1   **A Multiplex Real-Time PCR with High Resolution Melting Analysis for the**

2   **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.

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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73   growing interest in the development of NAATs that can supplement culture-based AMR

74   testing, enhance AMR surveillance and, ideally, be used to tailor individualized treatment for

75   gonorrhea patients (17).

76   Several nucleic acid amplification-based methods have been developed to identify the

77   presence of SNPs (18). One of these techniques is high resolution melting (HRM) analysis,

78   which relies on the detection of changes in the melting temperature (Tm) resulting from the

79   presence of mutations in a previously amplified target. This method is so sensitive that even

80   Tm shifts derived from one SNP can be detected (19). Moreover, strategic target design (i.e.,

81   distinct Tm of the amplicons) also allows multiplexing of more than one reaction per single

82   tube (20). However, only multiple-step (e.g., requirement of additional steps after nucleic acid

83   amplification for read-out) (21, 22), or single-antibiotic (e.g., only resistance to CIP or only

84   to AZM) NAAT-based methodologies to characterize AMR gonorrhea have been proposed in

85   the past (23-28).

86   In this study, we developed and evaluated a new SYBR-green based real-time PCR

87   method with HRM analysis to simultaneously detect *N. gonorrhoeae* and key mutations

88   associated with ESCs, AZM, CIP and SPC resistance in four closed-tube multiplex reactions.

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MATERIALS AND METHODS

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90 ***Design of the real-time PCR assay.*** Nine primer sets were designed with the Oligo Primer

91 Analysis software v4.0 (Molecular Biology Insights) to amplify specific sequences of the

92 targets described in Table 1. Primers were designed to flank the mutation site of interest in

93 *gyrA*, 23S rRNA, 16S rRNA and *rpsE* genes, and to amplify *penA* mosaic sequences (e.g.,

94 pattern XXXIV) around codons 501 and 545. Additionally, GC clamps were added at the 5'-

95 end of some oligonucleotides to shift the Tm of the resulting amplicons in order to separate

96 the peaks for easier interpretation of multiplex reactions. The nine primer sets generated ~40-

97 140 bp products and all operated at the same conditions both in single- and multiplex

98 reactions (Table 1).

99 *N. gonorrhoeae* isolates were grown on GC agar (bioMérieux) for 24 hrs at 35°C in a

100 humid 5% CO_2-enriched atmosphere. Genomic DNA extraction was performed using the

101 QIAamp DNA mini kit (QIAGEN). Each 20 µl reaction contained 0.3 µM of each primer, 1X

102 Meltdoctor Master Mix (Applied Biosystems), and 20 ng of genomic DNA (gDNA).

103 Experiments were run on a QuantStudio 7 Flex instrument (Applied Biosystems). The PCR

104 stage included a first denaturation step (95°C, 10 min), followed by 30 cycles of denaturation

105 (95°C, 15 sec), annealing (62°C, 10 sec), and extension (72°C, 10 sec). After amplification,

106 HRM analysis was performed using the following parameters: after 10 sec at 95°C and a 60°C

107 hold for 1 min, the fluorescence signal was collected, while the samples were heated up from

108 60°C to 95°C with a ramping time of 0.025°C/sec. Results were analyzed with the

109 QuantStudio 6 and 7 Flex Real-Time PCR Software v1.0 (Applied Biosystems). Overall,

110 starting from extracted DNA templates the results were available in <1.5 hrs (i.e., real-time

111 PCR amplification of <60 min followed by HRM analysis of <30 min). To assess the limit of

112 detection (LOD) of our molecular method, known quantities of gDNA copies/reaction were

113 tested in ten-fold serial dilutions.

114   *Neisseria spp. control strains.* A panel of 35 *N. gonorrhoeae* isolates was used to validate the

115   real-time PCR method. The panel included: 26 previously fully-characterized isolates with

116   known profiles of MICs and genetic resistance determinants (14); the fully sensitive reference

117   strain ATCC 49226;   WHO reference strains WHO K (carrying a mosaic X *penA* gene),

118   WHO L, WHO P, the SPC-resistant WHO O (with the 16S rRNA C1192T substitution; MIC

119   >1024 μg/ml) and WHO A (with the RPS5 Thr24Pro substitution; MIC, 128 μg/ml) (29);

120   two AZM-resistant strains, AZM-HLR (harboring four 23S rRNA alleles with the A2059G

121   mutation; MIC ≥256 μg/ml) and G07 (harboring four 23S rRNA alleles with the C2611T

122   mutation; MIC, 8 μg/ml); and the ESC-resistant strain F89 carrying a mosaic XXXIV *penA*

123   gene with an additional mutation in codon 501 leading to an Ala501Pro substitution (MICs

124   for CFX and CRO of 2 and 1.5 μg/ml, respectively) (5).

125   Nineteen non-gonococcal *Neisseria* spp. strains previously identified with the matrix-assisted

126   laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker

127   Daltonik) were also used to assess cross-reactivity. The panel included: *N. meningitidis* (n=5),

128   *N. mucosa* (n=3), *N. sicca* (n=2), *N. cinerea* (n=2), *N. lactamica* (n=2), *N. subflava* (n=1), *N.*

129   *flava* (n=1), *N. flavescens* (n=1), *N. elongata* (n=1), and *N. bacilliformis* (n=1).

130   ***Analysis of representative spiked negative and positive samples.*** Pharyngeal, rectal and

131   urethral clinical specimens were collected with ESwabs (Copan) and tested for *N.*

132   *gonorrhoeae* by APTIMA Combo 2 (Hologic). The QIAamp DNA Mini kit (Qiagen) was

133   used to extract total DNA from 200 μl of ESwabs with positive or negative APTIMA results.

134   For the assessment of negative spiked specimens, 2 μl of sample DNA obtained from ESwab

135   were spiked with additional 10^5, 10^4 or 10^3 gDNA copies of the appropriate control *N.*

136   *gonorrhoeae* strain per reaction for each multiplex. For the positive specimens, 2 μl of sample

137   DNA were used for each multiplex reaction. Culture isolates from the specimens were

138   obtained with standard microbiological methods and species identification (ID) was achieved

139   using the MALDI-TOF MS.

140   ***Analysis of gonococcal isolates and statistical analysis.*** We analyzed 193 *N. gonorrhoeae*

141   isolates collected during a 25-year period (1989-2014) in two microbiology laboratories

142   located in Switzerland (Institute for Infectious Diseases, University of Bern, Bern; Institute of

143   Medical Microbiology, University Hospital Zürich, Zürich) with both culture-based AST and

144   the new real-time PCR method.

145   ID was achieved using the MALDI-TOF MS. MICs for CFX, CRO, CIP, AZM and SPC were

146   obtained on GC agar plates (bioMérieux) (30) using the Etest method. MIC values for CFX,

147   CRO, CIP and SPC were categorized using the 2015 European Committee on Antimicrobial

148   Susceptibility Testing (EUCAST) criteria (31). For AZM, we defined moderate- and high-

149   level resistance as MICs >2 to 128 and ≥256 µg/ml, respectively, as previously published (9).

150   Positive results from the real-time PCR assay (based on both amplification and

151   melting temperature analysis) were interpreted as follow: *i*) *opa* and/or *porA*, strain identified

152   as *N. gonorrhoeae*; *ii*) *penA* encoding for Gly545Ser substitution and/or *penA* Ala501, strain

153   resistant to CFX and/or CRO; *iii*) 23S rRNA C2611T or A2059G mutations, strain

154   moderately or highly resistant to AZM, respectively; *iv*) *gyrA* encoding for Ser91Phe

155   substitution, strain non-susceptible to CIP; and *v*) *rpsE* encoding for Thr24Pro substitution or

156   16S rRNA C1192T mutation, strain resistant to SPC. Each sample was run in duplicate. Due

157   to small inter-assay variabilities of the Tm (Table 2), positive controls for each reaction (e.g.,

158   harboring the mutated AMR target sequence) were included to facilitate the interpretation of

159   the results. Inconsistent results were confirmed by repetition of the real-time PCR and

160   PCR/DNA sequencing.

161   For the 193 isolates, we calculated the sensitivity (with 95% CI) of the real-time PCR

162   with HRM analysis for the detection of *N. gonorrhoeae* compared with MALDI-TOF MS

163   used as the reference standard. We calculated sensitivity (with 95% CI) for the detection of

164   AMR to each antibiotic class as the percentage of isolates with a non-susceptible or resistant

165   MIC value that were correctly identified by a positive HRM result for the presence of the

166   correlated resistance determinant. We calculated specificity (with 95% CI) as the percentage

167   of isolates with a susceptible MIC value that were correctly identified by a negative HRM

168   result for the correlated resistance determinant.

169   Since the 193 isolates detected in Switzerland did not include the rare strains possessing the

170   mutations conferring fully resistance to CRO, AZM and SPC, sensitivity and specificity were

171   also calculated including the results for the 35 *N. gonorrhoeae* control strains and four

172   additional isolates provided by the WHO Collaborating Centre for Gonorrhoea and other STIs

173   (Örebro, Sweden). Those four included: the ESC-resistant strain A8806 harboring a mosaic

174   *penA* allele (MICs for CFX and CRO of 2 and 0.5 μg/ml, respectively) (32); the AZM-

175   resistant strains GC2 (33) and GC4 harboring the C2611T (AZM MIC of 8 μg/ml) and

176   A2059G (AZM MIC of $\geq$256 μg/ml) mutation in all four 23S rRNA alleles, respectively; and

177   the SPC-resistant strain GC3 harboring the 16S rRNA C1192T mutation (MIC for SPC of

178   **>**1024 μg/ml).

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179 **RESULTS AND DISCUSSION**

180 One triplex and three duplex reactions were designed to characterize target sequences specific

181 for *N. gonorrhoeae* identification (*opa* and *porA*) (34, 35), as well as for resistance to ESCs

182 (mosaic *penA* alleles), CIP (GyrA substitution), AZM (23S rRNA mutations), and SPC (16S

183 rRNA mutation or RPS5 substitution) (Table 1).

184 ***Validation of the method and limit of detection (LOD).*** As shown in Table 2, all 35 *N.*

185 *gonorrhoeae* control strains were correctly identified by the positive amplification of both *opa*

186 and *porA* reactions; amplicons had an average Tm of 76.98°C and 74.36°C, respectively, by

187 HRM analysis.

188 The *penA* reaction targeting Gly545Ser was relatively specific for mosaic *penA* patterns. Only

189 non-mosaic pattern XIX was cross-amplified, but all *N. gonorrhoeae* strains harboring a

190 mosaic *penA* allele (i.e., pattern XXXIV and X) were correctly identified by the presence of

191 the Gly545Ser, which caused a mean Tm shift of 0.46°C compared with the wild-type

192 sequence. Additionally, the Ala501 reaction only amplified mosaic *penA* patterns, but we

193 were not able to detect the mutation encoding the Ala501Pro substitution found in the ESC-

194 resistant F89 strain (Table 2) (5). This was probably because third class mutations (i.e., G to C

195 SNPs) are known to be difficult to detect by HRM, since the Tm shift resulting from such

196 nucleotide substitutions is very small (15). Nevertheless, we kept this reaction for

197 confirmation of the presence of mosaic *penA* alleles.

198 HRM analysis correctly identified the presence or absence of mutations associated with

199 resistance to ciprofloxacin, azithromycin and specitnomycin (Table 2). Strains harboring the

200 Ser91Phe substitution in GyrA generated discernible melting curves compared with the wild-

201 type isolates with a mean Tm difference (ΔTm) of 0.61°C. One strain (2121127) (14),

202 harbored an additional mutation in codon 92, which caused a further shift in the Tm when

203 compared with the wild-type sequence (ΔTm= 1.25°C). Strains with mutations A2059G or

204 C2611T in all four alleles of the 23S rRNA generated unique profiles compared with isolates

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205   harboring wild-type alleles, with mean ΔTm of 0.22°C and 0.75°C, respectively. Strains

206   harboring the target SNPs in *rpsE* or 16S rRNA exhibited a mean Tm shift of 0.68-0.69°C

207   compared with the wild-type sequences (Table 2).

208   Finally, when testing 10-fold dilutions of 10^7 to 10 gonococcal gDNA copies/reaction, a

209   starting quantity of at least 10^3-10^4 gDNA copies was needed to allow proper HRM analysis

210   in all four multiplex reactions (see examples in Figure S1). This is higher than available

211   commercial platforms (e.g., according to the manufacturer, the APTIMA Combo2 test claims

212   an analytical sensitivity of 50 cells/assay).

213   ***Cross-reaction with non-gonococcal Neisseria spp.*** The production of false-positive results

214   due to the presence of non-gonococcal *Neisseria* spp. commonly found in some specimen

215   types (e.g., pharyngeal and rectal samples) is a major challenge for the design of NAAT-

216   based diagnostic methods. In fact, several *Neisseria* spp. share with the gonococcus a high

217   sequence similarity for some of the targets (e.g., 23S rRNA and 16S rRNA genes). Moreover,

218   the *N. gonorrhoeae* mosaic *penA* allele is thought to be the result of horizontal gene transfer

219   of the commensal orthologues (36, 37). Therefore, in order to assess the level of cross-

220   reactivity for all nine genetic targets included in our multiplex real-time PCR platform, a

221   panel of ten different non-gonococcal *Neisseria* species (overall, 19 strains) was tested.

222   As shown in Table S1, none of these strains showed positive amplification for *opa* and

223   *porA*. This was expected, since both genetic regions were previously proven to be specific for

224   *N. gonorrhoeae* (34, 35). The GyrA Ser91Phe reaction was also specific for *N. gonorrhoeae*.

225   In contrast, several non-gonococcal species showed cross-reactions for all remaining target

226   sequences (Table S1). In only a few cases, cross-amplification could be distinguished from *N.*

227   *gonorrhoeae* by a different Tm (i.e., 23S rRNA A2059G), but for most targets the Tm of the

228   amplified commensal target matched the expected Tm of the gonococcal wild-type sequence

229   (e.g., 23S rRNA C2611, 16S rRNA C1192). However, none of the cross-reacting species had

230   a Tm equal to that of the mutated *N. gonorrhoeae* sequence for any of the targets, indicating

231 that false-positives deriving from the presence of commensals are unlikely. Even in the

232 presence of a positive *penA* A501 reaction, the lack of amplification of target sequence *penA*

233 Gly545Ser or the absence of the Gly545Ser substitution allowed the differentiation of the

234 gonococcal mosaic *penA* gene from its commensal counterpart, since this substitution is

235 mostly found in gonococcus. On the other hand, excessive amounts of wild-type amplification

236 due to commensal *Neisseria* spp. could potentially mask the presence of an AMR mutation in

237 *N. gonorrhoeae*, especially in clinical specimens with low load of the pathogen (i.e., in

238 pharyngeal samples) (38, 39).

239 ***Analysis of the representative spiked negative and positive samples.*** To assess the extent of

240 commensal interference on the detection of the AMR determinants in clinical specimens, four

241 pharyngeal and four rectal samples negative for *N. gonorrhoeae* were spiked with gDNA of

242 control strains possessing the mutations of interest for each multiplex reaction.

243 The results obtained from the pharyngeal specimens showed strong background amplification

244 of wild-type amplicons due to the presence of *Neisseria* spp. for most target reactions (e.g.,

245 23S rRNA C2611T, 16S rRNA C1192T, *rpsE* Thr24Pro). This background amplification

246 would cause false negative results especially in the presence of low amounts of gonococcus.

247 Additionally, nonspecific amplification strongly affected the melting curve interpretation of

248 the *gyrA* Ser91Phe and 23S rRNA A2059G reactions. Finally, two samples exhibited positive

249 amplification of the *penA* A501 reaction due to commensals (see examples in Figure S2 A-E).

250 On the other hand, for the spiked negative rectal specimens, only strong cross-amplification

251 of wild-type 16S rRNA C1192 was observed (see examples in Figure S3 A-D).

252 Taken together with the relatively high LOD needed for proper HRM analysis, these

253 limitations suggested that our method would not be suitable for direct screening of clinical

254 specimens. For this reason, total DNA extracted from four pharyngeal, four rectal and four

255 urethral clinical samples positive for *N. gonorrhoeae* was used to test the performance of our

256   method. Results were also compared to the gDNA extracted from *N. gonorrhoeae* strains

257   (when available) isolated from the specimens.

258   Our platform indicated that all four pharyngeal samples tested positive for the *opa* reaction

259   (Figure S4 A-D). Cross-amplification of commensals together with the relatively low

260   gonococcal load led to a false positive result for the presence of a mosaic *penA* in one sample.

261   Additionally, the melting curves of several reactions were not properly interpretable due to

262   low or nonspecific amplification (e.g., *gyrA* Ser91Phe, 23S rRNA A2059G, *rspE* Thr24Pro).

263   Similarly, low amplicon amounts strongly affected the melting curve interpretation of all four

264   multiplex reactions in the positive rectal (Figure S5 A-D) and urethral specimens (Figure S6

265   A-D), confirming that our method cannot be directly implemented for clinical specimens.

266   Nonetheless, it could be a valuable tool for rapid screening of large isolate collections, both

267   for surveillance and epidemiological purposes. For this reason, we compared our molecular

268   methodology with the standard culture-based AST Etest method for a panel of 193 Swiss

269   isolates.

270   ***Analysis of the 193 clinical isolates.*** As shown in Table 3, the real-time PCR platform

271   correctly identified all isolates as *N. gonorrhoeae*. Moreover, AMR characterization for CIP

272   had both sensitivity and specificity of 100%, whereas AZM and SPC had specificity of 100%.

273   In particular, our method correctly identified all isolates exhibiting resistance to CIP (58 out

274   of 58). No mutations associated to SPC resistance were observed in agreement with the

275   results obtained by phenotypic AST. Furthermore, none of the isolates tested positive for the

276   23S rRNA C2611T or A2059G mutations associated with moderate or high AZM resistance,

277   respectively. Consistently, none of the tested isolates exhibited AZM MICs >2 µg/ml. Finally,

278   all 7 strains showing CFX resistance by phenotypic AST were positive for the presence of a

279   mosaic *penA* allele. However, no resistance to CRO was observed. This was expected, since it

280   is known that the presence of a mosaic *penA* gene is typically associated to raised MICs for

281   ESCs, even if usually still in the susceptible range based on EUCAST criteria (40).

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282 Thus, we further explored the MIC distribution of CFX and CRO in isolates harboring mosaic

283 or non-mosaic *penA* patterns (Figure 1). Out of the 16 isolates positive for the presence of a

284 mosaic *penA* allele, seven were CFX resistant and five were only a two-fold dilution apart

285 from being resistant (MIC, 0.125 µg/ml). The remaining four strains with a mosaic *penA* gene

286 had raised CFX MICs of 0.064-0.094 µg/ml, whereas all other non-mosaic isolates tested

287 exhibited MICs of ≤0.047 µg/ml. Furthermore, all 16 strains harboring a mosaic *penA* allele

288 also showed raised CRO MICs in the range of 0.023 to 0.094 µg/ml, which were noticeably

289 higher compared to strains with non-mosaic patterns, in agreement with previous observations

290 (37, 40, 41).

291 ***Overall performance of the real-time PCR platform.*** Since some of the resistance mutations

292 were not included among the 193 Swiss isolates, we also evaluated the performance of our

293 test including the 35 control strains and 4 additional isolates harboring known, but very rare,

294 AMR determinants (Table 3).

295 Our platform accurately identified *N. gonorrhoeae* with a sensitivity and specificity of 100%.

296 However, strain GC2 tested positive only for the *opa* reaction. Notably, this strain was

297 previously reported to cause false-negative results in other *porA*-based PCRs due to the

298 acquisition of a meningococcal *porA* allele (33). For this reason, our dual-target approach

299 proved to be extremely valuable for the identification of even such exceptional isolates.

300 With regard to the AMR detection, the platform correctly predicted resistance to ciprofloxacin

301 in all 83 strains positive for a mutation in codon 91 of *gyrA*. Furthermore, the prediction of a

302 mosaic *penA* allele allowed the detection of two fully CRO-resistant strains (F89 and A8806),

303 as well as all isolates resistant to CFX with the execption of WHO L, which harbors a non-

304 mosaic *penA* allele with an additional substitution in amino acid 501. It is worth noting that

305 the mosaic *penA* allele of A8806 differs from the pattern XXXIV allele found in the high-

306 level CRO-resistant F89 strain. For this reason, no amplification of the *penA* Gly545Ser target

307 was observed for A8806. Nevertheless, the strain was correctly identified as harboring a

308   mosaic *penA* allele due to the positive *penA* Ala501 reaction. Finally, the identification of

309   either of the two mutations conferring resistance to AZM or SPC was correctly associated

310   with resistance to those antibiotics.

311   ***Conclusions.*** We developed and validated a new real-time PCR method coupled with HRM

312   analysis that accurately detected several important mutations associated with resistance to

313   antibiotics commonly used to treat gonorrhea. Cross-reactivity with commensal species and

314   high limit of detection suggested that our method is not suitable for direct screening of

315   clinical specimens. However, it proved to be a useful and rapid alternative to culture-based

316   methods to assess the AMR profiles for ESCs, AZM, CIP and SPC of a large collection of *N.*

317   *gonorrhoeae* isolates.

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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' opa_R 5'-aagggcggattatatcgggttcc-3'	56	*opa* (Species identification)	Triplex
porA	porA_F 5'-cagcaatttgttccgagtca-3' porA_R 5'-ggcgtataggcggacttg-3'	44	*porA* (Species identification)	Triplex
penA Gly545Ser	545_F 5'-*cccgcccccg*ccgactgcaaacggttacta-3' 545_R 5'-*cccgcccccg*cggccctgccactacacc-3'	61	Mosaic *penA* (Decreased susceptibility/resistance to ESCs)	Triplex
penA Ala501	501_F 5'-*cccgcccccgcc*gtcggcgcaaaaaccggtacg-3' 501_R 5'-*cccgcccccgcc*aatcgacgtaacgaccgttaaccaacttacg-3'	79	Mosaic *penA* (Decreased susceptibility/resistance to ESCs)	Duplex I
23S rRNA C2611T	C2611_F 5'-acgtcgtgagacagtttggtc-3' C2611_R 5'-caaacttccaacgccactgc-3'	49	23S rRNA C2611T (Moderate AZM resistance) [b]	Duplex I
23S rRNA A2059G	A2059_F 5'-ctacccgctgctagacgga-3' A2059_R 5'-cagggtggtatttcaaggacga-3'	142	23S rRNA A2059G (High AZM resistance) [b]	Duplex II
gyrA Ser91Phe	gyrA_S91_F 5'-taaataccaccccccacggcgatt-3' gyrA_S91_R 5'-atacggacgatggtgtcgtaaact-3'	47	GyrA Ser91Phe (CIP resistance)	Duplex II
rpsE Thr24Pro	S5_T24_F 5'-atggtcgcagttaaccgtgta-3' S5_T24_R 5'-aaagccataatgcgaccacc-3'	56	RPS5 Thr24Pro (SPC resistance)	Duplex III
16S rRNA C1192T	16S_1192_F 5'-*ccgcccccc*ggaggaaggtggggatga-3' 16S_1192_R 5'-*ccgcccccc*tggtcataagggccatgag-3'	64	16S rRNA C1192T (SPC resistance)	Duplex III

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)

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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]	n/i	100 (66-100)	100 (87-100
	Mosaic (n=9)	Mosaic (n=9)	83.59 - 84.35	84.17 ± 0.19			
gyrA Ser91Phe	GyrA Ser91 (tcc), Ala92 (gca) (n=11)	GyrA Ser91 (tcc), Ala92 (gca) (n=11)	77.97 - 78.16	78.08 ± 0.05		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	0.61 ± 0.06		
	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)

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71   **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	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)
						N. gonorrhoeae isolates collected during 1989-2014 (n=193)						Overall *N. gonorrhoeae* strains (n=232), including the 39 controls	
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) [e]	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	-		

72   **Note.** AST, antimicrobial susceptibility testing obtained with Etest; R, resistant; S, susceptible; CI, confidence interval; -, zero; n/a, not applicable
73   [a] Numbers are based on the results of the multiplex real-time PCR platform
74   [b] AST was categorized based on EUCAST criteria with exception for AZM (see below)
75   [c] AZM resistance were defined as > 2 µg/ml
76   [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
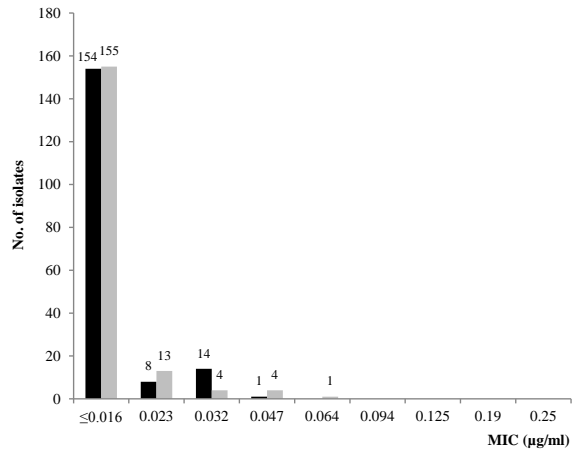77   real-time PCR
78   [e] For the evaluation of the "Species identification" we also included the 19 non-gonococcal *Neisseria* spp. strains
79   [f] Strain WHO L (non mosaic *penA* gene with an additional substitution in amino acid 501)

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).

A.

B.