Review

Recent advances in the development and use of molecular tests to predict antimicrobial resistance in *Neisseria gonorrhoeae*

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Abstract

Introduction: The number of genetic tests, mostly real-time PCRs, to detect antimicrobial resistance (AMR) determinants and predict AMR in *Neisseria gonorrhoeae* is increasing. Several of these assays are promising, but there are important shortcomings and few assays have been adequately validated and quality assured.

Areas covered: Recent advances, focusing on publications since 2012, in the development and use of molecular tests to predict gonococcal AMR for surveillance and for clinical use, advantages and disadvantages of these tests and of molecular AMR prediction compared with phenotypic AMR testing, and future perspectives for effective use of molecular AMR tests for different purposes.

Expert Commentary: Several challenges for direct testing of clinical, especially extra-genital, specimens remain. The choice of molecular assay needs to consider the assay target, quality controls, sample types, limitations intrinsic to molecular technologies, and specific to the chosen methodology, and the intended use of the test. Improved molecular- and particularly genome-sequencing-based methods will supplement AMR testing for surveillance purposes, and translate into point-of-care tests that will lead to personalized treatments, while sparing

the last available empiric treatment option (ceftriaxone). However, genetic AMR prediction will never completely replace phenotypic AMR testing, which detects also AMR due to unknown AMR determinants.

Keywords: *Neisseria gonorrhoeae*, antimicrobial resistance, molecular testing, NAAT, ceftriaxone, ciprofloxacin, azithromycin, *penA*, *gyrA*, 23S rRNA

1.Introduction

Gonorrhea is a major public health problem and in 2012 the WHO estimated 78 million new

annual cases among adults globally. The highest number of cases was in the WHO Western Pacific Region (35.2 million), followed by the WHO South-East Asian Region (11.4 million), WHO African Region (11.4 million), WHO American Region (11.0 million), WHO European Region (4.7 million), and WHO Eastern Mediterranean Region (4.5 million) [1]. These *Neisseria gonorrhoeae* (NG) infections can, if not appropriately treated, result in severe complications and sequelae, including pelvic inflammatory disease, ectopic pregnancy, and infertility, as well as enhance the transmission and acquisition of HIV [2,3]. Effective, accessible and affordable antimicrobial treatment is the mainstay for management and control of gonorrhea, i.e., supported by effective prevention, diagnostics, contact notification, and epidemiological surveillance.

It is a major concern that NG has developed resistance to all antimicrobials previously recommended for treatment of gonorrhea including sulphonamides, penicillins, tetracyclines, spectinomycin (SPC), fluoroquinolones, macrolides and early-generation as well as, more rarely, extended-spectrum cephalosporins (ESCs) [2]. The injectable ESC ceftriaxone (CRO) or dual antimicrobial therapy (usually CRO 250-500 mg plus azithromycin (AZM) 1-2 g) are currently the only appropriate options for empirical first-line therapy in most countries worldwide [3,4]. WHO has published a global action plan including numerous actions to mitigate the emergence and spread of AMR NG [3], strongly emphasizing the need for enhanced NG AMR surveillance globally. However, in many well-resourced settings nucleic acid amplifications tests (NAATs) have replaced culture for NG detection and in many less-resourced settings only syndromic management of gonorrhea is applied, resulting in a loss of capacity to culture NG and perform phenotypic AMR testing. Consequently, sensitive and specific tests for the molecular detection of AMR mechanisms or determinants to predict the NG AMR would be exceedingly valuable. These tests should ideally also be rapid and capable of being performed at the point-of-care (POC) to additionally guide personalized treatment of

each patient. Nevertheless, despite that many in-house- or laboratory-developed tests for NG AMR prediction were designed particularly during the last decade, no such test has yet become commercially available.

The objectives of this paper were to review recent advances, focusing on publications since 2012, in the development and use of molecular tests to predict gonococcal AMR for surveillance and for clinical use, advantages and disadvantages of these molecular tests and of molecular AMR prediction vs. phenotypic resistance testing, and finally provide some future perspectives for effective use of molecular tests to monitor NG AMR as well as to guide personalized treatment of gonorrhea at the POC.

2. Molecular mechanisms and determinants for antimicrobial resistance in *N. gonorrhoeae*

NG has shown an extraordinary ability to acquire or develop resistance to all antimicrobials used for treatment through *i)* enzymatic destruction/modification of the antimicrobial, *ii*) modification/protection of the target leading to decreased affinity to the antimicrobial, and *iii*) decreased influx and/or increased efflux of the antimicrobial. Some AMR determinants are directly linked to resistance to a particular antimicrobial, whereas others require a cumulative effect with other AMR determinants in order to achieve clinically relevant resistance. AMR mechanisms in NG were extensively reviewed recently [2].

The main AMR mechanisms and/or determinants conferring resistance to the previous or current gonorrhea treatment options ciprofloxacin (CIP), AZM, ESCs and SPC are summarized in Figure 1.

2.1. Ciprofloxacin (CIP) target resistance determinants

CIP resistance is mainly driven by mutations reducing fluoroquinolone affinity of the target enzymes DNA gyrase and topoisomerase IV. The main mutations associated with CIP resistance are found in the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene, encoding one of the two subunits forming the DNA gyrase heterotetramer. Initial non-synonymous mutations in codon 91 (most frequently encoding a GyrA S91F alteration) cause low- to intermediate resistance, with an additional mutation in codon 95 (e.g., GyrA D95N) further increasing the CIP MICs. Concomitant mutations in the *parC* gene, encoding one of the topoisomerase IV subunits, are required for high-level CIP resistance. The ParC D86N, S87N, S88P and E91A/K/G substitutions have been shown to increase CIP-resistance [2,5,6].

2.2. Azithromycin (AZM) target resistance determinants

The C2611T and A2059G target mutations in the four 23S rRNA alleles have been associated with moderate- to high-level AZM resistance, respectively, with the resistance level dependent on the number of mutated alleles [2,7-9]. It is important to detect strains with only one mutated allele since, under macrolide selective pressure, the 23S rRNA mutations can be transferred to the other alleles through recombination [8].

Additionally, rarely identified *erm* genes, encoding rRNA methylases, can lead to decreased susceptibility to low-level AZM resistance [2,8,10,11].

2.3. Extended-spectrum cephalosporin (ESC) target resistance determinants

Decreased susceptibility and resistance to ESCs are associated with the presence of mosaic *penA* alleles (the main ESC resistance determinant), encoding mosaic variants of the lethal target penicillin-binding protein 2 (PBP2) [2,12-16]. However, many different *penA* mosaic alleles exist, which result in widely different MICs of ESCs [2,13,17-19; https://ngstar.canada.ca/welcome/home]. NG strains harboring the mosaic *penA* alleles

XXXIV and X and with decreased susceptibility or resistance to ESCs have been associated with treatment failures in many parts of the world [2,13,20-22]. Notably, only one additional substitution in the mosaic *penA* XXXIV allele (i.e., A501P) results in high-level CRO resistance [19]. Nevertheless, ESC-resistant strains harboring a non-mosaic *penA* allele, with PBP2 A501 and G542/P551 alterations, have also been described, particularly in Asia [23-26].

2.4. Spectinomycin (SPC) target resistance determinants

High-level SPC resistance has mostly been caused by a C1192U mutation in the 16S rRNA target [2,27,28]. However, a Val25 deletion and K26E alteration in the ribosomal protein S5, encoded by the *rpsE* gene, are also associated with high-level SPC-resistance, whereas the T24P alteration confers low-level SPC resistance [28,29].

2.5. Increased efflux

Five efflux pump systems have been described in NG; MtrCDE, MacAB, NorM, MtrF and FarAB (Figure 2) [30-33]. The MtrCDE efflux pump has been shown to export several classes of antibiotics, including AZM and ESCs [30,34,35], whereas NorM and MacAB mainly export fluoroquinolones and macrolides, respectively [32,33].

Mutations causing an over-expression of the MtrCDE efflux pump have been associated with intermediate susceptibility and resistance to several antimicrobials. Among these, a mutation in the *mtrR* gene causing a G45D substitution in MtrR, a transcriptional repressor of the *mtrCDE* operon, is prevalent [36]. However, the most frequent mutations harbored by resistant isolates are located in the 13-bp inverted repeat sequence of the *mtrR* promoter, commonly a single A deletion [35].

2.6. Decreased influx

Mutations in the *porB1b* gene (the *penB* resistance determinant) encoding alterations in only amino acid 120 (e.g., G120K) or in combination with amino acid 121 (e.g., G120D/A121D) in PorB1b result in decreased influx and, accordingly, decreased susceptibility to e.g., ESCs [2,37]. These mutations affect the AMR phenotype only in strains with concomitant overexpression of the MtrCDE efflux pump [38].

3. Molecular tests for prediction of antimicrobial resistance in *N. gonorrhoeae*

Many methods for molecular prediction of NG AMR have been developed during the recent decade. The main characteristics of these molecular assays have been summarized in Table 1.

3.1. Ciprofloxacin (CIP)

As early as 2004, a duplex real-time (RT)-PCR assay was designed, which used Taqman probes to target the wild type (wt) sequence in the *gyrA* QRDRs, i.e. spanning codons 91 and 95, and *parC* QRDR including codons 86-88 [39]. Analysis of 80 NG isolates showed 100% agreement between RT-PCR results, sequencing, and CIP MICs. However, a main limitation, common also to other recently proposed molecular AMR methods, was that no internal control for the detection of NG DNA was included in the assay.

An improvement of this method included a marker for NG identification (*dcmH*) [40]. After validating the assay on 40 sets of linked urogenital samples and blinded testing of additional 33 NG positive urine specimens with paired NG isolates, sensitivity and specificity for the detection of CIP-susceptibility were 100% for the *gyrA* component of the assay, and 95.5% and 86.1% for the *parC* component. Conversely, failure to detect both *gyrA* and *parC* amplicons from the same DNA sample was 96.9% sensitive and 100% specific in predicting CIP-resistance. No false-positive amplification of either *gyrA* or *parC* targets in other bacterial species was observed among 21 paired sets of DNA extracts prepared from clinical

specimens of patients with non-gonococcal urethritis. Detection of NG by targeting *dcmH* showed 100% sensitivity and 97.9% specificity for isolates and clinical specimens. However, false-positive reactions for *dcmH* due to the presence of *Neisseria lactamica*, *Neisseria flavescens* and *Neisseria sicca* were previously observed in a *dcmH*-based commercial NAAT for NG, indicating the potential for cross-reaction with commensal *Neisseria* species for this target [41]. In fact, some cross-reactivity with *N. lactamica* was observed when 26 non-gonococcal isolates were further tested. Some false-positive results were also reported for the *parC* reaction [40]. No rectal and pharyngeal specimens were evaluated in the study, but these preliminary observations suggest that cross-reactivity for at least the *dcmH* and the *parC* reactions due to commensals may be expected.

Another probe-based RT-PCR targeting the same mutations in the QRDRs of *gyrA* and *parC* (positions *gyrA* S91, *gyrA* D95and *parC* D86/S87/S88), also including an internal control probe for each gene that should produce a positive signal in NG presence, was applied to 252 NG isolates. The assay showed 100% correct results for *gyrA* S91 and *parC* D86/S87/S88, and 99.6% for *gyrA* D95 [42]. The limits of detection (LODs) were 5 pg DNA/reaction for both *gyrA* reactions and 5 fg DNA/reaction for *parC*. The assay was subsequently validated using 24 APTIMA Combo 2 CT/NG urogenital and extra-genital NAAT specimens with matched NG isolates. The overall RT-PCR concordance for each genetic marker was 100% for *parC* and 83.3% for both *gyrA* assays. Seven samples were undetermined in the *gyrA* assays, indicating that the lower sensitivity for the *gyrA* reactions in the clinical specimens was mainly due to the higher LOD compared to the *parC* reaction. One rectal specimen gave a false-positive result for *gyrA* D95. The specificity of the assay was also assessed by testing 50 non-gonococcal isolates, showing some cross-reactivity (both wt and single nucleotide polymorphism [SNP]) for all three reactions. However, none of 24 tested NG-negative APTIMA specimens gave false-positive results for *gyrA*, and only two

pharyngeal swabs showed a cross-reaction for *parC* [42]. Nevertheless, additional testing of clinical specimens is crucial to thoroughly assess the potential for cross-reactivity.

Most recent RT-PCR based approaches detect only SNPs in *gyrA* codon 91. For instance, a Taqman probe-based RT-PCR assay for detection of the GyrA S91F substitution was validated using 108 clinical specimens with paired CIP-resistant NG and 101 with CIP-susceptible control strains [43]. The method showed a sensitivity and specificity of 100% and 99%, respectively, compared to phenotypic CIP susceptibility. No cross-reactivity was observed testing six other *Neisseria* and *Lactobacillus* species. However, the panel was limited and no negative clinical specimens were tested. Accordingly, additional testing to exclude cross-reactivity with other non-gonococcal species is crucial. Additionally, *gyrA* wt or other NG specific markers were not detected in the assay, i.e. to avert the risk of false negatives due to low NG DNA loads.

This limitation, i.e. lack of appropriate internal NG control, has been addressed in other assays. For instance, a Taqman-based RT-PCR assay that detects wt *gyrA* or the GyrA S91F alteration with two different probes showed 100% agreement with CIP susceptibility phenotype when 90 NG isolates were tested [44]. The method was further applied on urogenital (n=174) and extra-genital (i.e., pharyngeal and rectal; n=116) samples, NG-positive by the BD ProbeTec GC Qx Amplified DNA Assay providing a result for 90% (262/290) of the specimens, with most undetermined samples being extra-genital specimens. For the 234 clinical specimens with paired gonococcal isolates from the same anatomical site, the sensitivity and specificity for detecting CIP susceptibility was 95.8% and 100%, respectively. Accordingly, absence of the GyrA S91F SNP demonstrated 100% positive predictive value for CIP susceptibility in all clinical specimens tested [44]. Nevertheless, no NG-negative clinical specimens, and especially extra-genital samples, were tested to assess cross-reactivity.

The concern of cross-reactivity was addressed in another RT-PCR method in which primers with "non-template" bases at the 3'-end in order to increase the specificity were designed [45]. This probe-based assay correctly distinguished *gyrA* wt and the GyrA S91F alteration in 70 NG isolates tested, whereas all non-gonococcal isolates (n=55) and NG-negative clinical samples (n=171, including both rectal [n=27] and pharyngeal swabs [n=34]) gave negative results. When applied to 210 NG-positive clinical samples (including both rectal swabs (n=14) and pharyngeal swabs (n=7)) with paired NG isolates, 195 (92.9%) samples were successfully characterized, and for 194 samples the results were in agreement with phenotypic CIP susceptibility results. The single discordant sample harbored a mixed infection. Mixed infections in the same anatomical site were also discussed in a recent publication [46].

A RT-PCR with fluorescence resonance energy transfer (FRET)-probes coupled with melting curve analysis to detect *gyrA* wt or the GyrA S91F alteration was first published in 2007 [47]. Evaluation of 96 male urine specimens with paired NG isolates from matched urethral samples, found amplification in 95 (99.0%). Sensitivity and specificity to detect CIP resistance was 93.2% (41/44) and 100% (51/51), respectively. Additionally, a CIP susceptibility genotype was predicted in 72% (72/100) NG-positive female urine samples previously tested using APTIMA Combo 2, but 28 (28%) samples failed to amplify [47]. The assay was also applied on 13 urogenital specimens, of which 2 (15.4%) tested *gyrA* S91F positive, in a small substudy [48]. A further evaluation of this assay showed 100% concordance with CIP susceptibility phenotypes in 100 isolates. Among 76 urine and swab specimens that tested NG-positive by the Cobas 4800 CT/NG system, the assay correctly classified 54 (71%) as *gyrA* wt or mutant, and in 116 Cobas NG-negative specimens no amplification occurred [49]. Most of the undetermined samples were urine (n=17), confirming the suboptimal sensitivity for this specimen type. No pharyngeal specimens were evaluated.

3.2. Azithromycin (AZM)

Only one assay for detection of AZM resistance alone has been published [50]. This assay consisted of two probe-based RT-PCRs coupled with melting curve analysis targeting the 23S rRNA C2611T and A2059G mutations, respectively. To mitigate cross-reaction with other bacterial species, "non-template" bases were included at the 3'-end in the primer sequences, as previously described [45]. When testing 28 non-gonococcal isolates, including *Neisseria mucosa* (n=3), *Neisseria cinerea* (n=3), *Neisseria subflava* (n=11), *N. sicca* (n=3), *N. lactamica* (n=4), *N. flavescens* (n=1) and *N. meningitidis* (n=3), cross-reactivity with 26 and all 28 isolates could be observed for the 23S rRNA C2611T and A2059G targets, respectively. However, the cycle thresholds were higher, indicating that this strategy was able to limit the cross-reactivity. Nevertheless, when testing 90 NG-negative urogenital and extra-genital samples, 33% (7/21) of pharyngeal samples were false positive. Of 70 NG isolates, all were correctly characterized, i.e. no isolates contained the 23S rRNA A2059G mutation and three AZM-resistant isolates (AZM MIC>2 µg/ml) harbored the 23S rRNA C2611T mutation. Three AZM-susceptible isolates harbored both 23S rRNA wt and C2611T mutated alleles, which illustrates that ≥3 mutated alleles are usually required for moderate AZM resistance (MIC>2 µg/ml) [7,8]. Finally, 87% (266/306) of NG NAAT-positive clinical samples (no pharyngeal samples) could be characterized by both 23S rRNA PCRs, whereas 13% (40/306) could not be characterized by one or both assays, likely due to low NG DNA loads. A limitation of the study was that there was only one clinical sample positive for the 23S rRNA C2611T mutation and none for the 23S rRNA A2059G mutation. Accordingly, further evaluation would be valuable, i.e. with clinical samples including NG strains with these mutations or NG-negative specimens spiked with NG isolates harboring those mutations.

3.3. Extended-spectrum cephalosporins (ESCs)

Several assays to detect mosaic *penA* alleles associated with decreased susceptibility or resistance to ESCs have been developed.

In 2008, a Taqman-based RT-PCR assay with one primer designed to match the *Neisseria perflava*- and the other the *N. cinerea*-like sequences in the NG mosaic *penA* gene, was published [51]. Among 70 NG isolates, the assay correctly identified 47 mosaic *penA*-harboring isolates. The LOD of the assay was 10 copies/reaction. However, many mosaic *penA* alleles have now been described ([13]; https://ngstar.canada.ca/welcome/home), and it is unknown whether this method would detect all these mosaic *penA* alleles. Additionally, cross-reactivity with commensals was assessed by testing only one *N. cinerea* and one *N. perflava* strain, and *in silico* analysis suggests cross-reactivity with some *N. meningitidis* strains.

This mosaic *penA* PCR [51] has been used together with other RT-PCR assays for testing of bacterial strains and non-cultured clinical samples, NG-positive by an in-house PCR, in Australia [52,53]. Cross-reactivity with two *N. meningitidis* strains was confirmed when testing 100 non-gonococcal isolates. However, when applied to 192 NG-negative clinical specimens (142 urogenital and 50 pharyngeal samples), no false-positives were detected. This study also used two Taqman probe-based PCRs detecting the A501V PBP2 amino acid substitution and the A-deletion in the *mtrR* promoter region, respectively [52]. None of these newly designed assays showed any cross-reactivity when testing the 192 NG-negative clinical samples. The assays were also applied on 179 NG-positive clinical specimens with unknown sampling site. The A deletion in *mtrR* promoter was found in 24 samples, whereas all samples tested negative in the mosaic *penA* and A501V PCR assays [52]. Since the mosaic *penA* PCR and A501 PCR only detected mutant isolates, false-negative results due to limited NG DNA cannot be excluded.

The mosaic *penA* PCR developed in 2008 [51] has also been used in combination with a RT-PCR that specifically detects the mosaic *penA* XXXIV allele [53]. Fifty-nine (8.6%) isolates tested positive for a mosaic *penA* allele; 39 isolates possessed the mosaic *penA* XXXIV, four isolates the mosaic *penA* XXXVIII and 16 isolates a novel mosaic *penA* allele (LA-A). Fifteen (25%) of these isolates harboring a mosaic *penA* displayed an alert value of CRO MIC (≥0.125 μg/ml). The performance of the RT-PCR detecting the mosaic *penA* XXXIV was subsequently assessed by testing 71 NG isolates, 27 urine specimens (including three NG-culture-positive matched urethral swabs) and 20 NG-negative pharyngeal swab specimens. All thirty-nine isolates and three urine specimens with paired isolates containing the mosaic *penA* XXXIV allele were correctly identified. No cross-reactivity was seen for strains harboring the mosaic XXXVIII, LA-A or other *penA* alleles, or NG-negative urine and pharyngeal specimens. The sensitivity and specificity in detecting alert values of CRO MIC (≥0.125 μg/ml) was 100% and 57%, respectively [53]. These combined assays are valid tools for the detection of mosaic *penA* alleles, particularly mosaic *penA* XXXIV, in bacterial isolates. Nevertheless, for testing of clinical specimens further evaluations are recommended, including additional NG-positive and NG-negative samples, especially extra-genital specimens and additional mosaic *penA* alleles. Furthermore, the assay did not include detection of any NG-specific marker, so false negative results due to limited NG DNA cannot be excluded.

A more comprehensive probe-based RT-PCR assay included detection of SNPs in *penA*, *mtrR*, *porB*, *ponA*, and one NG-specific marker (*porA*) [54]. The *ponA* and *mtrR* assays contained probes targeting wt or mutated alleles, while the *porB* assay contained a probe matching the wt allele sequence and the *penA* assay three probes specific for the mosaic and non-mosaic alleles. The LODs were 50 fg/reaction for *porA*, *ponA*, *mtrR*, and *porB*, and 500 fg/reaction for *penA*. When initially testing 252 NG isolates, the agreement between the RT-

PCR and sequencing was 100% for *porA*, *ponA*, and *penA*, 99.6% for *mtrR*, and 95.2% for *porB* (all negatives contained the *porB1a* allele instead of the targeted *porB1b* allele). Specificity was further assessed by testing 50 non-gonococcal isolates, showing cross-reactivity with some non-gonococcal *Neisseria,* as well as other commensal species, for all markers except the NG-specific *porA* target. The cross-reactivity was further confirmed when testing 24 NG-negative urogenital and extra-genital specimens, i.e., three examined pharyngeal swabs showed cross-reactivity for the *penA* and *porB* targets. For 24 NG-positive APTIMA specimens with matched NG isolates, assay concordance was 100% for *porB*, 95.8% for *ponA* and *mtrR*, and 91.7% for *penA*. One rectal specimen gave indeterminate results for *ponA*, *mtrR*, and *penA*. One pharyngeal specimen also had indeterminate *penA* result, likely suggesting cross-reactivity with non-gonococcal *Neisseria* species [54]. Thus, particular care should be taken when testing extra-genital samples.

The report of the high-level CRO-resistant strain F89 [19], harboring a mosaic *penA* XXXIV plus an A501P substitution, subsequently resulted in the development of a modified FRET-probe-based RT-PCR with melting curve analysis to detect *penA* mosaic alleles, as well as *penA* mosaics with A501P or other A501 alterations [55]. Mosaic *penA* alleles including A501P and A501V could be distinguished from *penA* mosaics with A501 wt and A501T, however, the latter two could not be distinguished. When examining 33 NG isolates, all four mosaic *penA* isolates were identified and the A501 variants could be detected (i.e., three A501 wt and one A501V). The LOD of the method was comparable with that of the original *penA* mosaic-PCR assay [51]. This RT-PCR was also applied as a first screening tool for the detection of mosaic *penA* in 159 urogenital and extra-genital specimens (of which, only two pharyngeal) positive by an in-house PCR. Ten specimens (six rectal and four urine) provided positive results, which were subsequently shown to harbor *penA* A501 wt. However, when testing 58 non-gonococcal isolates, 22 provided melting curves in the mosaic501-

hybPCR with 19 called as mosaic/A501 variants [55]. It would be valuable to further assess specificity by testing NG-negative specimens, in order to determine the extent of cross-reactivity with other bacterial species. Additionally, both the mosaic PCR and the mosaic501-hybPCR were less sensitive than the in-house duplex assay used to detect NG [55]. Furthermore, the sensitivity of the mosaic501-hybPCR to detect the *penA* mosaic/A501P variant could not be estimated, since no such positive clinical samples were available and no spiked negative samples were included. Finally, in the developed modified hybridization probe format, the probes must remain separate from the reaction mix during PCR amplification to avoid inhibition of amplification, which is not effective and/or feasible with all RT-PCR systems.

Two Taqman-based RT-PCR assays to detect mosaic *penA* alleles and, specifically, the mosaic *penA* allele harbored by the high-level CRO-resistant strain H041 were also developed [56]. Both assays had comparable LODs and H041 could be distinguished among 67 NG isolates. However, when testing 100 non-gonococcal *Neisseria* and *Moraxella* isolates the H041-PCR1 showed cross-reaction with *N. subflava.* Furthermore, using the H041-PCR1 seven of 50 pharyngeal swabs negative for NG with an in-house PCR were false positive, and one of 59 clinical specimens, NG-positive by the same in-house PCR, was also false positive. Nevertheless, the H041-PCR2 was negative for all examined non-gonococcal isolates, as well as NG-positives (n=39) [56]. These assays share the main limitation of the F89-specific RT-PCR described above, i.e., no H041-positive samples were available, so further testing of spiked NG-negative clinical specimens to estimate sensitivity is recommended.

3.4. Multiple antimicrobials

Only a few molecular assays predicting resistance to multiple antimicrobials in NG in the same test have been reported.

The high-throughput Agena Bioscience SNP iPLEX genotyping technology, using mass spectrometry, was applied to characterize NG isolates in Australia [57]. The iPLEX-AMR method targeted 11 chromosomal AMR determinants, namely 23S rRNA A2059G, 23S rRNA C2611T, GyrA S91F, GyrA D95G/A, PBP1 L421P, *mtrR* adenine-deletion, *mtrR* thymine-insertion, MtrR G45D, PBP2 345A insertion, mosaic PBP2, and non-mosaic PBP2 A501T/V. This assay was applied together with the previously validated iPLEX-MLST [58] to characterize 2452 NG isolates, of which 2218 (90.4%) provided a result in both assays. Some samples failed to amplify due to variations in primer-target regions or low template DNA. Similar results were obtained when applying both iPLEX assays on additional 1625 NG isolates in a further study [59]. Overall, the MassARRAY system showed great potential for cost-effective, high-throughput surveillance studies, allowing to genotype up to 384 NG isolates per day. However, the applicability for testing clinical specimens is unknown and the sequences of the oligonucleotides required for the iPLEX-AMR assay are not available, preventing further implementation of the method.

A multiplex high resolution melting (HRM)-based RT-PCR assay has also been developed [60]. This assay included one triplex and three duplex reactions for detection of NG (*opa*, *porA*) and the 23S rRNA A2059G, 23S rRNA C2611T, GyrA S91F, mosaic *penA*, *rpsE* Thr24Pro and 16S rRNA C1192T alterations. The assay detected all AMR determinants in 39 well-characterized NG isolates, but the overall LOD for proper interpretation of the HRM curves was high (i.e., $\geq 10^3$ to 10^4 gDNA copies/reaction). Additionally, cross-reactivity was observed for several AMR determinants (e.g., 23S rRNA and 16S rRNA) when testing 19 non-gonococcal *Neisseria* isolates and eight NG-negative pharyngeal and rectal samples. The assay showed 100% sensitivity and 100% specificity for NG identification and in predicting phenotypic resistance to CIP, AZM and SPC in a further 193 NG isolates. Sixteen isolates with a mosaic *penA* allele and increased ESC MICs (≥0.064 µg/ml) were also correctly

identified. Accordingly, although not feasible for screening of clinical specimens, due to the high LODs and cross-reactivity with commensal *Neisseria* species, this assay can be useful for rapid characterization of clinical NG isolates.

The most comprehensive molecular NG AMR platform designed so far used a multiplex bead array suspension assay that included 31 NG-specific chromosomal mutations and plasmid genes associated with decreased susceptibility and resistance to PEN, CIP, ESC, TET, AZM, and SPC [61]. In six NG control strains a LOD of ~62 gDNA copies were required to detect all AMR determinants. The NG-specific marker *porA* correctly classified 20 NG-positive and 50 NG-negative cervicovaginal swabs based on previous in-house RT-PCR testing. In 250 NG-positive cervicovaginal swabs, 239 (95.6%) tested positive for *porA*. The detection of AMR determinants varied from 89% to 100%, compared to sequencing. Cross-reactivity was assessed by testing seven non-gonococcal *Neisseria* species, 33 other bacterial, fungal, and protozoan isolates commonly found in the vaginal environment, and 50 NG-negative cervicovaginal samples. All samples were negative for *porA*, but cross-reactivity was observed for several AMR determinants, particularly the 16S rRNA determinants. Accordingly, the specificity of testing clinical samples, particularly extra-genital specimens, can be suboptimal. Furthermore, no positive controls were available for some of the AMR determinants, so the sensitivity to detect these could not be determined. The assay requires equipment that remains rare in diagnostic laboratories in many settings, but its simplicity, relatively low price and flexibility could make it an effective confirmatory test for strain typing and predictive assessment of AMR.

Whole genome sequencing (WGS) allows more comprehensive analysis of both AMR prediction and genome-based molecular epidemiology in NG isolates, and costs are falling. WGS has been used for full characterization of all known AMR determinants, as well as strain typing by phylogenomic analysis and *in silico* NG multi-antigen sequence typing (NG-

MAST) and multi-locus sequence typing (MLST) [62-65]. A relatively high reliability of MIC prediction for five therapeutic gonorrhea antimicrobials in 681 NG isolates based on a WGS-based approach has been recently demonstrated [66].

The first attempt to apply WGS directly on NG-positive clinical specimens has also been recently published [67]. Thirteen NG-positive urine samples were sequenced with the Ion Torrent technology, obtaining an average depth of coverage based on the FA1090 reference genome of 6–130X for the 11 samples. Based on *rplF* gene and K-mer analysis, the presence of commensal bacteria was excluded. AMR determinants associated with resistance to, e.g., ESCs, CIP, TET and AZM, were detected. Additionally, new mutations in some AMR markers (i.e., *gyrA* and *mtrR*) were also identified, although their potential contribution to AMR was not confirmed. Finally, all 11 samples could be typed by both NG-MAST and MLST. A main limitation was that no paired NG isolates were available, so further studies should confirm the accuracy of this method. Nevertheless, this study shows that WGS can be successfully applied to obtain extensive typing information directly from clinical specimens, suggesting that WGS definitely represents a promising future tool for a more comprehensive analysis of both phylogenomics and AMR.

4. Advantages and disadvantages of molecular AMR prediction for *N. gonorrhoeae*

Many of the advantages of molecular methods for AMR prediction over culture-based methods are those of NAATs in general (Table 2). For example, molecular methods allow to dramatically shorten turnaround times from several days to a few hours. Additionally, NAATs can be easily automated to increase throughput and decrease hands-on time, which are valuable qualities for diagnostic routine settings. From the clinical perspective, molecular tests also have higher sensitivity compared with culture, particularly for frequently asymptomatic extra-genital infections and can be used to analyze culture negative samples

and non-viable previously cultured isolates. Non-invasive self-collected specimen types, such as urine and vaginal swabs, can also be used for testing. The improved performance and flexibility of sampling for molecular testing could substantially increase sample sizes for NG AMR surveillance globally. NAAT-based methods also have the potential to be further developed into rapid POC tests in the near future, which would be extremely valuable in remote and low technology settings and to guide personalized treatment in all settings. To date, no assay that has been developed to predict AMR in NG provides results in <1 hour, a definition that is widely applied to POC tests [68].

The major disadvantage of molecular prediction of AMR is that an assay will only detect known AMR determinants, whilst a culture-based assay will detect the exact level of susceptibility or resistance (MICs) that result from any known or new AMR determinants. A single molecular assay, with exception of WGS, will not be able to include markers of all known AMR determinants and the assay would need to be updated as soon as new AMR determinants or sequence variants arise. For these reasons, molecular tests will probably never be able to replace culture-based methods completely. Another intrinsic disadvantage of molecular AMR assays is that the presence of a molecular AMR determinant may not correlate strongly with the MIC of an antimicrobial, because the development of AMR can be multifactorial and result from the cumulative effects of several AMR determinants. For instance, the detection of a mosaic *penA* allele in a NG isolate has a relatively high sensitivity to predict resistance to ESCs because most ESC-resistant isolates harbor a mosaic *penA* allele. However, the detection of a mosaic *penA* allele in a NG isolate can also have relatively low specificity for the prediction of ESC resistance. Specificity can be relatively low because there are many different sequence types of mosaic *penA* alleles, which result in widely different MICs of ESCs, and most mosaic *penA* alleles do not result in ESC resistance in absence of additional ESC AMR determinants (see below). Accordingly, ESC AMR

determinants, i.e. mosaic *penA* alleles, *penB*, *mtrR* and Factor X, cumulatively increase the MICs of ESCs [2,57,63,69-72]. Notably, a vast majority of mosaic *penA*-harboring isolates reported to be resistant to ESCs belonged to successful strains that spread internationally, such as the NG-MAST genogroup 1407 spreading worldwide [2,19-22,73]. Additionally, also ESC-resistant strains harboring a non-mosaic *penA* allele have been described, which encode PBP2 with alterations in amino acids A501 and G542/P551 [2,23,24,26].

Conversely, the association between the main AMR determinants and phenotypic AMR appears to be much stronger for some other antimicrobial classes. For instance, the *gyrA* S91F alteration alone has proven to be highly predictive for intermediate susceptibility and resistance to CIP. The suitability of this AMR determinant to predict CIP resistance (or susceptibility when mutation is lacking) has been shown in several studies performed in many different countries worldwide (e.g., Canada [74], Brazil [75], Australia [76], USA [77], and Switzerland [78]), and further confirmed by the validation studies of the molecular CIP AMR methods mentioned above [44,45,47,60]. Similarly, mutations in at least three 23S rRNA alleles have shown to be a good predictor of moderate- to high-level AZM resistance (i.e., MIC>2 μg/ml), although low-level resistance may still be observed due to mutations in other AMR determinants (e.g., *mtrR*) [2,35].

Additional disadvantages common to most molecular AMR prediction methods, particularly for pharyngeal specimens, are that: *i)* sequence variations in primer/probe regions may lead to false positive or negative results; *ii)* several of the developed methods do not include an internal control to prevent false-negative results due to low NG DNA loads in the samples; and *iii)* the presence of potentially cross-reacting non-gonococcal *Neisseria* and other bacterial species, which makes the design of NG-specific primers and probes extremely complicated, especially for targets sharing a high sequence identity or similarity among different *Neisseria* species (e.g., 23S rRNA). The addition of an internal control to prevent

false-negative results due to low NG DNA loads in the samples and thorough validation of all molecular assays on extra-genital specimens are essential to increase the validity of existing and future assays.

In general, WGS represents a promising future approach in order to overcome many of the challenges with molecular AMR prediction methods.

5. Requirements for using molecular AMR prediction tests to enhance the gonococcal AMR surveillance and for guiding personalized treatment of gonorrhea

The molecular assays developed so far for AMR prediction in NG address a range of AMR determinants alone or in combination, might be used on a range of clinical samples and have differing performance characteristics. Technical requirements and cost mean that these tests are mainly accessible in well-resourced settings. Figure 3 shows that the key requirements of a molecular AMR prediction test will differ, depending on their usefulness for different applications [68]. Molecular tests will be most readily used for surveillance of NG AMR, because archived specimens can be batch-tested retrospectively, and ideal sensitivity and specificity are not critical because results will not be used to guide personalized treatment. Molecular assays that will be used to guide clinical management need to have high diagnostic and AMR predictive accuracy. In a pilot study, the FRET-probe-based RT-PCR for the S91F *gyrA* mutation was used to test NG-positive NAAT specimens to guide treatment for patients in California, USA [77]. During the study period, CRO use significantly declined and targeted CIP use increased. AMR prediction tests to be used at the POC have the most challenging requirements. The assay needs to accurately detect both NG and AMR determinants directly in clinical specimens within an hour. At the time of publication of this review, no test fulfils these requirements. Even rapid molecular tests for NG detection are not yet fast enough to be considered as rapid POC tests.

Mathematical modelling studies offer important insights about the potential effects of new diagnostic strategies that can help to inform priorities for test development. First, the promotion of increased use of screening tests without AMR testing to reduce the prevalence of gonorrhea could, paradoxically, increase the spread of resistance to at least some antimicrobials such as CIP. Notably, a recently developed mathematical model suggests that an increase in the treatment rate of gonorrhea can be the main driver of spread of CIP resistance, likely because the increase in empirical treatment imposes selection pressure for the emergence of AMR strains [79-81]. Second, POC tests that detect both NG and determinants of AMR could control the spread of AMR gonorrhea by allowing personalized specific antimicrobial treatment, provided that AMR surveillance continues also with culture-based phenotypic methods to detect new AMR determinants [81]. These examples highlight the importance of modelling studies that can show the trade-offs between strategies to reduce gonorrhea prevalence through early diagnosis, treatment and partner notification, and the spread of AMR.

6. Expert commentary

The publications reviewed in this article have refined our understanding of the AMR determinants in NG that can be identified easily and accurately and the assays that are most likely to be deployed for AMR surveillance, to inform treatment guidelines, and in the future to guide personalized treatment and for POC use.

In countries where levels of CIP resistance remain moderate, i.e. excluding settings in Asia with 90-100% resistance, identification of CIP susceptibility would be particularly valuable to guide personalized treatment and spare the use of dual antimicrobial therapy (CRO plus AZM). The detection of the GyrA S91F alteration has been proven to be highly indicative of a CIP-resistant phenotype in several validation and other epidemiological studies

[44,45,47,60,74-78]. However, considering the relatively limited number of well-characterized clinical, and especially extra-genital, samples tested so far, additional testing using molecular assays with appropriate controls would be advisable before applying those tests on all NG positive NAAT samples. Nevertheless, now is the time to investigate ideal implementation (where, when and how) of these assays in the clinical management of gonorrhea. Notably, one of these assays has already been introduced in a clinical setting, resulting in a significant reduction in CRO use with a concomitant increase in targeted therapy [77,82].

For the sole detection of AZM resistance determinants in the 23S rRNA gene, only one molecular assay has been recently published [50]. Specific mutations in the 23S rRNA, i.e. C2611T and A2059G, have been shown to be highly predictive of moderate- (MIC>2 μg/ml) and high-level (MIC≥256 μg/ml) resistance to AZM, respectively. However, low-level *in vitro* and clinical resistance to AZM can be due to other AMR mechanisms (e.g., frequently *mtrR* promoter or coding region mutations) [2,8-11,35,36,64]. This method can provide clinically relevant data to supplement and enhance culture-based AMR surveillance, and enable molecular-based surveillance for AZM resistance. However, since the prevalence of the targeted 23S rRNA mutations was very low in the materials tested, further testing of NG strains and NG-positive NAAT specimens harboring these mutations to appropriately validate the sensitivity of the assay is crucial. For this validation, clinical specimens spiked with different concentrations of mutated strains could also be used. Additionally, despite showing the potential for good discrimination of commensal bacterial species, some cross-reactivity was observed, which resulted in the exclusion of pharyngeal samples for further testing [50]. Thus, application of the method on extra-genital, and particularly pharyngeal, specimens requires further optimization and validation.

The emergence since the 1990s of mosaic *penA* alleles associated with decreased susceptibility and resistance to ESC has led to the development of several assays for the detection of different mosaic *penA* alleles. These methods provide important tools to monitor the spread of mosaic *penA*-harboring isolates, which gives an indication of the ESC susceptibility, and have shown promising results also for direct testing of clinical specimens. However, many different mosaic *penA* alleles exist globally and these result in widely different MICs of ESCs. Additional AMR determinants, such as non-mosaic *penA* alleles with alterations in PBP2 amino acids A501 and G542/P551, *penB*, *mtrR* and Factor X, also further increase the ESC MICs [2,23-26,57,63,69-72]. Furthermore, great care should be taken when examining extra-genital, especially pharyngeal, specimens, considering the high potential of cross-reaction with non-gonococcal *Neisseria* and even other bacterial species. Finally, further assessment of the sensitivity for different sample types is recommended, especially for those tests, for which no positive clinical specimen was available.

Assays targeting AMR determinants for multiple antimicrobial classes might have the broadest applicability, but are the most challenging to develop. A rapid HMR curve-based assay was developed to screen for the main AMR determinants associated with resistance to four different antimicrobial classes, but the limitations encountered during method validation prevent application directly on clinical specimens [60]. In contrast, a multiplex bead suspension array method appeared to work on clinical specimens [61], but a very limited number of samples has been tested so far. Similarly, the only attempt to apply WGS directly to urine samples [67] so far requires additional validation on more samples and specimen types.

Overall, several methodologies have been successfully developed in the last few years, showing promising results in predicting AMR to different classes of antimicrobials, both in isolated NG strains and NG-positive clinical samples. Nevertheless, cross-reactivity with

commensal species, particularly but not exclusively *Neisseria* species, as well as low NG DNA loads, especially in extra-genital specimens, still represent major challenges for the development of molecular tests sufficiently sensitive and specific for widespread clinical use. In this regard, it is also worth noting that several of the published methods do not include internal controls to prevent potential false negative samples. Thus, the addition of such controls is crucial when applying these methods to clinical, and especially extra-genital, specimens. The variability in the correlation between the presence of a particular AMR determinant and the expected AMR phenotype is also a main obstacle for several antimicrobial classes. For instance, while the GyrA S91F alteration has proven to be a very good predictor of phenotypic CIP resistance, ESC resistance is most frequently multifactorial at the genetic level and probably also linked to strain-specific genetic backgrounds or other still unknown factors. Finally, new AMR determinants or sequence variants will be identified. Consequently, some of the molecular AMR methods will require to be updated over time and this updating needs to be timely performed. This work needs to be fast-tracked by the manufacturer of the assays and regulatory bodies.

In conclusion, the choice of molecular assay needs to take into careful consideration the sample types to which it will be applied, potential limitations intrinsic to NAAT technologies, as well as specific to the chosen methodology, and the intended use of the test.

7. Five-year view

NAATs have become the methods of choice for the detection of NG in many particularly well-resourced settings. Their dominance is due to their advantages compared with NG culture, including the rapidity and the possibility of automation, dramatically shortening turnaround times and hands-on time, while increasing throughput. For these reasons, molecular methods for the prediction of AMR in NG will also be increasingly implemented in

the near future. Despite their inherent limitations, these molecular tests represent valuable tools to improve AMR surveillance by increasing the number of tested samples, and may even be soon implemented in diagnostics for personalized treatments.

Translational research to develop rapid and POC NG tests including AMR prediction, ultimately informing tailor-made treatment, will intensify. This research will need to be interdisciplinary to integrate highly accurate diagnostic assays with bioengineering solutions that allow DNA extraction and performance of the molecular assay including detection. For example, microfluidic and nanotechnology approaches are being developed to find low technology and even equipment-free assays. These assays will be particularly valuable in resource-poor settings where the burdens of AMR and gonorrhea are the highest.

Preliminary results also suggest that the development of WGS-based methods may in the future replace NAAT-based testing for NG AMR prediction, at least in well-resourced settings, offering the tempting possibility of a 360° genetic view of strains and samples. In fact, WGS displays the major advantage of detecting all known and not-previously discovered AMR determinants at the same time, as well as describing the phylogenomics and molecular epidemiology. Such methodology will also lead to a deeper understanding of the genetic background of the isolates, which in turn may increase our knowledge of potential additional factors contributing to increase AMR, virulence and/or fitness in internationally spreading clones (e.g., ST1407).

No methods for molecular prediction of AMR in NG will be able to completely replace phenotypic testing, since this would prevent detecting the emergence of new AMR determinants and their links to MICs of different antimicrobials, and the accuracy in phenotypic AMR prediction based on associated AMR determinants will continue to vary for different antimicrobials and NG strains.

In conclusion, improved NAAT- and WGS-based methods will continue to supplement AMR testing for surveillance purposes, and further translation into POC tests may lead to the golden end-goal of personalized treatment, sparing the use of the last available empiric treatment options (i.e., ESCs) in order to avert the threat of untreatable gonorrhea.

Key issues

- The number of genetic assays detecting AMR determinants in NG is increasing, which may enhance AMR surveillance and guide treatment guidelines
- Many genetic assays were relatively successfully applied for both cultured NG isolates and direct testing of clinical specimens
- The accuracy in the AMR phenotype prediction based on the presence of an AMR determinant varies dependent on antimicrobials and NG strains
- The main challenges for the application on extra-genital clinical specimens is cross-reactivity with other bacterial species and low NG DNA loads
- More clinical samples and specimen types (especially pharyngeal) should be tested to confirm sensitivity and specificity
- WGS-based approaches will be likely developed in the near future
- Genetic tests can and should supplement culture-based AMR testing, but will never completely replace it
- Further development of genetic AMR prediction methods into affordable POC tests will spare last-line antimicrobials and will be of greatest benefit in resource-poor settings

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***This paper describes the introduction of a GyrA S91 molecular assay in a clinical setting, resulting in a significant reduction in ceftriaxone use with a concomitant increase in targeted therapy.**

Figure legends

Figure 1. Main antimicrobial targets and mechanisms/determinants conferring resistance to ciprofloxacin, azithromycin, extended-spectrum cephalosporins and spectinomycin. Mutations or single nucleotide polymorphisms (SNPs): in *ponA* encoding PBP1 (PBP1 L421P), *penA* encoding PBP2 (PBP2 mosaic, or PBP2 A501 and/or G542/P551 alterations), *porB1b* encoding PorB1b ("*penB* alteration"; G120±A121 alterations of PorB1b), *gyrA* encoding GyrA (alterations in S91[±D95]), *parC* encoding ParC (alterations in D86, S87, S88 and/or E91), 16S rRNA gene (C1192T mutation), and 23S rRNA gene (C2611T or A2059G mutation). OM, outer membrane; PS, periplasmic space; IM, inner membrane

Figure 2. Membrane organization of the five known drug efflux pumps in *N. gonorrhoeae*. Main drug substrate(s) of NorM is fluoroquinolones; FarAB: fatty acids; MacAB: macrolides; MtrF: sulphonamides; and MtrCDE: macrolides, β-lactam antimicrobials (e.g., penicillins and cephalosporins) and tetracycline. OM, outer membrane; PS, periplasmic space; IM, inner membrane. Modified from Efflux-Mediated Antimicrobial Drug Resistance in Bacteria: Mechanisms, Regulation and Clinical Implications, M. Efflux pumps in Neisseria gonorrhoeae: contributions to antimicrobial resistance and virulence, 2016, 439-469, Shafer WM, Yu EW, Rouquette-Loughlin C, Golparian D, Jerse AE and Unemo Li X-Z, Elkins CA, Zgurskaya HI (Eds.) (© Springer International Publishing Switzerland 2016) with permission of Springer).

Figure 3. Key features of molecular tests for prediction of antimicrobial resistance (AMR) in *Neisseria gonorrhoeae*, according to intended use.

Table 1. Main characteristics of recently published (last five years) molecular methods for prediction of antimicrobial resistance in *Neisseria gonorrhoeae*.

Antimicrobial, Reference, year	Methodology	Targets	Highlights	Limitations/notes
CIP				
Magooa et al., 2013 [40]	Taqman-based RT-PCR	*gyrA* wt QRDR *parC* wt QRDR (+*dcmH* for ID)	Sensitivity (100%) and specificity (97.9%) for NG detection; Overall sensitivity and specificity (both 100%) of *gyrA* for CIP-susceptibility prediction; Overall sensitivity (96.9%) and specificity (100%) for CIP-resistance prediction.	Overall sensitivity (95.5%) and specificity (86.1%) of *parC* for CIP-susceptibility prediction; Cross-reaction with commensal species for *dcmH* and *parC*; No extra-genital samples tested.
Peterson et al., 2015 [42]	FRET-probe-based RT-PCR	*gyrA* wt S91 *gyrA* wt D95 *parC* wt (+2 internal CTRs)	Concordance with AMR testing of 100% for *gyrA* S91 and *parC* D86/ S87/S88, and 99.6% for *gyrA* D95 in isolates (n=252); Concordance with AMR testing of 100% for *parC* in NG-positive clinical specimens (n=24).	Undetermined results for both *gyrA* assays (overall, n=7) for the clinical specimens, affecting concordance (83.3%) with AMR testing; Potential cross-reaction with commensals (*parC*, n=2); Sample number for paired clinical specimen (n=24).
Zhao et al., 2012 [43]	Taqman-based RT-PCR	GyrA S91F	Sensitivity (100%) and specificity (99%) for CIP phenotype prediction in isolates and NG-positive clinical specimens (n=209)	Specimen type of the clinical samples unknown; No NG-negative specimens tested; No internal CTR.
Pond et al., 2016 [44]	Taqman-based RT-PCR	GyrA S91F *gyrA* wt	Concordance with AMR testing of 100% in isolates (n=70); NG-positive genital (n=174) and extra-genital (n=116) specimens tested; Sensitivity (95.8%) and specificity (100%) for CIP susceptibility prediction in typed NG-positive specimens.	262 of 290 (90%) of clinical specimens could be typed; No NG-negative clinical samples tested.
Buckley et al., 2016 [45]	Probe-based RT-PCR	GyrA S91F *gyrA* wt	"Non-template" bases at the 3'-end of primers to increase specificity; No cross-reactivity among non-NG isolates (n=55) and NG-negative clinical specimens (n=171, of which extra-genital samples, n=61); Concordance with AMR testing of 99% in typed NG-positive clinical samples (n=194/210).	195 of 210 (92.9%) of clinical specimens could be typed; Number of NG-positive extra-genital specimens (n=21, of which rectal, n=14; pharyngeal, n=7).
Hemarajata et al., 2016 [49]	FRET-probe-based RT-PCR	GyrA S91F *gyrA* wt	Further validation of [47]; Concordance with AMR testing of 100% for isolates (n=100); Specificity (100%) for NG-negative specimens (n=116).	54 of 76 (71%) of clinical specimens could be genotyped; No NG-negative pharyngeal samples tested.
AZM				
Trembizki et al., 2015 [50]	FRET-probe-based RT-PCR	23S rRNA C2611T 23S rRNA A2059G	"Non-template" bases at the 3'-end of primers to reduce cross-reactivity; Correct genotypic characterization of all tested NG isolates (n=70); Concordance with AMR testing of 97% in paired clinical specimens (n=64).	Cross-reactivity in NG-negative pharyngeal specimens (n=7/21); 266 of 306 (87%) of clinical specimens could be genotyped; No positive clinical specimens for both reactions with paired isolates; No internal CTR.
ESCs				

Goire et al., 2012 [52]	Taqman-based RT-PCR	Mosaic *penA* [51] *penA* A501V *mtrR* A-deletion in promoter	High concordance with sequencing results in NG (n=107) and non-NG (n=100) isolates for all three assays; No false positives in NG-negative clinical specimens (n=192, of which, urogenital, n=142; pharyngeal, n=50); 179 out 179 NG-positive clinical specimens could be typed.	Specimen type of the clinical samples unknown; No internal CTR for mosaic *penA* PCR and *penA* A501V PCR assays; Not all mosaic and non-mosaic *penA* alleles may be detected with the mosaic *penA* and *penA* A501 PCR, respectively; No paired isolates for the clinical specimens.
Gose et al., 2013 [53]	Taqman-based RT-PCR	Mosaic *penA* [51] Mosaic *penA* XXXIV	Concordance (100%) with sequencing results for the detection of mosaic *penA* and mosaic *penA* XXXIV alleles in both clinical isolates and positive urine specimens; Detection of a novel mosaic *penA* allele; Sensitivity (100%) to detect isolates with alert values of CRO MICs (≥0.125 µg/ml).	Only isolates positive by mosaic *penA* PCR were further tested; Urine specimens (n=3) with paired mosaic *penA* XXXIV isolates, no samples with paired non-mosaic *penA* isolates; Limited number of NG-positive and negative specimens and specimen types tested; Specificity (57%) to detect alert values of CRO MICs (≥0.125 µg/ml).
Peterson et al., 2015 [54]	Probe-based RT-PCR	Mosaic and non-mosaic *penA*, *porB* wt, *mtrR* wt/mutation and *ponA* (*porA* for ID)	Agreement with sequencing results of 100% for *porA*, *ponA*, and *penA*, 99.6% for *mtrR*, and 95.2% for *porB* in clinical isolates (n=252); Agreement with sequencing results of 100% for *porB*, 95.8% for *ponA* and *mtrR*, and 91.7% for *penA* in paired NG-positive clinical samples (n=24).	Cross-reactivity with non-NG isolates and extra-genital NG-negative clinical samples; Limited number of paired NG-positive (n=24, of which extra-genital n=8) and negative (n=24) specimens.
Goire et al., 2013 [55]	Modified-FRET-probe-based RT-PCR	Mosaic *penA* [51] Mosaic *penA* A501/A501V/ A501T/A501P	Detection of the mosaic *penA* XXXIV + A501P allele of strain F89; Concordance with sequencing results in clinical isolates (n=33); 10 of 159 clinical specimens were correctly identified as *penA* A501.	Mosaic *penA* A501 and A501 cannot be distinguished; 19 of 58 non-NG isolates were false positives for *penA* A501; Limited number of pharyngeal specimens tested (n=2); No NG-positive specimens for *penA* A501V/A501T/A501P; No internal CTR.
Goire et al., 2012 [56]	Taqman-based RT-PCR	H041-like mosaic *penA* allele	Detection of the mosaic *penA* allele of strain H041; No cross-reactivity in non-NG isolates and NG-negative clinical specimens for H041-PCR2.	Cross-reactivity in non-NG isolates and NG-negative clinical specimens for H041-PCR1; No internal CTR; No NG-positive specimens; Limited number of NG-positive (n=39) specimens tested.
Multiple classes				
Trembizki et al., 2012 [57]	iPLEX-AMR	11 AMR chromosomal mutations	Detection of CIP, ESC, AZM, and PEN AMR determinants; Applied together with iPLEX-MLST [58] for concomitant MLST; Large number of screened isolates (n=2452); Cost-effective high-throughput screening for surveillance.	No information about iPLEX-AMR method validation and primer sequences; Applicability to directly screen clinical specimens unknown; 2218 of 2452 (90.4%) isolates typed by both iPLEX-AMR and iPLEX-MLST.
Donà et al., 2016 [60]	SybrGreen-based RT-PCR with HRM analysis	7 chromosomal AMR mutations (+*opa* and *porA* for ID)	Detection of CIP, ESC, AZM, and SPC AMR determinants; Concordance (100%) with sequencing results for NG isolates (n=39); Sensitivity (100%) and specificity (100%) for NG identification and CIP-, AZM- and SPC-resistance prediction in clinical isolates (n=232); Sensitivity (100%) to predict CRO resistance.	Overall high LOD for proper interpretation of HRM curves; Cross-reactivity with non-NG isolates and NG-negative clinical specimens; Limitations do not allow direct screening of clinical specimens; Specificity (90%) to predict CRO-resistance; Sensitivity (92%) and specificity (94%) to predict CFX-resistance.

Balashov et al., 2013 [61]	Multiplex bead array suspension assay	31 AMR genes/ mutations	Comprehensive molecular platform detecting PEN, CIP, ESC, TET, AZM, and SPC AMR determinants; Sensitivity (95.6%) and specificity (100%) of *porA* in cervicovaginal specimens (n=250); Relative simplicity, low price and flexibility.	No positive CTR for all AMR determinants; Limited number of isolates (n=6) used for method validation showing a variable detection rate of AMR determinants ranging from 89% to 100%; Cross-reactivity, particularly for 16S rRNA reactions; No paired NG-positive specimen and other (e.g., extra-genital) sample types tested.
Graham et al., 2017 [67]	WGS (Ion Torrent)	Whole genome	Detection of ESCs, CIP, TET and AZM AMR determinants in NG directly from clinical specimens, including also new potential AMR mutations	Only a limited number of urines tested (n=13); No paired isolates available to confirm the presence of the detected AMR determinants.

CIP, ciprofloxacin; RT-PCR, real-time polymerase chain reaction; wt, wild type; QRDR, quinolone-resistance determining region; ID, identification; NG, *Neisseria gonorrhoeae*; CTR, control; AMR, antimicrobial resistance (phenotypic); FRET, Fluorescence resonance energy transfer; AZM, azithromycin; ESC, extended-spectrum cephalosporin; CRO, ceftriaxone; HRM, high-resolution melting; LOD, limit of detection; PEN, penicillin; MLST, multi-locus sequence typing; SPC, spectinomycin; CFX; cefixime; TET, tetracycline; WGS, whole-genome sequencing.

Table 2. Main advantages and disadvantages of molecular antimicrobial resistance prediction versus phenotypic AMR testing.

Characteristics	Molecular AMR prediction		Phenotypic AMR testing	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Test performance	Sensitivity higher than culture-based methods, especially for extra-genital specimens. Can be used on culture-negative specimens	Sub-optimal sensitivity and specificity for detection of AMR determinants in extra-genital specimens, owing to cross-reacting species	High sensitivity and specificity of AMR testing, including for extra-genital specimens, but limited by the lower sensitivity of culture	Sensitivity of culture lower than molecular tests, especially for extra-genital specimens
Methodology	Rapid, results available in hours Detection of potentially emerging AMR (first AMR determinants) Non-invasive specimen types (e.g., urine and vaginal samples)	AMR resistance can be multi-factorial, while inclusion of all known AMR determinants in one test is almost impossible [a] Mutations in primer/probe regions can lead to false-negatives [a] Presence of an AMR determinant might not strongly correlate with an AMR phenotype Only known AMR determinants can be detected	Exact level of resistance can be determined and is clinically relevant, i.e., can predict clinical treatment failure Detects AMR in strains with new AMR determinants before they have been characterized	Requires several days for strain isolation and AMR testing Invasive specimen types, e.g., urethral and endocervical
Clinical setting	Short turnaround time, same day if necessary Can be used to increase the number of specimens tested Can be easily automated	Requires more expensive instrumentation, kits and/or more advanced technical skills	Requires minimum material/training	High hands-on time and long turnaround time limit the number of samples processed Requires viable organisms

	Does not require viable organisms (important in remote settings) Can be further developed into point-of-care (POC) tests			

AMR, antimicrobial resistance

[a] Does not apply to whole-genome sequencing technologies