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Evaluation of the SpeeDx *ResistancePlus*® GC and SpeeDx GC 23S 2611 (beta) molecular assays for prediction of antimicrobial resistance/susceptibility to ciprofloxacin and azithromycin in *Neisseria gonorrhoeae*

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Background: Accurate molecular assays for prediction of antimicrobial resistance (AMR)/susceptibility in *Neisseria gonorrhoeae* (Ng) can offer individualized treatment of gonorrhoea and enhanced AMR surveillance.

Objectives: We evaluated the new *ResistancePlus*® GC assay and the GC 23S 2611 (beta) assay (SpeeDx), for prediction of resistance/susceptibility to ciprofloxacin and azithromycin, respectively.

Methods: Nine hundred and sixty-seven whole-genome-sequenced Ng isolates from 20 European countries, 143 Ng-positive (37 with paired Ng isolates) and 167 Ng-negative clinical Aptima Combo 2 (AC2) samples, and 143 non-gonococcal *Neisseria* isolates and closely related species were examined with both SpeeDx assays.

Results: The sensitivity and specificity of the *ResistancePlus*® GC assay to detect Ng in AC2 samples were 98.6% and 100%, respectively. *ResistancePlus*® GC showed 100% sensitivity and specificity for GyrA S91 WT/S91F detection and 99.8% sensitivity and specificity in predicting phenotypic ciprofloxacin resistance. The sensitivity and specificity of the GC 23S 2611 (beta) assay for Ng detection in AC2 samples were 95.8% and 100%, respectively. GC 23S 2611 (beta) showed 100% sensitivity and 99.9% specificity for 23S rRNA C2611 WT/C2611T detection and 64.3% sensitivity and 99.9% specificity for predicting phenotypic azithromycin resistance. Cross-reactions with non-gonococcal *Neisseria* species were observed with both assays, but the analysis software solved most cross-reactions.

Conclusions: The new SpeeDx *ResistancePlus*® GC assay performed well in the detection of Ng and AMR determinants, especially in urogenital samples. The GC 23S 2611 (beta) assay performed relatively well, but its sensitivity, especially for predicting phenotypic azithromycin resistance, was suboptimal and further optimizations are required, including detection of additional macrolide resistance determinant(s).

Introduction

Gonorrhoea remains a global public health concern due to increasing prevalence, particularly in many more-resourced settings and because *Neisseria gonorrhoeae* (Ng) has developed antimicrobial resistance (AMR) to all available therapeutic antimicrobials.[1,2] Capacity to culture and perform AMR testing of Ng is also declining worldwide because in more-resourced settings nucleic acid amplification tests (NAATs) are rapidly replacing culture for diagnosis, and in many less-well-resourced countries STI syndromic management is mainly practised without laboratory diagnostics. Sensitive and specific molecular assays for prediction of Ng AMR or antimicrobial susceptibility are imperative, to inform individualized treatment and for AMR surveillance, which is further highlighted by the WHO global action plan.[3–8]

Ciprofloxacin resistance is primarily due to mutations in the *gyrA* gene, encoding the GyrA subunit of DNA gyrase. The main

resistance mutation, which is highly predictive of ciprofloxacin resistance,[1,2,8–15] is the GyrA S91F mutation and mutations in GyrA D95 increase the ciprofloxacin resistance. Simultaneous mutation(s) in the *parC* gene (encoding the ParC subunit of topoisomerase IV) further increase the ciprofloxacin MIC.[1,3,9,11] Multiple molecular assays have been developed to detect ciprofloxacin resistance determinants, with high agreement between these assays and Ng phenotypic ciprofloxacin resistance testing.[3,5–8] However, many of these molecular assays have lacked an appropriate Ng internal control, only a few extragenital NAAT samples were evaluated and cross-reactivity with non-gonococcal *Neisseria* species, frequently colonizing the oropharynx in particular, was detected.[3,5,12–15] Regarding macrolides, target mutations in the 23S rRNA gene alleles, namely C2611T and A2059G mutations, are associated with moderate- and high-level azithromycin resistance, respectively. The level of azithromycin resistance is dependent on the number of the four 23S rRNA gene alleles that are mutated.[1,3,8,16,17] However, additional AMR determinants increase the MICs and can cause resistance to azithromycin, such as: *mtrR* mutations and mosaic *mtr* locus causing overexpression of the MtrCDE efflux pump; *erm* genes that encode rRNA methylases, which block the macrolide binding to the 23S rRNA; overexpression of the MacAB efflux pump; and the *mef*-encoded efflux pump.[1,18] To date, few molecular assays have been developed for detection of Ng azithromycin resistance in NAAT samples and cross-reactivity with non-gonococcal *Neisseria* species has also been observed in these assays.[3,8,19] Recently, the first commercially available Ng molecular resistance/susceptibility assay, *ResistancePlus*® GC (SpeeDx Pty Ltd, Sydney, Australia), was developed for detection of Ng and ciprofloxacin resistance/susceptibility and an evaluation of the beta version of this assay showed promising results.[20] This assay includes five targets: *opa* genes and *porA* pseudogene for detection of Ng, GyrA S91F and S91 WT, for prediction of ciprofloxacin resistance/susceptibility, and an internal control. A molecular assay for prediction of azithromycin resistance/susceptibility in Ng, herein named 'GC 23S 2611 (beta)', is under development by SpeeDx for commercial use. This assay currently includes three targets: *porA* pseudogene for Ng detection and 23S rRNA C2611T and C2611 WT for prediction of resistance/susceptibility to azithromycin.

We evaluated the new SpeeDx *ResistancePlus*® GC assay and the GC 23S 2611 (beta) assay for molecular prediction of resistance/susceptibility to ciprofloxacin and azithromycin, respectively, by examining a large collection of previously whole-genome sequenced Ng isolates from 20 European countries ($n = 967$)[9] and clinical Ng-positive ($n = 143$) and Ng-negative ($n = 167$) samples using the Aptima Combo 2 assay (AC2; Hologic, San Diego, CA, USA). Additionally, ciprofloxacin- and azithromycin-resistant and -susceptible isolates ($n = 143$) of more than eight non-gonococcal commensal *Neisseria* species ($n = 135$), *Neisseria meningitidis* ($n = 6$) and two closely related species ($n = 2$) were examined to substantially challenge the exclusivity of both assays.

Materials and methods

Bacterial isolates

Whole-genome sequenced Ng isolates cultured in 20 European countries in 2013 were included. These isolates represented 921 WGS genotypes, 103 MLST STs and 377 NG-MAST STs (including 249 singletons) (https://pathogen.watch/collection/eurogasp2013).[9] However, only 967 (91.7%; 967/1054) of these previously published isolates were viable and/or available for the present study [81 isolates from the UK and six isolates from Norway were not tested using the *ResistancePlus*® GC assay; and three additional isolates from the Netherlands were not tested with the GC 23S 2611 (beta) assay]. Using MIC gradient strip tests or agar dilution for susceptibility testing[9] and the EUCAST 2019 Ng breakpoints (http://www.eucast.org/clinical_breakpoints/), 446 of these isolates were susceptible, 1 was intermediate and 520 were resistant to ciprofloxacin; and 951 were susceptible and 13 resistant to azithromycin [23S rRNA C2611T ($n = 8$; 6 also had the characteristic *mtrR* promoter A-deletion[1,3]), only *mtrR* promoter A-deletion ($n = 3$) and unknown azithromycin resistance mutations ($n = 2$)], i.e. using the azithromycin epidemiological cut-off of MIC > 1 mg/L to indicate isolates with azithromycin resistance determinants (referred to as resistant in this study). Furthermore, isolates of more than eight non-gonococcal commensal *Neisseria* species ($n = 135$) and two closely related species ($n = 2$; *Moraxella catarrhalis*, *Moraxella osloensis*) were tested. Using Etest (BioMérieux, Marcy l'Étoile, France) and EUCAST 2019 Ng breakpoints (http://www.eucast.org/clinical_breakpoints/), because EUCAST lacks breakpoints for non-gonococcal *Neisseria* species, 42 of these non-gonococcal isolates were susceptible, 39 intermediate, and 56 resistant to ciprofloxacin; and 7 were susceptible and 130 resistant to azithromycin. Six *N. meningitidis* reference strains, susceptible to both ciprofloxacin and azithromycin (using EUCAST 2019 Ng breakpoints because of lack of azithromycin breakpoints for *N. meningitidis*), were also examined: serogroup A (CCUG 3269), B (CCUG 3270), C (CCUG 3271), W (CCUG 27650), X (CCUG 27645) and Y (CCUG 38303).

Clinical NAAT samples

One hundred and forty-three Ng-positive AC2 samples (one sample per gonorrhoea patient/episode), confirmed in culture ($n = 37$) or Aptima GC assay ($n = 106$), from November 2014 to May 2018 were examined. Of the 37 paired Ng isolates, 17 were susceptible and 20 were resistant to ciprofloxacin, and 36 were susceptible and 1 was resistant to azithromycin, using Etest. The Ng-positive AC2 samples included urine ($n = 54$), pharyngeal ($n = 43$), rectal ($n = 19$), vaginal ($n = 18$) and cervical swabs ($n = 9$). Ng-negative AC2 samples ($n = 167$) were also examined: cervical ($n = 65$), pharyngeal ($n = 48$), urine ($n = 20$), vaginal ($n = 20$) and rectal ($n = 14$).

DNA extraction and ResistancePlus® GC assay and GC 23S 2611 (beta) assay

Genomic DNA of all isolates was extracted using single colonies or cryobeads boiled in 100 µL of sterile distilled water for 10 min, followed by centrifugation at 2250 g for 10 min. The samples were diluted 1:400 for the *ResistancePlus*® GC assay and 1:100 for the GC 23S 2611 (beta) assay prior to analysis. In the case of indeterminate results, isolates were re-extracted using the QIAsymphony platform with the Virus/Pathogen Midi Kit (QIAGEN, GmbH, Hilden, Germany). All AC2 samples were extracted using the MagNA Pure Compact Nucleic Acid Isolation Kit Large Volume (Roche Diagnostics, Mannheim, Germany) on MagNA Pure Compact (Roche), including an internal control for the *ResistancePlus*® GC assay.

The *ResistancePlus*® GC assay and GC 23S 2611 (beta) assay (SpeeDx Pty Ltd, Sydney, Australia) were performed, according to the manufacturer's instructions, on a 7500 Fast Real-Time PCR System (Applied Biosystems). All data were analysed and reported using the ResistancePlus GC (7500) analysis software and GC 23S 2611 (beta) analysis software (still under development), respectively. Ng-positive AC2 samples and cross-reactive non-gonococcal *Neisseria* isolates in the *ResistancePlus*® GC assay were further analysed using an in-house Ng GyrA S91 WT/S91F PCR.[21] Indeterminate samples had an invalid internal control, similar amplification

of both AMR and WT targets in *gyrA* or 23S rRNA gene, or lack of detection of both Ng and AMR target.

Results

ResistancePlus® GC assay for ciprofloxacin resistance/susceptibility prediction

Detection of N. gonorrhoeae

The *ResistancePlus*® GC assay detected all of the 967 European Ng isolates[9] and 141 (98.6%) of the 143 Ng-positive AC2 samples. Furthermore, 135 (98.5%) of 137 non-gonococcal commensal isolates, all *N. meningitidis* isolates ($n = 6$) and all Ng-negative AC2 samples ($n = 167$) were negative for Ng; however, two Ng-negative AC2 samples were invalid and excluded from further calculations (Table 1). Compared with the AC2 assay, the overall sensitivity and specificity of *ResistancePlus*® GC for detection of Ng in AC2 samples were 98.6% and 100%, respectively.

Detection of GyrA S91 WT/S91F

The GyrA S91 WT/S91F targets were correctly identified by the *ResistancePlus*® GC assay in all of the 967 genome-sequenced European Ng isolates.[9] Of the 141 (98.6%) AC2 samples reported as Ng-positive by *ResistancePlus*® GC, indeterminate *gyrA* results were obtained for four (2.8%) samples. These four samples were detected with the in-house *gyrA* PCR.[21] Of the GyrA S91F-positive AC2 samples ($n = 57$) using the *ResistancePlus*® GC assay, the in-house *gyrA* PCR[21] confirmed all but two (indeterminate). Of the GyrA S91 WT AC2 samples ($n = 80$), the in-house *gyrA* PCR[21] confirmed all except four samples [GyrA S91F ($n = 1$) or indeterminate ($n = 3$)]. Accordingly, 92.9% (131/141) of the evaluable *gyrA* results were concordant between the *ResistancePlus*® GC and the in-house *gyrA* PCR assay.[21] The positive percentage agreement (PPA) and negative percentage agreement (NPA), after excluding samples with indeterminate results in any of the assays ($n = 9$), of *ResistancePlus*® GC and the in-house *gyrA* PCR assay[21] in the detection of GyrA S91F were 98.2% (55/56) and 100% (76/76),

Table 1. Evaluation of SpeeDx *ResistancePlus*® GC assay for ciprofloxacin resistance/susceptibility prediction, examining Ng isolates from 20 European countries,[9] clinical AC2 samples and non-gonococcal *Neisseria* and closely related isolates

		ResistancePlus® GC			In-house *gyrA* PCR		
Samples (*n*)	Ng (*opa* + *porA*)	GyrA S91 WT	GyrA S91F	Indeterminate	GyrA S91 WT	GyrA S91F	Indeterminate
European isolates (967)[9]	967	447 (46.2%)	520 (53.8%)	—	NA	NA	NA
Ng-positive AC2 samples (143)	141[a]	80 (55.9%)	57 (39.9%)	4 (2.8%)	79 (55.2%)	57 (39.9%)	7 (4.9%)[b]
Samples without confirmed culture (106)	104[c]	64 (60.4%)	37 (34.9%)	3 (2.8%)	63 (59.4%)	37 (34.9%)	6 (5.7%)
Samples with paired culture (37)	37	16 (43.2%)	20 (54.1%)	1 (2.7%)	16 (43.2%)	20 (54.1%)	1 (2.7%)
Ng-negative AC2 samples (167)	—	—[d]	—	2 (1.2%)[d]	—	—	—
N. meningitidis (6)	—	—	—	—	—	—	—
Non-Ng commensals (137)	2[e]	—[f]	—[f]	—	—	—	—
Neisseria flavescens (52)	—	—	—	—	—	—	—
Neisseria perflava (27)	—	—	—	—	—	—	—
N. macacae (9)	—	—	—	—	—	—	—
N. mucosa (6)	1[e]	—	—	—	—	—	—
N. sicca (4)	—	—	—	—	—	—	—
Neisseria cinerea (2)	—	—	—	—	—	—	—
N. animalis (1)	1[e]	—	—	—	—	—	—
Neisseria species (34)	—	—	—	—	—	—	—
M. osloensis (1)	—	—	—	—	—	—	—
M. catarrhalis (1)	—	—	—	—	—	—	—

NA, not applicable.
[a]Two AC2 samples (one urine sample and one rectal swab) were false-negative Ng compared with previous AC2 diagnostics.
[b]One sample had equally amplified GyrA S91 WT and GyrA S91F target.
[c]Two samples were only positive for the *opa* target, but reported as Ng positive by the analysis software.
[d]Eight samples amplified GyrA S91 WT and two samples (vaginal and rectal swabs) were excluded due to invalid internal control (probable inhibition).
[e]Positive for only the *opa* target, but reported as Ng positive by the analysis software. An additional eight non-gonococcal *Neisseria* isolates were positive for the *opa* target (*N. cinerea*, *N. macacae*, *N. perflava*, *N. flavescens*, *N. mucosa* and *Neisseria* species) and one isolate for *porA* (*N. flavescens*), but these were not reported as Ng using the analysis software.
[f]Amplification of GyrA S91 WT target was seen in *N. macacae* ($n = 2$), *N. mucosa* ($n = 1$), *N. sicca* ($n = 1$) and *Neisseria* species ($n = 1$). Dual amplification of both *gyrA* targets was seen in *N. macacae* ($n = 3$), *N. sicca* ($n = 1$) and *Neisseria* species ($n = 1$). However, these were not reported as Ng using the analysis software.

respectively. Regarding cross-reaction, GyrA S91 WT signal was visually observed in eight (4.8%) Ng-negative AC2 pharyngeal samples (all *gyrA* negative using the in-house *gyrA* PCR[21]) and GyrA S91 WT signal or amplification of both *gyrA* targets was observed in isolates of several non-gonococcal *Neisseria* species: *Neisseria macacae* (5/9 isolates), *Neisseria sicca* (2/4 isolates), *Neisseria* species (2/34 isolates) and *Neisseria mucosa* (1/6 isolates) (Table 1). However, all these samples were correctly reported as Ng negative using ResistancePlus GC analysis software. Two other non-gonococcal isolates (*N. mucosa* and *Neisseria animalis*) were reported as Ng positive but were only positive for the *opa* signal and showed no GyrA S91 call. All the cross-reactive non-pathogenic commensal *Neisseria* isolates were negative for the *gyrA* targets in the in-house Ng *gyrA* PCR.[21] Notably, many (81%) of the Ng-positive AC2 samples visually showed some level of amplification of both GyrA S91 WT and S91F, although the amplification of one of the targets was considerably stronger (lower cycle threshold value) than the other, illustrating the necessity of using the ResistancePlus GC analysis software. Almost all cultured Ng isolates showed some level of amplification of both *gyrA* targets, likely a result of the high bacterial load that was tested and possibly due to multiple Ng isolates in some samples.

For the 967 European Ng isolates[9] and the 37 Ng isolates paired with Ng-positive AC2 samples, both the sensitivity and specificity of the *ResistancePlus*® GC assay to predict phenotypic ciprofloxacin resistance by detection of GyrA S91F was 99.8%.

In total, 2.6% ($n = 8$) of all Ng-positive or Ng-negative AC2 samples were indeterminate in the *ResistancePlus*® GC assay due to invalid internal control ($n = 2$), indeterminate *gyrA* detection but with a valid Ng detection ($n = 4$), or the assay could not detect any of the Ng or *gyrA* targets ($n = 2$).

GC 23S 2611 (beta) assay for azithromycin resistance prediction

Detection of N. gonorrhoeae

The GC 23S 2611 (beta) assay detected Ng in all except one of the 964 European Ng isolates (99.9%).[9] This sample was repeatedly reported as negative by the analysis software, although a 23S rRNA gene signal was detected. For Ng-positive AC2 samples ($n = 143$), the assay detected Ng in 137 (95.8%) samples, missing Ng in three rectal samples, two urine samples and one pharyngeal sample. All Ng-negative AC2 samples and isolates of non-gonococcal *Neisseria* species were correctly identified as Ng negative (Table 2). The sensitivity and specificity of the assay for Ng detection in AC2 samples were 95.8% and 100%, respectively.

Detection of 23S rRNA C2611 WT/C2611T

Examining the 964 European Ng isolates,[9] the 23S rRNA gene C2611 WT target was detected by the GC 23S 2611 (beta) assay in 955 (99.1%) isolates, with all (100%) isolates in agreement with the WGS results, and the C2611T mutation in 9 (0.9%) isolates (Table 2), with 8 (88.9%) isolates in concordance with the WGS results. Of the 955 isolates with 23S rRNA C2611 WT, 647 had azithromycin MIC values of 0.016–1.0 mg/L, 5 had azithromycin MICs of 2 mg/L (likely due to their overexpressed MtrCDE efflux pump) and 303 with exact MIC lacking due to the use of agar dilution breakpoint technique in some European countries in 2013 (all susceptible, i.e. MICs $\leq$ 0.25 mg/L). The eight isolates with 23S rRNA gene C2611T had azithromycin MICs ranging from 2 to 12 mg/L and all had 3–4 mutated 23S rRNA gene alleles according to the previous WGS.[9] Among the Ng-positive AC2 samples detected as Ng positive ($n = 137$), the GC 23S 2611 (beta) assay reported 23S rRNA C2611 WT in 128 samples (89.5%, $n = 34$ with known azithromycin MICs of 0.016–1 mg/L), C2611T in 2 samples (1.4%, one with known azithromycin MIC of 8 mg/L) and 7 (4.9%) samples were indeterminate. Examining the Ng-negative AC2 samples, 23S rRNA gene C2611 WT signals were visually observed in 48 (28.7%) samples, i.e. pharyngeal ($n = 43$), cervical ($n = 3$) and vaginal ($n = 2$) samples; however, all these samples were reported as Ng negative by the analysis software of the GC 23S 2611 (beta) assay. Furthermore, 62 (45.3%) of the non-pathogenic commensal *Neisseria* isolates (azithromycin MIC: 1–24 mg/L) and all six *N. meningitidis* reference strains visually had C2611 WT signals but all these samples were reported as Ng negative by the analysis software of the GC 23S 2611 (beta) assay (Table 2). Notably, the GC 23S 2611 (beta) assay showed visual amplification of both the 23S rRNA gene C2611 WT and C2611T targets in 23.8% ($n = 34$) of the Ng-positive AC2 samples and 1.9% ($n = 18$) of the non-gonococcal *Neisseria* isolates including all *N. meningitidis* reference strains, emphasizing the requirement for the GC 23S 2611 (beta) analysis software in the analysis of raw results.

For the 964 European Ng isolates and the 37 Ng isolates paired with Ng-positive AC2 samples, the overall sensitivity and specificity of the GC 23S 2611 (beta) assay to predict azithromycin resistance by detection of 23S rRNA C2611T mutation compared with the phenotypic azithromycin resistance testing were 64.3% and 99.9%, respectively. However, the sensitivity and specificity of the GC 23S 2611 (beta) assay to detect the 23S rRNA C2611T mutation, compared with the WGS data, were 100% and 99.9%, respectively.

Discussion

Sensitive and specific molecular assays for prediction of Ng AMR or antimicrobial susceptibility are imperative, both to inform individualized treatment and for AMR surveillance, which is further highlighted by the WHO global action plan.[3–8] In the present study, the new commercially available *ResistancePlus*® GC assay showed high sensitivity and specificity in the detection of Ng, compared with the AC2 assay, and ability to detect and distinguish GyrA S91 WT and S91F. Both the sensitivity and specificity of the *ResistancePlus*® GC assay to predict ciprofloxacin resistance compared with phenotypic ciprofloxacin resistance testing were 99.8%, supporting the proposition that the assay can be effectively used for AMR surveillance and individualized treatment with ciprofloxacin, which is easily accessible and administered as a single 500 mg oral dose. Although signals for both the GyrA S91F resistance mutation (3.6%) and GyrA WT (7.3%) were visually observed in non-gonococcal commensal *Neisseria* isolates, all these samples were reported as Ng negative by the analysis software. Nevertheless, the high bacterial load of the non-gonococcal *Neisseria* isolates substantially challenging the assay likely seriously affected the probability of cross-reaction and, in future similar analytical evaluations, the bacterial load should ideally be standardized at more clinically relevant concentrations. These results regarding cross-reaction are comparable to those from

Table 2. Evaluation of SpeeDx GC 23S 2611 (beta) assay for azithromycin resistance/susceptibility prediction, examining Ng isolates from 20 European countries,[9] clinical AC2 samples and non-gonococcal *Neisseria* and closely related isolates

Samples (*n*)	Ng (*porA*)	23S rRNA C2611 WT	23S rRNA C2611T	Indeterminate
European isolates (964)[9]	963[a]	955 (99.1%)	9 (0.9%)[b]	—
Ng-positive AC2 samples (143)	137[c]	128 (89.5%)	2 (1.4%)	7 (4.9%)
Samples without confirmed culture (106)	101	94 (88.7%)	1 (0.9%)	6 (5.7%)
Samples with paired culture (37)	36	34 (91.9%)	1 (2.7%)	1 (2.7%)
Ng-negative AC2 samples (167)	—	48 (28.7%)[d]	—	—
N. meningitidis (6)	—	6 (100%)[e]	—	—
Non-Ng commensals (137)	—	62 (45.3%)[e]	—	—
N. flavescens (52)	—	23	—	—
N. perflava (27)	—	12	—	—
N. macacae (9)	—	5	—	—
N. mucosa (6)	—	4	—	—
N. sicca (4)	—	3	—	—
N. cinerea (2)	—	2	—	—
N. animalis (1)	—	1	—	—
Neisseria species (34)	—	12	—	—
M. osloensis (1)	—	—	—	—
M. catarrhalis (1)	—	—	—	—

[a]One isolate failed in the *porA* target but the 23S rRNA gene C2611 target was amplified.
[b]Eight isolates (azithromycin MIC 2–12 mg/L) had 23S rRNA C2611T according to previous WGS, but the remaining isolate (azithromycin MIC 0.5 mg/L) had 23S rRNA C2611 WT.
[c]Six AC2 samples were false-negative Ng compared with previous AC2 diagnostics.
[d]All reported as Ng negative by the analysis software.
[e]All six *N. meningitidis* strains and *N. flavescens* ($n = 3$), *N. macacae* ($n = 3$), *N. sicca* ($n = 2$), *N. cinerea* ($n = 2$), *N. animalis* ($n = 1$) and *Neisseria* species ($n = 1$) also showed a weak cross-reaction to the 23S rRNA C2611T target. However, all isolates were reported as Ng negative by the analysis software.

previous studies, including one evaluating the beta version of the *ResistancePlus*® GC assay that reported cross-reactions in non-pathogenic isolates and pharyngeal samples, as well as indeterminate *gyrA* calls.[13,20] Another study[22] showed that the *gyrA* genotype determination was significantly improved using the *ResistancePlus*® GC assay compared with an in-house PCR assay using high-resolution melt *gyrA* analysis. However, a proportion of samples remained indeterminate for *gyrA*, which is likely due to the low Ng load in some clinical NAAT samples and/or inhibition and cross-reactions with other *Neisseria* species.[22] In particular, extragenital sites, such as the pharynx, are challenging for molecular Ng AMR prediction, because these sites frequently harbour non-gonococcal *Neisseria* species as commensals and many DNA sequences, including AMR determinants, are identical or very similar in several *Neisseria* species.[1–3,18] Accordingly, the cross-reactive non-gonococcal *Neisseria* isolates and AC2 pharyngeal samples in the present study illustrate some of the main problems faced by the field of molecular prediction of AMR in Ng. Previous studies of molecular assays for prediction of ciprofloxacin AMR or susceptibility have reported an overall sensitivity of 95.8%–100% and a specificity of 97.9%–100%. However, several of these assays did not use any internal control in their molecular assay,[12,15] included a low number or no extragenital samples[13,21,23] or Ng-negative clinical NAAT samples,[15,24] which are essential for assessing cross-reactivity. Finally, the cost-effectiveness of using the *ResistancePlus*® GC assay or similar assays to predict ciprofloxacin resistance and inform individualized treatment in a setting depends on the local prevalence of ciprofloxacin resistance, the cost of the assay used, the proportion of NAAT samples that can be typed in regard to ciprofloxacin resistance/susceptibility, the testing frequency, the cost of ciprofloxacin locally and the cost of the empirical treatment used for ciprofloxacin-resistant or indeterminate cases.[25]

The GC 23S 2611 (beta) assay for detection of the 23S rRNA gene C2611 WT or C2611T resistance mutation and prediction of azithromycin resistance/susceptibility is still under development and not yet commercially available. This assay does not yet include any internal/inhibition control and contains only a single *porA* pseudogene target for detection of Ng. The proportion of indeterminate 23S rRNA C2611 results was 4.9% in Ng-positive AC2 samples, and many Ng-negative AC2 samples (28.7%), predominantly pharyngeal samples, and non-gonococcal *Neisseria* isolates (47.6%) visually showed some cross-reaction producing a C2611 WT signal; however, all these samples were reported as Ng negative by the analysis software. It was visually observed that the GC 23S 2611 (beta) assay frequently amplified both of the 23S rRNA gene targets, which also can be correct because it could be a mixed infection or only some of the four 23S rRNA gene alleles in the same gonococcal strain may be mutated,[9,16,17] and the GC 23S 2611 (beta) analysis software is imperative for analysis. The

detection of 23S rRNA targets in non-gonococcal *Neisseria* isolates may be mostly reflecting the high bacterial load tested. Similarly, a study by Donà *et al.*[26] also showed cross-reaction to C2611 WT with difficulty in the accurate prediction of C2611T AMR in extragenital samples. To reduce this type of cross-reaction, Trembizki *et al.*[19] used non-template bases in primers to distinguish the 23S rRNA gene amplification of Ng from commensals, with delayed amplification of the latter, in addition to melting curve analysis of the target. Similar to our findings, their 23S rRNA gene C2611 PCR assay mainly showed cross-reactivity with Ng-negative clinical pharyngeal NAAT samples and all had C2611 WT alleles.[19]

Importantly, azithromycin resistance can also be caused by other 23S rRNA gene mutations such as the A2059G mutation,[1,17,18,27] which results in high-level azithromycin resistance.[8,16,17] Furthermore, four copies of the 23S rRNA gene are present in the gonococcal genome and a higher number of mutated alleles causes higher MICs of azithromycin.[17,28] In addition, many other molecular AMR determinants such as *erm* genes and overexpressed MtrCDE efflux pump can also cause decreased susceptibility and resistance to azithromycin,[1,16–18,29,30] which is an inherent limitation of all Ng 23S rRNA-based molecular AMR assays. The GC 23S 2611 (beta) assay evaluated in the present study does not distinguish the number of mutated 23S rRNA gene alleles; however, this is likely not a problem because it has been shown that any mutated alleles will likely be shared, through recombination, as soon as the gonococcal strain is exposed to azithromycin or other similar macrolides.[1,16,17] Accordingly, independent of the azithromycin MIC of such gonococcal strains, 23S rRNA gene mutations should be detected and azithromycin should not be used for treatment of strains harbouring any mutated alleles.

In conclusion, the new SpeeDx *ResistancePlus*® GC assay performed well in the detection of Ng and AMR determinants. Our results support the proposition that the SpeeDx *ResistancePlus*® GC assay can be effectively used for individualized treatment, where patients with ciprofloxacin-resistant and indeterminate samples receive empirical treatment (for example, ceftriaxone plus azithromycin),[2,18] and AMR surveillance, particularly in urogenital samples. This type of individualized treatment will also reduce the use of empirical treatment (ceftriaxone and azithromycin), which is of importance to spare the last-line ceftriaxone, reduce azithromycin use and in general antimicrobial use, in accordance with antimicrobial stewardship strategies. The GC 23S 2611 (beta) assay performed relatively well, but its sensitivity for predicting phenotypic azithromycin resistance was suboptimal and further evaluation and optimizations are required, including detection of the 23S rRNA A2059G mutation as well as additional macrolide resistance determinant(s). Furthermore, adding detection of a multicopy gene, e.g. the *opa* genes, would increase the sensitivity of Ng detection. Cross-reactivity was visually observed with non-gonococcal commensal *Neisseria* isolates and foremost in clinical AC2 pharyngeal samples, particularly for the GC 23S 2611 (beta) assay, emphasizing the necessity to use the SpeeDx analysis software. It would be valuable to examine a larger collection of Ng-positive and Ng-negative clinical NAAT samples, with paired cultured Ng isolates and using both the *ResistancePlus*® GC assay and the GC 23S 2611 (beta) assay. Clearly, the high sensitivity and specificity of the *ResistancePlus*® GC assay and promising potential of the GC 23S 2611 (beta) assay encourage the further development of molecular AMR or antimicrobial susceptibility assays for Ng to complement culture-based methods in AMR surveillance and, ultimately, to inform individualized treatment of gonorrhoea. Nevertheless, it remains important to continuously strengthen culture-based phenotypic AMR surveillance for Ng, i.e. for national and international gonococcal antimicrobial surveillance programmes and to detect new AMR determinants.

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References

1 Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. *Clin Microbiol Rev* 2014; **27**: 587–613.

2 Unemo M, Lahra MM, Cole M *et al.* World Health Organization Global Gonococcal Antimicrobial Surveillance Program (WHO GASP): review of new data and evidence to inform international collaborative actions and research efforts. *Sex Health* 2019; **16**: 412–25.

3 Donà V, Low N, Golparian D *et al.* Recent advances in the development and use of molecular tests to predict antimicrobial resistance in *Neisseria gonorrhoeae*. *Expert Rev Mol Diagn* 2017; **17**: 845–59.

4 WHO. Global Action Plan to Control the Spread and Impact of Antimicrobial Resistance in *Neisseria gonorrhoeae*. https://apps.who.int/iris/handle/10665/44863.

5 Low N, Unemo M. Molecular tests for the detection of antimicrobial resistant *Neisseria gonorrhoeae*: when, where, and how to use? *Curr Opin Infect Dis* 2016; **29**: 45–51.

6 Goire N, Lahra MM, Chen M *et al.* Molecular approaches to enhance surveillance of gonococcal antimicrobial resistance. *Nat Rev Microbiol* 2014; **12**: 223–9.

7 Sadiq ST, Mazzaferri F, Unemo M. Rapid accurate point-of-care tests combining diagnostics and antimicrobial resistance prediction for *Neisseria gonorrhoeae* and *Mycoplasma genitalium*. *Sex Transm Infect* 2017; **93**: S65–8.

8 Hall CL, Harrison MA, Pond MJ *et al*. Genotypic determinants of fluoroquinolone and macrolide resistance in *Neisseria gonorrhoeae*. *Sex Health* 2019; **16**: 479–87.

9 Harris SR, Cole MJ, Spiteri G *et al*. Public health surveillance of multidrug-resistant clones of *Neisseria gonorrhoeae* in Europe: a genomic survey. *Lancet Infect Dis* 2018; **18**: 758–68.

10 Allan-Blitz LT, Wang X, Klausner JD. Wild-type gyrase A genotype of *Neisseria gonorrhoeae* predicts in vitro susceptibility to ciprofloxacin: a systematic review of the literature and meta-analysis. *Sex Transm Dis* 2017; **44**: 261–5.

11 Trees DL, Sandul AL, Peto-Mesola V *et al*. Alterations within the quinolone resistance-determining regions of GyrA and ParC of *Neisseria gonorrhoeae* isolated in the Far East and the United States. *Int J Antimicrob Agents* 1999; **12**: 325–32.

12 Giles J, Hardick J, Yuenger J *et al*. Use of Applied Biosystems 7900HT sequence detection system and Taqman assay for detection of quinolone-resistant *Neisseria gonorrhoeae*. *J Clin Microbiol* 2004; **42**: 3281–3.

13 Magooa MP, Müller EE, Gumede L *et al*. Determination of *Neisseria gonorrhoeae* susceptibility to ciprofloxacin in clinical specimens from men using a real-time PCR assay. *Int J Antimicrob Agents* 2013; **42**: 63–7.

14 Peterson SW, Martin I, Demczuk W *et al*. Molecular assay for detection of ciprofloxacin resistance in *Neisseria gonorrhoeae* isolates from cultures and clinical nucleic acid amplification test specimens. *J Clin Microbiol* 2015; **53**: 3606–8.

15 Zhao L, Zhao S. TaqMan real-time quantitative PCR assay for detection of fluoroquinolone-resistant *Neisseria gonorrhoeae*. *Curr Microbiol* 2012; **65**: 692–5.

16 Ng LK, Martin I, Liu G *et al*. Mutation in 23S rRNA associated with macrolide resistance in *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 2002; **46**: 3020–5.

17 Chisholm SA, Dave J, Ison CA. High-level azithromycin resistance occurs in *Neisseria gonorrhoeae* as a result of a single point mutation in the 23S rRNA genes. *Antimicrob Agents Chemother* 2010; **54**: 3812–6.

18 Unemo M, Seifert HS, Hook EW 3rd *et al*. Gonorrhoea. *Nat Rev Dis Primers* 2019; **5**: 79.

19 Trembizki E, Buckley C, Donovan B *et al*. Direct real-time PCR-based detection of *Neisseria gonorrhoeae* 23S rRNA mutations associated with azithromycin resistance. *J Antimicrob Chemother* 2015; **70**: 3244–9.

20 Ebeyan S, Windsor M, Bordin A *et al*. Evaluation of the ResistancePlus GC (beta) assay: a commercial diagnostic test for the direct detection of ciprofloxacin susceptibility or resistance in *Neisseria gonorrhoeae*. *J Antimicrob Chemother* 2019; **74**: 1820–4.

21 Buckley C, Trembizki E, Donovan B *et al*. A real-time PCR assay for direct characterization of the *Neisseria gonorrhoeae* GyrA 91 locus associated with ciprofloxacin susceptibility. *J Antimicrob Chemother* 2016; **71**: 353–6.

22 Allan-Blitz LT, Ellis OL, Wee R *et al*. Improved determination of *Neisseria gonorrhoeae* gyrase A genotype results in clinical specimens. *J Antimicrob Chemother* 2019; **74**: 2913–5.

23 Hemarajata P, Yang S, Soge OO *et al*. Performance and verification of a real-time PCR assay targeting the *gyrA* gene for prediction of ciprofloxacin resistance in *Neisseria gonorrhoeae*. *J Clin Microbiol* 2016; **54**: 805–8.

24 Pond MJ, Hall CL, Miari VF *et al*. Accurate detection of *Neisseria gonorrhoeae* ciprofloxacin susceptibility directly from genital and extragenital clinical samples: towards genotype-guided antimicrobial therapy. *J Antimicrob Chemother* 2016; **71**: 897–902.

25 Allan-Blitz LT, Hemarajata P, Humphries RM *et al*. A cost analysis of gyrase A testing and targeted ciprofloxacin therapy versus recommended 2-drug therapy for *Neisseria gonorrhoeae* infection. *Sex Transm Dis* 2018; **45**: 87–91.

26 Donà V, Smid JH, Kasraian S *et al*. Mismatch amplification mutation assay-based real-time PCR for rapid detection of *Neisseria gonorrhoeae* and antimicrobial resistance determinants in clinical specimens. *J Clin Microbiol* 2018; **56**: e00365–18.

27 Galarza PG, Abad R, Canigia LF *et al*. New mutation in 23S rRNA gene associated with high level of azithromycin resistance in *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 2010; **54**: 1652–3.

28 Unemo M, Golparian D, Hellmark B. First three *Neisseria gonorrhoeae* isolates with high-level resistance to azithromycin in Sweden: a threat to currently available dual-antimicrobial regimens for treatment of gonorrhea? *Antimicrob Agents Chemother* 2014; **58**: 624–5.

29 Roberts MC, Chung WO, Roe D *et al*. Erythromycin-resistant *Neisseria gonorrhoeae* and oral commensal *Neisseria* spp. carry known rRNA methylase genes. *Antimicrob Agents Chemother* 1999; **43**: 1367–72.

30 Wadsworth CB, Arnold BJ, Sater MRA *et al*. Azithromycin resistance through interspecific acquisition of an epistasis-dependent efflux pump component and transcriptional regulator in *Neisseria gonorrhoeae*. *mBio* 2018; **9**: e01419–18.