

**Antimicrobial susceptibility, resistance
determinants and molecular epidemiology of
Neisseria gonorrhoeae in Ireland**

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Summary

High-level resistance to and treatment failures with ceftriaxone and azithromycin, the first line agents for treatment of gonorrhoea are reported and antimicrobial-resistant *N. gonorrhoeae* is now an urgent public health threat. This study investigated rates of resistance to extended-spectrum cephalosporins (ESCs), azithromycin and other agents among gonococci in Ireland, resistance mechanisms and molecular epidemiology of the most resistant subset of isolates.

Six-hundred and nine isolates from 4 different tertiary referral hospitals in Ireland were recovered for susceptibility testing against extended-spectrum cephalosporins, azithromycin, ertapenem, ciprofloxacin, gemifloxacin, penicillin, tetracycline, spectinomycin, gentamicin and fosfomycin by gradient MIC strip. Forty-three isolates were selected for whole-genome sequencing based on elevated MICs, in particular to extended-spectrum cephalosporins and azithromycin. Sequencing libraries of *N. gonorrhoeae* genomic DNA were generated using the NexteraXT™ library preparation kit according to manufacturer's instructions and sequenced on an Illumina MiSeq instrument. The NG-STAR database, pubMLST tool and ARG-ANNOT database were employed for data analysis.

Seven high-level azithromycin resistant (HLAzi-R) isolates were identified, while no resistance to ceftriaxone was found. All isolates were susceptible to spectinomycin. 98.2%, 33% and 9.7% of isolates were non-susceptible to penicillin, ciprofloxacin and tetracycline, respectively. MIC₉₀ for ertapenem, gemifloxacin, gentamicin and fosfomycin were 0.032 mg/L, 2 mg/L, 8 mg/L and 32 mg/L, respectively.

Mosaic *penA* alleles XXXIV, X and non-mosaic XIII were associated with elevated ESC MICs. L421P mutation in *penA*, G120K plus A121N/D/G alterations in *penB*

(encoding PorB), H105Y alteration in *mtrR* and A deletions in the *mtrR* promoter were also associated with reduced susceptibility to ESCs. A2059G and C2611T mutations in 23s rRNA alleles were associated with HLAzi-R and gonococci with azithromycin MICs of 4-32 mg/L, respectively. The 43 isolates belonged to 31 NG-MAST STs and most prevalent MLST STs included ST1580, ST9396 and ST1901. All HLAzi-R isolates belonged to MLST ST1580, among which some clonal clustering was observed. When these isolates were compared to HLAzi-R isolates from a UK outbreak, analysed at a whole-genome sequence level, they differed significantly.

This is the largest genomic study of Irish *N. gonorrhoeae* performed to date.

Ceftriaxone remains a suitable empiric agent for treatment of gonorrhoea in Ireland. A number of potential alternative agents were identified. There was good correlation between previously described genetic mutations and phenotypic susceptibility categories for ESCs and azithromycin and *N. gonorrhoeae*. This work highlights the advantages and potential of whole-genome sequencing to be applied at scale in the surveillance of antibiotic resistant strains of *N. gonorrhoeae*, both locally and internationally.

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List of abbreviations

AL	Lysis buffer
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
ATL	Tissue lysis buffer
ATM	Amplicon Tagment mix
AW1	Wash buffer 1
AW2	Wash buffer 2
AZM	Azithromycin
BASHH	British Association for Sexual Health and HIV
BL	Beta-lactamase
BLAST	Basic local alignment search tool
CDC	Centre for Disease Control and Prevention
CFM	Cefixime
CFM-R	Cefixime-resistant
CFM-RS	Cefixime reduced susceptibility
CIP	Ciprofloxacin
CRO	Ceftriaxone
CRO-R	Ceftriaxone-resistant
CRO-RS	Ceftriaxone reduced susceptibility
CTX	Cefotaxime
CTX-R	Cefotaxime-resistant
CTX-RS	Cefotaxime reduced susceptibility
ddNTP	dideoxy nucleotide triphosphates
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid

ECDC	European Centre for Disease Prevention and Control
EQA	External quality assurance
ESC	Extended-spectrum cephalosporins
ETP	Ertapenem
ETP-R	Ertapenem-resistant
ETP-RS	Ertapenem reduced susceptibility
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EURO-GASP	European Gonococcal Antimicrobial Surveillance Programme
FOS	Fosfomycin
GEMI	Gemifloxacin
GENT	Gentamicin
HIV	Human Immunodeficiency virus
HLAzi-R	High-level azithromycin-resistant
HPSC	Health Protection Surveillance Centre
HPWR	Health Protection Weekly Report
IM	Intramuscular
IUSTI	International Union Against Sexually Transmitted Infections
MALDI ToF	Matrix-assisted laser desorption ionisation time-of-flight
MDR	Multidrug-resistant
MGW	Molecular grade water
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence type
MRHM	Midlands Regional Hospital Mullingar
MSM	Men who have sex with men
NAAT	Nucleic Acid Amplification Test
NG-MAST	<i>Neisseria gonorrhoeae</i> Multiantigen sequence type
NMH	National Maternity Hospital

NT	Not tested
PBP	Penicillin-binding protein
PEN	Penicillin
PHE	Public Health England
PID	Pelvic inflammatory disease
PO	Per oral
PPNG	Penicillinase-producing <i>Neisseria gonorrhoeae</i>
RBS	Resuspension buffer solution
RNA	Ribonucleic acid
SCH	St. Collumcille's Hospital
SJH	St. James's Hospital
SNP	Single nucleotide polymorphism
SOL	Solithromycin
SPC	Spectinomycin
TD	Tagment DNA
TET	Tetracycline
TOC	Test of cure
UHG	University Hospital Galway
UHL	University Hospital Limerick
UHW	University Hospital Waterford
UK	United Kingdom
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug-resistant

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Chapter I

Introduction

1.1 History and Microbiology of *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a fastidious gram-negative coccus, occurring intracellularly in pairs (diplococci) with opposing surfaces flattened. It displays twitching motility made possible by type IV pili. It is rapid oxidase positive (oxidising dimethyl- or tetramethyl-*p*-phenylenediamine). *N. gonorrhoeae* utilises glucose, but not maltose or sucrose.

The pathogen was discovered in 1879 by Neisser and in 1882 it was cultivated by Leistikow and Löffler (Mandell Douglas, 8th edition). It is susceptible to temperature change, drying, UV light and some other environmental changes. It grows best at 35-37°C in 5% CO₂ on chocolate agar. Selective agar containing antibiotics, e.g. Thayer-Martin medium, allow recovery from sites such where normal flora may overgrow *N. gonorrhoeae*, such as in the rectum or pharynx (Thayer *et al.*, 1964).

1.2 Cell surface structures

N. gonorrhoeae has a typical gram-negative cell envelope with an inner cytoplasmic membrane, a peptidoglycan cell wall and an outer membrane. Type IV pili extend from the cell surface through an outer membrane protein porin PilQ (coded for by the *pilQ* gene), and are important for host cell attachment and killing of neutrophils (Barry *et al.*, 2009).

Opa proteins, produced by *opa* genes, present in variable numbers on strains of *N. gonorrhoeae*, are responsible for the opaque appearance of colonies recovered from mucosal surfaces and play a role in adherence to and invasion of cells, as well as down regulating the immune response. The importance of LOS (lipid A and oligosaccharide)

lies in its endotoxin activity and its ability to resist anti-LOS antibodies and invade epithelial cells.

Por protein, encoded by *porB* gene, occurs in two antigenic forms, PorB1A and PorB1B and is the means by which solutes cross the outer membrane. *N. gonorrhoeae* has three penicillin binding proteins PBP1, 2 and 3. PBP2, coded for by the *penA* gene, is the main binding site for penicillins and cephalosporins. PBP1 is encoded by *ponA*. PBP3 is encoded by *dacB*.

1.3 Clinical aspects of infection with *Neisseria gonorrhoeae*

1.3.1 Types of clinical infections

The natural environment for *Neisseria gonorrhoeae* is in the human body. It is an obligate human pathogen, causing gonorrhoea, one of the most common sexually transmitted infections (STIs) (WHO 2016). Uncomplicated genital gonorrhoea manifests as purulent urethritis in male and cervicitis in females, but infection may be asymptomatic in $\leq 10\%$ of men and $\geq 50\%$ of women (Unemo *et al.*, 2016). Most rectal and the majority of pharyngeal infections are asymptomatic, but pharyngitis and proctitis may occur. Infection of the conjunctivae may also occur. Complications may include epididymitis in men and pelvic inflammatory disease with potential infertility (Unemo *et al.*, 2012a). Neonatal infection can occur in infants born to infected mothers. Ophthalmia neonatorum, if not diagnosed and treated appropriately, may result in scarring and blindness. Disseminated infection is rare but may lead to arthritis, endocarditis and meningitis (Unemo *et al.*, 2016).

1.3.2 Urogenital infection in women

Cervicitis is the most common infection associated with *N. gonorrhoeae* in women, but it may ascend and cause infection in the pelvis. Most cases of cervicitis are asymptomatic. Symptoms, if present, are non-specific and may include pruritus and mucopurulent discharge. The cervix may appear normal or friable with discharge. Urethral infection occurs in 90% of those with cervicitis, but uncommonly in isolation (Barlow *et al.*, 1978). Non-specific symptoms may include dysuria, frequency and urgency.

Pelvic inflammatory disease (PID) occurs in 10-20% of cases of cervical gonorrhoea (Walker *et al.*, 2011). Given the high percentage of asymptomatic infections in women, PID may be the first presentation with symptoms of pelvic pain, abnormal vaginal bleeding and dyspareunia. Perihepatitis (Fitz-Hugh-Curtis Syndrome) may occur in association with PID and gonococcal infection. Pleuritic right upper quadrant pain, nausea, vomiting and fever may be presenting symptoms. The incidence is unclear.

Bartholinitis may also occur resulting in symptoms of pain, swelling and discharge from the gland in up to 6% of women with cervical gonorrhoea (Rees, 1967).

1.3.3 Urogenital infection in men

Urethritis in men due to *N. gonorrhoeae* is mostly symptomatic, giving rise to copious purulent discharge and dysuria. Rare complications of urethritis include lymphangitis, oedema and periurethral abscess formation. Epididymitis may also be a complication of gonococcal infection, presenting with pain and swelling of the epididymis.

1.3.4 Extragenital infection

Infection of the rectum and pharynx secondary to *N. gonorrhoeae* may occur and are typically asymptomatic. Rectal infection in men is uncommon in heterosexuals, occurring mostly in men who have sex with men (MSM) due to practice of receptive anal intercourse. Anorectal infection may be the only site of infection in MSM.

Receptive anal intercourse is not necessary for anorectal infection to occur in women as infection may spread from vagina to rectum due to close proximity. If symptoms do occur, they may include tenesmus, anorectal pain, mucopurulent discharge or bleeding.

Pharyngeal infection is normally acquired by oral sexual exposure and is normally asymptomatic, but may present with sore throat, exudates or cervical lymphadenopathy.

The pharynx is felt to be the site where gene transfer frequently occurs between *N. gonorrhoeae* and commensal *Neisseria* species, of particular interest, genes conferring antimicrobial resistance (Unemo *et al*, 2012a). Pharyngeal infection is also important as an asymptomatic reservoir of infection and is a site that has proven more difficult for successful eradication of *N. gonorrhoeae*, especially with spectinomycin (Rompalo *et al.*, 1994), but with reports also of ceftriaxone treatment failures at this site (Golparian *et al.*, 2014).

Disseminated gonococcal infection is usually preceded by infection at a mucosal site and may present in two ways; acute purulent arthritis or a combination of tenosynovitis, dermatitis and polyarthralgias. Meningitis, endocarditis and osteoarthritis may also occur, albeit rarely.

Conjunctivitis may be aggressive with purulent discharge and periorbital oedema, progressing in some cases to corneal ulceration, perforation and blindness. Neonates

are mainly affected, following transmission from the untreated mother.

Autoinoculation from a urogenital source may occur in adults and teenagers.

1.4 Risk factors for gonorrhoea infection

Gonorrhoea is most common in the younger age group < 25 years (HPSC 2017b; ECDC 2017a; Weinstock *et al.*, 2000) and is also associated with men who have sex with men (MSM), illicit drug users, lower socio-economic classes and ethnic minorities (Staras *et al.*, 2011; Stoner *et al.*, 2000). Partner characteristics are an important factor also with adolescents whose partner has had an STI in the previous year being three times more likely to have have an STI (Staras *et al.*, 2009).

1.5 Historical and current treatment of gonorrhoea

Historical therapies for gonorrhoea include pepper and plant extracts (balsalm of Copaiva- extracted from a South American tree, 151,000 pounds of which was imported to Great Britain in 1859 for treatment of gonorrhoea), metallic compounds (arsenic, bismuth, gold and mercury) and heating the whole body to 41°C for four to six hours to kill the bacteria. Later targeted heating of the pelvis with heating elements inserted into the vagina or rectum achieved temperatures of 44°C and required only two hours of treatment for success (Benedeck 2017). Fortunately, introduction of sulfonamides allowed these treatments to be discontinued after 1937, but treatment failures quickly emerged. Penicillin was used in 1943 by the US army for cases of gonorrhoea which failed to respond to sulphonamide drugs. In 1946 four cases of penicillin resistant *N. gonorrhoeae* were reported and within a decade there was a steady increase in the

number of strains resistant to penicillin (Benedeck 2017). Over the following 70 years, *N. gonorrhoeae* developed resistance to all antibiotics introduced for treatment.

Tetracycline was used early in the treatment of gonorrhoea especially for penicillin-allergic patients and was discontinued due to high-level resistance in the 1980's (Morse *et al.*, 1980). Ciprofloxacin was the first line recommended empiric treatment for gonorrhoea in many countries in the 1980s, later being replaced by cephalosporins and azithromycin (MMWR April 2007) again because of high levels of resistance emerging.

Spectinomycin was introduced in the 1960's for the treatment of gonorrhoea (Cornelius CE. 3rd *et al.*, 1970) and by 1970's resistance had been described (Reyn *et al.*, 1973). In 1981 spectinomycin was introduced in Korea to treat gonorrhoea in the military personnel. Resistance and treatment failures ensued with 8 treatment failures in 124 cases, with 6/8 confirmed as highly resistant to spectinomycin (MIC $\geq$ 100 mg/L) (Boslego *et al.*, 1987). In the 1980s spectinomycin resistance was becoming a concern in the UK as well as in Korea. (Ison *et al.*, 1983; Easmon *et al.*, 1984; Boslego *et al.*, 1987). Since then, resistance to spectinomycin has not become more widespread. Interestingly, in Korea where spectinomycin is still used frequently in treatment of gonorrhoea, a study of 977 isolates from 2000 to 2006 found no resistant isolates. During those years, 52.6-58.1% of the patients treated for gonorrhoea received spectinomycin monotherapy (Lee *et al.*, 2011).

Current treatment guidelines for uncomplicated anogenital gonorrhoea in the US and Europe recommend combination therapy with intramuscular ceftriaxone (250-500 mg) and oral azithromycin (1-2g) (CDC 2015; Bignell *et al.*, 2012). A summary of the current treatment recommendations from various organisations is shown (Table 1.1). Dual antimicrobial therapy was introduced because of emergence of resistance to all

previously introduced agents (e.g. penicillin, tetracycline and quinolones), and most importantly to prevent resistance developing to either agent, i.e. ceftriaxone or azithromycin. In Europe the change to dual therapy, ceftriaxone and azithromycin, was introduced in 2012 (Bignell *et al.*, 2011). However, resistance has now been reported in some isolates to all recommended antimicrobials for the treatment of this infection (Unemo *et al.*, 2016).

Extended-spectrum cephalosporins (ESCs) used in treatment of gonococcal infection include oral cefixime and injectable ceftriaxone. Cefixime and ceftriaxone, are part of the recommended empiric treatment regimens for gonococcal infections worldwide (Table 1.1). Ceftriaxone is the first line recommendation (in combination with azithromycin) for anogenital or oropharyngeal infection given by the WHO, CDC, IUSTI, Irish national guidelines and BASHH (WHO 2016; CDC 2015; Bignell *et al.*, 2012; HPSC 2017a; BASHH 2011). The WHO, CDC and IUSTI also give an alternative option of cefixime plus azithromycin for anogenital infection only.

Recommended ceftriaxone or cefixime monotherapy options include ceftriaxone monotherapy for anogenital or oropharyngeal infection (WHO and IUSTI) and cefixime monotherapy for anogenital infection only (WHO and BASHH), keeping in mind that the latest update to the BASHH guidelines was in 2011. These organisations have established these latest recommendations because of antimicrobial resistance to penicillin, ciprofloxacin, macrolides and tetracycline as discussed above and also due to treatment failures reported with cefixime.

Spectinomycin is a drug with limited availability and poor activity in pharyngeal gonorrhoea, but it remains part of the empiric treatment guidelines for anogenital gonorrhoea as an alternative option (WHO, IUSTI and BASHH).

Organisation	Anogenital (DUAL THERAPY)	Anogenital (SINGLE THERAPY)	Oropharyngeal (DUAL THERAPY)	Oropharyngeal (SINGLE THERAPY)
WHO 2016	ceftriaxone 250 mg IM PLUS azithromycin 1 g PO or cefixime 400 mg PO PLUS azithromycin 1 g PO	ceftriaxone 250 mg IM or cefixime 400 mg PO or spectinomycin 2 g IM	As per anogenital	ceftriaxone 250 mg IM
CDC 2015	<u>Recommended:</u> ceftriaxone 250 mg IM PLUS azithromycin 1 g PO <u>Alternative:</u> cefixime 400 mg PO PLUS azithromycin 1 g PO	No recommendation for single therapy. State that monotherapy with azithromycin is no longer recommended.	ceftriaxone 250 mg IM PLUS azithromycin 1 g PO	No recommendation for single therapy
Ireland (HPSC) 2017	ceftriaxone 500 mg IM PLUS azithromycin 1 g PO	ciprofloxacin 500 mg PO (if susceptibility known) OR spectinomycin 2 g IM OR azithromycin 2 g PO (if susceptibility known)	ceftriaxone 500 mg IM PLUS azithromycin 1 g PO	ciprofloxacin 500 mg PO (if susceptibility known) OR azithromycin 2 g PO (if susceptibility known)

IUSTI 2012	<u>Recommended:</u> ceftriaxone 500 mg IM PLUS azithromycin 2 g PO <u>Alternative:</u> cefixime 400 mg PO PLUS azithromycin 2 g PO OR spectinomycin 2 g IM PLUS azithromycin 2 g PO	ceftriaxone 500 mg IM (only if azithromycin is unavailable or the patient is unable to take oral medications)	<u>Recommended:</u> ceftriaxone 500 mg IM PLUS azithromycin 2 g PO	ceftriaxone 500 mg IM (only if azithromycin is unavailable or the patient is unable to take oral medications) OR <u>Alternatives</u> (ONLY if history of penicillin or cephalosporin allergy and susceptibility is confirmed): ciprofloxacin 500 mg PO OR ofloxacin 400 mg PO OR azithromycin 2 g PO
BASHH 2011	ceftriaxone 500 mg IM PLUS azithromycin 1 g PO	cefixime 400 mg PO OR spectinomycin 2 g IM or cefotaxime 500 mg IM	ceftriaxone 500 mg IM PLUS azithromycin 1 g PO	ciprofloxacin 500 mg PO (if susceptibility known) OR ofloxacin 400 mg PO

Table 1.1. Current treatment recommendations for gonorrhoea (all are single dose regimens). WHO: the world health organisation. CDC: Centre for Disease Prevention and Control, USA. IUSTI: International union against sexually transmitted infections. Ireland: National guidelines for prevention and control of gonorrhoea and for minimising the impact of antimicrobial resistance in *Neisseria gonorrhoeae*. BASHH: British association for sexual health and HIV.

1.5.1 Adjuncts to antimicrobial therapy

Patients diagnosed with and treated for gonorrhoea infections require further attention in terms of follow up, test of cure (TOC), prevention of transmission and health promotion.

Routine test of cure (TOC) is recommended in the UK national treatment guidelines of gonorrhoea in adults, prioritising those with persisting signs or symptoms, pharyngeal infection or those who received an alternative to first-line treatment, if resources do not allow universal TOC. Culture should be used for TOC, if symptoms persist, at least 72 hours after completing therapy. For asymptomatic patients, nucleic acid amplification test (NAAT) two weeks after completion of therapy is indicated (BASHH 2011). In the US, CDC guidelines for gonococcal infections (2015) do not recommend routine TOC. They recommend TOC in those who have been treated with an alternative agent for pharyngeal infection, 14 days after treatment, using culture or NAAT. If NAAT is positive, then a sample for culture should be taken prior to retreatment (CDC 2015).

Prevention of transmission of gonorrhoea infection is of great public health importance given the large proportion of patients, especially female, who may be asymptomatic and act as a reservoir of ongoing transmission. Gonorrhoea infection increases the risk of human immunodeficiency virus (HIV) transmission five-fold (WHO 2012a; Wasserheit 1992) and so, it is clear that treatment and eradication of gonorrhoea infection has major public health implications.

1.6 Antimicrobial agents and resistance mechanisms

Resistance mechanisms employed by *N. gonorrhoeae* include enzyme production, decreased uptake or increased efflux of antimicrobials and changes in binding targets reducing affinity. Most of these mechanisms are chromosomal, but some are plasmid-mediated. Following the discovery and introduction of sulphonamides and penicillin for treatment of gonorrhoea, resistance developed within decades. Since then, *N. gonorrhoeae* has displayed the capacity to develop resistance to all agents so far used for treatment. Resistance generally appears to emerge in the WHO Western Pacific Region and spread globally through travel and sex tourism (Unemo *et al.*, 2012a). With the current ease of global travel, antimicrobial resistance in *N. gonorrhoeae* knows no boundary.

1.6.1 Penicillin

Penicillin is bactericidal, inhibiting bacterial cell wall synthesis. By targeting penicillin-binding-proteins (PBPs), penicillin inhibits transpeptidase enzymes from cross-linking peptidoglycan in the cell wall, which leads to cell lysis. Penicillin resistance in *N. gonorrhoeae* may be chromosomal (altered binding site PBP2 and PBP1 encoded by *penA* and *ponA*, respectively), or plasmid mediated (TEM-1 or TEM-135 β -lactamases). Other chromosomal mechanisms of resistance include increased efflux (*mtrR* mutations altering the expression of the MtrCDE (multiple-transferable resistance efflux pump) or decreased influx (*porB1b* mutations resulting in altered porin PorB) of antimicrobials.

Beta-lactamases encoded by *bla*_{TEM-1}, *bla*_{TEM-135} and *bla*_{TEM-220} have been reported in *N. gonorrhoeae* (Endimiani *et al.*, 2014; Gianecini *et al.*, 2015; Tribuddharat *et al.*, 2016). These enzymes are classified as Ambler class A β -lactamases and their functional groups are 2b, 2be and unknown classification, respectively (Lahey Clinic; Ambler RP., 1980). Functional group 2b and 2be hydrolyse penicillins plus cephalosporins and penicillins, narrow-/extended-spectrum cephalosporins plus monobactams, respectively (Bush *et al.*, 1995). These plasmid-mediated β -lactamase enzymes hydrolyse the cyclic amide bond of β -lactamase-susceptible β -lactam antibiotics, opening the β -lactam ring, thereby inactivating the agent.

1.6.2. Extended Spectrum Cephalosporins (ESCs)

ESCs also inhibit bacterial cell wall synthesis by binding to PBPs. Altered binding site (*penA*), increased efflux (*mtrR*) and decreased influx (*porB*) are also responsible for ESC resistance in *N. gonorrhoeae*. Mosaic *penA* alleles, with numerous amino acid substitutions, are particularly associated with resistance to cephalosporins, in particular specific alterations in *penA*. Three alterations initially thought to confer ESC resistance include G545S, I312M and V316T. In 2006 Takahata *et al.* reported that G545S alterations increase cephalosporin MICs 2-4 fold and I312M plus G545S or V316T plus G545S confer an 8-fold increase in cefixime MIC (Takahata *et al.*, 2006). Later it was shown that these alterations alone do not account for the increase in ESC MIC, but that other mutations in *penA* are also required and of the three, G545S is the most important in conferring resistance (Tomberg *et al.*, 2010). Among N512Y, A510V and F504L mutations, N512Y was found to be the most important in relation to ESC resistance in reversion to wild-type experiments (Tomberg *et al.*, 2010). A501V and F504L were

found in a non-mosaic high-level penicillin-resistant strain which is ESC-susceptible, *penA* IV (Tomberg *et al.*, 2010).

Alterations at A501 associated with decreased ESC susceptibility, especially ceftriaxone (Unemo *et al.*, 2012a) include A501V/T alterations in *penA* which were initially found in non-mosaic types and thereafter in mosaic types. Among 15 CRO-R isolates (from 2007, Nanjing, China) Chen *et al.* looked for specific resistance determinants. Six different *penA* alleles were found, all non-mosaic, but they found that A501 substitutions were present in 13/15, A501T (n = 7) and A501V (n = 6); these were associated with reduced susceptibility to ceftriaxone (Chen *et al.*, 2014). G542S and P551S substitutions were detected in 13 and 2 out of the 15 isolates in that study, respectively. Mean ceftriaxone MICs were higher for isolates with A501T or G542S vs wild-type *penA*, H105Y vs wild-type *mtrR*, A102D v wild-type *penB* (*porB*).

Shimuta *et al.* examined 193 isolates in Japan (2010-2012) (Shimuta *et al.*, 2013). Nineteen had a CRO MIC > 0.064 mg/L. Those with CRO MIC = 0.125 mg/L were all either mosaic XXXIV_P551S or XXXIV_A501V, i.e. they had additional alterations. Transformation experiments revealed that mosaic XXXIV_P551S or XXXIV_A501V were found to have similar effects as mosaic X, i.e. CRO/CFM MICs increased from 0.016/0.016 mg/L to 0.125/0.25 mg/L after transformation, whereas transformation with mosaic XXXIV *penA* increased MICs to only 0.0654/0.125 mg/L (Shimuta *et al.*, 2013). Similarly, additional alterations to mosaic XXXIV *penA* have resulted in high-level ESC resistance, e.g. XXXIV_A501P, now known as CI *penA* allele, in F89 (discussed further below) (Unemo *et al.*, 2011c).

The earliest CFM-R (mosaic X *penA*) and CFM-RS (non-mosaic XIII_A501V_P551S *penA*) isolates appear to have originated in Japan in 1995 and 1997, respectively, both

belonging to MLST ST 7363, discussed further (*Section 4.7.1.2*) . The first CFM-RS *N. gonorrhoeae* isolate with a mosaic XXXIV *penA* appears to have originated in Japan in 2001 (Shimuta *et al.*, 2015). The most prevalent MLST ST in Japan changed from ST7363 in 1998-2002 to ST1901 in 2003-2005. Shimuta *et al.* hypothesise that the change in the CFM-R/RS isolates from mosaic X *penA* (ST7363) to the mosaic XXXIV *penA* (ST1901) occurred by acquisition of resistance determinants, i.e. that mosaic XXXIV evolved from mosaic X *penA* (Shimuta *et al.* 2015). Bharat *et al.* report that mosaic X, XXXIV and XXXIV_E538G (mutation not seen in this study) increase MICs of cefixime by 27 to 53-fold and ceftriaxone by 16 to 21-fold (Bharat *et al.*, 2015).

1.6.3 Azithromycin

Azithromycin is a macrolide antibiotic which acts by binding the 50S subunit of the bacterial ribosome and thereby inhibits bacterial protein synthesis (Kanatani & Guglielmo 1994). Macrolide resistance in *N. gonorrhoeae* is due to mutations in the binding site of the 23s rRNA (particularly at specific bases A2059 and C2611 in *E. coli* numbering), L4 and L22 ribosomal protein alterations (encoded by *rplD* and *rplV*, respectively) and by post-transcriptional methylation of the 23s rRNA by methylases (encoded by *erm* genes), all leading to inhibition of protein synthesis (Gomes *et al.*, 2016). Efflux pump overexpression, especially MtrCDE and also MacAB (encoded by *mef* genes) are also important contributors to macrolide resistance. Esterases and phosphotransferases (encoded by *ere* and *mph* genes, respectively) are responsible for altering the structure of the macrolide, thereby inducing resistance (Gomes *et al.*, 2016). In the case of high-level azithromycin resistance (defined as minimum inhibitory concentration [MIC] > 256 mg/L), an A2059G mutation in the 23S rRNA gene is present (Chisholm *et al.*, 2010; Lynagh *et al.*, 2015; Unemo *et al.*, 2014; Galarza *et al.*, 2010; Allen *et al.*, 2014; Demczuk *et al.*, 2016).

1.6.4 Fluoroquinolones

Fluoroquinolones inhibit bacterial DNA gyrase and topoisomerase IV, both type-II topoisomerases, which are responsible for unlinking and relieving strain on the double stranded DNA as helicase unwinds it. Ciprofloxacin-resistance in *N. gonorrhoeae* may be due to mutations encoded by *gyrA* or *gyrB* (reduced ciprofloxacin affinity to DNA gyrase) or *parC* genes (topoisomerase IV).

NorM is an efflux pump localised only in the cytoplasmic membrane. It is a member of the multidrug and toxic compound extrusion (MATE) family of efflux systems and is reported to contribute to fluoroquinolone resistance in *N. gonorrhoeae* (Eyre *et al.*, 2017; Poole *et al.*, 2005)

1.6.5 Tetracyclines

Tetracyclines inhibit protein synthesis by binding to the 30S of the bacterial ribosome and preventing attachment of the aminoacyl-tRNA to the ribosome, blocking new amino acids from attaching. In tetracyclines, resistance may be plasmid-mediated by *tetM*, but this class is also affected by *porB1b* and *mtrR*. The tetM protein blocks the tetracycline target site on the 30S ribosomal subunit.

1.6.6 Other agents

Alternate agents which may be suitable for use in combination therapies include ertapenem, gentamicin, fosfomycin, spectinomycin and gemifloxacin, are discussed later (*Section 1.14*).

1.7 Risk Factors for Antimicrobial-Resistant *Neisseria gonorrhoeae* Infection

Certain characteristics are reported to be associated with antimicrobial resistance (AMR) in *N. gonorrhoeae*. In Europe cefixime resistance has been reported to be significantly associated with male and female heterosexuals and age > 25 years while isolates with a ciprofloxacin MIC > 0.5 mg/L is associated with male heterosexuals and age > 25 years (Cole *et al.*, 2014b). In the US, isolates of *N. gonorrhoeae* resistant to penicillin, tetracycline and ciprofloxacin were associated with male heterosexuals who had recently travelled (Goldstein *et al.* 2014). In Shanghai, older age and male gender were found to be associated with higher ceftriaxone MICs (Trecker *et al.*, 2014). Finally, in England and Wales an association between a ceftriaxone MIC > 0.015 mg/L and heterosexuals > 35 years who had a rapid partner turnover and recent sex abroad was found, while in men who have sex with men (MSM) the association was with a previous history of gonorrhoea and also asymptomatic infection (Town *et al.* 2017). Multiple exposures or infections with *N. gonorrhoeae* and importation of resistance may be important factors associated with AMR. The first reported case of gonorrhoea caused by penicillinase-producing *N. gonorrhoeae* (PPNG) was in Bristol in 1976 in a young male returned from South East Asia with urethral discharge who failed to respond to penicillin treatment (Ashford *et al.*, 1976). At the same time a case of PID in a female patient caused by PPNG was reported from London (Phillips, 1976).

1.8 Antimicrobial resistance and multidrug-resistance in *N. gonorrhoeae*

The currently recommended first-line treatment of gonorrhoea is being threatened by emergence of resistance in ceftriaxone and azithromycin. Isolates of *N. gonorrhoeae* resistant or showing reduced susceptibility to ceftriaxone ($\text{MIC} \geq 0.125 \text{ mg/L}$) and cefixime ($\text{MIC} \geq 0.125 \text{ mg/L}$) have been reported worldwide (Li *et al.*, 2014; Cole *et al.*, 2015; Martin *et al.*, 2012). Resistance to azithromycin ($\text{MIC} \geq 1 \text{ mg/L}$) is not uncommon (Cole *et al.* 2014a), but worryingly, high level resistance (azithromycin $\text{MIC} > 256 \text{ mg/L}$) has been reported in a number of countries, including Ireland (Chisholm *et al.*, 2010; Lynagh *et al.*, 2015; Unemo *et al.*, 2014; Galarza *et al.*, 2010; Allen *et al.*, 2014; Demczuk *et al.*, 2016).

1.8.1 ESC resistance (cefixime and ceftriaxone)

The first ever isolates of *N. gonorrhoeae* displaying cefixime-resistance ($\text{MIC} \geq 0.25 \text{ mg/L}$) cefixime-reduced susceptibility (CFM-RS) ($\text{MIC} = 0.125 \text{ mg/L}$) were detected in Japan in 1995 (Shimuta *et al.*, 2015). Shimuta *et al.* reviewed 690 isolates in Japan from 1995 to 2005 and found a peak cefixime resistance of 57.1% in 2002 (Shimuta *et al.*, 2015). They also found the first ceftriaxone-reduced susceptibility (CRO-RS) ($\text{MIC} = 0.125 \text{ mg/L}$) isolate in 2000 with a peak prevalence of CRO-RS in 2003 of 29.2%. In 2011 the first isolate of *N. gonorrhoeae* with high-level ceftriaxone resistance ($\text{MIC} = 2 \text{ mg/L}$) was reported, named H041, isolated from a female commercial sex worker in Kyoto in 2009 (Onishi *et al.*, 2011b). *N. gonorrhoeae* had now achieved ‘Superbug’ status. This prompted a later study by Shimuta *et al.* looking specifically at the Osaka and Kyoto areas in Japan in order to perform enhanced surveillance (Shimuta *et al.*, 2013). One-hundred and ninety-three isolates collected from five clinics in the two

areas were tested. Forty-eight (24.9%) were found to be cefixime-resistant (MIC > 0.125 mg/L) and for ceftriaxone, the MIC₉₀ was determined to be 0.094 mg/L, with no resistance being detected. The same year as the news of H041 from Japan was reported, F89 was reported from Quimper in France (Unemo *et al.*, 2011c). F89 was isolated from a urethral sample taken as test of cure following 200 mg PO x2 (taken 6 hours) in a male patient (MSM) in 2010. The cefixime and ceftriaxone MICs were determined to be 4 mg/L and 2 mg/L, respectively. In 2012, a year later, two high level cefixime- and ceftriaxone-resistant isolates were reported by Carnicer-pont *et al.* (Carnicer-pont *et al.*, 2012). These isolates from Spain (2011) came from two male partners (one urethral and one rectal) and had MICs of 1.5 mg/L for both cefixime and ceftriaxone. (Carnicer-pont *et al.*, 2012) This report also highlighted transmission of a strain with high-level ceftriaxone resistance between male partners.

In Australia a urogenital isolate from a young European female traveller was isolated in 2014 with a ceftriaxone MIC = 0.5 mg/L (A8806) (Lahra *et al.*, 2014) and in 2016 two ceftriaxone resistant isolates were reported from Japan. The first isolate GU140106 was cultured from a male patient who had received fellatio without a condom from a female sex worker in 2014. The urethral isolate had a ceftriaxone MIC = 0.5 mg/L (Deguchi *et al.*, 2016). The second, FC428, cultured from the urethra of a male patient, had a ceftriaxone MIC of 0.5 mg/L (Nakayama *et al.*, 2016). The ESC MICs and resistance determinants of these high-level ceftriaxone-resistant isolates are summarised (Table 1.2).

Molecular characteristics of high-level ceftriaxone-resistant <i>N. gonorrhoeae</i>											
Isolate (Year reported)	NG-MAST	MLST	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	<i>penA</i>	<i>penA</i> alterations	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
H041 (Japan 2011) Ohnishi et al., 2011b	4220	7363	4	8	8	mosaic C	A311V T316P A328T T484S	+	+	+	+
F89 (France 2011) Unemo et al., 2011c	1407	1901	2	4	4	mosaic CI	mosaic XXXIV_A501P	L421P	G101K A102N	ND	A13 del
Spanish (2012) Camara et al., 2012	1407	1901	1.5	1.5	1	mosaic XXXIV_A501P	A501P	L421P	G120K A121N	ND	A13 del
A8806 (Australia 2013) Lahra et al., 2014	4015	7363	0.5	ND	ND	mosaic	A311V T843S	ND	ND	ND	ND
GU140106 (Japan 2014) Deguchi et al., 2016	6543	7363	0.5	2	ND	mosaic	A311V T843S	ND	ND	ND	ND
FC428 (Japan 2015) Nakayama et al., 2016	3435	1903	0.5	1	ND	mosaic <i>penA</i> _{FC428}	A311V T843S	ND	G120K A121D	WT	A del

Table 1.2. Molecular characteristics of high-level ESC-resistant gonococci reported from Japan

(H041), France (F89) and Spain as well as CRO-R strains from Japan (GU140106 and FC428) and Australia (A8806). *ponA*, *penB* and *mtrR* resistance determinants are described as being present (+) for H041. Wild-type (WT), not described (ND).

1.8.2 Azithromycin resistance

HL-AziR *N. gonorrhoeae* was first reported in isolates from Scotland in 2004 (Young & Palmer, HPS weekly). Since then HL-AziR strains have been reported from a number of countries (Table 1.3). All of these isolates were susceptible to ceftriaxone (where tested), but many displayed resistance to penicillin, tetracycline and ciprofloxacin (Table 1.4).

An outbreak of ‘Super-gonorrhoea’ was reported in the UK by multiple news outlets in September 2015, following identification of 8 cases of high-level azithromycin-resistant *N. gonorrhoeae* in Northern England (Chisholm *et al.*, 2015). By April 2016 this outbreak had increased to 34 cases and by September to 48 cases (HPWR April 2016; HPWR Sept 2016). Public Health England (PHE) stated that contact tracing had been of limited success in controlling the outbreak and that WGS analysis of a subset of isolates suggested recent transmission events (HPWR April 2016). The same year, Fifer *et al.* reported the first failure of dual therapy for gonorrhoea (Fifer *et al.*, 2016). The isolate, following treatment failure, was resistant to ESCs (ceftriaxone [MIC 0.25µg/ml], cefotaxime, cefixime), azithromycin [MIC 1µg/ml], tetracycline, ciprofloxacin and penicillin.

Increasing resistance to the first line agents for treatment will undoubtedly mean more reports of treatment failures, which is of immense public health concern. In addition, high level resistance to azithromycin may diminish the usefulness of dual antimicrobial therapy and allow resistance to ESCs to develop more easily. As mentioned above, high level azithromycin resistance is present in Ireland already (Lynagh *et al.*, 2015), but the extent that these resistant strains are circulating is still unknown.

International reports of HLAzi-R isolates						
Country of origin	Number HLAziR isolates	Year (s) isolate(s) cultured	Author	Year published	Journal	Other susceptibilities
Scotland	2	2004	Young <i>et al.</i>	2005	HPS weekly	Not described
England and Wales	6	2007	Chisholm <i>et al.</i>	2009	JAC	All susceptible to Pen/Cip/SPC/CRO/CFM and resistant to Tet.
Italy	5	2001 & 2003	Starnino <i>et al.</i>	2009	JAC	One resistant to Pen/CipTet. One resistant to Cip/Tet
Argentina	2	2001 & 2003	Galarza <i>et al.</i>	2009	Sex Trans Dis	Not described
USA	1	2011	Katz <i>et al.</i>	2012	CID	Susceptible to CFM/CRO and resistant to Tet.
Canada	2	2010	Allen <i>et al.</i>	2014	AAC	One resistant to Ery/Pen/Tet/Cip. One resistant to Ery/Cip, intermediate to Pen/Tet. Both susceptible to CRO/SPC.
Sweden	3	2011 & 2012	Unemo <i>et al.</i>	2014	AAC	All susceptible to CRO/CFM/SPC and resistant to CIP.
Ireland	2	2008 & 2014	Lynagh <i>et al.</i>	2015	JAC	Both susceptible to CRO/CIP/SPC.
Australia	6	2011 & 2013	Stevens <i>et al.</i>	2015	JAC	All susceptible to CRO. Four resistant to Cip.
USA	1	2014	Gose <i>et al.</i>	2015	STD	Susceptible to Cip/CFM/CRO/SPC. Intermediate to Pen/Tet

Table 1.3. Summary of reports of HLAzi-R in *N. gonorrhoeae*. (Pen: penicillin. Cip: ciprofloxacin. SPC: spectinomycin. CRO: ceftriaxone. CFM: cefixime. Tet: tetracycline)

International HLAzi-R isolates: resistance determinants										
Country	Year	NG-MAST	AZM MIC (mg/L)	<i>mtrR</i>	<i>mtrR</i> promoter	L4 (<i>rplV</i>)	L22 (<i>rplD</i>)	23s rRNA (number of mutant alleles)	Other resistance determinants	Reference
Argentina	2001	649	> 256	G45K	WT	ND	ND	A2059G (4/4)	ND	Galarza <i>et al.</i> , 2009 Galarza <i>et al.</i> , 2010
USA	2011	649	> 512	WT	WT	ND	ND	A2059G (4/4)	<i>erm</i> A/B/C and <i>mefA</i> not detected	Katz <i>et al.</i> 2012
Canada	2010	4980	> 2058	G45D	A deletion	WT	WT	A2059G (4/4)	ND	Allen <i>et al.</i> , 2014
	2010	5343	> 2058	WT	A deletion	WT	WT	A2059G (4/4)	ND	
Sweden	2011	285	4096	<i>mtrR</i> resistance determinant detected in all three. No details given. ND		ND	ND	A2059G (3/4)	ND	Unemo <i>et al.</i> , 2014
	2012 (n = 2)	8727 8727	4096 (n = 2)			ND	ND	A2059G (4/4)	ND	

Ireland	2008	3311	> 256	WT	WT	ND	ND	A2059G (4/4)	<i>erm</i> A/B/C/f, <i>ere</i> A/B. <i>mef</i> A/E and <i>mphA</i> not detected	Lynagh <i>et al.</i> , 2015
	2014	649	> 256	WT	WT	ND	ND	A2059G (4/4)	<i>erm</i> A/B/C/f, <i>ere</i> A/B. <i>mef</i> A/E and <i>mphA</i> not detected	
Australia	2011 (n = 2)	5309 10572	> 256 (n = 2)	ND	A deletion A deletion	ND	ND	A2059G (4/4)	ND	Stevens <i>et al.</i> , 2015
	2013 (n = 4)	8917 649 649 10133	> 256 (n = 4)	ND	Adeletion WT WT A deletion	ND	ND	A2059G (4/4)	ND	
Canada	2004- 2012 (n = 5)	ND	> 256 (n = 5)	G45D	A deletion	WT	WT	A2059G (4/4)	ND	Demczuk <i>et al.</i> , 2016
Europe (Ireland n = 2, Italy, Sweden)	2011 2012 2011 2012	649 649 8527 4980	> 256 (n = 4)	G45D (n = 2)	A deletion (n = 2)	WT	WT	A2059G (4/4)	WT	Jacobsson <i>et al.</i> , 2016
USA (California)	2014	10844	> 256	G45D	ND	ND	ND	A2059G (4/4)	<i>erm</i> A/B/C and <i>mefA</i> not detected	Gose <i>et al.</i> , 2015

Table 1.4. Summary of resistance determinants in some internationally reported HLazi-R isolates. ND = not described

1.9 Treatment failures

1.9.1 ESC treatment failures (cefixime and ceftriaxone)

Both cefixime and ceftriaxone treatment failures have been reported. Verified and suspected cefixime treatment failures of pharyngeal, rectal and urethral gonorrhoea have been reported from numerous countries. While cefixime treatment failures have occurred frequently in cases where isolates were shown to have MICs at or above the EUCAST breakpoint for susceptibility (≥ 0.125 mg/L), many cases also had MICs in the susceptible range. Singh *et al.* reviewed cases from 4 STI clinics in Canada over almost 4 years (2010-2013) and reported 14 treatment failures with cefixime monotherapy (400 mg in 13 cases and 800 mg in one case) (Singh *et al.*, 2015). Nine of these cases were pharyngeal, 4 rectal and one urethral gonorrhoea. The MICs of the pre-treatment isolates showed MICs ranging from 0.008 to 0.125 mg/L, but the majority of the isolates (13/14) had an MIC of ≤ 0.032 mg/L with only one having an MIC of 0.125 mg/L (Singh *et al.*, 2015). Another retrospective review of cases of cefixime treatment failure of gonorrhoea was carried out in Toronto over 1 year (2010-2011) (Allen *et al.*, 2013). In this review, 9 treatment failures were identified (2 pharyngeal, 3 rectal and 4 urethral) out of 133 cases who returned for test of cure (TOC). They reported treatment failures in 25% (7/28) if the cefixime MIC was ≥ 0.125 mg/L and only 1.9% (2/105) if the cefixime MIC was < 0.125 mg/L (Allen *et al.*, 2013). The numbers of treatment failures are low, but show that elevated MICs are an obvious important factor. Site of infection in that cohort was also felt to be a risk factor given that treatment failure occurred in 28.6% (2/7), 5.26% (4/76) and 7.69% (3/39) of pharyngeal, urethral and rectal cases, respectively, who returned for test of cure. Also highlighting the importance of MIC value in treatment failures is a study in which a double dose regime of 200 mg of cefixime taken 6 hours apart for gonococcal urethritis

was reviewed (Deguchi *et al.*, 2003). Of 98 patients treated, 61 were evaluated for microbiological outcome. Of those, forty-five had an isolate of *N. gonorrhoeae* with a cefixime MIC of 0.06 mg/L and all of these were successfully eradicated. Eight of the remaining 16 cases had an isolate with a cefixime MIC of ≥ 0.125 mg/L and all of these had persistence of *N. gonorrhoeae* after the double-dose regime.

Individual cases of cefixime treatment failures in gonorrhoea are summarised (Table 1.5). It is important to note that these cases occurred during the time when oral cefixime 400 mg was the first line recommended empiric treatment for gonorrhoea. In the 2010 EUCAST clinical breakpoints (version 1.0) document clinical breakpoints for susceptibility to cefixime, cefotaxime and ceftriaxone was ≤ 0.12 mg/L, as it remains today. CLSI had no clinical breakpoints for gonorrhoea and cefixime/ceftriaxone, but stated that an MIC ≥ 0.5 mg/L implied reduced susceptibility (CLSI 2010). Current CLSI interpretive categories and breakpoints for cefixime and ceftriaxone are susceptible ≤ 0.25 mg/L and for cefotaxime, a higher breakpoint of ≤ 0.5 mg/L (CLSI 2017). The cases of cefixime treatment failures for which a pre-treatment MIC was available were all above this susceptible breakpoint, and for those without a pre-treatment MIC, the post-treatment MICs were elevated above 0.125 mg/L.

In August 2012, the CDC updated their 2010 sexually transmitted diseases treatment guidelines, no longer recommending oral cefixime as first-line therapy for gonorrhoea (MMWR August 2010). This change was prompted by the cases described here as well as increasing cefixime MICs across the USA from 2006 to 2011 (MMWR 2010). In the UK in 2011, the national guidelines for the treatment of gonorrhoea in adults (BASHH 2011) were updated to ceftriaxone 500 mg IM and azithromycin 1g PO first-line. Ceftriaxone had now superseded cefixime as the first-line recommended therapy and it was to be given in combination with oral azithromycin.

Cefixime Treatment Failures									
Number of cases	Isolate(s) dated from	Site of infection	Pre-treatment MICs	Post-treatment MICs	Treatment received	Treatment resulting in cure	Verified/suspected	Reference and Country	Comment
2	2010	Urethral	Case 1: CFM 0.19 mg/L CRO 0.064 mg/L AZM 0.25 mg/L	Case 1: CFM 0.19 mg/L CRO 0.047 mg/L AZM 1 mg/L	Case 1: CFM 400 mg PO then AZM 2 g PO on two occasions	CRO 250 mg IM	Case 1: Verified	Ison <i>et al.</i> , 2011 UK	Also exhibits verified AZM treatment failure
			Case 2: Not available	Case 2: CFM 0.25 mg/L CRO 0.064 mg/L	Case 2: CFM 400 mg PO + Doxy 100 mg BD x7d	CRO 250 mg IM	Case 2: Suspected		
2	2010	Urethral	Case 1: CFM 0.5 mg/L CRO 0.125 mg/L	Case 1: CFM 0.5 mg/L CRO 0.125 mg/L	CFM 400 mg PO	CRO 500 mg IM	Case 1: Verified	Unemo <i>et al.</i> , 2010 Norway	
			Case 2: CFM 0.25 mg/L CRO 0.125 mg/L	Case 2: CFM 0.5 mg/L CRO 0.125 mg/L	CFM 400 mg PO	CRO 500 mg IM	Case 2: Verified		
1	2011	Urethral	Not available	CFM 4 mg/L CRO 1-2 mg/L	CFM 200 mg PO x2 doses six hours apart	Gentamicin 160 mg IM	Suspected	Unemo <i>et al.</i> , 2011c France	F89 isolate from France with high level CFM and CRO resistance
1	2011	Urethral	Not available	CFM 1.0 mg/L CRO 0.5 mg/L	CFM 400 mg OD x7 days followed by CFM 400 mg OD for 14 days	AZM 2 g PO	Suspected	Unemo <i>et al.</i> , 2011b Austria	
1	2011	Urethral	Not available	CFM $\geq$ 0.25 mg/L CRO $\geq$ 0.125 mg/L AZM $\leq$ 1 mg/L	CFM 400 mg Po + AZM 1 g PO	CRO 500 mg IM	Suspected	Forsyth <i>et al.</i> , 2011 UK	
1	2012	Urethral	Not available	0.25 mg/L	Cefixime 400 mg PO on two occasions	Lost to follow up	Suspected	Lewis <i>et al.</i> , 2013 S. Africa	

Table 1.5. Summary of reported cases of cefixime treatment failures in gonorrhoea. CRO: ceftriaxone, CFM: cefixime, AZM: azithromycin.

Molecular characteristics of strains associated with cefixime treatment failures										
Reference Origin Year of isolate	Site	Pre-treatment CFM MIC (mg/L)	NG- MAST	MLST	<i>penA</i>	Verified	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
Ison <i>et al.</i> , 2011 UK 2010	Urethral	0.19 mg/L	3779	ND	mosaic	+	ND	ND	ND	ND
2010	Urethral	Not available	3431	ND	mosaic	-	ND	ND	ND	ND
Unemo <i>et al.</i> , 2010 Norway 2010	Urethral	0.5 mg/L	1407	ND	mosaic	+	ND	G120K A121N	ND	A del
2010	Urethral	0.25 mg/L	1407	ND	mosaic	+	ND	G120K A121N	ND	A del
Unemo <i>et al.</i> , 2011c France (F89) 2011	Urethral	Not available	1407	1901	mosaic XXXIV_ A501P (mosaic CI)	-	L421P	G101K A102N	ND	A13 del
Unemo <i>et al.</i> , 2011b Austria 2011	Urethral	Not available	1407	1901	mosaic novel	-	L421P	G120K A121N	ND	A del
Lewis <i>et al.</i> , 2013 South Africa 2012	Urethral	Not available	4822	1901	mosaic XXXIV	-	L421P	G101K A102N	ND	A del

Table 1.6. Molecular characteristics of *N. gonorrhoeae* strains responsible for cefixime treatment failures.

Ceftriaxone has also been associated with treatment failures. A number of cases have been published (Tables 1.7 & 1.8), showing pre-treatment MICs to ceftriaxone (where available) of 0.016-0.125 mg/L. All of these are cases of pharyngeal gonorrhoea, with the most recent being a suspected failure of ceftriaxone and azithromycin dual therapy (not verified) in 2016 in the UK (Fifer *et al.* 2016). Ceftriaxone is the last remaining antibiotic suitable for empiric monotherapy of gonorrhoea and as mentioned above, it remains part of the first-line empiric treatment in combination with azithromycin, with some organisations recommending a higher dose of 500 mg IM (BASHH 2011; HPSC 2017a; Bignell *et al.*, 2012) compared to 250 mg (CDC 2015; WHO 2016).

Ceftriaxone Treatment Failures									
Number of cases	Isolate(s) dated from	Site of infection	Pre-treatment MICs	Post-treatment MICs	Treatment received	Treatment resulting in cure	Verified/suspected	Reference and Country	Comment
2	2009	Pharyngeal	Case 1: CRO 0.03 mg/L	Case 1: CRO 0.03 mg/L	Case 1: D1: CRO 250 mg IM D17:AZM 1 g D24: CRO 500 mg IM	No final TOC detailed	Case 1: Suspected	Tapsall <i>et al.</i> , 2009a Australia	Clearance of associated urethral infection
			Case 2: CRO 0.016 mg/L	Case 2: Not available	Case 2: D1: CRO 250 mg IM	CRO 1 g IM	Case 2: Suspected		
1	2010	Pharyngeal	D1- NA D12:CRO 0.125 mg/L D36:CRO 0.125 mg/L D50:CRO 0.25 mg/L		D1: amoxicillin D12: CRO 250 mg IM D36: CRO 500 mg IM D50:CRO 1 g IM	CRO 1 g IM	Verified	Unemo <i>et al.</i> , 2011b Sweden	
1	2011	Pharyngeal	CRO 0.125 mg/L	CRO 0.125 mg/L	D1:CRO 250 mg IM D8: Doxy 100 mg BD PO (for treatment of concomittant chlamydia)	D30:CRO 250 mg IM plus AZM 1 g Po	Verified	Unemo <i>et al.</i> , 2012c Slovenia	
2	2011	Pharyngeal	Case 1: CRO 0.03 mg/L	Case 1: CRO 0.03 mg/L	Case 1: CRO 500 mg IM	Case 1: CRO 1 g IM plus AZM 2 g PO	Case 1: Verified	Read <i>et al.</i> , 2013 Australia	Clearance of concomittant urethral and rectal gonorrhoea with initial 500 mg IM CRO

			Case 2: CRO 0.03 mg/L	Case 2: CRO 0.06 mg/L	Case 2: CRO 500 mg IM	Case 2: CRO 1 g IM	Case 2: Suspected		Strongly suspected as the pre- and post-treatment isolates were genetically very closely related.
1	2010	Pharyngeal	CRO 0.03-0.06 mg/L	CRO 0.03-0.06 mg/L	D1:CRO 500 mg IM	AZM 2 g PO	Suspected	Chen <i>et al.</i> , 2013 Australia	
3	2013	Pharyngeal	Case 1: CRO 0.125 mg/L	Case 1: CRO 0.125 mg/L	Case 1: CRO 500 mg IM	Case 1: CRO 1 g IM	Case 1: Verified	Golparian <i>et al.</i> , 2014 Sweden	Reported also is a case of CTX treatment failures.
			Case 2: CRO 0.064 mg/L	Case 2: CRO 0.125 mg/L	Case 2: CRO 500 mg IM	Case 2: CRO 1 g IM	Case 2: Verified		
			Case 3: CRO 0.064 mg/L	Case 3: CRO 0.064 mg/L	Case 3: CRO 500 mg IM	Case 3 CRO 1 g IM:	Case 3: Verified		
1	2016	Urethral & pharyngeal	Not available	CRO 0.25 mg/L AZM 1 mg/L	CRO 500 mg IM plus AZM 1 g Po	CRO 1 g IM plus AZM 2 g Po	Suspected	Fife <i>et al.</i> , 2016 UK	Only case of suspected CRO failure to treat urethral gonorrhoea

Table 1.7. Summary of reported cases of ceftriaxone treatment failures in gonorrhoea. CRO: ceftriaxone, CFM: cefixime, AZM: azithromycin, CTX: cefotaxime.

Molecular characteristics of strains associated with ceftriaxone treatment failures										
Reference Origin Year of isolate	Site	Pre-treatment CRO MIC (mg/L)	NG- MAST	MLST	<i>penA</i>	Verified	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
Chen <i>et al.</i> , 2013 Australia 2010	Pharyngeal	0.03-0.06 mg/L	1407	ND	ND	-	ND	G120K A102N	ND	ND
Fifer <i>et al.</i> , 2016 UK 2016	Urethral Pharyngeal	Not available	12133	1901	mosaic X	-	ND	+	ND	A del
Golparian <i>et al.</i> , 2014 Sweden 2013	Pharyngeal	0.125 mg/L	4706	ND	mosaic XXXIV	+	ND	ND	ND	ND
		0.064 mg/L	3149	ND	mosaic XXXIV	+	ND	ND	ND	ND
		0.064 mg/L	3149	ND	mosaic XXXIV	+	ND	ND	ND	ND
Read <i>et al.</i> , 2013 Australia 2011	Pharyngeal	0.03 mg/L	225	1901	XII	+	ND	ND	ND	ND
Unemo <i>et al.</i> , 2011b Sweden 2010	Pharyngeal	0.125 mg/L	1407	1901	mosaic XXXIV	+	L421P	G120K A102N	ND	A del
Unemo <i>et al.</i> , 2012c Slovenia 2011	Pharyngeal	0.125 mg/L	1407	1901	mosaic XXXIV	+	ND	+	+	+

Table 1.8. Molecular characteristics of *N. gonorrhoeae* strains responsible for ceftriaxone treatment failures.

1.9.2 ESC treatment failures (cefotaxime)

Two verified treatment failures with cefotaxime have been reported in 2014, one from Sweden and one from Amsterdam. The patient in Sweden was treated with empirically with cefotaxime 500 mg IM for pharyngeal gonorrhoea, but remained culture positive 7 days later. He then received ceftriaxone 250 mg IM and azithromycin 1g PO and had a negative test of cure on day 26. Ceftriaxone/cefotaxime MICs pre- and post-treatment were 0.25/0.5 mg/L and 0.125/0.5 mg/L, respectively, i.e. resistance to cefotaxime and resistant/reduced susceptibility to ceftriaxone (Golparian *et al.*, 2014). The patient in Amsterdam was treated with cefotaxime 1g IM for urethral gonorrhoea and remained symptomatic and culture positive 6 days later. Clinical cure was achieved with ceftriaxone 500 mg IM. The pre- and post-treatment isolates had cefotaxime/ceftriaxone MICs of 0.25/0.064 mg/L, again resistant to cefotaxime and reduced-susceptibility to ceftriaxone. (van Dam *et al.*, 2014)

1.9.3 Azithromycin treatment failures

Azithromycin monotherapy has been associated with treatment failures in gonorrhoea infection. Verified treatment failures require a detailed clinical history excluding reinfection. Additionally, knowledge of the treatment regimens (including dosages) used, as well as MICs and discriminative genotyping of the isolates, prior to and following treatment, are required. (Tapsall *et al.*, 2009c). There are published reports of treatment failures in urogenital gonorrhoea associated with low azithromycin MICs i.e. two cases of verified treatment failure following 2g azithromycin orally for urogenital gonorrhoea (MICs pre-treatment of 0.25 mg/L and 1mg/L) (Ison *et al.*, 2011; Soge *et al.*, 2012). Similarly, a case of verified azithromycin treatment failure in a case of

concomitant urogenital and pharyngeal gonorrhoea infection following 2g azithromycin has been reported from Japan, with both pre-treatment isolates displaying slightly higher MICs of 4 mg/L (Morita-Ishihara *et al.*, 2014). There are also cases of treatment failures following 1g azithromycin for urogenital gonorrhoea associated with isolates displaying MICs to azithromycin of 0.125-0.25 mg/L (Tapsall *et al.*, 1998). More recently a case of failure of dual therapy of 1 g azithromycin and 500 mg ceftriaxone for pharyngeal gonorrhoea (with no pre-treatment isolate available for MIC determination) has been reported (Fifer *et al.*, 2016). The correlation between azithromycin MICs and treatment success is particularly unclear around the 0.5-2 mg/L range; hence the reluctance of CLSI to provide clinical breakpoints for *N. gonorrhoeae*. This drug should still be used in dual therapy for its roles in treatment of *N. gonorrhoeae* as well as its protective advantage for the accompanying ESC.

Resistance to and ease of development of resistance to azithromycin, along with documented treatment failures discussed above, means it is no longer suitable for use as empiric monotherapy and must be used in combination therapy with an ESC. The WHO recommends that a specific antimicrobial should achieve a cure rate of $\geq 95\%$ for it to be a suitable and effective option for empiric treatment of gonorrhoea (WHO 2016) and no longer recommend azithromycin as a suitable option for single therapy. They also recommend using local resistance data in determining the choice of agents in dual or single therapy, and considering the azithromycin-resistance found in Ireland in this study, azithromycin should not be used as empiric monotherapy for gonorrhoea. Irish national guidelines for prevention and control of gonorrhoea and for minimising the impact of antimicrobial resistance in *Neisseria gonorrhoeae* were published in 2017 (HPSC 2017a). These guidelines only recommend single agent azithromycin if the

isolate is confirmed susceptible, for patients allergic to cephalosporins and at a higher dose of 2 g. Test of cure is particularly important in these cases.

1.10 Laboratory diagnosis and antimicrobial susceptibility testing for *Neisseria gonorrhoeae*

Laboratory diagnosis has traditionally been isolation of *Neisseria gonorrhoeae* by culture on chocolate or selective media. Nucleic acid amplification tests (NAATs) are more sensitive than culture for the detection of gonorrhoea at both genital and extra genital sites and they are standard in many developed countries now (MMWR 2014). Without culture, susceptibility testing is not possible. Newer molecular techniques may make it possible for molecular identification of both the organism and resistance determinants without the need for culture, but this will only detect known resistance mechanisms and so culture must not be disregarded. Culture should be continued to allow surveillance of antimicrobial resistance as well as investigation of evolving resistance mechanisms for which this organism is infamous.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommend susceptibility testing by gradient minimum inhibitory concentration (MIC) methods (EUCAST 2017). Clinical breakpoints have been set for ceftriaxone, ciprofloxacin, penicillin, azithromycin, tetracycline, cefixime, cefotaxime and spectinomycin. They make no recommendations on zone diameters for disc diffusion.

1.11 Laboratory investigation of resistance mechanisms

1.11.2 Phenotypic

Beta-lactamase detection by cefinase (nitrocefin) disc test or by Hodge plate may be performed, but there are no other phenotypic tests in use for detection of resistance mechanisms (Hodge *et al.*, 1978; Ng & Martin, 2005). These tests identify isolates producing β -lactamase, which confers penicillin resistance even if the MIC is in the susceptible or intermediate range.

1.11.3 Molecular

While molecular methods are in use for detection of *N. gonorrhoeae*, as mentioned above, routine use of molecular techniques for detection of resistance determinants are not currently available. Known resistant determinant may easily be sought using end-point PCR specifically looking for the specific genes or mutations. There are many publications recently reporting assays for detection of *N. gonorrhoeae* plus genotypic detection of specific resistance determinants from samples, e.g. a *gyrA* (mutations at codon S91) genotypic assay was assessed in Los Angeles (hospital and primary care level) (Allan-Blits *et al.*, 2017). They successfully genotyped 121/176 (68.8%) of samples finding approximately 60% (72/121) were wild-type *gyrA* and the rest were mutant (approximately 40%, 49/121). Fifty-five could not be genotyped. Of 62 pharyngeal infections, 64.5% (n = 40) could not be genotyped compared to 9/114 (1.9%) of infections from other sites. The reason for such poor performance of the assay on pharyngeal samples was not elucidated (Allan-Blits *et al.*, 2017). It was used on all clinical samples positive for gonorrhoea and allowed an increase in ciprofloxacin

use for those cases in which the resistance determinant was not found (Allan-Blitz *et al.*, 2017). The concept behind this may be something that could be incorporated into routine clinical microbiology laboratories, but would require further investigation and a much wider variety of resistance determinants to be targeted. As technology advances rapidly, whole genome sequencing (WGS), only recently seen as a research tool, is now becoming much more accessible with prices becoming more competitive and performing the sequencing becoming more user friendly. Recently WGS has been incorporated into routine work in two reference laboratories in Ireland (carbapenemase-producing *Enterobacteriaceae* reference laboratory (CPERL) and in the Public Health Laboratory (PHL) for verotoxigenic *E. coli* (VTEC)). It has proven useful to identify resistance genes in *N. gonorrhoeae* (e.g. *penA*, *mtrR*, *porB*, *pilQ*, and *ponA*) (Grad *et al.*, 2014). While predicting phenotypic susceptibility category is useful, reports are now emerging regarding using this data to predict MICs of *N. gonorrhoeae* (Eyre *et al.*, 2017). Once resistance determinants were identified from WGS, multivariate linear regression models were used to calculate MICs. The MICs were predicted to the nearest doubling dilution in 53% (n = 1785), within ± 1 doubling dilution in 93% (n = 3147) and within ± 2 doubling dilutions in 98% (3314) of 3380 isolates tested from England, USA and Canada (Eyre *et al.* 2017). Importantly, this study reported low rates of very major errors (1.3%; phenotypically resistant, but predicted as susceptible) and major errors (1.7%; phenotypically susceptible, but predicted as resistant) which met regulatory thresholds for VME rates (except cefixime) and ME rates (except for tetracycline) (Eyre *et al.*, 2017). MIC data is more useful to allow identification of trends and development of resistance over time (Eyre *et al.*, 2017). WGS may be employed in the future for determining known and unknown resistance mechanisms in *N. gonorrhoeae* as part of routine work in microbiology.

1.12 Principles of Whole Genome Sequencing

Whole genome sequencing (WGS) is the process whereby the complete DNA sequence of an organism is determined. The full sequence can then be compared to reference sequences to allow identification of mutations. A number of platforms are available now for WGS and are known as ‘next or second-generation sequencing’ platforms as they differ from first-generation sequencing which was based on the Sanger method, or first-generation sequencing (Mardis, 2013). The Illumina MiSeq platform was used in this study. DNA extracted from the organism being studied is processed through a number of steps, including library preparation, cluster generation, sequencing and finally data analysis.

1.12.1 Library Preparation

Library preparation involves using transposomes to cleave dsDNA into a certain size fragments (insert size) and ‘tag’ adaptors (a segment of DNA) at each end in a process known as tagmentation. Adaptors allow for the incorporation of P5/P7 binding domains and sample specific index sequences through amplification of overhang primers. Bead based cleanup removes any fragments that are too small or any residual primers. At this point, double stranded DNA (dsDNA) is present and should be quantified. This can then be denatured and diluted in preparation for sequencing in a step called library normalisation, which ensures that all samples are represented equally.

1.12.2 Cluster Generation

Cluster generation is carried out on the flow cell, which is a glass slide with multiple channels coated with oligonucleotides complementary to the P5/P7 binding domains. Each cluster generated represents thousands of copies of the same piece of DNA in one spot (1 μm) on the flow cell. Single DNA libraries are hybridized to the primer lawn on the flow cell and each is extended by polymerisation. These dsDNA are denatured, the original template is washed away and the new strand is covalently attached to the flow cell surface. It then flips over to form a bridge by binding to an adjacent complementary primer and then is extended by polymerases (bridge amplification) so that a double-stranded bridge is formed. The double-stranded bridge is denatured forming two copies of covalently bound single-stranded templates. The bridge amplification cycle is repeated until there are multiple bridges formed. Linearization involves denaturing of all the formed dsDNA bridges. The reverse strands are cleaved and washed away. The remaining cluster is composed of forward strands only and represents identical sequences. A blocking step then prevents unwanted DNA priming of the free 3' ends of the linear strands in the cluster. Sequence primers are hybridized to each strand or adapter sequence (primer hybridization) in preparation for sequencing.

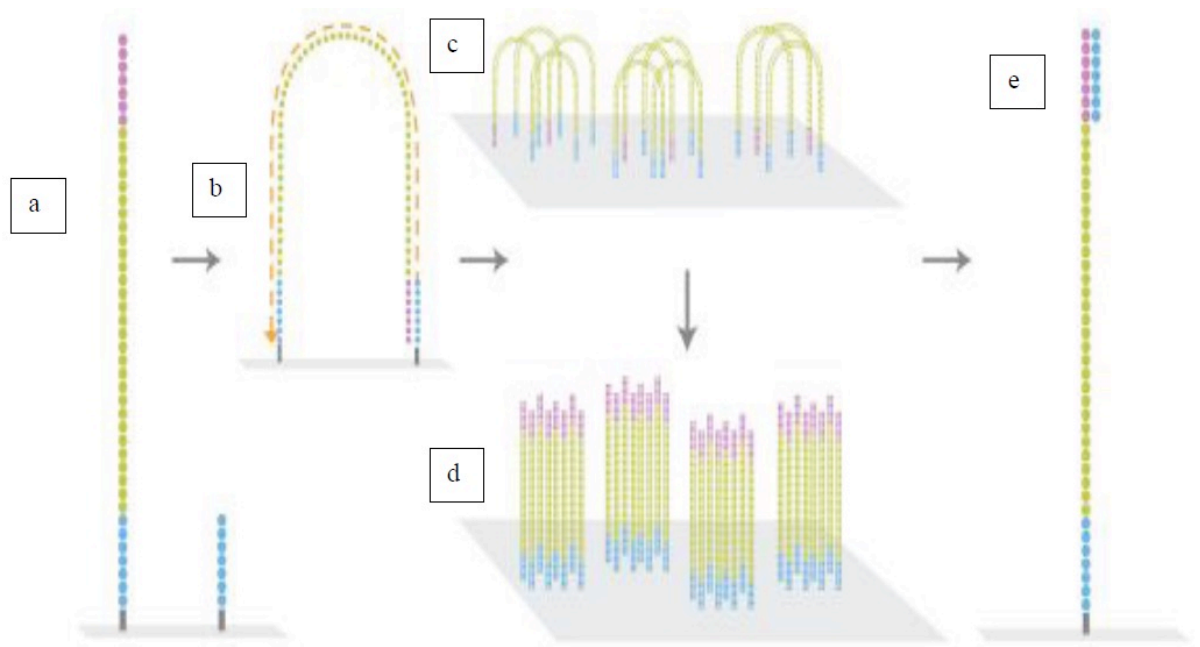


Figure 1.1. Depiction of cluster generation a) binding of the tagged sequence to the flow cell via complement adaptor, b) bridge formation and amplification, c) repeat bridge amplification, d) linearization and washing away of reverse strands and e) binding of sequence primers to each strand within the clusters.

1.12.3 Sequencing

Sequencing by synthesis is performed on covalently bound clusters (Figure 1.2). A mixture of four DNA base pairs or dideoxy Nucleotide Triphosphates (ddNTPs) (adenine, guanine, thymine and cytosine) is added to the flow cell. Each ddNTP has a terminator and fluorescent dye enabling PCR to progress one step before termination (unbound ddNTPs are washed away). Cluster fluorescence occurs with the colour of the incorporated ddNTP which is captured by a camera aided computer keeping track of each cluster position. Paired-end sequencing from both ends of input DNA aids bioinformatics assembly and allows for dual indexing. Sequenced DNA strands are stripped off. Lawn primers and 3'-ends of template strands are unblocked so the template loops over forming a bridge by hybridizing a lawn primer. The 3'-ends of the lawn primer are extended forming dsDNA. Bridges are linearized and the original forward template is cleaved. The free 3' ends of the reverse template and the lawn primers are blocked to prevent unwanted DNA priming. A sequencing primer is hybridized to the adapter sequence. Sequencing by synthesis is repeated (2nd read). The MiSeq analyser converts fluorescent images into FASTQ files in real time. FASTQ files contain the sequence identifiers, sequence letters and quality scores, but require significant bioinformatic analysis.

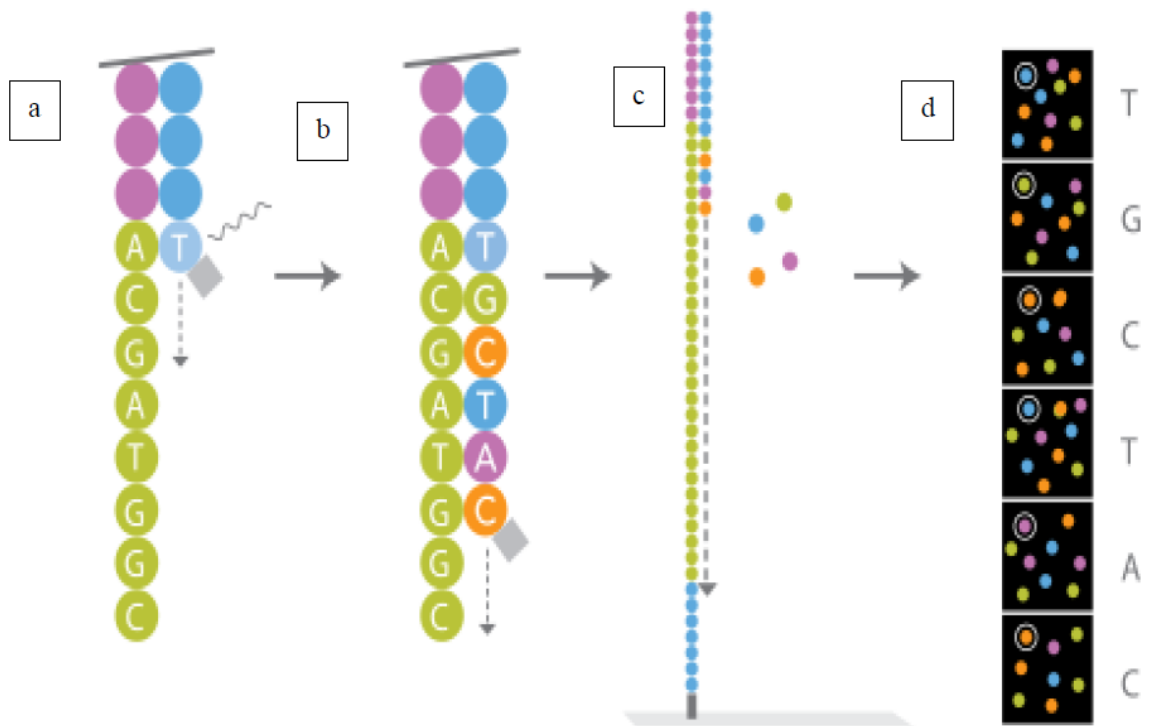


Figure 1.2. Sequencing by synthesis a) primer binding to the adaptor sequence, one complement ddNTP is added to the primer sequence and further binding is blocked, b) the block is removed and the cycle restarts to continue synthesis of the complement sequence, c) with each cycle the cluster fluoresces with the specific colour associated with the ddNTP that has been added, d) a computer aided camera tracks the position and colour of all the clusters with each cycle and images of each cycle are converted to FASTQ output files (Illumina user guide, 2014).

1.12.4 Data Analysis

Once the sequence reads (FASTQ files) have been generated, reconstruction of the whole genome sequence is required. This is performed using specific software and can be done by reference guided assembly (mapping) or by *de novo* assembly. The former is a relatively quick and easy means of reconstruction, while the latter is significantly more computationally demanding. In this study, reference guided assembly was performed which uses a single template genome against which all FASTQ reads are aligned. Only highly similar reads will be mapped to its relative position, typically >93% similarity for DNA genomes. Once assembly is complete, analysis and interrogation of the whole genome sequence is possible.

1.13 Molecular typing methods

Typing is an essential tool in surveillance and control of *N. gonorrhoeae* infections, allowing identification of patterns of transmissions and analysis of strain ancestry and clonality. *Neisseria gonorrhoeae* multiantigen sequence typing (NG –MAST) involves sequencing two hyper-variable regions of *porB* and *thp* genes, encoding outer membrane porin and the β subunit of transferrin-binding protein, respectively, two cell surface proteins exposed to human immune response and hence prone to antigenic change in response (Martin *et al.*, 2004; Shimuta *et al.*, 2013). It has been widely used in most areas of gonococcal epidemiology including investigation of outbreaks, treatment failures, AMR strains and strains from different regions and sexual networks since the early 2000's (Martin *et al.*, 2004; Palmer *et al.*, 2005). An online database (<http://www.ng-mast.net>) allows easy assignment of isolates to sequence types (STs)

and NG-MAST is long considered the most discriminatory typing method. It is deemed essential for short-term epidemiology of gonorrhoea infection (Unemo *et al.*, 2011a).

Multi-locus sequence typing (MLST) involves sequencing seven or more chromosomal house-keeping genes and generation of an allelic profile. Each different sequence (differing by one or more nucleotide) from each of the seven loci is assigned an allele and this generates the allelic profile (the combination of the seven allele numbers) (Unemo *et al.*, 2011a). Again there is an online resource (<http://www.mlst.net>) for assigning STs and this typing method allows unambiguous characterisation of strains, however, it is time-consuming, expensive and less discriminatory than NG-MAST for typing of *N. gonorrhoeae* and very few studies use MLST compared to NG-MAST (Unemo *et al.*, 2011a; Goire *et al.*, 2017; Shimuta *et al.*, 2013). The genes targeted in MLST are relatively conserved and so it is most suited for phylogenetic studies and long-term epidemiology of gonorrhoea infection (Unemo *et al.*, 2011a)

NG-MAST and MLST have been in widespread use for studying molecular epidemiology for many years, but WGS has recently proven more useful than either. Sequencing of a whole genome is a relatively recent advance, with *Haemophilus influenzae* being the first free-living organism to be completely sequenced (Fleischmann *et al.*, 1995). The complete genome of *N. gonorrhoeae* was initially reported in 2008 (Chung *et al.*, 2008) and WGS is currently overtaking NG-MAST and MLST as the preferred typing method. The increasing ease of operation and the falling cost associated with WGS makes it an attractive typing method for gonorrhoea. NG-MAST and MLST STs can be determined in silico from WGS data (Larsen *et al.*, 2011) and it has proven useful in identifying transmission events by assessing single nucleotide polymorphisms (SNPs) in *N. gonorrhoeae*, proving it to be a more discriminatory typing scheme (De Silva *et al.*, 2016), with higher resolution in

determining transmission events and phylogeny (De Silva *et al.*, 2016; Chisholm *et al.*, 2015; Grad *et al.*, 2014). WGS recently allowed detailed analysis and comparison of 4 isolates of high level azithromycin resistant *N. gonorrhoeae* from Ireland (2), Sweden (1) and Italy (1) and showed that they were diverse strains that had independently acquired resistance to azithromycin (Jacobsson *et al.*, 2016). This kind of detailed analysis is critical to fully comprehend the incidence, prevalence and spread of these resistant strains.

Although assessing the clonality of *N. gonorrhoeae* isolates is of great value, clinical epidemiological data is still required. The natural rate of mutation in the species is important, as well as how close the temporal relationship is, in determining what SNP difference is significant to determine whether isolates are related or unrelated. In cases where there are no SNP differences between two strains, direct transmission is not the only possibility; indirect transmission may also have occurred, especially if there is little time difference between the isolates (De Silva *et al.*, 2016).

1.14 Future treatment options – existing and novel drugs

The WHO global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae* was published in 2012. It came in response to increasing resistance, treatment failures and lack of alternative or novel antimicrobials for the treatment of gonorrhoea (WHO 2012c). They recommend that any drug being introduced for treatment of an STI should have: a cure rate of at least 95%; low financial cost; low toxicity; low level resistance or be unlikely to develop resistance; single dose; oral therapy; and safe for use in pregnancy and lactation.

Antibiotics currently on the market which may be of use as alternatives to the first-line gonorrhoea treatment options include ertapenem, gentamicin, fosfomycin, spectinomycin and gemifloxacin.

New drugs for treatment of *N. gonorrhoeae* infection are under investigation.

Solithromycin (macrolide), zoliflodacin (spiropyrimidinetrione) and gepotidacin (type II DNA topoisomerase inhibitor) are in various trials at present and may be of use for treatment of uncomplicated *N. gonorrhoeae* infection. New drugs should be considered for use in dual regimes.

1.14.1 Ertapenem

Ertapenem is part of the carbapenem class of β -lactam antibiotics, which are rapidly bactericidal. Carbapenems have a broad range of activity, especially against Gram-negative organisms. Ertapenem has not traditionally been used in the treatment of gonorrhoea, but in infections caused by multi-drug resistant gram negative organisms including pneumonia, urinary tract infections, intra-abdominal infections and so on.

Ertapenem is usually administered once daily intravenously, but may be given intramuscularly (Parakh *et al.*, 2009; Livermore *et al.*, 2004). Ertapenem has been compared to ceftriaxone in an *in vitro* study which found that the MIC₅₀/MIC₉₀ of the two drugs were similar, but for isolates with clinical resistance to ceftriaxone, the ertapenem MICs were lower, and hence advantageous (Unemo, *et al.*, 2012b). These favourable attributes make it a potentially useful drug for use in the treatment of gonorrhoea.

1.14.2 Gentamicin

Gentamicin, a commonly used aminoglycoside, is active against a range of Gram-negative organisms, including *N. gonorrhoeae*. It has been in use for many years in Malawi and is one antimicrobial that is proposed as an alternative to current treatment options or for more extensive use in the treatment of gonorrhoea (Lewis *et al.*, 2014). After many years of use in Malawi, > 95% of isolates remain susceptible (Kamanga *et al.*, 2010). In 1993 Malawi introduced gentamicin 240 mg IM in combination with doxycycline 100 mg BD PO for 7 days as first-line syndromic treatment of urethritis. The experience of use of gentamicin for treatment of gonorrhoea is mostly from that region. In 2007 after 14 years of use gentamicin susceptibility was examined in 126 isolates of *N. gonorrhoeae* from men with gonococcal urethritis in Malawi. One-hundred isolates were tested for gentamicin and 100% were found to be susceptible to gentamicin using the breakpoint ≤ 4 mg/L (Brown *et al.*, 2010). This study reported no clinical cure data unfortunately. Over 95% of gonococci tested against gentamicin in Malawi in 1993, 1996, 1998, 2001-2002 and 2007 were susceptible (Kamanga *et al.*, 2010). Clinical breakpoints have been proposed for *N. gonorrhoeae* of susceptible ≤ 4 mg/L, intermediate 8-16 mg/L and resistant ≥ 32 mg/L (Brown *et al.*, 2010) and a study has shown 95% of European isolates have MICs within a narrow range, 4-8 mg/L (Chisholm *et al.*, 2011) suggesting it may be a viable future treatment option as part of combination therapy. A randomised controlled trial is underway to compare ceftriaxone/azithromycin to gentamicin/azithromycin for treatment of genital, pharyngeal or rectal gonorrhoea (Brittain *et al.*, 2016).

1.14.3 Fosfomycin

Fosfomycin, a phosphonic acid derivative, was discovered in 1969 produced by *Streptomyces* spp. (Falagas *et al.*, 2016). It is a low molecular weight molecule with activity against *N. gonorrhoeae*, inhibiting phosphoenolpyruvate transferase involved in an early step in bacterial cell wall (peptidoglycan) synthesis (Falagas *et al.*, 2016; Tesh *et al.*, 2015). There are three formulations fosfomycin trometamol (oral), fosfomycin calcium (oral) and fosfomycin disodium (intravenous). The oral bioavailability of the two oral formulations are 40% and 12%, respectively, with urinary excretion of 30-60% and 9-18%, respectively and a half-life of about 4-8 hours (Michaelopoulos *et al.*, 2011). Intramuscular administration is reported to achieve concentrations 3-5 times higher than oral administration with 90% eliminated in urine (Gobernado *et al.*, 2003; Kirby *et al.*, 1977). Fosfomycin trometamol use has increased in Ireland in the past decade, specifically for the treatment of urinary tract infections with a single 3g oral dose and currently in Ireland it is recommended as first-line for treatment of uncomplicated UTI in women in the community (HSE 2017). Following administration of 3g PO fosfomycin trometamol high concentrations persist in urine and bladder (> 128 mg/L) for up to 48 hours (Falagas *et al.*, 2016).

Fosfomycin has been evaluated in clinical trials in the 1970s. López-Gracia *et al.* assessed fosfomycin in the treatment of 60 patients with acute or subacute gonococcal urethritis (< 2 weeks and > 2 weeks of symptoms, respectively) (López-Gracia *et al.*, 1977). There were three treatment groups. One received 4g IM fosfomycin (2 x 2g IM 4 hours apart), then second received 4g IM fosfomycin single dose (given as 2x 2g IM in each buttock) and the third group received 2g every 8 hours for 48 hours. The first two groups included patients with both acute and subacute presentations and the third group included subacute presentation only. In the first and second treatment groups,

11/12 and 37/43 patients were cured. All patients with subacute gonococcal urethritis who received multiple doses over 48 hours were cured (15 patients). Of the other 8 patients with subacute presentation receiving the other regimes, only one was cured, whereas all patients with acute gonococcal urethritis were cured (López-Gracia *et al.*, 1977). Rodríguez *et al.* again assessed IM fosfomycin in the treatment of gonococcal urethritis. A range of doses were used for the 87 men participating in this trial. Similar to the findings from Lopez-Gracia *et al.* above, patients with subacute gonococcal urethritis who received multiple doses (2g every 8 hours for 2 days) were all cured, but those who received 4g single dose all failed treatment. (Rodríguez *et al.*, 1977)

More recently Yuan *et al.* reported an open RCT comparing 3g PO fosfomycin trometamol given on days 1, 3 and 5 to standard therapy (ceftriaxone 250 mg IM plus azithromycin 1g PO) (Yuan *et al.*, 2016). One-hundred and twenty-six patients were in the per-protocol analysis, 5 of whom discontinued the intervention because of ongoing symptoms after receiving the assigned treatment and were prescribed different antibiotics from other doctors. The remaining 121 patients were assessed at day 7 and 14. Of those who received fosfomycin 60/62 (96.8%) had a clinical and microbiological cure at day 7, compared to 61/64 (95.3%) in the standard therapy group. At day 14 there were no recurrences detected (Yuan *et al.*, 2016).

Fosfomycin has also been tested in a number of combination therapies including ESCs and azithromycin as well as a range of other agents (Barbee *et al.*, 2014; Hauser *et al.*, 2014; Wind *et al.*, 2014). None of these studies found additive effect, synergy or antagonism with the combinations of fosfomycin plus ceftriaxone, cefixime, azithromycin or any other agent tested. Barbee *et al.* tested 32 isolates (28 clinical and 4 control strains) which had an MIC range of 8-24 mg/L and while no synergy was found, they felt that the combinations of cefixime or ceftriaxone with fosfomycin

trended towards synergy based on fractional inhibitory concentration indices (FICIs) (Barbee *et al.*, 2014). Hauser *et al.* tested 89 isolates from 1998 to 2012 finding fosfomycin MIC₅₀/MIC₉₀s and MIC range of 8/16 mg/L and 1-32 mg/L, respectively (Hauser *et al.*, 2014). This group also found no synergy or antagonism amongst the tested combinations of fosfomycin plus ceftriaxone or azithromycin, nor with ceftriaxone plus azithromycin. Time kill experiments reported in the same study showed that fosfomycin was bactericidal at twice the MIC, but regrowth occurred after > 24 hours with no regrowth occurring at 4 times the MIC. Ceftriaxone was bactericidal 0.5 times the MIC and interestingly the combination of ceftriaxone plus azithromycin showed slower killing effects than ceftriaxone alone, thought to be due to the bacteriostatic effect of azithromycin preventing the rapid growth phase of *N. gonorrhoeae* during which the ceftriaxone works (Hauser *et al.*, 2014). Finally Wind *et al.* tested fosfomycin in 65 combinations including with gentamicin, spectinomycin and ertapenem, but found no synergistic/antagonistic activity (Wind *et al.*, 2014).

1.14.4 Spectinomycin

The aminoglycoside, spectinomycin, was first isolated in the 1940s and used in treatment of gonorrhoea in the 1950s, but reports of resistance soon emerged (Davey *et al.*, 1957). It is not widely available in Europe and any future use would be as part of combination therapy given its reduced efficacy in clearing pharyngeal gonorrhoea (Lindberg *et al.*, 1982).

1.14.5 Gemifloxacin

Gemifloxacin is an oral 4th generation fluoroquinolone, administered usually in a once daily oral dose of 320 mg, which is active against *Streptococcus pneumonia*, *Haemophilus influenzae*, *Moraxella catarrhalis* and other atypical respiratory pathogens such as *Legionella*, *Mycoplasma* and *Chlamydia* spp. *In vitro* activity against *N. gonorrhoeae* has been examined against various sets of clinical isolates. Four hundred isolates from 12 regions in Spain (1992-1999) were tested and gemifloxacin was found to be active against all strains (Berron *et al.*, 2000). The MIC₅₀/MIC_{90s} and MIC range were 0.003/0.007 mg/L and 0.0005-0.12 mg/L, respectively and for comparison, for the same isolates, the MIC₅₀/MIC_{90s} and MIC range of ciprofloxacin were 0.003/0.001 mg/L and 0.001-0.25 mg/L, respectively (Berron *et al.*, 2000). Tanaka *et al.* compared isolates from two time periods 1992-1993 (94 isolates) and 1996-1997 (100 isolates) in Japan (Tanaka *et al.*, 2000). The gemifloxacin MIC₅₀/MIC_{90s} and MIC range increased from 0.008/0.003 mg/L and ≤ 0.001-0.5 mg/L, respectively in the earlier period to 0.006/0.25 mg/L and 0.002-8.0 mg/L, respectively in the second period. Ciprofloxacin MIC₅₀/MIC_{90s} and MIC range showed a similar increase between the two periods (Tanaka *et al.*, 2000). In 2000 Jones *et al.* reported results of gemifloxacin *in vitro* activity against 150 strains of *N. gonorrhoea* which included 50 that were resistant to ciprofloxacin (MIC > 0.06 mg/L) (Jones *et al.*, 2000). The highest gemifloxacin MIC found was 1 mg/L and the MIC₅₀/MIC_{90s} and MIC range were 0.004/0.008 mg/L and ≤ 0.002-0.003 mg/L, respectively for the ciprofloxacin susceptible isolates and 0.06/0.12 mg/L and 0.03-1 mg/L, respectively for those isolates resistant to ciprofloxacin, i.e. less than one doubling dilution higher MIC₅₀/MIC_{90s} in the ciprofloxacin resistant isolates (Jones *et al.*, 2000). Gemifloxacin has been found to be more potent than ciprofloxacin against

N. gonorrhoeae, but its MICs are higher for ciprofloxacin-resistant than for the ciprofloxacin-susceptible strains (Jones *et al.*, 2000; Pottumarthy *et al.*, 2006).

1.14.6 Solithromycin

Solithromycin is the first fluoroketolide antibiotic (fluorine atom on the lactone ring, C-2 position) with activity against both gram-negative and gram-positive organisms, selectively inhibiting bacterial translation (Llano-Sotelo *et al.* 2010). Solithromycin was developed to overcome resistance in macrolides and safety issues with ketolides (telithromycin). Macrolide resistance can result from an altered binding site (A2058 in the 23s rRNA) caused by methyltransferase encoded by *erm* genes and the fluorine atom in solithromycin is thought to allow tight binding to this altered site (Llano-Sotelo *et al.* 2010). Solithromycin has three targets on the bacterial ribosome. It is an oral formulation and has been evaluated in a Phase 2 trial and was found to be effective as a single dose for the treatment of genital, pharyngeal and rectal gonorrhoea (Hook *et al.*, 2015). It is also effective against *Chlamydia trachomatis* and *Mycoplasma genitalia*. It has a broad spectrum of activity and side effects did not affect efficacy in this trial. However, solithromycin is not likely to be useful for gonococci that display high-level azithromycin resistance (Unemo *et al.*, 2012a; Lewis *et al.*, 2014).

1.14.7 Gepotidacin

Gepotidacin is a new class of type II topoisomerase inhibitor which inhibits DNA gyrase and topoisomerase IV. It is a novel, first in class, triazaacenaphthylene. It does not

exhibit cross resistance with ciprofloxacin as it has a different binding mechanism (Jones *et al.*, 2016; Farrell *et al.*, 2017). *In vitro* evaluation is ongoing.

1.14.8 Zoliflodacin

Zoliflodacin is a novel, first in class, spiropyrimidinetrione. Its mechanism of action is by DNA topoisomerase II inhibition. In a large European study, resistance to ciprofloxacin did not confer resistance to zoliflodacin. (Su *et al.*, 2015; Unemo *et al.*, 2015).

Any agents being considered for the treatment of gonorrhoea should possibly be introduced as part of a dual therapy regimen. This may slow the emergence of resistance due to selective pressure and also protect the second agent. Additionally if a new drug is being introduced, then its use should be restricted, if possible, to the treatment of gonorrhoea, again in an attempt to reduce selective pressure from prescribing for other indication in the community.

1.15 Epidemiology of *N. gonorrhoea* infection in Ireland

In 2012, the World Health Organisation (WHO) estimated 78 million cases of gonorrhoea in adults aged 15-49 years worldwide (WHO 2016). Data for 2015 from the European Centre for Disease Prevention and Control (ECDC) shows that surveillance data across European countries is incomplete (Figure 1.3). Out of participating countries, Ireland (as well as only the UK, Denmark, Norway and Sweden) had a rate of ≥ 15 cases/100,000 population of reported confirmed gonorrhoea infection (Figure 1.3).

In Ireland, prior to 2013, data on numbers of cases is incomplete, but since then notification of cases from STI clinics and laboratories in Ireland has been carried out via the national computerised infectious disease surveillance system (CIDR) and so data on laboratory notified cases is now comprehensive. In 2016, the Health Protection Surveillance Centre (HPSC) reported 1,957 notified cases of gonorrhoea (1294 cases reported in 2013) (HPSC 2017b). In 2015, 1,709 (87.3%) cases were in males, with almost one third of cases (524, 27%) in those ages 20-24 years and 65% (n = 1,273) were ages 20-35 years. Mode of transmission was known for 1,249 cases (64%). Of those, 67% were men who have sex with men (MSM). In 2016, the Health Service Executive (HSE) area East (Dublin, Wicklow and Kildare) had the highest age-standardised notification rate of 73 per 100,000 population, which was significantly higher than the national rate of 41.1 per 100,000 population. From 2015 to 2016, there was an increase in gonorrhoea notifications from 27.2 to 41.1 per 100,000 population (51% increase). The upward trend in notification rates in Ireland is shown (Figure 1.4).

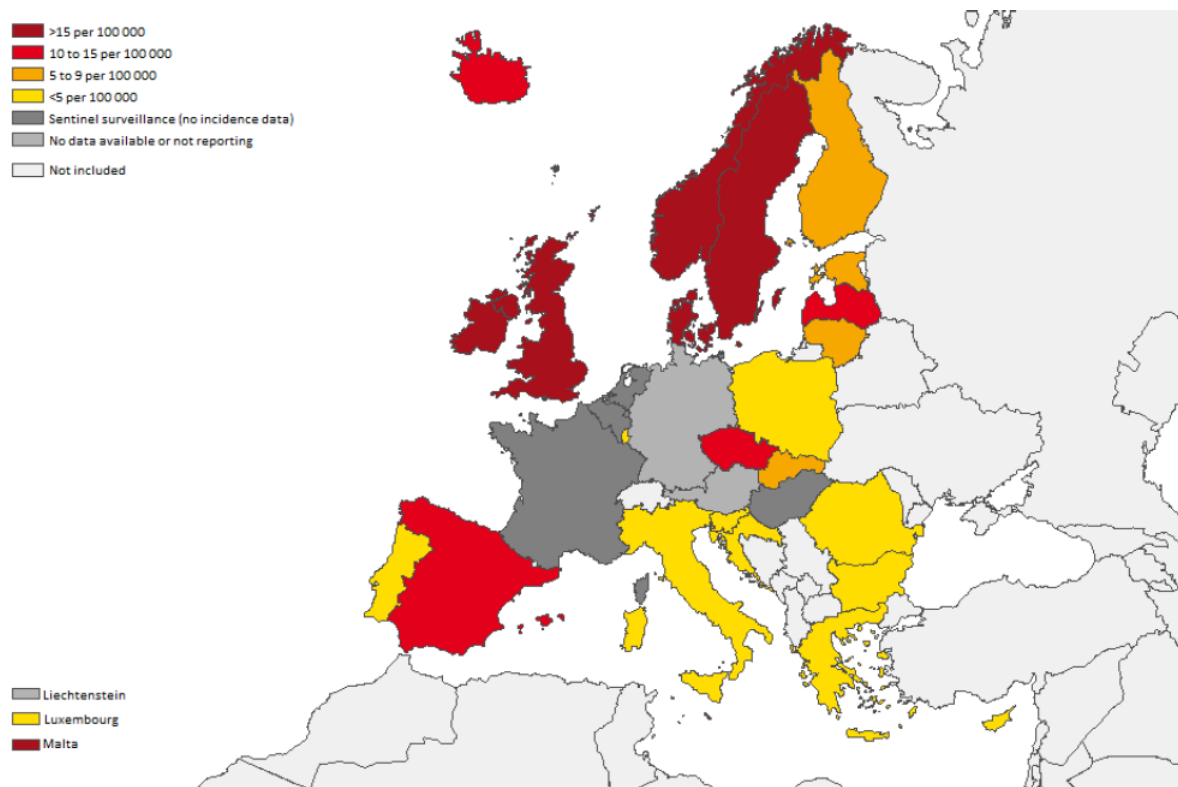


Figure 1.3. ECDC Annual epidemiological report 2015. Rate of reported confirmed gonorrhoea cases per 100,000 population.

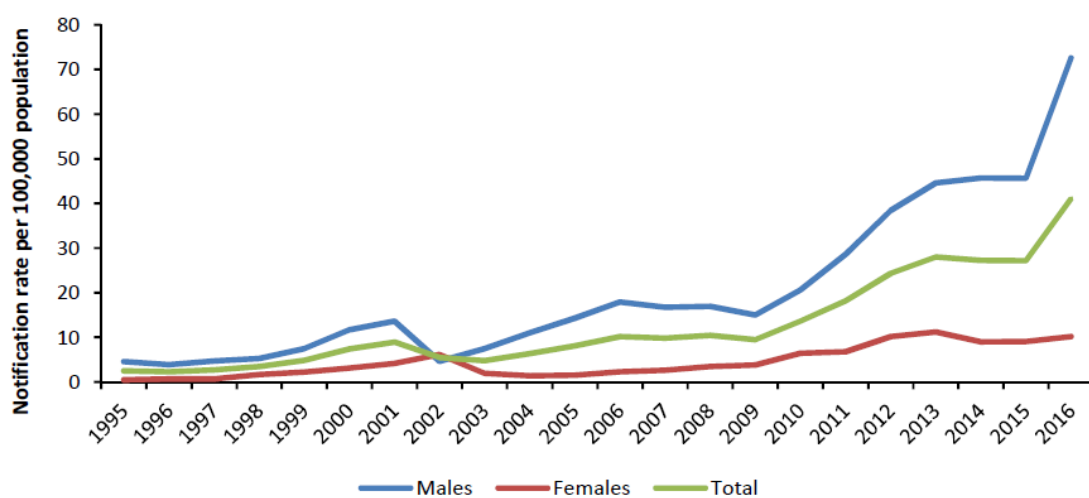


Figure 1.4. Notification rates per 100,000 for gonorrhoea in Ireland, 1995-2016.

1.16 Surveillance of antimicrobial resistance in *N. gonorrhoeae*

It is evident that surveillance of AMR in gonorrhoea is of immense importance in both treatment and control of this infection. In 2009 the European Gonococcal Antimicrobial Surveillance Programme (EURO-GASP) was established in order to perform sentinel surveillance of gonococcal susceptibility (a range of antimicrobials) and molecular epidemiology and link this data with epidemiological information. In addition they have implemented an external quality assurance (EQA) scheme for *N. gonorrhoeae* as well as providing training in susceptibility testing in gonorrhoea.

Isolates are submitted from the participating countries once per year and are selected from consecutive patients and from patients representing different patient groups and geographical regions within the country. The aim is to collect isolates representing approximately 10% of the number of reported cases for each participating country. Reports are then published on gonococcal antimicrobial susceptibility surveillance in Europe.

While sentinel data from EURO-GASP is both important and informative, it is not comprehensive. Comprehensive gonococcal AMR surveillance is unlikely to be possible in many countries where molecular methods are primarily used for diagnosis of gonorrhoea, but where possible, antimicrobial susceptibility testing should be performed to inform on local AMR rates.

Ireland has been participating in the EURO-GASP since 2010. Reports are available up to and including 2015. During this time, isolates from Ireland represented local coverage, not national, having been collected in one laboratory only (SJH). The sampling method was consecutive, but some isolates were selected for resistance phenotype. Over the 6 year period, of the total number of cases reported in Ireland each

year, the per cent of submitted was on average approximately 9% (range 7-14%).

Susceptibility to cefixime, ceftriaxone, azithromycin, ciprofloxacin and penicillin are reported each year at a European level, while resistance rates by country are reported for cefixime (from 2013), azithromycin, ciprofloxacin and penicillin (PPNG only).

Resistance rates for isolates from Ireland are summarised here from 2010 to 2015

(Table 1.9). These Irish data may be useful, they are unfortunately far from comprehensive representing only about 9% of reported cases in Ireland and originating from only one laboratory.

Year	Number of isolates tested (%)	Cefixime resistance		Azithromycin resistance		Ciprofloxacin resistance		Penicillin resistance (PPNG only)	
		Number	%	Number	%	Number	%	Number	%
2015	110 (9)	1	0.9	20	18.2	50	45.5	12	10.9
2014	101 (8)	0	0	38	37.6	35	34.7	2	2
2013	103 (8)	0	0	3	2.9	27	26.2	5	4.9
2012	80 (7)	NR	NR	7	8.8	18	22.5	2	2.5
2011	64 (8)	NR	NR	5	7.8	9	14.1	1	1.6
2010	88 (14)	NR	NR	1	1	31	35	6	7

Table 1.9. Resistance to cefixime, azithromycin, ciprofloxacin and penicillin (plasmid-mediated high-level resistance only, PPNG) by year in isolates from Ireland tested as part of EURO-GASP.

Apart from EURO-GASP data, there are no reports on gonococcal antimicrobial susceptibility rates in Ireland. Similarly, there are no data on gonococcal susceptibility to extended-spectrum cephalosporins in Ireland, nor are there any case reports describing resistance or treatment failures with extended-spectrum cephalosporins. EURO-GASP data above shows that azithromycin-resistant gonococci are not uncommon in Ireland, but the extent of this resistance is unclear.

Details of molecular characterisation of gonococcal resistance determinants and epidemiology in Ireland are limited to only two reports. The first two HLAzi-R *Neisseria gonorrhoeae* (isolated in 2008 and 2011) were reported in Ireland in 2015 (Lynagh *et al.*, 2015) and in 2017 the draft genome sequences for the first 14 azithromycin-resistant *Neisseria gonorrhoeae* reported in Ireland were reported (MacAogáin *et al.*, 2017). Many countries such as the UK, China, Switzerland, parts of the USA and Canada have published reports on molecular characterisation of resistance determinants and or molecular epidemiology of *Neisseria gonorrhoea* (De silva *et al.*, 2016; Chen *et al.*, 2014; Endimiani *et al.*, 2014; Gose *et al.*, 2013; Demczuk *et al.*, 2016), yet the extent of antimicrobial resistance, prevalence of specific resistance determinants and molecular epidemiology of *Neisseria gonorrhoeae* in Ireland is largely unknown.

1.17 Aims of the study

1. To assess resistance by susceptibility testing of 609 isolates of *N. gonorrhoeae* against first line treatment options (ceftriaxone and azithromycin), second line agents (cefotaxime, cefixime, penicillin, ciprofloxacin and tetracycline),

potential therapeutic options (fosfomycin, ertapenem, spectinomycin, gemifloxacin) and finally a new agent (solithromycin).

2. Determination of resistance mechanisms to β -lactams, in particular, to extended spectrum-cephalosporins (ESCs) by the characterisation of PBPs and if present, β -lactamases.
3. Furthermore, determination of resistance mechanisms to azithromycin, including alteration in ribosomal target or increased efflux (e.g. MtrCDE), and in the case of high-level azithromycin resistance, detection of the A2059G mutation in the 23S rRNA genes.
4. Identify known or potential resistance mechanisms to other antibiotics, including fluoroquinolones, aminoglycosides and fosfomycin, tetracycline and spectinomycin.
5. To study genetic relatedness among resistant strains of *N. gonorrhoeae* in Ireland, specifically in those displaying resistance to azithromycin and reduced susceptibility to extended-spectrum cephalosporins (ESCs).
6. To study molecular epidemiology of isolates of *N. gonorrhoeae*, collected from laboratories throughout Ireland, using whole genome sequencing (WGS).

Chapter II

Antimicrobial susceptibility testing of *Neisseria gonorrhoeae*

2.1 Introduction

N. gonorrhoeae has persisted and thrived over decades due to its ability to develop resistance to the antimicrobial drugs used to treat it. In Europe, surveillance of antimicrobial susceptibility of *N. gonorrhoeae* is currently coordinated by the European Centre for Disease Prevention and Control (ECDC) through the European Gonococcal Antimicrobial Surveillance Programme (Euro-Gasp). Reporting systems for gonococcal infection are not mandatory in all countries. The latest gonococcal antimicrobial surveillance in Europe report includes data from 24 European Union countries from 2015, but incidence data on gonorrhoea infection is incomplete, with no rates available for many countries, including France, Netherlands, Belgium, Germany, Italy, Austria and Hungary (ECDC 2017a). Ireland had one of the highest rates of reported confirmed gonorrhoea cases per 100,000 population, along with the UK, Denmark and Latvia, of ≥ 15 cases per 100,000 population (ECDC 2016). The Irish isolates were collected during one three-month period only and represented 9% of laboratory-confirmed cases that year (ECDC 2017a). Of those isolates tested in 2015, none displayed resistance to ceftriaxone, while 0.9%, 18.2% and 45.5% (compared to 0%, 37.6% and 34.7% in 2014) were resistant to cefixime, azithromycin and ciprofloxacin, respectively. Furthermore, the latter data were derived from isolates in one Irish tertiary-care centre, St. James's Hospital and therefore may not be representative of the true rates of antimicrobial resistance in Ireland. Comprehensive antimicrobial resistance (AMR) data on *N. gonorrhoeae* in Ireland are needed to update treatment guidelines and allow appropriate prescribing for infection due to *N. gonorrhoeae*.

As discussed (*Section 1.16*), antimicrobial resistance of *N. gonorrhoeae* is of global concern. Ceftriaxone- and azithromycin-resistant isolates have been reported in the

UK, Japan, France and Spain, and treatment failures with these first-line treatment options have been reported in the UK and France (Fifer *et al.*, 2016; Ohnishi *et al.*, 2011; Unemo *et al.*, 2011c; Cámara *et al.*, 2012). Resistance to cefixime emerged in Japan in the 1990's following years of oral cephalosporins use, often at subtherapeutic doses, as monotherapy for the treatment of gonorrhoea (Unemo *et al.*, 2012a). Reduced susceptibility to ceftriaxone emerged in Japan in the early 2000's (Shimuta *et al.*, 2015) and later in 2011; the first high-level ceftriaxone resistant isolates were reported from Japan and France (Ohnishi *et al.*, 2011a; Unemo *et al.*, 2011c). An outbreak of azithromycin-resistant *N. gonorrhoeae* was reported in South Australia in 2015-2016, with azithromycin-resistance increasing from < 5% to > 30% (Lahra *et al.*, 2017). Additionally, many countries have reported *N. gonorrhoeae* with high-level azithromycin resistance (HLAzi-R), including an outbreak in Leeds (UK) in 2015, where 8 cases of HLAzi-R were identified from November 2014 to March 2015 (Chisholm *et al.*, 2015). This outbreak spread to the West Midlands and south England, including London and increased to 34 cases by April 2016 (Health Protection Report April 2016). In the following 4 months, the number of cases had increased to 48 with additional areas in the UK affected (Health Protection Report Sept 2016). Further updates regarding this outbreak are awaited.

Alternative or new treatment options are required urgently as gonorrhoea may soon be an untreatable infection (Unemo *et al.*, 2016). The CDC list drug-resistant *N. gonorrhoeae* (especially cephalosporin resistant) as an urgent threat which is an "immediate public health threat" that requires "more monitoring and prevention activities" (CDC 2013). There are only two other organisms listed as urgent threats, *Clostridium difficile* and carbapenem-resistant *Enterobacteriaceae*. Alternative antimicrobials are in use in some countries, either because of financial costs or

antimicrobial resistance, e.g. in Malawi, gentamicin 240 mg IM once (combined with doxycycline 100 mg twice daily for 7 days) is used as first line syndromic treatment for urethritis (Brown *et al.*, 2010) and, more recently, gentamicin IM (combined with 2g Azithromycin orally) had been introduced as part of second line regimen in Australia (Lahra *et al.*, 2017). Other alternative antimicrobials include those previously used empirically for gonorrhoea such as ciprofloxacin or spectinomycin which may be of use again, i.e. ciprofloxacin as part of targeted therapy where the isolate is known to be susceptible or in the case of spectinomycin as part of an empiric dual antimicrobial regimen. Novel antimicrobials in the treatment of gonorrhoea include gemifloxacin, ertapenem and solithromycin (Lewis *et al.*, 2014; Unemo *et al.*, 2016). Knowledge of susceptibility data in Ireland for agents such as gentamicin is important in determining potentially useful alternative treatment options for gonorrhoea. Furthermore, the Irish data included in EuroGASP refers solely to isolates from one laboratory, St James's Hospital, and this may not represent the true status of *N. gonorrhoeae* in Ireland.

2.2 Aims

In this chapter, the study aimed to determine antimicrobial susceptibility profiles for clinical isolates of *N. gonorrhoeae* from laboratories throughout in Ireland over a three-year period. Selected antimicrobials tested included agents recommended in Ireland as first line treatment (ceftriaxone and azithromycin), agents used previously (penicillin, ciprofloxacin and cefixime), agents which may be of use in the future either alone or in combination therapies (tetracycline, spectinomycin, ertapenem, cefotaxime, gentamicin and fosfomycin) and novel therapeutic agents (solithromycin and gemifloxacin).

Trends in AMR in *N. gonorrhoeae* over the study period to extended-spectrum

cephalosporins (ESCs) and azithromycin were assessed. A second aim was to compare susceptibility of isolates to antimicrobials which have the same mechanisms of action, i.e. MICs of ESCs vs ertapenem, azithromycin vs solithromycin, and ciprofloxacin vs gemifloxacin. Thirdly, MIC distributions of selected antimicrobials were assessed according to body site to assess any variation in susceptibility that may occur between sites. Finally, a fourth aim of this study was to identify isolates of *N. gonorrhoeae* that are resistant to more than one antimicrobial and those that are classified as multi-drug resistant (MDR) or extensively-drug resistant (XDR).

2.3 Objectives

1. Determine MICs of approximately 600 isolates of *N. gonorrhoeae* by gradient MIC strip for 12 specific antimicrobials.
2. Determine solithromycin MICs of approximately 100 isolates of *N. gonorrhoeae* by agar dilution and gradient MIC strip.
3. Compare the proportion of isolates susceptible to azithromycin and ceftriaxone each year over the three-year study period, 2014-2016.
4. Compare the proportion of isolates susceptible to ciprofloxacin and gemifloxacin, two structurally different antimicrobials with similar mechanisms of action. Similarly for ESCs/ertapenem and azithromycin/solithromycin.
5. Compare the proportion of isolates susceptible to specific antimicrobials according to site of isolation from pharyngeal, rectal and urogenital samples.
6. Determine the number of isolates that meet proposed definitions for MDR and XDR status.

2.4 Materials and Methods

2.4.1 Sample selection

Bacterial isolates of *N. gonorrhoeae* from clinical samples which had been prospectively collected in St. James's Hospital (SJH) (2014-2016) and stored on Microbank™ cryobeads at -70°C were recovered for testing. Clinical isolates retrieved from or referred to SJH by other tertiary referral or regional hospitals in Ireland were also examined. These included University Hospitals Waterford (UHW), Galway (UHG) and Limerick (UHL), the National Maternity Hospital (NMH), the Midland Regional Hospital at Mullingar (MRHM) and St. Collumcille's Hospital, Dublin (SCH). A total of 609 isolates were recovered from frozen: 510 (83.6%) from SJH; 39 (6.4%) from UHW; 31(5.1%) from UGH; 26 (4.3%) from UHL; 2 (0.3%) from NMH; and one from each of MRHM and SCH (0.3%). The majority of isolates, 329 (53.9%) dated from 2016, followed by 166 (27.2%) from 2015 and 95 (15/6%) from 2014.

2.4.2 Culture

Each isolate was subbed from beads to chocolate agar, incubated at 35°C for 18-20 hours, followed by a second pass onto chocolate agar, prior to performing susceptibility testing.

2.4.3 Identification

Identification of all isolates was confirmed following recovery, by automated mass spectrometry system, matrix-assisted laser desorption ionisation time-of-flight

(MALDI ToF). An identification confidence value of $\geq 99.9\%$ was acceptable.

Gram's stain was performed on each culture to ensure purity. If required the culture was subbed to G.C. Sel. & V.C.A.T. agar (E & O Laboratories Ltd. Burnhouse Bonnybridge, FKA 2HH, Scotland.) to enable pure colonies of *N. gonorrhoeae* to be isolated. Susceptibility testing was performed on a pure culture taken from chocolate agar only.

2.4.4 Antimicrobial Susceptibility Testing (AST)

AST was performed using the gradient MIC method (Liofilchem® Roseto degli Abruzzi, Italy). This is a quantitative method which involves the application of an antimicrobial-impregnated strip (with predefined concentration gradient of the antimicrobial) onto agar pre-inoculated with a defined inoculum of the organism being tested. The lowest concentration of antimicrobial at which the growth is inhibited gives the MIC value.

After two passes from beads a bacterial suspension of 0.5 McFarland turbidity was made from each isolate of *N. gonorrhoeae* using Oxoid™ BHI broth (ThermoFischer Scientific). This suspension was used to inoculate GC sensitivity agar (Fannin, L.I.P Diagnostic Services, Galway, Ireland) using a sterile cotton swab, within 15 minutes of preparation. The agar plates were allowed to dry for 10-15 minutes before application of the MIC strips (penicillin, cefotaxime, ceftriaxone, cefixime, azithromycin, ciprofloxacin, tetracycline, gentamicin, spectinomycin, gemifloxacin, ertapenem and fosfomycin) (Table 2.1).

The agar plates were then incubated in the inverted position at 35°C in 5% CO₂ for 20-24 hours prior to determination of MIC. After incubation there is symmetrical inhibition of growth centred on the gradient strip. The MIC is determined by the point the ellipse intersects the gradient strip, which is marked with doubling concentrations of the antimicrobial in question, e.g. 1, 2, 4, 8, 16 mg/L etc. The gradient strip is also marked with half dilution values e.g. 1.5, 3, 6, 12 mg/L etc. The MIC for bacteriostatic drugs (tetracycline, azithromycin and solithromycin) is read at 80% inhibition, and for bactericidal drugs is read at 100% inhibition. If the ellipse intersects two values, the upper value is taken as the MIC. If the value determined from the gradient strip is a half dilution concentration, the MIC should be rounded up to the next upper two-fold concentration for interpretation.

Clinical breakpoints for susceptibility and resistance are set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The clinical breakpoint for susceptibility/resistance indicates the MIC at which the antimicrobial being tested is likely to be associated with clinical success/failure in treating infection caused the specified bacteria. EUCAST have set clinical breakpoints for *N. gonorrhoeae* and penicillin, ciprofloxacin, azithromycin, ceftriaxone, cefixime, cefotaxime, spectinomycin and tetracycline (EUCAST 2017) (Table 2.1). No clinical breakpoints are available for ertapenem, fosfomycin, gemifloxacin, gentamicin or solithromycin. For these antimicrobials the MIC range is given and the MIC₅₀ and MIC₉₀ are calculated. The MIC₅₀ represents the MIC value at which $\geq 50\%$ of the selected isolates are inhibited, and the MIC₉₀ is the value at which $\geq 90\%$ are inhibited (Schwarz *et al.*, 2010).

MIC Test Strip	Concentration gradient (mg/L)	ATCC strain for Quality Control (QC)	QC pass range (mg/L)	EUCAST interpretive criteria	
				MIC Breakpoint (mg/L)	
				S≤	R>
Ceftriaxone	0.002-32	<i>N. gonorrhoeae</i> ATCC 49226	0.004-0.015	0.125	0.125
Cefixime	0.016-256	<i>H. influenzae</i> ATCC 49766	0.016-0.06	0.125	0.125
Cefotaxime	0.002-32	<i>H. influenzae</i> ATCC 49247	0.12-0.5	0.125	0.125
Ertapenem	0.002-32	<i>H. influenzae</i> ATCC 49766	0.015-0.06	-	-
Azithromycin	0.016-256	<i>H. influenzae</i> ATCC 49766	4-16	0.25	0.5
Ciprofloxacin	0.002-32	<i>N. gonorrhoeae</i> ATCC 49226	0.001-0.008	0.03	0.06
Gemifloxacin	0.002-32	<i>H. influenzae</i> ATCC 49766	0.002-0.008	-	-
Penicillin	0.002-32	<i>N. gonorrhoeae</i> ATCC 49226	0.25-1	0.006	1
Tetracycline	0.016-256	<i>N. gonorrhoeae</i> ATCC 49226	0.25-1	0.5	1
Spectinomycin	0.064-1024	<i>N. gonorrhoeae</i> ATCC 49226	8-32	64	64
Gentamicin	0.016-256	<i>S. aureus</i> ATCC 29213	0.25-1	-	-
Fosfomycin	0.016-256	<i>E. coli</i> ATCC 25922	0.5-2.0	-	-
Solithromycin	0.002-32	<i>H. influenzae</i> ATCC 49247	1-4	-	-

Table 2.1. Concentration of MIC gradient strips. QC test strains and pass ranges (mg/L) and EUCAST Clinical Breakpoint interpretive criteria for antimicrobials where available.

2.4.5 Quality control

Quality control (QC) on GC sensitivity agar and MIC strips was performed weekly and with each new batch received. The following ATCC strains were used in QC: *S. aureus* ATCC 29213 for gentamicin; *H. influenzae* ATCC 49766 for azithromycin, cefixime, ertapenem and gemifloxacin; *H. influenzae* 49247 for cefotaxime and solithromycin; *E. coli* ATCC 25922 for fosfomycin; and *N. gonorrhoeae* ATCC 49226 for penicillin, ciprofloxacin, ceftriaxone, tetracycline and spectinomycin (Liofilchem 2016). A bacterial suspension of 0.5 McFarland turbidity was made from each of the above ATCC strains using BHI broth for *N. gonorrhoeae* and *H. influenzae* strains and 0.9% Saline for *S. aureus* and *E. coli*. These suspensions were used within 15 minutes to inoculate the following: GC sens agar for *N. gonorrhoeae*; Mueller-Hinton agar (BioMérieux, North Carolina, USA) for *S. aureus* and *E. coli*; and Mueller Hinton-Fastidious agar (BioMérieux, North Carolina, USA) for *H. influenzae*. After 10-15 minutes of drying time, the above mentioned gradient MIC strips were applied to the appropriate plate. The isolates of *N. gonorrhoeae* and *H. influenzae* were incubated in 5% CO₂ and the *S. aureus* and *E. coli* were incubated in air, all at 35°C for 20-24 hours. For the QC to pass and subsequent test results to be acceptable, the determined MIC for each drug/organism combination must fall within the concentration ranges specified by the manufacturer (Table 2.1) (Liofilchem 2016).

Each batch and lot number of GC sens agar was batch accepted according to the following method.

1. A bacterial suspension of 0.5 McFarland turbidity is prepared using *N. gonorrhoeae* ATCC 49226. This suspension is sequentially diluted.

2. Add 330 µl of the 0.5 McFarland suspension into 3 ml BHI broth to make a 1:10 solution
3. Add 330 µl of the 1:10 solution into 3 ml of BHI broth to make a 1:100 solution.
4. Finally, add 330 µl of the 1:100 solution into 3 ml of BHI broth to make a 1:1000 solution.
5. Inoculate 10 µl of the 0.5 McFarland suspension onto one GC sensitivity agar and one chocolate agar plate.
6. Inoculate 10 µl of the 1:1000 solution onto one GC sensitivity agar and one chocolate agar plate.
7. Incubate the 4 plates at 35°C in 5% CO₂ for 18-24.
8. For batch acceptance there must be 10-100cfu on all plates after 18-48 hours incubation.

Densitometers were calibrated prior to each use using DensiCHECK *plus* standards of 0.0, 0.5, 2.0 and 3.0 McFarland (BioMérieux, North Carolina, USA). The acceptable ranges are shown (Table 2.2). The densitometer in use is calibrated to 0.0 McFarland with each bijoux of BHI broth or each test tube of 0.9% Saline.

2.4.6 Phenotypic β -lactamase screening method

Two methods were employed to determine beta-lactamase production in *Neisseria gonorrhoeae*.

- Cefinase (nitrocefin) disc test for rapid detection of beta-lactamase production.
- Hodge plate.

2.4.6.1 Cefinase

The test uses paper discs impregnated with a chromogenic cephalosporin (nitrocefin). After allowing the cartridge to come to room temperature, the paper disc is moistened with sterile water. A few isolated colonies of the organism are spread across the surface of the disc. If the test organism produces beta-lactamase, it will hydrolyse the chromogenic cephalosporin releasing a red compound.

2.4.6.2 Hodge plate

A suspension of *S. aureus* ATCC 25923 is prepared in 3 ml of nutrient broth. This is swabbed over the entire surface of a chocolate agar plate. The plate is labelled with 4 inoculation lines from centre to edge. A heavy inoculum of the test organisms and control organisms are streaked along the inoculation lines from edge to centre. A Penicillin (2 μ g) disc is placed at the centre of the plate. Incubate at 35°C in 5% CO₂ 16-18 hours. The positive control is a penicillinase producing *Moraxella catarrhalis* (wild type) and the negative control used is *Haemophilus influenzae* ATCC 49247. A positive test result will show growth of *S. aureus* along the inoculum of the positive

control and the test organism, and no growth along the inoculum of the negative control.

2.4.7 Solithromycin susceptibility testing

2.4.7.1 Agar dilution

Solithromycin susceptibility testing was performed by agar dilution method following these steps (EUCAST 2000).

1. Preparation of stock solution: (10 mg/ml stock solution)
 - a. Dissolve 10mg of solithromycin in 1 ml 80% DMSO.
 - b. Vortex carefully intermittently if required, until the product visibly goes into solution and there is no haziness.
 - c. Once in solution, leave for 30 to 60 minutes at room temperature
2. Preparation of working solution
 - a. Dissolve the 10 mg/ml solution 1:40 to a working solution (50 µl antibiotic + 1950 µl dilution solution (using sterile water).
3. Preparation of plates
 - a. Nine bottles with 100 ml of 3.6% Oxoid GC Medium Agar Base (using 250 ml volume bottles) are prepared. GC agar base (3.6g) are added to 100ml of RO water, gently brought to the boil to dissolve the solution and autoclaved at 121°C for 15 minutes.
 - b. The sterilised agar is allowed to cool to 50-55 °C.
 - c. Specific volumes of the working solution are added to each bottle, leaving one without antibiotic as a control (Table 2.3).
 - d. Add 1% IsoVitalex (usually 1 ml) to each bottle and mix thoroughly.
 - e. Pour 19mL of molten agar to a sterile container.

- f. Pour the agar into pre-labelled sterile Petri dishes on a level surface.
- g. Allow the plates to set at room temperature

Standard	Acceptable ranges	
0.5 McFarland	0.44	0.56
2.0 McFarland	1.85	2.15
3.0 McFarland	2.79	3.21

Table 2.2. Acceptable ranges for using DensiCHECK *plus* standards (BioMérieux, North Carolina, USA)

Final agar concentration required	Working solution added to 100ml of agar
2 mg/L	800 µl
1 mg/L	400 µl
0.5 mg/L	200 µl
0.25 mg/L	100 µl
0.125 mg/L	50 µl
0.064 mg/L	25 µl
0.032 mg/L	12.5 µl
0.016 mg/L	6.25 µl
0.00 mg/L (drug-free control)	0.00 µl

Table 2.3. Preparation of dilutions from the working solution of solithromycin.

4. Preparation of inoculum

- a. Standardise the density of inoculum to give 10^4 CFU per spot on the agar, using four or five colonies of a pure culture to avoid selecting an atypical variant.
- b. The inoculum is prepared by emulsifying colonies from an overnight culture on chocolate agar.
- c. A 0.5McFarland solution in Muller-Hinton broth is prepared.
- d. Dilute the suspensions of organisms to give 10^7 CFU/mL.

5. Inoculation of the plate

- a. Inoculate the plates within 30min of standardising the inoculum.
- b. $1\mu\text{l}$ of the 10^7 CFU/mL suspension is inoculated to each plate

6. Incubation of the plates

- a. Plates are incubated at 35°C in 5% CO_2 for 20 to 24 hours.

7. Reading of results

- a. The MIC is the lowest concentration of that completely inhibits visible growth as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot.

Quality control guidelines for solithromycin agar dilution and *N. gonorrhoeae* are proposed by Riedel *et al.* (Reidel *et al.*, 2015). *N. gonorrhoeae* ATCC strain 49226 quality control range for solithromycin agar dilution is 0.03-0.25 mg/L (range accepted by CLSI) (Riedel *et al.*, 2015). Plates are also controlled for sterility and growth promotion, the latter with *N. gonorrhoeae* ATCC 49226 which resulted in an MIC of 0.064 mg/L, i.e. within the above proposed QC range.

2.4.7.2 Disc diffusion

Solithromycin disc diffusion was performed according to the following steps.

1. A bacterial suspension was prepared in Muller-Hinton broth and adjusted to 0.5 McFarland.
2. This suspension was lawned onto GC sensitivity agar and allowed to dry fully.
3. A 15 µg solithromycin disc (Liofilchem® Roseto degli Abruzzi, Italy) was placed in the centre of the plate.
4. A chocolate agar plate was also inoculated with the bacterial suspension to ensure purity.
5. The plates were incubated at 35°C in 5% CO₂ for 20 to 24 hours.
6. The zone of inhibition around the disc was measured and recorded.
7. Quality control was performed using *N. gonorrhoeae* ATCC 49226. *N. gonorrhoeae* ATCC strain 49226 quality control range for solithromycin disc diffusion is 33-43 mm (Riedel *et al.*, 2015).

2.5 Patient demographics

Data on baseline demographics (including age, sex), source of sample and body site of sample were collected for each isolate. A total of 609 isolates were recovered from frozen: 509 (83.6%) from SJH; 39 (6.4%) from UHW; 31(5.1%) from UGH; 26 (4.3%) from UHL; 2 (0.3%) from NMH; and one from each of MRHM and SCH (0.3%). The majority of isolates, 328 (53.9%) dated from 2016, followed by 166 (27.2%) from 2015 and 95 (15.6%) from 2014 (Table 2.4).

Five-hundred and sixteen samples (84.7%) originated from STI clinics and only 36 (5.9%) from general practice (GP). Of 609 clinical isolates, 553 (90.8%) were from male patients, 47 (7.7%) were from female patients and sex was unknown for 9 (1.5%) patients. The median age was 26 years (mean 28) with a range of 3 to 75 years. Age was not recorded for 3 of the clinical samples.

The majority of samples, 320/609 (52.5%), were from genital sites (urethral/cervical). Rectal and pharyngeal sites accounted for 162 (26.6%) and 96 (15.7%) samples, respectively. Five samples (0.8%) were labelled as “isolate” only, having been referred to SJH from another hospital, and site was unknown for these. There were 6 (1%) eye samples and 1 (0.2%) suprapratellar aspirate. The remaining 19 samples (3.1%) had no site recorded. (Figure 2.1)

2.6 Antimicrobial Susceptibility Results

Susceptibility results for individual antimicrobials are discussed below. A summary of the interpretation is included (Table 2.5) and the MIC₅₀, MIC₉₀ and MIC ranges for all antimicrobials tested are shown (Table 2.6). MIC results for all isolates may be found in *Appendix 1*. The percentage resistance of the isolates tested from 2014 to 2016 is shown (Figure 2.2).

Number of isolates by year and geographical location								
	SJH	UHW	UHG	UHL	NMH	MRHR	SCH	Total
2016	278	6	17	26	-	1	-	328
2015	147	9	9	-	1	-	-	166
2014	84	4	5	-	1	-	1	95
2013	-	17	-	-	-	-	-	17
2012	-	3	-	-	-	-	-	3
Total	509	39	31	26	2	1	1	609

Table 2.4. Number of isolates by year and geographical location.

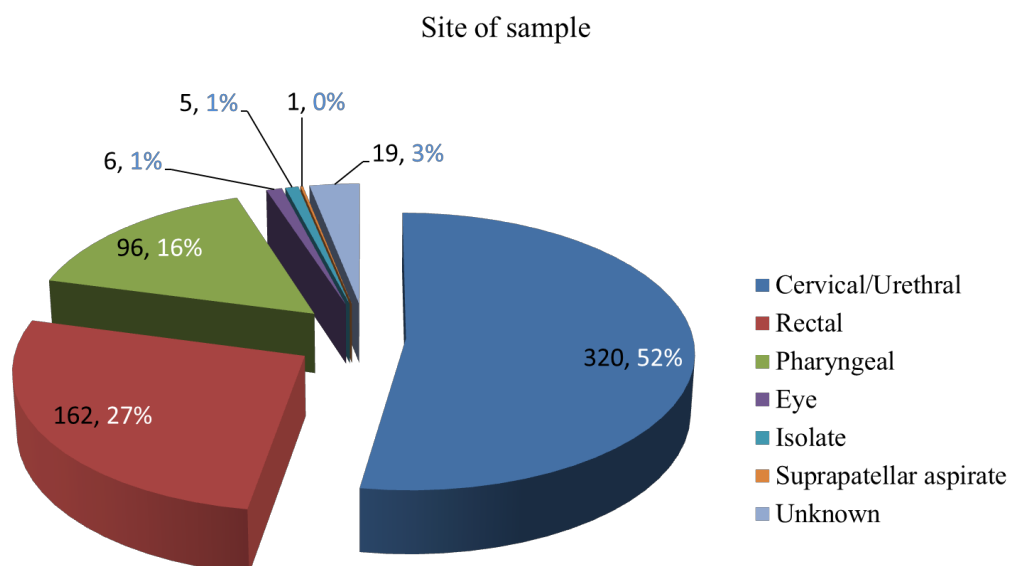


Figure 2.1. Sites of samples.

Antimicrobial	Susceptible	Intermediate	Resistant	Reduced susceptibility (ESCs)	High-level resistance (AZM)
Ceftriaxone	609 (100%)	-	-	11 (1.8%)	-
Cefixime	603 (99%)	-	6 (1%)	15 (2.5%)	-
Cefotaxime	597 (97.9%)		13 (2.1%)	46 (7.6%)	-
Azithromycin	341 (56%)	155 (25.5%)	106 (17.4%)	-	7 (1.1%)
Ciprofloxacin	408 (67%)	-	201 (33%)	-	-
Penicillin	11 (1.8%)	554 (91 %)	44 (7.2%)	-	-
Tetracycline	465 (76.3%)	84 (13.8%)	60 (9.9%)	-	-
Spectinomycin	609 (100%)	-	-	-	-

Table 2.5. Summary of susceptibility results interpretation. Reduced susceptibility defined as ceftriaxone MIC > 0.032 mg/L and cefixime/cefotaxime MIC = 0.125 mg/L

Antimicrobial	MIC ₅₀	MIC ₉₀	MIC range
Ceftriaxone	0.008 mg/L	0.016 mg/L	≤ 0.002-0.125 mg/L
Cefixime	0.016 mg/L	0.032 mg/L	≤ 0.016-0.25 mg/L
Cefotaxime	0.032 mg/L	0.064 mg/L	≤ 0.002-0.5 mg/L
Ertapenem	0.016 mg/L	0.032 mg/L	≤ 0.002-0.094 mg/L
Azithromycin	0.25 mg/L	1 mg/L	≤ 0.016-> 256 mg/L
Ciprofloxacin	0.008 mg/L	2 mg/L	≤ 0.002-> 32 mg/L
Gemifloxacin	0.008 mg/L	2 mg/L	≤ 0.002-16 mg/L
Penicillin	0.25 mg/L	1 mg/L	0.016-> 32 mg/L
Tetracycline	0.5 mg/L	1 mg/L	0.032-32 mg/L
Spectinomycin	8 mg/L	16 mg/L	2-32 mg/L
Gentamicin	4 mg/L	8 mg/L	1-16 mg/L
Fosfomycin	16 mg/L	32 mg/L	4-128 mg/L
Solithromycin	0.125 mg/L	0.25 mg/L	0.032-> 32 mg/L

Table 2.6. Summary of MIC₅₀, MIC₉₀ and MIC ranges for antimicrobials tested.

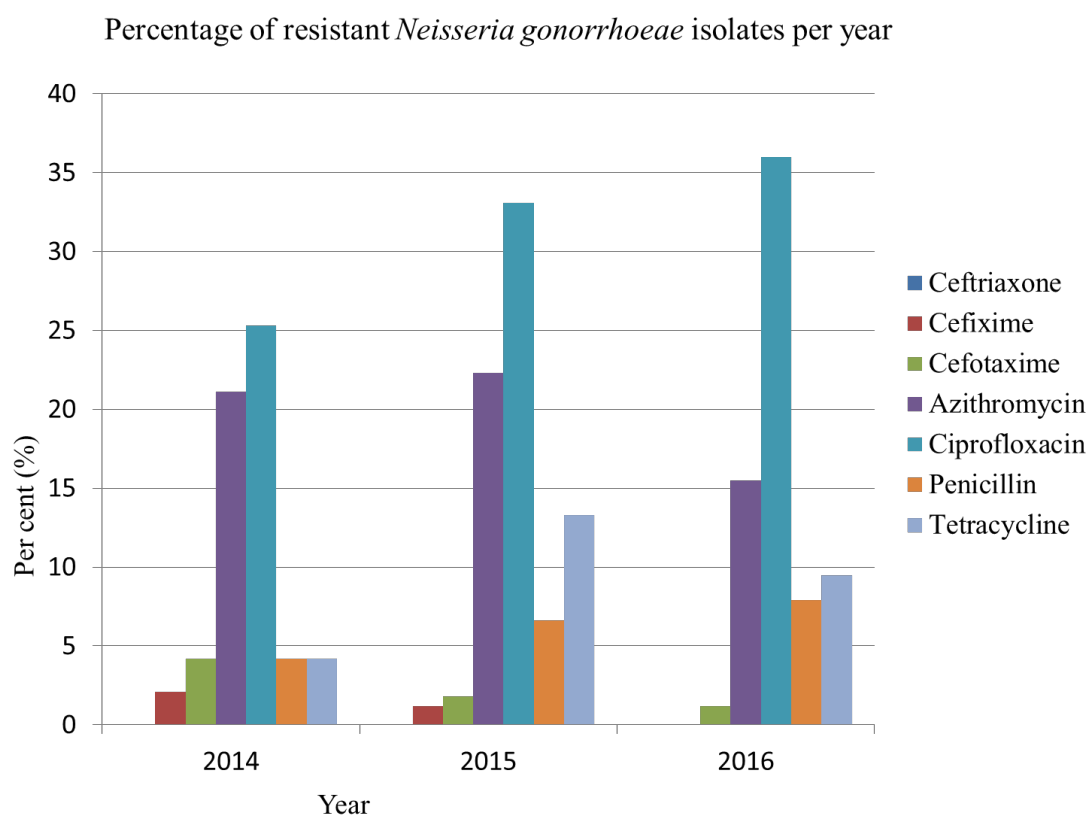


Figure 2.2. Percentage resistance of isolates per year from 2014 to 2016 for the antimicrobials for which there are clinical breakpoints.

2.6.1 Extended Spectrum Cephalosporins

In this study, susceptibility to ceftriaxone, cefixime and cefotaxime was determined in 609 gonococcal isolates.

2.6.1.1 Ceftriaxone

Ceftriaxone is the first-line agent recommended for gonorrhoea infection in combination with azithromycin. All 609 isolates were susceptible to ceftriaxone ($\text{MIC} \leq 0.125 \text{ mg/L}$). Reduced susceptibility to ceftriaxone is defined as $\text{MIC} > 0.032 \text{ mg/L}$ (Allen *et al.*, 2014) and was detected in 11 (1.8%) isolates. The MIC_{50} and MIC_{90} were 0.008 mg/L and 0.016 mg/L, respectively. The majority of isolates (480) fell into a narrow range of 0.004-0.016 mg/L, but the overall MIC range was ≤ 0.002 -0.125 mg/L (Figure 2.3). Over the three years the MIC distribution remains quite stable, but there appears to be some ‘MIC creep’ from 2014 compared to 2015 and 2016 (Figure 2.4). Slightly less isolates displayed reduced-susceptibility to ceftriaxone (CRO-RS) in 2015/2016 compared to 2014 (Figure 2.5).

2.6.1.2 Cefixime

Although cefixime has not been recommended first-line for treatment of gonorrhoea since 2011 in Ireland and the UK (BASH 2005 and 2011 guidelines), cefixime resistance ($\text{MIC} > 0.125 \text{ mg/L}$) was still detected in 6 (1%) isolates from 2014 to 2016. Five of these resistant isolates had MIC values of 0.19 mg/L and one had an MIC was 0.25 mg/L (Figure 2.6). The remaining isolates 603 (99%) tested susceptible to cefixime, however, of those, 15 displayed reduced susceptibility, defined as MIC equal

to 0.125 mg/L (Allen *et al.*, 2014) (Figure 2.6). The MIC₅₀ and MIC₉₀ were 0.016 mg/L and 0.032 mg/L, respectively. The MIC range was ≤ 0.016 -0.25 mg/L (Figure 2.6). Percentage susceptibility to cefixime by year, 2014 to 2016, was high, with low numbers of isolates each year being resistant or displaying reduced susceptibility and overall the trends appear to be towards higher susceptibility rates (Figure 2.7).

2.6.1.3 Cefotaxime

Cefotaxime resistance (MIC > 0.125 mg/L) was detected in 13 (2.1%) isolates. Of these, 4, 7 and 2 isolates had MICs of 0.19 mg/L, 0.25 mg/L and 0.38 mg/L, respectively. A further 46 (7.6%) isolates displayed reduced susceptibility to cefotaxime (MIC = 0.125 mg/L). The MIC distribution showed higher MICs compared to those of ceftriaxone and cefixime (Figure 2.8). The MIC₅₀ and MIC₉₀ were 0.032 mg/L and 0.064 mg/L, respectively, with an MIC range of ≤ 0.002 -0.5 mg/L (Figure 2.8). Percentage susceptibility to cefotaxime dropped from over 90% in 2014 and 2015 to just below 90% in 2016 (Figure 2.9).

2.6.1.4 Comparison of extended-spectrum cephalosporins (ESCs) MICs

Ceftriaxone, cefixime and cefotaxime have the same mechanism of action, but different levels of activity against *N. gonorrhoeae*. The MICs to cefotaxime tended to be higher than those of cefixime and ceftriaxone. Similarly, cefixime MICs tended to be higher than those of ceftriaxone (Figure 2.10), so among isolates with reduced susceptibility or resistance to cefixime and cefotaxime, ceftriaxone retained significant activity.

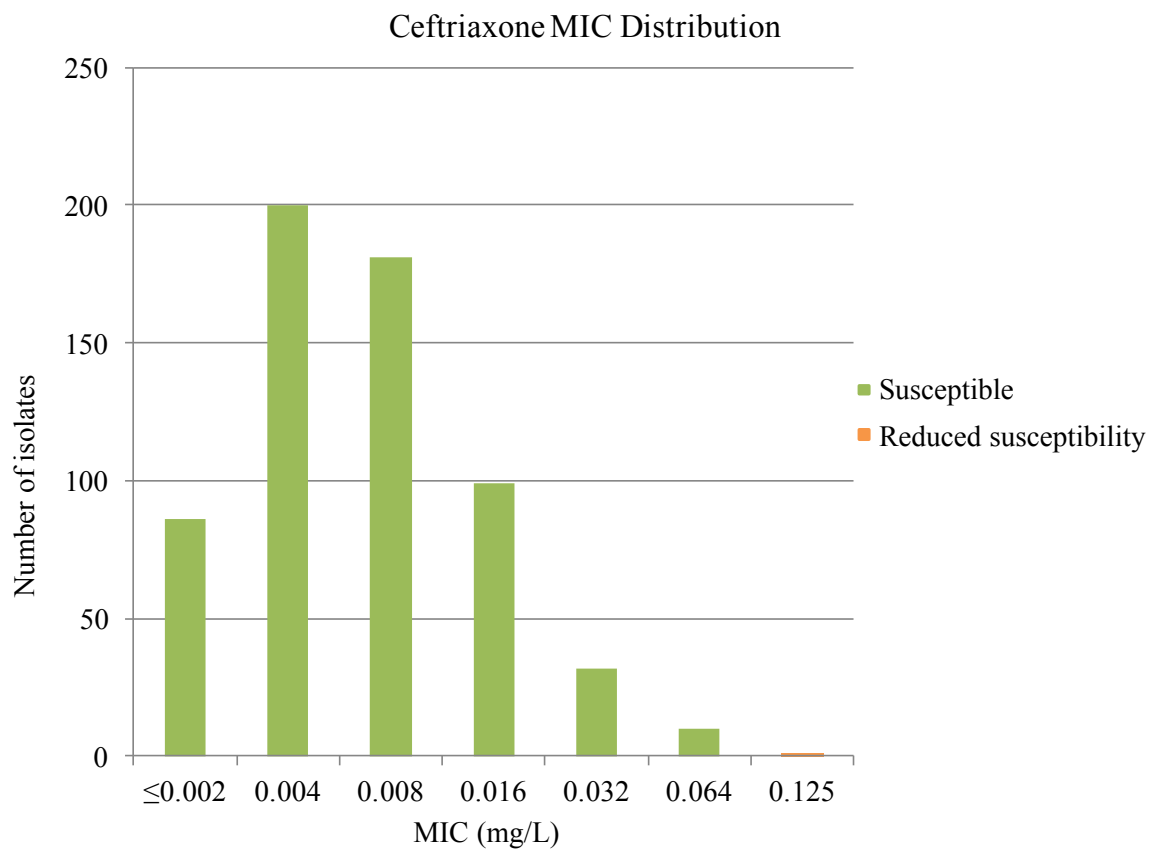


Figure 2.3. Ceftriaxone MIC distribution.

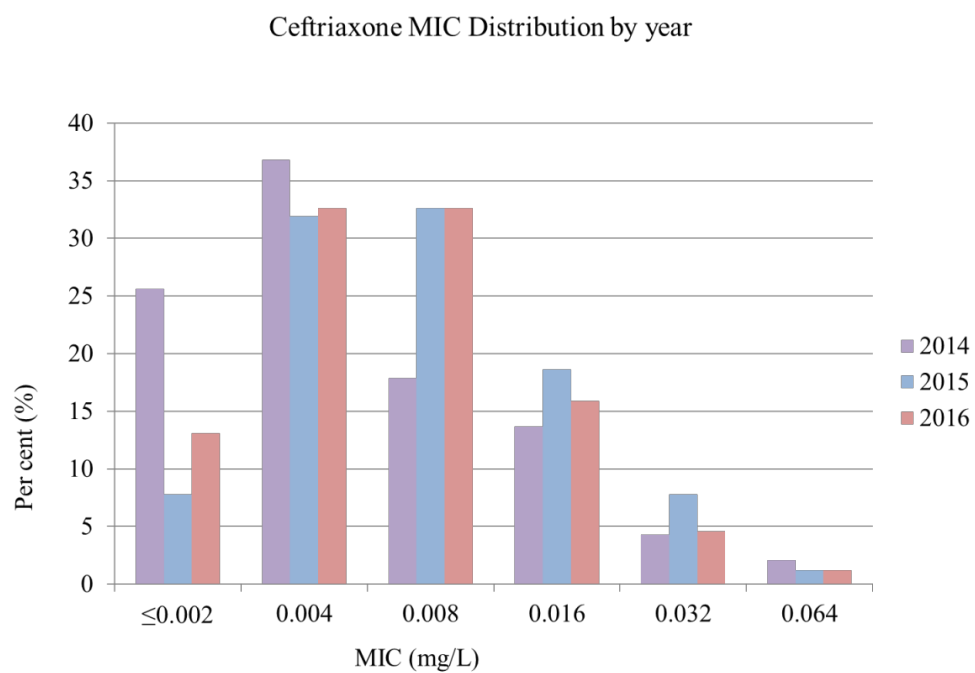


Figure 2.4. Ceftriaxone MIC distribution between years 2014-2016.

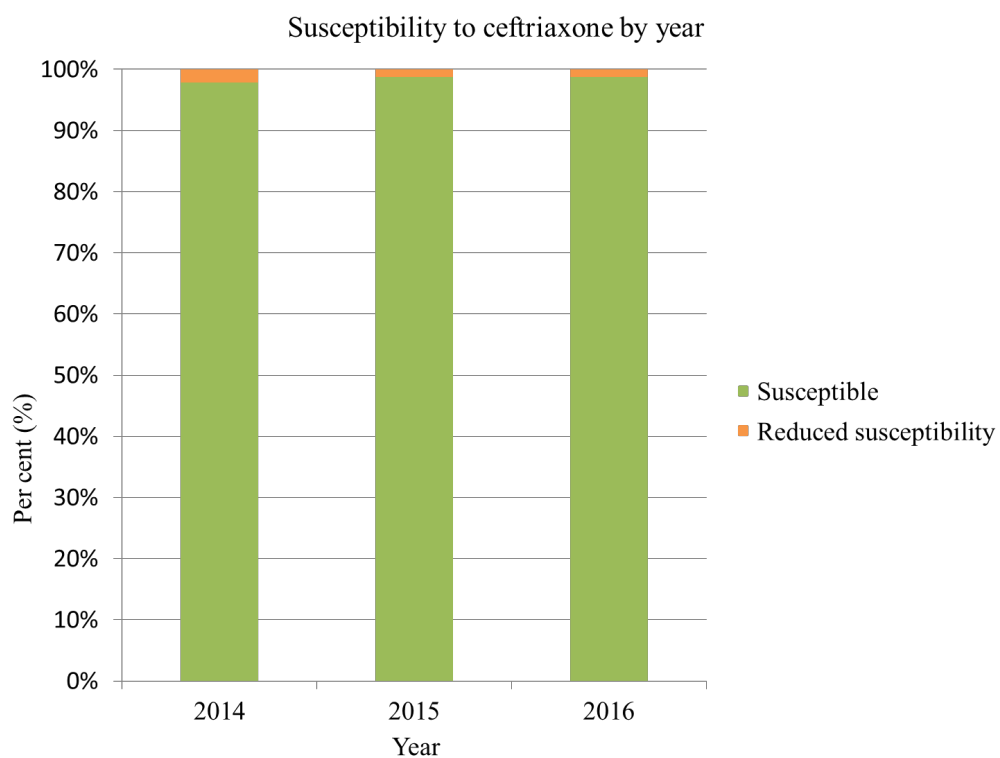


Figure 2.5. Ceftriaxone susceptibility, 2014-2016.

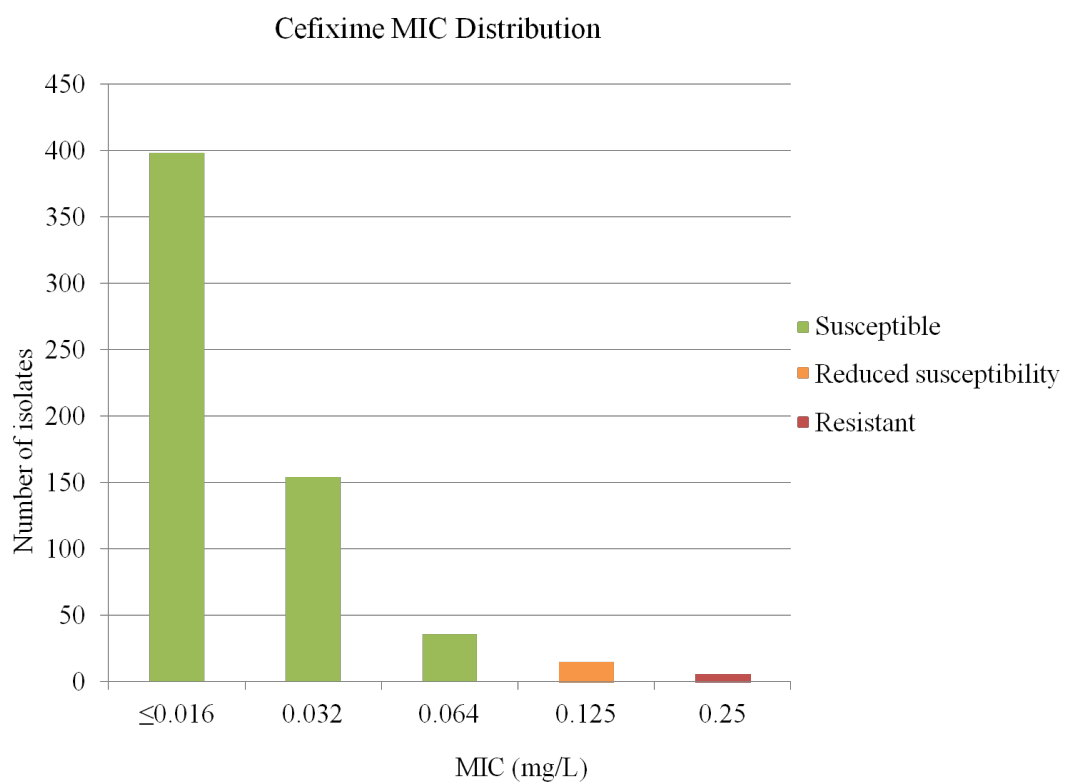


Figure 2.6. Cefixime MIC distribution.

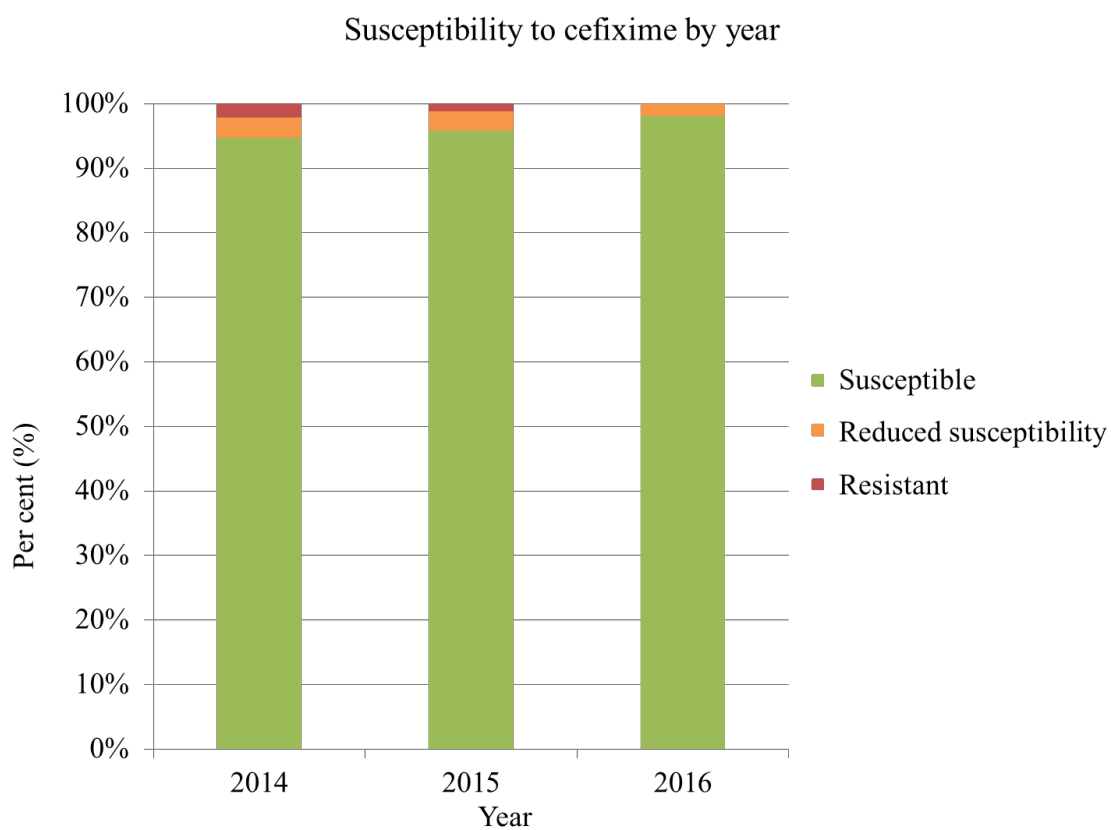


Figure 2.7. Cefixime susceptibility, 2014-2016.

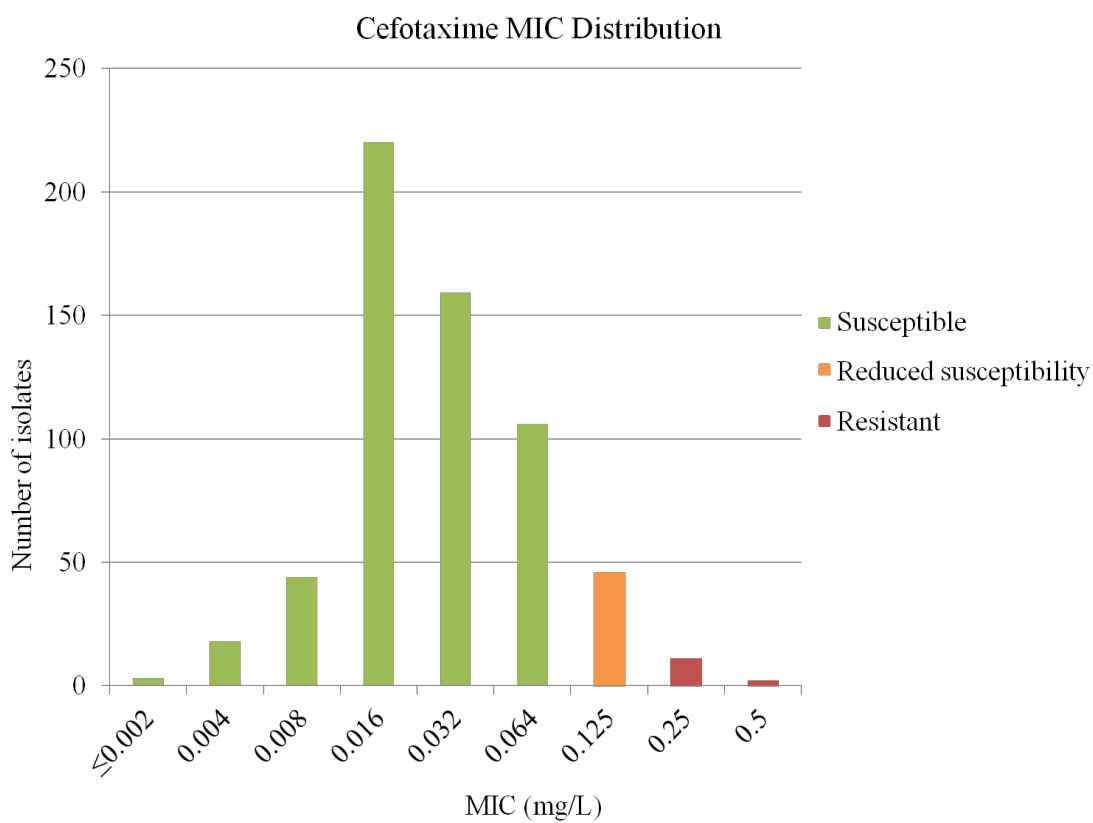


Figure 2.8. Cefotaxime MIC distribution.

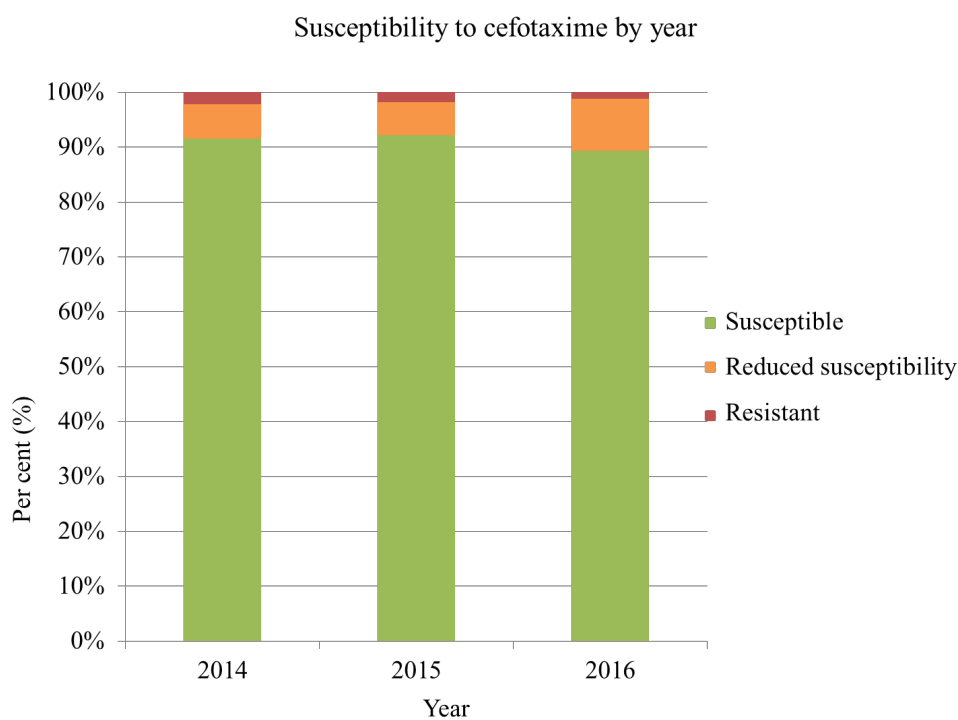


Figure 2.9. Cefotaxime susceptibility, 2014-2016.

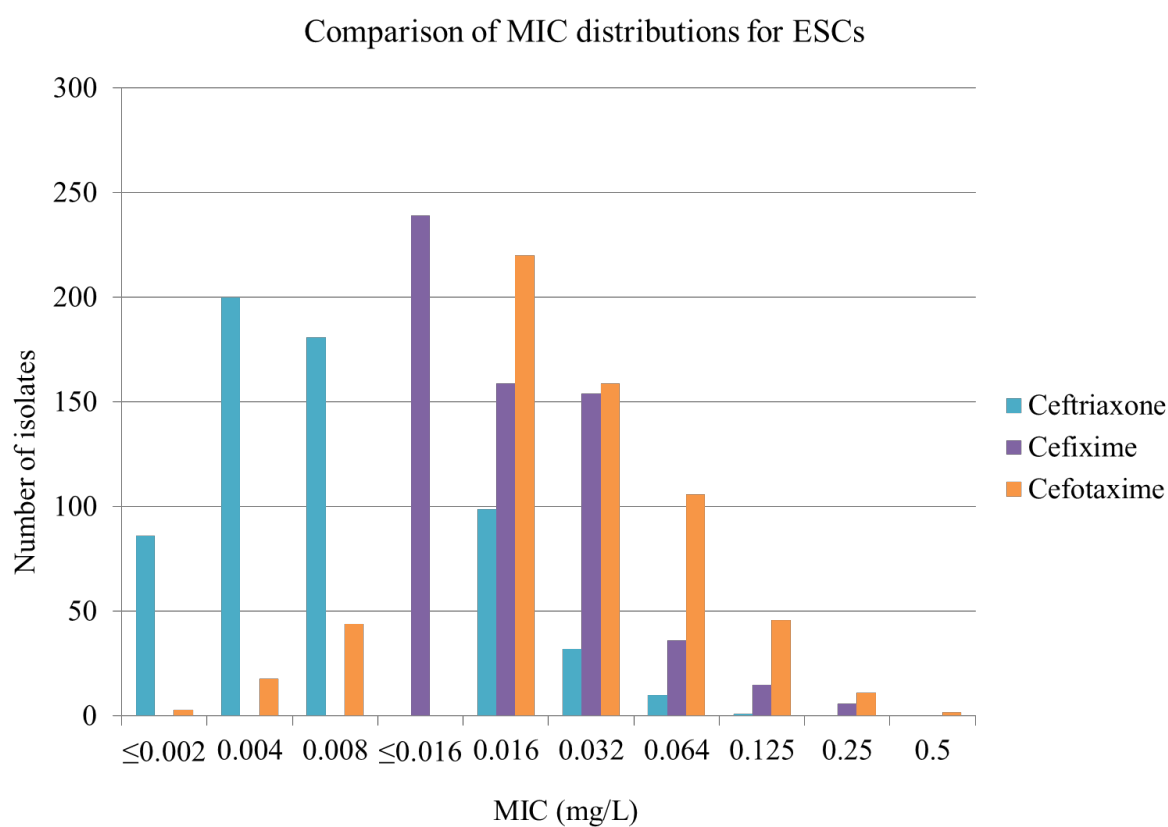


Figure 2.10. MIC distribution for ESCs, ceftriaxone, cefixime and cefotaxime. Of note, the lowest value on the cefixime MIC gradient strip is 0.016 mg/L.

2.6.2 Ertapenem

Ertapenem is an antimicrobial which may be a potential therapeutic option for gonorrhoea. There are no clinical breakpoints provided by EUCAST for ertapenem and *N. gonorrhoeae* and there have been no clinical evaluations of ertapenem for gonorrhoea. The range of MICs determined for the 609 isolates is < 0.002 mg/L to 0.094 mg/L, but over 90% of isolates have ertapenem MICs within a narrow range of 0.008 mg/L to 0.032 mg/L. The MIC₅₀ and MIC₉₀ are 0.016 mg/L and 0.032 mg/L, respectively (Figure 2.11). The ertapenem MIC distribution was compared to those of the three ESCs, ceftriaxone, cefixime and cefotaxime (Figure 2.12 and *Appendices 2-4*) and a comparison of MIC₅₀/MIC₉₀s of each of these antimicrobials was made (Table 2.7). These suggest that ertapenem retains activity against gonococci with reduced susceptibility or resistance to cefixime and cefotaxime, while it appears similarly or even slightly less active than ceftriaxone.

Six isolates were resistant to cefixime (MIC = 0.25 mg/L) and the ertapenem MICs for these isolates were 0.032 mg/L (n = 3), 0.064 mg/L (n = 2) and 0.125 mg/L (n = 1), i.e. mostly two doubling dilutions lower than cefixime. There were 15 isolates with a cefixime MIC at the breakpoint (0.125 mg/L) and the majority of these (11, 73.3%) had an ertapenem MIC of 0.032 mg/L, two doubling dilutions lower, while two and one had ertapenem MICs of 0.064 mg/L and 0.016 mg/L, respectively.

Thirteen isolates were resistant to cefotaxime (MIC > 0.125 mg/L) and for the eleven of these with an MIC of 0.25 mg/L, nine had ertapenem MICs two or three doubling dilutions lower (0.064mg/L (n = 3) and 0.032 mg/L (n = 6), respectively), while the remaining two isolates had ertapenem MICs of 0.016 mg/L (n = 1), and 0.125 mg/L (n = 1). The remaining two cefotaxime resistant isolates had an MIC of 0.5 mg/L and the

ertapenem MICs were 0.064 mg/L (n = 1) and 0.032 mg/L (n = 1), three and four doubling dilutions lower, respectively. Of the 46 isolates with a cefotaxime MIC at the breakpoint (0.125 mg/L) the majority (87%) had an ertapenem MIC of 0.016 mg/L or 0.032 mg/L, while 4 (8.7%) had an ertapenem MIC of 0.064 mg/L and one each had an ertapenem MIC of 0.004 mg/L and 0.008 mg/L.

2.6.3 Azithromycin

Azithromycin is recommended as a first-line treatment agent in combination with ceftriaxone. Over half of the isolates (56%) were susceptible to azithromycin (MIC $\leq$ 0.25 mg/L). One-hundred and fifty-five isolates (25.5%) displayed intermediate resistance, while 113 (18.5%) were resistant (MIC > 0.5 mg/L) according to EUCAST clinical breakpoints (Figure 2.13). (EUCAST 2017) Seven isolates displayed high level azithromycin resistance (HLAzi-R) (MIC > 256 mg/L) (Figure 2.13, Table 2.8). The MIC₅₀ and MIC₉₀ were 0.25 mg/L and 1.0 mg/L, respectively, while the MIC range was $\leq$ 0.016-> 256 mg/L. The distribution of MICs for azithromycin showed the majority of isolates falling in the narrow range of 0.125-0.5 mg/L (448/609, 73.6%), similar to findings in other countries (Figure 2.13). (Regnath Eurosurv 2016) While there was no significant change in the total number of resistant isolates between 2014 and 2016, HLAzi-R isolates were identified in 2015 and 2016 only (Figure 2.14).

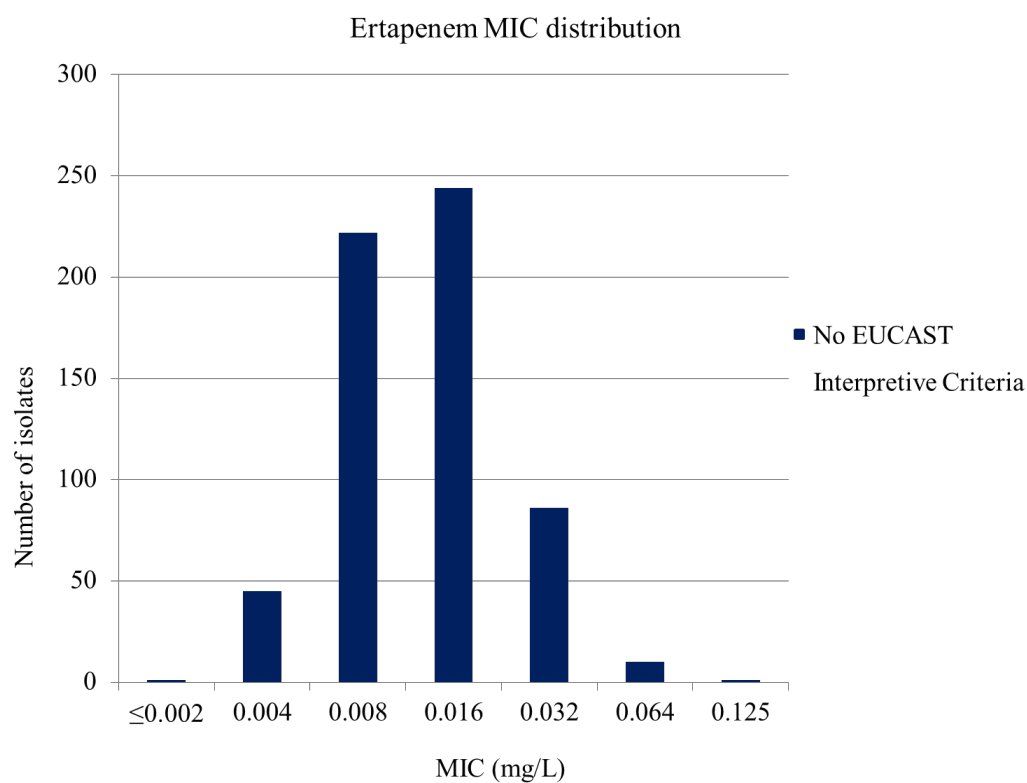


Figure 2.11. Ertapenem MIC distribution.

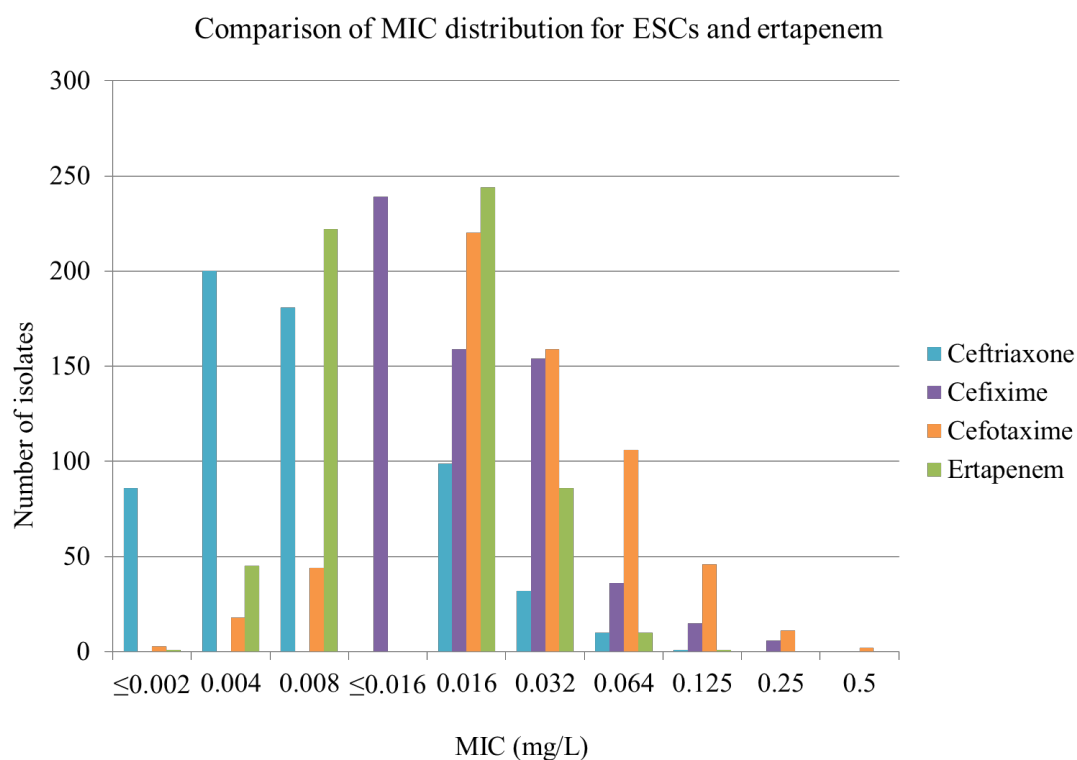


Figure 2.12. Ertapenem MIC distribution compared to those of ceftriaxone, cefixime and cefotaxime.

Antimicrobial	MIC ₅₀	MIC ₉₀	MIC range
Ertapenem	0.016 mg/L	0.032 mg/L	≤ 0.002-0.094 mg/L
Ceftriaxone	0.008 mg/L	0.016 mg/L	≤ 0.002-0.125 mg/L
Cefixime	0.016 mg/L	0.032 mg/L	≤ 0.016-0.25 mg/L
Cefotaxime	0.032 mg/L	0.064 mg/L	≤ 0.002-0.5 mg/L

Table 2.7. Ertapenem MIC₅₀, MIC₉₀ and MIC range compared to those of ceftriaxone, cefixime and cefotaxime.

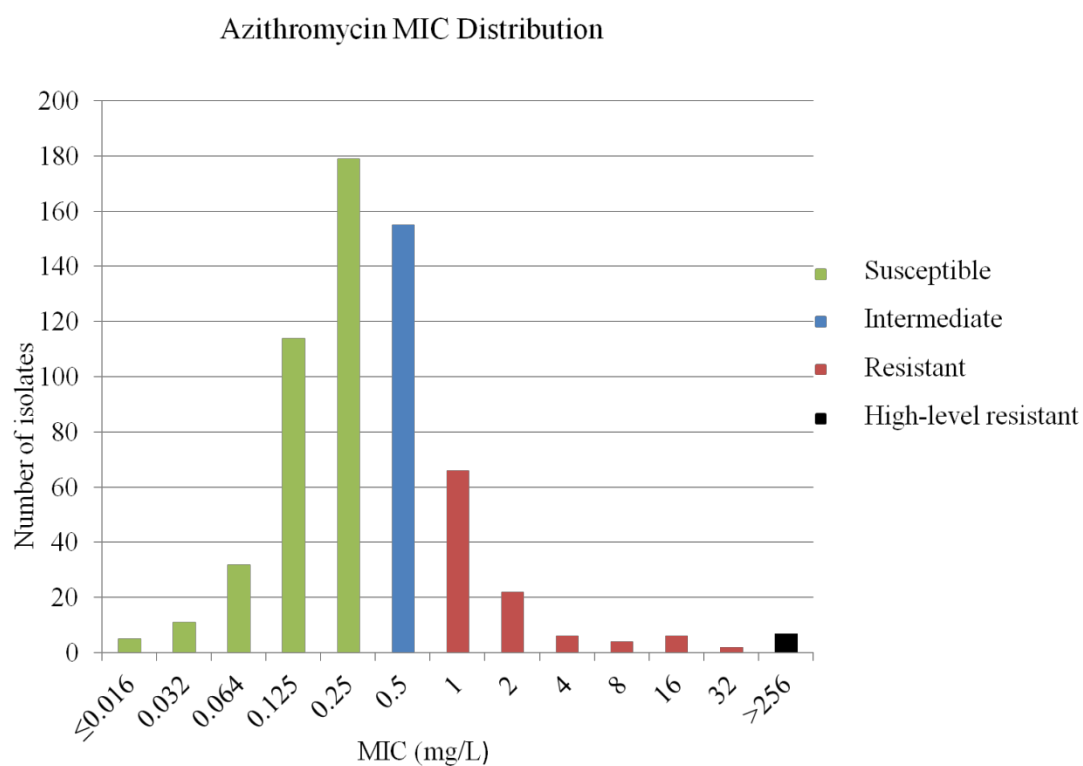


Figure 2.13. Azithromycin MIC distribution.

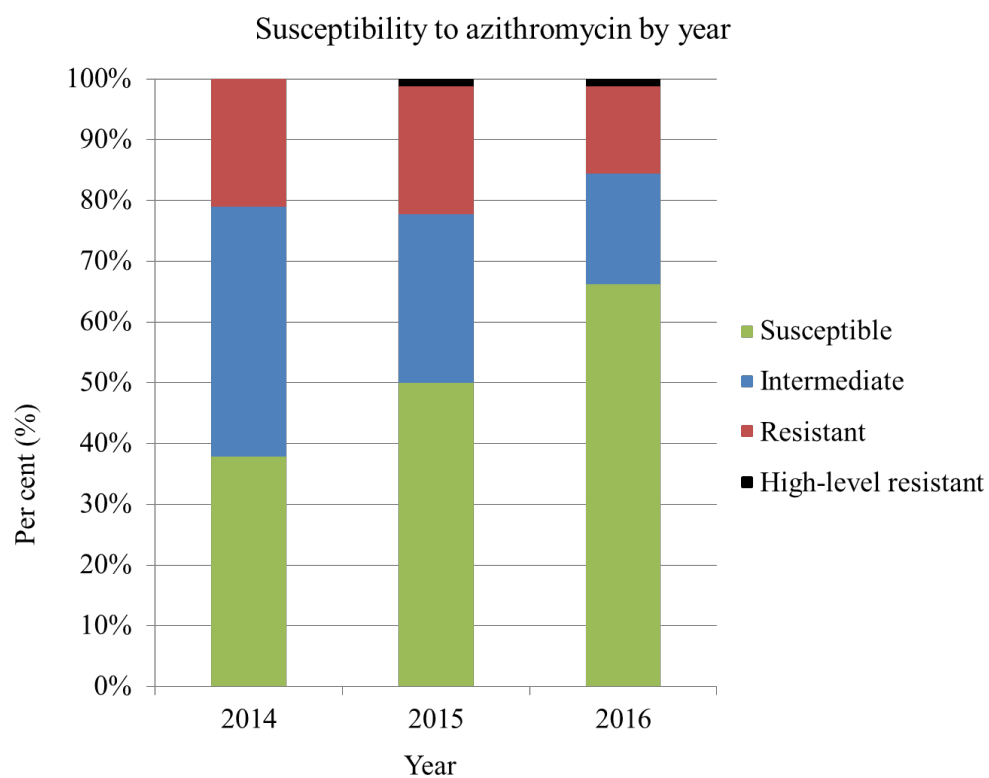


Figure 2.14. Azithromycin susceptibility by year, 2014-2016.

HL-AziR isolates: MICs to other antimicrobials													
Isolate	Pen	Cip	CRO	CFM	CTX	Tet	ERT	CN	GEM	SPC	FOS	SOL	BL
199856	0.125	0.004	0.002	≤ 0.016	0.004	0.38	0.008	3	≤ 0.002	8	8	0.5	Neg
196712	0.25	0.008	0.008	0.016	0.125	0.75	0.023	6	0.006	16	12	>32	Neg
193852	0.125	≤ 0.002	0.004	≤ 0.016	0.006	0.38	0.023	4	≤ 0.002	8	8	0.38	Neg
191587	0.25	0.008	0.008	0.032	0.064	1	0.016	3	0.004	8	12	>32	Neg
006	0.25	0.012	0.016	0.016	0.032	1	0.012	4	0.006	8	16	>32	Neg
181798	0.25	0.008	0.008	0.023	0.047	0.75	0.023	4	0.006	8	24	6	Neg
178487	0.19	0.008	0.006	0.023	0.047	0.5	0.032	4	0.004	6	12	1	Neg

Table 2.8. HLAzi-R isolates- MICs (mg/L) to penicillin (Pen), ciprofloxacin (Cip), ceftriaxone (CRO), cefixime (CFM), cefotaxime (CTX), tetracycline (Tet), ertapenem (Ert), gentamicin (CN), spectinomycin (Spc), fosfomycin (Fos), solithromycin (SOL) and β-lactamase (BL).

2.6.4 Ciprofloxacin

Rates of resistance to ciprofloxacin in *N. gonorrhoeae* are known to be high worldwide, so much so that again it is no longer a viable empiric treatment option for gonorrhoea. Approximately one-third of the isolates, 201 (33%), were resistant to ciprofloxacin (MIC > 0.064 mg/L). The remaining 67% (408) tested susceptible (MIC ≤ 0.032 mg/L). The distribution of MICs for ciprofloxacin showed a bimodal distribution (Figure 2.15) and the MIC₅₀ and MIC₉₀ values were 0.008 mg/L and 2 mg/L, respectively, while the MIC range was ≤ 0.002- > 32 mg/L.

2.6.5 Gemifloxacin

Gemifloxacin has the same mechanism of action as ciprofloxacin and better activity against *N. gonorrhoeae*, as discussed (Section 1.14.5), however, no clinical breakpoints are provided by EUCAST. The MIC range for the 609 isolates is ≤ 0.002 mg/L to 16 mg/L and the MIC₅₀ and MIC₉₀ are 0.008 mg/L and 2 mg/L, respectively (Figure 2.16). Similar to ciprofloxacin, gemifloxacin has a bimodal MIC distribution (Figures 2.15 & 2.16). The gemifloxacin MIC₅₀ and MIC₉₀ for ciprofloxacin-susceptible isolates are 250-fold lower compared to those for the ciprofloxacin-resistant isolates (Table 2.9).

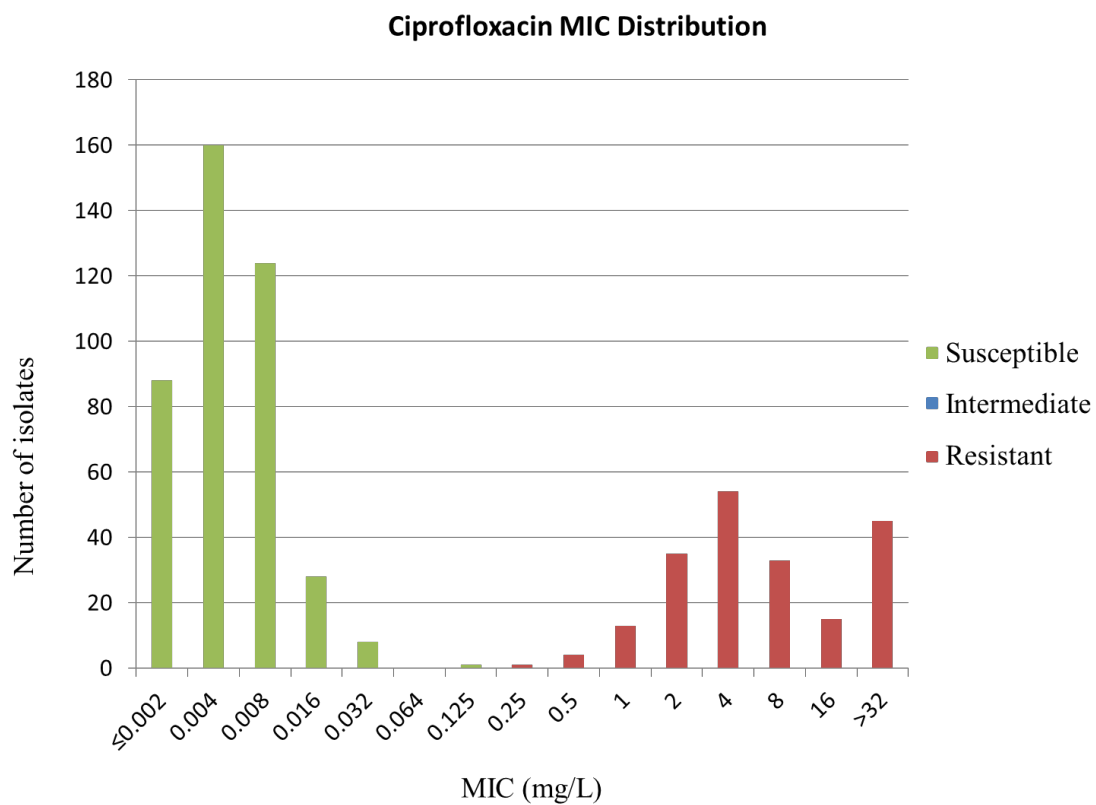


Figure 2.15. Ciprofloxacin MIC distribution.

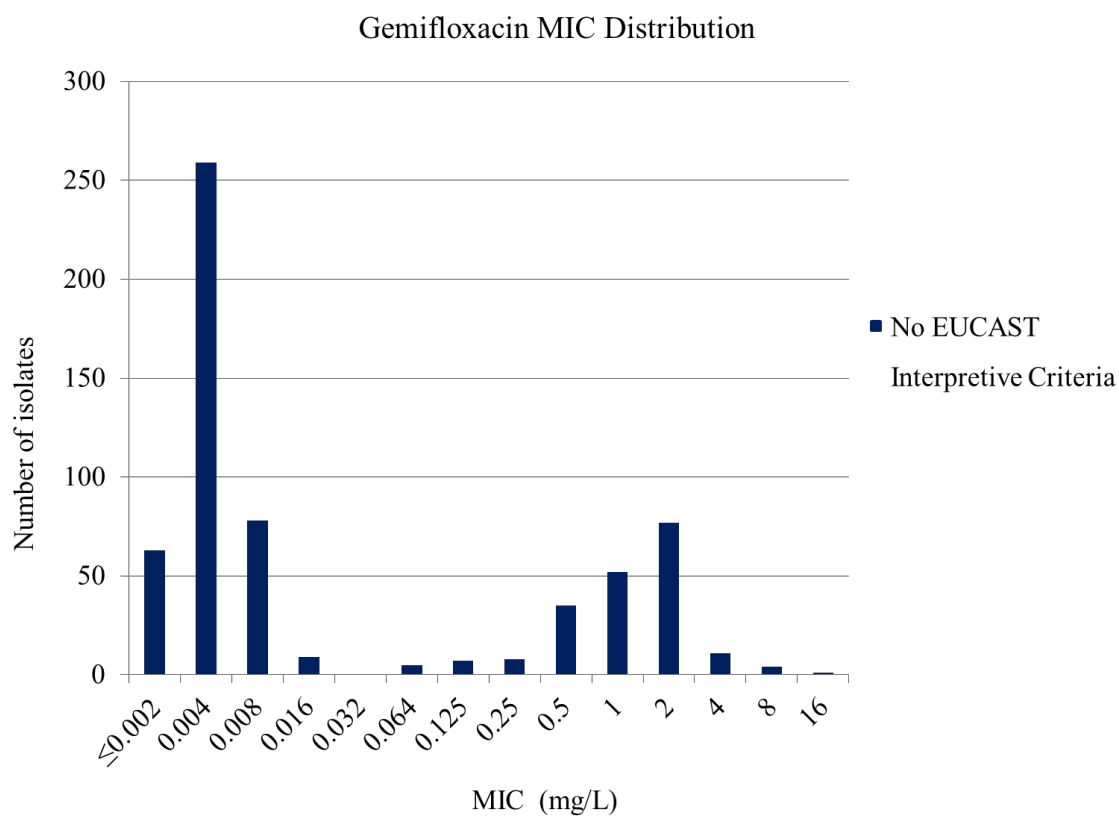


Figure 2.16. Gemifloxacin MIC distribution.

Isolates (number)	Gemifloxacin		
	MIC ₅₀	MIC ₉₀	MIC range
Ciprofloxacin susceptible (408)	0.004 mg/L	0.008 mg/L	≤ 0.002-1 mg/L
Ciprofloxacin resistant (201)	1.0 mg/L	2.0 mg/L	2-16 mg/L

Table 2.9. Gemifloxacin MIC₅₀, MIC₉₀ and MIC ranges for ciprofloxacin susceptible and resistant isolates.

2.6.6 Penicillin

Penicillin was one of the first successful antimicrobials used in the treatment of gonorrhoea. It is no longer part of gonococcal empiric treatment guidelines in Europe or the US given the rates of non-susceptibility (intermediate and resistant) now found in *N. gonorrhoeae*. Of the 609 isolates tested, 11 (1.8%) were found to be susceptible to penicillin ($\text{MIC} \leq 0.06 \text{ mg/L}$) (Figure 2.17). Forty (6.6%) were resistant ($\text{MIC} > 1 \text{ mg/L}$) and the remaining 558 (91.6%) were intermediate resistance. The MIC_{50} and MIC_{90} were 0.25 mg/L and 1 mg/L, respectively. The MIC range was 0.016- > 32 mg/L (Figure 2.17).

2.6.7 β -lactamase production

Thirty-seven isolates tested positive for beta-lactamase production. There was 100% correlation between the Hodge plate and cefinase disc tests. The range of penicillin MICs for these 37 isolates was 0.25 mg/L to > 32 mg/L (Figure 2.18). Four of these isolates had an MIC of 1 mg/L or less, categorising them as intermediately resistant, but a positive β -lactamase result indicated they were penicillin resistant (EUCAST ref). Therefore, the overall number of isolates tested categorised as resistant, intermediate resistance and susceptible to penicillin was 44 (7.2%), 554 (91%) and 11 (1.8%), respectively.

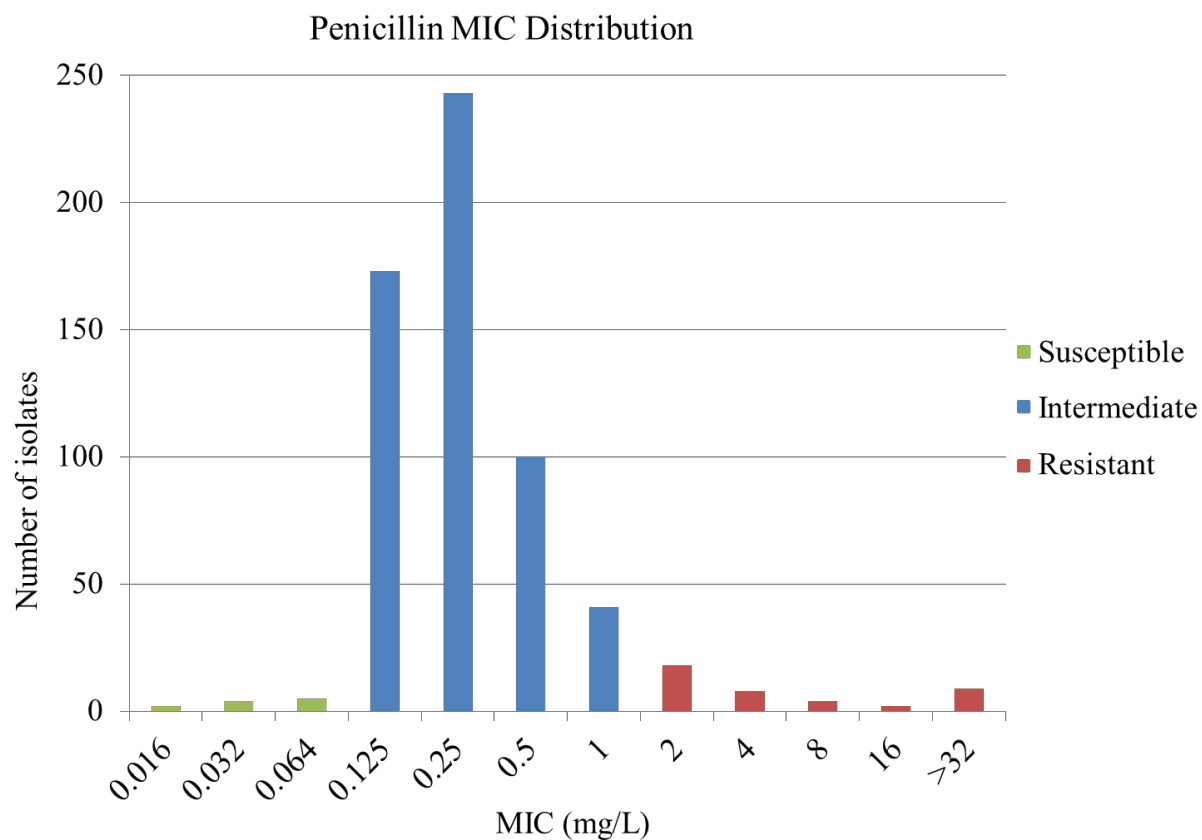


Figure 2.17. Penicillin MIC distribution

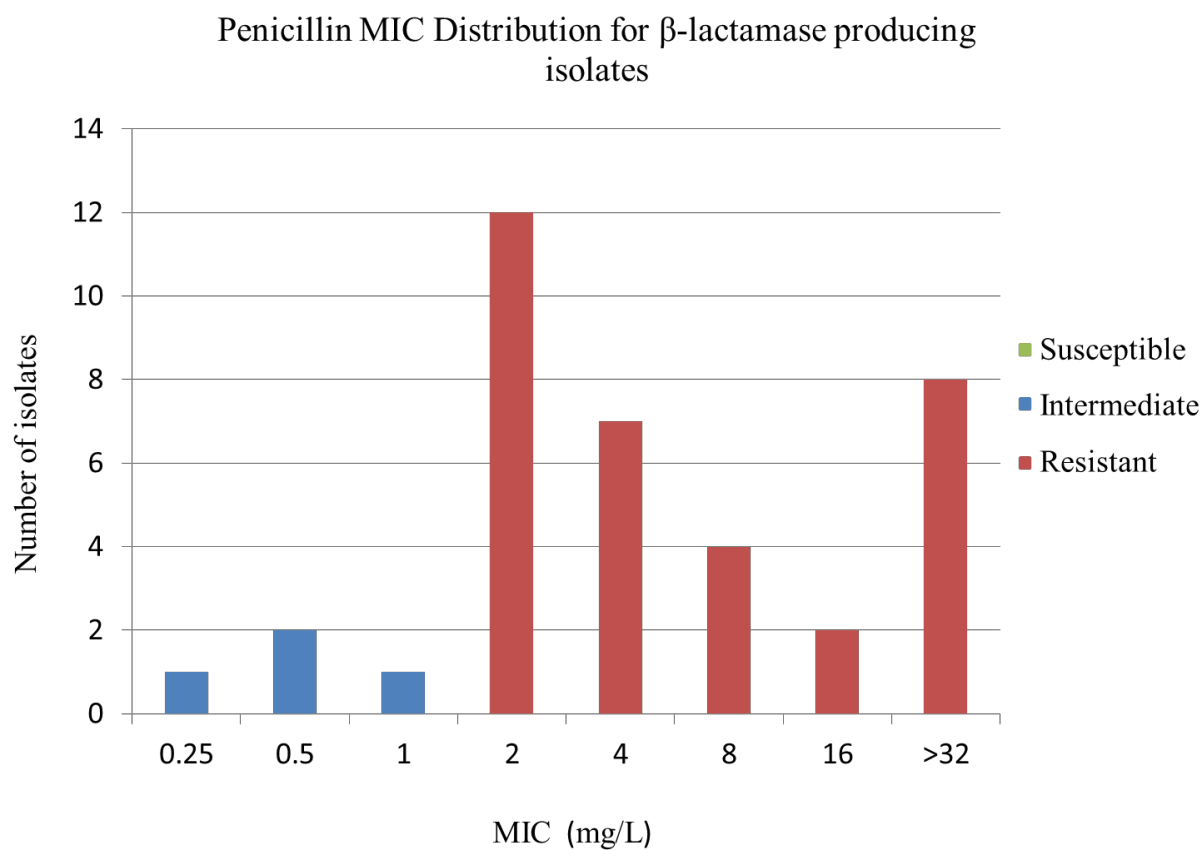


Figure 2.18. Penicillin MIC distribution for beta-lactamase producing isolates.

2.6.8 Tetracycline

Tetracycline is no longer used empirically in the treatment of gonorrhoea and the majority of isolates in this study tested susceptible (465/609, 76.3%) ($\text{MIC} \leq 0.5 \text{ mg/L}$). Sixty isolates (9.9%) were resistant ($\text{MIC} > 1 \text{ mg/L}$) and the remaining 84 (13.8%) isolates were of intermediate resistance. The MIC_{50} and MIC_{90} are 0.5 mg/L and 1 mg/L, respectively, while the MIC range is 0.032-32 mg/L (Figure 2.19).

2.6.9 Spectinomycin

Spectinomycin is rarely used in most European countries and often there is limited availability. All isolates were susceptible to spectinomycin ($\text{MIC} < 64 \text{ mg/L}$). The MIC_{50} and MIC_{90} were 8 mg/L and 16 mg/L respectively, with an MIC of range is 4-32 mg/L (Figure 2.20).

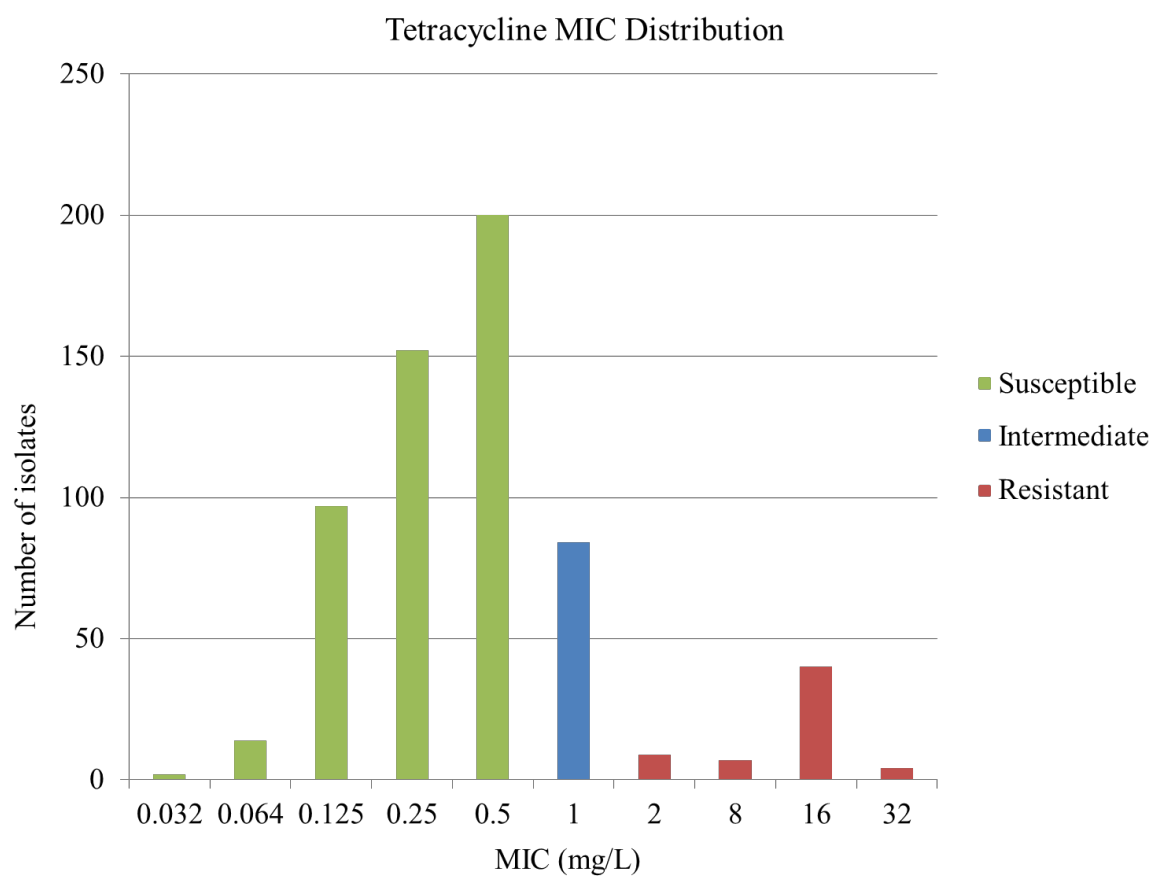


Figure 2.19. Tetracycline MIC distribution.

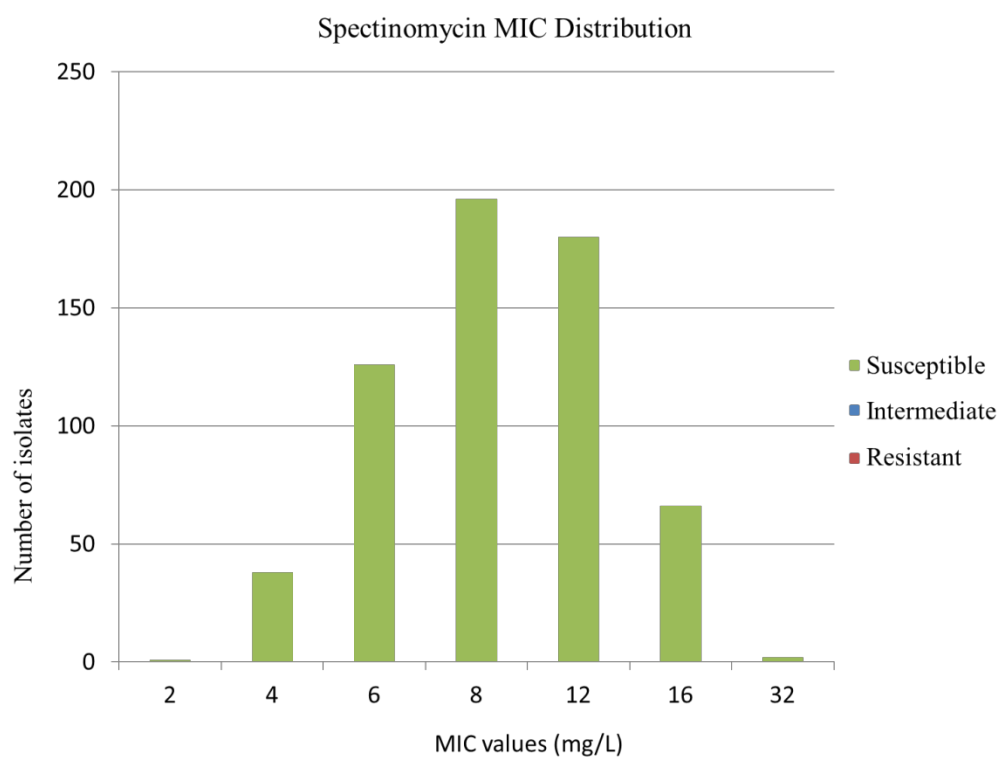


Figure 2.20. Spectinomycin MIC distribution.

2.6.10 Gentamicin

Gentamicin has been in use for many years in Malawi for syndromic management of urethritis in combination with doxycycline (Brown *et al.*, 2010). There have been some reports pertaining to clinical success of gentamicin specifically related to gonorrhoea urethritis (Lule 1994 GUM). However, there is unfortunately no literature regarding gentamicin MICs associated with treatment failures. There are no clinical breakpoints for gentamicin and *N. gonorrhoeae* provided by EUCAST or CLSI, but proposed breakpoints have been suggested, as discussed (*Section 1.14.2*). The MIC range for the isolates tested is 1-16 mg/L and the MIC₅₀ and MIC₉₀ are 4 mg/L and 8 mg/L, respectively (Figure 2.21).

2.6.11 Fosfomycin

While clinical trials have been performed assessing the clinical cure rate of various dosing regimens of fosfomycin in the treatment gonococcal urethritis, there are no reports of fosfomycin MICs associated with clinical failure and neither EUCAST nor CLSI provide clinical breakpoints for fosfomycin susceptibility testing of *N. gonorrhoeae*. The MIC range for the isolates tested is 4 mg/L to 128 mg/L and the MIC₅₀ and MIC₉₀ are 16 mg/L and 32 mg/L, respectively (Figure 2.22).

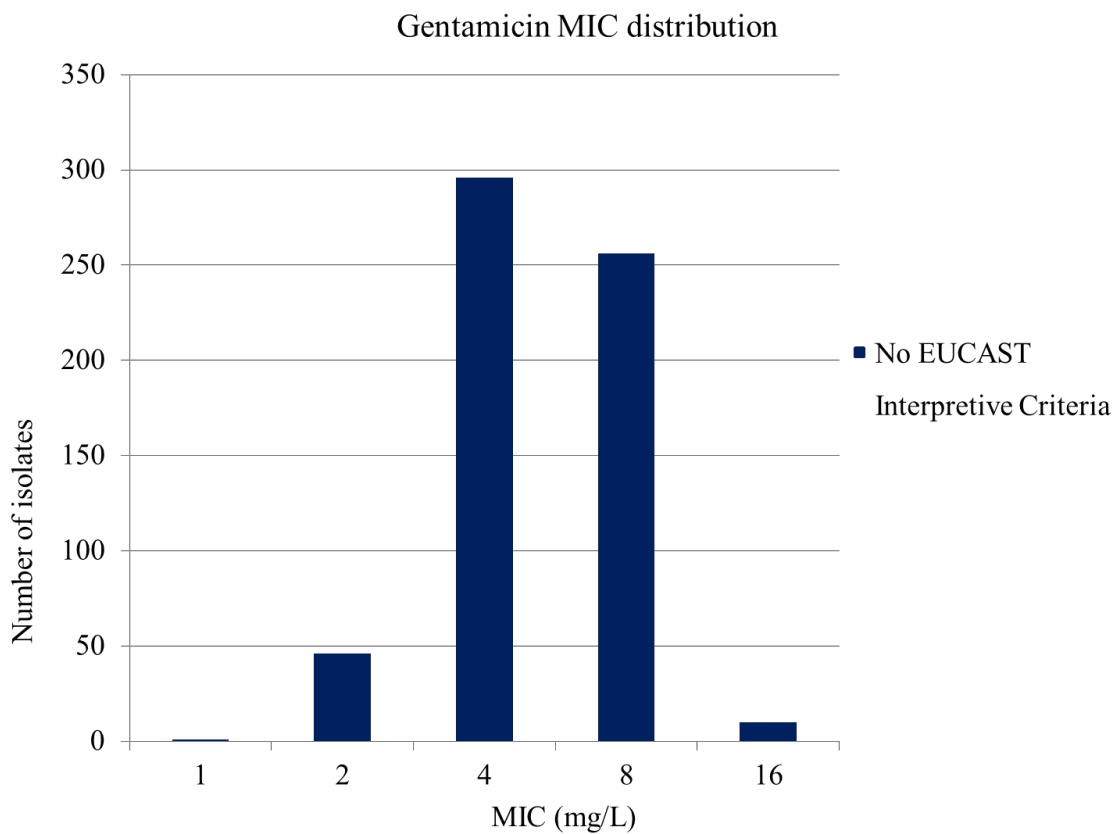


Figure 2.21. Gentamicin MIC distribution.

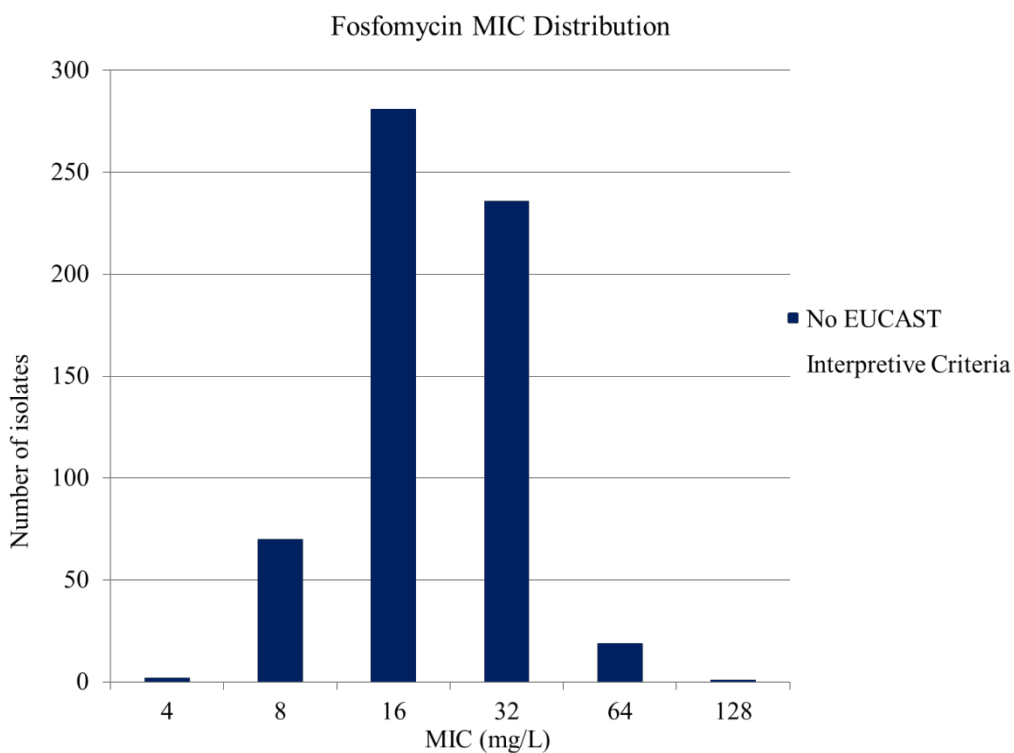


Figure 2.22. Fosfomycin MIC distribution

2.6.12 Solithromycin

Solithromycin, a novel fluoroketolide, has been investigated for use against *N. gonorrhoeae* as well as many other organisms, in particular those causing community acquired pneumonia (Zhanel *et al.*, 2016). As yet there is no commercial solithromycin MIC gradient strip available, but there is one available for research purposes only (Liofilchem® Roseto degli Abruzzi, Italy). Ninety-six selected isolates in this study had solithromycin MICs determined by agar dilution and gradient MIC strip. The same isolates also had zone diameters determined by disc diffusion. Gonococci with the highest azithromycin MICs in this study were selected for testing against solithromycin as it may be a useful alternative treatment agent for isolates already resistant to azithromycin (Golparian *et al.*, 2012). Agar dilution was performed as described above, but MIC results appeared very inconsistent with occasional poor growth on drug-free agar, so using *N. gonorrhoeae* ATCC 49226 control, one set of agar dilution plates were inoculated 12 times resulting in highly variable MICs with only 7 within the QC pass range (*Appendix 5*). The solithromycin agar dilution MIC results were therefore deemed unreliable (*Appendix 6*). Later a field safety notice was issued (June 29th 2017) regarding the GC agar base (Thermoscientific™ Oxoid™ CM0367) which stated that the combination of agar base and an isovitalex-type additive resulted in poor growth of *N. gonorrhoeae*. Therefore, solithromycin MICs as determined by MIC gradient strip only are shown here.

The azithromycin MIC₅₀/MIC₉₀ and MIC range of these 96 isolates tested were 1/16 mg/L and 0.75-> 256 mg/L, respectively, all resistant by EUCAST criteria. The solithromycin MIC₅₀/MIC₉₀ and MIC range of these isolates were 0.125/0.25 mg/L and 0.032-> 32 mg/L, respectively (Figure 2.23). The zone diameters ranged from 12 – 40

mm and the median was 33 mm (Figure 2.24). The solithromycin zone diameters correlate well with MICs. Seven HLAzi-R isolates were included and the solithromycin MICs (zone diameters) for these seven isolates were 0.38 mg/L (37 mm), 0.5 mg/L (31 mm), 1 mg/L (26 mm), 6 mg/L (18 mm), >32 mg/L (12 mm), >32 mg/L (12 mm), and >32 mg/L (12 mm). Overall the MICs of solithromycin were considerably lower than those of azithromycin (Figure 2.25) and it is a potential therapeutic option for gonorrhoea.

2.7 Comparison of antimicrobial susceptibility of pharyngeal, rectal and urethral *Neisseria gonorrhoeae* isolates

As discussed above (Section 2.5), there were 320, 162 and 96 isolates of *N. gonorrhoeae* from urethral, rectal and pharyngeal samples, respectively. The azithromycin MIC distribution (%) according to site of sample showed a trend towards increased MICs for isolates from the pharynx compared to those from urogenital sites (Figure 2.26). Twenty-six percent (25/96) of isolates sampled from the pharynx were resistant to azithromycin (MIC > 0.5 mg/L) compared to approximately 12% (20/162) and 18% (59/320) of those sampled from the rectum and ‘urogenital’, respectively (Figure 2.27).

The ceftriaxone, cefixime and cefotaxime MIC distributions (%) according to site samples were similar for each site sampled with no obvious trend towards higher MICs in isolates from any particular site (Figures 2.28-2.30).

The penicillin and tetracycline MIC distributions were similar for each site sampled, but there was a trend towards higher ciprofloxacin MICs for rectal and pharyngeal isolates (Figures 2.31-2.33).

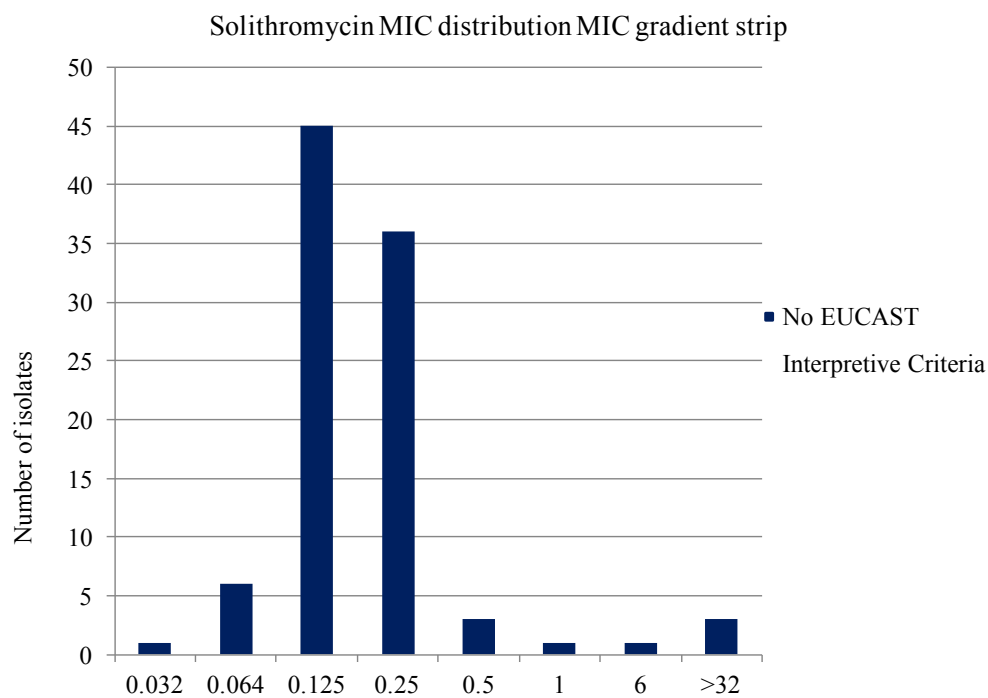


Figure 2.23. Solithromycin MIC distribution for 96 *N. gonorrhoeae* isolates.

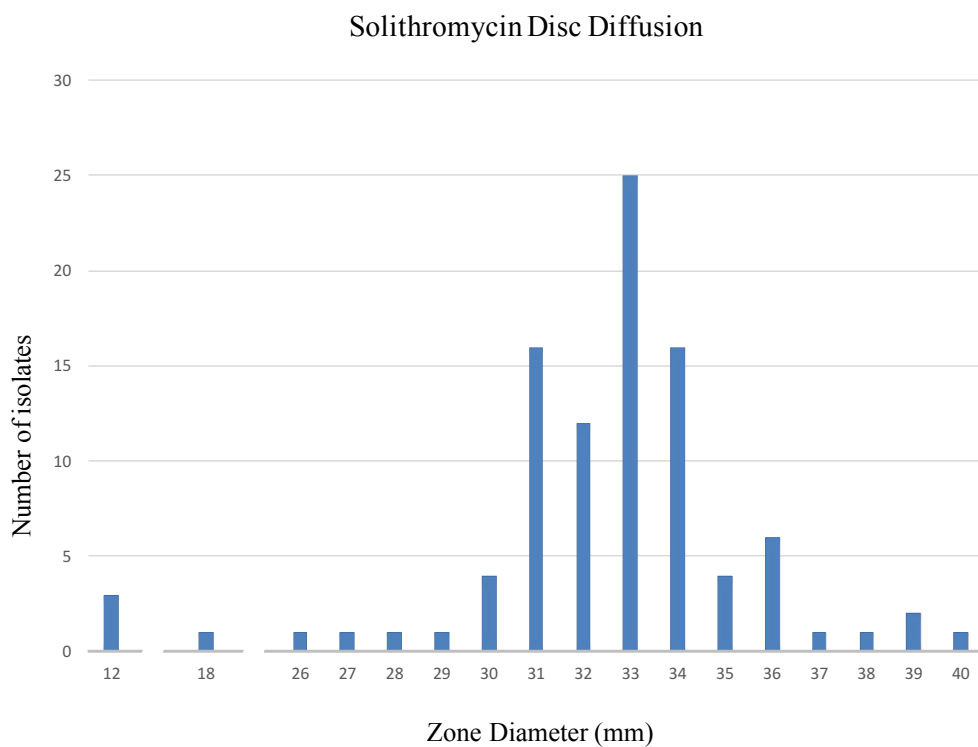


Figure 2.24. Solithromycin zone diameter distribution for 96 *N. gonorrhoeae* isolates.

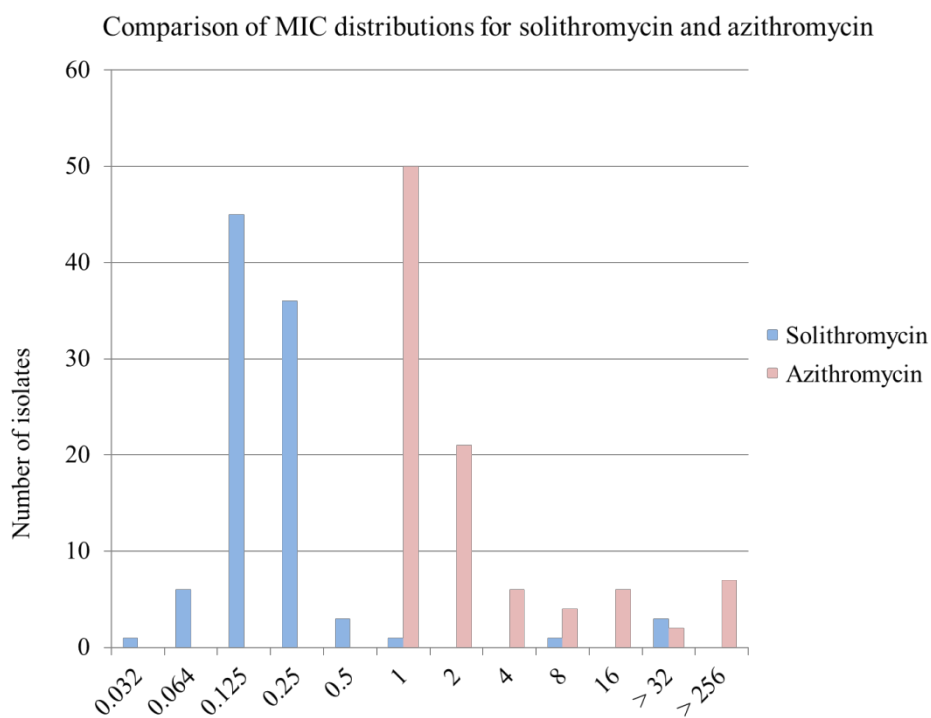


Figure 2.25. Solithromycin (gradient MIC) and azithromycin MIC distributions for 96 *N. gonorrhoeae* isolates.

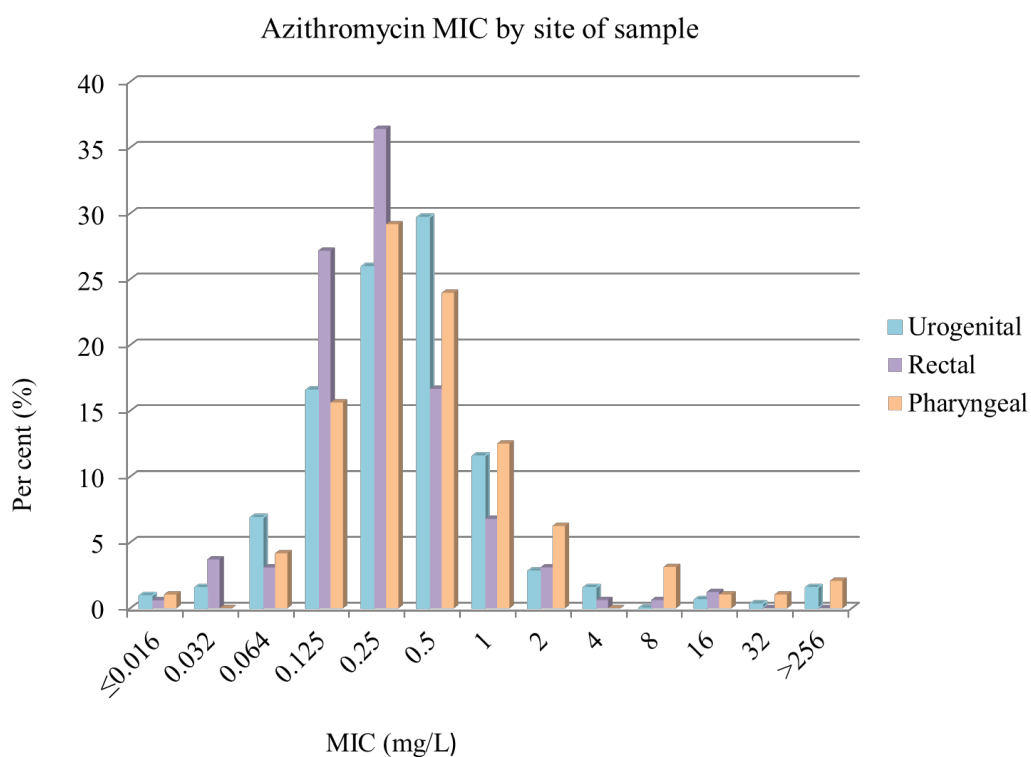


Figure 2.26. Azithromycin MIC by sample site.

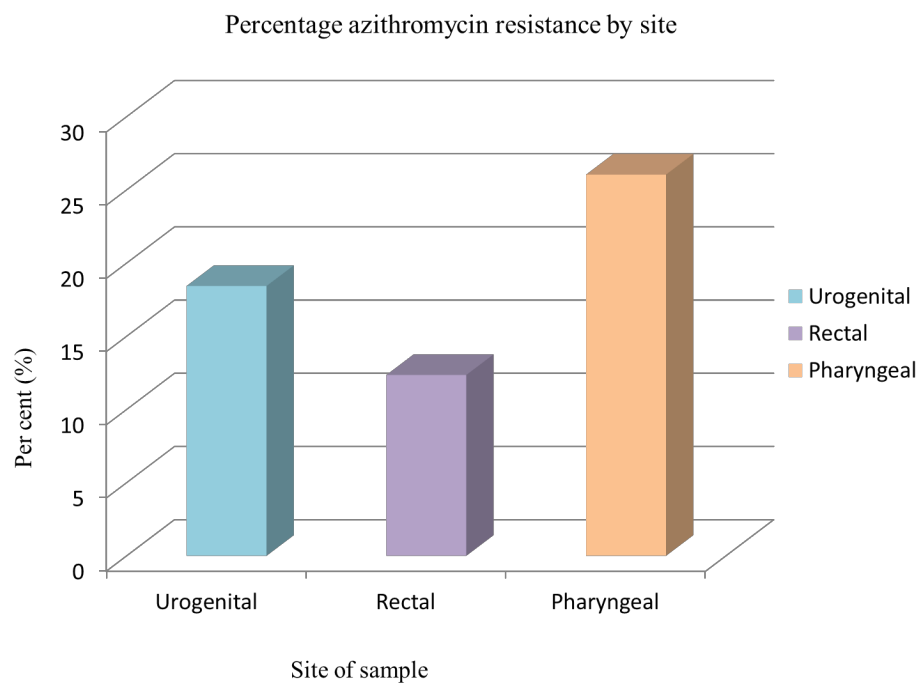


Figure 2.27. Percentage azithromycin resistance in urogenital, rectal and pharyngeal isolates.

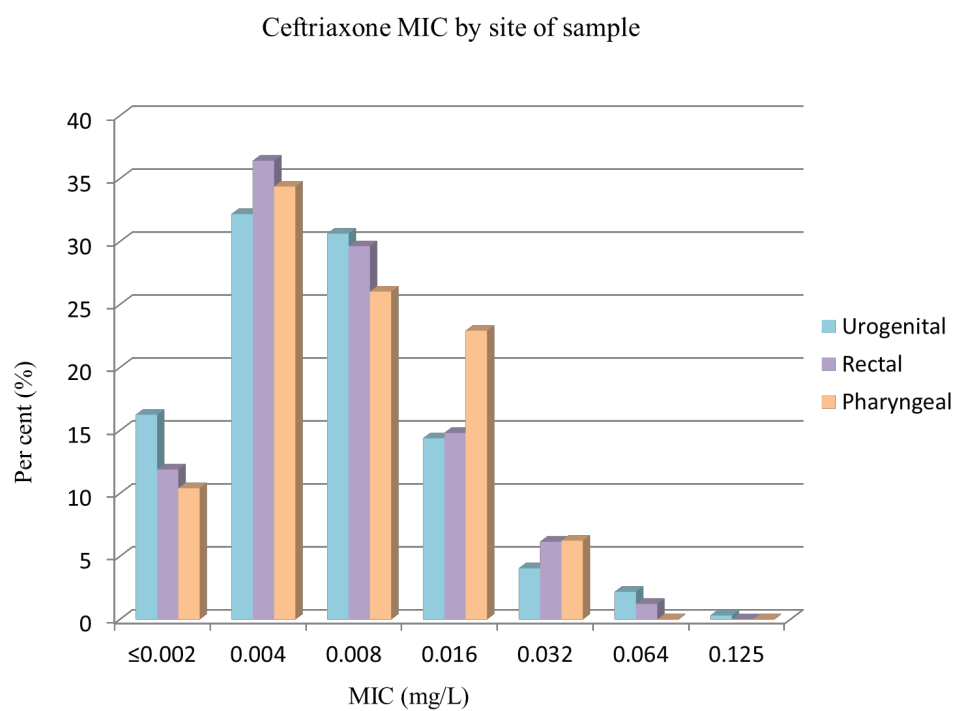


Figure 2.28. Ceftriaxone MIC distribution (%) according to site of sample.

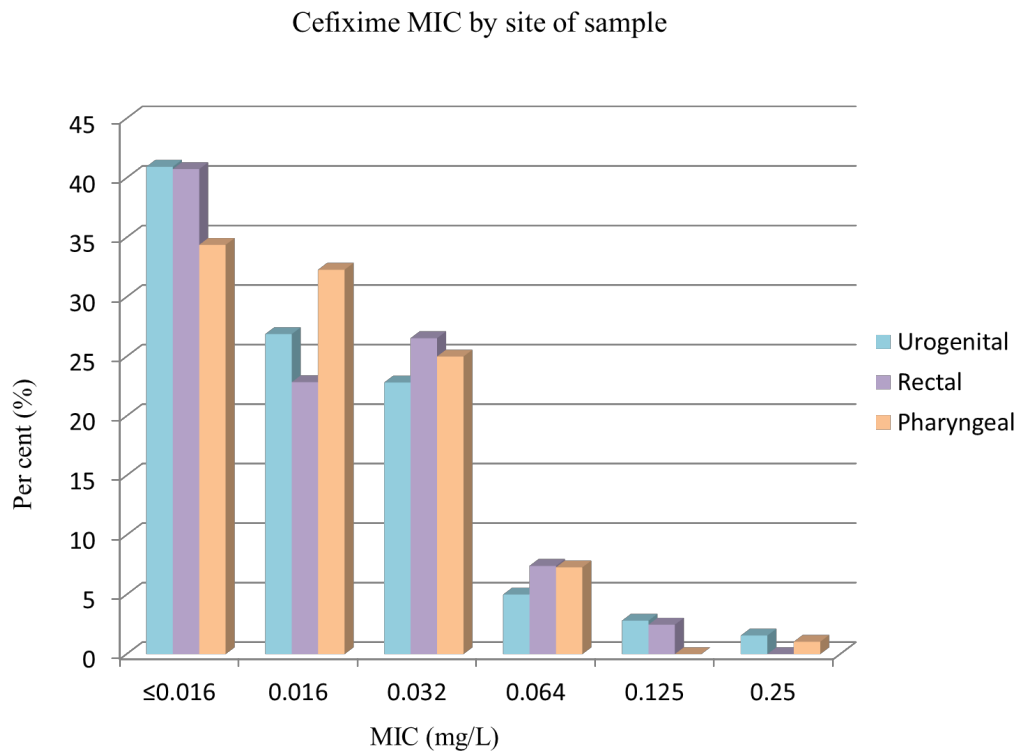


Figure 2.29. Cefixime MIC distribution (%) according to site of sample.

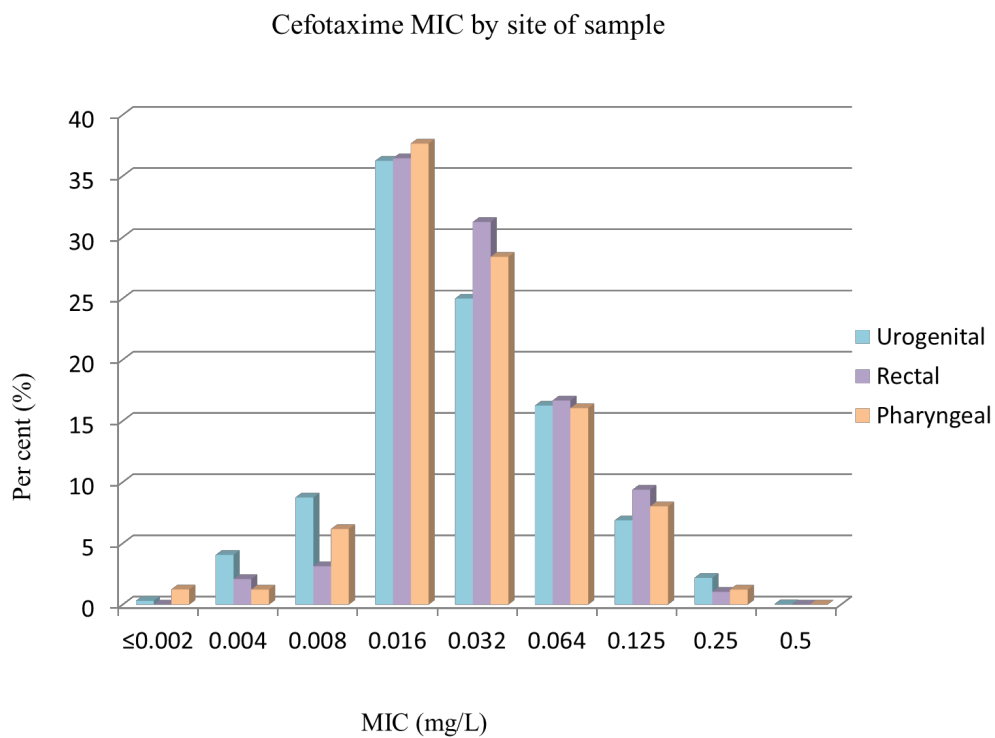


Figure 2.30. Cefotaxime MIC distribution (%) by site sampled.

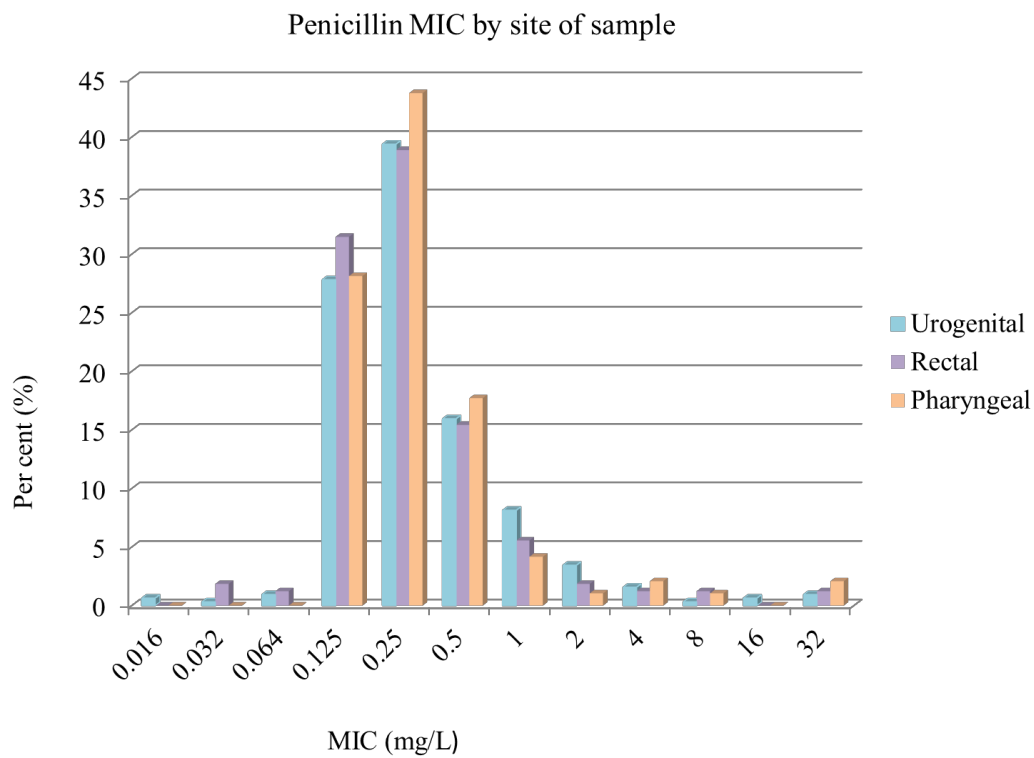


Figure 2.31. Penicillin MIC distribution (%) by site sampled.

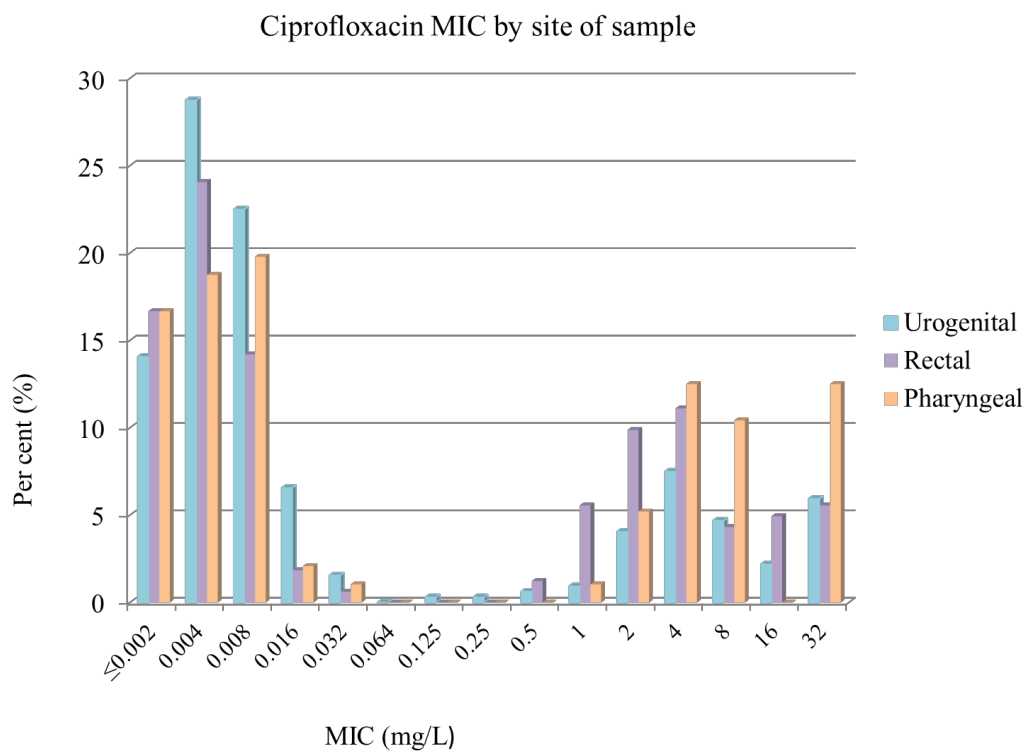


Figure 2.32. Ciprofloxacin MIC distribution (%) by site sampled.

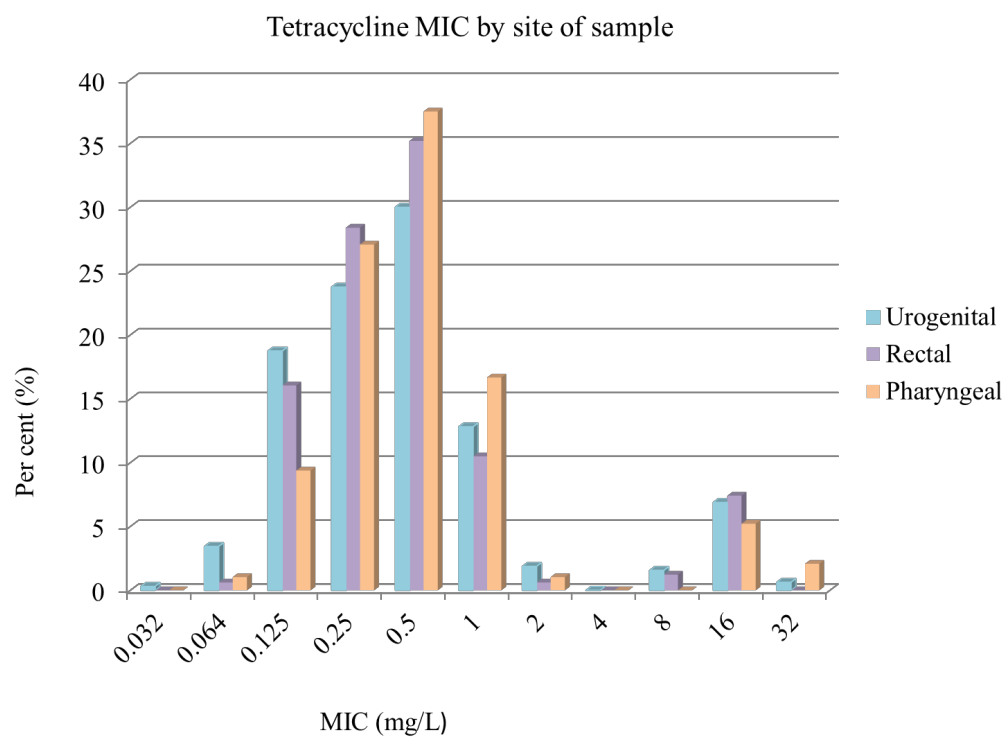


Figure 2.33. Tetracycline MIC distribution (%) by site sampled.

2.8 Resistance to more than one antimicrobial

A number of isolates in this study display resistance, reduced susceptibility or non-susceptibility to more than one antibiotic (Table 2.6). A summary of the number of isolates resistant to more than one antimicrobial is shown (Table 2.10). Reduced susceptibility to ceftriaxone, cefixime and cefotaxime were defined as MIC > 0.032 mg/L, MIC = 0.125 mg/L and MIC = 0.125 mg/L, respectively. Isolates non-susceptible (including those resistant) to penicillin (MIC > 0.064 mg/L), ciprofloxacin (MIC > 0.032 mg/L), azithromycin (MIC > 0.25 mg/L), tetracycline (MIC > 0.5 mg/L) were included. There was no resistance to spectinomycin detected in the isolates, therefore it was not included. Reduced susceptibility to ceftriaxone was detected in 11 isolates as discussed above; the MICs to the other antimicrobials tested are shown (Table 2.11). Of these, seven also display reduced-susceptibility or resistance to cefixime and cefotaxime. Importantly, 7 of these 11 isolates were also non-susceptible to azithromycin (MIC range 0.5-1.0 mg/L).

Seven of the 609 isolates display non-susceptibility to penicillin, azithromycin, ciprofloxacin, tetracycline as well as reduced susceptibility or resistance to ceftriaxone, cefixime and cefotaxime. Excluding ESCs, 54 isolates were non-susceptible to penicillin, ciprofloxacin, azithromycin and tetracycline.

Antimicrobials	Number (%)
PEN / CIP	199 (32.7)
PEN / CIP / AZM	71 (11.7)
PEN / CIP / AZM / CRO	8 (1.3)
PEN / CIP / AZM / CRO / CFM	7 (1.2)
PEN / CIP / AZM / CRO / CFM / CTX	6 (1)
PEN / CIP / AZM / CRO / CFM / CTX / TET	5 (0.8)
PEN / CIP / AZM / TET	54 (8.9)
CRO / CFM	8 (1.3)
CRO / CFM / CTX	7 (1.2)
AZM / CRO	7 (1.2)
BL / CIP	27 (4.4)
BL / AZM / CIP	5 (0.8)

Table 2.10. Numbers (%) of isolates non-susceptible (or reduced susceptibility/resistant in the case of ESCs) to multiple antimicrobials. PEN = penicillin non-susceptible (MIC > 0064 mg/L), CIP = ciprofloxacin non-susceptible (MIC > 0.032 mg/L), AZM = azithromycin non-susceptible (MIC > 0.25 mg/L), TET = tetracycline non-susceptible (MIC > 0.5 mg/L), CRO = ceftriaxone reduced susceptibility (MIC > 0.032 mg/L), CFM = cefixime reduced susceptibility and resistance (MIC ≥ 0.125 mg/L), CTX = cefotaxime reduced susceptibility and resistance (MIC ≥ 0.125 mg/L) and BL = β-lactamase positive.

Ceftriaxone reduced susceptibility isolates: MICs to other antimicrobials														
Isolate	PEN	CIP	AZM	CRO	CFM	CTX	TET	ERT	CN	GEM	SPC	FOS	SOL	BL
199020	0.5	4	0.125	0.064	0.064	0.125	1	0.016	6	0.5	6	6	ND	-
168628	1	2	0.25	0.064	0.064	0.25	1	0.012	4	0.38	12	8	ND	-
009	1	> 32	0.75	0.064	0.125	0.125	1.5	0.032	4	1.5	12	48	0.064	-
024	1.5	> 32	0.38	0.094	0.25	0.38	1	0.064	4	1.5	8	48	ND	-
016	2	> 32	1	0.047	0.19	0.25	1	0.032	6	1.5	12	32	0.094	-
035	0.75	> 32	1	0.047	0.125	0.125	0.75	0.032	4	1.5	6	24	0.047	-
180008	0.5	> 32	0.25	0.047	0.047	0.047	0.5	0.023	6	1.5	4	12	ND	-
177574	1	8	0.5	0.047	0.094	0.125	0.5	0.016	3	1	8	16	ND	-
71324	4	> 32	0.125	0.047	0.19	0.19	24	0.094	1.5	3	6	6	ND	+
76972	1.5	> 32	0.75	0.047	0.19	0.25	1	0.032	4	2	12	32	ND	-
422671	0.5	> 32	0.5	0.064	0.125	0.25	2	0.064	4	2	16	12	ND	-

Table 2.11. Ceftriaxone reduced susceptibility isolates. MICs (mg/L) to penicillin (PEN), ciprofloxacin (CIP), ceftriaxone (CRO), cefixime (CFM), cefotaxime (CTX), tetracycline (TET), ertapenem (Ert), gentamicin (CN), spectinomycin (SPC), fosfomycin (FOS), solithromycin (SOL) and β -lactamase (BL). Not determined (ND).

2.9 Discussion

2.9.1 Demographics

The majority of isolates in this study were from male patients, 90.8%, compared to only 7.7% from female patients. This finding is similar in many countries, such as, Italy, Hungary, China, Belarus, France, Germany, (Carannante *et al.*, 2014; Brunner *et al.*, 2014; Chen *et al.*, 2015; Lebedzeu *et al.*, 2015; Regnath *et al.*, 2016; La Ruche Eurosurv 2014) with reports ranging from 75.3% to 90% male patients (Regnath *et al.*, 2016; Carannante *et al.*, 2014). Unfortunately, data were not available in this study on the percentage of patients who are men who have sex with men (MSM). Of the 516 samples from STI clinics, 259 (50.2%) originated from a gay mens health clinic (all but 2 samples from men), which caters mostly for MSM, however assumptions cannot be made based on these figures. The age range (3-75 years) is also similar to other reports, with upper age ranges being reported at up to 76 (Regnath *et al.*, 2016; Brunner *et al.*, 2014) and 85 years of age (Chen *et al.*, 2015). Excluding the isolate from the three-year-old patient in this study, the youngest patient was aged 16 years. No clinical details were available for the three-year-old patient; an isolate for susceptibility testing was received without any further details regarding site of isolation or clinical history. The median age (26 years) is a little lower than reports of median ages of about approximately 30-33 years old in other studies (Carrannante *et al.*, 2014; Regnath *et al.*, 2016).

Genital, rectal and pharyngeal accounted for 52.5%, 26.6% and 15.7%, respectively, of samples in this study which is somewhat different from published reports. Carannante *et al.* (1430 samples, Italy) report 81.5% genital, 11% rectal, 1.2% pharyngeal and La Ruche *et al.* (13,400 samples, France) report 91% genital, 6% rectal and 1% pharyngeal

(Carannante *et al.*, 2014; La Ruche Eurosurv 2014). The proportion of pharyngeal samples in this study is higher than other reports. The majority of samples (84.7%) in this study came from STI clinics. STI clinics in Ireland routinely send pharyngeal samples from all patients in whom gonorrhoea is a presumed diagnosis, accounting for the finding in this study.

2.9.2 ESCs and ertapenem

In Ireland ESC resistance is currently not a significant problem since all isolates were susceptible to ceftriaxone and resistance to cefixime and cefotaxime was detected in a small number of isolates of *N. gonorrhoeae* in this study. While all 609 isolates were susceptible to ceftriaxone, 1.8% (11/609) had MICs consistent with reduced susceptibility (MIC > 0.032 mg/L), a proportion of 1.2% in each of 2015 and 2016, a decrease from 2.1% in 2014. The distribution of ceftriaxone MICs by year (Figure 2.4) showed minimal MIC creep from 2014 compared to 2015 and 2016, yet the MIC₉₀ for 2014, 2015 and 2016 was 0.016 mg/L, i.e. no change over time. Ceftriaxone MIC creep has been reported in Europe from Switzerland where Endimiani *et al.* compared *N. gonorrhoeae* isolates from 1998-2011 to isolates from 2009-2012 and showed the MIC₉₀ for ceftriaxone increased from 0.004 mg/L to 0.047 mg/L between the two periods and the cefixime MIC₉₀ increased from < 0.016 mg/L to 0.125 mg/L, but the number of isolates was very small, 24 in the first period compared to 36 in the second period (Endimiani *et al.*, 2015). Wind *et al.* found that the proportion of CRO-RS (MIC > 0.032 mg/L) in Amsterdam (3,151 isolates) increased from 3.6% in 2012 to 8.4% in 2015 (Wind *et al.*, 2017b). While in Germany Regnath *et al.* found no shift in cefixime or ceftriaxone MICs of 434 isolates spanning 2014 to 2015 (Regnath *et al.*, 2016).

Cefixime resistance in this study was 1% (6/609) with an additional 1.8% (11/609) having reduced susceptibility (MIC = 0.125 mg/L). There were no cefixime-resistant isolates found in 2016 and the MIC₉₀ decreased from 0.064 mg/L in 2014 and 2015 to 0.032 mg/L in 2016 which is similar to the findings in other studies, likely reflecting the withdrawal of cefixime from use in empiric treatment of gonorrhoea (Martin *et al.*, 2016; ECDC 2016). Overall in Europe, reports from the EURO-GASP have shown cefixime resistance decreased from 7.6% (145/1902) in 2011 to 4.7% (93/1994) in 2013, while ceftriaxone resistance changed from 0.5% (10/1902) to 0.4% (7/1994) between the same years, with 2011 being the first year that an isolate was found to be ceftriaxone resistant in the EURO-GASP study (Cole *et al.*, 2014a; Cole *et al.*, 2015).

The proportion of reduced susceptibility and resistance to ceftriaxone and cefixime found in this current study is difficult to compare to reports which use different MIC breakpoints to define these categories. In Canada, Martin *et al.* reviewed large numbers of isolates from 2010 to 2014 and found decreasing proportions of CFM-RS and CRO-RS (Martin *et al.*, 2016). They defined CFM-RS as MIC $\geq$ 0.25 mg/L and CRO-RS as $\geq$ 0.125 mg/L. The proportion of CFM-RS decreased from 3.3% (98/2970) in 2010 to 1.1% (42/3890) in 2014, while CRO-RS decreased from 7.3% (218/2970) to 2.7% (102/3809) during the same time. This coincided with the change in treatment guidelines to incorporate dual therapy. Different areas in China have reported varied rates of ESC resistance. In Nanjing, Chen *et al.* reviewed isolates from 2007 and from 2012 (Chen *et al.*, 2014). The overall rate of resistance to ceftriaxone was 5.4% (15/278) but all of these isolates were from 2007. Additionally they reported that the proportion of isolates with a ceftriaxone MIC on the breakpoint (0.125 mg/L) decreased from 39.4% in 2007 to 11.3% in 2012 (Chen *et al.*, 2014). While in Shanghai, Gu *et al.* looked at 3,153 isolates from 1998 to 2013 and found an overall ceftriaxone non-

susceptibility rate (defined in that report as MIC $\geq$ 0.125 mg/L) of 7.8%, with an increase from approximately 5% (1998) to 7-13% (2011-2013) (Gu *et al.*, 2014).

Compared to those studies using the same EUCAST clinical breakpoints, the rates in this current study are lower, but this may be related to the relatively low number of isolates.

2.9.2.1 Cefotaxime

Cefotaxime is not typically used in the treatment of gonorrhoea; however it is part of the BASHH empiric guidelines (last updated 2011) as an alternate monotherapy option (500mg IM) for anogenital gonorrhoea (BASHH 2011). Cefotaxime resistance (CTX-R) (MIC > 0.125 mg/L) was found in 13/609 (2.1%) isolates in this study with an additional 46 (7.5%) having reduced susceptibility (CTX-RS) (MIC = 0.125 mg/L).

The proportion of CTX-R isolates decreased from 4.2% in 2014 to 1.2% in 2016, while the proportion of CTX-RS increased from 4.2% to 9.45% in that period. Only one other study included data on cefotaxime susceptibility and had similar findings to the current study. Wind *et al.* reviewed 3,151 isolates (2012-2015, Amsterdam) and a proportion of 2.8% (89/3151) were found to be resistant to cefotaxime. This proportion was felt to be high and thought to be related to the use of cefotaxime as first line therapy in the Netherlands for several years up to 2006. The proportion of resistance to cefotaxime was found to decrease from 12% in 2008 to 2.7% in 2015. There was no consideration given to the timing of introduction of dual therapy of azithromycin and an ESC, which would have occurred in between those two years (Wind *et al.* 2017b).

Isolates of *N. gonorrhoeae* tested here had higher cefotaxime MICs compared to those of cefixime and ceftriaxone. The cefotaxime MIC₉₀ for all 609 isolates tested is 0.064 mg/L compared to 0.016 mg/L and 0.032 mg/L for ceftriaxone and cefixime,

respectively. These three antimicrobials have the same mechanism of action, but have different chemical structures which could result in different binding affinities and thereby different levels of activity against *N. gonorrhoeae*.

Cefotaxime gradient MIC strips are used in some laboratories in Ireland as a marker of ceftriaxone susceptibility, and vice versa. It is clear from the findings in this study that this practice is inappropriate for *N. gonorrhoeae* where cefotaxime would likely give spuriously high MICs for ceftriaxone. If those MICs were to be used for surveillance purposes without confirmatory testing it would result in falsely high rates of reduced susceptibility or even resistance to ceftriaxone.

Cefotaxime is not a suitable empiric option for treatment of gonorrhoea based on the results of this study. One potential use is for treatment cases with confirmed susceptibility to cefotaxime; however these cases are highly likely to be susceptible to ceftriaxone with a potentially lower MIC and hence more likely to be treated with ceftriaxone.

There was no resistance to ceftriaxone detected in this study and low levels of reduced susceptibility found in Ireland mean that ceftriaxone remains an appropriate choice for first-line empiric therapy for gonorrhoea.

2.9.2.2 Ertapenem

The findings in this study suggest that ertapenem confers no *in vitro* advantage over ceftriaxone for gonococci with ceftriaxone MICs ≤ 0.125 mg/L. Comparing ceftriaxone and ertapenem MICs for the 609 isolates in this study, ceftriaxone MICs trend towards lower values than those for ertapenem (Figure 2.12). In contrast, ertapenem MICs are

lower than those of cefotaxime, showing that it has greater activity against *N. gonorrhoeae* (Figure 2.12). Cefixime and ertapenem appear to have similar MIC distributions, but this comparison is limited by the lower concentration of the cefixime MIC gradient strip of 0.016 mg/L compared to 0.002 mg/L for ertapenem (Figure 2.12). As there were no CRO-R isolates in this study, it was not possible to assess ertapenem MICs in such isolates, but looking at isolates with cefixime or cefotaxime MICs ≥ 0.125 mg/L, 81% and 91.5%, respectively, had ertapenem MICs that were lower by two or more doubling dilutions.

Results in the current study are similar to those found in the few published reports on ertapenem MICs for clinical isolates and reference strains of *N. gonorrhoeae*.

Livermore *et al.* examined 652 isolates of *N. gonorrhoeae* which represented 30% of all gonococcal isolates collected under the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) 2003 for England and Wales (Livermore *et al.*, 2004). Ninety per cent of the isolates grouped into an MIC range of 0.002-0.06 mg/L and overall ceftriaxone had better activity than ertapenem. These isolates had ceftriaxone MICs of 0.00025 to 0.008 mg/L (Livermore *et al.*, 2004). Later Quaye *et al.* reviewed a smaller number of isolates (52 clinical and 10 control strains) dating from 2008-2011 and found that the ertapenem MICs were similar to those of cefixime, but overall the isolates were more susceptible to ceftriaxone than to ertapenem (Quaye *et al.*, 2014). The MIC₅₀/MIC₉₀s for ertapenem, cefixime and ceftriaxone were 0.12/0.25 mg/L, 0.06/0.012 mg/L and 0.016/0.032 mg/L, respectively. The one isolate included with high cefixime and ceftriaxone MICs (4 mg/L and 1-2 mg/L, respectively. F89 discussed above) had a much lower ertapenem MIC 0.032 mg/L (Quaye *et al.*, 2014).

Unemo *et al.* compared the *in vitro* efficacy of ertapenem to ceftriaxone for a range of isolates, including F89 and H04: isolates associated with cefixime and ceftriaxone treatment failures; clinical isolates dating from 2002 to 2011; and a range of international reference strains (Unemo *et al.* 2012b). The ertapenem MIC range and MIC₅₀/MIC₉₀s were 0.002-0.125 mg/L and 0.032/0.064 mg/L, respectively, compared to ceftriaxone MIC range and MIC₅₀/MIC₉₀s of $\leq$ 0.002-4 mg/L and 0.032/0.125 mg/L, respectively. The MICs of ertapenem and ceftriaxone were found to be similar overall, but the MIC range differed significantly. There were 4 isolates included with ceftriaxone resistance (MICs 0.5 to 4 mg/L) and the ertapenem MICs for these isolates ranged from 0.016-0.064 mg/L. Both H041 (CRO and CFM MICs = 4 mg/L and 8 mg/L, respectively) and F89 (CRO and CFM MICs = 2 mg/L and 4 mg/L, respectively) were included in this study and the ertapenem MICs were found to be 0.064 mg/L and 0.016 mg/L, respectively. Additionally isolates reported associated with cefixime treatment failures from Norway and Austria and associated with ceftriaxone treatment failure in Sweden, as discussed (*Section 1.9.1*), were also examined and the ertapenem MICs were 0.004- 0.125 mg/L compared to cefixime MICs of 0.25-1.0 mg/L and ceftriaxone MICs of 0.125-0.5 mg/L. As in the current study, they concluded that ertapenem had no apparent *in vitro* advantage over ceftriaxone for *N. gonorrhoeae* isolates with lower MICs to ceftriaxone, but for those isolates with high ceftriaxone MICs, the ertapenem MICs were much lower.

Many aspects of ertapenem make it a potentially suitable treatment option for gonorrhoea including the fact that it is more active against isolates with high ESC MICs. Clinical trials have yet to be performed to assess *in vivo* activity of ertapenem, but if ESC resistance becomes more widespread and treatment failures continue to

occur, clinicians may be forced to institute use of ertapenem without waiting for such clinical trials.

2.9.3 Azithromycin

Azithromycin is part of the empiric first-line dual therapy regimen (along with ceftriaxone) adopted by many countries for treatment of uncomplicated anogenital or pharyngeal gonorrhoea in adults, as discussed (*Section 1.5*). Of the 609 isolates tested here, the overall proportions resistant, intermediate and susceptible to azithromycin were 18.5%, 25.5% and 56.6% (Table 2.5). Although the resistance proportion of 18.5% found here was higher than the majority of reports from elsewhere in Europe, the distribution of MICs for azithromycin showed the majority of isolates falling in the narrow range of 0.125-0.5 mg/L (73.6%), similar to findings in other countries (Regnath *et al.*, 2016).

The level of high-level azithromycin resistance over the study period (2014-2016) remained very low (1.2%). HLAzi-R *N. gonorrhoeae* was first reported in Scotland in 2004 (Young & Palmer, 2005). Of the resistant isolates found in this study, a small proportion (n = 7) displayed high-level resistance (MIC > 256 mg/L) which is much higher than reported in other studies. In Canada, isolates from 2010 to 2013 (n = 2800) were examined for azithromycin resistance and 0.07% were found to be HLAzi-R isolates (n = 2) (Allen *et al.*, 2014). In the USA, 44,144 GISP isolates from 2005- 2013 were examined and only 1 isolate had an azithromycin MIC > 256 mg/L (0.002%) (Kirkcaldy *et al.*, 2015). In Guangzhou, China Liang *et al.* examined 485 gonococci from male urethritis from 2009-2013 and found no HLAzi-R by phenotypic or molecular methods (Liang *et al.*, 2016). Finally, in Europe, EURO-GASP data from

2011, 2013 and 2014 found 0.1% (n = 2/1902), 0.05% (1/1994, Ireland) and 0.04% (1/2151, Italy) HLAzi-R isolates, respectively (Cole *et al.*, 2014a; Cole *et al.*, 2015; ECDC 2016). In the current study, the 7 HLAzi-R isolates originated from SJH (4 in 2016 and 2 in 2015) and UHW (1 in 2013). The age range of these 7 patients is 18 to 36 years, with 4 males and 3 females. The site of these isolates was the pharynx (2) and urogenital (5). All seven of these isolates were intermediate to penicillin and susceptible ciprofloxacin, ceftriaxone, cefixime, cefotaxime and spectinomycin. Three were susceptible to tetracycline and four intermediate (Table 2.8). These resistance profiles are comparable to those reported internationally in other HLAzi-R isolates (Table 1.3). Current suitable therapeutic options for HLAzi-R isolates include ciprofloxacin, tetracycline and spectinomycin given the antimicrobial susceptibility profiles of the reported HLAzi-R isolates, but therapy should be individualised based on MIC results. All of these patients were treated empirically with 500 mg ceftriaxone and 1 g azithromycin and all responded to treatment. Information regarding risk factors for drug-resistant gonorrhoea, as discussed (*Section 1.7*), was not available for these patients.

Recorded resistance rates to azithromycin vary somewhat from country to country across the world. As part of the 2013 European surveillance of resistance in *N. gonorrhoeae* (Euro-GASP), Cole *et al.* report azithromycin resistance of 5.4% in 2013 (108 of 1985 isolates from 21 EURO-GASP participating countries) compared to 4.9% in 2012 (a non-significant increase) (Cole *et al.*, 2015). In that year, Ireland submitted 103 of the isolates, 3 (2.9%) of which were azithromycin resistant, including one HL-AziR (which was the only HL-AziR isolate detected). In the following year, 2014, the percentage of azithromycin resistance detected in all isolates (submitted from 23 Euro-GASP participating countries) was 7.9% (169/2147) (ECDC 2016). Out of those

submitted from Ireland, azithromycin resistance had risen to 37.6% (38/101). This was second only to that of Greece, 39.6% (42/106 isolates azithromycin-resistant). Again, these data were from isolates submitted from participating countries in one three-month period only and in addition, the Irish isolates sent for testing were from one centre and hence were unlikely to be representative of true resistance rates.

In Amsterdam from 2012 to 2015, of 3,151 isolates of *N. gonorrhoeae* tested, 90.1% (2838) were susceptible, 8.7% (275) intermediate and 1.2% (38) resistant to azithromycin. From 2012 to 2015, the proportion of isolates intermediate to azithromycin increased from 3.7% to 8.6%, while the proportion azithromycin-resistant increased from 1.3% to 1.5% (Wind *et al.*, 2017b). In South West Germany, azithromycin resistance increased significantly from the 2004-2009 period (5/168, 3%) to the 2010-2015 period (19/266, 7.1%) (Regnath *et al.*, 2016). The percentage of azithromycin-resistant *N. gonorrhoeae* isolates in Switzerland increased from 11.5% in 1998-2001 to 23.6% in 2009-2012 (Endimiani *et al.*, 2014). Other countries such as Hungary, Belarus and Japan, report azithromycin resistance rates of 15.9% (2013), 5% (2010-2013) and 3.6% (2010-2012), respectively (Brunner *et al.*, 2014; Lebedzeu *et al.*, 2015; Shimuta *et al.*, 2013).

2.9.4 Ciprofloxacin

Fluoroquinolone resistance emerged in Japan in the early 90's (Tanaka *et al.*, 1994) and quickly became widespread throughout the USA and other countries. By 2007 the resistance rates in the US lead to the removal of ciprofloxacin from the CDC treatment guidelines for gonorrhoea (MMWR 2007). In this study, although the overall proportion of resistant isolates rose from 24.3% in 2014 to 36% in 2016, the overall

proportion of isolates resistant to ciprofloxacin (33%) is similar to or lower than reports from other regions in Europe. For example, in Amsterdam, ciprofloxacin resistance was reported as 32.7% of 3,151 isolates from 2012 to 2015, also ciprofloxacin was the agent with the highest rate of resistance (Wind *et al.* 2017b). Cole *et al.* reporting results from the 2013 European surveillance of resistance in *N. gonorrhoeae* (Euro-GASP), found that of isolates tested from 21 countries, ciprofloxacin resistance had increased to 52.9% (1055/1994) in 2013 from 50.1% in 2012 (Cole *et al.*, 2015 BMC ID). In 2014, Euro-GASP reported 50.7% (1019/2151) ciprofloxacin resistance in isolates tested from 23 countries (REF ECDC AST 2014 document). In Italy, the resistance rate to ciprofloxacin was reported as 64% in 2012 (270 isolates tested) (Carannante *et al.*, 2014). In Greenland in 2014, of 88 culture positive gonorrhoea infections in Nuuk, 88% resistance to ciprofloxacin was detected (Skjerbæk Rolskov *et al.* 2015).

As in the current study ciprofloxacin had a bimodal distribution of MICs with a large gap between MIC₅₀ and MIC₉₀ (0.002 mg/L and 2 mg/L, respectively), with one group of isolates displaying very low MICs, and the other high MICs, and very few at or around the clinical breakpoint. This has been found in other countries also, e.g. South West Germany (Regnath *et al.*, 2016) and suggests two separate populations within the isolates selected. The presence or absence of resistance determinants is the explanation for this divide, as shown (Section 3.5.7-3.5.8).

2.9.5 Gemifloxacin

The MIC distribution for gemifloxacin was also bimodal with the MIC₅₀/MIC₉₀s and MIC range for gemifloxacin (0.008/2 mg/L and ≤ 0.002 -16 mg/L, respectively) similar

to those reported in other studies. The wide gap between MIC₅₀ and MIC₉₀ suggests two subpopulations, similar to that found for ciprofloxacin in this group of isolates. Comparing the gemifloxacin MIC₅₀/MIC₉₀s for isolates that are ciprofloxacin susceptible and resistant, there is a 250-fold increase from 0.004/0.008 mg/L to 1.0/2.0 mg/L (Table 2.9). Overall the ciprofloxacin MICs were a median of 1.5-fold higher (range 1.3- to 250-fold) than the gemifloxacin MICs for the 609 isolates.

Similar to findings in this study, Pottumarty *et al.* examined 250 isolates (200 from 2004 and 50 historic isolates) from Japan with high ciprofloxacin MICs and reported gemifloxacin MIC₅₀/MIC₉₀s and MIC range of 0.016/1.0 mg/L and ≤ 0.008 -2.0 mg/L, respectively, similar to this findings in this study (Pottumarty *et al.*, 2006) In 2014 Kirkcaldy *et al.* reported results from a randomised multisite open label non-comparative trial (Kirkcaldy *et al.*, 2014). There were 5 sites in North America involved. Two combination therapies were compared. Patients 15-60 years old presenting with uncomplicated anogenital gonorrhoea were included. One group received gentamicin 240mg IM plus azithromycin 2g PO and the other received gemifloxacin 320mg PO plus azithromycin 2g PO. The primary outcome was microbiological cure i.e. a negative culture at follow up 10-17 days after treatment. The gemifloxacin group had a cure rate of 99.5% (199 patients evaluated) which included 15/15 pharyngeal and 5/5 rectal cures. The pre-treatment MIC₅₀/MIC₉₀s and MIC range (for all 421 isolates) were 0.004/2 mg/L and 0.001-8 mg/L, respectively compared to those of ciprofloxacin 0.004/8 mg/L and 0.002 mg/L-32 mg/L, respectively, approximately 3 fold lower MIC₉₀ for gemifloxacin. Of note the MIC₅₀/MIC₉₀s and MIC range for azithromycin were 0.25/0.5 mg/L and 0.03-16 mg/L, respectively (Kirkcaldy *et al.*, 2014). It is difficult to assess the individual effectiveness of gemifloxacin when it is given in combination with azithromycin, but similarly, it is

prudent that any new drugs being introduced for the treatment of gonorrhoea be given in combination with another agent.

Cross resistance between fluoroquinolones does occur but each drug should be individually assessed for activity against *N. gonorrhoeae*. Gemifloxacin is a good viable option for treatment of gonorrhoea, especially in those in whom β -lactam allergies preclude cephalosporins and possibly carbapenems. In countries where gemifloxacin is not in use already, consideration should be given to reserving its use for gonorrhoea alone in an attempt to reduce the possibility of selective pressure resulting in more rapid emergence of resistance.

2.9.6 Penicillin

Penicillin was introduced for treatment of gonorrhoea in 1940's but resistance was reported soon after. Results from this study support the continued lack of utility of penicillin in the treatment of gonococcal infection. The majority of isolates tested in this study were intermediate to penicillin (MIC 0.125-1 mg/L) highlighted by the MIC₅₀/MIC₉₀s of 0.25/1 mg/L, with very few isolates testing susceptible. Penicillin resistance and non-susceptibility rates found in this study were 7.2% and 98.2%, respectively. While the resistance rate found was lower than reported from other countries, the non-susceptibility rate was as high or higher. Data from Austria showed that intermediate resistance rose from 0% in 1999 to 67% in 2007 (Stary *et al.* 2015). From 2010 to 2014 the proportion of isolates intermediate to penicillin in Austria was 40-60% and a low level of susceptibility was found (3548 isolates tested by agar dilution until 2012, then gradient MIC strip). In 2014, 51.2%, 31.8% and 17.0 % of isolates in Austria were intermediate, resistant and susceptible to penicillin,

respectively, which is a much lower non-susceptibility proportion compared to the current study. Furthermore, in Switzerland study of a small number of isolates from two time periods, 26 isolates from 1998 to 2001 and 34 isolates from 2009 to 2012 and found penicillin non-susceptibility/MIC₉₀ rising from 42.3%/3 mg/L to 85.3%/16 mg/L (Endimiani *et al.* 2014). Although the numbers were very small, a similar trend is reflected in many other studies. A report from Germany in 2014 reported penicillin non-susceptibility in 80% of 213 isolates tested from 23 centres in Germany over 15 months (Horn *et al.*, 2014). Regnath *et al.* examined 434 isolates from South West Germany dating from 2004 to 2015 and found 90.6% non-susceptible to penicillin (Regnath *et al.*, 2016).. Outside of Europe, resistance to penicillin has followed a similar trend. In Shanghai, China, rates of resistance to penicillin rose from 20-40% prior to 1998 to 70-90% in the period from 2000 to 2013 (Gu *et al.*, 2014). In that study, 3663 gonococcal isolates (1996 to 2013) were tested for susceptibility to penicillin and interpreted in this case according to the Clinical Laboratory Standards Institute (CLSI) clinical breakpoint (penicillin resistance ≥ 2 mg/L) which is higher than that given by EUCAST. Of those 3663 isolate 62% overall were determined to be penicillin resistant by these criteria.

2.9.6.1 β -lactamase production

Production of β -lactamase enzymes by *N. gonorrhoeae* confers penicillin resistance and EUCAST recommend always testing for β -lactamase production in gonococcal isolates. Isolates that have MICs below the clinical breakpoint for resistance but produce β -lactamase should be reported as resistant. In this study, 6% of isolates produced β -

lactamase, including four isolates which had MICs of 0.25, 0.5, 0.5 and 1 mg/L (intermediate category) and therefore are reported as resistant. The proportion of isolates producing β -lactamase in the current study is lower than the European average of 14.1% from the 2014 EURO-GASP data indicating less plasmid-mediated resistance in Ireland (ECDC 2016). The various types of β -lactamase enzymes and genes and their reported prevalence are discussed (*Section 3.6.4*).

2.9.7 Tetracycline

The rate of tetracycline resistance found in this study (9.9%) is considerably lower than any of the reports from Europe or elsewhere mentioned above. The MIC₅₀/MIC₉₀s of 0.5/1 mg/L are also markedly lower. The EURO-GASP reports do not include tetracycline resistance rates (Cole *et al.*, 2014a; Cole *et al.*, 2015), but there are reports from across Europe and the rest of the world which highlight the increase in resistance which occurred over the last few decades. From France La Ruche *et al.* report an increase in tetracycline resistance in *N. gonorrhoeae* isolates from 29% (43/150) in 2001 to 56% (611/1093) in 2012 (La Ruche *et al.*, 2014). In South-West Germany increase from 30.4% (of 62 isolates) in 2004-2009 to 55.6% (of 148 isolates) in 2010-2015 was reported (Regnath *et al.*, 2016). The MIC₅₀/MIC₉₀s and MIC range for these isolates was as 2/32 mg/L and 0.016-> 256 mg/L, respectively. Similarly in Italy, Carannante *et al.* found an increase from 16% (of 134 isolates) in 2014 to 50.5% (of 95 isolates) in 2009 (Carannante *et al.*, 2014). In Eastern Europe, a much higher rate of tetracycline resistance has been reported from Hungary. Over a four year period, 2010 to 2013, *N. gonorrhoeae* isolates from 582 patients were tested against tetracycline and the resistance rate was > 80% each year and overall was 86% (Brunner *et al.*, 2014).

Outside of Europe, very variable rates of tetracycline resistance have been reported. In Nanjing, China, tetracycline resistance has been reported in 97.7% (327/334) of isolates tested in a period from April 2011 to Dec 2012, with MIC₅₀/MIC_{90s} of 8 /> 16 mg/L (Li *et al.*, 2014). Much lower rates have been reported from Belarus and Pakistan, 36% and 51%, respectively, and MIC₅₀/MIC_{90s} of 0.38 /24 mg/L and 3mg/L/24 mg/L, respectively (Lebedzeu *et al.*, 2015; Jabeen *et al.*, 2016).

The reason for this difference is unclear. Tetracycline use in the community for other indications may be lower in Ireland than in the countries mentioned above, and hence lower overall exposure to it or possibly it may be due to the lower prevalence and circulation of plasmid mediated high-level resistance (*Section 3.5.10 and 3.6.5*).

Regardless of the reason why Ireland has such low levels of resistance to tetracycline, it should not be reintroduced into the empiric treatment guidelines because emergence of high-level resistance in the 1980s (Morse *et al.*, 1986) and current high rates of resistance. Many countries no longer perform routine susceptibility testing of tetracycline in *N. gonorrhoeae*, but it could be considered in cases where susceptibility is known if an alternate agent is required.

2.9.8 Spectinomycin

The findings of this study indicate absence of resistance to spectinomycin among gonococci, similar to the findings of other studies. A recent study of 210 isolates of *N. gonorrhoeae* in Korea from 2011-2013 again found no resistance to spectinomycin. In the preceding years the percentage of patients treated with spectinomycin was 72.8% in

2009 decreasing to 51.7% in 2011. The MIC range and MIC₅₀/MIC₉₀s for these isolates were ≤ 16 -32 mg/L and 32/32 mg/L, respectively (Lee *et al.*, 2015). Elsewhere, resistance to spectinomycin (MIC > 64 mg/L) is also extremely rare. Many published reports looking at susceptibility of *N. gonorrhoeae* to a range of antibiotics have found no resistance to spectinomycin. (Brunner *et al.*, 2014; Carannante *et al.*, 2014; Chen *et al.*, 2014; Endimiani *et al.*, 2014; Horn *et al.*, 2014; Jabeen *et al.*, 2016; Lebedzeu *et al.*, 2015; Regnath *et al.*, 2016; La Ruche *et al.*, 2014). In those reports using CLSI criteria which has a higher resistance breakpoint (MIC ≥ 128 mg/L) two studies from Nanjing, China and Japan found no resistance with MIC ranges up to 32 and 64 mg/L, respectively, (Li *et al.*, 2014; Zheng *et al.*, 2014) while a study from Shanghai, China which examined 3,153 isolates from 1995 to 2013 discovered 2 isolates resistant to spectinomycin (MIC ≥ 128 mg/L) and 213 (6.76%) intermediate to spectinomycin (MIC = 64 mg/L) (GU *et al.*, 2014). Those isolates deemed to be intermediate by CLSI standards would be susceptible according to EUCAST interpretive criteria.

Although spectinomycin resistance is rare nowadays, there have been treatment failures especially in pharyngeal gonorrhoea. A comparison of spectinomycin and ceftriaxone for uncomplicated gonorrhoea found spectinomycin cure rates of: 90% (36/40) in male urethral gonorrhoea; 100% (23/23) in cervical gonorrhoea; and 67% (8/12) in pharyngeal gonorrhoea. The ceftriaxone cure rates were 100% for each of the corresponding groups. There was no spectinomycin resistance in the pre-treatment isolates (Rompalo *et al.*, 1994). Introduction of spectinomycin monotherapy carries a risk of inadequately treating pharyngeal gonorrhoea as well as selection pressure leading to increased resistance. If spectinomycin is to be reintroduced in the future, it should be considered for use in dual therapy and issues with availability would need to be addressed if its use is foreseen.

2.9.9 Gentamicin

In this study, no resistance to gentamicin (according to the proposed breakpoints of susceptible ≤ 4 mg/L, intermediate 8-16 mg/L and resistant ≥ 32 mg/L) was found in the isolates tested and 56.3% and 43.7% were susceptible and intermediate, respectively. These results are similar to those reported in other studies. Recently Kirkcaldy *et al.* compared gentamicin 240mg IM plus azithromycin 2g PO to gemifloxacin 320mg PO plus azithromycin 2g PO, details as discussed above, in a randomised multisite open label non-comparative trial (Kirkcaldy *et al.*, 2014). The microbiological cure rate for gentamicin plus azithromycin was 100% (161/161 of fully evaluated patients), including 10/10 pharyngeal and 1/1 rectal cases of gonorrhoea. The pre-treatment isolates from all the enrolled patients (421) had MIC₅₀/MIC₉₀s and MIC range of 4/8 mg/L and 0.002-16 mg/L, respectively (Kirkcaldy *et al.*, 2014). One-hundred and ninety-three gonococci from 2010-2013 in Belarus tested against gentamicin were found to have an MIC range was 0.25-16 mg/L (Lebedzeu *et al.*, 2015). In Europe, Cole *et al.* reported EURO-GASP data to include modal MICs of 8 (2011 and 2013) and MIC ranges of 0.5-16 mg/L in 2011 and 1.0-16 mg/L in 2013 (Cole *et al.*, 2014a; Cole *et al.*, 2015). Chrisholm *et al.* reviewed 1366 gonococci from 17 regions in Europe from 2009 and found 95% were within an MIC range of 4-8 mg/L (overall range of 1.0-16 mg/L), with 79% and 16% having an MIC of 8 mg/L and 4 mg/L, respectively (Chrisholm *et al.*, 2011).

Gentamicin does not appear to be sufficiently efficacious for monotherapy. A systematic review by Hathorn *et al.* including 5 studies and 1063 participants (from 3 randomised controlled trials, one quasi random and one non-randomised trial with a comparator arm) was published in 2014 (Hathorn *et al.*, 2014). All the patients were

men with confirmed gonococcal urethritis who received single dose gentamicin IM. The cure rate was the primary outcome but the studies were too heterogeneous for a meta-analysis. The cure rates ranged from 62% to 98%. They concluded that gentamicin may not achieve the recommended $\geq 95\%$ cure rate (WHO), but the data available was insufficient (Hathorn *et al.*, 2014). Dowdall *et al.* published a systematic review plus meta-analysis of the three randomised controlled trials above and the outcome they focused on was patients with a negative culture after a single IM dose of gentamicin and reported a pooled percentage of 91.5% that achieved this. They concluded that gentamicin should not be recommended due to this low cure rate (Dowell *et al.*, 2013).

Gentamicin may be usefully employed as part of dual therapy for treatment of gonorrhoea. Further clinical trials would be beneficial. One such trial is enrolling at present and aims to compare IM ceftriaxone 500mg plus PO azithromycin 1g to IM gentamicin 240mg plus PO azithromycin 1g with a primary outcome being clearance of gonorrhoea (Brittain *et al.*, 2016). The results of this trial are awaited, but may augment the argument for gentamicin as part of a dual regimen in the treatment of gonorrhoea.

2.9.10 Fosfomycin

Fosfomycin has been tested against *N. gonorrhoeae* in a few studies. The majority (85%) of isolates in the current study had fosfomycin MICs of 16 or 32 mg/L, with an overall range of 4-128 mg/L and MIC₅₀/MIC₉₀ of 16/32 mg/L. Another study reported lower fosfomycin MIC₅₀/MIC₉₀s of 8/16 mg/L and a fosfomycin MIC range of 8-24 mg/L for 32 gonococci (Barbee *et al.*, 2014). Similarly, a further study looked at 89

isolates from 1998 to 2012 finding fosfomycin MIC₅₀/MIC₉₀s and MIC range of 8/16 mg/L and 1-32 mg/L, respectively (Hauser *et al.*, 2014).

The higher MIC₅₀/MIC₉₀s and MIC range for fosfomycin found in the current study is likely due to the significantly larger number of isolates tested (609 compared to 89 and 28, respectively). Another possible explanation is the potentially high frequency of fosfomycin trometamol used in the community for urinary tract infections, considering fosfomycins place in the primary care guidelines in this country as mentioned previously, however there are no data on fosfomycin consumption in Ireland.

There are no clinical breakpoints for fosfomycin and *N. gonorrhoeae*. EUCAST provide breakpoints for fosfomycin oral (applies only to *Enterobacteriaceae* and uncomplicated UTI only) and fosfomycin IV (*Enterobacteriaceae* and *Staphylococci* spp.) as susceptible ≤ 32 mg/L and resistant > 32 mg/L (EUCAST 2017). According to CLSI *Enterobacteriaceae* and enterococci are susceptible to fosfomycin ≤ 64 mg/L, intermediate = 128 mg/L and resistant > 256 mg/L (CLSI 2017). If the EUCAST clinical breakpoints are applied to the isolates in this study, 20/609 (3.3%) would be resistant to fosfomycin and the rest susceptible, while if CLSI breakpoints are applied, 1/609 (0.16%) would be intermediate to fosfomycin and the rest susceptible.

Considering the ease of administration of fosfomycin as a 3g oral single dose as well as the concentration achieved in the bladder its appeal in the treatment of gonorrhoea is clear, especially for uncomplicated gonococcal urethritis. While clinical cure has been shown to be achievable with 3 doses of 3g PO fosfomycin trometamol 48 hours apart as shown in the trial by Yuan *et al.*, contemporary clinical evidence is lacking, especially for single dose therapy which may be considered as part of a dual therapy regimens in the future (Yuan *et al.*, 2016). Intramuscular fosfomycin achieves higher serum

concentrations than oral administration and may be useful for urogenital and extragenital gonorrhoea, however further clinical trials are again required in this area to assess whether fosfomycin can achieve the target of > 95% cure desirable for introduction in the treatment of gonorrhoea.

2.9.11 Solithromycin

Solithromycin is a novel agent which has been studied for use in treating gonorrhoea. Of the seven HLAzi-R isolates in this study, 3 had solithromycin MIC values of > 32 mg/L while the remainder had better *in vitro* activity. These results imply that solithromycin MIC values need to be determined before using it for treatment of HLAzi-R infections. Although only seven isolates with HLAzi-R were studied, this is higher than the number of HLAzi-R published in the literature at the time of writing. Five HLAzi-R isolates had solithromycin MICs determined which range from 4-32 mg/L (Allen *et al.*, 2014; Golparian *et al.*, 2012; Mallegol *et al.*, 2013).

In vitro studies have shown solithromycin to be more potent than azithromycin against *N. gonorrhoeae* albeit it is unreliable against HLAzi-R isolates. These results suggest that solithromycin is a potential therapeutic alternative. Similarly, in other studies, 34 gonococcal isolates were examined and the solithromycin MIC₅₀/MIC₉₀s and MIC range were determined to be 0.06/0.12 mg/L and 0.03-0.25 mg/L, respectively, which were lower than those of azithromycin (0.25/0.5 mg/L and 0.6-2 mg/L, respectively) (Putnam *et al.*, 2010). More recently 196 isolates of *N. gonorrhoeae* in Canada were tested for susceptibility to solithromycin. The MIC₅₀/MIC₉₀s and MIC range for solithromycin (azithromycin) were 0.0625/0.125 mg/L (0.25/0.5 mg/L) and ≤ 0.015-8 mg/L (≤ 0.031-2048 mg/L), respectively (Mallegol *et al.*, 2013). In both studies the azithromycin MIC₅₀/MIC₉₀s and MIC ranges were lower than in the current study, i.e.

the isolates tested against solithromycin in the current study were more resistant to azithromycin than isolates in the other studies. Both studies also found solithromycin to have an MIC₉₀ of 4-fold lower than that of azithromycin which is less than that found in the isolates in the current study which had a solithromycin MIC₉₀ 64-fold lower than the azithromycin MIC₉₀, explained by the larger number of HLAzi-R isolates in the current study in comparison. Olsen *et al.* examined 108 isolates from 2011 (Vietnam) testing them against a range of antimicrobials including solithromycin. In this selection of isolates where 11% (12/108) and 27% (29/108) were resistant and intermediate to azithromycin, respectively, the solithromycin MIC₅₀/MIC₉₀s and MIC range were 0.064/0.125 mg/L and ≤ 0.016 -0.25 mg/L, respectively (Olsen *et al.*, 2013), lower than in the current study, but reflective of the lower azithromycin MICs in the selection of isolates tested. Golparian *et al.* looked at a collection of 246 isolates including clinical isolates, reference strains, H041 and F89 which displayed a range of resistance. The solithromycin (azithromycin) MIC₅₀/MIC₉₀s and MIC range were 0.125/0.25 mg/L (0.5/8 mg/L) and 0.001-32 mg/L (0.001-> 256 mg/L), respectively, similar to the current study (Golparian *et al.*, 2012). They also noted that the gonococcal isolates with ESC resistance remained susceptible to solithromycin. Those isolates resistant to cefixime and ceftriaxone displayed solithromycin MIC₅₀/MIC₉₀s and MIC range of 0.125/0.25 mg/L and 0.064-0.25 mg/L, respectively. The solithromycin MICs for HLAzi-R isolates were 4-32 mg/L, i.e. resistant to solithromycin if clinical breakpoints similar to those for azithromycin were to be implemented (Golparian *et al.*, 2012).

Solithromycin has not been tested by disc diffusion in any published reports on clinical isolates of *N. gonorrhoeae*. Disc diffusion quality control guidelines were published for solithromycin and *N. gonorrhoeae* in 2015 (Riedel *et al.*, 2015). *N. gonorrhoeae* ATCC 49226 was tested in multiple laboratories and the quality control zone diameter

was determined to range from 33 to 43 mm, while the quality control MIC (by agar dilution) ranged from 0.03 to 0.25 mg/L. In this study, the MIC determined by gradient MIC strip correlated well with the zone diameter resulting from disc diffusion testing and either may be useful in clinical laboratories once clinical breakpoints for solithromycin and *N. gonorrhoeae* are published in the future.

In 2015, a phase 2 trial was reported which assessed two doses of solithromycin in the treatment of uncomplicated urogenital gonorrhoea. Fifty-nine patients were recruited from 2013-2014 and assigned 1200 mg (first 28 patients) or 1000mg (last 31 patients) solithromycin as a single dose directly observed therapy for the treatment of suspected urogenital gonorrhoea. Overall 46 patients had the diagnosis confirmed on culture of the initial sample (24 in the 1200 mg group and 22 in the 1000 mg group) and there was 100% eradication at follow-up culture at days 7-10 (including 8 patients with pharyngeal and 4 patients with rectal gonorrhoea). The solithromycin (azithromycin) MIC₅₀/MIC₉₀s and MIC range of the pre-treatment isolates were 0.06/0.125 mg/L (0.25/0.5 mg/L) and < 0.008-0.125 mg/L (0.06-0.5 mg/L), respectively, isolates which were much more susceptible to azithromycin than those tested in the current study and hence the lower solithromycin MIC₅₀/MIC₉₀s and MIC range. The most common adverse effects included self-limiting diarrhoea, nausea and vomiting which did not diminish the efficacy of the drug and were more common with the higher dose (Hook *et al.*, 2015).

The findings of this study in addition to the reports discussed above show that solithromycin has good activity against *N. gonorrhoeae*, including those strains resistant to azithromycin and ESCs. Single dose oral therapy is an attractive feature for its use in treatment of gonorrhoea and its additional activity against *Mycoplasma genitalium* and *Chlamydia trachomatis* add to its appeal (Hook *et al.*, 2015). Results of a phase 3 trial

comparing 1000mg solithromycin against ceftriaxone 500mg IM plus azithromycin 1g PO in men and women with uncomplicated gonorrhoea will potentially strengthen the case for introduction of solithromycin in the treatment of gonorrhoea (Melinta Therapeutics 2018).

2.9.12 Comparison of antimicrobial susceptibility of pharyngeal, rectal and urethral *Neisseria gonorrhoeae* isolates

For most antimicrobials tested there was no obvious association between gonococcal antimicrobial susceptibility and the site of gonococcal isolation. Although MIC distributions for ceftriaxone, cefixime, cefotaxime, penicillin and tetracycline by site of sample (urogenital, rectal and pharyngeal) were similar, azithromycin MICs appeared higher for pharyngeal isolates compared to rectal or urogenital and ciprofloxacin MICs were higher for rectal and pharyngeal isolates compared to urogenital. Similar findings were reported in other studies. For example, in British Columbia, of 1,837 isolates (22% of reported cases) over a 5-year period (2006 to 2011), pharyngeal and rectal *N. gonorrhoeae* isolates were reported to be significantly associated with increased cephalosporin and azithromycin MICs compared to those from urogenital sites (Hottes *et al.*, 2011). However, Kidd *et al.* found no evidence of this in a set of 1,442 isolates over 21 months in North America (Kidd *et al.*, 2015). A more recent study (3,151 isolates from 2012 to 2015) found decreased susceptibility to ceftriaxone has to be significantly associated with pharyngeal infection in multivariable analysis (Wind *et al.*, 2017a). The isolates in this study showed a trend of increased azithromycin and ciprofloxacin MICs in pharyngeal isolates, but same was not true for penicillin, tetracycline and ESCs, for which there was a similar distribution of MICs for each of

rectal, pharyngeal and urethral samples which reflects the findings in the current study for azithromycin, but not for ciprofloxacin.

Site of gonococcal infection for a particular patient is important information for the clinician. It allows assessment as to whether the infection may be more difficult to treat or potentially associated with antimicrobial resistant *N. gonorrhoeae*, either of which would necessitate follow up and consideration of test of cure.

2.9.13 Combination resistance, MDR and XDR isolates

Many of the isolates included in this study are resistant to more than one antimicrobial. EUCAST set clinical breakpoints for penicillin, ciprofloxacin, azithromycin, tetracycline, ESCs (ceftriaxone, cefixime and cefotaxime) and spectinomycin and these antimicrobial are discussed here. Among the 609 isolates included in this study no resistance to spectinomycin or ceftriaxone was detected. However, reduced susceptibility to ceftriaxone was detected in 11 isolates as discussed (*Section 2.6.1.1*). Importantly, 7 of these 11 also had reduced susceptibility to azithromycin (MIC range 0.5-1.0 mg/L) the second agent used as first-line treatment for gonorrhoea. However, there was no evidence of treatment failures in these cases.

MDR (XDR) is defined as resistance to one (two) of injectable ESC, oral ESC or spectinomycin plus two (three) of penicillins, azithromycin, fluoroquinolones, aminoglycosides or carbapenems (Tapsall *et al.*, 2009c). Injectable ESCs include ceftriaxone and cefotaxime and oral ESCs include cefixime. As mentioned there are no clinical breakpoints for gentamicin or ertapenem, but according to this definition (using EUCAST clinical breakpoints for resistance) and considering only ceftriaxone,

cefotaxime, cefixime, penicillin, ciprofloxacin and azithromycin, 7 isolates (1.1%) in this study were MDR *N. gonorrhoeae*, two of which met the criteria for XDR *N. gonorrhoeae*. The MICs for these isolates are summarised in table 2.16.

It is very difficult to compare the prevalence of MDR/XDR *N. gonorrhoeae* with published data, because various definitions are used. Some authors defined MDR *N. gonorrhoeae* as resistance to ≥ 3 classes of antibiotic (Endimiani *et al.*, 2014) and XDR *N. gonorrhoeae* as those displaying high ESC MICs (Lewis *et al.*, 2014). Furthermore, many studies do not test the full range of antimicrobials needed to assign strains of *N. gonorrhoeae* as MDR versus XDR.

2.11 Limitations

This study has a number of limitations. Firstly, the isolates included are representative only, not comprehensive as it was not possible to retrieve isolates from all laboratories in Ireland. The retrospective nature of the study also meant that some frozen isolates were not retrievable. Similarly, epidemiological data was lacking with no information regarding sexual orientation of the patient, mode of transmission or response to treatment being available. Another limitation to this study is the small number of ESC-resistant gonococci available for testing, with no CRO-resistant (CRO MIC > 0.125mg/L) isolates available.

MICs of MDR and XDR isolates											
	Isolate	Hospital/ year	Age	Sex	Site	PEN	CIP	AZM	CRO	CFM	CTX
MDR	422102	UHG 2016	U	U	U	1	> 32	1	0.032	0.125	0.38
MDR	024	UHW 2012	26	M	Urethral	1.5	> 32	0.38	0.094	0.25	0.38
MDR	82432	SJH 2015	27	M	Rectal	0.75	> 32	1	0.023	0.125	0.25
MDR	178526	SJH 2015	26	M	Pharyngeal	1	> 32	1	0.032	0.19	0.25
MDR	71324	SJH 2014	36	M	Urethral	4	> 32	0.125	0.047	0.19	0.19
XDR	016	UHW 2013	27	M	Urethral	2	> 32	1	0.047	0.19	0.25
XDR	76972	SJH 2014	30	M	Urethral	1.5	> 32	0.75	0.047	0.19	0.25

Table 2.12. MICs (mg/L) of MDR and XDR *N. gonorrhoeae* to penicillin (PEN) ciprofloxacin (CIP), azithromycin (AZM), ceftriaxone (CRO), cefixime (CFM) and cefotaxime (CTX). Age sex and site are unknown (U) for isolate 422102. All isolates came from male (M) patients

2.12 Conclusion

Azithromycin and ceftriaxone dual therapy is the current first-line recommendation for the treatment of gonorrhoea in Ireland. This study found a gonococcal non-susceptibility rate to azithromycin of 44% which includes intermediate (25.5%), resistant (17.4%) and HLAzi-R (1.1%) isolates. In contrast, 100% of isolates were susceptible to ceftriaxone. The non-susceptibility rate to azithromycin does not render azithromycin useless; intermediate and low level resistance ($MIC \leq 4$ mg/L) do not preclude its use in treating gonorrhoea in dual therapy. The AMR trends in these two agents also appear stable and so the current first-line regimen remains suitable at present.

The results of this chapter indicate that there are numerous alternative antimicrobials which would be suitable should the first-line options become ineffective. Blanket susceptibility of gonococci in this study to spectinomycin suggests that it would be a suitable empiric agent for gonorrhoea, whereas tetracycline, ciprofloxacin and penicillin may be appropriate if susceptibility is known. Gentamicin and fosfomycin appear promising in the treatment of gonorrhoea, even if their efficacy is overshadowed somewhat by solithromycin which has shown formidable activity against *N. gonorrhoeae* in comparison to azithromycin in this study. Unfortunately, for HLAzi-R gonococci, the increased efficacy is not quite sufficient to suggest that solithromycin would be suitable for HLAzi-R.

This study has also shown that ESCs have different levels of activity against *N. gonorrhoeae*, with ceftriaxone being most active followed by cefixime and cefotaxime,

so it appears unlikely that either of the latter will have a role in the treatment of gonorrhoea in the near future. With the same mechanism of action, ertapenem seems to be as efficacious as cefixime and more active than cefotaxime, but it has no apparent advantage over ceftriaxone for the isolates in this study ($\text{MIC} \leq 0.125 \text{ mg/L}$), however it may become necessary to use ertapenem in treating gonorrhoea if high-level ceftriaxone resistance becomes widespread, against which it has been shown to have *in vitro* activity.

With a similar mechanism of action to that of ciprofloxacin, gemifloxacin overall had greater *in vitro* activity than ciprofloxacin against the isolates in this study. It is another potentially useful alternative agent for gonorrhoea, especially in patients who are cephalosporin allergic.

This chapter also compared gonococcal isolates from rectal, pharyngeal and urogenital sites to ascertain if any particular site is associated with AMR more than others. The majority of the antimicrobials assessed (ESCs, penicillin, ciprofloxacin and tetracycline) showed no obvious difference in MIC distributions according to body site, however azithromycin MICs were increased in pharyngeal isolates compared to urogenital and rectal, while ciprofloxacin MICs were increased in both rectal and pharyngeal isolates compared to urogenital.

Many of the isolates in this study were resistant to more than one antimicrobial, but only 5 and 2 met defined criteria for MDR and XDR gonococci. Such criteria cannot be applied to *N. gonorrhoeae* unless susceptibility testing to defined antimicrobials has been performed and for this to occur in a comprehensive manner, an appropriate reference laboratory is required.

The following chapter attempts to elucidate the reasons for AMR discussed in this chapter, by identifying resistance determinants associated with each of the individual antimicrobials assessed.

Chapter III

Molecular Characterisation of Antimicrobial Resistance

Determinants in *Neisseria gonorrhoeae*

3.1 Introduction

Antimicrobial resistance in *N. gonorrhoeae* is an ever evolving entity. *Neisseria* spp. have the capacity to readily alter their DNA by acquisition of genetic material from other organisms. All *N. gonorrhoeae* are naturally competent for DNA transformation. Type IV pili mediate this process as well as being involved in adherence and twitching motility. It has recently been proposed that a distinct pseudopilus similar in structure to the Type IV pilus is actually responsible for DNA transformation (Obergfell *et al.*, 2016). Regardless of the precise mechanism, DNA transformation is key to the acquisition of resistant determinants in *N. gonorrhoeae*. Most resistance determinants affecting gonococci are chromosomal apart from two which are plasmid mediated (*tetM* encoded protein and *bla_{TEM}* encoded β -lactamases) and spread by conjugation. Chromosomally encoded resistance determinants persist in the organism even when the specific antimicrobial is no longer used in the treatment of gonorrhoea (Unemo *et al.*, 2012a).

As discussed (Section 1.6), there are multiple mechanisms by which *N. gonorrhoeae* may become resistant to antimicrobials: genes encoding altered target sites; mutations leading to increased efflux and decreased influx of antimicrobials; and enzyme inactivation of target sites. Acquisition of individual resistance determinants may induce clinically non-important increases in MIC of *N. gonorrhoeae* to the specific antimicrobials, but their effect is additive and multiple resistance determinants will result in clinically significant resistance in the strain (Unemo *et al.*, 2016; Barry *et al.*, 2009). The following is a summary of known resistance mechanisms in *Neisseria gonorrhoeae*.

3.1.1 Mutations leading to alteration in binding sites or target enzymes

The primary binding site for β -lactams is the penicillin-binding protein-2 (PBP2) which is encoded by *penA*. Alterations in *penA* are characterised as mosaic or non-mosaic depending on the number of amino acid substitutions present (up to 70 in mosaic alleles), resulting in varying level of resistance with some mutations being associated with greater increases in MIC. An alternative or secondary binding site for β -lactam antimicrobials is PBP1, encoded by *ponA*. Mutations in *ponA* can lead to decreased binding affinity of β -lactams.

Fluoroquinolones target DNA gyrase II or topoisomerase IV, which are encoded by *gyrA* and *parC*, respectively. Mutations in these genes will lead to decreased affinity of fluoroquinolones for the target and thereby, phenotypic resistance (Redgrave *et al.*, 2014).

Plasmid-borne *tetM* encodes the TetM protein which blocks tetracycline from binding to the 30s ribosomal subunit thereby inhibiting its action (Mlynarczyk *et al.*, 2016).

3.1.2 Mutations leading to increased efflux

mtrR is a transcriptional repressor of the *mtrCDE* operon and mutations in it or its promoter region will increase expression of the MtrC-MtrD-MtrE efflux pump. The MtrCDE efflux pump spans the cell envelope and is of the resistance-nodulation-division (RND) family of efflux pumps (Poole *et al.*, 2005). RND efflux pumps work with periplasmic membrane fusion proteins as well as outer membrane proteins (Poole

et al., 2005). *mtrR* mutations will allow increased efflux of antimicrobials but also activates the *penB* resistance determinant, encoding an altered PorB which allows decreased influx of certain antimicrobials. A single nucleotide deletion in the 13-bp inverted repeat region of the *mtrR* promoter (adenine in the -35 to -10 region) also upregulates the *mtrCDE* operon resulting in reduced susceptibility to macrolides (Xia *et al.*, 2000).

The NorM efflux pump is a member of the multidrug and toxic compound efflux (MATE) pump family (Poole *et al.*, 2005). It is regulated by *norM* and mutations in this gene lead to overexpression of the efflux pump and increased MICs to fluoroquinolones in *N. gonorrhoeae*.

3.1.3 Mutations leading to decreased influx

penB (or *porB*) encodes the porin PorB, alterations of which will reduce the amount of penicillin entering the cell. The *penB* phenotype is only seen if there are concomitant alterations in *mtrR*, as mentioned above. *pilQ* encodes pore-forming secretin PilQ through which there influx into the cell is reduced, but these have only been found in laboratory isolates as they likely bear a fitness cost (Unemo *et al.*, 2016).

3.1.4 Modification by enzymes

Plasmid-encoded β -lactamase enzymes hydrolyse the cyclic amide bond of β -lactamase-susceptible β -lactam antibiotics, opening the β -lactam ring, thereby inactivating the drug. Other modifying enzymes are discussed later.

Many resistance determinants will affect a number of antimicrobials, e.g. mutations in *penA* will affect penicillin and extended-spectrum cephalosporins (ESCs) (especially mosaic *penA* alleles which occur by either recombination with *penA* from other *Neisseria* spp. or by amino acid substitutions) and mutations in *mtrR* will affect penicillins, tetracyclines, macrolides and ESCs. Fluoroquinolones resistance is mediated by *gyrA* and *parC* while macrolide resistance may be mediated by mutations in 23S rRNA.

Knowledge of resistance determinants is essential for monitoring evolution of resistance in *N. gonorrhoeae* as well as for future applications of advancing molecular technology. Many studies now evaluate the possibility of simultaneous detection of *N. gonorrhoeae* and known resistance determinants by molecular methods in a clinical sample (Allan-Blitz *et al.*, 2017; Graham 2017 STI; Pond *et al.*, 2016) and recently WGS has been assessed for prediction *N. gonorrhoeae* MIC (Eyre *et al.*, 2017). In the future, molecular techniques may supersede traditional microbiological methods; however, at this time culture and susceptibility testing are still essential and are suitably augmented by molecular detection of resistance mechanisms.

3.2 Aims

The first aim of this chapter was to identify resistance determinants in the selected isolates (43/609) and correlate those to the MIC results and clinical interpretations (where available) for the specific antimicrobial, as well as identifying resistance determinants present in MDR and XDR isolates identified in this study. A second aim

of this chapter was to specifically analyse the resistance determinants in isolates with reduced susceptibility or resistance to ESCs. Finally, to perform an in-depth analysis of resistance determinants found in isolates displaying various levels of resistance to azithromycin, i.e. low-level resistance and HLAzi-R.

3.3 Objectives

1. Extract DNA from isolates selected for WGS.
2. Library preparation and WGS of the selected isolates.
3. Identify specific genes or mutations in specific genes in silico which are known to be associated with resistance in *N. gonorrhoeae*.
4. Correlate MICs/clinical interpretations with presence or absence of resistance determinants.
5. Analyse isolates with reduced susceptibility or resistance to ESCs.
6. Identify resistance determinants associated with the various levels of azithromycin resistance identified (*Sections 2.6.1-2.6.12*).

3.4 Materials and Methods

Isolates were selected for DNA extraction ($n = > 200$) based on MIC results. These included isolates that displayed reduced susceptibility or resistance to ESCs, resistance to azithromycin, high-level resistance to penicillin, ciprofloxacin or tetracycline, or elevated MICs to ertapenem, gentamicin, gemifloxacin, fosfomycin, spectinomycin and solithromycin.

3.4.1 DNA Extraction

Over 200 isolates were selected based on susceptibility profiles for possible further investigation by molecular methods. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). This was carried out at the time of MIC determination and the template DNA was stored at -80°C for later use. Forty-three of these isolates were later selected for sequencing.

3.4.1.1 Method

1. A sweep of bacteria from a chocolate agar plate was inoculated into 180 µl ATL buffer and vigorously stirred.
2. 20 µl of proteinase K was added to the sample, mixed by vortexing for 15 seconds and incubated in a hot plate at 56°C for 30 minutes. The sample was vortexed intermittently during the 30 minutes.
3. The 1.5 ml microcentrifuge tube was briefly centrifuged at 8000 rpm to remove drops from the inside of the lid.
4. 200 µl of Buffer AL was added to the sample, mixed thoroughly by pulse-vortexing for 15 seconds, and incubated at 70°C for 10 min then briefly centrifuged to remove drops from inside the lid.
5. 200 µl of ethanol (96–100%) was added to the sample and mixed thoroughly by pulse-vortexing for 15 seconds. Again the sample was briefly centrifuged to remove drops from inside the lid. If a white precipitate formed on addition of ethanol, it was added to the QIAamp Mini spin column.

6. This mixture (including any precipitate) was transferred to the spin column (in a 2 ml collection tube) without wetting the rim and centrifuged at 8000 rpm for 1 min. The spin column was put in a clean 2 ml collection tube, and the filtrate discarded.
7. 500 µl of Buffer AW1 was added to the spin column without wetting the rim. This was centrifuged at 8000 rpm for 1 minute. The spin column was transferred to a clean 2 ml collection tube and the filtrate was discarded.
8. 500µl of Buffer AW2 was added to the spin column without wetting the rim. This was centrifuged at 14000 rpm for 3 minutes.
9. The spin column was transferred to a new 2ml collection tube and centrifuged and the filtrate was discarded. This collection tube was centrifuged at 14000 rpm for 1 minute to eliminate any remaining buffer.
10. The spin column was transferred to a clean 1.5ml microcentrifuge tube and the filtrate was discarded. 200 µl of molecular grade water was added to the spin column. This was incubated at room temperature for 1 minute then centrifuged at 8000 rpm for 1 minute.
11. Step 10 was repeated.
12. The eluate was transferred to a 1.5 ml tube for storage at -80°C.

3.4.2 Whole genome sequencing

The MICs of the DNA-extracted isolates above were examined to select for the most resistant strains. In total 43 isolates were selected for whole genome sequencing including; all of those resistant or with reduced susceptibility to ESCs; all HLAzi-R isolates and those with lower levels of azithromycin resistance; all isolates exhibiting

high level resistance to penicillin, ciprofloxacin and tetracycline; and elevated MICs to the other drugs as mentioned above.

3.4.2.1 Library preparation

Library preparation is a multi-stepped process required before any sample of DNA can be sequenced on the Illumina MiSeq Analyser. This process is carried out simultaneously on each sample prior to any run.

3.4.2.2. Illumina Experiment Manager

Illumina's experiment manager was downloaded from the Illumina website <http://www.illumina.com/index-c.ilmn>. A .csv file containing the sample name as well as i5 and i7 indices was created and exported to the MiSeq desktop analyser prior to sequencing.

3.4.2.3 Fluorometric Quantitation of Input DNA

The Nextera XT sample preparation kit requires the input of 1 ng of DNA. A highly sensitive fluorometric method of DNA measurement is advised by the Nextera XT protocol as opposed to the photometric methods such as the Nano-drop (NanoDrop Technologies, Wilmington USA) DNA was measured on the Qubit® fluorometer using Qubit® dsDNA HS assay kit (Thermo Fisher Scientific). Qubit™ working solution

was made by diluting the Qubit™ dsDNA HS reagent 1:200 in Qubit™ dsDNA HS buffer. Calibration standards were made up in individual tubes by adding 190 µl Qubit working solution to 10 µl of each of the standards. These standards were then used to calibrate the Qubit fluorometer. The DNA elute of each sample was then measured on the Qubit fluorometer at an absorbance of 260 nm in a dilution of 198 µl Qubit working solution and 2 µl DNA.

3.4.2.4 Tagmentation of Input DNA

Tagmentation is the simultaneous process of fragmenting and tagging both ends of the input DNA. This process was carried out using the Illumina Nextera® XT DNA Sample Preparation Kit utilising engineered transposome technology. The DNA concentration of each of the samples was measured using the fluorometric quantification and DNA was diluted to 0.2 ng/µl DNA concentration using nuclease-free water (NFW) as a diluent. Using the Illumina Experiment Manager work sheet for guidance, 5 µl of diluted sample elute (1ng total DNA) was added to its respective well position along with 10 µl Tagment DNA (TD) buffer and 5 µl Amplicon Tagment Mix (ATM). The plate was then sealed and centrifuged at 280 x g at 20°C for 1 minute. The plate was incubated in a Bio-Rad DNA Engine thermal cycler at 55°C for 5 minutes with a 10°C hold. Following tagmentation, 5 µl of neutralization buffer was added to each well, the plate was resealed and centrifuged at 280 x g at 20°C for 1 minute. The plate was left at room temperature for 5 minutes for neutralization of the transposome to occur.

3.4.2.5 Indexing of Input DNA

PCR amplification was used to insert unique S5 and N7 indices at either end of tagmented samples. The adaptor regions act as a primer binding sites for the S5 and N7 primers which also contain the unique overhanging sequences that become incorporated into the tagmented fragments during PCR. Nextera PCR Master Mix was added to each well along with 5 µl of 6 unique N7 primers to each row and 5 µl of 8 unique S5 primers to each column in the 96 well plate. This pattern ensures 96 different index primer combinations are used across each of the wells. The plate was sealed and centrifuged at 280 x g at 20°C for 1 minute. PCR was carried out on the Bio-Rad PTC 200 thermal cycler.

3.4.2.6 Clean-up of Tagmented DNA

Size selection and PCR clean up were carried out using the Agencourt AMPure XT 60ml kit (Beckman Coulter Genomics). Following PCR amplification, the 96 well plate was centrifuged at 280 x g at 20°C for 1 minute to collect any condensation. AMPure beads were vortexed for 30 seconds and 30 µl of the mixture was added to each well. This volume of AMPure mixture ensures size selection of tagmented products of roughly 300-500 bp in size. AMPure beads were left to incubate at room temperature for 5 minutes before being place on a magnetic stand for a future 2 minutes. The supernatant was then removed once it appeared clear. The beads were then washed in 200 µl of 80% ethanol for 30 seconds before removing the supernatant and repeating the wash. The plate was left to air dry for 15 minutes. The plate was removed from the

magnetic stand before re-suspending the beads in 52.5 µl of resuspension buffer (RBS). The solution was incubated at room temperature for 2 minutes before placing the plate back on the magnetic stand for 2 minutes. The Supernatant from each of the wells was transferred to their corresponding position in a clean 96 well plate and the magnetic beads disposed of. The cleaned library was sealed and stored at -20°C.

3.4.2.7 Library Normalisation

Fluorometric quantification for each tagmented samples was carried using Qubit. Each sample concentration was recorded and an adjusted volume for each sample was pooled into one solution. The majority of samples were calculated to contain 2.3% of the total DNA concentration of the pooled library.

3.4.2.8 Pooled Library Quantification

The pooled library was diluted 1:1000 using molecular grade water (MGW). The KAPA qPCR (KAPA Biosystems, Wilmington, Massachusetts) reagents were thawed and vortexed prior to creating master mix. Standards 1 to 6 along with the MGW and pooled library sample were tested in triplicate by adding 8 µl of master mix to 2 µl of each sample in a 96-well plate. The 96-well plate was covered in an adhesive seal and centrifuged briefly prior to loading the plate onto the ABI Prism® 7500 Sequence Detection System (Applied Biosystems, Foster City, California). The concentration of the diluted pooled library was quantified against the standard curve and multiplied by the dilution factor to calculate the concentration of the undiluted pooled library.

3.4.2.9 Whole genome sequencing

Sample DNA libraries were prepared using the procedure outlined (*Section 1.12.1*) and sequenced using the Illumina MiSeq bench top analyser. MiSeq reagent cartridge was removed from the fridge and allowed to thaw 30 minutes prior to starting the run. The pooled library was thawed, vortexed and centrifuged briefly to ensure the library was well mixed. Hybridization buffer was mixed with the pooled library to make a 600 µl volume of 20 pM final concentration for cluster generation. Dilute amplicon library was then vortexed and incubated at 96°C for 2 minutes to separate the double stranded DNA before being placed on ice for 5 minutes. The 600 µl dilute library loaded into the 'load here' well of the MiSeq reagent cartridge. The .csv file created on the Illumina Experiment Manager was downloaded onto the MiSeq analyser thus enabling sample indexing. The flow cell was washed with sterile water and loaded onto the MiSeq analyser along with reagent cartridge. The run was started using the on screen display. Data was uploaded to Illumina's basespace cloud network along with a summary of run parameters. Unprocessed sequence reads in the form of FastQ files for each sample were downloaded from Illumina's basespace cloud for bioinformatic analysis.

3.4.2.10 Data analysis

Reconstruction of raw FASTQ data was performed with SPADes de novo assembler using the DTU centre for genomic epidemiology 2.1 Assembler pipeline. Reconstructed contigs were then used to determine MLST type and genomic AMR alleles (ponA, porB, parC, parE, folP, mtrR, gryA, penA and rpoB) using pubMLST tool. PenA

alleles were assigned a genotype based on the NG-STAR database allocation.

Additional AMR genes were identified in isolate contig files using the publically available ARG-ANNOT tool. ARG-ANNOT contains a diverse database of AMR genes including the *ere*(A,B), *erm*(A,B,C), *mph*(A,B,C) and *mef* (C) genes, along with TetM and TEM plasmids reported in this study. Finally a local BLAST search was performed on de novo assemblies to determine NG-MAST type.

Reference guided assembly was also utilised on the Galaxy platform in order to identify and visualise specific point mutations associated chromosomally mediated AMR.

FASTQ files were uploaded to the online galaxy server and trimmed using the FASTQC and Trimmomatic tools. Reads were then mapped using BWA to the NCCP11945 reference genome. Assembled BAM files were cleaned up using SAMTools and visualised on IGV. Point mutations associated with resistance in the *rplD*, *rplV*, *rpsJ*, *rpsE* and *norM* genes along with those not identified by pubMLST in the aforementioned genes were identified visually on Galaxy. As there are four copies of the *rrl* (23S rRNA) gene within the *Neisseria gonorrhoeae* genome, reference mapping was performed separately to the whole genome alignment. Prevalence of the A to G mutations at position 2059 was rounded to its nearest quartile and reported as an estimate of overall allele prevalence within the genome.

3.5 Results

Of the 43 isolates selected for whole genome sequencing, one was collected in 2012, four in 2013, six in 2014, 13 in 2015, 18 in 2016 and the date is unknown for isolate number 422322. Sex is unknown for 2 patients, 6 were female and the remaining 35 were male. The age range was 18-60 years. The age was unknown for 2 patients and

the median age for the remainder of patients is 26 years. Eight isolates were from the pharynx, three from the rectum and twenty-eight were urogenital. The site was not recorded for the remaining 4 isolates. *Appendix 7* contains a summary of phenotypic and molecular characteristics of the 43 sequenced isolates. The MICs for the 43 sequenced isolates are summarised here (Table 3.1).

Number	CRO	CFM	CTX	ETP	Azm	Cip	Gemi	Pen	BL	Tet	SPC	Gent	FOS	SOL
016	0.047	0.19	0.25	0.032	1	> 32	1.5	2	N	1	12	6	32	0.094
81513	0.032	0.19	0.125	0.064	0.5	> 32	2	0.75	N	0.75	12	6	16	NT
178526	0.032	0.19	0.25	0.032	1	> 32	1	1	N	0.75	8	6	16	0.032
71324	0.047	0.19	0.19	0.094	0.125	> 32	3	4	P	24	6	1.5	6	NT
76972	0.047	0.19	0.25	0.032	0.75	> 32	2	1.5	N	1	12	4	32	NT
501373	0.032	0.125	0.125	0.023	0.5	16	2	0.25	N	1	8	6	32	NT
422102	0.032	0.125	0.38	0.032	1	> 32	2	1	N	1	16	8	32	0.094
191262	0.023	0.125	0.125	0.032	0.5	> 32	1.5	0.5	N	1	8	4	24	NT
009	0.064	0.125	0.125	0.032	0.75	> 32	1.5	1	N	1.5	12	4	48	0.064
035	0.047	0.125	0.125	0.032	1	> 32	1.5	0.75	N	0.75	6	4	24	0.047
82432	0.023	0.125	0.25	0.032	1	> 32	6	0.75	N	1	12	6	24	0.19
176561	0.016	0.125	0.19	0.032	0.5	> 32	1.5	0.75	N	0.5	8	6	12	NT
68931	0.032	0.125	0.25	0.047	0.5	> 32	1.5	1	N	1	12	8	12	NT
422671	0.064	0.125	0.25	0.064	0.5	> 32	2	0.5	N	2	16	4	12	NT
425204	0.016	0.125	0.125	0.032	0.5	0.016	0.006	0.25	N	0.125	16	4	12	NT
179501	0.016	0.094	0.094	0.023	0.25	3	0.75	0.25	N	0.25	8	8	12	NT
86606	0.008	0.094	0.064	0.047	0.5	0.006	0.004	0.25	N	0.38	8	6	24	NT
177574	0.047	0.094	0.125	0.016	0.5	8	1	1	N	0.5	8	3	16	NT
286008	0.023	0.094	0.19	0.032	0.38	> 32	0.75	0.75	N	1	16	6	24	NT
199856	0.002	≤ 0.016	0.004	0.008	> 256	0.004	≤ 0.002	0.125	N	0.38	8	3	8	0.5
197747	0.016	0.032	0.094	0.012	16	2	2	0.25	N	0.5	12	4	8	0.19
196712	0.008	0.016	0.125	0.023	> 256	0.008	0.006	0.25	N	0.75	16	6	12	> 32
166587	0.008	0.016	0.125	0.016	8	4	2	0.25	N	0.75	12	6	12	0.19
193852	0.004	≤ 0.016	0.006	0.023	> 256	≤ 0.002	≤ 0.002	0.125	N	0.38	8	4	8	0.38
191587	0.008	0.032	0.064	0.016	> 256	0.008	0.004	0.25	N	1	8	3	12	> 32
006	0.016	0.016	0.032	0.012	> 256	0.012	0.006	0.25	N	1	8	4	16	> 32
181798	0.008	0.023	0.047	0.023	> 256	0.008	0.006	0.25	N	0.75	8	4	24	6
179411	0.008	0.023	0.064	0.016	4	12	1	0.5	N	0.5	12	6	24	0.19
178575	0.003	0.016	0.012	0.012	32	0.004	0.003	0.19	N	0.38	6	6	16	0.19
178487	0.006	0.023	0.047	0.032	> 256	0.008	0.004	0.19	N	0.5	6	4	12	1
67818	0.012	0.023	0.064	0.012	6	> 32	1.5	0.19	N	0.38	8	3	8	0.125
425657	0.016	0.032	0.012	0.006	4	16	1.5	0.25	N	1	8	3	16	0.094
41277	0.003	≤ 0.016	0.012	0.008	12	0.004	0.003	0.125	N	0.25	6	4	12	0.125
422322	0.008	≤ 0.016	0.016	0.004	0.5	2	0.5	> 32	P	0.5	16	8	12	NT
008	0.008	≤ 0.016	0.023	0.006	0.5	3	0.75	> 32	P	0.5	12	6	12	NT
420132	0.008	≤ 0.016	0.008	0.012	0.064	2	0.5	> 32	P	0.5	12	8	12	NT
168628	0.064	0.064	0.25	0.012	0.25	2	0.38	1	N	1	12	4	8	NT
024	0.094	0.25	0.38	0.064	0.38	> 32	1.5	1.5	N	1	8	4	48	NT
190440	0.004	0.016	0.032	0.012	0.032	> 32	4	4	P	32	4	4	12	NT
180505	0.012	0.023	0.064	0.016	2	0.032	0.012	0.5	N	2	12	16	24	0.125
039	0.012	0.016	0.016	0.016	0.25	4	1.5	1.5	P	8	8	6	96	NT
193058	0.008	0.016	0.047	0.016	0.5	8	12	0.125	N	0.38	6	6	8	NT
194698	0.016	0.064	0.19	0.047	0.5	16	3	0.25	N	0.5	8	4	48	NT

Table 3.1. MICs (mg/L) of the 43 sequenced isolates to penicillin (PEN), ciprofloxacin (CIP), azithromycin (AZM), ceftriaxone (CRO), cefixime (CFM), tetracycline (TET), cefotaxime (CTX), ertapenem (ETP), gentamicin (GENT), gemifloxacin (GEMI), spectinomycin (SPC), fosfomycin (FOS) and solithromycin (SOL) where performed. The β -lactamase (BL) result is also included. Not tested (NT).

3.5.1 Ceftriaxone

Although all 43 isolates had ceftriaxone MICs below the EUCAST clinical breakpoint for susceptibility, nine of them had reduced susceptibility to ceftriaxone (CRO-RS), defined as CRO MIC > 0.032 mg/L. These 9 isolates had mosaic *penA* X (n = 1, MIC 0.047 mg/L), mosaic *penA* XXXIV (n = 6, MIC 0.047-0.094 mg/L) and non-mosaic *penA* XIII (n = 2, MICs 0.47-0.064 mg/L) alleles (Table 3.2). The amino acid substitutions associated with each *penA* type are shown (Table 3.3). Glycine to lysine at position 120 (G120K) plus alanine to glycine/asparagine/aspartate at position 121 (A121G/N/D) substitutions in *penB* were seen in the 9 isolates with reduced susceptibility to ceftriaxone. Each isolate also had L421P (leucine → proline) substitutions in *ponA* and 8 had A13 (adenine) deletions in the *mtrR* promoter region. All had alterations in the *mtrR* gene, mostly an H105Y (histidine → tyrosine) amino acid substitution (n = 6) or a G45D (glycine → aspartate) substitution (n = 2) and one had an A39T (alanine → threonine) substitution plus an internal stop codon.

The nine isolates (CRO MIC > 0.032 mg/L) share similar combinations of resistance determinants with some of the remaining 34 isolates (CRO MICs of 0.002-0.032 mg/L), but those with similar resistance determinants have CRO MICs 0.016-0.032 mg/L (Table 3.4). The 34 isolates had both mosaic *penA* (XXXIV) and non-mosaic *penA* (II, XLIX, XIX, XLIV, and XIV) types. *penB* alterations G120K/D and/or A121N/G/S (S = serine) were detected in 27 (79%) of these (Table 3.4). All isolates had at least one mutation in *mtrR*. Nineteen (56%) isolates have A13 deletions in the *mtrR* promoter.

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
71324 (2014)	0.047	0.19	0.19	0.094	mosaic X	L421P	G120K A121G	A39T ISC	WT
016 (2013)	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
035 (2016)	0.047	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
76972 (2014)	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
024 (2012)	0.094	0.25	0.38	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
009 (2013)	0.064	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422671 (2016)	0.064	0.125	0.25	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
177574 (2015)	0.047	0.094	0.125	0.016	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del
168628 (2016)	0.064	0.064	0.25	0.012	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del

Table 3.2. Molecular characteristics of 9 CRO-RS isolates (CRO MIC > 0.032 mg/L). WT = wild type.

<i>penA</i> alleles		Amino acid substitutions
Mosaic	X	I312M, V316T, D345A F504L, N512Y, G545S
	XXXIV	I312M, V316T, D345A, F504L, N512Y, G545S
Non-mosaic	II	345R insertion, F504L, A510V, I515V
	XLIX	345R insertion, F504L, A510V, I515V, A501T
	XLIV	345R insertion, A501T, P551L, F504L
	XIX	345R insertion, F504L, A510, I515V, H541N
	XIV	345R insertion, F504L, A510V, I515V, H541N
	XIII	345R insertion, A501V, P551S, F504L, A510V, I515V

Table 3.3. *penA* allele types and associated amino acid substitutions found among 43 isolates.

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
006 (2013)	0.016	0.016	0.032	0.012	non-mosaic II	WT	A121S	G45D	A12C
181798 (2015)	0.008	0.023	0.047	0.023	non-mosaic II	WT	A121S	G45D	WT
196712 (2016)	0.008	0.016	0.125	0.023	non-mosaic II	WT	A121S	G45D	WT
178487 (2015)	0.006	0.023	0.047	0.032	non-mosaic II	WT	A121S	G45D	WT
193852 (2016)	0.004	≤ 0.016	0.006	0.023	non-mosaic II	WT	A121S	G45D	A13 del
199856 (2016)	0.002	≤ 0.016	0.004	0.008	non-mosaic II	WT	A121S	G45D	WT
191587 (2016)	0.008	0.032	0.064	0.016	non-mosaic II	WT	A121S	G45D	WT
67818 (2014)	0.012	0.023	0.064	0.012	non-mosaic XLIX	L421P	G120K A121D	D79N T86A H105K	A13 del
190440 (2016)	0.004	0.016	0.032	0.012	non-mosaic XIX	L421P	G120K A121G	A39T ISC	WT
420132 (2016)	0.008	≤ 0.016	0.008	0.012	non-mosaic II	WT	WT	A39T	WT
286088 (2014)	0.023	0.094	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
176561 (2015)	0.016	0.125	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
501373 (2016)	0.032	0.125	0.125	0.023	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
194698 (2016)	0.016	0.064	0.19	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
191262 (2016)	0.023	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
179501 (2015)	0.016	0.094	0.094	0.023	mosaic XXXIV	L421P	A121S	H105Y	A13 del
422102 (2016)	0.032	0.125	0.38	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
81513 (2015)	0.032	0.19	0.125	0.064	mosaic XXXIV	L421P	G120K A121D	H105Y	A13 del
178526 (2015)	0.032	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
68931 (2014)	0.032	0.125	0.25	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del

425204 (2016)	0.016	0.125	0.125	0.032	mosaic XXXIV	WT	WT	A39T	WT
193058 (2016)	0.008	0.016	0.047	0.016	non-mosaic XLIV	L421P	WT	D79N T86A H105K	A13 del
197747 (2016)	0.016	0.032	0.094	0.012	non-mosaic XLIV	L421P	WT	A39T ISC	A13 del
425657 (2016)	0.016	0.032	0.012	0.006	non-mosaic XLIV	L421P	G120K A121D	D79N T86A H105K	A13 del
422322 (NA)	0.008	≤ 0.016	0.016	0.004	non-mosaic II	WT	G120D A121G	A39T ISC	WT
008 (2013)	0.008	≤ 0.016	0.023	0.006	non-mosaic II	WT	G120D A121G	H105Y	A13 del
166587 (2016)	0.008	0.016	0.125	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del
180505 (2015)	0.012	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	WT
178575 (2015)	0.003	0.016	0.012	0.012	non-mosaic II	WT	WT	A39T R44H	WT
41277 (2014)	0.003	≤ 0.016	0.012	0.008	non-mosaic II	WT	WT	A39T R44H	WT
179411 (2015)	0.008	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del
039 (2015)	0.012	0.016	0.016	0.016	non-mosaic XIV	WT	G120K A121N	D79N T86A H105K	WT
82432 (2015)	0.023	0.125	0.25	0.032	mosaic XXXIV	L421P	G120K A121D	A39T	A13 del
86606 (2015)	0.008	0.094	0.064	0.047	mosaic XXXIV	WT	WT	A39T R44H	WT

Table 3.4. Molecular characteristics of 34 isolates with ceftriaxone MIC ≤ 0.032 mg/L.

WT = wild type.

3.5.2 Cefixime

The MIC range for cefixime was ≤ 0.016 -0.25 with 6 resistant isolates (CFM-R, MIC > 0.125 mg/L) and a further 14 displayed reduced susceptibility (CFM-RS, MIC = 0.125 mg/L). Of the 6 CFM-R isolates, 5 had mosaic XXXIV *penA* alleles and one had a mosaic X *penA* allele (Table 3.5). All 6 resistant isolates had L421P substitution in *ponA* and G120K plus A121G/N/D substitutions in *penB*. Five had an A13 deletion in the *mtrR* promoter (one including an internal stop codon) and all isolates had alterations in *mtrR* (Table 3.5).

Among the 14 CFM-RS isolates (MIC = 0.125 mg/L) 13 had mosaic *penA* XXXIV allele and one had a non-mosaic XIII allele which had A501V (alanine→valine), P551S (proline→serine), F504L (phenylalanine→leucine), A510V and I515V (isoleucine→valine) amino acid substitutions (Tables 3.6 & 3.3). Twelve (86%) had L421P substitution in *ponA* as well as G120K plus A121N/D (n=11) or A121S substitutions in *penB* plus an A13 deletion in the *mtrR* promoter (Table 3.6). The other two isolates were wild-type *ponA*, *penB* and *mtrR* promoter (both mosaic XXXIV *penA*). All 14 isolates had amino acid substitutions in *mtrR* including H105Y (n = 10), A39T (n = 2), G45D (n = 1) and A39T plus R44H (arginine → histidine) (n = 1) (Table 3.6).

The remaining 23 isolates had MICs to cefixime of ≤ 0.064 mg/L. All of these had non-mosaic *penA* alleles except one which had a mosaic XXXIV *penA* allele and MICs to ceftriaxone, cefixime and cefotaxime of 0.016, 0.064 and 0.019 mg/L, respectively (Table 3.7). Eleven isolates (48%) had G120K plus A121N/G/D amino acid

substitutions in *penB* and a further 7 (30%) had single A121S (n = 7) or A121G (n = 1) substitutions. Five (22%) are wild-type *penB*. Seven had L421P substitutions in *ponA*, 7 had A13 deletion and one had an A12C (alanine→cysteine) substitution in the *mtrR* promoter region and all had alterations in *mtrR* (Table 3.7).

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
71324 (2014)	0.047	0.19	0.19	0.094	mosaic type X	L421P	G120K A121G	A39T ISC	WT
016 (2013)	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
76972 (2014)	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
024 (2012)	0.094	0.25	0.38	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
81513 (2015)	0.032	0.19	0.125	0.064	mosaic XXXIV	L421P	G120K A121D	H105Y	A13 del
178526 (2015)	0.032	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del

Table 3.5. Molecular characteristics of CFM-R (n = 6) isolates. WT = wild type.

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
035 (2016)	0.047	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
286088 (2014)	0.023	0.094	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
176561 (2015)	0.016	0.125	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
501373 (2016)	0.032	0.125	0.125	0.023	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
009 (2013)	0.064	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
191262 (2016)	0.023	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422671 (2016)	0.064	0.125	0.25	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
179501 (2015)	0.016	0.094	0.094	0.023	mosaic XXXIV	L421P	A121S	H105Y	A13 del
422102 (2016)	0.032	0.125	0.38	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
68931 (2014)	0.032	0.125	0.25	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
425204 (2016)	0.016	0.125	0.125	0.032	mosaic XXXIV	WT	WT	A39T	WT
177574 (2015)	0.047	0.094	0.125	0.016	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del
82432 (2015)	0.023	0.125	0.25	0.032	mosaic XXXIV	L421P	G120K A121D	A39T	A13 del
86606 (2015)	0.008	0.094	0.064	0.047	mosaic XXXIV	WT	WT	A39T R44H	WT

Table 3.6. Molecular characteristics of CFM-RS (n = 14) isolates. WT = wild type.

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
006 (2013)	0.016	0.016	0.032	0.012	non-mosaic II	WT	A121S	G45D	A12C
181798 (2015)	0.008	0.023	0.047	0.023	non-mosaic II	WT	A121S	G45D	WT
196712 (2016)	0.008	0.016	0.125	0.023	non-mosaic II	WT	A121S	G45D	WT
178487 (2015)	0.006	0.023	0.047	0.032	non-mosaic II	WT	A121S	G45D	Wt
193852 (2016)	0.004	≤ 0.016	0.006	0.023	non-mosaic II	WT	A121S	G45D	A13 del
199856 (2016)	0.002	≤ 0.016	0.004	0.008	non-mosaic II	WT	A121S	G45D	WT
191587 (2016)	0.008	0.032	0.064	0.016	non-mosaic II	WT	A121S	G45D	WT
67818 (2014)	0.012	0.023	0.064	0.012	non-mosaic XLIX	L421P	G120K A121D	D79N T86A H105K	A13 del
190440 (2016)	0.004	0.016	0.032	0.012	non-mosaic XIX	L421P	G120K A121G	A39T ISC	WT
420132 (2016)	0.008	≤ 0.016	0.008	0.012	non-mosaic II	WT	WT	A39T	WT
194698 (2016)	0.016	0.064	0.19	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
193058 (2016)	0.008	0.016	0.047	0.016	non-mosaic XLIV	L421P	WT	D79N T86A H105K	A13 del
197747 (2016)	0.016	0.032	0.094	0.012	non-mosaic XLIV	L421P	WT	A39T ISC	A13 del
425657 (2016)	0.016	0.032	0.012	0.006	non-mosaic XLIV	L421P	G120K A121D	D79N T86A H105K	A13 del
168628 (2016)	0.064	0.064	0.25	0.012	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del
422322 (NA)	0.008	≤ 0.016	0.016	0.004	non-mosaic II	WT	G120D A121G	A39T ISC	WT
008 (2013)	0.008	≤ 0.016	0.023	0.006	non-mosaic II	WT	G120D A121G	H105Y	A13 del
166587 (2016)	0.008	0.016	0.125	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del
180505 (2015)	0.012	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	WT
178575 (2015)	0.003	0.016	0.012	0.012	non-mosaic II	WT	WT	A39T R44H	WT
41277 (2014)	0.003	≤ 0.016	0.012	0.008	non-mosaic II	WT	WT	A39T R44H	WT
179411 (2015)	0.008	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del
039 (2015)	0.012	0.016	0.016	0.016	non-mosaic XIV	WT	G120K A121N	D79N T86A H105K	WT

Table 3.7. Molecular characteristics of CFM (n = 23) isolates with CFM MIC ≤ 0.064 mg/L.

3.5.3 Cefotaxime

Thirteen isolates were resistant to cefotaxime (MIC > 0.125 mg/L) and 11 displayed reduced susceptibility (CTX-RS, MIC = 0.125 mg/L). The MIC range for cefotaxime is 0.004-0.5 mg/L. The thirteen CTX-R isolates also exhibited resistance to cefixime (n = 5) and reduced susceptibility to ceftriaxone (n = 6). Overall, 11 of the 13 CTX-R isolates (85%) had mosaic XXXIV *penA* alleles and one had a mosaic X *penA* allele (Table 3.8). The remaining isolate had a non-mosaic XIII *penA* allele. All 13 had G120K plus A121N/G/D amino acid substitutions in *penB* and L421P substitutions in *ponA*. Twelve had A13 deletions in the *mtrR* promoter region, the exception being the isolate with the mosaic X *penA* allele which had an A39T substitution plus an internal stop codon in the *mtrR* gene. All of the other isolates also had amino acid substitutions H105Y (n = 11), G45D (n = 1) and A39T (n = 1) in the *mtrR* gene (Table 3.8).

Eleven isolates were CTX-RS which included 1 CFM-R, 7 CFM-RS and 3 CRO-RS isolates. Seven of these 11 CTX-RS isolates had mosaic XXXIV *penA* alleles and the remaining 4 had non-mosaic *penA* alleles (Table 3.9). Seven (64%) had double amino acid substitutions (G120K plus A121N/D) and two had single A121S substitutions in *penB*. Two were wild-type. Eight isolates (73%) had both L421P substitutions in *ponA* and A13 deletions in the *mtrR* promoter regions. All 11 isolates had alterations in *mtrR* (Table 3.9).

The remaining 19 isolates had MICs to cefotaxime of ≤ 0.064 mg/L. Eighteen (95%) had non-mosaic *penA* alleles and one had a mosaic XXXIV *penA* allele (MICs to ceftriaxone, cefixime and cefotaxime of 0.008, 0.094 and 0.064 mg/L, respectively) (Table 3.10). Eight (42%) had G120K plus A121N/D/G substitutions in *penB*, 6 (32%) had single A121S (n = 6) substitutions and 5 (26%) were wild-type. Four (21%) have L421P substitutions in *ponA*. All had alterations in the *mtrR* gene (Table 3.10) and 7

isolates had alterations in the *mtrR* promoter region, including A13 deletions (n = 6) and an A12C nucleotide substitution (n = 1).

3.5.4 Ertapenem

The ertapenem range for the 43 isolates was 0.004-0.125 mg/L. Seven of the isolates had an MIC > 0.032 mg/L. All of these were mosaic *penA* allele types (6 XXXIV and 1 X) (Table 3.11). Six had G120K plus A121N/D/G substitutions in *penB* and L421P substitutions in *ponA* and one was wild-type for both. Five isolates had A13 deletions in the *mtrR* promoter and all had alterations in *mtrR* (Table 3.11).

Seventeen isolates had an MIC of 0.032 mg/L to ertapenem. Of these, 13 (77%) had mosaic XXXIV *penA* alleles, the remaining 4 had non-mosaic II *penA* alleles (Table 3.12). Eleven (65%) had double amino acid substitutions in *penB* (G120K plus A121N/D) and 5 (29%) had single A121S substitutions. One isolate was wild-type *penB*. The 4 isolates with non-mosaic II *penA* alleles had wild-type *ponA*, 3 out of 4 had wild-type *mtrR* promoter and all 4 had a G45D amino acid substitution in *mtrR* (Table 3.12). Most of the 13 isolates with mosaic XXXIV *penA* alleles had L421P substitutions in *ponA* (n = 12), A13 deletions in the *mtrR* promoter region (n = 12) and an H105Y alteration (n = 11) in *mtrR* (Table 3.12).

The remaining 19 isolates had ertapenem MICs of 0.004-0.016 mg/L. All of these had non-mosaic *penA* allele types (Table 3.13). Eleven (58%) had double substitutions in *penB* (G120K plus A121N/D/G) and 3 (16%) had single A121S substitutions. Five (26%) were wild-type *penB*. Seven (37%) isolates had L421P substitutions in *ponA*

(Table 3.13). Nine (47%) had A13 deletions in *mtrR* promoter and 1 had an A12C substitution. The alterations in *mtrR* include various amino acid substitutions (Table 3.13).

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
71324 (2014)	0.047	0.19	0.19	0.094	mosaic type X	L421P	G120K A121G	A39T ISC	WT
016 (2013)	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
286088 (2014)	0.023	0.094	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
76972 (2014)	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
176561 (2015)	0.016	0.125	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
024 (2012)	0.094	0.25	0.38	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
194698 (2016)	0.016	0.064	0.19	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422671 (2016)	0.064	0.125	0.25	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422102 (2016)	0.032	0.125	0.38	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
178526 (2015)	0.032	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
68931 (2014)	0.032	0.125	0.25	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
168628 (2016)	0.064	0.064	0.25	0.012	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del
82432 (2015)	0.023	0.125	0.25	0.032	mosaic XXXIV	L421P	G120K A121D	A39T	A13 del

Table 3.8. Molecular characteristics of CTX-R (n = 13) isolates. WT = wild type.

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
196712 (2016)	0.008	0.016	0.125	0.023	non-mosaic II	WT	A121S	G45D	WT
035 (2016)	0.047	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
501373 (2016)	0.032	0.125	0.125	0.023	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
009 (2013)	0.064	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
191262 (2016)	0.023	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
179501 (2015)	0.016	0.094	0.094	0.023	mosaic XXXIV	L421P	A121S	H105Y	A13 del
81513 (2015)	0.032	0.19	0.125	0.064	mosaic XXXIV	L421P	G120K A121D	H105Y	A13 del
425204 (2016)	0.016	0.125	0.125	0.032	mosaic XXXIV	WT	WT	A39T	WT
197747 (2016)	0.016	0.032	0.094	0.012	non-mosaic XLIV	L421P	WT	A39T ISC	A13 del
177574 (2015)	0.047	0.094	0.125	0.016	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del
166587 (2016)	0.008	0.016	0.125	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del

Table 3.9. Molecular characteristics of CTX-RS (n = 11) isolates. WT = wild type.

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
006 (2013)	0.016	0.016	0.032	0.012	non-mosaic II	WT	A121S	G45D	A12C
181798 (2015)	0.008	0.023	0.047	0.023	non-mosaic II	WT	A121S	G45D	WT
178487 (2015)	0.006	0.023	0.047	0.032	non-mosaic II	WT	A121S	G45D	WT
193852 (2016)	0.004	≤ 0.016	0.006	0.023	non-mosaic II	WT	A121S	G45D	A13 del
199856 (2016)	0.002	≤ 0.016	0.004	0.008	non-mosaic II	WT	A121S	G45D	WT
191587 (2016)	0.008	0.032	0.064	0.016	non-mosaic II	WT	A121S	G45D	WT
67818 (2014)	0.012	0.023	0.064	0.012	non-mosaic XLIX	L421P	G120K A121D	D79N T86A H105K	A13 del
190440 (2016)	0.004	0.016	0.032	0.012	non-mosaic XIX	L421P	G120K A121G	A39T ISC	WT
420132 (2016)	0.008	≤ 0.016	0.008	0.012	non-mosaic II	WT	WT	A39T	WT
193058 (2016)	0.008	0.016	0.047	0.016	non-mosaic XLIV	L421P	WT	D79N T86A H105K	A13 del
425657 (2016)	0.016	0.032	0.012	0.006	non-mosaic XLIV	L421P	G120K A121D	D79N T86A H105K	A13 del
422322 (NA)	0.008	≤ 0.016	0.016	0.004	non-mosaic II	WT	G120D A121G	A39T ISC	WT
008 (2013)	0.008	≤ 0.016	0.023	0.006	non-mosaic II	WT	G120D A121G	H105Y	A13 del
180505 (2015)	0.012	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	WT
178575 (2015)	0.003	0.016	0.012	0.012	non-mosaic II	WT	WT	A39T R44H	WT
41277 (2014)	0.003	≤ 0.016	0.012	0.008	non-mosaic II	WT	WT	A39T R44H	WT
179411 (2015)	0.008	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del
039 (2015)	0.012	0.016	0.016	0.016	non-mosaic XIV	WT	G120K A121N	D79N T86A H105K	WT
86606 (2015)	0.008	0.094	0.064	0.047	mosaic XXXIV	WT	WT	A39T R44H	WT

Table 3.10. Molecular characteristics of 19 isolates with CTX MICs of 0.004-0.064 mg/L. WT = wild type.

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
71324 (2014)	0.047	0.19	0.19	0.094	mosaic type X	L421P	G120K A121G	A39T ISC	WT
024 (2012)	0.094	0.25	0.38	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
194698 (2016)	0.016	0.064	0.19	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422671 (2016)	0.064	0.125	0.25	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
81513 (2015)	0.032	0.19	0.125	0.064	mosaic XXXIV	L421P	G120K A121D	H105Y	A13 del
68931 (2014)	0.032	0.125	0.25	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
86606 (2015)	0.008	0.094	0.064	0.047	mosaic XXXIV	WT	WT	A39T R44H	WT

Table 3.11. Molecular characteristics of isolates with an ertapenem MIC > 0.032 mg/L.

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
181798 (2015)	0.008	0.023	0.047	0.023	non-mosaic II	WT	A121S	G45D	WT
196712 (2016)	0.008	0.016	0.125	0.023	non-mosaic II	WT	A121S	G45D	WT
178487 (2015)	0.006	0.023	0.047	0.032	non-mosaic II	WT	A121S	G45D	WT
193852 (2016)	0.004	≤ 0.016	0.006	0.023	non-mosaic II	WT	A121S	G45D	A13 del
016 (2013)	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
035 (2016)	0.047	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
286088 (2014)	0.023	0.094	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
76972 (2014)	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
176561 (2015)	0.016	0.125	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
501373 (2016)	0.032	0.125	0.125	0.023	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
009 (2013)	0.064	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
191262 (2016)	0.023	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
179501 (2015)	0.016	0.094	0.094	0.023	mosaic XXXIV	L421P	A121S	H105Y	A13 del
422102 (2016)	0.032	0.125	0.38	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
178526 (2015)	0.032	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
425204 (2016)	0.016	0.125	0.125	0.032	mosaic XXXIV	WT	WT	A39T	WT
82432 (2015)	0.023	0.125	0.25	0.032	mosaic XXXIV	L421P	G120K A121D	A39T	A13 del

Table 3.12. Molecular characteristics of isolates with an ertapenem MIC = 0.032 mg/L (n = 17).

WT = wild type.

Isolate (Year)	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
006 (2013)	0.016	0.016	0.032	0.012	non-mosaic II	WT	A121S	G45D	A12C
199856 (2016)	0.002	≤ 0.016	0.004	0.008	non-mosaic II	WT	A121S	G45D	WT
191587 (2016)	0.008	0.032	0.064	0.016	non-mosaic II	WT	A121S	G45D	WT
67818 (2014)	0.012	0.023	0.064	0.012	non-mosaic XLIX	L421P	G120K A121D	D79N T86A H105K	A13 del
190440 (2016)	0.004	0.016	0.032	0.012	non-mosaic XIX	L421P	G120K A121G	A39T ISC	WT
420132 (2016)	0.008	≤ 0.016	0.008	0.012	non-mosaic II	WT	WT	A39T	WT
193058 (2016)	0.008	0.016	0.047	0.016	non-mosaic XLIV	L421P	WT	D79N T86A H105K	A13 del
197747 (2016)	0.016	0.032	0.094	0.012	non-mosaic XLIV	L421P	WT	A39T ISC	A13 del
425657 (2016)	0.016	0.032	0.012	0.006	non-mosaic XLIV	L421P	G120K A121D	D79N T86A H105K	A13 del
177574 (2015)	0.047	0.094	0.125	0.016	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del
168628 (2016)	0.064	0.064	0.25	0.012	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del
422322 (NA)	0.008	≤ 0.016	0.016	0.004	non-mosaic II	WT	G120D A121G	A39T ISC	WT
008 (2013)	0.008	≤ 0.016	0.023	0.006	non-mosaic II	WT	G120D A121G	H105Y	A13 del
166587 (2016)	0.008	0.016	0.125	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del
180505 (2015)	0.012	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	WT
178575 (2015)	0.003	0.016	0.012	0.012	non-mosaic II	WT	WT	A39T R44H	WT
41277 (2014)	0.003	≤ 0.016	0.012	0.008	non-mosaic II	WT	WT	A39T R44H	WT
179411 (2015)	0.008	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del
039 (2015)	0.012	0.016	0.016	0.016	non-mosaic XIV	WT	G120K A121N	D79N T86N H105K	WT

Table 3.13. Molecular characteristics for isolates with an ertapenem MIC < 0.032 mg/L. WT = wild type.

3.5.5 Azithromycin

The azithromycin MIC range was 0.003-> 256 mg/L. Seven isolates had an azithromycin MIC > 256 mg/L (HLAzi-R) and 22 isolates were resistant (MIC > 0.5 mg/L). All seven HLAzi-R isolates had an A2059G (alanine→glycine) mutation in the peptidyltransferase loop in domain V of the 23S rRNA gene. Mutation A2059G was identified in all four alleles in the 23S rRNA gene in four isolates, while three alleles were mutation in two isolates and two mutations were found in one isolate (Table 3.14). Each of the seven HLAzi-R isolates also had a G45D substitution in the *mtrR* gene and one each had an A13 deletion or an A12C substitution in the *mtrR* promoter region (Table 3.14).

Seven isolates had azithromycin MICs of 4-32 mg/L. C2611T (cysteine→threonine) mutations in the 23S rRNA gene were detected in all four alleles in five of these isolates (MICs 4, 6, 12, 16 and 32 mg/L) and in 0 of the four alleles in the remaining 2 isolates (MICs 4 and 8 mg/L), both of which had a D79N (aspartate → asparagine) substitution in *mtrR* and an A13 deletion in the *mtrR* promoter (Table 3.15). The azithromycin MICs of these isolates were confirmed on repeat testing. In addition to the mutated 23S rRNA, the isolate with an azithromycin MIC of 32 mg/L had amino acid substitutions (A39T and R44H) in the *mtrR* gene and had a wild-type *mtrR* promoter while the isolates with azithromycin MICs of 12 and 16 mg/L had alterations in *mtrR* (A39T/R44H and A39T/internal stop codon, respectively) and the *mtrR* promoter (wild-type and an A13 deletion, respectively) (Table 3.15).

Twenty-nine isolates had azithromycin MICs of 0.032-2 mg/L. In only one of these was a C2611T mutation detected (in 1 out of 4 alleles) and this isolate had an azithromycin MIC of 0.125 mg/L (Table 3.16). This isolate also had an A39T substitution and an internal stop codon detected in the *mtrR* gene in addition (Table 3.16). The *mtrR* alterations present in the other isolates are summarised (Table 3.16). An A13 deletion in *mtrR* promoter was present in 21 (72.4%) isolates (MICs 0.25-1 mg/L).

None of *ereA*, *ereB*, *ermA*, *ermB*, *ermC*, *ermF*, *mefA*, *mphA*, *mphB* and *mphC* genes were detected in any of the isolates. L4 and L22 proteins (encoded by *rplD* and *rplV*, respectively) were not detected, however one isolate had a G71D (glycine→aspartate) substitution in *rplD* and an azithromycin MIC of 0.5 mg/L.

3.5.6 Solithromycin

Twenty-one of the 43 isolates had solithromycin MICs determined with a range of 0.032 - >32 mg/L. The seven HLAzi-R isolates corresponded to the highest solithromycin MICs (0.38 - > 32 mg/L) (Table 3.14). These seven isolates all had A2059G mutations in the 23S rRNA gene. The three isolates with solithromycin MIC > 32 mg/L have A2059G alterations (4/4 alleles) while the two isolates with solithromycin MICs of 1 and 6 mg/L had 3/4 mutated alleles, yet the remaining two isolates (solithromycin MICs 0.38 and 0.5 mg/L) had 1/4 and 4/4 mutated alleles. All of these 7 isolates had G45D alterations in *mtrR* and one each had an A12C nucleotide alteration and an A13 deletion, with solithromycin MICs of > 32 mg/L and 0.38 mg/L, respectively.

The remaining 14 isolates had solithromycin MICs of 0.032 – 0.19 mg/L. Of these, only five isolates have 4/4 C2611T mutated alleles in 23S rRNA and solithromycin MICs of 0.094 – 0.19 mg/L, while six isolates within the same MIC range had wild-type 23S rRNA (Tables 3.15 and 3.16). Similarly, there appears to be no definitive correlation between solithromycin MIC levels and mutations detected in *mtrR* and the *mtrR* promoter (Tables 3.15 and 3.16).

Isolate (Year)	AZM MIC (mg/L)	SOL MIC (mg/L)	<i>mtrR</i>	<i>mtrR</i> promoter	23S <i>rRNA</i>	<i>erm A/B/C/F</i> <i>ereA/B</i> <i>mefA</i> <i>mph A/B/C</i>
006 (2013)	> 256	> 32	G45D	A12C	A2059G (4/4)	-
181798 (2015)	> 256	6	G45D	WT	A2059G (3/4)	-
196712 (2016)	> 256	> 32	G45D	WT	A2059G (4/4)	-
178487 (2015)	> 256	1	G45D	WT	A2059G (3/4)	-
193852 (2016)	> 256	0.38	G45D	A13 del	A2059G (2/4)	-
199856 (2016)	> 256	0.5	G45D	WT	A2059G (4/4)	-
191587 (2016)	> 256	> 32	G45D	WT	A2059G (4/4)	-

Table 3.14. Molecular characteristics of 7 HLAzi-R isolates (MIC > 256 mg/L).

Isolate (Year)	AZM MIC (mg/L)	SOL MIC (mg/L)	<i>mtrR</i>	<i>mtrR</i> promoter	23S <i>rRNA</i>	<i>erm A/B/C/F</i> <i>ereA/B</i> <i>mefA</i> <i>mph A/B/C</i>
67818 (2014)	6	0.125	D79N T86A H105K	A13 del	C2611T (4/4)	-
197747 (2016)	16	0.19	A39T ISC	A13 del	C2611T (4/4)	-
425657 (2016)	4	0.094	D79N T86A H105K	A13 del	C2611T (4/4)	-
166587 (2016)	8	0.19	D79N	A13 del	WT	-
178575 (2015)	32	0.19	A39T R44H	WT	C2611T (4/4)	-
41277 (2014)	12	0.125	A39T R44H	WT	C2611T (4/4)	-
179411 (2015)	4	0.19	D79N	A13 del	WT	-

Table 3.15. Molecular characteristics of 7 isolates with azithromycin MICs 4-32 mg/L.

Isolate (Year)	AZM MIC (mg/L)	SOL MIC (mg/L)	<i>mtrR</i>	<i>mtrR</i> promoter	23S <i>rRNA</i>	<i>erm A/B/C/F</i> <i>ereA/B</i> <i>mefA</i> <i>mph A/B/C</i>
190440 (2016)	0.032	NT	A39T ISC	WT	WT	-
420132 (2016)	0.064	NT	A39T	WT	WT	-
71324 (2014)	0.125	NT	A39T ISC	WT	C2611T (1/4)	-
016 (2013)	1	0.094	H105Y	A13 del	WT	-
035 (2016)	1	0.047	H105Y	A13 del	WT	-
286088 (2014)	0.38	NT	H105Y	A13 del	WT	-
76972 (2014)	0.75	NT	H105Y	A13 del	WT	-
176561 (2015)	0.5	NT	H105Y	A13 del	WT	-
501373 (2016)	0.5	NT	H105Y	A13 del	WT	-
024 (2012)	0.38	NT	H105Y	A13 del	WT	-
009 (2013)	0.75	0.064	H105Y	A13 del	WT	-
194698 (2016)	0.5	NT	H105Y	A13 del	WT	-
191262 (2016)	0.5	NT	H105Y	A13 del	WT	-
422671 (2016)	0.5	NT	H105Y	A13 del	WT	-
179501 (2015)	0.25	NT	H105Y	A13 del	WT	-
422102 (2016)	1	0.094	H105Y	A13 del	WT	-
81513 (2015)	0.5	NT	H105Y	A13 del	WT	-
178526 (2015)	1	0.032	H105Y	A13 del	WT	-
68931 (2014)	0.5	NT	H105Y	A13 del	WT	-
425204 (2016)	0.5	NT	A39T	WT	WT	-
193058* (2016)	0.5	NT	D79N T86A H105K	A13 del	WT	-
177574 (2015)	0.5	NT	G45D	A13 del	WT	-
168628 (2016)	0.25	NT	G45D	A13 del	WT	-

422322 (NA)	0.5	NT	A39T ISC	WT	WT	-
008 (2013)	0.5	NT	H105Y	A13 del	WT	-
180505 (2015)	2	0.125	D79N	WT	WT	-
039 (2015)	0.25	NT	D79N T86A H105K	WT	WT	-
82432 (2015)	1	0.19	A39T	A13 del	WT	-
86606 (2015)	0.5	NT	A39T R44H	WT	WT	-

Table 3.16. Molecular characteristics of 29 isolates with azithromycin MICs 0.032-2 mg/L. WT = wild type.

* Isolate 193058 has a G71D amino acid substitution in *rpID*.

3.5.7 Ciprofloxacin

The MIC range for these 43 isolates for ciprofloxacin was $\leq 0.002 \rightarrow 32$ mg/L. Thirty-one (72.1%) isolates were resistant to ciprofloxacin (MIC > 0.064 mg/L). Seventeen of these had a ciprofloxacin MIC > 32 mg/L. These 17 isolates all had amino acid substitutions in *gyrA* and *parC* (Table 3.17). In *gyrA* all 17 (100%) had S91F (serine $\rightarrow$ phenylalanine) and D95A/G (aspartate $\rightarrow$ alanine/glycine) substitutions. One also had an additional A92P (alanine $\rightarrow$ proline) substitution. In *parC* the amino acid substitutions found included E91G (glutamate $\rightarrow$ glycine) (n = 1), S87R (serine $\rightarrow$ arginine) (n = 13), E91K (glutamate $\rightarrow$ lysine) plus S87N (serine $\rightarrow$ asparagine) (n = 2) and D86N (aspartate $\rightarrow$ asparagine) (n = 1) (Table 3.17). The majority of these isolates (ciprofloxacin MICs > 32 mg/L) had an H105Y substitution (13/17) in *mtrR* associated with an A13 deletion in the *mtrR* promoter (15/17) (Table 3.17).

There are an additional 14 isolates resistant to ciprofloxacin with MICs ranging from 2-16 mg/L. Similarly, all 14 (100%) of these isolates had S91F mutations in association with D95G/A substitutions in *gyrA* (Table 3.18). In *parC* the amino acid substitutions found were E91G (n = 4, one of which had an additional S87I substitution), S87R (n = 3), D86N (n = 6) and S87N plus E91Q (glutamate $\rightarrow$ glutamine) plus S87N (n = 1) (Table 3.18). There was no clear pattern between the MIC value and the specific amino acid substitution found for this group of isolates with ciprofloxacin MICs ranging from 2 mg/L to 16 mg/L (Table 3.18). These isolates (ciprofloxacin MICs 2-16 mg/L) all had *mtrR* mutations and 11/14 have an A13 deletion in the *mtrR* promoter (Table 3.18).

Twelve isolates were susceptible to ciprofloxacin with MICs ranging from ≤ 0.002 mg/L to 0.032 mg/L (Table 3.19). There were no amino acid substitutions detected in any of these isolates in either *gyrA* or *parC*, i.e. they were all wild-type. Ciprofloxacin susceptible isolates were wild-type *mtrR* promoter, except one isolate that had an A12C amino acid substitution which is of unclear significance and one that had an A13 deletion (Table 3.19). These isolates all had either G45D (n = 7), A39T (n = 1), D79N (n = 1), or A39T plus R44H (n = 3) substitutions in *mtrR* (Table 3.19).

3.5.8 Gemifloxacin

The gemifloxacin MIC range was ≤ 0.002 -12 mg/L. The 43 isolates form two groups according to gemifloxacin MIC; those with MICs ≤ 0.002 -0.012 mg/L and those with MICs 0.38-12 mg/L. The group with lower MICs corresponds to the isolates with ciprofloxacin MICs within the susceptible range and the group with higher gemifloxacin MICs correspond to those isolates resistant to ciprofloxacin. Isolates with lower gemifloxacin MICs, as above for ciprofloxacin susceptible isolates, had no amino acid substitutions in *gyrA* or *parC*. Isolates with higher gemifloxacin MICs had amino acid substitutions as described above for the ciprofloxacin-resistant isolates. Sequences of all isolates examined were wild-type *parE*.

Isolate (Year)	CIP MIC (mg/L)	GEMI MIC (mg/L)	<i>gyrA</i>	<i>parC</i>	<i>mtrR</i>	<i>mtrR</i> promoter
67818 (2014)	> 32	1.5	S91F D95G	E91G	D79N T86A H105K	A13 del
190440 (2016)	> 32	4	S91F A92P D95A	E91K S87K	A39T ISC	WT
71324 (2014)	> 32	3	S91F D95A	E91K S87K	A39T ISC	WT
016 (2013)	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
035 (2016)	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
286088 (2014)	> 32	0.75	S91F D95G	S87R	H105Y	A13 del
76972 (2014)	> 32	2	S91F D95G	S87R	H105Y	A13 del
176561 (2015)	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
024 (2012)	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
009 (2013)	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
191262 (2016)	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
422671 (2016)	> 32	2	S91F D95G	S87R	H105Y	A13 del
422102 (2016)	> 32	2	S91F D95G	S87R	H105Y	A13 del
81513 (2015)	> 32	2	S91F D95G	S87R	H105Y	A13 del
178526 (2015)	> 32	1	S91F D95G	S87R	H105Y	A13 del
68931 (2014)	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
82432 (2015)	> 32	6	S91F D95A	D86N	A39T	A13 del

Table 3.17. Phenotypic and molecular characteristics of 17 isolates with ciprofloxacin

MIC > 32 mg/L. WT = wild type.

Isolate (Year)	CIP MIC (mg/L)	GEMI MIC (mg/L)	<i>gyrA</i>	<i>parC</i>	<i>mtrR</i>	<i>mtrR</i> promoter
420132 (2016)	2	0.5	S91F D95G	E91G	A39T	WT
501373 (2016)	16	2	S91F D95A	S87R	H105Y	A13 del
194698 (2016)	16	3	S91F D95G	S87R	H105Y	A13 del
179501 (2015)	3	0.75	S91F D95G	S87R	H105Y	A13 del
193058 (2016)	8	12	S91F D95G	E91G S87I	D79N T86A H105K	A13 del
197747 (2016)	2	2	S91F D95G	E91G	A39T ISC	A13 del
425657 (2016)	16	1.5	S91F D95G	E91G	D79N T86A H105K	A13 del
177574 (2015)	8	1	S91F D95G	D86N	G45D	A13 del
168628 (2016)	2	0.38	S91F D95G	D86N	G45D	A13 del
422322 (NA)	2	0.5	S91F D95A	D86N	A39T ISC	WT
008 (2013)	3	0.75	S91F D95A	D86N	H105Y	A13 del
166587 (2016)	4	2	S91F D95A	D86N	D79N	A13 del
179411 (2015)	12	1	S91F D95A	D86N	D79N	A13 del
039 (2015)	4	1.5	S91F D95A	S87N E91Q	D79N T86A H105K	WT

Table 3.18. Phenotypic and molecular characteristics of 14 isolates, ciprofloxacin MIC 2-16 mg/L.

Isolate (Year)	CIP MIC (mg/L)	GEMI MIC (mg/L)	<i>gyrA</i>	<i>parC</i>	<i>mtrR</i>	<i>mtrR</i> promoter
006 (2013)	0.012	0.006	WT	WT	G45D	A12C
181798 (2015)	0.008	0.006	WT	WT	G45D	WT
196712 (2016)	0.008	0.006	WT	WT	G45D	WT
178487 (2015)	0.008	0.004	WT	WT	G45D	WT
193852 (2016)	≤ 0.002	≤ 0.002	WT	WT	G45D	A13 del
199856 (2016)	0.004	≤ 0.002	WT	WT	G45D	WT
191587 (2016)	0.008	0.004	WT	WT	G45D	WT
425204 (2016)	0.016	0.006	WT	WT	A39T	WT
180505 (2015)	0.032	0.012	WT	WT	D79N	WT
178575 (2015)	0.004	0.003	WT	WT	A39T R44H	WT
41277 (2014)	0.004	0.003	WT	WT	A39T R44H	WT
86606 (2015)	0.006	0.004	WT	WT	A39T R44H	WT

Table 3.19. Phenotypic and molecular characteristics of 12 ciprofloxacin susceptible isolates (MIC ≤ 0.032 mg/L).

3.5.9 Penicillin and β -lactamase

Of the 43 isolates sequenced 9 (20.9%) were resistant to penicillin (MIC > 1 mg/L). The MIC range for these isolates was 0.125-> 32 mg/L and three isolates had an MIC > 32 mg/L. Six of the 43 isolates produce β -lactamase. The three isolates with an MIC > 32 mg/L all had a non-mosaic *penA* II with a 345R insertion and A501V, F504L and I515V amino acid substitutions (Table 3.20). All three had plasmid encoded *bla*_{TEM-75} (n = 1) or *bla*_{TEM-141} (n = 2). No mutations in *ponA* encoding PBP1 were found in these three isolates. Two had G120D plus A121G substitutions in *penB* (encoding PorB) (Table 3.20). An A39T substitution deletion was present in *mtrR* in two isolates while the other isolate had a H105Y substitution in *mtrR* and A13 deletion in the *mtrR* promoter (Table 3.20).

In addition to the three isolates with penicillin MICs > 32 mg/L, a further six of the 43 isolates were also resistant to penicillin with MICs of 2 mg/L (n = 4) or 4 mg/L (n = 2) (Table 3.21). Five of these had L421P substitutions in *ponA* (encoding PBP1) and three had plasmids encoding *bla*_{TEM-198} or *bla*_{TEM-141}. These six isolates had 4 different *penA* (encoding PBP2) allele types, two non-mosaic types XIX and XIV and two mosaic types XXXIV and X, the latter two being associated with elevated ESC MICs in these isolates (Table 3.21). Three of these six isolates had A13 deletions in the *mtrR* promoter. The latter isolates had alterations in *mtrR* including three with H105Y substitutions and three with various alterations including two with internal stop codons (Table 3.21). In *penB* all had G120K plus A121G/N substitutions (Table 3.21).

Isolate (Year)	Penicillin MIC (mg/L)	BL	<i>penA</i>	<i>ponA</i>	<i>penB</i>	TEM	<i>mtrR</i>	<i>mtrR</i> promoter
420132 (2016)	> 32	+	non-mosaic II	WT	WT	TEM-75	A39T	WT
422322 (NA)	> 32	+	non-mosaic II	WT	G120D A121G	TEM-141	A39T	WT
008 (2013)	> 32	+	non-mosaic II	WT	G120D A121G	TEM-141	H105Y	A13 del

Table 3.20. Resistant determinants for three isolates with penicillin MIC > 32 mg/L.

Isolate (Year)	Penicillin MIC (mg/L)	BL	<i>penA</i>	<i>ponA</i>	<i>penB</i>	TEM	<i>mtrR</i>	<i>mtrR</i> promoter
190440 (2016)	4	+	non-mosaic XIX	L421P	G120K A121G	TEM-198	A39T ISC	WT
71324 (2014)	4	+	mosaic X	L421P	G120K A121G	TEM-198	A39T ISC	WT
016 (2013)	2	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13
76972 (2014)	1.5	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13
024 (2012)	1.5	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13
039 (2015)	1.5	+	non-mosaic XIV	WT	G120K A121N	TEM-141	D79N T86A H105K	WT

Table 3.21. Resistance determinants for isolates with penicillin MIC > 1-4 mg/L.

The remaining 34 isolates were intermediate to penicillin with MICs ranging from 0.125-1 mg/L (Table 3.22). Twenty of these had an L421P substitution in *ponA* and 23 had an A13 deletion in the *mtrR* promoter. One isolate had an A12C nucleotide substitution in the *mtrR* promoter (Table 3.22). Thirteen (38.2%) and 7 (20.6%) isolates had H105Y substitutions and G45D substitutions in *mtrR*, respectively. The remaining isolates had variable alterations in *mtrR* (Table 3.22). Alterations in *penB* in these isolates include A121S (n = 8) and G120K plus A121N/D (n = 20) with the remaining 6 being wild-type (Table 3.22). The *penA* types seen in these 34 isolates included non-mosaic II (n=12), non-mosaic XIII (n = 2), non-mosaic XLIV (n = 3), non-mosaic XLIX (n = 1) and mosaic XXXIV (n = 16) (Table 3.22). None of these isolates had plasmids encoding β -lactamases and neither did they phenotypically produce β -lactamase. The phenotypic and molecular characteristics of these 34 isolates are summarised in *Appendix 7*.

Overall 6 of the 43 isolates produced β -lactamase and genes encoding TEM β -lactamases were found in all 6 including *bla*_{TEM-198}, *bla*_{TEM-141} and *bla*_{TEM-75} (Tables 3.20 & 3.21).

Isolate (Year)	Penicillin MIC (mg/L)	BL	<i>penA</i>	<i>ponA</i>	<i>penB</i>	TEM	<i>mtrR</i>	<i>mtrR</i> promoter
006 (2013)	0.25	-	non-mosaic II	WT	A121S	-	G45D	A12C
181798 (2015)	0.25	-	non-mosaic II	WT	A121S	-	G45D	WT
196712 (2016)	0.25	-	non-mosaic II	WT	A121S	-	G45D	WT
178487 (2015)	0.19	-	non-mosaic II	WT	A121S	-	G45D	WT
193852 (2016)	0.125	-	non-mosaic II	WT	A121S	-	G45D	A13 del
199856 (2016)	0.125	-	non-mosaic II	WT	A121S	-	G45D	WT
191587 (2016)	0.25	-	non-mosaic II	WT	A121S	-	G45D	WT
67818 (2014)	0.19	-	non-mosaic XLIX	L421P	G120K A121D	-	D79N T86A H105K	A13 del
035 (2016)	0.75	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
286088 (2014)	0.75	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
176561 (2015)	0.75	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
501373 (2016)	0.25	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
009 (2013)	1	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
194698 (2016)	0.25	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
191262 (2016)	0.5	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
422671 (2016)	0.5	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
179501 (2015)	0.25	-	mosaic XXXIV	L421P	A121S	-	H105Y	A13 del
422102 (2016)	1	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
81513 (2015)	0.75	-	mosaic XXXIV	L421P	G120K A121D	-	H105Y	A13 del
178526 (2015)	1	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del

68931 (2014)	1	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13 del
425204 (2016)	0.25	-	mosaic XXXIV	WT	WT	-	A39T	WT
193058 (2016)	0.125	-	non-mosaic XLIV	L421P	WT	-	D79N T86A H105K	A13 del
197747 (2016)	0.25	-	non-mosaic XLIV	L421P	WT	-	A39T ISC	A13 del
425657 (2016)	0.25	-	non-mosaic XLIV	L421P	G120K A121D	-	D79N T86A H105K	A13 del
177574 (2015)	1	-	non-mosaic XIII	L421P	G120K A121D	-	G45D	A13 del
168628 (2016)	1	-	non-mosaic XIII	L421P	G120K A121D	-	G45D	A13 del
166587 (2016)	0.25	-	non-mosaic II	WT	G120K A121N	-	D79N	A13 del
180505 (2015)	0.5	-	non-mosaic II	WT	G120K A121N	-	D79N	WT
178575 (2015)	0.19	-	non-mosaic II	WT	WT	-	A39T R44H	WT
41277 (2014)	0.125	-	non-mosaic II	WT	WT	-	A39T R44H	WT
179411 (2015)	0.5	-	non-mosaic II	WT	G120K A121N	-	D79N	A13 del
82432 (2015)	0.75	-	mosaic XXXIV	L421P	G120K A121D	-	A39T	A13 del
86606 (2015)	0.25	-	mosaic XXXIV	WT	WT	-	A39T R44H	-

Table 3.22. Phenotypic and molecular characteristics of 34 penicillin susceptible and intermediate isolates. WT = wild type.

3.5.10 Tetracycline

The tetracycline MIC range was 0.125-32 mg/L, with six resistant isolates (MIC > 1 mg/L), 18 susceptible (MIC ≤ 0.5 mg/L) and the remainder, 19, intermediate. Three resistant isolates had MICs of 8 mg/L, 24 mg/L and 32 mg/L for tetracycline. These three isolates all carried plasmids with the tetM determinant, none had disruption in the *mtrR* promoter and all 3 had alterations in *mtrR*, including two with internal stop codons (Table 3.23). In the remaining 41 isolates (MICs 0.125-2 mg/L) no plasmid was detected. Amino acid substitution V57M (valine→methionine) was present in *rpsJ* in all but one isolate which had the lowest MIC for tetracycline of 0.125 mg/L (*Appendix 7*).

3.5.11 Spectinomycin

All 43 isolates were susceptible to spectinomycin and the MIC range is 4-16 mg/L. No mutations in the 16s rRNA gene were detected. A V25 deletion was detected in *rpsE* in all isolates sequenced (*Appendix 7*), but has not resulted in elevated spectinomycin MICs.

3.5.12 Gentamicin

The gentamicin MIC range is 0.5-16 mg/L. According to the proposed breakpoints by Brown *et al.* none were resistant, 22 were susceptible (MIC ≤ 4 mg/L) and 21 were intermediate (MIC > 4 and < 32 mg/L) (Brown *et al.*, 2010). No *aac* (encoding

aminoglycoside acetyltransferases), *ant* (encoding nucleotidyltransferases) or *aph* (encoding phosphotransferases) genes were present. No mutations in *rpsL*, encoding 30s ribosomal protein S12, were present which can also confer gentamicin resistance

Isolate (Year)	TET MIC (mg/L)	<i>tetM</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter	<i>rpsJ</i>
190440 (2016)	32	tetM	G120K A121G	A39T ISC	WT	V57M
71324 (2014)	24	tetM	G120K A121G	A39T ISC	WT	V57M
039 (2015)	8	tetM	G120K A121N	D79N T86A H105K	WT	V57M

Table 3.23. Molecular characteristics of isolates with tetracycline MIC values of 8-32 mg/L.

3.5.13 Fosfomycin

The fosfomycin MIC range is 6-96 mg/L. There are no clinical breakpoints for fosfomycin and *N. gonorrhoeae* but if EUCAST (CLSI) clinical breakpoints for *Enterobacteriaceae* are applied 4/43 (1/43) are resistant (intermediate). *fosA/B/C/X* genes which confer fosfomycin resistance were not detected. Similarly, no mutations in *murA* (involved in peptidoglycan synthesis), *glpT* or *uhpT* (encoding fosfomycin transporters) or *cyaA* and *ptsI* (regulating fosfomycin transporters) were present in any of the isolates in this current study (Falagas *et al.*, 2016).

3.5.14 MDR and XDR isolates

Isolates identified as MDR and XDR (Table 3.12), were included the sequenced isolates. The resistance determinants and MICs for all 13 antimicrobials tested are summarised (Table 3.24).

Isolate (Year)	MIC (mg/L)														<i>penA</i>	<i>ponA</i>	<i>penB</i>	TEM	<i>gyrA</i>	<i>parC</i>	<i>mtrR</i>	<i>mtrR</i> pr.	<i>tetM</i>	<i>rpld</i>	23S rRNA
	CRO	CFM	CTX	ETP	AZM	CIP	GEM	P	BL	TET	SPC	CN	FOS	SOL											
422102 (2016)	0.032	0.125	0.38	0.032	1	> 32	2	1	-	1	16	8	32	0.094	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT
024 (2012)	0.094	0.25	0.38	0.064	0.38	> 32	1.5	1.5	-	1	8	4	48	NT	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT
82432 (2015)	0.023	0.125	0.25	0.032	1	> 32	6	0.75	-	1	12	6	24	0.19	mosaic XXXIV	L421P	G120K A121D	-	S91F D95A	D86N	A39T	A13 del	-	WT	WT
178526 (2015)	0.032	0.19	0.25	0.032	1	> 32	1	1	-	0.75	8	6	16	0.032	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT
71324 (2014)	0.047	0.19	0.19	0.094	0.125	> 32	3	4	+	24	6	0.5	6	NT	mosaic type X	L421P	G120K A121G	TEM- 198	S91F D95A	E91K S87K	A39T ISC	WT	tetM	WT	C2611T (1/4)
016 (2013)	0.047	0.19	0.25	0.032	1	> 32	1.5	2	-	1	12	6	32	0.094	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT
76972 (2014)	0.047	0.19	0.25	0.032	0.75	> 32	2	1.5	-	1	12	4	32	NT	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT

Table 3.24. Phenotypic and molecular characteristics of MDR (n = 5) and XDR isolates (n = 2). CRO = ceftriaxone, CFM = cefixime, CTX = cefotaxime, ETP = ertapenem, AZM = azithromycin, CIP = ciprofloxacin, GEM = gemifloxacin, P = penicillin, BL = β -lactamase, TET = tetracycline, SPC = spectinomycin, CN = gentamicin, FOS = fosfomycin, SOL = solithromycin.

mtrR pr. = *mtrR* promoter

3.6 Discussion

3.6.1 Extended-spectrum cephalosporins (ESCs) and ertapenem: *penA* alleles

The major resistance determinant at which mutations are known to reduce susceptibility to ESC MICs is at gene *penA*. Of the 43 isolates sequenced, twenty-five were resistant or less susceptible to ESCs and ertapenem compared to the remaining eighteen. These 25 isolates were associated with mosaic *penA* alleles (80%, n = 20) more so than the remaining 18 ESC fully susceptible isolates (0%, n = 0) and belong to one or more of the following phenotypic groups: CRO-RS (CRO MIC > 0.032 mg/L, n = 9), CFM-R (CFM MIC > 0.125 mg/L, n = 6), CFM-RS (CFM MIC = 0.125 mg/L, n = 14), CTX-R (CTX MIC > 0.125 mg/L, n = 13), CTS-RS (CTX MIC = 0.125 mg/L, n = 11) or ETP-RS (ertapenem MIC > 0.032 mg/L, n = 7) which are discussed below.

3.6.1.1 CRO-RS: *penA* alleles

Three mosaic allele types identified in this study among CRO-RS isolates are known to be associated with reduced susceptibility to ESCs and are prevalent worldwide (Gose *et al.*, 2013; Lee *et al.*, 2015; Nguyen *et al.*, 2014; de Curarize *et al.*, 2016; Shimuta *et al.*, 2015). Most of the nine CRO-RS isolates had mosaic XXXIV *penA* alleles (n = 6). This *penA* allele type was also seen in thirteen other isolates (CRO MIC ≤ 0.032 mg/L). Ten of these (CRO MIC 0.016-0.32 mg/L) had the exact combination of resistance determinants as the CRO-RS isolates and while the CRO MICs were lower, the MICs of cefixime and cefotaxime were elevated with the majority (n = 9) having cefixime MICs ≥ 0.125 mg/L. The combination of resistance determinants seen in the CRO-RS isolates with mosaic XXXIV *penA* may not account fully for the elevation of ESC

MICs seen, there may be as yet undetermined resistance factors or ‘factor X’ may be responsible. Of the three remaining CRO-RS isolates, two had non-mosaic XIII (A501V) *penA* alleles and one had a mosaic X *penA* allele. Mosaic XXXIV *penA* is reported to be significantly associated with increased ESC MICs and transformation experiments have confirmed this (Gose *et al.*, 2013). It is also the *penA* allele that is associated with the majority of reported ESC treatment failures (Chen *et al.*, 2013; Allen *et al.*, 2013; Golparian *et al.*, 2014; Lewis *et al.*, 2013; Unemo *et al.*, 2012c; Unemo *et al.*, 2011d; van Dam *et al.*, 2014). In California, Nguyen *et al.* examined 718 gonococcal isolates. Seventeen isolates had a CRO MIC ≥ 0.0125 mg/L and all of these had a mosaic XXXIV *penA* allele (Nguyen *et al.*, 2014). In the US 116 of 118 CFM-RS isolates from 2009-2010 possessed a mosaic XXXIV *penA* (Grad *et al.*, 2014). Mosaic XXXIV *penA* has been also reported in Japan, Sweden and Canada associated with elevated ESC MICs (Shimuta *et al.*, 2015; Golparian *et al.*, 2014; Vidovic *et al.*, 2014 JCM).

Cefixime and ceftriaxone MICs are not affected to the same degree by a mosaic XXXIV *penA* allele. Grad *et al.* found that of all isolates with a mosaic XXXIV *penA* allele in a collection, 1% had a cefixime MIC ≤ 0.125 mg/L, whereas 21% had a ceftriaxone MIC ≤ 0.03 mg/L (Grad *et al.*, 2016). Similarly mosaic *penA* alleles have less of an effect on carbapenems than they do on ESCs.

Mosaic *penA* alleles with A501 alterations are known to be specifically associated with increased ESC MICs, as discussed (Section 1.6). In this current study, none of the mosaic *penA* alleles had A501 alterations, however, the non-mosaic XIII *penA* allele present in two CRO-RS isolates had an A501V mutation. These two isolates had a 345R insertion and P551S, F504L, A510V and I515V alterations (Table 3.3). Non-mosaic XIII *penA* has been reported by Lee *et al.* to be the most common allele type

(A501V and P551S alterations) in 56/95 isolates from Korea (2011-2013), which includes 21/25 ESC-resistant isolates (Lee *et al.*, 2015). The remaining 4/25 ESC-resistant isolates in that study had a mosaic X *penA* allele. They reported five alterations in non-mosaic XIII *penA*– A501V, F504L, A510V, A516G and P551S, which differs to our non-mosaic XIII *penA* described above which has an I515V instead of an A516G alteration and had an additional 345R insertion. The non-mosaic XIII *penA* allele has also reported by Li *et al.* in two out of 15 isolates that had CRO MIC $\geq$ 0.125 mg/L from Nanjing, China (2011-2012) (Li *et al.*, 2014).

In the current study, a mosaic X *penA* allele was present in the remaining CRO-RS isolate and it was very similar to XXXIV. Mosaic X *penA* allele is associated with ESC resistance and treatment failures (Shimuta *et al.*, 2015; Fifer *et al.*, 2016). In Japan 129/149 CFM-R gonococcal isolates (1995-2005) possessed mosaic X *penA* alleles (Shimuta *et al.*, 2015).

3.6.1.2 CFM-R/RS, CTX-R/RS, ETP-RS: *penA* alleles

Apart from the CRO-RS groups of isolates discussed above, the other more resistant phenotypic groups include CFM-R (n = 6), CFM-RS (n = 14), CTX-R (n = 13), CTS-RS (n = 11) and ETP-RS (n = 7). The *penA* alleles described above (mosaic XXXIV, X and non-mosaic XIII) were found in the majority of these isolates (Tables 3.5-6, 3.8-3.9 and 3.11) except for three (isolates 196712, 166587 and 197747) which were in the CTX-RS group only, i.e. CRO/ETP MICs $\leq$ 0.032 mg/L and CFM MIC $<$ 0.125 mg/L. These three isolates had non-mosaic II (n = 2) (345R insertion, F504L, A510V and I515V) and non-mosaic XLIV (n = 1) (345R insertion, A501T, P511L and F504L) *penA* alleles. These findings are similar to other studies in which non-mosaic alleles

have been reported in ceftriaxone resistant isolates and isolates with elevated ESC MICs and those containing A501 alterations are particularly associated with reduced susceptibility to ESCs (Chen *et al.*, 2014; Chen *et al.*, 2015). Non-mosaic II *penA* increase CFM MICs 2 to 5-fold and CRO MICs 5 to 11-fold in transformation experiments (Bharat *et al.*, 2015). Non-mosaic XLIV *penA* allele has not been reported previously. It was present in three isolates in this study, one of which had an elevated cefotaxime MIC of 0.094 mg/L which placed it in the CTX-RS group of isolates; however the MICs for ceftriaxone, cefixime and ertapenem were 0.016 mg/L, 0.032 mg/L and 0.012 mg/L, respectively. The second and third isolates with non-mosaic XLIV *penA* alleles had MICs to CRO, CFM, CTX and ETP of 0.008 mg/L, 0.016 mg/L, 0.047 mg/L and 0.016 mg/L and 0.016 mg/L, 0.032 mg/L, 0.012 mg/L and 0.006 mg/L, respectively. The mutations in this non-mosaic XLIV *penA* allele included a 345R insertion and A501T, P551L and F504L alterations and as mentioned above A501V/T alterations are associated with increased ESC MICs. A501V alterations in *penA* appear to be more prevalent than A501T alterations (101 v 68 out of 278 isolates in China [Chen *et al.*, 2014] and 33 v 4 out of 37 isolates in Europe with A501 alterations [Golparian *et al.*, 2010]). This is also reflected in the finding that one of the most common *penA* mutations arising during *in vitro* selection of resistance to ceftriaxone in gonococci is an A501V alteration (Gong *et al.*, 2016; Tomberg *et al.*, 2017). A501V/T alterations appear to confer similar levels of resistance in transformation experiments involving mosaic *penA* alleles, i.e. 2 to 3-fold increases in CFM and 2-fold increases in CRO MICs (Tomberg *et al.*, 2017). When transformed into a non-mosaic allele the increase in CFM/CRO MICs is 2-fold for A501V and 3-fold for A501T (Tomberg *et al.*, 2017).

Regarding carbapenems, non-mosaic alleles, with or without A501 mutations, increase ertapenem MICs 2-fold while a mosaic allele will increase the MIC 5 to 8-fold (Bharat *et al.*, 2015). Even so, ertapenem MICs overall remain lower than ESC MICs despite the presence of important resistance determinants. One intriguing finding from transformation experiments carried out by Bharat *et al.* is that the mosaic XXXIV_A501P (CI) allele (F89) has been shown to restore ertapenem susceptibility, thought to be related to the proline molecule preventing the action of the PBP2 on the carbapenem, but having a greater effect than that of either valine or threonine (Bharat *et al.*, 2015; Unemo *et al.*, 2011c).

3.6.1.3 Remaining 18 ESC fully susceptible isolates: *penA* alleles

The isolates which do not belong to any of the above mentioned ‘more resistant’ phenotypic groups are discussed here. Eighteen isolates had CRO/ETP MIC ≤ 0.032 and CFM/CTX MIC < 0.125 mg/L. All 18 had non-mosaic *penA* alleles including II (n = 13), XIV (n = 1), XIX (n = 1), XLIV (n = 2) and XLIX (n = 1). Non-mosaic *penA* II and XLIV alleles have been discussed above. Non-mosaic XIV, XIX and XLIX *penA* alleles were present in one isolate each. As the majority of publications relate to isolates displaying elevated ESC MICs there are few reports of these non-mosaic allele types, however, out of 25 isolates sequenced in Canada, non-mosaic XIX and XIV *penA* alleles were present in one isolate each originating from Chile (1982) and Saskatoon, Canada (2006), respectively (Vidovic *et al.*, 2014). Both isolates were susceptible to cefixime and ceftriaxone. Non-mosaic XIV *penA* allele has also been reported in a CRO-S isolate from China (Liang *et al.*, 2016). Furthermore, there are no reports of

non-mosaic XIX, XIV, XLIV, XLIX *penA* alleles associated with CRO-R isolates (Chen *et al.*, 2015).

3.6.1.4 ESC and ertapenem: *ponA*

Mutations in *ponA* add to reduced susceptibility to ESCs seen in *N. gonorrhoeae* by altering PBP1 binding sites and reducing binding. All CRO-RS isolates in this study had L421P alterations in *ponA* (encoding PBP1) resulting in reduced β -lactam acylation of PBP1. L421P in *ponA* was present in the majority of isolates in the CFM-R (6/6, 100%), CFM-RS (12/14, 86%), CTX-R (13/13, 100%), CTX-RS (8/11, 73%) and ETP-RS (6/7, 86%) phenotypic groups, whereas it was only seen in 3 of the remaining 18 ESC fully susceptible isolates (16.7%). Li *et al.* found an L421P alteration in *ponA* in 15/15 CRO-R isolates in China (Li *et al.*, 2014). It has also been found in the high-level ceftriaxone-resistant gonococci from Spain (Cámara *et al.*, 2012) and F89 from France (Unemo *et al.*, 2011c) and other ESC-resistant or reduced susceptibility isolates (de Curraize *et al.*, 2016; Lewis *et al.*, 2013).

3.6.1.5 ESC and ertapenem: *penB*

Mutations in *penB* result in reduced influx of antimicrobial in gonococci by altering porin PorB. In this study, G120K plus A121N/D/G (the majority were A121N) alterations in *penB* (encoding PorB) were present in 100% of isolates in the CRO-RS (9/9), CFM-R (6/6) and the CTX-R (13/13) phenotypic groups. These double alterations were also present in the CFM-RS (11/14, 79%), CTX-RS (7/11, 64%) and the ETP-RS (6/7, 86%) phenotypic groups. The remaining 18 isolates had double

G120K plus A121N/D/G in 8/18 isolates (44%), single A121S in 6/18 (33%), and the rest were wild-type (4/18, 22%). G102K and A121N/D/G mutations in *penB* are reported to be associated with resistance in *N. gonorrhoeae* (Endimiani *et al.*, 2014) and ceftriaxone resistance in China and Japan (Li *et al.*, 2014; Nakayama *et al.*, 2016). The high-level ceftriaxone-resistant gonococci from Spain (Cámara *et al.*, 2012) and France (F89) had G120K plus A121N alterations in *penB*. However, Grad *et al.* examined 1102 gonococcal isolates in the US (GISP 2000-2013) for resistance determinants and reported that the G120K mutations were not associated with reduced ESC susceptibility in the set of isolates examined, but A121N mutations had a positive predictive value of 72% and 82% for ESC resistance and reduced susceptibility, respectively. The A121S mutation is associated with a negative predictive value for ESC resistance and reduced susceptibility of 94% and 84%, respectively, in the same set of isolates (Grad *et al.*, 2016). No mutations in G101 or A102 were detected in the current study. These have previously been reported to be associated with CRO-R or CRO-RS isolates in China (especially the A102D alteration), France and various other European countries (Chen *et al.*, 2014; Chen *et al.*, 2015; de Curraize *et al.*, 2016; Golparian *et al.*, 2010)

3.6.1.6 ESC and ertapenem: *mtrR* and *mtrR* promoter region

In the current study, mutations in *mtrR* or the *mtrR* promoter region leading to increased expression of efflux pumps and increased efflux of antimicrobial were identified. The alterations seen in *mtrR* for the CRO-RS, CFM-R/RS, CTX-R/RS and ETP-RS phenotypic groups included combinations of H105Y, G45D, A39T, D79N and R44H alterations (Tables 3.4, 3.5/3.6, 3.8/3.9 & 3.11). The majority of the 25 isolates in the

above mentioned resistant or reduced susceptibility phenotypic groups had an H105Y alteration in *mtrR* (n = 16) and 13 of these also had an A deletion in the *mtrR* promoter. Only one isolate out of the remaining 18 had an H105Y alteration in *mtrR*. The MIC ranges for ceftriaxone, cefixime, cefotaxime and ertapenem for the 16 isolates (H105Y alteration) were 0.016-0.094 mg/L, 0.064-0.25 mg/L, 0.094-0.38 mg/L and 0.023-0.064 mg/L, respectively, showing an association with increased MICs. This is similar to the findings by Chen *et al.* who reported that a group of isolates with an H105Y alteration in *mtrR* had significantly higher mean ceftriaxone MICs compared to H105Y-wild-type isolates and also the proportion of isolates with H105Y was higher in the CRO-R/RS group than the susceptible group. They also report that an A deletion was present in *mtrR* promoter in 14/15 CRO-R isolates, however overall 272/278 isolates had the same deletion (Chen *et al.*, 2014).

The next most prevalent alteration in *mtrR* among the 25 CRO-RS, CFM-R/RS, CTR-R/RS and ETP-R isolates was a G45D alteration which was present in three isolates in the current study, two of which also had an A13 deletion in the promoter region. The MIC ranges for ceftriaxone, cefixime, cefotaxime and ertapenem were 0.008-0.064 mg/L, 0.016-0.094 mg/L, 0.125-0.25 mg/L and 0.012-0.023 mg/L, respectively, lower than the ranges seen above for isolates containing an H105Y alteration. Six out of the remaining 18 ESC fully susceptible isolates had a G45D alteration in *mtrR*. In a European study of 115 gonococcal isolates (all of which had either a mosaic *penA* or an A501 alteration) were examined for *mtrR* alterations, 93 (81%) were found to have a single A deletion in the promoter region, 8 (7%) a single G45 alteration and 9 (8%) had both alterations (Golparian *et al.*, 2010). G45D alterations in *mtrR* have been reported in ESC-susceptible isolates previously (Endimiani *et al.*, 2014) and as mentioned above, 6/18 of the ESC fully susceptible isolates in the current study possess this alteration.

The remaining *mtrR* alterations found in the CRO-RS, CFM-R/RS, CTX-R/RS and ETP-RS phenotypic groups (n = 25 isolates) occurred infrequently: A39T (n = 2); A39T plus an internal stop codon (n = 2); A39T plus R44H (n = 1); and a D79N (n = 1). These alterations were present in similar numbers in the 18 ESC fully susceptible isolates also and have been reported in ESC-susceptible gonococci previously (Endimiani *et al.*, 2014).

Four out of the 18 ESC fully susceptible isolates have a D79N, T86A and H105K alterations in *mtrR*. Three also have A13 deletions in the promoter region, but none had reduced susceptibility to ESCs or ertapenem.

3.6.2 Azithromycin and solithromycin

3.6.2.1 High-level azithromycin-resistant *N. gonorrhoeae*

An A2059G mutation in the peptidyltransferase loop in domain V in the 23S rRNA is associated specifically with HLAzi-R in *N. gonorrhoeae* when it is present in 3 or 4 alleles and has been reported worldwide (Allen *et al.*, 2014; Galarza *et al.*, 2010; Katz *et al.*, 2012; Lynagh *et al.*, 2015; Stevens *et al.*, 2015; Unemo *et al.*, 2014; Demczuk *et al.*, 2016; Jacobsson *et al.*, 2016). As described above, seven of the 43 isolates sequenced were HLAzi-R (MIC > 256 mg/L). One isolate in this study had an A2059G mutation in only 2 out of 4 alleles, the remaining isolates had 3/4 (n = 2) or 4/4 (n = 4) copies. No other specific mutation were found to be associated with HLAzi-R. The majority of internationally reported HLAzi-R isolates have A2059G mutations in 4 out of 4 alleles and in some cases 3 out of 4 alleles are mutated (Table 1.4). There have

been no previous reports of HLAzi-R isolates with only 2 alleles containing A2059G mutations, as was found in the current study. This isolate had a G45D alteration in *mtrR* as did each of the other HLAzi-R isolates as discussed below, however, it is the only HLAzi-R isolate which had an A13 deletion in the *mtrR* promoter region. The other HLAzi-R were wild type (n = 5) or had an A12C nucleotide alteration (n = 1) in the *mtrR* promoter, the latter being of unclear significance. Repeat analysis of the sequence and confirmation of MIC were performed with the same results and so it appears that A2059G mutations in 2 alleles, in conjunction with an A13 deletion in the *mtrR* promoter region, was sufficient to confer high-level resistance to azithromycin.

Mutations in *mtrR* and the *mtrR* promoter region contribute to azithromycin resistance in *Neisseria gonorrhoeae*. The 7 HLAzi-R isolates had mutations in *mtrR* (G45D) with only two of these having detectable mutations in the *mtrR* promoter; one with an A13 deletion and one with an A12C nucleotide substitution, the latter has not been reported previously and is of uncertain significance. Regarding *mtrR* alterations, at least 6 of the internationally reported HLAzi-R isolates (n = 25) (Table 1.4) have G45D amino acid substitutions in *mtrR* and at least 9 have an A deletion in the promoter region.

Demczuk *et al.* analysed AZM-R isolates in Canada by WGS and reported that the number of 23S rRNA alleles with A2059G, the presence of *mtrR* promoter mutations and *ermC* were strong contributors to increasing AZM MIC increments. They also state that G45D or A39T substitutions in *mtrR* and the presence of *ermB* had minimal effect on resistance (Demczuk *et al.*, 2016). This is reflected in the findings in the current study where mutations in 23S rRNA were the major predictor of azithromycin resistance. Similarly, Eyre *et al.* recently used WGS to predict MICs of *N. gonorrhoeae* to a range of antimicrobials and showed that 23S rRNA mutations are the strongest predictor of macrolide resistance. They found a 375-fold increase in azithromycin MIC

if 4/4 alleles have the A2059G (Eyre *et al.*, 2017). Other studies have reported a 100% positive predictive value for azithromycin resistance if there were 4 loci with A2059G mutations (Grad *et al.*, 2016).

3.6.2.2 Medium-level azithromycin-resistant *N. gonorrhoeae*

A second mutation, C2116T, in the 23S rRNA is also associated with resistance to azithromycin in *Neisseria gonorrhoeae*. Excluding HLAzi-R isolates, the remaining *gonococci* in this study formed two groups based on azithromycin MICs; those with azithromycin MICs ranging 4-32 mg/L and those ≤ 2 mg/L. The 7 isolates in the first group (MIC 4-32 mg/L, Table 3.15) had what I describe as medium-level resistance to azithromycin and five of these isolates had C2611T mutations in 4/4 alleles of the 23S rRNA.

This 23S rRNA mutation (C2611T) is widely reported to be associated with resistance to azithromycin in isolates with MICs ranging from 1-64 mg/L with the number of mutated alleles ranging from 1-4, but mostly 3 copies are associated with azithromycin MICs ≥ 4 mg/L (Lahra *et al.*, 2017; Allen *et al.*, 2014; Liang *et al.*, 2016; Shigemura *et al.*, 2015; Wind *et al.*, 2017a; Jacobsson *et al.*, 2016). Additionally there have been reports of isolates with 1 mutated allele (C2611T) associated with azithromycin MICs of 0.38 mg/L, 1 mg/L and 2 mg/L (Liang *et al.*, 2016; Wind *et al.*, 2017a). Eyre *et al.* state that each additional copy of a C2611T mutated allele will increase the azithromycin MIC by 2.9-fold (Eyre *et al.*, 2017). Grad *et al.* report a positive predictive value of 99% for reduced susceptibility to azithromycin if there are ≥ 2 mutated loci (C2611T) and interestingly having 1 mutated loci has the same negative predictive value for reduced susceptibility to azithromycin as wild-type 23S rRNA

(Grad *et al.*, 2016). In the current study one isolate (AZM MIC = 0.125 mg/L) had 1/4 mutated alleles. The five isolates with 4/4 C2611T mutated alleles in the current study also had alterations in either or both *mtrR* and the *mtrR* promoter region (Table 3.15).

The two isolates with all 4 wild-type 23S rRNA alleles (MICs 4-32 mg/L) (Table 3.15) both had a D79N substitution in *mtrR* and A13 deletion in *mtrR* promoter and azithromycin MICs of 8 mg/L and 4 mg/L. Overall, D79N substitutions in *mtrR* were present in 4 out of 7 medium-level azithromycin-resistant gonococci compared to 2 out of 29 isolates with azithromycin MICs ≤ 2 mg/L and none of the 7 HLAzi-R isolates. It has not been reported in other studies, however other substitutions at D79 have been reported in association with ESC reduced susceptibility or resistance (Chen *et al.* 2014).

Medium-level azithromycin-resistant gonococci with 4/4 wild-type 23S rRNA alleles have been reported previously. Chisholm *et al.* report 4 out of 26 gonococci (azithromycin MICs 2-8 mg/L) as wild-type while the remainder had 3 or 4 mutated 23S rRNA alleles (C2611T), however, it is unclear what the MICs of these 4 wild-type isolates were (Chisholm *et al.*, 2010). Again *erm*, *ere*, *mef* and *mph* genes were not detected in these isolates (AZM MIC 4-32 mg/L).

3.6.2.3 Low-level azithromycin-resistant and azithromycin-susceptible *N. gonorrhoeae*

The remaining 29 isolates (AZM MICs 0.032-2 mg/L) were susceptible, intermediate or low-level resistant to azithromycin. All of these isolates were wild-type 23S rRNA, except for one which had 1/4 C2611T mutated alleles, as discussed above. Of the 21 isolates with an A13 deletion in the *mtrR* promoter region, 17 also had an H105Y alteration in *mtrR*. Of the three isolates with the lowest azithromycin MICs (0.032

mg/L, 0.064 mg/L and 0.125 mg/L) two had internal stop codons in *mtrR* in association with A39T substitutions while the other had an A39T substitution. All three had wild-type *mtrR* promoter regions and as mentioned one has 1 out of 4 C2611T mutated 23S rRNA alleles (internal stop codon in *mtrR*). One additional isolate (AZM MIC = 0.5 mg/L) had an internal stop codon in *mtrR* (plus an A39T substitution) and had wild-type *mtrR* promoter and 23S rRNA. The presence of an internal stop codon in *mtrR* may be responsible for low azithromycin MICs in these isolates, by causing *mtrR* to revert to wild-type if transcription of an altered gene is prevented. Grad *et al.* suggested that the presence of an internal stop codon in *mtrR* is associated with azithromycin-susceptibility in *N. gonorrhoeae* (Grad *et al.*, 2016).

In the current study, A13 deletions in the *mtrR* promoter were present in 21/29 (72%), 4/7 (57%) and 1/7 (14%) of the three groups of isolates with MICs of 0.032-2 mg/L, 4-32 mg/L and > 256 mg/L, respectively. Single alterations in *mtrR* (not including internal stop codons as an alteration) were present in the majority of the isolates (40/43). Two or more alterations in *mtrR* were found in 3/29 (10%), 4/7 (57%) and 0/7 (0%) of these three groups, respectively. Liang *et al.* examined 485 *N. gonorrhoeae* isolates from 2009-2013 in China for resistance determinants and reported that the presence of a 13A deletion in the *mtrR* promoter region was similar across all levels of susceptibility (Liang *et al.*, 2016), whereas the more susceptible isolates in the current study had a higher proportion of this alteration, i.e. 72.4% (21/29) of isolates susceptible or with low-level resistance to azithromycin, 57% (4/7) of medium-level azithromycin-resistant isolates and only 14% (1/7) of HLAzi-R carried this alteration. In a study from Japan of 59 isolates, an A deletions in *mtrR* promoter was seen more frequently in isolates with MICs > 0.5 mg/L (35/37, 95%) compared to those with MICs ≤ 0.25 mg/L (4/22, 18%) (Shigemura *et al.*, 2015). The azithromycin MIC range in that

study was 0.06-16 mg/L and the distribution was ≤ 0.25 mg/L (n = 23), 0.5 mg/L (n = 33), 1 mg/L (n = 1) and 16 mg/L (n = 1), which for the most part matches the group of 29 isolates (MIC 0.032-2 mg/L) in the current study, where 2/6 (33%) isolates with azithromycin MIC ≤ 0.25 mg/L and 19/23 (83%) with MIC 0.5-2 mg/L had an A deletion in the *mtrR* promoter region. Liang *et al.* also found that double or triple alterations in *mtrR* were only present in isolates with azithromycin MICs of ≥ 2 mg/L (Liang *et al.*, 2016) similar to the findings in this study where the isolates with medium-level resistance to azithromycin (MICs 4-32 mg/L) had the highest proportion of double or triple alterations in *mtrR*.

The specific alterations in *mtrR* and the *mtrR* promoter region present in the isolates in the current study have been reported previously from China (A39T, T86A, H105Y, D79N substitutions in *mtrR*), Amsterdam (A39T and G45D substitutions in *mtrR*), Japan (A deletion in the promoter region) and Europe (G45D substitutions in *mtrR*) (Liang *et al.*, 2016; Wind *et al.*, 2017a; Shigemura *et al.*, 2015; Jacobsson *et al.*, 2016). These studies also report alterations that were not found in the current study including F62L and A40D substitutions in *mtrR* (Liang *et al.*, 2016; Shigemura *et al.*, 2015).

As mentioned above, L4 and L22 ribosomal proteins (encoded by *rplD* and *rplV*, respectively) also contribute to macrolide resistance. Only one isolate in this study had a G71D alteration in *rplD* (AZM MIC = 0.5 mg/L), however this isolate also had mutations in *mtrR* (D79N, T86A and H105K) and the *mtrR* promoter (A13 deletion). Grad *et al.* examined GISP gonococcal isolates from 2000-2013 in the US. They identified isolates with G68 (n = 9) and G70 (n = 57) alterations in *rplD* and found that almost 60% of these isolates had reduced susceptibility (MIC ≥ 1 in isolates prior to 2005 and MIC ≥ 2 mg/L in 2005 and after) to azithromycin (Grad *et al.*, 2016). In Europe *N. gonorrhoeae* with azithromycin MIC ≥ 2 mg/L (2009-2014) were examined

for resistance determinants. No mutations in *rplV* were detected, but 32/75 (43%) had alterations in *rplD* (V125A, A147G, R157Q and G68D) (Jacobsson *et al.*, 2016).

The majority of studies looking at resistance determinants for azithromycin in *N. gonorrhoeae* have not detected *erm*, *ere*, *mef* or *mph* genes, as is the case in the current study. Wind *et al.* detected *ermB* (AZM MIC = 0.5 mg/L) in one isolate out of 31 examined from STI clinics in Amsterdam (1999-2013). This isolate had wild-type 23S rRNA and an A39T alteration in *mtrR* (Wind *et al.*, 2017a). In Canada *N. gonorrhoeae* isolates with *ermC* (n = 1, AZM MIC = 16 mg/L) and *ermB* (n = 2, MICs 2 and 4 mg/L) have been reported (Demczuk *et al.*, 2016). Azithromycin resistance in *N. gonorrhoeae* induced by rRNA methylases, esterases, phosphoesterases or efflux via MacAB remain rare.

In the current study, solithromycin MICs correlated somewhat with mutations found in 23S rRNA and to azithromycin MICs of HLAzi-R and medium-level azithromycin-resistant isolates, but are not fully explained by the mutations in these isolates and it is likely that there are other unknown mutations affecting phenotypic solithromycin MIC. In Canada, Allen *et al.* examined gonococcal isolates and found 2 HLAzi-R isolates to have solithromycin MICs of 8 and 32 mg/L and 4/4 A2059G mutated alleles in 23S rRNA, while 17 isolates with azithromycin MICs of 2 – 8 mg/L had solithromycin MICs of ≤ 0.125 – 0.5 mg/L and mostly had C2611T mutations with no other known methods of macrolide resistance being found (Allen *et al.*, 2014). No other publications have determined methods of macrolide resistance in relation to solithromycin and *N. gonorrhoeae*. While 23S rRNA mutations appear to be a significant determinant of solithromycin MIC, it is likely from the results in this study that there are other determinants as yet unknown which increase the solithromycin MIC.

3.6.3 Ciprofloxacin and gemifloxacin

Resistance to fluoroquinolones is mediated by mutations in *gyrA* and *parC* which results in decreased binding affinity to DNA gyrase II or topoisomerase IV, respectively. In the current study, ciprofloxacin and gemifloxacin had bimodal MIC distributions. All ciprofloxacin-resistant isolates (n = 31, gemifloxacin MIC range 0.38-12 mg/L) had double amino acid substitutions in *gyrA*, S91F plus either D95G (n = 23) or D95A (n = 8) and *parC* alterations including S87R/K, E91K/G and D86N. All ciprofloxacin susceptible isolates (gemifloxacin MIC range ≤ 0.002 -0.012 mg/L) (n = 12) were wild-type *gyrA* and *parC*. The most common combination of *gyrA* and *parC* alterations for isolates with ciprofloxacin MIC > 32 mg/L was S91F/D95G plus S87R (13/17, 76.5%). This combination is reported to be associated with higher geometric mean MICs compared to other combinations (Grad *et al.*, 2016).

These alterations in *gyrA* and *parC* have been reported worldwide. In Switzerland, Endimiani *et al.* examined 60 isolates from 1998 and 2012 and found ciprofloxacin-resistant isolates to have S91F, D95G, D95A substitutions in *gyrA* and S87R, E91G in *parC* (Endimiani *et al.*, 2014). In Korea, 25 ciprofloxacin-resistant and 2 intermediate isolates from 2000-2006 were reviewed and the alterations found included S91 and A95 substitutions in *gyrA* and A86 and S87 substitutions in *parC*. All 25 ciprofloxacin-resistant isolates had S91P substitutions, 23 had A95G and 21 had S87A (Lee *et al.*, 2011). No S91P or S87A alterations were present in the isolates sequenced in this study.

It has been reported that *gyrA*95 alterations result in the greatest increases in ciprofloxacin MICs (Eyre *et al.*, 2017), an alteration which was found in all ciprofloxacin-resistant isolates sequenced in the current study, both those with a

ciprofloxacin MIC > 32 mg/L and MICs 2-16 mg/L. A negative interaction, in terms of resultant ciprofloxacin MIC, has been reported for the combination of mutations at amino acid residues 87 and 91 in *parC* (Eyre *et al.*, 2017). This combination was seen in three isolates in this study; two isolates whose ciprofloxacin (gemifloxacin) MICs were > 32 mg/L (4 mg/L) with E91K plus S87K alterations in *parC* and one isolate whose ciprofloxacin (gemifloxacin) MIC = 8 mg/L (12 mg/L) and had E91G plus S87I alterations in *parC*. S91F plus D95A/G alterations in *gyrA* were present in all three of these isolates. GISP CDC isolates (1102) from 2000-2013 from across USA were reviewed by Grad *et al.* Similar alterations in *parC* (D86N, S88P, E91G [only with *parC* 87S], E91K) were found, as reported above and in the current study (Grad *et al.*, 2016). They reported *gyrA* 91F and 95G as highly predictive of resistance (PPV 0.98 and 0.99, respectively). They also reported five ciprofloxacin-resistant isolates with no *parC* or *gyrA* variants meaning that alterations at other sites may potentially be responsible for fluoroquinolone resistance and may be associated with a fitness cost given their rarity (Grad *et al.*, 2016).

norM regulates the NorM efflux pump and mutations lead to its overexpression and increases MICs to fluoroquinolones, however in the current study, all 43 isolates had wild-type *norM*.

The contribution of *mtrR* and *mtrR* promoter mutations to fluoroquinolone resistance is unclear with *gyrA* mutations being associated with the greatest increases in MIC (Eyre *et al.*, 2017). In this study, there was no obvious association found between *mtrR* mutations and ciprofloxacin/gemifloxacin MICs (Table 3.17-3.19). Dewi *et al.* reported a good correlation between *mtrR* G45D alteration (but this was found in association with *gyrA* mutations in 93/131 or 71% of isolates tested) as well as A13 deletion in the promoter and ciprofloxacin/ofloxacin resistance in a study from Japan in 2004 (Dewi *et*

al. 2004). These results were referenced in a review by Poole *et al.* reporting that fluoroquinolone resistance in *N. gonorrhoeae* mediated by efflux systems is via the MtrCDE efflux pumps (Poole *et al.*, 2005). A study from New Dehli, India, reported *mtrR* mutations in relation to quinolone resistance, however this group did not examine the isolates for *gyrA* or *parC* (Kulkarni *et al.*, 2015). Castanheira *et al.* determined quinolone resistance mechanisms in *N. meningitides* and reported that alterations in the expression of MtrCDE efflux pump had little effect on ciprofloxacin MIC (Castanheira *et al.*, 2012). There are no reports of *mtrR*/*mtrR* promoter transformation experiments in *N. gonorrhoeae*. Contemporary studies and review articles do not consider overexpression of MtrCDE efflux pump an important contributor to fluoroquinolone resistance (Unemo *et al.*, 2016; Eyre *et al.*, 2017).

3.6.4 Penicillin and β -lactamase

Plasmid mediated TEM β -lactamases were present in the three isolates with high-level resistance to penicillin (MIC > 32 mg/L). In the current study *bla*_{TEM-141} (n = 2) and *bla*_{TEM-75} (n = 1) were present in these three isolates, yet only *bla*_{TEM-1}, *bla*_{TEM-135} and *bla*_{TEM-220} have been previously reported in *N. gonorrhoeae* (Endimiani *et al.*, 2014; Gianecini *et al.*, 2015; Tribuddharat *et al.*, 2016). *bla*_{TEM-198} was detected in two other isolates (penicillin MIC = 4 mg/L) discussed below, however all of the *bla*_{TEM} types in the current study, although novel in *Neisseria gonorrhoeae*, have 99% sequence similarity to *bla*_{TEM-135} which has been widely reported in the past. This may be explained by the enhanced discriminatory power of WGS compared to traditional methods.

In some countries penicillin resistance and PPNG rates are currently very high, while rates are low or unreported in others. For example, in Thailand, the rate of penicillin resistance is approximately 86% (2008-2014, 370 isolates) with PPNG accounting for 83.3% of isolates; *bla*_{TEM}-1 and *bla*_{TEM}-135 are present in approximately 70% and 30%, respectively, with *bla*_{TEM}-135 increasing significantly from 10% early in the study to 30% in the latter years (Tribuddharat *et al.*, 2016). In Italy, the rate of PPNG fell from 77% in 2003 to 7% in 2012 (1430 isolates tested), while in Switzerland (1998 & 2012) 8/26 isolates carried *bla*_{TEM} β -lactamases (Carannante *et al.*, 2014; Endimiani *et al.*, 2014) *bla*_{TEM} β -lactamases are responsible for the greatest increases in penicillin MICs (23.4-fold) out of all resistance determinants affecting penicillin (Eyre *et al.*, 2017).

Chromosomal penicillin resistance was identified in *N. gonorrhoeae* in this study. The three PPNG high-level penicillin-resistant isolates also had non-mosaic II *penA* alleles albeit including an A501V substitution which is reported to decrease penicillin MICs by 20-50% in the absence of penicillinase production (Tomberg *et al.*, 2010). The six other penicillin-resistant isolates (MIC > 1-4 mg/L) had mosaic XXXIV (n = 3, all without plasmid encoded β -lactamases), mosaic X (n = 1), non-mosaic XIX (n = 1) or XIV (n = 1) and 5/6 had L421P alterations in *ponA*. The mosaic XXXIV and X *penA* alleles have been shown to increase penicillin MICs, in transformation experiments, by an average of 2.1-fold, and the non-mosaic alleles by an average of 1.6-fold (Bharat *et al.*, 2015).

The L421P alteration in *ponA* is associated with high level penicillin resistance in isolates that also have alterations in *penA*, *mtrR* and *penB*, which were present in this study in all three penicillin-resistant isolates without plasmid-encoded β -lactamase (penicillin MICs = 2 mg/L). All penicillin-resistant isolates had an alteration in *mtrR* with or without A13 deletions in the *mtrR* promoter. Many studies have reported A13

deletions in the *mtrR* promoter region as well as the alterations found in this study in *mtrR* (Chen *et al.*, 2014; Endimani *et al.*, 2014; Liang *et al.*, 2016). An A deletion in the *mtrR* promoter region results in overexpression of the MtrCDE efflux pump. Of note, one isolate in this study had an internal stop codon in *mtrR* as well as an A39T substitution. Internal stop codons may prevent translation of the gene, essentially reverting it to wild-type.

Penicillin intermediate isolates in this study harboured similar *penA* alleles to the resistant strains, including mosaic XXXIV and non-mosaic *penA* alleles. All non-mosaic *penA* alleles had A501V/T mutations. A501V has been reported to reduce penicillin MICs in transformation experiments (Tomberg *et al.*, 2010), as mentioned above.

‘Factor X’ is an unknown resistance determinant which is non-transformable and is reported to play a role in elevating MICs of penicillin and ESCs (Unemo *et al.*, 2012a). While it is impossible to identify isolates carrying ‘Factor X’, it may have played a role in certain isolates in this study which had identical resistance determinants but varying MICs to penicillin or ESCs.

3.6.5 Tetracycline

Three out of 43 sequenced isolates in this study were found to harbour *tetM* which is reported to increase the tetracycline MICs significantly (126-fold) (Eyre *et al.*, 2017). All were tetracycline-resistant, MICs 8 mg/L, 24 mg/L and 32 mg/L. Tetracycline-resistant gonococci are prevalent worldwide and those with *tetM* generally have MICs $\geq$

16 mg/L (Mylarczyk ADA 2016), but there are isolates with MICs ≥ 6 mg/L reported that possess *tetM* (Endimiani *et al.*, 2014).

In this study, chromosomal determinants also conferred reduced susceptibility to tetracycline, including alterations in *rpsJ* encoding S10 ribosomal protein (involved in ribosomal binding and translation), *mtrR* and the *mtrR* promoter region and *penB* (Unemo *et al.*, 2016 Microbiol Spectre; Lewis *et al.*, 2014; Eyre *et al.*, 2017; The UniProt Consortium 2017) Three isolates in this study (MIC ≥ 8 mg/L) had alterations in *mtrR*, but two of these included an internal stop codon which may negate the effect of the amino acid substitutions. All three had wild-type *mtrR* promoter. In *penB*, the alterations found in these three isolates were G120K plus A121N/G. All three had a V57M alteration in *rpsJ*. V57M alters the structure of the rRNA near the tetracycline binding site (Hu *et al.*, 2005). This mutation has been reported to be associated with tetracycline non-susceptible gonococci only; a study in Italy of 58 strains of *N. gonorrhoeae* found V57M alteration in *rpsJ* in almost all of the tetracycline-resistant and intermediate strains, while it was not present in any of the susceptible strains (Starnino *et al.*, 2008 FEMS). In contrast, in a more recent study, 124 isolates in Russia were examined including 21 tetracycline-resistant and 10 intermediate isolates. V57M and V57L alterations in *rpsJ* were detected in 44 and 6 isolates, respectively, implying that tetracycline-susceptible isolates may also harbour the V57M alteration (Kubakov *et al.*, 2016).

The remaining 40 gonococci without *tetM* had tetracycline MICs of 0.125-2 mg/L and only one did not have a V57M alteration, i.e. the one with the lowest MIC of 0.125 mg/L. As mentioned above, V57M alterations may be present in tetracycline-susceptible isolates. These 40 isolates had a variety of alterations in *mtrR*, *mtrR* promoter and *penB* (Appendix 7). Seven of these had wild-type *penB* and the range of

MICs is 0.125-0.5 mg/L. Seventeen had G102K/A121N *penB* alterations and an MIC range of 0.5-2 mg/L. The variety of alterations seen in these genes in this group of 40 isolates is similar to results seen in previous studies also (Starnino *et al.*, 2008). Low levels of tetracycline resistance as seen in these isolates is due to accumulation of various combinations of resistance determinants, including mutations in *rpsJ*, *mtrR*, *mtrR* promoter and *penB* (Unemo *et al.*, 2016).

3.6.6 Spectinomycin

Spectinomycin resistance in *N. gonorrhoeae* is extremely rare, which was confirmed in this study. Spectinomycin MICs of the 43 sequenced isolates in this study ranged from 6 to 16 mg/L; all were susceptible to spectinomycin according to EUCAST interpretive criteria. In this study all isolates possessed a V25 deletion, but no K26E or T24P alterations were detected. Similarly all isolates sequenced have wild-type 16s rRNA. There are few reports of spectinomycin resistance in the literature, but resistance has been confirmed to be caused by a C1192U mutation in helix 34 of the 16s rRNA in *N. gonorrhoeae* (Galimand *et al.*, 2000). Additional mechanisms of resistance have been reported including the combination of a V25 deletion and a K26E alteration in *rpsE* (Unemo *et al.*, 2013) or a T24P alteration in *rpsE* (Ilina *et al.*, 2013).

3.6.7 Gentamicin

In general, aminoglycoside resistance may be mediated by a number of mechanisms including: modification and inactivation by aminoglycoside acetyltransferases, nucleotidyltransferases or phosphotransferases; increased efflux; decreased influx; and

binding site modifications (30s ribosomal protein) (Doi *et al.*, 2016). EUCAST do not provide clinical breakpoints for gentamicin and *N. gonorrhoeae*, but using the proposed breakpoints by Brown *et al.* the majority of gonococci in Malawi appear to remain susceptible to gentamicin even after previous exposure, as discussed (Section 1.6) (Brown *et al.*, 2010; Kamanga *et al.*, 2010).

The 43 sequenced isolates in the current study were either susceptible or intermediate to gentamicin (MIC range = 1-16 mg/L) (Brown *et al.*, 2010). In general, aminoglycoside resistance may be mediated by a number of mechanisms including: modification and inactivation by aminoglycoside acetyltransferases, nucleotidyltransferases or phosphotransferases; increased efflux; decreased influx; and binding site modifications (30s ribosomal protein) (Doi *et al.*, 2016). There is a paucity of reports relating specifically to gentamicin resistance mechanisms in *N. gonorrhoeae*, however, no genes (*aac*, *ant* and *aph*) encoding the aforementioned enzymes were identified in the isolates in the current study. Similarly, no mutations in *rpsL* (encoding 30s ribosomal protein S12 which is important in streptomycin resistance in *Mycobacterium tuberculosis*) were present, which may potentially interfere with gentamicin binding.

3.6.8 Fosfomycin

Neither EUCAST nor CLSI provide clinical breakpoints for fosfomycin and *N. gonorrhoeae* (EUCAST 2017; CLSI 2017). Mechanisms of resistance to fosfomycin in *Escherichia coli* include mutations in *murA* (the target gene for fosfomycin), *glpT* and *uhpT* (encode transport systems required for fosfomycin uptake), *cyaA* and *ptsI* (mutations result in down regulation of the transporters) and *fos* genes (encode fosfomycin-modifying enzymes) (Bi *et al.*, IJAA 2017; Silver *et al.*, 2017). None of the

isolates in this study possessed any of these resistance determinants on WGS analysis. For the four isolates with fosfomycin MICs > 32 mg/L (48 mg/L n = 3 and 96 mg/L n = 1) there was no explanation for the apparently elevated MICs. There are no publications on fosfomycin-resistant gonococcal isolates with which to compare sequence data generated in this study.

3.6.9 MDR and XDR isolates

Seven out of 43 isolates were categorised as MDR or XDR (Table 3.24). For the most part these seven isolates had very similar resistance determinants, e.g. all had mosaic *penA* alleles, L421P alteration in *ponA*, G120K plus A121N/G alterations in *porB*, and S91F plus D95G alterations in *gyrA*. The remaining genes had variable alterations or are wild-type. None of the HLAzi-R isolates met the defined criteria for MDR or XDR (Tapsall *et al.*, 2009c). This is often the case of reported HLAzi-R isolates (Table 1.3).

3.7 Limitations

The major limitation to this chapter was the number of isolates which were whole genome sequenced. WGS of all 609 isolates would have perhaps allowed for increased understanding of association of genetic resistance determinants and MIC values. Similarly, a greater number of ESC-R isolates may helped to elucidate specific combinations of resistance determinants.

3.8 Conclusion

The results in this chapter showed that there was good correlation between the previously described genetic mutations and the phenotypic susceptibility category for most of the antimicrobials above and *N. gonorrhoeae*. Some resistance determinants are specific for particular antimicrobials while others cause reduced susceptibility for many antimicrobials. The high-level resistant phenotypes typically carried specific resistance determinants, including plasmid-encoded β -lactamases or *tetM* for penicillin or tetracycline resistance, respectively, A2059G mutations in 23S rRNA for azithromycin resistance and S91F mutations in *gyrA* for ciprofloxacin resistance. For most antimicrobials, lower levels of resistance are often less definitively associated with specific mutations.

Ceftriaxone, the other first-line empiric antimicrobial for treating gonorrhoea is mainly affected by mosaic *penA* allele (less so than cefixime and cefotaxime) and in particular certain amino acid substitutions within it. Mosaic *penA* alleles acquiring additional amino acid substitutions have also resulted in increased ESC resistance, e.g. mosaic XXXIV acquiring an additional A501P lead to a novel *penA* allele, CI, in high-level ceftriaxone-resistant isolates and similarly, other novel alterations in *penA* such as A311V, T316P, A328T and T484S as found in H041 resulted in novel mosaic *penA* C.

Information on gonococcal resistance determinants generated from analysis of WGS data may be even more useful than current routine phenotypic susceptibility testing for certain antimicrobials. The current EUCAST azithromycin clinical breakpoints which state that *N. gonorrhoeae* with an MIC < 0.25 mg/L or > 0.5 mg/L should be considered susceptible or resistance, respectively, are possibly set too low. CLSI do not provide clinical breakpoints for *N. gonorrhoeae* and azithromycin, but state that the

epidemiological cut-off values (ECVs) (which separate gonococci into two populations - those with or without acquired resistance) for wild-type is ≤ 1 mg/L and for non-wild-type is ≥ 2 mg/L. Results in this study correlate with the CLSI statement on ECVs, given that A2059G (in ≥ 3 out of 4 alleles) and C2611T (in ≥ 3 out of 4 alleles) mutations in 23S rRNA are only found in isolates with MICs ≥ 4 mg/L. In future, absence of these mutations may provide a means of assessing whether azithromycin may be relied upon for treatment of gonorrhoea.

Other potential therapeutic options were investigated. Results of this study confirmed that ertapenem is less affected by *penA* resistance determinants *in vitro* compared to ESCs, especially considering its activity against ESC-resistant *N. gonorrhoeae*, in particular those strains with A501P alterations in *penA* which confer high-level ESC resistance but restore carbapenem susceptibility (Bharat *et al.*, 2015). It remains to be seen whether introduction of ertapenem for widespread treatment of gonorrhoea would cause selective pressure and result in development of resistance/reduced susceptibility to the same degree that is seen in ESCs.

Ciprofloxacin and gemifloxacin MICs correlated extremely well with the presence of resistance determinants (specific alterations in *gyrA* and *parC*) meaning that these fluoroquinolones are ideal candidates for molecular detection of resistance from clinical samples. Unfortunately, there is not such a clear harmony between penicillin and tetracycline resistance determinants and phenotypic susceptibility profile, meaning that predicting susceptibility from resistance determinants is more difficult, most likely due to multiple resistance mechanisms affecting these agents.

No resistance determinants were identified in this study for spectinomycin, gentamicin and fosfomycin, confirming the findings in Chapter II, suggesting that all may be

suitable options for gonorrhoea. Similarly, solithromycin may be suitable for use in gonorrhoeae, even in HLAzi-R if MICs have been predetermined and susceptibility is known.

The molecular epidemiology and clonal relatedness of specific isolates in this study are discussed in the following chapter.

Chapter IV

Molecular epidemiology of *Neisseria gonorrhoeae*

4.1 Introduction

The epidemiology of *N. gonorrhoeae* infection concerns the incidence, transmission, outbreaks and surveillance of this infection. Invaluable to the epidemiology of gonococcal infection are contact tracing and typing of isolates. Typing methods for *N. gonorrhoeae* have evolved from phenotypic and auxotyping/serotyping to pulsed-field gel electrophoresis (PFGE) to sequence based typing methods, NG-MAST, NG-MLST and WGS, as discussed (*Sections 1.12 – 1.13*).

Molecular typing of gonococcal isolates submitted to EURO-GASP in 2009 and 2010 was reported in 2013. NG-MAST was performed on 1066 isolates and 406 STs were identified. The predominant STs were ST1407, ST2992 and ST225. In Ireland ST2992 was the predominant ST accounting for 32% of the isolates typed. This ST was also prevalent in Norway and associated with MSMs (Chrisholm *et al.*, 2013). A second ECDC study on molecular typing of *N. gonorrhoeae* was performed on the 2013 EURO-GASP gonococcal isolates (n = 1218) and the report was published in June 2016 (ECDC 2017b). The second study utilised WGS as well as NG-MAST, compared to NG-MAST alone in the first. Again the predominant NG-MAST STs in the study were ST1407 and ST2992 with the latter being the most frequently observed ST in Ireland. This study reported an overall decrease in the proportion of NG-MAST ST1407 from 23.3% in 2009/2010 to 14.8% in 2013, with a concomitant decrease in cefixime resistance from 8.9% to 4.7%. Interestingly ST1407 was not found in Ireland or Iceland, but was present in all of the other 21 participating countries.

ST1407 emerged in Scotland in 2007 and quickly became established, accounting for 15.4% of strains in Scotland in 2009 (Eastwick, 2009). It is now the predominant strain in Europe (Golparian *et al.*, 2010; Carannante *et al.*, 2014; Horn *et al.*, 2014; ECDC

2017b), is associated with increased ceftriaxone MICs (Shimuta *et al.*, 2013) and is part of the globally predominant MLST ST1901 (Chrisholm *et al.*, 2013). De Silva *et al.* determined NG-MAST STs from WGS data on 1061 gonococcal isolates from Birmingham, UK from a 4 year period (2011-2015) (De Silva *et al.*, 2016). The most frequent STs were again ST2992 and ST1407. In California, ST2992 was reported to be the predominant ST among isolates from 2009 (Buono 2012 MDR).

MLST is performed much less frequently than NG-MAST in studies of *N. gonorrhoeae*. MLST STs were identified in silico from the WGS data of the latest ECDC molecular typing of *N. gonorrhoeae* study (ECDC 2017b). The most frequent STs of 1054 European isolates were ST1901, ST9363 and ST7363. MLST ST1901 was the predominant ST in 13 countries (65%) and it made up most of the NG-MAST ST1407s (83%). MLST was determined for 193 isolates in a study in Japan which aimed to increase surveillance after H041, the first strain with high-level ceftriaxone resistance, was reported (Shimuta *et al.*, 2013). The predominant NG-MAST was ST1407 (31/193, 16.1%) and MLST was ST1901 (79/193, 40.9%) with all but one of the ST1407s belonging to ST1901. Those ST1901 isolates subdivided into 30 NG-MAST STs and the authors concluded that NG-MAST has higher discriminatory power than MLST which was related to the variability within the sequenced *porB* fragment (Shimuta *et al.*, 2013).

WGS of 1054 EURO-GASP 2013 isolates found it to be more discriminating than NG-MAST finding a number of clades with specific evolutionary traits within the single NG-MAST ST1407 (ECDC 2017b). WGS also allows for in silico determination of NG-MAST and MLST STs, resistance determinants and generally a more discriminatory typing of all isolates.

4.2 Aims

The aims of this chapter were to assess the molecular epidemiology of the isolates selected for sequencing. The 43 isolates were selected based on MIC values to the antimicrobials tested, particularly ESCs and azithromycin, the first-line recommended agents. An additional 14 Irish *N. gonorrhoeae* isolates previously sequenced were also included to ascertain clonal relatedness. Of particular interest was whether HLAzi-R isolates were clonally related or were high-level azithromycin resistance arising in many different clones? Irish HLAzi-R strains were compared to those from an outbreak in the UK in 2014-2015 (Leeds, Chrisholm 2015 STI) to assess if they were related, implying international spread of a specific clone.

Gonococci with reduced susceptibility or resistance to ESCs were assessed for clonality also. An important part of the study was to ascertain if these strains represented a single clone circulating in the Irish population or multiple different clones acquiring the same resistance determinants.

4.3 Objectives

1. Analyse sequence data to assess clonal relatedness of *N. gonorrhoeae* isolates.
2. Examine specifically isolates with elevated ESC MICs.
3. Assess HLAzi-R isolates from Ireland in terms of clonality and also compare these to HLAzi-R isolates from the UK outbreak to see if these isolates are related.
4. Analyse sequence types to ascertain their association with AMR.

4.4 Materials and Methods

4.4.1 Method

Forty-three out of the 609 isolates in this study were selected for WGS. Fifteen additional isolates, sequenced previously as part of a different study were also included in the phylogenetic analysis for this chapter (Lynagh *et al.*, 2015; Mac Aogáin *et al.*, 2017).

4.4.2 DNA extraction and WGS

Extraction of genomic DNA from *N. gonorrhoeae* isolates, preparation of DNA sequencing libraries and high-throughput sequencing was performed as described (Section 3.4.1).

4.4.3 Data analysis

Reconstruction of the *N. gonorrhoeae* genome from raw FASTQ sequence reads was performed using the SPADES de novo assembler as integrated in the DTU centre for genomic epidemiology 2.1 Assembler pipeline. Reconstructed contigs were then used to determine MLST type using pubMLST tool. A local BLAST search was performed on de novo assemblies to determine NG-MAST type.

In parallel, sequencing reads were aligned to the *N. gonorrhoeae* NCCP11945 genome (CP001050) using the Burrows-Wheeler short-read aligner (BWA, version 0.7.12-r1039) and inter strain genetic variations, including those present in antibiotic resistance genes, were resolved using SAMtools (version 0.1.19-96b5f2294a) (Li & Durbin, 2009;

Li *et al.*, 2009b). Putative regions of recombination were masked by excluding any region with an average single nucleotide variants (SNV) per 100bp of > 5 as well as SNVs separated by less than 12 nucleotides. This led to the identification of 9182 variant sites within the core genome of all 58 isolates. Based on these sites a maximum-likelihood phylogenetic tree was constructed using the generalized time reversible (GTR) substitution model in PhyML (Guindon *et al.*, 2010).

4.5 Results

4.5.1 NG-MAST and MLST STs related to phenotypic resistance and resistance determinants

Extended-spectrum cephalosporins and ertapenem

Twenty-five of the forty-three isolates belong to one or more of the six phenotypic groups described (*Section 3.6.1*) (CRO-R, CFM-R, CFM-RS, CTX-R, CTX-R and ETP-RS). The results for these 25 isolates are summarised here (Table 4.1), as are the results for the remaining 18 isolates which do not belong to any of these 6 phenotypic groups (Table 4.2). Each of the 6 phenotypic groups is discussed individually thereafter.

The NG-MAST STs identified in the 43 isolates were highly diverse, but there were 9 clusters of ≥ 2 STs with the predominant being ST1407 ($n = 4$) followed by ST649 ($n = 3$), ST3806 ($n = 3$), ST3158 ($n = 2$), ST2318 ($n = 2$), ST12302 ($n = 2$), ST3311 ($n = 2$), ST5624 ($n = 2$) and ST2992 ($n = 2$). The remaining NG-MAST STs occurred singly in 17 isolates with an additional 4 for which there was no match (Tables 4.1 & 4.3).

There were 14 MLST STs, including 3 novel STs. Six of these MLST STs represent ≥ 2 isolates. The predominant type is ST1901 (n = 16) followed by ST1580 (n = 7), ST9363 (n = 5), ST1588 (n = 2), ST7363 (n = 3) and ST8143 (n = 2). There were 8 single MLST STs representing 1 isolate each, including the three novel MLST STs (ST13142, ST13143 and ST13144). ST1901 and ST7827 were found only in the more resistant 25 isolates, while ST8143 was only present in the 18 more susceptible isolates (Tables 4.1 & 4.2). ST1580, ST1588, ST7363 and ST9363 were present in both groups of isolates.

Molecular characteristics: CRO-RS, CFM-R/RS, CTX-R/RS and ETP-RS isolates											
Isolate	NG-MAST	MLST	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
196712	649	1580	0.008	0.016	0.125	0.023	non-mosaic II	no	A121S	G45D	WT
71324	1582	1588	0.047	0.19	0.19	0.094	mosaic X	L421P	G120K A121G	A39T ISC	WT
016	11645	1901	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
035	NM	1901	0.047	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
286008	3806	1901	0.023	0.094	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
66931	3806	1901	0.032	0.125	0.25	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
76972	3431	1901	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
176561	4936	1901	0.016	0.125	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
501373	5622	1901	0.032	0.125	0.125	0.023	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
024	1407	1901	0.094	0.25	0.38	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
009	1407	1901	0.064	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
194698	1407	1901	0.016	0.064	0.19	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
191262	1407	1901	0.023	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422671	3158	1901	0.064	0.125	0.25	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422102	3158	1901	0.032	0.125	0.38	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
179501	11690	1901	0.016	0.094	0.094	0.023	mosaic XXXIV	L421P	A121S	H105Y	A13 del
81513	2212	1901	0.032	0.19	0.125	0.064	mosaic XXXIV	L421P	G120K A121D	H105Y	A13 del
178526	8953	1901	0.032	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
425204	NM	7359	0.016	0.125	0.125	0.032	mosaic XXXIV	WT	WT	A39T	WT
197747	14449	7363	0.016	0.032	0.094	0.012	non-mosaic XLIV	L421P	WT	A39T ISC	A13 del
177574	2318	13143	0.047	0.094	0.125	0.016	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del

168628	2318	7827	0.064	0.064	0.25	0.012	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del
166587	12302	9363	0.008	0.016	0.125	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del
82432	7867	13142	0.023	0.125	0.25	0.032	mosaic XXXIV	L421P	G120K A121D	A39T	A13 del
86606	4244	11428	0.008	0.094	0.064	0.047	mosaic XXXIV	WT	WT	A39T R44H	WT

Table 4.1. Molecular characteristics of twenty five isolates which belong to phenotypic groups

CRO-R, CFM-R, CFM-RS, CTX-R, CTX-RS and ETP-RS. Clusters of ≥ 2 NG-MAST or MLST

STs are highlighted. Isolates sharing similar MLSTs or NG MASTs are highlighted in specific

colour throughout Tables 4.1 – 4.16.

Molecular characteristics of eighteen isolates with MICs CRO/ETP $\leq$ 0.032 mg/L and CFM/CTX $<$ 0.125 mg/L											
Isolate	NG-MAST	MLST	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
006	2475	1580	0.016	0.016	0.032	0.012	non-mosaic II	WT	A121S	G45D	A12C
181798	3311	1580	0.008	0.023	0.047	0.023	non-mosaic II	WT	A121S	G45D	WT
191587	3311	1580	0.008	0.032	0.064	0.016	non-mosaic II	WT	A121S	G45D	WT
178487	649	1580	0.006	0.023	0.047	0.032	non-mosaic II	WT	A121S	G45D	WT
199856	649	1580	0.002	$<$ 0.016	0.004	0.008	non-mosaic II	WT	A121S	G45D	WT
193852	1443	1580	0.004	$<$ 0.016	0.006	0.023	non-mosaic II	WT	A121S	G45D	A13 del
67818	2400	1587	0.012	0.023	0.064	0.012	non-mosaic XLIX	L421P	G120K A121D	D79N T86A H105K	A13 del
190440	3750	1588	0.004	0.016	0.032	0.012	non-mosaic XIX	L421P	G120K A121G	A39T ISC	WT
420132	NM	13144	0.008	$<$ 0.016	0.008	0.012	non-mosaic II	WT	WT	A39T	WT
193058	NM	7363	0.008	0.016	0.047	0.016	non-mosaic XLIV	L421P	WT	D79N T86A H105K	A13 del
425657	9184	7363	0.016	0.032	0.012	0.006	non-mosaic XLIV	L421P	G120K A121D	D79N T86A H105K	A13 del
422322	5624	8143	0.008	$<$ 0.016	0.016	0.004	non-mosaic II	WT	G120D A121G	A39T ISC	WT
008	5624	8143	0.008	$<$ 0.016	0.023	0.006	non-mosaic II	WT	G120D A121G	H105Y	A13 del
180505	3935	9363	0.012	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	WT
178575	2992	9363	0.003	0.016	0.012	0.012	non-mosaic II	WT	WT	A39T R44H	WT
41277	2992	9363	0.003	$<$ 0.016	0.012	0.008	non-mosaic II	WT	WT	A39T R44H	WT
179411	12302	9363	0.008	0.023	0.064	0.016	non-mosaic II	WT	G120K A121N	D79N	A13 del
039	NM	9903	0.012	0.016	0.016	0.016	non-mosaic XIV	WT	G120K A121N	D79N T86A H105K	WT

Table 4.2. Molecular characteristics of isolates with MICs CRO/ETP $<$ 0.032 mg/L and

CFM/CTX $<$ 0.125 mg/L (n = 18). Clusters of $>$ 2 NG-MAST or MLST STs are highlighted.

Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables

4.1 – 4.16.

4.5.2 Ceftriaxone reduced susceptibility (CRO-RS)

Nine isolates displayed reduced susceptibility to ceftriaxone (MIC > 0.032 mg/L).

Apart from NG-MAST ST1407 (n = 2) and ST2318 (n = 2), these 9 isolates were unique NG-MAST STs (Table 4.3). Six were MLST ST1901 (two of which are NG-MAST ST1407) and the remaining three were ST7827 (n = 1), ST13143 (n = 1, novel) and ST1588 (n = 1). The MLST types correlated well with the resistance determinants present as highlighted (Table 4.3). The MLST ST1901 isolates had mosaic XXXIV *penA* alleles, while ST7827 had non-mosaic XIII *penA* and ST1588 has mosaic X *penA* allele. The six ST1901 isolates had a H105Y alteration in *mtrR*, deletion in the *mtrR* promoter region, and G120K and A121N alterations in *penB*, while the ST1588 isolate had a A39T alteration in *mtrR*, wild-type *mtrR* promoter and G120K and A121G alterations in *penB* and the ST7827 and ST13143 isolates had G45D alterations in *mtrR*, a deletion in the *mtrR* promoter and G102K and A121D in *penB*.

4.5.3 Cefixime-resistance (CFM-R)

Six isolates were CFM-R, all of which were different NG-MAST STs. Five were MLST ST1902 and 1 was ST1588. The *penA*, *penB*, *mtrR* and *mtrR* promoter resistance determinants correlate well with the MLST STs among these isolates (Table 4.4) as discussed above.

Isolate (Year)	NG-MAST	MLST	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
71324 (2014)	1582	1588	0.047	0.19	0.19	0.094	mosaic X	L421P	G120K A121G	A39T ISC	WT
016 (2013)	11645	1901	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
035 (2016)	NM	1901	0.047	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
76972 (2014)	3431	1901	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
024 (2012)	1407	1901	0.094	0.25	0.38	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
009 (2013)	1407	1901	0.064	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422671 (2016)	3158	1901	0.064	0.125	0.25	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
177574 (2015)	2318	13143	0.047	0.094	0.125	0.016	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del
168628 (2016)	2318	7827	0.064	0.064	0.25	0.012	non-mosaic XIII	L421P	G120K A121D	G45D	A13 del

Table 4.3. Molecular characteristics of 9 CRO-RS isolates (CRO MIC > 0.032 mg/L). WT = wild

type. Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout

Tables 4.1 – 4.16.

Isolate (Year)	NG-MAST	MLST	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
71324 (2014)	1582	1588	0.047	0.19	0.19	0.094	mosaic type X	L421P	G120K A121G	A39T ISC	WT
016 (2013)	11645	1901	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
76972 (2014)	3431	1901	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
024 (2012)	1407	1901	0.094	0.25	0.38	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
81513 (2015)	2212	1901	0.032	0.19	0.125	0.064	mosaic XXXIV	L421P	G120K A121D	H105Y	A13 del
178526 (2015)	8953	1901	0.032	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del

Table 4.4. Molecular characteristics of CFM-R (n=6) isolates. WT = wild type. Isolates sharing

similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 – 4.16.

4.5.4 Cefixime reduced susceptibility (CFM-RS)

Fourteen isolates displayed reduced susceptibility to cefixime. The NG-MAST STs of these isolates were mostly single STs with two isolates belonging to ST1407. Again MLST ST1901 was the predominant MLST ST and the MLST STs correlated well again with resistance determinants (Table 4.5). Of note, MLST STs other than ST1901 also had mosaic XXXIV *penA* alleles, but these STs (ST7359 and two novel STs, ST13142 and ST11428) had differing resistant determinants in *penB*, *ponA*, *mtrR* and the *mtrR* promoter region (Table 4.5).

4.5.5 Cefotaxime resistance (CTX-R/CTX-RS)

Thirteen isolates were resistant to cefotaxime (MIC > 0.125 mg/L) and 11 displayed reduced susceptibility (CTX-RS, MIC = 0.125 mg/L). There were three clusters of 2 or more NG-MAST STs among these 13 CTX-R isolates: NG-MAST ST3806; ST1407; and ST3158 (Table 4.6). Ten were MLST ST1901 and the correlation with resistance determinants is shown (Table 4.6).

Eleven isolates were CTX-RS which includes one NG-MAST ST1407 cluster (n = 2) and 6 isolates were MLST ST1901 (Table 4.7).

4.5.6 Ertapenem

Seven of the isolates had an ertapenem MIC > 0.032 mg/L. Two of these isolates were NG-MAST1407 and the rest were single STs. Five of these were MLST ST1901 (Table 4.8).

Isolate (Year)	NG- MAST	MLST	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
035 (2016)	NM	1901	0.047	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
286088 (2014)	3806	1901	0.023	0.094	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
66931	3806	1901	0.032	0.125	0.25	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
176561 (2015)	4936	1901	0.016	0.125	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
501373 (2016)	5622	1901	0.032	0.125	0.125	0.023	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
009 (2013)	1407	1901	0.064	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
191262 (2016)	1407	1901	0.023	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422671 (2016)	3158	1901	0.064	0.125	0.25	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422102 (2016)	3158	1901	0.032	0.125	0.38	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
179501 (2015)	11690	1901	0.016	0.094	0.094	0.023	mosaic XXXIV	L421P	A121S	H105Y	A13 del
425204 (2016)	NM	7359	0.016	0.125	0.125	0.032	mosaic XXXIV	WT	WT	A39T	WT
177574 (2015)	2318	13143	0.047	0.094	0.125	0.016	non-mosaic type XIII	L421P	G120K A121D	G45D	A13 del
82432 (2015)	7867	13142	0.023	0.125	0.25	0.032	mosaic XXXIV	L421P	G120K A121D	A39T	A13 del
86606 (2015)	4244	11428	0.008	0.094	0.064	0.047	mosaic XXXIV	WT	WT	A39T R44H	WT

Table 4.5. Molecular characteristics of CFM-RS (n-14) isolates. WT = wild type. Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 – 4.16.

Isolate (Year)	NG- MAST	MLST	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
71324 (2014)	1582	1588	0.047	0.19	0.19	0.094	mosaic type X	L421P	G120K A121G	A39T ISC	WT
016 (2013)	11645	1901	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
286088 (2014)	3806	1901	0.023	0.094	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
68931 (2014)	3806	1901	0.032	0.125	0.25	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
76972 (2014)	3431	1901	0.047	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
176561 (2015)	4936	1901	0.016	0.125	0.19	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
024 (2012)	1407	1901	0.094	0.25	0.38	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
194698 (2016)	1407	1901	0.016	0.064	0.19	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422671 (2016)	3158	1901	0.064	0.125	0.25	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422102 (2016)	3158	1901	0.032	0.125	0.38	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
178526 (2015)	8953	1901	0.032	0.19	0.25	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
168628 (2016)	2318	7827	0.064	0.064	0.25	0.012	non-mosaic type XIII	L421P	G120K A121D	G45D	A13 del
82432 (2015)	7867	NM	0.023	0.125	0.25	0.032	mosaic XXXIV	L421P	G120K A121D	A39T	A13 del

Table 4.6. Molecular characteristics of CTX-R (n = 13) isolates. WT = wild type. Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 – 4.16.

Isolate (Year)	NG- MAST	MLST	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
196712 (2016)	649	1580	0.008	0.016	0.125	0.023	non-mosaic type II	WT	A121S	G45D	WT
035 (2016)	NM	1901	0.047	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
501373 (2016)	5622	1901	0.032	0.125	0.125	0.023	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
009 (2013)	1407	1901	0.064	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
191262 (2016)	1407	1901	0.023	0.125	0.125	0.032	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
179501 (2015)	11690	1901	0.016	0.094	0.094	0.023	mosaic XXXIV	L421P	A121S	H105Y	A13 del
81513 (2015)	2212	1901	0.032	0.19	0.125	0.064	mosaic XXXIV	L421P	G120K A121D	H105Y	A13 del
425204 (2016)	NM	7359	0.016	0.125	0.125	0.032	mosaic XXXIV	WT	WT	A39T	WT
197747 (2016)	14449	7363	0.016	0.032	0.094	0.012	non-mosaic typeXLIV	L421P	WT	A39T ISC	A13 del
177574 (2015)	2318	13143	0.047	0.094	0.125	0.016	non-mosaic type XIII	L421P	G120K A121D	G45D	A13 del
166587 (2016)	12302	9363	0.008	0.016	0.125	0.016	non-mosaic type II	WT	G120K A121N	D79N	A13 del

Table 4.7. Molecular characteristics of CTX-RS (n = 11) isolates. WT = wild type. Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 – 4.16.

Isolate (Year)	NG-MAST	MLST	CRO MIC (mg/L)	CFM MIC (mg/L)	CTX MIC (mg/L)	ETP MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> promoter
71324 (2014)	1582	1588	0.047	0.19	0.19	0.094	mosaic type X	L421P	G120K A121G	A39T ISC	WT
024 (2012)	1407	1901	0.094	0.25	0.38	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
194698 (2016)	1407	1901	0.016	0.064	0.19	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
422671 (2016)	3158	1901	0.064	0.125	0.25	0.064	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
81513 (2015)	2212	1901	0.032	0.19	0.125	0.064	mosaic XXXIV	L421P	G120K A121D	H105Y	A13 del
68931 (2014)	3806	1901	0.032	0.125	0.25	0.047	mosaic XXXIV	L421P	G120K A121N	H105Y	A13 del
86606 (2015)	4244	11428	0.008	0.094	0.064	0.047	mosaic XXXIV	WT	WT	A39T R44H	WT

Table 4.8. Molecular characteristics of isolates with an ertapenem MIC > 0.032 mg/L. Isolates

sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 –

4.16.

4.5.7 Azithromycin and solithromycin

Seven isolates had azithromycin MICs > 256 mg/L (HLAzi-R). Three were NG-MAST 649, two NG-MAST ST3311 and the remaining two were single NG-MAST STs (ST2475 and ST1443). All 7 HLAzi-R isolates grouped to MLST ST1580 (Table 4.9). This MLST ST was not found in any other isolates.

Seven isolates had azithromycin MICs of 4-32 mg/L. There were 2 of each NG-MAST ST2992 and ST12302 (all 4 are MLST ST9363). Of these 7 isolates, 4 were MLST ST9363 and two ST7363 (Table 4.10)

Twenty-nine isolates had azithromycin MICs of 0.032-2 mg/L. Sixteen of these 29 isolates were MLST ST1901 with 4 of these grouping to NG-MAST ST1407 (*Appendix 7*).

The next most common MLST ST was ST1588 (n = 3) which included the three isolates with the lowest azithromycin MICs (0.032, 0.064 and 0.125 mg/L).

Isolate (Year)	NG- MAST	MLST	AZM MIC (mg/L)	SOL MIC (mg/L)	<i>mtrR</i>	<i>mtrR</i> promoter	<i>23s rRNA</i>	<i>erm A/B/C/F</i> <i>ereA/B</i> <i>mefA</i> <i>mph A/B/C</i>
006 (2013)	2475	1580	> 256	0.032	G45D	A12C	A2059G (4/4)	-
196712 (2016)	649	1580	> 256	1	G45D	WT	A2059G (4/4)	-
178487 (2015)	649	1580	> 256	0.125	G45D	WT	A2059G (3/4)	-
199856 (2016)	649	1580	> 256	0.5	G45D	WT	A2059G (4/4)	-
193852 (2016)	1443	1580	> 256	0.032	G45D	A13 del	A2059G (2/4)	-
191587 (2016)	3311	1580	> 256	NT	G45D	WT	A2059G (4/4)	-
181798 (2015)	3311	1580	> 256	1	G45D	WT	A2059G (3/4)	-

Table 4.9. Molecular characteristics of 7 HLAzi-R isolates. Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 – 4.16.

Isolate (Year)	NG-MAST	MLST	AZM MIC (mg/L)	SOL MIC (mg/L)	<i>mtrR</i>	<i>mtrR</i> promoter	23s <i>rRNA</i>	<i>erm A/B/C/F</i> <i>ereA/B</i> <i>mefA</i> <i>mph A/B/C</i>
67818 (2014)	2400	1587	6		D79N T86A H105K	A13 del	C2611T (4/4)	-
197747 (2016)	14449	7363	16		ISC	A13 del	C2611T (4/4)	-
425657 (2016)	9184	7363	4		D79N T86A H105K	A13 del	C2611T (4/4)	-
178575 (2015)	2992	9363	32		A39T R44H	WT	C2611T (4/4)	-
41277 (2014)	2992	9363	12		A39T R44H	WT	C2611T (4/4)	-
179411 (2015)	12302	9363	4		D79N	A13 del	WT	-
166587 (2016)	12302	9363	8		D79N	A13 del	WT	-

Table 4.10. Molecular characteristics of 7 isolates with azithromycin MICs 4-32 mg/L. Isolates

sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 –

4.16.

4.5.8 Ciprofloxacin

Seventeen isolates had ciprofloxacin MICs > 32 mg/L. Eleven were MLST ST1901 and 3 and 2 were NG-MAST ST1407 and ST3806, respectively, the remaining isolates were unique NG-MAST STs (Table 4.11)

There were an additional 14 isolates resistant to ciprofloxacin with MICs ranging from 2-16 mg/L. MLST STs for these 14 isolates included ST1901 (n = 5), ST7363 (n = 3), ST8143 and ST9363 (n = 2 each) and ST9903, ST13143 and ST14144 (n = 1 each).

There were 4 NG-MAST STs represented by 2 isolates (ST12302, ST5624, ST2318 and ST3158) and the remaining STs were unique among this group of 14 isolates (Table 4.12).

Twelve isolates were susceptible to ciprofloxacin with MICs ranging from ≤ 0.002 mg/L to 0.032 mg/L. Of these 12 isolates, 7 belonged to MLST ST1580, three to ST9363 and the remaining two were unique STs. Three and 2 of the MLST ST1580 were NG-MAST ST649 and ST3311, respectively. Two of the MLST ST9363 isolates were NG-MAST ST2992.

4.5.9 Gemifloxacin

The gemifloxacin MIC range was ≤ 0.002 -12 mg/L. The 43 isolates formed two groups according to gemifloxacin MIC; those with MICs ≤ 0.002 -0.012 mg/L and those with MICs 0.38-12 mg/L (*Appendix 7 & Tables 4.11 & 4.12*). The group with lower MICs corresponded to the isolates with ciprofloxacin MICs within the susceptible range and the group with higher gemifloxacin MICs corresponded to those isolates resistant to ciprofloxacin.

Isolate (Year)	NG-MAST	MLST	CIP MIC (mg/L)	GEMI MIC (mg/L)	<i>gyrA</i>	<i>parC</i>	<i>mtrR</i>	<i>mtrR</i> promoter
67818 (2014)	2400	1587	> 32	1.5	S91F D95G	E91G	D79N T86A H105K	A13 del
190440 (2016)	3750	1588	> 32	4	S91F A92P D95A	E91K S87K	A39T ISC	WT
71324 (2014)	1582	1588	> 32	3	S91F D95A	E91K S87K	A39T ISC	WT
016 (2013)	11645	1901	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
035 (2016)	NM	1901	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
286088 (2014)	3806	1901	> 32	0.75	S91F D95G	S87R	H105Y	A13 del
68931 (2014)	3806	1901	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
76972 (2014)	3431	1901	> 32	2	S91F D95G	S87R	H105Y	A13 del
176561 (2015)	4936	1901	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
024 (2012)	1407	1901	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
009 (2013)	1407	1901	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
191262 (2016)	1407	1901	> 32	1.5	S91F D95G	S87R	H105Y	A13 del
422671 (2016)	3158	1901	> 32	2	S91F D95G	S87R	H105Y	A13 del
422102 (2016)	3158	1901	> 32	2	S91F D95G	S87R	H105Y	A13 del
81513 (2015)	2212	1901	> 32	2	S91F D95G	S87R	H105Y	A13 del
178526 (2015)	8953	1901	> 32	1	S91F D95G	S87R	H105Y	A13 del
82432 (2015)	7867	13142	> 32	6	S91F D95A	D86N	A39T	A13 del

Table 4.11. Phenotypic and molecular characteristics of 17 isolates with ciprofloxacin MIC > 32 mg/L. WT = wild type. Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 – 4.16.

Isolate (Year)	NG-MAST	MLST	CIP MIC (mg/L)	GEMI MIC (mg/L)	<i>gyrA</i>	<i>parC</i>	<i>mtrR</i>	<i>mtrR</i> promoter
420132 (2016)	NM	13144	2	0.5	S91F D95G	E91G	A39T	WT
501373 (2016)	5622	1901	16	2	S91F D95A	S87R	H105Y	A13 del
194698 (2016)	1407	1901	16	3	S91F D95G	S87R	H105Y	A13 del
179501 (2015)	11690	1901	3	0.75	S91F D95G	S87R	H105Y	A13 del
193058 (2016)	NM	7363	8	12	S91F D95G	E91G S87I	D79N T86A H105K	A13 del
197747 (2016)	14449	7363	2	2	S91F D95G	E91G	A39T ISC	A13 del
425657 (2016)	9184	7363	16	1.5	S91F D95G	E91G	D79N T86A H105K	A13 del
177574 (2015)	2318	13143	8	1	S91F D95G	D86N	G45D	A13 del
168628 (2016)	2318	7827	2	0.38	S91F D95G	D86N	G45D	A13 del
422322 (NA)	5624	8143	2	0.5	S91F D95A	D86N	A39T ISC	WT
008 (2013)	5624	8143	3	0.75	S91F D95A	D86N	H105Y	A13 del
166587 (2016)	12302	9363	4	2	S91F D95A	D86N	D79N	A13 del
179411 (2015)	12302	9363	12	1	S91F D95A	D86N	D79N	A13 del
039 (2015)	NM	9903	4	1.5	S91F D95A	S87N E91Q	D79N T86A H105K	WT

Table 4.12. Phenotypic and molecular characteristics of 14 isolates, ciprofloxacin MIC 2-16 mg/L.

Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 – 4.16.

4.5.10 Penicillin resistance related to β -lactamase production

Of the 43 isolates sequenced 9 (20.9%) were resistant to penicillin (MIC > 1 mg/L). Three isolates had MICs > 32 mg/L and two of these isolates were NG-MAST ST 5624 and MLST ST8143, the other isolate had no match NG-MAST type and was MLST ST13144, a novel ST (Table 4.13).

A further 6 of the 43 isolates were also resistant to penicillin with MICs of 2 mg/L (n = 4) or 4 mg/L (n = 2). There were 6 different NG-MAST STs for these 6 isolates (Table 4.14).

4.5.11 Tetracycline

The tetracycline MIC range was 0.125-32 mg/L, with 6 isolates resistant (MIC > 1 mg/L), 18 susceptible (MIC $\leq$ 0.5 mg/L) and the remainder, 19, were intermediate (*Appendix 7*). Three isolates had MICs of 8 mg/L, 24 mg/L and 32 mg/L for tetracycline and they belonged to MLST ST1588 (n = 2), ST9903 (n = 1) and three single NG-MAST STs (Table 4.15). In the current study the three *tetM*-positive gonococci were three different NG-MAST STs and two belonged to MLST ST1588. Similarly, a study from Switzerland found 9 different NG-MAST STs among the 9 *tetM*-positive gonococci examined (Endimiani *et al.*, 2014)

Isolate (Year)	NG-MAST	MLST	Penicillin MIC (mg/L)	BL	<i>penA</i>	<i>ponA</i>	<i>penB</i>	TEM	<i>mtrR</i>	<i>mtrR</i> promoter
420132 (2016)	NM	13144	> 32	+	non-mosaic type II	WT	WT	TEM-75	A39T	WT
422322 (NA)	5624	8143	> 32	+	non-mosaic type II	WT	G120D A121G	TEM-141	A39T	WT
008 (2013)	5624	8143	> 32	+	non-mosaic type II	WT	G120D A121G	TEM-141	H105Y	A13 del

Table 4.13. Resistant determinants for three isolates with penicillin MIC > 32 mg/L. Isolates

sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 –

4.16.

Isolate (Year)	NG-MAST	MLST	Penicillin MIC (mg/L)	BL	<i>penA</i>	<i>ponA</i>	<i>penB</i>	TEM	<i>mtrR</i>	<i>mtrR</i> promoter
190440 (2016)	3750	1588	4	+	non-mosaic type XIX	L421P	G120K A121G	TEM-198	A39T ISC	WT
71324 (2014)	1582	1588	4	+	mosaic type X	L421P	G120K A121G	TEM-198	A39T ISC	WT
016 (2013)	11645	1901	2	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13
76972 (2014)	3431	1901	1.5	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13
024 (2012)	1407	1901	1.5	-	mosaic XXXIV	L421P	G120K A121N	-	H105Y	A13
039 (2015)	Novel	9903	1.5	+	non-mosaic type XIV	WT	G120K A121N	TEM-141	D79N T86A H105K	WT

Table 4.14. Resistance determinants for isolates with penicillin MIC > 1-4 mg/L. Isolates sharing

similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 – 4.16.

Isolate (Year)	NG- MAST	MLST	TET MIC (mg/L)	<i>tetM</i>	<i>penB</i>	<i>mtrR</i>	<i>mtrR</i> <i>promoter</i>	<i>rpsJ</i>
190440 (2016)	3750	1588	32	tetM	G120K A121G	A39T ISC	WT	V57M
71324 (2014)	1582	1588	24	tetM	G120K A121G	A39T ISC	WT	V57M
039 (2015)	NM	9903	8	tetM	G120K A121N	D79N T86A H105K	WT	V57M

Table 4.15. Molecular characteristics of isolates with tetracycline MIC values of 8-32 mg/L.

Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables

4.1 – 4.16.

4.5.12 Spectinomycin

There was no resistance to spectinomycin and the NG-MAST and MLST STs are as discussed above (*Appendix 7*).

4.5.13 Gentamicin

According to the proposed clinical breakpoints (Brown *et al.*, 2010) there were 6 gonococci in this study which were intermediate to gentamicin. These isolates had 6 different NG-MAST STs. Three (50%) were MLST ST1901 and the remaining three were single STs (ST13144, ST8143 and ST9363), but this likely represents normal variability among the isolates present.

4.5.14 Fosfomycin

While there are no clinical interpretive criteria for fosfomycin in relation to *N. gonorrhoeae*, there were 4 sequenced isolates with fosfomycin MICs > 32 mg/L, the EUCAST clinical resistance breakpoint for *Enterobacteriaceae*. Three of these were NG-MAST ST1407 and MLST ST1901, while the remaining isolate had no NG-MAST ST match and was MLST ST9903.

4.5.15 MDR and XDR isolates

Five of the 7 MDR/XDR isolates belonged to MLST ST1901 and there were seven different NG-MAST STs, including one ST1407 (Table 4.16).

Isolate (Year)	NG- MAST	MLST	MIC (mg/L)														BL	penA	ponA	penB	TEM	gyrA	parC	mtrR	mtrR promoter	tet M	rpld	23s rRNA
			PEN	CIP	AZM	CRO	CFM	TET	CTX	ETP	CN	GEMI	SPC	FOS	SOL													
71324 (2014)	1582	1588	4	> 32	0.125	0.047	0.19	24	0.19	0.094	0.5	3	6	6	NT	+	mosaic type X	L421P	G120K A121G	TEM- 198	S91F D95A	E91K S87K	A39T ISC	WT	tet M	WT	C2611T (1/4)	
82432 (2015)	7867	13142	0.75	> 32	1	0.023	0.125	1	0.25	0.032	6	6	12	24	0.19	-	mosaic XXXIV	L421P	G120K A121D	-	S91F D95A	D86N	A39T	A13 del	-	WT	WT	
422102 (2016)	3158	1901	1	> 32	1	0.032	0.125	1	0.38	0.032	8	2	16	32	0.094	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT	
024 (2012)	1407	1901	1.5	> 32	0.38	0.094	0.25	1	0.38	0.064	4	1.5	8	48	NT	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT	
178526 (2015)	8953	1901	1	> 32	1	0.032	0.19	0.75	0.25	0.032	6	1	8	16	0.032	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT	
016 (2013)	11645	1901	2	> 32	1	0.047	0.19	1	0.25	0.032	6	1.5	12	32	0.094	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT	
76972 (2014)	3431	1901	1.5	> 32	0.75	0.047	0.19	1	0.25	0.032	4	2	12	32	NT	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	H105Y	A13 del	-	WT	WT	

Table 4.16. Phenotypic and molecular characteristics of MDR (n = 5, isolates 71324, 82432, 422102, 024 and 178526) and XDR isolates (n = 2, isolates 016 and 76972). NT = not tested. Isolates sharing similar MLSTs or NG MASTs are highlighted in specific colour throughout Tables 4.1 – 4.16.

4.6 Whole genome sequencing

4.6.1 Phylogeny of Irish *N. gonorrhoeae* isolates

To provide insight into the genetic relationship between our sequenced isolates, a phylogenetic tree based on whole genome sequencing data was constructed. Core variant sites from 58 sequenced strains identified with respect to the NCCP11945 reference genome were used as the basis for delineating strain phylogeny as illustrated (Figure 4.1).

Whole genome sequence phylogeny revealed isolates NGSJH11, 181798, 191587 and 178487 belonged to the same MLST group (ST-1580) while differing by <5 Single nucleotide variants at the whole genome level (Figure 4.1). This suggests potential linked transmission sources or the recent emergence of this ST1580 subtype in this region. The ST1580 isolates were distinguished by markedly higher resistance to azithromycin exhibiting the highest observed MICs of any MLST group (Figure 4.1).

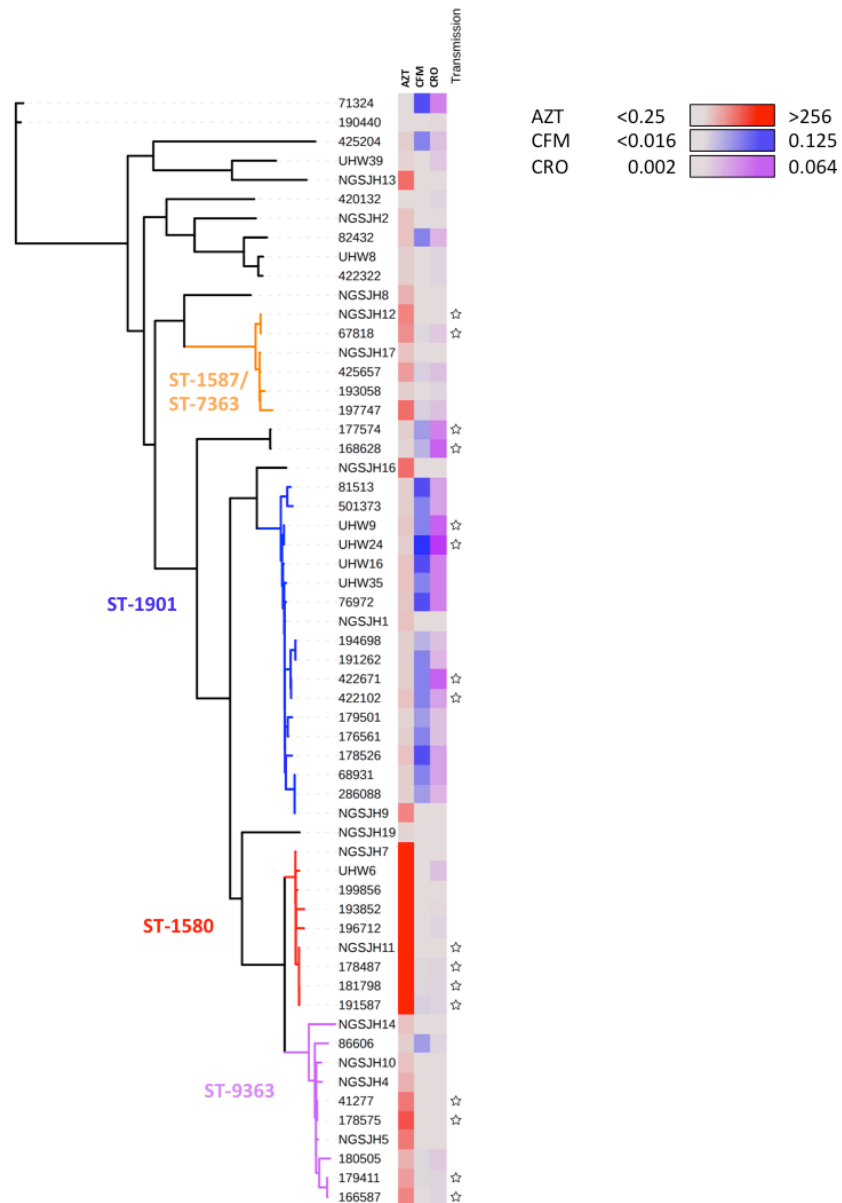


Figure 4.1. Phylogentic analysis of Irish *N. gonorrhoeae* isolates illustrating resistance to azithromycin (AZM), ceftriaxone (CRO) and cefixime (CFM) among identified clades. The most prevalent ST groups are identified by colour coding along with the antibiotic resistance level which is also colour-coded with respect to observed MIC levels as indicated in the colour scale to the right of the tree. Highly-related strains (<5 identified SNV differences) are highlighted by a star.

Isolates UHW24 and UH9 belonged to ST-1901 and were genetically indistinguishable (0 SNV differences) pointing to a shared source of transmission, while isolates 4226171 and 422102 (belonging to ST-1901) were too indistinguishable from each other though distinct from UH24 and UH9 (differing by 166 observed SNVs). Thus, whole genome sequence analysis revealed two potential transmission events within the ST1580 clade. The ST1580 MLST was also distinguished by reduced antibiotic susceptibility, in this case, to cephalosporin antibiotics. Isolates exhibiting CFM-R, CFM-RS and CRO-RS were observed in this clade with no high-level resistance to cephalosporins or azithromycin observed (Figure 4.1). Other ST groups where potential transmission events were supported included ST1587/ST7363 and ST9363 clades, where genetically-related isolates were observed while the two identified as ST7827 and ST13143 differed by only 11 SNVs. Overall, the largest potential transmission network of isolates ($n = 4$) was observed among ST1580 isolates which exhibited high-level azithromycin resistance.

Comparison of Irish ST1580 isolates in the current study were compared to other high-level azithromycin resistant ST1580 strains from an outbreak in the United Kingdom (European Nucleotide Archive accession PRJEB14933) to establish potential international spread of HLAzi-R ST1580 strains. Whole genome sequencing data from a previous report of highly resistant strains in the UK (PMID: 26601852) were compared to the ST1580 strains identified in Ireland (Figure 4.2).

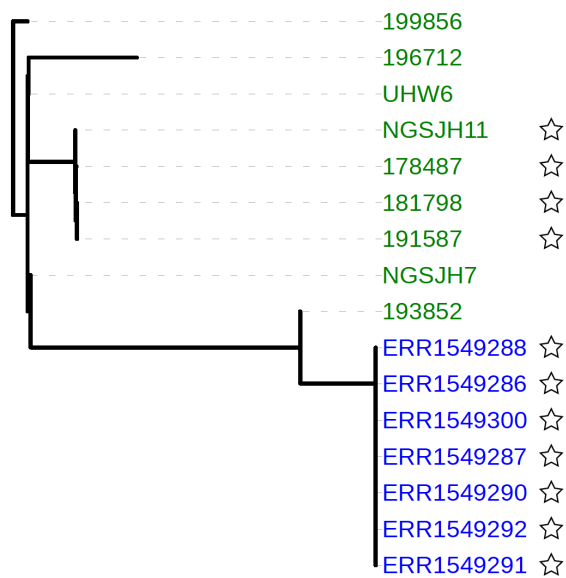


Figure 4.2. Whole-genome sequence comparison of Irish and UK HLAzi-R ST1580 strains. Irish and UK strains are indicated in green and blue font respectively. Stars indicate putative transmission events within specific clades based on observed SNV differences of < 5 among isolates. While geographically contained clonal clusters are observed, a single Irish isolate (193852) showed some similarity with the UK outbreak strains, but differed by > 160 SNVs.

4.7 Discussion

4.7.1 NG-MAST and MLST STs related to phenotypic resistance and resistance determinants: Extended-spectrum cephalosporins and ertapenem

4.7.1.1 CRO-RS

Of the 7 NG-MAST STs found in these 9 isolates, ST1407 (n = 2) is widely reported to be associated with reduced susceptibility and resistance to ESCs (Horn *et al.*, 2014; Shimuta *et al.*, 2013; Nguyen *et al.*, 2014; Buono 2012 MDR) and some high-level CRO-R isolates were NG-MAST ST1407 (Cámara *et al.*, 2012; Unemo *et al.*, 2011c). The second ECDC molecular typing study found NG-MAST ST1407 to be present in all of the 21 participating EU countries except Ireland and Iceland and it was the predominant ST in 6 countries (29%) (ECDC 2017b). The three *penA* allele types seen in these 9 CRO-RS isolates correlated with MLST ST1588 (mosaic X), ST1901 (mosaic XXXIV) and ST7827 (non-mosaic XIII). Similarly the *penB*, *mtrR* and *mtrR* promoter resistance determinants correlated well with MLST STs. As mentioned previously MLST STs are less frequently reported in publications than NG-MAST STs; however MLST ST1901 is associated with reduced susceptibility to ESCs (Shimuta *et al.*, 2013; Shimuta *et al.*, 2015; Fifer *et al.*, 2016), ceftriaxone treatment failures (Read *et al.*, 2013; Unemo *et al.*, 2012c; Fifer *et al.*, 2016) and it is the predominant MLST ST in Europe (ECDC 2017b).

4.7.1.2 CFM-R/RS, CTX-R/RS, ETP-RS: *penA* alleles

NG-MAST STs are highly diversified within each phenotypic group, but for each of these phenotypic groups MLST ST1901 is the most prevalent ST accounting for

approximately 55-83% of isolates in each group. The other STs are mostly single STs. ST7363 is reported to be associated with elevated ESC MICs including those with MICs to ceftriaxone of 0.5 mg/L and high-level ceftriaxone-resistant isolate, H041 (Shimuta *et al.*, 2013; Shimuta *et al.*, 2015; Deguchi *et al.*, 2016; Lahra *et al.*, 2014; Ohnishi *et al.*, 2011b).

4.7.1.3 Remaining 18 ESC fully susceptible isolates: *penA* alleles

Eighteen isolates had CRO/ETP MIC ≤ 0.032 and CFM/CTX MIC < 0.125 mg/L. There were 14 NG MAST STs in these 18 isolates, none of which are notably reported to be associated with elevated ESC MICs. These 18 isolates belonged to 8 different MLST STs with the two most frequently seen STs being ST1580 (n = 6) and ST9363 (n = 4), all of which had non-mosaic II *penA* alleles and are not reported to be associated with reduced susceptibility to ESCs. MLST ST7363 was seen in two isolates (non-mosaic XLIV *penA* alleles) which as mentioned above has been reported in association with ceftriaxone resistance (Shimuta *et al.*, 2015; Deguchi *et al.*, 2016; Lahra *et al.*, 2014; Ohnishi, 2011) and is the third most frequently occurring MLST ST in Europe (ECDE 2017b). There are no MLST ST1901s among these 18 ESC fully susceptible isolates.

4.7.2 Azithromycin and solithromycin

4.7.2.1 High-level azithromycin-resistant *N. gonorrhoeae*

As described above, seven of the 43 isolates sequenced were HLAzi-R (MIC > 256 mg/L). Four NG-MAST STs were found in the HLAzi-R isolates. Notably NG-MAST

ST649 has been found in HLAzi-R isolates reported from Ireland (Lynagh *et al.*, 2015), Australia (Stevens *et al.*, 2015), USA (Katz *et al.*, 2012), Argentina (Galarza *et al.*, 2010), England (Chisholm *et al.*, 2009) and Scotland (Palmer *et al.*, 2008), but in general there are a number of different STs reported in HLAzi-R isolates (Table 1.4) as was found in this study.

MLST ST1580 accounted for all of the HLAzi-R isolates and this ST was not seen in any other isolates in the study. Unfortunately MLST STs were not determined for the internationally reported HLAzi-R isolates (Table 1.3), except for the Irish isolates from 2008 and 2014 which were also ST1580 (Lynagh *et al.*, 2015).

4.7.2.2 Medium-level azithromycin-resistant *N. gonorrhoeae*

Seven isolates (MIC 4-32 mg/L) may be considered medium level resistant to azithromycin. These isolates had 5 different NG-MAST STs including ST2992 (n = 2) which is reported to be the predominant NG-MAST ST in Ireland and the second most common NG-MAST ST in Europe (Chisholm *et al.*, 2013; ECDC 2017b). Wind *et al.* also report NG-MAST ST2992 as the predominant ST in Amsterdam out of a collection of 31 isolates, accounting for 23% (7/31) (Wind *et al.*, 2017a). There is one other NG-MAST cluster which is ST 12302 (n = 2) which is not common in Europe (ECDC 2017b). MLST revealed 3 STs in this groups of isolates, ST9363 (n = 4), ST7363 (n = 2) and ST1587 (n = 1). Again Wind *et al.* report MLST ST9363 as the predominant ST (8/31, 26%) in the aforementioned study (Wind *et al.*, 2017a). MLST ST7363 is the third most common MLST ST in Europe (ECDC 2017b) and as mentioned previously, has been associated with high level ceftriaxone resistance (Table 1.2).

4.7.2.3 Low-level azithromycin-resistant and azithromycin-susceptible *N. gonorrhoeae*

The remaining 29 isolates (AZM MICs 0.032-2 mg/L) were susceptible, intermediate or low-level resistant to azithromycin. In this group of 29 isolates there were 20 different NG-MAST STs including three for which there was no match. NG-MAST typically results in a high number of STs in most groups of gonococcal isolates studied, e.g. in Scotland out of 3326 isolates 642 STs were found (Palmer *et al.*, 2008) and in China out of 77 isolates 67 different NG-MAST STs were identified (Liang *et al.*, 2016) with numerous novel ST reported. Regarding MLST, as mentioned 16 isolates (55%) were ST1901, the predominant ST and there were two clusters of ST1588 (n = 2) and ST8143 (n = 2) and there were 9 single STs. Again there is little published on MLST in *N. gonorrhoeae*. MLST ST1901 has been discussed above. ST1588 is a frequently occurring ST in Europe, accounting for almost 6% (61/1054) of isolates from EURO-GASP 2013 (ECDC 2017b). Of the single STs, ST9363 and ST7363 have been discussed above.

4.7.3 Ciprofloxacin and gemifloxacin

The predominant NG-MAST type among all 31 ciprofloxacin resistant isolates was ST1407 (n = 4) which is reported to be strongly associated with ciprofloxacin resistance (ECDC 2017b). There were 19 different NG-MAST STs (plus 4 which had no match) and 11 different MLST STs among the 31 ciprofloxacin resistant isolates sequenced. One MLST ST (ST1901) accounted for 16 of these isolates and, as mentioned is the predominant MLST ST in Europe (Jacobsson *et al.*, 2016; ECDC 2017b). The majority (76%) of the high-level ciprofloxacin resistant isolates (MIC > 32 mg/L) were MLST

ST1901, whereas among the isolates with lower level resistance to ciprofloxacin (MIC 2-16 mg/L) there were four MLST ST clusters of 2-3 isolates (Table 4.11 & 4.12). Among the 12 ciprofloxacin susceptible isolates there were 8 NG-MAST STs, including ST649 (n = 3), ST2992 (n = 2) and ST3311 (n = 2) and 4 MLST STs (including 7 ST1580 and 3 ST9363).

4.7.4 Penicillin resistance and β -lactamase

The NG-MAST and MLST types that were seen in the isolates with penicillin MICs > 1 mg/L correlated with the level of resistance (Tables 4.14 & 4.15). NG-MAST ST5624 (MLST ST 8143) represents 2/3 high-level penicillin-resistant isolates and MLST ST1588 and ST1901 appear to group isolates according to their resistance determinants including β -lactamase type, as well as penicillin MIC (Table 4.14 & 4.15). Of the NG-MAST STs found in these isolates, ST1407 has been discussed above and ST1582 has been reported to be associated with a cluster of PPNG gonococci in France (Micaëlo *et al.*, 2017). In that study the biggest clusters of PPNG belonged to ST1479 (18/176, 10.2%) and ST1582 (15/176, 8.5%) (Micaëlo *et al.*, 2017). ST1479 was not present in the isolates in this study.

4.7.5 Tetracycline

In this study the three *tetM*-positive gonococci were three different NG-MAST STs and two belonged to MLST ST1588. Similarly, a study from Switzerland found 9 different NG-MAST STs among the 9 *tetM*-positive gonococci examined (Endimiani *et al.*,

2014). In Korea, Lee *et al.* also found that 15 high-level tetracycline-resistant *N. gonorrhoeae* isolates (2010-2011) belonged to 15 different STs (Lee *et al.*, 2016).

4.7.6 Spectinomycin

The NG-MAST and MLST STs were diverse for the spectinomycin-susceptible gonococci in this study. As spectinomycin is rare, there are limited reports regarding STs associated with resistance. Unemo *et al.* reported a *N. gonorrhoeae* strain resistant to spectinomycin (MIC > 1024 mg/L) due to mutations in ribosomal protein S5, as discussed (*Section 3.6.6*) (Unemo *et al.*, 2013). This isolate was NG-MAST ST918, an ST which is not present in the isolates in this study. Ilina *et al.* also reported a spectinomycin-resistant strain from Russia which also had a ribosomal protein S5 mutation and belonged to NG-MAST ST5187, an ST which again was not detected among the isolates in this study.

4.7.7 Gentamicin

There was no clear association with STs and the 6 isolates with the highest gentamicin MICs (8-16 mg/L, intermediate according to Brown *et al.*) in this study (Brown *et al.*, 2010). There is little resistance to gentamicin reported (Brown *et al.*, 2010; Chisholm *et al.*, 2011), hence there are no published reports correlating gentamicin MICs and NG-MAST or MLST STs.

4.7.8 Fosfomycin

Four isolates had fosfomycin MICs > 32 mg/L, but no resistance determinants specific to fosfomycin were detected in these or any other isolates in this study. Again there are no publications relating to fosfomycin resistance and molecular typing.

4.7.9 MDR and XDR isolates

Five of the 7 MDR/XDR isolates belonged to MLST ST1901, which is prevalent worldwide and associated with resistance to ESCs and ciprofloxacin in particular. NG-MAST STs among these 6 isolates were 6 different STs including ST1407, which is again reported to be a successful clone worldwide associated with resistance, as discussed above.

4.7.10 Sequence types and antimicrobial resistance associations

The 43 isolates belonged to 31 NG-MAST STs, including 5 for which there was no ST match. There were 14 MLST STs including four novel STs. The most prevalent MLST STs are ST1901 (n = 16), ST1580 (n = 7) and ST9363 (n = 5). These three groups, in particular ST1901 and ST1580, correlated quite well according to the presence or absence of individual resistance determinants (*Appendix 7*). All ST1901 isolates were mosaic XXXIV *penA* and have identical mutations in *gyrA*, *parC*, *ponA*, *mtrR*, *mtrR* promoter and almost all have G120K plus A121N (n = 14) mutations in *penB*. Similarly, the ST1580 group isolates all had identical *penB*, *penA*, *gyrA*, *parC*, 23s rRNA and *mtrR*. The *mtrR* promoter and *ponA* had slight variations. A similar trend is seen for most MLST ST groups of isolates (*Appendix 7*). Critically, the 23s rRNA

A2059G allele is seen only in the ST1580 lineage, exhibiting high-level azithromycin resistance.

4.7.11 Phylogenetic analysis

Whole-genome analysis confirmed the most prevalent *N. gonorrhoeae* strain types in our study. These included the ST1580, ST9396, and ST1901 MLST lineages as well as the genetic diversity within these genetic clades. Four novel MLST STs were identified among the isolates tested (ST13142, ST13143, ST13144 and ST11428). Jacobsson *et al.* report NG-MAST ST2992 and MLST ST1901 to be the predominant STs out of 75 EURO-GASP isolates from 17 countries (2009-2014) (Jacobsson *et al.*, 2016), but in a study on a larger number of European isolates from 2013 (n = 1189) NG-MAST ST1407 and MLST ST1901 were the predominant STs, as mentioned previously. While significant genetic diversity was seen at the sub-strain level, a number of closely related isolates were observed suggesting putative transmission or shared sources, or, alternatively, the more recent emergence of these strains in this region. This highlights the potential of genomic sequencing approaches to track disease transmission at a fine-structure level. While this was not the primary focus of this study, larger and more systematic application of whole-genome sequencing in Ireland could better elucidate the emergence of *N. gonorrhoeae* strains at a national level. In terms of international surveillance, the portability of genomic data is also noteworthy. Here, we exploited this in the comparison of our highly resistant ST1580 strains with those from an outbreak in the UK. This highlighted the significant differences identified between the HLAzi-R ST1580 strains of the UK outbreak and those in this study. Thus, genomic data can be

integrated at scale to provide rapid comparisons of disparate databases toward greater international surveillance of disease.

4.8 Limitations

The analysis of clonal relatedness of isolates was limited by the number of isolates whole genome sequenced. Had all 609 isolates been sequenced many clusters of related isolates may have been seen.

4.9 Conclusion

This is the largest genomic study of Irish *N. gonorrhoeae* performed to date. Specific clones associated with resistance that are successful globally have been reported. NG-MAST ST1407 (MLST ST1901) is one of these, associated with both azithromycin and ESC resistance. Four isolates (MLST ST1901) in this study belonged to NG-MAST ST1407, one of which was an MDR strain (Tapsall *et al.*, 2009c). They displayed either reduced susceptibility or resistance to one or all of ceftriaxone, cefixime, cefotaxime and ertapenem. All four had azithromycin MICs ≤ 0.75 mg/L. In terms of MLST, ST1901 is an internationally successful clone which was prevalent in the isolates in this study (16/43) and particularly associated with elevated ciprofloxacin, gemifloxacin and ESC MICs. NG-MAST STs among the 43 isolates were numerous and diverse. High-level resistance to azithromycin was observed only in isolates belonging to MLST ST1580, all of which harboured the 23s rRNA A2059G. As observed in the UK outbreak of ST1580 HLAzi-R strains, some clonal clustering was observed, however, Irish and UK strains differed significantly when analysed at the

whole-genome level. This work highlights the advantages and potential of whole-genome sequencing to be applied at scale in the surveillance of antibiotic resistant strains of *N. gonorrhoeae*, both locally and internationally.

Chapter V

General Discussion

&

Conclusions

5.1 Introduction

This is the first extensive National Study of *N. gonorrhoeae* in Ireland. It provides an overview of the current situation in Ireland in relation to antimicrobial susceptibility, resistance determinants and molecular epidemiology of the isolates examined and allows comparison to international data.

5.2 Demographics

The isolates selected for this study represent gonococcal infections from many areas in Ireland including East, South-East, Mid-West and West of Ireland. Isolates included ranged from 2012 to 2016, but the majority were from 2014-2016. In Ireland in the latter 3 years 1302, 1320 and 1957 confirmed cases of gonorrhoea were reported in 2014, 2015 and, 2016, respectively. This means that the isolates in this study represent approximately 13% of the reported cases for that period.

In 2014 (83%), 2015 (83%) and 2016 (87%), the majority cases of gonorrhoea reported to the Health Protection Surveillance Centre (HPSC) occurred in male patients. The current study found a male preponderance of 90.8%, similar to the national picture.

The median age and age range in this study are similar those reported in 2014-2016 by the HPSC; median ages of 27 years and a range of 14-75. The proportion of samples from STI clinics / hospital outpatient clinics (STI/GUIDE) reported by the HPSC is 52-62.6% for 2014-2016, while in this study 84.7% of samples came from STI or GUIDE clinics. This may mean that data from this study is not fully representative of the general Irish population; however it is a massive step forward in terms of antimicrobial

surveillance of *N. gonorrhoeae*, a drug-resistant organism that is classified as an urgent threat to public health by the CDC.

5.3 Antimicrobial susceptibility

Susceptibility profiles of *N. gonorrhoeae* isolates are reported for 13 antimicrobials, including the not yet licensed novel agent, solithromycin. The results show that most of the agents previously used in the treatment of gonorrhoea (penicillin, ciprofloxacin and tetracycline) are still no longer suitable for empiric monotherapy given the high resistance rates shown, while others (spectinomycin) may be useful still. Non-susceptibility rates of the tested isolates to penicillin (98.2%), ciprofloxacin (33%) and tetracycline (23.7%), while lower than reported in some other countries, confirm that these three isolates no longer should be considered for empiric therapy of gonorrhoea. Spectinomycin resistance is not detected in the isolates in this study and as discussed above, is vanishingly rare in most countries, however the limited availability of this antibiotic is an issue and concerns of selection pressure if it is introduced make one cautious about celebrating the lack of resistance found.

5.3.1 Azithromycin and extended-spectrum cephalosporins

The current first-line agents, azithromycin and ceftriaxone have differing pictures. The azithromycin non-susceptibility rate of 44 % includes intermediate (25.5%), resistant (17.4%) and HLAzi-R (1.1%) isolates. Higher levels of resistance as well as isolates with HLAzi-R are not suitable for treatment with azithromycin monotherapy, but often infections caused by these strains will have been treated with azithromycin given in

combination with ceftriaxone empirically prior to determination of the MIC. Many will have respond to the ESC portion of the dual therapy, but test of cure in these patients is important to ensure eradication, as well as public health involvement to ensure comprehensive contact tracing.

In contrast to azithromycin, 100% of the isolates in this study are ceftriaxone susceptible. However, 11 (1.8%) displayed reduced susceptibility. While this may seem like small proportion, the concern is that this will lead to resistance following the trends seen in the WHO Western Pacific Regions, where historically most resistance in *N. gonorrhoeae* has emerged and spreads worldwide by means of travel and sex tourism (Unemo *et al.*, 2012a; Tapsall *et al.*, 2009b). The emergence of high level resistance to ceftriaxone (H041, F89 and the two Spanish isolates), as discussed (*Section 1.8.1*), is concerning, but fortunately dissemination of these isolates appears not to have occurred, perhaps due to fitness cost associated with the resistance. Even still, surveillance for such isolates and preparedness for widespread ESC resistance has to be a priority, as recommended by the CDC and WHO.

Trends in resistance/reduced susceptibility to azithromycin and ceftriaxone appear favourable in the set of isolates examined in this study. The proportion of azithromycin susceptible isolates increased in 2016 compared to 2015 and 2014, and the proportion of HLAzi-R remained stable in 2015 and 2016. Ceftriaxone reduced susceptibility also remained stable in 2015 and 2016, with a slight decrease from 2014.

Cefixime and cefotaxime resistance/reduced susceptibility are higher than that of ceftriaxone and means that empiric monotherapy with either of these agents should be avoided. Their role in the future treatment of gonorrhoea may be limited considering that if a cephalosporin is safe to use (i.e. no allergy) then ceftriaxone is the more

appropriate choice and if ceftriaxone treatment failure or resistance has been detected, then cefixime and cefotaxime will likely have proportionally higher MICs as seen in this study.

5.3.2 MDR and XDR *N. gonorrhoeae*

The number of MDR and XDR *N. gonorrhoeae* (Tapsall *et al.*, 2009c) detected in this set of isolates is small and it was not possible to assess trends from year to year, nor was it possible to compare the figures to international reports as the definition varies substantially. It is concerning that these 7 MDR (including 2 XDR) strains were dispersed over each year (2012-2016) from three major cities in Ireland. Given that the isolates included here represented approximately 13% of the reported cases, are these 7 isolates merely the tip of the iceberg?

5.4 Antimicrobial resistance determinants

There is good correlation between the previously described genetic mutations and the phenotypic susceptibility category for most of the antimicrobials above and *N. gonorrhoeae*. Some resistance determinants are specific for particular antimicrobials while others cause reduced susceptibility for many antimicrobials. As evident in the results from this study, often the highly resistant phenotypes will, in particular, harbour specific resistance determinants, including plasmid-encoded β -lactamases or *tetM* for penicillin or tetracycline resistance, respectively, A2059G mutations in 23S rRNA for azithromycin resistance and S91F mutations in *gyrA* for ciprofloxacin resistance. For

most antimicrobials, lower levels of resistance are often associated less definitively with specific mutations.

5.4.1 Azithromycin

This study showed good correlation between mutations in the 23s rRNA alleles, both A2059G and C2611TT, with phenotypic level of resistance to azithromycin, which may not be an ideal drug for treating gonorrhoea, regardless of whether it possesses resistance determinants or not. The long half-life of azithromycin means there is a tailing-off period of reducing serum concentrations as the drug is expelled, which may result in a selective pressure and development of resistance. It has been shown that gonococci with 1 out of 4 23s rRNA alleles with an A2059G mutation, if exposed to erythromycin will acquire the mutation in all 4 alleles and then display high-level resistance to azithromycin (Chisholm *et al.*, 2010). Wind *et al.* report that patients with recent exposure to azithromycin (within 30 days) had *N. gonorrhoeae* azithromycin MICs 2.7-fold higher than isolates from unexposed patients (Wind *et al.*, 2017a). Also A39T or G45D amino acid substitutions in *mtrR* were significantly more frequently seen in isolates from azithromycin-exposed patients compared to non-exposed (Wind *et al.*, 2017a). Despite the shortcomings of azithromycin, it remains one of the two drugs in use for first-line empiric therapy for gonorrhoea and must remain so until improved or alternate drugs are available.

5.4.2 Extended-spectrum cephalosporins

Ceftriaxone is the other first-line empiric antimicrobial for treating gonorrhoea and as discussed above *N. gonorrhoeae* has developed resistance to it also. The main resistance determinant affecting ceftriaxone and which has an even greater effect on cefixime and cefotaxime is a mosaic *penA* allele and in particular certain amino acid substitutions within it. The individual effect of many resistance determinants, including specific amino acid substitutions have been evaluated by transformation experiments to assess their contribution to resistance. These experiments are essential to allow compilation of a comprehensive catalogue of resistance determinants, so that should WGS be introduced in routine work in microbiology laboratories, MIC prediction from gonorrhoea-positive samples would be possible.

It is clear from this study and others mentioned above that ceftriaxone, cefixime and cefotaxime are not equally subject to the same degree of resistance conferred by an individual resistance determinant. Differences in structure of each of these antimicrobials is thought to account for this given that ceftriaxone has a longer C-3 side chain on the cephem skeleton and this may mean that it has greater affinity for PBP2 (Takahata *et al.*, 2006; Unemo *et al.*, 2012a; Lindberg *et al.*, 2007). This prompts the question, should the *N. gonorrhoeae* clinical breakpoints for ceftriaxone, cefixime and cefotaxime be revised to reflect this? Ceftriaxone has better activity or lower MICs for *N. gonorrhoeae* than either cefixime or cefotaxime and so neither are likely to be more useful than the current first-line choice of ceftriaxone. Results of this study and others discussed above confirm that ertapenem is even less affected by *penA* resistance determinants *in vitro* compared to ESCs and the reason is unclear. Is this related potentially to varying rates of expression of influx/efflux genes or unknown resistance determinants, such as factor X? It is an attractive antimicrobial for use in treatment of

gonorrhoea for many reasons and clinical trials have been performed, as discussed (Section 1.14.1). What makes ertapenem most appealing is its activity against ESC-resistant *N. gonorrhoeae*, especially those strains with A501P alterations in *penA* which confer high-level ESC resistance but restore carbapenem susceptibility (Bharat *et al.*, 2015). It remains to be seen whether introduction of ertapenem for widespread treatment of gonorrhoea would cause selective pressure and result in development of resistance/reduced susceptibility to the same degree that is seen in ESCs.

5.4.3 Potential therapeutic options for *N. gonorrhoeae*

Evidently, *N. gonorrhoeae* is quite efficient at altering its DNA and this is thought to primarily occur in the pharynx by transformation with commensal *Neisseria* spp. The concern surrounding introducing a new antimicrobial to treat gonorrhoea is that development of resistance to that drug will follow soon afterwards. The antimicrobials included in this study which are potential candidates to be used in the treatment of gonorrhoea include ertapenem as mentioned above, fosfomycin, gemifloxacin, gentamicin and the new agent solithromycin. Of these, gentamicin has been in use in Malawi for many years for syndromic treatment of urethritis (in combination with doxycycline), but the remaining drugs have not been specifically in use for gonorrhoea. Gentamicin has retained susceptibility to *N. gonorrhoeae* in Malawi after years of use and there is no literature regarding mechanisms of gentamicin resistance specifically in gonococci. Similarly there are no reports regarding fosfomycin resistance determinants peculiar to *N. gonorrhoeae*. Among the 43 isolates examined in this study no resistance determinants were detected which confer gentamicin or fosfomycin resistance in *E.coli*

or other Gram-negative organisms. General introduction of these two agents for the treatment of gonorrhoea would no doubt change those findings.

This study showed that gemifloxacin is generally more active against gonorrhoea compared to ciprofloxacin. This is especially true for strains with high-level resistance to ciprofloxacin (MIC > 32 mg/L) which harbour amino acid substitutions at S91 and D95 in *gyrA* as well as alterations in *parC*. It is also true for the other ciprofloxacin-resistant isolates (MICs 2-16 mg/L) except 2 which have ciprofloxacin and gemifloxacin MICs of 8 and 12 mg/L, respectively. Gemifloxacin MICs in this study are 4 to 42-fold (median 21-fold) lower than ciprofloxacin MICs when considering isolates with ciprofloxacin MIC > 32 mg/L only (n = 45) and 0.67 to 1333-fold (median 4-fold) lower when considering isolates with ciprofloxacin MICs 0.125-16 mg/L (n = 156). Among these 43 sequenced isolates, wild-type *gyrA* (S91 and D95) and *parC* (S87) (n = 12) are highly predictive of susceptibility to ciprofloxacin and low gemifloxacin MICs (≤ 0.002 -0.012 mg/L). This is reflective of other studies also (Grad *et al.*, 2016). This makes genotyping from clinical samples as discussed above, an extremely attractive strategy to increase ciprofloxacin use for treating gonorrhoeae (where appropriate) and thereby potentially reducing ceftriaxone use. Gemifloxacin may be considered susceptible by the same standard.

In addition to the previously used antimicrobials for treatment of gonorrhoea examined in this study and discussed above (ciprofloxacin and cefixime), penicillin, tetracycline and spectinomycin are drugs which are no longer in use, but may be still be useful in particular circumstances. Penicillin and tetracycline were discontinued due to accumulation of resistance determinants, including plasmid-mediated and spectinomycin has limited availability and poor cure rates in pharyngeal gonorrhoea. If penicillin and tetracycline are to be used again it would have to be based on

susceptibility results, given the current rates of resistance in both. This would mean a delay in treatment while awaiting the susceptibility results and running the risk of losing the patient to follow-up. Detection of resistance determinants directly from samples to predict penicillin or tetracycline susceptibility may overcome this problem for those patients not treated empirically, however because a number of different chromosomal resistance determinants add to resistance in these drugs a single PCR test would not be sufficient as was employed for ciprofloxacin above. WGS would be required to assess multiple resistance determinants at once and possibly even predict the MICs to allow reporting of susceptibility to these two drugs and any recommendations for treatment to be made.

Spectinomycin resistance in *N. gonorrhoeae* is exceedingly rare. No spectinomycin-resistant strain was found in this study and unsurprisingly all isolates have wild-type 16s rRNA. The alteration found in the *rpsE*, V25 deletion, is reported to cause resistance to spectinomycin if present in combination with K26E amino acid substitution, which was not present. Spectinomycin use seems favourable if looking at resistance rates, even as seen in Korea where it is used routinely, but as mentioned its poor success in pharyngeal gonorrhoea would mean that it would have to be used only in combination with another drug. Few mutations are associated with the limited spectinomycin resistance which has been reported, so genotyping directly from gonorrhoea-positive clinical samples would be a possibility.

5.5 Molecular epidemiology

Whole-genome analysis confirmed the most prevalent *N. gonorrhoeae* strain types in our study. These included the ST1580, ST9396, and ST1901 MLST lineages as well as

the genetic diversity within these genetic clades. Interestingly, four novel MLST STs were also identified among the isolates tested (ST13142, ST13143, ST13144 and ST11428). MLST ST1901 is an internationally successful clone, prevalent in the isolates in this study (16/43) and is particularly associated with elevated MICs to extended-spectrum cephalosporins, as well as ciprofloxacin and gemifloxacin.

MLST ST 1580 was found only in HLAzi-R isolates, while isolates with azithromycin MICs of 4-32 mg/L were associated with MLST ST7363 or ST9363. Phylogenetic analysis by whole genome sequencing found clonal clustering among the HLAzi-R isolates from Ireland, but significant differences when compared to HLAzi-R (also MLST ST1580) from a UK outbreak, proving the utility and increased discriminatory power of whole-genome sequencing for outbreak investigation in the clinical setting.

NG-MAST ST1407 is a specific clones, successful globally, that is associated with both azithromycin and ESC resistance. Four isolates in this study belong to NG-MAST ST1407, one of which is an MDR strain (Tapsall *et al.*, 2009c). The NG-MAST ST1407 isolates in this study had either reduced susceptibility or resistance to one or all of ceftriaxone, cefixime, cefotaxime and ertapenem. All 4 had azithromycin MICs $\leq$ 0.75 mg/L. NG-MAST STs among the 43 isolates are numerous and diverse. MLST STs are less numerous and where there are ≥ 2 isolates of the same ST, they are associated with gonococci with similar resistance determinants.

5.6 Recommendations and conclusion

Further investigation into mechanisms of resistance in *Neisseria gonorrhoeae* is warranted, particularly in relation to the rate of expression of genes encoding efflux

pumps and their contribution to increasing MICs to various antimicrobials. In the long term, a comprehensive surveillance system in Ireland for antimicrobial resistance in *N. gonorrhoeae* is urgently required. Ideally a large proportion of all cases of gonorrhoea would have susceptibility testing performed each year in Ireland to monitor trends, especially in ceftriaxone reduced-susceptibility, azithromycin resistance (including HLAzi-R) and MDR/XDR strains. A dedicated reference laboratory with highly-trained medical laboratory scientists, participating in an external quality control system as well as EURO-GASP is an appropriate aspiration. The results of this study will act as a foundation for such an endeavour to be undertaken in Ireland in the future.

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Appendices

Appendix 1. MIC results (609 isolates) for penicillin (PEN), ciprofloxacin (CIP), azithromycin (AZM), ceftriaxone (CRO), cefixime (CFM), tetracycline (TET), cefotaxime (CTX), ertapenem (ETP), gentamicin (GENT), gemifloxacin (GEMI), spectinomycin (SPC), fosfomycin (FOS) and solithromycin (SOL)(selected isolates only). B-lactamase result (BL) is also shown as positive (P) or negative (N).

Hospitals from which the isolates originated are listed including St. James's Hospital (SJH), University Hospital Waterford (UHW), University Hospital Galway (UHG), University Hospital Limerick (UHL), St. Columcilles (COL), Midlands Regional Hospital at Mullingar (MUL) and the National Maternity Hospital (NMH).

Age and sex are shown (male = M, female = F, not available = NA). Site of sample is also shown including STI male or female samples (STIM or STIF) with (+G) or without Gram stain performed or not. The source of samples includes from general practice (GP), GUIDE clinics (GUIDE), STI clinics (STI), Gay Mens Sexual Health Clinic (BS), emergency departments (ED), ophthalmology clinics (OPH) and surgical teams (SURG)

Isolate	Hospital	Date	Age	Sex	Site	Source	PEN	CIP	AZM	CRO	CFM	TET	CTX	ETP	GENT	GEMI	SPC	FOS	SOL	BL
199815	SJH	28/07/2016	35	M	Rectal	Guide	0.25	0.004	0.25	0.004	0.016	0.19	0.016	0.006	6	0.006	8	24		N
199769	SJH	28/07/2016	40	M	STIM	Guide	0.5	8	2	0.008	0.016	0.75	0.047	0.008	4	1.5	16	12	0.25	N
199678	SJH	26/07/2016	27	M	STIM	Guide	0.25	0.002	0.5	0.016	0.032	0.25	0.064	0.023	4	0.003	12	16		N
199838	SJH	28/07/2016	39	M	STIM (+G)	Guide	2	0.002	0.125	0.008	0.016	0.125	0.016	0.006	4	0.003	8	16		P
199856	SJH	28/07/2016	18	M	STIM (+G)	Guide	0.125	0.004	>256	0.002	≤0.016	0.38	0.004	0.008	3	≤0.002	8	8	0.5	N
199823	SJH	28/07/2016	19	F	Pharyngeal	Guide	0.5	0.008	1	0.032	0.064	0.25	0.064	0.032	3	0.004	12	12	0.19	N
199679	SJH	26/07/2016	31	F	STI F	Guide	0.25	0.004	0.5	0.032	0.064	0.38	0.047	0.016	2	0.003	12	12		N
173054	SJH	25/07/2016	36	M	Pharyngeal	BS	0.25	0.002	0.25	0.008	0.016	0.094	0.016	0.008	6	0.004	8	16		N
199684	SJH	26/07/2016	30	M	STIM	Guide	2	0.002	0.125	0.004	≤0.016	0.19	0.016	0.012	2	0.003	6	12		P
171121	SJH	06/07/2016	19	M	Urethral	BS	0.5	>32	2	0.016	0.016	1	0.094	0.023	6	8	16	16	0.25	N
170901	SJH	04/07/2016	31	M	Pharyngeal	BS	0.125	4	0.125	0.016	0.032	0.5	0.032	0.012	3	0.75	6	6		N
198813	SJH	07/07/2016	36	M	Rectal	Guide	0.25	0.002	0.25	0.004	0.016	0.125	0.016	0.012	8	0.004	16	12		N
198616	SJH	05/07/2016	27	M	Pharyngeal	Guide	0.25	0.008	0.38	0.012	0.032	0.5	0.064	0.016	4	0.003	12	16		N
172372	SJH	19/07/2016	26	M	Urethral	BS	0.5	≤0.002	0.125	0.016	0.032	0.5	0.032	0.012	2	0.003	8	12		N
172566	SJH	20/07/2016	23	M	Urethral	BS	0.5	0.008	1	0.016	0.032	0.5	0.094	0.047	4	0.006	16	12	0.25	N
171124	SJH	06/07/2016	26	M	Rectal	BS	1	0.008	0.25	0.016	0.032	0.5	0.094	0.016	6	0.006	8	8		N
198740	SJH	07/07/2016	35	M	STIM (+G)	Guide	0.25	0.002	0.125	0.004	0.016	0.19	0.016	0.008	4	0.004	12	16		N
170911	SJH	04/07/2016	23	M	Urethral	BS	1	0.5	0.125	0.008	0.032	12	0.047	0.016	6	0.094	12	12		N
199020	SJH	12/07/2016	20	M	Rectal	Guide	0.5	4	0.125	0.064	0.064	1	0.125	0.016	6	0.5	6	6		N
171582	SJH	12/07/2016	40	M	Urethral	BS	0.25	2	0.064	0.003	0.064	0.25	0.064	0.023	4	0.5	16	12		N
170599	SJH	04/07/2016	43	M	Rectal	GP	>32	1	0.25	0.008	≤0.016	0.38	0.023	0.012	6	0.5	16	16		P
172375	SJH	19/07/2016	26	M	Pharyngeal	BS	0.25	0.002	0.25	0.008	0.016	0.19	0.023	0.012	4	0.004	12	24		N
198532	SJH	04/07/2016	51	M	STIM	Guide	2	0.002	0.125	0.004	0.016	0.38	0.023	0.008	4	0.004	12	24		P
173058	SJH	26/07/2016	31	M	Rectal	BS	4	≤0.002	0.125	0.004	≤0.016	0.5	0.012	0.016	4	0.004	8	8		P
199544	SJH	22/07/2016	41	M	Rectal	Guide	8	2	0.125	0.008	0.032	16	0.032	0.016	4	0.75	8	16		P
198764	SJH	07/07/2016	19	M	Pharyngeal	Guide	0.25	0.002	0.5	0.016	0.032	0.38	0.023	0.023	8	0.004	16	16		N
171825	SJH	13/07/2016	20	M	Pharyngeal	BS	0.25	4	2	0.008	≤0.016	16	0.032	0.008	6	1.5	16	12	0.125	N
199193	SJH	15/07/2016	34	M	Rectal	Guide	0.5	1	0.125	0.032	0.032	0.25	0.125	0.023	6	0.75	8	8		N
173308	SJH	27/07/2016	26	M	Rectal	BS	0.125	2	0.25	0.004	≤0.016	0.25	0.016	0.006	6	0.5	8	24		N
173054	SJH	04/08/2016	36	M	Pharyngeal	BS	0.25	0.002	0.25	0.008	0.016	0.19	0.023	0.008	6	0.004	8	32		N
173059	SJH	26/07/2016	24	M	Urethral	BS	0.25	≤0.002	0.25	0.004	0.016	0.19	0.023	0.008	6	0.004	12	24		N
173061	SJH	26/07/2016	44	M	Urethral	BS	0.125	0.125	0.25	0.008	≤0.016	0.125	0.016	0.006	3	0.047	8	24		N

171118	SJH	06/07/2016	36	M	Rectal	BS	0.125	0.002	0.125	0.004	0.016	0.19	0.012	0.006	4	0.006	8	24		N
198797	SJH	07/07/2016	27	M	STIM (+G)	Guide	0.125	0.002	0.125	0.004	0.016	0.19	0.012	0.006	6	0.004	12	24		N
199041	SJH	12/07/2016	23	M	STIM	Guide	0.125	0.002	0.064	≤0.002	≤0.016	0.094	0.006	0.004	4	≤0.002	4	6		N
171821	SJH	13/07/2016	32	M	Rectal	BS	0.125	0.002	0.125	0.004	≤0.016	0.25	0.016	0.006	6	0.004	8	16		N
199464	SJH	21/07/2016	40	M	Rectal	Guide	0.25	0.004	0.25	0.008	≤0.016	0.19	0.012	0.008	4	0.004	8	16		N
199523	SJH	21/07/2016	19	M	Pharyngeal	Guide	0.25	2	0.125	0.016	≤0.016	0.25	0.016	0.004	6	0.38	8	24		N
199524	SJH	21/07/2016	19	M	Rectal	Guide	0.125	2	0.125	0.008	≤0.016	0.25	0.016	0.004	4	0.5	8	24		N
198880	SJH	11/07/2016	23	M	STIM (+G)	Guide	0.25	0.002	0.125	0.008	0.016	12	0.016	0.006	4	0.003	6	48		N
169403	SJH	21/06/2016	41	M	Rectal	BS	0.25	2	0.125	0.004	≤0.016	0.25	0.012	0.006	8	0.75	12	16		N
169394	SJH	21/06/2016	25	M	Rectal	BS	0.5	2	0.25	0.008	≤0.016	0.38	0.032	0.012	4	0.75	8	24		N
169399	SJH	21/06/2016	24	M	Rectal	BS	0.25	0.004	0.125	0.004	≤0.016	0.25	0.023	0.016	8	0.006	8	24		N
169662	SJH	22/06/2016	31	M	Urethral	BS	0.25	0.004	0.25	0.004	≤0.016	0.125	0.012	0.008	4	0.004	8	32		N
197813	SJH	20/06/2016	43	M	Pharyngeal	Guide	0.25	0.002	0.125	0.008	≤0.016	0.25	0.016	0.008	4	0.004	8	24		N
198225	SJH	28/06/2016	26	M	STIM	Guide	0.5	0.004	0.125	0.016	0.016	0.38	0.047	0.006	12	0.006	4	16		N
198215	SJH	28/06/2016	31	M	STIM (+G)	Guide	0.25	0.004	0.25	0.004	≤0.016	0.125	0.023	0.006	4	0.004	8	32		N
198051	SJH	24/06/2016	24	M	STIM (+G)	Guide	0.125	0.004	0.125	0.008	≤0.016	0.125	0.023	0.008	4	0.008	8	16		N
170191	SJH	28/06/2016	24	M	Pharyngeal	BS	0.125	0.004	0.125	0.008	≤0.016	0.25	0.012	0.006	12	0.004	8	32		N
170385	SJH	28/06/2016	27	M	Pharyngeal	BS	0.25	4	0.064	0.008	≤0.016	32	0.032	0.008	4	0.75	6	48		N
170382	SJH	29/06/2016	35	M	Pharyngeal	BS	0.25	0.004	0.25	0.004	≤0.016	0.094	0.016	0.004	4	0.003	6	24		N
170383	SJH	29/06/2016	43	M	Rectal	BS	0.25	0.004	0.25	0.004	≤0.016	0.25	0.008	0.004	3	0.004	6	12		N
170381	SJH	28/06/2016	32	M	Urethral	BS	0.125	1	0.25	0.004	≤0.016	0.19	0.016	0.004	3	0.5	4	8		N
198227	SJH	28/06/2016	26	M	Pharyngeal	Guide	0.5	0.008	0.25	0.016	0.016	0.25	0.016	0.008	2	0.004	6	8		N
198394	SJH	30/06/2016	27	M	Rectal	Guide	0.125	2	0.125	0.004	≤0.016	0.125	0.012	0.004	3	0.25	8	8		N
198527	SJH	04/07/2016	41	M	STIM	Guide	0.125	0.002	0.25	0.004	≤0.016	0.19	0.008	0.004	4	0.003	6	24		N
170600	SJH	04/07/2016	30	F	Endocervical	NA	0.25	2	0.125	0.008	≤0.016	0.25	0.016	0.004	8	0.5	6	24		N
170903	SJH	04/07/2016	27	M	Rectal	BS	0.125	1	0.125	0.004	≤0.016	0.25	0.016	0.006	4	0.38	6	24		N
170904	SJH	04/07/2016	27	M	Pharyngeal	BS	0.125	1	0.125	0.004	0.016	0.25	0.012	0.003	6	0.38	8	16		N
170905	SJH	04/07/2016	24	M	Rectal	BS	1	2	0.125	0.008	≤0.016	0.25	0.032	0.006	6	0.75	6	16		N
170908	SJH	05/07/2016	47	M	Urethral	BS	0.125	≤0.002	0.5	0.004	0.016	0.19	0.016	0.006	4	0.004	8	24		N
198610	SJH	05/07/2016	42	M	Rectal	Guide	0.25	1	0.25	0.004	≤0.016	0.25	0.012	0.006	6	0.5	8	32		N
170910	SJH	05/07/2016	28	M	Urethral	BS	0.125	≤0.002	0.125	0.004	0.016	0.19	0.016	0.004	6	0.004	12	24		N
170190	SJH	27/06/2016	26	M	Pharyngeal	BS	0.25	>32	0.5	0.004	≤0.016	12	0.016	0.016	3	3	8	3		N
170192	SJH	27/06/2016	24	M	Rectal	BS	0.25	0.004	0.5	0.008	0.016	0.5	0.023	0.012	6	0.003	8	16		N
198395	SJH	30/06/2016	26	M	Pharyngeal	Guide	0.25	0.008	1	0.016	≤0.016	0.75	0.064	0.023	6	0.008	16	16	0.25	N

169658	SJH	21/06/2016	23	M	Rectal	BS	0.5	16	0.5	0.032	0.064	0.5	0.125	0.032	2	0.75	6	24		N
198053	SJH	24/06/2016	30	M	Rectal	Guide	2	0.5	0.125	0.016	0.032	16	0.064	0.016	8	0.064	24	8		P
198365	SJH	30/06/2016	27	M	STIM (+G)	Guide	1	2	0.25	0.016	0.032	0.38	0.064	0.016	6	0.5	12	8		N
170189	SJH	27/06/2016	26	M	Rectal	BS	0.5	0.002	0.25	0.008	0.032	0.5	0.032	0.023	6	0.003	12	16		N
198436	SJH	01/07/2016	18	F	Pharyngeal	Guide	0.5	4	1	0.016	0.032	0.75	0.064	0.016	6	0.5	12	16	0.19	N
169657	SJH	22/06/2016	26	M	Urethral	BS	>32	2	0.064	0.008	≤0.016	0.25	0.016	0.012	3	0.064	6	6		P
198082	SJH	27/06/2016	31	M	STIM (+G)	Guide	2	0.25	0.125	0.016	0.032	12	0.032	0.008	3	0.064	8	12		P
169661	SJH	22/06/2016	22	M	Urethral	BS	2	0.004	0.25	0.004	0.016	0.38	0.023	0.012	6	0.004	16	24		P
169660	SJH	22/06/2016	26	M	Urethral	BS	0.25	0.004	0.5	0.008	≤0.016	0.5	0.032	0.016	4	0.006	12	24		N
169403	SJH	21/06/2016	41	M	Rectal	BS	0.25	2	0.125	0.004	≤0.016	0.38	0.016	0.012	6	0.38	12	12		N
197092	SJH	02/06/2016	26	M	Rectal	Guide	0.25	0.002	0.25	0.008	0.032	0.25	0.023	0.008	4	0.004	8	12		N
167337	SJH	01/06/2016	23	M	Urethral	BS	8	0.004	0.125	0.008	≤0.016	0.19	0.032	0.008	4	0.004	12	24		P
167148	SJH	31/05/2016	29	M	Urethral	BS	0.125	≤0.002	0.25	0.004	≤0.016	0.19	0.032	0.008	4	0.004	12	24		N
167147	SJH	31/05/2016	24	M	Pharyngeal	BS	0.25	2	0.25	0.008	≤0.016	0.5	0.064	0.012	6	1.5	12	16		N
168814	SJH	15/06/2016	33	M	Rectal	BS	0.5	0.004	0.25	0.016	0.032	0.38	0.064	0.023	3	0.003	12	8		N
196821	SJH	30/05/2016	18	M	Rectal	BS	>32	4	0.25	0.004	≤0.016	0.38	0.012	0.008	4	2	8	12		P
197582	SJH	15/06/2016	24	M	STIM	Guide	2	0.004	0.125	0.008	≤0.016	0.25	0.023	0.008	6	0.003	12	16		P
167155	SJH	31/05/2016	24	M	Urethral	BS	1	0.004	0.25	0.008	0.032	0.5	0.047	0.016	6	0.003	12	8		N
197747	SJH	17/06/2016	19	M	Pharyngeal	Guide	0.25	2	16	0.016	0.032	0.5	0.094	0.012	4	2	12	8	0.19	N
169395	SJH	21/06/2016	27	M	Rectal	BS	0.25	8	0.25	0.032	0.032	0.75	0.094	0.012	4	2	12	8		N
167767	SJH	07/06/2016	25	M	Pharyngeal	BS	2	≤0.002	0.125	0.008	≤0.016	0.5	0.032	0.012	4	0.004	16	16		P
168636	SJH	14/06/2016	36	M	Urethral	BS	0.5	0.004	0.25	0.008	0.032	0.5	0.064	0.023	6	0.002	12	12		N
168628	SJH	14/06/2016	24	M	Urethral	BS	1	2	0.25	0.064	0.064	1	0.25	0.012	4	0.38	12	8		N
197468	SJH	13/06/2016	33	M	STIM (+G)	Guide	0.25	8	0.125	0.032	0.032	0.75	0.094	0.016	4	1	8	8		N
196512	SJH	24/05/2016	24	M	STIM (+G)	Guide	0.125	0.002	0.064	0.004	≤0.016	0.19	0.016	0.008	8	0.004	6	32		N
196578	SJH	24/05/2016	35	M	Rectal	Guide	0.125	≤0.002	0.25	0.004	≤0.016	0.25	0.016	0.008	4	0.008	8	32		N
167326	SJH	01/06/2016	24	M	Rectal	BS	4	0.004	0.125	0.008	≤0.016	0.38	0.023	0.008	4	0.003	12	16		P
196892	SJH	31/05/2016	37	M	STIM (+G)	Guide	0.125	0.004	0.5	0.008	0.016	0.75	0.023	0.012	8	0.004	12	16		N
196746	SJH	26/05/2016	25	M	STIM	Guide	0.125	0.002	0.125	0.004	≤0.016	0.25	0.012	0.006	8	0.004	8	24		N
196797	SJH	30/05/2016	30	M	STIM (+G)	Guide	0.125	0.004	0.125	0.008	≤0.016	0.19	0.016	0.006	4	0.003	6	24		N
196859	SJH	30/05/2016	18	M	STIM (+G)	Guide	0.5	≤0.002	0.25	0.008	0.016	0.25	0.023	0.008	6	0.003	8	16		N
196601	SJH	25/05/2016	20	F	STIF	Guide	0.5	0.004	0.125	0.008	0.016	0.19	0.032	0.023	4	0.003	8	32		N
167768	SJH	07/06/2016	27	M	Urethral	BS	0.25	0.002	0.25	0.008	≤0.016	0.125	0.012	0.008	4	0.003	8	32		N

167329	SJH	01/06/2016	27	M	Rectal	BS	0.5	0.008	0.25	0.008	≤0.016	0.5	0.064	0.004	8	0.006	8	16		N
167766	SJH	07/06/2016	44	M	Pharyngeal	BS	0.25	0.002	0.25	0.004	≤0.016	0.125	0.023	0.006	4	0.004	8	32		N
197262	SJH	09/06/2016	19	M	STIM (+G)	Guide	0.125	0.004	0.125	0.008	≤0.016	0.125	0.016	0.006	6	0.006	6	12		N
197312	SJH	09/06/2016	28	M	Rectal	Guide	0.25	0.002	0.125	0.008	0.016	0.094	0.016	0.008	3	0.004	8	32		N
501373	SJH	30/08/2016	36	M	STIM	Guide	0.25	16	0.5	0.032	0.125	1	0.125	0.023	6	2	8	32		N
197338	SJH	09/06/2016	23	M	Rectal	Guide	0.125	0.002	0.064	0.004	≤0.016	0.125	0.016	0.006	4	≤0.002	6	24		N
197395	SJH	10/06/2016	27	M	Rectal	Guide	0.125	0.002	0.25	0.004	≤0.016	0.25	0.016	0.008	4	0.003	8	16		N
196555	SJH	24/05/2016	23	M	STIM (+G)	Guide	0.5	≤0.002	0.25	0.006	0.032	0.25	0.023	0.023	4	0.006	12	8		N
196750	SJH	26/05/2016	23	M	STIM (+G)	Guide	0.064	0.5	0.064	0.008	0.032	0.19	0.094	0.032	6	0.75	16	12		N
196712	SJH	26/05/2016	36	F	STI F	Guide	0.25	0.008	>256	0.008	0.016	0.75	0.125	0.023	6	0.006	16	12	32	N
169391	SJH	21/06/2016	25	M	Urethral	BS	0.25	8	0.25	0.004	≤0.016	0.25	0.012	0.006	3	1.5	6	16		N
197864	SJH	21/06/2016	24	M	Urethral	Guide	0.25	≤0.002	0.25	0.004	0.016	0.064	0.016	0.006	4	0.004	12	24		N
195440	SJH	28/04/2016	19	F	STI F	Guide	0.016	0.008	0.125	0.004	≤0.016	0.19	0.064	0.047	6	0.006	8	24		N
164179	SJH	04/05/2016	23	M	Urethral	BS	0.25	0.008	0.125	0.002	0.016	0.25	0.023	0.008	3	0.004	6	32		N
196325	SJH	19/05/2016	19	M	STIM (+G)	Guide	0.5	0.004	0.064	0.008	0.032	0.75	0.125	0.047	4	0.004	12	24		N
165894	SJH	20/05/2016	29	M	Urethral	GP	0.125	0.004	0.25	0.008	≤0.016	0.094	0.016	0.012	4	0.006	8	6		N
164180	SJH	04/05/2016	34	M	Urethral	BS	0.125	4	0.032	0.008	0.016	0.19	0.016	0.008	3	0.19	6	16		N
195743	SJH	09/05/2016	24	M	STIM (+G)	Guide	0.25	0.016	0.125	0.004	≤0.016	0.25	0.023	0.008	8	0.008	12	64		N
195750	SJH	09/05/2016	20	M	Rectal	Guide	0.5	4	0.25	0.016	≤0.016	0.38	0.064	0.006	6	0.75	6	16		N
195751	SJH	09/05/2016	23	M	STIM	Guide	1	4	0.25	0.016	0.016	0.19	0.064	0.012	4	0.5	8	24		N
195727	SJH	09/05/2016	27	M	STIM (+G)	Guide	0.25	0.016	0.125	0.004	≤0.016	0.25	0.023	0.008	12	0.006	12	24		N
166587	SJH	25/05/2016	20	M	Pharyngeal	BS	0.25	4	8	0.008	0.016	0.75	0.125	0.016	6	2	12	12	0.19	N
166589	SJH	25/05/2016	24	M	Urethral	BS	0.5	2	0.125	0.008	≤0.016	0.25	0.023	0.008	6	1.5	16	12		N
196274	SJH	19/05/2016	24	M	Rectal	Guide	2	0.5	0.125	0.008	0.032	16	0.032	0.006	6	0.25	12	12		P
195737	SJH	09/05/2016	28	M	STIM (+G)	Guide	0.25	0.004	0.25	0.002	≤0.016	0.125	0.016	0.006	4	0.004	8	32		N
195745	SJH	09/05/2016	20	M	Pharyngeal	Guide	1	8	0.125	0.008	0.016	0.19	0.032	0.008	4	0.5	8	16		N
195748	SJH	09/05/2016	17	F	Cervical	Guide	0.125	0.004	0.125	≤0.002	≤0.016	0.064	0.008	0.008	3	0.003	8	24		N
164973	SJH	11/05/2016	23	M	Pharyngeal	BS	0.5	0.008	0.25	0.008	0.016	0.25	0.047	0.016	4	0.002	6	12		N
164975	SJH	11/05/2016	28	M	Rectal	BS	0.125	0.008	0.064	0.004	≤0.016	0.25	0.023	0.008	4	0.004	6	16		N
164744	SJH	10/05/2016	22	M	Rectal	BS	0.5	0.032	1	0.008	≤0.016	1.5	0.094	0.016	6	0.012	12	12	0.19	N
163969	SJH	02/05/2016	26	M	Urethral	BS	>32	2	0.032	0.004	≤0.016	0.25	0.023	0.012	3	0.25	12	6		P
165624	SJH	16/05/2016	38	M	Rectal	BS	0.25	≤0.002	0.5	≤0.002	≤0.016	0.38	0.023	0.012	3	0.004	12	16		N
195986	SJH	12/05/2016	22	M	Rectal	Guide	0.125	1	0.125	0.008	0.064	0.38	0.064	0.032	6	1.5	12	16		N
196012	SJH	12/05/2016	42	M	STIM (+G)	Guide	0.5	16	0.25	0.012	≤0.016	12	0.047	0.008	6	0.064	8	12		N

165629	SJH	16/05/2016	29	M	Pharyngeal	Guide	0.125	0.002	0.064	≤0.002	≤0.016	0.094	0.016	0.004	3	0.003	4	24		N
165632	SJH	16/05/2016	24	M	Rectal	BS	0.25	0.004	0.25	0.008	≤0.016	0.25	0.016	0.008	4	0.003	8	24		N
165630	SJH	16/05/2016	24	M	Rectal	BS	0.5	2	0.25	0.008	≤0.016	0.25	0.032	0.012	4	0.5	4	16		N
165778	SJH	17/05/2016	28	M	Pharyngeal	BS	0.25	≤0.002	0.125	0.004	≤0.016	0.19	0.016	0.008	4	0.004	8	32		N
165779	SJH	17/05/2016	22	M	Pharyngeal	BS	0.125	≤0.002	0.125	0.004	≤0.016	0.19	0.016	0.008	12	0.003	12	12		N
195253	SJH	26/04/2016	29	M	Urethral	Guide	0.5	8	2	0.012	≤0.016	1	0.094	0.016	6	3	16	12		N
195655	SJH	05/05/2016	23	M	Rectal	Guide	2	1	0.125	0.016	0.032	16	0.047	0.012	6	0.125	12	12		N
196272	SJH	19/05/2016	56	M	STIM (+G)	Guide	0.25	0.002	0.25	0.004	≤0.016	0.125	0.016	0.004	4	0.004	8	32		N
196338	SJH	19/05/2016	35	M	Pharyngeal	Guide	0.25	≤0.002	0.25	0.004	≤0.016	0.094	0.032	0.012	6	0.004	6	24		N
166583	SJH	25/05/2016	35	M	Rectal	BS	0.064	≤0.002	0.016	≤0.002	≤0.016	0.19	0.008	0.008	6	≤0.002	4	12		N
157029	SJH	01/03/2016	26	M	Rectal	BS	0.125	0.002	1	0.008	0.016	0.19	0.016	0.008	12	0.004	12	24	0.125	N
158054	SJH	09/03/2016	31	M	Urethral	BS	0.125	0.004	0.25	0.004	0.016	0.094	0.023	0.004	6	0.004	8	12		N
192946	SJH	07/03/2016	23	M	Rectal	Guide	0.125	1	0.25	0.004	0.016	0.125	0.012	0.003	3	0.38	8	16		N
157031	SJH	01/03/2016	25	M	Urethral	BS	0.5	0.002	0.25	0.008	0.016	0.125	0.023	0.008	6	0.003	8	24		N
157274	SJH	02/03/2016	40	M	Rectal	BS	0.25	0.008	2	0.008	0.016	1	0.032	0.006	4	0.012	12	12	0.19	N
422102	UHG	19/09/2016	NA	NA	NA	NA	1	>32	1	0.032	0.125	1	0.38	0.032	8	2	16	32	0.094	N
158057	SJH	09/03/2016	24	M	Rectal	BS	0.25	4	0.125	0.004	0.016	0.094	0.016	0.004	3	0.003	4	6		N
158061	SJH	09/03/2016	20	M	Rectal	BS	0.25	0.002	0.125	0.008	0.016	0.38	0.032	0.012	4	0.003	6	16		N
158618	SJH	15/03/2016	23	M	Rectal	BS	0.125	0.004	0.125	0.004	≤0.016	0.25	0.023	0.012	4	0.003	6	24		N
157810	SJH	08/03/2016	28	M	Urethral	BS	0.25	0.004	1	0.008	0.032	0.5	0.023	0.016	4	0.006	12	16	0.125	N
158845	SJH	16/03/2016	37	M	Urethral	BS	0.25	0.002	0.25	0.004	≤0.016	0.125	0.016	0.006	3	0.003	8	24		N
193291	SJH	14/03/2016	22	M	Rectal	BS	0.25	≤0.002	0.125	0.004	0.016	0.125	0.016	0.008	4	0.006	6	8		N
193839	SJH	24/03/2016	21	M	Pharyngeal	Guide	0.125	8	≤0.016	0.016	0.016	0.5	0.032	0.006	4	1.5	6	16		N
157821	SJH	07/03/2016	32	M	Rectal	BS	1	0.008	0.5	0.016	0.016	1	0.032	0.016	6	0.008	8	8		N
157818	SJH	07/03/2016	20	M	Rectal	BS	0.125	0.002	16	0.004	0.016	0.38	0.012	0.008	6	0.004	8	24	0.19	N
160153	SJH	30/03/2016	37	M	Urethral	BS	0.25	0.004	0.5	0.004	≤0.016	0.5	0.032	0.012	4	0.006	6	16		N
193222	SJH	10/03/2016	24	M	STIM	Guide	1	0.008	0.5	0.016	0.016	0.75	0.032	0.016	2	0.008	6	6		N
158060	SJH	09/03/2016	18	M	Pharyngeal	BS	0.5	4	0.125	0.032	0.032	0.75	0.125	0.016	3	1	4	8		N
193058	SJH	08/03/2016	41	M	STIM (+G)	Guide	0.125	8	0.5	0.008	0.016	0.38	0.047	0.016	6	12	6	8		N
157813	SJH	08/03/2016	34	M	Pharyngeal	BS	4	0.002	0.25	0.004	0.016	0.38	0.016	0.016	8	0.004	12	24		P
157814	SJH	08/03/2016	36	M	Urethral	BS	2	0.004	0.25	0.004	0.016	0.38	0.016	0.012	8	0.004	8	24		P
157817	SJH	08/03/2016	29	M	Urethral	BS	0.25	4	0.5	0.004	0.016	12	0.032	0.012	4	2	6	6		N
193852	SJH	24/03/2016	19	M	STIM	Guide	0.125	≤0.002	>256	0.004	≤0.016	0.38	0.006	0.023	4	≤0.002	8	8	0.38	N

158840	SJH	14/03/2016	21	M	Rectal	BS	0.25	8	0.25	0.016	0.032	0.75	0.094	0.012	3	2	6	6		N
157820	SJH	07/03/2016	36	M	Pharyngeal	BS	0.25	0.004	1	0.004	0.016	0.38	0.023	0.016	6	0.008	8	24	0.19	N
159621	SJH	24/03/2016	58	M	Urethral	GP	0.125	≤0.002	0.25	0.004	≤0.016	0.064	0.016	0.004	4	0.003	4	24		N
192754	SJH	01/03/2016	25	F	STI F	Guide	0.25	0.002	4	0.008	≤0.016	0.25	0.016	0.006	6	0.003	6	12	0.25	N
193854	SJH	24/03/2016	40	M	STIM	Guide	0.125	0.004	0.064	0.008	≤0.016	0.5	0.032	0.012	4	0.004	6	24		N
194081	SJH	31/03/2016	43	M	STIM (+G)	Guide	0.125	0.004	0.125	0.004	≤0.016	0.19	0.016	0.003	4	0.003	4	16		N
159948	SJH	29/03/2016	35	M	Pharyngeal	BS	0.125	2	0.125	0.004	≤0.016	0.38	0.012	0.003	12	1.5	12	16		N
194194	SJH	04/04/2016	30	M	STIM	Guide	0.25	0.002	0.25	0.004	≤0.016	0.25	0.016	0.008	6	0.003	8	16		N
160755	SJH	06/04/2016	47	M	Pharyngeal	BS	0.125	0.032	0.25	0.012	≤0.016	0.38	0.016	0.006	8	0.006	12	16		N
195276	SJH	26/04/2016	19	F	Pharyngeal	Guide	0.25	>32	1	≤0.002	≤0.016	1	0.094	0.016	6	2	16	12	0.19	N
163285	SJH	26/04/2016	25	M	Pharyngeal	BS	0.25	8	2	0.008	≤0.016	1	0.064	0.012	6	2	16	24	0.19	N
160756	SJH	05/04/2016	21	M	Rectal	BS	0.125	≤0.002	0.25	0.002	≤0.016	0.19	0.016	0.006	4	0.003	12	32		N
194409	SJH	07/04/2016	23	M	Pharyngeal	Guide	1	>32	0.25	0.016	0.016	0.5	0.032	0.006	4	0.75	4	16		N
194455	SJH	07/04/2016	17	M	STIM	Guide	0.25	0.002	0.25	0.006	≤0.016	0.125	0.032	0.006	4	0.006	8	12		N
161522	SJH	11/04/2016	45	M	Pharyngeal	BS	0.125	0.002	1	0.004	0.016	0.25	0.023	0.008	6	0.004	8	32	0.19	N
194660	SJH	12/04/2016	26	M	STIM	Guide	0.125	0.002	0.125	0.002	0.016	0.125	0.012	0.003	6	0.003	6	12		N
194686	SJH	12/04/2016	25	M	STIM (+G)	Guide	1	0.008	0.016	0.008	0.016	0.19	0.032	0.008	3	0.003	4	24		N
161528	SJH	12/04/2016	26	M	Rectal	BS	0.5	4	0.25	0.008	0.016	0.5	0.064	0.006	4	0.5	6	12		N
178144	SJH	13/09/2016	28	M	Rectal	BS	0.5	16	4	0.032	0.032	1	0.064	0.016	6	1.5	16	24	0.19	N
178142	SJH	13/09/2016	33	M	Urethral	BS	0.5	8	2	0.032	0.032	1	0.064	0.012	8	1.5	16	32	0.25	N
178145	SJH	13/09/2016	37	M	Urethral	BS	0.5	4	2	0.016	0.032	2	0.064	0.012	6	1	16	24	0.19	N
163524	SJH	27/04/2016	24	M	Rectal	BS	0.25	12	1	0.008	≤0.016	1	0.047	0.008	6	1.5	12	12	0.125	N
161530	SJH	11/04/2016	31	M	Rectal	BS	0.25	0.004	0.125	0.004	≤0.016	0.125	0.023	0.006	6	0.006	12	48		N
161526	SJH	11/04/2016	26	M	Rectal	BS	0.25	4	0.125	0.008	0.016	0.25	0.023	0.004	2	0.75	6	12		N
194698	SJH	12/04/2016	27	M	STIM (+G)	Guide	0.25	16	0.5	0.016	0.064	0.5	0.19	0.047	4	3	8	48		N
157812	SJH	08/03/2016	34	M	Rectal	BS	0.25	4	0.25	0.002	0.032	0.5	0.047	0.008	3	2	6	6		N
194697	SJH	12/04/2016	35	M	Rectal	Guide	0.125	≤0.002	1	0.004	0.016	0.38	0.016	0.012	3	0.008	8	24	0.125	N
160757	SJH	05/04/2016	26	M	Rectal	BS	0.25	1	0.25	0.016	0.064	0.5	0.125	0.016	4	1	12	12		N
162513	SJH	19/04/2016	22	M	Urethral	BS	0.125	0.004	0.5	0.004	≤0.016	0.25	0.016	0.006	6	0.003	6	12		N
161666	SJH	13/04/2016	21	M	Rectal	BS	0.25	0.002	0.25	0.004	≤0.016	0.064	0.012	0.004	3	0.004	6	48		N
194765	SJH	14/04/2016	19	M	STIM (+G)	Guide	0.125	≤0.002	0.125	0.004	≤0.016	0.19	0.016	0.003	4	0.002	6	24		N
194771	SJH	14/04/2016	36	M	Rectal	Guide	0.125	≤0.002	0.125	0.008	≤0.016	0.125	0.032	0.004	4	0.004	8	12		N
162391	SJH	19/04/2016	37	M	Urethral	GP	0.5	16	0.5	0.016	0.064	0.5	0.125	0.032	3	2	6	24		N
161944	SJH	13/04/2016	41	M	STIM	Guide	0.125	2	0.25	0.002	≤0.016	16	0.016	0.006	6	1	12	16		N

162511	SJH	19/04/2016	33	M	Urethral	BS	0.25	1	0.25	0.004	≤0.016	0.19	0.016	0.008	8	0.5	6	24		N
162759	SJH	20/04/2016	28	M	Pharyngeal	BS	0.125	2	0.25	≤0.002	≤0.016	0.5	0.012	0.003	6	0.5	12	24		N
162760	SJH	20/04/2016	28	M	Rectal	BS	0.125	≤0.002	0.125	0.004	≤0.016	0.094	0.012	0.006	4	0.003	6	24		N
195077	SJH	21/04/2016	18	M	STIM (+G)	Guide	0.125	2	0.064	0.008	0.064	0.25	0.125	0.023	3	0.5	6	24		N
163282	SJH	26/04/2016	29	M	Rectal	BS	0.25	2	0.125	0.004	≤0.016	0.125	0.012	0.004	3	0.25	6	32		N
163287	SJH	26/04/2016	50	M	Rectal	BS	0.125	≤0.002	0.125	≤0.002	≤0.016	0.38	0.016	0.006	12	0.004	8	32		N
195165	SJH	25/04/2016	30	M	STIM (+G)	Guide	0.125	0.002	0.25	0.004	≤0.016	0.125	0.016	0.006	6	0.004	6	24		N
194308	SJH	05/04/2016	26	M	Pharyngeal	Guide	0.5	>32	0.5	0.016	0.032	0.75	0.064	0.016	6	3	8	6		N
162757	SJH	20/04/2016	22	M	Rectal	BS	0.125	0.004	0.5	0.004	0.016	0.38	0.016	0.012	6	0.004	12	16		N
194573	SJH	11/04/2016	29	M	Rectal	Guide	0.25	4	0.064	0.016	0.032	0.5	0.047	0.012	4	1.5	8	12		N
195266	SJH	26/04/2016	19	M	Urethral	Guide	0.094	≤0.002	0.064	≤0.002	≤0.016	0.047	0.004	0.004	2	0.002	6	12		N
163534	SJH	27/04/2016	48	M	Urethral	BS	0.25	0.004	0.125	0.004	≤0.016	0.5	0.032	0.006	8	1	12	16		N
193314	SJH	14/03/2016	27	M	STIM (+G)	Guide	0.5	0.002	0.125	0.004	0.016	0.38	0.023	0.008	4	0.003	8	12		N
158616	SJH	15/03/2016	49	M	Urethral	BS	0.25	4	0.125	0.006	≤0.016	0.5	0.032	0.006	6	0.5	6	24		N
422322	UHG	2016	NA	NA	NA	NA	>32	2	0.5	0.008	≤0.016	0.5	0.016	0.004	8	0.5	16	12		P
422321	UHG	2016	NA	NA	Urethral	NA	0.016	≤0.002	0.5	≤0.002	≤0.016	0.125	0.006	0.003	3	0.003	12	24		N
191356	SJH	01/02/2016	21	M	STIM (+G)	Guide	0.25	0.008	0.25	0.002	≤0.016	0.094	0.016	0.012	2	0.003	6	32		N
153792	SJH	03/02/2016	21	M	Rectal	Guide	0.125	0.004	0.25	0.004	≤0.016	0.094	0.012	0.012	3	0.004	8	32		N
191352	SJH	01/02/2016	22	M	STIM (+G)	Guide	0.25	4	1	0.002	≤0.016	12	0.032	0.006	3	1.5	12	8		N
153023	SJH	27/01/2016	29	M	Pharyngeal	BS	0.19	0.002	0.25	0.004	≤0.016	0.38	0.023	0.006	4	0.003	8	12		N
191702	SJH	08/02/2016	20	M	Rectal	Guide	0.25	4	0.25	0.016	0.016	0.5	0.094	0.003	4	1	4	24		N
191743	SJH	08/02/2016	30	M	Rectal	Guide	0.19	4	0.064	0.008	0.047	0.5	0.064	0.006	2	0.5	4	8		N
154272	SJH	09/02/2016	28	M	Rectal	BS	0.125	16	0.125	0.004	0.047	0.25	0.032	0.008	6	1.5	4	12		N
152118	SJH	20/01/2016	30	M	STIM	BS	0.25	0.008	0.125	0.004	≤0.016	16	0.016	0.008	6	0.004	12	64		N
157024	SJH	01/03/2016	24	F	Rectal	BS	0.125	0.002	1	0.008	0.016	0.25	0.012	0.008	4	0.004	12	24		N
157026	SJH	01/06/2016	35	M	Rectal	BS	0.064	2	0.125	0.002	0.016	12	0.012	0.006	2	0.5	8	6		N
157030	SJH	01/03/2016	25	M	Urethral	BS	0.5	0.002	0.25	0.008	0.016	0.38	0.032	0.008	4	0.003	4	24		N
155560	SJH	17/02/2016	48	M	Urethral	BS	0.125	2	0.125	0.004	≤0.016	12	0.032	0.008	8	0.5	6	8		N
156062	SJH	23/02/2016	25	M	Urethral	BS	0.125	≤0.002	0.125	0.002	≤0.016	0.125	0.012	0.006	2	0.003	6	12		N
191563	SJH	04/02/2016	29	M	Urethral	Guide	0.5	0.032	1	0.008	0.016	1	0.032	0.012	4	0.012	12	12		N
191615	SJH	04/02/2016	21	F	NA	Guide	0.25	0.008	2	0.006	0.023	0.75	0.023	0.023	4	0.006	6	24	0.125	N
191753	SJH	09/02/2016	19	M	Urethral	Guide	0.25	0.016	1	0.008	0.023	1.5	0.047	0.023	6	0.012	12	16		N
156416	SJH	24/02/2016	30	M	STI F	BS	0.25	0.002	0.5	0.004	≤0.016	0.5	0.012	0.012	8	0.003	16	16		N

191926	SJH	18/02/2016	38	M	Pharyngeal	Guide	0.125	≤0.002	0.5	≤0.002	≤0.016	0.38	0.012	0.012	4	0.004	8	16		N
152916	SJH	27/01/2016	26	M	STIM (+G)	GP	0.094	0.003	0.032	≤0.002	≤0.016	0.094	0.004	0.006	2	≤0.002	4	8		N
156533	SJH	26/02/2016	41	F	Endocervical	NA	0.25	0.002	0.5	0.008	0.032	0.5	0.064	0.032	6	0.004	12	16		N
191917	SJH	11/02/2016	35	M	Pharyngeal	Guide	0.125	0.004	8	0.004	≤0.016	0.19	0.023	0.016	6	0.008	8	8	0.125	N
153129	SJH	28/01/2016	27	M	Rectal	GP	0.023	0.002	0.023	≤0.002	≤0.016	0.125	≤0.002	≤0.002	1.5	≤0.002	3	3		N
191233	SJH	28/01/2016	29	M	Rectal	Guide	0.125	0.004	0.5	0.004	0.047	0.5	0.023	0.016	6	0.004	8	12		N
156131	SJH	23/02/2016	33	M	Rectal	BS	0.25	8	0.125	0.016	≤0.016	0.5	0.064	0.008	3	1.5	4	16		N
153800	SJH	03/02/2016	35	M	Rectal	BS	0.125	0.008	12	0.004	0.016	0.38	0.012	0.012	6	0.004	8	12	0.125	N
191587	SJH	04/02/2016	20	F	Pharyngeal	Guide	0.25	0.008	>256	0.008	0.032	1	0.064	0.016	3	0.004	8	12	32	N
155692	SJH	18/02/2016	29	M	Urethral	GP	0.25	≤0.002	0.25	≤0.002	≤0.016	0.094	0.012	0.008	3	0.003	8	32		N
192181	SJH	18/02/2016	36	M	STIM	Guide	0.25	≤0.002	0.25	0.004	≤0.016	0.094	0.016	0.008	4	0.003	8	32		N
190619	SJH	15/01/2016	32	M	STIM (+G)	Guide	0.5	0.004	0.25	0.008	0.032	0.38	0.047	0.023	6	0.004	8	24		N
190350	SJH	11/01/2016	25	M	Rectal	Guide	0.25	8	0.25	0.004	≤0.016	12	0.023	0.006	2	1.5	12	12		N
191821	SJH	09/02/2016	46	M	STIM	Guide	0.125	0.008	0.064	0.004	≤0.016	0.5	0.023	0.008	4	0.004	8	32		N
153511	SJH	02/02/2016	19	M	Rectal	BS	0.125	4	0.25	0.002	≤0.016	0.5	0.016	0.004	8	1	12	12		N
190622	SJH	15/01/2016	34	M	STIM (+G)	Guide	0.125	0.004	0.25	0.004	0.016	0.19	0.016	0.008	2	0.006	6	32		N
190767	SJH	19/01/2016	36	M	Rectal	Guide	0.125	0.004	0.25	0.004	≤0.016	0.094	0.016	0.006	4	0.004	8	32		N
151424	SJH	13/01/2016	23	M	Rectal	BS	0.25	0.004	0.25	0.004	≤0.016	0.125	0.012	0.004	6	0.003	6	32		N
192227	SJH	18/02/2016	22	M	Rectal	Guide	1	2	0.125	0.016	≤0.016	0.38	0.064	0.016	3	0.75	6	12		N
151999	SJH	19/01/2016	24	M	STIM (+G)	GP	0.5	4	0.064	0.008	0.023	0.5	0.032	0.016	2	0.5	8	12		N
190997	SJH	25/01/2016	27	M	Rectal	Guide	0.19	0.004	0.25	0.006	0.016	0.094	0.012	0.006	4	0.004	6	24		N
153020	SJH	27/01/2016	31	M	Urethral	BS	0.25	8	0.25	0.006	≤0.016	12	0.016	0.006	4	1.5	4	8		N
155183	SJH	15/02/2016	20	M	Urethral	GP	0.5	0.004	0.25	0.008	≤0.016	0.25	0.032	0.008	6	0.003	6	24		N
190029	SJH	04/01/2016	35	M	Rectal	Guide	0.5	12	2	0.008	0.023	0.75	0.047	0.012	6	2	6	24	0.25	N
190035	SJH	04/01/2016	23	M	STIM (+G)	Guide	0.25	0.004	0.5	0.003	0.016	0.19	0.016	0.012	4	0.006	8	32		N
191010	SJH	25/01/2016	25	M	STIM	Guide	0.125	0.004	0.064	0.002	≤0.016	0.38	0.006	0.012	3	≤0.002	6	16		N
151212	SJH	12/01/2016	19	M	Urethral	BS	0.125	0.008	1	0.008	0.032	0.5	0.023	0.012	6	0.006	8	16		N
190309	SJH	08/01/2016	28	M	STIM (+G)	Guide	0.5	>32	4	0.032	0.032	1	0.064	0.012	8	2	12	12	0.125	N
190325	SJH	11/01/2016	34	M	STIM (+G)	Guide	0.25	>32	0.5	0.016	0.047	1	0.064	0.016	6	1.5	12	8		N
150560	SJH	06/01/2016	19	M	Pharyngeal	BS	0.5	>32	2	0.006	≤0.016	24	0.016	0.016	2	6	8	12	0.125	P
150445	SJH	06/01/2016	31	M	STIM	BS	0.5	2	0.125	0.016	0.047	16	0.047	0.012	4	0.125	8	16		P
151213	SJH	12/01/2016	23	M	Urethral	BS	0.25	0.008	2	0.004	0.047	1	0.032	0.023	6	0.006	12	16	0.125	N
190440	SJH	12/01/2016	32	M	STIM (+G)	Guide	4	>32	0.032	0.004	0.016	32	0.032	0.012	4	4	4	12		P
190228	SJH	07/01/2016	40	M	Rectal	Guide	8	1	0.25	0.012	0.032	16	0.047	0.016	3	0.094	8	24		P

190253	SJH	07/01/2016	37	M	STIM (+G)	Guide	1	0.032	0.5	0.016	0.032	0.5	0.032	0.023	1.5	0.004	4	12		N
190251	SJH	07/01/2016	22	M	STIM (+G)	Guide	0.25	0.008	0.5	0.008	0.016	0.19	0.016	0.012	4	0.004	8	16		N
191657	SJH	05/02/2016	24	M	Rectal	Guide	0.25	0.008	1	0.004	0.032	0.5	0.023	0.016	6	0.006	8	16		N
153021	SJH	27/01/2016	36	M	Urethral	BS	0.5	0.032	1	0.008	0.023	1.5	0.047	0.016	6	0.012	12	12		N
152150	SJH	20/01/2016	30	M	Pharyngeal	BS	0.125	0.004	0.064	0.004	0.016	16	0.023	0.012	6	0.006	6	12		N
190766	SJH	19/01/2016	36	M	STIM (+G)	Guide	0.25	0.008	2	0.008	0.016	0.75	0.032	0.008	6	0.008	12	16	0.19	N
190692	SJH	18/01/2016	24	M	STIM (+G)	Guide	0.25	0.008	0.5	0.004	≤0.016	0.5	0.008	0.008	4	0.004	8	12		N
190642	SJH	18/01/2016	36	M	STIM (+G)	Guide	0.5	0.004	0.5	0.008	0.023	0.5	0.032	0.023	3	0.004	6	12		N
152376	SJH	20/01/2016	18	M	Urethral	BS	0.38	16	4	0.008	0.016	16	0.016	0.008	6	6	8	6	0.125	N
191262	SJH	28/01/2016	26	M	Rectal	Guide	0.5	>32	0.5	0.023	0.125	1	0.125	0.032	4	1.5	8	24		N
190904	SJH	21/01/2016	21	M	STIM (+G)	Guide	0.25	6	0.25	0.012	0.047	0.75	0.064	0.012	4	1.5	8	8		N
190250	SJH	07/01/2016	22	M	Rectal	Guide	0.25	0.012	0.5	0.008	0.016	0.125	0.016	0.008	6	0.006	8	24		N
152147	SJH	20/01/2016	28	M	Rectal	BS	0.25	0.004	0.25	0.002	0.016	0.125	0.023	0.006	4	0.004	8	32		N
003	UHW	08/08/2013	21	M	Urethral	STI	0.19	0.004	0.38	≤0.002	≤0.016	0.094	0.012	0.023	8	0.004	8	16		N
001	UHW	05/09/2013	47	M	Urethral	STI	0.023	0.003	0.125	≤0.002	≤0.016	6	0.004	0.003	2	0.002	6	16		N
005	UHW	10/07/2013	41	M	ASP	SURG	0.094	0.003	0.125	0.006	≤0.016	0.25	0.008	0.008	4	0.002	8	12		N
009	UHW	07/02/2013	24	M	Urethral	STI	1	>32	0.75	0.064	0.125	1.5	0.125	0.032	4	1.5	12	48	0.064	N
006	UHW	30/01/2013	21	M	Urethral	STI	0.25	0.012	>256	0.016	0.016	1	0.032	0.012	4	0.006	8	16	32	N
004	UHW	04/03/2013	21	M	Urethral	GP	0.125	0.004	0.125	0.002	≤0.016	0.064	0.008	0.016	2	0.004	6	32		N
002	UHW	16/08/2013	21	M	Urethral	GP	0.125	0.003	0.38	0.002	≤0.016	0.047	0.012	0.016	1.5	0.002	4	16		N
015	UHW	02/02/2016	27	M	Urethral	STI	0.094	0.006	16	0.006	≤0.016	0.19	0.008	0.003	6	0.006	8	16	0.19	N
008	UHW	24/01/2013	24	M	Throat swab	STI	>32	3	0.5	0.008	≤0.016	0.5	0.023	0.006	6	0.75	12	12		P
007	UHW	23/01/2013	19	M	Urethral	STI	0.19	0.004	0.5	0.004	0.016	0.094	0.016	0.023	4	0.004	12	24		N
010	UHW	16/01/2013	25	M	Urethral	STI	0.19	0.006	0.38	0.004	0.016	0.125	0.016	0.016	4	0.006	6	16		N
011	UHW	15/04/2015	20	M	Urethral	STI	0.19	0.008	0.5	0.006	≤0.016	0.19	0.016	0.023	4	0.004	8	48		N
012	UHW	12/03/2015	18	M	Urethral	GP	0.19	0.004	0.5	0.003	0.016	0.094	0.016	0.023	4	0.004	12	32		N
013	UHW	26/02/2015	27	M	Urethral	GP	0.19	0.006	1	0.012	0.016	0.38	0.023	0.008	4	0.004	8	16	0.125	N
014	UHW	06/01/2016	17	M	Corneal swab	Oph	0.38	0.004	0.38	0.008	≤0.016	0.38	0.023	0.008	6	0.003	12	24		N
024	UHW	27/12/2012	26	M	Urethral	GP	1.5	>32	0.38	0.094	0.25	1	0.38	0.064	4	1.5	8	48		N
016	UHW	30/09/2013	27	M	Urethral	GP	2	>32	1	0.047	0.19	1	0.25	0.032	6	1.5	12	32	0.094	N
017	UHW	18/12/2014	35	M	Urethral	STI	0.25	0.006	0.75	0.008	0.023	0.5	0.032	0.008	8	0.004	8	12	0.094	N
018	UHW	21/01/2015	25	M	Urethral	GP	1	4	0.125	0.006	0.016	12	0.012	0.006	3	1.5	6	64		P

025	UHW	23/11/2012	20	M	Urethral	GP	0.125	0.006	0.38	0.003	≤0.016	0.125	0.012	0.016	6	0.004	8	32		N
023	UHW	02/01/2013	20	F	Endocervical	STI	0.5	0.012	1	0.016	0.023	1	0.047	0.016	4	0.006	8	24	0.125	N
021	UHW	16/01/2013	23	F	Endocervical	STI	0.125	0.003	0.38	0.003	0.016	0.064	0.012	0.012	1	0.004	4	16		N
020	UHW	02/01/2013	21	M	Urethral	STI	0.125	0.003	0.25	0.002	≤0.016	0.094	0.012	0.016	2	0.003	6	24		N
019	UHW	02/01/2013	19	M	Urethral	STI	0.38	0.012	1	0.012	0.023	1.5	0.047	0.016	4	0.006	6	16	0.19	N
022	UHW	16/01/2013	23	M	Pharyngeal	STI	0.19	0.004	0.19	0.003	≤0.016	0.064	0.012	0.016	3	0.003	8	24		N
026	UHW	28/08/2014	27	M	Urethral	GP	0.19	0.004	0.5	0.003	≤0.016	0.094	0.008	0.016	2	0.002	4	16		N
027	UHW	23/11/2012	20	M	Urethral	GP	0.125	0.004	0.25	≤0.002	≤0.016	0.064	0.006	0.008	4	0.002	6	24		N
028	UHW	28/08/2014	27	M	Urethral	GP	0.19	0.004	0.38	0.003	0.016	0.094	0.016	0.016	4	0.003	8	24		N
029	UHW	02/10/2014	21	M	Urethral	GP	0.19	0.006	0.38	0.012	0.016	0.25	0.012	0.006	3	0.004	6	24		N
030	UHW	17/08/2015	18	M	Urethral	STI	0.125	0.003	0.094	0.008	≤0.016	0.38	0.008	0.016	4	≤0.002	8	16		N
031	UHW	30/09/2015	25	M	Urethral	STI	0.25	4	0.38	0.023	0.047	0.38	0.064	0.016	4	0.5	4	12		N
032	UHW	25/06/2015	29	M	Urethral	STI	4	0.75	0.032	0.006	≤0.016	8	0.004	0.008	3	0.094	4	16		P
033	UHW	23/01/2013	35	F	Endocervical	GP	0.25	0.004	0.38	0.008	0.016	0.38	0.016	0.012	4	0.004	8	16		N
034	UHW	11/09/2015	50	M	Urethral	STI	0.38	4	0.75	0.008	≤0.016	6	0.023	0.012	3	1.5	12	12	0.125	N
035	UHW	08/02/2016	23	M	Urethral	GP	0.75	>32	1	0.047	0.125	0.75	0.125	0.032	4	1.5	6	24	0.047	N
036	UHW	15/02/2016	24	M	Urethral	STI	0.19	0.006	0.38	0.003	≤0.016	0.125	0.016	0.016	6	0.004	12	24		N
037	UHW	06/01/2016	17	M	Corneal scrapings	Oph	0.38	0.004	0.5	0.023	0.032	0.5	0.047	0.032	6	0.003	8	32		N
038	UHW	16/03/2016	19	M	Urethral	STI	0.19	0.004	0.38	0.003	≤0.016	0.094	0.012	0.032	2	0.004	6	32		N
039	UHW	21/01/2015	25	M	Urethral	GP	1.5	4	0.25	0.012	0.016	8	0.016	0.016	6	1.5	8	96		P
90135	SJH	10/11/2015	28	M	Rectal	BS	0.125	≤0.002	0.032	0.006	≤0.016	0.125	0.006	0.008	4	≤0.002	6	24		N
181298	SJH	19/11/2015	19	M	STIM	Guide	0.125	0.002	0.125	0.004	≤0.016	0.125	0.004	0.012	4	≤0.002	6	24		N
181663	SJH	30/11/2015	57	M	STIM	Guide	0.064	0.004	0.016	0.006	≤0.016	12	0.004	0.008	2	≤0.002	6	8		N
91840	SJH	24/11/2015	31	M	Urethral	BS	1	0.008	0.25	0.023	0.032	0.38	0.047	0.016	4	0.008	6	16		N
91839	SJH	24/11/2015	29	M	Rectal	BS	0.19	0.004	0.25	0.008	≤0.016	0.19	0.016	0.012	3	0.004	8	32		N
91711	SJH	24/11/2015	50	M	STIM	BS	1	0.012	0.25	0.023	0.023	0.75	0.047	0.016	4	0.004	6	12		N
181716	SJH	01/12/2015	19	F	STI F	Guide	0.125	0.002	≤0.016	0.004	≤0.016	0.25	0.004	0.012	4	≤0.002	6	12		N
92523	SJH	01/12/2015	22	M	Pharyngeal	BS	0.38	8	0.125	0.023	0.032	0.38	0.047	0.016	4	0.75	6	12		N
181725	SJH	01/12/2015	35	M	Rectal	Guide	0.38	4	0.25	0.012	≤0.016	12	0.032	0.016	6	1.5	8	8		N
93987	SJH	15/12/2015	30	M	Rectal	BS	0.023	0.006	0.25	0.002	≤0.016	0.125	0.006	0.003	6	0.004	8	32		N
93236	SJH	08/12/2015	24	M	Rectal	BS	0.25	6	0.125	0.008	0.016	16	0.023	0.008	4	2	12	12		N
181825	SJH	03/12/2015	18	M	STIM	Guide	0.125	0.004	0.125	0.003	≤0.016	0.094	0.008	0.023	4	0.003	6	16		N
92761	SJH	02/12/2015	43	M	Pharyngeal	BS	0.25	8	0.25	0.032	0.047	0.75	0.094	0.023	6	1.5	8	16		N
180993	SJH	12/11/2015	34	M	STIM (+G)	Guide	0.19	0.006	0.5	0.004	0.016	0.5	0.012	0.016	8	0.004	12	32		N

91934	SJH	25/11/2015	25	M	STIM	BS	0.125	0.004	1	0.004	0.016	0.25	0.016	0.012	6	0.004	8	24	0.125	N
91125	SJH	18/11/2015	27	M	Rectal	BS	0.094	0.003	0.5	0.003	0.016	0.19	0.016	0.012	6	0.003	8	16		N
89478	SJH	04/11/2015	23	M	STIM	BS	0.19	0.004	16	0.002	≤0.016	0.38	0.016	0.012	6	0.004	8	24	0.19	N
180511	SJH	02/11/2015	29	M	Pharyngeal	Guide	0.5	0.016	2	0.008	0.016	1.5	0.094	0.012	8	0.016	12	24	0.19	N
93278	SJH	08/12/2015	22	M	STIM	BS	0.25	0.004	0.25	0.004	0.016	0.19	0.012	0.008	1.5	0.003	6	24		N
91838	SJH	24/11/2015	19	M	Pharyngeal	BS	0.19	0.006	1	0.006	0.023	0.75	0.016	0.012	8	0.006	8	24	0.125	N
91116	SJH	18/11/2015	53	M	STIM	BS	0.19	0.006	1	0.004	0.023	0.25	0.016	0.023	8	0.003	8	24	0.094	N
181413	SJH	23/11/2015	18	M	Pharyngeal	Guide	0.25	0.008	0.5	0.002	0.016	0.125	0.012	0.008	4	0.006	8	24		N
93526	SJH	09/12/2015	28	M	Pharyngeal	BS	0.125	0.006	0.5	0.004	0.023	0.38	0.023	0.008	4	0.004	6	48		N
181798	SJH	03/12/2015	20	F	Pharyngeal	Guide	0.25	0.008	>256	0.008	0.023	0.75	0.047	0.023	4	0.006	8	24	6	N
93358	SJH	08/12/2015	30	M	Rectal	BS	0.38	6	0.25	0.004	0.016	12	0.023	0.008	4	1.5	8	12		N
90947	SJH	17/11/2015	45	M	STIM	BS	0.19	0.006	0.5	0.006	0.023	0.38	0.023	0.023	4	0.004	12	24		N
182109	SJH	10/12/2015	20	M	Rectal	Guide	0.125	0.006	0.5	0.004	≤0.016	0.38	0.016	0.012	4	0.004	8	24		N
94019	SJH	15/12/2015	34	M	STIM	BS	0.125	0.006	0.5	0.006	0.023	0.38	0.023	0.012	8	0.003	8	16		N
94061	SJH	15/12/2015	30	M	Rectal	BS	0.125	12	0.125	0.016	0.032	0.5	0.047	0.012	3	1	6	8		N
93463	SJH	09/12/2015	24	M	Pharyngeal	BS	0.19	0.008	0.25	0.003	0.016	0.125	0.023	0.012	3	0.006	6	12		N
93978	SJH	15/12/2015	30	M	Rectal	BS	0.023	0.004	0.25	≤0.002	≤0.016	0.125	0.004	0.006	3	0.004	8	24		N
93488	SJH	09/12/2015	40	M	STIM	BS	0.25	0.006	0.5	0.003	0.016	0.125	0.016	0.016	6	0.006	8	32		N
87565	SJH	15/10/2015	75	M	Urethral	GP	0.19	4	0.064	0.008	0.016	16	0.012	0.012	8	1.5	8	24		N
180008	SJH	19/10/2015	31	M	Rectal	Guide	0.5	>32	0.25	0.047	0.047	0.5	0.047	0.023	6	1.5	4	12		N
88198	SJH	21/10/2015	21	M	Rectal	BS	0.094	0.002	0.032	0.008	0.016	0.38	0.008	0.016	4	≤0.002	6	24		N
180205	SJH	22/10/2015	22	m	Pharyngeal	NA	0.19	0.004	0.125	0.008	0.016	0.5	0.023	0.016	4	0.003	4	32		N
180212	SJH	23/10/2015	28	M	STIM (+G)	Guide	0.38	0.006	0.25	0.008	≤0.016	0.25	0.023	0.016	4	0.003	4	24		N
87912	SJH	20/10/2015	37	M	STIM	BS	0.25	0.006	0.5	0.012	0.032	0.5	0.023	0.016	8	0.004	12	24		N
179696	SJH	09/10/2015	31	M	Pharyngeal	Guide	0.25	0.004	1	0.008	0.023	0.25	0.016	0.012	6	0.006	8	24	0.094	N
179841	SJH	13/10/2015	26	M	Rectal	Guide	0.38	4	0.125	0.012	0.023	16	0.047	0.012	8	1.5	12	16		N
179842	SJH	13/10/2015	26	M	STIM	Guide	0.5	6	0.25	0.012	0.016	8	0.032	0.012	8	1.5	12	12		N
89346	SJH	03/11/2015	22	M	STIM	BS	0.19	0.004	0.125	0.006	≤0.016	0.094	0.016	0.004	4	0.004	4	24		N
180892	SJH	09/11/2015	34	M	STIM	Guide	0.38	4	0.25	0.008	≤0.016	0.25	0.016	0.012	8	0.75	8	24		N
90289	SJH	11/11/2015	26	M	Rectal	BS	0.094	≤0.002	0.032	0.006	≤0.016	0.19	0.006	0.008	4	≤0.002	4	12		N
90331	SJH	11/11/2015	31	F	STIM	BS	0.25	0.004	0.25	0.012	≤0.016	0.094	0.012	0.008	4	0.003	8	16		N
180704	SJH	05/11/2015	23	M	Rectal	Guide	0.25	4	0.25	0.012	≤0.016	8	0.016	0.012	4	0.75	8	8		N
180893	SJH	10/11/2015	24	M	STIM	Guide	0.125	0.002	0.064	0.004	≤0.016	0.19	0.006	0.012	3	≤0.002	6	16		N

90066	SJH	10/11/2015	28	M	Urethral	BS	0.25	0.003	0.125	0.004	≤0.016	0.19	0.012	0.004	2	0.002	6	32		N
90141	SJH	10/11/2015	24	M	Rectal	BS	0.125	0.003	0.125	0.004	≤0.016	0.094	0.012	0.006	8	0.003	6	16		N
88447	SJH	27/10/2015	35	M	Urethral	GP	0.5	8	0.25	0.012	0.016	0.5	0.032	0.008	4	0.75	12	24		N
88397	SJH	27/10/2015	26	M	Rectal	NA	0.5	3	0.125	0.016	0.023	0.19	0.023	0.008	8	0.75	12	24		N
180188	SJH	22/10/2015	52	F	STI F (+G)	Guide	0.19	0.006	1	0.006	0.023	0.25	0.012	0.016	8	0.004	12	32	0.094	N
179930	SJH	15/10/2015	55	M	STIM (+G)	Guide	16	4	0.125	0.004	0.023	16	0.023	0.008	4	1	8	48		P
179980	SJH	16/10/2015	28	M	STIM (+G)	Guide	0.19	0.004	1	0.003	0.016	0.38	0.016	0.006	6	0.004	6	24	0.125	N
180187	SJH	22/10/2015	55	M	STIM (+G)	Guide	0.19	0.006	0.5	0.008	0.032	0.38	0.032	0.016	8	0.006	8	24		N
179730	SJH	12/10/2015	39	M	STIM (+G)	Guide	0.19	0.003	0.5	0.002	≤0.016	0.19	0.016	0.012	4	0.006	8	32		N
88401	NMH	28/10/2015	18	M	Isolate	NMH	0.38	0.006	0.5	0.016	0.032	0.38	0.064	0.032	8	0.004	12	32		N
88116	SJH	21/10/2015	39	M	STIM	BS	0.19	0.006	0.5	0.006	0.023	0.38	0.023	0.008	6	0.003	6	24		N
180505	SJH	02/11/2015	35	M	STIM (+G)	Guide	0.5	0.032	2	0.012	0.023	2	0.064	0.016	16	0.012	12	24	0.125	N
88544	SJH	27/10/2015	28	M	STIM	BS	0.38	6	0.5	0.004	0.023	16	0.032	0.008	12	3	12	12		N
180296	SJH	27/10/2015	34	M	Rectal	Guide	0.25	0.006	0.5	0.004	≤0.016	0.19	0.023	0.008	4	0.004	12	48		N
180641	SJH	05/11/2015	33	F	STI F	Guide	0.5	>32	2	0.023	0.047	1	0.125	0.023	8	2	16	24	0.19	N
90030	SJH	10/11/2015	28	M	Urethral	GP	0.5	0.016	1	0.008	0.023	1	0.047	0.012	6	0.008	8	24	0.19	N
90299	SJH	11/11/2015	20	M	STIM	BS	0.19	0.006	0.5	0.004	0.032	0.5	0.023	0.016	6	0.004	8	32		N
87930	SJH	20/10/2015	25	M	Rectal	BS	0.125	0.006	0.5	0.006	0.023	0.5	0.023	0.016	6	0.006	12	24		N
85108	SJH	22/09/2015	28	M	Pharyngeal	BS	0.25	4	0.25	0.012	≤0.016	12	0.012	0.012	3	1.5	8	6		N
84652	SJH	16/09/2015	26	M	Rectal	BS	0.19	0.003	0.125	0.006	0.016	0.38	0.012	0.012	4	0.003	6	16		N
178540	SJH	14/09/2015	28	M	Rectal	Guide	0.25	>32	0.25	0.023	0.047	0.5	0.047	0.008	4	1.5	4	8		N
179122	SJH	28/09/2015	21	F	Cervical	Guide	0.5	>32	0.25	0.023	0.016	1	0.047	0.012	6	3	6	16		N
85104	SJH	22/09/2015	28	M	Pharyngeal	BS	0.5	>32	0.25	0.016	0.023	0.5	0.023	0.012	4	1.5	6	12		N
85106	SJH	22/09/2015	24	M	Rectal	BS	0.19	0.008	0.25	0.008	≤0.016	0.25	0.016	0.006	6	0.006	8	32		N
86089	SJH	30/09/2015	28	M	Pharyngeal	BS	0.19	0.008	0.25	0.008	0.016	0.125	0.023	0.008	6	0.006	6	8		N
179093	SJH	28/09/2015	24	M	STIM (+G)	Guide	0.094	0.004	0.064	0.004	≤0.016	0.25	0.006	0.006	8	≤0.002	6	24		N
85857	SJH	23/09/2015	45	M	Rectal	BS	0.094	0.003	0.125	0.006	≤0.016	0.19	0.012	0.008	2	0.002	6	16		N
179501	SJH	06/10/2015	26	F	STI F (+G)	Guide	0.25	3	0.25	0.016	0.094	0.25	0.094	0.023	8	0.75	8	12		N
86757	SJH	07/10/2015	30	M	STIM	BS	0.25	0.006	1	0.008	≤0.016	0.38	0.016	0.012	4	0.004	6	16	0.125	N
85798	SJH	29/09/2015	28	M	Rectal	BS	0.38	0.003	0.5	0.012	0.023	0.25	0.023	0.016	4	0.003	12	24		N
86789	SJH	07/10/2015	20	M	Rectal	BS	0.19	3	0.25	0.008	0.016	8	0.023	0.008	6	0.75	12	8		N
179652	SJH	08/10/2015	18	M	STIM (+G)	Guide	0.25	0.003	0.25	0.004	≤0.016	0.125	0.012	0.006	4	0.004	12	24		N
179296	SJH	01/10/2015	20	M	STIM (+G)	Guide	0.125	0.004	0.064	0.012	0.016	0.064	0.016	0.016	6	0.002	12	16		N
86607	SJH	06/10/2015	27	M	Rectal	BS	0.5	>32	0.25	0.023	0.047	0.75	0.064	0.012	4	1.5	6	16		N

179653	SJH	08/10/2015	18	M	Rectal	Guide	0.25	0.003	0.125	0.004	0.016	0.125	0.012	0.004	6	0.004	8	24		N
179487	SJH	06/10/2015	21	M	Rectal	Guide	0.5	>32	2	0.012	0.032	0.75	0.094	0.012	8	2	12	16	0.19	N
86788	SJH	07/10/2015	23	M	Pharyngeal	BS	0.25	6	0.5	0.008	0.016	16	0.016	0.006	6	3	12	6		N
179698	SJH	07/10/2015	23	F	STI F	Guide	16	3	0.064	0.004	0.016	16	0.016	0.008	8	0.75	6	32		P
84579	SJH	16/09/2015	23	M	STIM	BS	0.25	4	0.5	0.006	≤0.016	16	0.023	0.004	6	2	12	8		N
85105	SJH	22/09/2015	22	M	Pharyngeal	BS	0.25	0.006	2	0.004	0.016	0.5	0.023	0.012	4	0.008	12	24	0.38	N
86606	SJH	06/10/2015	28	M	STIM	BS	0.25	0.006	0.5	0.008	0.094	0.38	0.064	0.047	6	0.004	8	24		N
86090	SJH	30/09/2015	25	M	STIM	BS	1	0.012	0.5	0.012	0.023	0.5	0.047	0.016	4	0.006	6	12		N
86757	SJH	07/10/2015	30	M	STIM	BS	0.19	0.006	1	0.006	0.023	0.5	0.023	0.008	6	0.006	12	24		N
179411	SJH	05/10/2015	23	M	STIM (+G)	Guide	0.5	12	4	0.008	0.023	0.5	0.064	0.016	6	1	12	24	0.19	N
178696	SJH	17/09/2015	23	M	STIM	Guide	4	0.012	1	0.008	0.016	0.5	0.032	0.016	8	0.008	12	24	0.094	P
178575	SJH	15/09/2015	26	M	Pharyngeal	Guide	0.19	0.004	32	0.003	0.016	0.38	0.012	0.012	6	0.003	6	16	0.19	N
178919	SJH	22/09/2015	22	M	STIM (+G)	Guide	0.125	0.004	1	0.006	0.023	0.5	0.023	0.016	16	0.004	12	24	0.125	N
84537	SJH	16/09/2015	30	M	STIM	BS	0.25	0.004	1	0.004	0.032	0.25	0.016	0.008	4	0.004	8	24	0.125	N
179486	SJH	06/10/2015	21	M	Pharyngeal	Guide	0.5	>32	2	0.016	0.023	0.75	0.047	0.012	8	2	12	24	0.25	N
178608	SJH	15/09/2015	25	M	Rectal	Guide	0.19	0.004	1	0.006	0.023	0.38	0.023	0.016	6	0.004	8	24		N
178573	SJH	15/09/2015	26	M	STIM (+G)	Guide	0.125	0.004	32	0.004	0.016	0.38	0.012	0.008	4	0.004	8	12	0.19	N
177643	SJH	18/08/2016	33	m	STIM	Guide	0.75	0.016	0.25	0.012	0.032	0.75	0.047	0.016	4	0.006	12	12		N
81744	SJH	18/08/2015	28	M	Pharyngeal	BS	0.25	0.006	1	0.004	0.016	0.5	0.023	0.012	6	0.006	12	32	0.125	N
81765	SJH	18/08/2015	37	M	STIM	BS	0.25	6	0.5	0.003	≤0.016	0.38	0.016	0.008	6	2	12	24		N
177574	SJH	17/08/2015	19	M	STIM (+G)	Guide	1	8	0.5	0.047	0.094	0.5	0.125	0.016	3	1	8	16		N
81513	SJH	15/08/2015	60	M	STIM (+G)	GP	0.75	>32	0.5	0.032	0.19	0.75	0.125	0.064	6	2	12	16		N
82434	SJH	25/08/2015	41	M	Pharyngeal	BS	0.125	0.006	0.5	0.004	0.016	0.38	0.004	0.008	6	0.004	12	16		N
177829	SJH	24/08/2015	17	M	STIM	Guide	0.19	0.006	0.5	0.003	0.016	0.38	0.016	0.008	6	0.006	16	48		N
177850	SJH	24/08/2015	18	F	Urethral	Guide	0.125	0.006	0.5	0.004	0.016	0.25	0.016	0.012	6	0.004	16	16		N
83751	SJH	08/09/2015	31	M	STIM	BS	0.125	3	0.125	0.006	0.047	0.25	0.023	0.012	3	0.25	6	12		N
83064	SJH	01/09/2015	25	M	STIM	BS	0.38	0.006	1	0.006	0.023	0.38	0.047	0.016	6	0.006	12	24	0.19	N
178200	SJH	03/09/2015	28	M	Rectal	Guide	0.125	0.006	0.25	0.008	0.032	0.38	0.032	0.012	4	0.003	12	16		N
83085	SJH	01/09/2015	29	M	STIM	BS	0.125	0.006	0.25	0.003	0.016	0.094	0.016	0.008	6	0.004	8	16		N
178157	SJH	02/09/2015	25	M	Rectal	Guide	0.19	0.004	2	0.004	0.023	0.25	0.023	0.012	8	0.006	12	16	0.125	N
178367	SJH	09/09/2015	33	M	Rectal	Guide	0.125	3	0.125	0.006	0.023	0.19	0.023	0.008	4	0.25	8	12		N
178265	SJH	07/09/2015	29	M	STIM (+G)	Guide	0.5	>32	0.25	0.016	0.047	0.38	0.064	0.008	6	1.5	6	8		N
83954	SJH	09/09/2015	25	M	Rectal	BS	0.094	0.004	0.125	≤0.002	≤0.016	0.25	0.004	0.004	6	≤0.002	12	32		N

178487	SJH	11/09/2015	22	M	STIM (+G)	Guide	0.19	0.008	>256	0.006	0.023	0.5	0.047	0.032	4	0.004	6	12	1	N
178283	SJH	07/09/2015	28	M	Rectal	Guide	0.25	0.004	0.25	0.004	0.023	0.25	0.023	0.012	6	≤0.002	12	24		N
81743	SJH	18/08/2015	28	M	Rectal	BS	0.25	0.006	0.5	0.004	0.023	0.75	0.016	0.012	6	0.008	12	32		N
82432	SJH	25/08/2015	27	M	Rectal	BS	0.75	>32	1	0.023	0.125	1	0.25	0.032	6	6	12	24	0.19	N
177687	SJH	20/08/2015	23	M	STIM	Guide	0.19	4	0.5	0.003	≤0.016	16	0.016	0.012	6	1.5	12	12		P
177678	SJH	20/08/2015	32	M	Pharyngeal	Guide	0.125	0.006	0.5	0.004	0.023	0.38	0.016	0.016	4	0.004	12	24		N
78473	SJH	14/07/2015	27	M	Rectal	BS	0.125	0.006	0.5	0.003	0.016	0.38	0.016	0.008	8	0.002	6	16		N
78652	SJH	15/07/2015	29	M	Rectal	BS	0.25	0.006	0.25	0.002	≤0.016	0.125	0.012	0.008	3	0.002	8	16		N
79093	SJH	21/07/2015	22	M	STIM	BS	0.094	0.003	0.064	0.003	≤0.016	0.19	0.008	0.012	3	≤0.002	6	24		N
84397	SJH	15/09/2015	19	M	Rectal	BS	0.19	0.006	1	0.004	0.023	0.5	0.023	0.008	6	0.004	8	16	0.125	N
84414	SJH	15/09/2015	22	M	STIM	BS	0.5	>32	0.5	0.012	0.032	1	0.047	0.008	3	1	6	8		N
84444	SJH	15/09/2015	27	M	Rectal	BS	0.19	0.004	2	0.004	0.023	0.25	0.023	0.023	6	0.004	12	24	0.094	N
176624	SJH	20/07/2015	30	M	Rectal	Guide	0.38	12	0.25	0.016	0.064	0.38	0.094	0.016	3	0.5	4	12		N
78487	SJH	14/07/2015	23	M	STIM	BS	0.25	4	0.25	0.012	0.047	0.19	0.047	0.012	3	0.38	4	6		N
84117	SJH	11/09/2015	23	M	STIM (+G)	GP	4	0.012	1	0.008	0.016	0.5	0.032	0.012	6	0.004	12	48	0.064	N
178453	SJH	10/09/2015	22	F	Urethral	Guide	0.064	0.004	0.125	≤0.002	≤0.016	0.094	0.008	0.006	3	≤0.002	4	16		N
84372	SJH	15/09/2015	24	M	Pharyngeal	BS	0.38	6	0.25	0.006	0.016	0.25	0.023	0.012	8	0.38	8	16		N
78653	SJH	15/07/2015	30	M	STIM	BS	0.75	0.023	0.5	0.006	0.023	1	0.032	0.016	6	0.006	8	8		N
178488	SJH	14/09/2015	27	M	STIM (+G)	Guide	1	>32	0.5	0.008	0.016	0.75	0.032	0.008	4	1	6	12		N
176394	SJH	13/07/2015	27	M	Urethral	Guide	0.25	0.004	0.25	0.006	0.023	0.25	0.032	0.016	1.5	0.002	6	48		N
176561	SJH	16/07/2015	24	M	STIM	Guide	0.75	>32	0.5	0.016	0.125	0.5	0.19	0.032	6	1.5	8	12		N
176590	SJH	16/07/2015	25	M	Pharyngeal	Guide	>32	3	0.5	0.002	≤0.016	0.25	0.016	0.006	6	0.75	12	16		P
178526	SJH	15/07/2015	26	M	Pharyngeal	Guide	1	>32	1	0.032	0.19	0.75	0.25	0.032	6	1	8	16	0.032	N
177148	SJH	05/08/2015	21	M	STIM	Guide	1	>32	0.5	0.016	0.023	1	0.047	0.023	6	1.5	6	24		N
79822	SJH	28/07/2015	27	M	Pharyngeal	BS	0.125	0.004	0.25	0.003	0.016	0.19	0.012	0.008	6	0.004	6	24		N
79823	SJH	28/07/2015	32	M	Rectal	BS	0.75	2	0.25	0.016	0.064	0.38	0.094	0.008	3	0.75	8	12		N
80392	SJH	04/08/2015	31	M	Rectal	BS	0.19	0.004	0.5	0.003	0.016	0.25	0.012	0.008	6	0.003	6	24		N
176715	SJH	21/07/2015	25	M	Rectal	Guide	0.19	0.006	0.25	0.003	≤0.016	0.094	0.008	0.004	2	0.004	8	16		N
176805	SJH	23/07/2015	44	M	Rectal	Guide	0.125	0.004	0.064	0.004	0.023	0.38	0.012	0.008	4	≤0.002	12	24		N
176812	SJH	23/07/2015	27	M	STIM (+G)	Guide	1	0.016	0.5	0.012	0.032	0.38	0.047	0.012	3	0.006	6	12		N
79111	SJH	21/07/2015	24	M	Pharyngeal	BS	8	6	0.125	0.004	≤0.016	0.38	0.023	0.008	6	0.5	8	16		P
79824	SJH	28/07/2015	32	M	Pharyngeal	BS	0.38	3	0.25	0.023	0.064	0.5	0.094	0.012	6	0.75	8	12		N
79315	SJH	22/07/2015	35	M	STIM	BS	0.125	0.006	0.5	0.002	≤0.016	0.125	0.008	0.006	4	0.004	8	24		N
176835	SJH	23/07/2015	27	M	STIM (+G)	Guide	1	0.012	0.5	0.008	0.016	0.38	0.023	0.008	4	0.004	12	12		N

79119	SJH	21/07/2015	29	M	STIM	BS	0.094	0.004	0.25	0.003	0.016	0.38	0.006	0.008	4	≤0.002	6	24		N
68429	SJH	30/07/2014	26	M	Rectal	BS	0.094	0.004	0.38	0.006	0.023	0.38	0.032	0.016	6	0.003	8	12		N
68334	SJH	29/07/2014	23	M	Pharyngeal	BS	0.125	0.004	0.38	0.004	0.023	0.5	0.016	0.012	6	0.003	12	12		N
67810	SJH	22/07/2014	29	M	Pharyngeal	BS	0.38	0.016	0.75	0.012	0.047	0.5	0.064	0.016	8	0.006	12	16		N
68732	SJH	01/08/2014	23	F	EYE	NMH	1	>32	1	0.016	0.023	1	0.064	0.012	4	1	12	12	0.064	N
68430	SJH	30/07/2014	26	M	Pharyngeal	BS	0.125	0.004	0.25	0.002	0.023	0.38	0.016	0.012	4	0.012	12	16		N
68901	SJH	05/08/2014	32	M	STIM	BS	0.125	0.004	0.25	≤0.002	≤0.016	0.38	0.012	0.008	6	0.002	12	24		N
68335	SJH	29/07/2014	53	M	Pharyngeal	BS	0.125	0.004	0.5	0.004	0.023	0.38	0.023	0.023	6	0.002	12	16		N
286608	SJH	21/07/2014	20	M	Pharyngeal	Guide	0.094	0.004	0.5	0.004	0.016	0.25	0.016	0.016	4	≤0.002	6	12		N
67818	SJH	22/07/2014	31	M	Pharyngeal	BS	0.19	>32	6	0.012	0.023	0.38	0.064	0.012	3	1.5	8	8	0.125	N
66932	SJH	09/07/2014	27	M	STIM	BS	0.19	8	0.25	0.006	0.023	0.5	0.047	0.008	3	1	6	6		N
286008	SJH	03/07/2014	29	M	STIM	Guide	0.75	>32	0.38	0.023	0.094	1	0.19	0.032	6	0.75	16	24		N
66942	SJH	09/07/2014	20	M	STIM	BS	0.125	0.006	0.38	0.002	0.016	0.38	0.012	0.008	6	0.003	16	12		N
286514	SJH	17/07/2014	30	M	STIM (+G)	Guide	0.125	0.004	0.38	0.003	0.023	0.38	0.016	0.012	6	0.002	12	12		N
68922	SJH	05/08/2014	30	M	Rectal	BS	0.125	0.004	0.25	0.002	0.023	0.25	0.016	0.012	4	0.002	12	12		N
286745	SJH	24/07/2014	20	F	STI F	Guide	0.19	0.006	0.38	≤0.002	0.016	0.094	0.016	0.023	4	0.002	12	24		N
287671	SJH	25/08/2014	21	M	STIM	Guide	0.38	0.012	0.38	0.006	0.023	0.75	0.064	0.016	4	0.003	12	12		N
287593	SJH	21/08/2014	23	M	STIM (+G)	Guide	0.125	0.004	0.38	0.006	0.023	0.5	0.032	0.016	6	0.004	12	12		N
69988	SJH	19/08/2014	23	M	Rectal	BS	0.38	12	0.38	0.008	0.032	1	0.064	0.012	3	0.75	12	8		N
69989	SJH	19/08/2014	24	M	Rectal	BS	0.19	>32	8	0.008	0.023	0.5	0.064	0.012	1.5	0.75	12	8	0.094	N
70196	SJH	20/08/2014	26	M	STIM	BS	0.19	0.008	0.75	0.006	0.032	0.5	0.047	0.012	1.5	0.002	12	12	0.094	N
69517	SJH	12/08/2014	32	M	STIM	BS	0.125	0.003	0.38	0.004	0.032	0.38	0.023	0.016	4	0.002	12	12		N
70842	SJH	27/08/2014	45	M	STIM	BS	0.094	0.003	0.047	≤0.002	≤0.016	0.19	0.004	0.008	2	≤0.002	6	12		N
69899	SJH	18/08/2014	25	M	Urethral	GP	0.19	0.003	0.75	0.004	0.023	0.38	0.016	0.012	8	0.002	16	12	0.064	N
70646	SJH	26/08/2014	23	M	Rectal	BS	0.5	>32	0.25	0.023	0.125	1	0.064	0.032	4	0.004	12	12		N
70523	SJH	25/08/2014	26	M	Urethral	GP	0.25	0.012	0.75	0.006	0.023	0.5	0.047	0.032	6	0.003	12	12	0.094	N
287707	SJH	26/08/2014	23	M	STIM (+G)	Guide	0.125	0.006	0.25	≤0.002	≤0.016	0.094	0.006	0.023	2	0.002	8	16		N
71378	SJH	03/09/2014	33	M	Rectal	BS	0.19	0.006	0.38	0.004	0.016	0.38	0.012	0.012	1.5	0.003	16	16		N
70988	SJH	29/08/2014	48	M	Urethral	GP	0.19	0.004	0.75	0.004	0.016	0.25	0.016	0.008	6	0.003	12	12	0.125	N
70647	SJH	26/08/2014	25	M	Pharyngeal	BS	0.38	>32	0.5	0.016	0.047	1	0.094	0.023	3	0.75	12	12		N
71379	SJH	03/09/2014	33	M	Pharyngeal	BS	0.094	0.004	0.25	0.002	≤0.016	0.25	0.006	0.008	4	0.003	16	24		N
288421	SJH	16/09/2014	26	M	STIM (+G)	Guide	0.125	0.006	0.5	0.003	0.016	0.38	0.008	0.006	4	0.003	8	24		N
72411	SJH	16/09/2014	38	M	STIM	BS	0.125	0.004	0.25	0.004	0.023	0.38	0.016	0.008	4	0.003	8	12		N

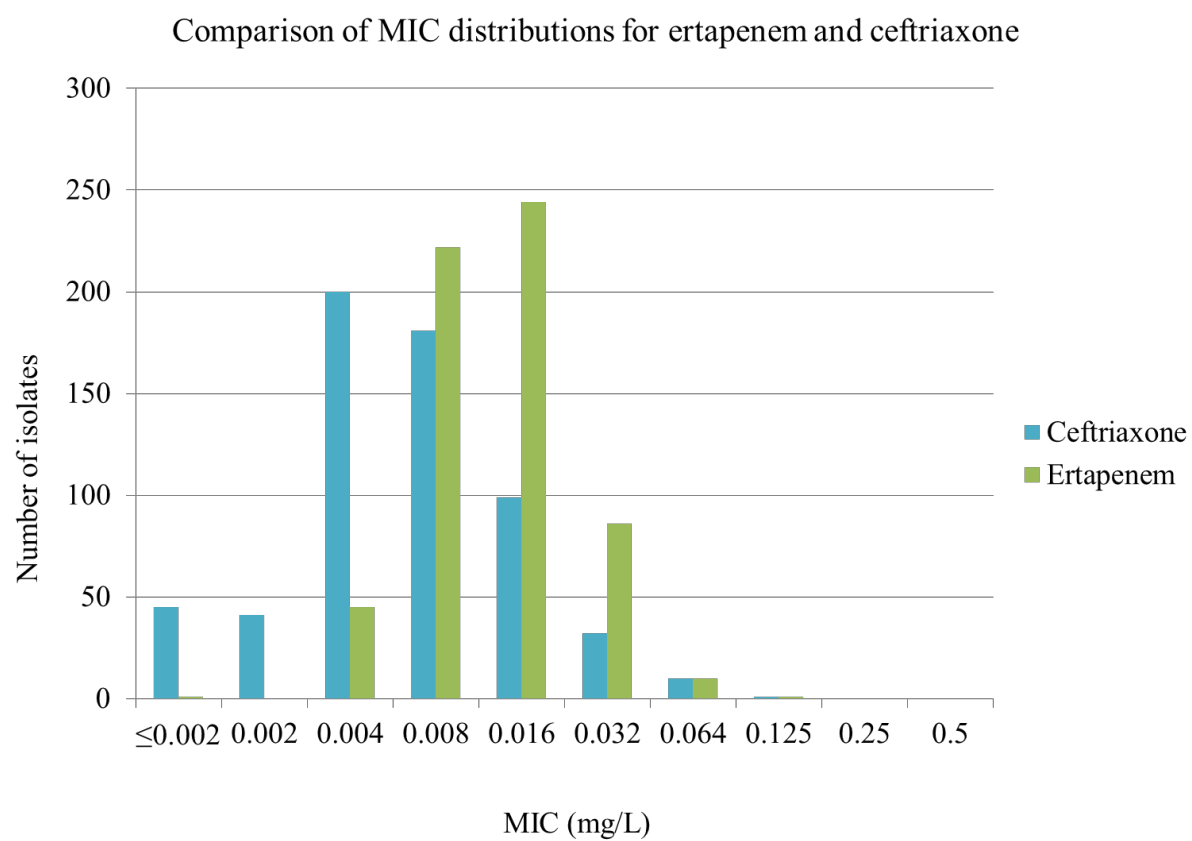
71672	SJH	09/09/2014	20	M	Rectal	BS	0.19	0.006	0.75	0.006	0.023	0.38	0.023	0.012	4	0.004	12	24	0.094	N
71670	SJH	09/09/2014	25	M	Pharyngeal	BS	1	>32	0.125	0.008	0.016	0.5	0.032	0.012	2	0.75	6	16		N
72518	SJH	17/09/2014	23	M	Rectal	BS	0.25	4	0.25	0.012	0.032	0.5	0.032	0.006	3	1.5	8	12		N
288483	SJH	18/09/2014	18	M	STIM	Guide	0.5	0.004	0.25	0.008	0.023	0.38	0.094	0.016	4	0.003	12	24		N
72431	SJH	16/09/2014	43	M	Pharyngeal	BS	0.125	3	0.25	0.003	≤0.016	0.25	0.008	0.004	6	0.75	12	16		N
72606	SJH	18/09/2014	32	M	Rectal	BS	0.25	0.006	0.38	0.004	0.016	0.125	0.016	0.008	8	0.006	12	16		N
288032	SJH	04/09/2014	38	M	Pharyngeal	Guide	0.19	4	0.064	0.003	0.016	0.125	0.004	0.008	2	0.25	6	8		N
73001	SJH	23/09/2014	25	M	Pharyngeal	BS	0.19	0.003	0.5	0.004	0.016	0.25	0.016	0.008	6	0.002	12	24		N
73070	SJH	23/09/2014	25	M	STIM	BS	0.19	0.003	0.75	0.004	0.023	0.38	0.016	0.008	2	0.002	16	24	0.125	N
72428	SJH	16/09/2014	28	M	Rectal	BS	1	0.016	0.38	0.006	0.023	1	0.032	0.008	3	0.004	12	12		N
72517	SJH	17/09/2014	23	M	Pharyngeal	BS	0.38	0.008	0.25	≤0.002	≤0.016	0.19	0.016	0.023	4	0.003	24	24		N
72127	SJH	12/09/2014	22	M	STIM (+G)	GP	0.094	3	0.064	≤0.002	≤0.016	0.19	≤0.002	0.006	4	0.094	6	12		N
72426	SJH	16/09/2014	22	M	Rectal	BS	0.75	0.016	0.5	0.012	0.023	0.5	0.047	0.008	4	0.006	12	12		N
288504	SJH	18/09/2014	25	M	STIM	Guide	0.094	0.006	0.125	≤0.002	≤0.016	0.094	0.004	0.006	2	0.002	6	6		N
71671	SJH	09/09/2014	29	M	Pharyngeal	BS	1.5	>32	0.5	0.016	0.023	0.75	0.094	0.012	4	1.5	8	32		N
71324	SJH	03/09/2014	36	M	STIM	BS	4	>32	0.125	0.047	0.19	24	0.19	0.094	1.5	3	6	6		P
70200	SJH	20/08/2014	40	M	Rectal	BS	0.125	0.004	0.19	0.002	0.016	0.19	0.012	0.008	3	≤0.002	12	16		N
287333	SJH	14/08/2014	25	F	STI F	Guide	0.125	0.006	0.25	≤0.002	≤0.016	0.064	0.008	0.023	1.5	0.003	8	24		N
69707	SJH	14/08/2014	21	M	Isolate	COL	0.25	0.012	1	0.012	0.023	0.75	0.094	0.032	6	0.004	8	16	0.094	N
68931	SJH	05/08/2014	38	M	Rectal	BS	1	>32	0.5	0.032	0.125	1	0.25	0.047	8	1.5	12	12		N
73828	SJH	01/10/2014	28	M	Rectal	BS	0.125	0.006	0.125	0.002	≤0.016	0.25	0.012	0.008	8	0.002	16	32		N
290029	SJH	04/11/2014	29	M	STIM (+G)	Guide	0.094	0.004	0.125	≤0.002	≤0.016	0.125	0.004	0.004	6	≤0.002	8	6		N
74324	SJH	07/10/2014	42	M	Pharyngeal	BS	0.125	0.003	0.38	0.012	0.047	0.25	0.023	0.016	4	0.003	12	24		N
76972	SJH	07/11/2014	30	M	STIM	GP	1.5	>32	0.75	0.047	0.19	1	0.25	0.032	4	2	12	32		N
289990	SJH	03/11/2014	26	M	STIM (+G)	Guide	0.19	0.006	0.25	0.002	≤0.016	0.125	0.012	0.023	4	0.004	8	24		N
290130	SJH	06/11/2014	27	F	STI F	Guide	0.19	0.006	0.5	≤0.002	≤0.016	0.125	0.012	0.023	4	0.004	12	32		N
76547	SJH	04/11/2014	23	M	STIM	BS	0.19	0.006	0.75	0.004	0.016	0.38	0.012	0.008	4	0.004	12	24		N
289160	SJH	07/10/2014	31	M	STIM (+G)	Guide	0.19	0.006	0.25	0.003	0.016	0.25	0.012	0.008	4	0.002	16	16		N
74323	SJH	07/10/2014	31	M	Urethral	BS	0.25	3	0.125	0.002	0.016	12	0.012	0.008	4	0.75	8	16		N
73644	SJH	30/09/2014	30	M	Pharyngeal	BS	0.25	6	0.38	0.012	0.032	0.75	0.064	0.006	6	0.75	12	24		N
289003	SJH	02/10/2014	20	F	Cervical	Guide	0.125	0.006	0.75	0.003	0.016	0.25	0.008	0.008	6	0.004	16	16		N
289139	SJH	08/09/2014	25	M	STIM (+G)	Guide	0.75	0.016	0.5	0.008	0.032	0.5	0.064	0.032	6	0.004	16	24		N
74325	SJH	07/10/2014	38	M	Pharyngeal	BS	0.125	0.006	0.75	0.002	≤0.016	0.25	0.012	0.012	6	0.004	16	24		N
73277	SJH	24/09/2014	27	M	Urethral	BS	0.19	0.006	0.19	0.003	≤0.016	16	0.016	0.008	6	0.002	16	32		N

73273	SJH	24/09/2014	23	M	Pharyngeal	BS	0.125	0.003	0.5	0.004	0.023	0.38	0.016	0.012	6	0.002	12	16		N
288587	SJH	22/09/2014	25	M	Pharyngeal	Guide	0.19	0.006	0.5	0.003	0.016	0.25	0.008	0.006	4	0.003	8	24		N
288997	SJH	02/10/2014	23	M	STIM (+G)	Guide	0.125	0.004	0.5	0.003	0.016	0.38	0.008	0.006	6	0.004	16	16		N
289368	SJH	13/10/2014	22	M	STIM	Guide	0.19	0.008	1	0.004	0.016	0.5	0.012	0.012	6	0.004	12	12		N
76054	SJH	28/10/2014	34	M	STIM	BS	0.19	0.006	0.25	0.004	0.016	0.25	0.023	0.012	6	0.003	16	24		N
289745	SJH	23/10/2014	26	M	Rectal	Guide	0.125	0.004	0.5	0.002	≤0.016	0.38	0.006	0.012	6	0.004	16	24		N
289595	SJH	20/10/2014	23	M	STIM	Guide	0.75	0.012	0.38	0.004	0.016	1	0.023	0.012	6	0.008	12	12		N
76216	SJH	29/10/2014	25	M	Pharyngeal	BS	0.38	6	0.25	0.012	0.047	0.5	0.064	0.012	3	1.5	12	16		N
75423	SJH	09/10/2014	21	M	Urethral	MUL	0.094	0.004	0.19	≤0.002	≤0.016	0.094	0.004	0.016	2	0.004	8	12		N
289472	SJH	16/10/2014	36	M	STIM (+G)	Guide	0.125	0.006	0.38	0.004	0.023	0.38	0.012	0.008	6	0.004	16	16		N
75746	SJH	22/10/2014	20	M	Rectal	BS	0.125	0.004	0.38	0.004	0.032	0.38	0.016	0.012	4	0.004	12	16		N
74608	SJH	08/10/2014	21	M	Rectal	BS	0.125	1.5	0.023	0.002	≤0.016	0.25	0.002	0.008	4	0.125	6	24		N
289755	SJH	23/10/2014	19	F	Cervical	Guide	0.094	0.006	0.25	≤0.002	≤0.016	0.064	0.008	0.023	3	0.002	8	24		N
289654	SJH	21/10/2014	27	M	Rectal	Guide	0.19	0.004	0.25	0.004	0.016	0.25	0.032	0.016	6	0.003	12	32		N
289655	SJH	21/10/2014	27	F	Cervical	Guide	0.25	0.006	0.38	0.006	0.032	0.38	0.032	0.016	4	0.003	12	16		N
76543	SJH	04/11/2014	35	M	STIM	BS	0.5	4	0.5	0.012	0.016	0.25	0.032	0.012	6	1.5	12	24		N
75180	SJH	15/10/2014	24	M	STIM	BS	0.25	0.006	0.25	0.003	0.016	0.5	0.016	0.008	6	0.004	12	16		N
75033	SJH	14/10/2014	23	M	Rectal	BS	0.19	0.006	0.75	0.006	0.023	0.38	0.016	0.016	6	0.006	12	16		N
75029	SJH	14/10/2014	29	M	Pharyngeal	BS	0.094	0.002	0.38	0.003	0.023	0.38	0.016	0.012	4	0.002	12	16		N
73274	SJH	24/09/2014	23	M	Rectal	BS	0.125	0.004	0.5	0.004	≤0.016	0.38	0.008	0.008	4	0.002	12	24		N
415428	UHL	21/04/2016	20	M	Rectal	NA	0.125	2	0.032	0.002	0.032	0.5	0.047	0.012	3	1.5	2	8		N
420132	UHL	08/08/2016	19	M	Urethral	NA	>32	2	0.064	0.008	≤0.016	0.5	0.008	0.012	8	0.5	12	12		P
421188	UHL	12/08/2016	18	M	Eye	NA	0.25	0.008	0.5	0.008	0.032	1	0.094	0.023	6	0.004	16	12		N
422671	UHG	28/09/2016	26	M	NA	NA	0.5	>32	0.5	0.064	0.125	2	0.25	0.064	4	2	16	12		N
421916	UHL	12/09/2016	20	M	Urethral	NA	0.25	4	0.25	0.016	0.016	1	0.047	0.012	6	0.75	8	12		N
423306	UHL	12/10/2016	21	M	Urethral	NA	0.125	0.004	0.5	0.008	0.032	0.5	0.023	0.008	6	0.004	16	16		N
414994	UHL	12/04/2016	18	F	Cervical	NA	0.5	0.008	0.5	0.016	0.064	0.5	0.094	0.032	4	0.004	16	16		N
417376	UHL	30/05/2016	28	M	Urethral	NA	0.25	0.004	0.25	0.004	≤0.016	0.25	0.012	0.012	3	0.002	16	8		N
418138	UHL	21/06/2016	21	M	Rectal	NA	0.25	2	0.25	0.004	≤0.016	0.25	0.008	0.006	6	1.5	8	12		N
422375	UHL	13/09/2016	29	M	Urethral	NA	0.25	≤0.002	0.5	0.008	≤0.016	0.25	0.023	0.012	4	0.004	16	24		N
422374	UHL	13/09/2016	18	F	Cervical	NA	0.5	0.008	0.5	0.016	0.016	1	0.125	0.032	4	0.006	16	16		N
424251	UHG	26/10/2016	22	NA	Urethral	NA	0.25	0.004	0.5	≤0.002	≤0.016	0.125	0.016	0.023	4	0.004	12	32		N
414993	UHL	12/04/2016	25	M	Urethral	NA	0.25	0.008	0.5	0.008	0.032	0.5	0.094	0.047	4	0.004	16	16		N

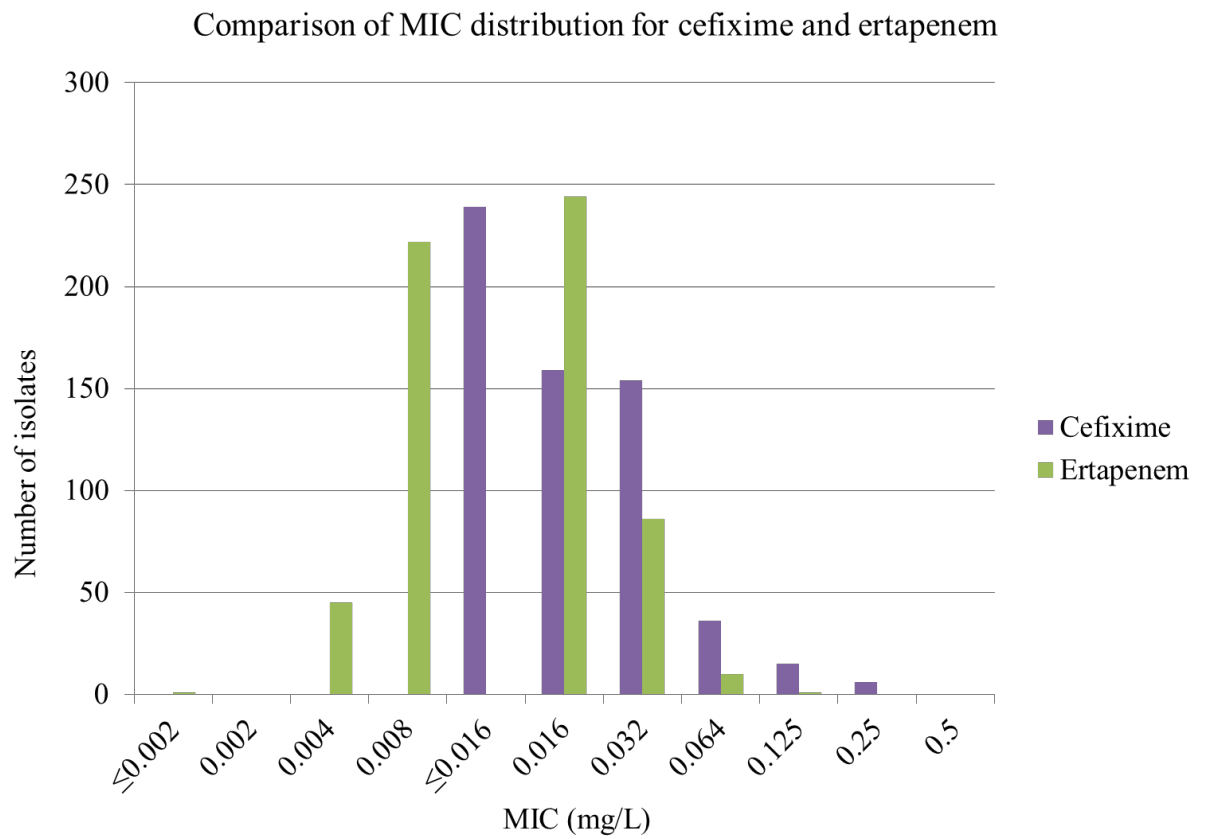
421976	MUL	13/09/2016	3	NA	NA	NA	0.25	0.008	0.5	0.016	0.016	1	0.047	0.023	3	0.003	8	8		N
423238	UHG	08/10/2016	19	F	Eye	NA	0.25	0.008	0.5	0.008	0.032	0.25	0.047	0.023	3	0.004	16	8		N
422670	UHG	16/09/2016	48	NA	Isolate	NA	0.5	>32	1	0.016	0.032	1	0.064	0.016	4	2	16	12	0.19	N
422751	UHG	21/09/2016	22	NA	Isolate	NA	0.25	0.004	0.125	0.006	0.016	1	0.047	0.016	3	0.003	8	48		N
423307	UHL	12/10/2016	19	M	Urethral	NA	0.25	0.004	0.25	≤0.002	≤0.016	0.125	0.008	0.023	2	0.003	16	24		N
421917	UHL	12/09/2016	21	F	Cervical	NA	0.25	2	0.25	0.032	0.032	1	0.125	0.016	6	1	16	12		N
424925	UHL	15/11/2016	20	F	Cervical	NA	0.25	0.016	0.25	0.008	0.016	0.5	0.064	0.023	6	0.004	16	16		N
424252	UHG	26/10/2016	19	F	Cervical	NA	0.125	0.004	0.064	≤0.002	≤0.016	0.5	0.004	0.023	4	≤0.002	16	16		N
424657	UHG	04/11/2016	20	NA	Urethral	NA	0.125	0.008	0.5	≤0.002	≤0.016	0.125	0.012	0.016	4	0.003	8	24		N
423877	UHG	26/10/2016	61	NA	Isolate	NA	0.25	0.008	0.5	0.004	0.032	0.5	0.012	0.008	4	0.004	8	16		N
424496	UHL	01/11/2016	36	M	Urethral	NA	0.125	0.004	0.125	0.002	≤0.016	0.125	0.008	0.004	6	0.003	8	12		N
424658	UHG	03/11/2016	20	M	Urethral	NA	0.125	0.008	0.5	≤0.002	≤0.016	0.125	0.016	0.023	6	0.004	8	24		N
424924	UHG	07/11/2016	23	M	Urethral	NA	0.125	0.004	0.5	0.002	≤0.016	0.125	0.047	0.016	4	0.004	8	12		N
423968	UHG	19/02/2016	21	M	Urethral	NA	0.25	0.008	0.5	0.004	≤0.016	0.25	0.012	0.008	6	0.004	16	16		N
425669	UHL	29/11/2016	18	M	Urethral	NA	0.125	0.004	0.125	≤0.002	≤0.016	0.125	0.016	0.032	3	0.004	16	32		N
425569	UHL	23/11/2016	55	M	Urethral	NA	0.125	8	0.25	0.008	0.032	0.5	0.032	0.008	4	2	8	12		N
422375	UHL	13/09/2016	29	M	Urethral	NA	0.25	≤0.002	0.5	0.008	≤0.016	0.25	0.016	0.008	4	0.004	16	16		N
425205	UHL	16/11/2016	43	M	Urethral	NA	0.125	0.004	0.5	≤0.002	≤0.016	0.125	0.012	0.023	3	0.003	8	32		N
422374	UHL	13/09/2016	18	F	Cervical	NA	0.5	0.008	0.5	0.016	0.016	1	0.064	0.032	4	0.004	16	12		N
425656	UHG	25/11/2016	24	M	Urethral	NA	0.25	0.008	0.25	≤0.002	≤0.016	0.125	0.064	0.016	6	0.004	8	8		N
424986	UHL	14/11/2016	19	M	NA	NA	0.125	0.004	0.25	≤0.002	≤0.016	0.125	0.012	0.023	3	0.003	8	24		N
425657	UHG	23/11/2016	27	M	Urethral	NA	0.25	16	4	0.016	0.032	1	0.012	0.006	3	1.5	8	16	0.094	N
425668	UHL	28/11/2016	33	M	Urethral	NA	0.25	0.008	0.5	≤0.002	≤0.016	0.25	0.016	0.006	3	0.004	16	32		N
425339	UHL	15/11/2016	43	M	Rectal	NA	0.25	8	0.5	0.004	≤0.016	16	0.032	0.006	6	3	16	32		N
425658	UHG	25/11/2016	20	F	Cervical	NA	0.5	0.016	1	0.004	≤0.016	0.5	0.032	0.016	4	0.004	16	16	0.064	N
423776	UHL	17/10/2016	43	M	Urethral	NA	0.25	0.004	0.25	0.002	≤0.016	0.032	0.016	0.008	6	0.006	8	12		N
425894	UHL	01/12/2016	19	F	Cervical	NA	0.25	0.008	0.25	≤0.002	≤0.016	0.125	0.016	0.016	3	0.003	16	48		N
425204	UHL	15/11/2016	20	F	Cervical	NA	0.25	0.016	0.5	0.016	0.125	0.125	0.125	0.032	4	0.006	16	12		N
54167	UHG	11/12/2015	19	F	NA	STI	0.25	0.004	0.25	0.008	0.032	0.5	0.047	0.023	4	0.003	12	32		N
47632	UHG	30/10/2015	28	M	NA	STI	1.5	0.023	1	0.016	0.032	1	0.064	0.023	6	0.008	12	12	0.094	N
46700	UHG	23/10/2015	19	M	NA	STI	0.25	0.004	0.25	0.004	0.016	0.38	0.016	0.016	6	0.003	12	16		N
35251	UHG	21/08/2015	46	M	NA	GP	0.125	0.004	0.25	0.004	0.016	0.25	0.016	0.012	4	0.006	12	32		N
54169	UHG	11/12/2015	18	F	NA	STI	0.125	0.004	0.25	≤0.002	≤0.016	0.047	0.008	0.016	1.5	0.003	4	24		N
54166	UHG	11/12/2015	19	M	NA	STI	0.094	0.003	0.19	≤0.002	≤0.016	0.032	0.004	0.012	1.5	0.003	4	12		N

32424	UHG	03/09/2014	26	M	NA	STI	0.19	0.006	0.75	0.006	0.023	0.38	0.016	0.016	8	0.004	12	32	0.094	N
33542	UHG	10/09/2014	28	M	NA	STI	0.5	>32	0.25	0.023	0.047	0.5	0.047	0.012	6	3	8	12		N
29806	UHG	15/08/2014	21	M	NA	GP	0.25	0.008	0.25	0.004	≤0.016	0.38	0.023	0.012	4	0.004	8	16		N
16460	UHG	22/04/2015	29	M	NA	STI	0.38	0.012	2	0.012	0.023	1	0.047	0.012	6	0.012	16	32	0.19	N
41277	UHG	29/10/2014	35	F	NA	STI	0.125	0.004	12	0.003	≤0.016	0.25	0.012	0.008	4	0.003	6	12	0.125	N
53105	UHG	04/12/2015	24	M	NA	STI	0.25	0.006	0.25	0.008	0.023	0.38	0.047	0.016	6	0.004	12	32		N
52612	UHG	01/12/2015	16	M	Corneal	Oph	0.19	0.006	0.38	0.002	0.016	0.094	0.012	0.023	4	0.004	12	32		N
31104	UHG	26/08/2014	47	M	NA	ED	1.5	6	0.064	0.002	≤0.016	12	0.008	0.012	6	2	12	16		P

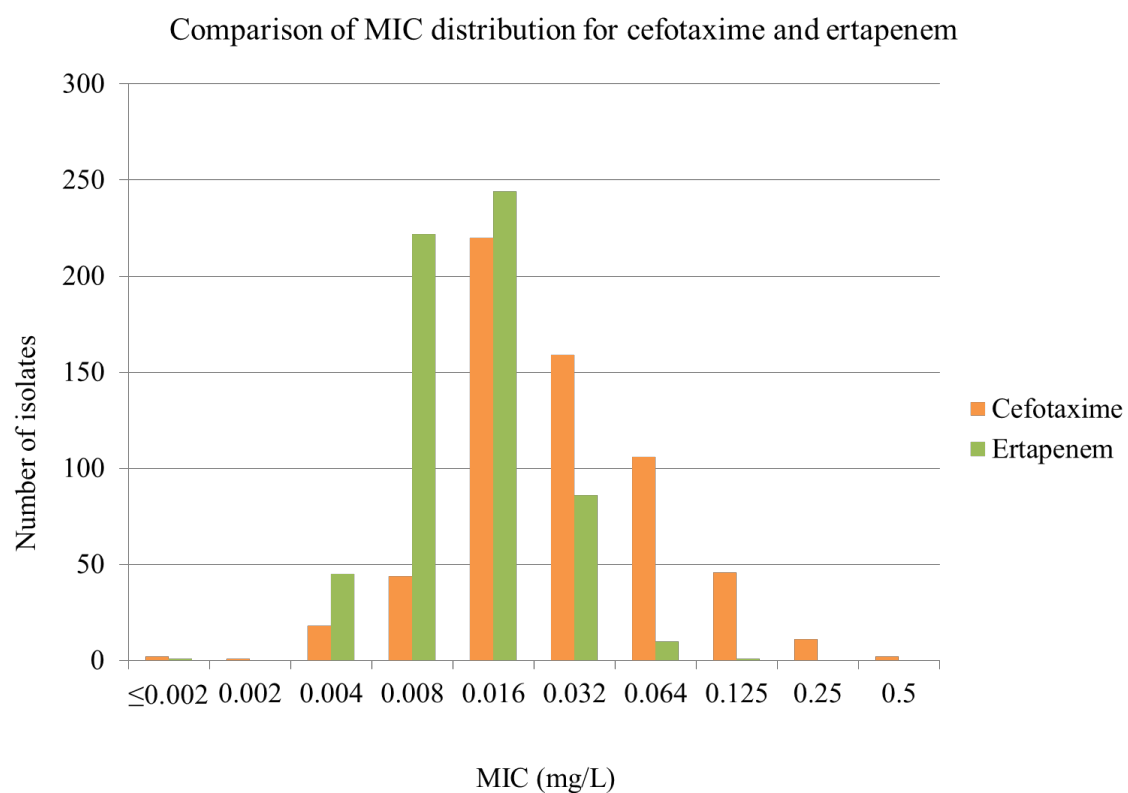
Appendix 2. Ertapenem MIC distribution compared to that of ceftriaxone.



Appendix 3. Ertapenem MIC distribution compared to that of cefixime.



Appendix 4. Ertapenem MIC distribution compared to that of cefotaxime.



Appendix 5. Solithromycin QC agar dilution MIC results *Neisseria gonorrhoeae* ATCC49226. One set of agar dilution plates were inoculated 12 times resulting in highly variable MICs with only 7 within the QC pass range (highlighted green).

QC ATCC 49226 <i>N. gonorrhoeae</i>	Solithromycin MIC (mg/L)
1	0.125
2	≤ 0.016
3	0.125
4	≤ 0.016
5	0.032
6	≤ 0.016
7	0.125
8	≤ 0.016
9	0.032
10	≤ 0.016
11	0.125
12	0.032

Appendix 6. Azithromycin gradient MICs compared to solithromycin gradient MIC, zone diameter by disc diffusion and agar dilution MIC for 96 isolates.

Number	AZM gradient MIC mg/L	Solithromycin gradient MIC mg/L	Solithromycin disc diffusion mm	Solithromycin Agar dilution mg/L
199769	2	0.25	31	0.125
199856	> 256	0.5	31	1
199823	1	0.19	28	0.5
171121	2	0.25	29	≤ 0.016
172566	1	0.25	32	≤ 0.016
171825	2	0.125	33	0.5
198395	1	0.25	32	≤ 0.016
198436	1	0.19	31	≤ 0.016
197747	16	0.19	32	0.5
196712	> 256	> 32	12	1
166587	8	0.19	31	0.125
164744	1	0.19	30	≤ 0.016
157029	1	0.125	34	0.032
157274	2	0.19	31	0.064
422102	1	0.094	32	≤ 0.016
157810	1	0.125	35	≤ 0.016
157818	16	0.19	34	0.032
193852	> 256	0.38	37	0.032
157820	1	0.19	31	0.032
192754	4	0.25	31	0.5
195276	1	0.19	31	0.032
163285	2	0.19	31	≤ 0.016
161522	1	0.19	33	≤ 0.016

178144	4	0.19	31	0.125
178142	2	0.25	32	0.5
178145	2	0.19	31	0.064
163524	1	0.125	31	≤ 0.016
194697	1	0.125	32	0.032
191615	2	0.125	31	-
191917	8	0.125	34	-
153800	12	0.125	34	--
191587	> 256	> 32	12	-
190029	2	0.25	32	-
190309	4	0.125	30	-
150560	2	0.125	33	-
151213	2	0.125	34	-
190766	2	0.19	33	-
152376	4	0.125	34	-
009	0.75	0.064	38	0.032
006	> 256	> 32	12	0.125
015	16	0.19	34	0.125
013	1	0.125	33	≤ 0.016
016	1	0.094	33	0.064
017	0.75	0.094	34	0.064
023	1	0.125	33	≤ 0.016
019	1	0.19	31	≤ 0.016
034	0.75	0.125	34	0.125
035	1	0.047	40	0.032
91934	1	0.125	33	0.032
89478	16	0.19	33	0.064

180511	2	0.19	30	0.064
91838	1	0.125	33	≤ 0.016
91116	1	0.094	33	0.064
181798	> 256	6	18	8
179696	1	0.094	36	≤ 0.016
180188	1	0.094	33	0.064
179980	1	0.125	35	0.032
180505	2	0.125	32	0.064
180641	2	0.19	33	0.125
90030	1	0.19	30	0.064
86757	1	0.125	33	0.032
179487	2	0.19	33	0.032
85105	2	0.38	27	0.125
179411	4	0.19	31	0.125
178696	1	0.094	33	≤ 0.016
178575	32	0.19	34	0.125
178919	1	0.125	34	≤ 0.016
84537	1	0.125	33	0.064
179486	2	0.25	31	0.032
178573	32	0.19	34	0.064
81744	1	0.125	33	0.064
83064	1	0.19	32	0.032
178157	2	0.125	33	0.032
178487	> 256	1	26	0.125
82432	1	0.19	33	0.032
84397	1	0.125	33	0.032
84444	2	0.094	33	0.032

84117	1	0.064	35	≤ 0.016
178526	1	0.032	36	≤ 0.016
68732	1	0.064	34	≤ 0.016
67818	6	0.125	39	≤ 0.016
69989	8	0.094	36	≤ 0.016
70196	0.75	0.094	33	≤ 0.016
69899	0.75	0.064	34	≤ 0.016
70523	0.75	0.094	36	≤ 0.016
70988	0.75	0.125	34	≤ 0.016
71672	0.75	0.094	32	0.032
73070	0.75	0.125	33	≤ 0.016
69707	1	0.094	36	0.032
422670	1	0.19	32	0.032
425657	4	0.094	39	0.032
425658	1	0.064	35	≤ 0.016
47632	1	0.094	36	≤ 0.016
32424	0.75	0.94	32	≤ 0.016
16460	2	0.19	33	0.125
41277	12	0.125	34	0.064

Appendix 7. Summary of MICs, NG-MAST STs, MLST STs and resistance determinants present in the 43 isolates selected for sequencing.

Isolate (Year)	NG- MAST	MLST	MIC (mg/L)													<i>penA</i>	<i>ponA</i>	<i>porB</i>	TEM	<i>gyrA</i>	<i>parC</i>	<i>mtrR</i>	<i>mtrR</i> promoter	<i>tet</i> <i>M</i>	<i>rplD</i>	23s rRNA	
			PEN	CIP	AZM	CRO	CFM	TET	CTX	ETP	CN	GEMI	SPC	FOS	SOL	BL											
006 (2013)	2475	1580	0.25	0.012	>256	0.016	0.016	1	0.032	0.012	4	0.006	8	16	> 32	-	non- mosaic type II	WT	A121S	-	WT	WT	G45D	A12C	-	WT	A2059G (4/4)
181798 (2015)	3311	1580	0.25	0.008	>256	0.008	0.023	0.75	0.047	0.023	4	0.006	8	24	6	-	non- mosaic type II	WT	A121S	-	WT	WT	G45D	WT	-	WT	A2059G (3/4)
196712 (2016)	649	1580	0.25	0.008	>256	0.008	0.016	0.75	0.125	0.023	6	0.006	16	12	> 32	-	non- mosaic type II	WT	A121S	-	WT	WT	G45D	WT	-	WT	A2059G (4/4)
178487 (2015)	649	1580	0.19	0.008	>256	0.006	0.023	0.5	0.047	0.032	4	0.004	6	12	1	-	non- mosaic type II	WT	A121S	-	WT	WT	G45D	WT	-	WT	A2059G (3/4)
193852 (2016)	1443	1580	0.125	≤0.002	>256	0.004	≤0.016	0.38	0.006	0.023	4	≤0.002	8	8	0.38	-	non- mosaic type II	WT	A121S	-	WT	WT	G45D	A13 del	-	WT	A2059G (2/4)
199856 (2016)	649	1580	0.125	0.004	>256	0.002	≤0.016	0.38	0.004	0.008	3	<0.002	8	8	0.5	-	non- mosaic type II	WT	A121S	-	WT	WT	G45D	WT	-	WT	A2059G (4/4)
191587 (2016)	3311	1580	0.25	0.008	>256	0.008	0.032	1	0.064	0.016	3	0.004	8	12	> 32	-	non- mosaic type II	WT	A121S	-	WT	WT	WT	WT	-	WT	A2059G (4/4)
67818 (2014)	2400	1587	0.19	>32	6	0.012	0.023	0.38	0.064	0.012	3	1.5	8	8	0.125	-	non- mosaic type 49	L421P	G120K A121D	-	S91F D95G	E91G	WT	A13 del	-	WT	C2611T (4/4)
190440 (2016)	3750	1588	4	>32	0.032	0.004	0.016	32	0.032	0.012	4	4	4	12	-	+	non- mosaic type XIX	L421P	G120K A121G	TEM- 198	S91F A92P D95A	E91K S87K	A39T	WT	tet M	WT	WT
420132 (2016)	NM	13144	>32	2	0.064	0.008	≤0.016	0.5	0.008	0.012	8	0.5	12	12	-	+	non- mosaic type II	WT	WT	TEM- 75	S91F D95G	E91G	A39T	WT	-	WT	WT
71324 (2014)	1582	1588	4	>32	0.125	0.047	0.19	24	0.19	0.094	0.5	3	6	6	-	+	mosaic type X	L421P	G120K A121G	TEM- 198	S91F D95A	E91K S87K	A39T	WT	tet M	WT	C2611T (1/4)
016 (2013)	11645	1901	2	>32	1	0.047	0.19	1	0.25	0.032	6	1.5	12	32	0.094	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
035 (2016)	NM	1901	0.75	>32	1	0.047	0.125	0.75	0.125	0.032	4	1.5	6	24	0.047	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
286088 (2014)	3806	1901	0.75	>32	0.38	0.023	0.094	1	0.19	0.032	6	0.75	16	24	-	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
76972 (2014)	3431	1901	1.5	>32	0.75	0.047	0.19	1	0.25	0.032	4	2	12	32	-	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
176561	4936	1901	0.75	>32	0.5	0.016	0.125	0.5	0.19	0.032	6	1.5	8	12	-	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G		WT	A13 del		WT	WT

(2015)	S87R																				-						
501373 (2016)	5622	1901	0.25	16	0.5	0.032	0.125	1	0.125	0.023	6	2	8	32	-	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95A	S87R	WT	A13 del	-	WT	WT
024 (2012)	1407	1901	1.5	>32	0.38	0.094	0.25	1	0.38	0.064	4	1.5	8	48	-	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
009 (2013)	1407	1901	1	>32	0.75	0.064	0.125	1.5	0.125	0.032	4	1.5	12	48	0.064	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
194698 (2016)	1407	1901	0.25	16	0.5	0.016	0.064	0.5	0.19	0.047	4	3	8	48	-	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	G71D	WT
191262 (2016)	1407	1901	0.5	>32	0.5	0.023	0.125	1	0.125	0.032	4	1.5	8	24	-	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
422671 (2016)	3158	1901	0.5	>32	0.5	0.064	0.125	2	0.25	0.064	4	2	16	12	-	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
179501 (2015)	11690	1901	0.25	3	0.25	0.016	0.094	0.25	0.094	0.023	8	0.75	8	12	-	-	mosaic XXXIV	L421P	A121S	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
422102 (2016)	3158	1901	1	32	1	0.032	0.125	1	0.38	0.032	8	2	16	32	0.094	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
81513 (2015)	2212	1901	0.75	>32	0.5	0.032	0.19	0.75	0.125	0.064	6	2	12	16	-	-	mosaic XXXIV	L421P	G120K A121D	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
178526 (2015)	8953	1901	1	>32	1	0.032	0.19	0.75	0.25	0.032	6	1	8	16	0.032	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
68931 (2014)	3806	1901	1	>32	0.5	0.032	0.125	1	0.25	0.047	8	1.5	12	12	-	-	mosaic XXXIV	L421P	G120K A121N	-	S91F D95G	S87R	WT	A13 del	-	WT	WT
425204 (2016)	NM	7359	0.25	0.016	0.5	0.016	0.125	0.125	0.125	0.032	4	0.006	16	12	-	-	mosaic XXXIV	WT	WT	-	WT	WT	WT	WT	-	WT	WT
193058 (2016)	NM	7363	0.125	8	0.5	0.008	0.016	0.38	0.047	0.016	6	12	6	8	-	-	non- mosaic type 44	L421P	WT	-	S91F D95G	E91G S87I	WT	A13 del	-	WT	WT
197747 (2016)	14449	7363	0.25	2	16	0.016	0.032	0.5	0.094	0.012	4	2	12	8	0.19	-	non- mosaic type 44	L421P	WT	-	S91F D95G	E91G	WT	A13 del	-	WT	C2611T (4/4)
425657 (2016)	9184	7363	0.25	16	4	0.016	0.032	1	0.012	0.006	3	1.5	8	16	0.094	-	non- mosaic type 44	L421P	G120K A121D	-	S91F D95G	E91G	G45D	A13 del	-	WT	C2611T (4/4)
177574 (2015)	2318	13143	1	8	0.5	0.047	0.094	0.5	0.125	0.016	3	1	8	16	-	-	non- mosaic type XIII	L421P	G120K A121D	-	S91F D95G	D86N	G45D	A13 del	-	WT	WT

168628 (2016)	2318	7827	1	2	0.25	0.064	0.064	1	0.25	0.012	4	0.38	12	8	-	-	non- mosaic type XIII	L421P	G120K A121D	-	S91F D95G	D86N	G45D	A13 del	-	WT	WT
422322 (NA)	5624	8143	>32	2	0.5	0.008	≤0.016	0.5	0.016	0.004	8	0.5	16	12	-	+	non- mosaic type II	WT	G120D A121G	TEM- 141	S91F D95A	D86N	A39T	WT	-	WT	WT
008 (2013)	5624	8143	>32	3	0.5	0.008	≤0.016	0.5	0.023	0.006	6	0.75	12	12	-	+	non- mosaic type II	WT	G120D A121G	TEM- 141	S91F D95A	D86N	WT	A13 del	-	WT	WT
166587 (2016)	12302	9363	0.25	4	8	0.008	0.016	0.75	0.125	0.016	6	2	12	12	0.19	-	non- mosaic type II	WT	G120K A121N	-	S91F D95A	D86N	WT	A13 del	-	WT	WT
180505 (2015)	3935	9363	0.5	0.032	2	0.012	0.023	2	0.064	0.016	16	0.012	12	24	0.125	-	non- mosaic type II	WT	G120K A121N	-	WT	WT	WT	WT	-	WT	WT
178575 (2015)	2992	9363	0.19	0.004	32	0.003	0.016	0.38	0.012	0.012	6	0.003	6	16	0.19	-	non- mosaic type II	WT	WT	-	WT	WT	A39T	WT	-	WT	C2611T (4/4)
41277 (2014)	2992	9363	0.125	0.004	12	0.003	≤0.016	0.25	0.012	0.008	4	0.003	6	12	0.125	-	non- mosaic type II	WT	WT	-	WT	WT	A39T	WT	-	WT	C2611T (4/4)
179411 (2015)	12302	9363	0.5	12	4	0.008	0.023	0.5	0.064	0.016	6	1	12	24	0.19	-	non- mosaic type II	WT	G120K A121N	-	S91F D95A	D86N	WT	A13 del	-	WT	WT
039 (2015)	NM	9903	1.5	4	0.25	0.012	0.016	8	0.016	0.016	6	1.5	8	96	-	+	non- mosaic type XIV	WT	G120N A121K	TEM- 141	S91F D95A	S87N E91Q	WT	WT	tet M	WT	WT
82432 (2015)	7867	13142	0.75	>32	1	0.023	0.125	1	0.25	0.032	6	6	12	24	-	-	mosaic XXXIV	L421P	G120K A121D	-	S91F D95A	D86N	A39T	A13 del	-	WT	WT
86606 (2015)	4244	11428	0.25	0.006	0.5	0.008	0.094	0.38	0.064	0.047	6	0.004	8	24	-	-	mosaic XXXIV	WT	WT	-	WT	WT	A39T	WT	-	WT	WT

Appendix 8. Antimicrobial resistance and molecular epidemiology using whole-genome sequencing of *Neisseria gonorrhoeae* in Ireland 2014-2016: focus on extended-spectrum cephalosporins and azithromycin. *European Journal of Clinical Microbiology and Infectious Diseases* (2018).



Antimicrobial resistance and molecular epidemiology using whole-genome sequencing of *Neisseria gonorrhoeae* in Ireland, 2014–2016: focus on extended-spectrum cephalosporins and azithromycin

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Abstract

High-level resistance and treatment failures with ceftriaxone and azithromycin, the first-line agents for gonorrhoea treatment are reported and antimicrobial-resistant *Neisseria gonorrhoeae* is an urgent public health threat. Our aims were to determine antimicrobial resistance rates, resistance determinants and phylogeny of *N. gonorrhoeae* in Ireland, 2014–2016. Overall, 609 isolates from four University Hospitals were tested for susceptibility to extended-spectrum cephalosporins (ESCs) and azithromycin by the MIC Test Strips. Forty-three isolates were whole-genome sequenced based on elevated MICs. The resistance rate to ceftriaxone, cefixime, cefotaxime and azithromycin was 0, 1, 2.1 and 19%, respectively. Seven high-level azithromycin-resistant (HLAzi-R) isolates were identified, all susceptible to ceftriaxone. Mosaic *penA* alleles XXXIV, X and non-mosaic XIII, and G120K plus A121N/D/G (PorB1b), H105Y (MtrR) and A deletion (*mtrR* promoter) mutations, were associated with elevated ESC MICs. A2059G and C2611T mutations in 23S rRNA were associated with HLAzi-R and azithromycin MICs of 4–32 mg/L, respectively. The 43 whole-genome sequenced isolates belonged to 31 NG-MAST STs. All HLAzi-R isolates belonged to MLST ST1580 and some clonal clustering was observed; however, the isolates differed significantly from the published HLAzi-R isolates from the ongoing UK outbreak. There is good correlation between previously described genetic antimicrobial resistance determinants and phenotypic susceptibility categories for ESCs and azithromycin in *N. gonorrhoeae*. This work highlights the advantages and potential of whole-genome sequencing to be applied at scale in the surveillance of antibiotic resistant strains of *N. gonorrhoeae*, both locally and internationally.

Keywords *Neisseria gonorrhoeae* · Ireland · Resistance determinants · MLST · NG-MAST · High-level azithromycin-resistant *Neisseria gonorrhoeae*

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Introduction

Gonorrhoea is one of the most prevalent sexually transmitted infections (STIs) globally, with an estimated 78 million cases of gonorrhoea in 15–49-year-olds in 2012 [1, 2]. The aetiological agent *Neisseria gonorrhoeae* causes both urogenital infections, mostly urethritis and cervicitis, and extragenital infections such as pharyngeal and rectal infections, and may result in severe complications such as ectopic pregnancy, infertility and increased HIV transmission [3, 4].

Gonococci have developed resistance to all first-line antimicrobials recommended for treatment of gonorrhoea since penicillin was introduced in the 1940s [5]. Antimicrobial-resistant *N. gonorrhoeae* is now an urgent public health threat

globally [1, 5–8]. Increasing resistance to and treatment failures with cefixime in the last decade lead to the introduction of dual therapy, mainly ceftriaxone 250–500 mg and azithromycin 1–2 g, in an attempt to avoid treatment failure, and most importantly to prevent development of resistance and/or spread of resistant strains [1, 8, 9]. However, resistance, including high-level resistance, has been reported to both ceftriaxone and azithromycin. High-level ceftriaxone resistance (MIC ≥ 2 mg/L) has been identified in four sporadic isolates in Japan, France and Spain [10–12]. High-level azithromycin-resistant (HLAzi-R) isolates (MIC ≥ 256 mg/L) have been identified in many countries worldwide, including Ireland [13–18]. Alternative treatment options will undoubtedly be necessary in the near future. Enhanced understanding of the national and international emergence and transmission of antimicrobial-resistant *N. gonorrhoeae* strains is a priority. Whole-genome sequencing (WGS) provides an ideal solution for microepidemiology, macroepidemiology and identification of molecular antimicrobial resistance determinants [19].

The aims of this study were to determine the rates of resistance to extended-spectrum cephalosporins (ESCs) and azithromycin among gonococci in Ireland, to identify antimicrobial resistance determinants and determine the molecular epidemiology using WGS in the most antimicrobial-resistant subset of isolates, including assessing relatedness of HLAzi-R isolates from Ireland and the UK.

Materials and methods

Sample collection and identification

N. gonorrhoeae isolates were collected from four University Hospitals in Ireland (East, South East, West and Midlands), with the majority of isolates (84.4%) originating from Dublin (East). Isolates from all clinical samples which had been prospectively collected at St. James's Hospital (SJH) (2014–2016) and stored on Microbank cryobeads at -70 °C were recovered for testing. Clinical isolates retrieved from or referred to SJH by other tertiary referral or regional hospitals in Ireland were also examined. A total of 609 isolates were recovered from being frozen: 513 (84.4%) from East, 39 (6.4%) from South East, 31 (5.1%) from West, and 26 (4.3%) from Midlands. Ninety isolates were not viable, and the majority of which were from 2014. The majority of isolates, 329 (53.9%) were collected in 2016, followed by 166 (27.2%) from 2015 and 95 (15.6%) from 2014. Isolates were cultured on chocolate agar prior to confirmation of *N. gonorrhoeae* (Gram-stained microscopy and MALDI-ToF mass spectrometry), antimicrobial susceptibility testing and DNA extraction. Fifteen additional isolates, sequenced previously as part of a different study, were also included in the phylogenetic analysis for this chapter [13, 20].

Antimicrobial susceptibility testing

MICs (mg/L) of ceftriaxone, cefixime, cefotaxime, azithromycin and ertapenem were determined using MIC Test Strips (Liofilchem Roseto degli Abruzzi, Italy), according to manufacturer's instructions. Quality control was performed using the *N. gonorrhoeae* reference strain ATCC 49226. EUCAST clinical breakpoints [21] were used to categorise isolates as resistant (susceptible): ceftriaxone/cefixime/cefotaxime MIC >0.125 mg/L (MIC ≤ 0.125 mg/L) and azithromycin MIC >0.5 mg (MIC ≤ 0.25 mg/L). No resistance breakpoints are published by any organisation for ertapenem, but the EUCAST epidemiological cut-off value (ECOFF) is 0.064 mg/L [22].

DNA extraction

Forty-three isolates (7.0%) were selected for WGS based on showing decreased susceptibility or resistance to ESCs and/or resistance or high-level resistance to azithromycin (MIC ≥ 256 mg/L). DNA extraction was performed using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The purified DNA was preserved at -80 °C prior to WGS.

Whole-genome sequencing

Sequencing libraries of *N. gonorrhoeae* genomic DNA were generated using the NexteraXT library preparation kit (Illumina, Eindhoven, the Netherlands), according to manufacturer's instructions, and sequenced on an Illumina MiSeq instrument at the TrinSeq sequencing lab (Trinity College Dublin) using MiSeq v3 reagents. All short-read data obtained in this study has been deposited in the Sequence Read Archive (SRA); project accession number PRJNA473385.

Read mapping and phylogenetic analysis

Delineation of *N. gonorrhoeae* isolate phylogeny was achieved by read mapping to the *N. gonorrhoeae* WHO reference genome WHO N (LT591910.1) and subsequent analysis of core single nucleotide polymorphisms (SNPs) identified using the variant calling tool Snippy (<https://github.com/tseemann/snippy>). Genome alignments were generated and masked for recombination using Gubbins (<https://github.com/sanger-pathogens/gubbins>) and a subsequent maximum-likelihood phylogenetic tree was constructed using the generalised time reversible (GTR) substitution model in PhyML [23, 24]. A pairwise comparison table comparing total SNP differences between each isolate pair was generated using a custom python script ('vcf-to-pairwise-distance2.py'). To reproduce the described phylogenetic analysis, a Docker image has been made available at the following link:

<https://hub.docker.com/r/nsilico/tree-service/>. The code to make this image is available here: <https://github.com/blawlor/snippygubbins>.

Bioinformatic analysis of antimicrobial resistance determinants

De novo genome assembly was performed using the DTU centre for genomic epidemiology 2.1 assembler pipeline (<http://www.genomic epidemiology.org/>), which implements the SPAdes genome assembly algorithm [25]. Using genome assembly data, *penA* alleles were assigned a genotype based on the NG-STAR database [26]. The pubMLST tool was employed to determine MLST type and detect antimicrobial resistance determinants (<https://pubmlst.org/neisseria/>) while additional AMR genes were identified by cross-reference of the ARG-ANNOT database, which contains additional data on AMR (e.g. *ereA*, *ereB*, *ermA*, *ermB*, *ermC*, *mphA*, *mphB*, *mphC*, *mefC*) and plasmid associated genes reported in this study [26, 27]. As there are four copies of the *rrl* (23S rRNA) gene within the *N. gonorrhoeae* genome, reference mapping was performed separately and the number of reads supporting mutations such as the A to G mutations at position 2059 was rounded to the nearest quartile and reported as an estimate of overall allele prevalence within the genome. Finally, a local blast search was performed on de novo assemblies to determine NG-MAST types [28].

Ethics approval

The research proposal was reviewed and approved by the SJH/AMNCH Research and Ethics Committee.

Results

Five-hundred and sixteen isolates (84.6%) originated from STI clinics and only 36 (5.9%) from general practice (GP). The remaining isolates were referred from other hospitals ($n = 45$), emergency department ($n = 1$), surgical team ($n = 1$), ophthalmology ($n = 3$), and the source was unknown for 7 isolates. Over 90% ($n = 553$) of isolates were from male patients. The median age was 26 years (mean 28) with a range of 3 to 75 years. Over half the isolates (52.5%, $n = 320$, of which 35 were female) were from genital sites (urethral/cervical), followed by rectal (26.6%, $n = 162$, of which 1 was female) and pharyngeal (15.7%, $n = 96$, of which 5 were female).

No resistance to ceftriaxone was identified, but 1.8% of isolates ($n = 11$) displayed decreased susceptibility to ceftriaxone (MIC > 0.032 mg/L, CRO-DS), 1% ($n = 6$) were resistant to cefixime (CFM-R), and 2.1% ($n = 13$) resistant to cefotaxime (CTX-R). A further 2.5% ($n = 15$) displayed decreased susceptibility to cefixime (MIC = 0.125 mg/L, CFM-DS)

and 7.6% ($n = 46$) displayed decreased susceptibility to cefotaxime (MIC = 0.125 mg/L, CTX-DS). The MIC₅₀ and MIC₉₀ of cefotaxime were also higher than corresponding measures for ceftriaxone and cefixime (Table 1).

Forty-four percent of gonococci ($n = 268$) were non-susceptible to azithromycin (MIC > 0.25 mg/L), including 7 (1.1%) isolates with high-level resistance (MIC > 256 mg/L), 18 (3%) with medium-level resistance (MICs 4–32 mg/L), 88 (14.4%) with low-level resistance (MICs from 1–2 mg/L) and 155 (25.5%) with intermediate susceptibility.

Of the 43 isolates (7%), whole-genome sequenced, 25 belonged to one or more of the following phenotypic groups: CRO-DS (CRO MIC > 0.032 mg/L, $n = 9$), CFM-R (CFM MIC > 0.125 mg/L, $n = 6$), CFM-DS (CFM MIC = 0.125 mg/L, $n = 14$), CTX-R (CTX MIC > 0.125 mg/L, $n = 13$) and CTX-DS (CTX MIC = 0.125 mg/L, $n = 11$). The remaining 18 isolates had MICs lower than the above groups but were included based on MICs of azithromycin. The 25 isolates with resistance or decreased susceptibility to ESCs were associated with mosaic *penA* alleles (80%, $n = 20$) while none (0%) of the 18 isolates showing susceptibility to all ESCs had a mosaic *penA* allele. The majority ($n = 6$) of the nine CRO-DS isolates carried the mosaic *penA* XXXIV allele ($n = 6$), while the remainder had mosaic *penA* X ($n = 1$) or non-mosaic *penA* XIII ($n = 2$), containing the A501V mutation, alleles. The majority of CFM-DS/R and CTX-R/DS isolates also had these three *penA* allele types: of 14 CFM-DS isolates, 13 were mosaic XXXIV and 1 was non-mosaic XIII; of 6 CFM-R isolates, 5 were mosaic XXXIV and 1 was mosaic X; of 13 CTX-R isolates, 11 were mosaic XXXIV, 1 was mosaic X and 1 non-mosaic XIII; and of 11 CTX-DS isolates, 7 were mosaic XXXIV and 1 was non-mosaic XIII. The 18 isolates with lower ESC MICs contained mainly non-mosaic *penA* II, XIV, XIX, XLIV and XLIX. Additional antimicrobial resistance determinants, NG-MAST STs and MLST STs of all isolates are summarised in Table 2.

All HLAzi-R (MIC > 256 mg/L) isolates had the characteristic A2059G mutation in 2–4 alleles of the 23S rRNA gene, i.e. two mutated alleles in one isolate, three in two isolates and all four alleles mutated in four isolates. All seven HLAzi-R isolates also had an azithromycin resistance mutation in *mtrR*

Table 1 MIC₅₀, MIC₉₀ and MIC ranges for five antimicrobials when examining Irish *Neisseria gonorrhoeae* isolates ($n = 609$) from 2014 to 2016

Antimicrobial	MIC ₅₀	MIC ₉₀	MIC range
Ceftriaxone	0.008 mg/L	0.016 mg/L	≤0.002–0.125 mg/L
Cefixime	0.016 mg/L	0.032 mg/L	≤0.016–0.25 mg/L
Cefotaxime	0.032 mg/L	0.064 mg/L	≤0.002–0.5 mg/L
Ertapenem	0.016 mg/L	0.032 mg/L	≤0.002–0.094 mg/L
Azithromycin	0.025 mg/L	1 mg/L	≤0.016–>256 mg/L

Table 2 Summary of MICs, NG-MAST STs, MLST STs and antimicrobial resistance determinants for the 43 sequenced isolates

Isolate (year)	NG-MAST	MLST	PEN	MIC (mg/L)	CIP	AZM	CRO	CFM	TET	CTX	ETP	CN	GEMI	SPC	FOS
006 (2013)	2475	1580	0.25	0.012	> 256	0.016	0.016	0.016	1	0.032	0.012	4	0.006	8	16
181798 (2015)	3311	1580	0.25	0.008	> 256	0.008	0.008	0.023	0.75	0.047	0.023	4	0.006	8	24
196712 (2016)	649	1580	0.25	0.008	> 256	0.008	0.008	0.016	0.75	0.125	0.023	6	0.006	16	12
178487 (2015)	649	1580	0.19	0.006	> 256	0.006	0.006	0.023	0.5	0.047	0.032	4	0.004	6	12
193852 (2016)	1443	1580	0.125	≤ 0.002	> 256	0.004	0.004	≤ 0.016	0.38	0.006	0.023	4	≤ 0.002	8	8
199856 (2016)	649	1580	0.125	0.004	> 256	0.002	0.002	≤ 0.016	0.38	0.004	0.004	3	< 0.002	8	8
191587 (2016)	3311	1580	0.25	0.008	> 256	0.008	0.008	0.032	1	0.064	0.016	3	0.004	8	12
67818 (2014)	2400	1587	0.19	> 32	6	0.012	0.012	0.023	0.38	0.064	0.012	3	1.5	8	8
190440 (2016)	3750	1588	4	> 32	0.032	0.004	0.004	0.016	32	0.032	0.012	4	4	4	12
420132 (2016)	NM	13144	> 32	2	0.064	0.008	0.008	≤ 0.016	0.5	0.008	0.012	8	0.5	12	12
71324 (2014)	1582	1588	4	> 32	0.125	0.047	0.047	0.19	24	0.19	0.094	0.5	3	6	6
016 (2013)	11645	1901	2	> 32	1	0.047	0.19	0.19	1	0.25	0.032	6	1.5	12	32
035 (2016)	NM	1901	0.75	> 32	1	0.047	0.125	0.094	0.75	0.125	0.032	4	1.5	6	24
286088 (2014)	3806	1901	0.75	> 32	0.38	0.023	0.023	0.094	1	0.19	0.032	6	0.75	16	24
76972 (2014)	3431	1901	1.5	> 32	0.75	0.047	0.19	0.19	1	0.25	0.032	4	2	12	32
176561 (2015)	4936	1901	0.75	> 32	0.5	0.016	0.016	0.125	0.5	0.19	0.032	6	1.5	8	12
501373 (2016)	5622	1901	0.25	16	0.5	0.032	0.032	0.125	1	0.125	0.023	6	2	8	32
024 (2012)	1407	1901	1.5	> 32	0.38	0.094	0.094	0.25	1	0.38	0.064	4	1.5	8	48
009 (2013)	1407	1901	1	> 32	0.75	0.064	0.064	0.125	1.5	0.125	0.032	4	1.5	12	48
194698 (2016)	1407	1901	0.25	16	0.5	0.016	0.016	0.064	0.5	0.19	0.047	4	3	8	48
191262 (2016)	1407	1901	0.5	> 32	0.5	0.023	0.023	0.125	1	0.125	0.032	4	1.5	8	24
422671 (2016)	3158	1901	0.5	> 32	0.5	0.064	0.064	0.125	2	0.25	0.064	4	2	16	12
179501 (2015)	11690	1901	0.25	3	0.25	0.016	0.016	0.094	0.25	0.094	0.023	8	0.75	8	12
422102 (2016)	3158	1901	1	32	1	0.032	0.032	0.125	1	0.38	0.032	8	2	16	32
81513 (2015)	2212	1901	0.75	> 32	0.5	0.032	0.19	0.125	0.75	0.125	0.064	6	2	12	16
178526 (2015)	8953	1901	1	> 32	1	0.032	0.19	0.125	0.75	0.25	0.032	6	1	8	16
68931 (2014)	3806	1901	1	> 32	0.5	0.032	0.19	0.125	1	0.25	0.047	8	1.5	12	12
425204 (2016)	NM	7359	0.25	0.016	0.5	0.016	0.016	0.125	0.125	0.125	0.032	4	0.006	16	12
193058 (2016)	NM	7363	0.125	8	0.5	0.008	0.008	0.016	0.38	0.047	0.016	6	12	6	8
197747 (2016)	14449	7363	0.25	2	16	0.016	0.016	0.032	0.5	0.094	0.012	4	2	12	8
425657 (2016)	9184	7363	0.25	16	4	0.016	0.016	0.032	1	0.12	0.006	3	1.5	8	16
177574 (2015)	2318	13143	1	8	0.5	0.047	0.047	0.094	0.5	0.125	0.016	3	1	8	16
168628 (2016)	2318	7827	1	2	0.25	0.064	0.064	0.064	1	0.25	0.012	4	0.38	12	8
422322 (NA)	5624	8143	> 32	2	0.5	0.008	0.008	≤ 0.016	0.5	0.016	0.004	8	0.5	16	12
008 (2013)	5624	8143	> 32	3	0.5	0.008	0.008	≤ 0.016	0.5	0.023	0.006	6	0.75	12	12
166587 (2016)	12302	9363	0.25	4	8	0.008	0.008	0.016	0.75	0.125	0.016	6	2	12	12
180505 (2015)	3935	9363	0.5	0.032	2	0.012	0.012	0.023	2	0.064	0.016	16	0.012	12	24
178575 (2015)	2992	9363	0.19	0.004	32	0.003	0.003	0.016	0.38	0.012	0.012	6	0.003	6	16
41277 (2014)	2992	9363	0.125	0.004	12	0.003	0.003	≤ 0.016	0.25	0.012	0.008	4	0.003	6	12
179411 (2015)	12302	9363	0.5	12	4	0.008	0.008	0.023	0.5	0.064	0.016	6	1	12	24
039 (2015)	NM	9903	1.5	4	0.25	0.012	0.012	0.016	8	0.016	0.016	6	1.5	8	96
82432 (2015)	7867	13142	0.75	> 32	1	0.023	0.023	0.125	1	0.25	0.032	6	6	12	24
86606 (2015)	4244	11428	0.25	0.006	0.5	0.008	0.008	0.094	0.38	0.064	0.047	6	0.004	8	24

Table 2 (continued)

Isolate (year)	MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>porB</i>	TEM	<i>gyrA</i>	<i>parC</i>	<i>mttR</i>	<i>mttR</i> promoter	<i>tetM</i>	<i>rpld</i>	23S rRNA
BL												
Isolate (year)	MIC (mg/L)	<i>penA</i>	<i>ponA</i>	<i>porB</i>	TEM	<i>gyrA</i>	<i>parC</i>	<i>mttR</i>	<i>mttR</i> promoter	<i>tetM</i>	<i>rpld</i>	23S rRNA
BL												
006 (2013)	–	Non-mosaic type II		WT	A121S	–	WT	WT	G45D	A12C	–	A2059G (4/4)
181798 (2015)	–	Non-mosaic type II		WT	A121S	–	WT	WT	G45D	WT	–	A2059G (3/4)
196712 (2016)	–	Non-mosaic type II		WT	A121S	–	WT	WT	G45D	WT	–	A2059G (4/4)
178487 (2015)	–	Non-mosaic type II		WT	A121S	–	WT	WT	G45D	WT	–	A2059G (3/4)
193852 (2016)	–	Non-mosaic type II		WT	A121S	–	WT	WT	G45D	A13 del	–	A2059G (2/4)
199856 (2016)	–	Non-mosaic type II		WT	A121S	–	WT	WT	G45D	WT	–	A2059G (4/4)
191587 (2016)	–	Non-mosaic type II		WT	A121S	–	WT	WT	WT	WT	–	A2059G (4/4)
67818 (2014)	–	Non-mosaic type 49		L421P	G120K A121D	–	S91F D95G	E91G	WT	A13 del	–	C2611T (4/4)
190440 (2016)	+	Non-mosaic type XIX		L421P	G120K A121G	TEM-198	S91F A92P D95A	E91K S87K	A39T	WT	tetM	WT
420132 (2016)	+	Non-mosaic type II		WT	WT	TEM-75	S91F D95G	E91G	A39T	WT	–	WT
71324 (2014)	+	Mosaic type X		L421P	G120K A121G	TEM-198	S91F D95A	E91K S87K	A39T	WT	tetM	C2611T (1/4)
016 (2013)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
035 (2016)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
286088 (2014)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
76972 (2014)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
176561 (2015)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
501373 (2016)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95A	S87R	WT	A13 del	–	WT
024 (2012)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
009 (2013)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
194698 (2016)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
191262 (2016)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
422671 (2016)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
179501 (2015)	–	Mosaic XXXIV		L421P	A121S	–	S91F D95G	S87R	WT	A13 del	–	WT
422102 (2016)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
81513 (2015)	–	Mosaic XXXIV		L421P	G120K A121D	–	S91F D95G	S87R	WT	A13 del	–	WT
178526 (2015)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
68931 (2014)	–	Mosaic XXXIV		L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT
425204 (2016)	–	Mosaic XXXIV		WT	WT	–	WT	WT	WT	WT	–	WT
193058 (2016)	–	Non-mosaic type 44		L421P	WT	–	S91F D95G	E91G S87I	WT	A13 del	–	WT
197747 (2016)	–	Non-mosaic type 44		L421P	WT	–	S91F D95G	E91G	WT	A13 del	–	C2611T (4/4)

Table 2 (continued)

Isolate (year)	MIC (mg/L)	penA	ponA	porB	TEM	gyrA	parC	mitrR	mitrR promoter	tetM	rpld	23S rRNA
BL												
425657 (2016)	–	Non-mosaic type 44	L421P	G120K A121D	–	S91F D95G	E91G	G45D	A13 del	–	WT	C2611T (4/4)
177574 (2015)	–	Non-mosaic type XIII	L421P	G120K A121D	–	S91F D95G	D86N	G45D	A13 del	–	WT	WT
168628 (2016)	–	Non-mosaic type XIII	L421P	G120K A121D	–	S91F D95G	D86N	G45D	A13 del	–	WT	WT
422322 (NA)	+	Non-mosaic type II	WT	G120D A121G	TEM-141	S91F D95A	D86N	A39T	WT	–	WT	WT
008 (2013)	+	Non-mosaic type II	WT	G120D A121G	TEM-141	S91F D95A	D86N	WT	A13 del	–	WT	WT
166587 (2016)	–	Non-mosaic type II	WT	G120K A121N	–	S91F D95A	D86N	WT	A13 del	–	WT	WT
180505 (2015)	–	Non-mosaic type II	WT	G120K A121N	–	WT	WT	WT	WT	–	WT	WT
178575 (2015)	–	Non-mosaic type II	WT	WT	WT	WT	WT	A39T	WT	–	WT	C2611T (4/4)
41277 (2014)	–	Non-mosaic type II	WT	WT	WT	WT	WT	A39T	WT	–	WT	C2611T (4/4)
179411 (2015)	–	Non-mosaic type II	WT	G120K A121N	–	S91F D95A	D86N	WT	A13 del	–	WT	WT
039 (2015)	+	Non-mosaic type XIV	WT	G120N A121K	TEM-141	S91F D95A	S87N E91Q	WT	WT	tetM	WT	WT
82432 (2015)	–	Mosaic XXXIV	L421P	G120K A121D	–	S91F D95A	D86N	A39T	A13 del	–	WT	WT
86606 (2015)	–	Mosaic XXXIV	WT	WT	–	WT	WT	A39T	WT	–	WT	WT

(G45D), and one of the isolates also had a single-base pair (A) deletion in the repeated sequence of the *mitrR* promoter. All seven HLAzi-R isolates were assigned as MLST ST1580. Three of the HLAzi-R isolates belonged to NG-MAST ST649, two were of NG-MAST ST3311 and the remaining two were single NG-MAST ST2475 and ST1443 (Table 3).

The remaining isolates formed two groups based on azithromycin MICs, those with azithromycin MICs ranging from 4 to 32 mg/L ($n = 7$) and those with MICs ≤ 2 mg/L ($n = 29$). Five of the seven isolates with medium-level resistance to azithromycin (MIC 4–32 mg/L) had a C2611T mutation in all four alleles of the 23S rRNA gene, and two were wild type (MIC = 4 mg/L and 8 mg/L). These isolates were assigned as MLST ST9363 ($n = 4$), ST 7363 ($n = 2$) and ST1587 ($n = 1$) and NG-MAST ST2992 ($n = 2$), ST12302 ($n = 2$), ST2400 ($n = 1$), ST1449 ($n = 1$) and ST9184 ($n = 1$) (Table 4).

All isolates with azithromycin MICs < 2 mg/L ($n = 29$) had wild-type 23S rRNA, except for one isolate which had a C2611T mutation in one of the four 23S rRNA gene alleles (azithromycin MIC = 0.125 mg/L). Sixteen of these 29 isolates were MLST ST1901 with four of these isolates NG-MAST ST1407. The second most common MLST ST was ST1588 ($n = 3$).

A single-base pair (A) deletion in the repeated sequence of the *mitrR* promoter was present in 21/29 (72%), 4/7 (57%) and 1/7 (14%) of the isolates with azithromycin MICs of 0.032–2 mg/L, 4–32 mg/L and > 256 mg/L, respectively. No *erm*, *ere*, *mef* or *mph* genes were detected.

A phylogenetic tree based on the whole-genome sequence data was constructed. Core SNP sites in 58 whole-genome sequenced strains compared to the NCCP11945 genome were used as the basis for delineating strain phylogeny (Fig. 1).

The phylogeny revealed that HLAzi-R isolates NGSJH11, 181798, 191587 and 178487 belonged to the same MLST ST (ST1580) while differing by < 5 SNPs in their genome sequences (Fig. 1). This suggests potential linked transmission sources or the recent emergence of ST1580 in Ireland, although the latter is unlikely since it has circulated in Ireland since at least 2008 possibly reflecting a very conserved genome [13]. NG-MAST STs in these seven HLAzi-R isolates included ST649 ($n = 3$), ST3311 ($n = 2$), ST2475 ($n = 1$) and ST1443 ($n = 1$). Isolates UHW24 and UH9 belonged to MLST ST1901 and were genetically indistinguishable (0 SNP differences) pointing to a shared source of transmission, while isolates 4226171 and 422102 (also belonging to ST1901) were closely related to each other but differed by 166 SNPs from UHW24 and UH9. The MLST ST1901 was also distinguished by decreased susceptibility to the ESCs. Isolates exhibiting CFM-R ($n = 5$), CFM-DS ($n = 10$) and CRO-DS ($n = 6$) were observed in this clade but no high-level resistance to ESCs or azithromycin was found (Fig. 1). Other MLST STs where potential transmission events were supported included ST1587/ST7363 (isolates 67818 and

Table 3 Summary of NG-MAST, MLST and antimicrobial resistance determinants of seven *Neisseria gonorrhoeae* isolates with high-level resistance to azithromycin (MICs > 256 mg/L)

Isolate (year)	NG-MAST	MLST	AZM MIC (mg/L)	<i>mtrR</i>	<i>mtrR</i> promoter	23S rRNA	<i>erm A/B/C/F</i> <i>ereA/B</i> <i>mefA</i> <i>mph A/B/C</i>
006 (2013)	2475	1580	> 256	G45D	A12C	A2059G (4/4)	–
196712 (2016)	649	1580	> 256	G45D	WT	A2059G (4/4)	–
178487 (2015)	649	1580	> 256	G45D	WT	A2059G (3/4)	–
199856 (2016)	649	1580	> 256	G45D	WT	A2059G (4/4)	–
193852 (2016)	1443	1580	> 256	G45D	A13 del	A2059G (2/4)	–
191587 (2016)	3311	1580	> 256	G45D	WT	A2059G (4/4)	–
181798 (2015)	3311	1580	> 256	G45D	WT	A2059G (3/4)	–

NGSJH12) and ST9363 (isolates: 178575/41277 and 166587/179411) clades, where genetically related isolates were observed while the two identified as ST7827 and ST13143 differed by only 11 SNPs.

The Irish HLAzi-R isolates (all MLST ST1580) identified in the current study were also compared to HLAzi-R MLST ST1580 isolates from an ongoing outbreak in the UK (Sequence Read Archive accession PRJEB14933) to establish potential international spread of HLAzi-R ST1580 strains (Fig. 2). There was significant difference between these isolates (> 100 SNP).

Discussion

Regular and quality-assured surveillance of gonococcal antimicrobial susceptibility is essential for empiric treatment guidelines. In Ireland, ESC resistance is currently not a significant

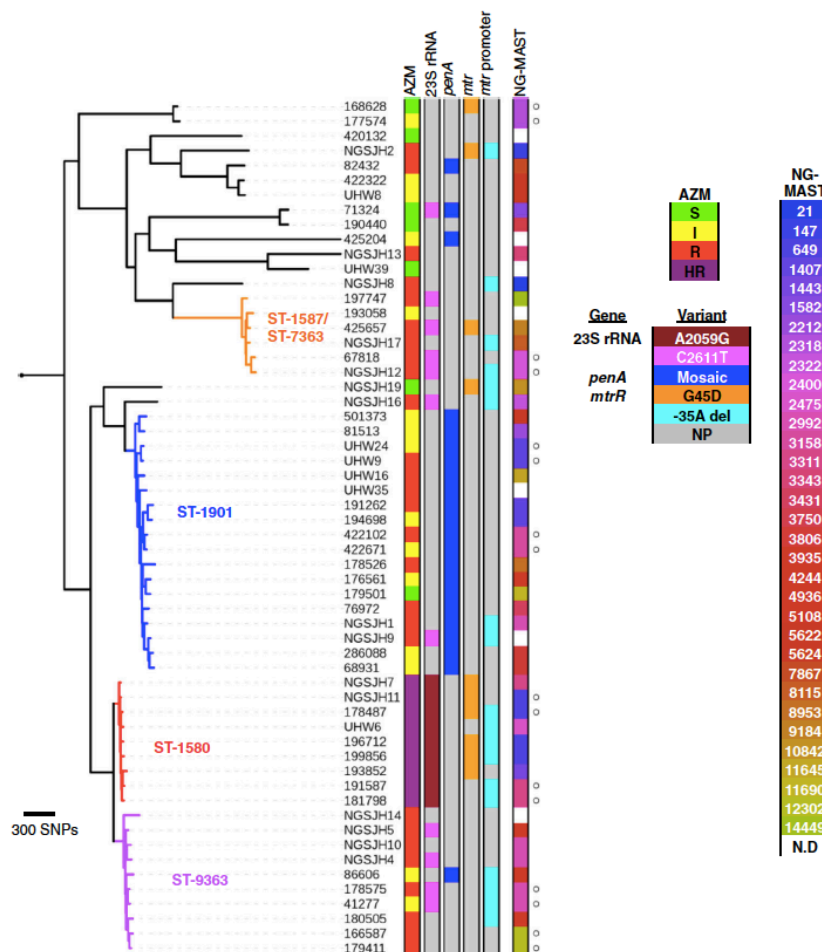
problem since all isolates in this study were susceptible to ceftriaxone, and resistance to cefixime and cefotaxime was relatively rare, i.e. 1 and 2.1%, respectively. Reports from the European Gonococcal Antimicrobial Surveillance Programme (EURO-GASP) have shown that in 2015, 1.7% of isolates were cefixime resistant compared to 2.0% in 2014, while ceftriaxone resistance was detected in only one (0.05%) isolate in 2015, compared with five (0.2%) in 2014 [29, 30].

Azithromycin is part of the empiric first-line dual therapy regimen (along with ceftriaxone) adopted by many countries for treatment of uncomplicated anogenital or pharyngeal gonorrhoea in adults [8]. Although the resistance proportion of 18.6% found in the current study was higher than the majority of reports from elsewhere in Europe, the MIC distribution for azithromycin showed the majority of resistant isolates had MICs of 1–2 mg/L (low-level resistance) and, in general, most isolates had MICs falling in the narrow range of 0.125–0.5 mg/L (73.6%) as previously reported [31].

Table 4 Summary of NG-MAST, MLST and antimicrobial resistance determinants of seven isolates displaying medium-level azithromycin resistance (MICs = 4–32 mg/L)

Isolate (year)	NG-MAST	MLST	AZM MIC (mg/L)	<i>mtrR</i>	<i>mtrR</i> promoter	23S rRNA	<i>erm A/B/C/F</i> <i>ereA/B</i> <i>mefA</i> <i>mph A/B/C</i>
67818 (2014)	2400	1587	6	D79N T86A H105K	A13 del	C2611T (4/4)	–
197747 (2016)	14449	7363	16	Stop codon	A13 del	C2611T (4/4)	–
425657 (2016)	9184	7363	4	D79N T86A H105K	A13 del	C2611T (4/4)	–
178575 (2015)	2992	9363	32	A39T R44H	WT	C2611T (4/4)	–
41277 (2014)	2992	9363	12	A39T R44H	WT	C2611T (4/4)	–
179411 (2015)	12302	9363	4	D79N	A13 del	WT	–
166587 (2016)	12302	9363	8	D79N	A13 del	WT	–

Fig. 1 Phylogenetic analysis based on whole-genome sequences of Irish *N. gonorrhoeae* isolates (PRJNA473385) illustrating resistance to azithromycin (AZM), identified resistance mutations and NG-MAST type. The most prevalent MLST ST groups are identified by colour coding along with the AZM resistance level (S; ≤ 0.25 mg/L, I; > 0.25 – ≤ 0.5 mg/L, R; > 0.5 mg/L, HR; ≥ 256 mg/L). The presence of detected mutations in the 23S rRNA gene (A2059G and C2611T) and mosaic *penA* alleles and mutations in the *mtrR* coding (G45D) and promoter regions ($\sim 35A$ del) are indicated by coloured strips according to the key to the right of the figure (NP, 'not present'). Identified NG-MAST ST types are also colour-indicated (ND, 'not determined'). Highly related strains (< 20 SNPs) are highlighted by open circles. Genome sequences from 15 additional Irish *N. gonorrhoeae* isolates, which can be accessed in the Sequence Read Archive (accession PRJNA275092), were included in the tree



Of the azithromycin-resistant isolates found in this study, a small proportion ($n = 7$, 1.1%) displayed high-level resistance to azithromycin (MIC > 256 mg/L). However, this is a higher proportion than reported in other countries or regions that frequently examined larger numbers of isolates, such as 0 and 18% in China ($n = 0/485$, 2009–2013 and $n = 21/118$, 2011–2012, respectively), 0.002% in the USA ($n = 1/44,144$, 2005–2013), and 0.04–0.2% in Europe ($n = 2/1902$, $n = 1/1994$, $n = 1/2151$ and $n = 5/2134$ in 2011, 2013, 2014 and 2015, respectively) and 0.7% in Canada ($n = 2/2800$, 2010–2013) [29–37].

In general, the recorded resistance rates to azithromycin vary somewhat from country to country across the world which is due to differences in true resistance rates but may also reflect application of different resistance breakpoints and that many circulating gonococcal strains have MICs close to the resistance breakpoint. In this study, the proportion of isolates resistant to azithromycin was higher than older reports

from elsewhere in Europe, including Amsterdam (1.2%; 2012–2015) and South-West Germany (7.1%; 2010–2015) [31, 38], but was lower or similar to other countries such as Switzerland (23.6%; 2009–2012) and Hungary (15.9%; 2013) [39, 40]. The rate of azithromycin resistance found in the 2015 EURO-GASP surveillance is similar to the rates found in South-West Germany, at 7.1% [30].

The major resistance determinant at which mutations are known to decrease susceptibility to ESC MICs is the *penA* gene and particularly the presence of a mosaic *penA* gene. Two mosaic *penA* allele types were identified in this study among the CRO-DS isolates and both these mosaic *penA* alleles are associated with decreased susceptibility to ESCs and are prevalent worldwide [19, 41–45]. Most ($n = 6$) of the nine CRO-DS isolates had the mosaic *penA* XXXIV allele. The mosaic *penA* XXXIV has been shown to be associated with increased ESC MICs [19, 41]. This is also the *penA* allele that

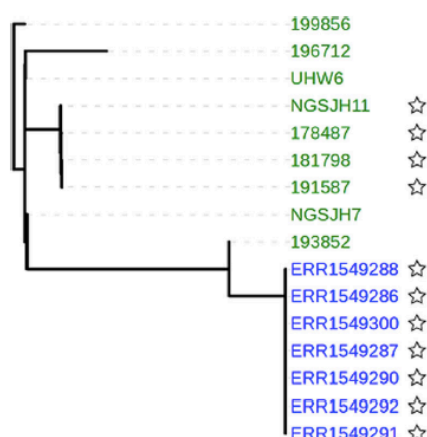


Fig. 2 Whole-genome sequence comparison of Irish and UK high-level azithromycin-resistant (HLAzi-R) ST1580 isolates. Irish and UK strains are indicated in green and blue font respectively. Stars indicate putative transmission events within specific clades based on observed SNP differences of < 5 among isolates. While geographically contained clonal clusters are observed, a single Irish isolate (193852) showed some similarity with the UK outbreak strains, but differed significantly (> 100 SNPs)

has been associated with the majority of verified ESC treatment failures [46–51]. Of the three remaining CRO-DS isolates, two had the non-mosaic *penA* XIII (A501V) allele and one had a mosaic *penA* X allele. The mosaic *penA* X allele has also been associated with ESC resistance and treatment failures [19, 45, 52]. In 1995–2005 in Japan, 129/149 CFM-R gonococcal isolates were shown to possess the mosaic *penA* X alleles [45]. Non-mosaic *penA* XIII (containing the A501V and P551S resistance mutations) was the most common allele type in 56/95 isolates from Korea (2011–2013), which included 21/25 of the ESC-resistant isolates [42].

Other mutations found to be associated with elevated ESC MICs in this study included the additional ESC resistance mutations G120K plus A121N/D/G alterations in PorB1b, and an A deletion in the inverted repeated sequence of the *mtrR* promoter. These mutations have been associated with increased MICs to ESCs [39, 53–55].

N. gonorrhoeae HLAzi-R is specifically associated with an A2059G mutation in the peptidyltransferase loop in domain V in the 23S rRNA, when it is present in 3 or 4 alleles and this has been reported worldwide [13, 14, 16–18, 35, 56, 57]. As described in this study, seven of the 43 isolates sequenced were HLAzi-R, one of which had an A2059G mutation in only 2 out of the 4 23S rRNA gene alleles, the remaining isolates had 3/4 ($n=2$) or 4/4 ($n=4$) mutated alleles. HLAzi-R gonococcal isolates with only 2 alleles containing the A2059G mutation have rarely been reported [58]. This isolate also had a G45D alteration in *mtrR* that all the seven HLAzi-R isolates had; however, it was the only HLAzi-R isolate which also had an A deletion in the inverted repeat

sequence of the *mtrR* promoter. Of 25 internationally reported HLAzi-R isolates, at least six had a G45D substitution in *mtrR* and at least nine had an A deletion in the promoter region [13, 14, 16–18, 35, 56, 57, 59].

The 23S rRNA mutation C2611T found in the medium-level azithromycin-resistant isolates in the current study has been widely reported to be associated with resistance to azithromycin, MICs ranging from 1 to 64 mg/L with the number of mutated alleles ranging from 1 to 4, but mostly three copies have been associated with azithromycin MICs ≥ 4 mg/L [13, 19, 35, 37, 57, 60–62]. One azithromycin-susceptible isolate in the current study (AZM MIC = 0.125 mg/L) had one C2611T mutated 23S rRNA allele, but there were other reports of gonococcal isolates with one C2611T mutated allele, associated with AZM MICs of 0.38, 1 and 2 mg/L [25, 62]. Grad et al. reported a positive predictive value of 99% for decreased susceptibility to azithromycin if there were ≥ 2 C2611T mutated loci (C2611T), and interestingly, having one C2611T mutated loci had the same negative predictive value for decreased susceptibility to azithromycin as wild-type 23S rRNA [63].

Given that A2059G (in ≥ 3 of the 4 alleles) and C2611T (in ≥ 3 of the 4 alleles) mutations in the 23S rRNA gene are only found in gonococcal isolates with AZM MICs ≥ 4 mg/L, absence of these mutations may provide a means of assessing whether azithromycin may be relied upon for treatment of gonorrhoea if molecular methods are employed rather than phenotypic susceptibility testing.

In the current study, the A deletion in the *mtrR* promoter was present more often in gonococci with lower AZM MICs than in those with higher MICs. In other studies, the presence of this *mtrR* mutation region has been similar across all levels of susceptibility to azithromycin [19, 37, 57]. No *erm*, *ere*, *mef* or *mph* genes were detected in the current study, which is in accordance with the majority of studies investigating azithromycin resistance in *N. gonorrhoeae*.

The genomic analysis confirmed the most prevalent *N. gonorrhoeae* strain types in our study. These included the MLST ST1580, ST9396 and ST1901 lineages as well as the genetic diversity within these genetic clades. Three novel MLST STs were identified among the isolates tested (ST13142, ST13143 and ST13144). Jacobsson et al. reported NG-MAST ST2992 and MLST ST1901 to be the predominant STs out of 75 azithromycin-resistant (MIC > 2 mg/L) isolates from 17 EURO-GASP countries (2009–2014) [57], but in another genomic study examining a larger number of consecutive European isolates from 20 countries in 2013 ($n=1054$), NG-MAST ST1407 and MLST ST1901 were the predominant STs [19]. While significant genetic diversity was seen, a number of closely related isolates were observed suggesting putative transmission or shared environmental sources, or, alternatively, the more recent emergence of these strains in Ireland. This highlights the potential of whole-

genome sequencing-based approaches to track disease transmission at a finer and more accurate level.

While this was not the primary focus of the current study, larger and more systematic application of whole-genome sequencing in Ireland could elucidate the emergence and spread of *N. gonorrhoeae* strains at a national level. In terms of international surveillance, the portability of whole-genome sequencing data and possibility for international comparison of whole-genome sequencing data are also noteworthy [19]. In the current study, we exploited this in the comparison of our HLAzi-R MLST ST1580 isolates with those from an ongoing outbreak in the UK. This highlighted the significant differences identified between the HLAzi-R MLST ST1580 isolates in the current study and those of the UK outbreak. Thus, genomic data can be integrated at scale to provide rapid comparisons of disparate databases toward greater international surveillance of gonorrhoea. A more recent UK study reported a sustained transmission of a clonal outbreak of HLAzi-R *N. gonorrhoeae* over several years, the majority of isolates belonging to ST9768 which they stated may be a descendant of ST649 [58]. This study also found that HLAzi-R may emerge quite quickly from azithromycin-susceptible or low-level azithromycin-resistant strains, making WGS essential for identifying transmission events [58].

The limitations of the current study included that the isolates were not representative of all gonococcal isolates cultured in Ireland during the study time period (2014–2016), as it was not possible to obtain isolates from all laboratories in Ireland and some frozen isolates were not retrievable ($n = 90$). Similarly, because other tertiary hospitals referred isolates to SJH, this may have led to an overrepresentation of azithromycin resistance. Furthermore, epidemiological data were mainly lacking and no information regarding sexual orientation of the patient, mode of transmission or response to antimicrobial treatment was available. Finally, the study was limited by the small number of ESC-resistant gonococcal isolates available and the small number of isolates that were whole-genome sequenced.

This is the largest antimicrobial resistance study, linked to whole-genome sequencing, of Irish *N. gonorrhoeae* isolates performed to date and highlights the importance of continued antimicrobial susceptibility surveillance and important advantages and potential of whole-genome sequencing to be applied at scale in the surveillance of antimicrobial-resistant strains of *N. gonorrhoeae*, both locally and internationally.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Ethics approval was granted by the Tallaght University Hospital/St. James's Hospital Joint Research Ethics Committee.

Informed consent Not applicable.

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