SEXUAL HEALTH

Genotypic determinants of fluoroquinolone and macrolide resistance in Neisseria gonorrhoeae

Journal:	*Sexual Health*
Manuscript ID	SH18225
Manuscript Type:	Research Paper
Date Submitted by the Author:	29-Nov-2018
Complete List of Authors:	Hall, Catherine; St George's, University of London, Applied Diagnostic Research and Evaluation Unit Harrison, Mark; St George's, University of London, Applied Diagnostic Research and Evaluation Unit Pond, Marcus; St George's, University of London, Applied Diagnostic Research and Evaluation Unit Chow, Christine; St George's, University of London, Applied Diagnostic Research and Evaluation Unit Harding-Esch, Emma; St George's, University of London, Applied Diagnostic Research and Evaluation Unit; Public Health England, National Infection Service Sadiq, Tariq; St George's, University of London, Applied Diagnostic Research and Evaluation Unit; Public Health England, National Infection Service; St George's University Hospitals NHS Foundation Trust
Keyword:	Gonorrhea, Diagnostics, Resistance

Genotypic determinants of fluoroquinolone and macrolide resistance in *Neisseria gonorrhoeae*

Short Title: Diagnostic AMR markers in *Neisseria gonorrhoeae*

Catherine L. Hall[1], Mark A. Harrison[1], Marcus J. Pond[1], Christine Chow[1], Emma M. Harding-Esch[1, 2] and S. Tariq Sadiq[1, 2 3].

[1]Applied Diagnostic Research and Evaluation Unit, St George's University of London, Institute for Infection & Immunity, London, UK.

[2] National Infection Service, Public Health England, London, UK

[3] St George's University Hospitals NHS Foundation Trust, London, UK

Abstract

Background

High rates of antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* hinder effective treatment, but molecular AMR diagnostics may help address the challenge. We aimed to appraise the literature for resistance-associated genotypic markers linked to fluoroquinolones and macrolides, to identify and review their use in diagnostics.

Methods and Findings

Medline and EMBASE databases were searched and data pooled to evaluate associations between genotype and phenotypic resistance. Diagnostic accuracy estimates were limited by data availability, differences in mutations typed, and methods for reporting MIC. 1) S91 and D95 mutations in gyrA protein independently predicted ciprofloxacin resistance and used together gave 98.6% (95% confidence interval (CI) 98.0-99.0%) sensitivity and 91.4% (95% CI 88.6-93.7%) specificity; 2) the number of 23S rRNA gene alleles with C2611T or A2059G mutations was highly correlated with azithromycin resistance, with mutation in any allele giving sensitivity and specificity of 66.1% (95% CI 62.1-70.0%) and 98.9% (95% CI 97.5-99.5%), respectively. Estimated negative (NPV) and positive predictive values for a 23S rRNA diagnostic were 98.6% (95% CI 96.8-99.4%) and 71.5% (95% CI 68.0-74.8%) respectively; 3) mutations at amino acid positions G45 and H105 in MtrR protein independently predicted azithromycin resistance, however, when combined with 23S rRNA mutations, improved NPVs only marginally.

Conclusions

Viable candidates for markers of resistance detection for incorporation into laboratory and point-of-care diagnostics were demonstrated. Such tests may enhance antibiotic stewardship and treatment options.

Introduction

Patients attending sexual health clinics (SHCs) will often present with non-specific genital infections, associated with a number of common symptoms resulting from a diverse range of aetiologies. The number of potential causative agents can hinder effective management, which is highly dependent on an accurate and rapid diagnosis to inform prompt treatment. Difficulty in treatment is further compounded by increasing antimicrobial resistance (AMR) rates within sexually transmitted pathogens [1], threatening the efficacy of current treatment regimens, exemplified by recent UK reports of gonorrhoea resistant to azithromycin, part of the current first line treatment [2,3].

Current diagnostics commonly employ nucleic acid amplification tests (NAATs), favoured due to high sensitivity of detection and a quicker time to result than culture [4]. However, culture remains the only means of determining AMR profiles for gonococcal infection, but its use is in decline and results are often unavailable at time of diagnosis, meaning empirical management of genital syndromes is common [5]. Although empirical therapy helps to reduce time to treatment, onward transmission of infection and the possible loss to follow-up, it also risks induction and further spread of AMR. It also removes the opportunity for recycling older antibiotics that are likely to still be effective in many cases, but are no longer recommended for empirical use [6,7].

These challenges could in part be addressed by the development and deployment of NAAT technologies that identify both infection and AMR susceptibility in the laboratory and at the point of care (PoC), thereby enabling immediate administration of specific antibiotic therapy when patients are diagnosed ("precision medicine"). Calls for such novel diagnostics have been increasing in response to rising AMR rates [8,9] yet have been restricted in part by incomplete understanding of the relationship between bacterial genotype and treatment response.

Neisseria gonorrhoeae is a common sexually transmitted infection (STI) with high rates of AMR [10,11]. Fluoroquinolone and macrolide antibiotics, which represent both current and previously

recommended treatments for gonorrhoea [11], are effective in eradicating susceptible strains and are associated with specific genetic mechanisms of resistance. These typically centre on either alteration of the macrolide binding site through mutation or methylation of specific sites in the 23S rRNA for macrolide resistance[12], or mutations within the quinolone resistance-determining region (QRDR) of DNA gyrase (*gyrA*) or DNA topoisomerase IV (*parC*) for fluoroquinolone resistance[13, 14]. *N. gonorrhoeae* harbours up to four copies of the 23S rRNA allele, and the number of alleles carrying mutations can vary [15]. The activity of multi-drug efflux pumps can also influence minimum inhibitory concentration (MIC) to varying degrees [16].

As part of a body of work developing PoC tests for fluoroquinolone and macrolide resistance in *M. genitalium* and *N. gonorrhoeae* (www.preciseresearch.co.uk), this review aimed to appraise the literature on AMR genotype for *N. gonorrhoeae* in relation to fluoroquinolone and macrolide antibiotics and to test the strength of genotypic-phenotypic associations when pooling data from included publications. We also aimed to appraise the diagnostic accuracy of detecting AMR using genotypic markers identified in the review, in order to assess their suitability for inclusion on diagnostic platforms for AMR prediction.

Methods

Publication search strategy and screening criteria

Two separate searches were performed to reflect the aims: (1) macrolide resistance in *N. gonorrhoeae* and (2) fluoroquinolone resistance in *N. gonorrhoeae*. Preliminary review of the literature informed search term format, including the organism name, region associated with resistance (e.g. 23S rRNA for macrolides or *gyrA* and *parC* for fluoroquinolones) and a broader component comprising variations on “genotype” and the target antibiotic. This approach was used in an effort to ensure more general or emerging resistance mechanisms could still be detected (S1 Text). Publication screening and data extraction were shared between two people and performed in September 2016 using OvidSP to search both EMBASE and Medline databases.

Abstracts were screened to determine publication suitability for inclusion in the review (Table 1), with full text searched where necessary. Included publications had to target organism of interest and antibiotic of interest, and report resistance-associated genotype in relation to antibiotic of interest, with no date restriction applied. Exclusion criteria were: publications that were not available in English language or reviews and conference abstracts where results were available in full publication. Due to variations in data provision and quality, further exclusion criteria were applied to limit publications to those providing the level of detail required for data analysis of phenotypic-genotypic relationships. Publications excluded at this stage were limited to the literature review only and used as a source of additional information for included papers, especially if two publications were linked. Exclusion criteria for data analysis were: mutation listed to gene level only; number of isolates/samples with each mutation not stated; reference strains and laboratory strains only; and repeat data sets. Some publications containing data constituting an exclusion criteria (e.g. reference strains), were extracted if this was clearly differentiated from the usable sample set. Publications included for data analysis (S2 Text) and relevant reviews underwent reference and citations checking.

Data Extraction

Optimal data capture from each eligible publication included: genotyping methodology; coverage and capacity to detect all mutations (e.g. targeted single nucleotide polymorphisms (SNPs) or sequencing of relevant gene regions); whether samples were randomly selected or stratified by phenotypic characteristics; susceptibility phenotype and/or treatment outcome (including test of cure methodology and timing); position of mutation; new base and/or amino acid following mutation; treatment regimen and dose; and whether the study had pre- and post-treatment data available. All samples/isolates were treated as separate cases except repeat data sets and those identified as pre- and post- treatment samples (S3 and S4 Data).

If the minimum inhibitory concentration (MIC) to a number of fluoroquinolones or macrolides was reported, analysis centred on ciprofloxacin and azithromycin, respectively, as these are either in use or have been recommended for treatment previously. Numbering of nucleotide bases or amino acid residue positions are in *Escherichia coli* numbering for macrolide resistance and *N. gonorrhoeae* numbering for fluoroquinolone resistance.

Data Analysis

Variation in the level of detail provided in each publication made comparison of the entire database unreliable so set criteria were defined for each analysis (Table 2), with studies or samples/isolates only included if these were met.

In order to standardise the thresholds for resistance and susceptibility for isolates of *N. gonorrhoeae*, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [17] were referenced to assign resistance phenotypes based on MICs described in each publication. EUCAST guidelines define ciprofloxacin resistance in *N. gonorrhoeae* as a MIC >0.06 mg/L, which was used for this analysis. However, many studies used ≤0.06 mg/L as a susceptibility threshold, therefore not differentiating between intermediate (0.06 mg/L) and sensitive (≤0.03 mg/L) isolates, as defined by EUCAST. Therefore, all isolates with an MIC of ≤0.06 mg/L were assigned as non-resistant. A similar approach was taken for azithromycin resistance in *N. gonorrhoeae*, with an MIC of >0.5 mg/L assigned as resistant and ≤0.5 mg/L as non-resistant with resistant isolates further categorised as low-level resistant (>0.5 mg/L and <2 mg/L), moderate-level resistance (≥2 mg/L and <256 mg/L) and high-level resistant (≥256 mg/L).

Statistical Methods

All statistical analysis was performed in Stata/IC 14 (Stata Corp, Texas). χ^2 and univariate logistic regression analyses were performed for macrolide and fluoroquinolone resistance in *N. gonorrhoeae* to determine if presence of a mutation was significantly higher in isolates/samples with resistant MICs

or treatment failure. This was followed by multivariate logistic regression analyses using univariate variables at $p<0.05$ in a forward stepwise approach to determine the strength of each genotype as independent markers of resistance. The correlation between the numbers of mutated 23S rRNA alleles for *N. gonorrhoeae* and level of macrolide resistance was analysed using Spearman's rank-order correlation coefficient. Sensitivity and specificity of resistance detection for the genotypic markers of AMR determined in this review were calculated using the following definitions: presence of AMR marker in resistant isolates/samples as true positive, in non-resistant isolates/samples as false positive, their absence (wild-type) in non-resistant isolates/samples as true negative and in resistant isolates/samples as false negative. Calculated sensitivities and specificities were applied to the number of *N. gonorrhoeae* infections and the prevalence of resistance for 2016 in England and Wales acquired from Public Health England (PHE) STI and Gonococcal Resistance to Antimicrobial Surveillance Program (GRASP) data sets [11, 18], to determine positive predictive values (PPV) and negative predictive values (NPV). Wilson score interval was used to calculate 95% confidence intervals (CI).

Results

Macrolide resistance

In *N. gonorrhoeae*, mutations within domain V of the 23S rRNA gene, A to G or C to T substitution at A2059 and C2611, respectively, are associated with azithromycin resistance ($p<0.001$). Of the 366 isolates harbouring 23S rRNA mutations with the specific number of mutated alleles reported, five were non-resistant and each had only one allele mutated. All isolates with two or more mutated alleles from this review were resistant (n=359) (Table 3).

We found data for 1015 isolates for which the numbers of 23S rRNA mutated alleles were recorded and for which an azithromycin resistance category (i.e. non-resistant, low-level, moderate-level and high-level resistant could be allocated) (Figure 1). A strong correlation was found between MIC and

number of mutated alleles, in isolates where MIC was defined as an integer (for example, not as a range) and the number of mutated alleles was specifically reported (n=571, r_s=0.7846; p=<0.001).

A mutation in L22 was only reported once and mutations in L4 were not significantly associated with resistance. Methylase (*erm*) genes, *mac* efflux pump and *ere* genes were investigated but were either not associated with resistance, very rare in resistant isolates, or present with 23S rRNA mutations or where no other resistance associated region was typed.

Mutations in the MtrCDE transporter were found in both the repressor protein, MtrR, and its promoter. Mutations were identified at 20 positions within MtrR, but only mutations at H105 and G45 were associated with resistance (p<0.05). These could not be assessed in multivariate logistic regression analysis with the 23S rRNA mutations as, in the samples in which both 23S rRNA and *mtrCDE* mutations were described, absence of 23S rRNA mutations was only found in non-resistant samples. Furthermore, mutations at H105 and G45 were only present in 17.9% (145/812) and 35.0% (52/149) of all resistant isolates screened, respectively. Included publications described a number of rare alterations to the *mtr* promoter region including: *Neisseria meningitidis* like promoter; mosaic promoter; and a range of insertions, deletions and substitutions. Most frequently reported alterations were an adenine deletion (DelA), a thymidine insertion, and an adenine to cytosine substitution within the *mtr* promoter region but these changes were found in a number of both resistant and non-resistant isolates and none were determined to be independent markers of resistance by univariate analysis.

Consequently, this review only indicated the A2059G and C2611T mutations within the 23S rRNA gene to be independent markers of azithromycin resistance, with higher levels of resistance more likely with increasing numbers of mutated alleles. However, the independent role of MtrR mutations impacting on resistance could not be discounted. As a diagnostic marker of azithromycin resistance, use of the presence of either C2611T or A2059G 23S rRNA mutations (n=1015) within at least one allele gave a sensitivity and specificity of 66.1% (95% CI 62.1 – 70.0%) and 98.9% (95% CI 97.5% -

99.5%), respectively; when mutations in two or more alleles were used this gave 65.8% (95% CI 61.7 – 69.6%) and 100% (95% CI 99.2 – 100%), respectively. When presence of either at least one mutated 23S rRNA allele or the MtrR mutations G45 or H105 were considered, in a smaller sample set of 207, sensitivity was 78.9% (95% CI 70.8 – 85.2%) and specificity 94.0% (95% CI 86.8 – 97.4%). The sensitivity and specificity of the combined putative diagnostic was applied to the 36,244 [19] diagnoses of gonorrhoea made in England and Wales in 2016, using a prevalence of macrolide resistance (MIC >0.5 mg/L) of 10% [11].This gave a positive predictive value (PPV) of 59.5% (95% CI 58.2 – 60.9%) and a negative predictive value (NPV) of 97.6% (95% CI 97.4 – 97.7%). However, when applying the lower margin of the 95% CI of sensitivity and specificity estimates, a PPV and NPV of 37.4% (95% CI 36.2% – 38.5%) and 96.4% (95% CI 96.2– 96.6%) were obtained, respectively. Interestingly, applying the lower margins of accuracy of a single 23S rRNA mutant allele to this same dataset gave a PPV and NPV of 73.6% (95% CI 72.0 - 75.2%) and 95.9% (95% CI 95.6 – 96.1), respectively.

Fluoroquinolone resistance

Studies investigating fluoroquinolone resistance sequenced the QRDR for *gyrA* and *parC,* encoding the major subunits of DNA gyrase and DNA topoisomerase IV, respectively [13]. The minor subunits of these proteins, encoded by *gyrB* and *parE*, respectively, were investigated in some cases and mutations detected. No mutations were found in *gyrB,* and in *parE* mutations were only present in isolates also harbouring *gyrA* mutations.

N. gonorrhoeae isolates frequently harboured multiple mutations in both genes with a total of 7 and 9 amino acid changes within the QRDR of *gyrA* and *parC*, respectively. Of these, mutations at S91 and D95 from *gyrA* and D86 and S87 from *parC* were significantly higher in resistant isolates ($p<0.05$), with wild-type S88 and E91 always found in non-resistant isolates. Mutations at other residues were present in less than 1% of the total number of resistant isolates where the QRDR was typed. Importantly, only one resistant isolate harboured a *parC* mutation (E91G) without an S91

and/or D95 mutation in *gyrA*. Multivariate analyses revealed only S91 and D95 to be significantly associated with resistance, with 98.5% of resistant isolates harbouring one or both of these mutations (Table 4). Of those isolates with an S91 and D95 genotype and accompanying phenotypic susceptibility, 2.5% of resistant and 5.6% of non-resistant isolates harboured a D95 mutation only. Within this same sample set but using only those isolates where S91 is wild-type, D95 mutations were found in 64% of resistant isolates as opposed to 5.6% of non-resistant isolates. When used together, diversion from the wild-type at S91 and/or D95 gave a 98.6% (95% CI 98.0 – 99.0%) sensitivity and 91.4% (95% CI 88.6 – 93.7%) specificity for resistance detection.

As with macrolide resistance, the MtrCDE efflux pump was investigated for association with ciprofloxacin resistance in *N. gonorrhoeae*, although in fewer studies. Of the 10 mutations reported within MtrR, only the G45D polymorphism was significantly higher in resistant isolates ($p<0.001$), as was the DelA *mtr* promoter mutation ($p<0.001$). The presence of wild-type at H105 in MtrR was only found in non-resistant samples and therefore could not go forward into multivariate analysis but of all resistant isolates typed at H105, only 16.0% (43/269) carried the mutation. Both G45D and DelA mutations were no longer significantly associated with resistance in multivariate analysis when tested with *gyrA* S91.

Discussion

The spread of antimicrobial resistance threatens to undermine management of many infectious diseases. Interventions proposed to address this challenge include a more intelligent use of antibiotics, partly enabled by novel diagnostic technologies that can predict AMR rapidly [1]. These promise more accurate treatments and the potential to recycle older antibiotics, thus improving antibiotic stewardship [20]. In this review we sought to appraise the literature for genotypic candidates associated with macrolide and fluoroquinolone resistance for *N. gonorrhoeae* and assess the potential accuracy of these candidate markers if included in AMR diagnostic platforms.

In *N. gonorrhoeae,* fluoroquinolones have been largely discontinued for empirical therapy because of high levels of resistance globally, yet in many regions a large proportion of gonococcal strains remain phenotypically susceptible to ciprofloxacin [11]. Our analysis confirmed the central role of two mutations in the *gyrA* gene, representing amino acid changes at S91 and D95, which were both independently predictive of ciprofloxacin resistance. The data in this review gave a combined S91/D95 diagnostic a sensitivity and specificity of 98.6% (95% CI 98.0 – 99.0%) and 91.4% (95% CI 88.6 – 93.7%), respectively, for detection of ciprofloxacin resistance. The GRASP report for 2016, uses MIC ≥1.0 mg/L as the definition for ciprofloxacin resistance [11]. As the EUCAST defined MIC >0.06 mg/L was used in this review, the 29% prevalence of resistance from GRASP could not be applied to the 36,244 gonococcal diagnoses reported in England and Wales in 2016. However, speculating a resistance prevalence of 50% by our definition is not unreasonable and applying the lower 95% CI margins of sensitivity and specificity to the dataset gives a PPV and NPV of 89.5% (95% CI 89.1 - 90.0%) and 97.7% (95% CI 97.5 - 98.0%), respectively. In this virtual scenario, of the 18,122 ciprofloxacin "non-resistant" diagnoses of gonorrhoea, the S91/D95 diagnostic test would potentially identify 16,047. The test would also report around 370 diagnoses to be genotypically "non-resistant" when in fact phenotypically resistant and around 2075 genotypically resistant when in fact phenotypically non-resistant. Although minimising the first of these errors is more important clinically, the second error represents both missed opportunities to identify patients for whom ciprofloxacin could be used and diagnostic test wastage. Clearly these predictions are dependent on the availability of an AMR genotypic result at the point of diagnosis prior to treatment, population prevalence of resistance as well as the molecular accuracy of marker detection. Evaluating these diagnostic approaches prospectively for accuracy, cost-effectiveness and impact on resistance spread will be an important factor in future development. However, the data suggest that an S91/D95 *gyrA* diagnostic may well be of value, even in particularly high ciprofloxacin resistance prevalence settings, but more work on the determinants of resistance is required to improve both sensitivity and specificity.

Although critical for resistance in *E. coli*, D95 mutation was reported in borderline ciprofloxacin resistant *N. gonorrhoeae* in transformation studies or when induced *in vitro*, but MIC was 16-fold greater than the wild-type parental strain [21, 22]. Of those isolates wild-type at S91 and resistant in this review, 64% carried a mutation at D95, compared to just 5.6% in non-resistant strains, with inclusion of D95 raising sensitivity of detecting AMR from 96.0% (95% CI 95.1 – 96.8%) to 98.5% (95% CI 98.0 – 99.0%). Inclusion of D95 reduces specificity from 97.1% (95% CI 95.2 – 98.3%) to 91.4% (95% CI 88.6 – 93.7%), meaning more isolates will be labelled resistant when in fact non-resistant, but as ciprofloxacin is no longer recommended for treatment of *N. gonorrhoeae*, these people would not have received this treatment anyway, highlighting this as an added value approach.

In this review, we demonstrated a clear association between 23S rRNA mutations at positions A2059 and C2611 and azithromycin resistance in *N. gonorrhoeae*, particularly if two or more alleles of the mutated gene were carried. Although using 23S rRNA markers was associated with sensitivity of only 66.1% (95% CI 62.1 – 70.0%) for detecting azithromycin resistance, this could be explained by the association of mutated alleles with moderate/high-level resistance, combined with the fact that a significant proportion of resistant samples were low-level resistant (Figure 1). We also found azithromycin resistance to be associated with mutations in the MtrR protein at H105 and G45, but could not test the independence of these associations.

Applying the lower margin of the 95% CI interval of sensitivity and specificity estimates for a combined 23S rRNA-G45-H105 test, where any deviation from wild-type is regarded as genotypically resistant, we obtained a PPV and NPV of 37.4% (95%CI 36.2 – 38.5%) and 96.4% (95%CI 96.2 – 96.6%), respectively. Interestingly, applying the lower margins of accuracy of a single 23S rRNA mutant allele to this same dataset gave a PPV and NPV of 73.6% (95% CI 72.0 – 75.2%) and 95.9% (95% CI 95.6% –

96.1%), respectively. The estimates suggest that for macrolide resistance, some value may be obtained by using the markers to identify azithromycin susceptible cases genotypically but that this would come at a significant cost in terms of test wastage as a result of misidentifying susceptible cases as resistant. Again, further work to better understand the genotypic-phenotypic relationships of macrolide AMR may improve the accuracy of predictions.

Overexpression of MtrCDE can increase MIC to macrolides [15] and has been associated with fluoroquinolone resistance when in combination with *gyrA* or *parC* mutations [23, 24]. However, this review found mutations within MtrCDE and its promoter unreliable as targeted independent markers of azithromycin and ciprofloxacin resistance due to their prevalence in non-resistant isolates, or association with more definitive resistance markers such as S91. This demonstrates the complicated nature of designing AMR detection systems when a number of mechanisms can influence the resistance phenotype and a limitation of using specific targets for resistance detection. Alternative methods such as sequencing may be able to screen a number of regions associated with resistance and use wild-type across the entire region as a marker of susceptibility. Furthermore, variation in MIC reporting necessitated resistance cut-offs which may mean smaller borderline increases or intermediate MICs associated with certain resistance mechanisms, are missed.

Rapid NAAT diagnostics have been integrated into SHC clinical care pathways [25] but in the UK there remains no licenced diagnostic for dual detection of infection and susceptibility at the PoC, although these are under investigation, for example the Precise study (www.preciseresearch.co.uk) and SpeeDx assay (https://plexpcr.com/resistanceplus-gc/). Furthermore, use and trials of laboratory detection of fluoroquinolone resistance in *N. gonorrhoeae* are underway [26]. However, implementation and technology choice for the detection of resistance markers presents a series of challenges. SNP-based resistance detection may require coverage of multiple alleles, such as the 23S rRNA region in *N. gonorrhoeae* [15], or account for the occurrence of synonymous SNPs to avoid

false calling of resistant isolates. Test efficacy is also highly dependent on active surveillance programmes, monitoring patterns of resistance and associated genotypes, ideally on a global scale to capture the influence of regional differences in antibiotic usage.

This review provides a critical appraisal of genetic determinants of resistance to fluoroquinolones and macrolides in *N. gonorrhoeae*, however, data reporting varied greatly between publications with differences in both resistance mechanisms and specific mutations selected to screen for and report MIC. Included publications are also subject to common limitations including sensitivity and specificity of tests, particularly for studies investigating new methods for resistance detection, although the majority of publications used sequencing or established PCR based assays.

Molecular detection of antibiotic resistance offers the potential to significantly enhance NAAT diagnostics. Providing susceptibility profiles at the PoC enables a more prompt and accurate treatment, an invaluable tool in aiding antibiotic stewardship, a key approach to reduction of AMR. However, such tests should be employed to enhance testing and be performed in conjunction with culture and sequencing to monitor susceptibility profiles and circulating and emerging genotypes.

346 References

347 1. O'Neill J., The Review on Antimicrobial Resistance. Tackling drug-resistant infections
348 globally: Final Report and Recommendations; 2016. Available from http://amr-
349 review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf.
350
351 2. Chisholm SA, Wilson J, Alexander S, Tripodo F, Al-Shahib A, Schaefer U, et al. An
352 outbreak of high-level azithromycin resistant Neisseria gonorrhoeae in England. Sexually
353 transmitted infections. 2016;92(5):365-7. Epub 2015/11/26. doi: 10.1136/sextrans-2015-
354 052312. PubMed PMID: 26601852.
355
356 3. Eyre DW, Sanderson ND, Lord E, Regisford-Reimmer N, Chau K, Barker L et al.
357 Gonorrhoea treatment failure caused by a Neisseria gonorrhoeae strain with combined
358 ceftriaxone and high-level azithromycin resistance, England, February 2018. Euro
359 Surveill. 2018;23(27):pii=1800323
360
361 4. Low N, Unemo M. Molecular tests for the detection of antimicrobial resistant Neisseria
362 gonorrhoeae: when, where, and how to use? Current opinion in infectious diseases.
363 2016;29(1):45-51. Epub 2015/12/15. doi: 10.1097/qco.0000000000000230. PubMed
364 PMID: 26658656.
365
366 5. Mohammed H, Ison CA, Obi C, Chisholm S, Cole M, Quaye N, et al. Frequency and
367 correlates of culture-positive infection with Neisseria gonorrhoeae in England: a review
368 of sentinel surveillance data. Sexually transmitted infections. 2015;91(4):287-93. Epub
369 2014/10/30. doi: 10.1136/sextrans-2014-051756. PubMed PMID: 25352692.
370
371 6. Manhart LE, Gillespie CW, Lowens MS, Khosropour CM, Colombara DV, Golden MR, et al.
372 Standard treatment regimens for nongonococcal urethritis have similar but declining
373 cure rates: a randomized controlled trial. Clinical infectious diseases : an official
374 publication of the Infectious Diseases Society of America. 2013;56(7):934-42. Epub
375 2012/12/12. doi: 10.1093/cid/cis1022. PubMed PMID: 23223595; PubMed Central
376 PMCID: PMCPmc3588116.
377
378 7. Sadiq ST, Dave J, Butcher PD. Point-of-care antibiotic susceptibility testing for
379 gonorrhoea: improving therapeutic options and sparing the use of cephalosporins.
380 Sexually transmitted infections. 2010;86(6):445-6. Epub 2010/10/14. doi:
381 10.1136/sti.2010.044230. PubMed PMID: 20940156.
382
383 8. Gaydos C, Hardick J. Point of care diagnostics for sexually transmitted infections:
384 perspectives and advances. Expert review of anti-infective therapy. 2014;12(6):657-72.
385 Epub 2014/02/04. doi: 10.1586/14787210.2014.880651. PubMed PMID: 24484215;
386 PubMed Central PMCID: PMCPMC4065592.
387
388 9. Unemo M. Challenges with antimicrobial susceptibility testing for Neisseria gonorrhoeae
389 in the era of extensively drug-resistant gonorrhoea — molecular antimicrobial resistance
390 testing crucial. Pathogens and Global Health. 2014;108(5):214-5. doi:
391 10.1179/2047772414Z.000000000216. PubMed PMID: PMC4153821.
392
393 10. Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, et al. Is Neisseria
394 gonorrhoeae Initiating a Future Era of Untreatable Gonorrhea?: Detailed

Characterization of the First Strain with High-Level Resistance to Ceftriaxone. Antimicrobial Agents and Chemotherapy. 2011;55(7):3538-45. doi: 10.1128/AAC.00325-11. PubMed PMID: PMC3122416.

11. Public Health England. GRASP 2016 Report. Surveillance of antimicrobial resistance in Neisseria gonorrhoeae; 2016.

12. Leclercq R. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2002;34(4):482-92. Epub 2002/01/18. doi: 10.1086/324626. PubMed PMID: 11797175.

13. Belland RJ, Morrison SG, Ison C, Huang WM. Neisseria gonorrhoeae acquires mutations in analogous regions of gyrA and parC in fluoroquinolone-resistant isolates. Molecular microbiology. 1994;14(2):371-80. Epub 1994/10/01. PubMed PMID: 7830580.

14. Redgrave LS, Sutton SB, Webber MA, Piddock LJ. Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. Trends in microbiology. 2014;22(8):438-45. Epub 2014/05/21. doi: 10.1016/j.tim.2014.04.007. PubMed PMID: 24842194.

15. Unemo M, Shafer WM. Antimicrobial resistance in Neisseria gonorrhoeae in the 21st century: past, evolution, and future. Clinical microbiology reviews. 2014;27(3):587-613. Epub 2014/07/02. doi: 10.1128/cmr.00010-14. PubMed PMID: 24982323; PubMed Central PMCID: PMCPMC4135894.

16. Chitsaz M, Brown MH. The role played by drug efflux pumps in bacterial multidrug resistance. Essays in biochemistry. 2017;61(1):127-39. Epub 2017/03/05. doi: 10.1042/ebc20160064. PubMed PMID: 28258236.

17. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1. Available http://www.eucast.org. 2017.

18. Public Health England. Sexually Transmitted Infections and Chlamydia Screening in England, 2016. Volume 11, Number 20; 2016.

19. Zaman S, Fitzpatrick M, Lindahl L, Zengel J. Novel mutations in ribosomal proteins L4 and L22 that confer erythromycin resistance in Escherichia coli. Molecular microbiology. 2007;66(4):1039-50. doi: 10.1111/j.1365-2958.2007.05975.x. PubMed PMID: PMC2229831.

20. Bissonnette L, Bergeron MG. Infectious Disease Management through Point-of-Care Personalized Medicine Molecular Diagnostic Technologies. Journal of Personalized Medicine. 2012;2(2):50-70. doi: 10.3390/jpm2020050. PubMed PMID: PMC4251365.

21. Tanaka M, Sakuma S, Takahashi K, Nagahuzi T, Saika T, Kobayashi I, et al. Analysis of quinolone resistance mechanisms in Neisseria gonorrhoeae isolates in vitro. Sexually transmitted infections. 1998;74(1):59-62. PubMed PMID: PMC1758082.

22. Heisig P. Genetic evidence for a role of parC mutations in development of high-level fluoroquinolone resistance in Escherichia coli. Antimicrobial agents and chemotherapy. 1996;40(4):879-85.

23. Ilina EN, Vereshchagin VA, Borovskaya AD, Malakhova MV, Sidorenko SV, Al-Khafaji NC, et al. Relation between genetic markers of drug resistance and susceptibility profile of clinical Neisseria gonorrhoeae strains. Antimicrobial Agents and Chemotherapy. 2008;52(6):2175-82.

24. Balashov S, Mordechai E, Adelson ME, Gygax SE. Multiplex bead suspension array for screening Neisseria gonorrhoeae antibiotic resistance genetic determinants in noncultured clinical samples. The Journal of molecular diagnostics : JMD. 2013;15(1):116-29.

25. Gaydos CA. Review of use of a new rapid real-time PCR, the Cepheid GeneXpert(R) (Xpert) CT/NG assay, for Chlamydia trachomatis and Neisseria gonorrhoeae: results for patients while in a clinical setting. Expert review of molecular diagnostics. 2014;14(2):135-7. Epub 2014/01/24. doi: 10.1586/14737159.2014.871495. PubMed PMID: 24450867; PubMed Central PMCID: PMCPMC4061495.

26. Allan-Blitz LT, Humphries RM, Hemarajata P, Bhatti A, Pandori MW, Siedner MJ, et al. Implementation of a Rapid Genotypic Assay to Promote Targeted Ciprofloxacin Therapy of Neisseria gonorrhoeae in a Large Health System. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2017;64(9):1268-70. Epub 2016/12/31. PubMed PMID: 28034887; PubMed Central PMCID: PMCPMC5399946.

Supporting Information

S1 Text. Search term details.

S2 Text. List of publications included for data analysis.

S3 Data. Data extracted from publications concerning fluoroquinolone resistance in *N. gonorrhoeae.*

S4 Data. Data extracted from publications concerning macrolide resistance in *N. gonorrhoeae.*

Funding Statement

This work was supported by the UK Clinical research Collaboration (Medical Research Council) (http://www.ukcrc.org/) Translation Infection Research Initiative Consortium (grant number G0901608) and by the National Institute for Health Research (NIHR) i4iProgramme (https://www.nihr.ac.uk/about-us/how-we-aremanaged/boards-and-panels/programme-boards436and-panels/invention-for-innovation/)(grant number II-LB-0214-20005). The views

485 expressed are those of the authors and not necessarily those of the NIHR, the NHS or the
486 Department of Health. Both grants were awarded to STS.

487 Conflicts of interest

488 Ms. Hall, Dr Pond and Ms Chow disclose having received funding outside the submitted work from:
489 Atlas Genetics, Alere, Cepheid and Sekisui. Mr. Harrison, Dr. Harding-Esch and Dr. Sadiq disclose
490 having received funding outside the submitted work from: Atlas Genetics, Alere, Cepheid, SpeedDx,
491 Mologic, and Sekisui. Dr Harding-Esch discloses their membership of the Becton- Dickinson
492 "Provision of Sexual Health in the UK" Advisory Board.

493

494

495

496

497

498

499

500 **Table 1. Number of publications included in literature review and data analysis following screening of results from the literature search.**

Inclusion/Exclusion Criteria		Macrolide resistance in *Neisseria gonorrhoeae*	Fluoroquinolone resistance in *Neisseria gonorrhoeae*
	Number of studies following literature search, duplicate deletion and reference and citation checking	**688**	**613**
Inclusion criteria not met	Targets *Neisseria gonorrhoeae*	52	49
	Discusses macrolide or fluoroquinolone resistance	243	137
	Reports resistance-associated genotype in relation to antibiotic of interest	153	239
Exclusion criteria (literature review)	Not available in English language	21	24
	Review data (bibliography and citation check still performed if relevant)	52	22
	Conference abstracts where results are listed in a full publication	1	8
	Number of studies in literature review	**62**	**133**
Exclusion criteria (data analysis)	Studies listing mutations at gene level only (unless resistance is result of gene presence e.g. *erm*)[a]	3	8
	Non-clinical samples e.g. laboratory and reference strains[a]	10	21
	Repeat data sets	7	23
	Cannot determine number of isolates for each genotype	0	3
Number of studies in literature review used in data analysis		**42**	**79**

501 [a]These included strains selected due to known mutation profiles e.g. for testing of new methodology or antibiotics

502 **Table 2. Criteria used to determine publication suitability for analysis**

	Data required from each study for inclusion in analysis									
Analysis conducted	All isolates genotyped	Region/SNPs genotyped	Treatment outcome or MIC	Treatment regimen	Treatment dose	Position of mutation and any change from wild-type detected	Genotype after mutation	Sequence of entire region available	Associated figure or table in this review	Comments
Proportion of resistant isolates with *gyrA/parC* QRDR mutations in *N. gonorrhoeae*	Yes or a random selection	QRDR of *gyrA* or *parC*	Yes	Ciprofloxacin only	No	Yes	No	Yes	None	
Association between ciprofloxacin resistance and fluoroquinolone resistance determinants in *N. gonorrhoeae*	No	See comments	Yes	Ciprofloxacin only	No	Yes (MtrR mutations only recorded to mutate to one residue so these were included)	No	Only mutations being investigated	None	Potential resistance determinants were investigated individually: *gyrA* and *parC* QRDR mutations and Mtr promoter and protein mutations.
Determining independent markers of ciprofloxacin resistance in *N. gonorrhoeae*	No	S91 and D95 in *gyrA* and D86 and S87 in *parC* and DelA and G45D from the Mtr	Yes	Ciprofloxacin only	No	Yes	No	No	None	Those mutations significant in the univariate were analysed in the multivariate, firstly using S91, D95 from *gyrA* with Mtr mutations then S91, D95 with *parC* mutations.
Sensitivity and specificity of ciprofloxacin resistance markers for *N. gonorrhoeae*	No	S91 and D95 in *gyrA*	Yes	Ciprofloxacin only	No	Yes (S91 and D95)	No	S91 and D95 only	Table 4	
Association with azithromycin resistance for macrolide resistance determinants in *N. gonorrhoeae*	No	See comments	Yes	Azithromycin only	No	Yes (MtrR mutations only recorded to mutate to one residue were included)	No	Only mutations being investigated	None	Potential resistance determinants were investigated individually: *erm* genes; 23S rRNA mutations; L4 and L22 mutations and Mtr promoter and protein mutations
Azithromycin resistance and number of mutated *23S rRNA* alleles in *N. gonorrhoeae*	No	A2059 and C2611	Yes and possible to categorise as low, moderate or high	Azithromycin only	No	Yes and number of alleles mutated	No	No	Table 4 and Figure 1.	

			level resistant							
Sensitivity and specificity of *23S rRNA* mutations as azithromycin resistance markers for *N. gonorrhoeae*	No	A2059 and C2611, number of *23S rRNA* alleles mutated	Yes	Azithromycin only	No	Yes	No	A2059 and C2611 only	None	
Sensitivity and specificity of *23S rRNA*, MtrR G45 and H105 mutations as azithromycin resistance markers for *N. gonorrhoeae*	No	A2059 and C2611 of *23S rRNA*, number of *23S rRNA* alleles mutated, G45 and H105 of MtrR	Yes	Azithromycin only	No	Yes	No	A2059 and C2611 of *23S rRNA*, G45 and H105 of MtrR all required	None	

503 QRDR, quinolone resistance determining region.

504

505

Number of alleles harbouring a C2611T or A2059G mutation	Number of azithromycin resistant isolates- n=546 (%)	Number of azithromycin non-resistant isolates- n=469 (%)
0	185	464
1	2	5
2	21	0
3	26	0
4	312	0

Table 3. Number of mutated alleles of the 23S rRNA gene and azithromycin resistance in *Neisseria gonorrhoeae.*

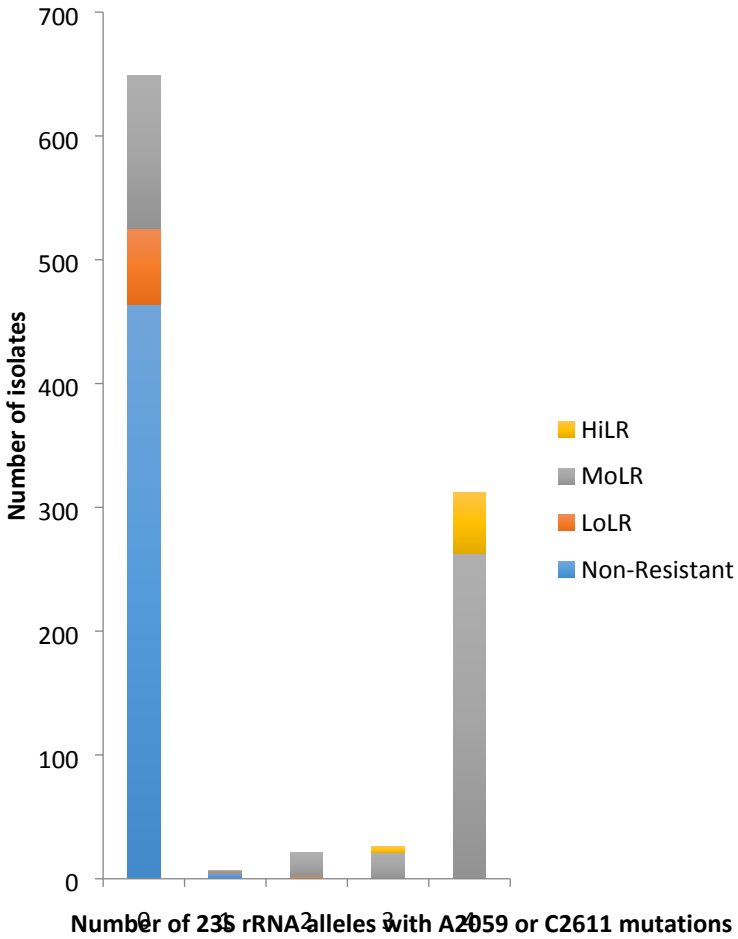

Figure 1. Level of azithromycin resistance with number of mutated 23S rRNA alleles in *Neisseria gonorrhoeae.* Only isolates that could be categorised in to macrolide resistance levels are included in the figure (n=1015). Non-Resistant: MIC≤0.5 mg/L; LoLR= low-level resistance: MIC>0.5mg/L and <2mg/L; MoLR=moderate level resistance: MIC ≥2mg/L and <256mg/L; HiLR=high level resistance: MIC ≥256mg/L. Spearman rank correlation coefficient (r_s) between actual MIC and numbers of mutated alleles =0.7846; p=0.0000005 (n=571)

	gyrA S91 and D95 genotype and ciprofloxacin susceptibility (n=2678)	
Genotype	Number of resistant isolates (%). n=2211	Number of non-resistant isolates (%). n=467
S91 and D95 mutation	1702 (77.0)	6 (1.3)
S91 mutation only	421 (19.0)	8 (1.7)
D95 mutation only	56 (2.5)	26 (5.6)
Wild-type at both residues	32 (1.5)	427 (91.4)

Table 4. Mutations within DNA gyrase at codons S91 and D95 and association with fluoroquinolone resistance in *Neisseria gonorrhoeae*.

Supplement 1

Search terms for the four searches were constructed and performed in OvidSP. Both Medline and EMBASE databases were searched which differ slightly in terms for mapping to subject heading so the details for each are listed.

1. Fluoroquinolone resistance in *Neisseria gonorrhoeae*

MEDLINE

1. exp Neisseria gonorrhoeae/
2. Neisseria gonorrh?eae.ti,ab.
3. 1 or 2
4. exp DNA Gyrase/
5. exp DNA Topoisomerase IV/
6. gyrase.ti,ab.
7. (topoisomerase IV or topoisomerase 4).ti,ab.
8. (parC or parE or gyrA or gyrB).ti,ab.
9. 4 or 5 or 6 or 7 or 8
10. exp Genetics/
11. (gene* or genotyp* or genom*).ti,ab.
12. 10 or 11
13. exp Fluoroquinolones/
14. (fluoroquinolone* or ciprofloxacin).ti,ab.
15. 13 or 14
16. 12 and 15
17. 9 or 16
18. 3 and 17

EMBASE

1. exp Neisseria gonorrhoeae/
2. Neisseria gonorrh?eae.ti,ab.
3. 1 or 2
4. exp DNA topoisomerase IV/
5. exp "DNA topoisomerase (ATP hydrolysing)"/
6. gyrase.ti,ab.
7. (topoisomerase IV or topoisomerase 4).ti,ab.
8. (parC or parE or gyrA or gyrB).ti,ab.
9. 4 or 5 or 6 or 7 or 8
10. exp genetics/
11. (gene* or genotyp* or genom*).ti,ab.
12. 10 or 11
13. exp quinolone derivative/
14. (fluoroquinolone* or ciprofloxacin).ti,ab.
15. 13 or 14
16. 12 and 15
17. 9 or 16
18. 3 and 17

2. Macrolide resistance in *Neisseria gonorrhoeae*

MEDLINE

1. exp Neisseria gonorrhoeae/
2. neisseria gonnorhoeae.ti,ab.
3. 1 or 2
4. exp RNA, Ribosomal, 23S/
5. (23S or rrna or ribosomal RNA).ti,ab.
6. 4 or 5
7. exp Methyltransferases/
8. (ermB or ermF or methylase or methyltransferase).ti, ab.
9. 7 or 8
10. 6 or 9
11. exp Genetics/
12. (gene? or genetic? genotype? or genotypic or genome? or genomic?).ti,ab.
13. 7 or 8
14. exp Macrolides/
15. (macrolide? or azithromycin).ab,ti.
16. 10 or 11
17. 9 and 12
18. 6 or 13
19. 3 and 14

EMBASE

1. exp Neisseria gonorrhoeae/
2. neisseria gonnorhoeae.ti,ab.
3. 1 or 2
4. exp RNA 23S/
5. (23S or rrna or ribosomal RNA).ti,ab.
6. 4 or 5
7. exp RNA methyltransferase/
8. (ermB or ermF or methylase or methyltransferase).ti, ab.
9. 7 or 8
10. 6 or 9
11. exp genetics/
12. (gene* or genotyp* or genom*) .ti,ab.
13. 7 or 8
14. exp macrolide/
15. (macrolide* or azithromycin).ab,ti.
16. 10 or 11
17. 9 and 12
18. 6 or 13
19. 3 and 14