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Exploration of the *Neisseria* Resistome Reveals Resistance Mechanisms in Commensals That May Be Acquired by *N. gonorrhoeae* through Horizontal Gene Transfer

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Abstract: Nonpathogenic *Neisseria* transfer mutations encoding antibiotic resistance to their pathogenic relative *Neisseria gonorrhoeae*. However, the resistance genotypes and subsequent phenotypes of nonpathogens within the genus have been described infrequently. Here, we characterize the minimum inhibitory concentrations (MICs) of a panel of *Neisseria* (n = 26)—including several commensal species—to a suite of diverse antibiotics. We furthermore use whole genome sequencing and the Comprehensive Antibiotic Resistance Database Resistance Gene Identifier (RGI) platform to predict putative resistance-encoding mutations. Resistant isolates to all tested antimicrobials including penicillin (n = 5/26), ceftriaxone (n = 2/26), cefixime (n = 3/26), tetracycline (n = 10/26), azithromycin (n = 11/26), and ciprofloxacin (n = 4/26) were found. In total, 63 distinct mutations were predicted by RGI to be involved in resistance. The presence of several mutations had clear associations with increased MIC such as DNA gyrase subunit A (*gyrA*) (S91F) and ciprofloxacin, tetracycline resistance protein (*tetM*) and 30S ribosomal protein S10 (*rpsJ*) (V57M) and tetracycline, and TEM-type β-lactamases and penicillin. However, mutations with strong associations to macrolide and cephalosporin resistance were not conclusive. This work serves as an initial exploration into the resistance-encoding mutations harbored by nonpathogenic *Neisseria*, which will ultimately aid in prospective surveillance for novel resistance mechanisms that may be rapidly acquired by *N. gonorrhoeae*.

Keywords: resistome; commensal bacteria; horizontal gene transfer; antibiotic resistance; microbiome; *Neisseria*

1. Introduction

The emergence of antibiotic resistance in pathogenic bacteria presents a challenge for successful treatment of infections and is a global threat to public health. In the United States alone, antibiotic-resistant bacteria cause an estimated 2.8 million infections and 35,000 deaths each year [1]. While resistance can arise in bacteria via de novo mutations, it can also be horizontally transferred from environmental [2–4], animal [5–7], or human-associated [8–10] microbial communities. Thus, profiling the resistome, or the collection of all antibiotic resistance mechanisms available to particular bacterial species [4], is an important step in prospective surveillance for novel resistance-encoding mutations that may be rapidly acquired by pathogens.

There are a number of mechanisms that are employed by bacteria to circumvent the efficacy of antibiotics. These include but are not limited to decreased drug influx, target modification, antibiotic degradation, and increased efflux through pumps [11]. The threat of rapid resistance acquisition via horizontal gene transfer (HGT) of all these mechanisms is exponentially amplified in bacteria that are naturally competent and highly recombinogenic such as the *Neisseria*. The genus *Neisseria* is composed of several closely related Gram-negative species, which are typically isolated from the naso- and oropharynx of humans and animals. While most species are considered commensals or "accidental pathogens", only one, *N. gonorrhoeae*, is an obligate human pathogen which colonizes the additional sites of the urogenital tract and rectum and causes the sexually transmitted infection gonorrhea. Concerningly, antimicrobial resistance is an increasing problem within *N. gonorrhoeae*, with over half of all 550,000 reported infections in the U.S. in 2017 resistant to at least one antibiotic [1] and with treatment failing for the recommended azithromycin and ceftriaxone combination therapy reported internationally as of 2018 [12,13].

One of the likely reasons for the high prevalence of resistance in *N. gonorrhoeae* is due to its natural competence for transformation, preferentially with *Neisseria*-specific DNA, allowing for extensive intragenus gene exchange and quick access to new adaptive solutions [14]. Gene acquisition from commensal *Neisseria* is evidenced by widespread genomic mosaicism in *N. gonorrhoeae*, whereby specific mutations or haplotypes have been shown to have been inherited from its close relatives [15–19]. Furthermore, mutations encoding resistance to both azithromycin [10,20] and third-generation cephalosporins [21,22] have been demonstrated to have been transferred from nonpathogenic *Neisseria* to *N. gonorrhoeae*. Ultimately, these documented cases of widespread gene exchange between the *Neisseria* highlights the importance of surveying nonpathogenic members of the genus for the resistance mechanisms that they harbor and may potentially share with their pathogen relative.

In this study, we set out to document both phenotypic and genotypic resistance across a panel of commensal *Neisseria* acquired from the Centers for Disease Control and Prevention (CDC) and Food and Drug Association's (FDA) Antibiotic Resistance (AR) Isolate Bank, a bacterial strain collection resource for studying antibiotic resistance [23]. We phenotype this panel (n = 26) to multiple classes of antimicrobials (beta-lactams, a macrolide, fluoroquinolone, and tetracycline) and report their minimum inhibitory concentrations (MIC). To link any observed increases in resistance to the possible underlying genetic contributors, we furthermore sequence the genomes of these isolates and use the Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) platform to predict possible causal resistance-encoding mutations.

2. Results

2.1. Characterization of Phenotypic Resistance in the AR Isolate Bank Neisseria Panel

A total of 26 *Neisseria* isolates were obtained from the CDC and FDA's AR Isolate Bank from the *Neisseria* species MALDI-TOF verification panel (Table 1). Characterization of antimicrobial susceptibility to penicillin, ceftriaxone, cefixime, tetracycline, azithromycin, and ciprofloxacin was conducted using the Etest method (Table 1). For the gonococci within the panel (n = 6), three were resistant to multiple antibiotic classes. *N. gonorrhoeae* AR Bank # 0936 was resistant to tetracycline and ciprofloxacin and was just below the reduced susceptibility threshold for azithromycin (1.5 μg/mL with the breakpoint at 2 μg/mL); *N. gonorrhoeae* AR Bank # 0937 was resistant to penicillin and ciprofloxacin and was just below the reduced susceptibility threshold for tetracycline (1.5 μg/mL with the breakpoint at 2 μg/mL); and *N. gonorrhoeae* AR Bank # 0938 was resistant to penicillin, tetracycline, and ciprofloxacin.

Table 1. Minimum inhibitory concentrations of *Neisseria* spps. measured by Etest.

		Minimum Inhibitory Concentration (MIC) [a,b]					
AR Bank #	**Species**	**Penicillin (PEN)**	**Ceftriaxone (CRO)**	**Cefixime (CFX)**	**Tetracycline (TET)**	**Azithromycin (AZI)**	**Ciprofloxacin (CIP)**
AR-0933	*Neisseria gonorrhoeae*	0.19	0.004	<0.016	1.5	0.25	0.004
AR-0934	*Neisseria gonorrhoeae*	0.38	0.016	0.023	1.5	0.125	0.006
AR-0935	*Neisseria gonorrhoeae*	0.19	0.006	<0.016	0.75	0.064	0.006
AR-0936	*Neisseria gonorrhoeae*	0.38	0.047	<0.016	**2**	1.5	**>32**
AR-0937	*Neisseria gonorrhoeae*	**>32**	0.016	<0.016	1.5	0.38	**6**
AR-0938	*Neisseria gonorrhoeae*	**>32**	0.008	<0.016	**32**	0.094	**4**
AR-0943	*Neisseria bacilliformis*	**32**	**16**	**0.5**	**4**	**4**	**6**
AR-0944	*Neisseria cinerea*	0.38	0.094	<0.016	**2**	**8**	0.032
AR-0945	*Neisseria elongata*	0.25	0.094	0.032	0.38	0.5	0.25
AR-0946	*Neisseria lactamica*	0.75	0.023	0.25	0.75	1.5	0.008
AR-0947	*Neisseria oralis*	1.5	0.047	0.064	1.5	1.5	0.016
AR-0948	*Neisseria canis*	0.25	0.008	<0.016	0.5	0.38	0.008
AR-0949	*Neisseria macacae*	1.5	0.064	0.064	**2**	**8**	0.125
AR-0950	*Neisseria macacae*	**3**	0.047	0.125	**3**	**8**	0.032
AR-0951	*Neisseria mucosa*	0.25	0.032	0.125	1	**3**	0.023
AR-0952	*Neisseria macacae*	0.38	0.023	0.047	**2**	0.5	0.012
AR-0953	*Neisseria subflava*	1.5	0.019	0.38	0.5	**2**	0.75
AR-0954	*Neisseria subflava*	**3**	0.25	**0.5**	**48**	**4**	0.047
AR-0955	*Neisseria subflava*	1	0.032	0.064	**6**	**12**	0.125
AR-0956	*Neisseria subflava*	1.5	0.125	0.25	1	**6**	0.75
AR-0957	*Neisseria subflava*	1	0.047	0.064	**4**	**8**	0.064
AR-0958	*Neisseria weaveri*	0.38	0.064	0.047	0.5	0.25	0.006
AR-0959	*Neisseria weaveri*	1	0.19	0.023	0.75	0.75	0.023
AR-0960	*Kingella denitrificans*	0.32	0.064	0.25	0.75	**2**	0.094
AR-0961	*Moraxella catarrhalis*	0.19	**1**	**2**	0.5	0.25	0.094
AR-0962	*Moraxella catarrhalis*	0.094	0.012	0.064	0.75	0.25	0.125

[a] Reported minimum inhibitory concentrations (MICs) are the mode of $n = 3$ replicates. [b] Isolates with reduced susceptibilities are indicated by bold and underlined MIC values (Clinical & Laboratory Standards Institute (CLSI) breakpoints: Penicillin (PEN) ≥ 2 μg/mL; Ceftriaxone (CRO) ≥ 0.5 μg/mL; Cefixime (CFX) ≥ 0.5 μg/mL; Tetracycline (TET) ≥ 2 μg/mL; Azithromycin (AZI) ≥ 2 μg/mL; and Ciprofloxacin (CIP) ≥ 1 μg/mL).

Of the typically human-associated commensal *Neisseria* within the panel ($n = 11$), eight displayed reduced susceptibly to at least one of the tested antibiotics (Table 1). Two isolates were resistant to penicillin (*N. bacilliformis* AR Bank # 0943 and *N. subflava* AR Bank # 0954), one was resistant to ceftriaxone (*N. bacilliformis* AR Bank # 0943), two were resistant to cefixime (*N. bacilliformis* AR Bank # 0943 and *N. subflava* AR Bank # 0954), five were resistant to tetracycline (*N. bacilliformis* AR Bank # 0943, *N. cinerea* AR Bank # 0944, *N. subflava* AR Bank # 0954, *N. subflava* AR Bank # 0955, and *N. subflava* AR Bank # 0957), eight were resistant to azithromycin (*N. bacilliformis* AR Bank # 0943, *N. cinerea* AR Bank # 0944, *N. mucosa* AR Bank # 0951, and *N. subflava* AR Bank # 0953 through 0957), and one was resistant to ciprofloxacin (*N. bacilliformis* AR Bank # 0943). Only one isolate was resistant to all tested antibiotics, *N. bacilliformis* AR Bank # 0943, which was the only representative of *N. bacilliformis* in the panel. This isolate also had a high MIC to ceftriaxone of 16 μg/mL, with recorded MICs for *N. gonorrhoeae*, typically ≤1 μg/mL [22]. Two out of the three isolates of the other represented human-associated *Neisseria* (*Moraxella* and *Kingella*) showed reduced susceptibility to azithromycin (*K. denitrificans* AR Bank # 0960) or to ceftriaxone and cefixime (*M. catarrhalis* AR Bank # 0961).

For the *Neisseria* typically associated with animals (*N. canis*, *N. macacae*, and *N. weaveri*; $n = 6$), three were resistant to at least one antibiotic (Table 1); *N. macacae* AR Bank # 0949 was resistant to tetracycline and azithromycin and was just below the reduced susceptibility threshold for penicillin (1.5 μg/mL with the breakpoint at 2 μg/mL); *N. macacae* AR Bank # 0950 was resistant to penicillin, tetracycline, and azithromycin; and *N. macacae* AR Bank # 0952 was resistant to tetracycline.

2.2. Genome Assemblies and Computational Analyses

Of the 26 isolates within our panel, we were able to generate genomic libraries for 24 of them (Table 2). For the remaining isolates, *N. cinerea* AR Bank # 0944 and *K. denitrificans* AR Bank # 0960, we were unable to generate sufficient DNA for sequencing. Genome assemblies ranged from 1.76 to 2.9 Mbp long and contained between 1684 to 2749 predicted open reading frames. Coverages

ranged from 214x to 921x. All of the sequenced AR Bank isolates clustered with representatives of their own species as assessed via a phylogeny generated from 16S rRNA gene sequences (Figure 1). However, representatives of *N. mucosa* ($n = 3$) and *N. macacae* ($n = 3$) formed a single monophyletic cluster.

Table 2. Genome assembly statistics for sequenced strains.

Isolate	SRA Read Accession	Genome Size (bp)	GC Content (%)	No. of Contigs	Estimated Coverage (x)	No. of ORFs	No. of tRNAs
AR-0933	SAMN15454039	2,100,787	52.6	65	438	2059	52
AR-0934	SAMN15454040	2,102,184	52.7	64	458	2081	49
AR-0935	SAMN15454041	2,215,614	52.35	54	470	2224	51
AR-0936	SAMN15454042	2,158,950	52.38	71	380	2117	50
AR-0937	SAMN15454043	2,138,399	52.53	69	498	2102	49
AR-0938	SAMN15454044	2,152,496	52.5	71	491	2124	49
AR-0943	SAMN15454045	2,342,297	59.34	91	421	2151	52
AR-0945	SAMN15454046	2,572,594	53.81	45	214	2511	55
AR-0946	SAMN15454047	2,145,323	52.48	53	366	1994	54
AR-0947	SAMN15454048	2,497,075	52.8	19	247	2302	56
AR-0948	SAMN15454049	2,399,311	48.41	42	341	2261	52
AR-0949	SAMN15454050	2,938,382	50.84	98	228	2749	58
AR-0950	SAMN15454051	1,762,877	51.47	18	344	2184	55
AR-0951	SAMN15454052	2,580,111	51.1	98	295	2330	54
AR-0952	SAMN15454053	2,446,961	51.04	66	319	2216	53
AR-0953	SAMN15454054	2,201,301	49.57	16	921	2096	54
AR-0954	SAMN15454055	2,359,025	49.13	57	390	2276	54
AR-0955	SAMN15454056	2,188,966	49.37	16	327	2033	55
AR-0956	SAMN15454057	2,238,415	49.02	34	397	2106	55
AR-0957	SAMN15454058	2,187,919	49.37	15	326	2029	55
AR-0958	SAMN15454059	2,268,810	49.1	30	478	2132	49
AR-0959	SAMN15454060	2,148,848	49.01	24	382	1962	49
AR-0961	SAMN15454061	1,882,811	41.81	13	577	1728	44
AR-0962	SAMN15454062	1,843,431	41.64	12	432	1684	43

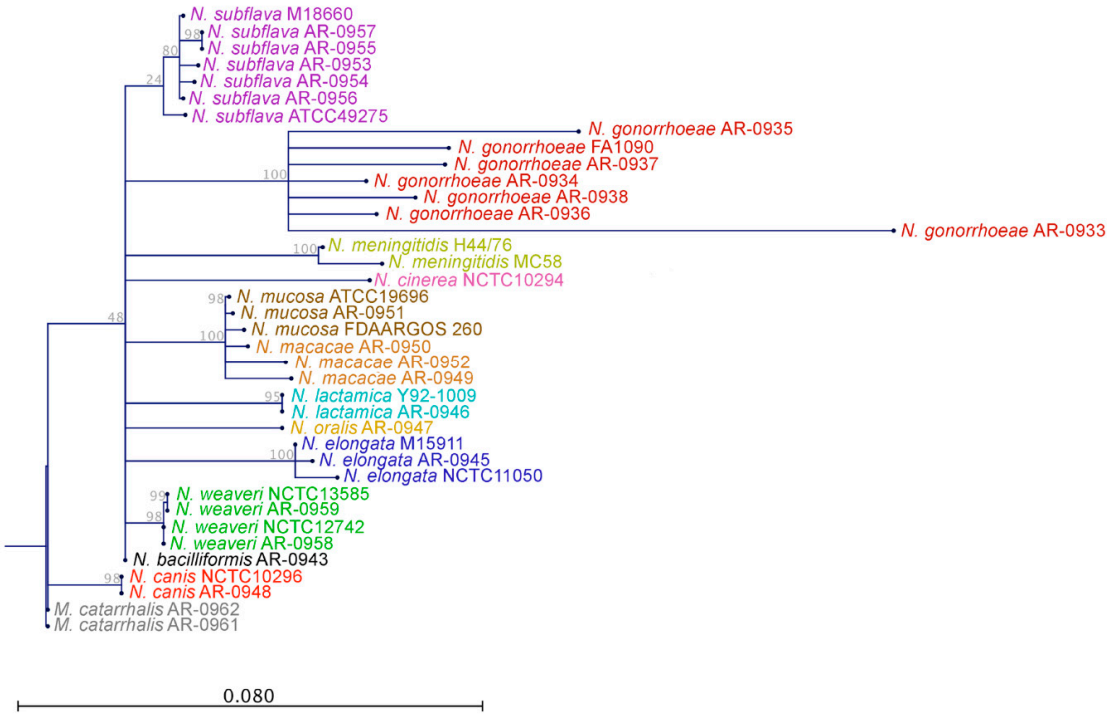

Figure 1. Maximum likelihood phylogenetic tree of 38 *Neisseria* isolates based on the 16S rRNA gene: species are coded by unique colors. The scale bar represents 0.08 substitutions per nucleotide site. Antibiotic Resistance (AR) Bank # 0961, one of the *Moraxella catarrhalis* in the *Neisseria* species MALDI-TOF verification panel, was used as an outgroup.

In total, 279 total mutations or haplotypes predicted to encode resistance were found across all sequenced isolates—consisting of 63 unique genetic mechanisms (Figure 2 and Supplementary Table S1). These polymorphisms were predicted to encode resistance to 14 different classes of antimicrobial compounds and included predicted phenotypic resistance to all tested drugs within this study.

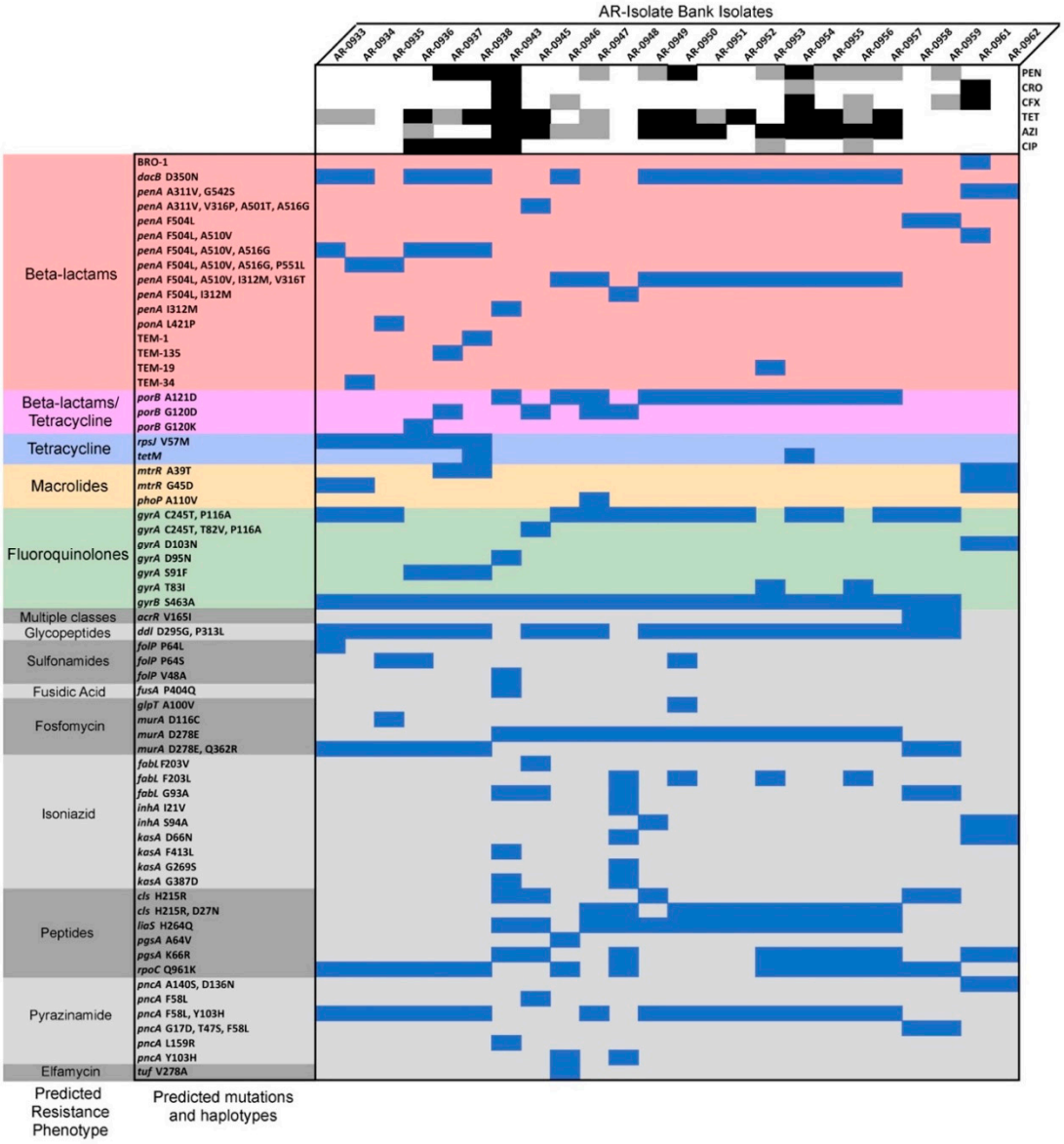

Figure 2. Heatmap showing the distribution of Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) predicted resistance-encoding mutations for all sequenced isolates (n = 24). Upper panel: *Neisseria* species' susceptibility phenotypes as quantified by MIC. Black denotes loss of susceptibility (CLSI standards), grey denotes one dilution below the reduced susceptibility breakpoint, and white denotes susceptibility to the antibiotics listed on the right (PEN, penicillin; CRO, ceftriaxone; CFX, cefixime; TET, tetracycline; AZI, azithromycin; and CIP, ciprofloxacin). Lower panel: presence of each recovered mutation is indicated by blue fill. CARD-predicted resistance phenotypes for sets of mutations are denoted for beta-lactam resistance (red shading), beta-lactam and tetracycline resistance (purple), tetracycline (blue), macrolides (orange), fluoroquinolones (green), and resistance to other antibiotics (grey).

2.3. Quinolone Reduced Susceptibility

Resistance to quinolones like ciprofloxacin in gonococci is mediated by mutations that reduce binding affinity to the primary drug target, DNA gyrase subunit A (GyrA)—a type II topoisomerase that negatively supercoils ds-DNA. Specifically, these mutations include either a missense mutation at codon 91 in *gyrA* (S91F) or codon 95 (D95N), which have nearly perfect sensitivity and specificity for the prediction of resistance to this drug in clinical *N. gonorrhoeae* specimens [22,24–27]. In our panel, we documented four isolates with reduced susceptibility to ciprofloxacin (*N. gonorrhoeae* AR Bank # 0936 through 0938 and *N. bacilliformis* AR Bank # 0943), and unsurprisingly, they all had either the GyrA S91F or D95N substitutions (Figure 2). However, interestingly, we also found two *N. subflava* isolates (AR Bank # 0953 and AR Bank # 0956) with ciprofloxacin MICs less than one dilution below the reduced susceptibility threshold (Table 1) which did not harbor these mutations. Instead, they had a missense mutation at codon 83 in *gyrA* (T83I), which is located in a quinolone resistance-determining region (QRDR) and has been shown to moderately increase quinolone resistance in *Pseudomonas aeruginosa* [28].

2.4. Macrolide Resistance

Macrolide class antibiotics obstruct protein synthesis by binding to the 50S ribosomal subunit, and within *N. gonorrhoeae*, several types of mutations have been documented to be involved in resistance. These mutations include substitutions in the 23S rRNA azithromycin binding sites (C2611T and A2059G) [29,30], the presence of mosaic *multiple transferable resistance* (*mtr*) efflux pump alleles acquired from commensal *Neisseria* species [10,20,22,31], mutations that increase the expression of *mtrCDE* [32–34], mutations in *rplD* [22], *rplV* tandem duplications [22], and variants of the rRNA methylase genes *ermC* and *ermB* [35]. However, we found no isolates in this panel with any of these mutations above the Clinical & Laboratory Standards Institute (CLSI) reduced susceptibility breakpoint (Figure 2). We did find four *N. gonorrhoeae* isolates with missense mutations at codons 39 (A39T) and 45 (G45D) of the repressor of the Mtr efflux pump (MtrR), which have been shown to enhance *mtrCDE* expression by introducing radical amino acid substitutions in the DNA-binding motif of the repressor and by ablating its promoter-binding function [32,36]. However, these isolates did not have elevated MICs to azithromycin.

2.5. Reduced Susceptibility to Tetracycline

Tetracycline is a broad-spectrum polyketide antibiotic that binds the bacterial 30S ribosomal subunit and blocks incoming aminoacyl tRNAs from entering the ribosome acceptor site. Here, we found two isolates *N. gonorrhoeae* AR Bank # 0938 and *N. subflava* AR Bank # 0954 with high-level tetracycline resistance (≥32 μg/mL; Table 1). High-level tetracycline resistance in *Neisseria* has been demonstrated to be a direct result of inheritance of a class M tetracycline resistance determinant (encoded by *tetM*), which after binding to the ribosome triggers the release of tetracycline due to its resemblance to elongation factor G (EF-G). Both *N. gonorrhoeae* AR Bank # 0938 and *N. subflava* AR Bank # 0954 had a *tetM* gene present (Figure 2), further supporting prior literature suggesting that *tetM* circulates within commensal *Neisseria* communities [37] in addition to gonococcal populations [38,39]. Lower level tetracycline resistance has been shown to be mediated by mutations that decrease the influx of tetracycline through porin [40,41], mutations in *mtr* that increase pump expression [41–43], or structure-modifying mutations in the ribosome [44]. Several isolates had mutations in the porin (PorB G120K, A121D, or A121N) and/or mutations in the ribosomal protein RpsJ (V57M), which have previously been associated with reduced susceptibility to tetracycline in previous studies [40,44]; however, inheritance of these mutations were not perfectly correlated with reduced susceptibility (Figure 2).

2.6. Resistance to β-Lactams

Penicillins (penicillin G) and cephalosporins (ceftriaxone and cefixime) are β-lactam antibiotics which inhibit cell wall biosynthesis by binding the transpeptidase enzymes (penicillin-binding proteins (PBPs)) that form the peptidoglycan cross-links in the bacterial cell wall. High-level penicillin resistance in gonococci is typically mediated by the presence of a TEM-1-type β-lactamase encoded by the bla_{TEM-1} gene, which acts through degradation of the four-atom β-lactam ring of penicillin [45]. Of the isolates within this panel with penicillin MICs ≥ 32 μg/mL, two out of three had a TEM-type β-lactamase present. Lower level penicillin resistance can be modulated through multiple mutations including those in *penA* [41,46], *mtr* and its regulatory components [41–43], *porB* [40,41], *ponA* [47], and *pilQ* [47,48], which often contribute additively to one another [47]. Notably within the commensals in this panel, we found at least one of these mutations present in all isolates (i.e., *porB* G120K, A121D, or A121N; *ponA* L421P; or several mutations in *penA* (Figure 2)), suggesting their widespread availability for horizontal exchange.

In contrast to the multiple mutations that give rise to penicillin resistance, reduced susceptibility to cephalosporins in *Neisseria* is most frequently mediated by mutations in the PBP targets of the drug which decrease their acylation rate. Commensal *Neisseria* spps. are proven sources of alleles conferring cephalosporin resistance for *N. gonorrhoeae*, and within this panel, we found *penA* alleles with several amino acid substitutions that have been proven or associated with increased resistance including A311V, I312M, V316T, V316P, A501T, G542S, and P551L [21,49–51] (Figure 2). Finally, we found a BRO-1 β-lactamase in one of the *Moraxella catarrhalis* isolates (AR Bank # 0961) within the panel which also was resistant to both cefixime and ceftriaxone (Table 1 and Figure 2). The BRO-1 β-lactamase has previously been associated with resistance to β-lactams and is present in over 90% of all *M. catarrhalis* isolates [52,53], though it is unclear if this β-lactamase can be transferred to the other *Neisseria*.

3. Discussion

The significance of the commensal *Neisseria* as reservoirs of antibiotic resistance for gonococci has been repeatedly demonstrated [10,20–22,49–51], which emphasizes the importance of characterizing mutations encoding reduced susceptibility across the entirety of this species consortium. Our results offer an initial exploration into the *Neisseria* resistome and clearly demonstrate resistance-encoding mutations that are known in gonococci (*gyrA* (S91F), *tetM*, and TEM-type β-lactamases) to also circulate in commensal communities, suggesting their widespread availability for horizontal transfer. However, it is also clear that we have not captured all mutations involved in producing reduced susceptibility across this panel. This is evidenced by isolates with the same reported resistance haplotypes displaying variation in MIC. For example, *N. subflava* AR Bank # 0954 and 0955 had MICs to penicillin of 3 and 1 μg/mL, respectively, despite having the same mutations in *penA* and *porB* (Table 1 and Figure 2). Similarly, *N. gonorrhoeae* AR Bank # 0936 and 0937 isolates had ciprofloxacin MICs of 32 and 6 μg/mL, respectively, yet harbored the same *gyrA* (S91F) substitution (Table 1 and Figure 2). Furthermore, there are cases of unexplained resistance for which no putative resistance mutations had been identified—especially for the drug azithromycin. However, this is not necessarily surprising for macrolide antibiotics, as much of the genetic basis of resistance remains unclear in gonococci [22], suggesting that many mutational steps are required and that there may be many different paths to reduced susceptibility. Ultimately, these discrepancies between phenotype and genotype point to the main limitations of our approach, which include the dependence on a database that contains the full universe of polymorphisms within a given species or genus that may give rise to resistance, sufficient sequence homology between database entries and query sequences for alignment, and knowledge of epistatic modulators of resistance that may not directly impact MIC on their own but only in combination with other mutations.

Though lab-based efforts will be needed to confirm the causality of our CARD-nominated mutations in reduced susceptibility in addition to the elucidation of the genetic underpinnings that to contribute to the unexplained MIC variance, our combined approaches employing both experimental

quantification of MIC data coupled with genomic sequencing is a key first step in exploring the possible resistance mechanisms harbored by commensal populations. Ultimately, these types of studies will provide the foundation for prospective surveillance of novel resistance determinants that may be rapidly acquired by pathogens of critical importance across a wide range of genera.

4. Materials and Methods

4.1. Bacterial Strains and Culture Conditions

All isolates were cultured on GC agar base medium (Becton Dickinson Co., Franklin Lakes, NJ, USA) supplemented with 1% Kellogg's solution (GCB-K plates) [54] at 37 °C in a 5% CO_2 incubator. Stocks for all bacteria were stored at −80 °C in trypticase soy broth containing 50% glycerol.

4.2. Minimum Inhibitory Concentration Testing

Antimicrobial susceptibility testing was conducted using Etest strips on GCB-K plates, according to the manufacturer specifications (bioMérieux, Durham, NC, USA), which have been shown to have comparable MIC values to the agar dilution method, with the exception of cefixime for which Etests systemically report lower MICs [55]. In brief, cells from overnight plates were suspended in trypticase soy broth to a 0.5 McFarland standard and inoculated onto new GCB-K plates. Etest strips were subsequently placed on the surface of the inoculated plates. Following 18–24 h of incubation at 37 °C in a 5% CO_2 incubator, MICs were determined by reading the lowest concentration that inhibited growth, and reduced susceptibility was determined using CLSI guidelines (CLSI breakpoints: penicillin (PEN) ≥ 2 μg/mL; ceftriaxone (CRO) ≥ 0.5 μg/mL; cefixime (CFX) ≥ 0.5 μg/mL; tetracycline (TET) ≥ 2 μg/mL; azithromycin (AZI) ≥ 2 μg/mL; and ciprofloxacin (CIP) ≥ 1 μg/mL) [56]. Intermediate MIC values are defined for penicillin, tetracycline, and ciprofloxacin at PEN ≥ 0.12 μg/mL, TET ≥ 0.5 μg/mL, and CIP ≥ 0.12 μg/mL, respectively [56]. MICs were read by at least two independent researchers, and the mode of three tests was reported.

4.3. Library Preparation and Genomic Sequencing

Genomic DNA was isolated by lysing growth from overnight plates in TE buffer (10 mM Tris (pH 8.0), 10 mM EDTA) with 0.5 mg/mL lysozyme and 3 mg/mL proteinase K (Sigma-Aldrich Corp., St. Louis, MO, USA). To obtain sufficient DNA from AR BANK # 0943 and AR BANK # 0958, which were difficult to swab off of GCB-K plates, we instead inoculated liquid GCB broth media (7.5 g protease peptone #3, 0.5 g soluble starch, 2 g dibasic K_2HPO_4, 0.5 g monobasic KH_2PO_4, 2.5 g NaCl, and ddH_2O to 500 mL; Becton Dickinson) supplemented with 1% Kellogg's solution and incubated overnight at 37 °C. After 24 h, cultures were centrifuged for 10 min at 14,000 rpm. The supernatant was discarded, and the same method as described above was used to isolate DNA.

DNA was purified using the PureLink Genomic DNA Mini kit (Thermo Fisher Corp., Waltham, MA, USA), treated with RNase A, and stored in water. Sequencing libraries were prepared using the Nextera XT kit as per the manufacturer's instructions (Illumina Corp., San Diego, CA, USA). Samples were uniquely dual-indexed, pooled (n = 17–18 libraries per pool), and sequenced using a V3 600 cycle cartridge (2 × 300 bp) on an Illumina MiSeq platform at the Rochester Institute of Technology Genomics Core.

4.4. Genome Assembly and Bioinformatic Analyses

Sequencing quality of each paired-end read library was assessed using FastQC v0.11.9 [57]. Trimmomatic v0.39 [58] was used to trim adapter sequences and to remove bases with phred quality score < 15 over a 4-bp sliding window. Reads < 36 bp long or those missing a mate were also removed from subsequent analysis. Trimmed reads were assembled using SPAdes v.3.7.0 [59], and the resultant de novo assemblies were evaluated using the Quality Assessment Tool for Genome Assemblies (QUAST) v.4.1 [60]. Prokka v.1.11 [61] was used to annotate assemblies. Resistance-encoding mutations

were predicted using the Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) v5.1.0 [62]. Gene presence or absence was not considered, unless *tetM* or TEM beta lactamases were recorded, which are most often harbored on plasmids [63].

To assess evolutionary relationships between isolates, we used the annotated 16s rRNA gene in FA1090 (AE004969.1) as a reference and subset the homologous region in each assembly with blastn. We then used CLC Main Workbench v20.0.4 [64] to align sequences and to reconstruct a maximum likelihood phylogeny using 100 bootstrap replicates and the Jukes Cantor substitution model.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6382/9/10/656/s1, Table S1: CARD RGI predicted resistance mutations for each sequenced isolate in the study (n = 24), Table S2: Accessions for the *Neisseria* used in the Figure 1 16S phylogeny.

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