Detection and Molecular Epidemiology of ciprofloxacin-resistant *Neisseria gonorrhoeae,* using a real-time polymerase chain reaction (PCR).

by

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Submitted in partial fulfilment of the requirements for the degree of

Master of Science

M.Sc. (Medical Microbiology)

FACULTY OF HEALTH SCIENCES

in the

School of Pathology
University of the Witwatersrand,
Johannesburg

September 2010

DECLARATION

"I certify that the thesis hereby submitted to the University of the Witwatersrand for the degree M.Sc. (Medical Microbiology) is my own work and has not been submitted previously by me in respect of a degree at any other tertiary institution."

Signature:…….

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ABSTRACT

Emergence and spread of resistance to ciprofloxacin among *Neisseria gonorrhoeae* strains has reduced the options of effective treatment for gonococcal infections and has become a concern worldwide. Up until 2008, ciprofloxacin was recommended first-line therapy for treatment of presumptive *N. gonorrhoeae* infections in South Africa. At the time this MSc project was conceived, ciprofloxacin was still used as first-line therapy for presumptive gonococcal infections.

A real-time polymerase chain reaction (PCR) assay was used to detect ciprofloxacin-resistant *N. gonorrhoeae* in DNA extracted from non-invasive urine samples collected as part of the national microbiological surveillance (NMS) programme during 2006-2007. The molecular epidemiology of ciprofloxacin-resistant *Neisseria gonorrhoeae* was investigated by sequencing the quinolone resistance determining regions (QRDR) of the *gyrA* and *parC* genes *of N. gonorrhoeae* and performing *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST).

As part of the NMS program for sexually transmitted infections (STIs) urine and urethral swabs were collected from men presenting with urethral discharge at primary health care clinics in Johannesburg (Gauteng), Cape Town (Western

Cape) and Kimberley (Northern Cape). Urine samples and cultured *N. gonorrhoeae* isolates from 2006-2007 were stored at -70^{0}C and available for this study. Gonococci, previously isolated from urethral swabs, were subcultured directly onto New York City media. Isolate identity was re-confirmed by typical colony morphology and biochemical tests. Urine samples from Johannesburg were tested in order to develop the real-time PCR protocol. Subsequently, paired urethral swab DNA and *N. gonorrhoeae* cultures were tested from NMS patients recruited in Kimberley and Cape Town. Where possible, the PCR assay results were compared with paired antibiotic susceptibility data for ciprofloxacin.

Quinolone resistance determining regions (QRDR) for *gyrA* and *parC* were screened for known point mutations associated with resistance to ciprofloxacin. Detection of mutations by the real-time PCR assay generally agreed with the phenotype of either decreased susceptibility or resistance to ciprofloxacin. All ciprofloxacin resistant gonococcal isolates had the same *gyrA* and *parC* mutations, which initially suggested that quinolone resistant *N. gonorrhoeae* (QRNG) in Kimberley, Cape Town and Johannesburg, may be attributed to the spread of a single clone. The use of a more discriminatory typing scheme, *Neisseria gonorrhoeae* Multi-Antigen Sequence Typing (NG-MAST) genotyping, revealed that ciprofloxacin resistant gonococcal isolates in Johannesburg and Cape Town were heterogeneous, with sequence type (ST) 217 being most prevalent in both cities (5/16, Johannesburg; 7/11, Cape Town). In contrast, all eight QRNG isolates from Kimberley were typed as ST 533.

The use of molecular methods allowed ciprofloxacin antimicrobial susceptibility determination by PCR in non-invasive specimens. This is useful in situations where bacterial cultures are unavailable or die before antimicrobial susceptibility testing can be performed. Molecular assays to detect ciprofloxacin resistance may guide physicians as to the most ideal antimicrobial combinations for individual patient treatment.

As a result of emerging widespread resistance gonococci to ciprofloxacin, in 2008, the Department of Health recommended that ciprofloxacin be removed as a first line therapy in the South African national sexually transmitted infections treatment guidelines for treatment of urethritis, cervicitis and their complications. Although ciprofloxacin is no longer used as a first-line therapy to treat gonorrhoea within our country, it may still be used in cases of severe penicillin allergy or as part of multi-drug therapy for gonococcal infections in the future. The ability to detect ciprofloxacin resistance by real-time PCR will be a useful technique in such situations.

Acknowledgements

I would sincerely like to thank the following people:

1. Prof D A Lewis, Head of the STI Reference Centre at National Institute for Communicable Diseases (NICD), National Health Laboratory Services (NHLS), for his astuteness and clarity of thought, which reflect his experience as both a researcher and supervisor of research projects. I am also grateful for his belief in my ability, his patience and guidance as my co-supervisor.

2. Dr E Müller, Senior Medical Scientist at the STI Reference Centre at NICD/NHLS for his knowledge and practical assistance of molecular techniques. I am also grateful for his patience and guidance as my co-supervisor.

3. Mrs. L Gumede, Medical Technologist at the STI Reference Centre at NICD/NHLS for her knowledge and practical assistance of bacteriology techniques.

4. Dr O Perovic, Consultant Pathologist of the Microbiology Laboratory at Johannesburg General Hospital, for her helpful assistance in supplying urine samples from patients with urinary tract infections.

5. Ms Ruth Mpembe, Medical Technologist at the Respiratory and Meningeal Pathogens Reference Unit at NICD/NHLS for their helpful assistance in providing the non gonococcal Neisseria isolates

6. The staff of the STI Reference Centre at NICD/NHLS for their time and support.

7. I am most grateful to my son, Linda, for his support and understanding through out my study. Thanks for all the fun we have shared together.

Presentations arising from this thesis

1. Magooa MP., Muller E., Lewis DA. Detection and Molecular Epidemiology of ciprofloxacin-resistant *Neisseria gonorrhoeae*, using real-time PCR. University of Witwatersrand, Faculty of Health Academic Day. 15 August 2008, Johannesburg.
2. Magooa MP., Muller E., Lewis DA. Detection and Molecular Epidemiology of ciprofloxacin-resistant *Neisseria gonorrhoeae*, using real-time PCR. National Institute for Communicable Diseases (NICD) Academic Day, 11 November 2008, Johannesburg.
3. Magooa MP., Muller E., Lewis DA. Molecular Analysis of Quinolone resistant Gonococci in South Africa. 18th International Society for STD research. 28th June-1st July 2009, London.

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LIST OF ABBREVIATIONS

µl	Microliter
µg	Microgram
AAC	Aminoglycoside acetyltransferase
AME	Aminoglycoside-modifying enzyme
ANT	Aminoglycoside nucleotidyltransferase
APH	Aminoglycoside phosphotransferase
ATCC	American Type Culture Collection
bp	Base pairs
CDC	Centers for Disease Control and Prevention
CipS	Ciprofloxacin susceptible
CipR	Ciprofloxacin resistant
CipI	Ciprofloxacin intermediately susceptible
CLSI	Clinical Laboratory Standards Institute
CMRNG	Chromosomally-mediated resistant *Neisseria gonorrhoeae*
Ct value	Is the value where the threshold line crosses the amplification curve
DGI	Disseminated gonococcal infection
DI	Simpson's index of diversity (Discriminatory index)
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EF	Elongation factor
ET	Electrophoretic type
*f*mol	Femtomoles
GTP	Guanosine-5"-triphosphate
I	Intermediate
MAb	Monoclonal antibody

MDa	Mega Dalton
MIC	Minimum inhibitory concentration
ml	Milliliter
ML	Martin-Lewis
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
M-PCR	Multiplex Polymerase chain reaction
MTM	Modified Thayer-Martin
mRNA	Messenger ribonucleic acid
NCCLS	National Committee for Clinical Laboratory Standards
NG MAST	*N. gonorrhoeae* Multi-Antigen Sequence Typing
NMS	National Microbiological Surveillance
NYC	New York City
OMP	Outer membrane protein
PABA	ρ-aminobenzoic acid
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PHC	Primary Health Care
PFGE	Pulsed-field gel electrophoresis
PID	Pelvic inflammatory disease
PMQR	Plasmid-mediated quinolone resistance
POMP	Principal outer membrane protein
PPNG	Penicillinase-producing *N. gonorrhoeae*
QC	Quality control
QRDR	Quinolone resistance determining region
QRNG	Quinolone resistant *Neisseria gonorrhoeae*
R	Resistant
REA	Restriction endonuclease analysis

S	Susceptible
ST	Sequence type
STI	Sexually transmitted infection
TBE	Tris-boric acid buffer
tRNA	Transfer ribonucleic acid
TRNG	Tetracycline resistant *N. gonorrhoeae*
UV	Ultraviolet
WHO	World Health Organization
WT	Wild type

CHAPTER 1: INTRODUCTION

Neisseria gonorrhoeae, a Gram negative bacterium, is the aetiological agent of gonorrhoea, which remains the most frequent cause of the male urethritis syndrome (MUS) in South Africa (107).

Up until August 2008, the South African national guidelines for the management of sexually transmitted infections (STIs) recommended ciprofloxacin 500 mg as a single oral dose for the treatment of presumptive gonococcal infections among men with MUS and scrotal swelling. In the revised guidelines, ciprofloxacin is still indicated for patient with severe penicillin allergy presenting to primary healthcare clinics (175).

Within South Africa, STIs are managed syndromically (221). Integral to this approach is the requirement for periodic surveillance to assess both the microbiological causes of the various STI syndromes (aetiological surveillance) and antimicrobial resistance testing for key STI pathogens, notably *Neisseria gonorrhoeae*. Ciprofloxacin-resistant gonorrhoea was first reported as an emergent problem in South Africa's Kwa-Zulu Natal Province in 2003 (126). Subsequent to that, the National Department of Health and National Institute for Communicable Diseases, in conjunction with a number of South African university laboratories, undertook a survey in 2004 to assess the prevalence of ciprofloxacin resistant gonorrhoea across the country (101,105).

The most prevalent mechanism contributing to fluoroquinolone resistance in the gonococcus involves mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene and, often additional point mutations in the analogous region of the *parC* gene on the bacterial chromosome (52). Therefore, to understand the mechanisms of quinolone resistance in *N. gonorrhoeae*, analyses of both the *gyrA* and *parC* genes are important and necessary.

The volatile nature of antimicrobial resistance in gonococci means that surveillance of resistance for public health purposes must be optimal, both in terms of obtaining a sufficiently large and representative sample of gonococcal isolates as well as using appropriate tools to identify resistance (188). Increasing use of nucleic acid amplification assays in industrialised countries and the widespread application of syndromic management principles in less developed countries has increasingly restricted the availability of gonococcal isolates for phenotypic detection of resistance rates (47,218). Logistic problems with gonococcal storage and transport and the intrinsic fragility of *N. gonorrhoeae* also impact adversely on viable isolate availability (47, 218).

Molecular tools are being used to provide an understanding of the genetic basis of resistance as well as to supplement phenotypic antimicrobial susceptibility testing (63, 170, 207). These tools have not yet advanced to a stage where they can be used in place of phenotypic testing, mainly because the genetic determinants of resistance to most antibiotics are not yet fully known. In addition

resistance to several antibiotics in *N. gonorrhoeae* is potentially mediated by a number of independent genetic mechanisms. Nevertheless, for well characterised resistance mechanisms, molecular tools offer an accurate and objective means of detection and systems have been described for the detection of pivotal resistance determinants for public health purposes where viable isolates are not required (207).

Typing of *Neisseria gonorrhoeae* has many applications including the definition of sexual networks, interventions to arrest disease outbreaks by defining the contacts of outbreak strains, confirmation or exclusion of possible treatment failures, monitoring the spread of subtypes of gonococci with altered diagnostic features, and the detection of the emergence and subsequent spread of antibiotic resistant gonococci (162). Gonococcal typing methods have progressed from single (e.g. auxotyping) to combination phenotypic systems (e.g. combined auxotyping and serovar determination). Genotyping systems are, however now regarded as being more sensitive than phenotypic methods (144).

1.2 Aims and objectives of the study

There were two working hypotheses and three specific objectives.

Hypothesis 1: that invasive specimen can be used to detect antimicrobial resistance.

Hypothesis 2: that the information on gonococcal strains circulating in a community can be used to identify patterns of transmission of gonorrhea.

To explore these two hypotheses within a population, the aim of the research presented in this dissertation was to detect and describe molecular epidemiology of ciprofloxacin-resistant *Neisseria gonorrhoeae*.

The objectives were as follows:

1. To establish a real-time PCR assay at the STI Reference Centre to detect quinolone resistance in gonococcal DNA extracted from male urine and swabs.

2. To identify and compare point mutations in the quinolone resistance determining regions of the *gyrA* and *parC* genes of ciprofloxacin resistant gonococcal DNA.

3. To use the high discriminatory power of NG-MAST to molecular subtyping of ciprofloxacin resistant strains with identical mutation patterns in QRDRs of the *gyrA* and *parC* genes.

CHAPTER 2: LITERATURE REVIEW

2.1. HISTORICAL PERSPECTIVE

Neisseria gonorrhoeae, the gonococcus, is a Gram-negative diplococcus which causes the sexually transmitted disease gonorrhoea. Reference to the contagious nature of gonococcal infection dates back to biblical times (Leviticus 15:1-15,19), making gonorrhoea one of the oldest recorded human diseases. Today, gonococcal infection remains a major global health problem, as more than sixty million cases are reported annually worldwide (31).

The first usage of the term “gonorrhoea”, by Galen in the second century, implied a “flow of seed”. For centuries thereafter, gonorrhoea and syphilis were confused, resulting from the fact that the two diseases were often present together in infected individuals. Paracelsus (1530) thought that gonorrhoea was an early symptom of syphilis. The confusion was further heightened by the classic blunder of English physician John Hunter, in 1767. Hunter intentionally inoculated himself with pus from a patient with symptoms of gonorrhoea and wound up giving himself syphilis. The causative agent of gonorrhoea, *N. gonorrhoeae*, was first described by Albert Neisser (90) in 1879 in the pustular exudates of a case of gonorrhoea. The organism was grown in pure culture in 1885, and its aetiological relationship to human disease was later established using human volunteers in order to fulfil the experimental requirements of Koch’s postulates (76).

2.2 TRANSMISSION

Maintenance and transmission of gonorrhoea are related to a subset of “core transmitters” who have unprotected sex with multiple partners and either are asymptomatic or choose to ignore the symptoms (119,155, 227). Both social (i.e., low socioeconomic status, urban residence, lack of education, limited access to health care, unmarried status,) and behavioral risk factors (i.e., commercial sex work, previous sexually transmitted infections, male homosexuality, early onset of sexual activity, unprotected sex, multiple partners, other high risk partners, drug use) have been identified for targeting by outreach/intervention and sexual transmitted infection control programs (10, 19, 103).

The risk of acquiring gonorrhoea is multifactorial and is related to the number and sites of exposure. For heterosexual males, the risk of acquiring urethral infection from an infected female is about 20% for a single exposure and up to 80% for four exposures (76, 177). Due to anatomical considerations, the risk of infection for the female genital tract from a single exposure to an infected male is probably significantly higher. Transmission of rectal infection is also quite efficient, and recent studies among homosexual/bisexual men have demonstrated that urethral infection following fellatio with an infected partner may account for as much as 26% of urethral infections diagnosed in this population (103). Among women, use of hormonal contraceptive methods is associated with an increased risk of gonococcal infection while barrier methods such as condoms and diaphragms

used with spermicidal foams and gels exert a protective effect against infection (76).

2.3. CLINICAL MANIFESTATIONS

Gonorrhoea is generally a disease of mucous membranes, involving the urethra, endocervix, pharynx, rectum and conjunctiva. Gonorrhoea often presents as a copious discharge of pus, more apparent in the male than in the female (76). Asymptomatic infections are important from the public health perspective and enable on-going transmission.

2.3.1. GONOCOCCAL INFECTION OF THE MALE URETHRA

N. gonorrhoeae infection of men most commonly occurs as an acute urethritis with dysuria and urethral discharge (Fig 2.1) (76, 178). The incubation period between organism acquisition and onset of symptoms averages 2 to 7 days (range, 1 to 14 days) (85). During this time gonococci are quiescent and cannot be cultured from the urethra for up to 40 hours after the initiation of infection, after which a purulent exudative process begins (54). These data suggest that gonococci enter a protective environment early in disease where they survive and replicate. It was proposed that, in men, the urethral epithelial cell is this sanctuary. In vitro infection assays and microscopic analyses of patient exudates indicate that gonococci are released from epithelial cells and that infected epithelial cells are subsequently shed from the mucosal surface to the urethral lumen (54).

After infection, 95 to 99% of men experience a urethral discharge that may be purulent (Fig 2.1), cloudy, or mucoid; the consistency of the discharge at presentation is affected by the length of time that the infection has been incubating and whether the patient has recently urinated (85). About 2.5% of men with gonorrhoea presenting to sexual transmitted disease clinics are truly asymptomatic (85), but the prevalence of asymptomatic urogenital gonorrhoea in men in high risk community-based populations may be as high as 4.7% (106). Men with asymptomatic urethritis are an important reservoir for transmission. In addition, such men and those who ignore their symptoms are at increase risk for developing complications. Ascending infection may result in gonococcal epididymo-orchitis, prostatitis, periurethral abscess, or urethral stricture (85),

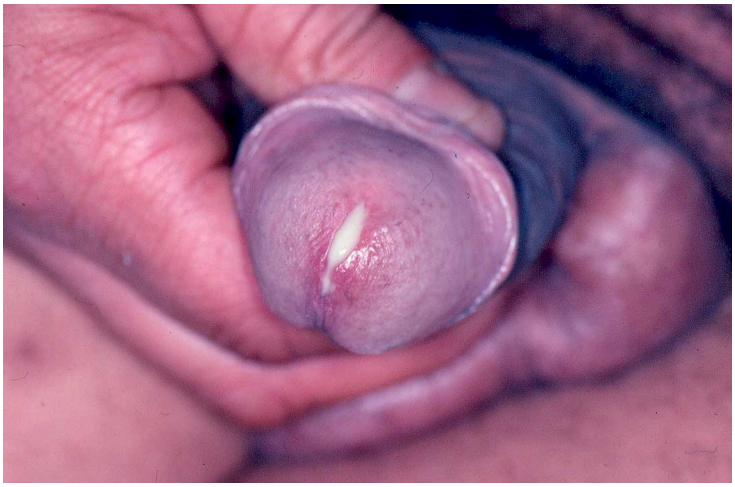


Fig 2.1: Purulent urethral discharge due to gonorrhoea *(Photograph credit: D. Lewis)*

2.3.2. INFECTION OF THE LOWER FEMALE GENITAL TRACT

Endocervical infection is the most common form of uncomplicated gonorrhoea in women (119). Although up to 50% of cases may be asymptomatic, symptomatic women may present with vaginal discharge, lower abdominal pain and sometimes dysuria (because of coexistent urethritis). After an incubation period of eight to ten days, patients may present with cervico-vaginal discharge (Fig 2.2) or inter-menstrual bleeding, and abdominal or pelvic pain; the presence of pain may suggest the presence of upper genital tract disease (178). Asymptomatic disease in females occurs worldwide at an estimated rate of over one million cases per year (178).

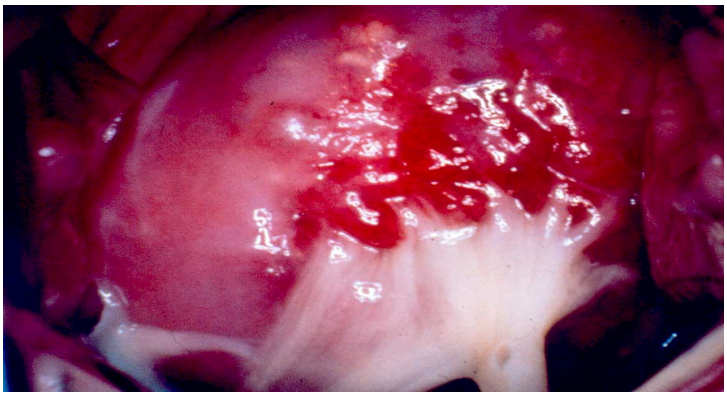


Fig 2.2: Purulent endocervical exudate in gonococcal cervicitis *(Photograph credit: C. Rodgers)*

The most common form of infection in prepubertal girls is a diffuse vaginitis with a secondary vulvitis (18). Gonococcal infection of the vaginal squamous epithelium of postpubertal women is uncommon, and in women with hysterectomies, the urethra is the most common primary site of infection (85). Symptoms of uncomplicated endocervical infection often resemble those of other conditions, such as cystitis or vaginal infections, and the symptoms of

gonococcal endocervicitis are clouded by frequent coinfection with *Chlamydia trachomatis, Trichomonas vaginalis,* and/or *Candida albicans*. Between 20 and 75% of women are presented with a mucopurulent endocervical discharge (85). Endocervical gonorrhoea may also complicate pregnancy and is a recognised co-factor for spontaneous abortion, chorio-amnionitis, premature rupture of membranes, and premature delivery (178)

2.3.3. INFECTION OF THE UPPER FEMALE GENITAL TRACT

Ascending gonococcal infection may occur in 10 to 20% of infected women and can result in acute pelvic inflammatory disease (PID) that may manifest as salpingitis (infection of the fallopian tubes), endometritis, and/or tubo-ovarian abscess, all of which can lead to scarring, ectopic pregnancies, sterility, and chronic pelvic pain (76, 178) (Fig 2.3). Ascent to the upper female genital tract may be facilitated through the ability of gonococci to exhibit twitching motility, in conjunction with hormonal changes which influence the expression of complement and molecules serving as gonococcal receptors within the female genital tract. The involvement of fallopian tubes or ovaries may result in sterility (54).

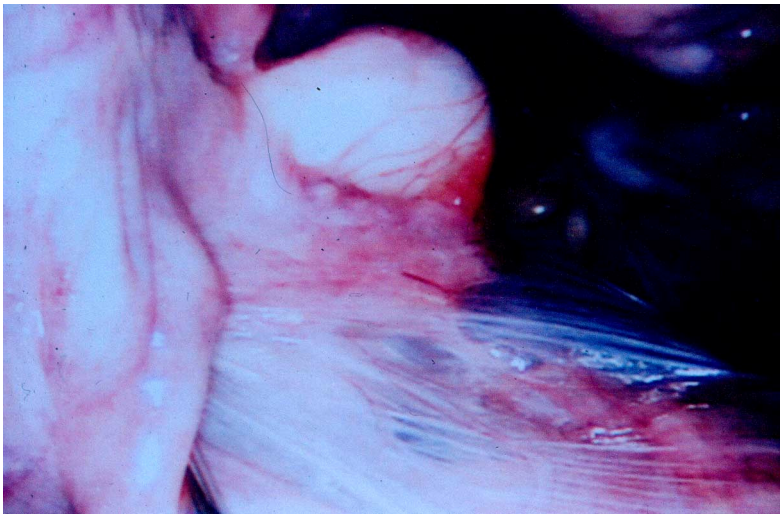


Fig 2.3: Pelvic inflammatory disease due acute gonococcal infection *(Photograph credit: A. Amar)*

PID caused by *N. gonorrhoeae* generally occurs early, rather than late, in infection and often during or shortly after the onset of menstruation (85). In pregnant women, gonococcal infection is associated with increase risk of complications, including premature labour, premature rupture of the foetal membranes, spontaneous abortion, and infant morbidity (85).

2.3.4. PHARYNGEAL, ANORECTAL AND CONJUNCTIVAL INFECTIONS IN ADULTS

N. gonorrhoeae may also cause pharyngeal and anorectal infections. Rectal gonorrhoea (proctitis) is most prevalent among homosexual men and heterosexual women (94). Unlike homosexual men who acquire gonococcal infection by penile-anal and oro-anal forms of sexual intercourse (and are more often symptomatic), most ano-rectal infections in women are considered to result from the inoculation of the anorectal mucosa with infectious vaginal discharge

and are rarely symptomatic (94). Rectal gonococcal infections are often asymptomatic, although some individuals experience acute proctitis with anorectal pain and itching, a mucopurulent discharge, bleeding, tenesmus, and constipation 5 to 7 days following infection (153). The rectum is relatively rich in inhibitory hydrophobic molecules since faeces contain 4-5% lipid consisting of fatty acids, sterols and bile acids (120). The gonococcal outer membrane is particularly permeable to fatty acids (123). Rectal isolates from homosexual men and heterosexual women, however, are more resistant to faecal lipids than cervical or urethral isolates (120). This then suggest that the host environment plays a role in the selection of gonococcal strains.

Oropharyngeal gonococcal infection is seen in homosexual and bisexual men and heterosexual women who acquire the infection by engaging in orogenital sexual contact with an infected partner. Pharyngeal gonorrhoea is also seen occasionally in heterosexual men as a result of performing cunnilingus with an infected partner. Some reports have suggested that gonococci infection may cause acute pharyngitis or tonsillitis (178). Over 90% of oropharyngeal gonococcal infections are asymptomatic and are diagnosed by culture of the organism from the throat (76, 178). Since oral sex has been associated with urethral gonorrhoea, asymptomatic pharyngeal infection can serve as an unrecognised reservoir for transmission.

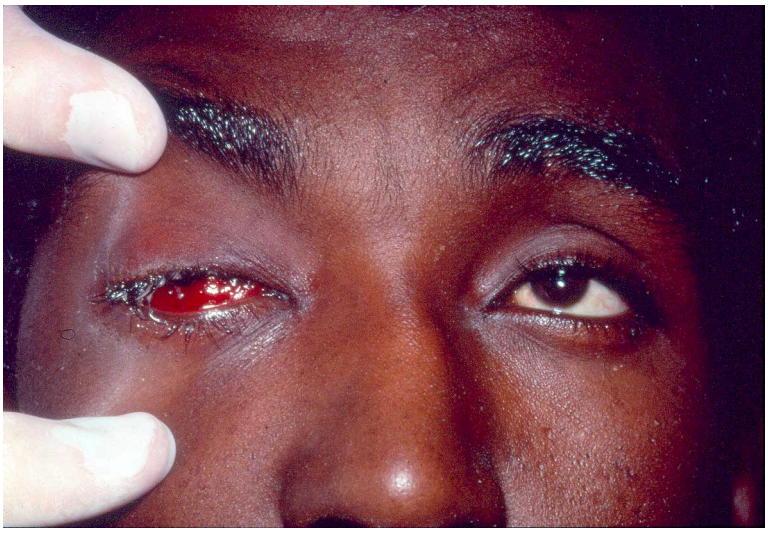


Fig 2.4: Adult gonococcal conjunctivitis *(Photograph credit: D. Lewis)*

Eye involvement in adults occurs by autoinoculation of gonococci into the conjunctival sac from a primary site of infection, such as the genitals. The most common form of presentation is a purulent conjunctivitis, which may rapidly progress to panophthalmitis and loss of the eye unless promptly treated (Fig 2.4) (18).

2.3.5 NEONATAL INFECTIONS

N. gonorrhoeae infections in neonates commonly follow transmission of the organism from the infected mother to the infant during passage through the birth canal. Prolonged rapture of the membranes and consequent chorio-amnionitis in women infected with *N. gonorrhoeae* are also predisposing factors to neonatal disease. On rare occasions, children born by caesarian section may be infected with *N. gonorrhoeae*, but this usually follows prolonged rupture of the membranes (18).

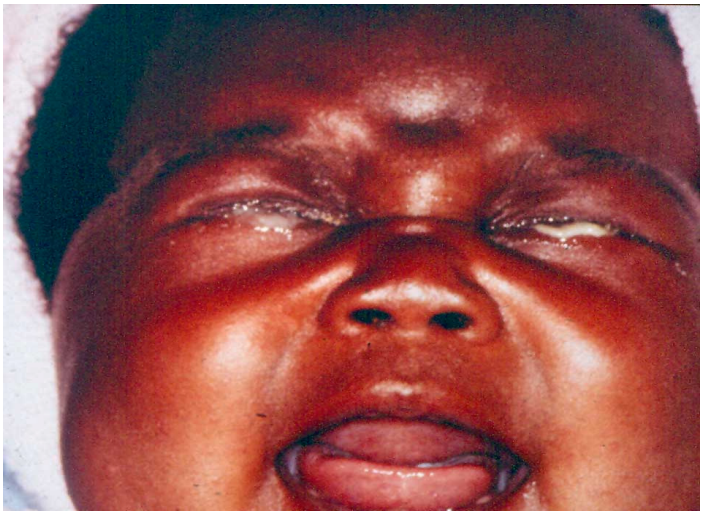


Fig 2.5: Gonococcal ophthalmia neonatorum *(Photograph credit: R. Ballard)*

Babies of infected women are potentially subject to a series of complications associated with, if not directly caused by, *N. gonorrhoeae*. These complications include foetal distress, neonatal distress, abortion, premature delivery, gonococcal conjunctivitis ("ophthalmia neonatorum") (Fig. 2.5) and pharyngeal gonococcal infection (18).

Neonatal prophylaxis may be directed against both gonococcal ophthalmia and chlamydial conjunctivitis. Instillation of a prophylactic agent into the eyes of all newborn infants is recommended. These days, antibiotic treatment with chloramphenicol ointment is the prophylactic method of choice. Tetracycline ointment, used in the past, is no longer recommended due to the high prevalence of tetracycline resistant gonorrhoea. Silver nitrate was also used in the past but has been associated with conjunctival scarning and is no longer recommended (18).

2.3.6. DISSEMINATED GONOCOCCAL INFECTION (DGI)

In a small percentage (approximately 0.5 to 3%) of infected individuals, gonococci invade the blood stream, resulting in disseminated gonococcal infection (DGI) (14, 18). Disseminated disease may also develop following infection at genital or extragenital sites, and repeated bouts of DGI have been observed in individuals with certain complement deficiencies (i.e., C7, C8 or C9) (54). DGI is more common in women than in men (18). Approximately 75% of the cases occur in women in association with menstruation or infection during the second and third trimesters of pregnancy. In men, DGI can follow either homosexual or heterosexual sexual activity. In 30 to 40% of cases, organisms from the bloodstream may localise in one or more joints to cause a purulent and destructive gonococcal arthritis. The most common forms of disseminated infection are the skin lesions and dermatitis-arthritis syndrome (75).

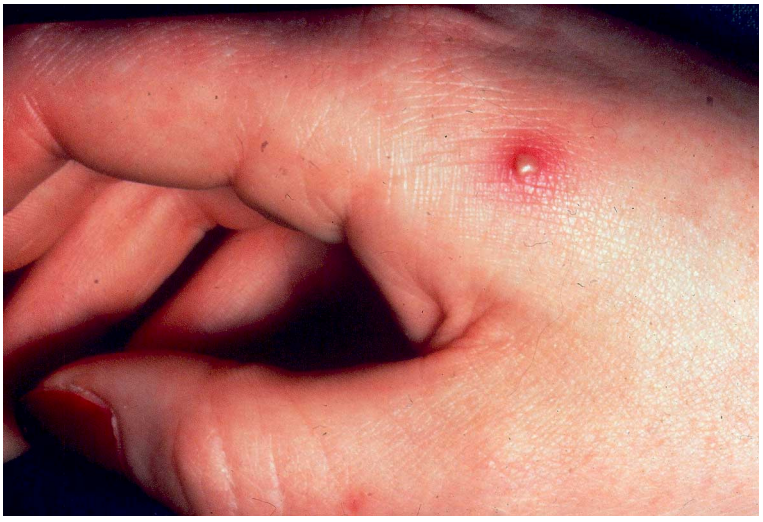


Fig 2.6: Skin lesion of disseminated gonorrhoea *(Photograph credit: J.W Harris)*

Skin lesions begin as erythematous macules, commonly 1-3mm in diameter (Fig 2.6). Typically, the skin lesions are found on the distal parts of the arms and legs. They frequently occur on the skin near the small joints of the toes or fingers and on the feet and hands. Microscopic examination of skin lesion biopsies will show superficial ulcers with pus formation and diffuse inflammation in the dermis and subcutaneous tissues. Patients with arthritis have multiple joints involved simultaneously or sequentially, and this is termed migratory polyarthritis. Knees, joints of the wrists and hands, ankles and elbows are most frequently involved. Joints which are readily accessible for examination, such as the knee, will show diffuse redness and swelling with demonstrable fluid, and needle aspiration will yield thick pus fluid (18). *Neisseria gonorrhoeae* may be isolated from blood, joint pus and/or rarely, skin lesions

2.4 MICROBIOLOGY

2.4.1. Taxonomy of the Family *Neisseriaceae*

The genus *Neisseria* belongs to the family *Neisseriaceae*, which has undergone many taxonomic changes (16). The genus *Neisseria* was assigned to the family *Coccaceae* until 1948 when the term Neisseriaceae was introduced (131). The family *Neisseriaceae* at that time also contained the strictly anaerobic *Veillonella* spp. The family *Neisseriaceae* now contains the genera *Neisseria*, *Moraxella*, *Acinetobacter*, and *Kingella* (16)**,** which are differentiated from each other by cell morphology, oxidase and catalase reactions, the presence of carbonic

anhydrase, the production of acid from glucose, the ability to reduce nitrite, the presence of thymidine phosphorylase, nucleoside deoxyribosyl transferase, and thymidine kinase, and the presence of true waxes in the cell wall (16).

The genus *Neisseria* contains species that are isolated from humans and other animals. The *Neisseria* species infecting humans have undergone few taxonomic changes. The most notable change in the taxonomy of the family has been a result of genetic studies. These led to the reassignment of *N. catarrhalis* to the genus *Branhamella* (26) and the inclusion of *B. catarrhalis* as a subgenus in the genus *Moraxella* (16). Because subgenus and subspecific epithets are not used (176)**,** strains of *B. catarrhalis* should correctly be called *Moraxella catarrhalis*. However, because *B. catarrhalis* is distinctly different from the *Moraxella* spp. in cell morphology and has recently been recognised as a pathogen, the name *B. catarrhalis* is commonly used although no formal request has been made to have the name conserved taxonomically.

2.4.2. Taxonomy of *Neisseria* spp.

The genus *Neisseria* contains 12 species and biovars isolated from humans (172). They can be identified by many characteristics, including their patterns of acid production from carbohydrates and their ability to reduce nitrate and to produce polysaccharide from sucrose. Although several human *Neisseria* species were described in the late 1800s, most were described in 1906, when von Lingelsheim cultured specimens to determine the etiology of meningitis (56).

Studies to characterise the *Neisseria* spp. were undertaken to determine the etiology of influenza, colds, and meningitis and to classify the species objectively (56, 214). During these studies, problems in identifying commensal *Neisseria* spp. were noted. It was found that colonial cell morphology could not be used for the classification of *Neisseria* spp. and that reproducible patterns of acid production from carbohydrates could not be obtained from subcultures of the same strain or from strains tested in different media (214). Attempts to classify *Neisseria* spp. were also hampered by a lack of differential tests such as the oxidase, nitrate reduction, and polysaccharide production tests (99). Because the oxidase reaction was not used as a differential test, oxidase-negative species were included in the family *Neisseriaceae* until 1974 (172). Consequently, the taxonomy of the genus has been confused and the data in many early publications on *Neisseria* spp. must be interpreted cautiously.

The human *Neisseria* spp. can be divided into two major groups. The first group includes *N. gonorrhoeae, N. meningitidis, N. lactamica, N. cinerea, N. flavescens, N. polysaccharea,* and *N. gonorrhoeae* subsp*. kochii*. Species belonging to this group generally grow as non-pigmented, translucent colonies. The yellow-pigmented species, *N. flavescens*, is the only exception to this rule. The second group of species includes the saccharolytic commensal *Neisseria* species, *N. subflava* (including the *N. subflava* biovars *perflava* and *flava*, which can be referred to as *N. perflava* and *N. flava*), *N. sicca*, and *N. mucosa*.

Colonies of these species are generally opaque, although some strains of *N. perflava* grow as transparent, non-pigmented colonies.

2.4.2.1 Morphology and basic structure of the pathogenic *Neisseria*

The *Neisseria* resemble other Gram-negative organisms in terms of cell wall structure but are simpler versions with respect to genome size and other specialised structures (18) (Fig.2.7, 2.8). These organisms are structurally distinguished from other cocci by their 'kidney-shaped' diplococcal forms. This physical attribute is acquired during the septation of cells in a longitudinal plane at cell division, so that they appear microscopically with a flattened edge rather than a rounded edge between the two cells. *Neisseria* are commonly arranged in tetrads or pairs and occasionally can be seen as individual cells (18). Gonococci do not express a true polysaccharide capsule despite several early reports to the contrary (178). Other investigators have demonstrated that capsule production appeared to be dependent on the medium composition and other conditions in which the organism are grown (69). Also that capsules may be lost by manipulation of the organism prior fixation (69). However, Noegel et al (138) have reported that gonococci do produce a surface polysaccharide capsule, including provision of a hydrophilic and negatively charged cell surface. But the function of the capsule in gonococcal biology is largely undetermined.

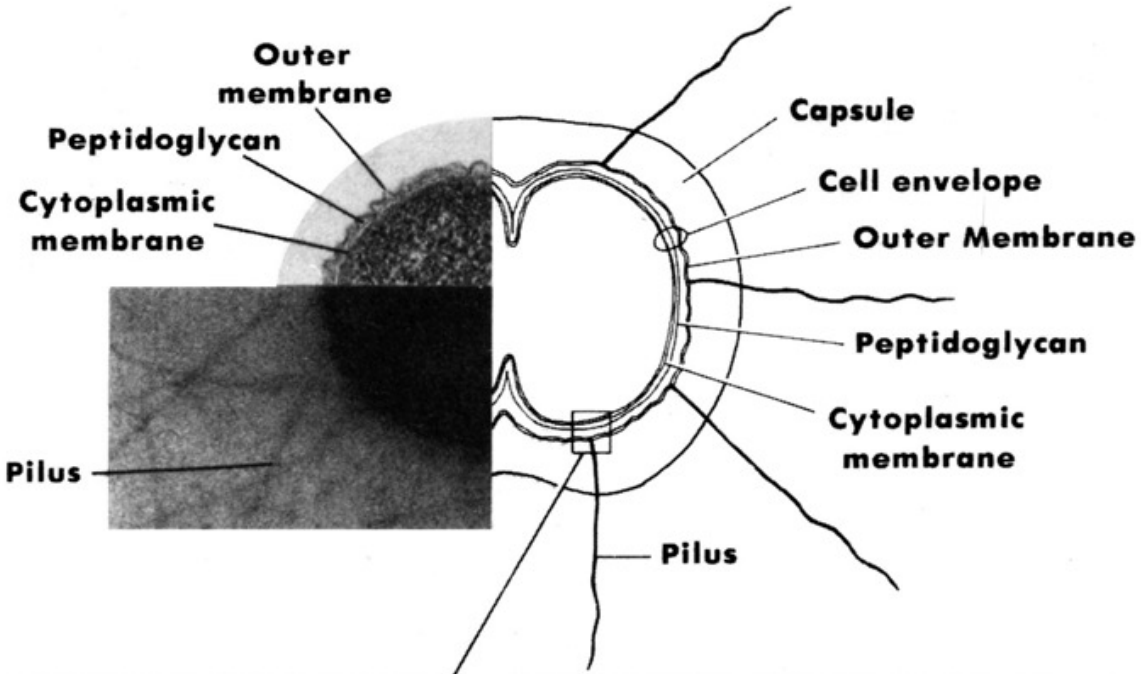


Fig. 2.7 Diagrammatic and electron micrographic representation of *Neisseria*. In the upper section of the left quadrant is a thin section transmission electron micrograph, which shows the morphology of the cell membrane. The lower left quadrant is a phosphotungstic acid negative-stain transmission electron micrograph where the cell stains black, and the pili can be seen extending from the cell. The pili are much longer than shown and would extend off the page. The right half drawing is developed from the electron micrograph and other data (18).

The cell membrane of the *Neisseria* is composed of outer membrane proteins, lipids, lipopolysaccharides and has an inner peptidoglycan matrix (Fig.2.8). Porin proteins cover the cell membrane and form aqueous channels enabling the exchange of ions, dyes, antibiotics and other larger compounds to occur (Fig. 2.8). Therefore, the cell membrane is a dynamic structure that is constantly adapting to changes within its external environment (18, 68).

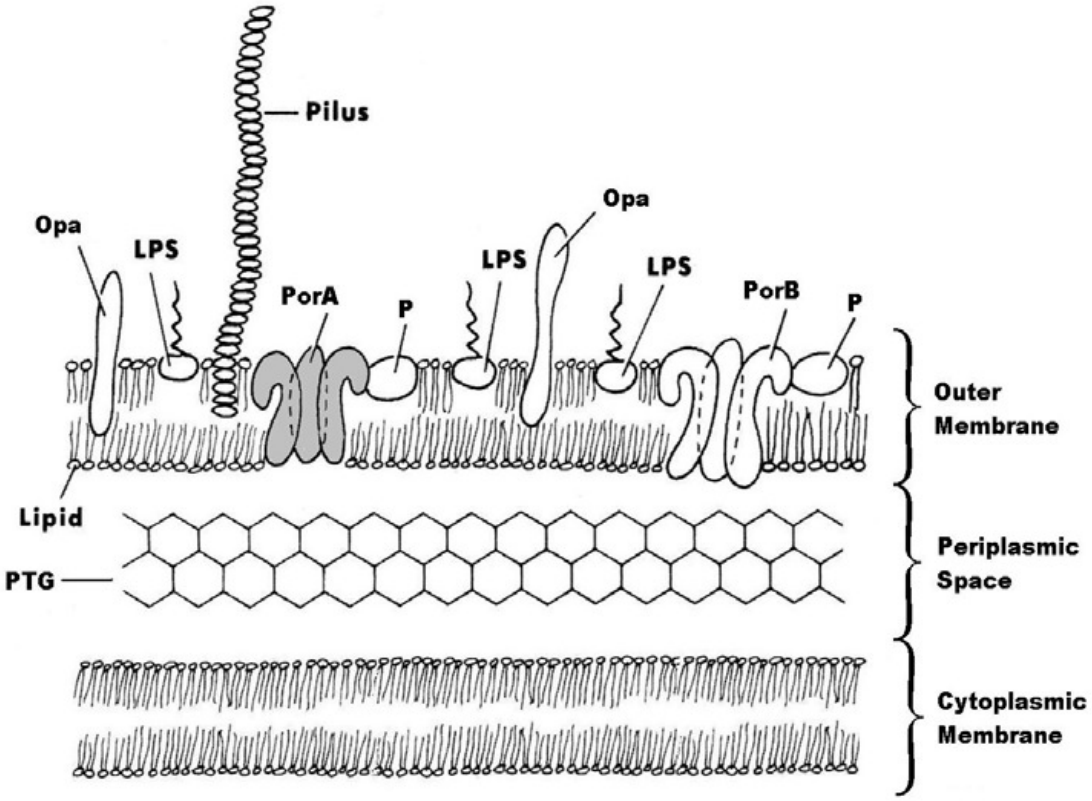


Fig. 2.8 The membrane structure of *Neisseria* showing the structural features of the outer membrane, periplasmic membrane and cytoplasmic membrane. Opa, opacity proteins; LPS, lipopolysaccharides; PorA, class 1 proteins (porin proteins); PorB, class 2, 3 proteins (porin proteins); P, proteins; PTG, peptidoglycan. This diagram was modified from Brooks *et al* (18).

2.4.3. MICROBIOLOGICAL CHARACTERISTICS OF *N. gonorrhoeae*

Neisseria gonorrhoeae is a non-motile, non-spore-forming, Gram-negative coccus that characteristically grows in pairs (diplococci) with adjacent sides flattened. As mentioned above, within clinical specimens *N. gonorrhoeae* is typically intracellular and this is an important criterion for microscopic diagnosis of gonorrhoeae in clinical setups (Fig 2.9). Growth is best for most strains at 35^{o}C

to 37°C, and many freshly isolated strains have a relative or absolute requirement for atmospheric CO_2 in concentration around 5%. The atmosphere should be moist, and, with candle jars, moisture evaporating from the medium during incubation is usually sufficient for organism growth. All strains are strictly aerobic under usual growth conditions, but the organism grows anaerobically when nitrite is provided as an electron acceptor. Colonies appear in 24 to 48 hours, but on most media viability is rapidly lost after 48 hours because of autolysis (68).

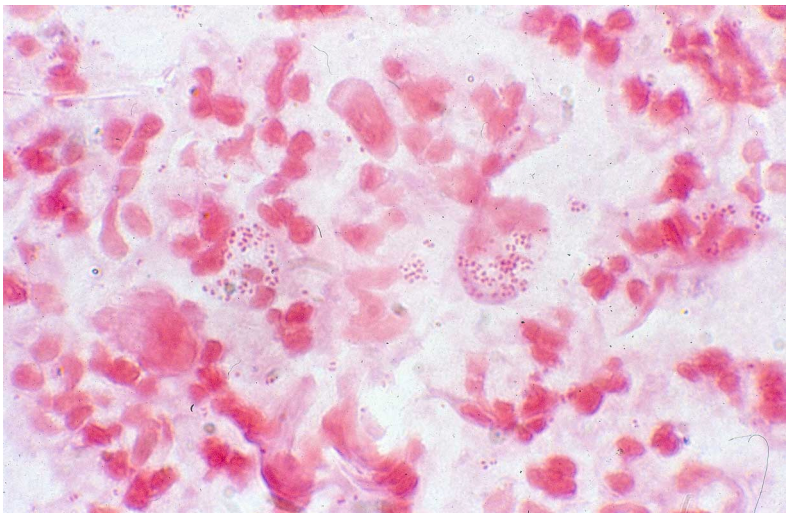


Fig. 2. 9: Gram-stained smear of urethral exudates showing Gram-negative intracellular diplococci. *(Photograph credit: D. Lewis)*

While most *Neisseria* species are not exacting in their nutritional requirements for growth, the pathogenic species, and *N. gonorrhoeae* in particular, are more nutritionally demanding. *N. gonorrhoeae* does not grow in the absence of the amino acid cysteine and a usable energy source (i.e. glucose, pyruvate or lactate). Some strains display requirements for amino acids, pyrimidines and purines as a result of defective or altered biosynthetic pathways. Demonstration

of amino acid growth requirements forms the basis of a strain typing method for gonococcal isolates called auxotyping (27).

For clinical purposes, a satisfactory growth medium is chocolate agar enriched with glucose and other defined supplements. Isolation of gonococci from sites that normally contain high concentrations of saprophytic microorganisms, especially the pharynx, rectum, and cervix, may be difficult because of overgrowth of the hardier normal flora, a problem that is largely overcome by use of media containing antimicrobial agents that inhibit most nonpathogenic *Neisseria* and other species but permit growth of most strains of *N. gonorrhoeae*, *N. meningitidis* and *N. lactamica* (190).

A variety of enriched selective media for culture of *N. gonorrhoeae* are available and include modified Thayer-Martin (MTM) medium, Martin-Lewis (ML) medium, GC-Lect medium (BD Biosciences), and New York City (NYC) medium. MTM, ML, and GC-Lect media are chocolate agar based media that are supplemented with GC agar base and haemoglobin for the growth of fastidious microorganisms, whereas NYC medium is a clear peptone-corn starch agar-based medium containing yeast dialysate, citrated horse plasma, and lysed horse erythrocytes (178). These media contain antimicrobial agents that inhibit other microorganisms and allow the selective recovery of *N. gonorrhoeae, N. meningitidis* and *N. lactamica*. Vancomycin and colistin, antimicrobials present in all four formulations, inhibit Gram-positive and Gram-negative bacteria (including

saprophytic *Neisseria* species), respectively. Trimethoprim is added to inhibit the swarming of Proteus spp. present in rectal and, occasionally, in cervicovaginal specimens. Nystatin, amphotericin B, or anisomycin is added to inhibit yeasts and molds. NYC medium also supports the growth of genital mycoplasmas and ureaplasmas (178).

2.4.3.1 Examples of genetic adaptability in *N. gonorrhoeae*

The linked features of adaptability and transmissibility are key elements for survival in these highly evolved bacteria. Examples include the genetic and phenotypic hypervariability of the cell surface structures important in mucosal cell adherence and attachment. The genetic hypervariability seen in these structures contributes significantly to the 'non-clonal' nature of the pathogenic *Neisseria* and include the pilin, porin and the outer membrane proteins (OMP) (Figs. 2.7 and 2.8) (36).

2.4.3.1.1 Pili

Pili are microscopic hair-like structures surrounding the bacterial cell and the pilus assembly apparatus is activated for the transfer of DNA (17). Piliated cells also facilitate the expression of certain proteins to the cell surface necessary for the processes of transformation and the uptake of DNA (121). Each pilus is composed of repeating protein subunits and with hypervariable regions exposed to the cell surface (Fig. 2.8). Pili gain their variability from the rearrangement of

the expressed gene together with numerous copies of 'silent' genes. These silent genes are distributed throughout the genome as resources for adaptation (121).

2.4.3.1.2 Porin protein 1

PorB is the Omp1 equivalent porin in gonococci and is present as two mutually exclusive alleles, IA or IB. Its role is to function as a co-factor in the invasion of epithelial mucosa by forming a cell-to-cell channel regulating calcium ion intake (Fig.2.8). Calcium ion influx is a stimulatory signal for gonococcal entry and invasion (121). Porin proteins are comprised of highly conserved outer membrane sequences interspersed with hypervariable surface regions**.** These hypervariable regions allow protein PorB to undergo antigenic shifts via allelic variation, which can furthermore assist in the evasion of host responses (20). For example, gonococci possessing porin protein 1 of the protein 1A type, which are classically associated with disseminated gonococcal infections (DGIs), have the advantage of resisting serum-mediated killing of the organism (36, 164).

2.4.3.1.3 Opacity proteins

Opacity proteins (Opa) govern the colonial morphology of gonococci when cultivated on special media (opaque or transparent) and contribute to the mucosal binding and invasion of host epithelial cells. They are highly heterogenous proteins with approximately eleven gene copies present in the gonococcus (Fig. 2.8). *Opa* gene expression is independently controlled by a site-specific genetic mechanism (termed 'slipped strand mispairing') so that

within a few generations a single bacterium can simultaneously produce several, one, or none of these Opa proteins. Consequently, this genetic variation produces heterogenous populations, which are hypervariable in antigenic nature and opacity proteins (180).

2.4.3.1.4 Antibiotic resistance

Another important resource for bacterial survival is the acquisition of antibiotic resistance genes and those encoding antibiotic resistance may be of chromosomal or extrachromosomal (plasmid) origin. However, it would appear gonococci are different from other Gram-negative bacteria, in that their most resistant phenotype is not preferentially expressed. Rather, the organism seems to select and express at random an appropriate phenotype in response to its host and environmental influences (20). The sudden emergence of two gonococcal phenotypes (Mtr and Env) in response to the toxic nature of long chain fatty acids and bile salts present in the bowel, illustrates this well (20). The Mtr phenotype (multiple transferable resistance) expresses broad-spectrum resistance to these fatty acids, dyes and unrelated drugs, while Env (envelope), the antithesis, expresses hypersensitivity to precisely the same agents. Both of these phenotypes enhance bacterial survival in different environmental conditions.

2.4.4 Diagnostic and laboratory-based tests

Sexually transmitted infections (STIs) are most easily diagnosed using laboratory tests. These tests, however, require sophisticated equipment which most health

care settings in resource-poor countries can hardly afford. Factors determining the choice of diagnostic test for *N. gonorrhoeae* include test sensitivity and specificity, ability to assess antimicrobial susceptibility, ease of specimen collection, cost, biological site tested, tolerance of possible non-culture false positive results, specimen transport and laboratory capability.

2.4.4.1. Gram Stain

Gram stain is the key tool for the diagnosis of gonococcal urethritis in men, but its application to screening for asymptomatic infection is limited because of the requirement for uncomfortable endourethral swab specimen. A Gram stain of the presumptive diagnosis of *N. gonorrhoeae* infection is performed on thin smears of urethral exudates from men and is presumptively positive if the smear contains typical Gram-negative diplococci within polymorphonuclear (PMN) leukocytes (Fig.2.9). Unfortunately, other *Neisseria* species have similar morphological appearances which negate the use of microscopy for pharyngeal specimens (see below). Although commensal *Neisseria* species are not normal flora of anogenital sites, isolates of *Neisseria meningitidis* and non-pathogenic *Neisseria* species have been reported occasionally from anogenital sites among both men and women (178).

In men, Gram stain of urethral smears has a sensitivity of >95% in symptomatic patients, and lower in asymptomatic patients (50-75%) (178). Gram stain of endocervical smears in women has a sensitivity of between 30-50%. In the

hands of an experienced technician this method results in a sensitivity of >99% (65). However, in less experienced hands the specificity may fall to about 50% (65). The normal flora of the female genital tract often includes Acinetobacter species which are Gram-negative and may look like gonococci. Acinetobacter can yield false positive Gram stain smears of cervical exudates (65).

Direct smears of the pharynx and rectum are not considered to be reliable specimens primarily because the normal flora in these areas obscures gonococci or yield a high frequency of false-positive results. In particular, the diagnosis from direct stains of pharyngeal specimens is inappropriate owing to the variety and numbers of non-pathogenic *N. meningitidis* and commensal *Neisseria* species that colonise this area (50). The Gram stain can only be used as a near-patient test to provide an immediate presumptive diagnosis of gonorrhoea in symptomatic genital infections.

2.4.4.2. Culture Tests

Methods of gonococcal culture involves streaking specimens on a selective (e.g., Thayer-Martin or Martin-Lewis) or nonselective (e.g., chocolate agar) medium if specimens are from nonsterile or sterile sites, respectively. Inoculated media are incubated at 35°C-36.5°C in an atmosphere supplemented with 5% CO_2 and examined at 24 hour intervals for up to 72 hours (49, 178). Supplemental CO_2 can be supplied by a CO_2 incubator, candle-extinction jar, or CO_2 –generating tablets (86). As described in section 2.4.3, media for *N. gonorrhoeae* isolation

usually contains a supplemented base medium and may be made selective by the inclusion of antibiotics.

A presumptive identification of *N. gonorrhoeae* isolates recovered from a genital specimen on selective medium can be made with a Gram stain and oxidase test. A presumptive identification indicates only that a Gram-negative, oxidase positive diplococcus (e.g. a *Neisseria* species or *Moraxella catarrhalis*) has been isolated. A confirmed laboratory diagnosis of *N. gonorrhoeae* cannot be made on the basis of these tests alone. A presumptive test result is sufficient to initiate antimicrobial therapy in the context of clinically compatible symptoms, but additional tests must be performed to confirm the identity of an isolate as *N. gonorrhoeae* (82).

The advantages of culture are high sensitivity and specificity, low cost, suitability for use with different types of specimens, and the ability to retain the isolate for additional testing. Retention of the isolate might be indicated for medicolegal purposes, antimicrobial susceptibility determination, and subtyping of isolates. The major disadvantage of culture for *N. gonorrhoeae* is that the specimen must be transported under conditions adequate to maintain the viability of organisms. Another disadvantage is that a minimum of 24-72 hours is required from specimen collection to the report of a presumptive culture result (82).

2.4.4.3. Culture Confirmation Tests

2.4.4.3a. Sugar Utilisation Testing

The traditional method of identification of this pathogen relies on its unique pattern of carbohydrate utilisation in cysteine trypticase agar (CTA) sugars (93). *N. gonorrhoeae* can be differentiated from other oxidase-positive Gram-negative diplococci as it produces acid from glucose alone, whereas other members of the *Neisseria* genus such as *Neisseria lactamica* and *Neisseria meningitidis* produce acid from both glucose and maltose. CTA method is based on fermentative species and is not sensitive enough to detect acid from oxidative species. CTA sugars are a cost-effective method of identification, but they require a heavy inoculum and prolonged incubation and can be difficult to interpret. Additionally, glucose negative *N. gonorrhoeae* isolates have been reported raising concerns about false-negative results, because CTA sugars have difficulty in differentiating between *N. gonorrhoeae* and *N. cinerea* (99). Some of these difficulties have been circumvented by the development of alternative rapid methods based on the presence of preformed enzymes for carbohydrate degradation. The rapid carbohydrate test is a nongrowth-dependent method for the detection of acid production from carbohydrates by Neisseria species. The four carbohydrates utilisation test systems widely used are: Neisseria Kwik test (Micro Bio Logics, St. Cloud, Minn.); Rapid Identification Method for Neisseria (RIM-N; Austin Biological Laboratories, Inc., Austin, Tex.); Gonobio Test (I.A.F. Production Inc., Lava, Quebec, Canada) and Minitek kit (Miniaturized Microbiology Differentiation System; BBL Microbiology System, Cockeysville) (49). The evaluations of the

Rapid Identification Method for Neisseria have compared well with the conventional method, but may also not differentiate between *N. gonorrhoeae* and *N. cinerea* (85). The rapid non-growth tests have a specificity of 99% to 100% and are more sensitive than the CTA sugars. Some of the commercial tests include not only acid production tests, but also for other biochemical characteristics (such as enzyme production), including DNase and nitrate reduction. All tests must include appropriate control strain (85).

2.4.4.3b. Immunological Testing

The three most widely used immunological kits (49) are the MicroTrak *N. gonorrhoeae* culture confirmation test (An immunofluorescence test; Trinity Biotech, Bay, Ireland), the Phadebact Monoclononal GC test (A co-agglutination test; Pharmacia, Uppsala, Sweden) and GonoGen II (A membrane immunoassay; Key Scientific, Columbia, Texas), all of which employ monoclonal antibodies raised to specific epitopes on the two types of the major outer-membrane protein, PIA and PIB (36). Whilst the specificity of these kits has been shown to be very high, *N. gonorrhoeae* strains giving negative results have been reported (49, 163).

2.4.4.3c. Biochemical Testing

One of the most common method routinely used in clinical microbiology settings for the identification of *N. gonorrhoeae* is the detection of preformed enzymes. The use of preformed enzymes as a tool for the differentiation of *Neisseria spp*

was first described by D'Amato et al (50), who reported the production of the enzymes gamma glutamyl transferase (GGT) by *N. meningitidis* and proline aminopeptidase (Pip/ProA) by *N. gonorrhoeae* (50). Molecular cloning and characterisation of the *N. gonorrhoeae pip* gene was performed in 1993 where it was confirmed that *pip* was a single copy gene and, while non-essential for growth *in vitro* was present in virtually all isolates of *N. gonorrhoeae* (1). Biochemical tests that detect the presence of preformed enzymes should be interpreted with caution because proline aminopeptidase (Pip/ProA)-negative *N. gonorrhoeae* have been reported (3). Consequently Pip/ProA negative gonococcal isolates generate ambiguous identifications ranging from "doubtful *N. gonorrhoeae"* to "presumptive *Kingella kingea*" when examined using these kits**.** False positive *N. gonorrhoeae* identification can also be generated when using these kits as many non-pathogenic *Neisseria spp* are Pip/ProA positive and some have been shown to grow well on selective media (97). However despite these reports the use of preformed enzyme based kits remains popular and they are widely used for the routine identification of *N. gonorrhoeae*.

A range of commercially available biochemical kits are also widely used, including the *Neisseria* preformed Enzyme Test (PET, Key Scientific, Columbia, Texas), Gonochek II (E-Y Laboratories, SanMateo, CA), RapID NH (Remel, Lenexa, KS) and API NH (bioMérieux, l"Etoile, France). The *Neisseria* PET and Gonochek II are both single-use tubes containing chromogenic substrates that detect the presence of three preformed enzymes, each of which is indicative of a pathogenic Neis*s*eria species, namely *N. lactamica*, *N. meningitidis* and *N.*

gonorrhoeae. The API NH and RapID NH kits employ a battery of tests, combining carbohydrate utilisation and preformed enzymes.

2.4.4.4. Nucleic Acid amplification Tests (NAATs)

In the early 1990s, nucleic acid tests became available for routine use. These include both nucleic acid hybridisation assays and nucleic acid amplification tests (NAATs). The hybridisation assays include the Gen-Probe PACE II (Gen-Probe, San Diego, CA)) and the Digene Hybrid Capture II assays (Digene Corp., Beltsville, MD). These assays use a specific oligonucleotide probe to hybridise directly to *N. gonorrhoeae* nucleic acid present within a specimen. Reported sensitivity and specificity values of the hybridisation assays showed that these may be below that of bacterial culture (102). To date, there have been four main commercial *N. gonorrhoeae* NAAT assays, including the Roche Cobas Amplicor (Roche Molecular Systems, Branchburg, NJ), the Gen-Probe APTIMA Combo 2 (AC2; Gen-Probe), the Becton Dickinson ProbeTec assay (Becton Dickinson, Sparks, MD), and the Abbott Ligase Chain Reaction (LCx) (Abbott Laboratories). All of these use duplex NAAT assays, targeting both *C. trachomatis* and *N. gonorrhoeae*. In addition, each of these duplex assays has used a unique *N. gonorrhoeae* gene target and amplification technology. The Abbott LCx has previously been recalled because of manufacturing issues (30). In addition to the commercial assays, numerous in-house *N. gonorrhoeae* NAAT assays have also been described (33, 58, 182, 213). These have primarily used polymerase chain

reaction (PCR), have targeted various *N. gonorrhoeae* genes, and for the most part, have not been multiplexed with other assays.

Real-time PCR assays have been widely used for the detection of Neisseria gonorrhoeae (182). The real-time PCR system is based on the detection and quantitation of a fluorescent reporter (35). The signal increases in direct proportion to the amount of PCR product in a reaction. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. There are four main fluorescent-monitoring systems for DNA amplification: (i) Taqman Probes; (ii) Molecular Beacons; (iii) Scorpions and (iv) SYBR® Green (35)

TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the 5' end of probe which contains the reporter dye (35). Then the activity of quencher (no FRET) and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye.

Molecular beacons are similar to TaqMan probes but are not designed to be cleaved by the 5' nuclease activity of Taq polymerase (35). These probes have a fluorescent dye on the 5' end and a quencher dye on the 3' end of the

oligonucleotide probe. A region at each end of the molecular beacon probe is designed to be complementary to itself, so at low temperatures, the ends anneal, creating a hairpin structure. This integral annealing property positions the two dyes in close proximity, quenching the fluorescence from the reporter dye. The central region of the probe is designed to be complementary to a region of the PCR amplification product (35).

The scorpion probe maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also contains sequence that is complementary to the extension product of the primer. This sequence is linked to the 5' end of a specific primer via a non-amplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicons thus opening up the hairpin loop. This prevents the fluorescence from being quenched and a signal is observed. Thus, with Scorpion primer/probes, sequence-specific priming and PCR product detection is achieved using a single oligonucleotide.

Hybridization probes are two DNA probes designed to anneal next to each other in a head-to-tail configuration on the PCR product (35). The upstream probe has a fluorescent dye on the 3' end and the downstream probe has an acceptor dye on the 5'end. If both probes anneal to the target PCR product, fluorescence from the 3'dye is absorbed by the adjacent acceptor dye on the 5' end of the second

probe. The second dye is excited and emits light at a third wavelength and this third wavelength is detected. If the two dyes do not align together because there is no specific DNA for them to bind, then FRET does not occur between the two dyes because the distances between the dyes are too great. A design detail of hybridization probes is the 3' end of the second (downstream) probe is phosphorylated to prevent it from being used as a primer by Taq during PCR amplification (35).

There are several advantages of *N. gonorrhoeae* NAATs. First, they offer improved sensitivity compared with bacterial culture. When compared with *N. gonorrhoeae* NAATs, gonococcal culture ranges in sensitivity from 85 to 95% for acute infections and may fall as low as 50% for females with chronic infection (11). The increased sensitivity of NAATs makes them particularly suitable for screening, enabling accurate diagnosis of both symptomatic and asymptomatic gonococcal infections, which is critical to control of the disease (88). Secondly, specimens collected for NAAT assays do not require the organism to be viable for detection and so require less stringent transport conditions compared with those collected for bacterial culture. Finally, NAATs can be used effectively on noninvasive specimens such as urine and self-collected specimens. This is particularly useful for patients in remote regions where sexual health services may not be available and for special populations where religious or cultural restrictions otherwise restrict opportunities for specimen collection (61).

N. gonorrhoeae NAATs do have some limitations. These include the typical problems associated with the use of NAAT protocols, such as high cost (88), carryover contamination (88); inhibition of the reaction (191); high quality control requirements (22); and the absence of antibiotic resistance data (59). More importantly, there are sequence-related limitations that are unique to *N. gonorrhoeae* NAATs and include the generation of both false-negative and false positive results. This is because target sequences may either be absent in some *N. gonorrhoeae* subtypes or otherwise present in some commensal *Neisseria* strains. These limitations have lead to recommendations that *N. gonorrhoeae* NAAT-positive results should be confirmed by a 2nd NAAT before a positive result is reported (173). Overall, there is a broad range of technical challenges associated with the successful implementation of *N. gonorrhoeae* NAATs.

Since *N. gonorrhoeae* is genetically a highly diverse species it should therefore be considered that, no single gonococcal NAAT target may be sufficiently conserved across all gonococci (12). For this reason, it may be prudent to also use a 2-target system for routine NAAT detection of *N. gonorrhoeae*. In addition, the 2-target system of the assay decreases the potential for sequence-related false negatives and can provide simultaneous confirmation of positive results.

2.5 TYPING METHODOLOGIES

Knowledge of gonococcal strains circulating in a community and of temporal changes in prevalent strains can identify patterns of transmission of gonorrhoea

and guide prevention and control efforts (208). A number of typing methods have been developed for *N. gonorrhoeae* based on phenotypic and genotypic characterisation of the bacterium. The ability of a typing scheme to distinguish between unrelated isolates measures its discriminatory power (80, 171). The discriminatory power is determined by the number of types defined by the test method and the relative frequencies of these types. Hunter and Gaston (80) proposed a single numerical index of discrimination, based on the probability that two unrelated isolates would be placed into different typing groups. This probability can be calculated from Simpson's index of diversity. If typing results are to be interpreted with confidence, a discriminatory index (DI) of greater than 0.90 is desirable (80,171).

2.5.1 Phenotypic characterisation of *N. gonorrhoeae* isolates

2.5.1.1 Auxotyping

Auxotyping is the characterisation of gonococcal strains according to their nutritional requirements. The method was first developed by Catlin (27), where a chemically defined media was developed for *N. gonorrhoeae* growth requirements and used for gonococcal typing. Gonococci exhibited a diversity of nutritional requirements; strains were subdivided into distinct clusters (auxotypes) on the basis of their patterns of growth responses to thiamine, proline, arginine, methionine, isoleucine, and hypoxanthine (27).

Subsequently, other auxotyping systems have been developed which differ with respect to medium composition and the requirements determined (70). A large number of gonococcal auxotypes have been described and their geographical distribution has been extensively studied (70, 204).

Gonococcal isolates that have no nutritional requirements are designated non-requiring (NR), Zero, prototrophic (Proto), or wild type and those that require proline (Pro) are prevalent world wide. Arginine-requiring (Arg) isolates are widely distributed geographically but are less numerous. Isolates with multiple requirements have been isolated. These include, most notably, the arginine-, hypoxanthine-, plus uracil-requiring (AHU) and the proline-, citrulline- (arginine-) plus uracil-requiring (PCU or PAU) isolates (162).

The AHU isolates, which were isolated infrequently prior to the 1950s, were found most frequently in the mid-1970s, when they accounted for as many as 50% of isolates in cities in the United States and Denmark (98). AHU isolates were frequently found in areas geographically adjacent to the cities in which they were prevalent (95). In 1977 to 1978, PAU isolates accounted for approximately 40% of isolates in Ontario, Canada (70). The PAU isolates have been spread to a limited number of geographical areas; they occurred frequently in other cities in Canada, the United States, Europe, and Japan (95).

Strains belonging to certain auxotypes were found to be associated with specific disease syndromes and antibacterial resistance. AHU isolates were frequently isolated from disseminated gonococcal infection (DGI) patients in many cities in the 1970s (128). AHU isolates are serum resistant (164) and highly susceptible to penicillin (128). PAU isolates have been associated with disseminated and asymptomatic gonococcal infections in Winnipeg, Manitoba, Canada (70). In contrast to the AHU isolates, PAU isolates are less susceptible to antimicrobial agents (70).

In summary, auxotyping which is based on the nutrient growth requirement of strains, is complicated, labour-intensive, and time consuming.

2.5.1.2 Plasmid profiles

Bacterial plasmids are units which replicate independently of the bacterial chromosome. They are generally less than 1/20 the size of the bacterial chromosome, and also contain the information for self replication (57). The plasmids of *N. gonorrhoeae* have been described in relation to their mobilities in agarose gels. The cryptic 2.6-MDa plasmids from *N. gonorrhoeae* were first described in 1972 (57). This plasmid occurs in a majority of clinical isolates [96%], excluding isolates of the proline-, citrulline-, and uracil-requiring auxotype are plasmid free, and yet no function has been associated with the cryptic plasmid (48). Six types of β-lactamase plasmids, the 4.4-MDa (Asia), 3.2-MDa (Africa), 2.9-MDa (Rio), 3.05-MDa (Toronto), 4.0-MDa (Nîmes) and 6.5 MDa

(New Zealand) plasmids, have been identified in penicillinase-producing *N. gonorrhoeae* (PPNG) strains (142). Two conjugative plasmids, a 24.5-MDa and a 25.2-MDa *tetM*-containing plasmid (possessed by high-level tetracycline resistant *N. gonorrhoeae* or TRNG, strains), have also been described (62).

Plasmid profiles, in conjunction with auxotyping and more recently with serological classification, have permitted the characterisation of isolates from different geographical areas and the documentation of temporal changes in their distribution and prevalence (204). However, plasmid profiling is of limited value when a common plasmid or a common combination of plasmids is present (41). Also, since plasmids are mobile genetic elements, they may be lost or acquired independently of chromosomal genetic change. For example, the loss of resistance plasmids has previously been associated with fluoroquinolone use (89).

2.5.1.3 Serological classification of gonococcal strains

2.5.1.3.3 **Serological classification with polyvalent antibodies**.

Serological typing methods for *N. gonorrhoeae* have been developed and refined during the last decade. Wang *et al*. (210) developed a microimmunofluorescence test with polyvalent antibodies against formalinised whole gonococcal cells that divided gonococcal strains into three groups, designated A, B, and C (Fig 2.10). Subsequently, a coagglutination test permitted Sandström and Danielsson to divide gonococci into three serologically distinct groups, designated WI, WII, and

WIII, that corresponded to the Wang serogroups A, B, and C, respectively (Fig 2.10) (159,161).

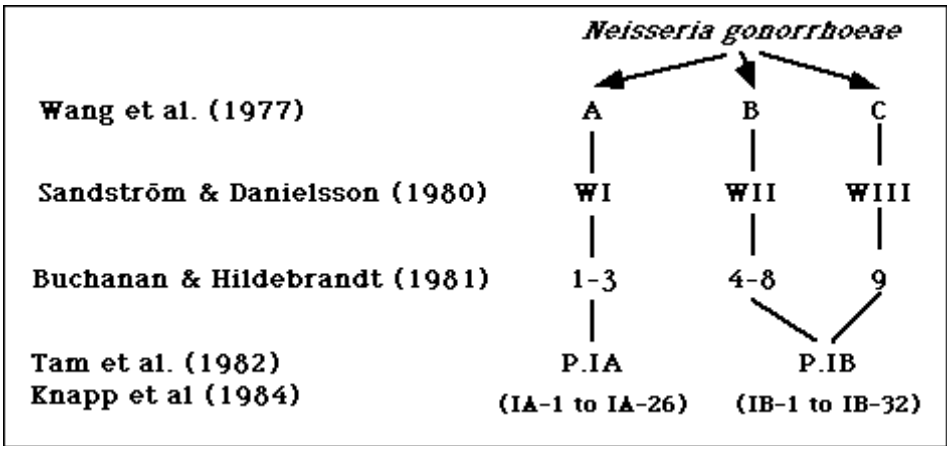


Fig. 2.10 Development of serological schemes for *N. gonorrhoeae* classification (http://www.cdc.gov/std/Gonorrhea/lab/sero.htm)

In 1981, Buchanan and Hildebrandt developed an enzyme linked immunosorbent assay (ELISA) with partially purified gonococcal protein I (outer membrane protein, formally designated P.I but now designated Por) and divided gonococcal strains into nine principal outer membrane protein (POMP) serotypes (Fig 2.10) (21). Serotypes 1 to 3 corresponded to serogroup WI, serotypes 4 to 8 corresponded to serotype WII and serotype 9 corresponded to serogroup WIII, respectively.

The proportion of isolates belonging to the different W serogroups varied among geographical areas worldwide. For example, both penicillinase-producing *N. gonorrhoeae* (PPNG) and non-penicillinase-producing *N. gonorrhoeae* (non-PPNG) isolates belonging to serogroup WII and WIII were generally more

resistant to other antibiotics than were isolates belonging to serogroup WI (23). Gonococcal strains of serogroup WII have been found to be associated with homosexually acquired rectal infections, whereas in one study WII/III strains were observed more often in women with concomitant rectal infection than in those without (37, 149).

2.5.1.3.2 Serological classification with monoclonal antibodies.

Tam *et al.* (183) developed monoclonal antibodies against gonococcal outer membrane proteins. Antibodies specific for epitopes on Porin molecules, P.IA or P.IB, were selected by screening against W-serogrouping reference strains in coagglutination tests and confirmed by radioimmune precipitation assays (Fig 2.10) (183).

Numerous serovars exist, reactive to various monoclonal antibodies. A standard panel of six P.IA-specific and six P.IB-specific monoclonal antibody reagents was subsequently selected (96). Strains characterised serologically by their reaction patterns with these reagents were designated as serovars (96). For example, by employing a set of monoclonal antibodies against PorA strains and another set against PorB strains, one can subdivide each of the serogroups into a wide variety of serovars (e.g., P.IA-6, P.IB-1), differ in their ability to react to certain members of the panel of monoclonal antibodies. Today, hundreds of specific serovars have been defined by these techniques (96).

The widely used serotyping of *N. gonorrhoeae* has some limitations. Over long periods of time, the reproducibility of this technique was less than 100% (64). Previous studies have also shown that serotyping may not be sufficiently discriminatory; because, some strains are nontypable (205).

2.5.1.4 A/S classification of *N. gonorrhoeae*.

Both auxotyping and serological classifications lack the discriminatory power to differentiate gonococcal isolates. To overcome the limitations of either of these methods, an auxotype-serovar (A/S) classification system has been proposed (96). This dual classification system, based on two independent phenotypic characteristics that are stable *in vitro*, provide a greater resolution among gonococcal isolates than does a system based on one phenotypic characteristic. For example, an isolate that requires proline and belong to serovar IB-4 is assigned to the A/S class Pro/IB-4. The A/S classification of *N. gonorrhoeae* has been used alone or in conjunction with plasmid profiles and antimicrobial susceptibilities to perform detailed analyses of gonococcal strain populations. The A/S classification provides a discriminatory classification system for gonococcal isolates but does not always distinguish between epidemiologically related and unrelated isolates (141).

2.5. 2 Genotypic characterisation of *N. gonorrhoeae* isolates

2.5.2.1 Restriction endonuclease analysis (REA)

The first employed genotypic technique was restriction endonuclease (RE) analysis, which consists of extracting DNA from the organism and digesting the DNA *in vitro* with an appropriate restriction enzyme, followed by electrophoretic separation of the DNA fragments in either agarose gel or polyacrylamide gel. REs that recognise and cleave double stranded DNA at specific sites are used to digest DNA strands into a unique set of fragments. These patterns constitute a characteristic fingerprint for any particular DNA examined. Although several extraction procedures are available, problems such as mechanical shearing are often encountered during DNA extraction/isolation. Thus RE digestion patterns generated from such sheared DNA may not be through reflection of specific site cleavages by the REs used (147). Also resolution of DNA bands may be difficult unless the technique employs enzymes that cut rare DNA sequences.

2.5.2.2 Random-primed PCR

Another method is termed random-primed PCR which uses short DNA primers that bind to multiple sites to generate a polymerase chain reaction-based ladder of DNA products, the sizes of which depend on the spacing of the homologous sequences scattered about the chromosome (24).

2.5.2.3 OPA typing

The most extensively tested and discriminatory of the molecular techniques is the *opa* gene typing method (OPA typing) of O'Rouke *et al. (*141). For this method the 11 *opa* genes are amplified with a single pair of primers, the products are

digested with frequently cutting restriction enzymes, the restriction fragments are separated on polyacrylamide gels, and the patterns of bands produced by different strains are compared. The principal limitation of this method, as well as the other genetic typing methods based on the analysis of band patterns, is that the restriction fragment patterns are difficult to analyse objectively and are not easily stored in a data base.

2.5.2.4 *por* sequencing

The *N. gonorrhoeae* outer membrane protein PorB is universally present, is constitutively expressed at the cell surface, and does not undergo high frequency variation during the course of infection in smaller groups of sexual contacts (202). The antigenic expression of PorB within a strain is stable: however, diversities between strains form the basis for serogroup and serovar determination with monoclonal antibodies (MAbs) (96, 160).

Attempts to map the epitopes of PorB recognised by serovar-specific MAbs by using amino acid alignments of the mature proteins as well as synthetic peptides have been published, but many epitopes remain unidentified (38). Thus, for several of the widely used MAbs, the exact antigenic epitopes of PorB are not identified. A two dimensional structural model of the topology of the porin PorB within the outer membrane has previously been predicted (202). The model predicts eight surface-exposed loops, which exhibit an extensive variation in

length and amino acid sequence and which are interspaced with nine more conserved predominately transmembrane or interspacing regions.

The PorB proteins are classified into two different groups, PorB1a or PorB1b, on the basis of immunological and sequence homology. Any individual strain expresses only one of the groups, either PorB1a or PorB1b (202). However, naturally occurring strains that express PorB1a/PorB1b hybrids have been identified (38). The proteins PorB1a and PorB1b are encoded by mutually exclusive alleles of the *porB* gene, *porB1a* and *porB1b*, respectively.

POR sequencing is based on the PCR amplification and sequencing of the entire *porB* gene followed by the analysing of exclusively shorter highly variably regions of the gene comprise a powerful method for genetic typing of *N. gonorrhoeae*. However sequencing of the whole *por* gene would be labour intensive, and most of the variation within *por* is likely to be captured by sequencing an internal fragment of the gene, which requires a single sequencing reaction for each DNA strand (199). Pyrosequencing, a recently described fast real-time DNA sequence analysis technology, has been used to sequence and analyse exclusively shorter highly variable regions of the *porB* gene (148).

2.5.2.5 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) was developed by Maiden *et al.* (114) for the naturally transformable Gram-negative pathogen *Neisseria meningitidis* but has

since been applied to many pathogenic species (201). The procedure is essentially an updated version of multilocus enzyme electrophoresis (MLEE), which indexes variation within multiple core metabolic ('housekeeping') genes on the basis of differing electrophoretic mobilities of the gene products (166). A serious drawback of MLEE, and other gel-based methods such as pulsed-field gel electrophoresis (PFGE), is that it is often difficult to compare results between laboratories. This problem does not arise with MLST because variation within a sample of housekeeping genes is indexed directly by the nucleotide sequencing of internal gene fragments.

The advantage of nucleotide sequencing is that it is a generic technology, the results of which are easily validated, stored and shared electronically. For MLST analysis, all unique sequences for a given locus are assigned an allele number in order of discovery; this is equivalent to the designation of 'electromorphs' in MLEE. The alleles present at each of the MLST loci for a given isolate are combined into an allelic profile and assigned a sequence type (ST) designation, equivalent to the 'electrophoretic type' (ET) designation used in MLEE (114, 166). The higher discrimination of nucleotide sequencing means that compared with MLEE, MLST can attain similar levels of discrimination with fewer loci (201). Relationships among isolates are apparent by comparisons of allelic profiles. Closely related isolates have identical STs, or STs that differ at a few loci, whereas unrelated isolates have unrelated STs.

Application of the MLST scheme to *N. gonorrhoeae* is therefore advantageous as it can be used to analyse genetic relationships among gonococcal isolates, as well as among the *Neisseriae*. Another advantage of MLST is its ability to discriminate among species, facilitating species identification, and the detection of mixed bacterial cultures (12).

2.5.2.6 *N. gonorrhoeae* Multi-Antigen Sequence Typing (NG MAST)

NG MAST technique is based on the sequencing of the internal fragments of two highly polymorphic antigen-encoding loci, *por* and *tbpB*, thereby generating a simple numerical sequence type (ST) from the combined sequence data of the two genes by means of an internationally accessible web based data analysis system http://www.ng-mast.net (116, 133).

As mentioned previously, the *por* gene encodes the gonococcal outer membrane porin. A typing method based on the complete sequence of this highly variable *por* gene has been evaluated and provide a substantial level of discrimination between isolates (148, 199). The *tbpB* gene encodes the β subunit of the transferring-binding protein, a surface-exposed peripheral component of the outer membrane that binds to the human iron binding protein transferrin (116).

NG MAST produces unambiguous data that can easily be compared via the internet and is suitable for the identification of linked cases of gonorrhoea and the timely identification of transmission of antibiotic resistant strains, even within

large cities (133). Compared to OPA typing which relies on interpretation of bands in a gel, DNA sequence data (from the *porB* DNA sequence analysis, MLST and NG MAST) offer the advantage of being objective and easily shared with other investigators. Both *porB* DNA sequence analysis and NG MAST analysis have high discrimination powers sufficient to distinguish *N. gonorrhoeae* isolates and identify circulating clusters of strains. However NG MAST can further differentiate *porB* types due to sequence variations present in *tbpB* alleles which can contribute to different NG MAST alleles (108).

2.5.2.7 Lipotyping

The pathogenic *Neisseria* species *N. meningitidis* and *N. gonorrhoeae* possess an outer membrane protein (OMP) designated H.8, with conserved epitope recognised by H.8 monoclonal antibody (MAb) (73). Most nonpathogenic *Neisseria* do not bind the H.8 MAb. Additionally the H.8 OMP is immunogenic in humans during Neisserial disease (14) and has immunoprophylactic potential. The apparent molecular mass of H.8 OMP demonstrates strain variation, with a size range of 18 to 30 kDa (73). Studies have indicated that pathogenic *Neisseria* possess at least two genes encoding proteins that bind H.8 MAb (66). One of these genes encodes a lipoprotein with two domains: an N-terminal domain consisting of five imperfect repeats of the sequence Ala-Ala-Glu-Ala-Pro (AAEAP) and a C-terminal domain very similar to that of the azurins of other bacterial genera (217). Hence the predicted H.8 OMP is a lipoprotein 71 amino acids in length, composed of 13 to 19 repeats of a consensus sequence AAEAP with perfect 5-residue periodicity.

Lipoprotein subtyping is therefore based on the amplification of the *lip* gene by PCR and sequencing to determine the repeat number and sequence (196). Sequences of all Lip types or sequences are provided means of an internationally accessible web based data analysis system http://www.cdc.gov/nicdod/dastlr/gcdir/liptyping.html (29) to allow other investigators to compare the Lip patterns previously reported. It should be noted, however, that Lip subtyping is not sufficiently discriminatory to be used without additional gonococcal subtyping methods, such as A/S classification, TetM subtyping, β-lactamase plasmid profiles, or GyrA-ParC mutation analysis in fluoroquinolone-resistant isolates.

2.6. ANTIMICROBIAL THERAPY AND RESISTANCE

Antimicrobial chemotherapy has played a vital role in the treatment of human infectious diseases in the 20th century. Whilst antimicrobial agents have proven invaluable in the management of bacterial infectious diseases, resistance to these agents actually predates the introduction of first true antibiotic (penicillin) into clinical usage, and resistance continues to compromise the use of old and new antimicrobial agents alike (78). The clinical impact of resistance is immense, characterised by increased cost, length of hospital stay, disease-related complications (morbidity) and mortality, often as a result of inappropriate initial antimicrobial therapy.

2.6.1 Overview of the mechanisms of antimicrobial resistance

Resistance to antibiotics can be caused by a variety of mechanisms**:**

(i) the presence of an enzyme that inactivates the antimicrobial agent (β-actamase, *tetX* gene);

(ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent (aminoglycosides);

(iii) a mutation in the antimicrobial agent's target, which reduces the binding of the antimicrobial agent (ciprofloxacin; macrolides);

(iv) post-transcriptional or post-translational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent (macrolides);

(v) reduced uptake of the antimicrobial agent (tetracycline-the *penB* determinant);

(vi) active efflux of the antimicrobial agent (tetracycline- the *mtrR* mutation);

(vii) overproduction of the target of the antimicrobial agent (sulphonamide).

Genetically these changes may be mediated by either chromosomal or extra-chromosomal elements (plasmids) (189). In the case of *N. gonorrhoeae* example of all or just some antimicrobial resistance mechanism have been described below.

2.6.2 Sulphonamides and Trimethoprim

Sulphonamides became available for clinical use between 1936 and 1937 (25). They are derived from sulfanilamide, which has chemical similarities to ρ-aminobenzoic acid (PABA), a factor essential for bacterial folic acid synthesis.

Various substitutions at the sulphonyl radical attached to the benzene ring nucleus enhance the antibacterial activity and also determine the pharmacologic properties of the drug. Numerous studies have been made of the antibacterial action of sulphonamides and of the development of bacterial resistance (25).

Resistance of *N. gonorrhoeae* to sulfonamides results from one of two mechanisms (87): i) oversynthesis of PABA, which effectively dilutes the effect of sulfonamides; and ii) genetic alteration of dihydropteroate synthetase, resulting in a mutant enzyme with a reduced affinity for sulfonamide.

The discovery by Woods that sulphonamide competitively inhibits bacterial utilisation of PABA was followed by the recognition that PABA is an essential component of folic acid (215). Folic acid coenzymes function as carriers of single carbon units in the biosynthesis of methionine and several other amino acids, and also of purines and thymine. Thus, the bacteriostatic action of sulphonamide is due to the ultimate shortage of the essential end products of folic acid metabolism (216). Various factors enhance the capacity of a given bacterial strain to multiply in the presence of a concentration of sulphonamide higher than the minimal inhibitory concentration (MIC) established for that strain under standard conditions.

In the absence of any genetic change, the bacteriostatic action of sulphonamide may be reduced by exogenous supplementation of the bacterial environment with

PABA or with one or more of the essential products of folate metabolism. Genetic changes resulting in increased endogenous supplies of PABA, or in alteration of a PABA-utilising enzyme with consequent decreased affinity for sulphonamides, have also been proposed to account for increased resistance to sulphonamides (216).

2.6.3 Penicillins

Penicillins are a group of natural and semi-synthetic antibiotics containing the chemical nucleus 6-aminopenicillanic acid, which consists of a β-lactam ring fused to a thiazolidine ring. Penicillin and other β-lactam antibiotics exert their action by covalently binding to, and inactivating, penicillin binding proteins (PBPs), enzymes located in the cell envelope that participate in cell wall metabolism (51). Alterations in PBP-2 and PBP-1 decrease their affinity for the penicillins, and thus the susceptibility of the organism. PBP-1 and PBP-2 are encoded by the *ponA* and *penA* locus respectively (177).

Changes in other loci such as *mtr* and *penB* produce additive effects. The *mtr* locus mediates resistance to a wide range of antibiotics, detergents and dyes, through an active efflux system (67). Mutations in the *penB* locus, which encodes for the PorB1-b porin, results in reduced permeability of the cell envelope to hydrophilic antibiotics and other compounds (87)

The combined effects of *penA* mutations, *penB* mutations and increased expression of *mtr* is said to increase the MIC of penicillins by 120 fold (177). Gonococci exhibiting these changes are termed chromosomally-mediated penicillin resistant *N. gonorrhoeae* (CMPR).

Resistance to penicillins is also mediated by a plasmid-borne and inducible TEM-1 type β-lactamase (8). This enzyme hydrolyses the β-lactam ring of penicillins, thus inactivating them. In contrast to the slow evolution and incremental increase in resistance associated with chromosomal changes, acquisition of the plasmid confers resistance in a single step and plasmids can be rapidly disseminated among *N. gonorrhoeae* strains.

Penicillinase-producing *N. gonorrhoeae* (PPNG) were detected in 1976 in the United Kingdom and the USA (8, 145, 146). The United Kingdom-derived strain contained a 3.2 MDa “African” plasmid, which was isolated from a man who was diagnosed with gonorrhoea in Liverpool, UK but who had recently returned from Africa. The USA-derived strain contained a 4.4 MDa “Asia” plasmid, and was isolated from a US soldier who had acquired gonorrhoea in Philippines. Subsequently other TEM-1 type β-lactamase-containing plasmids have been described, for example the New Zealand (6.5 MDa), Nîmes (4.0 MDa), Toronto (3.05 MDa) and the Rio (2.9 MDa) plasmids (142).

Transmission of the resistance by conjugation required the presence of another mobilising plasmid, which was already present in the original Asian PPNG isolate but was not found in the African strains until 1981 (203). Thus the Asian strain initially disseminated more widely and more quickly. β-lactamase production (PPNG) and chromosomal changes (CMRNG) can co-exist in the same isolate. This is relevant because of the clinical use of penicillins in combination with β-lactamase inhibitors, such as clavulanic acid.

2.6.4 Aminoglycosides

Aminoglycosides such as gentamicin, tobramycin, amikacin and streptomycin are commonly used antimicrobial agents in the treatment of infections by both Gram-negative and Gram-positive organisms.

The process of bacterial killing by aminoglycosides is multifactorial. The principal target of aminoglycosides is the 30S subunit of ribosomes (124). This binding prevents the elongation of the growing peptide chain by causing mis-reading or premature termination during peptide synthesis. By interfering with the translation of mRNA, protein production is altered, aberrant proteins are inserted in the cell membrane, cell permeability is increased, more aminoglycosides are taken up into the cell, and cell death ensues (124).

Resistance to aminoglycosides emerges by one of four mechanisms: (i) alterations in the target site (ribosome) that prevent binding; (ii) loss of cell

permeability; (iii) expulsion by efflux pumps and (iv) enzymatic inactivation by aminoglycoside-modifying enzymes (AMEs) (124).

Resistance to these antimicrobial agents is wide spread with more than 50 AMEs described. Depending on their type of modification, these enzymes are classified as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferases (also named aminoglycoside nucleotidyltransferases [ANT]), and aminoglycoside phosphotransferases (APH) (124). Aminoglycosides modified at aminogroup by AAC enzymes or at hydroxyl groups by ANT or APH enzymes lose their ribosome binding ability and thus no longer inhibit protein synthesis (124).

In *N. gonorrhoeae*, resistance to spectinomycin or to aminoglycosides usually occurs via a single-step, chromosomal mutation, resulting in high-level resistance (115). The different ribosomal genes involved in spectinomycin and aminoglycoside resistance are linked. For example the loci responsible for resistance to streptomycin (*str*) and spectinomycin (*spc*) are genetically linked. Resistance due to mutations in *str* and *spc* loci results from alterations in the sensitivity of the 30S subunit of the gonococcal ribosome to streptomycin or spectinomycin, respectively (87).

2.6.5 Tetracyclines

Tetracyclines were discovered in the 1940s and have been used clinically to treat a variety of infections since the 1950s and are still widely used today for treatment of chlamydial and other infections (152). Tetracyclines penetrate the

bacterial cell by passive diffusion and act by inhibiting the attachment of aminoacyl-tRNA to the ribosome acceptor site, resulting in the inhibition of protein synthesis (165). A growing number of bacterial species have acquired resistance to the bacteriostatic activity of tetracycline. Most of the resistance genes code for one of the two important mechanisms of tetracycline resistance, either by efflux or ribosomal protection. These two widespread mechanisms of bacterial resistance to tetracycline do not destroy the compound.

Efflux is mediated by an energy dependent efflux pump. The efflux proteins exchange a proton for a tetracycline-cation complex and are anti-reporter systems. Efflux determinants from Gram-negative bacteria (Tet A to Tet E, Tet G and Tet H) have a have a common genetic organisation that is different from that in Gram-positive bacteria. They all contain structural gene and a repressor gene that are expressed in opposite directions from overlapping operator regions (165).

Protection of the ribosome from the action of tetracycline as a mechanism of tetracycline resistance was discovered in streptococci (165). Tetracycline resistance can result from the production of a protein that interact with the ribosome such that protein synthesis is unaffected by the presence of the antibiotic. These proteins exhibit homology to elongation factors EF-Tu and EF-G and exhibit ribosome-dependent GTPase activity (165). They act by binding to

the ribosome, thereby changing its conformation and inhibiting the binding of tetracycline. Tet M and Tet O are the best characterised of these proteins.

The *tetX* gene codes for an enzyme which inactivate tetracyclines. This enzyme is a novelty because it is the first and the only enzyme described which inactivates tetracycline. However it does not seem to have much clinical relevance since it requires oxygen to function and is found only in strict anaerobes. The *tetX* genes have been identified on transposable elements found in anaerobic bacteria of the genus *Bacteroides* (225).

Tetracycline resistance in *Neisseria gonorrhoeae* is mediated by two major mechanisms: expression of a plasmid-encoded TetM protein and mutations in endogenous genes (chromosomally mediated resistance). *tetM* in *N. gonorrhoeae* exists as two slightly different “Dutch’’ and ‘’American’’ types located on a large self-mobilising 25.2 MDa plasmid (62). A study of the molecular epidemiology of the tetM gene suggests that the Dutch type may have originated in the Far East and the American type on the African continent. The TetM plasmid is widely dispersed in the normal genital tract flora; the mobility of the plasmid and the selective pressure created by use of tetracyclines to treat other STIs has contributed to the widespread dispersal of the tetracycline resistant *N. gonorrhoeae* (TRNG) phenotype (225).

High-level chromosomally mediated resistance to tetracycline in *N. gonorrhoeae*, is mediated by a combination of three gene mutations (79): (i) the *mtrR* mutation, which results in over-expression of an nonspecific efflux pump (MtrC-MtrD-MtrE) that promotes the efflux of a range of hydrophobic agents and detergents; (ii) the *penB* determinant, which is a mutated porin IB that decreases the influx of tetracycline into the cell, and (iii) the *rpsJ1* allele, which results in altering the rRNA-binding site for tetracycline, thus lowering the affinity of the antibiotic for the ribosome. Although the combination of these mutations does not confer a level of tetracycline resistance as high as that observed with tetracycline-specific efflux pumps or the TetM determinant, the *mtrR-penB-rpsJ1* gene triad is highly effective and provides levels of resistance above those clinically achievable at the site of infection (79).

2.6.6 Macrolides

Macrolide antibiotics are chemically distinct inhibitors of bacterial protein synthesis (187). Macrolides inhibit protein synthesis in susceptible organisms by binding reversibly to the peptidyl-tRNA binding region of the 50S ribosomal subunit, inhibiting the translocation of a newly synthesised peptidyl-tRNA molecule from acceptor site on the ribosome to the peptidyl (donor site) (137). Intrinsic resistance to macrolide antibiotics in Gram-negative bacilli is due to low permeability of the outer membrane to these hydrophobic compounds. The enzymes EreA and EreB, encoded by the *ereA* and *ereB* genes, which hydrolyse the lactone ring of the macrocyclic nucleus, and the phosphotransferases types I

and II which inactivate macrolides by introducing a phosphate on the 2'-hydroxyl-group of the amino sugar have been found in members of the family Enterobacteriaceae (211).

Three different mechanisms of acquired macrolide resistance have been found in Gram-positive bacteria (211) namely post-transcriptional modification of the 23S rRNA, efflux and enzymatic inactivation. Post-transcriptional modification of the 23S rRNA by the adenine-N6-methyltransferase was the first mechanism of macrolide resistance to be described. Target modifications alter a site in 23S rRNA common to the binding of macrolide antibiotics. Modification of the ribosomal target confers cross resistance to macrolides antibiotics and remains the most frequent mechanism of resistance. A number of different antibiotic resistance genes code for efflux proteins, which pump the antibiotic out of the cell or the cellular membrane, keeping intracellular concentrations low and ribosomes free from antibiotic (211). The *mef* (macrolide efflux) genes have been found in a variety of Gram-positive genera (112). Many of these genes are associated with conjugative elements located in the chromosome and are readily transferred conjugally across species and genus barriers (112). Unlike target modification, which causes resistance to structurally distinct antibiotics, enzymatic inactivation confers resistance mostly only to structurally related drugs. As with Enterobacteriaceae, *ereA* and *ereB* genes have been identified in *Staphylococcus aureus* (211).

The MtrR repressor-regulated MtrC-MtrD-MtrE efflux system of *N. gonorrhoeae* exports macrolides. Increased efflux may occur by deletion or insertional inactivation of either the *mtrR* gene or the *mtrR* promoter (168).The *mef* (macrolide efflux) genes has been detected in some isolates, although its contribution to gonococcal macrolides resistance remains unclear (113). Expression of several 23S rRNA methylases, encoded by the *ermB*, *ermC* and *ermF* genes, is responsible for modification of the gonococcal ribosomal target (40). These methylases genes are associated with conjugative transposons which facilitate interbacterial spread. Mutations in the peptidyltransferese loop of domain V of 23S rRNA also confer gonococcal resistance to macrolides (137).

2.6.7 Quinolones

Quinolone drugs are widely used class of synthetic antibacterial compounds (53). First generation (acidic) quinolones include nalixidic acid and oxolinic acid. Subsequent generations have been modified to increase spectrum and potency. The most significant modification has been the addition of a fluorine atom at position C-6 in fluoroquinolones such as ciprofloxacin, which result in considerable increase in activity. The newer drugs also commonly contain a secondary amine in addition to the carboxylic acid group common to most quinolones, making the drug amphoteric rather than acidic (6). More recent modifications that increase drug potency include the presence of a methoxy group at C-8 (53).

Fluoroquinolones have a broad spectrum of antibacterial activity, commonly used in both clinical and veterinary medicine. Their strong activity against Gram-negative bacteria, excellent diffusion throughout tissue, and especially the ease of their oral administration, justify this widespread adoption (53). To date, three mechanisms of resistance to quinolones are currently recognized: mutations that alter the drug targets, mutations that reduce drug accumulation and plasmids that protect cells from the lethal effects of quinolones (77). For *N. gonorrhoeae*, the main mechanism involves mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene and, occasionally additional point mutations in the analogous region of the *parC* gene on the bacterial chromosome.

2.6.7.4 Target alterations

Quinolones act by inhibiting the action of type II topoisomerases, DNA gyrase and DNA topoisomerase IV (52). The subunits of DNA gyrase are GyrA, a 97-kDa protein encoded by the *gyrA* gene, and GyrB, a 90-kDa protein encoded by the *gyrB* gene. The main function of this enzyme is to catalyse the negative supercoiling of DNA, to remove both positive and negative supercoils, and to catenate and decatenate closed circular molecules. Topoisomerase IV is an A_2B_2 enzyme as well, encoded by *parC* and *parE* (referred to as *grlA* and *grlB* in *S. aureus*). The *parC* and *parE* subunits are highly homologous to *gyrA* and *gyrB,* respectively. The main aim of topoisomerase IV seems to be associated with decatenating the daughter replicons (77). The two enzymes, DNA gyrase and topoisomerase IV, work together in the replication, transcription,

recombination, and repair of DNA. The enzymes transiently break both strands of double-stranded DNA, and, in an ATP-dependent reaction, pass a second DNA double helix through the break, which is then resealed (91).

Quinolones block the reaction and trap gyrase or topoisomerase IV as a drug-enzyme-DNA complex, with subsequent release of lethal, double-stranded DNA breaks (71). A few bacteria are able to function with only DNA gyrase, but most bacteria have both enzymes. In gram-negative bacteria, gyrase is more susceptible to inhibition by quinolones than is topoisomerase IV (60), whereas, in gram-positive bacteria, topoisomerase IV is usually the prime target, and gyrase is intrinsically less susceptible (55,136). Each of the target enzymes has a quinolone resistance determining region (QRDR), a region of DNA encoding a portion of the DNA-binding surface of the enzyme, at which amino acid substitution can diminish quinolone binding.

Alteration of the GyrA subunits of DNA gyrase, particularly at Ser-91 and Asp-95, appears to play a central role in conferring high-level quinolone resistance in *N. gonorrhoeae*. Mutations in codons 67, 81, 82, 83, 84, 87 and 106 of *gyrA* have been observed to be responsible for the development of quinolone resistance in *Escherichia coli* (60). The presence of a single mutation in the above-mentioned positions of the QRDR of *gyrA* usually results in high-level resistance to nalidixic acid, but to obtain high levels of resistance to fluoroquinolones, the presence of additional mutations in *gyrA* and/or in another target such as *parC* is required. In

gyrB of *E. coli*, substitutions resulting in resistance to quinolones have been described at positions 426 (Asp-426 to Asn) and 447 (Lys-447 to Glu) (228). Substitutions at position 426 seem to confer resistance to all quinolones, whereas those at position 447 result in an increased level of resistance to nalidixic acid, but a greater susceptibility to fluoroquinolones (228).

Conversely, in *S. aureus* or *S. pneumoniae,* the initial target mutations occur more frequently in *parC,* whereas, in highly resistant strains, additional mutations are found in *gyrA* and *parE* (55,136). The role of amino acid substitutions in *parE*, resulting in the development of quinolone resistance in clinical isolates appears to be irrelevant in *N. gonorrhoeae* (156). However one substitution (Leu-445 to His) has been described in *parE* of a single quinolone-resistant *in vitro* mutant of *E. coli*. This mutation only seems to affect the quinolone MIC value in the presence of a concomitant mutation in *gyrA* (174).

Once a first-step mutation has reduced the susceptibility of DNA gyrase in a gram-negative organism, additional mutations in *gyrA* or mutations in *gyrB* or *parC* can further augment resistance, although, by themselves, they would be ineffective in a bacterial cell with wild-type GyrA, because the most-susceptible target sets the level of susceptibility. A plausible mechanism for how these substitutions decrease susceptibility is that they reduce drug affinity. In support of this model, single and double substitutions in the QRDR of *E. coli* gyrase have been shown to reduce the binding of quinolones to the enzyme-DNA complex

(9). Alternatively, mutations may marginally impair target enzyme function and, thus, reduce the formation of enzyme-DNA complexes and lethal double-stranded breaks in DNA (81).

2.6.7.5 Decreased uptake

To reach their targets, quinolones must cross the cell wall and cytoplasmic membrane of gram-positive bacteria; in gram-negative bacteria, quinolones must traverse an additional outer membrane barrier. Quinolones may cross the outer membrane in two different ways: through specific porins or by diffusion through the phospholipid bilayer (32). The degree of diffusion of a quinolone is greatly associated with, and dependent on, its level of hydrophobicity. All quinolones may cross the outer membrane through the porins, but only those with a greater level of hydrophobicity may diffuse through the phospholipid bilayer.

Decreased quinolone uptake may be associated with two factors: an increase in the bacterial impermeability to these antibacterial agents or the over-expression of efflux pumps (2, 209). Alterations in the composition of porins and/or in the lipopolysaccharides may alter bacteria susceptibility profiles. In lipopolysaccharide-defective mutants, increased susceptibility to hydrophobic quinolones has been described, without alterations in the level of resistance to the hydrophobic quinolones (72). Alterations in membrane permeability are usually associated with decreased expression of porins. This has been described both in *E. coli* and other Gram-negative bacteria (72). Both mechanisms of

resistance are mutational, arising in an individual organism and then passing vertically to surviving progeny. Neither mechanism seems to transfer effectively on mobile genetic elements (157).

2.6.7.6 Transferability of quinolone resistance

Plasmid-mediated resistance to nalidixic acid was first reported in 1987 from clinical isolates of *S. dysenteriae* (129), although, the plasmid involvement was later refuted (7). However, a re-visit to this literature cannot fully rule out the possibility of plasmid involvement in the quinolone resistance, since there seems no alternative interpretation for the findings that plasmid-carrying strains had higher survival advantage under nalidixic acid stress and that they generated 1000-fold higher nalidixic acid-resistant mutants than their parental plasmid-free strains even though it was suspected that the plasmid might act as a mutator factor specific for nalidixic acid resistance (7).

In 1998, Martinez-Martinez *et al*. made an inadvertent but crucial discovery during a study of a plasmid, pMG252 that produced an unusual multidrug resistance phenotype including resistance to quinolones, β-lactams, aminoglycosides, sulphonamides, trimethoprim and chloramphenicol (118). Plasmid pMG252 came initially from a ciprofloxacin-resistant strain of *K. pneumoniae* isolated in 1994 from the urine of a patient at the University of Alabama in the USA (118). This identical plasmid was also found in two additional strains of *K. pneumoniae* and one strain of *E. coli* from patients in the

same institution over a period of 5 months. The plasmid had a broad host range and was transferred by conjugation from *E. coli* to *Citrobacter freundii*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*. The plasmid-bearing conjugants displayed enhanced resistance, with 4- to 16-fold increases in minimum inhibitory concentration (MIC) values of nalidixic acid, norfloxacin, ciprofloxacin, clinafloxacin, levofloxacin, pefloxacin and trovafloxacin, confirming for the first time the involvement of a plasmid in quinolone resistance (118). Cloning and nucleotide sequence analysis of plasmid pMG252 revealed a gene responsible for the plasmid mediated quinolone resistance (PMQR) (192). This gene, named *qnr* (GenBank accession number AY070235), encodes a protein, Qnr, of 218 amino acid residues. Qnr belongs to the pentapeptide repeat family, in which almost every fifth amino acid is either leucine or phenylalanine and each pentapeptide repeat likely forms a sheet that is important for protein–protein interactions (223). Purified Qnr protein was shown to bind to and protect both DNA gyrase and topoisomerase IV from inhibition by ciprofloxacin (193).

Two more kinds of PMQR determinants have been described in *E. coli*, namely AAC(6)-Ib-cr and QepA (224). AAC(6)-Ib-cr is a variant of AAC(6)-Ib and is responsible for reduced susceptibility to ciprofloxacin or norfloxacin by N-acetylation of a piperazinyl amine. QepA is a quinolone efflux pump protein and shows a considerable similarity to the MFS types of efflux pumps belonging to the 14-transmembrane segment family of environmental actinomycetes.

The development of plasmid mediated quinolone resistance (PMQR) through decreased drug accumulation has not yet been described in *N. gonorrhoeae*.

2.6.8 Cephalosporins

Brotzu discovered cephalosporins in 1945 as naturally occurring substances produced by the fungus *Cephalosporium acremonium,* now known as *Acremonium chrysogenum* (158). The first widely used cephalosporin was cephalothin, introduced to the market in 1962 for parenteral use. Similar to penicillins, cephalosporins act by binding to penicillin-binding protein (PBPs) of susceptible organisms, thereby interfering with the synthesis of peptidoglycan of the bacterial cell wall. In addition, these β-lactam agents may produce bactericidal effects by triggering autolytic enzymes in the cell envelope.

Cephalosporins are usually classified by 'generations' which roughly correspond to the time of their introduction and antibacterial activity. The first–generation (narrow-spectrum) drugs, exemplified by cephalothin and cefazolin, have good Gram-positive activity and relatively modest Gram-negative activity. The second-generation (expanded-spectrum) cephalosporins are stable against certain β–lactamases found in Gram-negative bacteria and as a result, have increased activity against Gram-negative organisms. Third-generation (broad-spectrum) cephalosporins are generally less active than the narrow-spectrum agents against Gram-positive cocci (134, 158). Their potent broad spectra of Gram-

negative activity are due to their stability to β–lactamases and their ability to pass through the outer cell envelopes of Gram-negative bacilli (134).

The increase in QRNG resulted in the cessation of fluoroquinolones therapy and the increased use of third-generation cephalosporins, such as cefixime and ceftriaxone, as the treatment of choice for gonorrhoea. The most active of this group of 'extended spectrum' cephalosporins is ceftriaxone. This high intrinsic activity on *N. gonorrhoeae*, together with a long half-life, success in eradicating the organism from all sites of infection and availability as a cheaper generic preparation has recently seen it widely adopted globally as an effective treatment (135). Disadvantages of ceftriaxone are that it is given as an intramuscular injection that is locally painful and requires co-administration of a local anaesthetic. There are also practical reasons for avoiding injectable agents in less resourced settings with high rates of HIV. For these reasons, the use of a number of oral formulations of third generation cephalosporins has been explored. The most widely recommended oral third-generation cephalosporin is cefixime, although some countries have made use of other oral agents in this group including ceftibuten, cefozopran, cefdinir and cefpodoxime (158).

Recently, there have been an increasing number of studies of treatment failure in gonorrhoea treated with oral third-generation cephalosporin regimens, most notably cefixime and ceftibuten (111). Treatment failures with oral cephalosporins were first reported in Japan in 2001 and continue to be recorded in Australia and elsewhere (4, 111). The clinical treatment failures with oral cephalosporins have

been paralleled by laboratory data showing increasing *in-vitro* resistance to these antibiotics in *N. gonorrhoeae* (as measured MIC) (130). Although affected strains also have decreased susceptibility *in vitro* to ceftriaxone, this is not yet at MIC levels that translate into loss of clinical efficacy.

A number of chromosomal genes have now been regarded as relevant to the increased MICs reported for both oral and parenteral cephalosporins. Earlier studies paid considerable attention to alterations in penicillin-binding protein 2 (PBP2), encoded by the *pen A* gene, and in particular to the presence of a mosaic PBP2 in gonococci from treatment failures with oral cephalosporins (5). PBP2 is the major target of β-lactam antibiotics in *N. gonorrhoeae*, having a 10-fold greater affinity for penicillin than the other major target site, PBP1, which is encoded by ponA. Many have suggested that the presence of a mosaic PBP2 is a pivotal requirement for decreased susceptibility to both the oral and parenteral cephalosporins and is associated with decreased affinity for oral cephalosporins (110, 139). A number of specific loci within the mosaic PBP2 are said to be relevant to the development of this 'resistance' (184). However it should be noted that, whilst most of the *N. gonorrhoea* strains that are resistant to oral cephalosporins possess mosaic *penA* genes, the mosaic pattern is also seen in a small proportion of susceptible strains (139).

Other genetic changes associated with increases in MICs for penicillins, cefixime and ceftriaxone include the de-repression of an efflux pump inhibitor by

mutations in mtr. This change is almost always being combined with an alteration in penB, which encodes for the porin PorBI-b, and changes in *ponA*, which encodes PBP1 (167). Other lesions have also been described including mutations in pilQ (formerly known as penC) (154). If a mosaic PBP2 is then added to this mix of different genetic combinations, different levels of cephalosporin resistance will be detected, as was the case in the study by Ito *et al.* (84). These combinations of changes were said to define an unambiguous association between penA mosaic alleles, polymorphisms in genes and raised MICs to cefixime and ceftriaxone (110). These different mutations and combinations of genetic changes, however, have differential effects on the oral and parenteral cephalosporins *in vitro* (in terms of MICs) and *in vivo* (in terms of clinical outcomes). It appears that the full range of genetic changes and the interactions between the different alterations has yet to be elucidated.

2.6.9 Syndromic Management

Within South Africa, STI care at the primary level is being achieved with a strategy called "syndromic management." This approach is based on identifying the major groups of signs and symptoms (syndromes) commonly associated with certain infections (132). Through the syndromic management approach, patients are diagnosed and treated on the basis of these syndromes, rather than on the basis of specific STIs. STI control programs need to identify the common syndromes in the area, the organisms responsible for them, and effective antibiotics. Simple standard guidelines in the form of flow charts for each

syndrome should then be developed for use by primary level health care workers (Fig 2.11, Fig 2.12).

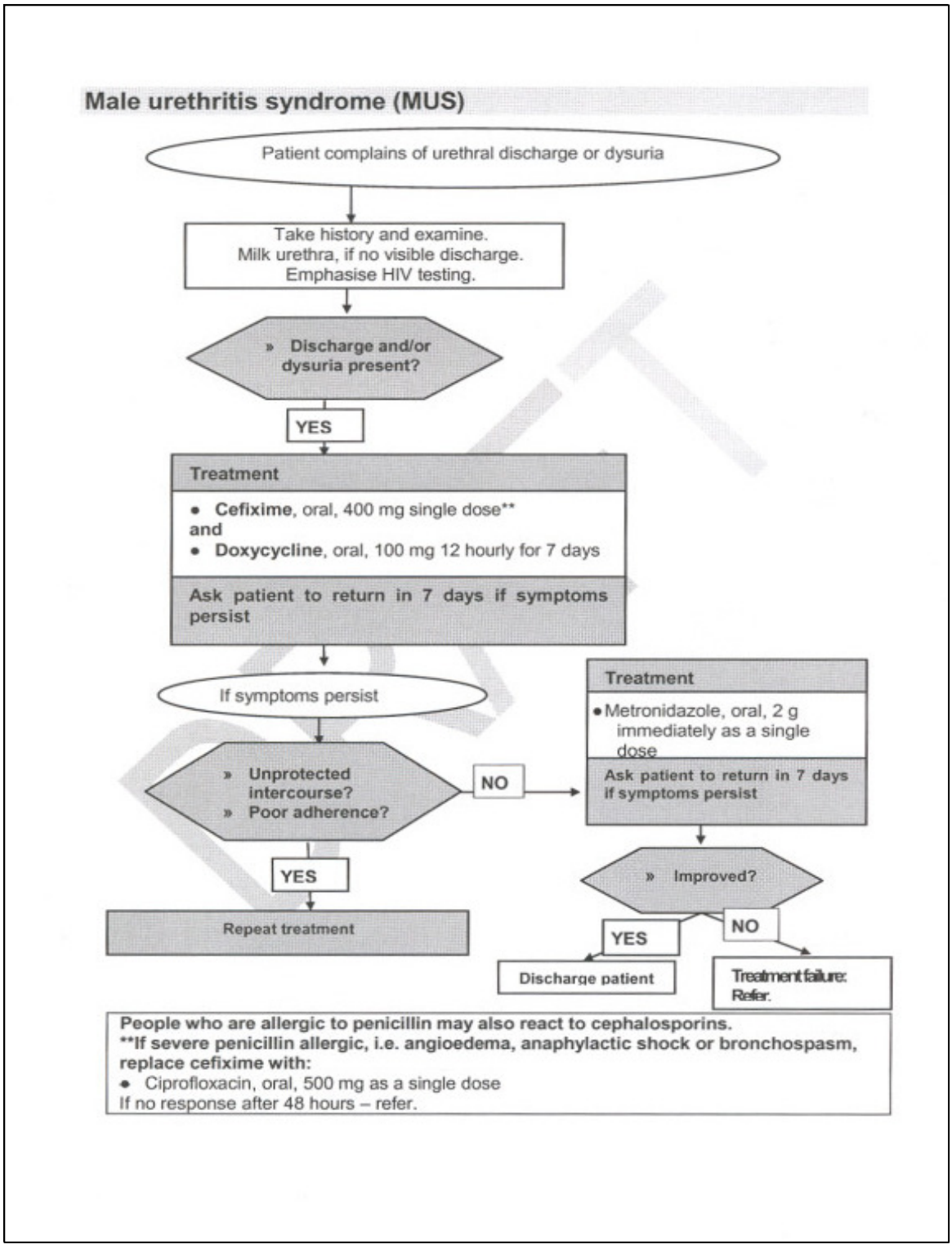


Fig. 2.11 Syndromic treatment regimen for male urethritis syndrome (175).

Treatment is prescribed to deal with the infections commonly associated with the syndrome in the region (Fig 2.11, Fig 2.12) (104). The syndromic approach has been researched and tested in several countries and is developed and recommended by WHO and other international agencies (219). This approach facilitates rapid diagnosis and treatment without requiring sophisticated, time-consuming laboratory tests or advanced medical skills.

As the syndromic approach is based on self-reported symptoms, it does not detect or treat patients with asymptomatic infections (15). Another disadvantage is that no single algorithm is appropriate for every setting. For example, in Papua New Guinea, where chancroid is rare and most ulcers are caused by *Klebsiella granulomatis*, treatment should be directed at the latter rather than at both chancroid and syphilis, as practiced in East Africa (151). Also in view of changing antimicrobial susceptibility patterns and continuing research, algorithms will need to be regularly updated.

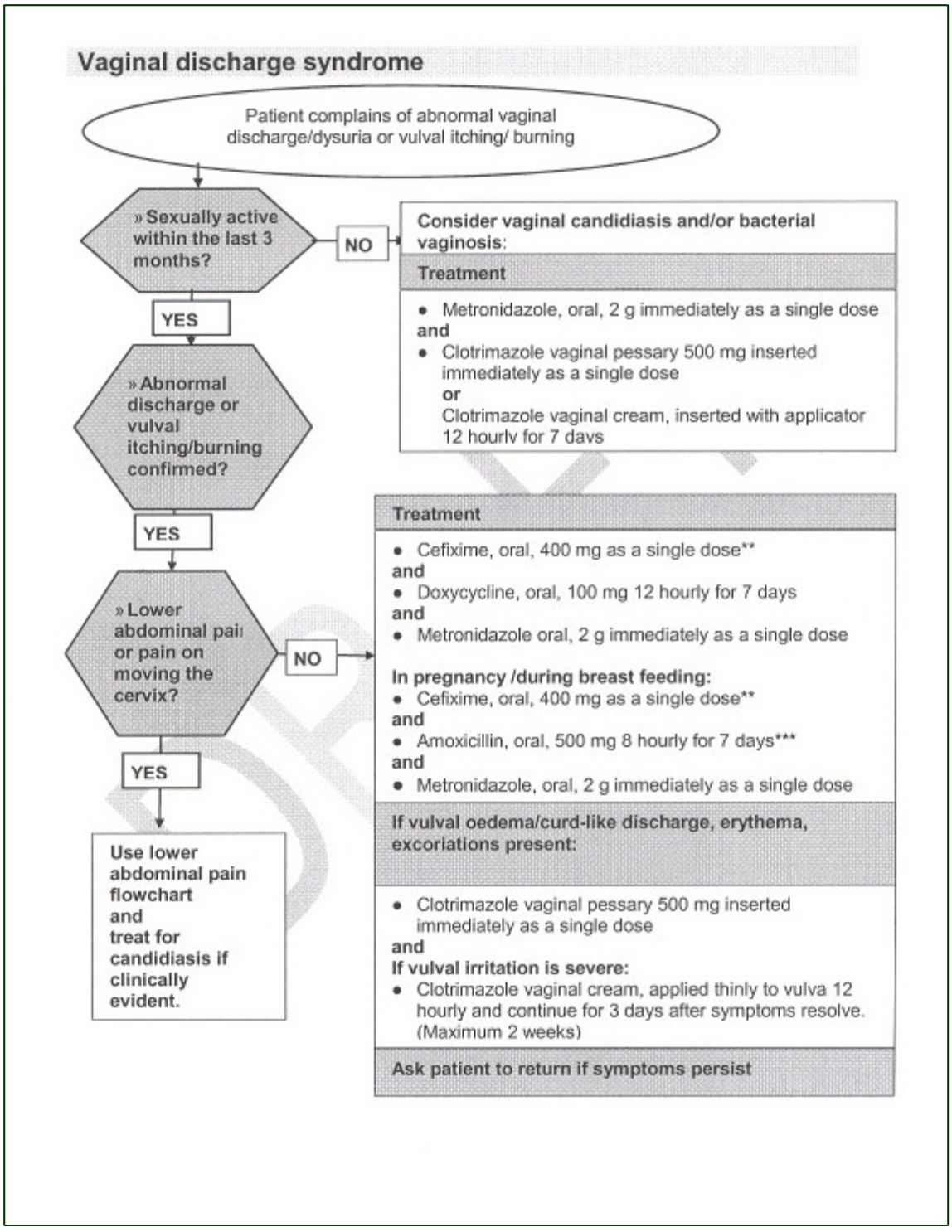


Fig. 2.12 Syndromic treatment regimen for vaginal discharge syndrome (175).

Over-diagnosis and over-treatment are the major disadvantages of syndromic management (15). Over-treatment in female patients with virginal discharge is especially common, where cervicitis (due to gonorrhoea and/or chlamydial infection) is not the predominant cause of the discharge. In order to assess the effectiveness of the syndromic approach, it is necessary to carry out regular evaluations of the accuracy of diagnoses and patient satisfaction by using laboratory tests. Cheaper and more effective laboratory approaches for STI diagnosis and screening (for both symptomatic and asymptomatic individuals) are required to ensure quality of care in STI clinics in resource-poor settings.

2.7 Laboratory-based methods for detecting antimicrobial resistance

The control of gonorrhoea caused by resistant gonococcal strains is facilitated, in part, by the rapid laboratory identification of resistant strains infecting patients for whom therapy is unsuccessful and of strains isolated during epidemics associated with the rapid spread of resistant strains (100). Laboratory methods for susceptibility testing of gonococci are similar to those of other bacteria. However, *N. gonorrhoeae* has specialised growth requirements and efforts to handle this fastidious organism have led to the development of a plethora of tests, with numerous variations in methodology. The susceptibility of an isolate to an antimicrobial agent is generally expressed as the MIC, i.e., the minimal concentration of an antimicrobial agent required to inhibit growth of the isolate (150).

2.7.1 Agar dilution methods

The agar dilution MIC is the definitive susceptibility test (150). It is a labour intensive method and is only performed in specialised laboratories, but it is relatively inexpensive when large numbers of strains are tested in batches. If susceptibility testing of *N. gonorrhoeae* is performed for epidemiological purposes, rather than for individual case management, delay in treatment is not an issue. A simplified 'breakpoint' method, using a smaller number of antibiotic concentrations, is useful for screening large numbers of strains when the frequency of resistance is expected to be low. Tests of this type require experienced staff and access to antibiotic powders of known potency. Strains must be stored, which involves extra handling and subculture and resources.

Although Mueller-Hinton medium has been controlled for antimicrobial susceptibility testing and is recommended for testing non-fastidious bacterial species, it does not support satisfactory growth of all strains of *N. gonorrhoeae* (13). Unlike Mueller-Hinton agar, which shows good lot-to-lot reproducibility for susceptibility testing, lot-to-lot variations of different media (e.g., Proteose peptone or GC agar base) used to grow *N. gonorrhoeae* have been noted (179). These media variations may disproportionately affect MIC results for antimicrobial agents (15). Thus, different lots of the medium base (or components) must be evaluated to ensure adequate growth of isolates and reproducibility of results of susceptibility tests for reference strains. With use of a suitable lot of medium, reproducible results may be obtained if procedures,

including preparation and storage of plates, growth and preparation and storage of inoculum, and incubation conditions, are followed precisely.

The World Health Organization Scientific Group formerly recommend that agar-dilution susceptibility test be performed on a “chocolate” agar medium consisting of Proteose peptone no 3 (Difco, Detroit, MI) agar medium supplemented with 1% (vol/vol) hemoglobin solution (Difco) and 1% (vol/vol) IsoVitaleX(BBL) and containing $\log_2$ dilutions of antimicrobial agents (220, 222). The same medium without added antimicrobial agent is inoculated to test for adequate growth of isolates. For routine use, plates can be stored for two weeks at 4^{o}C in a sealed container that prevents desiccation of the medium. However it should be noted that the use of hemoglobin-supplemented antibiotic medium is not recommended for agar dilution MIC testing.

For a number of years the CDC now recommends that agar-dilution susceptibility testing of strains of *N. gonorrhoeae* should be performed using GC-agar base (BBL) supplemented with 1% (vol/vol) IsoVitaleX (28). The same media is recommended by the Clinical Laboratory Standards Institute (CLSI) (34). Media should be inoculated with 10^4 cfu/ml, which is in accordance with the recommendations of the CLSI (34). Preliminary results suggest that MICs obtained with this procedure are generally higher than those obtained on supplemented chocolate agar inoculated with 10^3 cfu.

In summary, agar dilution methods currently in use are not uniform, and different MIC values expressed in mg/l may be obtained when the same strains are tested in different laboratories or on different media in the same laboratory (100). Variables include type of growth medium (basal medium and supplements), inoculum size, incubation conditions and incubation time. As in any test method, the one variable most difficult to control is reading the endpoint, which is subjective. However, the extent of variability in endpoint determination can be reduced by including standard strains in each batch of tests for quality control QC.

2.7.2 Disc diffusion methods

Disc-diffusion testing is most applicable for rapid screening of small numbers of gonococcal isolates and may be limited to those antimicrobial agents being used for therapy. Test results can be useful to guide selection of therapy for individual patients or to focus disease intervention activities for outbreak control. Results for disc-diffusion susceptibility tests may be available within 24 hours of the isolation of the strain. Sizes of the zones of inhibition are measured with calipers or with a millimeter ruler. Inhibition zone sizes for the interpretation of disc-diffusion susceptibility results have been recommended for various antimicrobial agents and discs of different concentrations (220). As zone sizes of different international standards vary, most investigators use the CLSI guidelines for the interpretation of disc-diffusion susceptibility results (13, 34).

The media for disc-diffusion susceptibility testing must also be controlled for lot-to-lot variation by use of strains with known susceptibilities. Disc-diffusion testing depends on the diffusion into the medium of the antimicrobial agent contained in the disc that is placed on the medium. The rate of diffusion of the antibiotic will vary with the molecular weight of the antibiotic and medium and incubation conditions (e.g., type of medium, moisture content of medium, depth of medium and humidity of incubation chamber). The test results obtained with use of an un-calibrated procedure must be interpreted cautiously and should be confirmed with a calibrated susceptibility test system. In addition, the inhibition zone sizes obtained by other procedures (e.g., chocolate agar) may not be the same as those obtained on supplemented GC-base medium, and such procedures should be independently controlled using reference strains with known susceptibilities (100).

2.7.3 E-test

Antimicrobial susceptibility testing with the E-test® antimicrobial gradient strip is technically as simple to perform as the disc diffusion test, but provides semiquantitative MIC results (150). The strip is impregnated with a standard gradient of antimicrobial agent and the front of the strip has MIC values that are to be read in correspondence with inhibition of growth on the plate after incubation.

Antimicrobial susceptibility testing of *N. gonorrhoeae* is performed on GC base medium plus 1% defined growth supplement. The standardisation of the inoculum and methods for the inoculation of the test plate are the same for the E-test® as they are for the disc diffusion test for *N. gonorrhoea*. Strict quality control practices are of extreme importance in order for the proper performance and appropriate interpretation of the antimicrobial susceptibility test.

The strip is placed on the surface of an inoculated plate and the endpoint (MIC) is determined by reading the point where the inhibition zone intersects the strip (Fig 2.13).

Fig. 2.13 Detection of ciprofloxacin resistant *N. gonorrhoeae* using E-test (MIC=3mg/l) *(Photograph credit: D. Lewis)*

MICs obtained with this method in reference laboratories tend to be slightly lower than those obtained by conventional agar dilution methods (150). Endpoint interpretation poses the same problem as for disc diffusion test, i.e. determining the precise edge of the inhibition zone. The ease of use of the E-test makes it attractive as a potential standard method. However, the test strips are very costly, particularly when testing susceptibility to multiple antibiotics for epidemiological purposes.

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacterial isolates

3.1.1 *N. gonorrhoeae* isolates

Gonococci were isolated from urethral swabs taken from men presenting with urethral discharge, to primary health care (PHC) clinics in Gauteng (Johannesburg), Northern Cape (Kimberley) and Western Cape (Cape Town) provinces as part of the National Microbiological Surveillance (NMS) programme coordinated at the NICD's STI Reference Centre (Table 3.1). At the time of the study, microbiological surveillance was only conducted in the three provinces and 49 isolates were chosen based on the increasing MIC values and budget limitations.

Table 3.1. Characteristics of the population from which samples were collected

Category	Johannesburg, Gauteng	Kimberley, Northern Cape	Cape Town, Western Cape
Months of survey	Jan-April 2007	March-Aug 2006	Nov 2006, Jan-Feb 2007
No. of MUS enrolled	217	158	290
Total gonorrhoea cases	157	59	259
GC M-PCR Pos	154	50	247
GC Culture Pos	151	35	243
No of isolates (%) selected for study	24 (16%)	9 (26%)	16 (7%)
Susceptibility phenotypes of selected isolates	16R,2I,6S	4R,5S	11R,5S

S = Susceptible; I = Intermediate/Reduced Susceptibility; R = Resistant

A total of 35 resistant isolates, 2 isolates with intermediate susceptibility and 12 fully susceptible isolates were consecutively selected to validate and assess the performance of the real-time PCR in order to detect quinolone resistant *Neisseria gonorrhoeae* (QRNG) (Table 3.2). Of these, 24 isolates (i.e. 16 resistant, 2 intermediate and 6 susceptibility) were consecutively chosen from the Johannesburg strain collection, 9 isolates (i.e. 4 resistant and 5 susceptibility) from the Kimberley strain collection, and 16 isolates (i.e. 11 resistant and 5 susceptibility) from the Cape Town strain collection(Tables 3.2 and 3.3).

Table 3.2. Description of specimen used on different assays

Category	Specimen type	Johannesburg, Gauteng	Kimberley, Northern Cape	Cape Town, Western Cape
Assay validation	Urines	24 (16R,2I,6S)	–	–
	Swabs	24 (16R,2I,6S)	–	–
	Cultures	24 (16R,2I,6S)	–	–
Testing of clinical specimens	Swabs	–	9 (4R,5S) and 18 (no MIC data)	16 (11R,5S)
	Cultures	–	9 (4R,5S)	16 (11R,5S)
Sequencing	Cultures	24 (16R,2I,6S)	9 (4R,5S)	16 (11R,5S)
	Swabs*	–	5 (2R,3S)	–
NG MAST	Cultures	24 (16R,2I,6S)	9 (4R,5S)	16 (11R,5S)
	Swabs*	–	5 (2R,3S)	–

S = Susceptible; I = Intermediate/Reduced Susceptibility; R = Resistant

* Swabs only tested for Kimberley patients where a discrepancy existed between the real-time PCR assay results for cultures and swabs

Table 3.3. Number of isolates selected and the susceptibility phenotype by Province

Previous susceptibility phenotype	City and Province		
	Johannesburg, Gauteng	Kimberley, Northern Cape	Cape Town, Western Cape
Susceptible	GP-07-MUS-125	NC-06- MUS- 045	WC-06-MUS- 181
Susceptible	GP-07-MUS-136	NC-06-MUS- 079	WC-06-MUS- 182
Susceptible	GP-07-MUS-149	NC-06-MUS- 085	WC-06-MUS- 189
Susceptible	GP-07- MUS-151	NC-06-MUS- 103	WC-06-MUS- 200
Susceptible	GP-07- MUS-159	NC-06-MUS- 105	WC-06-MUS- 228
Susceptible	GP-07- MUS-170		
Intermediate	GP-07- MUS-148		
Intermediate	GP-07- MUS-187		
Resistant	GP-07- MUS- 002	NC-06-MUS- 056	WC-06-MUS- 010
Resistant	GP-07- MUS- 006	NC-06-MUS- 100	WC-06-MUS- 012
Resistant	GP-07- MUS- 016	NC-06-MUS-135	WC-06-MUS- 013
Resistant	GP-07- MUS- 018	NC-06-MUS- 144	WC-06-MUS- 022
Resistant	GP-07- MUS- 026		WC-06-MUS- 050
Resistant	GP-07- MUS- 029		WC-06-MUS- 071
Resistant	GP-07- MUS- 031		WC-06-MUS- 105
Resistant	GP-07- MUS- 036		WC-06-MUS- 131
Resistant	GP-07- MUS- 038		WC-06-MUS- 196
Resistant	GP-07- MUS- 040		WC-06-MUS- 239
Resistant	GP-07- MUS- 064		WC-06-MUS- 243
Resistant	GP-07- MUS- 097		
Resistant	GP-07- MUS-120		
Resistant	GP-07- MUS- 166		
Resistant	GP-07- MUS- 168		
Resistant	GP-07- MUS- 171		
Total Isolates	**24**	**9**	**16**

Four control strains were used to validate the E-test procedure, namely *Neisseria gonorrhoeae* ATCC 49226 (ciprofloxacin MIC < 0.002 μg/ml, CipS), WHO A (ciprofloxacin MIC < 0.002 μg/ml, CipS), MAL058 (ciprofloxacin MIC = 0.25 μg/ml, CipI) and ESN306 (ciprofloxacin MICs, ≥1 μg/ml, CipR). In house and WHO controls (222) were also used for both sequencing and real-time PCR and are as follows: WHO A, MAL058 and ESN306 (Table 3.4). In house controls, MAL058 and ESN306 were selected based on their phenotypic characteristics, and previous sequencing data was not available.

Table 3.4. Susceptibility to ciprofloxacin of *N. gonorrhoeae* control strains and associated *gyrA* and *parC* alterations

Control Strain	Ciprofloxacin MIC (μg/ml)	GyrA		ParC
		Ser91 (TCC)	Asp95 (GAC)	Asp86 (GAC)
Neisseria gonorrhoeae ATCC 49226	< 0.002	Ser91 (TCC)	Asp95 (GAC)	Asp86 (GAC)
WHO A	< 0.002	Ser91 (TCC)	Asp95 (GAC)	Asp86 (GAC)
MAL058	0.25	Phe91 (TTC)	Gly95 (GGC)	Asp86 (GAC)
ESN306	≥1	Phe91 (TTC)	Gly95 (GGC)	Asn86 (AAC)

3.1.1.1 Other Neisseria isolates

Three different non gonococcal Neisseria cultures were provided by Ms. Ruth Mpembe from the Respiratory and Meningeal Pathogens Reference Unit (RMPRU) at NICD/NHLS to be used for sensitivity and specificity of the primers

used in the real time PCR assay for the detection of QRDR mutations. One of each non gonococcal Neisseria species received was namely: *Neisseria lactamica* (ATCC4418), *Neisseria meningitidis* (ATCC13077) and *Neisseria mucosa* (ATCC19696).

3.1.1.2 Urinary Tract Infection isolates

Urines from 17 patients were collected as part of routine clinical investigation at the Johannesburg General Hospital, and cultured isolates were provided by Dr Olga Perovic. A total of 6 bacteria species were isolated from the 17 cultured urines: 10x *Escherichia coli*, 2x *Klebsiella pneumoniae*, 1x *Proteus mirabilis*, 2x *Pseudomonas auruginosa*, 1x *Acinetobacter baumannii* and 1x *Klebsiella oxytoca*.

3.1.2 Culture

The frozen gonococcal cultures, in Microbank™ vials (Pro-Lab Diagnostic, Richmond Hill, Canada) kept at -70^{o}C, were thawed and subcultured directly onto New York City media [Diagnostic Media Products (DMP), National Health Laboratory Services (NHLS), Johannesburg, South Africa]. The inoculated plates were incubated immediately at 35-37^{o}C in a humid atmosphere containing 3-10% CO_2 in a candle jar. Plates were incubated for at least 48 hours and examined after 24-48 hours. Typical colonies, 0.5 - 1mm in diameter, varied from grey to white in colour, transparent to opaque and with convex or flat profiles. Suspect colonies were subcultured onto non-selective chocolate agar to ensure purity and

to provide sufficient growth for other tests. Typical colonies were picked for Gram staining and microbiological examination, as well as oxidase testing.

3.1.3 Confirmatory tests for *Neisseria gonorrhoeae*

3.1.3.1 Gram staining

N. gonorrhoeae isolates were Gram stained to confirm typical Neisserial morphology (131). Single colonies were emulsified in a drop of saline on a glass slide, dried, and stained. The fixed or dried smears were covered with crystal violet (DMP, NHLS; Johannesburg, South Africa) for 1 min and rapidly washed with running water. The slides were flooded with iodine (DMP, NHLS; Johannesburg, South Africa) for 1 min, and then rinsed gently with running water. The smears were decolourized with acetone-alcohol (DMP, NHLS, Johannesburg, South Africa) until the drops falling off the slides were no longer blue. The decolouration was stopped by rinsing the slides quickly in running water and draining off the excess water. The smears were counterstained with safranin (DMP, NHLS; Johannesburg, South Africa) for 1 minute. The slides were rinsed with running water, gently blotted with absorbent paper and air-dried. The slides were read with a 100x objective using a light microscope with immersion oil. Typical Gram-negative diplococci with flattened adjacent sides were consisted with the presence of *N. gonorrhoeae.*

3.1.3.2 Oxidase test

The identity of *N. gonorrhoeae* isolates was confirmed by the oxidase reaction (131). A few drops of oxidase reagent (tetramethyl-p-phenylene diamine hydrochloride) (Davis Diagnostics, Brampton, Canada) were placed on a piece of filter paper and a part of a colony was transferred onto the filter paper with a wooden stick or platinum loop. Gonococcal colony material turned pink and then rapidly deepened to purple indicating the presence of oxidase.

3.1.3.3 Phadebact monoclonal antibody test

N. gonorrhoeae isolates were also reconfirmed by a co-agglutination technique called the Phadebact Monoclonal GC OMNI test (Pharmacia, Uppsala, Sweden) (131). The test was carried out according to manufacturer's instructions. A light suspension of the suspect colony was made in saline. The suspension was heated in a boiling water bath for 5 min and allowed to cool at room temperature. At least one drop of the WI reagent and one drop of the WII/III reagent was added onto a test card. A drop of heated suspension was added to each reagent. The reagents on the card were mixed gently and the card was rotated for one minute before reading. Visible agglutination indicated the presence of *N. gonorrhoeae*.

A reaction in either WI or WII/III gonococcal reagents constituted a positive result. A positive reaction of the same strength in both reagents was an equivocal result. For equivocal results, the treated suspension was diluted 2-4x

with 0.9% saline and tested again. Lack of reaction in both WI and WII/III gonococcal reagents constituted a negative result. A negative result strongly suggested that the bacteria tested were not *N. gonorrhoeae*.

3.1.3.4 BBL crystal™ Neisseria*/Heamophilus* (N/H) identification kit

N. gonorrhoeae isolates were initially identified by a fluorogenic and chromogenic substrate utilizing test called the BBL Crystal™ Identification System (Becton, Dickson and Company, Maryland, USA) (131). The procedure was carried out according to manufacturer's instructions. An inoculum fluid tube was labeled with the specimen number. Using aseptic techniques, presumptive *N. gonorrhoeae* colonies were picked from the 24 h culture media. Colonies were suspended in a tube of BBL Crystal ANR, GP, RGP, N/H ID Inoculum Fluid. The tube was re-capped and vortexed for approximately 10–15 sec. The turbidity of the suspension was equivalent to a McFarland No. 3 standard. The entire content of the inoculum fluid tube was transferred into the target area of the BBL Crystal base. The inoculum was rolled gently along the tracks until all of the wells were filled. The BBL Crystal N/H ID panel lid was aligned, so that the labeled end of the lid was on top of the target area of the base. The lid was pushed down until a slight resistance was felt. The BBL Crystal N/H ID panel contained 29 enzymatic and biochemical substrates (Table 3.5).

Table 3.5. Substrates used in the BBL crystal™ N/H identification system

Panel location	Substrate/Enzyme tested	Positive Reaction	Negative Reaction	Typical *N. gonorrhoeae* response
4A	Fluorescent negative control (FC)	blue fluorescence >FCT well	blue fluorescence ≤FCT well	—
2A	4MU-phosphate	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Negative (0)
1A	L-proline-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (1)
4B	L-serine-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (4)
2B	LYS-ALA-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (2)
1B	L-tryptophan-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (1)
4C	L-phenylalanine-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (4)
2C	N-succinyl-ALA-PRO-ALA-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Negative (0)
1C	ALA-ALA-PHE-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (1)
4D	L- glutamic acid-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Negative (0)
2D	L-arginine-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (2)
1D	Ornithine-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (1)/ Negative (0)
4E	Glycine-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (4)
2E	GLY-PRO-AMC	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Positive (2)/ Negative (0)
1E	4MU-β-D-galactose	blue fluorescence >FCT well	blue fluorescence ≤FCT well	Negative (0)
4F	Saccharose	Gold/Yellow	Orange/Red	Negative (0)
2F	Maltotriose	Gold/Yellow	Orange/Red	Negative (0)

1F	Carubinose	Gold/Yellow	Orange/Red	Negative (0)
4G	Pyranose	Gold/Yellow	Orange/Red	Positive (4)/ Negative (0)
2G	Maltobiose	Gold/Yellow	Orange/Red	Negative (0)
1G	Dissacharide	Gold/Yellow	Orange/Red	Negative (0)
4H	Riberol	Gold/Yellow	Orange/Red	Negative (0)
2H	Levulose	Gold/Yellow	Orange/Red	Negative (0)
1H	p-nitrophenyl-phosphorylcholine	Yellow	Colourless	Negative (0)
4I	γ-L-glutamyl-p-nitroanilide	Yellow	Colourless	Negative (0)
2I	p-nitrophenyl-phosphate	Yellow	Colourless	Negative (0)
1I	o-nitrophenyl-β-D-galactoside (OPNG)	Yellow	Colourless	Negative (0)
4J	Urea	Aqua/Blue	Yellow/Green	Negative (0)
2J	Resazurin	Pink	Blue/Purple	Negative (0)
1J	Ornithine	Purple	Yellow/Gray	Positive (1)/ Negative (0)

The inoculated panels were placed in incubation trays. All panels were incubated face down (larger windows facing up; label facing down) in a non-CO_2 incubator with 40 – 60% humidity. The incubation time for panels was 4 h at 35 – 37°C. After the recommended period of incubation, the panels were removed from the incubator. All panels were read face down using the BBL Crystal Panel Viewer. The colour reaction chart and Table 3.6 was used for the interpretation of the reactions. The results pad was used to record reactions. The regular (white) light source was used to read columns F thru J first. The UV light source in the panel viewer was used to read columns A thru E (fluorescent substrates). A fluorescent substrate well was considered positive only if the intensity of the fluorescence

observed in the well was greater than the negative control well (4A). Each positive test result (except 4A) was given a value of 4, 2, or 1, corresponding to the row where the test was located (Table 3.6). A value of 0 (zero) was given to any negative result. The values resulting from each positive reaction in each column were then added together. A 10-digit number was generated; this was the profile number (Table 3.6).

Table 3.6. Calculation of BBL crystal™ N/H identification kit profile number

Example	A	B	C	D	E	F	G	H	I	J
4	*	+	+	–	+	–	–	–	–	–
2	–	+	–	+	–	–	–	–	–	–
1	+	+	+	–	–	–	–	–	–	–
Profile	**1**	**7**	**5**	**2**	**4**	**0**	**0**	**0**	**0**	**0**

*(4A) = fluorescent negative control

The resulting profile number and cell morphology were entered on a PC in which the BBL crystal™ N/H identification system Electronic Codebook has been installed to obtain the tested isolate identification. According to the manufacturer's information, out of 513 isolates tested, the sensitivity of the BBL crystal™ N/H identification system without supplemented tests was 89.5%, whereas with supplemented tests the sensitivity was 93.6%. The specificity of the BBL crystal™ N/H identification system was 94.9%.

3.1.4 Epsilon Test (E-test®)

E-test® is a quantitative technique for determining the anti-microbial susceptibility of both non-fastidious Gram negative and Gram positive aerobic bacteria. The

system comprises a predefined antimicrobic gradient which is used to determine the MIC, in µg/ml, of individual agents against micro-organisms as tested on agar media. The E-test was performed as specified in the manufacturer's product package insert (AB Biodisk, Solna Sweden). An inoculum of *N. gonorrhoeae*, with a density of a 0.5 McFarland standard (equivalent to $1.5x10^8$ cfu/ml), was prepared in saline directly from a fresh overnight subculture and applied to the surface of a culture plate GC agar base plus 1% IsoVitalex, by using a cotton swab and then allowed to dry. Four control strains namely *Neisseria gonorrhoeae* ATCC 49226, WHO A, MAL058 and ESN306 were used to validate the test as a necessary component of the study design.

Ciprofloxacin E-test strips were allowed to reach room temperature prior to use. When the inoculated agar surface was completely dry, the ciprofloxacin E-test package was opened. Using sterile forceps, the ciprofloxacin E-test strip was applied to the inoculated agar surface, ensuring that the MIC scale was facing upwards and that the concentration maximum was nearest the rim of the plate. The whole length of the strip was completely in contact with the agar surface. Air pockets were removed, if necessary, by pressing gently on the strip with forceps, always moving from the minimum concentration upwards. Small bubbles under the strip did not affect the results. Once applied, the strip was not moved because of the instantaneous release of antibiotic into the agar.

The inoculated plates were incubated immediately at 35-37^oC in a >70% humid

atmosphere containing 3-10% CO_2 in a candle jar. Plates were incubated for at least 48 hours and examined after 24-48 hours. Bacterial growth was distinctly visible after the required period of incubation. The MIC value was read at the point of intersection between the inhibition ellipse edge and the E-test strip (Fig 2.12). When the inhibition ellipse was below the strip i.e. the zone edge does not intersect the strip, the MIC was reported as less than (<) the lowest value on the reading scale. Clinical Laboratory Standards Institute (CLSI) (34) guidelines were used for the interpretation of E-test results for *N. gonorrhoeae* (Table 3.7).

Table 3.7. CLSI interpretation criterion for ciprofloxacin susceptibilities of the *N. gonorrhoeae* isolates (34)

Category of strain	**MIC (μg/ml) ranges**
Fully susceptible (cip^s)	≤0.06 μg/ml
Less/Intermediately (cip^i)	>0.6 μg/ml to <1.0μg/ml
Resistant (cip^r)	greater or equal to than 1.0μg/ml

3.2 Specimens for molecular experiments

Specimens were selected to assist with the development of a real-time PCR assay to detect quinolone-susceptibility in DNA extracted from genital specimen (see Section 3.4.2). Once developed, this PCR assay was used to test DNA extracted from NMS patients' urethral swabs and culture.

3.2.1 Gauteng specimens

N. gonorrhoeae culture and both urine and urethral swab specimens were also collected from men presenting with gonorrhoea at a PHC clinic in Johannesburg, Gauteng Province in 2007 as part of the NMS programme coordinated at the NICD's STI Reference Centre. A total of 24 consecutively paired urine and urethral swab specimens from NMS patients, in whom *N. gonorrhoeae* has been previously isolated were selected based on established ciprofloxacin MIC results (i.e. 16 resistant, 2 intermediate susceptible and 6 susceptible). The 24 urine specimens were used to validate the real-time PCR assay.

3.2.2 Northern Cape specimens

Urethral swabs were collected from males presenting with gonorrhoea at PHC clinics in Kimberley, Northern Cape Province in 2006 as part of the NMS programme. A total of 38 isolates were initially grown and then stocked in Kimberley (NHLS Laboratories). Only 17 of these were viable when re-cultured for MIC determination at NICD's STI Reference Centre in 2007. The ciprofloxacin susceptibility profiles for the remaining 21 gonococci were unknown. The real-time PCR assay, once developed, was used to determine susceptibility to ciprofloxacin for 9 of the previously cultured 17 strains (5 resistant and 4 susceptible) based on urethral swab DNA extracted previously for prior aetiological studies. The remaining 8 gonococci isolates were not viable on subculture at the time of the current work. The protocol was further used on

swabs from 21 patients with unknown MICs to determine mutations in *gyrA* and *parC* genes.

3.2.3 Western Cape specimens

Urethral swab specimens were collected from men presenting with gonorrhoea attending a PHC clinic in Cape Town, Western Cape Province in 2006 as part of the NMS programme. A total of 16 swab specimens, from patients known to be *N. gonorrhoeae* culture positive, were consecutively selected based on known MIC results of these previously isolated strains. These 16 swabs come from 11 patients with known ciprofloxacin resistant gonorrhoea and from 5 patients with known ciprofloxacin susceptible gonorrhoea. These 16 urethral swabs were also processed using the developed protocol for the detection of quinolone susceptibility by real-time PCR assay.

3.2.4 Specimen used to assess the sensitivity and specificity of the QRDR real-time PCR assay

In order to detect cross reactivity of the real-time PCR, urine and swab specimens were selected for testing from 21 male patients who had non-gonococcal urethral disease. These men were recruited as part of NMS activities but were determined to be both culture and PCR negative for *N. gonorrhoeae*. These 21 men were infected either singly (X14) or in combination (X5) with *Chlamydia trachomatis* (CT), *Trichomonas vaginalis* (TV) and/or *Mycoplasma genitalium* (MG); two of these men had no pathogens detected.

Cultures from 17 patients with urinary tract infections were also processed using real-time PCR (see section 3.1.1.2) in order to assess the specificity of the assay.

The PCR assay was also run without probes, in order to see if the primers can bind to *gyrA* and *parC* genes of other bacteria for example *C. trachomatis*, *T. vaginalis, M. genitalium*, *N. lactamica*, *N. meningitidis*, *N. mucosa* and the six urinary tract infection-causing organisms. The sensitivity and specificity of the primers was validated using DNA isolated from the following specimens: urine and swabs of patient with non-gonococcal urethral discharge, cultures from patients with urinary tract infections and cultures from non-gonococcal Neisseria species. To visualize PCR products, 1µl of PCR products, including the 1000bp marker were run on an Agilent 2100 Bioanalyzer (Chemetrix, Midrand, South Africa). The expected product size was 72bp for both *gyrA* and *parC* PCR.

3.3 Nucleic acid preparation

Nucleic acids were extracted from cultures, swabs and urines, using either manual or automated extraction methods. Pure genomic DNA for the positive controls used in the M-PCR assay were received from the American Type Culture Collection (ATCC). Positive controls included genomic DNA from *Neisseria gonorrhoeae* (ATCC-700825), *Chlamydia trachomatis* (VR-885), *Trichomonas vaginalis* (ATCC-30001) and *Mycoplasma genitalium* (ATCC-33530).

3.3.1 Nucleic acid extraction using the QIAamp Viral RNA Kit

Nucleic acids were extracted by using the QIAamp Viral RNA kit (Qiagen, Doncaster Vic, Australia) and performed according to the manufacturer's instructions. Briefly, 140 µl samples were lysed by adding 560 µl lysis buffer and incubated at room temperature for 10 min. After incubation, 560 µl of absolute ethanol was added and the lysate was loaded onto a QIAamp spin column. The salt and the pH conditions ensured that RNA/DNA binded to the silica-gel column during two configuration steps. Nucleic acids were washed free of contaminants using two different buffers. Nucleic acids were eluted in 100 µl Buffer AE or elution buffer and stored at -70^{o}C for further use.

3.3.2 Nucleic acid extraction using an automated extractor

The isolation of nucleic acids from cultures, swabs and urines were performed by using an automated DNA extractor, X-tractor Gene^{TM} kit (Corbett Robotics, Eight Mile Plains, Australia), following the manufacturer's guidelines and eluting in 100µl of elution buffer.

The configuration of the extraction process was stepped through with the aid of the X-Tractor Gene^{TM} Wizard. Sample information was either entered or imported into the run file to complete the software setup. A template was created from the initial run enabling rapid set-up time for subsequent extractions.

Samples (200µl), reagents and consumables (e.g. filtered tips) were then loaded onto the workstation. A 180µl volume of sample was added to lysis buffer and mixed. The lysed sample was loaded onto a capture plate containing a glass fibre matrix to which nucleic acids would specifically bind. Unbound waste was removed during successive wash steps and the samples were dried under vacuum.

The capture plate was automatically moved to the Elution station and 100µl of nucleic acid was eluted under vacuum into individual cluster tubes (1.2 mL or 0.65 mL volume capacity) in a 96-well rack format. Eluted nucleic acid was stored at -70^{o}C until used for PCR.

3.4 Real-Time PCR

Real-time PCR technology was used to detect pathogens causing male urethral discharge and also to detect susceptibility in the QRDRs of the *gyrA* and *parC* genes. Taqman probes were used in the real-time PCR assay, in order to bind to the amplification products and fluorescence resonance energy transfer chemistry is used to specifically detect the amplification product.

3.4.1 Detection of *N. gonorrhoeae* DNA using Multiplex PCR (M-PCR)

A multiplex PCR (M-PCR) is a single PCR test which incorporates multiple primer pairs each targeting a specific gene, resulting in multiple amplification products. The M-PCR protocol, recently published by Mhlongo S, et al (122), was originally

developed from CDC and the method was transferred to NICD STI Reference Centre in the year 2005. The M-PCR procedure detected the presence of STI discharge causing organisms (e.g. *N. gonorrhoeae* (GC), *C. trachomatis* (CT), *T. vaginalis* (TV) and *M. genitalium* (MG). This M-PCR amplified the DNA from the cytosine DNA methyltransferase gene of *N. gonorrhoeae*, the 7.4 Kb cryptic plasmid of *C. trachomatis*, the repeated DNA sequence of *T. vaginalis* and the dihydrolipoamide dehydrogenase gene of *M. genitalium.* All real-time PCR amplification reactions were conducted using the RotorGene 3000 instrument, (Corbett Research, Mortlake, Australia) which is comprised of a fluorometer and a thermal cycler for the detection of fluorescence during the cycling process. The primers and probes were manufactured and obtained from the University of Cape Town and Southern Cross Biotechnology (Cape Town, South Africa) respectively (Table 3.8).

PCR amplification was performed in a 25µl reaction mixture containing the following constituents: 5µl 10X PCR Buffer (Applied Biosystems, Foster City, USA), 8µl of 25 mM $MgCl_2$ (Applied Biosystems, Foster City, USA), 0.4µl of 50mM dNTP w/dUTP (Bioline, London, UK), 8.2µl of sterile water, 0.2µl of each set of forward and reverse primers per each organism (0.2µM final concentration of each), 0.2µl of each probe (0.2µM final concentration of each), 1µl of 5U/µl Amplitaq Gold (Applied Biosystems, Foster City, USA) and 25µl of DNA template. The cycling started with a 2 min hold at 50^{o}C and a 10 min Taq activation step at 95^{o}C followed by 50 cycles of denaturation at 95^{o}C for 20 sec, and

annealing/extension at 60°C for 60 sec. The fluorescence signal was measured once in each cycle at the end of the extension step.

Table 3.8. Primers and probes for the detection of STI discharge causing organisms

MPCR primer, probe and fluorophore	Primer name	Sequence (5' to 3')
Primers for GC		
Forward	GC-019	GGA TAC GAC GTA ACC TTG ACT ATG G
Reverse	GC-020	CCG ATG TAG AAG ACC CTT TTG C
Probe for GC	GC-023	CA ACG CCA AAG ACT ACG GTG TAG CAC AG
Fluorophore	Rox[a]	
Primers for CT		
Forward	CT-008	GGA TTG ACT CCG ACA ACG TAT TC
Reverse	CT-009	ATC ATT GCC ATT AGA AAG GGC ATT
Probe for CT	CT-010	TT ACG TGT AGG CGG TTT AGA AAG CGG [
Fluorophore	6-FAM[b]	
Primers for TV		
Forward	TV-001	AAA GAT GGG TGT TTT AAG CTA GAT AAG G
Reverse	TV-002	TCT GTG CCG TCT TCA AGT ATG C
Probe for TV	TV-011	AG TTC ATG TCC TCT CCA AGC GTA AGT
Fluorophore	CY5[c]	
Primers for MG		
Forward	MG-041:	CGG ATC AAG ACC AAG ATA CTT AAC TTT
Reverse	MG-042:	AGC TTG GGT TGA GTC AAT GAT AAA C
Probe for MG	MG-048	CC AGG GTT TGA AAA AGC ACA ACA AGC TG
Fluorophore	Joe[d]	

[a] Rox emits light at 602 nm
[b] 6-FAM (6-carboxyflourescein) emits light at 520 nm
[c] CY5 emits light at 667 nm.
[d] Joe emits light at 548 nm

3.4.2 Real-Time PCR assay for the detection of QRDR

The real-time PCR assay used to detect susceptibility of QRDR in DNA extracted from urine and swabs were based on a recently published article (63), which was developed for gonococcal isolates rather than DNA extracted from non-invasive samples. The primers and probes were manufactured and obtained from the University of Cape Town and Southern Cross Biotechnology (Cape Town, South Africa) respectively (Table 3.9). Probes encompass the DNA regions encoding for amino acids 91 and 95 of GyrA and amino acids 86, 87 and 88 of ParC, the loci most often associated with resistance. The sequence of the *gyrA* probe (see Table 3.9) is backward and will bind TCC and GAC. The assay was modified by the inclusion of an internal control. The *cytosine methyltransferase* gene was used to confirm the presence of *N. gonorrhoeae* in all samples (see section 3.4.1).

Table 3.9. Primers and probes for the ABI QRNG QRDR susceptibility detection system

QRDR primer, probe and fluorophore	**Primer and probe name**	**Sequence (5' to 3')**
gyrA		
Primers		
Forward	GyraABI1	TTG-CGC-CAT-ACG-GAC-GAT
Reverse	GyraABI2	GCG-ACG-TCA-TCG-GTA-AAT-ACC-A
Probe	GyrAWT91.95	TGT-CGT-AAA-CTG-CGG-AA
Fluorophore	6-FAM[a]	
parC		
Primers		
Forward	ParCABI1	TGA-GCC-ATG-CGC-ACC-AT
Reverse	ParCABI2	GGC-GAG-ATT-TTG-GGT-AAA- TAC-CA
Probe	ParCWT86.87.88	CGG-AAC-TGT-CGC-CGT
Fluorophore	Joe[b]	
cytosine methyltransferase		
Primers		
Forward	GC-019	GGA TAC GAC GTA ACC TTG ACT ATG G
Reverse	GC-020	CCG ATG TAG AAG ACC CTT TTG C
Probe	GC-023	CA ACG CCA AAG ACT ACG GTG TAG CAC AG
Fluorophore	Rox[c]	

[a] 6-FAM (6-carboxyflourescein) emits light at 520 nm
[b] Joe emits light at 548 nm.
[c] Rox emits light at 602 nm

PCR amplification was performed in a 25µl reaction mixture containing the following constituents: 2.5µl 10X PCR Buffer (Roche Diagnostics, Mannheim, Germany), 4µl of 50 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany) , 0.2µl of 50mM dNTP mix (Roche Diagnostics, Mannheim, Germany), 6.9µl of sterile water, 0.1µl of each set of forward and reverse primers (0.2µM final concentration of each), 0.25µl of each probe (0.2µM final concentration of each),

0.5µl of 5U/µl Amplitaq Gold and 10µl of DNA template. The cycling started with a 2 min hold at 50°C and a 10 min denaturation step at 95°C followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The fluorescence signal was measured once in each cycle at the end of the extension step.

Controls included a known wild type (WT) QRDR DNA sequence (WHO A) and two QRNG strain with known QRDR mutations in either the *gyrA* gene alone (MAL 058) or in both the *gyrA* and *parC* genes (ESN306). The amplification plot for a WT strain showed exponential signal increase. This indicated that the *gyr*A and *par*C loci of WT strains were amplified. For mutant strains, no exponential increases in fluorescence were observed for either locus. Strains with intermediate resistance showed signal amplification for the *par*C locus only, indicating the presence of *gyrA* mutation. The amplification plot of all the controls showed an exponential fluorescence increase for the cytosine methyltransferase gene confirming the presence of *N. gonorrhoeae*.

3.5 QRDR amplification and direct sequencing

3.5.1 *gyrA* and *parC* PCR amplification for DNA sequencing

PCR was performed to amplify the *gyrA* and *parC* genes of the gonococcal isolates, following which these genes were sequenced to determine the presence of mutations. Published oligonucleotide primers (43, 45**,** 63) for the PCR amplification are documented in Table 3.10.

Table 3.10. Sequences and location of oligonucleotide primers for the amplification of QRDR of the *gyrA* and *parC*

Primer	Primer name	Nucleotide Sequence (5' to 3')	Nucleotide Position
gyrA			
Forward	NG-GYRA-Z	ATG TGA GAT TTT CGC CAT GCG G	2332-2353
Reverse	NG-GYRA-B	CAA ATT CGC CCT CGA AAC CCT	2702-2722
			391bp product
parC			
Forward	NG-PARC-Z	CAG CGG CGC ATT TTG TTT GC	145-164
Reverse	NG-PARC-B	AAC TAC GAC GGC GCG TTT GA	454-473
			329bp product

QRDR's were amplified from culture DNA extracts using methodologies previously described for gonococcal isolates (42, 44). QRDRs were also amplified from the urethral swabs, in cases where the results of real-time PCR assay to detect altered QRDRs for urethral swabs did not agree with the results using culture-based DNA extracts. PCR was performed by adding 5µl of DNA extract into a PCR master mix making the total volume to 25µl. PCR was performed using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, USA). The PCR master mix contained: 12.5 µl of 2X ImmoMix containing Immolase^{TM} DNA Polymerase and Ultra-pure dNTPs (Bioline, London, UK), 0.5 µl of $MgCl_2$ (2.5 mM final concentration) 6.8 µl of sterile water and 0.1 µl each of forward and reverse primers (0.2µM final concentration of each). The primers were manufactured by and obtained from the University of Cape Town (Cape Town, South Africa). PCR cycling conditions were as follows: 35 cycles of 94^{o}C for 30 sec, 55^{o}C for 30 sec and 72^{o}C for 45 sec. Depending on the size of the fragment to be analyzed, 5µl of PCR products, including the 100bp marker, were

electrophoresed on a 2% agarose gel containing ethidium bromide at a concentration of 1µg/ml. Gels were run in 1X Tris-boric acid buffer (TBE) (Promega, Madison, USA) at 120 volts for 1h. The expected product sizes were 391 bp for *gyrA* and 329 bp for *parC*. DNA bands were visualized on an ultraviolet (UV) transluminator (Sygene, Maryland, USA) and their sizes estimated by comparison with a 100 bp DNA molecular weight marker (Promega, Madison, USA).

3.5.2 Direct Sequencing

Direct DNA sequencing was performed to identify mutations in the *gyrA* and *parC* genes of the gonococcal isolates.

3.5.2.1 Purification and concentration of PCR products from PCR reactions

PCR products with the correct *gyrA* and *parC* molecular weight were purified by using the MSB® Spin PCRapace kit (Invitek, Berlin, Germany). A spin filter was placed into a 2.0 ml receiver tube. A 250µl volume of binding buffer was added to the PCR sample and mixed by pipetting or vortexing. The sample was completely transferred onto a corresponding spin filter and centrifuged for 3 min at 12 000 rpm with the cap closed. After centrifugation the spin filter was placed into a new 1.5 ml receiver tube. At least 10µl of elution buffer was added directly onto the center of the spin filter and incubated at room temperature for 5 min with cap closed. The spin filter tube was centrifuged for 1 min at 10 000 rpm. The

spin filter was then discarded and the filtrate or the purified PCR product was processed immediately.

3.5.2.2 Cycle Sequencing

At least 2µl of the purified PCR product was used to determine the DNA concentration (in ng/µl) using the NanoDrop (Nanodrop Technologies, Wilmington, USA) spectrophotometer. Purified PCR products were diluted to a concentration of 25 *f*mol which was used for cycle sequencing. The reaction mixture for the cycle sequencing contained: 1µl of the Big Dye terminator (Applied Biosystems, Foster City, USA), 1µl of the 5 µM forward primer, 1.5 µl of 5X Big Dye sequencing Buffer (Applied Biosystems, Foster City, USA), 4.5 µl of deionised water and 2µl of the purified PCR product. PCR was performed using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, USA). PCR cycling conditions were as follows: 94^{o}C for 1 min followed by 25 cycles of 95^{o}C for 30 sec, 50^{o}C for 20 sec, and 60^{o}C for 4 min.

3.5.2.3 Removal of DyeDeoxy™ terminators from DNA cycle sequencing reactions of PCR products

Cycle sequencing products were further purified by using the MSB® Spin PCRapace kit (Invitek, Berlin, Germany). A spin filter was placed into a 2.0 ml receiver tube. A 500µl of binding buffer was added to the completed cycle sequencing reaction and mixed by pipetting or vortexing. The sample was completely transferred onto a corresponding spin filter and centrifuged for 4 min

at a maximum speed with the cap closed. After centrifugation, the spin filter was placed into a new 1.5 ml receiver tube. At least 5µl of elution buffer was added directly onto the center of the spin filter and incubated at room temperature for 5 min with the cap closed. The spin filter tube was centrifuged for 1 min at 10 000 rpm. The spin filter tube was discarded and the filtrate or the purified cycle sequencing product was processed immediately.

3.5.2.4 DNA Sequencing

At least 13 µl of Hi-Di Formamide (Applied Biosystems, Foster City, USA) was added into 2µl of purified cycling sequencing product and mixed by pipetting. The purified cycle sequencing product was denatured at 94^{o}C for 2 min and immediately cooled on ice for 2 min. The products were sequenced, using an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, USA). Data were aligned with QRDR DNA sequences corresponding to amino acids 91 to 95 of *GyrA* (GenBank accession no. U08817) and amino acids 86 to 92 of *ParC* (GenBank accession no. U08907). The three different DNA sequence profiles identified were the wild type S; mutant I; mutant R and were corresponding to the observed ciprofloxacin MIC of the corresponding gonococcal isolates using E tests (S, susceptible; I, intermediate resistance; R, resistant).

3.6 *N. gonorrhoeae* Multi-Antigen sequence typing (NG MAST)

If the DNA sequence patterns of the QRNG strains were identical, further characterization was performed by using the *N. gonorrhoeae* Multi-Antigen Sequence Typing (NG-MAST) technique (116). Primers used for PCR and sequencing are shown in Table 3.11. These primers amplified sequences within two gonococcal genes, *por* and *tbpB*.

Table 3.11. Sequences and location of primers used for NG MAST.

Primer	**Nucleotide Sequence (5' to 3')**	**Nucleotide Position**
por		
Forward	CAA GAA GAC CTC GGC AA	350-366
Reverse	CCG ACA ACC ACT TGG T	1086-1071
		737bp product
tbpB		
Forward	CGT TGT CGG CAG CGC GAA AAC	1098-1118
Reverse	TTC ATC GGT GCG CTC GCC TTG	1686-1666
		589bp product

3.6.1 *por* PCR

The PCR reaction of the *por* gene fragment was performed in a reaction volume of 50µl, using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, USA). The reaction mixture contained the following: 5µl of 10X PCR buffer (Roche, Basel, Switzerland), 0.5µl of 2.5U Taq polymerase (Roche, Basel, Switzerland), 5µl of DNA lysate**,** 5µl of each 0.2mmol/l dNTP (Invitrogen, California, USA), 0.5µl of each 100pmol forward and reverse *por* primer (Invitrogen, California, USA), and deionised water to a volume of 50µl. The PCR cycle involved an initial denaturation of 4 min at 95^{o}C, followed by 25 cycles of 30

sec at 95°C, 30 sec at 58°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C and cooling to 4°C.

3.6.3 *tbpB* PCR

The PCR reaction of the *tbpB* gene fragment was performed in a reaction volume of 50µl, using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, USA). The reaction mixture contained the following: 5µl of 10X PCR buffer (Roche, Basel, Switzerland), 0.5µl of 2.5U Taq polymerase (Roche, Basel, Switzerland), 5µl of DNA lysate**,** 5µl of each 0.2mmol/l dNTP (Invitrogen, California, USA), 1µl of each 50pmol forward and reverse tb*pB* primer (Invitrogen, California, USA), and deionised water to a volume of 50µl. The PCR cycle involved an initial denaturation of 4 min at 95°C, followed by 25 cycles of 30 sec at 95°C, 30 sec at 69°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C and cooling to 4°C.

3.6.3 Agarose gel electrophoresis

Five microlitres of PCR products including the 100bp marker were electrophoresed on a 2% agarose gel (Seaken® LE Agarose, Lonza, Rockland, USA) containing ethidium bromide at a concentration of 1µg/ml in order to separate the DNA fragments by size. Gels were run in 1X TBE (Promega, Madison, USA) at 120 volts for 1 h. The expected product size for *por* was 737 bp and for *tbpB* was 589 bp. DNA bands were visualized on an UV transluminator (Syngene, Maryland, USA) and their size estimated by

comparison with a 100 bp DNA molecular weight marker (Promega, Madison, USA).

3.6.4 Purification of PCR products and DNA Sequencing

PCR products with the correct *por* and *tbpB* molecular weight were purified by using the MSB® Spin PCRapace kit (Invitek, Berlin, Germany) (see section 3.5.2.1). Purified PCR products were diluted to a concentration of 25 *f*mol which was used for cycle sequencing (see section 3.5.2.2). Cycle sequencing products were further purified by using the MSB® Spin PCRapace kit (Invitek, Berlin, Germany) (see section 3.5.2.3). Both strands of DNA amplified from the *por* and *tbpB* genes were sequenced by using the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, USA) (see section 3.5.2.4). The trace files from the forward sequencing reactions were analysed to the correct length. For *por*, a sequence of 490bp was used to define the alleles, starting at the conserved sequence TTGAA and for *tbpB* a sequence of 390bp was selected for defining alleles, starting at the conserved sequence CGTCTGAA.

3.6.5 Data analysis

The edited and trimmed *por* and *tbpB* sequences were initially compared with each other by use of the Non-Redundant Database (available at http://www.mlst.net) (133) and each allele numbers were assigned to each different *por* and *tbpB*. Sequence and the corresponding sequence type (ST) were assigned on the basis of the combination of the alleles at the 2 loci by using

the NG MAST website (http://www.ng-mast.net) (116). Clusters of isolates, defined as more than one isolate having the same ST, and single types were identified by ST number assignment.

3.7 Handling of Data

Data were entered, cleaned and analysed using a Microsoft Office Excel 2003 database. A chi-squared (χ^2) test was used to determine associations between the NG MAST ST and the ciprofloxacin resistance phenotype with the level of significance set at p=0.05.

3.8 Ethics

The Human Research Ethics Committee of the University of Witwatersrand has approved the collection of clinical specimen as part of the national STI NMS programme (Protocol M051024). In addition, approval was also been given for this current project, using stored DNA extracts from urine specimens and stored gonococci isolated from urethral swabs of male participants in various cities as part of the surveillance programme (Protocol M060508).

CHAPTER 4: RESULTS

4.1. Strain identification

In total, 49 *Neisseria gonorrhoeae* isolates were re-cultured from stock vials, 24 from Gauteng (GP), 9 from the Northern Cape (NC) and 16 from the Western Cape (WC). The identities of all 49 gonococcal isolates were confirmed by typical colony morphology and Gram stain appearance, oxidase positivity, and by immunological testing using the Phadebact® Monoclonal GC test. These isolates were identified as Gram-negative, oxidase positive and all isolates belong to the WII/III serogroup (Table 4.1). All isolates were also correctly identified using the BBL Crystal™ *Neisseria/Heamophilus* (N/H) Identification System. The E-test method for MIC determination was repeated on all isolates in order to confirm the ciprofloxacin susceptibility profile. On the basis of the susceptibility criteria based on those of CLSI (28), the susceptibility criteria of all isolates from Gauteng and Western Cape had the same susceptibility profile as previously determined. Gonococcal isolates from Northern Cape had different ciprofloxacin MIC results from those reported by the original microbiology surveillance team working at Northern Cape in 2007. Of the nine viable gonococcal isolates, from Northern Cape, selected for this study, five were originally reported as susceptible to ciprofloxacin (MIC≤0.003) and four were resistant (MIC≥1.0). On repeat of the susceptibility testing, using stock isolates, eight isolates were determined to be resistant to ciprofloxacin (MIC ≥1.0) and only one isolate was susceptible (MIC ≤

0.003) (Table 4.1).The MIC testing was repeated three time and the same susceptibility phenotype was found each time.

Table 4.1: Strain identification and ciprofloxacin susceptibility profiles

Patient Specimen ID	Gram-negative cocci present	Oxidase Test	Phadebact	BBL Profile	Ciprofloxacin MIC (µg/ml)	Susceptibility to Ciprofloxacin
GP-07-MUS-002	Yes	Pos	WII/WIII	*N. gono* 98.99%	2	Resistant
GP-07-MUS-006	Yes	Pos	WII/WIII	*N. gono* 99.51%	4	Resistant
GP-07-MUS- 016	Yes	Pos	WII/WIII	*N. gono* 99.89%	3	Resistant
GP-07-MUS -018	Yes	Pos	WII/WIII	*N. gono* 99.89%	3	Resistant
GP-07-MUS- 026	Yes	Pos	WII/WIII	*N. gono* 98.99%	4	Resistant
GP-07-MUS-029	Yes	Pos	WII/WIII	*N. gono* 99.89%	1	Resistant
GP-07-MUS-031	Yes	Pos	WII/WIII	*N. gono* 99.89%	3	Resistant
GP-07-MUS-036	Yes	Pos	WII/WIII	*N. gono* 99.08%	4	Resistant
GP-07-MUS-038	Yes	Pos	WII/WIII	*N. gono* 97.00%	3	Resistant
GP-07-MUS-040	Yes	Pos	WII/WIII	*N. gono* 99.89%	4	Resistant
GP-07-MUS-064	Yes	Pos	WII/WIII	*N. gono* 98.99%	2	Resistant
GP-07-MUS-097	Yes	Pos	WII/WIII	*N. gono* 99.98%	1	Resistant
GP-07-MUS-120	Yes	Pos	WII/WIII	*N. gono* 99.93%	6	Resistant
GP-07-MUS-125	Yes	Pos	WII/WIII	*N. gono* 99.93%	<0.002	Susceptible
GP-07-MUS-136	Yes	Pos	WII/WIII	*N. gono* 99.93%	<0.002	Susceptible
GP-07-MUS-148	Yes	Pos	WII/WIII	*N. gono* 98.99%	0.064	Intermediate
GP-07-MUS-149	Yes	Pos	WII/WIII	*N. gono* 94.19%	<0.002	Susceptible
GP-07-MUS-151	Yes	Pos	WII/WIII	*N. gono* 97.73%	<0.002	Susceptible
GP-07-MUS-159	Yes	Pos	WII/WIII	*N. gono* 99.86%	0.006	Susceptible
GP-07-MUS-166	Yes	Pos	WII/WIII	*N. gono* 99.51%	3	Resistant
GP-07-MUS-168	Yes	Pos	WII/WIII	*N. gono* 98.99%	3	Resistant
GP-07-MUS-170	Yes	Pos	WII/WIII	*N. gono* 94.91%	<0.002	Susceptible
GP-07-MUS-171	Yes	Pos	WII/WIII	*N. gono* 98.99%	1.5	Resistant
GP-07-MUS-187	Yes	Pos	WII/WIII	*N. gono* 98.99%	0.094	Intermediate
NC-06-MUS-045	Yes	Pos	WII/WIII	*N. gono* 79.69%	2	Resistant
NC-06-MUS-056	Yes	Pos	WII/WIII	*N. gono* 98.99%	1	Resistant

Patient Specimen ID	Gram-negative cocci present	Oxidase Test	Phadebact	BBL Profile	Ciprofloxacin MIC (µg/ml)	Susceptibility to Ciprofloxacin
NC-06-MUS-079	Yes	Pos	WII/WIII	*N. gono* 99.98%	1	Resistant
NC-06-MUS-085	Yes	Pos	WII/WIII	*N. gono* 99.08%	1	Resistant
NC-06-MUS-100	Yes	Pos	WII/WIII	*N. gono* 99.98%	1	Resistant
NC-06-MUS-103	Yes	Pos	WII/WIII	*N. gono* 99.51%	2	Resistant
NC-06-MUS-105	Yes	Pos	WII/WIII	*N. gono* 99.89%	0.003	Susceptible
NC-06-MUS-135	Yes	Pos	WII/WIII	*N. gono* 97.88%	2	Resistant
NC-06-MUS-144	Yes	Pos	WII/WIII	*N. gono* 99.89%	4	Resistant
WC-06-MUS-010	Yes	Pos	WII/WIII	*N. gono* 94.28%	4	Resistant
WC-06-MUS-012	Yes	Pos	WII/WIII	*N. gono* 99.89%	2	Resistant
WC-06-MUS-013	Yes	Pos	WII/WIII	*N. gono* 98.99%	2	Resistant
WC-06-MUS-022	Yes	Pos	WII/WIII	*N. gono* 99.99%	2	Resistant
WC-06-MUS-050	Yes	Pos	WII/WIII	*N. gono* 99.86%	8	Resistant
WC-06-MUS-071	Yes	Pos	WII/WIII	*N. gono* 99.51%	4	Resistant
WC-06-MUS-105	Yes	Pos	WII/WIII	*N. gono* 98.99%	8	Resistant
WC-06-MUS-131	Yes	Pos	WII/WIII	*N. gono* 98.99%	4	Resistant
WC-06-MUS-181	Yes	Pos	WII/WIII	*N. gono* 99.86%	<0.002	Susceptible
WC-06-MUS-182	Yes	Pos	WII/WIII	*N. gono* 95.13%	<0.002	Susceptible
WC-06-MUS-189	Yes	Pos	WII/WIII	*N. gono* 99.86%	<0.003	Susceptible
WC-06-MUS-196	Yes	Pos	WII/WIII	*N. gono* 98.99%	8	Resistant
WC-06-MUS-200	Yes	Pos	WII/WIII	*N. gono* 97.00%	<0.002	Susceptible
WC-06-MUS-228	Yes	Pos	WII/WIII	*N. gono* 94.68%	<0.002	Susceptible
WC-06-MUS-239	Yes	Pos	WII/WIII	*N. gono* 99.86%	4	Resistant
WC-06-MUS-243	Yes	Pos	WII/WIII	*N. gono* 97.03%	8	Resistant

GP= Gauteng isolates; NC= Northern Cape isolates; WC= Western Cape isolates

4.2. Real-time PCR results

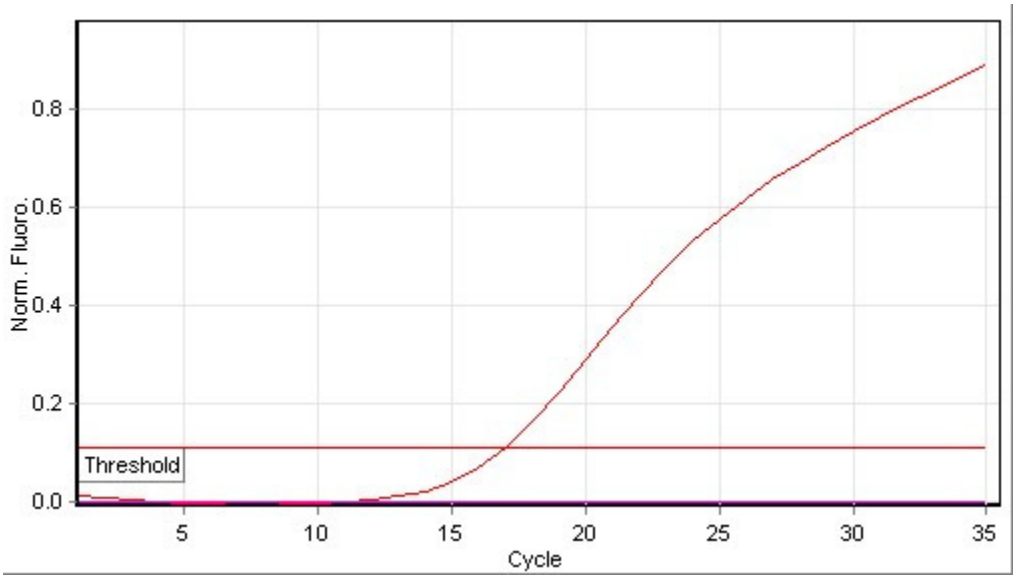
4.2.1. Real-time QRDR detection PCR results: Controls

Results from the real-time PCR assay developed to detect susceptibility in the QRDRs for the controls used in this study correlated 100% with the ciprofloxacin MICs (Figures 4.1, 4.2 and 4.3). All control strains showed exponential signal increase of the *cytosine methyltransferase*, which confirms the presence of *N. gonorrhoeae* (Table 4.3).

The amplification plot for a WT ciprofloxacin susceptible (CipS) strain (WHO A) showed exponential signal increase. This indicates WT strains were positively amplified, with a Ct of 16.98 cycles and 15.31 cycles for the *gyrA* and *parC* loci, respectively (Figures 4.1 and 4.2).

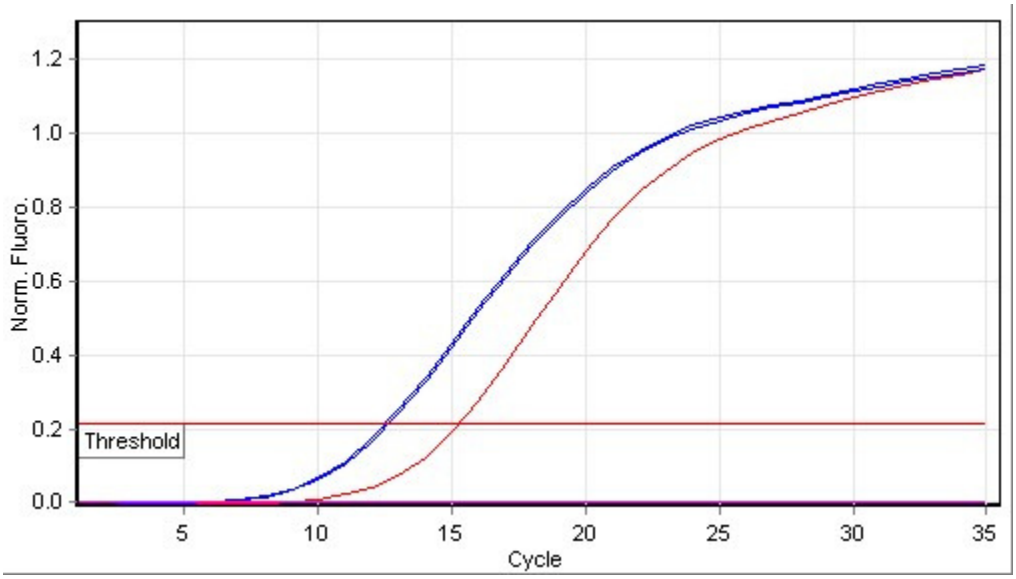
For strains with reduced ciprofloxacin susceptibility (CipI) and strains with ciprofloxacin resistant (CipR), no exponential fluorescence increases were observed (Figure 4.1). The CipI control *N. gonorrhoeae* strain (MAL058) showed signal amplification for the *parC* locus but nor the *gyrA* locus (with a mean Ct of 12.61 cycles for *parC*).

CipR control *N. gonorrhoeae* strains (ESN306) failed to show signal amplification at either locus (Figures 4.1 and 4.2). Curves were analogous when either one or two mutations were present in *gyrA* or *parC* genes.



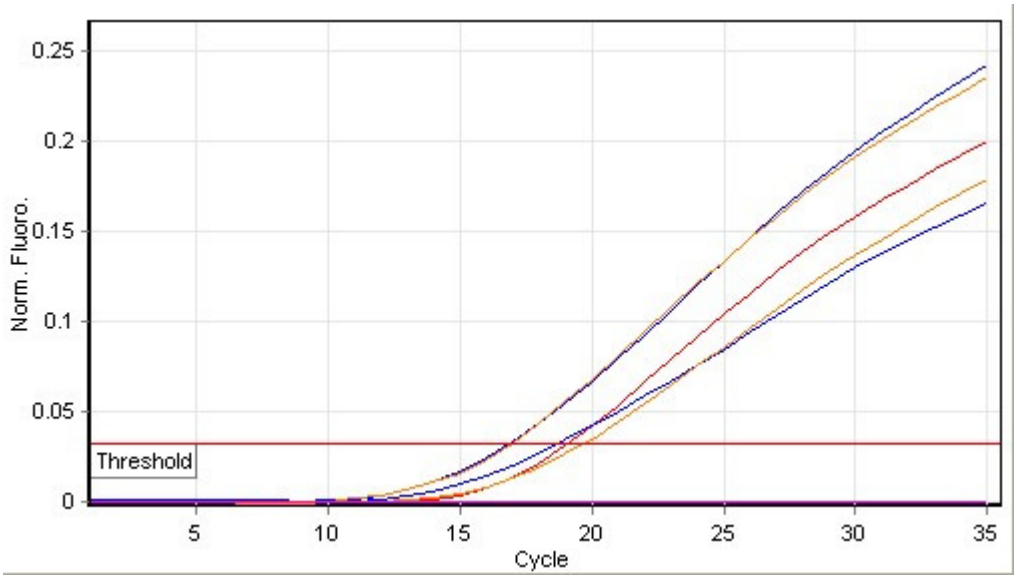
No.	Colour	Name	Type	Ct
1		WHO A	Positive Control	16.98
2		MAL- 058	Unknown	NEG (NTC)
3		MAL- 058	Unknown	NEG (NTC)
4		ESN- 306	Unknown	NEG (NTC)
5		ESN- 306	Unknown	NEG (NTC)
6		Neg ct	Unknown	NEG (NTC)

Figure 4.1: Quantitation data for *gyrA*: The WT (WHO A) strain was positively amplified for the *gyrA* loci and whereas no amplification was observed for the CipI (MAL-058) and CipR (ESN-306) strains for the *gyrA* loci.



No.	Colour	Name	Type	Ct
1		WHO A	Positive Control	15.31
2		MAL- 058	Unknown	12.66
3		MAL- 058	Unknown	12.56
4		ESN- 306	Unknown	NEG (NTC)
5		ESN- 306	Unknown	NEG (NTC)
6		Neg. ct	Unknown	NEG (NTC)

Figure 4.2: Quantitation data for *parC*: The WT (WHO A) and CipI (MAL-058) strains were positively amplified for the *parC* loci and whereas no amplification was observed for the CipR (ESN-306) strain for the *parC* loci.



No.	Colour	Name	Type	Ct
1		WHO A	Positive Control	19.07
2		MAL- 058	Unknown	18.68
3		MAL- 058	Unknown	16.80
4		ESN- 306	Unknown	16.94
5		ESN- 306	Unknown	19.65
6		Neg ct	Unknown	NEG (NTC)

Figure 4.3: Quantitation data for *cytosine methyltransferase*: All control strains were positively amplified for the *cytosine methyltransferase gene.*

4.2.2 Specificity of the Real-Time PCR

The specificity and cross reactivity of the primers and probes were tested on DNA extracted from 21 paired urine and urethral swab specimens from men with urethral discharge but for whom culture and PCR assays were negative for *N.*

gonorrhoeae (Table 4.2). The real-time M-PCR assay for the detection of STI discharge causing organisms was able to detect *T. vaginalis, C. trachomatis* and *M. genitalium* whereas the real-time PCR for the detection of gonococcal *gyrA* and *parC* genes showed no signal amplification in both urine and swabs even after 35 cycles.

The PCR assay was also run without probes, in order to see if the primers can bind to *gyrA* and *parC* genes of other bacteria. The primers bound to both QRNG controls and *N. gonorrhoeae* positive urine specimens, in the absence of probes, producing a 72bp amplicon size for *gyrA* and *parC* (Fig 4.4). These results showed that the primers were able to bind to the *gyrA* and *parC* genes of *N. gonorrhoeae*. All six urinary tract causing organisms, *N. lactamica*, *N. meningitidis* and *N. mucosa* as well as *T. vaginalis, C. trachomatis* and *M. genitalium* were all positive for the 72bp amplicon size (Fig.4.5 and 4.6), indicating that the primers were also able to bind to *gyrA* and *parC* genes of other bacteria. This experiment demonstrated the non-specificity of the primers and confirms that the specificity of the assay is dependant upon the probes.

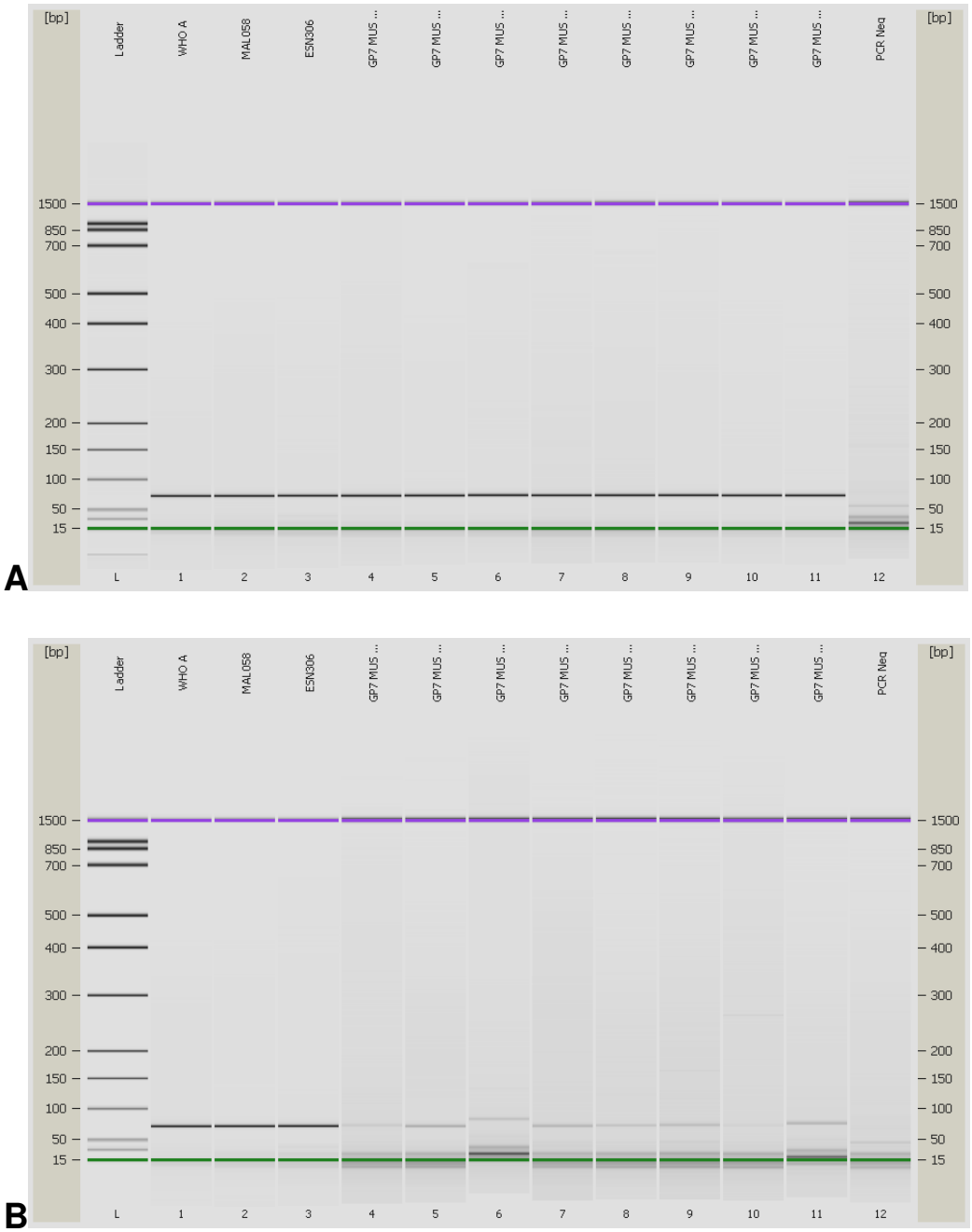
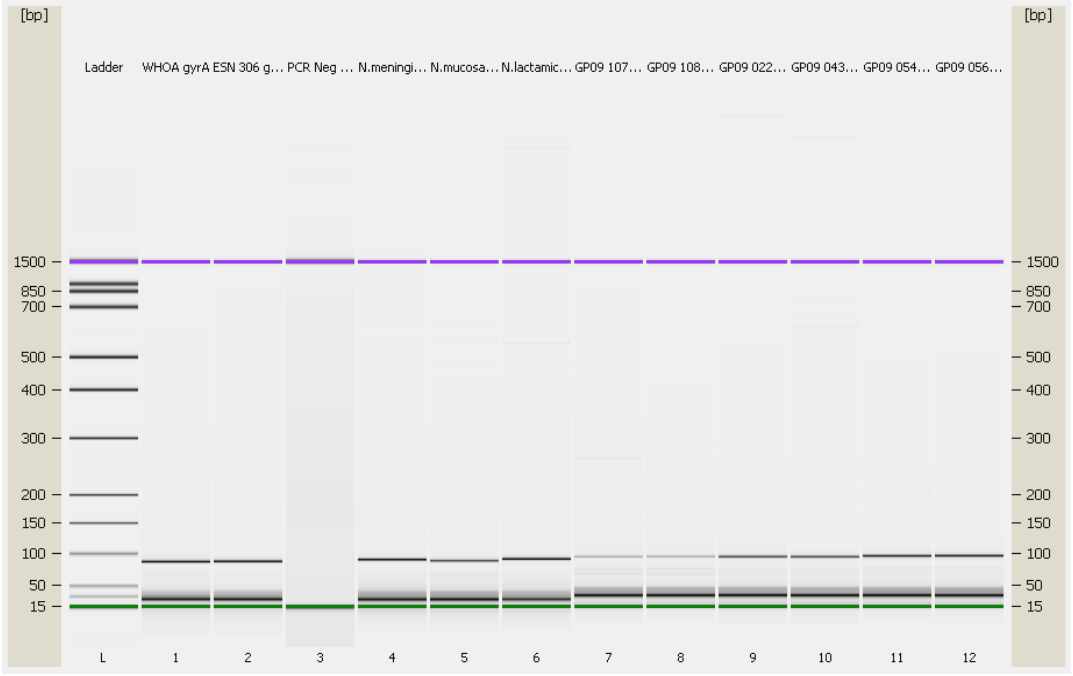


Fig.4.4: Electrophoresis for a *gyrA* (A) *and parC* (B) PCR run without probes on *N.gonorrhoeae* positive controls (lanes 1 to 3), PCR negative control (lane 12 with non specific bands, due to primer dimers**), *N. gonorrhoeae* positive urine specimens (lane 4-11) with a 1000bp ladder (L).** Note: The marker mixture for the DNA1000 Lab Chip contains lower and upper molecular size markers (10 and 1,500 bp) which the Bioanalyzer uses as references when sizing DNA fragments.

C



D

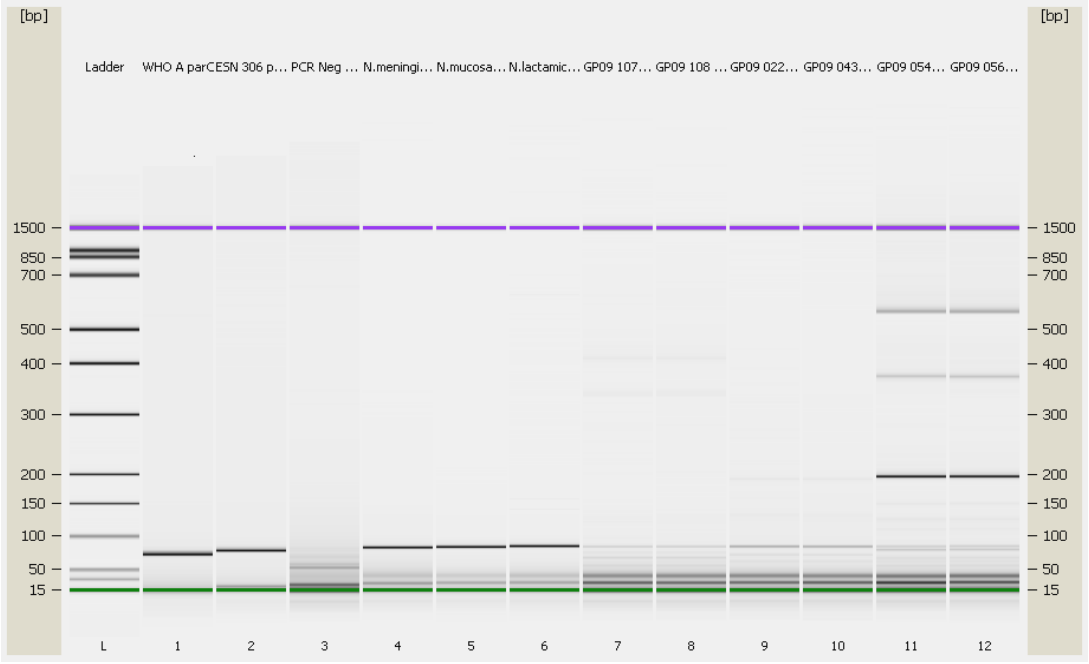
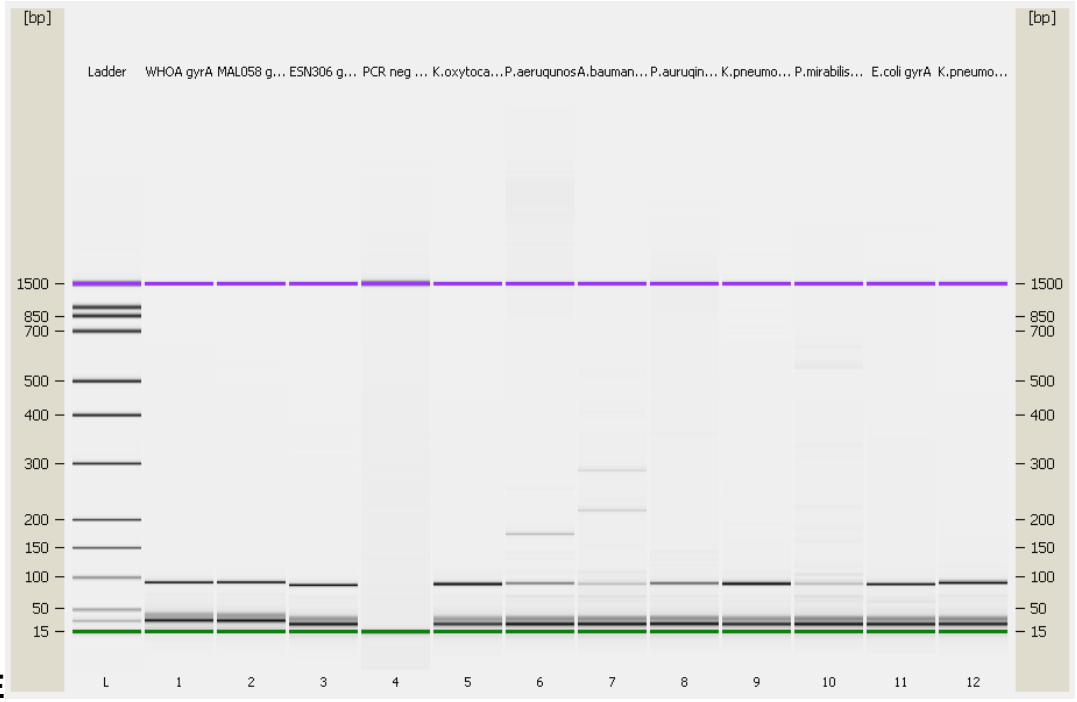
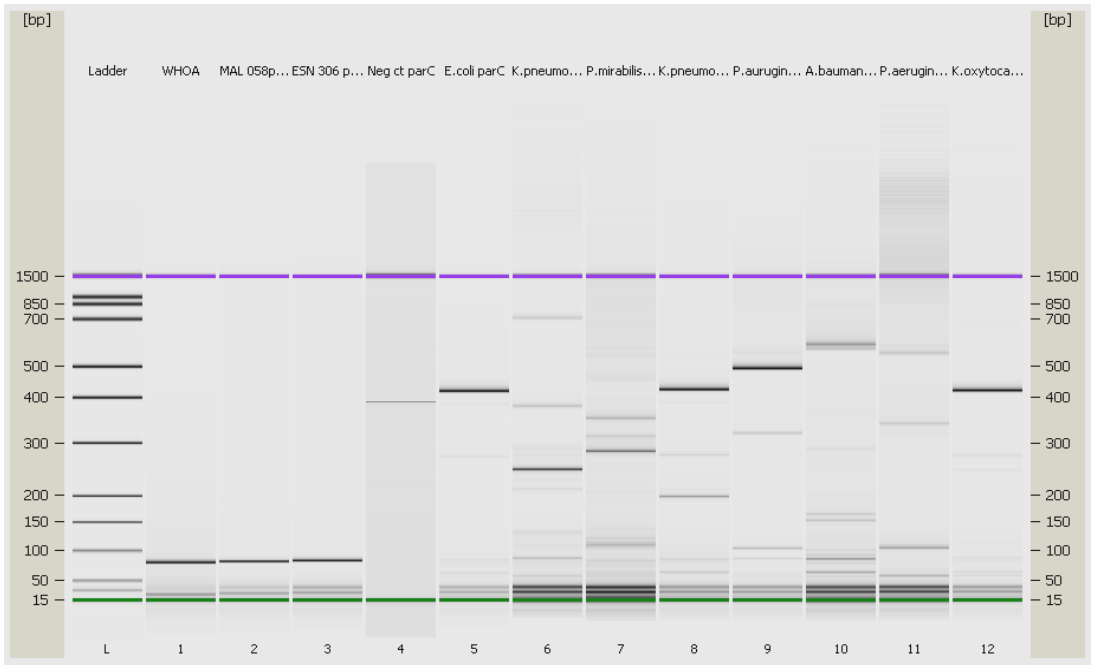


Fig. 4.5: Electrophoresis for a *gyrA* (C) and *parC* (D) PCR run without probes on other Neisseria species. *N.gonorrhoeae* positive controls (lanes 1 to 2), PCR negative control (lane 3), three non-gonococcal Neisseria species (lanes 4 to 6), CT (lanes 7to 8), TV (lanes 9 to10) and MG (lanes 11 to 12) positive urine specimens and a 1000bp ladder (L).



E



F

Fig.4.6: Electrophoresis for a *gyrA* (E) and *parC* (F) PCR run without probes on UT organisms. *N.gonorrhoeae* positive controls (lanes 1 to 3); PCR negative control (lane 4); six urinary tract causing organisms (lanes 5 to 12) and with a 1000bp ladder (L).

Table 4.2: Real-Time PCR results for the detection of gonococcal *gyrA* and *parC* genes in paired urine and urethral swab specimen from 21 MUS patients with non-gonococcal urethral discharge

Patient Specimen ID	Bacteriology			Pathogens detected by Real-Time Multiplex-PCR	Real-time PCR products for wild type gonococcal *gyrA* and *parC* detected			
	Microscopy		Culture		Urines		Urethral swabs	
	GNDC present on urethral smear	Pus cells on urethral smears	*N. gonorrhoeae culture*		*gyrA*	*parC*	*gyrA*	*parC*
GP-07-MUS-022	Negative	1+	Negative	TV	No	No	No	No
GP-07-MUS-043	Negative	0	Negative	TV	No	No	No	No
GP-07-MUS-048	Negative	3+	Negative	CT+MG	No	No	No	No
GP-07-MUS-054	Negative	1+	Negative	MG	No	No	No	No
GP-07-MUS-055	Negative	1+	Negative	CT+TV+MG	No	No	No	No
GP-07-MUS-056	Negative	0	Negative	MG	No	No	No	No
GP-07-MUS-063	Negative	0	Negative	TV	No	No	No	No
GP-07-MUS-078	Negative	2+	Negative	MG	No	No	No	No
GP-07-MUS-088	Negative	2+	Negative	CT+MG	No	No	No	No
GP-07-MUS-100	Negative	3+	Negative	MG	No	No	No	No
GP-07-MUS-105	Negative	0	Negative	TV	No	No	No	No
GP-07-MUS-107	Negative	0	Negative	CT	No	No	No	No
GP-07-MUS-108	Negative	0	Negative	CT	No	No	No	No

Patient Specimen ID	Bacteriology			Pathogens detected by Real-Time Multiplex-PCR	Real-time PCR products for wild type gonococcal *gyrA* and *parC* detected			
	Microscopy		Culture		Urines		Urethral swabs	
	GNDC present on urethral smear	Pus cells on urethral smears	*N. gonorrhoeae culture*		*gyrA*	*parC*	*gyrA*	*parC*
GP-07-MUS-112	Negative	1+	Negative	None	No	No	No	No
GP-07-MUS-115	Negative	1+	Negative	CT+MG	No	No	No	No
GP-07-MUS-135	Negative	0	Negative	TV	No	No	No	No
GP-07-MUS-154	Negative	2+	Negative	TV	No	No	No	No
GP-07-MUS-156	Negative	0	Negative	CT	No	No	No	No
GP-07-MUS-157	Negative	0	Negative	None	No	No	No	No
GP-07-MUS-163	Negative	1+	Negative	CT	No	No	No	No
GP-07-MUS-193	Negative	0	Negative	CT+TV	No	No	No	No

1 Urethral smears were scored as follows: 0= less than 5 pus cells/hpf
1+= 5-9 pus cells/hpf
2+= 10-15 pus cells/hpf;
3+= greater than 15 pus cell/hpf
hpf= high power field oil immersion x100

2. Abbreviations are as follows: GNDC= Gram negative diplococci
TV= *Trichomonas vaginalis*
CT=*Chlamydia trachomatis*
MG=*Mycoplasma genitalium*

Further evaluation of the specificity was conducted by performing the real-time PCR on cultures obtained from 17 patients with urinary tract pathogens (Table 4.3). PCR results showed no exponential fluorescence increases for these strains, hence no cross-reactivity observed. These results further confirmed the specificity of our assay.

Table 4.3: Real-Time PCR results for the detection of *gyrA* and *parC* genes in DNA extracted from 17 cultured Gram-negative urinary tract pathogens

Patient Specimen ID	Organism	Real-Time PCR products for wild type gonococcal *gyrA* and *parC* detected	
		gyrA	***parC***
TJG4361408	*Escherichia coli*	No	No
TJG4355953	*Escherichia coli*	No	No
TJG4354696	*Escherichia coli*	No	No
TJG4356637	*Escherichia coli*	No	No
TJG4364852	*Escherichia coli*	No	No
TJG4364045	*Escherichia coli*	No	No
TJG4370758	*Escherichia coli*	No	No
TJG4370628	*Escherichia coli*	No	No
TJG4362808	*Escherichia coli*	No	No
TJG4363790	*Escherichia coli*	No	No
TJG4362360	*Klebsiella pneumoniae*	No	No
TJG4356283	*Proteus mirabilis*	No	No
TJG4355387	*Klebsiella pneumoniae*	No	No
TJG4356801	*Pseudomonas aeruginosa*	No	No
TJG4367884	*Acinetobacter baumannii*	No	No
TJG4370166	*Pseudomonas aeruginosa*	No	No
TJG4370652	*Klebsiella oxytoca*	No	No

4.2.3 Results of the Real-Time PCR assay to detect susceptibility in the QRDRs of surveillance specimens

The assay successfully amplified the selected portion of cytosine methyltransferase, *gyrA* and *parC* genes from DNA extracted from swab and gonococcal culture samples from participants in the Gauteng, Northern Cape and Western Cape microbiological surveillance programme (Table 4.4, 4.5 and 4.6), and also from DNA extracted from participants' urine samples from Gauteng (Table 4.4). All 49 samples showed exponential signal increase of the cytosine methyltransferase, which confirms the presence of *N. gonorrhoeae* in the samples (Table 4.4, 4.5 and 4.6).

All sixteen ciprofloxacin resistant samples from Gauteng, had *gyrA* and *parC* gene mutations, whereas five of six susceptible isolates had no detectable mutations *gyrA* or *parC* genes (Table 4.4). One of the ciprofloxacin susceptible isolates (i.e. GP-07-MUS-159) had a detectable mutation in *parC* only. Both isolates with reduced ciprofloxacin susceptibility (GP-07-MUS-148 and GP-07-MUS-187) had detectable mutations in the *gyrA* gene and no mutation in the *parC* gene.

Table 4.4: Real-Time PCR results for urine, urethral swab and cultured gonococci from 24 MUS patients recruited in Gauteng

Patient Specimen ID	Ciprofloxacin (µg/ml)	Real-Time PCR								
		Urine			Urethral Swab			Urethral Culture		
		gyrA	*par C*	*cytosine methyltransferase*	*gyrA*	*par C*	*cytosine methyltransferase*	*gyrA*	*par C*	*cytosine methyltransferase*
Ciprofloxacin Susceptible										
GP-07-MUS -125	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
GP-07-MUS -136	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
GP-07-MUS -149	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
GP-07-MUS -151	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
GP-07-MUS -159	0.008	**YES**	NO	**YES**	**YES**	NO	**YES**	**YES**	NO	**YES**
GP-07-MUS -170	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
Ciprofloxacin Intermediate										
GP-07-MUS -148	0.064	NO	**YES**	**YES**	NO	**YES**	**YES**	NO	**YES**	**YES**
GP-07-MUS -187	0.125	NO	**YES**	**YES**	NO	**YES**	**YES**	NO	**YES**	**YES**
Ciprofloxacin Resistance										
GP-07-MUS -2	4	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -6	8	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -16	8	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -18	1.5	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -26	4	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -29	1	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -31	3	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -36	2	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -38	3	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -40	6	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -64	1.5	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -97	1.5	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -120	4	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**

Patient Specimen ID	Ciprofloxacin (µg/ml)	Real-Time PCR								
		Urine			Urethral Swab			Urethral Culture		
		gyrA	*par C*	*cytosine methyltransferase*	*gyrA*	*par C*	*cytosine methyltransferase*	*gyrA*	*par C*	*cytosine methyltransferase*
Ciprofloxacin Resistance										
GP-07-MUS -166	4	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -168	4	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**
GP-07-MUS -171	1.5	NO	NO	**YES**	NO	NO	**YES**	NO	NO	**YES**

No mutations were detected in the samples (cultures and swabs) from patient with susceptible *N. gonorrhoeae* isolates from the Western Cape (n=5) and Northern Cape (n=1) (Table 4.5). All eleven resistant samples (culture and swabs) from the Western Cape, had *gyrA* and *parC* mutations. Three of the eight resistant samples from the Northern Cape had *gyrA* and *parC* mutations in DNA extracted from both cultured *N. gonorrhoeae* and a paired urethral swab. However, results of the real-time PCR analysis of DNA extracted from the remaining five paired *N. gonorrhoeae* cultures and urethral swabs were not in agreement. The cultures had a ciprofloxacin resistant phenotype and failed to produce an amplified product using the real-time PCR, consistent with a CipR genotype. The QRDR of these fine culture-based DNA extracts were subsequently shown by DNA sequencing to possess the same *gyrA* and *parC* QRDR mutations as the rest of the ciprofloxacin resistant specimen (Table 4.5). The urethral swab real-time PCR results were consistent with a CipS (WT) genotype, suggesting that the *N. gonorrhoeae* culture stock and urethral swabs were from different patients. This was confirmed by DNA sequencing of the *gyrA* and *parC* genes.

Table 4.5: Real-Time PCR results for urethral swabs and cultured gonococci from patient with urethral discharge from the Western Cape (16) and from the Northern Cape (9)

Patient Specimen ID	Ciprofloxacin (µg/ml)	Real-Time PCR					
		Swabs			Cultures		
		gyrA	***parC***	*cytosine methyltransferase*	***gyrA***	***parC***	*cytosine methyltransferase*
Ciprofloxacin Susceptible							
NC-06-MUS-105	0.003	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
WC-06-MUS-181	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
WC-06-MUS-182	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
WC-06-MUS-189	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
WC-06-MUS-200	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
WC-06-MUS-228	<0.002	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
Ciprofloxacin Resistance							
NC-06-MUS-045	2	NO	NO	**YES**	NO	NO	**YES**
NC-06-MUS-056	1	NO	NO	**YES**	NO	NO	**YES**
NC-06-MUS-079*	1	Yes	Yes	**YES**	NO	NO	**YES**
NC-06-MUS-085*	1	Yes	Yes	**YES**	NO	NO	**YES**
NC-06-MUS-100	1	NO	NO	**YES**	NO	NO	**YES**
NC-06-MUS-103*	2	Yes	Yes	**YES**	NO	NO	**YES**
NC-06-MUS-135+	2	Yes	Yes	**YES**	NO	NO	**YES**
NC-06-MUS-144+	4	Yes	Yes	**YES**	NO	NO	**YES**
WC-06-MUS-010	12	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-012	4	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-013	16	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-022	3	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-050	8	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-071	12	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-105	6	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-131	16	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-196	8	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-239	2	NO	NO	**YES**	NO	NO	**YES**
WC-06-MUS-243	6	NO	NO	**YES**	NO	NO	**YES**

* = original MICs on isolated gonococci, determined in Kimberley at the time of the surveillance (prior to stocking cultures), were 0.002µg/ml for patient's NC-06-MUS-079; NC-06-MUS-085 and NC-06-MUS-103.

+ = original MICs on isolated gonococci were >1µg/ml for patient's NC-06-MUS-135 and NC-06-MUS-144.

The assay successfully amplified the selected portion of *gyrA* and *parC* swabs samples among the strains with unknown antimicrobial resistant profile from the Northern Cape. Fifteen isolates had no detectable mutations in *gyrA* and *parC* and six isolates had mutation in *gyrA* and *parC* (Table 4.6).

Table 4.6: Real-time PCR results from the Northern Cape urethral swabs with unknown MIC results

Material Tested	***Gyr A***		***Par C***		cytosine methyltransferase	
	No. Pos	No. Neg.	No. Pos	No. Neg.	No. Pos	No. Neg.
Swabs	15	0	15	0	21	0
	0	6	0	6	0	0

All 35 ciprofloxacin-resistant (CipR) gonococcal isolates had mutations in both *gyrA* and *parC* QRDRs (Table 4.7). The two Gauteng isolates with reduced ciprofloxacin susceptibility (CipI) had mutations in *gyrA*. All samples (swabs and culture) tested had identical *gyrA* and *parC* mutations, except the five urethral swabs collected from Northern Cape (i.e. NC-06-MUS-079; NC-06-MUS-085; NC-06-MUS-103; NC-06-MUS-135 and NC-06-MUS-144).

Table 4.7: Summary of real-time PCR results for urines (Gauteng only), urethral swab and cultured gonococci from 49 men attending clinics in Gauteng, the Northern Cape and the Western Cape

Ciprofloxacin susceptibility profile	Specimen Tested	Number tested	*gyrA* PCR Product		*parC* PCR Product	
			No. Pos	No. Neg	No. Pos	No. Neg
Susceptible	Cultures	12	12	0	11	1
	Urines	6†	6	0	5	1
	Swabs	12	12	0	11	1
Intermediate	Cultures	2	0	2	2	0
	Urines	2†	0	2	2	0
	Swabs	2	0	2	2	0
Resistant	Cultures	35	0	35	0	35
	Urines	16†	0	16	0	16
	Swabs	35*	5	30	5	30

* The real-time PCR results obtained using urethral swabs from 5/8 resistant gonorrhoea cases are in doubt (see discussion).

† Urines were only taken from patients recruited in Gauteng.

4.3. Sequencing results

Results of *gyrA* and *parC* QRDR sequencing are summarized in Tables 4.8, 4.9 and 4.10. The mutations within the QRDRs of *gyrA* and *parC* of 35 isolates of *N. gonorrhoeae* are shown, by ciprofloxacin susceptibility category, *gyrA* and *parC* QRDR alteration, and geographical origin of isolates. The two CipI isolates from Gauteng exhibited the identical point mutations in the *gyrA* gene only (TCC to TTC, Ser91→ Phe; GAC to GGC, Asp95→ Gly), at amino acid positions 91 and 95. CipR isolates from Gauteng, Northern Cape and Western Cape, exhibited

the same point mutation pattern in *gyrA* (TCC to TTC, Ser91→ Phe; GAC to GGC, Asp95→ Gly) as well as an additional point mutation (GAC to AAC, Asp86→Asn) in the *parC* gene. All CipS isolates had wild type QRDR DNA sequences with the exception of the (GP-07-MUS-159) isolate which had a mutation in *parC* at the codon encoding for amino acid 86(GAC to AAC, Asp86→Asn). Sequencing results of the five paired *N. gonorrhoeae* cultures and urethral swabs from Northern Cape were still inconsistent which agreed with the real-time PCR findings (Table 4.9).

Table 4.8: Sequencing results for *gyrA* and *parC* QRDRs of *N. gonorrhoeae* isolates from Gauteng

Ciprofloxacin susceptibility phenotype	Amino acid codon at indicated position in:														
		GyrA							ParC						
		90	91	92	93	94	95	96	86	87	88	89	90	91	92
	WT	Asp GAT	Ser TCC	Ala GCA	Val GTT	Tyr TAC	Asp GAC	Thr ACC	Asp GAC	Ser AGT	Ser TCC	Ala GCC	Tyr TAT	Glu GAG	Ala GCG
S	GP-07-MUS-125	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S	GP-07-MUS-136	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S	GP-07-MUS-149	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S	GP-07-MUS-151	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S	GP-07-MUS-159	—	—	—	—	—	—	—	Asn AAC	—	—	—	—	—	—
S	GP-07-MUS-170	—	—	—	—	—	—	—	—	—	—	—	—	—	—
I	GP-07-MUS-148	—	Phe TTC	—	—	—	Gly GGC	—	—	—	—	—	—	—	—
I	GP-07-MUS-187	—	Phe TTC	—	—	—	Gly GGC	—	—	—	—	—	—	—	—
R	GP-07-MUS-002	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	GP-07-MUS-006	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	GP-07-MUS-016	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	GP-07-MUS-018	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	GP-07-MUS-026	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	GP-07-MUS-029	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	GP-07-MUS-031	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	GP-07-MUS-036	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—

Ciprofloxacin susceptibility phenotype	Amino acid codon at indicated position in:														
		GyrA							ParC						
		90	91	92	93	94	95	96	86	87	88	89	90	91	92
	WT	Asp GAT	Ser TCC	Ala GCA	Val GTT	Tyr TAC	Asp GAC	Thr ACC	Asp GAC	Ser AGT	Ser TCC	Ala GCC	Tyr TAT	Glu GAG	Ala GCG
			Phe				Gly		Asn						
R	GP-07-MUS-038	—	TTC	—	—	—	GGC	—	AAC	—	—	—	—	—	—
			Phe				Gly		Asn						
R	GP-07-MUS-040	—	TTC	—	—	—	GGC	—	AAC	—	—	—	—	—	—
			Phe				Gly		Asn						
R	GP-07-MUS-064	—	TTC	—	—	—	GGC	—	AAC	—	—	—	—	—	—
			Phe				Gly		Asn						
R	GP-07-MUS-097	—	TTC	—	—	—	GGC	—	AAC	—	—	—	—	—	—
			Phe				Gly		Asn						
R	GP-07-MUS-120	—	TTC	—	—	—	GGC	—	AAC	—	—	—	—	—	—
			Phe				Gly		Asn						
R	GP-07-MUS-166	—	TTC	—	—	—	GGC	—	AAC	—	—	—	—	—	—
			Phe				Gly		Asn						
R	GP-07-MUS-168	—	TTC	—	—	—	GGC	—	AAC	—	—	—	—	—	—
			Phe				Gly		Asn						
R	GP-07-MUS-171	—	TTC	—	—	—	GGC	—	AAC	—	—	—	—	—	—

S = Susceptible; I = Intermediate/Reduced Susceptibility; R = Resistant

Table 4.9: Sequencing results for the *gyrA* and *parC* QRDRs of paired *N. gonorrhoeae* isolates and urethral swabs from the Northern Cape

Ciprofloxacin susceptibility phenotype	Real-Time PCR genotype	Source of *N. gonorrhoeae*		Amino acid (codon) at indicated position in:														
				GyrA							ParC							
				90	91	92	93	94	95	96	86	87	88	89	90	91	92
			WT	Asp (GAT)	Ser (TCC)	Ala (GCA)	Val (GTT)	Tyr (TAC)	Asp (GAC)	Thr (ACC)	Asp (GAC)	Ser (AGT)	Ser (TCC)	Ala (GCC)	Tyr (TAT)	Glu (GAG)	Ala (GCG)
S	S	CULTURE	NC-06-MUS-105	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	S	SWAB	NC-06-MUS-105	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R	R	CULTURE	NC-06-MUS-045	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
	R	SWAB	NC-06-MUS-045	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
R	R	CULTURE	NC-06-MUS-056	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
	R	SWAB	NC-06-MUS-056	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
R	R	CULTURE	NC-06-MUS-079	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
	S	SWAB	NC-06-MUS-079	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R	R	CULTURE	NC-06-MUS-085	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
	S	SWAB	NC-06-MUS-085	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R	R	CULTURE	NC-06-MUS-100	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
	R	SWAB	NC-06-MUS-100	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
R	R	CULTURE	NC-06-MUS-103	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—

Ciprofloxacin susceptibility phenotype	Real-Time PCR genotype	Source of *N. gonorrhoeae*		Amino acid (codon) at indicated position in:														
				GyrA							ParC							
				90	91	92	93	94	95	96	86	87	88	89	90	91	92
			WT	Asp (GAT)	Ser (TCC)	Ala (GCA)	Val (GTT)	Tyr (TAC)	Asp (GAC)	Thr (ACC)	Asp (GAC)	Ser (AGT)	Ser (TCC)	Ala (GCC)	Tyr (TAT)	Glu (GAG)	Ala (GCG)
	S	SWAB	NC-06-MUS-103	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R	R	CULTURE	NC-06-MUS-135	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
	S	SWAB	NC-06-MUS-135	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R	R	CULTURE	NC-06-MUS-144	—	Phe (TTC)	—	—	—	Gly (GGC)	—	Asn (AAC)	—	—	—	—	—	—
	S	SWAB	NC-06-MUS-144	—	—	—	—	—	—	—	—	—	—	—	—	—	—

S = Susceptible ; R = Resistant

Table 4.10: Sequencing results for *gyrA* and *parC* QRDRs of *N. gonorrhoeae* isolates from the Western Cape

Ciprofloxacin susceptibility phenotype		Amino acid codon at indicated position in:													
		GyrA							ParC						
		90	91	92	93	94	95	96	86	87	88	89	90	91	92
	WT	Asp GAT	Ser TCC	Ala GCA	Val GTT	Tyr TAC	Asp GAC	Thr ACC	Asp GAC	Ser AGT	Ser TCC	Ala GCC	Tyr TAT	Glu GAG	Ala GCG
S	WC-06-MUS-181	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S	WC-06-MUS-182	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S	WC-06-MUS-189	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S	WC-06-MUS-200	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S	WC-06-MUS-228	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R	WC-06-MUS-010	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-012	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-013	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-022	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-050	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-071	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-105	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-131	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-196	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-239	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—
R	WC-06-MUS-243	—	Phe TTC	—	—	—	Gly GGC	—	Asn AAC	—	—	—	—	—	—

S = Susceptible; R = Resistant

4.4. NG-MAST Results

The primers amplified the internal fragments of *por* and *tbpB* from all of the isolates, from which trimmed sequences were obtained for both genes (Table 4.11). There were 18 different *por* alleles and 17 different *tbpB* sequences, resulting in 28 different 2-locus allelic profiles. Each allelic profile was assigned a different sequence type (ST) number. The correctly trimmed sequences of all known *por* and *tbpB* alleles are available at the NG-MAST web site (http://www.ng-mast.net).

A total of 16 QRNG isolates from Gauteng and were resolved into 12 STs, 7 of which were represented by two different clusters of ST217 (n=5) and ST3226 (n=2). Two isolates from Gauteng with reduced susceptibility to ciprofloxacin were resolved into two unique STs (ST2670 and ST3238). A total of six isolates from Gauteng with susceptibility to ciprofloxacin were resolved into five new STs (Table 4.11).

All eight QRNG isolates from Northern Cape had an identical NG MAST profile (ST533). One isolate from Northern Cape with susceptibility to ciprofloxacin was resolved into one unique ST. In order to investigate the inconsistent genotypic results between swabs and culture, an attempt to generate *tbpB* and *por* gene amplicons was attempted for NG MAST analysis. Unfortunately, the PCR assay failed due to the DNA concentration being too low as a result of the samples being too old. Hence, the NG MAST for the five urethral swabs was not done due

to the fact that the PCR targeting the *tbpB* and *por* gene did not produce sufficient PCR product for sequencing.

A total of 11 QRNG isolates from Western Cape were resolved into 5 STs, consisting of four unique STs and a clone of 7 isolates (ST217). All five ciprofloxacin susceptible isolates from the Western Cape were resolved into unique STs, of which two known ST (ST502 and ST607) and three new ST (ST3312, ST3318, ST3325) (Table 4.11). Among the 49 isolates, the most common alleles were *por*-158 and *tbpB*-4, which were present in 13 and 30 QRNG isolates, respectively. The most common ST was ST-217, which possessed the most common alleles at each locus (*por*-158 and *tbpB*-4), and was represented by 12 QRNG isolates (Table 4.11).

Table 4.11: NG-MAST genotypes of *N. gonorrhoeae* isolates from three South African provinces

Gauteng Province (n= 24)

No.	Ciprofloxacin susceptibility phenotype	Patient ID	*por* Allele	*tbpB* Allele	Sequence Type (ST)
1	**S**	GP-07-MUS-125	121	165	2652
2	**S**	GP-07-MUS-136	1662	747	3317
3	**S**	GP-07-MUS-149	1662	616	2659
4	**S**	GP-07-MUS-151	35	713	3173
5	**S**	GP-07-MUS-159	121	61	2660
6	**S**	GP-07-MUS-170	1662	616	2659
7	**I**	GP-07-MUS-148	1953	729	3238
8	**I**	GP-07-MUS-187	123	4	2670
9	**R**	GP-07-MUS-002	1649	4	2640
10	**R**	GP-07-MUS-006	158	4	217
11	**R**	GP-07-MUS-016	158	4	217
12	**R**	GP-07-MUS-018	1922	616	3226
13	**R**	GP-07-MUS-026	158	4	217
14	**R**	GP-07-MUS-029	158	4	217

15	R	GP-07-MUS-031	1922	616	3226
16	R	GP-07-MUS-036	1952	4	3239
17	R	GP-07-MUS-038	121	728	3253
18	R	GP-07-MUS-040	1922	719	3237
19	R	GP-07-MUS-064	361	4	524
20	R	GP-07-MUS-097	368	4	534
21	R	GP-07-MUS-120	1655	4	2647
22	R	GP-07-MUS-166	220	4	2661
23	R	GP-07-MUS-168	158	4	217
24	R	GP-07-MUS-171	121	4	531

Northern Cape Province (n= 9)

No.	Ciprofloxacin susceptibility phenotype	Patient ID	*por* Allele	*tbpB* Allele	Sequence Type (ST)
1	S	NC-06 -MUS -105	121	49	189
2	R	NC-06 -MUS -045	367	4	533
3	R	NC-06 -MUS -056	367	4	533
4	R	NC-06 -MUS -079	367	4	533
5	R	NC-06 -MUS -085	367	4	533
6	R	NC-06 -MUS -100	367	4	533
7	R	NC-06 -MUS -103	367	4	533
8	R	NC-06 -MUS -135	367	4	533
9	R	NC-06 -MUS -144	367	4	533

Western Cape Province (n= 16)

No.	Ciprofloxacin susceptibility phenotype	Patient ID	*por* Allele	*tbpB* Allele	Sequence Type (ST)
1	S	WC-07-MUS-181	2010	750	3325
2	S	WC-07-MUS-182	406	21	607
3	S	WC-07-MUS-189	251	165	502
4	S	WC-07-MUS-200	123	745	3312
5	S	WC-07-MUS-228	123	746	3318
6	R	WC-07-MUS-010	158	4	217
7	R	WC-07-MUS-012	35	612	2797
8	R	WC-07-MUS-013	158	650	2798
9	R	WC-07-MUS-022	1667	4	2668
10	R	WC-07-MUS-050	158	4	217
11	R	WC-07-MUS-071	158	4	217
12	R	WC-07-MUS-105	158	4	217
13	R	WC-07-MUS-131	158	4	217
14	R	WC-07-MUS-196	158	4	217
15	R	WC-07-MUS-239	361	4	524
16	R	WC-07-MUS-243	158	4	217

Typing of the 49 isolates revealed 5 ST clusters: ST 217 (12 isolates), ST 533 (8 isolates), ST 524 (2 isolates), ST 2659 (2 isolates), and ST 3226 (2 isolates) (Fig 4.7.). Three of the five ST clusters (ST217, ST524 and ST533) within this dataset have been previously described and are documented at the NG-MAST website (133) and the remaining two clusters (ST2659 and ST3266) were new. The remaining 23 STs comprised single isolates, of which 4 STs (ST189, ST502, ST534 and ST607) have been previously described and are documented at the NG-MAST website (133) and the remaining 19 STs were new.

Isolates of a given ST were typically identical or closely related with regard to antibiotic-resistance profile and region of isolation. The largest cluster of the *N. gonorrhoeae* in this study isolates belong to ST217, which was significantly associated with QRNG (p=0.0109). The presence quinolone resistance in the Northern Cape and the Western Cape was significantly associated with ST533 (p=0.0001) and ST217 (p=0.0397) respectively. There was no significant association between Gauteng province and any of the 5 ST clusters, due to small sampling size.

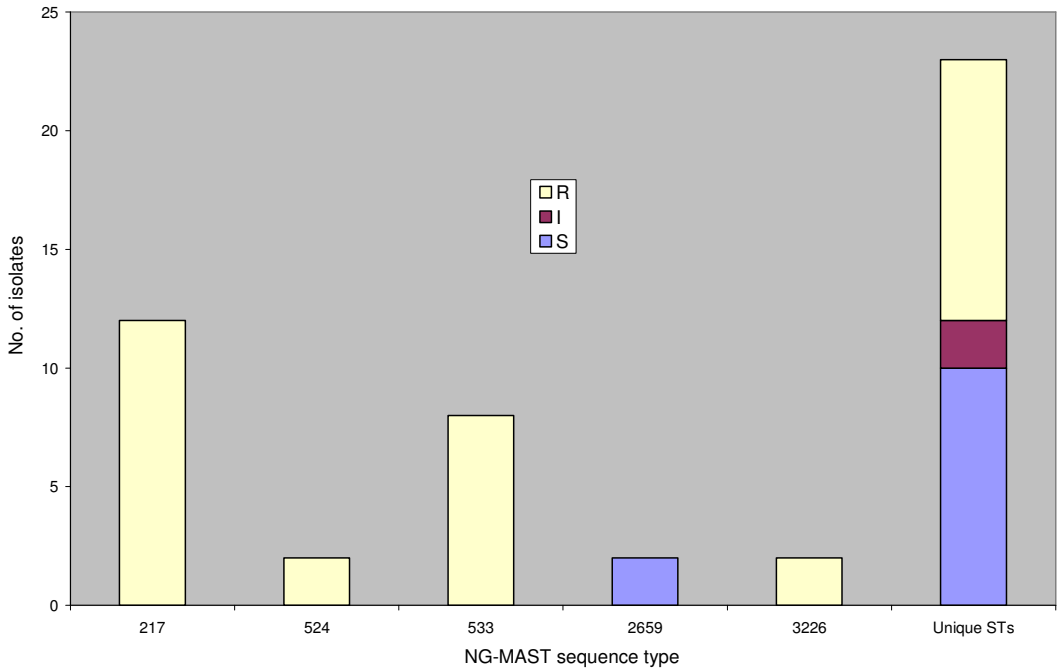


Figure 4.7: Distribution on *N. gonorrhoeae* isolates according to NG-MAST sequence type for the five ST clusters and the unique ST
S = Susceptible; I = Intermediate/Reduced susceptibility; R = Resistant

4.5 Summary of results

4.5.1 Assay validation

In summary, the assay successfully amplified the selected portion of *gyrA* and *parC* of all samples from Gauteng. All point mutations were easily discriminated and the mutation status obtained by real-time PCR generally agreed with the phenotype of either decreased susceptibility or resistance to ciprofloxacin (Table: 4.12).

4.5.2 Testing of clinical isolates

The assay successfully amplified the selected portion of *gyrA* and *parC* of all samples from Northern Cape and Western Cape. The real-time PCR results

agreed with the antimicrobial susceptibility phenotypes of the Western Cape samples. However, inconsistent results of the paired *N. gonorrhoeae* cultures and urethral swabs from Northern Cape were observed.

4.5.3 Sequencing

All quinolone resistant isolates (QRNG) had identical mutations; for *gyrA*, Ser (TCC) to Phe (TTC) at amino acid (AA) position 91 and Asp (GAC) to Gly (GGC) at AA position 95, and for *parC* Asp (GAC) to Asn (AAC) at AA position 86. The two intermediate isolates had the same two *gyrA* mutations but no mutations in the *parC* gene. Eleven of twelve susceptible isolates had wild type *gyrA* and *parC* genes.

4.5.4 NG MAST

NG MAST has demonstrated heterogeneity among the strains in Johannesburg and Cape Town; in contrast, all eight QRNG isolates from Kimberley has the same sequence type (Table: 4.12). The prevalent STs were ST217 and ST533.

Table: 4.12: Summary of the results from three South African provinces

Category		Johannesburg, Gauteng (x 24)			Kimberley, Northern Cape (x 9 MIC data)		Cape Town, Western Cape (x16)	
		Urine	Swab	Culture	Swab	Culture	Swab	Culture
Susceptibility phenotype				16R, 2S,6S		8R, 1S		11R, 5S
Assay validation	*gyrA*	6xPos; 18xNeg.	6xPos; 18xNeg.	6xPos; 18xNeg.				
	parC	7xPos; 17xNeg.	7xPos; 17xNeg.	7xPos; 17xNeg.				
Testing of clinical specimens	*gyrA*	–	–	–	6xPos; 3xNeg.	1xPos; 8xNeg.	5xPos; 11xNeg.	5xPos; 11xNeg.
	parC	–	–	–	6xPos; 3xNeg.	1xPos; 8xNeg.	5xPos; 11xNeg.	5xPos; 11xNeg.
Sequencing	*gyrA*	–	–	18x (Ser91→Phe) & (Asp95→Gly)	3x (Ser91→Phe) & (Asp95→Gly)*	8x (Ser91→Phe) & (Asp95→Gly)		11x (Ser91→Phe) & (Asp95→Gly)
	parC	–	–	17x (Asp86→Asn)	3x (Asp86→Asn)*	8x (Asp86→Asn)		11x (Asp86→Asn)
NG MAST				4X known ST; 16x new ST		2x known ST		4x known ST; 6x new ST

S = Susceptible; I = Intermediate/Reduced Susceptibility; R = Resistant

CHAPTER 5: DISCUSSION

Gonococci are fastidious organisms and are difficult to culture successfully especially in resource poor settings. Advances in DNA amplification technology have resulted in improved performance of molecular detection and allow use of non-invasive samples, such as urine. Nucleic acid amplification tests (NAATs) also allow the testing of non-viable organisms, overcoming the transport difficulties encountered when trying to collect viable gonococcal isolates in countries like South Africa. Real-time PCR assays enable rapid results to be produced in the laboratory setting, enabling prompt clinical decisions to be made in the field. Real time PCR assays also lend themselves to approaches incorporating multiplexing technology, allowing simultaneous detection of more than one target.

In this study, the real-time fluorometric PCR amplified the *gyr*A and *par*C genes, the two regions in the *N. gonorrhoeae* genome associated with quinolone resistance. The primers used for the detection of QRDR successfully amplified these two genes in three different non gonococcal Neisseria species namely: *N. lactamica*, *N. meningitidis* and *N. mucosa* but when the probes were included, no amplification was observed. The *gyrA* primers were able to amplify urinary tract causing organism (which are *E. coli*, *K. pneumoniae*, *P.mirabilis*, *P. auruginosa*, *A. baumannii* and *K. oxytoca*) and also on NMS patient infected with *C. trachomatis*, *T. vaginalis* and/or *M. genitalium*, showing an amplicon size of 72bp. However with *parC* primers, the urinary tract causing organism had very faint bands for the 72bp amplicon

(but darker on other amplicons), including the CT positive specimen. When the probes were included, no amplification was observed with urinary tract causing organism, CT, TV, and MG positive specimens. Hence, the QRDR PCR run with probes was found to be more specific to *Neisseria gonorrhoeae*.

For all surveillance samples from patients with gonorrhoea recruited in Gauteng and the Western Cape, the mutation results inferred from the real-time fluorometric PCR were completely consistent with the results obtained from conventional sequencing analysis and that of the susceptibility testing. These results corresponding with the results of the conventional sequencing confirm the reliability of the real-time fluorometric PCR protocol for susceptibility analysis. The observed association between mutation in the *gyrA* gene and resistance to fluoroquinolones was consistent with that reported in previous studies (43; 109).

However, results of the real-time PCR analysis of DNA extracted from the remaining five of the nine paired *N. gonorrhoeae* cultures and urethral swabs from the Northern Cape were not in agreement. The QRDR of these fine culture-based DNA extracts were subsequently shown by DNA sequencing to possess the same *gyrA* and *parC* QRDR mutations as the rest of the ciprofloxacin resistant specimen. This may be due to technical issues such as mislabeling of swabs during sample collection or mislabeling of stock cultures during cultures storage. Attempts to resolve the issue by NG MAST failed due to insufficient DNA template availability. Fifteen isolates with unknown antimicrobial resistant profile from the Northern Cape had no detectable mutations in *gyrA* and *parC* and six isolates had mutation in *gyrA* and *parC*.

Based upon these results we were able to predict that 29% of *N.gonorrhoeae* isolates in the Northern Cape were resistant ciprofloxacin.

The real-time PCR assay originally developed for cultured *N. gonorrhoeae* isolates, by Giles *et al*. (63), with the inclusion of the internal control, might prove valuable in settings where rapid determination of quinolone resistance to *N. gonorrhoeae* is desired from the clinical specimen. It is a faster method than that of culture and traditional agar-dilution or disc susceptibility testing and does not require expensive sequencing equipment. Furthermore, in the age of nucleic acid amplification testing for gonococcal and chlamydial infection, using urine as a specimen for drug resistance genotyping is a valuable capability, precluding laborious culture and isolation methods and supplanting a requirement for urethral swab specimens. However, this current real-time PCR assay has some disadvantages. Gonococci could potentially harbour *gyrA* and *parC* mutations outside the probe binding regions or other mechanisms of resistance e.g. efflux. These genetic changes would not be detected by the real-time PCR assay.

Sequencing of QRDRs in *gyrA* and *parC* were chosen for this analysis, on the basis of the findings of previous studies, which indicate this is the sole mechanism of quinolone resistance in *N. gonorrhoeae* at the present time (226). Consistent QRDR mutation patterns associated with fluoroquinolone resistance and clinical treatment failure have been reported in multiple investigations (186, 196,197). Therefore, these QRDR mutation patterns are potential molecular markers that can be used as a tool for QRNG surveillance.

In the present study, the molecular characterization of QRDRs (for both Gauteng and Western Cape) was in complete agreement with susceptibility to ciprofloxacin, as previously determined for these strains. The QRDRs of our resistant isolates showed mutation patterns that were consistent with previously reported patterns (39,186,196,197). The literature suggests that CipR isolates had more than one mutations in the *gyrA* QRDR and one mutation in the *parC* QRDR. A combination of *N. gonorrhoeae* QRDR mutations at *gyrA* Ser91→Phe and Asp95→Gly, with a *parC* mutation at either Asp86→Gly or Ser87→Arg, has been associated with high levels of resistance (181). Trees *et al*. (194) identified double mutation patterns at *gyrA* 91/95, in combination with either *parC* 87/91 or *parC* 87/116, by use of restriction enzyme analysis of strains with MICs that ranged from 16 to 64µg/ml.

In the present study, the two isolates that had double mutations at codon 91 and 95 of *gyrA* alone had intermediate resistance, while the isolates that had mutations at codons 91 and 95 of *gyrA*, with an additional mutation at codon 86 of *parC,* were resistant to ciprofloxacin. These results are consistent with the hypothesis proposed by Trees *et al.* (194), which indicated that, although mutations in the *gyrA* QRDR can afford low level resistance, additional mutations within the *parC* QRDR facilitate high level resistance to fluoroquinolones (194).

This study also supports previous results (109) indicating that strains containing identical *gyrA*/*parC* QRDR alteration patterns frequently exhibit different MICs to ciprofloxacin. Examples in the current study are the 35

isolates that exhibited *gyrA* point mutations in the codon 91 and 95 and *parC* point mutations in the codon 86. These isolates had a ciprofloxacin MIC range of 1.0 to 8.0 μg/ml and the two isolates with only *gyrA* point mutations in the codon 91 and 95 had a ciprofloxacin MIC range of 0.064 to 0.125 μg/ml. It appears that the presence of *parC* QRDR alterations facilitates high ciprofloxacin MICs, but the actual MIC may be influenced by other characteristics of the strain, such as reduced intracellular drug accumulation, as described by Tanaka *et al.* (185).

All except one gonococcal isolates susceptible to ciprofloxacin had no mutations in *gyrA* and *parC*. The one strain (GP7 MUS 159) with mutation at *parC* gene only, had a ciprofloxacin MIC of 0.006 μg/mL. This then suggest that the presence of mutation in *parC* only, does not lead to decreased susceptibility of gonococci to ciprofloxacin.

The association between ciprofloxacin MICs and QRDR alterations, reflecting clinical resistance, was generally well accepted by other investigators namely Giles, et al, Ison, et al, Shigemura, et al and Trees, *et al.* (63,83,169,195). There were however, three studies that were unable to discern such a correlation namely Lindback, *et al.*, Vereshchagin, *et al*. and Yang, *et al*. (109,206, 226). Some specific examples from the Russian study include high level ciprofloxacin resistance in the absence of *gyrA* and *parC* changes expressed in four strains and little distinction in terms of ciprofloxacin MICs was observed for QRNGs with double *gyrA* changes only and those with an additional *parC* change (MIC range 4 to ≥ 32g/mL for both) (206).

Furthermore, the amino acid substitution Ser-87→Arg in *parC*, normally associated with high levels of ciprofloxacin resistance was not a characteristic of these QRNGs (206). On closer examination it was apparent that there were problems inherent in the experimental design of these studies, such as, QRNGs in the advanced stages of fluoroquinolone resistance (ciprofloxacin MICs ≥ 4g/mL) were examined only, so that an evolving resistance corresponding to QRDR change would not have been apparent (109,206, 226). A set of control strains representing each different level of resistance was also omitted, casting some doubt on the validity of the results. Nonetheless, the conclusions of Yang *et al*. (226) agreed with the general principles presented in our sequencing results for molecular changes relating to quinolone resistance in gonococci, namely that:- *parC* changes are associated with higher ciprofloxacin MICs; *parC* changes are not the primary determinant specifying resistance to ciprofloxacin; and *gyrA* mutations are necessary for the quinolone resistance phenotype.

The present study also has important implications for public health. The sequence data presented in this study indicate the existence of identical *gyrA* and *parC* mutations among gonococcal strains expressing ciprofloxacin resistance, which result in amino acid changes in *gyrA* (Phe-91 and Gly-95) and *parC* (Asn-86). These data also raised the possibility that the observed increase in the relative prevalence of CipR strains may have been due to the appearance of a single-strain outbreak. In order to further address this question, highly discriminatory genotyping by NG-MAST was performed. When these isolates were further genotyped by NG-MAST, several different

sequence types were observed. The NG-MAST genotyping data do not support the hypothesis of a single strain outbreak at the time of the collection of the specimens. However, given the possibility of the development of further mutations in the *por* and *tbpB* genes overtime the typing results cannot be used to comment upon whether the first ciprofloxacin resistant isolates in South Africa were clonal or not. In addition, the initial recognition of ciprofloxacin resistance in South Africa appeared in Durban, a city in Kwa-Zulu Natal Province that is geographically distant to Johannesburg, Cape Town and Kimberly (126,127).

Recognition of endemic QRNG as a partially clonal event suggests that control efforts similar to those used in other outbreaks of infectious disease could be considered as a public health strategy (92,198). Rapid identification of endemic transmission of QRNG within a community, combined with tools that identify strain and resistance markers, could allow a redirection of resources to identify and treat contacts and extended sexual networks. However, such an approach is very costly and would not be affordable in the South African context.

A gene sequence-based typing method, NG-MAST, with high discriminatory power was applied in this study, to all 49 *N. gonorrhoeae* isolates recovered from patients in Gauteng, Western Cape and Northern Cape Provinces. This typing method was also used to distinguish between the QRNG from the three South African provinces. The NG-MAST web site (http://www.ng-mast.net), which is currently hosted by Imperial College London, maintains the central database that includes the sequences of all known alleles at each locus and

details of the known STs. Investigators can assign their alleles and STs by interrogating the web site and can obtain allele numbers and ST numbers for new alleles or strains, which then become available to others via the public database. In this way, isolates recovered in one city can be compared with those from other cities or other countries. The ability to compare gonococcal isolates from different cities or countries may be less important for gonorrhea than for many other diseases, but is likely to be useful in the case of antibiotic resistant strains that may be imported into several countries from a common source.

In this study, there was more clustering among the ciprofloxacin-resistant isolates than among the susceptible and intermediate resistance isolates. The large ST clusters (ST217 and ST533) suggested that multiple clonal transmissions existed in Gauteng, Western Cape and Northern Cape Provinces. The remaining twenty three STs had only one or two isolate each, which may be due to the limited strain collection, or the local emergence of new STs, or the recent introduction of foreign STs.

The presence of QRNG in the Northern Cape was significantly associated with ST533 ($p<0.0001$), however concrete epidemiological conclusions were limited because genotyping was performed on a limited number of isolates. ST217 constitutes the largest cluster of QRNG isolates from Gauteng and Western Cape, however the presence of QRNG in the Western Cape was significantly associated with ST217 ($p=0.0397$), but not in Gauteng ($p=0.7416$).The ease of travel around the world allows for the introduction of foreign strains into a community. Cape Town, which is in the Western Cape

Province, is Africa's most popular destination for tourism. The Gauteng goldmines and its economic centre in Johannesburg also draw its workforce in the form of migrant workers from the different cities in South Africa and facilitate bidirectional spread of isolates.

QRNG isolates with ST 217 have previously been reported from Europe as described on the database (133), Durban (125) and Pretoria (46). De Jongh et al (46) has demonstrated that typing of 10 QRNG isolates from Pretoria can be resolved into five new STs and a cluster of four isolates of ST217. Whereas, in Durban, typing of 35 QRNG isolates by Moodley et al (125) has revealed two clusters: ST217 (20 isolates), and ST524 (4 isolates). The remaining eleven STs comprised of single isolates. Although isolates with ST 217 have previously been seen in Europe, it cannot be determined whether the resistance seen in South Africa is the result of the introduction of already resistant strains into the area with subsequent spread, independent resistance development in the area, or both.

In Scotland, genotyping of the 2002 isolates by NG MAST showed that ST147 was one of the main sequence types associated with ciprofloxacin resistance (147). However, a high number of QRNG strains belonging to ST225 were identified in Sweden during 2005 (140), England from 2003-2004 (117), Scotland from 2004-2006 (143), in Australia in 2005 (212) and in Denmark from 2005-2006 (74).

The NG MAST data presented in this study was cross sectional and the longitudinal data is required to address the issue of spread or clonal selection

of ciprofloxacin resistant genotypes within any population. One of the longitudinal data presented by Martin et al (117) has demonstrated that typing isolates by NG-MAST, in conjunction with the demographic and behavioral data, has shown that the transmission of QRNG in London appears to have changed over the course of 4 years. In 2000, QRNG was predominantly isolated from heterosexuals in London, had unique sequence types (STs) and were associated with foreign travel. From 2001 onward, QRNG was more frequently isolated from MSM and from 2003 onward, QRNG was no longer significantly associated with heterosexuals. In 2002 and 2003, 3 of the 4 largest ST clusters were associated with MSM. Another study by Palmer et al (116) has demonstrated that typing isolates by NG-MAST, in combination with the epidemiological data, has revealed sustained transmission of several gonococcal strains predominately within the largest metropolitan area of Scotland. Clusters of isolates were associated with transmission within the United Kingdom, whereas isolates with unique STs were associated with foreign travel. Hence more extensive typing of national and international *N gonorrhoeae* isolates needs to be performed to inform on the transmission patterns of resistant organisms in South Africa.

In conclusion, our study has shown that the real-time PCR assay may assist in detection of presumptive QRNG isolates by showing failure to detect susceptibility to ciprofloxacin. This approach has the potential to enhance public health-based surveillance of antimicrobial resistance in gonococci. Further, this molecular tool provides a convenient method for detecting fluoroquinolone resistance in non-invasive samples, which is of advantage in

resource-poor and rural settings. This study has also confirmed that the NG-MAST analysis has a higher discrimination power than *gyrA* and *parC* DNA sequence analysis, and that this typing technique is able to distinguish *N. gonorrhoeae* isolates and to identify circulating clusters of strains.

CHAPTER 6: REFERENCE

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