A Multiplex Real-Time PCR with High Resolution Melting Analysis for the Characterization of Antimicrobial Resistance in *Neisseria gonorrhoeae*

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ABSTRACT

Resistance to antibiotics used against *Neisseria gonorrhoeae* infections is a major public health concern. Antimicrobial resistance (AMR) testing relies on time-consuming culture-based methods. Development of rapid molecular tests for detecting AMR determinants could provide valuable tools for surveillance, epidemiological studies and to inform individual case management. We developed a fast (<1.5 hrs) SYBR-green based real-time PCR method with high resolution melting (HRM) analysis. One triplex and three duplex reactions included two sequences for *N. gonorrhoeae* identification and seven determinants of resistance to extended-spectrum cephalosporins (ESCs), azithromycin, ciprofloxacin, and spectinomycin. The method was validated by testing 39 previously fully-characterized *N. gonorrhoeae* strains, 19 commensal *Neisseria* spp., and an additional panel of 193 gonococcal isolates. Results were compared with culture-based AMR determination. The assay correctly identified *N. gonorrhoeae* and the presence or absence of the seven AMR determinants. There was some cross-reactivity with non-gonococcal *Neisseria* species and the detection limit was 10^3-10^4 gDNA copies/reaction. Overall, the platform accurately detected resistance to ciprofloxacin (sensitivity and specificity, 100%), ceftriaxone (sensitivity 100%, specificity 90%), cefixime (sensitivity 92%, specificity 94%), azithromycin and spectinomycin (both sensitivity and specificity, 100%). In conclusion, our methodology accurately detects mutations generating resistance to antibiotics used to treat gonorrhea. Low assay sensitivity prevents direct diagnostic testing of clinical specimens but this method can be used to screen collections of gonococcal isolates for AMR more quickly than with current culture-based AMR testing.

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INTRODUCTION

Gonorrhea is the second most common bacterial sexually transmitted infection worldwide, with an estimated 78 million new cases in 2012 (1). Moreover, *Neisseria gonorrhoeae* has developed resistance to most current and past treatment options. Antimicrobial resistant (AMR) gonorrhea is a major public health concern about which the World Health Organization (WHO) emphasizes the importance of global surveillance to identify emerging resistance, monitor trends, and inform revisions of treatment guidelines (2, 3).

At a molecular level, the mechanisms which confer resistance to the most common treatment options have been well characterized. For instance, the acquisition of mosaic *penA* alleles, with or without substitutions at amino acid position 501 of the encoded penicillin-binding protein 2 (PBP2), has been linked to decreased susceptibility or resistance to the extended-spectrum cephalosporins (ESCs) cefixime (CFX) and ceftriaxone (CRO) (4, 5). In particular, strains harboring a mosaic XXXIV *penA* gene, including the internationally-spreading *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) genogroup 1407, have been responsible for ESC treatment failures in several countries worldwide (5-8). The mutations A2059G or C2611T in the 23S rRNA alleles are associated with resistance to azithromycin (AZM) (9, 10), whereas a Ser91Phe substitution in GyrA results in ciprofloxacin (CIP) non-susceptibility (11). Single nucleotide polymorphisms (SNPs) in the 16S rRNA or in the ribosomal protein S5 (RPS5) encoding gene *rpsE* (12, 13) confer spectinomycin (SPC) resistance. However, we should note that while the CIP-resistant *N. gonorrhoeae* isolates are frequently observed, those fully resistant to ESCs, AZM and SPC are still sporadically found (14, 15).

Nucleic acid amplification testing (NAAT) has already replaced culture-based detection of *N. gonorrhoeae* in many settings, but these methods do not provide any information about AMR (16). On the other hand, antimicrobial susceptibility testing (AST) is usually performed with time-consuming culture methods (16). For this reason, there has been

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growing interest in the development of NAATs that can supplement culture-based AMR testing, enhance AMR surveillance and, ideally, be used to tailor individualized treatment for gonorrhea patients (17).

Several nucleic acid amplification-based methods have been developed to identify the presence of SNPs (18). One of these techniques is high resolution melting (HRM) analysis, which relies on the detection of changes in the melting temperature (Tm) resulting from the presence of mutations in a previously amplified target. This method is so sensitive that even Tm shifts derived from one SNP can be detected (19). Moreover, strategic target design (i.e., distinct Tm of the amplicons) also allows multiplexing of more than one reaction per single tube (20). However, only multiple-step (e.g., requirement of additional steps after nucleic acid amplification for read-out) (21, 22), or single-antibiotic (e.g., only resistance to CIP or only to AZM) NAAT-based methodologies to characterize AMR gonorrhea have been proposed in the past (23-28).

In this study, we developed and evaluated a new SYBR-green based real-time PCR method with HRM analysis to simultaneously detect *N. gonorrhoeae* and key mutations associated with ESCs, AZM, CIP and SPC resistance in four closed-tube multiplex reactions.

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MATERIALS AND METHODS

Design of the real-time PCR assay. Nine 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. Primers were designed to flank the mutation site of interest in *gyrA*, 23S rRNA, 16S rRNA and *rpsE* genes, and to amplify *penA* mosaic sequences (e.g., pattern XXXIV) around codons 501 and 545. Additionally, GC clamps were added at the 5'-end of some oligonucleotides to shift the Tm of the resulting amplicons in order to separate the peaks for easier interpretation of multiplex reactions. The nine primer sets generated ~40-140 bp products and all operated at the same conditions both in single- and multiplex reactions (Table 1).

N. gonorrhoeae isolates were grown on GC agar (bioMérieux) for 24 hrs at 35°C in a humid 5% CO_2-enriched atmosphere. Genomic DNA extraction was performed using the QIAamp DNA mini kit (QIAGEN). Each 20 µl reaction contained 0.3 µM of each primer, 1X Meltdoctor Master Mix (Applied Biosystems), and 20 ng of genomic DNA (gDNA). Experiments were run on a QuantStudio 7 Flex instrument (Applied Biosystems). The PCR stage included a first denaturation step (95°C, 10 min), followed by 30 cycles of denaturation (95°C, 15 sec), annealing (62°C, 10 sec), and extension (72°C, 10 sec). After amplification, HRM analysis was performed using the following parameters: after 10 sec at 95°C and a 60°C hold for 1 min, the fluorescence signal was collected, while the samples were heated up from 60°C to 95°C with a ramping time of 0.025°C/sec. Results were analyzed with the QuantStudio 6 and 7 Flex Real-Time PCR Software v1.0 (Applied Biosystems). Overall, starting from extracted DNA templates the results were available in <1.5 hrs (i.e., real-time PCR amplification of <60 min followed by HRM analysis of <30 min). To assess the limit of detection (LOD) of our molecular method, known quantities of gDNA copies/reaction were tested in ten-fold serial dilutions.

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Neisseria spp. control strains. A panel of 35 *N. gonorrhoeae* isolates was used to validate the real-time PCR method. The panel included: 26 previously fully-characterized isolates with known profiles of MICs and genetic resistance determinants (14); the fully sensitive reference strain ATCC 49226; WHO reference strains WHO K (carrying a mosaic X *penA* gene), WHO L, WHO P, the SPC-resistant WHO O (with the 16S rRNA C1192T substitution; MIC >1024 µg/ml) and WHO A (with the RPS5 Thr24Pro substitution; MIC, 128 µg/ml) (29); two AZM-resistant strains, AZM-HLR (harboring four 23S rRNA alleles with the A2059G mutation; MIC ≥256 µg/ml) and G07 (harboring four 23S rRNA alleles with the C2611T mutation; MIC, 8 µg/ml); and the ESC-resistant strain F89 carrying a mosaic XXXIV *penA* gene with an additional mutation in codon 501 leading to an Ala501Pro substitution (MICs for CFX and CRO of 2 and 1.5 µg/ml, respectively) (5).

Nineteen non-gonococcal *Neisseria* spp. strains previously identified with the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonik) were also used to assess cross-reactivity. The panel included: *N. meningitidis* (n=5), *N. mucosa* (n=3), *N. sicca* (n=2), *N. cinerea* (n=2), *N. lactamica* (n=2), *N. subflava* (n=1), *N. flava* (n=1), *N. flavescens* (n=1), *N. elongata* (n=1), and *N. bacilliformis* (n=1).

Analysis of representative spiked negative and positive samples. Pharyngeal, rectal and urethral clinical specimens were collected with ESwabs (Copan) and tested for *N. gonorrhoeae* by APTIMA Combo 2 (Hologic). The QIAamp DNA Mini kit (Qiagen) was used to extract total DNA from 200 µl of ESwabs with positive or negative APTIMA results. For the assessment of negative spiked specimens, 2 µl of sample DNA obtained from ESwab were spiked with additional 10^5, 10^4 or 10^3 gDNA copies of the appropriate control *N. gonorrhoeae* strain per reaction for each multiplex. For the positive specimens, 2 µl of sample DNA were used for each multiplex reaction. Culture isolates from the specimens were obtained with standard microbiological methods and species identification (ID) was achieved using the MALDI-TOF MS.

Analysis of gonococcal isolates and statistical analysis. We analyzed 193 *N. gonorrhoeae* isolates collected during a 25-year period (1989-2014) in two microbiology laboratories located in Switzerland (Institute for Infectious Diseases, University of Bern, Bern; Institute of Medical Microbiology, University Hospital Zürich, Zürich) with both culture-based AST and the new real-time PCR method.

ID was achieved using the MALDI-TOF MS. MICs for CFX, CRO, CIP, AZM and SPC were obtained on GC agar plates (bioMérieux) (30) using the Etest method. MIC values for CFX, CRO, CIP and SPC were categorized using the 2015 European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (31). For AZM, we defined moderate- and high-level resistance as MICs >2 to 128 and ≥256 µg/ml, respectively, as previously published (9).

Positive results from the real-time PCR assay (based on both amplification and melting temperature analysis) were interpreted as follow: *i*) *opa* and/or *porA*, strain identified as *N. gonorrhoeae*; *ii*) *penA* encoding for Gly545Ser substitution and/or *penA* Ala501, strain resistant to CFX and/or CRO; *iii*) 23S rRNA C2611T or A2059G mutations, strain moderately or highly resistant to AZM, respectively; *iv*) *gyrA* encoding for Ser91Phe substitution, strain non-susceptible to CIP; and *v*) *rpsE* encoding for Thr24Pro substitution or 16S rRNA C1192T mutation, strain resistant to SPC. Each sample was run in duplicate. Due to small inter-assay variabilities of the Tm (Table 2), positive controls for each reaction (e.g., harboring the mutated AMR target sequence) were included to facilitate the interpretation of the results. Inconsistent results were confirmed by repetition of the real-time PCR and PCR/DNA sequencing.

For the 193 isolates, we calculated the sensitivity (with 95% CI) of the real-time PCR with HRM analysis for the detection of *N. gonorrhoeae* compared with MALDI-TOF MS used as the reference standard. We calculated sensitivity (with 95% CI) for the detection of AMR to each antibiotic class as the percentage of isolates with a non-susceptible or resistant MIC value that were correctly identified by a positive HRM result for the presence of the

correlated resistance determinant. We calculated specificity (with 95% CI) as the percentage of isolates with a susceptible MIC value that were correctly identified by a negative HRM result for the correlated resistance determinant.

Since the 193 isolates detected in Switzerland did not include the rare strains possessing the mutations conferring fully resistance to CRO, AZM and SPC, sensitivity and specificity were also calculated including the results for the 35 *N. gonorrhoeae* control strains and four additional isolates provided by the WHO Collaborating Centre for Gonorrhoea and other STIs (Örebro, Sweden). Those four included: the ESC-resistant strain A8806 harboring a mosaic *penA* allele (MICs for CFX and CRO of 2 and 0.5 µg/ml, respectively) (32); the AZM-resistant strains GC2 (33) and GC4 harboring the C2611T (AZM MIC of 8 µg/ml) and A2059G (AZM MIC of ≥256 µg/ml) mutation in all four 23S rRNA alleles, respectively; and the SPC-resistant strain GC3 harboring the 16S rRNA C1192T mutation (MIC for SPC of >1024 µg/ml).

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RESULTS AND DISCUSSION

One triplex and three duplex reactions were designed to characterize target sequences specific for *N. gonorrhoeae* identification (*opa* and *porA*) (34, 35), as well as for resistance to ESCs (mosaic *penA* alleles), CIP (GyrA substitution), AZM (23S rRNA mutations), and SPC (16S rRNA mutation or RPS5 substitution) (Table 1).

Validation of the method and limit of detection (LOD). As shown in Table 2, all 35 *N. gonorrhoeae* control strains were correctly identified by the positive amplification of both *opa* and *porA* reactions; amplicons had an average Tm of 76.98°C and 74.36°C, respectively, by HRM analysis.

The *penA* reaction targeting Gly545Ser was relatively specific for mosaic *penA* patterns. Only non-mosaic pattern XIX was cross-amplified, but all *N. gonorrhoeae* strains harboring a mosaic *penA* allele (i.e., pattern XXXIV and X) were correctly identified by the presence of the Gly545Ser, which caused a mean Tm shift of 0.46°C compared with the wild-type sequence. Additionally, the Ala501 reaction only amplified mosaic *penA* patterns, but we were not able to detect the mutation encoding the Ala501Pro substitution found in the ESC-resistant F89 strain (Table 2) (5). This was probably because third class mutations (i.e., G to C SNPs) are known to be difficult to detect by HRM, since the Tm shift resulting from such nucleotide substitutions is very small (15). Nevertheless, we kept this reaction for confirmation of the presence of mosaic *penA* alleles.

HRM analysis correctly identified the presence or absence of mutations associated with resistance to ciprofloxacin, azithromycin and specitnomycin (Table 2). Strains harboring the Ser91Phe substitution in GyrA generated discernible melting curves compared with the wild-type isolates with a mean Tm difference (ΔTm) of 0.61°C. One strain (2121127) (14), harbored an additional mutation in codon 92, which caused a further shift in the Tm when compared with the wild-type sequence (ΔTm= 1.25°C). Strains with mutations A2059G or C2611T in all four alleles of the 23S rRNA generated unique profiles compared with isolates

harboring wild-type alleles, with mean ΔTm of 0.22°C and 0.75°C, respectively. Strains harboring the target SNPs in *rpsE* or 16S rRNA exhibited a mean Tm shift of 0.68-0.69°C compared with the wild-type sequences (Table 2).

Finally, when testing 10-fold dilutions of 10^7 to 10 gonococcal gDNA copies/reaction, a starting quantity of at least 10^3-10^4 gDNA copies was needed to allow proper HRM analysis in all four multiplex reactions (see examples in Figure S1). This is higher than available commercial platforms (e.g., according to the manufacturer, the APTIMA Combo2 test claims an analytical sensitivity of 50 cells/assay).

Cross-reaction with non-gonococcal Neisseria spp. The production of false-positive results due to the presence of non-gonococcal *Neisseria* spp. commonly found in some specimen types (e.g., pharyngeal and rectal samples) is a major challenge for the design of NAAT-based diagnostic methods. In fact, several *Neisseria* spp. share with the gonococcus a high sequence similarity for some of the targets (e.g., 23S rRNA and 16S rRNA genes). Moreover, the *N. gonorrhoeae* mosaic *penA* allele is thought to be the result of horizontal gene transfer of the commensal orthologues (36, 37). Therefore, in order to assess the level of cross-reactivity for all nine genetic targets included in our multiplex real-time PCR platform, a panel of ten different non-gonococcal *Neisseria* species (overall, 19 strains) was tested.

As shown in Table S1, none of these strains showed positive amplification for *opa* and *porA*. This was expected, since both genetic regions were previously proven to be specific for *N. gonorrhoeae* (34, 35). The GyrA Ser91Phe reaction was also specific for *N. gonorrhoeae*. In contrast, several non-gonococcal species showed cross-reactions for all remaining target sequences (Table S1). In only a few cases, cross-amplification could be distinguished from *N. gonorrhoeae* by a different Tm (i.e., 23S rRNA A2059G), but for most targets the Tm of the amplified commensal target matched the expected Tm of the gonococcal wild-type sequence (e.g., 23S rRNA C2611, 16S rRNA C1192). However, none of the cross-reacting species had a Tm equal to that of the mutated *N. gonorrhoeae* sequence for any of the targets, indicating

that false-positives deriving from the presence of commensals are unlikely. Even in the presence of a positive *penA* A501 reaction, the lack of amplification of target sequence *penA* Gly545Ser or the absence of the Gly545Ser substitution allowed the differentiation of the gonococcal mosaic *penA* gene from its commensal counterpart, since this substitution is mostly found in gonococcus. On the other hand, excessive amounts of wild-type amplification due to commensal *Neisseria* spp. could potentially mask the presence of an AMR mutation in *N. gonorrhoeae*, especially in clinical specimens with low load of the pathogen (i.e., in pharyngeal samples) (38, 39).

Analysis of the representative spiked negative and positive samples. To assess the extent of commensal interference on the detection of the AMR determinants in clinical specimens, four pharyngeal and four rectal samples negative for *N. gonorrhoeae* were spiked with gDNA of control strains possessing the mutations of interest for each multiplex reaction.

The results obtained from the pharyngeal specimens showed strong background amplification of wild-type amplicons due to the presence of *Neisseria* spp. for most target reactions (e.g., 23S rRNA C2611T, 16S rRNA C1192T, *rpsE* Thr24Pro). This background amplification would cause false negative results especially in the presence of low amounts of gonococcus. Additionally, nonspecific amplification strongly affected the melting curve interpretation of the *gyrA* Ser91Phe and 23S rRNA A2059G reactions. Finally, two samples exhibited positive amplification of the *penA* A501 reaction due to commensals (see examples in Figure S2 A-E). On the other hand, for the spiked negative rectal specimens, only strong cross-amplification of wild-type 16S rRNA C1192 was observed (see examples in Figure S3 A-D).

Taken together with the relatively high LOD needed for proper HRM analysis, these limitations suggested that our method would not be suitable for direct screening of clinical specimens. For this reason, total DNA extracted from four pharyngeal, four rectal and four urethral clinical samples positive for *N. gonorrhoeae* was used to test the performance of our

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method. Results were also compared to the gDNA extracted from *N. gonorrhoeae* strains (when available) isolated from the specimens.

Our platform indicated that all four pharyngeal samples tested positive for the *opa* reaction (Figure S4 A-D). Cross-amplification of commensals together with the relatively low gonococcal load led to a false positive result for the presence of a mosaic *penA* in one sample. Additionally, the melting curves of several reactions were not properly interpretable due to low or nonspecific amplification (e.g., *gyrA* Ser91Phe, 23S rRNA A2059G, *rspE* Thr24Pro). Similarly, low amplicon amounts strongly affected the melting curve interpretation of all four multiplex reactions in the positive rectal (Figure S5 A-D) and urethral specimens (Figure S6 A-D), confirming that our method cannot be directly implemented for clinical specimens. Nonetheless, it could be a valuable tool for rapid screening of large isolate collections, both for surveillance and epidemiological purposes. For this reason, we compared our molecular methodology with the standard culture-based AST Etest method for a panel of 193 Swiss isolates.

Analysis of the 193 clinical isolates. As shown in Table 3, the real-time PCR platform correctly identified all isolates as *N. gonorrhoeae*. Moreover, AMR characterization for CIP had both sensitivity and specificity of 100%, whereas AZM and SPC had specificity of 100%. In particular, our method correctly identified all isolates exhibiting resistance to CIP (58 out of 58). No mutations associated to SPC resistance were observed in agreement with the results obtained by phenotypic AST. Furthermore, none of the isolates tested positive for the 23S rRNA C2611T or A2059G mutations associated with moderate or high AZM resistance, respectively. Consistently, none of the tested isolates exhibited AZM MICs >2 µg/ml. Finally, all 7 strains showing CFX resistance by phenotypic AST were positive for the presence of a mosaic *penA* allele. However, no resistance to CRO was observed. This was expected, since it is known that the presence of a mosaic *penA* gene is typically associated to raised MICs for ESCs, even if usually still in the susceptible range based on EUCAST criteria (40).

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Thus, we further explored the MIC distribution of CFX and CRO in isolates harboring mosaic or non-mosaic *penA* patterns (Figure 1). Out of the 16 isolates positive for the presence of a mosaic *penA* allele, seven were CFX resistant and five were only a two-fold dilution apart from being resistant (MIC, 0.125 µg/ml). The remaining four strains with a mosaic *penA* gene had raised CFX MICs of 0.064-0.094 µg/ml, whereas all other non-mosaic isolates tested exhibited MICs of ≤0.047 µg/ml. Furthermore, all 16 strains harboring a mosaic *penA* allele also showed raised CRO MICs in the range of 0.023 to 0.094 µg/ml, which were noticeably higher compared to strains with non-mosaic patterns, in agreement with previous observations (37, 40, 41).

Overall performance of the real-time PCR platform. Since some of the resistance mutations were not included among the 193 Swiss isolates, we also evaluated the performance of our test including the 35 control strains and 4 additional isolates harboring known, but very rare, AMR determinants (Table 3).

Our platform accurately identified *N. gonorrhoeae* with a sensitivity and specificity of 100%. However, strain GC2 tested positive only for the *opa* reaction. Notably, this strain was previously reported to cause false-negative results in other *porA*-based PCRs due to the acquisition of a meningococcal *porA* allele (33). For this reason, our dual-target approach proved to be extremely valuable for the identification of even such exceptional isolates.

With regard to the AMR detection, the platform correctly predicted resistance to ciprofloxacin in all 83 strains positive for a mutation in codon 91 of *gyrA*. Furthermore, the prediction of a mosaic *penA* allele allowed the detection of two fully CRO-resistant strains (F89 and A8806), as well as all isolates resistant to CFX with the execption of WHO L, which harbors a non-mosaic *penA* allele with an additional substitution in amino acid 501. It is worth noting that the mosaic *penA* allele of A8806 differs from the pattern XXXIV allele found in the high-level CRO-resistant F89 strain. For this reason, no amplification of the *penA* Gly545Ser target was observed for A8806. Nevertheless, the strain was correctly identified as harboring a

mosaic *penA* allele due to the positive *penA* Ala501 reaction. Finally, the identification of either of the two mutations conferring resistance to AZM or SPC was correctly associated with resistance to those antibiotics.

Conclusions. We developed and validated a new real-time PCR method coupled with HRM analysis that accurately detected several important mutations associated with resistance to antibiotics commonly used to treat gonorrhea. Cross-reactivity with commensal species and high limit of detection suggested that our method is not suitable for direct screening of clinical specimens. However, it proved to be a useful and rapid alternative to culture-based methods to assess the AMR profiles for ESCs, AZM, CIP and SPC of a large collection of *N. gonorrhoeae* isolates.

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REFERENCES

1. **Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, Stevens G, Gottlieb S, Kiarie J, Temmerman M.** 2015. Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. PLoS One **10:**e0143304.

2. **Unemo M, Shafer WM.** 2011. Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. Ann N Y Acad Sci **1230:**E19-28.

3. **World Health Organization.** 2012. Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*, World Health Organization, Geneva, Switzerland.

4. **Ohnishi M, Watanabe Y, Ono E, Takahashi C, Oya H, Kuroki T, Shimuta K, Okazaki N, Nakayama S, Watanabe H.** 2010. Spread of a chromosomal cefixime-resistant *penA* gene among different *Neisseria gonorrhoeae* lineages. Antimicrob Agents Chemother **54:**1060-1067.

5. **Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P.** 2012. High-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: novel *penA* mosaic allele in a successful international clone causes treatment failure. Antimicrob Agents Chemother **56:**1273-1280.

6. **van Dam AP, van Ogtrop ML, Golparian D, Mehrtens J, de Vries HJ, Unemo M.** 2014. Verified clinical failure with cefotaxime 1g for treatment of gonorrhoea in the Netherlands: a case report. Sex Transm Infect **90:**513-514.

7. **Unemo M, Golparian D, Potocnik M, Jeverica S.** 2012. Treatment failure of pharyngeal gonorrhoea with internationally recommended first-line ceftriaxone verified in Slovenia, September 2011. Euro Surveill **17**.

8. **Unemo M, Golparian D, Stary A, Eigentler A.** 2011. First *Neisseria gonorrhoeae* strain with resistance to cefixime causing gonorrhoea treatment failure in Austria, 2011. Euro Surveill **16**.

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9. **Chisholm SA, Dave J, Ison CA.** 2010. High-level azithromycin resistance occurs in *Neisseria gonorrhoeae* as a result of a single point mutation in the 23S rRNA genes. Antimicrob Agents Chemother **54:**3812-3816.

10. **Ng LK, Martin I, Liu G, Bryden L.** 2002. Mutation in 23S rRNA Associated with Macrolide Resistance in *Neisseria gonorrhoeae*. Antimicrobial Agents and Chemotherapy **46:**3020-3025.

11. **Shultz TR, Tapsall JW, White PA.** 2001. Correlation of in vitro susceptibilities to newer quinolones of naturally occurring quinolone-resistant *Neisseria gonorrhoeae* strains with changes in GyrA and ParC. Antimicrob Agents Chemother **45:**734-738.

12. **Sigmund CD, Ettayebi M, Morgan EA.** 1984. Antibiotic resistance mutations in 16S and 23S ribosomal RNA genes of *Escherichia coli*. Nucleic Acids Res **12:**4653-4663.

13. **Ilina EN, Malakhova MV, Bodoev IN, Oparina NY, Filimonova AV, Govorun VM.** 2013. Mutation in ribosomal protein S5 leads to spectinomycin resistance in *Neisseria gonorrhoeae*. Front Microbiol **4:**186.

14. **Endimiani A, Guilarte YN, Tinguely R, Hirzberger L, Selvini S, Lupo A, Hauser C, Furrer H.** 2014. Characterization of *Neisseria gonorrhoeae* isolates detected in Switzerland (1998-2012): emergence of multidrug-resistant clones less susceptible to cephalosporins. BMC Infect Dis **14:**106.

15. **Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, Wittwer C.** 2004. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. Clin Chem **50:**1156-1164.

16. **Whiley DM, Tapsall JW, Sloots TP.** 2006. Nucleic acid amplification testing for *Neisseria gonorrhoeae*: an ongoing challenge. J Mol Diagn **8:**3-15.

17. **Low N, Unemo M, Skov Jensen J, Breuer J, Stephenson JM.** 2014. Molecular diagnostics for gonorrhoea: implications for antimicrobial resistance and the threat of untreatable gonorrhoea. PLoS Med **11:**e1001598.

18. **Gibson NJ.** 2006. The use of real-time PCR methods in DNA sequence variation analysis. Clin Chim Acta **363:**32-47.

19. **Vossen RH, Aten E, Roos A, den Dunnen JT.** 2009. High-resolution melting analysis (HRMA): more than just sequence variant screening. Hum Mutat **30:**860-866.

20. **Seipp MT, Pattison D, Durtschi JD, Jama M, Voelkerding KV, Wittwer CT.** 2008. Quadruplex genotyping of F5, F2, and MTHFR variants in a single closed tube by high-resolution amplicon melting. Clin Chem **54:**108-115.

21. **Balashov S, Mordechai E, Adelson ME, Gygax SE.** 2013. Multiplex bead suspension array for screening *Neisseria gonorrhoeae* antibiotic resistance genetic determinants in noncultured clinical samples. J Mol Diagn **15:**116-129.

22. **Lawung R, Cherdtrakulkiat R, Charoenwatanachokchai A, Nabu S, Suksaluk W, Prachayasittikul V.** 2009. One-step PCR for the identification of multiple antimicrobial resistance in *Neisseria gonorrhoeae*. J Microbiol Methods **77:**323-325.

23. **Magooa MP, Muller EE, Gumede L, Lewis DA.** 2013. Determination of *Neisseria gonorrhoeae* susceptibility to ciprofloxacin in clinical specimens from men using a real-time PCR assay. Int J Antimicrob Agents **42:**63-67.

24. **Zhao L, Zhao S.** 2012. TaqMan real-time quantitative PCR assay for detection of fluoroquinolone-resistant *Neisseria gonorrhoeae*. Curr Microbiol **65:**692-695.

25. **Siedner MJ, Pandori M, Castro L, Barry P, Whittington WL, Liska S, Klausner JD.** 2007. Real-time PCR assay for detection of quinolone-resistant *Neisseria gonorrhoeae* in urine samples. J Clin Microbiol **45:**1250-1254.

26. **Li Z, Yokoi S, Kawamura Y, Maeda S, Ezaki T, Deguchi T.** 2002. Rapid detection of quinolone resistance-associated *gyrA* mutations in *Neisseria gonorrhoeae* with a LightCycler. J Infect Chemother **8:**145-150.

27. **Lindback E, Rahman M, Jalal S, Wretlind B.** 2002. Mutations in *gyrA*, *gyrB*, *parC*, and *parE* in quinolone-resistant strains of *Neisseria gonorrhoeae*. APMIS **110:**651-657.

28. **Buckley C, Trembizki E, Donovan B, Chen M, Freeman K, Guy R, Kundu R, Lahra MM, Regan DG, Smith H, Whiley DM, investigators Gs.** 2015. A real-time PCR assay for direct characterization of the *Neisseria gonorrhoeae* GyrA 91 locus associated with ciprofloxacin susceptibility. J Antimicrob Chemother.

29. **Unemo M, Fasth O, Fredlund H, Limnios A, Tapsall J.** 2009. Phenotypic and genetic characterization of the 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. J Antimicrob Chemother **63:**1142-1151.

30. **Clinical and Laboratory Standards Institute.** Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement., CLSI document M100-S25, Wayne, PA.

31. **EUCAST.** 2015. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 5.0, 2015. http://www.eucast.org.

32. **Lahra MM, Ryder N, Whiley DM.** 2014. A new multidrug-resistant strain of Neisseria gonorrhoeae in Australia. The New England journal of medicine **371:**1850-1851.

33. **Golparian D, Johansson E, Unemo M.** 2012. Clinical *Neisseria gonorrhoeae* isolate with a *N. meningitidis porA* gene and no prolyliminopeptidase activity, Sweden, 2011: danger of false-negative genetic and culture diagnostic results. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin **17**.

34. **Tabrizi SN, Chen S, Tapsall J, Garland SM.** 2005. Evaluation of *opa*-based real-time PCR for detection of *Neisseria gonorrhoeae*. Sex Transm Dis **32:**199-202.

35. **Whiley DM, Buda PJ, Bayliss J, Cover L, Bates J, Sloots TP.** 2004. A new confirmatory *Neisseria gonorrhoeae* real-time PCR assay targeting the *porA* pseudogene. Eur J Clin Microbiol Infect Dis **23:**705-710.

36. **Tanaka M, Nakayama H, Huruya K, Konomi I, Irie S, Kanayama A, Saika T, Kobayashi I.** 2006. Analysis of mutations within multiple genes associated with resistance in a clinical isolate of *Neisseria gonorrhoeae* with reduced ceftriaxone susceptibility that shows a multidrug-resistant phenotype. Int J Antimicrob Agents **27:**20-26.

37. **Ameyama S, Onodera S, Takahata M, Minami S, Maki N, Endo K, Goto H, Suzuki H, Oishi Y.** 2002. Mosaic-like structure of penicillin-binding protein 2 Gene (*penA*) in

clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime. Antimicrob Agents Chemother **46:**3744-3749.

38. **Knapp JS, Hook EW, 3rd.** 1988. Prevalence and persistence of *Neisseria cinerea* and other *Neisseria* spp. in adults. J Clin Microbiol **26:**896-900.

39. **Bissessor M, Tabrizi SN, Fairley CK, Danielewski J, Whitton B, Bird S, Garland S, Chen MY.** 2011. Differing *Neisseria gonorrhoeae* bacterial loads in the pharynx and rectum in men who have sex with men: implications for gonococcal detection, transmission, and control. J Clin Microbiol **49:**4304-4306.

40. **Lindberg R, Fredlund H, Nicholas R, Unemo M.** 2007. *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in *penA*, *mtrR*, *porB1b*, and *ponA*. Antimicrob Agents Chemother **51:**2117-2122.

41. **Osaka K, Takakura T, Narukawa K, Takahata M, Endo K, Kiyota H, Onodera S.** 2008. Analysis of amino acid sequences of penicillin-binding protein 2 in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime and ceftriaxone. J Infect Chemother **14:**195-203.

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458

459 **TABLE 1.** Target genes, primer sequences, amplicon lengths, mutations and affected antibiotics, and multiplex combinations of the real-time PCR platform

Target, mutation	Primer name and oligonucleotide sequences [a]	Amplicon length	Associated target and antibiotic affected	Multiplex
opa	opa_F 5'-gttcatccgccatattgtgttga-3'	56	*opa* (Species identification)	Triplex
	opa_R 5'-aagggcggattatatcgggttcc-3'			
porA	porA_F 5'-cagcaatttgttccgagtca-3'	44	*porA* (Species identification)	Triplex
	porA_R 5'-ggcgtataggcggacttg-3'			
penA Gly545Ser	545_F 5'-*cccgcccccg*ccgactgcaaacggttacta-3'	61	Mosaic *penA* (Decreased susceptibility/resistance to ESCs)	Triplex
	545_R 5'-*cccgcccccg*cggccctgccactacacc-3'			
penA Ala501	501_F 5'-*cccgcccccgccg*tcggcgcaaaaaccggtacg-3'	79	Mosaic *penA* (Decreased susceptibility/resistance to ESCs)	Duplex I
	501_R 5'-*cccgcccccgcc*aatcgacgtaacgaccgttaaccaacttacg-3'			
23S rRNA C2611T	C2611_F 5'-acgtcgtgagacagtttggtc-3'	49	23S rRNA C2611T (Moderate AZM resistance) [b]	Duplex I
	C2611_R 5'-caaacttccaacgccactgc-3'			
23S rRNA A2059G	A2059_F 5'-ctacccgctgctagacgga-3'	142	23S rRNA A2059G (High AZM resistance) [b]	Duplex II
	A2059_R 5'-cagggtggtatttcaaggacga-3'			
gyrA Ser91Phe	gyrA_S91_F 5'-taaataccacccccacggcgatt-3'	47	GyrA Ser91Phe (CIP resistance)	Duplex II
	gyrA_S91_R 5'-atacggacgatggtgtcgtaaact-3'			
rpsE Thr24Pro	S5_T24_F 5'-atggtcgcagttaaccgtgta-3'	56	RPS5 Thr24Pro (SPC resistance)	Duplex III
	S5_T24_R 5'-aaagccataatgcgaccacc-3'			
16S rRNA C1192T	16S_1192_F 5'-*ccgcccccc*ggaggaaggtggggatga-3'	64	16S rRNA C1192T (SPC resistance)	Duplex III
	16S_1192_R 5'-*ccgcccccc*tggtcataagggccatgag-3'			

460 **Note.** ESCs, extended spectrum cephalosporins; AZM, azithromycin; CIP, ciprofloxacin; SPC, spectinomycin
461 [a] GC clamps, which were added to the 5'-end of some primers to allow multiplexing, are shown in italics.
462 [b] They confer moderate- to high-level resistance to AZM (i.e., MIC > 2 µg/ml) when at least 3 out of 4 copies are mutated (9)

463

464 **TABLE 2.** Results of the method validation using the 35 well-characterized *N. gonorrhoeae* isolates

Target sequence	Sequence type of the 35 control isolates by:		Tm (°C)		Mean ΔTm ± SD (°C)	Sensitivity, [c] % (95% CI)	Specificity, [c] % (95% CI)
	DNA sequencing	Real-time PCR/HRM analysis	Range	Mean ± SD			
opa	Positive (n=35)	Positive (n=35)	76.63 - 77.22	76.98 ± 0.13	n/a	100 (90-100)	n/a [d]
	Negative (n=0)	Negative (n=0)	n/a	n/a			
porA	Positive (n=35)	Positive (n=35)	73.79 - 74.88	74.36 ± 0.20	n/a	100 (90-100)	n/a [d]
	Negative (n=0)	Negative (n=0)	n/a	n/a			
penA Gly545Ser	Non-mosaic (n=23)	Non-mosaic (n=23)	n/a [a]	n/a [a]	0.46 ± 0.05	100 (66-100)	100 (87-100)
	Non-mosaic Gly545 (ggc) (n=3)	Non-mosaic Gly545 (ggc) (n=3)	85.05 - 85.23 [a]	85.14 ± 0.08 [a]			
	Mosaic Gly545Ser (agc) (n=9)	Mosaic Gly545Ser (agc) (n=9)	84.09 - 84.72	84.47 ± 0.20			
penA Ala501	Non-mosaic (n=26)	Non-mosaic (n=26)	n/a [b]	n/a [b]		100 (66-100)	100 (87-100
	Mosaic (n=9)	Mosaic (n=9)	83.59 - 84.35	84.17 ± 0.19	n/i		
gyrA Ser91Phe	GyrA Ser91 (tcc), Ala92 (gca) (n=11)	GyrA Ser91 (tcc), Ala92 (gca) (n=11)	77.97 - 78.16	78.08 ± 0.05	0.61 ± 0.06	100 (86-100)	100 (72-100)
	GyrA Ser91Phe (ttc), Ala92 (gca) (n=23)	GyrA Ser91Phe (ttc), Ala92 (gca) (n=23)	77.29 - 77.59	77.47 ± 0.07			
	GyrA Ser91Phe (ttc), Ala92Ser (tca) (n=1)	GyrA Ser91Phe (tcc), Ala92Ser (tca) (n=1)	76.15 - 76.17	76.16 ± 0.02	1.25 ± 0.01		
23S rRNA A2059G	A2059 (n=34)	A2059 (n=34)	81.33 - 81.52	81.44 ± 0.03	0.22 ± 0.02	100 (3-100)	100 (90-100)
	A2059G (n=1)	A2059G (n=1)	81.61 - 81.70	81.67 ± 0.03			
23S rRNA C2611T	C2611 (n=34)	C2611 (n=34)	75.69 - 76.33	76.12 ± 0.16	0.75 ± 0.05	100 (3-100)	100 (90-100)
	C2611T (n=1)	C2611T (n=1)	75.08 - 75.55	75.30 ± 0.20			
rpsE Thr24Pro	Thr24 (acc) (n=34)	Thr24 (acc) (n=34)	73.87 - 74.34	74.08 ± 0.07	0.68 ± 0.01	100 (3-100)	100 (90-100)
	Thr24Pro (ccc) (n=1)	Thr24Pro (ccc) (n=1)	74.66 - 74.94	74.76 ± 0.09			
16S rRNA C1192T	C1192 (n=34)	C1192 (n=34)	81.38 - 81.72	81.56 ± 0.08	0.69 ± 0.01	100 (3-100)	100 (90-100)
	C1192T (n=1)	C1192T (n=1)	80.74 - 80.94	80.82 ± 0.09			

465 **Note.** Tm, melting temperature; ΔTm, melting temperature difference between wild-type and mutated sequence**;** n/a, not applicable; n/i, not interpretable
466 [a] Only non-mosaic pattern XIX (with *penA* Gly545) showed cross-amplification
467 [b] No amplification was observed for all other non-mosaic *penA* pattern tested
468 [c] Sensitivity is the probability that an isolate was correctly identified as positive by HRM analysis for the target sequence (species ID, mosaic or mutation); specificity was the probability that an
469 isolate was correctly identified as negative by HRM analysis for the target sequence (species ID, mosaic or mutation).
470 [d] Specificity was 100% considering that all 19 non-gonococcal control strains were correctly characterized as non-*N. gonorrhoeae* (see Table S1)

TABLE 3. Performance of the real-time PCR platform in characterizing the collection of 193 *N. gonorrhoeae* isolates alone and combined with the 39 *N. gonorrhoeae* control strains

Phenotypic target	Target sequence	*N. gonorrhoeae* isolates collected during 1989-2014 (n=193)						Overall *N. gonorrhoeae* strains (n=232), including the 39 controls					
		Test result	No. of isolates [a]	AST [b] S	AST [b] R	Sensitivity [d] % (95% CI)	Specificity [d] % (95% CI)	Test result	No. of strains [a, e]	AST [b] S	AST [b] R	Sensitivity [d] % (95% CI)	Specificity [d] % (95% CI)
Species identification	*opa* and/or *porA*	Positive	193	n/a	n/a	100 (97-100)	n/a	Positive [e]	232	n/a	n/a	100 (98-100) [e]	100 (82-100) [e]
		Negative	-					Negative [e]	19				
Ceftriaxone (CRO)	*penA* Gly545Ser and/or *penA* Ala501	Positive	16	16	-	n/a	92 (87-95)	Positive	26	24	2	100 (16-100)	90 (85-93)
		Negative	177	177	-			Negative	206	206	-		
Cefixime (CFX)	*penA* Gly545Ser and/or *penA* Ala501	Positive	16	9	7	100 (47-100)	95 (91-98)	Positive	26	14	12	92 (64-100)	94 (90-96)
		Negative	177	177	-			Negative	206	205	1 [f]		
Azithromycin (AZM) [c]	23S rRNA A2059G or 23S rRNA C2611T	Positive	-	-	-	n/a	100 (97-100)	Positive	4	-	4	100 (40-100)	100 (98-100)
		Negative	193	193	-			Negative	228	228	-		
Ciprofloxacin (CIP)	*gyrA* Ser91Phe	Positive	58	-	58	100 (91-100)	100 (96-100)	Positive	83	-	83	100 (96-100)	100 (98-100)
		Negative	135	135	-			Negative	149	149	-		
Spectinomycin (SPC)	*rpsE* Thr24Pro or 16S rRNA C1192T	Positive	-	-	-	n/a	100 (97-100)	Positive	3	-	3	100 (29-100)	100 (98-100)
		Negative	193	193	-			Negative	229	229	-		

Note. AST, antimicrobial susceptibility testing obtained with Etest; R, resistant; S, susceptible; CI, confidence interval; -, zero; n/a, not applicable

[a] Numbers are based on the results of the multiplex real-time PCR platform

[b] AST was categorized based on EUCAST criteria with exception for AZM (see below)

[c] AZM resistance were defined as > 2 µg/ml

[d] Sensitivity was the probability that an isolate categorized as resistant was identified as positive by real-time PCR; specificity was the probability that an isolate categorized as sensitive was identified as negative by real-time PCR

[e] For the evaluation of the "Species identification" we also included the 19 non-gonococcal *Neisseria* spp. strains

[f] Strain WHO L (non mosaic *penA* gene with an additional substitution in amino acid 501)

480 **LEGEND TO FIGURE 1**

481 Ceftriaxone (black bars) and cefixime (grey bars) MIC distribution of the 193 gonococcal
482 isolates. **A,** isolates harboring a non-mosaic *penA* gene (n=177); **B,** isolates carrying a mosaic
483 *penA* gene (n=16).

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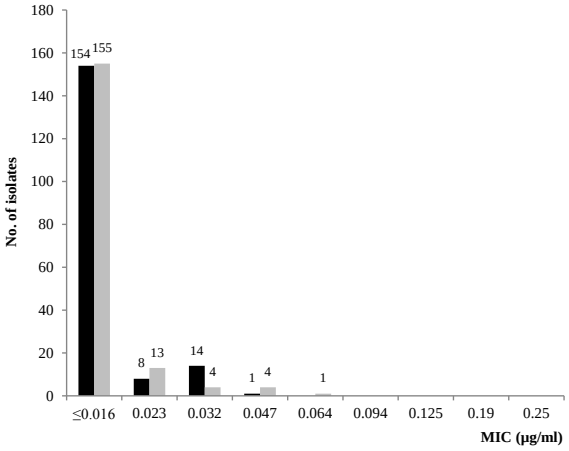
A.



B.