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

Kimberley V. Sukhum1,2,* Sophonie Jean2,3,* Meghan Wallace2, Neil Anderson2, Carey-Ann D. Burnham2,4,5,6, Gautam Dantas1,2,6,7#

1 The Edison Family Center for Genome Sciences and Systems Biology, Washington University School of Medicine in St Louis, St Louis, MO, USA
2 Department of Pathology and Immunology, Washington University School of Medicine in St Louis, St Louis, MO, USA
3 Department of Pathology & Laboratory Medicine, Nationwide Children’s Hospital, Columbus, OH, USA
4 Department of Medicine, Washington University School of Medicine in St Louis, St Louis, MO, USA
5 Department of Pediatrics, Washington University School of Medicine in St Louis, St Louis, MO, USA
6 Department of Molecular Microbiology, Washington University School of Medicine in St Louis, St Louis, MO, USA
7 Department of Biomedical Engineering, Washington University in St Louis, St Louis, MO, USA

*These authors contributed equally to this work. KVS is listed before SJ, as KVS did primary organizing and submission of manuscript during drafting phases.

#Corresponding authors: Carey-Ann Burnham cburnham@wustl.edu; Gautam Dantas dantas@wustl.edu

Running head: *Neisseria meningitidis* from urine

Keywords: *Neisseria*, urethritis, whole genome sequencing, antibiotic resistance

Submission: J Clin Microbiol

Word count: Abstract—249, Manuscript—4344
Abstract

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

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

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

The genus of *Neisseria* has evolved mechanisms that result in a high frequency of horizontal gene transfer (HGT), both within and between species, with up to 10% of the Nm genome made up of mobile genetic elements (9, 10). Co-localization of Nm and Ng in the urogenital system may result in increased transfer of virulence or antibiotic resistance genes (11). Recent studies have identified a strain of Nm that has been isolated from urogenital system and is associated with urethritis (12-16). As this strain becomes a more prominent emerging pathogen in areas with high Ng infection rates, it is essential to assess the ability of identification tools to discriminate between Nm and Ng urogenital isolates (13, 14, 17).
Following implementation of a total laboratory automation system for culture-based microbiology (BD Kiestra TLA; Beckton Dickinson), the clinical microbiology laboratory at Barnes Jewish Hospital in St. Louis MO observed significant increases in the recovery of \(N.\ gonorrhoeae\) and, more recently, \(N.\ meningitidis\) incidentally from urine specimens submitted for routine culture-based testing (18). In this study, we characterize consecutively recovered Nm and compare them to urethritis and Nm invasive strains reported elsewhere.

**Materials and Methods**

*Clinical isolates and human studies approval*

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

*Laboratory characterization*

Frozen Nm and Ng isolates were sub-cultured to chocolate (CHC) agar (Hardy Diagnostics, Santa Maria CA), incubated at 35°C and 5% \(\text{CO}_2\) and passaged twice prior to additional testing. For phenotypic characterization, 10 \(\mu\)L of a 0.5 McFarland (McF) suspension of each isolate was cross-struck to CHC and Modified Thayer-Martin (MTM) (Hardy Diagnostics, Santa Maria, CA) to achieve less subjective interpretation and quadrant struck to a third CHC plate with 10 \(\mu\)g colistin disk (BD BBL™, Sparks, MD). Following incubation at 35°C and 5% \(\text{CO}_2\) for 18-20 h, colony-forming units were enumerated and colistin zone size to the nearest millimeter was recorded. For biochemical characterization, the RapidID™ NH system (Remel, Lenexa, KS) was used per manufacturer’s instructions. Briefly, biochemical strips were inoculated with 3 McF
suspensions of each isolate and incubated at 35°C for 4 h in air. Following incubation, biochemical reactions were read, scored, and microcodes interpreted by the ERIC™ system to obtain organism identifications. For molecular characterization with Matrix-Assisted-Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-ToF MS), 2 commercially available systems were utilized: Bruker BioTyper (Bruker, Billerica, MA) and VITEK MS (bioMérieux, Durham, NC). Briefly, single colonies of pure growth were spotted to target slides and overlaid with matrix prior to analysis on each instrument per manufacturer’s instructions. For Nm isolates, target slides were spotted with organism and matrix and fully dried inside a BSC before removal for loading onto the MALDI-ToF MS instrument. For analysis with commercial in vitro diagnostic (IVD) nucleic acid amplification tests (NAATs), both contrived swab and urine specimens of Nm and Ng isolates were tested to confirm the lack of matrix-specific effects. Swabs from Aptima Vaginal and Xpert Vaginal/Endocervical Specimen collection kits were inserted into a 0.5 McF suspension of each isolate for 10 s diluting or 0.5 McF isolate suspensions was diluted10-fold with remnant urine specimens previously determined to be negative for Chlamydia trachomatis and Neisseria gonorrhoeae. Both contrived swab and urine specimens were tested with the Aptima Combo 2 CT/NG (A-CT/NG) on the Tigris GTS system (Hologic, Inc., San Diego, CA) and the Xpert CT/NG (X-CT/NG) assay on the GeneXpert Infinity system (Cepheid, Sunnyvale, CA) per manufacturer’s instructions.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) of Nm and Ng isolates was performed by disk diffusion and gradient diffusion strips and MIC and/or disk diffusion zone size interpreted according to the Clinical & Laboratory Standards Institute (CLSI) M100 29th edition (19). For Nm isolates, 0.5 McF suspensions of test isolates were inoculated to Mueller Hinton Agar with 5% sheep blood and incubated with penicillin, azithromycin, ceftaxone ETESTs (bioMérieux, Durham, NC) and ciprofloxacin (5 µg), rifampin (5 µg), minocycline (30 µg) and trimethoprim-sulfamethoxazole disks (1.25/23.75 µg) (BD BBL™, Sparks, MD) at 35°C and 5% CO₂, for 20-24 h. For Ng isolates, 0.5 McF suspensions of test isolates were inoculated to GC
agar base with 1% defined growth supplement and incubated with penicillin, azithromycin, ceftriaxone Etests (bioMérieux, Durham, NC) and ciprofloxacin (5µg) and rifampin (5µg) disks (BD BBL™, Sparks, MD) at 35°C and 5% CO₂, for 20-24 h. MIC doubling dilutions and zone sizes to the nearest millimeter were read with reflected light and interpreted per CLSI M100 29th edition guidelines (19). AST categorical results were visualized using pheatmap (R) with color strips used to indicate source of isolate, species, and SNP pairwise distance-based grouping.

Whole genome sequencing

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

Data availability

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

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

Phylogenetic analysis

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

Isolate groupings based on SNP pairwise distances

Snippy v4.3.8 was used to map forward and reserve reads for Nm isolates to the Nm MC58 type strain complete genome assembly (ID) and to call SNPs (36). To determine groupings, we compared pairwise SNP
distances between each Nm isolate pair. Isolates were grouped into perfectly reciprocal groups at every pairwise
distance cutoff between Nm isolates using igraph v1.2.4.1 as described previously (37). Groupings are
visualized with a SNP cutoff of 2000.

*Antibiotic resistance mutations identification and analysis*

Targeted analysis of acquired antibiotic resistance mutations against β-lactams (*bla*TEM, *penA*, *porA*,
*ponA*, *mtrR*), macrolides/lincosamides/streptogramins (*23S rRNA*, *mtrR*) and quinolones (*gyrA*, *parC*) was
performed as a result of phenotypic AST findings using PointFinder (38). The presence/absence matrix of
ARGs was visualized in pheatmap (R). Associated meta-data was displayed as a color strip to represent
bacterial isolate identification and Aptima CT results. We further validated PointFinder results for key
resistance gene mutations using BLASTn with MUSCLE alignment and maximum likelihood tree visualization
(Figure S3).

**Results**

*Nm urinary isolates can cause false-positive Ng molecular test result*

Consecutive Nm isolates and a random selection of Ng isolates recovered from clinical specimens
during the same time period were characterized by phenotypic and molecular methods routinely used to identify
*Neisseria* species in clinical microbiology laboratories including MALDI-ToF MS, and commercial
biochemical and molecular tests. Detailed demographic information was not available for these isolates but
limited information including patient age, gender, and isolate source are summarized in Table 1. All urogenital
Nm and Ng isolates were correctly identified using Bruker Biotyper and VITEK MS MALDI-ToF MS
platforms (Table 1). One *Neisseria* isolate from a respiratory source (NM12) was incorrectly identified using
MALDI-ToF MS and biochemical tests as Nm (Bruker Biotyper) or *N. polysacchareal/N. meningitidis* (VITEK
MS) and Ng (RapidID NH). However, this isolate was phenotypically consistent with non-pathogenic *Neisseria*
species with no growth on MTM media and a zone of inhibition when incubated with a 10 µg colistin disk on solid media, and was identified using WGS methods as *N. polysaccharea*.

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

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

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

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

Ribosomal RNA (rRNA) classification is used in the APTIMA COMBO2 assay, with the specific loci being proprietary, (41) and in 16S rRNA gene sequence classification to determine bacterial operational taxonomic unit (OTU) or amplicon sequence variant (ASV) (28). Thus, we classified and compared 16S rRNA gene sequences across *Neisseria* isolates. For all STL-collected isolates, 16S rRNA gene sequences were submitted to EZ BIoCloud taxonomic database for classification (28). 16S rRNA gene classification correlated
with MLST for all isolates (Table 1). An approximate maximum likelihood tree with NM12 as the outgroup shows Ng sequences form a monophyletic clade distinct from Nm sequences with Nm that tested positive for Ng using the A-CT/NG falling within the Nm sequences (Figure S1).

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

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

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

Nm urinary isolates are highly related to other urogenital isolates and not respiratory isolates.

SNP distance across whole genomes have been found to provide higher resolution of phylogenetic distances than core genome comparisons (37). Thus, to further investigate genomic similarity of STL-collected Nm isolates, we calculated pairwise SNP distances by mapping quality filtered reads from Nm isolates to the Nm type strain. To find groupings, we used a grouping technique, “clique” (37) on STL-collected Nm isolates. We compared pairwise SNP distances between Nm isolate pairs and iterated through each unique SNP distance cutoff to filter the isolate pairwise network list (Figure 3a). For each cutoff, we found reciprocal groups and recorded the number of groups and isolates per group. Then groups were defined as complete subgraphs, where each node in the group was connected to every other node in the group. Number of Nm groups rose initially from 1 to 3 groups as SNP distances increased from 357 to 6269. Only a single SNP distance of 5624 SNPs had 4 groups, and immediately after this peak, groups decreased again to 3 with a decline in group size to 1 after 20,000 SNPs. Figure 3a shows the groups, which corresponds to a SNP cutoff that includes only highly related Nm isolates with less than 2000 SNP distances.

Urogenital Nm isolates primarily formed a single grouping (Figure 3b). The first grouping includes 8/10 urogenital isolates and all ST-11 urethritis isolates. This grouping fell entirely with the ST-11 urogenital clade described in the core genome phylogeny (Figure 2). A second grouping included a respiratory (NM11) and a urogenital isolate (NM09). These groupings suggest that while ST-11 urethritis isolates are highly related with between 9.5-9.6x10^4 pairwise SNPs/genome length, not all urogenital isolates fall into the grouping, and one isolate shares high similarity (4.8x10^4 pairwise SNPs/genome length) with a respiratory isolate.

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

**Discussion**

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

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

Accurate identification of *Neisseria* isolates is important as AST profiles vary between Ng and Nm isolates. Though rising rates of reduced susceptibility to azithromycin in Ng has been reported across the US (43), we found that the Ng isolates test in our study were primarily susceptible to azithromycin (n=4/5), while urogenital Nm isolates collected over the same time period as Ng isolates were more likely to be azithromycin non-susceptible (n= 7/10). Our observation of largely azithromycin-susceptible Ng is consistent with a previous study of a larger cohort of Ng isolates recovered from the same institution that reported that azithromycin non-susceptibility was rare (< 2%) (44). Interestingly, all Nm isolates that tested falsely-positive for Ng were azithromycin non-susceptible (n= 3/3). Currently single dose of intramuscular ceftriaxone (250mg) plus a single dose of oral azithromycin (1g) is the primary treatment recommendation for uncomplicated gonococcal infection (45). Although dual-therapy was primarily aimed at treatment of uncomplicated chlamydial co-
infections, routine combination therapy may hinder development of antimicrobial resistance in Ng particularly in light of increased cephalosporin resistance in the US (46). Given the rare reports of ceftriaxone-resistant Nm (47-49), ceftriaxone plus azithromycin dual therapy is likely effective for treatment of urogenital Nm infection. However, if azithromycin non-susceptibility is common among urogenital Nm isolates and in the setting of reduced susceptibility to 3rd-generation cephalosporins, current gonococcal treatment guidelines may be suboptimal for urogenital Nm infection. Interestingly, despite recent reports of ciprofloxacin-resistant, beta-lactamase-producing Nm serogroup Y (50), all of the Nm isolates evaluated in this study were ciprofloxacin and ceftriaxone susceptible (n=13/13) though most were non-susceptible to penicillin (n= 10/13); beta-lactamase testing was not performed.

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

Studies have indicated that invasive Nm isolates from MSM are associated with colonization of the urethra or rectum (2), and that Nm urethritis outbreaks are closely related to cases of invasive Nm in MSM populations, suggesting that urethral colonization may contribute to invasive disease (11). In our study, the sister clade to all ST-11 urethritis isolates included one respiratory isolate and three meningitis-associated isolates from MSM patients. This phylogeny suggests a common ancestor between MSM meningitidis and the origin of urogenital Nm isolates. However, as this study is focused on urogenital Nm isolates, only a subset of...
54 Nm meningitis isolates were used for comparison with a focus on Nm isolates within ST-11. A more exhaustive study of Nm meningitis isolates may find additional clades related to the urogenital NM isolates. Further, in this data set, we do not see evidence for closely related urethritis and invasive Nm isolates. It is possible that increasing the collection and analysis of invasive and urogenital Nm isolates from meningitis patients may expand further on this issue.

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

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

Acknowledgements
This work was supported in part by awards to G.D. through the National Institute of Allergy and Infectious Diseases and the Eunice Kennedy Shriver National Institute of Child Health & Human Development of the National Institutes of Health (NIH) under award numbers R01AI123394 and R01HD092414, respectively. K.V.S is supported by the Society for Healthcare Epidemiology of America Research Scholar Award. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies. The authors thank the Edison Family Center for Genome Sciences & Systems Biology at WUSM staff, Eric Martin, Brian Koebbe, Jessica Hoisington-López, and MariaLynn Crosby for technical support in high-throughput sequencing and computing.
References


36. Seemann T. Snippy: Rapid haploid variant calling and core genome alignment.


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

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

---

Abbreviations: M- male, F- female, y- years, Sp- sputum, TA- tracheal aspirate, BAL- bronchoalveolar lavage, BW- bronchial wash, Nm- *Neisseria meningitidis*, Ng- *Neisseria gonorrhoeae*, Np- *Neisseria polysaccharea*, Mo-
Moraxella osloensis, ND- not done, N/A, not applicable, R- resistant, S- susceptible, Pos- positive, Neg- negative for all targets.

*Serogroup determination or confirmatory testing performed by Missouri State Public health Laboratory (MSPHL) Jefferson City, MO.
† Confirmed by repeat testing in remnant urine matrix.

Figure 1.

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

Figure 2

Urogenital Nm isolates primarily fall within a single, highly related clade. An approximate maximum likelihood tree of core genome alignment of St. Louis and select Neisseria isolates with tree branch lengths > 0.001 shown. Two urogenital isolates collected from St. Louis fall outside of this clade and are distantly related to the ST-11 clonal complex. All isolates that tested positive for Ng fall into a clade with other urogenital Nm isolates. Source is indicated by color of tip, and Aptima test results are indicated by shape of tip.

Figure 3

Urogenital Nm isolates primarily form a single SNP pairwise distance-based grouping. A) Histogram of pairwise SNP distances indicate three modes of pairwise distances. The first corresponds to within clade, the second to within species, and the third to between species. We define variant threshold as variant pairwise distances that fall before 2000 (black line). B) Groupings are visualized on Nm core genome phylogeny. Group
1 includes 8 of 10 urogenital isolates. Group 2 includes a respiratory and a urogenital isolate. Source is indicated by color of tip, and Aptima test results are indicated by shape of tip.

**Figure 4.**

**Antibiotic susceptibility profile varies between Ng isolates and urogenital Nm isolates.** A) Heatmap of AST profiles for each isolate shows Nm and Ng isolates organized by 16S phylogenetic gene tree, with major differences being that Nm isolates are resistant to azithromycin and intermediate to penicillin, while Ng isolates are largely susceptible to both antibiotics. NM12 isolate has a distinct antibiotic susceptibility profile that varies from both Ng and Nm isolates. Color strips indicate source of isolate, species identification by ANI, and SNP pairwise distance based grouping. B) Distributions of MIC and zone diameter for Nm urogenital isolates and Ng respiratory isolates for azithromycin, rifampin, ciprofloxacin, and trimethoprim-sulfamethoxazole.
<table>
<thead>
<tr>
<th>Clade</th>
<th>Aptima test</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nm Clade 1</td>
<td>-</td>
<td>N. meningitidis</td>
<td>Urogenital</td>
</tr>
<tr>
<td>Nm Clade 2</td>
<td>-</td>
<td>N. polysaccharea</td>
<td>Respiratory</td>
</tr>
<tr>
<td>N/A</td>
<td>-</td>
<td>N. gonorrhoeae</td>
<td>-</td>
</tr>
</tbody>
</table>

### Antibiotic Susceptibility

- **Rifampin**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A

- **Ciprofloxacin**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A

- **Trimethoprim-sulfamethoxazole**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A

- **Azithromycin**
  - MIC (µg/mL)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible

- **Penicillin**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A

- **Ceftriaxone**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A

- **Rifampin**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A

- **Ciprofloxacin**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A

- **Trimethoprim-sulfamethoxazole**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A

- **Azithromycin**
  - MIC (µg/mL)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible

- **Penicillin**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A

- **Ceftriaxone**
  - Zone Diameter (mm)
  - Resistant/Not susceptible
  - Intermediate
  - Susceptible
  - N/A