

**REAL-TIME POLYMERASE CHAIN
REACTION SPECIES SPECIFIC FOR
*NEISSERIA GONORRHOEAE***

MASTER'S THESIS IN MOLECULAR BIOLOGY

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SUMMARY

Gonorrhoea is a sexually transmitted disease with both medical and social implications, thus clinicians urge for rapid, specific and sensitive diagnostic tools. Phenotypic and genotypic approaches have been tested with variable outcome.

Today the gold standard for diagnosing gonorrhoea is cultivation combined with phenotypic tests. The challenge is that these methods require living bacterial cells and the fastidious *N. gonorrhoeae* survives poorly outside its human host.

To increase the sensitivity, nucleic acid amplification tests (NAATs) targeting *N. gonorrhoeae* have been developed and are commercially available. However, studies evaluating specificity in several of these methods, have uncovered cross reactivity causing false positive results which may have major impacts for the individual.

The overall aim of this study was to establish and clinically validate a qualitative in-house real time Polymerase Chain Reaction (PCR) method targeting *porA* pseudogene in *N. gonorrhoeae*. Reference strains (n= 32) and clinical isolates (n= 168) of *N. gonorrhoeae* and commensal *Neisseria* (n= 66) were examined as well as other micro organisms able to colonize gonorrhoea sampling sites (n= 46).

Functional and specific primers for the real-time PCR were designed from the *porA* sequence and the in-house real-time PCR amplification was evaluated, and the technical performance was found to be both specific and sensitive. The in-house PCR detected all *N. gonorrhoeae* strains included, and showed no cross reactivity with other microbes.

The Universal Transport Medium (UTM-RT) system was tested for its suitability in transporting samples for PCR.

The clinical validation was performed on 360 samples and compared the outcome from the *porA* PCR and the phenotypic techniques for gonococcal identification. The *porA* PCR was found to be more sensitive than the traditional phenotypic tests, especially in samples from sites other than urethra and cervix.

It can be concluded that the *porA* pseudogene real-time PCR is species specific for *N. gonorrhoeae* and will be a valid supplement in the diagnosis of gonorrhoea.

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1 INTRODUCTION

Gonorrhoea is a sexually transmitted disease caused by bacteria called gonococci. The disease was described in ancient times, but until the late nineteenth century it was mixed up with syphilis. Eventually microscopy could differ between the organisms and give a reliable diagnosis. Human is the only known reservoir for gonorrhoea. It is transferred during all acts of sexual activity, and an infection cause characteristic symptoms if established. Reliable diagnostic tools are important not only because gonorrhoea is a severe disease but also because the means of spreading will always have a negative social impact on people given the diagnosis. Gonococci are fastidious bacteria, and survive poorly outside its human host. Thus diagnostic methods have been challenged by the problem of retrieving living bacteria for phenotypic tests. There have been several attempts to identify gonococci by specific characteristic in its genome, but these have been less successful. Due to its fluctuating genome it has been difficult to find a specific target for the gonococci. Here I present an attempt to establish a reliable diagnostic tool for reproducible detection and specific identification of gonococci in samples from different body sites.

1.1 The genus *Neisseria*

Neisseria gonorrhoeae belongs to the family *Neisseriaceae* in the genus *Neisseria*. The bacteria in the genus are Gram-negative diplococci (except *N. elongata* which is rod shaped), and the only human pathogens are *N. gonorrhoeae*, causing gonorrhoea, and *N. meningitidis*, causing meningococcal meningitis. *N. meningitidis* may occur in a carrier state in the pharynx, but *N. gonorrhoeae* is never part of the normal human flora, and causes disease whenever colonized in its human host.

N. gonorrhoeae primarily infects the mucosa membrane in the male urethral tract and the cervical mucosa membrane in females, but the bacteria may also colonize the mucosa membrane in the pharynx, rectum and even cornea of the eye. The commensal *Neisseria* (i.e. *N. lactamica*, *N. sicca*, *N. cinerea*, *N. flavescens* and *N. elongata*) and the *N. meningitidis* are most common in the mucosa of the pharynx. But there are reports that *N. cinerea*, *N. lactamica*, *N. sicca* and *N. subflava* also have been isolated from the genital mucosa^{1,2}.

1.2 Physiology and characteristic structures in *N. gonorrhoeae*

N. gonorrhoeae is a fastidious aerobic bacterium. It requires nutritious media for growth and prefers humid atmosphere that contain approximately 5 % carbon dioxide. The bacteria have an optimal growth temperature of 35-37°C, and have minimal chances to survive at low temperatures³. It produces oxidase, catalase and oxidizes glucose if available. *N. gonorrhoeae* do not expose flagella and produces no endospores³.

Examined in the microscope, the bacterial cells have a characteristic coffee bean shape with adjacent side flattened in the microscope. In older colonies of *N. gonorrhoeae*, the cells may appear swollen and round due to autolysis.

The cell wall structure of *N. gonorrhoeae* is similar to other Gram negative bacteria with an inner cytoplasmic membrane and an outer membrane composed of proteins, phospholipids, and lipopolysaccharide (LPS), and a thin peptideoglycan layer between the inner cytoplasmic membrane and the outer membrane. The LPS in the *N. gonorrhoeae* are referred to as lipooligosaccharides (LOS), due to that they consist of a shorter saccharide chain compared to the LPS. Several proteins are exposed on the cell surface of the *N. gonorrhoeae*, pili, porin proteins (PorB), Rmp proteins (Reduction modifiable protein) and Opa (opacity protein) proteins. They are all thought to have different roles in invasion of the host and virulence activity^{3,11}.

Figure 1 is a schematic drawing of the cell wall structure in *N. gonorrhoeae*.

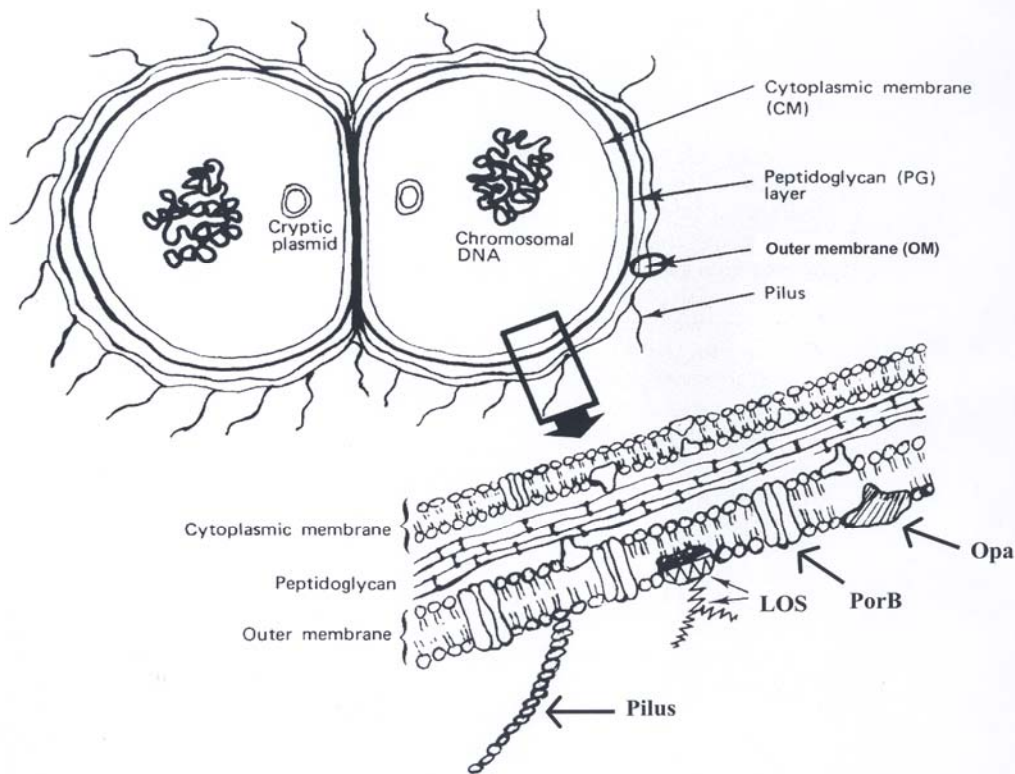


Figure 1: Modified schematic drawing from Danielsson D (Biology of *Neisseria gonorrhoeae*, 1986) kindly provided by Magnus Unemo¹³ showing *N. gonorrhoeae* as a characteristic diplococci. The cell wall, bacterial chromosome, cryptic plasmid and some of the interesting structures and proteins of the outer membrane (pilus, lipooligosaccharide (LOS), PorB, and Opa protein) are shown.

The genomic similarity among the *Neisseria* species is well reported for the pathogenic species. *N. meningitidis* and *N. gonorrhoeae* share 80-90% homology in their genomic DNA, and their housekeeping genes may show 98% sequence identity^{4,5}. Despite their high genomic similarity there are cellular differences between the two species. One major difference is the polysaccharide capsule which only *N. meningitidis* expresses.

In 2000 the *N. gonorrhoeae* strain FA 1090 had its entire chromosome fully sequenced 2,153,922 base pairs (bp) long (www.ncbi.nlm.nih.gov/sites/entrez). Several plasmids have been found in *N. gonorrhoea*, and different types of plasmids circulate among the strains. A cryptic plasmid has been found in a majority of strains, and the rapid spreading of beta-lactam resistance is thought to be plasmid mediated⁶.

Transformation is the main mechanism for horizontal genetic exchange transfer in *N. gonorrhoeae*, but extra-chromosomally DNA can be transferred by conjugation.

N. gonorrhoeae is competent throughout the entire lifecycle and donate genetic material to the environment by active secretion or by autolysis. With a type IV secretion system, similar to type IV found in *E. coli*, the bacterium can secret DNA to its neighboring cells. The factor that triggers the secretion mechanism is poorly documented. Autolysis occurs when the bacterium is under unfavorable growth conditions, like lack of nutrients and change in pH. A specific enzyme is thought to be responsible for the autolysis process ⁷. A successful transformation is dependent on several factors, and the presence of pili is thought to be important in DNA uptake ⁸. However, transformation in *N. gonorrhoeae* occurs only if the DNA contains a specific sequence, called DNA Uptake Sequence (DUS) ⁷. The sequence comprises of 10 base pairs 5'-GCCGTCTGAA-3' and is frequently distributed in the *Neisseria* genome⁷. The above mentioned *N. gonorrhoeae* FA 1090 has 1965 DUS present in its genome ³⁰.

It has been reported in several studies that genetic exchange occurs between commensal *Neisseria* and pathogenic *Neisseria* ^{9,10}. A frequent exchange of *tbpB* sequences (gene encodes the TbpB, transfer binding protein) from commensal *Neisseria* has contributed to antigenic variations in *N. meningitidis* ⁹. A great number of virulence genes found in pathogenic *Neisseria* have also been found in commensal *Neisseria* ¹⁰.

1.3 Pathogenesis and clinical manifestations of gonorrhoea

Expression of pili is one of many virulence mechanisms in the *N. gonorrhoeae*. *N. gonorrhoeae* attach to the epithelial cells of the mucosa membrane by its pili, which is thought to be the initial step in gonococcal invasion. Figure 2 gives an overview of the *Neisseria* attachment. The OPA proteins can be expressed by a variety of alleles. These proteins are responsible for both a tighter attachment and the migration into the host cell. Inside the cell the bacteria multiply. Usually they remain in the epithelial layers, but the bacteria can cross over into the bloodstream causing disseminated infection. LOS in *N. gonorrhoeae* has endotoxin activity and stimulates the inflammatory response in the host. The Rmp proteins are believed to protect other surface antigens due to its production of antibodies that block bactericidal activity ^{3,11}.

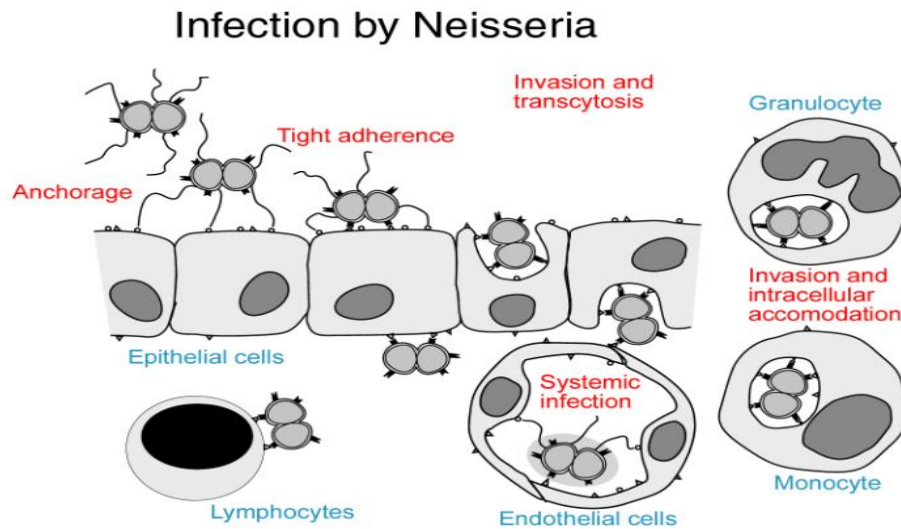


Figure 2: The figure illustrates the primary attachment of *Neisseria* to the surface of mucosal epithelial cells mediated by the pilus (thin filament). Opa proteins (black dots) cause a tighter interaction. Once attached, the bacteria are able to pass through the epithelial layer and exit into bloodstream causing disseminated infections. The image is kindly provided by Professor Scott D. Gray-Owen.

An invasion of *N. gonorrhoeae* primarily affects the urethral mucosa membrane, but mucosa membrane in body site as cervix, rectum and pharynx can also be invaded. Invasion of pharynx and rectum are often asymptomatic, and asymptomatic carriers can act as a reservoir for gonococcal infection but they also risk complicated gonorrhea infection. Gonococcal infection of urogenital sites is more frequently symptomatic than asymptomatic. If infections are untreated the symptoms often clear within weeks, and an asymptomatic “carrier state” can be established^{3,11}.

Symptoms of infection occur within 2-7 days and are often more appearing in male than females. Gonorrhea is manifested by urethritis, dysuria and purulent discharge from urethra³. In male complicated infections may lead to inflammation of the prostate and testis. And in

female, infection of the endocervix is most common, and may cause vaginal discharge. If the infection invades further it may extend through the uterus to the fallopian tubes causing inflammation in the tubes and subsequent in the ovaries³. And a severe infection may cause infertility. If *N. gonorrhoeae* crosses the epithelial layer and enters the bloodstream, this may result in septicaemia, which is manifested by pustular skin lesion on extremities and can lead to complication as endocarditis, meningitis and arthritis³. Another complication caused by *N. gonorrhoeae* is eye infections in newborns infected during passage of the birth canal, untreated this can lead to scarring of the cornea³. Once this was a major cause of blindness, but today this is rare due to screening and prophylaxis.

1.4 Antibiotic treatments

Historically, Penicillin was a common antibiotic in the treatment of gonorrhea. Nowadays, Penicillin is no longer used because many strains of *N. gonorrhoea* have reduced susceptibility to penicillin. Plasmid encoded β -lactamase production is one common resistance mechanism⁶. The spread of such plasmids have contributed to a rapid increase in number of resistant strains. New antibiotics or combinations of drugs must be used to treat these resistant strains. According to Norwegian guidelines, ciproxin, ciprofloxacin or ofloxacin are antimicrobial agents for treatment of uncomplicated gonorrhoeae. However, an increased resistance to ciproxin/ciprofloxacin has been observed during an ongoing test of cure study (Hjelmevoll et al., to be published).

1.5 Epidemiology

In Norway the reported incidence of gonorrhea peaked in the years 1975 and 1976 with about 15 000 cases of gonorrhea annually. Since then the number of annually reported cases has decreased, and today the incidence of gonorrhea is considered low compared to other countries. The number of gonorrhoeae infections in 2007, stated by the Norwegian institute of public health, was 238. Of the 238 cases 160 were from heterosexuals, while 77 cases were reported from homosexuals. Of the 160 heterosexuals, 131 were males and 29 females, and Norway was stated as location of infection in 40 % of the males and 31 % of the females. Antibiotic resistant gonococci were found in 53 % of the 238 gonorrhea cases.

Eye infection caused by gonococci is most common in newborns, but today gonococcal eye infections are rare in Norway. The last reported case of eye infection was stated in 1991 by Norwegian institute of public health.

As mentioned, *N. gonorrhoeae* can cause disseminated infections. However, there are few cases reported. Approximately 0.1 – 0.5% of all gonococcal infections may lead to a disseminated infection (www.nfi.no, home page of Norwegian institute of public health).

1.6 Detection of *N. gonorrhoeae*

There are several ways to identify *N. gonorrhoeae*, both phenotypic and genotypic. The gold standard is still cultivation and microscopy of smear from urogenitalia. The microscopy of smear in females is less reliable, then for males, and must be confirmed by culture. A number of phenotypic diagnostic techniques have been developed based on; growth requirements of specific nutrient and cofactors (auxotyping), enzymatic activity (i.e. Api NH) and serological reactivity against surface antigens (i.e. Phadebact monoclonal Test). These methods have in common that they require living bacterial cells, and this is one of the major problems when using phenotypic techniques in diagnosing of gonorrhoea. The *N. gonorrhoeae* survives poorly outside its human host, and tend to have a low recovery rate from extra genital samples¹⁵. Another problem is due to overgrowth of normal flora, especially in sample sites such as pharynx, cervix and rectum, which also decreases the sensitivity.

In the attempt to improve diagnosing of gonorrhoea there have been developed nucleic acid amplification tests (NAATs) as a supplement to the diagnostics. However, the qualities of many of these methods have been evaluated during the last years, i.e. targets as the *cppB* gene on cryptic plasmid has been reported missing from several strains, causing a potentially false negative diagnosis¹⁸, many of the studies have shown variability specificity^{1,17,18}. Even so, detection of nucleic acid may increase sensitivity.

1.7 The *porA* pseudogene

Porins are the most represented outer membrane proteins in the pathogenic *Neisseria*. *N. meningitidis* is the only species known to express both PorA and PorB, while *N. gonorrhoeae* is known to only express PorB. However, *N. gonorrhoeae* possesses the *porA* gene despite of not expressing the protein, and the *porA* has been proposed as pseudogene in *N. gonorrhoeae*⁵. Phylogenetically it seems like these two species share common ancestor, and during evolution *N. gonorrhoeae* had no need for expressing the PorA protein when invading the host, leading to a “silencing” of the gene. The lack of PorA expression may indicate that the PorA protein is not essential to the survival of the bacteria¹².

“Silencing” of the gene is caused by an inactivation mutation in the promoter region, and frame shift mutations in the amino acid coding region of the gene¹³. Due to diversity in the *porA* gene between the pathogens, the *porA* pseudogene has been proposed as a suitable target for nucleic detection of the *N. gonorrhoeae*¹²⁻¹⁴

1.8 Background

In our laboratory diagnosing gonorrhoeae is based on cultivation and phenotypic identification. In optimized conditions this has a high specificity and fairly good sensitivity for detection of *N. gonorrhoeae*. However, the recovery of *N. gonorrhoeae* from clinical samples is influenced by pre-analytic risk factors such as, sampling, sample site, temperature variations during transport, transport duration and transport media.

The agar plate used for cultivation in our laboratory is selective for *N. gonorrhoeae*, but several other bacteria and fungi find nourishment in this agar. This complicates the detection of gonococcal colonies. Furthermore the cultivation combined with the phenotypic detection tools can be time consuming.

N. gonorrhoeae sampled from pharynx and rectum have a lower recovery rate compared to samples from urogenitalia when cultivated¹⁵. The transport media consists of a charcoal agar, and a purified cotton swab (Amies Charcoal, COPAN, Italy), but this agar is not optimal for the nutritional requirements of *N. gonorrhoeae*. The viability of the bacteria is partly maintained if the samples arrives the laboratory within 24 hours¹⁶, but this is not always the case for samples sent by practitioners from rural areas.

A genotypic approach to diagnose gonorrhoea is proposed as a supplement to increase the sensitivity. As mentioned, i.e. nucleic amplification tests (NAATs) require only intact DNA. This might reduce the impact of the pre-analytic risk factors. Furthermore compared to cultivation the NAAT might decrease the time from receiving the sample to diagnosing gonorrhoea.

There are several commercial NAATs available, but studies have reported low specificity in many of these; some auxotypes or individual strains are not identified as *N. gonorrhoeae*, and some *Neisseria* species can be misclassified as *N. gonorrhoeae*^{1,17,18}. Cross reactivity is specially the case when sampling from pharynx, where commensal *Neisseria* species share habitat with the human pathogen *Neisseria* species.

One of the major problems in developing NAATs for detection of *N. gonorrhoeae* was finding a specific target sequence. *N. gonorrhoeae* is naturally competent and the genetic exchange that occurs between the *Neisseria* species makes this difficult^{9,10}.

2 AIMS OF STUDY

The overall aim was to establish a validated species specific qualitative real-time Polymerase Chain Reaction (PCR) assay for detection of *N. gonorrhoeae*.

- I. To design species specific primers to detect *N. gonorrhoeae*.
- II. To perform a technical evaluation of the in-house PCR by determining efficiency and detection limit for the PCR and also its analytic specificity and sensitivity.
- III. To find and test the suitability of a transport media containing a buffer that preserves the DNA and comprises of swabs suitable for different sample sites; cervix, urethra, rectum and pharynx. In addition it should be suitable for the transport of other bacteria and viruses, with already established in-house PCR in our laboratory.
- IV. To perform a clinical validation of the in-house PCR by comparing it to phenotypic identification techniques.

3 MATERIALS

3.1 International *Neisseria* reference strains

In total we analyzed DNA from 44 International *Neisseria* reference strains with the in-house PCR. They 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) in Denmark. These reference strains included *Neisseria gonorrhoeae* (n=32), *Neisseria meningitidis* (n=4), *Neisseria sicca* (n=2), *Neisseria subflava* (n=1), *Neisseria flavescens* (n=1), *Neisseria mucosa* (n=2), *Neisseria lactamica* (n=1), and *Neisseria cinerea* (n=1). The *N. gonorrhoeae* reference strains originated from different geographic areas worldwide and have been isolated during the last four decades. And in 29 of the 32 *N. gonorrhoeae* reference strains the entire *porA* pseudogene has been sequenced. Isolation of DNA was performed as described in chapter 4.3.1 Isolation of DNA.

Table 1: Reference strains and reference number, year of isolation are stated where available.

Species	Reference ID	Year
<i>N. gonorrhoeae</i>	ATCC 23051	1966
<i>N. gonorrhoeae</i>	ATCC 19424	1966
<i>N. gonorrhoeae</i>	CCUG 42289	1973
<i>N. gonorrhoeae</i>	CCUG 42290	1973
<i>N. gonorrhoeae</i>	CCUG 42287	1978
<i>N. gonorrhoeae</i>	CCUG 13572	1983
<i>N. gonorrhoeae</i>	CCUG 13574	1983
<i>N. gonorrhoeae</i>	CCUG 13576	1983
<i>N. gonorrhoeae</i>	CCUG 13577	1983
<i>N. gonorrhoeae</i>	CCUG 13578	1983

Species	Reference ID	Year
<i>N. gonorrhoeae</i>	CCUG 13579	1983
<i>N. gonorrhoeae</i>	CCUG 13583	1983
<i>N. gonorrhoeae</i>	CCUG 13584	1983
<i>N. gonorrhoeae</i>	CCUG 13586	1983
<i>N. gonorrhoeae</i>	CCUG 15821	1984
<i>N. gonorrhoeae</i>	CCUG 15823	1984
<i>N. gonorrhoeae</i>	ATCC 31426	1990
<i>N. gonorrhoeae</i>	CCUG 13581	1995
<i>N. gonorrhoeae</i>	CCUG 33978	1995
<i>N. gonorrhoeae</i>	CCUG 33979	1995
<i>N. gonorrhoeae</i>	CCUG 34327	1995
<i>N. gonorrhoeae</i>	CCUG 34447	1995
<i>N. gonorrhoeae</i>	CCUG 43069	1995
<i>N. gonorrhoeae</i>	CCUG 41813	1996
<i>N. gonorrhoeae</i>	CCUG 36701	1996
<i>N. gonorrhoeae</i>	CCUG 36702	1996
<i>N. gonorrhoeae</i>	ATCC 49226	1997
<i>N. gonorrhoeae</i>	WHO-D	1998
<i>N. gonorrhoeae</i>	CCUG 41810	1999
<i>N. gonorrhoeae</i>	CCUG 41811	1999
<i>N. gonorrhoeae</i>	CCUG 41812	1999
<i>N. gonorrhoeae</i>	CCUG 42285	NA
<i>N. cinera</i>	CCUG 2156	1973
<i>N. flavescens</i>	NCTC 8263	1952
<i>N. lactamica</i>	CCUG 5853	1976
<i>N. meningitidis</i>	ATCC 13090	NA
<i>N. meningitidis</i>	ATCC 13102	NA
<i>N. meningitidis</i>	CCUG 41446	1998
<i>N. meningitidis</i>	CCUG 41447	1998
<i>N. mucosa</i>	NCTC 10774	1971
<i>N. mucosa</i>	NCTC 10777	1971
<i>N. sicca</i>	ATCC 9913	1985
<i>N. sicca</i>	CCUG 34456	1995
<i>N. subflava</i>	ATCC 19243	1985

NA, Not available

3.2 Clinical isolates

In total we examined 168 clinical isolates of *N. gonorrhoeae*, and 54 clinical isolates of other *Neisseria* species. DNA from all the clinical isolates was analyzed by the *porA* PCR.

Of the 168 *N. gonorrhoeae* isolates 76 were cultured in Archangelsk Russia in 2004. These isolates had initially been cultured on non selective media (NPO Microgen, Stavropol, Russia) and identified as *N. gonorrhoeae* by characteristic colony morphology, Gram staining and oxidase reaction¹⁹. In addition they were also confirmed as *N. gonorrhoeae* at the Department of Microbiology and Infection control, University Hospital of North Norway, by cultivation on selective media, oxidase reaction, sugar oxidation and a specific monoclonal antibodies test. This is described in chapter 4.2 Phenotypic identification of *N. gonorrhoeae*.

Furthermore 14 were isolated at Department of Microbiology and Infection control, University Hospital of North Norway, in 2003-2004 and 9 isolates were received from Norwegian Organization for Surveillance of Antibiotic Resistant Micro organisms (NORM). The isolates were identified as mentioned above.

We received 13 isolates from Lene Berthelsen at SSI in Denmark, identified by cultivation on selective medium and MINIBACT-N and 5 isolates provided by Helen Palmer¹ and 51 Swedish isolates from 1998-2001 with known *porA* pseudogene sequence were provided by Magnus Unemo¹³.

In addition clinical isolates (n=54) of other *Neisseria* species were included. *N. gonorrhoeae* subspecies *kochii* (n=4), *Neisseria meningitidis* (n=7), *Neisseria sicca* (n=7), *Neisseria subflava* (n=11), *Neisseria flavescens* (n=3), *Neisseria mucosa* (n=5), *Neisseria lactamica* (n=7), *Neisseria cinerea* (n=7), *Neisseria caviae* (n=1), *Neisseria animalis* (n=1), and *Neisseria polysaccharea* (n=1). These isolates were identified by sugar oxidation, oxidase reaction and API NH (BioMerieux, La Balme-les-Grottes, France). Isolation of DNA was performed as described in chapter 4.3.1 Isolation of DNA.

3.3 Heterogeneous control strains

In total we examined DNA from 47 other micro organisms as well as human DNA. The heterogeneous control strains were used to uncover cross-reaction with DNA from other organisms than *Neisseria* species. The in-house PCR was tested with DNA from the following organisms: Gram negative bacteria (n=18), Gram positive bacteria (n=23), fungus (n=1), viruses (n=4) and human DNA. More details of the strains are listed in Table 2. Isolation of DNA was performed as described in chapter 4.3.1 Isolation of DNA. Bacteria and viruses commonly found in the normal flora in sites as cervix, pharynx, rectum and urethra, were selected for specificity testing. In addition, bacteria and viruses with already established in-house PCRs were added to the test panel as they are at risk of contaminate the environment of our laboratory.

Table 2: Heterogeneous control strains used in this study; media and culture conditions

Heterogenous control strains	ID	Media** and culture condition
<i>Acinetobacter baumannii</i>	ATCC 19606	Lactose agar (37°C)
<i>Actinomyces odontolyticus</i>	ATCC 17929	Anaerobic agar (37°C anaerobic milieu)
<i>Bordetella pertussis</i>	ATCC 8467	Chocolate agar (35°C CO ₂)
<i>Candida albicans</i>	ATCC 10231	Blood agar (37°C)
<i>Chlamydia trachomatis</i>	Tebu bio *	Purified genomic DNA from manufacture
<i>Citrobacter freundii</i>	ATCC 43864	Lactose agar (37°C)
<i>Corynebacterium diptheriae</i>	ATCC 19409	Chocolate agar (37°C)
<i>Eikenella corrodens</i>	CCUG 2138	Blood agar (37°C)
<i>Enterobacter sakazakii</i>	ATCC 29544	Lactose agar (28°C)
<i>Enterococcus casseliflavus</i>	ATCC 25788	Blood agar (37°C)
<i>Enterococcus faecalis</i>	ATCC 19433	Blood agar (37°C)
<i>Enterococcus faecium</i>	ATCC 19434	Blood agar (37°C)
<i>Enterococcus flavescens</i>	ATCC 49996	Blood agar (37°C)
<i>Enterococcus gallinarum</i>	ATCC 49608	Blood agar (37°C)
<i>Enterococcus saccharolyticus</i>	ATCC 43076	Blood agar (37°C)
<i>Escherichia coli</i>	ATCC 25922	Lactose agar (37°C)
<i>Gardenella vaginalis</i>	ATCC 14018	Blood agar (35°C CO ₂)
<i>Haemophilus influenzae</i>	ATCC 35039	Chocolate agar (35°C CO ₂)
<i>Helicobacter pylori</i>	ATCC 43504	Chocolate agar (37°C microaerophilic milieu)

Heterogenous control strains	ID	Media** and culture condition
<i>Klebsiella pneumoniae</i>	ATCC 13883	Lactose agar (37°C)
<i>Lactobacillus plantarum</i>	ATCC 4008	Blood agar (37°C)
<i>Legionella pneumophila</i>	ATCC 33153	Chocolate agar (35°C CO ₂ Humide milieu)
<i>Listeria monocytogenes serot. 1</i>	ATCC 19111	Blood agar (37°C mikroaer)
<i>Micrococcus diversus</i>	ATCC 25590	Blood agar (28°C)
<i>Micrococcus luteus</i>	ATCC 9341	Blood agar (37°C)
<i>Moraxella catarrhalis</i>	ATCC 23246	Blood agar (37°C)
<i>Moraxella osloensis</i>	ATCC 10973	Blood agar (37°C)
<i>Mycoplasma pneumoniae</i>	MI83p27	Purified DNA donated by Linda B.Heide
<i>Proteus mirabilis</i>	ATCC 35659	Lactose agar (37°C)
<i>Pseudomonas aeruginosa</i>	ATCC 27853	Lactose agar (37°C)
<i>Rhodococcus equi</i>	ATCC 6939	Blood agar (37°C)
<i>Staphylococcus aureus</i>	ATCC 25923	Blood agar (37°C)
<i>Staphylococcus aureus (MRSA)</i>	ATCC 43300	Blood agar (37°C)
<i>Staphylococcus epidermidis</i>	ATCC 12228	Blood agar (37°C)
<i>Staphylococcus lugdunensis</i>	ATCC 49576	Blood agar (37°C)
<i>Staphylococcus saprophyticus</i>	ATCC 15305	Blood agar (37°C)
<i>Streptococcus gruppe B</i>	ATCC 12386	Blood agar (37°C)
<i>Streptococcus mutans</i>	ATCC 25175	Blood agar (37°C)
<i>Streptococcus pneumoniae</i>	ATCC 6305	Blood agar (37°C)
<i>Streptococcus pyogenes gr.A</i>	ATCC 19615	Blood agar (37°C)
<i>Streptococcus salivarius</i>	ATCC 13419	Blood agar (37°C)
<i>Ureaplasma urealyticum</i>	ATCC 27618	Mycoplasma Broth (37°C anaer.)
<i>Herpesvirus 1</i>	Patient samples	Provided by DNA by Linda B.Heide
<i>Herpesvirus 2</i>	Patient samples	Provided by DNA by Linda B.Heide
<i>Metapneumovirus</i>	Patient samples	Provided by DNA by Linda B.Heide
<i>Rhinovirus</i>	Patient samples	Provided by DNA by Linda B.Heide
Human DNA	Patient samples	Provided by DNA by Linda B.Heide

*(Le Perray en Yvelines, France).

**All media were produced in-house at the Department of Microbiology and Infection control, University Hospital of North Norway. Contents of the media are listed in table 4.

3.4 Clinical samples

In total 360 samples from 242 individuals were included in the study, with 284 samples from 185 males and 76 samples from 57 females. The distribution of samples and sample sites among males and females are listed in Table 3. All clinical samples were analyzed by the *porA* PCR and phenotypic diagnostic tools.

All individuals with suspected infection of gonorrhoea were included, in total 269 individuals participated.

Table 3: Distribution of samples and sample sites in males and females.

Sex	Urethra	Rectum	Pharynx	Cervix	Total
Male	95	80	109	0	284
Female	14	4	10	48	76
Total	109	84	119	48	360

The samples were collected in a period from January 2006 through May 2006, and were taken from varying localizations; urethra, rectum, pharynx and cervix, depending on the case history. The recruiting of patients and sample collection were performed by the Olafia clinic in Oslo, Norway. The samples were brought to the Department of Microbiology Ullevål University Hospital in Oslo, Norway (located 3 km in distance from Olafia) for phenotypic diagnostics and to the Department of Microbiology and Infection control, University Hospital of North Norway in Tromsø (located 1640 km in distance from Olafia), for analysis by *porA* PCR.

The samples for PCR were collected using appropriate swabs that were placed and transported in 3 ml Universal Transport Medium UTM-RT transport media (Copan, Italy). The urethral flocked swab was used for sampling in the urethra, and the endocervical flocked swab was used to take samples from the cervix, rectum and pharynx. All samples were stored at 4°C before shipping them with regular mail.

In parallel identical swabs were taken from the same sites and placed in a M40 transport media (Copan) for phenotypic diagnostic. The samples were stored maximum 3 hours at room temperature before the laboratory received them.

The samples for phenotypic diagnostics were consistently taken before the samples for PCR to avoid interference with the validated routine diagnostic.

3.5 UTM-RT system

The UTM-RT transport system (Copan, Italy) consists of a polypropylene container with 3 mL transport buffer in combination with one of several flocked swabs available for different sample sites. After sampling, the respective swabs should be placed in the buffer. According to the manufacture the buffer contains the following ingredients; Modified Hank's Balanced Salts, Bovine Serum Albumin, Gelatin, Sucrose, L-glutamic acid, Hepes, Phenol Red, Amphotericin and Colistin. Phenol Red works as a pH indicator. Hepes works as a buffer. The product should be stored in its original container at 2-25°C until used.

4 METHODS

4.1 Growth media

All International *Neisseria* reference strains and clinical isolates of *Neisseria* species were cultivated for 24 to 48 hours at 37°C in 5% CO₂ on non-selective chocolate agar.

The bacteria and fungi, which constitute the heterogeneous control strains, were cultivated from frozen stocks (-70°C) by using suitable media and incubation conditions as described in table 2.

Table 4: Contents in agar and broth.

Agar	Contents
Anaerobic agar	Anaerobic Basal Agar (Oxoid Ltd, Basingstoke, UK) defibrinated horse blood, distilled water.
Blood agar	Blood Agar Base No2 (Oxoid Ltd, Basingstoke, UK), distilled water.
Chocolate agar	GC-Agar Base Medium CM367B (Oxoid Ltd. Basingstoke, UK), distilled water, defibrinated horse blood, glucose solution 25 % and Vitox solution (Oxoid Ltd, Basingstoke, UK).
Lactose agar	Tryptose blood agar base (Oxoid Ltd, Basingstoke, UK) Lactose LP0070, bromtymolblue.
Modified Thayer-Martin agar	GC-Agar Base Medium CM367B (Oxoid Ltd. Basingstoke, UK), distilled water, defibrinated horse blood, glucose solution 25 % and Vitox solution (Oxoid SR090H and SR090A). Colistin 6.0, Lincomycin 1.0 µg/mL, Trimetophrim 6.5 µg/mL and Amfotericin B 2.0 µg/mL
Mycoplasma broth	PPLO Broth (Difco, USA), Yeast Autolysate(Oxoid Ltd, Basingstoke, UK), phenolred 0.2 %, Horse serum (Gibco, USA), Urea (Merck, USA) Ampicillin 100 mg/mL, Amphotericin 5 mg/mL, 5 MHCL, Manganese reagent.

4.2 Phenotypic identification of *N. gonorrhoeae*.

The following protocols are adapted from the Department of Microbiology and Infection control, University Hospital of North Norway, which uses them as a part of the routine diagnosis of gonorrhoea. In addition they perform antibiotic susceptibility testing but this was not performed in this study, and therefore the testing is not described.

4.2.1 Modified Thayer-Martin agar plate

Inoculated plates are incubated for 24 to 48 hours at 37°C in 5% CO₂. *N.gonorrhoeae* grows in this agar by medium sized grayish colonies²⁰. Contents of the agar plate are listed in table 2.

4.2.2 Oxidase protocol

Oxidase reduction is based on the bacteria's ability to produce enzymatic oxidase. In the presence of enzyme, the reagent tetramethyl-p-phenylenediamine (Oxoid) oxidises, and this is visualized by change of colour of the reagent. In this study, the reagent containing phenol blue, form a complex with the oxidase enzyme and a bright blue colour appear²¹.

Procedure:

1. Add a drop of reagent on filter paper.
2. Inoculated the suspected colony.
3. Instant appearance of blue color indicates presence of oxidase.

Evaluation:

N. gonorrhoeae reacts instantly and blue colour should appear within seconds.

4.2.3 Gram staining protocol

Gram staining is based on bacteria's ability to retain color in the bacteria cell wall. The reagents are Crystal violet, Gram's iodine, Safranin (Becton Dickinson) 96% ethanol and saline. Crystal violet and Gram's iodine interact and produces a complex inside the cell. The cell wall of gram positive bacteria contains a thick peptideglycan layer, and they do not decolorize by alcohol. The cell of gram positive bacteria is stained violet. The cell wall of the gram negative bacteria has a different structure, and will decolorize when exposed to alcohol. The gram negative bacteria are stained Safranin red²².

Procedure:

1. Add one drop of saline solution on a slide.
2. Harvest and blend suspect colony in the saline solution.
3. Air dry the slide, and then heat it to firmly mount the bacteria on the slide.
4. Add the primary stain crystal violet and incubate 1 minute. This step stains all cells violet.
5. Add Gram's iodine, for 30 seconds. This fixes the crystal violet to the bacterial cell wall. All cells remain violet.
6. Wash with ethanol to decolorize.
7. Add the secondary stain, safranin, and incubate 1 min
8. Wash with water for a maximum of 5 seconds.
9. Gently wipe the slide to remove excess water.
10. Use a microscope to evaluate the result.

Evaluation: The Gram positive bacteria stain purple, while the Gram negative bacteria stain pink. In the microscopy the *N.gonorrhoeae* are pink diplococci, coffee bean shaped.

4.2.4 Phadebact Monoclonal GC Test protocol

Phadebact Monoclonal GC Test (Bactus AB, Sweden) is a co agglutination test. The antibodies in the WI and WII/WIII reagents react with group specific parts of a membrane protein called Protein I. When a sample containing *N. gonorrhoeae* is mixed with the reagent, antigens on the surface of the gonococci bind to the corresponding antibodies. The agglutination is visible to the naked eye²³. The reagents are WI Gonococcal Reagent (In test Kit), WII/WIII Gonococcal Reagent (In test Kit) and 0.9 % saline.

Procedure:

1. Make a suspension of the suspected *N. gonorrhoeae* colonies in 1.5 ml saline in a tube.
2. Mix the suspension lightly.
3. The suspension is heated in boiling water for at least 5 minutes.
4. Cool suspension to room temperature.
5. Place drops of the heat treated suspension on the slide.
6. Blend in the reagents and rock the slide for 1 minute while observing for an agglutination reaction.

Evaluation:

Positive result: Agglutination in the WI or/and WII/WIII-reagent.

Negative result: No agglutination with the any of the reagents.

4.2.5 Carbohydrate metabolism protocol

The species in the *Neisseria* metabolize different carbohydrates, and the metabolic product is acid. Suspected colonies are plated on different agars containing; GC-Agar Medium (Oxoid Ltd, Basingstoke, UK), distilled water, NaOH, phenolred, L-glutamin (Sigma- Aldrich, USA), Iron-nitrate (Merck, USA) and a carbohydrate (glucose, maltose, lactose and saccharose). When a carbohydrate is oxidized acid is the product, and this is visualized by a pH-indicator, phenolred. *N. gonorrhoeae* only utilizes glucose²⁴.

Procedure:

1. Harvest colonies after 18-24 hour of incubation.
2. Place the suspect colonies on the different agar plates.
3. Place the inoculated agar plates in a humid compartment.
4. Incubate 18-24 hour in 37 °C.

Evaluation:

Positive reaction: Yellow color in the agar surrounding the colonies.

Negative reaction: No color change of the agar.

4.2.6 API NH protocol

API NH (BioMérieux, La Balme-les-Grottes, France) is used to identify *Neisseria* species, *Haemophilus* species og *Moraxella catarrhalis*. It is based on biochemical properties in the metabolism of the bacteria. The test strip has 10 wells which contain different dehydrated substrates, and are able to perform 12 identification tests (enzymatic reactions or sugar fermentation), and in addition detect penicillinase production. According to manufacture; after incubation with bacteria suspension, a metabolic reaction is visualized as change of color in the well. Evaluation of the test result can be performed manually or by using the API LAB software (ATB). The reagents are API NH test-kit, 0.9 % saline, JAMES reagent, ZYM B and Mineral oil (BioMérieux, La Balme-les-Grottes, France).

Procedure:

1. Harvest colonies and makes a 4.0 McFarland suspension in 3 ml saline.
2. Tilt the test strip and add 50 µl to the wells from PEN including URE.
3. Add additionally mineral oil from PEN including URE.
4. Add 150 µl to the wells from LIP including β-GAL.
5. Seal of the strip with the enclosed lid and place the strip in humidity chamber.
6. Incubate for 2 hours in 37°C.
7. Compare the color change according to manufactures instructions.

8. When LIP reaction is positive, the well ProA is predicted negative and no reagents are added.
9. When LIP reaction negative add 1 drop of ZYM B to well LIP including well PAL, and add 1 drop of James reagent to well β -GAL.

Evaluation:

1. Compare the color change according to manufacture's instructions.
2. Transfer the result to the API LAB software for prediction and identification of the bacteria.

4.3 Genotypic identification of *N. gonorrhoeae*

4.3.1 Isolation of genomic DNA

Two different robots were used to isolate genomic DNA; BioRobot M48 from Qiagen (Hilden, Germany) and TECAN MiniPrep-75 automated Robot (Genpoint, Norway). In general the principle for isolation of genomic DNA is incubation of a suspension of bacteria cells which are mixed with a lysis buffer, magnetic beads, and washing solutions. After lysis of the bacteria cells the DNA is bound to the beads and is then washed to remove potential contaminants. The DNA is released from the beads and resuspended in elution buffer (i.e. sterile water). However, the isolation procedure in the TECAN MiniPrep differs from this by an additionally step of binding of cells to magnetic beads, before wash and lysis of the cells. Thereafter, the DNA is bound to magnetic beads and washed before resuspension in sterile water. Isolation for both robots was performed as recommended from manufacture.

I. BioRobot M48.

All International *Neisseria* reference strains, clinical isolates and heterogeneous control strains were initially suspended in 200 μ l 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 (Gibco, Carlsbad Calif), Hepes buffer (Gibco) and Gentamycin (Gibco). Isolation of DNA was performed using the MagAttract DNA tissue kit with the Infectious disease protocol and an elution volume set to 100 μ l. A known positive

processing control (PPC) containing cell suspension of ATCC 19424 *N. gonorrhoeae* and a known negative processing control (NPC) containing water was included in each isolation to verify the isolation process.

II. TECAN MiniPrep-75 automated Robot.

Isolation of DNA from clinical samples in UTM RT was performed with TECAN MiniPrep automated Robot by using the Bugs'n Beads kits. Isolation volume was 700 µl and elution volume was 100 µl. The robot also performed the set up for the PCR reaction, with 11.5 µl template and 13.5 µl reaction solutions (Total 25 µl) for each PCR sample. As mentioned above, processing control, PPC and NPC were also included in the Tecan isolation process.

4.3.2 Real-time PCR assay

In general PCR is amplification of a specific DNA sequence by using primers which bind to complementary sequences in the target DNA. By real-time PCR it is possible to monitor the progress of the PCR, since data is collected throughout the process. TaqMan® or SYBR® Green chemistry can be used to detect PCR Product (amplicon).

In this study we used TaqMan® chemistry combined with the ABI™ SDS 7900 HT Fast Real-Time PCR System (Applied Biosystems Foster city, California).

TaqMan® chemistry is based on measuring increase of fluorescence. The target probe has a fluorescent dye (reporter) bound in the 5'- position and a quenching dye (quencher) in the 3' position. If the probe anneals to a specific target sequence, it is cleaved by the 5' nuclease activity in the Taq® DNA Polymerase as the primer is extended. And during accumulation of the PCR product the fluorescence intensity from the probe increases. If the probe does not anneal the reporter dye is quenched by the quenching dye. The increase in fluorescence is proportional to the amount of amplicons.

Fast real-time PCR (often called fast cycle PCR) differs from Standard real time PCR due to properties in the polymerase used in the Fast Universal PCR Master Mix (reaction solution). The polymerase does not require a long activation step and with increased ramping of temperature the Fast real time PCR protocol runs 40 cycles within 35-55 minutes (Standard

real-time PCR protocol often runs 40 cycles within 90 minutes). However, the principle remains the same; denaturation of template DNA occurs due to high temperature, and then by lowering the temperature primers hybridize to the complementary DNA sequence. DNA polymerase will extend the annealed primers by deoxy nucleoside triphosphates (dNTPs) present in the reaction solution. A series of 25-40 cycles results in an exponential amplification of the specific DNA sequence flanked by specific primers. In a 100 % efficient, PCR the DNA copies will increase by 10-fold in 3.33 cycles.

4.3.3 Design of primers and probes

The *porA* PCR targets a specific sequence in the region of the *porA* pseudogene in the genome of *N. gonorrhoeae*. The design of primers and probes was performed by Stig Ove Hjelmevoll²⁵ In general the procedure of primer/probe design is finding a sequence that is specific for the target of interest (in this case *N. gonorrhoeae*). By comparing (BLASTn) the sequence of the entire gene to all genetic sequences deposited in genome databases (GenBank[®]), a specific region of the gene can be identified. This specific sequence is then used to design primers and probe. It is recommended to design primers and probe in a region with low Guanine/Cytosine content (20-80 %). The primers should be close to the probe and amplify short sequences within the target sequence, 50 to 150 bp in amplicon size. The melting temperature (T_m) for the primers should be 58-60°C and 68-70°C T_m for the probe. (Guidelines from Applied biosystems, Foster city, California.)

However, the *porA* primers and probe were designed as a TaqMan[®] assay by using Primer Xpress[™] 2.0 (Applied Biosystems) according to manufacturer's guidelines. The primers and probe sequences and the amplicon were compared to *porA* pseudogene sequences from a previous study¹³ as well as all the genetic sequences deposited in GenBank[®]. Table 5 shows the sequences of the primers and probe used for targeting the *porA* pseudogene in this study.

Table 5: Primer and probe sequences used for targeting the *N. gonorrhoeae porA* pseudogene.

Name primer/probe	Sequence 5'-3'
Forward primer <i>porA</i> -F	GTTTCAGCGGCAGCATTCA
Revers primer <i>porA</i> -R	CCGGAACTGGTTTCATCTGATT
Probe <i>porA</i> -P	*FAM-CGTGAAAGTAGCAGGCGTATAGCGGACTT-BHQ-1

*FAM is the fluorescent reporter dye and BHQ-1 (BlackHole Quencher one) is the quenching dye.

4.3.4 PCR conditions

The *porA* reaction solution contained TaqMan® Fast Universal PCR Master Mix (2×) No AmpErase® UNG and in addition RNase Free water, primers, probes and an internal amplification control (IAC). The *porA* primers and probe were optimized as recommended by Applied Biosystems, and a final concentration of 900 nM of each primer and 200 nM of the *porA* probe was found to be optimal. In addition an internal amplification control (IAC) and an IAC probe (100 nM final concentration) were added to the reaction solution. The IAC function as control of inhibition of the PCR reaction, and was expected to be amplified in all negative samples. The IAC is described in detail below in chapter 4.3.5

For the bacterial strains and the clinical isolates, the reaction volume was 25 µl, with 20 µl reaction solution and 5 µl template. For the clinical samples the reaction volume was also 25 µl, with 13.5 µl reaction solution and 11.5 µl template. All templates were run in parallel, and in addition each PCR analysis was run with a known positive template control (PTC) containing Quantified *N. gonorrhoeae* DNA (Tebu-bio, Le Perray en Yvelines, France) and a known negative template control (NTC) containing water to test the reaction solution.

The FAST-PCR protocol was run by:

- 20 seconds at 95°C to activate the Taq DNA polymerase.
- 1 second in 50 cycles of 95° to denaturated DNA.
- 20 seconds in 60° C to anneal and extend primers.

The FAST-PCR protocol is run within 45 minutes. The primers amplify a 102-bp fragment of the *porA* pseudogene.

4.3.5 Internal Amplification Control

The internal amplification control (IAC) is a PCR product added to the reaction solution to uncover the presence of inhibitors of the PCR reaction. The *porA* primers recognise and amplify the IAC, creating a 225 bp amplicon (figure 1). The amplicon is detected using a TaqMan® probe (IAC-P) which anneals specifically to an internal part of the IAC sequence.

In all negative samples, or in samples with very low amount of target DNA, the IAC would be amplified. If the IAC was not amplified in gonorrhoea negative sample, the samples were diluted 1:2 and then retested diluted and undiluted. To avoid primer competition between the IAC and target DNA, the amount of IAC was kept as low as possible in the reaction solution. The IAC was not quantified but optimized to give cycle threshold values from 36 to 39.

The IAC was made by using composite primers based on the primers for the *porA* pseudogene. These composite primers is constructed by a *porA* primer sequence (*porA* forward primer or reverse primer) in the 5'-end, and in the 3'-end a sequence from GEM®-luc plasmid (Promega Madison, Wi). To amplify the selected region of the plasmid we used 50 nM of the composite primers, and added 5 µl of a 10⁻⁴ dilution of the pGEM®-luc plasmid in a total volume of 50 µl reaction solution with no UNG. The IAC-PCR protocol was run at GeneAmp® PCR system 9700 (Applied biosystems) and contains the following steps:

- 10 minutes activation step in 95°C.
- 15 seconds denaturation step in 40 cycles of 95°C.
- 1 minute annealing in 60°C.

This makes a 181 bp fragment of the Beta-lactamase coding region in the GEM®-luc plasmid²⁵.

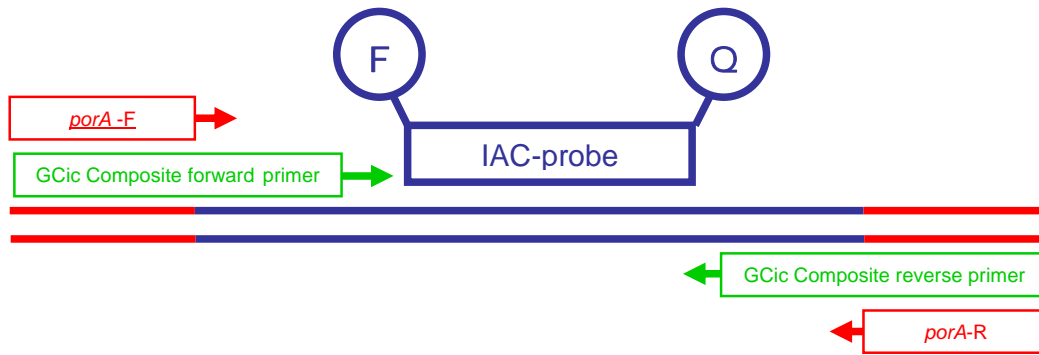


Figure 3: A schematic view illustrating the detection of IAC in the reaction solution. The IAC (blue and red parallel lines) and the IAC probe and the *porA*- primers. The GCic composite primers are not present in the reaction solution but shown to illustrate the connection to the IAC and the target primers. F: Fluorophore, Q: Quencher. Image is kindly provided by Stig Ove Hjelmevoll.

Table 6: Primers and probe sequences for production and detection of the IAC.

Name Primer/Probe	Sequence 5'-3'
Forward primer GCic-F	GTTTCAGCGGCAGCATTTCATGGTCTGACAGTTACCAATGCTTAA
Revers primer GCic-R	CCGGAAGTGGTTTCATCTGATTGGCTGGCTGGTTTATTGCTG
Probe IAC-P*	Yakima yellow-CCATAGTTGCCTGACTCCCCGTCG-DarkQuencher

* The probe is used to detect IAC product added in the *porA* reaction solution.

4.3.6 Efficiency of PCR

For efficiency (E) calculation we used a dilution series of quantified *N. gonorrhoeae* DNA from Tebu-bio (Le Perray en Yvelines, France). Based on the dilution series, a standard curve was generated by the software in ABI SDS 7900 HT® (Applied Biosystems, Foster city, Calif.). Efficiency was calculated using the formula: $E = 10^{-1/\text{slope}} - 1^{26}$, where slope refers to the slope of the standard curve.

Furthermore the efficiency of the DNA isolation methods was estimated. The ATCC 19424 *N.gonorrhoeae* was suspended in phosphate buffered saline to 0.5 McFarland (approximately 1.5×10^8 Colony Forming Units (CFU)/ml³).

The suspension was then diluted 10^{-1} through 10^{-7} and from each of the dilution:

- 100 μ l was plated on modified Thayer Martin Medium and incubated overnight at 37°C in the presence of 5% CO₂.
- 100 μ l was transferred to 3 mL UTM RT buffer (Copan, Brescia, Italy) and Stored overnight at 4°C. The next day 200 μ l was used to prepare DNA on the BioRobot M48, and 700 μ l was used to prepare DNA on the TECAN MiniPrep automated Robot.

4.3.7 Detection limit of PCR

The detection limit of the *porA* pseudogene PCR (*porA* PCR) was determined by using quantified *N. gonorrhoeae* DNA from Tebu-bio, containing 1.3×10^4 DNA copies/ μ l. The quantified DNA was diluted in TE-buffer in a dilution series of 1:10 to find the lowest detectable concentration. This was performed three times.

4.3.8 Robustnes of PCR

We tested robustness of the PCR by keeping the reaction solution volume constant at 13.5 μ l, and varying the sample volume from 9 μ l to 15 μ l in 1 μ l increments.

4.3.9 Interpretation of PCR results

The results from the *porA* PCR were interpreted according to the software guidelines from the manufacture (Software ABI™ SDS 7900 HT Fast Real-Time PCR System, Applied Biosystems). However, all reactions with signal from the target *porA* probe were assigned positive. All reactions without signal from the target probe and with amplified IAC were assigned negative. Reactions without signal from both the target probe and the IAC probe, were retested undiluted and diluted 1:2, before new isolation of DNA.

4.4 Clinical validation of the *porA* PCR

The clinical validation of the *porA* PCR method were performed in collaboration with

- Olafia clinic, Oslo, Norway.
- The Department of Microbiology Ullevål University Hospital, Oslo, Norway.
- The Department of Microbiology and Infection control, University Hospital of North Norway, Tromsø, Norway.
- National Reference Laboratory for Pathogenic Neisseria, Department of Clinical Microbiology, Örebro University Hospital, Örebro, Sweden.

The Olafia clinic was responsible for recruiting pre-selected patients, and the collection and distribution of clinical samples in this study. The Department of Microbiology Ullevål University hospital was responsible for diagnosing gonorrhoeae from the clinical samples by using their routine culture diagnostic tools. The Department of Microbiology and Infection control developed and performed the *porA* in-house PCR, and was responsible for the collection of data and evaluation of the clinical validation.

Test results from each laboratory were compared after enclosure of the study. The clinical validation was performed by comparing the results from the *porA* PCR with the results from the phenotypic diagnostic. In cases of discrepancy between the results, an aliquot of the clinical samples was sent to Örebro for sequencing analysis.

4.5 Interpretation of diagnostic data

An evaluation of the analytic sensitivity of the *porA* PCR was done before running the PCR with clinical samples. After the clinical validation was ended the diagnostic sensitivity and specificity was calculated for both *porA* PCR and phenotypic diagnostic techniques. In addition the positive predicative value (PPV) and negative predicative value (NPV) was calculated for both methods.

- Analytic sensitivity is the number of samples identified as positive by the method.
- Diagnostic sensitivity is the number of individuals with a disease that is detected by the method.

- Analytic specificity is the number of true negative samples detected by the method. Diagnostic specificity is the number of healthy individuals detected by the method.
- PPV indicates how often test positives are positive.
- NPV predict how often test negatives are negative.
- Prevalence indicates the distribution of disease within a study population.

All calculations were performed by using reference:

(www.chestx-ray.com/Statistics/TwoByTwo).

5 RESULTS

5.1 *porA* pseudogene primers

The alignments and design of primers and probes were performed by Stig Ove Hjelmvoll²⁵. The primers, probe and amplicon were aligned to all available sequences deposited in GenBank[®]. The forward primer shared greatest homology to *Yersinia* species, while the reverse primer showed similarity to *Homo sapiens* chromosome 11 clone fa0692. The probe had a 23 of 30 bp similarity to *N. meningitidis*. Blasting the entire amplicon showed a homology in 58 of 102 bp of the *porA* gene found in *N. meningitidis*. As shown in figure 4 the *porA* gene found in *N. meningitidis* and *porA* pseudogene found in *N. gonorrhoeae* is sufficiently different and can be used to discriminate the two bacteria. In addition this figure shows that the primers and probe is located in a conserved region of the *porA* pseudogene.

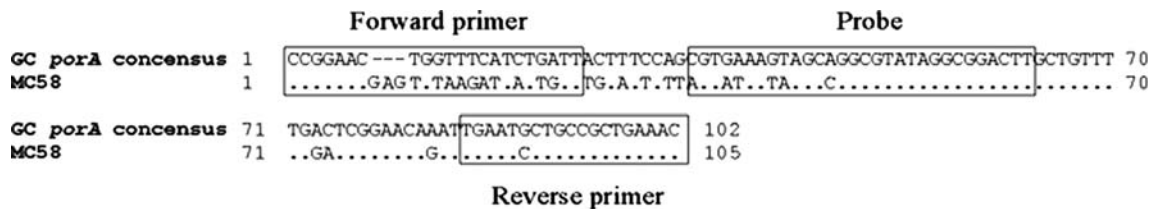


Figure 4: The image show a nucleotide sequence alignment of a partial *N. gonorrhoeae* consensus *porA* pseudogene compiled from 87 sequences and the corresponding *N. meningitidis* sequence. The boxed sequences on the flanks are the forward and reverse primer location, respectively. The boxed sequence in the middle is the location of the probe. The MC58 sequence is the partial *porA* sequence of the previously published whole-genome sequenced *N. meningitidis* strain MC58. The image is kindly provided by Stig Ove Hjelmvoll²⁵.

5.2 Technical performance of *porA* PCR

As previous described, the *porA* primers amplify a 102 base pair (bp) fragment of the *porA* pseudogene. The *porA* primers and probe were optimized as recommended by Applied Biosystem, and a final concentration of 900 nM primer and 200 nM probe was found to be optimal. The final concentration of the IAC probe was 100 nM. The amount of IAC added in the *porA* reaction solution was not quantified, but optimized to give approximately a cycle threshold value form 36 to 39 in known negative samples.

To test efficiency of the optimized reaction solution, quantified *N. gonorrhoeae* DNA, was diluted in a ten-fold dilution series to generate a standard curve by using the software available in SDS ABI 7900 HT. This is illustrated in figure 5. The slope value in the figure is approximately 3.3 and this indicates a very efficient PCR.

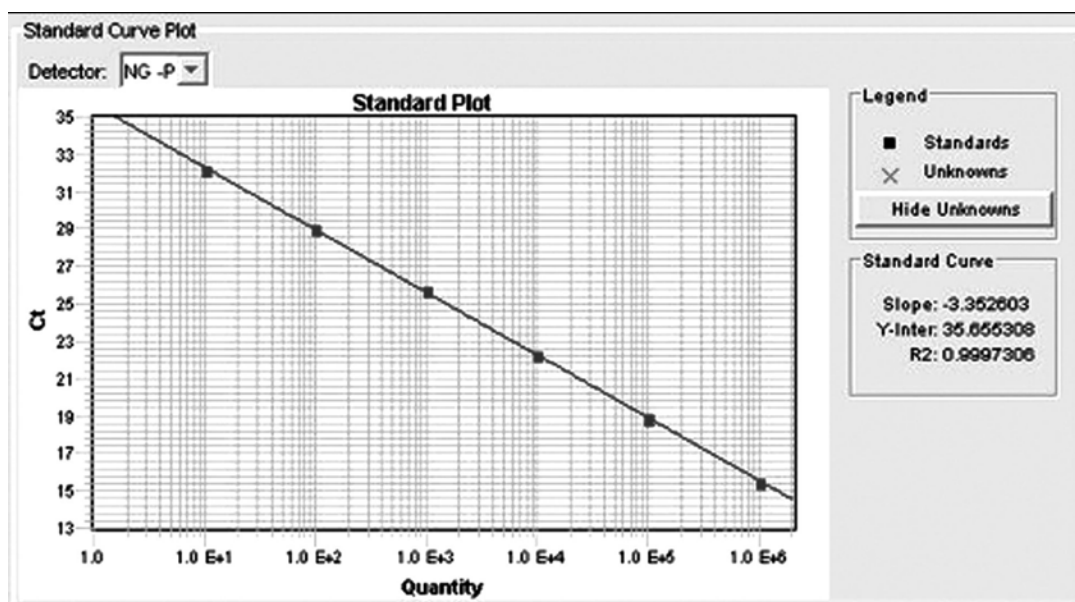


Figure 5: Standard curve made by using the software for ABI SDS 7900 HT® based on data from dilution series of the quantified *N.gonorrhoeae* DNA.

The efficiency of the *porA* PCR, when IAC was added to reaction solution, showed an amplification efficiency of 99.3%, and the amplification efficiency without the IAC was 94.9 %. Figure 6 shows the difference between the reaction solution with and without IAC, the 4.4 % difference in amplification efficiency is insignificant. The curves in the plot are approximately equal in each dilution and this indicates that the presence of IAC in the reaction solution does not influence the efficiency of the *porA* PCR.

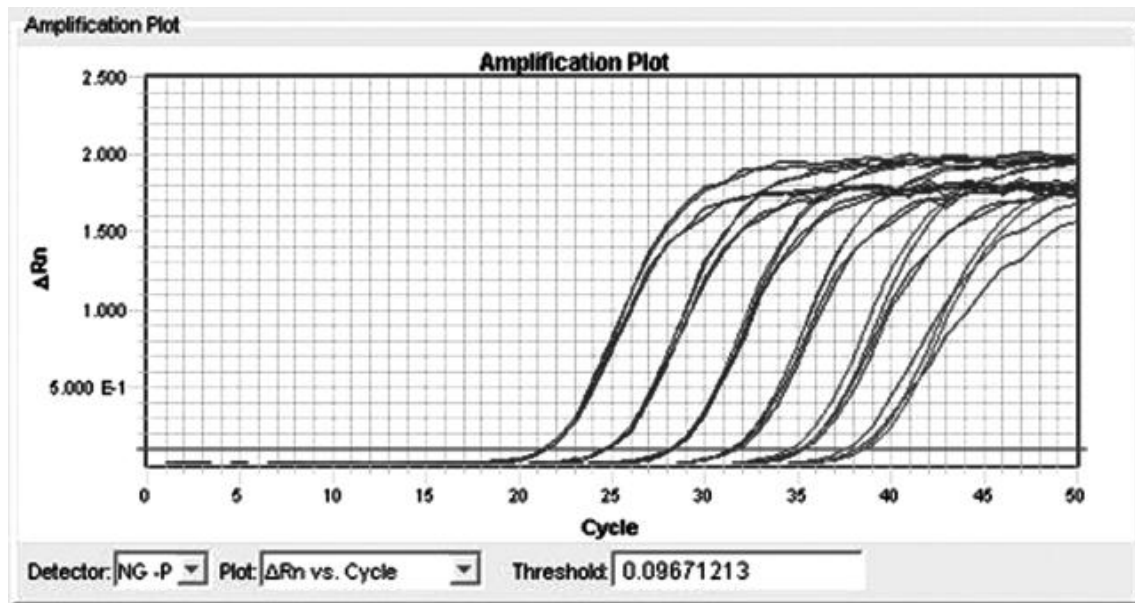


Figure 6: Curves generated from making a dilution series of quantified *N. gonorrhoeae* DNA and two different reactions solutions with and without IAC. There are four parallels for each dilution, hence two parallels for each reaction solution. There were insignificant differences in the amplification of DNA.

Detection limit was also determined by using dilution series of the quantified *N. gonorrhoeae* DNA from Tebu-bio. As an example, figure 7 shows the results of a dilution series of the quantified DNA and according to the curves the PCR has a good linearity. In 5 of 5 parallel reactions the *porA* PCR detected 7.5 genome equivalents (Geq)/PCR reaction, and this was concluded as the lower detection limit. No upper limit was determined for the *porA* PCR. However, the *porA* PCR also detected 1.5 Geq/PCR reaction in 3 of 5 parallel reactions, indicating that the *porA* PCR is able to amplify even lower amounts of *N. gonorrhoeae* DNA than the settled detection limit.

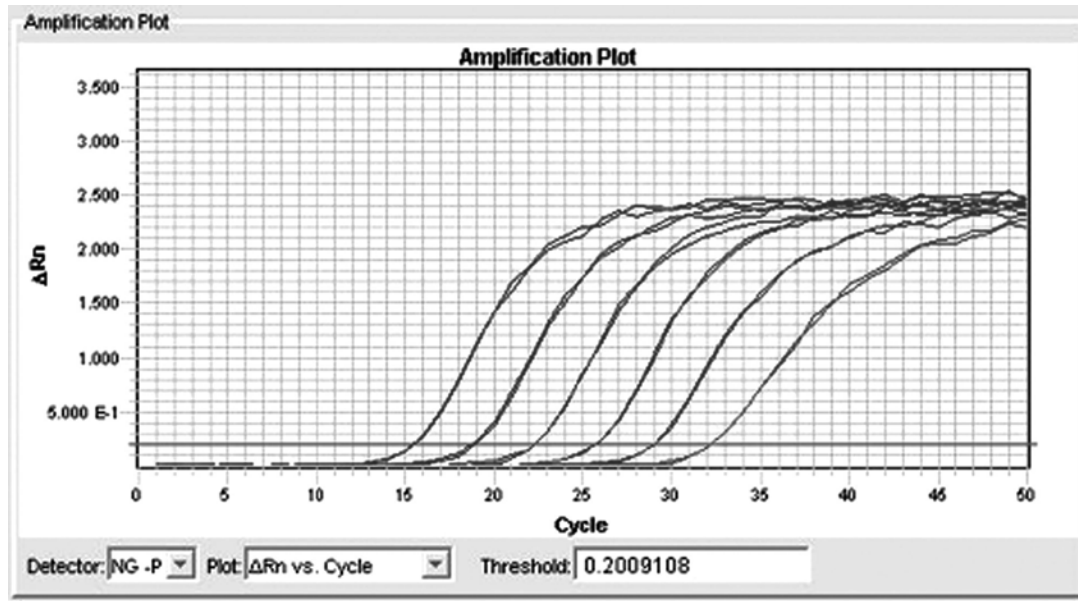


Figure 7: The figure illustrates the linearity of a dilution series of quantified *N. gonorrhoeae* DNA from Tebu-bio with the *porA* PCR.

To determine the robustness of the *porA* PCR, the reaction solution volume was held constant at 13.5 μl while the template volume varied from 9 to 15 μl . When using a low and a high concentration of quantified *N. gonorrhoeae* DNA and a NTC as shown in figure 8 the varying template volume showed insignificant differences in the cycle threshold values. This indicates that the PCR reaction will be equally efficient even if the template volume increase to 15 μl , or decrease to 9 μl .

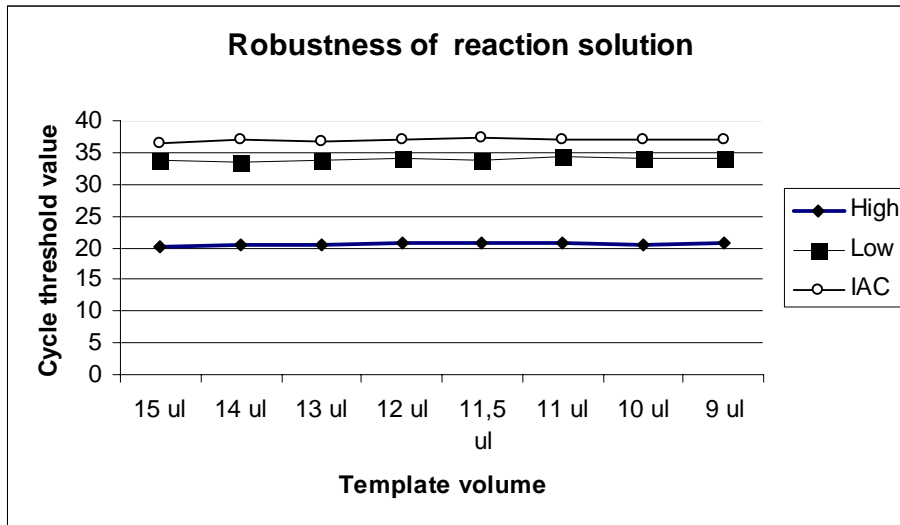


Figure 8: Plotted cycle threshold values for IAC, low and high quantified *N. gonorrhoeae* DNA with varying template volumes.

To evaluate the recovery of DNA during the isolation procedure a dilution series of *N. gonorrhoeae* spiked in UTM-RT medium were plated in duplicates and the PCR was also run in parallels. The number of colony forming units/ml was 80 in the first experiment, and the second experiment resulted in 53. When translated to colony forming units/ μ l DNA eluate this gives 0.13 CFU/ μ l DNA eluate, meaning a 100 % recovery in the DNA isolation ²⁵.

Before running the *porA* PCR with clinical samples, the DNA from several international *Neisseria* reference strains, clinical isolates and heterogeneous control strains were analyzed. This was done to estimate the analytic sensitivity and specificity of the *porA* PCR.

The PCR results from 44 *Neisseria* references strains, 168 clinical isolates and 46 heterogeneous control strains listed in table 7, 8 and 9.

A positive result indicates amplification by the *porA* primers, and presence of the *porA* pseudogene in the DNA. A negative result indicates no amplification by the *porA* primers and no *porA* pseudogene detected.

Table 7: International *Neisseria* reference strains analyzed with the *porA* PCR and PCR results.

Reference strain*	Number of strains	Positive PCR Results	Negative PCR Results
<i>Neisseria gonorrhoeae</i>	32	32	0
<i>Neisseria meningitides</i>	4	4	0
<i>Neisseria sicca</i>	2	2	0
<i>Neisseria subflava</i>	1	1	0
<i>Neisseria flavescens</i>	1	1	0
<i>Neisseria mucosa</i>	2	2	0
<i>Neisseria lactamica</i>	1	1	0
<i>Neisseria cinera</i>	1	1	0
Total	44	44	0

*The reference number for each strain is previous listed in table 1.

Table 8: Clinical isolates analyzed with the *porA* PCR and PCR results.

Clinical isolate	Number of isolates	Positive PCR Results	Negative PCR Results
<i>Neisseria gonorrhoeae</i>	168	168	0
<i>Neisseria gonorrhoeae</i> subspecies <i>kochii</i>	4	4	0
<i>Neisseria meningitides</i>	7	0	7
<i>Neisseria sicca</i>	7	0	7
<i>Neisseria subflava</i>	11	0	11
<i>Neisseria flavescens</i>	3	0	3
<i>Neisseria mucosa</i>	5	0	5
<i>Neisseria lactamica</i>	7	0	7
<i>Neisseria cinera</i>	7	0	7
<i>Neisseria cavicae</i>	1	0	1
<i>Neisseria animalis</i>	1	0	1
<i>Neisseria polysaccharea</i>	1	0	1
Total	222	172	50

Table 9: Heterogeneous control strains analyzed with the *porA* PCR and PCR results.

Heterogeneous strains*	Number of isolates	PCR results Positive	PCR results Negative
Gram negative bacteria	18	0	18
Gram positive bacteria	23	0	23
Fungus	1	0	1
Viruses	4	0	4
Total	46	0	46

*More details about the isolates are previously listed in table 2.

None of the reference strains, clinical isolates or the heterogeneous control strains analyzed by the *porA* PCR gave unexpected results. All of the reactions containing *N. gonorrhoeae* DNA were amplified by the primers indicating that all of the tested *N. gonorrhoeae* strains possess the *porA* pseudogene. The DNA from strains and isolates, other than *N. gonorrhoeae*, were not amplified. The IAC was amplified in all negative reactions and indicated no inhibition in any of the PCR reactions. However, it unlikely that eluated DNA from the above mentioned material contains inhibitory contaminants, considering they all are pure isolates.

In each DNA isolation and PCR set up, controls were used to monitor the protocols. ATCC 19424 *N. gonorrhoeae* as PPC, and water as NPC. In addition each PCR set up was run by including a purified DNA from *N. gonorrhoeae* as PTC, and water as NTC. PPC, NPC, PTC and NTC were consistently evaluated before the other results, and if they passed, an evaluation of the other reactions followed. The PPC, NPC, PTC and NTC gave expected results in each run, and this indicates that the isolation and PCR were accurate.

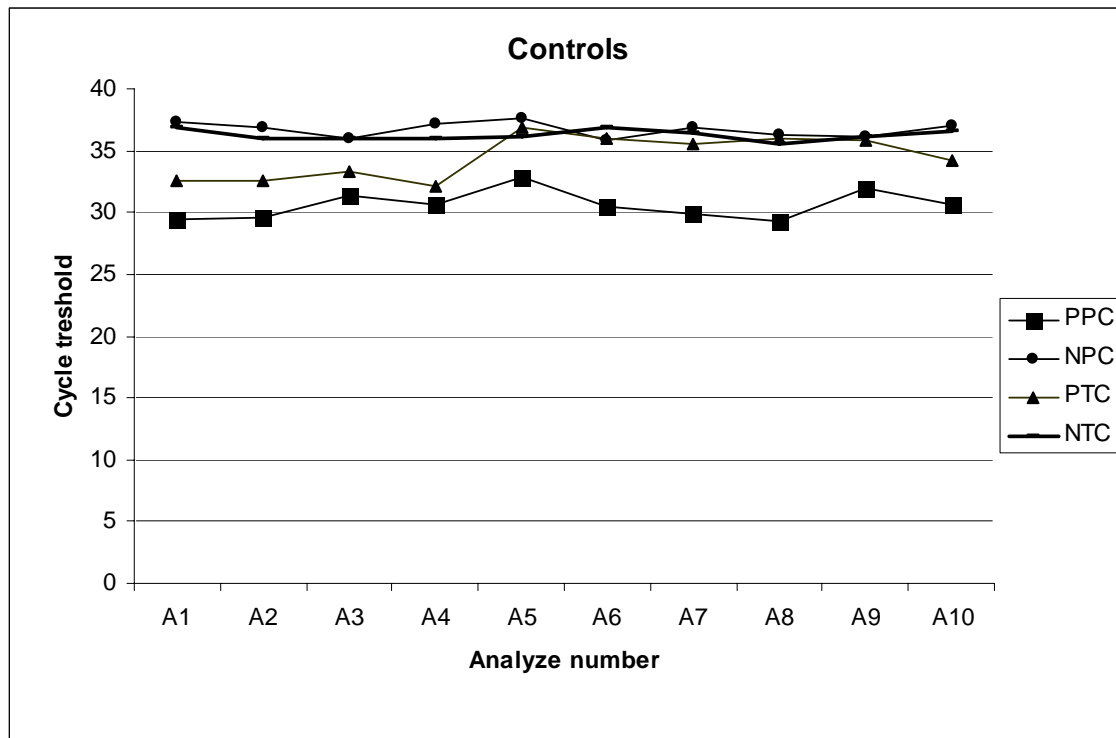


Figure 9: Cycle threshold values from PPC, NPC, PTC and NTC from 10 independent analyses.

In figure 9 the cycle threshold values (average value of 2 parallels) from PPC, NPC, PTC and NTC from 10 independent analyses of clinical samples are plotted (Isolation of DNA and PCR was performed within the same day). Three different batches of reaction solution are used, but same batches of PPC, NPC and NTC was used in all 10 analyses. A new batch of PTC was used after analysis A4, and explains the increase in cycle threshold value. The plot of NPC and NTC should preferably achieve equal cycle threshold values, since reagents and contaminants during the isolation process should not interfere with the pureness in the DNA eluate. The plot indicates stability in both isolation process and PCR set up.

In overall, the *porA* pseudogene *porA* PCR has shown a 100 % analytic sensitivity and specificity. It also has proved to be robust, rapid and reproducible.

5.3 Functionality of UTM-RT system

To test the suitability for the transport media, a low and high dilution of the ATCC 19424 (*N. gonorrhoeae*) were added to the buffer. After storage in different temperatures, isolation of DNA and *porA* PCR were performed after 1, 3 and 7 days. The results are presented in Table 10.

Table 10: Cycle threshold values achieved for the ATCC 19424 *N. gonorrhoeae* inoculated in UTM RT.

ATCC 19424 inoculated in UTM-RT	1 day Cycle threshold value*	3 days Cycle threshold value*	7 days Cycle threshold value*
Dilution 10 ⁻¹ Room temperature	27,8	27,9	27,9
Dilution 10 ⁻¹ + 4°C	26,9	27,5	27,6
Dilution 10 ⁻⁴ Room temperature	37,8	36,8	37,6
Dilution 10 ⁻⁴ + 4°C	38,3	39,1	37,2

*The cycle threshold value is an average value from three reactions.

There were insignificant variations in cycle threshold values for the low and high dilution of the ATCC 19424 when analyzed after 1, 3 and 7 days. This indicates that the UTM-RT is a suitable transport media for *N. gonorrhoeae* when analyzed with the *porA* PCR.

5.4 Diagnostic validity

In total 360 samples from 242 individuals were included in this study, 185 males and 57 females²⁷. The sample sites and distribution of samples are shown I figure 11.

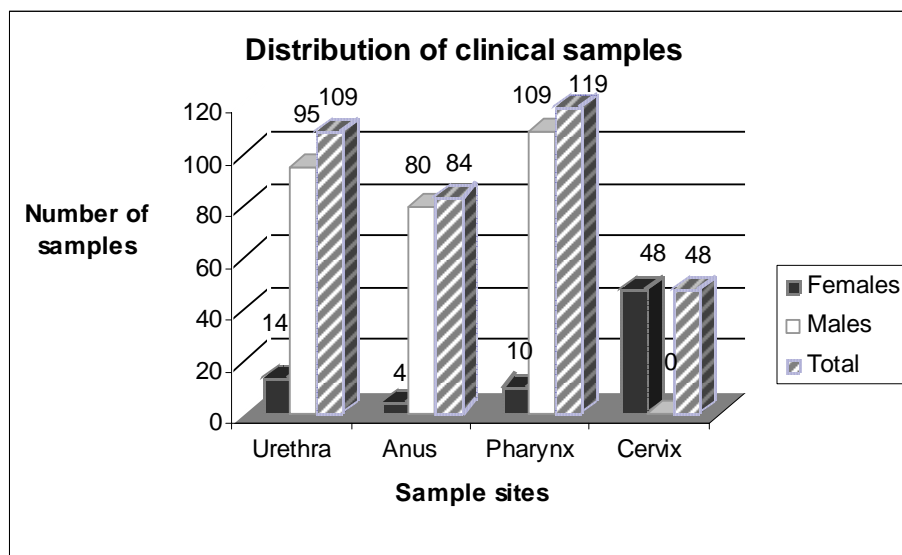


Figure 11: Distribution of different sample sites in male and female included in this study.

The *porA* PCR identified *N. gonorrhoeae* in 52 samples while phenotypic techniques identified *N. gonorrhoeae* in 37 samples. All 15 samples in discrepancy were confirmed as true positive by sequencing the 16S *rRNA* and the *porA* pseudogene. Table 11 shows the distribution of samples with *N. gonorrhoeae* positive *porA* PCR, compared with results from phenotypic identification.

In other words, based on the results from *porA* PCR, phenotypic diagnostic tools and the results from the sequencing, 308 samples were concluded as true negatives and 52 samples, hence 39 males and 3 females were confirmed as true positive.

Table 11: Distribution of *porA* PCR positive samples in different sample sites.

Sex	PCR	Ph. Tech.*	Urethra	Rectum	Pharynx	Cervix	Total
Male	Positive	Positive	26	5	2	0	33
	Positive	Negative	1	6	8	0	15
Female	Positive	Positive	1	0	0	3	4
Total			28	11	10	3	52

* Phenotyp. Tech = Phenotypic techniques

Based on this data the *porA* PCR has a 100 % diagnostic sensitivity and specificity, and respectively 100 % for the PPV and NPV. The prevalence of gonorrhoea in the study population was 14.78 %.

Approximately 76% of the included individuals were males, and the prevalence for gonorrhoea among males was 17.4%. The prevalence in tested females was 5 %, but the number of tested females has no good statistical basis due to limited individuals tested.

In comparison, the cultivation techniques did not identify 14 individuals as gonorrhoea positive. The sensitivity was 71 %, while specificity was 100 %. The PPV was 100 % and the NPV 93 %.

The internal amplification control was amplified in all of the 308 negative samples except for 35.

Table 12: Distribution of samples with inhibition.

Sex	Urethra	Rectum	Pharynx	Cervix	Total
Male	11	6	11	0	28
Female	2	0	1	4	7
Total	13	6	12	4	35

Table 12 show number of samples with inhibition during this study, and the sampling sites; urethra (n =13), rectum (n= 6), pharynx (n =12), and cervix (n =4). These samples were isolated once more undiluted and diluted 1:2 and then retested. All samples were concluded as

true negative after retesting them, there were no detection of *N. gonorrhoeae* and the IAC was amplified.

However, 42 samples from 27 patients were excluded in the study. The reasons for exclusion were:

- Samples sent for test of cure after treatment (n=21).
- Samples sent for Herpes simplex virus testing (n=7).
- Mechanical failure of the DNA preparation robot, Tecan miniprep-75 (n=14).

As mentioned, PPC, NPC, PTC and NTC were also included during clinical validation. The controls were consistently evaluated before sample results, and if they passed an evaluation of the results followed. The PPC, NPC, PTC and NTC gave expected results in each run, indicating accuracy in the isolation and the PCR process, as shown in figure 9

6 DISCUSSION

The results from the technical and clinical validation of *N. gonorrhoeae porA* pseudogene real time PCR²⁷, indicate that the method is species specific for *N. gonorrhoeae*. All of the 200 *N. gonorrhoeae* reference strain and gonococcal clinical isolates were detected by the *porA* PCR. In addition neither the heterogeneous control strains nor the commensal *Neisseria* was amplified. The *porA* PCR achieved 100 % specificity and sensitivity in the material tested here in comparison to culture and phenotypical diagnostics.

6.1 A species specific target

The success of designing a species specific PCR depends on finding an appropriate target sequence. The primers and probes showed low similarity to other sequences deposited in GenBank[®]. Despite of the great similarity between *N. gonorrhoeae* and *N. meningitidis*, the alignments of the *N. gonorrhoeae porA* pseudogene consensus sequence and *porA* gene found in *N. meningitidis* showed a sufficient diversity within the genes to discriminate the two bacteria. Even so, when using sequencing data to find regions of local similarity between genomic sequences, it is important to consider that the results purely depend on the information stored in the databases. And as for the *Neisseria*, it is important to bear in mind that several commensal *Neisseria* species are not fully sequenced, and that they are highly transformable organisms.

However, the primers and the probe were located in a conserved region of the *porA* pseudogene. This is beneficial since conserved regions may indicate that they have been maintained during species evolution. All *N. gonorrhoeae* examined in this study were amplified by the *porA* primers, indicating they all possess the *porA* pseudogene. Furthermore, the analyzed *N. gonorrhoeae* strains had been isolated over 5 decades (from 1960 to 2006). This indicates that the target region of the *porA* pseudogene has been stable over the past 5 decades.

Other studies have reported presence of *porA* pseudogene in *N. gonorrhoeae* from different geographical regions, as well as absence in commensal *Neisseria*²⁸, and suggesting that it is a suitable target for identifying *N. gonorrhoeae*. In addition, previous sequencing of the entire *porA* pseudogene has showed a distinct diversity from the *porA* gene in *N. meningitidis*¹³. The lack of *porA* pseudogene expression in *N. gonorrhoeae* may protect this gene from changes since it is not subjected to evolutionary pressure. However, bacteria are known to

only retain genomic material that is beneficial. Why the *N. gonorrhoeae* possess this pseudogene is unknown, but there might be a reason for keeping it.

6.2 Assay validity

There are other aspects influencing the development of an in-house PCR, such as finding optimal primer and probe concentrations, and achieving an acceptable efficiency and detection limit.

Regarding the PCR efficiency two aspects of the PCR protocol should be considered, the components in the reaction solution and the equipment. Insufficient concentrations of primers and probe, long amplicons and primer dimers will influence the efficiency. To reveal primer dimer, the target primers were tested by using SYBR® green (data not shown in the thesis), and non unspecific products were observed. In this study a majority of components in the TaqMan® Fast Universal PCR Master Mix were already optimized by the manufacture, making the optimization less laborious. All primer/probe concentrations and amplicon size were according to manufacture guidelines, and the optimized reaction solution was considered both robust and efficient.

The diagnostic value of an assay depends on the ability to produce result of certain stability. By including known control parameters in each step of the assay the robustness was monitored. Processing controls was included in the isolation of DNA, both positive and negative controls. In addition positive and negative template controls was analyzed in each PCR setup. However, all controls showed stable values (in this case cycles threshold values) during the study, indicating robustness in the machinery as well as in the reaction solution. The use of known positive controls is crucial when analysing only negative samples. 308 samples were concluded as true negatives in this study, and in several isolation processes and following PCR setups there were no positive clinical samples.

In addition the robustness of the reaction solution was also tested by using a low and high concentration of purified *N. gonorrhoeae* DNA. Variations in the template volume had insignificant impact on the cycle threshold value, for both low and high concentration.

Also, the IAC controls the PCR reaction solution which function is to detect the presences of inhibitory contaminants. Inhibition of the PCR reaction was observed in 35 of 308 negative clinical samples, but after retesting they were concluded as true negatives.

The amount of IAC added in the reaction solution was not quantified, but it was optimized to give a cycle threshold value from 36 to 39 in known negative samples. The attempt was to keep the amount as low as possible to avoid competition of target primers in samples containing low amount of target DNA. Since the IAC is a PCR product it might influence the efficiency of the PCR, hence efficiency was determined with and without the presence of IAC. As shown, the *porA* PCR efficiency in the presence of IAC were 99.3%, while without IAC it was 94.9%²⁷, indicating that the IAC has little influence on the efficiency.

Regarding the amount of target DNA, the detection limit of the assay decides whether it is detected or not. The *porA* PCR had a detection limit of 7.5 Geq/PCR reactions, and this is considered low. The limit of detection was reproduced in three separate runs. As a curiosity the *porA* PCR was also able to detect 1.5 Geq/PCR reaction, but the detection was not consistent. Even so, this indicates the possibility to detect even lower amounts of DNA than the settled detection limit. Samples containing large amount of DNA will inhibit the PCR reaction and this would be uncovered by the IAC in reaction solution, hence an upper detection limit was not determined.

Several pathogenic and non-pathogenic micro-organisms as well as *N. gonorrhoeae* reference strains and gonococcal isolates were included to evaluate the specificity of the assay. We attempted to include representative organisms found in the normal flora in locations *N. gonorrhoeae* colonizes; cervix, urethra, rectum and pharynx. In addition we tested DNA from organisms, which already had established NAATs in our laboratory. None of the bacteria and viruses examined was amplified by the *porA* primers, and all of the 200 *N. gonorrhoeae* reference strains and gonococcal isolates examined in this study were identified by the *porA* PCR.

With the above mentioned satisfying results, the *porA* PCR showed potential for being specific for *N. gonorrhoeae*.

6.3 DNA preservation

The molecular approach in the attempt of diagnosing gonorrhoea demanded a different transport media than what already existed in our laboratory. The UTM-RT was suitable for pathogens with already established NAATs, but had not been evaluated to transport *N. gonorrhoeae* by the manufacture. Regarding the variation in temperatures during transport and also the transport duration, inoculated UTM was stored both at room temperature and at 4°C. Further it was tested after 1, 3 and 7 days of storage. The UTM-RT was found suitable for preservation of gonococcal DNA, and the DNA was not degraded even when stored for 7 days. Unfortunately *N. gonorrhoeae* does not recover from the UTM-RT, so a separate sample-swab for phenotypic detection and susceptibility testing is still necessary.

6.4 Diagnostic validity

The analytic sensitivity and specificity of the *porA* PCR was 100 %. Even so, the use of reference strains and isolates to determine these parameters can never replace the value of testing biological samples. Clinical samples contribute to testing of biological variation in the sample (host), which can not be done when using pure isolates. To achieve significant statistical values, the number individuals in the study population are of importance. In this study 360 samples from 242 individuals were analysed. Statistically it would have strengthened the clinical validation if a greater number of individuals had been possible to include. In addition the samples were taken from individuals attending only one clinic, from the same geographic region, and a possible diversity among gonococcal strains from other regions was not considered. To include more individuals in the study population and sampling from different geographic areas would improve the validation of *porA* PCR. However gonorrhoeae is rare in Norway and the population available is therefore limited.

The *porA* PCR showed a 100 % diagnostic sensitivity and specificity, while the phenotypic identification achieved a sensitivity of 71 %, and the specificity was equal to the *porA* PCR's specificity. The clinical validation was in favor of the *porA* PCR which achieved a greater sensitivity than the cultivation techniques. The *porA* PCR identified further 14 more cases of gonorrhoeae. To resolve discrepancy, all samples were examined by sequencing of the entire *porA* pseudogene to state the presence of gonococci, and since the PCR targets the *porA*

pseudogene, sequencing of the *16S rRNA* was also performed ²⁷ to strengthen the discrepant resolution. In the end, all discrepant samples were concluded as true positives.

However, the discrepant samples were sampled from pharynx and rectum, and *N. gonorrhoeae* recover poorly from these sample sites compared to sampling from urogenitalia ¹⁵. The cause of false-negatives from the cultivation techniques could also have been due to transport conditions. But the sampling clinic (Olafia clinic) attempt to store samples for cultivation maximum 3 hours in room temperature before sending them to microbiology laboratory. In addition, the laboratory which performed the phenotypic identification (Ullevål University Hospital) is located only 3 km in distance from the clinic. This is an advantage regarding the survival of *N. gonorrhoeae*, but this is rarely the situation for general practitioners or clinics, which in Norway often are located far from the laboratory. Another reason to the false negative results can be explained by possible overgrowth of other micro organisms from the normal flora³¹. This is often the problem when cultivation from pharynx and rectum. Regarding gonococcal infection in these sites, they are often asymptomatic, and valid diagnostic tools are essential to identify *N. gonorrhoeae*. Our results indicate that the *porA* PCR can improve diagnosis of gonorrhea in these sites.

Nevertheless, false-positive diagnosis of gonorrhea might occur particularly from pharynx samples. The commensal *Neisseria* species and *N. meningitidis* are most common in the pharyngeal mucosa, which gives a greater risk for assay cross reactivity when analyzing samples from pharynx. But there has been reports of *N. cinerea*, *N. lactamica*, *N. sicca* and *N. subflava* isolated from genital mucosa ^{1,29}, indicating a possibility of false-positive diagnosis if the method does not adequately distinguish gonorrhoeae from commensal *Neisseria*, even in genital samples. False positive results have also been reported for many of the commercial NAATs ^{1,14,29}, and this was also the case when we tested other genes as targets for detection of *N. gonorrhoeae*. Before choosing the *porA* pseudogene, TraG and TraH, dcmG, carbonic anhydrase, and pJDI ²⁵ were tested but none of them were species specific. Their either misclassified commensal *Neisseria* as *N. gonorrhoeae* or the *N. gonorrhoeae* was not identified. A misdiagnosing of gonorrhoeae can have a major impact on the individual. A false positive diagnosis can be social devastating for individuals living in a partnership, while a false negative diagnosed individual will continue as a reservoir for the gonococci.

A good discriminatory tool for diagnosing gonorrhoea is crucial, to avoid false positive as well as false negatives result. The evaluation of the *porA* PCR has so far shown that is suitable for diagnosing gonorrhoeae; it is reproducible, fast and specific.

7 CONCLUSION

The major objective in this study was to establish a sensitive and specific PCR for detection of *N. gonorrhoeae*.

The specificity of the primers showed that the design was successful. Choosing the *porA* pseudogene as target and locating the primers in a conserved region of the gene resulted in amplification of only DNA from *N. gonorrhoeae*.

The technical validation of the *porA* PCR gave an acceptable result. The optimized reaction solution had a final concentration of 900 nM primers, 200 nM *porA* probe and 100 nM of the IAC probe, and efficiency was 99.3 % even in the presence of an IAC. The 7.5 Geq/PCR reaction detection limit is considered low. And 100 % analytic sensitivity and specificity is acceptable. All of the 200 different strains and isolates of *N. gonorrhoeae* were amplified by the *porA* primers, and neither of commensal *Neisseria* strains and other micro organisms tested was identified as *N. gonorrhoeae*.

The UTM-RT system is already in use as transport system for *Chlamydia trachomatis*, *Herpes Simplex I/II* and Adenoviruses. We found it suitable for preserving *N. gonorrhoeae* DNA after 7 days and in different temperatures. The system comprises of a variety of swab suitable for different gonococcal samples sites.

The clinical validation resulted in 100 % specificity and sensitivity for the PCR in comparison to culture (71 % sensitivity). The real-time PCR assay may therefore increase sensitivity in supplement to culture. The PPV and NPV were also 100 % in comparison to culture in this study population. A high PPV and NPV are important, especially in low prevalence populations such as Norway.

In overall, the work presented show when analyzing samples from the selected population the *porA* PCR can increase the sensitivity in sites as pharynx and rectum compared to cultivation techniques. Throughout the study the *porA* PCR has shown sufficient assay stability, it is rapid and reproducible. The *porA* pseudogene has so far proved to be highly species specific for *N. gonorrhoeae* and is therefore a valid supplement in diagnosing gonorrhea.

8 FUTURE PERSPECTIVES

The *porA* pseudogene PCR has so far proved to be a valid supplement in diagnosing gonorrhoeae. However, it would be of interest to further evaluate the PCR. A thorough validation by including study populations from different geographical areas over a long time period is necessary. In fact, the method is in use at several places around the world and was recommended by centers for disease control and prevention (Atlanta, Georgia) to their collaborators in Thailand. In addition, after antibiotic treatment of gonorrhoeae, a test of cure is done, prompting the need to find the appropriate time for a test of cure when diagnosing with PCR.

9 REFERENCES

1. Palmer,H.M., Mallinson,H., Wood,R.L., & Herring,A.J. Evaluation of the specificities of five DNA amplification methods for the detection of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* 41, 835-837 (2003).
2. Telfer Brunton,W.A., Young,H., & Fraser,D.R. Isolation of *Neisseria lactamica* from the female genital tract. A case report. *Br J Vener Dis* 56, 325-326 (1980).
3. Murray,P.R., Baron,E.J., & Jorgensen,J.H. *Manual of clinical microbiology*. (ASM Press American Society for Microbiology,(2003).
4. Perrin,A., Nassif,X., & Tinsley,C. Identification of regions of the chromosome of *Neisseria meningitidis* and *Neisseria gonorrhoeae* which are specific to the pathogenic *Neisseria* species. *Infect. Immun.* 67, 6119-6129 (1999).
5. Tinsley,C.R. & Nassif,X. Analysis of the genetic differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae*: two closely related bacteria expressing two different pathogenicities. *Proc. Natl. Acad. Sci. U. S. A* 93, 11109-11114 (1996).
6. Roberts M.C. Plasmids of *Neisseria gonorrhoeae* and other *Neisseria* species. *Clinical Microbiology Reviews* 18-23 (1989).
7. Hamilton,H.L. & Dillard,J.P. Natural transformation of *Neisseria gonorrhoeae*: from DNA donation to homologous recombination. *Mol. Microbiol.* 59, 376-385 (2006).
8. Biswas,G.D., Thompson, S.A. & Sparling,P.F. Gene transfer in *Neisseria gonorrhoeae*. *Clinical Microbiology Reviews* S24-S28 (1989).
9. Linz,B., Schenker,M., Zhu,P., & Achtman,M. Frequent interspecific genetic exchange between commensal *Neisseriae* and *Neisseria meningitidis*. *Mol. Microbiol.* 36, 1049-1058 (2000).
10. Snyder,L.A. & Saunders,N.J. The majority of genes in the pathogenic *Neisseria* species are present in non-pathogenic *Neisseria lactamica*, including those designated as 'virulence genes'. *BMC. Genomics* 7, 128 (2006).
11. Murray, P.R., Rosenthal, K.S, Kobayashi, G.S., Pfaller, M.A. *Medical Microbiology* 4th ed. Mosby INC 2002 p256-261
12. Feavers,I.M. & Maiden,M.C. A gonococcal *porA* pseudogene: implications for understanding the evolution and pathogenicity of *Neisseria gonorrhoeae*. *Mol. Microbiol.* 30, 647-656 (1998).

13. Unemo,M., Norlèn,O., & Fredlund,H. The *porA* pseudogene of *Neisseria gonorrhoeae*-- low level of genetic polymorphism and a few, mainly identical, inactivating mutations. *APMIS* 113, 410-419 (2005).
14. Whiley,D.M., Buda,P.J., Bayliss,J., Cover,L., Bates,J., & Sloots,T.P. A new confirmatory *Neisseria gonorrhoeae* real-time PCR assay targeting the *porA* pseudogene. *Eur. J. Clin. Microbiol. Infect. Dis.* 23, 705-710 (2004).
15. Olsen,C.C., Schwebke,J.R., Benjamin,W.H., Jr., Beverly,A., & Waites,K.B. Comparison of direct inoculation and Copan transport systems for isolation of *Neisseria gonorrhoeae* from endocervical specimens. *J. Clin. Microbiol.* 37, 3583-3585 (1999).
16. Graver,M.A. & Wade,J.J. Survival of *Neisseria gonorrhoeae* isolates of different auxotypes in six commercial transport systems. *J. Clin. Microbiol.* 42, 4803-4804 (2004).
17. Boel,C.H., van Herk,C.M., Berretty,P.J., Onland,G.H., & van den Brule,A.J. Evaluation of conventional and real-time PCR assays using two targets for confirmation of results of the COBAS AMPLICOR *Chlamydia trachomatis/Neisseria gonorrhoeae* test for detection of *Neisseria gonorrhoeae* in clinical samples. *J. Clin. Microbiol.* 43, 2231-2235 (2005).
18. Tapsall,J.W., Limnios,E.A., Nguyen,N.L., Carter,I., Lum,G., Freeman,K., Tabrizi,S.N., Garland,S.M., Whiley,D.M., Sloots,T.P., & Chambers,I.W. Cryptic-plasmid-free gonococci may contribute to failure of *cppB* gene-based assays to confirm results of BD ProbeTEC PCR for identification of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* 43, 2036-2037 (2005).
19. Vorobieva,V., Firsova,N., Ababkova,T., Leniv,I., Haldorsen,B.C., Unemo,M., & Skogen,V. Antibiotic susceptibility of *Neisseria gonorrhoeae* in Arkhangelsk, Russia. *Sex Transm. Infect.* 83, 133-135 (2007).
20. Martin,J.E., Jr. & Lester,A. Transgrow, a medium for transport and growth of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *HSMHA. Health Rep.* 86, 30-33 (1971).
21. McFadden J.F test for identification of medical bacteria. (Williams & Wilkins, Baltimore, MD; 1980).
22. Kruczak-Filipov P. & Shively R.G. in *Clin. Microbiol. Proc. handbook*, Edn. 1. ed. Isenberg,H.D. 1.5.1-1.5.18 (American Society for Microbiology, Washington, DC; 1992).
23. Anand,C.M., Gubash,S.M., & Shaw,H. Serologic confirmation of *Neisseria gonorrhoeae* by monoclonal antibody-based coagglutination procedures. *J Clin. Microbiol.* 26, 2283-2286 (1988).

24. Sneed J. in Clin. Microbiol. Proc. handbook, Edn. 1. ed. Isenberg,H.D. 1.14.7-1.14.21 (American Society for Microbiology, Washington, DC; 1992).
25. Hjelmevoll,S.O., Olsen,M.E., Sollid,J.U., Haaheim,H., Unemo,M., & Skogen,V. A fast real-time polymerase chain reaction method for sensitive and specific detection of the *Neisseria gonorrhoeae* porA pseudogene. *J. Mol. Diagn.* 8, 574-581 (2006).
26. Rutledge,R.G. & Cote,C. Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Res.* 31, e93 (2003).
27. Hjelmevoll,S.O., Olsen,M.E., Sollid,J.U., Haaheim,H., Melby,K.K., Moi,H., Unemo,M., & Skogen,V. Clinical Validation of a Real-Time Polymerase Chain Reaction Detection of *Neisseria gonorrhoeae* porA Pseudogene Versus Culture Techniques. *Sex Transm. Dis.* 35, 517-520 (2008).
28. Whiley,D.M., Anderson,T.P., Barratt,K., Beaman,M.H., Buda,P.J., Carter,M., Freeman,K., Hallsworth,P., Limnios,E.A., Lum,G., Merien,F., Vernel-Pauillac,F., Tapsall,J.W., Witt,M.J., Nissen,M.D., & Sloots,T.P. Evidence that the gonococcal porA pseudogene is present in a broad range of *Neisseria gonorrhoeae* strains; suitability as a diagnostic target. *Pathology* 38, 445-448 (2006).
29. Brunton,W.A.T., Young,H., & Fraser,D.R. Isolation of *Neisseria lactamica* from the female genital tract. *Br. J. Vener. Dis.* 56, 325-326 (1980).
30. Tonje Davidsen, T., Rødland, E.,A., Lagesen,K., Seeberg,E., Rognes,T., & Tønjum, T. Biased distribution of DNA uptake sequences towards genome maintenance genes. *Nucleic Acids Res.* 2004; 32(3): 1050–1058.
31. Van Dyck E MAPP. Laboratory diagnosis of sexually transmitted diseases. *World Health Organization*, 1999:1-21.