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Article

Antimicrobial resistance profiles of human commensal *Neisseria* species

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Abstract: Pathogenic *Neisseria gonorrhoeae* causes the sexually-transmitted infection gonorrhea. *N. gonorrhoeae* has evolved high levels of antimicrobial resistance (AR) leading to therapeutic failures even in dual-therapy treatment with azithromycin and ceftriaxone. AR mechanisms can be acquired by genetic transfer from closely related species, such as naturally-competent commensal *Neisseria* species. At present, little is known about the antimicrobial resistance profiles of commensal *Neisseria.* Here, we characterized the phenotypic resistance profile of four commensal *Neisseria* species (*N. lactamica*, *N. cinerea*, *N. mucosa*, and *N. elongata*) against 10 commonly used antibiotics, and compared their profiles to 4 *N. gonorrhoeae* strains, using disk diffusion and minimal inhibitory concentration assays. Overall, we observed that 3 of the 4 commensals were more resistant to several antibiotics than pathogenic *N. gonorrhoeae* strains. Next, we compared the penicillin-binding-protein 2 (PBP2) sequences between commensal and *N. gonorrhoeae* strains. We found mutations in PBP2 known to confer resistance in *N. gonorrhoeae* also present in commensal *Neisseria* sequences. Our results suggest that commensal *Neisseria* have unexplored antibiotic resistance gene pools that may be exchanged with pathogenic *N. gonorrhoeae,* possibly impairing drug development and clinical treatment.

Keywords: Commensal bacteria; *Neisseria*; antimicrobial resistance; multidrug resistance

1. Introduction

Neisseria gonorrhoeae, the etiologic agent of gonorrhea, is the second most commonly reported bacterial sexually-transmitted infection in the US [1], and a worldwide public health concern. The World Health Organization (WHO) estimates there were 87 million new cases globally of *N. gonorrhoeae* in 2016 [2]; this incidence is increasing in many countries including the USA [3]. The Centers for Disease Control and Prevention (CDC) classifies *N. gonorrhoeae* as an "urgent threat" due to the emergence of antibiotic resistance (AR) and multidrug resistance (MDR) [4-9]. The spread of MDR has led to increasing rates of untreatable gonorrhea [10], including reports of untreatable and harder to treat pharyngeal *N. gonorrhoeae*, acquired through oral sex [11-13], where *N. gonorrhoeae* shares the environment with closely related commensal *Neisseria*.

Pathogenic and commensal *Neisseria* species exchange and transfer genes via natural competence and transformation [14]. *Neisseria* will exchange and transfer genes at high rates, as long as they share an identical or similar DNA uptake Sequences (DUS) and the corresponding DNA import complex [15-18]. Hence, in this study, we characterize the antimicrobial resistance profiles of commensal *Neisseria* species and explore their potential role as antibiotic resistance gene reservoirs for pathogenic *Neisseria* species. We selected and characterized commensal *Neisseria* species across a spectrum of genetic relatedness, including *N. lactamica*, *N. elongata, N. cinerea,* and *N. mucosa*. Of these, *N. lactamica* is the closest relative to the pathogens *N. gonorrhoeae* and *N. meningitidis*, while *N. elongata* appears to be the most distant relative to the pathogens [19-21]. *N. cinerea* is more closely related to *N. lactamica*; *N. mucosa* is closely related to the common

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ancestor of *N. lactamica* and *N. cinerea*. We also used the *N. gonorrhoeae* FA19 strain [22] as our reference strain, susceptible to antibiotics, while strains MS11, H041, and F89 are representatives of resistant strains for several antibiotics. We tested commensal and pathogenic strains against a panel of 10 antibiotics that are commonly or previously used, or likely to be used in a therapeutic context. We used disk diffusion and minimal inhibitory concentration assays, and show that commensal *Neisseria* display increased antimicrobial resistance profiles to widely used antibiotics, including the first line dual-therapy drugs, azithromycin and ceftriaxone, compared to pathogenic *N. gonorrhoeae* strains. Notably, when analyzing the *penA* sequences, which encode the Penicillin Binding Protein 2 (PBP2), we identified several mutations in some commensal *Neisseria* species that are known to cause resistance in *N. gonorrhoeae*. We discuss these findings in light of the potential for commensal *Neisseria* to function as *de facto* reservoirs of antibiotic resistance genes for the pathogenic *N. gonorrhoeae*.

2. Results

2.1. Commensal Neisseria display increased resistance levels to several antibiotics, detected by disk diffusion assays

In order to assess the levels of antibiotic resistance of commensal *Neisseria*, we performed disk diffusion assays (DDA) with 10 commonly used antibiotics, including 3 beta-lactams and 7 protein synthesis inhibitors as described in the Methods. Our DDA results revealed that some commensal *Neisseria* species were particularly resistant to azithromycin and ceftriaxone (Fig 1 and Supplementary Table 1), the first-line treatment drugs against *N. gonorrhoeae*, as well as to erythromycin, the antibiotic applied on newborns' eyes to prevent gonococcal conjunctivitis. All commensal species displayed increased resistance to azithromycin as evidenced by the smaller zone of inhibition (ZoI) diameters (ZoI ranging 14.7 - 21.2 mm) than any of the *N. gonorrhoeae* strains (ZoI ranging 29.2 - 34.1 mm), including the highly resistant strains *N. gonorrhoeae* F89 and H041. For ceftriaxone, only *N. lactamica* displayed susceptibility levels similar to *N. gonorrhoeae* strains, with ZoI of 36.7 mm and ranging 30.8 - 43.0 mm, respectively. Commensals *N. cinerea, N. mucosa,* and *N. elongata* were found to be more resistant to ceftriaxone (ZoI ranging 21.3 - 28.5 mm) than any of the *N. gonorrhoeae* tested. According to the CLSI guidelines, ZoI > 35 mm suggest that *N. gonorrhoeae* were susceptible to ceftriaxone [32]; hence, only *N. lactamica* and *N. gonorrhoeae* FA19 can be considered susceptible to ceftriaxone; all other strains tested displayed resistance to it. The resistance of commensals to erythromycin resembled azithromycin's pattern where *N. cinerea*, *N. mucosa* and *N. elongata* displayed increased erythromycin resistance levels (ZoI ranging 13.5 - 15.0 mm), while *N. lactamica* was more susceptible (ZoI at 19.0 mm). Nonetheless, all the commensals tested were less susceptible to erythromycin than the 4 strains of *N. gonorrhoeae* tested (ZoI ranging 23.8 - 30.5 mm).

Commensal *Neisseria* species displayed increased resistance to penicillin and ampicillin (ZoI ranging 20.3 - 31.0 mm and 21.8 - 28.5 mm, respectively) compared to the susceptible *N. gonorrhoeae* FA19 strain (ZoI 41.7 mm and 38.5 mm, respectively). *N. lactamica* appears again more susceptible to these antibiotics than the other commensal *Neisseria* tested. According to the interpretive standards published by the CLSI [32], *N. gonorrhoeae* were considered resistant to penicillin when ZoI < 26 mm, such as *N. cinerea, N. elongata, N. gonorrhoeae* MS11 and H041.

Similarly, three commensals, but not *N. lactamica*, displayed higher resistance to chloramphenicol (ZoI ranging 21.3 - 24.5 mm) than the susceptible *N. gonorrhoeae* FA19 (ZoI of 38.7 mm). The other *N. gonorrhoeae* strains (MS11, F89, H041) displayed ZoI to chloramphenicol similar to the commensal species. Commensal *Neisseria* strains were more resistant to tetracycline (ZoI ranging 21.2 - 28.7 mm) than *N. gonorrhoeae* FA19 (ZoI of 34.8 mm). Streptomycin was the only antimicrobial tested for which commensals displayed slightly higher susceptibility levels (ZoI ranging 12.5 - 17.7 mm) than the susceptible *N. gonorrhoeae* FA19 (ZoI of 14.9 mm). Only two commensals, *N. cinerea* and *N. elongata* displayed kanamycin resistance levels (ZoI of 20.5 and 21.3 mm respectively) higher than *N.*

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gonorrhoeae FA19 (ZoI of 31.3 mm). It is important to note that the ZoI of *N. cinerea, N. mucosa,* and *N. elongata* were found to be at least 14 mm smaller than those of *N. gonorrhoeae* FA19, for ampicillin, ceftriaxone, azithromycin, erythromycin, and chloramphenicol, reflecting a wide gap in resistance levels between these 3 commensals and the susceptible *N. gonorrhoeae* FA19 strain.

Interestingly, both groups of commensal and pathogenic strains displayed similar levels of resistance to gentamicin (ZoI ranging 17.8 - 21.8 mm and 17.5 - 20.2 mm, respectively), a possible alternative to treat *N. gonorrhoeae* (Fig. 1 and Supplementary Table 1). These ZoI to gentamicin were slightly larger than the ZoI ≥ 16 mm categorized as the limit for susceptibility to gentamicin [33]. This suggests that the *Neisseria* species tested do not currently display antibiotic resistance against gentamicin, nor should they be considered a reservoir of gentamicin resistance. However, the small difference between the threshold of susceptibility (16 mm) and the range of ZoI observed (ranging 17.5 - 21.8 mm) suggests that a mutation, even if slightly decreasing the susceptibility level to gentamicin, could render these species resistant.

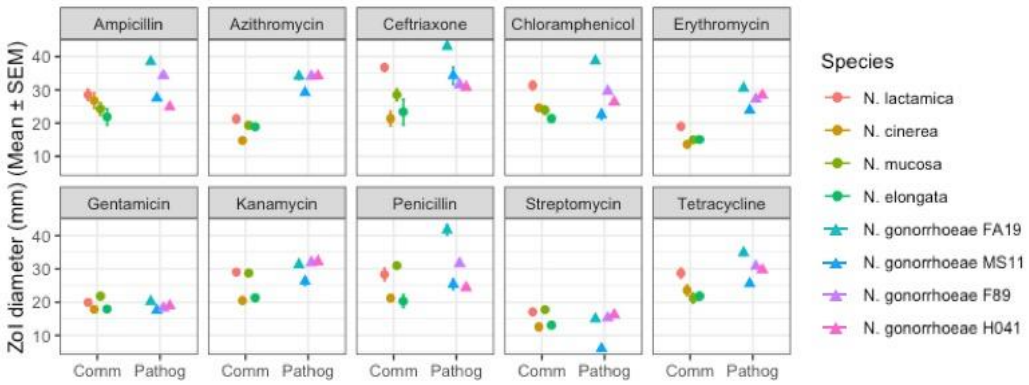

Figure 1: Antibiotic disk diffusion assays (DDA) demonstrate antibiotic resistance profiles to 10 antibiotics of *Neisseria* species, grouped as commensal (Comm, circles) and pathogenic (Pathog, triangles). Means (± SEM) of zone of inhibition (ZoI) diameters (mm) from 3 independent experiments, each performed in three biological replicates are shown. Comm, commensal; Pathog, pathogenic.

Overall, *N. cinerea, N. mucosa, and N. elongata* appeared more resistant to antibiotics than *N. lactamica*, which is more closely related to *N. gonorrhoeae* and *N. meningitidis.* As shown in Figure 1, *N. lactamica* was the most susceptible of the four commensals to seven of the 10 antibiotics tested and the second-most susceptible in the three remaining antibiotics (see Figure 1); and therefore *N. lactamica* was most similar in its antibiotic susceptibility profile to the susceptible pathogenic *Neisseria gonorrhoeae* FA19. This suggests that the other commensal strains - *N. cinerea, N. mucosa, and N. elongata* - may have distinct antimicrobial resistance mechanisms than *N. gonorrhoeae* FA19. Indeed, the profile of resistance for these 3 commensal species was very different from *N. lactamica* and the 4 *N. gonorrhoeae* strains, possibly suggesting that these commensals carry mutations and/or genes that confer increased antimicrobial resistance, in ways that have not been observed in *N. gonorrhoeae*. This requires further exploration of genomic data of commensal *Neisseria* species, both in known antimicrobial resistance genes and in regions that are not known to confer antimicrobial resistance.

2.2. Commensal Neisseria display increased resistance levels to several antibiotics, detected by minimal inhibitory concentrations

Next, we performed minimal inhibitory concentration (MIC) assays using a 2-fold serial dilution of azithromycin, ceftriaxone, penicillin, erythromycin, chloramphenicol, and gentamicin (see materials and methods for concentration ranges). We compared our

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results to susceptible and resistant strains of *N. gonorrhoeae* with values reported in the literature. Figure 2 shows differences in MIC to these 6 antibiotics for the commensal *Neisseria* species tested in the lab. Similarly to our DDA analysis, *N. lactamica* appeared susceptible compared to the other commensal *Neisseria* species (Fig. 2 and Supplementary Table 2). Indeed, *N. cinerea, N. mucosa,* and *N. elongata* showed increased ceftriaxone MIC values (0.128 μg/mL, 0.064 μg/mL, 0.064 μg/mL respectively), reflecting increased resistance, than *N. lactamica* (0.008 μg/mL) or a susceptible *N. gonorrhoeae* strain (≤0.015 μg/mL). *N. cinerea* displayed ceftriaxone MIC values 16 times higher than *N. lactamica*; *N. mucosa* and *N. elongata* displayed ceftriaxone MIC values 8 times higher than *N. lactamica*. However, these values of MIC for commensal *Neisseria* were still below the MIC breakpoint for ceftriaxone described by Kirkcaldy et al. [34]. The MIC values for azithromycin in commensal *Neisseria* were lower (0.25-0.5 μg/mL) than the MIC breakpoint for resistant *N. gonorrhoeae* (≥2 μg/mL) described by Kirkcaldy et al. [34]; hence, commensals appeared sensitive to azithromycin, detected by MIC assays. All commensals were overall more resistant to penicillin than the susceptible strain of *N. gonorrhoeae*. *N. elongata* and *N. mucosa* appeared more resistant than *N. cinerea* and *N. lactamica* towards chloramphenicol and erythromycin; we did not find MIC values to these antibiotics for *N. gonorrhoeae* susceptible and resistant strains. These observations reinforce the need to analyze genomic sequences of commensal *Neisseria* to identify possible antimicrobial resistance genes and mutations.

We also noted that there were key differences in the relative resistance between commensal and pathogenic species when contrasting DDA to MIC assays. Contrary to our DDA observations, commensal and pathogenic species displayed differences in resistance to gentamicin. Detected by MIC, commensal species appeared more susceptible than a susceptible *N. gonorrhoeae* strain. Currently, we cannot explain the discrepancies in our observations between DDA and MIC data, except that MIC can identify extreme behaviors of resistance as values reflect growth of a reduced number of individuals (0.01% of the population) under high antibiotic levels. These observations were less likely to be detected in DDA.

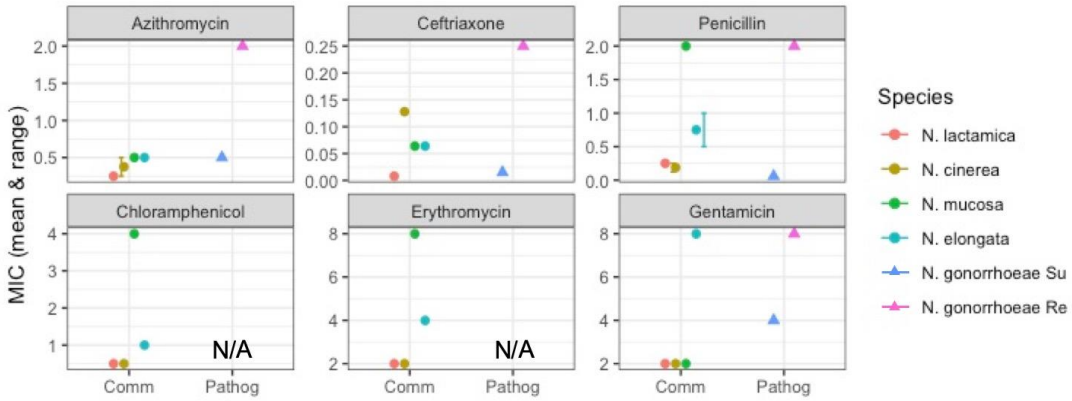

Figure 2: Minimal Inhibitory Concentrations (μg/mL) of several antibiotics in commensal (Comm, circles) *Neisseria* species compared to *N. gonorrhoeae* (Pathog, triangles) susceptible (Su) and resistant (Re) strains. MIC were performed 3 times independently, using 2-fold serial dilutions of the antibiotics. When necessary, ranges (as color-coded bars) of MIC are displayed for specific data points that are plotted as a midpoint of the range. N/A, Not available.

2.3. Mutations in penicillin-binding protein 2 (PBP2) sequences can partially explain resistance in commensal species

In order to explore the potential mechanistic basis for variation in resistance, we compared sequence variation amongst commensal and pathogenic strains, focusing on previously identified genes associated with antibiotic resistance. One of these genes - the *penA* gene - encodes the penicillin-binding-protein-2 that is known to modify penicillin and other beta-lactam drugs [35]. In *N. gonorrhoeae*, known mutations in *penA* lead to increased

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resistance [36]. *N. gonorrhoeae* resistant strains often contain point mutations leading to amino acid changes, and/or contain a mosaic sequence of *penA*, which is a recombination of *penA* genes from *N. perflava* and *N. cinerea* [37-40], both commensal species regularly carried in the human oro- and nasopharynx, where other commensal *Neisseria* also reside, and where *N. gonorrhoeae* may be found in cases of oral sex gonococcal infections.

The *penA* sequences of commensal *Neisseria* species reveal genetic polymorphism. We aligned amino acid sequences of PBP2 from the different commensal *Neisseria*, *N. gonorrhoeae* susceptible strain LM306 (wild-type sequence, Ngo_WTSu) and resistant strain NG-3 (mosaic sequence, Ngo_mosaic), and 2 outgroup sequences from *Eikenella corrodens* (Eik) and *Kingella oralis* (Kor) (from the family Neisseriaceae), using BLASTp [41] and Clustal Omega [42] algorithms. We observed that mutations in PBP2 known to increase antibiotic resistance in *N. gonorrhoeae*, were present in *N. mucosa* (Nmu), *N. elongata* (Nel), and *N. cinerea* (Nci) (Fig. 3). On the other hand, *N. lactamica* (Nla) conserved the amino acids present in the susceptible *N. gonorrhoeae* strain LM306 (highlighted in yellow). Other described mutations were not present in the commensal *Neisseria* species (hence, are not shown in Fig. 3), namely the insertion of an aspartate after position 345 of the wild-type susceptible sequence [38,43], the mutation A501V or A501P [25,44], and the mutation G545S [45].

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Kor_QMT43252.1            PGSVMKPFIIAKALDDGKIGRNSTFNTRPYAIGDKTIRDTHDYPSLTTQGILQKSSNVGT  368
Nel_WP_107971226.1        FGSVLKPFPIAKALDDGKISTRSHFDTRPYNVGGHPVRDTHLYPSLDVRGIMQKSSNVGT  452
Eik_SNW07260.1            PGSAMKPFPIAKALDSGKVNENMVFNTNTYNIGPATVRDTHNYPSLTLRGIMQKSSNVGV  367
Nmu_EFC88110.1            PGSAMKPFTIAKALDSGKVGVADRFNTMPYKIGPATVRDTHVYPTLDVRGIMQKSSNVGT  382
Ngo_mosaic_BAB86942.1     PGSAMKPFTIAKALDSGKVDATDTFNTLPYKIGSATVQDTHVYPTLDVRGIMQKSSNVGT  367
Nci_WP_003676738.1        PGSAIKPFVIAKALDADKTNLNERLNTQPYKIGPAQVRDTHVYPSLDVRGIMQKSSNVGT  367
Ngo_WTSu_AAA25463.1       PGSAIKPFVIAKALDAGKTDLNERLNTQPYKIGPSPVRDTHVYPSLDVRGIMQKSSNVGT  367
Nla_WP_003709943.1        PGSAIKPFVIAKALDAGKTDVNERLNTQPYKIGPAPVRDTHVYPSLDVRGIMQKSSNVGT  367
                           **.:*** ****** .* .    ::*  * :*   ::*** **:*  :**:*******.

Kor_QMT43252.1            VAGPAFREIMAGGLKKLGVKPTYVNTEPAANVAKKR  583
Nel_WP_107971226.1        VAGPAFKGIMAGTLNILGVHPTNAVKAVDLAAK---  665
Eik_SNW07260.1            VAGPVFKDIMAGSLNILGVTPTKPVQQVAAK-----  578
Nmu_EFC88110.1            VAGPVFKQVMGGSLNILGVSPTKPLTNVAAVKTPS-  597
Ngo_mosaic_BAB86942.1     VTGPVFKQVMGGSLNILGVSPTKPLTNVAAVKTPS-  582
Nci_WP_003676738.1        VAGPVFKQVMGGSLNILGVSPTKPLTNVAAVKTPS-  582
Ngo_WTSu_AAA25463.1       VAGPPFKKIMGGSLNILGISPTKPLTAAA-VKTPS-  581
Nla_WP_003709943.1        VAGPPFKKIMGGSLNILGVSPTKPLTAAA-VKTPS-  581
                          *:** *: :*.* *: **: **
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Figure 3: Multiple sequence alignment using BLASTp of portions of the PBP2 sequences from 2 *N. gonorrhoeae* species, 4 commensal *Neisseria* species, and 2 non-*Neisseria* species from the Neisseriaceae family. The sequence for *N. gonorrhoeae* LM306 susceptible strain is bolded. Amino acid positions identified as relevant for antibiotic resistance (highlighted in yellow) that are mutated in more than one commensal are highlighted in orange (known mutations), or blue (undescribed mutations). Kor, *Kingella oralis*, Nel, *N. elongata*; Eik, *Eikenella corrodens*; Nmu, *N. mucosa*; Ngo, *N. gonorrhoeae*; Nci, *N. cinerea;* Nla, *N. lactamica*; WTSu, wild-type susceptible.

The differences observed among PBP2 sequences of commensal *Neisseria* species may partially explain the higher resistance levels observed in these species for beta-lactam antibiotics, penicillin and ceftriaxone. Further analyses of other known antimicrobial

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resistance genes are necessary to explain antibiotic resistance for non-beta-lactam antibiotics, such as azithromycin, chloramphenicol, and erythromycin.

3. Discussion

In this study, we characterized the antimicrobial resistance profiles of 4 commensal *Neisseria* species, closely related to pathogenic *Neisseria*, and clinically relevant given their natural niche, the human oral and nasal pharynx (ONP) [46]. We compared the resistance levels of *N. lactamica*, *N. cinerea, N. mucosa,* and *N. elongata* to 4 *N. gonorrhoeae* strains (FA19, MS11, F89, H041). We observed that *N. cinerea, N. mucosa,* and *N. elongata* generally displayed higher resistance levels than *N. gonorrhoeae* FA19 or *N. lactamica*. Given the high antimicrobial resistance (AR) profiles observed for the commensals, it is possible that commensals present AR genes, mutations, and/or mechanisms not yet identified in *N. gonorrhoeae*. Hence, commensals are likely antimicrobial resistance gene reservoirs for *N. gonorrhoeae*.

Among the ten antibiotics tested, ceftriaxone and azithromycin are particularly relevant as they are the current line of treatment against *N. gonorrhoeae*. Indeed, *N. gonorrhoeae* is treated with a dual therapy of oral azithromycin (1 g, single dose) and injectable intramuscular ceftriaxone (0.25 g, single dose) (or oral cefixime) [47], to which certain gonococcal strains display resistance, such as *N. gonorrhoeae* F89 and H041 from France and Japan, respectively [10,25]. While antibiotic resistance to this dual treatment is increasing, the rates of resistance are still below the 5% threshold needed to recommend new guidelines by the CDC. However, if this treatment fails, clinicians require a time- and resource-consuming antibiogram to assess the antibiotic susceptibility panel of the infectious strain. Gentamicin was the only antibiotic for which all strains displayed a similar level of susceptibility and were categorized as susceptible according to EUCAST and Bala et al. [33]. Gentamicin could be a possible drug of choice to treat extreme drug resistant *N. gonorrhoeae*, as all strains tested in our study show ZoI larger than 16 mm, breakpoint for susceptibility to gentamicin [33]. Additionally, new data associated with gentamicin shows that *N. gonorrhoeae* WHO reference strains present an MIC of 4 μg/mL (dispersion 2-8 μg/mL) [33], which is also consistent with our MIC results for commensal *Neisseria*. However, gentamicin is an aminoglycoside used to treat Gram-negative infections causing bone, urinary tract and respiratory infections, endocarditis, meningitis, and pelvic inflammatory disease. Often, these infections have limited treatment alternatives. Knowing how easily *N. gonorrhoeae* acquires resistance whether through mutation or natural competence, it is absolutely indispensable that scientists and clinicians reflect on and model the outcome of prescribing gentamicin as a future drug of choice for *N. gonorrhoeae*.

In order to explore a potential genetic mechanism for variation in antibiotic resistance profiles, we analyzed protein sequences of PBP2, encoded by the *penA* gene in the four commensal strains along with the focal *N. gonorrhoea* strain. We observed that mutations known to increase antibiotic resistance to beta-lactams were found in *N. cinerea*, *N. mucosa*, and *N. elongata*. However, these mutations were not found in *N. lactamica* which conserves the amino acids present in susceptible strains of *N. gonorrhoeae* strain LM306. These differences may partially explain the reduced susceptibility to beta-lactams observed in the commensals studied. This suggests that genomic analyses of commensal *Neisseria* species for antimicrobial resistance mechanisms could reveal known and new candidate genes and mutations involved in antimicrobial resistance, in particular to azithromycin and ceftriaxone, two antibiotics currently used to treat *N. gonorrhoeae* infections. Further work is needed to explore the potential influence of plasmid-mediated penicillin resistance in commensal *Neisseria* species, which is highly prevalent in *N. gonorrhoeae* strains.

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As with PBP2, several other genes are involved in antimicrobial resistance, such as *mtrR, penA* (PBP2)*, penC, ponA* (PBP1)*, tetM, pilTQ, folP, mtrC, 23S rRNA, rpsJ, 16S rRNA, gyrB, gyrA, parC, prld, porB* (PIB)*.* Genetic analysis of these sequences for commensal *Neisseria* both at the nucleic acid and protein levels could help inform the antimicrobial resistance profile of the pathogen. Antibiotic resistance in commensals has been observed in several genera, often through transformation with plasmids carrying antibiotic resistance genes [48,49]. Antibiotic resistance is widely spread in *N. gonorrhoeae*, and researchers have focused their studies on genomic analyses of AR genes in pathogenic *Neisseria* [11] given the potential for transfer of AR genes between pathogenic *Neisseria* species [50-54]. However, our results and those of Fiore et al. [55] emphasize the need to further investigate AR gene pools in commensal *Neisseria* species. Fiore et al. [55] initiated this work by performing a genomic and phenotypic study of the CDC AR panel of *Neisseria* species, using 6 antibiotics (penicillin, cefixime, ceftriaxone, tetracycline, azithromycin, and ciprofloxacin). Our study further complements that work by analyzing the antibiotic resistance profile of 8 *Neisseria* strains to 10 antibiotics. Together, these studies provide a gateway to understanding bacterial mechanisms of resistance and help to identify putative genes involved in the expression and regulation of these mechanisms.

Moving forward, given observations that antibiotic resistance levels were higher in *N. cinerea, N. mucosa,* and *N. elongata*, it would be critical to further compare genomic sequences of these species, to identify candidate genes involved in resistance mechanisms not yet observed in *N. gonorrhoeae*, and to continue the analysis of phenotypic antibiotic resistance level characterization of other commensal *Neisseria* species.

4. Materials and Methods

Bacterial strains:

Commensal and pathogenic *Neisseria* species were kindly provided by W. M. Shafer (Emory University School of Medicine, Atlanta, GA) and E. Aho (Concordia College, Moorhead, MN). *N. lactamica* strain NRL 36016 [23], *N. cinerea* strain ATCC 14685, *N. mucosa* strain NRL 9297 [23], and *N. elongata* strain ATCC 25295, *N. gonorrhoeae* FA19 [22], *N. gonorrhoeae* MS11[24], *N. gonorrhoeae* F89 [25], *N. gonorrhoeae* H041[10] were stored at -80˚C in GCB broth containing 30% glycerol. Bacteria were plated on GCB agar with Supplements I and II [15], and incubated overnight at 37˚C, in a 5% CO_2 atmosphere. When cultured in liquid broth, GCB liquid media was supplemented with supplements I and II, and 0.043%(w/v) of $NaHCO_3$.

Disk Diffusion Assays:

Neisseria strains plated on GCB agar plates, overnight, at 37˚C in 5% CO_2, were harvested with a sterile loop and suspended in supplemented GCB broth at OD_{600nm} 0.2 UA. Bacteria were spread on the plate using CLSI guidelines [26]. Briefly, a sterile cotton swab was dipped in the suspension and spread in one direction on the plate. The procedure (dip and spread) was repeated two additional times, every time rotating the plate by 120 degrees, to obtain a homogeneous lawn of bacterial growth throughout the plate. Plates were then allowed to dry for 10 min, and antibiotic disks were applied. To prevent overlay of antibiotics or zones of inhibitions (ZoI), we applied 3 antibiotics per plate. Zones of inhibition were measured using AntibiogramJ [27] and ImageJ[28]. Averages and standard deviation (SD) values were obtained from 3 independent experiments, each containing 3 biological replicates. All strains (4 commensal strains and 4 *N. gonorrhoeae* strains (FA19, MS11, F89 and H041), were tested against a panel of 10 antibiotics (Penicillin 10 UI, Kanamycin 30 μg, Streptomycin 10 μg, Azithromycin 15 μg, Ceftriaxone 30 μg, Erythromycin 15 μg, Tetracycline 30 μg, Ampicillin 10 μg, Chloramphenicol 30 μg, Gentamicin 10 μg), on pre-loaded disks (6 mm diameter) purchased from Hardy Diagnostics (Santa Maria, CA), stored at -20’C when not in use.

Minimal Inhibitory Concentrations:

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The minimal inhibitory concentration (MIC) was determined as the concentration of antimicrobial inhibiting 99.99% (or a 4-$\log_{10}$ decrease) of bacterial growth. We used the plate dilution technique to quantify inhibition, following CLSI guidelines [29]. Briefly, three biological replicates of each bacterial species grown overnight on supplemented GCB plates at 37°C, in 5% CO_2, were harvested with sterile plastic loops, and suspended in supplemented GCB broth, at OD_{600nm} 0.2 UA. Five µL of each suspension were plated in triplicates (technical replicates), on supplemented GCB agar plates containing a range of antibiotics, in 2-fold serial dilutions. Plates contained 0.006 µg/mL to 2 µg/mL of Penicillin G, or 4 ng/mL to 512 ng/mL of Ceftriaxone, or 0.031 µg/mL to 4 µg/mL of Azithromycin, or 2 µg/mL to 256 µg/mL of Erythromycin, or 0.125 µg/mL to 16 µg/mL of Chloramphenicol, or 1 µg/mL to 64 µg/mL of Gentamicin. Plates were stored at 4°C and used at most 5 days after plating. Three independent experiments were performed. The concentrations of antibiotics on plates were considered the minimal inhibitory concentrations where less than 4 colonies were observed per spot, as it suggests 99.99% growth inhibition.

Penicillin-binding protein 2 (PBP2) sequence alignment:

Sequences from penicillin-binding protein 2 (PBP2) of several *Neisseria* and non-*Neisseria* Neisseriaceae sequences were obtained from NCBI. Amino acid sequences were aligned using ClustalOmega and BLASTp platforms, using the default parameters.

Nucleotide sequence accession numbers used were *N. gonorrhoeae* LM306 (AAA25463), *N. gonorrhoeae* NG-3 (BAB86942), *N. lactamica* (WP_003709943), *N. cinerea* (WP_003676738), *N. mucosa* (EFC88110), *N. elongata* (WP_107971226), *Kingella oralis* (QMT43252), *Eikenella corrodens* (SNW07260).

Data analysis:

Data were analyzed using R and RStudio [30], and plots were made using ggplot2 [31]. All experiments were performed at least 3 times independently. Each experiment contained 3 biological replicates of each species. Means and standard error of the means (SEM) were reported where applicable on the charts and tables.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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