Mismatch Amplification Mutation Assay (MAMA)-Based Real-Time PCR for Rapid Detection of Neisseria gonorrhoeae and Antimicrobial Resistance Determinants in Clinical Specimens

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Running title: Rapid detection of antimicrobial resistant gonococcus

Key words: Gonococcus, antimicrobial resistance, NAAT, real-time PCR, clinical samples, diagnostics

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Molecular methods are often used for Neisseria gonorrhoeae (NG) detection, but complete definition of antimicrobial resistance (AMR) patterns still requires phenotypic tests. We developed an assay that both identifies NG and detects AMR determinants in clinical specimens. We designed a mismatch amplification mutation assay (MAMA)-based SYBR Green real-time PCR targeting: one NG-specific region (opa); mosaic penA alleles (Asp345 deletion, Gly545Ser) associated with decreased susceptibility to cephalosporins; alterations conferring resistance to ciprofloxacin (GyrA: Ser91Phe), azithromycin (23S rRNA: A2059G and C2611T) and spectinomycin (16S rRNA: C1192T). We applied the real-time PCR to 489 clinical specimens, of which 94 had paired culture isolates, and evaluated its performance by comparison with commercial diagnostic molecular and phenotypic tests. Our assay exhibited a sensitivity/specificity of 93%/100%, 96%/95%, 90%/91%, 100%/100% and 100%/90% for the detection of NG directly from urethral, rectal, pharyngeal, cervical and vaginal samples, respectively. The MAMA strategy allowed the detection of AMR mutations by comparing cycle threshold values with the reference opa reaction. The method accurately predicted the phenotype to four antibiotic classes when compared with the MIC values obtained from 94 paired cultures (sensitivity/specificity for cephalosporins, azithromycin, ciprofloxacin and spectinomycin resistance: 100%/95%, 100%/100%, 100%/100% and not applicable (NA)/100%, respectively, in genital specimens; NA/72%, NA/98%, 100%/97%, and NA/96%, respectively, in extra-genital specimens). False-positive results, particularly for the penA Asp345del reaction were observed predominantly in pharyngeal specimens. Our real-time PCR assay is a promising rapid method to identify NG and predict AMR directly in genital specimens, but further optimization for extra-genital specimens is needed.
INTRODUCTION

*Neisseria gonorrhoeae* (NG) is the causative agent of the sexually transmitted infection gonorrhea. More than 90% of the estimated 78 million new cases of gonorrhea each year occur in low and middle income countries where access to diagnosis and treatment are often limited (1). Despite international efforts to promote preventive strategies and ensure adequate treatment, NG may develop resistance mechanisms to all antimicrobials that have been recommended for empirical first-line treatment (2-4).

Commercial nucleic acid amplification tests (NAATs) have replaced culture-based methods for diagnosis of gonococcal infections in many settings. However, a recognized disadvantage of these assays is that they do not provide any information about antimicrobial resistance (AMR) patterns (5). Culture methods are time-consuming and limited by stringent sampling, transport and culture conditions but they remain essential for surveillance to identify changes in AMR trends, detect emerging resistance patterns, and inform prompt revision of treatment guidelines. (6, 7). The importance of developing NAATs that detect and predict AMR determinants has been clearly recognized, both to enhance culture-based AMR surveillance and, potentially, support clinicians to provide individualized therapies (5, 8-10).

Most key molecular mechanisms that confer resistance to currently and previously recommended antimicrobials for gonorrhea are known. The presence of mosaic *penA* alleles encoding the penicillin binding protein 2 (PBP2) has been associated with decreased susceptibility and resistance to extended-spectrum cephalosporins (ESCs). For instance, the mosaic *penA* XXXIV allele, present in the successful internationally-spreading clone with NG multiantigen sequence typing (NG-MAST) sequence type (ST) 1407, as well as in other strains belonging to the same genogroup (11-14), has resulted in clinical treatment failures with ESCs worldwide (15). A Ser91Phe substitution in GyrA is present in all documented ciprofloxacin (CIP) resistant gonococcal strains (16). Moderate- and high-level resistance to azithromycin (AZM) is associated with the single nucleotide polymorphisms (SNPs) C2611T
or A2059G in at least three of the four 23S rRNA alleles (17, 18). The SNP C1192T in the
16S rRNA gene confers spectinomycin (SPC) resistance (19).

Many PCR-based tests to detect molecular determinants predicting resistance to a
single antibiotic have been developed, but there have been only few published attempts to
develop assays that predict AMR to multiple drugs (20). We previously developed a multiplex
high-resolution melting (HRM) based real-time PCR to detect NG and seven genetic AMR
determinants in clinical isolates (21). However, cross-amplification of Neisseria spp. and the
relatively high limit of detection (LOD) suggested that our assay would not be suitable for
direct testing of clinical specimens.

To overcome the limitations of our previously developed assay (20), we developed a new
rapid SYBR Green-based real-time PCR method based on a mismatch amplification mutation
assay (MAMA) strategy (22). This approach relies on strategic primer design to selectively
amplify a target sequence only in the presence of the AMR mutation of interest while
suppressing wild-type amplification. The objectives of this study were to describe our new
MAMA-based SYBR Green real-time PCR and report on its performance for the
identification (ID) of NG and detection of gonococcal AMR determinants directly in clinical
specimens.
MATERIALS AND METHODS

Design of the real-time PCR assay. Eight primer sets were designed with the Oligo Primer Analysis software v4.0 (Molecular Biology Insights) to amplify specific sequences of the targets described in Table 1. For ID, primers that amplify the gonococcal upstream regions of *opa* and *porA* were designed (21).

For the AMR determinants, one primer was designed to match at its 3'-end the mutations of interest in the *penA*, *gyrA*, 23S rRNA and 16S rRNA genes and a mismatch in the second last bp at the 3'-end was added. This strategy was also applied to the lower primer of the 16S rRNA reaction to improve discrimination from other *Neisseria* spp. For both mosaic *penA* reactions (*penA* Asp345del and *penA* Gly545Ser), primer design also took advantage of mosaic/allele-specific regions in *penA*.

*Neisseria spp. control strains for method validation.* Extraction of genomic DNA (gDNA) from culture strains for validation of the real-time PCR method was performed using the QIAamp DNA Mini kit (Qiagen). Each 20 µl reaction contained 0.2 µM of each primer, 1X Meltdoctor Master Mix (Applied Biosystems) containing the SYBR Green-derivative Syto, and 20 ng (or defined copy numbers) of gDNA. Each sample was tested in duplicate.

Experiments were run on a QuantStudio 7 Flex instrument (Applied Biosystems). The PCR program parameters included an initial denaturation step (95°C, 10 min), followed by 40 cycles of denaturation (95°C, 15 sec), annealing (62°C, 10 sec), and extension (72°C, 10 sec). Results were analyzed with the QuantStudio 6 and 7 Flex Real-Time PCR Software v1.0 (Applied Biosystems). To assess the LOD of the method, known quantities of gDNA copies/reaction were tested in ten-fold serial dilutions.

We tested a panel of 45 NG strains that included: 26 previously fully-characterized strains with known profiles of MICs and genetic AMR determinants (23); GC3 (with the 16S rRNA C1192T mutation; SPC MIC >1024 µg/ml); GC4 (harboring four 23S rRNA alleles with the A2059G mutation; AZM MIC ≥256 µg/ml); and G07 (harboring four 23S rRNA
alleles with the C2611T mutation; AZM MIC, 8 µg/ml) (21); the fully susceptible reference strain ATCC 49226; WHO reference strains (24): WHO A, WHO F, WHO G, WHO L, WHO K and WHO W (both carrying a mosaic penA X allele; MIC of cefixime (CFM) of 0.25 and 0.5 µg/ml, respectively); WHO M, WHO N, WHO O (with the 16S rRNA C1192T mutation; SPC MIC >1024 µg/ml), WHO P, WHO U (harboring four 23S rRNA alleles with the C2611T mutation; AZM MIC, 4 µg/ml), WHO V (harboring four 23S rRNA alleles with the A2059G mutation; AZM MIC ≥256 µg/ml), WHO Y (carrying a mosaic penA XXXIV with an additional Ala501Pro alteration; MICs for CFM and ceftriaxone (CRO) of 2 and 1.5 µg/ml, respectively), WHO X and WHO Z (both carrying a mosaic penA allele, MICs for CFM and CRO of 4 and 2 µg/ml, and 2 and 0.5 µg/ml, respectively) (24, 25). All NG isolates were grown on chocolate agar PolyVitex plates (bioMérieux) for 24 hrs at 35°C in a humid 5% CO₂-enriched atmosphere.

Twenty clinical non-gonococcal Neisseria spp. strains previously identified with the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics) were also tested to assess cross-reactivity. The panel included: N. meningitidis (n=3), N. sicca (n=3), N. flavescens (n=3), N. mucosa (n=2), N. cinerea (n=2), N. subflava (n=2), N. lactamica (n=1), N. flava (n=1), N. weaveri (n=1), N. polysaccharea (n=1) and N. bacilliformis (n=1).

NG-positive and NG-negative clinical specimens. From May 2015 to June 2016, we collected 819 clinical specimens using ESwabs™ (Copan Diagnostics Inc.) from 261 patients at three study centers (Checkpoint Zurich, Zurich; Ambulatorium Kanonengasse, Zurich; Department of Infectious Diseases, Bern University Hospital, Bern) in Switzerland. Our study population consisted of 240 (92.0%) men, of which 230 (88.1%) were men who have sex with men (MSM), and 21 (8.0%) women.

These samples were tested for NG by culture and by APTIMA Combo 2 (Hologic Inc.) (n=238 patients) at the laboratory Laborgemeinschaft 1 (Zurich, Switzerland), or by Cobas™
CT/NG (Roche Molecular Diagnostics) (n=23 patients) at the Clinical Microbiology Laboratory of the Institute of Infectious Diseases, University of Bern, Bern, Switzerland (Table S1). After routine testing and storage at -80°C, residual ESwabs and cultures were transported periodically (i.e., approximately every two months for samples from the laboratory of Laborgemeinschaft 1) to the Institute for Infectious Diseases (University of Bern; Bern, Switzerland) and stored at -80°C before implementation of the new MAMA-based SYBR Green real-time PCR.

The QIAamp DNA Mini kit (Qiagen) was used to extract total DNA from 200 µl of the residual ESwab specimens, which met the minimum requirements for further testing with our method. Reasons for exclusion included: <200 µl sample left, delayed transport/delivery, inability to test the samples immediately after DNA extraction, and/or inability to test the extra-genital samples immediately after arrival. For each PCR reaction, 4 µl of extracted total DNA was used and the real-time PCR was performed as described above.

Establishment of cycle threshold (Ct) ranges for the detection of AMR determinants. Detection of mutations in the target genes was based on the difference in cycle threshold values (ΔCt) between the reference *opa* reaction and each AMR determinant reaction. We used the NG control isolates (tested in duplicate) to determine the optimal ΔCt for the detection of each AMR determinant using the Youden index, which maximized the sum of sensitivity and specificity (26). We then rounded to the nearest whole number and added one to account for inter-sample variability. Adding one minimized the number of false-negatives observed for culture isolates (data not shown).

Establishment of the Ct cut-off point for the detection of NG in clinical specimens. We used the results of the commercial NAATs as the reference standard and determined the cut-off point for the *opa* Ct value to classify positive and negative results. In particular, we examined the distributions of Ct values for specimens with positive and negative reference test results...
and determined the optimal cut-off point that maximized the sum of sensitivity and specificity for the detection of NG (26).

**Analysis of the real-time PCR results in clinical specimens.** For NG identification, results from the *opa* reaction were interpreted as positive if the Ct value was below the cut-off point. We calculated sensitivity and specificity with 95% confidence intervals (CI, determined using the Clopper Pearson method) compared with commercial NAAT-testing as the reference standard (27).

For the detection of AMR determinants, the results were interpreted as follows: i) positive penA Asp345del reaction (indicating the presence of a mosaic penA allele) without or with positive penA Gly545Ser reaction (indicating the presence of a penA mosaic XXXIV), strain with decreased susceptibility to CFM and/or CRO; ii) 23S rRNA C2611T or A2059G mutations, strain moderately or highly resistant to AZM, respectively; iii) gyrA encoding for Ser91Phe substitution, strain non-susceptible to CIP; and iv) 16S rRNA C1192T mutation, strain resistant to SPC.

We calculated the sensitivity and specificity with 95% CI of the real-time PCR AMR reactions using as the reference standard either their molecular characterization or the antimicrobial susceptibility based on the MICs for CRO, CFM, AZM, CIP and SPC of culture isolates. MICs were obtained with the Etest (bioMérieux) method on chocolate agar PolyVitex plates (bioMérieux) and categorized using the 2016 European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria except for AZM, for which a breakpoint of MIC>2 µg/ml was used, as described previously (21, 28-30).
RESULTS AND DISCUSSION

Method design and validation using control strains. To detect SNPs in the 23S rRNA, gyrA and 16S rRNA genes associated with resistance to AZM, CIP and SPC (16-19), respectively, we used the MAMA method to design primers to selectively amplify mutated alleles while suppressing wild-type amplification (Table 1) (22). Briefly, the sequence of one primer matched at the 3-end the mutation of interest, and a non-template base was introduced in the second last base at the 3’-end. Based on previous observations (22), this was expected not to influence amplification for a mutated allele, while strongly inhibiting wild-type amplification.

To assess the presence or absence of a particular AMR determinant, particularly when testing clinical specimens with unknown gonococcal DNA concentration, we established the opa reaction for species ID as the reference reaction to determine the presence of an AMR mutation of interest. We expected that, in the presence of the target mutation, the Ct of the reference reaction (opa) and of the AMR determinant reaction would be similar, whereas the Ct in the presence of a wild-type sequence would be substantially delayed. When testing the panel of 45 fully-characterized gonococcal isolates, the Ct values of the AMR reactions in the presence of the target determinants were close to the Ct values of the opa reference reaction (i.e., $\Delta Ct \leq 0.8$, $\Delta Ct \leq 1.8$, $\Delta Ct \leq 5.8$ and $\Delta Ct \leq 9.4$ for the 23S rRNA A2059G, 23S rRNA C2611T, GyrA S91F and 16S rRNA C1192T reactions, respectively). Conversely, in the presence of a wild-type sequence we observed a delay of at least 8 additional Cts in all four AMR reactions (Table S2; examples in Figure S1).

At the time of primer design, mosaic penA patterns X and XXXIV were the most frequently identified mosaic alleles. Therefore, we designed a reaction (penA Asp345del) targeting both these alleles, i.e., the penA mosaic region including an Asp345 deletion in the PBP2 compared to non-mosaic penA alleles (31), and a reaction (penA Ser545Gly) targeting specifically the penA region of the Gly545Ser substitution of the penA mosaic XXXIV allele (32) in order to discriminate mosaic penA XXXIV from mosaic penA X alleles.
Consistently, when testing the penA Asp345del reaction, amplification in the presence of a mosaic penA allele X or XXXIV occurred with a ΔCt≤2.6 compared to the reference opa reaction, while for non-mosaic penA alleles we observed a ΔCt≥16.9. For the penA Gly545Ser reaction, amplification in the presence of the mosaic penA XXXIV allele occurred with a ΔCt≤3.2 compared to the opa reaction, in contrast to the mosaic penA X allele for which a significant delay in amplification was observed (ΔCt≥19.3). However, both reactions were considered negative for mosaic penA harboring-strains WHO X and WHO Z (Ct>40 for the penA Ser545Gly reaction and ΔCt≥9.7 for the penA Asp345del reaction; Table S2), indicating that such rare H041-like mosaic penA alleles cannot be identified. Nevertheless, isolates harboring these alleles have remained rare (33-35). A posterior in silico analysis of the 22 mosaic penA alleles available in the NG-STAR database (36) in February 2018 also revealed that mosaic penA patterns XXXV, 37, 38, 59, 60, 62, 63, and 74 might not be detected with the penA Asp345del reaction (data not shown). For the penA Gly545Ser reaction, we would expect that, in addition to mosaic penA XXXIV, mosaic penA patterns 42, 51, 52, 55 and 67 would also yield a positive result, owing to 100% sequence homology in the primer sequences.

Using the Youden index, we determined the maximum ΔCt within which the AMR reaction was considered positive (Table 2); samples with a higher ΔCt were considered not to possess the AMR determinant (examples in Figure S2). The analytical LOD for all reactions was 10-100 genomic DNA (gDNA) copies/reaction in the presence of the mutation of interest, whereas wild-type amplification within 40 cycles was only observed when testing ≥10^5 gDNA copies (23S rRNA C2611T and A2059G, gyrA S91F), and ≥10^7 gDNA copies (16S 1192T).

Using these cut-offs, our method showed 100% sensitivity and specificity for the prediction of phenotypic resistance to AZM and CIP in the 45 gonococcal control strains in less than one hour (less than two hours including DNA extraction) (Table S3). It should be
noted that our method only detects the presence of the AZM-associated resistance mutations and not the number of mutated alleles, so we might detect AZM susceptible gonococci with only 1-2 mutated alleles. However, since these strains are at risk of quickly mutating more alleles, especially if exposed to AZM selective pressure (18), it is still relevant to detect such strains and treat them as potentially AZM-resistant.

Prediction of ESC resistance was less accurate (sensitivity 62% and specificity 86%) for three main reasons: i) the H041-like penA alleles harbored in strains WHO X and WHO Z were not detected; ii) the ESC-resistant strain WHO L possessed a non-mosaic penA allele; iii) the presence of a mosaic penA allele is often associated with raised MICs for ESCs, which are still in the susceptible range based on EUCAST criteria (21, 37). Our assay also did not predict SPC resistance in strain WHO A, which has a resistance mutation in rpsE. This single amino acid alteration confers low-level SPC resistance and has only been rarely described (38). The assay successfully detected the more common 16S rRNA C1192T mutation in strains WHO O and GC3, which is associated with high-level SPC resistance.

**Cross-reaction with non-gonococcal Neisseria spp.** Cross-reactivity with *N. meningitidis* and commensal *Neisseria* spp. naturally found in the pharyngeal flora poses a great challenge in the development of molecular diagnostic tests for detecting AMR determinants in NG (20, 39). We expected that, even in the presence of 100% homology with the wild-type allele in a gonococcal species, the MAMA method would strongly inhibit background amplification from commensal sequences.

The MAMA strategy was also applied to the lower primer of the 16S rRNA reaction to improve discrimination from non-gonococcal species, as described previously (39). Little or no cross-amplification was observed for the penA Gly545Ser and 16S rRNA C1192T reactions, when $10^7$ gDNA copies/reaction of 20 strains from different cultured *Neisseria* spp. were tested (Table S4). Nevertheless, cross-reactivity at high Ct (comparable to NG wild-type sequences) was observed for the penA Asp345del and both 23S rRNA reactions. This was due...
to the high similarity of the target sequences among different *Neisseria* spp.; notably, mosaic penA alleles most likely arose through recombination with the commensal orthologs (40, 41).

**Testing of the NG-positive and NG-negative clinical specimens.** During our study, we collected a total of 891 ESwab samples from 261 patients, with 159 paired culture isolates from 128 patients. Of the 891 ESwab samples, 489 (54.9%), including 94 with paired culture isolates, met the requirements for further testing with our method (Table S1 and Table S5). The 489 samples included urethral (n=177), pharyngeal (n=158), rectal (n=130), vaginal (n=13), and cervical (n=11) specimens (Table S1), of which 160 were NG-positive (pharyngeal, n=59; rectal, n=48; urethral, n=46; cervical, n=4; vaginal, n=3) and 329 were NG-negative (urethral, n=131; rectal, n=82; pharyngeal, n=99; cervical, n=7; vaginal, n=10) by previous commercial NAAT testing.

When testing the NAAT-negative samples, non-specific amplification of the *opa* reaction was observed in most samples of all specimen types (i.e., samples with *opa* Ct>40 were 11.1%, 18.3%, 18.3%, 25% and 0% for pharyngeal; urethral, rectal, vaginal, and cervical specimens, respectively), possibly due to sample degradation, sub-optimal primer specificities/PCR conditions and/or background amplification from commensals. The potential of background amplification for *opa* was also confirmed when testing the panel of non-gonococcal control strains at 10^7 gDNA copies/reaction (i.e., cross-reactivity was observed for *N. meningitidis*, *N. mucosa* and *N. weaveri*; Table S4). For these reasons, we analyzed the distributions of the *opa* Ct values for specimens with positive and negative commercial NAAT results in order to determine the optimal cut-off point (i.e., the maximum Ct value for which the sample will be considered positive for the presence of NG). We initially evaluated the addition of the *porA* reaction as a confirmatory identification reaction. However, we eventually dismissed it since this reaction did not perform well and led to inconsistent results with the *opa* reaction (data not shown).
Overall, using the optimal cut-offs for *opa* (e.g., Ct <26.1 for urethral samples; Table 3), our method showed good agreement with the reference NAATs for the detection of NG in genital specimens (e.g., sensitivity 93% and specificity 100% for urethral samples), but additional testing on different sample sets will be required in the future to confirm the accuracy of the established cut-offs. The real-time PCR was less accurate in identifying NG in extra-genital specimens. In particular, the low specificity resulting in a low positive predictive value indicates that further optimization of the method is needed to increase the accuracy for these sample types (Table 3).

All 94 samples from 77 patients for which paired culture isolates were available tested positive for NG by using our real-time PCR (Table S1 and Table S5). Additionally, our method accurately predicted (directly from the clinical ESswab samples) the susceptibility profile for CIP, AZM, SPC and ESCs of the paired culture isolates, when compared to the MICs as gold standard (Table 4).

Our assay predicted CIP resistance with 100% sensitivity and 98% specificity. One clinical sample that tested positive for the mosaic *penA* reaction (*penA* Asp345del) had a paired isolate that carried a mosaic *penA* X allele and was phenotypically resistant to CFM (28). This isolate was subjected to PCR/sequencing (23), confirming the presence of a mosaic *penA* X allele. Similarly, all three samples that tested positive for both *penA* Asp345del and *penA* Gly545Ser) had paired isolates that contained the *penA* mosaic XXXIV, despite being phenotypically susceptible to ESCs according to the EUCAST criteria. This is not surprising, since strains harboring this particular allele often only show raised ESC MICs, which are still in the susceptible range (37). Nevertheless, the detection of such strains is still of clinical relevance, since isolates harboring this mosaic *penA* variants, (e.g., the internationally-spreading clone NG-MAST ST1407) have been associated with CFM treatment failures, despite being susceptible based on standard phenotypic testing (42). Overall, our assay showed 100% sensitivity and 98%-100% specificity for the prediction of the AMR phenotype.
except for the penA Asp345del reaction (82% specificity). False-positives were mainly associated with the penA Asp345del reaction in extra-genital specimens (i.e., likely due to background amplification of other Neisseria spp.). In fact, the specificities of the penA Asp345del reaction observed for genital and extra-genital specimens were 95% and 72%, respectively (Table 5 and Table 6).

A main limitation of the present study was that we did not identify clinical samples positive for some AMR mutations (i.e., 23S rRNA A2059G and 16S rRNA C1192T), since they are still very rare (15), and only a few positives for additional AMR determinants (i.e. 23S rRNA C2611T and mosaic penA alleles) were recorded. In this context, the results obtained from the remaining 55 samples, for which no culture isolates were available, also suggest that the number of samples testing positive for ESCs, AZM and SPC resistance overestimate the presence of these resistance determinants in extra-genital specimens (Table S6). This finding likely results from false positive results due to cross-reactivity with other Neisseria spp. (Tables S4). Additionally, lower gonococcal loads in extra-genital specimens, typically associated also with the inability to recover culture isolates, may also lead to a higher opa Ct and thus a decreased ΔCt (Figure S3, Table S7). Finally, despite being in transport medium, samples were processed at different times after collection and could not be tested fresh, potentially allowing degradation.

Overall, further evaluations will be beneficial to confirm the performance of our method in genital specimens and, particularly, more sample sets with different AMR determinants should be included. Our results also show that the method does not accurately predict AMR in extra-genital samples. We designed a SYBR Green approach since it is cheap, it requires only specific primers for the different reactions, and it is more feasible for application in low-income settings, as well as advantageous for translation into a point-of-care test. However, in light of our results, the implementation of molecular probes to increase the specificity may represent a useful future optimization strategy.
Conclusions

We developed a rapid multi-target SYBR Green-based method that was successfully applied to accurately identify and predict NG antimicrobial phenotype directly from urogenital samples, which can be also used to concomitantly perform culture isolation. However, our data showed that the method cannot be used for some extra-genital specimens, particularly pharyngeal samples, because of the lower gonococcal load, the higher risk of DNA degradation, and the cross-reactivity with other Neisseria spp. Nevertheless, owing to its ease of use and rapidity, this method might be a promising candidate for further development into a rapid test. Such a test, if deployed at the point of care, could contribute to individually tailored gonorrhea treatments in the future.

ACKNOWLEDGMENTS

We thank Prof. H. Furrer, Dr. C. Hauser (Dept. of Infectious Diseases, Bern University Hospital, Bern), Dr. S. Droz, Dr. M. Gorgievski, Ms. R. Tinguely (Institute for Infectious Diseases, Bern), Dr. M. Grubenmann, Dr. B. Bertisch, Mrs. B. Savic (Laborgemeinschaft 1, Zurich), and Dr. M. Kluschke (Checkpoint Zurich, Zurich) for their contributions to the study. This study was funded by the SwissTransMed initiative (Translational Research Platforms in Medicine, project number #25/2013: Rapid Diagnosis of Antibiotic Resistance in Gonorrhoea, RaDAR-Go) from the Rectors’ Conference of the Swiss Universities (CRUS).
REFERENCES


TABLE 1. Primers implemented for the real-time PCR assay, target mutations and corresponding affected antibiotics

<table>
<thead>
<tr>
<th>Target,</th>
<th>Primer name and sequences *</th>
<th>Associated target (purpose or antibiotic affected)</th>
</tr>
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<tbody>
<tr>
<td>spa</td>
<td>opa_F 5'-GTTCATCCGCCATATTTGTTGA-3'</td>
<td>spa (Neisseria gonorrhoeae identification)</td>
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<tr>
<td></td>
<td>opa_R 5'-AAGGGCGAGTTATATACCGGATTCC-3'</td>
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<tr>
<td>porA</td>
<td>porA_F 5'-CTATGCTCCATGTTGGATCTTTG-3'</td>
<td>porA (N. gonorrhoeae identification, confirmatory)</td>
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<td>porA_R 5'-CATAGCTGTAGTTCGCGTTTC-3'</td>
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<td>penA Gly545Ser</td>
<td>545_F 5'-TGGTTAAGGCTGTTACTGATT-3'</td>
<td>Mosaic penA (Decreased susceptibility/resistance to ESCs)</td>
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<td></td>
<td>545_R 5'-GGCCCTGCCACTACACCCTT-3'</td>
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<tr>
<td>penA A345del</td>
<td>345_F 5'-GGCAAAGTGGATGCAAGCTT-3'</td>
<td>Mosaic penA (Decreased susceptibility/resistance to ESCs)</td>
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<td>345_R 5'-GATAAACGTTGGATACTTGTACCC-3'</td>
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<tr>
<td>23S rRNA C2611T</td>
<td>C2611_F 5'-AACGTGAGTACGACAGTTGTTT-3'</td>
<td>23S rRNA C2611T (Moderate AZM resistance)</td>
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<td>C2611_R 5'-GAACTTAGCTACCGCTATGGA-3'</td>
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<tr>
<td>23S rRNA A2059G</td>
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<td>23S rRNA A2059G (High AZM resistance)</td>
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<td>gyrA Ser91Phe</td>
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<td>GyrA Ser91Phe (CIP resistance)</td>
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<td>16S rRNA C1192T</td>
<td>16S_1192_F 5'-CTGTCAATTAGTGGCCATATCTTCA-3'</td>
<td>16S rRNA C1192T (SPC resistance)</td>
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<td>16S_1192_R 5'-TAAGGCCATGAGGACTTGATAA-3'</td>
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Note. ESCs, extended-spectrum cephalosporins; AZM, azithromycin; CIP, ciprofloxacin; SPC, spectinomycin

* All non-template bp compared to the wild-type sequence are underlined.
Table 2. Establishment of the ΔCt for each antimicrobial resistance (AMR) reaction based on the results of the Neisseria gonorrhoeae control isolates

<table>
<thead>
<tr>
<th>Target</th>
<th>No. of wild-type strains</th>
<th>ΔCt range wild-type strains</th>
<th>No. of mutant strains</th>
<th>ΔCt range mutant strains</th>
<th>Established ΔCt for positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>penA Gly545Ser</td>
<td>37</td>
<td>[15.65 to &gt;25.8]</td>
<td>8</td>
<td>[0.30 to 3.17]</td>
<td>4</td>
</tr>
<tr>
<td>penA A345del</td>
<td>35 a</td>
<td>[9.65 to &gt;25.25]</td>
<td>10</td>
<td>[-0.21 to 2.64]</td>
<td>4</td>
</tr>
<tr>
<td>23S rRNA C2611T</td>
<td>43</td>
<td>[17.60 to 12.84]</td>
<td>2</td>
<td>[-0.12 to -0.82]</td>
<td>1</td>
</tr>
<tr>
<td>23S rRNA A2059G</td>
<td>43</td>
<td>[9.35 to 15.20]</td>
<td>2</td>
<td>[0.73 to 0.76]</td>
<td>2</td>
</tr>
<tr>
<td>gyrA Ser91Phe</td>
<td>15</td>
<td>[17.07 to 16.93]</td>
<td>30</td>
<td>[1.01 to 5.78]</td>
<td>7</td>
</tr>
<tr>
<td>16S rRNA C1192T</td>
<td>43</td>
<td>[17.07 to 24.12]</td>
<td>2</td>
<td>[6.67 to 9.35]</td>
<td>10</td>
</tr>
</tbody>
</table>

Note. ΔCt, difference between the Ct of the AMR reaction and the opa reaction.

* Results for WHO X and WHO Z carrying a rare mosaic penA allele were considered negative and included in the range of wild-type isolates.

* When Ct >40, the upper range could not be estimated precisely.
### TABLE 3. Optimal opa cycle threshold (Ct) cut-off value and performance of the RT-PCR for Neisseria gonorrhoeae identification compared to commercial NAATs

<table>
<thead>
<tr>
<th>Sample site</th>
<th>opa Cut-off</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
<th>Total</th>
<th>Sensitivity (95% CI) *</th>
<th>Specificity (95% CI) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urethra</td>
<td>26.1</td>
<td>43</td>
<td>0</td>
<td>3</td>
<td>131</td>
<td>177</td>
<td>0.93 (0.82, 0.99)</td>
<td>1.00 (0.97, 1.00)</td>
</tr>
<tr>
<td>Rectum</td>
<td>33.7</td>
<td>46</td>
<td>12</td>
<td>2</td>
<td>70</td>
<td>130</td>
<td>0.96 (0.86, 0.99)</td>
<td>0.85 (0.76, 0.92)</td>
</tr>
<tr>
<td>Pharynx</td>
<td>31.3</td>
<td>53</td>
<td>9</td>
<td>6</td>
<td>90</td>
<td>158</td>
<td>0.90 (0.79, 0.96)</td>
<td>0.91 (0.83, 0.96)</td>
</tr>
<tr>
<td>Cervix</td>
<td>28.4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>11</td>
<td>1.00 (0.4, 1.00)</td>
<td>1.00 (0.59, 1.00)</td>
</tr>
<tr>
<td>Vagina</td>
<td>33.4</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>13</td>
<td>1.00 (0.29, 1.00)</td>
<td>0.90 (0.55, 1.00)</td>
</tr>
<tr>
<td>All sites combined</td>
<td>33</td>
<td>149</td>
<td>22</td>
<td>11</td>
<td>307</td>
<td>489</td>
<td>0.92 (0.87, 0.96)</td>
<td>0.91 (0.87, 0.94)</td>
</tr>
</tbody>
</table>

* CI, confidence interval; TP, true positive; FP, false positive; FN, false negative; TN, true negative
* * Sensitivity and specificity were calculated using the results of the commercial NAATs as gold standard.
TABLE 4. Performance of the real-time PCR in predicting antimicrobial resistance to four antibiotic classes directly from *Neisseria gonorrhoeae* positive clinical specimens with paired *N. gonorrhoeae* isolates

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Associated antibiotic</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
<th>Total</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>penA Ala345del +/- penA Gly545Ser</td>
<td>ESCs</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>76</td>
<td>94</td>
<td>1.00 (0.03, 1.00)</td>
<td>0.82 (0.72, 0.89)</td>
</tr>
<tr>
<td>23S rRNA C2611T and/or 23S rRNA A2059G</td>
<td>AZM</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>92</td>
<td>94</td>
<td>1.00 (0.03, 1.00)</td>
<td>0.99 (0.94, 1.00)</td>
</tr>
<tr>
<td>gyrA Ser91Phe</td>
<td>CIP</td>
<td>41</td>
<td>1</td>
<td>0</td>
<td>52</td>
<td>94</td>
<td>1.00 (0.91, 1.00)</td>
<td>0.98 (0.90, 1.00)</td>
</tr>
<tr>
<td>16S rRNA C1192T</td>
<td>SPC</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>92</td>
<td>94</td>
<td>NA</td>
<td>0.98 (0.93, 1.00)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sensitivity and specificity were calculated based on the susceptibility phenotype (MICs) of the paired culture isolates

<sup>b</sup> This isolate harbored a penA mosaic X allele and was correctly identified as ESC-resistant by the penA Ala345del reaction

<sup>c</sup> Three isolates were correctly predicted to harbor a mosaic penA allele based on the molecular characterization, but were susceptible to ESCs

Note. CI, confidence interval; TP, true positive; FP, false positive; FN, false negative; TN, true negative; ESCs, extended-spectrum cephalosporins; AZM, azithromycin; CIP, ciprofloxacin; SPC, spectinomycin; NA, not applicable

Note: CI, confidence interval; TP, true positive; FP, false positive; FN, false negative; TN, true negative; ESCs, extended-spectrum cephalosporins; AZM, azithromycin; CIP, ciprofloxacin; SPC, spectinomycin; NA, not applicable

* This isolate harbored a *penA* mosaic X allele and was correctly identified as ESC-resistant by the *penA* Ala345del reaction

* Three isolates were correctly predicted to harbor a mosaic *penA* allele based on the molecular characterization, but were susceptible to ESCs
TABLE 5. Performance of the real-time PCR in predicting antimicrobial resistance to four antibiotic classes directly from Neisseria gonorrhoeae positive genital specimens with paired N. gonorrhoeae isolates

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Associated antibiotic</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
<th>Total</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>penA A345del +/-</td>
<td>ESCs</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>37</td>
<td>40</td>
<td>1.00 (0.03, 1.00)</td>
<td>0.95 (0.83, 0.99)</td>
</tr>
<tr>
<td>23S rRNA C2611T and/or 23S rRNA A2059G</td>
<td>AZM</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>39</td>
<td>40</td>
<td>1.00 (0.03, 1.00)</td>
<td>1.00 (0.91, 1.00)</td>
</tr>
<tr>
<td>gyrA Ser91Phe</td>
<td>CIP</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>40</td>
<td>1.00 (0.81, 1.00)</td>
<td>1.00 (0.85, 1.00)</td>
</tr>
<tr>
<td>16S rRNA C1192T</td>
<td>SPC</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>40</td>
<td>NA</td>
<td>1.00 (0.91, 1.00)</td>
</tr>
</tbody>
</table>

Note. CI, confidence interval; TP, true positive; FP, false positive; FN, false negative; TN, true negative; ESCs, extended-spectrum cephalosporins; AZM, azithromycin; CIP, ciprofloxacin; SPC, spectinomycin; NA, not applicable

<sup>a</sup> Sensitivity and specificity were calculated based on the susceptibility phenotype (MICs) of the paired culture isolates

<sup>c</sup> This isolate harbored a penA mosaic X allele and was correctly identified as ESC-resistant by the penA Ala345del reaction

<sup>c</sup> One isolate was correctly predicted to harbor a mosaic penA allele based on the molecular characterization, but was susceptible to ESCs


<table>
<thead>
<tr>
<th>Reaction</th>
<th>Associated antibiotic</th>
<th>TP</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
<th>Total</th>
<th>Sensitivity (95% CI) *</th>
<th>Specificity (95% CI) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>penA A345del +/- penA Gly545Ser</td>
<td>ESCs</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>39</td>
<td>54</td>
<td>NA</td>
<td>0.72 (0.58, 0.84)</td>
</tr>
<tr>
<td>23S rRNA C2611T and/or 23S rRNA A2059G</td>
<td>AZM</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>53</td>
<td>54</td>
<td>NA</td>
<td>0.98 (0.90, 1.00)</td>
</tr>
<tr>
<td>gyrA Ser91Phe</td>
<td>CIP</td>
<td>23</td>
<td>1</td>
<td>0</td>
<td>30</td>
<td>54</td>
<td>1.00 (0.85, 1.00)</td>
<td>0.97 (0.83, 1.00)</td>
</tr>
<tr>
<td>16S rRNA C1192T</td>
<td>SPC</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>52</td>
<td>54</td>
<td>NA</td>
<td>0.96 (0.87, 1.00)</td>
</tr>
</tbody>
</table>

Note. CI, confidence interval; TP, true positive; FP, false positive; FN, false negative; TN, true negative; ESCs, extended-spectrum cephalosporins; AZM, azithromycin; CIP, ciprofloxacin; SPC, spectinomycin; NA, not applicable

* Sensitivity and specificity were calculated based on the susceptibility phenotype (MICs) of the paired culture isolates

b Two isolates were correctly predicted to harbor a mosaic penA allele based on the molecular characterization, but were susceptible to ESCs