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Applications of genomics to slow the spread of MDR *Neisseria gonorrhoeae*

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Abstract

Infections with *Neisseria gonorrhoeae*, a sexually transmitted pathogen that causes urethritis, cervicitis, and more severe complications, are increasing. Gonorrhea is typically treated with antibiotics; however, *N. gonorrhoeae* has rapidly acquired resistance to many antibiotic classes, and lineages with reduced susceptibility to the currently recommended therapies are emerging worldwide. In this review, we discuss the contributions of whole genome sequencing (WGS) to our understanding of resistant *N. gonorrhoeae*. Genomics has illuminated the evolutionary origins and population structure of *N. gonorrhoeae* and the magnitude of horizontal gene transfer within and between *Neisseria* species. WGS can be used to predict susceptibility of *N. gonorrhoeae* based on known resistance determinants, track the spread of these determinants throughout the *N. gonorrhoeae* population, and identify novel loci contributing to resistance. WGS has also allowed more detailed epidemiological analysis of transmission of *N. gonorrhoeae* between individuals and populations than previously used typing methods. Ongoing *N. gonorrhoeae* genomics will complement other laboratory techniques to understand the biology and evolution of the pathogen, improve diagnostics and treatment in the clinic, and inform public health policies to limit the impact of antibiotic resistance.

Graphical Abstract

Over the 20 years since the first gonococcal genome was sequenced, 15 finished genomes, 413 draft genome assemblies, and 4795 sequencing runs have been made publicly available. Here, we review the background on gonorrhea, resistant gonococcus, and the ways in which researchers are exploring how to integrate these data to advance clinical care and public health management of gonorrhea.

Keywords

gonorrhea; whole genome sequencing; antibiotic resistance; epidemiology

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Competing interests

The authors declare no competing interests

Introduction

Gonorrhea, caused by *Neisseria gonorrhoeae*, is the second most common sexually transmitted infection (STI) with 468,514 reported cases in the United States in 2016[1] and an estimated 78 million cases worldwide annually[2]. *N. gonorrhoeae* infection rates in the United States have increased yearly since 2013, and rate increases are larger in men than women[1]. *N. gonorrhoeae* is particularly prevalent in men who have sex with men (MSM), and 37.8% of isolates were collected from MSM during surveillance in the United States in 2016[3].

Bacterial pathogens are becoming increasingly resistant to antibiotics, and *N. gonorrhoeae* is no exception. *N. gonorrhoeae* has developed resistance to every antibiotic used for treatment through a variety of mechanisms (Table 1), and dual therapy with ceftriaxone and azithromycin is currently recommended based on the hypothesis that the two drugs together will help to curb the spread of resistant lineages[4]. With the dearth of antibiotic options for gonorrhea and concern for rising levels of resistance in this highly prevalent infection, the Centers for Disease Control and Prevention (CDC) highlighted *N. gonorrhoeae* resistant to cephalosporins as one of three urgent threats among antibiotic resistant bacteria[5].

The World Health Organization (WHO) has identified several strategies to control the spread of antibiotic resistant *N. gonorrhoeae*, centering on improved methods for diagnosis, strengthened detection and surveillance of resistance, and identification of new treatment strategies[2]. Current challenges include rapid identification of gonococcal infection and antibiotic susceptibility, determination of transmission links between individuals and populations, and tailoring treatment and intervention strategies to optimally slow or contain the spread of antibiotic resistance (Box 1).

Box 1

Current challenges and contributions of WGS at different scales

***Neisseria gonorrhoeae* populations**

- Identification of novel resistance and compensatory mutations
- Characterize selective pressures and fitness costs
- Identify targets for vaccines
- Frequency of AMR acquisition and interspecies mosaicism
- Roles of major and minor within-host populations in transmission and susceptibility

In the clinic

- Rapidly identify infection and colonization
- Identify susceptibility of strains and appropriate antibiotic treatment
- Identify clinically relevant mixed infections

Local transmission networks

- Identify links among individuals
- Tailor antibiotic use at local scale
- Translation to local policies and interventions

Global Scales

- Identify links among populations
- Tailor antibiotic use at global scale
- Translation to global policies and interventions

Whole genome sequencing (WGS), along with other methodological and technical innovations, will enable new approaches to address each of these challenges. WGS can be used to identify investigate outbreaks[6], enhance diagnostics by pathogen and antibiotic resistance detection[7–9], and perform pathogen surveillance[10]. Over the 20 years since the first gonococcal genome was sequenced, 15 finished genomes, 413 draft genome assemblies, and 4795 sequencing runs have been made publicly available (as of December 10, 2017). Here, we review the background on gonorrhea, resistant gonococcus, and the ways in which researchers are exploring how to integrate these data to advance clinical care and public health management of gonorrhea.

Neisseria gonorrhoeae: clinical manifestations and origins

The Gram negative diplococcus *N. gonorrhoeae* is a major cause of urethritis as well as of cervicitis and pelvic inflammatory disease (PID)[11]. *N. gonorrhoeae* also causes disease outside of the urogenital tract, including pharyngeal and rectal infection, which tend to be asymptomatic, as well as conjunctivitis, disseminated gonococcal infection with associated mono-and oligoarticular arthritis, and Fitz-Hugh-Curtis syndrome (perihepatitis)[12]. Antibiotic therapy cures gonococcal infections and is indicated for both symptomatic and asymptomatic infections.

N. gonorrhoeae is part of the *Neisseria* genus, with most other species considered commensal oropharyngeal flora. The clinical manifestations of *N. gonorrhoeae* lead to the definition of gonococcus along with *Neisseria meningitidis* (the meningococcus)—the cause of meningococcal meningitis and septicemia—as the pathogenic *Neisseria* (Figure 1)[13]. The standard pigeonholing of these species—while a convenient shorthand—belies the clinical observations that not all the Gram negative diplococci that cause “gonorrhea-like” symptoms are *N. gonorrhoeae.* Meningococcus and *N. lactamica* have been isolated in urethritis cases[14], and a recent outbreak of meningococcal urethritis renewed interest in the question of whether adaptation to the urogenital niche may happen periodically[15–17]. *N. gonorrhoeae* is not as diverse as other *Neisseria* (Figure 1), suggesting the species has arisen relatively recently[18–21] and likely as an offshoot from meningococcus. These observations invite speculation about the origins and ongoing evolution of *Neisseria*, raising the possibility that contact between the oropharynx and other mucosa present opportunities for Neisseria to

adapt to and inhabit new niches, and the confluence of the right genetic and behavior contexts to promote transmission may facilitate emergence of strains of *Neisseria*.

Population structure analyses based on Bayesian methods have identified 5–12 clusters in *N. gonorrhoeae* populations, depending on the data set, and these clusters and major clades defined by phylogenetic analysis have little to no geographic structure[21–24]. In *Neisseria meningitidis*, clade specific restriction modification systems, competition between lineages, and selection by the immune system contribute to the population structure[25,26], but the factors that maintain population structure in *N. gonorrhoeae* have not been identified. The global distribution of these major groups suggests that international transmission of *N. gonorrhoeae* is relatively common.

Acquisition of antibiotic resistance

Antibiotic exposure drives emergence of resistance in gonococcus. These exposures derive from treatment for gonorrhea and potentially through “bystander effect”, during treatment for other infections in individuals who are asymptomatically infected with gonococcus. The variable tissue penetration of antibiotics into the pharynx, a site of gonococcal infections, is one likely source of resistance, as the dose needed to clear urethral or cervical infection may yield a relatively low pharyngeal antibiotic concentration inadequate for eradication and thereby promote resistance emergence. A recent epidemiological study relating annual antibiotic consumption and gonococcal resistance suggested that the pressure from bystander selection may be small to none[27], whereas a study from the Netherlands indicated that previous exposure to azithromycin is associated with higher levels of azithromycin resistance, perhaps reflecting the long half-life of azithromycin and the observation that individuals previously infected with gonorrhea are at higher risk for subsequent infection[28].

In addition to *de novo* acquisition of resistance due to antibiotic exposure, genes and alleles can easily move between *N. gonorrhoeae* lineages, providing another mechanism for acquisition of antibiotic resistance. *N. gonorrhoeae* harbors several mobile genetic elements. Almost all gonococcal isolates encode a small, cryptic plasmid, which can also be integrated into the chromosome[29,30]. A conjugative plasmid can also be found, and a transposon encoding *tetM*, which mediates tetracycline resistance, has inserted into this plasmid and mobilized among *N. gonorrhoeae* (Dutch type, so called because the initially described plasmid of this type was encoded by an isolate from the Netherlands) [3132]. A distinct *tetM* determinant can be found on another plasmid backbone (American type, as it was first described in an isolate from the United States) [31,32], suggesting two introductions of *tetM* mediated resistance into the *N. gonorrhoeae* population[31,33]. A diverse set of plasmids encoding bla_{TEM} beta lactamase are transmitted among *N. gonorrhoeae* populations; however, they are all derivatives of the Asia-type plasmid arising by repeat mediated deletions or duplications[34]. This plasmid likely originated in *Haemophilus* species after a transposon insertion into a cryptic plasmid and was horizontally transferred to *N. gonorrhoeae*[35]. Finally, many *N. gonorrhoeae* contain the gonococcal genetic island (GGI)[36,37], which encodes machinery for a Type IV secretion system (T4SS) that secretes DNA into the environment, as well as proteins of unknown function[38]. Similar T4SSs can be found in other proteobacteria; however, flanking regions are not conserved[39]. DNA secretion

may facilitate horizontal gene transfer, including resistance acquisition, and biofilm formation[36,37,40]. The T4SS can also enable intracellular survival of *N. gonorrhoeae* via iron acquisition by an unknown mechanism, which is independent of DNA secretion[41]. These mobile elements are found in diverse *N. gonorrhoeae* strains and are not confined to a particular clade. Besides the cryptic plasmid, plasmids and the GGI are found at intermediate frequencies in the *N. gonorrhoeae* population, suggesting that a fitness cost to carrying these mobile elements exists in some niches.

Neisseria species are naturally competent [42], with a preference for DNA carrying a DNA uptake sequence (DUS)[43]. In addition to exchanging alleles within the *N. gonorrhoeae* population, *N. gonorrhoeae* will uptake genetic material from other *Neisseria*, including *N. meningitidis, N. lactamica,* and other commensals[44], further implicating the oropharynx as a potential site of resistance acquisition. Mosaicism with other *Neisseria* species has also been an important source for the acquisition of antibiotic resistance in *N. gonorrhoeae.* Most notably, mosaic sequences in *penA* have resulted in resistance to penicillin and reduced susceptibility to extended spectrum cephalosporins (ESCs)[45,46], and a mosaic *mtr* operon has been shown to be associated with azithromycin resistance in the United States[22,47] and Australia[48,49]. In a dataset of *N. gonorrhoeae* isolated in the United States, six percent of recombinant tracts matched sequences found in other *Neisseria*, including an acquisition of *tbpB* from *N. meningitidis*[50]. Multiple *N. gonorrhoeae* lineages have acquired *porA* sequences from *N. meningitidis*, replacing the gonococcal *porA* pseudogene that is occasionally used for diagnostics[51]. Exchange among *Neisseria* species is not unidirectional, and urethritis-associated *N. meningitidis* isolates contain recombinant tracts originating from *N. gonorrhoeae*[17].

Despite being a potential reservoir of resistance determinants for pathogenic species, the prevalence and mechanisms of antibiotic resistance in commensal *Neisseria* is largely unknown. Interspecies exchange of *penA* sequences has long been recognized as contributing to penicillin resistance in both pathogenic and commensal *Neisseria*[52–54]. Transformation of *N. gonorrhoeae* with *penA* sequences from a ceftriaxone resistant *Neisseria cinerea* isolate resulted in transformants with MICs to ESCs similar to the donor and a mosaic *penA* similar to alleles found in clinical *N. gonorrhoeae* isolates with high level ceftriaxone resistance[55]. It is unknown if the *penA* sequences originated in *N. cinerea* or were horizontally transferred to both *N. cinerea* and *N. gonorrhoeae* from an unknown source. The only survey of commensal *Neisseria* resistance to ESCs showed that 93% and 100% of a *Neisseria subflava* sample from Japan were susceptible to cefixime and ceftriaxone, respectively[56]. Resistance to penicillin, tetracycline, and ciprofloxacin was also identified; however, the genetic mechanisms of resistance were not determined. Commensal *Neisseria* have also been shown to encode *erm* genes, which mediate macrolide resistance[57].

From pathogen to superbug: treatment and resistance

N. gonorrhoeae has routinely developed resistance to each of the antibiotics used to treat it [58]. Resistance to sulfonamides and low level resistance to penicillin emerged in the 1940s[59,60]. Penicillin continued to be the most common treatment for gonorrhea for several decades despite slowly increasing resistance due to chromosomal mutations. High-level

resistance to penicillin via plasmid and chromosomally mediated mechanisms were reported in 1976 and 1986, respectively[61,62]. Tetracycline resistance can also be mediated by both determinants on plasmids or the chromosome, and *N. gonorrhoeae* harboring *tetM* encoding plasmids were first described in 1986[32]. Clinical trials to study the effectiveness of ciprofloxacin to treat gonorrhea began in 1983[63], and, though it was not recommended as therapy in the United States until 1993[64], reduced susceptibility to ciprofloxacin had already been reported as early as 1990[65]. In 2007, with rapidly rising prevalence of resistance, the CDC recommended that ciprofloxacin no longer be used for gonorrhea treatment[66]. In Southeast Asia and the Western Pacific, rates of ciprofloxacin resistance remain extremely high (> 90% in 2014)[67]. Even as first line antibiotic therapies have changed, resistance to older therapies remains high in *N. gonorrhoeae* populations across the globe[68–72]. For example, 44.1% of isolates collected by GISP in 2016 were resistant to penicillin, tetracycline, and/or ciprofloxacin[1]. With the idea that dual therapy would be helpful to halt the development of resistance to the remaining effective antibiotics, the CDC currently recommends ceftriaxone and azithromycin dual therapy[73]. However, resistance to azithromycin and ESCs is emerging[74], and treatment failure with this regimen has been reported[75].

Since susceptibility is not routinely measured in the clinic, the monitoring of antibiotic resistance prevalence falls to surveillance programs, which measure levels of resistance in the *N. gonorrhoeae* population and inform treatment guidelines. In the United States, the Gonococcal Isolates Surveillance Project (GISP) collects the first 25 urethral isolates each month from sentinel clinics for susceptibility testing. This project is being enhanced (eGISP) to include isolates from additional body sites and collect additional demographic and behavioral data from patients through the STD Surveillance Network (SSuN). Similarly, the European Gonococcal Antibiotic Surveillance Programme (GASP) monitors rates of gonorrhea and antibiotic resistance in 27 European countries. There are also active surveillance efforts in China (China GASP), Australia (Australia GSP), and the United Kingdom (GRASP, Sexually Transmitted Bacteria Reference Unit (STBRU)). In a partnership between CDC and WHO, the Enhanced Gonorrhea Antimicrobial Surveillance Project (EGASP) was initiated in 2015 to monitor antibiotic resistance worldwide, particularly in high risk populations[76]. Currently, surveillance data is particularly sparse in Central America, Africa, and Central Asia[67].

Since there is no point-of-care antibiotic susceptibility testing, antibiotic selection for gonorrhea treatment is based on recommended drugs and doses per national or regional guidelines. A recent review of *N. gonorrhoeae* treatment guidelines show that organizations suggest 250–500 mg of ceftriaxone intramuscularly and 1–2 g azithromycin orally for treatment of uncomplicated infections; 400–800 mg cefixime orally is provided as an alternative to ceftriaxone in WHO and Canadian guidelines[77]. The WHO also recommends ceftriaxone single therapy. However, this review was limited in scope to guidelines published in English, and guidelines from Africa, Asia, and South America were not included beyond WHO recommendations. To limit empiric treatment failures, it has been the practice to switch the recommended first-line antibiotics when prevalence of resistance exceeds 5%, though it is worth noting that more nuanced strategies for optimizing antibiotic use are needed.

The clonality and number of times drug resistant lineages have emerged varies by drug and mechanism of resistance. Chromosomal mutations mediating low level resistance to beta-lactams and tetracycline are common in *N. gonorrhoeae* isolates, and they are widespread across the phylogeny[22]. While azithromycin resistance is less common than resistance to previously used drugs, resistance or reduced susceptibility has arisen across a large number of genomic backgrounds[22,78,79]. In contrast, reduced susceptibility to ESCs is primarily clonal, and the majority of ESC-RS isolates belong to only a few major lineages[22,50]. In isolates from the United States, azithromycin reduced susceptibility is estimated to have been acquired 75 times, and reversion to susceptibility is also relatively frequent (42 times)[22]. In contrast, ciprofloxacin resistance has only been acquired 11 times, but there are far more ciprofloxacin resistant isolates in the data set (594 vs. 294 AZI-RS)[22].

An impediment to the emergence of antibiotic resistant bacteria is the fitness cost incurred by resistance in environments without antibiotic selective pressure. Resistant bacteria have been shown to be less fit than their susceptible counterparts both *in vitro* and *in vivo* for several bacterial species, and resistant bacteria can mediate these costs by acquiring compensatory mutations[80]. However, fitness costs of resistance and compensatory mutations in *N. gonorrhoeae* are understudied. The continued prevalence of penicillin, tetracycline, and ciprofloxacin resistant lineages despite discontinued use of these antibiotics has been cited as evidence that there may be little to no fitness costs for these resistance determinants. Experimental work shows that *mtr* mutants have increased fitness in a mouse model[81,82]. The most common mutations in *gyrA* conferring ciprofloxacin resistance also do not have a cost in the mouse model, but the addition of *parC* mutations which appear in clinical isolates does result in lower fitness[83]. A modelling study by Whittles and colleagues using data on rates of cefixime susceptibility and cefixime use for gonorrhea treatment in England suggests that cefixime resistance imparts a fitness cost compared to susceptible lineages[84]. Finally, reversion from resistant to susceptible phenotypes have been observed in *N. gonorrhoeae*, which may indicate a fitness cost for resistance[22].

Interactions between resistance loci may also influence the emergence of multi-drug resistant lineages. Resistance correlates across antibiotics, and many *N. gonorrhoeae* isolates are resistant to multiple drugs (17.6% of isolates collected by GISP in 2016)[85,86]. Resistance determinants in *N. gonorrhoeae* have been shown to have additive effects. For example, successive mutations in *penA*, *mtr*, *porB*, *ponA,* and *pilQ* contribute to high level penicillin resistance[62,87,88]. Of particular concern currently are lineages that have reduced susceptibility to both azithromycin and ESCs. In isolates sampled across the United States from 2000–2013, high level AZI resistance and ESC reduced susceptibility appeared to be anti-correlated, and depending on the genomic background, the presence of 23S rRNA mutations lowered MICs for other antibiotics[22]. Isolates with reduced susceptibility to both azithromycin and ESCs are circulating in Canada[89], and a cluster of isolates with high level resistance to azithromycin and reduced susceptibility to ESCs was identified in Hawaii, suggesting ongoing transmission of this lineage[90]. Interestingly, these isolates were more closely related to an isolate associated with failed azithromycin and ceftriaxone dual therapy in England[75] than other contemporaneous isolates from Hawaii.

Azithromycin resistance and ESC reduced susceptibility have emerged on several genomic backgrounds. However, some clones are highly successful and have spread across the globe, while others seem to result in few descendants or resistance is lost[22]. One of the two major ESC-RS clades in the United States, for example, seems to have ceased transmission after 2011[22]. Some lineages encoding azithromycin resistance are associated with international transmission. For example, an azithromycin resistant *N. gonorrhoeae* lineage has emerged in Scotland[91] and spread to England and Wales[92] causing outbreaks[78]. This lineage has also been observed elsewhere in Europe[79] and in Australia[49]. Whether success is attributable to mutations facilitating acquisition and maintenance of resistance determinants and sustained transmission remains unclear. Understanding the interactions between fitness costs of resistance, compensatory mutations, and transmission of resistant lineages is an important step in controlling the spread of antibiotic resistance in *N. gonorrhoeae.*

Opportunities for addressing AMR gonorrhea: rapid diagnosis and antibiotic susceptibility assessment

The majority of gonorrhea cases are diagnosed via a nucleic acid amplification test (NAAT), through which *N. gonorrhoeae* genetic material is detected in patient samples. Culture is rarely done, though it remains the only basis for assessing the minimum inhibitory concentrations (MIC) for anti-gonococcal antibiotics.

Gonorrhea is currently treated empirically based on the extent of resistance described in surveillance systems. While resistance to previously effective antibiotics remains high in many areas of the world, a large fraction of isolates remain susceptible to older antibiotics and in some regions,[68], most isolates remain susceptible to all drugs. This has led to the idea that a point-of-care diagnostic (POC) that detects antibiotic resistance—akin to the use of detection of *rpoB* mutations to rapidly identify resistant TB[93,94]—would increase treatment options, permitting use of antibiotics shelved because of concern for the high fraction of empiric treatment failures.

A real-time PCR based assay targeting *gyrA* has been shown to accurately detect ciprofloxacin resistance from patient samples (sensitivity: 95.8%, specificity: 100%)[95]. POCs have also been developed for penicillin susceptibility; however, accurate predictions require assessment of multiple genetic loci [96,97]. Modeling suggests that point of care testing for antibiotic resistance has the potential to slow the spread of resistance[98]; however, a test for ciprofloxacin alone will not delay the increases in resistance to azithromycin and ESCs, whereas a POC for all three antibiotics would delay the rising levels of resistance[99].

The increasingly portable and rapid turnaround of sequencing technologies may make sequencing in the clinic more practical. Just as nanopore technology has been used for real time epidemiological investigations for recent Ebola and Zika outbreaks[100,101], preliminary work has demonstrated the feasibility of using the Oxford Nanopore MinION system as a gonococcal diagnostic, sequencing and predicting antibiotic susceptibility of *N. gonorrhoeae* isolates in less than 2 hours[102].

One potential advantage of WGS in a diagnostic test is that it can assay for multiple antibiotic resistance determinants at the same time. Several tools have been developed to predict antibiotic resistance from WGS data[8,9]. Mykrobe[8] and ARIBA[9] can identify resistance determinants directly from sequencing reads, eliminating the need for genome assembly. Eyre and colleagues (2017) found that the MICs for antibiotics commonly used to treat gonorrhea can be predicted from WGS data for 52% of isolates, and MICs within 2 dilutions of the observed MIC were predicted for 98% of isolates[103].

Ability to predict susceptibility profiles of clinical isolates is dependent on the comprehensiveness and quality of resistance allele databases as well as understanding of how loci interact to generate resistance. Positive and negative predictive values of known resistance alleles vary by antibiotic [37]; for example, mutations associated with ciprofloxacin resistance are highly predictive, but in a recent WGS study of resistant isolates in the United States, 36% of reduced susceptibility to azithromycin could not be explained by known mutations[22]. Continued surveillance and identification of novel resistance mutations should be an ongoing effort.

Genomic epidemiology approaches to reducing the transmission of AMR gonorrhea

Prior to the advent of WGS, several typing schemes based on DNA sequences were developed to define genetic relationships between *N. gonorrhoeae* isolates and track the emergence and spread of drug resistant gonorrhea. Multi-locus sequence typing (MLST), a technique used for many bacterial species, uses the sequence of defined portions of a small number of genes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm* in *Neisseria* species) to assign an isolate to a sequence type (ST)[104]. *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), another typing scheme, is based on the sequence of the hypervariable portions of *porB* and *tbpB*[105]. Recently, targeted sequencing of known resistance determinants has also been utilized, and typing based on resistance profiles and housekeeping genes has been proposed (NG-STAR)[106]. Online resources for typing based on these schemes are available at https://pubmlst.org/, http://www.ng-mast.net, and https://ngstar.canada.ca/.

Typing of bacterial strains has long been a complement to typical epidemiological practices when investigating an outbreak. While WGS is generally concordant with traditional typing methods like MLST and NG-MAST, it can provide increased resolution and rule out potential transmission links that may be inferred by these methods[107]. For example, in a study of *N. gonorrhoeae* specimens from Brighton, UK, 8.8% of isolates collected within 28 days of each other had the same NG-MAST type, but only 3.6% of isolates were compatible with direct or indirect transmission according to WGS results[24]. In British Columbia, monitoring of a database of HIV genotypes allowed for identification of a growing transmission cluster and timely public health follow-up[108]; *N. gonorrhoeae* WGS may similarly allow for identification of expanding lineages and prevention of further onward transmission.

Since *N. gonorrhoeae* has a calculable substitution rate at short genetic distances, the WGS data can be combined with dates of collection to infer likely transmission links[24]. Moreover,

the consistency of estimates of the *N. gonorrhoeae* substitution rate suggests that the transmission nomogram calculated by De Silva and colleagues (based on a substitution rate of 3.55 SNPs per genome per year) could be applicable to transmission of gonorrhea in other settings. Hypermutator strains—which would have a faster substitution rate—have been observed in other pathogens, including epidemic clones of *N. meningitidis*[109] and antibiotic resistant *Pseudomonas aeruginosa*[110], but gonococcal hypermutators have not been described.

Combined with patient metadata, WGS can reveal information about the demographics of contact networks. For example, clusters likely associated with transmission in Brighton, UK contain isolates from both HIV positive and HIV negative patients[111], and clusters associated with heterosexual women in Australia contain patients across a range of ages[112]. In the United States, lineages with reduced susceptibility to cefixime were found to be primarily circulating among men who have sex with men (MSM), and transmission from MSM to men who have sex with women (MSW) occurred more often than MSW to MSM transmission[50]. WGS of *N. gonorrhoeae* populations in New Zealand shows that the majority of clusters contain isolates from both men and women, suggesting the absence of clones associated with exclusively MSM transmission[23]. While the *N. gonorrhoeae* global population does not show geographic structure, examining WGS of isolates from smaller scales (i.e. London) show that transmission links are associated with shorter geographic distances [24]. Beyond inferring likely transmission links, phylogenetic analyses utilizing a molecular clock enable additional inferences. Calculating the time to most recent common ancestor (TMRCA) of isolates from pairs of known contacts in Sheffield, UK enabled estimation of the average duration of infection in this primarily heterosexual population to be 3.4 months[107].

WGS can also be used to track the spread of resistance across geographic boundaries. For example, a cluster of cefixime resistant gonococcus mediated by mosaic *penA* XXXIV spread from the west coast to the east coast of the United States[50]. The TMRCA of this cluster was estimated to be 1997, and the addition of subsequently sequenced isolates from the UK confirmed this estimate[24]. Nine percent of infections in Brighton, UK were found in transmission clusters with US isolates, indicating that intercontinental spread of *N. gonorrhoeae* is common, which is also supported by the lack of geographic structure in *N. gonorrhoeae* populations[24]. Frequent international transmission of resistant strains poses a challenge to local control efforts.

Ongoing areas of research

Even as we understand many of the most common genetic pathways to antibiotic resistance, our catalog of resistance mechanisms and pathways remains incomplete (Table 2). Genomic data provide a rich source of information that can be used to define the variants that cause resistance and their interaction with other loci in the genome. Bacterial genome wide association studies (GWAS)[113–115] have successfully identified known and potentially novel antibiotic resistance mutations in other bacterial pathogens like *Mycobacterium tuberculosis* and *Escherichia coli*[114,116]. Genomic studies of *N. gonorrhoeae* have identified isolates without known resistance determinants (Table 2), suggesting that in addition to GWAS other

methods, such as RNAseq and Tn-Seq, may be needed to identify the cellular responses to and essential genes required under exposure to antibiotics[117–120]. While the selective pressures applied in the laboratory are unlikely to fully replicate those pressures in the context of human infection and transmission, analysis of clinical *N. gonorrhoeae* isolates can identify loci under purifying or diversifying selection in the natural environment.

Drug resistance alleles are likely to interact with the genomic background and other resistance determinants. For example, the presence of a mosaic *penA* sequence is not a perfect predictor for reduced susceptibility to cefixime and ceftriaxone, suggesting that additional variants contribute to this phenotype[22]. In a model for MIC prediction, some resistance alleles had synergistic interactions where the presence of both alleles increased MIC more than the combination of their individual effects while other combinations did not increase MIC above levels associated with a single allele[103]. For example, *penB* and *mtrR* promoter mutations appear to have a synergistic effect, and conversely, the addition of *rpsJ* or *mtrR* mutations do not increase tetracycline MICs above levels of resistance conferred by *tetM* alone. GWAS may be helpful to identify additional variants that interact with resistance alleles, including compensatory and enabling mutations that allow resistant lineages to successfully compete with their susceptible counterparts.

WGS of patient samples may also illuminate the extent of within-host diversity and the impact of mixed infections on pathogenicity, transmission, and drug resistance. Horizontal gene transfer and recombination among *N. gonorrhoeae* provide evidence of mixed infections, and several studies of genetic loci and genome sequencing have further supported mixed infections. Martin and Ison found evidence of mixed infection using *opa* typing, which is based on restriction enzyme digestion of PCR amplified *opa* genes[121,122]. Different *opa* profiles were found in urethral and cervical swabs but not in cultured samples. Using DNA-DNA hybridization of *porB*, Lynn and colleagues found evidence for mixed infection in 21% of samples[123]. Using WGS, De Silva found evidence for distinct strains at different anatomical sites in 13% of pairs when multiple samples were obtained from the same patient[24]. The clinical importance of mixed infections remains unclear, though a concern about DNA-based resistance prediction is that these tests may have insufficient sensitivity to detect clinically meaningful resistant subpopulations. Within-host diversity may also interfere with reconstruction of transmission networks when the genome of a single isolate per patient is sequenced[124]. The amount of diversity that accumulates during *N. gonorrhoeae* infection and the reduction of diversity that occurs during transmission are unknown. The impact of diversity on transmission inference is likely to be higher in networks containing asymptomatic patients where duration of infection is longer.

Many public health labs are now incorporating WGS into pipelines to identify and type *N. gonorrhoeae* and other pathogens. However, WGS of every patient sample may not be feasible in all settings, and other technologies for POC testing may prove to be faster and more economical for routine care. Further research is needed to determine the optimal role of WGS in public health, including establishing how to sample the isolates for sequencing [58]. For WGS to contribute to goals to reduce the burden of AMR *N. gonorrhoeae*, results from WGS—including reconstruction of transmission networks and inferences about

connections between demographic and geographic groups—must be reliable and translatable to cost-effective interventions.

Further, routine WGS in clinical and public health laboratories comes with a new set of technical and ethical challenges. Introducing WGS into clinical labs will require development of user-friendly tools to analyze sequence data and/or bioinformatics training for clinical scientists; it will be important to ensure that sequencing platforms and training on how to use them are widely available. WGS directly from patient samples has similar ethical challenges to human microbiome research, as sequences from both the patient as well as other pathogens may be captured[125]. Additionally, during outbreak investigations, WGS can provide more precise information than older molecular techniques, and unreported transmission links may be discovered [24]. Guidelines for how to interpret and report incidental findings from sequence data to patients and public health authorities will need to be established. Rapid sharing of genomic data from *N. gonorrhoeae* outbreaks will be key to enact timely interventions, but sequence data must be carefully de-identified and cleaned of human sequence data to protect patient privacy.

As a gonococcal vaccine would be a transformational intervention, much effort is being put into development of candidates. However, efforts to date have been stymied by *N. gonorrhoeae*'s evasion of the immune system through extensive variation in surface antigens. Two vaccines have proceeded to clinical trials in humans but were unsuccessful[126]. Recently, a retrospective study found that the outer membrane vesicle (OMV) meningococcal B vaccine showed some effectiveness against infection by *N. gonorrhoeae* in New Zealand; vaccination reduced incidence of gonorrhea by an estimated 31% compared to the unvaccinated population[127]. Reverse vaccinology, or the use of microbial genomic information to identify vaccine targets[128], has been successfully used to develop vaccines for *N. meningitidis* serogroup B, a bivalent fHbp vaccine and a multicomponent vaccine[129–131]. Two of the three main antigens from the multicomponent vaccine are also present in *N. gonorrhoeae*: factor H binding protein (fHbp) and neisserial heparin-binding antigen (NHBA)[132]; however, some *N. gonorrhoeae* isolates contain premature stop codons in these genes[133]. The efficacy of OMV-containing meningococcal B vaccines against *N. gonorrhoeae* infection remain unknown but are of significant interest. Information gleaned from genomics could be used to identify novel vaccine targets that are conserved across the *N. gonorrhoeae* population. Other efforts to develop vaccines, including from non-protein antigens, offer promising avenues of research[126].

Conclusion

With the rise of multidrug resistant *N. gonorrhoeae*, we urgently need to develop new tools for management of gonococcal infections—a need that extends from the development of new therapeutics to improved diagnostics and public health interventions and strategies for how to implement these tools optimally. WGS has the potential contribute to each of these efforts by improving our understanding of *N. gonorrhoeae* at multiple scales, from the biology of the microbe to global evolution and transmission dynamics. GWAS has the potential to identify new resistance determinants as they emerge, and increased understanding of epistatic interactions and fitness costs will inform best practices for

treatment of gonorrhea. WGS of patient samples can improve diagnostics and illuminate the role of intra-host diversity. Finally, gonorrhea surveillance incorporating WGS can more accurately capture transmission dynamics within outbreaks and between regions, which will inform public health strategies to control the spread of antibiotic resistance.

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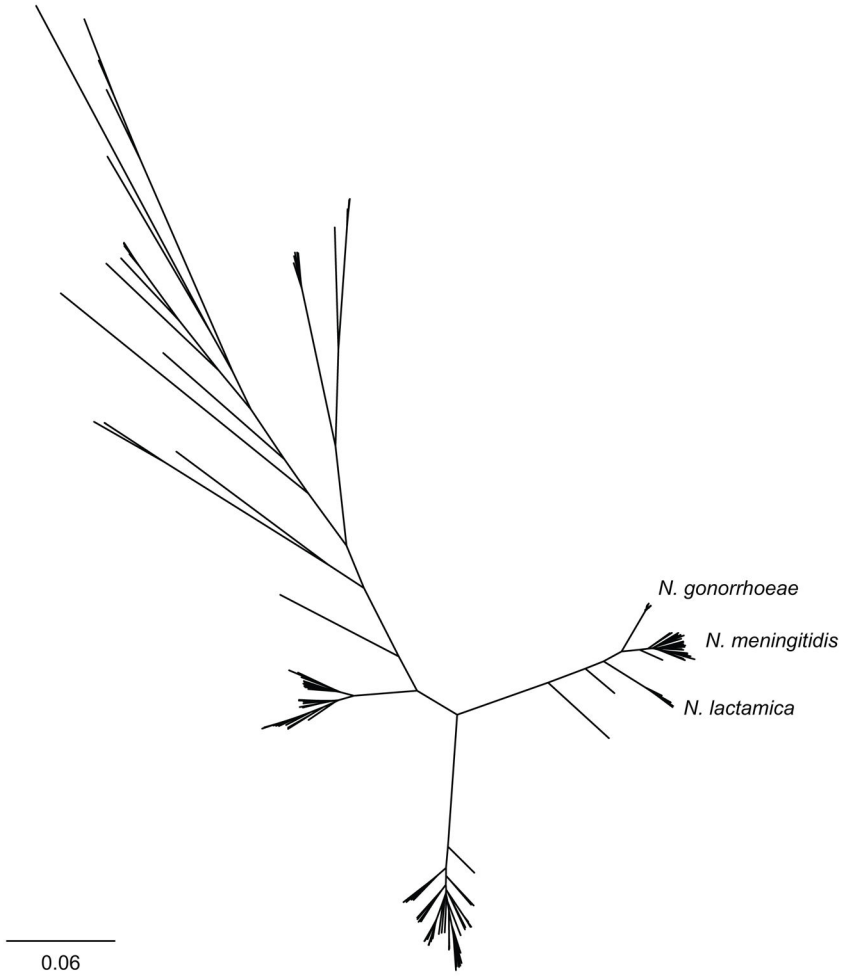

Figure 1.

Phylogeny of *Neisseria*. All publicly available genomes from the *Neisseria* genus were downloaded from NCBI (as of December 30, 2017) and annotated with Prokka v 1.12[134]. *N. gonorrhoeae* and *N. meningitidis* were subsampled to 20 genomes. Core genes (n = 236) were identified and aligned with Roary v 3.12.0[135]. Approximate maximum likelihood phylogenetic analysis was performed used FastTree v 2.1.9[136]. The phylogeny was visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Branches are scaled by substitutions per site. Data are available at 10.6084/m9.figshare.5877486.

Table 1

Mechanisms of antibiotic resistance in *Neisseria gonorrhoeae*

Drug	MIC breakpoints[137] (μg/mL) S, R	Gene (codon/allele)	Mechanism	Year
Sulfonamides		*folP* (R228)[138,139]	TM	2005
Penicillin (PEN)	≤0.06, ≥2	*penA*[45,140]	TM	1975
		porB (G120K, A121D/N)[140,141]	P	1975
		mtrR (A39T, G45D, 1 bp del in promoter)[140,142–144]	E	1975
		bla_{TEM}[61]	I	1976
		ponA (L421P)[87]	TM	2002
		pilQ (E666K)[87,88]	P	2002
Tetracycline (TET)	≤0.25, ≥2	*porB* (G120K, A121D/N)[140,141]	P	1975
		mtrR (A39T, G45D, 1 bp del in promoter)[140,142–144]	E	1975
		tetM[32]	TM	1986
		pilQ (E666K)[87,88]	P	2002
		rpsJ (V57M)[145,146]	TM	1974
Ciprofloxacin (CIP)	≤0.06, ≥1	*gyrA* (S91F, D95A/N/G)[147,148]	TM	1994
		parC (D86N, S87R/N, S88P, E91K)[147,149]	TM	1994
		parE (G410V)[148]	TM	2002
		norM (−35 promoter sequence, RBS)[150]	E	2003
Spectinomycin	≤32, ≥128	*rpsE* (T24P, deletion V27, A82G)[151,152]	TM	2013
		16S rDNA (C1187)[153]	TM	2000
Azithromycin (AZI)		*mtrR* (A39T, G45D, 1 bp del in promoter)[140,142–144]	E	1975
		mosaic mtr operon[47]	E	2016
		ermBCF[57]	I	1999
		23S rDNA (C2611T, A2059G)[154,155]	TM	2002
		mef[156]	E	2000
		macAB (−10 promoter sequence)[150]	E	2003
		rplV (3′ tandem duplications)[22]	TM	2016
		rplD (G68, G70)[22]	TM	2016
Cefixime (CFX)	≤0.25, -	mosaic *penA*[46,157]	TM	2002
Ceftriaxone (CRO)	≤0.25*, -	mosaic *penA*[157]	TM	2005
		porB[158]	P	2009
		mtrR[158]	E	2009

TM = target modification, P = permeability, E = efflux, I = inactivation.

*Reduced susceptibility to CRO is also described as ≥0.125 μg/mL

Table 2

Genomic studies of gonococcus

Location	Year	*n*	Resistant isolates without known mechanisms	Reference	Accession
United States	2009–2010	236	-	Grad *et al.* 2014[50]	PRJEB2999
Canada	1982–2008	25	-	Vidovic *et al.* 2014[159]	-
Canada	1989–2013	169	27 CRO-RS without mosaic *penA*	Demczuk *et al.* 2015[160]	PRJNA266539
Global	1982–2013	61	1 CFX-RS 1 AZI-RS 1 TET-R	Ezewudo *et al.* 2015[21]	SRA099559
England	2014–2015	15	-	Chisholm *et al.* 2016[78]	-
France	2010–2014	4	1 AZI-RS	de Curraize *et al.* 2016[161]	PRJEB13093
Canada	1989–2014	236	-	Demczuk *et al.* 2016[162]	SRP065041
UK	2004–2015	1842	-	De Silva *et al.* 2016[24]	PRJNA315363
UK	1995–2000	237	-	Didelot *et al.* 2016[107]	PRJEB2124
United States	2000–2013	1102	106 AZI-RS 5 CIP-R	Grad *et al.* 2016[22]	PRJEB7904
Global		289	-	Harrison *et al.* 2016[37]	Previously published
Europe	2009–2014	75	-	Jacobsson *et al.* 2016[79]	PRJNA322768
Global		14	-	Unemo *et al.* 2016[139]	PRJEB14020
UK	2014–2015	100	-	Peters *et al.* 2017[111]	Previously published
US, Brazil		804 + 118	-	Vidyaprakash *et al.* 2017[163]	Previously published
Ireland	2008–2014	14	-	Mac Aogáin *et al.* 2017[164]	PRJNA275092
New Zealand	2014–2015	398	2 CIP-R 6 Spec-RS 1 CFX-RS	Lee *et al.* 2017[23]	PRJNA394216
US (Hawaii)	2011–2016	61	-	Papp *et al.* 2017[90]	-
Australia	2005–2014	94		Kwong *et al.* 2017[165]	PRJEB17738
Brazil	2006–2015	116	3 PEN-R	Costa-Lourenço *et al.* 2018[166]	-
Australia	2012–2014	94	-	Buckley *et al.* 2018[112]	PRJNA392203
UK	2014–2017	180	-	Fifer *et al.* 2018[167]	PRJEB23008
Australia	2011–2013	59	-	Al Suwayyid *et al.* 2018[168]	-

Location	Year	*n*	Resistant isolates without known mechanisms	Reference	Accession
Kenya	2010–2015	103	-	Cehovin et al. 2018[169]	PRJEB10104
Japan, Australia	2015, 2017	4	-	Lahra et al. 2018[170]	PRJNA416507

Studies were included when > 1 isolate was sequenced. -R = resistant, -RS = reduced susceptibility.