Characterization of *Neisseria gonorrhoeae* strain differences in patients with multi-site infection

Original Research

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Running title:
Multi-site gonococcal infection

Summary:
We performed antimicrobial susceptibility testing (AST) on gonococcal isolates from 41 patients with multi-site infection. Whole genome sequencing based typing was performed on a subset of gonococcal isolates from patients with divergent AST profile differences. Multi-strain carriage was found in 14.6% (6/41) of our population, 4/6 of these due to infection with distinct strains.

Key words:
Neisseria gonorrhoeae, multisite infection, antimicrobial resistance, whole genome sequencing

ABSTRACT

Background

Neisseria gonorrhoeae, the etiological agent of gonorrhoea, is an increasing global health priority due to high levels of antimicrobial resistance (AMR). It is estimated that up to 42% of patients are infected at multiple anatomical sites simultaneously. Previous studies identified that 7-40% of those with multisite infection have different strains infecting the different sites, with potentially different antimicrobial susceptibility profiles. This study aims to estimate the proportion of patients with multi-site infection through differential AST profiles and sequence based molecular methods.

Methods

This was a cross-sectional study of multisite gonococcal isolates provided by three National Health Service (NHS) laboratories. Minimum inhibitory concentrations (MICs) for cefixime, ceftriaxone, azithromycin, ciprofloxacin, tetracycline and spectinomycin were determined. Possible multi-strain infections were defined as isolates with a significant difference in minimum inhibitory concentration to at least one antimicrobial. Whole genome sequencing (WGS) was performed to determine multi-strain infection through *NG*-MAST, *NG*-STAR, MLST, SNP phylogeny and to compare AST profiles with identified AMR genes.

Results

Ninety-one isolates were collected from 41 patients with multi-site infections. Six (14.6%) of 41 patients had *N. gonorrhoeae* isolates with discordant MICs and WGS based typing confirmed that 4/6 patients were infected with different gonococcal strains. The relatedness of isolates with the same MLST across multiple patients were able to be differentiated using SNP based analysis and included the identification of a potential transmission event. WGS based AMR prediction for all antimicrobials tested correlated well with the phenotypic data.

Conclusion

This study demonstrates that potentially a significant proportion of patients with multi-site infections are infected with multiple gonococcal strains, with differing AST profiles, at different anatomical sites. This has implications for patient sampling, susceptibility testing protocols, AMR surveillance, and potentially appropriate antibiotic therapy.

Key Messages

What is already known on this topic. Studies thus far have reported 7-40% of individuals tested having multiple strains but have used outdated typing methods or have not used a comprehensive set of MIC data. Mixed gonococcal infections have also been found in single sites (1.3-2.3%).

What this study adds. This study adds newer data on multi-site gonococcal infection. To our knowledge this is the first study on multi-site infection to include molecular typing and comprehensive MIC data on all isolates.

How this study might affect research, practice or policy. This study further supports the policy on enhanced patient sampling, particularly in multiple anatomical sites. It also informs potential guidance on susceptibility testing of multi-site gonococcal isolates and encourages more research to determine how multi-site infection impacts AMR surveillance.

INTRODUCTION

Neisseria gonorrhoeae has become a public health priority due to high levels of antimicrobial resistance (AMR) and an increasing number of treatment failures[1]. In 2016, an estimated 87 million cases of gonorrhoea occurred globally, an increase from 78 million in 2012[2].

N. gonorrhoeae can infect most mucosal sites, including the urethra, cervix, conjunctiva, rectum, and pharynx and up to 42% of patients are infected at multiple anatomical sites simultaneously[3]. Rectal and pharyngeal infections are largely asymptomatic, which makes clinical diagnosis and testing decisions challenging[4]. Thus, enhanced screening of multiple anatomical sites is now widely advocated and can detect up to 77% more infections in certain populations compared to single site sampling[5]. Based on previous research, there is evidence of 7-40% of patients with multi-site infection carrying different strains at different anatomical sites[6–11]. However, most strain typing studies pre-date 2014, used depreciated methods such as auxotyping, or did not report minimum inhibitory concentrations (MICs) to all antimicrobials tested by national surveillance schemes[6–11].

Evidence that the pharynx plays an important role in the development and spread of gonococcal antimicrobial resistance further highlights the importance of detecting of extra-genital infections[12,13]. Studies have also reported that pharyngeal isolates exhibited higher MICs than extra-pharyngeal isolates[10,14]. Furthermore, patients with pharyngeal gonorrhoea are also more likely to fail treatment with extended spectrum cephalosporins than those with extra-pharyngeal infection[15–17].

There is no formal guidance outlining how laboratories should perform and report antimicrobial susceptibility testing (AST) from patients with multi-site gonococcal infection, with individual laboratories operating on local protocols. It is also unclear how this may impact surveillance of AMR, for example the England Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) processes only a single isolate from multi-site infection, favouring the pharyngeal isolates[18]. The main aim of this study was to investigate the proportion of patients with multi-site infections carrying multiple strains of

gonorrhoea across their different anatomical sites, by MIC testing and modern molecular typing.

METHODS

Study design

This was an exploratory cross-sectional study of antimicrobial susceptibility profiles and sequence types in multi-site *N. gonorrhoeae* infection.

Gonococcal isolates

Gonococcal isolates from multi-site infection (defined as infection in more than one anatomical site simultaneously in a single patient) were provided by three National Health Service (NHS) laboratories within the Royal Free London NHS Foundation Trust (RFH), Maidstone and Tunbridge Wells NHS Trust (MTW) and St George's University Hospital NHS Foundation Trust (SGH). Anatomical sites included were urethra, cervix, rectum and pharynx. The isolates were collected from consecutive patients attending genitourinary medicine (GUM) clinics served by those laboratories for one year between 2014-2015. Each laboratory identified the gonococcal isolates based on local diagnostic protocols and stored them at -80ºC. Each isolate was provided with the following information: laboratory number, anatomical site and patient sex. Isolates were cultured from storage vials on VCAT gonococcal selective media (Oxoid, Basingstoke, England) and identified by gram stain and oxidase. Pharyngeal isolates were further confirmed biochemically by API NH (Biomerieux, Marcy-l'Étoile, France) to differentiate between *N. gonorrhoeae* and contaminating commensal *Neisseria* species. Purified isolates were stored in 20% glycerol brain heart infusion (BHI) broth (Oxoid) at -70°C until further testing.

Antimicrobial susceptibility testing

Antimicrobial MICs for cefixime, ceftriaxone, azithromycin, tetracycline and spectinomycin were determined by agar dilution using the Clinical Laboratory Standards Institute (CLSI) methodology[19] and MICs for penicillin and ciprofloxacin were determined by gradient strip (Biomerieux). Both methodologies used Gonococcal Medium Base (GCMB) (Beckton-Dickinson, Franklin Lakes, New Jersey, US) supplemented with 1% Vitox (Oxoid). Gonococcal isolates resistant to penicillin were tested for β-lactamase production with a nitrocefin disc (Oxoid). Eight WHO reference strains (F, G, K, L, M, N, O and P) were included in all MIC testing for quality control [24]. Clinical and control strains of *N. gonorrhoeae* were subjected to no more than two subcultures before AST. In patients that carried isolates with the same AST profile in multiple anatomical sites, just one of the isolates was used to calculate the cohort's resistance rates.

Multi-site Infection

Gonococcal isolates from a multi-site patient were considered to be possible different strains if there was a difference of ≥ 2 $\log_2$ MIC for at least one antimicrobial, or if they had discrepant β-lactamase results. Discordant MIC results were confirmed by repeat testing (supplementary table S6). Sequencing and molecular typing were performed on gonococcal strains with differing AST profiles.

N. gonorrhoeae Sequencing and Typing

Genomic DNA was extracted with the Archive Pure kit (5PRIME, Dusseldorf, Germany) following the gram-negative bacteria protocol. *NG*-MAST was performed manually, as previously described [20]. In addition to manual *NG*-MAST, WGS libraries were prepared using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, USA) as per the manufacturer's instructions. The libraries were sequenced using the MiSeq platform

(Illumina) set to generate 2 x 251 base-pair paired end reads. Additional sequencing was performed by United Kingdom Health & Security Agency (UKHSA Colindale, UK) on a HiSeq (Illumina) generating 2 x 101 bp paired end reads. Raw fastq data was assessed and trimmed using Trimmomatic v0.39. Trimmed reads were assembled into contigs using Spades v3.13.0. Pilon was used to improve genome assembly and contigs were ordered using ABACAS with the *N. gonorrhoeae* NCCP11945 (NC_011035) genome[21] as the reference, and then annotated using PROKKA with a bespoke database. Multilocus sequencing typing (MLST) was determined *in silico* using MLST script (v2.10) (Seemann T, MLST, Github https://github.com/tseemann/mlst). Antimicrobial genotype prediction was performed using Abricate v0.8.2 (Seemann T, Abricate, Github https://github.com/tseemann/abricate) with the CARD and NCBI databases. *NG*-STAR typing, and AMR prediction were obtained using the PathogenWatch website (https://pathogen.watch/). Single nucleotide mutations (SNPs) and core SNPs were defined using SNIPPY (v4.6.0) (https://github.com/tseemann/snippy) against the *N. gonorrhoeae* NCCP11945 genome using default settings. The phylogeny was determined using the FastTree[22] approximately-maximum-likelihood for alignments of SNP nucleotides using default settings with the SNIPPY SNPs alignment and viewed in GrapeTree[23]

RESULTS

Patients and gonococcal isolates

A total of 101 isolates from 46 patients with multi-site infections were received from the three laboratories. Three patients with two isolates each from RFH, one patient with two isolates from MTW and 95 isolates from 42 patients from SGH. The isolates from MTW (n=2) and two patients' isolates from RFH (n=4) were non-viable on arrival. Further, pharyngeal isolates from two patients from SGH were subsequently identified as *Kingella* species. All

isolates from these five patients were excluded from further analysis. This left 91 isolates from 41 patients that were included in the analysis: two isolates (one patient) from RFH and 89 isolates (40 patients) from SGH (supplementary table S1). Thirty (73.2%) of 41 patients were male. The anatomical distribution of multi-site infections were rectal-pharyngeal in 14 (34.1%) of 41 patients, urethral-pharyngeal in four (9.8%), urethral-rectal in nine (22%), urethral-cervical in six (14.6%), cervical-pharyngeal in two (4.9%), urethral-pharyngeal-rectal in five (12.2%), and urethral-cervical-pharyngeal-rectal in one (2.4%).

Antimicrobial susceptibility of *N. gonorrhoeae* isolates

To calculate resistance rates, 44 of 91 isolates with the same intra-patient MIC profile were removed as duplicates at random, i.e. no anatomical site was prioritised. For the remaining 47 deduplicated isolates resistance rates for penicillin, ciprofloxacin and tetracycline were 10.6% (5/47), 27.7% (13/47) and 23.4% (11/47) respectively (table 1). Resistance to ceftriaxone, azithromycin, cefixime and spectinomycin was not detected. All penicillin resistant isolates tested positive for β-lactamase production (10.6%). The full MIC data for all isolates tested can be found in supplementary table S1.

Table 1. Susceptibility of 47 deduplicated study isolates according to CLSI breakpoints.

	PEN	CFX	CRO	AZI	CIP	TET	SPE
Median MIC (mg/L)	0.125	0.004	0.004	0.008	0.008	0.5	16
IQR	0.064-0.25	0.004-0.16	0.002-0.008	0.008-0.016	0.008-2	0.25-0.5	8-16
Range	0.006-64	0.002-0.062	0.002-0.032	0.002-0.061	0.004-64	0.063-16	4-64
Modal MIC (mg/L)	0.094	0.004	0.002	0.004	0.008	0.5	16
MIC breakpoint (> mg/L)*	1	0.25	0.25	1	0.5	1	64
Resistance rate	10.6%	0%	0%	0%	27.7%	23.4%	0%

CLSI; Clinical & Laboratory Standards Institute, MIC; Minimum inhibitory concentration, IQR; interquartile range, PEN; penicillin, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin. * indicates MIC for 'CLSI resistant' classification

Multi-site infection gonococcal strain differences

Antimicrobial susceptibility differences

Six (14.6%, 95% CI; 6.8%, 28.4%) of 41 patients had isolates from different anatomical sites with divergent MICs to at least one antimicrobial, suggesting these patients were carrying different gonococcal strains in different anatomical sites (table 2, supplementary table S6). These included two patients with rectal-pharyngeal infection, two with urethral-rectal infection, one with urethral-pharyngeal infection and one with urethral-pharyngeal-rectal infection (table 2).

Three patients (25, 36 and 40) had MIC differences in two antibiotics (cefixime/ciprofloxacin, penicillin/tetracycline and ceftriaxone/tetracycline respectively), one patient (38) had differences in penicillin, ciprofloxacin and tetracycline, one patient (20) had differences in penicillin, cefixime, azithromycin and ciprofloxacin and one patient (16) had different MICs for all antimicrobials tested apart from penicillin and spectinomycin (table 2, supplementary table S6). Two patients (20 and 38) also had discordant β-lactamase results (table 2). Discordant MICs were further confirmed at least once (supplementary table S6).

Table 2. MIC results and molecular typing for gonococcal discordant isolates.

Patient	Isolate number	Site	PEN	CFX	CRO	AZI	CIP	TET	SPE
16	GC16P	PH	0.016	***0.016***	***0.016***	***0.064***	***0.023***	***2***	32
	GC16U	UR	0.016	***0.004***	***0.002***	***0.004***	***0.006***	***16***	16
20	GC20P	PH	***0.19***	***0.064***‡	0.016	***0.016***	***0.016***	16	16
	GC20R	RE	***>32***§	***0.008***	0.008	***0.004***	***>32***	16	16
25	GC25U	UR	0.125	***0.016***	0.002	0.004	***8***	16	16
	GC25R	RE	0.125	***0.004***	0.002	0.004	***0.064***	16	16
36	GC36U	UR	***2***§	0.004	0.002	0.002	0.008	***0.5***	8
	GC36R	RE	***6***§	0.008	0.002	0.004	0.008	***0.125***	8
38	GC38R	RE	***8***§	0.008	0.002	0.008	***0.008***	***0.125***	8
	GC38P	PH	***0.19***	0.008	0.002	0.008	***3***	***16***	8
	GC38U	UR	***0.25***	0.004	0.002	0.016	***6***	***16***	8
40	GC40P	PH	0.25	0.016	***0.002***	0.016	4	***8***	8
	GC40R	RE	0.25	0.016	***0.008***	0.008	2	***0.25***	4

All patients with discordant isolates were male.

Results in ***bold italics*** indicate different MICs (≥ 2 MIC doubling dilutions for agar dilution method or ≥ 2 MIC gradations with gradient strip method). PH; pharynx, UR; urethra, RE; rectum, MIC; Minimum inhibitory concentration, PEN; penicillin, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin, §β-lactamase positive, ‡mosaic *penA*,

Comparative genomics

Draft genomes were assembled with a mean length of 2,194,125bp (SD ±101,178bp), 52.40% (±0.14%) GC content and 2,151 (±100) CDSs (supplementary table S2). The MLST and *NG*-MAST data indicated that patients 16, 20, 38 and 40 had different strains in different anatomical sites, whereas the ST's indicated patients 25 and 36 had the same strain in both sites (table 2 and supplementary table S3).

Phylogenies of the 13 isolates from the above six patients were generated using SNP data (figure 1). SNP analysis showed that the isolates that differed at MLST and *NG*-MAST level also differed significantly with this higher resolution analysis (e.g. GC16U and GC16P, figure 1). Conversely, isolates with the same ST from a single patient were highly similar at SNP level (e.g. GC25R and GC25U, figure 1).

For the three ST_{MLST}1584 isolates across two patients (GC36U, GC36R and GC38R), *NG*-MAST and *NG*-STAR differentiated GC36U and GC36R ($ST_{NG\text{-}MAST}$19451) from GC38R ($ST_{NG\text{-}MAST}$26) (figure 2, supplementary tables S3 and S4). This was also reflected in the phylogeny with the GC36 isolates clustering together but GC38R was separate but related (figure 1).

Patient 25 isolates were both ST_{MLST}1599 and $ST_{NG\text{-}MAST}$11461 and demonstrated to be highly similar by SNP differences yet differed in susceptibility to cefixime and ciprofloxacin (table 2). Analysis of the draft genomes between the two isolates demonstrated that there were no differences in *gyrA, parC* (figure 2). Further, both isolates carried a 14.001 non-mosaic *penA* allele and a 346D *penA* insertion leading to a penicillin intermediate result and *tet*(M) causing tetracycline resistance (supplementary tables S4 and S5).

Overall, there was good agreement between the presence of genotypic markers of resistance and phenotypic resistance (figure 2). All isolates with a positive β-lactamase test carried a TEM-1 (GC20R, GC36U, GC36R and GC38R) (table 2, figure 2 and supplementary table S5) and were associated with a penicillin MIC ≥2 mg/L. Ciprofloxacin

resistance was associated with a S91F/D95A or S91F/D95G single nucleotide polymorphism (SNP) in *gyrA* and a S87N or D86N SNP in *parC*, leading to MIC ≥2 mg/L (GC20R, GC38P, GC38U, GC40P, GC40R), except for GC25U which was phenotypically resistant (MIC 8 mg/L) without any identifiable genetic resistance determinants in *gyrA* or *parC* (table 2, figure 2, supplementary table S5). Isolates that carried *tet*(M) were associated with tetracycline MIC ≥8 mg/L (GC16U, GC20R, GC25U, GC25R, GC38P, GC38U, GC40P) (table 2, figure 2, supplementary table S5). Isolate GC20P was the only isolate with a mosaic *penA* allele (supplementary table S5).

DISCUSSION

As *N. gonorrhoeae* has become resistant to many first line antimicrobials it is important to ensure the standardisation of AMR surveillance and AST practices. This study found that six (14.6%) of 41 patients with multisite gonococcal infection carried gonococcal isolates with different AST profiles at different sites. For four of six patients these differences were associated with different strains infecting the different anatomical sites. This suggests performing AST on all isolates in multi-site infection would provide the most accurate AMR surveillance estimates. In this study urethral and cervical samples (U-C) from the same patient were considered as multi-site isolates. However, these sites could be considered as a single female urogenital site, as urethral swabs are usually taken to increase the detection rate of cervical infection, rather than acquired by separate sexual contact[24]. All seven U-C isolates in this study had the same MIC (+/- 1 dilution) for all antimicrobials suggesting the same strain in both sites. If these samples are excluded, the proportion of patients with multi-site AST differences increases to 17% (6/35). Previous studies describing divergent isolates in multi-site infection using older methods such as auxotyping and RFLP, reported a prevalence between 7-40%[6–8]. Mixed strains in single anatomical sites have also been detected. In a study by Goire *et al*, an estimated 3.2% (2/63) of samples contained mixed

cultures of *Ng* detected by AST and MLST[25]. A similar proportion of mixed infection (1.3%, 4/298) was found in a subsequent study, determined by *porB* sequencing[26].

Although AST profiles can be valuable in suggesting strain differences, especially in the routine clinical microbiology setting, molecular typing provides more accurate and granular differentiation. This was evident with patients 25 and 36 (table 2) who had strains with different MICs to two or more antimicrobials but were the same strain type by *NG*-MAST, MLST and *NG*-STAR, and were essentially identical by SNP distances (figure 1). The ciprofloxacin MIC difference in patient 25 was particularly striking, 8 mg/L and 0.064 mg/L in the urethral and rectal isolates respectively. On further re-testing, the MIC for both isolates were 0.032 mg/L, indicating that perhaps GC25 contained a mixed culture of *N. gonorrhoeae*, as described previously[25,26]. Understanding the impact of divergent MICs and mixed infections on AMR surveillance is key, as there have been conflicting reports on susceptibility of isolates from different anatomical sites. For example, some studies have reported higher cefixime resistance in pharyngeal compared to extrapharyngeal isolates[27], while others report similar susceptibilities in all anatomical sites[28,29].

High resolution SNP phylogeny identified a potential transmission event involving the $ST_{NG\text{-}MAST}$10421 / ST_{MLST}7822 isolates from the pharynx and urethra of patient 38 and the pharynx of patient 40 (figure 2). Conversely, SNP phylogeny, *NG*-MAST and *NG*-STAR differentiated the ST_{MLST}1584 isolates present in patient 36 and rectal isolate of patient 38 as relatively distinct strains (figure 1, figure 2). Although both *NG*-MAST and MLST are used in gonococcal molecular epidemiology, it is proposed that MLST is more suitable for long-term, large-scale epidemiology whereas *NG*-MAST is more suitable to micro-epidemiology[30]. Ultimately, however, WGS based typing methods provides best resolution for determining relationship between isolates.

Our study is not without limitations. Firstly, our sample size is small, limiting the accuracy of our estimates of occurrence of multi-strain multi-site infections. Most of the samples (97.8%) also originated from a single laboratory, meaning the data is not necessarily representative

of London or a wider population. Further research on a wider and local epidemiology should be conducted to confirm the results of this study in a more recent setting. This is important to capture whether the epidemiology of multi-strain gonococcal infections is evolving over time, considering the data presented in this study is from 2014. This research should include estimation of cost implications that additional AST may have on local and national laboratories. These studies can be further stratified by core transmission groups and other demographic and epidemiological factors such as age, number of sexual partners and travel history. Further research should also be conducted to ascertain whether the cervix and urethra in patients with female anatomy should be considered as the same or different anatomical sites. Isolate sequencing was also limited to those with AST profile differences, limiting the phylogenetic analysis and preventing detection of strain differences among isolates with similar AST profiles. Sequencing all study isolates would have enabled us to further understand the relationship between ST and MICs and more accurately determine the proportion of multi-site infections with strain differences. Access to patient metadata such as sexual orientation, date of collection and further testing results would have been added further context to our results.

Despite these limitations, isolates with differing AST profiles were able to be identified in different anatomical sites within individual patients. This suggests that performing AST on all isolates in multi-site infection would increase the detection of resistant strains, provide the most accurate AMR surveillance estimates and in the event of AST guided treatment could lead to optimised therapy.

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Supplementary Data: All data relevant to the study are included in the article or uploaded as supplementary information.

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FIGURE LEGENDS

Figure 1. Phylogeny of study isolates. Single nucleotide mutations were determined against the *N. gonorrhoeae* NCCP11945 genome using snippy. An approximately-maximum-likelihood phylogeny was estimated using fasttree. Multisite samples are coloured by patient ID and defined by GCn designation, U; urethral, P; pharyngeal, R; rectal. Branch lengths are shown.

Figure 2. *NG*-MAST, *NG*-STAR and MLST of sequenced isolates from six multisite patients with differing antibiograms along genotypic markers of resistance phenotypic susceptibility profiles to penicillin, ciprofloxacin and tetracycline. Indicated is the presence or absence of genotypic resistance markers and susceptibility of the isolates to the antimicrobials stated.

NG-MAST; *N. gonorrhoeae* multiantigen sequence type, *NG*-STAR; *N. gonorrhoeae* sequence typing for antimicrobial resistance, MLST; multilocus sequence type, [a] novel ST [*penA*(34.001), *mtrR*(9), *porB*(11), *ponA*(100), *gyrA*(100), *parC*(100), 23S(100)]; [b] novel ST[*penA*(19.001), *mtrR*(38), *porB*(1), *ponA*(1), *gyrA*(7), *parC*(55), 23S(100)