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1 Genomic characterization of emerging bacterial uropathogen *Neisseria meningitidis* misidentified as *Neisseria*
2 *gonorrhoeae* by nucleic acid amplification testing
3

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26 **Abstract**

27 *Neisseria meningitidis* (Nm) and *Neisseria gonorrhoeae* (Ng) are pathogenic bacteria that can cause human
28 infections. While Nm infections are associated with bacterial meningitis and bacteremia, a strain of Nm,
29 isolated from the urogenital system, has recently been associated with urethritis. As this strain is becoming
30 prominent as an emerging pathogen, it is essential to assess identification tools for Nm and Ng urogenital
31 isolates. Consecutive Nm isolates recovered from urogenital cultures of symptomatic patients with presumptive
32 diagnoses of gonorrhea and a random selection of Ng isolates recovered from the same population within the
33 same time frame were characterized with routine identification systems, antimicrobial susceptibility testing, and
34 whole genome sequencing. MALDI-ToF MS, multilocus sequence typing, 16S rRNA gene sequence, and
35 average nucleotide identity methods accurately identified 95% (18/19) of Nm and Ng isolates. 30% (3/10) of
36 Nm isolates were misidentified as Ng with Aptima Combo 2 CT/NG but no misidentifications were found with
37 the Xpert CT/NG NAAT. Phylogenetic core genome and SNP-based grouping analyses showed that urogenital
38 Nm isolates were highly related, and phylogenetically distinct from Ng and respiratory Nm isolates but similar
39 to urogenital Nm isolates from patients with urethritis in the US. Urogenital Nm isolates were predominantly
40 azithromycin resistant while Ng isolates were azithromycin susceptible. These data indicate that urogenital
41 isolates of Nm can cause false-positive detections with Ng diagnostic assays. Misidentification of urogenital
42 Nm isolates may confound public health-related activities for gonorrhea and future studies are needed to
43 understand the impact on clinical outcome of Nm urogenital infection.

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51 **Introduction**

52 *Neisseria gonorrhoeae* (Ng) and *Neisseria meningitidis* (Nm) are human bacterial pathogens that can
53 occupy different niches in the body (1, 2). Ng is the causative agent of the sexually transmitted infection,
54 gonorrhea, which impacts 78 million people worldwide (3). As an obligate pathogen, Ng primarily colonizes the
55 genital mucosa and has evolved virulence factors that allow it to survive and evade the host immune system (1).
56 Recently, Ng has received increased public health attention and drug-resistant Ng has been categorized as an
57 urgent threat by the US Centers for Disease Control and Prevention due to resistance to commonly used
58 antibiotics, limiting treatment in patients (4-6). Molecular point-of-care and sample-to-answer assays have been
59 developed to rapidly and accurately identify the presence of Ng in clinical specimens (7). While development of
60 these assays are important for patient treatment, there is evidence for sporadic false-positive molecular results
61 due to cross reactivity between *Neisseria* species (8).

62 Nm is found as a commensal in the respiratory system with ~10% of healthy adults and 40% of men
63 who have sex with men (MSM) demonstrate naso/oro pharyngeal carriage (2). Nm is also a leading cause of
64 bacterial meningitis and causes significant morbidity and mortality in children and young adults with an
65 estimated 1.2 million cases of meningococcal infection per year worldwide (9). The virulence of Nm is
66 determined by host factors (i.e. complement deficiency) and several virulence genes that facilitate adherence
67 and survival in the respiratory system and invasion of the blood stream (9).

68 The genus of *Neisseria* has evolved mechanisms that result in a high frequency of horizontal gene
69 transfer (HGT), both within and between species, with up to 10% of the Nm genome made up of mobile genetic
70 elements (9, 10). Co-localization of Nm and Ng in the urogenital system may result in increased transfer of
71 virulence or antibiotic resistance genes (11). Recent studies have identified a strain of Nm that has been isolated
72 from urogenital system and is associated with urethritis (12-16). As this strain becomes a more prominent
73 emerging pathogen in areas with high Ng infection rates, it is essential to assess the ability of identification
74 tools to discriminate between Nm and Ng urogential isolates (13, 14, 17).

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75 Following implementation of a total laboratory automation system for culture-based microbiology (BD
76 Kiestra TLA; Beckton Dickinson), the clinical microbiology laboratory at Barnes Jewish Hospital in St. Louis
77 MO observed significant increases in the recovery of *N. gonorrhoeae* and, more recently, *N. meningitidis*
78 incidentally from urine specimens submitted for routine culture-based testing (18). In this study, we characterize
79 consecutively recovered Nm and compare them to urethritis and Nm invasive strains reported elsewhere.
80

81 **Materials and Methods**

82 *Clinical isolates and human studies approval*

83 Consecutive *Neisseria meningitidis* (Nm) and a random selection of *Neisseria gonorrhoeae* (Ng) isolates
84 recovered from March 2018 to March 2019 from clinical specimens submitted for routine testing to the Barnes
85 Jewish Hospital Clinical Microbiology Laboratory in St. Louis, MO were included in this study. Previous
86 studies from our laboratory have found increased recovery of Ng and Nm isolates incidentally from urine
87 cultures submitted for routine testing when incubated with the Kiestra Total Laboratory Automation (TLA)
88 (18). Study isolates were de-identified but patient age, gender, and isolate source were documented. This study
89 was approved by the Human Research Protection Office of Washington University School of Medicine.
90

91 *Laboratory characterization*

92 Frozen Nm and Ng isolates were sub-cultured to chocolate (CHC) agar (Hardy Diagnostics, Santa Maria
93 CA), incubated at 35°C and 5% CO_2 and passaged twice prior to additional testing. For phenotypic
94 characterization, 10 µL of a 0.5 McFarland (McF) suspension of each isolate was cross-struck to CHC and
95 Modified Thayer-Martin (MTM) (Hardy Diagnostics, Santa Maria, CA) to achieve less subjective interpretation
96 and quadrant struck to a third CHC plate with 10 µg colistin disk (BD BBL™, Sparks, MD). Following
97 incubation at 35°C and 5% CO_2 for 18-20 h, colony-forming units were enumerated and colistin zone size to the
98 nearest millimeter was recorded. For biochemical characterization, the RapidID™ NH system (Remel, Lenexa,
99 KS) was used per manufacturer's instructions. Briefly, biochemical strips were inoculated with 3 McF

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100 suspensions of each isolate and incubated at 35°C for 4 h in air. Following incubation, biochemical reactions
101 were read, scored, and microcodes interpreted by the ERIC™ system to obtain organism identifications. For
102 molecular characterization with Matrix-Assisted-Laser Desorption Ionization-Time of Flight Mass
103 Spectrometry (MALDI-ToF MS), 2 commercially available systems were utilized: Bruker BioTyper (Bruker,
104 Billerica, MA) and VITEK MS (bioMérieux, Durham, NC). Briefly, single colonies of pure growth were
105 spotted to target slides and overlaid with matrix prior to analysis on each instrument per manufacturer's
106 instructions. For Nm isolates, target slides were spotted with organism and matrix and fully dried inside a BSC
107 before removal for loading onto the MALDI-ToF MS instrument. For analysis with commercial *in vitro*
108 diagnostic (IVD) nucleic acid amplification tests (NAATs), both contrived swab and urine specimens of Nm
109 and Ng isolates were tested to confirm the lack of matrix-specific effects. Swabs from Aptima Vaginal and
110 Xpert Vaginal/Endocervical Specimen collection kits were inserted into a 0.5 McF suspension of each isolate
111 for 10 s diluting or 0.5 McF isolate suspensions was diluted10-fold with remnant urine specimens previously
112 determined to be negative for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. Both contrived swab and
113 urine specimens were tested with the Aptima Combo 2 CT/NG (A-CT/NG) on the Tigris GTS system (Hologic,
114 Inc., San Diego, CA) and the Xpert CT/NG (X-CT/NG) assay on the GeneXpert Infinity system (Cepheid,
115 Sunnyvale, CA) per manufacturer's instructions.

116

117 *Antimicrobial susceptibility testing*

118 Antimicrobial susceptibility testing (AST) of Nm and Ng isolates was performed by disk diffusion and
119 gradient diffusion strips and MIC and/or disk diffusion zone size interpreted according to the Clinical &
120 Laboratory Standards Institute (CLSI) M100 29th edition (19). For Nm isolates, 0.5 McF suspensions of test
121 isolates were inoculated to Mueller Hinton Agar with 5% sheep blood and incubated with penicillin,
122 azithromycin , ceftriaxone ETESTs (bioMérieux, Durham, NC) and ciprofloxacin (5 µg), rifampin (5 µg),
123 minocycline (30 µg) and trimethoprim-sulfamethoxazole disks (1.25/23.75 µg) (BD BBL™, Sparks, MD) at
124 35°C and 5% CO_2, for 20-24 h. For Ng isolates, 0.5 McF suspensions of test isolates were inoculated to GC

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125 agar base with 1% defined growth supplement and incubated with penicillin, azithromycin, ceftriaxone Etests
126 (bioMérieux, Durham, NC) and ciprofloxacin (5µg) and rifampin (5µg) disks (BD BBL™, Sparks, MD) at
127 35°C and 5% CO_2, for 20-24 h. MIC doubling dilutions and zone sizes to the nearest millimeter were read with
128 reflected light and interpreted per CLSI M100 29th edition guidelines (19). AST categorical results were
129 visualized using pheatmap (R) with color strips used to indicate source of isolate, species, and SNP pairwise
130 distance-based grouping.
131

132 *Whole genome sequencing*

133 Total genomic DNA was extracted from cell cultures suspended in 1 mL of deionized water using the
134 QIAamp BiOstic Bacteremia DNA kit (QIAGEN, Germantown, MD, USA). We quantified DNA concentration
135 using Qubit dsDNA assays (ThermoFisher Scientific). Illumina sequencing libraries were prepared using 5
136 ng/uL of isolate DNA in a modified Nextera kit protocol (Illumina, San Diego, CA, USA). We then pooled and
137 sequenced libraries on a NextSeq HighOutput platform (Illumina) to obtain ~ 2 million 2 x 150 bp reads. The
138 reads were demultiplexed by barcode, had adapters removed with Trimmomatic v.36, and contaminating human
139 sequences were removed with Deconseq v.4.3 (20, 21). We assembled processed reads into draft genomes using
140 the de-novo assembler SPAdes v3.11 (22). Quality of draft genomes was assessed using QUAST v4.5 and
141 checkM (23, 24). Assembles were considered to have passed quality control when assembly length represented
142 in contigs <1kb was less than 10%, number of contigs greater than 500bp was less than 5000, completeness was
143 greater than 90%, and contaminated reads were less than 5%. Draft genomes were annotated using Prokka
144 v1.12 (25).
145

146 *Data availability*

147 All assembles are uploaded to NCBI under BioProject PRJNA643774
148 (http://www.ncbi.nlm.nih.gov/bioproject/643774).
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150 *Genomic taxonomic identification*

151 Following draft genome assembly, we determined genomic taxonomic identification by average
152 nucleotide identity (ANI), 16S rRNA gene identification, and multi-locus sequence typing (MLST). Assembled
153 scaffolds were submitted to the Neisseria Multi Locus Sequence Typing website (https://pubmlst.org/neisseria/)
154 to determine MLST and clonal complex (26). For all isolates, 16S rRNA gene sequences were identified using
155 RNAmmer v1.2 and submitted to EZ BIoCloud taxonomic database for classification (27, 28). Using ANI
156 analysis, species were determined if the genome in question had >95% ANIm with the type genome (Nm:
157 NM_MC58; Ng: NG_ref_FA_1090) using dnadiff (29). Pairwise ANI for each isolate was clustered and
158 visualized using pheatmap (R) (30).

159

160 *Phylogenetic analysis*

161 To phylogenetically compare isolate sequences, 16S rRNA gene sequences identified by RNAammer
162 were aligned using MUSCLE and an approximate maximum likelihood tree was built with FastTree (31, 32).
163 FastTree uses a heuristic variant of neighbor joining to construct a rough topology, reduces the length of the tree
164 using a mix of nearest-neighbor interchanges and subtree-prune-regraft moves, and improves the tree with
165 maximum-likelihood rearrangements(32). Branch length precision was rounded to 0.0001 substitutions per site.
166 The output newick files were visualized and annotated with isolate source using ggtree (R) (33, 34). To
167 compare isolate genomes, .gff files produced by prokka were used to construct a core genome alignment with
168 Roary v3.8.0 for Nm(35). Roary alignments were used to create an approximate maximum likelihood tree with
169 fasttree (32). The output newick files were visualized and annotated with isolate source using ggtree (R) (33,
170 34).

171

172 *Isolate groupings based on SNP pairwise distances*

173 Snippy v4.3.8 was used to map forward and reserve reads for Nm isolates to the Nm MC58 type strain
174 complete genome assembly (ID) and to call SNPs (36). To determine groupings, we compared pairwise SNP

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175 distances between each Nm isolate pair. Isolates were grouped into perfectly reciprocal groups at every pairwise
176 distance cutoff between Nm isolates using igraph v1.2.4.1 as described previously (37). Groupings are
177 visualized with a SNP cutoff of 2000.
178
179 *Antibiotic resistance mutations identification and analysis*
180 Targeted analysis of acquired antibiotic resistance mutations against β-lactams (*bla_{TEM}*, *penA, porA,*
181 *ponA, mtrR*), macrolides/lincosamides/streptogramins (*23S rRNA*, *mtrR*) and quinolones (*gyrA, parC*) was
182 performed as a result of phenotypic AST findings using PointFinder (38). The presence/absence matrix of
183 ARGs was visualized in pheatmap (R). Associated meta-data was displayed as a color strip to represent
184 bacterial isolate identification and Aptima CT results. We further validated PointFinder results for key
185 resistance gene mutations using BLASTn with MUSCLE alignment and maximum likelihood tree visualization
186 (Figure S3).
187
188 **Results**
189 *Nm urinary isolates can cause false-positive Ng molecular test result*
190 Consecutive Nm isolates and a random selection of Ng isolates recovered from clinical specimens
191 during the same time period were characterized by phenotypic and molecular methods routinely used to identify
192 *Neisseria* species in clinical microbiology laboratories including MALDI-ToF MS, and commercial
193 biochemical and molecular tests. Detailed demographic information was not available for these isolates but
194 limited information including patient age, gender, and isolate source are summarized in Table 1. All urogenital
195 Nm and Ng isolates were correctly identified using Bruker Biotyper and VITEK MS MALDI-ToF MS
196 platforms (Table 1). One *Neisseria* isolate from a respiratory source (NM12) was incorrectly identified using
197 MALDI-ToF MS and biochemical tests as Nm (Bruker Biotyper) or *N. polysaccharea*/*N. meningitidis* (VITEK
198 MS) and Ng (RapidID NH). However, this isolate was phenotypically consistent with non-pathogenic *Neisseria*

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199 species with no growth on MTM media and a zone of inhibition when incubated with a 10 µg colistin disk on
200 solid media, and was identified using WGS methods as *N. polysaccharea*.

201 Organism suspensions of each isolate were also tested with the Aptima CT/NG Combo 2 assay (A-
202 CT/NG) on the Tigris DTS system and Xpert CT/NG (X-CT/NG) on the GeneXpert system. All Ng isolates
203 were detected by both systems, while all Nm isolates were not detected by X-CT/NG. Importantly, urinary
204 isolates of Nm (NM04, NM07, NM08) tested positive for Ng with A-CT/NG. This result was confirmed with
205 remnant urine specimens spiked with NM04, NM07, NM08 NM09, NM12 and NM14 (Table 1).

206

207 *Urogenital Nm classified as Nm by MLST, 16S rRNA gene classification, and average nucleotide identity.*

208 We performed Illumina whole genome sequencing (WGS) on all Saint Louis, MO (STL)-collected
209 isolates. After draft genome assembly, scaffolds were submitted to the *Neisseria* MLST website
210 (https://pubmlst.org/neisseria/) to determine MLST and clonal complex (26). For 18 of 19 *Neisseria* isolates,
211 MLST species classification agreed with MALDI-ToF MS classification. One respiratory isolate (NM12), that
212 MALDI-ToF MS was unable to classify to a single species, was characterized as *N. polysaccharea.* MLST
213 clonal complex indicated 7 of 10 STL-collected urogenital Nm isolates fell into the ST-11 clonal complex
214 (Table 1).

215 To determine the phylogenetic context of isolates, we downloaded a series of Nm genomes from NCBI
216 and PubMLST: 28 Nm isolates from UTIs in the US (13, 14), 3 Nm ST-11 isolates from cases of meningitis in
217 MSM in the US (MSM) (39), 29 Nm ST-11 isolates from a meningitis epidemic in Africa (40), and 8 Nm
218 isolates from non-ST-11 meningitis cases (Table S1).

219 Ribosomal RNA (rRNA) classification is used in the APTIMA COMBO2 assay, with the specific loci
220 being proprietary, (41) and in 16S rRNA gene sequence classification to determine bacterial operational
221 taxonomic unit (OTU) or amplicon sequence variant (ASV) (28). Thus, we classified and compared 16S rRNA
222 gene sequences across *Neisseria* isolates. For all STL-collected isolates, 16S rRNA gene sequences were
223 submitted to EZ BIoCloud taxonomic database for classification (28). 16S rRNA gene classification correlated

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224 with MLST for all isolates (Table 1). An approximate maximum likelihood tree with NM12 as the outgroup
225 shows Ng sequences form a monophyletic clade distinct from Nm sequences with Nm that tested positive for
226 Ng using the A-CT/NG falling within the Nm sequences (Figure S1).

227 Finally, we used ANI for genomic species classification. Species were determined if the genome in
228 question had >95% pairwise ANI with the type genome (Figure 1). All Nm or Ng isolates from urogenital
229 samples that were identified by MALDI-ToF MS and MLST were also identified as Nm or Ng, respectively, by
230 ANI. NM12 did not fall above the cut off for Nm, Ng, or *N. polysaccharea* type strains. Pairwise ANI of all Nm
231 isolates and select reference Nm genomes indicated that ST-11 isolates form a distinct cluster with ANI above
232 99% (Figure 1). This cluster included all urogenital Nm isolates for which Ng was detected by A–CT/NG and
233 one Nm respiratory isolate, NM13. Thus, MALDI-ToF MS, MLST, 16S rRNA, and ANI agree on classification
234 for 18 of 19 *Neisseria* isolates.

235

236 *Nm urogenital isolates form a primary lineage that is distinct from ST-11 meningitis isolates.*

237 To determine genomic similarity across Nm genomes, we used a core genome alignment of 1057 genes
238 at 95% identity of all Nm isolates, using NM12 as an outgroup. The phylogenetic tree of this alignment shows
239 that urogenital Nm isolates primarily fall within a single lineage (Figure 2). All (3 of 3) STL-collected Nm
240 isolates for which the A-CT/NG test detected Ng formed a single clade within STL-collected ST-11 urogenital
241 Nm isolates, suggesting a recent common ancestor. Of the STL-collected urogenital Nm isolates, 8/10 cluster
242 together and form a sister clade to other urogenital Nm isolates. This similarity suggests a single common
243 ancestor for 93% (31 of 33) of urogenital Nm isolates. Two urogenital STL-collected Nm isolates, NM09 and
244 NM15, did not cluster with other urogenital isolates, and NM09 was instead highly related to a respiratory Nm
245 isolate. Both NM09 and NM15 isolates were non-groupable using serotyping methods, did not fall into ST-11
246 clonal complex, and were misclassified by RapID NH--NM15 had been misclassified as Ng and NM09 was
247 misclassified as *Moraxella osloensis* (Table 1). ST-11 urethritis isolates were sister clade to a lineage that
248 included one STL-collected respiratory isolate and all 3 of the MSM meningitis isolates. This clade was sister

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249 clade to the African ST-11 meningitis isolates. In contrast to Nm urinary isolates, Nm respiratory isolates were
250 highly diverse and distantly related.
251
252 *Nm urinary isolates are highly related to other urogenital isolates and not respiratory isolates.*
253 SNP distance across whole genomes have been found to provide higher resolution of phylogenetic
254 distances than core genome comparisons (37). Thus, to further investigate genomic similarity of STL-collected
255 Nm isolates, we calculated pairwise SNP distances by mapping quality filtered reads from Nm isolates to the
256 Nm type strain. To find groupings, we used a grouping technique, "clique" (37) on STL-collected Nm isolates.
257 We compared pairwise SNP distances between Nm isolate pairs and iterated through each unique SNP distance
258 cutoff to filter the isolate pairwise network list (Figure 3a). For each cutoff, we found reciprocal groups and
259 recorded the number of groups and isolates per group. Then groups were defined as complete subgraphs, where
260 each node in the group was connected to every other node in the group. Number of Nm groups rose initially
261 from 1 to 3 groups as SNP distances increased from 357 to 6269. Only a single SNP distance of 5624 SNPs had
262 4 groups, and immediately after this peak, groups decreased again to 3 with a decline in group size to 1 after
263 20,000 SNPs. Figure 3a shows the groups, which corresponds to a SNP cutoff that includes only highly related
264 Nm isolates with less than 2000 SNP distances.
265 Urogenital Nm isolates primarily formed a single grouping (Figure 3b). The first grouping includes 8/10
266 urogenital isolates and all ST-11 urethritis isolates. This grouping fell entirely with the ST-11 urogenital clade
267 described in the core genome phylogeny (Figure 2). A second grouping included a respiratory (NM11) and a
268 urogenital isolate (NM09). These groupings suggest that while ST-11 urethritis isolates are highly related with
269 between 9.5-9.6x10^{-4} pairwise SNPs/genome length, not all urogenital isolates fall into the grouping, and one
270 isolate shares high similarity (4.8x10^{-4} pairwise SNPs/genome length) with a respiratory isolate.
271
272 *Nm isolates have a distinct antibiotic susceptibility profile from Ng isolates*

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273 To consider clinical implications of misidentified Nm isolates, we performed phenotypic AST on all
274 STL-collected isolates. AST was performed against azithromycin, penicillin, ceftriaxone, rifampin,
275 ciprofloxacin, minocycline, trimethoprim-sulfamethoxazole, and colistin. AST profiles varied between Ng
276 isolates and urogenital Nm isolates (Figure 4a). Most Nm isolates tested non-susceptible to azithromycin with
277 $MIC_{50/90}$ of 4 µg/mL (range: 0.5 to 4 ug/mL) compared to Ng isolates which were mostly susceptible to
278 azithromycin with MIC_{50} of 0.125 µg/mL and MIC_{90} of 2 µg/mL (range: 0.064 to 2 ug/mL). The respiratory
279 isolate, NM012, has a unique AST profile distinct from both Ng and Nm isolates in that it tested resistant to
280 azithromycin, penicillin, ciprofloxacin, and trimethoprim-sulfamethoxazole. We also evaluated chromosomal
281 point mutations that may account for antibiotic resistance in *Neisseria* using both PointFinder and by
282 individually validating mutations of known interest in specific genes (Table S2, Figure S3). Point mutations for
283 resistance were primarily shared by species (Figure S2). One prominent point mutation in Nm isolates was in
284 non-mosaic penA allele p.N512Y. This point mutation is associated with mosaic penA, which can contribute to
285 decreased susceptibility to expanded-spectrum cephalosporins (42); however all isolates in this study were
286 ceftriaxone susceptible with MIC $\leq$ 0.016 µg/mL. While all urogenital Nm isolates and 1/3 respiratory Nm
287 isolates had this mutation, this mutation was not present in any Ng isolate. Isolates within the same SNP
288 pairwise distance-based grouping had identical resistance mutation profiles (Figure S2, Table S2, Figure S3).
289

290 **Discussion**

291 As Nm becomes an increasingly recognized pathogen in the urogenital system, accurate species
292 identification of Nm and Ng urogenital isolates may be important for clinical care. Thus, it is essential to assess
293 tools used for identification and compare Nm and Ng urogenital isolates. In this study, we demonstrate that 30%
294 (3/10) of urogenital Nm isolates were misidentified as Ng with the A-CT/NG NAAT, and that these urogenital
295 Nm isolates were predominantly non-susceptible to azithromycin. We found specific identification using
296 MALDI-ToF MS, MLST, 16S rRNA gene sequence, and ANI methods was 100% accurate for both urogenital
297 Nm and Ng isolates. However, our data indicate that some urogenital isolates of Nm can cause false-positive

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298 detections with Ng-specific molecular tests and that some commensal *Neisseria* strains can be identified as Nm
299 by MALDI-ToF MS.

300 While NAAT tests such as the Aptima CT/NG Combo 2 or the Xpert CT/NG are the standard of care for
301 detection of Ng from urine and genital specimens in clinical laboratories, there is evidence that other *Neisseria*
302 species can cause false-positive Ng detections (7, 8). A previous report suggested these false-positive results
303 were sporadic and low level as no isolate tested positive twice in their study (8). In contrast, our findings were
304 not sporadic as 3 unique urogenital Nm isolates tested as Ng-detected both as pure isolate suspensions in saline
305 and when spiked into urine. The Xpert CT.NG NAAT has two Ng-specific targets, both of which must be
306 detected to return a Ng positive result, while the Aptima CT/NG Combo 2 NAAT targets a region of the 16S
307 rRNA to detect Ng. Since the exact locus of both the Aptima CT/NG Combo 2 and the Xpert CT/NG tests are
308 proprietary, we are unable to directly test for sequence differences that may result in misidentifications.
309 However, genomic characterization of these isolates demonstrated that Nm isolates that test falsely-positive for
310 Ng form a distinct clade based on a core genome phylogeny, suggesting a common ancestor and indicating a
311 genomic component rather than random chance or a sporadic error in the NAAT test is responsible for the false
312 positive Ng result.

313 Accurate identification of *Neisseria* isolates is important as AST profiles vary between Ng and Nm
314 isolates. Though rising rates of reduced susceptibly to azithromycin in Ng has been reported across the US (43),
315 we found that the Ng isolates test in our study were primarily susceptible to azithromycin (n=4/5), while
316 urogenital Nm isolates collected over the same time period as Ng isolates were more likely to be azithromycin
317 non-susceptible (n= 7/10). Our observation of largely azithromycin-susceptible Ng is consistent with a previous
318 study of a larger cohort of Ng isolates recovered from the same institution that reported that azithromycin non-
319 susceptibility was rare (< 2%) (44). Interestingly, all Nm isolates that tested falsely-positive for Ng were
320 azithromycin non-susceptible (n= 3/3). Currently single dose of intramuscular ceftriaxone (250mg) plus a single
321 dose of oral azithromycin (1g) is the primary treatment recommendation for uncomplicated gonococcal
322 infection (45). Although dual-therapy was primarily aimed at treatment of uncomplicated chlamydial co-

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323 infections, routine combination therapy may hinder development of antimicrobial resistance in Ng particularly
324 in light of increased cephalosporin resistance in the US (46). Given the rare reports of ceftriaxone-resistant Nm
325 (47-49), ceftriaxone plus azithromycin dual therapy is likely effective for treatment of urogenital Nm infection.
326 However, if azithromycin non-susceptibility is common among urogenital Nm isolates and in the setting of
327 reduced susceptibility to 3rd-generation cephalosporins, current gonococcal treatment guidelines may be
328 suboptimal for urogenital Nm infection. Interestingly, despite recent reports of ciprofloxacin-resistant, beta-
329 lactamase-producing Nm serogroup Y (50), all of the Nm isolates evaluated in this study were ciprofloxacin and
330 ceftriaxone susceptible (n=13/13) though most were non-susceptible to penicillin (n= 10/13); beta-lactamase
331 testing was not performed.

332 One hypothesis for conflicting identification of Nm isolates may be increased HGT between Ng and
333 Nm. However, our 16S rRNA gene sequence, MLST, and whole genome analyses indicate that urogenital Nm
334 isolates are not more similar to Ng than other Nm isolates. The majority of urogenital isolates for which we
335 performed genomic analyses (n= 35/37) share a recent common ancestor. This suggests that most cases of Nm
336 urethritis are due to the spread of urethritis-associated Nm ST-11 and not due to translocation and subsequent
337 infection of respiratory or meningitis-associated Nm isolates to the genitourinary tract consistent with previous
338 reports (11, 15). However, we did observe two instances where urogenital Nm isolates were not from the ST-11
339 urethritis clade, and was in one case, a respiratory Nm isolate highly related to a urinary Nm isolate. This
340 suggests urogenital Nm isolates do not derive exclusively from the ST-11 urethritis clade and that transmission
341 between body sites may be possible.

342 Studies have indicated that invasive Nm isolates from MSM are associated with colonization of the
343 urethra or rectum (2), and that Nm urethritis outbreaks are closely related to cases of invasive Nm in MSM
344 populations, suggesting that urethral colonization may contribute to invasive disease (11). In our study, the
345 sister clade to all ST-11 urethritis isolates included one respiratory isolate and three meningitis-associated
346 isolates from MSM patients. This phylogeny suggests a common ancestor between MSM meningitidis and the
347 origin of urogenital Nm isolates. However, as this study is focused on urogenital Nm isolates, only a subset of

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348 54 Nm meningitis isolates were used for comparison with a focus on Nm isolates within ST-11. A more
349 exhaustive study of Nm meningitis isolates may find additional clades related to the urogenital NM isolates.
350 Further, in this data set, we do not see evidence for closely related urethritis and invasive Nm isolates. It is
351 possible that increasing the collection and analysis of invasive and urogenital Nm isolates from meningitis
352 patients may expand further on this issue.

353 Public health-related activities associated with gonococcal infection such as contact-tracing and
354 expedited partner therapy maybe indicated and initiated following notification of this reportable infection.
355 However, misidentification of Nm can confound these activities, particularly if identification of Ng and/or Nm
356 is inconsistent across currently available diagnostic tests. Increased vigilance surrounding these
357 (mis)identifications will be required for a more complete understanding of the scope, epidemiology,
358 susceptibility, and clinical outcomes associated with Nm urogenital infections, as has been previously suggested
359 (51).

360 Overall, our findings demonstrate that some urogenital Nm isolates are incorrectly identified as Ng by
361 the Aptima CT/NG NAAT despite being correctly identified as Nm by other molecular methods including
362 MALDI-ToF MS, MLST, 16S rRNA gene sequencing, and ANI analysis. Further, these urogenital Nm isolates
363 are related to the ST-11 urethritis-associated Nm isolates from across the US. These isolates may be recovered
364 from routine urine cultures with increasing frequency as laboratories transition to automated inoculation and
365 incubation systems. Finally, our studies have found that accurate identification of Nm and Ng may be
366 important due to implications for public-health related activities and potential differences in susceptibility
367 profiles. Future studies further describing the scope, epidemiology, clinical course, and outcomes of Nm-
368 mediated urogenital infection compared to gonococcal infection will be needed to justify strategies to identify
369 and/or differentiate Nm from Ng urogenital specimens.

370

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529 **Figure and Table Legends**

530 **Table 1.** ***Neisseria meningitidis*** **(Nm) and** ***Neisseria gonorrhoeae*** **(Ng) isolate characterization.**

Isolate	Age (y), Gender	Source	Serogroup*	Colistin (10 µg) disk	RapID NH™	VITEK MS	Bruker Biotyper	Aptima CT/NG	Xpert CT/NG	16S rRNA ID	MLST CC
NM02	43 y, M	Urine	Non-groupable	R- 6 mm	Nm	Nm	Nm	Neg	Neg	Nm	ST-11
NM03	31 y, M	Urine	ND	R- 6 mm	Nm	Nm	Nm	Neg	Neg	Nm	No type
NM04	27 y, M	Urine	Non-groupable	R- 6 mm	Nm	Nm	Nm	Ng Pos†	Neg†	Nm	ST-11
NM05	18 y, F	Urine	ND	R- 6 mm	Nm	Nm	Nm	Neg	Neg	Nm	ST-11
NM06	31 y, M	Urine	W135	R- 6 mm	Nm	Nm	Nm	Neg	Neg	Nm	ST-11
NM07	43 y, M	Urine	Non-groupable	R- 6 mm	Nm	Nm	Nm	Ng Pos†	Neg†	Nm	ST-11
NM08	23 y, M	Urine	Non-groupable	R- 6 mm	Nm	Nm	Nm	Ng Pos†	Neg†	Nm	ST-11
NM09	22 y, M	Urine	Non-groupable	R- 6 mm	Mo	Nm	Nm	Neg	Neg	Nm	ST-32
NM10	29 y, M	Urine	ND	R- 6 mm	Nm	Nm	Nm	Neg	Neg	Nm	ST-11
NM11	46 y M	Respiratory-Sp	Non-groupable	R- 6 mm	Nm	Nm	Nm	Neg	Neg	Nm	No type
NM12	8 y, F	Respiratory-TA	N/A	S- 20 mm	Ng	Nm/ Np	Nm	Neg	Neg†	Np	No type
NM13	21 y, F	Respiratory-BAL	Non-groupable	R- 6 mm	Nm	Nm	Nm	Neg	Neg	Nm	ST-11
NM14	71 y, M	Respiratory-BW	B	R- 6 mm	Mo	Nm	Nm	Neg†	Neg†	Nm	ST-11
NM15	32 y, M	Genital	Non-groupable	R- 6 mm	Nm	Nm	Nm	Neg	Neg	Nm	ST-4821
NG ATCC® 49226		NA	ND	R- 6 mm	Ng	Ng	Ng	Ng Pos	Ng Pos	Ng	No type
NG01	35y, M	Urine	ND	R- 6 mm	Ng	Ng	Ng	Ng Pos	Ng Pos	Ng	No type
NG02	34y, M	Urine	ND	R- 6 mm	Ng	Ng	Ng	Ng Pos	Ng Pos	Ng	No type
NG03	31y, M	Urine	ND	R- 6 mm	Ng	Ng	Ng	Ng Pos	Ng Pos	Ng	No type
NG04	29y, M	Urine	ND	R- 6 mm	Ng	Ng	Ng	Ng Pos	Ng Pos	Ng	No type
NG05	23y, M	Urine	ND	R- 6 mm	Ng	Ng	Ng	Ng Pos	Ng Pos	Ng	No type

531 Abbreviations: M- male, F- female, y- years, Sp- sputum, TA- tracheal aspirate, BAL- bronchoalveolar lavage,
532 BW- bronchial wash, Nm- *Neisseria meningitis*, Ng- *Neisseria gonorrhoeae*, Np- *Neisseria polysaccharea*, Mo-

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533 *Moraxella osloensis,* ND- not done, N/A, not applicable, R- resistant, S- susceptible, Pos- positive, Neg-
534 negative for all targets.
535 *Serogroup determination or confirmatory testing performed by Missouri State Public health Laboratory
536 (MSPHL) Jefferson City, MO.
537 † Confirmed by repeat testing in remnant urine matrix.
538
539
540 **Figure 1.**

541 **Identification of clinical *N. meningitidis* (Nm) and *N. gonorrhoeae* (Ng) by ANI analysis.** All Nm and Ng
542 isolates from urogenital samples in St. Louis (STL) were characterized by pairwise ANI with all other isolates
543 from STL and a subset of meningitis and ST-11 meningitis genomes. Isolates cluster within 96% ANI for all but
544 one Ng and Nm isolates. A single isolate (NM12) from a respiratory sample did not fall above a 96% cut off of
545 either Nm or Ng type strain. Color strips indicate source of isolate, clonal complex identified my PubMLST,
546 and Aptima test results.
547
548 **Figure 2**

549 **Urogenital Nm isolates primarily fall within a single, highly related clade.** An approximate maximum
550 likelihood tree of core genome alignment of St. Louis and select *Neisseria* isolates with tree branch lengths >
551 0.0001 shown. Two urogenital isolates collected from St. Louis fall outside of this clade and are distantly
552 related to the ST-11 clonal complex. All isolates that tested positive for Ng fall into a clade with other
553 urogenital Nm isolates. Source is indicated by color of tip, and Aptima test results are indicated by shape of tip.
554
555 **Figure 3**

556 **Urogenital Nm isolates primarily form a single SNP pairwise distance-based grouping**. A) Histogram of
557 pairwise SNP distances indicate three modes of pairwise distances. The first corresponds to within clade, the
558 second to within species, and the third to between species. We define variant threshold as variant pairwise
559 distances that fall before 2000 (black line). B) Groupings are visualized on Nm core genome phylogeny. Group

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560 1 includes 8 of 10 urogenital isolates. Group 2 includes a respiratory and a urogenital isolate. Source is
561 indicated by color of tip, and Aptima test results are indicated by shape of tip.

562

563 **Figure 4.**

564 **Antibiotic susceptibility profile varies between Ng isolates and urogenital Nm isolates.** A) Heatmap of AST
565 profiles for each isolate shows Nm and Ng isolates organized by 16S phylogenetic gene tree, with major
566 differences being that Nm isolates are resistant to azithromycin and intermediate to penicillin, while Ng isolates
567 are largely susceptible to both antibiotics. NM12 isolate has a distinct antibiotic susceptibility profile that varies
568 from both Ng and Nm isolates. Color strips indicate source of isolate, species identification by ANI, and SNP
569 pairwise distance based grouping. B) Distributions of MIC and zone diameter for Nm urogenital isolates and Ng
570 respiratory isolates for azithromycin, rifampin, ciprofloxacin, and trimethoprim-sulfamethoxazole.

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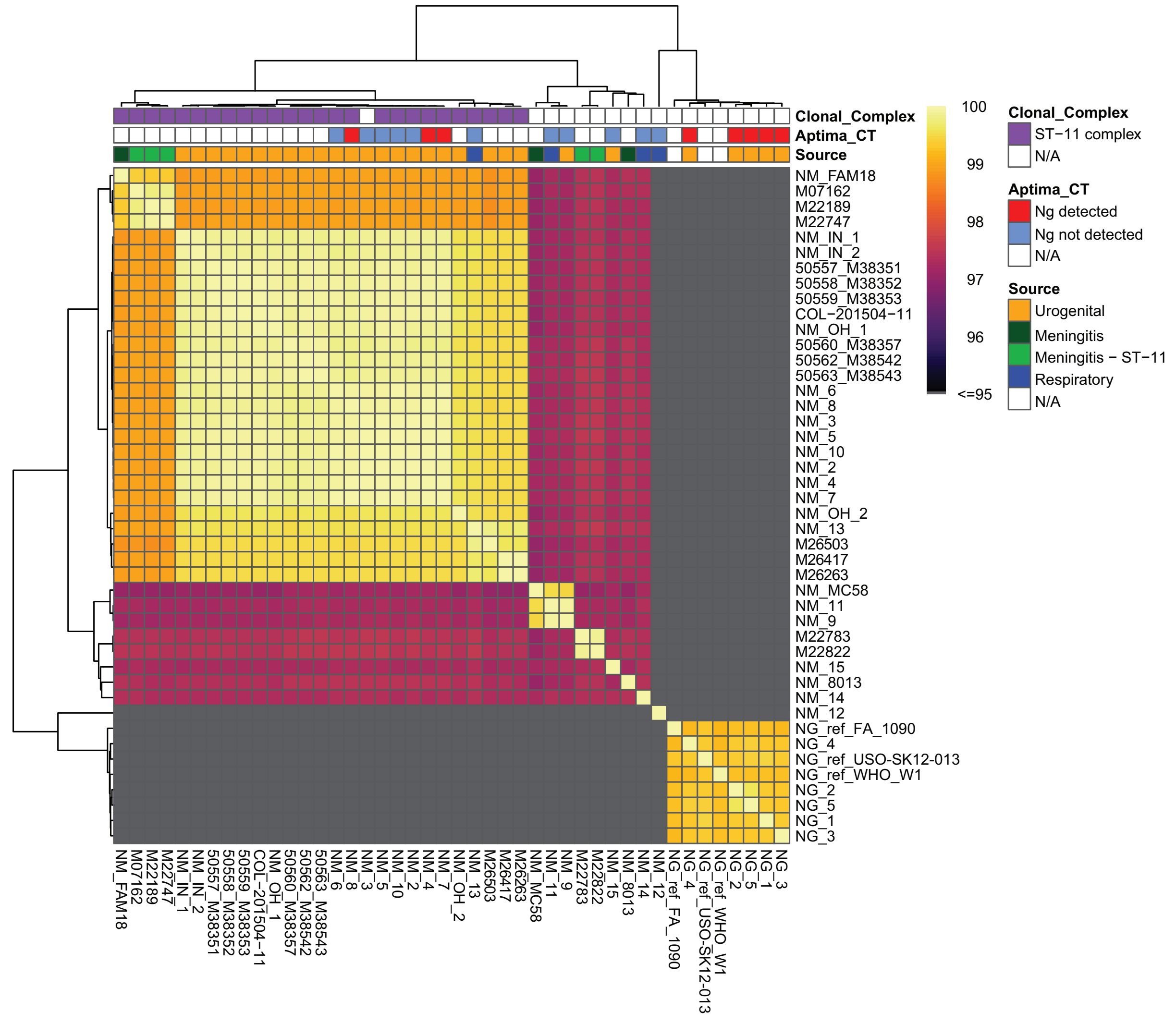

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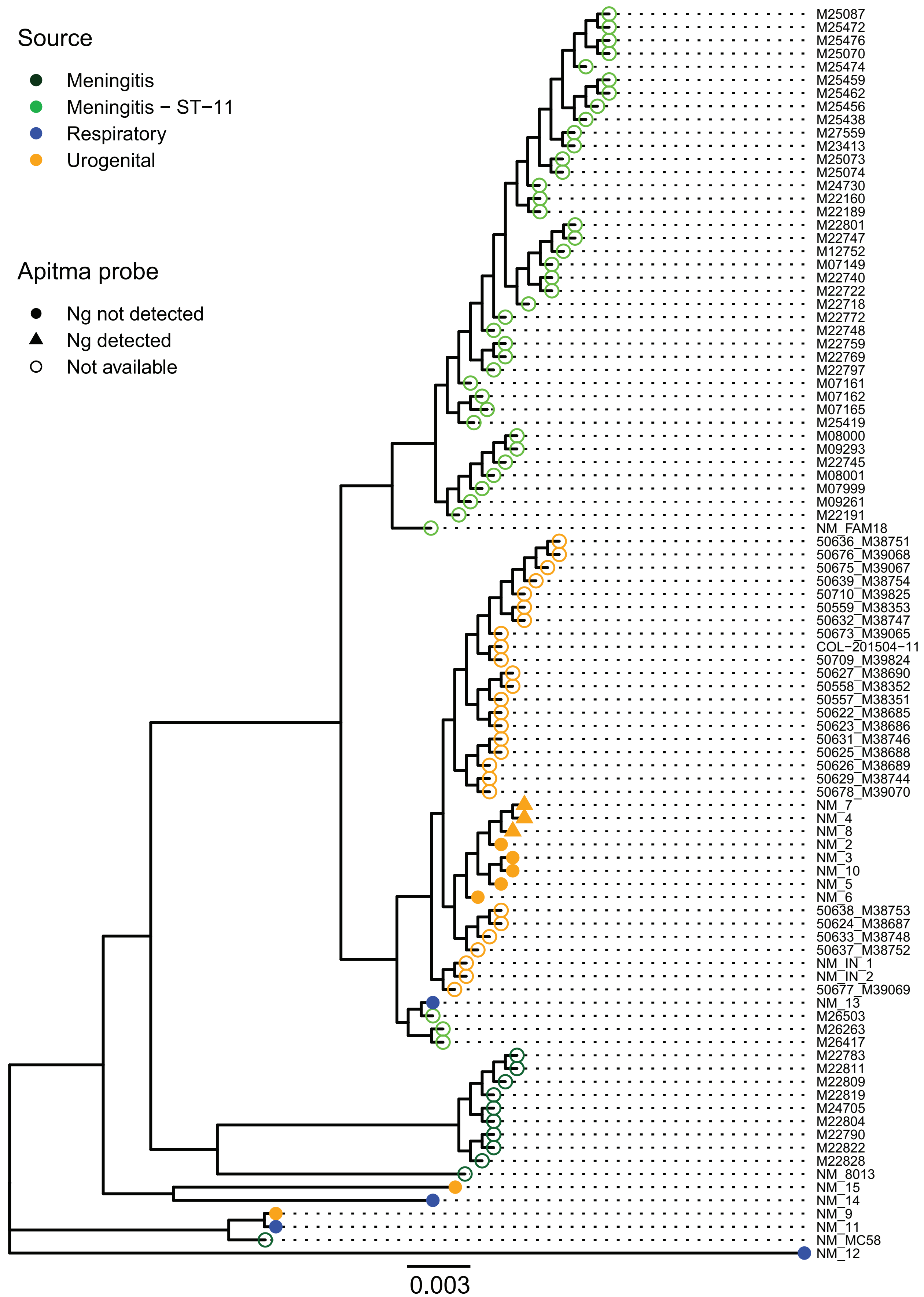

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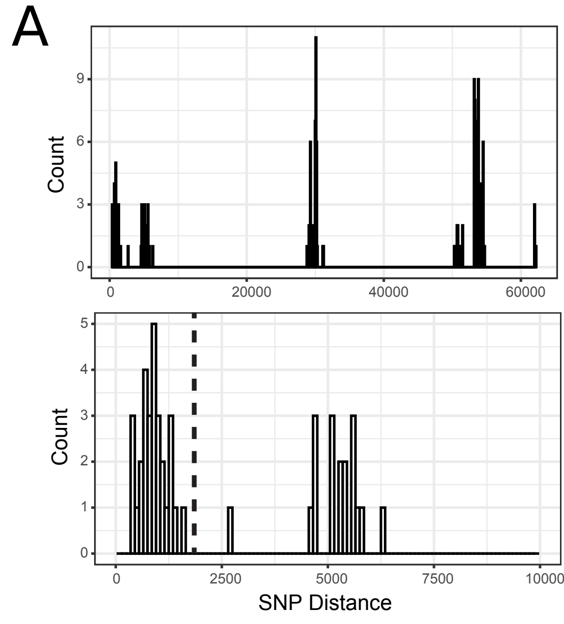

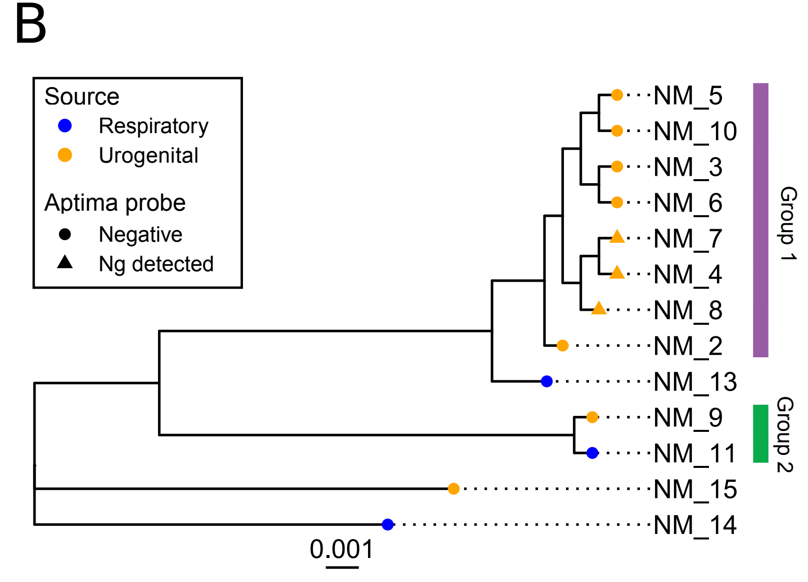

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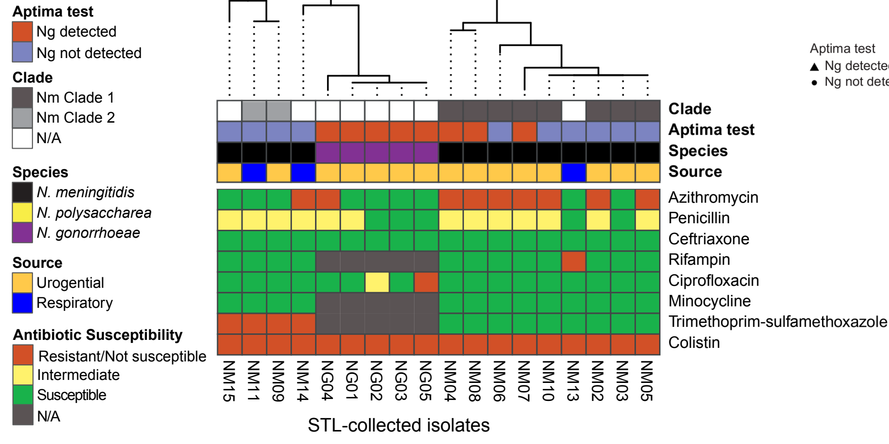

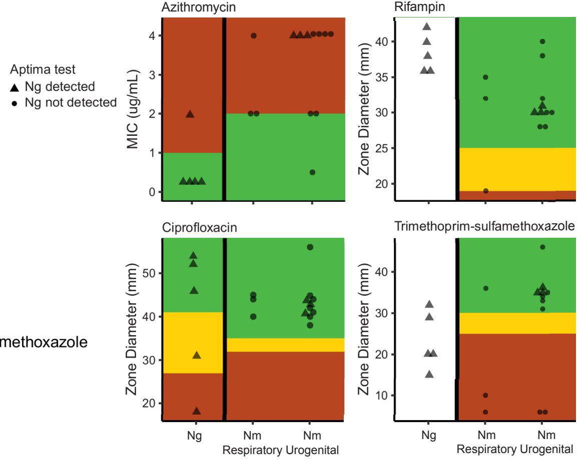

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