Molecular diagnostics and characterization of *Neisseria gonorrhoeae*

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1 Preface
The study was conducted primarily at the Department of Microbiology and Infection Control at the University Hospital of North Norway, Tromso, Norway. Patient samples were collected at Olafsklinikken in Oslo, Norway for paper II and paper III. For paper IV, patient samples were collected at different laboratories in Norway. Some analyses were performed at the Swedish Reference Laboratory for Pathogenic Neisseria, Örebro, Sweden for paper IV as well as discrepancy analysis for paper II.

The study was initiated to ensure appropriate diagnostics of *N. gonorrhoeae* in Northern Norway where general practitioners commonly treat patients for sexually transmitted infections and times for transportation of samples can be long.
2 Acknowledgements
I want to thank my inspirational, supportive and skilful co-workers at the Department of Microbiology and Infection control, University Hospital of North Norway. You make it interesting and enjoyable to come to work.

My supervisors Vegard Skogen, Magnus Unemo and Johanna Ulirica Ericson Sollid, get my sincere admiration for their patience and willingness to share of their vast wisdom with me. Vegard has been a guide and support in life, science and work-ethics, and for this I am eternally grateful. The research experience you have given me during this project goes far beyond gonorrhoea diagnostics. Magnus, it has been an honour to work and have fun with you. Thanks to Johanna for believing in me and guiding me through the writing of my thesis.

I thank all my friends for having the patients to listening to my preaching about sexually transmitted infections, and your support (Somehow my work is beneficial for you too.....). Thanks also for providing me with alternative activities such as floor ball, skiing, social activities and motorcycle adventures. This has been important for my mental health. Special thanks to Håkon Haaheim, for believing in me and continue to hire me – most recently as CEO in our joint adventure.

To my good friends in Forskerforbundet I am grateful for their support, good spirit and common believe in Science and Higher education and their relentless battle for this idea.

I thank my collaborating partners at hospitals and laboratories that contributed to my research, without them I would not have been able to do this research. Harald Moi at Olafisklinikken has been especially helpful for paper II and III, but also as a general source of knowledge – Thank you. I am also deeply grateful to the patients who in a vulnerable point in their life, agreed to be part of our studies.
Most of all, I need to thank my family for allowing me to spend more time doing my research than I ought to. My oldest son, Sondre was the reason I got my Masters degree. Sebastian, my youngest son, has been an inspiration and support for me during the work on this thesis. You are two very smart boys, and you can do or be anything you want to. I have seen you both excel at academic and physical challenges, be it at school, biking, motor crossing or snowmobiling.

I also like to thank my wife, Heidi. I was always very proud of you, but lately you have really shown the enormous strength that lies within you – good luck on your master-degree, and teaching career. I envy the kids who will have you for a teacher, the noblest of professions. I also like to thank my parents, who supported most my endeavours growing up. I am sorry that my father never got to see me finish my thesis - you are greatly missed.

Stig Ove Hjelmevoll

Tromsø, October 2011
3 List of papers


4 List of Abbreviations

AIDS – acquired immune deficiency syndrome
AMR – antimicrobial resistance
AMS – antimicrobial susceptibility
BHQ – black hole quencher
BP – base pairs
CAH – carbonic anhydrase
CARE – Core facility for Automated Real-Time PCR & Extraction
CDC – Centers for Disease Control and Prevention
DCMG – site-specific DNA-methyltransferase (cytosine-specific)
NgoVII
DFA – direct fluorescent antibody
DGI – disseminated gonococcal infection
DNA – deoxyribonucleic acid
DUS – DNA uptake sequence
EIA – enzyme immunoassay
ESC – extended-spectrum cephalosporin
EUCAST – The European Committee on Antimicrobial Susceptibility Testing
FDA – U.S Food and Drug Administration
FP – false positive
HIV – human immunodeficiency virus
IAC – internal amplification control
IUSTI – International Union against Sexually Transmitted Infections
JD1 – unknown protein on cryptic plasmid PJD1
LCR – ligase chain reaction
LOS – lipoooligosaccharides
LPS – lipopolysaccharides
MDR – multidrug resistant
MSIS – Norwegian surveillance system for communicable diseases
MSM – men who have sex with men
NAAT - nucleic acid amplification test
NGO – B protein on cryptic plasmid PJD1
NPV – negative predictive value
NG-MAST – *N. gonorrhoeae* multiantigen sequence typing
nM – nano molar
Opa – opacity protein
ORF – open reading frame
PCR – polymerase chain reaction reaction
PID – pelvic inflammatory disease
POC – point of care
*porA* – porin A gene (silent)
*porB* – porin B gene
PPNG – penicillinase (β-lactamase) producing *N. gonorrhoeae*
PPV – positive predictive value
QRNG – quinolone resistant *N. gonorrhoeae*
RNA – ribonucleic acid
rRNA – ribosomal ribonucleic acid
SDA – strand displacement amplification
ST – sequence type
STI – Sexually Transmitted Infections
TMA – transcription mediated amplification
TOC – test of cure
TP – true positive
TraG – conjugal coupling protein
TraH – conjugative relaxosome accessory transposon protein
UTM-RT – universal transport medium – room temperature
WHO – World Health Organization
WW1 – World War One
WW2 – World War Two
5 Introduction
Sexually transmitted infections (STIs) are a global burden (1-3), with increasing number of new infections. Gonorrhoeae is the second most prevalent bacterial STI with a global estimated incidence of 88 million people annually (2005) (1,4,5). Effective condom use, diagnosis and timely adequate treatment of patients and their sexual contacts are the best available means to prevent spread of the infection in the absence of a vaccine.

Unfortunately effective treatment is getting increasingly problematic due to rapidly developing antimicrobial resistance, and gonorrhoea may soon become untreatable in certain circumstances (6-8). The gold standard for diagnosing gonococcal infections has for many decades been culture, which however has limited sensitivity, in particular, in samples from rectum and pharynx. These extra-genital sites are also more problematic to treat, may commonly be asymptomatic, and act as reservoir for infection even in apparently successfully treated patients (9-12). Extra-genital samples can on the other hand be diagnosed using nucleic acid amplification test (NAAT) with improved sensitivity, but historically with poor specificity due to closely related commensal Neisseria species or Neisseria meningitidis.

Historical background
Ancient Chinese, Egyptian, Greek and Roman literature as well as the Bible, all describe symptoms related to gonorrhoea (13), and the term gonorrhoea stems from ancient Greek and was first used by Galen in 130 A.D, meaning flow of seed. Albert Neisser was the first to identify the aetiological agent of gonorrhoeae in 1879 in microscopy of stained smears from vaginal, urethral and conjunctival exudates (14) and termed it Micrococcus gonorrhoeae. Many other terms have historically been used for Neisseria gonorrhoeae; Gonococcus neisseri, Diplococcus gonorrhoeae, Micrococcus gonococcus, Micrococcus gonorrhoeae, Merismopedia gonorrhoeae, Micrococcus der gonorrhoe, Gonococcus neisseri (Lindau, 1898), Diplococcus gonorrhoeae (15,16) and Micrococcus gonococcus (Schroeter, 1886).
Prior to introduction of effective antimicrobial treatment of *N. gonorrhoeae*, urethral irrigation, abstinence from alcohol & sexual activity, rest and systemic treatment with various balsams were common treatment regimens (17). During World War One (WW1), prophylactic packets were handed out to soldiers, containing condoms, calomel ointment and Argyrol (18).

Leistikow and Loeffler (Leistikow, 1882) successfully cultured *N. gonorrhoeae* and with the introduction of Gram staining (Gram, 1884), and carbohydrate oxidation test (Elser and Huntoon, 1909), diagnosis of gonorrhoea improved significantly. The discovery of sulphonamides for treatment of gonorrhoea (17), and later the discovery of penicillin (19,20) gave hope of eradicating gonorrhoea. As it turned out, *N. gonorrhoeae* has a remarkable ability to adapt to and survive any antimicrobial treatment, and continues as a major STI and public health concern worldwide. New phenotypic and genotypic knowledge about *N. gonorrhoeae* has elucidated the bacterium’s effective ability to evade the host immune system and environmental threats like antimicrobials. Gonorrhoea is here to stay.

**The bacterium**

The Neisseriaceae family are beta-proteobacteria which consists of Gram-negative aerobic bacteria from thirty genera including *Neisseria, Eikenella*, and *Kingella* (22). *N. gonorrhoeae* and *N. meningitidis* are the human pathogenic members of the Neisseria genus, which further includes several human and animal commensals. Commensal *Neisseria* and *N. meningitidis* are frequently part of the normal flora of especially the human oro- and nasopharynx (see table 1 for a list of Neisseria species). All *Neisseria* species are non-motile, (despite some twitching motility, using their pili), aerobic, capnophilic, non-sporeulating and inhabit the mucous membrane surface of warm blooded hosts (23). The bacteria are coccoidally shaped with exception of *N. elongate* and approximately 0.6 to 1.0 µm in diameter. They are usually seen in pairs with adjacent flattened sides, which gives them a characteristic kidney appearance in microscopy. *N. gonorrhoeae* is frequently found intracellular in polymorphonuclear leukocytes
(neutrophil granulocytes) of the gonorrhoea pustular exudate. It possesses a typical Gram-negative outer membrane composed of proteins, phospholipids, and lipopolysaccharide (LPS). However, the neisserial LPS, with its highly branched basal oligosaccharide structure and the absence of repeating O-antigen subunits, differs from enteric bacteria. Because of these differences, neisserial LPS is referred to as lipo-O-oligosaccharide (LOOS). *N. gonorrhoeae* is a fragile organism, susceptible to temperature changes, desiccation, UV-light, and many other environmental conditions (24). This contributes to the reduced sensitivity of culture diagnostics in comparison to NAATs as the sampling conditions and transport conditions (temperature and time) substantially affect the viability of gonococci.

Despite being genetically similar, with 80-90% sequence homology in the genomic deoxyribonucleic acid (DNA), major cellular and molecular differences exist between the two pathogenic neisserial species. Both pathogens can colonize a variety of body sites and produce different symptoms. However, while *N. meningitidis* infections predominantly cause meningitis or septicaemia with a high mortality and low prevalence, *N. gonorrhoeae* typically causes urethritis and cervicitis with a high prevalence, but low mortality. *N. meningitidis* possesses a polysaccharide capsule which is not expressed by *N. gonorrhoeae* (25), and *N. gonorrhoeae* has up to 12 opacity (Opa) proteins, whereas *N. meningitidis* has three to four Opa proteins (26-29). *N. meningitidis* expresses two different porins (30-33); PorA and PorB, while *N. gonorrhoeae* only expresses PorB. The *porA* gene was long thought to be exclusive to *N. meningitidis* and forms the basis for genosubtyping of *N. meningitidis* (34). However, the gene was identified in *N. gonorrhoeae* (31,35), but a frame shift mutation in the coding region of the gene abolishes its expression (31,35). Nevertheless, this gonococcal *porA* pseudogene is enough different from the meningococcal *porA* gene for using it as specific target in genetic diagnostics of *N. gonorrhoeae*. The PorA protein is accordingly not essential for colonising the urogenital tract, and may even be disadvantageous. Hence the loss of expression of *porA* has
been suggested to reflect a step in the divergence into the two separate species of pathogenic *Neisseria* (31).

The *Neisseria* species are very promiscuous and readily exchanges DNA with its surroundings. They harbour DNA uptake sequences (DUS), which are required for efficient natural genetic transformation (36), surrounding and within coding regions of their genes. DUS are typically 9 – 10mer sequences, and ~1900 are scattered throughout the Neisseria genome (36-39). The highest density of DUS is found within and in close proximity to genes involved in DNA repair, recombination, restriction modification and replication. *N. gonorrhoeae* are naturally competent in all phases of the growth cycle resulting in high frequency of horizontal transfer of genetic material between *N. gonorrhoeae* strains, and other *Neisseria* species (40-45).

Mutations and homologous recombination’s also contribute to the genetic heterogeneity of *N. gonorrhoeae* (29,46-53). Most recombination will be conservative in its nature, and thus it is primarily a mechanism for genome repair and conservation. However recombination can occasionally give rise to diversity, some of which are beneficial to the bacterium.

A high degree of recombination between chromosomal genetic loci causes antigenic variability, a key feature of panmictic or non-clonal pathogens (54-56). The resulting high level of genotypic variability (incorporation of new genetic material acquired, in particular, by transformation) and phenotypic variability (differential expression of existing parts of the genome) is important for evasion or adaptation to the immune response of the host. This also aids the development of, or spread of antibiotic resistance mechanisms.

Combined with *N. gonorrhoeae*’s ability to produce mildly symptomatic or asymptomatic infection, the high level of genotypic and phenotypic variability also aids the bacteria in persistence without severely damaging the host.
Table 1. Different Neisseria species, their natural hosts and known pathogenic status.

<table>
<thead>
<tr>
<th>Commensal</th>
<th>Human host (22)</th>
<th>Other mammal host (57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. weaver</td>
<td>N. canis (Cat)</td>
<td>N. denitrificans (Guinea pig)</td>
</tr>
<tr>
<td>N. lactamica</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. polysaccharea</td>
<td>N. weaverii (Dog)</td>
<td></td>
</tr>
<tr>
<td>N. cinerea</td>
<td>N. iguanae (Iguanid lizards)</td>
<td></td>
</tr>
<tr>
<td>N. flavescens</td>
<td>N. ovis (Cattle)</td>
<td></td>
</tr>
<tr>
<td>N. sicca</td>
<td>N. caviae (Guinea pig)</td>
<td></td>
</tr>
<tr>
<td>N. mucosa</td>
<td>N. cuniculi (Rabbit)</td>
<td></td>
</tr>
<tr>
<td>N. baciliformis</td>
<td>N. macacae (Rhesus monkey)</td>
<td></td>
</tr>
<tr>
<td>N. subflava</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Biovar subflava</td>
<td></td>
<td>N. animalis</td>
</tr>
<tr>
<td>• Biovar flava</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Biovar perflava</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. elongate</td>
<td>N. dentiae (Cows)</td>
<td></td>
</tr>
<tr>
<td>Subspecies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• N. elongata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• N. glycolytica</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• N. nitroreducens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathogenic</td>
<td>N. gonorrhoeae</td>
<td></td>
</tr>
<tr>
<td>Subspecies</td>
<td>N. kochii</td>
<td></td>
</tr>
<tr>
<td>N. meningitidis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Clinical manifestation – symptoms**

For adults, *N. gonorrhoeae* infections are primarily contracted through sexual contact. The main infection-sites are urethral mucous membranes in men and the endocervix and urethra in women, but the oropharynx, conjunctiva, and rectum can also be infected. Transmission to neonates during birth can cause conjunctivitis (58).

The incubation period is typically 1-7 days, even though it may vary. *N. gonorrhoeae*
infections have a variety of clinical presentations, all commonly also caused by other organisms. The infection gonorrhoea is, if symptomatic, usually attributed as a specific type of urethritis/cervicitis resulting in copious discharge of pus, more apparent in men than women. The gonococcal infection may however infect also other tissues such as mucosa in anorectal tract, oropharynx and conjunctiva. In men, untreated gonococcal infections may lead to urethral stricture and epididymitis, and ultimately infertility. In women, the infections are more often asymptomatic (50%) than for men (10-20%) and mostly result in symptoms such as dysuria, vaginal discharge and sometimes irregular bleeding. If left untreated the fallopian tubes and uterus can get infected, with further complications such as pelvic inflammatory disease (PID), ectopic pregnancy and infertility.

For Human immunodeficiency virus (HIV) positives, a gonococcal infection may also lead to dramatically increased shedding and accordingly transmission of HIV (59), probably through an increase of the viral load in the semen (60) or cervico-vaginal fluids (60,61). An underlying N. gonorrhoeae infection or other symptomatic STI in the recipient may also cause elevated number of CD4 lymphocytes to be available for the HIV (62).

Disseminated gonococcal infection (DGI) is a rare complication, were a systemic spread of the bacteria in the bloodstream may cause, e.g., dermatitis, arthritis, septicaemia, endocarditis, and meningitis. Only 0.5-3% of infected individuals develops DGI (63), and a combination of certain strains of N. gonorrhoeae and individual’s deficient in, for example, complement factors C7, C8 and C9 appear to be predisposing (64).

**Epidemiology**

Gonorrhoea remains a major STI worldwide, and in some countries it is as prevalent as Chlamydia trachomatis (1,4,5). In 1999, the global incidence of gonorrhoea was estimated at
62 million cases (65). In 2005 the estimates were 88 million cases (1). This increase may not all be indicative of a true rise in prevalence, because new optimised models and algorithms were used for the 2005 estimates. Accordingly, the 2005 estimates are believed to me more accurate than the 1999 estimates and clearly the incidence remains high in many less-resourced countries and also increasing in several developed, industrialised countries. In Norway, compared to previous years there was a substantial increase in the number of diagnosed \textit{N. gonorrhoeae} cases in 2010 (n=411) due to the increased use of the NAAT presented in this thesis. This increase was predominantly due to the diagnosis of more than twice the number of pharyngeal and rectal gonorrhoea among men who have sex with men (MSM) patients diagnosed in the capital city of Norway – Oslo (www.msis.no).
Figure 1. Incidence of *N. gonorrhoeae* in Norway from 1922 to 2010 (number of new infections per 100 000 population). Figures for 2011, are not ready when this thesis is submitted, by September 237 gonorrhoeae cases were reported.
Table 2. Epidemiology of *N. gonorrhoeae* diagnosed in Norway past 10 years.

*In this table, other indicates that patients have not reported sexual preference.*

<table>
<thead>
<tr>
<th>Year</th>
<th>Domestically contracted</th>
<th>Contracted abroad</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homo</td>
<td>Hetero</td>
<td>Mother Child</td>
</tr>
<tr>
<td>2010</td>
<td>176</td>
<td>80</td>
<td>1</td>
</tr>
<tr>
<td>2009</td>
<td>84</td>
<td>74</td>
<td>1</td>
</tr>
<tr>
<td>2008</td>
<td>76</td>
<td>105</td>
<td>-</td>
</tr>
<tr>
<td>2007</td>
<td>66</td>
<td>68</td>
<td>-</td>
</tr>
<tr>
<td>2006</td>
<td>58</td>
<td>78</td>
<td>-</td>
</tr>
<tr>
<td>2005</td>
<td>66</td>
<td>98</td>
<td>-</td>
</tr>
<tr>
<td>2004</td>
<td>96</td>
<td>74</td>
<td>-</td>
</tr>
<tr>
<td>2003</td>
<td>55</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>2002</td>
<td>70</td>
<td>85</td>
<td>-</td>
</tr>
<tr>
<td>2001</td>
<td>54</td>
<td>192</td>
<td>-</td>
</tr>
<tr>
<td>2000</td>
<td>59</td>
<td>101</td>
<td>-</td>
</tr>
</tbody>
</table>

Gonococcal infections have been voluntarily recorded in Norway since 1922, and mandatory from 1975. Historically, reported clinical infections in Norway show three major peaks, i.e. one after WW1, one after WW2 and one corresponding to the sexual revolution that started in the 1960ies. Only Sweden has longer recorded historical data than Norway and has reported similar data. The decline starting in the late 1970s in Norway is similar as the ones reported in the rest of the Scandinavian countries and the USA (66) and was probably due to the decreased size of the 18- to 24-year age group, changed sexual behaviour, improved diagnostic methods, adequate antibiotic treatment, effective contact tracing and fear of HIV and acquired immunodeficiency syndrome (AIDS) from the mid-1980s and onward (66,67).
However, it is important to keep in mind that the incidences are influenced by several factors like patterns of sexual behaviour, population demographics, economic and social conditions, and biases in the quality and quantity of the epidemiological data (67,68).

**Antimicrobial treatment & resistance**

*N. gonorrhoeae* is natively susceptible to many antimicrobials such as sulphonamides, penicillins, tetracyclines, aminoglycolides, macrolides, cephalosporins, and fluoroquinolones. The choice of antimicrobial agent depends on site of infection and susceptibility of the bacteria. Pharyngeal infections are more difficult to treat than urogenital infections, due to different bioavailability of the drug (69). Currently extended-spectrum cephalosporins (ESCs) are the most widely recommended antimicrobials for treatment of gonorrhoea, either as a single oral dose or injected dose (intramuscularly most common).

The promiscuous nature (ease of acquisition of resistance genes from other bacteria) and relatively high mutational rate in many genes encoding resistance determinants of the bacteria has resulted in a global increase in antimicrobial resistance (AMR) in *N. gonorrhoeae*. An effective AMR surveillance program is essential to optimize standard treatments. In numerous countries, including many with high disease rates, such surveillance is often lacking or of a poor quality (6).

The introduction of sulphonamides as an effective treatment for gonorrhoea, soon led to resistance (70). However, penicillin was already in 1943 shown to be an effective drug for treatment of the widely spread sulphonamide-resistant *N. gonorrhoeae* strains (70). Penicillins became the drugs of choice, and a slow but steady rise of chromosomally-mediated decreased susceptibility led to increased doses during several decades (71). Penicillinase producing *N. gonorrhoeae* (PPNG; causing plasmid-mediated penicillin resistance) spread rapidly after they were first described in 1976 (72). The spread of PPNG resulted in increased use of tetracycline,
and later quinolones in many countries, and both tetracycline resistant and quinolone (e.g., ciprofloxacin and ofloxacin) resistant (QRNG) *N. gonorrhoeae* are now widespread and these drugs are therefore not recommended for first-line treatment (73,74).

ESCs are now the most widely used drugs for treatment of gonorrhoea. However, during the recent decade there have been numerous reports of decreasing *in vitro* susceptibility worldwide and, most recently, also verified treatment failures with orally administered ESCs (cefixime) in Japan, Norway and United Kingdom (69,70,75-78); Futhermore, the reported cases of treatment failures with cefixime, are unfortunately caused by MDR-NG (multidrug resistant *N. gonorrhoeae*) with decreased susceptibility or resistance to, e.g., quinolones, azithromycin and penicillins as well. The susceptibility to ceftriaxone has the last decade also decreased worldwide and recently a case of clinical failure treating pharyngeal gonorrhoea was described in Europe (7). Worryingly, most recently the first gonococcal strain with high-level resistance to ceftriaxone (2-4 mg/L), related to a treatment failure, was found in Kyoto, Japan and characterised in detail (8). This calls for more frequent follow up and test of cure (TOC) worldwide, especially with lack of regular AMR surveillance in many countries that also needs to be substantially strengthen, increasing use of NAAT diagnostics and strictly receptive oral- and/or rectal sex in subpopulations of MSM.

So far there are no other reports on treatment failure for intravenously administered ESCs such as ceftriaxone. Consequently all cases of genital and pharyngeal gonorrhoea are now treated with high dose of intravenous ceftriaxone in Japan (75). The emergence of MDR-NG with ESC resistance in the Pacific Rim is thought to spread rapidly throughout the rest of the world in light of past experience with similar pattern of spread (6).

The competent nature of *N. gonorrhoeae* and proximity to other closely related *Neisseria* species in the pharynx, treatment difficulties and predominantly asymptomatic infection in the
pharynx (69) and incorrect use of antimicrobial treatment, make the pharynx a possible hotspot for emergence of new types of resistance as well as a reservoir for infection and its further spread (9). Commensal *Neisseria* commonly found in the pharynx, may also acquire resistance coding genetic elements from other bacteria colonizing the pharynx and later spread this to *N. gonorrhoeae*. 
Table 3. Antimicrobial classes, mechanisms of action and genetic location of resistance for antimicrobials commonly used for treating gonorrhoea. The table is a simplified and updated reprint from Lewis DA, 2010 (18).

<table>
<thead>
<tr>
<th>Antimicrobial class</th>
<th>Antimicrobial</th>
<th>Mechanism of action</th>
<th>Chromosomal or plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphonamides</td>
<td>Trimethoprim/sulpha-methoxazole</td>
<td>Inhibition of folic acid synthesis</td>
<td>Chromosomally-mediated</td>
</tr>
<tr>
<td>Penicillin</td>
<td>Ampicillin, Amoxicillin, Penicillin G</td>
<td>Inhibition of cell wall synthesis</td>
<td>Chromosomally-mediated or Plasmid-mediated</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Cefotaxime, Cefixime, Ceftriaxone</td>
<td>Inhibition of cell wall synthesis</td>
<td>Chromosomally-mediated</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Doxycycline, Tetracycline</td>
<td>Inhibition of protein synthesis</td>
<td>Chromosomally-mediated or Plasmid-mediated</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Spectinomycin, Kanamycin, Gentamicin</td>
<td>Inhibition of protein synthesis</td>
<td>Chromosomally-mediated</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Erythromycin, Azithromycin</td>
<td>Inhibition of protein synthesis</td>
<td>Chromosomally-mediated</td>
</tr>
<tr>
<td>Fluroquinolones</td>
<td>Ofloxacin, Ciprofloxacin</td>
<td>Inhibition of nucleic acid synthesis (supercoiling)</td>
<td>Chromosomally-mediated</td>
</tr>
</tbody>
</table>

*Neisseria gonorrhoeae sampling and diagnostics*

5.1.1 Sampling

Samples for *N. gonorrhoeae* diagnostics may be taken by urethral swab, urine, cervical swab, vaginal swab, rectal swab, pharyngeal swab, conjunctival swab and in rare DGI cases from skin wounds, synovial fluid and blood. Different transport medium are traditionally used for culture diagnostics than for NAATs. Copan has however developed a new transport medium, the M40,
which is a liquid transport medium suitable for both culture and NAAT diagnostics.

5.1.2 Diagnostic challenges

The antigenic variability of the gonococcus is a survival mechanism in this one-host bacterium and has contributed to the difficulties to produce an effective vaccine. Maintaining effective diagnostics is therefore important in controlling the spread of *N. gonorrhoeae*. The ideal diagnostic test would be cheap, rapid, non-invasive, and have 100% sensitivity and specificity while also providing an AMR profile. Such a test does not exist despite decades of research and development efforts and a US$ 1 million reward offered by the Rockefeller Foundation in 1995, which was later withdrawn. At present, culture diagnostics can be considered as the most complete test as it can provide an AMR profile in addition to detection. On the other hand, the sensitivity of culture diagnostics is largely dependent on sampling, transportation of samples and culture procedures. In addition sample types like pharyngeal swabs and rectal swabs frequently contain large quantity of other bacterial species, which outcompete the *N. gonorrhoeae* on the agar plate. In a study by Bachmann et al (79), culture diagnostics had a sensitivity of 50-65% compared to NAATs.

Microscopy is a fast, cheap and reliable diagnostic method in skilled hands, but only for urethral swabs from symptomatic men. The low sensitivity in samples from asymptomatic men, cervix, rectum and pharynx makes microscopy time-consuming and expensive resulting in only presumptive positive results (80), because a negative result cannot exclude infection. Culture diagnostics has for long been the gold standard method for diagnosing *N. gonorrhoeae*. This is largely because it has exceedingly high specificity, and can provide a measure of AMR. It is performed on selective agar medium but still requires subsequent species-verifying test to confirm that it is *N. gonorrhoeae*. This method is time consuming and even under optimal conditions cultures has a relative low sensitivity compared to NAATs. This is due to
overgrowth of other bacteria (especially in pharyngeal and rectal samples) and low viability of the bacteria outside the host.

NAATs are very sensitive and work well in any sample type used (i.e. urine, rectal swabs, pharyngeal swabs, urethral swabs, cervical swabs, and conjunctival). By using NAATs, AMR testing is not possible and hence assessing treatment outcome is hard to do, especially for patients not presenting symptoms upon initiation of treatment. The genetic relatedness between \(N.\ gonorrhoeae\), \(N.\ meningitidis\) and the commensal Neisseria species, coupled with extensive exchange of genetic elements, make it difficult to find DNA sequences conserved and unique for \(N.\ gonorrhoeae\). For this reason, specificity is a major concern, especially in extra-genital specimens. Many Neisseria species are genetically very similar and exchange DNA promiscuously, making molecular assays prone to reduced specificity (81). Both commercial and in-house NAAT assays have documented specificity problems (table 4).
Table 4. Select Nucleic acid amplification tests (NAATs), their target regions used for detection of *N. gonorrhoeae* and cross-reactions.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Assay Name</th>
<th>Amplification Technology</th>
<th>Gene Target</th>
<th>Reported cross reaction*&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbott</td>
<td>RealTime CT/NG Test</td>
<td>Real-time PCR</td>
<td>Opa (multi-copy)</td>
<td><em>N. meningitidis</em>&lt;sup&gt;b&lt;/sup&gt;, <em>N. Mucosae</em>&lt;sup&gt;d&lt;/sup&gt; Both unconfirmed.</td>
</tr>
<tr>
<td>Becton Dickinson</td>
<td>ProbeTec GC Qx Amplified DNA assay</td>
<td>Strand displacement amplification (SDA)</td>
<td>Pilin (multi-copy; different sequence from BD ProbeTEC ET)</td>
<td><em>N. cinerea</em>&lt;sup&gt;a,b&lt;/sup&gt;, <em>N. lactamica</em>&lt;sup&gt;a,b&lt;/sup&gt;, <em>N. sicca</em>&lt;sup&gt;a&lt;/sup&gt;, <em>N. flavescens</em>&lt;sup&gt;b&lt;/sup&gt;, <em>N. meningitidis</em>&lt;sup&gt;b&lt;/sup&gt;, <em>N. mucosa</em>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gen-Probe</td>
<td>Aptima Combo 2 (AC2)</td>
<td>Transcription mediated amplification</td>
<td>16S rRNA (multi-copy)</td>
<td><em>N. meningitidis</em>&lt;sup&gt;b&lt;/sup&gt;, <em>N. sicca</em>&lt;sup&gt;b&lt;/sup&gt; Both unconfirmed.</td>
</tr>
<tr>
<td>Gen-Probe</td>
<td>Aptima GC</td>
<td>Transcription mediated amplification</td>
<td>16S rRNA (different to AC2 target)</td>
<td><em>N. meningitidis</em>&lt;sup&gt;b&lt;/sup&gt;,</td>
</tr>
<tr>
<td>Becton Dickinson</td>
<td>COBAS AMPLICOR CT/NG TEST</td>
<td>PCR with endpoint detection</td>
<td>Cytosine DNA methyltransferase (single-copy)</td>
<td><em>N. cinerea</em>&lt;sup&gt;a,b&lt;/sup&gt;, <em>N. lactamica</em>&lt;sup&gt;a,b&lt;/sup&gt;, <em>N. subflava</em>&lt;sup&gt;a,b&lt;/sup&gt;, <em>N. sicca</em>&lt;sup&gt;b&lt;/sup&gt;, <em>N. polysaccharae</em>&lt;sup&gt;a&lt;/sup&gt;, <em>N. pharyngis</em>&lt;sup&gt;b&lt;/sup&gt;, <em>N. meningitidis</em>&lt;sup&gt;a&lt;/sup&gt;, <em>N. caviae</em>&lt;sup&gt;b&lt;/sup&gt;, <em>N. animalis</em>&lt;sup&gt;b&lt;/sup&gt;, <em>Moraxella catarrhalis</em>&lt;sup&gt;b&lt;/sup&gt;, <em>M. osloensis</em>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Roche</td>
<td>COBAS 4800 CT/NG Test</td>
<td>Real-time PCR</td>
<td>Direct repeat region DR9 (multi-copy)</td>
<td><em>N. lactamica</em>&lt;sup&gt;b&lt;/sup&gt;, <em>N. subflava</em>&lt;sup&gt;b&lt;/sup&gt; Both unconfirmed.</td>
</tr>
<tr>
<td>University hospital of North Norway</td>
<td>In-house experimental assay</td>
<td>Real-time PCR</td>
<td>Conjugative relaxosome accessory transposon protein TraH</td>
<td><em>N. meningitidis group C</em>&lt;sup&gt;c&lt;/sup&gt;, <em>N. sicca</em>&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>University hospital of North Norway</td>
<td>In-house experimental assay</td>
<td>Real-time PCR</td>
<td>Conjugative coupling protein TraG</td>
<td><em>N. meningitides gr.</em>&lt;sup&gt;B&lt;/sup&gt;, <em>N. sicca</em>&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>University hospital of North Norway</td>
<td>In-house experimental assay</td>
<td>Real-time PCR</td>
<td>NGO</td>
<td><em>N. sicca</em>&lt;sup&gt;c&lt;/sup&gt;, <em>M. osloensis</em>&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>University hospital of North Norway</td>
<td>In-house experimental assay</td>
<td>Real-time PCR</td>
<td>B protein on Cryptic plasmid PJD1</td>
<td><em>N. sicca</em>&lt;sup&gt;c&lt;/sup&gt;, <em>N. flavescens</em>&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
University hospital of North Norway

In-house experimental assay

Real-time PCR
CAH Carbonic anhydrase
N. sicca

University hospital of North Norway

In-house experimental assay

Real-time PCR
JD1 Orf1, unknown protein on Cryptic plasmid
N. subflava, N. lactamica,


Real-time PCR
Opa (multi-copy)
None reported

Chui, L. et al 2008

PCR
cppB gene
N. cinerea, N. flavescens,
N. lactamica, N. subflava,
and N. sicca

Chui, L. et al 2008

PCR
Gyr
N. mucosa and N. heidelgerensi

SDA
PivN8
N. Flavescens, N. lactamica,
N. subflava and N. cinera

Goire, N. et al 2008

Real-time PCR
omp
Just report false positive

Op
Just report false positive

\(^a\) Previously reported cross-reacting species are from reference Palmer, H. et al 2003(82)

\(^b\) Reported cross reacting species are from reference Tabrizi, Z.N. et al 2011(81).

\(^c\) Reported cross reacting species are from unpublished in-house results.

\(^d\) Reported cross reacting species are from Tabrizi, Z. N. et al 2005 (83,84)

\(^e\) Reported cross reacting species are from Chui, L. et al 2008 (85)

\(^f\) Reported cross reacting species are from Goire, N. et al 2008 (86)

5.1.3 Diagnostics

Diagnosis of gonorrhoea based solely on clinical manifestations is very difficult and is only suggestive. This is due to the fact that the bacteria can cause both symptomatic and asymptomatic genital as well as extra genital tract infection with a broad spectrum of symptoms, many of which are similar to those of other STIs.

5.1.3.1 Direct microscopy

Gram staining followed by direct microscopy of sample smears from urethra, cervix, rectum and vagina from men and women can easily be prepared at the point of care (POC), searching
for Gram negative diplococci intracellular within polymorphonuclear leukocytes. Performed by a trained professional, this can be a good preliminary diagnosis of gonorrhoea. This method is a rapid diagnosis with sensitivity approaching culture diagnostics for urethral samples in symptomatic men (≥90%), which makes it possible to even provide a definitive diagnosis of gonorrhoea for these samples (73,74,87). However the method is relatively insensitive and does not provide a definitive diagnosis for specimens from asymptomatic men, women and extra-genital sites from both sexes (≤50% sensitivity). For extra genital sites, the specificity is also suboptimal due to presence of commensal Neisseria species and/or N. meningitidis. In any case, negative microscopy does not exclude infection for these samples. Both CDC (74) and the IUSTI/WHO (73) guidelines accept the use of microscopy for definitive diagnosis of gonorrhoea in urethral samples from symptomatic men.

5.1.3.2 Culture diagnostics
Culture diagnostics requires optimization of every step from specimen collection, transportation, inoculation and incubation to maintain high sensitivity. To maintain high specificity, the subsequent species verifying assays are important.

It is recommended to use selective agar medium for N. gonorrhoeae. There are many known selective culture media; Martin-Lewis, Modified Thayer-Martin, GC-Lect, and New York City. These have a growth factors and antimicrobial agents in an agar or equivalent base. The antimicrobial agents (i.e. vancomycin, colistin, nystatin, and trimethoprim) prevent growth of Gram-positive bacteria, nongonococcal Gram-negative bacteria, fungi and swarming proteus species. For species verification of N. gonorrhoeae, microscopy, rapid oxidase production, carbohydrate utilization test, and rapid biochemical, substrate co-agglutination test, immunofluorescence assay or chromogenic enzyme substrate tests are typically used. NAATs can also be used to verify positive cultures as N. gonorrhoeae.
5.1.3.3 **Antimicrobial resistance testing**
Minimum inhibitory concentration (MIC) determination by agar dilution method is the reference method for antimicrobial susceptibilities determination in *N. gonorrhoeae* isolates. However this is a laborious method and a quality assured Etest® (Biomerieux, Marcy l'Etoile, France) is just as good, but easier to perform. The agar disc diffusion test however is not recommended.

When clinical failures to respond to recommended therapies with specific antimicrobial agents, a resistant category is established with breakpoints for *in-vitro* determination of resistance after testing a variety of resistant isolates. In Europe a standardized set of breakpoints are set by the European committee on Antibacterial Susceptibility Testing (Eucast; www. Eucast.org)

5.1.3.4 **Nucleic acid amplification test**
All NAATs use specific primers sequences to guide enzymes to amplify specific sequences in the genome of the pathogen. The principle of amplification, enzymes and mode of detection of the amplified oligonucleotides vary between different methods. There is also a difference in what kind of nucleic acid target that is used. Both RNA (ribonucleic acid) and DNA (chromosomal or plasmid) are used as targets. RNA-based methods have a sensitivity advantage as one can target rRNA/mRNA, which can be present in 10-1000 copies. Plasmid targets have similar advantage due to possible multiple copies of plasmids. However, RNA is a more fragile nucleic acid and preserving buffers are needed for reliable results. Plasmids are not always evenly distributed during cell division, and may also be lost and readily exchanged. It is sometimes possible to find a multi-copy genomic DNA target gene, but such genes are often preserved between species and hence prone to false-positive test results.

Polymerase chain reaction (PCR) utilizes a thermostable DNA polymerase enzyme to extend primers and thus amplify specific parts of DNA. Running an agarose gel electrophoresis and staining with ethidium bromide verifies the specific amplified products. This type of
conventional PCR is however not widely used as a diagnostic method for *N. gonorrhoeae*, but some laboratories use diagnostic conventional PCR method(s) for verifying culture positive samples, and some commercial such assays are available in resource poor settings as it requires less sophisticated equipment and are generally less expensive.

Real-time PCR is the predominant NAAT used worldwide for microbiological diagnostics, in the format of in-house methods or commercially available kits. For *N. gonorrhoeae*, real-time PCR is used as in-house diagnostic assays (88-91), verification assays (92) and in commercial diagnostic assays such as, VERSANT CT/GC DNA 1.0 Assay (Siemens, Deerfield, IL, USA), COBAS TaqMan48 and 4800 (CT/NG) Test (Roche Molecular systems inc., San Diego, Calif) and RealTime CT/NG test (Abbott Laboratories, Abbot Park, Illinois, USA). Real-time PCR methods differ from conventional methods by detecting amplified products as an increase in emitted light, instead than after agarose gel electrophoresis. Specific regions of DNA are amplified using two primers, defining the outer borders of the region to be amplified, to mark starting points for the polymerase. A target specific probe complementary to a region between the primer sites is used for detection. The probe carries a quencher and a fluorophore to give a measure of increased DNA as increased light. A number of different probing systems are used, such as; TaqMan probes, Molecular beacons and Fret Probes. The most common probe used is the TaqMan probe or dual labelled hydrolytic probe. This probe binds the target quicker and harder than the primers as a design default and when bound to target is degraded by the polymerase as it elongates the primers. When degraded the fluorophore and quencher are separated and light emitted by the fluorophore will be measurable as an increase in light of specific wavelengths.

Ligase chain reaction (LCR) (93,94) was used in the Abbot LCx assays (Abbot Laboratories, Abbot Park, Il, USA) for duplex detection of *N. gonorrhoeae* and *C. trachomatis*. The principle of LCR is based on ligation of two adjacent primers using a thermostable ligase enzyme, for
each of the strand in the two-stranded target DNA. Each of the ligated pair of primers will in a
cycling reaction act as new templates for other primers. However, the Abbott LCx was
previously recalled due to specificity as well as sensitivity problems (95). Developed at Gen-
Probe (Gen-Probe Inc., San Diego, Calif, USA), the transcription mediated amplification
(TMA) technology is used in their APTIMA Combo 2 assay. The method uses two primers and
two enzymes: RNA polymerase and reverse transcriptase. One of the primers contains a
promoter sequence for RNA polymerase, which hybridizes to the target rRNA. Reverse
transcriptase then makes a DNA copy of the target rRNA by extension from the 3’-end of the
promoter primer. The RNase activity of the reverse transcriptase then degrades the RNA in the
resulting RNA:DNA duplex. Next, the second primer binds to the DNA copy, and reverse
transcriptase elongates the primer, making a new strand of DNA, resulting in a double-stranded
DNA molecule. RNA polymerase recognizes the promoter sequence in the DNA template and
initiates transcription. Each of the newly synthesized RNA amplicons re-enters the TMA
process and serves as a template for a new round of replication.

Strand displacement amplification (SDA) (96,97) and fluorescent energy transfer is used by
Becton Dickinson in their BD ProbeTec ET Chlamydia trachomatis Amplified DNA Assay and
/or the BD ProbeTec ET Chlamydia trachomatis (CT) and Neisseria gonorrhoeae (GC)
Amplified DNA Assays.

SDA is commonly explained as two steps; the generation step that creates the structure feeding
into the second face – the exponential amplification step.

The denatured target DNA is primed with two primers (S1 and S2), two bumpers (B1 and B1),
and a detector probe. The primers have a target specific 3’ end, and a non hybridizing 5’ end
with a BsoB1 recognition site between them.

A polymerase and restriction enzyme extends primers and bumpers. When extending the
bumpers, the polymerase displaces the primer extension product, which then hybridizes to a complementary primer and bumper before another extension and displacement, and so on. The resulting products contain the annealing region for the detector probe flanked by \textit{BsoBI} sites. Incorporation of alpha thio-dCTP in the complement to the restriction site ensures that \textit{BsoBI} only nicks the extension products. The polymerase will extend from these nicks, resulting in exponential amplification. The amplified products are continuously detected using a detector probe with a target specific 3’ end and a hairpin in the 5’ end with a \textit{BsoBI} recognition site in the loop. When the probe is annealed to correct target, the hairpin is linearized and a cleavable restriction site is made. When \textit{BsoBI} cleaves at this site, the acceptor and fluorophore are distally separated, resulting in fluorescence.

\textbf{5.1.3.5 Other diagnostics}
Other diagnostic methods for diagnosing \textit{N. gonorrhoeae} are direct fluorescent antibody (DFA) test, enzyme immunoassay (EIA), nucleic acid hybridization and syndromic diagnosis. Apart from nucleic acid hybridization, these methods have a very low sensitivity and commonly suboptimal specificity.

The Digene Hybrid Capture II (Digene Corp., Beltsville MD, USA) (98) is the most common nucleic hybridization test for detecting \textit{N. gonorrhoeae} and \textit{C. trachomatis} and this method uses signal amplification to increase sensitivity. The sensitivity and specificity of Hybrid Capture II is lower than for optimized culture diagnostics (99-101), and do not provide AMR profiles.

POC tests are often limited by their low sensitivity and should be used only for populations unlikely to return for follow-up. In these settings, rapid POC diagnostics, followed by immediate treatment of positive patients, with lower sensitivity than reference test are shown in several studies to lead to treatment of more patients where patients have to return for results.
and treatment (100,102).

Where appropriate diagnostic facilities are not available, the World Health Organization (WHO) recommends the use of syndromic approach for diagnostic and treatment of bacterial STIs (especially urethritis and cervicitis) in both men and women. In regard to diagnosing gonorrhoea, syndromic management generally works well for urethritis in men. However, the low sensitivities and specificities for diagnosis of other gonococcal infections result in many false diagnoses (positives and negatives), and massive over-treatment as well as many STIs remain untreated (103).

5.1.3.6 **Test of cure**

Test of cure (TOC) is used in Norway to control the treatment outcome for both *C. trachomatis* and *N. gonorrhoeae*. As many gonococcal infections, especially in women are asymptomatic, a laboratory diagnostic test is required to assess treatment success. Since NAATs will detect nucleic acids also from non-viable pathogens and Chlamydial nucleic acid has been shown to reside for up to 2-3 weeks (104) after treatment, time for clearance of amplifiable nucleic acid had to be determined when introducing NAAT testing. We know from experience that far less than one week after effective treatment is enough to have a negative result using culture diagnostics for *N. gonorrhoeae*. Determination of appropriate time to do TOC with NAAT may depend on both the NAAT used for diagnostics, antibiotic used for treatment, and anatomical site of infection.

Previous studies on TOC by Bachmann et al in 2002 (105), concluded that full clearance of gonococcal DNA after appropriate treatment of uncomplicated gonorrhoea was achieved within a week, but recommended TOC after 2 weeks for all type of gonococcal infections. Since both the diagnostic and treatment was different in their study, compared to Norwegian guidelines
and settings, we decided to investigate the appropriate time for TOC in the conditions relevant for Norway.

The use of TOC for *N. gonorrhoeae* is debated and the literature and recommendations vary between no TOC for uncomplicated gonorrhoea and after 3-6 months for expedited partner therapy (4,73,74,106-108). CDC (4,74) recommends retest after 3-12 months rather than TOC for uncomplicated *N. gonorrhoeae* infection with the rationale that positives after treatment are likely to be re-infections when recommended treatment is provided. The IUSTI/WHO guidelines state that TOC is not routinely necessary for anogenital infection if a recommended treatment has been given, but an assessment to confirm compliance with treatment is recommended (73), looking at resolution of symptoms and partner notification. They do however recommend TOC in case of persistence of symptoms, re-exposure to infection, possibility of resistance to therapy, pharyngeal infections and if stipulated by national practice or guidelines (the latter being the case in Norway). Manavi et al. recommends TOC for pharyngeal infections (109) because of higher rate of treatment failure. Pharyngeal gonococcal infections can be exceedingly difficult to treat (10,109,110) and are often asymptomatic resulting in potential reservoirs for infection and its further spread.

The most support for not performing TOC is for uncomplicated urogenital gonococcal infections diagnosed with culture diagnostics and subsequent AMR testing performed (4,74,106-108). Culture diagnostics has a lower sensitivity for rectal samples due to overgrowth of other bacteria, and pharyngeal infections, in addition to lower diagnostic sensitivity, can be difficult to treat despite AMR testing indicating appropriate antibiotic, and hence TOC is more relevant for extra-genital samples. NAATs are rapidly replacing culture for detection of *N. gonorrhoeae*, and adequate evidence-based recommendations for appropriate time to perform TOC are lacking for non-culture diagnostics of gonorrhoea (105).
In Norway, the prevalence of *N. gonorrhoeae* is still low and the empiric treatment recommendation for gonococcal infection remains ciprofloxacin, making TOC crucial to perform to control the spread of gonorrhoea.

TOC as part of gonorrhoea management may be controversial in different national, regional and international guidelines. However, it might be an important tool in the future to manage gonorrhoea, due to the increasing resistance to all recommended antibiotics for treatment, the importance of diagnosing and treating pharyngeal and rectal samples, and the increasing use of NAATs in diagnosing gonorrhoea. For asymptomatic patients, TOC is the only means of assessing treatment outcome.

### 5.1.3.7 Molecular epidemiologic typing methods

To understand the patterns of disease transmission, identify and target high-risk groups within communities, and to control outbreaks of antibiotic-resistant gonorrhoea, we can use either partner notification, or molecular typing. Molecular typing methods assume that the bacterial isolate from individuals infected in a short transmission chain are genotypically indistinguishable (29,77). A carefully validated molecular typing method will recognize the relatedness of isolates by how similar they are to form the bases to construct a sexual network. Many different genes and methods have been explored for discriminatory power and ease of use (111-114).

*N. gonorrhoeae* multiantigen sequence typing (NG-MAST) is so far the preferred method, providing means of comparison between laboratories via the Internet (115,116). NG-MAST sequences internal fragments of two highly polymorphic antigen-encoding loci, the *porB* and *tbpB* genes (115,116).
6 Aims of the present thesis

The main aims of the thesis were:

I. To develop and clinically validate a robust, sensitive and specific real-time PCR for detection of *N. gonorrhoeae* to be used on a variety of sample types (paper I & II).

II. To determine appropriate time for test of cure when diagnosing *N. gonorrhoeae* with NAAT (paper III).

III. To characterize phenotypic and genotypic properties of *N. gonorrhoeae* isolates in Norway (paper IV).
7 Materials & Methods

Clinical *N. gonorrhoeae* isolates and reference strains

7.1.1 Paper I

To develop a *porA* real-time PCR, several international *Neisseria* reference strains (n=48) were collected from American Type Culture Collection (ATCC), Culture Collection University of Gothenburg (CCUG), National Collection of Type Cultures (NCTC), World Health Organization (WHO), Swedish Reference Laboratory for Pathogenic Neisseria, and Statens Serum Institut (SSI), Denmark. These reference strains included *N. gonorrhoeae* (n=34), *N. meningitidis* (n=4), *Neisseria sicca* (n=2), *Neisseria subflava* (n=1), *Neisseria flavescens* (n=2), *Neisseria mucosa* (n=2), *Neisseria lactamica* (n=2), and *Neisseria cinerea* (n=1). The *N. gonorrhoeae* reference strains originated from different geographic settings worldwide and were isolated during the last four decades.

In addition to the international reference strains, we examined 168 clinical *N. gonorrhoeae* isolates, including 76 isolates cultured in Archangelsk, Russia in 2004, 14 isolates cultured at University Hospital of North Norway 2003-2004, 9 isolates from Norwegian Organization for Surveillance of Antibiotic Resistant Microorganisms (NORM), 13 isolates from SSI in Denmark, 5 confirmed *N. gonorrhoeae* isolates donated by Helen (82), and 51 genetically different Swedish *N. gonorrhoeae* isolates from 1998-2001 with known *porA* pseudogene sequence (35). In the paper we erroneously state that we examined 176 clinical samples, however 8 of these samples were not included in the following text. This means that everything, except the total number of samples examined, is correct.

Furthermore, clinical isolates (n=54) of other *Neisseria* species were included. These comprised *N. gonorrhoeae subspecies kochii* (n=4), *N. meningitidis* (n=7), *N. sicca* (n=7), *N.
subflava (n=11), N. flavescens (n=3), N. mucosa (n=5), N. lactamica (n=7), N. cinerea (n=7), Neisseria caviae (n=1), Neisseria animalis (n=1), and Neisseria polysaccharea (n=1).

Specificity was tested using a panel of Gram-negative bacteria (n=18), Gram-positive bacteria (n=23), fungus (n=1) and viruses (n=4) as well as human DNA.

7.1.2 Paper II

To clinically validate the porA real-time PCR, a total of 284 samples from 242 consenting patients attending Olafiaklinikken in Oslo, Norway from January 2006 through May 2006 with suspected gonorrhoea where examined. Samples were collected using either a urethral flocked swab (Copan, Brescia, Italy) or an endocervical flocked brush (Copan, Brescia, Italy). The urethral swab was used for sampling in the urethra. The endocervical brush was used to take samples from the cervix, rectum and pharynx.

7.1.3 Paper III

To determine proper time for TOC, 234 men and 23 women were recruited at Olafiaklinikken in Oslo from June 2006 through January 2007. Samples for PCR were collected using either a urethral-flocked swab (Copan, Brescia, Italy) or an endocervical-flocked swab (Copan, Brescia, Italy). The urethral swab was used for sampling the urethra. The endocervical swab was used for sampling the cervix, rectum and pharynx. In total 669 clinical samples were collected from the 257 patients. Patients with positive samples who did not return for first TOC within 2 weeks were excluded from the study.

7.1.4 Paper IV

To genetically and phenotypically characterize circulating Norwegian N. gonorrhoeae isolates, a total of 114 viable clinical isolates were collected from six university hospitals during 2009. These isolates comprised 42% of the total number of gonorrhoea cases in Norway in 2009, and
were initially cultured at the University Hospital of North Norway, Tromsø (n=3); St. Olavs Hospital, Trondheim University Hospital, Trondheim (n=6); Stavanger University Hospital, Stavanger (n=16); Haukeland University Hospital, Bergen (n=25); Oslo University Hospital, Ullevål Hospital, Oslo (n=60); and Akershus University Hospital, Oslo (n=4).

DNA extraction

7.1.5 Paper I

Genomic DNA from the bacteria was isolated with a BioRobot M48 from Qiagen (Hilden, Germany) using the MagAttract DNA tissue kit with the Infectious Disease protocol and an elution volume of 100 μl, according to the manufacturers specifications. All bacteria and the fungus were initially suspended in 200 μl TE-buffer, pH 8 (Ambion, Austin TX, USA) to approximately 1.5 × 10^8 Colony Forming Units (CFU)/ml before isolation of DNA. All viruses were suspended in a virus in-house transport medium made from Minimum Essential Medium, Hepes buffer and Gentamicin (Gibco, Carlsbad, Calif, USA).

7.1.6 Paper II & III

Seven hundred μl of the UTM-RT sample was put in a Tecan Miniprep-75 (Tecan, Männedorf, Switzerland) modified by NorDiag AS (Oslo, Norway), to perform DNA preparation with the BUGS´n BEADS STI kit (Genpoint). The principle of the kit is to immobilise bacteria onto a paramagnetic bead and remove the sample material. Then the bacterial cells are lysed and the nucleic acid is precipitated back to the magnetic bead and washed. The purified nucleic acid is then eluted for downstream use. The Tecan Miniprep-75 was modified to perform this procedure automatically. To avoid human error, the Tecan also prepare the PCR reaction by adding mastermix and DNA eluate.
7.1.7 **Paper IV**

Genomic DNA was isolated using the BUGS’n BEADS STI-fast kit (CE/IVD) (NorDiag ASA, Oslo, Norway) on the NorDiag Bullet according to manufacturer’s instructions. The principle of the kit is the same as for paper II and III.

**Real-time PCR**

Primers and probe against the *N. gonorrhoeae* *porA* pseudogene were designed for a TaqMan assay by using Primer Xpress 2.0 (Applied Biosystems, Foster city, Calif., USA) according to manufacturer’s guidelines. The forward and reverse primer sequences for *porA* pseudogene were 5’-CCG-GAA-CTG-TCA-TCT-GAT-T-3’ (22 bp) and 5’-GTT-TCA-GCG-GCA-GCA-TTC-A-3’ (19 bp) respectively. The sequence for the TaqMan probe for the *porA* pseudogene was 5’-FAM-CGT-GAA-AGT-ACTG-GAG-ATG-GGC-GGA-CTT-BHQ-1-3’. The primer and probe sequences, and the amplicon (255 bp) were compared with *porA* pseudogene sequences from a previous study (35) as well as all the genetic sequences deposited in GenBank.

For the *N. gonorrhoeae* specific PCR we used TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG on a 7900 HT Fast Real-Time PCR System (Applied Biosystems Foster city, Calif., USA). The Fast-PCR was run with 20 seconds activation of the polymerase at 95°C, followed by 50 cycles of 95°C for 1 second and 60°C for 20 seconds. We used 11.5 µl template added to 13.5 µl reaction mix for each PCR sample. We also tested the robustness of the method by varying the sample volume in the PCR between 9 and 15 µl, while keeping the reaction mix constant at 13.5 µl. The primers amplify a 102 base pair (bp) fragment of the *porA* pseudogene. The *porA* primers and probe were optimized as recommended by Applied Biosystems and final concentrations of 900 nM primers and 200 nM probe were found to be
optimal.

For inhibition control, an Internal Amplification Control (IAC) was constructed by using composite primers that have *N. gonorrhoeae* specific 5’-end and a pGEM-luc plasmid (Promega Madison, Wi., USA) specific 3’-end, which amplify a 181 bp fragment of the Beta-lactamase coding region (position 2988 – 3169). The forward and reverse primer sequences for making the IAC were 5´-GTT-TCA-GCG-GCA-ATG-GTC-TGA-CAG-TTA-CCA-ATG-CTT-AA-3’ and 5´-CCG-GAA-CTG-GTT-TCA-TCT-GAT-TGC-TGG-CTG-GTT-TAT-TGC-TG-3’-3’ respectively. The internal control probe sequence was (24 bp): 5’-Yakima Yellow-CCA-TAG-TTG-CCT-GAC-TCC-CG-TGC-Darkquencer3’.

The five µl of a 10^{-4} dilution of the pGEM-luc plasmid was used as a template in a total volume of 50 µl reaction mix with no UNG. Fifty nM of each of the composite primers were used. First it was heated to 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute followed.

**Sequencing**

**7.1.8 16s rRNA sequencing (Paper II)**

In cases of discrepant results, the samples were sent to the National Reference Laboratory for Pathogenic Neisseria, Department of Laboratory Medicine, Clinical Microbiology, Örebro University Hospital, Örebro, Sweden, for discrepant analysis using sequencing of the 16S rRNA gene (primers used for PCR: 5´-TGA-TCC-ARC-CGC-ASS-TTC-3’, 5´-AGA-GTT-TGA-TCY-TGG-YTY-AG-3’; primers used for sequencing: the two PCR primers, and 5´-GTG-CCA-GCA-GCC-GCG-GTA-A-3’, 5´-TTA-CCG-CGG-CTG-CTG-GCA-C-3’, 5´-GCA-ACG-AGC-GCA-ACC-C-3’, 5´-AGG-GTT-GCG-CTC-GTT-G-3’).
7.1.9 porA sequencing (Paper II)

The entire porA pseudogene was sequenced as previously described (35). The porA pseudogene, was amplified using the primers from Feavers (5′-CGA-AGC-CCA-CAT-CAA-ACA-GGG-3′ and 5′-AAT-GAA-GGC-AAG-CCG-TCA-AAA-ACA-3′). The amplified porA pseudogene was sequenced using primers from Feavers (5′-AAT-GAA-GGC-AAG-CCG-TCA-AAA-ACA-3′, 5′-AAA-CAG-GGC-AAA-ATC-TAT-GTC-3′, 5′-AAT-ACG-AGG-GCG-GTA-AGT-TTT-T-3′, and 5′-ATG-CGA-AAA-AAA-CTT-ACC-GCC-CTC-3′) and Suker (5′-AAC-GGA-TAC-GTC-TTG-CTC-3′, 5′-GAG-CAA-GAC-GTA-TCC-GTT-3′, 5′-GGC-GAG-ATT-CAA-GCC-GCC-3′, and 5′-GGC-GGC-TTG-AAT-CTC-GCC -3′) (31,33,35). The reactions were performed in 96-well plates in a GeneAmp PCR system 2700 (Applied Biosystems). The nucleotide sequences were determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer’s instructions. The sequence of each strand of each compiled sequence was determined (35).

7.1.10 porB sequencing (Paper IV):

The coding region of the porB gene was amplified using the PorBU (5′-CCG-GCC-TGC-TTA-AAT-TTC-TTA-3′) and PorBL (5′-ATT-AGA-ATT-TGT-GGC-GCA-G-3′) primers described by Unemo et al (50). The 50 μl reaction mixture contained 1.0 U DNA polymerase, 2.5 mM MgCl2, 0.1 mM deoxynucleoside triphosphates, 0.5 μM concentration of each primer, and 1 μl of the genomic DNA template. The cycling parameters: an enzyme activation step at 94°C for 10 min, followed by 30 sequential cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. At the end of the final cycle, an extension phase at 72°C for 4 min was included before storage at 4°C.

The same primers were then used for cycle sequencing of the porB amplicon on an ABI PRISM
3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). The sequence of each strand of each compiled sequence was determined.

7.1.11 Neisseria gonorrhoeae multiantigen sequence typing (NG-MAST) (Paper IV)

The more variable internal segments of \( \text{porB} \) (490 bp) and \( \text{tbpB} \) gene (390 bp), where sequenced as described previously (115,117). The sequenced fragments are uploaded to the NG-MAST web site (www.ng-mast.net) assigned different allele numbers and sequence types (STs).

Culture diagnostics (Paper II & III)

The culture diagnostics was performed at Ullevål University Hospital as part of their routine diagnostic services by identification of characteristic colonies on chocolate agar medium supplemented with inhibitory antimicrobials, i.e. 5 µg/ml trimethoprim lactate, 100 IE/ml colistin, 0.5 µg/ml lincomycin and 1 µg/ml Amphotericin B. For thorough species confirmation, oxidase test, identification of Gram-negative diplococcic in microscopy, sugar oxidation tests, and Phadebact GC Monoclonal test (Bactus AB, Solna, Sweden) were used.

Antimicrobial resistance testing (Paper III-IV)

In paper II and III, the isolates where tested for their susceptibility to ampicillin, erythromycin, spectinomycin, ciprofloxacin, ceftriaxone, cefotaxime, and tetracycline using Etest® according to manufacturers instruction.

In paper IV, the isolates where tested for susceptibility to penicillin G, erythromycin, spectinomycin, ciprofloxacin, ceftriaxone, azithromycin, tetracycline, cefixime, and gentamicin
using Etest® according to manufacturers instruction. β-lactamace production was investigated with nitrocefin solution (Oxoid, Vasingsoke, Hants, England).

Interpretative criteria from the European Committee on Antimicrobial Susceptibility testing (EUCAST, http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.3_pdf.pdf) were used for paper III and IV, whereas national breakpoints from Norwegian Working Group for Antibiotics (NWGA) was used for paper II by the investigating laboratory

**Direct microscopy (Paper II & III)**

All smears were methylene blue- or Gram-stained and examined for typical intracellular, i.e. within polymorphonuclear leukocytes, diplococci in high power field microscopy using oil-immersion.
8 General Results

Paper I & II

The developed PCR method amplified all of the different *N. gonorrhoeae* international reference strains (*n* = 34) and *N. gonorrhoeae* clinical isolates (*n* = 176) but none of the isolates of the 13 different non-gonococcal *Neisseria* species (*n* = 68) available for testing. A panel of Gram-negative bacteria (*n* = 18), Gram-positive bacteria (*n* = 23), fungus (*n* = 1), and viruses (*n* = 4) as well as human DNA, was also negative. The limit of detection was determined to be less than 7.5 genome equivalents/PCR reaction.

Of the 360 clinical samples (from 242 patients) included in paper II 37 samples were positive by both culture diagnostic and PCR, however the PCR method identified 15 additional positive samples (from 14 symptomatic patients). All 15 culture-negative/PCR positive samples were confirmed positive by both 16S and *porA* pseudogene sequencing. The PCR method showed a sensitivity, specificity, positive predictive value, and negative predictive value of 100% in this study. The population investigated were preselected on the basis of being at high risk of having a gonococcal infection, or as contacts of *N. gonorrhoeae* positive patients. This pre-selection accounted for an *N. gonorrhoeae* prevalence of 17.4%.

Paper III

Thirty *N. gonorrhoeae* positive patients that were diagnosed by culture (*n* = 27) and/or NAAT (*n* = 30), in at least one clinical specimen, were included in the study. The gender distribution was 2 women (mean age: 24.5 years, range: 21 to 28 years) and 28 men (mean age: 37.4 years, range: 22 to 58 years), of which 50% (*n*=14) were MSM. Seven clinical specimens (divergent anatomical sites) from seven different patients were positive using NAAT, but negative with culture. Three of these patients (10% of all positive patients) did not have any positive culture
sample from other anatomical sites and, accordingly, would have been false negative if culture were the only diagnostic test used.

Twenty-five patients were diagnosed with gonorrhoea at a single genital site, two patients had only extra-genital gonorrhoea (pharyngeal and rectal), and three patients displayed multiple infected sites. All patients diagnosed with genital gonorrhoea reported symptoms such as discharge and dysuria, while all the extra-genital infections were asymptomatic.

Nineteen patients (63%) returned for TOC within day seven (day four to seven) after treatment, and 16 (84%) of these were negative using NAAT. Two of the remaining positive patients, which had their initial TOC taken on day four and day six, deposited a negative sample within day 14 (day 11 for both patients). The last patient did not return before day 19, but then had a negative TOC. There were eleven (37%) patients who did not return for their initial TOC before day eight to 14 after treatment, and they were all negative.

AMR testing was performed on *N. gonorrhoeae* isolates for 25 culture positive patients. Seventeen (68%) of these 25 isolates were ciprofloxacin resistant; however, no isolate was resistant to ceftriaxone or spectinomycin.

**Paper IV**

The mean age was 25 years (median age: 23 years, range: 18-53 years) and 35 (median age: 33 years, range: 19-60 years) for women (n=17; 15%) and men (n=97; 85%), respectively. Heterosexual infection was reported for 81 patients (64 men, 17 women), homosexual infection was reported for 31 men and two men did not disclose their sexual preference.

Seven of the seventeen (41%) women were infected by their steady partner, whereas only 13 (13%) men were infected by their steady partner. Commercial female sex workers were given as source of infection by 9 (9.3%) men.
The results of the AMR testing of all *N. gonorrhoeae* isolates (n=114) are summarized in Table 7. In total 78% (n=89) of the isolates were resistant, and one isolate (0.9%) had intermediate susceptibility to ciprofloxacin, which is the recommended first-line antibiotic for treatment of gonorrhoea in Norway. Four (3.5%) isolates were intermediate susceptible/resistant to cefixime (0.19-0.38 mg/L), two isolates (1.8%) displayed intermediate susceptibility/resistance to ceftriaxone (0.9 mg/L), and 12 (11%) isolates were resistant to azithromycin. β-lactamase production causing high-level resistance to penicillins, was found in 41 (36 %). A penA mosaic allele was found in 17 (15%) of the isolates, including all isolates with intermediate susceptible/resistant to cefixime (n=4).
Table 5. Antibiotic susceptibility of *Neisseria gonorrhoeae* isolates (n=114) cultured in 2009 in Norway

<table>
<thead>
<tr>
<th>Antimicrobial (Breakpoints)</th>
<th>In vitro resistance (%)</th>
<th></th>
<th>Intermediate (%)</th>
<th>Susceptible (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td>Total</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Ciprofloxacin (S≤0.03/R&gt;0.06)</td>
<td>75</td>
<td>14</td>
<td>89 (78)</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>Ceftriaxone (S≤0.12/R&gt;0.12)</td>
<td>2</td>
<td>0</td>
<td>2 (1.8)</td>
<td>0</td>
</tr>
<tr>
<td>Cefixime (S≤0.12/R&gt;0.12)</td>
<td>4</td>
<td>0</td>
<td>4 (3.5)</td>
<td>0</td>
</tr>
<tr>
<td>Azithromycin (S≤0.25/R&gt;0.5)</td>
<td>10</td>
<td>2</td>
<td>12 (11)</td>
<td>0</td>
</tr>
<tr>
<td>Spectinomycin (S≤64/R&gt;64)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>MIC range: 1.5 to 8 mg/L</td>
</tr>
</tbody>
</table>

<sup>a</sup>Breakpoints for susceptible (S≤x mg/L) and resistant (R>y mg/L) according to the breakpoints stated by The European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org/Clinical breakpoints/ Clinical breakpoints - bacteria (v 1.3)).

<sup>b</sup>No breakpoints have yet been stated for gentamicin.

Among the 31 MSMs, 24 (77%) had strains resistant to ciprofloxacin while 64 of the 81 heterosexuals (79%) had ciprofloxacin-resistant strains. No significant difference was found (p
All the four isolates displaying intermediate susceptibility/resistance to cefixime were cultured from males (2 MSM) in the age of 39-46 years infected in Oslo (n=3) and France (n=1). These four (all NG-MAST ST1407) isolates all contained a penA mosaic allele, which is a predictive indicator of decreased susceptibility to ESCs, especially cefixime (117-119). Further 13 isolates contained a penA mosaic allele, and excluding the four isolates mentioned above, these had the highest MIC of cefixime (0.094-0.125 mg/L). Of the 17 isolates with the penA mosaic allele, 11 were determined as ST1407 (10 of these were isolated from MSM). All but two penA mosaic allele positive isolates were from gonorrhoea cases contracted in Norway, 14 of them in Oslo. The two foreign infections were one MSM and one heterosexual, both infected in France (ST1407 and ST3168, respectively).

The NG-MAST results in total showed 72 different STs, of which 37 have not been previously described. The most frequent STs were ST1407 (9.6% of isolates), ST292 (6.1%) and ST5030 (5.3%), which has not been described previously. Sixty-two STs (54%) and eight STs (7.0%) were represented by one isolate and two isolates, respectively.

In total, 38 (39%) of the men were infected abroad, and Thailand was the most frequent place for contracting N. gonorrhoeae. For women, 29% of the patients were infected abroad. Symptoms were the reason for seeking medical care for 92 (94.8%) of the men and 9 (52.9%) of the women.

Of the domestically contracted isolates and the isolates contracted abroad, 55/68 (81%) and 34/45 (76%) respectively were ciprofloxacin resistant. Seventy-one percent (5/7) of the women infected abroad had ciprofloxacin resistant isolates, and nine (90%) of the women infected domestically had ciprofloxacin resistant isolates. For men, 29 (27 heterosexual)/38 (76%) of those infected abroad and 46 (23 heterosexual)/58 (79%) of those infected domestically had ciprofloxacin resistant isolates. No significant difference was found ($p = 0.729$).
The two isolates displaying intermediate susceptibility/resistance to ceftriaxone were both from MSM infected domestically. Two of the four isolates showing intermediate susceptibility/resistance to cefixime were from domestically infected MSM, one from a domestically infected heterosexual man and one heterosexual man infected in another European country. Among the 12 azithromycin resistant strains two were from women (1 domestically infected), one was from a heterosexual male infected abroad and nine from men (five of these were MSM) infected domestically.
9 Discussion

General background and summary

The Department for Microbiology and Infection Control at the University Hospital of North Norway is one of two laboratories, which provides diagnostic services to the three most northern Norwegian counties with a total size of 112,960 km$^2$, an area almost the size of England (130,000 km$^2$), with a population of 465,000 (England has a population of almost 50 million). This makes diagnosis of \textit{N. gonorrhoeae} by culture difficult with great distances for transport of clinical samples from the consulting general practitioner to the laboratory. In addition, the incidence of gonorrhea in Norway is low – 8.4/100,000 (2010). This background makes it especially important to improve the diagnostics by establishing a sensitive and specific, clinically validated diagnostic NAAT for diagnosing \textit{N. gonorrhoeae}.

The positive predictive value (PPV) of a test is a function of true positives (TP)/ (TP + false positives (FP)), making the specificity of the assay as well as the prevalence of infection determining factors for PPV. The disease prevalence influences the PPV by influencing the true positive and false positive rates as seen in Table 6.

\textbf{Table 6. Demonstration of the effect of prevalence on probability of true positive using different sensitivities and specificities.}

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity 96.4%</th>
<th>Sensitivity 98.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>97.9%</td>
<td>99.7%</td>
</tr>
<tr>
<td>Positive Predictive Value (PPV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% prevalence</td>
<td>84%</td>
<td>97%</td>
</tr>
<tr>
<td>5% prevalence</td>
<td>73%</td>
<td>95%</td>
</tr>
<tr>
<td>1% prevalence</td>
<td>35%</td>
<td>77%</td>
</tr>
</tbody>
</table>
By using stringent inclusion criteria for who should be tested for *N. gonorrhoeae*; such as symptoms, sexual contact with known positive, risk behaviour, or risk group we create a population of patients actually tested where the prevalence is much higher than in the general population. This will increase the PPV markedly in low prevalence populations.

Culture diagnostics has for decades been the gold standard internationally, despite suboptimal sensitivity, and NAATs have traditionally been hampered with poor specificity. The latest generation commercial NAATs have a substantially higher specificity, even though false positives still occur (81). The great distances and long transport times in Northern Norway made us suspect that culture diagnostics resulted in unacceptable high number of false negatives and we decided to develop a real-time PCR (Paper I). The results were very good (Paper I) and we therefore chose to clinically validate the method with both genital and extra-genital samples (Paper II). No diagnostic assay is complete without an evidence-base for when a TOC should be sampled, especially with the currently recommended empirical treatment in Norway (ciprofloxacin), and we investigated this in paper III. As the empirical treatment with ciprofloxacin is certainly suboptimal, we chose to genotypically and phenotypically characterize the circulating Norwegian *N. gonorrhoeae* strains in paper IV. This work substantially strengthened the evidence-base for changing the national treatment guidelines and demonstrated a widespread import of important international strains and antimicrobial resistance patterns.

**Development and clinical validation of a real-time PCR for diagnostics**

The real-time PCR developed for this study has so far proven to be 100% specific in all comparisons to culture and sequencing, as well as direct testing of known commensal *Neisseria* species, *N. meningitidis* and other closely related bacteria. The method is in use by several laboratories (domestically and abroad) with excellent performance characteristics. Combined with the practice of applying stringent inclusion criteria, the method described in paper I and II,
offers a highly sensitive and specific NAAT for diagnosing gonorrhoea.

Appropriate, quality assured NAATs are the preferred method for screening for and diagnosing *N. gonorrhoeae* in Europe and USA because of the superior sensitivity and ease of use compared to culture diagnostics. Meanwhile it is necessary to monitor AMR trends in every region/nation to maintain effective treatment guidelines. The ability of *N. gonorrhoeae* to take up, accumulate and maintain exogenous DNA, including AMR determinants, and the genetic similarity to other *Neisseria* species, makes it difficult to design reliable NAATs (6,59,81). This is exemplified by the finding by Whiley, et al. (120), showing a clinical *N. gonorrhoeae* harbouring an *N. meningitidis* porA sequence. Increased sequencing capacity has improved design prospects as ever increasing *Neisseria* genomes become available in databases. A potentially fruitful design approach could be to align DUS-poor regions of all *Neisseria* species, looking for *N. gonorrhoeae* unique sequences. This opens up possibilities to make dual target NAATs for diagnosing *N. gonorrhoeae*. Such self-confirming assays would be in compliance with current and upcoming guidelines recommending confirmation of *N. gonorrhoeae* positives in screening or diagnostics using NAATs, and could make it possible to get NAAT tests approved for extra-genital samples. Such an assay would also affect the PPV positively, making it more feasible and reliable to screen low prevalent populations for *N. gonorrhoeae*. Sequentially testing with two different PCR targets will also improve PPV.

The design of our real-time PCR proved to give a diagnostic sensitivity and specificity needed to reliably diagnose gonorrhoea in scarcely populated areas with low prevalence (Paper I and II).

Pharyngeal and rectal gonococcal infections can be difficult to treat (10,109,110) and are often asymptomatic resulting in potential reservoirs for infection. The ability to diagnose rectal and pharyngeal samples with NAAT is therefore of great value. The pharynx is also regarded as an
important site for recombination as several commensal *Neisseria* species are typically found there. The low number of pharyngeal and rectal samples available in each of the studies conducted in the present thesis was insufficient to fully conclude that our PCR method is highly reliable for these samples. However the total number of cases examined in paper I-III, provides good evidence to recommend using the method for extra-genital samples also. The ongoing use of the method as supplemental diagnostics for culture at Olafaklinikken and at the University Hospital North Norway also supports the high sensitivity and specificity for both urogenital and extra-genital specimens.

Improved diagnostics of *N. gonorrhoeae* and STIs in general can help limit the spread of HIV as it has been shown in several studies that an underlying STI can increase HIV transmission 5-10 times (121). Furthermore it is critical to make a correct diagnosis including all body site infected, and administer effective therapy. Adequate treatment is important to prevent emergence of AMR as we are running out of treatment options for gonococcal infections (7,8,122,123). For the same reason representative and timely AMR surveillance programs are important to ensure that the most effective drugs are recommended and used in each setting.

Even though increased sensitivity is a major driving force for the increased use of NAAT for diagnosis of *N. gonorrhoeae*, the social stigma and potential catastrophic family implications of a false positive diagnosis, have made specificity a priority over sensitivity. In cases of child-abuse, in many countries, culture diagnostics is the only approved diagnostic method.

It appears that the *porA* pseudogene is exceedingly conserved over time, probably due to lack of selective pressure, as it is not an expressed gene. There has not been identified any known functions for the dormant gene, but one may speculate that a gene without any function would at some point be deleted from the gonococcal genome if the metabolic burden is noticeable. However, no isolate of *N. gonorrhoeae* lacking the *porA* pseudogene, have been described. The
DUS sequence – ATG CCG TCT GAA (also called DUS10) – was found in one copy 9 kb upstream and two copies 18 and 30 bp downstream of the \textit{porA} pseudogene (3011 kb downstream from the reverse primer). The search was performed on one whole genome (NC_002946) and revealed 1522 DUS10 in the genome, an average of 1 DUS10/1415 bp.

Future work will involve finding a second target with similar specificity as the \textit{porA} pseudogene assay to make a self-confirmatory assay to meet present and future regulatory requirements and to be prepared for possible isolates with a deletion or alteration in the \textit{porA} gene or the advent of commensal \textit{Neisseria} species acquiring a \textit{porA} gene or pseudogene. Furthermore fitness studies to determine metabolic cost of maintaining the dormant \textit{porA} pseudogene and knockout studies to look for unknown functions of the pseudogene could shed new light on the likelihood of emergence of \textit{porA} pseudogene-free gonococcal isolates.

**Empirical determination of appropriate time for TOC**

In paper III all, but one individual (who did not deposit the second TOC until day 19), were negative inNAAT within two weeks after treatment. In that respect, we managed to find an appropriate evidence-based time for TOC on patients diagnosed with our NAAT and treated with cefixime. The AMR test results for the patients with a culture positive \textit{N. gonorrhoeae} isolate showed that 17/28 (68\%) were ciprofloxacin resistant. This secondary result of the study further supports previous reports (88,89,124) that the level of ciprofloxacin resistance in Norwegian isolates is too high to support continuing use of ciprofloxacin as empiric treatment.

In paper III we also debate the need for TOC in the Norwegian gonorrhoea management in context of the internationally supported abolishing of TOC. Our approach to make it feasible to get patients back for TOC was to use the same time intervals as was normally used for culture diagnostics – one week. This gives a lower time-resolution than was achieved by Bachmann et
al, who used fiscal incentives to have patients do self-sampling everyday in form of urine specimens. Experience from Paper II indicated that weekly testing for clearance was practical as we could expect longer clearing time than one week. The Bachmann, et al. Study (105) reported shorter clearance time, with a difference for urine in men (mean of 1.6 days), urine in women (mean of 1.7 days), and vaginal specimens (mean of 2.8 days).

The sampling method, treatment regimen and diagnostic method used are factors which explain why our study concluded that 2 weeks is appropriate for TOC, while Bachmann, et al. (105) observed full clearance by day 6 for male urine and day 9 for female vaginal swabs. However we managed to find an appropriate time for TOC valid for all sample types with the current diagnostics and treatment in the Norwegian population (primarily citizens of the capital). There is some concern that any later TOC than two weeks might be re-infection rather than treatment failure and should therefore be treated as such by doing contact-tracing again.

Empiric treatment recommendation for gonococcal infection is still ciprofloxacin in Norway (125). However, we did not follow Norwegian guidelines treating our study population. This is based on antimicrobial surveillance reports (NORM), previous findings (89) and international reports (126-130). The high proportion of ciprofloxacin resistant gonococcal isolates found in this study, indicates that there is an urgent need to change Norwegian guideline for gonorrhoea treatment.

The use of TOC seems reasonable in Norway, bearing in mind the Norwegian treatment guidelines and the high level of ciprofloxacin resistant isolates circulating in Norway. Despite this, Norway is still a low incidence country (124), further supporting the use of TOC (due to its ease). This study and a previous study (89) also showed that culture fails to detect several positive patients in comparison with NAATs, even under optimal sampling and transport conditions. The general practitioners (GPs) have a major role in diagnoses and treatment of STI
in Norway and several other countries. The discrepancies between culture and NAAT diagnostics might even be larger in routine diagnostic among GPs due to long transportation time to laboratory. Increasing use of NAATs and the increasing number of circulating multi-resistant \textit{N. gonorrhoeae} isolates might warrant a future revision of international guidelines for management and follow-up of gonorrhoea, including recommendations of substantially more frequent use of TOC.

**Molecular characterization of Norwegian gonococcal isolates**

In paper IV, we collected all viable \textit{N. gonorrhoeae} strains from six university hospitals in Norway to characterize the phenotypic and genotypic properties of circulating strains. These data were then coupled with epidemiological data from Norwegian surveillance system for communicable diseases (MSIS). The AMR testing revealed exceptionally high resistance level to the recommended empiric treatment in Norway – ciprofloxacin (78%). The work done for paper III reported 68% resistance two years prior to collecting isolates for paper IV. This shows a very frightening reluctance to change treatment regimens in light of empirical evidence. The present study also showed that the level of ciprofloxacin resistance was higher among isolates from individuals infected domestically (59%) compared to abroad (40%). According to the WHO, the empirical first-line treatment should be changed when the resistance exceeds 5% in the general population (and mainly any resistance in a high-risk group of frequent transmitters).

As this thesis is finalized, changes to the guidelines have been announced (personal communication). Furthermore, some isolates displayed intermediate susceptibility/resistance to cefixime and ceftriaxone which have also been used in Norway for treatment of patients attending Olafiahklinikken in Oslo.

The high number of resistance to ciprofloxacin Norway fits well with the international situation
There are also increasing number of reports on verified clinical failures with cefixime from Norway (76), England (77) and Japan (131). Recently, the first N. gonorrhoeae strain with high-level clinical resistance to ceftriaxone was identified in Japan and subsequently characterised in detail (8).

Forty-one (36%) of the isolates analyzed in paper IV were β-lactamase producing, and 17 (15%) displayed penA mosaic alleles. NG-MAST analysis revealed a highly diverse population with 72 different N. gonorrhoeae multiantigen sequence types. Thirty-seven of the sequence types (51.4%) had previously not been described. ST1407 has been found in Italy(132), England(133), Whales(133), Japan(134), and Sweden (118). The large number of new sequence types might be expected, as there has never been a similar molecular mapping of circulating Norwegian strains. A large import of gonorrhoea (39% of the isolates) also helps explain the heterogeneous population of N. gonorrhoeae. The most commonly found ST was ST1407 (n=11), containing a penA mosaic allele, a predictor of decreased susceptibility to ESCs, especially cefixime (117-119). This ST1407 clone, including its genetically highly related variants, has been shown to spread in many countries worldwide and account for a substantial proportion of the multidrug resistant gonococci (118,133,134). In the present study, the proportion of isolates containing a penA mosaic allele was higher among MSM (39%) than in heterosexuals (6.2%), which also caused the higher level of isolates with intermediate susceptibility/resistance to cefixime found among MSM, partly explained by the circulating ST1407. The susceptibility to these ESCs, the last remaining treatment options in many countries, is also decreasing worldwide (6,18,69).
10 Conclusions
We managed to establish a sensitive diagnostic NAAT while maintaining high specificity for diagnosing \textit{N. gonorrhoeae} in a low prevalence population. The method was clinically validated and has worked well (present thesis and use in routine diagnostics in several laboratories) on both genital and extra-genital samples. Appropriate evidence-based time for TOC was established.

New official figures from Norway show an increase from 95 to 215 of infected MSM from 2009 to 2010. There is no indication that this increase only constitutes an increase in incidence. Two major clinics in Oslo changed their sampling routines during this period to include more rectal and pharyngeal samples and supplement analysis using the PCR method described in paper I-III. This translates to an increase in positives on a national level from 269 in 2009 to 412 in 2010. Hopefully this enables better diagnostics of asymptomatic men who predominantly practice receptive oral and/or rectal sex, and that this in turn reduces spread of the infection domestically. The import of gonorrhoea and domestic heterosexual spread continues to be a challenge, especially with reports of treatment failures using ECSs (7,8,76).

Furthermore, this thesis helped provide strong evidences to force a change in the Norwegian guidelines for treatment of gonorrhoea.

A large study on the sensitivity and specificity of the latest generation commercially available NAAT for gonorrhoea diagnostics (81), confirms, especially for extra-genital specimens, the need for supplementary testing of gonorrhoea positive samples diagnosed with NAATs. Additional work remains to include such a secondary target in our method, even though our \textit{porA} pseudogene based method has yet to produce false positive results. NAATs detecting \textit{N. gonorrhoeae} specific DNA is an important supplement to traditional culture methods, but should be used with caution to avoid reporting false positive results. Implementation of a secondary, confirmatory assay or a self-confirming assay with two targets, is needed to get
international acceptance to use NAAT for also extra-genital samples.
11 References


11. **Ota, K. V., D. N. Fisman, I. E. Tamari, M. Smieja, L. K. Ng, K. E. Jones, A.**


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12 Papers
Paper II
Paper III
Paper IV
Science is a lot like sex. Sometimes something useful comes of it, but that’s not the reason we’re doing it.
- Richard Feynman-