Oropharyngeal *Neisseria gonorrhoeae*

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Oropharyngeal *Neisseria gonorrhoeae*:

the key for surveillance

Brian M.J.W. van der Veer

Oropharyngeal *Neisseria gonorrhoeae*: the key for surveillance
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Oropharyngeal *Neisseria gonorrhoeae*:

the key for surveillance

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Brian Marinus Johannus Wilhelmus van der Veer

Promotor

Prof. dr. Christian J.P.A. Hoebe

Copromotores

Dr. Lieke B. van Alphen

Dr. ir. Petra F.G. Wolffs

Beoordelingscommissie

Prof. dr. Paul Savelkoul (voorzitter)

Prof. dr. Jochen Cals

Dr. Sylvia Bruisten (GGD Amsterdam)

Dr. Janneke Heijne (RIVM, Bilthoven)

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General introduction

Background

Sexually transmitted infections (STI) are a major global public health challenge [1]. The four most common curable bacterial STIs are *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, and *Treponema pallidum*. The World Health Organization (WHO) estimated that 376 million infections of these four STI occur each year with *N. gonorrhoeae* (gonorrhea) being the second most common bacterial STI with an estimated global incidence of 64 million new cases annually [2]. Gonorrhea can infect the urogenital tract in both women and men but also the mucosa of extra-genital sites such as the rectum and oropharynx [3]. Another route of transmission is from mother to child during birth that causes an ocular infection.

Many gonorrhea infections remain hidden to care because of stigma and their asymptomatic nature, especially in women [4-7]. Therefore, these infections may remain undiagnosed and untreated that can lead to ongoing transmission and complications [8]. In women, gonorrhea can cause pelvic inflammatory disease, infertility, ectopic pregnancy, and maternal death [4]. In men, urethral infections are frequently symptomatic and can cause urethritis with pus-like discharge, dysuria, and pain or swelling in one testicle [9]. In addition, infection with gonorrhea has been associated with an increased risk of transmission and acquisition of human immunodeficiency virus (HIV) [3, 10].

Epidemiology

Gonorrhea is the second most common bacterial STI worldwide and this is also the case in the Netherlands [2, 11]. There were 7,362 gonorrhea cases diagnosed at an STI clinic and an estimated 9,500 cases in general practice in the Netherlands in 2018 [11]. The reported positivity rate in STI clinics was 1.6% in women, 1.8% in heterosexual men, and 11.0% in men who have sex with men (MSM). Figure 1 shows the positivity rate and number of tests in STI clinics from 2009-2018. The positivity rates of these groups remained stable since 2015 because triage and number of tests has not changed. This is because of the implementation of the latest Dutch STI guidelines and budget cap

(ASG-regeling) in 2015 [12]. In the guidelines several sexual risk-groups for gonorrhea infection acquisition are described such as MSM, sex workers, and young people [4, 12].

Figure 1. Total number of tests and positivity rate of gonorrhea by sexual risk-group, 2009-2018. Source: Slurink et al. 2019. Sexually transmitted infections in the Netherlands 2018. National Institute for Public Health and Environment.

Biology of *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a Gram-negative diplococcus that is part of the genus *Neisseria* of which most species are commensal and colonize the nasopharynx [3]. These *Neisseria* species are a substantial part of the human nasal and oropharyngeal microbiome. Other *Neisseria* species colonize a range of other hosts such as cats and dogs [13]. *Neisseria gonorrhoeae* and *N. meningitidis* are the two pathogenic species, with the second causing bacterial meningitis [14]. As both commensal and pathogenic *Neisseria* species share the same niches they compete in colonization and nutrition [3].

As gonorrhea is an obligate human pathogen it cannot survive outside the host [3]. Therefore, transmission relies on unprotected sexual intercourse [15]. Gonorrhea is easily transmitted from men to women as it attaches to sperm and high bacterial load is observed in ejaculates [16-18]. However, the transmission efficiency from women to men is less clear [3, 19]. This might be because gonorrhea must be desialylated to infect the male urinal tract but gonorrhea is sialylated during infection in women [20]. After transmission, gonorrhea needs to adhere to the mucosal epithelium, in which type IV pili

and opacity (Opa) proteins are essential in both women and men [3]. Type IV pili is responsible for the initial adherence and micro-colony formation but is also involved in the natural competence and immune evasion [21-23]. Opa proteins mediate adherence by multigene phase variation that also results in antigenic variation to avoid the immune system [24, 25]. Another mechanism to avoid the immune system is binding to components of the complement system (C4BP and factor H) by *porB* to suppress the neutrophil response [26]. This suppresses the inflammatory response and result in an asymptomatic infection [3]. However, the modulation of the immune system is still under debate as gonorrhea can also deliberately activate and attract neutrophils and macrophages that might facilitate transmission [3, 27].

Diagnoses and treatment

Gonorrhea can be cultured on agar plates like chocolate agar medium and was considered the golden standard [15, 28]. Culture of gonorrhea is inexpensive to perform but is not ideal for routine diagnostics because of the demanding nutritional and environmental conditions. These issues also lead to frequent failure of gonorrhea culture as it requires viable gonorrhea after transport to the laboratory. Still, culture is performed to date but mainly for surveillance of antimicrobial resistance (AMR), in which culture remains to be considered as the golden standard [28, 29].

In modern routine diagnostics Nucleic Acid Amplification Testing (NAAT) is performed to detect gonorrhea RNA or DNA [11, 15, 30]. These NAATs are the recommended tests as they outperform culture in sensitivity, do not require viable gonorrhea to be present in the sample, and can be automated but have a lower specificity compared to culture [9, 15]. The first generation of NAATs for gonorrhea diagnostics presented issues with false-positive results by detection of commensal *Neisseria* species in a sample that was negative for gonorrhea [31]. As a direct consequence none of the first generation NAATs are approved for extra-genital (anorectal and oropharyngeal) testing as commensal *Neisseria* species are commonly present at those anatomical sites [28, 31]. The second generation of NAATs vastly improved on this aspect but still almost all NAATs are not

officially approved for extra-genital testing. Nevertheless, in practice, NAATs are widely used for extra-genital testing nowadays.

Uncomplicated gonorrhea is treated with a single dose of 500mg ceftriaxone intramuscular in the Netherlands [12]. Resistance of gonorrhea towards the last first-line treatment of ceftriaxone has been reported by different countries worldwide but not in the Netherlands [11, 32]. Therefore, international guidelines recommended a dual-therapy of 500mg ceftriaxone with 1g of azithromycin to slow down AMR in gonorrhea [4, 30]. However, gonorrhea developed azithromycin resistance rapidly thereby limiting the dual-therapy strategy [11, 33, 34]. In addition, the susceptibility of gonorrhea towards ceftriaxone is decreasing. Therefore, other antibiotics with adverse events like gentamycin have to be considered but also novel antimicrobials should be developed [32, 35].

Prevention and screening

Identification and effective treatment of patients infected with gonorrhea can interrupt onward transmission and development of serious complications but many gonorrhea infections are asymptomatic and therefore the patient may not actively seek care [4, 5, 7]. This lead to several control strategies to reduce gonorrhea prevalence and prevent AMR: (I) education and counseling on safer sexual practices, (II) identification of both asymptomatic and symptomatic patients, (III) evaluation, treatment and counseling of sex partners, (IV) effective diagnosis and treatment, and (V) gonococcal resistance surveillance and stewardship [4, 36].

Since 2015 a budget cap and triage (ASG-regeling) was implemented by the Dutch government [12]. The triage is based on research in which populations at risk to acquire gonorrhea infections were identified. Examples are MSM, sex workers, individuals notified for an STI, and young people (<25) for which free of charge testing is provided at an STI clinic. Furthermore, testing is recommended for individuals presenting with symptoms related to gonorrhea like vaginal discharge in women and urethritis in men. Patients are tested urogenitally for gonorrhea at each STI clinic consultation but based on sexual risk group and risk factors additional extra-genital samples are tested. The STI

clinic guideline covers different test policies for women, heterosexual men, and MSM. MSM are routinely and systematically tested at both anorectal and oropharyngeal sites (extra-genital). Heterosexual patients are only tested extra-genitally when reporting symptoms, anal or oral sexual behavior in the last six months. However, the need for extra-genital testing and clinical relevance of these infections in heterosexuals is under debate [37, 38]. One of the key questions is whether oropharyngeal gonorrhea has sufficient transmission potential to infect others as it is believed that the oropharynx is a reservoir for AMR development for gonorrhea [32, 39, 40]. A possible measure for transmission potential is the load of gonorrhea as this has been described in other STIs [41-43].

Oropharyngeal gonorrhea

Recent epidemiological studies demonstrate that oropharyngeal NG is common in MSM and women [44, 45]. Prevalence of oropharyngeal NG was 5.5% in MSM and 1.4% in women when routine universal testing was performed and shows that half of the oropharyngeal NG infections were oropharyngeal-only infections. As oropharyngeal gonorrhea infections are common, frequently asymptomatic, and are linked to AMR development it is a very important anatomical site of infection [32, 44-47]. The oropharyngeal site is potentially a reservoir for resistance development as commensal *Neisseria* species are commonly present that can exchange DNA with gonorrhea [32, 47]. In addition, kissing, oral sex, and saliva use as lubricant are common sexual practices in both heterosexuals and MSM [38, 46, 48]. This can result in unnoticed onward transmission and facilitate the development of multi-drug resistant gonorrhea strains. A key problem for surveillance of oropharyngeal gonorrhea is that infections of this particular anatomical site are difficult to culture and thus limits characterization of strains [11, 28]. However, the exact role of oropharyngeal gonorrhea infections is unknown. The transmission potential of oropharyngeal gonorrhea might be lower compared to genital and urethral infections in MSM but the published studies are relatively small and no data exists of women and heterosexual men [39, 40, 49]. It is also unknown if resistance development in the oropharynx is spontaneous or rare with clonal spread of resistant strains [32].

Antimicrobial resistance

During the pre-antimicrobial era, treatment of gonorrhea was not quick and easy. Treatment involved having a healthier lifestyle with abstinence of sexual activity and alcohol but also urethral irrigation and hyperthermia could be used [32]. Initially, when antibiotics were discovered and applied to treat gonorrhea, most gonorrhea cases were cured with sulfonamides [50]. However, resistance towards sulfonamides developed rapidly, leading to discontinued use as a first-line treatment [50, 51]. Many other classes of antibiotics were developed and initially effective to treat gonorrhea infections but AMR was developed for all classes of antibiotics (figure 2) [32]. This limits the options for treatment of gonorrhea that could become untreatable with current antibiotics [32, 52]. To complicate things further, insufficient new antibiotics are in development thus preventing gonorrhea to become untreatable is imperative [4, 53]. Gonorrhea has developed resistance via various AMR mechanisms like enzymatic destruction or increased export of antimicrobials [32]. A very important mechanism is target alteration by creating a mosaic gene variant of *penA*, which encodes for the protein penicillin-binding protein-2 (PBP2), leading to ceftriaxone resistance [54-56]. As ceftriaxone is the final empirical first-line treatment and used worldwide, it is imperative to understand the development of mosaic *penA* genes. A likely cause is via horizontal-gene-transfer in the oropharynx as *Neisseria* species are naturally competent and commensal *Neisseria* species are commonly present in this anatomical site [57-60]. Therefore, oropharyngeal gonorrhea infections are considered as a reservoir for AMR development but it is unknown whether mosaic genes are spontaneously formed *in vivo* [32].

Figure 2. Historical overview of recommended antimicrobials and resistance in *N. gonorrhea*.

Source: Unemo et al. 2014. Antimicrobial Resistance in *Neisseria gonorrhoeae* in the 21st Century: Past, Evolution, and Future. Clinical Microbial Reviews.

Surveillance of gonorrhea

As gonorrhea is frequently asymptomatic and could become untreatable it is essential to implement surveillance to monitor transmission and dissemination of resistant strains [4, 61]. This lead to national and international Gonococcal Antimicrobial Surveillance Programs (GASP) [4, 62]. In the Netherlands this program is called Gonokokken Resistentie tegen Antibiotica Surveillance (GRAS) [11]. In principle, the surveillance programs aim to gain insight in the occurrence of AMR in gonorrhea by characterizing many cultured strains. The surveillance programs should monitor trends in resistance to react to emerging resistant strains. The cultured strains in surveillance are characterized by using techniques like Multi-Locus Sequence Typing (MLST), *Neisseria Gonorrhea* Multi-Antigen Sequence Typing (NG-MAST), and Whole-Genome Sequencing (WGS) [63-65]. MLST has the lowest discriminatory power, then NG-MAST, and WGS has the highest but is also the most expensive one. A major issue in surveillance is that all techniques require a cultured isolate but culture is known to frequently fail [11, 66]. Also, typically only a single colony is characterized thus missing potential mixed gonococcal infections

[62, 67]. These infections do occur and could have an impact on the treatment of the infection as the uncultured strain could be resistant [68-70]. In addition, if mixed gonococcal infections are not considered, the transmission network is not well characterized, thereby limiting opportunities for interventions.

Aim of thesis

The aim of this thesis is to provide insight in the clinical relevance of extra-genital gonorrhea infections, particularly oropharyngeal infections, and surveillance of gonorrhea. To assess the clinical relevance of extra-genital infections we validated the Cobas 4800 CT/NG test for oropharyngeal samples and determined the bacterial load at different anatomical sites in different sexual risk groups. For surveillance of gonorrhea we developed various culture-independent methods to assess the unculturable and mixed gonococcal infections. Furthermore, nanopore-sequencing was performed to gain insight in the oral *Neisseria* resistome with a focus on the *penA* gene.

In **chapter 2** we validated the cobas 4800 CT/NG test for oropharyngeal samples and provide insight in potentially missed infections.

Chapter 3 evaluates the clinical relevance of (extra) genital gonorrhea infections in both women and men by assessing the bacterial load. The bacterial load of different anatomical sites was compared and also related to sex and symptoms.

In **chapter 4** we technically validated the culture-free NG-MAST method that is a culture-independent variant of NG-MAST. This method was also used to gain insight in the extent of patients infected with different gonococcal strains at separate anatomical sites.

Chapter 5 describes the occurrence of mixed gonococcal infections in a selected group of MSM by combining culture-free NG-MAST with next-generation sequencing. The data of this chapter and **chapter 4** were evaluated to review the relevance of multiple NG isolates in a patient, either mixed or at separate anatomical sites.

In **chapter 6**, the added value of culture-independent surveillance was tested by comparing culture-positive and culture-negative samples subjected to culture. Furthermore, the implications for current culture-dependent surveillance are given per sample material.

A potential gonococcal outbreak in young heterosexuals in South-Limburg, the Netherlands was characterized in **chapter 7**. We applied culture-free NG-MAST to genotype the gonococcal strains to identify outbreak clusters. Furthermore, we describe determinants associated with these clusters, the added value of culture-independent genotyping, and discuss challenges associated with gonococcal outbreak detection and management.

Chapter 8, the oropharyngeal *Neisseria* resistome is assessed by characterizing *penA* genes and their flanking regions with nanopore-sequencing. Despite ceftriaxone resistance in gonorrhea does not exist in the Netherlands, mosaic *penA* genes were observed in gonorrhea. These mosaic genes were compared with commensal *Neisseria* species in the sample to test the possibility of recombination during the infection.

Lastly, in **chapter 9**, the main findings are discussed of the presented work and directions for future research are given.

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2

Despite excellent test characteristics of the cobas®4800 CT/NG assay, detection of oropharyngeal *Chlamydia trachomatis* and *Neisseria gonorrhoeae* remains challenging

Julius M. van Niekerk*, Brian M.J.W. van der Veer*, Christian J.P.A. Hoebe,

Jeroen van de Bovenkamp, Christel van Herk, Inge H.M. van Loo,

Lieke B. van Alphen, Petra F.G. Wolffs

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* Contributed equally

Abstract

Objectives

Oropharyngeal *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) infections are common but few commercial nucleic acid amplification tests (NAATs) specify extragenital samples as intended use. The test characteristics of the cobas®4800 CT/NG assay are evaluated for oropharyngeal swabs.

Methods

The technical validation includes analysis of the specificity, sensitivity, dynamic range, linearity, efficiency and precision. The probability of detection curve combined with historical data enables estimation of potentially missed diagnoses. A clinical evaluation has been performed on a subset of 2798 clinical samples available from routine diagnostics. Results of the cobas®4800 were compared with *in-house* CT/NG PCR assays. Discrepant samples were tested with resolver assays and these results were considered decisive.

Results:

No cross-reactivity was seen in the analytical specificity analysis. High linearity ($\geq 0.983 R^2$), efficiency (89%-99%), and precision (0.1-0.9 C_t -value) were seen for both CT/NG. The limit of detection in oropharyngeal samples was 3.2×10^2 IFU/mL for CT and 6.7×10^2 CFU/mL for NG. Estimates on potentially missed diagnoses were up to 7.2% for CT and up to 24.7% for NG. Clinical sensitivity and specificity were evaluated with 25 CT-, 86 NG positive and 264 negative samples, resulting in 100% and 99.6% for CT and 100% and 96.7% for NG respectively.

Conclusion:

The findings in this study demonstrate the utility of the cobas®4800 CT/NG assay for oropharyngeal samples. Despite being a highly accurate test, the range of reported C_t -values especially for NG suggests relatively low oropharyngeal loads. Hence, consistent detection over the full range of oropharyngeal loads could be impaired.

Introduction

Chlamydia trachomatis (CT) and *Neisseria gonorrhoeae* (NG) are the most common diagnosed bacterial sexual transmitted infections (STI) globally (1). When left untreated, these STI may have significant health implications (2), including chronic pelvic pain, ectopic pregnancy, tubal infertility, pelvic inflammatory disease, and Fitz-Hugh-Curtis syndrome in women, and epididymitis, or epididymo-orchitis in men (3, 4).

Previous studies demonstrated the importance of screening of extragenital sites for CT/NG infections, as up to 6.6% of CT and up to 36.4% of NG can be missed in men who have sex with men when oropharyngeal sampling is not performed (5), and up to 92.2% and 73.8% of oropharyngeal CT/NG have negative urethral tests (6).

Few of the currently commercially available nucleic acid amplification tests (NAATs) specify oropharyngeal samples for their intended use. The FDA has granted clearance of both the Xpert CT/NG to Cepheid, and Aptima Combo 2 Assay to Hologic for extragenital testing (7). Concerns about oropharyngeal testing are mainly related to the ability of assays to cross-react with non-gonococcal *Neisseria* species, leading to false-positive results (8-11). Complicating matters even more is that oropharyngeal CT/NG loads have been demonstrated to be lower than urogenital and rectal loads (12, 13). Nevertheless, current laboratory standards recommend NAATs for the detection of oropharyngeal CT/NG if a laboratory has established specifications for performance characteristics in accordance to present regulations (8).

Recent efforts by Roche have shown promising results for the cobas®6800 for CT/NG screening (Roche Diagnostics, Indianapolis, IN, USA) of oropharyngeal and anorectal swab specimens, and are the first CE-IVD test with a claim for the detection of CT/NG from extragenital samples (14). However, the commonly used cobas®4800 (Roche) has not been validated for the detection of oropharyngeal CT/NG, despite its high analytical (9, 15) and clinical specificity in urogenital (15-19) and rectal samples (20), and the cobas®CT/NG assay being a FDA-cleared NAAT (8). Although the more recently launched cobas®6800 uses an optimized protocol, it is stated that the primer and probe design are identical for the cobas®4800 (14). Therefore, the aim of this study is to assess the validity of the cobas®4800 for oropharyngeal swabs for the detection of CT/NG. The

secondary goal of the study is to provide an estimate of missed low level positives, based on load-related probability of detection.

Methods

Technical validation

The cobas®4800 is a qualitative NAAT for CT/NG detection on an automated system that implements DNA isolation, polymerase chain reaction (PCR) preparation, and qPCR of the cryptic plasmid and outer-membrane protein A for CT detection, including the detection of the Swedish new variant strain and the genomic target DR9-region (wild-type or highly conserved sequence variant) for NG (15). The technical validity is based on the analysis of the dynamic range (linearity and efficiency), the probability of detection (POD) curve, the limit of detection (LOD), precision (intra- and inter-assay variation) and analytical specificity.

The dynamic range was determined using three CT serovars (D, E, F) and three NG strains (NG-MAST ST2992, ST11461, ST14764) cultured as described previously (21, 22). Dilution series in cobas® PCR Media were made ranging from $1.6\text{--}4.3 \times 10^8$ till $1.6\text{--}4.3 \times 10^0$ IFU/mL for CT and $10^7\text{--}10^0$ CFU/mL for NG in triplicate (23). Linear regression was performed to determine the dynamic range, defined as the range in which at least one replicate was positive, and efficiency in GraphPad Prism version 5.03 (GraphPad Software, San Diego, USA). The strain with the shortest dynamic range was spiked in triplicate in aliquots of a CT/NG negative oropharyngeal swab pool, resulting in concentration series of $10^5\text{--}10^3\text{--}10^2\text{--}10^1\text{--}10^0$ IFU/mL or CFU/mL. The series were tested at seven separate days resulting in 21 measurements per concentration to calculate the proportion of positive samples and a sigmoidal curve fit was used to construct a POD-curve. The LOD, defined as 95% detection, was interpolated from the POD curve. Precision of the assay is described as the standard deviation (SD) as a measure of variation of which ≤ 1 C_t-value is considered acceptable. This was determined on three separate days by adding two additional samples for low (10^2 IFU/CFU/mL), moderate (10^3 IFU/CFU/mL), and high (10^5 IFU/CFU/mL) concentrations. The analytical specificity of the cobas®4800 was tested with a panel of 39 bacterial strains based on genetic similarity with CT/NG or potential presence in the oropharynx ($\geq 10^6$ IFU/mL or CFU/mL), not

CT/NG, including *C. muridarum* (1), *C. pneumoniae* (1), *E. coli* (1), *H. influenzae* (2), *M. catarrhalis* (1), *N. cinerea* (1), *N. denitrificans* (1), *N. elongata* (1), *N. flavescens* (1), *N. lactamica* (2), *N. meningitidis* (3), *N. mucosa* (7), *N. perflava* (1), *N. polysaccharea* (1), *N. subflava* (14), *S. aureus* (1).

Clinical evaluation

The clinical evaluation is based on a retrospective sample selection from 24 August 2017 until 19 June 2018. In routine diagnostics, 2798 oropharyngeal swab samples (collected and stabilized with cobas® PCR media) were screened for CT/NG in using the cobas®4800 and stored at -20°C. Based on routine diagnostic results, 28 were CT positive and 92 NG positive resulting in a positivity rate of 1.0% and 3.3% respectively. All CT and NG positive samples were included and 264 consecutive CT/NG negative oropharyngeal samples from 28 August 2017 till 2 October 2017. The original results of the cobas® were used for this study. Of nine samples, no residual sample was available and were therefore excluded, leaving 25 CT positive and 86 NG positive samples for analysis. Residual sample material of routine diagnostics was used in comparator tests targeting the cryptic plasmid for CT and *porA* pseudogene for NG (24, 25). QPCRs were performed as described previously (25). Discrepancy analysis was performed by resolver tests; the Versant kPCR CT/GC (Siemens Healthcare Diagnostics, Tarrytown, USA) in conjunction with an *in-house opa* qPCR for NG, by an independent technician (CvH) (26). All tests were performed within 2 years, which has previously been validated to hardly impact CT DNA detection (27). The comparator assay results were considered decisive, thereby classifying discrepant samples as true-positive or true-negative. After discrepancies were resolved sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were determined.

Estimation of undetected CT/NG

Load values of interpolated cobas® C_t-values of CT or NG positive oropharyngeal swabs (n=796) between 2012 till 26 June 2019 were binned with intervals of 0.2 log₁₀ and observed frequencies were plotted in a histogram. These frequencies were divided by

the mean POD per bin and subtracted by the observed frequency. This provides a rough estimate of missed low level positives, based on load-related probability of detection.

Standard curves *Chlamydia trachomatis*

Standard curves *Neisseria gonorrhoeae*

Figure 1 Standard curves of CT and NG. The dots represent the mean C_t -value and the error bars the standard deviation of the three independent replicates. In both CT and NG standard curves none of the 10^0 were positive.

Results

Technical validation

The dynamic range was $1.6\text{--}4.3 \times 10^8$ till $1.6\text{--}4.3 \times 10^1$ IFU/mL for CT and $10^7\text{--}10^1$ CFU/mL for NG (Figure 1). In 8/18 CT samples with a load $>10^7$ IFU/mL false-positive NG results with high C_t -values (range C_t -value: 36.9–41.4) were observed. The assay demonstrated high linearity (≥ 0.983 R^2) and efficiency (89%–99%) for both CT/NG. Serovar F and ST14764 were used for the POD curve as only 1/3 replicates was positive at 10^1 IFU/CFU/mL in contrast with the other CT serovars and NG strains (2/3). All POD curve replicates ($n=21$) of 10^3 and 10^5 (IFU or CFU per mL) were detected by the assay (Figure 2). At 10^2 IFU/mL, 76.2% (16/21) of the CT replicates were positive, whereas at 10^2 CFU/mL only 57.1% (12/21) of the NG replicates were positive. Only 9.5% (2/21) for both CT and NG at 10^1 and none at 10^0 were positive. The interpolated LOD was 3.2×10^2 IFU/mL for CT and 6.7×10^2 CFU/mL for NG in oropharyngeal samples (Figure 2). Variation of qPCR results of oropharyngeal samples was tested with 10^3 , 10^5 IFU or CFU/mL at three separate days; 10^2 was excluded as it is below the LOD. The assay demonstrated high precision for both CT and NG as the demonstrated SD ranged from 0.2–0.7 C_t -value for all concentrations

and similar results were found for the repeatability (SD 0.1-0.9 C_t -value). A 100% specificity was seen as none of the strains in the test panel showed positive amplification.

Figure 2 CT and NG POD curve of oropharyngeal samples. Dots (CT) and squares (NG) represent the proportion of positive replicates per concentration. The LOD was interpolated from the sigmoidal curve fit at 95% detection. For CT the LOD was 3.2×10^2 IFU/mL and 6.7×10^2 CFU/mL for NG.

Clinical evaluation

Sensitivity and specificity for CT detection were 100% and 99.6% and for NG 100% and 96.7% respectively. Almost all CT results (288/289) were concordant except one C_t -value 40.1 which was negative in the comparator assays and therefore considered false positive (Table 1). Most (326/350) of the NG results were concordant but 24 NG positive samples (C_t -value range 31.4-39.6) were discordant in the *in-house porA* qPCR. Fifteen of the 24 discordant NG positive samples were confirmed with the resolver assay, whereas the remaining nine were not, and considered false positive. One sample had a C_t -value above the LOD and was Sanger sequenced, confirming the NG positive result of the cobas®4800.

Table 1 Clinical validity for CT and NG detection of the cobas®4800

		Cobas®4800		
<i>Chlamydia plasmid</i>		Positive	Negative	
and Versant kPCR	Positive	24	0	24
	Negative	1	264	265
		25	264	289
<i>N. gonorrhoeae porA,</i>		Positive	Negative	
Versant kPCR and <i>opa</i>	Positive	77	0	77
	Negative	9 ^a	264	273
		86	264	350

^a Contains one Sanger sequence confirmed sample

Estimated undetected CT/NG in oropharyngeal swabs

Theoretical missed oropharyngeal infections were estimated using the POD curve and the observed CT/NG bacterial loads (Figure 3). For CT, up to 7.2% of oropharyngeal infections could go undetected and up to 24.7% for NG.

Figure 3 Potentially missed oropharyngeal CT/NG diagnoses. Absolute frequencies of oropharyngeal CT/NG diagnoses divided by probability of detection per bin. Loads are divided in bins of 0.2 log10. In red: potentially missed diagnoses. Percentage potentially missed: amount of missed diagnoses / expected frequency. A) Potentially missed CT diagnoses 7.2% ($13.5/(174+13.5)$). B) Potentially missed NG diagnoses 24.7% ($261/(796 + 261)$).

Discussion

For CT, the cobas®4800 results demonstrate excellent technical performance characteristics, as evidenced by the high precision, the LOD, and the analytical specificity analysis. When assessing the technical performance with CT spiked samples, false positive NG results were observed in samples with $>10^7$ IFU/mL (C_t 20.4). This phenomenon is likely caused by the high intensity fluorescent signal from the CT reporter dye overlapping into the NG detection channel. To estimate the impact of these potential false-positive results, we analysed all clinical routine diagnostic results in our laboratory between 2012-June 2019, but CT loads required to cause this phenomenon have not occurred. Therefore, spectral bleed-through is considered to be of limited clinical relevance. The clinical evaluation has also shown very good assay performance, with sensitivity for CT of 100%, specificity of 99.6%, PPV of 96%, and NPV of 100%. Although, the technical performance characteristics of the comparator assays are unknown for oropharyngeal samples. Lastly, the true specificity is likely underestimated as only a limited number of negative samples within the reported time period has been included, as further discussed under the limitations of the study.

Accordingly, the assay displays a very good performance for *Neisseria gonorrhoeae*. The analytical sensitivity demonstrates a LOD of 6.65×10^2 CFU/mL (C_t 36.5), with the lower limit of the dynamic range being 10^1 CFU/mL. Importantly, no cross-reactivity was observed in the analytical specificity analysis. Likewise, a high precision has been observed. The clinical evaluation exhibited an excellent sensitivity (100%) and NPV (100%). Nine cobas® positive samples could not be confirmed with the comparator assays. Eight of these samples had low loads (C_t -values 38.7-39.3). Non-detection in this range of loads can be a matter of probability, as illustrated in Figure 2. The effects of DNA degradation could contribute in non-detection. However, the extent of degradation is likely limited, as specimens are collected and stabilized with cobas® PCR media, and stored at -20°C . The remaining unconfirmed cobas® positive sample had a C_t -value of 36.4. Identification through Sanger sequencing demonstrated a NG nonetheless, disproving cross-reactivity. Hence, the definite specificity and PPV should be 97.1% and 90.7% respectively, instead of the reported 96.7% and 89.5% in Table 1.

However, the results on specificity and PPV should be interpreted in accordance with the limitations discussed below.

Previous studies on urine and endocervical swabs have shown equal performance characteristics of the cobas® CT/NG assay in comparison to two FDA approved NAATs, with sensitivities and specificities ranging between 89.1-97.6% and 99.5-100% for CT, and 95.6-100% and 99.7-100% for NG (16, 28). To best of our knowledge, only one study has performed a head-to-head comparison of the analytical sensitivity of the cobas®4800 CT/NG assay to three FDA approved NAATs, for *Chlamydia trachomatis* (29). Self-collected vaginal swabs and first void urine were spiked with a CT dilutions series. This study showed a comparable sensitivity to the Abbott RealTime CT/NG assay and BD Probetec CT/NG, and possibly a sensitivity disadvantage compared to the Gen-Probe Aptima Combo 2 (AC2). However, this disadvantage in sensitivity compared to the AC2 did not become statistically apparent in the aforementioned clinical evaluations (16, 28). Unfortunately, no comparisons of analytical sensitivity are available for NG. With the limited available data, there are no indications of underperformance of the cobas® compared to the other FDA approved NAATs. In addition, NAATs are the recommended test for the detection of CT/NG infections, due to their overall sensitivity and specificity compared to other tests available, including culture (8).

Taking into account previous research indicating lower oropharyngeal CT/NG loads compared to urogenital and anorectal sites (12, 13), historical data on oropharyngeal CT/NG positive samples were reviewed. Of note, the NG load distribution curve peaks between C_t 38-39, with 469/797 samples (58.8%) having a C_t -value ≥ 36.5 (load bin <2.8), and 301 samples ≥ 38.0 (37.8%, load bin <2.4). Given that the probability of detection declines rapidly with lower loads, the true number of positive samples in the range below the LOD are underrepresented. To estimate missed low level positives, a POD-curve was constructed (Fig. 2), which enables division of observed frequencies by their respective probability of detection, which provides a rough estimate of potentially missed diagnoses. These results suggest that up to 7.2% of oropharyngeal CT could go undetected, and possibly up to 25% of NG. As exemplified by the load distribution

curves, it could be argued that the probability to consistently detect oropharyngeal CT/NG across the full range of loads might be a more common problem, likely across multiple platforms. Although the true extent of potentially missed diagnoses is subject to a large degree of measurement uncertainty discussed under the limitations, this study aims to point out that a considerable number of CT/NG could go undetected. If CT/NG loads were to be clinically relevant across the entire range of loads, clinical diagnostics and public health would benefit from more sensitive methods and/or controlling for pre-analytical factors that could improve sample collection.

Lastly, there is an ongoing debate on whether or not confirmatory testing is required for oropharyngeal NG results (18). The IUSTI guideline recommends confirmatory testing for extragenital samples, alongside a strict local evaluation of the performance on extragenital samples, before introduction of a NAAT. If the NAAT's PPV does not exceed 90%, confirmatory testing is recommended (4). The CDC recommends that consideration should be given to retest samples with an alternate target assay, when NAATs are used which are able to cross-react with non-gonococcal *Neisseria* species (8). From the perspective of oropharyngeal NG loads as presented in this study, the authors would argue that load-related inability to confirm with a secondary test must be taken into consideration in this debate, and might be under-recognized in this discussion.

This study has several limitations. First, only a fraction of negative samples within the reported time period has been assessed in this study (<10%, i.e. 264/2773 for CT, and 264/2712 for NG). This especially impacts the specificity, causing an underestimation. Hence, it is important to note that specificity data in this study cannot be extrapolated to different populations with known disease prevalence to calculate NPV or PPV. The PPV in this study is unaffected, as all cobas® positive samples were included. The impact on sensitivity and NPV is probably small, as the prior probability of disease is small. Hence, the majority of negative samples will be 'true negatives'. Second, there are several factors that could cause significant measurement uncertainty in estimating the potentially missed diagnoses: (i) the adequacy of sample collection, (ii) the estimation of the CT/NG loads used in the dilution series required to establish the

calibration curves, (iii) not estimating the calibration curves with multiple strains, (iv) the sigmoidal curve fit. Lastly, no LODs or POD-curves have been established for either of the comparator assays used in this study.

Taken together, the findings in this study demonstrate the utility of the cobas®4800 CT/NG assay for oropharyngeal samples. This study consists of a comprehensive technical and clinical evaluation of the CT/NG assay, taking into account probability of detection, and offers estimations of potentially missed diagnoses. Despite being a highly accurate test, the range of C_t -values especially for NG suggest relatively low oropharyngeal loads. Hence, consistent detection over the full range of oropharyngeal loads could be impaired, possibly for multiple platforms and more sensitive methods are called upon.

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3

Men and Women Have Similar *Neisseria gonorrhoeae* Bacterial Loads: a Comparison of Three Anatomical sites

Brian M.J.W. van der Veer, Christian J.P.A. Hoebe, Nicole H.T.M. Duker-Muijters,

Lieke B. van Alphen, Petra F.G. Wolffs

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Abstract

Neisseria gonorrhoeae is a common bacterial sexually transmitted infection (STI). Currently, there are limited data on the bacterial load in both men and women and on both genital and extragenital sites. Therefore, we quantified *N. gonorrhoeae* bacterial loads in a large population of women, heterosexual men, and men who have sex with men (MSM) at three different anatomical sites. *N. gonorrhoeae*-positive samples ($n=1265$) of STI clinic consultations ($n=944$) were tested for *N. gonorrhoeae* with the Roche Cobas 4800 system, and quantification cycle (C_q) values were used as an inversely proportional measure for *N. gonorrhoeae* bacterial load after interpolation from a standard curve. Bacterial loads were compared between sample materials and sexes using t tests. The following mean *N. gonorrhoeae* loads were observed: urine, $4.5 \pm 1.0 \log_{10}$ CFU/ml; vaginal swabs, $4.3 \pm 1.1 \log_{10}$ CFU/ml; anorectal swabs (women), $4.0 \pm 1.2 \log_{10}$ CFU/ml; anorectal swabs (men), $4.5 \pm 1.3 \log_{10}$ CFU/ml; oropharyngeal swabs (women), $2.8 \pm 0.9 \log_{10}$ CFU/ml; and oropharyngeal swabs (men), $3.2 \pm 1.0 \log_{10}$ CFU/ml. Oropharyngeal swabs had a significantly lower *N. gonorrhoeae* load ($P < 0.001$) than genital and anorectal samples. Loads did not differ between men and women. This is the first study that determined *N. gonorrhoeae* load in both women and men at three anatomical sites. The substantial *N. gonorrhoeae* load at all sample sites suggest that all sites may have transmission potential. However, the oropharyngeal site presents the lowest bacterial load. Men and women have a similar *N. gonorrhoeae* loads on separate anatomical sites, arguing for similar transmission potential and similar clinical relevance.

Neisseria gonorrhoeae accounts for 87 million of the estimated 376 million new curable sexually transmitted infection (STI) cases worldwide every year (1). Limiting the transmission of *N. gonorrhoeae* is a major public health challenge. Many infections remain hidden to care, especially in women, because of stigma and their asymptomatic nature (2–5). Untreated *N. gonorrhoeae* infections can affect the vagina, cervix, urethra, the rectum, or the throat and could lead to severe sequelae. In women, *N. gonorrhoeae* can cause pelvic inflammatory disease, infertility, ectopic pregnancy, and maternal death (2). In men, urethral infections are frequently symptomatic and can cause urethritis with pus-like discharge, dysuria, and pain or swelling in one testicle (6). Current guidelines recommend urogenital testing for *N. gonorrhoeae* in both men and women (7–9). The need for extragenital testing is recognized for men who have sex with men (MSM) but not for women and heterosexual men. Aside from MSM, extragenital testing is only recommended for patients with an indication, such as a history of direct exposure by anal or oral sex or symptoms. The prevalence of extragenital *N. gonorrhoeae* is 3% to 21% in MSM but substantially lower in heterosexual men and women (0% to 3%) (10, 11). However, as current health care is mainly focused on genital testing, many *N. gonorrhoeae* infections remain hidden to care. Thus, 29% to 30% of *N. gonorrhoeae* infections in women and 14% of *N. gonorrhoeae* infections in heterosexual men will be missed if extragenital testing is not performed (11, 12). Hidden *N. gonorrhoeae* infections may facilitate ongoing transmission, although the extent of underdiagnosis and undertreatment of extragenital *N. gonorrhoeae* infections remains unknown (6, 10–13). In addition, oropharyngeal infections are thought to have a key role in antimicrobial resistance (AMR) development in *N. gonorrhoeae* because of the common presence of commensal *Neisseria* species that readily exchange DNA (14). Aside from poor access to health care and high-risk behavior, the transmissibility of *N. gonorrhoeae* can also be linked to bacterial load, as a higher inoculum dose in an experimental infection study resulted in higher infection probability (2, 15). The site of infection and symptoms could also be associated with *N. gonorrhoeae* bacterial load. Previous studies have demonstrated a higher *N. gonorrhoeae* load in urethral infections of symptomatic men and anorectal infections of symptomatic MSM (16, 17). Lower *N. gonorrhoeae* load was

observed in oropharyngeal swabs of MSM than in urethral and anorectal swabs (16, 17). However, these studies were relatively small, and no study has assessed the *N. gonorrhoeae* bacterial load in women in relation to symptoms. Therefore, the aim of this study was to assess the bacterial load of *N. gonorrhoeae* at three different anatomical sites (urogenital, anorectal, and oropharyngeal) in a large population of women, heterosexual men, and MSM visiting a large STI clinic. Bacterial loads were compared between sample materials and associations with symptoms were assessed. Results might provide insights in the transmission potential of different anatomical sites in women and men and might also inform testing guidelines for clinical practice.

Material and methods

Study population and procedures.

The South Limburg public health service STI clinic offers STI testing for patients with high-risk of acquiring an STI free of charge. Between January 2012 and July 2017, *N. gonorrhoeae* was diagnosed in 923 individuals, yielding data from 1,095 consultations for retrospective analysis. The *N. gonorrhoeae* positivity rates in this time frame were 1.7% in women, 1.7% in heterosexual men, and 12.0% in MSM. Patients were tested urogenitally for *N. gonorrhoeae* using first-void urine (men) or a self-collected vaginal swab (women). According to regular STI clinic guidelines, MSM were routinely and systematically tested at both anorectal and oropharyngeal sites (18). However, heterosexual men and women were tested extragenitally when reporting symptoms or receptive anal sex (women) or oral sex in the last 6 months (18). Patients were requested to collect a self-taken anorectal swab or a nurse-taken oropharyngeal swab for extragenital testing. A standardized medical and sexual history was routinely taken by nurses for all patients, including self-reported symptoms and sexual behavior in the last 6 months. Urogenital symptoms were defined as one or more of the following: genital discharge, bleeding, itching, swelling, pain, burning sensation, and more frequent urination. Anal symptoms were defined as rectal discharge, bleeding, pain, and itching. Any oropharyngeal symptoms were defined as symptomatic. We excluded samples ($n = 215$) from consultations that had missing data on the Cobas 4800 CT/NG test (Roche Diagnostics, Basel, Switzerland) quantification cycle (C_q) value, sexual risk group,

symptoms, and number of sex partners in the previous 6 months, leaving 944 consultations (800 individuals) and 1,265 samples for analysis (see Fig. S1 in the supplemental material).

***N. gonorrhoeae* diagnosis and bacterial load.**

All samples were tested for *N. gonorrhoeae* with the Cobas 4800 CT/NG test. This assay targets a direct repeat region called DR-9 that is specific for *N. gonorrhoeae* (19). The Cobas test utilizes amplification using two sets of primers and probes to detect two highly conserved variations within the DR-9 region. Little variation is expected between *N. gonorrhoeae* strains, because this assay is not prone to copy number variation and the targets are highly conserved. Therefore, bacterial load can be quantified by interpolation of *C_q* values to a standard curve with known concentrations of *N. gonorrhoeae*. For quantification, a standard curve was made for each specimen type (e.g., swab or urine) as the Cobas 4800 CT/NG test requires different volumes of input material (20). *N. gonorrhoeae* suspension from log-phase culture was spiked (100 μ l) and serially diluted 10-fold. A part of the suspension was plated to determine the amount of *N. gonorrhoeae* by counting the colonies resulting in *n* CFU per ml. To resemble urine samples, 5ml of an *N. gonorrhoeae*-negative male urine pool was added and stabilized before *N. gonorrhoeae* was spiked.

Data analysis.

We performed a retrospective data analysis of the bacterial loads of 1,265 *N. gonorrhoeae*-positive samples from 944 routine diagnostic consultations stratified by sample material and sexual risk group (data available in Data Set S1). We binned the number of sex partners in the previous 6 months in tertiles (0 to 2, 3 to 7, and ≥ 8). Descriptive statistics were used to describe the proportion of concurrent *N. gonorrhoeae* infections at separate anatomical sites, symptoms, and number of sex partners per sexual risk group and sample material. Bacterial load values were $\log_{10}$ transformed for analyses and compared between sample materials using Student's *t* tests (all combinations of two sample materials). Using multivariable linear regression, the

bacterial load comparison between sample materials was adjusted for age, sexual risk group, symptoms, and number of sex partners. Bacterial loads were also compared per sample material between sexes with a Student's *t* test. Using multivariable linear regression, the bacterial load comparison between sample materials and sexes was adjusted for age, sexual risk group, symptoms, and number of sex partners. Next, associations between symptoms (i.e., the dependent variable with the asymptomatic group as the reference group) and bacterial load (i.e., the outcome) were assessed. Therefore, univariable and multivariable linear regression analyses were performed on data stratified by sample material and sexual risk group. These models were adjusted for age and number of sex partners in multivariable linear regression. A *P* value of less than 0.05 was considered statistically significant. All analyses were performed using R statistical software, version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria). Figures were made using the R package ggplot2 version 3.1.0.

Ethics.

The Medical Ethical Committee of Maastricht University Medical Centre (MUMC+; number METC 2017-2-0251) approved the study protocol, as only deidentified retrospective data from standard care were used. Consultations were part of routine STI clinic procedures where individuals did not object to the use of their samples and data for research. For this study, we used two retrospective databases from the STI clinic and laboratory, with data collected within standard care.

Results

Proportion of extragenital positivity.

N. gonorrhoeae was detected in 944 routine diagnostic consultations with 1,265 *N. gonorrhoeae*-positive samples, among which, 603 belonged to MSM, 144 to heterosexual men, and 197 to women (Table 1). In most patients, only one sample was *N. gonorrhoeae* positive, irrespective of sexual risk group (73.6% of women, 98.6% of heterosexual men, and 64.8% of MSM) (Table 1). Some patients were *N. gonorrhoeae* positive at all three tested anatomical sites (55/944 [5.8%]). Extragenital-only, anorectal, or oropharyngeal *N. gonorrhoeae*-positive samples were observed in 31.0% (61/197) of women, 22.9% (33/144) of heterosexual men, and 73.8% (445/603) of MSM who were diagnosed (Table 1). Of these, most of the women (49/61 [80.3%]) and all heterosexual men were *N. gonorrhoeae*-positive only at the oropharyngeal site.

Table 1 Characteristics of the study population by sexual risk group.

Characteristic	Women (n=197)	Heterosexual men (n=144)	MSM ^a (n=603)	Total (n=944)
Age (yr) (median [interquartile range])	31 (22-44)	30 (23-47)	32 (24-45)	31 (23-45)
No of <i>N. gonorrhoeae</i> -positive samples within one patient (% [n]) ^b				
1	73.6 (145)	98.6 (142)	64.8 (391)	71.8 (678)
2	20.3 (40)	1.4 (2)	28.0 (169)	22.4 (211)
3	6.1 (12)	0.0 (0)	7.1 (43)	5.8 (55)
<i>N. gonorrhoeae</i> diagnosis (% [n])				
Genital only	43.7 (86)	75.7 (109)	6.8 (41)	25.0 (236)
Anorectal only	5.1 (10)	0.0 (0)	32.8 (198)	22.0 (208)
Oropharyngeal only	24.9 (49)	22.9 (33)	25.2 (152)	24.8 (234)
Genital – anorectal	14.2 (28)	0.0 (0)	10.8 (65)	9.9 (93)
Genital – oropharyngeal	5.1 (10)	1.4 (2)	1.5 (9)	2.2 (21)
Anorectal – oropharyngeal	1.0 (2)	0.0 (0)	15.8 (95)	10.3 (97)
All three sites	6.1 (12)	0.0 (0)	7.1 (43)	5.8 (55)
Symptoms (any site) (% [n])				
Asymptomatic	40.1 (79)	38.9 (56)	46.9 (283)	44.3 (418)
Symptomatic	59.9 (118)	61.1 (88)	53.1 (320)	55.7 (526)
Number of sex partners (% [n])				
0-2	31.5 (62)	34.7 (50)	18.2 (110)	23.5 (222)
3-7	34.0 (67)	47.9 (69)	43.6 (263)	42.3 (399)
≥8	34.5 (68)	17.4 (25)	38.1 (230)	34.2 (323)

^a MSM, men who have sex with men^b n, number of consultations

***N. gonorrhoeae* bacterial loads compared between sample materials and sexes.**

In total, 269 urine samples (genital site, men), 136 vaginal swabs (genital site, women), 453 anorectal swabs, and 407 oropharyngeal swabs were available for analysis (Table 2). The mean bacterial loads per sample material were as follows: urine samples, $4.5 \pm 1.0 \log_{10}$ CFU/ml; vaginal swabs, $4.3 \pm 1.1 \log_{10}$ CFU/ml; anorectal swabs, $4.4 \pm 1.3 \log_{10}$ CFU/ml; and oropharyngeal swabs, $3.1 \pm 1.0 \log_{10}$ CFU/ml. Oropharyngeal swabs had a lower mean load (16- to 25-fold lower) than all other sample materials ($P < 0.001$, t tests), while no statistically significant difference was observed between the other sample materials. The observation of lower load in oropharyngeal swabs persisted even when corrected for age, sexual risk group, symptoms, and number of sex partners in a linear regression model ($P < 0.001$).

Table 2 Characteristics of the study population by sample material.

Category	% (no.) of samples				
	Urine (n=269)	Vaginal (n=136)	Anorectal (n=453)	Oropharyngeal (n=407)	Total (n=1265)
Sexual risk group					
Women	NA ^a	100 (136)	11.5 (52)	17.9 (73)	20.6 (261)
Heterosexual men	41.3 (111)	NA	0.0 (0)	8.6 (35)	11.5 (146)
MSM ^b	58.7 (158)	NA	88.5 (401)	73.5 (299)	67.8 (858)
Symptoms					
Asymptomatic	23.0 (62)	31.6 (43)	75.1 (340)	88.9 (362)	63.8 (807)
Symptomatic	77.0 (207)	68.4 (93)	24.9 (113)	11.1 (45)	36.2 (458)

^a NA, not applicable, as no samples were collected as part of routine diagnostic procedures.

^b MSM, men who have sex with men.

Stratified by sex, the mean bacterial loads were as follows: urine samples, $4.5 \pm 1.0 \log_{10}$ CFU/ml; vaginal swabs, $4.3 \pm 1.1 \log_{10}$ CFU/ml; anorectal swabs of women, $4.0 \pm 1.2 \log_{10}$ CFU/ml; anorectal swabs of men, $4.5 \pm 1.3 \log_{10}$ CFU/ml; oropharyngeal swabs of women, $2.8 \pm 0.9 \log_{10}$ CFU/ml; and oropharyngeal swabs of men, $3.2 \pm 1.0 \log_{10}$ CFU/ml (Fig. 1). Anorectal and oropharyngeal swabs from women had a significantly lower load than those from men, 2.8-fold ($P = 0.02$) and 2.3-fold ($P = 0.002$), respectively. However, these differences were not statistically different when corrected for age, symptoms, and number of sex partners ($P = 0.09$ and $P = 0.06$, respectively).

FIG 1 *Neisseria gonorrhoeae* bacterial loads stratified by sample material and sex. Oropharyngeal NG bacterial load was lower than genital and anorectal *N.gonorrhoeae* bacterial loads ($P < 0.001$). No significant difference was observed between the other sample materials. The small differences between women and men can be explained by differences in age, symptoms, and number of sex partners.

Association between symptoms and *N. gonorrhoeae* bacterial load.

Most anorectal (75%) and oropharyngeal (89%) samples were from asymptomatic infections, but only a minority of the genital samples (23% to 32%) were from asymptomatic infections (Table 2). Genital symptoms were associated with a 3- to 5-fold higher bacterial load in urine samples from men (heterosexual men, $P < 0.001$; MSM, $P =$

0.03) but not in vaginal swabs from women ($P = 0.26$), in both univariable and multivariable linear regression models (Tables 3 to 5). Associations of oropharyngeal symptoms with *N. gonorrhoeae* bacterial load could not be assessed in heterosexual men, because no symptoms were reported (Table 4). In both women ($P = 0.007$) and MSM ($P < 0.001$), anorectal symptoms were associated with a higher *N. gonorrhoeae* bacterial load, 4- and 47-fold, respectively. In contrast, oropharyngeal symptoms were not associated with a higher *N. gonorrhoeae* load in either women ($P = 0.99$) or MSM ($P = 0.06$). The results remained similar in analyses that were restricted to women (97/197) or MSM (595/603) who were tested at both genital and extragenital sites, including in an analysis corrected for the *N. gonorrhoeae* load at the concurrent sites (see Tables S1 and S2 in the supplemental material).

Table 3 Mean *N. gonorrhoeae* bacterial load and association of symptoms with *N. gonorrhoeae* bacterial load in women per sample material

		CFU/ml			
Category	n	Mean log ₁₀ (SD)	Δlog ₁₀ (95%CI) ^a	Adjusted Δlog ₁₀ (95%CI)	Factor load
Vaginal					
Asymptomatic	43	4.1 (±1.1)	Ref ^b	Ref	Ref
Symptomatic	93	4.4 (±1.0)	0.28 (-0.11-0.66)	0.22 (-0.17-0.61)	1.7
Anorectal					
Asymptomatic	48	3.9 (±1.2)	Ref	Ref	Ref
Symptomatic	4	5.4 (±0.8)	1.42 (0.20-2.65) ^c	1.67 (0.47-2.87) ^d	46.8
Oropharyngeal					
Asymptomatic	67	2.8 (±0.9)	Ref	Ref	Ref
Symptomatic	6	3.0 (±0.9)	0.16 (-0.57-0.89)	0.01 (-0.69-0.71)	1.0

a CI, confidence interval.

b Ref, reference.

c $P < 0.05$.

d $P < 0.01$.

Table 4 Mean *N. gonorrhoeae* bacterial load and association of symptoms with *N. gonorrhoeae* bacterial load in heterosexual men

Category	n	CFU/ml		Adjusted $\Delta\log_{10}$ (95%CI)	Factor load
		Mean $\log_{10}$ (SD)	$\Delta\log_{10}$ (95%CI) ^a		
Urine					
Asymptomatic	33	3.9 (±1.3)	Ref ^b	Ref	Ref
Symptomatic	78	4.7 (±0.9)	0.72 (0.31-1.14) ^c	0.74 (0.31-1.16) ^c	5.4
Oropharyngeal					
Asymptomatic	35	3.1 (±1.0)	NA ^d	NA	NA
Symptomatic	0	NA	NA	NA	NA

a CI, confidence interval.

b Ref, reference.

c $P < 0.001$.

d NA, not applicable, as no oropharyngeal symptoms were reported.

Table 5 Mean *N. gonorrhoeae* bacterial load and association of symptoms with *N. gonorrhoeae* bacterial load in MSM per sample material

Category	n	CFU/ml		Adjusted $\Delta\log_{10}$ (95%CI)	Factor load
		Mean $\log_{10}$ (SD)	$\Delta\log_{10}$ (95%CI) ^a		
Urine					
Asymptomatic	29	4.3 (±1.0)	Ref ^b	Ref	Ref
Symptomatic	129	4.6 (±0.8)	0.36 (0.00-0.72) ^c	0.41 (0.04-0.77 ^c	2.5
Anorectal					
Asymptomatic	292	4.3 (±1.3)	Ref	Ref	Ref
Symptomatic	109	4.9 (±1.2)	0.57 (0.29-0.84) ^d	0.57 (0.30-0.85) ^d	3.8
Oropharyngeal					
Asymptomatic	260	3.1 (±1.0)	Ref	Ref	Ref
Symptomatic	39	3.5 (±1.0)	0.34 (-0.00-0.67)	0.31 (-0.02-0.64)	2.1

a CI, confidence interval.

b Ref, reference.

c $P < 0.05$.d $P < 0.001$.

Discussion

This is the first study that determined the *N. gonorrhoeae* bacterial load in a large group of women, heterosexual men, and MSM at three different anatomic sample sites (urogenital, anorectal, and oropharynx). Oropharyngeal swabs had a significantly lower bacterial load (16- to 25-fold) than all other sample materials, whereas the other sample materials had comparable bacterial loads. Also, *N. gonorrhoeae* bacterial load was comparable in women and men per anatomical sample site. Genital symptoms were associated with a higher *N. gonorrhoeae* bacterial load in urine samples from men but not in vaginal swabs from women. Furthermore, anorectal symptoms were associated with a higher bacterial load in anorectal swabs from both women and MSM, but no difference was seen in oropharyngeal swabs. More than 70% of *N. gonorrhoeae* infections were single site infections, of which, 31% were anorectal only and 35% oropharyngeal only. This implies a need for extragenital testing, at least in MSM. Moreover, >60% of *N. gonorrhoeae* infections were asymptomatic, with a wide range between genital (23% to 32%), anorectal (75%), and oropharyngeal (89%) infections.

We demonstrated that oropharyngeal swabs have on average a 16- to 25-fold lower bacterial load than all other sample materials, which is in line with previous studies (16, 17). Next to sample material, *N. gonorrhoeae* load was associated with genital symptoms (18-fold) in men and anorectal symptoms in MSM (20-fold) in previous studies (16, 17). We observed associations of only 3- to 5-fold higher *N. gonorrhoeae* loads in urine samples from men with genital symptoms and 4- to 47-fold higher *N. gonorrhoeae* loads in anorectal swabs from women and MSM with anorectal symptoms. No significant association in women reporting genital symptoms was found, possibly due to the difficulty of recognizing genital symptoms compared to that for men (7). The large difference in results between our study and previous studies could be explained by differences in analytical methods, sample materials, and study population. The strong association of *N. gonorrhoeae* load with symptoms in anorectal swabs from women in our study (47-fold) is likely due to the low number of samples from women reporting anal symptoms ($n = 4$), resulting in a large variation in the estimates (Table 3).

To interpret the *N. gonorrhoeae* load of this study, in our opinion, the only relevant comparison could be with the reported infectious doses of experimental gonococcal infection studies. Hobbs et al. described that a high *N. gonorrhoeae* inoculum load is associated with higher infection probability in experimental gonococcal infections in male volunteers (15). *N. gonorrhoeae* was inoculated in the urethra by instillation of 0.2 to 0.3 ml *N. gonorrhoeae* suspension. A 50% infectious dose (ID_{50}), defined as the concentration of an infectious dose of a single exposure needed to infect half of the volunteers, of $3.3 \log_{10}$ CFU for the MS11mkC strain and one of $5 \log_{10}$ CFU for the FA1090 strain were estimated. To compare the *N. gonorrhoeae* loads of the present study with the reported ID_{50} , the ID_{50} dose was divided by the volume of the suspension (average of 0.25 ml), resulting in $3.9 \log_{10}$ CFU/ml for the MS11mkC strain and $5.6 \log_{10}$ CFU/ml for the FA1090 strain. This would imply that all three anatomical sites have sufficient transmission potential, as the mean *N. gonorrhoeae* load (3.1 to $4.5 \log_{10}$ CFU/ml) is comparable with the ID_{50} doses. It would also mean that asymptomatic *N. gonorrhoeae* infections would have a *N. gonorrhoeae* load that is sufficient for transmission, as the mean bacterial load was 2.8 to $4.3 \log_{10}$ CFU/ml in both women and men (Tables 3 to 5). These comparisons and ID_{50} doses should be interpreted with caution, because the reported inocula of the experimental infection needed for infection varied between studies, most likely because of the inoculation procedures (21). Also, the large difference in ID_{50} s between the MS11mkC and FA1090 strains found in the study of Hobbs et al. suggests that infection is not solely driven by bacterial load (15, 22). In addition, we determined the *N. gonorrhoeae* load of an anatomical site, but this does not necessarily reflect the transmitted *N. gonorrhoeae* load per sexual act.

In our study, we observed that 65% of *N. gonorrhoeae* infections in MSM were single site infections, of which, 33% were anorectal only and 25% oropharyngeal only, confirming the need for extragenital testing in MSM. Potentially, we demonstrate the need for testing in women and heterosexual men, as high proportions of oropharyngeal-only infections were found (Table 1). However, women and heterosexual men were not systemically tested at genital and extragenital sites but on indication such as a history of

direct exposure by anal or oral sex or symptoms. This could introduce bias and elevate the proportion of extragenital-only infections in women and heterosexual men. Moreover, >60% of *N. gonorrhoeae* infections are asymptomatic, with a wide range between genital (23% to 32%), anorectal (75%), and oropharyngeal (89%) infections. Asymptomatic infections may potentially lead to unnoticed transmission of *N. gonorrhoeae* and would be unnoticed when routine testing is not applied (as in heterosexual men and in women) (23). Even though the oropharyngeal site might have a lower transmission potential per sexual exposure due to the lower mean *N. gonorrhoeae* bacterial load, an oropharyngeal *N. gonorrhoeae* infection could have a profound role in transmission (16, 24, 25). First, oral sex is a common sexual practice in women, heterosexual men, and MSM (11). Second, kissing and saliva use as lubricant have also been proposed as a plausible route of transmission, as substantial amounts of *N. gonorrhoeae* were detected in the saliva of MSM (24, 26). In addition, oropharyngeal infections are thought to have a key role in the AMR development in *N. gonorrhoeae* because of the common presence of commensal *Neisseria* species that readily exchange DNA (14). As the estimated median duration of oropharyngeal *N. gonorrhoeae* infection is at least 15 weeks, it could be a reservoir for onward transmission and antimicrobial-resistant strains (14, 27, 28).

One of the strengths of this study is the diversity of the study population, achieved by including women and heterosexual men along with MSM and the routine extragenital testing in MSM. One of its weaknesses is that women and heterosexual men were not routinely tested extragenitally, thereby potentially underestimating the relative number of extragenital *N. gonorrhoeae* infections. Generalizability might be difficult, as only high-risk individuals for acquiring an STI were studied (10). In addition, the volume of first-void urine taken per consultation can vary, and that could have an impact on the determined *N. gonorrhoeae* bacterial load in a sample. This could also explain the large range of observed bacterial load in urine samples in Fig. 1.

In conclusion, our results suggest that all anatomical sample sites may have a role in transmission because of the substantial *N. gonorrhoeae* load. Oropharyngeal *N. gonorrhoeae* infections might have a smaller role, as the mean bacterial load is lower. It would also imply that a *N. gonorrhoeae* infection is equally relevant in women and men, as there is no difference in bacterial load. Oropharyngeal infections should be monitored, since they are frequently single infections, mostly asymptomatic, and could be involved in the AMR development of *N. gonorrhoeae*. Furthermore, extragenital testing should be systematically implemented in routine diagnostics, at least in MSM, to reduce missed infections and help improve *N. gonorrhoeae* control.

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Supplemental files

Supplemental figure 1 Flowchart, including *Neisseria gonorrhoeae* positive samples for analysis

Supplemental table 1 Mean NG bacterial load and association of symptoms with NG bacterial load in women per sample material. Women included in the analysis (97/197) were tested at genital and extra-genital sites. NG load of NG negative samples was set at 0.

Women	<i>n</i>	Mean log ₁₀ CFU/mL (SD)	Δlog ₁₀ CFU/mL (95%CI)	Adjusted Δlog ₁₀ CFU/mL (95%CI)	Adjusted Δlog ₁₀ CFU/mL (95%CI) including NG load concurrent site	Factor load
Vaginal						
Asymptomatic	17	3.7 (±1.3)	Ref	Ref	Ref	Ref
Symptomatic	28	4.3 (±1.0)	0.58 (-0.11-1.27)	0.48 (-0.22-1.19)	0.60 (-0.11-1.31)	4.0
Anorectal						
Asymptomatic	39	4.0 (±1.2)	Ref	Ref	Ref	Ref
Symptomatic	1	5.7	1.74 (-0.70-4.18)	2.01 (-0.22-4.25)	1.91 (-0.42-4.25)	82.1
Oropharyngeal						
Asymptomatic	56	2.8 (±0.9)	Ref	Ref	Ref	Ref
Symptomatic	6	3.0 (±0.9)	0.17 (-0.57-0.91)	-0.05 (-0.76-0.67)	-0.06 (-0.81-0.69)	0.9

Abbreviations: CFU, Colony forming units; SD, standard deviation, CI, confidence interval, Ref, reference

Supplemental table 2 Mean NG bacterial load and association of symptoms with NG bacterial load in MSM per sample material. MSM included in the analysis (595/603) were tested at genital and extra-genital sites. NG load of NG negative samples was set at 0.

MSM	<i>n</i>	Mean log ₁₀ CFU/mL (SD)	Δlog ₁₀ CFU/mL (95%CI)	Adjusted Δlog ₁₀ CFU/mL (95%CI)	Adjusted Δlog ₁₀ CFU/mL (95%CI) including NG load concurrent site	Factor load
Urine						
Asymptomatic	29	4.3 (±1.0)	Ref	Ref	Ref	Ref
Symptomatic	128	4.6 (±0.8)	0.36 (-0.00-0.72)	0.41 (0.04-0.77)*	0.40 (0.03-0.77)*	2.5
Anorectal						
Asymptomatic	288	4.3 (±1.3)	Ref	Ref	Ref	Ref
Symptomatic	106	4.9 (±1.2)	0.58 (0.30-0.86)***	0.59 (0.31-0.87)***	0.56 (0.28-0.83)***	3.6
Oropharyngeal						
Asymptomatic	256	3.1 (±1.0)	Ref	Ref	Ref	Ref
Symptomatic	36	3.5 (±1.0)	0.33 (-0.02-0.68)	0.30 (-0.04-0.64)	0.31 (-0.03-0.65)	2.1

* = p<0.05; *** = p<0.001

Abbreviations: CFU, Colony forming units; SD, standard deviation, CI, confidence interval, Ref, reference

4

Culture-free genotyping of *Neisseria gonorrhoeae*
revealed distinct strains at different anatomical
sites in a quarter of patients, the Netherlands,
2012 to 2016

Brian M.J.W. van der Veer, Petra F.G. Wolffs, Christian J.P.A. Hoebe,

Nicole H.T.M. Duker-Muijrs, Lieke B. van Alphen

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Abstract

Background: Genotyping of *Neisseria gonorrhoeae* (NG) is essential for surveillance to monitor NG transmission and dissemination of resistant strains. Current genotyping methods rely on bacterial culture which frequently fails.

Aim: Our aim was to develop a culture-free genotyping method that is compatible with the widely used *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) database, which facilitates genotyping of NG detected at separate anatomical sites in individual patients.

Methods: Specific primers for both PCR targets *porB* and *tbpB* were designed and technically validated by assessing the analytical sensitivity, cross-reactivity with 32 non-gonococcal *Neisseria* species, and concordance with NG-MAST. Clinical application was assessed on 205 paired samples from concurrent NG infections at different anatomical sites of 98 patients (81 men who have sex with men and 17 women) visiting our sexually transmitted infections clinic.

Results: Typing could be consistently performed on samples with a PCR quantification cycle (Cq) value <35. Furthermore, the method showed no cross-reactivity and was concordant with NG-MAST. Culture-free NG-MAST improved the typing rate from 62% (59/95) for cultured samples to 94% (89/95) compared with culture-dependent NG-MAST. Paired samples of 80 of 98 patients were genotyped, revealing distinct NG strains in separate anatomical sites in 25% (20/80) of the patients.

Conclusions: This NG-specific genotyping method can improve NG surveillance as it facilitates genotyping of non-culturable and extra-genital samples. Furthermore, 25% of patients were infected with multiple NG strains, which is missed in current culture-dependent surveillance. Including non-culturable and concurrent NG infections in surveillance informs actions on dissemination of multidrug-resistant NG strains.

Introduction

Neisseria gonorrhoeae (NG) is one of the most common bacterial sexually transmitted infections [1]. The World Health Organization (WHO) estimates that more than 100 million new cases of NG occur each year, even though testing for NG and diagnostics have improved [1,2]. Detection of NG allows empirical treatment that results in cure in at least 95% of cases, and rapid cure subsequently limits transmission [1]. However, increasingly resistant strains of NG have been reported in the last decades, which could complicate empirical treatment [3]. Therefore, gaining insight in transmission and antimicrobial resistance (AMR) of NG is important. NG can be detected by culture or nucleic acid amplification test (NAAT) but both methods have limitations [2]. Culture is known to be less sensitive because NG requires demanding nutritional and environmental conditions, leading to a low percentage of culture-confirmed diagnoses [4]. In contrast, NAAT are more sensitive but cannot determine the AMR profile [2,5].

Surveillance of NG is essential to monitor transmission and dissemination of resistant strains. NG multi-antigen sequence typing (NG-MAST) is a widely used genotyping method to monitor transmission and outbreaks [6,7]. This method has a higher discriminatory power than multilocus sequence typing (MLST) and is more cost-effective than highly discriminatory whole genome sequencing [8,9]. In addition, some of the NG-MAST sequence types (ST) are associated with AMR [7,10]. The currently used NG-MAST protocol requires culture because the primers cross-react with other *Neisseria* species [11]. To date, only two studies have genotyped non-cultured clinical samples with NG-MAST [6,11]. Whiley et al. demonstrated that NG-MAST can be applied to non-cultured urogenital samples but not to samples from extra-genital sites (oropharynx and rectum) because of the presence of commensal *Neisseria* species [11]. They showed that mainly *N. lactamica*, *N. meningitidis* and *N. polysaccharea* strains lead to the cross-reactivity. Furthermore, it appeared that successful application of NG-MAST to non-cultured samples was linked to the quantification cycle (Cq) of the PCR-positive sample because four of the five failed samples had a high Cq value (>35).

Previous studies have shown that patients can be NG-positive at extra-genital sites and have concurrent NG infections at different anatomical sites [12,13]. Most of the extra-genital and concurrent infections are observed in risk groups, for example in men who have sex with men (MSM). Extra-genital sites may act as a reservoir for AMR genes as the present commensal *Neisseria* species, potentially harbouring AMR genes, readily exchange DNA with NG [14]. Typing the oropharyngeal site using culture-dependent methods is especially difficult because the bacterial load is lower than at other anatomical sites and this appears to be linked to culture success [15]. In previous studies, concurrent NG infections were studied with various genotyping methods [6,11,16-18]. Distinct NG strains per anatomical site have been observed and some strains demonstrated discordant antibiotic susceptibility profiles [6,16-18]. The observed distinct NG strains could be explained by high-risk sexual behaviour and patients being part of different transmission chains [16,18]. However, these studies were small (fewer than 10 patients), focussed on cultured isolates, used a single-position single nucleotide polymorphisms (SNP) and/or used non NG-specific primers [6,16-18]. Therefore, we aimed to develop a culture-free NG-MAST genotyping method that does not cross-react with other *Neisseria* species and is compatible with the NG-MAST database. Furthermore, we aimed to gain more insight in the frequency of distinct NG strains at separate anatomical sites in individual patients.

Methods

This study was designed to test the clinical application of the culture-free NG-MAST method to non-culturable clinical samples and use these data to compare ST of separate anatomical sites within a patient. The method was technically validated by assessing analytical sensitivity, specificity and concordance with NG-MAST.

Clinical samples

All NG-positive clinical samples (n = 1,110) from different anatomical sites were retrieved from 814 consultations (further referred to as number of patients) from 642 individual patients. NG positivity was based on NG detection by the Cobas 4800 CT/NG NAAT assay (Roche Diagnostics, Basel, Switzerland), between January 2012 and May 2016 from our

sexually transmitted infections (STI) clinic (South Limburg Public Health Service). These samples were from MSM (n = 769 samples), women (n = 254 samples) and heterosexual men (n = 87 samples). Samples with a Cq value of 35 or higher did not consistently yield PCR products in dilution series (see technical validation). Therefore, clinical samples with a Cq value of ≥ 35 were excluded (n = 418), leaving 692 samples for analysis. Of the remaining 692 NG-positive samples, we included only paired samples from separate anatomical sites belonging to a single STI clinic visit of a patient (n = 228). Different pairs of any combination of genital, anorectal or oropharyngeal NG positivity were observed. A total of 108 patients were NG-positive at two or three anatomical sites (90 MSM and 18 women who reported anal sex or symptoms and who were systematically tested on all three anatomical sites). The remaining amount of sample material was not sufficient for typing for 10 patients (nine MSM and one woman) and therefore these patients were excluded, leaving 98 patients with paired samples for analysis. In total, 205 Cobas NAAT clinical samples were included: 57 urine, 17 vaginal, 92 anorectal and 39 oropharyngeal samples. With these samples, we assessed the clinical application of the culture-free NG-MAST method and the presence of distinct STs within a patient. Data on culture success were retrieved by routine diagnostics because NG culture is mostly performed as part of the national NG resistance surveillance since NAAT diagnosis of NG is the primary diagnostic procedure. All patients were treated with a single dose of ceftriaxone, the primary choice of treatment because no resistance exists in the Netherlands [4]. An additional swab or urine sample for routine NG culture is taken at the treatment visit at the STI clinic only when treatment has not already been provided at the diagnostic visit based on symptoms. For this study, data of this routine culture was available for all patients if culture was performed.

DNA isolation clinical samples

Total DNA was isolated from 400 μ L Cobas 4800 clinical samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and eluted in 50 μ L Milli-Q water (MQ). To increase elution yield, we extended the incubation time to 10 min. The eluate was stored at -20°C .

DNA isolation cultured gonococcal and non-gonococcal *Neisseria* strains

Gonococcal and non-gonococcal clinical and reference *Neisseria* strains were inoculated on chocolate agar with IsoVitalEx or blood agar (Becton Dickinson, Sparks, United States (US)) and incubated over night at 37 °C in 5% CO₂. Morphology of the colonies was checked and a single colony was subcultured before DNA isolation. Bacterial suspensions were prepared in sterile saline solution from two or three colonies (depending on the size of the colonies) picked with a pre-wetted sterile swab. The bacteria were pelleted by centrifugation at 2,000 g for 5 min and washed once. The pellet was resuspended in 500 µL MQ and boiled for 10 min. Cell debris was pelleted by centrifugation at 8,000 g for 2 min and the supernatant was stored at –20 °C.

NG-MAST genotyping

PCR for both targets was performed in 50 µL reaction volumes using the Biometra T3000 Thermal Cycler (Labrepco Inc., US). Each reaction per target (*porB* and *tbpB*) contained 50 pmol of the NG-MAST forward and reverse primer for the respective target (Table 1), 2.5 U HotStar polymerase (Qiagen), 1× Qiagen PCR buffer, 0.2 mmol/L dNTP, 5 µL DNA lysate and MQ to a volume of 50 µL. The PCR protocol of Martin et al. was used to amplify the targets but cycles were increased to 30 [7].

Table 1. Overview of primers used in PCR and sequencing reactions for NG-MAST and culture-free NG-MAST

	NG-MAST [7]	Culture-free NG-MAST
PCR primers <i>porB</i>		
Forward	5'-CAA GAA GAC CTC GGC AA-3'	5'-GTT AAT CCG CTA TAA CCC CC-3'
Reverse	5'-CCG ACA ACC ACT TGG T-3'	5'-CCG ACA ACC ACT TGG T-3'
PCR primers <i>tbpB</i>		
Forward	5'-CGT TGT CGG CAG CGC GAA AAC-3'	5'-TTC CTT CCA AAA AAC CGG AAG CCC G-3'
Reverse	5'-TTC ATC GGT GCG CTC GCC TTG-3'	5'-CAT TGC CCG GAT AGG CAA ACC A-3'
Sequence primers <i>porB</i>		
Forward	5'-CAA GAA GAC CTC GGC AA-3'	5'-CAA GAA GAC CTC GGC AA-3'
Reverse	5'-CCG ACA ACC ACT TGG T-3'	5'-CCG ACA ACC ACT TGG T-3'
Sequence primers <i>tbpB</i>		
Forward	5'-CGT TGT CGG CAG CGC GAA AAC-3'	5'-CGT TGT CGG CAG CGC GAA AAC-3'
Reverse	5'-TTC ATC GGT GCG CTC GCC TTG-3'	5'-TTC ATC GGT GCG CTC GCC TTG-3'

The amplicons were precipitated with 50 µL 20% polyethylene glycol 8000 and 2.5 mol/L sodium chloride at 37 °C for 15 min. Precipitated amplicons were centrifuged at 15,000 × g for 15 min and washed twice with ice-cold 80% ethanol. The pellet was allowed to dry and resuspended in 25 µL MQ. The *porB* and *tbpB* fragments were sequenced with their respective forward and reverse primer using the BigDye Terminator v1.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, Massachusetts, US). The sequence protocol has an initial denaturation step of 1 min at 96 °C, followed by 25 cycles of 10 s at 96 °C, 10 s at 55 °C (*porB*) or 65 °C (*tbpB*), and 3 min at 60 °C.

Primer design culture-free NG-MAST

The genome sequences of all NG reference strains published by the WHO (n = 14) were downloaded from GenBank and used for multiple alignments with Clustal Omega [19]. A 2 kb flanking region of the aligned NG-MAST *porB* and *tbpB* primers were selected to identify conserved regions. Each flanking region was aligned and conserved regions were tested for in silico specificity using basic-local alignment search tool (BLAST). A specific sequence was identified that could be used as the forward primer for *porB* but no specific sequence was identified for the reverse primer, therefore the NG-MAST reverse primer was used which resulted in a fragment of ca 1.2 kb (Table 1). Two specific sequences were identified for *tbpB* which could be used as a forward and reverse primer, resulting in a fragment of ca 1.8 kb (Table 1).

Culture-free NG-MAST genotyping

This method was similar to the NG-MAST method apart from the initial PCR. Each reaction per target (*porB* and *tbpB*) contained 50 pmol of the culture-free NG-MAST forward and reverse primer for the respective target (Table 1), 0.2 µL AccuPrime Taq DNA Polymerase High Fidelity (Thermo Fisher), 1× AccuPrime PCR buffer II, 15 µL DNA isolated from a clinical sample and MQ to a volume of 50 µL. The PCR protocol had an initial denaturation step of 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 60 s at 58 °C (*porB*) or 69 °C (*tbpB*), 2.5 min at 68 °C, and a final extension of 10 min at 68 °C. The *porB* and *tbpB* amplicons were sequenced with NG-MAST primers (Table 1). The culture-free method was therefore compatible with the NG-MAST online database because we

characterised the same fragments of *porB* and *tbpB* genes.

Technical validation of culture-free NG-MAST method

Analytical sensitivity was determined using dilution series ranging from 1.3×10^6 to 1.3×10^2 colony-forming units (CFU)/mL. Concordance of culture-free NG-MAST method with NG-MAST was tested with seven randomly selected isolates cultured from four urine samples, two anorectal swabs and one oropharyngeal swab, and their respective unculturable Cobas 4800 screening samples between January and March 2017. The isolates were subjected to NG-MAST genotyping whereas the clinical samples were subjected to the culture-free NG-MAST method. The analytical specificity was tested with a panel of 32 non-gonococcal *Neisseria* species strains, including *N. cinerea* (n = 1), *N. denitrificans* (n = 1), *N. elongata* (n = 1), *N. flavescens* (n = 1), *N. lactamica* (n = 2), *N. meningitidis* (n = 3), *N. mucosa* (n = 7), *N. perflava* (n = 1), *N. polysaccharea* (n = 1) and *N. subflava* (n = 14).

Data analysis

The trace files were assembled, trimmed and edited using Bionumerics (version 7.6, Applied Maths, Sint-Martens-Latem, Belgium). The starting trimming patterns for *porB* and *tbpB* and lengths were used as described in Martin et al. [7]. Alleles and ST were called according to the NG-MAST online curated database. Phylogenetic trees of *porB*, *tbpB* and concatenated sequences were constructed using multiple alignment and unweighted pair group method with arithmetic mean (UPGMA) clustering using default settings with gap penalty at 100%.

Ethical statement

The study protocol was approved as a scientific study not done in humans by the Medical Ethical Committee of Maastricht University Medical Centre (MUMC+; number METC 2017–2-0251) as it concerned a laboratory and observational study using anonymous data and leftover diagnostic samples only. This was part of an STI clinic procedure where patients did not object to the use of their data and samples anonymously for research purposes.

Results

Analytical sensitivity, specificity and concordance of culture-free NG-MAST

Dilution series in triplicate showed that culture-free NG-MAST consistently yielded PCR products for both *porB* and *tbpB* in samples with a Cq value <35. None of the tested 32 non-gonococcal *Neisseria* strains were PCR-positive for either *porB* or *tbpB* in the culture-free NG-MAST PCR reactions. The seven randomly selected cultured isolates had identical ST as their respective unculturable Cobas 4800 screening sample but distinct ST were observed between the selected isolates.

Paired clinical samples

In total, 90.2% (185/205) of the selected paired clinical samples were successfully genotyped with the culture-free NG-MAST method. The *porB* fragment was successfully sequenced in 95.6% (196/205) of samples and *tbpB* in 93.7% (192/205). Failure of both targets in a sample does not appear to be related to the Cq value because both low (<30) and higher (30–35) Cq values show comparable failure rates (data not shown). We observed 36 different *porB* and 22 *tbpB* alleles, resulting in 45 ST. Among the samples, *porB*-1808 and *tbpB*-29 were the most common alleles, present in 51 and 49 samples, respectively. Furthermore, we found five previously unidentified *porB* and two *tbpB* alleles which all had the highest identity with NG using a BLAST search. The most prevalent ST were ST2992 (n = 36), ST11461 (n = 30), and ST5441 (n = 26), and 15 new STs were found.

Routine culture was performed for 95 of the 205 paired clinical samples and 59 (62.1%) were culture-positive. Typically, only one anatomical site was sampled for culture, and the majority of the culture-positive samples were collected from the genital site (44/59). Culture-free NG-MAST applied to the non-culturable clinical material (Cobas 4800 sample material) of samples sent in for culture (including culture-negative samples) showed that 93.7% (89/95) were genotyped successfully. However, four samples negative in culture-free NG-MAST were culture-positive. Of the remaining 110 uncultured clinical samples, 98 (89.1%) could be genotyped.

Sequence diversity within *porB* and *tbpB* alleles

High sequence diversity was observed for both *porB* and *tbpB* in this study population (Supplement Figures S1 and S2). Two *porB* alleles (90 and 2723) were divergent, with more than 50% dissimilarity, from all other observed alleles. The newly identified *tbpB* allele with 91% similarity with *tbpB*-1251 was divergent from all other observed alleles with more than 60% dissimilarity. In addition, the average dissimilarity between *tbpB* alleles appeared to be greater than between *porB* alleles.

Sequence types of samples from separate anatomical sites in a patient

In this dataset of clinical samples, we genotyped 169 paired samples (taken from a single patient at separate anatomical sites) from 80 patients (66 MSM and 14 women) (Supplement Table). We observed distinct concurrent ST in a quarter (20/80) of the patients. They had the following combinations of sample material: urine-anorectal (n = 6), urine-orpharyngeal (n = 1), anorectal-orpharyngeal (n = 8), urine-anorectal-orpharyngeal (n = 1), vaginal-anorectal (n = 3), and vaginal-anorectal-orpharyngeal (n = 1) (Table 2). Similar proportions of distinct concurrent ST were observed in MSM (16/66) and women (4/14). Interestingly, a single patient (patient 32) was NG-positive with a distinct NG strain at all three tested anatomical sites (Table 2).

Table 2. Characteristics of patients with concurrent *Neisseria gonorrhoeae* infection with distinct sequence types, including age, risk group, multiple sequence types and NG-MAST results per sample site, the Netherlands, January 2012–May 2016 (n = 42)

Patient	Age (years)	Risk group	Urine			Vaginal			Anorectal			Oropharyngeal		
			porB	tbpB	ST	porB	tbpB	ST	porB	tbpB	ST	porB	tbpB	ST
9	26	Women ^a	-	-	-	1808	91%- 1251	New ST1	1808	29	2992	-	-	-
10	22	MSM	-	-	-	-	-	-	3031	33	4995	1489	33	10257
24	27	MSM	-	-	-	-	-	-	7272	33	New ST4	99%- 7988	110	New ST3
27	24	Women ^a	-	-	-	301	29	359	2723	110	4431	301	29	359
28	20	MSM	-	-	-	-	-	-	908	27	3588	2723	27	15046
29	41	MSM	6720	188	11461	-	-	-	1808	137	11084	-	-	-
31	44	MSM	7988	110	13902	-	-	-	908	110	1407	-	-	-
32	42	MSM	182	74	1247	-	-	-	1808	29	2992	1808	836	New ST6
33	21	Women ^a	-	-	-	99%- 6405	74	New ST7	6720	188	11461	-	-	-
37	25	MSM	1808	188	New ST8	-	-	-	182	74	1247	-	-	-
39	20	MSM	-	-	-	-	-	-	3031	33	4995	4288	4	9382
48	21	MSM	-	-	-	-	-	-	6720	188	11461	4288	188	New ST9
53	41	MSM	30	18	5441	-	-	-	3059	29	5049	-	-	-
54	25	MSM	-	-	-	-	-	-	1808	29	2992	30	18	5441
55	36	Women ^a	-	-	-	1808	29	2992	6720	188	11461	-	-	-
58	49	MSM	30	18	5441	-	-	-	-	-	-	1808	29	2992
59	26	MSM	-	-	-	-	-	-	1808	29	2992	30	18	5441
60	42	MSM	30	18	5441	-	-	-	1808	29	2992	-	-	-
79	20	MSM	-	-	-	-	-	-	4199	29	New ST14	4199	4	New ST12
80	19	MSM	6720	188	11461	-	-	-	30	29	298	-	-	-

NG-MAST: *Neisseria gonorrhoeae* multi-antigen sequence typing; ST: sequence type.

^aWomen reporting anal sex or symptoms.

Sample material that was NG-negative or not sampled as part of routine diagnostics is indicated with a hyphen.

The Figure presents the dissimilarity of concatenated sequences of *porB* and *tbpB* between STs. For the majority of the patients with distinct concurrent STs, a large (>15%) dissimilarity was observed between the concatenated sequences. Patients 31 and 48 had only 1% dissimilarity between the concatenated sequences. In both patients, the *tbpB* allele was identical between the distinct ST but the *porB* allele showed >1% dissimilarity, meaning the two ST did not belong to the same genogroup. When assigning ST to genogroups, we identified two genogroups that consisted of more than five samples: G2992 (n = 32) and G11084 (n = 6). Furthermore, we identified three samples belonging to G1407 (one ST1407 and two ST2212) of which only one (ST2212) was culture-positive and susceptible for ceftriaxone.

Figure. Dendrogram constructed by multiple alignment of concatenated *porB* and *tbpB* sequences clustered with unweighted pair group method with arithmetic mean (UPGMA) algorithm, the Netherlands, January 2012–May 2016 (n = 169)

Sequence type (ST) of each sample is shown at the tips of the dendrogram and samples of patients with distinct ST are coloured. The branch length depicts the percentage of dissimilarity.

Discussion

In this study we show that the culture-free NG-MAST method can readily be used to genotype NG in clinical samples including extra-genital samples. In addition, the method is compatible with the online NG-MAST database. The culture-free NG-MAST method was technically validated by assessing the NG analytical specificity using non-gonococcal *Neisseria* species; it demonstrated good specificity for NG as no cross-reactivity was observed. Furthermore, concordance with NG-MAST was demonstrated by comparing typing results of non-culturable clinical samples (Cobas 4800 sample material) with cultured isolates, which were taken less than 2 weeks apart. In this time frame, identical genotypes were expected based on the study by Martin et al. [7]. With culture-free NG-MAST, we genotyped 90% of the selected paired clinical samples with sufficient bacterial load (Cq value <35). Extrapolating this genotyping rate to all NG positive samples (n = 1,110) would result in successful typing of 56% (624/1,110) of all NG-positive samples. Among all samples sent in for culture, culture-free NG-MAST showed a higher typing rate of 94% (89/95) compared with the culture-dependent method with 62% (59/95). However, four of the 59 culture-positive samples were negative in culture-free NG-MAST. The clinical samples testing negative in culture-free NG-MAST could be caused by PCR-inhibitory substances in the clinical material. The majority of the culture-positive samples were collected from the genital site (44/59); that could be explained by the sampling strategy, but the low sensitivity of extra-genital NG culture could also have contributed [2]. This highlights the importance of culture-free genotyping as the current surveillance data would be biased towards genital samples. With our method, we were able to genotype 33 of the 36 culture-negative samples, which were mainly extra-genital samples (25/33).

Our results show that both sexes were frequently infected with distinct NG strains in a quarter of patients (20/80) which is higher than most previous studies [6,11,17,18]. The studies of Whiley et al. (0/4) and Carannante et al. (1/8) assessed, respectively, only four and eight patients with paired samples, which could explain the lower proportion [6,11]. Pond et al. (3/71) developed a real-time PCR assay to predict ciprofloxacin resistance

with the detection of a resistance-associated SNP [17]. This method uses a single position to identify distinct strains of NG, leading to a lower resolution than NG-MAST where two internal fragments (490 bp and 390 bp) of highly polymorphic genes are analysed. De Silva et al. (26/206) performed whole genome sequencing only on cultured strains and therefore may have missed distinct strains from samples that were NAAT-positive but culture-negative [18]. A higher percentage of distinct strains in paired clinical samples was reported in a study by Kolader et al. (52/130) which applied *por-opa* restriction fragment length polymorphism typing [16]. The authors hypothesised that the observed high frequency could be the result of high-risk sexual behaviour or also of recombination in the *opa* genes.

High-risk behaviour and sex with multiple sex partners on the same occasion may explain the frequently observed distinct ST in our study as we included MSM and women reporting anal sex or symptoms attending our STI clinic, who are considered as risk groups [1,16]. Another possible reason could be DNA exchange with commensal *Neisseria* species or other NG strains [11,16]. Patients colonised with multiple NG strains could have different AMR profiles, potentially resulting in under-treatment which could allow dissemination of resistant strains [6,17]. However, the impact of multiple strain infections on treatment needs to be addressed in future research to answer questions of the effect on AMR development and dissemination of resistant strains.

The observed concurrent infections with distinct strains in our study would be overlooked in routine diagnostics as Dutch and European (European Centre for Disease Prevention and Control) NG resistance surveillance guidelines recommend culture of only one anatomical site [2,20,21]. Without typing data for concurrent NG infections, surveillance data are incomplete and potential transmission links or associations between ST and AMR can be missed. This potentially results in dissemination of unrecognised resistant types. Therefore, early detection and improved surveillance of ST that are linked to AMR could minimise sequelae and prevent dissemination of multidrug-resistant strains.

We observed high variability in both alleles and ST in our study population, which could be due to sampling over a prolonged time period and from different risk groups (MSM and women reporting anal sex or symptoms). For example, ST2212, ST2992, ST5441 and ST5793 are more prevalent in MSM than heterosexual men or women [10,22,23]. In our study, these STs were mainly found in MSM, but eight samples with ST2992 and one with ST5793 were from women. Interestingly, we found three samples which belong to the genogroup G1407 (ST1407 (n = 1) and ST2212 (n = 2)) linked to decreased susceptibility to the last first-line treatment with ceftriaxone [10,23]. Only one of the three could be cultured (ST2212) and was susceptible to ceftriaxone. Furthermore, ST359, ST2992, ST3588 and ST4995 are linked to azithromycin resistance which is the recommended dual-therapy treatment with ceftriaxone in case of a *Chlamydia trachomatis* co-infection [1,23-25]. In addition, this dual therapy is applied to slow down emerging resistance or where local resistance data are not available [1,5,20]. In the Netherlands, a single treatment with ceftriaxone is applied because no resistance has yet been found in the Netherlands [4]. However, a multidrug-resistant isolate was recently found in the United Kingdom that showed high-level resistance to both ceftriaxone and azithromycin, thereby highlighting the need for improved surveillance [26]. In our study population, we found a high prevalence of ST belonging to genogroup G2992 (19.5%), which is in line with earlier data from the Netherlands (16.1%), while genogroups G1407 (1.7%) and G359 (1.1%) were less frequent (respectively 7.7% and 6.3% in the study of Wind et al.) [23]. The genogroup G2992 is also frequently observed in most other countries in Europe [27] and G1407 prevalence is higher in most European countries than in the Netherlands.

Even though many NG-MAST ST are linked to resistance profiles in NG, this does not necessarily imply that the strain is phenotypically resistant [7,10]. Additional tests that can identify mutations leading to resistance, for example azithromycin resistance, could give more insight into those strains that cannot be cultured [28]. A limitation of this study is that we only included samples with a higher NG load (Cq value <35); therefore the typing rate of samples with a lower bacterial load is unknown. However, as culture success is also associated with bacterial load, culture-dependent methods are expected

to perform worse than our culture-free method in samples with a low NG load (Cq value ≥ 35). This hypothesis is strengthened because only 10% (18/188) of the samples with a Cq value ≥ 35 were culture-positive in routine diagnostics. A nested PCR approach might improve genotyping of samples with a low bacterial load as has been applied for medico legal purposes to allow typing from a piece of clothing [29].

Conclusion

The culture-free NG-MAST method can genotype NG from most non-culturable clinical samples, including extra-genital samples, as cross-reactivity with commensal *Neisseria* species was not observed. Compared with culture-dependent NG-MAST, culture-free NG-MAST has a higher typing rate and does not have high demands on sample conditions. Applying culture-free NG-MAST to clinical samples revealed frequent concurrent infections with distinct ST at separate anatomical sites in MSM and women reporting anal sex or symptoms. These distinct concurrent ST and extra-genital NG infections would be missed in the current European surveillance strategy possibly allowing dissemination of resistant NG strains. Including non-culturable and concurrent NG infections in surveillance informs actions to contain the dissemination of multidrug-resistant NG strains.

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Supplemental files

Supplemental figure 3 Dendrogram constructed by multiple alignment of *porB* sequences (n=185) clustered with Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. Sequence type of each sample is shown at the tips of the dendrogram and samples are coloured according to the respective *porB* allele. The branch length depicts the percentage of dissimilarity.

Supplemental figure 4 Dendrogram constructed by multiple alignment of *tbpB* sequences (n=185) clustered with Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. Sequence type of each sample is shown at the tips of the dendrogram and samples are coloured according to the respective *tbpB* allele. The branch length depicts the percentage of dissimilarity.

Supplemental table 1 Characteristics of patients with concurrent NG infection, including age, risk group, multiple sequence types, and NG-MAST results per sample site.

Oropharyngeal			Anorectal			Vaginal			Urine			Distinct	Risk	Age	Patient
ST	tbpB	porB	ST	tbpB	porB	ST	tbpB	porB	ST	tbpB	porB	STs	group		
-	-	-	2652	165	121	2652	165	121	-	-	-	No	Women ^a	24	1
2992	29	1808	2992	29	1808	-	-	-	-	-	-	No	MSM	24	2
-	-	-	2992	29	1808	-	-	-	2992	29	1808	No	MSM	35	3
2992	29	1808	2992	29	1808	-	-	-	-	-	-	No	MSM	52	4
2992	29	1808	2992	29	1808	-	-	-	-	-	-	No	MSM	36	5
-	-	-	2992	29	1808	2992	29	1808	-	-	-	No	Women ^a	34	6
-	-	-	2212	110	1388	-	-	-	2212	110	1388	No	MSM	30	7
10257	33	1489	10257	33	1489	-	-	-	-	-	-	No	MSM	21	8
-	-	-	2992	29	1808	New ST1	91%-1251	1808	-	-	-	Yes	Women ^a	26	9
10257	33	1489	4995	33	3031	-	-	-	-	-	-	Yes	MSM	22	10
51	27	39	51	27	39	-	-	-	-	-	-	No	MSM	20	11
5118	32	3040	5118	32	3040	-	-	-	5118	32	3040	No	MSM	25	12
10015	33	5898	10015	33	5898	-	-	-	10015	33	5898	No	MSM	36	13
11461	188	6720	11461	188	6720	-	-	-	-	-	-	No	MSM	23	14
4995	33	3031	4995	33	3031	-	-	-	-	-	-	No	MSM	32	15
-	-	-	10015	33	5898	-	-	-	10015	33	5898	No	MSM	53	16
2992	29	1808	-	-	-	-	-	-	2992	29	1808	No	MSM	24	17
8435	33	1808	8435	33	1808	-	-	-	-	-	-	No	MSM	19	18
-	-	-	12729	75	7421	12729	75	7421	-	-	-	No	Women ^a	23	19
-	-	-	4643	39	1873	-	-	-	4643	39	1873	No	MSM	40	20
10015	33	5898	10015	33	5898	-	-	-	10015	33	5898	No	MSM	30	21
-	-	-	5553	29	3361	5553	29	3361	-	-	-	No	Women ^a	36	22
-	-	-	New ST2	836	99%-6405	New ST2	836	99%-6405	-	-	-	No	Women ^a	28	23
New ST3	110	99%-7988	New ST4	33	7272	-	-	-	-	-	-	Yes	MSM	27	24
-	-	-	2992	29	1808	-	-	-	2992	29	1808	No	MSM	39	25
5441	18	30	5441	18	30	-	-	-	-	-	-	No	MSM	37	26
359	29	301	4431	110	2723	359	29	301	-	-	-	Yes	Women ^a	24	27
15046	27	2723	3588	27	908	-	-	-	-	-	-	Yes	MSM	20	28
-	-	-	11084	137	1808	-	-	-	11461	188	6720	Yes	MSM	41	29
-	-	-	New ST5	29	99%-5504	New ST5	29	99%-5504	-	-	-	No	Women ^a	34	30
-	-	-	1407	110	908	-	-	-	13902	110	7988	Yes	MSM	44	31

New ST6	836	1808	2992	29	1808	-	-	-	-	1247	74	182	Yes	MSM	42	32
-	-	-	11461	188	6720	New ST7	74	99%-6405	-	-	-	-	Yes	Women ^a	21	33
-	-	-	New ST2	836	99%-6405	-	-	-	-	New ST2	836	99%-6405	No	MSM	46	34
-	-	-	2992	29	1808	-	-	-	-	2992	29	1808	No	MSM	30	35
5441	18	30	5441	18	30	-	-	-	-	-	-	-	No	MSM	45	36
-	-	-	1247	74	182	-	-	-	-	New ST8	188	1808	Yes	MSM	25	37
11084	137	1808	11084	137	1808	-	-	-	-	-	-	-	No	MSM	20	38
9382	4	4288	4995	33	3031	-	-	-	-	-	-	-	Yes	MSM	20	39
-	-	-	9382	4	4288	-	-	-	-	9382	4	4288	No	MSM	52	40
-	-	-	11084	137	1808	-	-	-	-	11084	137	1808	No	MSM	33	41
-	-	-	11461	188	6720	-	-	-	-	11461	188	6720	No	MSM	22	42
-	-	-	2992	29	1808	-	-	-	-	2992	29	1808	No	MSM	31	43
-	-	-	2992	29	1808	2992	29	1808	-	-	-	-	No	Women ^a	59	44
New ST9	188	4288	11461	188	6720	-	-	-	-	-	-	-	Yes	MSM	21	48
5441	18	30	5441	18	30	-	-	-	-	-	-	-	No	MSM	46	49
-	-	-	2992	29	1808	2992	29	1808	-	-	-	-	No	Women ^a	46	50
-	-	-	11461	188	6720	-	-	-	-	11461	188	6720	No	MSM	26	51
11461	188	6720	11461	188	6720	-	-	-	-	11461	188	6720	No	MSM	37	52
-	-	-	5049	29	3059	-	-	-	-	5441	18	30	Yes	MSM	41	53
5441	18	30	2992	29	1808	-	-	-	-	-	-	-	Yes	MSM	25	54
-	-	-	11461	188	6720	2992	29	1808	-	-	-	-	Yes	Women ^a	36	55
11461	188	6720	11461	188	6720	-	-	-	-	11461	188	6720	No	MSM	21	56
11461	188	6720	-	-	-	-	-	-	-	11461	188	6720	No	MSM	21	57
2992	29	1808	-	-	-	-	-	-	-	5441	18	30	Yes	MSM	49	58
5441	18	30	2992	29	1808	-	-	-	-	-	-	-	Yes	MSM	26	59
-	-	-	2992	29	1808	-	-	-	-	5441	18	30	Yes	MSM	42	60
-	-	-	5441	18	30	-	-	-	-	5441	18	30	No	MSM	17	61
New ST10	98%-460	1164	New ST10	98%-460	1164	-	-	-	-	New ST10	98%-460	1164	No	MSM	24	62
-	-	-	5624	953	90	-	-	-	-	5624	953	90	No	MSM	36	63
5624	953	90	5624	953	90	-	-	-	-	-	-	-	No	MSM	26	64
-	-	-	5441	18	30	-	-	-	-	5441	18	30	No	MSM	26	65
-	-	-	5441	18	30	-	-	-	-	5441	18	30	No	MSM	38	66

-	-	-	-	11461	188	6720	-	-	-	11461	188	6720	No	MSM	38	67
5441	18	30	5441	18	30	30	-	-	-	-	-	-	No	MSM	18	68
13069	1634	3957	-	-	-	-	-	-	-	13069	1634	3957	No	MSM	28	69
-	-	-	5441	18	30	30	-	-	-	5441	18	30	No	MSM	27	70
-	-	-	New ST11	18	98%-1797	98%-1797	-	-	-	New ST11	18	98%-1797	No	MSM	32	71
New ST12	4	4199	New ST12	4	4199	4199	-	-	-	New ST12	4	4199	No	MSM	20	72
-	-	-	New ST13	188	266	266	New ST13	188	266	-	-	-	No	Women ^a	51	73
-	-	-	5441	18	30	30	-	-	-	5441	18	30	No	MSM	49	74
-	-	-	New ST13	188	266	266	-	-	-	New ST13	188	266	No	MSM	52	75
-	-	-	New ST13	188	266	266	New ST13	188	266	-	-	-	No	Women ^a	52	76
-	-	-	12141	1579	7105	7105	-	-	-	12141	1579	7105	No	MSM	45	77
New ST12	4	4199	-	-	-	-	-	-	-	New ST12	4	4199	No	MSM	20	78
New ST12	4	4199	New ST14	29	4199	4199	-	-	-	-	-	-	Yes	MSM	20	79
-	-	-	298	29	30	30	-	-	-	11461	188	6720	Yes	MSM	19	80

5

Metagenomic analysis shows distinct *Neisseria gonorrhoeae* strains at separate anatomical sites occur more commonly than mixed strain infections: implications for surveillance

EMBARGO

Brian M.J.W. van der Veer, Petra F.G. Wolffs, Christian J.P.A. Hoebe,

Nicole H.T.M. Duker-Muijrs, Lieke B. van Alphen

Submitted

6

Culture-free Genotyping Improves
Surveillance of *Neisseria gonorrhoeae*,
Especially in Oropharyngeal Samples, the
Netherlands, 2017 to 2018

EMBARGO

Michiel H.C. Slaats, Brian M.J.W. van der Veer, Lieke B. van Alphen,
Christian J.P.A. Hoebe, Nicole H.T.M. Dukers-Muijrsers, Petra F.G. Wolffs

Submitted

7

Potential *Neisseria gonorrhoeae* outbreak in
young heterosexuals (<25); culture-independent
genotyping revealed three clusters, the
Netherlands, October 2017 to March 2019

Brian M.J.W. van der Veer, Petra F.G. Wolffs, Christian J.P.A. Hoebe,
Juliën N.A.P. Wijers, Gènevieve A.F.S. van Liere, Marita I.L.S. Werner,
Amanja Verhaegh, Nicole H.T.M. Duker-Muijrs, Lieke B. van Alphen

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Abstract

In the last quarter of 2017, seven young (<25) women were diagnosed with genital *Neisseria gonorrhoeae* (NG) at the STI clinic in South-Limburg (the Netherlands), compared to only five young women previously in the year 2017. As triage and testing had not been changed this could indicate a potential outbreak. Further outbreak investigation demonstrated that 81 cases of young heterosexuals were observed between October 2017 to March 2019 (4.5 per month) compared to 30 young heterosexuals between January 2016 to September 2017 (1.4 per month) which was considered as baseline. Culture-independent genotyping of NG was performed to assess the genetic relatedness as only 21 outbreak cases were culture confirmed. This analysis revealed three independent outbreak clusters G2 (n=18), G13113 (n=11), and G NewST (n=24). None of the clusters were geographically linked. Number of sex partners reported by men and *Chlamydia trachomatis* co-infection were associated with cluster G2 and G NewST respectively. No indication of bridging from MSM networks was found as possible introduction of NG in the young heterosexual population. Targeted interventions were difficult as information on sex partners was limited. Therefore, prospective culture-independent typing and an additional questionnaire were implemented to inform further control measures.

Background

Neisseria gonorrhoeae (NG) is a common bacterial sexually transmitted infection (STI) that can infect genital, anorectal, oropharyngeal, and ocular mucosa [1-3]. Many NG infections, especially anorectal and oropharyngeal infections, are asymptomatic which could lead to unnoticed transmission [4, 5]. Therefore, it is a major public health challenge as the incidence rate remains high despite improved diagnostics [1, 6]. NG is commonly transmitted during unprotected sexual activity but can also be transmitted from mother to child during birth [2, 3, 7]. Another potential route of transmission is through contact with saliva [8, 9]. Key populations with a higher risk to acquire NG such as men who have sex with men (MSM) have been identified [4]. This is reflected in a higher positivity rate among MSM (11.0%) compared to women (1.6%) and heterosexual men (1.8%) in the Netherlands [10].

Molecular epidemiology can have an important contribution in the elucidation of sexual networks, in addition to collected epidemiological data [11]. NG multi-antigen sequence typing (NG-MAST) and whole-genome sequencing have been applied to elucidate transmission networks and outbreaks of NG [12-19]. These studies revealed sustained transmission of some lineages in both MSM and heterosexuals resulting in strain clusters [12, 13, 15-17, 19]. Also, bridging between MSM and heterosexual sexual networks has been demonstrated previously [16]. Furthermore, some studies reveal outbreaks of NG in distinct sexual networks or geographical areas [12, 13, 18].

Strain clusters of NG in heterosexuals have been described previously in the UK [12, 13]. Significant differences in behaviour and demographics of individuals between strain clusters were found [12]. Therefore, the authors hypothesize that strain clusters could represent localised transmission by distinct sexual networks. Some of the strain clusters identified by Risley et al. were associated with a geographical area [13]. The strain clusters that were not associated with a geographical area had a longer mean duration in the population and therefore might represent endemic strains.

Outbreak detection

We noticed a potential outbreak of NG in the young heterosexual population visiting our STI clinic after we recognized an increasing number of young women with genital NG compared with an increasing number of young heterosexual men, while triage and the number of tests did not change since 2015 [20]. In the last quarter of 2017 seven young (<25) women were diagnosed with genital NG visiting our STI clinic while in the previous three quarters of 2017, only five young women were diagnosed with genital NG. Here, we report the main findings of molecular outbreak investigation and demonstrate the added value of culture-independent genotyping and discuss challenges associated with NG outbreak detection and management.

Methods

Case definition and data collection

Confirmed cases related to this outbreak were defined as young (<25) heterosexuals (women and men) living in the region South-Limburg in the Netherlands with a genital NG infection from October 2017 till March 2019. Extra-genital samples were not included as heterosexuals are not routinely tested at those anatomical sites as recommended by the Dutch STI guidelines [20]. NG and *Chlamydia trachomatis* (CT) infections were diagnosed in routine diagnostics using the Cobas® 4800 CT/NG test using a self-collected vaginal swab (women) or first-void urine sample (men). As part of routine diagnostic procedures, a standardised questionnaire regarding the medical and sexual history of the last six months was collected by trained nurses. Data on sexual risk group, symptoms, number of sex partners, educational level, residence (four-digit postcode), and ethnicity were used in this study. The following were considered as urogenital symptoms: genital discharge, bleeding, itching, swelling, pain, burning sensation and more frequent urination. Educational level was defined as low/medium (no education, primary school or vocational) and high (higher education (secondary school) or university). Ethnicity was categorised as western and non-western background according to the Dutch consultation manual [20]. All missing data were handled as unknown and were not included in the analyses.

Outbreak investigation

There were 81 confirmed cases (57 women and 24 men) in the timeframe of October 2017 – March 2019 compared to 30 young heterosexuals (17 women and 13 men) with genital NG from January 2016 to September 2017 which was considered as baseline. This resulted in a threefold higher number of genital NG-positive young heterosexuals per month during the outbreak (4.5 per month) compared to baseline (1.4 per month). Three outbreak cases were excluded due to lack of residual material for genotyping, leaving 78 cases (56 women and 22 men), of which 21 (26.9%) were culture confirmed, available for analysis. Residual samples of routine diagnostics were genotyped using culture-free NG-MAST to assess the genetic relatedness of the NG strains [21]. For comparison, residual NG-positive genital material of young heterosexuals at baseline was genotyped to determine the occurrence of clustering in this population. In addition, residual NG-positive genital material of MSM, including bisexual men, in 2018 (n = 47) were genotyped to check for potential introduction of NG in the heterosexual population via bridging.

Culture-independent genotyping

Culture-free NG-MAST was performed as previously described, with minor modifications [21]. PCR protocols were modified as follows to improve sensitivity. The *porB* PCR protocol: 5 min at 95°C, 12 cycles of 30s at 95°C, 3 min at 68°C (decreasing by 0.5°C per cycle), 30 cycles of 30s at 95°C, 1 min at 58°C, 2.5 min at 68°C, and a final extension of 10 min at 68°C. The *tbpB* PCR protocol: 5 min at 95°C, 12 cycles of 30s at 95°C, 3 min at 72°C (decreasing by 0.5°C per cycle), 30 cycles of 30s at 95°C, 1 min at 65°C, 2.5 min at 68°C, and a final extension of 10 min at 68°C.

Data analysis

The Sanger sequence data analysis was performed as described previously [21]. Genogroups were assigned if ≥ 10 cases had a shared identical allele and the other $\geq 99\%$ similarity and were considered as a cluster [22]. Genogroups were used to ensure robust statistical analysis as proposed by Chisholm et al. and because some known sexual contacts do not have identical NG-MAST sequence types [22, 23].

Geospatial coordinates (residence) of outbreak cases were extracted using the R-package “nlgeocoder” version 0.1.3. We visualized residence areas of patients, based on their 4 digit postcode, per outbreak cluster in a geographical map using Qgis 2.18.28 [24]. In addition, the coordinates were used to test whether outbreak clusters, based on NG-MAST, were associated with a geographical area using SatScan (version 9.6) using the multinomial model [25].

Differences in epidemiological characteristics (sex, CT co-infection, educational level, notified for an STI, symptoms, and ethnicity) between strain clusters were pair-wise compared with the fisher-exact test. Student’s T-test was used to test associations with number of sex-partners between strain clusters and between women and men within a strain cluster. These analyses were performed using R statistical software version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria).

Ethical statement

The data in this study were obtained from medical records from routine care of our STI clinic in a de-identified manner. None of the researchers had access to patient identifying information. The STI clinic uses the ‘no objection’ informed consent and persons who refused use of data or samples are not included in this study. The study protocol was exempt from formal medical-ethical approval under prevailing laws in the Netherlands as it concerns only an retrospective observational study using anonymous data only (as stated by the National Central Committee for Human Studies: www.ccmo.nl and in the conduct of good behaviour in research www.federa.org).

Results

Descriptive epidemiology

A total of 81 cases were identified (57 women and 24 men) in the timeframe of October 2017 – March 2019 (figure 1A). As the increase in genital NG cases in young women was notified in the last quartile of 2017, all young heterosexuals with genital NG from January 2016 till September 2017 were considered baseline (n = 30, 17 women and 13 men)(figure 1A). Of the potential outbreak cases three were excluded due to lack of residual material for genotyping but none of the baseline.

A

B

Figure 1 A. Epidemiological curve of genital NG cases in young heterosexuals in South-Limburg the Netherlands. B. Epidemiological curve of genotyped samples of young heterosexuals in South-Limburg the Netherlands. Bars are coloured according to the availability of cultured isolate.

Three independent outbreak clusters

In total, 128/155 (82.6%) of genital NG-positive samples were successfully genotyped with culture-free NG-MAST, 20/30 (66.7%) baseline, 70/78 (89.7%) of confirmed outbreak cases, and 38/47 (80.9%) of MSM. Of the genotyped samples only 57/128 (44.5%) were culture positive. Of the 27 samples that failed, 5 showed potential mixed strain infections (4 culture-positive) and 8 had a low bacterial load ($<2.8 \log_{10}$ CFU/mL, no culture-positives). The remaining 14 samples (2 culture-positive) could have failed due to PCR inhibition. The majority of the genotyped samples at baseline and during outbreak were not culture confirmed and thus could only be typed by culture independent methods (figure 1B). Figure 2 presents the dissimilarity of concatenated *porB* and *tbpB* sequences revealing three independent outbreak clusters. Namely G2 (n = 18), G13113 (n = 11), and G NewST (n = 24). Of these outbreak clusters only 5/18, 2/11, and 6/24 of cases had a cultured isolate and therefore would not have met the outbreak cluster criteria if a culture-dependent method was used. All genogroups, G2 (n = 1), G13113 (n = 1) and G NewST (n = 3), were already present in the young heterosexual population before the increase of genital NG, in January 2016 (G13113) and between May and September 2017 (G2 and G NewST) respectively (figure 3). No genogroups were seen in young heterosexual cases pre-outbreak and MSM cases (figure 2).

Figure 2 Dendrogram of concatenated *porB* and *tbpB* sequences of outbreak (red) and reference (green) cases. Brackets indicate a genogroup and are named by the most abundant sequence type. The branch length depicts the percentage of dissimilarity.

Figure 3 Epidemiological curve of genotyped samples of young heterosexuals in South-Limburg the Netherlands. Bars are coloured according to genogroup.

Geographical mapping

The four-digit postcode of patients per outbreak cluster were used to explore possibilities for targeted interventions. Figure 4 shows that the cases per outbreak cluster are quite scattered in South-Limburg. Despite the small numbers, geographical clustering analysis was performed using SatScan but no significant geographical clustering was observed (Supplemental table).

Figure 4 Geographical maps of cases per outbreak cluster.

Association epidemiological characteristics with outbreak clusters

As no clear geographical clustering was observed, other explanatory variables could provide insight in the epidemiology of the outbreak. Therefore, we analysed whether a significant association with sex, sex partner, CT co-infection, educational level, notified for an STI, symptoms, and ethnicity could be assessed. Fisher-exact tests showed that CT

co-infection was significantly associated with cluster G NewST (92%, $p<0.03$) (table 1). Higher number of sex partners was reported by heterosexual men in cluster G2 compared to the other clusters ($p=0.03$, table 1). In addition, women in cluster G2 and G13113 report lower number of sex partners compared to heterosexual men in the respective cluster ($p=0.03$ and $p=0.02$).

Table 1 Characteristics of the outbreak clusters. Data are reported as percentages (number of cases) except sex partners as mean (standard deviation).

		G2 (n = 18)	G13113 (n = 11)	G NewST (n = 24)
Sex	Women	66.7 (12)	81.8 (9)	83.3 (20)
	Men	33.3 (6)	18.2 (2)	16.7 (4)
Sex partners reported by women		2.3 (± 1.2)	2.1 (± 1.3)	2.6 (± 2.1)
Sex partners reported by men		11.3 (± 7.3) ^a	5.5 (± 0.7) ^b	2.8 (± 2.4)
CT co-infection	CT negative	38.9 (7)	54.5 (6)	8.3 (2)
	CT positive	61.1 (11)	45.5 (5)	91.7 (22) *
Educational level	Low/medium	66.7 (12)	90.9 (10)	66.7 (16)
	High	33.3 (6)	9.1 (1)	20.8 (5)
	Unknown	0 (0)	0 (0)	12.5 (3)
Notified for an STI	Not notified	77.8 (14)	45.5 (5)	62.5 (15)
	Notified	22.2 (4)	45.5 (5)	37.5 (9)
	Unknown	0.0 (0)	9.1 (1)	0.0 (0)
Symptoms	Asymptomatic	16.7 (3)	27.3 (3)	45.8 (11)
	Symptomatic	83.3 (15)	72.7 (8)	54.2 (13)
Ethnicity	Western	88.9 (16)	81.8 (9)	95.8 (23)
	Not-western	11.1 (2)	18.2 (2)	4.2 (1)

* = $p<0.05$

^a = Men report more sex partners than women ($p=0.03$)

^b = Men report more sex partners than women ($p=0.02$)

No introduction of NG by bridging

The relatively high number of NG cases in the young heterosexual population could be the result of acquisition of NG by bridging with a high-risk population. As MSM, including bisexual men, are a risk group for acquiring a NG infection, bisexual men could introduce NG strains in the young heterosexual population. None of the cases of MSM,

including bisexual men, clustered together with any of the three independent outbreaks (figure 2).

Outbreak control measures

Implementation of control measures to stop further transmission of NG within this population proved difficult, due to limited information on sexual partners and no obvious association with a geographical area. A prospective approach was taken in which an additional questionnaire is implemented, which will be collected upon return to the clinic for treatment or via telephone when empirically treated. This questionnaire covers detailed information of all sex partners, sexual practices per sex partner, and where and when did they meet their sex partner(s). Furthermore, prospective culture-independent genotyping of NG-positive heterosexuals was implemented as it proved essential for outbreak clusters identification. This prospectively collected data of the additional questionnaire and genotyping can be used to perform network analyses for targeted interventions to control future NG outbreaks.

Discussion

Between October 2017 – March 2019 a three-fold higher number of genital NG-positive young heterosexuals per month was seen (4.5 per month) compared to January 2016 – September 2017 (1.4 per month, considered as baseline), indicating an potential outbreak of NG among young heterosexuals while NG is mostly diagnosed in MSM. Therefore, culture-independent genotyping was performed and revealed three independent outbreak clusters of only heterosexual individuals. Using culture-independent genotyping was essential to identify the three outbreak clusters as only 5/18 (G2), 2/11 (G13113), and 6/24 (G NewST) of cases had a cultured isolate available for typical culture-dependent genotyping. Analysis of these clusters showed a link with number of sex partners reported by men for cluster G2 and cluster G NewST with CT co-infection. The three independent strain clusters were not introduced by bridging with MSM sexual networks as these strains were not identified in samples of MSM.

The three independent outbreak clusters (genogroups) were only found in heterosexual individuals. As these genogroups were not identified in samples of MSM, including bisexual men, our results make introduction of NG in the young heterosexual population by bridging from other sexual networks unlikely. However, bridging and mixed sexual risk groups have been described previously in strain clusters [12, 16]. Some strains in each genogroup of this study were also found in a study by Choudhury et al. and were also associated with heterosexuals likely confirming the observed clusters in this study [12]. Choudhury et al. described significant differences in behaviour and demographics of individuals between strain clusters that could indicate localised transmission clusters. In this study number of sex partners reported by men and CT co-infection were significantly different between the clusters. Geographical mapping in this study did not reveal clear localised clusters possibly due to low absolute numbers. In contrast, Risley et al. did observe strain clusters to be associated with a geographical area and considered these as localised outbreaks and persisted shorter compared to strains that were not associated with a geographical area [13]. In this study only one strain (G13113) was observed in 2016 (considered pre-outbreak), while the other genogroups (G2 and G NewST) were not observed in 2016 (figure 3). Therefore, it is possible that the three clusters represent localised outbreaks in separate sexual networks but with similar epidemiological characteristics. For instance, the network could have an origin in dating app usage or attending the same school rather than location of residence.

Culture-independent molecular typing was essential for epidemiological analyses as in total 63/155 samples had a cultured isolate available. The outbreak clusters might not have been identified without this method, as only 5/18 (G2), 2/11 (G13113), and 6/24 (G NewST) of cases had a cultured isolate available for typical culture-dependent genotyping. Aside from the low sensitivity of NG culture, nucleic acid amplification tests (NAAT), which use non-culturable samples, are generally used for NG detection in routine diagnostics and are not always confirmed by culture [3, 6]. This could lead to underestimation of linked individuals and delayed identification of outbreaks. Culture-independent molecular typing on residual NAAT samples could provide links between individuals rapidly to inform STI clinics.

Even though three independent outbreak clusters were identified, targeted control measures were difficult to setup because partner information from regular partner notification to identify transmission networks of the clusters was limited. Prospective genotyping in combination with intensive contact tracing could provide the necessary data to prevent transmission in sexual networks and control NG outbreaks. However, the highly feasible and cost-effective prospective genotyping could be implemented in many places and is available for future prospective studies.

Our study does have limitations. First, the outbreak cases were analysed and genotyped retrospectively, and therefore we were hampered in gathering additional information. Second, only genital NG cases of clients attending the STI clinic in South-Limburg were genotyped but not clients of general practitioners thereby potentially underestimating the magnitude of the outbreak clusters. However, groups at risk to acquire a NG infection are mainly tested at STI-clinics [10].

In conclusion, within the strong increase of NG cases in a short period of time, three independent outbreak clusters of closely related NG strains were identified using culture-free NG-MAST. This highlights the potential added value of culture-independent genotyping for outbreak investigations. As detailed partner information was limited and we did not observe clear associations within a geographical area, additional control measures were difficult to setup. Prospective culture-independent genotyping in combination with detailed epidemiological data could provide the data needed for additional control measures to halt NG transmission and control NG outbreaks.

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Supplemental file

Supplemental table Geographical cluster detection analysis results using the multinomial model in SatScan.

	Radius (km)	<i>n</i>	RR each genogroup (G2, G13113, G NewST)	<i>p</i> -value
Cluster 1 (Sittard-Geleen)	7.88	10	(0.29, 7.35, 0.40)	0.07
Cluster 2 (Heerlen)	4.89	5	(0.00, 0.00, 2.61)	0.69
Cluster 3 (Maastricht)	2.73	6	(0.45, 0.00, 2.13)	0.99
Cluster 4 (Kerkrade)	3.91	7	(2.47, 0.64, 0.29)	1.00

n is number of cases. RR is relative risk of the genogroup being inside the geographical area versus outside.

8

Despite no ceftriaxone resistance in the Netherlands many *Neisseria gonorrhoeae* isolates have a mosaic *penA* gene

EMBARGO

Brian M.J.W. van der Veer, Christian J.P.A. Hoebe, Nicole H.T.M. Duker-Muijters,

Lieke B. van Alphen, Petra F.G. Wolffs

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9

Summary and general discussion

Neisseria gonorrhoeae (NG) is one of the most commonly diagnosed bacterial sexually transmitted infections (STI) worldwide [1]. The introduction of nucleic acid amplification tests (NAATs) revolutionized the ability to diagnose NG infections as non-invasive samples can be used [2]. Modern NAATs are highly sensitive and specific for NG detection and are automated. Hence, NAATs have replaced NG culture for routine screening diagnostics. However, current NAATs cannot determine the antimicrobial resistance (AMR) profile of a NG isolate [2]. As NG has developed resistance towards all classes of antibiotics, surveillance is critical to avoid untreatable NG [3, 4]. Current surveillance requires a cultured NG isolate to monitor transmission and AMR [2, 5, 6]. However, NG culture is complicated as it requires logistics and technical expertise because NG requires demanding nutritional and environmental conditions for growth [2]. Despite the improved diagnostics and screening strategies, the incidence of NG remains high [1-3, 7]. Therefore, surveillance needs to be improved, and transmission dynamics and AMR acquisition of NG better understood. This in particular for extra-genital sites of infection, especially the oropharynx, as they are considered a reservoir of AMR genes for NG [8, 9]. This is because commensal *Neisseria* species are commonly present and easily exchange DNA with NG.

Several reports of high-level resistance in NG isolates prompted the World Health Organization (WHO) to develop a global action plan with recommendations to stop AMR and transmission of NG [3]. Strategies suggested included: effective diagnosis and NG control, systemic monitoring of treatment failures, strengthening of AMR surveillance, research into new molecular methods for surveillance, and new treatment options. With the studies in this thesis we aimed to gain further knowledge on effective diagnosis, transmission, improving surveillance, and further understanding of AMR in NG, including the potential pivotal role of the oropharynx and commensal *Neisseria* species as reservoir for resistance. In this final chapter, the main conclusions of the studies in this thesis are discussed in the light of existing literature. Furthermore, recommendations for future research are provided.

The clinical relevance of extra-genital NG infections

The need for extra-genital testing is recognized for men who have sex with men (MSM) but not for women and heterosexual men [2, 5, 10]. Aside from MSM, extra-genital testing is only recommended for patients with an indication, such as a history of direct exposure by anal or oral sex or symptoms. This is linked to the difference in prevalence of extra-genital NG in MSM (3% to 21%) and heterosexual men and women (0% to 3%) [11, 12]. Recent epidemiological studies demonstrate that oropharyngeal NG is common in MSM and women [13, 14]. Prevalence of oropharyngeal NG was 5.5% in MSM and 1.4% in women when routine universal testing was performed and shows that half of the oropharyngeal NG infections were oropharyngeal-only infections. An additional issue with extra-genital NG infections is the ongoing discussion of correct diagnoses of these infections [2, 15-17]. In first-generation NAATs false-positive results were the underlying issue. This has been solved in currently used modern NAATs, however these are not approved for extra-genital testing. Nevertheless, current laboratory standards recommend NAATs for the detection of extra-genital NG if a laboratory has established specifications for performance characteristics in accordance to present regulations [2]. Previous studies showed that the cobas 4800 CT/NG test could be used for anorectal swab testing and is highly specific [15, 18]. Based on these results we argued that the cobas 4800 CT/NG test would perform well in oropharyngeal samples. In **chapter 2**, we assessed the validity of the cobas 4800 CT/NG test for detection of *C. trachomatis* (CT) and NG in oropharyngeal swabs. The cobas 4800 demonstrated very good performance for NG detection. The limit of detection, defined as 95% detection, was 6.65×10^2 CFU/mL, but more importantly no cross-reactivity and high specificity (97.1%) were observed. These results are in line with previous studies and demonstrate that NG is correctly diagnosed in extra-genital samples, including the oropharynx [15, 18]. However, our study also raises a potential important issue as in theory up to 25% of oropharyngeal NG infections could be missed due to an average low NG bacterial load at this anatomical site. As there are no indications that the cobas 4800 CT/NG test is underperforming compared to other NAATs, this could be a common problem, likely across multiple

platforms [19-21]. Therefore, more sensitive NAATs should be developed to avoid missed oropharyngeal NG infections that might otherwise be transmitted unnoticed.

The transmissibility of vaginal and extra-genital (anorectal and oropharyngeal) NG is not completely understood [9]. The load of a pathogen has been linked to transmission and clinical outcome in other STIs [22, 23]. Therefore, the NG bacterial load could be linked to transmissibility and clinical outcome like symptoms. Previous studies have shown higher NG load in urethral infections of symptomatic men and anorectal infections of symptomatic MSM [24, 25]. Lower NG load was observed in oropharyngeal swabs of MSM compared to urethral and anorectal swabs. However, these studies were relatively small and no data exist of NG load in women in relation to symptoms. The NG load in a diverse population was determined in **chapter 3**, including genital and extra-genital samples. Loads were compared between sample material and sex, and associations of NG load with symptoms per sexual risk group to acquire an STI and sample material were tested. Our data demonstrate that men and women have a similar NG load in genital and extra-genital sites. This would imply that a NG infection is equally relevant in women and MSM. Our results also suggest that all anatomical sites may have a role in transmission because of the substantial NG load. Oropharyngeal NG infections might have a smaller role as the mean load is lower (16 to 25-fold). Still, oropharyngeal NG infections are important as they are frequently single infections, mostly asymptomatic, and could be involved in AMR development of NG [4]. Hence, systematical extra-genital testing in routine diagnostics might reduce the incidence of NG. However, because of costs, systematical extra-genital testing will not be implemented in heterosexual men and women, as the prevalence is low [11, 12, 26, 27]. In low-income countries this would be even less applicable. In many of those countries a syndromic approach based on symptoms linked with a NG infection is used [2, 28, 29]. As we demonstrate in **chapter 3** that asymptomatic infections have sufficient load for transmission, these asymptomatic infections can cause more sequelae. For example, NG infections are linked with increased risk to acquire HIV [9].

The need for improved NG surveillance

Despite improved diagnostics and prevention efforts the incidence rate of NG infections remains high [1-3, 9]. Some key populations at risk to acquire a NG infection have been identified such as MSM, young people, and sex workers [3, 5, 10, 27]. Especially in these key populations, surveillance is essential to stop transmission and detect potential outbreaks [3]. Current surveillance relies on cultured isolates for genotyping to assess the genetic relatedness [5, 6]. However, NG culture often fails as NG requires demanding nutritional and environmental conditions, leading to a low percentage of culture-confirmed cases [30]. Hence, the WHO advocates in their global action plan to increase culture capacity and new molecular methods for surveillance [3]. Therefore, we believe that there is a need for culture-independent genotyping methods to overcome the issue with NG culture, thereby improving NG surveillance. We have developed a culture-independent method based on the widely used NG multi-antigen sequence typing (NG-MAST) in **chapter 4**, called “culture-free NG-MAST” [31-33]. Crucially, this method did not cross-react with any of the tested non-gonococcal *Neisseria* species. Therefore, the culture-free NG-MAST method can readily be used to genotype NG in clinical samples including extra-genital samples. In contrast, the culture-dependent NG-MAST cannot be used in uncultured extra-genital samples as it cross-reacts with commensal *Neisseria* species leading to wrongly assigned sequence types (ST) [34]. Furthermore, culture-free NG-MAST demonstrated a higher typing rate (94%) compared to the culture-dependent method (62%) in samples sent in for culture. Thus, the ability of including non-culturable and extra-genital samples, combined with the increased typing rate, can improved NG surveillance. However, one should note that (culture-free) NG-MAST is mainly applicable to identify transmission clusters [32]. Even though many NG-MAST STs are linked to resistance profiles in NG, this does not necessarily imply that the strain is phenotypically resistant [32, 35]. To date culture remains the ‘golden-standard’ for AMR surveillance [2].

In **chapters 6 and 7**, the potential added value of culture-free NG-MAST in surveillance and outbreak management was studied. In **chapter 6**, all samples (n=196) subjected to routine culture between January 2017 till September 2018 from our STI clinic (South Limburg Public Health Service) were genotyped with culture-free NG-MAST. In total, 152/196 (77%) were successfully genotyped and of the 44 samples that failed, 11 showed a possible mixed infection. Of the 196 samples, 115 were culture-positive which reflects an overall typing rate of 59%. This is higher than the national average, likely as we only advise culture in case of high bacterial load [30]. Nevertheless, culture-free NG-MAST demonstrates an overall higher typing rate (77% versus 59%) and this rate is similar in culture-positive (80%) versus negative samples (74%). When stratified by sample material, the typing rate of urine samples did not differ between methods. However, culture-free NG-MAST vastly improved the typability of the other sample materials. In particular, the oropharyngeal site as only 6.3% (1/16) could be typed with culture-based methods, while with culture-free NG-MAST 81% (13/16) was genotyped. This improved typability demonstrated to be essential in a NG outbreak investigation (**chapter 7**). We noticed a potential outbreak of NG in the young (<25) heterosexual population visiting our STI clinic between October 2017 till March 2019. A three-fold higher number of genital NG-positive young heterosexuals per month was seen (4.5 per month) compared to January 2016 – September 2017 (1.4 per month, considered as baseline). Using culture-free NG-MAST, three independent outbreak clusters were identified and this would not have been observed using culture-based methods. Still, implementation of control measures to stop further transmission of NG within the clusters proved difficult. This was due to limited information on sexual partners and no obvious association with a geographical area (residence). It could be possible that residence is not a good predictor because the individuals within a cluster could attend the same school or use the same dating app. Previous studies also show that molecular epidemiology, using NG-MAST, can help in identifying large clusters and newly emerging strains which we have also seen in both **chapters 6 and 7** [36, 37]. Hence, we believe that culture-free NG-MAST has an added value in NG surveillance, which will be discussed in future directions.

Impact of multiple NG isolates in a patient

As typically one isolate of a single anatomical site is characterized in surveillance, NG infections with multiple isolates like mixed infections are missed [2, 5, 6]. There are two types of multiple isolate infections in a patient; 1) distinct strains at separate anatomical sites, and 2) multiple isolates in one anatomical site (mixed infection). Both of these types were observed in previous studies but the frequency varies greatly between studies [31, 34, 38-44]. As many of these studies rely on culture, used less discriminatory methods than NG-MAST, or could not detect low abundant isolates (<10%), we developed a targeted metagenomic method that can detect low abundant isolates and is highly discriminatory. We employed this to gain further knowledge on the frequency of multiple NG isolates in a patient. This metagenomic method is based on our highly discriminatory culture-free NG-MAST method [32]. In **chapter 4** we included 81 MSM and 17 women with paired NG-positive samples from separate anatomical sites. In total, paired samples of 80/98 patients were genotyped in which distinct concurrent NG isolates at separate anatomical sites was observed in a quarter (20/80) of patients. This proportion is higher than most previous studies, likely because of study size, higher discriminatory method used, and inclusion of culture-negative samples [31, 34, 38, 39]. As the proportion of distinct NG isolates at separate anatomical sites was high, this needed further investigation to understand the high occurrence. The distinct NG strains at separate anatomical sites could be the result of mixed infections but only the dominant strain of an anatomical site was characterized. Our hypothesis is that also additional NG strains, previously undetected, could be present because of mixed infections. Therefore, we performed a follow-up study in **chapter 5** of the samples of MSM with distinct NG strains at separate anatomical sites (n=33) of **chapter 4**. In **chapter 5** a metagenomic genotyping method was developed by combining culture-free NG-MAST with next-generation sequencing and an analysis pipeline. Despite the selected MSM with distinct NG strains at separate anatomical sites, most samples (79%, 25/33) show a single NG infection. Our study provides further evidence that mixed NG infections do occur but likely at low prevalence [40-44]. Possible explanations of the

apparent low occurrence of mixed NG infections and implications of multiple NG isolates for surveillance are discussed below.

Based on our research in **chapters 4 and 5** it appears that mixed NG infections are less frequent than distinct NG isolates at separate anatomical sites. There are several explanations like competition between NG strains, separate introduction of NG, and tissue tropism. In an murine infection model, fitness and competitive co-culture were studied of ceftriaxone resistant NG strains by Vincent et al. (2018) [45]. The authors report significant differences in fitness and in some competitive co-cultures with wild-type NG, no ceftriaxone resistant NG bacteria were recovered. This overgrowth of a more fit, in this case the wild-type NG, increased over time. As asymptomatic patients are tested at earliest 14 days after potential exposure, as described in the Dutch STI guidelines, this could explain a part of the low frequency of mixed NG infections [27]. The MSM in **chapter 5** reported a median of 10 (6-18, inter quartile range) sex partners in the previous six months. Hence, they could be exposed to NG at different points in time. Combined with competition between NG isolates, an established infection of NG might prevent colonization of a newly introduced NG isolate. However, reporting multiple sex partners does not reflect multiple potential exposures of NG at an anatomical site. Studies that assessed sexual behavior show that sexual practices per sex partner can vary greatly, including condom use and type of sexual practice per sex partner [46, 47]. Lastly, tissue tropism might contribute to the low occurrence of mixed infections as some isolates might infect certain anatomical sites poorly. However, many cell adherence proteins can undergo phase variation to mediate and adept adherence to various human cells [48-50]. Also, NG can adept to different environmental conditions by an array of responsive transcriptional factors [51]. Therefore, NG isolates should be able to adjust to an anatomical site and likely does not explain the low frequency of mixed infections. Still, these mixed infections do occur and could be relevant in treatment as a potential resistant NG isolate in a mixed infection might lead to treatment failure. Also, mixed infections could be relevant for AMR development as AMR genes can be exchanged between NG isolates [9]. As discussed before, typically one isolate of a single anatomical site is characterized in surveillance [2, 5, 6]. Therefore, infections with

multiple NG isolates would be missed in the current surveillance strategies possibly allowing dissemination of resistant NG strains. Hence, distinct NG isolates at separate anatomical sites should be included in surveillance as these infections occur frequently.

The oropharyngeal site as a reservoir for antimicrobial resistance

Mosaic genes can occur in the genome of NG and lead to resistance towards various antimicrobials [4, 8]. As ceftriaxone is the current first-line empirical treatment of NG and not many treatment options are left, AMR research is essential to avoid untreatable NG [3, 28]. Mosaic *penA* genes can result in ceftriaxone resistance [4, 9]. These mosaic genes are considered to have emerged by DNA recombination with DNA of commensal *Neisseria* species that commonly reside the oropharynx. Hence, the oropharynx is considered to be a reservoir for AMR genes of NG [52, 53]. In a study by Igawa et al. (2018), *in vitro* recombination of NG with *N. cinerea* DNA has been demonstrated [54]. However, this does not necessarily reflect the process *in vivo* as for example the regulation of DNA donation is unclear [55]. Mostly *N. cinerea* and *N. subflava* group are involved in mosaic *penA* genes in NG. Therefore, the presence of *N. cinerea* and *N. subflava* in the oropharynx during a NG infection would be a prerequisite for recombination. To study the presence of *Neisseria* species and potential recombination in NG during an oropharyngeal infection, we developed a metagenomics method that uses nanopore sequencing of amplicons to characterize the *penA* genes and their flanking regions in **chapter 8**. The flanking regions of the *penA* gene were used to identify the species. Hence, we could compare *penA* genes and identify potential recombination events. In this study 64 NG-positive oropharyngeal samples of MSM were characterized of which 61 were *penA* PCR positive. In almost half of these samples (28/61) a NG contig was observed and 8/28 were mosaic. All except one had amino acid substitutions I312M and V316T linked to ceftriaxone resistance [4]. We demonstrate that recombination during a NG infection could be possible as in all samples (61/61) *N. subflava* and in more than half of the oropharyngeal samples (35/61) *N. cinerea* were present. In addition, in all samples with a mosaic *penA* gene in NG, the mosaic part of NG was similar to either *N. cinerea* or *N. subflava* was seen. Though resistance is low in the Netherlands, in 29% (8/28) of NG contigs a mosaic *penA* was observed and this is in line

with the recent study of de Laat et al. (2019) [30, 56]. Recent epidemiological studies in other countries report mosaic *penA* gene in 2.3% to 27% of tested isolates [57-61]. Worrisome is that the proportion of strains with mosaic *penA* appears to be increasing [59, 61]. Also, A501 mutations in non-mosaic *penA*, linked to ceftriaxone resistance, is commonly reported [56, 57, 59]. In our study from January 2017 to June 2018, only one NG isolate has the mutation A501V and is non-mosaic. To summarize this part, it is clear that commensal *Neisseria* species commonly reside the oropharynx also during a NG infection. As *Neisseria* species readily exchange DNA and the mosaic part of NG was similar to commensal *Neisseria* species, recombination *in vivo* could have a pivotal role in AMR development in NG [62, 63]. Still, there are many unknowns like efficiency in DNA donation and uptake, fitness cost of recombination, AMR in commensal *Neisseria* species, and potential killing of NG by commensal *Neisseria* species DNA [64, 65].

Concluding remarks and future perspectives

Despite many efforts to detect and treat NG infections the incidence remains high. Guidelines recognize the need for extra-genital testing in MSM and specific key populations [2, 5, 10]. In this thesis we observed many single oropharyngeal NG infections which are mostly asymptomatic. Though the cobas 4800 CT/NG test demonstrates very good performance to detect NG in oropharyngeal samples, in theory, up to 25% could be missed. Because there is no indication that the cobas 4800 underperforms compared to other NAATs, more sensitive tests are needed. Future studies should address the impact of this potential issue. For example, repeated testing of NG-negative oropharyngeal could be performed to estimate the initially missed infections.

One of the goals of this thesis was to improve surveillance of NG. We demonstrated that culture-independent typing was essential to identify NG outbreak clusters. As mentioned several times, diagnostics and surveillance has vastly improved in the past decades but NG remains a global issue. We believe that a prospective typing in combination with intensive contact tracing could aid to limit NG transmission and provide early detection of outbreaks. With this data, sexual networks should readily be

identified for a targeted intervention like intensified testing and counseling. With unlimited resources, ideally, all NG positive samples should be typed to have the highest resolution to identify clusters. Together with metadata from the public health services an interactive NG infection monitoring tool for clinicians, epidemiologists, and microbiologist could be created. In this tool a warning system could be implemented to highlight regions or populations that might need additional attention. For instance, a potential NG outbreak could be early identified to take action to stop transmission. Unfortunately, resources are limited and the estimated costs for typing are €40 using culture-free NG-MAST, including a technician [27]. A pilot study using the proposed approach should be conducted that (hopefully) shows clusters to allow interventions that result in a decrease in incidence of NG.

Gonorrhea is a major public health challenge today, not only because of the high incidence but also because of increasing resistance. Based on previous results and the results in **chapter 8** commensal *Neisseria* species appear to have a pivotal role in AMR development of NG. However, data on commensal *Neisseria* species is limited. Hence, future research should focus more on the commensal *Neisseria* rather than only target NG. For instance, little is known about the minimal inhibitory concentration (MIC) values in these species. This is particularly relevant for AMR in NG because commensal *Neisseria* could become resistant after multiple exposures to antibiotics and could exchange the AMR genes with NG. Therefore, it is important to better understand the dynamics of the commensal *Neisseria*. For example, a longitudinal study in which individuals are sampled regularly could provide further knowledge. Thereby addressing, but not limited to, the composition of the oropharyngeal *Neisseria* species, mosaic genes, MIC values, and whole-genome sequences of isolates.

Though there is a lack of novel antibiotics for treatment of NG, we could improve care and limit ceftriaxone usage to potentially limit AMR development. By using point-of-care tests or new NAATs that can also identify NG isolates that are still susceptible to previously used antibiotics like spectinomycin or ciprofloxacin, and treat the patient with one of these antibiotics.

In summary, with the introduction of NAATs the diagnoses of an infection with NG became more sensitive and with antibiotics the treatment became easier. However, NG proved to be a highly adaptive pathogen that is increasingly resistant to all classes of antibiotics. Therefore, surveillance programs were established to monitor and prevent resistant NG. Still, much remains to be understood like the role of commensal *Neisseria* species and the persistent high incidence of NG globally. The research presented in this thesis elucidates a part of the puzzle to understand transmission dynamic but also provide tools to improve NG surveillance.

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Addendum

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Samenvatting en algemene discussie

Neisseria gonorrhoeae (NG) is een van de meest voorkomende bacteriële seksueel overdraagbare aandoeningen (SOA) wereldwijd. De introductie van nucleïnezuur amplificatietests (NAATs) zorgde voor een revolutie waarop NG infecties gediagnosticeerd kunnen worden, vanwege de mogelijkheid om niet invasieve samples te gebruiken. Moderne NAAT's zijn zeer gevoelig en specifiek voor NG detectie en zijn geautomatiseerd. Hierdoor hebben NAAT's de NG kweek vervangen in routine screeningsdiagnostiek. De huidige NAAT's kunnen het antimicrobiële resistentie (AMR) profiel van een NG-isolaat echter niet bepalen. Aangezien NG resistentie heeft ontwikkeld tegen alle antibioticaklassen, is surveillance cruciaal om onbehandelbare NG te voorkomen. Huidige surveillance vereist een gekweekte NG isolaat om transmissie en AMR te kunnen monitoren. De kweek van NG is echter complex wat logistieke en technische expertise vereist, vanwege de veeleisende voedings- en omgevingsomstandigheden voor groei van NG. Ondanks de verbeterde diagnostiek en screeningsstrategieën blijft de incidentie van NG hoog. Daarom moet surveillance verbeterd worden en is het noodzakelijk om de transmissiedynamiek en AMR-acquisitie van NG beter te begrepen. Dit is met name belangrijk voor extragenitale infecties, vooral de orofarynx, aangezien deze worden beschouwd als een reservoir van AMR-genen voor NG. Dit komt doordat commensale *Neisseria* soorten vaak aanwezig zijn in de orofarynx en makkelijk DNA uitwisselen met NG.

De Wereldgezondheidsorganisatie (WHO) werd door verschillende meldingen van zeer resistente NG isolaten aangezet tot een zo gehete 'global action plan' om het ontstaan en verspreiding van resistente NG te voorkomen. De voorgestelde strategieën omvatten: effectieve diagnose en NG surveillance, systematische monitoring van behandelfalen, verbeteren van AMR surveillance, onderzoek naar nieuwe moleculaire surveillance methoden en nieuwe behandelingsopties. Met de onderzoeken in dit proefschrift wilden we meer kennis verwerven over effectieve diagnose, transmissie, verbeteren van surveillance en AMR beter te begrijpen bij NG. Hierbij bekeken we ook de potentiële centrale rol van de orofarynx en commensale *Neisseria* als reservoir voor resistentie. In dit hoofdstuk worden de belangrijkste conclusies van de studies in dit proefschrift

besproken in perspectief van de bestaande literatuur. Daarnaast worden er aanbevelingen gedaan voor toekomstig onderzoek.

De klinische relevantie van extragenitale NG infecties

De noodzaak om extragenitaal te testen is erkend voor mannen die seks hebben met mannen (MSM), maar niet voor vrouwen en heteroseksuele mannen. Afgezien van MSM worden extragenitale testen alleen aanbevolen voor patiënten met een indicatie, zoals een voorgeschiedenis van directe blootstelling door anale en/of orale seks en/of symptomen. Dit is gelinkt aan het verschil in prevalentie van extragenitale NG bij MSM (3% tot 21%) en heteroseksuele mannen en vrouwen (0% tot 3%). Recente epidemiologische studies tonen aan dat orofaryngeale NG veel voorkomt bij MSM en vrouwen. Wanneer er universeel routinematig werd getest, was de prevalentie van orofaryngeale NG 5,5% bij MSM en 1,4% bij vrouwen, waarbij de helft van de patiënten alleen een orofaryngeale infectie was. Een discussie rondom extragenitale NG infecties betreft de juistheid van de diagnose van deze infecties. Dit komt met name doordat de eerste generatie NAAT's vals-positieve resultaten konden geven door kruisreactiviteit met commensale *Neisseria*. Dit is opgelost in de momenteel gebruikte moderne NAAT's, maar deze zijn niet goedgekeurd voor extragenitale testen. Desondanks worden NAAT's aanbevolen voor de detectie van extragenitale NG mits het laboratorium specificaties voor prestatiekenmerken heeft opgesteld en deze overeenkomen met de huidige regelgeving. In eerdere studies is aangetoond dat de cobas 4800 CT/NG test zeer specifiek is en deze gebruikt kan worden voor anorectale swabs. Gebaseerd op deze resultaten veronderstellen wij dat de cobas 4800 CT/NG test ook goed zou presteren met orofaryngeale swabs. In **hoofdstuk 2** hebben we de validiteit van de cobas 4800 CT/NG test voor detectie van *C. trachomatis* (CT) en NG in orofaryngeale swabs onderzocht. De cobas 4800 presteerde zeer goed om NG in orofaryngeale swabs te detecteren. De detectielimiet, gedefinieerd als 95% detectie, was $6,65 \times 10^2$ CFU/ml, maar belangrijker was de hoge specificiteit (97,1%) en dat er geen kruisreactiviteit is waargenomen. Deze resultaten zijn in lijn met eerdere onderzoeken en tonen aan dat NG correct wordt gediagnosticeerd in extragenitale samples, waaronder de orofaryngeale swab. Echter, in onze studie wordt ook een potentieel belangrijk

probleem gevonden, omdat er in theorie tot 25% van de orofaryngeale NG infecties gemist zouden kunnen worden door een gemiddeld lage NG bacteriële load. Aangezien er geen aanwijzingen zijn dat de cobas 4800 CT/NG test ondermaats presteert ten opzichte van andere NAAT's, zou dit een veelvoorkomend probleem kunnen zijn, waarschijnlijk bij meerdere platformen. Vandaar dat er gevoeligere NAAT's ontwikkeld zouden moeten worden om gemiste orofaryngeale NG infecties te voorkomen die anders onopgemerkt zouden kunnen verspreiden.

De overdraagbaarheid van vaginale en extragenitale (anorectale en orofaryngeale) NG is nog niet geheel duidelijk. De load (hoeveelheid) van een pathogeen is gelinkt aan de overdraagbaarheid en klinische uitkomst bij andere SOA's. Vandaar dat NG bacteriële load gelinkt zou kunnen worden aan klinische uitkomst zoals symptomen. Voorgaande studies hebben een hogere NG load geobserveerd in urethrale infecties bij symptomatische mannen en anorectale infecties bij symptomatische MSM. Een lagere NG load werd waargenomen in orofaryngeale swabs van MSM ten opzichte van urethrale en anorectale swabs. Deze onderzoeken waren echter relatief klein en er is geen data over NG load bij vrouwen in relatie tot symptomen. Daarom is de NG load van genitale en extragenitale samples van een diverse populatie bepaald in **hoofdstuk 3**. In deze studie werden loads vergeleken tussen samplemateriaal en geslacht. Daarnaast werden er ook associaties tussen NG load en symptomen getest per seksuele risicogroep om een SOA te krijgen en sample materiaal. Uit onze data blijkt dat mannen en vrouwen een vergelijkbare NG load hebben op zowel genitale als extragenitale anatomische locaties. Dit impliceert dat een NG infectie even relevant zou zijn bij vrouwen als bij MSM. Onze data suggereren ook dat alle anatomische locaties een rol kunnen hebben in transmissie vanwege de aanzienlijke NG load. Wel zouden orofaryngeale NG infecties mogelijk een kleinere rol hebben gezien de lagere gemiddelde NG load (16 tot 25-voud). Desondanks zijn orofaryngeale NG infecties zeer belangrijk, omdat het vaak enkele infecties betreft, merendeel asymptomatisch is en waarschijnlijk betrokken bij AMR ontwikkeling van NG. Daarom zou systematisch extragenitaal testen in routine diagnostiek de incidentie van NG kunnen verlagen. Echter, vanwege de kosten zal dit niet geïmplementeerd worden voor heteroseksuele mannen en vrouwen, omdat de

prevalentie lager is. In lage-inkomenslanden zou dit zelfs nog minder toepasbaar zijn. In veel lage-inkomenslanden wordt een syndromale aanpak toegepast op basis van symptomen die gelinkt zijn aan een NG infectie. Omdat we in **hoofdstuk 3** hebben aangetoond dat asymptomatische infecties voldoende load hebben voor transmissie, kunnen deze asymptomatische infecties meer schade veroorzaken. NG infecties zijn bijvoorbeeld gelinkt aan een verhoogd risico om geïnfecteerd te worden met HIV.

De noodzaak om NG surveillance te verbeteren

Ondanks verbeterde diagnostiek en preventie blijft de incidentie van NG infecties hoog. Enkele belangrijke populatiegroepen die risico lopen om geïnfecteerd te worden met NG zijn geïdentificeerd, zoals MSM, jongeren en sekswerkers. Vooral bij deze belangrijke populaties is surveillance essentieel om transmissie te stoppen en mogelijke uitbraken op te sporen. De huidige surveillance is afhankelijk van gekweekte isolaten om te kunnen genotypering waarmee genetische verwantschap kan worden vastgesteld. De kweek van NG mislukt echter vaak vanwege de veeleisende voedings- en omgevingsomstandigheden, wat leidt tot een laag percentage kweek bevestigde casussen. Daarom pleit de WHO in hun 'global action plan' om de kweekcapaciteit te vergroten en nieuwe moleculaire methoden voor surveillance te ontwikkelen. Hierdoor denken wij dat er behoefte is aan kweekonafhankelijke genotyperingsmethoden om het probleem met NG-kweek te vermijden, waardoor de surveillance van NG wordt verbeterd. We hebben een kweekonafhankelijke methode ontwikkeld op basis van de veelgebruikte NG multi-antigen sequentietypering (NG-MAST) in **hoofdstuk 4**, genaamd "culture-free NG-MAST". Onze methode vertoonde geen kruisreactiviteit met niet-gonokokken *Neisseria* isolaten. Hierdoor kan de culture-free NG-MAST methode gebruikt worden om NG te genotyperen in klinische samples, inclusief extragenitale samples. Daarentegen kan de kweekafhankelijke NG-MAST niet worden gebruikt voor niet gekweekte extragenitale samples, omdat deze methode wel kruisreageert met commensale *Neisseria*, wat kan leiden tot verkeerde sequentietypen (ST). Bovendien heeft culture-free NG-MAST een hogere typeerbaarheid van 94% ten opzichte van de kweekafhankelijke methode met 62%, in samples die ingezonden werden voor kweek. Doordat niet-kweekbare en extragenitale samples getypeerd kunnen worden in

combinatie met de verhoogde typeerbaarheid zou de NG surveillance verbeterd kunnen worden. Wel moet men rekening houden dat (culture-free) NG-MAST vooral toepasbaar is om transmissieclusters te identificeren. Desondanks worden wel veel NG-MAST STs gekoppeld aan resistentie in NG, maar dit betekent niet perse dat de desbetreffende stam ook fenotypisch resistent is. Tot op heden blijft NG-kweek de 'gouden standaard' voor AMR-surveillance.

In de **hoofdstukken 6 en 7** werd de potentiële meerwaarde van culture-free NG-MAST voor surveillance en uitbraakmanagement onderzocht. In **hoofdstuk 6** zijn alle samples (n = 196) die tussen januari 2017 en september 2018 routinematig gekweekt werden van onze SOA poli (GGD Zuid-Limburg), gegenotypeerd met culture-free NG-MAST. In totaal werden 152/196 (77%) succesvol gegenotypeerd en van de 44 samples die mislukte, vertoonden er 11 een mogelijke gemixte infectie (≥ 2 NG isolaten). Van de 196 samples waren er 115 kweekpositief, wat overeenkomt met een typeerbaarheid van 59%. Dit is hoger dan het landelijk gemiddelde, waarschijnlijk doordat we alleen kweek adviseren bij een hoge bacteriële load. Desondanks heeft culture-free NG-MAST een hogere typeerbaarheid (77% versus 59%) en dit percentage is vergelijkbaar in kweekpositieve (80%) versus kweeknegatieve samples (74%). Wanneer we stratificeren op sampl materiaal was er geen verschil in typeerbaarheid van urinesamples tussen de methoden. Echter, zagen we een sterk verbeterde typeerbaarheid bij de andere sampl materialen wanneer culture-free NG-MAST werd gebruikt. In het bijzonder de orofaryngeale site, aangezien slechts 6,3% (1/16) kon worden getypeerd met kweek gebaseerde methoden, terwijl met culture-free NG-MAST 81% (13/16) werd gegenotypeerd. Deze verbeterde typeerbaarheid bleek essentieel te zijn in een NG-uitbraakonderzoek (**hoofdstuk 7**). Hierdoor identificeerde we een mogelijke uitbraak van NG in de jonge (<25) heteroseksuele populatie welke onze SOA poli bezocht tussen oktober 2017 en maart 2019. We zagen een driemaal hoger aantal genitale NG infecties bij jonge heteroseksuelen per maand (4,5 per maand) ten opzichte van januari 2016 - september 2017 (1,4 per maand, beschouwd als normale incidentie). Met behulp van culture-free NG-MAST werden drie onafhankelijke uitbraakclusters geïdentificeerd wat niet gedetecteerd zou zijn met kweek gebaseerde methoden. Ondanks de identificatie

bleek implementatie van maatregelen om verdere verspreiding van NG te voorkomen moeilijk te zijn. Dit was te wijten aan beperkte informatie over seksuele partners en geen duidelijke associatie met een geografisch gebied (woonplaats). Het kan zijn dat de woonplaats geen goede voorspeller is, omdat de individuen in een cluster naar dezelfde school gaan of bijvoorbeeld dezelfde datingapp gebruiken. Eerdere studies tonen ook aan dat moleculaire epidemiologie, met behulp van NG-MAST, kan helpen bij het identificeren van grote clusters en nieuwe opkomende stammen, wat we ook hebben gezien in **hoofdstukken 6 en 7**. Daarom vinden wij dat culture-free NG-MAST een meerwaarde heeft voor NG surveillance, dit zal in meer detail toegelicht worden in de toekomst visie.

De gevolgen van meerdere NG isolaten bij een patiënt

Normaal gesproken wordt slechts één isolaat van een enkele anatomische locatie gekarakteriseerd in surveillance, waardoor NG infecties met meerdere isolaten, zoals gemixte infecties, over het hoofd worden gezien. Er zijn twee soorten infecties met meerdere NG isolaten; 1) verschillende stammen op afzonderlijke anatomische locaties, en 2) meerdere isolaten in één anatomische locatie (gemixte infectie). Beide typen infecties met meerdere NG isolaten werden waargenomen in eerdere onderzoeken, maar de frequentie van dit soort infecties varieert sterk tussen onderzoeken. Omdat veel van deze studies afhankelijk zijn van kweek, minder onderscheidende methoden gebruikten dan NG-MAST, of weinig voorkomende isolaten niet konden detecteren (<10% in een mix), hebben we een targeted metagenomische methode ontwikkeld die weinig voorkomende isolaten kan detecteren en zeer onderscheidend is. We hebben deze methode gebruikt om meer inzicht te krijgen in de frequentie van meerdere NG isolaten bij een patiënt. Deze metagenomische methode is gebaseerd op onze zeer onderscheidende culture-free NG-MAST methode. In **hoofdstuk 4** hebben we 81 MSM en 17 vrouwen met gepaarde NG positieve samples van afzonderlijke anatomische locaties geïncubeerd in onze studie. In totaal werden van 80/98 patiënten de gepaarde samples gegenotypeerd waarbij een kwart van de patiënten (20/80) geïnfecteerd waren met verschillende NG isolaten op afzonderlijke anatomische locaties op hetzelfde tijdstip. De geobserveerde proportie is hoger dan bij de meeste voorgaande

onderzoeken mogelijk vanwege de omvang van het onderzoek, door de gebruikte meer discriminatoire methode en de inclusie van kweeknegatieve samples. Omdat het aandeel van verschillende NG-isolaten op afzonderlijke anatomische locaties hoog was, moest dit verder onderzocht worden om dit beter te begrijpen. De verschillende NG-stammen op afzonderlijke anatomische plaatsen zouden het resultaat kunnen zijn van gemixte infecties, maar alleen de dominante stam van een anatomische locatie werd gekarakteriseerd in **hoofdstuk 4**. Onze hypothese is dat er ook additionele NG-stammen, die voorheen niet werden ontdekt, aanwezig zouden kunnen zijn vanwege gemixte infecties. Daarom hebben we een vervolgstudie uitgevoerd in **hoofdstuk 5** van de samples van MSM met verschillende NG-stammen op afzonderlijke anatomische locaties ($n = 33$) van **hoofdstuk 4**. In **hoofdstuk 5** werd een metagenomische genotyperingsmethode ontwikkeld door culture-free NG-MAST te combineren met next-generation sequencing en een analysepijplijn. Ondanks de geselecteerde MSM met verschillende NG-stammen op afzonderlijke anatomische locaties, laten de meeste samples (79%, 26/33) een enkele NG-infectie zien. Onze studie levert additioneel bewijs dat gemixte NG-infecties voorkomen, maar waarschijnlijk bij een lage prevalentie. Mogelijke verklaringen voor het schijnbaar lage voorkomen van gemengde NG-infecties en de implicaties van meerdere NG-isolaten voor surveillance worden hieronder besproken.

Op basis van ons onderzoek in de **hoofdstukken 4 en 5** blijkt dat gemixte NG-infecties minder vaak voorkomen dan verschillende NG-isolaten op afzonderlijke anatomische locaties. Er zijn verschillende verklaringen mogelijk zoals concurrentie tussen NG-stammen, afzonderlijke introductie van NG en weefseltropisme. In een muisinfectiemodel werden fitheid en competitieve co-kweek van ceftriaxon-resistente NG-stammen bestudeerd door Vincent et al. (2018). De auteurs rapporteren significante verschillen in fitheid en in sommige competitieve co-kweken met wild-type NG werden geen ceftriaxon-resistente NG-bacteriën teruggevonden. Deze overgroei van een fittere, in dit geval de wild-type NG, nam in de loop van de tijd toe. Aangezien asymptomatische patiënten op zijn vroegst 14 dagen na mogelijke blootstelling worden getest, zoals beschreven in de Nederlandse SOA-richtlijnen, zou dit een deel van de lage

frequentie van gemixte NG-infecties kunnen verklaren. De MSM in **hoofdstuk 5** rapporteerden een mediaan van 10 (6-18, interkwartielbereik) sekspartners in de voorgaande zes maanden. Hierdoor is het mogelijk dat ze op verschillende tijdstippen aan NG zijn blootgesteld. Gecombineerd met competitie tussen NG-isolaten, zou een gevestigde NG-infectie van een nieuw geïntroduceerd NG-isolaat kunnen voorkomen. Het rapporteren van meerdere sekspartners weerspiegelt echter niet meerdere mogelijke blootstellingen van NG op een anatomische locatie. Uit onderzoek naar seksueel gedrag blijkt dat seksuele handelingen per sekspartner sterk kunnen variëren, inclusief condoomgebruik en soort seksuele handelingen per sekspartner. Ten slotte kan weefseltropisme bijdragen aan het lage voorkomen van gemixte infecties, aangezien sommige isolaten bepaalde anatomische plaatsen mogelijk slecht zouden kunnen infecteren. Echter ondergaan veel eiwitten fasevariatie die betrokken zijn bij de aanhechting van cellen. Dit resulteert in adaptatie om de adhesie aan verschillende menselijke cellen mogelijk te maken. Daarnaast kan NG zich aanpassen aan verschillende omgevingsomstandigheden door een reeks transcriptiefactoren. Hierdoor veronderstellen we dat NG-isolaten zich aan kunnen passen aan een anatomische locatie en is weefseltropisme waarschijnlijk geen verklaring voor de lage frequentie van gemixte infecties. Toch komen deze gemixte infecties voor en ze kunnen relevant zijn voor de behandeling, omdat een potentieel resistente NG-isolaat bij een gemixte infectie kan resulteren in behandelfalen. Daarnaast kunnen gemixte infecties relevant zijn voor de ontwikkeling van AMR, aangezien AMR-genen kunnen worden uitgewisseld tussen NG-isolaten. Zoals eerder besproken, wordt normaal gesproken één isolaat van een enkele anatomische locatie gekarakteriseerd in surveillance. Hierdoor zouden infecties met meerdere NG-isolaten worden gemist in de huidige surveillancestrategieën die potentieel de verspreiding van resistente NG-stammen mogelijk maken. Vandaar dat verschillende NG-isolaten op afzonderlijke anatomische locaties in de surveillance opgenomen zouden moeten worden, aangezien deze infecties vaak voorkomen.

De orofarynx als een reservoir voor antimicrobiële resistentie

Mozaïekgenen kunnen voorkomen in het genoom van NG en leiden tot resistentie tegen verschillende antibiotica. Aangezien ceftriaxon de huidige eerstelijns empirische behandeling van NG is en er niet veel behandelingsopties over zijn, is AMR-onderzoek essentieel om onbehandelbare NG te voorkomen. Mozaïek *penA*-genen kunnen resulteren in ceftriaxonresistentie. Er wordt verondersteld dat deze mozaïek genen ontstaan door DNA-recombinatie met DNA van commensale *Neisseria*-soorten die doorgaans in de oropharynx voorkomen. Hierdoor wordt de orofarynx beschouwd als een reservoir van AMR-genen voor NG. In een studie van Igawa et al. (2018), is *in vitro* recombinatie van NG met *N. cinerea* DNA aangetoond. Dit weerspiegelt echter niet per definitie het proces *in vivo*, omdat bijvoorbeeld de regulatie van DNA-donatie onduidelijk is. Meestal zijn *N. cinerea* en *N. subflava*-groep betrokken bij mozaïek *penA*-genen in NG. Daarom zou de aanwezigheid van *N. cinerea* en *N. subflava* in de orofarynx tijdens een NG-infectie een voorwaarde zijn voor recombinatie. Om de aanwezigheid van *Neisseria*-soorten en mogelijke recombinatie in NG tijdens een orofaryngeale infectie te bestuderen, hebben we een metagenomische-methode ontwikkeld waarbij gebruik wordt gemaakt van nanopore-sequencing om amplicons van *penA*-genen en de flankerende regio's te karakteriseren (**hoofdstuk 8**). De flankerende regio's van het *penA*-gen werden gebruikt om de soort te kunnen identificeren. Hierdoor konden we *penA*-genen vergelijken en mogelijke recombinatiegebeurtenissen identificeren. In deze studie werden 64 NG-positieve orofaryngeale samples van MSM gekarakteriseerd, waarvan 61 *penA* PCR-positief waren. In bijna de helft van deze samples (28/61) werd een NG-contig waargenomen en 8/28 waren mozaïek. Op één na hadden alle mozaïek *penA*-genen aminozuursubstituties I312M en V316T die gelinkt zijn aan ceftriaxonresistentie. In deze studie tonen we aan dat recombinatie tijdens een NG-infectie mogelijk zou kunnen zijn, omdat in alle samples (61/61) *N. subflava* en in meer dan de helft van de orofaryngeale samples (35/61) *N. cinerea* aanwezig waren. Bovendien was in alle samples met een mozaïek *penA*-gen in NG het mozaïekgedeelte van NG vergelijkbaar met *N. cinerea* of *N. subflava*. Hoewel de resistentie in Nederland laag is, werd bij 29% (8/28) van NG contigs een mozaïek *penA* waargenomen en dit is in

lijn met de recente studie van de Laat et al. (2019). Recente epidemiologische studies in andere landen rapporteren het mozaïek *penA*-gen in 2,3% tot 27% van de geteste isolaten. Zorgwekkend is dat de proportie stammen met mozaïek *penA* lijkt toe te nemen. Daarnaast worden A501-mutaties in niet-mozaïek *penA* vaak gerapporteerd die gelinkt zijn aan ceftriaxonresistentie. In onze studie van januari 2017 tot juni 2018 heeft slechts één NG-isolaat de mutatie A501V en is niet-mozaïek. Samengevat is het duidelijk dat commensale *Neisseria*-soorten die doorgaans voorkomen in de orofarynx ook tijdens een NG-infectie aanwezig zijn. Omdat *Neisseria*-soorten gemakkelijk DNA uitwisselen en het mozaïekgedeelte van NG vergelijkbaar was met commensale *Neisseria*-soorten, zou recombinatie *in vivo* een centrale rol kunnen spelen in de ontwikkeling van AMR in NG. Toch zijn er veel onbekende factoren, zoals de efficiëntie in DNA-donatie en opname, fitnesskosten van recombinatie, AMR in commensale *Neisseria* en mogelijke doding van NG door commensaal DNA.

Afsluitende opmerkingen en toekomstperspectieven

Ondanks vele inspanningen om NG-infecties op te sporen en te behandelen, blijft de incidentie hoog. Richtlijnen erkennen de noodzaak van extragenitale tests bij MSM en specifieke populaties. In dit proefschrift hebben we veel enkele orofaryngeale NG-infecties waargenomen die meestal asymptomatisch zijn. Hoewel de cobas 4800 CT/NG-test zeer goede prestaties vertoont om NG in orofaryngeale samples te detecteren, zou in theorie tot 25% kunnen worden gemist. Omdat er geen aanwijzingen zijn dat de cobas 4800 ondermaats presteert in vergelijking met andere NAAT's, zijn er gevoeligere testen nodig. Toekomstige studies moeten de impact van dit potentiële probleem bekijken. Herhaaldelijk testen van NG-negatieve orofarynx kan bijvoorbeeld worden uitgevoerd om de aanvankelijk gemiste infecties in te schatten.

Een van de doelen van dit proefschrift was om de surveillance van NG te verbeteren. We hebben aangetoond dat kweekonafhankelijke typering essentieel was om NG-uitbraakclusters te identificeren. Zoals verschillende keren vermeld, zijn diagnostiek en surveillance de afgelopen decennia enorm verbeterd, maar NG blijft een wereldwijd probleem. Wij zijn van mening dat een prospectieve typering in combinatie met

intensief bron en contact onderzoek zou kunnen helpen om NG-transmissie te beperken en uitbraken vroegtijdig op te sporen. Met deze gegevens zouden seksuele netwerken makkelijker geïdentificeerd kunnen worden om gerichte interventies zoals intensiever testen. Met onbeperkte middelen zouden idealiter alle NG-positieve samples moeten worden getypeerd om de hoogste resolutie te verkrijgen waarmee clusters geïdentificeerd kunnen worden. Samen met metadata van de GGD zou een interactieve monitoringtool voor NG-infecties voor klinici, epidemiologen en microbiologen kunnen worden gemaakt. In deze tool kan een waarschuwingssysteem worden geïmplementeerd om regio's of populaties te markeren die mogelijk extra aandacht nodig hebben. Zo kan bijvoorbeeld een mogelijke NG-uitbraak vroegtijdig worden geïdentificeerd om de transmissie proberen te stoppen. Helaas zijn de middelen beperkt en worden de kosten voor het typeren geschat op € 40 bij gebruik van cultuur-free NG-MAST, inclusief een analist. Een pilotstudie met de voorgestelde aanpak zou moeten worden uitgevoerd waar (hopelijk) clusters worden gezien om interventies mogelijk te maken wat uiteindelijk resulteert in een afname van de incidentie van NG.

Gonorrroe is tegenwoordig een grote uitdaging voor de volksgezondheid, niet alleen vanwege de hoge incidentie, maar ook vanwege de toenemende resistentie. Gebaseerd op eerdere resultaten en de resultaten in **hoofdstuk 8** lijken commensale *Neisseria*-soorten een cruciale rol te spelen in de ontwikkeling van AMR van NG. De data over commensale *Neisseria*-soorten zijn echter beperkt. Daarom zou toekomstig onderzoek zich meer moeten richten op de commensale *Neisseria* dan alleen op NG. Zo is er weinig bekend over de minimale inhiberende concentratie (MIC) waarden bij deze soorten. Dit is met name relevant voor AMR in NG, omdat commensale *Neisseria* resistent zou kunnen worden na meerdere blootstellingen aan antibiotica en zouden dan AMR-genen kunnen uitwisselen met NG. Daarom is het belangrijk om de dynamiek van de commensale *Neisseria* beter te begrijpen. Zo zou een longitudinaal onderzoek waarbij individuen regelmatig worden gesampled, meer kennis op kunnen leveren. Daarbij wordt ingegaan op, maar niet beperkt tot, de samenstelling van de orofaryngeale *Neisseria*-soorten, mozaïekgenen, MIC-waarden en sequenties van het hele genoom van isolaten.

Hoewel er een gebrek is aan nieuwe antibiotica voor de behandeling van NG, kunnen we de zorg verbeteren en het gebruik van ceftriaxon beperken om de ontwikkeling van AMR mogelijk te vertragen. Door point-of-care-testen of nieuwe NAAT's te gebruiken die ook NG-isolaten kunnen identificeren die nog steeds behandeld kunnen worden met eerder gebruikte antibiotica zoals spectinomycine of ciprofloxacine. Hierdoor kan de patiënt behandeld worden met een van deze antibiotica in plaats van met ceftriaxon.

Samenvattend, met de introductie van NAATs werd de diagnose van een infectie met NG gevoeliger en met antibiotica werd de behandeling makkelijker. NG bleek echter een zeer adaptieve pathogeen te zijn die steeds resistenter wordt tegen alle antibiotica klassen. Daarom zijn er surveillanceprogramma's opgezet om resistente NG te monitoren en te voorkomen. Toch is er nog steeds veel onduidelijk, zoals de rol van commensale *Neisseria* en de aanhoudend hoge incidentie van NG wereldwijd. Het onderzoek dat in dit proefschrift wordt gepresenteerd, verduidelijkt een deel van de puzzel om de transmissiedynamiek te begrijpen, maar biedt ook tools om NG-surveillance te verbeteren.

Impact paragraph

The World Health Organization estimates that 87 million new cases of *Neisseria gonorrhoeae* (NG) occur annually worldwide. An infection with NG can cause reproductive tract complications like pelvic inflammatory disease, infertility, ectopic pregnancy, and maternal death in women and urethritis and dysuria in men. Hence, preventing spread of sexually transmitted NG is a global health priority. Another major issue is that NG is increasingly resistant to all classes of antibiotics. Therefore, the main goals of this thesis were to improve surveillance, and to gain further knowledge on transmission dynamics and antimicrobial resistance acquisition in NG.

Bacterial load, the amount of bacteria, could be an indication for the transmissibility of a pathogen, in this case NG. We observed that all tested anatomical sites (genital, anorectal, and oropharynx) have sufficient bacterial load for transmission of NG. The observed bacterial loads were similar between men and women indicating that an infection with NG is equally relevant.

Current surveillance of NG characterizes only one isolate of one anatomical site, though many individuals can be infected concurrently with NG in separate anatomical sites. Studies presented in this thesis showed that in a quarter of patients distinct NG isolates were identified in separate anatomical sites. This would be missed with the current surveillance strategy and might have severe consequences. For example, this possibly allows dissemination of antimicrobial resistant (AMR) NG.

AMR is a global issue in NG that can lead to treatment failure and ultimately in untreatable NG. AMR can occur if DNA is exchanged by bacteria. In this exchange AMR genes can be donated by resistant NG isolates to susceptible ones. Another possibility is DNA donation by commensal *Neisseria* species that commonly reside in the oropharynx. A study in this thesis demonstrated that during all tested oropharyngeal infections with NG, commensal *Neisseria* were present. As these commensal *Neisseria* species readily exchange DNA with NG, the oropharynx is considered to be a reservoir for AMR development in NG.

The studies presented in this thesis provide further knowledge on transmission dynamics and surveillance of NG. Some results can be readily applied to improve NG surveillance. Current surveillance of NG relies on culture that frequently fails because NG requires demanding nutritional and environmental conditions. In this thesis a surveillance method was developed that does not rely on culture. Studies presented in this thesis demonstrate the improved surveillance using our method and show that this method was essential to detect a potential outbreak of NG in young heterosexual in South Limburg, the Netherlands. The improved surveillance of this method could aid in unraveling of sexual networks to prevent transmission of NG. Potentially the improved detection of transmission and outbreaks might ultimately result in a lower incidence of NG, thereby reducing sequelae and costs.

The results presented in this thesis provide insights for, but not limited to, microbiologists, epidemiologists, and policy makers. As discussed above, transmission dynamics of NG was investigated with the use of NG bacterial load. Surveillance methods were developed in this thesis and demonstrate the potential added value in surveillance. Also, the occurrence and impact of multiple distinct NG isolates in an individual is investigated in detail. Hence, our results can be used for modeling studies of transmission of NG and to improve surveillance guidelines.

Sharing study results and interdisciplinary collaborations helps to understand all aspects of a pathogen. Therefore, all studies presented in this thesis will be published in international journals, including datasets. Also, preliminary results were presented at national and international conferences to share knowledge and potentially start new collaborations. The studies presented in this thesis were performed in collaboration with local epidemiologists of the Public Health Service of South Limburg. Based on our results new studies are designed but also the culture-independent surveillance is being implemented.

To conclude, this thesis provides further understanding of transmission dynamics and AMR acquisition of NG, and offers tools to improve surveillance. The culture-independent surveillance method can be readily implemented in surveillance, thereby aiding in NG transmission prevention and outbreak detection.

List of publications

In this thesis

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Curriculum vitae

Brian van der Veer werd geboren op 15 juni 1991 te Boxmeer. Hij voltooide de HAVO in 2008 aan de scholengemeenschap Stevensbeek. Daarna werd de Bachelor opleiding 'Hoger Laboratorium Opleiding Biochemie' aan Hogeschool van Arnhem en Nijmegen succesvol afgerond in 2013, hierbij verkreeg hij de titel Bachelor of Applied Sciences. De titel Bachelor of Science werd in hetzelfde jaar verkregen aan de Radboud Universiteit. Hierop volgde de research Master 'Medical Biology' aan de Radboud Universiteit waar de titel Master of Science bene meritum werd behaald in 2015. Zijn promotieonderzoek ging van start in juli 2016 bij de afdeling Medische Microbiologie van het Maastricht Universitair Medisch Centrum+ (MUMC+) in samenwerking met GGD Zuid Limburg, onder leiding van Prof. dr. Christian Hoebe, Dr. Lieke van Alphen en Dr. Petra Wolffs. Per november 2020 is hij werkzaam als bioinformaticus bij dezelfde afdeling van het MUMC+, waar hij onderzoek verricht naar uitbraken en het voorkomen van (nieuwe) varianten van SARS-CoV-2.

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