Australia, and the United States [10].

Detection of antimicrobial resistant gonococci using molecular methods is a necessary innovation [2,6,11,12] because nucleic acid amplification tests (NAAT) are now the most widely used assays for gonorrhoea diagnosis in many countries [11,13,14▪▪,15]. The WHO global action plan to mitigate the spread of multidrug-resistant gonococci calls for increased surveillance of antimicrobial resistant *N. gonorrhoeae* globally, strengthened capacity for bacterial culture and research into molecular methods to monitor and detect resistance [1]. We searched the US National Library of Medicine (Ovid Medline) using the medical subject headings '*Neisseria gonorrhoeae*' or 'gonorrhea', 'drug resistance,

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KEY POINTS

- Molecular methods are increasingly used for gonorrhoea diagnosis but, despite the global threat of antimicrobial resistance, commercially available diagnostic assays do not detect gonococcal resistance determinants.
- The requirements of molecular tests to detect gonococcal resistance depend on the purpose of the assay; to enhance surveillance of antimicrobial resistance, or to guide the clinical management of gonorrhoea.
- Molecular detection of resistance determinants in genital and rectal specimens is most accurate; cross-reactivity with nongonococcal *Neisseria* species particularly in the pharynx reduces assay specificity.
- Detection of ciprofloxacin resistance or susceptibility to spare the use of extended-spectrum cephalosporins is the strategy that has advanced the furthest to date, but is not a long-term solution.
- Now is the time to exploit the molecular technologies used for diagnosis and to invest in the development of molecular gonococcal resistance tests that can be implemented for public health good.

bacterial', and 'nucleic acid amplification techniques' in September 2015. We selected articles from this search, reference lists of review articles [3,4▪,6,14▪▪,16] and abstracts of the World STI & HIV Congress 2015. We found no articles about commercially available molecular assays that detect genetic resistance determinants of *N. gonorrhoeae*, but the number of reports of laboratory-developed assays is increasing [17▪,18–20,21▪,22–26,27▪,28–38]. This review examines the requirements, recent progress and challenges for the molecular detection of antimicrobial resistant *N. gonorrhoeae* in clinical specimens from the point of view of the two main functions of resistance testing: to enhance surveillance [14▪▪] and to guide decisions about individual patient management [15]. We focus on publications in the last 2 years about assays developed to detect genetic determinants of *N. gonorrhoeae* resistance in clinical specimens and for which results have been compared with minimum inhibitory concentrations (MICs) for specific antimicrobials.

The need for molecular detection of antimicrobial resistant *Neisseria gonorrhoeae*

The molecular technologies have changed patterns of gonorrhoea diagnosis in high-income countries. NAAT are recommended because they have high sensitivity, robust specimen handling conditions, can be automated with high throughput and can be used on noninvasively collected samples like first-void urine and vaginal swabs [39]. The rapid adoption of NAAT has, however, raised two main concerns about the ability to detect, monitor, and manage antimicrobial resistance [1,6,11,14▪▪,15]. First, commercial NAAT specimens provide no viable organism for antimicrobial susceptibility testing. Second, as the number of specimens for culture falls the microbiological skills required for isolation and antimicrobial susceptibility testing are being lost [1]. Countries that have poor healthcare infrastructure and rely on syndromic management of genitourinary symptoms and/or microscopy of Gram-stained smears have a particularly urgent need for better diagnostics and knowledge of gonococcal antimicrobial resistance patterns [40].

Molecular technology is redefining expectations about the detection of antimicrobial resistant gonorrhoea. The paradigm for sexually transmitted infection treatment reflects the clinical priority to treat symptomatic patients on presentation [3]. In the absence of results of antimicrobial susceptibility testing, the first-line treatment regimen should cure at least 95% of infections [3]. Bacterial culture and antimicrobial susceptibility testing underpin the success of this strategy and have many advantages [11,14▪▪]. First, culture on selective media directly provides the biological material for assessing a strain's susceptibility to multiple antimicrobials simultaneously [3]. Second, breakpoints for the MIC that determines resistance correspond with clinical treatment failure for many antimicrobials. Third, organised surveillance programmes can monitor changes in the resistance prevalence for many antimicrobials [41–43]. Fourth, clinicians can use the antimicrobial susceptibility profile to alter therapy, or choose an antimicrobial for an untreated patient. The use of NAATs alone precludes monitoring of antimicrobial resistance, but has also highlighted the limitations of culture. First, considerable technical skills and time are required to culture the fastidious *N. gonorrhoeae* and test for antimicrobial resistance. Second, culture has poor sensitivity for extragenital sites such as the pharynx [3], where horizontal transfer of resistance determinants from commensal *Neisseria* species is thought to be the origin of resistance to extended-spectrum cephalosporins [4▪,6].

Requirements of molecular tests to detect antimicrobial resistant *N. gonorrhoeae*

Ideally, molecular resistance testing should reflect the exact MICs of different antimicrobials.

Table 1. Main genes associated with antimicrobial resistance in *Neisseria gonorrhoeae*

Antimicrobial	Principal gene(s) and position[a]	Other genes with verified impact on resistance in clinical strains[a,c]
Ciprofloxacin	*gyrA* S91 (D95)	*parC*
Ceftriaxone	*penA* mosaic allele (A501-altered *penA* alleles)	*mtrR*, *porB*
Azithromycin	23S rRNA gene: C2611 (lower level of resistance) and A2059 (high-level resistance)[b]	*mtrR*, *mefA*, *erm* genes
Spectinomycin	16S rRNA gene: C1192	*rpsE*
Tetracycline	*tetM*-carrying plasmid (high-level resistance) and *rpsJ* V57 (lower level of resistance)	*mtrR*, *porB*
Penicillin	β-lactamase plasmid (bla_{TEM} gene; high-level resistance) and *penA* (D345 insertion or mosaic allele; lower level of resistance)	*mtrR*, *porB*, *ponA*

[a]With the exception of the *tetM*-carrying plasmid and the β-lactamase plasmid, listed genes, and positions are involved in chromosomally-mediated resistance. Wild type positions are listed, which can have many different mutations resulting in resistance.
[b]There are four alleles of the 23S rRNA gene in each gonococcal strain. *Escherichia coli* numbering used.
[c]There are additional efflux pumps, for example, MacAB and NorM, in *N. gonorrhoeae* that affect the minimum inhibitory concentrations of several antimicrobials.

Resistance to many antimicrobials in *N. gonorrhoeae*, however, is affected by multiple genes. Different mutations and an accumulation of these mutations result in the high MIC of the specific antimicrobial [4▪]. This characteristic makes it exceedingly difficult to use molecular assays to predict exact MICs of antimicrobials, but by targeting the main resistance determinants the resistance/susceptibility phenotypes might be predicted. The principal genes associated with gonococcal antimicrobial resistance are shown in Table 1.

Most publications about molecular tests to detect gonococcal resistance determinants report their intended use for surveillance purposes [17▪,18–20,21▪,22–26,27▪,28–31]. Fewer mention the need or potential for guiding individual patient management [18,27▪,31–35]. There are many similarities in the requirements for assays for both purposes. For example, the capacity for direct detection in clinical specimens [17▪,18,19,21▪,24,26,27▪, 29–32,44▪] is critical if the assays are to be used in situations where bacterial culture has not been performed. Table 2 lists similarities and differences between test requirements for surveillance and clinical management.

Enhancing surveillance of antimicrobial resistant *N. gonorrhoeae*

Experts agree that molecular tests can enhance but not replace culture-based surveillance [1,4▪,6,14▪▪]. The lack of correspondence between genetic resistance determinants, MICs and clinical treatment failure, particularly for evolving resistance in extended-spectrum cephalosporins, remains a critical stumbling block [6]. Nevertheless, molecular assays to enhance surveillance can advance more quickly than those for clinical management partly because the assays can be solely laboratory-based, short reaction times are not essential and sensitivity and specificity can be suboptimal [26,31] (as long as they allow consistent monitoring of trends) because the results will not influence individual patient management (Table 2). As new resistant strains emerge or levels of recognised resistance increase, culture-based surveillance can be broadened to confirm the findings of molecular tests.

Some assays have been used in real-life settings. A real-time polymerase chain reaction (PCR) assay was developed to detect penicillinase producing gonococci [30] in remote regions of Australia where resistance to penicillin remains below 5%. The molecular assay had 100% sensitivity and 98.7% specificity compared with phenotypic tests. When applied subsequently to *N. gonorrhoeae* positive NAAT specimens from Western Australia, the assay detected penicillinase producing gonococci in 15/915 (1.6%) of specimens from remote areas and 34/303 (11.2%) of specimens from more heavily populated areas [44▪]. As a result, the treatment regimen of amoxicillin, probenecid, and azithromycin was continued in remote areas and, in populous areas, changed from ceftriaxone alone to ceftriaxone plus azithromycin [44▪]. This assay has been further optimised to include additional β-lactamase plasmid targets [19]. Molecular tests for surveillance can also be used to monitor specific strains. For example, real-time PCR assays have been developed to screen clinical specimens for extensively drug resistant gonococcal strains (‘superbugs’) that were first detected in Japan (H041) [29] and subsequently in Europe (F89) [24].

An antibiogram gives information about susceptibility to multiple antimicrobials. Most molecular assays detect resistance determinants and relate to a single antibiotic (penicillin [19,30,44▪];

Table 2. Requirements of molecular tests to detect antimicrobial resistant *Neisseria gonorrhoeae*

		Clinical management	
Characteristic	**Surveillance**	**Asymptomatic patient**	**Symptomatic patient**
Specimen type	Culture or clinical specimen	Culture or clinical specimen	Clinical specimen
Location of test equipment	Laboratory	Laboratory or point-of-care	Point-of-care
Time from sample collection to reporting result	Not critical	Same as or quicker than culture-based testing	Minimal
Technical skill required	Skilled laboratory technician	Skilled laboratory technician or minimal	Minimal
Skill required to interpret results	Minimal–high	Minimal–intermediate	Minimal
Accuracy	Intermediate–high	High	High
Cost	Low–high	Low–intermediate	Low
Scalable	Not critical	Desirable	Critical
Multiplex	Not critical	Desirable	Critical

ciprofloxacin [18,31,32]; azithromycin [21▪,45]; extended-spectrum cephalosporins [17▪]); or a single strain [24,29]. Molecular assays aiming to detect resistance to several antimicrobials have been run in series [26] or as multiplex reactions [27▪,36], but their analytical sensitivity was too low to be used in clinical specimens [36] or, if assessed on clinical specimens, was not validated against a culture-based MIC reference standard [26,27▪].

Laboratory-based molecular tests to guide individual patient management

Laboratory-based molecular assays could have advantages if they produce faster results and have greater sensitivity than bacterial culture, particularly when used on extragenital specimens for NAAT testing. Laboratory-based assays will usually not help, however, in the management of patients who present with symptoms or who need same-day treatment for other reasons.

The strategy that has advanced furthest aims to spare overuse of ceftriaxone plus azithromycin (the only remaining first-line empirical treatment in many countries) by targeting ciprofloxacin to patients with ciprofloxacin-sensitive gonococcal strains before treatment is initiated. Several laboratory-based molecular assays for use in clinical specimens detect one or more chromosomal mutations in gonococci that are associated with ciprofloxacin resistance [18,26,27▪,31,32]. Siedner and colleagues [31] designed a real-time PCR assay to detect resistance using a single target mutation in urine specimens. A single nucleotide polymorphism in amino acid codon S91 in the *gyrA* gene is found in all ciprofloxacin resistant strains, even though additional mutations in *gyrA* and other loci (Table 1) contribute to higher levels of resistance [4▪,46]. The prototype assay amplified the target sequence in only 85% of all urine NAAT specimens tested [31]. Further optimisation of this assay has improved sensitivity in cultured isolates, which should be validated in clinical specimens [37]. The suboptimal diagnostic test accuracy of real-time PCR assays to detect ciprofloxacin resistance in clinical specimens presents ongoing challenges. In a study that tested 24 NAAT specimens, real-time PCR assays correctly identified only 4/6 ciprofloxacin-resistant specimens with S91 mutations and 16/18 ciprofloxacin-sensitive specimens [18]. Three of four specimens that failed were from the pharynx or rectum. Cross-reactivity occurred with *N. meningitidis*, *N. lactamica*, *N. subflava,* and *N. polysaccharea*. In another study, real-time PCR failed in 10% (28/290) of clinical specimens, particularly those from nongenital sites [38].

An alternative strategy is to identify wild type *gyrA* sequences that predict ciprofloxacin susceptibility rather than resistance [32]. To exclude cross-reactivity, a *N. gonorrhoeae* specific target (*dcmH*) was also detected in this molecular assay. When used in male urine NAAT specimens in South Africa, the assay correctly identified 15/15 ciprofloxacin-sensitive and 18/18 strains with intermediate susceptibility or resistance [32].

Point-of-care molecular tests to guide individual patient management

Point-of-care tests have a rapid turnaround time that guides clinical decisions and allows results and treatment to be given to the patient at the same visit [47]. None of the molecular assays developed so far to detect antimicrobial resistance in *N. gonorrhoeae* is a ‘transformative point-of-care diagnostic test that will conserve antibiotics for future generations…

(and is) accurate, rapid, affordable, easy-to-use and available to anyone, anywhere in the world' [48]. These criteria, set by the Longitude Prize, aim to stimulate innovation in diagnostic test development for all infectious diseases. Even the GeneXpert (Cepheid, Sunnyvale, Washington, USA) tests for gonorrhoea/chlamydia diagnosis are not rapid point-of-care tests because the NAAT takes 90 min, rather than a 30-min benchmark set by international groups, and their high cost precludes widespread use [40,48,49].

The requirements of a point-of-care test focusing on accurate and cost-effective detection of a limited number of targets without the need for separate DNA extraction methods [50] need to be differentiated from tests for surveillance, which might detect many resistance determinants in multistage multiplex assays [27▪] (Table 2). Advances in bioengineering and nanotechnology, such as microfluidics, will help to adapt laboratory-based systems to clinic-based formats [40,51]. Target product profiles that define the user, patient population, and point-of-care device requirements are essential [47]. Evaluation trials should use clinically relevant endpoints [40,52], such as prediction of resistant phenotypes and reduced antibiotic prescribing. The ideal system would detect both *N. gonorrhoeae* and its main resistance determinants at the point-of-care and allow individualised antimicrobial treatment to help slow the spread of antimicrobial resistance, particularly in resource-poor settings.

Molecular detection of extended-spectrum cephalosporin resistance

Resistance to extended-spectrum cephalosporins remains the most urgent threat. The development of molecular assays to detect any resistance to extended-spectrum cephalosporins in clinical specimens is inherently challenging because multiple mechanisms are involved and are still evolving [4▪]. Mosaic alleles of the *penA* gene encoding penicillin-binding protein 2 (PBP2) and nonmosaic *penA* alleles with A501 mutations are the main determinants of decreased susceptibility and resistance to extended-spectrum cephalosporins [4▪]. The gonococcal 'superbug' F89 strain is a good example of how resistance to extended-spectrum cephalosporins evolves. This strain contains a *penA* mosaic allele (type XXXIV), which is common worldwide [4▪,5–7], with an additional A501P alteration, resulting in high-level ceftriaxone resistance [9]. Molecular test specificity is a major challenge, particularly for pharyngeal specimens that frequently harbour nongonococcal *Neisseria* species with similar *penA* sequences. Several assays have examined the presence of mosaic *penA* allele sequences in cultured isolates [25,50,53]. One real-time PCR assay that detects *N. gonorrhoeae* (*porA* pseudogene), mosaic *penA* alleles and mutations in additional resistance-determining loci (*mtrR*, *porB*, and *ponA*) was tested on 24 gonococcal-negative NAAT specimens and 34 gonococcal-positive NAAT specimens [17▪]. The assay detected a mosaic *penA* allele in one specimen with the highest ceftriaxone MIC (0.25 μg/ml), but lacked specificity in the prediction of decreased susceptibility to extended-spectrum cephalosporins (in clinical specimens and culture isolates). This suboptimal specificity will be exceedingly difficult to overcome because this assay and other similar assays detect many different mosaic *penA* alleles which result in highly divergent MICs of the extended-spectrum cephalosporins [4▪,6,8]. Furthermore, cross-reactivity in the *penA*, *mtrR*, *porB*, and *ponA* targets with commensal *Neisseria* isolates or in clinical specimens was identified. All three clinical cross-reactive specimens were from the pharynx [17▪].

Molecular detection of azithromycin resistance

Azithromycin alone is not a first-line treatment for gonorrhoea, but detecting resistance is important because of its use in dual treatment regimens. Two mutations in the 23S ribosomal RNA genes (A2059G and C2611T) are associated with azithromycin resistance with MICs depending on how many of the four alleles are mutated [45,54]. Two real-time PCR assays could characterise the 23S rRNA 2059 and 2611 positions as wild type or mutated in 87% (266/306) of genital and rectal specimens. However, cross-reactivity was observed with both assays testing commensal *Neisseria* species and in 33% (7/21) of pharyngeal samples [21▪]. Furthermore, among 64 samples with MIC results, 3% (2/64) with raised MICs (1 μg/ml), which indicates resistance, were identified as 23S rRNA wild type.

Challenges for molecular detection of antimicrobial resistant *N. gonorrhoeae*

The number of assays and techniques used to detect genetic determinants of antimicrobial resistant *N. gonorrhoeae* is increasing and some assays are beginning to be adopted for surveillance [14▪▪]. Progress in molecular assays to guide clinical management, however, is lagging. In addition to the challenges to developing usable and commercially viable point-of-care tests [47], there are many remaining roadblocks for the development of diagnostic tools to contain antimicrobial resistance in general [40] and for gonorrhoea in particular. First, molecular tests can only detect known targets and new mutations and resistance mechanisms will develop for both extended-spectrum cephalosporins and other antimicrobials. Adaptable assays and maybe new technologies such as whole genome sequencing might be required to overcome this challenge. Second, incomplete understanding of the relationship between genetic resistance determinants and phenotypic resistance [4▪] needs to be overcome to improve the validity of evaluation studies. Third, diagnostic evaluation studies that compare detection of genetic resistance determinants with MICs are small and their methodology is subject to biases that overestimate test performance [52]. Larger field studies with blinded evaluation of a range of specimen types and results are needed. Fourth, test sensitivity and specificity are still suboptimal for clinical specimens, particularly for pharyngeal specimens. Some cross-reactivity with nongonococcal *Neisseria* species that carry the same genetic sequences cannot be overcome but advances in primer and assay design might improve test accuracy. Finally, diagnostic tests can help to implement and monitor treatment strategies to spare first-line empirical therapy with ceftriaxone plus azithromycin. But targeting ciprofloxacin treatment, using azithromycin as co-therapy and other actions are short-term solutions with unknown consequences for the spread of antimicrobial resistant *N. gonorrhoeae*. In the medium term, new therapeutic antimicrobials are crucial. A gonococcal vaccine [55] is probably the only long-term solution.

CONCLUSION

Progress with the development of molecular tests to detect gonococcal resistance is significantly more advanced for enhancing surveillance than for guiding clinical decision making but substantial challenges remain. Gonococcal culture and antimicrobial susceptibility testing remain essential tools for surveillance and as a reference standard for assay validation. The prioritisation of molecular resistance determinants for inclusion in assays needs to consider both short and longer term antimicrobial resistance threats. In addition, cross-reactivity with nongonococcal *Neisseria* species, particularly in the pharynx, is a challenge for all assays and suggests that assay development should focus first on genital and rectal clinical specimens. Now is the time to exploit the molecular technologies used for diagnosis and to invest in the development of molecular gonococcal resistance tests that can be implemented for public health good.

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Conflicts of interest

N.L. and M.U. are co-investigators in the RaDAR-Go project which aims to develop a point-of-care test to detect antimicrobial resistance in N. gonorrhoeae.

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