Understanding the role of the pharynx as a driver for antimicrobial resistance in *Neisseria gonorrhoeae*

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I. ABSTRACT

Introduction: *Neisseria gonorrhoeae* (*Ng*) is the cause of the second most common bacterial sexually transmitted infection (STI) worldwide and a priority antimicrobial resistance (AMR) pathogen. It is widely accepted that pharyngeal infection plays an important role in the development and transmission of AMR and is a key site for control strategies. Ceftriaxone (CRO), alone or combined with azithromycin, is the current empirical treatment of choice in most regions. However, treatment failures (TFs) have already been reported in the literature, especially at pharyngeal infection sites. The main resistance mechanism to CRO is the development of *penA* mosaic genes acquired from commensal *Neisseria* (*N*c) species during pharyngeal infection. Additionally, pharyngeal infection is more difficult to treat than other sites due to the pharmacokinetic limitations of many antimicrobials, leading to lower pharyngeal concentrations. This thesis aims to contribute to evidence relating to the surveillance of gonococcal and *N*c AMR, the relationship between extended spectrum cephalosporin (ESC) TFs and minimum inhibitory concentrations (MICs), and the evaluation of novel treatment options relating to the pharyngeal site.

Methods: The work was carried out by a mixed methods approach; a systematic review was performed to summarise the relationship between treatment failures in pharyngeal and non-pharyngeal isolates. A cross-sectional study of 41 genitourinary clinic (GUM) patients was also undertaken to characterise gonococcal strains in multisite infection and estimate the proportion of patients carrying different strains between anatomical sites. Another cross-sectional study of 50 London School of Hygiene & Tropical Medicine (LSHTM) volunteers was employed to determine the pharyngeal carriage and AMR burden of *N*c species. Lastly, the *in vitro* susceptibility of *Ng* and *N*c strains to chlorhexidine (CHX), an antiseptic compound, were determined using laboratory methods.

Results: The systematic review identified that the pooled *Ng* MICs for pharyngeal TFs were lower than extra-pharyngeal TFs for both cefixime and ceftriaxone and lower than both the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for reduced susceptibility. Of the TFs for ceftriaxone, 66.7% (24/36)

were in the pharynx, and 31% (11/36) had multisite infection that included pharyngeal infection but only failed treatment in the pharynx. Of 41 participants with multisite *Ng* infection, 14.6% (6/41; 95% [CI]; 6.88%, 28.4%) had different strains between anatomical sites, as determined by MICs. Molecular typing confirmed differing strains in four out of the six patients. Carriage rates of *N*c species in our study participants were 86% (43/50; 95% [CI]; 73.8%, 93%) and AMR rates for cefixime and ceftriaxone were both higher than the reported *Ng* rates for that year. Lastly, clinical and control *Ng* strains showed high susceptibility to CHX. Additionally, CHX eradicated *Ng* strains within one minute.

Conclusions: *N. gonorrhoeae* remains a critical priority in the fight against AMR, necessitating innovative public health strategies. This thesis proposes several key interventions: a) ongoing and improved monitoring and reporting of TFs with or without the review of AMR breakpoints for pharyngeal infection, b) enhanced antimicrobial susceptibility testing processes for multisite isolates, c) surveillance of the AMR burden in *N*c species, and d) exploring treatments beyond traditional antimicrobials. Together, these approaches can support efforts to control the development and transmission of AMR in *Ng*.

II. DECLARATION

I, Victoria Miari, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Victoria Miari, October 2024

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VII. ABBREVIATIONS

AGSP	Australian Gonococcal Surveillance Programme
AMR	Antimicrobial Resistance
AMRHAI	Antimicrobial Resistance and Healthcare-Associated Infections
AMRSTI	Antimicrobial Resistance in STIs
AMS	Antimicrobial Susceptibility
API NH	Analytical Profile Index
AST	Antimicrobial Susceptibility Testing
BHI	Brain Heart Infusion
BSHSH	Blood Safety, Hepatitis, Sexually Transmitted Infections and HIV
CDC	Centres for Disease Control & Prevention
cgMLST	Core genome Multilocus Sequence Type
CHX	Chlorhexidine
CLSI	Clinical Laboratory Standards Institute
CPH	Clinical and Public Health
DE	Dey-Engley
DST	Diagnostic Sensitivity Agar
DUS	DNA Uptake Sequence
ECDC	European Centre for Disease Control
ECOFF	Epidemiological Cut-off values
EGASP	Enhanced Gonococcal Antimicrobial Surveillance Programme
ESAG	Enhanced Surveillance of Antimicrobial Resistant Gonorrhoea
ESC	Extended Spectrum Cephalosporin
EUCAST	European Committee for Antimicrobial Susceptibility Testing
Euro-GASP	European Gonococcal Antimicrobial Surveillance Programme
FICI	Fractional Inhibitory Concentration Index
GASP	Gonococcal Antimicrobial Surveillance Programme
GCMB	Gonococcal Medium Base
GNDC	Gram Negative Diplococci
GGI	Gonococcal Genetic Island
GISP	Gonococcal Isolate Surveillance Programme
GLASS	Global Antimicrobial Resistance and Use Surveillance System
GRASP	Gonococcal Resistance to Antimicrobials Surveillance Programme
GUM	Genitourinary Medicine Clinic
HGT	Horizontal Gene Transfer
LBVT.SNR	Luria-Bertani Vancomycin Trimethoprim Sucrose Neutral Red
LMIC	Low- and Middle-Income Country
LSHTM	London School of Hygiene & Tropical Medicine
MALDI-ToF	Matrix Assisted Laser Desorption Ionisation Time of Flight
MBC	Minimum Bactericidal Concentration
MDR	Multidrug Resistant
MF	MacFarland
MIC	Minimum Inhibitory Concentration
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Type
MSM	Men who have Sex with Men
NAAT	Nucleic Acid Amplification Test
*N*c	Commensal *Neisseria spp*
NCTC	National Collection of Type Cultures
Ng	*Neisseria gonorrhoeae*

NG-MAST	*Neisseria gonorrhoeae* Multi-Antigen Sequence Type
NG-STAR	*Neisseria gonorrhoeae* Sequence Typing for Antimicrobial Resistance
OMV	Outer Membrane Vesicles
PAGE	Polyacrylamide Gel Electrophoresis
PID	Pelvic Inflammatory Disease
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PMF	Peptide Mass Fingerprint
PMNL	Polymorphonuclear Leucocyte
POC	Point of Care
PPV	Positive Predictive Value
RE	Restriction Enzyme
RM	Restriction Modification
rMLST	Ribosomal Multilocus Sequence Type
rRNA	Ribosomal RNA
SD	Standard Deviation
SDA	Strand Displacement Assay
SE	Standard Error
SGSS	Second Generation Surveillance System
SHS	Sexual Health Service
SNP	Single Nucleotide Polymorphism
ssDNA	Single-stranded DNA
STI	Sexually Transmitted Infection
T4SS	Type 4 Secretion System
TBE	Tris-Borate Ethylenediaminetetraacetic acid
TF	Treatment Failure
TM	Theyer-Martin
TMA	Transcription-Mediated Amplification
TMPPD	N,N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride
TOC	Test Of Cure
TRNG	Tetracycline Resistant *N. gonorrhoeae*
UK	United Kingdom
UKHSA	United Kingdom Health & Security Agency
UTI	Urinary Tract Infection
VCAT	Vancomycin Colistin Amphotericin Trimethoprim
WGS	Whole Genome Sequencing
WHO	World Health Organisation

VIII. DEFINITIONS OF KEY TERMS

Antimicrobial synergy. When the effect of a combination of antimicrobials is greater than the effect of the antimicrobials alone.

Antimicrobial working concentration. The concentration of an antimicrobial that is used for antimicrobial susceptibility testing.

Batch. A reagent or media that is prepared at any one time as an individual quantity. For example, two bottles of media prepared on separate days were considered two separate batches.

Epidemiological cut-off value (ECOFF). A laboratory term used to define the highest minimum inhibitory concentration (MIC) of a bacterial isolate considered to be part of a "wild-type" population, meaning it lacks detectable resistance mechanisms to a specific antibiotic.

Extrapharyngeal. Relating to *Neisseria gonorrhoeae*; infection not at a pharyngeal site, i.e. urethral, rectal, cervical or eye.

Evolutionary pressure. An external or selective pressure that reduces or increases the reproductive success of a population.

Horizontal gene transfer. The movement of genetic material between bacterial cells and incorporation via homologous recombination or insertion.

Minimum bactericidal concentration (MBC). The minimum concentration of an antimicrobial required to kill at least 99.9% of a bacterial inoculum.

Minimum inhibitory concentration (MIC). The minimum concentration of an antimicrobial required to inhibit the visual growth of bacteria.

MIC breakpoint. The concentration of an antimicrobial that defines whether the organism tested is clinically susceptible or resistant. For example, if an MIC breakpoint of a bacterial isolate is 1 mg/L, isolates with MICs of <1 mg/L are considered susceptible, whereas isolates with MIC ≥1 mg/L are considered resistant.

Multisite infection. Concomitant infection in multiple anatomical sites.

Reduced susceptibility. A term describing decreased sensitivity of an organism to an antimicrobial indicating a potential trend towards resistance, in the absence of established clinical breakpoints.

Treatment failure. Persistent infection after appropriate treatment, where re-infection has been excluded.

CHAPTER 1

INTRODUCTION

1.1. Overview of *Neisseria gonorrhoeae* antimicrobial resistance challenges

Neisseria gonorrhoeae (*Ng*) is one of the top ten global antimicrobial resistance (AMR) threats[1]. Public health organisations globally have published action plans to control the development, spread and impact of antimicrobial resistant gonorrhoea[2–5]. In the United Kingdom (UK), the Health and Social Care Act 2008 recognises AMR as a Government priority and highlights a) infection prevention and b) improvement of antimicrobial stewardship as key components of its strategy[6]. A paradigm shift is thus needed to reduce evolutionary pressure toward antimicrobial resistance, while seeking novel antimicrobials.

Gonorrhoea is treated empirically, meaning that patients receive antibiotics before the susceptibility profile is known[7]. Empirical treatment relies on the World Health Organisation (WHO) standard of using an antimicrobial only if greater than 95% of local gonococcal strains are susceptible (also referred to as "5% resistance threshold")[7]. The treatment of choice in the UK is currently intramuscular 1 g ceftriaxone[8] and is the only remaining antibiotic that can be used reliably to treat gonorrhoea. Worryingly, ceftriaxone resistance and treatment failures (TFs) have already been reported, meaning that evaluating alternative therapeutic agents is a public health priority.

It is generally accepted that pharyngeal gonorrhoea contributes to the development and dissemination of AMR[9]. Pharyngeal gonorrhoea is a major driver of transmission, functioning as a hidden reservoir that maintains the infection prevalence in the population[9]. The development of AMR, particularly to extended spectrum cephalosporins (ESCs) in pharyngeal infection is complex, but can be influenced by a combination of three main factors that can work co-dependently to create the 'perfect storm' of AMR:

i. The asymptomatic nature of the infection leading to lack of timely detection, treatment, and transmission prevention[10].
ii. The poor penetration of β-lactams into the pharyngeal mucosa leading to sub-therapeutic concentrations and selection of resistant strains[11].

iii. The horizontal gene transfer (HGT) of AMR genes from commensal *Neisseria* (*N*c) species during pharyngeal infection[12].

Enhanced understanding of the processes involved in the evolution of AMR *Ng* is critical to apply appropriate control efforts and reduce the emergence of future resistant strains.

1.2. *Neisseria* species: an overview

1.2.1. Introduction to the genus

The genus *Neisseria* belongs to the family *Neisseriaceae*, order *Neisseriales*, class *beta-proteobacteria*[13]. Other genera of medical importance that belong to the *Neisseriaceae* family include *Kingella* and *Eikenella*[13]. *Neisseria* species are Gram-negative, aerobic, non-motile cocci, except for *N. elongata, N bacilliformis* and *N. weaveri* which have a rod-like appearance[14]. *Neisseria* species colonise the human oropharynx and upper respiratory tract of humans and animals, apart from *Ng* that primarily infects mucosal surfaces of urogenital sites[14]. Ten species are thought to be associated with human colonisation, eight commensal (*N. lactamica, N. cinerea, N. polysaccharea, N. mucosa, N. oralis, N. subflava, N. elongata* and *N. bacilliformis*), and two pathogenic species, *Ng* and *N. meningitides*, the causative agents of gonorrhoea and meningitis respectively[15]. Recent metagenomic studies describe *N*c as being part of the "core" oropharyngeal flora, as they are the most abundant genus within *beta-proteobacteria*[15].

The first *Neisseria* species to be discovered was *Ng*, identified by Albert Ludwig Sigesmund Neisser in 1879, when he observed small diplococci in exudates of men and women with gonorrhoea[16]. This was followed by the discovery of *N. meningitides* by Anton Weichselbaum in 1887 which was isolated from the cerebrospinal fluid of patients with meningitis[17]. Alexander von Lingelsheim isolated the first *N*c which he named *Micrococcus cinereus*, now known as *N. cinerea*, in 1906 and subsequently described *N. sicca, N. flava* and *N. subflava*[17]. There are currently at least 44 published *Neisseria* species by the List of Prokaryotic names with Standing in Nomenclature[18] (LPSN; accessed 19 August 2024) and 47 by the National Center for Biotechnology Information (NCBI; accessed 19 August 2024)[19].

1.2.2. Taxonomy and phylogeny of Neisseria

The phylogeny of *Neisseria* species is continuously being redefined and evolving alongside advancing technologies. Before the introduction of molecular technologies, taxonomic changes were implemented using phenotypic techniques, specifically the production of pigment, the ability to ferment specific sugars (Table 1), superoxol (30% H_2O_2) reaction, the ability to reduce nitrite, the requirement for CO_2 for growth and growth on different types of media[20]. The genus itself belonged to the family *Coccaceae* until 1948 when it was reassigned as the 'type' genus *Neisseriaceae* and included the genera *Moraxella, Acinetobacter*, and *Kingella*[20]. Early attempts at taxonomy and phylogeny placed *Neisseria* species in two groups; the first group included *Ng, N. meningitidis, N. lactamica, N. cinerea, N. flavescens, N. polysaccharea*, and *Ng* subsp. *kochii*. The second group included *N. subflava, N. perflava, N. flava, N. sicca*, and *N. mucosa*[20].

In the 1980s, 16S ribosomal RNA (16S rRNA) sequencing became a new standard for molecular speciation of bacteria[21]. The 16S rRNA gene is comprised of conserved and species-specific hypervariable regions (V1-V9), making it possible to design universal primers that complement the conserved regions of the gene and sequence the variable regions[22]. Sequences generated can be compared to curated databases to determine the species of the organism[21]. A study in 1999 identified five groups of *Neisseria* using 16s rRNA: group one contained *N. lactamica*, N*. meningitidis, N. gonorrhoeae* and *N. polysaccharea*, group two included *N. subflava*, *N. flavescens*, *N. perflava* and *N. mucosa*. The third group included only *N. cinerea* strains. The fourth group contained *N. pharyngis, N. sicca and N. flava* and the fifth group contained *N. elongata* species[23]. Sequencing of 50S ribosomal protein L6 (*rplF)* gene and ribosomal multilocus sequence typing (rMLST) have been suggested to have higher phylogenetic resolution than 16S rRNA[21]. Ribosomal multilocus sequence typing is a scheme based on the sequences of 53 ribosomal loci and can be used to define species grouping and strain types[24]. The introduction of whole genome sequencing has greatly improved the taxonomical categorisation of *Nc* [25] and has led to the reclassification of known species as well as the discovery of new ones. A study using core genome MLST (cgMLST) (1.4.5) suggested the re-classification of *N. sicca* to a variant of *N. musoca* and *N. subflava, N. flava, N. flavescens* and *N. perflava* to a single *N. subflava* biovar[26]. A study in 2013 that performed

phylogenetic analysis of 51 *Neisseria* isolates by cgMLST suggested that *N. mucosa* var. *heidelbergensis* should be renamed *N. oralis*[27]. A further study in 2019 identified seven putative novel *Neisseria* species by cgMLST; *N. bergeri, N. maigaei, N. uirgultaei, N. basseii, N. blantyrii, N. viridiae* and *N. benectictiae*[28].

The focus of this thesis is on *Ng* and *N*c species; therefore, *N. meningitidis* will not be discussed further in terms of a public health problem, as it is beyond the scope of this work.

Table 1. Summary of key phenotypic properties of commensal *Neisseria* species. Adapted from Knapp and Hook[20]

Species	Acid from					Nitrate reduction	Superoxol (30% H_2O_2)
	Glu	***Mal***	***Suc***	***Fru***	***Lac***		
N. gonorrhoeae	+	-	-	-	-	-	+
N. meningitidis	+	+	-	-	-	-	-
N. lactamica	+	+	-	-	+	-	-
Kingella dentrificans	+	-	-	-	-	+	-
N. cinerea	-	-	-	-	-	-	-
N. sublfava	+	+	-	-	-	-	-
N. flava	+	+	-	+	-	-	-
N. perflava	+	+	+	+	-	-	-
N. sicca	+	+	+	+	-	-	-
N. mucosa	+	+	+	+	-	+	-
N. flavescens	-	-	-	-	-	-	-

+; >90% strains positive, -; >90% strains negative
Glu; glucose, Mal; maltose, Suc; sucrose, Fru; fructose, Lac; lactose
H_2O_2; hydrogen peroxide

1.3. Clinical presentation, management, and epidemiology of *Neisseria* species

1.3.1. N. gonorrhoeae

N. gonorrhoeae infect the mucosal surfaces of the cervix, urethra, rectum, eye and pharynx. Approximately 20% of genital infections in men and 50% in women are asymptomatic, but extra-genital infection is almost always asymptomatic[8]. In women, genital gonorrhoea can present as mucopurulent cervicitis with symptoms of increased vaginal discharge, post-coital bleeding, intra-menstrual bleeding, or pain during sex. In men, it most commonly presents as gonococcal urethritis with symptoms of urethral discharge, inflammation and pain on urination. When symptomatic, rectal, and pharyngeal infections present as proctitis and pharyngitis respectively[8]. Complications of gonorrhoea include pelvic inflammatory disease (PID) and ectopic pregnancy in women. In men, the most common complications include epididymo-orchitis, urethral stricture and reduced fertility. Disseminated gonococcal infection can lead to severe life-threatening infections including endocarditis and systemic complications such as perihepatitis and reactive arthritis[8].

Gonorrhoea is treated 'empirically' with patients receiving antibiotics before the susceptibility profile of the infecting strain is known[7]. Empirical treatment relies on the WHO standard of using an antimicrobial only if greater than 95% of local gonococcal strains are susceptible[7]. The recommended treatment in the UK is intramuscular 1g ceftriaxone[8] (Table 2). A test of cure (TOC), taken up to 14 days post-treatment, is recommended on all patients, with a particular focus on those a) with persistent symptoms or signs, b) with pharyngeal infection, c) treated with anything other than first line recommended regimen when the antimicrobial susceptibility of the isolate is unknown or d) who acquired the infection in the Asia-Pacific region when the antimicrobial susceptibility is unknown[8].

Table 2. Summary of uncomplicated gonorrhoea treatment guidelines in different geographical regions as of August 2024.

Country/Region	First choice	Alternative
UK[8]	Ceftriaxone 1 g IM	Ciprofloxacin 500 mg PO*
USA[29]	Ceftriaxone 500 mg IM	Gentamicin 240 mg IM *plus* azithromycin 2 g PO or Cefixime 800 mg PO
Europe[30]	Ceftriaxone 1 g IM *plus* azithromycin 2 g PO‡	Spectinomycin 2 g IM *plus* azithromycin 2 g PO
Australia[31]	Ceftriaxone 500 mg IM *plus* azithromycin 1 g PO	None
Africa[32]	Ceftriaxone 500 mg IM§	Cefixime 800 mg PO
Japan[33]	Ceftriaxone 1 g DI	Spectinomycin 2 g IM

IM; intramuscular, PO; orally, DI; drip infusion
**If proven susceptibility to ciprofloxacin only*
‡Azithromycin can be omitted in certain clinical settings
§Increase dose to 1 g if confirmed pharyngeal infection.

Although gonorrhoea can infect all population groups, certain patient groups are disproportionately affected. For example, in England, 48% of all gonorrhoea diagnoses were in men who have sex with men (MSM) and 68% of all diagnoses in men were in MSM[34]. Transmission in MSM occurs in dense sexual networks where there are high rates of sex partner change or concurrency and can lead to localised outbreaks[33]. Persons of black Caribbean ethnicity are also disproportionately affected as they are more likely to be associated with deprivation and poor access to healthcare services, higher incidence of risky sexual behaviour and transmission within dense sexual networks[35]. Asymptomatic infection, particularly in the pharynx is key in the widespread transmission of gonorrhoea; these patients are colonized for longer periods which facilitates optimal conditions for transmission[36].

An estimated 87 million cases of gonorrhoea occurred globally in 2016 (0.9% prevalence), an increase from 78 million cases in 2012[37]. In England 82,592 (146.1 per 100,000) infections were reported in 2022, an increase of 50% from 2021 and 165% from 2013, and 2022 saw the highest number of gonorrhoea diagnoses on record. While England has rigid surveillance systems for monitoring the incidence of gonorrhoea and AMR trends, this is not the case in most regions globally, especially in low and middle-income (LMIC) countries. The most recent global estimates

(2016) of gonorrhoea incidence and prevalence were generated from data from individual prevalence studies using Bayesian meta-analysis (Fig. 1). The highest incidence was in the WHO African region, with 41 cases per 1,000 women and 50 per 1,000 men, followed by the WHO region of the Americas, with 23 cases per 1,000 women and 32 per 1,000 men; the lowest incidence was in the WHO European region, with 7 cases per 1,000 women and 11 per 1,000 men[37]. Although surveillance data from African countries is sparse, despite the highest estimated burden, a systematic review estimated a pooled *Ng* prevalence of 3.28% (95% confidence interval 2.61%, 3.94%) among women[38].

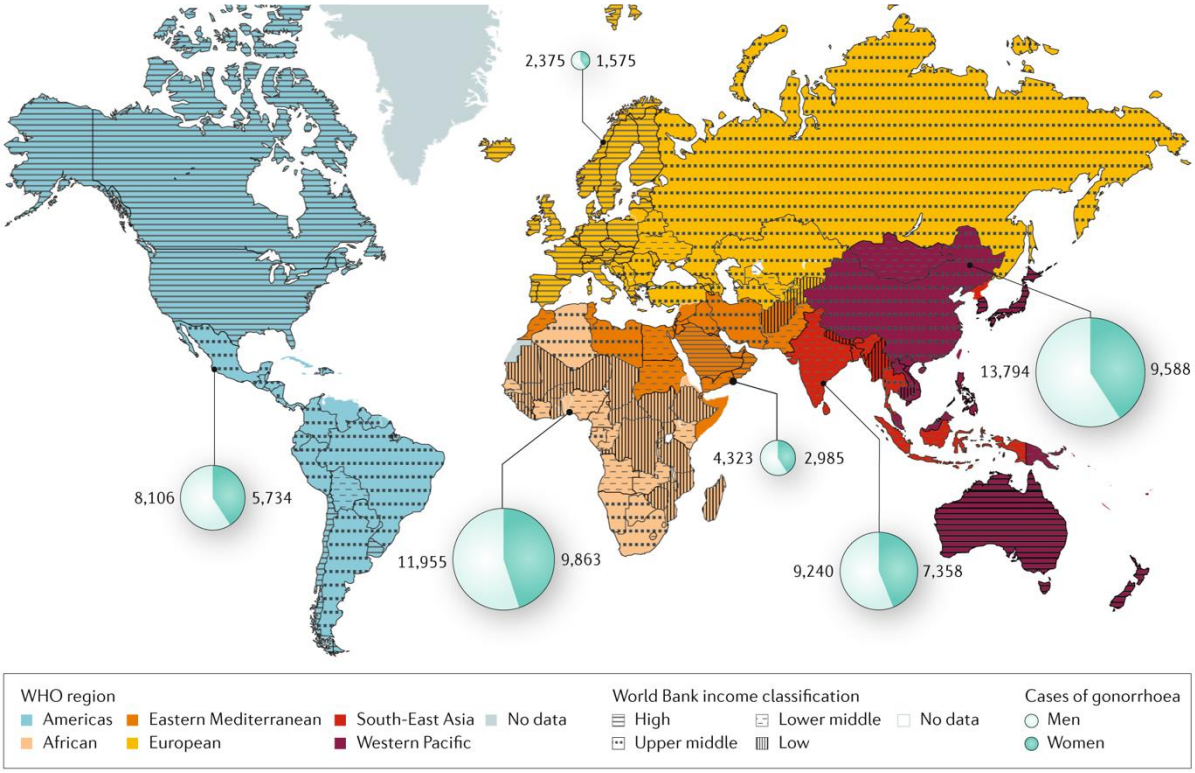

Figure 1. Estimated numbers (in millions) of incident cases of gonorrhoea in adults (15–49 years of age) by WHO region[39]. Image reproduced from Rowley *et al* (2019)[37] (CC BY 3.0).

1.3.2. Multisite N. gonorrhoeae infection

Multisite gonorrhoea can be defined as a concomitant gonococcal infection in more than one anatomical site in an individual patient. In 2022, the Gonococcal Resistance to Antimicrobials

Surveillance Programme (GRASP) reported that 38% of patients had multisite infection in England and Wales during the surveillance period[40]. However, the literature reports multisite infection in 24-42% of patients[41–43] and colonisation by one or more strains in the different anatomical sites[44–47]. For example, a study of 84 individuals with multisite infection found that 3.6% (3/84) of patients carried strains with differing penicillin minimum inhibitory concentrations (MICs), and 3.6% had strains with differing auxotypes (1.4.5), with one patient having both differing MICs and auxotypes[44] (Table 3). Similarly, another study of 69 individuals showed 8.7% (6/69) of their patients had isolates with differing antibiograms, with 2/6 having different auxotypes[46]. A study in Japan that assessed two individuals with urethral and pharyngeal infection showed that in one individual, the urethral isolate had differing auxotype, pulsed-field gel electrophoresis (PFGE) profile and MICs to a penicillin, cefixime, tetracycline and spectinomycin[48]. Three other studies in the Netherlands and the UK assessed strain type only, using auxotyping, *Ng* multiantigen sequence typing (*NG*-MAST), and *por-opa* restriction fragment length polymorphism (RFLP), and found that 7%, 9% and 40%, respectively of multisite infections had different strains at the different sites[46,47,49] (Table 3). Mixed infections can also occur in a single anatomical site; an estimated 3.2% of samples in an Australian population were identified as having more than one strain when tested by MIC and MLST[50]. Five of these studies have used outdated typing techniques such as auxotyping. However, there is limited evidence of the ecology of multisite infection; the most recent study was published in 2013 and only used *NG*-MAST[47]. Further, there is no formal guidance on how laboratories should process multisite isolates, especially regarding antimicrobial susceptibility testing (AST). Currently, the absence of NHS guidelines leads laboratories to either perform AST on each isolate individually or test one isolate and extrapolate the results to isolates from the other sites (personal observation). The latter practice risks overlooking resistant strains, potentially impacting patient management and AMR surveillance. Generating more evidence on multisite infections can lead to enhanced patient sampling, improved antimicrobial stewardship practices, and refined treatment and surveillance strategies for gonococcal infections and AMR.

Table 3. Summary of studies describing gonococcal strain differences in multisite infection.

Author	Year	Country	Number of patients tested	Number of isolates	Proportion of different strains (%)	Lab methods used	Antimicrobials Tested
Catlin, BW *et al*[44]	1977	U.S.	84	181	7%	Auxotyping MIC	PEN, RIF, STR, TET, CHL, OXA, ERY
Noble, RC[45]	1980	U.S.	69	140	8.7%	Auxotyping Acrylamide gel electrophoresis MIC	PEN, AMP, TET, SPE
Ansink-Schipper, MC *et al*[49]	1985	The Netherlands	50	100	7%	Auxotyping MIC	PEN, TET, ERY, SPE, CFR, TPH
Saika T, *et al*[48]	2001	Japan	2	4	50%	Auxotyping PFGE MIC	PEN, CFX, TET, SPE, CIP, SPA
Kolader ME *et al*[46]	2006	The Netherlands	130	287	40%	RFLP	NT
Eastick K‡[47]	2013	UK	410	860	9.8%	*NG*-MAST	NT
Miari *et al*	2024	UK	41	97	14.1%	*NG*-MAST MLST SNP phylogeny MIC	PEN, CFX, CRO, AZI, CIP, TET, SPE

MIC; minimum inhibitory concentration, PFGE; pulse-filed gel electrophoresis, RFLP; restriction fragment length polymorphism, NG-MAST; Neisseria gonorrhoeae multiantigen sequence type, MLST; multilocus sequence type, SNP; single nucleotide polymorphism, PEN; penicillin, RIF; rifampicin, STR; streptomycin, CHL; chloramphenicol, OXA; oxacillin, ERY; erythromycin, AMP; ampicillin, TET; tetracycline, SPE; spectinomycin, CFR; cefuroxime, TPH; thiamphenicol, CFX, cefixime, CIP; ciprofloxacin, SPA; sparfloxacin, CRO; ceftriaxone, AZI; azithromycin
‡Conference proceedings

1.3.3. Significance of pharyngeal infection

The significance of pharyngeal gonorrhoea relating to patient morbidity has been questioned, particularly whether it can be considered a true infection or transient colonisation[51]. There is, however, clinical and scientific consensus that pharyngeal gonorrhoea is a major driver of transmission and development of AMR, functioning as a hidden reservoir that maintains the infection prevalence in the population[9,52,53]. Pharyngeal gonorrhoea disproportionally affects MSM, with rates increasing over time; one Australian study found an increase in positivity by 183% between 2011 to 2015, compared to a 39% and 87% increase in urogenital and anorectal positivity respectively[54]. The cycle of gonococcal acquisition, development of AMR and transmission is complex and can be attributed to several factors. Firstly, over 90% of pharyngeal gonorrhoea infections are asymptomatic meaning that patients are less likely to recognise the infection, seek care and therefore continue the transmission chain[55,56]. This is further exacerbated by the ability of pharyngeal gonorrhoea to persist in the pharynx for up to three months[57]. *N. gonorrhoeae* can also be isolated from saliva[58] providing evidence for the transmission of gonorrhoea through oral sex which has implications for the risk of transmission by common sexual practices, previously perceived to be harmless, such as kissing[59]. For example, a recent study found that the use of saliva as a lubricant for anal sex was associated with rectal gonorrhoea[59] and concluded that rectal gonorrhoea could be reduced by half by eliminating this practice. This has influenced the review of the traditional gonococcal transmission paradigm, that the male urethra has a central role in transmitting gonorrhoea to the pharynx and anorectal sites, especially in MSM patients. However, in 2017, Fairley *et al* proposed that instead, the pharynx is the major source of onward transmission, and the urethra is the primary recipient[10] (Fig. 2). This was further substantiated with a study of 60 MSM couples (120 men) that found a high partner-to-partner gonococcal transmission even in the absence of urethral infection[60]. This is further applicable in heterosexual couples, as pharyngeal gonorrhoea in women and men is common; a study estimated that sampling the pharynx in addition to the urogenital sites increased gonorrhoea detection by 38%[61]. Additionally, 27% of the study patients were positive only at the pharynx so would have otherwise been missed by urogenital-only screening[61].

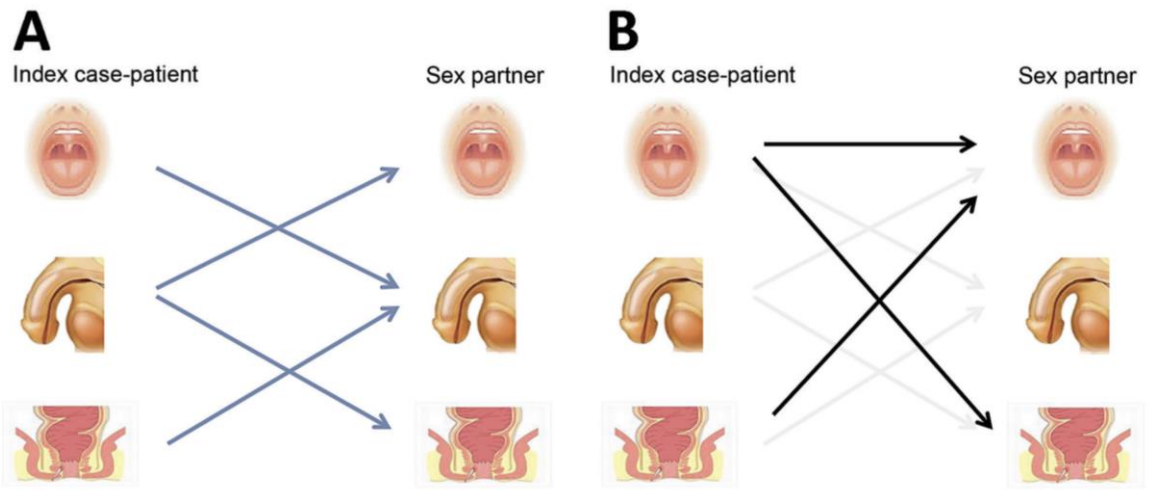

Figure 2. Traditional and proposed transmission models for gonorrhoea in men who have sex with men (M). A) Generally accepted transmission routes (arrows) for gonorrhoea between sites in MSM from an infected index case-patient to an uninfected sexual partner. B) Additional proposed transmission routes (dark arrows) compared with accepted transmission routes (light arrows). MSM, men who have sex with men. Figure acquired from Fairley *et al* 2017[10] (Image without copyright).

The pharynx also provides the optimal environment for the development of AMR through the uptake of bacterial DNA released into the environment by *Nc* species and other organisms[62]. This ability generates genetic and antigenic diversity and enables the acquisition and spread of AMR[63–65]. Of particular importance is the acquisition of *penA* from ESC resistant commensal species leading to gonococcal mosaic *penA* genes and β-lactam resistance or MIC increases[66]. This is facilitated by gonococcal type IV pili that recognise unique DNA sequences termed DNA Uptake Sequences (DUS) and incorporate them into its genome via homologous recombination (1.5.5)[67]. Lastly, pharyngeal gonorrhoea is associated with treatment failure more than genital sites, especially for ESCs[68–71]. In a 2013 study, treatment failures with cefixime occurred in 28.6% of pharyngeal infections compared to 5.26% and 7.69% of urethral and rectal infections, respectively[71]. To date, 66.7% (24/36) of reported ceftriaxone clinical failures have occurred in pharyngeal infection, compared to 19.4% (7/36) in urethral and 16.7% in rectal infection (1.5.2). Importantly, in 11 patients with simultaneous pharyngeal and extra-pharyngeal infection, pharyngeal infection persisted whereas extra-pharyngeal infection was successfully treated (1.5.2). Furthermore, in these reported cases, almost 64% (7/11) cases were infected with

phenotypically ceftriaxone susceptible strains (MIC 0.016-0.064 mg/L). This is believed to be due to the complex pharmacokinetic activity of β-lactam antibiotics in oropharyngeal tissue[11]. Studies have shown that serum concentration of cefixime was five-fold higher than salivary levels[72]. It is estimated that the serum concentration of β-lactams should be at least four times the organism's MIC for successful treatment which can be challenging as there is variability in antimicrobial clearance and almost 90% of ESCs can be protein bound[11]. Due to these factors (Fig. 3), the pharynx is a key site to target in the AMR control efforts.

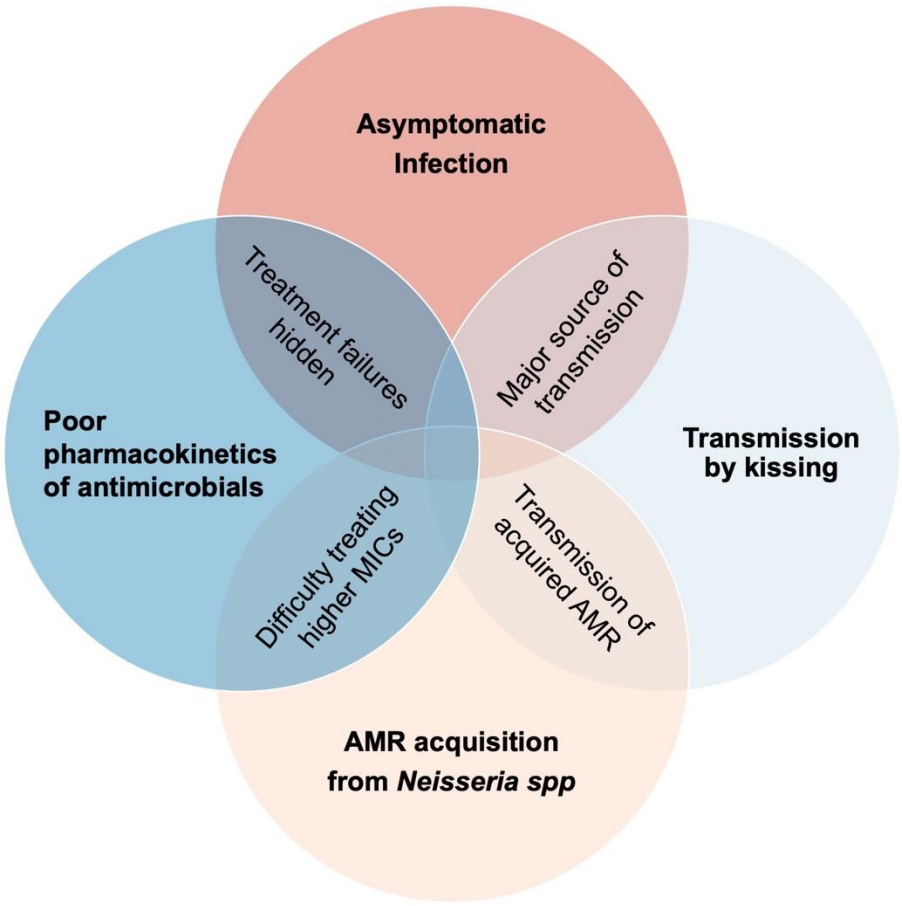

Figure 3. Factors contributing to the importance of pharyngeal gonorrhoea in the development and spread of AMR.

1.3.4. Clinical significance of commensal Neisseria *species*

Commensal *Neisseria* species are ubiquitous organisms, found in the nasopharynx of humans and animals[73]. Although *N*c are usually non-pathogenic in healthy hosts, there have been reports of unusual infections, mainly in immunocompromised patients[13]. The most common infections reported are septicaemia, endocarditis and respiratory tract infections, by commensals such as *N. lactamica, N. mucosa* and *N. flavescens*[13] (Fig 4). Overall, however, colonisation with *N*c species is thought to be more beneficial than harmful. For example, a study showed that *N. lactamica* had a protective effect against *N. meningitidis* colonisation in adults, specifically, meningococcal carriage was reduced from 24% to 14% in patients who were colonised by *N. lactamica*[74]. Further, it has been suggested that *N. elongata* can kill *Ng in vitro* by donating DNA with a different methylation status to that of *Ng*[75]. The most important role of *N*c is thought to be as donors of antimicrobial resistance genes, particularly *penA*, leading to the development of ESC AMR in pathogenic *Neisseria* species. A recent analysis of 1,700 *penA* alleles across 15 *Neisseria* species found that *Ng* acquires mosaic *penA* from *N. cinerea* and *N. subflava*, whereas *N. meningitidis* acquires mainly from *N. lactamica*. As such, it has been suggested that surveillance of *N*c species can contribute to the strategy of delaying the spread of AMR in pathogenic *Ng*[15].

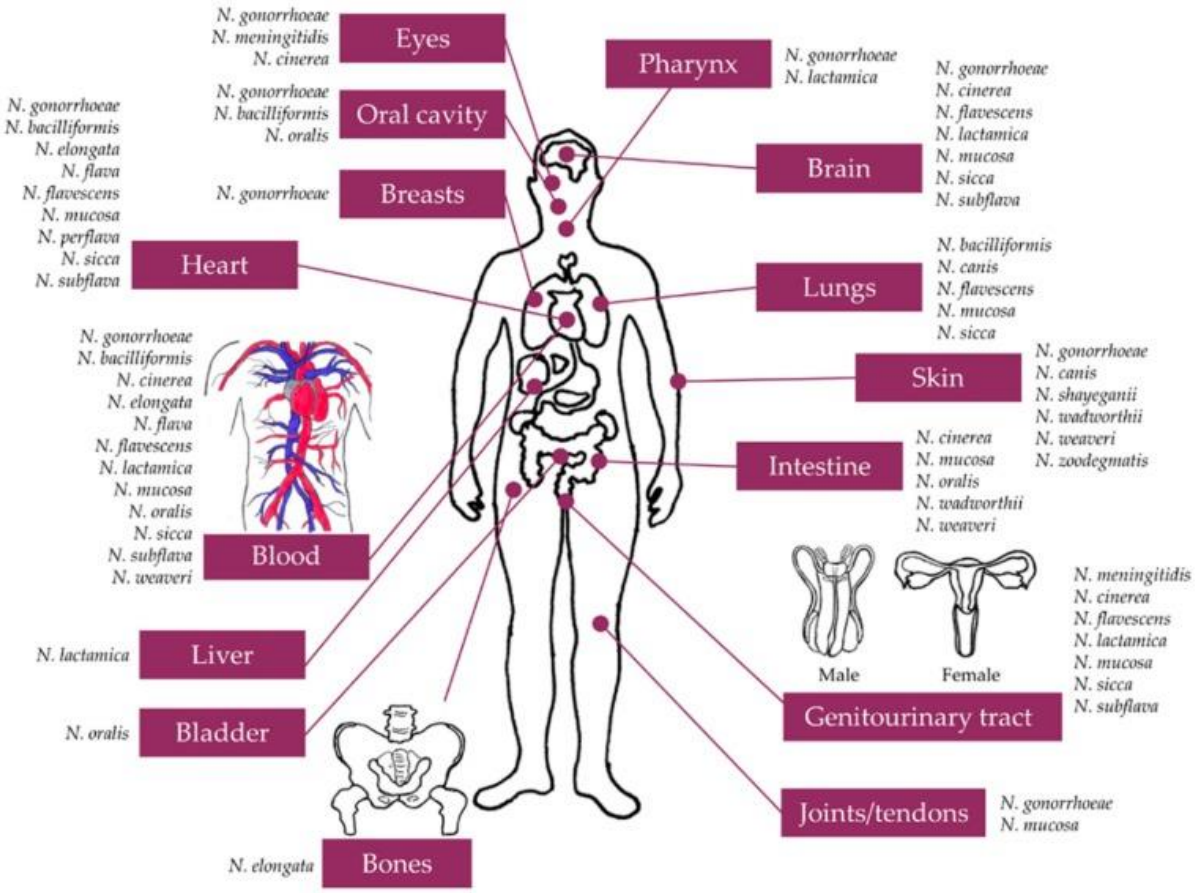

Figure 4. Infections by *Neisseria* species previously recorded in the literature. Image taken from Humbert and Christodoulides, 2019[13] (CC BY 4.0).

1.3.5. Carriage of commensal Neisseria in the human oropharynx

Despite sparse literature on the subject, it is estimated that the prevalence of *N*c in the pharynx is high. Eight published studies between 1988 and 2023 have examined the prevalence of *N*c in the human oropharynx (Table 4). The first study in 1988 tested pharyngeal samples from 209 patients and detected commensal *Neisseria* in 96.6% (202/209)[76]. Commensal *Neisseria* were isolated on selective agar and speciated using colonial morphology, oxidase, sugar utilisation and reduction of nitrate. The most common species was *N. perflava-N. sicca,* present in 96% (194/202) of patients carrying *N*c species. The species *N. perflava* and *N. sicca* were clustered together as they couldn't be differentiated biochemically. This was followed by *N. cinerea* (28.2%, 57/202) and *N. flava* (26.2%, 53/202)[76]. Colonisation with multiple species was common; 69 (33%) patients were colonised with two species, 38 (18.2%) by three species and five (2.4%) by four species[76]. Similar

results were reported by Saez who reported a 100% colonisation rate in 40 participants, including multicolonisation in 22 participants (55%)[77] (Table 4). The most common species isolated in this study was also *N. perflava-N. sicca* (92.5%) followed by *N. mucosa* (25%) and *N. flava* (20%)[77]. Subsequent studies reported high carriage rates in Vietnam (100%)[78] and Belgium (68%)[79] (Table 4). Some studies however have reported lower *N*c carriage rates, for example, a study of 45,847 participants in Burkina Faso reported a colonisation rate of 18.2%[80] and a study of 46,034 participants in the African meningitis belt reported a 10.2% colonisation rate[81] (Table 4). These differences may be explained by the different patient populations studied; the two African studies were focused on colonisation of *N. meningitidis* and particularly vaccinated individuals, and it has been suggested that *N*c carriage can be negatively impacted by recent meningococcal vaccination[81]. Also, both these studies used Theyer-Martin (TM) media for pathogenic *Neisseria* species, whereas the growth of some *N*c species such as *N. cinerea, N. subflava* and *N. mucosa* is not supported by this media[20,82].

Table 4. Summary of studies investigating the prevalence of commensal *Neisseria* carriage in the general population. Shown is overall prevalence of commensal *Neisseria* species as well as the prevalence of the individual species found in each study.

Author	**Knapp et al** [76]	**Le Saux et al**[83]	**Saez et al**[77]	**Kristiansen et al** [80]	**Diallo et al**[81]	**Dong et al** [78]	**Laumen et al**[79]	**Gaspari et al**[84]
Year	1988	1992	1998	2012	2016	2020	2022	2023
Country/Region	US	Canada	Spain	Burkina Faso	Africa*	Vietnam	Belgium	Italy
N	202	2116	40	45847	46034	218	96	195
Isolation Media	LBVT.SNR	NYC	LBVT. SNR	M-TM	M-TM	ChocP Choc	M-TM, CBA	M-TM, CBA
Identification	Ph-Bio	Ph-Bio	Ph-Bio PFGE	Ph-Bio MLST	Ph *rplF*	Ph MALDI	Ph MALDI	Ph MALDI
Prevalence	96.6%	11.6%	100%	21.4%	10.2%	100%	68%	100%
N. lactamica	1%	11.2%	5%	18.2%	5.6%	0.4%	2.1%	0.8%
N. meningitidis	8.2%	4.%	5%	0%	3.6%	4.9%	27.1%	0%
N. polysaccharea	0%	0%	0%%	0%	0.6%	0%	0%	0%
N. bergeri	0%	0%	0%	0%	0.2%	0%	0%	0%
N. subflava	0%	0%	2.5%	0%	0.05%	21.5%	65.6%	59.7%
N. cinerea	28.2%	0%	10%	0%	0%	2.6%	3.1%	0%
N. flavescens	0%	0%	5%	0%	0%	47.2%	0%	28%
N. gonorrhoeae	0%	0%	0%	0%	0%	3.4%	1%	0%
N. macacae	0%	0%	0%	0%	0%	4.5%	0%	3.2%

N. mucosa	24.8%	0%	25%	0%	0%	2.6%	14.6%	2%
N. oralis	0%	0%	0%	0%	0%	1.5%	8.3%	0%
N. perflava	96%	0%	92.5%	0%	0%	11.3%	0%	3.2%
N. flava	26.2%	0%	20%	0%	0%	0%	0%	0%
N. elongata	0%	0%	0%	0%	0%	0%	3.1%	0%
N. bacilliformis	0%	0%	0%	0%	0%	0%	1%	0%
N. sicca	0%	0%	0%	0%	0%	0%	0%	1.2%
Neisseria spp.	0%	0%	0%	3.26%	0%	0%	0%	1.6%

Nc; commensal Neisseria species, N; number of isolates
**Included countries: Chad, Ethiopia, Ghana, Mali, Niger and Senegal*
LBVT.SNR; Luria-Bertani Vancomycin Trimethoprim Sucrose Neutral Red, NYC; New York City agar, M-TM; Modified Theyer Martin agar, ChocP; chocolate agr with Polyvitalex, Choc; chococlate agar, CBA; Columbia agar with blood, PFGE; pulse field gel electrophoresis, MLST; multilocus sequence type, MALDI; matrix assisted laser desorption ionisation (time-of-flight), cgMLST; core genome multilocus sequence type, rplF; sequencing of 50S ribosomal protein L6
Ph; phenotypic methods of identification that include Gram stain and colonial morphology
Bio; biochemical methods of identification that include sugar utilisation, oxidase and nitrite reduction

1.4. Diagnosis and susceptibility testing of *Neisseria species*

1.4.1. Clinical diagnosis of gonorrhoea

The diagnosis of gonorrhoea at the genitourinary medicine (GUM) clinic relies on the recognition of clinical symptoms, clinical examination and Gram stain smears of affected sites[8]. Microscopy is performed on symptomatic patients with urogenital infection and allows a two-fold diagnosis: the presence of ≥2 polymorphonuclear leukocytes (PMNLs) in a field of view (x1000) indicates urethritis or cervicitis, the presence of Gram-negative diplococci (GNDC) within PMNLs suggests gonococcal urethritis or cervicitis[8,85] (Fig. 5). The sensitivity of microscopy from a symptomatic penile urethra is 90-95%, whereas cervical microscopy has a much lower sensitivity of 37-50%[8]. Direct microscopy of pharyngeal and rectal specimens is not recommended because morphologically identical *N*c are found in the pharynx, and rectal specimens are often heavily contaminated with gut microbiota, making the visualization of *Ng* challenging[8].

UK national guidelines recommend that nucleic acid amplification testing (NAAT) specimens should be taken for all patients[8]. The recommended specimen types for NAAT testing are first void urine for male urogenital infection, vulvovaginal or cervical swabs for female urogenital infection, and rectal and pharyngeal swabs[8] and the relevant anatomical sites are sampled according to clinical history. Swabs for gonococcal culture are taken in patients who have had a positive NAAT test, are a contact of a known positive patient or there is a clinical suspicion of *Ng* infection, for example, GNDC are seen in the Gram stain. The sensitivity of gonococcal culture depends on the site sampled, for example in one study the recovery rate from the pharynx was 21.7% (78/359) compared to 67% (99/147) in rectal and 71.4% (5/7) in male urethral infection[86]. It is therefore recommended that inoculation of culture specimens takes place at the clinic as it can increase gonococcal recovery from 41% to 57% before transporting to the diagnostic laboratory[8,87]. A TOC is recommended in all patients treated for gonorrhoea to detect treatment failures and resistant strains[8].

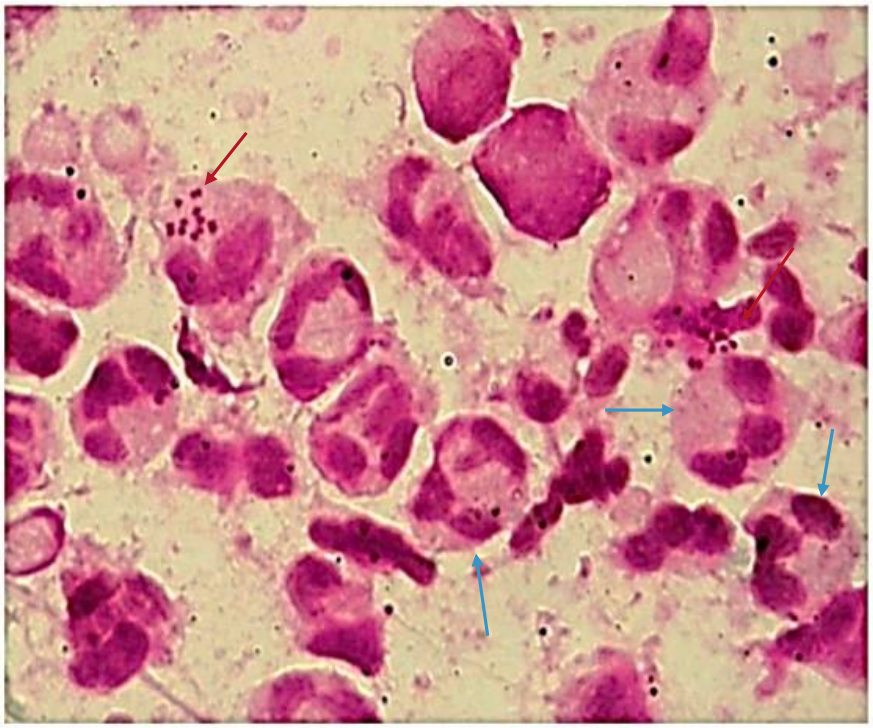

Figure 5. Gram negative diplococci (red arrows) within polymorphonuclear leucocytes (blue arrows) seen in a urethral Gram smear. Image taken from Meyer and Buder (2020)[88] (CC BY 4.0).

1.4.2. Laboratory diagnosis of gonorrhoea

The laboratory detection of *Ng* follows two workstreams, NAAT testing and culture depending on the type of specimen under investigation. The gold standard for the detection of *N. gonorrhoeae* from clinical specimens are NAATs due to their high sensitivity (>95%) and specificity (>99%)[88,89]. The reason for the high sensitivity of NAATs is two-fold. First, they can detect very low levels of nucleic acid and second, they can detect nonviable gonococcal cells reducing the need for strict transport requirements compared to culture[88]. Laboratories most commonly use commercial platforms that employ technologies such as polymerase chain reaction (PCR), strand displacement assays (SDA) and transcription-mediated amplification (TMA)[88]. Common gonococcal targets for NAAT detection include 16S rRNA, Opa and pilin genes[88] and some platforms detect multiple gonococcal targets and some offer simultaneous detection of other sexually transmitted infections (STIs) such as *Chlamydia trachomatis* and *Mycoplasma genitalium*[88]. Despite the high sensitivity of these molecular platforms, there are disadvantages. Firstly, most of these platforms are not

validated for extragenital samples and diagnostic implementation requires in-house validation of such samples[88]. Secondly, there is the issue of confirmatory testing which can be complex. Confirmatory testing for *Ng* is key to reducing the risk of false positives and ideally should be detected by a different molecular target to the primary test[89]. Positive tests from rectal and urogenital specimens should be repeated where the positive predictive value (PPV) of the primary test is <90%, but all pharyngeal specimens should be confirmed due to high cross-reactivity with *N*c species in the pharynx[89]. Lastly, the performance of NAATs should be monitored continuously as single nucleotide polymorphisms (SNPs) in the assay's genetic target can lead to false negative results. For example, in 2011 a high number of false negative gonococcal NAATs were identified in Australia due to the acquisition of a meningococcal *porA* sequence[90] that spread internationally[91]. The role of gonococcal culture is primarily to perform AST which cannot be achieved from NAAT testing[92]. The first step of gonococcal culture is to inoculate specimens onto selective media such as vancomycin, colistin, amphotericin, trimethoprim (VCAT) media, usually at point of care (POC)[8,82] (Fig. 6). Selective media is designed to allow the growth of the pathogen of interest while simultaneously suppressing background contaminating bacterial microbiota. Gram stain and oxidase tests are performed on all morphologically distinct colonies that grow on selective media for the presumptive identification of *Neisseria spp* (GNDC and oxidase positive)[82]. These are subsequently confirmed as *Ng* by biochemical methods, for example API NH, immunological testing, for example slide agglutination, NAATs and matrix-assisted laser desorption/deionisation time-of-flight mass spectrometry (MALDI-ToF MS)[88,93]. The most common confirmatory method in most UK laboratories is MALDI-ToF, as it is easy to use, rapid and has an estimated PPV of 99.3%[82,94].

The introduction of MALDI-ToF MS revolutionised microbiology diagnostics in the past 10 years by reducing diagnostic turnaround times and simplifying the diagnostic workflow[95]. This technique uses the principles of mass spectrometry, identifying key bacterial proteins through their mass-to-charge ratio, and the time it takes for charged proteins to reach the analyser. This produces a unique peptide mass fingerprint (PMF) that is specific to an organism[95]. The organism's PMF, comprised mainly of ribosomal proteins is compared to a database from a collection of reference

strains to find the closest match. Depending on the quality of the sample, the software will give a genus level or species level identification[95]. Identification of pathogenic *Neisseria* species with MALDI-ToF systems can be up to 100% accurate for *Ng*[82,94,96], but there have been reports of some commensal species such as *N. cinerea*[97], *N. elongata*[98] and *N. polysaccharea*[99] being misidentified as *N. meningitidis*. Speciation of *N*c is even less accurate; one study that compared MALDI-ToF identification of *N*c to 16s rRNA found that depending on the instrument and incubation time, between 86.2% (25/29) and 93.1% (27/29) of tested isolates were identified correctly[98]. Both instruments performed better when the isolates were incubated for 24h compared to 48h[98]. As the taxonomy of *N*c continues to evolve, MALDI-ToF databases should continue to be updated to ensure accurate identification of all *Neisseria* species. A summary of the diagnostic workflow for both clinical and laboratory detection of *Ng* is outlined in Fig. 7.

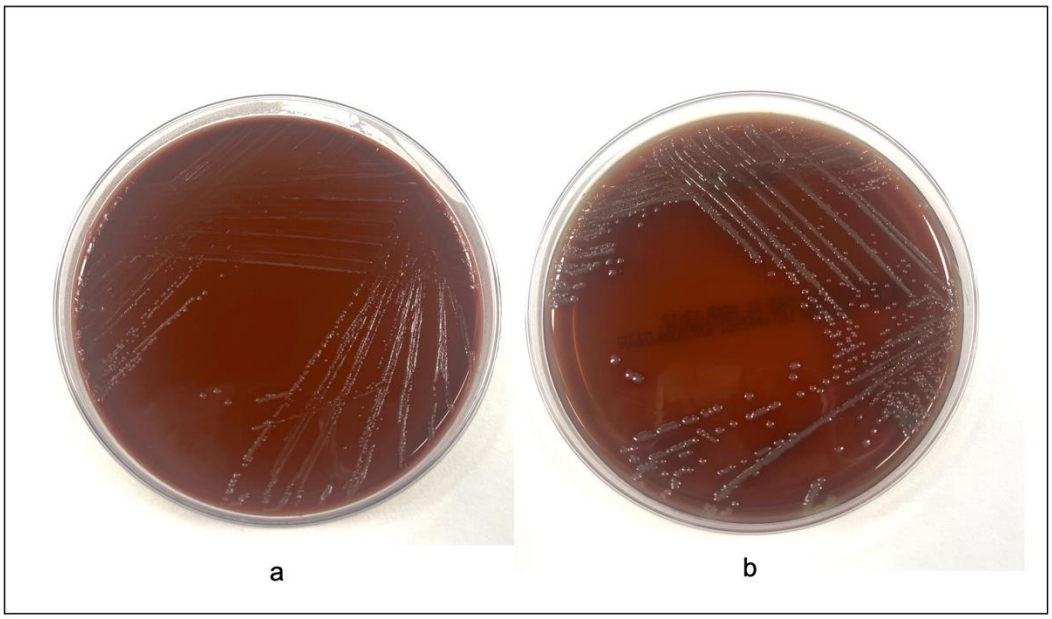

Figure 6. *Neisseria gonorrhoeae* on non-selective chocolate agar (a) and selective VCAT agar (b). VCAT contains vancomycin, colistin, amphoteracin and trimethoprim to suppress background bacterial and fungal microbiota. *Ng* are intrinsically resistant to the above antimicrobials.

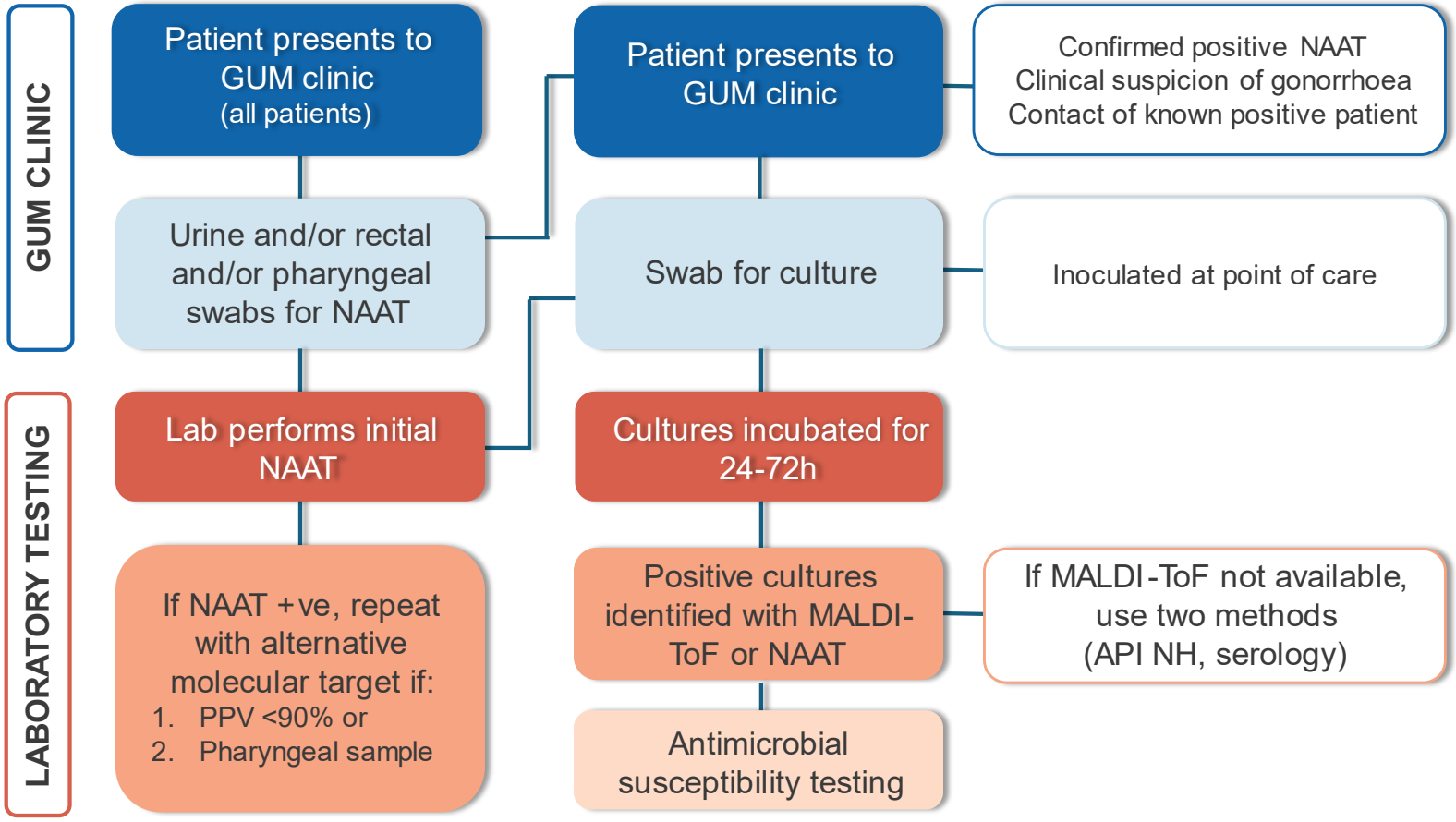

Figure 7. Summary of the clinical and laboratory diagnostic workflow for *N. gonorrhoeae*.
GUM; genitourinary medicine clinic, MALDI-ToF; matrix assisted laser desorption ionisation time-of-flight, NAAT; nucleic acid amplification test, PPV, positive predictive value

1.4.3. Phenotypic and biochemical identification of commensal Neisseria

Commensal *Neisseria* species can be grown on a range of non-selective media such as nutrient, blood or chocolate agars. The only selective agar supporting *N*c species was first developed by Knapp and Hook in 1984, the Luria-Bertani Vancomycin Trimethoprim Sucrose Neutral Red (LBVT.SNR) medium[100]. The medium contains the antibiotics vancomycin and trimethoprim, as *N*c are intrinsically resistant to, but selectively inhibit competing oral microbiota. Additionally, the media contains sucrose and the pH indicator neutral red to distinguish sucrose fermenters from non-sucrose fermenters. If an organism utilises sucrose, it will produce acid as part of the fermentation process which will, in turn, change the pH of the media and therefore the colour of neutral red (Fig. 8). Sucrose fermenters such as *N. perflava*, and *N. mucosa* will change the colour of the media to pink (Table 1), whereas non-sucrose fermenters will remain orange/yellow. The texture of colonies is also a useful indicator, with some colonies presenting as dry and wrinkled; other *N*c may appear smooth and shiny[76,100]. It is important to note that the pathogenic *N. gonorrhoeae* and *N. meningitidis* are unable to grow on this media due to lack of nutrient enrichment[20].

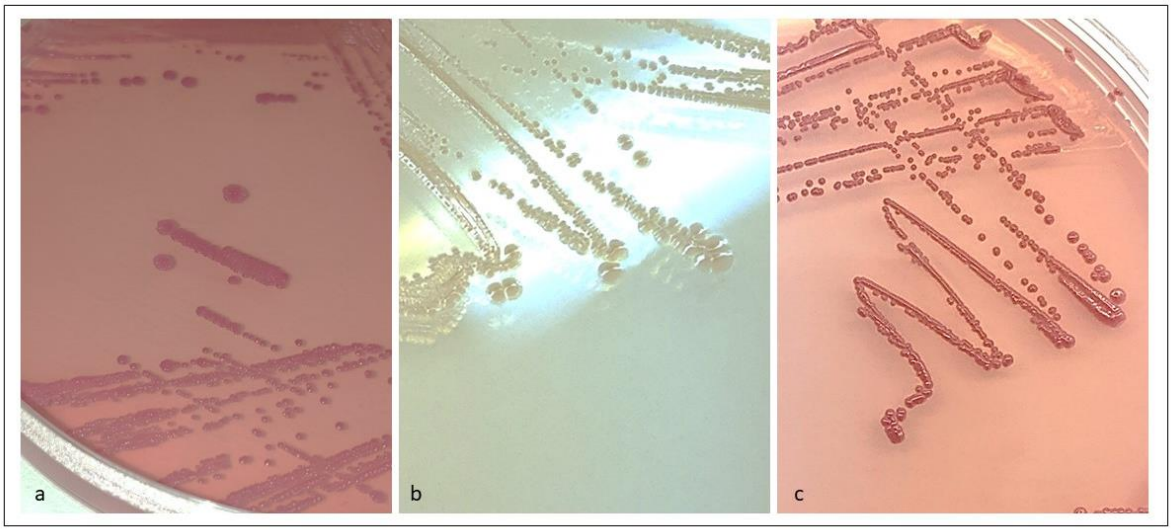

Figure 8. Examples of commensal Neisseria species colonial morphology on LBVT.SNR selective agar. Rough sucrose fermenter (a), smooth non-sucrose fermenter (b) and smooth sucrose fermenter (c).

Biochemical identification has been traditionally used alongside macroscopic phenotypes to identify bacteria. Oxidase and catalase (3% H_2O_2) tests are both positive in *Neisseria spp,* but only *N. gonorrhoeae* is positive for superoxol (30% H_2O_2) (Table 1). A nitrate reduction test differentiates species on their ability to reduce nitrate to nitrite – this is positive in *N. mucosa* but negative for other *N*c species (Table 1). *Neisseria* species can also be identified by their ability to ferment glucose, maltose, lactose and sucrose, for example, *N. gonorrhoeae* can only ferment glucose, whereas *N. perflava* can ferment all four sugars (Table 1). This was traditionally determined by using cystine trypticase agar medium containing 1% of each sugar separately[101] but now is more commonly performed with rapid biochemical identification panels such as Analytical Profile Index for *Neisseria* and *Haemophilus* (API NH) or RapID™ NH test. Identification of *Neisseria* species by biochemical methods can accurately detect pathogenic species but have low specificity in the differentiation of commensals, so phenotypic identification should be used in combination with molecular methods[93].

1.4.4. Antimicrobial susceptibility testing of gonorrhoea

Susceptibility profiles do not usually influence individualised treatment due to gonorrhoea being treated empirically and before a culture is obtained, but AST has a major role in the surveillance of AMR and the detection of resistant strains. Various methodologies are available to perform AST, depending on the outcome of interest, time and cost restrictions. For example, in the clinical laboratory, it is often sufficient to know whether the infective microorganism is susceptible or resistant to a particular antibiotic and as such, disk diffusion testing is sufficient[102]. Disc diffusion involves placing cellulose discs impregnated with the antimicrobials of interest at a standardised concentration, on a bacterial lawn on an agar plate. The susceptibility result is interpreted by measuring the diameter of each antimicrobial's zone of inhibition and using published breakpoints such as the European Committee for Antimicrobial Susceptibility Testing (EUCAST) and Clinical Laboratory Standards Institute (CLSI)[102,103] to translate this into susceptibility or resistance[102]. However, the gold standard for gonococcal MIC testing is currently the agar dilution methodology[103,104] and this is used by reference laboratories when performing AMR surveillance or confirming primary clinical laboratory AST results[104]. Gradient strips are also frequently used

and have a good correlation with agar dilution results[105]. The antimicrobials recommended by all guidelines include ceftriaxone, cefixime, penicillin, ciprofloxacin, tetracycline and azithromycin[106,107]. However, antimicrobial susceptibility testing for *Ng* remains controversial. Whereas EUCAST and CLSI guidelines agree on the antimicrobials that should be tested, MIC breakpoints are less standardised. For example, CLSI does not have a breakpoint for cefixime and ceftriaxone resistance but considers MICs ≤0.25 mg/L as reduced susceptibility, whereas EUCAST has a resistance breakpoint of >0.125 mg/L (Table 5). There are also MIC breakpoint differences for ciprofloxacin, spectinomycin and tetracycline (Table 5). These differences could be explained by differences in AST media; for example, the GRASP use diagnostic sensitivity testing (DST) agar, which may affect the MICs of pH-sensitive antimicrobials such as tetracyclines and macrolides[108]. Further, EUCAST zone size breakpoints for all antimicrobials are still undefined and only epidemiological cut-off values (ECOFF) MIC breakpoint criteria for azithromycin are published for both CLSI and EUCAST[106]. Lastly, EUCAST does not define recommended media for MIC testing yet, whereas CLSI recommends gonococcal medium base (GCMB) supplemented with a defined growth supplement such as Isovitalex, Vitox or Kellog's supplement[103].

Table 5. Comparison of CLSI and EUCAST MIC breakpoints for gonococcal antimicrobial resistance

Antimicrobial	CLSI (≤S mg/L)	CLSI (≥R mg/L)	EUCAST (≤S mg/L)	EUCAST (>R mg/L)
Cefixime	0.25	n/a	0.125	0.125
Ceftriaxone	0.25	n/a	0.125	0.125
Ciprofloxacin	0.06	1	0.03	0.06
Azithromycin*	1	-	-	-
Spectinomycin	32	128	64	64
Penicillin	0.06	2	0.06	1
Tetracycline	0.25	2	0.5	0.5

CLSI; Clinical and Laboratory Standards Institute, EUCAST; European Committee on Antimicrobial Susceptibility Testing, S; susceptible, R; resistant
**Only used in conjunction with another effective agent, therefore only epidemiological cut-off of ≤1 mg/L (susceptible only) have been published*

1.4.5. Strain typing of N. gonorrhoeae

Several methods have been used for the typing of *Ng*, including phenotypic, serological and molecular methods.

Auxotyping. This method was developed in 1973 and is based on *Ng's* nutritional requirements for amino acids, purines, pyrimidines and vitamins[109]. The specific substrates included in auxotyping are L-proline, L-arginine, L-ornithine, L-methionine, hypoxanthine, uracil, thiamine, and thiamine pyrophosphate. An auxotype is defined as a strain with a characteristic pattern of growth responses from media containing these substrates[109]. This technique is reproducible even after several subcultures; however, it has low discriminatory power compared to other methods, such as DNA-based methods[110].

Serotyping. Serotyping of *Ng* is based on the gonococcal outer membrane protein I, otherwise known as PorB[111]. The serovar is determined by challenging *Ng* strains with a panel of 12 monoclonal antibodies and observing patterns of agglutination to assign *Ng* strains into three groups: WI, WII or WIII, based on the presence of protein IA or IB[111]. Outside of serotyping for epidemiological purposes, this technique was also used as a confirmatory test for the identification of *Ng* in routine microbiology laboratories[82,93].

Gel-based DNA typing methods. The first DNA typing method was multilocus enzyme electrophoresis (MLEE), which involved indexing variations in gonococcal housekeeping genes. However, this had low discriminatory power due to the uniformity of the genes examined and laborious nature[112]. Subsequently, MLEE was replaced by *opa* typing, which involves amplifying 11 *opa* genes by PCR, digesting the products with restriction enzymes (for example, *Taq*I and *Hha*I) and separating them by PAGE to produce an *opa* type by visualising the pattern of individual amplicons produced[112]. Although highly discriminatory, *opa* typing is laborious and operator-dependent, so results between laboratories cannot be compared easily due to the lack of standardisation[113]. Restriction fragment length polymorphism (RFLP)[114] was introduced in 1984 for *Ng* typing and used the *Hind*III restriction enzyme (RE) to digest the whole genome into fragments that are separated into distinct bands by PAGE[115]. This method was subsequently improved by applying PFGE, a variation of gel electrophoresis that uses an electric field that

periodically changes direction, allowing the separation of larger DNA bands[110]. Apart from MLEE, gel-based typing methods have high discriminatory power; however, the interpretation of gel patterns can be subjective, require technical expertise and cannot be easily implemented in a high throughput laboratory[110].

DNA sequence-based typing methods. Multilocus sequence typing and *NG*-MAST are now considered gold standard methodologies, as they are simple to perform, high throughput and have online databases that assign sequence types. Maiden *et al* in 1998[116] first described MLST for *Neisseria* species and is based on observing genetic variations of seven gonococcal housekeeping genes [*abcZ* (putative ABC transporter), *adk* (adenylate kinase), *aroE* (shikimate dehydrogenase), *fumC* (fumarate hydratase), *gdh* (glucose-6-phosphate dehydrogenase), *pdhC* (pyruvate dehydrogenase subunit) and *pgm* (phosphoglucomutase)]. Similarly, *NG*-MAST is based on genetic variations in two hypervariable genes, the outer membrane porin (*porB*) and β-subunit of the transferrin binding protein (*tbpB*)[113]. Although both molecular typing methodologies are highly discriminatory, they have different advantages. MLST is considered to be more suited for large-scale outbreaks or long-term studies[113,117], whereas *NG*-MAST is suitable for local outbreaks, as it has higher discriminatory power than MLST[118]. Therefore, when performing both typing methods on a population, it is common for MLST STs to include several *NG*-MAST STs (Fig. 9). In 2017, a new typing scheme based on antimicrobial resistance, *Ng* Sequence Typing for Antimicrobial Resistance (*NG*-STAR) was developed[119]. This typing scheme uses allelic identifications for seven genes associated with cephalosporin, macrolide and fluoroquinolone AMR in *Ng*: *penA, mtrR, porB, ponA, gyrA, parC* and 23S rRNA.

With the advancements of sequencing technologies, MLST, *NG*-MAST and *NG*-STAR can be derived from whole genome sequences (WGS) to avoid the laborious process of multiple PCR's and Sanger sequencing[120]. Data from WGS can also be used to examine cgMLST, which is a typing scheme that analyses approximately 1668 loci (depending on the scheme) from the *Ng* core genome[121].

Figure 9. Relationship between MLST, *NG*-STAR and *NG*-MAST sequence types. MLST is shown in the inner circle, followed by *NG*-STAR and *NG*-MAST in the middle and outer circles respectively. Image adapted from the 2023 GRASP report[40] (Image in public domain).

1.5. Antimicrobial resistance in *Neisseria* species

1.5.1. Antimicrobial resistance history of N. gonorrhoeae

While gonorrhoea infections are increasing globally, our ability to successfully treat them is decreasing, as AMR to most antibiotics such as penicillin, ciprofloxacin, azithromycin and tetracycline has emerged and spread. Penicillin was introduced for the treatment of gonorrhoea in 1943 and was initially very successful, until the 1960s when strains with reduced susceptibility started to emerge[122]. In the 1970s, plasmid-mediated β-lactamases were detected, and by the 1980s, due to widespread resistance, penicillin was no longer recommended for empirical treatment[123]. Tetracycline was introduced in the 1950s, primarily as an alternative treatment option for penicillin-allergic patients until resistance developed and was no longer recommended by the mid-1980s when high-level resistance was detected. Spectinomycin was introduced in the 1970s solely for the treatment of gonorrhoea but was abandoned very quickly due to the rapid AMR development and restricted availability[124]. Ciprofloxacin and azithromycin were introduced in the 1980s and quickly replaced penicillin and tetracycline. They were used worldwide and without official recommended dosages, but resistance developed quickly to ciprofloxacin. In England, ciprofloxacin was officially recommended in the 1990s but was replaced in 2004. Extended spectrum cephalosporins were shown to have good clinical activity against gonorrhoea in the late 1980s and were the last class of antimicrobials introduced[122] (Fig. 10).

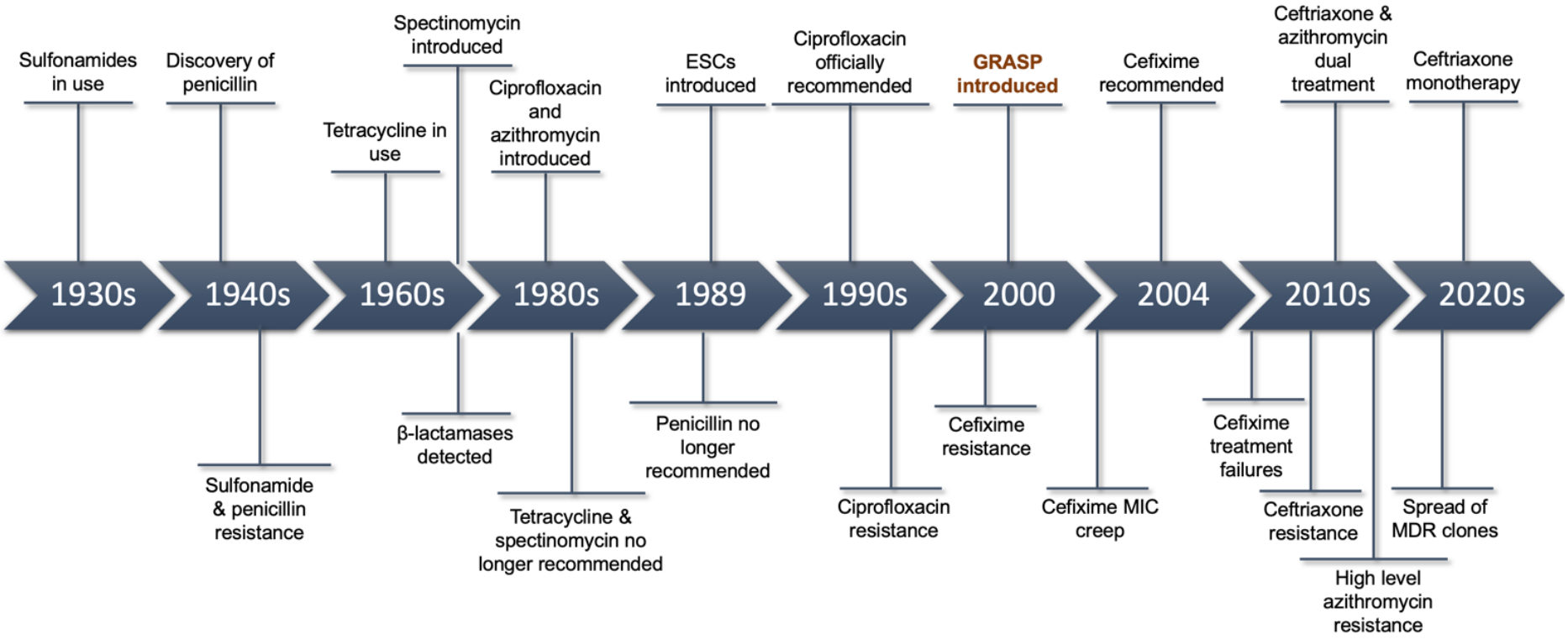

Figure 10. History of antimicrobial use and antimicrobial resistance in *N. gonorrhoeae*. Adapted from Unemo & Shafer[125]. *GRASP*; gonococcal resistance to antimicrobials surveillance programme, *ESC*; extended spectrum cephalosporin, *MIC*; minimum inhibitory concentration, *MDR*; multidrug resistance

In the past 20 years, empirical therapy has changed three times in England, following increasing rates of resistance, on average every seven years[12]. In 2004, first-line therapy changed from ciprofloxacin to cefixime, after ciprofloxacin resistance surpassed the 5% WHO threshold[57]. Even though cefixime was used successfully, in 2010 GRASP reported strains with higher MICs and a shift in the modal MIC[126]; 17.1% of isolates had a MIC ≥0.125 mg/L and 6.3% had a MIC ≥0.25 mg/L[127]. In the meantime, reduced cefixime susceptibility was reported in several European countries such as Spain, Greece and the Netherlands and internationally in the U.S. and Southeast Asia[128]. Further, cefixime TFs were reported internationally including Australia[129], Norway[123], Austria[130] and the UK[131]. Genomic epidemiological studies showed that reduced susceptibility to ESCs was strongly correlated with isolates within genogroup 1407 (G1407) which was widespread in Europe among MSM and the most prevalent genogroup in Europe in 2010 (248/1066, 23%), with $ST_{NG\text{-}MAST}$1407 being the most common ST within G1407[132]. Genogroups are clusters of *Ng* $ST_{NG\text{-}MAST}$ that have >99% similarity in both alleles from the main $ST_{NG\text{-}MAST}$[118]. These events triggered the update of treatment guidelines to dual treatment of 500mg ceftriaxone and 1 g azithromycin[57], as a strategy to delay AMR, especially as ceftriaxone was the last remaining reliable antimicrobial. Even so, resistance to ceftriaxone was reported as early as 2009, when a resistant gonococcal strain (H401, $ST_{NG\text{-}MAST}$4220) was isolated in Japan from the pharynx of a sex worker[133]. This was followed by a cluster in France and Spain (F89, $ST_{NG\text{-}MAST}$1407)[134,135] and thought to be the first detected transmission of ceftriaxone resistant gonorrhoea. It is important to note that the strains from both instances were also resistant to all other classes of antibiotics apart from spectinomycin and that both had mosaic *penA* alleles responsible for ceftriaxone resistance. Further ceftriaxone TFs, which are described in 1.5.2, were reported worldwide. Despite the use of dual therapy, azithromycin resistance developed rapidly. In England, it was first recognised in 2007 when six high-level resistant isolates (MIC ≥256 mg/L) were identified from the national surveillance programme[136,137], and was then reported in Scotland[138] and Italy[139]. This worrying trend resurfaced in 2015 when an outbreak of gonorrhoea highly resistant to azithromycin was identified in Leeds[140]. At the same time, the first TF was reported in a patient receiving ceftriaxone and azithromycin in England[141] and the novel multidrug-resistant (MDR) clone FC428 ($ST_{NG\text{-}}$

$_{MAST}$3435, ST_{MLST}1903) harbouring a mosaic *penA*-60.001 allele was isolated in Japan[142]. The FC428 lineage has become one of the most successful clones by disseminating internationally in Australia, Canada, Denmark, Ireland, China and the UK and was still being detected in 2023[143]. The *penA*-60.001 allele has even spread across previously susceptible strains that have been detected in China[144], South Korea[145], Austria[146], France[147], Sweden[148] and the US[149]. In 2018, the UK updated the treatment guidelines again, by removing azithromycin and increasing ceftriaxone from 500 mg to 1 g[8]. This was implemented for two main reasons, a) resistance to azithromycin increased from 0.5% in 2011 to 9.3% in 2017[150], above the WHO 5% threshold and b) to avoid the selection of resistance in other STIs, such as *M. genitalium* and *Treponema pallidum*[8]. However, MDR strains such as the FC428 clone are persisting and TFs continue to occur, the last reported one in 2022 in Austria[146]. In the same year, a ceftriaxone resistant strain (MIC 0.5-1 mg/L) with a novel mosaic *penA*-237.001 allele was reported in the UK[151] along with nine other cases harbouring *penA*-6001 and France[152,] thought to be linked to travel to Vietnam. In 2022, a systematic review of *Ng* AMR in Africa identified 40 studies including a total of 7961 isolates over 22 years (1995 – 2017)[153]. Antimicrobial resistance has increased over time, for example ciprofloxacin resistance increased from 22% in 2003 to 69% in 2016[153]. Penicillin resistance also increased from 15% in 1995 to 30% in 1997 and tetracycline resistance increased from 3% in 1997 to 51% in 1999[153]. Reported AMR to azithromycin was variable; two studies found rates of 68% (2014) and 4.3% (2008) respectively[153]. Fortunately, resistance to ESCs was found to be low[153]. Given the increase in resistance and multiple changes to empirical therapy, resistance to current therapeutic options may occur in the next few years, leading to very limited treatment options.

1.5.2. Extended spectrum cephalosporin treatment failures

Treatment failure can be defined as persisting gonococcal infection despite appropriate treatment, where reinfection has been excluded, ideally by comparing the genomes of the pre-and post-failure isolates (Table 6), but this is not always possible as initial infection is diagnosed by NAATs[11]. Since monitoring TFs is a key strategy to gonococcal AMR control efforts, countries such as the UK, U.S. and Australia have published guidance to manage ESC resistant *Ng* and TFs. In the UK this is currently coordinated by UKHSA and occurs via a collaborated effort between sexual health

clinicians, general practitioners, microbiologists and health protection teams with the coordinating centre[3]. Stakeholders are required to report any ceftriaxone resistant isolate and/or treatment failure to the UKHSA for confirmation and continued surveillance of gonococcal AMR. Similarly, the Centres for Disease Control & Prevention (CDC) coordinates the monitoring of TFs in the U.S. by providing a consultation form for healthcare providers[5].

The first ESC TF recorded in the literature was in Japan in 2007, in four patients with urethral infection treated with 200 mg cefixime. The isolates were cefixime resistant (MIC 0.5-1 mg/L) and were eventually treated successfully with 1 g ceftriaxone[154]. Subsequently, there have been 29 further cefixime TFs reported in Norway[155], Austria[130], Canada[68,71], France[135] and the latest being in 2022 in the UK[151]. Cefixime TFs have been reported in pharyngeal, urogenital and rectal sites and both in phenotypically susceptible and resistant strains; 63% (12/19) of resistant isolates were reported in urogenital sites whereas 60% (9/15) of susceptible isolates were reported in the pharynx. The presence of mosaic *penA* genes and molecular typing were not reported consistently, but mosaic genes such as *penA*-XXXVI and *penA*-60.001 were present in isolates tested[130,135,151]. The only *NG*-MAST ST reported in Europe (n=4) was the drug-resistant ST1407[130,135,155], all associated with a cefixime MIC >0.25 mg/L, in line with local molecular epidemiology[118]. The most common ST in Canada was ST3935 (5/23), but ST1407 was the second most common with three isolates reported[68,71].

Table 6. Definitions of confirmed and probable cephalosporin gonococcal treatment failures. Adapted from the ECDC 'Response plan to control and manage the threat of multi- and extensively drug-resistant gonorrhoea in Europe' report[156].

Confirmed	**Probable**	1	A gonorrhoea patient who returns for test of cure or who has persistent genital symptoms after having received treatment for laboratory-confirmed gonorrhoea with a recommended cephalosporin regimen (ceftriaxone or cefixime in an appropriate dose) AND
		2	Remains positive for one of the following tests for *N. gonorrhoeae*: • Presence of intracellular Gram-negative diplococci on microscopy taken at least 72 hours after completion of treatment OR • Isolation of *N. gonorrhoeae* by culture taken at least 72 hours after completion of treatment OR • Positive nucleic acid amplification test (NAAT) taken two to three weeks after completion of treatment. AND
		3	Denies sexual contact during the post-treatment follow-up period. AND
		4	Decreased susceptibility to cephalosporin used for treatment*: • Cefixime MIC>0.12 mg/L** • Ceftriaxone MIC>0.12 mg/L**
**Ideally, the pre-and post-treatment isolates should be examined with an appropriate and highly discriminatory molecular epidemiological typing method to establish if isolates are indistinguishable. **These thresholds are in accordance with EUCAST tentative breakpoints. Reporting of probable treatment failures where MICs are lower than the EUCAST breakpoints will be essential to evaluate if current breakpoints are clinically relevant.*			

The first ceftriaxone TF was reported in 2009, from the pharynx of two patients in Australia[157] (Table 7). This was followed by the first high-level ceftriaxone resistant isolate (MIC 2 mg/L) due to the novel mosaic *penA*$_{H401}$ reported in Japan from the pharynx of a woman[133]. Further ceftriaxone TFs have been reported in Slovenia[158], Australia[129,159–161], Sweden[162], the U.S.[163], France[164], Canada[146,165] and Austria[146] (Table 7). In 2016 the first global dual ceftriaxone (MIC 0.25 mg/L) and azithromycin (MIC 1 mg/L) treatment failure case study was reported in the UK by an isolate harbouring mosaic *penA*-X[141]. It's important to note that this patient had pharyngeal and

urethral infection, but only the pharyngeal site failed treatment. This a common trend among patients with multisite infection; 11/12 patients treated with ceftriaxone failed at the pharyngeal site but not the urogenital or rectal sites and one patient that failed at both sites was due to an isolate with high-level ceftriaxone resistance (MIC 1 mg/L) (Table 7). In 2018, the first TF due to a gonococcal strain with combined ceftriaxone (MIC 0.5 mg/L) and high azithromycin resistance (MIC >256 mg/L) was detected in the UK, harbouring the globally disseminated mosaic *penA*-60.001[166]. The most common mosaic was the *penA*-60.001 which was found in isolates with ceftriaxone MICs 0.25-1 mg/L (Table 7). Ceftriaxone treatment failures due to $ST_{NG\text{-}MAST}$1407 were not as frequent as for cefixime, as they were only found in two patients, in Slovenia[158] and Australia[160]. However, the most common MLST was ST1901, found in 44% (4/9) isolates where MLST was reported, in Slovenia in 2012[158], Australia in 2013[167] and the UK in 2016[141] (Table 7).

Table 7. Summary of ceftriaxone treatment failures in the literature

Author	Year	Country	Sample size	Sex	Anatomical site*	MIC CRO (mg/L)	Mosaic *penA*	*NG*-MAST	MLST
Tapsall J, *et al*.[157]	2009	Australia	1	Male	Urethral and **pharyngeal**	0.03‡	No (VII)	5	NR
			1	Female	Urogenital and **pharyngeal**	0.016‡	No (V)	2740	NR
Ohnishi M, *et al*.[133]	2011	Japan	1	Female	Pharyngeal	2	*penA* H041	4220	7363
Unemo M *et al*.[158]	2012	Slovenia	1	Female	Pharyngeal	0.125	XXXIV	1407	1901
Hustig A, *et al*.[159]	2013	Australia	5	Male	Pharyngeal	<0.03‡	NR	NR	NR
			1	Male	Pharyngeal	<0.03‡	NR	NR	NR
Chen, MY, *et al*.[160]	2013	Australia	1	Male	Rectal and **pharyngeal**	0.03-0.06‡	XXXIV	1407	NR
Read PJ, *et al*.[167]	2013	Australia	1	Male	Urethral and **pharyngeal**	0.03‡	No (XII)	225	1901
			1	Male	**Pharyngeal** and rectal	0.03‡	No (XII)	225	1901

Golparian D, *et al.* [162]	2014	Sweden	1	Female	**Pharyngeal** and urogenital	0.125	XXXIV	4706	NR
			1	Male	**Pharyngeal** and urogenital	0.064‡	XXXIV	3149	NR
			1	Female	**Pharyngeal** and urogenital	0.064‡	XXXIV	3149	NR
Bissessor M, *et al* [161]	2015	Australia	1	Male	Rectal	<0.008‡	NR	NR	NR
			3	Male	Rectal	0.03‡	NR	NR	NR
			1	Male	Rectal	0.06‡	NR	NR	NR
			2	Male	Pharyngeal	0.06‡	NR	5333	NR
			1	Male	Pharyngeal	<0.008‡	NR	NR	NR
			1	Male	Pharyngeal	0.016‡	NR	NR	NR
			1	Male	Pharyngeal	0.03‡	NR	NR	NR
Fifer H, *et al.* [141]	2016	UK	1	Male	Urethral and **pharyngeal**	0.25	X	12133	1901

			1	Male	Urethral	0.003‡	NR	NR	NR
Okah E, *et al*.[163]	2018	USA	1	Male	Urethral	0.012‡	NR	NR	NR
			1	Male	Urethral	0.023‡	NR	NR	NR
			1	Male	Urethral	0.047‡	NR	NR	NR
Eyre DW, *et al*.[166]	2018	UK	1	Male	Urethral and **pharyngeal**	0.5	60.001	16848	12039
Poncin T, *et al.*[164]	2018	France	1	Female	Urogenital and **pharyngeal**	0.5	60.001	3435	1903
Smyczek P, *et al*.[165]	2019	Canada	1	Male	Urethral	0.5	60.001	3435	1903
Eyre DW, *et al*.[143]	2019	UK	1	Female	**Urogenital** and **rectal**	1	60.001	1614	1903
Pleininger S, *et al.*[146]	2022	Austria	1	Male	**Urethral**	0.25	60.001	Novel	16406

**For patients infected with multiple anatomical sites, site in bold failed treatment, ‡phenotypically susceptible strain*

MIC; minimum inhibitory concentration, CRO; ceftriaxone, AZI; azithromycin, DOX; doxycycline, ERT; ertapenem, NR; not recorded

NG-MAST; Neisseria gonorrhoeae multiantigen sequence type, MLST; multilocus sequence type

1.5.3. Surveillance of N. gonorrhoeae antimicrobial resistance

Gonococcal AMR surveillance is an integral part of control efforts. There are several surveillance schemes worldwide that contribute to AMR data and inform policy changes on empirical treatment guidance. In England and Wales, AMR surveillance is currently performed by GRASP, which is an annual sentinel surveillance programme established in 2000 (Table 8). It is coordinated by the Blood Safety, Hepatitis, Sexually Transmitted Infections and HIV (BSHSH) Division, part of the Clinical and Public Health (CPH) group at the UK Health Security Agency (UKHSA), Antimicrobial Resistance in STIs (AMRSTI) section, part of the Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, and 27 sexual health services (SHS) that are linked to 21 laboratories across England and Wales[104]. Isolates are collected from patients attending the 27 SHS during the months of July to September. Not all isolates from patients with multisite infection are tested by GRASP, instead, they follow a hierarchy of testing; rectal isolates were prioritised before 2021, with pharyngeal isolates currently prioritised, followed by rectal and then urogenital isolates[104]. In addition to the GRASP surveillance scheme, AST data are supplemented by the Second-Generation Surveillance System (SGSS) provided by primary diagnostic laboratories across England[104]. Until January 2020, the United Kingdom also contributed to the European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP), an annual sentinel surveillance scheme coordinated by the European Centre for Disease Control (ECDC)[168] (Table 8). This surveillance scheme was established in 2009 and incorporated data from 23 countries across Europe[168]. In the United States, Canada and Australia, gonococcal AMR surveillance is performed by the Gonococcal Isolate Surveillance Project (GISP)[169], Enhanced Surveillance of Antimicrobial resistant Gonorrhea (ESAG)[170], and Australian Gonococcal Surveillance Programme (AGSP) respectively[171] (Table 8). These coordinating centres collaborate with the WHO to publish global into the Gonococcal Antimicrobial Surveillance Programme (GASP), and the Enhanced Gonococcal Antimicrobial Surveillance Programme (EGASP)[172] (Table 8). The GASP was established in 1990 and consists of reference laboratories globally that are networked with other international and national programs, incorporating data from 68 participating countries in six global regions[172]. The WHO EGASP was established in 2021 to allow participating

countries to adapt to their context and is aligned with the WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS)[172]. By the end of 2023, EGASP was implemented in a total of 10 countries, although the most recent report includes data only from Thailand, the Philippines, Cambodia and Uganda[172] (Table 8).

Table 8. Summary of gonococcal antimicrobial surveillance schemes and latest reported resistance rates for tested antimicrobials.

Scheme	**GRASP**[40]	**Euro-GASP**[168]	**GISP**[169]	**ESAG**[170]	**AGSP**[171]	**EGASP**[172]
Region	England	Europe	U.S.	Canada	Australia	Worldwide*
Latest Report	2023	2022	2024	2024	2023	2024
Breakpoints	EUCAST	EUCAST	CLSI	CLSI	AGSP	EUCAST
PEN	13.6%	NR	12%	3.9%	38.8%	NR
CFX	0.8%	0.5%	0.2%	3.4%	NR	1.7%
CRO	0%	0.03%	0.1%	0.2%	5.6%‡	0.8%
AZI	20.4%	11%	4.6%	1.9%	3.9%	0.5%
CIP	58.6%	57.7%	32.8%	63.1%	63.3%	93.3%
TET	61.8%	NR	20.6%	58.6%	45%	NR
SPE	0%	NR	NR	0%	0%	NR

**Contains data from Thailand, the Philippines, Cambodia and Uganda, ‡resistance rate reported for MIC of 0.06%, resistance rate for MIC ≥0.125 mg/L was 0.5%*
GRASP; Gonococcal Resistance to Antimicrobials Surveillance Programme, Euro-GASP; European Gonococcal Antimicrobial Surveillance Programme, GISP; Gonococcal Isolate Surveillance Programme, ESAG; Enhanced Surveillance of Antimicrobial resistance Gonorrhea, AGSP; Australian Gonococcal Surveillance Programme, EGASP; Enhanced Gonococcal Surveillance Programme, EUCAST; European Committee for Antimicrobial Susceptibility Testing, CLSI; Clinical and Laboratory Standards Institute, PEN; penicillin, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin, NR; not reported

The latest GRASP data reported that resistance rates to all antimicrobials apart from ESCs and spectinomycin were above the 5% WHO threshold (Table 8)[40]. In the UK, resistance rates to ciprofloxacin increased from 29.3% to 58.6% and for azithromycin 1.6% to 20.4% in the past 10 years [40,173] (Fig. 11). In contrast, since the switch from cefixime to ceftriaxone in the 2011 UK treatment guidelines, cefixime resistance has decreased from a peak of 17.1% in 2010 to 0.8% in 2024 (Fig. 11)[40,173]. A similar trend was seen in Europe; in the past 10 years ciprofloxacin and azithromycin resistance increased by 13.8% and 39% respectively and cefixime resistance decreased from 2% to 0.5%[168]. In the U.S. resistance rates to β-lactams were similar to the UK but differed in the other antimicrobials tested[169], which may reflect differences in breakpoints and AST methodology. The EGASP surveillance scheme detected low resistance rates to ESCs and azithromycin, but ciprofloxacin resistance was 93.3%[172], however, the report contains data from only four countries so may not be representative of global data, as in China it is estimated that up to 16% and 38% of gonococcal isolates tested have reduced susceptibility to ceftriaxone and cefixime respectively[174].

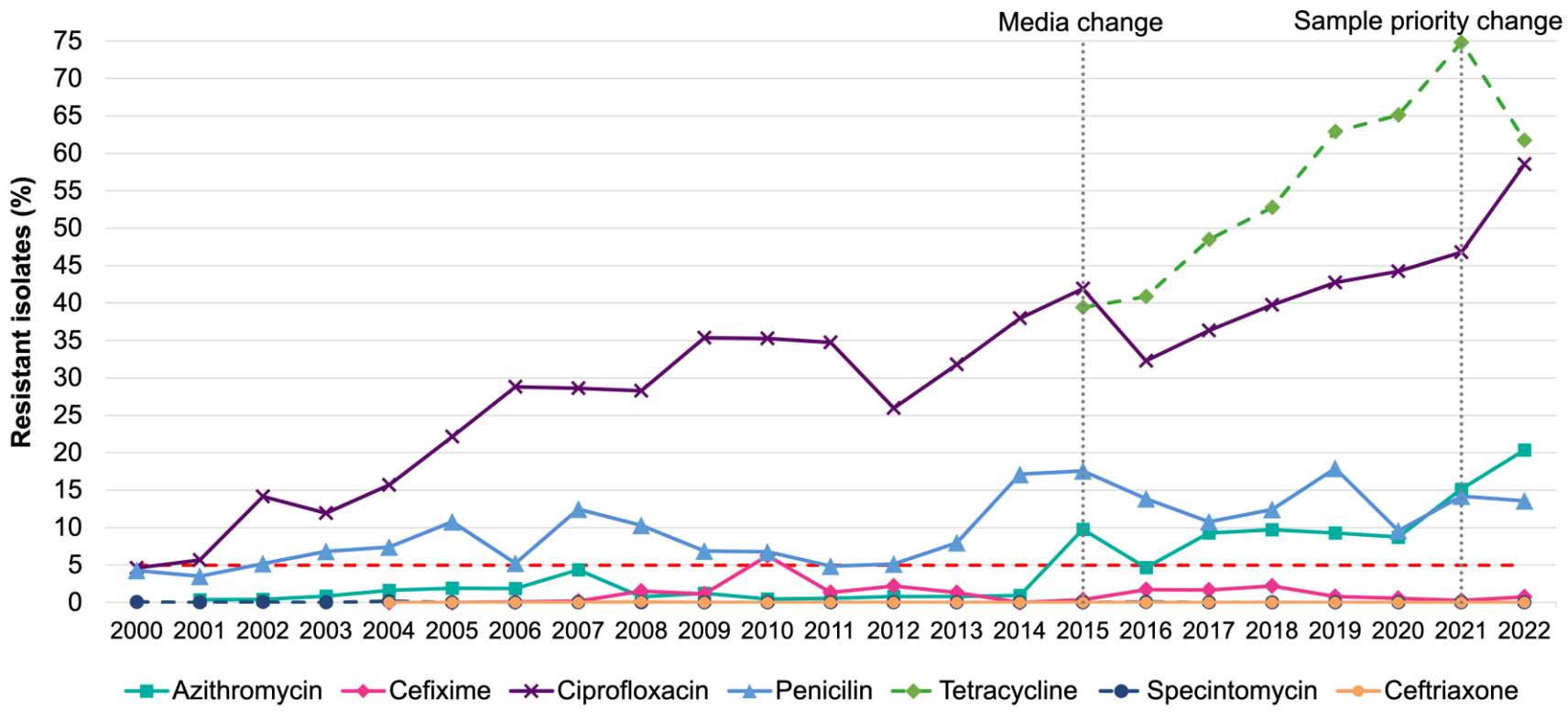

Figure 11. *N. gonorrhoeae* resistance rates to selected antimicrobials in the GRASP 2022 surveillance report[40] (Image in public domain). Sample priority change indicates switch from priority of rectal isolates to pharyngeal isolates.

1.5.4. Mechanisms of antimicrobial resistance in N. gonorrhoeae

N. gonorrhoeae employs several mechanisms to mediate AMR, including enzymatic inactivation (e.g. bla_{TEM-1}), alteration of the target site (e.g. *gyrA*), porin mutations (e.g. *porB*) and overexpression of efflux pumps (e.g. MtrCDE) (Fig. 12)[125]. Most resistance determinants are chromosomal except for bla_{TEM} and *tetM* which are located on conjugative plasmids.

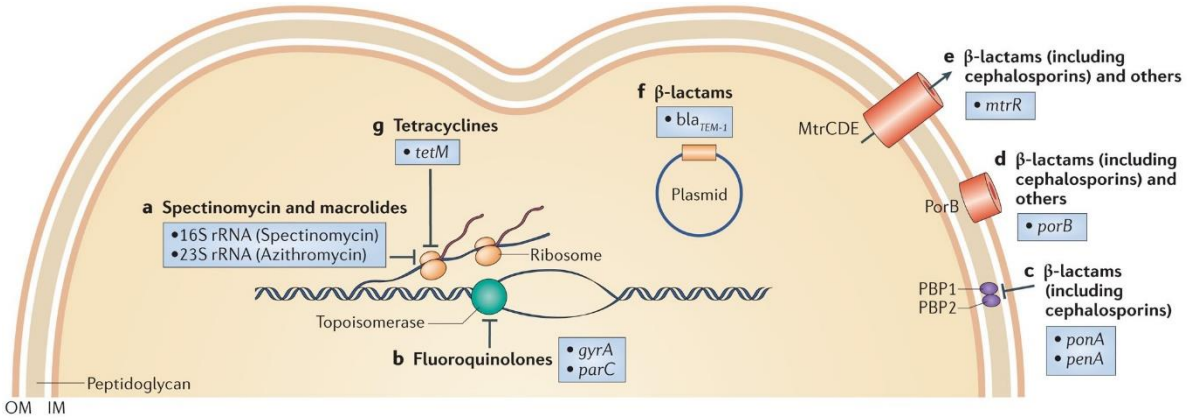

Figure 12. Summary of *N. gonorrhoeae* antimicrobial resistance mechanisms to previously used treatment options. Taken from Goire *et al*[175] (*Reproduced with permission from Springer Nature,* Licence Number 5854730090633).

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Penicillins and ESCs are β-lactam antimicrobials that bind to the bacterial transpeptidase enzyme, otherwise known as penicillin-binding protein (PBP), inhibiting cell wall synthesis and leading to cell death. Penicillin resistance occurs as a result of both chromosomal and plasmid-mediated resistance determinants. Plasmid-mediated resistance is due to a conjugative plasmid that encodes TEM-1 or TEM-135 type penicillinases that inactivate penicillins through enzymatic action and lead to high-level resistance[66]. It has been suggested that there are three main β-lactamase plasmid variants termed p*bla*.1, p*bla*.2 and p*bla*.3, associated with distinct gonococcal lianeages[176]. Chromosomal penicillin resistance occurs through mutations in *penA* and *ponA* leading to target alterations in PBP2 and PBP1 respectively, *porB* that lead to porin alternations and reduced permeability and the *mtrCDE* operon leading to overexpression of the MtrCDE efflux pump[66,125]. Gonococci also acquire segments from the *penA* of *N*c species through HGT, which

can form mosaic *penA* alleles, resulting in reduced affinity of β-lactams and therefore MIC increases, AMR and treatment failures[125]. These mosaic alleles are a major resistance mechanism for ESCs and are found in most resistant strains but can affect susceptibility in all β-lactams[125]. In *Ng*, approximately 36 variations of *penA* have been described and before the implementation of *NG*-STAR, were classified using the Roman numeric system as alleles I-XXXVI, based on substitutions at 82 amino acid positions[133] (Fig. 13). The *NG*-STAR scheme assigns whole numerical values to *penA*, followed by sequential decimals for each new amino acid change detected[118]. Non-mosaic SNPs in *penA* such as A501V and A501T, which can also exist in mosaic *penA* alleles, can also decrease susceptibility to ESCs[125]. Lastly, an unknown, non-transformable resistance mechanism affecting penicillin and ESCs termed 'Factor X', has been described in the literature and can increase MICs two to fourfold[123,125].

Macrolides such as erythromycin and azithromycin inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit and cause ribosomes to release incomplete polypeptides[66]. Resistance to macrolides is mainly due to alterations of the ribosomal target, either by SNPs or rRNA methylases, but can also be conferred through overexpression of efflux pumps. Macrolide resistance due to SNPs varies according to the nature and position of the mutations and how many of the four 23rRNA alleles contain the specific SNP. For example, C2611T leads to low to moderate-level resistance (MIC 2-8 mg/L) and A2059G leads to high-level resistance with MICs ≥256 mg/L[136] which have been spreading across England and worldwide[137–140,177,178]. The methylation of 23S is a rarer cause of resistance conferred by *erm* genes, present on conjugative transposons and leads to azithromycin MICs of 1-4 mg/L[66,136]. Lastly, similarly to β-lactams mutations in *mtrR* lead to overexpression of the MtrCDE efflux pump but other efflux pumps such as MacA-MacB encoded by *mef*, have also been implicated in macrolide resistance[123,136].

Fluoroquinolones such as ciprofloxacin inhibit the activity of DNA gyrase (encoded by *gyrA* and *gyrB*) and/or Topoisomerase IV (encoded by *parC* and *parE*) resulting in cell death[122]. Gonococcal resistance to fluoroquinolones develops through SNPs primarily in *gyrA* and accessory SNPs in *gyrB, parC* and *parE* and lead to alterations in the antimicrobial binding site[66,125]. The S91F

mutation in *gyrA* is highly predictive of ciprofloxacin resistance and has been used as a specific molecular marker for the detection of resistant strains, with a PPV of up to 100%[179].

Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit, therefore preventing the binding of aminoacyl-tRNA to the mRNA/ribosome complex[122]. Resistance to tetracyclines can be plasmid or chromosomally mediated. Plasmid-mediated resistance is due to the acquisition of a *tetM*-containing conjugative plasmid that encodes the protein TetM which results in competitive binding to the bacterial ribosome.[66]. Gonococcal strains with *tetM* have high MICs (>8 mg/L) to tetracycline and are commonly referred to as high-level tetracycline-resistant *Ng* (TRNG) strains[40]. Chromosomally mediated tetracycline resistance in gonococci can be due to mutations in the *rpsJ* gene leading to alterations in the antimicrobial target and similarly to β-lactams, loss of permeability due to mutations in PorB and overexpression of MtrCDE efflux pump[125].

Aminoglycosides such as spectinomycin and gentamicin inhibit protein synthesis by binding to the 30S ribosomal unit[125]. Resistance to spectinomycin can be due to a C1192U SNP in 16S rRNA (MIC >1,024 mg/L) or due to mutations in *rpsE* that encode the S5 protein within 30S[66]. Spectinomycin is no longer routinely used for the treatment of gonorrhoea, due to lack of availability and rapid development of resistance[123]. Gentamicin resistance hasn't been comprehensively studied; however, it is thought to be due to mutations in *fusA* which encodes elongation factor-G (EF-G) and therefore prevents the binding of the antimicrobial[180].

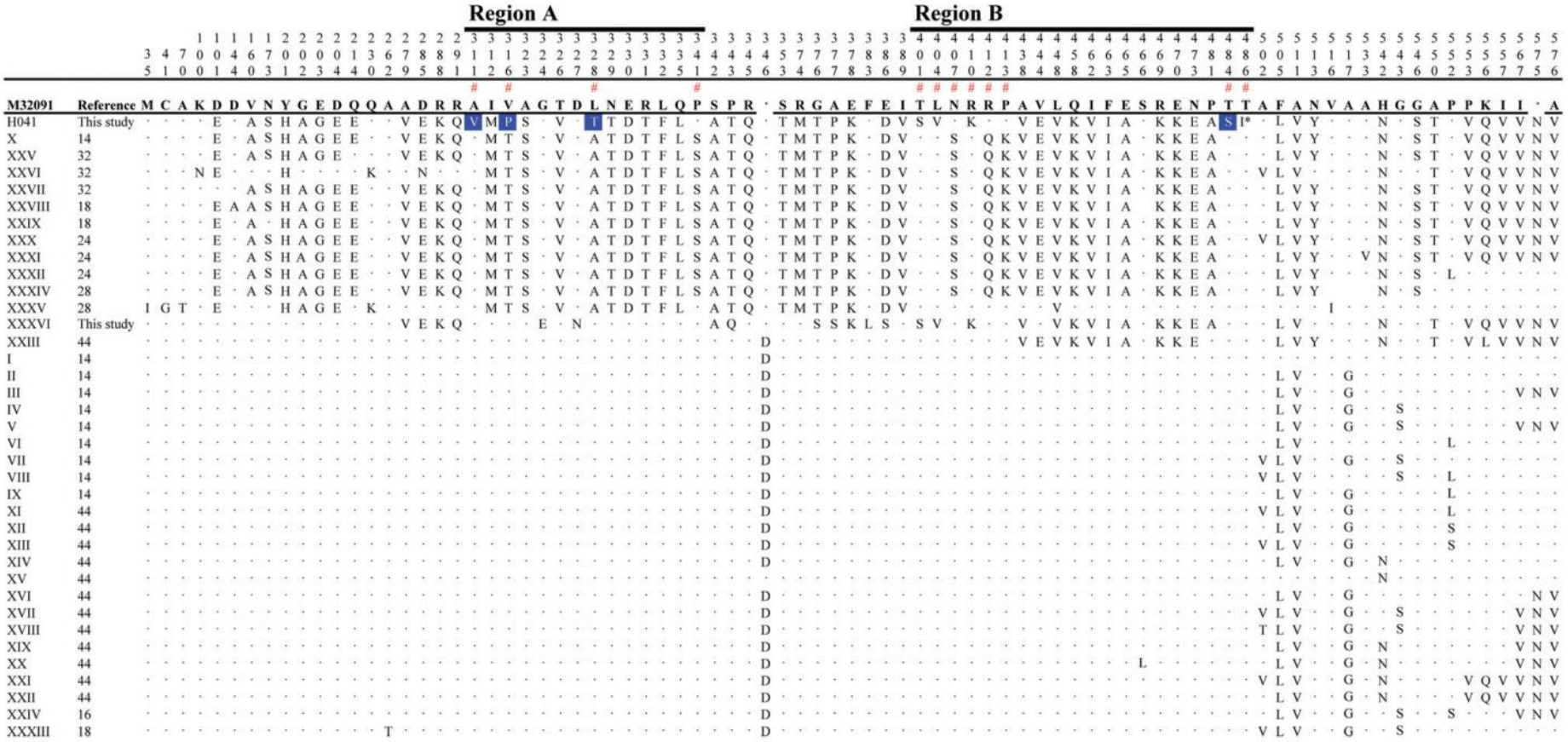


Figure 13. Numbering system for mosaic *penA* alleles, prior to the introduction of *NG*-STAR typing, from Ohnishi *et al* ,2011[133]. (*Reproduced with permission by the American Society for Microbiology*, Licence Number P2024.16).

1.5.5. Neisseria DNA Uptake Mechanisms

Transformation is a mechanism of acquiring genetic material from an organism's environment that is utilised by over 80 bacterial species, for example, *Haemophilus influenzae, Streptococcus pneumoniae* and *Bacillus subtilis*[181]. *Neisseria* species are also considered naturally competent, meaning they can acquire genetic material mainly from other species of the same genus in all growth phases[182]. For *Ng*, it is hypothesised that transformation is used to create and spread antigenic variation, genetic diversity and advantageous alleles[67]. The uptake of DNA through transformation is a technique used to exchange DNA between *Neisseria spp* and is a four-step process involving DNA donation, DNA binding, DNA uptake and homologous recombination[67] (Fig. 14). Donation of DNA by neighbouring *Neisseria spp* can occur by autolysis or by a type IV secretion system (T4SS), which is a mechanism employed by Gram-negative bacteria to secrete molecules across the cell envelope[67]. The gonococcal T4SS, encoded on the gonococcal genetic island (GGI) is present in approximately 80% of *Ng* and secretes single-stranded DNA (ssDNA) to facilitate the transformation of recipient cells[183]. The uptake of donor DNA relies on the ability of the recipient cell to produce type IV pili[184], which are structures of repeating units of pilin, mainly PilE and have further functions in bacterial adherence and motility[182]. Binding of donor DNA is facilitated by pilus-related proteins such as PilE, PilF and PilG[67]. A unique feature of *Neisseria* and *Haemophilus* species is that they require a specific DNA uptake sequence (DUS) to be present in the donor DNA, recognised by Type IV pili, for successful transformation[185]. The *Neisseria* DUS is a 10-base pair sequence (DUS10 5′-GCCGTCTGAA) which occurs over 2,000 times in the gonococcal genome and approximately 80% of DUS10 are contained within a 12-mer DUS (DUS12 5′- ATGCCGTCTGAA) which enhances transformation efficiency [181]. A variant DUS (vDUS 5'-GTCGTCTGAA-3') present in *N*c has also been described, with some species such as *N. mucosa* having >3,000 copies[186]. Once bound, donor DNA is transported through the periplasmic space into the cytoplasm by several proteins such as ComP, ComL and ComA (Fig. 14)[67]. The DNA is then processed; dsDNA is converted to ssDNA restriction-modification enzymes (RM) after which it is bound by cytoplasmic RecA, which mediates homologous recombination into the gonococcal chromosome[67].

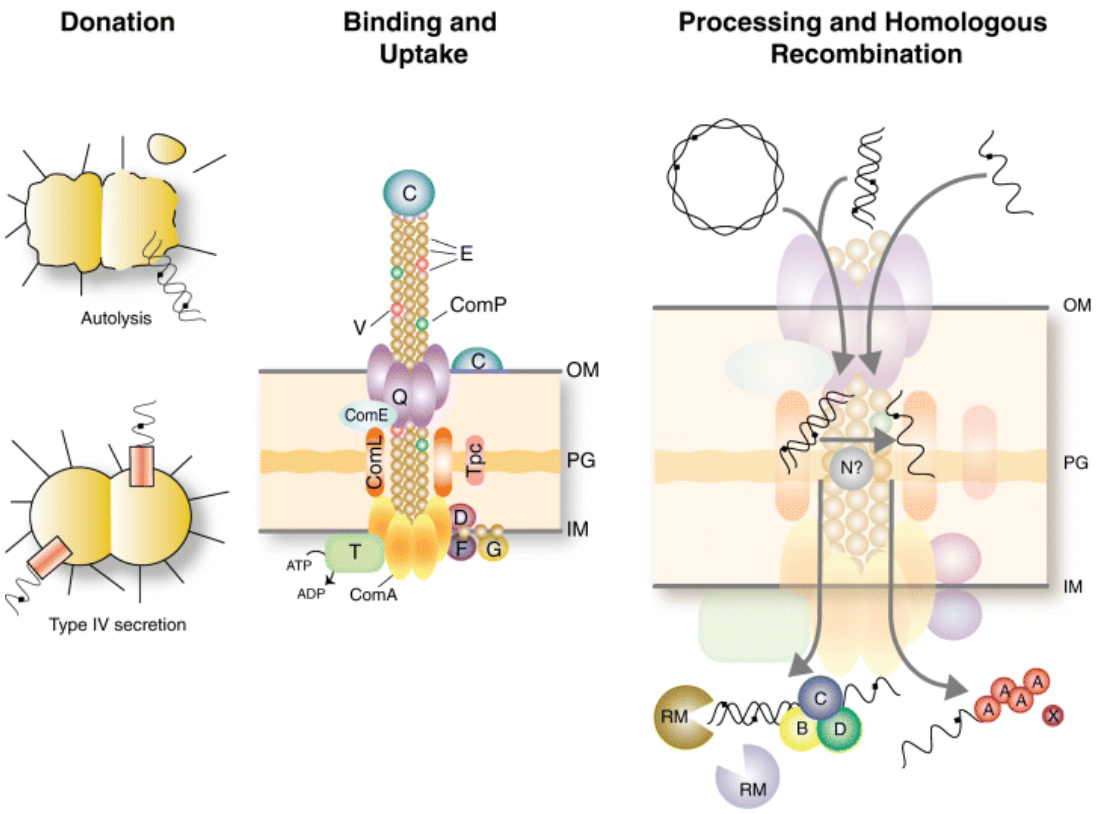


Figure 14. Mechanism employed by *N. gonorrhoeae* to acquire, uptake and incorporate DNA from donor bacteria. Image from Hamilton *et al*[67] (*Reproduced with permission from John Wiley and Sons Molecular Microbiology*, Licence Number 5854730443598).

1.5.6. Antimicrobial resistance in commensal Neisseria spp

The first study that examined the susceptibility of *N*c species performed MIC testing of a range of antimicrobials on *N. lactamica* isolates and found that all isolates were susceptible to ESCs but intermediate to penicillin[187] (Table 9). A further survey of 45 *N. subflava* isolates from Japanese men and women in 2007 detected 28.9% were resistant to tetracycline and 31.1% to ciprofloxacin, whereas 89% of isolates were intermediate to penicillin and all isolates were susceptible to ESCs[188]. Increased MICs to β-lactams were seen in 2011 in *N*c, the same year that *Ng* H401 was detected, when a study on neutropenic patients found high median MICs to penicillin (1 mg/L), ampicillin (1 mg/L) and cefotaxime (0.064 mg/L) and detected a bla_{TEM-1} β-lactamase in four isolates[189]. More recently, a Vietnamese study of oropharyngeal *Neisseria* carriage in 207 MSM reported that 31%, 28% and 78% of 265 isolates had reduced susceptibility to cefixime, ceftriaxone

and cefpodoxime respectively[78]. This study also examined the association between recent antimicrobial therapy and the MICs of *Neisseria* species isolated and found that antimicrobial use within the past month was strongly associated with having increased MICs to ESCs, compared to patients who received antimicrobials between one and six months prior, suggesting the short-term selection of resistant strains[78]. However, this is in contrast with a study of 96 MSM in Belgium who did not find a correlation between antimicrobial therapy and MIC to ESCs[79]. The authors also reported that *N*c species from MSM had lower susceptibility to azithromycin and ciprofloxacin compared to the general population but had comparable MICs to ceftriaxone (0.047 mg/L and 0.034 mg/L respectively)[79]. The most recent study reported high resistance rates to azithromycin (91%, 224/246), ciprofloxacin (57.5%, 142/246), cefotaxime (13.4%, 33/246) and ceftriaxone (29/246)[84].

Table 9. Summary of studies examining resistance profiles of commensal *Neisseria* species from the general population.

Author	Arreaza et al[187]	Furuya et al[188]	Mechergui et al[189]	Dong et al [78]	Laumen et al*[79]	Gaspari et al[84]
Year	2002	2007	2011	2020	2022	2023
Country	Spain	Japan	Tunisia	Vietnam	Belgium	Italy
AST Method	AD	AD	NS	GS	GS	GS
AZI	NT	NT	NT	NT	3	91.1%
CRO	NT	0%	NT	28%	0.047	11.7%
CIP	0%	31%	NT	93%	0.032	57.7%
CFX	NT	0%	NT	31%	NT	NT
CPO	NT	NT	NT	78%	NT	NT
PEN	0%‡	8.8%	34%	NT	NT	NT
AMP/AMX	5%	NT	9%	NT	NT	NT
CTX	0%	NT	0%	NT	NT	13.4%
RIF	0%	NT	NT	NT	NT	NT
TET	NT	29%	NT	NT	NT	NT
GEN	NT	NT	NT	NT	NT	NT

**Authors reported median MIC only.*
‡All isolates were intermediate
AST; antimicrobial susceptibility testing, AD; agar dilution, GS; Gradient strip, AZI; azithromycin, CRO; ceftriaxone, CIP; ciprofloxacin, CFX; cefixime, CPO; cefpodoxime, PEN; penicillin, AMP; ampicillin, AMX; amoxycillin, CTX; cefotaxime, RIF; rifampicin, TET; tetracycline, GEN; gentamicin, NS; not specified, NT; Not tested

1.6. Control strategies for *N. gonorrhoeae*

The 2022 WHO Global Health Strategies on HIV and STIs aims to reduce global gonorrhoea cases from 82.3 million to 8.23 million cases annually in 15–49-year-olds by 2030[190]. A specific action from this strategy relating to gonorrhoea is to monitor the AMR patterns to inform appropriate treatment recommendations and policies and therefore recognise that these efforts should be aligned with WHO and other gonococcal AMR action plans[2–5]. The WHO, CDC and ECDC published respective action plans in 2012[2,4,5], with the Health Protection Agency (now UKHSA) publishing supplementary guidance to the ECDC in 2013[3]. In the absence of an effective vaccine, the published action plans focus on the following thematic areas (Table 10):

i. Advocacy and education of healthcare professionals and the public
ii. Public health actions and prevention
iii. Monitoring of treatment failures
iv. Optimising antimicrobial treatment
v. Improving surveillance of AMR
vi. Improvement of laboratory detection
vii. Research in AMR molecular detection
viii. Research and introduction of new and novel treatments.

The importance of monitoring TFs and AMR surveillance has been acknowledged in all strategy documents, highlighting the importance of these factors in control efforts[2–5] (Table 10). The need for standardised TF definitions and improved monitoring of TFs and AMR rates are considered key priorities. Advocacy for awareness and education of patients and healthcare providers on the gonococcal AMR problem were also considered key priorities in WHO, ECDC and UKHSA strategies (Table 10). Importantly, apart from ECDC, all other guidance documents highlight the importance of laboratory testing and molecular testing for resistance. Lastly, only the WHO and CDC recognise the need for new treatment options for gonorrhoea. However, new treatment options alone are not sufficient as resistance is expected to develop after a period of time. New and novel treatments should be introduced with an antimicrobial stewardship strategy. For example, a mathematic modelling study compared four antimicrobial introduction strategies to determine which strategy delayed the development of 5% resistance the longest and found that

combination therapy or 50-50 random allocation therapy delayed resistance the longest[191]. These findings however should be interpreted with caution; combination therapy for gonorrhoea was introduced in 2011 and resistance to azithromycin developed rapidly leading to the switch to monotherapy in 2018[8].

Table 10. Summary of strategies from WHO, ECDC, UKHSA and CDC action plans to control the threat of antimicrobial resistant gonorrhoea.

	Author	WHO[2]	ECDC[4]	UKHSA[3]	CDC[5]
	Year	2012	2012	2013	2012
	Region	Global	Europe	England & Wales	US
Strategy themes	Advocacy and Education	Advocacy for increased awareness on correct use of antibiotics among healthcare providers and the consumer, particularly in key populations including men who have sex with men (MSM) and sex workers.	Establishing a communication strategy to increase awareness and disseminate the results from AMR surveillance in order to inform authorities, professional societies, physicians and potential patients about the threat of MDR NG.	Communicate to relevant healthcare professionals and populations with higher rates of gonorrhoea diagnoses to raise awareness of the threat of untreatable gonorrhoea.	None
	Public health actions & prevention	Effective prevention, diagnosis and control of gonococcal infections, using prevention messages, and prevention interventions, and recommended adequate diagnosis and appropriate treatment regimens.	None	Promote prevention messages to enhance public health control of gonorrhoea.	To outline the recommended public health actions to be implemented at the national, state, and local levels following detection of suspect or probable Ceph-R NG cases.
	Monitoring of treatment failures	Systematic monitoring of treatment failures by developing a standard case definition of treatment failure, and protocols for verification, reporting and management of treatment failure.	Implement treatment failure monitoring to inform national and international authorities and professional societies in order to develop treatment guidelines and design national interventions.	Provide support to allow rapid detection of treatment failures.	Improve detection and monitoring of treatment failures.
	Antimicrobial treatment	Effective drug regulations and prescription policies.	None	Advise on appropriate changes to the national guidelines for the management of gonorrhoea.	None

	AMR Surveillance	Strengthened AMR surveillance, especially in countries with a high burden of gonococcal infections, other STIs and HIV.	Strengthening surveillance to obtain AMR profiles in a timely manner and with sufficient epidemiological information to inform national interventions.	Provide robust and timely surveillance data on gonococcal AMR in England and Wales.	To establish enhanced surveillance for patients with suspect or probable Ceph -R gonococcal infections.
	Improved laboratory detection	Capacity building to establish regional networks of laboratories to perform gonococcal culture, with good-quality control mechanisms.	None	Give technical advice to clinical microbiologists on appropriate methods for detection of resistant gonococcal isolates. in the laboratory.	Expansion of culture and AST capacity.
	Molecular detection of AMR	Research into newer molecular methods for monitoring and detecting AMR.	None	None	None
	New and novel treatment s	Research into, and identification of, alternative effective treatment regimens for gonococcal infections.	None	None	Need for alternative treatment options.

***NG**; Neisseria gonorrhoeae, **Ceph-R**; Cephalosporin resistant, **AMR**; Antimicrobial resistance, **MDR**; Multidrug resistance, **WHO**; World health organisation, **ECDC**; European centre for disease control, **UKHSA**; United Kingdom Health Security Agency, **CDC**; Center for disease control and prevention, **STI**; Human immunodeficiency virus, **AST**; Antimicrobial susceptibility testing*

1.7. Novel options for the treatment of gonorrhoea

The rise in ESC MICs and the emergence of MDR gonococcal strains has led to the clinical evaluation of existing alternative antimicrobial therapeutic agents, such as gentamicin, gemifloxacin, zoliflodacin, delafloxacin and solithromycin[192–194]. However, these agents are associated with shortcomings; for example, solithromycin and delafloxcin showed inferiority compared to current treatment regimens[194], gentamicin and zoliflodacin have poor gonococcal clearance at extragenital sites and gepotidacin was only tested at urogenital sites[194]. Although these options may provide a temporary solution to AMR gonorrhoea, novel ways of using antimicrobials should be evaluated. Topical antiseptics may have a role in the topical treatment of gonorrhoea, especially in the pharynx.

1.7.1. Chlorhexidine

Chlorhexidine (CHX), a widely used antiseptic may be an option. The antibacterial and antifungal efficacy of chlorhexidine has been known since the 1950s[195]. Chlorhexidine gluconate was first introduced in the UK as a disinfectant and topical antiseptic but since the 1970s its main use has been in oral microbiology for the treatment of periodontitis and gingivitis[196]. It exists as the acetate (diacetate), gluconate and hydrochloride salts, is bacteriostatic in low concentrations to many Gram-positive and Gram-negative bacteria and bactericidal in higher concentrations[197]. The mode of action is well characterized; in low concentrations it disrupts the bacterial membrane by releasing potassium ions, altering the osmotic pressure of the cell[197] and has some ATPase activity. In high concentrations it precipitates cytoplasmic proteins and nucleic acids causing cell death[197]. Chlorhexidine gluconate has a good safety profile as it is free of systemic toxicity in oral use and has an LD_{50} value of 1800 mg/kg[195].

Chlorhexidine is widely used in the routine clinical setting as a topical disinfectant and oral antimicrobial agent. It is used to disinfect skin via hand or body washing to eradicate AMR organisms[198,199], to disinfect surfaces for medical interventions such as mechanical ventilation[200,201] and to prevent or treat dental disease[198–203]. There are numerous published systematic reviews supporting the clinical efficacy of chlorhexidine for various applications[198–203],

especially oral use, supporting the evidence for the utility of CHX against pharyngeal *Ng*. A study by Tomas *et al* showed that the use of single 0.12% and 0.2% CHX mouthwashes for as little as 30 seconds reduced the bacterial viability of salivary microbiota by up to 95%, taking up to seven hours to return to normal[204]. Other studies also showed that the two CHX concentrations have comparable results[205]. The antimicrobial efficacy of CHX against *Ng* has also been demonstrated. A study showed that CHX-containing gel had a gonococcal minimum bactericidal concentration (MBC) between 12.5 mg/L and 250 mg/L, depending on the pH of the media and the presence of organic matter in the growth media[206]. Other studies determined the effect of CHX-containing mouthwashes on gonococcal suspensions *in vitro* and found a 0.2% concentration reduced the number of cfu to zero.[207,208]

The CHX solution Corsodyl (GlaxoSmithKline, Brentford, UK) is widely used and commercially available. It contains a CHX concentration of 0.2% w/v and 7% v/v ethanol, which have been shown to have a synergistic effect[209,210]. The concentration of CHX in Corsodyl is markedly higher than the MBC determined by the study above, making it a good candidate for a clinical trial for the treatment of pharyngeal gonorrhoea.

1.7.2. Evidence and rationale for antiseptic mouthwashes

Antiseptic mouthwashes for the treatment of gonorrhoea are already being evaluated. A clinical trial in 2016 by Chow *et al*, evaluated the mouthwash Listerine, which has ethanol as an active ingredient, on gonococcal viability[211]. This showed that gonococcal viability after a single gargle in the treatment arm was lower than the control arm, providing an important proof of principle for the use of antiseptic gargles for the treatment of pharyngeal gonorrhoea. However, two subsequent clinical trials that followed up patients for 12 weeks showed that a daily Listerine gargle did not reduce pharyngeal incidence compared to the control solution[212] nor conventional antimicrobial therapy[213]. A further clinical trial assessing the efficacy of Corsodyl on pharyngeal gonorrhoea had to stop early as they did not observe a reduction in gonococcal viability in five participants after seven days[214].

1.8. Summary

The imminent threat of untreatable gonorrhoea calls for a combination of different strategies to control the emergence and spread of AMR. Pharyngeal gonorrhoea has a unique role in exacerbating this public health problem, so research is required to understand the biological, molecular, epidemiological and clinical dynamics of gonococcal infection at this site. This can be achieved by optimising current gonococcal AMR surveillance by including isolates from multisite patients, introducing *N*c AMR surveillance and improving the monitoring of TFs. Together with this, having alternative therapeutic options, especially for pharyngeal gonorrhoea will ensure we can develop appropriate antimicrobial stewardship protocols to delay the emergence of pan-resistant gonorrhoea.

1.9. Aims and objectives

This thesis aims to contribute to evidence relating to the surveillance of gonococcal and *N*c AMR, understanding the relationship between ESC MICs and TF, and the evaluation of novel treatment options (Fig. 15).

The objectives of this work are to:

i. Complete a systematic review of gonococcal ESC TFs and compare pharyngeal with extra-pharyngeal MICs.
ii. To determine the proportion of patients with multisite *Ng* infection who harbour strains with differing MICs between anatomical sites.
iii. To describe the carriage and AMR profiles of *N*c species carried by a subset of the general population.
iv. To explore alternative treatment options for treating pharyngeal gonorrhoea.

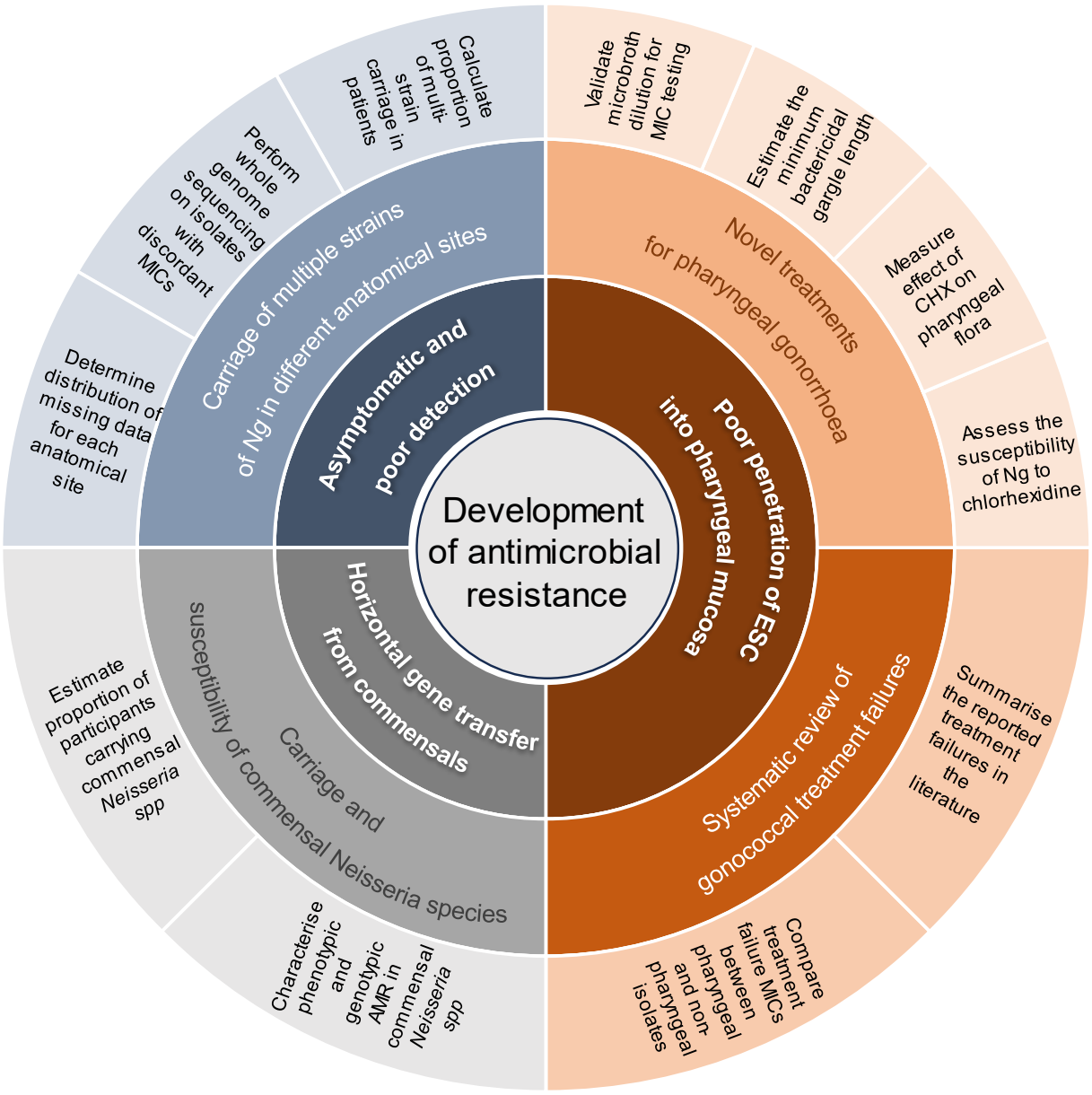


Figure 15. Summary of thesis and how the research fits with the current knowledge of how pharyngeal gonorrhoea facilitates the development of antimicrobial resistance. The three inner segments summarise three components thought to contribute to the development of AMR in *N. gonorrhoeae*; the four middle segments are research themes presented in this thesis, mapped to each of the three components to which they contribute new research. The outer segments outline the objectives of each results chapter onto these research themes.

1.10. Thesis structure

This work is organised and presented in four distinct results chapters. Below is an outline of the research that is presented in this thesis.

Chapter 3. In this chapter I summarise ESC TFs reported in the literature and characterise the differences between the MICs in pharyngeal and non-pharyngeal TFs, which may be used to inform the review and standardisation of gonococcal AMR breakpoints, especially for pharyngeal infection.

Chapter 4. In this chapter I explore differences in gonococcal strain carriage in multisite patients and how this may impact laboratory AST practices and gonococcal AMR surveillance. I also examine how missing laboratory data may impact data integrity.

Chapter 5. In this chapter, I present estimates of *N*c carriage and AMR burden, by species, in the pharynx of a subset of the general population. I also explore how phenotypic identification compares with WGS phylogeny and measure three dialects of DUS from the genomes of the *N*c.

Chapter 6. In this chapter, I determine the susceptibility of clinical and control *Ng* strains to CHX. I also assess the effect of a CHX gargle on the pharyngeal microbiota of study participants.

CHAPTER 2

MATERIALS AND METHODS

2.1. Agars, broths and reagents

2.1.1. Chocolate agar

Chocolate agar was prepared using Columbia agar base (Oxoid, Basingstoke, UK), according to manufacturer instructions. For every litre, 39 g of Columbia agar base powder was added to 950 mL distilled water. The media was autoclaved and allowed to cool to approximately 50°C, after which 50 mL (5%) defibrinated horse blood (Oxoid) was added to the agar. The media was placed in a boiling water bath (Grant Instruments, Royston, UK) and mixed periodically, to ensure uniform lysis of red blood cells. The chocolatised agar was poured into petri dishes (ThermoFisher Scientific, Dartford, UK) (20 mL each petri dish) and allowed to solidify before storing at 4°C until further use.

2.1.2. Gonococcal Medium Base (GCMB Agar)

This agar was prepared using GCMB agar (Beckton-Dickinson, Franklin Lakes, New Jersey, US), as described in the WHO GASP protocol[103].

For agar dilution MIC, 3.6 g GCMB powder was added to 89 mL distilled water. The media was autoclaved and cooled down to 50°C, to which 1 mL of Vitox supplement (Oxoid) (2.1.10) and 10 mL of antimicrobial solution (2.6) or sterile distilled water was added.

For gradient strip MIC, 36 g GCMB powder was added to 990 mL distilled water. The media was autoclaved and cooled down to 50°C, to which 10 mL of Vitox supplement (Oxoid) (2.1.10) was added. The agar was poured into petri dishes (25 mL each dish) and allowed to solidify before storing at 4°C.

2.1.3. Gonococcal Broth

Gonococcal broth (GC) was prepared as described previously[215]. Briefly, for every litre, GC broth was prepared by combining 15 g proteose peptone (Oxoid), 4 g potassium phosphate dibasic (K_2HPO4) (Sigma-Aldrich), 1 g potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich), 1 g potato starch and 5 g sodium chloride (NaCl) (WVR, Radnor, Pennsylvania, US) with 980 mL distilled water. The broth was mixed using a magnetic stirrer (Stuart Scientific, Cambridgeshire, UK) until fully dissolved and autoclaved. The broth was allowed to cool to approximately 50°C and

10 mL Vitox supplement (Oxoid) (2.1.10) and 10 mL 4.3% w/v $NaHCO_3$ (Sigma-Aldrich) (2.1.9) were added. The GC broth was stored at 4°C until further use.

2.1.4. Commensal Neisseria selective agar (LBVT.SNR)

Luria-Bertani Vancomycin Trimethoprim Sucrose Neutral Red agar was prepared as described by Knapp and Hook (1988)[100]. For every litre, 10 g tryptone (Oxoid), 5 g yeast extract (Oxoid), 5 g sodium chloride (Sigma-Aldrich) and 1.5 g Bacteriological Agar (Oxoid) was added to 965 mL water and autoclaved. Separately, 10 g sucrose was dissolved in 10 mL distilled water and filter sterilised. After autoclaving the molten agar was cooled to 50°C, to which the sucrose (VWR) solution, trimethoprim (2.6.2) (Sigma-Aldrich) and vancomycin (2.6.2) (Sigma-Aldrich) to a final concentration of 3 mg/L and 5 mL of neutral red indicator (Sigma-Aldrich) was added. The LBVT.SNR was poured into Petri dishes (20 mL each petri dish) and allowed to solidify before storing at 4°C until further use.

2.1.5. 20% Glycerol Brain Heart Infusion Broth

Brain Heart Infusion (BHI) broth was made according to manufacturer instructions; briefly, for every litre, 200 mL glycerol (VWR) was added to 800 mL distilled water and mixed thoroughly. Subsequently, 37 g dehydrated BHI powder (Oxoid) was added to the 20% (v/v) glycerol mixture and mixed. This was autoclaved, aliquoted into 1 mL cryovials (Simport, Quebec, Canada) and stored at -70°C until further use.

2.1.6. Agarose gel

A solution of 1X Tris-Borate-Ethylenediaminetetraacetic Acid (TBE) buffer was prepared by adding 100 mL 10X TBE (VWR) to 900 mL distilled water and mixing thoroughly. A 1.5% agarose solution was prepared by adding 1.65 g agarose (Sigma-Aldrich) to 110 mL 1X TBE buffer and mixing. The agarose solution was placed in a microwave oven and heated until the gel was clear and boiling. This was cooled down to approximately 50°C, after which 5.5 µL ethidium bromide (Sigma-Aldrich) was added.

2.1.7. Oxidase reagent

Oxidase reagent was prepared by combining N,N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride (TMPPD) reagent with 0.1% ascorbic acid solution. In a 50 mL Falcon tube (StarLab, Milton Keynes, UK), 0.02 g ascorbic acid (Sigma-Aldrich) was added to 20 mL distilled water and mixed thoroughly by vortexing until the powder was fully dissolved (0.1% ascorbic acid solution). Subsequently, 0.2 g TMPDD powder was added to this solution and mixed thoroughly until fully dissolved, to create a 1% TMPDD reagent. This was stored at 4°C until further use.

2.1.8. Gram stain reagents

Crystal violet, Lugol's iodine and safranin stains were purchased from Pro-Lab (Pro-Lab, Merseyside, UK) as a 10X concentrate. To prepare the ready-to-use Gram stain reagents, the concentrated vial of each stain (containing 100 mL) was added to 900 mL distilled water. Acetone was purchased ready-to-use from WVR.

2.1.9. 4.3% Sodium bicarbonate

In a 200 mL glass beaker, 4.3 g sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich) was added to 100 mL distilled water and mixed thoroughly using a magnetic stirrer (Stuart Scientific) until the powder was fully dissolved. In a class II safety cabinet (Biopharma Group, Winchester, UK), the sodium bicarbonate solution was filter sterilised. This was achieved by passing the solution through a 2 μm filter (Pall Corporation, Cornwall, UK) with a 50 mL syringe (Beckton-Dickinson). The solution was aliquoted in 25 mL sterile Falcon tubes (StarLab) and stored at 4°C until further use.

2.1.10. Vitox supplement

The Vitox supplement (Oxoid) was prepared according to manufacturer instructions. Briefly, a vial each of Vitox powder and reconstitution solution were transferred to a class II safety cabinet. The contents of the reconstitution solution were transferred to the vial containing the Vitox powder using a serological pipette (Phoenix Instrument, Garbsen, Germany) and mixed gently by pipetting up and down. When the powder was fully reconstituted and dissolved, the Vitox solution was aliquoted into smaller volumes and stored at -20°C until further use.

2.1.11. *Phosphate buffered saline*

The PBS was prepared according to manufacturer instructions. Briefly, one PBS tablet (Oxoid, UK) was added for every 100 mL distilled water and mixed thoroughly using a magnetic stirrer until fully dissolved. The PBS was aliquoted into smaller volumes, in glass bijoux or universals and autoclaved. These were stored at room temperature until further use.

2.1.12. *0.8% Porcine mucin solution*

A porcine mucin solution at 0.8% was prepared by adding 0.8 g porcine mucin (Sigma-Aldrich) to 100 mL sterile water. This was mixed thoroughly and autoclaved. The solution was stored at 4°C until further use.

2.1.13. *Dey-Engley neutralising broth*

The Dey-Engley (DE) neutralising broth was prepared according to manufacturer instructions. Briefly, for every litre 39 g of DE powder (ThermoFisher Scientific, San Diego, California, US) was added to 1000 mL distilled water and mixed thoroughly until fully dissolved, using a magnetic stirrer. This was autoclaved and stored at 4°C until further use.

2.2. Gonococcal Strains

2.2.1. *Source of isolates*

Gonococcal isolates were provided by kind donation from two London NHS microbiology laboratories, St George's University Hospitals NHS Foundation Trust (Dr Tim Planche and Dr Julie Johnson) and Barts Health NHS Trust (Dr Jayshree Dave and Dr Derren Ready). The isolates were collected from patients attending GUM clinics served by those laboratories in the period 2013-2015. Each laboratory identified the gonococcal isolates based on local diagnostic protocols and stored them at -70ºC in glycerol broth (2.1.5) or beads (Pro-Lab, UK).

WHO reference strains (F, G, K, L, M, N, O, P, X, V and Y)[216] were included in MIC testing for quality control purposes. Reference strains G, K, N, O, F, N and P were provided by the UKHSA and strains L, M, X, Y and V were purchased from the UKHSA National Collection of Type Cultures (NCTC).

2.3. Culturing, maintenance, and storage of bacterial isolates

2.3.1. *Culturing isolates from frozen stock*

Isolates were cultured from frozen storage vials on purchased pre-poured VCAT gonococcal selective media (Oxoid, UK) or chocolate agar (2.1.1). A rigid bacteriological loop (Thermo-Fisher, UK) was used to transfer a small amount of inoculated glycerol broth (2.1.5) onto a VCAT or chocolate agar plate. The same loop was used to streak the inoculated plate for discrete colonies. The inoculated and streaked plates were incubated as per 2.3.2.

2.3.2. *Incubation conditions*

Agar plates and inoculated broths were incubated at 37°C in 5% CO_2 for 24 hours unless otherwise stated.

2.3.3. *Inoculation of broth culture*

A 0.5 McFarland (MF) suspension (2.5.1) of the organism was prepared in PBS (2.1.11) or GC broth (2.1.3). An appropriate volume from the 0.5 MF suspension (Table 11) was added to GC broth to achieve a final concentration of approximately 5×10^5 cfu/mL or 10^6 cfu/mL. After the addition of the bacterial suspension, the inoculated GC broth was mixed thoroughly before further testing or incubating as per (2.3.2).

Table 11. Preparation of bacterial suspension dilutions used to inoculate broth cultures.

Final concentration (cfu/mL)	GC broth volume (mL)	Volume of 0.5 MF added (mL)
5 x 10^5	25	0.125
5 x 10^5	50	0.25
10^6	25	0.25
10^6	50	0.5

MF; McFarland

2.3.4. *Storage of bacterial stocks*

The organism of interest was isolated on VCAT or chocolate agar and inspected visually for contaminants. Bacterial colonies of interest were removed with a sterile swab (Medical Wire, Corsham, UK) and emulsified in 1 mL glycerol broth (2.1.5). The vial was labelled appropriately and stored at -70°C until further use.

2.4. Bacterial colony counts

Bacterial colony counts were performed using the adapted Miles and Misra method[217]. A tenfold dilution was performed on a bacterial suspension or inoculated GC broth (2.1.3), by transferring 500 μL of suspension to 4.5 mL PBS (2.1.11) and vortexing thoroughly for 10 seconds. This was repeated until a 10^{-9} dilution was reached. Pre-warmed chocolate agar plates (2.1.1) were divided into quarters and labelled with a dilution, as prepared previously. Using a P20 pipette (Gilson, Lewis Centre, Ohio, US), three 20 μL spots of each dilution were transferred to the respective labelled agar quarter and incubated as per 2.3.2, but for 48 hours (Fig. 16).

The inoculated spots were examined and counted. The colony forming unit (cfu) counts from each spot were calculated using the formula below:

$$cfu/mL = 50 \times (cfu \times 10^{n}),$$

Where 'cfu' is the number of colonies counted and 'n' is the reciprocal of the dilution factor of the dilution used to count the colonies. The colonies from each spot within the same dilution factor were counted separately and an average was calculated.

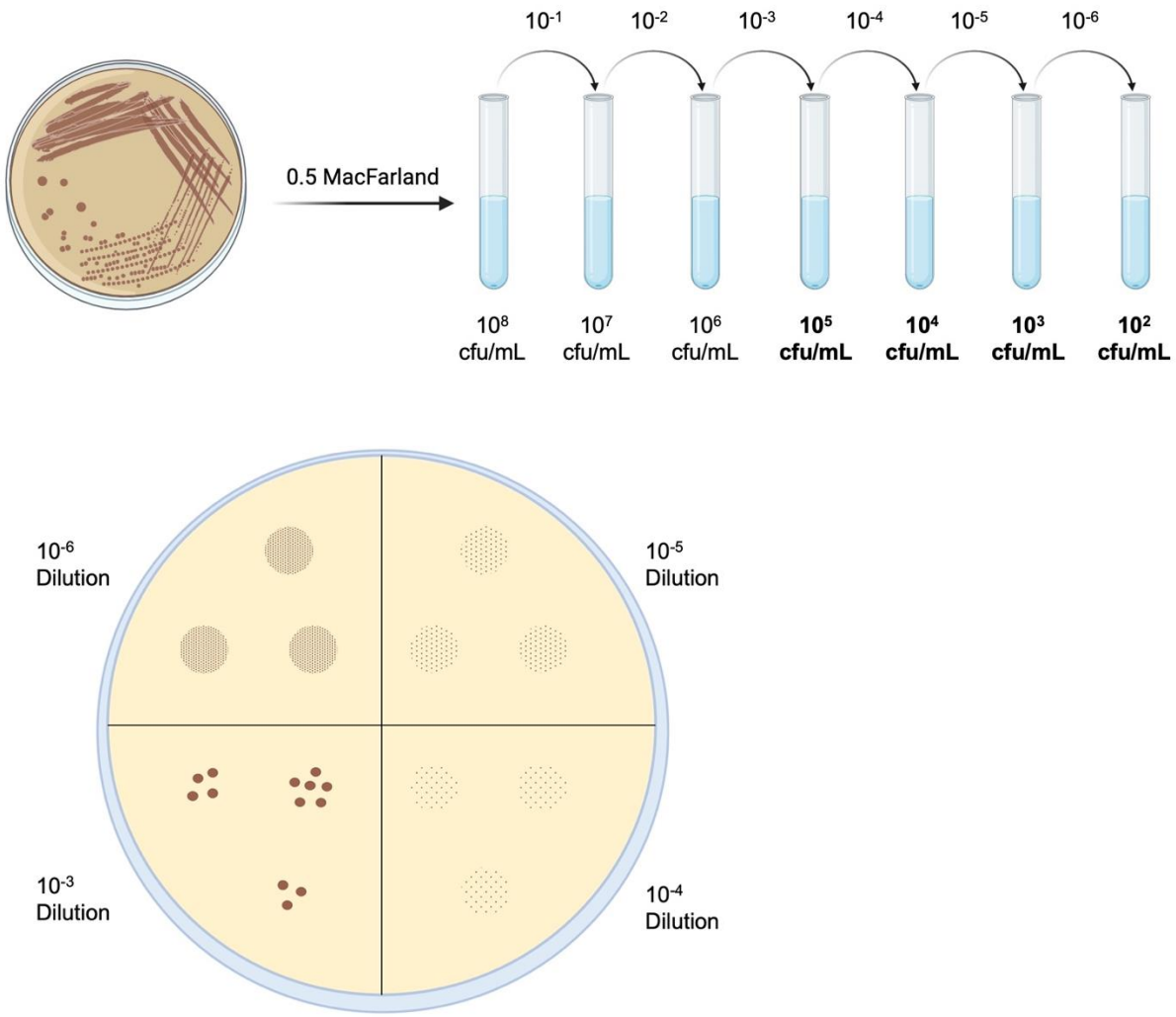


Figure 16. Miles and Misra colony counting method template. Each 'spot' contains 20 µL of each serial dilution.

2.5. Laboratory identification of bacterial isolates

2.5.1. Preparation of McFarland suspensions

A sterile cotton swab (Medical Wire) was used to transfer and emulsify several colonies of interest into sterile PBS (2.1.11). The swab containing the colonies was placed into a glass bijoux containing sterile PBS and rolled against the inside of the wall of the bijoux to express the organisms in the PBS until it reached the desired MF turbidity (Fig. 17). The emulsified suspension

was then inspected visually and compared to a commercial MF standard (Pro-Lab). The turbidity is proportional to the concentration of organisms in the suspension (Table 12)

Table 12. Approximate bacterial concentrations (cfu/mL) in each McFarland standard.

MacFarland Standard	Bacterial cfu/mL (approximate)
0.5	1.5×10^8
1	3×10^8
2	6×10^8
3	9×10^8
4	12×10^8

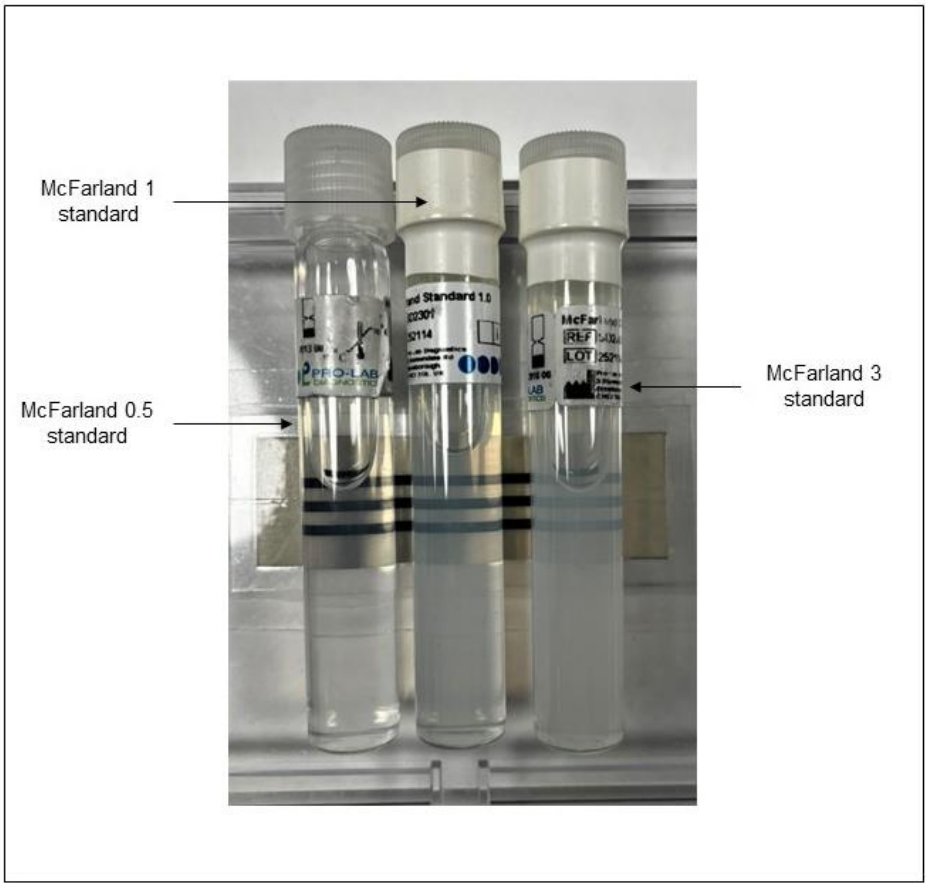


Figure 17. Pro-Lab McFarland 0.5, 1.0 and 3.0 turbidity standards used to estimate the bacterial concentration in suspensions.

2.5.2. *Oxidase test*

To perform the oxidase test, a sterile cotton swab (Medical Wire) was placed in the TMPDD reagent (2.1.7) until moistened. The moistened swab was used to touch a colony of interest; the development of a purple colour within 10 seconds indicated a positive result, and no colour change indicated a negative result (Fig. 18).

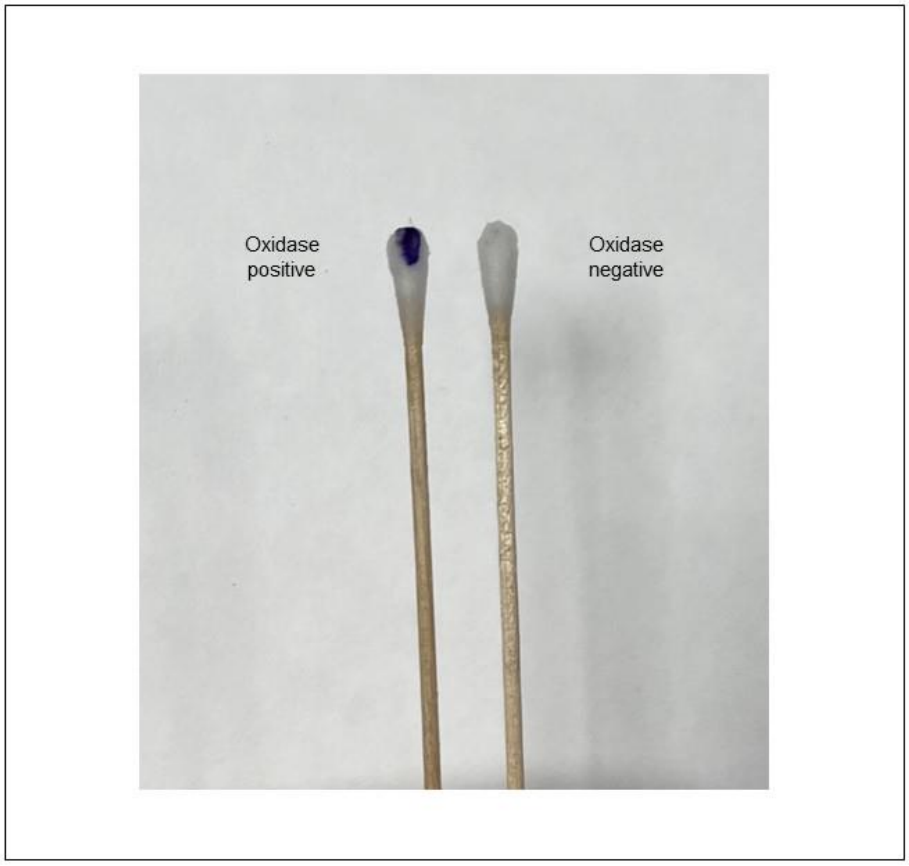


Figure 18. Positive and negative oxidase test results.

2.5.3. *Gram stain*

A drop of sterile saline was placed on a glass microscope slide (Scientific Laboratory Supplies, Dublin, Ireland) using a 10 µL bacteriological loop (Medical Wire). A colony of interest was emulsified in the saline and spread on the slide into a larger surface area. The slide was placed on a slide drying bench (Electrothermal, Basildon, UK) set at 55°C until the bacterial emulsion was fully dried. The slide was placed on a sink rack and flooded with crystal violet stain (Pro-Lab) for one minute (2.1.8). The slide was washed with tap water and replaced with Lugol's iodine (Pro-

Lab) for one minute. The iodine was rinsed with tap water thoroughly, the slide was washed with acetone (VWR) for 2-3 seconds and rinsed again with tap water. Finally, the slide was flooded with safranin (Pro-Lab) for 30 seconds and rinsed again with tap water. The slide was blotted dry with Wypall paper (Kimberely-Clark, Irving, Texas, US).

The Gram stains were examined with a light microscope (Leica, Wetzlar, Germany). Briefly, a drop of immersion oil (Sigma-Aldrich) was placed on the stained slide and placed on the microscope stage. The stage was adjusted so that the x100 lens touched the immersion oil. This was further adjusted finely to bring the Gram stain into focus. The Gram (positive or negative), shape, size and arrangement of the bacterial cells were noted (Fig. 19).

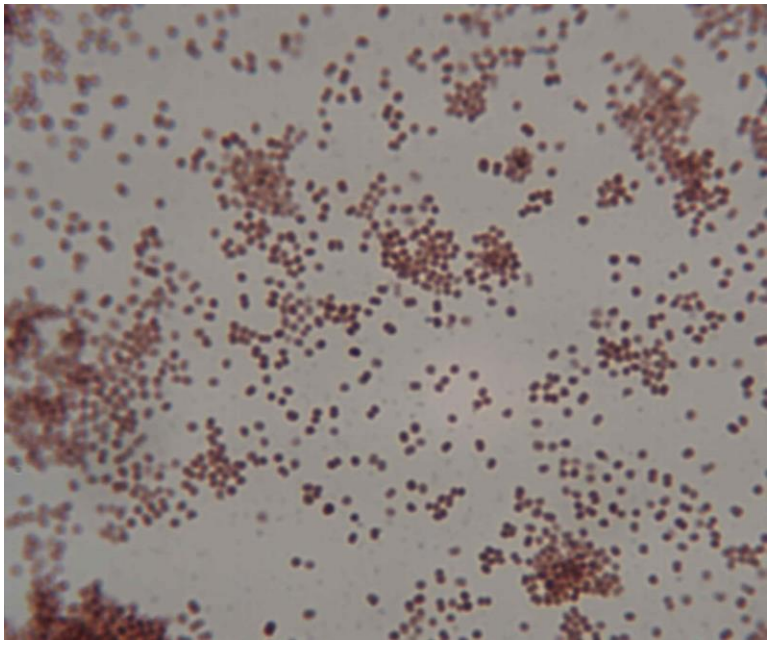


Figure 19. Gram stain of *Neisseria gonorrhoeae*, showing characteristic Gram-negative diplococci.

2.5.4. Analytical Profile Index (API) NH test

The API NH test was performed according to manufacturer instructions. A MF 4 suspension (2.5.1) of a pure 24-hour culture of the organism was prepared in a 2 mL 0.85% saline ampoule provided in the API NH kit (Biomerieux, Marcy-l'Étoile, France). Subsequently, 5 mL sterile water was placed in the bottom of the plastic incubation box. The API strip was removed from the packaging and inserted in the incubation box. Using a sterile Pasteur pipette (Scientific Laboratory Supplies), the organism suspension was transferred into the individual wells of the strip. Mineral oil (Biomerieux) was added to the first seven wells (Fig. 20a). The plastic lid of the incubation box was placed on the strip, and it was incubated for 2 hours, at 37°C, in normal atmosphere.

After incubation, wells PEN - β GAL were interpreted as positive or negative using the guide in Table 13 and noted in the API NH results slip provided in the kit (Fig. 21). On the result sheet, the tests were separated into groups of three and a value 1, 2 or 4 was assigned to each. The scores for the positive wells (except for PEN) were added together to produce a 4-digit numerical profile (Fig. 21 and Fig. 20b). One drop of ZYM B reagent (provided in the kit) was added to the LIP and PAL wells, for the proline arylamidase and γ-glutamyltransferase results respectively. One drop of JAMES reagent (provided in the kit) was added to the IND well for the β galactosidase result (Fig. 20c). The last three results were added to the results sheer to produce the final numerical profile of the tested organism. This was checked on the API Web software database (Biomerieux), which in turn gave an identification.

Table 13. Interpretation guide for the Biomerieux API NH biochemical identification kit.

Well	Test	Reagents	Positive	Negative
PEN	Production of penicillinase	n/a	Yellow / Yellow-green	Blue
GLU	Acidification of glucose	n/a	Yellow / orange	Red
FRU	Acidification of fructose	n/a	Yellow / orange	Red
MAL	Acidification of maltose	n/a	Yellow / orange	Red
SAC	Acidification of sucrose	n/a	Yellow / orange	Red
ODC	Ornithine decarboxylase	n/a	Blue	Yellow/green
URE	Urease	n/a	Fuchsia	Yellow
LIP	Lipase	n/a	Blue	Colourless
PAL	Alkaline phosphatase	n/a	Yellow	Colourless
β GAL	β galactosidase	n/a	Yellow	Colourless
ProA	Proline arylamidase	ZYM B	Orange	Yellow / Pale orange
GGT	γ-glutamyltransferase	ZYM B	Orange	Yellow / Pale orange
IND	Indole	JAMES	Pink/Red	colourless

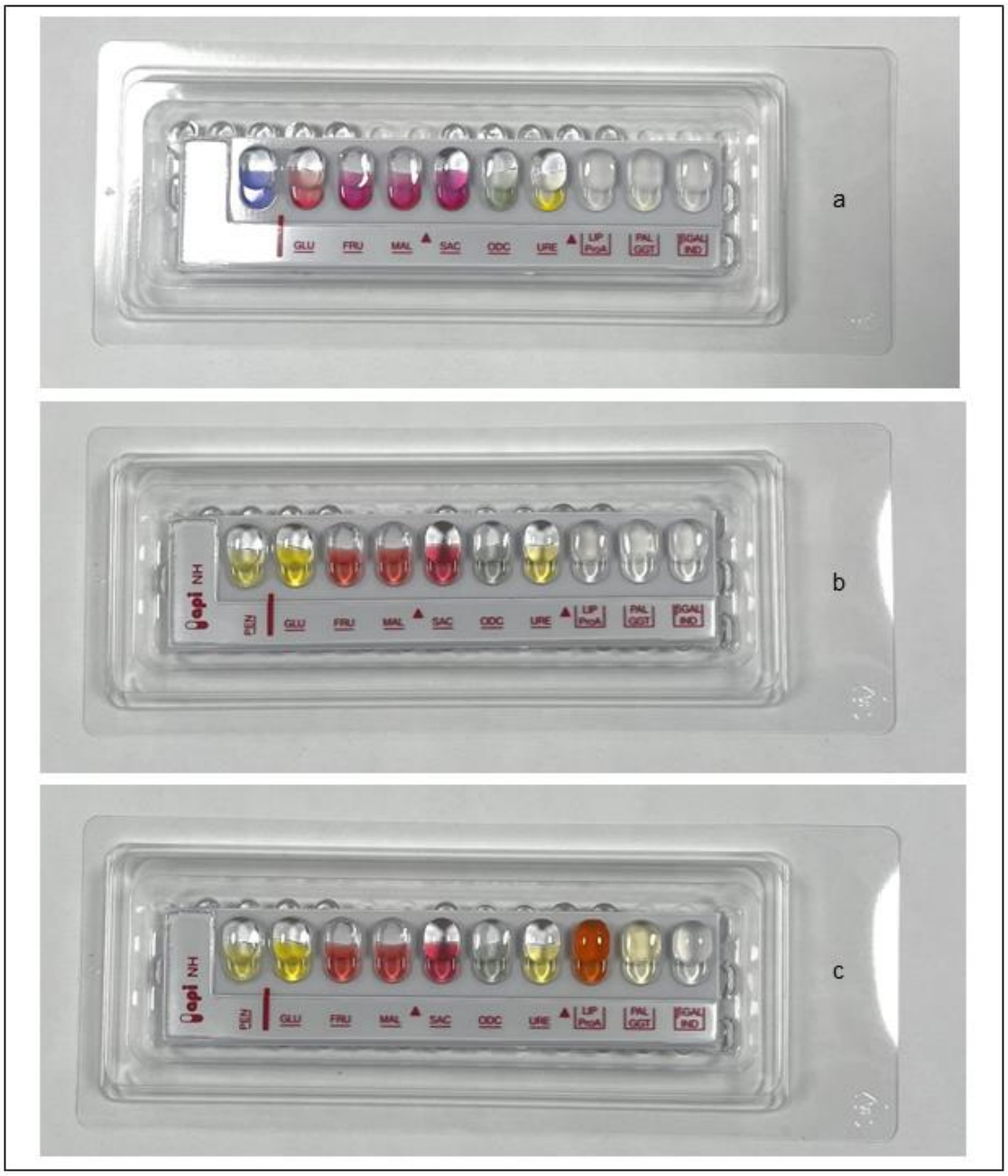


Figure 20. Image of API NH kit, after inoculation (a), 2h incubation (b) and after the addition of reagents (c). JAMES reagent was added to the βGAL/IND well and ZYMB reagent was added to the LIP/ProA and PAL/GGT wells.

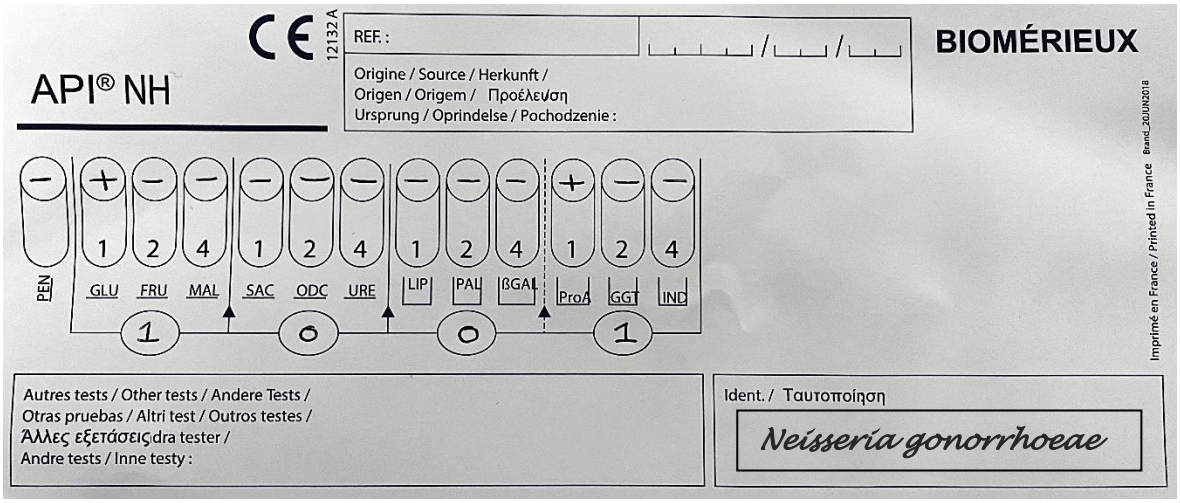


Figure 21. API NH results sheet after final interpretation of test kit.

2.5.5. RapID™ NH Test

The RapID™ NH test (Oxoid) was performed according to manufacturer instructions. A MF 3 suspension (2.5.1) of a pure 24-hour culture of the organism was prepared in 1 mL RapID inoculation fluid (Oxoid). The lid of the RapID NH panel was peeled back, where stated on the test kit. Using a Pasteur pipette, the bacterial suspension was transferred to the inoculating trough while tilting the test kit backwards (Fig. 22a). The kit was carefully rocked to evenly distribute the bacterial suspension (Fig. 22b). While maintaining a level, horizontal position, the panel was carefully tilted forwards to inoculate the reaction wells with the bacterial suspension (Fig. 22c). The panel was incubated for one hour at 37°C in normal atmosphere.

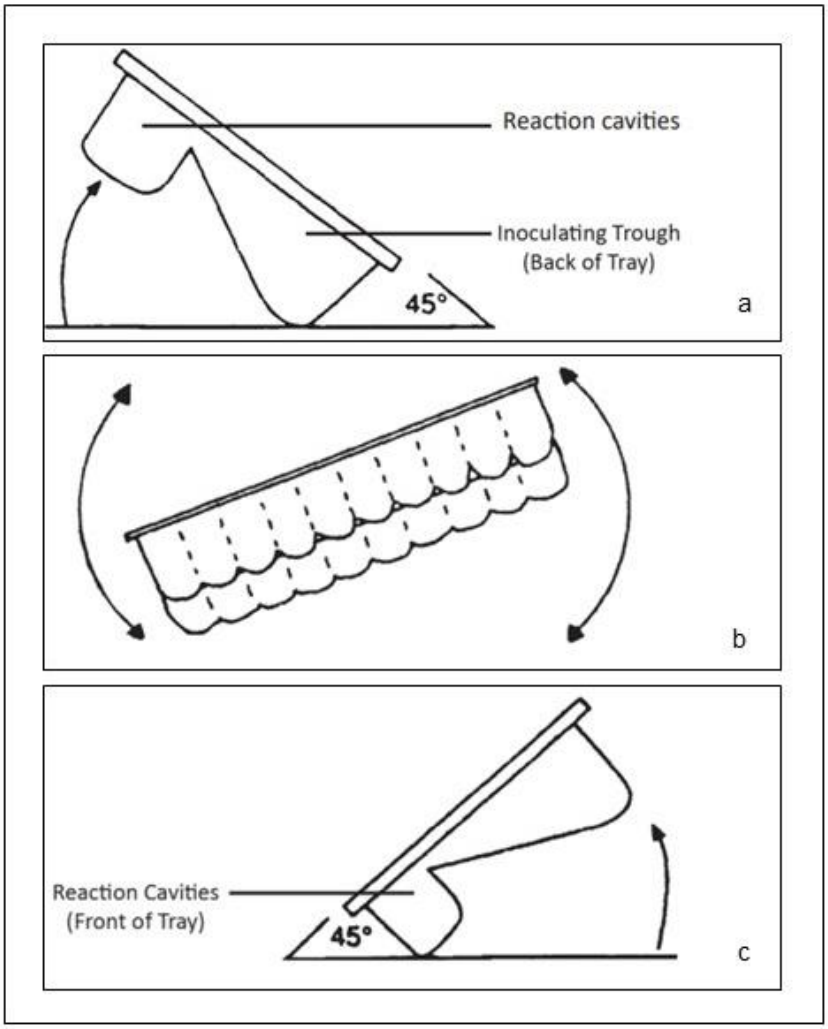


Figure 22. Inoculation method of RapID NH panel. Image provided in the kit insert.

After incubation, the reaction wells PRO – URE were interpreted (Fig. 23b) and scored (Fig. 24, Fig. 25). Two drops each of RapID Nitrate A and Nitrate B reagents (Oxoid) were added to wells PO_4/NO_2 and ORN/NO_3. Two drops of RapID Indole reagent (Oxoid) were added to the URE/IND well. After one minute a colour change was noted and added to the scoring (Fig. 23c, Fig. 24, Fig. 25).

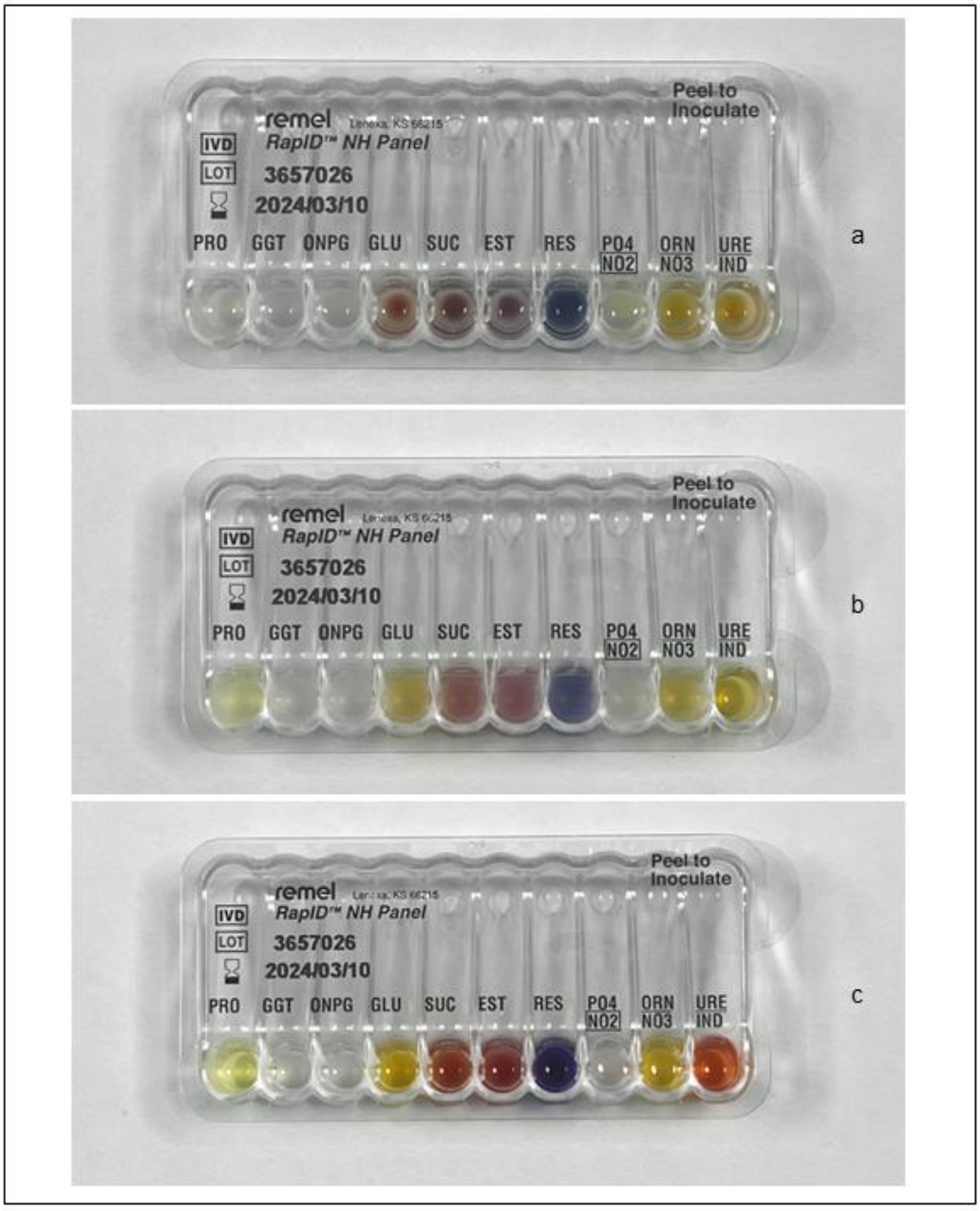


Figure 23. Image of Remel Rapid NH identification test kit after inoculation (a), 1h incubation (b) and after the addition of reagents (c). Two drops each of RapID Nitrate A and Nitrate B reagents were added to wells PO4/NO2 and ORN/NO3. Two drops of RapID Indole reagent were added to the URE/IND well.

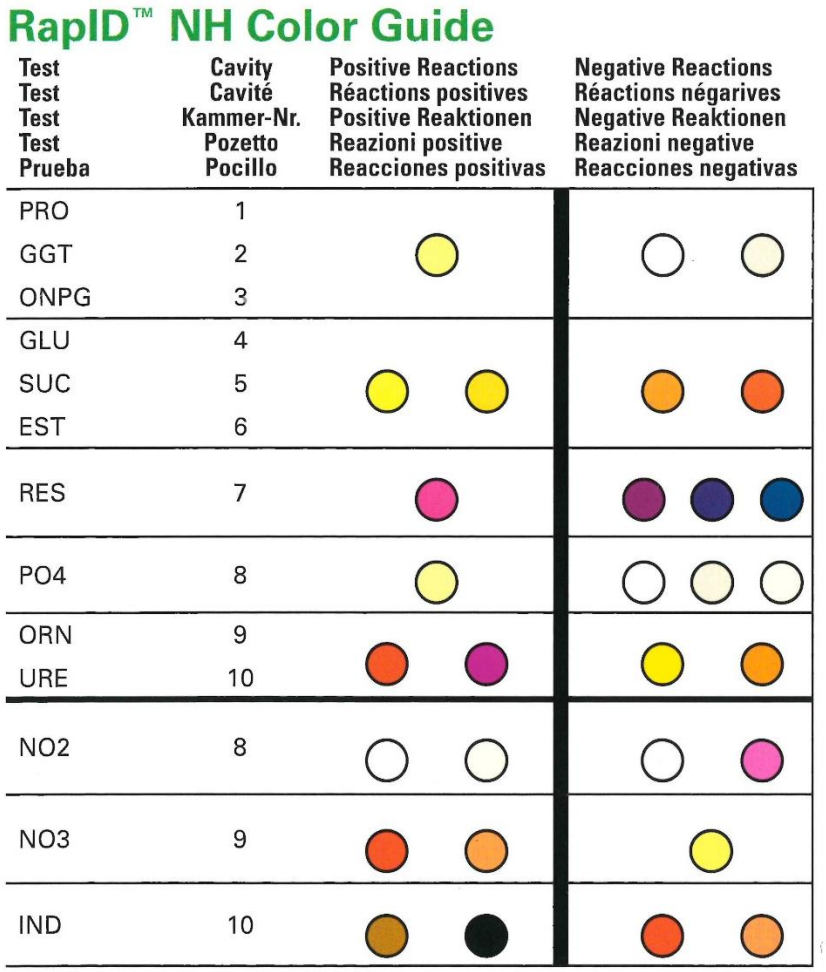
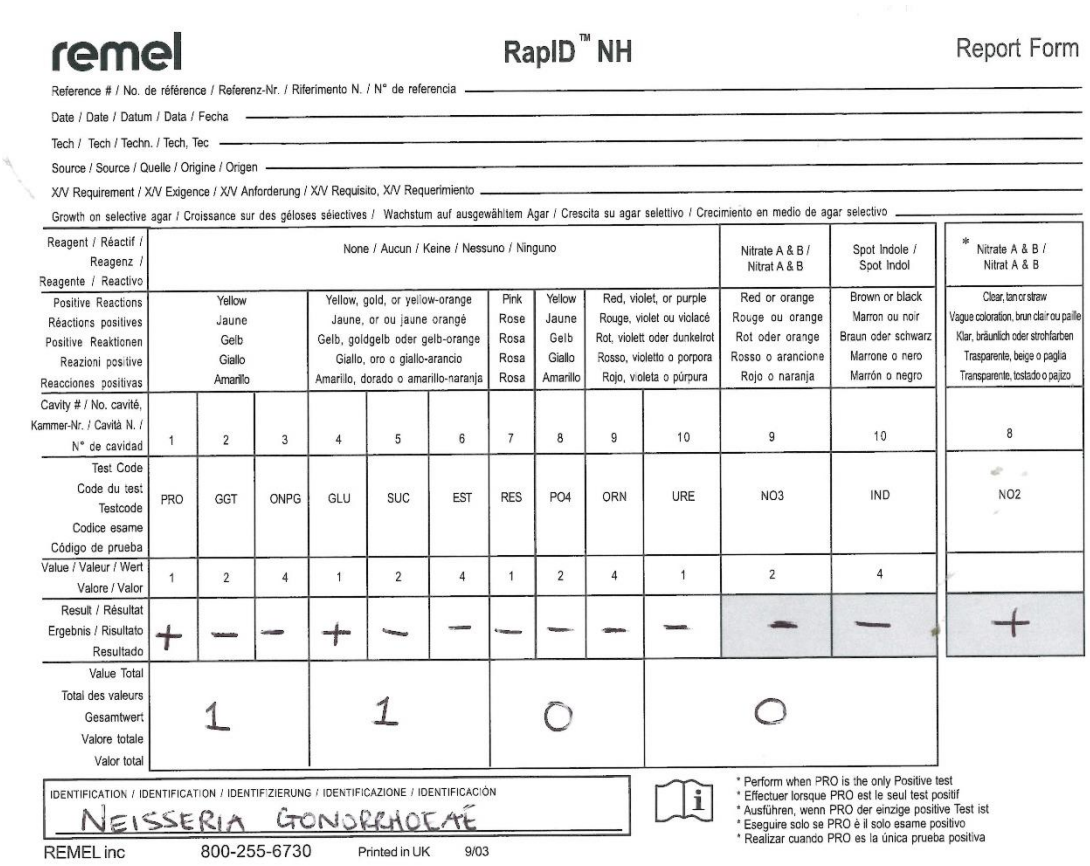


Figure 24. Rapid NH colour guide for the interpretation of the inoculated strip.



remel — RapID™ NH — Report Form

Reference # / No. de référence / Referenz-Nr. / Riferimento N. / N° de referencia

Date / Date / Datum / Data / Fecha

Tech / Tech / Techn. / Tech, Tec

Source / Source / Quelle / Origine / Origen

X/V Requirement / X/V Exigence / X/V Anforderung / X/V Requisito, X/V Requerimiento

Growth on selective agar / Croissance sur des géloses sélectives / Wachstum auf ausgewähltem Agar / Crescita su agar selettivo / Crecimiento en medio de agar selectivo

Reagent / Réactif / Reagenz / Reagente / Reactivo	None / Aucun / Keine / Nessuno / Ninguno										Nitrate A & B / Nitrat A & B	Spot Indole / Spot Indol	* Nitrate A & B / Nitrat A & B
Positive Reactions / Réactions positives / Positive Reaktionen / Reazioni positive / Reacciones positivas	Yellow / Jaune / Gelb / Giallo / Amarillo			Yellow, gold, or yellow-orange / Jaune, or ou jaune orangé / Gelb, goldgelb oder gelb-orange / Giallo, oro o giallo-arancio / Amarillo, dorado o amarillo-naranja			Pink / Rose / Rosa / Rosa / Rosa	Yellow / Jaune / Gelb / Giallo / Amarillo	Red, violet, or purple / Rouge, violet ou violacé / Rot, violett oder dunkelrot / Rosso, violetto o porpora / Rojo, violeta o púrpura		Red or orange / Rouge ou orange / Rot oder orange / Rosso o arancione / Rojo o naranja	Brown or black / Marron ou noir / Braun oder schwarz / Marrone o nero / Marrón o negro	Clear, tan or straw / Vague coloration, brun clair ou paille / Klar, bräunlich oder strohfarben / Trasparente, beige o paglia / Transparente, tostado o pajizo
Cavity # / No. cavité, Kammer-Nr. / Cavità N. / N° de cavidad	1	2	3	4	5	6	7	8	9	10	9	10	8
Test Code / Code du test / Testcode / Codice esame / Código de prueba	PRO	GGT	ONPG	GLU	SUC	EST	RES	PO4	ORN	URE	NO3	IND	NO2
Value / Valeur / Wert / Valore / Valor	1	2	4	1	2	4	1	2	4	1	2	4	
Result / Résultat / Ergebnis / Risultato / Resultado	+	−	−	+	−	−	−	−	−	−	−	−	+
Value Total / Total des valeurs / Gesamtwert / Valore totale / Valor total	1			1			0				0		

IDENTIFICATION / IDENTIFICATION / IDENTIFIZIERUNG / IDENTIFICAZIONE / IDENTIFICACIÓN

NEISSERIA GONORRHOEAE

* Perform when PRO is the only Positive test
* Effectuer lorsque PRO est le seul test positif
* Ausführen, wenn PRO der einzige positive Test ist
* Eseguire solo se PRO è il solo esame positivo
* Realizar cuando PRO es la única prueba positiva

REMEL inc 800-255-6730 Printed in UK 9/03

Figure 25. Rapid NH result sheet after the interpretation of the inoculated strip.

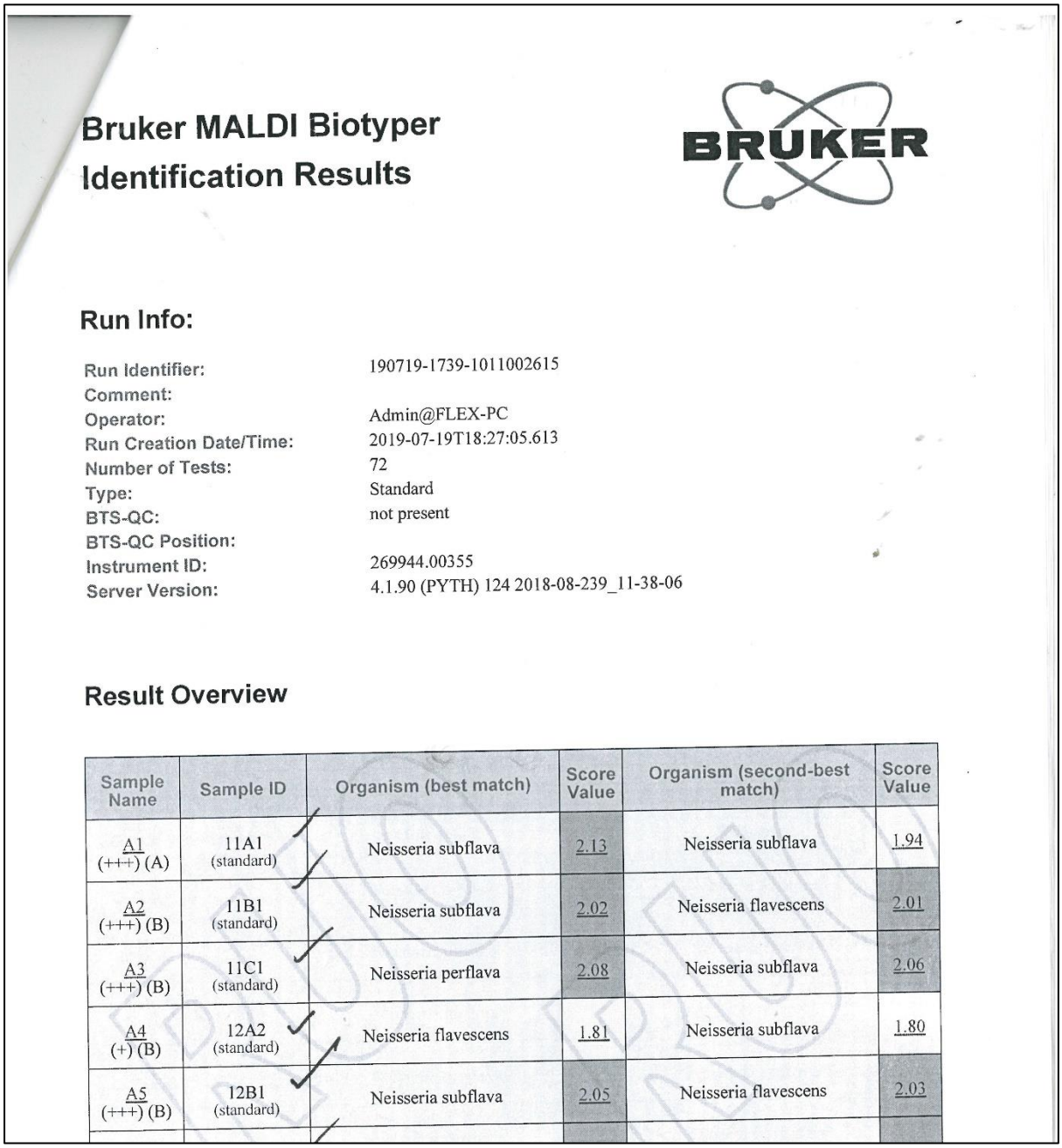
2.5.6. *Phenotypic identification of commensal Neisseria species*

Cultured isolates were first observed for colonial morphology including evidence of sucrose fermentation, texture, and size. Morphologically distinct colonies from the LBVT.SNR agar (2.1.4) were sub-cultured on chocolate agar and labelled with morphological characteristics for further processing and identification. Examples of colonial morphologies are shown in Fig. 8 (1.4.3). Colonies were screened using oxidase (2.5.2) and Gram staining (2.5.3). Oxidase-positive organisms and Gram-negative diplococci were considered as presumptive *Nc species*. Isolates were stored in glycerol broth at -70ºC.

2.5.7. Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALDI-ToF MS)

An extraction matrix was created by combining equal volumes of TA30 solvent [30:70(v/v) acetonitrile: 0.1% Trifluoroacetic acid (TFA) in water] (Bruker Daltonics, Massachusetts, US) and α-Cyano-4-hydroxy cinnamic acid (HCAA) (Bruker). A discrete colony was spread on a numbered position on a ground steel target plate using a wooden toothpick, after which 1 μL extraction matrix was placed on each smeared colony and left to air dry, before transporting to the Whittington Hospital Microbiology Laboratory for testing. Each plate was inserted into the Bruker MALDI Biotyper™ (Bruker Daltonics) for identification.

The output (Fig. 26) gives probable matches for the species of each isolate, with a numbered value that determines the reliability of the result. Values of 2.0 are considered high-confidence identifications (ID), values 1.7 – 1.99 are considered low-confidence IDs and values under 1.7 do not give a species ID (Fig. 27). These criteria were used to determine to species of each isolate. Isolates with values below 2.0 and isolates where no species was given were repeated once.



Bruker MALDI Biotyper Identification Results

BRUKER

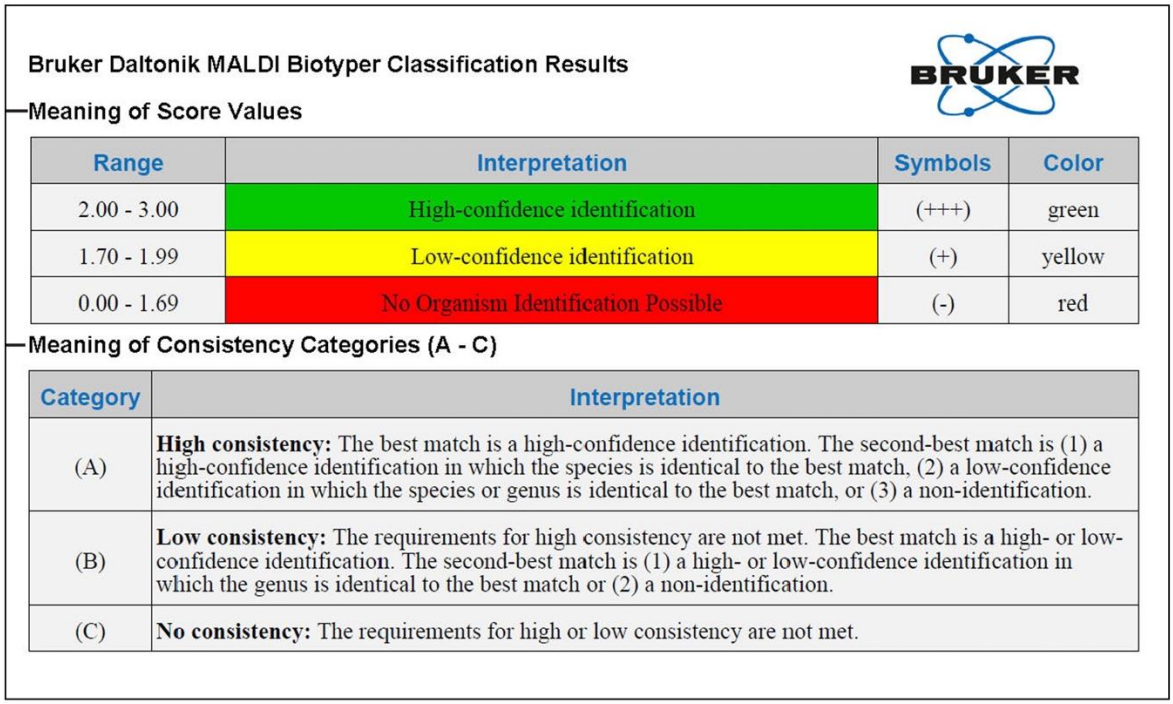
Run Info:

Run Identifier:	190719-1739-1011002615
Comment:	
Operator:	Admin@FLEX-PC
Run Creation Date/Time:	2019-07-19T18:27:05.613
Number of Tests:	72
Type:	Standard
BTS-QC:	not present
BTS-QC Position:	
Instrument ID:	269944.00355
Server Version:	4.1.90 (PYTH) 124 2018-08-239_11-38-06

Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A1 (+++) (A)	11A1 (standard)	Neisseria subflava	2.13	Neisseria subflava	1.94
A2 (+++) (B)	11B1 (standard)	Neisseria subflava	2.02	Neisseria flavescens	2.01
A3 (+++) (B)	11C1 (standard)	Neisseria perflava	2.08	Neisseria subflava	2.06
A4 (+) (B)	12A2 (standard)	Neisseria flavescens	1.81	Neisseria subflava	1.80
A5 (+++) (B)	12B1 (standard)	Neisseria subflava	2.05	Neisseria flavescens	2.03

Figure 26. Example MALDI-ToF results output, outlining sample ID and organism scores.



Bruker Daltonik MALDI Biotyper Classification Results

Meaning of Score Values

Range	Interpretation	Symbols	Color
2.00 - 3.00	High-confidence identification	(+++)	green
1.70 - 1.99	Low-confidence identification	(+)	yellow
0.00 - 1.69	No Organism Identification Possible	(-)	red

Meaning of Consistency Categories (A - C)

Category	Interpretation
(A)	**High consistency:** The best match is a high-confidence identification. The second-best match is (1) a high-confidence identification in which the species is identical to the best match, (2) a low-confidence identification in which the species or genus is identical to the best match, or (3) a non-identification.
(B)	**Low consistency:** The requirements for high consistency are not met. The best match is a high- or low-confidence identification. The second-best match is (1) a high- or low-confidence identification in which the genus is identical to the best match or (2) a non-identification.
(C)	**No consistency:** The requirements for high or low consistency are not met.

Figure 27. Interpretation of MALDI-ToF scores (taken from Bruker Daltonics website, (https://www.microbiologics.com/core/media/media.nl?id=1245512&c=915960&h=f324a4c9632c04e3a6ee&_xt=.pdf)

2.6. Preparation of antimicrobial stocks

2.6.1. Preparation of antimicrobial stocks for MIC Testing

The antimicrobial stock concentrations were prepared as described in the WHO GASP protocol[103] and the solvents used as stated by the manufacturer. All antimicrobial powders were purchased from Sigma-Aldrich. Stock concentrations of 1,000 mg/L or 10,000 mg/L were prepared by adding 0.01 g or 0.1 g respectively to 10 mL of the appropriate solvent and mixed until fully dissolved. The stocks were aliquoted into smaller volumes and stored at -70°C until further use. The full details of the stock concentrations prepared and solvents for each antimicrobial are summarised in Table 14.

Table 14. Concentrations, solvent details and volumes used for each antimicrobial stock prepared for agar dilution MIC.

Antibiotic	Stock Concentration (mg/L)	Solvent	Volume of solvent (mL)	Weight of antimicrobial (g)
Cefixime	1,000	Methanol (VWR)	10	0.01
Ceftriaxone	1,000	Water	10	0.01
Penicillin	10,000	Water	10	0.1
Ciprofloxacin	10,000	0.1N HCl (VWR)	10	0.1
Tetracycline	10,000	Methanol	10	0.1
Spectinomycin	10,000	Ethanol (VWR)	10	0.1
Azithromycin	10,000	Ethanol	10	0.1
Gentamicin	10,000	Water	10	0.1

For agar dilution MIC, 10 mL antimicrobial solution was added to 90 mL GCMB agar (2.1.2), so the serial dilutions prepared were 10-fold more concentrated than the final concentrations in the agar. These will be referred to as 'working concentrations'. The highest working concentration for each antimicrobial was prepared by adding an appropriate volume of the stock (Table 15) to a final volume of 20 mL sterile distilled water as outlined in Table 15 and vortexing for 10 seconds. Two-fold serial dilutions were prepared by transferring 10 mL of the highest working concentration to 10 mL sterile distilled water and vortexing for 10 seconds. This was repeated so that there was one 10 mL working dilution for each final agar dilution in the range tested (Table 16). From the final lowest dilution, 10 mL was removed and discarded. The prepared antimicrobial working dilutions were added to GCMB agar as per 2.1.2.

Table 15. Preparation of antimicrobial highest working concentrations to be used for serial dilutions and subsequent agar dilution MIC.

Antimicrobial	Working Concentration (mg/L)	Highest Agar Dilution Concentration (mg/L)	Volume of stock	Volume of water
Cefixime	20	2	0.4 mL	19.6 mL
Ceftriaxone	20	2	0.4 mL	19.6 mL
Penicillin	1280	128	2.56 mL	17.44 mL
Ciprofloxacin	640	64	1.28 mL	18.72 mL
Tetracycline	640	64	1.28 mL	18.72 mL
Spectinomycin	2560	256	5.12 mL	14.88 mL
Azithromycin	320	32	0.64 mL	19.36 mL
Gentamicin	2560	256	5.12 mL	14.88 mL

Table 16. Range of each antimicrobial tested in agar dilution MIC.

Antimicrobial	Range tested (mg/L)
Cefixime	0.002 - 4
Ceftriaxone	0.002 - 2
Penicillin	0.002 - 64
Ciprofloxacin	0.002 - 64
Tetracycline	0.064 - 64
Spectinomycin	4 - 256
Azithromycin	0.032 - 32
Gentamicin	2 - 32

2.6.2. Preparation of antimicrobial stocks for LBVT.SNR agar

A 3,000 mg/L suspension of trimethoprim stock was prepared by adding 0.03 g trimethoprim powder (Sigma-Aldrich) to 10 mL dimethylsulfoxide (Sigma-Aldrich) and mixed thoroughly until fully dissolved. A 10^{-1} dilution of the stock was performed in sterile distilled water to create a working stock of 300 mg/L. The trimethoprim stock solutions were stored at room temperature until further use.

A 1,000 mg/L suspension of vancomycin (Sigma-Aldrich) was prepared by aseptically reconstituting a vial containing 10 mg vancomycin powder with 10 mL sterile distilled water and mixing thoroughly until fully dissolved. A 300 mg/L working stock was further prepared by adding 3 mL of the 1,000 mg/L vancomycin stock to 7 mL sterile distilled water. The vancomycin stocks were stored at 4°C until further use.

2.6.3. Preparation of chlorhexidine digluconate stock and working concentrations

Chlorhexidine digluconate 20% (0.2 g/mL) solution (Sigma-Aldrich) was used to produce a stock solution of 20 mg/mL (2%; 20,000 mg/L) by adding 1 mL 20% solution to 9 mL sterile distilled water. Working stocks of 2,000 mg/L (0.2%) or 200 mg/L (0.02%) were prepared by performing 10^{-1} and 10^{-2} dilutions respectively of the stock solution in sterile distilled water. A 0.4% (4,000 mg/L) chlorhexidine solution was prepared by adding 2mL of 2,000 mg/mL stock to 8 mL sterile distilled water. A 0.12% (1,200 mg/L) solution was prepared by adding 0.6 mL of 2% chlorhexidine to 9.4 mL sterile distilled water. The highest working concentrations of the assays performed have been summarised in Table 17. All stock solutions were stored at 4°C until further use.

Table 17. Summary of how chlorhexidine digluconate working stocks were prepared for assays performed.

Assay	Highest working concentration (mg/L)	Stock concentration (mg/L)	Volume of stock (mL)	Volume of diluent (mL)	Diluent
Agar Dilution	80	2,000	0.8	19.2	SDW
Microbroth Dilution	16	2,000	0.16	19.84	GC broth
Checkerboard	32	2,000	0.64	39.36	GC broth
Timed kill study (A)	2,000	20,000	1	9	SDW
Timed kill study (B)	2,000	4,000	10	10	0.8% mucin
Timed kill study (C)	600	20,000	0.3	9.7	SDW
Timed kill study (D)	600	1,200	10	10	0.8% mucin

SWD; sterile distilled water, GC; gonococcal, (A); 0.2% chlorhexidine, (B); 0.2% chlorhexidine and 0.4% porcine mucin, (C); 0.06% chlorhexidine, (D); 0.06% chlorhexidine and 0.4% porcine mucin

2.7. Antimicrobial Susceptibility Testing

2.7.1. Inoculation of agar plates for AST

Suspensions equal to 0.5 MF (2.5.1) for each organism were prepared in sterile PBS (2.1.11). A sterile cotton swab (Medical Wire) was placed in the suspension and used to inoculate an agar plate by streaking the plate in three directions, to create a bacterial lawn of semi-confluent growth with no gaps (Fig. 28). The inoculated agar plates were allowed to fully dry at room temperature before antibiotic disks or gradient strips were added onto the surface.

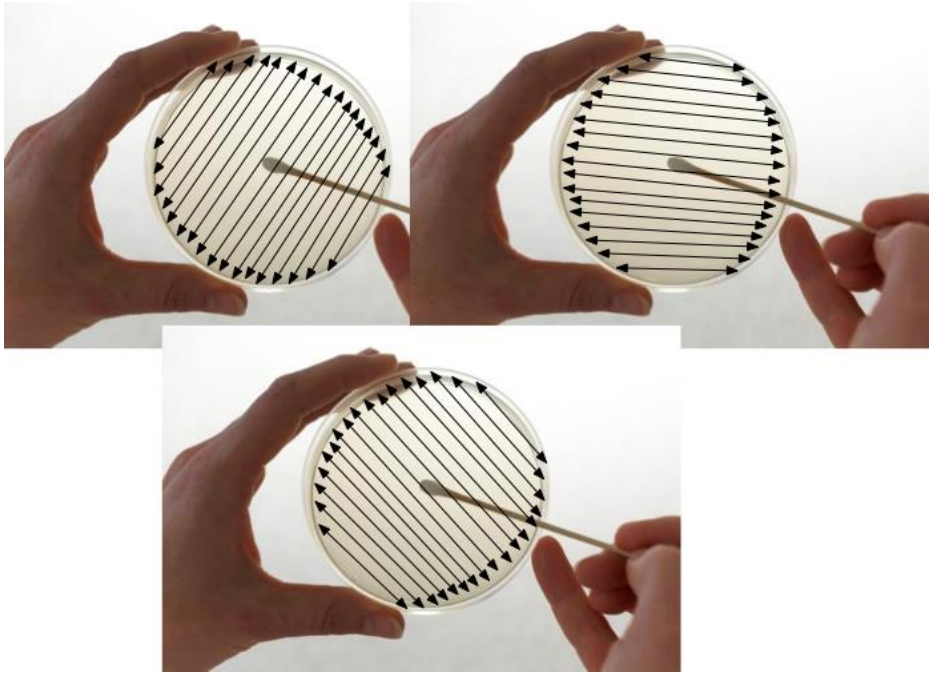


Figure 28. Visual representation of agar plate inoculation for antimicrobial susceptibility testing (image reproduced from LSHTM teaching resources, with permission)

2.7.2. Beta lactamase detection

Gonococcal isolates were tested for β-lactamase production using nitrocefin discs (Oxoid). Nitrocefin disks were placed on the lid of the agar plate used to culture the organism of interest. The disks were moistened with one drop of sterile water and colonies of interest were transferred onto the nitrocefin disks with a sterile loop (Medical Wire). A positive result was interpreted by the development of a dark orange colour within 30 seconds of inoculation (Fig. 29).

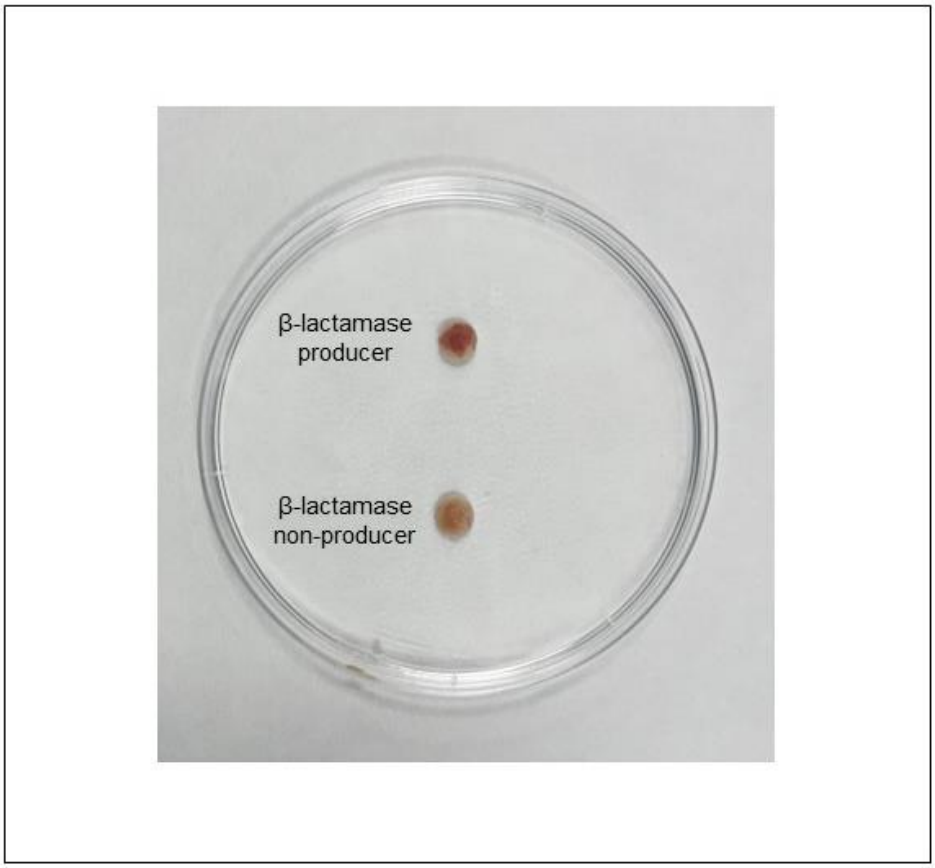


Figure 29. Image of positive and negative β-lactamase nitrocefin test.

2.7.3. Agar dilution minimum inhibitory concentration testing

Minimum inhibitory concentrations were determined using the WHO GASP method[103]. Gonococcal medium base agar was prepared as per 3.2.2 and the respective 10 mL antimicrobial solution was added according to 3.8.1. A 0.5 McFarland suspension of each gonococcal isolate was prepared (2.5.1) and further diluted 10^{-1} in PBS. Each suspension was added to a separate well of a multipoint inoculator template (Fig. 30). A multipoint inoculator (Denley, Colchester, UK) containing 21 pins was used to inoculate 1 µL of each organism from the template onto each plate in the respective antimicrobial agar dilution series (Fig. 30), so that approximately 10^4 cfu of each organism was inoculated, as previously described[103]. A total of 21 isolates were inoculated onto each plate and incubated as per 2.3.2. Gonococcal medium base agar containing no antibiotic was used as a growth control. The MIC of each organism was interpreted as the minimum concentration

to inhibit visible growth of the inoculated spot. The MIC was used to determine the susceptibility of each organism according to WHO breakpoints (Table 18).

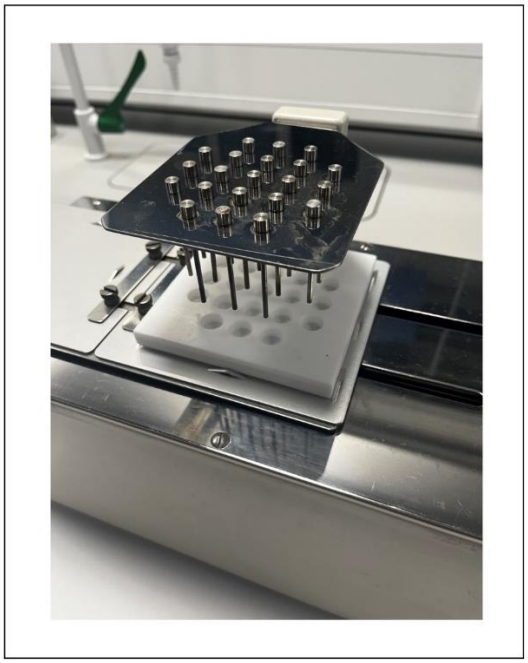


Figure 30. Multipoint inoculator showing the 21 inoculation pins, each transferring 1 μL organism suspension and isolate template. Each isolate suspension tested is transferred to individual wells of the template.

Table 18. *N. gonorrhoeae* susceptibility agar dilution breakpoints for penicillin, ceftriaxone, cefixime, azithromycin, tetracycline, ciprofloxacin and spectinomycin according to CLSI breakpoints.

Antimicrobial	S≤ (mg/L)	I (mg/L)	R≥ (mg/L)
Penicillin	0.06	0.12-1.0	2.0
Ceftriaxone	0.25	n/a	n/a
Cefixime	0.25	n/a	n/a
Azithromycin	n/a	n/a	2.0
Tetracycline	0.25	0.5-1.0	2.0
Ciprofloxacin	0.06	0.12-0.5	1.0
Spectinomycin	32	64	128

S, susceptible; I, intermediate; R, resistant; n/a, not applicable

2.7.4. *Gradient strip minimum inhibitory concentration testing*

Gradient strip MICs were performed according to the WHO GASP method[103] . A 0.5 MF suspension (2.5.1) of each isolate was inoculated as per 2.7.1 onto an antimicrobial-free GCMB agar plate (2.1.2). Sterile forceps were used to place a gradient strip (Biomerieux) of the respective antimicrobial on the surface of the agar and incubated as per 2.3.2. The MIC of the organism was interpreted as the number where the bacterial growth intercepted the gradient strip (Fig. 31). The susceptibility of the organism to each antimicrobial was interpreted as outlined in Table 18.

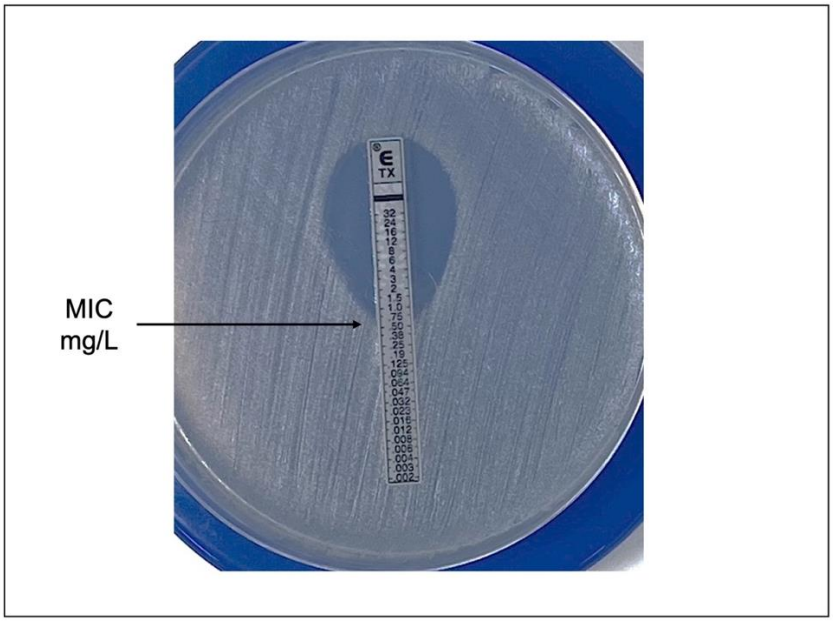


Figure 31. Ceftriaxone gradient strip demonstrating a MIC of 0.5 mg/L

2.7.5. *Microbroth dilution minimum inhibitory concentration testing*

Gonococcal broth was prepared according to 2.1.3 and antimicrobial solutions were prepared as per 2.6.1 and 2.6.3. Working concentrations of the antimicrobials tested were prepared in 50 mL GC broth, by adding an appropriate volume of the antimicrobial stock to GC broth in a Falcon tube (SLS) to create the highest working concentration and mixed thoroughly by vortexing for 10 seconds. The working concentrations of the antimicrobials prepared were two-fold higher than the final concentrations in the microbroth dilution. Two-fold serial dilutions of the antimicrobials were

prepared in 25 mL GC broth so that there was one working stock for each tested concentration. The GC broth containing the lowest antimicrobial concentration was poured into a sterile reservoir (ThermoFisher Scientific) and an 8-well multichannel pipette (Gilson) was used to transfer 50 μL to each well in column 8 of a 96-well flat microtitre plate (ThermoFisher Scientific) (Fig. 32). This was repeated for the remaining working stocks so that the highest concentration was in column 2. A total of 50 μL and 100 μL sterile uninoculated GC broth was also inoculated in columns 1 and 9, to be used as growth and sterility controls respectively.

Bacterial suspensions were prepared according to 2.3.3 (Table 11) so that the inoculated wells contained a final concentration of approximately 5×10^5 cfu/mL. A baseline count of the inoculum was prepared as per 2.4. A P100 pipette (Gilson) was used to transfer 50 μL of each organism to a distinct row of the 96-well flat microtitre plate (Fig. 32), apart from the sterility control. A maximum of eight isolates were tested in each plate (one isolate to each row). The inoculated microtitre plates were incubated as per 2.3.2. After 24 hours, growth was first observed visually by examining the turbidity of the broth in the wells and the MIC recorded. The MIC was interpreted as the lowest concentration of antimicrobial that inhibited visual gonococcal growth (turbidity) within the inoculated wells. Subsequently, 10 μL Deep Blue viability dye (BioLegend, San Diego, California, US) was added to each broth-containing well of the microtitre plate and incubated for 2 hours at 37°C CO_2. The MIC was recorded once more by considering pink wells as positive for viability and blue wells as negative.

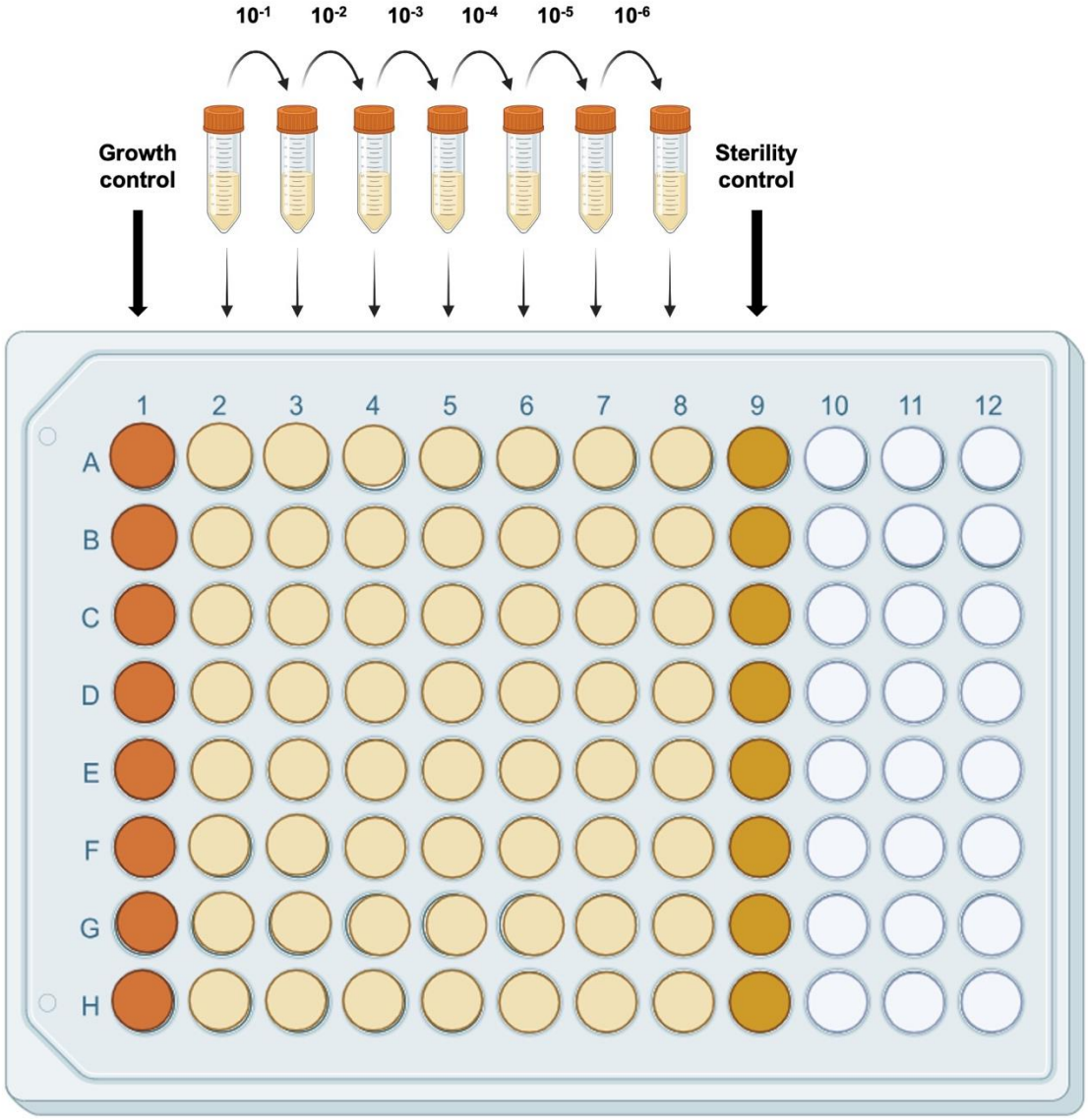


Figure 32. Microbroth dilution MIC template. Serial dilutions of the tested compound were performed in the microtitre plate columns. Each isolate was tested in one row. Image produced in BioRender.com.

2.7.6. Minimal bactericidal concentration testing

Minimum bactericidal concentrations were determined from 2.7.5, specifically from the wells without visible turbidity. Excluding the sterility control, 100 μL of each non-turbid well was transferred to a chocolate agar plate (2.1.1) with a P100 pipette (Gilson), spread on the entire agar surface using a spreader (Medical Wire) and incubated as per 2.3.2 but for 48 hours. After incubation, individual cfu were counted from all inoculated plates.

The MBC was interpreted as the lowest concentration of antimicrobial that reduced the baseline gonococcal cfu by 3-logs or 99.9%[218]. For example, a 3-log reduction of a 5×10^5 baseline inoculum of 99.9% reduction would equate to 50 cfu on the agar plate:

$$3 \log reduction = 0.1 \times (\frac{x \times 10^n}{10^3}),$$

Where 'χ' is the number of cfu counted from the baseline and '*n*' is the reciprocal of the dilution factor counted.

2.7.7. *Checkerboard assay*

Gonococcal broth was prepared according to 2.1.3 and antimicrobial solutions were prepared as per 2.6.1 and 2.6.3. Working concentrations of 32 mg/L for both ceftriaxone and CHX were prepared. Serial dilutions of the antimicrobial compounds were performed as per 2.6.1, to create working concentration ranges of 0.063 – 32 mg/L for ceftriaxone and 0.5 – 32 mg/L for CHX.

The GC broth containing 0.5 mg/L CHX was poured into a sterile reservoir (ThermoFisher) and a multichannel pipette (Gilson) was used to transfer 25 μL to wells B1 to B11 of a flat 96-well microtitre plate (Fig. 33). This was repeated for the remaining working stocks of CHX so that the highest concentration (32 mg/L) was in row H and the lowest (0.5 mg/L) in row B.

The GC broth containing 0.063 mg/L ceftriaxone was poured into a sterile reservoir (ThermoFisher) and a multichannel pipette was used to transfer 25 μL to all wells in column 2. The same was performed for the remaining ceftriaxone concentrations so that the highest concentration (32 mg/L) was in column 11 and the lowest concentration (0.063 mg/L) was in column 2. Sterile GC broth (25 μL) was added to wells B1-H1 and A2-A11, 50 μL was added to well A1 and 100 μL to well H12. Bacterial suspensions were prepared according to 2.3.3 and 50 μL was added to all wells except the sterility control (H12), so that the final concentration was approximately 5×10^5 cfu/mL and incubated as per 2.3.2.

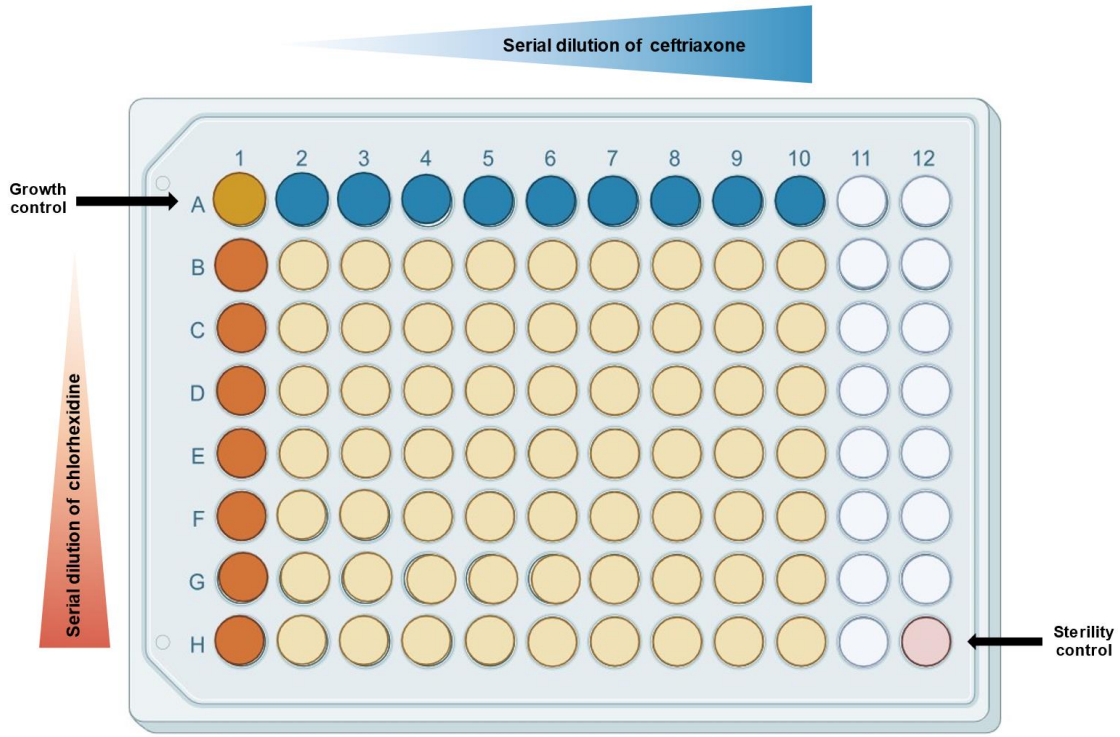


Figure 33. Checkerboard synergy assay template. Wells B2 to H10 contain a unique combination of chlorhexidine and ceftriaxone. Wells A2-A10 and B1-H1 contain serial dilutions of ceftriaxone and chlorhexidine respectively. Wells A1 and H12 are growth and sterility controls respectively. The tested isolate (50 μL) was added to wells A1-H10 to a final concentration of approximately 5 x 10^5 cfu/mL. Image produced in BioRender.com.

After 24 hours the plates were examined initially for visual turbidity. Subsequently, 10 μL Deep Blue (BioLegend) was added to each well and the plates were placed back in the incubator for 2 hours, after which the plates were examined for colour change (blue to pink).

The fractional inhibitory concentration index (FICI) was calculated using the MICs produced by the checkerboard assays using the formula:

$$FICI = \frac{MIC_A\ combination}{MIC_A\ alone} + \frac{MIC_B\ combination}{MIC_B\ alone}$$

The combined effect of the compounds tested was interpreted using the criteria outlined in Table 19.

Table 19. Interpretation criteria for checkerboard assay synergy testing

FICI	Interpretation
≤0.5	Synergistic
>0.5 - <1	Additive
1 - 4	Indifferent
>4	Antagonistic

FICI; Fractional Inhibitory Concentration Index

2.8. Antiseptic efficacy assay

2.8.1. *Sterility testing*

The sterility of the mucin solution (2.1.12) and DE neutralising broth (2.1.13) were determined by spreading 100 μL of each onto a chocolate agar plate (2.1.1) using a spreader (Medical Wire) and incubating as per 2.3.2. The agar plates were inspected visually for bacterial growth.

2.8.2. *Toxicity testing*

A 0.5 MF suspension of a bacterial isolate was prepared as per 2.5.1 and 100 μL was added each to 900 μL DE neutraliser (2.1.13) and 900 μL 0.4% porcine mucin (2.1.12), in a sterile bijou (Sterilin, Cheshire, UK). This was incubated at room temperature for 30 seconds or 2 minutes, after which 100 μL was spread onto a chocolate agar plate (2.1.1) using a plate spreader. The plates were incubated as per 2.3.2. After 24 hours, the plates were examined visually for bacterial growth.

2.8.3. *Neutraliser efficacy testing*

A 0.5 MF suspension of a bacterial isolate was prepared as per 2.5.1. Five bijoux tubes containing 800 μL DE neutraliser and two bijoux containing 800 μL sterile distilled water were used for the neutraliser efficacy testing. A total of 100 μL 0.2% and 0.06% were added to two bijoux each containing the DE neutraliser. This was repeated with the two tubes containing sterile water. Separately, 100 μL sterile water was added to the fifth tube containing DE neutraliser. Finally, 100 μL of the bacterial suspension was added to two tubes containing chlorhexidine and neutraliser, water and neutraliser, and chlorhexidine and water. The tubes were incubated at room temperature

for 30 seconds to neutralise the disinfectant, after which 100 μL from each tube was spread onto a chocolate agar plate (2.1.1) using a plate spreader. The plates were incubated as per 2.3.2. After 24 hours, the plates were examined visually for bacterial growth. The combination of efficacy testing components is summarised in Table 20.

Table 20. Summary of components and controls for the DE disinfectant neutraliser tests.

Container	Component 1	Component 2	Component 3	Purpose
1	800 μL DE	100 μL 0.2% CHX	100 μL H_2O	Sterility of components
2	800 μL DE	100 μL 0.06% CHX	100 μL H_2O	Sterility of components
3	800 μL DE	100 μL 0.2% CHX	100 μL 0.5 MF	Neutraliser testing
4	800 μL DE	100 μL 0.06% CHX	100 μL 0.5 MF	Neutraliser testing
5	800 μL DE	100 μL H_2O	100 μL 0.5 MF	Growth control
6	800 μL H_2O	100 μL 0.2% CHX	100 μL 0.5 MF	Disinfectant efficacy
7	800 μL H_2O	100 μL 0.06% CHX	100 μL 0.5 MF	Disinfectant efficacy

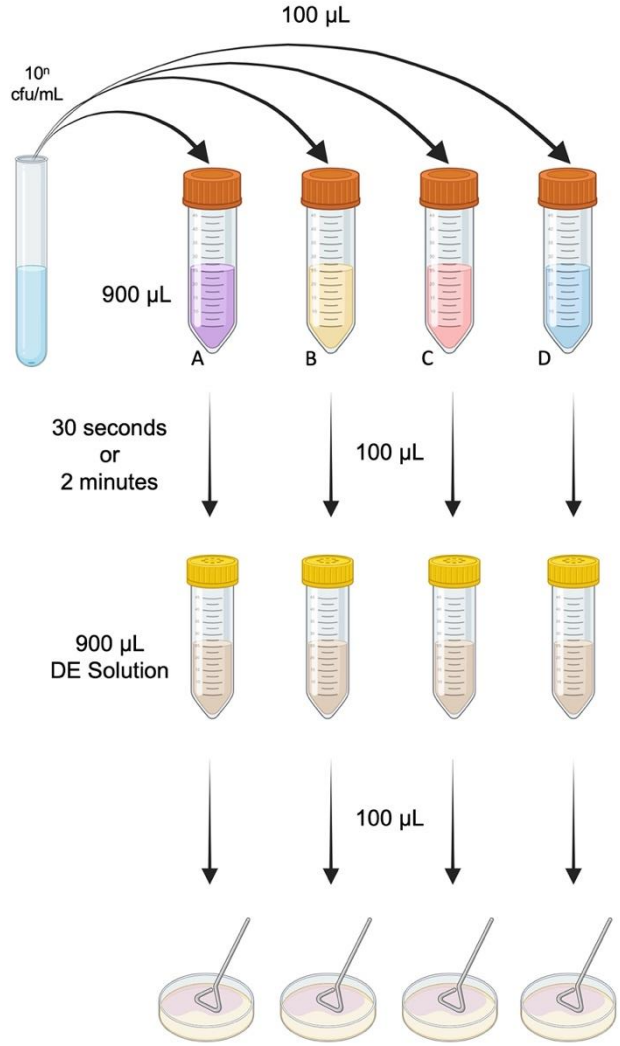
DE; Dey-Engley, CHX; chlorhexidine, MF; McFarland,

2.8.4. *Timed kill assay*

The time-kill assay procedure was performed using a modified suspension test as described by Kawamura-Kato[219], based on the European standard EN 1040[220]. The bactericidal activity of chlorhexidine was tested at two concentrations, 0.2% (2,000 mg/L; 2.6.3) and 0.06% (600 mg/L) and 0.4% porcine mucin (2.1.12) was used as the organic material. A 0.5 MF suspension of a bacterial isolate (2.5.1) was serially diluted as per 2.4. From the dilutions containing 10^5 – 10^7 cfu/mL, 100 μL was transferred to two bijoux each containing:

- 900 μL 0.2% chlorhexidine, or
- 900 μL 0.4% chlorhexidine and 0.8% mucin at a 1:1 ratio, or
- 900 μL 0.06% chlorhexidine, or
- 900 μL 0.12% chlorhexidine and 0.8% mucin at a 1:1 ratio.

Each of the above tested will be referred to as a '*set*'. Each set tested was incubated for either 30 seconds or two minutes, after which 100 μL was transferred to another bijoux containing 900 μL DE neutraliser (2.1.13) and mixed thoroughly by vortex mixing. The mixtures were incubated at room temperature for 30 seconds to neutralise the disinfectant, after which 100 μL was spread onto a chocolate agar plate (2.1.1) (Fig. 34) and incubated as per 2.3.2, but for 48 hours. Each isolate was tested in triplicate. Baseline counts of the initial inoculum were performed as described previously (2.4).



A = 0.2% CHX

B = 0.4% CHX + 0.8% mucin (1:1)

C = 0.06% CHX

D = 0.12% CHX +0.8% mucin (1:1)

Contact time of either 30 seconds or 2 minutes.

Solutions were neutralised for 30 seconds.

Inoculated on agar and incubated.

Figure 34. Timed kill studies protocol. Variables tested were organism concentration, chlorhexidine concentration, presence of porcine mucin and contact time. Image created in BioRender.com. CHX; chlorhexidine

2.9. Nucleic acid extraction

2.9.1. *Archive Pure kit method*

Genomic material was extracted with the Archive Pure kit (5PRIME, Dusseldorf, Germany) following the protocol for gram-negative bacteria. All reagents except isopropanol and ethanol were provided in the 5PRIME kit.

A 1 MF bacterial suspension as per section 2.5.1 was prepared and 1 mL was transferred to a sterile 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany). This was centrifuged at 15,000 x*g* for five seconds. The supernatant was removed using a P200 pipette (Gilson) and discarded, the pellet was re-suspended with 300 μL Lysis Buffer and incubated at 80°C for five minutes, in a heated block (DLAB, Beijing, China). Subsequently, 1.5 μL RNAse A was added to the solution, mixed by inverting 25 times and incubated at 37°C for 30 minutes in a water bath (Grant). The suspensions were cooled on ice for one minute, and then 100 μL protein precipitation solution was added. This was vortexed vigorously for 25 seconds and centrifuged at 15,000 x*g* for three minutes. The supernatant was transferred to a fresh 1.5 mL microcentrifuge tube (Eppendorf) containing 300 μL isopropanol (VWR). The mixture was inverted 50 times and centrifuged at 15,000 x*g* for one minute. The supernatant was removed with a P200 pipette, discarded, and 300 μL 70% ethanol (VWR) was added. The mixture was further centrifuged for one minute at 15,000 x*g* and the supernatant discarded. The microcentrifuge tubes were incubated at room temperature with the lids open until the ethanol fully evaporated, after which the DNA pellet was re-suspended in 50 μL nuclease-free water (VWR) and rehydrated for one hour at 65°C. The extracts were stored at -20°C until further use.

2.9.2. *Invitrogen DNA mini kit method*

Nucleic acid extraction was performed using Invitrogen DNA mini kit (ThermoFisher Scientific) according to the protocol for Gram negative bacteria. Briefly, 1 mL of a 4 MF suspension (2.5.1) of each isolate was prepared and transferred to a 1.5 mL microcentrifuge tube. The suspension was centrifuging at 10,000 *x*g to pellet the cells. The supernatant was removed with a P200 pipette, the pellet was resuspended in 180 μL Genomic Digestion buffer and 20 μL Proteinase K and mixed

by vortexing for 10 seconds. The mixture was incubated at 55°C for two hours. The mixture was then snap cooled on ice and 20 μL RNAse A was added, mixed by vortexing, and incubated at room temperature for two minutes. Subsequently 200 μL Genomic Lysis/Binding Buffer was added and mixed by vortexing, after which 200 μL ethanol was added and vortexed for five seconds. The resulting lysate was added to a spin column and centrifuged at 10,000 x*g* for one minute, and the supernatant discarded with a P1000 pipette. A total of 500 μL Wash Buffer 1 was added and centrifuged at 10,000 x*g* for one minute and the supernatant discarded as previously, after which 500 μL of Wash Buffer 2 was added and centrifuged at maximum speed for three minutes. The supernatant was finally discarded. Subsequently, 25 μL nuclease free water (VWR) was added, and incubated for one minute at room temperature, before being centrifuged at maximum speed for one minute, and then repeated with another 25 μL of elution buffer. The eluant containing the purified DNA was stored at -20°C.

2.10. *NG*-MAST Typing

2.10.1. Preparation of dNTP mix

The dNTP mix was prepared as per the manufacturer's instructions. Each dNTP (A, T, C, G) (ThermoFisher) was provided in separate vials, each containing 100mM of the respective dNTP. A combined dNTP mix was prepared by adding 10 μL of each dNTP to 460 μL of nuclease-free water (VWR). The mix was divided into 50 μL aliquots and stored at -20°C until further use.

2.10.2. Preparation of primer stocks

The primers were purchased by Eurofins Genomics (Eurofins, Ebesberg, Germany) as lyophilised pellets. A 100 μM stock of each primer was prepared by adding an appropriate volume of nuclease-free water to the lyophilised pellet (Table 21) and mixing thoroughly by vortexing. A 10 μM working stock was further created by performing a 10-fold dilution of the primer stock and vortexing thoroughly. The working stock was divided into 50 μL aliquots and stored at -20°C until further use.

Table 21. Preparation of primer stocks from purchased lyophilised pellets.

Primer	Yield (nmol)	Volume of nuclease-free water for 100 mM (μL)
***porB* Forward**	39.2	392
***porB* Reverse**	42.5	425
***tbpB* Forward**	23.3	233
***tbpB* Reverse**	30.1	301

2.10.3. PCR Amplification

NG-MAST was performed by sequencing the gonococcal outer membrane porin (*porB*) and the β subunit of the transferrin-binding protein (*tbpB*) genes, as described previously[221] (Table 22). Each PCR reaction was performed in a 50 μL volume in a 0.2 mL reaction tube (Eppendorf) and consisted of 5 μL 10X DreamTaq buffer, 5 μL dNTP mix containing 0.2 μM of each nucleotide, 1.25U DreamTaq DNA polymerase (all ThermoFisher Scientific, US), 0.2 μM of respective forward and reverse primers (Eurofins) (Table 23), 1 μL of DNA extract or nuclease-free water and 36.75 μL of nuclease-free water (Sigma-Aldrich). One negative control (nuclease-free water) was included in each PCR run. If more than one reaction was performed in the same cycle run, a PCR mastermix was performed by multiplying the volumes needed per reaction by the number of reactions, plus two more:

$$MMX\ Volume\ per\ reagent = Volume\ per\ individual\ reaction\ \times\ (n\ +2),$$

Where 'MMX' is the PCR mastermix and 'n' is the number of PCR reactions performed.

Table 22. Forward and reverse primers for the *porB* and *tbpB* polymerase chain reactions.

Primer name	Sequence
porB forward	5′-^{350}CAA-GAA-GAC-CTC-GGC-AA^{366}-3’
porB reverse	5′-^{1086}CCG-ACA-ACC-ACT-TGG-T^{1071}-3′
tbpB forward	5′-^{1098}CGT-TGT-CGG-CAG-CGC-GAA-AAC^{1118}-3′
tbpB reverse	5′-^{1686}TTC-ATC-GGT-GCG-CTC-GCC-TTG^{1666}-3′

Table 23. Components, volumes and final concentrations of the *NG*-MAST PCR mix.

Component	Volume added (μL)	Final concentration
10X DreamTaq Buffer	5	1X
2 mM dNTP mix	5	0.2 mM
5 U/μL DreamTaq DNA Polymerase	0.25	1.25 U
10 μM Forward Primer	1	0.2 μM
10 μM Reverse Primer	1	0.2 μM
DNA Extract	1	n/a
Nuclease Free Water	36.75	n/a
Final Volume	50	n/a

The PCR amplification was adapted from Martin *et al*[221] with the following modifications: the cycling conditions for the *porB* PCR were as follows: denaturation step at 95°C for four minutes followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, elongation at 78°C for one minute, and a final one-minute extension step at 72°C. The *tbpB* cycle was the same except for an annealing temperature of 60°C.

2.10.4. Agarose gel electrophoresis

PCR products were visualised on 1.5% agarose gel, prepared as per 2.1.6. The molten agarose was poured into an electrophoresis tank (Life Technologies, Carlsbad, California, US) and a comb with 14 wells was placed in the gel. This was left at room temperature until solidified. The electrophoresis tank was flooded with 1X TBE (2.1.6) until the anode and cathode were submerged

after which the comb was carefully removed. Each amplicon or negative control was mixed with loading dye in a 1:5 ratio by adding 1 μL of amplicon to 5 μL loading dye (Bioline, London, UK) on a piece of parafilm (Bemis, Neenah, Wisconsin, US) and mixed carefully by pipetting up and down. A total of 5 μL mixed amplicon was added to each well. For each gel, 5 μL of 1Kb HypperLadder™ (Bioline) was added to one well (Figure 35). Gels were run at 100V for 60 minutes and the amplicons were visualized with the GeneGenius imaging system (Syngene, Cambridge, UK).

Figure 35. Agarose gel loading template. Each gel contained 14 wells, one well each for the ladder and negative control and remaining wells were loaded with sample amplicon.

2.10.5. PCR clean-up

PCR amplicons were transferred to individual wells of a PCR reaction plate (Applied Biosystems, Waltham, Massachusetts, US). To each well, 30 μL of a 20% polyethylene glycol (PEG) / 2.5M NaCl solution was added (provided by Dr Richard Stabler). Plates were subsequently centrifuged at 2750 *x* g for one hour at 4°C, after which the PEG-NaCl supernatant was removed with a P200 pipette. The DNA pellets were washed by adding 150 μL 70% ethanol (VWR) and centrifuged at 2750 *x* g for 10 minutes. The ethanol was removed with a P200 pipette (Gilson), and the plates were air-dried at room temperature for 30 minutes. Finally, the DNA pellets were re-suspended by adding 20 μL nuclease-free water (VWR) and stored at 4°C.

2.10.6. Sequencing porB and tbpB fragments

Each sequencing reaction mix was made up to 20 μL in 200 μL reaction plates, consisting of 4 μL of the respective forward or reverse primer (Table 22) at a final concentration of 0.2 μM, 1 μL of DNA from 2.10.5, 8 μL of ABI Prism Terminator Ready Reaction Mix (Applied Biosystems), and 7 μL of nuclease-free water (VWR). Two sequencing reactions were prepared per amplicon, each containing either the forward or reverse primer for the gene of interest. The sequencing cycling conditions were as follows: 96°C for 10 seconds, 50°C for five seconds, and 60°C for two minutes, for a total of 25 cycles.

The amplified products were precipitated by adding 3 μL 3M sodium acetate, 62.5 μL 100% ethanol (VWR) and 24.5 μL nuclease-free water. The plate was covered with sticky foil (Applied Biosystems), vortex mixed briefly and placed on ice for 20 minutes. The plates were then centrifuged at 3000 *x* g for one hour at 4°C, after which the foil was removed, and the plates were inverted on blue roll and allowed to drain. The plate was then centrifuged upside down at 50 *x* g for 50 seconds, after which 50 μL ice-cold 70% ethanol was added. The plate was covered with fresh foil, inverted five times, and centrifuged 3000 *x* g at 4°C for 10 minutes. The plate was inverted again to drain as described above. Then 10.5 μL of Hi-Di formamide (Applied Biosystems) was added to each well and left overnight at 4°C. Sequences were produced by the ABI 3730 DNA analyser (Applied Biosystems). The chromatograms were viewed using BioEdit (Informer Technologies, Los Angeles, California, US) software and exported as text files. Each *porB* and *tbpB* sequence was manually trimmed to 490 base pairs starting from the conserved TTGAA sequence and 390 base pairs starting from the conserved CGTCTGAA sequence respectively. Trimmed *porB* and *tbpB* sequences were input into the *NG*-MAST database (www.ngmast.net) to determine the respective alleles and ST.

2.11. Statistical Analyses

Data management and descriptive statistics were performed in Microsoft Excel (Microsoft, Redmond, Washington, United States). Statistical analyses for significance were performed with Stata 18 (StataCorp LLC, College Station, Texas, US) (chapters 3 and 5), or the Social Science Statistics Calculator (https://www.socscistatistics.com/) (chapters 4 and 6). For ordinal and continuous datasets, the normality of data was assessed using the Kolmogorov-Smirnov test; if the test produces a p-value <0.05 the data is not normally distributed. For datasets that were distributed normally, a parametric test was used, and a non-parametric test was used for non-normally distributed data. Statistical significance was set at $p < 0.05$.

The following statistical tests were used in this thesis:

Random effects models. A common test used in meta-analyses, when the values tested across studies cannot be assumed to be functionally equivalent. (3.7)

Kruskall-Wallis test. A test used for comparing two or more *independent* groups, with numerical datasets. This test is the non-parametric equivalent of the ANOVA test. (5.9, 6.9.2)

Mann-Whitney U test. A test used to compare the distribution of variables between two *independent* groups, when the values are numerical. It is a non-parametric equivalent of the Student's t-test. (6.7.3, 6.9.1)

Fisher's exact test. A test used to determine if there is a difference between the proportions of the categories in two nominal group variables. It is an alternative to the chi-square test that can be used on small sample sizes. (6.9.3)

Paired t-test. A test that compares the means of variables from two *dependent* groups, for example, samples that have been tested by two different laboratory tests. (6.7.3, 6.9.1)

Wilcoxon rank-sum test. A non-parametric equivalent of the paired t-test, used to compare variables from two *dependent* groups. (6.9.4)

McNemar test. A test used to compare *paired* nominal data, for example, the presence or absence of Nc before and after a gargle. (6.9.4)

Spearman's rank correlation test. A test used to test the correlation between two variables, for example the relationship between MICs of different antimicrobials. It is a non-parametric equivalent of the Pearson test (6.8).

Where averaged data are plotted, standard deviation (SD) was used to show variability in a population with single measurements (for example total microbiota counts), whereas standard error (SE) was used when there were repeated measurements for each sample (samples tested in replicates).

2.12. Overview of methods used

The following table (Table 24) summarises the key methods used in each results chapter.

Table 24. Summary of key methods used in each results chapter.

Method	Chapter/s	Section/s
Bacterial colony counts	6	6.7.1, 6.9.1, 6.9.3
Processing of pharyngeal swabs	5, 6	5.9, 6.9.4
Oxidase test	4, 5	4.7, 5.9
Gram stain	4, 5	4.7, 5.9
API NH	4	4.7
Rapid NH	4	4.7
MALDI-ToF	5	5.9
β-lactamase testing	4, 5	4.7
Agar dilution	4, 5, 6	4.7, 5.9, 6.9.1, 6.9.2
Gradient strip	4, 5	4.7, 5.9
Microbroth dilution	6	6.7.2, 6.7.3, 6.9.1
MBC	6	6.9.1
Checkerboard assay	6	6.9.5
Antiseptic efficacy assay	6	6.9.3
Nucleic acid extraction	4, 5	4.7, 5.9
PCR	4	4.7
NG-MAST	4	4.7

CHAPTER 3

SYSTEMATIC REVIEW OF EXTENDED-SPECTRUM CEPHALOSPORIN *NEISSERIA GONORRHOEAE* TREATMENT FAILURES

3.1. Introduction

The treatment of pharyngeal gonorrhoea is challenging, with TFs having occurred in infections with phenotypically susceptible strains, mainly due to pharmacokinetic limitations of first-line antimicrobials[11]. This is more evident in patients with multisite infection who have had TFs at the pharyngeal site but not extrapharyngeal sites, despite being infected by the same strain in all anatomical sites (Table 7, 1.5.2). Treatment failures have occurred with different ESC treatment regimens such as cefixime and ceftriaxone and different dosages. Furthermore, in reports of TFs, antimicrobial susceptibility has been determined by both agar dilution and gradient strip methods, however, research has shown conflicting results on the accuracy of gradient strips compared to agar dilution. For example, one study showed that ceftriaxone E-test had >90% agreement with agar dilution[105], whereas a different study reported that >20% of E-test MIC values were above those generated by agar dilution[222].

Considering the high discrepancy in the efficacy of ESCs in different anatomical sites, comparing the MICs between pharyngeal and extrapharyngeal site TFs may inform the revision of clinical breakpoints for pharyngeal infection. For other bacteria such as *Escherichia coli* and *Staphylococcus aureus,* there are different clinical breakpoints for different infection sites, for example, the co-amoxiclav clinical breakpoint for urinary tract infections (UTIs) caused by *E. coli* is 32 mg/L, whereas for infections other than UTIs it is 8 mg/L[102]. Further, there is a need for a standardised and globally acceptable method of reporting TFs, but this cannot be achieved without reviewing reported failures to date. This systematic review aims to fill some of the above knowledge gaps regarding treatment failures and provide a comparison of pharyngeal and extrapharyngeal infections using reported.

This research is published in Sexually Transmitted Diseases (doi: 10.1097/OLQ.0000000000002116) and the manuscript can be found in Appendix E1.

3.2. Research questions

Research Question 1. Are MIC values of pharyngeal isolates from ESC TFs different to extrapharyngeal isolates?

Research Question 2. What is the difference in MIC of isolates tested with agar dilution compared to gradient strip?

3.3. Hypothesis

Overall, gonococcal isolates from pharyngeal TFs have lower MICs than extra-pharyngeal, and it is potentially useful to review and standardise resistance breakpoints.

3.4. Aims

This systematic review aims to summarise the published ESC TFs of *Ng* and the associated MIC values. The review also compares the ESC TF MIC values between pharyngeal and extra-pharyngeal isolates.

3.5. Objectives

i. Identify sources from the available literature that describe treatment failures of ESCs.
ii. Systematically review the literature according to inclusion criteria
iii. Summarise reported treatment failures according to sample type, geographical region, patient sex, age and sexual orientation
iv. Compare cefixime and ceftriaxone MIC values of pharyngeal and extrapharyngeal isolates, including sub-group analyses by dosage and secondary antimicrobial treatment, using random effects models.
v. Compare cefixime and ceftriaxone MIC values of isolates tested by agar dilution and gradient strip through sub-group analyses by dosage, using random effects models.

3.6. Ethical considerations

Ethical approval was granted by the London School of Hygiene & Tropical Medicine (LSHTM) Research Ethics Committee. Approval was granted on 02/06/2020 (Ref: 17126) (Appendix A1).

3.7. Systematic review methodology

3.7.1. Search strategy and selection criteria

I used PRISMA (preferred reporting items for systematic reviews and meta-analyses) guidelines and registered the study with PROSPERO ID: CRD42020189101 (Appendix A2). EMBASE and PubMed databases were searched as well as the Eurosurveillance journal, on 17 June 2023. Search terms were selected to account for differences in spellings and treatment regimens. Title and free-text terms included, “treatment failure,” “gonorrh*,” “cephalosporin,” “cefixime,” “cefotaxime,” and “ceftriaxone.” I imported records from PubMed, EMBASE and Eurosurveillance into EndNote 20. I then examined potential data sources first by title and abstract, and then full text to establish eligibility. Studies were included if they reported the: (1) gonococcal infection was confirmed by culture or NAAT; (2) treatment was administered with ESC at first presentation; (3) MIC of an initial isolate; (4) patient returned for a TOC and had continuing clinical symptoms, positive gonococcal NAAT or gonococcal culture; (5) patient reported no sexual activity between initial treatment and TOC; and (6) MICs of first and second isolates had no more than one dilution factor difference. These definitions adhered to guidelines from the UKHSA and ECDC for probable or confirmed treatment failures (1.5.2).

A co-author (Jonna Mosoff) extracted data points from studies that met inclusion criteria (Appendix A3) which were then reviewed independently by myself. Other variables of interest included: (i) initial treatment or combination of treatments, (ii) dosage of third-generation cephalosporin, (iii) demographic information on treatment failure cases, (iv) the MIC reported, and (v) the geographic region of cases. I extracted information about gender and sexual orientation as reported and noted the method of MIC testing used.

3.7.2. Statistical analysis

Individual treatment failure cases were initially recorded in Excel (Microsoft, Redmond, Washington, US). To account for the non-linear nature of MIC data, a logarithmic transformation of each MIC value was applied to calculate the mean and standard error for each study. I used Stata 18 (StataCorp LLC) to pool data by applying random-effects restricted maximum likelihood (REML) models to account for heterogeneity between studies. I produced forest plots organised by anatomical site of sample collection, dosage and study publication year. I then conducted sub-group analyses based on binary anatomical site of infection, initial treatment, and dosages. I performed separate sub-group analyses comparing log-transformed MICs by antimicrobial treatment and MIC testing methods. If studies reported multiple failures at different anatomical sites and/or after different treatment regimens, I included them in the corresponding group analysis as individual data points.

3.7.3. Study quality and bias assessment

The Joanna Briggs Institute case report (Appendix A4), cross-sectional and cohort study critical appraisal checklists were used to assess data quality based on completeness of clinical history, demographic detail, and treatment description[223]. The Cochrane Review Manager 5.4 was used to summarise quality and risk of bias.

3.8. Results

3.8.1. Study selection and characteristics

A total of 23 eligible studies were identified by systematic review (Fig. 36). Most data points were case reports of 69 treatment-failure cases (cefixime n=33, ceftriaxone n=36) involving 71 sites [37 (52%) pharyngeal, 22 (31%) urogenital and 12 (17%) rectal]. Of cases with demographic data, 85.5% (59/69) were among men; of reports describing failed treatment after cefixime administration, eight had sufficient data for meta-analysis. Overall, 40.6% of treatment failures were from the Americas region (n=28) followed by the Western Pacific region with 37.7% (n=26). Full details of studies included are in Tables 25 and 26.

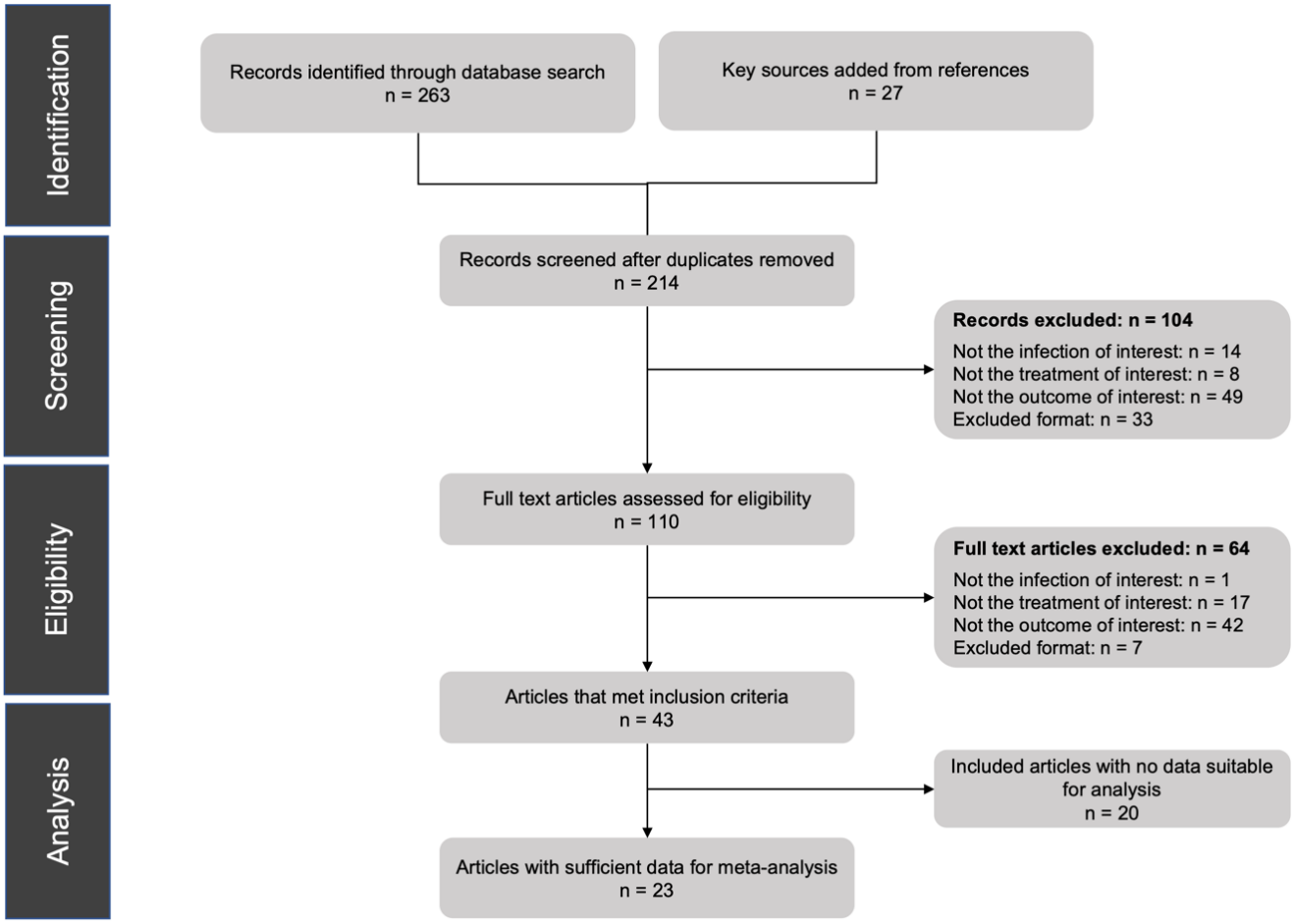


Figure 36. PRISMA flowchart

Table 25. List of studies describing cefixime treatment failures.

Authors	Year	Study Region	Country	Sample size	Age group	Sex	Sexual Orientation	Anatomical site	First treatment	Successful Treatment	MIC CFM (mg/L)	MIC Method	Dual therapy susceptibility*	Other STI
Yokoi S, et al[154]	2007	Western Pacific	Japan	2	16-64	M	NS	Urethral	200 mg CFM	1g CRO	0.5	Agar dilution	n/a	None
				2	16-64	M	NS	Urethral	200 mg CFM	1g CRO	1	Agar dilution	n/a	None
Unemo M, *et al.*[155]	2010	Europe	Norway	2	25-44	M	Heterosexual	Urethral	400 mg CFM	500 mg CRO	0.5	Gradient Strip	n/a	NS
Unemo M, *et al.*[130]	2011	Europe	Austria	1	NS	M	MSM	Urethral	400 mg CFM	1g AZI	1	Gradient Strip	n/a	Chlamydia
Forsyth S, *et al*.[224]	2011	Europe	UK	1	25-44	M	MSM	Urethral	400mg CFM plus 1 g AZI	500 mg CRO	>0.25	Gradient Strip	S	Suspected Chlamydia
Unemo M and Sednaoui P *et al*.[135]	2012	Europe	France	1	45-64	M	MSM	Urethral	200 mg CFM	160 mg GEN	4	Gradient Strip	n/a	NS
Allen VG, *et al*.[71]	2013	Americas	Canada	1	25-44	M	NS	Urethral	400 mg CFM plus	250 mg CRO	0.12	Agar Dilution	R	Suspected Chlamydia

						100mg DOX				
	1	25-44	M	NS	Urethral	400 mg CFM plus 100mg DOX	800 mg CFM	0.12	Agar Dilution	I	Suspected Chlamydia
	1	16-24	M	NS	Urethral	400 mg CFM plus 100mg DOX	800 mg CFM	0.12	Agar Dilution	R	Suspected Chlamydia
	1	25-44	M	NS	Urethral	400 mg CFM plus 100mg DOX	250mg CRO	0.06	Agar Dilution	R	Suspected Chlamydia
	1	25-44	M	NS	Rectal	400 mg CFM	250mg CRO	0.12	Agar Dilution	n/a	None
	1	25-44	M	NS	Rectal	800 mg CFM	250mg CRO	0.12	Agar Dilution	n/a	Suspected Chlamydia
	1	45-64	M	NS	Rectal	400 mg CFM	250mg CRO	<0.03	Agar Dilution	n/a	None
	1	25-44	F	NS	Pharyngeal	400 mg CFM	800 mg CFM	0.12	Agar Dilution	n/a	None

				1	16-24	M	NS	Pharyngeal	800 mg CFM plus 1g AZI	250 mg CRO	0.12	Agar Dilution	S	Suspected Chlamydia
Singh AE, *et al*.[225]	2015	Americas	Canada	1	25-44	M	MSM	Pharyngeal	400 mg CFM	125 mg CRO	0.03	Agar Dilution	n/a	NS
				1	16-24	M	MSM	Rectal	400 mg CFM	250 mg CRO	0.12	Agar Dilution	n/a	NS
				4	16-24	M	MSM	Pharyngeal	400 mg CFM	250 mg CRO	0.03	Agar Dilution	n/a	NS
				1	25-44	M	MSM	Rectal	400 mg CFM	125 mg CRO	0.03	Agar Dilution	n/a	NS
				1	16-24	M	MSM	Pharyngeal	400 mg CFM	125 mg CRO	0.03	Agar Dilution	n/a	NS
				1	25-44	M	MSM	Rectal	400 mg CFM	250 mg CRO	0.03	Agar Dilution	n/a	NS
				1	0-15	F	Heterosexual	Rectal	400 mg CFM	400 mg CFM	0.03	Agar Dilution	n/a	NS
				1	16-24	M	MSM	Pharyngeal	400 mg CFM	250 mg CRO	0.016	Agar Dilution	n/a	NS
				1	25-44	F	Heterosexual	Pharyngeal	400 mg CFM	250 mg CRO	0.008	Agar Dilution	n/a	NS

				1	45-64	M	MSM	Urethral	400 mg CFM	200 mg CRO plus 1 g AZI	0.016	Agar Dilution	n/a	NS
				1	25-44	F	Heterosexual	Pharyngeal	800 mg CFM	250 mg CRO	0.016	Agar Dilution	n/a	NS
Day M, *et al*.[151]	2022	Europe	UK	1	45-64	M	Heterosexual	Urethra and pharynx	CFM and AZI (dose unknown)	CRO 1g	1	Gradient Strip	R	NS

Studies that met inclusion criteria and had sufficient information for analysis are included.

Each row represents one study or case report, if multiple instances of treatment failure were reported, the number is listed under sample size

Regions are WHO defined

MIC: minimum inhibitory concentration mg/L, MSM: men who have sex with men. n/a: nor applicable, NS: not specified

CFM cefixime; CRO ceftriaxone; AZI azithromycin; SPT spectinomycin; ETP ertapenem; DOX doxycycline, GEN; gentamicin.

****Dual therapy indicates that the first treatment included multiple antimicrobials, the susceptibility of the non-cephalosporin is listed in this column: R resistant; I intermediate; S susceptible***

Table 26. List of studies describing ceftriaxone treatment failures.

Authors	Year	Study Region	Country	Sample size	Age Group	Sex	Sexual Orientation	Anatomical site*	First treatment	Successful Treatment	MIC CRO (mg/L)	MIC Method	Dual therapy susceptibility**	Other STI
Tapsall J, et al.[157]	2009	Western Pacific	Sydney	1	25-44	Male	MSM	Urethral and **pharyngeal**	250 mg CRO	500 mg CRO	0.03	Agar Dilution	n/a	Chlamydia
				1	25-44	Female	Heterosexual	Urogenital and **pharyngeal**	250 mg CRO	1 g CRO	0.016	Agar Dilution	n/a	None
Ohnishi M, *et al*.[133]	2011	Western Pacific	Japan	1	25-44	Female	Unknown	Pharyngeal	1g CRO	1g CRO	2	Agar Dilution	n/a	None
Unemo M *et al*.[158]	2012	Europe	Slovenia	1	25-44	Female	Bisexual	Pharyngeal	250 mg CRO	250 mg CRO and 1 g AZI	0.125	Gradient Strip	n/a	Confirmed Chlamydia
Hustig A, *et al.*[159]	2013	Western Pacific	Australia	5	NS	Male	MSM	Pharyngeal	250 mg CRO	250 mg CRO	<0.03	Agar Dilution	n/a	None
				1	NS	Male	MSM	Pharyngeal	250 mg CRO and 1g AZI	250 mg CRO	<0.03	Agar Dilution	NS	Confirmed Chlamydia
Chen, MY, *et al*.[160]	2013	Western Pacific	Australia	1	NS	Male	MSM	Rectal and **pharyngeal**	500 mg CRO	2g AZI	0.03-0.06	Agar Dilution	n/a	NS

											and E-test		
Read PJ, *et al*.[167]	2013	Western Pacific	Australia	1	25-44	Male	MSM	Urethral and **pharyngeal**	500mg CRO	1g CRO and 2 g AZI	0.03	Agar Dilution	n/a	None
				1	25-44	Male	MSM	**Pharyngeal** and rectal	500mg CRO	1g CRO	0.03	Agar Dilution	n/a	None
Golparian D, *et al.*[226]	2014	Europe	Sweden	1	25-44	Female	Heterosexual	**Pharyngeal** and urogenital	500 mg CRO	1g CRO	0.125	Gradient Strip	n/a	NS
				1	45-64	Male	Heterosexual	**Pharyngeal** and urogenital	500 mg CRO	1g CRO	0.064	Gradient Strip	n/a	NS
				1	45-64	Female	Heterosexual	**Pharyngeal** and urogenital	500 mg CRO	1g CRO	0.064	Gradient Strip	n/a	NS
Okah E, *et al*. §[163]	2018	Americas	USA	1	NS	Male	MSM	Urethral	250mg CRO & 1g AZI	NS	0.003	NS, presumed Gradient Strip	S	NS
				1	NS	Male	MSM	Urethral	250mg CRO & 1g AZI	500mg CRO & 2g AZI	0.012	NS, presumed	S	NS

				1	NS	Male	MSM	Urethral	250mg CRO & 1g AZI	NS	0.023	Gradient Strip NS, presumed Gradient Strip	S	NS
				1	NS	Male	MSM	Urethral	250mg CRO & 1g AZI	NS	0.047	Gradient Strip NS, presumed Gradient Strip	S	NS
Eyre DW, *et al*.[166]	2018	Europe	UK	1	NS	Male	Heterosexual	Urethral and **pharyngeal**	1g CRO and 100mg DOX	1g ETP	0.5	Gradient Strip	R	None
Poncin T, *et al.*[164]	2018	Europe	France	1	16-24	Female	Heterosexual	Urogenital and **pharyngeal**	250mg CRO and 100mg DOX	Loss to follow up	0.5	Gradient Strip	R	None
Smyczek P, *et al*.[165]	2019	Americas	Canada	1	25-44	Male	Heterosexual	Urethral	250mg CRO & 1g AZI	250mg CRO & 1g AZI	0.5	Agar Dilution	S	None
Eyre DW, *et al*.[143]	2019	Europe	UK	1	NS	Female	Heterosexual	**Urogenital** and **rectal**	1g CRO	1g ERT	1	Gradient Strip	n/a	NS

Fifer H, *et al.*[141]	2016	Europe	UK	1	NS	Male	Heterosexual	Urethral and **pharyngeal**	500mg CRO and 1g AZI	1g CRO and 2g AZI	0.25	Gradient Strip	R	NS
Bissessor M, *et al.*[161]	2015	Western Pacific	Australia	1	NS	Male	MSM	Rectal	500mg CRO plus 1g AZI	NS	<0.008	Agar Dilution	S	NS
				3	NS	Male	MSM	Rectal	500mg CRO plus 1g AZI	NS	0.03	Agar Dilution	S	NS
				1	NS	Male	MSM	Rectal	500mg CRO plus 1g AZI	NS	0.06	Agar Dilution	S	NS
				2	NS	Male	MSM	Pharyngeal	500mg CRO plus 1g AZI	NS	0.06	Agar Dilution	S	NS
				1	NS	Male	MSM	Pharyngeal	500mg CRO plus 1g AZI	NS	<0.008	Agar Dilution	S	NS
				1	NS	Male	MSM	Pharyngeal	500mg CRO plus 1g AZI	NS	0.016	Agar Dilution	S	NS

				1	NS	Male	MSM	Pharyngeal	500mg CRO plus 1g AZI	NS	0.03	Agar Dilution	S	NS
Pleininger S, *et al.*[146]	2022	Europe	Austria	1	45-64	Male	Heterosexual	**Urethral**	CRO 1g and AZI 1.5g	AMC 1g BID 7 days	0.25	Gradient Strip	R	None

Studies that met inclusion criteria and also had sufficient information for analysis are included below.

Each row represents one study or case report, if multiple instances of treatment failure were reported, the number is listed under sample size

Regions defined by WHO

MIC: minimum inhibitory concentration mg/L, MSM: men who have sex with men. n/a: nor applicable, NS: not specified

CFM cefixime; CRO ceftriaxone; AZI azithromycin; SPT spectinomycin; ETP ertapenem; DOX doxycycline.

****Dual therapy indicates that the first treatment included multiple antimicrobials, the susceptibility of the non-cephalosporin is listed in this column: R resistant; I intermediate; S susceptible***

[§]Seven treatment failures described but only four had MIC data.

3.8.2. *Meta-analysis of cefixime treatment failures*

Published reports described 33 cases of treatment failure, one of which failed at two anatomical sites (urogenital and pharyngeal), leading to a total of 34 treatment failure sites. For cefixime, urogenital infections accounted for 44.1% of treatment failures (15/34), followed by pharyngeal at 35.3% (12/34). The pooled mean MIC of all cefixime treatment-failure isolates was 0.17 mg/L (95% Confidence Interval [CI]: 0.07, 0.41) (Fig. 37). The pooled estimate for extra-pharyngeal isolates cefixime was 0.29 mg/L (95% [CI]: 0.11, 0.81). Pharyngeal treatment failures yielded a pooled estimate of 0.05 mg/L (95% [CI]: 0.02, 0.14).

3.8.3. *Meta-analysis of ceftriaxone treatment failures*

Of the reports describing failures following treatment with ceftriaxone, 15 had sufficient data for analysis. There were 36 failure cases, one of which failed at two anatomical sites (urogenital and rectal), leading to a total of 37 treatment failure sites. Pharyngeal infections accounted for 67.6% of treatment failures (25/37). The overall pooled mean MIC for ceftriaxone treatment-failure isolates was 0.10 mg/L (95% [CI]: 0.05, 0.22) (Fig. 38). The pooled mean MIC for pharyngeal isolates was 0.09 mg/L (95% [CI]: 0.03, 0.22) and for extra-pharyngeal isolates was 0.14 mg/L (95% [CI]: 0.03, 0.73) (Fig. 38). Of the patients with ceftriaxone treatment failure, 33.3% (12/36) were infected at multiple sites, but only failed at the pharyngeal site. Seven of these pharyngeal cases grew a phenotypically susceptible isolate (MIC Range: 0.016-0.03 mg/L).

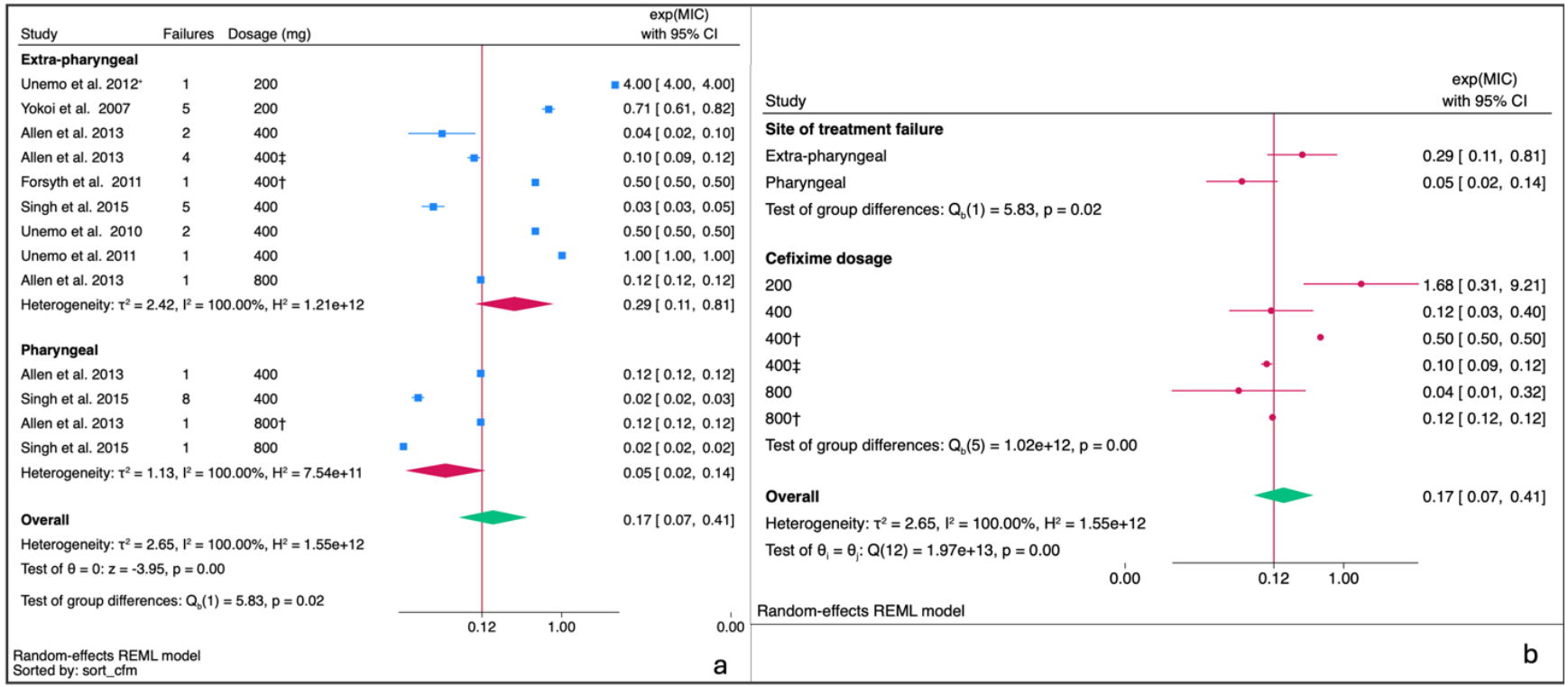


Figure 37. Random-effects restricted maximum likelihood model for cefixime treatment failure of included studies (a) and sub-group analyses by anatomical site (pharyngeal and extra pharyngeal) and treatment dosage (b). MIC = minimum inhibitory concentration (mg/L).
All dosages in milligrams (mg).
a) Random effects models generated with data from 8 included studies that ranged in dosage 200-800 mg, grouped by site of infection and ordered by dosage and year.
b) Data from 8 studies used for random effects models sub-group analysis.
‡ 100mg Doxycycline
† 1g Azithromycin
+ Unemo M and Sednaoui P *et al.*

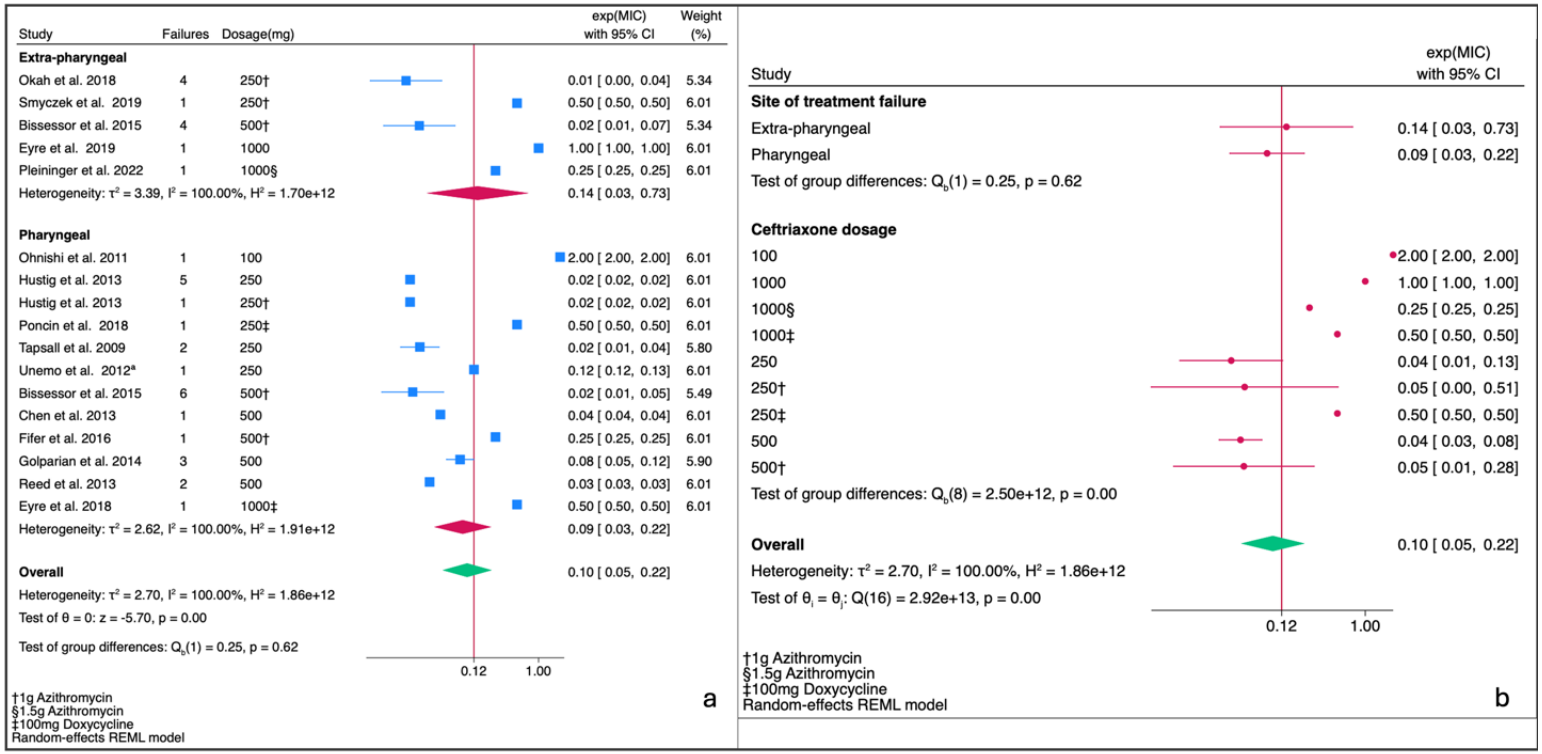


Figure 38. Random-effects restricted maximum likelihood model for ceftriaxone treatment failure of included studies (a) and sub-group analyses by anatomical site (pharyngeal and extra pharyngeal) and treatment dosage (b). MIC = Minimum Inhibitory Concentration (mg/L). All dosages in milligrams (mg).

a) Random effects models generated with data from 15 included studies, range in dosage 250-1000 milligrams, grouped by site of infection and ordered by dosage and year.

b) Data from 15 studies used for random effects models sub-group analysis.

‡ 100mg Doxycycline

† 1g Azithromycin

§ 1.5g Azithromycin

a Unemo M and Jeverica S *et al.*

3.8.4. *Treatment failures with dual antimicrobial treatment*

Of the 69 cases, 58% (40/69) were treated with the ESC alone. Of cases given dual-therapy, 75.8% (22/29) were treated with ESC and azithromycin, whereas the remaining received doxycycline alongside the given ESC (Tables 25 and 26). Of the 33 patients treated with cefixime, 78.8% (26/33) were treated with the ESC alone. Of the cases given dual-therapy, four received doxycycline and three were given azithromycin. The strains from patients treated with azithromycin were susceptible, whereas strains from patients treated with doxycycline were either intermediate or resistant to doxycycline (Tables 25 and 26). Of the 36 patients treated with ceftriaxone, 17 (47.2%) also received a second antibiotic, 88.2% (15/17) of whom received azithromycin and the remaining received doxycycline. Of the patients treated with azithromycin, 84.2% (16/19) carried phenotypically susceptible strains (Table 26).

3.8.5. *Treatment failures by antimicrobial susceptibility testing*

Of the patients treated with cefixime, the pooled mean MIC for those tested by agar dilution was 0.08 mg/L (95% [CI]: 0.04, 0.16), whereas the pooled mean MIC for those tested by gradient strip was 0.17 mg/L (95% [CI]: 0.07, 0.41) (Fig. 39). Of the patients treated with ceftriaxone, the pooled MIC for those tested by agar dilution and gradient strips was 0.05 mg/L (95% [CI]: 0.01, 0.2) and 0.21 mg/L (95% [CI]: 0.09, 0.49) respectively (Fig. 40).

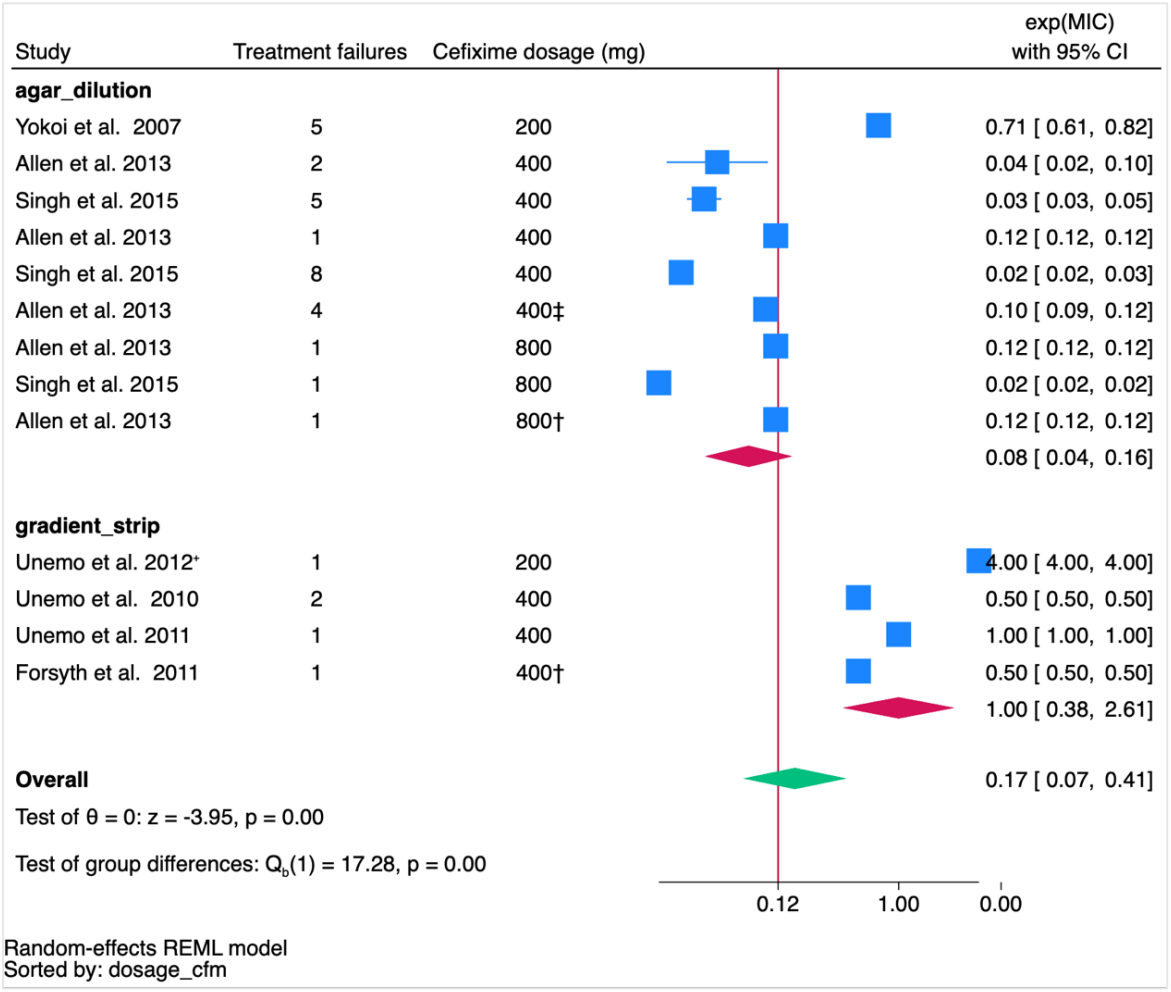


Figure 39. Random-effects restricted maximum likelihood model for cefixime treatment failures of included studies by testing method (agar dilution and gradient strip)

MIC = minimum inhibitory concentration (mg/L).
All dosages in milligrams (mg).
‡ 100mg Doxycycline
† 1g Azithromycin
+ Unemo M and Sednaoui P et al.[135]

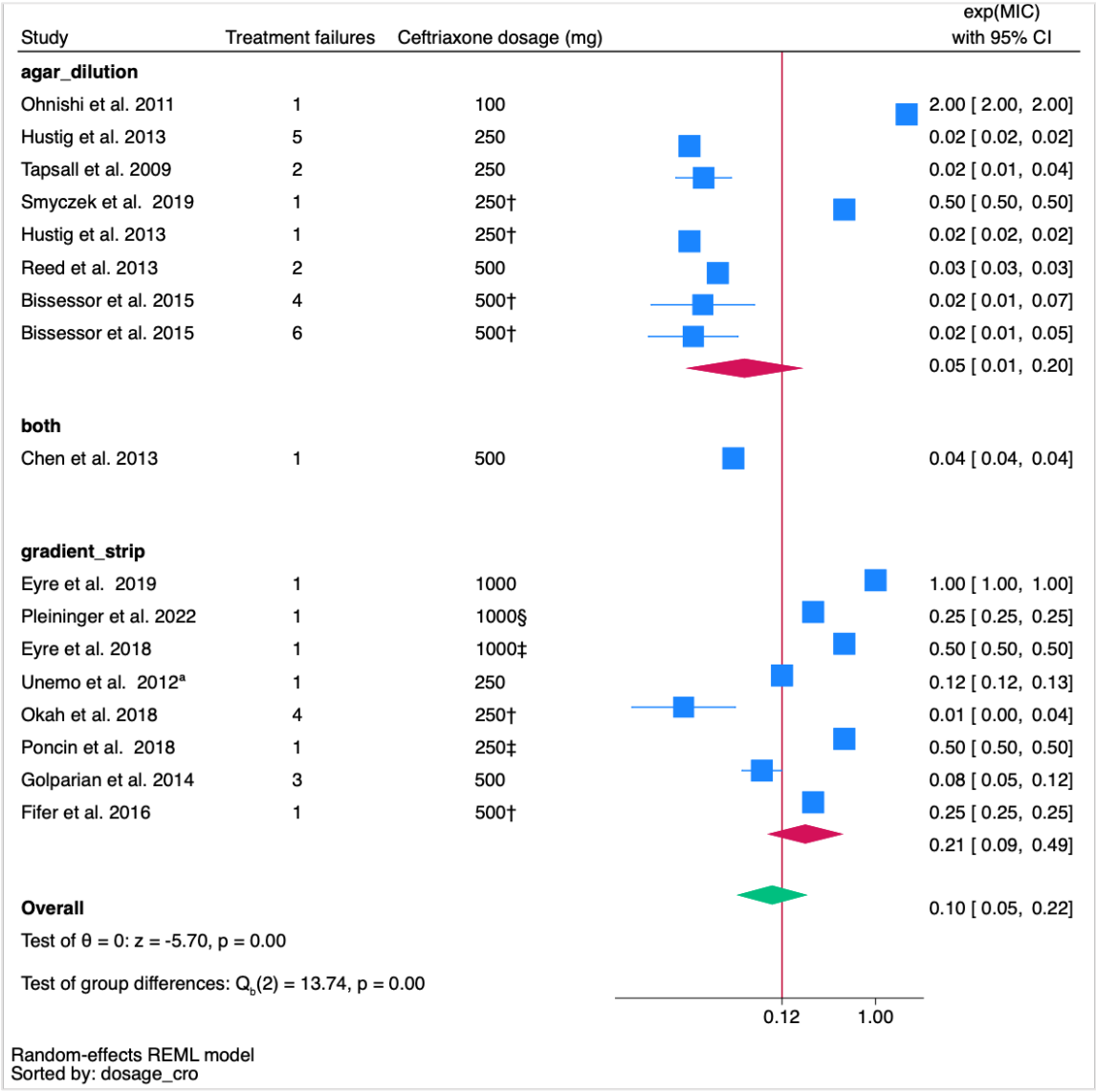


Figure 40. Random-effects restricted maximum likelihood model for ceftriaxone treatment failures of included studies by testing method (agar dilution and gradient strip)

MIC = minimum inhibitory concentration (mg/L).
All dosages in milligrams (mg).
‡ 100mg Doxycycline
† 1g Azithromycin
§ 1.5g Azithromycin
[A] Unemo M and Jeverica S et al.[158]

3.8.6. *Risk of bias*

Most treatment failures were reported as case reports, many of which listed only one instance of failure. These reports were generally of high quality, but some lacked sufficient demographic data and others contained incomplete description of methods. The risk of bias checklist for case reports is presented as a quality summary table and figure (Appendix A5). We generated bias figures based on all included studies.

3.9. Discussion

In this study we describe gonococcal treatment failures after ESC therapy and compare the characteristics of pharyngeal and non-pharyngeal failures, particularly the MICs of gonococcal strains. Treatments administered varied regionally, over time, and by anatomical site of infection. Cefixime and ceftriaxone were the most common, but cefotaxime, cefdinir and ceftibuten were also reported. In addition, treatment regimens varied, ranging from 200 to 800 mg of cefixime and 250 mg to 1 g of ceftriaxone. Due to co-infection with more than one STI or concerns about resistance, combination treatments with other antimicrobials were common. For example, a patient was treated with ceftriaxone, doxycycline, and spectinomycin before eventually clearing infection with 1 g ertapenem[227]. In addition to having unique targets, antimicrobials may also have different modes of administration that result in differing levels of bioavailability. Cefixime, unlike ceftriaxone, is an oral antibiotic historically delivered as inpatient partner-therapy without the discomfort of an intramuscular injection and with no requirement for trained personnel and sterile injection equipment [228].

Our results should be interpreted with caution given the variability in antibiotic use, dosages administered, and small sample sizes. Moreover, MIC may be measured by different methods, leading to different results. Gradient strips were more commonly used in included sources (53%), but many older reports, and reports from regions outside Europe, used agar dilution. Studies validating gradient strips against the standard of agar dilution showed good agreement, but it merits noting that methods were not standard across sources[229]. Most sources did, however, report their breakpoints for resistance.

Overall, the mean MICs of isolates from patients treated with cefixime and ceftriaxone were 0.17 mg/L (95% [CI] 0.07, 0.41) and 0.1 mg/L (95% [CI]: 0.05, 0.22), respectively. However, there were several reports where patients were infected in multiple anatomical sites and treated with ceftriaxone, but only experienced treatment failure with the sample from the pharyngeal site, despite gonococcal isolates being phenotypically susceptible. This further adds to the evidence that pharyngeal gonorrhoea is particularly difficult to treat and resistance breakpoints for pharyngeal infection should be reviewed.

It is well-documented that *β*-lactam concentrations in oropharyngeal tissue are lower than serum levels and this may contribute to the higher rate of treatment failure at this site[230]. In treatment failure observed after cefixime exposure, the mean MIC for pharyngeal isolates was 0.05 mg/L (95% [CI]: 0.02, 0.14) based on 11 patients, whereas for extra-pharyngeal isolates it was 0.29 mg/L (95% [CI]: 0.11, 0.81) generated from 22 patients. Importantly, unlike the extra-pharyngeal isolates, the MIC and 95% CIs for pharyngeal isolates falls below the EUCAST breakpoint of 0.125 mg/L with the upper CI at just above the breakpoint. The mean pharyngeal MIC after ceftriaxone treatment was marginally lower than the mean extra-pharyngeal MIC, although this was not statistically significant. The mean MIC for the 11 extra-pharyngeal isolates was 0.14 mg/L (95% [CI]: 0.03, 0.73) and for the 25 pharyngeal isolates the MIC was 0.09 mg/L (95% [CI]: 0.03, 0.22). The point estimate of the pharyngeal mean MIC is still lower than for the extra-pharyngeal isolates, but just under the EUCAST breakpoint, although the CIs between pharyngeal and extra-pharyngeal sites do overlap. Ceftriaxone 1 g is now the recommended treatment for pharyngeal infections in most countries[7]. There were four patients treated with 1 g ceftriaxone, all of whom had isolates with phenotypic resistance and MICs between 0.25 mg/L and 2 mg/L. This suggests that treatment failure after the increased dose of ceftriaxone may be a more accurate predictor of phenotypic resistance.

Interestingly, we found a difference in pooled MIC between isolates tested by agar dilution and gradient strip for both antimicrobials, more evidently for ceftriaxone. Given our small sample size, it is difficult to know whether testing method influences the susceptibility estimates that we generated using the log-transformed MICs. For example, the pooled MIC for isolates tested by

gradient strip after cefixime treatment was 1 mg/L (95% [CI]: 0.38, 2.61), compared to agar dilution which was 0.08 mg/L (95% [CI]: 0.04, 0.16) (Fig. 39). Although the CIs do not overlap, two factors may have influenced these results. Firstly, the number of isolates tested by both methods were unequal (28 by agar dilution and five by gradient strip) and secondly a single isolate MIC of 4 mg/L in the gradient strip group may artificially increase the pooled estimate, compared to the agar dilution pooled MIC (Fig.39). For ceftriaxone, the pooled MIC for isolated tested by gradient strip was fourfold higher than those tested by agar dilution (0.21 mg/L and 0.05 mg/L respectively) (Fig. 40). Previous studies report conflicting results on the accuracy of gradient strips versus agar dilution. For example, a study by Papp *et al* showed that ceftriaxone E-test had >90% agreement with agar dilutions[105], whereas a study by Gose *et al* reported that >20% of E-test MIC values were above agar dilution values[222]. Both studies, however, suggested criteria that are method-specific for interpreting breakpoints.

Our meta-analysis has limitations. There was considerable variability among studies included due to the range of treatments administered, anatomical sites tested, definition of treatment failure and patient characteristics. We stratified results by sub-group to account for these differences, but this made for smaller sample sizes and wider CIs. In some instances, sample collection skewed towards a particular anatomical site; for example, 70% of the reported ceftriaxone failures occurred in the pharynx. Further stratifications would also have been useful, for example by dual treatment. However, our sample size precluded further sub-group analyses. We recognise that our data are based on reported treatment failures at the given ESC dosages and we do not have denominator data to compare successfully treated gonococcal infections with the same MICs. Consequently, the meta-analysis does not include all representative outcomes for each MIC. Additionally, some sources may not have reported details on reinfection which may have impacted their inclusion. Further research such as cross-sectional studies that include all outcomes from each MIC and denominator data should be performed to corroborate this research. Another limitation is the inclusion of mainly case reports which may introduce publication bias. A risk of bias analysis is outlined in Appendix A5. The level of heterogeneity in the information presented in the sources further demonstrates the necessity for global surveillance systems and guidance on reporting

gonococcal treatment failures. Despite these limitations the findings of this study are in line with the WHO global action plan to control the spread and impact of gonococcal AMR which calls for the "systematic monitoring of treatment failures by developing a standard case definition of treatment failure, and protocols for verification, reporting and management of treatment failure"[231]. Further, surveillance is key to combatting gonococcal AMR and must be strengthened in all contexts. High-resource settings with the ability to sequence pre- and post-treatment isolates must contribute to knowledge about resistance mechanisms and adaptations. In low-resource settings, where syndromic management is the current policy, periodic surveillance is necessary to ensure that treatment, and the larger policy of which it is a part, remains effective. Special attention must be paid to key marginalised populations to lessen the high burden of disease and new technology should be made available in all regions to ensure that data is accurate and representative.

3.10. Conclusions

Global surveillance and reporting of treatment failure remain important for control efforts of gonorrhoea, particularly pharyngeal gonorrhoea. Our study presents data that may inform breakpoint revisions for different anatomical sites, findings that align with the WHO global action plan to control the spread and impact of gonococcal AMR. There is an urgent need to establish common standards for breakpoints, including an internationally agreed MIC testing method to foster improved reporting of treatment failures and surveillance practices that are key to informing appropriate public health responses.

CHAPTER 4

CHARACTERISATION OF *NEISSERIA GONORRHOEAE* STRAINS FROM PATIENTS WITH MULTISITE INFECTION

4.1. Introduction

N. gonorrhoeae can infect multiple anatomical sites including the pharynx, rectum and urogenital sites and up to 42% of patients are estimated to have concomitant infection in multiple sites. While these can be independent transmission events from different partners, these are often primarily presumed as the same isolate. Currently, there is no guidance on how microbiology laboratories should process gonococcal isolates from multisite infection; some laboratories only test a single isolate and extrapolate the results to the other sites. If different isolates present in different sites are not assessed, this could lead to incorrect assessment of AMR and suboptimal detection of resistant strains. Further, accurate estimations of multistrain carriage can be impacted by missing data such as missing isolates, particularly due to the low gonococcal culture recovery rate[86] It is therefore important to acknowledge and measure missing data where possible to ensure meaningful public health action recommendations. For example, missing data may affect comparisons of gonococcal antimicrobial susceptibility testing (AMS) between anatomical sites, with a bias against pharyngeal isolates as they have the lowest recovery rates.

In this chapter, I present a cross-sectional study of patients with multisite infection in two parts; the first part includes data on the proportion of patients infected with multiple gonococcal strains and subsequent molecular typing and phylogenetic analyses (published in Sexually Transmitted Infections, doi: 10.1136/sextrans-2024-056297, Appendix E2). The second part includes additional analyses regarding additional molecular typing not included in the published manuscript (Appendix E2), a description of missing data and MIC comparisons between anatomical sites.

4.2. Research Questions

Research Question 1. What proportion of multisite patients have *Ng* isolates with differing antibiograms between anatomical sites?

Research Question 2. Does molecular typing confirm that *Ng* isolates with antibiogram differences are due to separate STs?

Research Question 3. How many potential *Ng* isolates are missing from these analyses?

4.3. Hypothesis

A higher proportion of the general population than previously thought are infected with multiple strains of *Ng*.

4.4. Aims

To identify the proportion of patients with multisite infections carrying different *Ng* strains between anatomical sites and to determine the number of missing data points by anatomical site.

4.5. Objectives

i. Determine the MICs of gonococcal strains from patients with multisite infection to a range of antimicrobials.
ii. Calculate the proportion of patients infected with multiple gonococcal stains between anatomical sites.
iii. Perform *NG*-MAST, WGS and phylogenetic analyses on isolates from patients with discordant MICs.
iv. Determine missing data by analysing additional metadata provided by home laboratories.

4.6. Ethical considerations

Ethical approval was granted by the LSHTM Research Ethics Committee. Approval was granted on 09/04/2014 (Ref: 7604) (Appendix B1).

4.7. Gonococcal isolates

Gonococcal isolates from multisite infection were provided by three 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) (2.2.1). Anatomical sites included were urethra, cervix, rectum and pharynx. The isolates were collected from consecutive patients attending 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 -80^{o}C. Each isolate was provided with the following information: laboratory number, anatomical site and patient sex. Isolates were cultured from storage vials on VCAT agar (Oxoid,) and identified by Gram stain (2.5.3) and oxidase (2.5.2). Pharyngeal isolates were further confirmed biochemically by API NH (2.5.4) (Biomerieux) or RapidTM NH (2.5.5) (Oxoid) to differentiate between *N. gonorrhoeae* and contaminating commensal *Neisseria* species. Purified isolates were stored in 20% glycerol BHI broth (Oxoid) (2.1.5) at -70°C until further testing.

4.8. Antimicrobial susceptibility testing

Antimicrobial MICs for cefixime, ceftriaxone, azithromycin, tetracycline and spectinomycin were determined by agar dilution (2.7.3) and MICs for penicillin and ciprofloxacin were determined by gradient strip (2.7.4). Gonococcal isolates resistant to penicillin were tested for β-lactamase production with a nitrocefin disc (2.7.2). Eight WHO reference strains (F, G, K, L, M, N, O and P) were included in all MIC testing for quality control (2.2.1). Clinical and control strains of *Ng* 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.

4.9. Definition of multisite 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 (Appendix B4). Sequencing and molecular typing were performed on gonococcal strains with differing AST profiles.

4.10. *N. gonorrhoeae* sequencing and molecular typing

Genomic DNA was extracted with the Archive Pure kit (5PRIME) following the Gram-negative bacteria protocol (2.9.1) and *NG*-MAST was performed manually (2.10). 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 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 *Ng* NCCP11945 (NC_011035) genome[232] as the reference, and then annotated using PROKKA with a bespoke database. Multilocus sequencing typing 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 and core SNPs were defined using SNIPPY (v4.6.0) (https://github.com/tseemann/snippy) against the *Ng* NCCP11945 genome using default settings. The phylogeny was determined using the FastTree[233] approximately-maximum-likelihood for

alignments of SNP nucleotides using default settings with the SNIPPY SNPs alignment and viewed in GrapeTree[234]. All WGS analysis was performed by Dr Richard Stabler.

4.11. Analysis of gonococcal isolate distribution and antimicrobial resistance

4.11.1. 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 (Appendix B2). 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%).

4.11.2. 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 27). 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 Appendix B2.

Table 27. 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*

4.12. Analysis of discordant gonococcal isolates from patients with multisite infection

4.12.1. Multisite infection 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 28, Appendix B4). 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 28).

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 28, Appendix B4). Two patients (20 and 38) also had discordant β-lactamase results (Table 28). Discordant MICs were further confirmed at least once (Appendix B4).

Table 28. 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,*

4.12.2. Multisite infection 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 (Table 29). 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 28 and Table 29).

Phylogenies of the 13 isolates from the above six patients were generated using SNP data (Fig. 41). 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, Fig. 41). Conversely, isolates with the same ST from a single patient were highly similar at SNP level (e.g. GC25R and GC25U, Fig. 41).

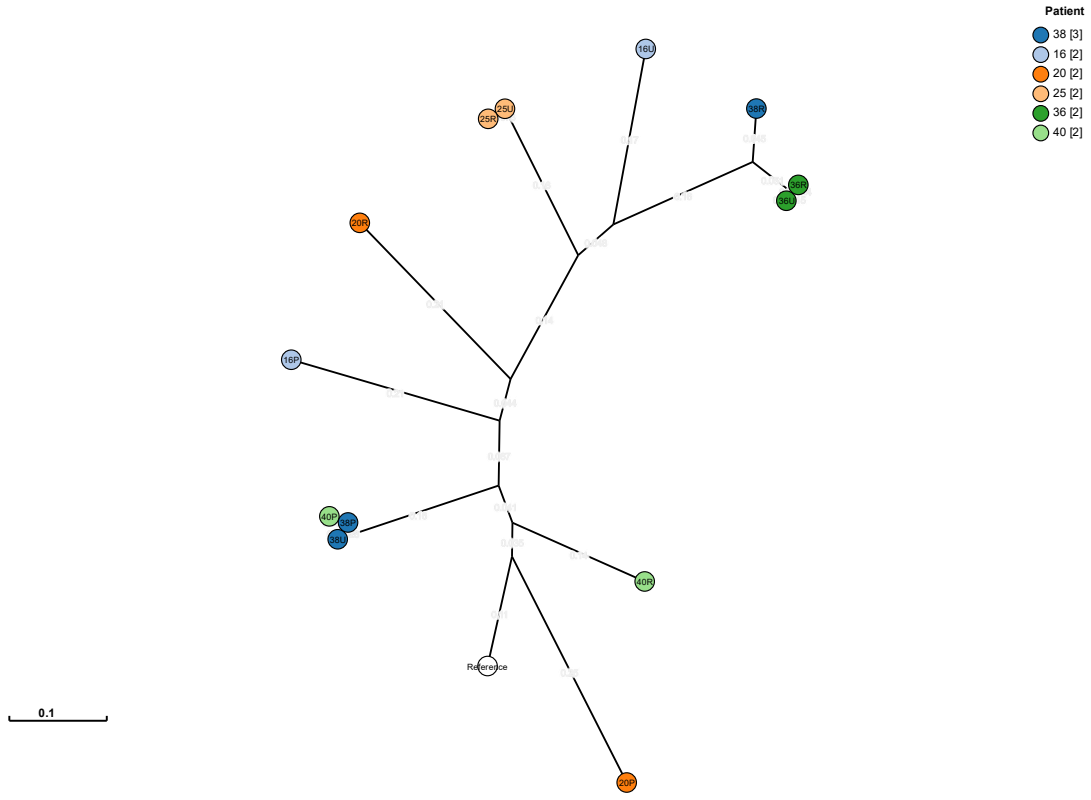


Figure 41. Phylogeny of study isolates. Single nucleotide mutations were determined against the N. gonorrhoeae NCCP11945 genome using snippy. An approximately-maximum-likelihood phylogeny was estimated using fasttree. Multisite samples are coloured by patient ID.

Table 29. Whole genome sequencing data.

Patient	Site	Code	Reads(pe)	length(bp)	GC%	CDS
16	Pharynx	GC16P	6,788,756	2,445,172	52.20	2389
	Urethra	GC16U	4,409,944	2,359,616	52.28	2315
20	Pharynx	GC20P	770,540	2,093,978	52.61	2039
	Rectum	GC20R	1,480,964	2,123,997	52.62	2078
25	Urethra	GC25U	1,276,254	2,172,592	52.37	2135
	Rectum	GC25R	1,382,844	2,175,313	52.37	2132
36	Urethra	GC36U	4,328,924 + 87,350	2,158,533	52.37	2123
	Rectum	GC36R	4,266,848 + 1,079,108	2,173,750	52.36	2140
38	Rectum	GC38R	3,260,850 + 1,254,808	2,169,216	52.38	2128
	Pharynx	GC38P	3,690,662 + 925,030	2,188,800	52.35	2150
	Urethra	GC38U	4,510,396 +1,212,456	2,190,603	52.34	2153
40	Pharynx	GC40P	4,540,194 + 947,676	2,197,570	52.33	2160
	Rectum	GC40R	2,839,536 + 822,330	2,074,488	52.68	2022

Reads; total paired end (pe) reads, for some isolates two different libraries were made and sequenced on separate MiSeq runs. CDS; coding sequences (number of genes)

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) (Fig. 42, Table 30 and Table 31). This was also reflected in the phylogeny with the GC36 isolates clustering together but GC38R was separate but related (Fig. 41).

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 30). Analysis of the draft genomes between the two isolates demonstrated that there were no differences in *gyrA, parC* (Fig. 42). 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 (Table 31 and Appendix B3).

Overall, there was good agreement between the presence of genotypic markers of resistance and phenotypic resistance (Fig. 42). All isolates with a positive β-lactamase test carried a TEM-1

(GC20R, GC36U, GC36R and GC38R) (Table 28, Fig. 42 and Appendix B3) 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 28, Fig. 42, Appendix B3). Isolates that carried *tet*(M) were associated with tetracycline MIC ≥8 mg/L (GC16U, GC20R, GC25U, GC25R, GC38P, GC38U, GC40P) (Table 28, Fig. 42, Appendix B3). Isolate GC20P was the only isolate with a mosaic *penA* allele (Appendix B3).

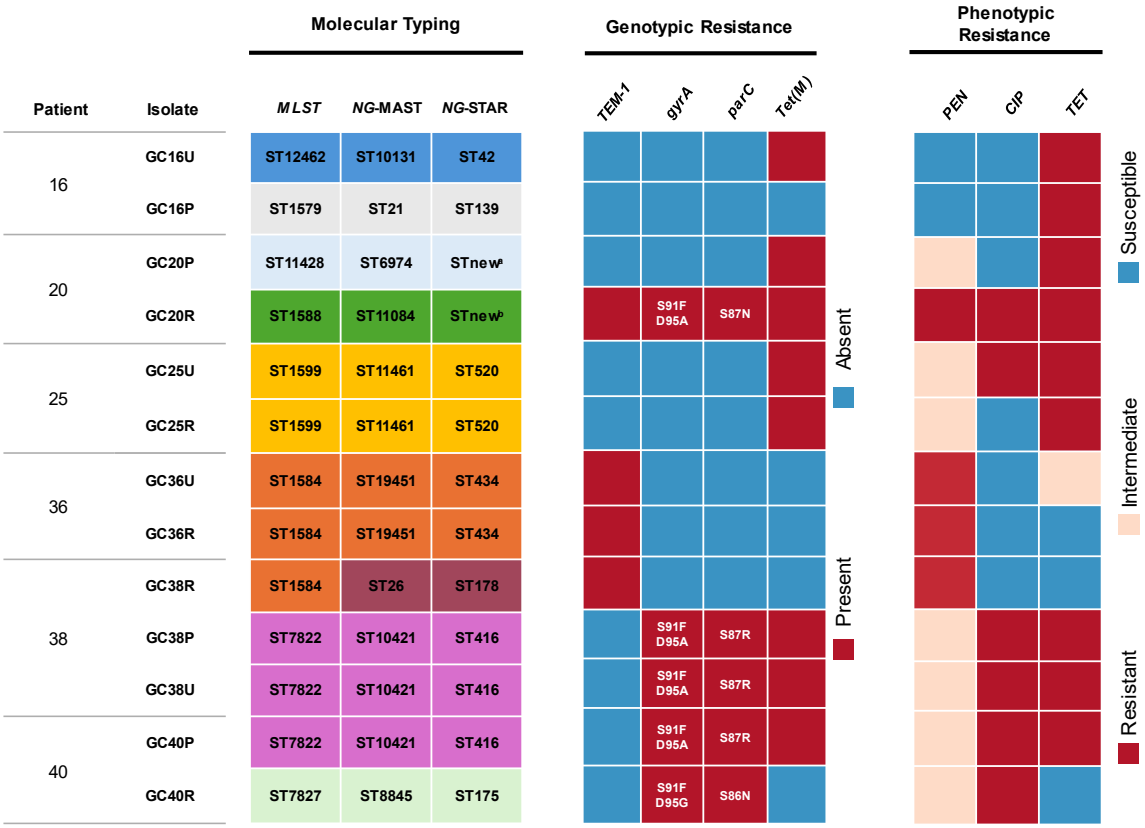


Figure 42. *NG*-MAST, *NG*-STAR and MLST of sequenced isolates from six multisite patients with differing antibiograms along genotypic markers of resistance phenotypic susceptibility profiles to penicillin, ciprofloxacin and tetracycline. Indicated is the presence or absence of genotypic resistance markers 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)

Table 30. *NG*-MAST and MLST typing information for sequenced multisite isolates

Patient	Site	Isolate Number	*NG*-MAST	*porB*	*tbpb*	MLST	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*
16	Pharynx	GC16P	21	*14*	*33*	1579	*109*	*39*	*67*	*111*	*148*	*153*	*65*
	Urethra	GC16U	10131	*3575*	*1802*	12462	*59*	*39*	*785*	*157*	*188*	*153*	*65*
20	Pharynx	GC20P	6974	*908*	*137*	11428	*126*	*39*	*170*	*238*	*734*	*153*	*133*
	Rectum	GC20R	11084	*1808*	*137*	1588	*59*	*39*	*67*	*158*	*148*	*71*	*65*
25	Urethra	GC25U	11461	*6720*	*188*	1599	*59*	*39*	*67*	*157*	*148*	*153*	*65*
	Rectum	GC25R	11461	*6720*	*188*	1599	*59*	*39*	*67*	*157*	*148*	*153*	*65*
36	Urethra	GC36U	19451	*11281*	*4*	1584	*59*	*39*	*67*	*156*	*150*	*153*	*65*
	Rectum	GC36R	19451	*11281*	*4*	1584	*59*	*39*	*67*	*156*	*150*	*153*	*65*
38	Rectum	GC38R	26	*19*	*24*	1584	*59*	*39*	*67*	*156*	*150*	*153*	*65*
	Pharynx	GC38P	10421	*6094*	*33*	7822	*126*	*39*	*170*	*111*	*148*	*153*	*65*
	Urethra	GC38U	10421	*6094*	*33*	7822	*126*	*39*	*170*	*111*	*148*	*153*	*65*
40	Pharynx	GC40P	10421	*6094*	*33*	7822	*126*	*39*	*170*	*111*	*148*	*153*	*65*
	Rectum	GC40R	8845	*1808*	*4*	7827	*59*	*39*	*67*	*158*	*148*	*153*	*65*

NG-MAST; *Neisseria gonorrhoeae* multi-antigen sequence typing, MLST; multilocus sequence typing

Table 31. *NG*-STAR full profiles of sequenced isolates

Patient	Site	Isolate Number	*NG*-STAR	*penA*	*mtR*	*porB*	*ponA*	*gyrA*	*parC*	*23S*
16	Pharynx	GC16P	139	9.001	1	8	1	100	2	100
	Urethra	GC16U	42	14.001	10	3	100	100	1	100
20	Pharynx	GC20P	new[a]	34.001‡	9	11	100	100	100	100
	Rectum	GC20R	new[b]	19.001	38	1	1	7	55	100
25	Urethra	GC25U	520	14.001	130	3	100	100	2	100
	Rectum	GC25R	520	14.001	130	3	100	100	2	100
36	Urethra	GC36U	434	14.001	50	1	100	100	7	100
	Rectum	GC36R	434	14.001	50	1	100	100	7	100
38	Rectum	GC38R	178	14.001	29	1	100	100	7	100
	Pharynx	GC38P	416	2.002	19	3	1	7	3	100
	Urethra	GC38U	416	2.002	19	3	1	7	3	100
40	Pharynx	GC40P	416	2.002	19	3	1	7	3	100
	Rectum	GC40R	175	13.001	19	1	1	1	9	100

‡ *Mosaic penA, new[a] & new[b] are novel combinations of known alleles, NG-STAR; Neisseria gonorrhoeae Sequence Typing for Antimicrobial Resistanc*

4.13. Analyses of additional antimicrobial susceptibility testing, *NG*-MAST typing and whole genome sequencing

4.13.1. Comparison of NG-MAST typing and whole genome sequencing

NG-MAST typing (2.10) was performed on isolates from multisite *Ng* infection with divergent antibiograms and confirmed *in silico* through WGS (4.10). From traditional *NG*-MAST typing (2.10), amplification of *porB* and *tbpB* showed the expected amplicon sizes of 737bp and 589pb respectively (Fig. 43). The results of traditional *NG*-MAST for all isolates tested agreed with *in silico* *NG*-MAST (Table 32).

Table 32. Comparison of *NG*-MAST results from traditional and WGS methods.

Patient	*Study Number*	Traditional			WGS		
		porB	*tbpB*	*NG-MAST*	*porB*	*tbpB*	*NG-MAST*
16	GC16P	14	33	21	14	33	21
	GC16U	3575	1802	10131	3575	1802	10131
20	GC20P	908	137	6974	908	137	6974
	GC20R	1808	137	11084	1808	137	11084
25	GC25U	6720	188	11461	6720	188	11461
	GC25R	6720	188	11461	6720	188	11461
36	GC36U	11281	4	19451	11281	4	19451
	GC36R	11281	4	19451	11281	4	19451
38	GC38R	19	24	26	19	24	26
	GC38P	6094	33	10421	6094	33	10421
	GC38U	6094	33	10421	6094	33	10421
40	GC40P	6094	33	10421	6094	33	10421
	GC40R	1808	4	8845	1808	4	8845

NG-MAST; Neisseria gonorrhoeae multiantigen sequence type; WGS; whole genome sequencing
Study numbers ending in P, U and R denote pharyngeal, urethral and rectal isolates respectively.

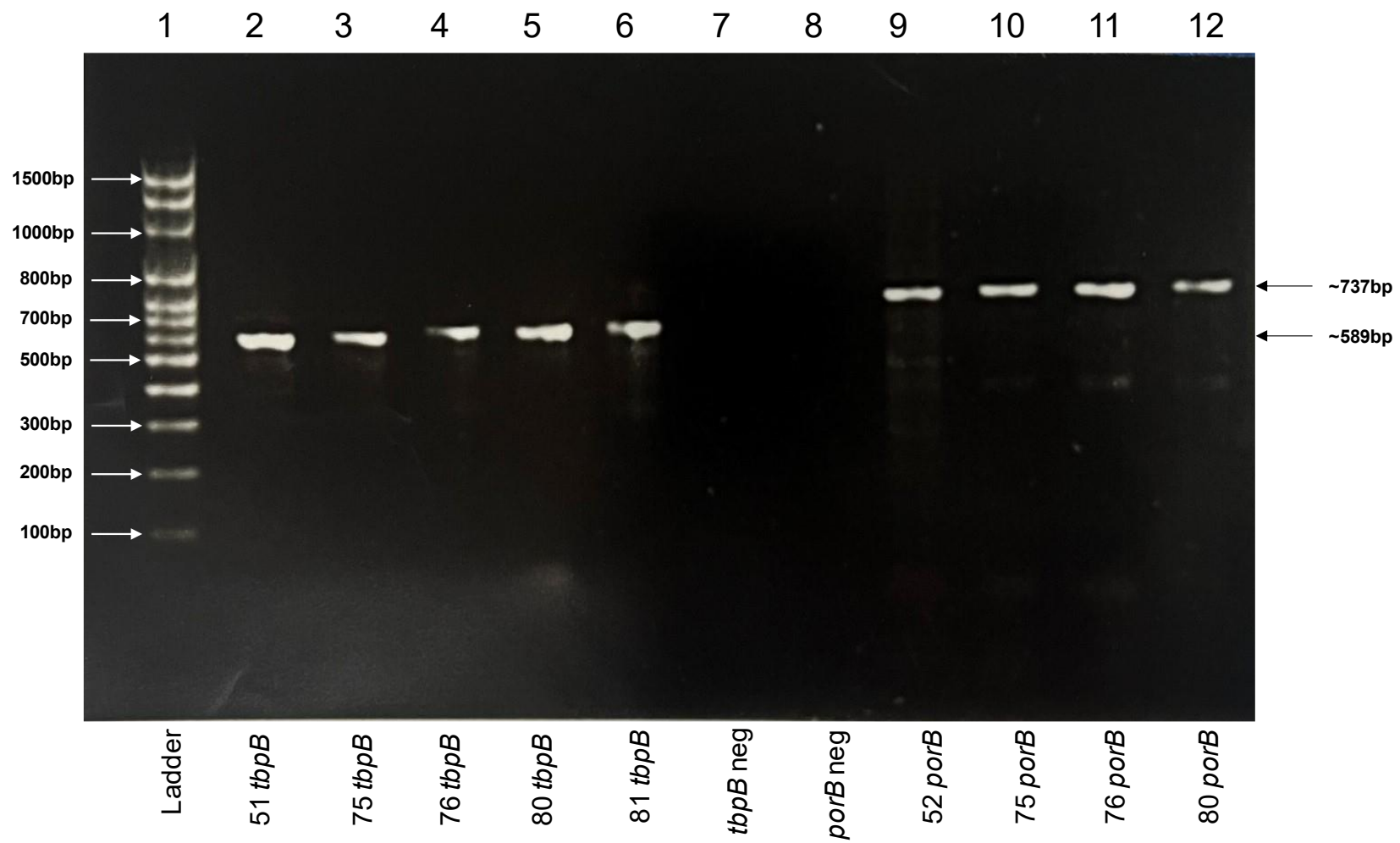


Figure 43. Example *NG*-MAST agarose gel; *tbpB* amplicons with the expected size of ~589bp (lanes 2-6) and *porB* amplicons with the expected size of ~737bp (lanes 9-12). Negative controls are molecular grade water (lanes 7 & 8). Bioline Ladder (lane 1)

4.13.2. *Additional NG-MAST typing on non-discordant gonococcal isolates from patients with multisite infection*

Typing by *NG*-MAST was also performed on eight patients' multisite isolates that on initial testing had >1 $\log_2$ difference in MICs fulfilling our definition of divergent MICs, but on confirmatory testing, were within ±1 $\log_2$ (Appendix B2). Full *NG*-MAST profiles (*tbpB* and *porB*) were generated for all isolates from patients 3 ($ST_{NG\text{-}MAST}$9368), 8 ($ST_{NG\text{-}MAST}$6360), 29 ($ST_{NG\text{-}MAST}$4244) and 43 ($ST_{NG\text{-}MAST}$14611) and all had the same $ST_{NG\text{-}MAST}$ in the different anatomical sites infected (Table 33). A full *NG*-MAST profile was generated for isolate GC17P from patient 17 ($ST_{NG\text{-}MAST}$4995), and only the *tbpB* allele (partial *NG*-MAST) for GC17C from the same patient was successfully sequenced (*tbpB*33) (Table 33). All isolates from patient 7 contained *tbpB*16, patient 10 isolates contained *tbpB*403 and all isolates from patient 13 contained *tbpB*893; *porB* sequencing was unsuccessful on these isolates (Table 33) and was not attempted, as further MIC testing showed non-divergent MICs.

Table 33. Antimicrobial MICs and *NG*-MAST information performed on gonococcal isolates from multisite patients

Patient	Study Number	MIC (mg/L)							*porB*	*tbpB*	*NG*-MAST
		PEN	CFX	CRO	AZI	CIP	TET	SPE			
3	GC3U	0.008	0.008	0.004	0.061	0.016	0.5	16	2703	893	9368
	GC3R	0.012	0.008	0.004	0.061	0.016	0.5	16	2703	893	9368
7	GC7U	0.064	0.002	0.002	0.002	0.008	0.125	4	UN	16	NP
	GC7C	0.094	0.004	0.002	0.004	0.006	0.25	4	UN	16	NP
8	GC8P	0.125	0.008	0.008	0.008	>32	0.5	8	3957	563	6360
	GC8R	0.125	0.004	0.008	0.008	>32	0.5	8	3957	563	6360
10	GC10U	0.004	0.002	0.004	0.004	0.006	0.063	16	UN	403	NP
	GC10C	0.008	0.002	0.002	0.002	0.006	0.063	16	UN	403	NP
13	GC13P	0.125	0.061	0.008	0.031	0.006	0.5	32	UN	893	NP
	GC13R	0.125	0.031	0.008	0.031	0.006	0.5	32	UN	893	NP
17	GC17C	0.032	0.002	0.002	0.016	16	16	>32	UN	33	NP
	GC17P	0.032	0.002	0.002	0.016	12	16	>32	3031	33	4995
29	GC29P	0.094	0.064	0.008	0.016	0.008	0.5	16	1808	893	4244
	GC29R	0.25	0.125	0.016	0.031	0.008	0.5	16	1808	893	4244
43	GC43C	0.25	0.008	0.016	0.016	1.5	1	8	1808	2003	14611
	GC43P	0.25	0.016	0.008	0.016	2	0.5	8	1808	2003	14611
	GC43U	0.5	0.016	0.008	0.008	2	0.5	8	1808	2003	14611

MIC; minimum inhibitory concentration, UN; unsuccessful, NP; not possible, NG-MAST; Neisseria gonorrhoeae multiantigen sequence type, PEN; penicillin, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin, Study numbers ending in P, U and R denote pharyngeal, urethral and rectal isolates respectively

4.14. Investigation of missing data

Missing data were defined as *Ng* isolates from multisite patients not included in this study, as identified from additional metadata provided by the diagnostic laboratories. Specifically, the laboratories provided the following metadata for each patient: a) whether a culture swab was collected for each anatomical site (pharynx, urethra, rectum and cervix), b) whether the culture swab was positive or negative, c) whether a NAAT test was performed for each anatomical site and d) the result of the NAAT test.

Two reasons were identified for missing data: isolates were not sent by the diagnostic laboratories (n=4), or gonococcal recovery from culture swabs was not possible (positive by NAAT but culture negative) (n=8) (Table 34) (Appendix B6). Overall, there were seven missing data points for pharyngeal, four for rectal and one for cervical isolates (Table 34). Four patients (3, 6, 33 and 43, one male, three female) grew *Ng* isolates that were not sent to LSHTM for testing, consisting of one pharyngeal, one cervical and two rectal isolates (Appendix B6). None of these patients had divergent MIC values between anatomical sites and one patient (patient 3) had $ST_{NG\text{-}MAST}$9368 in both sites (Table 34). Eight further patients (4, 11, 17, 22, 23, 25, 28 and 36, six male, and two female) had a negative culture result at a NAAT-positive site (Appendix B6). This consisted of six pharyngeal and two rectal sites (Table 34). Two patients (25 and 36) were previously identified as having divergent MICs (4.12.1) (Appendix B6).

Table 34. Summary of gonococcal isolates missing from the study by anatomical site

	Cervix	Rectum	Pharynx	Urethra	Total
Cultures positive at home laboratory	12	30	27	26	95
Not sent by laboratory	1	2	1	0	4
NAAT positive / culture negative	0	2	6	0	8
Total missing data points	1	4	7	0	12

NAAT; nucleic acid amplification test

The gonococcal recovery rate rates were also calculated for isolates with a positive NAAT test by anatomical site, as follows:

$$Recovery\ rate = \frac{Culture^{Positive}}{NAAT^{Positive}} \times 100$$

A total of 94 specimens were NAAT positive, for which a culture swab was taken, consisting of 33 pharyngeal, 31 rectal, 10 cervical and 20 urethral specimens. Overall, the culture recovery rate was 90.4% (85/94). The recovery rate for pharyngeal and rectal swabs was 78.8% (26/33) and 93.5% (29/31) respectively, whereas for cervical and urethral isolates, the recovery rate was 100% (10/10, 20/20, respectively).

4.15. Discussion

As *Ng* 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 multisite 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[235]. All seven U-C isolates in this study had the same MIC (+/- 1 $\log_2$) 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 multisite infection using older methods such as auxotyping and RFLP, reported a prevalence between 7-40%[44,45,48]. 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[50]. A similar proportion of mixed infection (1.3%, 4/298) was found in a subsequent study, determined by *porB* sequencing[236].

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 28) 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 (Fig. 42, Table 30, table 31). 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 *Ng*, as described previously[50,236]. 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[235], while others report similar susceptibilities in all anatomical sites[237,238].

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 (Fig. 41, Fig. 42). 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 (Fig. 41, Fig. 42). 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[118]. Ultimately, however, WGS based typing methods provides best resolution for determining relationship between isolates.

In this study, *NG*-MAST typing was performed initially manually and subsequently *in silico* through WGS. Sequence types generated by both methods were the same, for all isolates tested (Table 32). These results agree with previous comparisons between traditional *NG*-MAST and *in silico* *NG*-MAST, which have shown 100% concordance[120]. Although the two methods show high concordance, WGS can add increased resolution for phylogenetic analyses and transmission tracking as well as output flexibility, for example, additional typing like MLST, *NG*-STAR and

cgMLST[239]. Traditional *NG*-MAST typing was attempted on multisite isolates from eight further patients that on initial testing, had divergent MIC, but on confirmatory testing, were within 1 $\log_2$ difference and were subsequently excluded as multistrain patients. All patients with full *NG*-MAST patients included in this secondary analysis had the same strain between anatomical sites (Table 32). Ideally, all isolates in the study would have undergone *NG*-MAST to explore whether multisite patients carried isolates with the same antibiogram but differing STs. Even though this was not possible due to financial reasons, previous studies have examined agreement between AMR profiles and STs; for example, in a previous study, concordance between *NG*-MAST and susceptibility to penicillin, ciprofloxacin, azithromycin, and tetracycline was 97.1%, 99.5%, 97.5%, and 92.0% respectively[240].

Estimates for the proportion of patients with divergent MICs may have been biased by missing data, particularly false negative cultures which may result from suboptimal sampling technique, especially from the pharynx[241], improper transport and agar inoculation[242], or low gonococcal loads in the sampled site[243]. In this study, two reasons for missing data were identified: isolates were not stored for study purposes (n=4), or the gonococcal isolate failed to grow (n=8). This led to missing data points from 12/41 patients (29%); considering that in our study, approximately 1:7 patients (6/41) had divergent MICs, we can estimate that with the complete data set, we would have been able to identify one more patient with divergent MICs.

The culture recovery of pharyngeal isolates was lower than that of extrapharyngeal isolates. The challenge of recovering *Ng* from clinical samples, especially from the pharynx, is widely acknowledged in the literature. The sensitivity of gonococcal culture compared to NAATs has been estimated as 39% in pharyngeal isolates compared to 53% in rectal isolates[243]. Further, gonococcal recovery by culture from pharyngeal specimens reported in one study was 27%, compared to rectal (40-67%) and urethral (71.4-91%) specimens[86,242]. There are several reasons why gonococcal culture is less sensitive than NAAT testing; firstly, *Ng* is a fastidious organism, very susceptible to changes in temperature, pH and desiccation[242]. Delays in agar inoculation, inability to maintain a cold chain during transport and use of incorrect swab types may impact the viability of *Ng* and lead to false negative gonococcal cultures[242]. In contrast, NAATs are not

affected by organism viability as they detect nucleic acid. Secondly, gonococcal culture is thought to have a higher detection limit than NAATs. Previous research showed that gonococcal culture positivity from pharyngeal and rectal specimens was strongly associated with higher bacterial loads compared to negative cultures, although this research study did not report a specific limit of detection[243]. This is further exacerbated by the fact that gonococcal loads in the pharynx have been estimated to be lower (median 2.1×10^2 copies per swab) compared to rectal loads (1.9×10^4 copies per swab)[243] and urethral loads from both symptomatic (3.7×10^6 copies per swab) and asymptomatic (2.0×10^5 copies per swab) men[244]. Lastly, the sampling technique can influence the recovery of *Ng* from pharyngeal specimens. A recent study showed that sampling from the tonsils had a higher recovery rate than oropharyngeal sampling, but recovery was highest when both sites were sampled[241]. Improving the recovery rate of *Ng* from clinical specimens, especially from the pharynx, is imperative to enable consistent AST and monitoring of MICs and phenotypic AMR. Further work to develop gonococcal culture enrichment is needed to facilitate this.

Our study is not without limitations. Firstly, our sample size is small, limiting the accuracy of our estimates of occurrence of multistrain multisite 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 on this study in a more recent setting. This is important to capture whether 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. Further, molecular typing and phylogenetic analyses of isolates from these patients identified a potential transmission event; specimen collection dates and information on sexual partners would have solidified this hypothesis.

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.

CHAPTER 5

CHARACTERISATION OF COMMENSAL *NEISSERIA* SPECIES IN THE HUMAN PHARYNX AND THEIR ANTIMICROBIAL SUSCEPTIBILITY PROFILES

5.1. Introduction

Commensal *Neisseria* species are considered a significant reservoir of AMR genes that can be transferred to pathogenic *Neisseria species* via HGT[15]. For *Ng*, this is facilitated by the recognition of DUS sequences in the donor DNA[67,84]. It has recently been proposed that AMR surveillance in *N*c species may help quantify circulating resistance determinants in *N*c, correlate AMR genotypes with transferable resistance determinants and generate data that may enable the prediction of future HGT events and AMR in pathogenic *Neisseria* species[15]. Further, Kenyon *et al*. have proposed a "pan-*Neisseria*" approach to the selection of antimicrobials for the treatment of pathogenic *Neisseria* infections, which should limit the selection of resistant *N*c[245].

There have been several surveys of *N*c incidence and carriage of AMR, however almost all have limitations; some studies focus on a single *N*c species, for example, *N. lactamica*[80,83,187], or perform AST on a limited range of antimicrobials[78,79,189], very few have performed WGS and phylogenetic analyses and none have described DUS repeats in the *N*c genomes. Furthermore, no such surveys have been carried out in the UK. To fill these knowledge gaps, I performed an exploratory cross-sectional study of 50 participants at LSHTM. In this chapter, I determine the *N*c incidence by species, describe phenotypic and genotypic AMR determinants for a wide range of antimicrobials, perform phylogenetic analyses and describe the DUS content in the *N*c genomes.

This chapter has been published in Scientific Reports (https://doi.org/10.1038/s41598-024-75130-9) and the manuscript can be found in Appendix E3.

5.2. Research questions

Research question 1. What proportion of participants are colonised with *N*c species?

Research question 2. What is the AMR burden carried by *N*c?

Research question 3. What is the relatedness of the species isolated sing WGS methods?

Research question 4. Which DUSs are present in the genomes of *N*c and how many repeats of each do they contain?

5.3. Hypothesis

The prevalence of *Nc* in the pharynx is high and can be a reservoir of AMR genetic material to pathogenic *Neisseria*.

5.4. Aims

This study aims to determine the proportion of 50 participants colonised by *Nc* in their pharynx and to characterise these isolates using phenotypic and genotypic methods.

5.5. Objectives

i. Obtain pharyngeal swabs from 50 participants.
ii. Isolate *Nc* using selective LBVT.SNR media.
iii. Use MALDI-ToF to identify *Nc* to species level
iv. Determine the MICs of *Nc* to penicillin, ceftriaxone, cefixime, azithromycin, ciprofloxacin, tetracycline and gentamicin by agar dilution.
v. Perform WGS on a subset of isolates
vi. Use WGS to determine the species and AMR determinants.
vii. Perform phylogenetic analyses to ascertain the relatedness of *Nc*.
viii. Characterise the dialects and frequency DUS within *Nc* genomes.

5.6. Ethical considerations

Ethical approval was granted by the LSHTM Research Ethics Committee. Approval was granted on 14/06/2019 (Ref: 17126) (Appendix C1).

5.7. Sample size calculation

According to the mean prevalence of *Nc* species in previous studies [(75%), Table 4, 1.3.5], the ideal sample size would have been 289 to have a confidence level of 95% and a ±5% margin of error. The confidence level is a measure of certainty regarding how accurately a sample reflects the population being studied. The margin of error refers to the degree to which research results may differ from the true value within the general population. However, our study showed a carriage prevalence of 86% (5.9) which adjusts the ideal sample size to 186. In this study, a sample size of 50 participants was used based on a realistic success rate resulting in a margin of error of ±9.6%, with a 95% confidence level.

5.8. Participant recruitment and specimen processing

5.8.1. Participant Recruitment

A total of 50 staff and students from the LSHTM were recruited in this study. In total, four recruitment sessions were conducted, one session per week; three were in the Keppel Street building, with a further session in the Tavistock Square building.

Recruited participants were informed of the study procedure, requirements, aims of the study and outcome measures. An eligibility checklist (Appendix C2) was also carried out to determine the suitability of each participant. The study exclusion criteria were as follows:

1. Any antibiotic use within the month leading up to the recruitment date.
2. Usage of antiseptic mouthwash (e.g. Corsodyl or Listerine) on the date of the recruitment.
3. Allergy to chlorhexidine.
4. Participants who were taking immunosuppressants such as methotrexate.

An information leaflet was provided to eligible participants, who were offered a total of five minutes to read this. A written consent form was then given to the participants to sign (Appendix C3). The participants were asked to indicate their age and sex alongside their anonymous participant number.

5.8.2. *Participant Sampling*

An anonymized swab was taken with a sterile DrySwab (MWE - Wiltshire, England) from the peritonsillar area of each participant. The participants were subsequently instructed to gargle with 10 mL of Corsodyl for 60 seconds. After the gargle, a second swab was used to swab the same peritonsillar area of the participant. The swab was labelled with a participant number paired with the pre-gargle specimen to indicate both samples were from the same participant. A summary of the recruitment and sampling procedure is described in Fig. 44.

5.8.3. *Specimen Processing*

Swabs were expressed by vortexing rigorously in 1 mL of sterile PBS (2.1.11) and then labelled with the participant number. A total of 50 µL of the participant's expressed sample was streaked onto an LBVT.SNR agar plates (2.1.4). A 10^{-3} dilution was prepared from the expressed participant sample and 100 µL was spread onto the surface of a chocolate agar plate, using a sterile spreader. Agar plates were incubated as per 2.3.2. The expressed swabs were stored for one week at 4°C, after which they were disposed of. After incubation, the number of discrete colonies on the chocolate agar were counted on each plate. The presence of *Nc* species on the LBVT.SNR agar was also noted.

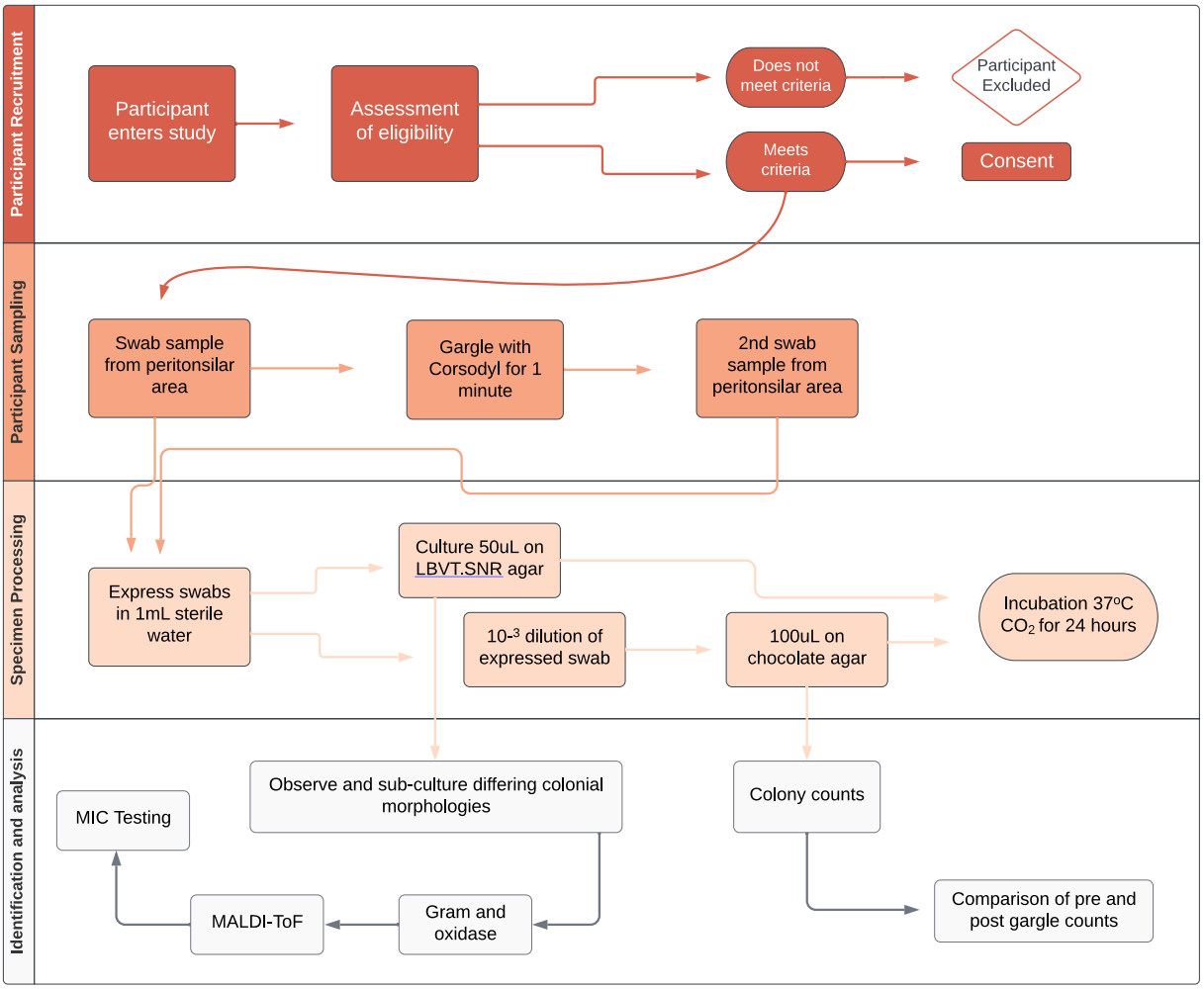


Figure 44. Participant recruitment, intervention and laboratory processing of participant specimens for the cross-sectional study. Image created with Lucidchart.com.

5.9. Confirmation of *Nc* identity and antimicrobial susceptibility testing

Cultured isolates were first observed for colonial morphology, including colour, texture, and size. Morphologically distinct colonies from the LBVT.SNR agar (2.1.4) were sub-cultured on chocolate agar (2.1.1) for further identification and AST. Oxidase (2.5.2) and Gram staining (2.5.3) were performed on colonies of interest. Oxidase positive, Gram-negative cocci were considered as presumptive *Neisseria species*. Isolates were stored in 20% glycerol BHI broth (2.1.5) at -70˚C until further testing. Identification to species level was determined by MALDI-ToF MS, using a Bruker MALDI Biotyper (2.5.7). Identification values of 2.0 or over were accepted, while values under 2.0 were repeated once.

Minimum inhibitory concentrations for penicillin, ceftriaxone, ciprofloxacin, azithromycin, tetracycline, and gentamicin were all determined by agar dilution (2.7.3). Cefixime MICs were obtained by E-test (Biomerieux) (2.7.4). Gonococcal WHO controls K, G, V, F, X and Y [216] (2.2.1) were included in the AST, due to the lack of *N*c control strains. Isolates with a penicillin MIC >1 mg/L were tested for β-lactamase production using a cefinase disk (2.7.2).

As there are no MIC breakpoints for *N*c, calculated rates of reduced susceptibility (referred to as resistance for ease) used the CLSI[103] and EUCAST v.13.1 recommended breakpoints[246]. Gentamicin breakpoints used epidemiological values suggested previously[247]. Resistance rates to all antimicrobials were calculated for all *N*c overall and for each species individually.

5.10. Whole genome sequencing and bioinformatic analyses

Total genomic DNA was extracted using the PureLink Genomic DNA Mini Kit extraction kit (2.9.2) and quantified using the Qubit dsDNA BR assay kit (Invitrogen). The Nextera XT library (2 x 151 bp) prep kit (Illumina, San Diego, California, US) was used to prepare the sequence libraries as per manufacturer's protocol. The samples were sequenced on a MiSeq System (Illumina) as per

the recommended protocol. Additional Illumina (2 x 251bp) sequencing was performed at MicrobesNG (MicrobesNG, Birmingham, UK). Raw sequence data were quality controlled using Trimmomatic v0.38[248] with the following specifications: Leading:3 Trailing:3 SlidingWindow:4:20 Minlen:36. Quality control (QC) checks were performed using FastQC v0.11.8[249]. Fastq reads were mapped against reference sequences using BWA MEM with default settings[250] and viewed in Artemis and ACT[251,252]. *De novo* sequence assemblies were performed using Spades v3.13[253] with default settings, a coverage cut-off of 20 and k-mer lengths of 21, 33, 55, 77, 99 and 111. Draft genome multi-fasta files were evaluated using Quast assessment tool v5.0.2[254]. Contigs were ordered against a *N. meningitidis* MC58 (accession AE002098) using ABACAS v1.3.1 using -dmbc settings[255]. Non-matching contigs were appended to the ordered contigs. The resulting assemblies were polished using Pilon v1.22 with default settings[256] and annotation using Prokka v1.13 in Gram negative mode[257].

The assembled contigs were screened for AMR genes using ABRicate[258] v1.0.1 and CARD[259], and NCBI AMRFinderPlus[260] databases and combined. Putative plasmid replicons were identified using the ABRicate with the PlasmidFinder database[261]. MLST profiles were determined using the software package MLST v2.16.1 from the draft assemblies[262,263]. Kraken2 using draft assemblies and the minikraken_8Gb_20200312 database[263] was used to predict species. The BSR-Based Allele Calling Algorithm (chewBBACA)[264] and predetermined *Neisseria* schema was used to generate cgMLST profiles and paralog removal using alleles present in 95%[121]. Allele profile data was used to generate a MSTree in Grapetree using --wgMLST and default settings[234]. Heatmaps was generated using Morpheus website (software.broadinstitute.org) with hierarchical clustering using Euclidean distance, average linkage method. All WGS analysis was performed by Dr Richard Stabler.

5.11. Deduplication of commensal *Neisseria* isolates

The MIC values generated were used to deduplicate isolates within individual patients, using the following criteria:

1) Isolates with the same phenotypic appearance on LBVT.SNR agar, and

2) Isolates with same species ID by MALDI-ToF, or WGS where MALDI-ToF did not give an ID, and

3) Isolates with at least five out of seven antibiotic matching MICs, within 1 $\log_2$ MIC.

5.12. Statistical analysis

All statistical analyses were performed with STATA 18. Prevalence and 95% confidence intervals (CI) were calculated for each of the *N*c species. The MICs between *N*c species was compared using the Kruskal-Wallis rank sum test. To enable statistical testing, MICs above the maximum or below the minimum range tested were converted to the dilution before or after the limit of detection, as previously described[79]. For example, azithromycin MIC>256 mg/L was expressed as 512 mg/L.

5.13. Results

5.13.1. Participant demographics and Neisseria isolates

Fifty participants were recruited with 37 (74%) females and median age was 35 (range 17 to 81). The number of participants colonised with *N*c was 43/50, generating an estimated population prevalence of 86% (95% CI; 73.8%, 93%). In total, there were 143 morphologically distinct *N*c isolates cultured from the 43 participants. A total of 42 isolates were removed as duplicates, leading to a final total of 101 isolates from the 43 participants that grew *N*c.

5.13.2. Neisseria species prevalence and characterisation

The most common *N*c species detected by MALDI-ToF was *N. subflava* (62/101, 61.4%) (Appendix C4). The second most prevalent species was *N. flavescens* (12 isolates, 11.9%), then *N. perflava* (10, 9.9%), *N. macacae* (6, 5.9%) and *N. mucosa* (3, 2.9%) (Appendix C4). Twenty isolates

(19.8%) were identified by MALDI-ToF as either one of two probable species, both having an index of over 2.0 (high confidence identification); the isolate with the highest index was considered as the primary ID (Appendix C4). No ID was possible on eight isolates by MALDI-ToF; these were classified as *Neisseria spp* (Appendix C4).

N. subflava had the highest incidence among the participants, with 74% (37/50 participants) carrying this species. This was followed by *N. flavescens* (20%, n=10), *N. perflava* (18%, n=9), *N. macacae* (10%, n=5) and *N. mucosa* (6%, n=3). Ten participants (20%) harboured a single *N*c species, however, some participants harboured multiple isolates; 18 (32%) participants were colonised by two isolates, 11 (22%) by three isolates, 2 (4%) by five isolates and 1 (2%) each were colonised by four and eight isolates (Fig. 45).

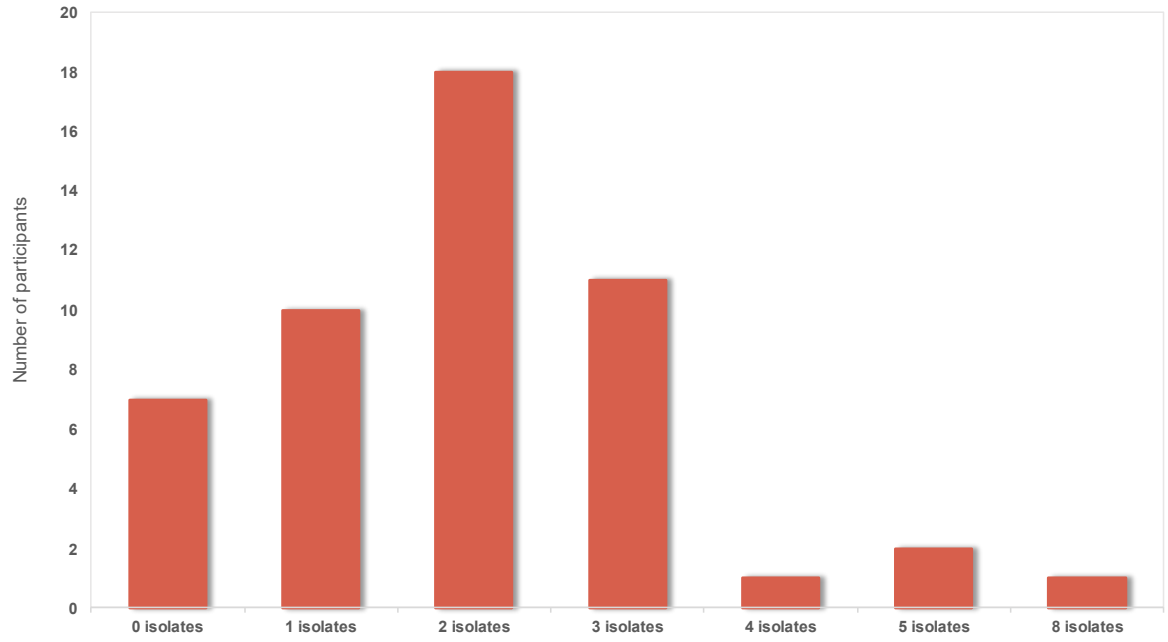


Figure 45. Number of commensal *Neisseria* isolates present in the pharynx of 50 study participants. Analysis was performed with results obtained from MALDI-ToF MS.

5.13.3. *Antimicrobial susceptibility of commensal Neisseria species*

After deduplication of isolates, the following MIC data were analysed: penicillin and ceftriaxone MICs for 101 and 100 isolates respectively and for cefixime, ciprofloxacin, azithromycin, gentamicin and tetracycline, 91 isolates MICs (Table 35). The median MICs for penicillin, ceftriaxone, cefixime, ciprofloxacin, tetracycline, azithromycin and gentamicin were 1 mg/L, 0.06 mg/L, 0.064 mg/L, 0.032 mg/L, 0.5 mg/L, 0.5 mg/L and 4 mg/L respectively (Table 35, Fig. 46 and Appendix C5). No isolates produced a detectable β-lactamase. The proportion of isolates overall resistant to penicillin and azithromycin according to both CLSI and EUCAST breakpoints was 26.7% (27/101) and 29.3% (27/92) respectively (Appendix C5). Of the penicillin resistant isolates, 10 were also resistant to azithromycin. *N. subflava* had the highest number of resistant isolates to both antibiotics (PEN; n=15/59 [25.4%], AZI; n=15/58 [25.9%]) (Table 36), with seven isolates being resistant to both antimicrobials. According to CLSI breakpoints, the proportion of isolates resistant to ceftriaxone, cefixime, ciprofloxacin and tetracycline were 5%, 4.3%, 16.3% and 22.8% respectively. The proportion of isolates resistant to these antibiotics differed by EUCAST breakpoints; they were 13.0%, 5.4%, 45.7% and 37%. No isolates were resistant to gentamicin.

The Kruskal-Wallis H was performed only on *N. subflava, N. macacae, N. perflava* and *N. flavescens* (Table 35). The test demonstrated no statistically significant difference in MIC values between the four *Neisseria* species (Table 35).

Table 35. Summary of minimum inhibitory concentration characteristics by commensal *Neisseria* species and relationship between species and MIC.

Antimicrobial	PEN	CRO	CFX	CIP	TET	AZI	GEN
Median MICs (mg/L)							
Neisseria* all *spp	1	0.06	0.064	0.032	0.5	0.5	4
N. flavescens	1	0.06	0.047	0.032	0.5	0.25	4
N. macacae	1.5	0.125	0.064	0.5625	1.5	0.5	3
N. mucosa	0.5	0.06	0.047	0.016	0.5	0.125	4
N. perflava	0.5	0.07	0.064	0.016	0.5	1	4
N. subflava	1	0.06	0.023	0.032	0.5	0.375	4
MIC^{N}	101	100	92	92	92	92	92
Modal MIC	1	0.06	0.064	0.016	0.5	0.032	4
Range	0.03-4	0.015-8	0.002-0.5	0.008-32	0.032-32	0.016-512	0.5-16
IQR Range	0.5-2	0.06-0.125	0.047-0.094	0.016-0.5	0.25-1	0.06-1.5	2-4
Geometric mean	0.7	0.07	0.06	0.09	0.81	0.37	3.47
Kruskall-Wallis^{N}	90	88	86	86	86	86	86
H score	2.56	2.94	3	4.57	2.9	0.61	2.03
p	0.464	0.4	0.39	0.21	0.41	0.89	0.57

N; number of isolates, IQR; Interquartile Range, PEN; penicillin, CRO; ceftriaxone, CFX; cefixime, CIP; ciprofloxacin, TET; tetracycline, GEN; gentamicin

Table 36. Resistance rates of commensal *Neisseria* species to the tested antimicrobials, interpreted by CLSI and EUCAST breakpoints for *Neisseria gonorrheae*.

	CLSI N^R/N^T (%)						
	PEN	**CRO**	**CFX**	**CIP**	**TET**	**AZI***	**GEN§**
Breakpoint (R>)	**1**	**0.25**	**0.25**	**0.5**	**1**	**1**	**16**
Overall	27/101 (26.7%)	5/100 (5%)	4/92 (4.3%)	15/92 (16.3%)	21/92 (22.8%)	27/92 (29.3%)	0/92 (0%)
N. flavescens	3/15 (20%)	0/15 (0.0%)	1/13 (7.7%)	1/13 (7.7%)	2/13 (15.4%)	5/13 (38.5%)	0/13 (0%)
N. macacae	3/6 (50%)	0/6 (0.0%)	0/6 (0.0%)	3/6 (50%)	3/6 (50%)	2/6 (33.3%)	0/6 (0%)
N. mucosa	1/3 (33.3%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0%)
N. perflava	4/10 (40%)	1/10 (10%)	0/9 (0.0%)	1/9 (11.1%)	1/9 (11.1%)	2/9 (22.2%)	0/9 (0%)
N. subflava	15/59 (25.4%)	4/59 (6.8%)	3/58 (5.1%)	10/58 (17.2%)	15/58 (25.9%)	15/58 (25.9%)	0/59 (0%)
Neisseria spp (NO ID)	1/7 (14.2%)	0/7 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	3/3 (100%)	0/2 (0%)
	EUCAST N^R/N^T (%)						
	PEN	**CRO**	**CFX**	**CIP**	**TET**	**AZI***	**GEN§**
Breakpoint (R>)	**1**	**0.125**	**0.125**	**0.06**	**0.5**	**1**	**16**
Overall	27/101 (26.7%)	13/100 (13%)	5/92 (5.4%)	42/92 (45.7%)	34/92 (37%)	27/92 (29.3%)	0/92 (0%)
N. flavescens	3/15 (20%)	3/15 (20%)	1/13 (7.7%)	6/13 (46.2%)	5/13 (38.5%)	5/13 (38.5%)	0/13 (0%)
N. macacae	3/6 (50%)	1/6 (16.7%)	1/6 (16.7%)	6/6 (100%)	4/6 (66.7%)	2/6 (33.3%)	0/6 (0%)
N. mucosa	1/3 (33.3%)	0/3 (0.0%)	0/3 (0.0%)	1/3 (33.3%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0%)
N. perflava	4/10 (40%)	2/10 (20%)	0/9 (0.0%)	4/9 (44.4%)	2/9 (22.2%)	2/9 (22.2%)	0/9 (0%)
N. subflava	15/59 (25.4%)	6/59 (10.1%)	3/58 (5.1%)	24/58 (41.3%)	23/58 (39.7%)	15/58 (25.9%)	0/59 (0%)
Neisseria spp (NO ID)	1/7 (14.2%)	1/7 (14.2%)	0/3 (0.0%)	1/3 (33.3%)	0/3 (0.0%)	3/3 (100%)	0/2 (0%)
***N. gonorrhoeae* (%R)‡**	17.9	0	0.8	42.7	62.9	4.2	n/a

NR; number of resistant isolates, NT; total number of isolates tested, n/a; not applicable

PEN; penicillin, CRO; ceftriaxone, CFX; cefixime, CIP; ciprofloxacin, TET; tetracycline, AZI; azithromycin; GEN; gentamicin

**Azithromycin based on ECOFF of S< 1mg/L, §Gentamicin based on previous recommended breakpoint[247]*

‡Data from Gonococcal Resistance to Antimicrobials Surveillance Programme, 2020[150]

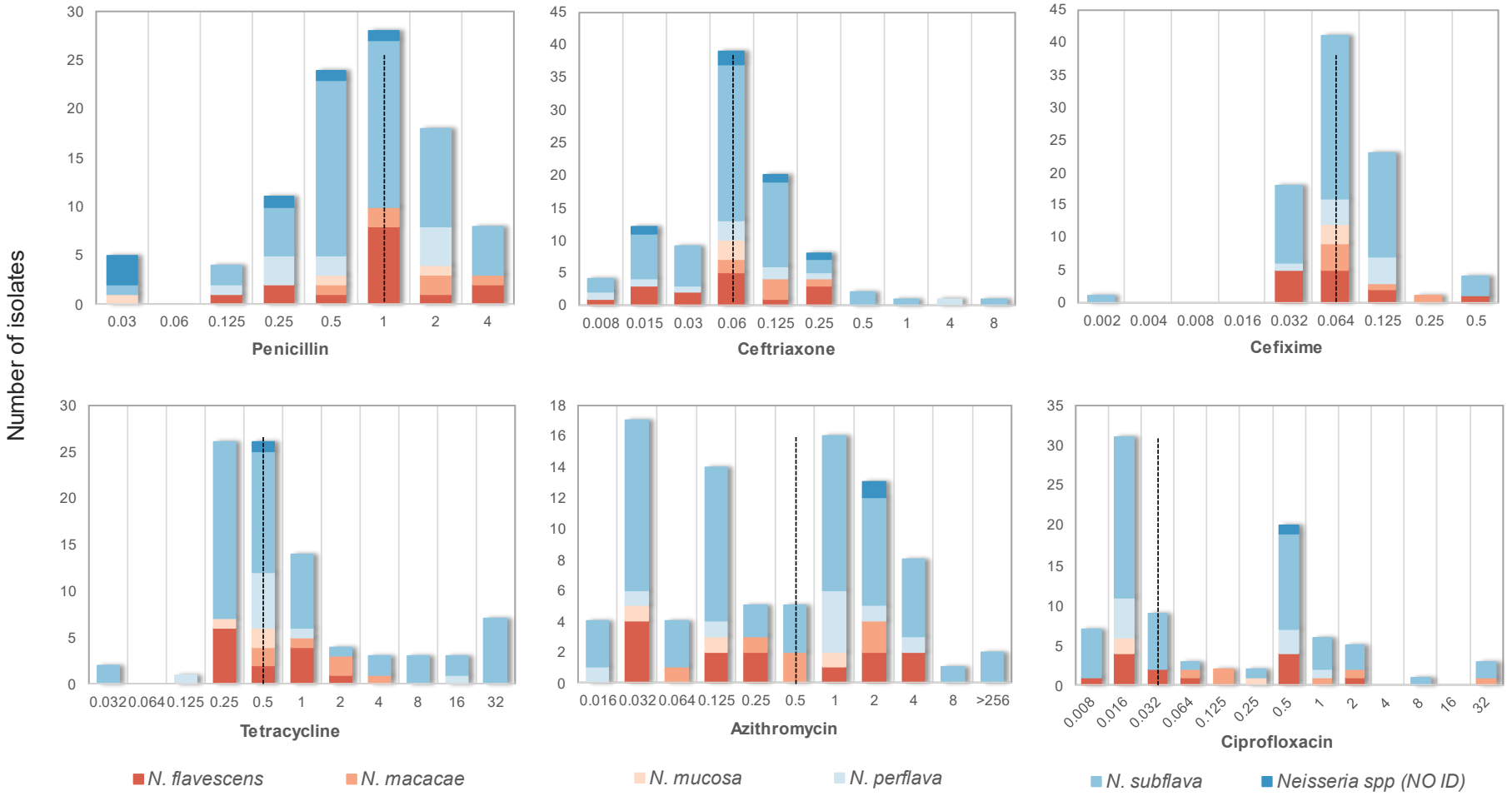


Figure 46. Minimum inhibitory concentration distribution of commensal *Neisseria* species to penicillin, ceftriaxone, cefixime, ciprofloxacin, azithromycin and tetracycline, performed by agar dilution. The dotted line indicates the median MIC for each antimicrobial.

5.13.4. Genomic analysis and relatedness

Thirty isolates were selected for WGS, covering isolates with ceftriaxone MICs ≥0.125 mg/L (15 isolates) and <0.125 mg/L (four isolates), at least one of each species from the MALDI-ToF identification (six isolates) and three isolates where MALDI-ToF identification was not possible (Appendix C8). The genomic data from the study isolates, along with 61 *Neisseria* reference genomes (Appendix C6), was used to generate cgMLST neighbour joining phylogeny.

The 91 *Neisseria* isolates clustered in approximately five clusters (Fig. 47). As previously described, *N. meningitidis* and *N. gonorrhoeae* isolates clustered together with *N. lactamica* and *N. polysaccharea*[4] however *N. bergeri* and *N. cinerea* were also present within the cluster. No study isolates were present in the *N. meningitidis/N. gonorrhoeae* cluster (Appendix C6 and Appendix C7). The *N. bacilliformis* group also contained *N. bacilliformis, N. animaloris*, and 8 other species but no study isolates (Appendix C6 and Appendix C7). MLST analysis of *N. perflava* CCH10-H12, which clustered with *N. mucosa* isolates only matched three alleles in the database: *abcZ*233, adk178 and pdhC561 (Appendix C6). This combination of alleles was only found together in ST-16693 but this ST was not associated with any isolates in the database. *abcZ*233 was present in ST-3706 (*N. mucosa*), ST-9926 (*N. perflava*), ST-10150 (*N. mucosa*), ST-16006 (*N. mucosa*), ST-16037 (*N. mucosa*), ST-16480 (*N. mucosa*). *Adk*178 was present in ST-3706 (N*. mucosa*) and *pdhC*561 was present in ST-12049 (*N. mucosa*).

The *N. flavescens* cluster contained 3/4 *N. flavescens*, a single *N. subflava* and 10 study isolates. The *N. subflava* cluster contained 4/5 *N. subflava* and 2/2 *N. perflava* plus 16 study isolates. Finally, the *N. macacae* cluster contained 1/1 *N. macacae*, 3/3 *N. elongata*, 3/3 *N. sicca* and 1/1 *N. mucosa* plus four study isolates (Appendix C6 and Appendix C7).

We compared the first and second species identification given by MALDI-ToF and Kraken2 from the genome sequence, excluding the three isolates with no MALDI-ToF ID. A total of 16/26 (61.5%) isolates had ID concordance between the primary MALDI-ToF ID and Kraken2 and 22/26 (84.6%) had concordance between any MALDI-ToF ID and Kraken2 (Appendix C8). The three isolates with no MALDI-ToF ID were predicted as *N. subflava* by Kraken2. All isolates identified as *N. subflava,*

N. perflava or *N. flavescens* by MALDI-ToF were predicted as *N. subflava* by Kraken2. The isolates identified as *N. macacae* by MALDI-ToF were predicted as *N. mucosa* by Kraken2.

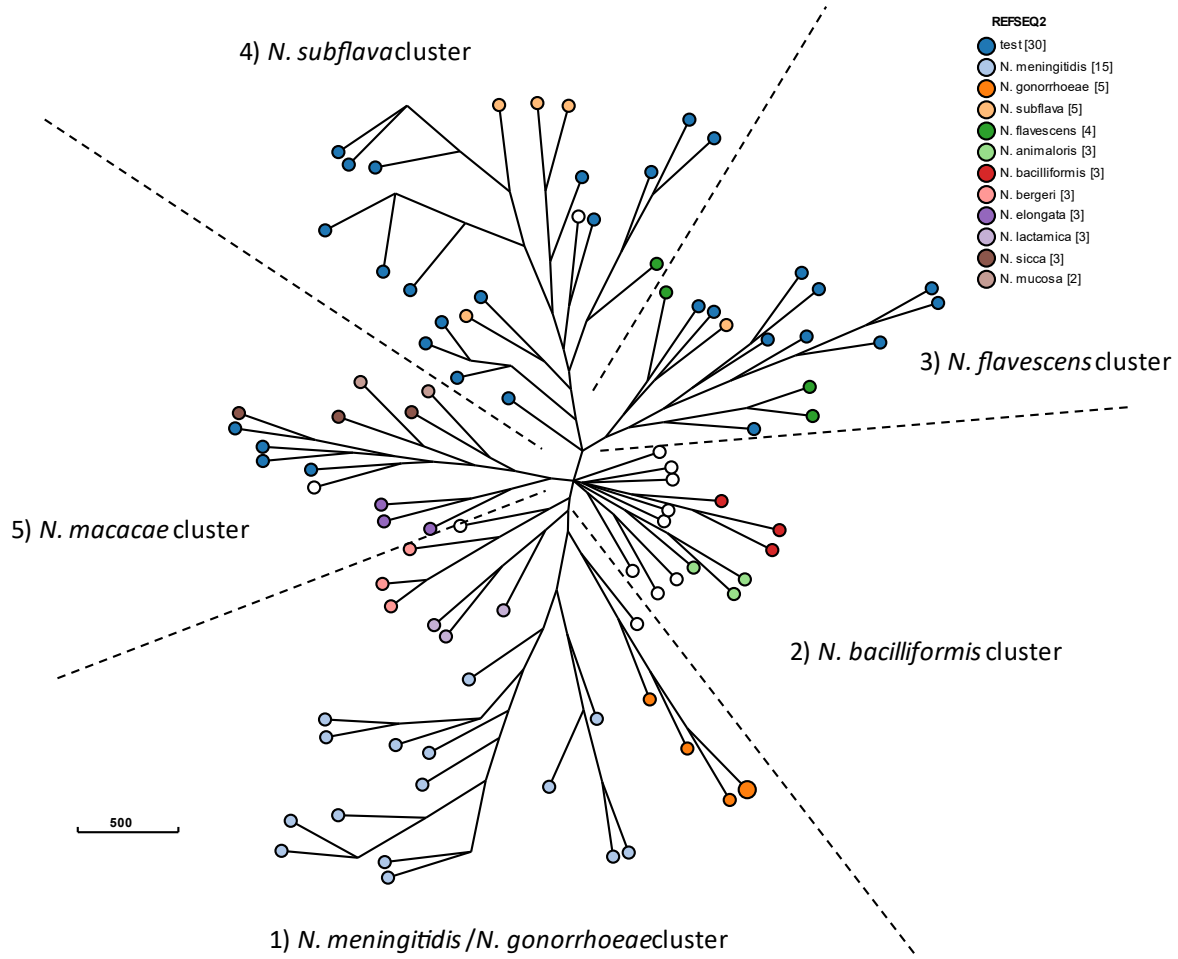


Figure 47. Core genome multi-locus sequence typing (cgMLST) nearest neighbour phylogeny. cgMLST phylogeny derived from 842 gene alleles conserved within 95% of 30 commensal *Neisseria* plus 61 reference *Neisseria* species. Nodes coloured by reference species, study isolates coloured dark blue.

5.13.5. Genotypic antimicrobial resistance

One isolate (49A) produced a poor assembly so was removed from further analysis. Analysis of the remaining 29 *N*c genomes for AMR related genes identified five matches (min 80% identity, 80% coverage) with the CARD database, three with ResFinder, eight with MEGARes additionally 14 virulence related genes with matched against VFDB (Fig. 48).

The MacAB-TolC tripartite macrolide efflux complex consists of *macA, macB* and *tolC*. *macB* was present in most isolates except 12/14 of the *N. bacilliformis* cluster isolates and *N. perflava* CCH10-H12, however *macA* was only identified in *N. meningitidis/N. gonorrhoeae* cluster isolates and *N. macacae* group isolates plus 49A[265] (Fig. 48). Similarly, *mtrC* and *mtrD*, along with *mtrE,* encode a multidrug efflux complex but while mtrCD were conserved within *N. meningitidis/N. gonorrhoeae* cluster [cluster 1], these genes differentiated the *N. mucosa/sicca/macacae* (present) from *N. elongata* and *N. perflava* CCH10-H12 (absent) within the *N. macacae* cluster[28]. *mtrCD* was also completely absent from the *N. bacilliformis* cluster[19]. Within the *N. flavescens* and *N. subflava* cluster all isolates except *N. flavescens* ERR2764931 had *mtrD* but only five isolates also had *mtrC*.

PenA, linked to β-lactam resistance, was only present in the *N. meningitidis/N. gonorrhoeae*, *N. flavescens* (except *N. flavescens* ERR2764931) and *N. subflava* clusters. TetM, a ribosomal protection protein that confers tetracycline resistance, was present in seven isolates: *N. subflava* C2007002879, 1A, 10A, 14B, 18B, 35A and 48B, which were spread evenly across *N. flavescens* and *N. subflava* clusters (Fig. 48). Isolates 14B and 18B had tetracycline MICs of 0.5 mg/L and 1A, 10A & 35A had MICs of 16-32 mg/L (Appendix C5). Tetracycline MIC testing was not performed on isolate 48B as it was nonviable on resuscitation.

Capsule polysaccharide modification proteins (LipA/LipB) and capsule polysaccharide export ATP-binding protein (CtrD) were present in all *N. meningitidis* (except *N. meningitidis* alpha14) but absent from *N. gonorrhoeae* and *N. lactamica.* Additionally, all three genes were conserved within the majority of *N. flavescens/N. subflava* clusters (*lipA*: 34/36, *lipB*: 32/36, *ctrD*: 22/36) but absent from *N. bergeri, N. polysaccharea* and *N. cinerea*.

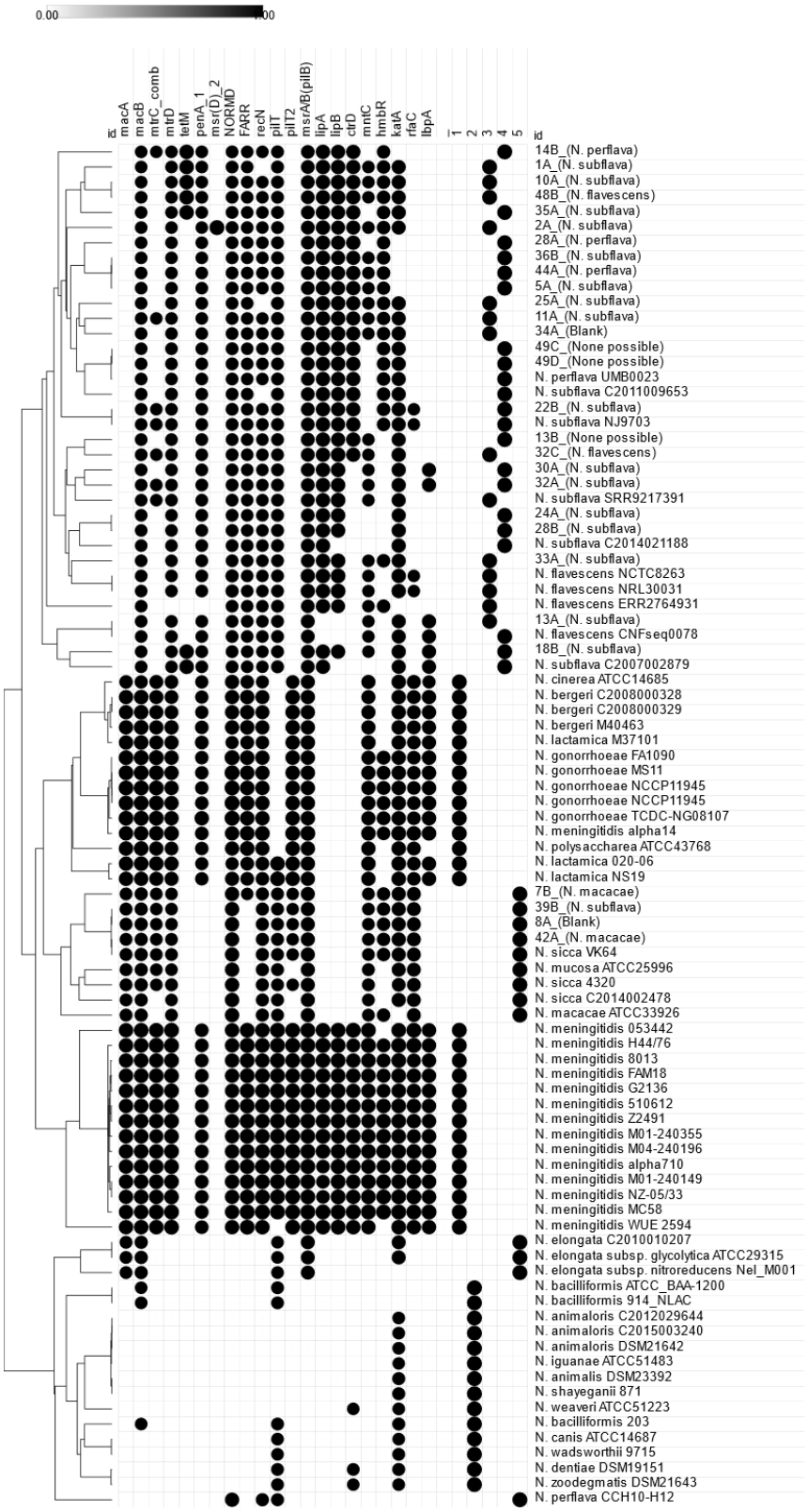


Figure 48. AMR and virulence genes. Draft genomes were analysed for AMR genes (CARD, ResFinder and Megares databases) and virulence (VFDB) genes using Abricate (min ID/coverage 80%). Circles represent the presence of gene, scaled to %ID. Similar profiles were grouped using Euclidean hierarchical clustering using average linkage algorithm in Morpheus. Study isolates are given with MALDI-ToF identification in parenthesis. 1 to 5 indicate cgMLST clustering group; 1 = *N. meningitidis*/*N. gonorrhoeae* cluster, 2 = *N. bacilliformis* cluster, 3 = *N. flavescens* cluster, 4 = *N. subflava* cluster, 5 = *N. macacae* cluster.

5.13.6. *Analysis of DNA transfer mechanisms*

The *N*c genomes were screened for the presence of gcDUS (5'-GCCGTCTGAA-3'), AT-DUS (5'-ATGCCGTCTGAA-3') and vDUS (5'-GTCGTCTGAA-3'). All three DUS dialects were found in the *N*c genomes. Overall, the *N. subflava* complex (*N. subflava, N. perflava* and *N. flavescens*) isolates had more gcDUS repeats than vDUS whereas the opposite was seen with *N. macacae*. The *N. subflava* complex isolates had 2738-2990 gcDUS, 144-192 AT-DUS and 158-276 vDUS repeats. *N. macacae* isolates carried 247-292 gcDUS, 29-40 AT-DUS and 3608-3802 vDUS repeats (Appendix C9). No genetic plasmid markers were identified; however, *tetM* has previously been identified as plasmid mediated [48]. Raw reads from all *tetM* positive isolates were mapped against pEP5289 (GU479466, 'Dutch' *tetM*) and pEP5050 (GU479464, 'American' *tetM* genetic load area) which showed no mapped reads except to the *tetM* gene. Subsequent analysis identified a cryptic 40kb plasmid in isolate 8A (*N. macacae*) that had 95% coverage, 99.7% identity to a *Ng* plasmid (CP048906) however this plasmid did not contain any AMR genes.

5.14. Discussion

The value of monitoring carriage and the AMR reservoir of *N*c from the human oropharynx is becoming increasingly evident, not only to prevent the development of AMR in *Nm* and *Ng*, but also the assess the risk of oropharyngeal colonisation and persistence of the pathogenic *Neisseria* species. Not only is there transmission of AMR genes between *Neisseria* species, there is also evidence *N*c are shared between intimate partners[266], further exacerbating the problem of AMR transmission. In this study we characterised the carriage, genomic relatedness and antimicrobial susceptibility profiles of *N*c species, acquired from the pharynx of 50 LSHTM volunteers.

In this study, 84% of the study population were colonised with at least one *N*c species. This finding aligns with recent studies reporting *N*c carriage of 68%[79] and 100%[78]. However, our findings contrasted with those found by Diallo *et al*[81] and Le Saux *et al*[83] who found a *N*c prevalence of 10.2% and 11.6% respectively. These studies were focused on colonisation of *N. meningitidis* and specifically vaccinated individuals, and it has been suggested that both *N. meningitidis* and *N*c carriage can be negatively associated with recent meningococcal vaccination, especially with the

MenAfriVac® vaccine[81]. Also, both these studies used Theyer-Martin (TM) media for pathogenic *Neisseria* species, whereas some *N*c species such as *N. cinerea, N. subflava* and *N. mucosa* do not grow very well on this media[20]. This was confirmed by the lack of growth of study *N*c on *Ng* selective VCAT agar. LBVT.SNR media, formulated specifically for the isolation of *N*c[76], aligns with two older studies that used the same media and identified high prevalence of 96.6%[76] and 100%[77]. Additionally, the study by Sáez *et al* that found 100% prevalence used both LBVT.SNR and TM media, the latter added specifically to ensure the recovery of *N. meningitidis* and *N. lactamica*[77]. Interestingly, a study by Laumen *et al* suggested that MSM have a lower *N*c colonisation rate (51.6%) than non-MSM (100%)[79].

The most common *N*c species found in this study was *N. subflava*, with 61.4% and 74% of participants colonised by this species. The colonisation rate of *N. subflava* was similar to two recent studies[78,79], especially when combined with *N. flavescens* and *N. perflava* as previously described[26]. Surprisingly, no *N. lactamica* were identified from the study participants, however this was likely due to omission of selective media for pathogenic *Neisseria* species. In fact, as part of our quality control checks, a *N. lactamica* laboratory reference strain grew very poorly on SBVT.SNR media. Carriage of *N. lactamica* seems to be variable depending on the population; the prevalence of *N. lactamica* in previous studies ranged from 0.4%[78] to 17.3%[80]. Interestingly, some studies showed that young children carry *N. lactamica* at much higher rates than adults[80,81], which could further explain the lack of recover in our study.

Concordance between MALDI-ToF species identification and Kraken2 prediction was just 65.2% when considering the primary species ID. This further demonstrates the challenge of accurate identification in this homogeneous genus, due to the limitations of both technologies. The accuracy of these techniques is only as good as the curation of the database itself demonstrated by several reports of misidentification of Nc by MALDI-ToF[97,98,267]. Similarly, genomic identification is limited by the high genetic recombination of *Neisseria* species[25,268–270] coupled with the lack of an internationally accepted genomic identification scheme.

The introduction of more advanced techniques such as WGS, rMLST and cgMLST have led to several re-classifications of existing species and the discovery of novel species[24,26,28]. In this study,

the isolates clustered into three distinct groups, the *N. flavescens, N. perflava* and *N. macacae* clusters, in line with previous findings. The clustering agreed with previously suggested re-classifications of *N. perflava* and *N. subflava* into different variants of *N. subflava*[28]. Similarly, it has been suggested that *N. macacae* and *N. mucosa* can be merged into a single *N. mucosa* group[25], which our cgMLST cluster analysis supports.

Resistance to all antimicrobials except gentamicin and cefixime was high according to both CLSI and EUCAST breakpoints. The median MIC to ceftriaxone was 0.06 mg/L, which although phenotypically susceptible according to both CLSI and EUCAST breakpoints is just 1-2 $\log_2$ MIC lower than the 0.125-0.25 mg/L breakpoint with one isolate having an MIC of 8 mg/L. This translates to resistance rates of 5% (CLSI) and 13% (EUCAST) compared to *Ng* resistance rates of 0% for the same year in England[150] but lower than *N*c resistance rates of 28% reported in Vietnam[78]. Differing AMR rates could be due to differences in study populations, as the study in Vietnam included only men MSM[78]. This patient group are described as having a higher likelihood of repeated gonococcal infection and exposure to ceftriaxone, leading to AMR selection pressures on *N*c[78].

Commensal *Neisseria* species with high ESC MICs pose a significant reservoir for transfer of resistance and development of mosaic genes in pathogenic *Neisseria* species. Although other antimicrobials are no longer used as empirical treatment, resistance to these should not be overlooked, as there has been evidence of macrolide, tetracycline and fluoroquinolone AMR transfer[271]. Investigations of the *Neisseria* resistome have found high resistance to β-lactams, fluoroquinolones encoded by mutations in *gyrA*, tetracylines due to *tetM* as well as TEM-type β-lactamases[272]. Importantly, a recent study demonstrated *in vitro* transformation of zoliflodacin resistance, a new DNA replication inhibitor evaluated for treatment of *Ng*, from *N*c to *N*g, suggesting important implications for the introduction of new antimicrobials[273]. In this study, 30 *N*c isolates genomes were analysed for genotypic markers of acquired resistance and we identified several acquired resistance genes. For example, *msr*(D) responsible for high level macrolide resistance (>256 mg/L)[274], was present in 2A which had an MIC of >256 mg/L. Macrolide resistance has also been associated with overexpression of the MtrCDE efflux pump, which also confers

resistance to β-lactams, tetracyclines and fluoroquinolones[274]. The MtrCDE efflux pump is commonly found in *Ng*[274] and other *Neisseria* species, however correlation between presence of *mtrCDE* and any macrolide resistances was not identified. Similarly, most of our *N*c isolates had *macB*, another efflux pump complex also found in *Ng*[275], but there was no correlation with phenotypic resistance. Antimicrobial resistance due to overexpression of efflux pumps are associated with specific mutations[276] and the presence of efflux pumps genes do not necessarily translate to phenotypic resistance.

Transfer of AMR genes between isolates provides a rapid solution to antibiotic treatment compared to accumulation of new genes through evolutionary purposes. *N*c are proposed as a possible source of horizontally acquired AMR genes in pathogenic *Neisseria*, for example horizontal gene transfer of *penA* from *N. lactamica, N. macacae, N. mucosa* and *N. cinerea* to *Ng*[65,269,270,277]. *Neisseria* are naturally competent and therefore naked DNA is a primary method of acquiring new DNA. The Neisseria DUS sequences enhance this DNA uptake. Members of the *N. subflava* and *N. flavescens* clusters had more copies of gcDUS than vDUS and the opposite was true for the *N. macacae* cluster (Appendix C9). These findings agree with previous published data [10], [69] and suggest that DNA incorporation into *Ng* and *N. meningitidis* would be more efficient from *N. subflava* and N. flavescens clusters than *N. macacae* cluster isolates. Even though *N*c have fewer copies of AT-DUS that enhances transformation efficiency, these findings demonstrate the high likelihood of HGT between *N*c and pathogenic *Neisseria* species, not just relating to AMR, but also virulence and niche adaptation[278]. Plasmids also can transfer AMR genes in *Neisseria* for example *tetM* was associated with tetracycline resistance in six of our isolates (1A, 10A, 14B, 18B, 35A and 48B), three of which had tetracycline MICs of 16-32 mg/L (1A, 10A and 35A) and two had an MIC of 0.5 mg/L (14B and 18B) (Appendix C5). Tetracycline resistance due to *tetM* is usually coded on a conjugative plasmid in *Ng*, resulting in MICs of 16-64 mg/L[279]. No plasmid markers or known *tetM* carrying plasmids were detected suggesting *tetM* may be present in the chromosome of some *N*c species. Interestingly, a single plasmid was identified in a *N. macacae* isolate that had previously been sequenced in a *Ng* isolate. While this supports transfer between pathogenic and commensal Neisseria no AMR genes were present on this plasmid.

In our study we performed comprehensive phenotypic and genotypic analysis of both *N*c carriage, speciation, and AMR determinants, but it is not without limitations. Firstly, our sample size was small, which limited statistical power in some analyses, such as exploring the relationship between *N*c and AMR. For example, we were not able to analyse *N*c carriage by age, even though we collected this data. This would have been a worthwhile analysis, as some research suggests that age is one of the most important drivers in microbiota changes within the nasopharynx[280]. Additionally, we did not use *Ng* selective agar, which may have enabled us to recover *N. lactamica* due to the possibility of isolating *Ng/N. meningitidis* which was outside the scope of the project and had additional ethical considerations. There is currently no gold standard for speciation of *N*c; the accuracy of genomic and MALDI-ToF analyses are reliant on the accuracy of published reference genomes and identification databases. The nomenclature and speciation of *N*c is evolving, with species reclassified and new species being discovered, meaning that taxonomic errors in reference databases have been discovered[25]. This issue also extends to phenotypic and genotypic analysis of AMR. Firstly, there are no guidelines or resistance breakpoints for *N*c and most published literature have used CLSI or EUCAST breakpoints for *Ng*. This also means there are no international control strains for *N*c susceptibility testing which impacts the accuracy of both phenotypic and genotypic testing. Published fully susceptible *N*c reference genomes will enable detection of single nucleotide polymorphisms and mosaic genes as well as acquired resistances.

This study demonstrated high pharyngeal colonisation rates in our population with higher AMR rates than *Ng*. Although more research in needed to understand the mechanisms of HGT *in vivo*, monitoring *N*c may help us predict the rates of *Ng* resistant strains occurring in the future, especially relating to ESCs and other newly introduced antimicrobials.

CHAPTER 6

EVALUATION OF NOVEL TREATMENT OPTIONS FOR PHARYNGEAL GONORRHOEA

6.1. Introduction

The declining effectiveness of first-line therapeutic agents, along with the rise of MDR gonococcal strains and TFs with ESCs, highlights the urgent need for the evaluation of novel antimicrobials. This chapter presents the *in vitro* activity of CHX against *Ng*. The compound CHX is a readily available antiseptic, commonly used in the field of dentistry at concentrations of 0.06% or 0.2%[195,197]. The mode of action is well characterized; in low concentrations it disrupts the bacterial membrane by releasing potassium ions, altering the osmotic pressure of the cell[197] and has some ATPase activity. In high concentrations it precipitates cytoplasmic proteins and nucleic acids causing cell death[197]. Chlorhexidine gluconate has a good safety profile as it is free of systemic toxicity in oral use and has an LD_{50} value of 1800 mg/kg[195].

Recently there has been clinical interest in CHX gargles for the treatment of pharyngeal gonorrhoea, with *in vitro* studies and a clinical trial published[208,214]. The results of these trials have been variable, however clinical trial assessing the efficacy of Corsodyl on pharyngeal gonorrhoea had to stop early as they did not observe a reduction in gonococcal viability in five participants after seven days[214].

Before implementation of a new antimicrobial, it is useful to measure baseline susceptibility of the target organism, to determine the therapeutic potential of the compound. I have therefore determined the susceptibility of *Ng* to CHX using a variety of methods. As CHX is a topical agent, determination of bactericidal concentrations and minimum bactericidal contact time is essential, so I validated a microbroth dilution method for the determination of MICs and MBCs. This was a key step in the experimental process; agar dilution is the gold standard for MIC testing as *Ng* does not grow well in broth, however MBCs require a broth-based MIC method. Relevant to this, it can be challenging to determine gonococcal visual turbidity in broth due to poor growth, therefore I validated a resazurin-based microbroth MIC method that was used for both MICs and synergy testing. Microbroth dilution MIC is not widely used due to the fragility of *Ng*, however rapid microbroth dilution methods using resazurin have been developed[281]. Resazurin is a blue dye used to measure cell viability. Metabolically active cells internalise the dye and reduce it to resorufin, changing the colour of the inoculated broth pink, whereas inactive or nonviable cells remain blue[282].

Laboratory validation is a process by which the reproducibility, accuracy, analytical sensitivity and specificity of a new or existing assay are determined[283]. Usually, the new assay is compared to an existing or gold standard methodology, although this is not always necessary. It is recommended that a laboratory validation should contain at least 40 samples (referred to as data points) that are generated by testing and can be qualitative (detected / not detected) or quantitative (numerical value)[283]. Qualitative assay data are compared to a gold standard method by using a 2 x 2 contingency table and calculating the kappa coefficient, whereas quantitative data are compared using the R^2 coefficient of determination. The kappa coefficient is a statistical test used to measure the level of agreement between two or more datasets or observers. For both kappa and R^2 coefficients, values of or approaching 1 indicate very good agreement between methods.

Lastly, I assessed whether chlorhexidine and ceftriaxone are synergistic. Patients with multisite infections including the pharynx will need to be treated with ceftriaxone in addition to a CHX gargle, so it is key to assess whether these compounds are synergistic.

6.2. Research questions

Research question 1. What is the susceptibility of *Ng* to CHX as determined by MICs and MBCs?

Research question 2. What is the susceptibility of *N*c to CHX as determined by MICs?

Research question 3. What is the minimum contact time for CHX to eradicate *Ng in vitro*?

Research question 4. Does a one-minute CHX gargle reduce total pharyngeal microbiota and by how much?

Research question 5. Does a one-minute CHX gargle remove *N*c from the pharynx?

6.3. Hypothesis

First, we hypothesise that *Ng* is highly susceptible to CHX. Additionally, we hypothesise that CHX can eradicate *Ng* within one minute, *in vitro*. Lastly, that CHX can significantly reduce the pharyngeal microbiota and the presence of *N*c after gargling for one minute.

6.4. Aims

This study aims to determine the susceptibility of *Ng* strains to CHX and to determine whether a CHX gargle will significantly reduce pharyngeal microbiota.

6.5. Objectives

i. To determine whether GC broth sustains the growth of *Ng*.
ii. To evaluate a resazurin microbroth dilution assay for the determination of *Ng* susceptibility to CTX.
iii. To determine the CHX MICs of clinical and control *Ng* strains by agar dilution and resazurin microbroth dilution.
iv. To determine the CHX MBCs of clinical *Ng* strains.
v. To determine whether CHX can eradicate *Ng* in 30 and 60 seconds and whether porcine mucin has an inhibitory effect.
vi. To determine whether CHX and ceftriaxone are synergistic, by performing checkerboard assays.
vii. To the change in the total pharyngeal microbiota count and *N*c after a one-minute CHX gargle.

6.6. Ethical considerations

Ethical approval was granted by the LSHTM Research Ethics Committee. Approval was granted on 14/06/2019 (Ref: 17126) (Appendix C1) and 27/06/2019 (Ref: 17680) (Appendix D1).

6.7. Laboratory method validations

6.7.1. GC broth validation

Gonococcal broths (2.1.3) were inoculated into flat 96-well microtitre plates (100 μL) as per 2.3.3, to contain a final concentration of approximately 5 x 10^5 cfu/mL. The gonococcal strains tested were WHO control strains G, V, Y, F, N, X, K and P^{216}, across five batches of GC broth (B1-B5) (Table 37). All containers were incubated as per 3.4.2. The concentration of the 0.5 MF gonococcal suspension was determined using Miles and Misra (2.4). After 24 hours incubation, the gonococcal concentration in each container was determined again using Miles and Misra (2.4). In total, the GC broth validation generated 161 data points for analysis, fulfilling the minimum requirement for validation.

Table 37. Summary of gonococcal broth validations. Outlined are the number of WHO control *N. gonorrhoeae* strains tested on each gonococcal broth batch.

Isolate	Batch B1	Batch B2	Batch B3	Batch B4	Batch B5
WHO G	✓			✓	✓
WHO V	✓	✓		✓	✓
WHO Y	✓			✓	✓
WHO F		✓	✓		
WHO N			✓		
WHO X			✓		
WHO K				✓	✓
WHO P				✓	✓

The mean gonococcal inoculum was 5.1 $\log_{10}$ (range: 4.5, 6, SD ±0.49) leading to a mean 24-h growth of 8.6 $\log_{10}$ (range: 7.2, 9.7, SD ±0.58). On average, the growth of the gonococcal strains increased by 3.4 $\log_{10}$ (SD ±0.69) over 24h. This ranged from 2.4 $\log_{10}$ (strains V and Y, batch B4) to 4.5 $\log_{10}$ (strain Y, batch B1) (Fig. 49, Fig. 51). Gonococcal broth batch B1 had the highest overall 24-hour growth (4.3 $\log_{10}$, SD±0.14) and batch B4 had the lowest (2.8 $\log_{10}$, SD±0.58). All strains grew to at least 8 $\log_{10}$, except strain Y which grew to 7.2 $\log_{10}$ in batch B4, and strain P which grew to 7.5 $\log_{10}$ in batch B5.

The 24-hour growth of strains V, G and Y increased by a mean of 3.2 log10 (SE±0.36), 3.8 log_{10} (SE±0.29) and 3.7 log_{10} (SE±0.64) respectively, and had the highest log_{10} increase in batch B1, by 4.1 log_{10}, 4.3 log_{10} and 4.5 log_{10} respectively (Fig 49, Fig 50, Fig. 51). The mean 24-hour growth of strains K, F and P was 3.1 (SE±0.1), 4 (SE±0.15) and 2.5 (SE±0.05) log_{10} respectively (Fig. 52, Fig 53) and had the highest 24-hour growth in batches B4, (3.1 $log_{10,}$), B3 (4.1 log_{10}) and B5 (2.5 log_{10}) respectively. Strains X and N grew by 3.4 log_{10} (SE±0.08) and 3.1 log_{10} (SE±0.02) in batch B3 (Fig 53).

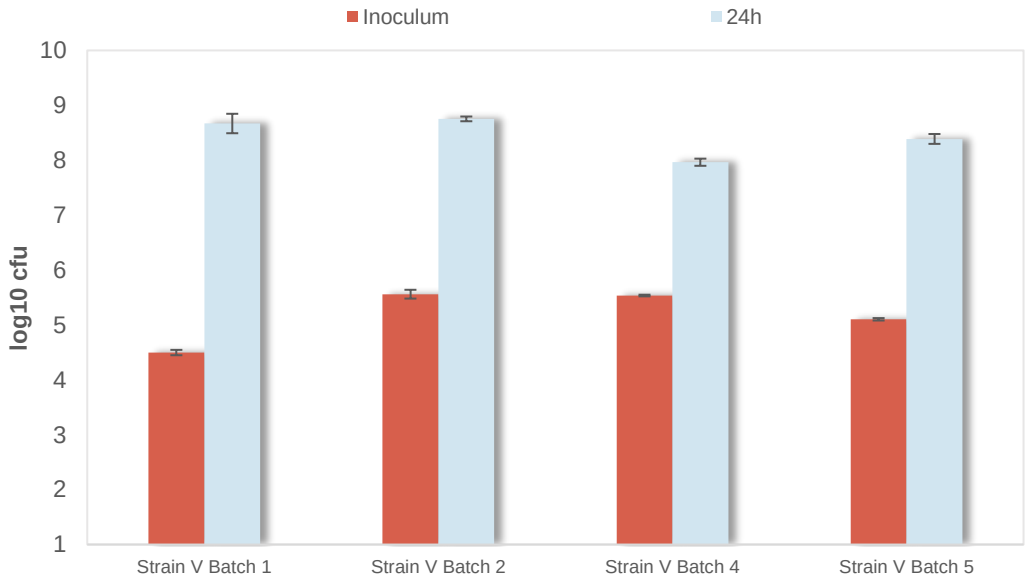


Figure 49. 24h-growth of WHO control strain V, across four batches of GC broth (n=12 in total, n=3 for each batch). Error bars represent standard error of the mean.

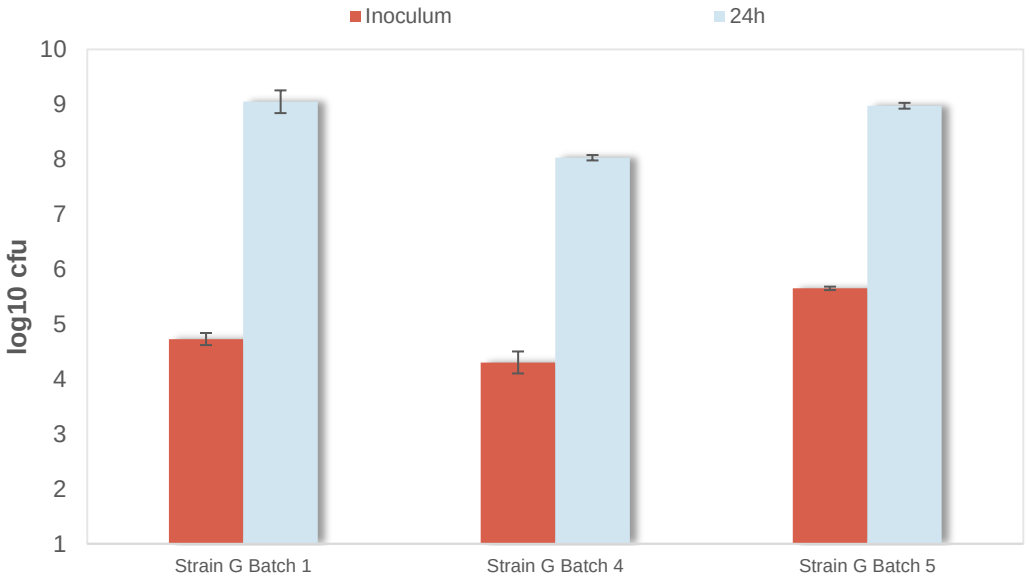


Figure 50. 24h-growth of WHO control strain G, across three batches of GC broth (n=9 in total, n=3 for each batch). Error bars represent standard error of the mean.

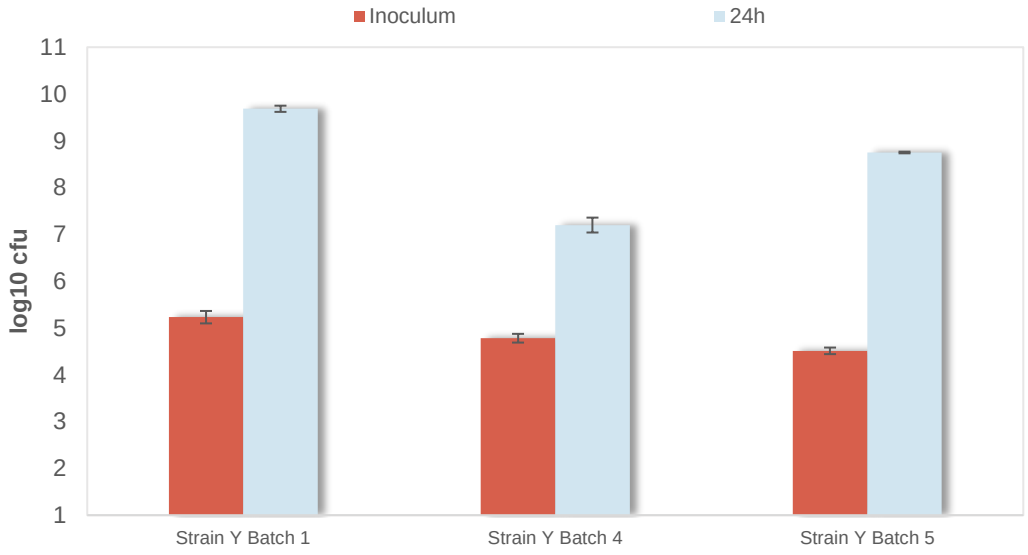


Figure 51. 24h-growth of WHO control strain Y, across three batches of GC broth (n=9 in total, n=3 for each batch). Error bars represent standard error of the mean.

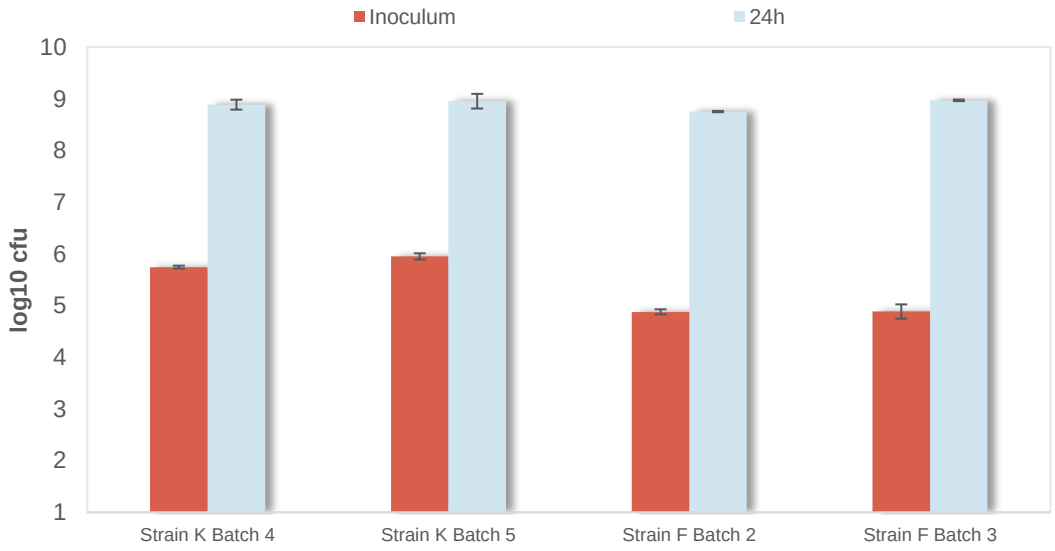


Figure 52. 24h-growth of WHO control strains K and F, across two batches of GC broth each (n=6 in total, n=3 for each batch, for each strain). Error bars represent standard error of the mean.

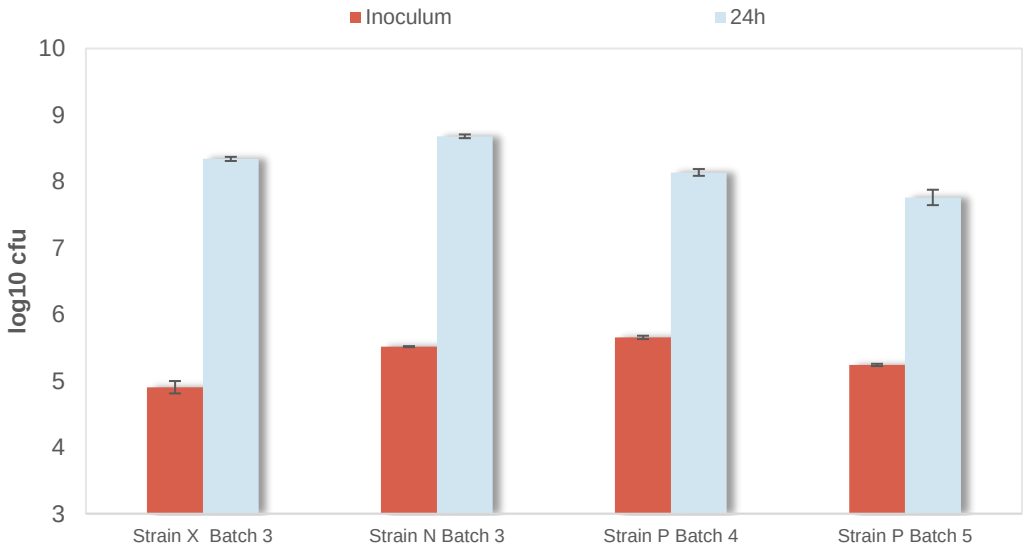


Figure 53. 24h-growth of WHO control strains X, N and P (n=3 for each strain). Error bars represent standard error of the mean.

6.7.2. *Microbroth MICs compared to gold standard agar dilution*

Microbroth dilution CHX MICs (2.7.5) of WHO control strains M, G, N, O, K, V, X, Y and L by visual turbidity were compared to gold standard agar dilution (2.7.3) across five batches of GC broth (M1-M5).

For each batch, strains were tested in triplicate (Table 38), leading to 84 data points and fulfilling the minimum requirement for validation. All control strains apart from L were tested with batches M1 and M2, strains K, V, X and Y were tested with batch M3, and strains K, X, Y and L were tested with batches M4 and M5 (Table 38).

The modal microbroth MIC for strains M, N and K was 1 mg/L and for G, V, X, Y and L it was 0.5 mg/L. Strain O had three replicates with a CHX MIC of 0.5 mg/L and three replicates with a MIC of 1 mg/L. Apart from strain K, all MICs were consistently reproducible within 1 $\log_2$ MIC. The CHX MICs for strain K ranged between 0.5 – 2 mg/L, all MICs were within 1 $\log_2$ of the modal MIC (Table 38). Modal microbroth MICs for all strains were within 1 $\log_2$ compared to agar dilution (Table 38). Overall essential agreement between microbroth MICs and agar dilution was 96.4% (81/84) and the R^2 coefficient was 0.8 (p<0.00001). The layout of the CHX MB dilution plate can be found in Appendix D2.

Table 38. Testing of WHO *N. gonorrhoeae* control strains for the validation of microbroth dilution MIC for chlorhexidine

Broth Batch	Replicate	Control Strain MIC (mg/L)								
		M	G	N	O	K	V	X	Y	L
M1	1	1	0.5	0.5	1	1	0.5	0.5	0.5	NT
	2	1	0.5	0.5	1	1	0.5	1	0.5	NT
	3	1	0.5	1	1	1	0.5	0.5	0.5	NT
M2	1	1	0.5	1	0.5	1	0.5	0.5	0.5	NT
	2	1	0.5	1	0.5	1	0.5	0.5	0.5	NT
	3	1	0.5	1	0.5	1	0.5	0.5	0.5	NT
M3	1	NT	NT	NT	NT	0.5	0.5	0.5	0.5	NT
	2	NT	NT	NT	NT	0.5	0.5	0.5	0.5	NT
	3	NT	NT	NT	NT	0.5	0.5	0.5	0.5	NT
M4	1	NT	NT	NT	NT	2	NT	0.5	0.5	0.5
	2	NT	NT	NT	NT	2	NT	0.5	0.5	0.5
	3	NT	NT	NT	NT	2	NT	0.5	0.5	0.5
M5	1	NT	NT	NT	NT	1	NT	0.5	0.5	0.5
	2	NT	NT	NT	NT	1	NT	0.5	0.5	0.5
	3	NT	NT	NT	NT	1	NT	0.5	0.5	0.5
Modal MIC (mg/L)		1	0.5	1	n/a	1	0.5	0.5	0.5	0.5
Agar Dilution MIC (mg/L)		2	1	1	1	2	1	1	1	1

NT; not tested, MIC; minimum inhibitory concentration

6.7.3. Resazurin microbroth MICs compared to visual and spectrophotometric readings

Microbroth dilution MICs on WHO strains Y, F, O, V and G were performed as per 2.7.5. The MICs were recorded as follows: first turbidity was determined visually, then by spectrophotometer (OD_{595nm}) (BioTek, Winooski, Vermont, U.S.) and finally by 10% Deep Blue (BioLegend) (2.7.5). The *Ng* strains were tested in multiple replicates across five batches of GC broth (R1-R5), leading to 108 data points (Table 39), fulfilling the minimum requirement for validation.

Table 39. Summary of validation testing performed on *N. gonorrhoeae* control strains across five batches of GC broth.

Control strain	Batch R1	Batch R2	Batch R3	Batch R4	Batch R5	Total replicates
Y	4 replicates	4 replicates	4 replicates	NT	NT	12
F	4 replicates	4 replicates	4 replicates	8 replicates	8 replicates	28
O	4 replicates	4 replicates	4 replicates	8 replicates	8 replicates	28
V	4 replicates	4 replicates	4 replicates	NT	NT	12
G	4 replicates	4 replicates	4 replicates	8 replicates	8 replicates	28
Total replicates	20	20	20	24	24	108

NT; not tested

Resazurin MICs compared to visual interpretation.

Minimum inhibitory concentration values by visual and resazurin interpretation had 97.2% (105/108) essential agreement and an R^2 coefficient of 0.82 (p<0.00001). The MIC values that did not agree were for one replicate of strain Y and two replicates of strain V, tested with GC broth batch 3; all MICs were 4 mg/L by resazurin compared to 2 mg/L interpreted visually. A paired t-test showed that there was no significant difference between MIC values generated by the two interpretative methods (p=0.08). The resazurin modal MICs of control strains Y and V were 1 mg/L, and the modal MICs of control strains F, G and O were 2 mg/L (Table 40). The range of MICs for strains V and O was 1-2 mg/L, whereas for Y and G it was 1-4 mg/L. Strain F had the highest range of MICs generated by resazurin (0.5-4 mg/L) (Table 40).

Table 40. Resazurin MIC values generated for WHO control *N. gonorrhoeae* strains Y, F, V, G and O, tested across five batches of GC broth.

Control Strain	MIC (mg/L)				Total MIC values	Median MIC	Modal MIC
	0.5	1	2	4			
Y	0	6	3	3	12	1.5	1
F	1	8	16	3	28	2	2
V	0	8	4	0	12	1	1
G	0	11	16	1	28	2	2
O	0	9	19	0	28	2	2

MIC; minimum inhibitory concentration

Resazurin MICs compared to spectrophotometric reading

The ODs generated from each well of the MIC microbroth dilution were divided into two groups: ODs with a negative resazurin result and ODs with a positive resazurin result. A total of 863 data points were included in this analysis, 395 ODs that generated a positive resazurin result and 468 ODs that generated a negative resazurin result. This comprised 108 each of growth and sterility control ODs, 287 positive resazurin test wells and 360 negative resazurin test wells (Table 41).

Table 41. Summary of OD_{595} results by control and test wells and resazurin result. Comparisons between positive and negative OD readings were performed with the Mann-Whitney test.

	Control wells		Test wells		All wells	
	Positive resazurin	Negative resazurin	Positive resazurin	Negative resazurin	Positive resazurin	Negative resazurin
N	108	108	287	360	395	468
Range	0.04-0.262	0.049-0.112	0.075-0.253	0.039-0.069	0.04-0.262	0.032-0.112
Median	0.138	0.045	0.13	0.047	0.133	0.046
Mean	0.151	0.045	0.154	0.046	0.153	0.046
SD	0.056	0.009	0.052	0.004	0.053	0.005
p	<0.00001		<0.00001		<0.00001	

N; number of tests, SD; standard deviation

The OD_{595} values clustered well into the positive and negative resazurin groups, with 1.04% overlap (9/863) (Fig 54). The range of OD readings from the positive and negative resazurin groups were 0.04-0.262 (mean; 0.153, SD; ±0.053) and 0.032-0.112 (mean; 0.046, SD ±0.005) (Table 41). The OD overlap was driven by the readings in the control wells; there was no overlap seen in the test wells (Table 41) (Fig 54). A Mann-Whitney U test showed that OD_{595} readings between positive and negative resazurin wells were significantly different (p<0.00001) (Table 41).

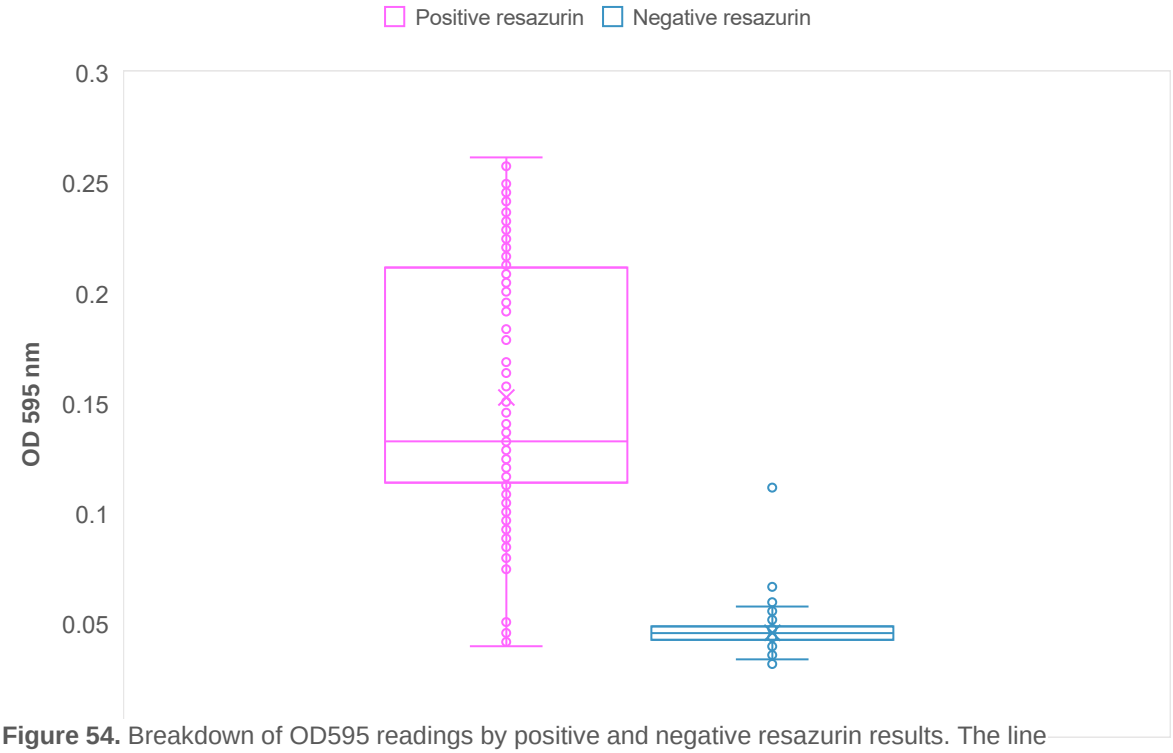


Figure 54. Breakdown of OD595 readings by positive and negative resazurin results. The line inside the box indicates the median OD and the x indicates the mean OD.

6.8. Activity of chlorhexidine on Neisseria species and pharyngeal microbiota

6.8.1. *Susceptibility of N. gonorrhoeae to chlorhexidine*

A total of 74 clinical *Ng* isolates were tested against CHX by agar dilution (2.7.3) (Appendix D3), alongside WHO control strains M, N, O, F, X, Y, V, L and K (Appendix D4). The MIC of M, N, O, V, X, Y and L was 1 mg/L, and the MICs of strains M and K was 2 mg/L (Table 42). The MICs of the clinical *Ng* isolates ranged between 0.25 mg/L and 2 mg/L (Table 42) and had a normal distribution. The median and modal chlorhexidine MIC from the clinical strains was 1 mg/L (IQR: 0.5-1) and the geometric mean was 0.98 mg/L (Table 42). A total of three (4.1%), 17 (23%), 48 (64.9%) and six (8.1%) isolates had an MIC of 0.25 mg/L, 0.5 mg/L, 1 mg/L and 2 mg/L respectively (Fig. 54, Appendix D3).

Table 42. Summarised chlorhexidine minimum inhibitory concentration results of clinical *N. gonorrhoeae* strains tested by agar dilution and microbroth dilution and minimum bactericidal concentrations.

	Agar Dilution MIC (mg/L)	Microbroth Dilution MIC (mg/L)	MBC (mg/L)
Range	0.25-2	0.5-2	0.5-8
Median	1	1	2
IQR	0.75-1	1-2	1-2
Mode	1	1	2
Geometric Mean	0.98	1.13	1.74

MBC; minimum bactericidal concentration, IQR; interquartile range

The chlorhexidine MICs of 69/74 isolates tested by agar dilution were further determined by microbroth dilution (2.7.5) as well as MBCs (2.7.6) (Fig. 55). The microbroth dilution MICs ranged from 0.5 – 2 mg/L (Table 42) (Fig. 54). The median and modal chlorhexidine MIC from the clinical strains was 1 mg/L (IQR: 1-2) and the geometric mean was 1.13 mg/L (Table 42). A total of nine (13%), 39 (56.5%), and 21 (30.4%) isolates had an MIC of 0.5 mg/L, 1 mg/L and 2 mg/L respectively (Fig. 54, Appendix D3). Compared to agar dilution, 29/69 (42%) of isolates had the

same CHX MIC by microbroth dilution, 10/69 (14.5%) had 1 log_2 lower MIC by microbroth dilution, 24/69 (16.3%) had a 1 log_2 higher MIC, 5/69 (7.2%) had 2 log_2 higher MIC higher and 1/69 (1.5%) had a 3 log_2 higher MIC (Table 43). When accounting for the acceptable MIC margin of error of ±1 log_2 dilution[284], microbroth dilution MICs had 87% (63/69) essential agreement with agar dilution (Table 43). A paired t-test showed that the MIC values generated by agar dilution and microbroth dilution were significantly different (p = 0.02) and the R^2 coefficient was 0.32 (p<0.00001).

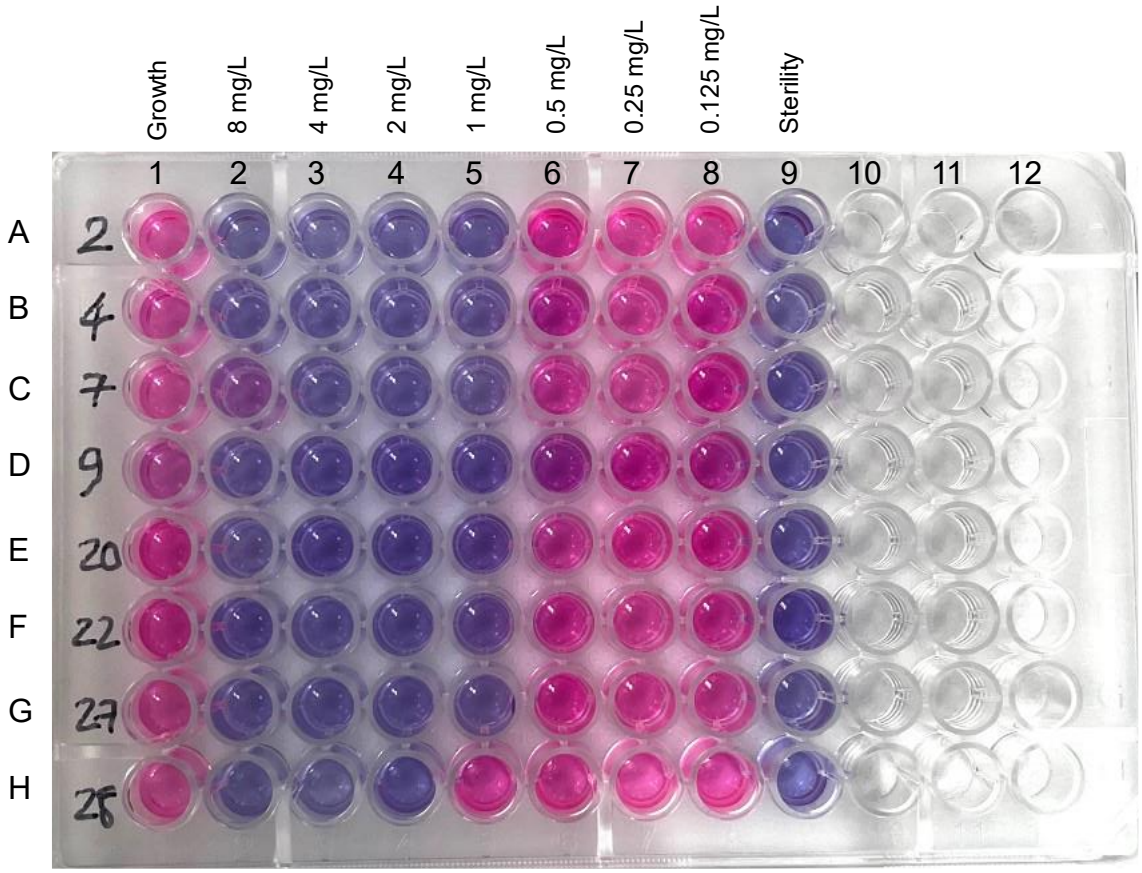


Figure 55. Example of chlorhexidine microbroth MIC with resazurin. Wells in column 1 are growth controls (pink = positive) and wells in column 9 are sterility controls (blue = negative) for each organism. The MICs of organisms in rows A-G is 1 mg/L and the MIC of the organism in row H is 2 mg/L.

Table 43. Number of clinical isolates tested for chlorhexidine susceptibility, with given combinations of agar dilution and microbroth dilution MICs.

Agar Dilution MIC (mg/L)	*Microbroth Dilution MIC (mg/L)*			*Total no. of isolates*
	0.5	*1*	*2*	
0.25	1	1	1	3
0.5	1	9	4	14
1	7	26	13	46
2	0	3	3	6
Total	9	39	21	69

CHX; chlorhexidine, MIC; minimum inhibitory concentration, dark blue cells denote same MIC, light grey cells denote ±1 log_2 difference, light orange cells denote ±2 log_2 difference and dark orange denotes ±3 log_2 difference

The MBCs ranged from 0.5 – 8 mg/L (Table 42). The median and modal chlorhexidine MBC from the clinical strains was 2 mg/L (IQR: 1-2) and the geometric mean was 1.78 mg/L (Table 42). A total of 21 (30.4%), 35 (50.7%) and seven (10.1%) isolates had an MBC of 1 mg/L, 2 mg/L and 4 mg/L respectively (Fig. 56, Appendix D3). Three isolates each (4.3%) had an MBC of 0.5 mg/L and 8 mg/L. On average, the MBC of the isolates was 0.64 log_2 higher than microbroth dilution MICs. The majority of isolates had the same MBCs (36/69, 52.2%) as the microbroth dilution MICs, whereas 33.3% (23/69) were 1 log_2 higher, 13% (9/69) were 2 log_2 higher and 1.4% (1/69) were 3 log_2 higher.

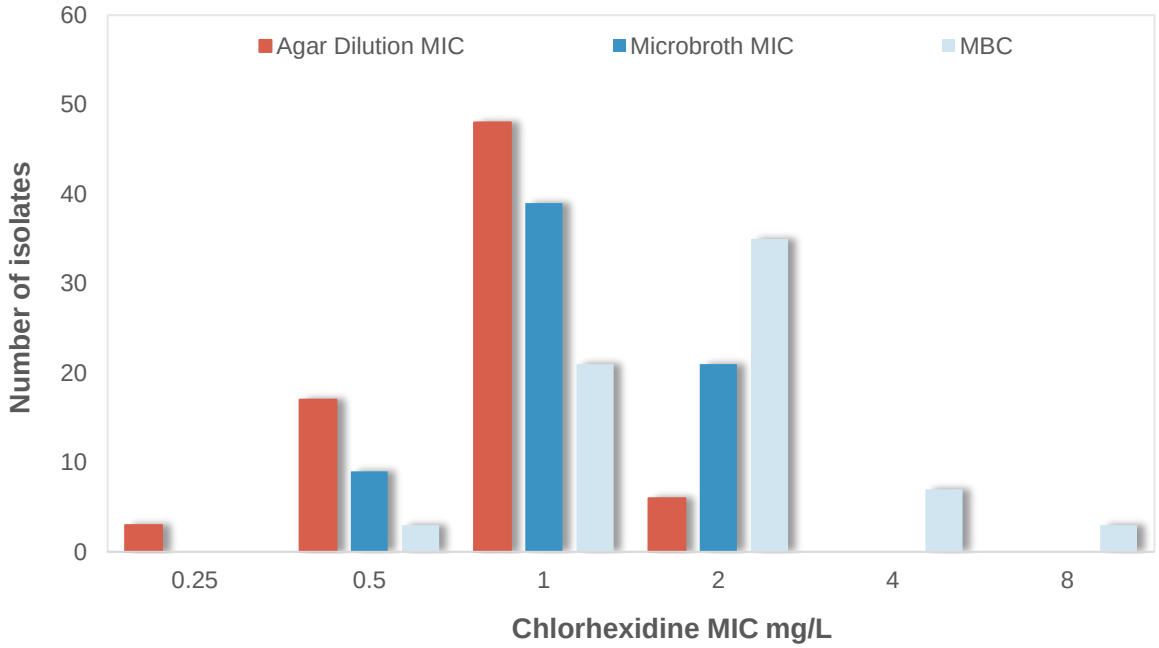


Figure 56. Distribution of chlorhexidine minimum inhibitory concentrations in 74 clinical isolates of *N. gonorrhoeae*, tested by agar dilution. MIC; minimum inhibitory concentration.

6.8.2. *Susceptibility of commensal Neisseria species to chlorhexidine*

The *N*c species were acquired from 50 participants, as described in 5.8. A total of 98 *N*c isolates were tested against CHX, by agar dilution (2.7.3), consisting of 14 *N. flavescens*, six *N. macacae*, three *N. mucosa*, 10 *N. perflava*, 59 *N. subflava* and six *Neisseria spp* with no ID by MALDI-ToF (Table 44). The CHX median and geometric mean of all *Neisseria* species were 16 mg/L (IQR; 8-16 mg/L) and 10.7 mg/L respectively (Table 44).

Table 44. Summary of chlorhexidine minimum inhibitory concentration characteristics by commensal *Neisseria* species.

Species	N	Range	Median	IQR	Modal MIC	Geometric mean
N. flavescens	14	4 - 16	16	8 - 16	16	11.3
N. macacae	6	4 - 32	16	16 - 16	16	13.9
N. mucosa	3	16 - 32	32	n/a	32	25.4
N. perflava	10	1 - 32	16	4 - 16	16	9.2
N. subflava	59	1 - 32	8	8 - 16	16	10
N. species	6	8 - 16	12	8 - 16	n/a	11.3
All *Neisseria*	98	1 - 32	16	8 - 16	16	10.7

A total of four, one, 11, 27, 44 and 11 *Neisseria* isolates had a CHX MIC of 1 mg/L, 2 mg/L, 4 mg/L, 8 mg/L, 16 mg/L and 32 mg/L respectively (Fig 57). The Kruskal-Wallis test was performed only on *N. subflava, N. macacae, N. perflava and N. flavescens,* as the number of *N. mucosa* was below the minimum threshold required for statistical testing, and *Neisseria spp.* cannot be assigned to a specific species. The test demonstrated no statistically significant difference in MIC values between the four *Neisseria* species (p = 0.71). The full MIC data and characteristics of the *N*c species can be found in Appendix D6.

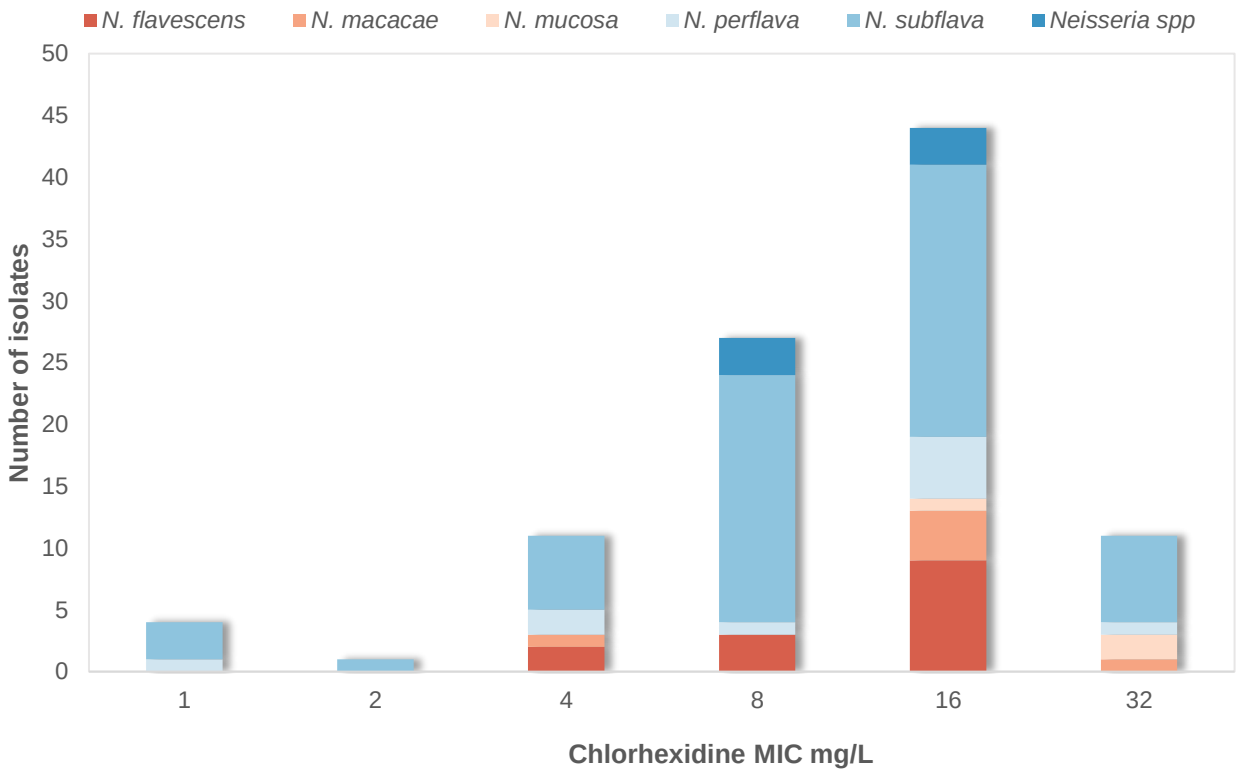


Figure 57. Distribution of minimum inhibitory concentrations to chlorhexidine in 98 commensal *Neisseria* species, tested by agar dilution**.**

6.8.3. *Chlorhexidine time-kill studies*

Time-kill studies were performed on WHO control strains F, N and X (2.2.1), and clinical strains GC20P, GC20R, GC25U and GC40P in triplicate, as described in 2.8. The strains were selected to represent a range of susceptibility to CHX (Table 45). The time-kill assays were performed with and without 0.4% mucin to determine if salivary mucous will inhibit the efficacy of CHX (Appendix D7). All reagents passed quality control testing (2.8.1, 2.8.2, 2.8.3).

The kill studies showed that both 0.06% and 0.2% CHX reduced the *Ng* load from a mean 8.1 $\log_{10}$ (SD±0.42) to zero in 30 and 60 seconds, in all gonococcal strains tested (Fig 58). The gonococcal inoculums challenged ranged from 1.9 x 10^7 cfu/mL to 4.5 x 10^8 cfu/mL (Appendix D7). The presence of 0.4% mucin did not have an inhibitory effect on CHX, as gonococcal loads were reduced to zero in all variables tested

Table 45. Minimum inhibitory concentrations of *N. gonorrhoeae* strains tested by kill-assays to historical antimicrobials and chlorhexidine. MICs in mg/L.

	PEN	CFX	CRO	AZI	CIP	TET	SPE	CHX
WHO F	0.032	<0.016	<0.002	0.125	0.004	0.25	16	2
WHO N	>32	<0.016	0.004	0.25	4	16	16	1
WHO X	4	4	2	0.5	>32	2	16	1
GC20P	0.19	0.064	0.016	0.016	0.016	16	16	2
GC20R	>32	0.008	0.008	0.004	>32	16	16	1
GC25U	0.125	0.016	0.002	0.004	8	16	16	1
GC40P	0.25	0.016	0.002	0.016	4	8	8	1

WHO; world health organisation, PEN; penicillin, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin, CHX; chlorhexidine

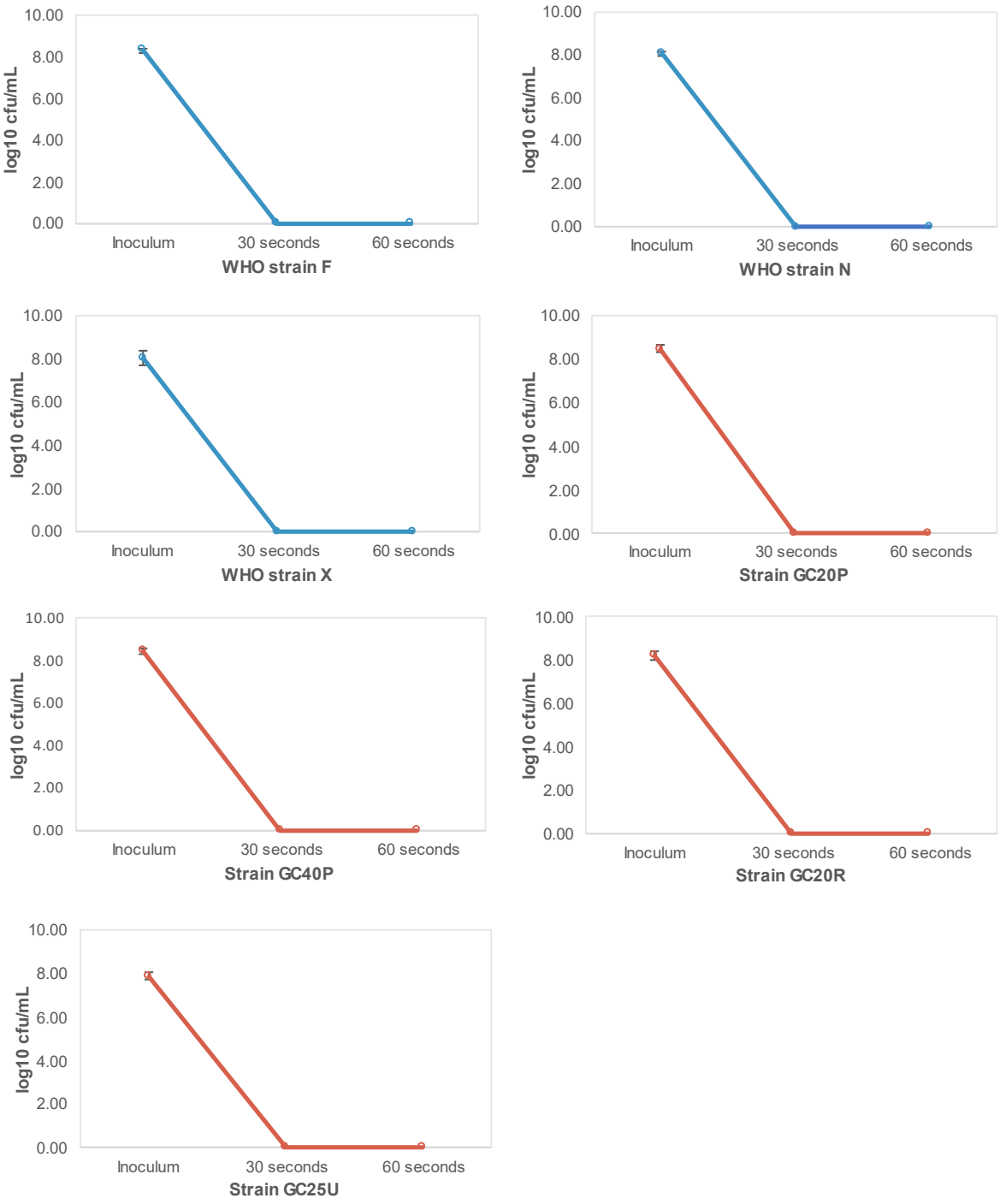


Figure 58. Timed-kill studies for WHO control and clinical strains of *N. gonorrhoeae*. 0.06% and 0.2% chlorhexidine were tested with and without 0.4% mucin. All variables had identical results for both 30 and 60-second contact time. Blue lines indicate WHO control *Ng* strains and orange lines indicate clinical *Ng* strains. Error bars represent standard error of the mean.

6.8.4. *Effect of chlorhexidine on pharyngeal microbiota*

The pharynx of 50 participants was sampled before and after a one-minute CHX (Corsodyl) gargle (5.8). The effect of CHX on pharyngeal bacterial microbiota was determined by comparing a) the total cfu of participants before and after the gargle from chocolate agar and b) recording the presence of *Nc* from all participants using LBVT.SNR agar. For the total count comparison, data from 36/50 participants were analysed. Counts from 14 participants were not performed, as the samples were lost. The detection limit for colony counting was 10^4 cfu/mL due to a) an initial 10^{-3} dilution of the original expressed sample and b) 10^{-1} mL was transferred to each agar plate. Less than 1 cfu, would equate to <1 x 10^4 cfu/mL, so no growth was recorded as <10^4 cfu/mL.

The range of bacterial microbiota total counts detected in the participants before the gargle was <10^4 cfu/mL to 4.7 x 10^6 cfu/mL, whereas the range after the gargle was <10^4 cfu/mL to 2.5 x 10^6 cfu/mL (Appendix D8). The CHX gargle reduced the mean number of colonies cultured from the swabs by 49%. The mean colony count before and after the gargle was 9.5 x 10^5 cfu/mL (SD ±1.4x10^6) (5.98 $\log_{10}$) and 4.8 x 10^5 cfu/mL (SD ±7.8x10^5) (5.68 $\log_{10}$) respectively (Fig 59, Fig. 60). A total of 5/36 (13.9%) participants (17, 18, 27, 39, 48) had no growth (<10^4 cfu/mL) on the post-gargle sample, from a mean of 8 x 10^5 cfu/mL (SD ±1.7x10^6) (5.9 $\log_{10}$) (Fig. 59) (Appendix D8). A total of 6/36 (16.7%) of participants (22, 23, 24, 26, 28, 33) had a higher total count in the post-gargle sample, the mean number of cfu increasing by 140% (from 4.8x10^5 cfu/mL, SD ±5x10^5 to 1.2x10^6, SD ±8.8x10^5) (Appendix D8) (Fig. 60). Two participants (42, 43) had no growth on both pre-and post-gargle samples (Fig 59). A Wilcoxon signed-rank test was used to compare the difference between colony counts, before and after the chlorhexidine gargle, which determined the reduction was significant (p = 0.001).

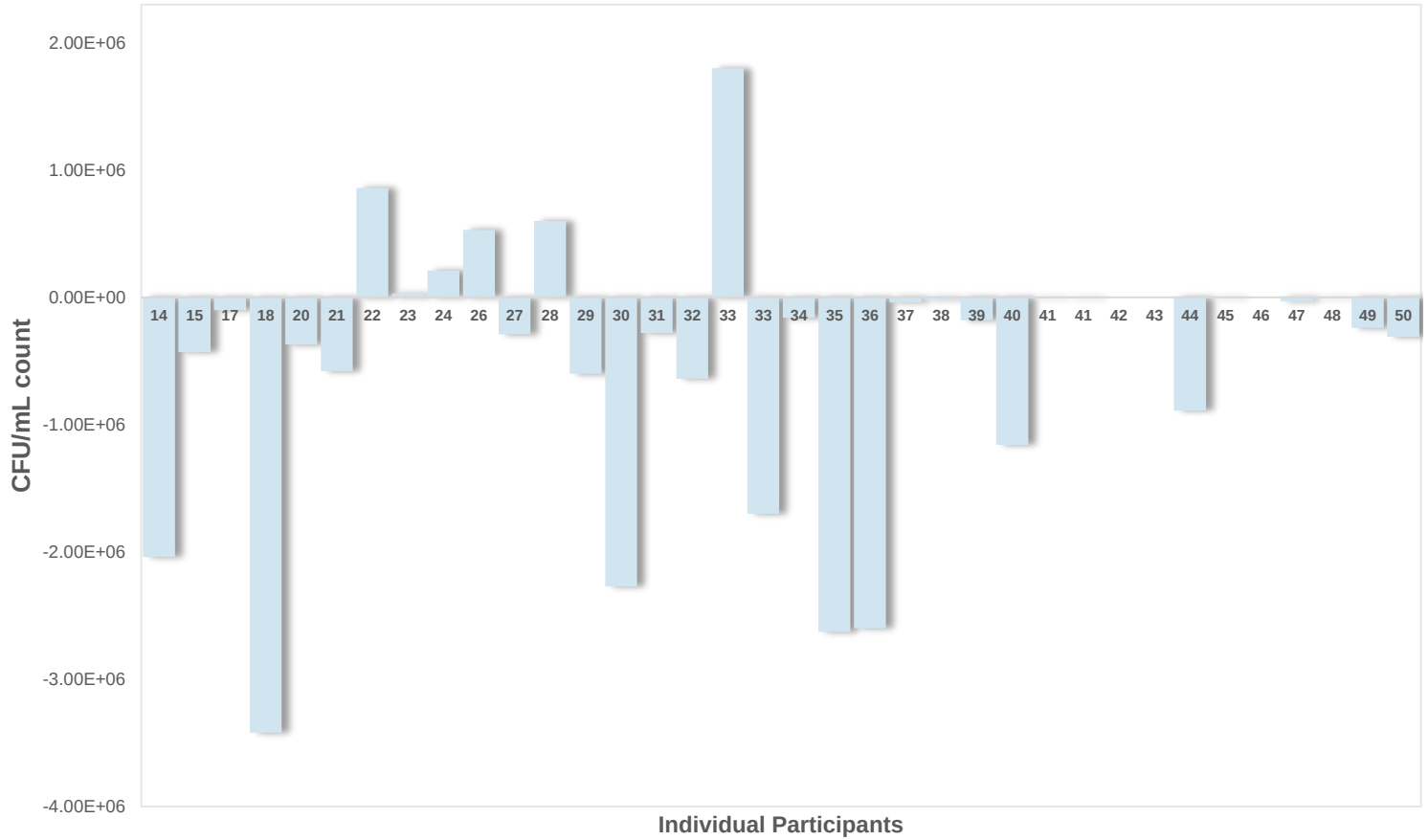


Figure 59. Total colony count difference in pharyngeal swabs of participants, after a one-minute Corsodyl gargle.

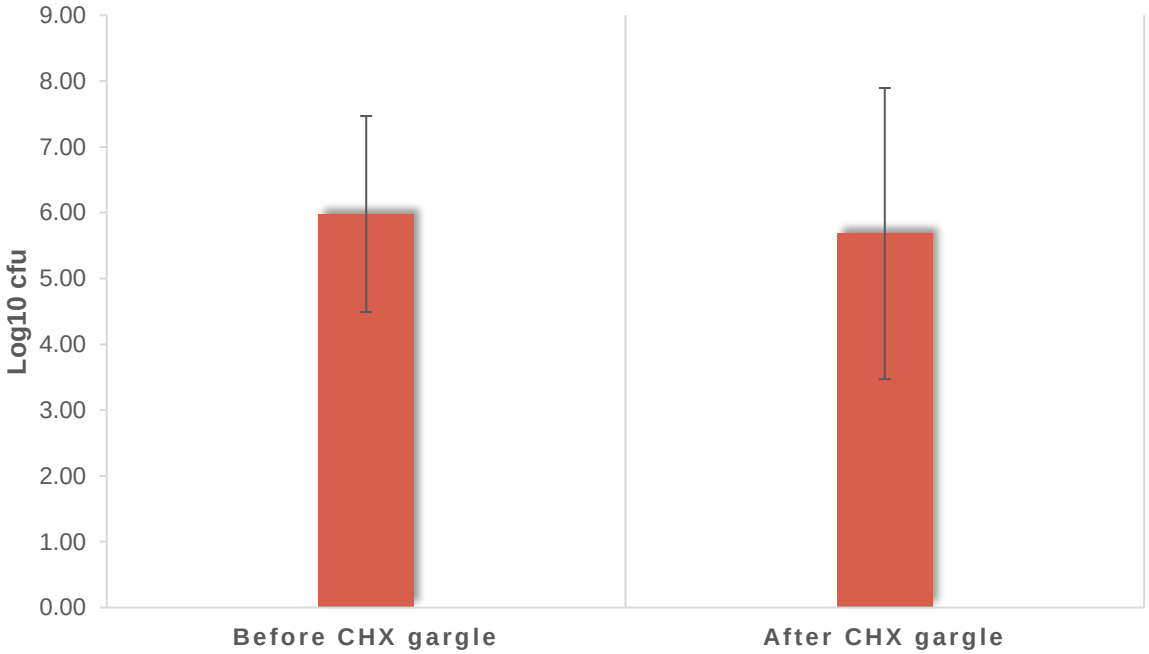


Figure 60. Comparison of mean log_{10} colony counts (cfu/mL) before and after a one-minute chlorhexidine gargle. Error bars represent standard deviation.

The presence of *N*c before and after the gargle was recorded to determine whether CHX significantly reduced the number of *N*c isolates in the pharynx of the participants (Appendix D8). Before the gargle, 84% of participants (43/50) carried at least one *N*c isolate, whereas this number decreased to 42% (21/50) after the gargle (Fig. 61). A McNemar's test determined that CHX significantly reduced the presence of *N*c (p = 0.0001). The median CHX MICs of isolates from patients that cleared *N*c post-gargle was the same as those that did not (16 mg/L, IQR; 8 – 16 mg/L) and geometric means were 11.3 mg/L and 10.3 mg/L respectively. A Mann-Whitney test was performed to compare the CHX MICs between the two groups, which showed no significant difference (p = 0.9). To determine whether a reduction in total microbiota count was associated with the elimination of *N*c, a Fisher's Exact test was performed which showed there was no association (p = 0.05).

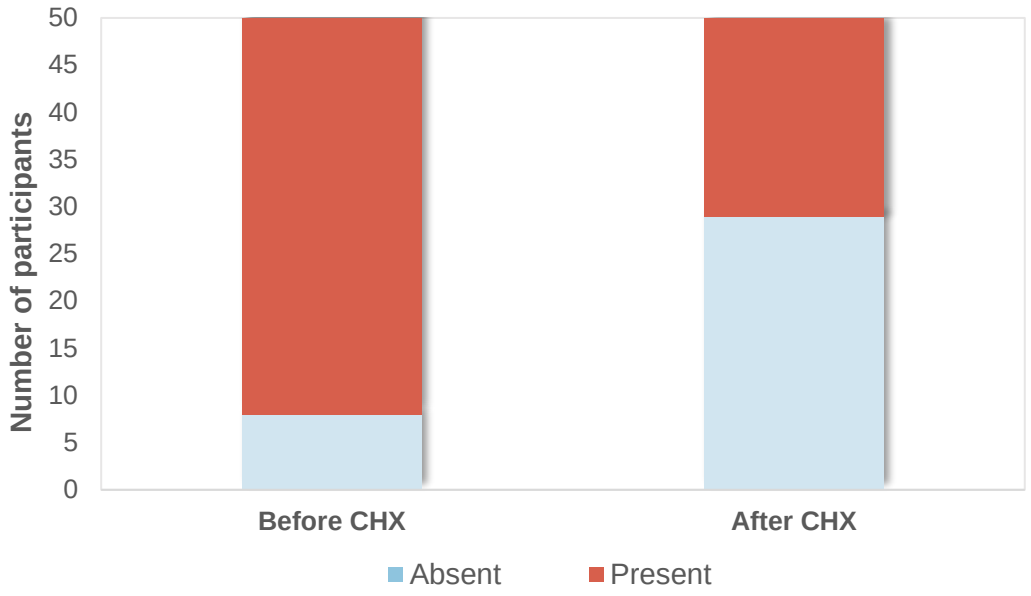


Figure 61. Number of participants with presence or absence of commensal *Neisseria* in their pharynx, before and after a chlorhexidine gargle.

6.8.5. *Synergy between chlorhexidine and ceftriaxone*

Synergy between CHX and ceftriaxone was determined for control strains X, Y, K and L by checkerboard assay (2.7.7), in five replicates (Fig. 62, Appendix D5). These strains were selected due to their high ceftriaxone MICs. The FICI for strains X and Y were 1.5 and 2 respectively, leading to an indifferent result (Table 46, Table 47). The FICI for strain K was additive (0.75) (Table 48) and synergistic for strain Y (0.5) (Table 49). For strains X and K, CHX reduced the CRO MIC by 1 $\log_2$ MIC, whereas for strain Y, it reduced it by 2 $\log_2$ MIC and for strain L, no reduction was seen.

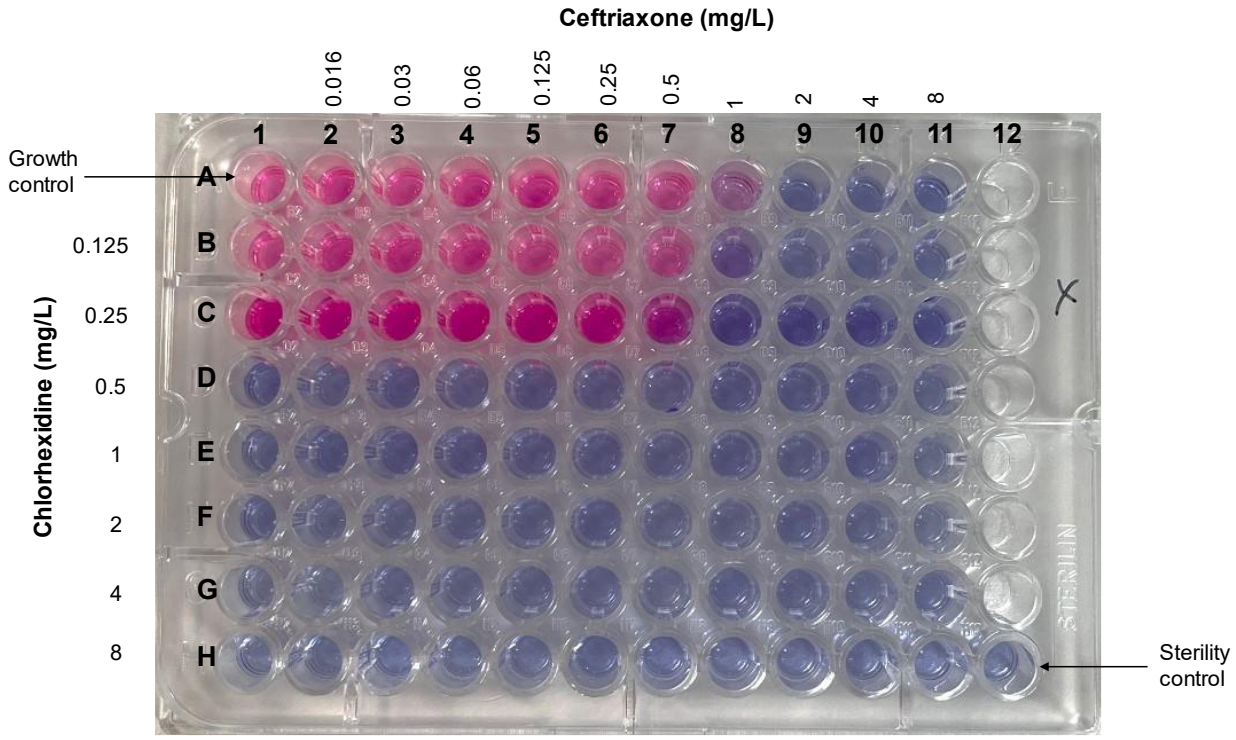


Figure 62. Example checkerboard assay, for *N. gonorrhoeae* control strain X. Direction of arrows is from lowest to highest dilution. A9 is MIC for ceftriaxone (2mg/L), D1 is MIC for chlorhexidine (0.5 mg/L), C8 is combination MIC for ceftriaxone (1 mg/L) and D7 is combination MIC for chlorhexidine (0.5 mg/L).

Table 46. Checkerboard assay results for WHO *N. gonorrhoeae* control strain X. MIC and FIC in mg/L.

Control strain X	Replicate					FIC		FICI	Interpretation
	1	2	3	4	5	CHX	CRO		
CHX MIC	0.5	0.5	0.5	0.5	0.5				
CRO MIC	2	2	2	2	2				
CHX MIC Comb	0.5	0.5	0.5	0.5	0.5	1	0.5	1.5	Indifferent
CRO MIC comb	1	1	1	1	1				

CHX; chlorhexidine, CRO; ceftriaxone, MIC; minimum inhibitory concentration, FIC; fractional inhibitory concentration, FICI; fractional inhibitory concentration index

Table 47. Checkerboard assay results for WHO *N. gonorrhoeae* control strain Y. MIC and FIC in mg/L.

Control strain Y	Replicate					FIC		FICI	Interpretation
	1	2	3	4	5	CHX	CRO		
CHX MIC	0.5	0.5	0.5	0.5	0.5				
CRO MIC	1	1	1	1	1				
CHX MIC Comb	0.125	0.125	0.125	0.125	0.125	0.25	0.25	0.5	Synergistic
CRO MIC comb	0.25	0.25	0.25	0.25	0.25				

CHX; chlorhexidine, CRO; ceftriaxone, MIC; minimum inhibitory concentration, FIC; fractional inhibitory concentration, FICI; fractional inhibitory concentration index

Table 48. Checkerboard assay results for WHO *N. gonorrhoeae* control strain K. MIC and FIC in mg/L.

Control strain K	Replicate					FIC		FICI	Interpretation
	1	2	3	4	5	CHX	CRO		
CHX MIC	2	2	2	2	2				
CRO MIC	0.032	0.032	0.032	0.032	0.032				
CHX MIC Comb	0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.75	Additive
CRO MIC comb	0.016	0.016	0.016	0.016	0.016				

CHX; chlorhexidine, CRO; ceftriaxone, MIC; minimum inhibitory concentration, FIC; fractional inhibitory concentration, FICI; fractional inhibitory concentration index

Table 49. Checkerboard assay results for WHO *N. gonorrhoeae* control strain L. MIC and FIC in mg/L.

Control strain L	Replicate					FIC		FICI	Interpretation
	1	2	3	4	5	CHX	CRO		
CHX MIC	0.5	0.5	0.5	0.5	0.5				
CRO MIC	0.125	0.125	0.125	0.125	0.125				
CHX MIC Comb	0.5	0.5	0.5	0.5	0.5	1	1	2	Indifferent
CRO MIC comb	0.125	0.125	0.125	0.125	0.125				

CHX; chlorhexidine, CRO; ceftriaxone, MIC; minimum inhibitory concentration, FIC; fractional inhibitory concentration, FICI; fractional inhibitory concentration index

6.9. Discussion

The need for the evaluation and introduction of novel treatments for pharyngeal gonorrhoea is becoming increasingly evident; the pharynx can be difficult to treat with current regimens and is more likely to facilitate HGT of AMR genes between *Neisseria* species. So far, existing alternative antimicrobial therapeutic agents, such as gentamicin, gemifloxacin, zoliflodacin, delafloxacin and solithromycin[192–194] have been evaluated, however, they are either inferior compared to current treatment regimens[192,194], have poor gonococcal clearance at extragenital sites[194], or not evaluated for pharyngeal infection[193]. As such, it is essential to continue evaluating new compounds and novel ways of treating pharyngeal gonorrhoea. Topical antiseptics may have a role as an adjunctive topical treatment, alone or in combination with current treatment regimens. In this study, I evaluated the susceptibility of clinical and control strains of *Ng* to CHX. I further measured the effect of a one-minute CHX gargle on pharyngeal microbiota and determined the susceptibility of *N*c to CHX.

The CHX MICs were determined by agar dilution, MICs and MBCs by microbroth dilution, and the minimum bactericidal contact time were determined with timed-kill assays. Due to the fastidious nature of *Ng*, the gold standard method for MIC determination is agar dilution, which is recommended by both CLSI[103] and EUCAST[106]. There are, however, some limitations to agar dilution; it can be laborious to set up and does not allow for MBC testing[285]. Therefore, there is a need to evaluate the ability of *Ng* to grow in liquid media and assess the performance of microbroth MIC against agar dilution. There are several examples of liquid media being evaluated for the growth of *Ng* such as BHI[286], tryptic soy broth[287] and fastidious broth[285]. The first version of GC broth was developed in 1967[288], based on the formulation of GCMB and has been modified several times until its final simplified form[287,289–291]. The most recent gonococcal broth to be developed is the Wade-Graver medium, which allows growth of *Ng* to reach >5 $\log_{10}$ cfu/mL over 33 hours[292]. All media previously described have been able to allow growth of *Ng* to > 7 $\log_{10}$ cfu/mL, which is comparable to the findings of the validation presented in this chapter (mean; 8.9 $\log_{10}$, SD ±0.66, range; 6.7 - 9.9 $\log_{10}$).

The inoculum chosen for the GC broth validation was approximately 5 x 10^5 cfu/mL, to reflect the recommended inocula for MB dilution assays[284] and ensure successful visualisation of MICs. This was a key step for the subsequent validation of MB dilution MICs against agar dilution. As GC broth and GCMB have the same formulation except for the addition of agar to GCMB, GC broth was chosen as the most suitable medium for MIC and MBC testing. The decision was based on the observation that even minor changes to media formulations such as pH and differences in supplementation can alter the growth of *Ng* and susceptibility to antimicrobials[108]. Essential agreement between microbroth and agar dilution CHX MICs was 96.4% for the initial validation and 92.8% for the *Ng* clinical strains tested, which were above the recommended limitation set by CLSI (≥90%)[293]. While there have been no comparisons between CHX microbroth and agar dilution, MICs published in the wider literature, agreement between the two methods varies depending on the antimicrobial tested. For example, Shapiro *et al* found that spectinomycin had the highest agreement (100%, 22/22), whereas penicillin (64%, 14/22) and doxycycline (63%, 15/22) had the lowest[294]. Agreement for ceftriaxone has shown to be >90%, but <90% for ciprofloxacin and azithromycin[295,296]. There is still however lack of standardisation for microbroth dilution protocols; previous studies used different variations of GC broth, inoculum preparations and incubation conditions[294–296].

As inoculum preparations of 5 x 10^5 cfu/mL allowed *Ng* to grow to approximately 10^8 cfu/mL which is at the lower limit of visual turbidity, I validated the use of resazurin to improve the interpretation of MICs generated by traditional microbroth dilution. The initial validation showed that resazurin-based MICs had a high essential agreement with visual readings (97.2%) and clustered well by spectrophotometric readings (Fig. 54). Although automated readings are not the gold standard for the interpretation of MB dilution MICs[102], they have been used in the literature [284,294,295] and can give more granular quantitative data for validation purposes. Resazurin-based MIC testing is not routinely used for *Ng;* one study compared an 8h resazurin-based assay to gradient strip results and found good correlation, apart from ESC MICs[281]. Further research on the use and function of resazurin is needed, for example, to determine the limit of detection. Expanding on this research would allow us to understand whether resazurin was over or underestimating MICs compared to

visual interpretation, which has a detection limit of approximately 10^8 cfu/mL. This enhanced understanding of the relationship between gonococcal inoculum and resazurin positivity may facilitate the development of further rapid MIC assays for CHX and other antimicrobials.

Chlorhexidine is not in clinical use to treat pharyngeal *Ng* and there are no resistance breakpoints for *Ng*; ECOFFs are usually helpful to establish such microbiological breakpoints, and bimodal distributions indicate sub-populations with resistance to antimicrobials and biocides[297]. The tested isolates produced a normal distribution suggesting that they may be considered susceptible wild-type strains with no obvious resistance, but a larger study would be needed to establish an ECOFF. Chlorhexidine has not been studied extensively and there are no similar studies to date in the literature, however, previous time-kill studies showed that 0.2% chlorhexidine was able to inactivate 10^6 cfu/mL *Ng* at 30 seconds[207]. This was comparable with both 0.06% and 0.2% CHX tested which inactivated 10^7 cfu/mL in 30 seconds. The presence of 0.4% porcine mucin, used to replicate the potential inactivating effect of human mucus, did not negatively affect the bactericidal effect of CHX. This suggests a recommended one-minute CHX gargling time should be sufficient to eradicate *Ng* in the pharynx[243].

Although time-kill studies are more relevant to the immediate effect of a gargle, MICs and MBCs can give additional context to the residual activity of CHX which can remain in the saliva for 12 hours at concentrations of 36.2 mg/L (SD ±23.3) and for six hours at 7.3 mg/L (SD ±7.3)[298]. The CHX MBCs reported in this chapter were 0.5 - 8 mg/L meaning that the CHX concentration at six hours post gargle is 4.5 times higher than the bactericidal dose. This may explain why previous clinical trials failed to eradicate *Ng* from the pharynx of participants; the clinical trial by van Dijck *et al*, reported that gargling with Corsodyl, which contains 0.2% CHX, at 12h intervals failed to eradicate *Ng* in 3/3 participants [214]. Going forward, it would be worthwhile assessing whether gargling at six-hour intervals is more efficacious, however, this may impact adherence. More research on the effect of CHX on pharyngeal microbiota may also give some insight into whether the reasons for the poor clinical efficacy are biological or behavioural, but also for assessing the impact of CHX on oral health. For example, a recent study that assessed the impact of antiseptic gargles for a period of 12 weeks found a significant change in the composition of the participants'

pharyngeal microbiomes[299]. Ideally, a treatment should successfully eradicate the pathogen of interest while disrupting the bacterial microbiota as little as possible.

Data presented in this chapter showed that a one-minute Corsodyl gargle reduced pharyngeal microbiota count by 50% (0.3 $\log_{10}$) (Fig 58). There was great variability on the effect of the Corsodyl gargle; 19.4% (7/36) of participants reduced their total count to $<10^4$ cfu/mL (Fig 58), 16.7% (6/36) increased their count and most decreased it but did not eliminate it, but there was variability in the reduction in these participants also (SD ± $8.8x10^5$) (Fig 58, Appendix D7). It is unclear why there is such variability in these participants; one reason could be the composition of pharyngeal microbiota. Some organisms can cause biofilms which makes them less susceptible to biocide and antimicrobials, or bacteria in some participants may have had higher CHX MICs making eradication more difficult. The starting bacterial load is unlikely to be a factor, as some participants had a low bacterial load on the first sample which stayed almost the same after, whereas some participants had a high bacterial load that was eradicated. The reason for increasing post-gargle counts is also unclear; gargling for some participants was challenging and it is possible suboptimal gargling technique caused the mechanical release of microbiota from mucosal surfaces without appropriate contact with the active compound. Another reason for this could be the variation in biofilm formation within the pharynx or each participant which may have provided some protective effect to the microbiota community. A limitation of this study was that anaerobic bacteria were not counted, which may have led to different results. The oral microbiome is composed of a high proportion of anaerobic bacteria[299] and in future studies both types should be counted along with metagenomic sequencing which will also give additional information on the oropharyngeal resistome. Further, noting gargling technique would have enabled additional context to this variability and a 'gargle effectiveness score' would be helpful in future studies. Additionally, it would have been helpful to measure the concentration of CHX in the post-gargle sample by mass spectrometry to determine whether the gargle was performed effectively. A phenotypic method of CHX detection was attempted in this study but was unsuccessful.

There was a 50% reduction in the detection of *N*c seen in participants which was not associated with a reduction in pharyngeal microbiota. However, a total count of *N*c pre- and post-gargle would

have added additional context to these analyses, for example, to examine whether lower CHX MICs are associated with a reduction in *N*c counts. The reason for measuring *N*c was as a proxy for pathogenic *Neisseria* species due to high phenotypic and genetic similarity[300] as suggested previously[301], but further research should be carried out to determine if this is feasible in the context of this study.

Finally, this chapter presented data on the combined effect of CHX and ceftriaxone, which found results were dependent on the *Ng* strain; for strains X (FICI=1.5) and L (FICI=2) were indifferent, for strain K (FICI=0.75) it was additive and for strain Y (FICI=0.5) slightly synergistic (Tables 46-49). There are no similar published studies in the wider literature, however, studies performed on other bacterial pathogens have conflicting results with other β-lactams. A study on *Acinetobacter baumanii* found synergy when CHX was tested with meropenem but indifference when tested with imipenem[302], but for *Pseudomonas aeruginosa*, CHX and meropenem were antagonistic[303]. Interestingly, both these studies found synergy between CHX and ciprofloxacin[302,303]. Although pharyngeal gonorrhoea is common, especially in MSM, 36% of patients with gonorrhoea have single-site pharyngeal infection[41], meaning that a CHX gargle without standard treatment would be appropriate for only a small proportion of patients. According to the data in this study, CHX would not inhibit the action of ceftriaxone and may slightly enhance it. Even if CHX gargles are not successful as a standalone treatment, there may be potential to add it as an adjunctive, particularly for *Ng* strains with high ceftriaxone MICs.

The studies in this chapter are not without limitations. Firstly, the performance of GC broth was only assessed after 24h growth, to reflect the timepoints for MIC determination. Growth curves would have added additional context to the doubling time of *Ng* and could help finetune the MIC method, by estimating the exact timepoint MICs should be determined. Growth curves were attempted several times; however, they were unsuccessful due to loss of *Ng* viability or contamination. When validating resazurin, OD_{595} readings were compared to visual determination of resazurin positivity; using a spectrophotometer to detect fluorescence would have enabled more granular analysis, however, I did not have access to the necessary equipment. Further, determining the detection limit of resazurin in cfu/mL would have given more context to the

discordant visual and resazurin CHX MICs, as the lower limit of visual turbidity is approximately 10^8 cfu/mL. Lastly, to finetune this microbroth MIC method, the effect of resazurin on cell viability should be measured, as some research suggests that resazurin compounds can have bactericidal activity against *Ng*[304].

When performing timed-kill assays, 0.4% mucin was used as a proxy for salivary mucin. However, saliva includes more protein components, unfortunately, I did not have the means to create an accurate saliva alternative. In previous studies, donor saliva from healthy volunteers was used[207]. For the CHX gargle study, it would have been beneficial to include a negative control group to ascertain whether any potential reduction was due to CHX or the mechanical action of gargling. A negative control group (saline gargle) was originally included in the study protocol, however, was removed at the request of the LSHTM Research Governance team. Additionally, quantitation of *N*c total counts and by species would have enabled further analyses, for example, to determine whether the post-gargle elimination of *N*c was correlated with CHX MICs or specific species of *N*c. Lastly, additional synergy testing with azithromycin, ciprofloxacin and gentamicin would have provided a more complete dataset to estimate the potential effect of CHX on the action of other antimicrobials. Despite these limitations, this chapter provides novel data that is not otherwise available in the literature, or preliminary data that can be expanded on.

CHAPTER 7

FINAL DISCUSSION AND CONCLUSIONS

7.1. Summary and further application of findings

N. gonorrhoeae remains an AMR public health priority, as identified by the WHO[1]. An estimated 87 million cases of gonorrhoea occurred globally in 2016, an increase from 78 million cases in 2012[37]. In England, 82,592 infections were reported in 2022, an increase of 50% from 2021 and 165% from 2013[40]. A major cause for concern is that decreasing susceptibility to ESCs has led to an increase in the number of clinical treatment failures[2]. Additionally, in 2022, the number of ceftriaxone resistant isolates reported in England was higher than in the past seven years, since the first reported case in 2015[40]. Pharyngeal infection has an important role in the development and dissemination of AMR, due to its asymptomatic nature, HGT of resistance determinants from *N*c and suboptimal pharmacokinetic ability of ESCs, which can lead to TFs even in seemingly phenotypically susceptible strains[10–12]. The control of AMR gonorrhoea requires a multifaceted approach that includes continued monitoring of TFs, improving AMR surveillance globally, improving the laboratory detection of *Ng*, research and introduction of novel treatments and further research on developing molecular testing, especially for the rapid detection of AMR determinants[2,5]. These factors when applied together can enable the optimisation of antimicrobial stewardship and minimise the emergence of resistant strains. This thesis presents research that contributes to the knowledge gaps identified by the above guidance, using a mixed methods approach: a systematic review of ESC treatment failures, a cross-sectional study of multistrain *Ng* carriage, a cross-sectional study of *N*c carriage and AMR burden and *in vitro* evaluation of Ng susceptibility to CTA and CHX. A summary of the key findings from each chapter is outlined in Fig 63.

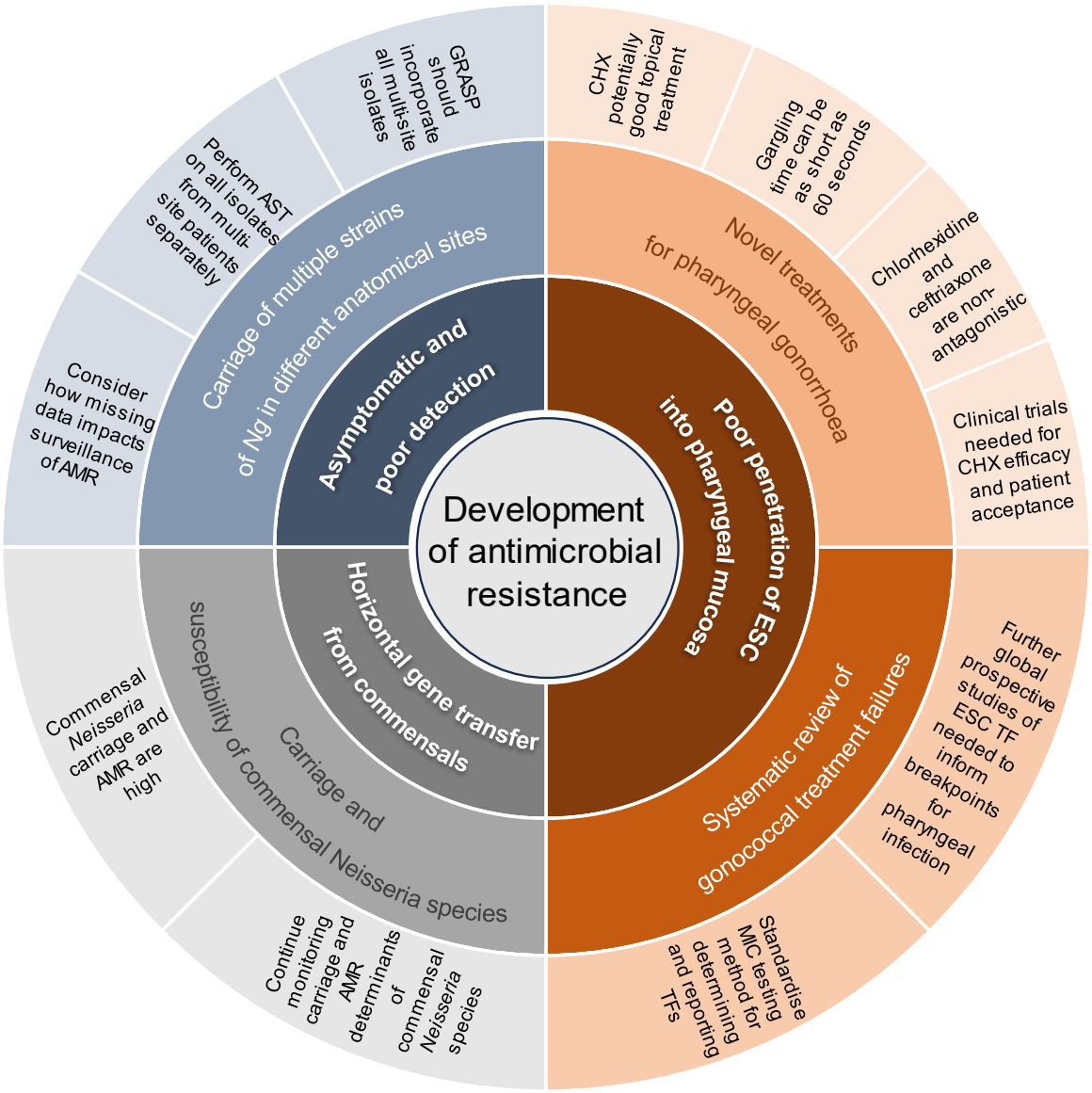


Figure 63. Summary of findings from the research presented.

The WHO recognises the importance of strengthened surveillance programmes including AMR surveillance, that relies on the ability of laboratories to detect resistant strains as accurately and early as possible. This is confounded by the lack of standardisation among laboratories, especially relating to AST testing and the lack of guidance on how laboratories should process multisite isolates. For example, in 2022, GRASP surveillance analysed 1460 *Ng* isolates in 2022, one isolate per patient and 555 patients (38%) had multisite infection. We reported that a higher proportion of study patients than expected based on previous research (14.6%, 6/41) had different *Ng* MICs

between anatomical sites. A crude extrapolation of our finding would translate to a total of 78 isolates being excluded from the 2022 GRASP surveillance scheme due to a single isolate from multisite patients being processed. It is unclear whether the MICs of these missing isolates would impact the AMR rates identified by GRASP surveillance, and more research should be carried out to ascertain this. Currently, for patients with multisite infection, GRASP uses a hierarchy of testing based on anatomical site; pharyngeal isolates are prioritised, followed by rectal, urethral or cervical and then any other site[40]. The rationale is based on concerns that resistance is more likely to develop at the pharyngeal site[40]. In our multisite study, among the four patients with divergent MICs who had pharyngeal infection, the pharyngeal isolates from three patients had higher ceftriaxone MICs and two had the same MICs. Furthermore, the only mosaic *penA* detected in this study was from a pharyngeal isolate, showcasing the importance of *N*c in the development of *Ng* AMR. However, there is conflicting information in the literature on whether there are MIC differences between pharyngeal and extrapharyngeal isolates; for example, a study by Kidd *et al* (2014) found no difference in MICs between sites of infection[238], whereas Quilter *et al* found that ESC MICs were significantly higher (p <0.05) in pharyngeal isolates[305]. These studies did not specify how the isolates were selected and whether they originated from patients with multisite infection; the fact that up to 42% of patients may have multisite infection calls into question the study design of these surveys, highlighted by our finding that 85.4% (35/41) of multisite patients had the same strain between anatomical sites.

Regardless of whether pharyngeal isolates have higher MICs, the question is whether the detection of resistant strains should be the function of a surveillance scheme (as GRASP does) or whether it should primarily rely on diagnostic laboratories, as it may have direct implications on patient management and detection of TFs. For example, monitoring and detection of TFs, detected during the TOC process, is a critical part of control efforts, and it appears that the site of infection influences how *Ng* responds to treatment; specifically pharyngeal infections are more challenging to treat successfully; the systematic review showed that of the patients with ceftriaxone treatment failure, 12/36 were infected at multiple sites, but only failed at the pharyngeal site with seven of these cases growing phenotypically susceptible isolates. Importantly, the mean cefixime and

ceftriaxone MICs for pharyngeal isolates fell below the phenotypic EUCAST resistance breakpoint of 0.125 mg/L, whereas the mean MICs for extrapharyngeal isolates fell above the breakpoint. The lack of standardisation of AST practices across laboratories is impacting control efforts, as AMR surveillance relies heavily on the ability of laboratories to detect *Ng* including TFs and report AST data[2]. This variation in AST testing methods, such as the use of different breakpoints and differences in processing multisite isolates makes it challenging to compare data between laboratories and regions. For example, in the systematic review, we found differences in ESCs MICs between gradient strip and agar dilution methodologies. The WHO, ECDC and UKHSA recognise the importance of accurately verifying and reporting TFs, by producing clear definitions, however, there is no strong correlation between TF and phenotypic resistance[2,4]. The WHO states that where TF is confirmed, the gonococcus should be considered resistant, irrespective of the MIC value[2], however, this means that MICs <0.125 mg/L can be considered both susceptible and resistant depending on the infection site. Perhaps the improvement of these definitions can be achieved by further research to determine whether there should be site-specific *Ng* resistance breakpoints, as defined in other bacteria such as *E. coli* and *S. aureus*. The results presented in the systematic review reinforce the narrative that pharyngeal gonorrhoea is more challenging to treat and that further research to inform the revision of resistance breakpoints is warranted. A key patient group to include in this research is patients with multisite infection, considering the discrepancy in treatment success between pharyngeal and extrapharyngeal infection. Data from these patients will be valuable for studying the differences in post-treatment gonococcal clearance across the different anatomical sites, particularly if infected by the same strain, as each patient can be their own control. These patients are estimated to account for up to 38% of total patients infected with gonorrhoea[40,41] and almost 80% of multisite patients are estimated to be infected in the pharynx[41].

Since the introduction of 1 g ceftriaxone as empirical therapy, there appears to be an improved correlation between MIC and treatment success, as all TFs were caused by *Ng* strains with MICs above both CLSI and EUCAST resistance breakpoints. However, this may introduce further challenges by applying evolutionary pressure and selecting for more resistant *N*c species that in

turn will act as reservoirs of AMR genes that can be acquired by *Ng*, continuing the cycle. We found *N*c estimated population prevalence of 86%, in agreement with previous studies of similar study design[78,79]. Importantly, *Nc* resistance to ESCs and azithromycin was higher than *Ng* resistance rates of the same year, approaching or exceeding the 5% WHO threshold for empirical therapy. Additionally, all strains carried multiple gcDUS repeats highlighting their ability to donate DNA and potentially resistance genes. A recent systematic review of the global epidemiology of *N*c AMR reported that overall, MICs have increased over time for all antimicrobials tested and above the level found in pathogenic *Neisseria* species[306]. There has therefore been a recent interest in establishing *N*c AMR surveillance, with the view to measuring the AMR reservoirs available to *Ng*, which this research supports. This could be combined with an improved understanding of the molecular mechanisms and efficiency of the *Neisseria* transformation process to build prediction models for further ESC resistance in *Ng*. Furthermore, multisite patients with pharyngeal infection, especially those with the same *Ng* strain between sites could be a useful tool to detect HGT events *in vivo.* Another way to prevent acquisition of AMR genes is to eliminate the source of these genes, by potentially developing a vaccine against *N*c. However, this approach may lead to other adverse outcomes. For example, it has been proposed that *N*c have some protective effect against colonisation by pathogenic *Neisseria* species. *N. lactamica* is thought to protect against *N. meningitidis* colonisation[74] and *N. elongata* is thought to kill *Ng*[75]. Furthermore, *N*c are an integral part of the oropharyngeal microbiome and plays a role in the regulation of nitric oxide which in turn protects against cardiovascular disease and hypertension[307].

In the meantime, novel therapeutics for pharyngeal gonorrhoea are needed and CHX may be an effective option; it is already used as a mouthwash, it is easy to use and quickly eradicates *Ng in vitro*. Unfortunately, the rapid bactericidal effect of CHX has not translated well into clinical efficacy. A recent (2022) clinical trial found that a twice-daily CHX gargle over six days was not successful in eradicating *Ng* in the pharynx of three patients[214]. Several reasons were hypothesised for this; firstly, it is possible that CHX cannot reach the areas within which *Ng* resides, for example within the crypts of mucosal membranes[214]. There is no strong evidence that *Ng* resides intracellularly in PMNLs, such as urogenital infection[308], however, this cannot be discounted. There is also the

possibility that *Ng* creates biofilms in the oropharyngeal mucosa, that enables them to resist the effect of CHX[214]. Lastly, although bactericidal concentrations remain in saliva for approximately seven hours, its activity is attenuated by food[204]. However, the clinical trial used a very small sample size (n=3) and the exact effect of CHX on the gonococcal count over time has not been established. For example, daily gonococcal counts by culture and quantitative PCR from multiple pharyngeal sites would determine post-gargle *Ng* load peaks and inform the recommended frequency of a CHX gargle. Further, there is the question of whether *Ng* eradication should be the desired clinical outcome or whether transmission prevention would be sufficient, which can be achieved by modelling the transmission risk between sexual partners. Lastly, the application of CHX as pre- or post-exposure prophylaxis is worthwhile considering. Realistically, even if CHX was clinically effective it would only be used as a standalone treatment on patients with only pharyngeal gonorrhoea which can be challenging; not only is gonorrhoea treated empirically, it is also treated before knowing which sites are infected[7]. Further, only a small proportion of patients (approximately 36%) with gonorrhoea have single-site pharyngeal infection which means that most patients will receive standard treatment[41]. I determined that CHX and ceftriaxone together were additive, indifferent or slightly synergistic, depending on the *Ng* strain tested. Although the strains tested were limited the results suggest that CHX would not inhibit the effect of ceftriaxone in these patients.

It is important to be mindful that new antimicrobials introduce the risk of further AMR. Additionally, considering CHX is used commonly in dentistry, monitoring of CHX resistance in *N*c is key to assessing the impact on the transfer of AMR genes to *Ng*. Resistance to CHX in *N*c has not been described in the literature, however, we demonstrated that the median MICs to CHX were higher in *N*c than in *Ng*. *In vitro* studies have shown that CHX MIC increases in *Ng* are associated with mutations in the genes coding the MtrCDE efflux pump, MlaA (maintenance of lipid asymmetry system) and NorM (Na^+-drug antiporter] proteins)[215]. Previous evidence of mtrCDE transfer from *N*c to *Ng*[309] means there are implications for the acquisition of CHX resistance. Despite this, the CHX concentration in clinical formulations of CHX (2,000 mg/L) is 250-fold higher than the *Ng*

highest MBC (8 mg/L) and 62.5-fold higher concentration than the highest *N*c MIC (32 mg/L), found in our study.

This thesis demonstrated that combined efforts of improved AST practices and AMR surveillance, coupled with enhanced understanding of *N*c AMR and innovative application of topical therapeutics will contribute to *Ng* AMR control efforts, as outlined by the WHO.

7.2. Future work

This work contributes to knowledge gaps identified by the WHO global action plan to control the spread and impact of AMR in *Ng*, particularly pertaining to the relationship between ESC MICs and TFs, optimisation of AMR surveillance and evaluation of novel compounds for the treatment of *Ng*[2]. The findings of the research presented have the potential to be directly applied to public health control efforts, however, the work can be developed further.

One of the challenges of the systematic review was differentiating true TFs from reinfection. Although we used the UKHSA and ECDC definitions of possible and probable TF, it was not always possible to differentiate true TF from reinfection, even if the pre- and post-TF STs were the same. One of the reasons for this could be that the time between treatment and TOC can be up to two weeks, providing more time for reinfection to occur. Furthermore, TOC testing is performed using a NAAT test which does not guarantee the *Ng* is viable, as it can detect residual DNA. Instead, a viability NAAT to be used as TOC could be developed, which would reduce the TOC period. Viability PCR uses propidium monoazide (PMA) to distinguish viable from non-viable cells using an existing NAAT[310] and in-house preliminary testing has shown promising results for *Ng*. Further research should be conducted to determine the optimum time for a TOC using this method. This can also be applied to future clinical trials or assessment of a CHX gargle. Daily viability NAAT testing will enable the quantitation of *Ng* loss of viability post gargle and fine-tuning of gargling frequency. Furthermore, more research should be conducted on the patient acceptability of CHX and the application of CHX as pre- or post-exposure prophylaxis.

Following the multisite study presented in this work, another key area for development is reviewing UK laboratory AST practices for *Ng*. To make effective recommendations for standardizing AST, it

is essential to first assess the current variability in laboratory practices. A national audit, facilitated by UKHSA and other stakeholders, should collect data on factors such as the AST methods used, types of agar employed, the antimicrobials tested, and how laboratories handle multisite isolates. This comprehensive approach will help address existing knowledge gaps.

Lastly, to further the *N*c study, understanding the molecular mechanisms of transformation through *in vitro* transformation studies would be useful. I have demonstrated that different clusters of *N*c contain different variants of the DUS sequence; specifically, the *N. subflava* cluster has a high number of gcDUS repeats which suggests that these species in particular are more able to donate DNA to *Ng*. However, DNA transformation from *N*c to *Ng* has yet to be demonstrated *in vitro*. I would like to assess transformation frequency and efficacy by using combinations of several *N*c species and *Ng* strains as well as determine whether transformation is enhanced by the presence of antimicrobials. Along with the data from the *N*c carriage cross-sectional study, transformation data can be incorporated into a mathematical model to predict future HGT events and ESC resistance.

7.3. Development of *N. gonorrhoeae* AMR control efforts

The current global, European and UK action plans for the control of AMR *Ng* propose a multifaceted approach that encompasses patient and healthcare education, monitoring of TFs and AMR, improvement in diagnostics and evaluation of novel compounds. One of the reasons for this approach is the lack of a gonococcal vaccine. Development of a gonococcal vaccine has been challenging, as natural infection does not offer protective immunity and reinfection is very common[311]. Whether this is due to immune evasion or failure to mount the correct type of immune response remains unclear[311]. However, recent evidence suggests that a meningococcal vaccine containing outer membrane vesicles (OMV) of *N. meningitidis* serogroup B (MenB) may provide *Ng* cross-protection in certain individuals. This was first identified in 2004 when a MenB outbreak led to the mass vaccination of children and young adults, after which a decline in gonorrhoea cases was noted[312]. This has been followed by several studies in New Zealand, Canada and Norway

which suggested a MenB OMV vaccine efficacy of 22-46%[313]. However, this falls short of the impact model that calculated a vaccine efficacy of 70% would most significantly prevent the spread of *Ng* and the development of AMR and is unlikely to offer satisfactory protection[314]. Recommended aspects of control efforts rely on the improved a) detection of *Ng*, AMR and TFs, b) AMR surveillance and c) antimicrobial stewardship. This can be achieved by evaluating and finetuning several aspects of current *Ng* management protocols. Firstly, the 5% WHO threshold for empirical therapy could be reviewed to determine if it is still relevant to the current *Ng* epidemiology. This could be achieved by applying several thresholds to mathematical models based on current AMR rates and predicted future resistance events. Information from *N*c carriage and AMR burden can also feed into this mathematical model that can be reviewed periodically. The development and evaluation of new antimicrobials is crucial; however, existing antimicrobials should not be forgotten. For example, molecular assays that detect ciprofloxacin resistance have been described in the literature and have been implemented in some laboratories[179]. Widespread implementation of resistance testing will detect patients that are suitable for ciprofloxacin rather than ESCs, reducing the evolutionary pressure to develop AMR to the last remaining antimicrobial. Furthermore, other antimicrobials not previously used can be considered. The latest GRASP surveillance reported that 97.5% (193/198) of penicillin resistance was due to a β-lactamase[40] which can be treated successfully with a β-lactamase inhibitor combination such as co-amoxiclav. If β-lactamase positive isolates are excluded and no secondary resistance mechanisms are present, penicillin resistance falls to 0.34% (5/1460), which is below the 5% WHO threshold for empirical therapy. However, such targeted treatment is currently challenging as most patients are treated before the test result or susceptibility profile is known. The development of rapid diagnostics, preferably incorporating resistance detection would lead to improvement in antimicrobial stewardship for the control of *Ng*, that perhaps would not rely on the need for empirical treatment.

Lastly, there is an urgent need for the standardization of AST. The current EUCAST guidelines lack specific disk diffusion breakpoints and do not recommend an appropriate agar for AST.

Addressing these critical gaps is essential for enhancing the detection of AMR and improving AMR surveillance efforts.

In summary, this thesis contributes valuable insights to public health strategies aimed at combating *Ng*, aligning with the WHO's action plan to address AMR. As treatment options continue to decline and emerging antimicrobials fall short of current standards, the urgency for effective control measures is becoming increasingly important. Future efforts must be comprehensive, leveraging innovative technologies and optimizing existing diagnostic protocols and treatment regimens.

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CHAPTER 8

APPENDICES

Appendix A1: Systematic review ethics letter

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LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

MSc Research Ethics Committee

MSc Student
Control of Infectious Diseases
LSHTM

2 June 2020

Study Title: A systematic review of in vitro and in vivo evidence of Neisseria gonorrhoeae third generation cephalosporin sensitivity

LSHTM MSc Ethics ref: 21953

Thank you for submitting your application for the above MSc research project.

As your projectis a systematic/literature review only, it was assessed by the Research Governance & Integrity Office as not requiring ethical approval from the MSc ethics committee. It is the student's responsibility to ensure that all other required approvals are in place before starting the research project.

Any subsequent changes to the application must be submitted to the Committee via an Amendment form on the ethics online applications website: http://leo.lshtm.ac.uk .

Best of luck with your project.

Yours sincerely,

[redacted]

Rebecca Carter

Research Governance Coordinator

MScEthics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

Appendix A2: Prospero submission information

□R□ □□□R□

Adapted from the PROSPERO registration template accessible through: https://www.crd.york.ac.uk/PROSPERO/

Re□ie□ Title: A systematic review of *in vitro* and *in vivo* evidence of *Neisseria gonorrhoeae* third generation cephalosporin sensitivity

Anticipated □tart □ate: 15 June 2020

Anticipated Completion □ate: 2 September 2020

□rgani□ational A□iliation: The London School of Hygiene and Tropical Medicine

Re□ie□ Team Mem□ers: Jonna Mosoff, Victoria Miari, Matthew Chico; LSHTM

Re□ie□ Question: What is the current evidence of *in vitro* minimum inhibitory concentration (MIC) of *N. gonorrhoeae* and *in vivo* treatment failures in lab-confirmed cases treated with third generation cephalosporins? Do the treatment failure MICs in these instances differ between anatomical sites?

□earches: Sources will be identified from PubMed, MEDLINE, and EMBASE and from reference lists of eligible studies. MeSH and free text will be searched by terms listed below. Results will be restricted to English language and will be re-run before final analysis.

UR□ to search strategy: PubMed, MEDLINE and EMBASE using Medical Subject Headings (MeSH) and free-text terms: *Neisseria gonorrhoeae*, treatment failure, resistance, penA, PBP1, PBP2, (oro)pharyngeal, urogenital, minimum inhibitory concentration, cefixime, ceftriaxone, cephalosporin.

Condition or domain □eing studied: Third generation cephalosporin treatment failures of *N gonorrhoeae* in oropharyngeal and urogenital sites.

□opulation: Adults with lab confirmed *N. gonorrhoeae,* oropharyngeal and extrapharyngeal, and documented treatment failures after third generation cephalosporin treatment.

□nter□entions□exposures: Treatment efficacy of *N gonorrhoeae* pertains to instances of persistent infection, as documented at test of cure, after a completed treatment regimen. For this review, treatment is defined as third generation cephalosporin antibiotics.

Comparators□controls: susceptible *in vitro* MIC (< 0.125 mg/L); MIC of *N. gonorrhoeae* in extra-oropharyngeal as compared to oropharyngeal sites.

Types o□study to □e included: Cohort, case-control and cross-sectional studies will be included in addition to case reports; reviews and other non-primary source data will be excluded.

Context: Studies will be restricted to those with confirmation of *N. gonorrhoeae*, restricting contexts to those with available laboratory resources. All countries and years will be included.

Main outcome: Third generation cephalosporin treatment failure of *N. gonorrhoeae* in oropharyngeal and extra-oropharyngeal sites including MIC values and anatomical site differences.

□ata □xtraction: One reviewer will conduct the review and another will independently screen records and check decisions to ensure a proper selection processes. A data extraction form will be used to extract relevant demographic information, identify presence or absence of inclusion/exclusion criteria and record relevant demographic and statistical data.

Risk o Bias: Characteristics of the study will be assessed for quality and data extraction will include relevant population characteristics and risk factors so as to allow stratification of results at the analysis stage. If sufficient data meets the inclusion criteria to calculate a pooled estimate, formal and informal tests for bias will also be conducted.

trategy or ata ynthesis: Results will be identified with the above search strategy and by strict inclusion and exclusion criteria. Analysis will be conducted to compare both *in vitro* MIC and *in vivo* treatment efficacy. Additionally, a sub-group analysis of oropharyngeal and extra-oropharyngeal sites will be conducted. A meta-analysis with pooled estimates of MIC will be calculated if sufficient data are found.

Analysis o su groups: Subgroups based on anatomical site of infection will be investigated as there has been preliminary evidence of higher prevalence of *in vivo* treatment failures with different *in vitro* MIC in oropharyngeal sites compared to extra-oropharyngeal sites. The types of studies included will remain consistent across the subgroups and an appropriate analytical approach will be chosen based on sample size.

ey ords: Systematic review, *Neisseria gonorrhoeae*, oropharyngeal, treatment failure, cephalosporin, ceftriaxone, resistance

Appendix A3: Systematic review data extraction form

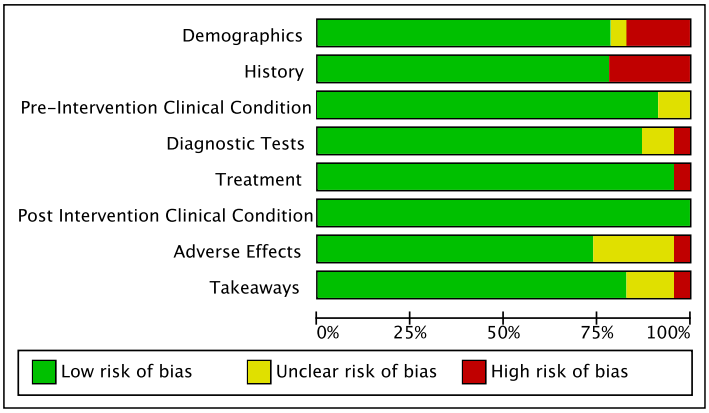
GENERAL INFO						
Study Title						
First Author						
Year of Publication						
Journal						
Database:	1. PubMed/MEDLINE	2. EMBASE	3. Eurosurveillance			
Other:	1. Reference List	2. suggestion				
STUDY ELIGIBILITY						
Region	1. European	2. Americas	3. Eastern Mediterranean	4. Western Pacific	5. Africa	6. Southeast Asia
Country						
Study type	1. Case Study	2. Observational Study	3. Other			
No. study participants						
Language	1. English	2. Other:				
Eligibility criteria met?	Yes	No				
If no, reason for exclusion	Not N. gonorrhoaeae	Not outcome of interest	Not antibiotic class of interest	other		
CASE DEMOGRAPHICS						
Country/Region						
Age						
Gender Identity	Male	Female	Other			
Sexual Orientation	Heterosexual	MSM	Bisexual	Other		
CLINICAL INFORMATION						
Location	1. Hospital	2. Clinic				
Diagnostic Test	C&S	NAAT	Other			
Culture performed?	1. Yes	2. No				
MIC						
MIC Method	1. Agar dilution	2. E-test	Other			
MIC > 0.125 microg/mL	1. Yes	2. No				
Anatomical Site	1. Urogenital	2. Anorectal	3. Oropharyngeal			
Treatment failure	1. Yes	2. No				
Primary Abx	cefixime	ceftriaxone	cefotaxime	Other		
Secondary/additional Abx	cefixime	ceftriaxone	cefotaxime	Other		
Non-cephalosporin used?	1. Yes	2. No				
Resistance gene identified?	1. Yes	2. No				
Other						
Notes						

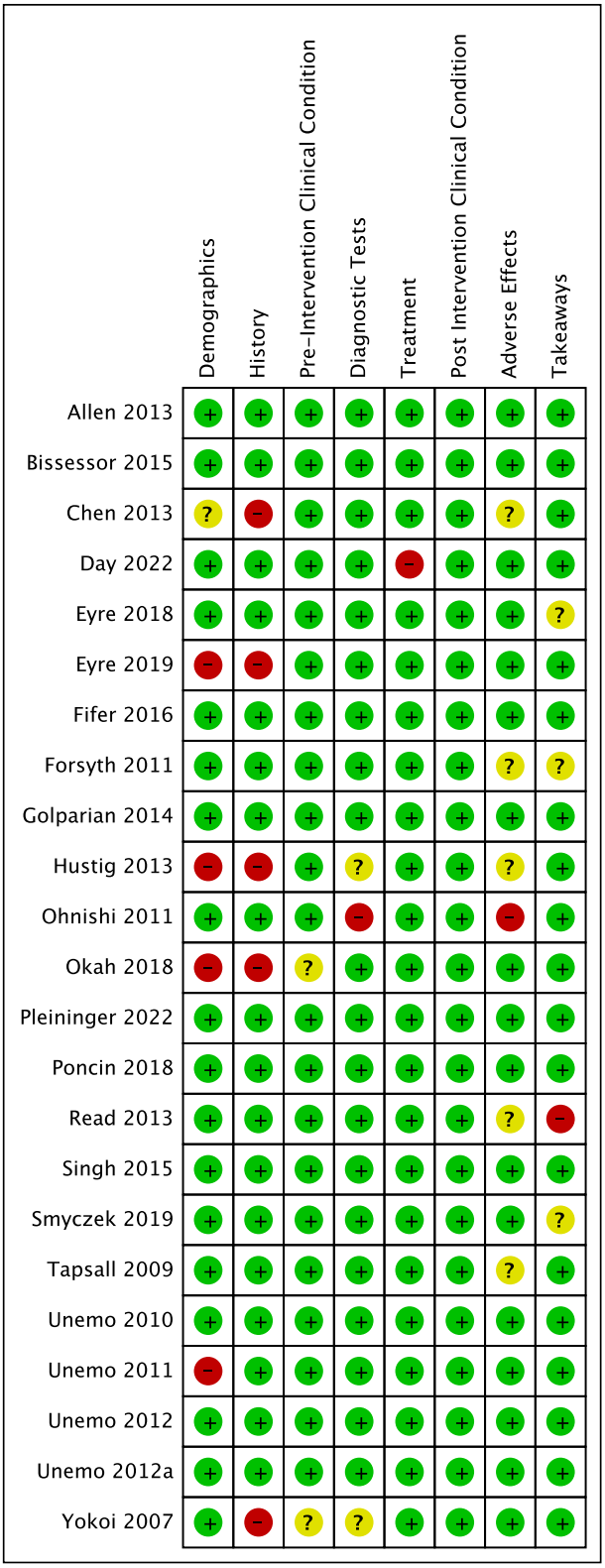
Appendix A4: Joanna Briggs Institute (JBI) checklist

JBI Critical Appraisal Checklist for Case Reports. Criteria used to rate quality and bias in included studies. Taken and adapted from JBI.

	Yes	No	Unclear	Not applicable
Were patient's demographic characteristics clearly described?	□	□	□	□
Was the patient's history clearly described and presented as a timeline?	□	□	□	□
Was the current clinical condition of the patient on presentation clearly described?	□	□	□	□
Were diagnostic tests or assessment methods and the results clearly described?	□	□	□	□
Was the intervention(s) or treatment procedure(s) clearly described?	□	□	□	□
Was the post-intervention clinical condition clearly described?	□	□	□	□
Were adverse events (harms) or unanticipated events identified and described?	□	□	□	□
Does the case report provide takeaway lessons?	□	□	□	□

Appendix A5. Risk of bias assessment using Joanna Briggs Institute checklist





Appendix B1: Multi-site study ethics letter

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LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

MSc Research Ethics Committee

Matthew Blakiston
MSc Student
MSc Medical Microbiology
LSHTM

9 April 2014

Dear Matthew,

Study Title: Relationship between multisite Neisseria gonorrhoeae infection, N. gonorrhoeae multi-antigen sequence typing (NG-MAST), and antimicrobial resistance

LSHTM MSc Ethics ref: 7604

Thank you for your application for the above MSc research project.

As your project is a lab-based project using pathogens or other organisms that are not in human tissue, ethical approval from the MSc Research Ethics Committee is not required for your project.

Please ensure that the patient identifiers are removed *before* the samples are sent to you, otherwise you will have identifiable data (Q42a states that identifiers will be removed subsequently). Please also check local R&D requirements for the study as they will likely need to approve prior to sending out the samples from each Trust.

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an Amendment form on the ethics online applications website: http://leo.lshtm.ac.uk .

Best of luck with your project.

Yours sincerely,

[redacted]

Professor Tim Rhodes
Chair

MScEthics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

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Appendix B2: Identification and susceptibility testing results of study isolates.

All isolates identified as *Neisseria gonorrhoeae* by home diagnostic laboratory. LSHTM identification confirmed by oxidase and gram stain; with pharyngeal isolates further confirmed by Analytical Profile Index (API, Biomerieux). Minimum inhibitory concentrations (MIC) for penicillin and ciprofloxacin were determined by E-test (Biomerieux) and for remaining antimicrobials, agar dilution was used. β-lactamases were detected by cefinase on any isolate with a penicillin MIC ≥1 mg/L.

Values in ***bold italics*** indicate patients with multiple gonococcal strains.

Patient	Isolate Number	Infection Site	Gram	Ox	API	PEN	β-lac	CFX	CRO	AZI	CIP	TET	SPE
1	GC1U	Urethra	GNDC	Pos	NT	0.064	NT	0.004	0.004	0.004	0.004	0.5	16
	GC1C	Cervix	GNDC	Pos	NT	0.064	NT	0.008	0.004	0.004	0.004	0.5	16
2‡	GC2C	Cervix	GNDC	Pos	NT	0.032	NT	0.016	0.004	0.002	0.004	0.063	4
	GC2P	Pharynx	GNR	Pos	*Kingella**	0.06	NT	0.064	0.002	0.032	0.064	0.063	4
3	GC3U	Urethra	GNDC	Pos	NT	0.008	NT	0.008	0.004	0.061	0.016	0.5	16
	GC3R	Rectum	GNDC	Pos	NT	0.012	NT	0.008	0.004	0.061	0.016	0.5	16
4	GC4U	Urethra	GNDC	Pos	NT	>32	Pos	0.004	0.004	0.061	12	0.25	16
	GC4R	Rectum	GNDC	Pos	NT	>32	Pos	0.004	0.004	0.031	8	0.25	16
5	GC5R	Rectum	GNDC	Pos	NT	0.006	NT	0.004	0.004	0.031	0.004	0.5	16
	GC5P	Pharynx	GNDC	Pos	*Ng*	0.006	NT	0.004	0.004	0.031	0.004	0.25	16
6	GC6P	Pharynx	GNDC	Pos	*Ng*	0.064	NT	0.008	0.004	0.031	0.006	0.25	8
	GC6R	Rectum	GNDC	Pos	NT	0.064	NT	0.004	0.004	0.031	0.004	0.125	8
7	GC7U	Urethra	GNDC	Pos	NT	0.064	NT	0.002	0.002	0.002	0.008	0.125	4
	GC7C	Cervix	GNDC	Pos	NT	0.094	NT	0.004	0.002	0.004	0.006	0.25	4
8	GC8P	Pharynx	GNDC	Pos	*Ng*	0.125	NT	0.008	0.008	0.008	>32	0.5	8
	GC8R	Rectum	GNDC	Pos	NT	0.125	NT	0.002	0.008	0.008	>32	0.5	8
9	GC9U	Urethra	GNDC	Pos	NT	0.094	NT	0.008	0.004	0.004	0.008	0.125	16
	GC9P	Pharynx	GNDC	Pos	*Ng*	0.094	NT	0.004	0.004	0.004	0.008	0.125	16

	GC9R	Rectum	GNDC	Pos	NT	0.12	NT	0.004	0.002	0.004	0.006	0.125	8
10	GC10U	Urethra	GNDC	Pos	NT	0.003	NT	0.002	0.004	0.004	0.006	0.063	16
	GC10C	Cervix	GNDC	Pos	NT	0.008	NT	0.002	0.002	0.002	0.006	0.063	16
11	GC11U	Urethra	GNDC	Pos	NT	0.25	NT	0.008	0.004	0.031	>32	0.25	16
	GC11R	Rectum	GNDC	Pos	NT	0.25	NT	0.008	0.008	0.031	>32	0.25	16
12	GC12P	Pharynx	GNDC	Pos	*Ng*	0.047	NT	0.002	0.002	0.004	0.006	0.25	16
	GC12R	Rectum	GNDC	Pos	NT	0.047	NT	0.002	0.002	0.004	0.006	0.25	16
13	GC13P	Pharynx	GNDC	Pos	*Ng*	0.125	NT	0.061	0.008	0.031	0.006	0.5	32
	GC13R	Rectum	GNDC	Pos	NT	0.125	NT	0.031	0.008	0.031	0.006	0.5	32
14[§]	GC14P	Pharynx	GNDC	Pos	*Ng*	0.064	NT	0.004	0.004	0.004	0.008	0.25	8
	GC14R	Rectum	GNDC	Pos	NT	0.064	NT	0.004	0.004	0.004	0.008	0.25	8
15	GC15R	Rectum	GNDC	Pos	NT	0.064	NT	0.016	0.016	0.031	>32	1	8
	GC15P	Pharynx	GNDC	Pos	*Ng*	0.094	NT	0.016	0.016	0.031	>32	1	8
16	***GC16P***	***Pharynx***	***GNDC***	***Pos***	***Ng***	***0.016***	***Neg***	***0.016***	***0.016***	***0.061***	***0.023***	***2***	***32***
	GC16U	***Urethra***	***GNDC***	***Pos***	***NT***	***0.016***	***NT***	***0.004***	***0.002***	***0.004***	***0.006***	***16***	***16***
17	GC17C	Cervix	GNDC	Pos	NT	0.032	NT	0.002	0.002	0.016	16	16	32
	GC17P	Pharynx	GNDC	Pos	*Ng*	0.032	NT	0.002	0.002	0.016	12	16	32
18	GC18U	Urethra	GNDC	Pos	NT	0.047	NT	0.004	0.004	0.004	0.006	0.25	16
	GC18P	Pharynx	GNDC	Pos	*Ng*	0.064	NT	0.004	0.004	0.004	0.006	0.25	16
19	GC19P	Pharynx	GNDC	Pos	*Ng*	0.25	NT	0.032	0.016	0.008	6	0.5	16
	GC19R	Rectum	GNDC	Pos	NT	0.25	NT	0.032	0.008	0.008	8	0.5	16
20	***GC20P***	***Pharynx***	***GNDC***	***Pos***	***Ng***	***0.19***	***NT***	***0.064***	***0.016***	***0.015***	***0.016***	***16***	***16***
	GC20R	***Rectum***	***GNDC***	***Pos***	***NT***	***>32***	***Pos***	***0.008***	***0.008***	***0.004***	***>32***	***16***	***16***
21	GC21R	Rectum	GNDC	Pos	NT	>32	Pos	0.004	0.002	0.008	>32	0.5	16
	GC21P	Pharynx	GNDC	Pos	*Ng*	>32	Pos	0.004	0.002	0.008	>32	0.5	16
22	GC22U	Urethra	GNDC	Pos	NT	0.023	NT	0.004	0.002	0.008	0.012	0.5	16
	GC22P	Pharynx	GNDC	Pos	*Ng*	0.016	NT	0.004	0.002	0.008	0.016	0.5	16
23	GC23U	Urethra	GNDC	Pos	NT	0.032	NT	0.002	0.004	<0.002	0.008	0.25	16
	GC23R	Rectum	GNDC	Pos	NT	0.032	NT	0.004	0.004	0.008	0.008	0.5	16
24	GC24U	Urethra	GNDC	Pos	NT	0.38	NT	0.008	0.008	0.032	0.023	2	16
	GC24C	Cervix	GNDC	Pos	NT	0.38	NT	0.008	0.008	0.015	0.032	2	32

25	***GC25U***	***Urethra***	***GNDC***	***Pos***	***NT***	***0.125***	***NT***	***0.016***	***0.002***	***0.004***	***8***	***16***	***16***
	GC25R	***Rectum***	***GNDC***	***Pos***	***NT***	***0.125***	***NT***	***0.004***	***0.002***	***0.004***	***0.064***	***16***	***16***
26	GC26C	Cervix	GNDC	Pos	NT	0.125	NT	0.004	0.002	0.015	0.012	0.125	16
	GC26U	Urethra	GNDC	Pos	NT	0.125	NT	0.004	0.002	0.008	0.016	0.125	16
27	GC27R	Rectum	GNDC	Pos	NT	0.094	NT	0.004	0.002	0.008	0.008	0.5	16
	GC27U	Urethra	GNDC	Pos	NT	0.094	NT	0.004	0.002	0.008	0.008	0.5	16
28	GC28U	Urethra	GNDC	Pos	NT	0.032	NT	0.002	0.002	0.008	0.006	0.25	16
	GC28C	Cervix	GNDC	Pos	NT	0.032	NT	0.002	0.002	0.015	0.008	0.25	16
	GC28R	Rectum	GNDC	Pos	NT	0.064	NT	0.002	0.002	0.004	0.008	0.5	16
29	GC29P	Pharynx	GNDC	Pos	*Ng*	0.094	NT	0.064	0.008	0.015	0.008	0.5	16
	GC29R	Rectum	GNDC	Pos	NT	0.25	NT	0.125	0.016	0.031	0.008	0.5	16
30	GC30R	Rectum	GNDC	Pos	NT	0.064	NT	0.004	0.004	0.015	0.016	0.5	16
	GC30P	Pharynx	GNDC	Pos	*Ng*	0.064	NT	0.004	0.004	0.015	0.012	0.5	16
31	GC31R	Rectum	GNDC	Pos	NT	0.125	NT	0.004	0.002	0.004	0.004	0.5	4
	GC31P	Pharynx	GNDC	Pos	*Ng*	0.125	NT	0.004	0.002	0.004	0.004	0.5	4
	GC31U	Urethra	GNDC	Pos	NT	0.125	NT	0.004	0.002	0.004	0.004	0.25	8
32	GC32U	Urethra	GNDC	Pos	NT	0.25	NT	0.008	0.002	0.008	0.004	0.5	8
	GC32C	Cervix	GNDC	Pos	NT	0.19	NT	0.004	0.002	0.004	0.004	0.5	4
33	GC33R	Rectum	GNDC	Pos	NT	0.047	NT	0.004	0.002	0.008	0.004	0.5	4
	GC33U	Urethra	GNDC	Pos	NT	0.047	NT	0.004	0.002	0.015	0.008	0.5	8
34	GC34P	Pharynx	GNDC	Pos	*Ng*	0.5	NT	0.004	0.002	0.004	0.003	0.5	4
	GC34R	Rectum	GNDC	Pos	NT	0.5	NT	0.008	0.004	0.004	0.004	0.5	8
35	GC35U	Urethra	GNDC	Pos	NT	1.5	neg	0.008	0.008	0.016	>32	1	8
	GC35P	Pharynx	n/a	Pos	*Ng*	0.5	NT	0.008	0.004	0.008	>32	1	4
36	***GC36U***	***Urethra***	***GNDC***	***Pos***	***NT***	***2***	***pos***	***0.004***	***0.002***	***0.002***	***0.008***	***0.5***	***8***
	GC36R	***Rectum***	***GNDC***	***Pos***	***NT***	***6***	***pos***	***0.008***	***0.002***	***0.004***	***0.008***	***0.125***	***8***
37‡	GC37P	Pharynx	GNR	Pos	*Kingella**	0.06	NT	0.125	0.016	0.125	0.032	0.063	8
	GC37R	Rectum	GNDC	Pos	NT	>32	pos	0.008	0.004	0.008	0.25	8	4
38	***GC38R***	***Rectum***	***GNDC***	***Pos***	***NT***	***8***	***pos***	***0.008***	***0.002***	***0.008***	***0.008***	***0.125***	***8***
	GC38P	***Pharynx***	***GNDC***	***Pos***	***Ng***	***0.19***	***NT***	***0.008***	***0.002***	***0.008***	***3***	***16***	***8***
	GC38U	***Urethra***	***GNDC***	***Pos***	***NT***	***0.25***	***NT***	***0.004***	***0.002***	***0.016***	***6***	***16***	***8***

39	GC39R	Rectum	GNDC	Pos	NT	0.19	NT	0.016	0.002	0.004	0.006	0.125	4
	GC39C	Cervix	GNDC	Pos	NT	0.125	NT	0.016	0.004	0.008	0.008	0.125	4
	GC39U	Urethra	GNDC	Pos	NT	0.125	NT	0.016	0.004	0.008	0.008	0.125	4
	GC39P	Pharynx	GNDC	Pos	*Ng*	0.094	NT	0.016	0.004	0.008	0.006	0.25	8
40	***GC40P***	***Pharynx***	***GNDC***	***Pos***	***Ng***	***0.25***	***NT***	***0.016***	***0.002***	***0.016***	***4***	***8***	***8***
	GC40R	***Rectum***	***GNDC***	***Pos***	***NT***	***0.25***	***NT***	***0.016***	***0.008***	***0.008***	***2***	***0.25***	***4***
41	GC41R	Rectum	GNDC	Pos	NT	0.094	NT	0.008	0.002	0.004	0.006	0.063	4
	GC41P	Pharynx	GNDC	Pos	*Ng*	0.094	NT	0.016	0.004	0.008	0.006	0.125	8
	GC41U	Urethra	GNDC	Pos	NT	0.19	NT	0.008	0.002	0.004	0.003	0.063	8
42	GC42R	Rectum	GNDC	Pos	NT	1	neg	0.016	0.008	0.032	0.023	2	8
	GC42U	Urethra	GNDC	Pos	NT	1	neg	0.016	0.008	0.032	0.023	0.5	8
	GC42P	Pharynx	GNDC	Pos	*Ng*	0.5	NT	0.016	0.008	0.032	0.016	2	8
43	GC43C	Cervix	GNDC	Pos	NT	0.25	NT	0.008	0.016	0.016	1.75	1	8
	GC43P	Pharynx	GNDC	Pos	*Ng*	0.25	NT	0.064	0.008	0.016	1.75	0.125	8
	GC43U	Urethra	GNDC	Pos	NT	0.5	NT	0.016	0.008	0.008	1.75	0.5	8
44‡§	GC44U	Urethra	RIP										
	GC44P	Pharynx	RIP										
45‡§	GC45U	Urethra	RIP										
	GC45R	Rectum	RIP										
46‡⁎	GC46U	Urethra	RIP										
	GC46R	Rectum	RIP										

§Isolates from Royal Free London NHS Foundation Trust, ⁎isolates from Maidstone and Tunbridge Wells NHS Trust, all other isolates from St George's University Hospital NHS Foundation Trust

*‡Not included in the analysis, *Confirmed by whole genome sequencing. GNDC; Gram negative diplococci, GNR; Gram negative rod, Ng; Neisseria gonorrhoeae, NT; Not tested, Pos; positive, Neg; negative, Ox; Oxidase test. β-lac; β-lactamase test (cefinase), PEN; Penicillin, CFX; Cefixime, CRO; Ceftriaxone, AZI; Azithromycin, CIP; Ciprofloxacin, TET; Tetracycline, SPE; Spectinomycin. RIP; Unable to resuscitate on arrival*

Appendix B3: Genotypic resistance determinants and SNPs in genes associated with antimicrobial resistance. Information determined by Pathogen Watch.

Isolate Number	PEN	β-lac	CFX	CRO	AZI	CIP	TET	SPE
GC16P	*mtrR*_promoter_a-57del; *penA*_ins346D; *ponA*1_L421P; *porB1b*_G120K/A121D	None	None	None	None	None	*mtrR*_promoter_a-57del; *rpsJ*_V57M	None
GC16U	*mtrR*_A39T; *penA*_ins346D	None	None	None	None	None	*mtrR*_A39T; *rpsJ*_V57M; *tetM*	None
GC20P	*mtrR*_A39T; *penA*_I312M/ V316T/ G545S; *porB1b*_G120K/A121N	None	*penA*_I312M/ V316T/G545S‡	None	None	None	*mtrR*_A39T; rpsJ_V57M	None
GC20R	*mtrR*_disrupted/A39T; *penA*_ins346D; *ponA1*_L421P	TEM-1	None	None	None	*gyrA*_S91F/D95A; *parC*_S87N	*mtrR*_disrupted/A39T; *rpsJ*_V57M; *tetM*	None
GC25U	*mtrR*_A39T; *penA*_ins346D	None	None	None	None	None	*mtrR*_A39T; *rpsJ*_V57M; *tetM*	None
GC25R	*mtrR*_A39T; *penA*_ins346D	None	None	None	None	None	*mtrR*_A39T; *rpsJ*_V57M; *tetM*	None

GC36U	*penA*_ins346D	TEM-1	None	None	None	None	*rpsJ*_V57M	None
GC36R	*penA*_ins346D	TEM-1	None	None	None	None	*rpsJ*_V57M	None
GC38R	*penA*_ins346D	TEM-1	None	None	None	None	*rpsJ*_V57M	None
GC38P	*mtrR*_G45D; *mtrR*_promoter_a-57del; *penA*_ins346D; *ponA1*_L421P	None	*mtrR*_G45D*	None	*mtrR*_G45D; *mtrR*_promoter_a-57del*	*gyrA*_S91F/D95A; *parC*_S87R	*mtrR*_G45D; *mtrR*_promoter_a-57del; rpsJ_V57M; *tetM*	None
GC38U	*mtrR*_G45D; *mtrR*_promoter_a-57del; *penA*_ins346D; *ponA1*_L421P	None	*mtrR*_G45D*	None	*mtrR*_G45D; *mtrR*_promoter_a-57del*	*gyrA*_S91F/D95A; *parC*_S87R	*mtrR*_G45D; *mtrR*_promoter_a-57del; rpsJ_V57M; *tetM*	None
GC40P	*mtrR*_G45D; *mtrR*_promoter_a-57del; *penA*_ins346D; *ponA1*_L421P	None	*mtrR*_G45D*	None	*mtrR*_G45D; *mtrR*_promoter_a-57del*	*gyrA*_S91F/D95A; *parC*_S87R	*mtrR*_G45D; *mtrR*_promoter_a-57del; *rpsJ*_V57M; *tetM*	None
GC40R	*mtrR*_G45D; *mtrR*_promoter_a-57del; *penA*_ins346D/A501V/P551S; *ponA1*_L421P	None	*mtrR*_G45D*	None	*mtrR*_G45D; *mtrR*_promoter_a-57del*	*gyrA*_S91F/D95G; *parC*_D86N	*mtrR*_G45D; *mtrR*_promoter_a-57del; *rpsJ*_V57M	None

**Does not infer phenotypic resistance, ‡Mosaic penA*

PEN; penicillin, β-lac; beta lactamase, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin

Appendix B4: Antimicrobial susceptibility testing of multi-site isolates.

Antibiotic sensitivity tested by agar dilution unless otherwise stated.

Replicate	PEN‡		CFX		CRO		AZI		CIP‡		TET		SPE	
	GC16P	**GC26U**	**GC16P**	**GC26U**	**GC16P**	**GC26U**	**GC16P**	**GC26U**	**GC16P**	**GC26U**	**GC16P**	**GC26U**	**GC16P**	**GC26U**
1	0.016	0.016	0.016	0.004	0.016	0.002	0.063	0.004	0.023	0.006	2	16	32	16
2	NT	NT	0.016	0.004	0.016	0.002	0.063‡	<0.016‡	0.032	0.006	2	16	NT	NT
	GC20P	**GC20R**	**GC20P**	**GC20R**	**GC20P**	**GC20R**	**GC20P**	**GC20R**	**GC20P**	**GC20R**	**GC20P**	**GC20R**	**GC20P**	**GC20R**
1	0.19	>32§	0.064	0.008	0.016	0.008	0.015	0.004	0.016	>32	16	16	16	16
2	0.25	>32§	0.094‡	0.016‡	0.012‡	0.004‡	0.03‡	0.004‡	4	>32	NT	NT	NT	NT
	GC25U	**GC25R**	**GC25U**	**GC25R**	**GC25U**	**GC25R**	**GC25U**	**GC25R**	**GC25U**	**GC25R**	**GC25U**	**GC25R**	**GC25U**	**GC25R**
1	0.125	0.125	0.016	0.004	0.002	0.002	0.004	0.004	8	0.064	16	16	16	16
2	0.19	0.125	0.016	0.004	0.004‡	0.003‡	0.008	0.004	4	0.064	16	16	8	8
3	0.25	0.125	<0.016‡	<0.016‡	NT	NT	0.008	0.004	4	0.032	NT	NT	NT	NT
4	0.25	0.25	0.016‡	<0.016‡	NT	NT	NT	NT	0.032	0.032	NT	NT	NT	NT
	GC36U	**GC36R**	**GC36U**	**GC36R**	**GC36U**	**GC36R**	**GC36U**	**GC36R**	**GC36U**	**GC36R**	**GC36U**	**GC36R**	**GC36U**	**GC36R**
1	2§	6§	0.004	0.008	0.002	0.002	0.002	0.004	0.008	0.008	0.5	0.125	8	8
2	1§	4§	<0.016‡	<0.016‡	NT	NT	0.002	0.004	NT	NT	0.5	0.125	4	8
3	2§	2§	NT	NT	41mm*	45mm*	NT	NT	32mm*	40mm*	30mm*	23mm*	15mm*	15mm*
	GC38R	**GC38P** ***GC38U***	**GC38R**	**GC38P** ***GC38U***	**GC38R**	**GC38P** ***GC38U***	**GC38R**	**GC38P** ***GC38U***	**GC38R**	**GC38P** ***GC38U***	**GC38R**	**GC38P** ***GC38U***	**GC38R**	**GC38P** ***GC38U***
1	8§	0.19 *0.25*	0.008	0.008 *0.004*	0.002	0.002 *0.002*	0.008	0.008 *0.016*	0.008	3 *6*	0.125	16 *16*	8	8 *8*
2	8§	0.16 *0.5*	0.008	0.008 *0.008*	0.002	0.002 *0.002*	NT	NT *NT*	NT	NT *NT*	NT	NT *NT*	NT	NT *NT*
3	NT	NT	NT	NT	42mm*	43mm*	NT	NT	38mm*	0mm*	29mm*	0mm*	15mm*	15mm*

		NT		*NT*		*40mm**		*NT*		*0mm**		*0mm**		
	GC40P	**GC40R**	**GC40P**	**GC40R**	**GC40P**	**GC40R**	**GC40P**	**GC40R**	**GC40P**	**GC40R**	**GC40P**	**GC40R**	**GC40P**	**GC40R**
1	0.25	0.25	0.016	0.016	0.002	0.008	0.016	0.008	4	2	8	0.25	8	4
2	0.25	0.5	0.016	0.016	0.002	0.008	0.016	0.008	NT	NT	16	0.25	8	4
3	NT	NT	NT	NT	41mm*	45mm*	NT	NT	0mm*	10mm*	0mm*	31mm*	15mm*	20mm*

*PEN; penicillin, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin, NT; Not tested. §; β-lactamase positive, ‡; E-test, *; Disk diffusion.*

Appendix B5: Complete MIC testing results for additional isolates from multi-site study

Complete MIC testing and confirmation for gonococcal isolates from patients 3, 7, 8, 10, 13, 17, 29 and 43

Patient 3	Study Number	MIC (mg/L)						
		PEN	CFX	CRO	AZI	CIP	TET	SPE
Test 1	GC3U	0.016	0.008	0.004	0.061	***0.008***	NT	NT
	GC3R	0.012	0.008	0.004	0.061	***0.002***	NT	NT
Test 2	GC3U	0.008	0.008	0.004	0.061	0.016	0.5	16
	GC3R	0.012	0.008	0.004	0.061	0.008	0.5	16
Test 3	GC3U	0.008	0.008	0.004	0.061	0.016	0.5	16
	GC3R	0.012	0.008	0.004	0.061	0.016	0.5	16

Patient 7	Study Number	MIC (mg/L)						
		PEN	CFX	CRO	AZI	CIP	TET	SPE
Test 1	GC7U	0.064	***0.002***	0.004	<0.016	0.008	NT	NT
	GC7C	0.094	***0.008***	0.004	<0.016	0.006	NT	NT
Test 2	GC7U	0.064	0.002	0.004	<0.016	0.008	0.125	4
	GC7C	0.094	0.004	0.004	<0.016	0.006	0.25	4
Test 3	GC7U	0.064	0.002	0.004	<0.016	0.008	0.125	4
	GC7C	0.094	0.004	0.004	<0.016	0.006	0.25	4

Patient 8	Study Number	MIC (mg/L)						
		PEN	CFX	CRO	AZI	CIP	TET	SPE
Test 1	GC8P	0.125	***0.008***	0.008	<0.016	>32	NT	NT
	GC8R	0.125	***0.002***	0.004	<0.016	>32	NT	NT
Test 2	GC8P	0.125	0.008	0.008	0.008	>32	0.5	8
	GC8R	0.125	0.004	0.004	0.008	>32	0.5	8
Test 3	GC8P	0.125	0.008	0.008	0.008	>32	0.5	8
	GC8R	0.125	0.004	0.008	0.008	>32	0.5	8

Patient 10	Study Number	MIC (mg/L)						
		PEN	CFX	CRO	AZI	CIP	TET	SPE
Test 1	GC10U	***0.002***	<0.002	0.002	<0.016	0.006	NT	NT
	GC10C	***0.016***	<0.002	0.002	<0.016	0.006	NT	NT
Test 2	GC10U	0.008	0.002	0.004	0.004	0.006	0.063	16
	GC10C	0.008	0.002	0.002	0.002	0.006	0.063	16
Test 3	GC10U	0.004	0.002	0.004	0.004	0.006	0.063	16
	GC10C	0.008	0.002	0.002	0.002	0.006	0.063	16

Patient 13	Study Number	MIC (mg/L)						
		PEN	CFX	CRO	AZI	CIP	TET	SPE
Test 1	GC13P	0.064	***0.06***	0.008	0.031	0.006	NT	NT
	GC13R	0.064	***0.016***	0.008	0.031	0.006	NT	NT
Test 2	GC13P	0.125	0.06	0.008	0.031	0.006	0.25	32
	GC13R	0.064	0.03	0.008	0.031	0.006	0.5	32
Test 3	GC13P	0.125	0.06	0.008	0.031	0.006	0.5	32
	GC13R	0.125	0.03	0.008	0.031	0.006	0.5	32

Patient 17	Study Number	MIC (mg/L)						
		PEN	CFX	CRO	AZI	CIP	TET	SPE
Test 1	GC17C	0.016	<0.002	0.002	0.031	***16***	NT	NT
	GC17P	0.016	<0.002	0.002	0.031	***4***	NT	NT
Test 2	GC17C	0.032	0.002	0.002	0.016	16	16	>32
	GC17P	0.032	0.002	0.002	0.031	12	16	>32
Test 3	GC17C	0.032	0.002	0.002	0.016	16	16	>32
	GC17P	0.032	0.002	0.002	0.016	12	16	>32

Patient 29	Study Number	MIC (mg/L)						
		PEN	CFX	CRO	AZI	CIP	TET	SPE
Test 1	GC29P	0.094	***0.064***	***0.016***	0.016	0.008	NT	NT
	GC29R	0.125	***0.25***	***0.25***	0.031	0.008	NT	NT
Test 2	GC29P	0.094	0.064	0.008	0.016	0.008	0.5	16
	GC29R	0.25	0.125	0.016	0.031	0.008	0.5	16
Test 3	GC29P	0.094	0.064	0.008	0.016	0.008	0.5	16
	GC29R	0.25	0.125	0.016	0.031	0.008	0.5	16

Patient 43	Study Number	MIC (mg/L)						
		PEN	CFX	CRO	AZI	CIP	TET	SPE
Test 1	GC43C	0.25	0.032	0.016	0.016	***1.5***	***1***	8
	GC43P	0.25	0.016	0.008	0.016	***2***	***0.125***	8
	GC43U	0.5	0.008	0.008	0.016	***4***	***0.5***	8
Test 2	GC43C	0.25	0.016	0.016	0.016	1.5	1	8
	GC43P	0.5	0.016	0.008	0.016	2	0.5	8
	GC43U	0.5	0.008	0.008	0.008	2	0.5	8
Test 3	GC43C	0.25	0.008	0.016	0.016	1.5	1	8
	GC43P	0.25	0.016	0.008	0.016	2	0.5	8
	GC43U	0.5	0.016	0.008	0.008	2	0.5	8

MIC; minimum inhibitory concentration, NT; not tested, PEN; penicillin, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin, Study numbers ending in P, U and R denote pharyngeal, urethral and rectal isolates respectively

Appendix B6: Details of missing data

Missing data details from patients who had a positive nucleic acid amplification test and negative gonococcal culture. Cells in red indicate missing isolates.

MICs in mg/L/

Patient	Sex	Site	Culture Swab taken	Culture Result	NAAT swab taken	NAAT result	PEN	CFX	CRO	AZI	CIP	TET	SPE
		Urethra	Yes	Positive	Yes	Positive	>32	0.004	0.004	0.061	12	0.25	16
4	Male	Rectum	Yes	Positive	Yes	Positive	>32	0.004	0.004	0.031	8	0.25	16
		Throat	**Yes**	**Negative**	**Yes**	**Positive**							
		Urethra	Yes	Positive	Yes	Positive	0.25	0.008	0.004	0.031	>32	0.25	16
11	Male	Rectum	Yes	Positive	Yes	Positive	0.25	0.008	0.008	0.031	>32	0.25	16
		Throat	**Yes**	**Negative**	**Yes**	**Positive**							
		Cervix	Yes	Positive	Yes	Positive	0.032	0.002	0.002	0.016	16	16	>32
17	Female	Throat	Yes	Positive	YES	Positive	0.032	0.002	0.002	0.016	12	16	>32
		Rectum	**YES**	**Negative**	**Yes**	**Positive**							
		Urethra	Yes	Positive	YES	Positive	0.023	0.004	0.002	0.008	0.012	0.5	16
22	Male	Throat	Yes	Positive	Yes	Positive	0.016	0.004	0.002	0.008	0.016	0.5	16
		Rectum	**YES**	**Negative**	**YES**	**Positive**							
		Urethra	Yes	Positive	YES	Positive	0.032	0.002	0.004	<0.002	0.008	0.25	16
23	Male	Rectum	Yes	Positive	YES	Positive	0.032	0.004	0.004	0.008	0.008	0.5	16
		Throat	**YES**	**Negative**	**YES**	**Positive**							
		Urethra	Yes	Positive	YES	Positive	0.125	0.016	0.002	0.008	>32	2	16
25	Male	Rectum	Yes	Positive	YES	Positive	0.125	0.004	0.002	0.004	0.064	16	16
		Throat	**Yes**	**Negative**	**YES**	**Positive**							
		Urethra	Yes	Positive	No	n/a	0.032	0.002	0.002	0.008	0.006	0.25	16
28	Female	Cervix	Yes	Positive	Yes	Positive	0.032	0.002	0.002	0.015	0.008	0.25	16
		Rectum	Yes	Positive	Yes	Positive	0.064	0.002	0.002	0.004	0.008	0.5	16

		Throat	**Yes**	**Negative**	**Yes**	**Positive**							
		Urethra	Yes	Positive	Yes	Positive	1	0.004	0.002	0.004	0.008	0.5	8
36	Male	Rectum	Yes	Positive	Yes	Positive	6	0.008	0.002	0.004	0.008	0.125	8
		Throat	**Yes**	**Negative**	**Yes**	**Positive**							

n/a; not available, PEN; penicillin, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin

Missing data details for isolates that were not sent by the primary diagnostic laboratories. Cells in red indicate missing isolates.MICs in mg/L.

Patient	Sex	Site	Culture Swab taken	Culture Result	NAAT swab taken	NAAT result	PEN	CFX	CRO	AZI	CIP	TET	SPE
	Male	Urethra	Yes	Positive	Yes	Positive	0.008	0.008	0.004	0.061	0.016	0.5	16
3		Rectum	Yes	Positive	Yes	Positive	0.012	0.008	0.004	0.061	0.016	0.5	16
		Throat	**Yes**	**Positive**	**Yes**	**Positive**							
		Urethra	Yes	Positive	Yes	Positive	0.25	0.008	0.002	0.008	0.004	0.5	8
6	Female	Cervix	Yes	Positive	No	n/a	0.19	0.004	0.002	0.004	0.004	0.5	4
		Throat	No	n/a	No	n/a							
		Rectum	**Yes**	**Positive**	**Yes**	**Positive**							
		Rectum	Yes	Positive	No	n/a	0.047	0.004	0.002	0.008	0.004	0.5	4
33	Female	Urethra	Yes	Positive	No	n/a	0.047	0.004	0.002	0.015	0.008	0.5	8
		Throat	No	n/a	No	n/a							
		Cervix	**Yes**	**Positive**	**Yes**	**Positive**							
		Cervix	Yes	Positive	Yes	Positive	0.25	0.032	0.016	0.016	1.5	1	8
43	Female	Throat	Yes	Positive	Yes	Positive	0.25	0.064	0.008	0.016	2	0.125	8
		Urethra	Yes	Positive	No	n/a	0.5	0.016	0.008	0.008	4	0.5	8
		Rectum	**yes**	**Positive**	**No**	**n/a**							

n/a; not available, PEN; penicillin, CFX; cefixime, CRO; ceftriaxone, AZI; azithromycin, CIP; ciprofloxacin, TET; tetracycline, SPE; spectinomycin

Appendix C1: Commensal *Neisseria* ethics letter

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LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Research Ethics Committee

Mr Wesley Bonnin

MSc Student

LSHTM

27 June 2019

Dear Wesley ,

Study Title: Prevalence of commensal Neisseria species and antimicrobial resistance in the general population: pilot study.

LSHTM Ethics ref: 17126-01

Thank you for submitting your amendment for the above research project.

Your amendment has been assessed by the Research Governance & Integrity Office and has been approved as a non-substantial change. The amendment does not require further ethical approval from the MSc ethics committee.

List of documents reviewed:

Document Type	File Name	Date	Version
Other	Neisseria Study - Information sheet - v2.2	27/06/2019	v2.2

Any subsequent changes to the application must be submitted to the Committee via an Amendment form on the ethics online applications website: http://leo.lshtm.ac.uk .

Best of luck with your project.

Yours sincerely,

[redacted]

Rebecca Carter

Research Governance Coordinator

Ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

Appendix C2: Study eligibility checklist

Investigators: Victoria Miari/Wesley Bonnin
Participant checklist

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Participant eligibility criteria

Title of Project: 'Investigating the number of commensal Neisseria bacteria in the throat of staff/students at LSHTM'

Before you read the patient information leaflet, this is to inform you of the eligibility criteria in this study. Some conditions or activities may alter the results of our study, and therefore we would ask that you are able to fulfil these before participating,

- ☐ **I am employed or studying at the LSHTM.**
- ☐ **I have not taken antibiotics for one month prior to this date.**
- ☐ **I have not used any dental mouthwashes in the last three days prior to participation (such as Corsodyl or Listerine).**
- ☐ **I am not allergic to chlorhexidine.**
- ☐ **I do not have a condition or take medication which causes immunosuppression (weakened immune system), such as being on chemotherapy.**

If you fulfil these criteria you can participate in our study. Feel free to read the patient information leaflet and speak to one of the researchers who will talk through the steps involved. Thank you for your participation!

Participant checklist v1.0
Date: 14/05/19

Page **1** of **1**

Appendix C3: Consent Form

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

PARTICIPANT CONSENT FORM

Title of Project: 'Investigating the number of commensal Neisseria bacteria in the throat of staff/students at LSHTM'

Principle investigator: Victoria Miari

Co-investigator: Wes Bonnin

Statement	Please initial each box
I confirm that I have read the information sheet dated_____ (version_____) for the above-named study. I have had the opportunity to consider the information, ask questions and have these answered satisfactorily.	
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	
I understand that data about/from me/the participant may be shared directly with other researchers, and that I will not be identifiable from this information	
(Include if applicable) I understand that the bacterial isolates collected from me/the participant will be used to support other research in the future, and may be shared anonymously with other researchers, for their ethically approved projects.	
I confirm that I am not immunosuppressed, and I have not taken antibiotics in the past month, or used a dental mouthwash in the past two days.	
I understand that no personal information will be collected in this study, apart from age and gender.	
I understand that the specimen acquired will not be used to detect an infectious agent or disease.	
I agree to take part in the above-named study.	
I understand I will be randomised to either a control (water gargle) or intervention group (Corsodyl)	

Printed name of participant	Signature of participant	Date

Printed name of person obtaining consent	Signature of person obtaining consent	Date

A copy of this informed consent document has been provided to the participant.

Version number: 1.2
Date: 21/06/19
Informed Consent Form

Appendix C4: Commensal *Neisseria* species as reported by MALDI-ToF MS, and phenotypic characteristics on LBVT.SN media.

Peritonsillar swabs from 50 participants were spread onto LBVT.SNR agar. Morphologically distinct colonies were sub-cultured onto chocolate agar. Gram stain, oxidase and antimicrobial sensitivity testing were performed on each isolate. Gram-negative, oxidase positive cocci were considered as presumptive *Neisseria spp*. and species determined by MALDI-ToF.

MALDI ID 1; Primary identification (best species match), MALDI ID 2; Secondary identification

MALDI score >1.99; high confidence ID, MALDI score 1.7-1.99; low confidence ID, MALDI score <1.7; No organism ID possible.

Isolate	MALDI ID 1	MALDI Score 1	MALDI ID 2	MALDI Score 2	Sucrose Fermenter	Colonial Morphology
1E	*N. subflava*	2.05	*N. subflava*	2.05	No	Smooth
1A	*N. subflava*	2.2	*N. subflava*	2.18	No	Smooth
1B	*N. subflava*	2.22	*N. subflava*	2.19	Yes	Smooth
2A	*N. subflava*	1.97	*N. subflava*	1.8	No	Smooth
2B	*N. macacae*	2.31	*N. subflava*	1.91	Yes	Dry
2C	*Neisseria spp*	ND	NP	NP	No	Smooth
3A	*Neisseria spp*	ND	NP	NP	No	Smooth
3B	*N. subflava*	2.05	*N. subflava*	2.03	Yes	Smooth
4A	*N. subflava*	1.89	*N. subflava*	1.87	No	Smooth
4B	*N. subflava*	2.07	*N. subflava*	2.06	Yes	Dry
5A	*N. subflava*	2.24	*N. subflava*	2.18	No	Dry
5B	*N. subflava*	2.21	*N. subflava*	2.16	Yes	Smooth
5C	*N. subflava*	2.02	*N. subflava*	1.98	Yes	Smooth

5D	*N. flavescens*	2.05	*N. subflava*	2.03	No	NR
5E	*N. flavescens*	2.13	*N. subflava*	2.1	Yes	Smooth
7A	*N. subflava*	1.99	*N. subflava*	1.98	No	Smooth
7B	*N. macacae*	2.26	*N. mucosa*	1.93	Yes	Smooth
8E	*N. subflava*	1.91	*N. flavescens*	1.88	No	Smooth
8A	*N. macacae*	2.14	*N. subflava*	1.8	Yes	Dry
8D	*N. subflava*	2.31	*N. subflava*	2.31	No	Dry
8F	*N. subflava*	1.97	*N. subflava*	1.9	NR	Dry
8G	*N. subflava*	1.91	*N. flavescens*	1.89	NR	NR
8H	*N. macacae*	2.14	*N. mucosa*	1.93	No	Dry
8I	*N. flavescens*	2.16	*N. subflava*	2.15	No	Smooth
8J	*Neisseria spp*	ND	None	1.5	No	Dry
9A	*N. subflava*	1.99	*N. flavescens*	1.96	No	Dry
9B	*N. flavescens*	2.07	*N. perflava*	1.82	No	Smooth
10A	*N. subflava*	1.94	*N. subflava*	1.89	No	Smooth
10B	*N. subflava*	2.2	*N. subflava*	2.16	Yes	Smooth
11A	*N. subflava*	2.13	*N. subflava*	1.94	No	Smooth
11B	*N. subflava*	2.02	*N. flavescens*	2.01	Yes	Smooth
12A	*N. subflava*	2.16	*N. subflava*	2.11	No	Smooth
12B	*N. subflava*	2.12	*N. subflava*	2.05	Yes	Dry
13A	*N. subflava*	2.08	*N. subflava*	2.03	Yes	Smooth
13B	NP	NP	NP	1.21	No	Smooth
14A	*N. flavescens*	2.04	*N. perflava*	2.02	No	NR
14B	*N. perflava*	2.17	*N. flavescens*	2.03	No	Smooth
15A	*N. perflava*	2.04	*N. subflava*	2.08	No	Dry

15B	*N. subflava*	2.19	*N. subflava*	2.08	No	Smooth
15C	*N. subflava*	2.1	*N. subflava*	2.1	NR	Dry
18A	NP	NP	NP	NP	No	Smooth
18A	*N. subflava*	2.11	*N. perflava*	2.09	No	Smooth
19A	*N. mucosa*	1.92	*N. cinera*		No	NR
20A	*N. subflava*	2.2	*N. perflava*	2.19	Yes	Smooth
21A	*N. perflava*	2.15	*N. flavescens*	2.1	Yes	Dry
21B	*N. perflava*	2.08	*N. flavescens*	2.06	Yes	Smooth
21C	*N. subflava*	2.15	*N. subflava*	2.11	Yes	Dry
22A	*N. subflava*	2.15	*N. subflava*	2.07	Yes	Dry
22B	*N. subflava*	2.03	*N. flavescens*	1.96	No	Smooth
22C	*N. flavescens*	2.07	*N. flavescens*	2.07	Yes	Smooth
24A	*N. subflava*	2.36	*N. subflava*	2.32	No	NR
24B	*N. flavescens*	1.97	*N. flavescens*	1.97	No	Smooth
24C	*N. perflava*	2.09	*N. flavescens*	1.98	No	Smooth
25A	*N. subflava*	2.18	*N. subflava*	2.12	No	Smooth
26A	*N. perflava*	2.03	*N. subflava*	1.96	No	Dry
26B	*N. mucosa*	2.01	*N. subflava*	1.97	No	Smooth
27A	*N. subflava*	2.05	*N. subflava*	2.01	No	Dry
28A	*N. perflava*	2.16	*N. flavescens*	2.14	No	Smooth
28B	*N. subflava*	2.28	*N. subflava*	2.27	Yes	Smooth
29A	*N. flavescens*	2.08	*N. subflava*	2.08	Yes	Smooth
30A	*N. subflava*	2.09	*N. subflava*	2.05	No	Smooth
30B	*N. subflava*	2.19	*N. subflava*	2.18	Yes	Dry
30C	*N. subflava*	2.27	*N. subflava*	2.18	Yes	Smooth

32A	*N. subflava*	2.22	*N. subflava*	2.17	NR	Dry
32B	*N. subflava*	2.23	*N. subflava*	2.22	No	Smooth
32C	*N. flavescens*	2.13	*N. subflava*	2.12	Yes	Smooth
33A	*N. subflava*	2.18	*N. subflava*	2.12	No	Smooth
34A	*N. subflava*	2.08	*N. subflava*	2.03	Yes	Dry
35B	*N. subflava*	2.36	*N. subflava*	2.3	Yes	Smooth
35A	*N. subflava*	2.21	*N. subflava*	2.2	Yes	Smooth
36A	*N. subflava*	2.15	*N. subflava*	2.09	Yes	Smooth
36B	*N. subflava*	2.22	*N. subflava*	2.15	No	Smooth
38A	*N. subflava*	2.01	*N. subflava*	1.89	No	Smooth
38B	*N. subflava*	1.76	NP	1.69	Yes	Smooth
39A	*N. subflava*	2.07	*N. subflava*	1.99	No	Smooth
39B	*N. macacae*	1.81	*N. mucosa*	1.75	Yes	Smooth
40A	*N. flavescens*	2.01	*N. subflava*	1.84	No	NR
40B	*N. subflava*	2.16	*N. subflava*	2.06	No	Dry
40C	*N. subflava*	2.12	*N. flavescens*	2.03	Yes	Smooth
42A	*N. macacae*	2.15	*N. mucosa*	2.02	Yes	Smooth
43A	*N. subflava*	2.1	*N. subflava*	2.05	No	Smooth
44E	*Neisseria spp*	ND	NP	ND	No	Smooth
44A	*N. perflava*	2.14	*N. subflava*	2.12	No	NR
44B	*N. subflava*	1.95	*Neisseria spp*	1.89	No	Smooth
44C	*N. subflava*	2.14	*N. subflava*	2.14	No	NR
45A	*N. subflava*	2.18	*N. subflava*	2.17	Yes	Smooth
45B	*N. subflava*	2.1	*N. subflava*	1.92	No	Smooth
46A	*N. subflava*	1.93	*N. flavescens*	1.73	No	Smooth

47A	*N. flavescens*	2.11	*N. subflava*	2	No	Smooth
47B	*N. flavescens*	2.18	*N. subflava*	2.16	Yes	NR
48A	*N. subflava*	2.24	*N. subflava*	2.18	No	Smooth
48B	*N. flavescens*	1.9	*N. subflava*	1.87	Yes	Smooth
48C	*N. flavescens*	1.95	*N. flavescens*	1.92	No	NR
49E	*N. subflava*	2.23	*N. subflava*	2.22	Yes	NR
49A	*N. mucosa*	2.11	*N. macacae*	2.05	No	Smooth
49C	NP	NP	NP	1.54	Yes	Smooth
49D	NP	NP	NP	1.59	Yes	Smooth
49F	*N. flavescens*	1.83	*N. subflava*	1.77	No	Smooth
49B	*N. subflava*	2.24	*N. subflava*	2.18	Yes	Smooth
50A1	*Neisseria spp*	ND	NP	1.33	No	NR
50B1	*N. perflava*	1.73	*N. flavescens*	1.72	NR	Smooth
50C1	*Neisseria spp*	ND	NP	1.43	Yes	NR

ND; none detected, NP; no ID possible, NR; not reported

Appendix C5: Full MIC data (mg/L) for commensal *Neisseria spp*

Cefixime MIC by gradient strip, all other MICs by agar dilution on gonococcal medium base.

Isolate	PEN	CRO	CIP	AZI	GEN	TET	CFX
1E	2	0.015	0.016	4	4	32	0.032
1A	0.5	0.125	1	0.125	4	32	0.064
1B	0.5	0.125	0.016	0.125	4	32	0.094
2A	0.5	0.125	0.5	512	4	8	0.047
2B	0.5	0.06	0.125	0.25	4	2	0.047
2C	0.03	NV	NV	NV	NV	NV	NV
3A	0.25	0.125	0.5	2	4	0.5	0.023
3B	0.25	0.015	0.016	0.125	2	0.25	0.38
4A	1	0.125	0.016	0.25	4	1	0.094
4B	0.5	1	0.032	0.125	4	4	0.064
5A	2	0.5	0.016	1	4	1	0.047
5B	1	0.03	0.5	1	4	0.5	0.032
5C	0.5	0.125	0.016	0.125	4	0.25	0.5
5D	0.25	0.06	0.5	4	2	2	0.023
5E	0.25	0.03	0.064	0.25	4	1	0.032
7A	0.5	0.125	0.016	1	4	1	0.094
7B	1	0.125	0.125	2	4	4	0.125
8E	4	0.015	32	0.125	4	1	0.064
8A	1	0.125	0.064	0.5	4	0.5	0.19
8D	2	0.015	0.032	8	8	0.5	0.064
8F	4	8	32	0.125	4	1	0.047
8G	0.5	0.08	0.5	0.032	2	0.5	0.032
8H	4	0.06	32	0.063	2	0.5	0.064
8I	2	0.25	0.016	0.032	4	1	0.023
8J	0.03	0.25	NV	NV	NV	NV	NV
9A	1	0.06	0.016	0.125	4	0.25	0.047
9B	1	0.06	0.008	0.032	2	0.25	0.064
10A	2	0.125	0.5	1.5	4	32	0.125
10B	4	0.125	2	0.5	4	32	0.032
11A	2	0.06	0.5	1	4	1	0.064

11B	1	0.06	0.016	0.125	2	0.25	0.064
12A	1	0.06	0.016	0.064	4	0.25	0.023
12B	1	0.03	0.032	0.25	4	0.25	0.094
13A	4	0.06	0.5	2	4	1	0.023
13B	2	0.015	0.032	1.5	8	1	0.094
14A	0.5	0.25	0.032	0.25	4	0.5	0.064
14B	0.5	0.25	0.016	0.125	4	0.5	0.094
15A	2	0.06	1	1	2	0.5	0.047
15B	0.5	0.06	1	1	4	0.5	0.032
15C	0.25	0.125	1	1	4	32	0.125
18A	0.125	0.03	0.016	2	4	0.25	0.047
18B	0.125	0.008	0.008	0.75	2	0.5	0.016
19A	2	0.06	0.016	1	4	0.25	0.047
20A	1	0.06	0.016	4	8	32	0.064
21A	0.25	4	0.5	1	2	0.5	0.047
21B	0.25	0.08	0.5	1	2	0.5	0.032
21C	0.125	0.08	0.5	0.75	2	0.25	0.047
22A	1	0.06	0.008	2	4	0.25	0.047
22B	0.25	0.015	0.016	0.5	4	0.25	0.032
22C	1	0.015	0.016	0.125	4	0.25	0.032
24A	0.5	0.03	2	0.06	2	0.5	0.094
24B	4	0.03	0.5	0.75	4	0.5	0.125
24C	0.5	0.06	0.5	0.75	4	1	0.125
25A	0.5	0.06	0.5	1	4	0.5	0.032
26A	2	0.125	0.016	4	4	0.5	0.094
26B	0.03	0.06	0.016	0.125	4	0.5	0.064
27A	2	0.06	0.5	1.5	4	0.25	0.047
28A	2	0.03	0.016	1.5	4	0.125	0.064
28B	2	0.06	0.016	4	4	0.5	0.094
29A	0.125	0.08	0.016	1.5	4	0.25	0.094
30A	1	0.125	0.064	2	4	0.25	0.064
30B	0.5	0.03	0.016	2	4	0.25	0.094
30C	2	0.125	2	512	2	2	0.047
32A	2	0.06	0.032	0.063	8	0.5	0.064
32B	1	0.06	0.008	0.032	4	0.25	0.064
32C	1	0.015	0.032	0.125	4	0.25	0.064
33A	0.5	0.25	8	0.032	4	0.032	0.5

34A	4	0.25	0.032	0.125	4	0.5	0.125
35B	0.125	0.03	0.032	0.032	4	16	0.094
35A	0.25	0.125	0.016	0.032	4	16	0.064
36A	0.25	0.06	0.008	0.032	0.5	0.032	0.002
36B	0.5	0.5	0.016	0.5	4	0.25	0.047
38A	0.5	0.06	0.016	0.032	4	0.25	0.047
38B	0.5	0.06	0.5	0.032	4	8	0.047
39A	0.25	0.06	0.016	0.032	8	0.25	0.032
39B	2	0.125	2	2	2	2	0.064
40A	1	0.06	0.5	0.032	2	0.25	0.047
40B	1	0.06	0.25	0.016	2	0.25	0.094
40C	1	0.06	1	0.032	2	8	0.125
42A	2	0.25	1	0.5	2	1	0.064
43A	1	0.06	0.008	0.016	4	0.5	0.094
44A	1	0.06	NV	NV	NV	NV	NV
44E	2	0.06	0.016	0.016	4	0.5	0.125
44A	0.5	0.06	0.016	0.032	4	0.25	0.032
44B	2	0.06	0.008	0.016	4	0.5	0.094
45A	1	0.06	0.016	1.5	4	4	0.047
45B	0.5	0.03	0.032	3	4	0.25	0.047
46A	0.5	0.125	0.008	1	2	16	0.125
47A	1	0.06	0.016	1.5	4	0.25	0.032
47B	1	0.25	2	4	16	1	0.5
48A	1	0.125	NV	NV	NV	NV	NV
48B	4	0.125	NV	NV	NV	NV	NV
48C	1	0.015	NV	NV	NV	NV	NV
49E	1	0.06	0.5	0.032	2	1	0.064
49A	0.5	0.06	0.25	0.032	2	0.5	0.047
49C	0.03	0.015	0.5	1	2	0.5	0.064
49F	1	0.06	0.5	0.032	2	1	0.047
49B	1	0.015	0.016	3	4	0.5	0.023
50A	0.5	0.06	NV	NV	NV	NV	NV
50B	0.125	0.015	NV	NV	NV	NV	NV
50C	0.03	0.015	NV	NV	NV	NV	NV

PEN; penicillin, CRO; ceftriaxone, CIP; ciprofloxacin, AZI; azithromycin, GEN; gentamicin, TET; tetracycline, CFX; cefixime,

NV; Not tested as non-viable upon resuscitation.

Appendix C6: Commensal *Neisseria* reference genomes

High quality reference genomes used in this study. Multilocus sequence type (MLST) designated sequence type (ST) is derived from unique combination of *abcZ, adk, aroE, fumC, gdh, pdhC, pgm* alleles. ~n denotes a novel full-length allele with ≥ 95% identity to allele number 'n'. n? denotes a partial match to known allele with ≥ 10% coverage and identity ≥ 95%. – denotes no match to existing alleles with ≥ 10% coverage and identity ≥ 95%. Reference and study isolates were used to generate a whole genome MLST (wgMLST) schema and a nearest neighbour phylogeny consisting of 5 main clusters labelled; 1. *Nm/Ng*, 2. *N. bacilliformis*, 3. *N. flavescens*, 4. *N. subflava*, 5. *N. macacae*.

Accession ID	Species	Strain	ST	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	wgMLST group
RKRJ01	*N. animalis*	*N. animalis DSM 23392*	-	528	357	621	-	551	552	551	*N. bacilliformis*
MTBN01	*N. animaloris*	*N. animaloris DSM 21642*	-	-	-	-	-	-	-	-	*N. bacilliformis*
POXR01	*N. animaloris*	*N. animaloris C2012029644*	-	-	-	-	-	-	771	-	*N. bacilliformis*
POYC01	*N. animaloris*	*N. animaloris C2015003240*	-	-	-	-	-	-	-	-	*N. bacilliformis*
AFAY01	*N. bacilliformis*	*N. bacilliformis* ATCC BAA-1200	9330	552	353	605	529	589	548	545	*N. bacilliformis*
JUOC01	*N. bacilliformis*	*N. bacilliformis 914_NLAC*	-	552?	374	605?	553	~589	~586	~568	*N. bacilliformis*
JVQC01	*N. bacilliformis*	*N. bacilliformis* 203	-	552?	~359	~664	~525	~638	~586	641	*N. bacilliformis*
MTBL01	*N. canis*	*N. canis ATCC 14687*	-	540	377	625	-	574	571	570	*N. bacilliformis*
MTBO01	*N. dentiae*	*N. dentiae DSM 19151*	9335	539	376	624	554	572	570	569	*N. bacilliformis*
PXYY01	*N. iguanae*	*N. iguanae ATCC 51483*	-	-	-	-	-	-	-	-	*N. bacilliformis*
AGAY01	*N. shayeganii*	*N. shayeganii* 871	-	596	417	-	-	-	656	656	*N. bacilliformis*
AGAZ01	*N. wadsworthii*	N. wadsworthii 9715	-	595	416	669	-	643	623	655	*N. bacilliformis*
AFWR01	*N. weaveri*	*N. weaveri* ATCC 51223	-	529	358	618	533	587	~553	552?	*N. bacilliformis*
MTBM01	*N. zoodegmatis*	N. zoodegmatis DSM 21643	-	~746	~516	791?	~752	757?	~769	552?	*N. bacilliformis*
ACEN01	*N. flavescens*	*N. flavescens* NRL30031	3576	244	172	296	285	270	259	276	*N. flavescens*
UGQV01	*N. flavescens*	*N. flavescens NCTC8263*	3576	244	172	296	285	270	259	276	*N. flavescens*
CAJZIH01	*N. flavescens*	*N. flavescens* ERR2764931_	-	~822	-	~307	~879	272	~573	~270	*N. flavescens*

		bin.5_metaWRAP_v1.1_MAG									
LAEK01	*N. flavescens*	*N. flavescens CNF seq0078*	-	~271	538?	~511	~854	~276	~792	~270	*N. flavescens*
CAJPLX01	*N. subflava*	*N. subflava* SRR9217391-mag-bin.22	-	527?	~171	~883	86?	~566	~512	~502	*N. flavescens*
ADBF01	*N. elongata*	*N. elongata* subsp. *glycolytica* ATCC 29315	9806	493	330	604	491	584	542	540	*N. macacae*
JAGJWT01	*N. elongata*	*N. elongata subsp. nitroreducens Nel_M001*	-	~776	~328	~604	~692	~588	~534	~540	*N. macacae*
POXH01	*N. elongata*	*N. elongata C2010010207*	-	~312	~328	~607	~487	~586	~550	~546	*N. macacae*
AFQE01	*N. macacae*	*N. macacae* ATCC 33926	9339	525	354	577	530	548	549	547	*N. macacae*
ACDX02	*N. mucosa*	*N. mucosa* ATCC 25996	8082	492	329	546	490	520	513	503	*N. macacae*
AEPF01	*N. sicca*	*N. sicca* 4320	3707	236	170	317	268	274	288	264	*N. macacae*
AJMT01	*N. sicca*	*N. sicca* VK64	10254	526	355	578	531	549	613	548	*N. macacae*
POXX01	*N. sicca*	*N. sicca C2014002478*	-	~236	~367	583	~743	~520	~804	~547	*N. macacae*
LSIT01	*N. perflava*	*N. perflava CCH10-H12*	-	233	178	-	-	-	561	-	*N. subflava*
PKJQ01	*N. perflava*	*N. perflava UMB0023*	-	770	543	820	~299	785	794	810	*N. subflava*
ACEO02	*N. subflava*	*N. subflava* NJ9703	9805	490	345	306	488	269	277	505	*N. subflava*
POWV01	*N. subflava*	*N. subflava C2007002879*	-	~19	~538	~244	895	~269	~308	~898	*N. subflava*
POXL01	*N. subflava*	*N. subflava C2011009653*	-	685?	~578	~883	296?	~823	~568	844	*N. subflava*
POYB01	*N. subflava*	*N. subflava C2014021188*	-	~836	~542	~883	~487	~272	~308	~272	*N. subflava*
POWY01	*N. bergeri*	*N. bergeri C2008000328*	3558	225	166	23	255	278	269	271	*Nm/Ng*
POWZ01	*N. bergeri*	*N. bergeri C2008000329*	3558	225	166	23	255	278	269	271	*Nm/Ng*
QQHX01	*N. bergeri*	*N. bergeri M40463*	12190	495	257	23	29	538	28	529	*Nm/Ng*
ACDY02	*N. cinerea*	*N. cinerea* ATCC 14685	3579	247	167	284	298	283	284	274	*Nm/Ng*
AE004969	*N. gonorrhoeae*	*N. gonorrhoeae* FA 1090	1899	109	39	67	190	147	71	65	*Nm/Ng*
CP001050	*N. gonorrhoeae*	*N. gonorrhoeae* NCCP11945	1901	109	39	170	111	148	153	65	*Nm/Ng*
NC_011035	*N. gonorrhoeae*	*N. gonorrhoeae* NCCP11945	1901	109	39	170	111	148	153	65	*Nm/Ng*
CP003909	*N. gonorrhoeae*	*N. gonorrhoeae* MS11	6959	126	39	67	78	146	153	133	*Nm/Ng*
CP002440	*N. gonorrhoeae*	*N. gonorrhoeae* TCDC-NG08107	7363	59	39	67	78	148	153	65	*Nm/Ng*
FN995097	*N. lactamica*	*N. lactamica* 020-06	640	84	49	48	50	92	46	45	*Nm/Ng*
AEPI01	*N. lactamica*	*N. lactamica* NS19	12442	61	511	328	66	625	218	196	*Nm/Ng*
QQLL01	*N. lactamica*	*N. lactamica M37101*	-	83	37	87	45	90	44	~168	*Nm/Ng*
AL157959	*N. meningitidis*	*N. meningitidis serogroup A Z2491*	4	1	3	3	1	4	2	3	*Nm/Ng*

FR774048	*N. meningitidis*	*N. meningitidis* WUE 2594	5	1	1	2	1	3	2	3	*Nm/Ng*
CP007524	*N. meningitidis*	*N. meningitidis 510612*	7	1	1	2	1	3	2	19	*Nm/Ng*
CP002419	*N. meningitidis*	*N. meningitidis* G2136	8	2	3	7	2	8	5	2	*Nm/Ng*
AM421808	*N. meningitidis*	*N. meningitidis* serogroup C FAM18	11	2	3	4	3	8	4	6	*Nm/Ng*
CP002420	*N. meningitidis*	*N. meningitidis* H44/76	32	4	10	5	4	6	3	8	*Nm/Ng*
CP002421	*N. meningitidis*	*N. meningitidis* M01-240149	41	3	6	9	5	9	6	9	*Nm/Ng*
CP002424	*N. meningitidis*	*N. meningitidis* NZ-05/33	42	10	6	9	5	9	6	9	*Nm/Ng*
AM889136	*N. meningitidis*	*N. meningitidis* alpha14	53	16	2	6	25	17	25	22	*Nm/Ng*
AE002098	*N. meningitidis*	*N. meningitidis* MC58	74	4	10	5	4	5	3	2	*Nm/Ng*
CP001561	*N. meningitidis*	*N. meningitidis* alpha710	136	27	6	9	3	9	6	16	*Nm/Ng*
FM999788	*N. meningitidis*	*N. meningitidis* 8013	177	7	8	10	38	10	1	20	*Nm/Ng*
CP002422	*N. meningitidis*	N. meningitidis M01-240355	213	7	5	1	13	36	53	15	*Nm/Ng*
CP002423	*N. meningitidis*	*N. meningitidis* M04-240196	269	4	10	15	9	8	11	9	*Nm/Ng*
CP000381	*N. meningitidis*	*N. meningitidis* 053442	4821	222	3	58	275	30	5	255	*Nm/Ng*
ADBE01	*N. polysaccharea*	*N. polysaccharea* ATCC 43768	3557	106	66	40	46	219	43	261	*Nm/Ng*

Appendix C7: Commensal *Neisseria* study genomes

Multilocus sequence type (MLST) designated sequence type (ST) is derived from unique combination of abcZ, adk, aroE, fumC, gdh, pdhC, pgm alleles. ~n denotes a novel full-length allele with ≥ 95% identity to allele number 'n'. n? denotes a partial match to known allele with ≥ 10% coverage and identity ≥ 95%. – denotes no match to existing alleles with ≥ 10% coverage and identity ≥ 95%. PubMLST denotes Neisseria species associated with the given MLST ST in the PubMLST database (accessed Aug 2022). Match6 denotes Neisseria species that share 6 out 7 alleles in the PubMLST database. Match5 denotes Neisseria species that share 5 out 7 alleles. Match4 denotes Neisseria species that share 4 out 7 alleles. MALDI-ToF was the species predicted by matrix-assisted laser desorption/ionization time of flight analysis.

Reference and study isolates were used to generate a whole genome MLST (wgMLST) schema and a nearest neighbour phylogeny consisting of 5 main clusters labelled; 1. *Nm/Ng*, 2. *N. bacilliformis*, 3. *N. flavescens*, 4. *N. subflava*, 5. *N. macacae*.

NOP; No organism possible

Isolate	ST	abcZ	adk	aroE	fumC	gdh	pdhC	pgm	PubMLST	Match6	Match5	Match4	MALDI Primary ID	wgMLST group
10A	-	~57	~453	-	~634	~823	~800	~555					*N. subflava*	*N. flavescens*
11A	-	495?	~382	~883	612?	303?	~308	~555					*N. subflava*	*N. flavescens*
13A	-	536	~431	~581	~269	~695	~512	~557					*N. subflava*	*N. flavescens*
13B	-	~822	~159	581?	~905	~350	790	~566					*NOP*	*N. subflava*
14B	-	495?	180	~848	488	269	277	260				*N subflava*	*N. perflava*	*N. subflava*
18A	-	685	453	738	488?	~707	308	~809					*N. subflava*	*N. subflava*
1A	-	~57	~382	~883	~777	303?	~800	~393					*N. subflava*	*N. flavescens*

22B	-	~537	~615	~441	~488	~269	~568	~267			*N. subflava*	*N. subflava*
24A	-	~237	~174	~816	~363	~850	~551	~283			*N. subflava*	*N. subflava*
25A	-	770	~382	~883	~777	303?	~800	~393			*N. subflava*	*N. flavescens*
28A	-	231	180	~848	~296	269	277	260		*N. subflava*	*N. perflava*	*N. subflava*
28B	-	~382	538	~816	286?	~545	800	~898			*N. subflava*	*N. subflava*
2A	-	836	~393	~883	895	276?	857	~554			*N. subflava*	*N. flavescens*
30A	-	685	453	738	698	707	711	566?	*N. subflava*		*N. subflava*	*N. subflava*
32A	-	685	453	738	698	707	711	893?	*N. subflava*		*N. subflava*	*N. subflava*
32C	-	~233	~225	~813	~269	~545	~666	~797			*N. flavescens*	*N. flavescens*
33A	-	785	~597	~587	794	~545	800	822?			*N. subflava*	*N. flavescens*
34A	-	~267	~431	~883	~488	303?	~800	~283			*N. subflava*	*N. flavescens*
35A	-	~386	180	~307	~488	269	277	~505			*N. subflava*	*N. subflava*
36B	-	~783	~180	~883	286?	269	277	260			*N. subflava*	*N. subflava*
39B	-	~591	~354	~585	~86	~520	~561	~547			*N. macacae*	*N. macacae*
42A	-	~236	~438	311?	~692	~267	~707	~262			*N. macacae*	*N. macacae*
44A	-	231	180	306	~488	269	277	260	*N. subflava*		*N. perflava*	*N. subflava*
48B	12596	785	554	833	794	798	800	822	*N. subflava*		*N. flavescens*	*N. flavescens*

49A	-	~19	174?	~848	86?	~275	~793	~502			*N. mucosa*	*N. subflava*
49C	-	~19	174?	~848	86?	~275	~793	~502			*NOP*	*N. subflava*
49D	-	~19	174?	~848	86?	~275	~793	~502			*NOP*	*N. subflava*
5A	-	762	180	306	488	778	277	260	*N. subflava*		*N. subflava*	*N. subflava*
7B	-	~638	438	~311	~620	~701	707	642?			*N. macacae*	*N. macacae*
8A	-	526	~355	578	~696	549	613	~691		*N. mucosa*	*N. macacae*	*N. macacae*

Appendix C8: Agreement of MALDI-ToF with Kraken2 identifications

Agreement of MALDI-ToF with Kraken2 identifications. MALDI ID 1; Primary identification (best species match), MALDI ID 2; Secondary identification. MALDI score >1.99; high confidence ID, MALDI score 1.7-1.99; low confidence ID, MALDI score <1.7; No organism ID possible. MIC in mg/L.
N/A; Not applicable
**Poor genome assembly*

Isolate	MALDI-ToF ID 1	MALDI-ToF 1 SCORE	MALDI-ToF ID 2	MALDI-ToF 2 SCORE	Kraken2 ID	Agreement with MALDI-ToF ID 1	Agreement with at least 1 MALDI-ToF ID	Reason for sequencing
1A	*N. subflava*	2.2	*N. subflava*	2.18	*N. subflava*	Y	Y	CRO MIC ≥0.125
2A	*N. subflava*	1.97	*N. subflava*	1.8	*N. subflava*	Y	Y	CRO MIC ≥0.125
5A	*N. subflava*	2.24	*N. subflava*	2.18	*N. subflava*	Y	Y	CRO MIC ≥0.125
7B	*N. macacae*	2.26	*N. mucosa*	1.93	*N. mucosa*	N	Y	CRO MIC ≥0.125
8A	*N. macacae*	2.14	*N. subflava*	1.8	*N. mucosa*	N	N	CRO MIC ≥0.125
10A	*N. subflava*	1.94	*N. subflava*	1.89	*N. subflava*	Y	Y	CRO MIC ≥0.125
11A	*N. subflava*	2.13	*N. subflava*	1.94	*N. subflava*	Y	Y	Representative of *N. subflava*
13A	*N. subflava*	2.08	*N. subflava*	2.03	*N. subflava*	Y	Y	Participant with >1 isolates
13B	*No organism possible*	1.23	*No organism possible*	1.21	*N. subflava*	N/A	N/A	No ID by MALDI-ToF
14B	*N. perflava*	2.17	*N. flavescens*	2.03	*N. subflava*	N	N	CRO MIC ≥0.125
18A	*N. subflava*	2.11	*N. perflava*	2.09	*N. subflava*	Y	Y	CRO MIC <0.125
22B	*N. subflava*	2.03	*N. flavescens*	1.96	*N. sublfava*	Y	Y	CRO MIC ≥0.125
24A	*N. subflava*	2.36	*N. subflava*	2.32	*N. subflava*	Y	Y	Representative of *N. subflava*
25A	*N. subflava*	2.18	*N. subflava*	2.12	*N. subflava*	Y	Y	CRO MIC <0.125
28A	*N. perflava*	2.16	*N. flavescens*	2.14	*N. subflava*	N	N	Representative of *N. perflava*

28B	*N. subflava*	2.28	*N. subflava*	2.27	*N. subflava*	Y	Y	CRO MIC <0.125
30A	*N. subflava*	2.09	*N. subflava*	2.05	*N. subflava*	Y	Y	CRO MIC ≥0.125
32A	*N. subflava*	2.22	*N. subflava*	2.17	*N. subflava*	Y	Y	Participant with >1 isolates
32C	*N. flavescens*	2.13	*N. subflava*	2.12	*N. subflava*	N	N	Representative for *N. flavescens*
33A	*N. subflava*	2.18	*N. subflava*	2.12	*N. subflava*	Y	Y	CRO MIC ≥0.125
34A	*N. subflava*	2.08	*N. subflava*	2.03	*N. subflava*	Y	Y	CRO MIC ≥0.125
35A	*N. subflava*	2.21	*N. subflava*	2.2	*N. subflava*	Y	Y	CRO MIC ≥0.125
36B	*N. subflava*	2.22	*N. subflava*	2.15	*N. subflava*	Y	Y	CRO MIC ≥0.125
39B	*N. macacae*	1.81	*N. mucosa*	1.75	*N. mucosa*	N	Y	CRO MIC ≥0.125
42A	*N. macacae*	2.15	*N. mucosa*	2.02	*N. mucosa*	N	Y	Representative of *N. macacae*
44A	*N. perflava*	2.14	*N. subflava*	2.12	*N. subflava*	N	Y	CRO MIC <0.125
48B	*N. flavescens*	1.9	*N. subflava*	1.87	*N. subflava*	N	Y	CRO MIC ≥0.125
49A	*N. mucosa*	2.11	*N. macacae*	2.05	*None**	Y	Y	Representative of *N. mucosa*
49C	*No organism possible*	1.6	*None*	1.54	*N. subflava*	n/a	N/A	No ID by MALDI-ToF
49D	*No organism possible*	1.62	*No organism possible*	1.59	*N. subflava*	N/A	N/A	No ID by MALDI-ToF

Appendix C9: DNA Uptake Sequence Information

***AT-DUS**; Neisseria gonorrhoeae 12 base pair DNA uptake sequence - 5'-AT-GCCGTCTGAA-3'*

***vDUS**; Commensal Neisseria variant DNA uptake sequence (vDUS) – 5'-GTCGTCTGAA-3'*

***gcDUS**; Neisseria gonorrhoeae 10 base pair DNA uptake sequence (gcDUS) - 5'-GCCGTCTGAA-3'*

Isolate	Species by MALDI	Species by Kraken2	AT-DUS	vDUS	gcDUS
10A	*N. subflava*	*N. subflava*	165	203	2766
11A	*N. subflava*	*N. subflava*	168	169	2754
13A	*N. subflava*	*N. subflava*	174	276	2641
13B	*No ID*	*N. subflava*	173	176	2717
14B	*N. perflava*	*N. subflava*	168	167	2822
18B	*N. subflava*	*N. subflava*	177	209	2749
1A	*N. subflava*	*N. subflava*	164	179	2740
22B	*N. subflava*	*N. subflava*	159	165	2759
24A	*N. subflava*	*N. subflava*	152	183	2720
25A	*N. subflava*	*N. subflava*	161	173	2733
28A	*N. perflava*	*N. subflava*	169	181	2761
28B	*N. subflava*	*N. subflava*	144	217	2727
2A	*N. subflava*	*N. subflava*	192	158	2766
30A	*N. subflava*	*N. subflava*	177	194	2620
32A	*N. subflava*	*N. subflava*	184	218	2737
32C	*N. flavescens*	*N. subflava*	178	198	2560
33A	*N. subflava*	*N. subflava*	167	205	2774
34A	*N. subflava*	*N. subflava*	158	185	2705
35A	*N. subflava*	*N. subflava*	157	181	2691
36B	*N. subflava*	*N. subflava*	158	173	2753
39B	*N. macacae*	*N. mucosa*	32	3801	250
42A	*N. macacae*	*N. mucosa*	29	3795	220
44A	*N. perflava*	*N. subflava*	172	165	2741
48B	*N. flavescens*	*N. subflava*	165	180	2785
49C	*No ID*	*N. subflava*	165	228	2758
49D	*No ID*	*N. subflava*	170	235	2796
5A	*N. subflava*	*N. subflava*	165	173	2696
7B	*N. macacae*	*N. macacae*	39	3608	208
8A	*N. macacae*	*N. mucosa*	35	3802	257

Appendix D1: Chlorhexidine susceptibility ethics letter

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LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

MSc Research Ethics Committee

Mr Cameron Ward
MSc Student
Control of Infectious Diseases
LSHTM

27 June 2019

Dear Mr Cameron Ward

Study Title: N. gonorrhoeae chlorhexadine susceptibility

LSHTM MSc Ethics ref: 17680

Thank you for submitting your application for the above MSc research project.

As your project is a lab-based project using pathogens or other organisms that are not in human tissue, it was assessed by the Research Governance & Integrity Office as not requiring ethical approval from the MSc ethics committee. It is the student's responsibility to ensure that all other required approvals are in place before starting the research project.

Any subsequent changes to the application must be submitted to the Committee via an Amendment form on the ethics online applications website: http://leo.lshtm.ac.uk .

Best of luck with your project.

Yours sincerely,

[redacted]

Rebecca Carter

Research Governance Coordinator

MScEthics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

Appendix D2: Microbroth dilution layout

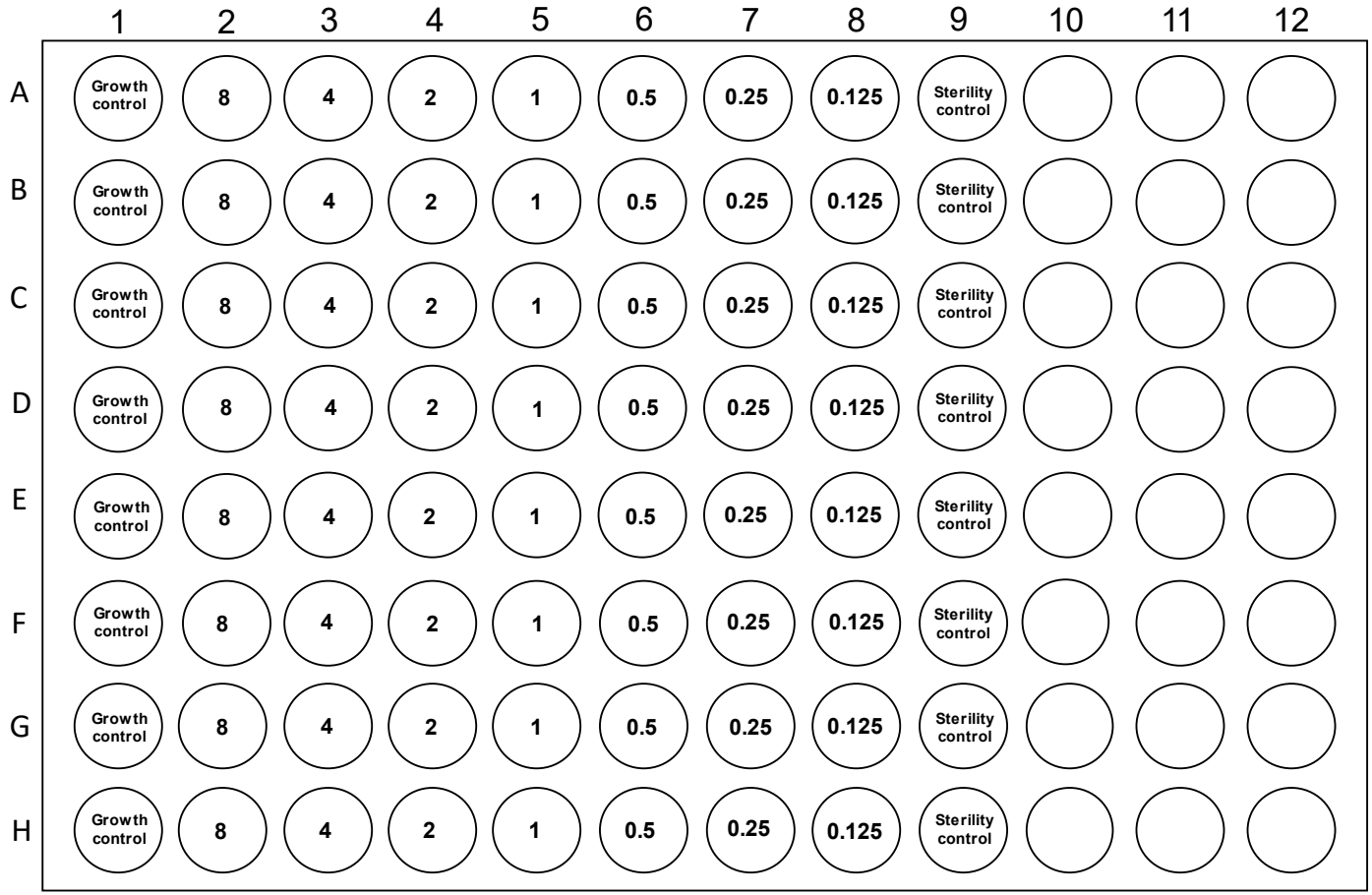

Figure D2. Microbroth dilution layout for chlorhexidine MIC testing (mg/L). Each row was inoculated with a separate *Neisseria gonorrhoeae* isolate.

Appendix D3: *Neisseria gonorrhoeae* CHX MIC and MBC data

Neisseria gonorrhoeae chlorhexidine MICs tested by agar dilution and microbroth dilution, and chlorhexidine MBCs. All in mg/L.
Study numbers ending in C indicate cervical isolate, R a rectal isolate, P a pharyngeal isolate, and N is an unknown site.

Study Number	CHX MIC AD	CHX MIC MB	CHX MBC
GC1C	1	2	2
GC2C	0.5	1	2
GC3R	1	1	1
GC4R	0.5	1	1
GC5R	1	1	2
GC6P	2	1	4
GC7C	0.5	1	2
GC8R	0.5	2	4
GC9U	1	1	2
GC12R	1	2	2
GC13R	1	1	2
GC14R	1	0.5	2
GC16U	1	1	1
GC17C	1	0.5	0.5
GC18P	1	2	2
GC19R	1	2	2
GC20P	2	2	2
GC20R	1	1	2
GC21R	1	2	2
GC22P	1	1	2
GC23R	1	1	2
GC24C	1	1	2
GC25U	1	2	2
GC25R	1	0.5	2
GC26C	1	1	2
GC27U	1	1	2
GC28C	1	1	2
GC29R	2	2	2
GC30R	1	1	1
GC31R	1	1	1
GC32C	1	0.5	2
GC33R	2	1	1
GC34R	1	1	1
GC35P	2	2	4
GC36U	0.25	0.5	2
GC36R	1	0.5	1
GC38R	1	2	2

GC38P	1	1	1
GC39R	1	1	1
GC40P	1	0.5	0.5
GC40R	1	1	1
GC43C	1	2	2
GC47N	1	1	2
GC48N	1	1	1
GC49N	1	rip	rip
GC50N	1	1	1
GC51N	0.5	rip	rip
GC52N	1	1	1
GC53N	1	2	4
GC54N	1	2	8
GC55N	0.5	0.5	0.5
GC56N	0.5	rip	rip
GC57N	1	1	1
GC58N	0.5	1	1
GC59N	1	2	8
GC60N	0.5	1	1
GC61N	0.5	1	1
GC62N	2	1	2
GC63N	0.5	2	4
GC64N	1	2	2
GC65N	1	rip	rip
GC66N	1	1	2
GC67N	1	1	8
GC68N	0.5	1	2
GC69N	0.5	1	4
GC70N	0.5	1	2
GC71N	0.5	rip	rip
GC72N	1	2	4
GC73N	0.5	2	2
GC74N	0.5	2	2
GC75N	0.25	1	1
GC76N	0.25	2	2
GC77N	1	0.5	1
GC78N	1	1	1

CHX; chlorhexidine, MIC; minimum inhibitory concentration, AD; agar dilution, MB; microbroth dilution, MBC; minimum bactericidal concentration, rip; unable to resuscitate

Appendix D4: WHO *Neisseria gonorrhoeae* MIC information

Minimum inhibitory concentrations to historical antimicrobials and chlorhexidine of WHO control strains. All MICs in mg/L.

WHO strain	PEN	CFX	CRO	AZI	CIP	TET	SPE	CHX
F	0.032	<0.016	<0.002	0.125	0.004	0.25	16	NT
G	0.5	<0.016	0.008	0.25	0.125	32	16	1
K	2	0.25	0.064	0.25	>32	2	16	2
L	2	0.125	0.25	0.5	>32	2	16	1
M	>32	<0.016	0.016	0.25	2	2	16	2
N	>32	<0.016	0.004	0.25	4	16	16	1
O	>32	0.016	0.032	0.25	0.008	2	>1024	1
P	0.25	<0.016	0.004	4	0.004	1	8	NT
V	>32	<0.016	0.064	>256	>32	4	16	1
X	4	32	2	0.5	>32	2	16	1
Y	1	8	1	1	>32	4	16	1

NT; not tested

Appendix D5: Checkerboard assay layout

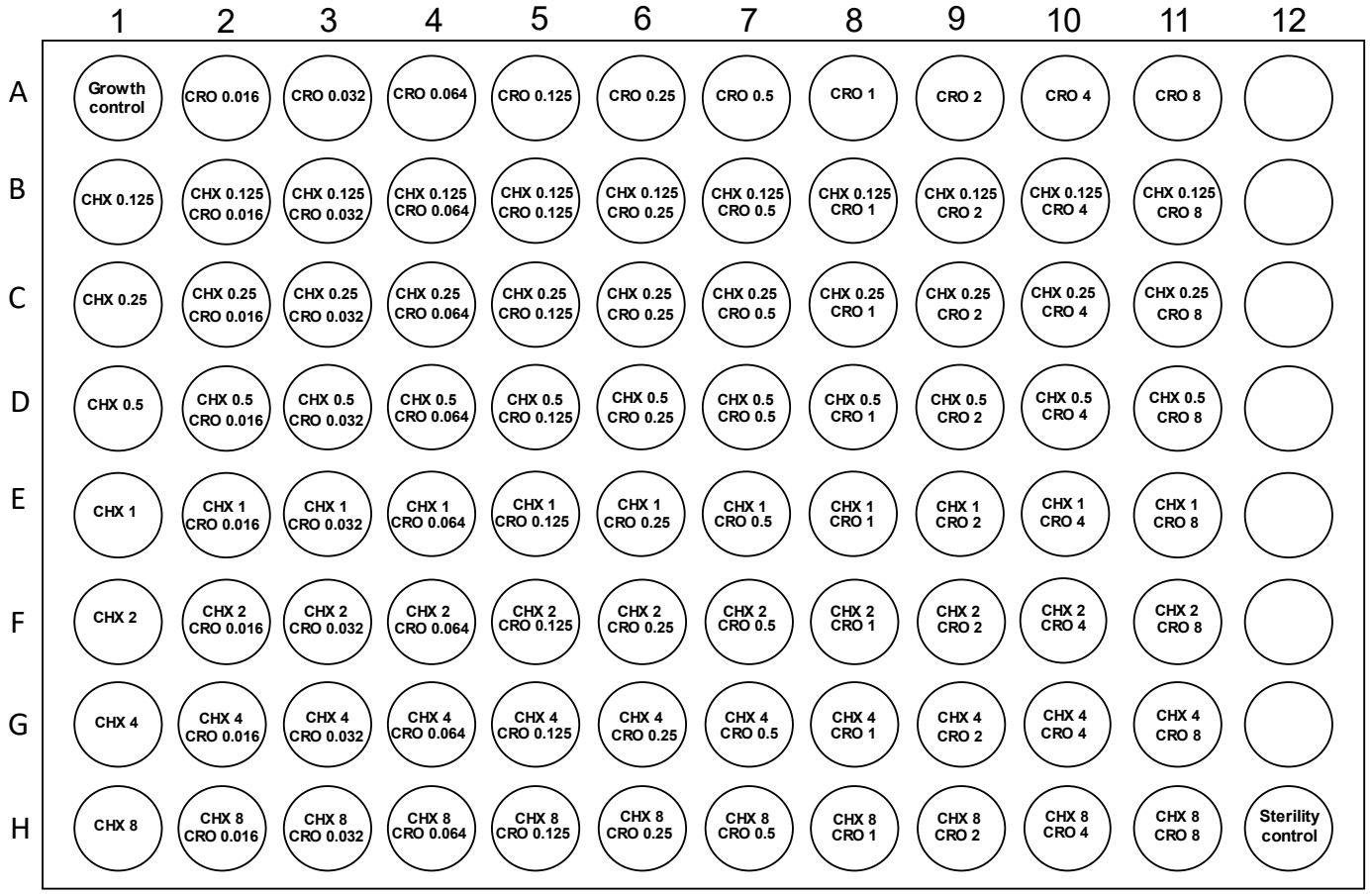

Figure D5. Plate layout for checkerboard assay, assessing synergy between chlorhexidine and ceftriaxone. All concentrations in mg/L.

Appendix D6: Commensal *Neisseria* CHX MIC data

Chlorhexidine MICs of commensal *Neisseria* species, acquired from cross-sectional study.

Colony number	MIC CHX (mg/L)	Colony number	MIC CHX (mg/L)	Colony number	MIC CHX (mg/L)
1E1	8	**14B1**	16	**38B1**	16
1A1	16	**15A1**	16	**39A1**	4
1B1	16	**15B1**	16	**39B1**	32
2A1	16	**15B2**	16	**40A1**	16
2B1	16	**18A1**	16	**40B1**	16
2A2	16	**18B1**	16	**40C1**	8
3A1	16	**19A1**	16	**42A1**	16
3B1	16	**20A1**	8	**43A1**	8
4A1	16	**21A1**	4	**44E1**	8
4A2	16	**21B1**	1	**44A1**	4
5A1	16	**21A2**	1	**44B1**	4
5C1	8	**22A1**	8	**44C1**	8
5D1	16	**22B1**	16	**45A1**	16
5E1	16	**22C2**	16	**45B1**	16
5F1	16	**24A1**	32	**46A1**	4
7A1	16	**24B2**	16	**47A1**	8
7B1	16	**25A1**	8	**47B1**	16
8E1	2	**26A2**	16	**48A1**	16
8A1	16	**26B2**	32	**48B1**	16
8D1	4	**27A1**	8	**48C1**	16
8F1	4	**28A1**	32	**49E1**	8
8G1	1	**28B1**	32	**49A1**	32
8A2	4	**29A1**	4	**49C1**	**1**
8B2	8	**30A1**	32	**49F1**	4
8D2	8	**30B1**	16	**49B2**	8
9A1	8	**30C1**	16	**50A1**	16
9A2	8	**32A1**	32	**50B1**	8
10A1	8	**32B1**	32	**50B2**	8
10B1	8	**32C1**	16		
11A1	8	**33A2**	32		
11B1	8	**34A1**	32		
12A1	8	**35B1**	16		
12B1	8	**35A2**	16		
13A1	8	**36A1**	16		
13B1	8	**36B1**	16		
14A1	16	**38A1**	4		

Appendix D7: Time-kill assay colony counts

Colony counts (cfu/mL) of gonococcal time kill assays at 30- and 60-seconds chlorhexidine contact time, in the presence and absence of 0.4% porcine mucin.

Strain	Replicate	Inoculum (cfu/mL)	0.06% CHX				0.2% CHX			
			No mucin 30s	No mucin 60s	0.4% mucin 30s	0.4% mucin 60s	No mucin 30s	No mucin 60s	0.4% mucin 30s	0.4% mucin 60s
F	1	1.31E+08	0	0	0	0	0	0	0	0
	2	2.55E+08	0	0	0	0	0	0	0	0
	3	2.16E+08	0	0	0	0	0	0	0	0
N	1	9.70E+07	0	0	0	0	0	0	0	0
	2	8.50E+07	0	0	0	0	0	0	0	0
	3	1.63E+08	0	0	0	0	0	0	0	0
X	1	1.05E+07	0	0	0	0	0	0	0	0
	2	1.40E+08	0	0	0	0	0	0	0	0
	3	7.80E+07	0	0	0	0	0	0	0	0
GC20P	1	3.52E+08	0	0	0	0	0	0	0	0
	2	1.10E+08	0	0	0	0	0	0	0	0
	3	4.46E+08	0	0	0	0	0	0	0	0
GC20R	1	1.01E+08	0	0	0	0	0	0	0	0
	2	3.02E+08	0	0	0	0	0	0	0	0
	3	1.19E+08	0	0	0	0	0	0	0	0
GC25U	1	4.00E+07	0	0	0	0	0	0	0	0
	2	3.60E+07	0	0	0	0	0	0	0	0
	3	1.71E+08	0	0	0	0	0	0	0	0
GC40P	1	1.17E+08	0	0	0	0	0	0	0	0
	2	4.19E+08	0	0	0	0	0	0	0	0
	3	1.59E+08	0	0	0	0	0	0	0	0

CHX; chlorhexidine

Appendix D8: Colony counts before and after one-minute CHX gargle

Total microbiota counts and presence of commensal *Neisseria* species before and after a one-minute chlorhexidine gargle.

Participant	Total count of microbiota				Presence of *Neisseria* species	
	Before CHX (cfu/mL)	Before CHX log10	After CHX (cfu/mL)	After CHX log10	Before CHX	After CHX
1	NT	NT	NT	NT	Present	Absent
2	NT	NT	NT	NT	Present	Present
3	NT	NT	NT	NT	Present	Absent
4	NT	NT	NT	NT	Present	Present
5	NT	NT	NT	NT	Present	Absent
6	NT	NT	NT	NT	Absent	Absent
7	NT	NT	NT	NT	Present	Absent
8	NT	NT	NT	NT	Present	Present
9	NT	NT	NT	NT	Present	Present
10	NT	NT	NT	NT	Present	Present
11	NT	NT	NT	NT	Present	Absent
12	NT	NT	NT	NT	Present	Present
13	NT	NT	NT	NT	Present	Absent
14	4.58E+06	6.66	2.54E+06	6.40	Present	Present
15	7.10E+05	5.85	2.80E+05	5.45	Present	Present
16	NT	NT	NT	NT	Absent	Absent
17	1.00E+05	5.00	1.00E+00	0	Absent	Absent
18	3.42E+06	6.53	1.00E+00	0	Present	Absent
19	5.40E+05	5.73	1.70E+05	5.23	Present	Absent
20	7.90E+05	5.90	2.10E+05	5.32	Present	Absent
21	2.80E+05	5.45	1.14E+06	6.06	Present	Present
22	1.30E+05	5.11	1.60E+05	5.20	Present	Present
23	3.70E+05	5.57	5.80E+05	5.76	Absent	Absent
24	1.10E+05	5.04	6.40E+05	5.81	Present	Present
25	2.90E+05	5.46	1.00E+00	0	Present	Absent
26	1.45E+06	6.16	2.05E+06	6.31	Present	Present
27	7.50E+05	5.88	1.50E+05	5.18	Present	Absent
28	3.13E+06	6.50	8.60E+05	5.93	Present	Present
29	3.50E+05	5.54	7.00E+04	4.85	Present	Absent
30	2.77E+06	6.44	2.13E+06	6.33	Present	Present
31	2.33E+06	6.37	6.30E+05	5.80	Absent	Absent
32	5.60E+05	5.75	2.36E+06	6.37	Present	Present
33	1.70E+05	5.23	1.00E+04	4	Present	Present
34	4.66E+06	6.67	2.03E+06	6.31	Present	Absent
35	3.67E+06	6.56	1.07E+06	6.03	Present	Present
36	9.00E+04	4.95	5.00E+04	4.70	Present	Present

37	3.00E+04	4.48	1.00E+04	4	Absent	Absent
38	1.80E+05	5.26	1.00E+00	0	Present	Absent
39	1.17E+06	6.07	1.00E+04	4	Present	Absent
40	2.00E+04	4.30	1.00E+04	4	Present	Present
41	2.00E+04	4.30	1.00E+04	4	Absent	Absent
42	1.00E+00	0.00	1.00E+00	0	Present	Absent
43	1.00E+00	0.00	1.00E+00	0	Present	Absent
44	9.80E+05	5.99	9.00E+04	4.95	Present	Present
45	2.00E+04	4.30	1.00E+04	4	Present	Absent
46	1.00E+04	4.00	1.00E+04	4	Present	Absent
47	5.00E+04	4.70	2.00E+04	4.30	Present	Absent
48	1.00E+04	4.00	1.00E+00	0.00	Present	Absent
49	2.60E+05	5.41	2.00E+04	4.30	Present	Present
50	3.40E+05	5.53	3.00E+04	4.48	Present	Absent

NT; not tested

Appendix E1: Manuscript - Minimum inhibitory concentrations of extended spectrum cephalosporins: A systematic review and meta-analysis of *Neisseria gonorrhoeae* treatment failures

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SECTION A – Student Details

Student ID Number	187548	**Title**	Miari
First Name(s)	Victoria		
Surname/Family Name	Miari		
Thesis Title	Understanding the role of the pharynx as a driver for antimicrobial resistance in Neisseria gonorrhoeae		
Primary Supervisor	Dr Richard Stabler		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Sexually Transmitted Diseases		
When was the work published?	December 2024		
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I conceptualised the project and peformed the study design. I wrote the PROSPERO protocol and sumbitted it. I provided search terms for the sytematic review. I performed secondary screening of the sources and made the final decision on which studies will be included in the study. I performed descriptive analyses and produced the following tables and figures: Table S2.1, Table S.2.2, Table S3, Figure S2 Finally, I wrote the manuscript submitted for publication, which was reviewed by the co-authors.

SECTION E

Student Signature	Victoria Miari
Date	10/01/25

Supervisor Signature	Richard Stabler
Date	10/01/25

Minimum Inhibitory Concentrations of Extended-Spectrum Cephalosporins: A Systematic Review and Meta-Analysis of *Neisseria gonorrhoeae* Treatment Failures

Victoria F~~otini~~ << Q1 - Query: Please check if authors name are correctly captured for given name s (in red) and surnames (in blue) for indexing after publication. Ans: **Victoria.miari@lshtm.ac.u k:** R. Matthew Chico ORCID ID:https://orcid.org/0000-0003-1795-7100Victoria F Miari ORCHi D: https://orcid.org/0000-0002-5995-9422 >>Miari, PhD,* Jonna Messina Mosoff, MSc,† and R. Matthew Chico, PhD†

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Author Contributions. V.F.M. initiated the project, provided key references, and performed data analysis. J.M.M. performed the initial data extraction and data analysis, and produced the figures. V.F.M. and J.M.M. equally contributed to the manuscript and supplementary data. R.M.C. supervised the study design and interpretation of results, and contributed to the manuscript.

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We conducted a systematic review and meta-analysis of gonococcal treatment failures and associated minimum inhibitory concentrations after treatment with third-generation

cephalosporins. Our findings provide data to inform the review of resistance breakpoints for pharyngeal infection and to recommend establishment of an internationally agreed-upon MIC testing method.

Background

Neisseria gonorrhoeae is one of the recognized global antimicrobial resistance priorities. Extended-spectrum cephalosporins, the last remaining reliable antimicrobial, increasingly fail to clear *N. gonorrhoeae* infections, especially pharyngeal gonorrhea, leading to limited future treatment options.

Methods

We conducted a systematic review and meta-analysis of gonococcal treatment failures and compared the minimum inhibitory concentrations (MICs) of isolates from pharyngeal and extra-pharyngeal anatomical sites (PROSPERO registration: CRD42020189101).

Results

The overall pooled mean MIC for cefixime was 0.17 mg/L (95% confidence interval [CI], 0.07–0.41 mg/L), and that for ceftriaxone was 0.10 mg/L (95% CI, 0.05–0.22 mg/L). For cefixime, the mean MIC estimates for pharyngeal and extrapharyngeal treatment failures were 0.05 mg/L (95% CI, 0.02–0.14 mg/L) and 0.29 mg/L (95% CI, 0.11–0.81 mg/L), and those for ceftriaxone were 0.09 mg/L (95% CI, 0.03–0.22 mg/L) and 0.14 mg/L (95% CI, 0.03–0.73 mg/L), respectively. The pooled mean MICs for pharyngeal isolates are below the phenotypic European Committee on Antimicrobial Susceptibility Testing resistance breakpoint for both antimicrobials (>0.125 mg/L).

Conclusions

Our findings underscore the need to review the current resistance breakpoints used for pharyngeal infection, ~~and the urgency~~ to establish international standards for MIC testing, and to advance efforts of the World Health Organization's global action plan to control the spread and impact of antimicrobial resistance in *N. gonorrhoeae*. Ongoing susceptibility testing of gonococcal isolates and surveillance of treatment failures are central to informing appropriate public health responses.

There were an estimated 82.7 million incident cases of gonorrhea in 2020, an estimate the World Health Organization (WHO) aims to reduce by 90% before the end of the decade as stated in the

Global Health Sector Strategies 2022–2030.[1] One difficulty in achieving this target is the alarming increase in gonococcal resistance to extended-spectrum cephalosporins (ESC), including ceftriaxone, which is presently the last remaining licensed antibiotic for gonorrhea treatment. Apart from the public health threat of incurable gonococcal infection, at the individual level, untreated gonorrhea may lead to serious reproductive health complications including a range of adverse pregnancy outcomes, pelvic inflammatory disease, and infertility.[2] Pharyngeal gonorrhea is a key site for control efforts, as it is a major driver of transmission, functioning as a hidden reservoir that perpetuates onward transmission.[3] More than 90% of gonococcal infections are asymptomatic[4]; thus, few infected individuals know to seek care and may consequently continue to expose new or existing sexual partners.[3] The pharynx also provides an ideal environment for antimicrobial resistance (AMR) to develop, as *Neisseria gonorrhoeae* is able to acquire DNA from commensal *Neisseria* species by horizontal gene transfer to produce mosaic genes.[5] The pharyngeal site may then contain multiple gonococcal populations, each with distinct susceptibility profiles, some of which may be resistant to antimicrobial therapy.[6] Thus, even in the absence of human sexual contact, pharyngeal gonorrhea can develop AMR. In addition, successful treatment of pharyngeal gonorrhea is difficult due to the limited pharmacokinetic activity conferred by many antibiotics in oropharyngeal tissue.[5] For example, the concentration of cefixime in the saliva is 5-fold lower than serum levels.[7] This has also been reported among other β-lactam antibiotics, including penicillin, amoxicillin, and ceftriaxone.[8] These biological issues make pharyngeal gonorrhea particularly difficult to manage, sometimes leading to treatment failure among seemingly phenotypically susceptible isolates. For example, one study in Canada showed cefixime (the previous first-line antibiotic) failed to clear 28.6% (n = 2 of 7) of pharyngeal infections, compared with 5.26% (n = 4 of 76) and 7.69% (n = 3 of 39) of urethral and rectal infections, respectively.[9] A recent clinical trial found that aztreonam cleared just 33% (n = 2 of 6) of pharyngeal infections, compared with 75% (n = 3 of 4) of rectal and 100% (n = 11 of 11) of urethral infections.[10] Empirical treatment of gonorrhea has changed over the years, informed by antimicrobial resistance trends. Oral cephalosporins or dual treatment with ceftriaxone and azithromycin was recommended until recently.[11] In the United Kingdom and the United States, the first-line treatment for gonorrhea is now 1 g[4] and 500 mg[12] of ceftriaxone, respectively, whereas a high dose of both 1 g ceftriaxone plus 2 g azithromycin is standard in Europe.[13]

Although there is no widely held consensus on the definition of gonococcal treatment failure, it can briefly be described as experiencing continuous clinical symptoms or gonococcal growth after appropriate treatment with the exclusion of reinfection.[5,14–16] Ideally, pretreatment and posttreatment gonococcal isolates are available to compare susceptibility profiles and molecular

sequence types.

The aim of our study was to summarize the published ESC treatment failures of *N. gonorrhoeae* and the associated minimum inhibitory concentration (MIC) values, as well as to characterize the relationship between treatment failure and MIC values of ESC treatment on pharyngeal and extrapharyngeal (urogenital and anorectal) isolates.

METHODS

Search Strategy and Selection Criteria

We used PRISMA (preferred reporting items for systematic reviews and meta-analyses) guidelines and registered our study with PROSPERO ID: CRD42020189101. We searched 2 databases, EMBASE and PubMed, as well as the Eurosurveillance journal on June 17, 2023. Search terms were selected to account for differences in spellings and treatment regimens. Title and free-text terms included "treatment failure," "gonorrh*," "cephalosporin," "cefixime," "cefotaxime," and "ceftriaxone." We imported records from PubMed, EMBASE, and Eurosurveillance into EndNote 20. We examined potential data sources first by title and abstract, and then full text to establish eligibility. We included studies if they reported the following: (1) gonococcal infection was confirmed by culture or nucleic acid amplification test; (2) treatment was administered with ESC at first presentation; (3) MIC of an initial isolate; (4) patient returned for a test of cure and had continuing clinical symptoms, positive gonococcal nucleic acid amplification test, or gonococcal culture; (5) patient reported no sexual activity between initial treatment and test of cure; and (6) MICs of first and second isolates had no more than 1 dilution factor difference. These definitions adhere to guidelines from the United Kingdom Health & Security Agency and European Centre for Disease Control for probable or confirmed treatment failures (Supplementary Fig. S2 and Supplementary Table S3, http://links.lww.com/OLQ/B164).

Coauthor J.M.M. extracted data points from studies that met the inclusion criteria (Supplementary Table S1, http://links.lww.com/OLQ/B164), which were then reviewed independently by V.F.M. Other variables of interest included (i) initial treatment or combination of treatments, (ii) dosage of third-generation cephalosporin, (iii) demographic information on treatment failure cases, (iv) the MIC reported, and (v) the geographic region of cases. We extracted information about gender and sexual orientation as reported and noted the method of MIC testing used.

Statistical Analysis

Individual treatment failure cases were initially recorded in Excel (Microsoft, Redmond, WA). To account for the nonlinear nature of MIC data, we applied a logarithmic transformation of each MIC value to calculate the mean and standard error for each study. We used Stata 18 (StataCorp LLC, College Station, TX) to pool data by applying random-effects restricted maximum likelihood models to account for heterogeneity between studies. We produced forest plots organized by anatomical site of sample collection, dosage, and study publication year. We then conducted subgroup analyses based on binary anatomical site of infection, initial treatment, and dosages. We performed separate subgroup analyses comparing log-transformed MICs by antimicrobial treatment and MIC testing methods. If studies reported multiple failures at different anatomical sites and/or after different treatment regimens, we included them in the corresponding group analysis as individual data points.

Study Quality and Bias Assessment

We used the Joanna Briggs Institute case report, cross-sectional, and cohort study critical appraisal checklists to assess data quality based on completeness of clinical history, demographic detail, and treatment description.[17] We used Cochrane Review Manager 5.4 to summarize quality and risk of bias.

RESULTS

Study Selection and Characteristics

We identified 23 eligible studies by systematic review (Fig. 1). Most data points were case reports of 69 treatment-failure cases (cefixime, n = 33; ceftriaxone, n = 36) involving 71 sites (37 [52%] pharyngeal, 22]31%] urogenital, and 12 [17%] rectal). Of cases with demographic data, 85.5% (59 of 69) were among men; of reports describing failed treatment after cefixime administration, 8 had sufficient data for meta-analysis. Overall, 40.6% of treatment failures were from the Americas region (n = 28) followed by the Western Pacific region with 37.7% (n = 26). Full details of studies included are in Supplementary Tables S2.1, S2.2, and S4, http://links.lww.com/OLQ/B164.

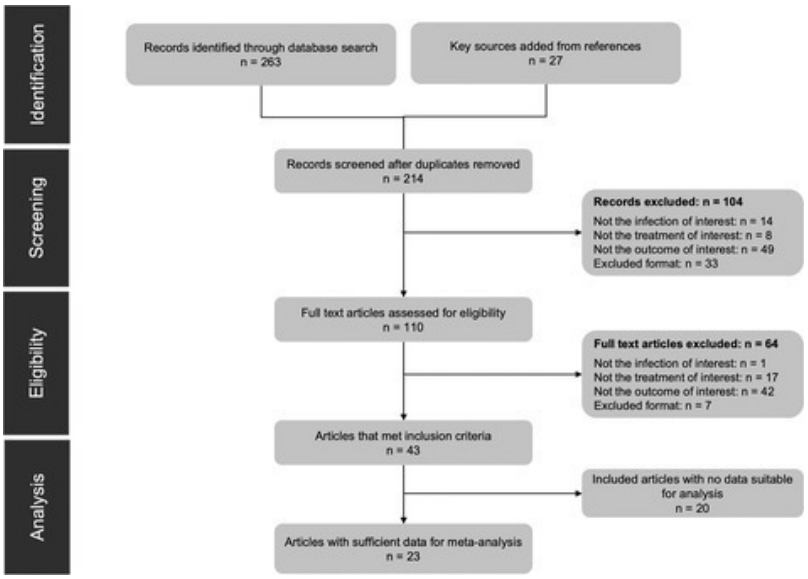

Figure 1 << Q2 - Query: Tables or figures that are not the author's own original work or are adapted from published content require permission letters in order to publish. Please supply the letters granting permission to publish Figures 1, 2 and 3 , or confirm they are the authors' own creations for this article. Ans: **Victoria.miari@lshtm.ac.uk:** Tables and figures were all produced by the coauthors. >>PRISMA flowchart.

Meta-Analysis of Cefixime Treatment Failures

Published reports described 33 cases of treatment failure, 1 of which failed at 2 anatomical sites (urogenital and pharyngeal), leading to a total of 34 treatment failure sites. For cefixime, urogenital infections accounted for 44.1% of treatment failures (15 of 34), followed by pharyngeal at 35.3% (12 of 34). The pooled mean MIC of all cefixime treatment-failure isolates was 0.17 mg/L (95% confidence interval [CI], 0.07–0.41 mg/L; Fig. 2A). The pooled estimate for extrapharyngeal isolates cefixime was 0.29 mg/L (95% CI, 0.11–0.81 mg/L). Pharyngeal treatment failures yielded a pooled estimate of 0.05 mg/L (95% CI, 0.02–0.14 mg/L).

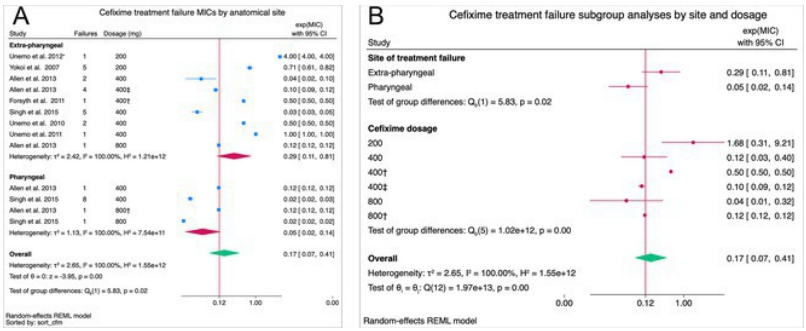

Figure 2 Random-effects restricted maximum likelihood model for cefixime treatment failure of included studies (A) and subgroup analyses by anatomical site (pharyngeal and extra pharyngeal) and treatment dosage (B). MIC indicates minimum inhibitory concentration (mg/L). All dosages in milligrams. Extrapharyngeal includes urogenital and anorectal infections. A, Random-effects models generated with data from 8 included studies that ranged in dosage from 200 to 800 mg, grouped by site of infection and ordered by dosage and year. Full details of studies included can be found in Supplementary Table 2.1, http://links.lww.com/OLQ/B164. B, Data from 8 studies used for random-effects models subgroup analysis. ‡100 mg doxycycline. †1 g azithromycin. ⁺Unemo and Sednaoui et al<< Q3 - Query: Please check if "Unemo and Sednaoui et al" should be considered as reference citations. If so, please provide complete reference details for inclusion in the reference list. Ans: **Victoria.miari@lshtm.ac.uk:** This is reference 5 in the suppementary files (Supplementary Table S4), can we link this ref to this rather than add a new reference to the manuscript? It will be more confusing having two references for the same paper. ThanksFull reference for Unemo and Sednaoui et al: Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P. High-level cefixime- and ceftriaxone-resistant Neisseria gonorrhoeae in France: novel penA mosaic allele in a successful international clone causes treatment failure. Antimicrob Agents Chemother. 2012 Mar;56(3):1273-80. doi: 10.1128/AAC.05760-11. Epub 2011 Dec 12. PMID: 22155830; PMCID: PMC3294892. >>.

Meta-Analysis of Ceftriaxone Treatment Failures

Of the reports describing failures following treatment with ceftriaxone, 15 had sufficient data for analysis. There were 36 failure cases, 1 of which failed at 2 anatomical sites (urogenital and rectal), leading to a total of 37 treatment failure sites. Pharyngeal infections accounted for 67.6% of treatment failures (25 of 37). The overall pooled mean MIC for ceftriaxone treatment-failure

isolates was 0.10 mg/L (95% CI, 0.05–0.22 mg/L; Fig. 3A). The pooled mean MIC for pharyngeal isolates was 0.09 mg/L (95% CI, 0.03–0.22 mg/L), and that for extrapharyngeal isolates was 0.14 mg/L (95% CI, 0.03–0.73 mg/L; Fig. 3B). Of the patients with ceftriaxone treatment failure, 33.3% (12 of 36) were infected at multiple sites, but only failed at the pharyngeal site. Seven of these cases grew a phenotypically susceptible isolate (MIC range, 0.016–0.03 mg/L).

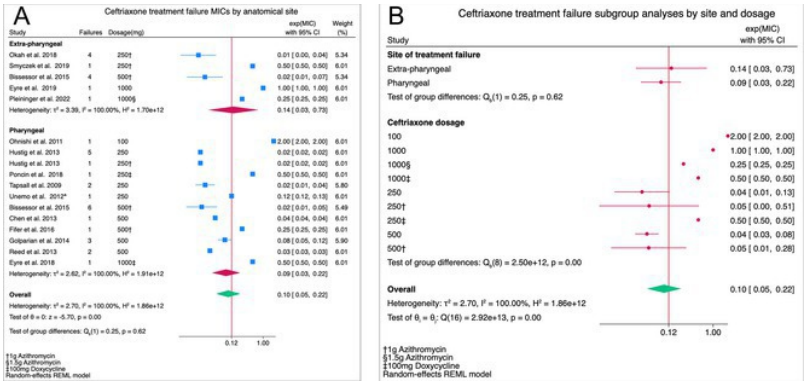

Figure 3 Random-effects restricted maximum likelihood model for ceftriaxone treatment failure of included studies (A) and subgroup analyses by anatomical site (pharyngeal and extra pharyngeal) and treatment dosage (B). MIC indicates minimum inhibitory concentration (mg/L). All dosages in milligrams. Extrapharyngeal includes urogenital and anorectal infections. A, Random-effects models generated with data from 15 included studies that ranged in dosage from 250 to 1000 mg, grouped by site of infection and ordered by dosage and year. Full details of studies included can be found in Table S2.1b in Supplementary Table 2.1, http://links.lww.com/OLQ/B164. B, Data from 15 studies used for random-effects models subgroup analysis. ‡100 mg doxycycline. †1 g azithromycin. §1.5 g azithromycin. [a] Unemo and Jeverica et al<< Q4 - Query: Please check if “Unemo and Jeverica et al” should be considered as reference citations. If so, please provide complete reference details for inclusion in the reference list. Ans: **Victoria.miari@lshtm.ac.uk:** This is reference 11 in the suppementary files (Supplementary Table S4), can we link this ref to this rather than add a new reference to the manuscript? It will be more confusing having two references for the same paper. Thanks Full reference for Unemo and Jeverica et al:Unemo M, Golparian D, Potočnik M, Jeverica S. Treatment failure of pharyngeal gonorrhoea with internationally recommended first-line ceftriaxone verified in Slovenia, September 2011. Euro Surveill. 2012 Jun 21;17(25):20200. PMID: 22748003. >>.

Treatment Failures With Dual Antimicrobial Treatment

Of the 69 cases, 58% (40 of 69) were treated with the ESC alone. Of cases given dual-therapy, 75.8% (22 of 29) were treated with ESC and azithromycin, whereas the remaining received doxycycline alongside the given ESC (Supplementary Tables S2.1 and S2.2, http://links.lww.com/OLQ/B164). Of the 33 patients treated with cefixime, 78.8% (26 of 33) were treated with the ESC alone. Of the cases given dual therapy, 4 received doxycycline and 3 were given azithromycin, 6 had suspected chlamydia coinfection, and for 1 patient, coinfection was not specified. The strains from patients treated with azithromycin were susceptible, whereas strains from patients treated with doxycycline were either intermediate or resistant to doxycycline (Supplementary Tables S2.1 and S2.2, http://links.lww.com/OLQ/B164). Of the 36 patients treated with ceftriaxone, 17 (47.2%) also received a second antibiotic, 88.2% (15 of 17) of whom received azithromycin and the remaining received doxycycline. Of these cases, 1 had confirmed chlamydia coinfection, and for 1 patient, coinfection was not specified. Of the patients treated with azithromycin, 84.2% (16 of 19) carried phenotypically susceptible strains (Supplementary Table S2.2, http://links.lww.com/OLQ/B164).

Treatment Failures by Antimicrobial Susceptibility Testing Methodology

Of the patients treated with cefixime, the pooled mean MIC for those tested by agar dilution was 0.08 mg/L (95% CI, 0.04–0.16 mg/L), whereas the pooled mean MIC for those tested by gradient strip was 0.17 mg/L (95% CI, 0.07–0.41 mg/L; Supplementary Fig. S3.1, http://links.lww.com/OLQ/B164). Of the patients treated with ceftriaxone, the pooled MIC for those tested by agar dilution and gradient strips was 0.05 mg/L (95% CI, 0.01–0.2 mg/L) and 0.21 mg/L (95% CI, 0.09–0.49 mg/L), respectively (Supplementary Fig. S3.2, http://links.lww.com/OLQ/B164).

Risk of Bias

Most treatment failures were reported as case reports, many of which listed only one instance of failure. These reports were generally of high quality, but some lacked sufficient demographic data and others contained incomplete description of methods. The risk of bias checklist for case reports is presented as a quality summary table and figure (Supplementary Figs. S1a and S1b, http://links.lww.com/OLQ/B164). We generated bias figures based on all included studies.

DISCUSSION

In this study, we describe gonococcal treatment failures after ESC therapy and compare the characteristics of pharyngeal and extrapharyngeal failures, particularly the MICs of gonococcal strains. Treatments administered varied regionally, over time, and by anatomical site of infection. Cefixime and ceftriaxone were the most common, but cefotaxime, cefdinir, and ceftibuten were also reported. In addition, treatment regimens varied, ranging from 200 to 800 mg of cefixime and 250 mg to 1 g of ceftriaxone. Because of coinfection with more than 1 STI or concerns about resistance, combination treatments with other antimicrobials were common. In one instance, a patient was treated with ceftriaxone, doxycycline, and spectinomycin before eventually clearing infection with 1 g ertapenem.[18] In addition to having unique targets, antimicrobials may also have different modes of administration that result in differing levels of bioavailability. Cefixime, unlike ceftriaxone, is an oral antibiotic historically delivered as inpatient partner therapy without the discomfort of an intramuscular injection and with no requirement for trained personnel and sterile injection equipment.[19]

Our results should be interpreted with caution given the variability in antibiotic use, dosages administered, and small sample sizes. Moreover, MIC may be measured by different methods, leading to different results. Gradient strips were more commonly used in included sources (53%), but many older reports and reports from regions outside Europe used agar dilution. Studies validating gradient strips against the standard of agar dilution showed good agreement, but it merits noting that methods were not standard across sources.[20] Most sources did, however, report their breakpoints for resistance.

Overall, the mean MICs of isolates from patients treated with cefixime and ceftriaxone were 0.17 mg/L (95% CI, 0.07–0.41 mg/L) and 0.1 mg/L (95% CI, 0.05–0.22 mg/L), respectively. However, 7 of 11 (63.6%) patients infected in multiple anatomical sites and treated with ceftriaxone experienced treatment failure only at the pharyngeal site, despite gonococcal isolates being phenotypically susceptible.

It is well documented that β-lactam concentrations in oropharyngeal tissue are lower than serum levels, and this may contribute to the higher rate of treatment failure at this site.[6] In treatment failure observed after cefixime exposure, the mean MIC for pharyngeal isolates was 0.05 mg/L (95% CI, 0.02–0.14 mg/L) based on 11 patients, whereas for extrapharyngeal isolates, it was 0.29 mg/L (95% CI, 0.11–0.81 mg/L) generated from 22 patients. Importantly, unlike the extrapharyngeal isolates, the MIC and 95% CIs for pharyngeal isolates fall below the phenotypic

European Committee on Antimicrobial Susceptibility Testing breakpoint of 0.125 mg/L with the upper CI at just above the breakpoint. It is important to note that the Clinical and Laboratory Standards Institute has not established *N. gonorrhoeae* resistance breakpoints for ESCs but categorizes isolates with MICs of ≤0.25 mg/L as susceptible.[21] A difference in MIC after ceftriaxone treatment between pharyngeal and extrapharyngeal sites was also observed. The mean MIC for the 11 extrapharyngeal isolates was 0.14 mg/L (95% CI, 0.03–0.73 mg/L), and for the 25 pharyngeal isolates, the MIC was 0.09 mg/L (95% CI, 0.03–0.22). The point estimate of the pharyngeal mean MIC is still lower than for the extrapharyngeal isolates, but just under the European Committee on Antimicrobial Susceptibility Testing breakpoint, although the CIs between pharyngeal and extrapharyngeal sites do overlap. This further adds to the evidence that pharyngeal gonorrhea is particularly difficult to treat and resistance breakpoints for pharyngeal infection should be reviewed, especially for countries recommending treatment with <1 g ceftriaxone. This is currently practiced with other organisms such as *Escherichia coli*, where there are different clinical breakpoints for different infection sites. For example, the co-amoxiclav clinical breakpoint for urinary tract infections caused by *E. coli* is 32 mg/L, whereas for infections other than UTIs, it is 8 mg/L.[22] Ceftriaxone 1 g is now the recommended treatment for pharyngeal infections in most countries.[11] There were 4 patients treated with 1 g ceftriaxone, all of whom had isolates with phenotypic resistance and MICs between 0.25 and 2 mg/L. This suggests that treatment failure after the increased dose of ceftriaxone may be a more accurate predictor of phenotypic resistance.

Interestingly, we found a difference in pooled MIC between isolates tested by agar dilution and gradient strip for both antimicrobials, more evidently for ceftriaxone. Given our small sample size, it is difficult to know whether testing method influences the susceptibility estimates that we generated using the log-transformed MICs. For example, the pooled MIC for isolates tested by gradient strip after cefixime treatment was 1 mg/L (95% CI, 0.38–2.61 mg/L), compared with agar dilution, which was 0.08 mg/L (95% CI, 0.04–0.16 mg/L; Supplementary Fig. S3.1, http://links.lww.com/OLQ/B164). Although the CIs do not overlap, 2 factors may have influenced these results. First, the number of isolates tested by both methods was unequal (28 by agar dilution and 5 by gradient strip), and second, a single isolate MIC of 4 mg/L in the gradient strip group may artificially increase the pooled estimate, compared with the agar dilution pooled MIC (Supplementary Fig. S3.1, http://links.lww.com/OLQ/B164). For ceftriaxone, the pooled MIC for isolated tested by gradient strip was 4-fold higher than those tested by agar dilution (0.21 and 0.05 mg/L, respectively; Supplementary Fig. S3.2, http://links.lww.com/OLQ/B164). Previous studies report conflicting results on the accuracy of gradient strips versus agar dilution. For

example, studies by Papp et al.[23] and Raphael et al.,[24] showed that ESC E-test had >90% agreement with agar dilutions, whereas a study by Gose et al.[25] reported that >20% of E-test MIC values were above agar dilution values. Both studies, however, suggested criteria that are method-specific for interpreting breakpoints.

Our meta-analysis has limitations. There was considerable variability among studies included due to the range of treatments administered, anatomical sites tested, definition of treatment failure, and patient characteristics. We stratified results by subgroup to account for these differences, but this made for smaller sample sizes and wider CIs. In some instances, sample collection skewed toward a particular anatomical site; for example, 70% of the reported ceftriaxone failures occurred in the pharynx. Further stratifications would also have been useful, for example, by dual treatment. However, our sample size precluded further subgroup analyses. We recognize that our data are based on reported treatment failures at the given ESC dosages, and we do not have denominator data to compare successfully treated gonococcal infections with the same MICs. Consequently, the meta-analysis does not include all representative outcomes for each MIC. In addition, some sources may not have reported details on reinfection, which may have impacted their inclusion. Another limitation is the inclusion of mainly case reports, which may introduce publication bias. We have provided risk of bias analysis in Supplementary Figures S1a and S1b, http://links.lww.com/OLQ/B164. The level of heterogeneity in the information presented in the sources further demonstrates the necessity for global surveillance systems and guidance on reporting gonococcal treatment failures. Despite these limitations, the findings of this study are in line with the WHO global action plan to control the spread and impact of gonococcal AMR, which calls for the "…systematic monitoring of treatment failures by developing a standard case definition of treatment failure, and protocols for verification, reporting and management of treatment failure."[26] Furthermore, surveillance is key to combatting gonococcal AMR and must be strengthened in all contexts. Accurate clinical resistance definitions directly impact AMR surveillance, which in turn informs treatment guidelines, highlighting the importance of reviewing resistance breakpoints for pharyngeal infection. High-resource settings with the ability to sequence pretreatment and posttreatment isolates must contribute to knowledge about resistance mechanisms and adaptations. In low-resource settings, where syndromic management is the current policy, periodic surveillance is necessary to ensure that treatment, and the larger policy of which it is a part, remains effective. Special attention must be paid to key marginalized populations to lessen the high burden of disease, and new technology should be made available in all regions to ensure that data are accurate and representative. Fundamentally, setting separate pharyngeal and extrapharyngeal breakpoints can serve as a critical catalyst for more holistic screening of patients across anatomical

sites and help advance gonococcal treatment guidelines updates that are tailored to presence as well as location of infection. Lastly, the implementation of different resistance breakpoints between anatomical sites may be logistically complex, especially in low-income countries and in patients with infections at multiple anatomical sites. The feasibility and economic impact of the proposed changes within this study should be assessed in multiple settings, before implementation of national or local guidance.

CONCLUSIONS

Global surveillance and reporting of treatment failure remain important for control efforts of gonorrhea, particularly pharyngeal gonorrhea. Our study presents data that may inform breakpoint revisions for different anatomical sites, findings that align with the WHO global action plan to control the spread and impact of gonococcal AMR. There is an urgent need to establish common standards for breakpoints, including an internationally agreed MIC testing method to foster improved reporting of treatment failures and surveillance practices that are key to informing appropriate public health responses.

REFERENCES

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Appendix E2: Manuscript - Characterization of *Neisseria gonorrhoeae* strain differences in patients with multi-site infection

Published in Sexually Transmitted Infections, October 2024

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RESEARCH PAPER COVER SHEET

Please note that a cover sheet must be completed for each research paper included within a thesis.

SECTION A – Student Details

Student ID Number	187548	**Title**	Miss
First Name(s)	Victoria		
Surname/Family Name	Miari		
Thesis Title	Understanding the role of the pharynx as a driver for antimicrobial resistance in Neisseria gonorrhoeae		
Primary Supervisor	Dr Richard Stabler		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Sexually Transmitted Infections		
When was the work published?	October 2024		
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I conceptualised, deigned and initiated the project. I performed antimicrobial susceptibility testing and nucleic acid extraction on the majority of gonococcal isolates. I performed NG-MAST sequencing. I also performed all data analysis, apart from whole genome sequencing and phylogenetic analyses which were performed by Dr Richard Stabler. I produced all figures except for Figure 1 in the manuscript. Lastly, I wrote the manuscript which was reviewed by the co-authors.

SECTION E

Student Signature	Victoria Miari
Date	10/01/25

Supervisor Signature	Richard Stabler
Date	10/01/25

Characterisation of *Neisseria gonorrhoeae* strain differences in patients with multisite infection

Victoria F Miari, Matthew R Blakiston, Priya Solanki, Ozan Gundogdu, Richard A Stabler

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/sextrans-2024-056297).

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ABSTRACT

Background *Neisseria gonorrhoeae*, the aetiological 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 different sites, with potentially different antimicrobial susceptibility profiles. This study aims to estimate the proportion of patients with multisite infection through differential antimicrobial susceptibility testing (AST) profiles and sequence-based molecular methods.

Methods This was a cross-sectional study of multisite gonococcal isolates provided by three National Health Service laboratories. Minimum inhibitory concentrations (MICs) for cefixime, ceftriaxone, azithromycin, ciprofloxacin, tetracycline and spectinomycin were determined. Possible multistrain infections were defined as isolates with a significant difference in MIC to at least one antimicrobial. Whole genome sequencing (WGS) was performed to determine multistrain infection through *N. gonorrhoeae* multiantigen sequence typing (*NG*-MAST), *N. gonorrhoeae* sequence typing for antimicrobial resistance (*NG*-STAR), multilocus sequencing typing (MLST) and single nucleotide polymorphism (SNP) phylogeny, and to compare AST profiles with identified AMR genes.

Results Ninety-one isolates were collected from 41 patients with multisite infections. Of these 41 patients, 6 (14.6%) had *N. gonorrhoeae* isolates with discordant MICs. WGS-based typing confirmed that four out of six patients were infected with different gonococcal strains. The relatedness of isolates with the same MLST across multiple patients was differentiated using SNP-based analysis, and this 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 multisite 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.

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 minimum inhibitory concentration (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 multisite gonococcal infection.
⇒ To our knowledge, this is the first study on multisite 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 at multiple anatomical sites.
⇒ The study also informs potential guidance on susceptibility testing of multisite gonococcal isolates and encourages more research to determine how multisite infection impacts antimicrobial resistance 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 with single-site sampling.[5] Based on previous research, there is evidence of 7%–40% of patients with multisite infection carrying different strains at different anatomical sites.[6–11] However, most strain typing studies predating 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 AMR further highlights the importance of detecting extragenital infections.[12 13] Studies have also reported that pharyngeal isolates exhibited higher

MICs than extrapharyngeal isolates.[10 14] Furthermore, patients with pharyngeal gonorrhoea are also more likely to fail treatment with extended-spectrum cephalosporins than those with extrapharyngeal infection.[15–17]

There is no formal guidance outlining how laboratories should perform and report antimicrobial susceptibility testing (AST) from patients with multisite 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 processes only a single isolate from multisite infection, favouring the pharyngeal isolates.[18] The main aim of this study was to investigate the proportion of patients with multisite 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 multisite *N. gonorrhoeae* infection.

Gonococcal isolates

Gonococcal isolates from multisite 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 Hospitals NHS Foundation Trust (SGH). Anatomical sites included were urethra, cervix, rectum and pharynx. The isolates were collected from consecutive patients attending genitourinary medicine clinics served by those laboratories for 1 year between 2014 and 2015. Each laboratory identified the gonococcal isolates based on local diagnostic protocols and stored them at −80°C. Each isolate was provided with the following information: laboratory number, anatomical site and patient sex. Isolates were cultured from storage vials on Vancomycin, Colistin, Amphoteracin, Trimethoprim (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 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 methodology,[19] and MICs for penicillin and ciprofloxacin were determined by gradient strip (Biomerieux). Both methodologies used gonococcal medium base (Becton Dickinson, Franklin Lakes, New Jersey, USA) 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.[20] Clinical and control strains of *N. gonorrhoeae* were subjected to no more than two subcultures before AST. In patients who carried isolates with the same AST profile in multiple anatomical sites, only one of the isolates was used to calculate the cohort's resistance rates.

Multisite infection

Gonococcal isolates from a multisite 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 (online supplemental 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 (5 PRIME, Dusseldorf, Germany) following the Gram-negative bacteria protocol. *N. gonorrhoeae* multiantigen sequence typing (*NG*-MAST) was performed manually, as previously described.[21] In addition to traditional *NG*-MAST, whole genome sequencing (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 2×251 base-pair (bp) paired-end reads. Additional sequencing was performed by the UK Health Security Agency (Colindale, UK) on a HiSeq (Illumina) generating 2×101 bp paired-end reads. Raw FASTQ data were assessed and trimmed using Trimmomatic V.0.39. Trimmed reads were assembled into contigs using Spades V.3.13.0. Pilon was used to improve genome assembly, and contigs were ordered using ABACAS with the *N. gonorrhoeae* NCCP11945 (NC_011035) genome[22] as the reference, and then annotated using PROKKA with a bespoke database. Multilocus sequencing typing (MLST) was determined in silico using MLST script (V.2.10) (T Seemann, MLST, Github; https://github.com/tseemann/mlst). Antimicrobial genotype prediction was performed using Abricate V.0.8.2 (T Seemann, Abricate, Github; https://github.com/tseemann/abricate) with the Comprehensive Antibiotic Resistance Datsbase (CARD) and National Centre for Biotechnology Information (NCBI) databases. *N. gonorrhoeae* sequence type for antimicrobial resistance (*NG*-STAR) and AMR prediction were obtained using the PathogenWatch website (https://pathogen.watch/). Single nucleotide polymorphisms (SNPs) and core SNPs were defined using SNIPPY (V.4.6.0) (https://github.com/tseemann/snippy) against the *N. gonorrhoeae* NCCP11945 genome using default settings. The phylogeny was determined using the FastTree[23] approximately maximum likelihood for alignments of SNP nucleotides using default settings with the SNIPPY SNP alignment and viewed in GrapeTree.[24]

RESULTS

Patients and gonococcal isolates

A total of 101 isolates from 46 patients with multisite infections were received from the three laboratories. Three patients had 2 isolates each from RFH, 1 patient with 2 isolates from MTW and 42 patients with 95 isolates from SGH. The isolates from MTW (n=2) and two patients' isolates from RFH (n=4) were nonviable 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: 2 isolates (1 patient) from RFH and 89 isolates (40 patients) from SGH (online supplemental table S1). Of these 41 patients, 30 (73.2%) were male. The anatomical distribution of multisite infections was rectal-pharyngeal in 14 (34.1%) of 41 patients, urethral-pharyngeal in 4 (9.8%), urethral-rectal in 9 (22%), urethral-cervical in 6 (14.6%), cervical-pharyngeal in

Table 1 Susceptibility of 47 deduplicated study isolates according to Clinical Laboratory Standards Institute 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

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

2 (4.9%), urethral-pharyngeal-rectal in 5 (12.2%) and urethral-cervical-pharyngeal-rectal in 1 (2.4%).

Antimicrobial susceptibility of *N. gonorrhoeae* isolates

To calculate resistance rates, 44 of 91 isolates with the same intrapatient MIC profile were removed as duplicates at random, that is, no anatomical site was prioritised. For the remaining 47 deduplicated isolates, the resistance rates for penicillin, ciprofloxacin and tetracycline were 10.6% (5 of 47), 27.7% (13 of 47) and 23.4% (11 of 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 online supplemental table S1.

Multisite infection gonococcal strain differences

Antimicrobial susceptibility differences

Of the 41 patients, 6 (14.6%, 95% CI 6.8%, 28.4%) had isolates from different anatomical sites with divergent MICs to at least one antimicrobial, suggesting these patients were carrying different gonococcal strains at different anatomical sites (table 2 and online supplemental 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 (patients 25, 36 and 40) had MIC differences in two antibiotics (cefixime/ciprofloxacin, penicillin/tetracycline and ceftriaxone/tetracycline, respectively), one patient (patient 38) had differences in penicillin, ciprofloxacin and tetracycline, one patient (patient 20) had differences in penicillin, cefixime, azithromycin and ciprofloxacin, and one patient (patient 16) had different MICs for all antimicrobials tested apart from penicillin and spectinomycin (table 2 and online supplemental table S6). Two patients (patients 20 and 38) also had discordant β-lactamase results (table 2). Discordant MICs were further confirmed at least once (online supplemental table S6).

Comparative genomics

Draft genomes were assembled with a mean length of 2 194 125 bp (SD ±101 178 bp), 52.40% (±0.14%) GC content and 2151 (±100) coding sequences (CDS) (online supplemental table S2). The MLST and *NG*-MAST data indicated that patients 16, 20, 38 and 40 had different strains at different anatomical sites, whereas the sequence types (STs) indicated patients 25 and 36 had the same strain at both sites (table 2 and online supplemental 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 levels also differed significantly with this higher-resolution analysis (eg,

Table 2 MIC (mg/L) 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 indicate different MICs (≥2 MIC doubling dilutions for agar dilution method or ≥2 MIC gradations with gradient strip method).
*β-lactamase-positive.
†Mosaic *penA*.
AZI, azithromycin; CFX, cefixime; CIP, ciprofloxacin; CRO, ceftriaxone; MIC, minimum inhibitory concentration; PEN, penicillin; PH, pharynx; RE, rectum; SPE, spectinomycin; TET, tetracycline; UR, urethra.

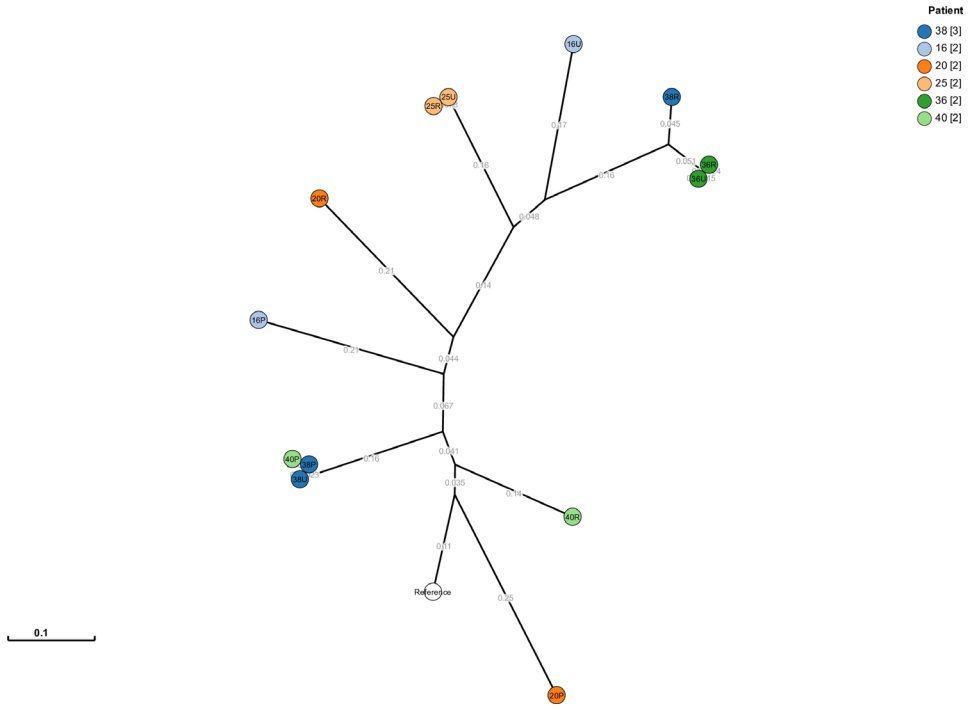

Figure 1 Phylogeny of study isolates. Single nucleotide mutations were determined against the *Neisseria gonorrhoeae* NCCP11945 genome using SNIPPY. An approximately maximum likelihood phylogeny was estimated using FastTree. Multisite samples are coloured by patient ID and defined by designation: P,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

GC16U and GC16P; figure 1). Conversely, isolates with the same ST from a single patient were highly similar at the SNP level (eg, 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

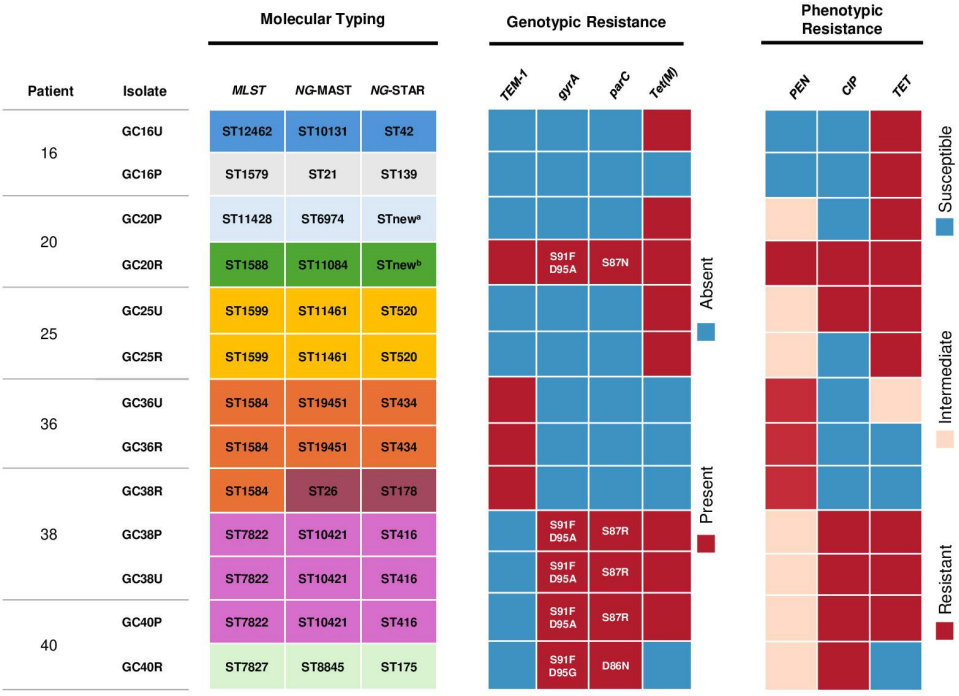

Figure 2 *NG*-MAST, *NG*-STAR and MLST of sequenced isolates from six multisite patients with differing antibiograms along the genotypic markers of resistance phenotypic susceptibility profiles to penicillin (PEN), ciprofloxacin (CIP) and tetracycline (TET). Indicated is the presence or absence of genotypic resistance markers and susceptibility of the isolates to the antimicrobials stated. [a]Novel Sequence Type (*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)). MLST, multilocus sequence type; *NG*-MAST, *Neisseria gonorrhoeae* multiantigen sequence type; *NG*-STAR, *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance.

($ST_{NG\text{-}MAST}$26) (figure 2 and online supplemental tables S3 and S4). This was also reflected in the phylogeny, with the GC36 isolates clustering together, while 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* and *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 (online supplemental 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 online supplemental table S5) and were associated with a penicillin MIC of ≥2 mg/L. Ciprofloxacin resistance was associated with a S91F/D95A or S91F/D95G SNP in *gyrA* and an S87N or D86N SNP in *parC*, leading to an MIC of ≥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 and online supplemental 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 and online supplemental table S5). GC20P was the only isolate with a mosaic *penA* allele (online supplemental 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 6 (14.6%) of 41 patients with multisite gonococcal infection carried gonococcal isolates with different AST profiles at different sites. In four of these six patients, these differences were associated with different strains infecting different anatomical sites. This suggests performing AST on all isolates in a multisite 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 multisite 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.[20] 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 multisite AST differences increases to 17% (6 of 35). Previous studies describing divergent isolates in multisite infection using older methods such as auxotyping and restriction fragment length polymorphism (RFLP) reported a prevalence of between 7% and 40%.[6–8] Mixed strains in single anatomical sites have also been detected. In a study by Goire *et al*,[25] an estimated 3.2% (2 of 63) of samples contained mixed cultures of *N. gonorrhoeae* detected by AST and MLST. A similar proportion of mixed infection (1.3%, 4 of 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 of 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 retesting, the MIC for both isolates was 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 the susceptibility of isolates from different anatomical sites. For example, some studies have reported higher cefixime resistance in pharyngeal compared with extrapharyngeal isolates,[27] while others report similar susceptibilities at 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 (figures 1 and 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 microepidemiology.[30] Ultimately, however, WGS-based typing methods provide the best resolution for determining the relationship between isolates.

Our study is not without limitations. First, our sample size is small, limiting the accuracy of our estimates of occurrence of multistrain, multisite infections. Most of the samples (97.8%) also originated from a single laboratory, meaning the data are 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 multistrain gonococcal infections is evolving over time, considering the data presented in this study are 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 multisite infections with strain differences. Access to patient metadata such as sexual orientation, date of collection and further testing results would have added further context to our results.

Despite these limitations, isolates with differing AST profiles were able to be identified at different anatomical sites within

individual patients. This suggests that performing AST on all isolates in multisite 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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Contributors VFM: conceptualisation, methodology, validation, formal analysis, writing—original draft. RAS: methodology, validation, formal analysis, investigation, visualisation. All authors: investigations, writing—review and editing. RAS is responsible for the overall content as guarantor for this study.

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Appendix E3: Manuscript - Carriage and antimicrobial susceptibility of commensal *Neisseria* species from the human oropharynx

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First Name(s)	Victoria		
Surname/Family Name	Miari		
Thesis Title	Understanding the role of the pharynx as a driver for antimicrobial resistance in Neisseria gonorrhoeae		
Primary Supervisor	Dr Richard Stabler		

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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I conceptualised, designed and initiated the study. I co-wrote the participant information sheet and consent forms with my MSc project student. With my MSc project student, I also recruited participants, took oropharyngeal swabs and processed them in the laboratory. I inoculated MALDI-ToF plates for identification. I performed antimicrobial suscptibility testing for cefixime, tetracycline, ciprofloxacin, gentamicin and azithromycin. I extracted nucleic acid for whole genome sequencing. I also performed all data analysis, apart from whole genome sequencing and phylogenetic analyses which were performed by Dr Richard Stabler. I produced all figures except for Figures 3 and 4 in the manuscript. Lastly, I wrote the manuscript which was reviewed by the co-authors.

SECTION E

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Carriage and antimicrobial susceptibility of commensal *Neisseria* species from the human oropharynx

Victoria F. Miari[1]✉, Wesley Bonnin[1], Imogen K. G. Smith[1], Megan F. Horney[1], Samer J. Saint-Geris[2] & Richard A. Stabler[1]

Commensal *Neisseria* (*Nc*) mainly occupy the oropharynx of humans and animals. These organisms do not typically cause disease; however, they can act as a reservoir for antimicrobial resistance genes that can be acquired by pathogenic *Neisseria species*. This study characterised the carriage and antimicrobial susceptibility profiles of *Nc* from the oropharynx of 50 participants. Carriage prevalence of *Nc* species was 86% with 66% of participants colonised with more than one isolate. Isolates were identified by MALDI-ToF and the most common species was *N. subflava* (61.4%). Minimum inhibitory concentrations (MICs) to penicillin, ceftriaxone, ciprofloxacin, azithromycin, tetracycline, and gentamicin were determined by agar dilution and E-test was used for cefixime. Using *Ng* CLSI/EUCAST guidelines, *Nc* resistance rates were above the WHO threshold of 5% resistance in circulating strains for changing the first line treatment empirical antimicrobial: 5% (CLSI) and 13 (EUCAST) for ceftriaxone and 29.3% for azithromycin. Whole genome sequencing of 30 *Nc* isolates was performed, which identified AMR genes to macrolides and tetracycline. Core gene MLST clustered *Nc* into three main groups. Gonococcal DNA uptake sequences were identified in two *Nc* clusters. This suggests that *Nc* have the potential AMR gene pool and transfer sequences that can result in resistance transfer to pathogenic *Neisseria* within the nasopharyngeal niche.

Keywords Commensal, *Neisseria*, Antimicrobial resistance, Whole genome sequencing

Neisseria species are gram-negative aerobic cocci, part of the β-proteobacteria class. *Neisseria* colonise the mucosal surfaces of humans and animals, mainly the oral cavity and nasopharynx. To date, there are at least 43 published *Neisseria* species by the List of Prokaryotic names with Standing in Nomenclature (LPSN; accessed 4 May 2024)[1] and 47 by the National Center for Biotechnology Information (NCBI; accessed 4 May 2024)[2]. The predicted phylogeny of *Neisseria* species is continuously evolving. Studies performed using 16s rRNA sequencing and conserved housekeeping genes identified five separate groups of *Neisseria*[3]. Group one contained *Neisseria gonorrhoeae (Ng)*, *Neisseria meningitidis (Nm)*, *N. polysaccharea*, and *N. lactamica*, group two included *N. subflava*, *N. flavescens*, and *N. mucosa*. The third group included only *N. cinerea* strains. The fourth and fifth groups contained *N. phayngis* and *N. elongata* species respectively[3]. More recent studies however have suggested the re-classification of certain species into single clusters, for example *N. perflava*, *N. subflava* and *N. flava* are now thought to belong to the *N. flavescens* group[4]. Genomic relatedness among *Neisseria* species has been examined by several methods, but core genome MLST (cgMLST) is now commonly used[5,6].

The ability of *Neisseria* species to uptake DNA and integrate it into their genome is a common feature among the genus leading to a high degree of genetic variation, which is crucial to survival and adaption to their host[7]. Uptake of DNA in *Ng* is regulated by the presence of the 10-base pair DNA uptake sequence (DUS) 5'-GCCGTC TGAA-3'[8]. More recently, a revised 12-base pair sequence was identified (AT-DUS: 5'-AT-GCCGTCTGAA-3'), which enhances transformation efficiency[9]. A variant DUS (vDUS 5'-GTCGTCTGAA-3') present in commensal *Neisseria* (*Nc*) has also been described, with some species such as *N. mucosa* having > 3,000 copies[10].

Commensal *Neisseria* are important reservoirs of transferable antimicrobial resistance (AMR) for pathogenic species[11,12]. The transfer of β-lactam resistance, including extended spectrum cephalosporins (ESC) is of particular importance; *Nm* and *Ng* strains resistant to β-lactams have been shown to harbour mosaic *penA* genes,

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acquired from *Nc* species such as *N. cinerea* and *N. perflava*[13,14]. As such, it has been suggested that surveillance of *Nc* species can contribute to delaying the spread in AMR in pathogenic *Neisseria* species[15].

The prevalence of *Nc* in the oropharynx and associated AMR is understudied compared to pathogenic *Neisseria* species. However, *Nc* prevalence has been estimated between 10.2% and 100%[16–20], with some studies reporting individuals' colonisation by up to four different species[17,18,20]. Susceptibility of *Nc* to ceftriaxone is low, with reported median minimum inhibitory concentrations (MICs) of 0.047 mg/L[21], 0.002 mg/L[22] and 0.03 mg/L[23], although the last two studies were limited to only *N. lactamica* and *N. subflava* respectively. Additionally, resistance rates to ceftriaxone and cefixime among *Nc* has been estimated as 28% and 31% respectively[17].

To our knowledge, this is the first study to report *Nc* prevalence combined with penicillin, ceftriaxone, ciprofloxacin, azithromycin, tetracycline, and gentamicin MICs and genomic analyses, and the only one to date performed in the United Kingdom. This study highlights that *Nc* have the potential AMR gene pool and transfer sequences that can result in resistance transfer to *Ng* and *Nm* within the nasopharyngeal niche.

Methods

Participant recruitment and sample processing

A cross-sectional study of staff and students from the London School of Hygiene & Tropical Medicine (LSHTM) was undertaken between June and July 2019. Any participant over the age of 17 years old was eligible for inclusion, with the following exclusion criteria: antibiotic use within one month, usage of antiseptic mouthwash in the past week and participants who are taking steroids or immunosuppressant therapy. The aims of the study were explained to all participants, after which informed consent was obtained. All subsequent experiments were performed in accordance with the relevant guidelines and regulations.

A DrySwab device (MWE, Nottingham, UK) was used to sample the peritonsillar areas of participants. Swabs were expressed in 1mL of sterile saline by vortexing vigorously, and 50 µL inoculated onto a Luria-Bertani Vancomycin Trimethoprim Sucrose Neutral Red (LBVT.SNR) agar, as previously described[18]. Briefly, LBVT.SNR agar consisted of 1% tryptone (Oxoid, Basingstoke, UK), 0.5% yeast extract (Oxoid), 0.5% sodium chloride (Sigma-Aldrich, St. Louis, Missouri, U.S.), 1.5% Bacteriological Agar Number 1 (Oxoid), 1% w/v sucrose (VWR International, Radnor, Pennsylvania, US), 3 mg/L trimethoprim (Sigma-Aldrich), 3 mg/L vancomycin (Sigma-Aldrich) and 0.3% neutral red indicator (Sigma-Aldrich). Inoculated plates were incubated at 5% CO_2 at 37°C for 48 h.

Bacterial identification

Cultured isolates were first observed for colonial morphology, including colour, texture, and size. Morphologically distinct colonies from the LBVT.SNR agar were sub-cultured on chocolate agar (Oxoid) for further identification and antimicrobial susceptibility testing (AST). Oxidase and gram staining were performed on colonies of interest; oxidase positive, gram-negative cocci were considered as presumptive *Neisseria species*. Isolates were stored in 20% glycerol brain heart infusion (BHI) broth (Oxoid) at -70°C until further testing.

Identification to species level was determined by Matrix-Assisted Laser Desorption/Ionisation – Time-of-Flight mass spectrometry (MALDI-ToF MS), using a Bruker MALDI Biotyper (Bruker Daltonics, Billerica, Massachusetts, US). Identification values of 2.0 or over were accepted, while values under 2.0 were repeated once.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations for penicillin, ceftriaxone, ciprofloxacin, azithromycin, tetracycline, and gentamicin (Sigma-Aldrich) were all determined by agar dilution in line with the Clinical and Laboratory Standard Institute protocol[24], using gonococcal medium base (GCMB) agar (BD Difco, Franklin Lakes, New Jersey, US). Cefixime MICs were obtained by E-test (Biomerieux, Marcy-l'Étoile, France), on GCMB. Gonococcal WHO controls K, G, V, F, X and Y[25] were included in the AST, due to the lack of *Nc* control strains. Isolates with a penicillin MIC > 1 mg/L were tested for β-lactamase production using a cefinase disk (Oxoid), according to manufacturer's instructions. As there are no MIC breakpoints for *Nc*, calculated rates of reduced susceptibility (referred to as resistance for ease) used the Clinical & Laboratory Standards Institute (CLSI)[24] and European Committee on Antimicrobial Susceptibility Testing (EUCAST v.13.1)[26]. Gentamicin breakpoints used epidemiological values suggested previously[27] (Table 2). Resistance rates to all antimicrobials were calculated for all *Nc* overall and for each species individually (Table 2).

The MIC values generated were used to deduplicate isolates within individual patients, using the following criteria:

(1) Isolates with the same phenotypic appearance on LBVT.SNR agar, and.
(2) Isolates with same species ID by MALDI-ToF, or whole genome sequencing (WGS) where MALDI did not give an ID, and.
(3) Isolates with at least five out of seven antibiotic matching MICs, within 1 $\log_2$ MIC.

Whole genome sequencing and bioinformatic analysis

Total genomic DNA was extracted using the PureLink Genomic DNA Mini Kit extraction kit (Invitrogen, Waltham, Massachusetts, US) and quantified using the Qubit dsDNA BR assay kit (Invitrogen). The Nextera XT library (2×151 bp) prep kit (Illumina, San Diego, California, US) was used to prepare the sequence libraries as per manufacturer's protocol. The samples were sequenced on a MiSeq System (Illumina) as per the recommended protocol. Additional Illumina (2×251 bp) sequencing was performed at MicrobesNG (MicrobesNG, Birmingham, UK). Raw sequence data were quality controlled using Trimmomatic v0.38[29] with the following specifications: Leading:3 Trailing:3 SlidingWindow:4:20 Minlen:36. Quality control (QC) checks

were performed using FastQC v0.11.8[30]. Fastq reads were mapped against reference sequences using BWA MEM with default settings[31] and viewed in Artemis and ACT[32,33]. *De novo* sequence assemblies were performed using Spades v3.13[34] with default settings, a coverage cut-off of 20 and k-mer lengths of 21, 33, 55, 77, 99 and 111. Draft genome multi-fasta files were evaluated using Quast assessment tool v5.0.2[35]. Contigs were ordered against a *N. meningitidis* MC58 (accession AE002098) using ABACAS v1.3.1 using -dmbc settings[36]. Non-matching contigs were appended to the ordered contigs. The resulting assemblies were polished using Pilon v1.22 with default settings[37] and annotation using Prokka v1.13 in gram negative mode[38].

The assembled contigs were screened for AMR genes using ABRicate[39] v1.0.1 and CARD[40], and NCBI AMRFinderPlus[41] databases and combined. Putative plasmid replicons were identified using the ABRicate with the PlasmidFinder database[42]. MLST profiles were determined using the software package MLST v2.16.1 from the draft assemblies[43]. Kraken2 using draft assemblies and the minikraken_8Gb_20200312 database[44] was used to predict species. The BSR-Based Allele Calling Algorithm (chewBBACA)[45] and predetermined *Neisseria* schema was used to generate cgMLST profiles and paralog removal using alleles present in 95%[46]. Allele profile data was used to generate a MSTree in Grapetree using --wgMLST and default settings[47]. Heatmaps was generated using Morpheus website (software.broadinstitute.org) with hierarchical clustering using Euclidean distance, average linkage method.

Statistical analysis

All statistical analyses were performed with STATA 18 (StataCorp LLC, College Station, Texas, US). Prevalence and 95% confidence intervals (CI) were calculated for each of the *Nc* species. The MICs between *Nc* species was compared using the Kruskal-Wallis rank sum test. To enable statistical testing, MICs above the maximum or below the minimum range tested were converted to the dilution before or after the limit of detection, as previously described[21]. For example, azithromycin MIC > 256 mg/L was expressed as 512 mg/L.

Results

Participant demographics and *Neisseria* isolates

Fifty participants were recruited with 37 (74%) females and median age was 35 (range 17 to 81). The number of participants colonised with *Nc* was 43/50, generating an estimated population prevalence of 86% (95% CI; 73.8%, 93%). In total, there were 143 morphologically distinct *Nc* isolates cultured from the 43 participants. A total of 42 isolates were removed as duplicates, leading to a final total of 101 isolates from the 43 participants that grew *Nc*.

Neisseria species prevalence and characterisation

The most common *Nc* species detected by MALDI-ToF was *N. subflav*a (62/101, 61.4%) (Supplementary Table S1). The second most prevalent species was *N. flavescens* (12 isolates, 11.9%), then *N. perflava* (10, 9.9%), *N. macacae* (6, 5.9%) and *N. mucosa* (3, 2.9%) (Supplementary Table S1). Twenty isolates (19.8%) were identified by MALDI-ToF as either one of two probable species, both having an index of over 2.0 (high confidence identification); the isolate with the highest index was considered as the primary ID (Supplementary Table S1). No ID was possible on eight isolates by MALDI-ToF; these were classified as *Neisseria spp* (Supplementary Table S1).

N. subflava had the highest incidence among the participants, with 74% (37/50 participants) carrying this species. This was followed by *N. flavescens* (20%, $n = 10$), *N. perflava* (18%, $n = 9$), *N. macacae* (10%, $n = 5$) and *N.*

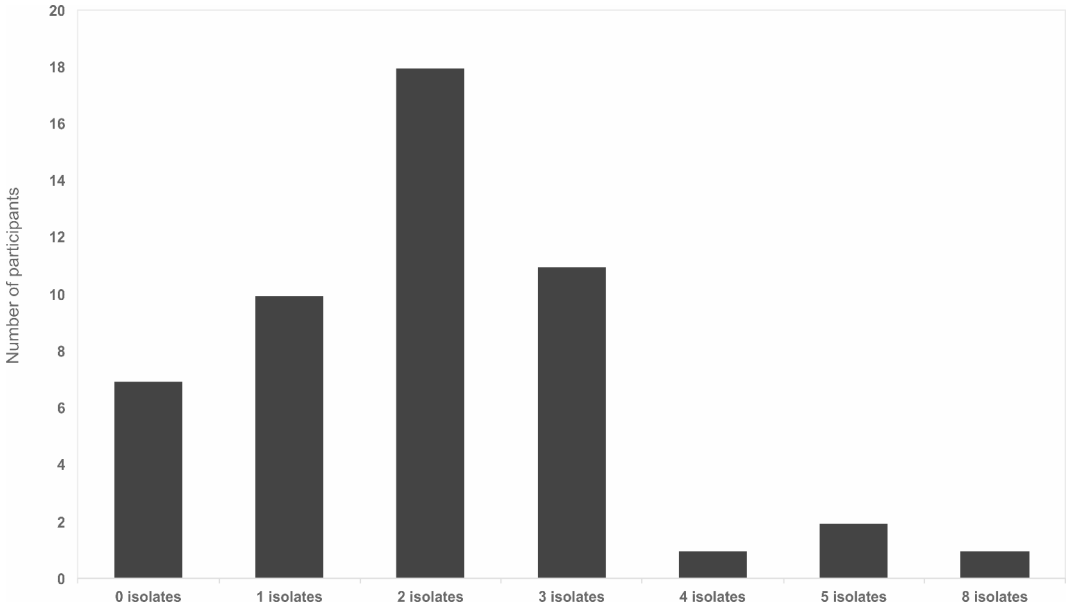

Fig. 1. Number of *Neisseria* isolates present in the oropharynx of 50 study participants. Analysis was performed with results obtained from MALDI-ToF MS.

mucosa (6%, $n=3$). Ten participants (20%) harboured a single *Nc* species, however, some participants harboured multiple isolates; 18 (32%) participants were colonised by two isolates, 11 (22%) by three isolates, 2 (4%) by five isolates and 1 (2%) each were colonised by four and eight isolates (Fig. 1).

Susceptibility of commensal *Neisseria* species

After deduplication of isolates, the following MIC data were analysed: penicillin and ceftriaxone MICs for 101 and 100 isolates respectively and for cefixime, ciprofloxacin, azithromycin, gentamicin and tetracycline, 91 isolates MICs (Table 1). The median MICs for penicillin, ceftriaxone, cefixime, ciprofloxacin, tetracycline, azithromycin and gentamicin were 1 mg/L, 0.06 mg/L, 0.064 mg/L, 0.032 mg/L, 0.5 mg/L, 0.5 mg/L and 4 mg/L respectively (Table 1; Fig. 2 and Supplementary Table S2). No isolates produced a detectable β-lactamase. The proportion of isolates overall resistant to penicillin and azithromycin according to both CLSI and EUCAST breakpoints was 26.7% (27/101) and 29.3% (27/92) respectively (Supplementary Table S2). Of the penicillin resistant isolates, 10 were also resistant to azithromycin. *N. subflava* had the highest number of resistant isolates to both antibiotics (PEN; $n=15/59$ [25.4%], AZI; $n=15/58$ [25.9%]) (Table 2), with seven isolates being resistant to both antimicrobials. According to CLSI breakpoints, the proportion of isolates resistant to ceftriaxone, cefixime, ciprofloxacin and tetracycline were 5%, 4.3%, 16.3% and 22.8% respectively. The proportion of isolates resistant to these antibiotics differed by EUCAST breakpoints; they were 13.0%, 5.4%, 45.7% and 37%. No isolates were resistant to gentamicin.

The Kruskal-Wallis H was performed only on *N. subflava*, *N. macacae*, *N. perflava* and *N. flavescens* (Table 1). The test demonstrated no statistically significant difference in MIC values between the four *Neisseria* species. (Table 1).

Genomic analysis and relatedness

Thirty isolates were selected for whole genome sequencing (WGS), covering isolates with ceftriaxone MICs ≥ 0.125 mg/L (15 isolates) and <0.125 mg/L (four isolates), at least one of each species from the MALDI-ToF identification (six isolates) and three isolates where MALDI-ToF identification was not possible (Supplementary Table S5). The genomic data from the study isolates, along with 61 *Neisseria* reference genomes (Supplementary Table S3), was used to generate cgMLST neighbour joining phylogeny.

The 91 *Neisseria* isolates clustered in approximately five clusters (Fig. 3). As previously described, *N. meningitidis* and *N. gonorrhoeae* isolates clustered together with *N. lactamica* and *N. polysaccharea*[4] however *N. bergeri* and *N. cinerea* were also present within the cluster. No study isolates were present in the *N. meningitidis*/*N. gonorrhoeae* cluster (Supplementary Tables S3 and S4). The *N. bacilliformis* group also contained *N. bacilliformis*, *N. animaloris*, and 8 other species but no study isolates (Supplementary Table S3 and S4). MLST analysis of *N. perflava* CCH10-H12, which clustered with *N. mucosa* isolates only matched 3 alleles in the database: *abcZ233*, *adk178* and *pdhC561* (Supplementary Table S3). This combination of alleles was only found together in ST-16,693 but this ST was not associated with any isolates in the database. *abcZ233* was present in ST-3706 (*N. mucosa*), ST-9926 (*N. perflava*), ST-10,150 (*N. mucosa*), ST-16,006 (*N. mucosa*), ST-16,037 (*N. mucosa*), ST-16,480 (*N. mucosa*). *Adk178* was present in ST-3706 (*N. mucosa*) and *pdhC561* was present in ST-12,049 (*N. mucosa*).

The *N. flavescens* cluster contained 3/4 *N. flavescens*, a single *N. subflava* and 10 study isolates. The *N. subflava* cluster contained 4/5 *N. subflava* and 2/2 *N. perflava* plus 16 study isolates. Finally, the *N. macacae* cluster contained 1/1 *N. macacae*, 3/3 *N. elongata*, 3/3 *N. sicca* and 1/1 *N. mucosa* plus four study isolates (Supplementary Tables S3 and S4).

We compared the first and second species identification given by MALDI-ToF and Kraken2 from the genome sequence, excluding the three isolates with no MALDI-ToF ID. A total of 16/26 (61.5%) isolates had ID concordance between the primary MALDI-ToF ID and Kraken2 and 22/26 (84.6%) had concordance between any MALDI-ToF ID and Kraken2 (Supplementary Table S5). The three isolates with no MALDI-ToF ID were predicted as *N. subflava* by Kraken2. All isolates identified as *N. subflava*, *N. perflava* or *N. flavescens* by MALDI-ToF were predicted as *N. subflava* by Kraken2. The isolates identified as *N. macacae* by MALDI-ToF were predicted as *N. mucosa* by Kraken2.

Genotypic antimicrobial resistance

One isolate (49 A) produced a poor assembly so was removed from further analysis. Analysis of the remaining 29 *Nc* genomes for AMR related genes identified five matches (min 80% identity, 80% coverage) with the CARD database, three with ResFinder, eight with MEGARes additionally 14 virulence related genes with matched against VFDB (Fig. 4).

The MacAB-TolC tripartite macrolide efflux complex consists of *macA*, *macB* and *tolC*. *macB* was present in most isolates except 12/14 of the *N. bacilliformis* cluster isolates and *N. perflava* CCH10-H12, however *macA* was only identified in *N. meningitidis*/*N. gonorrhoeae* cluster isolates and *N. macacae* group isolates plus 49 A[48] (Fig. 4). Similarly, *mtrC* and *mtrD*, along with *mtrE*, encode a multidrug efflux complex but while *mtrCD* were conserved within *N. meningitidis*/*N. gonorrhoeae* cluster [cluster 1], these genes differentiated the *N. mucosa*/*sicca*/*macacae* (present) from *N. elongata* and *N. perflava* CCH10-H12 (absent) within the *N. macacae* cluster[5]. mtrCD was also completely absent from the *N. bacilliformis* cluster[2]. Within the *N. flavescens* and *N. subflava* cluster all isolates except *N. flavescens* ERR2764931 had *mtrD* but only five isolates also had *mtrC*.

PenA, linked to β-lactam resistance, was only present in the *N. meningitidis*/*N. gonorrhoeae*, *N. flavescens* (except *N. flavescens* ERR2764931) and *N. subflava* clusters. TetM, a ribosomal protection protein that confers tetracycline resistance, was present in 7 isolates: *N. subflava* C2007002879, 1 A, 10 A, 14B, 18B, 35 A and 48B, which were spread evenly across *N. flavescens* and *N. subflava* clusters (Fig. 4). Isolates 14B and 18B had

Antimicrobial	PEN	CRO	CFX	CIP	TET	AZI	GEN
Median MICs (mg/L)							
Neisseria all *spp*	1	0.06	0.064	0.032	0.5	0.5	4
N. flavescens	1	0.06	0.047	0.032	0.5	0.25	4
N. macacae	1.5	0.125	0.064	0.5625	1.5	0.5	3
N. mucosa	0.5	0.06	0.047	0.016	0.5	0.125	4
N. perflava	0.5	0.07	0.064	0.016	0.5	1	4
N. subflava	1	0.06	0.023	0.032	0.5	0.375	4
Neisseria spp (NO ID)							
MIC^{N}	101	100	92	92	92	92	92
Modal MIC	1	0.06	0.064	0.016	0.5	0.032	4
Range	0.03-4	0.015-8	0.002-0.5	0.008-32	0.032-32	0.016-512	0.5–16
IQR range	0.5-2	0.06–0.125	0.047–0.094	0.016-0.5	0.25-1	0.06–1.5	2–4
Geometric mean	0.7	0.07	0.06	0.09	0.81	0.37	3.47
Kruskall-Wallis^{N}	90	88	86	86	86	86	86
H score	2.56	2.94	3	4.57	2.9	0.61	2.03
p	0.464	0.4	0.39	0.21	0.41	0.89	0.57

Table 1. Summary of minimum inhibitory concentration characteristics by commensal *Neisseria* species and relationship between species and MIC. N, numbr of isolates; IQR, interquartile range; PEN, penicillin; CRO, ceftriaxone; CFX, cefixime; CIP, ciprofloxacin; TET, tetracycline; GEN, gentamicin.

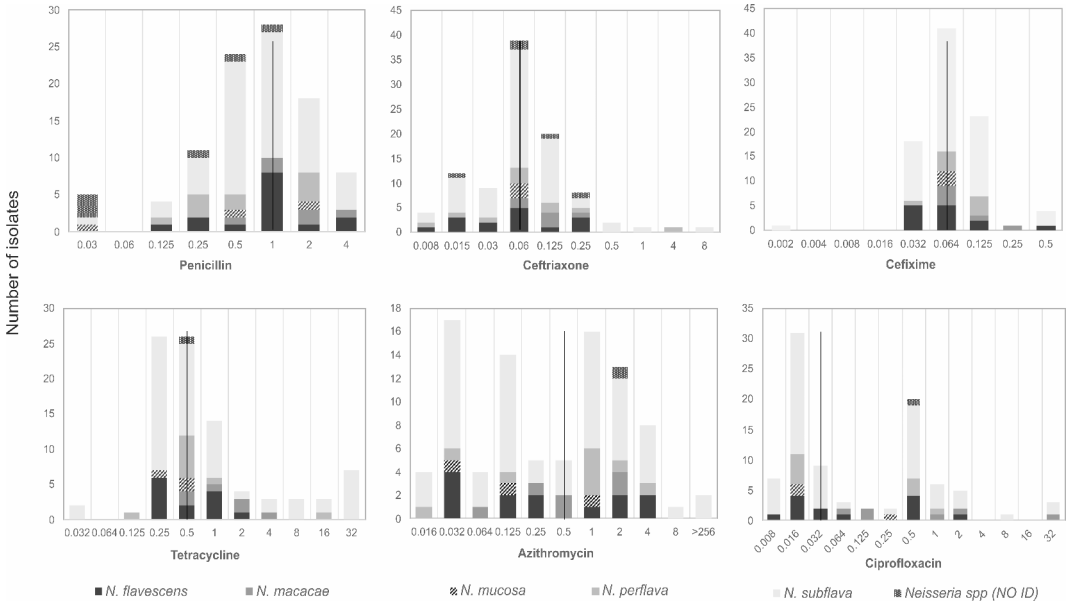

Fig. 2. Minimum inhibitory concentration distribution of commensal *Neisseria* species to penicillin, ceftriaxone, cefixime, ciprofloxacin, azithromycin and tetracycline, performed by agar dilution. The dotted line indicates the median MIC for each antimicrobial.

tetracycline MICs of 0.5 mg/L and 1 A, 10 A & 35 A had MICs of 16–32 mg/L (Supplementary Table S2). Tetracycline MIC testing was not performed on isolate 48B as it was nonviable on resuscitation.

Capsule polysaccharide modification proteins (LipA/LipB) and capsule polysaccharide export ATP-binding protein (CtrD) were present in all *N. meningitidis* (except *N. meningitidis* alpha14) but absent from *N. gonorrhoeae* and *N. lactamica* Additionally, all three genes were conserved within the majority of *N. flavescens/N. subflava* clusters (*lipA*: 34/36, *lipB*: 32/36, *ctrD*: 22/36) but absent from *N. bergeri*, *N. polysaccharea* and *N. cinerea*.

Analysis of DNA transfer mechanisms

The *Nc* genomes were screened for the presence of gcDUS, AT-DUS and vDUS. All three DUS dialects were found in the *Nc* genomes. Overall, the *N. subflava* complex (*N. subflava*, *N. perflava* and *N. flavescens*) isolates had more gcDUS repeats than vDUS whereas the opposite was seen with *N. macacae*. The *N. subflava* complex isolates had 2738–2990 *Ng* DUS, 144–192 AT-DUS and 158–276 vDUS repeats. *N. macacae* isolates carried

	CLSI N^R/N^T (%)						
	PEN	CRO	CFX	CIP	TET	AZI*	GEN§
Breakpoint (R >)	**1**	**0.25**	**0.25**	**0.5**	**1**	**1**	**16**
Overall	27/101 (26.7%)	5/100 (5%)	4/92 (4.3%)	15/92 (16.3%)	21/92 (22.8%)	27/92 (29.3%)	0/92 (0%)
N. flavescens	3/15 (20%)	0/15 (0.0%)	1/13 (7.7%)	1/13 (7.7%)	2/13 (15.4%)	5/13 (38.5%)	0/13 (0%)
N. macacae	3/6 (50%)	0/6 (0.0%)	0/6 (0.0%)	3/6 (50%)	3/6 (50%)	2/6 (33.3%)	0/6 (0%)
N. mucosa	1/3 (33.3%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0%)
N. perflava	4/10 (40%)	1/10 (10%)	0/9 (0.0%)	1/9 (11.1%)	1/9 (11.1%)	2/9 (22.2%)	0/9 (0%)
N. subflava	15/59 (25.4%)	4/59 (6.8%)	3/58 (5.1%)	10/58 (17.2%)	15/58 (25.9%)	15/58 (25.9%)	0/59 (0%)
Neisseria spp (NO ID)	1/7 (14.2%)	0/7 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0.0%)	3/3 (100%)	0/2 (0%)
	EUCAST N^R/N^T (%)						
	PEN	CRO	CFX	CIP	TET	AZI*	GEN§
Breakpoint (R >)	**1**	**0.125**	**0.125**	**0.06**	**0.5**	**1**	**16**
Overall	27/101 (26.7%)	13/100 (13%)	5/92 (5.4%)	42/92 (45.7%)	34/92 (37%)	27/92 (29.3%)	0/92 (0%)
N. flavescens	3/15 (20%)	3/15 (20%)	1/13 (7.7%)	6/13 (46.2%)	5/13 (38.5%)	5/13 (38.5%)	0/13 (0%)
N. macacae	3/6 (50%)	1/6 (16.7%)	1/6 (16.7%)	6/6 (100%)	4/6 (66.7%)	2/6 (33.3%)	0/6 (0%)
N. mucosa	1/3 (33.3%)	0/3 (0.0%)	0/3 (0.0%)	1/3 (33.3%)	0/3 (0.0%)	0/3 (0.0%)	0/3 (0%)
N. perflava	4/10 (40%)	2/10 (20%)	0/9 (0.0%)	4/9 (44.4%)	2/9 (22.2%)	2/9 (22.2%)	0/9 (0%)
N. subflava	15/59 (25.4%)	6/59 (10.1%)	3/58 (5.1%)	24/58 (41.3%)	23/58 (39.7%)	15/58 (25.9%)	0/59 (0%)
Neisseria spp (NO ID)	1/7 (14.2%)	1/7 (14.2%)	0/3 (0.0%)	1/3 (33.3%)	0/3 (0.0%)	3/3 (100%)	0/2 (0%)
N. gonorrhoeae (%R)‡	17.9	0	0.8	42.7	62.9	4.2	n/a

Table 2. Resistance rates of commensal *Neisseria* species to the tested antimicrobials, interpreted by CLSI and EUCAST breakpoints for *Neisseria gonorrheae*. N^R, number of resistant isolates; N^T, total number of isolates tested; n/a, not applicable. *PEN;* penicillin, *CRO*; ceftriaxone, *CFX*; cefixime, CIP; ciprofloxacin, TET; tetracycline, AZI; azithromycin; GEN; gentamicin. *Azithromycin based on ECOFF of S < 1 mg/L, §Gentamicin based on previous recommended breakpoint[27]. ‡Data from Gonococcal Resistance to Antimicrobials Surveillance Programme, 2020[28].

247–292 *Ng* DUS, 29–40 AT-DUS and 3608–3802 vDUS repeats (Supplementary Table S6). No genetic plasmid markers were identified; however, *tetM* has previously been identified as plasmid mediated[49]. Raw reads from all *tetM* positive isolates were mapped against pEP5289 (GU479466, 'Dutch' *tetM*) and pEP5050 (GU479464, 'American' *tetM* genetic load area) which showed no mapped reads except to the *tetM* gene. Subsequent analysis identified a cryptic 40 kb plasmid in isolate 8 A (*N. macacae*) that had 95% coverage, 99.7% identity to a *Ng* plasmid (CP048906) however this plasmid did not contain any AMR genes.

Discussion

The value of monitoring carriage and the AMR reservoir of *Nc* from the human oropharynx is becoming increasingly evident, not only to prevent the development of AMR in *Nm* and *Ng*, but also the assess the risk of oropharyngeal colonisation and persistence of the pathogenic *Neisseria* species. Not only is there transmission of AMR genes between *Neisseria* species, there is also evidence *Nc* are shared between intimate partners[50], further exacerbating the problem of AMR transmission. In this study we characterised the carriage, genomic relatedness and antimicrobial susceptibility profiles of *Nc* species, acquired from the oropharynx of 50 LSHTM volunteers.

In this study, 84% of the study population were colonised with at least one *Nc* species. This finding aligns with recent studies reporting *Nc* carriage of 68%[21] and 100%[17]. However, our findings contrasted with those found by Diallo *et al.*[16] and Le Saux *et al.*[19] who found a *Nc* prevalence of 10.2% and 11.6% respectively. These studies were focused on colonisation of *Nm* and in particular vaccinated individuals, and it has been suggested that both *Nm* and *Nc* carriage can be negatively associated with recent meningococcal vaccination[16]. Also, both these studies used Theyer-Martin (TM) media for pathogenic *Neisseria* species, whereas some *Nc* species such as *N. cinerea*, *N. subflava* and *N. mucosa* do not grow very well on this media[51]. This was confirmed by the lack of growth of study *Nc* on *Ng* selective VCAT agar. LBVT.SNR media, formulated specifically for the isolation of *Nc*[18], aligns with two older studies that used the same media and identified high prevalence of 96.6%[18] and

100%[20]. Additionally, the study by Sáez *et al*. that found 100% prevalence used both LBVT.SNR and TM media, the latter added specifically to ensure the recovery of *Nm* and *Nl*[20].

The most common *Nc* species found in this study was *N. subflava*, with 61.4% and 74% of participants colonised by this species. The colonisation rate of *N. subflava* is similar found in two recent studies[17,21], especially when combined with *N. flavescens* and *N. perflava* as previously described[4]. Surprisingly, *N. lactamica* were not isolated from the study participants, however this was likely due to omission of selective media for pathogenic *Neisseria* species. In fact, as part of our quality control checks, a *N. lactamica* laboratory reference strain grew very poorly on SBVT.SNR media. Carriage of *N. lactamica* seems to be variable depending on the population; the prevalence of *N. lactamica* in previous studies ranged from 0.4%[17] to 17.3%[52]. Interestingly, some studies showed that young children carry *N. lactamica* at much higher rates than adults[16,52], which could further explain the lack of recover in our study.

Concordance between MALDI-ToF species identification and Kraken2 prediction was just 65.2% when considering the primary species ID. This further demonstrates the challenge of accurate identification in this homogeneous genus, due to the limitations of both technologies. The accuracy of these techniques is only as good as the curation of the database itself demonstrated by several reports of misidentification of *Nc* by MALDI-ToF[53–55]. Similarly, genomic identification is limited by the high genetic recombination of *Neisseria* species[6,28,56–58] coupled with the lack of an internationally accepted genomic identification scheme.

The introduction of more advanced techniques such as WGS, rMLST and cgMLST have led to several re-classifications of existing species and the discovery of novel species[4–6]. In this study, the isolates clustered into three distinct groups, the *N. flavescens*, *N. perflava* and *N. macacae* clusters, in line with previous findings. The clustering agreed with previously suggested re-classifications of *N. perflava* and *N. subflava* into different variants of *N. subflava*[5]. Similarly, it has been suggested that *N. macacae* and *N. mucosa* can be merged into a single *N. mucosa* group[59], which our cgMLST cluster analysis supports.

Resistance to all antimicrobials except gentamicin and cefixime was high according to both CLSI and EUCAST breakpoints. The median MIC to ceftriaxone was 0.06 mg/L, which although phenotypically susceptible according to both CLSI and EUCAST breakpoints is just 1–2 $\log_2$ MIC lower than the 0.125–0.25 mg/L breakpoint with one isolate having an MIC of 8 mg/L. This translates to resistance rates of 5% (CLSI) and 13% (EUCAST) compared to *Ng* resistance rates of 0% for the same year in England[28], but lower than *Nc* resistance rates of 28% reported in Vietnam[17]. Differing AMR rates could be due to differences in study populatons, as the study in Vietnam included only men who have sex with men (MSM)[17]. This patient group are described as having a higher likelihood of repeated gonococcal infection and exposure to ceftriaxone, leading to AMR selection pressures on *Nc*[17].

Commensal *Neisseria* species with high ESC MICs pose a significant reservoir for transfer of resistance and development of mosaic genes in pathogenic *Neisseria* species. Although other antimicrobials are no longer used as empirical treatment, resistance to these should not be overlooked, as there has been evidence of macrolide, tetracycline and fluoroquinolone AMR transfer[57]. Investigations of the *Neisseria* resistome have found high resistance to β-lactams, fluoroquinolones encoded by mutations in *gyrA*, tetracylines due to *tetM* as well as TEM-type β-lactamases[60]. Importantly, a recent study demonstrated in vitro transformation of zoliflodacin resistance, a new DNA replication inhibitor evaluated for treatment of *Ng*, from *Nc* to *Ng*, suggesting important implications for the introduction of new antimicrobials[61]. In this study, 30 *Nc* isolates genomes were analysed

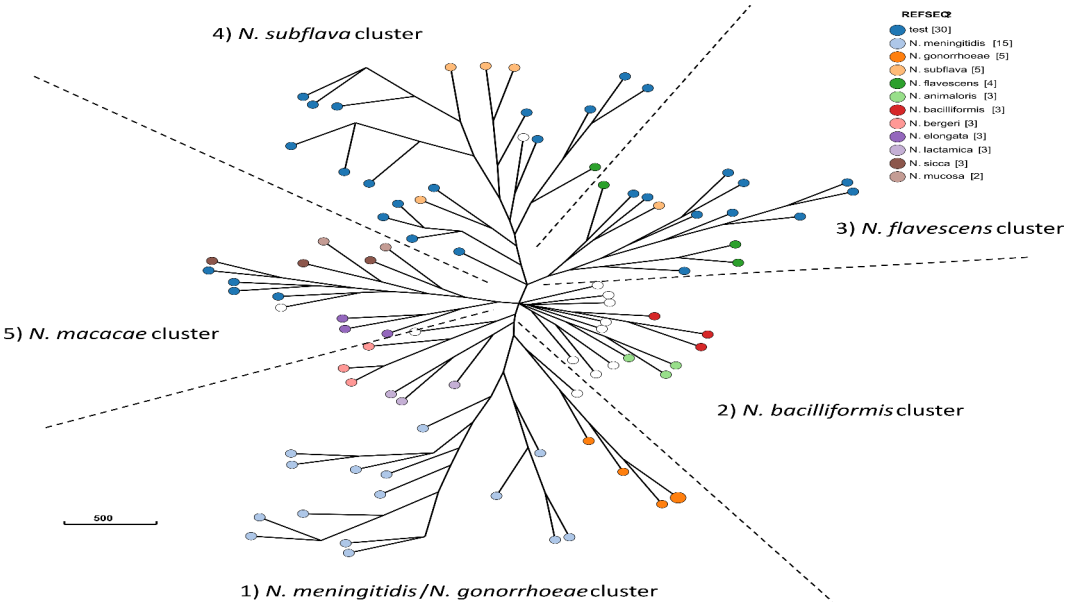

Fig. 3. Core genome multi-locus sequence typing (cgMLST) nearest neighbour phylogeny. cgMLST phylogeny derived from 842 gene alleles conserved within 95% of 30 commensal *Neisseria* plus 61 reference *Neisseria* species. Nodes coloured by reference species, study isolates coloured dark blue.

Fig. 4. AMR and virulence genes. Draft genomes were analysed for AMR genes (CARD, ResFinder and Megares databases) and virulence (VFDB) genes using Abricate (min ID/coverage 80%). Circles represent the presence of gene, scaled to %ID. Similar profiles were grouped using Euclidean hierarchical clustering using average linkage algorithm in Morpheus. Study isolates are given with MALDI-ToF identification in parenthesis. 1 to 5 indicate cgMLST clustering group; 1 = *N. meningitidis*/*N. gonorrhoeae* cluster, 2 = *N. bacilliformis* cluster, 3 = *N. flavescens* cluster, 4 = *N. subflava* cluster, 5 = *N. macacae* cluster.

for genotypic markers of acquired resistance and we identified several acquired resistance genes. For example, *msr(D)* responsible for high level macrolide resistance (> 256 mg/L)[57], was present in 2 A which had an MIC of > 256 mg/L. Macrolide resistance has also been associated with overexpression of the MtrCDE efflux pump, which also confers resistance to b-lactams, tetracyclines and fluoroquinolones[62]. The MtrCDE efflux pump is commonly found in *Ng*[62] and other *Neisseria* species, however correlation between presence of *mtrCDE* and any macrolide resistances was not identified. Similarly, most of our *Nc* isolates had *macB*, another efflux pump complex also found in *Ng*[63], but there was no correlation with phenotypic resistance. Antimicrobial resistance due to overexpression of efflux pumps are associated with specific mutations[64] and the presence of efflux pumps genes do not necessarily translate to phenotypic resistance.

Transfer of AMR genes between isolates provides a rapid solution to antibiotic treatment compared to accumulation of new genes through evolutionary purposes. Nc are proposed as a possible source of horizontally acquired AMR genes in pathogenic Neisseria, for example horizontal gene transfer of *penA* from *N. lactamica*, *N. macacae*, *N. mucosa* and *N. cinerea* to Ng[58,65–67]. *Neisseria* are naturally competent and therefore naked DNA is a primary method of acquiring new DNA. The Neisseria DUS sequences enhance this DNA uptake. Members of the *N. subflava* and *N. flavescens* clusters had more copies of gcDUS than vDUS and the opposite was true for the *N. macacae* cluster (Supplementary Table S6). These findings agree with previous published data[10,68] and suggest that DNA incorporation into *Ng* and *Nm* would be more efficient from *N. subflava* and *N. flavescens* clusters than *N. macacae* cluster isolates. Even though *Nc* have fewer copies of AT-DUS that enhances transformation efficiency, these findings demonstrate the high likelihood of HGT between *Nc* and pathogenic *Neisseria* species, not just relating to AMR, but also virulence and niche adaptation[68]. Plasmids also can transfer AMR genes in Neisseria for example *tetM* was associated with tetracycline resistance in six of our isolates (1 A, 10 A, 14B, 18B, 35 A and 48B), three of which had tetracycline MICs of 16–32 mg/L (1 A, 10 A and 35 A) and two had an MIC of 0.5 mg/L (14B and 18B) (Supplementary Table S2). Tetracycline resistance due to *tetM* is usually coded on a conjugative plasmid in *Ng*, resulting in MICs of 16–64 mg/L[69]. No plasmid markers or known *tetM* carrying plasmids were detected suggesting *tetM* may be present in the chromosome of some *Nc* species. Interestingly, a single plasmid was identified in a N. macacae isolate that had previously been sequenced in a Ng isolate. While this supports transfer between pathogenic and commensal Neisseria no AMR genes were present on this plasmid.

In our study we performed comprehensive phenotypic and genotypic analysis of both *Nc* carriage, speciation, and AMR determinants, but it is not without limitations. Firstly, our sample size was small, which limited statistical power in some analyses, such as exploring the relationship between *Nc* and AMR. Additionally, we did not use *Ng* selective agar, which may have enabled us to recover *N. lactamica* due to the possibility of isolating *Ng/Nm* which was outside the scope of the project and had additional ethical considerations. There is currently no gold standard for speciation of *Nc*; the accuracy of genomic and MALDI-ToF analyses are reliant on the accuracy of published reference genomes and identification databases. The nomenclature and speciation of *Nc* is evolving, with species reclassified and new species being discovered, meaning that taxonomic errors in reference databases have been discovered[59]. This issue also extends to phenotypic and genotypic analysis of AMR. Firstly, there are no guidelines or resistance breakpoints for *Nc* and most published literature have used CLSI or EUCAST breakpoints for *Ng*. This also means there are no international control strains for *Nc* susceptibility testing which impacts the accuracy of both phenotypic and genotypic testing. Published fully susceptible *Nc* reference genomes will enable detection of single nucleotide polymorphisms and mosaic genes as well as acquired resistances.

This study demonstrated high pharyngeal colonisation rates in our population with higher AMR rates than *Ng*. Although more research in needed to understand the mechanisms of HGT in vivo, monitoring *Nc* may help us predict the rates of *Ng* resistant strains occurring in the future, especially relating to ESCs and other newly introduced antimicrobials.

Data availability

The whole genome datasets presented in this study can be found online at https://www.ebi.ac.uk/ena under study PRJEB67528. Any additional datasets are available from the corresponding author upon reasonable request.

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Author contributions

VM designed the study, recruited participants, performed laboratory work, and wrote the manuscript. WB recruited participants and performed laboratory work. IS and MH performed MIC testing. SG assisted with MALDI-ToF identification. RS performed WGS, genomic analyses and prepared Figs. 3 and 4. All authors reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

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Additional information

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