Neisseria gonorrhoeae: testing, typing and treatment in an era of increased antimicrobial resistance
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CHAPTER 1

Introduction
Neisseria gonorrhoeae – A brief history

Neisseria gonorrhoeae is probably as ancient as mankind; although *N. gonorrhoeae* was not yet recognized as the causative agent of gonorrhoea, the disease itself was already described in early literature from different parts of the world. The main symptom of gonorrhoea (mucopurulent discharge) and its association with disease were mentioned in the Old Testament, and by the Greek physician Galen of Pergamon. The latter is credited to have given the disease its name: gonorrhoea, which translates from ancient Greek to “flow of seed.” Not for several centuries did it become clear that the discharge is caused by leucocytes and not semen.

Like many other sexually transmitted infections (STIs), gonorrhoea has been linked to specific populations. Historically it was considered a disease of prostitutes, sailors, and soldiers. In the 14th century it was also known as “the clap”, named after Les Clapiers, an old red-light district in Paris. In 1879 Albert Neisser discovered that gonorrhoea was caused by a bacterial infection. This bacterium was subsequently named *Neisseria gonorrhoeae* after him. As part of the family of *Neisseriaceae*, it is related to *Neisseria meningitidis* (a major causative agent of meningitis), and to different commensal *Neisseria* species that reside in the throat. *Neisseria* species are fastidious, aerobic, and Gram-negative diplococci (round- or kidney-shaped bacteria, clustering in pairs). This specific shape of *N. gonorrhoeae* provided the alternative name “gonococcus”. It infects only human mucosa, and survival outside the body is very limited; it can be grown only in a strictly regulated environment of growth medium at 37°C and 5% CO₂. After an infection with *N. gonorrhoeae* no immunity for the disease occurs, and repeated infections are common among risk groups.

Epidemiology

Gonorrhoea is the second most common bacterial STI. The most recent estimates from 2012 suggested 78 million infections annually worldwide. In the Netherlands, the number of gonorrhoea diagnoses is estimated at more than 10,000 each year, and the incidence has increased since several years. Patients in the Netherlands are most often diagnosed by general practitioners (6,716 diagnoses estimated in 2014), or at designated STI clinics (5,391 in 2015). Gonorrhoea is in current times still strongly linked to populations with high-risk behaviour. The nationwide positivity rate recorded at STI clinics in 2015, was 10.7% among men who have sex with men (MSM), 6.2% among male or female swingers (sharing partners), and 3.3% among female commercial sex workers.
The STI clinic in Amsterdam is the largest STI clinic in the Netherlands, with around 40,000 annual consultations. The clinic is government funded, and provides free STI testing and treatment to high-risk populations, such as people under 25 years old, commercial sex workers, MSM, people with STI related complaints, or people who were notified of an STI by a sex partner. The most recent numbers from 2014, show 1,557 consultations that resulted in a gonorrhoea diagnosis. The positivity rate was 1.3% among all heterosexuals, and 11.4% among MSM. Because *N. gonorrhoeae* infects human mucosa, most infections are of urogenital origin. Due to changing sexual behaviour extra-genital infections of the rectum and the pharynx are increasingly common, especially among MSM. Ocular and systemic infections, such as gonococcal arthritis are rare. In addition to sexual transmission, vertical transmission from mother to child during delivery is possible. This can result in an infection of the eyes of the newborn: ophthalmia neonatorum.

**Disease, diagnosis, and antimicrobial susceptibility**

Depending on the anatomical site of infection, patients can experience a range of symptoms. Urogenital infections in men are symptomatic in more than 90% of cases. Reported symptoms are urethral discharge and dysuria, and start within a few days of infection. Urogenital infections in women are often asymptomatic. Less than 50% of infected women report vaginal discharge, and less than 25% report abdominal pain, dysuria or vaginal bleeding. Rectal and pharyngeal infections are also mostly asymptomatic. If symptoms do occur they are unspecific, and consist of abdominal discomfort or a sore throat. Historically, symptoms were the basis for a gonorrhoea diagnosis, a method that is still used widely in low-income countries without access to modern laboratory facilities. However, because infections are often asymptomatic, the symptoms themselves unspecific, or caused by a different infection, such as *Chlamydia trachomatis* or *Mycoplasma genitalium*, many will be missed or misdiagnosed using this syndromic approach.

Three main types of laboratory tests exist to diagnose gonorrhoea: direct microscopy using Gram-stained smears, culture, and molecular testing. Gram-stained smears are easy to perform, and give the best results (i.e. sensitivity ≥95%) in males when urethral discharge is present. A sample from the infected site is stained and examined under a microscope. Because *N. gonorrhoeae* is Gram-negative, the bacteria will appear as pink diplococci located inside polymorphonuclear leucocytes. However, other bacteria can resemble this morphology and misdiagnosis can occur. Rectal and pharyngeal
infections, and urogenital infections in females are usually less abundant in leukocytes and bacteria, and are less easily identified with this method.4

The second type of diagnostic is culture. This is also easy to perform, but requires more complex laboratory logistics. A patient sample is obtained and inoculated onto a selective growth medium, such as GC-agar, by a health care professional. Incubation is at 37°C and 5% CO₂ for approximately 48 hours, after which *N. gonorrhoeae* can be diagnosed by morphology, Gram-staining, and phenotypic or genotypic methods. The sensitivity of this method is estimated at around 80%, depending on the anatomical site of infection, and the occurrence of symptoms in the patient.16,17 A major advantage of this method is the possibility to determine antimicrobial susceptibility.

The susceptibility of *N. gonorrhoeae* to an antimicrobial drug is expressed as the minimum inhibitory concentration (MIC). This is the minimum concentration (in mg/L) of the drug that is sufficient to stop bacterial growth. The higher the MIC, the more antibiotic is needed, and thus the more resistant the bacterial strain is against the drug. At this moment, antimicrobial susceptibility in *N. gonorrhoeae* can only be determined if there is a successful culture, and this is usually performed by either of two methods.18 By using agar dilution, different concentrations of antimicrobial drugs are dissolved into growth medium (agar). The *N. gonorrhoeae* from culture are grown on these agar plates containing different concentrations of antibiotic, and after 24 hours the lowest concentration that inhibits growth can be determined.19,20 As agar dilution can be very labour intensive, a new method was developed. Using this method, *N. gonorrhoeae* from culture are inoculated on GC-agar plates, on which Etests are subsequently placed. Etests are paper or plastic strips, lined on one side with a concentration gradient of an antibiotic, and on the other side with a scale listing the concentrations. After incubation for 20–24 hours the minimum concentration that inhibits growth can be read from the scale. Both methods are used by different laboratories, and results can sometimes be slightly different.18 The MICs are checked against internationally published breakpoints. These breakpoints are predetermined MIC cutoff values that categorize strains into susceptible, intermediately resistant, or fully resistant.19,21

The third, most sensitive and recommended method is molecular testing using nucleic acid amplification tests (NAATs). Patient samples, consisting of either swabs or urine, are obtained by a health care professional or through self-collection.22 Samples are tested for the presence of specific parts of *N. gonorrhoeae* ribonucleic acid (RNA)
or deoxyribonucleic acid (DNA). This method is usually highly sensitive (>99%) and specific (>99%), even in asymptomatic infections, it is fast, and can handle many samples at once.\(^4,23\) Moreover, many NAATs can test for \emph{N. gonorrhoeae} and \emph{C. trachomatis} simultaneously. Depending on the test performed, cross-reaction with other \emph{Neisseria} species may occur. NAATs are costly and therefore not available in all settings, and most importantly, do not provide antimicrobial susceptibility data.

**Complications and antimicrobial management**

If left untreated, gonorrhoea can have serious sequelae. Complications in men consist of epididymitis or prostatitis.\(^12\) In women complications consist of pelvic inflammatory disease. In those cases the infection spreads to the ovarian tubes and ovaries, where scarring of the ovarian tubes can occur, causing infertility or ectopic pregnancy. Systemic infections, where \emph{N. gonorrhoeae} enters the bloodstream, such as in disseminated gonococcal infection (DGI), are rare (<1%) and more often seen among women than among men.\(^4,24\) DGI presents most often with septic arthritis, mainly of the knee, with skin lesions such as pustules or papules, or with tenosynovitis. Rarely it causes fever or meningitis.\(^12,24,25\) When neonates are infected during birth, they can develop ophthalmia neonatorum. This severe conjunctivitis can result in blindness of the newborn.\(^12\) In addition to systemic sequelae, \emph{N. gonorrhoeae} infections can increase the transmission of human immunodeficiency virus (HIV) in both men and women.\(^26,27\)

To prevent these complications of gonorrhoea, treatment with antimicrobial agents is necessary. Before the era of antibiotics, gonorrhoea was treated with remedies such as pepper, silver nitrate, or ‘irrigation’ of the urethra.\(^5,28-30\) In the 1930s, treatment with sulphonamides was initiated.\(^31\) In the early 1940s, the first antibiotic drug, penicillin (discovered by Alexander Fleming in 1928), was used to treat gonorrhoea on a large scale.\(^5\) Since then, due to the development of antimicrobial resistance, different antimicrobial drugs, such as spectinomycin, tetracycline, and ciprofloxacin, have become first-line treatment options for gonorrhoea. Finally, in the late 1990s third-generation cephalosporins, such as cefixime (for oral administration), cefotaxime, or ceftriaxone (both for intramuscular administration), were used as first-line treatment globally. In the 2000s, ceftriaxone as a single 500 mg dose was the recommended first-line drug to treat both genital and extra-genital gonorrhoea.\(^5\) Around 2012, several international guidelines changed their recommendations from monotherapy to a combination therapy of ceftriaxone (250–500 mg) plus azithromycin (1000–2000 mg).\(^3,32\) In the Netherlands, the currently recommended treatment is ceftriaxone 500
mg monotherapy. Azithromycin (a single 1000 mg dose) is added in case of suspected or proven urogenital coinfection with *Chlamydia trachomatis*, whereas doxycycline (100 mg twice daily for at least 7 days) is added for rectal chlamydia. In 2016, the World Health Organization (WHO) updated their *N. gonorrhoeae* treatment guidelines, and recommended dual therapy consisting of ceftriaxone 250 mg plus azithromycin 1000 mg. However, single therapy with ceftriaxone is also recommended, depending on the availability of local antimicrobial resistance data showing no resistance to this antibiotic.

**Antimicrobial resistance**

The reason all these different antibiotic drugs have been used since penicillin became available, lies in the development of antimicrobial resistance (AMR). Most bacteria have innate defence mechanisms to protect themselves against their environment so they can survive. Because most active components of antibiotics are substances that naturally occur in the environment (for instance the fungus producing penicillin as discovered by Fleming), bacteria can obtain resistance against antimicrobial drugs. *N. gonorrhoeae* has a remarkable capacity to develop resistance against many drugs, in a relatively short space of time. It can become resistant in different ways; through exchange of resistance genes with other *N. gonorrhoeae* strains, or with other *Neisseria* species, such as the commensals in the pharynx. In addition, specific mutations causing resistance can occur spontaneously, leading to improved survival in the presence of antibiotics.

Even before the use of penicillin, AMR against sulphonamides already occurred as a result of changes in the drug’s target on *N. gonorrhoeae*. Resistance to penicillin was acquired by *N. gonorrhoeae* on different levels. Like many other bacteria, *N. gonorrhoeae* can produce β-lactamase, an enzyme that can open the β-lactam ring of penicillin, which then loses its activity. In addition, mutations can occur in the genes (e.g. *penA* and *ponA*) coding for penicillin binding proteins (PBPs), and as a consequence, penicillin can no longer bind to *N. gonorrhoeae*. Lastly, mutations in the *mtrR* gene cause an up-regulation of the *mtrCDE*-efflux pump, and thereby increased efflux of penicillin (and also other antibiotics) out of *N. gonorrhoeae* bacteria. The first sign of AMR in *N. gonorrhoeae* was the continuous need of higher penicillin dosages to cure the infection. By the 1970s, high-level resistance to penicillin was spreading rapidly around the world, and penicillin turned obsolete for the treatment of gonorrhoea not long afterwards. Around the same time both tetracycline and spectinomycin were used in patients with penicillin allergy. Like for penicillin, AMR occurred within several years, and both
tetracycline and spectinomycine were no longer recommended by the late 1980s. Next in line was ciprofloxacin (a fluoroquinolone), but dosages had to be increased after approximately 10 years of use. Resistance spread fast, and by 2007 also ciprofloxacin was no longer recommended as a first-line drug for gonorrhoea.5

Another type of drug, azithromycin (a macrolide) is often used empirically in patients who are suspected of having an STI, in settings where no laboratory diagnostics are available. This practice of syndromic management is still used widely in low-income countries.9,13,14 Because azithromycin is effective against both gonorrhoea and chlamydia, this is often the drug of choice.35-37 However, already by the 1990s resistance of N. gonorrhoeae to azithromycin was reported.5,38 Azithromycin is active by binding to 23S rRNA of N. gonorrhoeae, and thereby blocking the protein synthesis and replication of bacteria. Different point mutations in the 23S rRNA gene (rrl) have been identified, and are associated with resistance to azithromycin. Some mutations (C2611 T) are associated with medium-level resistance, while others (A2058G and A2059G) are associated with high-level resistance.39,40 Every N. gonorrhoeae bacterium has four 23S rRNA alleles, and the number of mutated alleles is related to the level of resistance.39,40

Finally, extended-spectrum cephalosporins (ESC), were introduced. Especially the oral drug cefixime was recommended, due to the ease of administration. In the Netherlands, cefixime has never been widely available, and its use was therefore limited in this country. In the early 2000s, resistance and treatment failures to cefixime were reported, and by 2006 only the injectable drug ceftriaxone remained.1,5 In 2011, the first reports of resistance and treatment failures for ceftriaxone emerged.41-43 Ceftriaxone is a β-lactam antibiotic, like penicillin, and therefore can be rendered inactive by β-lactamase producing N. gonorrhoeae strains. Especially important in resistance to ESC are mutations of the penA gene, coding for PBP2. As a result of transferring genetic material between N. gonorrhoeae and commensal Neisseria species, a mosaic type of the penA gene has occurred.44 This mosaic gene is associated with increased MICs to both cefixime and ceftriaxone, leading in some strains to overt resistance against cefixime. Resistance to ceftriaxone has been described in a few strains due to the presence of additional point mutations, including at position A501.5,42 Since the first reports of ceftriaxone resistance, multi-resistant strains have also been isolated.45,46

As mentioned before, to halt resistance international guidelines abandoned monotherapy and recommend dual therapy of ceftriaxone and azithromycin.4,9,32
Some reports suggested synergy between the drugs, and others suggested that in case of resistance at least one of the two would be sufficient. However, resistance to either of those drugs is increasing. Furthermore, in the United Kingdom an epidemic of high-level azithromycin resistance emerged in 2015, despite the use of dual therapy. Moreover, in 2016 the first treatment failure of dual therapy has been reported.

These developments do not bode well for the future treatment of gonorrhoea. The WHO advises to refrain from using an antimicrobial drug as first-line treatment, if more than 5% of isolates in the population are resistant to that specific drug. In some countries this limit has already been reached for ceftriaxone (Vietnam), or for azithromycin (Vietnam and Hungary). If ceftriaxone can no longer be recommended, and resistance is not halted by dual therapy, there are very few options left. The number of expected new antimicrobial drugs is extremely limited, and many existing drugs have already been used, or are evidently not effective. Therefore AMR in *N. gonorrhoeae* should be high on the medical scientific agenda. New methods are needed in a number of fields. We need better diagnostic methods that can determine AMR, and improved surveillance of resistance globally. Most importantly, alternative therapies are vital to ensure the treatment of future patients with gonorrhoea. To aid in these goals, we performed several studies, which are described in this thesis.

**Outline of this thesis**

In part I of this thesis our efforts to improve diagnostics of gonorrhoea are described. **Chapter 2** provides a new method to combine NAAT with targeted deferred culture. Using this method, it is possible to determine AMR by culture in settings that do not have the required laboratory logistics. **Chapter 3** provides unique data on the time from treatment to clearance of RNA and DNA of *N. gonorrhoeae*. This information is highly useful to policy makers and clinicians to determine if treatment failures have occurred. **Chapter 4** reports on a subset of patients coinfected with *N. gonorrhoeae* and *C. trachomatis* from **Chapter 3**. It provides unique data on the time to clearance of RNA and DNA of *C. trachomatis* in patients coinfected with gonorrhoea. This is important because recommended gonorrhoea dual therapy includes azithromycin, which is also recommended for urogenital chlamydia. Although no resistance of *C. trachomatis* has been reported yet, some concern exists about the effectiveness of azithromycin. In addition, many patients with gonorrhoea are coinfected with chlamydia, and most NAATs provide results for both *N. gonorrhoeae* and *C. trachomatis*. 
Part II focuses on AMR, and specifically on the components of dual therapy: azithromycin and ceftriaxone. Chapter 5 shows the trends of AMR in Amsterdam from 2012 through 2015, and determinants associated with decreased susceptibility to azithromycin or ceftriaxone. Chapter 6 describes the lack of synergy in many different antimicrobial dual combinations, especially for azithromycin and ceftriaxone. In Chapter 7 we report on the effect of recent exposure to azithromycin of patients with *N. gonorrhoeae*, in regard to the susceptibility to azithromycin. Chapter 8 shows the genetic epidemiology, molecular markers of resistance, and clustering of *N. gonorrhoeae* isolates that are either susceptible or resistant to azithromycin.

In Chapter 9 the results of the studies in this thesis are compared to recent literature, and their added value is discussed. In addition, recommendations for future research and management regarding *N. gonorrhoeae* are made.
REFERENCES


Chapter 1