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Evaluation of the ResistancePlus GC (beta) assay: a commercial diagnostic test for the direct detection of ciprofloxacin susceptibility or resistance in *Neisseria gonorrhoeae*

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Objectives: To evaluate the performance of the ResistancePlus GC (beta) assay for the simultaneous detection of *Neisseria gonorrhoeae* and *gyrA* S91 markers of resistance (S91F) and susceptibility (WT) to ciprofloxacin, from both clinical specimens and isolates.

Methods: Performance was assessed on several sample banks, including *N. gonorrhoeae* isolates ($n = 822$), non-gonococcal isolates ($n = 110$), *N. gonorrhoeae*-positive clinical specimens ($n = 402$) and *N. gonorrhoeae*-negative specimens ($n = 290$). Results were compared with previous testing data, including S91 genotyping and phenotypic resistance profiles.

Results: Overall, the assay demonstrated 100% sensitivity for *N. gonorrhoeae* detection in clinical isolates. For *gyrA* S91 mutation detection in clinical isolates, the assay showed 100% sensitivity/specificity compared with the genotype, and >99%/>97% sensitivity/specificity when compared with phenotype. For positive clinical specimens, the assay demonstrated >96% sensitivity for *N. gonorrhoeae* detection and 100% sensitivity/specificity for *gyrA* S91 mutation detection. The assay demonstrated >99% specificity for *N. gonorrhoeae* detection against non-gonococcal isolates and 100% specificity for negative clinical specimens.

Conclusions: The ResistancePlus GC (beta) assay is suitable for the detection of *N. gonorrhoeae* and *gyrA* markers associated with resistance/susceptibility to ciprofloxacin directly in clinical samples. This assay could be implemented for the individualized treatment of gonorrhoea infections as well as to enhance current antimicrobial resistance surveillance methods.

Introduction

Antimicrobial-resistant (AMR) *Neisseria gonorrhoeae* is a global public health concern.[1,2] Under current management practices gonorrhoea is treated empirically, with treatment guided by available AMR surveillance data. Based on this model, when resistance rates approach the WHO-defined threshold of 5% an alternative empirical treatment is recommended.[3] However, this approach is fast becoming unsustainable as alternative treatment options are exhausted, with *N. gonorrhoeae* now having developed resistance to all previously recommended therapies including, in recent years, ceftriaxone.[1] New antimicrobials are still several years away from being available and we are therefore in a situation where utilizing current antibiotics more effectively is necessary to preserve current therapies. One potential approach that has been suggested is individualized treatment, where a diagnostic test is used to test for antibiotic susceptibility at an individual level, and then this information is used to guide selection of appropriate treatment for that individual.[4] There are several potential benefits of using this approach for gonorrhoea, including that it has the potential to reinstigate use of antibiotics that are no longer recommended because they have breached the WHO 5% threshold; at the same time, the method will foster antibiotic stewardship by, in the short term, sparing the use of ceftriaxone and, in the long term, sparing the use of novel therapies.[4,5]

Individualized antibiotic treatment relying on culture and traditional susceptibility testing methods is not always practical in clinical settings, being both time consuming and technically challenging for many pathogens. Using nucleic acid amplification test (NAAT)-based methods, individualized antibiotic treatment has so far become a reality for infections such as TB[6] and more

recently *Mycoplasma genitalium*.[7] Moreover, there is now evidence highlighting the feasibility of using a ciprofloxacin-based individualized treatment for gonorrhoea.[8] Ciprofloxacin, a fluoroquinolone antibiotic, ceased to be used as a front-line treatment for gonorrhoea in most parts of the world by the mid-2000s.[9] In fact, with few exceptions, ciprofloxacin resistance rates now exceed the WHO 5% threshold in most parts of the world (where such data are available)[1] and so it cannot be used empirically in most settings. Despite this, there are compelling reasons why ciprofloxacin is well suited for individualized treatment. These include: (i) the large proportions of gonorrhoea cases that remain susceptible to ciprofloxacin, with recent data indicating 53% in Europe[10] and >70% in Australia;[11] (ii) ciprofloxacin has an excellent pharmacokinetic profile for both urogenital and extra-genital infections (when the infecting strain is susceptible); (iii) it requires simple oral administration; (iv) it has relatively few side effects; (v) molecular targets for *N. gonorrhoeae* ciprofloxacin susceptibility are well described, with codon 91 of the *gyrA* gene identified as being highly predictive[5,12] (although resistance to ciprofloxacin has also been linked to mutations in the *gyrA* 95 codon, mutations in this position are rarely seen in the absence of *gyrA* 91 mutations, with a recent European surveillance study only finding 1/549 isolates with a single *gyrA* 95 mutation capable of raising the MIC[10]); and (vi) various in-house molecular assays have been described for direct detection of this *gyrA* 91 target.[13–16] Moreover, preliminary results from a pilot study in the USA demonstrate the feasibility of using an in-house PCR assay to guide ciprofloxacin treatment, where 100% of patients with WT *gyrA* 91 *N. gonorrhoeae* ($n = 25$) had a negative result at test of cure, including both genital and non-genital infections.[8,17]

To facilitate individualized ciprofloxacin treatment of *N. gonorrhoeae* infections in a broader range of settings, commercially available tests are required. To address this need, the ResistancePlus® GC (beta) multiplex real-time PCR assay was developed (SpeeDx Pty Ltd). The assay was designed to detect both the *gyrA* S91F mutation as a highly predictive marker for resistance to ciprofloxacin, as well as the *gyrA* WT 91 codon, which is highly predictive of susceptibility. The multiplex also included two *N. gonorrhoeae* detection targets, *opa* and *porA*. In this study, the sensitivity and specificity of the assay were evaluated on both characterized clinical isolates and clinical specimens collected from routine hospital testing and surveillance programmes.

Materials and methods

Assay design, reaction mixture and cycling conditions

The ResistancePlus GC (beta) test (SpeeDx Pty Ltd, Sydney, Australia) utilizes PlexPrime® and PlexZyme® technology[18] to enable high-level multiplexing of targets including antimicrobial resistance markers. The assay was performed as described by the manufacturer, where 10 μL of processed sample was added to 20 μL of master mix [15 μL of Plex Mastermix, 1.5 μL of GC gyrA oligonucleotide mix, 1.5 μL of control oligonucleotide mix, 1.5 μL of diluted Amplification Control (SpeeDx Pty Ltd), 0.5 μL of PCR grade H_2O] for a final reaction volume of 30 μL. Amplification and detection was performed using the Applied Biosystems® 7500 Fast Dx instrument, on 96-well plates and with the following thermocycling parameters: 95°C for 2 min, followed by 10 cycles of 95°C for 5 s, 61°C for 30 s (−0.5°C per cycle), 50 cycles of 95°C for 5 s and 52°C for 40 s. The assay reports detection across five channels: (i) Cy5 for the detection of *N. gonorrhoeae opa* gene; (ii) JOE for the detection of *N. gonorrhoeae porA* gene; (iii) FAM for the detection of *gyrA* wild-type S91; (iv) Texas Red for the detection of *gyrA* S91F mutant; and (v) TAMRA for the detection of an external control to monitor extraction and PCR inhibition. Data analysis was performed using the ResistancePlus GC (7500) (beta) analysis software. The assay reports the following results: *N. gonorrhoeae* not detected; *N. gonorrhoeae* detected *gyrA* mutant; *N. gonorrhoeae* detected, *gyrA* mutant not detected (WT); and *N. gonorrhoeae* detected, *gyrA* indeterminate.

Table 1. Bank 3 non-gonococcal isolates

Neisseria and *Moraxella* isolates	No. tested
Moraxella catarrhalis	7
Moraxella osloensis	2
Neisseria cinerea	4
Neisseria elongata	1
Neisseria flavescens	1
Neisseria lactamica	16
Neisseria mucosa	1
Neisseria polysaccharea	4
Neisseria sicca	4
Neisseria subflava	14
Neisseria weaveri	1
N. meningitidis	55
Total	110

N. gonorrhoeae and non-gonococcal isolates (Banks 1–3)

The performance of the assay was assessed on characterized *N. gonorrhoeae* and non-gonococcal isolate banks including: Bank 1, *N. gonorrhoeae* isolates ($n = 782$) collected from New South Wales (Australia) in 2014 and consisting of 108 unique genotypes (Table S1, available as Supplementary data at *JAC* Online);[19] Bank 2, *N. gonorrhoeae* isolates ($n = 40$) consisting of the 40 most common strains from Australia in 2012 (Table S2);[20] and Bank 3, a convenience sample of non-gonococcal isolates consisting of *Neisseria* spp. and *Moraxella* ($n = 55$) as well as *Neisseria meningitidis* isolates ($n = 55$) (Table 1). Both the *N. gonorrhoeae* isolate Banks 1 and 2 were previously characterized using iPLEX-MLST and iPLEX-AMR typing as well for phenotypic antimicrobial susceptibility profile using standard bacterial culture methods as part of the Australian Gonococcal Surveillance Programme (AGSP).[19,20] For testing with the ResistancePlus GC (beta) assay, isolates were prepared to 1.0 McFarland standard in distilled water and boiled at 100°C for 8 min to release any nucleic acids, before being briefly spun down to capture any droplets and supernatant added to the PCR reaction.

N. gonorrhoeae-positive clinical samples (Banks 4 and 5)

The ResistancePlus GC (beta) assay was evaluated on two clinical sample banks to assess detection of *N. gonorrhoeae* as well as characterization of *gyrA*. Sample Bank 4 was collected from Royal Darwin Hospital Pathology (Western Australia) in 2014 ($n = 140$) and has previously been described;[13] these samples were positive for *N. gonorrhoeae* by both the Siemens Versant CT/NG assay and bacterial culture and had parallel antibiotic susceptibility testing (AST) performed. The remnant DNA extracts from the Siemens Versant CT/NG assay were used for the ResistancePlus GC (beta) assay. The second clinical sample bank (Bank 5) comprised routinely collected clinical samples from Pathology Queensland (Australia) in the years 2014 and 2016–17 ($n = 262$); these samples were *N. gonorrhoeae* positive

Table 2. Specimen type information for *N. gonorrhoeae*-positive clinical samples

	Bank 4: Royal Darwin Hospital Pathology				Bank 5: Pathology Queensland			
	male	female	unknown	total	male	female	unknown	total
Anal swab	1	1	–	2	–	–	–	–
Unspecified aspirate	1	–	–	1	–	–	–	–
Cervical swab	–	24	–	24	–	63	–	63
Unspecified genital swab	–	2	–	2	–	4	–	4
Penile swab	1	–	–	1	27	–	–	27
Pharyngeal swab	1	1	–	2	73	8	–	81
Urethral swab	24	–	1	25	44	–	1	45
Urine	61	1	–	62	–	–	–	–
Vaginal swab	–	21	–	21	–	42	–	42
Total	89	50	1	140	144	117	1	262

Values are given as number of samples.

by cobas® 4800 CT/NG and an in-house real-time PCR targeting the *opa* and *porA* gene regions.[21] The remnant DNA extracts from the cobas® 4800 CT/NG were used for the ResistancePlus GC (beta) assay. A breakdown of specimen types in Banks 4 and 5 is shown in Table 2. All samples from Banks 4 and 5 were also characterized for the S91F *gyrA* mutation via a previously described in-house PCR assay,[13] which reported results as either *gyrA* mutant, *gyrA* WT or 'NC' for samples for which no call could be determined.

N. gonorrhoeae-negative clinical samples (Bank 6)

The specificity of the assay was further challenged with 290 cobas® 4800 CT/NG (Roche Diagnostics, Australia) negative specimens, collected from routine gonorrhoea testing at Pathology Queensland in 2017. The remnant DNA extracts from the cobas® 4800 CT/NG were used for the ResistancePlus GC (beta) assay. These consisted of 110 pharyngeal, 103 cervical, 64 vaginal, 4 urethral and 9 genital site unspecified swabs.

Results

For Bank 1, 100% of isolates were correctly detected as *N. gonorrhoeae* by the ResistancePlus GC (beta) assay and provided a *gyrA* call (383 S91F, 399 WT). No isolates provided an indeterminate call for *gyrA* (Table S1). All isolates had previously available information for the *gyrA* 91 codon via iPLEX genotyping, and 781/782 had culture-based AST results for ciprofloxacin (isolate NSW-006 did not have AST results and was not able to be regrown). Overall the ResistancePlus GC (beta) assay demonstrated 100.0% sensitivity and specificity (95% CI 99.04%–100.0% and 99.08%–100.0%, respectively) for detection of the S91F mutation and S91 WT, respectively, when compared with iPLEX-AMR genotyping, and 99.7% (95% CI 98.55%–99.99%) sensitivity and 99.61% (95% CI 98.9%–99.99%) specificity when compared with the phenotypic-based susceptibility data. Two isolates showed discordant results between the ResistancePlus GC (beta) assay and phenotype. One NSW isolate (NSW-002; Bank 1, Table S1) was S91F positive by the ResistancePlus GC (beta) assay and previous iPLEX genotyping but was categorized as less susceptible to ciprofloxacin. A second NSW isolate (NSW-008; Bank 1, Table S1) was WT by both ResistancePlus GC (beta) assay and previous iPLEX genotyping but was phenotypically resistant to ciprofloxacin. All other isolates from the same genotype groups as the above discordant isolates provided a concordant phenotype with the ResistancePlus GC (beta) assay and iPLEX *gyrA* genotyping results (Table S1; where there were 121 isolates with NSW-002 genotype and 44 isolates with NSW-008), suggesting there may have been some sampling error during AST testing for these two isolates. Isolates NSW-002 and NSW-008 were re-grown and susceptibility testing was repeated. The NSW-002 isolate remained discordant with genotyping and the ResistancePlus GC (beta) assay, with an MIC of 0.25 mg/L corresponding to a less susceptible phenotype; the NSW-008 isolate, however, was seen to be susceptible to ciprofloxacin (MIC 0.008 mg/L) on repeat and became concordant with genotyping and the assay.

All Bank 2 isolates were correctly detected as *N. gonorrhoeae* by the ResistancePlus GC (beta) assay, with 100.0% sensitivity and specificity (95% CI 75.3%–100.0% and 87.2%–100.0%, respectively) compared with iPLEX-AMR genotype and 100.0% sensitivity and specificity (95% CI 75.3%–100.0% and 87.2%–100.0%) when compared with culture-based phenotypic results for ciprofloxacin.

For Bank 3, 10/55 *Neisseria* spp. and *Moraxella* isolates, as well as the majority of the *N. meningitidis* isolates (52/55), produced a WT *gyrA* signal with the ResistancePlus GC (beta) assay; however, the analysis software has been designed to only report *gyrA* status if *opa* and/or *porA* are detected. Hence, the ResistancePlus GC (beta) assay correctly identified all non-gonococcal isolates as *N. gonorrhoeae* negative, except for one *N. meningitidis* isolate that returned the result of *N. gonorrhoeae* detected, *gyrA* WT, where the *N. gonorrhoeae opa* target was detected. The specificity of the ResistancePlus GC (beta) assay for Bank 3 was, therefore, 99.1% (95% CI 95.0%–100.0%).

For sample Bank 4, 100% of samples were correctly detected as *N. gonorrhoeae* by the ResistancePlus GC (beta) assay and 138/140 samples provided a *gyrA* call. Performance for *gyrA* S91 mutation detection was first compared with the in-house *gyrA* PCR and second to the phenotypic antimicrobial susceptibility profile. A total of 130/140 samples were included in the comparison between PCR assays, as 10 samples were NC with the in-house assay and/or *gyrA* indeterminate with the ResistancePlus (beta) assay. The ResistancePlus GC (beta) test showed 100.0% sensitivity and specificity (95% CI 83.9%–100.0% and 96.7%–100.0%, respectively) for *gyrA* S91F detection from the remaining samples compared with the in-house PCR (Table S3). A total of 138/140

samples were included in the comparison with AST results, where ResistancePlus GC (beta) detection of S91F demonstrated 100.0% sensitivity (95% CI 83.2%–100.0%) and 98.3% specificity (95% CI 94.0%–99.7%) compared with AST-defined resistance or susceptibility to ciprofloxacin, respectively (Table S4). Two samples were discordant between the ciprofloxacin phenotypic profile and the assay; both samples were susceptible to ciprofloxacin (albeit less susceptible, with MIC values of 0.125 and 0.25 mg/L) but were *gyrA* mutants by both the ResistancePlus GC (beta) assay and the in-house PCR.

For sample Bank 5, 254/262 (96.9%) of samples were correctly identified as *N. gonorrhoeae* detected by the ResistancePlus GC (beta) assay. False-negative samples included six female (three vaginal, two cervical and one throat) and two male (one penile and one urethral) swab specimens; all eight of these samples also failed to produce a result with the in-house *gyrA* PCR assay. Two samples (one penile swab and one vaginal swab) produced an invalid internal control (IC) call with the ResistancePlus (beta) assay, indicative of potential PCR inhibition, and could not be re-extracted for retesting. A summary of *gyrA* results from all samples is shown in Table S5. A total of 194/262 samples were included in the assessment of *gyrA* S91 mutation detection, as 68 samples were NC with the in-house *gyrA* assay and/or *gyrA* indeterminate with the ResistancePlus (beta) assay (Table S5). These 194 samples consisted of 51 pharyngeal, 48 cervical, 23 penile, 40 urethral, 28 vaginal and 4 genital swabs of unspecified site. For these 194 samples, the in-house *gyrA* PCR results correlated 100% with the *gyrA* results of the ResistancePlus GC (beta) assay, providing 100.0% sensitivity and specificity (95% CI 92.1%–100.0% and 97.6%–100.0%, respectively).

For sample Bank 6, the ResistancePlus GC (beta) assay demonstrated 100% specificity for *N. gonorrhoeae*, with all 290 *N. gonorrhoeae*-negative clinical specimens correctly identified as *N. gonorrhoeae* not detected. It was observed that 7/290 samples (2 cervical and 5 pharyngeal swabs) showed detection of a *gyrA* WT signal; however, the analysis software correctly identified these as negative as the *porA* and *opa* targets produced no signal.

Discussion

Overall the ResistancePlus GC (beta) assay proved highly suitable for simultaneous *N. gonorrhoeae* detection and *gyrA* characterization for both isolates and clinical samples. Notably, sensitivity and specificity data for *gyrA* S91 mutation detection was 100% for clinical samples, indicating the assay could be confidently used for informing ciprofloxacin treatment. This performance compares favourably to other published *gyrA* PCR assays[5,12,13,15,16,22] that have targeted the same *gyrA* markers for susceptibility and/or resistance to ciprofloxacin.

The overall characterization rates (i.e. number of *N. gonorrhoeae*-positive clinical samples providing a *gyrA* call) were high, exceeding 98% for Bank 4 and 84% for Bank 5, where only a small number of samples returned a *gyrA*-indeterminate call (Tables S3–S5). As Bank 4 consisted of culture-positive samples, *N. gonorrhoeae* loads would have therefore been on average higher than for Bank 5, which consisted of routinely collected *N. gonorrhoeae* NAAT-positive samples. Other published assays have reported *gyrA*-indeterminate rates between 7% and 29%, with assay sensitivity and low *N. gonorrhoeae* DNA loads as well as interference from non-gonococcal *Neisseria* spp. in samples attributed as potential causes.[13,15,23] The impact of a *gyrA* indeterminate call on clinical management is believed to be minimal, as a patient with a *N. gonorrhoeae* infection of unknown ciprofloxacin status would still receive standard of care and the observed indeterminate rate with this assay is low, meaning the assay remains effective for diagnostic use.

For the non-gonococcal isolates (Bank 3), WT *gyrA* signal was detected in 56.4% of isolates, indicating that the *gyrA* WT component of the test is not specific to *N. gonorrhoeae*. This type of cross-reaction is a well-recognized problem for *N. gonorrhoeae* NAAT methods, including diagnostic methods,[24] but is particularly problematic for AMR methods given markers of resistance are also shared between gonococcal and non-gonococcal *Neisseria* spp. To counteract this issue, the ResistancePlus GC (beta) analysis software requires detection of *opa* or *porA* genes within a pre-determined Cq range before the *gyrA* results are analysed, thereby limiting the potential for *gyrA* false-positive results. It should also be noted that the isolate preparations used here were at high loads, which likely contributed to the high rates of cross-reaction observed for the commensal isolates. This is further highlighted by the fact that only 2.4% of *N. gonorrhoeae*-negative clinical samples (Bank 6) produced a *gyrA* WT signal. Notably, this bank comprised 110 pharyngeal samples (of which only 4 generated a *gyrA* signal) in which commensal *Neisseria* species and strains are ubiquitous. Likewise, for Banks 4 and 5, there was no evidence of interference from non-gonococcal organisms impacting the gonococcal *gyrA* calls, with the ResistancePlus GC (beta) assay correlating 100% with the in-house *gyrA* assay.

The ResistancePlus GC (beta) assay can be readily implemented in the diagnostic laboratory, as a reflex test, on *N. gonorrhoeae*-positive samples to confirm *N. gonorrhoeae* as well as provide additional resistance information that can be used for patient management. This allows individualized treatment of patients with ciprofloxacin-susceptible infections and is a step towards improved antibiotic stewardship and conservation of last-line treatment options. The ResistancePlus GC (beta) assay is, however, still a PCR-based test, requiring access to specialized laboratory equipment and appropriately skilled staff, and because of this would primarily be used to inform treatment of asymptomatic patients returning for treatment on the basis of a positive NAAT result. The translation of this assay into a point-of-care or near-patient format, which can be performed within the timeframe of a patient's visit to a clinic, would further reduce result turn-around times and facilitate treatment of symptomatic patients who would otherwise receive empirical syndromic treatment.

In conclusion, the ResistancePlus GC (beta) assay, which simultaneously detects the presence of *N. gonorrhoeae* and the *gyrA* S91F mutant and S91 WT markers of ciprofloxacin resistance/susceptibility, demonstrated sensitive and specific detection across a range of gonococcal isolates and directly in clinical samples. The ResistancePlus GC (beta) test can be used to individualize patient treatment and could also be used as a molecular surveillance tool to enhance culture-based susceptibility testing.

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Supplementary data

Tables S1–S5 are available as Supplementary data at *JAC* Online.

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