EVALUATION OF MEDIA FOR SUSCEPTIBILITY TESTING OF *NEISSERIA GONORRHOEAE*

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SUMMARY

Due to its fastidious nature, susceptibility testing of *N. gonorrhoeae* has always been problematic. As there is no current European Committee of Antimicrobial Susceptibility Testing (EUCAST) standardised method for susceptibility testing of *Neisseria gonorrhoeae,* this MPhil aims to evaluate all appropriate commercially available agar plates from several manufacturers, along with evaluation of two gradient strip manufacturers, bioMérieux Etest and Liofilchem MTS to develop a Minimum Inhibitory Concentration (MIC) gradient strip method. Standard procedures of inocula preparation and primary isolation agar plates will also be investigated. All data will be analysed using the appropriate ISO standards.

The outcomes of this MPhil will be to establish the most reliable agar plate and gradient strip manufacturers to perform a gradient strip MIC method, and to provide recommendations for routine diagnostic microbiology laboratories in the UK and Europe. Guidance will be published on the British Society of Antimicrobial Chemotherapy and EUCAST website.

The results of this MPhil will further inform which agar plate should be used to develop a disc diffusion method in conjunction with the EUCAST Developmental Laboratory in Sweden.

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COMMUNICATIONS

Conferences/presentations

Poster presentation: P1644 – Evaluation of media for minimum inhibitory concentration testing of *N. gonorrhoeae*, ECCMID 2024, 27 – 30th April 2024, Barcelona, Spain

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ABBREVIATIONS

AMRHAI – Antimicrobial Resistance and Healthcare Associated Infections

AMRSTI – Antimicrobial Resistance in Sexually Transmitted Infections

AZ – Azithromycin

BASHH – British Association for Sexual Health and HIV

BD – Becton Dickinson

BSAC – British Society of Antimicrobial Chemotherapy

CA – Categorical agreement

CLSI – Clinical Laboratory Standards Institute

CRI – Cardiff Royal Infirmary

CRO – Ceftriaxone

DHB – Defibrinated horse blood

DST – Diagnostic Sensitivity Test

EA – Essential agreement

EAGLE – Efficacy of Antimicrobial Gepotidacin Evaluated

ECDC – European Centre for Disease Prevention and Control

EDL – EUCAST Developmental Laboratory

EoR – Ease of Reading

EU/EEA – European Union/European Economic Area

EUCAST – European Committee of Antimicrobial Susceptibility Testing

Euro-GASP – European Gonococcal Antimicrobial Surveillance Programme

FAA – Fastidious Anaerobe Agar

FIX – Cefixime

GC – Gonococcus

GISP – Gonococcal Isolate Surveillance Project

GRASP – Gonococcal Resistance to Antimicrobials Surveillance Programme

GS – Gradient strip

GSK – GlaxoSmithKline

GUM – Genitourinary Medicine

HCCA – Hydroxycinnamic acid

ISO – IsoSensitest agar

LPS – Lipopolysaccharides

MALDI-ToF – Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry

MHB – Mueller Hinton Broth

MHF – Muller-Hinton Fastidious

MIC – Minimum inhibitory concentration

mRNA – Messenger ribonucleic acid

MTS – MIC test strip

NAAT – Nucleic acid amplification test

NAD – β-nicotinamide adenine dinucleotide

NICE – National Institute for Health and Care Excellence

PCR – Polymerase Chain Reaction

PENGU – Pathogen Genomics Unit

rRNA – Ribosomal ribonucleic acid

SACU – Specialist Antimicrobial Chemotherapy Unit

ST – Sequence types

TX – Ceftriaxone

UHW – University Hospital of Wales

UKHSA – United Kingdom Health Security Agency

WGS – Whole genome sequencing

WHO – World Health Organisation

WoG – Weight of Growth

CHAPTER 1 –

INTRODUCTION

1.1 OVERVIEW

Neisseria gonorrhoeae are Gram-negative diplococci bacteria, first isolated in 1879 by Albert Neisser. *N. gonorrhoeae* is the causative agent of the sexually transmitted disease gonorrhoea.(1, 2) It typically causes infection in the genitourinary tract and is the second most common sexually transmitted infection in the UK and the rest of the world with the World Health Organisation (WHO) estimating 82.4 million new infections worldwide in 2020.(3, 4) In 2017 the WHO placed *N. gonorrhoeae* on the Global Priority Pathogens List, placing it in the high antibiotic resistance category.(5) Left untreated, *N. gonorrhoeae* can cause a wide range of serious medical problems for individuals including pelvic inflammatory disease, ectopic pregnancy, infertility, miscarriage, premature labour and birth, sepsis, blindness in babies and infection in the testicles and prostate gland and more.(6, 7) Recommendations of treatment by the WHO, and the National Institute for Health and Care Excellence (NICE) in the UK both aim to provide efficient treatment for patients and prevent further spread of antimicrobial resistant *N. gonorrhoeae.*(7) Therefore, clinical laboratory testing of *N. gonorrhoeae* is vitally important in providing accurate, reproducible, and affordable susceptibility testing results.

1.2 MORPHOLOGY OF *N. GONORRHOEAE*

N. gonorrhoeae is a Gram-negative coccus bacterium, 0.6 to 1 µm in diameter, the bacteria are typically arranged in pairs with adjacent sides flattened and often look coffee bean shaped under the microscope. On primary isolation media, they appear as uniform smooth grey/brown colonies and require an overnight culture up for to 48 hours in 5 – 10 % CO_2 at 35 – 37 °C.(2, 8) The Gram-negative cell envelope is made up of three layers; the outer membrane, the cell wall and the cytoplasmic or inner membrane. The outer membrane cell is composed of proteins, phospholipids and lipopolysaccharides (LPS), and serves as a protective barrier to the cell. Hair-like appendages called pili extend from the cell surface, with Gram-negative bacteria such as *N. gonorrhoeae* expressing type IV pili. The pili promote bacterial colonisation and pathogenesis by boosting the ability of the cell to adhere to the host cells and tissues. The cell wall is made up of peptidoglycan which sits in the periplasmic space below the outer membrane, providing rigidity and structure to the cell. The

cytoplasmic membrane is a phospholipid bilayer, forming a permeable barrier and regulating the passage of solutes between the cell and the outer environment. Inside of the plasma membrane is the cytoplasm, containing ribosomes, the sites of protein synthesis, and the DNA of the cell.(7-10)

1.3 CLINICAL TESTING OF *N. GONORRHOEAE*

Laboratory testing of *N. gonorrhoeae* is of vital importance, starting with a good quality sample from the patient. Traditionally culture has been the "gold standard" for detection and identification of *N. gonorrhoeae* and is also necessary for antimicrobial susceptibility testing.(11) As antimicrobial resistance in *N.* gonorrhoeae continues to evolve and spread, antimicrobial susceptibility testing becomes of increased importance.(12)

N. gonorrhoeae infection in women usually affects the cervix and is often asymptomatic, however it can cause dysuria, genital itching, and an increased vaginal discharge. Endocervical or urethral swabs are the preferred specimens for women. Whereas in men, patients commonly present with symptomatic gonococcal infections such as urethritis with a purulent urethral discharge and dysuria. For men the best specimen is expressed urethral exudate, however in men who are asymptomatic, a urethral swab is taken. For both women and men infection of the pharynx by *N. gonorrhoeae* is often characterised by the absence of symptoms; it is usually only tested because of antibiotic treatment failures of genital site infections or due to contact tracing, then a pharyngeal swab is recommended. (12-14)

The time taken between sample collection and processing in the laboratory is crucial when testing for *N. gonorrhoeae,* as this species does not survive outside of the body for very long. Collected samples should be immediately inoculated on the appropriate primary culture media such as Gonococcus (GC) selective agar and incubated promptly. If there are no facilities for immediate incubation, then the sample should be placed in a charcoal transport medium and transported to the laboratory as soon as possible in appropriate CE marked containers, so they can be cultured in the laboratory. Dry swabs should also be sent as charcoal transport medium is not ideal for microscopy smears.(14)

Samples from patients with suspected *N. gonorrhoeae* infection entering the microbiology laboratory are processed by microscopy, culture, susceptibility testing and nucleic acid amplification test (NAAT). UK laboratories differ in which tests are performed to identify *N. gonorrhoeae,* some perform all of the above tests, others a combination of one or two. Male urethral exudate and male urethral swabs are smeared on a clean microscope slide for Gram staining. Due to low sensitivity, microscopy is not recommended for female urethra and endocervical swabs, rectal, and pharyngeal swabs.(12)

Gram staining differentiates organisms according to their cell wall structure. A thin smear from the patient swab or exudate is prepared on a microscopy slide and heated to fix. Once dried the slide is flooded with 0.5 % crystal violet stain for 30 seconds, rinsed with water and flooded with 1 % Lugol's iodine for 30 seconds then washed again and 95 – 100 % acetone applied. A further wash is followed by the addition of carbol fuschin for 30 seconds and then the slide is washed and dried. The slide is examined with immersion oil under a microscope. *N. gonorrhoeae* appear as Gram-negative cocci stained pink (carbol fuschin) which denotes lack of a thicker cell wall, seen in Gram-positive bacteria, which traps the initial crystal violet stain.(15)

Primary culture media, such as GC selective agar usually consists of GC agar base supplemented with lysed or chocolatised horse blood with or without the addition of VitoX or IsoVitaleX. A cocktail of selective antimicrobials is included, typically vancomycin or lincomycin, colistin, trimethoprim and nystatin or amphotericin to inhibit any contaminating microorganisms contained in the swab. The agar plate is incubated at 35 – 37 °C in an atmosphere of 5 – 10 % CO_2 for 18 – 48 hours. Identification of *N. gonorrhoeae* should be performed with a combination of test procedures to identify the organism and exclude other *Neisseria* species. *N. gonorrhoeae* forms smooth, round, moist, uniform grey/brown colonies, similar to *Neisseria meningitidis* on primary isolation media, therefore confirmatory testing is essential to discriminate between the two species. There are several ways to confirm the presence of *N. gonorrhoeae* including the use of gonococcal specific antibodies, the use of Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-ToF) or molecular confirmation using Polymerase Chain Reaction (PCR) using specific DNA found in *N. gonorrhoeae*.(1)

To identify organisms such as *N. gonorrhoeae* on the MALDI-ToF, pure and freshly cultured isolates are required to provide accurate results, cultures should be 24 – 48 hours old. The direct transfer method is recommended for identification of *N. gonorrhoeae*. A small amount of a single colony of suspected *N. gonorrhoeae* is smeared onto the MALDI-ToF Biotarget 96 target plate using a wooden cocktail stick to create a thin and homogenous distribution of the sample. A second spot is smeared using the same wooden cocktail stick without reloading the organism to give a thinner film, the sample is allowed to air dry and 1 μL of α-Cyano-4-hydroxycinnamic acid (HCCA) matrix containing organic solvent is pipetted onto each culture spot and again allowed to air dry. The target plate is loaded onto the MALDI-ToF once the sample has dried, and the matrix has crystalised on the spots. Once loaded onto the instrument the microbial molecules are vapourised and ionised by a short laser pulse that is absorbed by the HCCA matrix. The ions are then accelerated and separated by their mass-to-charge ratio in a vacuum tube, and the time it takes for the ions to reach the detector at the end of the tube is recorded and used to calculate their mass-to-charge ratio. The spectrum is compared with the bacterial library containing many reference spectra, providing an accurate identification of the bacteria.(16)

Some laboratories confirm the MALDI-ToF culture result with Nucleic Acid Amplification Tests. Molecular methods used by laboratories can be performed with either an in-house assay detecting *porA* and *opa* genes, or a commercial platform such as the Cobas CT/NG test.(1) NAATs has been shown to be more sensitive than culture in the identification of *N. gonorrhoeae* directly from samples, especially for pharyngeal and rectal sites of infection. As a result of this increased sensitivity, even though NAATs are not licenced for use in extra genital sites, they are recommended in diagnostic laboratories. Another benefit to using NAATs is that they do not require the *N. gonorrhoeae* organism to be viable for detection, so less stringent transport conditions of the sample are required compared to those needed for culture.(11, 12, 17)

1.4 *N. GONORRHOEAE* INFECTION

1.4.1 SYMPTOMS OF *N. GONORRHOEAE* INFECTION

Symptoms of infection by *N. gonorrhoeae* typically develop with two weeks, although they can sometimes take months to appear. Five in 10 women and 1 in 10 men experience no symptoms at all causing the infection to remain untreated for some time therefore risking further transmission.(18, 19)

Symptoms of *N. gonorrhoeae* infection in women can include an abnormal vaginal discharge, which may be thin or watery and green or yellow in colour or pain or burning sensation when urinating. Less common symptoms include pain or tenderness in the lower abdominal region or bleeding between periods, heavier periods, and bleeding after sexual intercourse. Symptoms experienced by women are more likely to go unnoticed as most women may experience vaginal discharge or abdominal pain as part of their monthly hormonal changes, especially women who experience heavy periods routinely.(18, 19)

In men, symptoms of *N. gonorrhoeae* can include an unusual white, yellow, or green discharge from the tip of the penis, pain or burning sensation when urinating, or inflammation of the foreskin. Less common in men is pain or tenderness in the testicles. Asymptomatic infection is less common in men as inflammation occurs in the same niche as urination and is likely to be painful.(18, 19)

N. gonorrhoeae infection can also occur in extra genital sites such as rectal, throat or eyes in both women and men through unprotected anal or oral sex.(6) Conjunctivitis can develop when infected semen or vaginal fluid encounters the eyes, causing irritation, pain, swelling and discharge. Rectal infection is mostly asymptomatic but has been known to cause discomfort, pain, or discharge.(20) Infection in the throat sometimes causes a sore throat but is usually asymptomatic and has been shown to contribute to the spread of the disease.(19, 21)

Infected mothers can pass on *N. gonorrhoeae* infection to babies during childbirth. Infections typically presenting within two weeks of birth, displaying as red and swollen eyes with a thick pus like discharge.(22)

1.4.2 COMPLICATIONS AND LONG-TERM PROBLEMS CAUSED BY *N. GONORRHOEAE* INFECTION

Antimicrobial resistance notwithstanding, *N. gonorrhoeae* is essentially easily treated with antibiotics. However, asymptomatic infection, reluctance to get appropriate testing, or a delay in treatment can cause complications in both women and men.(20) Complicated infections arise when *N. gonorrhoeae* spreads from the primary infection site, such as the urethra or endocervix to the upper genital tract.(12)

In women, if left untreated *N. gonorrhoeae* can cause pelvic inflammatory disease, which in turn can cause chronic pelvic pain, infertility, and ectopic pregnancy.(19) Pelvic inflammatory disease is an infection induced inflammation of the female upper reproductive tract i.e., the endometrium, fallopian tubes, ovaries, or pelvic peritoneum. Over 85% of pelvic inflammatory disease infections are caused by *N. gonorrhoeae,* chlamydia, or bacterial vaginosis that have spread to the upper genital tract from the vagina or cervix.(23) Pelvic inflammatory disease can cause scarring and narrowing of the fallopian, making it more difficult for the eggs to pass from the ovaries into the womb, increasing the chances of an ectopic pregnancy or possible infertility if left untreated.(24)

During pregnancy, untreated *N. gonorrhoea* has been linked to miscarriage, premature labour and birth, premature rupture of membranes, and chorioamnionitis.(25) Chorioamnionitis or intraamniotic infection is an acute inflammation of the membranes and chorion of the placenta, typically due to ascending polymicrobial bacterial infection.(22, 26) During birth, *N. gonorrhoeae* can pass from an infected mother to the baby causing infection of the eyes, where it can cause ulceration of the cornea, perforation of the globe of the eye, and permanent blindness if left untreated. Up to 48 % of infants born to mothers infected with *N. gonorrhoeae* develop ophthalmia neonatorum, and up to 10 % of those who receive antibiotic prophylaxis still develop infection.(27)

In men, *N. gonorrhoeae* has been linked to genitourinary tract inflammation and obstruction causing a pain in the testicles and prostate gland.(28) Pain in the testicles or prostate gland due to inflammation of the small coiled tube at the rear of the testicle can affect the sperm ducts located in the epididymis or a painful abscess in the interior of the penis. *N.*

gonorrhoeae infection can also have an effect on the prostate by causing scarring in the urethra, leading to difficulties in urinating, and potentially infertility if left untreated.(29, 30)

N. gonorrhoeae infection in both women and men also comes with an increased risk of HIV transmission. A sore or inflammation due to a sexually transmitted disease such as *N. gonorrhoea* may allow infection with HIV that would have been stopped by intact skin.(11, 22, 31)

Infrequently, *N. gonorrhoeae* invades the bloodstream through the mucosal membranes of the urethra, cervix, rectum, oropharynx, or conjunctivae, causing disseminated gonococcal infection.(32) Patients with disseminated gonococcal infection can have a wide range of complications such as tenosynovitis, dermatitis, arthritis, liver inflammation and even fatal infections including endocarditis, meningitis, and osteomyelitis.(33-35)

1.5 SUSCEPTIBILITY TESTING OF *N. GONORRHOEAE*

1.5.1 HISTORY OF SUSCEPTIBILITY TESTING OF *N. GONORRHOEAE*

Antimicrobial susceptibility testing is an important part of diagnostics in clinical microbiology laboratories for all bacteria, including *N. gonorrhoeae*. In 2001 the British Society of Antimicrobial Chemotherapy (BSAC) in the UK developed one of the first standardised disc susceptibility testing methods for bacteria. A standardised test is essential for providing reproducible results. The BSAC standardised method included clinical breakpoints used to categorise different bacteria against different antibiotics, predicting clinical outcome of the patient. BSAC included breakpoints for *N. gonorrhoeae* for the antimicrobials in use at the time, spectinomycin, penicillin, cefuroxime, tetracycline, and rifampicin. The BSAC method was performed using IsoSensitest agar plates, or for fastidious organisms such as *N. gonorrhoeae* IsoSensitest agar with the addition of 5 % defibrinated horse blood, or 5 % defibrinated horse blood and 20 mg/L β-nicotinamide adenine dinucleotide (ISON). For *N. gonorrhoeae* a 0.5 McFarland suspension of the bacteria was made in either IsoSensitest broth or distilled water and using a cotton swab the suspension spread without dilution in three directions to cover the ISON agar plate. Antibiotic discs added to the surface of the

agar once the plate dried, and incubated at 35 °C in CO_2 for 18 – 20 hours.(36) However, anecdotal evidence from the BSAC working party suggests that *N. gonorrhoeae* susceptibility testing with ISON was not without issues, with reports of about 85 % of *N. gonorrhoeae* clinical isolates tested having the growth supported and 15 % failing to grow. (M. Wootton, personal communication, 31st January 2023).

The BSAC Standing Committee on Antimicrobial Susceptibility Testing along with several European national breakpoint committees formed European Committee of Antimicrobial Susceptibility Testing (EUCAST) and agreed in 2002 to harmonise clinical minimum inhibitory concentration breakpoints. Before 2002, different breakpoints were employed for the same drug/species combination in different countries. This meant that for example an *E. coli* with a cefuroxime MIC of 4 mg/L would be considered susceptible in the UK but resistant in Sweden and France.(37) Harmonisation of breakpoints was completed in 2010. In 2011, EUCAST then developed its own standardised disc diffusion method, which was adopted by many laboratories in Europe. BSAC replaced support for its own disc diffusion method with support for the EUCAST method from January 2016.(36)

The EUCAST method for susceptibility testing of fastidious organisms uses Mueller-Hinton agar, supplemented with 5 % mechanically defibrinated horse blood and 20mg/L NAD, known as Muller-Hinton Fastidious (MHF) agar. However, EUCAST did not develop a disc diffusion method with MHF agar for use with *N. gonorrhoeae* as it was shown to be inadequate at supporting growth.(38) Although there are breakpoints available for minimum inhibitory concentration (MIC) testing, there is no advice on which susceptibility testing agar to use, the recommendation is to follow the instructions of the manufacturer of the antibiotic gradient strips used.(39)

Post 2016, most laboratories in the UK were adopting the EUCAST disc method for most bacteria, however because there was no standardised disc method for susceptibility testing of *N. gonorrhoeae,* laboratories continued to use the BSAC disc susceptibility method even though it had ceased to be supported. BSAC recommended UK laboratories to use the unsupported BSAC *N. gonorrhoeae* disc test for 2 years after recommending the change to EUCAST disc method because it was considered very important in the UK to continue to susceptibility test *N. gonorrhoeae* as antibiotic resistance was increasing.(40) In recent years

(2018 - 2020) however, laboratories were advised by BSAC to move to an MIC method, generally gradient strips, for testing *N. gonorrhoeae* using EUCAST breakpoints.(41) Both gradient strip manufacturers available in the UK, bioMérieux and Liofilchem, recommend the use of supplemented GC agar (GC agar base + defined supplements, Mueller Hinton + 1% IsoVitaleX + 1% haemoglobin), and Mueller Hinton Chocolate agar with Mueller Hinton broth for the inoculum.(42, 43) However, at this time (2018 - 2020) most, if not all, of this recommended media were unavailable in the UK.

With ambiguity surrounding the recommended media called "supplemented GC agar" laboratories used several different agars that were available from commercial manufacturers. These media had not been verified as providing accurate and reproducible MIC results, which could have led to potential treatment failure or inappropriate treatment. Further, an MIC method is far more costly than a disc diffusion test, putting financial stress on underfunded laboratories. In summary, the unavailability of some or all of the recommended agars meant that the susceptibility testing of *N. gonorrhoeae* in UK laboratories was not performed by a standardised susceptibility test and so poor or unreproducible results were probably reported, possibly leading to treatment failure or inappropriate treatment as well as poorer quality surveillance data. (Personal experience from working in a routine microbiology laboratory).

1.5.2 SUSCEPTIBILITY TESTING WITH GRADIENT STRIPS

There are several ways to perform an antibiotic susceptibility test, in this study the focus was on minimum inhibitory concentration (MIC) testing using gradient strips and agar plates. There are currently two manufacturers of gradient strip available in the United Kingdom, Etest by bioMérieux (France) and MTS by Liofilchem (Italy). There are slight differences between the two products from the two manufacturers.

bioMérieux – The Etest gradient strip consists of a thin, inert, non-porous plastic strip. On the rear of the strips there is an antibiotic gradient, and on the front of the strip is a minimum inhibitory concentration reading scale in mg/L printed, corresponding to the

antibiotic gradient. This gradient consists of 15 x two-fold dilutions, for example, 0.002 – 32 mg/L.(42)

Liofilchem – The MIC test strip (MTS) gradient strip is made of a high-quality paper which is impregnated with an antibiotic gradient of 15 x two-fold dilutions. The dilutions are printed on the front of the strip, as for the bioMérieux Etest gradient strip.(43)

Both strips perform in the same way. Once the agar plate is inoculated with a bacterial suspension and allowed to dry, for up to 15 minutes, the gradient strip can be applied aseptically to the plate with forceps. Once placed, the gradient strip cannot be moved, this is because the antibiotic is released from the strip immediately after contact with surface of the agar. The agar plate with the attached strip is promptly incubated in suitable conditions. For *N. gonorrhoeae* the agar plate should be incubated in an atmosphere of 5 % CO_2 at 35 °C ± 1 °C for 20 – 24 hours for bioMérieux Etest gradient strips and for Liofilchem MTS gradient strips 5 % CO_2 at 36 °C ± 1 °C for 20 – 24 hours. An effective antibiotic diffuses through the agar plate creating an ellipse of inhibition. Where the growth of the bacteria intersects with the strip correlates to the MIC of the bacteria, if the intersection is between two MICs, then it is read at the higher number.(42, 43)

1.6 CURRENT TREATMENT REGIMENS FOR *N. GONORRHOEAE*

1.6.1 FIRST- AND SECOND-LINE THERAPY AND OTHER RECOMMENDED TREATMENTS OF *N. GONORRHOEAE*

Treatment of *N. gonorrhoeae* is usually administered by clinicians before any laboratory susceptibility testing results are available.(44) According to British Association for Sexual Health and HIV (BASHH) for uncomplicated ano-genital and pharyngeal infections, the first line treatment in the UK is ceftriaxone 1 g given intramuscularly as a single dose. Previous guidelines from BASHH listed ciprofloxacin as first-line treatment for *N. gonorrhoeae* if the antimicrobial susceptibility result was known prior to treatment. The current BASHH guidelines have removed that recommendation due to safety concerns and stated that ciprofloxacin only be used if deemed appropriate by clinicians. (12, 45, 46)

The first line treatment of ceftriaxone monotherapy is a major change from the 2011 UK national guidance for the management of infection with *N. gonorrhoeae* where the first-line treatment was dual therapy 500 mg ceftriaxone and 1 g azithromycin.(12)

There are alternative regimens for empirical uncomplicated *N. gonorrhoeae* infections that may be given due to allergy, needle phobia or other contraindications. According to current BASHH guidelines: Cefixime 400 mg orally followed by 400 mg 6 – 12 hours later plus azithromycin 2 g orally, only advisable if an intramuscular injection is contraindicated or refused by the patient; gentamicin 240 mg intramuscularly as a single dose plus azithromycin 2 g orally; azithromycin 2 g orally as divided doses of 1 g followed 6 – 12 hours later with another 1 g dose to reduce gastrointestinal side effects; and finally ciprofloxacin 500 mg orally as a single dose. Spectinomycin is no longer a recommended alternative treatment for *N. gonorrhoeae* by BASHH as it the antimicrobial is no longer available in the UK.(12, 46)

For complicated *N. gonorrhoeae* infections ceftriaxone 1 g intramuscularly as a single dose can be given in the treatment of pelvic inflammatory disease, inflammation of the epididymis and gonococcal conjunctivitis.(12, 46)

For disseminated gonococcal infection the following is recommended: ceftriaxone 1 g intramuscularly or intravenous every 24 hours. This therapy should continue for seven days for arthritis but may be switched 24 – 48 hours after symptoms improve to one of the following oral regimens: cefixime 800 mg twice daily, or ciprofloxacin 500 mg twice daily. For gonococcal meningitis should be treated with 1 – 2 g ceftriaxone IV every 12 – 24 hours for 10 – 14 days, and gonococcal endocarditis treated with 1 – 2 g IV every 12 – 24 hours for a minimum of four weeks. Dissemination only occurs in 0.5 to 3 % of *N. gonorrhoeae* infections from any site, with females at a 4-fold higher risk than males, especially if they are menstruating or pregnant.(12, 33, 46)

Ceftriaxone 1 g intramuscularly as a single dose is the recommended treatment during pregnancy and breast-feeding. Azithromycin 2 g should only be used in the absence of adequate alternatives, and if the *N. gonorrhoeae* isolate is known to be susceptible. It is recommended that pregnant and breast-feeding individuals should not be treated with

quinolone antimicrobials due to concerns regarding foetal malformations and carcinogenesis in animals. Therefore, treatment with ciprofloxacin 500 g is not advised for pregnant individuals.(12, 45, 46)

1.6.2 ANTIMICROBIAL RESISTANCE IN *N. GONORRHOEAE*

N. gonorrhoeae has developed antimicrobial resistance to all antibiotics available in the UK and around the world, causing the WHO to recognise it as a serious global threat.(4) As there are no new antibiotics available to replace the current treatment regimes, understanding current antibiotic resistance trends is important.(20, 47) The antibiotics currently recommended for use in the UK by BASHH are ceftriaxone, ciprofloxacin, azithromycin, cefixime, and gentamicin.(12, 46)

Ceftriaxone, the current first-line monotherapy for *N. gonorrhoeae* infection in the UK, is a third-generation cephalosporin antibiotic.(12, 48) This antimicrobial is not available orally and is usually administered intravenously or intramuscularly.(12) Ceftriaxone attaches itself to penicillin binding proteins on the surface of the cell to interrupt cell wall synthesis, causing lysis of the cell and death.(49) The current clinical breakpoint for *N. gonorrhoeae* and ceftriaxone determined by EUCAST is 0.125 mg/L.(39) According to the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) surveillance report, a decrease in reduced susceptibility, where the MIC is greater than 0.03 mg/L, was reported in ceftriaxone from 1.4 % in 2020 to 0.07 % in 2021, remained low at 0.21 % in 2022, increasing to 0.91 % in 2023. Although reassuringly low, caution should be heeded as referrals of ceftriaxone non-susceptible *N. gonorrhoeae* isolates to the United Kingdom Health Security Agency (UKHSA) Antimicrobial Resistance in Sexually Transmitted Infections (AMRSTI) laboratory have increased in recent years: 0 in 2020, 2 in 2021 and 23 in 2022 – 2023.(12, 45, 50, 51)

Cefixime is also a third-generation cephalosporin antibiotic that attaches to penicillin binding proteins and disrupts cell wall synthesis.(52) From 2005 – 2010 in the UK, cefixime was the recommended first line treatment for infection with *N. gonorrhoeae* and during this

time resistance increased. The current EUCAST clinical breakpoint for cefixime is 0.125 mg/L.(39) Data from GRASP surveillance shows resistance at almost zero in 2005 to over 25 % in men who have sex with men (MSM) in 2010.(53) Cefixime resistance in Wales and England declined from 0.6 % in 2020 to 0.3 % in 2021. However, resistance to cefixime increased to 0.8 % in 2022, and increased again in 2023 to 5.6 %.(45, 50, 51)

Resistance to third generation cephalosporins such as ceftriaxone and cefixime in *N. gonorrhoeae* is thought to have originated in commensal *Neisseria* species. Areas such as the pharynx provide reservoirs of commensal *Neisseria* species which contain antimicrobial resistance genes. As pharyngeal gonorrhoea infection is typically asymptomatic *N. gonorrhoeae* and commensal *Neisseria* species can coexist for some time in this reservoir, sharing genetic material such as antimicrobial resistance genes (e.g., mosaic *penA* alleles).(11, 44) As a result, surveillance programmes such as GRASP made the decision in 2021 to prioritise pharyngeal infections over the other sites in patients suffering from multi-site *N. gonorrhoeae* infections.(45) Since 2015 there have been world-wide concerns about the long-term effectiveness of ceftriaxone therapy due to the spread of the ceftriaxone resistant clone FC428, which is characterised by a novel mosaic *penA* 60.001. This mosaic *penA* allele contains two key ceftriaxone resistance mutations A311V and T483S. The FC248 clone has been widely reported around the world, originating in Japan, and spreading to Denmark, South Korea, Ireland, Canada, Australia, Singapore, France, and the UK.(54, 55)

Azithromycin belongs to the macrolide class of antibiotics. Until a change in prescribing in 2018, dual therapy of azithromycin 1 g orally plus ceftriaxone 500 mg intramuscularly was the first line treatment in the UK for infection by *N. gonorrhoeae* leading to a rise in resistance. Most recently in Wales and England, resistance to azithromycin increased from 8.7 % in 2020, to 15.2 % in 2021, and 20.4 % in 2022. Unfortunately there was no reported azithromycin resistance in the most recent GRASP report due to a laboratory issue.(12, 45, 50, 51) EUCAST no longer has a set breakpoint for azithromycin (previously 0.5 mg/L), instead replacing it with an epidemiological cut-off (> 1 mg/L).(39) A clinical breakpoint is used to predict the clinical outcome of patient treatment, whereas an epidemiological cut off provides a method for categorising isolates as wild type isolates with no phenotypical resistance, and non-wild type isolates with acquired resistance mechanisms.(56) It should be

noted that in the 2024 GRASP report they noted an error in their susceptibility testing agar dilution method affecting the azithromycin results potentially going back to 2018. They are currently reviewing the process and their susceptibility results. Therefore, these listed resistance rates should be read with caution.(51)

Azithromycin exercises its antimicrobial effect by binding to the 23S ribosomal ribonucleic acid (rRNA) of the 50S ribosome, inhibiting protein synthesis in the cell.(57) Isolates of *N. gonorrhoeae* can be referred to as high-level azithromycin resistant, those with MICs > 256 mg/L, or low-level azithromycin resistant, MICs > 1 mg/L to 256 mg/L.(58) Alteration of 23S rRNA by either a mutation or by enzymatic modification is thought to contribute to low-level resistance. The presence of enzymes such as *ermB* and *ermF* modify the 23S rRNA, preventing macrolides from binding and contribute to low-level azithromycin resistance, (greater than 0.5 mg/L).(59, 60) It is thought a mutation in the peptidyl transferase loop of domain V of the 23S rRNA (C2611T) gene first seen in two Canadian *N.* gonorrhoeae strains contributes to low-level azithromycin resistance.(61) Efflux pumps are proteins that actively pump substances such antimicrobials out of the cell, the MtrCDE efflux pump can contribute to low-level azithromycin resistance when there is a mutation in either *mtrR*, *mtrD* or the promoter region causing overexpression of the efflux pump.(60, 62) *N. gonorrhoeae* contains 4 copies of the 23S rRNA gene, and it is thought that if at least three if these alleles contain the mutation A2059G in domain V, high-level azithromycin resistance is expected.(55, 60)

Ciprofloxacin 500 mg is a fluoroquinolone class of antibiotic and given to patients as first line therapy for *N. gonorrhoeae* as directed by the 2018 BASHH guidelines. As resistance remained high, (increasing from 44.3 % in 2020 to 46.9 % in 2021 and 58.6 % in both 2022 and 2023), it was only administered if the susceptibility result is known.(12, 45, 50, 51) Ciprofloxacin is a bactericidal drug that inhibits topoisomerase II (known as DNA gyrase) and topoisomerase IV, essential components of bacterial genetic replication whose function is to break the existing double DNA strand and replicate a new DNA strand.(63, 64) It has been reported that ciprofloxacin inhibits subunit A of the DNA gyrase from resealing the DNA double strand.(65) Bacterial resistance to ciprofloxacin in *N. gonorrhoeae* is caused by mutations S91F, D95A and D95G in *gyrA*, causing reduced quinolone binding to the DNA

gyrase. Mutations in *parC* such as S87R, S88P and E91K lessen the binding of quinolones to topoisomerase IV. Mutations in *gyrB* and *parE* genes appear to have no effect on ciprofloxacin resistance in *N. gonorrhoeae*.(55, 63, 65) According to EUCAST *N. gonorrhoeae* isolates with a ciprofloxacin MIC greater than 0.064 mg/L are resistant.(39) Due to a change in the British Association for Sexual Health and HIV (BASHH) guidelines in April 2025, ciprofloxacin is no longer recommended as first-line treatment. It is however still possible to use ciprofloxacin in the treatment of *N. gonorrhoeae* if deemed appropriate by clinicians.(46)

Gentamicin is an aminoglycoside antimicrobial that is an alternative treatment for *N. gonorrhoeae* infection. It is used in many developing countries as first-line treatment due to its low cost and efficacy.(66) Aminoglycosides such as gentamicin bind to the 16s rRNA at the 30s ribosomal subunit, disrupting messenger ribonucleic acid (mRNA) leading to the inhibition of protein synthesis.(5) Low-level gentamicin resistance, with MICs greater than or equal to 32 mg/L, but less than 128 mg/L in *N. gonorrhoeae,* is thought to be because of amino acid alterations in the elongation factor G, involved in protein synthesis, especially A563V, and also G564D and V651F. High-level gentamicin resistance, with MICs greater than or equal to 128 mg/L, is proposed to be due to mutations in the binding site to the 30S ribosome, specifically (M520I) in elongation factor G domain IV.(39, 67)

Another aminoglycoside, spectinomycin, was listed as an alternative treatment for *N. gonorrhoeae* albeit rarely until the new BASHH guidelines published in 2025. Its mode of action is the same as gentamicin, binding to the 16S rRNA of the 30S ribosome, leading to inhibition of protein synthesis, and ultimately to death of the cell.(5) In *N. gonorrhoeae,* spectinomycin high-level resistance, with an MIC greater than or equal to 2048 mg/L, can be due to a single mutation C1192U in 16S rRNA, whilst another novel resistance mechanism identified in Norway, is a mutation in the S5 protein also causing high-level spectinomycin resistance.(39, 68, 69)

1.7 POTENTIAL OTHER TREATMENT POSSIBILITIES FOR *N. GONORRHOEAE*

1.7.1 OLD/REPURPOSED ANTIMICROBIALS FOR TREATMENT OF *N. GONORRHOEAE*

As antimicrobial resistance for the most used antimicrobials against *N. gonorrhoeae* continues to increase and few new antimicrobials are entering the market quickly enough, healthcare scientists and clinicians have been looking at older antimicrobials that can be repurposed and be used in the treatment of *N. gonorrhoeae* infection.(70)

A randomised control trial conducted in Amsterdam, Netherlands in 2021 investigated the efficacy of some older alternative antimicrobials such as ertapenem, gentamicin, and fosfomycin against ceftriaxone susceptible, uncomplicated *N. gonorrhoeae* infections. These antimicrobials were tested in comparison with ceftriaxone, the current recommended antimicrobial therapy for many countries worldwide. The study found that although fosfomycin was not an appropriate alternative, single dose ertapenem treatment was found to be "non-inferior" when compared with single dose ceftriaxone treatment on ceftriaxone susceptible *N. gonorrhoeae*. The study advised further work to investigate how well single dose ertapenem performed when tested against ceftriaxone resistant *N. gonorrhoeae*. Although this study sounds promising, the use of ertapenem to treat uncomplicated *N. gonorrhoeae* infections may not be the best course of action. Increasing rates of antimicrobial resistance to carbapenems such as ertapenem is a global problem in the treatment of Enterobacterales, *Pseudomonas* spp., and *Acinetobacter* spp. infections, with clinicians and healthcare scientists advising minimising the use of these antimicrobials to prevent the increasing spread of resistance.(71)

1.7.2 NEW ANTIMICROBIALS FOR TREATMENT OF *N. GONORRHOEAE*

As the risk of antimicrobial resistance continues to increase for the antimicrobials currently in circulation for the treatment of *N. gonorrhoeae* infections, there is a pressing need for new antimicrobials. However, the discovery and testing required for new antimicrobials is time consuming and expensive. With the development and implementation taking as many

as 15 years and costing millions of pounds. Therefore, the development of new antimicrobials to treat infections such as *N. gonorrhoeae* are few.(70)

In recent years there are a few antimicrobials new to the market that have antimicrobial activity against *N. gonorrhoeae*, they are solithromycin, gepotidacin and zoliflodacin, with all three belonging to new classes of antimicrobials.(70)

Solithromycin is the first of the fluoroketolide class of antimicrobials. Fluoroketolides are a new class of antibiotics that are structurally related to ketolides, and ketolides are structurally related to macrolides. Macrolides' mode of action is the inhibition of protein synthesis by binding to the 23S rRNA of the 50S ribosome. Solithromycin binds to three binding sites on the ribosome compared to older macrolides that only bind to two binding sites, suggesting resistance will be less likely. Several trials have been performed to assess use of solithromycin in the treatment of *N. gonorrhoeae*. One trial, SOLITAIRE-U, compared solithromycin to the usual treatment of single dose azithromycin and ceftriaxone. The SOLITAIRE-U trial consisted of patients with *N. gonorrhoeae* infections; 123 patients were in the solithromycin group and 129 patients in the ceftriaxone plus azithromycin group. Due to a lower percentage of patients with eradication at day 7, the SOLITAIRE-U trial found that treatment of *N. gonorrhoeae* with a single dose of 1000 mg solithromycin was not an adequate alternative to single dose azithromycin and ceftriaxone. The trial also noted an elevated level of gastrointestinal adverse events in the solithromycin arm of the trial, with 44 % of participants in this arm reporting gastrointestinal disorders ranging from mild to moderate in their severity. This is important to note as in this trial a single dose of 1000 mg solithromycin was an inferior treatment, however increasing the dosage could lead to an even higher level of adverse events for the patients.(70, 72, 73)

Gepotidacin is a novel triazaacenaphthylene antimicrobial that inhibits bacterial DNA replication. It inhibits two of the type IIA topoisomerases, the B subunit of DNA gyrase and topoisomerase IV. GlaxoSmithKline (GSK) is currently performing clinical trials testing the efficacy of gepotidacin. The Efficacy of Antimicrobial Gepotidacin Evaluated phase III programme (EAGLE), one of which, EAGLE-1, is a non-inferiority urogenital gonorrhoea trial. EAGLE-1 is comparing the efficacy and safety of gepotidacin against ceftriaxone and azithromycin in the treatment of uncomplicated urogenital *N. gonorrhoeae* infections. The

results are promising with gepotidacin reported as non-inferior to treatment with ceftriaxone and azithromycin. However, the study showed that for pharyngeal site infections the gepotidacin group had a 78 % success rate whereas the ceftriaxone and azithromycin group had a 94 % success rate. This Phase III trial reported that gepotidacin was tolerated well by patients, with only some patients reporting mild gastrointestinal side effects.(70, 74-76)

Zoliflodacin belongs to the new class of antimicrobials, spiropyrimidinetrione. Zoliflodacin inhibits type II topoisomerases, by binding in the DNA gyrase of the bacteria, preventing replication of the cell. There is a Phase III clinical trial by the Global Antibiotic Research & Development Partnership (GARDP), in collaboration with Innovia Speciality Therapeutics that is reporting very promising results. They reported that single dose oral zoliflodacin was non-inferior when tested in comparison with intramuscular injection of ceftriaxone and oral azithromycin, and the trial reported no serious adverse events. Encouragingly zoliflodacin was shown to be active against strains of *N. gonorrhoeae* that were resistant to ceftriaxone and azithromycin.(70, 77)

With so few new antimicrobials that are appropriate for the treatment of *N. gonorrhoeae* infections being brought to the market the burden still lies with the antimicrobials currently recommended for treatment. Therefore, accurate susceptibility testing is a must for these antimicrobials as it informs on resistance trends and advising clinicians and healthcare scientists whether the first-line therapy needs to be changed to prevent the continued increase of antimicrobial resistance.

1.7.3 GONOCOCCAL VACCINES

Researchers around the world have stated that as the options for treatment of *N. gonorrhoeae* are hampered by antimicrobial resistance, the only sustainable solution for the management of *N. gonorrhoeae* is a gonococcal vaccine.(17, 44) In the UK the Bexsero vaccine is going to be offered to individuals considered at high-risk of *N. gonorrhoeae* infection starting August 2025. The Bexsero vaccine is a meningococcal group B vaccine that

has been used in the immunisation of babies since 2015 and has effectively reduced meningococcal B infection by 75 %. It is shown to also offer 30 – 40 % protection against *N. gonorrhoeae* infection, this is a huge breakthrough and will reduce transmission of *N. gonorrhoeae* in the community. However, even with the protection of the vaccine patients are still at risk of infection of *N. gonorrhoeae* so an effective susceptibility testing method is required.(78)

1.8 WHOLE GENOME SEQUENCING OF *N. GONORRHOEAE*

1.8.1 USING WHOLE GENOME SEQUENCING TO PREDICT ANTIMICROBIAL SUSCEPTIBILITY

As technology progresses in the field of whole genome sequencing, it is far easier and quicker to produce a whole genome sequence now than it has ever been in the past.(79, 80) These whole genome sequences can be utilized in a variety of ways in reference to susceptibility testing.(81) Many of the international *N. gonorrhoeae* surveillance projects employ whole genome sequencing to inform on the clones currently in circulation.(51, 82, 83) By knowing which clones of *N. gonorrhoeae* are prevalent at any one time we can better understand the effectiveness of the current first line treatment, and make changes as required.

It is thought that whole genome sequencing of *N. gonorrhoeae* can be used to predict the susceptibility profile of the bacteria. Allowing the clinician to tailor patient treatment depending on the sequence of *N. gonorrhoeae*. As previously discussed, mutations in the whole genome sequence can identify if certain antimicrobials will not be effective against a particular *N. gonorrhoeae* strain.(81)

As it has been proven that a whole genome sequence can inform as to the antimicrobial resistance of some antibiotics present in the isolate, the practicalities of this process in routine diagnostics for the treatment of patients is questionable. This technology, although widely used for certain bacteria (e.g. *Mycobacterium tuberculosis*), is still an expensive process, especially when compared to current gradient strip or disc susceptibility testing methods.(84) Typically, those patients who present to a clinic with symptomatic *N.*

gonorrhoeae infection are treated before susceptibility results are available, whole genome sequencing of the isolate would therefore not be of benefit to the patient.(44)

Any change in processing of patient samples, diagnosis or treatment of patients needs to be superior to the current method, whether it be by saving money, time but still providing the same level of accuracy or giving a more accurate result. Whole genome sequencing can be a useful tool in national and international surveillance projects but currently is of little benefit to patients as part of the routine diagnostic and susceptibility testing process.

1.9 EFFECT OF THE COVID-19 PANDEMIC ON *N. GONORRHOEAE* TRANSMISSION

On 30th January 2020 the World Health Organisation (WHO) declared the novel COVID-19 outbreak, "a public health emergency of international concern, WHO's highest level of alarm".(85) As a result of this declaration many countries enabled strict lockdowns, closing their borders from international travel and promoting social distancing measures such as mask wearing and increased hand washing procedures. Due to these interventions many healthcare workers and scientists in the healthcare sector expected the transmission of sexually transmitted infections such as *N. gonorrhoeae* to reduce significantly, however this was not the case in many countries around the world.(86-89)

In Queensland, Australia, public health measures due to the COVID-19 pandemic went into effect on 19th March 2020 with limits on in-person gatherings, both indoors and outside. On 23rd March 2020 all non-essential businesses were instructed to close, and further border restrictions were instated on 26th March 2020, restricting travel from alternative states or territories and the implementation of a self-quarantine for 14 days for travellers. A high percentage of *N. gonorrhoeae* infections in Australia, 44 %, are because of foreign travel or contact with an overseas traveller. Therefore, it comes as a surprise that even with the application of these strict measures including the closure of the borders, Queensland noted an increase in *N. gonorrhoeae* infections during the pandemic.(86)

The state of California went into lockdown 19th March 2020, with the Californian governor issuing a shelter-in-place order to mitigate the transmission of the virus, this order closed

schools, restaurants and most workplaces. However, a study in San Diego, California looking at the COVID-19 pandemic and the association between where men who have sex with men meet sexual partners and *N. gonorrhoeae* infection found the prevalence of STIs such as *Chlamydia trichomatis* and *N. gonorrhoeae* increased during the COVID-19 pandemic, compared with pre COVID-19 levels.(87)

Lockdown in Finland began on 16th March 2020, with the closure of schools, entertainment venues, limiting public meetings and border restrictions including a 2-week quarantine for those returning to Finland. However, even with these measures in place, Finland reported a similar number of *N. gonorrhoeae* diagnoses in March 2020 when compared to diagnoses in reference years 2015 – 2019. This is surprising as it is reported that 50 % of *N. gonorrhoeae* infections in Finland are acquired outside of the country.(89)

The increase or stable rates of *N. gonorrhoeae* infections during the pandemic is surprising. With strict lockdowns in place in almost all countries, and foreign travel seriously disrupted for most of 2020, it could be assumed that the rates of infection would dramatically decrease. The increase could be due to several factors, including, populations with low adherence to lockdown measures and continuing to meet up with sexual partners. Another reason could be due to many healthcare clinics and hospitals around the world redeploying staff from sexual health clinics to help with the COVID-19 response. As a result, routine screening of sexually transmitted infections was dramatically reduced with some sexual health clinics were only seeing patients that had symptomatic *N. gonorrhoeae* infections. Therefore, patients infected with *N. gonorrhoeae* but were asymptomatic continued to engage in sexual behaviours during lockdown and unknowingly spreading the infection to others.(86-89)

1.10 WORLDWIDE SURVEILLANCE PROJECTS FOR *N. GONORRHOEAE*

Around the world there are many surveillance projects specifically looking at *N. gonorrhoeae* infections, these give us a global picture of the rates of infection amongst different populations and the level of resistance prevalent for the antimicrobials tested. These

surveillance projects are vital in the fight against antimicrobial resistance, as we can more easily track the spread of resistance and use the information to better inform which antimicrobials are appropriate for first line treatment for patients.(51, 82, 83)

1.10.1 GONOCOCCAL RESISTANCE TO ANTIMICROBIALS SURVEILLANCE PROGRAMME

For over 20 years in the United Kingdom GRASP has collected isolates of *N. gonorrhoeae* from laboratories in Wales and England. In 2022, 26 sexual health services, 2 in Wales and 24 in England, provided isolates and additional data for this important surveillance project. This sentinel surveillance programme collects consecutive *N. gonorrhoeae* isolates from a variety of sites over a two- or three-month period, typically July to September, each year. Since 2021, the hierarchy of site of specimen collection has listed pharyngeal as the highest priority, vitally important for accurate rates of resistance. GRASP performs susceptibility testing by agar dilution on ceftriaxone, azithromycin, cefixime, ciprofloxacin, tetracycline and spectinomycin. Each isolate is whole genome sequenced, highlighting clones of *N. gonorrhoeae* that are circulating each year and driving antimicrobial resistance. This surveillance project collects important information that can inform of any increase in resistance leading to a change of the first-line antimicrobial treatment in the UK.(45, 50, 51)

1.10.2 EUROPEAN GONOCOCCAL ANTIMICROBIAL SURVEILLANCE PROGRAMME

Since 2009 the European Centre for Disease Prevention and Control (ECDC) has co-ordinated the European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP). Euro-GASP is a yearly surveillance project that requires member countries to collect *N. gonorrhoeae* isolates during September to November. The susceptibility testing is decentralised, with each of the European Union/European Economic Area (EU/EEA) Member States performing either gradient strip testing or agar dilution on ceftriaxone, cefixime, azithromycin, and ciprofloxacin. In 2020 there were 23 EU/EEA Member States that participated in this surveillance project.(83)

1.10.3 GONOCOCCAL ISOLATE SURVEILLANCE PROJECT

In the United States of America there is the Gonococcal Isolate Surveillance Project (GISP), which was established in the 1980s for the purpose of monitoring antimicrobial resistance trends in *N. gonorrhoeae*. GISP asks laboratories based across the United States of America to collect 25 samples per month from men diagnosed with *N. gonorrhoeae* of the urethra. Susceptibility testing is performed on these isolates with the following antimicrobials, azithromycin, cefixime, ceftriaxone, ciprofloxacin, gentamicin, penicillin, and tetracycline. As this surveillance only requires samples from the male urethra it does not give a true representation of the vast range of *N. gonorrhoeae* circulating in the population, as a result, in recent years GISP has been expanded to include *N. gonorrhoeae* isolated from women and from sites such as pharynx, endocervix and rectum.(82) It is vital that pharyngeal samples are included in this type of surveillance as the pharynx may be a reservoir of antimicrobial resistance mechanisms which are able to be transferred from commensal *Neisseria* to *N. gonorrhoeae* and often is the cause of many treatment failures.(44)

1.11 ANTIMICROBIAL RESISTANCE SEEN IN WALES AND THE UK

1.11.1 CEFTRIAXONE RESISTANCE ON THE RISE

There has been a recent report in the press of 15 confirmed cases of *N. gonorrhoeae* isolated from patients in England that are resistant to ceftriaxone in the previous two years up to June 2024. This is a worrying development as ceftriaxone is the current first-line treatment for patients infected with *N. gonorrhoeae* in the UK. Of these 15 cases, 5 have been confirmed as multidrug resistant. Most of these patients have a history of travel to the Asia-Pacific region.(90, 91)

In Wales there were two documented ceftriaxone resistant *N. gonorrhoeae* strains seen in 2022. These strains were isolated from 1 male and 1 female patient of Southeast Asian descent. The patients were in a monogamous relationship, limiting the spread of this ceftriaxone resistant strain.(92)

We are seeing in real time the transmission of ceftriaxone resistant *N. gonorrhoeae* strains from Southeast Asia to other countries around the world. Again, the importance of swift identification and accurate antimicrobial susceptibility testing cannot be stated. As the current first-line therapy in the UK is ceftriaxone, patients with ceftriaxone resistant strains need to be called back into clinic as soon as possible to receive a more appropriate therapy before further transmission occurs.(93)

1.11.2 AZITHROMYCIN RESISTANCE

Azithromycin resistance, both high and low-level, has been seen in the UK.(94) In 2022 laboratories in Wales were asked to submit any high-level azithromycin resistant *N. gonorrhoeae* to the Specialist Antimicrobial Chemotherapy Unit (SACU) of Public Health Wales for confirmation so we could keep a track of the numbers circulating locally. During this time four high-level azithromycin resistant, > 256 mg/L, strains of *N. gonorrhoeae* were submitted to SACU. Further investigation on these strains is described in chapter 6. (M. Wootton, personal communication, 31st October 2023)

1.12 AIMS OF THE PROJECT

The impact of *Neisseria gonorrhoeae* infection is a far-reaching global concern. With high risk of the infection spreading from person to person, burden on health services and increasing antimicrobial resistance especially in underdeveloped countries around the world, the importance of accurate, cheap, and readily available susceptibility testing cannot be stated high enough.

Generally, *N. gonorrhoeae* culture is highly challenging, with isolates failing to grow on laboratory media, however recently molecular methods have improved diagnosis. Susceptibility testing methods for *N. gonorrhoeae* have always been problematic, mainly due to the fastidious nature of the bacteria, with many common laboratory media unable to support growth. In the past 5 years in the UK, *N. gonorrhoeae* susceptibility testing methods

have been hampered by lack of availability of recommended media for MIC testing by gradient strip. This project will evaluate all commercially available appropriate culture media available from several manufacturers, along with two gradient strip manufacturers for frequently used antimicrobials in the UK: ceftriaxone, cefixime, azithromycin and ciprofloxacin. The media will be evaluated for their ability to support sufficient growth of *N. gonorrhoeae* to enable a susceptibility test and accuracy of MICs to all antimicrobial agents. Inter and intra-laboratory differences will be established as well as reproducibility plus common variations in methods such as inoculum prepared in saline as opposed to Muller Hinton Broth. The results will establish the most reliable methods, which will provide recommendations for culture media and methodology for routine diagnostic microbiology laboratories in the UK and Europe for susceptibility testing by gradient strip, with the guidance published on the British Society of Antimicrobial Chemotherapy and European Committee of Antimicrobial Susceptibility Testing website. This study will further inform which culture media should be used in developing a disc diffusion method in conjunction with the EUCAST Developmental Laboratory in Sweden.

1.12.1 MAIN OBJECTIVES

The main objectives of this study were as follows:

1. Evaluate seven commercially available agar plates for minimum inhibitory concentration determination of *N. gonorrhoeae*.
2. Evaluate two different commercially available gradient strips for antimicrobial susceptibility testing.
3. Compare inoculum preparation using saline and Mueller Hinton Broth.
4. Compare the effect of primary culture media used for strain recovery from frozen storage on gradient strip method.
5. Provide guidance for UK laboratories through the BSAC website.
6. Collaborate with EUCAST to develop a disc diffusion method for *N. gonorrhoeae*.
7. Investigate local rates of incidence and antimicrobial resistance of *N. gonorrhoeae* in comparison with a national *N. gonorrhoeae* surveillance programme such as GRASP.

8. Investigate anomalous azithromycin gradient strip results seen in Wales.

These objectives were devised due to the lack of published literature on the currently available media for susceptibility testing of *N. gonorrhoeae* in the UK. Previously published studies either included media that is no longer available, media that is not available in the UK, or only tested gradient strips from one manufacturer, bioMérieux.(95-98) This thesis includes the most comprehensive evaluation of UK media for susceptibility testing of *N. gonorrhoeae.*

CHAPTER 2 – MATERIALS AND METHODS

The laboratory work in this project was done in conjunction with the Antimicrobial Resistance in Sexually Transmitted Infections (AMRSTI) section of the Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Laboratory at the United Kingdon Health Security Agency (UKHSA). I performed the susceptibility testing in SACU (Laboratory 1) and one technician in AMRSTI (Laboratory 2).

To address objectives listed in the introduction this study was split into three stages, described below.

Note: The terms agar plates and media are used interchangeably in routine diagnostic laboratories.

2.1 MATERIALS

2.1.1 BACTERIAL STRAINS

The study was divided into 3 stages, using different isolates for each stage. The clinical isolates used in stages 2 and 3 were all anonymised and no patient information was attached, therefore the study did not require ethical approval.

2.1.1.1 Stage 1

Stage 1 is a viability assessment of the chosen media, to determine whether they support the growth of *N. gonorrhoeae*.

Four World Health Organisation (WHO) control strains were used to assess viability on various commercial agar plates, seen in table 1.

Table 1: WHO quality control strains used throughout the project, with associated reference numbers, equivalent gradient strip (GS) MIC ranges, and to which stage they are assigned

SACU number	WHO Control strain	NCTC culture collection	Azithromycin GS MIC range	Ceftriaxone GS MIC range	Cefixime GS MIC range	Ciprofloxacin GS MIC range	Stage 1	Stage 2	Stage 3
34807	WHO F	13477	0.064 - 0.25	< 0.002	< 0.016	0.002 - 0.008	✓		✓
34808	WHO G	13478	0.125 – 0.5	0.004 - 0.016	< 0.016	0.064 - 0.25	✓	✓	✓
34809	WHO K	13479	0.125 - 0.5	0.032 - 0.125	0.125 - 0.5	> 32	✓	✓	✓
34810	WHO L	13480	0.25 - 1	0.125 - 0.5	0.064 - 0.25	> 32			✓
34811	WHO M	13481	0.125 - 0.5	0.008 - 0.032	< 0.016	1 - 4	✓		✓
34812	WHO P	13484	2 - 8	0.002 - 0.008	< 0.016	0.002 - 0.008			✓
34814	WHO V	13818	> 256	0.032 - 0.125	< 0.016	> 32			✓
34815	WHO W	13819	0.25 - 1	0.032 - 0.125	0.125 - 0.5	> 32			✓
34816	WHO X	13820	0.25 - 1	1 - 4	2 - 8	> 32			✓
34817	WHO Y	13821	0.5 - 2	0.5 - 2	1 - 4	> 32			✓
34818	WHO Z	13822	0.5 - 2	0.25 - 1	1 - 4	> 32			✓

The quality control strains listed in table 1 are well established laboratory isolates, with robust growth patterns.

2.1.1.2 Stage 2

Stage 2 is the media assessment of all agar plates tested with clinical strains.

Forty-six clinical *N. gonorrhoeae* isolates, previously submitted to AMRSTI as part of the Gonococcal Resistance to Antimicrobial Surveillance Programme (GRASP) by the Specialist Antimicrobial Chemotherapy Unit (SACU) Cardiff, were tested, seen in table 2. These strains were chosen as both laboratories (SACU and AMRSTI) had access to them, therefore reducing the risk of potential laboratory errors and isolates dying during transportation between sites. These clinical isolates are representative of *N. gonorrhoeae* isolates within the Cardiff area. WHO quality control strains G and K were also tested alongside the clinical isolates, selected as they are well categorised with respect to published antibiograms for the antimicrobials tested in this study, seen in table 1.(99)

Table 2: GRASP clinical isolates used in stage 2

SACU number	GRASP collection year
26333	2017
26334	2017
26335	2017
26336	2017
26337	2017
26340	2017
26342	2017
26343	2017
26344	2017
26345	2017
26346	2017
26347	2017
26348	2017
26350	2017
26351	2017
26352	2017
26353	2017
26354	2017
28581	2018
28582	2018
28583	2018
28584	2018
28585	2018

SACU number	GRASP collection year
28586	2018
28587	2018
28588	2018
28589	2018
28590	2018
28591	2018
28592	2018
28593	2018
28594	2018
28595	2018
28596	2018
28597	2018
28598	2018
28599	2018
28600	2018
28601	2018
28605	2018
28607	2018
28608	2018
28609	2018
28610	2018
28611	2018
28612	2018
28613	2018

2.1.1.3 Stage 3

Stage 3 aimed to determine the optimum medium/gradient strip combination for susceptibility testing. This stage is to determine the best combination of media and gradient strip when tested against a diverse set of clinical and quality control strains.

For stage 3, twenty clinical strains were provided to SACU by AMRSTI. These strains were submitted to AMRSTI as part of their reference service from around the UK. The clinical strains of *N. gonorrhoeae* were chosen as they cover fully susceptible antibiograms but include isolates which exhibit resistances to the most common antimicrobial agents currently used for treatment seen in table 3.

Also tested alongside the clinical isolates were eleven well categorised WHO quality control strains provided to SACU by AMRSTI, containing resistance to a variety of clinically relevant antimicrobial agents.(99) These strains are well established reference results, shown in table 1.

Table 3: Clinical isolates provided by AMRSTI exhibiting resistance to clinically relevant antibiotics tested in stage 3

SACU number	AMRSTI phenotype
34837	Cefixime resistant
34827	Azithromycin and ciprofloxacin resistant
34828	Azithromycin resistant
34829	Azithromycin resistant
34830	Azithromycin resistant
34819	Azithromycin resistant
34820	Cefixime, ceftriaxone, ciprofloxacin resistant
34821	Cefixime, ceftriaxone, ciprofloxacin resistant
34832	Ciprofloxacin resistant
34835	Azithromycin and ciprofloxacin resistant
34833	Ciprofloxacin resistant
34831	Fully susceptible
34834	Ciprofloxacin resistant
34836	Cefixime and ciprofloxacin resistant
34838	Cefixime and ciprofloxacin resistant
34839	Fully susceptible
34822	Fully susceptible
34823	Fully susceptible
34824	Fully susceptible
34825	Ciprofloxacin resistant
34826	Ciprofloxacin resistant

Antimicrobials tested in stage 3 were azithromycin, ceftriaxone, cefixime, and ciprofloxacin.

2.1.2 ISOLATE STORAGE

All bacterial strains listed above were stored in the SACU laboratory at the University Hospital of Wales, Cardiff using the Protect bead storage (Technical Service Consultants) system and kept at - 80 °C until cultured.

2.1.3 PRIMARY CULTURE

For stage 1, different primary culture media were used in each laboratory to culture isolates from frozen storage. The media used at the two testing laboratories: GC + vitox (product code: PO0982A Thermo Fisher Oxoid, Basingstoke, UK) in AMRSTI, Chocolate agar (product code: PO5090A Thermo Fisher Oxoid, Basingstoke, UK) in SACU. In stages 2 and 3, Oxoid GC + vitox was used in both laboratories.

2.1.4 SUSCEPTIBILITY TESTING AGAR PLATES

Seven commercial agar plates were tested, including five that are recommended by gradient strip manufacturers: Oxoid GC + vitox, Oxoid Chocolate + vitox, Becton Dickinson (BD) GC Chocolate, E&O Laboratories GC chocolate + 10 % Defibrinated Horse Blood (DHB), and E&O Laboratories GC Non-selective.

The recommended media for both gradient strip manufacturers were as follows:

Etest (bioMérieux) media recommendations: GC agar base + defined supplements (Clinical Laboratory Standards Institute (CLSI) in USA) and Mueller Hinton + 1% IsoVitaleX (Becton Dickinson) + 1% haemoglobin (outside USA).(100)

MTS (Liofilchem) media recommendations: GC agar base + defined supplements (CLSI) or Mueller Hinton Chocolate agar.(43)

Recently, EUCAST have developed a disc diffusion method for anaerobic bacteria using a modified Fastidious Anaerobe Agar (FAA) medium (4 mm depth with 5 % defibrinated horse blood)(101). FAA media from two manufacturers were included in the study, with the potential that it may be useful in developing a EUCAST disk diffusion method for *N. gonorrhoeae* in the future. These were E&O Laboratories fastidious anaerobe agar with 5 % defibrinated horse blood (DHB) and Liofilchem fastidious anaerobe agar with horse blood.

All test media are listed in table 4 with corresponding product codes.

Table 4: Commercial agar plates used for susceptibility testing in stages 1 and 2

Manufacturer	Agar plate	Product code
Thermo Fisher Oxoid	GC + vitox	PO0982A
	Chocolate + vitox	PO5090A
Becton Dickinson (BD)	GC chocolate	254060
E&O Laboratories	GC chocolate + 10 % DHB	PP2311
	GC non-selective	PP2071
	Fastidious anaerobe agar with 5 % DHB	PP1564
Liofilchem	Fastidious anaerobe agar with horse blood	10062

2.1.5 GRADIENT STRIPS

Gradient strips from two manufacturers were used: bioMérieux and Liofilchem. The antibiotics tested are listed in table 5.

Table 5: Gradient strips tested throughout project

Antibiotic	Liofilchem Product Code	bioMérieux Product Code
Azithromycin	92030	412257
Cefixime	92060	412275
Ceftriaxone	92943	412303
Ciprofloxacin	92045	412311

2.1.6 INOCULA PREPARATION

For stages 1, 2 and 3, different inocula diluents were used to assess the effect on MIC in the different laboratories: 0.9% saline (product code: EB0334) in AMRSTI and Mueller Hinton Broth (MHB) (product code: T3462) in SACU. MHB was chosen as it is the preferred method of inocula preparation for both gradient strip manufacturers, and saline is most commonly used in routine diagnostic laboratories for inocula preparation of most other bacteria.(43, 100) It should be noted that when tests were performed in duplicate in SACU, the inocula were performed as if done in a separate laboratory, that is, one repeat was processed with MHB and labelled as laboratory 1 and one with saline and labelled as laboratory 2.

2.2 METHODS

All culture viability and gradient strip testing were performed in two laboratories where possible: Specialist Antimicrobial Chemotherapy Unit (SACU) in Cardiff and the Antimicrobial Resistance in Sexually Transmitted Infections section (AMRSTI) of the Antimicrobial Resistance and Healthcare Associated Infections Reference Laboratory at UKHSA in Colindale, London. However, due to reduced capacity at AMRSTI some tests were performed in duplicate at SACU. One technician performed the susceptibility testing in AMRSTI

(Laboratory 2) and I performed the susceptibility testing in SACU (Laboratory 1), and any duplicate testing required.

The project was split into three stages, the flowchart in figure 1 summarises quality control strains, clinical strains tested, and agars tested.

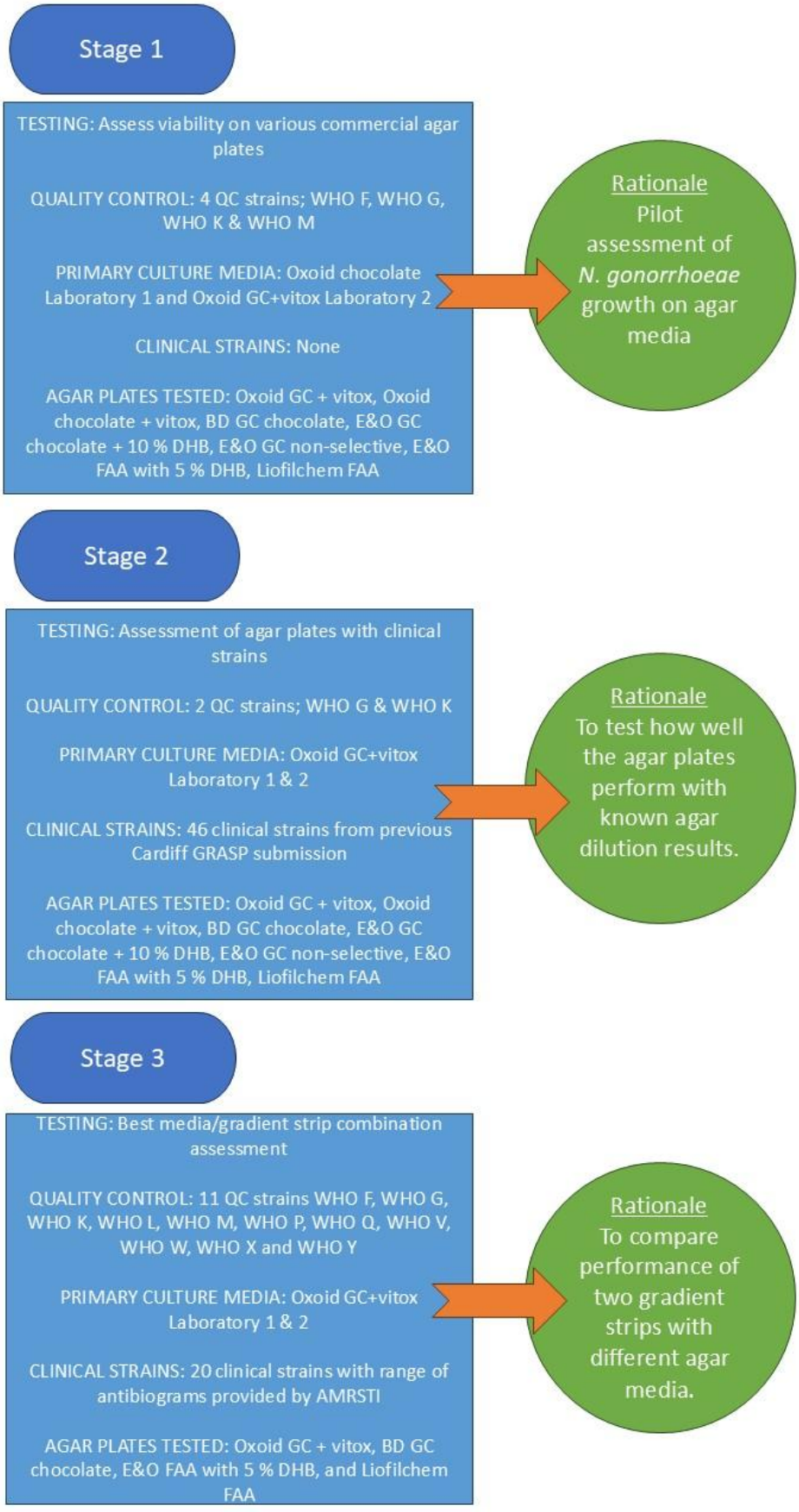


Figure 1: Flowchart of laboratory work for stages 1, 2, and 3.

2.2.1 PRIMARY CULTURE

Each bead vial of *N. gonorrhoeae* was taken from the -80 °C freezer, and beads removed from the vial using a 10 µL loop. The beads were inoculated onto either chocolate agar or GC + vitox and spread for single colonies, figure 2. All agar plates were incubated overnight in 5 - 10 % CO_2 at 35 to 37 °C.

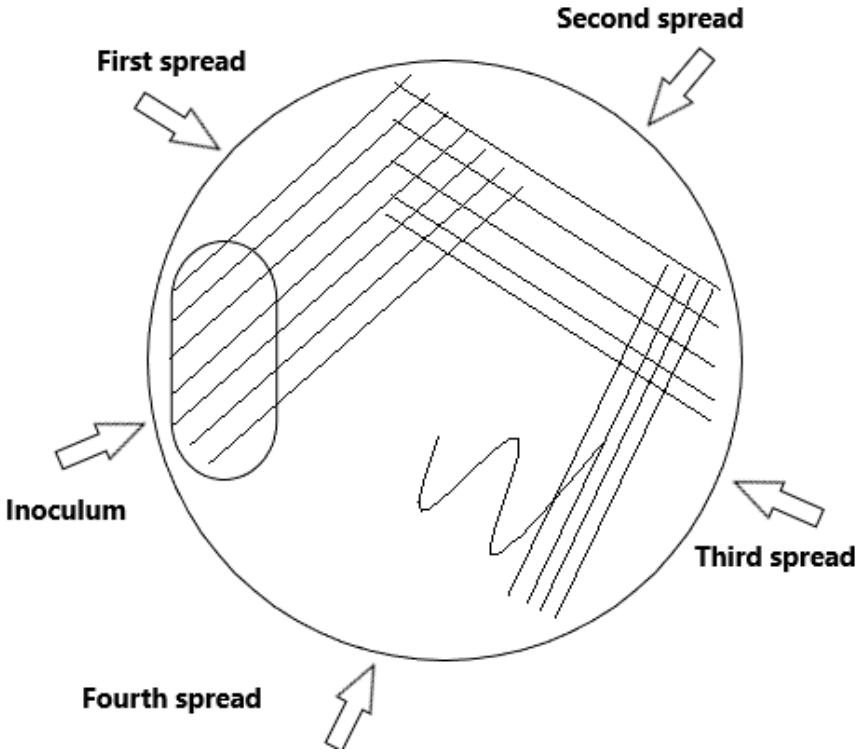


Figure 2: Standardised plate spreading method for single colony culture.

For stage 1, SACU (Laboratory 1) used Oxoid chocolate agar for primary culture and AMRSTI (Laboratory 2) used Oxoid GC + vitox.

Reference minimum inhibitory concentrations (MICs) for clinical strains were provided by AMRSTI from previous work resulting from their GRASP surveillance using agar dilution.

GRASP agar dilution is performed on a range of antimicrobials using Diagnostic Sensitivity Test (DST) agar plus 5 % lysed horse blood and 1 % vitox.(102)

The evaluation was structured into three stages to support ease of testing and allowing for discussion between laboratories about which agar plates to take through to the following stage.

2.2.2 STAGE 1 – VIABILITY ASSESSMENT

The viability assessment is a proof-of-concept project to investigate whether the agar plates chosen will support the growth of *N. gonorrhoeae* sufficiently to perform an MIC method test. Summarised in figure 1.

Agar plates used for the primary culture were examined to see whether they influenced the viability and weight of growth on the primary culture plate. To investigate this each laboratory used different agar plates for primary culture, SACU used Oxoid chocolate agar and AMRSTI used Oxoid GC + vitox, detailed in table 6.

Vials of WHO control strains F, G, K and M listed in table 1 were removed from the -80 °C freezer and two beads inoculated with a 10 µL loop onto Oxoid GC + vitox in AMRSTI, and Oxoid chocolate agar in SACU and spread for single colonies as in figure 2.

Culture viability was initially assessed in SACU and AMRSTI by quantifying the inoculation of 0.5 McFarland suspensions for four well characterised control strains: WHO F, WHO G, WHO K, and WHO M.

Seven commercially available agar plates were tested: Oxoid GC + vitox, Oxoid chocolate + vitox, Becton Dickinson GC chocolate, E&O Laboratories GC chocolate with 10 % defibrinated horse blood, E&O Laboratories GC Non-selective agar, E&O Laboratories fastidious anaerobe agar with 5 % defibrinated horse blood and Liofilchem fastidious anaerobe agar, listed in table 7.

Inocula of WHO G and K were prepared from an overnight culture using two methods, a 0.5 McFarland in Mueller Hinton Broth in SACU on Oxoid chocolate agar and a 0.5 McFarland in sterile 0.9 % saline in AMRSTI on Oxoid GC + vitox. Using a sterile swab 3 - 6 colonies were removed from the overnight culture and emulsified into either MHB or saline. Once prepared, a sterile swab was dipped into the inocula and spread onto the agar, as if performing the gradient strip method (complete surface coverage as opposed to spreading for single colonies). No gradient strips were applied, and the agar plates were incubated in 5 % CO_2 at 35 °C ± 2 °C for 20 - 24 hours.

All seven test agars supported the growth of *N. gonorrhoeae,* so these were then assessed for weight of growth, a standard method of quantifying bacterial growth in a microbiology laboratory. Inocula were prepared in either MHB or saline as previously described. For each strain, a 10 µL loop was used to dip into the inocula and spread onto the agar plate to create an inoculum and a new 10 µL loop used to spread for single colonies, figure 1. After overnight incubation the weight of growth of each agar plate was recorded (seen in figure 2) as follows; 0.5 - growth in inoculum/first spread only, 1 - growth up to second spread, 2 - growth up to third spread, 3 growth up to fourth spread. This is standard procedure for calculating weight of growth in microbiology laboratories. The scores of the WHO isolates were added together to give an overall weight of growth score for each agar plate.

Using a sterile swab the same inocula of WHO G and K was used to inoculate another set of agar plates to perform the gradient strip method, once the surface of the plate was dry, azithromycin, ceftriaxone and cefixime gradient strips from bioMérieux were aseptically applied using a sterile forceps and incubated in 5 % CO_2 at 35 °C ± 2 °C for 20 - 24 hours. The agar plates were assessed for ease of reading of the gradient strips. This allows the visibility of the growth and the contrast to the zone of inhibition to be calculated, especially where the ellipse intersects the strip at the MIC. One replicate of WHO G and K was tested. Ease of reading was scored follows: 1 - Very fuzzy zone of inhibition edge, difficult to read, 2 – moderate growth with slightly fuzzy zone of inhibition edge, 3 - easy to read, good contrast between growth and zone of inhibition.

Table 6: Summary of culture and inocula preparation in stage 1

	SACU	AMRSTI
Primary culture	Oxoid Chocolate agar	Oxoid GC + vitox
Inoculation preparation	Oxoid Muller Hinton Broth	Oxoid Saline

Table 7: Summary of agar media used by each laboratory in stage 1 and 2

Agar plate	Tested by
Oxoid GC + vitox	SACU & AMRSTI
Oxoid chocolate + vitox	SACU & AMRSTI
BD GC chocolate	SACU & AMRSTI
E&O Laboratories GC chocolate with 10 % DHB	SACU on 2 separate occasions
E&O Laboratories GC Non-selective agar	SACU on 2 separate occasions
E&O Laboratories fastidious anaerobe agar with 5 % DHB	SACU on 2 separate occasions
Liofilchem fastidious anaerobe agar	SACU on 2 separate occasions

2.2.3 STAGE 2 – MEDIA ASSESSMENT

Forty-six clinical isolates submitted from SACU to AMRSTI for the GRASP programme along with WHO G and WHO K control strains were tested by SACU and AMRSTI. Azithromycin and ceftriaxone MICs using gradient strips from bioMérieux on the commercial agar plates listed in table 7 were compared to reference MICs performed with the gold standard method of agar dilution provided by AMRSTI.(103) Inocula were prepared as described above using Mueller Hinton Broth in SACU and sterile saline in AMRSTI, gradient strips applied using a sterile forceps, and agar plates incubated in 5 % CO_2 at 35 °C ± 2 °C for 20 - 24 hours.

2.2.4 STAGE 3 – OPTIMUM MEDIA/GRADIENT STRIP COMBINATION ASSESSMENT

After completion of stage 2, the results were analysed, and it was decided four agars would proceed to stage 3, table 8.

Two gradient strip manufacturers readily available in the UK, Etest from bioMérieux and MTS from Liofilchem were evaluated on Oxoid GC + vitox, Becton Dickinson GC chocolate, E&O Laboratories Fastidious anaerobe agar and Liofilchem Fastidious anaerobe agar.

MICs were determined for twenty clinical isolates with a range of antibiograms and quality control strains, WHO F, G, K, L, M, P, Q, V, W, X, and Y by SACU and AMRSTI as stated in Table 10. Strains were tested on the four best performing agars from stage 2 against gradient strips azithromycin, cefixime, ceftriaxone and ciprofloxacin from two manufacturers listed in table 5. Summarised in figure 1.

Table 8: Summary of agar plates used in each laboratory for stage 3

Agar plates	Tested by
Oxoid GC + vitox	SACU & AMRSTI
Becton Dickinson GC chocolate	SACU & AMRSTI
E&O Laboratories Fastidious anaerobe agar with 5 % horse blood	SACU on 2 separate occasions
Liofilchem Fastidious anaerobe agar	SACU on 2 separate occasions

Inocula and application of gradient strips was performed as described above using sterile saline, and agar plates were incubated in an atmosphere of 5 % CO_2 at 35 °C ± 1 °C for 20 – 24 hours for bioMérieux Etest gradient strips and for Liofilchem MTS gradient strips 5 % CO_2 at 36 °C ± 1 °C for 20 – 24 hours.(43, 100)

CHAPTER 3 –

RESULTS

3.1 STAGE 1 – VIABILITY ASSESSMENT

Consistent and reliable growth of this fastidious organism, *N. gonorrhoeae*, is crucial to performing susceptibility testing and determining accurate results.

Firstly, culture viability of WHO quality control strains F, G, K and M was assessed initially by two laboratories, SACU (laboratory 1) and AMRSTI (laboratory 2) for each of the following agars: Oxoid chocolate + vitox, Oxoid GC + vitox, Becton Dickinson (BD) GC chocolate, E&O GC non-selective, E&O GC chocolate with 10 % defibrinated horse blood (DHB), E&O fastidious anaerobe agar with 5 % defibrinated horse blood and Liofilchem fastidious anaerobe agar. This aimed to investigate whether they support the growth of *N. gonorrhoeae* sufficiently enough to perform susceptibility tests. In each laboratory, inocula were prepared of four internationally accepted reference WHO strains to 0.5 McFarland in either Muller Hinton Broth (MHB) in laboratory 1 or 0.85 % sterile saline in laboratory 2. The inocula were spread with a sterile swab onto all agar plates as if performing a gradient strip method (i.e., to give confluent growth) and incubated as described in Materials and Methods. It should be noted that when tests were performed in duplicate in SACU the inocula were performed as if done in a separate laboratory, that is, one repeat was processed with MHB and labelled laboratory 1, and one with MHB labelled laboratory 2. On all agar plates, confluent growth of *N. gonorrhoeae* was observed, see table 11. Confluent growth signifies that individual colonies of the bacteria cannot be identified, there is a complete lawn of growth over the plate. Examples of confluent growth can be seen in figure 3, with the addition of gradient strips.

Table 9: Results of culture viability test on all seven agar plates for stage 1

Agar plates	Laboratory 1 (using MHB for inoculum)	Laboratory 2 (using saline for inoculum)
	Confluent Growth / No Growth	
Oxoid Chocolate + vitox	Confluent Growth	Confluent Growth
Oxoid GC + vitox	Confluent Growth	Confluent Growth
BD GC Chocolate agar	Confluent Growth	Confluent Growth
E&O GC Non-Selective	Confluent Growth	Confluent Growth
E&O GC Chocolate with 10 % DHB	Confluent Growth	Confluent Growth
E&O FAA 5 % DHB	Confluent Growth	Confluent Growth
Liofilchem FAA	Confluent Growth	Confluent Growth

Next, quality control strains WHO G and WHO K were used to determine if there was any difference in the support of growth between the different agar plates when the amount of inoculum was reduced. A typical way of showing this is to spread for single colonies, a practice which is common in primary culture of pathogens in diagnostic laboratories.

As previously stated in the Materials and Methods, a 0.5 McFarland inocula was prepared for each WHO strain in MHB in laboratory 1 and saline in laboratory 2, then using a 10 µL loop each of the agar plates was spread for single colonies (Figure 2). After incubation, the bacterial growth of each *N. gonorrhoeae* strain on each agar plate was used to calculate the Weight of Growth (WoG) of the organism. The weight of growth scoring was calculated as follows; WoG = 0.5 - growth in inoculum/first spread only, 1 - growth up to second spread, 2 - growth up to third spread, 3 growth up to fourth spread. This is standard procedure for calculating weight of growth in microbiology laboratories.

The same inoculum was then used to inoculate each of the agar plates as if for gradient strip testing, and azithromycin, ceftriaxone and cefixime gradient strips from bioMérieux were applied. Following incubation, as stated in Materials and Methods, the plates were assessed for Ease of Reading (EoR). This allows the visibility of the growth and the contrast to the zone of inhibition to be calculated, especially where the ellipse intersects the strip (i.e., the MIC). Ease of reading scoring was calculated as follows: 1 - Very fuzzy zone of inhibition edge, difficult to read, 2 – moderate growth with slightly fuzzy zone of inhibition edge, 3 - easy to read, good contrast between growth and zone of inhibition.

Both laboratories used a different scale for WoG and EoR, but the individual scores correlated for each agar plate. As the same consensus was achieved from both laboratories as to which agar plates performed the best, and the judgement that all seven agar plates also be used in stage 2 testing, it was decided upon for this section only, that is ease of reading and weight of growth would be scored by SACU only using MHB.

One replicate each of WHO G and K was tested, and as both WHO G and K behaved similarly the results of weight of growth and ease of reading were combined, seen in table 10.

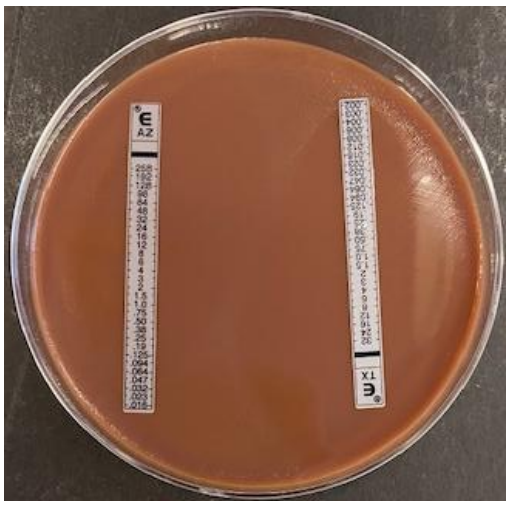
Table 10: Combined (WHO G and K) Weight of growth and Ease of Reading MIC scores in stage 1

Agar plates	WoG score	EoR score
Oxoid GC + vitox	18	15
E&O FAA with 5 % DHB	12	15
BD GC chocolate	12	14
Oxoid chocolate + vitox	12	11
Liofilchem FAA	10	12
E&O GC non-selective	6	8
E&O GC choc DHB	6	7

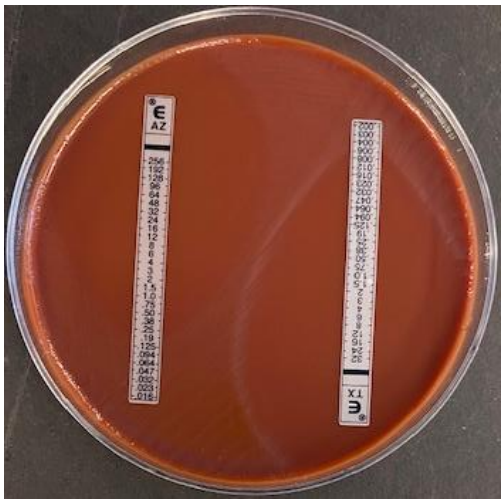
The maximum score achievable for each agar plate was 18 for weight of growth, and 18 for ease of reading. With only one agar plate, Oxoid GC + vitox, achieving the maximum score for weight of growth.

Seen in table 10 the three agar plates achieving the highest scores were Oxoid GC + vitox (WoG 18, EoR 15), E&O FAA with 5 % defibrinated horse blood (WoG 12, EoR 15) and BD GC chocolate (WoG 12, EoR 14). WoG and EoR scores were 12/11 for Oxoid chocolate + vitox, 10/12 for Liofilchem FAA, 6/8 for E&O GC Non-selective and 6/7 for E&O GC chocolate with 10 % defibrinated horse blood.

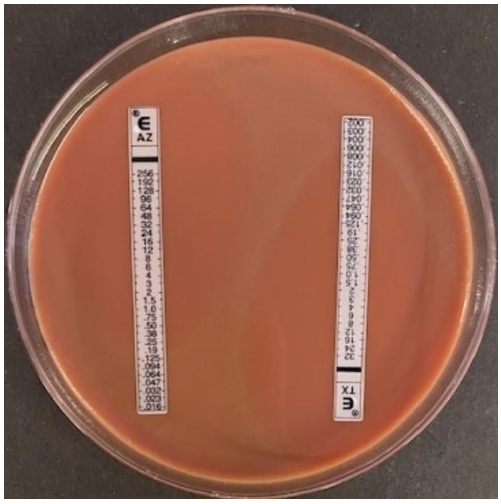
Pictures of each of the seven agar plates inoculated with WHO G, with azithromycin and ceftriaxone gradient strips were taken as a visual representation of the WHO strains tested, this is to emphasise the quality of growth, along with azithromycin and ceftriaxone gradient strips illustrated in figure 3. To limit the variability the same 0.5 McFarland inocula made in Muller Hinton Broth of WHO G was used on all seven agar plates.



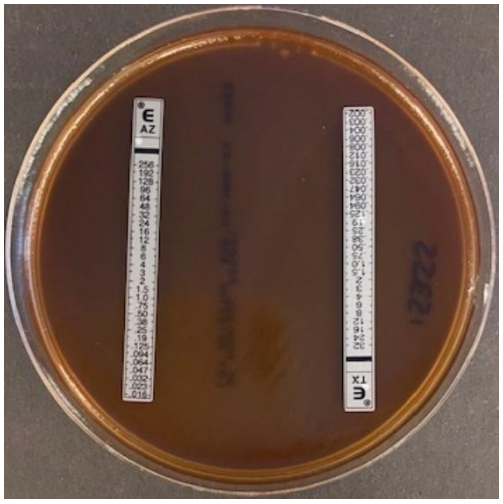
a. Oxoid chocolate + vitox (WoG 12/EoR 11)



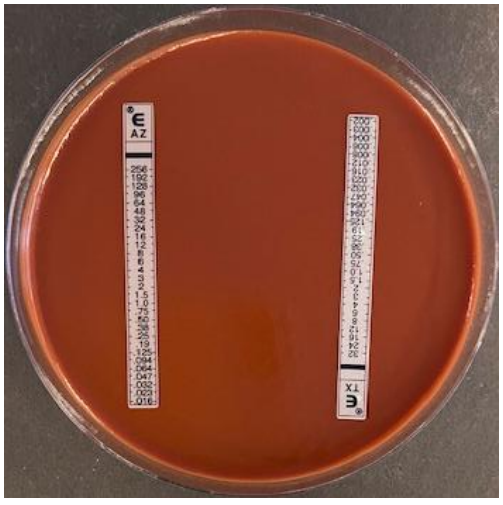
b. Oxoid GC + vitox (WoG 18/EoR 15)



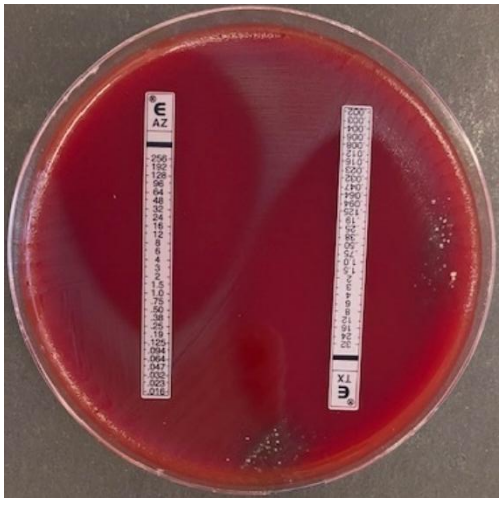
c. BD GC chocolate (WoG 12/EoR 14)



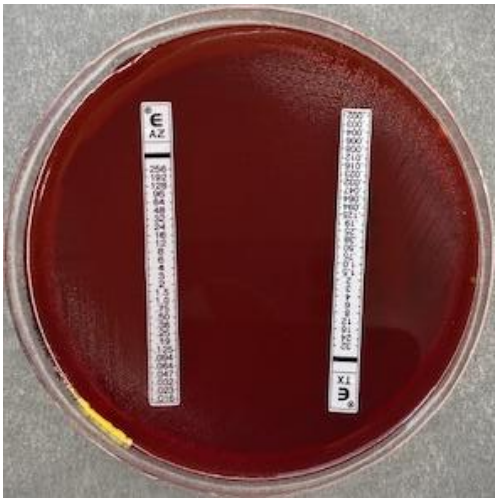
d. E&O GC Non-selective (WoG 6/EoR 8)



e. E&O GC choc w/ 10 % DHB (WoG 6/EoR 7)



f. E&O FAA w/ 5 % DHB (WoG 12/EoR 15)



g. Liofilchem FAA (WoG 10/EoR 12)

Figure 3: Agar plate images illustrating EoRand WoG of WHO G quality control strain with azithromycin (AZ) and ceftriaxone (TX) bioMérieux gradient strips on the seven agar plates, inoculated using Mueller Hinton Broth. WoG and EoR scores are for combined WHO G and K.

The pictures in figure 3 illustrate the poor growth on E&O GC Non-selective (d) and E&O GC chocolate with 10 % DHB (e), there is growth on these plates but as it is so faint it does not photograph well. The contrast between growth and the zone of inhibition was very poor, making reading the MIC interpretation difficult and more prone to errors. The Oxoid GC + vitox agar showed the best growth of *N. gonorrhoeae* and had good confluent growth required for gradient strip testing when using a 0.5 McFarland inoculum, making the reading of the gradient strips easier, and so less likely to be read incorrectly.

Next, minimum inhibitory concentrations (MICs) of azithromycin, ceftriaxone, and cefixime for WHO strains F, G, K and M were compared to the reference MICs published by Unemo.(99) These target MICs were determined by Unemo using bioMérieux Etest gradient strips using agar plates containing 3.6 % Difco GC Medium base from Becton Dickinson supplemented with 1 % haemoglobin from Becton Dickinson and 1 % IsoVitalex from Becton Dickinson in the European reference lab for GC. Each strain was tested in triplicate on different batches of agar so that a consensus MIC could be determined. This consensus MIC was the target MIC used for any subsequent testing.(99) As previously stated, reference

results should be tested with the appropriate reference method, however in this case the MICs were determined by gradient strip.

For susceptibility testing with an MIC method there is an internal error of ± 1 doubling dilution, which means, for example, if the MIC of an isolate is 1 mg/L, when that isolate is repeatedly tested in the laboratory with either gradient strips or agar dilution then the MICs expected could be 0.5 mg/L, 1 mg/L or 2 mg/L. The results 0.5 mg/L or 2 mg/L are not incorrect, rather they are due to the natural variance in the agar plates or the gradient strips etc. Therefore, the target MICs described by Unemo,(99) can be extrapolated to a target range, which is plus or minus 1 x doubling dilution, referred to as reference result QC ranges in tables 11 and 12. When these strains are used for quality control purposes these ranges help to determine whether the tests that are performed are accurate.

Table 11: MIC results of WHO F, G, K and M with azithromycin (azith), ceftriaxone (CRO), and cefixime (FIX) gradient strips in Laboratory 1, on seven agar plates for stage 1

	Laboratory 1 (mg/L)											
	WHO F			WHO G			WHO K			WHO M		
	Azith MIC	CRO MIC	FIX MIC	Azith MIC	CRO MIC	FIX MIC	Azith MIC	CRO MIC	FIX MIC	Azith MIC	CRO MIC	FIX MIC
Oxoid chocolate + vitox	0.125	< 0.002	< 0.016	0.25	0.002	< 0.016	0.25	0.032	0.125	0.25	0.008	< 0.016
Oxoid GC + vitox	0.125	< 0.002	< 0.016	0.25	0.004	< 0.016	0.25	0.064	0.25	0.5	0.008	< 0.016
BD GC chocolate agar	0.125	< 0.002	< 0.016	0.25	0.004	< 0.016	0.25	0.064	0.25	0.5	0.008	< 0.016
E&O GC Non-Selective	0.125	< 0.002	< 0.016	0.125	0.008	< 0.016	0.125	0.064	0.25	0.25	0.004	< 0.016
E&O GC chocolate with 10 % DHB	0.064	< 0.002	< 0.016	0.25	0.004	< 0.016	0.25	0.032	0.125	0.25	0.008	< 0.016
E&O FAA with 5 % DHB	0.25	< 0.002	< 0.016	0.25	0.008	< 0.016	0.5	0.064	0.25	0.5	0.008	< 0.016
Liofilchem FAA	0.25	< 0.002	< 0.016	0.125	0.004	< 0.016	0.5	0.064	0.25	0.25	0.004	< 0.016
Reference result QC ranges	0.064 – 0.25	< 0.002	< 0.016	0.125 – 0.5	0.004 – 0.016	< 0.016	0.125 – 0.5	0.032 – 0.125	0.125 – 0.5	0.125 – 0.5	0.008 – 0.032	< 0.016

All agar plates tested in SACU (laboratory 1).

Cells coloured in red identify MICs outside of the quality control range.

Table 12: MIC results of WHO F, G, K and M with azithromycin (Azith), ceftriaxone (CRO), and cefixime (FIX) gradient strips in Laboratory 2, on seven agar plates for stage 1

	Laboratory 2 (mg/L)											
	WHO F			WHO G			WHO K			WHO M		
	Azith MIC	CRO MIC	FIX MIC	Azith MIC	CRO MIC	FIX MIC	Azith MIC	CRO MIC	FIX MIC	Azith MIC	CRO MIC	FIX MIC
Oxoid chocolate + vitox*	0.125	< 0.002	< 0.016	0.25	0.004	< 0.016	0.25	0.032	0.125	0.25	0.008	< 0.016
Oxoid GC + vitox*	0.125	< 0.002	< 0.016	0.25	0.008	< 0.016	0.25	0.125	0.25	0.5	0.008	< 0.016
BD GC chocolate agar*	0.25	< 0.002	< 0.016	0.25	0.008	< 0.016	0.125	0.125	0.25	0.5	0.008	< 0.016
E&O GC Non-Selective▫	0.125	< 0.002	< 0.016	0.125	0.004	< 0.016	0.125	0.064	0.25	0.25	0.004	< 0.016
E&O GC chocolate with 10 % DHB▫	0.125	< 0.002	< 0.016	0.25	0.004	< 0.016	0.125	0.032	0.125	0.25	0.008	< 0.016
E&O FAA with 5% DHB▫	0.25	< 0.002	< 0.016	0.25	0.008	< 0.016	0.5	0.032	0.25	0.5	0.008	< 0.016
Liofilchem FAA▫	0.25	< 0.002	< 0.016	0.125	0.004	< 0.016	0.5	0.032	0.25	0.25	0.004	< 0.016
Reference result QC ranges	0.064 - 0.25	< 0.002	< 0.016	0.125 - 0.5	0.004 - 0.016	< 0.016	0.125 - 0.5	0.032 - 0.125	0.125 - 0.5	0.125 - 0.5	0.008 - 0.032	< 0.016

* Tested in AMRSTI
▫ Tested in SACU
Cells coloured in red identify MICs outside of the quality control range.

As seen in tables 11 and 12 most MICs for the antimicrobials tested were within the accepted QC ranges, with 64.9 % of all tests in both laboratories having the same MIC as the target. 10.1 % of MICs were 1 x doubling dilution higher than the target MIC, and 22 % were 1 x doubling dilution lower than the target MIC, but still within the quality control range. Only 3 % of the MICs were out of the quality control range (highlighted in red in tables 11 and 12), all occurring when testing with ceftriaxone with WHO G and WHO M.

3.1.1 INTRA- AND INTER-LABORATORY VARIATION

As stated in the Materials and Methods due to time constraints and low staffing levels, AMRSTI only provided MIC results for three of the agars tested: Oxoid GC + vitox, Oxoid chocolate + vitox and BD GC Chocolate, the other four agars were tested twice on separate occasions in SACU using MHB (laboratory 1) and sterile saline (laboratory 2), weeks apart to mimic a second laboratory test. One technician in each laboratory, SACU and AMRSTI performed the testing.

Oxoid chocolate + vitox, Oxoid GC + vitox and Becton Dickinson Chocolate agar, were tested in SACU and AMRSTI, and so inter-laboratory variation can be assessed. MICs in tables 11 and 12 show MICs for azithromycin, ceftriaxone and cefixime were all within range except for one ceftriaxone MIC for WHO G in laboratory 1. The inter-laboratory differences were within 1 x doubling dilution, the natural error rate of the method, with these agars except for the out-of-range ceftriaxone MIC.

Agar plates E&O GC Non-selective, E&O GC chocolate with 10 % DHB, E&O FAA with 5 % DHB and Liofilchem FAA were tested in SACU only but tested on two separate occasions. The intra-laboratory variation can be seen in tables 11 and 12. The MICs for all agents and strains on each occasion tested were all within 1 x doubling dilutions, the natural error rate of the methodology. Both manufacturers of FAA agar plates, E&O and Liofilchem produced MICs on the upper limit of the reference range for azithromycin in three out of the four WHO strains tested. MICs to azithromycin on E&O GC chocolate with 10 % DHB plate for each of the four

WHO strains were on the lower limit of the quality control range for azithromycin, ceftriaxone and cefixime.

Ideally all agars would be tested in separate laboratories. However, the intra- and inter-laboratory variation results show that of the three agar plates tested in each laboratory the differences in MIC results between these laboratories were the same as the natural error rate of the methodology and therefore any variation would be attributable to the agar plates used. These 2 analyses will show that any differences in MICs will be attributed to the agar plates and strips and not the methodology and technical differences.

3.1.2 EFFECT OF PRIMARY AGAR PLATES

Initially, retrieval of *N. gonorrhoeae* isolates from the freezer were plated on Oxoid GC + vitox in AMRSTI and Oxoid chocolate agar in SACU, as per local protocols. Discussion between laboratories on completion of stage 1 suggested that the growth of *N. gonorrhoeae* after 24 hours on Oxoid chocolate plates were insufficient to perform a 0.5 McFarland inoculum on each of the four WHO control strains tested. An additional 24 hours of incubation was required for sufficient growth of *N. gonorrhoeae* isolates to enable testing. It was therefore decided for subsequent stages 2 and 3, that SACU and AMRSTI would both use Oxoid GC vitox for the primary subculture from the -80 °C freezer.

3.1.3 EFFECT OF INOCULA PREPARATION MEDIUM

Inocula were prepared as per gradient strip recommendations, with MHB in SACU and using saline in AMRSTI. The MICs for all WHO strains performed in both laboratories seen in tables 13 and 14 were within the natural error rate of the method (± 1 x doubling dilution). However, as so few strains were used in stage 1, and the absence of any clinical isolates tested, there was no definitive answer as to which preparation of inocula, if any, was preferred. It was decided that SACU would continue to use Mueller Hinton Broth for 0.5 McFarland inocula preparation and AMRSTI would continue to use 0.85 % sterile saline in

stages 2 and 3. Allowing to investigate if there was any difference in MIC between methods when testing clinical *N. gonorrhoeae* isolates.

3.1.4 STAGE 1 SUMMARY

Stage 1 results illustrate that the seven agar plates perform well enough to support the confluent growth of *N. gonorrhoeae* and generally provide MICs within the acceptable quality control ranges but more importantly, within the natural error rate of the methodology. However, three plates gave out of range MICs for the quality control isolate, Oxoid chocolate + vitox, E&O GC non-selective and Liofilchem FAA. The three exceptions, where quality control MICs were outside of the stated range were for ceftriaxone only, one for WHO G and two for WHO M. These out-of-range MICs were within 1 doubling dilution of the lower end of the range which would indicate that the growth of the bacteria was insufficient to accurately perform the MIC.

It was intended that the results of stage 1 would inform which of the best performing agar plates should be continued through to stage 2. However, as results were comparable for all seven agar plates in both laboratories, results mostly within the accepted quality control ranges and within the natural error rate of the methodology, it was decided that they would all be tested in stage 2. The three exceptions, where MICs were out of the quality control range were for ceftriaxone only.

Discussions between SACU and AMRSTI after completion of stage 1 suggested that there was better growth of the primary culture using GC + vitox at AMRSTI than on chocolate agar at SACU, leading to easier inoculation preparation of the 0.5 McFarland. Therefore, both laboratories for the rest of the study used Oxoid GC + vitox for the primary culture of all *N. gonorrhoeae* strains.

Stage 2 comprises of a set of clinical strains, therefore giving a better understanding of how the agar plates would perform in a real world setting of the clinical laboratory with *N. gonorrhoeae* isolated from patients, rather than well categorised control strains that are known to perform consistently.

3.2 STAGE 2 – MEDIA ASSESSMENT OF CLINICAL ISOLATES OF *N. GONORRHOEAE*

Forty-six clinical isolates previously submitted to the Gonococcal Resistance in Antimicrobial Susceptibility Programme (GRASP) alongside two control strains, WHO G and WHO K were tested. This group of isolates was tested in both SACU and AMRSTI, however due to staffing levels in AMRSTI some agars were tested twice, on separate occasions in SACU. As described previously three of the agar plates were tested in both laboratories and the four remaining agar plates were tested in SACU on two separate occasions mimicking a second laboratory test. Preparation of the plates and incubation conditions remains the same as before, with all strains tested with ceftriaxone and azithromycin gradient strips from bioMérieux. Ceftriaxone and azithromycin MICs for the clinical strains were compared to the MIC performed using the gold standard method of agar dilution. These agar dilution reference MICs were available due to the isolates having been previously submitted to GRASP, and the results kindly shared by AMRSTI, seen in tables 13 - 16.

Azithromycin and ceftriaxone MICs performed by gradient strip on quality control strains WHO G and K were compared to target reference MICs determined by Unemo as follows; WHO G: azithromycin target 0.25 mg/L, ceftriaxone target 0.008 mg/L, and WHO K: azithromycin target 0.25 mg/L, ceftriaxone target, 0.064 mg/L. As for the previous stage the target MICs reported by Unemo were extrapolated to a target range for each antimicrobial seen in tables 13 - 16.(99)

Table 13: MIC results of WHO G and K and forty-six clinical isolates with azithromycin gradient strips in laboratory 1 on seven agar plates for stage 2

		Laboratory 1 azithromycin MIC (mg/L)						
Reference number	**Reference result**	**Oxoid chocolate + vitox**	**Oxoid GC + vitox**	**BD GC chocolate agar**	**E&O GC Non-selective**	**E&O GC chocolate w 10 % DHB**	**E&O FAA 5 % DHB**	**Liofilchem FAA**
WHO G	0.125 - 0.5	0.25	0.25	0.125	0.125	0.064	0.125	0.125
WHO K	0.125 - 0.5	0.25	0.25	0.25	0.125	0.25	0.25	0.25
26333	≤ 0.064	0.125	0.125	0.125	0.064	0.125	0.125	0.125
26334	0.125	0.25	0.25	0.125	0.064	0.125	0.25	0.064
26335	0.5	0.25	0.5	0.5	0.125	0.5	0.5	0.5
26336	0.25	0.5	0.5	0.5	0.125	0.25	0.5	0.25
26337	≤ 0.064	0.125	0.25	0.25	0.25	0.125	0.25	0.25
26340	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.5
26342	0.5	1	1	0.5	0.25	0.25	0.25	0.5
26343	0.25	0.5	0.5	0.25	0.125	0.25	0.5	0.5
26344	0.25	0.5	0.5	0.5	0.25	0.5	0.25	0.5
26345	0.5	0.5	1	1	0.25	0.5	0.5	0.5
26346	0.125	0.25	0.125	0.25	0.125	0.125	0.25	0.25
26347	0.5	0.25	0.25	0.25	0.125	0.25	0.5	0.25
26348	0.5	0.25	0.25	0.25	0.125	0.25	0.25	0.5
26350	0.25	0.25	0.5	0.25	0.25	0.5	0.5	0.5
26351	≤ 0.064	0.064	0.064	0.064	0.032	0.25	0.064	0.125
26352	0.125	0.125	0.064	0.064	0.064	0.064	0.125	0.125
26353	0.5	0.25	0.25	0.25	0.125	0.25	0.5	0.25
26354	1	0.5	0.5	0.25	0.25	0.25	0.25	0.25
28581	≤ 0.064	0.125	0.5	0.25	0.125	0.25	0.25	0.25
28582	0.5	0.5	0.5	0.5	0.125	0.5	0.5	0.5
28583	0.25	0.5	0.25	0.5	0.125	0.25	0.5	1
28584	0.125	0.25	0.25	0.125	0.125	0.125	0.25	0.25
28585	0.5	0.5	0.5	0.5	0.125	0.25	1	1

Table 13: Continued

		Laboratory 1 azithromycin MIC (mg/L)						
Reference number	**Reference result**	**Oxoid chocolate + vitox**	**Oxoid GC + vitox**	**BD GC chocolate agar**	**E&O GC Non-selective**	**E&O GC chocolate w 10 % DHB**	**E&O FAA 5 % DHB**	**Liofilchem FAA**
28586	0.5	0.25	0.25	0.25	0.125	0.25	0.5	0.25
28587	0.5	0.5	0.25	0.25	0.125	0.25	0.5	0.5
28588	0.125	0.25	0.125	0.125	0.125	0.125	0.25	0.5
28589	4	2	2	2	0.5	1	2	2
28590	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.25
28591	0.125	0.125	0.125	0.125	0.064	0.125	0.125	0.125
28592	0.125	0.125	0.064	0.125	0.032	0.064	0.125	0.064
28593	0.5	0.5	0.25	0.5	0.125	0.25	0.5	0.5
28594	0.125	0.25	0.125	0.25	0.125	0.064	0.25	0.25
28595	≤ 0.064	0.125	0.125	0.125	0.064	0.125	0.25	0.125
28596	0.25	0.25	0.125	0.25	0.125	0.125	0.25	0.125
28597	0.5	0.5	0.5	0.5	0.25	0.25	1	1
28598	0.125	0.25	0.25	0.25	0.125	0.125	0.25	0.25
28599	0.5	0.5	0.25	0.25	0.25	0.25	0.5	0.125
28600	0.25	0.5	0.5	0.5	0.25	0.5	0.5	0.25
28601	0.25	0.25	0.25	0.125	0.064	0.125	0.25	0.25
28605	0.25	0.25	0.25	0.25	0.125	0.125	0.5	0.5
28607	≤ 0.064	0.016	0.032	0.032	0.016	0.032	0.032	0.064
28608	≤ 0.064	0.064	0.064	0.064	0.064	0.064	0.125	0.125
28609	0.125	0.125	0.125	0.125	0.064	0.125	0.25	0.25
28611	0.125	0.125	0.25	0.25	0.25	0.25	0.5	0.5
28612	≤ 0.064	0.064	0.032	0.032	0.032	0.032	0.064	0.064
28613	0.125	0.125	0.25	0.25	0.25	0.25	0.25	0.5

All agar plates tested in SACU.

Cells highlighted in red are outside of the quality control range, cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 14: MIC results of WHO G and K and forty-six clinical isolates with azithromycin gradient strips in laboratory 2 on seven agar plates for stage 2

		Laboratory 2 azithromcyin MIC (mg/L)						
Reference number	**Reference result**	**Oxoid chocolate + vitox***	**Oxoid GC + vitox***	**BD GC chocolate agar***	**E&O GC Non-selective▫**	**E&O GC chocolate w 10 % DHB▫**	**E&O FAA 5 % DHB▫**	**Liofilchem FAA▫**
WHO G	0.125 - 0.5	0.25	0.25	0.125	0.125	0.125	0.25	0.125
WHO K	0.125 - 0.5	0.25	0.5	0.5	0.125	0.125	0.25	0.25
26333	≤ 0.064	0.125	0.5	0.25	0.064	0.125	0.125	0.125
26334	0.125	0.25	0.25	0.125	0.064	0.125	0.25	0.064
26335	0.5	0.25	0.5	0.5	0.25	0.5	0.5	0.5
26336	0.25	0.5	0.5	0.5	0.125	0.25	0.5	0.125
26337	≤ 0.064	0.064	0.5	0.5	0.125	0.25	0.25	0.25
26340	0.25	0.25	0.5	0.5	0.125	0.25	0.5	0.5
26342	0.5	1	0.5	1	0.25	0.25	0.25	0.5
26343	0.25	0.25	0.25	0.25	0.125	0.25	0.5	0.5
26344	0.25	0.5	0.5	0.25	0.25	0.5	0.25	0.5
26345	0.5	1	0.5	0.5	0.25	0.5	0.25	0.5
26346	0.125	0.25	0.25	0.25	0.125	0.125	0.25	0.25
26347	0.5	0.25	0.5	0.5	0.125	0.25	0.5	0.5
26348	0.5	0.25	0.5	0.5	0.25	0.25	0.125	0.5
26350	0.25	0.25	0.5	0.5	0.25	0.5	0.25	0.5
26351	≤ 0.064	0.064	0.064	0.064	0.032	0.25	0.064	0.125
26352	0.125	0.064	0.125	0.125	0.064	0.064	0.125	0.125
26353	0.5	0.5	0.25	0.5	0.125	0.25	0.5	0.25
26354	1	0.5	0.5	1	0.125	0.25	0.5	0.25
28581	≤ 0.064	0.125	0.25	0.25	0.125	0.25	0.25	0.125
28582	0.5	0.5	0.5	0.5	0.125	0.25	0.25	0.25
28583	0.25	0.25	0.25	0.25	0.125	0.125	0.5	0.5
28584	0.125	0.25	0.25	0.25	0.125	0.125	0.5	0.25
28585	0.5	0.25	0.5	0.5	0.25	0.25	0.5	0.5
28586	0.5	0.25	0.5	0.25	0.125	0.25	0.5	0.25

Table 14: Continued

		Laboratory 2 azithromcyin MIC (mg/L)						
Reference number	**Reference result**	**Oxoid chocolate + vitox***	**Oxoid GC + vitox***	**BD GC chocolate agar***	**E&O GC Non-selective▫**	**E&O GC chocolate w 10 % DHB▫**	**E&O FAA 5 % DHB▫**	**Liofilchem FAA▫**
28587	0.5	0.25	0.5	0.5	0.125	0.25	0.5	0.25
28588	0.125	0.125	0.25	0.25	0.125	0.125	0.25	0.5
28589	4	1	2	2	0.5	1	2	2
28590	0.5	0.5	0.5	1	0.25	0.25	0.25	0.25
28591	0.125	0.125	0.25	0.5	0.064	0.064	0.125	0.125
28592	0.125	0.064	0.125	0.125	0.032	0.064	0.125	0.064
28593	0.5	0.25	0.5	0.5	0.125	0.25	0.5	0.5
28594	0.125	0.25	0.125	0.125	0.125	0.064	0.25	0.125
28595	≤ 0.064	0.064	0.125	0.064	0.064	0.125	0.125	0.064
28596	0.25	0.125	0.25	0.25	0.064	0.125	0.25	0.25
28597	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5
28598	0.125	0.25	0.25	0.25	0.125	0.125	0.25	0.25
28599	0.5	0.5	0.25	0.25	0.25	0.25	0.5	0.25
28600	0.25	0.5	0.5	0.25	0.25	0.25	0.5	0.25
28601	0.25	0.25	0.25	0.125	0.125	0.125	0.25	0.25
28605	0.25	0.25	0.5	0.5	0.125	0.125	0.5	0.5
28607	≤ 0.064	0.016	0.032	0.032	0.016	0.032	0.032	0.064
28608	≤ 0.064	0.064	0.064	0.064	0.064	0.064	0.125	0.125
28609	0.125	0.125	0.125	0.125	0.064	0.125	0.25	0.25
28611	0.125	0.125	0.25	0.5	0.25	0.25	0.5	0.5
28612	≤ 0.064	0.064	0.032	0.125	0.032	0.032	0.064	0.064
28613	0.125	0.125	0.5	0.5	0.25	0.25	0.5	0.5

* Tested in ARMSTI.

▫ Tested in SACU.

Cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 15: MIC results of WHO G and K and forty-six clinical isolates with ceftriaxone gradient strips in laboratory 1 on seven agar plates for stage 2

		Laboratory 1 ceftriaxone MIC (mg/L)						
Reference number	**Reference result**	**Oxoid chocolate + vitox**	**Oxoid GC + vitox**	**BD GC chocolate agar**	**E&O GC Non-selective**	**E&O GC chocolate w 10 % DHB**	**E&O FAA 5 % DHB**	**Liofilchem FAA**
WHO G	0.004 - 0.016	0.004	0.008	0.008	0.008	< 0.002	0.008	0.004
WHO K	0.032 - 0.125	0.032	0.064	0.125	0.064	0.032	0.032	0.032
26333	0.008	0.002	0.004	0.008	0.004	0.004	0.004	0.002
26334	0.008	0.004	0.032	0.064	0.004	0.032	0.064	0.004
26335	0.016	0.008	0.016	0.016	0.016	0.008	0.016	0.008
26336	0.032	0.064	0.064	0.25	0.125	0.032	0.032	0.004
26337	≤ 0.004	< 0.002	0.004	0.004	< 0.002	0.004	0.004	0.004
26340	≤ 0.004	0.002	0.004	0.008	0.002	0.004	0.004	0.008
26342	0.064	0.064	0.125	0.25	0.125	0.064	0.125	0.032
26343	0.008	0.008	0.004	0.008	0.004	0.004	0.008	0.004
26344	0.032	0.016	0.032	0.032	0.032	0.016	0.016	0.008
26345	0.016	0.008	0.032	0.032	0.008	0.008	0.016	0.008
26346	0.016	0.004	0.008	0.016	0.008	0.008	0.008	0.008
26347	0.016	0.004	0.008	0.016	0.008	0.004	0.008	0.008
26348	0.016	0.008	0.016	0.016	0.008	0.004	0.004	0.004
26350	0.016	0.008	0.016	0.032	0.008	0.008	0.016	0.016
26351	≤ 0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.002
26352	0.008	0.002	0.002	0.004	< 0.002	< 0.002	0.002	< 0.002
26353	0.008	0.002	0.004	0.004	0.002	0.002	0.004	0.004
26354	0.008	0.004	0.004	0.008	0.004	0.002	0.004	0.004
28581	0.008	0.002	0.004	0.008	0.004	0.004	0.008	0.004
28582	0.016	0.004	0.008	0.008	0.004	0.004	0.004	0.008
28583	0.032	0.004	0.016	0.032	0.008	0.008	0.008	0.016
28584	0.008	< 0.002	0.002	0.002	< 0.002	< 0.002	0.004	0.004
28585	0.008	0.004	0.004	0.008	0.004	0.004	0.004	0.008
28586	0.032	0.002	0.016	0.032	0.008	0.008	0.016	0.008

Table 15: Continued

		Laboratory 1 ceftriaxone MIC (mg/L)						
Reference number	**Reference result**	**Oxoid chocolate + vitox**	**Oxoid GC + vitox**	**BD GC chocolate agar**	**E&O GC Non-selective**	**E&O GC chocolate w 10 % DHB**	**E&O FAA 5 % DHB**	**Liofilchem FAA**
28587	0.008	0.002	0.004	0.004	0.002	< 0.002	0.008	0.004
28588	0.008	0.002	0.004	0.008	0.002	0.004	0.004	0.008
28589	0.016	0.004	0.008	0.016	0.004	0.004	0.008	0.016
28590	0.032	0.016	0.032	0.032	0.032	0.032	0.032	0.016
28591	0.008	< 0.002	0.004	0.004	0.002	< 0.002	0.004	0.004
28592	0.016	0.004	0.016	0.032	0.008	0.008	0.008	0.002
28593	0.008	< 0.002	0.004	0.004	< 0.002	< 0.002	0.004	0.004
28594	0.008	< 0.002	0.002	0.004	0.002	0.002	0.004	0.002
28595	0.016	0.002	0.008	0.008	0.002	0.008	0.004	0.002
28596	0.016	0.008	0.008	0.016	0.008	0.008	0.008	0.008
28597	0.008	< 0.002	0.004	0.004	< 0.002	< 0.002	0.002	0.008
28598	0.016	0.004	0.008	0.016	0.008	0.008	0.008	0.008
28599	0.032	0.016	0.032	0.064	0.032	0.032	0.016	0.016
28600	0.032	0.016	0.032	0.032	0.032	0.032	0.016	0.016
28601	0.016	< 0.002	0.002	0.004	< 0.002	< 0.002	0.004	0.008
28605	0.016	< 0.002	0.008	0.008	0.008	0.008	0.008	0.008
28607	0.008	< 0.002	0.002	0.002	0.002	< 0.002	0.004	0.004
28608	≤ 0.004	0.004	0.004	0.008	0.004	0.004	0.004	0.002
28609	0.008	0.002	0.002	0.004	0.002	0.002	0.004	0.004
28611	0.008	< 0.002	0.004	0.004	< 0.002	< 0.002	0.004	0.004
28612	≤ 0.004	0.016	0.004	0.004	0.004	0.004	0.002	0.004
28613	0.008	0.002	0.008	0.008	< 0.002	< 0.002	0.008	0.004

All agar plates tested in SACU.

Cells highlighted in red are outside of the quality control range, cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 16: MIC results of WHO G and K and forty-six clinical isolates with ceftriaxone gradient strips in laboratory 2 on seven agar plates for stage 2

		Laboratory 2 ceftriaxone MIC (mg/L)						
Reference number	**Reference result**	**Oxoid chocolate + vitox***	**Oxoid GC + vitox***	**BD GC chocolate agar***	**E&O GC Non-selective▫**	**E&O GC chocolate w 10 % DHB▫**	**E&O FAA 5 % DHB▫**	**Liofilchem FAA▫**
WHO G	0.004 - 0.016	0.004	0.008	0.008	0.004	< 0.002	0.004	0.004
WHO K	0.032 - 0.125	0.032	0.125	0.125	0.064	0.032	0.064	0.016
26333	0.008	0.002	0.004	0.004	0.004	0.004	0.004	0.002
26334	0.008	0.004	0.008	0.008	0.004	0.032	0.064	0.004
26335	0.016	0.008	0.008	0.016	0.016	0.008	0.016	0.008
26336	0.032	0.064	0.032	0.032	0.125	0.032	0.016	0.008
26337	≤ 0.004	< 0.002	0.004	0.004	0.002	0.004	0.004	0.004
26340	≤ 0.004	0.002	0.004	0.004	0.002	0.004	0.004	0.004
26342	0.064	0.064	0.064	0.064	0.125	0.032	0.125	0.064
26343	0.008	0.008	0.008	0.004	0.002	0.004	0.004	0.004
26344	0.032	0.016	0.032	0.032	0.016	0.016	0.016	0.016
26345	0.016	0.008	0.016	0.016	0.008	0.008	0.016	0.008
26346	0.016	0.004	0.008	0.016	0.008	0.004	0.008	0.008
26347	0.016	0.004	0.008	0.016	0.008	0.004	0.008	0.004
26348	0.016	0.008	0.016	0.032	0.004	0.008	0.004	0.004
26350	0.016	0.008	0.032	0.064	0.008	0.008	0.016	0.016
26351	≤ 0.004	0.004	0.004	0.004	0.002	0.004	0.004	0.002
26352	0.008	0.002	0.004	0.008	<0.002	< 0.002	0.002	< 0.002
26353	0.008	0.004	< 0.002	0.004	0.002	0.002	0.004	0.004
26354	0.008	0.004	0.002	0.004	0.002	0.002	0.004	0.002
28581	0.008	< 0.002	0.004	0.004	0.002	0.004	0.004	0.002
28582	0.016	0.002	0.004	0.004	0.004	0.004	0.004	0.004
28583	0.032	0.008	0.016	0.032	0.008	0.008	0.008	0.008
28584	0.008	< 0.002	0.004	0.004	< 0.002	< 0.002	0.002	0.002
28585	0.008	0.002	0.004	0.016	0.004	0.004	0.004	0.008
28586	0.032	0.002	0.016	0.032	0.008	0.008	0.016	0.008

Table 16: Continued

		Laboratory 2 ceftriaxone MIC (mg/L)						
Reference number	**Reference result**	**Oxoid chocolate + vitox***	**Oxoid GC + vitox***	**BD GC chocolate agar***	**E&O GC Non-selective▫**	**E&O GC chocolate w 10 % DHB▫**	**E&O FAA 5 % DHB▫**	**Liofilchem FAA▫**
28587	0.008	< 0.002	0.004	0.004	< 0.002	< 0.002	0.004	0.008
28588	0.008	0.002	0.004	0.008	0.002	0.002	0.004	0.004
28589	0.016	0.004	0.016	0.016	0.004	0.004	0.008	0.016
28590	0.032	0.016	0.064	0.064	0.032	0.032	0.016	0.016
28591	0.008	< 0.002	0.004	0.004	< 0.002	< 0.002	0.004	0.008
28592	0.016	0.004	0.016	0.016	0.008	0.004	0.008	0.002
28593	0.008	< 0.002	0.004	0.004	< 0.002	< 0.002	0.004	0.004
28594	0.008	< 0.002	0.002	0.002	0.002	0.002	0.004	0.002
28595	0.016	0.002	0.004	0.008	0.004	0.008	0.004	0.002
28596	0.016	0.002	0.008	0.016	0.008	0.004	0.016	0.008
28597	0.008	< 0.002	0.004	0.004	< 0.002	< 0.002	0.004	0.004
28598	0.016	0.004	0.016	0.016	0.008	0.008	0.008	0.008
28599	0.032	0.016	0.032	0.032	0.032	0.032	0.016	0.016
28600	0.032	0.016	0.032	0.064	0.032	0.032	0.016	0.016
28601	0.016	< 0.002	0.002	0.004	< 0.002	< 0.002	0.004	0.008
28605	0.016	0.004	0.008	0.016	0.008	0.008	0.008	0.016
28607	0.008	< 0.002	0.004	0.004	0.002	< 0.002	0.004	0.004
28608	≤ 0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
28609	0.008	< 0.002	0.004	0.004	0.002	0.002	0.002	0.004
28611	0.008	< 0.002	0.004	0.008	< 0.002	< 0.002	0.004	0.008
28612	≤ 0.004	0.008	0.004	0.002	0.004	0.004	0.002	0.004
28613	0.008	0.002	0.004	0.004	< 0.002	< 0.002	0.008	0.004

* Tested in AMRSTI.

▫ Tested in SACU.

Cells highlighted in red are outside of the quality control range, cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

It should be noted that reference MICs denoted as ≤ 0.064 mg/L for azithromycin and ≤ 0.004 mg/L for ceftriaxone mean that the actual MIC could be 0.064 mg/L or lower for azithromycin and 0.004 mg/L or lower for ceftriaxone, this is the lowest dilution performed for the agar dilution for these antibiotics. Ceftriaxone gradient strips read as < 0.002 mg/L denotes strains where the bacterial growth forms an ellipse that is beneath the gradient strip. An example of bacterial growth forming an ellipse below bottom of the gradient strip can be seen in figure 4.

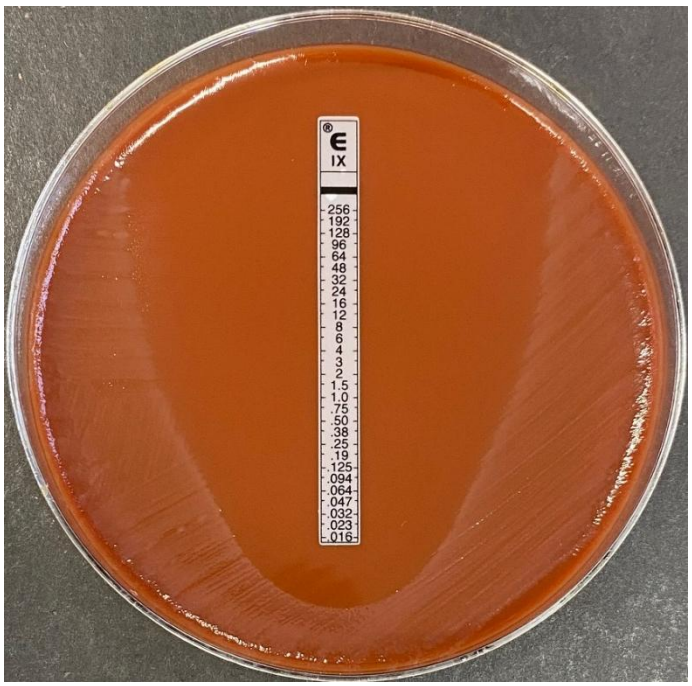


Figure 4: Example of ellipse of bacterial growth beneath bioMérieux gradient strip.

3.2.1 OUT OF RANGE RESULTS FOR QUALITY CONTROL STRAINS WHO G AND WHO K

Seen in tables 13 – 16 E&O GC chocolate with 10 % DHB, when tested with azithromycin in laboratory 1 was out of range with WHO G, 0.064 mg/L, which is 1 x doubling dilution below the quality control range. WHO G was also out of range for ceftriaxone in both laboratories with E&O GC chocolate with 10 % DHB, < 0.002 mg/L, but his time 2 x doubling dilutions

below the acceptable quality control range. The remaining six agars produced azithromycin MICs within the acceptable range for WHO G and WHO K.

Liofilchem FAA was 1 x doubling dilution below the quality control range for ceftriaxone when tested with WHO K, 0.016 mg/L. All other agar plates performed within the quality control ranges for WHO G and K.

3.2.2 VARIANCE OF MICS FOR SPECIFIC ISOLATES ACROSS ALL AGAR PLATES FOR STAGE 2

In tables 13 – 16 some isolates appear to have a large variety of MICs to each antimicrobial across the agar plates, these are highlighted in orange. For example, isolate 26351 had azithromycin MICs ranging from 0.032 mg/L up to 0.25 mg/L, a 3 x doubling dilution difference in both laboratories. Other isolates with a 3 x doubling dilution difference in one laboratory for azithromycin were 26333, 26337, 26354, 28583, and 28591.

There was a greater instance of variance of MICs when looking at ceftriaxone gradient strips, with one isolate, 26336 having MICs ranging from 0.004 mg/L up to 0.25 mg/L, that is a 6 x doubling dilution difference. Isolates in one or both laboratories which have a 4 x doubling dilution range of MICs are 26334, 26342, 28586, and 28592. Isolates in one or both laboratories which have a 3 x doubling range of MICs are WHO G, WHO K, 26350, 26352, 28583, 28585, 28587, 28592, 28596, 28597, 28601, 28605, 28611, 28612, and 28613.

3.2.3 STAGE 2 QUALITY CONTROL RESULTS

In stage 2 of this project only two quality control strains were tested, a more comprehensive assessment of the quality control strains can be seen in the stage 3 results.

3.2.4 INTERPRETATION OF STAGE 2 CLINICAL RESULTS

According to ISO document 20776 – 2 (2021, second edition) there are two ways to evaluate a commercial antimicrobial susceptibility test such as a gradient strip, by essential agreement and bias. Essential agreement measures whether the gradient strip is within one doubling dilution above or below the reference result, in this case, agar dilution. Bias is used to determine whether the test results are heavily leaning in a particular direction, either higher or lower than the reference result.(104)

For this study it was also decided to include the categorical agreement assessment of the test results. Categorical agreement is described in ISO standard 20776 – 2 (2007, first edition) and is the calculation of whether an MIC result from a commercial test such as a gradient strip yields the same categorical interpretation (i.e., susceptible, susceptible increased exposure or resistant) as the reference result, in this case agar dilution. 20776 – 2 (2007) was replaced with 20776 – 2 (2021), the categorical agreement calculation was replaced with bias.(104, 105) Categorical agreement is useful in the assessment of whether the test results are giving the correct interpretation. This is important in the treatment of patients, for example if a test is reported as susceptible but when tested with the reference method the result is in fact is resistant, the patient given that antibiotic would result in treatment failure, resulting in worsening outcome.

3.2.5 ESSENTIAL AGREEMENT

From the gradient strip results recorded for the clinical strains in tables 13 - 16 above, essential agreement can be established, this calculates how well the test performs against the reference MICs performed by agar dilution. Acceptable essential agreement, according to ISO 20776 – 2 (2021), is greater than or equal to 90 %.(104) Table 17 shows the essential agreement (EA) calculation of the MICs performed by gradient strip for both antimicrobials, tested in both laboratories, and compared with the MICs performed using the reference method of agar dilution. Cells highlighted red denote essential agreement which is unacceptable.

Table 17: Essential agreement between forty-six clinical gradient strip MICs and reference result MICs against all seven agars for stage 2

Agar plate	AZITHROMYCIN		CEFTRIAXONE	
	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	EA	EA	EA	EA
Oxoid chocolate + vitox	100 %	97.8 %	41.3 %	41.3 %
Oxoid GC + vitox	95.7 %	91.3 %	84.8 %	87.0 %
BD GC chocolate agar	93.5 %	87.0 %	87.0 %	91.3 %
E&O GC Non-Selective	69.6 %	78.3 %	43.5 %	47.8 %
E&O GC chocolate with 10 % DHB	91.3 %	89.1 %	54.4 %	47.8 %
E&O FAA 5 % DHB	89.1 %	87.0 %	82.6 %	80.4 %
Liofilchem FAA	82.6 %	89.1 %	80.4 %	69.6 %

Cells coloured red denote EA which is unacceptable (< 90 %)

3.2.5.1 Azithromycin

Seen in table 17, Oxoid chocolate + vitox and Oxoid GC + vitox were the only two agars that had an acceptable level of essential agreement for both laboratories when tested against azithromycin. BD GC chocolate agar and E&O GC chocolate with 10 % DHB only had an acceptable essential agreement for one of the laboratories tested, laboratory 1, with the second laboratory just falling short of the cut off, 87.0 % and 89.1 % respectively. E&O FAA with 5 % DHB had less than 90 % essential agreement for both laboratories, 89.1 % in laboratory 1 and 87.0 % in laboratory 2, as did Liofilchem FAA, 82.6 % in laboratory 1 and 89.1 % in laboratory 2. E&O GC Non-selective performed poorly against azithromycin, with essential agreement of 69.6 % in laboratory 1 and 78.3 % in laboratory 2.

3.2.5.2 Ceftriaxone

As seen in table 17, all agars performed poorly when tested against ceftriaxone, with only one agar in one laboratory achieving greater than 90 % essential agreement. BD GC chocolate agar performed the best of all agars with ceftriaxone with essential agreement of 87.0 % in laboratory 1 and 91.3 % in laboratory 2. Oxoid GC + vitox was the next best performing for ceftriaxone, only slightly under the cut off for acceptable essential agreement, 84.8 % in laboratory 1 and 87.0 % in laboratory 2. The next four agars performed poorly against ceftriaxone, E&O FAA 82.6 % and 80.4 % in laboratory 1 and 2 respectively, Liofilchem FAA 80.4 % and 69.6 %, E&O GC Non-selective 43.5 % and 47.8 %, E&O GC chocolate with 10 % DHB 54.4 % and 47.8 %. The Oxoid chocolate + vitox agar plate, which exhibited one of the highest essential agreements with azithromycin showed the lowest essential agreement with ceftriaxone, 41.3 % in both laboratories.

3.2.6 BIAS

Bias was calculated according to the International Standard ISO 20776 – 2 (2021) and is calculated to assess the variation in MICs performed by commercial kits such as gradient strips, when compared to the reference method MIC. Positive bias denotes that the MIC performed by the commercial kit produces MICs generally higher than the reference method MIC. Whilst a negative bias signifies that the MIC produced by the commercial kit is lower than that by the reference method. According to 20776 – 2 (2021), bias should be within plus or minus 30 %, bias outside of this is unacceptable.(104)

Bias was calculated on the MICs of all forty-six clinical strains and is shown in Table 18, with unacceptable bias highlighted in red.

Table 18: Bias of forty-six clinical gradient strip MICs against reference result MICs for all seven agars for stage 2

Agar plate	AZITHROMYCIN		CEFTRIAXONE	
	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	Bias	Bias	Bias	Bias
Oxoid chocolate + vitox	+ 16.4 %	- 5.5 %	- 88.3 %	- 88.3 %
Oxoid GC + vitox	+ 3.2 %	+ 28.6 %	- 59.6 %	- 61.5 %
BD GC chocolate agar	+ 4.1 %	+ 26.4 %	- 17.0 %	- 30.6 %
E&O GC Non-Selective	- 62.4 %	- 65.0 %	- 78.6 %	- 81.0 %
E&O GC chocolate with 10 % DHB	- 28.3 %	- 38.3 %	- 83.2 %	- 85.6 %
E&O FAA 5 % DHB	+ 39.5 %	+ 25.1 %	- 68.8 %	- 78.6 %
Liofilchem FAA	+ 21.5 %	+ 12.8 %	- 85.6 %	- 80.5 %

Cells coloured red denote bias which is unacceptable (> plus or minus 30 %)

3.2.6.1 Azithromycin

As seen in table 18, Oxoid chocolate + vitox has the best overall acceptable bias for azithromycin, with laboratory 1 showing positive bias, + 16.4 % and laboratory 2 showing negative bias, - 5.5 %, meaning for azithromycin the MICs are higher or lower compared to the reference depending on the laboratory testing.

BD GC chocolate agar exhibited positive bias against azithromycin, + 4.1 % and + 26.4 % in laboratory 1 and 2 respectively, suggesting this agar gives higher results than the reference method for azithromycin.

Oxoid GC + vitox MICs are demonstrating acceptable positive bias against azithromycin in both laboratories, + 3.2 % and + 28.6 %. These results show that the test azithromycin MICs are slightly above the reference MICs this agar plate.

Liofilchem FAA providing MICs higher than the reference method for azithromycin, with positive bias of + 21.5 % in laboratory 1 and 12.8 % in laboratory 2.

E&O GC chocolate with 10 % DHB exhibits negative bias in both laboratories, - 28.3 % in laboratory 1. Unacceptable negative bias was reported in laboratory 2, - 38.3 %. Implying azithromycin MICs were lower than the reference method.

E&O FAA with 5 % DHB has acceptable positive bias, + 25.1 % for laboratory 2 with azithromycin, and laboratory 1 having an unacceptable bias level of + 39.5 %, giving MICs for both laboratories higher than the reference method.

E&O GC non-selective performed poorly against azithromycin, with high levels of unacceptable negative bias, – 62.4 % in laboratory 1 and – 65.0 % in laboratory 2, indicating MICs for azithromycin tested with E&O non-selective agar were much lower than the reference method.

3.2.6.2 Ceftriaxone

As shown in table 18, BD GC chocolate agar had negative bias when tested with ceftriaxone gradient strips, - 17.0 % acceptable bias in laboratory 1 and – 30.6 % unacceptable bias in laboratory 2. This suggests that BD GC chocolate agar gives lower MIC results than the reference method for ceftriaxone.

Oxoid GC + vitox MICs to ceftriaxone are above the acceptable threshold of negative bias, - 59.6 % in laboratory 1 and – 61.5 % in laboratory 2. These results show that the test ceftriaxone MICs are lower than the reference MIC.

E&O FAA with 5 % DHB when tested against ceftriaxone has high negative bias – 68.8 % and – 78.6 % in laboratory 1 and 2 respectively, suggesting MICs much lower than the reference method.

Ceftriaxone MICs when tested on Liofilchem FAA were far lower than the reference method, with negative bias of – 85.6 % and - 80.5 % in laboratory 1 and 2.

MICs for ceftriaxone tested with E&O non-selective agar were much lower than the reference method, -78.6 % in laboratory 1 and – 81.0 % in laboratory 2.

E&O GC chocolate with 10 % DHB exhibits unacceptably high negative bias, - 83.2 % in laboratory 1 and – 85.6 % in laboratory 2 against ceftriaxone. The negative bias implying ceftriaxone MICs were much lower than the reference method.

Oxoid chocolate + vitox when tested with ceftriaxone exhibits incredibly high negative bias, -88.3 % in both laboratories. Implying for Oxoid chocolate + vitox the ceftriaxone MICs are lower when tested by gradient strip than the reference method.

For the three agars tested in different laboratories the bias seen was consistent for Oxoid GC + vitox and BD GC chocolate agar. Positive bias was seen in both laboratories for azithromycin and negative bias in both laboratories for ceftriaxone. However, for Oxoid chocolate + vitox when tested with azithromycin there was positive bias seen in laboratory 1 (SACU) and negative bias in laboratory 2 (AMRSTI). This suggests that for azithromycin gradient strips, SACU results were higher than the reference method, whereas AMRSTI results were lower than the reference method. When tested with ceftriaxone both laboratories were consistent with very high levels of negative bias.

Agar plates tested within the same laboratory on separate occasions were consistent producing similar levels of either positive or negative bias.

One agar, BD GC chocolate agar, achieved the best levels of acceptable bias out of all seven agar plates for both antibiotics tested in both laboratories.

3.2.7 CATEGORICAL AGREEMENT

Categorical agreement (CA) was calculated for susceptibility tests for all forty-six clinical isolates (table 19). Acceptable categorical agreement is classed as those results greater than 95 %, according to 20776 – 2 (2007).(105)

Categorical agreement also looks at whether major errors or very major errors (false susceptibility) occurred in testing. Very major errors occur when there is a category difference, an isolate is reported as susceptible by gradient strip but resistant by agar dilution. Whist major errors (false resistance) are when an isolate is reported as resistant by gradient strip but it susceptible by agar dilution. This can have a direct impact on patient treatment and outcomes, for example, if their isolate has tested susceptible by gradient strip and they are treated accordingly, when the true result, if done by the reference method is resistant. This could result in treatment failure.

Table 19: Categorical agreement between forty-six clinical strain gradient strip MICs and reference result MICs against all seven agars for stage 2

	AZITHROMYCIN		CEFTRIAXONE	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	CA	CA	CA	CA
Oxoid chocolate + vitox	100 %	97.8 %	100 %	100 %
Oxoid GC + vitox	100 %	100 %	100 %	100 %
BD GC chocolate agar	100 %	100 %	95.7 %	100 %
E&O GC Non-Selective	97.8 %	97.8 %	100 %	100 %
E&O GC chocolate with 10 % DHB	97.8 %	97.8 %	100 %	100 %
E&O FAA 5 % DHB	100 %	100 %	100 %	100 %
Liofilchem FAA	100 %	100 %	100 %	100 %

MICs for azithromycin and ceftriaxone have acceptable CA for all agars and in both laboratories.

Oxoid chocolate + vitox had CA of 97.8 % in laboratory 2 which equates to one very major error, where one isolate tested resistant by the reference method, and susceptible by gradient strip for azithromycin. E&O GC non-selective and E&O GC chocolate with 10 % DHB

both had CA of 97.8 % in both laboratories, which equated to very major errors for one isolate tested with azithromycin gradient strips, testing susceptible when the reference test was resistant.

Only one agar, BD GC chocolate had CA of 95.7 % for ceftriaxone gradient strips. This equated to a very major error where 2 isolates tested resistant by gradient strip in laboratory 1, but susceptible by the reference method.

Only one of the clinical isolates tested was resistant according to EUCAST version 15 breakpoint tables to azithromycin by the reference method. None of the clinical isolates were resistant to ceftriaxone by the reference method according to the current EUCAST breakpoint tables.(39) Including more resistant isolates you could expect to see more variation in categorical agreement. In stage 3, isolates with a variety of antibiograms was included to address this.

3.2.8 SUMMARY OF STAGE 2 RESULTS

To calculate the overall best performing agar plate, overall essential agreement and overall categorical agreement for all antimicrobial agents were calculated, tables 20 and 21.

Table 20: Overall essential agreement for seven agar plates for stage 2

Agar plate	Total EA
Oxoid chocolate + vitox	70 %
Oxoid GC + vitox	89.7 %
BD GC chocolate agar	89.7 %
E&O GC Non-Selective	59.8 %
E&O GC chocolate with 10 % DHB	70.7 %
E&O FAA 5 % DHB	84.8 %
Liofilchem FAA	80.4 %

Table 21: Overall categorical agreement for seven agar plates for stage 2

Agar plate	Total CA
Oxoid chocolate + vitox	100 %
Oxoid GC + vitox	100 %
BD GC chocolate agar	98.9 %
E&O GC Non-Selective	98.9 %
E&O GC chocolate with 10 % DHB	98.9 %
E&O FAA 5 % DHB	100 %
Liofilchem FAA	100 %

Considering overall essential and categorical agreement seen in tables 20 and 21, the four best performing agar plates when tested against bioMérieux gradient strips were, Oxoid GC + vitox, BD GC chocolate, E&O FAA with 5 % DHB and Liofilchem FAA. These four agars were subsequently selected for stage 3 experiments, where they'll be tested against a diverse group of clinical and quality control *N. gonorrhoeae* isolates containing a variety of antibiograms. MICs will be determined using four different antimicrobial agents: azithromycin, cefixime, ceftriaxone and ciprofloxacin, the recommended antibiotics for treatment of *N. gonorrhoeae*, and using gradient strips from two manufacturers: bioMérieux and Liofilchem.

3.3 STAGE 3 – ASSESSMENT OF GRADIENT STRIP MANUFACTURERS ON THE BEST PERFORMING AGAR PLATES

Twenty clinical isolates and WHO quality control strains F, G, K, L, M, P, V, W, X, Y, and Z were kindly provided by AMRSTI from their extensive *N. gonorrhoeae* collection. The isolates were tested in SACU and AMRSTI for two agars, Oxoid GC + vitox and BD GC chocolate, and in SACU on two occasions for E&O FAA with 5 % DHB and Liofilchem FAA. As stated in Materials and Methods, 0.5 McFarland inocula were prepared in MHB in SACU, as per manufacturers recommendations, and 0.85 % sterile saline in AMRSTI, mimicking what the majority of clinical laboratories use for inoculum preparation.

Azithromycin, cefixime, ceftriaxone and ciprofloxacin gradient strips from two manufacturers available in the UK, bioMérieux and Liofilchem, were tested on the four recommended agars from stage 2. In stage 2, MICs for each of the eleven WHO quality control isolates were compared to target reference MICs determined by Unemo for azithromycin, ceftriaxone, cefixime, and ciprofloxacin, seen in table 22.(99) MIC ranges for quality control isolates are usually determined through data from multiple laboratory testing; ranges normally cover a 2 x doubling dilution range, an example of this is: 0.032 – 0.125 mg/L. For this example, the target MIC is the median of the values, 0.064 mg/L, all laboratories should aim to achieve the target MIC when performing a susceptibility test.(39)

Table 22: Target MICs of azithromycin, ceftriaxone, cefixime, and ciprofloxacin for eleven WHO strains for stage 3 as described by Unemo

	Azithromycin mg/L	Ceftriaxone mg/L	Cefixime mg/L	Ciprofloxacin mg/L
WHO F	0.125	< 0.002	< 0.016	0.004
WHO G	0.25	0.008	< 0.016	0.125
WHO K	0.25	0.064	0.25	> 32
WHO L	0.5	0.25	0.125	> 32
WHO M	0.25	0.016	< 0.016	2
WHO P	4	0.004	< 0.016	0.004
WHO V	> 256	0.064	< 0.016	> 32
WHO W	0.5	0.064	0.25	> 32
WHO X	0.5	2	4	> 32
WHO Y	1	1	2	> 32
WHO Z	1	0.5	2	> 32

The target MICs in table 22 were extrapolated to an MIC quality control range, 1 x doubling dilution above and below the target for each antimicrobial, these are seen in tables 23 – 30.(99) An MIC range is what you would expect to find in the EUCAST quality control document for example.(39)

3.3.1 WHO QUALITY CONTROL STRAINS

The MIC results of the WHO quality control strains against all antimicrobial agents tested using two gradient strips from different manufacturers, and on the four best performing plates can be seen in tables 23 – 30. Quality control ranges seen in tables 23 – 30 were extrapolated from the target reference MICs determined by Unemo, which are detailed in table 22.

Table 23: MIC results of WHO F, G, K, L, M, P, V, W, X, Y and Z tested with azithromycin gradient strips in laboratory 1, on four agar plates for stage 3

		Laboratory 1 azithromycin MIC (mg / L)							
WHO strain	**Reference result QC range**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
WHO F	0.064 – 0.25	0.125	0.125	0.125	0.125	0.25	0.25	0.25	0.25
WHO G	0.125 - 0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
WHO K	0.125 – 0.5	0.5	0.25	0.5	0.25	0.25	0.25	0.5	0.5
WHO L	0.25 – 1	0.5	0.25	0.5	0.25	0.5	0.5	0.5	0.5
WHO M	0.125 – 0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.5	1
WHO P	2 - 8	4	4	4	4	8	8	8	4
WHO V	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
WHO W	0.25 – 1	0.5	0.25	0.25	0.25	0.25	0.25	0.5	0.25
WHO X	0.25 – 1	0.5	0.5	0.5	0.25	0.5	0.25	0.5	0.5
WHO Y	0.5 - 2	0.25	0.25	0.25	0.25	0.5	0.5	0.5	0.5
WHO Z	0.5 - 2	1	0.5	1	1	1	1	1	1

All tests performed in SACU.

Cells highlighted in red are outside of the reference result quality control range.

Table 24: MIC results of WHO F, G, K, L, M, P, V, W, X, Y and Z with azithromycin gradient strips in laboratory 2, on four agar plates for stage 3

WHO strain	Reference result QC range	Laboratory 2 azithromycin MIC (mg / L)							
		Oxoid GC + vitox*		BD GC chocolate agar*		E&O FAA w/ 5% DHB▫		Liofilchem FAA▫	
		bioMérieux	Liofilchem	bioMérieux	Liofilchem	bioMérieux	Liofilchem	bioMérieux	Liofilchem
WHO F	0.064 – 0.25	0.125	0.125	0.125	0.25	0.25	0.5	0.25	0.5
WHO G	0.125 – 0.5	0.25	0.25	0.5	0.25	0.125	0.25	0.25	0.25
WHO K	0.125 – 0.5	0.5	0.5	0.5	0.5	0.25	0.25	0.25	1
WHO L	0.25 – 1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
WHO M	0.125 – 0.5	0.5	1	0.5	0.5	0.25	0.5	0.5	0.5
WHO P	2 – 8	4	4	4	8	8	8	4	8
WHO V	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
WHO W	0.25 – 1	0.5	0.5	0.5	0.5	0.25	0.5	0.5	0.5
WHO X	0.25 – 1	0.5	0.5	0.5	0.5	0.25	0.5	0.5	0.5
WHO Y	0.5 – 2	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5
WHO Z	0.5 – 2	1	1	1	1	1	0.5	1	1

* Tested in AMRSTI, ▫ Tested in SACU.

Cells highlighted in red are outside of the reference result quality control range.

Table 25: MIC results of WHO F, G, K, L, M, P, V, W, X, Y and Z with ceftriaxone gradient strips in laboratory 1, on four agar plates for stage 3

		Laboratory 1 ceftriaxone MIC (mg / L)							
WHO strain	**Reference result QC range**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
WHO F	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002
WHO G	0.004 – 0.016	0.008	0.008	0.008	0.008	0.016	0.004	0.008	0.008
WHO K	0.032 – 0.125	0.125	0.064	0.125	0.064	0.016	0.016	0.064	0.032
WHO L	0.125 – 0.5	0.25	0.25	0.25	0.5	0.125	0.064	0.125	0.064
WHO M	0.008 – 0.032	0.008	0.008	0.008	0.008	0.004	0.002	0.008	0.004
WHO P	0.002 – 0.008	0.004	0.002	0.004	0.002	0.004	0.002	0.008	0.002
WHO V	0.032 – 0.125	0.032	0.032	0.032	0.032	0.008	0.004	0.016	0.004
WHO W	0.032 – 0.125	0.064	0.032	0.064	0.064	0.032	0.032	0.064	0.032
WHO X	1 – 4	1	2	1	1	0.5	0.5	1	1
WHO Y	0.5 – 2	0.5	1	0.5	1	0.5	0.5	1	1
WHO Z	0.25 - 1	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.25

All tests performed in SACU.

Cells highlighted in red are outside of the reference result quality control range, cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 26: MIC results of WHO F, G, K, L, M, P, V, W, X, Y and Z with ceftriaxone gradient strips in laboratory 2, on four agar plates for stage 3

		Laboratory 2 ceftriaxone MIC (mg / L)							
WHO strain	**Reference result QC range**	**Oxoid GC + vitox***		**BD GC chocolate agar***		**E&O FAA w/ 5% DHB▫**		**Liofilchem FAA▫**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
WHO F	< 0.002	0.002	0.002	0.002	0.002	< 0.002	< 0.002	< 0.002	< 0.002
WHO G	0.004 – 0.016	0.008	0.004	0.016	0.008	0.008	0.004	0.008	0.008
WHO K	0.032 – 0.125	0.125	0.125	0.125	0.125	0.016	0.016	0.064	0.032
WHO L	0.125 – 0.5	0.25	0.25	0.25	0.5	0.125	0.064	0.125	0.064
WHO M	0.008 – 0.032	0.016	0.008	0.016	0.008	0.004	0.004	0.008	0.008
WHO P	0.002 – 0.008	0.004	0.004	0.004	0.004	0.004	0.002	0.008	0.004
WHO V	0.032 – 0.125	0.064	0.064	0.064	0.064	0.008	0.004	0.016	0.004
WHO W	0.032 – 0.125	0.064	0.064	0.125	0.064	0.032	0.032	0.064	0.064
WHO X	1 – 4	2	2	2	2	0.5	0.5	1	1
WHO Y	0.5 – 2	1	2	1	1	0.5	0.5	0.5	1
WHO Z	0.25 - 1	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5

* Tested in AMRSTI, ▫ Tested in SACU.

Cells highlighted in red are outside of the reference result quality control range, cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 27: MIC results of WHO F, G, K, L, M, P, V, W, X, Y and Z with cefixime gradient strips in laboratory 1, on four agar plates for stage 3

		Laboratory 1 cefixime MIC (mg / L)							
WHO strain	**Reference result QC range**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
WHO F	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016
WHO G	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	0.016	< 0.016	0.016	0.016
WHO K	0.125 – 0.5	0.25	0.25	0.25	0.5	0.125	0.125	0.125	0.125
WHO L	0.064 – 0.25	0.25	0.25	0.25	0.25	0.064	0.064	0.064	0.064
WHO M	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016
WHO P	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	0.016	< 0.016
WHO V	< 0.016	0.016	0.016	0.032	0.032	< 0.016	< 0.016	0.016	< 0.016
WHO W	0.125 – 0.5	0.125	0.25	0.25	0.25	0.25	0.25	0.25	0.25
WHO X	2 – 8	4	8	4	8	4	4	4	4
WHO Y	1 – 4	2	2	2	4	2	2	2	4
WHO Z	1 – 4	1	2	1	2	2	2	2	2

All tests performed in SACU.

Cells highlighted in red are outside of the reference result quality control range.

Table 28: MIC results of WHO F, G, K, L, M, P, V, W, X, Y and Z with cefixime gradient strips in laboratory 2, on four agar plates for stage 3

		Laboratory 2 cefixime MIC (mg / L)							
WHO strain	**Reference result QC range**	**Oxoid GC + vitox***		**BD GC chocolate agar***		**E&O FAA w/ 5% DHB▫**		**Liofilchem FAA▫**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
WHO F	< 0.016	0.016	0.016	0.016	0.016	< 0.016	< 0.016	< 0.016	< 0.016
WHO G	< 0.016	0.016	0.016	0.016	0.016	< 0.016	< 0.016	< 0.016	< 0.016
WHO K	0.125 – 0.5	0.5	0.25	0.5	0.25	0.125	0.125	0.125	0.125
WHO L	0.064 – 0.25	0.125	0.125	0.25	0.125	0.064	0.032	0.064	0.064
WHO M	< 0.016	0.016	0.016	0.016	0.016	< 0.016	< 0.016	< 0.016	< 0.016
WHO P	< 0.016	0.016	0.016	0.016	0.016	< 0.016	< 0.016	< 0.016	< 0.016
WHO V	< 0.016	0.032	0.032	0.064	0.032	< 0.016	< 0.016	< 0.016	< 0.016
WHO W	0.125 – 0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
WHO X	2 – 8	4	8	4	8	2	4	4	4
WHO Y	1 – 4	2	2	4	4	2	2	2	2
WHO Z	1 – 4	2	2	2	2	1	2	1	1

* Tested in AMRSTI, ▫ Tested in SACU.

Cells highlighted in red are outside of the reference result quality control range, cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 29: MIC results of WHO F, G, K, L, M, P, V, W, X, Y and Z with ciprofloxacin gradient strips in laboratory 1, on four agar plates for stage 3

		Laboratory 1 ciprofloxacin MIC (mg / L)							
WHO strain	**Reference result QC range**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
WHO F	0.002 – 0.008	< 0.002	< 0.002	< 0.002	< 0.002	0.004	0.002	0.002	0.002
WHO G	0.064 – 0.25	0.064	0.064	0.125	0.125	0.064	0.064	0.064	0.064
WHO K	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
WHO L	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
WHO M	1 – 4	1	1	1	1	1	1	1	1
WHO P	0.002 – 0.008	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
WHO V	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
WHO W	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
WHO X	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
WHO Y	> 32	8	8	8	8	16	8	> 32	8
WHO Z	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32

All tests performed in SACU.

Cells highlighted in red are outside of the reference result quality control range, cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 30: MIC results of WHO F, G, K, L, M, P, V, W, X, Y and Z with ciprofloxacin gradient strips in laboratory 2, on four agar plates for stage 3

		Laboratory 2 ciprofloxacin MIC (mg / L)							
WHO strain	**Reference result QC range**	**Oxoid GC + vitox***		**BD GC chocolate agar***		**E&O FAA w/ 5% DHB▫**		**Liofilchem FAA▫**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
WHO F	0.002 – 0.008	0.002	0.002	0.004	0.004	0.002	0.002	0.002	0.002
WHO G	0.064 – 0.25	0.064	0.064	0.125	0.125	0.064	0.064	0.064	0.064
WHO K	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
WHO L	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
WHO M	1 – 4	1	1	1	2	1	1	1	1
WHO P	0.002 – 0.008	0.004	0.004	0.004	0.008	0.002	0.004	0.004	0.004
WHO V	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
WHO W	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
WHO X	> 32	> 32	> 32	> 32	> 32	> 32	8	> 32	> 32
WHO Y	> 32	32	16	32	> 32	8	8	8	8
WHO Z	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32

* Tested in AMRSTI, ▫ Tested in SACU.

Cells highlighted in red are outside of the reference result quality control range, cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

3.3.2 VARIANCE OF QUALITY CONTROL MICS ACROSS AGAR PLATES FOR STAGE 3

When looking at the MICs in tables 23 – 30 some isolates appear to have a large variety of MICs to some antimicrobials across the agar plates, highlighted in orange.

Ceftriaxone gradient strips saw a 3 x doubling dilution range of MICs against WHO K and WHO L in both laboratories, WHO V had 3 x doubling dilution range of MICs in laboratory 1 and a 4 x doubling dilution range laboratory 2.

For cefixime gradient strips, one quality control isolate, WHO L had a 3 x doubling dilution range of MICs in laboratory 2.

Ciprofloxacin gradient strips saw a 3 x doubling dilution range of MIC for WHO Y in both laboratories. WHO X had a 3 x doubling dilution range of MICs in laboratory 2.

3.3.3 ANALYSIS OF WHO QUALITY CONTROL STRAINS FOR STAGE 3

Using the MICs from tables 23 – 30, the percentage of MICs for the quality control isolates found within the quality control ranges and the percentage of MICs that met the target MICs was calculated, shown in tables 31 and 32.

Table 31: Percentage MICs within the reference result quality control range for stage 3

	AZITHROMYCIN BIOMERIEUX		AZITHROMYCIN LIOFILCHEM		CEFTRIAXONE BIOMERIEUX		CEFTRIAXONE LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	In range	In range	In range	In range	In range	In range	In range	In range
Oxoid GC + vitox	90.9 %	100 %	90.9 %	90.9 %	100 %	100 %	100 %	100 %
BD GC Chocolate agar	90.9 %	100 %	90.9 %	100 %	100 %	100 %	100 %	100 %
E&O FAA 5% DHB	100 %	90.9 %	100 %	81.8 %	63.6 %	63.6 %	54.5 %	54.5 %
Liofilchem FAA	100 %	100 %	90.9 %	81.8 %	90.9 %	90.9 %	72.7 %	81.8 %

	CEFIXIME BIOMERIEUX		CEFIXIME LIOFILCHEM		CIPROFLOXACIN BIOMERIEUX		CIPROFLOXACIN LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	In range	In range	In range	In range	In range	In range	In range	In range
Oxoid GC + vitox	100 %	90.9 %	100 %	90.9 %	81.8 %	100 %	81.8 %	90.9 %
BD GC Chocolate agar	90.9 %	90.9 %	90.9 %	90.9 %	81.8 %	100 %	81.8 %	100 %
E&O FAA 5% DHB	100 %	100 %	100 %	90.9 %	90.9 %	90.9 %	90.9 %	81.8 %
Liofilchem FAA	100 %	100 %	100 %	100 %	100 %	90.9 %	90.9 %	90.9 %

Table 32: Percentage of quality control MICs on target for stage 3 as described by Unemo(99)

	AZITHROMYCIN BIOMERIEUX		AZITHROMYCIN LIOFILCHEM		CEFTRIAXONE BIOMERIEUX		CEFTRIAXONE LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	On target	On target	On target	On target	On target	On target	On target	On target
Oxoid GC + vitox	72.7 %	72.7 %	54.5 %	72.7 %	54.5 %	81.8 %	63.6 %	63.6 %
BD GC Chocolate agar	63.6 %	63.6 %	54.5 %	54.5 %	54.5 %	72.7 %	54.5 %	72.7 %
E&O FAA 5% DHB	63.6 %	45.4 %	45.4 %	54.5 %	18.2 %	27.3 %	9.1 %	9.1 %
Liofilchem FAA	54.5 %	72.7 %	54.5 %	54.5 %	54.5 %	45.4%	27.3 %	54.5 %

	CEFIXIME BIOMERIEUX		CEFIXIME LIOFILCHEM		CIPROFLOXACIN BIOMERIEUX		CIPROFLOXACIN LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	On target	On target	On target	On target	On target	On target	On target	On target
Oxoid GC + vitox	63.6 %	45.4 %	72.7 %	45.4 %	63.6 %	63.6 %	63.6 %	63.6 %
BD GC Chocolate agar	72.7 %	27.3 %	54.5 %	36.4 %	72.7 %	81.8 %	72.7 %	90.9 %
E&O FAA 5% DHB	72.7 %	63.6 %	81.8 %	81.8 %	72.7 %	54.5 %	63.6 %	54.5 %
Liofilchem FAA	54.5 %	72.7 %	63.6 %	72.7 %	72.7 %	63.6 %	63.6 %	63.6 %

3.3.3.1 Oxoid GC + vitox

3.3.3.1.1 *Azithromycin*

Seen in tables 31 and 32, in laboratory 1 90.9 % of bioMérieux azithromycin MICs for all WHO strains were within the quality control range and in laboratory 2, 100 % of the WHO MICs were within range for Oxoid GC + vitox agar plates. 72.7 % of the WHO azithromycin MICs were on target when tested with bioMérieux gradient strips in both laboratories. 90.9 % of Liofilchem azithromycin MICs were in range in both laboratories. In laboratory 1, 54.5 % of Liofilchem azithromycin gradient strip MICs met the target, and in laboratory 2, 72.7 % of the MICs were on target. Azithromycin MICs for WHO Y were out of range when using both gradient strip manufacturers in laboratory 1. Laboratory 2 recorded out of range MICs for WHO M when tested with Liofilchem MIC strips, reporting an MIC 1 x doubling dilution higher than the acceptable range.

3.3.3.1.2 *Ceftriaxone*

There were no out of range results recorded for either manufacturer of ceftriaxone gradient strips when tested on Oxoid GC + vitox in both laboratories. bioMérieux gradient strips performed slightly better with 54.5 % of ceftriaxone MICs meeting the target in laboratory 1 and 81.8 % in laboratory 2. With ceftriaxone Liofilchem gradient strips, 63.6 % of MICs met the target in both laboratory 1 and 2, seen in tables 31 and 32.

3.3.3.1.3 *Cefixime*

Seen in tables 31 and 32 cefixime bioMérieux gradient strips recorded no out-of-range results for laboratory 1 and 90.9 % of MICs were within range in laboratory 2, the same was seen for cefixime Liofilchem gradient strips. Liofilchem gradient strips performed a little better in laboratory 1, 72.7 % of the MICs exactly on target, whereas for bioMérieux, 63.6 % of MICs were on target. Laboratory 2 recorded 45.5 % of results on target for both

bioMérieux and Liofilchem gradient strips. Laboratory 1 recorded out of range results for WHO V for both bioMérieux and Liofilchem gradient strips when tested on Oxoid GC + vitox.

3.3.3.1.4 *Ciprofloxacin*

In tables 31 and 32, 81.8% of ciprofloxacin MICs using bioMérieux gradient strips were within range for laboratory 1 and 100 % within range in laboratory 2. Liofilchem ciprofloxacin gradient strips reported 81.8 % of MICs in range for laboratory 1, and 90.9 % in range for laboratory 2. Laboratory 1 recorded out-of-range MICs for ciprofloxacin with WHO F and WHO Y on Oxoid GC + vitox with both gradient strip manufacturers, and laboratory 2 recorded an out-of-range MIC for WHO Y when tested with Liofilchem ciprofloxacin gradient strips only. 63.6 % of ciprofloxacin MICs were on target for bioMérieux and Liofilchem gradient strips in laboratory 1 and laboratory 2.

3.3.3.2 BD GC chocolate agar

3.3.3.2.1 *Azithromycin*

Seen in tables 31 and 32, when tested on BD GC chocolate agar laboratory 1, 90.9 % of azithromycin MICs are within the quality control range for both bioMérieux and Liofilchem gradient strips. In laboratory 2 100 % of bioMérieux and Liofilchem MICs were within the quality control range. Of these MICs, 63.6 % of MICs with bioMérieux azithromycin gradient strips in both laboratories matched the target MIC. 54.5 % of the Liofilchem azithromycin gradient strips MICs were on target in laboratory 1 and laboratory 2. For BD GC chocolate agar, there were out-of-range MICs recorded only in laboratory 1 for WHO Y with both gradient strip manufacturers for azithromycin with MICs 1 x doubling dilution below the threshold.

3.3.3.2.2 *Ceftriaxone*

Ceftriaxone gradient strips from both bioMérieux and Liofilchem gave MICs which were 100 % within the quality control range in both laboratories. When tested in laboratory 1, 54.5 % of bioMérieux and Liofilchem ceftriaxone MICs were the same as the target MIC. In laboratory 2 72.7 % of bioMérieux and Liofilchem MICs were exactly as the target ceftriaxone MIC on BD GC chocolate agar, as seen in tables 31 and 32.

3.3.3.2.3 *Cefixime*

Seen in tables 31 and 32, 90.9 % of cefixime MICs in both laboratories with both gradient strip manufacturers were within range. In Laboratory 1, 72.7 % of bioMérieux and 54.5 % of Liofilchem MICs met the target MIC. 27.3 % of bioMérieux cefixime MICs in laboratory 2 and 36.4 % of Liofilchem MICs were the same as the target MIC.

3.3.3.2.4 *Ciprofloxacin*

There were out-of-range MICs recorded in laboratory 1 for WHO F and WHO Y, with both gradient strip manufacturers for ciprofloxacin. MICs were 1 x doubling dilution below the range for WHO F and at least 2 x doubling dilutions below the quality control range for WHO Y. 81.8 % of ciprofloxacin MICs were within range with bioMérieux and Liofilchem gradient strips in laboratory 1 and 100 % for both manufacturers in laboratory 2. 72.7 % of ciprofloxacin MICs in laboratory 1 were the same as the target MIC for both manufacturers of gradient strip. In laboratory 2, 81.8 % of bioMérieux ciprofloxacin MICs and 90.9 % of Liofilchem ciprofloxacin MICs matched the target MIC on BD GC chocolate agar, seen in tables 31 and 32.

3.3.3.3 Liofilchem FAA

3.3.3.3.1 *Azithromycin*

From tables 31 and 32, on Liofilchem FAA, 100 % of bioMérieux azithromycin MICs were within the quality control range in both laboratories. For Liofilchem azithromycin gradient strips, 90.9% and 81.8% of MICs were within range for laboratories 1 and 2 respectively. 54.5 % of azithromycin bioMérieux gradient strips recorded MICs on target in laboratory 1 and 72.7 % in laboratory 2. Liofilchem recorded 54.5 % of MICs on target in both laboratory 1 and 2. Laboratory 1 recorded one out of range result for WHO M when tested with Liofilchem azithromycin gradient strips, recording a result 1 x doubling dilution above the acceptable quality control range. In laboratory 2, two out of range results for WHO F and WHO K, were recorded, both results being 1 x doubling dilution above the top of the quality control range.

3.3.3.3.2 *Ceftriaxone*

Seen in tables 31 and 32, for ceftriaxone, 90.9 % of bioMérieux ceftriaxone MICs were within range for laboratory 1 and 2 when tested on Liofilchem FAA. Whilst for Liofilchem ceftriaxone gradient strips, 72.7 % of MICs in laboratory 1, and 81.8 % of MICs in laboratory 2 were within range. In laboratory 1, 54.5 % of bioMérieux gradient strips MICs and 27.3 % of Liofilchem gradient strip MICs were the same as the target MIC. On Liofilchem FAA in laboratory 2, 45.4 % of bioMérieux MICs and 54.5 % of Liofilchem MICs were exactly as the target. Laboratory 1 and 2 recorded out-of-range MICs for Liofilchem ceftriaxone gradient strips for WHO L, the MICs were 1 x doubling dilution below the bottom of the range. In laboratory 1 and 2, both manufacturers exhibited MICs which were out-of-range for WHO V, bioMérieux gradient strips were 1 x doubling dilution below the range, and Liofilchem gradient strips were 3 x doubling dilutions below the quality control range. Laboratory 1 also recorded out-of-range results for Liofilchem gradient strips with WHO M, 1 x doubling dilution below the bottom of the range.

3.3.3.3.3 *Cefixime*

All cefixime MICs were within range for laboratory 1 and 2, with bioMérieux and Liofilchem gradient strips. In laboratory 1 54.5 % of bioMérieux and 63.6 % of Liofilchem MICs were the same as the target MIC. 72.7 % of MICs in laboratory 2 with bioMérieux and Liofilchem gradient strips met the target MIC, seen in tables 31 and 32.

3.3.3.3.4 *Ciprofloxacin*

In tables 31 and 32, when testing ciprofloxacin gradient strips, in laboratory 1, 100 % of bioMérieux MICs were within the quality control range, compared to 90.9 % of Liofilchem MICs. In laboratory 2, 90.9 % of bioMérieux and Liofilchem gradient strip MICs were within range. Laboratory 1 saw 72.7 % of bioMérieux MICs match the target MIC, whereas 63.6 % of Liofilchem MICs were the same as the target. 63.6 % of laboratory 2 MICs for both bioMérieux and Liofilchem were exactly as the target. Out-of-range ciprofloxacin MICs were recorded for WHO Y with Liofilchem gradient strips in laboratory 1 and both manufacturers in laboratory 2. MICs were recorded 3 x doubling dilutions below the range.

3.3.3.4 E&O FAA with 5 % Defibrinated Horse Blood

3.3.3.4.1 *Azithromycin*

As seen in tables 31 and 32, E&O FAA with 5 % DHB when tested with azithromycin in laboratory 1 saw 100 % of the MICs within range for bioMérieux and Liofilchem gradient strips. In laboratory 2, 90.9 % of bioMérieux gradient strips and 81.8 % of Liofilchem gradient strip MICs were within range. 63.6 % of MICs were the same as the target MIC for bioMérieux and 45.4 % for Liofilchem in laboratory 1. In laboratory 2, 45.4 % of bioMérieux MICs and 54.5 % of Liofilchem MICs met the target. There were out-of-range MICs recorded in laboratory 2 with both manufacturers of gradient strip with WHO Y, the results were 1 x

doubling below the quality control range. There were out-of-range results also recorded for WHO F, where MICs were 1 x doubling dilution above the range.

3.3.3.4.2 *Ceftriaxone*

According to tables 31 and 32, 63.6 % of ceftriaxone MICs tested on E&O FAA with 5 % DHB were within the quality control range for bioMérieux gradient strips in laboratory 1 and 2. 54.5 % of Liofilchem ceftriaxone gradient strip MICs were in range in both laboratories. In laboratory 1, 18.2 % of ceftriaxone bioMérieux MICs, and 27.3 % in laboratory 2 were the same as the target MIC. Only 9.1 % of Liofilchem ceftriaxone MICs in laboratories 1 and 2 met the target MIC. There were many out-of-range results recorded for both laboratories with bioMérieux and Liofilchem ceftriaxone gradient strips on E&O FAA with 5 % DHB. WHO K, L, M, V, and X recorded results ranging from 1 x doubling dilution to 3 x doubling dilutions below the quality control range.

3.3.3.4.3 *Cefixime*

Seen in tables 31 and 32, when tested on E&O FAA with 5 % DHB, 100 % of bioMérieux and Liofilchem cefixime gradient strip MICs were within the quality control range in laboratory 1. In laboratory 2, 100 % of bioMérieux cefixime MICs and 90.9 % of Liofilchem cefixime MICs were in range. For bioMérieux cefixime gradient strip MICs, 72.7 % in laboratory 1 and 63.6 % in laboratory 2 met the target MIC. For Liofilchem cefixime MICs, both laboratories recorded 81.8 % of MICs the same as the target. There was one out-of-range cefixime MIC recorded in laboratory 2 with Liofilchem gradient strips with WHO L, the MIC was 1 x doubling dilution below the bottom of the range.

3.3.3.3.4 *Ciprofloxacin*

As seen in tables 31 and 32, 90.9 % of ciprofloxacin MICs in laboratory 1 with bioMérieux and Liofilchem gradient strips and laboratory 2 with bioMérieux gradient strips were within the quality control range. In laboratory 2, 81.8 % of Liofilchem ciprofloxacin gradient strip MICs were within range. Of these results, 72.7 % of bioMérieux ciprofloxacin gradient strips MICs, and 63.6 % of Liofilchem ciprofloxacin gradient strip MICs were the same as the target MIC in laboratory 1. In laboratory 2, 54.5 % of bioMérieux and Liofilchem gradient strips MICs met the target MIC. When tested on E&O FAA with 5 % DHB out-of-range results were recorded in laboratory 1 and 2 for WHO Y with both gradient strip manufacturers, where results were 2 to 3 x doubling dilution below the acceptable quality control range. Laboratory 2 also recorded an out-of-range results for Liofilchem gradient strips with WHO X, 3 x doubling dilutions below the bottom of the quality control range. E&O FAA with 5 % DHB was the worst performing agar for the quality control strains with both manufacturers of gradient strip.

3.3.4 Summary of stage 3 quality control results

3.3.4.1 *Observations*

For the agar plates tested in separate laboratories, Oxoid GC + vitox and BD GC chocolate agar, the results were mostly within 1 x doubling dilution of each other. This is the same as the natural error of the method, meaning the MICs are reliable when tested on different occasions by different individuals using varied lot numbers of agar plates. E&O FAA with 5 % DHB and Liofilchem FAA MICs that were performed in the same laboratory on different occasions again recorded results which were within the natural error rate of the method, with two exceptions that were 2 x doubling dilutions different.

3.3.4.2 *Overall Stage 3 quality control MICs summary*

To calculate the overall best performing agar plate and gradient strip manufacturer for the quality control strains, the overall percentage of MICs in-range and percentage of MICs on target for gradient strips and agar plates was calculated, seen in tables 33 to 36.

Table 33: Overall percentage of MICs within the quality control range for gradient strips for stage 3

AZITHROMYCIN BIOMÉRIEUX	AZITHROMYCIN LIOFILCHEM	CEFTRIAXONE BIOMÉRIEUX	CEFTRIAXONE LIOFILCHEM
In range	In range	In range	In range
96.6%	90.9%	88.6%	82.9%

CEFIXIME BIOMÉRIEUX	CEFIXIME LIOFILCHEM	CIPROFLOXACIN BIOMÉRIEUX	CIPROFLOXACIN LIOFILCHEM
In range	In range	In range	In range
96.6%	95.5%	92%	88.6%

As seen in table 33, bioMérieux gradient strips have a higher percentage of MICs within the quality control range than Liofilchem gradient strips for each antimicrobial tested.

Table 34: Overall percentage of quality control MICs on target for gradient strips for stage 3

AZITHROMYCIN BIOMÉRIEUX	AZITHROMYCIN LIOFILCHEM	CEFTRIAXONE BIOMÉRIEUX	CEFTRIAXONE LIOFILCHEM
On target	On target	On target	On target
63.6%	55.5%	51.1%	44.3%

CEFIXIME BIOMÉRIEUX	CEFIXIME LIOFILCHEM	CIPROFLOXACIN BIOMÉRIEUX	CIPROFLOXACIN LIOFILCHEM
On target	On target	On target	On target
59.1%	63.6%	68.2%	67%

Table 34 shows that bioMérieux azithromycin, ceftriaxone, and ciprofloxacin gradient strips have a higher percentage of on target MICs than the equivalent Liofilchem gradient strips. Cefixime gradient strips by Liofilchem have a higher percentage of MICs on target than bioMérieux gradient strips.

Table 35: Overall percentage of quality control MICs within the quality control range for agar plates for stage 3

Agar plate	In range
Oxoid GC + vitox	94.3 %
BD GC Chocolate agar	94.3 %
E&O FAA 5% DHB	84.6 %
Liofilchem FAA	92.6 %

Table 36: Overall percentage of quality control MICs the same as the quality control target MIC for agar plates for stage 3

Agar plate	On target
Oxoid GC + vitox	63.6 %
BD GC Chocolate agar	62.5 %
E&O FAA 5% DHB	51.1 %
Liofilchem FAA	59.1 %

As seen in tables 35 and 36, Oxoid GC + vitox has the highest percentage of MICs in range, 94.3 % and on target, 63.6 % for the quality control strains tested.

The comparisons of the two best performing agar plates tested against the WHO quality control strains are summarised in table 37.

Table 37: Observations of four agar plates against WHO quality control strains in stage 3

	Oxoid GC + vitox	BD GC chocolate	Performance
Azithromycin QC in range	90.9 % (Lab 1) 100 % (Lab 2)	90.9 % (Lab 1) 100 % (Lab 2)	Excellent for both agar plates
Azithromycin meeting target MIC	bioMeriéux 72.7 % Liofilchem up to 72.2 %	bioMeriéux 63.6 % Liofilchem 54.5 %	Oxoid performed slightly better
Ceftriaxone QC in range	100 %	100 %	Excellent for both agar plates
Ceftriaxone meeting target MIC	54.5 % - 81.8 %	54.5 % - 72.7 %	Poor performance for both agar plates
Cefixime QC in range	90.9 % - 100 %	90.9 %	Excellent for both agar plates
Cefixime meeting target MIC	63.6 %	72.7 % - 90.9 %	BD GC chocolate performed slightly better
Out-of-range MICs	WHO Y & M for azithromycin WHO F & Y for ciprofloxacin	WHO Y for azithromycin WHO F & Y for ciprofloxacin	Discrepancies for both agar plates

Considering overall percentage of quality control strains in range and on target shown in tables 33 – 36, and table 37, the recommended agar and gradient strip manufacturer combination for susceptibility testing of *N. gonorrhoeae* control strains are Oxoid GC + vitox and bioMérieux gradient strips.

3.3.5 CLINICAL STRAINS

The MIC results of the clinical strains against all antimicrobial agents tested using two gradient strip manufacturers and on the four best performing plates can be seen in tables 38 – 45, alongside the equivalent reference agar dilution result as provided by AMRSTI.

Table 38: MIC results of twenty clinical isolates with azithromycin gradient strips from two manufacturers on four agar plates in laboratory 1 for stage 3

		Laboratory 1 azithromycin MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34819	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
34820	0.5	0.25	0.25	0.25	0.25	0.5	0.5	0.5	0.25
34821	0.125	0.25	0.25	0.125	0.125	0.125	0.125	0.125	0.125
34822	0.125	0.125	0.125	0.125	0.25	0.032	0.032	0.25	0.25
34823	≤ 0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.064
34824	0.5	0.5	0.25	0.5	0.5	0.125	0.125	0.25	0.25
34825	1	0.5	0.5	0.5	0.5	0.25	0.25	0.5	1
34826	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.5
34827	16	8	8	8	8	16	8	8	32
34828	2	1	1	1	1	1	1	2	2
34829	8	8	8	8	4	16	32	8	16
34830	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
34832	8	16	32	8	16	8	32	8	8
34833	0.25	0.5	0.5	0.25	0.25	1	0.5	0.5	0.5
34834	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.5

Table 38: Continued

		Laboratory 1 azithromycin MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34835	0.5	0.25	0.25	0.25	0.5	0.25	0.25	0.25	0.25
34836	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
34837	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
34838	0.25	0.125	0.125	0.125	0.125	0.25	0.25	0.5	0.25
34839	≤ 0.064	0.032	0.064	0.032	0.032	0.125	0.25	0.125	0.125

All results performed in SACU.

Cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 39: MIC results of twenty clinical isolates with azithromycin gradient strips from two manufacturers on four agar plates in laboratory 2 for stage 3

		Laboratory 2 azithromycin MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox***		**BD GC chocolate agar***		**E&O FAA w/ 5% DHB▫**		**Liofilchem FAA▫**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34819	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
34820	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.5	0.5
34821	0.125	0.25	0.25	0.25	0.5	0.125	0.125	0.125	0.125
34822	0.125	0.25	0.25	0.125	0.125	0.064	0.064	0.25	0.25
34823	≤ 0.064	0.064	0.125	0.125	0.125	0.125	0.125	0.125	0.125
34824	0.5	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5
34825	1	1	1	1	1	0.25	0.5	0.5	0.5
34826	0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.5	1
34827	16	8	16	8	16	16	16	4	16
34828	2	2	2	2	2	1	1	2	2
34829	8	8	16	8	16	16	32	16	32
34830	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
34832	8	16	32	8	16	16	32	16	16
34833	0.25	0.5	0.5	0.5	0.5	1	1	0.5	0.5
34834	0.25	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5

Table 39: Continued

SACU number	Reference result	Laboratory 2 azithromycin MIC (mg/L)							
		Oxoid GC + vitox*		BD GC chocolate agar*		E&O FAA w/ 5% DHB▫		Liofilchem FAA▫	
		bioMérieux	Liofilchem	bioMérieux	Liofilchem	bioMérieux	Liofilchem	bioMérieux	Liofilchem
34835	0.5	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5
34836	0.5	1	1	2	1	1	0.5	0.5	0.5
34837	0.5	1	0.5	1	0.5	0.5	1	0.5	1
34838	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
34839	≤ 0.064	0.064	0.064	0.064	0.064	0.125	0.125	0.125	0.125

* Tested in AMRSTI, ▫ Tested in SACU

Cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 40: MIC results of twenty clinical isolates with ceftriaxone gradient strips from two manufacturers on four agar plates in laboratory 1 for stage 3

		Laboratory 1 ceftriaxone MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34819	0.032	0.016	0.016	0.016	0.016	0.032	0.016	0.016	0.008
34820	> 0.25	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5
34821	> 0.25	0.5	0.5	0.5	0.5	0.25	0.25	0.25	0.25
34822	0.016	0.004	0.004	0.004	0.004	0.004	0.008	0.016	0.008
34823	≤ 0.004	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002
34824	0.032	0.032	0.032	0.032	0.032	0.016	0.008	0.016	0.016
34825	0.064	0.032	0.032	0.064	0.064	0.016	0.016	0.064	0.032
34826	0.032	0.032	0.032	0.032	0.016	0.016	0.008	0.032	0.016
34827	0.032	0.032	0.032	0.032	0.032	0.008	0.004	0.032	0.008
34828	0.016	0.004	0.004	0.004	0.004	0.004	0.004	0.008	0.004
34829	0.008	0.002	< 0.002	0.002	< 0.002	0.004	< 0.002	0.002	< 0.002
34830	0.016	0.016	0.016	0.016	0.016	0.004	< 0.002	0.016	0.004
34832	0.032	0.016	0.016	0.032	0.032	0.016	0.008	0.032	0.016
34833	≤ 0.004	0.002	< 0.002	0.002	0.002	0.004	0.002	0.004	0.002
34834	≤ 0.004	0.004	0.004	0.004	0.004	0.004	< 0.002	0.004	< 0.002

Table 40: Continued

		Laboratory 1 ceftriaxone MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34835	0.016	0.016	0.016	0.016	0.016	0.008	0.004	0.008	0.008
34836	0.064	0.064	0.064	0.125	0.064	0.032	0.016	0.064	0.016
34837	0.064	0.064	0.064	0.125	0.064	0.064	0.032	0.064	0.064
34838	0.064	0.064	0.064	0.064	0.064	0.032	0.016	0.064	0.064
34839	0.016	0.004	0.002	0.004	0.004	0.008	0.004	0.004	0.004

All results performed in SACU.

Cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 41: MIC results of twenty clinical isolates with ceftriaxone gradient strips from two manufacturers on four agar plates in laboratory 2 for stage 3

		Laboratory 2 ceftriaxone MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox***		**BD GC chocolate agar***		**E&O FAA w/ 5% DHB▫**		**Liofilchem FAA▫**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34819	0.032	0.032	0.016	0.032	0.032	0.016	0.008	0.016	0.008
34820	> 0.25	0.5	0.5	0.5	0.5	0.25	0.25	0.25	0.5
34821	> 0.25	0.5	0.5	0.5	0.5	0.125	0.25	0.25	0.25
34822	0.016	0.008	0.008	0.008	0.004	0.016	0.008	0.032	0.016
34823	≤ 0.004	0.002	0.002	0.002	0.002	< 0.002	< 0.002	< 0.002	< 0.002
34824	0.032	0.064	0.064	0.064	0.064	0.016	0.008	0.032	0.016
34825	0.064	0.064	0.064	0.064	0.125	0.032	0.016	0.032	0.032
34826	0.032	0.032	0.032	0.032	0.032	0.016	0.008	0.032	0.032
34827	0.032	0.064	0.032	0.064	0.032	0.016	0.008	0.032	0.008
34828	0.016	0.008	0.008	0.008	0.008	0.008	0.004	0.008	0.004
34829	0.008	0.004	< 0.002	0.004	< 0.002	0.002	< 0.002	0.004	0.004
34830	0.016	0.032	0.032	0.032	0.032	0.004	0.004	0.008	0.004
34832	0.032	0.064	0.032	0.064	0.064	0.016	0.008	0.032	0.016
34833	≤ 0.004	0.004	0.004	0.004	0.004	0.004	0.002	0.004	0.002
34834	≤ 0.004	0.004	0.004	0.004	0.004	0.004	< 0.002	0.004	0.002

Table 41: Continued

SACU number	Reference result	Laboratory 2 ceftriaxone MIC (mg/L)							
		Oxoid GC + vitox*		BD GC chocolate agar*		E&O FAA w/ 5% DHB▫		Liofilchem FAA▫	
		bioMérieux	Liofilchem	bioMérieux	Liofilchem	bioMérieux	Liofilchem	bioMérieux	Liofilchem
34835	0.016	0.032	0.032	0.032	0.032	0.008	0.004	0.008	0.008
34836	0.064	0.125	0.125	0.125	0.125	0.032	0.016	0.064	0.032
34837	0.064	0.125	0.125	0.125	0.125	0.064	0.032	0.125	0.125
34838	0.064	0.064	0.064	0.125	0.064	0.032	0.016	0.064	0.032
34839	0.016	0.008	0.004	0.008	0.004	0.004	0.002	0.008	0.004

* Tested in AMRSTI, ▫ Tested in SACU

Cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 42: MIC results of twenty clinical isolates with cefixime gradient strips from two manufacturers on four agar plates in laboratory 1 for stage 3

		Laboratory 1 cefixime MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34819	0.125	0.016	0.032	0.032	0.032	0.125	0.032	0.016	0.032
34820	> 0.5	1	2	2	2	1	1	2	2
34821	> 0.5	1	2	2	2	0.5	1	1	1
34822	0.064	< 0.016	< 0.016	< 0.016	< 0.016	0.064	0.016	0.032	0.016
34823	≤ 0.004	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016
34824	0.25	0.125	0.125	0.125	0.125	0.064	0.032	0.064	0.064
34825	0.25	0.125	0.125	0.125	0.25	0.125	0.064	0.125	0.064
34826	0.25	0.064	0.064	0.064	0.064	0.032	0.032	0.064	0.125
34827	0.5	0.064	0.125	0.064	0.125	0.032	0.032	0.064	0.064
34828	0.032	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016
34829	0.032	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016
34830	0.125	0.016	0.032	0.032	0.032	< 0.016	< 0.016	0.016	< 0.016
34832	0.125	0.064	0.125	0.064	0.125	0.064	0.032	0.064	0.032
34833	0.008	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016
34834	0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016	< 0.016

Table 42: Continued

		Laboratory 1 cefixime MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34835	0.064	0.016	0.032	0.016	0.032	< 0.016	< 0.016	< 0.016	< 0.016
34836	0.25	0.25	0.25	0.25	0.25	0.125	0.064	0.125	0.125
34837	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
34838	0.25	0.125	0.25	0.25	0.25	0.125	0.125	0.25	0.125
34839	0.032	< 0.016	0.016	< 0.016	0.016	0.016	<0.016	< 0.016	< 0.016

All results performed in SACU.

Cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 43: MIC results of twenty clinical isolates with cefixime gradient strips from two manufacturers on four agar plates in laboratory 2 for stage 3

		Laboratory 2 cefixime MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox***		**BD GC chocolate agar***		**E&O FAA w/ 5% DHB▫**		**Liofilchem FAA▫**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34819	0.125	0.032	0.032	0.064	0.032	0.064	0.064	0.032	< 0.016
34820	> 0.5	2	2	2	2	1	1	1	1
34821	> 0.5	1	2	2	2	1	1	1	1
34822	0.064	0.016	0.016	0.016	0.016	0.016	0.016	0.032	0.032
34823	≤ 0.004	0.016	0.016	0.016	0.016	< 0.016	< 0.016	< 0.016	< 0.016
34824	0.25	0.125	0.125	0.25	0.125	0.064	0.032	0.064	0.064
34825	0.25	0.25	0.125	0.25	0.25	0.064	0.064	0.125	0.125
34826	0.25	0.064	0.064	0.125	0.064	0.064	0.064	0.064	0.125
34827	0.5	0.125	0.064	0.125	0.125	0.032	0.064	0.064	0.064
34828	0.032	0.016	0.016	0.016	0.016	< 0.016	< 0.016	< 0.016	< 0.016
34829	0.032	0.016	0.016	0.016	0.016	< 0.016	< 0.016	< 0.016	< 0.016
34830	0.125	0.064	0.032	0.125	0.125	< 0.016	< 0.016	< 0.016	< 0.016
34832	0.125	0.125	0.064	0.125	0.125	0.032	0.064	0.125	0.064
34833	0.008	0.016	0.016	0.016	0.016	< 0.016	< 0.016	< 0.016	< 0.016
34834	0.016	0.016	0.016	0.016	0.016	< 0.016	< 0.016	< 0.016	< 0.016

Table 43: Continued

		Laboratory 2 cefixime MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox***		**BD GC chocolate agar***		**E&O FAA w/ 5% DHB▫**		**Liofilchem FAA▫**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34835	0.064	0.016	0.016	0.032	0.032	< 0.016	< 0.016	< 0.016	< 0.016
34836	0.25	0.25	0.25	0.5	0.25	0.125	0.064	0.125	0.064
34837	0.25	0.5	0.25	0.5	0.25	0.25	0.125	0.25	0.25
34838	0.25	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25
34839	0.032	0.016	0.016	0.016	0.016	0.016	< 0.016	0.016	< 0.016

* Tested in AMRSTI, ▫ Tested in SACU

Cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 44: MIC results of twenty clinical isolates with ciprofloxacin gradient strips from two manufacturers on four agar plates in laboratory 1 for stage 3

		Laboratory 1 ciprofloxacin MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34819	0.016	0.004	0.008	0.008	0.008	0.008	0.008	0.008	0.008
34820	> 0.5	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
34821	> 0.5	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
34822	≤ 0.008	0.004	0.004	0.004	0.004	0.004	0.002	0.004	0.004
34823	≤ 0.008	0.002	< 0.002	0.002	0.002	< 0.002	< 0.002	< 0.002	< 0.002
34824	> 0.5	32	16	32	16	8	4	16	16
34825	> 0.5	16	8	> 32	12	16	8	> 32	> 32
34826	> 0.5	8	4	8	8	8	8	16	8
34827	> 0.5	16	8	> 32	12	16	8	16	8
34828	≤ 0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.016
34829	≤ 0.008	0.002	0.002	0.002	0.004	0.002	0.002	0.002	< 0.002
34830	≤ 0.008	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.008
34832	> 0.5	16	16	12	12	16	8	16	16
34833	≤ 0.008	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004
34834	> 0.5	1	1	1	1	2	2	2	2

Table 44: Continued

		Laboratory 1 ciprofloxacin MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox**		**BD GC chocolate agar**		**E&O FAA w/ 5% DHB**		**Liofilchem FAA**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34835	> 0.5	16	16	16	8	16	16	32	8
34836	> 0.5	16	8	8	8	16	8	16	8
34837	> 0.5	16	16	12	8	>32	> 32	> 32	> 32
34838	> 0.5	8	8	8	8	>32	16	> 32	16
34839	≤ 0.008	< 0.002	0.002	< 0.002	0.002	0.002	< 0.002	0.002	0.002

All results performed in SACU.

Cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

Table 45: MIC results of twenty clinical isolates with ciprofloxacin gradient strips from two manufacturers on four agar plates in laboratory 2 for stage 3

		Laboratory 2 ciprofloxacin MIC (mg/L)							
SACU number	**Reference result**	**Oxoid GC + vitox***		**BD GC chocolate agar***		**E&O FAA w/ 5% DHB▫**		**Liofilchem FAA▫**	
		bioMérieux	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**	**bioMérieux**	**Liofilchem**
34819	0.016	0.008	0.008	0.004	0.008	0.008	0.008	0.008	0.008
34820	> 0.5	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
34821	> 0.5	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
34822	≤ 0.008	0.004	0.004	0.004	0.008	< 0.002	0.002	0.004	0.004
34823	≤ 0.008	< 0.002	0.002	0.004	0.004	0.002	0.002	< 0.002	0.002
34824	> 0.5	16	16	16	16	12	8	16	8
34825	> 0.5	16	16	32	32	12	8	>32	8
34826	> 0.5	16	8	16	8	8	4	16	4
34827	> 0.5	> 32	> 32	> 32	> 32	12	8	16	16
34828	≤ 0.008	0.016	0.016	0.016	0.032	0.008	0.008	0.008	0.008
34829	≤ 0.008	0.002	0.004	0.004	0.004	0.002	0.004	0.002	0.002
34830	≤ 0.008	0.004	0.008	0.008	0.008	0.004	0.004	0.004	0.008
34832	> 0.5	> 32	> 32	> 32	> 32	16	16	> 32	16
34833	≤ 0.008	0.002	0.004	0.004	0.004	0.004	0.004	0.002	0.004
34834	> 0.5	1	1	2	2	2	2	2	2

Table 45: Continued

SACU number	Reference result	Laboratory 2 ciprofloxacin MIC (mg/L)							
		Oxoid GC + vitox*		BD GC chocolate agar*		E&O FAA w/ 5% DHB▫		Liofilchem FAA▫	
		bioMérieux	Liofilchem	bioMérieux	Liofilchem	bioMérieux	Liofilchem	bioMérieux	Liofilchem
34835	> 0.5	32	16	32	32	16	> 32	16	16
34836	> 0.5	16	16	32	16	16	8	8	8
34837	> 0.5	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
34838	> 0.5	> 32	> 32	> 32	> 32	16	8	> 32	16
34839	≤ 0.008	0.002	0.002	0.004	0.004	0.002	0.002	0.002	0.004

* Tested in AMRSTI, ▫ Tested in SACU

Cells highlighted in orange have a 3 x doubling dilution variance or greater across all agar plates.

3.3.6 VARIANCE OF MICS ACROSS AGAR PLATES FOR STAGE 3

When looking at tables 38 – 45 some isolates appear to have a broad range of MICs to each antimicrobial across the agar plates, highlighted in orange.

For azithromycin gradient strips, three isolates, 34822, 34829 and 34839 had a 3 x doubling dilution range of MICs in laboratory 1.

Ceftriaxone gradient strips saw a 3 x doubling dilution range of MICs in many isolates, especially in laboratory 2. One isolate in laboratory 1, 34830, had a 4 x doubling dilution range of MICs.

When testing cefixime gradient strips, one isolate, 34830 had a 4 x doubling dilution range of MICs. Four isolates, 34819, 34822, 34824, and 34836 have a 3 x doubling dilution range of MICs.

Ciprofloxacin gradient strips saw a 4 x doubling dilution range of MIC for one isolate, 34826. Five isolates had a 3 x doubling dilution range of MICs.

3.3.7 INTERPRETATION OF STAGE 3 RESULTS

As with stage 2, the clinical isolates in stage 3 were analysed according to ISO 20776 – 2 (2021), by calculating essential agreement and bias. Categorical agreement described in ISO standard 20776 – 2 (2007), will also be included.(104, 105)

3.3.8 ESSENTIAL AGREEMENT

Essential agreement was calculated for each antimicrobial agent, table 46. Essential agreement informs how well the test performs against the reference method, in this case, agar dilution. MICs performed by the test method, in this instance by gradient strip, are

deemed acceptable if the essential agreement is greater than or equal to 90 %, those below this threshold are highlighted in red in table 48.(104)

Table 46: Essential agreement between twenty clinical gradient strip MICs and reference result MICs against four agars for stage 3

	AZITHROMYCIN BIOMÉRIEUX		AZITHROMYCIN LIOFILCHEM		CEFTRIAXONE BIOMÉRIEUX		CEFTRIAXONE LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	EA	EA	EA	EA	EA	EA	EA	EA
Oxoid GC + vitox*	100 %	100 %	95 %	95 %	85 %	100 %	85 %	95 %
BD GC chocolate agar*	100 %	95 %	100 %	95 %	85 %	100 %	100 %	90 %
E&O FAA w/ 5 % DHB▫	80 %	90 %	70 %	85 %	75 %	85 %	45 %	40 %
Liofilchem FAA▫	100 %	95 %	95 %	95 %	95 %	100 %	70 %	75 %

	CEFIXIME BIOMÉRIEUX		CEFIXIME LIOFILCHEM		CIPROFLOXACIN BIOMÉRIEUX		CIPROFLOXACIN LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	EA	EA	EA	EA	EA	EA	EA	EA
Oxoid GC + vitox*	70 %	75 %	75 %	70 %	100 %	100 %	100 %	100 %
BD GC chocolate agar*	70 %	90 %	75 %	80 %	100 %	100 %	100 %	95 %
E&O FAA w/ 5 % DHB▫	75 %	60 %	50 %	60 %	100 %	100 %	100 %	100 %
Liofilchem FAA▫	70 %	70 %	60 %	55 %	100 %	100 %	100 %	100 %

*Tests performed in SACU and AMRSTI, ▫Tests performed in SACU on two separate occasions

Analysis in sections 3.3.8.1 to 3.3.8.4 refers to table 46.

3.3.8.1 Azithromycin

When tested on BD GC chocolate agar, azithromycin gradient strips from both bioMérieux and Liofilchem achieved essential agreement of 100 % in laboratory 1 and 95 % in laboratory 2 respectively.

Essential agreement was acceptable for azithromycin gradient strips from both manufacturers when tested on Oxoid GC + vitox. Essential agreement for azithromycin was 100 % in both laboratories when using bioMérieux gradient strips and 95 % in both laboratories when using Liofilchem gradient strips.

For azithromycin tested on E&O FAA with 5 % DHB, there was acceptable essential agreement in laboratory 2 when tested with bioMérieux gradient strips, 90 %, however laboratory 1 recorded 80 % essential agreement with bioMérieux gradient strips. Both laboratories recorded unacceptable essential agreement with Liofilchem azithromycin gradient strips, 70 % and 85 % in laboratory 1 and 2 respectively.

bioMérieux azithromycin gradient strips achieved 100 % essential agreement in laboratory 1 and 95 % in laboratory 2 when tested on Liofilchem FAA. Liofilchem azithromycin gradient strips when tested on Liofilchem FAA had 95 % essential agreement in both laboratories.

3.3.8.2 Ceftriaxone

Liofilchem ceftriaxone gradient strips tested on BD GC chocolate agar scored acceptable essential agreement in laboratory 1, 100 % and laboratory 2, 90 %. bioMérieux ceftriaxone gradient strips tested on the same agar plates recorded 100 % essential agreement in laboratory 2, but only 85 % in laboratory 1, falling below the acceptable range.

Essential agreement for both manufacturers of gradient strip was unacceptable when tested on Oxoid GC + vitox in laboratory 1, at 85 % for both bioMérieux and Liofilchem. Laboratory 2 recorded acceptable essential agreement at 100 % and 95 % for bioMérieux and Liofilchem gradient strips respectively.

For ceftriaxone gradient strips tested on E&O FAA with 5 % DHB essential agreement was recorded below the threshold of 90 %. bioMérieux ceftriaxone gradient strips recorded essential agreement of 75 % and 85 % in laboratory 1 and 2 respectively, however Liofilchem ceftriaxone gradient strips were particularly poor, 45 % essential agreement in laboratory 1, and 40 % essential agreement in laboratory 2.

bioMérieux ceftriaxone gradient strips tested on Liofilchem FAA recorded essential agreement of 95 % in laboratory 1 and 100 % in laboratory 2. Liofilchem gradient strips tested on Liofilchem FAA fell short of the acceptable threshold, 70 % essential agreement in laboratory 1 and 75 % in laboratory 2.

3.3.8.3 Cefixime

bioMérieux cefixime gradient strips tested on BD GC chocolate agar had an acceptable essential agreement, 90 % in laboratory 2, but with laboratory 1 scoring below the threshold at 70 % essential agreement. Liofilchem cefixime gradient strips tested on BD GC chocolate agar had unacceptable essential agreement in both laboratories, 75 % in laboratory 1 and 80 % in laboratory 2.

bioMérieux cefixime gradient strips when tested on Oxoid GC + vitox scored essential agreement of 70 % in laboratory 1 and 75 % in laboratory 2. Liofilchem cefixime gradient strips tested similarly on the same agar, 75 % in laboratory 1 and 70 % in laboratory 2. Both manufacturers of gradient strip recording essential agreement below the acceptable threshold.

Cefixime gradient strips performed poorly with E&O FAA with 5 % DHB in both laboratories, with both manufacturers of gradient strip. bioMérieux gradient strips recording essential

agreement of 75 % in laboratory 1 and 60 % in laboratory 2, whereas Liofilchem gradient strips had an essential agreement of 50 % in laboratory 1 and 60 % in laboratory 2.

Both manufacturers of gradient strip performed poorly on Liofilchem FAA. bioMérieux gradient strips performed below the acceptable essential agreement level, 70 % in both laboratory 1 and 2. Liofilchem gradient strips performing slightly worse, essential agreement 60 % in laboratory 1 and 55 % in laboratory 1.

3.3.8.4 Ciprofloxacin

Ciprofloxacin gradient strips from both manufacturers performed very well on all agar plates. bioMérieux ciprofloxacin gradient strips have an essential agreement of 100 % in laboratory 1 and laboratory 2 when tested on BD GC chocolate agar. Liofilchem gradient strips had an essential agreement of 100 % in laboratory 1 and 95 % in laboratory 2.

When tested on Oxoid GC + vitox, E&O FAA with 5 % DHB, and Liofilchem FAA, bioMérieux and Liofilchem ciprofloxacin gradient strips scored 100 % essential agreement in laboratory 1 and 2.

3.3.9 BIAS

Bias was calculated on all twenty clinical strains. ISO standard 20776 – 2 (2021) states bias greater than 30 % whether positive, or negative is unacceptable, these are shown in red in table 47. Positive bias highlighting the gradient strip MIC is greater than the gold standard reference result, and negative bias illustrating gradient strip results are lower than the reference result.(104)

Table 47: Bias of twenty clinical gradient strip MICs against reference result MICs for four agars for stage 3

	AZITHROMYCIN BIOMÉRIEUX		AZITHROMYCIN LIOFILCHEM		CEFTRIAXONE BIOMÉRIEUX		CEFTRIAXONE LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	Bias	Bias	Bias	Bias	Bias	Bias	Bias	Bias
Oxoid GC + vitox*	- 22.2 %	+ 33.3 %	- 27.8 %	+ 38.9 %	- 41.2 %	+ 15.4 %	- 41.2 %	- 1.6 %
BD GC Chocolate agar*	- 38.9 %	+ 22.2 %	- 27.8 %	+ 33.3 %	- 18.3 %	+ 20.9 %	- 35.3 %	+ 26.5 %
E&O FAA 5% DHB▫	- 16.7 %	- 5.6 %	- 16.7 %	0 %	- 83.3 %	- 88.2 %	- 88.2 %	- 100 %
Liofilchem FAA▫	+ 5.6 %	+ 27.8 %	+ 16.7 %	+ 44.4 %	- 41.2 %	- 41.8 %	- 82.4 %	- 82.4 %

	CEFIXIME BIOMÉRIEUX		CEFIXIME LIOFILCHEM		CIPROFLOXACIN BIOMÉRIEUX		CIPROFLOXACIN LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	Bias	Bias	Bias	Bias	Bias	Bias	Bias	Bias
Oxoid GC + vitox*	- 68.4 %	- 47.1 %	- 57.9 %	- 63.2 %	- 7.7 %	- 7.7 %	- 7.7 %	- 7.7 %
BD GC Chocolate agar*	- 63.2 %	- 31.0 %	- 52.6 %	- 47.4 %	- 7.7 %	- 7.7 %	- 7.7 %	+ 4.8 %
E&O FAA 5% DHB▫	- 68.4 %	- 73.7 %	- 73.7 %	- 84.2 %	- 7.7 %	- 7.7 %	- 7.7 %	- 7.7 %
Liofilchem FAA▫	- 68.4 %	- 63.2 %	- 73.7 %	- 63.4 %	- 7.7 %	- 7.7 %	+ 4.8 %	- 7.7 %

*Tests performed in SACU and AMRSTI, ▫Tests performed in SACU on two separate occasions. Cells highlighted red are below the acceptable threshold of 90 %

Analysis in sections 3.3.9.1 to 3.3.9.4 refers to table 47.

3.3.9.1 Azithromycin

Azithromycin gradient strips tested on Oxoid GC + vitox have acceptable negative bias for laboratory 1 when tested with bioMérieux gradient strips, - 22.2 % and Liofilchem gradient strips, - 27.8 %, suggesting MICs lower than the reference method. However, when tested in laboratory 2, unacceptable positive bias was noted with bioMérieux and Liofilchem gradient strips, + 33.3 % and + 38.9 % respectively, suggesting MICs greater than the reference method.

bioMérieux azithromycin gradient strips tested on BD GC chocolate agar showed positive and negative bias depending on the laboratory performing the testing. Laboratory 1 having unacceptable negative bias, - 38.9 % with bioMérieux gradient strips, and - 27.8 % with Liofilchem gradient strips. Laboratory 2 reporting acceptable positive bias with bioMérieux azithromycin gradient strips, + 22.2 % and unacceptable positive bias with Liofilchem gradient strips, + 33.3 %.

Both manufacturers of azithromycin gradient strips performed well on E&O FAA with 5 % DHB. bioMérieux gradient strips showing acceptable levels of negative bias of - 16.7 % in laboratory 1 and - 5.6 % in laboratory 2. Liofilchem gradient strips performed slightly better on E&O FAA with 5 % DHB, with - 16.7 % negative bias in laboratory 1, and 0 % bias in laboratory 2.

bioMérieux azithromycin gradient strips exhibited acceptable positive bias in both laboratories on Liofilchem FAA, + 5.6 % in laboratory 1 and + 27.8 % in laboratory 2. Laboratory 1 reported acceptable positive bias with Liofilchem azithromycin gradient strips, + 16.7, but laboratory 2 had unacceptable positive bias with Liofilchem gradient strips, + 44.4 %.

3.3.9.2 Ceftriaxone

Ceftriaxone gradient strips when tested in laboratory 1 on Oxoid GC + vitox showed unacceptable negative bias with both bioMérieux, - 41.2 % and Liofilchem, - 41.2 % gradient strips. Laboratory 2 noted acceptable positive bias with bioMérieux gradient strips, + 15.4 %, and negative bias with Liofilchem gradient strips, - 1.6 %.

Ceftriaxone gradient strips from bioMérieux in both laboratories had acceptable levels of bias when tested on BD GC chocolate agar, - 18.3 % in laboratory 1 and, + 20.9 % in laboratory 2. However, with Liofilchem gradient strips laboratory 1 reported unacceptable negative bias, - 35.3 %, but acceptable positive bias in laboratory 2, + 26.5 %.

Both manufacturers of ceftriaxone gradient strips performed poorly on E&O FAA with 5 % DHB. bioMérieux ceftriaxone gradient strips with bias of - 83.3 % in laboratory 1 and - 88.2 % in laboratory 2, and Liofilchem ceftriaxone gradient strips, - 88.2 % and - 100 %, in laboratory 1 and 2 respectively. For both ceftriaxone gradient strip manufacturers, the MICs were much lower than the reference method on E&O FAA with 5 % DHB.

Ceftriaxone gradient strips performed on Liofilchem FAA exhibited unacceptable negative bias, - 41.2 % and - 41.8 % for bioMérieux gradient strips in laboratory 1 and 2 respectively. Liofilchem gradient strips in both laboratories scored - 82.4 % bias.

3.3.9.3 Cefixime

Cefixime gradient strips from both bioMérieux and Liofilchem performed poorly on all agar plates tested with regards to bias.

Cefixime gradient strips tested on Oxoid GC + vitox reported high negative bias for both laboratories and gradient strip manufacturers, suggesting MICs much lower than the reference method. bioMérieux cefixime gradient strips reported bias - 68.4 % in laboratory 1 and, - 47.1 % in laboratory 2. Liofilchem gradient strips, - 57.9 % in laboratory 1 and - 63.2 % in laboratory 2.

bioMérieux and Liofilchem cefixime gradient strips in both laboratories reported high negative bias when tested on BD GC chocolate agar. Laboratory 1 noted bias - 63.2 % and laboratory 2, - 31.0 % for bioMérieux gradient strips. Liofilchem gradient strips showed negative bias of - 52.6 % in laboratory 1 and - 47.4 % in laboratory 2.

Both manufactures of cefixime gradient strips performed poorly on E&O FAA with 5 % DHB. bioMérieux cefixime gradient strips, - 68.4 % in laboratory 1 and - 73.7 % in laboratory 2, and Liofilchem cefixime gradient strips, - 73.7 % and - 84.2 % in laboratory 1 and 2 respectively. The gradient strip MICs were much lower than the reference method.

Cefixime gradient strips tested on Liofilchem FAA resulted in high unacceptable negative bias, - 68.4 % and - 63.2 % in laboratory 1 and 2 for bioMérieux gradient strips and - 73.7 % and - 63.4 % in laboratory 1 and 2 for Liofilchem gradient strips.

3.3.9.4 Ciprofloxacin

For ciprofloxacin, regardless of which manufacturer of gradient strip was used, or which laboratory performed the testing, very low levels of bias were seen on all agar plates. This suggests that all agars performed well, with the gradient strip MICs very close to the reference method MICs. However, the reference testing that was performed used a very narrow range of dilutions, 0.008 to 0.5 mg/L. This meant that most of the clinical strain MICs landed in the less than 0.008 mg/L or greater than 0.5mg/L categories, giving a false representation. If the reference method was repeated, for ciprofloxacin a suggested range of dilutions would be 0.002 – 32 mg/L. Thus, enabling a true calculation of bias and mimicking the range of dilutions seen on both bioMérieux and Liofilchem ciprofloxacin gradient strips.

3.3.9.5 Observations of bias of stage 3 clinical isolates

Cefixime gradient strips consistently recorded high negative bias, suggesting MICs much lower than the reference method regardless of which agar plate or gradient strip

manufacturer was used, or which laboratory performed the testing. This suggests poor correlation between the gradient strip and the reference method.

As Oxoid GC + vitox and BD GC chocolate agar were tested in separate laboratories, SACU and AMRSTI, it is worth noting that a pattern can be discerned from testing of azithromycin and ceftriaxone gradient strips regardless of the manufacturer. Laboratory 1 (SACU) consistently recorded MICs lower than the reference MIC, whereas laboratory 2 (AMRSTI) on all but one occasion consistently records gradient strip MICs higher than the reference MIC. E&O FAA with 5 % DHB and Liofilchem were tested in the same laboratory by the same person on different occasions and these results remain consistent with regards to bias, which is to be expected.

3.3.10 CATEGORICAL AGREEMENT

Categorical agreement is described in ISO standard 20776 – 2 (2007), as the calculation of whether a gradient strip MIC result yields the same categorical interpretation (i.e., susceptible, susceptible increased exposure or resistant) as the reference result, in this case agar dilution.(105)

Categorical agreement was calculated on twenty clinical isolates, seen in table 48. Acceptable categorical agreement is classed as those results with greater than 95 % concordance between the gradient strip and agar dilution reference method. Those below this cut off are highlighted in red.

Table 48: Categorical agreement between twenty clinical gradient strip MICs and reference result MICs against four agars for stage 3

	AZITHROMYCIN BIOMÉRIEUX		AZITHROMYCIN LIOFILCHEM		CEFTRIAXONE BIOMÉRIEUX		CEFTRIAXONE LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	CA	CA	CA	CA	CA	CA	CA	CA
Oxoid GC + vitox*	95 %	100 %	95 %	100 %	100 %	100 %	100 %	100 %
BD GC chocolate agar*	95 %	95 %	95 %	100 %	100 %	100 %	100 %	100 %
E&O FAA with 5 % DHB▫	95 %	95 %	95 %	95 %	100 %	95 %	100 %	100 %
Liofilchem FAA▫	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %

	CEFIXIME BIOMÉRIEUX		CEFIXIME LIOFILCHEM		CIPROFLOXACIN BIOMÉRIEUX		CIPROFLOXACIN LIOFILCHEM	
Agar plate	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method	Lab 1 v Ref method	Lab 2 v Ref method
	CA	CA	CA	CA	CA	CA	CA	CA
Oxoid GC + vitox*	75 %	85 %	80 %	80 %	100 %	100 %	100 %	100 %
BD GC chocolate agar*	80 %	90 %	85 %	85 %	100 %	100 %	100 %	100 %
E&O FAA with 5 % DHB▫	70 %	70 %	70 %	70 %	100 %	100 %	100 %	100 %
Liofilchem FAA▫	75 %	75 %	70 %	75 %	100 %	100 %	100 %	100 %

*Tests performed in SACU and AMRSTI, ▫Tests performed in SACU on two separate occasions

Cells highlighted red are below the acceptable threshold of 90 %

Acceptable categorical agreement is classed as those results of greater than or equal to 95 % where the MIC is in concordance with the reference MIC, those below this cut off are highlighted in red. Categorical agreement takes into consideration major errors (false

resistance) and very major errors (false susceptibility). Of the clinical isolates tested, six were resistant to azithromycin, two were resistant to ceftriaxone, nine were resistant to cefixime, and twelve were resistant to ciprofloxacin by the reference method according to version 15 of the EUCAST breakpoint tables.(39)

Analysis in sections 3.3.10.1 to 3.3.10.4 refers to table 48.

3.3.10.1 Azithromycin

Azithromycin gradient strips tested on Oxoid GC + vitox scored 95 % categorical agreement in laboratory 1 for both bioMérieux and Liofilchem gradient strips, with one very major error recorded for each manufacturer. Laboratory 2 recorded 100 % categorical agreement for bioMérieux and Liofilchem gradient strips.

bioMérieux azithromycin gradient strips reported 95 % categorical agreement in laboratory 1 and laboratory 2 when tested on BD GC chocolate agar, with one very major error in each laboratory. Liofilchem azithromycin gradient strips scored 95 % categorical agreement in laboratory 1, with one very major error, and 100 % categorical agreement in laboratory 2.

Azithromycin gradient strips tested on E&O FAA with 5 % DHB reported categorical agreement of 95 % for both laboratories and both gradient strips manufacturer. One very major error recorded for each gradient strip manufacturer and laboratory.

Tested on Liofilchem FAA, bioMérieux and Liofilchem gradient strips scored 100 % categorical agreement for both laboratories.

3.3.10.2 Ceftriaxone

Ceftriaxone gradient strips from bioMérieux and Liofilchem in laboratory 1 and 2 achieved 100 % categorical agreement for the following agar plates: Oxoid GC + vitox, BD GC chocolate agar and Liofilchem FAA.

Liofilchem ceftriaxone gradient strips had 100 % categorical agreement in both laboratories for E&O FAA with 5 % DHB. In laboratory 1 bioMérieux gradient strips had 100 % categorical agreement, and laboratory 2 had 95 % categorical agreement, with one very major error for E&O FAA with 5 % DHB.

3.3.10.3 Cefixime

When tested with cefixime gradient strips, the categorical agreement was poor for all agars, in both laboratories with bioMérieux and Liofilchem gradient strips.

When tested on Oxoid GC + vitox, bioMérieux cefixime gradient strips reported categorical agreement of 75 % in laboratory 1 and 85 % in laboratory 2, with five and three very major errors respectively. Liofilchem cefixime gradient strips had 80 % categorical agreement in both laboratories, with four very major errors in each laboratory.

Liofilchem gradient strips tested on BD GC chocolate agar had categorical agreement of 85 % in laboratory 1 and 2, with three very major errors in each laboratory. Whereas bioMérieux cefixime gradient strips had a categorical error of 80 % in laboratory 1, with 4 very major errors and 90 % in laboratory 2, with 2 very major errors.

Cefixime gradient strips from both bioMérieux and Liofilchem performed poorly on E&O FAA with 5 % DHB, 70 % categorical agreement for both laboratories, six very major errors in each laboratory.

bioMérieux cefixime gradient strips tested on Liofilchem FAA recorded categorial agreement of 75 % in laboratory 1 and 2, 5 very major errors in each laboratory. Liofilchem gradient

strips tested on Liofilchem FAA had categorical agreement of 70 % in laboratory 1, six very major errors, and 75 % in laboratory 2, 5 very major errors.

3.3.10.4 Ciprofloxacin

Ciprofloxacin gradient strips from bioMérieux and Liofilchem reported categorical agreement of 100 % across the board for laboratory 1 and 2 on Oxoid GC + vitox, BD Gc chocolate, E&O FAA with 5 % DHB and Liofilchem FAA. There were zero very major errors across the board.

3.3.11 SUMMARY OF STAGE 3 RESULTS

To calculate the overall best performing agar plate and gradient strip manufacturer, the overall essential agreement and categorical agreement for gradient strip manufacturers and agar plates must be calculated, see tables 49 to 52 for the combined percentages.

The cells highlighted in green identify the highest combined percentage for each manufacturer and gradient strip combination, and each agar plate.

Table 49: Overall essential agreement for gradient strip manufacturers for stage 3

AZITHROMYCIN BIOMÉRIEUX	AZITHROMYCIN LIOFILCHEM	CEFTRIAXONE BIOMÉRIEUX	CEFTRIAXONE LIOFILCHEM
EA	EA	EA	EA
95 %	91.9 %	90.6 %	75 %

CEFIXIME BIOMÉRIEUX	CEFIXIME LIOFILCHEM	CIPROFLOXACIN BIOMÉRIEUX	CIPROFLOXACIN LIOFILCHEM
EA	EA	EA	EA
72.5 %	65.6%	100 %	99.4 %

As seen in table 49, bioMérieux gradient strips, percentages highlighted in green, had the best essential agreement for each of the antimicrobials tested.

Table 50: Overall categorical agreement for gradient strip manufacturers for stage 3

AZITHROMYCIN BIOMÉRIEUX	AZITHROMYCIN LIOFILCHEM	CEFTRIAXONE BIOMÉRIEUX	CEFTRIAXONE LIOFILCHEM
CA	CA	CA	CA
97.5 %	97.5 %	99.4 %	100 %

CEFIXIME BIOMÉRIEUX	CEFIXIME LIOFILCHEM	CIPROFLOXACIN BIOMÉRIEUX	CIPROFLOXACIN LIOFILCHEM
CA	CA	CA	CA
77.5 %	76.9 %	100 %	100 %

Overall categorical agreement does not distinguish the gradient strip manufacturers, with both performing the same for azithromycin, 97.5 % and ciprofloxacin, 100 %. bioMérieux

performing better for cefixime gradient strips, and Liofilchem performing better for ceftriaxone gradient strips.

Table 51: Overall essential agreement for four agar plates for stage 3

Agar plate	Total EA
Oxoid GC + vitox	90.3 %
BD GC Chocolate agar	92.2 %
E&O FAA 5 % DHB	75.9 %
Liofilchem FAA	86.6 %

Table 52: Overall categorical agreement for four agar plates for stage 3

Agar plate	Total CA
Oxoid GC + vitox	94.4 %
BD GC Chocolate agar	95.6 %
E&O FAA 5 % DHB	90.9 %
Liofilchem FAA	93.4 %

As seen in table 51 and 52, BD GC chocolate agar had the best overall essential agreement, 92.2 %, and categorical agreement, 95.6 %.

Considering overall essential and categorical agreement calculations of clinical strains shown in tables 49 – 52, the recommended agar and gradient strip manufacturer combination for susceptibility testing of *N. gonorrhoeae* are BD GC chocolate agar and bioMérieux gradient strips.

Taking in to account the best performing agar plates and gradient strips for quality control and clinical strains, the recommendation is either Oxoid GC + vitox or Becton Dickinson GC chocolate agar in combination with bioMérieux gradient strips.

CHAPTER 4 –

COMPARISON OF LOCAL CARDIFF *N. GONORRHOEAE* DATA TO THE UK-WIDE DATA FROM THE GONOCOCCAL RESISTANCE TO ANTIMICROBIALS SURVEILLANCE PROGRAMME (GRASP)

Susceptibility testing is an important part of clinical microbiology as is informs the treatment of patients and on resistance trends present in the community. Throughout this MPhil project I have learnt about the increase in *N. gonorrhoeae* infections globally and in turn the increase of antimicrobial resistance in *N. gonorrhoeae* in circulation. Therefore, I decided to use data from our routine diagnostic microbiology laboratory here in Cardiff to see how our increased prevalence compares with the UK as a whole and whether we were also seeing increased antimicrobial resistance in *N. gonorrhoeae* locally.

4.1 BACKGROUND

Prevalence of *N. gonorrhoeae* infection has increased in recent years globally, and the same has been seen here locally in Cardiff.(4) As GRASP routinely reports on the prevalence of infection with *N. gonorrhoeae* and antimicrobial resistance of *N. gonorrhoeae* each year, this data was compared to the prevalence of infection and antimicrobial resistance of *N. gonorrhoeae* in Cardiff to see how it compares to the rest of the UK.

GRASP is a surveillance project investigating *N. gonorrhoeae* infections in the UK. It is a collaboration between the UK Health Security Agency (UKHSA), the Antimicrobial Resistance in STIs (AMRSTI) section of the Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit and sexual health clinics based in Wales and England. GRASP collects *N. gonorrhoeae* isolates from sexual health clinics in Wales and England, usually during July to September, and has been running since 2000. GRASP also collects patient demographic, clinical, and behavioural information. The aim of the programme is to monitor antimicrobial susceptibility trends and how it changes over time, identifying links between resistance and patient demographic and using this information to inform national treatment guidelines in the UK.(51)

In the participating laboratories, *N. gonorrhoeae* isolates are collected from patient samples, stored on cryogenic beads at – 80 °C at participating laboratories in Wales and England until the end of the collection period when they are then shipped on dry ice by courier to UKHSA for analysis. Patient information is also collected along with the samples such as gender, age,

ethnicity, and sexual orientation, amongst others. Additional patient information such as site of infection, presence of symptoms, previous gonorrhoea infection, and HIV status is gathered from patient medical records, thus enhancing the data.(51, 102)

The 2023 GRASP report includes isolates collected between 1st July and 30th September 2022, and the 2024 GRASP report includes isolates collected between 1st July and 31st August 2023.(51, 58)

4.2 COMPARING CARDIFF PREVALENCE OF *N. GONORRHOEAE* DATA TO GRASP REPORTS IN 2023 AND 2024

Local Cardiff data on *N. gonorrhoeae* isolates was downloaded from our local Datastore program for any patient seen at the Cardiff Royal Infirmary (CRI) Genitourinary Medicine (GUM) clinic in 2022 and 2023 that tested positive for *N. gonorrhoeae*. From that dataset there were 637 patients with a positive *N gonorrhoeae* result in 2022 compared to 787 in 2023. Of these, 402 (63.1 %) of these cases were from male patients in 2022, and 469 (59.6 %) in 2023 seen in figures 5 and 6. This is compared to 1,147 (78.6 %) male patients in the GRASP 2023 report and 1,762 (77 %) in the GRASP 2024 report.(51, 58)

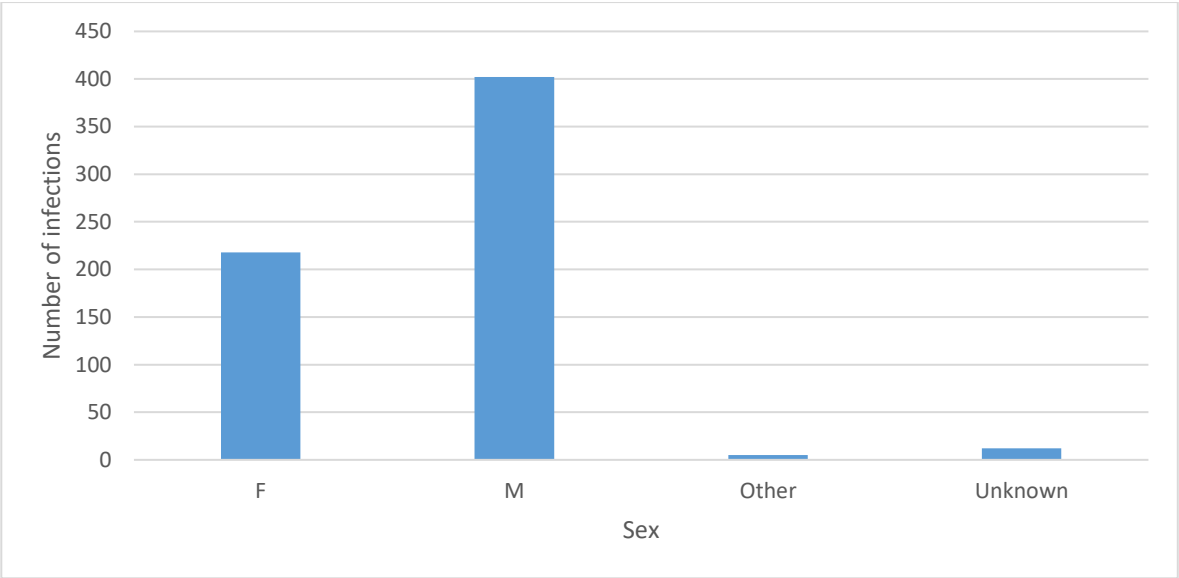


Figure 5: Distribution of *N. gonorrhoeae* infections by sex in Cardiff in 2022.

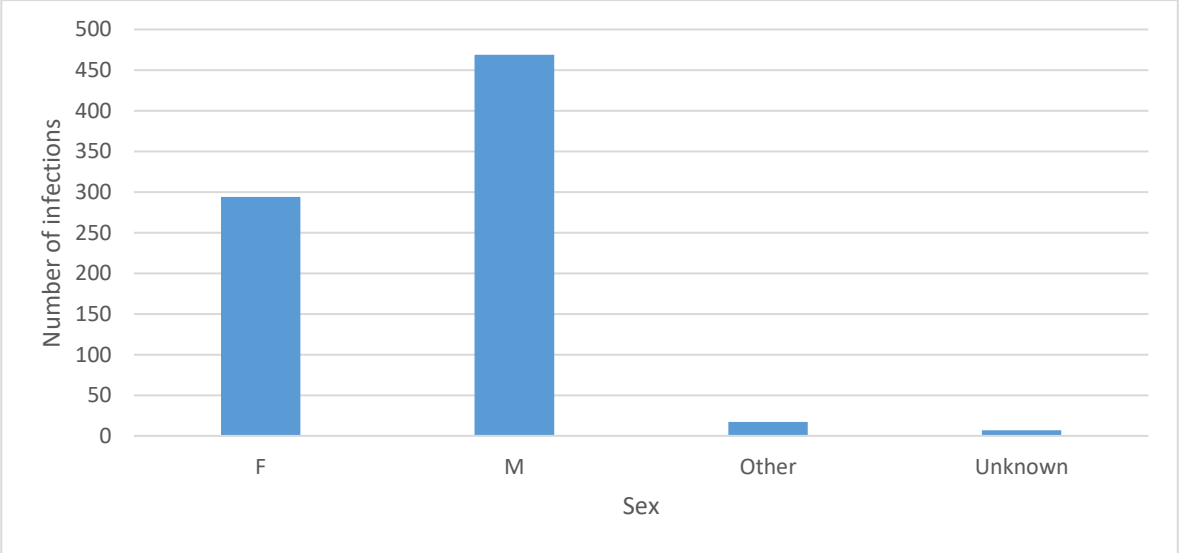


Figure 6: Distribution of *N. gonorrhoeae* infections by sex in Cardiff in 2023.

The GRASP report looks at the modal age group for men and women, this is the age group with the highest incidence of *N. gonorrhoeae* infection. GRASP 2024 reports the modal age group for men was 25 to 35 years, and for women the modal age was 25 to 34 years. Seen in figure 7, the modal age group for men in Cardiff in 2023 was 25 to 34 years, the same as the

GRASP 2024 report. However, the modal age group for women was lower in Cardiff, at 20 to 24 years of age, compared to the GRASP 2024 report. In Cardiff in 2023, the ages of individuals infected with *N. gonorrhoeae* ranged from 14 to 76 years, very similar to the GRASP 2024 report which was 15 to 73 years.(51)

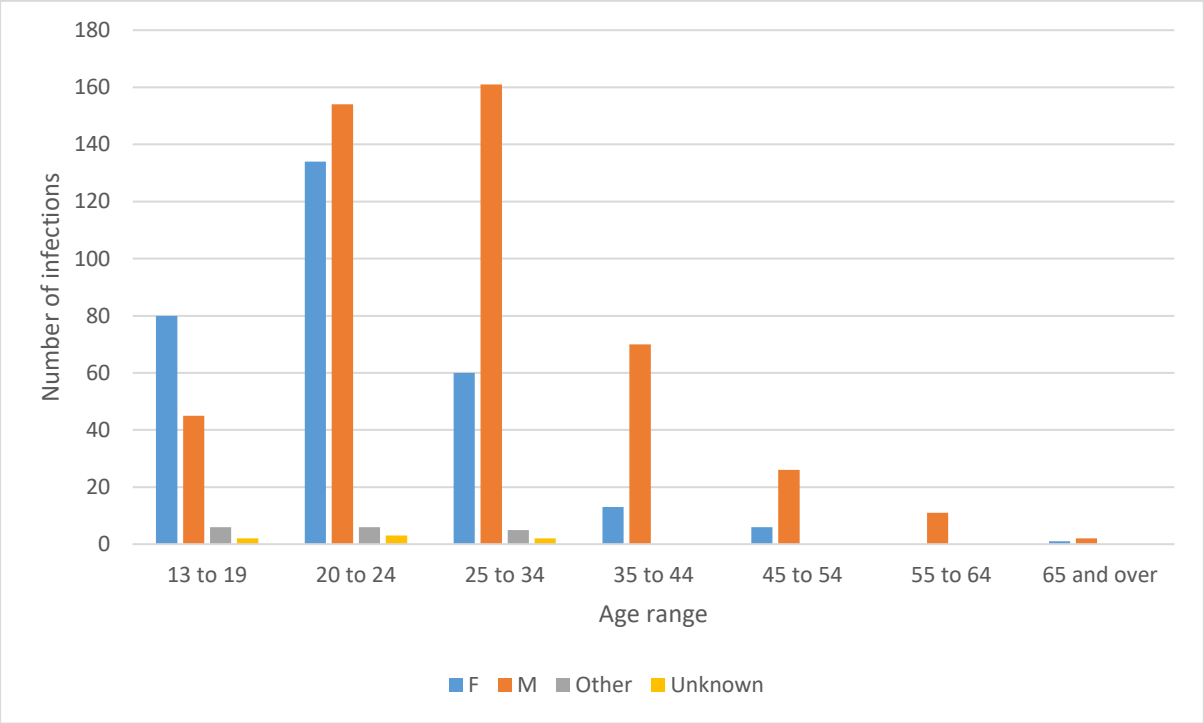


Figure 7: Distribution of *N. gonorrhoeae* infections in Cardiff in 2023 by age range and sex.

During routine diagnosis of *N. gonorrhoeae* infections at the Cardiff Royal Infirmary GUM clinic patient information such as ethnicity, sexual orientation of the patient or HIV status is not recorded onto the microbiology report, therefore these data could not be compared to data points in the GRASP 2023 and 2024 reports.

4.3 ANTIMICROBIAL RESISTANCE OF *N. GONORRHOEAE* IN CARDIFF COMPARED TO GRASP

The following graph, figure 8, shows the percentage resistance to azithromycin, ceftriaxone and ciprofloxacin for years 2018 to 2023 in Cardiff and GRASP.

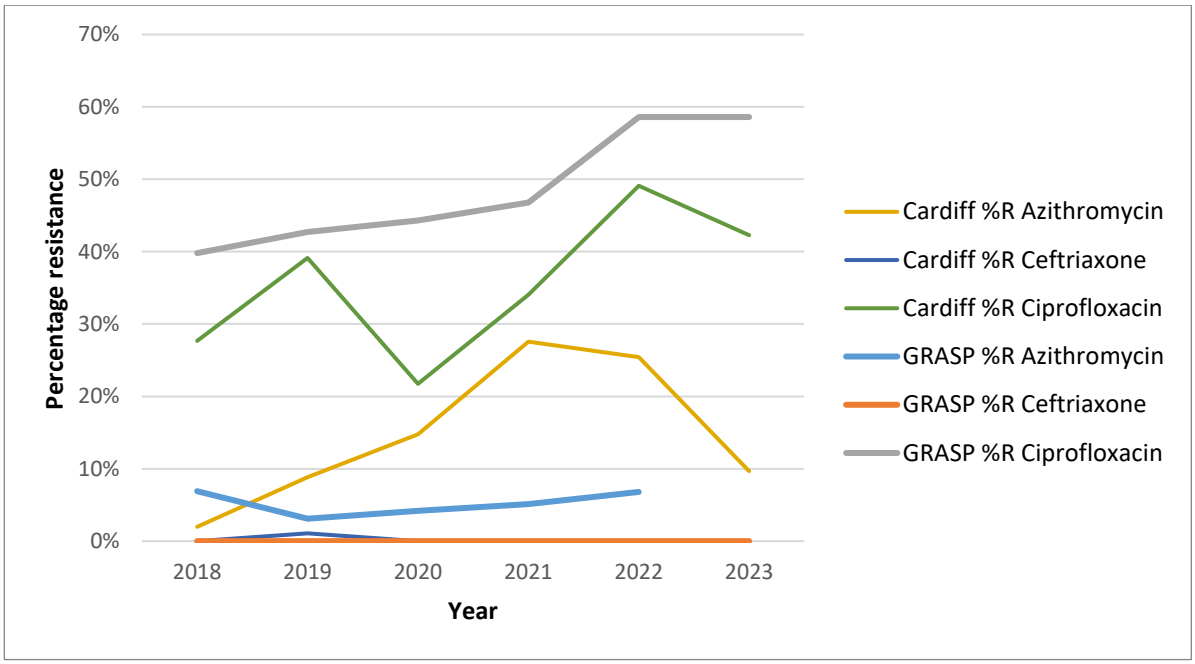


Figure 8: Percentage antimicrobial resistance prevalent in Cardiff and reported in GRASP, 2018 – 2023 to azithromycin, ceftriaxone, and ciprofloxacin.

Ceftriaxone resistance was rarely seen in Cardiff during the period 2018 - 2023, there was a small increase in 2019 where 1 % of the isolates received that year tested resistant by a gradient strip susceptibility test, figure 8. This is reassuring as ceftriaxone is the current first line treatment when the susceptibility test result is unknown for patients in Wales. Since 2019 the percentage resistance has returned to zero. This is in line with what is seen nationally from the most recent GRASP report for the same period, up to 2023 seen in figure 8.(51)

Ciprofloxacin resistance in Cardiff has been on an upward trajectory since 2019 seen in figure 8, with a decrease in 2020, probably due to a decrease in samples being taken and tested during the COVID-19 pandemic. Since 2020, ciprofloxacin resistance has peaked at almost 50 % in 2022 but recently has begun to drop in 2023 to 42 %. This is a slight change to what is seen in GRASP, figure 8, as ciprofloxacin resistance nationally has increased to almost 60 % in 2023 from 40 % in 2018.(51) This high resistance rate is the reason ciprofloxacin was not given as first line treatment, as it would fail in almost half of the patients treated. Ciprofloxacin was recommended as treatment of *N. gonorrhoeae* only if a susceptibility test has been performed and the result is susceptible, until a change in the BASHH guidance stated there were safety concerns attributed to this treatment. The updated BASHH guidance states that ciprofloxacin must only be used if clinically appropriate.(12, 46) Ceftriaxone is the first-line treatment empirically as there is currently zero or very low resistance observed in the UK currently.(51)

Azithromycin resistance increased from 2 % in 2018 up to 28 % in 2021 in Cardiff shown in figure 8, this is concerning as an increase of almost 30 % translates to potential treatment failures in a third of all patients treated in Cardiff. Azithromycin was the recommended first-line treatment for *N. gonorrhoeae* until 2018 when the prescribing guidelines were changed in favour of ceftriaxone. This could account for the decrease in resistance seen in Cardiff after 2021, as there is often a lag between a change of prescribing guidelines, the implementation of said guidelines and development or loss of resistance. Azithromycin resistance in Cardiff decreases to below 10 % in 2023. The most recent GRASP 2024 report shown in figure 8 differs from what is seen in Cardiff where azithromycin resistance stays steady at around 10 % from 2018 to 2020 where it then increases up to around 20 % in 2022.(51)

It should be noted that the most recent GRASP report details a potential error in their agar dilution method for MIC testing of azithromycin. The results as far back as 2018 are currently being checked and this could account for the discrepancy between Cardiff and the wider UK azithromycin resistance of *N. gonorrhoeae*.(51)

4.4 NUMBER OF *N. GONORRHOEAE* ISOLATED IN CARDIFF 2018 TO 2023

As reported in recent news, there has been a notable increase in the number of *N. gonorrhoeae* infections in the UK. With over 82,000 cases seen in England in 2022 which is an increase of greater than 50 % on the previous year, this is a cause of concern for the UK as a whole.(106) As seen in figure 9, in Cardiff we have also seen a notable increase in the number of *N. gonorrhoeae* infections since 2018 (254 cases). The number of cases decreased in 2020 to 184 cases, possibly as a result of the COVID-19 pandemic and returning to pre-pandemic numbers in 2021 (287 cases). However, since 2021 we have seen a dramatic increase of 174 % compared to 2023 (787 cases).

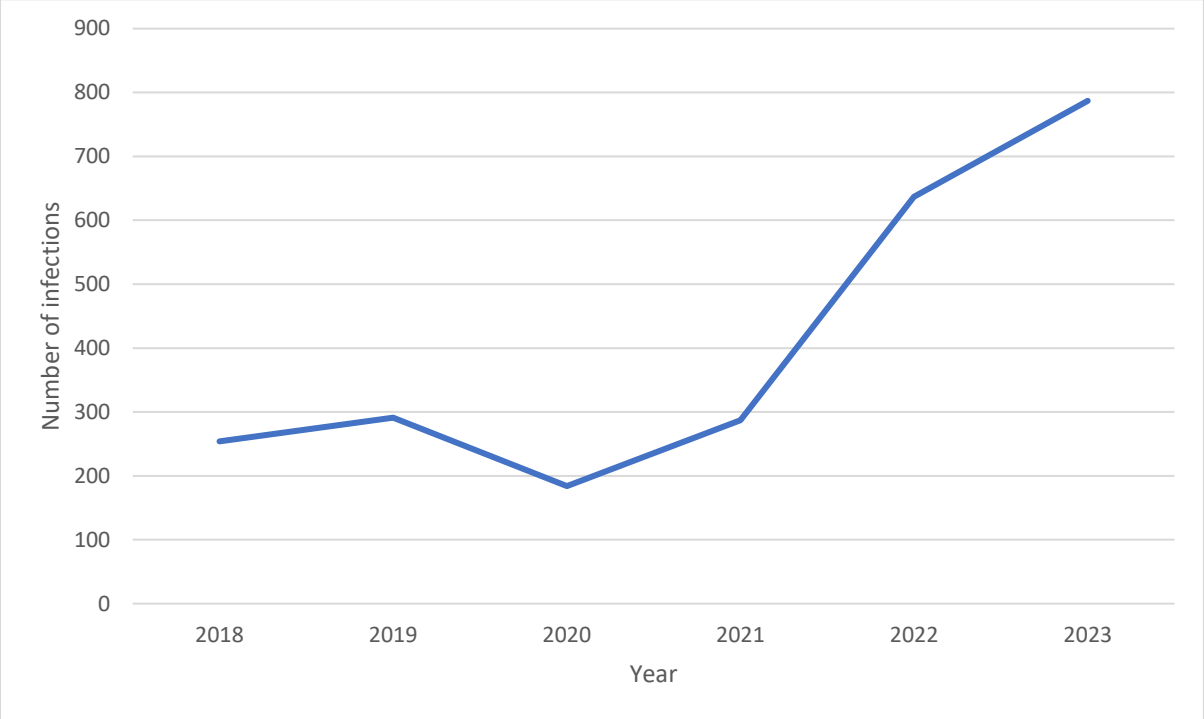


Figure 9: Distribution of *N. gonorrhoeae* isolated in Cardiff from 2018 to 2023.

In recent years the number of *N. gonorrhoeae* infections has greatly increased for women, over 320 %, from 69 cases in 2021 to 294 cases in 2023. Infections in men has also seen a considerable increase of 115 %, from 218 cases in 2018 to 469 cases in 2023, figure 10.

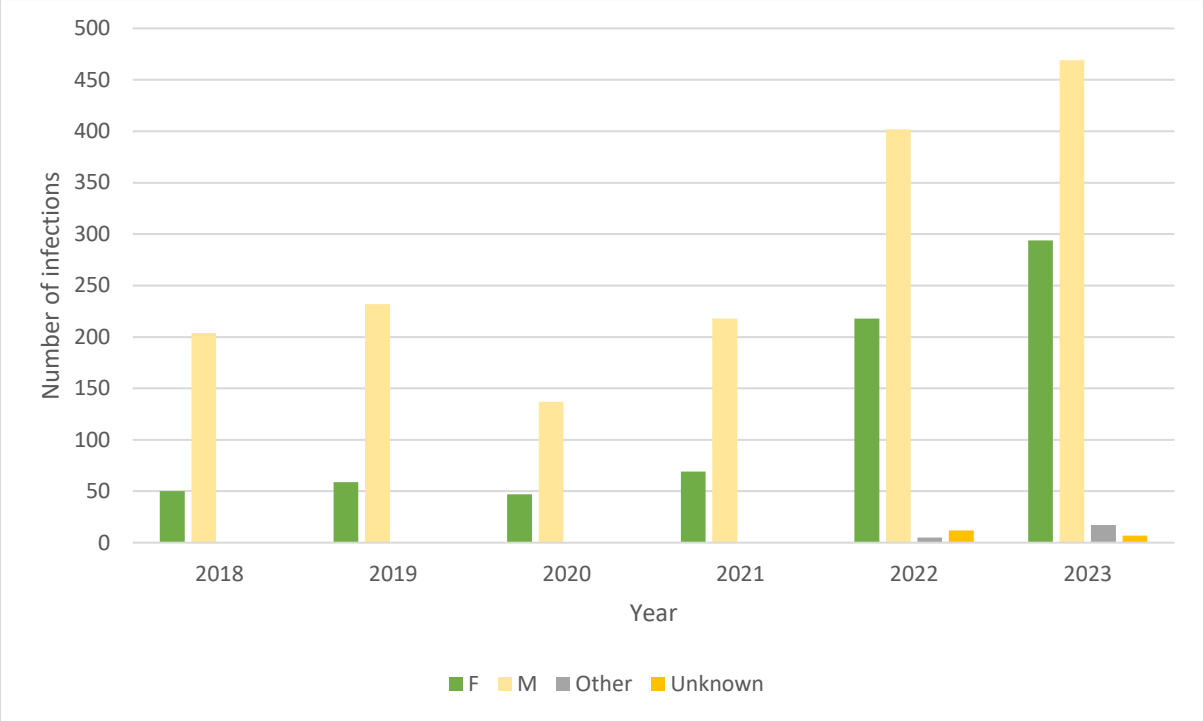


Figure 10: Distribution of *N. gonorrhoeae* infections in Cardiff by sex from 2018 to 2023.

As GRASP only captures data from 2 or 3 months each year there is no data to compare the total number of infections UK wide from GRASP.

4.5 WHOLE GENOME SEQUENCING OF *N. GONORRHOEAE* IN CARDIFF

Unfortunately, *N. gonorrhoeae* isolates that are sent to the Microbiology diagnostic laboratory in Cardiff are not routinely whole genome sequenced, as there is no immediate or direct benefit to the patient and these tests are costly. Therefore, there cannot be a comparison made between the sequence types seen in Cardiff and those that were in circulation in Wales and England as identified by GRASP. A request was made to UKHSA/AMRSTI for the Cardiff sequencing data from isolates submitted to GRASP 2018 - 2023, but due to staffing issues they were not able to share data at this time.

4.6 SUMMARY OF *N. GONORRHOEAE* PRESENT IN CARDIFF

The increasing number of *N. gonorrhoeae* infections in Cardiff, particularly post covid correlates with the wider UK and reinforces the importance of a standardised susceptibility test for accurate results. Clinicians and laboratory staff require a robust susceptibility testing method so any increase of antimicrobial resistance to the first- and second-line treatment options for *N. gonorrhoeae* is highlighted in a timely manner. Not treating *N. gonorrhoeae* infections promptly and effectively means that infections can spread easily. A quick, easy and accurate susceptibility test is essential to effectively reduce *N. gonorrhoeae* infections in the community.

CHAPTER 5 –

DIFFERING AZITHROMYCIN MINIMUM INHIBITORY CONCENTRATIONS DEPENDING ON MANUFACTURER OF GRADIENT STRIP SEEN IN WALES

5.1 BACKGROUND

The Specialist Antimicrobial Chemotherapy Unit (SACU), based at the University Hospital of Wales (UHW) in Cardiff, UK is an antimicrobial resistance reference laboratory providing susceptibility testing and antimicrobial resistance gene detection to referring diagnostic laboratories across Wales. As part of the service, laboratories in Wales can refer any aerobic bacteria with an unusual resistance to SACU, where resistance can be confirmed and an alternative therapy tested. In 2021 there appeared to be an increase in high-level azithromycin resistant *N. gonorrhoeae* in Wales. To investigate, SACU asked all Welsh laboratories to submit any azithromycin high-level resistant *N. gonorrhoeae* isolates (those with an MIC of > 256 mg/L) to SACU, where the result could be confirmed.

All laboratories in Wales use the same methods to susceptibility test bacteria, providing consistent results throughout Wales. For *N. gonorrhoeae,* laboratories currently use the gradient strip method for susceptibility testing, using either Beckton Dickinson GC chocolate or Oxoid GC + vitox agar plates as per this study's findings. In SACU, bioMérieux azithromycin gradient strips are used on Oxoid GC + vitox agar plates.

Between 2021 and 2022, four *N. gonorrhoeae* isolates from the Microbiology department at UHW, Cardiff were referred to SACU. All four had MICs to azithromycin greater than 256 mg/L (high-level resistant) in the local laboratory. However, when tested in SACU the MICs were much lower, between 8 and 16 mg/L, MICs greater than 1 mg/L to 256 mg/L are said to be low-level resistant. After investigation, it was discovered that the referring laboratories across Wales, including UHW use azithromycin MTS gradient strips from Liofilchem for susceptibility testing of *N. gonorrhoeae*, whereas SACU uses azithromycin Etest gradient strips from bioMérieux.

SACU uses azithromycin gradient strips from bioMérieux, this is the same as the *N. gonorrhoeae* reference laboratory in London, AMRSTI. Upon further investigation it was found that AMRSTI also noted a discrepancy between manufacturers, with azithromycin MTS from Liofilchem giving much higher MICs than Etest from bioMérieux for certain strains of *N. gonorrhoeae*.(94) As SACU and AMRSTI perform the same AST method for *N. gonorrhoeae* it was thought that the lower of the MICs seen in SACU using bioMérieux Etest was the correct

result, making these isolates low-level resistant to azithromycin rather than high-level resistant.

Through research for this MPhil, it was discovered that there may be a way to definitively say if these isolates were high or low-level resistant to azithromycin. By utilising whole genome sequencing we can identify whether there are genes or mutations present causing different levels of azithromycin resistance and confirm whether the MICs performed in SACU using bioMérieux gradient strips were correct. As discussed in Chapter 1 – Introduction, if the isolates were truly high-level resistant we could expect to see mutation A2059G (*Escherichia coli* numbering) in at least three out of four copies of the 23S rRNA, as this can cause azithromycin MICs to be > 256 mg/L.(55, 60) If the isolates were low-level resistant to azithromycin then in the sequences we could expect to see any of the following: mutation C2611T (*E. coli* numbering) in the peptidyl transferase loop of domain V of the 23S rRNA, mutation of *mtrR, mtrD* or in the promoter region of the efflux pump causing overexpression of the efflux pump, or the presence of *ermB* and *ermF* which modify the 23S rRNA binding site.(59-62)

All azithromycin MICs were interpreted using the current EUCAST breakpoint tables.(39)

5.2 INVESTIGATION – PART 1

The preliminary investigation was to repeat the azithromycin MICs using gradient strips from both manufacturers to confirm the differing results. The strains were taken from storage at -80 °C, subcultured and incubated at 35 to 37 °C overnight in 5 to 10 % CO_2. From the overnight culture a McFarland 0.5 inoculum prepared in Mueller Hinton Broth was spread onto GC + vitox (Oxoid, Thermo Fisher), and azithromycin gradient strips applied (described in materials and methods, chapter 2). Both manufacturers of azithromycin gradient strip were placed on the same Oxoid GC + vitox plate for each isolate to minimise variability.

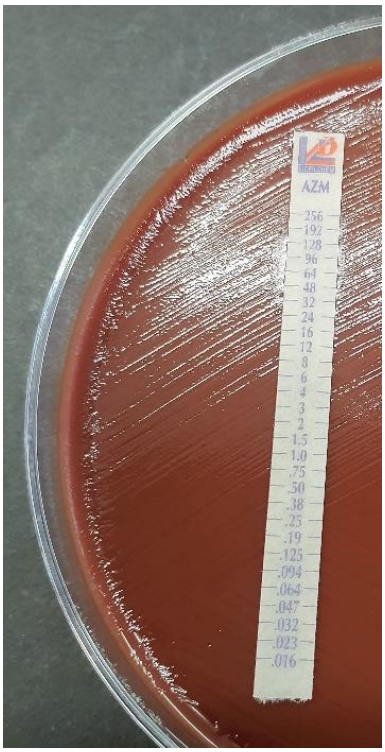
The MIC results obtained by the referring laboratories and the initial investigation by SACU can be seen in table 53.

Table 53: Original azithromycin MICs from referring laboratories and the initial investigation MICs for four strains tested with Liofilchem and bioMérieux gradient strips

SACU number	Original Liofilchem Azithromycin MIC	SACU bioMérieux Azithromycin MIC	Repeat Liofilchem Azithromycin MIC	Repeat bioMérieux Azithromycin MIC
37829	> 256 mg/L	8 mg/L	24 mg/L	12 mg/L
38093	> 256 mg/L	16 mg/L	> 256 mg/L	16 mg/L
38094	> 256 mg/L	12 mg/L	> 256 mg/L	16 mg/L
38688	> 256 mg/L	8 mg/L	> 256 mg/L	12 mg/L

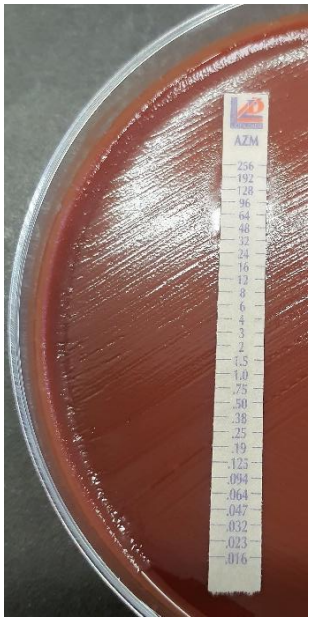
As can be seen in table 53, the repeat azithromycin MIC for isolate 37829 was 24 mg/L with the Liofilchem gradient strips, which is only 1 x doubling dilution higher than the bioMérieux gradient strip at 12 mg/L. Isolate 37829 is no longer showing high-level resistance (> 256 mg/L) with the Liofilchem gradient strip and no discrepancy between gradient strip manufacturers. An explanation for this is that the discrepancy could have been due to a reading error in the referring laboratory, or perhaps the inoculum was too high when the isolate was tested, resulting in a higher MIC. As the MIC is consistent between the manufacturers it was decided that we would not sequence this isolate.

The remaining strains, 38093, 38094 and 38688, all gave the same MIC results to the original MICs (> 256 mg/L), when tested with Liofilchem gradient strips, and between 12 and 16 mg/L when tested with bioMérieux gradient strips. This is illustrated in the pictures shown in figure 11.



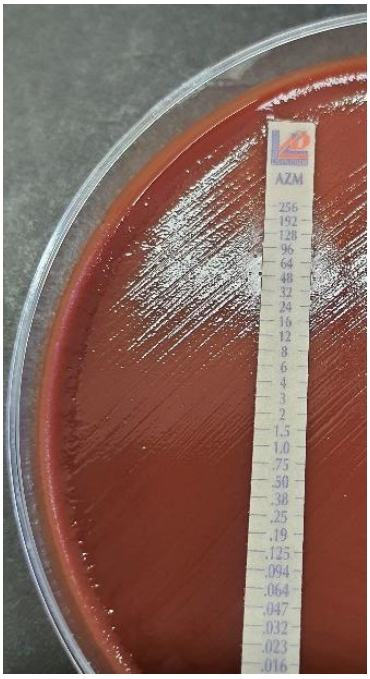
38093 Liofilchem MTS

38093 bioMérieux Etest



38094 Liofilchem MTS

38094 bioMérieux Etest



38688 Liofilchem MTS 38688 bioMérieux Etest

Figure 11: Three strains of *N. gonorrhoeae*, 38093, 38094 and 38688, tested with azithromycin gradient strips from Liofilchem and bioMérieux.

As seen in figure 11, there was a clear difference of the ellipse of inhibition between azithromycin gradient strips from bioMérieux Etest and Liofilchem MTS.

As the three isolates of *N. gonorrhoeae* 38093, 38094 and 38688, maintained the phenotypic MIC discrepancy when tested with different gradient strips, they were processed for whole genome sequencing (WGS), as described below.(107)

5.2.1 A BRIEF DESCRIPTION OF THE WGS METHOD

A colleague from SACU, Massimo Mentasti and the team at the Pathogen Genomics Unit (PENGU) kindly performed the WGS on my behalf as described below.

In SACU isolates stored on frozen beads at -80 °C were cultured onto Oxoid GC + vitox agar plates and incubated at 35 to 37 °C in 5 to 10 % CO_2 overnight. 1 µL loop of each isolate was emulsified into 190 µL of lysis buffer and 10 µL proteinase K and incubated for 30 minutes at 56 °C, then sent to colleagues in PENGU for DNA extraction using the EMAG platform (bioMérieux) following manufacturers' instructions. Extracted DNA was then quantified and normalised; libraries were then prepared using the Nextera Kit (Illumina), quantified and then loaded onto a MiSeq platform (Illumina) for sequencing.(107) Once sequenced the assembled genomes of the three isolates were uploaded onto the Resfinder website (http://genepi.food.dtu.dk/resfinder) to identify presence of azithromycin resistance markers. ResFinder is a free online resource that enables identification of antimicrobial resistance genes or point mutations in assembled genomes.

Table 54: Results of repeat azithromycin MICs performed and WGS results

SACU number	Repeat Liofilchem Azithromycin MIC	Repeat bioMérieux Azithromycin MIC	Sequence type	Resistance genes/mutations
38093	> 256 mg/L	16 mg/L	1901	C2611T
38094	> 256 mg/L	16 mg/L	1901	C2611T
38688	> 256 mg/L	12 mg/L	11422	C2611T

Illustrated in table 54 ResFinder did not identify any high-level azithromycin resistance genes or mutations in any of the isolates. However, the C2611T mutation in the 23S Ribosomal DNA associated with low-level azithromycin resistance was identified in all three isolates, confirming the bioMérieux Etest results.(61)

The WGS also enabled identification of the sequence types (STs) for the isolates. Knowing the sequence type can identify which strains are circulating in our local community, and whether they have any antimicrobial resistance. With 38093 and 38094 having an identical sequence type, the demographics of the patients were investigated. As these sample types are anonymised it is not possible to know whether the two patients were from the same

geographical area, what is known is that these strains were both isolated from cervical swabs from two different female patients on the same day. The third isolate, 38688 was an oral pharyngeal swab from a male patient taken three months later.

5.3 INVESTIGATION – PART 2

To further prove that WGS is a reliable tool for resolving susceptibility testing anomalies it was decided that a selection of WHO quality control strains with known resistance markers and MICs and clinical strains with known reference MICs that were tested in stage 3 of the project would be sequenced to verify the investigation. WHO quality control strains M, P, V and Y, and clinical strains 34826, 34828, 34829 and 34830 were chosen as these strains give a range of susceptibilities to azithromycin, susceptible, low-level resistant (LLR) and high-level resistant (HLR).

These isolates were processed in the same way for WGS as the isolates in part 1 of the investigation by colleagues in SACU and PENGU, and the assembled genomes uploaded to ResFinder. Table 55 details the MICs, susceptibility, sequence type and resistance genes/mutations identified for each isolate.

Table 55: WHO and clinical strains sequenced

SACU number	WHO quality control / clinical strain	Azithromycin reference MIC	Azithromycin interpretation	Sequence type	Azithromycin resistance genes or mutations
34811	WHO M	0.25 mg/L	Susceptible	7367	N/A
34812	WHO P	4 mg/L	LLR	8127	*mtrR* and *mtrD*
34814	WHO V	> 256 mg/L	HLR	10314	A2059G
34817	WHO Y	1 mg/L	Susceptible	1901	N/A
34826	Clinical strain	0.5 mg/L	Susceptible	7360	N/A
34828	Clinical strain	2 mg/L	LLR	9363	*mtrR* and *mtrD*
34829	Clinical strain	8 mg/L	LLR	9363	C2611T
34830	Clinical strain	> 256 mg/L	HLR	1580	A2059G

Seen in table 55, as expected for the susceptible isolates 34811, 34817, and 34826, the sequences did not contain any resistance genes or mutations associated with either low-level or high-level azithromycin resistance.

For isolates 34812 and 34828, sequences showed carriage of *mtrR* and *mtrD* mutations confirming the low-level azithromycin resistance MICs.(60, 62)

High-level azithromycin resistant isolates 34814 and 34830 were identified as having a 23S rRNA A2059G mutation which is associated with high-level azithromycin resistance.(60)

Isolate 34829 has a 23S rRNA C2611T mutation associated with low-level azithromycin resistance. The ResFinder output detailed the mutations present in the four 23S rRNA alleles for isolate 34829. It should be noted that these mutations are different numbers from the C2611T mutation, with copy 1 C2604T, copy 2 C2603T, copy 3 C2603T and copy 4 C2610T. This is because the numbering system is based on *Escherichia coli* numbering and the ResFinder output details the *N. gonorrhoeae* numbering. Nomenclature discrepancies are a common issue in WGS.(61)

5.4 OUTCOMES OF INVESTIGATION

These whole genome sequencing results corroborate the initial conclusions from part 1 of the investigation, showing that the bioMérieux Etest result gives the correct MIC and the Liofilchem MTS result of > 256 mg/L is incorrect. The incorrect Liofilchem MTS gradient strip results have patient implications, a high-level resistant azithromycin result would mean this antimicrobial would not be considered a treatment option, whereas azithromycin can still be used in combination to treat low-level *N. gonorrhoeae* strains.

It should be noted that both manufacturers of gradient strip, bioMérieux and Liofilchem have removed azithromycin from the package insert for testing of *N. gonorrhoeae*. As azithromycin is commonly susceptibility tested in clinical microbiology laboratories against *N. gonorrhoeae*, results should be interpreted with extreme caution.

Due to the anomalous results found when using Liofilchem azithromycin MTS gradient strips it is recommended when susceptibility testing azithromycin against *N. gonorrhoeae* with gradient strips, bioMérieux Etest should be used. All information will be available to laboratories when the guidance document for susceptibility testing of *N. gonorrhoeae* is posted to the BSAC website.

CHAPTER 6 – DISCUSSION

Neisseria gonorrhoeae infections are increasing across the globe, and alongside the rate of antimicrobial resistance to these bacteria is also on the rise.(4) These increases highlighting the need for laboratories to continue to susceptibility test to enable resistance detection, preferably using an accurate and reliable susceptibility testing method. Some laboratories in the UK, and most laboratories in Europe, use NAATs (Nucleic Acid Amplification Tests) to detect *N. gonorrhoeae* in clinical samples and do not susceptibility test the isolate.(51) Most UK microbiology laboratories use the EUCAST disc diffusion method for susceptibility testing of bacteria; however, this method does not presently extend to *N. gonorrhoeae*. Current EUCAST advice is to use a minimum inhibitory concentration (MIC) method, with corresponding manufacturer's instructions.(39) The frequently used MIC method in diagnostic laboratories is a gradient strip; however, gradient strip manufacturer instructions suggest media which is not available or of limited supply in the UK. Therefore, there is a need to establish which media can be used with gradient strips in diagnostic laboratories which provide accurate and reproducible results for *N. gonorrhoeae*. This research aimed to address the issues associated with susceptibility testing of *N. gonorrhoeae* by evaluating appropriate commercially available agar plates and gradient strip manufacturers. Finding an appropriate commercial agar plate that supports growth of this fastidious organism, gives accurate and reproducible results, and within range quality control results was a priority in this project. These commercial media had to be readily available within the UK and by known manufacturers. Seven agar plates were included in stage 1 and stage 2 to provide a comprehensive evaluation, before the choice was narrowed down to the four best performing agar media for stage 3.

In addition to the main project there was a comparison of *N. gonorrhoeae* infections in Cardiff and those reported in recent GRASP reports performed, and an investigation in discrepancies of azithromcyin results from gradient strips from two manufacturers.

The main project was performed in three stages to evaluate different aspects of the study:

Stage 1: Evaluate the agar plates for viability of isolates (weight of growth) and quality of the MIC test reading. Performed in two laboratories.

Stage 2: Evaluate the accuracy and reproducibility of two commonly used antibiotics by gradient strips (one manufacturer) on seven agar plates. Performed in two laboratories.

Stage 3: Evaluate the performance of two gradient strips from two manufacturers using identified media from stage 2. Performed in two laboratories.

The following objectives were addressed in this thesis.

1. Establish the best (most accurate and reproducible) commercially available agar media for MIC determination of *N. gonorrhoeae*.

2. Establish whether gradient strips from both manufacturers provide accurate and reproducible results (MICs).

3. Establish if laboratories can use saline (commonly used in diagnostic laboratories) or Mueller Hinton Broth (as recommended by gradient strip manufacturers) for inoculum preparation.

4. Compare effect of primary culture media used for strain recovery from frozen storage on MIC.

5. Provide guidance for UK clinical laboratories through the BSAC website.

6. Collaborate with EUCAST to develop disc methods for *N. gonorrhoeae*.

7. Investigate local rates of incidence and antimicrobial resistance of *N. gonorrhoeae* in comparison with a national *N. gonorrhoeae* surveillance programme such as GRASP.

8. Investigate anomalous azithromycin gradient strip results seen in Wales.

6.1 ESTABLISH THE BEST (MOST ACCURATE AND REPRODUCIBLE) COMMERCIALLY AVAILABLE AGAR PLATE FOR MIC DETERMINATION OF *N. GONORRHOEAE*

Before any testing was performed, choosing agar plates for testing that supported the growth of *N. gonorrhoeae* was the first objective. The most common susceptibility test in UK laboratories is the EUCAST disc diffusion test, and usually for any fastidious bacteria the EUCAST guidelines recommend Mueller Hinton Fastidious (MHF) agar. However, when the EUCAST disc diffusion method was first developed it was noted that MHF did not sufficiently support the growth of *N. gonorrhoeae*. This resulted in EUCAST omitting a disc diffusion method for *N. gonorrhoeae* from their breakpoint tables and instead told laboratories to use an MIC method and the manufacturers guidance. As MHF was unsuitable for growth of *N. gonorrhoeae*, it was decided to follow the advice given by EUCAST and to look at the instructions given by the gradient strip manufacturers, which was to use a supplemented GC agar base medium.(38, 43, 100) The most common agars recommended by both gradient strip manufacturers were chocolated Mueller Hinton agar, which is currently unavailable in the UK, and GC agar with vitox.(43, 100) However, media manufacturers offer a variety of media for *N. gonorrhoeae*, therefore, the following five agar plates were chosen for testing: Oxoid chocolate + vitox, Oxoid GC + vitox, Becton Dickinson GC chocolate agar, and E&O GC chocolate with 10 % defibrinated horse blood. These five agars are readily available to purchase by UK laboratories.

EUCAST has recently developed a disk diffusion method for anaerobic bacteria. This method was developed using modified Fastidious Anaerobe Agar (FAA), a readily available medium in the UK and throughout Europe which supports the growth of fastidious species. The anaerobic disc diffusion method has been widely appreciated and accepted as a standardised method.(101) As anaerobic bacteria are extremely fastidious it was thought that this FAA could be appropriate for *N. gonorrhoeae* too. It was decided that due to the success of the development of the anaerobe disc diffusion method using modified FAA by EUCAST that two manufactures of modified FAA would be included in the project, E&O Laboratories and Liofilchem.

When considering all the results (both laboratories, all antimicrobial agents tested), the best performing agar plate for the quality control strains was Oxoid GC + vitox and for the clinical

strains BD GC chocolate agar performed the best. Both agars were considered the best as they both provided good growth to all *N. gonorrhoeae* strains tested, including quality control and clinical strains. They both gave reproducible results for most of the antimicrobial agents tested and a high number of the quality control strains were within range. All of these are the qualities of a good antimicrobial susceptibility testing method. Advice to laboratories is summarised in Table 55 below, showing laboratories what the best combination of agar plate and gradient strip manufacturer is, when they are limited to choice.

Overall, E&O GC Non-selective agar was the poorest performing agar with the gradient strip tests. Overall essential agreement was 59.8 %, overall categorical agreement was 98.9 %, and bias was unacceptable (> 30 %). Azithromycin bias was greater than 60 % negative bias in both laboratories and ceftriaxone bias was greater than 78 % negative bias in both laboratories. This is important as ceftriaxone is the first line agent used to treat gonorrhoea in the UK and poor-quality susceptibility testing results may impact the patient. High negative bias in ceftriaxone gradient strips means that the results are much lower than the true MIC, if the strain of *N. gonorrhoeae* happens to have an MIC close to the breakpoint, which means that if the isolate is resistant, it could be reported as susceptible when using E&O GC Non-selective agar. When a laboratory has a resistant ceftriaxone result, the sexual health clinic is notified, and the patient can be recalled to clinic and given a more appropriate treatment. Sending out potentially incorrect results from using this agar has many implications; patients remain infected with *N. gonorrhoeae* when they assume the treatment has worked and they potentially infect others, and patients could also develop disseminated gonococcal infection. Incorrect susceptibility results could also skew *N. gonorrhoeae* surveillance data. Both types of FAA (Liofilchem and E&O Laboratories) were the worst performing agars of the final four agar plates tested in stage 3. Liofilchem FAA had poor essential agreement with cefixime for both gradient strip manufacturers, 55 – 70 %, and Liofilchem ceftriaxone gradient strips, 70 – 75 %. E&O FAA with 5 % DHB was the worst performing of all with the poorest essential agreement for azithromycin, ceftriaxone and cefixime.

Due to time constraints and logistical issues both laboratories bought agar plates independently at different times, resulting in different lot numbers being used. From my 19

years' experience working in a diagnostic microbiology laboratory there can be a slight variance between lot numbers of most agar types, and this can have an impact on results. This variance in results highlights the importance of performing a quality control strain alongside the clinical strains whenever there is a change in lot number of the agar plates. Using a quality control strain will inform you as to whether the lot number of the agar plates in use is performing to the expected standard by giving you an in-range result.

6.2 ESTABLISH WHETHER GRADIENT STRIPS FROM BOTH MANUFACTURERS PROVIDE ACCURATE AND REPRODUCIBLE RESULTS (MICs)

In the UK there are two manufacturers of gradient strips readily available, bioMérieux and Liofilchem. Both manufacturers of gradient strip generally produced reproducible results, and there was no difference between manufacturers of gradient strip with regards to categorical agreement, i.e. both strips gave either susceptible, susceptible increased exposure or resistant (S, I or R) results similar to those using the reference method. However, overall, the best gradient strip manufacturer was bioMérieux as these strips had the higher essential agreement for azithromycin, ceftriaxone, cefixime and ciprofloxacin. Essential agreement is a best sign of accuracy for a susceptibility test as it is an indicator of how similar the MIC of your test is to the reference MIC. Categorical agreement is a poorer indicator of accuracy of an MIC and is more useful when calculating the impact on changes in category (S/I/R); it can also be useful if MICs of the test are around the clinical breakpoint. Categorical agreement is no longer part of the analysis of MICs, and it was recently removed from the ISO 20776 – 2 document.(104, 105) Categorical agreement was used in analysis of this data as it does add value, as stated above.

It is interesting to note that in the analyses of ciprofloxacin gradient strips (both manufacturers) there was excellent essential agreement (95 - 100%), low levels of bias and 100 % categorical agreement for clinical strains. This implies that both gradient strip manufacturers performed well on all agars. However, when the agar dilution reference method was performed by AMRSTI the range of MICs tested was 0.008 – 0.5 which is quite a narrow range with only seven dilutions. Typically, there would be 15 x doubling dilutions in a reference method test. This range was probably chosen as it spans the clinical breakpoint: susceptible ≤ 0.03 mg/L, resistant > 0.06 mg/L, according to EUCAST version 15 breakpoint tables for *N. gonorrhoeae*.(39) As a result of this narrow range, 95 % of the MIC results were in either the ≤ 0.008 mg/L or > 0.5 mg/L categories. This means that for ciprofloxacin the resultant analysis showing excellent essential agreement should be viewed with caution. If this project was to be repeated, then a doubling dilution range of 0.002 mg/L to 32 mg/L for the agar dilution method would be more appropriate for ciprofloxacin. This range is the same as the range of both gradient strip manufacturers for ciprofloxacin, better for a direct

comparison between the reference method and gradient strip test method. A greater range would enable a more accurate essential agreement, categorical agreement, and bias to be calculated for ciprofloxacin gradient strips.

Quality control strains are a good guide to accuracy of MICs. During testing some of the control strains used gave 'out-of-range' results, and in a routine diagnostic laboratory such quality control results would also invalidate any clinical results for that day, and both would need to be repeated immediately. In the event of repeat out-of-range quality control results then it would need to be investigated, with each element of the process changed one at a time to discern which part of the process was causing the anomalous result. For example, there could be issues with the agar, inoculum preparation, gradient strips, or the incubation conditions. However, in this study any out-of-range results were not repeated so the number of out-of-range quality control results could be assessed in each laboratory when only performed once. As multiple quality control strains were used, it ensured the methods were performed correctly.

As detailed in Chapter 5 – Differing azithromycin minimum inhibitory concentrations depending on manufacturer of gradient strip seen in Wales, Liofilchem azithromycin MTS strips can give incorrect MICs for certain strains of *N. gonorrhoeae*. With the Liofilchem MTS strips reporting isolates of *N. gonorrhoeae* as high-level resistant when they are actually low-level resistant. Azithromycin can still be used to treat low-level azithromycin resistant strains of *N. gonorrhoeae,* usually in combination with ceftriaxone. It is therefore important this result is accurate; reporting as high-level resistant would deny appropriate treatment to be considered. It is essential that these commonly used gradient strips give accurate results so that patients can be treated appropriately. I will be contacting Liofilchem with the results of the azithromycin discrepancies and discuss with them whether this is a known problem and what they are doing to resolve it.

6.3 ESTABLISH IF LABORATORIES CAN USE SALINE (COMMONLY USED IN DIAGNOSTIC LABORATORIES) OR MUELLER HINTON BROTH (AS RECOMMENDED BY GRADIENT STRIP MANUFACTURERS) FOR INOCULUM PREPARATION

Diagnostic laboratories in the UK mostly perform the EUCAST disc diffusion method to determine antimicrobial susceptibility in aerobic bacteria. Laboratories are busy environments with high workloads and so benefit from the use of common consumables. Most laboratories use the EUCAST recommended saline to make the inocula for susceptibility testing, however gradient strip manufacturers recommend Mueller Hinton Broth (MHB), a consumable not always readily available in a diagnostic laboratory.(39, 43, 100) This study aimed to establish if laboratories could use the cheaper and more readily available saline rather than MHB.

The minimal difference between using saline or MHB was shown when inter and intra-laboratory difference was examined in stage 2, where the difference of MIC between laboratories was between single, or 1 doubling dilution. It was concluded that the difference between laboratories testing was small and within the natural error range of the test. However, in the final stage of testing there appeared to be a stark difference in percentage of quality control MICs which were on target when performed in laboratory 1 compared with laboratory 2. As the inter and intra-laboratory differences were proven to be minimal in stage 2, it was concluded this variance could be due to variance in lot numbers of the agar plates and not due to the use of saline or MHB.

During each stage of testing there was no discernible difference in results between the two laboratories, one using saline, one MHB that could be attributed to the preparation of inoculum. This will therefore allow labs to use saline for susceptibility testing of *N. gonorrhoeae*, alongside other bacteria, thus reducing overall costs and negating the need for purchase of MHB.

6.4 COMPARE THE EFFECT OF PRIMARY CULTURE ON MIC

N. gonorrhoeae is a fastidious organism, with specific growth requirements. From my 19 years of experience working in a clinical microbiology laboratory poor growth affects susceptibility testing and so the health of the initial or primary culture may affect subsequent growth on an AST plate. If the growth is poor on the primary plate, then when it is challenged in a susceptibility test the antibiotic may have an enhanced effect, presenting as false susceptibility.

In stage 1 of this study AMRSTI used Oxoid GC + vitox for primary culture and SACU used Oxoid chocolate agar. Oxoid chocolate agar was used in SACU as this was the agar used by the diagnostic Bacteriology laboratory in Cardiff for the isolation of *N. gonorrhoeae*, AMRSTI laboratory usually used Oxoid GC + vitox so it was decided that they would continue to do so. By using different agar plates for the primary isolation of *N. gonorrhoeae* the effect on the susceptibility testing results could be investigated. Once stage 1 of the project had been completed SACU noted that there was poor initial growth after 24-hour incubation when the isolates had been subcultured from frozen storage, sometimes isolates needed 48 hours incubation before there was sufficient growth to prepare an adequate 0.5 McFarland suspension. AMRSTI did not encounter this issue, they noted good growth of the primary culture on Oxoid GC + vitox after 24-hour incubation, allowing them to easily prepare a 0.5 McFarland suspension. It was decided that both laboratories for stage 2 and 3 would use Oxoid GC + vitox for the primary culture of the *N. gonorrhoeae* isolates.

A vital part of susceptibility testing is the performance of control strains, which determine whether the test is being performed correctly. Results of control strain testing shows how well the test is being performed and can therefore be an indicator for any clinical *N. gonorrhoeae* tested. Typically, laboratories have a stock of each of their control strains that are kept on cryogenic beads frozen at -80 °C. Due to the fastidious nature of *N. gonorrhoeae*, the cryogenic bead should not be allowed to fully defrost when subculturing onto appropriate agar plates, as repeat freeze/thawing will cause the strain to become unviable.

The choice of agar plate for the initial culture of any *N. gonorrhoeae* strain once removed from the freezer is an important step. From consultation with AMRSTI and personal

experience working in SACU, it is recommended that *N. gonorrhoeae* should be plated on to Oxoid GC + vitox or BD GC chocolate, these agar plates will provide sufficient nutrients for good bacterial growth, so the user is able to perform a susceptibility test after 24 hours initial incubation. Most diagnostic laboratories use chocolate agar plates to purity plate suspected *N. gonorrhoeae* isolates from the original clinical sample. These plates perform adequately for most *N. gonorrhoeae* strains. However as evidenced in stage 1, for some strains the chocolate agar plates do not provide the necessary nutrients to support the growth of *N. gonorrhoeae* where growth can be insufficient for a susceptibility test after 24 hours incubation. This leads to an extended incubation of 48 hours, which in turn potentially delays results for the patient. Therefore, Oxoid GC + vitox or BD GC chocolate is recommended for the initial purity plate.

For both quality control and clinical samples, the time between plating of *N. gonorrhoeae* onto appropriate agar plates and incubation into a CO_2 incubator should be minimal, as the fastidious nature of the bacteria can cause it to die off very quickly when left on the laboratory bench in room temperature conditions.

6.5 PROVIDE GUIDANCE FOR UK CLINICAL LABORATORIES THROUGH THE BSAC WEBSITE

UK laboratories look to the BSAC for guidance on susceptibility testing in general but specifically for any difficult to test bacteria or antimicrobials which are not covered by the EUCAST breakpoint tables. Since BSAC stopped supporting the BSAC disc diffusion method and advised UK laboratories to adopt EUCAST methods, the initial advice given to UK laboratories was to perform an MIC, following gradient strip manufacturer's instructions. However, after completion of stages 1 and 2 of this study, the BSAC advice was updated to include a recommendation of agar. It is planned that the data from the final thesis will allow publication of further detailed advice on which agar plates and gradient strip manufacturers perform the best when susceptibility testing *N. gonorrhoeae.*

As laboratories in the UK usually have tender agreements lasting several years with manufacturers of agar, this makes it difficult to use an alternative manufacturer's agar whilst

under contract. Therefore, laboratories may not be able to use the best recommended agar plate for susceptibility testing of *N. gonorrhoeae*. The following table 55, can highlight to laboratories where they can expect to see bias depending on which combination of agar plate and gradient strip they have available. This will help give evidence for laboratories to apply for exclusion from the tender contract for *N. gonorrhoeae* and use the best media manufacturer for antimicrobial susceptibility testing, irrespective of tender agreement.

Table 56 below is a guide for laboratories summarising this study's findings. Cells highlighted in green are acceptable, essential agreement (EA) and categorical agreement (CA) ≥ 90 %, and bias ≤ 30 %, those labelled predominantly acceptable had MICs from one laboratory with acceptable bias, and one laboratory just over the acceptable bias threshold. Cells in red are unacceptable. Cells in orange labelled moderately acceptable identify where one laboratory had MICs with acceptable bias and one laboratory had MICs a little over the acceptable threshold.

Table 56: Final summary table of this study's findings

		AZITHROMYCIN		CEFTRIAXONE		CEFIXIME		CIPROFLOXACIN	
		BIOMÉRIEUX	LIOFILCHEM	BIOMÉRIEUX	LIOFILCHEM	BIOMÉRIEUX	LIOFILCHEM	BIOMÉRIEUX	LIOFILCHEM
Oxoid GC + vitox	**EA**	100 %	95 %	92.5 %	90 %	72.5 %	72.5 %	100 %	100 %
	BIAS	Predominantly acceptable	Predominantly acceptable	Moderately acceptable	Moderately acceptable	Unacceptable	Unacceptable	Acceptable	Acceptable
	CA	97.5 %	97.5 %	100 %	100 %	80 %	80 %	100 %	100 %
BD GC chocolate agar	**EA**	97.5 %	97.5 %	92.5 %	95 %	80 %	77.5 %	100 %	97.5 %
	BIAS	Predominantly acceptable	Predominantly acceptable	Acceptable	Predominantly acceptable	Unacceptable	Unacceptable	Acceptable	Acceptable
	CA	95 %	97.5 %	100 %	100 %	85 %	85 %	100 %	100 %
E&O FAA 5 % DHB	**EA**	85 %	77.5 %	80 %	42.5 %	67.5 %	55 %	100 %	100 %
	BIAS	Acceptable	Acceptable	Unacceptable	Unacceptable	Unacceptable	Unacceptable	Acceptable	Acceptable
	CA	95 %	95 %	97.5 %	100 %	70 %	70 %	100 %	100 %
Liofilchem FAA	**EA**	97.5 %	95 %	97.5 %	72.5 %	70 %	57.5 %	100 %	100 %
	BIAS	Acceptable	Moderately acceptable	Unacceptable	Unacceptable	Unacceptable	Unacceptable	Acceptable	Acceptable
	CA	100 %	100 %	100 %	100 %	75 %	72.5 %	100 %	100 %

As seen in table 56, those laboratories using Oxoid GC + vitox could use either manufacturer of gradient strip for azithromycin and ciprofloxacin as they have the same acceptable essential agreement, bias, and categorical agreement. Oxoid GC + vitox also performs the same with both manufacturers of gradient strip, acceptable EA and CA and moderately acceptable bias.

Laboratories using BD GC chocolate agar could use either gradient strip manufacturer for azithromycin, ceftriaxone, and ciprofloxacin as all had acceptable EA and CA, and either acceptable or predominantly acceptable bias.

Laboratories using E&O FAA with 5 % DHB could use either gradient strip manufacturer for ciprofloxacin. However, it would not be advisable to test this agar plate with ceftriaxone gradient strips from bioMérieux or Liofilchem as there was unacceptable EA and bias.

Any laboratory using Liofilchem FAA could use either bioMérieux or Liofilchem ciprofloxacin gradient strips as they both performed the same. It would be advisable to use azithromycin gradient strips from bioMérieux as they had acceptable EA, bias, and CA, whereas Liofilchem gradient strips had acceptable EA and CA but moderately acceptable bias. Liofilchem FAA performed slightly better with bioMérieux ceftriaxone gradient strips, with acceptable EA and CA but unacceptable bias, as Liofilchem ceftriaxone gradient strips had acceptable CA but unacceptable EA and bias.

Regardless of which agar plate was used cefixime gradient strips did not perform well with either gradient strip manufacturer, unacceptable EA, CA, and bias. Due to the poor performance of this test the recommendation for cefixime would be not to susceptibility test with gradient strips.

From the results of this project no agar plate and gradient strip combination is perfect for all antimicrobials. But if a choice had to be made then the recommendation would be Oxoid GC + vitox, this agar performed consistently for all antimicrobials.

The results of this MPhil project are already having a real-world impact. It was decided in collaboration with Welsh microbiology laboratories either Oxoid GC + vitox or Becton Dickinson GC chocolate would be used for susceptibility testing of clinical *N. gonorrhoeae*. These agar plates were the best performing for ceftriaxone gradient strips, the first-line treatment in Wales, and also performed adequately for azithromycin gradient strips which is second-line treatment in Wales. The information is further being used to provide guidance to laboratories in the rest of the UK and around Europe detailing the best performing agar plates with which to susceptibility test *N. gonorrhoeae* using gradient strips. This will be achieved by susceptibility testing guidance that is currently under consultation and soon to be placed on the BSAC website and the EUCAST website, ensuring maximum dissemination of information to laboratories in the UK, Europe and around the world.

6.6 COLLABORATE WITH EUCAST TO DEVELOP A DISC SUSCEPTIBILITY METHOD FOR *N. GONORRHOEAE*

The results of this MPhil project have been shared with the EUCAST Developmental Laboratory (EDL) in Sweden and are being used to inform a current collaboration with them to develop a disc diffusion method for *N. gonorrhoeae*. Even though the best performing agars were Oxoid GC + vitox and BD GC chocolate, the results of the FAA testing were of most interest. FAA has recently been successfully used to developed anaerobe disc diffusion method by EUCAST.(101) The EDL wanted to collaborate on evaluating whether FAA would be appropriate for a standardised *N. gonorrhoeae* disc diffusion method. Through discussions between our laboratories, it was decided that I would perform agar dilution method with FAA and EDL would perform the disc diffusion method on modified FAA agar plates.

I have recently performed agar dilution on 100 *N. gonorrhoeae* strains with FAA from two manufacturers, E&O Laboratories and Neogen. Both agar manufacturers have been tested against 10 antimicrobials: penicillin, ampicillin, amoxicillin, cefixime, cefotaxime, ceftriaxone, ertapenem, ciprofloxacin, ofloxacin, azithromycin, tetracycline, and spectinomycin. These MIC results are the reference results that the disc diffusion results performed in EDL Sweden

will be compared to. Once analysis is complete, providing there are reliable and reproducible results, the new disc diffusion method for *N. gonorrhoeae* will be added to the next EUCAST breakpoint table in 2026 for use by laboratories in the UK, and Europe.

6.7 INVESTIGATE LOCAL RATES OF INCIDENCE AND ANTIMICROBIAL RESISTANCE OF *N. GONORRHOEAE* IN COMPARISON WITH A NATIONAL *N. GONORRHOEAE* SURVEILLANCE PROGRAMME SUCH AS GRASP

The increasing rates of *N. gonorrhoeae* infections in Cardiff does correlate with the increase of infections seen in the UK. Low antimicrobial resistance rates for ceftriaxone are the same for local Cardiff results and the wider UK, which is reassuring due to this being the first-line treatment.(12) There are slight differences in antimicrobial resistance rates for ciprofloxacin and azithromycin, but this could be due to the error reported in the reference method by the GRASP team.(51)

The increase in *N. gonorrhoeae* infections in both Cardiff and the UK highlight the importance of this project in providing the optimal media and gradient strips to enable clinical bacteriology laboratories to provide good quality susceptibility testing results so any increase in antimicrobial resistance can highlighted quickly.

6.8 INVESTIGATE ANOMALOUS AZITHROMYCIN GRADIENT STRIP RESULTS SEEN IN WALES

This investigation determined that azithromycin gradient strips from Liofilchem gave incorrect results for some isolates of *N. gonorrhoeae*. As a result of this investigation a guidance document distributed by BSAC in due course advising laboratories of the problems due to these gradient strips.

6.9 FINAL SUMMARY

From completion of this MPhil project, the optimal agar and gradient strip combination was determined.

Oxoid GC + vitox and BD GC chocolate were the best performing agar plates, with Oxoid GC + vitox performing consistently better for all antimicrobials. This agar had high quality control compliance, with most MICs within the quality control range. Oxoid GC + vitox had better QC target matching for azithromycin, ceftriaxone and cefixime compared to BD GC chocolate. Alongside these agars this MPhil project analysis demonstrated that the bioMérieux gradient strip was the best performing gradient strip, with a higher percentage of MICs within the quality control range for all antimicrobials, and a higher percentage of quality control MICs on target for azithromycin, ceftriaxone, and ciprofloxacin.

The results of this MPhil determined that the worst performing agar was E&O FAA with 5 % DHB. This agar plate had numerous out-of-range quality control MICs for both manufacturers of gradient strip, bioMérieux and Liofilchem, the poorest essential agreement, bias, and categorical agreement. However, it should be noted that the quality control ranges are based on testing performed on different media. Clinical microbiology laboratories should refrain from using this agar plate for susceptibility testing of *N. gonorrhoeae*. Analysis of the results revealed that Liofilchem gradient strips were the worst performing, with lower percentage of MICs within the quality control range and only cefixime gradient strips having a higher percentage of quality control MICs on target compared to bioMérieux, and clinical microbiology laboratories should use caution if using them.

6.10 CONTINUATION OF RESEARCH

Work is already in progress to develop a disc diffusion method for *N. gonorrhoeae* in collaboration with the EUCAST Developmental Laboratory in Sweden. The results are currently being analysed, with the hope to publish in the new EUCAST breakpoint tables which will be released early 2026. This will be useful to clinical microbiology laboratories in the UK and Europe as a disc diffusion method is significantly cheaper than the current gradient strip method.

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