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Genomic sequencing of *Neisseria gonorrhoeae* to respond to the urgent threat of antimicrobial-resistant gonorrhea

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Abstract

The development of resistance of *Neisseria gonorrhoeae* to available first-line antibiotics, including penicillins, tetracyclines, fluoroquinolones and cephalosporins, has led to the circulation of multidrug-resistant gonorrhea at a global scale. Advancements in high-throughput whole-genome sequencing (WGS) provide useful tools that can be used to enhance gonococcal detection, treatment and management capabilities, which will ultimately aid in the control of antimicrobial resistant gonorrhea worldwide. In this minireview, we discuss the application of WGS of *N. gonorrhoeae* to strain typing, phylogenomic, molecular surveillance and transmission studies. We also examine the application of WGS analyses to the public health sector as well as the potential usage of WGS-based transcriptomic and epigenetic methods to identify novel gonococcal resistance mechanisms.

One sentence summary:

This minireview examines the application of whole-genome sequencing methodologies to respond to the urgent threat of antimicrobial-resistant gonorrhea.

Keywords

Neisseria gonorrhoeae; gonorrhea; whole-genome sequencing; antimicrobial resistance

INTRODUCTION

The discovery of antibiotics led to a revolutionary era of medicine that provided a simple and effective way to combat both common and deadly diseases. While the development of resistance to antimicrobial agents is an adaptive mechanism, the extensive and improper use of antibiotics in both medical and agriculture communities has ushered in a period of significant and problematic antimicrobial resistance (AMR) (Khachatourians 1998; Andersson and Levin 1999). Moreover, the extent of AMR has led to the evolution of multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogens that are

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commonly referred to as superbugs, including resistant strains of *Klebsiella pneumonia, Escherichia coli, Staphylococcus aureus, Clostridium difficile* and *Neisseria gonorrhoeae* (NG) (Brazier 2008; Ippolito *et al.* 2010; Unemo and Shafer 2014; WHO 2017). *Neisseria gonorrhoeae* is the etiological agent that causes the sexually transmitted infection gonorrhea, and both MDR and XDR strains of this pathogen have been identified (Tapsall *et al.* 2009; Ohnishi *et al.* 2011; Unemo *et al.* 2012). Tapsall *et al.* (2009) defined MDR NG isolates as those that are resistant to either extended-spectrum cephalosporins (ESCs) or spectinomycin (i.e. category I antibiotics), plus at least two of the following antibiotic classes: penicillins, fluoroquinolones, azithromycin, aminoglycosides and carbapenems (i.e. category II antibiotics). XDR isolates are defined as those that are resistant to category I antibiotics and three or more category II antibiotics. The identification of both MDR and XDR strains emphasizes the critical need to continually monitor NG antimicrobial susceptibility patterns (CDC 2016).

Gonorrhea is the second most commonly reported notifiable disease in the USA (CDC 2016), and the World Health Organization (WHO) estimated 78 million new cases (among adults aged 15–49 years) worldwide in 2012 (Newman *et al.* 2015). Since initial reports of acquired resistance to sulfonamides in the early 1940s, NG strains have readily developed resistance to each monotherapeutic antibiotic treatment (Unemo, Del Rio and Shafer 2016). Moreover, the development of NG resistance to available first-line antibiotics, including penicillins, tetracyclines, fluoroquinolones and cephalosporins, has been mediated by both plasmid- and chromosome-mediated mechanisms (Tapsall 2001). NG is naturally competent for DNA transformation and the uptake and incorporation of foreign DNA into *Neisseria* genomes can occur during all growth phases (Biswas *et al.* 1977). This characteristic enables the spread of AMR determinants, and oral commensal *Neisseria* species are likely significant contributors. Oral commensal species are repeatedly exposed to antimicrobial agents, and this establishes an environment that facilitates the acquisition of resistance genes (Lewis 2015). These resistance genes are likely acquired by NG associated with asymptomatic pharyngeal infections. Therefore, the development of plans to combat and control AMR gonococcal strains is critical.

The development of resistance to available first-line antibiotics led the Centers for Disease and Prevention (CDC) to recommend a dual-use treatment of ceftriaxone with either azithromycin or doxycycline as treatment for uncomplicated gonorrhea (Workowski and Bolan 2015), and similar guidelines were established by other countries, regions and organizations, including Australia, Canada, Europe, and the WHO (Bignell and Unemo 2013; PHAC 2013; Australian Sexual Health Alliance 2016; WHO 2016). Furthermore, countries and organizations have implemented surveillance projects such as the Gonococcal Isolate Surveillance Project (GISP) and the STD Surveillance Network in the USA, the surveillance program run by the Public Health Agency of Canada (PHAC), the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) in the United Kingdom and the Gonococcal Antimicrobial Surveillance Programme (GASP) run by the WHO, which monitors several regions worldwide (WHO 2012; Cole *et al.* 2014; PHAC 2015; CDC 2016; Chen *et al.* 2016; Kubanov *et al.* 2016; Public Health England 2016; Trembizki *et al.* 2016). Increased surveillance in combination with retrospective studies has elucidated patterns of resistance in several countries. For instance, gonorrhea surveillance in the USA, based on

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GISP data, indicated that the prevalence of NG isolates exhibiting reduced susceptibility to ceftriaxone (MIC ≥ 0.125 μg/mL) was 0.1%–0.4% between 2007 and 2015 (CDC 2016). The prevalence of reduced susceptibility to cefixime (MIC ≥ 0.25 μg/mL) declined from 1.4% to 0.5% between 2011 and 2015, but increased to 0.8% in 2014 (CDC 2015, 2016). Therefore, these data indicate that resistance to cephalosporins is currently evolving in US populations. Regarding azithromycin resistance, the percentage of resistant isolates increased from 0.6% to 2.6% between 2013 and 2015 (CDC 2016). The lack of novel antimicrobial treatments in the development pipeline makes the study and surveillance of resistance to previously used antibiotics necessary. An examination of resistance to spectinomycin, ciprofloxacin, penicillin and tetracycline indicated that all isolates collected through GISP in 2014 were susceptible to spectinomycin (CDC 2015), and 39.6% of isolates collected in 2015 were resistant to penicillin, tetracycline or ciprofloxacin (CDC 2016). Furthermore, an analysis of isolates collected in 2011 through Euro-GASP, representing 21 European countries, found that 7.6%, 5.3% and 48.7% of the examined isolates exhibited reduced susceptibility to cefixime (MIC ≥ 0.12 μg/mL), azithromycin (MIC ≥ 0.5 μg/mL) and ciprofloxacin (MIC ≥ 0.5 μg/mL), respectively (Cole *et al.* 2014). The observed percentages indicated minor decreases in the prevalence of reduced susceptibility to cefixime (down from 8.7% in 2010) and significant decreases in resistance to azithromycin (down from 13.2% in 2009) and ciprofloxacin (down from 62.7% in 2009) (Cole *et al.* 2014). However, it is also important to note that the number of countries reporting isolates with reduced susceptibility to cefixime increased from 10 in 2009 to 17 in 2011. Studies of NG isolates from Japan have identified several isolates with reduced susceptibility to ESC, including the XDR HO41 isolate, which exhibited high-level resistance to ceftriaxone (Ohnishi *et al.* 2011; Shimuta *et al.* 2013, 2015). Furthermore, an examination of isolates collected from Korea indicated that 3%, 9% and 5% of the studied isolates exhibited resistance to ceftriaxone, cefixime and azithromycin, respectively (Lee *et al.* 2015b). Although extensive surveillance studies have not been conducted in all countries and regions, reports of resistance to various antibiotics continue to be published from localities around the world (Ali *et al.* 2016; Hamze *et al.* 2016; Hananta *et al.* 2016; Costa-Lourenço *et al.* 2017), thus indicating that the spread of AMR NG is a persistent global threat that warrants continued monitoring. While the presence of resistance determinants does not always translate into treatment failure, several reports of treatment failures to ESCs and azithromycin have also appeared (Unemo 2015), including an incident of dual treatment failure (ceftriaxone plus azithromycin) (Fifer *et al.* 2016), thus providing additional evidence that the presence of AMR gonorrhea poses a significant risk to global health.

Advances in high-throughput whole-genome sequencing (WGS) have drastically reduced the cost of sequencing and have increased the amount of available data (Park and Kim 2016). The availability of WGS data will greatly impact both our understanding of the evolution of AMR and the way that gonorrhea is diagnosed and treated (Seth-Smith and Thomson 2013; Baquero *et al.* 2015). This review will examine advances in molecular techniques used to type susceptible and resistant NG strains and how WGS has been utilized to characterize isolates and associated transmission patterns. Moreover, potential applications and implications of WGS to public health issues, including the determination of AMR profiles

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and patient treatment options will be considered. Lastly, the potential application of WGS-based transcriptomic and epigenetic methodologies to gonococcal studies will be discussed.

ADVANCES IN MOLECULAR TECHNIQUES USED FOR THE CHARACTERIZATION AND SURVEILLANCE OF NG ISOLATES

A complete understanding of the mechanisms associated with the development of AMR gonorrhea requires the ability to adequately characterize and classify specific isolates or strains that are involved in resistance outbreaks. In addition to non-sequence-based typing methods such as antimicrobial susceptibility testing, serovar determination and auxotyping, several sequenced-based methods are also utilized to compare isolates of interest (Table 1; Unemo and Dillon 2011). Before Sanger sequencing was readily accessible, non-sequence-based methods such as restriction fragment length polymorphism, ribotyping and pulse field gel electrophoresis (PFGE) methods were used to type and characterize NG isolates (Unemo and Dillon 2011). PFGE has been successfully employed in molecular epidemiology analyses that examined the clustering of antibiotic-resistant NG isolates (Sosa *et al.* 2003; Yong *et al.* 2004; Morris *et al.* 2009; Starnino and Stefanelli 2009). However, while PFGE analyses can be used to detect and delineate clusters of resistant isolates, information about specific resistance mutations cannot be determined without the inclusion of sequence data.

Sequence-based methods such as NG multiantigen sequence typing (NG-MAST) and multilocus sequence typing (MLST), which assess the sequence variation of specific genetic loci, have been widely adopted for gonococcal strain typing, the assessment of relatedness, and the establishment and maintenance of respective databases that can be used to track specific sequence types (Unemo and Dillon 2011). NG-MAST analyses are based on the variable internal fragments of the highly polymorphic porin B (*porB*) and transferrin-binding protein B (*tbpB*) genes (Martin *et al.* 2004), and the most commonly used MLST scheme is based on the detection of variation between the sequences of seven conserved housekeeping genes (putative ABC transporter (*abcZ*), adenylate kinase (*adk*), shikimate dehydrogenase (*aroE*), fumarate hydratase (*fumC*), glucose-6-phosphate dehydrogenase (*gdh*), pyruvate dehydrogenase subunit (*pdhC*) and phosphoglucomutase (*pgm*)) (Bennet *et al.* 2007). Both methods are highly reproducible, and they provide high discriminatory power. However, the utilization of these methods often requires the additional isolation and sequencing of resistance genes to provide a basis for discriminatory analyses, and the results of alternative methods are often included to support the results of these methods (Ohnishi *et al.* 2010; Unemo and Dillon 2011). Attempts to utilize NG-MAST and MLST typing methods to predict antimicrobial susceptibility patterns at a regional scale have been met with some success (Palmer *et al.* 2008; Thakur *et al.* 2014).

The advancement of sequencing technology has led to the availability of high-throughput sequencing methods, including commonly used short- and long-read technologies such as Illumina and PacBio, respectively (Reuter, Spacek and Snyder 2015). Moreover, the decreased costs associated with these technologies have resulted in the sequencing of thousands of NG genomes, which are available as complete or draft genomes in publically accessible databases such the Sequence Read Archive maintained by the National Center for

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Biotechnology Information and the European Nucleotide Archive. These sequences represent a plethora of data that can be used to determine genome-wide variation between isolates (e.g. indels, single-nucleotide polymorphisms (SNPs), recombination breakpoints, etc.), structural variation, and transcriptomic and methylomic profiles (Flusberg *et al.* 2010; Reuter, Spacek and Snyder 2015). Identified SNPs can be aligned and phylogenetically analyzed, providing enhanced discriminatory power regarding relatedness and transmission at both regional and global scales. For instance, Vidovic *et al.* (2014) used Illumina-derived data to extract SNPs from nine housekeeping genes and *penA* alleles, and these data were subsequently used to elucidate the population structure and phylogenetic relationships of several NG isolates. While most WGS studies use Illumina and PacBio technologies, it important to note that researchers also utilize less common technologies, including 454 and Ion Torrent technologies, to characterize AMR NG isolates (de Curraize *et al.* 2016; Graham, Doyle and Jennison 2017).

WGS not only allows the identification and tracking of resistance genes, but it also facilitates the identification of novel resistance mutations. Mutations in several genes are well described as being predictive of resistance, including mutations in genes associated with resistance to cephalosporins (e.g. penicillin-binding protein 2 (*penA*), multiple transferable resistance repressor (*mtrR*) and *porB*), azithromycin (e.g. *mtrR* and its promoter region and 23S rRNA) and fluoroquinolones (e.g. *gyrA* and *parC)* (Table 2; Goire *et al.* 2014; Unemo and Shafer 2014). While previous methods required the isolation, PCR amplification and sequencing of individual genes to detect novel resistance determinants, comparative WGS analyses can be used to compare variation throughout the genome, resulting in the discovery of novel mutations. For instance, WGS analyses of NG sequences from the USA resulted in the identification of a novel mosaic *penA* allele and mosaic *mtrR* mutations that conferred resistance to cefixime and azithromycin, respectively (Grad *et al.* 2014, 2016). Therefore, while the utilization of molecular-based typing methods is beneficial, WGS-based methods provide a superior tool for typing and relatedness analyses, and the use of these data also allow the identification and characterization of specific resistance genes and associated mutations without the need for the isolation and sequencing of individual genes. However, despite its prevalence in several countries, WGS capabilities are not available worldwide, resulting in the continued use of MLST and NG-MAST typing methods. Therefore, until WGS becomes ubiquitous, the characterization of NG isolates using these methods will likely continue. However, recent studies that utilized WGS in conjunction with MLST and/or NG-MAST methodologies reported discordance between the results gleaned from the two methods in that isolates assigned to a specific sequence type were distributed among different phylogenetic clades (Demczuk *et al.* 2015; Jacobsson *et al.* 2016). Thus, the use of NG-MAST and MLST analyses can lead to confusion regarding the phylogenetic relatedness of isolates. This result is expected given that NG-MAST analyses examine less than 1% of the gonococcal genome, while WGS analyses provide enhanced resolution since they consider genome-wide variation. However, until WGS-based methods are established as the gold standard, the use of WGS in combination with NG-MAST/MLST analyses will likely persist as a way to compare isolates that were typed using different methods.

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APPLICATION OF WGS TO THE ELUCIDATION OF GONOCOCCAL PHYLOGENOMIC RELATIONSHIPS AND TRANSMISSION PATTERNS

In addition to enhanced typing capabilities, WGS-based analyses provide tools that can be used to elucidate phylogenetic relationships among isolates, to identify novel resistant lineages and to estimate transmission patterns. WGS data have been successfully used to study a variety of bacterial species with regard to molecular epidemiology, AMR determination, AMR surveillance and genomic characterization (Lee *et al.* 2015a; Tyson *et al.* 2015; Metcalf *et al.* 2016; Nair *et al.* 2016; Zhao *et al.* 2016), and the methods used to examine those species have been, or will likely be, key to our understanding of the mechanisms that drive the evolution and dissemination of AMR in NG populations. Regarding general phylogenetic relationships among NG isolates, studies have examined the phylogenetic patterns of multiple isolates collected from individual countries or regions (Ezewudo *et al.* 2015; Grad *et al.* 2016). For instance, Ezewudo *et al.* (2015) utilized WGS data to characterize the phylogenetic relationships of 76 NG isolates from Australia, Austria, Chile, Canada, Japan, Pakistan, the Philippines, Norway, Sweden and the USA, and the results indicated that the isolates did not cluster geographically. In addition, the researchers performed population structure analyses and determined that the samples represented at least five distinct subpopulations. Thus, this study provides an example of various WGS applications that will further our understanding of the genetic relatedness, spread, and population structures of NG isolates, which cannot be easily garnered from traditional sequencing methods. Grad *et al.* (2016) utilized WGS data from 1102 isolates collected in the USA from 2000 to 2013 to examine the genetic relatedness and the population structure of isolates that exhibited resistance to cephalosporins, macrolides and fluoroquinolones. The results of the phylogenomic analyses suggested that cephalosporin resistance is largely clonal and that resistance to both macrolides and fluoroquinolones has emerged multiple times in the USA. Although this study examined isolates that were systematically collected over a long period of time, the application of similar phylogenomic analyses to target isolates collected outside of a formal surveillance system will provide invaluable information about the origin and spread of resistance mechanisms at regional and global scales.

In addition to utilizing WGS data to obtain increased resolution of phylogenetic relationships, several studies focused on determining the genetic relatedness of isolates with similar resistance profiles (Demczuk *et al.* 2015, 2016; Grad *et al.* 2016; Jacobsson *et al.* 2016). Jacobsson *et al.* (2016) utilized WGS to analyze the molecular resistance mechanisms and the spread of azithromycin-resistant isolates in Europe. The results of the analysis indicated that the 75 isolates (collected from 17 countries) fell into five distinct clades, thus indicating that the clonal spread of azithromycin resistance determinants was limited to relatively few strains. Moreover, the results of the comparative WGS analysis indicated that most of the isolates exhibited mutations in *mtrR* and its promoter, and that the four isolates with high-level resistance to azithromycin (MIC > 256 μg/mL) represented three separate emergent events of that specific phenotype, which was associated with 23S rRNA mutations. Comparative WGS methods also allowed the researchers to examine variation in nine additional genes that are associated with macrolide resistance without having to individually

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isolate and amplify the specific genes. A retrospective study of azithromycin-resistant isolates from Canada, which were collected between 1997 and 2014, also found that isolates with high-level azithromycin resistance were phylogenetically diverse, and the associated mutations were found in 23S rRNA (Demczuk *et al.* 2016). Furthermore, the results indicated that low-level resistance was typically associated with *mtrR* promoter mutations, thus the general results were consistent with those of Jacabsson *et al.* (2016). However, Grad *et al.* (2016) found that while azithromycin resistance was associated with mutations in *mtrR*, 23S rRNA and ribosomal protein L22 (*rplv*), a resistance mechanism was not determined for 36% of the tested isolates. Therefore, additional analyses must be conducted to determine the unknown genes and mutations that confer resistance to azithromycin, and the availability of WGS data will make the difficult task of identifying those unknown resistance mechanisms tractable.

Researchers have also utilized WGS methods to explicitly examine the transmission of NG isolates (Grad *et al.* 2014; De Silva *et al.* 2016; Didelot *et al.* 2016). For instance, Grad *et al.* (2014) examined 236 isolates collected through GISP between 2009 and 2010, and phylogeographic and Bayesian analyses were used to infer the transmission of NG isolates across time, space and sexual networks (men who have sex with men (MSM) and men who have sex with women) in the USA. The results of the analyses indicated that reduced susceptibility to cephalosporins was primarily spread eastward through MSM sexual networks. Moreover, the study found several instances of introductions into heterosexual populations by MSM. De Silva *et al.* (2016) conducted a similar study that focused on the transmission of approximately 1400 NG isolates collected in the UK between 2011 and 2015, and these isolates were also compared to the data examined by Grad *et al.* (2014). The study attempted to elucidate transmission patterns at local, national and international scales. The results of the analysis indicated that most infections could be linked to direct or indirect transmission events at a local scale, and a small proportion of the isolates could be linked to cases outside of the local area (Brighton, UK) and to cases that originated in the USA. It is important to note that both Grad *et al.* (2014) and De Silva *et al.* (2016) incorporated MLST and NG-MAST data, respectively, and WGS-based analyses provided enhanced resolution compared to the alternative methods.

APPLICATION OF WGS TO PUBLIC HEALTH

WGS has been applied to a diverse array of clinical issues, including infection management, clinical diagnostics, drug development, prenatal testing and cancer treatment development (Dunne, Westblade, and Ford 2012; Price *et al.* 2013; Chrystoja and Diamandis 2014; Wyres *et al.* 2014; Kwong *et al.* 2015; Nakagawa *et al.* 2015; Swaminathan *et al.* 2016). The molecular diagnostics used by clinicians to detect gonorrhea are generally limited to the use of nucleic acid amplification tests (NAATs), which determine the presence or absence of the pathogen in clinical samples. Although widely used, non-culture-based NAATs do not provide any information about the susceptibility/resistance profiles of the detected isolate. To address this concern, a WGS pilot study, funded by the CDC's Advanced Molecular Detection Initiative and coordinated by the Division of STD Prevention, was initiated at two public health laboratories in the USA to develop a WGS-based genotype to phenotype assay. As a part of the collaboration, the public health laboratories currently perform antimicrobial

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susceptibility testing and WGS on select AMR alerts collected by GISP, and these data are then sent to the CDC for genomic characterization and phylogenomic analyses. Moreover, the WGS data are then used to inform the development of a bioinformatics pipeline that will utilize raw sequence data and calculated positive and negative predictive values (Grad *et al.* 2016) associated with resistance determinants to estimate the susceptibility/resistance profiles of clinical isolates, with the development of WGS-based point-of-care tests as the ultimate goal. However, the development of WGS-based point-of-care tests is dependent on the advancement of faster and more efficient WGS technologies such as the single-molecule based Oxford Nanopore technology, which was recently used to sequence Ebola isolates in real-time in the field (Hoenen *et al.* 2016). Ideally, real-time sequencing technologies and a genotype to phenotype assay will eventually be available to clinicians for the relatively rapid diagnosis and treatment of gonococcal infections. The complete implementation of WGS analyses at clinical laboratories is dependent upon the resolution of several issues associated with the lack of user-friendly software and the interpretation of results by clinicians (Wyres *et al.* 2014). While several user-friendly tools have been developed to assess WGS data in non-clinical environments, the analysis of WGS data in clinical settings remains complicated. However, the development of a streamlined pipeline that requires minimal input from clinicians to generate simple, comprehensible results will help remedy this issue by automating the bioinformatics analyses.

Despite the current inability to perform real-time WGS in a clinical setting, the WGS data from the aforementioned pilot study were used to characterize the resistance determinants and phylogenomic characteristics of a cluster of NG isolates that exhibited reduced susceptibility to cephalosporins and azithromycin in Hawaii (Papp *et al.* 2017). Moreover, an expansion of WGS of NG at public health laboratories across the USA is being established through the Combating Antimicrobial Resistant Bacteria initiative. An increased focus on combining WGS and epidemiological data will further inform our understanding of the evolution and transmission of AMR gonococcal isolates, which will greatly enhance the management of this pathogen.

APPLICATION OF WGS TO GENE EXPRESSION AND EPIGENETIC ANALYSES

It is important to note that the applications of WGS are not limited to analyses that assess typing, relatedness and transmission at the nucleotide level. In fact, WGS-based data can also be used to examine differential expression and epigenetic characteristics of various bacterial strains. RNA-Seq is a method that uses the high-throughput sequencing of RNA transcripts (converted to cDNA) to determine and quantify changes in gene expressions levels (Wang, Gerstein and Snyder 2009). RNA-Seq analyses were previously employed to predict a set of 827 essential gonococcal genes, including 133 genes that lacked a known function (Remmele *et al.* 2014). McClure *et al.* (2015) utilized the method to examine gene expression patterns in the lower genital tract of females during natural gonococcal infection, and the results indicated that over 65% of the gonococcal genome was transcribed during the infection. Moreover, the results of the analysis showed that *mtrCDE* genes were upregulated and *mtrR* (a known AMR gene) was downregulated in antibiotic-resistant NG strains. The

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association between differential expression and AMR has been examined in other bacterial species. For instance, Suzuki, Horinouchi and Furusawa (2014) performed microarray analyses to examine differential expression patterns of *E. coli* isolates that were cultured under various drug treatment conditions, and the resulting gene expression profiles of a small number of genes were successfully used to predict resistance. Wright *et al.* (2015) utilized RNA-Seq analyses and resulting expression profiles to identify three genetic mechanisms that were associated with colistin resistance in *Klebsiella pneumoniae* isolates. Considering that some gonococcal resistance determinants have yet to be identified using traditional methods, the application of WGS-based transcriptome analyses will likely play a major role in the discovery of resistance determinants, and comparative WGS methods can be used to identify the specific mutations in the genes that confer resistance. In addition, evidence suggests that AMR is a multilocus phenomenon that could result from the combined effects of mutations in different genes (Day and Gandon 2012), so the results of RNA-Seq analyses might also help elucidate the interactions between various resistance genes.

Epigenetics refers to heritable changes in gene expression that do not alter the genetic code, and advances in this field will greatly impact our understanding of gonococcal AMR. Most studies of epigenetic mechanisms in bacteria have focused on the role of DNA and RNA methylation and corresponding effects on cell regulation, virulence and antibiotic resistance (Casadesus and Low 2006; Doi and Arakawa 2007). The development of single-molecule real-time (SMRT) sequencing via the PacBio platform also led to advancements in DNA methylation detection without the use of traditional bisulfite methods. PacBio SMRT sequencing uses variation in polymerase kinetics (based on fluorescent pulses) to detect methylated nucleotides (m4A, m4C and m5C methylation) (Flusberg *et al.* 2010), and this methodology has been used to characterize the methylomes of several bacterial species and strains (Murray *et al.* 2012; Davis, Chao and Waldor 2013; Powers *et al.* 2013; Lee *et al.* 2015c; Pirone-Davies *et al.* 2015; Zhu *et al.* 2015). Evidence supports the conclusion that both DNA and RNA methylation have an effect on AMR in bacteria. For instance, DNA methylation can lead to differential gene expression, and if that expression affects the antibiotic susceptibility of a bacterial strain in the presence of a particular antibiotic, selection could favor the evolution and spread of a resistant phenotype (Adam *et al.* 2008; Baquero 2013). Moreover, RNA methylation of antibiotic targets (e.g. ribosomes) by rRNA methyltransferases can result in high-level resistance to antibiotics such as macrolides (Maravic and Flogel 2004). Therefore, it is imperative that epigenomic analyses of AMR gonococcal isolates be conducted, because the results of these analyses could shed light on novel resistance mechanisms in this bacterial species. Moreover, once the epigenetic patterns are detected, transcriptomic analyses can be used to quantify differences in the expression of identified genes.

CONCLUSIONS

The development of high-throughput WGS has revolutionized our ability to characterize and analyze microbial isolates and communities by impacting typing methods, epidemiologic surveillance, genetic relatedness analyses and transmission studies. The application of WGS tools to the study of AMR gonococcal isolates has already improved our understanding of

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the evolution of AMR in NG and the transmission of resistant strains at local, national and global scales. WGS is not only applicable to research-based studies, but the implications of the results gleaned from WGS analyses will also influence the way in which surveillance is conducted, strain typing is assessed and treatment options are determined.

However, current applications of WGS have not fully elucidated the mechanisms that influence AMR in NG. As previously mentioned, Grad *et al.* (2016) recently found that the mechanisms associated with azithromycin resistance in 36% of the examined isolates could not be determined based on current knowledge. Furthermore, researchers have long postulated the existence of a non-transformable resistance determinant known as “factor X” that is associated with increased resistance to penicillin and ESCs, but this mechanism has not been identified (Unemo and Shafer 2014). The presence of unknown resistance mechanisms, in conjunction with the ease at which NG obtains novel resistance mechanism (resulting from its natural competence for DNA transformation), suggests that innovative techniques will be needed to determine both cryptic and novel resistance determinants. Although traditional methods have failed to identify these mechanisms, the use of WGS-based transcriptomic and epigenetic analyses might provide novel ways to detect and describe unknown resistance mechanisms. Further WGS-based studies of gonococcal isolates and the development of innovative WGS-based analyses will enhance our gonococcal detection, treatment and management capabilities, which will ultimately aid in the control of AMR gonorrhea worldwide.

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Table 1.

Methods used to type and characterize NG isolates.

Typing method	Category	Description[a]
Antimicrobial susceptibility testing	Phenotypic	Analysis based on the antimicrobial susceptibility profiles of strains, allowing the identification of AMR phenotypes.
Auxotyping	Phenotypic	Analysis based on the specific nutrient requirements of strains (i.e. growth or lack of growth), allowing strain differentiation when used in combination with serological methods.
Serology	Phenotypic	Analysis based on the antigenic properties of PorB (an outer membrane porin), allowing strain differentiation when used in combination with auxotyping methods.
RFLP	Non-sequence-based	Analysis based on the digestion of the bacterial genome by restriction endonucleases followed by fragment separation via electrophoresis (e.g. polyacrylamide or pulse-field gel electrophoresis), allowing strain differentiation and characterization.
Ribotyping	Non-sequence-based	Analysis based on the digestion of chromosomal DNA by restriction endonucleases followed by the hybridization of rRNA fragments to specific probes, allowing strain differentiation and characterization.
PFGE	Non-sequence-based	Analysis based on the digestion of the bacterial genome by restriction endonucleases followed by fragment separation via pulse-field gel electrophoresis, allowing the identification and characterization of AMR clusters.
MLST	Sequence-based	Analysis based on the detection of variation between the sequences of seven conserved housekeeping genes, allowing the identification and characterization of AMR clusters and associated transmission patterns.
NG-MAST	Sequence-based	Analysis based on the detection of variation between internal fragments of the highly polymorphic *porB* and *tbpB* genes, allowing the identification and characterization of AMR clusters and associated transmission patterns.
WGS	Sequence-based	Analysis based on the detection of variation throughout the bacterial genome (using genomic, transcriptomic, and/or epigenomic analyses), allowing the identification and characterization of AMR clusters and associated transmission patterns and resistance determinants.

RFLP: restriction fragment length polymorphism; PFGE: pulse-field gel electrophoresis; MLST: multilocus sequence typing; NG-MAST: NG multiantigen sequence typing; WGS: whole-genome sequencing.

[a]See Unemo and Dillon (2011) for a thorough review of NG-typing methods.

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Table 2.

Antibiotics to which NG exhibits resistance or reduced susceptibility and resistance markers that are detectable using WGS methodologies.

Antibiotic	Resistance markers	References
Penicillin	*bla*$_{TEM-1}$ (plasmid-mediated)	Ashford, Golash and Hemming (1976);
	penA (D354-insertion)	Dowson *et al.* (1989); Veal, Nicholas and Shafer (2002)
Tetracycline	*tetM* (plasmid-mediated) *rpsJ* (V57M, V57L, V57Q)	Morse *et al.* (1986); Hu *et al.* (2005)
Spectinomycin	16S rRNA (C1192U) *rpsE* (V25-deletion; K26E)	Galimand, Gerbaud and Courvalin (2000); Unemo *et al.* (2013)
Quinolones	*gyrA* (D95G, D95A, D95N, S91F) *parC* (S87R, S87N, S87I)	Shultz, Tapsall and White (2001); Grad *et al.* (2016)
Macrolides	*mtrR* (G45D) *mtrR* promoter (A-deletion, interspecies mosaic alleles) 23S rRNA (C2611T, A2059G; >1 copy of each)	Zarantonelli *et al.* (1999); Ng *et al.* (2002); Cousin, Whittington and Roberts (2003); Galarza *et al.* (2010); Grad *et al.* (2016); Johnson *et al.* (2016)
Cephalosporins	*penA* (mosaic *penA* XXXIV) *ponA* (L421P) *porB* (A121N)	Olesky, Hobbs and Nicholas (2002); Unemo *et al.* (2012); Golparian *et al.* (2014); Grad *et al.* (2014)