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MECHANISMS OF RESISTANCE TO CIPROFLOXACIN IN *NEISSERIA GONORRHOEAE*

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ABSTRACT

A few years ago, most strains of *Neisseria gonorrhoeae* were susceptible to ciprofloxacin, but after introduction as a first line therapy, resistant strains emerged. Ciprofloxacin inhibit two enzymes necessary for DNA replication. Mutations in the quinolone resistance determining regions (QRDR) of genes encoding the subunit GyrA of the target enzyme DNA gyrase and ParC of topoisomerase IV are mechanisms of resistance in *N. gonorrhoeae*. These alterations do not explain why the minimum inhibitory concentrations (MICs) of ciprofloxacin in resistant strains vary so widely. The target enzymes also contain the subunits GyrB and ParE. Their role and other mechanisms of resistance in *N. gonorrhoeae*, such as increased efflux out of the cell, decreased uptake or competition of quinolone binding sites by protection, have not been fully investigated in *N. gonorrhoeae*. Several commercial kits for molecular diagnosis of *N. gonorrhoeae* are available and can be analyzed in a duplex PCR with *Chlamydia trachomatis*, but these methods are known to produce false positive results.

Results obtained with AMPLICOR *N. gonorrhoeae* polymerase chain reaction (PCR) (Roche Diagnostics) were compared to cultivation results in 956 samples. In positive samples species verification of the 16S rRNA gene was compared to pyrosequencing of QRDR the *gyrA* gene, which was also evaluated as an indicator of ciprofloxacin susceptibility. Culture and the molecular method verified in *gyrA* produced two and one false negative result respectively and the molecular method verified in 16S rRNA produced four false positive results. QRDR of all eleven urine samples positive in AMPLICOR *N. gonorrhoeae* PCR, with corresponding isolates as well as 46 *N. gonorrhoeae* strains, were correctly diagnosed according to susceptibility to ciprofloxacin compared to MICs. Pyrosequencing of QRDR of *gyrA* of 40 isolates of nine other *Neisseria* spp. showed that QRDR in *gyrA* is not unique for *N. gonorrhoeae*.

Sequencing of QRDR of *gyrA, gyrB, parC,* and *parE* in 25 highly ciprofloxacin resistant and five susceptible strains of *N. gonorrhoeae*, showed that all the resistant strains had two mutations in *gyrA.* Fourteen strains also had an additional mutation in *parC*, and 17 strains had an additional mutation in *parE*. No alterations were found in *gyrB* in any strain. In transformation experiments an alteration in GyrA was introduced in a ciprofloxacin susceptible *N. gonorrhoeae* strain (MIC 0.008 mg/L) and MIC increased to 0.064 mg/L. Two alterations, together, increased MIC to 0.125 - 0.25 mg/L. Introduction of alterations in major outer membrane porin, PorB1b, and probably other alterations, in a moderately ciprofloxacin resistant strain (MIC 0.25 mg/L) gave transformants with MICs of ciprofloxacin 0.5-16 mg/L. In one transformant an alteration in ParE was also introduced.

We conclude that verification of a molecular method by pyrosequencing in *gyrA* gene is superior to verification by PCR in 16S rRNA. The gene *gyrA* is not unique for *N. gonorrhoeae*. However, whether this region is possible to use also for verification also depends on the specificity of the primary method. QRDR of *gyrA* is a strong indicator of ciprofloxacin resistance in *N. gonorrhoeae*. Two alterations in gyrA only increases MIC of ciprofloxacin to 0.125 – 0.25 mg/L. Additional alterations in QRDR of *parC* and *parE* as well as alterations in *porB1b* also contribute to ciprofloxacin resistance.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Lindbäck E**, Rahman M, Jalal S, Wretlind B. 2002. Mutations in *gyrA*, *gyrB*, *parC* and *parE* in quinolone-resistant strains of *Neisseria gonorrhoeae*. APMIS; 110:651-7

II. **Lindbäck E**, Gharizadeh B, Ataker F, Airell Å, Jalal S, Nyrén P, Wretlind B. 2005. DNA Gyrase gene in *Neisseria gonorrhoeae* as indicator for resistance to ciprofloxacin and species verification. Int J STD AIDS; 16:142-7

III. Airell Å, **Lindbäck E**, Ataker F, Jalakas-Pörnull K, Wretlind B. 2005. Verification of clinical samples positive in AMPLICOR. *Neisseria gonorrhoeae* PCR by 16S rRNA and *gyrA* compared to culture. Int J STD AIDS; 16:415-9

IV. **Lindbäck E**, Unemo M, Akhras M, Gharizadeh B, Fredlund H, Pourmand N, Wretlind B. 2006. Sequence of the quinolone determining region of the DNA gyrase gene in *Neisseria*. Submitted manuscript

V. **Lindbäck E**, Islam S, Unemo M, Lang C, Wretlind B. 2006. Transformation of ciprofloxacin resistant *Neisseria gonorrhoeae gyrA*, *parE* and *porB1b* genes. Submitted manuscript

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ABBREVIATIONS

ATCC	American Type Culture Collection
bp	Base pairs
CCD	Charge-coupled device
CCUG	Culture Collection University of Göteborg
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
dcw	Division cell wall
DHPLC	Denaturing high-performance liquid chromatography
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
HIV	Human immunodeficiency virus
I	Intermediate
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
LOS	Lipooligosaccharides
LPS	Lipopolysaccharides
MATE	Multidrug and toxic compound extrusion
MF	Major facilitator
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
Mtr	Multiple transferable resistance
NAAT	Nucleic acid amplification test
NCCLS	The National Committee on Clinical Laboratory Standards
NG-MAST	*N. gonorrhoeae* multiantigen sequence typing
OD	Optical density
Opa	Opacity
PBP	Penicillin binding protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
PID	Pelvic inflammatory disease
PPNG	Penicillinase producing *Neisseria gonorrhoeae*
QRDR	Quinolone resistance determining region
QRNG	Quinolone resistant *Neisseria gonorrhoeae*
R	Resistant
RFLP	Restriction fragment length polymorphism
RND	Resistance-nodulation-division (RND)
S	Susceptible
SMI	Swedish Institute of Infectious Disease Control
spp.	Species
SRGA	The Swedish Reference Group for Antibiotics
ST	Sequence type
STI	Sexually transmitted infection
wt	Wild type

1 INTRODUCTION

Gonorrhea is a major sexually transmitted infection (STI), and ciprofloxacin is an oral drug that can be used to treat it. Soon after the drug was introduced in the 1980s it became a first line therapy, and since the mid 1990s ciprofloxacin resistant strains have emerged worldwide [1-3].

1.1 THE GENUS *NEISSERIA*

Neisseria is named after Albert Neisser, who in 1879 published "Ueber eine der Gonorrhoe eigentümliche Microccusform", where he established the bacterial etiology of gonorrhea [4].

The genus *Neisseria* belongs to the family *Neisseriaceae*. *Neisseriaceae* consists of the "true neisseriae" and three species (spp.) only found in animals (*Neisseria ovis*, *N. cavie* and *N. cuniculi*). The "true neisseriae" colonizing humans are *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. cinerea*, *N. polysaccharea*, *N. subflava*, *N. sicca*, *N. mucosa*, *N. flavescens* and *N. elongata*. *Neisseriaceae* is classified in the ß-subgroup of *Proteobacteria* which also includes *Kingella*, *Eikenella*, *Simmonsiella*, *Alysiella*, *N. weaveri*, *N. elongata* subsp. *nitroreducens* [5].

N. gonorrhoeae, which causes gonorrhea, and *N. meningitidis*, which causes meningitis or septicemia, are the main human pathogens among the *Neisseriaceae*. *N. gonorrhoeae* subsp. *kochii* was reported in 1986 as a new subspecies found in conjunctival cultures from children in Egypt [5, 6]. Also *N. cinerea* is known to cause infections of the eye [5]. The other *Neisseria* spp. are often part of the normal flora in humans, but can cause opportunistic infections. *N. meningitidis* frequently colonizes the human mucosa without any symptoms or signs of disease. *N. gonorrhoeae*, however, although it might cause no symptoms, is still considered a pathogen, and if it is found, this should always lead to antibiotic treatment.

1.2 BIOLOGY OF N. GONORRHOEAE

N. gonorrhoeae is a Gram-negative coccus, often seen in the microscope as diplococci shaped like a coffee bean. *N. gonorrhoeae* is aerobic, requires CO_2 and shows optimal growth at 35-37ºC in a humid atmosphere. *N. gonorrhoeae* produces acids from carbohydrates by oxidation, and does not grow without glucose, pyruvate or lactate as energy source. The amino acid cysteine is essential, and some strains also require other amino acids, pyrimidines or purines [5, 7].

Comared to other bacterial species, *N. gonorrhoeae* has a small to medium sized genome of approximately 2.15 million base pairs (bp). Plasmid content varies from strain to strain, and plasmids may also be absent. A cryptic plasmid is present in a majority of strains, but the plasmids can also be partly or fully integrated in the chromosome. Plasmids are known to carry antibiotic resistance genes [8, 9].

1.3 VIRULENCE AND COMPETENCE

N. gonorrhoeae adhere to and invade human mucosal epithelia. To escape the human immune system *N. gonorrhoeae* utilizes phase variation and antigenic variation. In phase variation the control of expression is "on" or "off" and in antigenic variation mutations lead to protein changes in the cell wall. The cell wall of *N. gonorrhoeae* is highly homologous to that of other Gram-negative bacteria, with a bilayered outer membrane, a cytoplasmatic bilayered phospholipid membrane, and a periplasmatic space between the outer and cytoplasmatic membranes with a thin layer of peptidoglycan. The outer membrane contains pili, opacity (opa) proteins, porins and lipooligosaccharides (LOS), all of which are involved in virulence [10].

In the 1960s the correlations between virulence and clinical strains, as well as loss of virulence due to in vitro passages, were established [11-13], as was a correlation between colony morphology and virulence [11]. Piliated *N. gonorrhoeae* are competent and naturally transformable [10, 14], which means the bacterium is able to take up external genetic components. Sparling found that naturally occurring, virulent clones of *N. gonorrhoeae* had greater competence than laboratory strains, and gave transformation rates of up to 1% [13].

Horizontal genetic transfer is an important strategy for the adaptation and evolution of *Neisseria* spp. [15-17]. Natural transformation occurs within the species due to the frequency of the 10 bp species-specific signal sequence, which, for example appear in 1965 times in the genome of *N. gonorrhoeae* strain FA1090 [18]. The signal sequence appears in other *Neisseria* spp. and there are some copies in *Haemophilus influenzae* [16]. The sequenced genome of *N. gonorrhoeae* strain FA1090 has at least six genetic islands presumably acquired via transformation from various bacterial species [16].

A majority of *N. gonorrhoeae* carry a gene encoding a type IV secretion system. This system is involved in providing DNA for genetic transfer without cell lysis [19].

The division cell wall (dcw) cluster is a group of genes found in several bacteria, encoding proteins involved in peptidoglycan synthesis and cellular division. Snyder et al. found that the *dcw* cluster of *N. gonorrhoeae* and *N. meningitidis* includes three extra genes within the *dcw* cluster. One of these genes, *dca*, was found to be associated to competence in gonococcal but not in meningococcal strains [15].

1.3.1 Type IV pili

Type IV pili are long hair-like filaments on the surface of *N. gonorrhoeae*. They are involved in adherence, motility and host cell responses. Pili undergo phase variation may be expressed or not. Type IV pili are composed of several components; the outer layer of the pili is hypervariable and the core is highly conserved [10]. Expression of pili is highly related to degree of competence.

1.3.2 Opa proteins

The Opa proteins are expressed in about eleven *opa* loci. The name "opa" originates from the former name opacity proteins. Phase and antigenic variation in the *opa* genes

changes the color and opacity of the gonococcal colony. Opa proteins are involved in attachment to and uptake into host cells [10].

1.3.3 Porins

The major outer membrane protein in *N. gonorrhoeae*, PorB1, is important in pathogenesis and is involved in uptake of gonococci in epithelial cells [20]. Its function is mainly to transport ions and small nutrients between the bacteria and the environment [21].

PorB1 consists of eight loops and all *N. gonorrhoeae* strains express either PorB1a or PorB1b, encoded by *porB1a* and *porB1b* respectively [10, 21-23].The gene encoding PorB is a single copy gene [24]. The loops of PorB exposed on the surface of the outer cell membrane are hypervariable [22, 24-26]. Although the membrane-bound parts of the protein are conserved, PorB1b is less well conserved than PorB1a [22]. Both the Pharmacia [27] and Genetic Systems [28] panels of monoclonal antibodies target PorB and several epidemiological studies have been carried out based on sequencing of *porB* [26, 29-31].

Alterations in PorB are also associated with antibiotic resistance. Low-level resistance to penicillins and tetracycline have been associated with the genetic locus named *penB* [32, 33], later found to be located in loop 3 of PorB [34, 35].

Unlike Opa proteins and type IV pili, PorB does not undergo phase variation, which means that PorB is always expressed during infection and under laboratory conditions [25, 33].

Fudyk et al. found that PorB from strains isolated from gonorrhea high-frequency transmitter core groups had more mosaicism and sequence polymorphism than other strains, implying that recombination probably occurs, especially in core groups who are more prone to have a double infection [24].

1.3.4 Lipooligosaccharides (LOS)

Neisseria produce LOS, potent endotoxins that activate the immune response and cause tissue damage. LOS in *Neisseria* equals lipopolysaccharides (LPS) in other Gram-negative bacteria. Both LOS and LPS consist of lipid A and a core oligosaccharide, but the neisserial LOS has a distal polysaccharide, often called the O-antigen, that is not repeated as in most other Gram-negative bacteria [36].

1.4 GONORRHEA

Gonorrhea is an STI. In males the classical symptoms of gonorrhea are acute urethritis with dysuria and purulent discharge. The incubation period averages from 2 to 7 days. About 95% of males with urethritis due to gonococcal infection have symptoms. Untreated gonorrhea may lead to an ascending infection such as epididymitis, epididymo-orchitis, prostatitis, periurethral abscess, or urethral stricture and may lead to infertility [5].

In females a gonococcal infection presents as cervicovaginal discharge, bleeding, abdominal or pelvic pain. About 70-90% of infected women have a concomitant urethral infection [5, 37].The incubation period averages from 8 to 10 days. Ascending gonococcal infection may result in acute pelvic inflammatory disease (PID) that can manifest as salpingitis, endometritis, or tubo-ovarian abscess. It may also result in scarring, ectopic pregnancies, infertility and chronic pain [5]. In pregnant women gonorrhea increases the risk of complications. The symptoms in women are often milder than in men, or even non-existent. Women without symptoms are an important reservoir for *N. gonorrhoeae* [5].

Via contamination from genital secretions, *N. gonorrhoeae* may cause eye infections, presenting as a massive purulent discharge. If not treated adequately these infections have a high risk of keratitis and blindness. If the mother has gonorrhea during vaginal delivery, the newborn is at high risk of getting ophthalmia neonatorum, a gonococcal eye infection, or an infection in the pharynx. Other *Neisseria* spp. as *N. cinerea* and *N. gonorrhoeae* subsp. *kochii* are also known to cause purulent eye infections, also in children [5, 38].

N. gonorrhoeae also causes infections in pharynx and rectum. Infections at these sites are often asymptomatic, often concomitant with urethral or endocervical infections, and may serve as a reservoir for *N. gonorrhoeae* [5].

Gonococcal infection may also cause septicemia, developing from infection at any site. Gonococcal septicemia often presents as low-grade fever, painful hemorrhagic skin lesions developing into pustules, tenosynovitis, migratory polyarthralgias and arthritis [5].

1.5 EPIDEMIOLOGY

1.5.1 Sweden

The Swedish Communicable Diseases Act states that each case of gonorrhea has to be reported, by both the clinician and the laboratory, to the Swedish Institute of Infectious Disease Control (SMI). The patients are by the same Act obliged to undertake treatment. Gonorrhea has been carefully monitored in Sweden since 1912. The incidence peaked in 1970 with 487/100 000 inhabitants [39, 40]. The years after that saw an almost unbroken decline until 1996, when the incidence was 2.4/100 000. Since 1997 the incidence has again increased, rising in 2003 to 6.7/100 000 inhabitants, which was 596 cases [41]. In 2004 there were 569 cases [42]. The reemergence of gonorrhea in Sweden is due to an increase of domestic cases, and heterosexual teenagers as well as homosexual men were identified as core groups [39].

As a gender aspect, in Sweden in 2004 14% of reported cases were women, 40% were heterosexual men and 44% were homosexual men. However, among those who were tested for gonorrhea, women represented 62%, and 0.3% of the women tested were diagnosed positive compared to 1.2% of the men, in a few cases, the person's sex was not reported [42].

1.5.2 Global epidemiology

Gonorrhea is worldwide one of the most frequent bacterial STI second only to *Chlamydia trachomatis* infections. The risk factors for transmission of human immunodeficiency virus (HIV), as an STI, are the same as for gonorrhea. It is a major concern that a concomitant gonococcal infection increases the risk of HIV transmission [43-46]. In this perspective, gonorrhea is considered an important problem in developing countries, especially in countries with a high prevalence of HIV infections.

The prevalence of gonorrhea in Western Europe is similar to the Swedish numbers [42]. In the United States the prevalence of gonorrhea was 116/100 000 inhabitants in 2003 [47], but the prevalence varies a lot in different parts of the country and in different ethnic groups [48]. That the prevalence of the disease varies widely within and between countries reflects differences in social, economic and cultural factors, and in the age structure of the population, as well as access to diagnostics and adequate treatment.

As much as gonorrhea can be considered to be a local problem in subpopulations, international sexual contacts highlight the global aspect of the same problem. In Sweden, in 2004, 36% of patients with gonococcal infections had been infected abroad [42]. The vast majority were heterosexual men, infected in South East Asia, mainly in Thailand, but gonococcal infections acquired in 40 different countries were reported in 2004 [42].

High prevalence in developing countries in Africa and Asia reflects many of the above mentioned aspects. In addition, the numbers are frequently underestimated due to limited resources for surveillance as well as laboratory diagnostics. In 1998, 35.5% of 226 female sex workers attending a clinic in Dhaka, Bangladesh, were culture positive for *N. gonorrhoeae* [49]. In Kupang, Indonesia in 1999, prevalence of gonorrhea among female sex workers was 31% [50]. In a township in Carletonville, South Africa in 1998, the prevalence of gonorrhea among 834 women was 9.8% [45]. In Mbeya region, Tanzania, in the year 2000, the prevalence was 22% among 600 female bar workers [51].

1.6 EPIDEMIOLOGICAL TYPING

Epidemiological typing of strains supports clinical epidemiological data and is an important tool in characterizing core groups and outbreaks. All Swedish isolates are now typed by one of two serological methods. Both methods are easy to perform, and discriminative enough for clinical epidemiological purposes. However, it has become a problem to find monoclonal antibodies of high quality, and the number of strains that cannot be typed in these systems is increasing, due to genetic drift in the *porB1* gene [29, 52].

Sequencing methods are objective, highly reproducible and the results from different laboratories can easily be compared. They are more discriminative than the serological methods in use, but also more labor intensive [25].

Many methods have been developed for epidemiological purposes. Auxotyping, which identifies strains based on nutritional requirements, used to be an important method [7], but it is time consuming and new molecular methods are more discriminative and less laborious.

1.6.1 Serological typing

Serological typing is a phenotypic characterization. The methods are based on the two serogroups WI and WII/III and IA and IB, both corresponding to PorB1a and PorB1b membrane proteins respectively, combined with determining serovar by a coagglutination test using a panel of monoclonal antibodies to PorB1. Originally two different systems with different nomenclature were developed. The system developed by Pharmacia Diagnostics [28] is now called Phadebact Monoclonal GC Test (Boule Diagnostics AB, Huddinge, Sweden). The other system developed by Genetic Systems [27], is now called GonoGen I (New Horizons Diagnostics, Columbia, MD, USA). The two systems have been compared and serovar WI and WII/WII corresponds to serovar IA and IB respectively, but serotypes are not translatable into the other system [53].

1.6.2 Pulsed-field gel electrophoresis

The pulsed-field gel electrophoresis (PFGE) method allows restriction enzymes to digest the genome, and presents the resulting fragments as a pattern of bands, differing from strain to strain. This method is highly discriminative but laborious, and therefore expensive, and there are also difficulties in comparing results from different laboratories. The method is not suitable for clinical use on all strains, but can be highly useful in short-term epidemiological research, for example to study an individual outbreak, and will probably remain a reference method [54, 55].

1.6.3 Multilocus sequence typing

Multilocus sequence typing (MLST) is a widespread typing method used on numerous bacterial species, among them *Neisseria.* It involves sequencing of seven house-keeping genes and alignment with the relevant species database at the web site http://pubmlst.org/. Any change in sequence in any of the sequenced genes defines a new sequence type (ST) and a clonal complex is defined as a group of STs where every ST shares at least five of seven identical alleles with at least one other ST in the complex. The *Neisseria* MLST data-base is somewhat unique in that it contains sequences of several species of the genus *Neisseria.* It can be accessed at the web site http://pubmlst.org/neisseria/. MLST is useful in long-term epidemiological studies. This technique has revealed that in a highly genetically variable genus such as *Neisseria,* there is no clear-cut difference between species, especially between *N. meningitidis* and *N. lactamica* which both colonize the human pharynx [56].

1.6.4 *N. gonorrhoeae* multiantigen sequence typing

N. gonorrhoeae multiantigen sequence typing (NG-MAST) is based on the highly variable internal segment of the two genes *porB1* and *tbpB*, which are analyzed in the NG-MAST protocol [30], and then compared at the web site http://test3.mlst.net/. The NG-MAST method is interpreted in the same way as the MLST. Differences in sequence define different STs, but instead of seven house-keeping genes two highly

variable genes are analyzed. This makes the NG-MAST useful for short-term epidemiology.

1.6.5 Sequencing of the porB1 gene

Sequencing of all or part of the hyper-variable gene *porB1,* is a highly discriminative method [29, 31], giving results comparable to those obtained with NG-MAST, but the NG-MAST method has the advantage of relying on two different genes.

1.6.6 Opa-typing

Opa-typing is based on PCR of the eleven copy *opa* gene with a single pair of primers. The amplified fragments are digested with restriction enzymes, and the fragments are then fractioned on polyacrylamide gel. Different opa-types become visible through fixation and exposure to X-ray. Again, this yields a banding pattern that is not very suitable for comparing results from different laboratories, and the method is considered dated since it uses both polyacrylamide and X-ray and is less discriminative than strict molecular methods [57].

1.7 SUSCEPTIBILITY

A bacterial strain is defined as susceptible (S) if it has a minimum inhibitory concentration (MIC) that is represented within the wild type (wt) population and the wt can successfully be treated with the antibiotic in question. Resistant (R) strains have higher MIC than the susceptible wt population and can also, especially if resistance represents one major mechanism of resistance, form a population. Between the S and R populations there is often a zone called intermediate (I). From a clinical perspective, it is important that the isolates defined as I are as few as possible (http://www.srga.org/Eucastwt/eucastdefinitions.htm, access date 060315).

1.8 CIPROFLOXACIN

Ciprofloxacin is a synthetically derived antibacterial agent in the family of quinolones. Nalidixic acid was first discovered in the 1960s as antibiotic agent against urinary infections. Later, different side chains were added to the original two-ring structure [58]. These quinolones were named fluoroquinolones, as they each contained a fluorine atom attached to the nucleus at position 6 [59]. Ciprofloxacin was introduced in the 1980s; it is a fluoroquinolone and the clinically most used quinolone.

All quinolones are well absorbed from the gastrointestinal tract, except norfloxacin. Co-administration of multivitamins containing iron or zinc or antacids containing aluminum, magnesium or calcium reduces the absorption. Quinolones penetrate to lungs, kidney, bone and intestinal wall. Only some quinolones penetrate to CNS, and ciprofloxacin is not one of them. Thus ciprofloxacin should only be used as post exposure prophylaxis, and not for treatment of meningococcal meningitis. Quinolones penetrate well into cells and have excellent activity against intracellular bacteria and *N. gonorrhoeae* [59].

There is some confusion in the terms of generations of quinolones, basically founded in which core structure the drug was developed from. Nalidixic acid has a naphthridone structure and is often considered a first generation quinolone, active only against Gram-

negative bacteria. It is no longer in clinical use, but is used in some laboratories to screen strains for resistance. Second generation quinolones are active against both Gram-negative and Gram-positive bacteria. Ciprofloxacin is one of them, and has the fluoroquinolone structure. Later generations of quinolones have increased activity against Gram-positive and anaerobic bacteria, and are often named third or fourth generation quinolones. Moxifloxacin and sparfloxacin have the fluoroquinolone structure, whereas gemifloxacin, for example shares the naphthridone structure with nalidixic acid [58, 59].

1.9 MECHANISMS OF ACTION

Quinolones have a bactericidal effect when they bind their target enzymes, DNA gyrase and topoisomerase IV, both essential for DNA replication. Defect enzymes lead to strand cuts, which will kill the bacteria [59, 60].

DNA-gyrase has four main functions, the first one being that it introduces negative supercoils into DNA. The enzyme binds and unwinds DNA into a positive supercoil, one region of DNA is cut and passes through another, and then the DNA is rejoined. The negative supercoil activates the chromosome for all processes involving strand separation. The second function is to facilitate transcription by inserting negative supercoils ahead of transcription. The third function is to remove knots from DNA, and the fourth function is to bend and fold DNA [59, 60].

Topoisomerase IV works in a similar way as DNA-gyrase. Topoisomerase IV, however, does not wrap DNA, but recognizes DNA crossovers, which facilitates intermolecular crossovers rather than intramolecular crossovers, which are the result of wrapping [59, 60]. DNA-gyrase consists of two GyrA and two GyrB subunits, encoded by the genes *gyrA* and *gyrB*. Topoisomerase IV, which is highly homologous to gyrase, consists of two ParC and two ParE subunits, encoded by the genes *parC* and *parE* [60].

1.10 MECHANISMS OF RESISTANCE

In analogy to other Gram-negative species, such as *Pseudomonas aeruginosa* and *Enterobacteriaceae* [61, 62], as well as Gram-positive species, such as *Streptococcus pneumoniae* [63, 64] mutations in the quinolone determining region (QRDR) of the subunits are major mechanisms of resistance to ciprofloxacin also in *N. gonorrhoeae* [65-69]. QRDR of *gyrA* was defined by Yoshida et al. in 1990 in *Escherichia coli*, ranging from amino acids 67 to 106 [70]. Belland et al. states that QRDR of GyrA and ParC in *N. gonorrhoeae* correspond to amino acids 55-110 and 66-119 respectively [71]. The QRDRs of the target enzymes show a high degree of homology in Gram-negatives [61, 71-74]. Several reports support that mutations in the subunits of DNA-gyrase are more important in Gram-negatives [61, 62, 66, 74], and that on the other hand mutations in topoisomerase IV play a more important role in Gram-positives [63, 64, 74]. However, mutations in *gyrA* and *parC* do not alone explain the wide range of MIC in quinolone resistant strains [65, 66].

The alterations conferring resistance to quinolones in *E. coli* are at the dimer interfaces, indicating that these sites are the quinolone binding sites [75]. Hypothetically the

altered targets block quinolones from binding, leading to unaffected enzymatic action and bacterial replication.

In other species also increased efflux out of the cell [76], decreased uptake [77] and protection by competitive binding to target enzymes [78, 79], have caused ciprofloxacin resistance.

1.10.1 Alterations in GyrA

In *N. gonorrhoeae* FA1090 GyrA is a 916 amino acid long protein, beginning at the genomic position 618439 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome&cmd=Retrieve&dopt=Protein+Table&list_uids=635, access date 060315). As early as 1995, Deguchi et al. described the alterations in position Ser91 and Asp95 in *N. gonorrhoeae* corresponding to Ser83 and Asp87 in *E. coli* [72] (Table 1).

Table 1. References showing different GyrA alterations in quinolone resistant strains of *N. gonorrhoeae* MIC of quinolones (norfloxacin, ofloxacin or ciprofloxacin) ≥ 1 mg/L

Position	Substitution	References
Ser91	Phe	[65-68, 71, 72, 80-94]
	Tyr	[66-68, 80, 82, 87, 90-92, 95]
Asp95	Gly	[65-68, 80, 81, 83, 85-92, 94]
	Asn	[65-68, 71, 72, 80-95]
	Ala	[81, 85, 87, 88, 92]
	Tyr	[82, 87, 91]
	His	[94]

Although these mutations are most common, alterations in other positions have been reported. Three different single mutations in GyrA were reported at Ala67 to Ser, Ala75 to Ser and Ala 84 to Pro, all with wild type ParC and MICs of ciprofloxacin 0.004-0.063 mg/L; however, since strains with alterations at position 91 or 95 were included, it is not possible to state a correlation between resistance and mutations at other positions [96]. An Ala92 to Pro substitution in addition to a Ser91 to Phe substitution was also reported [87]. An isolate with a Ser91 to Cys substitution had MIC of ciprofloxacin of 0.008 mg/L [80]. A single mutation at position Ser91 to Ile was reported together with a *parC* Ser87 to Arg mutation, the strain had MIC of ciprofloxacin 0.25 mg/L [94].

Belland et al. showed in transformation studies that two mutations at position 91 and 95 corresponded to MIC of ciprofloxacin of 0.5 mg/L [71]. A single mutation in QRDR of *gyrA* with wild type *parC* seems to generate MICs of ciprofloxacin of 0.03 - 0.13 mg/L (Table 2). Double mutations in QRDR *gyrA* with wild type *parC* seem to generate MICs of ciprofloxacin of ≥ 0.13 mg/L (Table 2).

Table 2. References of lowest reported MIC of ciprofloxacin in *N. gonorrhoeae* with wild type parC correlating number of mutations in positions 91 and 95 in gyrA

Substitutions	MIC of ciprofloxacin mg/L	References
91 or 95	0.03	[80, 97]
	0.06	[67, 69, 85, 95, 98]
	0.13	[68, 81, 83, 90]
91 and 95	0.13	[69, 97]
	0.25	[81]
	0.5	[80]

1.10.2 Alterations in GyrB

In *N. gonorrhoeae* FA1090 GyrB is a 796 amino acid long protein, beginning at genomic position 1738581 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome &cmd=Retrieve&dopt=Protein+Table&list_uids=635, access date 060227).

Nalidixic acid resistant *N. gonorrhoeae* mutants were induced by growing *N. gonorrhoeae* on agar plates containing increasing concentrations of nalidixic acid. The responsible gene was cloned and transformation experiments resulted in transformants with increased MIC of nalidixic acid. The cloned gene showed a 70% homology to *E. coli gyrB* gene [73]. Clinical strains have not shown any mutations in *gyrB*, and such mutations do not seem to be of importance in quinolone resistance in clinical strains of *N. gonorrhoeae* [65, 99].

1.10.3 Alterations in ParC

In *N. gonorrhoeae* FA1090 ParC is a 767 amino acid long protein, beginning at genomic position 1210524 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome &cmd=Retrieve&dopt=Protein+Table&list_uids=635, access date 060315).

Most of the reported alterations associated to resistance of ciprofloxacin are at positions Asp86, Ser87, Ser88 and Glu91 and Ala92 but alterations in other positions have been described.

Table 3. References showing different ParC alterations in addition to alterations in QRDR of GyrA, in quinolone resistant strains of *N. gonorrhoeae* MIC of quinolones (norfloxacin, ofloxacin or ciprofloxacin) ≥ 1 mg/L

Position	Substitution	References
Asp86	Asn	[66-69, 80, 82, 83, 85-92, 94]
	Ile	[91]
	His	[91]
Ser87	Asn	[69, 83, 85, 87, 88, 90, 92]
	Ile	[66, 67, 80, 87, 100]
	Arg	[66, 86-88, 90, 92-94]
Ser88	Pro	[66, 67, 71, 80, 84, 87, 88, 90, 92, 94]
Glu91	Lys	[66, 69, 71, 80, 82, 83, 88, 90, 92]
	Gly	[65-67, 80, 81, 85, 87, 88, 93, 100]
	Gln	[65, 66, 80, 81, 84, 87, 90]
	Ala	[87]
Ala92	Gly	[66, 80]
	Val	[94]

Tanaka et al. described one in vitro assessed mutant with a substitution at position Glu91 to Val [95]. An atypical double mutation in ParC with changes in Arg 116 to Leu and Gly85 to Cys and an additional strain with a single mutation at Arg116 to Leu were reported by Trees et al. [69]. A substitution to His has also been reported in position 116 [80]. Shigemura et al. sequenced a strain with a Gly85 change to Asp [94]. All the strains with atypical ParC alterations also had double or single mutations in QRDR of GyrA [69, 80, 94, 95].

1.10.4 Alterations in ParE

In *N. gonorrhoeae* FA1090 ParE is a 661 amino acid long protein, beginning at genomic position 1291428 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome&cmd=Retrieve&dopt=Protein+Table&list_uids=635, access date 060315).

In *E. coli,* Breines et al. have described a Leu445 to His mutation, associated to a one-dilution-step increase of MIC to ciprofloxacin [101]. A mutation in *parE* in *N. gonorrhoeae* was found at position 439, but did not seem to be involved in a high degree of resistance to ciprofloxacin in [65].

1.10.5 Uptake

In *N. gonorrhoeae* FA1090 PorB is a 348 amino acid long protein, beginning at the genomic position 1788698 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome&cmd=Retrieve&dopt=Protein+Table&list_uids=635, access date 060315). The length of the protein varies between strains.

Tanaka et al. studied uptake and accumulation of norfloxacin in a total of 10 strains of *N. gonorrhoeae* exhibiting both quinolone susceptibility as well as resistance. Data

indicated that both reduced uptake and active efflux may be present in the studied strains [102].

There are a few reports on the relation between PorB1 and ciprofloxacin susceptibility in *N. gonorrhoeae*, and changes in *porB1* in ciprofloxacin resistant strains of *N. gonorrhoeae* have been reported in loop 3 (Gly120 to Asn and Ala121 to Asp), as well as changes in loop 5. The reports originated from studies of outbreaks of gonorrhea in Israel and Greece [89, 98]. Vereshchagin et al. sequenced *porB1* in 33 *N. gonorrhoeae* strains and found no correlation between amino acid substitutions in *porB1* position 120 and 121 and resistance to fluoroquinolones [93]. Corkill et al. found a reduced uptake of ciprofloxacin in a resistant strain of *N. gonorrhoeae* [103].

1.10.6 Efflux systems

Efflux systems active against erythromycin, tetracycline and ß-lactams have been described in the gonococcus [91, 104]. The efflux pump NorM has been reported to export ciprofloxacin, but probably has only a limited effect in ciprofloxacin resistant strains [105].

There are several types of efflux systems described in Gram-negative bacteria. The complete systems are the resistance-nodulation-division (RND) family and ABC-transporters. Both are built up by a cytoplasmic membrane transporter, a periplasmic linker and an outer membrane channel. They utilize different energy sources and ABC-transporters prefer proteins as substrates [105, 106]. The major facilitator (MF) and multidrug and toxic compound extrusion (MATE) families are cytoplasmic membrane transporters dependent on other channels [105, 106].

The multiple transferable resistance (Mtr) locus later characterized as efflux system *mtr*CDE was described by Maness and Sparling [107, 108] and exports hydrophobic agents among them fatty acids, penicillin, tetracycline and macrolides [91, 104, 109, 110] and belongs to the RND-family [105]. MtrR represses expression of *mtrCDE* [111]. Mutations in the *mtrR* promotor region has been shown to increase expression of the pump proteins [109, 110, 112]. Loss of MtrD resulted in gonococci hyper-susceptible to toxic fatty acids. Active efflux may promote growth of gonococci in mucosal sites rich in toxic free fatty acids as for example in rectum [113]. Despite several studies no clear correlation between *mtr*CDE and resistance to ciprofloxacin has been established [89, 91, 93, 113].

NorM belongs to the MATE-family and exports compounds harboring a quaternary ammonium on an aromatic ring e.g. ethidium bromide, acriflavine hydrochloride, 2-*N*-methylellipticinium and beriberine. Rouquette-Loughlin et al. stated that ciprofloxacin was a poor substrate for this efflux pump [105].

The efflux system FarAB belongs to the MF-family and exports free fatty acids and bile salts. It is dependent on the *mtrE* outer membrane compound and is positively regulated by *mtrR* [114] and repressed by *marR* [115]. Active efflux of toxic fatty acids may promote growth of gonococci in mucosal sites rich in toxic free fatty acids as for

example in rectum [116]. Jerse et al. found in a mouse model that progesterone might play an inhibitory role in vivo [116].

MacA-MacB is an ABC-transporter and exports macrolides in *N. gonorrhoeae*, and could enhance *mtrCDE* induced resistance to macrolides [117].

1.11 ANTIBIOTIC TREATMENT

N. gonorrhoeae is not considered to be part of a normal human flora. Thus, a laboratory finding of *N. gonorrhoeae* should always lead to antibiotic treatment, regardless of whether the patient shows symptoms or not. All isolates of *N. gonorrhoeae* should be tested for susceptibility to several antibiotics, because *N. gonorrhoeae* is known to have an extremely variable susceptibility pattern, and is also known to adapt to new treatment regimens, by developing mechanisms of resistance. Fortunately after a period with high prevalence of resistant strains, *N. gonorrhoeae* has now once more become susceptible to both penicillin and tetracycline [66]. When an antibiotic is no longer in use as treatment, the mechanisms of resistance are probably a genetic burden, and susceptible strains are selected.

Sulphonamides, introduced in 1930s, were the first antibiotic drugs used to combat gonorrhea. A decade later when penicillin was introduced as an effective treatment, the sulphonamide resistant strains of *N. gonorrhoeae* were wide-spread. With time, chromosomally mediated resistance gave the strains decreased susceptibility to penicillin and increased doses were needed for curative treatment. In 1976 the first strains with high level of plasmid mediated resistance to penicillins were described. The penicillinase producing *N. gonorrhoeae* (PPNG) strains spread rapidly. As PPNG strains became frequent, the use of tetracyclines increased, but soon both chromosomally and plasmid mediated resistance to tetracyclines was also reported.

Spectinomycin, azithromycin and third generation cephalosporins such as cefixime and ceftriaxone are today widely used as treatment. Strains with decreased susceptibility to azithromycin and third generation cephalosporins have been reported, but are still uncommon [66, 109, 118, 119]. Resistance to spectinomycin is rare [66, 120]. In Sweden, ciprofloxacin, cefixime, ceftriaxone, cefotaxime, spectinomycin and azithromycin are considered drugs of choice for gonorrhea.

1.11.1 Epidemiology of ciprofloxacin resistance

Quinolones were introduced in the 1980s and a decade later they had become the drug of choice for gonorrhea more or less worldwide. From the mid 1990s the reports of quinolone resistant *N. gonorrhoeae* (QRNG) strains became frequent [87, 121-125] and today resistance is common [1-3, 126]. Xia et al. made study of ciprofloxacin resistant isolates of *N. gonorrhoeae* from different parts of the world using PGFE and concluded that they were not a clonal spread [127].

Reports from several Asian countries also show that resistance to ciprofloxacin has emerged since the mid 1990s. In Hong Kong in 1996, 24% of the isolates were already resistant to ciprofloxacin and the rate increased to 80% in 2000 [87]. In Japan the ciprofloxacin resistant strains increased from 7% in 1993/1994 to 74% in 2002 [66]. In

Guangzhou, China, resistance to ciprofloxacin rose from 17.6% in 1996 to 72.7% in 2001 [128]. Ray et al. report QRNG prevalence from New Delhi, India, and Bangladesh of more than 70% in 2000. Interestingly, their report showed that QRNG prevalence in Sri Lanka was stable around 10% 1996-2000. This was maybe due to differences in treatment strategies [2].

In the United States the CDC report of increases in QRNG strains among men who have sex with men reflects that the prevalence of QRNG in 2003 is much higher in this group (4.9%) than in heterosexual men (0.4%). The prevalence of QRNG was also higher in Hawaii and California. Overall QRNG prevalence increased from 0.7% in 2001 to 4.2% in 2003. In Hawaii resistant isolates increased from 1.4% in 1997 to 9.5% in 2002 [129].

Reports from several European countries show the epidemiological pattern of emergence of ciprofloxacin resistance. According to the database ResNet in Sweden, 10% of the isolates of *N. gonorrhoeae* were resistant to ciprofloxacin in 1998 and in 2004 that figure had increased to 48% (http://www4.smittskyddsinstitutet.se/ResNet /index.jsp, access date 060219). In Denmark ciprofloxacin resistance rose from 6.9% to 13.2% between 1995 and 1998 [92]. In France the first strain with resistance to ciprofloxacin was reported in 1997 and in 1998-2000 the resistant strains increased to 5.2% [130]. From 2001 to 2002 ciprofloxacin resistant isolates increased from 3.1% to 9.8% in England and Wales [131]. In Austria QRNG increased from 3.9% in 1999 to 59.4% in 2002 [132].

Reports from African countries are not frequent. In an STI clinic in Durban, South Africa, all isolates of *N. gonorrhoeae* were susceptible to ciprofloxacin up until 2002, but by January 2005 56% of the isolates were resistant [1].

1.12 PREVENTION

Silver nitrate eye drops, the first prophylactic treatment against gonococcal infection, was introduced in 1881 by Karl Credé. This treatment prevented ophthalmia neonatorum in neonates. It was used in Sweden until 1983 [38]. So far no successful vaccine to prevent gonorrhea has been developed.

Surveillance and education about the importance of using condoms may result in changed sexual behavior and thereby decrease the prevalence of STIs and gonorrhea [133-136].

1.13 LABORATORY DIAGNOSTICS

A trained clinician, for example in an STI clinic, can diagnose gonorrhea by a direct microscopic examination of urethral discharge stained with methylene blue or Gram stain. However, this method is not equally suitable for endocervical discharges, pharyngeal or rectal samples, mainly because of a rich normal flora. If the stained sample shows intracellular diplococci in the leucocytes, this is a presumptive diagnosis of gonorrhea. Nonetheless, samples from all patients, positive or negative in direct microscopy, should be sent for a laboratory diagnosis.

1.13.1 Culture

In Sweden, as all over the world, culture is the gold standard for laboratory diagnosis of *N. gonorrhoeae*. Clinical samples are sent to the laboratory on swabs in a transport medium. Studies have shown an increased sensitivity in culture if the agar medium is inoculated at bedside and then promptly transported to the laboratory [137, 138].

All swabs are cultured on selective and non-selective agar media in 5% CO_2 at 37°C over night. The overnight cultures are examined. Colonies with morphology consistent with *N. gonorrhoeae*, positive in oxidase test and showing Gram-negative diplococci in the microscope, are further analyzed in a sugar oxidation test [5]. Production of ß-lactamase is detected with nitrocefin [139]. Serogroup and serovar are determined by an agglutination test. All strains are susceptibility tested by Etest to a variety of antimicrobial agents.

The culture method is considered to have excellent specificity, but since *N. gonorrhoeae* is vulnerable and demanding in terms of growth conditions, sensitivity can be decreased by prolonged transportation time. An advantage of culture is that susceptibility tests can be performed on all isolates. Negative results can be ready the next day, but positive results demand at least two overnight incubations, one for the sample from the swab, and the next for sugar oxidation tests. If susceptibility tests are not done the same day as the sugar oxidation test, they will take yet another day to incubate.

1.13.2 Molecular methods

Nucleic acid hybridization tests and nucleic acid amplification tests (NAATs) detect genomic parts of *N. gonorrhoeae* from a clinical specimen. The advantages are that results are obtained fast and the reactions are performed in commercial kits designed to detect *N. gonorrhoeae* and *C. trachomatis* in the same specimen in a duplex reaction. Other advantages are that they are fast, that urine samples can be used and that also dead bacteria can be detected. Several studies show that patients prefer the molecular methods because urine samples can be used, since both urethra and cervix swabs are considered invasive [140-142]. In addition, if urine samples are sufficient, the tests need not be done by highly trained staff, they are less time consuming and do not require gynecological equipment for the sample collection. The molecular methods have been evaluated as an alternative for use outside clinical settings [143].

The disadvantages are that antimicrobial susceptibility tests and serological tests for epidemiological purposes can only be performed from strains. So far all commercial tests have suffered from low specificity and several confirmational genes have been evaluated among them 16SrRNA, *gyrA* and *cppB* genes [8, 81, 144-147]. For this purpose the 16SrRNA is the most studied gene and strains of *N. cinerea* as well as *Lactobacillus* spp. have given positive results in confirmation [148, 149]. The plasmid borne *cppB* gene is not present in all strains, and thus not suitable as a target for confirmation [8, 147]. A double infection may be present, and a problem of competition between simultaneous amplification in duplex PCR methods has been addressed [150]. This problem must be solved because double infections with both

targets are not uncommon [151]. Culture still has the advantage of being the gold standard and is considered as highly specific [5].

1.13.3 Nucleic acid amplification tests (NAATs)

In NAATs a specific pair of primers are designed to amplify the target DNA of *C. trachomatis* and *N. gonorrhoeae* in a duplex polymerase chain reaction (PCR) [5].

Two commercial methods use this technique for detection of *N. gonorrhoeae*, all as duplex PCR additional to detection of *C. trachomatis*. Roche AMPLICOR (Roche Diagnostics, Indianapolis, IN, USA) targets the 201-base pair (bp) sequence of the cytosine methyltransferase gene [152]. COBAS AMPLICOR is a more automated version of the same method. BDProbe Tec ET (BD Biosciences, Sparks, MD, USA) targets the multicopy pilin gene [5]. Abbott LCx test (Abbott Laboratories Inc., Abbott Park, IL, USA) targeted a 48-bp sequence of the *opa* gene, a multicopy gene present in up to 11 copies, but due to reproducibility problems this method was recalled [153, 154]. Targeting multicopy genes enhance sensitivity of the tests. Since all NAATs amplify the target RNA the sensitivity is considered increased compared to nucleic acid hybridization tests. Nucleic acid hybridization tests are considered less sensitive, since they hybridize but not amplify target rRNA. Only Hybrid Capture II assay (Digene Corp., Gaithersburg, MD, USA) is commercially available [5]. Other companies than those mentioned here have kits for molecular detection of *N. gonorrhoeae*.

Several studies have evaluated commercial molecular diagnostics of *N. gonorrhoeae.* The conclusions are that for specimens from pharynx and rectum, molecular diagnostics could not be recommended, because of high risk of false positive results [147]. To increase sensitivity, confirmation tests may be needed for all positive results [147]. The advantages of molecular diagnostics still remain and further investigations were needed [138, 149, 155-157]. Two real time PCR methods, both targeting the *opa* gene, have been evaluated and the results are promising [154, 158]. The real time PCR technique is now available for commercial tests of *C. trachomatis*.

2 AIMS OF THE STUDY

STUDY I

The aim of study I was to determine the correlation between MIC of ciprofloxacin in 25 highly resistant *N. gonorrhoeae* strains from Bangladesh and mutations in *gyrA*, *gyrB*, *parC* and *parE*.

STUDY II

The aims of the study II were to show that the sequence of QRDR of *gyrA* in *N. gonorrhoeae* is an indicator of resistance to ciprofloxacin and discriminates *N. gonorrhoeae* from other *Neisseria* spp. also in samples without living bacteria.

STUDY III

The aims of study III were to investigate how AMPLICOR *N. gonorrhoeae* PCR (Roche Diagnostics) performs compared to culture in clinical samples, and to compare confirmation by PCR of 16S rRNA as recommended by Roche Diagnostics, to confirmation by sequencing of QRDR in *gyrA*.

STUDY IV

The aims of study IV were to investigate if the *N. gonorrhoeae* ciprofloxacin susceptibility method, based on pyrosequencing of QRDR of the *gyrA* gene, can be used as indicator of susceptibility to ciprofloxacin in most *Neisseria* spp. and if this QRDR sequence is suitable also for species confirmation of *N. gonorrhoeae*.

STUDY V

The aim of study V was to determine if alterations in gonococcal porin Por1Bb are involved in resistance to ciprofloxacin.

3 METHODS

3.1 BACTERIAL STRAINS

The strain collection from Dhaka, used in studies I, II, IV and V consisted of 25 *N. gonorrhoeae* strains, highly resistant to ciprofloxacin, with a range of MIC from 4 mg/L to >32 mg/L, and two susceptible strains, isolated from female sex workers in Dhaka in 1997. The strains originated from two studies made by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) [49, 159].

The strain collection from Stockholm, used in studies II, IV and V, consisted of 33 *N. gonorrhoeae* strains isolated in Sweden at Karolinska University Hospital, Huddinge, in 2000-2002, with decreased susceptibility to ciprofloxacin with a range of MIC from 0.125 mg/L to >32 mg/L, and three clinical ciprofloxacin susceptible *N. gonorrhoeae* strains.

The clinical study comparing culture and molecular diagnostics with two different verification methods, used in studies II and III, included 956 swabs, from urethra, cervix/urethra, rectum or pharynx, for *N. gonorrhoeae* culture, accompanied by urine, or cervix swabs in urine, rectum or pharynx, for AMPLICOR *C. trachomatis* PCR. The samples for AMPLICOR *C. trachomatis* PCR were also analyzed using AMPLICOR *N. gonorrhoeae* PCR (Roche Diagnostics) with duplex PCR. All samples were collected with informed consent from the patients, and the study was approved by the local ethical committee.

In study IV, strains of *N. lactamica, N. meningitidis, N. subflava, N. flavescens, N. cinerea, N. mucosa, N. sicca, N. elongata, N. gonorrhoeae* subsp. *kochii* and *N. gonorrhoeae* were examined. Twenty-five *N. gonorrhoeae* strains as well as isolates (n=40) of *Neisseria* spp. from Örebro University Hospital, Department of Clinical Microbiology, were added. The *N. gonorrhoeae* strains from studies I and II were also included in study IV.

In studies I-IV, reference strains from CDC (Centers for Disease Control and Prevention), ATCC (American Type Culture Collection) and CCUG (Culture Collection University of Göteborg) were analyzed. In study III, a type strain of *E. coli* and a mix of clinical strains of *C. trachomatis* were also analyzed. In paper V, a new clinical *N. gonorrhoeae* recipient strain was added.

All strains were stored at -70°C.

3.2 MEDIA AND CHARACTERIZATION

All clinical *N. gonorrhoeae* strains were identified as described in the introduction. The isolates from Dhaka in study I were also genotyped by PCR restriction fragment length polymorphism (RFLP) of the *por* gene. The amplified PCR products were digested by *Msp*A1I and *Cfo*I (Promega, Madison, WI, USA).

In study IV the species identification of the clinical isolates of *Neisseria* spp. was based on their ability to grow under various incubation conditions, colony morphology and

pigmentation on different culture media, reduction of nitrate, sugar oxidation tests (glucose, maltose, fructose, and lactose), RapID NH (Remel, Lenexa, KS, USA) and/or API NH v2.0 (BioMérieux, Lyon, France), and sequencing of the 16S rRNA gene (Unemo et al., to be published).

3.3 ANTIMICROBIAL SUSCEPTIBILITY

In studies I-V, MIC of ciprofloxacin was determined by Etest (AB Biodisk, Solna, Sweden) [160].

3.4 MOLECULAR DIAGNOSTICS

In study II, both the COBAS AMPLICOR *C. trachomatis* PCR and COBAS AMPLICOR *N. gonorrhoeae* PCR (Roche Diagnostics) were performed according to the manufacturer's instructions [161]. The COBAS AMPLICOR method is an automated PCR, used for designed kits, and results in a color change read in an ELISA like process. A positive sample will change color resulting in an increased optical density (OD) value.

3.5 PRIMERS AND PCR

In studies I, II and V, primers were designed in Macintosh software OLIGO 4.0 (National Biosciences, Inc., Plymouth, MN, USA). The primers were synthesized by INTERACTIVA Biotechnologie (Ulm, Germany). PCR protocols were developed according to the characteristics of each primer pair. In studies III, IV and V, already published primers were used.

3.6 DNA SEQUENCING

Sequencing in studies I, II, V was done by capillary electrophoresis in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and in Paper I also in an ABI PRISM 377 Automated DNA Sequencer (Applied Biosystems). PCR products were purified by the Qiaquick-spin PCR purification kit (Qiagen Inc., Chatsworth, CA, USA).

Sequencing in studies II-IV was done by a pyrosequencing method in an automated bench-top PSQ 96 system (Pyrosequencing AB, Uppsala, Sweden). DNA in samples positive in AMPLICOR *N. gonorrhoeae* PCR were purified with QIAamp viral RNA mini kits (Qiagen Inc.), and DNA from strains was purified by the QIAamp DNA mini kit (Qiagen Inc.).

3.6.1 Fluorescence-based Sanger sequencing

The Sanger technique is based on the four different nucleotides labeled with four different fluorescent dyes. During a cycle sequencing PCR a copy of the template is elongated until termination by incorporation of a fluorescent labeled nucleotide. This is performed in 25 cycles to get detectable amounts of extension products. The four different nucleotides are then either separated by capillary electrophoresis or by gel electrophoresis. All four fluorescent dyes emit a different wavelength and a charge-coupled device (CCD) camera detects the fluorescence [162].

3.6.2 Pyrosequencing

Single-strand DNA sample preparation prior to sequencing was performed automatically on a Magnatrix 1200 robot (Magnetic Biosolutions, Stockholm, Sweden). The biotinylated PCR product was immobilized onto streptavidin-coated super paramagnetic beads (Dynabeads M280; Dynal, Oslo, Norway). Single stranded DNA was obtained by incubating the immobilized PCR product in NaOH.

Pyrosequencing method is a real time sequencing-by-synthesis DNA sequencing technique that specifically analyzes the sequence of the amplified DNA fragment. The principle relies on an iterative addition of the individual nucleotides to a substrate consisting of single strand template and a sequencing primer in the presence of a DNA polymerase. By incorporation of complementary nucleotides, sequence results in the form of a quantitative release of pyrophosphate and its consequent conversion to light through a cascade of enzymatic reactions. The light is detected by a CCD camera. The intensity of the light correlates to the height of the peaks in the form of a pyrogram. The heights of the peaks reveal the number of the same nucleotides incorporated next to each other. The whole process is monitored in real time. Nucleotides that are not complementary, are instead inactivated by the enzyme apyrase, before the next nucleotide is dispensed [163]. A maximum of 30-50 nucleotides can be sequenced by Pyrosequencing, compared to up to 700 by the more time-consuming Sanger method.

3.7 SEQUENCE ANALYSIS

The results of sequencing are presented in the software EditView (Perkin Elmer, Palo Alto, CA, USA) and were in paper V further analyzed in Finch TV version 1.3.1 (available at http://www.geospiza.com/finchtv/index.htm, access date 060315).

All sequences were aligned in ClustalW, Interactive Multiple Sequence Alignment at European Bioinformatics Institute (available at:http://www.ebi.ac.uk/clustalw/, access date 060218). Sequences were translated into amino acids in ExPASy Molecular Biology Server at the Swiss Institute of Bioinformatics, Geneva, Switzerland (available at http://www.expasy.ch/tools/dna.html, access date 060315).

The complete sequenced genome of *N. gonorrhoeae* FA1090 (*N. gonorrhoeae* ATCC 700825) (available at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome&cmd=Retrieve&dopt=Protein+Table&list_uids=635, access date 060315) was used as the basis for evaluation.

3.8 TRANSFORMATION STUDIES

This protocol was developed from the protocols of Goodman and Scocca and Anticgnac et al. [164, 165]. Genomic DNA from whole cells of donor strains was extracted with the QIAamp DNA mini kit (Qiagen Inc.). Recipient strain colonies, the size of a rice grain, were taken after 18 hours of incubation on a chocolate agar plate and subcultured in 10 mL tryptic soy broth for 18 hours in cell culture bottles (37°C; 5% CO_2). Then approximately 3.0 µg of extracted whole cell DNA was added to the broth and incubated for 6 hours. Thereafter, the culture was transferred to a 10 mL tube and spun down. Supernatant was discharged, the pellet was resuspended in the remaining broth, and 150 µL of the cell suspension was cultured on each chocolate agar

plate without and with selected concentrations of ciprofloxacin. In all transformation experiments we used this protocol for the controls, except without adding the donor DNA.

4 RESULTS AND DISCUSSION

4.1 SUSCEPTIBILITY

MIC breakpoints for *N. gonorrhoeae* of ciprofloxacin vary. The Swedish Reference Group for Antibiotics (SRGA) (http://www.srga.org/MICTAB/MICTAB2.htm, access date 060315) as well as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.srga.org/eucastwt/MICTAB/index.html, access date 060315) currently give the MIC breakpoints in *N. gonorrhoeae* to ciprofloxacin as S $\leq$ 0.03 and R > 0.06 mg/L. CDC refer to the Clinical and Laboratory Standards Institute (CLSI), former National Committee on Clinical Laboratory Standards (NCCLS), and currently give the MIC breakpoints in *N. gonorrhoeae* to ciprofloxacin as S $\leq$ 0.06 and R $\geq$ 1.0 mg/L [166]. The Clinical Bacteriology Laboratory at Karolinska University Hospital, Huddinge, uses the MIC breakpoints S $\leq$ 0.016 and R > 0.06 mg/L. This clearly illustrates that national and regional breakpoints vary, and they have varied even more over time. Interestingly, MIC breakpoints vary far more than the actual wild type population; this is evident from a search for *N. gonorrhoeae* and ciprofloxacin by year in the Swedish database ResNet (available at http://www4.smittskyddsinstitutet.se /ResNet/index.jsp, access date 060315).

4.2 MECHANISMS OF RESISTANCE

Alterations in target enzymes are the major mechanisms of resistance to ciprofloxacin in all studied species [60]. In *N. gonorrhoeae* alterations in GyrA are the major mechanism of resistance, and additional alterations in ParC also contribute to resistance to ciprofloxacin [167]. The roles of GyrB and ParE are not fully investigated.

Changes in porin composition or production are a known mechanism of resistance to antimicrobial agents, as in *P. aeruginosa* where loss of outer membrane protein OprD results in imipenem resistance [168]. In *E. coli,* Neves et al have. shown that all quinolones, and especially the newer ones with better activity against Gram-positives, have good affinity to outer membrane protein F, OmpF, but their study did not include ciprofloxacin resistant strains [169]. The homologues to OmpF in *Klebsiella pneumoniae* are OmpK35 and OmpK36. Wild type *K. pneumoniae* expresses both proteins, but rare strains produce neither of them. Loss of OmpK36 is associated with a moderate increase of fluoroquinolone resistance in addition to alterations in target enzymes or active efflux [77]. Uptake of ciprofloxacin in *N. gonorrhoeae* is not fully investigated as a mechanism of resistance.

Efflux is a known mechanism of resistance to ciprofloxacin in other species [76, 170]. *N. gonorrhoeae* has known efflux systems including MtrCDE, NorM, MacA-MacB and Far but none of them seems to export ciprofloxacin as a preferred substrate [91, 105, 113].

Protection has been described as a mechanism of resistance to quinolones in *E. coli* where the protein Qnr is thought to protect DNA gyrase from self inhibition by the bacterially produced microcin B17, a peptide that blocks DNA replication [78]. By binding to DNA Qnr probably reduces the amount of targets for quinolones [79]. This

mechanism has been described in *K. pneumoniae* and *E. coli* but not yet for resistance to ciprofloxacin in *N. gonorrhoeae*.

4.2.1 Alterations in GyrA

The results of studies I-III and V support that reduced susceptibility to ciprofloxacin correlates to mutations at position 91 and 95 in *gyrA.* All sequenced strains in study I-V with MIC ≥ 2 mg/L had both mutations at positions 91 and 95 in *gyrA*. In studies II and V we found that all strains with MIC ≥ 0.064 mg/L had at least one of the mutations at position 91 or 95 in *gyrA*. Results of studies II and V also indicate that mutations at only one of the two positions were most frequent in strains with MIC 0.125 - 0.5 mg/L. One strain in study II with MIC 1.0 mg/L had only one mutation at position 91. Even though the ranges of MICs in the strains containing one or both the mutations overlap, there is a correlation between one mutation and lower MICs and two mutations and higher MICs.

At Ser91, all strains in study I-V have a substitution to Phe, and at Asp95 the alterations were to either Asn, Ala or Gly. No correlation between changes in amino acids in positions 91 and 95 and the degree of resistance to ciprofloxacin was established. Mutations at position 91 and 95 seem to contribute equally and additively to ciprofloxacin resistance.

In study V, the MICs of the first generation transformants support the findings of presence of mutations in QRDR of *gyrA* in clinical strains in study II, generating a MIC of ciprofloxacin of 0.064 - 0.25 mg/L. These data support the transformation study performed by Belland et al. [71]. They showed that two mutations at position 91 and 95 corresponded to MIC of ciprofloxacin of 0.5 mg/L. The correlation of presence of mutations in QRDR of *gyrA* and *parC* and resistance to ciprofloxacin is evident in all studies with sequenced strains (Tables 1 and 3). The susceptible strains sequenced in study I-V all had the wild type *gyrA*, as in other studies including susceptible strains.

Numerous strains of *N. gonorrhoeae* have been analyzed according to mutations in QRDR of *gyrA* and are available in the literature. So far, only three publications have been found describing strains with reduced susceptibility to ciprofloxacin and lack alterations in GyrA [91, 93, 97].

In conclusion, mutations in position 91 and 95 in QRDR of GyrA have additive effects on MIC, and since the strains with two mutations have a much wider range of MIC, and since ParC alterations occur most frequently in strains with double mutations in GyrA, additional mechanisms such as decreased uptake or efflux probably also occur more frequently in strains containing both of the mutations.

4.2.2 Alterations in GyrB

In study I we did not find any alterations in QRDR of *gyrB*, but we found an insertion of 14 amino acids. Both the absence of mutations and the insertion are consistent with the report by Deguchi et al. concluding that mutations in *N. gonorrhoeae gyrB* are probably not a common finding among clinical ciprofloxacin resistant strains of *N. gonorrhoeae* [99]. Stein et al. found correlations between low-level nalidixic acid

resistance in laboratory-induced mutants of *N. gonorrhoeae* and alterations in *gyrB*, however in this study the whole gene was cloned and transformed, so the specific positions of the presumed mutations were not revealed [73].

4.2.3 Alterations in ParC

In studies I and II we found a mutation in position Glu91 in *parC*. In our material we only found mutations in strains with MIC ≥4 and the alterations were present at both positions 91 and 95 in GyrA, which is consistent with other studies. Study I and II as well as numerous publications support that mutations in *parC* are only found in quinolone resistant strains, and thus contribute to resistance to ciprofloxacin in *N. gonorrhoeae* (Table 3).

4.2.4 Alterations in ParE

In study I we found a mutation at Pro439 to Ser in 17 highly resistant strains of *N. gonorrhoeae*, but this mutation was not found in any of the five susceptible strains. To investigate the role of this mutation, we performed transformation studies with both PCR product of *parE* as well as whole cell transformation. Only two colonies grew on the agar-plate containing ciprofloxacin at 8 mg/L, but these did not contain the *parE* mutation, and were discharged as non-*parE* transformants. The negative result of this transformation may be due to suboptimal conditions in choosing recipient strains. The transformation protocol for transferring the TRNG plasmid conferring to resistance to tetracycline as control was performed in the Dhaka laboratory, but the recipient strains #6 and #20 were dry frozen when transported to Stockholm, which probably made them less competent. The conclusion drawn from the negative result was that this mutation did not cause a significant increase in MIC to ciprofloxacin. This might still be true, but the result of study V, with a co-transformation of *porB* and *parE* only in one of the most ciprofloxacin resistant transformants, indicates that the alteration in ParE probably contributes to ciprofloxacin resistance.

4.2.5 Alterations in PorB

There are a few reports on PorB and ciprofloxacin susceptibility in *N. gonorrhoeae*, but the absence of a defined wild type PorB in *N. gonorrhoeae* complicates conclusive correlations to ciprofloxacin resistance. Corkill et al. have shown a reduced uptake of ciprofloxacin in a resistant strain and transformation of resistance to other strains, but neither the resistant strain nor the transformants were sequenced in *gyrA* and resistance to chloramphenicol and tetracycline was not co-transformed [103]. The study by Tanaka et al. indicating reduced uptake or active efflux was not conclusive and done from a limited material consisting of only 10 strains [102].

Gill et al. state that the genomic locus *penB* equals alterations in PorB1b loop 3, i.e. Gly101 to Asp and Ala 102 to Asp, reducing PorB1b permeability to hydrophilic antibiotics as penicillin and tetracycline in *N. gonorrhoeae* [34]. Olesky et al. also found that *penB* equals mutations in PorB1b loop 3 in position Gly120 and Ala 121, i.e. to single mutation Gly120 to lysine or double mutations to charged amino acids that contributed to chromosomally-mediated penicillin and tetracycline resistance. On the basis of GenBank studies, they stated that Lys and Asp mutations in position 120 and/or 121 occur naturally in *N. gonorrhoeae* [35]. Despite the different position

numbers, these amino acids are likely to be the same, since Gill et al. probably excluded the 19 amino acid long signal sequence in their numbering. Olesky et al. did not include ciprofloxacin susceptibility as an aspect in their study. Donor strains A and B, used in study V, have the 120Lys 121Asp and the 120Lys 121Gly sequence respectively, and both were ciprofloxacin resistant. The gene *porB* is known to be highly variable in the sequence that includes loop 3 [22, 24, 26].

Ciprofloxacin resistant strains of *N. gonorrhoeae* have been reported to have alterations in loop 3, Gly120 to Asn and Ala121 to Asp, as well as changes in loop 5. The report originated from a study of a gonorrhea outbreak, which implied that the studied isolates were related [89]. In epidemiological studies mutations in individual genes are difficult to evaluate, since other mechanisms are often impossible to rule out. Vereshchagin et al. sequenced *porB* in 33 *N. gonorrhoeae* strains but found no correlation between amino acid substitutions in *porB* position 120 and 121 and resistance to fluoroquinolones [93]. This implies that the relation between PorB and ciprofloxacin resistance may be more complex than specific changes in position 120 and 121. In the second generation transformants in study V the whole *porB1b* genes were transformed and MIC of ciprofloxacin increased from 0.25 to 0.5 - 16 mg/L and 0.5 - 2 mg/L respectively. In the transformation experiments in study V we can compare sequences of recipient and donor strains, which make us less dependent on defining a wild type.

It is possible that *porB* was cotransformed with another gene, responsible for fluoroquinolone resistance. To address this, the genome was searched upstream and downstream of porB but only putative LysR-family transcriptional regulator was found, the next gene downstream of *porB*, as a possible regulator of an efflux system. Since no mutations were detected in putative *lysR*, in donors, recipients or transformants, we conclude that putative LysR was not involved as a mechanism of resistance in these strains. In addition, *porB* was transformed repeatedly in two different transformation experiments, which speaks in favor of this gene being the one that confers resistance.

In studies I-V no ciprofloxacin resistant strain lacking alterations in QRDR of GyrA was found, but a few such strains have been reported elsewhere [91, 93, 97]. Alterations in porB1b might be an explanation.

In conclusion, *N. gonorrhoeae* transformants comprising donor *porB1b* also had increased MICs to ciprofloxacin, and in analogy to other hydrophilic antimicrobial agents, an alteration in outer membrane protein reduces the uptake. This mechanism of resistance to ciprofloxacin in *N. gonorrhoeae* seems mainly to be of importance in combination with alterations in GyrA, and might explain some of the wide range of MIC of ciprofloxacin exhibited in resistant strains.

4.3 OTHER MECHANISMS OF CIPROFLOXACIN RESISTANCE

Strains of *N. gonorrhoeae* with reduced susceptibility to ciprofloxacin exhibit a wide range of MICs to ciprofloxacin, implying that also other mechanisms than mutations in QRDR of the target enzymes and porins, as efflux are involved in the most resistant strains. There are also reports of ciprofloxacin resistant strains of *N. gonorrhoeae* with

the wild type sequence in *gyrA* and *parC* genes [91, 93, 97]. Alterations in porins or active efflux might be an explanation of resistance in these strains.

Protection has shown to be involved in ciprofloxacin resistance in other species as *K. pneumoniae* [171] and *E. coli* [78, 79] by the plasmid gene *qnr* [78, 79]. In *Mycobacterium smegmatis* as well as in *M. tuberculosis,* a similar mechanism of protection was described by a plasmid encoded protein [172, 173]. Since the gene *qnr* was not found in any of the donor strains in study V, we excluded this mechanism of resistance as introduced in the recipient strain. Efflux is a known mechanism of resistance to ciprofloxacin in several bacteria, and is probably also involved in ciprofloxacin resistance in a yet not defined way.

4.4 LABORATORY DIAGNOSTICS

4.4.1 Sensitivity and specificity in molecular diagnostics

Culture is the gold standard for laboratory diagnostics of *N. gonorrhoeae*. This method has been used for a long time and works adequately. In addition, culture of a strain provides an opportunity to test its susceptibility to a variety of antibiotics and to agglutination tests. Susceptibility testing is important in *N. gonorrhoeae* since strains are highly variable. On the other hand, culture requires living bacteria. This is a problem because *N. gonorrhoeae* is vulnerable, and prolonged pre-culture time affects sensitivity. Bedside inoculation of agar plates has been shown to increase sensitivity of culture test [137, 138]. Culture also requires skilled laboratory technicians, which can be a problem, especially when it comes to low prevalence diseases. The fact that every laboratory uses a local definition of sensitivity was clearly illustrated in a study by Martin et al., where sensitivity was influenced by what is described as “exceptionally poor performance” of *N. gonorrhoeae* culture in one of the six laboratories [155]. The same conclusion was drawn by van Dyck et al. when comparing the sensitivity of *N. gonorrhoeae* culture, which was described as “extremely low”, in two of the three laboratories in their study [146]. The other end of the range is demonstrated by the excellent sensitivity of *N. gonorrhoeae* culture performed by Higgins et al. [138].

In cultivation on agar plates, colonies with morphology compatible with *N. gonorrhoeae* must be recognized and investigated. Sensitivity in molecular methods is more standardized, but is nonetheless an issue. Sensitivity in molecular tests can be reported as excellent compared to culture [132, 174] but the possibility of false positives must be considered. Several *Neisseria* spp. are known to colonize the human pharynx, and several commercial tests have been reported to produce false positives [132, 144, 145, 147]. Confirmational tests are recommended by manufacturers, the published literature and the CDC [144, 145, 161, 175]. The Roche test, for example, has not been cleared by the FDA for samples from pharynx [5]. Comparing results obtained using different methods can result in erroneous conclusions if the experimental test has a higher sensitivity or specificity than the gold standard [176]. Screening in low-prevalence settings could also result in a greater proportion of false positive results [175, 177].

Roche has recognized a problem in low specificity and recommends a confirmatory test in another part of the genome. The Roche test seems to be one of the most frequently used tests in scientific research and many studies concerning confirmation have been performed to confirm the results of their commercial test. Since most laboratories are running commercial tests for *C. trachomatis,* they are probably more prone to test the molecular *N. gonorrhoeae* method developed by the same company, as was the basis of decision of which commercial test to evaluate in study III.

Scrutiny of studies that have compared the results of commercial tests with the results obtained using culture or other methods of verification reveals that the study design is influenced by whether or not the group carrying out the study believes in molecular diagnostics. Those who are skeptical to molecular diagnostics design studies that include confirmatory tests, and those who believe in molecular diagnostics rerun the analysis, using the same technique if the first results were borderline. There are also groups whose strategy falls between these two: they use confirmatory tests under certain conditions, such as when repeat runs give discrepant results, when cultures are negative, or if the patient shows no symptoms (Table 4). These differences in strategy will affect sensitivity and specificity. Moreover, such strategies would probably only be used in a designed (experimental) study. Since the literature seems to contain more studies than there are laboratories actually running the methods, it would appear that most clinical bacteriologists are still in doubt about molecular diagnostics. Another speculation is that true believers are more prone to run the test than to design a study. Be this as it may, Roche Diagnostics is releasing a new method where quantitative PCR is used, and they have already released a new quantitative method for *C. trachomatis*. These new methods will probably inspire further studies.

Table 4. Studies evaluating molecular tests of *N. gonorrhoeae* according to how the study design achieves confirmation of positive results

Study design		References
Confirmation		[138, 142, 144-146, 155, 157, 174]
Conditional confirmation:	if discrepant results	[149, 178, 179]
	if asymptomatic	[155]
	if culture negative	[155, 174]
Rerun in same method		[155]

A future molecular *N. gonorrhoeae* test with acceptable specificity probably will be considered as a diagnostic tool also in populations with a low prevalence of gonorrhea. For example, when the clinician today chooses to test only for *C. trachomatis*, or when a patient with risk behavior has no symptoms, but wishes to be tested, a molecular test also for *N. gonorrhoeae* could add valuable information. The test is fast, especially for negative results, and if urine samples can be used, the patients will find the test less invasive than those based on culture methods. Patients who have symptoms that qualify them for treatment before the result of the laboratory diagnosis are ready should still be diagnosed by culture, and, if possible, patients with positive results from molecular diagnostics should be retested by culture, to obtain a susceptibility test to different antimicrobial agents. Since pharynx specimens are known to produce false positive

results in molecular methods, such specimens need to be validated for molecular diagnostics.

Screening for *C. trachomatis* has been reported to be cost effective among young sexually active women [180, 181], but cost effectiveness depends on prevalence and the definition of a population [143, 182], which implies that all settings must be evaluated separately. Although most guidelines recommend screening for *C. trachomatis* only, a future molecular test with acceptable specificity should be interesting to evaluate in a cost-benefit analysis in certain populations, because the additional cost for a second test in a duplex PCR should be negligible.

In all studies it is obvious that sensitivity and specificity are also affected by the type of sample and whether it is from a male or a female, and it is also difficult to compare the genital samples (cervix, urine, urethra, vaginal swabs and cervix samples transported in urine). For females, Airell et al. have shown that testing of cervix samples in urine gives results superior to those obtained with either separate urine or cervix samples [37]. For testing of genital *C. trachomatis* infection the Karolinska University Hospital today recommends taking urine for males and cervix samples in urine for females. Probably this sample collection would work also for *N. gonorrhoeae*. It is of importance that the future test has an acceptable sensitivity also in urine samples from females; this sampling technique has been shown to have decreased sensitivity [149, 155, 157], but urine or vaginal swab in urine might prove sufficient for screening purposes.

In conclusion, culture has a sensitivity problem and molecular diagnostics has a problem in specificity. When the outcome is digital, presence of *N. gonorrhoeae* or not, specificity is certainly of extreme importance. Laboratory diagnostics will always face the problem of false negative results, but as in study III, false positive results are a major problem, and far less acceptable, not least the in STI-diagnostics, where the profound psychosocial implications together with legal aspects must also be considered.

4.5 MOLECULAR CIPROFLOXACIN SUSCEPTIBILITY TEST

Molecular susceptibility tests to ciprofloxacin have been evaluated for strains of *Mycobacterium* spp. [183-185], *E. coli* [186, 187] and *N. gonorrhoeae* (Table 5). These tests make the correlation between mutations in QRDR of *gyrA* and ciprofloxacin resistance solid, but exceptions in *N. gonorrhoeae* have been reported [91, 93, 97]. To make a molecular indication of resistance to ciprofloxacin in *N. gonorrhoeae*, testing for alterations in *parC* or *parE* will not add valuable information about the susceptibility, since these alterations are additional to GyrA-alterations.

A rapid method for detection of chromosomally mediated penicillin resistance targeting the *ponA* gene in *N. gonorrhoeae* was developed using a real time PCR assay [188]. Molecular methods have been evaluated for use on strains and cerebrospinal fluid for detection of *penA* alterations correlating to penicillin resistance in *N. meningitidis* [189] as well as in *N. flavecsens* and *N. lactamica* [190]. The *penA* gene encodes penicillin binding protein (PBP) 2, which is the major PBP responsible for penicillin resistance in

gonococci, PBP 1, encoded by *ponA*, has lower affinity for penicillin and PBP3 is not involved in resistance [191]. The limitation is that detection of plasmid mediated resistance is not possible through these methods [188, 191, 192], however, this limitation could be overcome by an additional test targeting TEM β-lactamases. Together with a ciprofloxacin molecular susceptibility test, the opportunity to make a duplex molecular susceptibility test should be considered.

Table 5. References of *N. gonorrhoeae* ciprofloxacin molecular susceptibility tests

References	Method	Sample
Study II	Pyrosequencing	Urine
[193]	Pyrosequencing	Strains
[194]	Real time PCR	Urethral swab
[195]	Real time PCR	Strains
[196, 197]	PCR	Strains
[198]	Microarray	Cervical/urethral swab
[88, 199]	Microarray	Strains
[200]	ICAN-hybrid chromatostrip	Strains
[201]	DHPLC	Strains

Study II is one of few studies of molecular susceptibility in *N. gonorrhoeae* made directly on the specimen, and urine must be considered by far the most interesting specimen. Gharizadeh et al. also used the pyrosequencing method, which should detect all variants of mutations, but they used strains and not clinical specimens [193].

The study by Li et al. was performed on urethral swab specimens and not on urine, and they do not specify what amino acid alterations they detected in position Asp95. Since the method of Li et al. was based on detection of changes in melting points, presumably the three mutants presented are not representatives for all different amino acid changes, since they have the same melting point [194]. Giles et al. have used a real time PCR; their study was done on strains and does not include all different variants of substitutions in position 95 [195]. Sultan et al. worked with strains and used a PCR mismatch amplification mutation assay that does not detect all variants of mutations correlating to ciprofloxacin resistance [196]. The early test developed by Deguchi et al. and presented in 1996 does not detect all variants; it was supposedly developed for research matters [197].

Booth et al., Ng et al. and Zhou et al. used different microarray methods [88, 198, 199]; the methods seemed to be accurate, but not suitable for clinical use. Only Zhou et al. used clinical specimens [198]. The ICAN-hybrid chromatostrip method used with strains method is not described in detail [200]. Shigemura et al. worked with strains and used a denaturing high-performance liquid chromatography (DHPLC) method, which seems sound [201].

A molecular susceptibility test will add valuable information to any commercial molecular method. It is crucial that the method can be used on clinical specimen types, including urine, and also detects all possible alterations in positions 91 and 95. Tanaka

et al. showed in an extensive material seven strains with alterations only at Ala67, Ala75 or Ala81 and MIC of ciprofloxacin 0.004 - 0.06 mg/L; however, the strains were presented in a group that also included strains with single mutations at positions 91 and 95. These alterations are not detectable with the pyrosequencing primers; however, they appear not to contribute to resistance to quinolones [96].

Tanaka et al. and Horii et al. have described strains with alterations in one of the positions Ser91 and Asp95 and MIC of ciprofloxacin ranging from 0.004 to 0.06 mg/L [96, 200]. In this aspect the molecular method might be superior to MIC determination, in predicting therapy failure in strains with alterations in GyrA. Avoiding ciprofloxacin treatment in these strains is probably one way to avoid development of further resistance.

In slowly growing species as *Mycobacterium* spp., molecular tests on strains may be useful in a clinical setting [183-185]. The tests for *E. coli* were probably developed for research purposes [186, 187]. However, in *N. gonorrhoeae* a molecular susceptibility test that can be used on clinical specimens will add valuable information to any commercial molecular test.

4.6 GYRA IN *NEISSERIA* SPECIES

Interestingly, the sequenced part of QRDR of gyrA in the *Neisseria* spp. examined in study IV did not seem to correlate to susceptibility to ciprofloxacin other species than *N. gonorrhoeae* and *N. meningitidis* [202-205]. This finding mirrors what Tanaka et al. describe as "surprising", that the region of the transpeptidase domain of PBP2 of a *N. gonorrhoeae* strain was similar to that in strains of *N. subflava*, *N. flavescens* and *N. sicca* [206]. It was proposed that genetic exchange of *penA* gene from *N. flavescens* to *N. lactamica* [190] and then from *N. lactamica* to *N. meningitidis* resulted in intermediate resistance to penicillin [190, 207]. A mosaic PBP2 composed of fragments from *N. cinerea* and *N. perflava* resulted in decreased susceptibilities to cephalosporins [208, 209].

The close relation between *N. lactamica* and *N. meningitidis*, but also between several *Neisseria* spp., was confirmed by MLST by Hanage et al. [56]. The results of study IV imply that a genetic exchange of QRDR of *gyrA* between *N. cinerea*, *N. elongata*, *N. mucosa* and *N. sicca* to *N. gonorrhoeae* could result in a ciprofloxacin resistant sequence variant in *N. gonorrhoeae*. Vázquez el al. found a meningococcus comprising a gonococcal PorB1b [210].

In study II, we found verification of *N. gonorrhoeae* in *gyrA* superior to verification in 16SrRNA. The results of sequencing *gyrA* in *Neisseria* spp. show that *gyrA* is probably not suitable for species verification of *N. gonorrhoeae*, but whether this method is feasible depends on the specificity of the primary commercial molecular method used. Other genes have been evaluated for verification, for example *cppB* [144]. This method was not considered suitable, because the plasmid was not present in all strains [8]. Probably confirmation in another part of the genome will remain the gold standard of molecular diagnostics for *N. gonorrhoeae*, at least until this is proven unnecessary.

N. gonorrhoeae strain FA1090 is the only gonococcal strain for which the entire genome has been sequenced, and is often used as a reference strain. This sequence has at least six genetic islands presumably acquired via transformation from different bacterial species [16], which complicates definition of a wild type. The genetic fuzziness of *Neisseria* spp. is certainly a challenge both when designing a molecular diagnostic test and in mechanisms of antibiotic resistance in *Neisseria* spp.

5 CONCLUSIONS

In *Neisseria* spp., only QRDR of *gyrA* in *N. gonorrhoeae* is a strong indicator of resistance to ciprofloxacin. The pyrosequencing method works with accuracy and is possible to perform on material from clinical specimens. QRDR of *gyrA* in *N. gonorrhoeae* is not a unique sequence and is therefore probably unsuitable for species verification.

An alteration in GyrA position 91 or 95 in *N. gonorrhoeae* correlates to MIC of ciprofloxacin of 0.03 - 0.13 mg/L and simultaneous presence of both alterations correlate to MIC of ciprofloxacin of 0.13 - 0.5 mg/L.

In addition to GyrA alterations in *N. gonorrhoeae,* a ParE Pro439 to Ser alteration probably contributes to resistance to ciprofloxacin.

Alterations in gonococcal porin PorB1b contribute to resistance to ciprofloxacin in *N. gonorrhoeae*, probably by decreasing the uptake.

The roles of both ParE and PorB in ciprofloxacin resistant *N. gonorrhoeae* need further investigation.

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