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Kimberley V Sukhum

Washington University School of Medicine in St. Louis

Sophonie Jean

Washington University School of Medicine in St. Louis

Meghan Wallace

Washington University School of Medicine in St. Louis

Neil Anderson

Washington University School of Medicine in St. Louis

C A Burnham

Washington University School of Medicine in St. Louis

See next page for additional authors

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Authors

Kimberley V Sukhum, Sophonie Jean, Meghan Wallace, Neil Anderson, C A Burnham, and Gautam Dantas

Genomic Characterization of Emerging Bacterial Uropathogen *Neisseria meningitidis*, Which Was Misidentified as *Neisseria gonorrhoeae* by Nucleic Acid Amplification Testing

Kimberley V. Sukhum,^{a,b} Sophonie Jean,^{b,c*} Meghan Wallace,^b Neil Anderson,^b Carey-Ann D. Burnham,^{b,d,e,f} Gautam Dantas^{a,b,f,g}

^aThe Edison Family Center for Genome Sciences and Systems Biology, Washington University School of Medicine in St. Louis, St. Louis, Missouri, USA

^bDepartment of Pathology and Immunology, Washington University School of Medicine in St. Louis, St. Louis, Missouri, USA

^cDepartment of Pathology & Laboratory Medicine, Nationwide Children's Hospital, Columbus, Ohio, USA

^dDepartment of Medicine, Washington University School of Medicine in St. Louis, St. Louis, Missouri, USA

^eDepartment of Pediatrics, Washington University School of Medicine in St. Louis, St. Louis, Missouri, USA

^fDepartment of Molecular Microbiology, Washington University School of Medicine in St. Louis, St. Louis, Missouri, USA

^gDepartment of Biomedical Engineering, Washington University in St. Louis, St. Louis, Missouri, USA

Kimberley V. Sukhum and Sophonie Jean contributed equally to this work. Kimberley V. Sukhum is listed before Sophonie Jean, as Kimberley V. Sukhum did primary organizing and submission of the manuscript during drafting phases.

ABSTRACT *Neisseria meningitidis* and *Neisseria gonorrhoeae* are pathogenic bacteria that can cause human infections. While *N. meningitidis* infections are associated with bacterial meningitis and bacteremia, a strain of *N. meningitidis*, 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 *N. meningitidis* and *N. gonorrhoeae* urogenital isolates. Consecutive *N. meningitidis* isolates recovered from urogenital cultures of symptomatic patients with presumptive diagnoses of gonorrhea and a random selection of *N. gonorrhoeae* isolates recovered from the same population within the same time frame were characterized with routine identification systems, antimicrobial susceptibility testing, and whole-genome sequencing. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), multilocus sequence typing, 16S rRNA gene sequence, and average nucleotide identity methods accurately identified 95% (18/19) of *N. meningitidis* and *N. gonorrhoeae* isolates. With the Aptima Combo 2 CT/NG test, 30% (3/10) of *N. meningitidis* isolates were misidentified as *N. gonorrhoeae*, but no misidentifications were found with the Xpert CT/NG nucleic acid amplification test (NAAT). Phylogenetic core genome and single nucleotide polymorphism (SNP)-based grouping analyses showed that urogenital *N. meningitidis* isolates were highly related and phylogenetically distinct from *N. gonorrhoeae* and respiratory *N. meningitidis* isolates but similar to urogenital *N. meningitidis* isolates from patients with urethritis in the United States. Urogenital *N. meningitidis* isolates were predominantly azithromycin resistant, while *N. gonorrhoeae* isolates were azithromycin susceptible. These data indicate that urogenital isolates of *N. meningitidis* can cause false-positive detections with *N. gonorrhoeae* diagnostic assays. Misidentification of urogenital *N. meningitidis* isolates may confound public health-related activities for gonorrhea, and future studies are needed to understand the impact on clinical outcome of *N. meningitidis* urogenital infection.

KEYWORDS *Neisseria*, urethritis, whole-genome sequencing, antibiotic resistance

Neisseria gonorrhoeae and *Neisseria meningitidis* are human bacterial pathogens that can occupy different niches in the body (1, 2). *N. gonorrhoeae* is the causative

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Address correspondence to Carey-Ann D. Burnham, cburnham@wustl.edu, or Gautam Dantas, dantas@wustl.edu.

*Present address: Sophonie Jean, Department of Pathology & Laboratory Medicine, Nationwide Children's Hospital, Columbus, Ohio, USA.

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agent of the sexually transmitted infection gonorrhea, which impacts 78 million people worldwide (3). As an obligate pathogen, *N. gonorrhoeae* primarily colonizes the genital mucosa and has evolved virulence factors that allow it to survive and evade the host immune system (1). Recently, *N. gonorrhoeae* has received increased public health attention, and drug-resistant *N. gonorrhoeae* has been categorized as an urgent threat by the U.S. 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 *N. gonorrhoeae* in clinical specimens (7). While the development of these assays is important for patient treatment, there is evidence for sporadic false-positive molecular results due to cross-reactivity between *Neisseria* species (8).

N. meningitidis is found as a commensal in the respiratory system, and ~10% of healthy adults and 40% of men who have sex with men (MSM) demonstrate naso/oropharyngeal carriage (2). *N. meningitidis* 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 *N. meningitidis* 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 bloodstream (9).

The genus *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 *N. meningitidis* genome made up of mobile genetic elements (9, 10). Colocalization of *N. meningitidis* and *N. gonorrhoeae* in the urogenital system may result in increased transfer of virulence or antibiotic resistance genes (11). Recent studies have identified a strain of *N. meningitidis* that has been isolated from the urogenital system and is associated with urethritis (12–16). As this strain becomes a more prominent emerging pathogen in areas with high *N. gonorrhoeae* infection rates, it is essential to assess the ability of identification tools to discriminate between *N. meningitidis* and *N. gonorrhoeae* urogenital isolates (13, 14, 17).

Following the implementation of a total laboratory automation system for culture-based microbiology (BD Kiestra total laboratory automation (TLA) system; 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 *N. meningitidis* and compare them to *N. meningitidis* urethritis and invasive strains reported elsewhere.

MATERIALS AND METHODS

Clinical isolates and human study approval. Consecutive *Neisseria meningitidis* and a random selection of *Neisseria gonorrhoeae* 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 found increased recovery of *N. gonorrhoeae* and *N. meningitidis* isolates incidentally from urine cultures submitted for routine testing when incubated with the Kiestra total laboratory automation (TLA) (18). Study isolates were deidentified, 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 *N. meningitidis* and *N. gonorrhoeae* isolates were subcultured to chocolate (CHC) agar (Hardy Diagnostics, Santa Maria, CA), incubated at 35°C and 5% CO₂, and passaged twice prior to additional testing. For phenotypic characterization, 10 µl 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 was quadrant struck to a third CHC plate with 10 µg colistin disk (BD BBL, Sparks, MD). Following incubation at 35°C and 5% CO₂ for 18 to 20 h, CFU were enumerated and the colistin zone size to the nearest millimeter was recorded. For biochemical characterization, the RapidID NH system (Remel, Lenexa, KS) was used per the 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 and scored, and microcodes were interpreted using 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 the manufacturer's instructions. For *N. meningitidis* isolates, target slides were spotted with organism and matrix and fully dried inside a biosafety cabinet (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 *N. meningitidis* and *N. gonorrhoeae* 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, or 0.5-McF isolate suspensions were diluted 10-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 the manufacturer's instructions.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing (AST) of *N. meningitidis* and *N. gonorrhoeae* isolates was performed using disk diffusion and gradient diffusion strips, and the MIC and/or disk diffusion zone size was interpreted according to the Clinical and Laboratory Standards Institute (CLSI) M100, 29th edition (19). For *N. meningitidis* isolates, 0.5 McF suspensions of test isolates were inoculated to Mueller-Hinton Agar with 5% sheep blood and incubated with penicillin, azithromycin, ceftriaxone 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 *N. gonorrhoeae* 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 (5 µg) and rifampin (5 µg) disks (BD BBL, Sparks, MD) at 35°C and 5% CO₂, for 20 to 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 pheamap (R) with color strips used to indicate the source of the 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 the DNA concentration using Qubit double-stranded DNA (dsDNA) assays (Thermo Fisher 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 high-output platform (Illumina) to obtain ~2 million 2 × 150-bp reads. The reads were demultiplexed by barcode, adapters were 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). The quality of draft genomes was assessed using QUAST v.4.5 and CheckM (23, 24). Assemblies were considered to have passed quality control when the assembly length represented in contigs of <1 kb was less than 10%, the number of contigs greater than 500 bp was less than 5,000, completeness was greater than 90%, and contaminated reads were less than 5%. Draft genomes were annotated using Prokka v.1.12 (25).

Genomic taxonomic identification. Following draft genome assembly, we determined genomic taxonomic identification by average nucleotide identity (ANI), 16S rRNA gene identification, and multilocus sequence typing (MLST). The assembled scaffolds were submitted to the *Neisseria* multilocus sequence typing website (<https://pubmlst.org/neisseria/>) to determine MLST and clonal complex (26). For all isolates, 16S rRNA gene sequences were identified using RNAmmer v.1.2 and submitted to the EZ BioCloud taxonomic database for classification (27, 28). Using ANI analysis, species were determined if the genome in question had >95% ANI with the type genome (*N. meningitidis*, NM_MC58; *N. gonorrhoeae*, NG_ref_FA_1090) using dnadiff (29). Pairwise ANI for each isolate was clustered and visualized using pheamap (R) (30).

Phylogenetic analysis. To phylogenetically compare isolate sequences, 16S rRNA gene sequences identified by RNAmmer 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 v.3.8.0 for *N. meningitidis* (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 v.4.3.8 was used to map forward and reserve reads for *N. meningitidis* isolates to the *N. meningitidis* MC58 type strain complete genome assembly (ID) and to call SNPs (36). To determine groupings, we compared pairwise SNP distances between each *N. meningitidis* isolate pair. Isolates were grouped into perfectly reciprocal groups at every pairwise distance cutoff between *N. meningitidis* isolates using igraph v.1.2.4.1 as described previously (37). Groupings were visualized with a SNP cutoff of 2,000.

Antibiotic resistance mutation identification and analysis. Targeted analysis of acquired antibiotic resistance genes/mutations (ARGs) 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 pheamap (R). Associated metadata were displayed as a color strip to represent bacterial isolate

TABLE 1 *Neisseria meningitidis* and *Neisseria gonorrhoeae* isolate characterization^a

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

^aAbbreviations: M, male; F, female; 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; NA, not applicable; R, resistant; S, susceptible; Pos, positive; Neg, negative for all targets.

^bSerogroup determination or confirmatory testing performed by Missouri State Public Health Laboratory (MSPHL) Jefferson City, MO.

^cConfirmed by repeat testing in remnant urine matrix.

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 (see Fig. S3 in the supplemental material).

Data availability. All assemblies are uploaded to the NCBI under BioProject [PRJNA643774](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA643774).

RESULTS

***N. meningitidis* urinary isolates can cause false-positive *N. gonorrhoeae* molecular test result.** Consecutive *N. meningitidis* isolates and a random selection of *N. gonorrhoeae* 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 and gender, and isolate source is summarized in Table 1. All urogenital *N. meningitidis* and *N. gonorrhoeae* isolates were correctly identified using the 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 *N. meningitidis* (Bruker Biotyper) or *Neisseria polysaccharea*/*N. meningitidis* (Vitek MS) and *N. gonorrhoeae* (RapID NH). However, this isolate was phenotypically consistent with nonpathogenic *Neisseria* species with no growth on MTM medium and a zone of inhibition when incubated with a 10-µg colistin disk on solid medium and was identified using whole-genome sequencing (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 *N. gonorrhoeae* isolates were detected by both systems, while all *N. meningitidis* isolates were not detected by X-CT/NG. Importantly, urinary isolates of *N. meningitidis* (NM04, NM07, NM08) tested positive for *N. gonorrhoeae* with A-CT/NG.

This result was confirmed with remnant urine specimens spiked with NM04, NM07, NM08, NM09, NM12, and NM14 (Table 1).

Urogenital *N. meningitidis* classified as *N. meningitidis* 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), which MALDI-TOF MS was unable to classify to a single species, was characterized as *N. polysaccharea*. MLST clonal complex indicated that 7 of 10 STL-collected urogenital *N. meningitidis* isolates fell into the sequence type 11 (ST-11) clonal complex (Table 1).

To determine the phylogenetic context of isolates, we downloaded the following series of *N. meningitidis* genomes from NCBI and PubMLST: 28 *N. meningitidis* isolates from urinary tract infections (UTIs) in the United States (13, 14), 3 *N. meningitidis* ST-11 isolates from cases of meningitis in MSM in the United States (39), 29 *N. meningitidis* ST-11 isolates from a meningitis epidemic in Africa (40), and 8 *N. meningitidis* isolates from non-ST-11 meningitis cases (Table S1).

Ribosomal RNA (rRNA) classification is used in the Aptima Combo 2 assay, with the specific loci being proprietary (41), and in 16S rRNA gene sequence classification to determine the 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 the 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 *N. gonorrhoeae* sequences form a monophyletic clade distinct from *N. meningitidis* sequences with *N. meningitidis* that tested positive for *N. gonorrhoeae* using the A-CT/NG falling within the *N. meningitidis* sequences (Fig. 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 (Fig. 1). All *N. meningitidis* or *N. gonorrhoeae* isolates from urogenital samples that were identified by MALDI-TOF MS and MLST were also identified as *N. meningitidis* or *N. gonorrhoeae*, respectively, by ANI. NM12 did not fall above the cutoff for *N. meningitidis*, *N. gonorrhoeae*, or *N. polysaccharea* type strains. Pairwise ANI of all *N. meningitidis* isolates and select reference *N. meningitidis* genomes indicated that ST-11 isolates form a distinct cluster with ANI above 99% (Fig. 1). This cluster included all urogenital *N. meningitidis* isolates for which *N. gonorrhoeae* was detected by A-CT/NG and one *N. meningitidis* respiratory isolate, NM13. Thus, MALDI-TOF MS, MLST, 16S rRNA, and ANI agree on classification for 18 of 19 *Neisseria* isolates.

***N. meningitidis* urogenital isolates form a primary lineage that is distinct from ST-11 meningitis isolates.** To determine genomic similarity across *N. meningitidis* genomes, we used a core genome alignment of 1,057 genes at 95% identity of all *N. meningitidis* isolates, using NM12 as an outgroup. The phylogenetic tree of this alignment shows that urogenital *N. meningitidis* isolates primarily fall within a single lineage (Fig. 2). All (3 of 3) STL-collected *N. meningitidis* isolates for which the A-CT/NG test detected *N. gonorrhoeae* formed a single clade within STL-collected ST-11 urogenital *N. meningitidis* isolates, suggesting a recent common ancestor. Of the STL-collected urogenital *N. meningitidis* isolates, 8/10 cluster together and form a sister clade to other urogenital *N. meningitidis* isolates. This similarity suggests a single common ancestor for 93% (31 of 33) of urogenital *N. meningitidis* isolates. Two urogenital STL-collected *N. meningitidis* isolates, NM09 and NM15, did not cluster with other urogenital isolates, and NM09 was, instead, highly related to a respiratory *N. meningitidis* isolate. Both NM09 and NM15 isolates were nongroupable using serotyping methods, did not fall into the ST-11 clonal complex, and were misclassified by RapID NH-; NM15 had been misclassified as *N. gonorrhoeae* and NM09 was misclassified as *Moraxella osloensis*

FIG 1 Identification of clinical *N. meningitidis* (Nm) and *N. gonorrhoeae* (Ng) by ANI analysis. All *N. meningitidis* and *N. gonorrhoeae* 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 *N. gonorrhoeae* and *N. meningitidis* isolate. A single isolate (NM12) from a respiratory sample did not fall above a 96% cutoff for either *N. meningitidis* or *N. gonorrhoeae* type strain. Color strips indicate source of isolate, clonal complex identified by PubMLST, and Aptima test results.

(Table 1). ST-11 urethritis isolates were a sister clade to a lineage that included one STL-collected respiratory isolate and all 3 of the MSM meningitis isolates. This clade was a sister clade to the African ST-11 meningitis isolates. In contrast to *N. meningitidis* urinary isolates, *N. meningitidis* respiratory isolates were highly diverse and distantly related.

***N. meningitidis* urinary isolates are highly related to other urogenital isolates and not respiratory isolates.** SNP distances across whole genomes have been found to provide higher resolution of phylogenetic distances than core genome comparisons (37). Thus, to further investigate the genomic similarity of STL-collected *N. meningitidis* isolates, we calculated pairwise SNP distances by mapping quality-filtered reads from *N. meningitidis* isolates to the *N. meningitidis* type strain. To find groupings, we used a grouping technique, “clique” (37), on STL-collected *N. meningitidis* isolates. We compared pairwise SNP distances between *N. meningitidis* isolate pairs and iterated

FIG 2 Urogenital *N. meningitidis* 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 of >0.0001 is 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 *N. gonorrhoeae* fall into a clade with other urogenital *N. meningitidis* isolates. The source is indicated by color of the tip, and Aptima test results are indicated by the shape of tip. Ng, *N. gonorrhoeae*.

FIG 3 Urogenital *N. meningitidis* isolates primarily form a single SNP pairwise distance-based grouping. (A) Histogram of pairwise SNP distances indicates 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 2,000 (black line). (B) Groupings are visualized on the *N. meningitidis* core genome phylogeny. Group 1 includes 8 of 10 urogenital isolates. Group 2 includes a respiratory and a urogenital isolate. The source is indicated by color of the tip, and Aptima test results are indicated by the shape of the tip. Ng, *N. gonorrhoeae*.

through each unique SNP distance cutoff to filter the isolate pairwise network list (Fig. 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. The number of *N. meningitidis* groups rose initially from 1 to 3 groups as SNP distances increased from 357 to 6,269. Only a single SNP distance of 5,624 SNPs had 4 groups, and immediately after this peak, groups decreased again to 3, with a decline in group size to 1 after

FIG 4 The antibiotic susceptibility profile varies between *N. gonorrhoeae* isolates and urogenital *N. meningitidis* isolates. (A) The heatmap of AST profiles for each isolate shows *N. meningitidis* and *N. gonorrhoeae* isolates organized by 16S phylogenetic gene tree, with major differences being that *N. meningitidis* isolates are resistant to azithromycin and intermediate to penicillin, while *N. gonorrhoeae* isolates are largely susceptible to both antibiotics. Isolate NM12 has a distinct antibiotic susceptibility profile that varies from both *N. gonorrhoeae* and *N. meningitidis* isolates. Color strips indicate the source of isolate, species identification by ANI, and SNP pairwise distance-based grouping. (B) Distributions of MIC and zone diameter for *N. meningitidis* urogenital isolates and *N. gonorrhoeae* respiratory isolates for azithromycin, rifampin, ciprofloxacin, and trimethoprim-sulfamethoxazole. Ng, *N. gonorrhoeae*; Nm, *N. meningitidis*.

20,000 SNPs. Figure 3A shows the groups, which correspond to a SNP cutoff that includes only highly related *N. meningitidis* isolates with less than 2,000 SNP distances.

Urogenital *N. meningitidis* isolates primarily formed a single grouping (Fig. 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 (Fig. 2). A second grouping included a respiratory (NM11) and a urogenital (NM09) isolate. These groupings suggest that while ST-11 urethritis isolates are highly related with between 9.5 and 9.6×10^{-4} pairwise SNPs/genome length, not all urogenital isolates fall into the grouping, and one isolate shares high similarity (4.8×10^{-4} pairwise SNPs/genome length) with a respiratory isolate.

***N. meningitidis* isolates have an antibiotic susceptibility profile distinct from *N. gonorrhoeae* isolates.** To consider clinical implications of misidentified *N. meningitidis* 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 *N. gonorrhoeae* isolates and urogenital *N. meningitidis* isolates (Fig. 4A). Most *N. meningitidis* isolates tested nonsusceptible to azithromycin with MIC_{50/90} of $4 \mu\text{g/ml}$ (range, 0.5 to $4 \mu\text{g/ml}$) compared to *N. gonorrhoeae* isolates, which were mostly susceptible to azithromycin with MIC₅₀ of $0.125 \mu\text{g/ml}$ and MIC₉₀ 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 *N. gonorrhoeae* and *N. meningitidis* 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* both by using PointFinder and by individually validating mutations of known interest in specific genes (Table S2, Fig. S3). Point mutations for resistance were primarily shared by species (Fig. S2). One prominent point mutation in *N. meningitidis* isolates was in nonmosaic 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 MIC of $\leq 0.016 \mu\text{g/ml}$. While all urogenital *N. meningitidis* isolates and 1/3 respiratory *N. meningitidis* isolates had this mutation, this mutation was not present in any *N. gonorrhoeae* isolate. Isolates within the same SNP pairwise distance-based grouping had identical resistance mutation profiles (Fig. S2 and S3, Table S2).

DISCUSSION

As *N. meningitidis* becomes an increasingly recognized pathogen in the urogenital system, accurate species identification of *N. meningitidis* and *N. gonorrhoeae* urogenital isolates may be important for clinical care. Thus, it is essential to assess tools used for identification and compare *N. meningitidis* and *N. gonorrhoeae* urogenital isolates. In this study, we demonstrate that 30% (3/10) of urogenital *N. meningitidis* isolates were misidentified as *N. gonorrhoeae* with the A-CT/NG NAAT and that these urogenital *N. meningitidis* isolates were predominantly nonsusceptible to azithromycin. We found that specific identification using MALDI-TOF MS, MLST, 16S rRNA gene sequence, and ANI methods was 100% accurate for both urogenital *N. meningitidis* and *N. gonorrhoeae* isolates. However, our data indicate that some urogenital isolates of *N. meningitidis* can cause false-positive detections with *N. gonorrhoeae*-specific molecular tests and that some commensal *Neisseria* strains can be identified as *N. meningitidis* 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 *N. gonorrhoeae* from urine and genital specimens in clinical laboratories, there is evidence that other *Neisseria* species can cause false-positive *N. gonorrhoeae* 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 *N. meningitidis* isolates were detected as *N. gonorrhoeae* both when tested as pure isolate suspensions in saline and when spiked into urine. The Xpert CT/NG NAAT has two *N. gonorrhoeae*-specific targets, both of which must be detected to return an *N. gonorrhoeae*-positive result, while the Aptima CT/NG Combo 2 NAAT targets a region of the 16S rRNA to detect *N. gonorrhoeae*. 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 *N. meningitidis* isolates that test falsely positive for *N. gonorrhoeae* 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 *N. gonorrhoeae* result.

Accurate identification of *Neisseria* isolates is important, as AST profiles vary between *N. gonorrhoeae* and *N. meningitidis* isolates. Though rising rates of reduced susceptibility to azithromycin in *N. gonorrhoeae* have been reported across the United States (43), we found that the *N. gonorrhoeae* isolates tested in our study were primarily susceptible to azithromycin ($n = 4/5$), while urogenital *N. meningitidis* isolates collected over the same time period as *N. gonorrhoeae* isolates were more likely to be azithromycin nonsusceptible ($n = 7/10$). Our observation of largely azithromycin-susceptible *N. gonorrhoeae* is consistent with a previous study of a larger cohort of *N. gonorrhoeae* isolates recovered from the same institution that reported that azithromycin nonsusceptibility was rare (<2%) (44). Interestingly, all *N. meningitidis* isolates that tested falsely positive for *N. gonorrhoeae* were azithromycin nonsusceptible ($n = 3/3$). Currently, a single dose of intramuscular ceftriaxone (250 mg) plus a single dose of oral azithromycin (1 g) is the primary treatment recommendation for uncomplicated gonococcal infection (45). Although dual therapy was primarily aimed at treatment of uncomplicated chlamydial coinfections, routine combination therapy may hinder development of antimicrobial resistance in *N. gonorrhoeae*, particularly in light of increased cephalosporin resistance in the United States (43). Given the rare reports of ceftriaxone-resistant *N. meningitidis* (46–48), ceftriaxone plus azithromycin dual therapy is likely effective for treatment of urogenital *N. meningitidis* infection. However, if azithromycin nonsusceptibility is common among urogenital *N. meningitidis* isolates and in the setting of reduced susceptibility to 3rd-generation cephalosporins, current gonococcal treatment guidelines may be suboptimal for urogenital *N. meningitidis* infection. Interestingly, despite recent reports of ciprofloxacin-resistant, beta-lactamase-

producing *N. meningitidis* serogroup Y (49), all of the *N. meningitidis* isolates evaluated in this study were ciprofloxacin and ceftriaxone susceptible ($n = 13/13$), though most were nonsusceptible to penicillin ($n = 10/13$); beta-lactamase testing was not performed.

One hypothesis for conflicting identification of *N. meningitidis* isolates may be increased HGT between *N. gonorrhoeae* and *N. meningitidis*. However, our 16S rRNA gene sequence, MLST, and whole-genome analyses indicate that urogenital *N. meningitidis* isolates are not more similar to *N. gonorrhoeae* than other *N. meningitidis* 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 *N. meningitidis* urethritis are due to the spread of urethritis-associated *N. meningitidis* ST-11 and not due to translocation and subsequent infection of respiratory or meningitis-associated *N. meningitidis* isolates to the genitourinary tract, consistent with previous reports (11, 15). However, we did observe two instances where urogenital *N. meningitidis* isolates were not from the ST-11 urethritis clade, and in one case, a respiratory *N. meningitidis* isolate was highly related to a urinary *N. meningitidis* isolate. This suggests urogenital *N. meningitidis* 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 *N. meningitidis* isolates from MSM are associated with colonization of the urethra or rectum (2) and that *N. meningitidis* urethritis outbreaks are closely related to cases of invasive *N. meningitidis* 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 meningitis and the origin of urogenital *N. meningitidis* isolates. However, as this study is focused on urogenital *N. meningitidis* isolates, only a subset of 54 *N. meningitidis* meningitis isolates was used for comparison, with a focus on *N. meningitidis* isolates within ST-11. A more exhaustive study of *N. meningitidis* 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 *N. meningitidis* isolates. It is possible that increasing the collection and analysis of invasive and urogenital *N. meningitidis* 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, may be indicated and initiated following notification of this reportable infection. However, misidentification of *N. meningitidis* can confound these activities, particularly if identification of *N. gonorrhoeae* and/or *N. meningitidis* 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 *N. meningitidis* urogenital infections, as has been previously suggested (50).

Overall, our findings demonstrate that some urogenital *N. meningitidis* isolates are incorrectly identified as *N. gonorrhoeae* by the Aptima CT/NG NAAT despite being correctly identified as *N. meningitidis* by other molecular methods, including MALDI-TOF MS, MLST, 16S rRNA gene sequencing, and ANI analysis. Further, these urogenital *N. meningitidis* isolates are related to the ST-11 urethritis-associated *N. meningitidis* isolates from across the United States. 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 *N. meningitidis* and *N. gonorrhoeae* 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 *N. meningitidis*-mediated urogenital infection compared to gonococcal infection will be needed to justify strategies to identify and/or differentiate *N. meningitidis* from *N. gonorrhoeae* urogenital specimens.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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