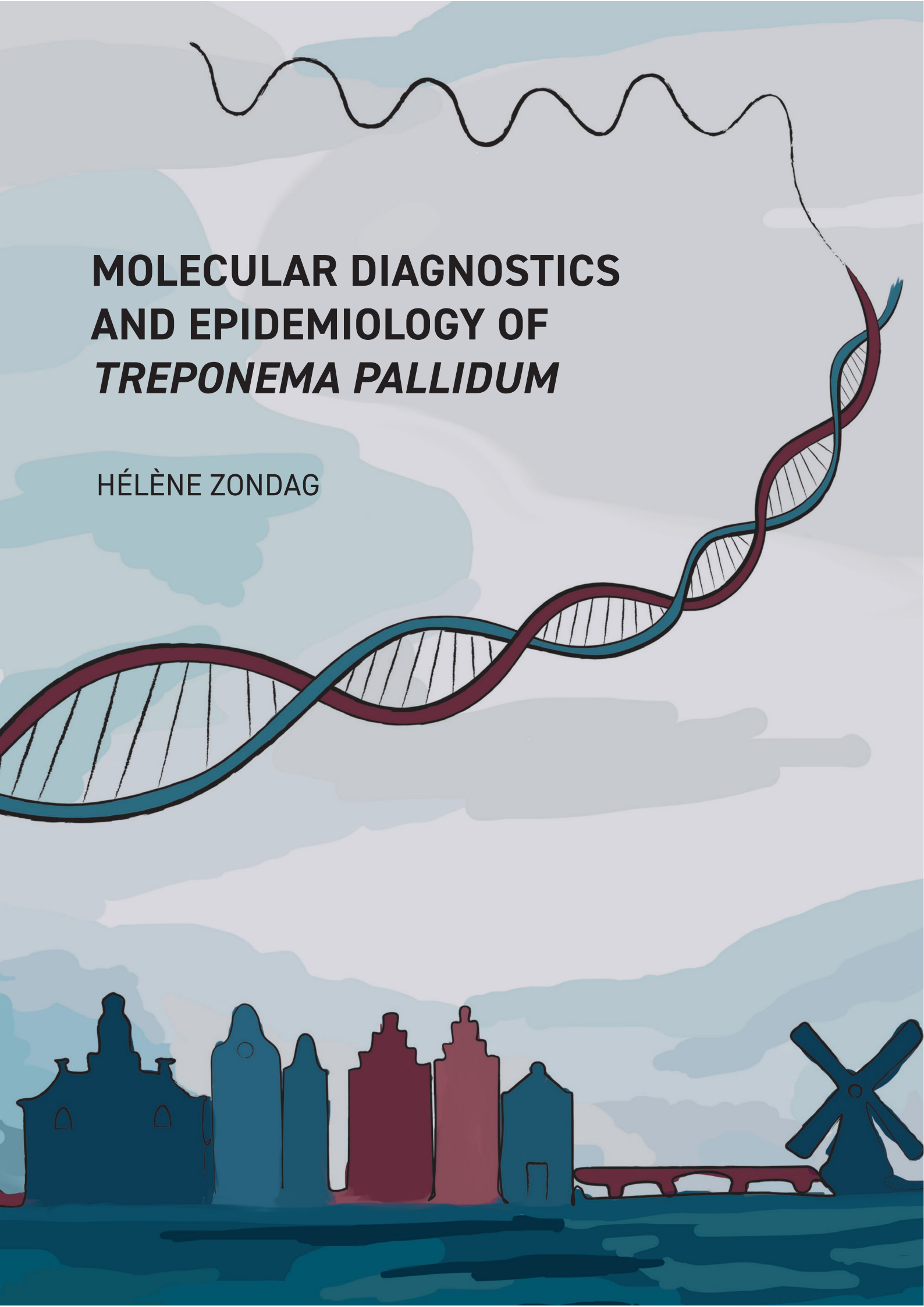
MOLECULAR DIAGNOSTICS AND EPIDEMIOLOGY OF *TREPONEMA PALLIDUM*

HÉLÈNE ZONDAG



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Hélène Zondag

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Molecular Diagnostics and Epidemiology of *Treponema pallidum*

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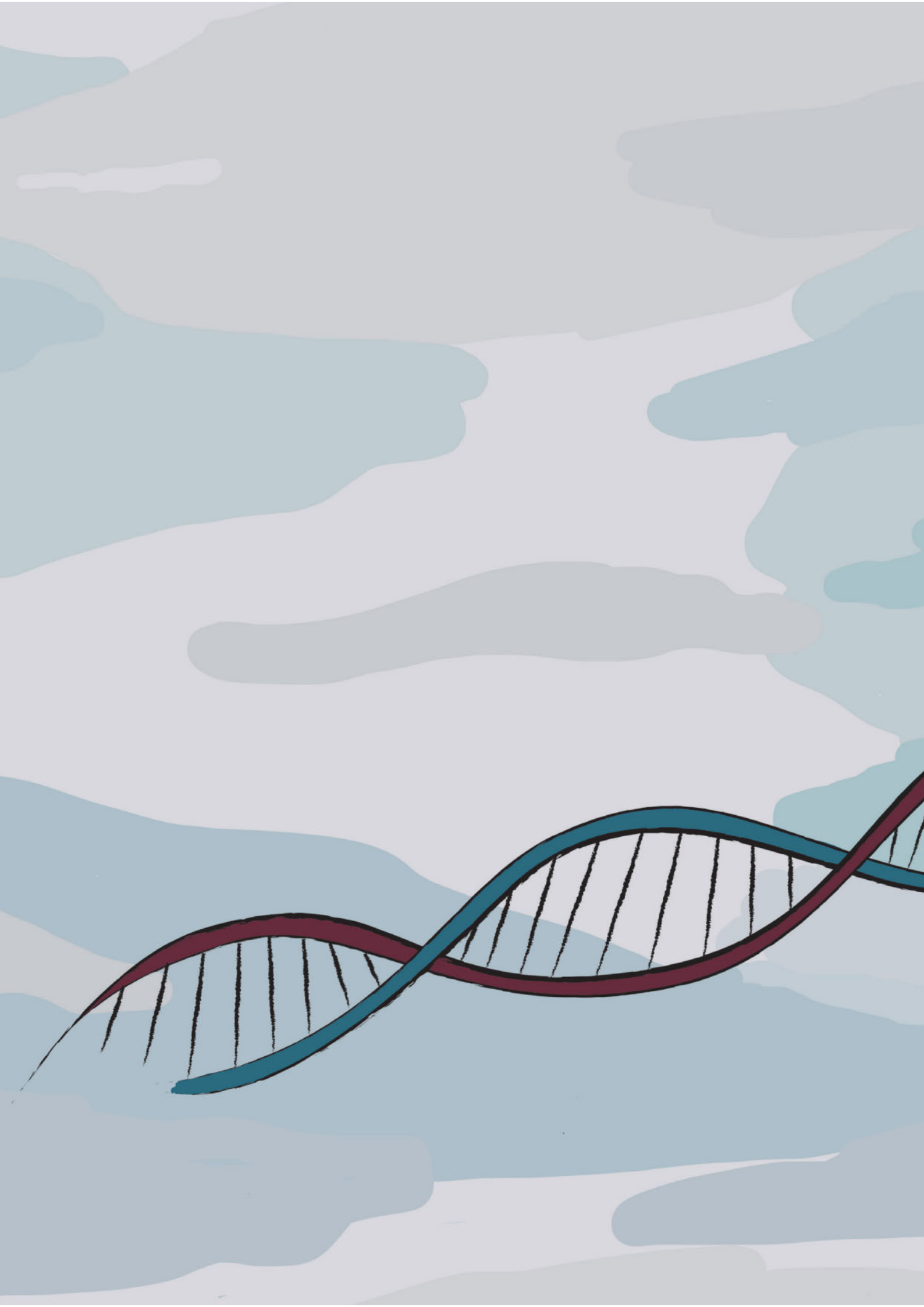

Chapter 1

General Introduction

Micro-organisms and viruses are all around us. Some cause disease in humans or other species and are called pathogens. Infectious pathogens that transmit between human hosts can be decreased in prevalence or even eradicated by timely detection and treatment. Insights in their geographical spread, physiology, pathogenicity, virulence, transmissibility and interaction with their hosts aids this goal. Globally, around 1 million people are infected every day with one of the main bacterial sexually transmitted infections (STI); syphilis, gonorrhea and chlamydia, among people between 15 – 49 years old (1, 2). Yearly, 7.1 million cases are caused by infection with *Treponema pallidum* subspecies *pallidum* (*T. pallidum*), the causative agent of syphilis, and 82.3 million are by *Neisseria gonorrhoeae* (*N. gonorrhoeae*), causing gonorrhea.

Since the discovery of penicillin by Alexander Fleming in 1928 and its implementation as treatment for syphilis in 1943, syphilis incidence has decreased drastically. However, despite the effective treatment syphilis remains prevalent worldwide (Figure 1). In high-income countries, men who have sex with men (MSM) are disproportionally burdened with syphilis. In the Netherlands, MSM accounted for 96% of the syphilis cases in 2021 (3). The World Health Organization (WHO) put together a global health strategy plan with ambitious goals to take steps towards ending curable STI epidemics by 2030 (1). The importance of strong surveillance systems were highlighted, also by the Dutch health authorities (RIVM) (1, 4).

Molecular epidemiology and diagnostics play an important role in surveillance. It allows monitoring and identifying populations at increased risk of acquiring STI, to rapidly treat cases accordingly and prevent further transmission. Most chapters in this thesis are on *T. pallidum,* which is the focus of this thesis and will be discussed in more detail.

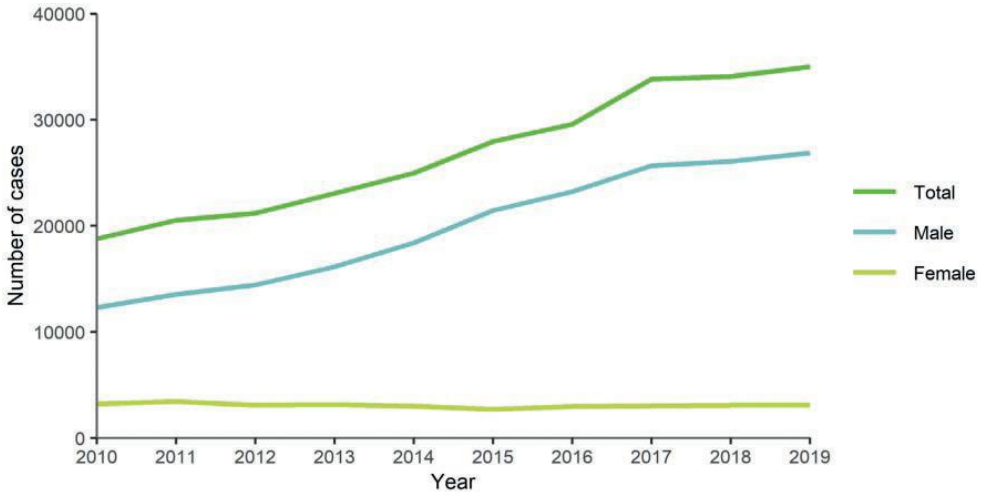

Figure 1 – Number of syphilis cases in EU/EEA countries reporting consistently to the European Centre for Disease Prevention and Control (ECDC) between 2010 – 2019* (5).
** Belgium, Bulgaria, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, the Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, the United Kingdom.*

BACKGROUND

The discovery of a pathogen and the identification of its association with a certain infectious disease allows more efficient research into diagnostics, treatment and epidemiology.

History of syphilis

Syphilis is a STI caused by the spirochete *Treponema pallidum* subspecies *pallidum* (*T. pallidum*). Syphilis has been around for at least 500 years (6). The origin of treponematoses has been a controversial topic in many areas of expertise for decades. The most common theory is the Columbian theory which describes syphilis coming to Europe with the troops of Christopher Columbus in 1492 (7, 8). Another theory that has gained interest is the pre-Columbian theory that asserts the presence of syphilis in Europe before 1493 (7, 9). Paleopathology researchers have attempted to elucidate the truth of the origin of syphilis and support different theories highlighting the need for more systemic approaches. However, often these analyses lack either specific diagnostic indicators to verify treponemal disease or accuracy in site date (8). Another challenge is subspecies determination between *T. pallidum* subspecies *pallidum, endemicum* or *pertenue*, causing venereal syphilis, bejel or endemic syphilis and yaws, respectively, or even the more distantly related *Treponema carateum* causing pinta. They are morphologically and antigenically indistinguishable, but are considered to be geographically distinct (10, 11). Transmission of these pathogens occur by direct contact of infectious lesions and clinical manifestations may be similar. Epidemiological differences, such as prevalence and age at infection, have been used to carefully differentiate between the subspecies. Not only historical records are subjected to this challenge, by molecular typing recently some patients previously diagnosed with syphilis in Cuba and Japan have been found to have been infected with *endemicum* subspecies (12-14). These challenges call for an interdisciplinary approach between research fields like modern paleopathology (15), epidemiology, biomedicine and more (16). Recent developments in sequencing techniques allowed sequencing of ancient DNA and contribute to uncovering the origin and evolution of treponematoses with higher resolution methods in the close future (6, 17, 18).

T. pallidum discovery

In 1905, 400 years after the first written reports about the syphilis epidemic, the causative pathogen was found by Fritz Schaudinn and Erich Hoffmann in Berlin, Germany (Figure 2) (19-21). With the invention of the dark-field microscope, *T. pallidum* was easier to visualize. The next step to uncovering more information was through culture. Using *in vitro* culture more information can be gained on the physiology, pathogenicity, immunology characteristics and antimicrobial susceptibility testing of a microorganism. *T. pallidum* proved difficult to grow *in vitro*, even though some studies described *in vitro* culture, the experiments were either not reproducible or showed contamination with a different, commensal, *Treponema* species (22). For years cultivation of *T. pallidum* relied on *in vivo* propagation in rabbits, which greatly

slowed down research (22-25). Extensive efforts were done to attempt a continuous *in vitro* culture using microaerophilic conditions and a support cell-line of cottontail rabbit epithelial cells (Sf1Ep) (24, 25). Despite these efforts *T. pallidum* multiplication and survival was limited to about 2 weeks in these culture systems (22). In 2018, the first long-term culture system was described (22).

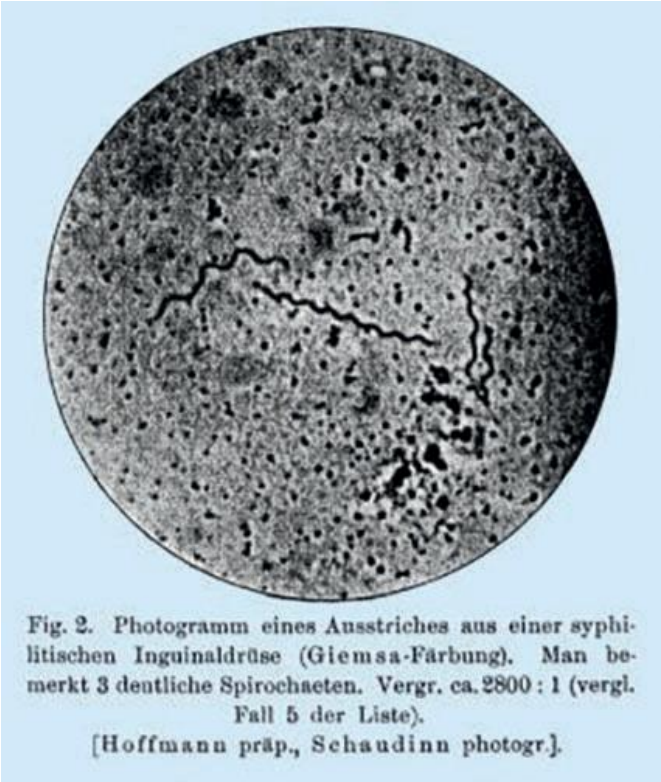

Fig. 2. Photogramm eines Ausstriches aus einer syphilitischen Inguinaldrüse (Giemsa-Färbung). Man bemerkt 3 deutliche Spirochaeten. Vergr. ca. 2800 : 1 (vergl. Fall 5 der Liste).
[Hoffmann präp., Schaudinn photogr.].

Figure 2 – First documentation of the observation by microscopy of *T. pallidum* by Fritz Schaudinn and Erich Hoffmann in 1905. Translation of figure legend: "Fig 2. Photograph of a smear from a syphilitic inguinal gland (Giemsa stain). One notices 3 distinct spirochaetes. Enlargement approximately 2800:1 (Comparison of case 5 in the list). [Preparation by Hoffmann, Photograph by Schaudinn]." Figure from (21) reproduced with permission of SNCSC.

TRANSMISSION AND INFECTION WITH *T. PALLIDUM*

The complexity of *T. pallidum* does not stop at its history. By understanding the modes of infection of an infectious pathogen, preventive strategies can be formulated.

Transmission and dissemination

Syphilis can be transmitted through contact of infected lesions (26). After infection, *T. pallidum* becomes systemic and may be found in various anatomical locations within a patient besides the location of initial infection (27, 28). Even urine samples have been found to contain *T. pallidum* DNA (27, 29, 30). Transmission between patients may occur from any anatomical location in which *T. pallidum* resides (31). The spirochetes first traverses through mucosal layers and membranes and once it crosses the epithelium, it can multiply

and quickly disseminate through the lymphatic system and bloodstream causing a systemic disease (10, 11, 26). The highly invasive nature of *T. pallidum* allows invading of endothelial cell monolayers, blood barriers and the placenta to cause a multi stage disease, which, if left untreated, may lead to life-threatening diseases (Figure 3) (32, 33).

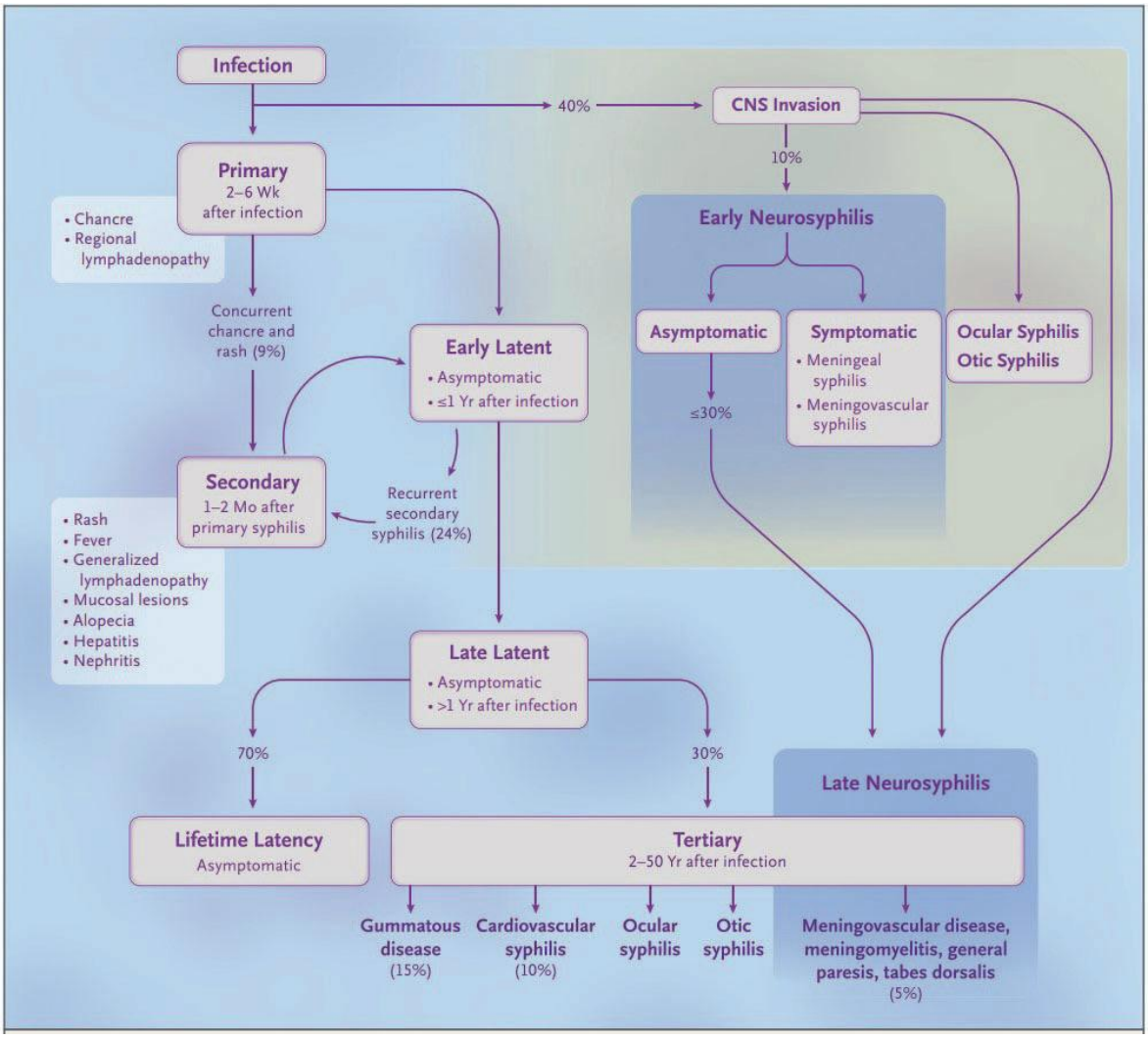

Figure 3 – Flowchart of syphilis stages and typical clinical manifestations during untreated infection with *T. pallidum*. Reproduced with permission from (33), Copyright Massachusetts Medical Society

T. pallidum can also be vertically transmitted, by traversing the placenta, from a pregnant person to the fetus, and is known as congenital syphilis. Congenital syphilis is a serious public health threat being one of the leading causes of adverse pregnancies and stillbirths worldwide (34, 35). The WHO set up a strategy to globally eradicate congenital syphilis, which has been successful in lowering congenital syphilis rates worldwide and eradication in Cuba (36). However, we are far from worldwide eradication (36, 37). High-income countries have included prenatal screening of *T. pallidum* serological markers to screen all pregnant persons. This intervention allows early detection of *T. pallidum* and treatment. However, most countries do not have the resources to implement the prenatal screening and would benefit

from other strategies like dual HIV and syphilis testing of people to tackle both important infectious diseases (38-41).

Course of disease

Clinical manifestations of syphilis vary widely and typical signs and symptoms often occur inconspicuous (10, 26). Replication of *T. pallidum* at the site of infection leads to the activation of the host immune system, which causes the formation of an ulcer within 10 – 90 days (mean 21 days) after infection (31). The presence of an ulcer is characteristic of primary syphilis and is typically painless. However, the ulcer may go unnoticed, especially when located in the anus or pharynx, which occurs frequently in MSM (10, 26, 28). After 3 - 6 weeks, the ulcer heals, but is in 15% of the cases still perceptible in secondary stage syphilis (26).

Secondary syphilis is a generalized infection and clinically recognizable based on a disseminated mucocutaneous rash with or without lymphadenopathy, or mucosal lesions such as condylomata lata (Figure 3). These clinical manifestations may be subtle and occur within 3 months of infection (26). Also these secondary syphilis manifestations heal and disappear after which an asymptomatic period follows. This period is known as latent syphilis and is divided into two stages, early latent and late latent syphilis, based on time from initial infection and serological assays. The European Centers for Disease Control and Prevention (ECDC) defines early latent as an infection within 1 year from acquisition whereas the WHO defines early latent when the infection took place within 2 years (31, 42, 43). During the early latent stage, infected patients may experience relapsing secondary symptoms (Figure 3) (33). Sexual transmission in the late latent syphilis stage is considered unlikely even with serological assays indicating an infection with *T. pallidum* (26). Infectious syphilis stages consist of the primary, secondary and early latent syphilis stages (33).

Immune evasion

The immune system of the host responds to the infection with *T. pallidum* by producing anti-treponemal antibodies. Despite high levels of antibodies, especially in secondary and early latent syphilis, *T. pallidum* survives by evading the immune system throughout the infection (Figure 3) (11, 28). It is unsure how the infection progresses to asymptomatic latency (11). Treating a syphilis infection does not prevent reinfection with *T. pallidum*. MSM living with HIV and the absence of STI symptoms during diagnosis are factors that have been found to be associated with increased risk of a recurrent syphilis infection (44-47). Individuals with recurrent syphilis infections have subsequently less symptoms (48).

(MOLECULAR) DIAGNOSTICS AND TREATMENT

Early detection and treatment of an infectious pathogen reduces its transmission and spread.

Syphilis diagnostics

To diagnose a syphilis infection the clinical observation and laboratory tests come together (10, 49). Clinical observations of typical signs and symptoms of suspected syphilis infections are always backed up with laboratory testing. In addition, some clinical practices include dark-field microscopy on serous fluid of an ulcer.

Nucleic acid amplification tests (NAAT), like PCR, have been found to be an important addition to syphilis diagnostics in detecting *T. pallidum* syphilitic lesions (50-53). Especially in the early stage of the infection as serological responses might not yet be measurable (Figure 4). Most laboratories have implemented PCRs targeting the *polA* or the *tpp47* gene, which are equally sensitive (54).

Serological assays remain the primary method for syphilis diagnostics. There are two types of serological tests; non-treponemal and treponemal tests. The first non-treponemal serological assay was developed in 1906 by Wassermann and colleagues in Berlin, Germany and was known as the Wassermann test (55). Non-treponemal tests measure immunoglobulin M (IgM) and IgG antibodies to non-treponemal antigens from damaged host cells after infection with syphilis (10, 56). Examples of current frequently used non-treponemal tests are rapid plasma reagin (RPR) and the venereal disease research laboratory test (VDRL) (49). These tests indicate an infection with *T. pallidum* and can be used to monitor treatment response. In addition, a four-fold titer increase in non-treponemal tests indicates relapse or reinfection in patients with a previous syphilis infection (57). However, tissue damage due to a concurrent infection or autoimmune disease, may result in false positive results in non-treponemal tests (26). This is one of the reasons why the non-treponemal assays always need to be confirmed using a treponemal assay (57, 58).

Treponemal assays, like chemiluminescent immunoassay (CLIA), are assays that detect antibodies to *T. pallidum* proteins and are easily implemented in high-throughput laboratories (59, 60). The disadvantage of this assay is that once the test becomes positive in a person it will remain positive in subsequent tests, making it impossible to discriminate between a persisting infection, a reinfection or an effectively treated infection (49).

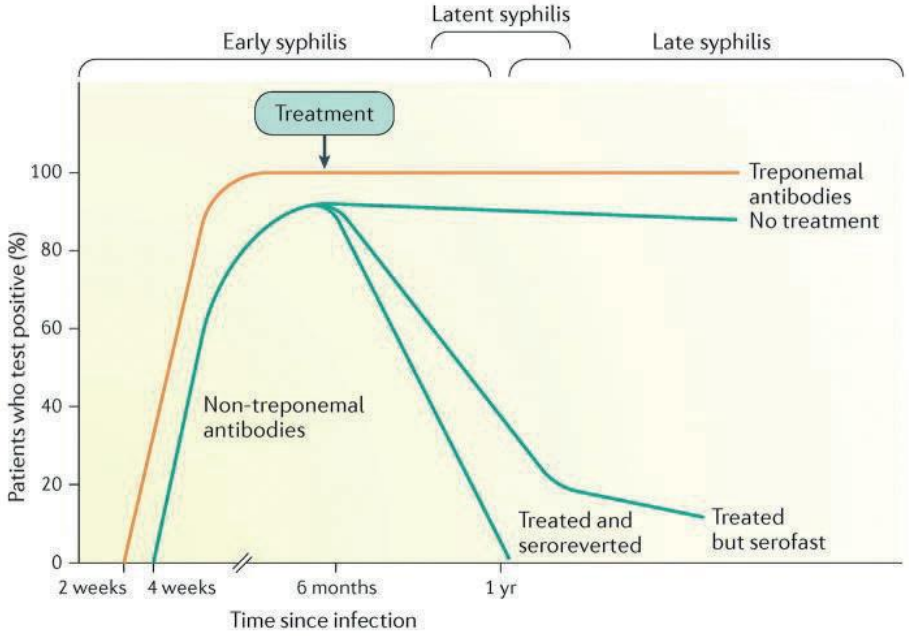

Figure 4 – Serological response in patients with syphilis. Treponemal antibodies appear first in patients soon after infection with *T. pallidum*. A few weeks later non-treponemal antibodies appear. After treatment treponemal antibodies remain, while non-treponemal antibodies decline. Figure from (10). Reproduced with permission from Springer Nature.

Diagnostics of clinical syphilis stages

At the public health service of Amsterdam, a CLIA is performed during screening of all clients without a known previous syphilis infection. After which, two confirmatory tests are performed; the non-treponemal RPR test and the immunoblot. The immunoblot is only performed if the CLIA value is equivocal or positive below 20. A previous analysis in our department showed that immunoblots were always positive in sera with a CLIA value >20 (unpublished data). If a client has already been positive in the CLIA before, only a RPR assay is done. In addition, suspected syphilitic ulcers are swabbed using a dry swab and tested for the presence of *T. pallidum* DNA using a *polA* targeted polymerase chain reaction (PCR) (53, 61). Dark-field microscopy is also performed provided that there is enough serous fluid in the ulcer.

The clinical syphilis stages are defined based on the presence of clinical symptoms and results of molecular and serological tests (31, 49). Primary syphilis is defined when an ulcer is present and the dark-field microscopy shows spirochetes or *T. pallidum* DNA is detected by PCR targeting the *polA* gene of *T. pallidum* (61). Secondary syphilis is diagnosed based on clinical manifestations such as a rash with or without lymphadenopathy, or mucosal lesions such as condylomata lata, in combination with a RPR titer of 1:4 or higher. Ulcers may also occur in secondary stage patients (26, 31). Early latent syphilis is defined in an asymptomatic patient based on a seroconversion of the CLIA with a positive immunoblot, a RPR titer of 1:32 or higher, or a 4-fold or higher rise in RPR titer. In a patient previously known to have treponemal

antibodies, the diagnosis of early latent syphilis is only based on RPR titers. Late latent syphilis is defined in asymptomatic patients without previous syphilis diagnosis or without previous serological data and a positive CLIA and a RPR titer lower than 1:32. Infectious syphilis stages consist of the primary, secondary and early latent syphilis stages (33).

The magic bullet and penicillin

The first effective antibiotic against syphilis was Arsphenamine, also known as Salvarsan, which was developed in 1906 in Paul Ehrlich's laboratory and introduced in 1910. Its anti-syphilitic effects were discovered by Sahachiro Hata in 1908. This drug was the first 'magic bullet' of Paul Ehrlich (62) and its discovery pioneered the modern pharmaceutical research by combining chemistry, biology and medicine for the development and discovery of targeted antimicrobial drugs. Due to the severe side effects a less toxic version was introduced in 1913, Neosalvarsan. However, both drugs had to be stored in sealed vials to prevent oxidation, which makes it difficult to handle.

Penicillin was discovered in 1928 by Alexander Fleming and used to treat syphilis from 1943 onward (63, 64). This caused a drastic decrease in the number of syphilis cases. Despite the presence of this effective antibiotic, with which to this day any syphilis infection can be successfully treated, syphilis remains steadily prevalent worldwide.

After 80 years of use, no antibiotic resistance has been reported against penicillin. However, episodic shortages are being reported worldwide, especially affecting lower and middle income countries (65). Benzathine penicillin is also used to treat congenital syphilis in order to prevent adverse pregnancies and to treat newborns with syphilis. A large amount of penicillin is necessary to combat congenital syphilis, especially in countries with less resources that carry the highest burden (66). Alternative treatment for congenital syphilis, suggested by the WHO, is erythromycin, azithromycin or ceftriaxone (67). For the treatment of other syphilis infections when penicillin cannot be used, doxycycline is the preferred second option, then ceftriaxone or, in special circumstances, azithromycin (43). However, macrolide resistance in *T. pallidum* is found widely.

Antibiotic resistance

T. pallidum antibiotic resistance against penicillin has not yet been identified. However, treatment failure and genotypic resistance has been reported widely for macrolides, which is another antibiotic that is frequently used to treat STIs (68). Macrolides such as azithromycin bind to the 23S rRNA locus. Macrolide resistance is on the rise in multiple bacterial STI causing pathogens (69). Mutations in the 23S rRNA loci of *T. pallidum* renders this antibiotic ineffective by a A2058 or A2059 single-nucleotide polymorphism (SNP) (70). These mutations are called macrolide resistance associated mutations (MRAM) (71) and are increasingly prevalent in *T. pallidum* over time (68, 72, 73). Some studies found that the MRAM prevalence in *T. pallidum*

derived from women or men who have sex with women (MSW) is higher than in MSM . In the Netherlands the MRAM prevalence is higher than 80% in all affected populations (73, 74).

Other STI causing pathogens, like *Neisseria gonorrhoeae* causing gonorrhea, also experience antibiotic pressure. *N. gonorrhoeae* has limited treatment options due to its capability to develop resistance against antibiotics (75). Ceftriaxone is the last resort antibiotic option to treat gonorrhea and resistance has already been reported (76, 77). Genomic studies focus on monitoring emerging strains that harbor antimicrobial resistance markers. Studies show increased spread and prevalence of azithromycin resistant *N. gonorrhoeae* strains which have an impact on dual therapy treatment, which was introduced to lower the antibiotic pressure and slow down resistance against the last resort antibiotic, ceftriaxone (78, 79).

MOLECULAR EPIDEMIOLOGY AND GENOMICS

Population dynamics of microorganisms can be monitored through molecular epidemiology. Strain typing is a powerful tool for determining the diversity of pathogens and epidemiology of infections.

The role of molecular epidemiology in public health

Molecular epidemiology is the addition of molecular strain techniques to epidemiological data. This allows for a more precise picture of pathogenic outbreaks, transmission networks, antimicrobial resistance monitoring and surveillance of the overall distribution of a microorganism and more (80, 81). At the start of the COVID-19 pandemic, molecular tools were implemented worldwide to have a close to real-time map of the circulating strains (82). One year later over 1 million SARS-CoV-2 sequences could be found on multiple public platforms (83). The high number of sequences together with epidemiological data allowed quick communication to governments about upcoming variants and allowed vaccines to be tailored accordingly. To put that in perspective, 25 years after the first sequenced *T. pallidum* genome, 1672 *T. pallidum* genomes have been uploaded into Genbank (accessed in Feb, 2023). Major events like a pandemic also have an impact on other infectious diseases, which can again be investigated by applying molecular tools (84).

T. pallidum

A complete genome of *T. pallidum* was sequenced in 1998 (85). However, only a few full genomes followed for a long time as whole genome sequencing of *T. pallidum* required *in vivo* propagation in rabbits. *T. pallidum* has a genome of 1.1 mega basepairs long. The *T. pallidum* genome is highly conserved. The genomes of *T. pallidum* and other subspecies, *endemicum* and *pertenue*, share over 99.7% identical genomes (86, 87). Therefore SNP level genomic analyses of *T. pallidum* are necessary. A large *T. pallidum* core-genome analysis

using *T. pallidum* samples from 23 countries showed very low and often zero SNP diversity of *T. pallidum* within and between countries, especially when sampling was high for those countries (88). *T. pallidum* genomes fall into two main lineages called SS14 and Nichols, of which SS14 contains the majority of typed isolates (6, 88-91). These lineages are separated with approximately 95 SNPs (88). For molecular typing techniques and especially for whole genome sequencing (WGS), a high bacterial load is required and therefore ulcer swab samples are mostly used in these studies (88, 92, 93). Several DNA target enrichment techniques have succeeded in investigating *T. pallidum* genomes (6, 88, 90, 94-96). Unfortunately, most clinical samples contain a low *T. pallidum* bacterial load as compared to the host DNA and performing WGS remain mostly unsuccessful (93). Other techniques, such as the use of host DNA removal techniques and selective whole genome amplification, are investigated to overcome this challenge (97).

T. pallidum also has variable genetic regions which have been the focus in research regarding molecular typing, virulence and immune evasion (94, 98-101). These analyses allow researchers to include more diverse sample types. *T. pallidum* DNA can often be found in multiple anatomical locations and sample types within patients, but the bacterial load is highest in ulcer swab samples, increasing the success of typing (28, 93, 101).

Molecular typing techniques

Using molecular typing techniques investigation on the geographical distribution and virulence of strains may be identified (99). In 1998, Pillay and colleagues from the Centre for Disease Control developed the first widely used molecular typing method for *T. pallidum*, called the CDC method, for which genetic regions *arp*, acidic repeat protein, and *tpr*, *T. pallidum* repeat gene subfamily II, were chosen (102). The *arp* gene encodes for acidic repeat protein, which contains repeats of 60 bp each, and was analyzed by counting the number of repeats on agarose gel. The *tpr* genes are multiple highly polymorphic genes (*tprE*, *tprG*, and *tprJ*) that were analyzed using a restriction enzyme, Mse1, digestive pattern. The CDC method was enhanced in 2010, by Marra et al., with the addition of the partial amplification and sanger sequencing of a genetic region of the *tp0548* gene (enhanced CDC)(99). Unfortunately, some reports suggested that the tpr and arp loci were too variable for this use as multiple variants were found from multiple samples within one patient (74, 103). Another highly polymorphic *tpr* gene, *tprK*, was found to be unsuited for sequencing with the goal of uncovering *T. pallidum* diversity between patients of a population (104).

In order to type *T. pallidum* with a higher resolution, Grillova et al. 2018 developed a multi-locus sequence typing (MLST) scheme (105). Generally, MLST methods use housekeeping genes of microorganisms to amplify, sequence and analyze them after which a specific sequence type (ST) can be determined (106). The MLST scheme by Grillova et al. 2018 is based on three genetic regions, *tp0136, tp0548* and *tp0705. Tp0136* and *tp0548* both encode

for outer membrane proteins while *tp0705* encodes for a penicillin-binding protein (105). Every SNP in the genetic regions in the MLST scheme refers to a different allelic variant, which together constitute an allelic profile or type. Using this method, sequence types can be analyzed, subspecies can be determined and corresponding lineages may be identified. A recent study, from the same research group that developed the enhanced CDC typing method, found no differences in the discriminatory power between the enhanced CDC and MLST methods (107). However, since the development of the *T. pallidum* MLST typing scheme more molecular typing studies applying this technique have been performed as it holds several advantages, including the ability to create a phylogenetic tree to analyze the SNP level variation between the allelic profiles that are found. Also, the addition of the pubMLST Bigsdb database for *T. pallidum* allows for convenient incorporation of important epidemiological data, data sharing and reuse of data for epidemiological analyses (108).

THIS THESIS

Aims

Molecular diagnostics and epidemiology are important topics for monitoring, understanding and surveillance of infectious diseases. The aim of this thesis is to contribute to these topics by investigating beyond current diagnostics and monitoring of circulating strains. In addition, this thesis aimed to contribute by increasing knowledge on the molecular diversity and spread of *T. pallidum* in different populations and within patients. Lastly, a study on *Neisseria gonorrhoeae* was included, which aimed to investigate the impact of the first COVID-19 lockdown on the molecular diversity of *Neisseria gonorrhoeae*. This chapter adds to the illustration of the potential of molecular epidemiological tools.

Outline

This thesis is divided into two parts; molecular diagnostics and molecular epidemiology. The first part consists two studies that each touch upon molecular diagnostics of STIs. **Chapter 2**, "Detection of *Treponema pallidum* DNA During Early Syphilis Stages in Peripheral Blood, Oropharynx, Ano-Rectum and Urine as a Proxy for Transmissibility" describes *T. pallidum* DNA detection by PCR in various sample types from different anatomical locations in patients with different early infectious syphilis stages. In the second chapter of this part, **Chapter 3, "**Timely diagnosis of incubating syphilis infections using *Treponema pallidum* Transcription Mediated Amplification assay", a research-use-only developed assay for fast and high-throughput detection of *T. pallidum* was clinically validated in a large-scale setting at the public health service of Amsterdam by adding this assay to the routine diagnostics for participants of the national PrEP program.

The second part called “molecular epidemiology” consists of five studies. In **Chapter 4 “**Molecular diversity of *Treponema pallidum* subspecies *pallidum* isolates in Amsterdam, the Netherlands” the molecular diversity of *T. pallidum* isolates is mapped in Amsterdam using the enhanced CDC typing method which includes a variety of molecular techniques for different polymorphic genes. In **Chapters 5, 6 and 7** the multi-locus sequence typing (MLST) method is used to look at the molecular diversity of *T. pallidum*. In **Chapter 5 “***Treponema pallidum* strains from different anatomical locations within patients per infectious syphilis stage” this molecular technique is applied on *T. pallidum* isolates from different anatomical locations and different sample types. **Chapter 6** “*Treponema pallidum* strains among women and men who have sex with women in Amsterdam, the Netherlands and Antwerp, Belgium between 2014 – 2020” describes the use of the technique to map circulating *T. pallidum* strains in a less well investigated population, being women and men who have sex with women, in Amsterdam, the Netherlands and Antwerp, Belgium. In **Chapter 7** “No bejel among Surinamese, Antillean and Dutch syphilis diagnosed patients in Amsterdam between 2006 – 2018 evidenced by multi-locus sequence typing of *Treponema pallidum* isolates”, *T. pallidum* subspecies were typed and identified in a retrospective selection of samples from patients with Surinamese, Antillean or Dutch ethnicity diagnosed with a syphilis infection over a time-period of 12 years. Lastly, **Chapter 8** describes the impact of the restriction measures during the COVID-19 pandemic in the first half of 2020 on the molecular diversity of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) by whole genome sequencing and MLST analyses of *N. gonorrhoeae* isolates from patients included from two time periods (before and during the first lockdown in the Netherlands).

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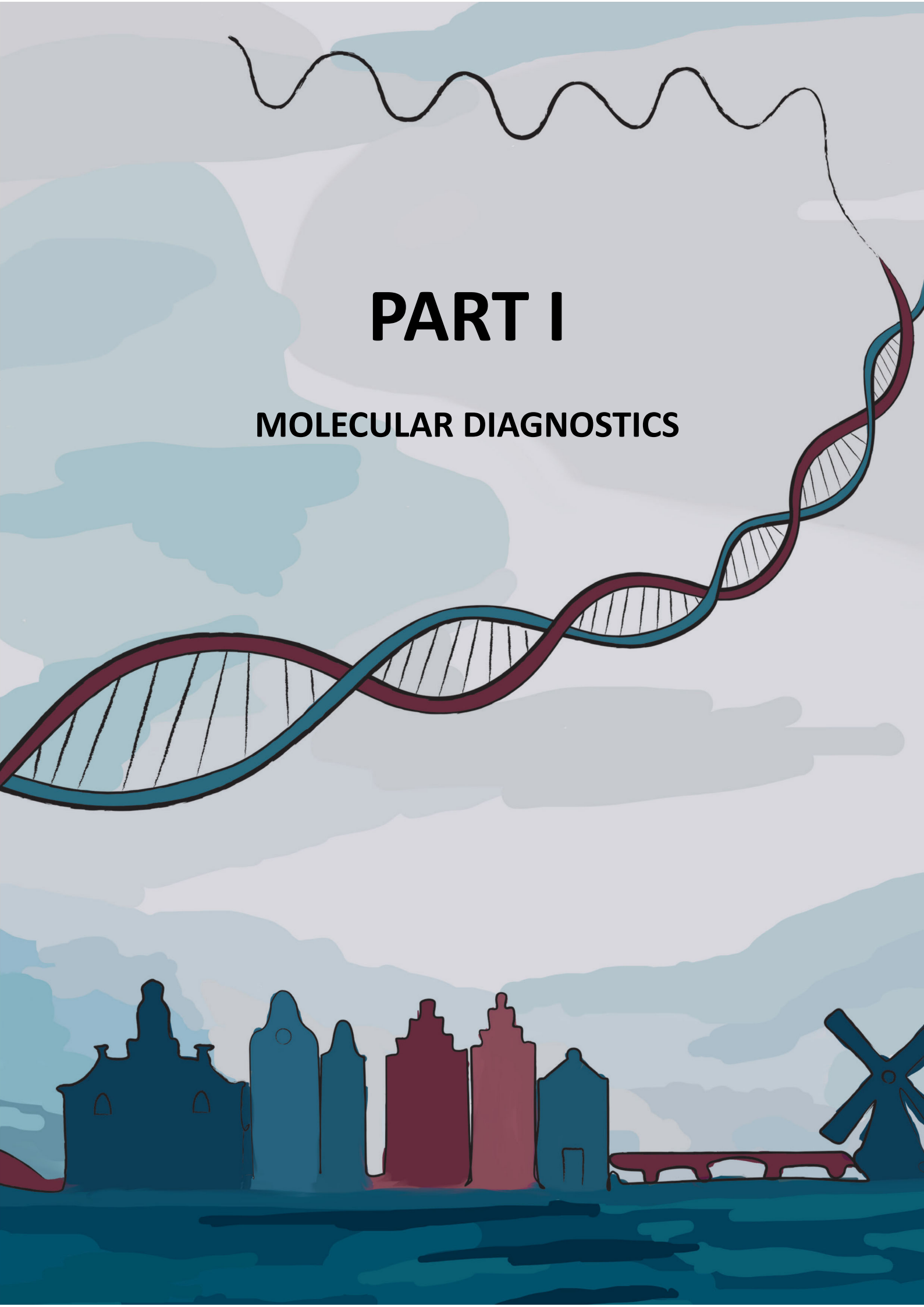

PART I

MOLECULAR DIAGNOSTICS

Chapter 2

Detection of *Treponema pallidum* DNA during early syphilis stages in peripheral blood, oropharynx, ano-rectum and urine as a proxy for transmissibility

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ABSTRACT

Background

Syphilis diagnosis may be challenging, especially in the asymptomatic and early clinical stages. We evaluated the presence of *Treponema pallidum* DNA (TP-DNA) in various sample types to elucidate transmissibility during various syphilis stages.

Methods

The study was conducted at the Amsterdam Centre for Sexual Health. We included adult men who have sex with men (MSM), who were suspected of having syphilis. The 2020 European guidelines definitions were followed for the diagnosis and staging of syphilis. Using a polymerase chain reaction (PCR) targeting the *polA* gene of *Treponema pallidum* (TP-PCR), we tested the following study samples on TP-DNA: peripheral blood, oropharyngeal swab, ano-rectal swab, and urine.

Results

From November 2018 to December 2019 we included 293 MSM. Seventy clients had primary syphilis, 73 secondary syphilis, 86 early latent syphilis, 14 late latent syphilis, 23 treated syphilis, and 27 had no syphilis. TP-DNA was detected in at least 1 study sample in 35/70 clients with primary syphilis (2/70 peripheral blood, 7/70 oropharynx, 13/70 ano-rectum, and 24/70 urine); in 62/73 clients with secondary syphilis (15/73 peripheral blood, 47/73 oropharynx, 37/73 ano-rectum, and 26/73 urine); and in 29/86 clients with early latent syphilis (5/86 peripheral blood, 21/86 oropharynx, 11/86 ano-rectum, and 6/86 urine). TP-DNA was not detected in clients with late latent syphilis or treated syphilis, nor in clients without syphilis.

Conclusions

TP-DNA was frequently detected in various sample types in the absence of lesions. This is in line with the high transmission rate of syphilis and opens diagnostic opportunities for early presymptomatic syphilis stages.

INTRODUCTION

Syphilis is a multistage sexually transmitted disease (STD) caused by the bacterium *Treponema pallidum* subspecies *pallidum* (TP) (1). Worldwide syphilis rates are on the rise (2). Like in many countries in the Western world, in the Netherlands syphilis is predominantly found in men who have sex with men (MSM), and especially in MSM living with HIV (3, 4).

Early syphilis is defined as the infectious stage within the first year after acquisition. Early syphilis can be subdivided in primary syphilis, characterized by the presence of a chancre, secondary syphilis, which is clinically polymorphous, and early latent syphilis, which is symptomless (5). After the first year, spirochetes can persist in untreated patients and cause late symptoms of syphilis. However, the spirochete burden at this late stage is considered to be very low and patients are regarded as not sexually infectious (6). Sexual syphilis acquisition is believed to occur via exposure to infectious genital lesions, but also via lesions in body orifices involved in sexual contact such as the anus and mouth. These lesions are considered highly infectious, with an efficiency of transmission estimated at approximately 30% per sexual act (7). The high incidence of early syphilis among MSM emphasizes the need to detect infected individuals as early as possible to prevent ongoing transmission.

The diagnosis of syphilis remains challenging. A combination of clinical findings, direct demonstration of TP by dark field microscopy or polymerase chain reaction (PCR) tests, or indirect serological tests form the basis for a syphilis diagnosis (8). However, in primary syphilis, serological tests may be negative due to the window period between transmission and seroconversion. Therefore, PCR tests for the detection of *T. pallidum* have been introduced as routine diagnostics in several medical microbiology laboratories worldwide (9-12), including in our public health laboratory in Amsterdam, the Netherlands (13, 14).

The sensitivity of *T. pallidum* PCR (TP-PCR) tests is high in primary syphilitic chancres and secondary stage condyloma lata, but low when used on samples from stage 2 roseoles and peripheral blood samples (9, 14-18). Positive TP-DNA results have also been seen in anal (19) and pharyngeal swabs in patients with primary ulcers on these locations (20, 21). In addition, TP-DNA has been found in blood and urine samples from patients with syphilis (22-26). A recent study performed in Australia showed that TP-DNA was frequently found in lesional samples, but less often in nonlesional samples (27) .

Although it is known that primary and secondary syphilis are highly infectious, it is not well understood how transmission to other patients occurs (28). So far, the extent of secondary spirochetal spread to distant mucosal locations has not been systematically evaluated in patients. The aim of this study is to evaluate the presence of TP-DNA in peripheral blood and body orifices such as the oropharynx, ano-rectum, and urethra (urine used as a proxy for urethra) in patients with early syphilis. This to improve syphilis diagnostic options and elucidate transmissibility during various syphilis stages.

METHODS

Study population and study design

The Center for Sexual Health of the Amsterdam Public Health Service, the Netherlands, is a low-threshold sexually transmitted infection (STI) clinic performing approximately 50,000 consultations annually. Consultations are at the client's own initiative, anonymous and free of charge. From November 2018 through December 2019 participants were recruited among clients aged 18 years and older visiting the STI clinic.

Clients were eligible for participation if they were MSM and (1) had signs and symptoms suggestive of primary syphilis or secondary syphilis, or (2) were diagnosed with early latent or late latent syphilis. Clients belonging to the first group were invited on their first visit (Supplement Figure 1). Patients belonging to the second group were invited at their follow-up clinic visit.

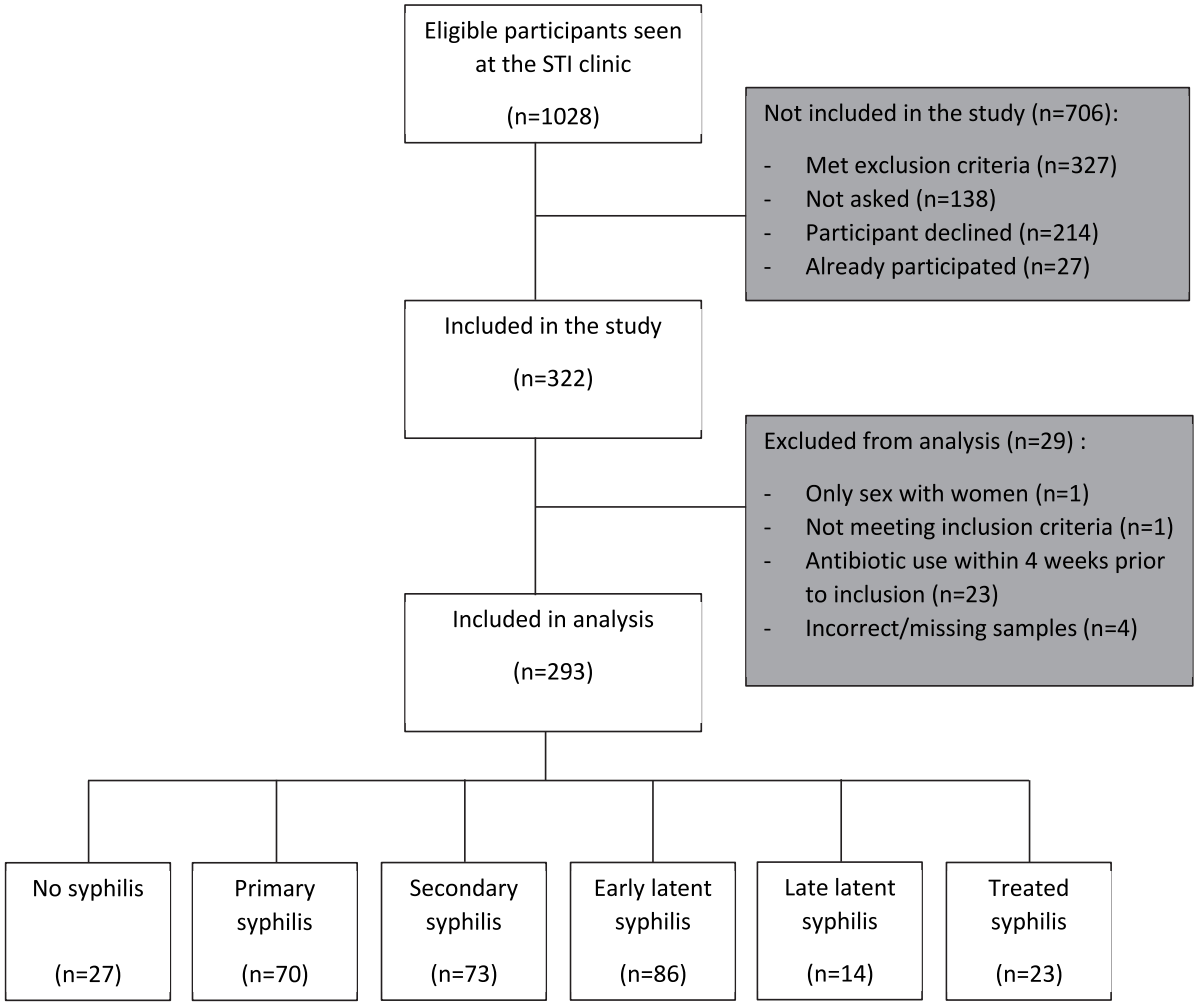

Figure 1 – Flowchart of the inclusion and exclusion of MSM included in the study, Amsterdam, the Netherlands, November 2018 – December 2019.
Abbreviations: MSM, men who have sex with men; STI, sexually transmitted infection.

Upon consent, we collected peripheral blood, client-obtained oropharyngeal and ano-rectal swab, and urine (as proxy for urethral mucosa) for TP-DNA analysis, in addition to the routine diagnostic samples, often including a genital ulcer swab. According to the 2020 European guideline for syphilis (29), participants were allocated into groups based on clinical signs and symptoms, and serological and routine molecular test results using the following algorithm:

1. Primary syphilis: oro- or ano-genital ulcerative disease and a positive dark field microscopy (DFM) or a positive PCR result of the ulcer swab.
2. Secondary syphilis: a rash with or without lymphadenopathy, or mucosal lesions such as condylomata lata, and a rapid plasma reagin (RPR) ≥ 1:4.
3. Early latent syphilis: no symptoms and a seroconversion of the chemoluminence immunoassay (CLIA), or a RPR ≥ 1:32, or a 4-fold or higher RPR titer rise.
4. Late latent syphilis/latent syphilis of unknown duration: no symptoms and no previous syphilis diagnosis and a positive CLIA and a RPR with a titer of <1:32.
5. Findings compatible with treated syphilis (hereafter: treated syphilis): history of previous diagnosis and treatment for syphilis, and not meeting criteria for groups 1 – 4.
6. No syphilis: signs and symptoms compatible with syphilis, but CLIA negative and not meeting criteria for groups 1 – 5.

In the event of a symptomatic participant with a negative initial DFM, negative PCR ulcer swabs and negative serology, but 1 or more positive PCR results in the study samples, such patients were followed up to exclude incubating syphilis. Follow-up would in that case be at 3, 6 and 12 weeks after inclusion with tests at each visit by PCR on peripheral blood, oropharyngeal swab, ano-rectal swab, urine and by serology.

Every client included in this study was examined by a physician for the staging of syphilis. The oral cavity was examined under direct visualization. External examination of the anus was done. If clinically indicated, proctoscopy was done.

Treponema pallidum diagnostics

In patients with suspected primary syphilis, DFM on ulcer exudate was performed. Moreover, an ulcer swab for DNA extraction and real-time PCR targeting the *polA* gene was routinely collected (13, 14). This TP-PCR was considered positive when the fluorescent signal was clearly visible and the Cycle threshold (Ct) was <36. For a Ct value between 36 and 40 the DNA extraction was repeated, and both the first and the second DNA extract were (re)tested in the TP-DNA assay. When the Ct value was ≤40, the result was positive for *Treponema pallidum* DNA detection. A Ct value >40 was considered a negative result.

Routine diagnostics at the Public Health Laboratory in Amsterdam include 3 routine serological tests: a treponemal antibody test (CLIA; Diasorin) used for screening and 2 confirmatory tests: the non-treponemal quantitative rapid plasma reagin (RPR) flocculation test (RPR-Nosticon

II; bioMérieux) and the immunoblot (Inno-LIA). The immunoblot was only done if the CLIA value was ≤30. A previous analysis in our department showed that immunoblots were always positive in serum samples with a CLIA value >30 (unpublished data). All serological tests were performed according to the specifications of the manufacturers.

DNA isolation and PCR testing

DNA was extracted from oropharyngeal and ano-rectal dry swab samples using isopropanol precipitation after which the DNA pellet was dissolved in 50 µL of T10 buffer (10 mM Tris-HCl, pH 8.0) (30). From March 2019, the DNA from the swabs was isolated using the MagNA Pure 24 (Roche Molecular Systems), according to the specifications of the manufacturers, after in-house validation to ensure fully comparable results.

DNA from urine and peripheral blood samples was extracted within 24 hours of collection. From the urine samples 1ml was centrifuged at 14000 rpm for 10 minutes. The residual fluid was removed and the pellet resuspended with 200µl PBS and 500 µL easyMAG Lysisbuffer (BioMerieux) containing 0.2% glycogen after which isopropanol precipitation was performed (31). DNA was extracted from peripheral blood using the QIAamp Blood Mini Kit following the protocol from the manufacturer (Qiagen).

All extracted DNA samples were tested using the TP-DNA PCR assay. Simultaneously, all samples were spiked with phocine herpesvirus 1 (PhHV) and tested as inhibition control in the PCR. PCR on DNA extracted from anal swabs frequently showed inhibition and was therefore always simultaneously tested in a 1:10 dilution.

Statistical analysis

We described categorical variables using count data and proportions. Continuous variables were described using their median and interquartile range (IQR). The Fisher exact test was used to compare proportions within categorical variables. The Wilcoxon rank-sum test was used to compare the distribution of continuous variables between groups. We estimated odds ratios (OR) and their 95% confidence intervals (CI) using univariable and multivariable logistic regression to assess associations between determinants and TP-DNA detection in at least 1 sample type. We carried out analyses using Stata (v15.1, StataCorp, College Station, TX, USA).

Ethical statement

This study was approved by the Medical Ethics Committee of the Amsterdam University Medical Center (NL66419.018.18, 2018_236).

RESULTS

Between November 2018 and December 2019, we included 322 participants in the study, of whom 29 participants did not meet the inclusion criteria (Figure 1). Thus 293 MSM were included in the analysis. Among these participants 27 (9%) had no syphilis, 70 (24%) were diagnosed with primary syphilis, 73 (25%) with secondary syphilis, 86 (29%) with early latent syphilis, 14 (5%) with late latent syphilis, and 23 (8%) with treated syphilis. There were no participants with incubating syphilis. Median age was 40 years (IQR 31-48) (Table 1). Of the 293 participants, 167 (57%) were born in the Netherlands. Among the 103 men living with HIV (35%), 93 (90%) used antiretroviral therapy. Of the 190 HIV-negative men, 27 (14%) had used pre-exposure prophylaxis (PrEP) in the preceding year. Thirty-one (11%) of the 293 participants had been notified for syphilis by a sexual partner.

For 35/70 (50%) participants with primary syphilis, TP-DNA was detected in at least 1 study sample: 2 (3%) peripheral blood, 7 (10%) pharyngeal, 13 (19%) anal, and 24 (34%) urine samples (Table 2, Figure 2). For 62/73 (85%) participants with secondary syphilis, TP-DNA was detected in at least 1 study sample: 15 (21%) peripheral blood, 47 (64%) pharyngeal, 37 (51%) anal and 26 (36%) urine samples. For 29/86 (34%) participants with early latent syphilis, TP-DNA was detected in at least 1 study sample: 5 (6%) peripheral blood , 21 (24%) pharyngeal, 11 (13%) anal and 6 (7%) urine samples. No TP-DNA was detected in participants with late latent syphilis or treated syphilis, nor those without syphilis.TP-DNA was detected in 2 or more sample types in 7 (10%) participants with primary syphilis, in 39 (53%) participants with secondary syphilis and in 9 (11%) participants with early latent syphilis (Supplementary Figure 2). Irrespective of syphilis stage, TP-DNA was less frequently detected in peripheral blood samples than in pharyngeal, anal or urine samples (Figure 2). In primary and early latent syphilis the median RPR titer in TP-DNA positive participants was higher than in TP-DNA negative participants, but this difference was significant only in participants with early latent syphilis (p=0.006) (Supplementary Figure 3).

Table 1 – Socio-demographic and clinical characteristics of male patients included in the Trepoli study (n=293) by syphilis stage, Amsterdam, the Netherlands, November 2018 – December 2019

	Syphilis stage											
	No syphilis (n=27)		**Primary (n=70)**		**Secondary (n=73)**		**Early latent (n=86)**		**Late latent (n=14)**		**Treated syphilis (n=23)**	
	n[1]	*%*[1]	*n*[1]	*%*[1]	*n*[1]	*%*[1]	*n*[1]	*%*[1]	*n*[1]	*%*[1]	*n*[1]	*%*[1]
Demographics												
Age (yrs)												
Median [IQR]	35	[27-43]	41	[33-50]	39	[33-48]	40	[30-52]	35	[26-42]	41	[35-48]
<35 years	13	48%	22	31%	24	33%	30	35%	7	50%	5	22%
35 – 44 years	8	30%	21	30%	25	34%	21	24%	6	43%	9	39%
≥45 years	6	22%	27	39%	24	33%	35	41%	1	7%	9	39%
Country of origin												
The Netherlands	16	59%	46	66%	36	50%	51	59%	5	36%	13	57%
Other	11	41%	24	34%	36	50%	35	41%	9	64%	10	43%
Education												
None/primary/secondary	2	8%	15	23%	16	27%	20	25%	4	3%	3	15%
College/university	24	92%	49	75%	41	68%	60	74%	8	67%	16	80%
Other	0	0%	1	2%	3	5%	1	1%	0	0%	1	5%
Sexual behaviour												
Gender of sex partners												
Men	26	96%	68	97%	73	100%	85	99%	13	93%	22	96%
Men and women	1	4%	2	3%	0	0%	1	1%	1	7%	1	4%
No. of sexual partners (6mo)[2]												
Median [IQR]	4	[3-15]	8	[4-20]	6	[4-15]	10	[5-20]	8	[4-12]	8	[5-15]
<5	14	52%	19	27%	23	32%	16	20%	5	36%	5	22%
5-9	4	15%	18	26%	18	25%	21	26%	2	14%	7	30%

10-14	2	7%	11	16%	11	15%	14	18%	4	29%	3	13%
≥15	7	26%	22	31%	21	29%	29	36%	3	21%	8	35%
Health and biometrics												
HIV status												
Negative	21	78%	52	74%	44	60%	49	57%	13	93%	11	48%
Positive	6	22%	18	26%	29	40%	37	43%	1	7%	12	52%
cART use[3]												
No	1	17%	0	0%	3	11%	2	6%	0	0%	0	0%
Yes	5	83%	18	100%	25	89%	33	94%	1	100%	11	100%
Most recent CD4 count (cells/µl)[3]												
<350	0	0%	0	0%	1	5%	0	0%	0	0%	0	0%
350-499	0	0%	0	0%	2	10%	1	3%	0	0%	0	0%
≥500	4	100%	14	100%	17	85%	29	97%	1	100%	8	100%
PrEP use[4]												
No	20	95%	44	85%	37	84%	42	86%	11	85%	9	82%
In the past 3 months	1	8%	8	15%	7	16%	6	12%	1	8%	2	18%
In the past 4-12 months	0	0%	0	0%	0	0%	1	2%	1	8%	0	0%
Chlamydia diagnosis[5]												
No	23	85%	65	93%	69	95%	86	100%	13	93%	19	83%
Yes	4	15%	6	7%	4	5%	0	0%	1	7%	4	17%
LGV diagnosis[5]												
No	24	89%	69	99%	73	100%	86	100%	14	100%	22	96%
Yes	3	11%	1	1%	0	0%	0	0%	0	0%	1	4%
Gonorrhea diagnosis[5]												
No	22	81%	64	91%	67	92%	84	98%	13	93%	19	83%
Yes	5	19%	6	9%	6	8%	2	2%	1	7%	4	17%

Table 1 – Continued

Herpes simplex diagnosis[5]												
No	21	78%	70	100%	72	99%	84	98%	14	100%	18	78%
Yes	6	22%	0	0%	1	1%	2	2%	0	0%	5	22%
Notified for syphilis												
No	23	85%	58	83%	62	85%	86	100%	14	100%	19	83%
Yes	4	15%	12	17%	11	15%	0	0%	0	0%	4	17%
RPR												
Median [IQR]	NA	NA	2	[0-8]	16	[16-32]	8	[4-32]	6	[0-8]	0	[0-1]
Negative	27	100%	21	30%	0	0%	10	12%	4	29%	17	74%
Low (1:1-1:4)	NA	NA	24	34%	4	5%	13	15%	3	21%	5	22%
Middle (1:8-1:16)	NA	NA	15	21%	38	52%	39	45%	7	50%	1	4%
High (1:32-1:128)	NA	NA	10	14%	31	42%	24	28%	0	0%	0	0%

Abbreviations: cART, combination antiretroviral therapy; HIV, human immunodeficiency virus; IQR, interquartile range; LGV, lymphogranuloma venereum; no., number; PrEP, pre-exposure prophylaxis; yrs, years; RPR, rapid plasma reagin

Data missing for: country of origin (n=1), education level (n=29), number of sexual partners (n=6), ART use (n=4), most recent CD4 count (n=26)

1. Unless otherwise stated
2. In the 6 months before the consultation
3. In patients with HIV
4. In HIV negative men only
5. At current visit

Table 2 – TP-DNA detection in Peripheral blood, throat and anal swabs and urine, by syphilis stage, in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	Syphilis stage											
	No syphilis (n=27)		**Primary (n=70)**		**Secondary (n=73)**		**Early latent (n=86)**		**Late syphilis (n=14)**		**Treated syphilis (n=23)**	
	n	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
Anatomical location												
Peripheral blood	0	0%	2	3%	15	21%	5	6%	0	0%	0	0%
Throat	0	0%	7	10%	47	64%	21	24%	0	0%	0	0%
Anus	0	0%	13	19%	37	51%	11	13%	0	0%	0	0%
Urine	0	0%	24	34%	26	36%	6	7%	0	0%	0	0%
Number of locations positive												
0	27	100%	35	50%	11	15%	57	66%	14	100%	23	100%
1	0	0%	28	40%	23	32%	20	23%	0	0%	0	0%
2	0	0%	4	6%	21	29%	6	7%	0	0%	0	0%
3	0	0%	2	3%	12	16%	1	1%	0	0%	0	0%
4	0	0%	1	1%	6	8%	2	2%	0	0%	0	0%
≥1 location positive	0	0%	35	50%	62	85%	29	34%	0	0%	0	0%

Abbreviations: MSM, men who have sex with men; No., number; TP-DNA, *Treponema pallidum* DNA.

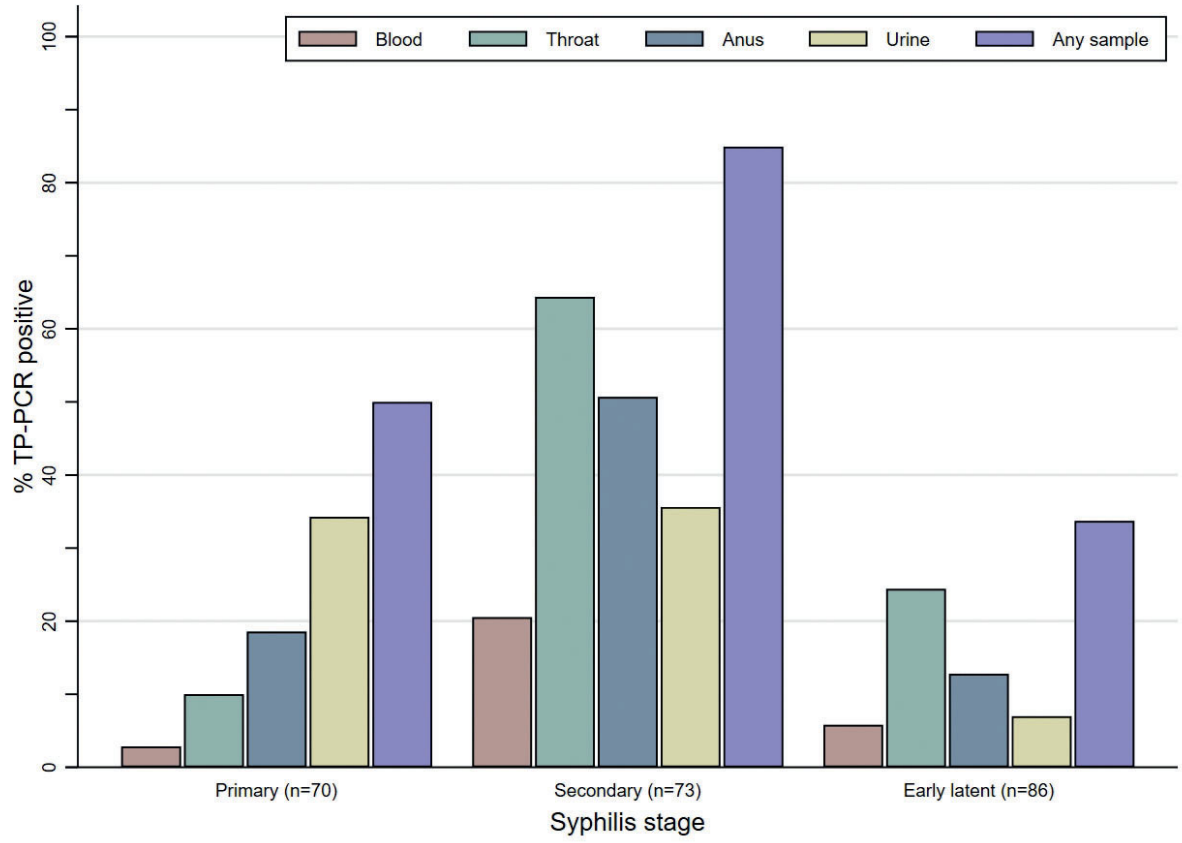

Figure 2 – TP-DNA detection in peripheral blood, throat and anal swabs, and urine in MSM with early syphilis, by stage, Amsterdam, the Netherlands, November 2018 – December 2019.
Abbreviations: MSM, men who have sex with men; TP-DNA, *Treponema pallidum* DNA; TP-PCR, *Treponema pallidum* polymerase chain reaction.

To assess whether TP-DNA positivity in oropharyngeal and ano-rectal swabs and in urine was due to TP-DNA from lesions in patients with ulcerative disease at the site of collection, we separately analyzed those with and without localized ulcerative disease. Among primary syphilis patients without a penile ulcer, 8/29 (28%) urine samples were TP-DNA positive; among secondary syphilis patients without a penile ulcer this was 22/68 (39%) (Table 3, Supplementary Figure 4). Among primary syphilis patients without an anal ulcer, 7/60 (12%) ano-rectal samples were TP-DNA positive; among secondary syphilis patients without an anal ulcer this was 32/68 (47%). Among primary syphilis patients without a pharyngeal ulcer, 6/70 (9%) pharyngeal samples were TP-DNA positive; among secondary syphilis patients without a pharyngeal ulcer this was 46/71 (65%).

Table 3 – Urine, anal and pharyngeal TP-PCR positivity in the presence or absence of ulcers in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	Penile ulcer present		**Penile ulcer absent**	
	N	**TP-DNA positive** *n (%)*	*N*	**TP-DNA positive** *n (%)*
Urine				
Primary syphilis	41	16 (39%)	29	8 (28%)
Secondary syphilis	5	4 (80%)	68	22 (33%)
Early latent syphilis	0	0 (0%)	86	6 (7%)
	Anal ulcer present		**Anal ulcer absent**	
	N	**TP-DNA positive** *n (%)*	*N*	**TP-DNA positive** *n (%)*
Anus				
Primary syphilis	10	6 (60%)	60	7 (12%)
Secondary syphilis	5	5 (100%)	68	32 (47%)
Early latent syphilis	1	0 (0%)	85	11 (13%)
	Pharyngeal ulcer present		**Pharyngeal ulcer absent**	
	N	**TP-DNA positive** *n (%)*	*N*	**TP-DNA positive** *n (%)*
Pharyngeal				
Primary syphilis	1	1 (100%)	69	6 (9%)
Secondary syphilis	2	1 (50%)	71	46 (65%)
Early latent syphilis	2	1 (50%)	84	20 (24%)

Abbreviations: MSM, men who have sex with men; No., number; TP-DNA, *Treponema pallidum* DNA.

We assessed the determinants of TP-DNA detection in one or more sample types. In the multivariable model secondary syphilis was significantly associated with TP-DNA positivity (adjusted odds ratio [aOR] 6.83 (95% CI 2.94-15.90, Table 4). Also, fewer sex partners and a negative HIV status were significantly associated with TP-DNA positivity. There were no significant associations between the Ct value (as a measure of sample TP-DNA content) of the various sample types and syphilis stage. The TP-DNA content of the peripheral blood samples however was markedly lower (higher Ct values) than those of the other sample types (Supplementary Table 1). We assessed study sample TP-DNA positivity by duration of symptoms (dichotomized into ≤2 weeks or >2 weeks) in the primary and secondary syphilis subgroups. In almost all combinations of stage and sample type TP-PCR positivity was higher in participants with symptoms lasting >2 weeks, reaching statistical significance for anal TP-PCR positivity in participants with primary syphilis (p=0.042) and throat TP-PCR positivity in participants with secondary syphilis (p=0.038, Supplementary Table 2).

Table 4 – Univariable and multivariable analysis of determinants of TP-DNA detection in one or more sample types in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	TP-DNA detected ≥1 sample type	**Univariable model**			**Multivariable model**		
	n/N (%)[1]	*OR*	*95% CI*	*p-value*	*aOR*	*95% CI*	*p-value*
Syphilis stage							
Primary	35/70 (50%)	REF		<0.001	REF		<0.001
Secondary	62/73 (85%)	5.64	2.55-12.47		6.83	2.94-15.90	
Early latent	29/86 (34%)	0.51	0.27-0.97		0.55	0.27-1.12	
Age, median [IQR][2]	38 [31-48]	0.81	0.03-1.36	0.084	0.86	0.65-1.14	0.304
Age							
<35 years	48/76 (63%)	REF		0.140			
35-44 years	37/67 (55%)	0.72	0.37-1.41				
≥45 years	41/86 (48%)	0.53	0.28-1.00				
Country of birth[3]							
The Netherlands	74/133 (56%)	REF		0.770	REF		0.246
Other	51/95 (54%)	0.92	0.54-1.57		0.68	0.36-1.31	
Number of sex partners, median [IQR] [4]	6 [4-15]	0.65	0.47-0.89	0.008	0.69	0.48-1.00	0.046
Number of sex partners							
<5	39/58 (67%)	REF		0.105			
5-9	32/57 (56%)	0.62	0.29-1.33				
10-14	19/36 (53%)	0.54	0.23-1.28				
≥15	33/72 (46%)	0.41	0.20-0.85				
HIV status							
Negative	88/145 (61%)	REF		0.024	REF		0.041
Positive	38/84 (45%)	0.54	0.31-0.92		0.50	0.25-0.98	
Any *C. trachomatis* infection							
No	120/220 (55%)	REF		0.468	REF		0.657
Yes	6/9 (67%)	1.67	0.41-6.83		1.46	0.27-7.85	
Any *N. gonorrhoeae* infection							
No	118/215 (55%)	REF		0.869	REF		0.433
Yes	8/14 (57%)	1.09	0.37-3.27		0.59	0.16-2.19	

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; HIV, human immunodeficiency virus; IQR, interquartile range ; MSM, men who have sex with men; No., number; OR, odds ratio; Ref, reference; TP-DNA, *Treponema pallidum* DNA.

1. Unless otherwise indicated
2. OR per 10 year increase in age
3. 1 missing
4. OR for each (log+1) increase in number of partners

DISCUSSION

In this study we detected TP-DNA in anal and pharyngeal swabs, in urine samples (as a proxy of urethral mucosa) and in peripheral blood in patients with early stages of syphilis (primary, secondary and early latent syphilis). These findings support the following assumptions about the nature of syphilis: (1) The presence of TP-DNA in mucosal tissue and body fluids of patients with early latent syphilis supports the notion that syphilis is transmissible in the absence of signs or symptoms. (2) In contrast, the absence of TP-DNA in patients with late latent syphilis is in line with the assumption that the late stages are considered not infectious (5). (3) The presence of TP-DNA in peripheral blood samples in all early syphilis stages is in line with the assumption that hematogenous dissemination of the infection occurs soon after inoculation (31, 32). (4) In comparison to patients with either primary or early latent syphilis, patients with secondary syphilis significantly more often had at least 1 or more TP-DNA positive study sample, significantly more often a TP-DNA positive oropharyngeal and peripheral blood sample. This finding supports the notion that secondary syphilis is the most contagious stage of syphilis. (5) Finally, the absence of TP-DNA in patients with treated syphilis and those without syphilis confirms the specificity of the test used to detect TP-DNA. (14) In none of the study participants an extra diagnosis of syphilis was found based on TP-DNA positivity of study samples, compared to routine diagnostics.

Several studies have shown that PCR can be used to detect TP-DNA in whole blood at various syphilis stages (15-17, 24, 25). Here, we used peripheral blood and confirmed TP-PCR positivity, although this was infrequently detected. The presence of TP-DNA in pharyngeal and anal samples in the presence of a lesion has been reported before (19-21). In this study, we also detected TP-DNA in the absence of a lesion. Oral and anal shedding could therefore play a role in the transmission of syphilis. We also detected TP-DNA in urine samples as was reported previously (23, 27). In both Dubourg et al. and Towns et al, urine samples were positive for TP-DNA in respectively 4/25 (16%) and 12/198 (6%) samples. Although urine samples are easy to obtain, they are hardly ever used for syphilis diagnosis, as judged by the low number of studies. Whether TP-DNA in urine originates from uro-genital tract lesions, such as penile ulcers, or from renal filtration or asymptomatic mucosal lesions is unknown.

Towns et al. (27) reported the presence of TP-DNA in both lesion and nonlesional samples. In their study, oral rinses, urine and anal swabs were also analyzed, as well as semen from some participants. However, they did not analyze peripheral blood samples. Similar to our study, they frequently detected TP-DNA in the oral (44%) and anal (29%) cavity of patients with secondary syphilis, although we found higher proportions (64% and 51%, respectively). We also more frequently detected TP-DNA in extra-lesional samples of participants with primary syphilis (50% in our study vs 30% by Towns et al.) and early latent syphilis (34% in our study vs 8% by Towns et al.). We could not confirm a relation between RPR titre and extra-lesional

TP-DNA as found by Towns et al. They did not include late latent and treated infections nor persons with a negative syphilis diagnosis. In addition, compared to Towns et al. we diagnosed fewer patients with extra-genital ulcers which may explain their lower positivity rates.

The strength of this study was that we systematically screened peripheral blood, oropharynx and ano-rectum samples, and urine which may play a role in the transmission of *Treponema pallidum*. This study also has several limitations. The detection of TP-DNA does not prove the presence of viable and potentially infectious bacteria. Future studies should investigate the viability of TP-DNA positive samples, especially found in the oral and anal cavity. Presently this has not been possible because TP culture of direct patient samples has proven to be very difficult. Furthermore, we found that fewer sex partners and a negative HIV status were associated with TP-DNA positivity. This counterintuitive finding could possibly be explained assuming that those with fewer sex partners and/or a negative HIV status seek care at an earlier (i.e. more infectious) stage and respond better to partner notification calls. In patients with early syphilis, we might have missed lesions. Proctoscopy was only done if clinically indicated (e.g. proctitis or intra-anal lesion) and during throat examination ulcers might have been overlooked. Furthermore, the number of patients without syphilis was small, so the demonstrated precision of the specificity of the test is limited. In addition, this study was conducted among an MSM population; it would be worthwhile to examine whether women may have a different pattern of TP-DNA shedding. Finally, we do not know if patients with 2 or more positive TP-DNA samples acquired this from the same sexual partner. However, we found intra-patient homogeneity in an investigation of the *Treponema pallidum* molecular variation within patients (33).

Performing TP- PCR on extra-genital samples could be useful as an additional diagnostic tool, especially to identify early incubating infections within the serological window phase. Blood samples are rarely PCR positive and thus may not be useful in routine screening. Urine samples, and anal and pharyngeal swabs are frequently routinely collected for STI screening for molecular diagnosis of *C. trachomatis* and *N. gonorrhoeae* infections. An additional TP-NAAT assay could possibly help to diagnose early syphilis infections in patients with non-reactive serological test results, who are nevertheless suspected for having syphilis (34). The diagnostic value of TP-DNA assays in routine STI screening of asymptomatic persons at increased risk for syphilis should be further evaluated in larger cohorts and other routine clinic settings.

In conclusion, we frequently found TP-DNA in peripheral blood, oropharyngeal and ano-rectal swabs, and urine collected from MSM diagnosed with early syphilis. Detection of TP-DNA in these sample types provide a better understanding of the infectious nature of early syphilis stages.

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Patient consent Patient consent was obtained for every individual participating in this study

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Conflicts of interest The authors declare that this study was funded by the Public Health Service of Amsterdam. A current study on syphilis at the Public Health Service has received research use only *Treponema pallidum* Transcription Mediated Assay kits by the company HOLOGIC.

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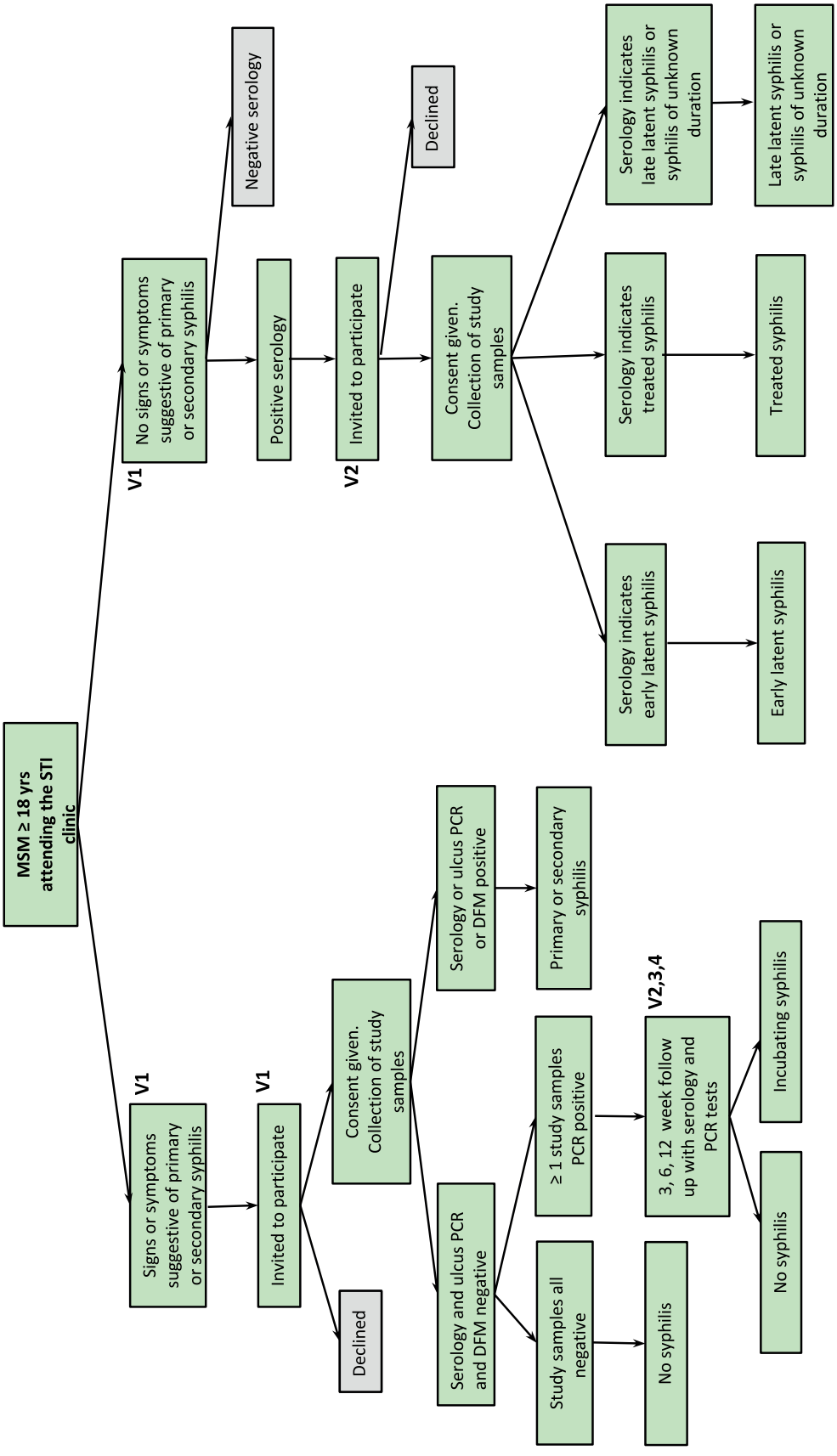
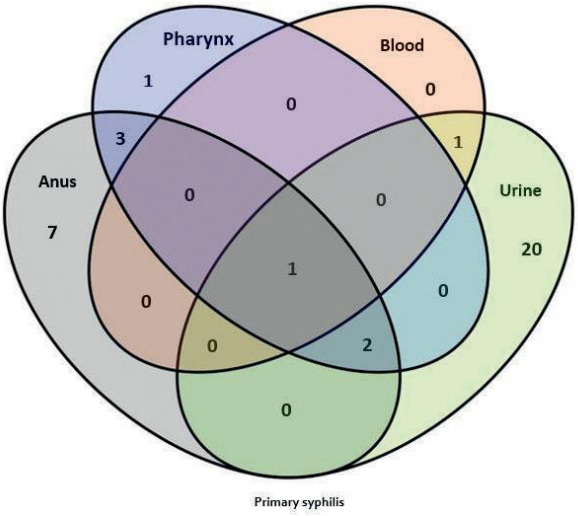
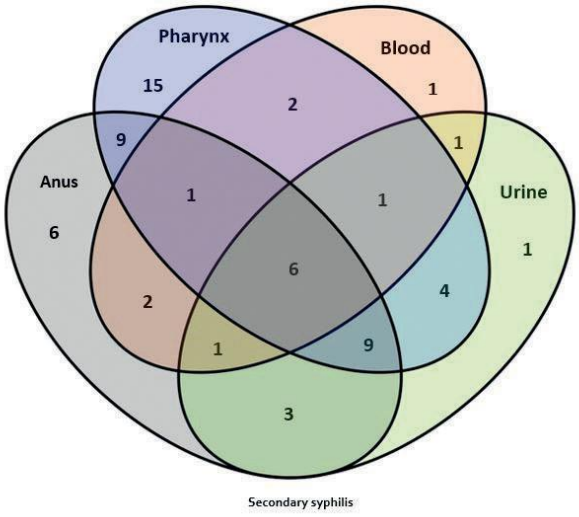
SUPPLEMENTARY DATA



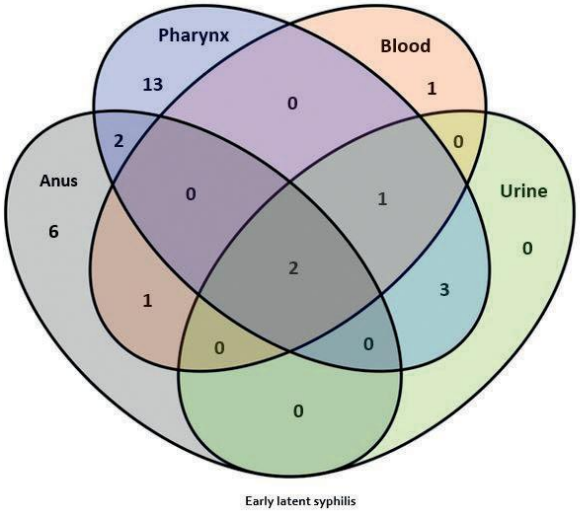
Supplementary Figure 1 – Study design flowchart. Six groups (no syphilis, primary syphilis, secondary syphilis, early latent syphilis, treated syphilis and late latent syphilis or syphilis of unknown duration) were created based on routine diagnostics. DFM: Dark-field microscopy;V: visit.



Primary syphilis

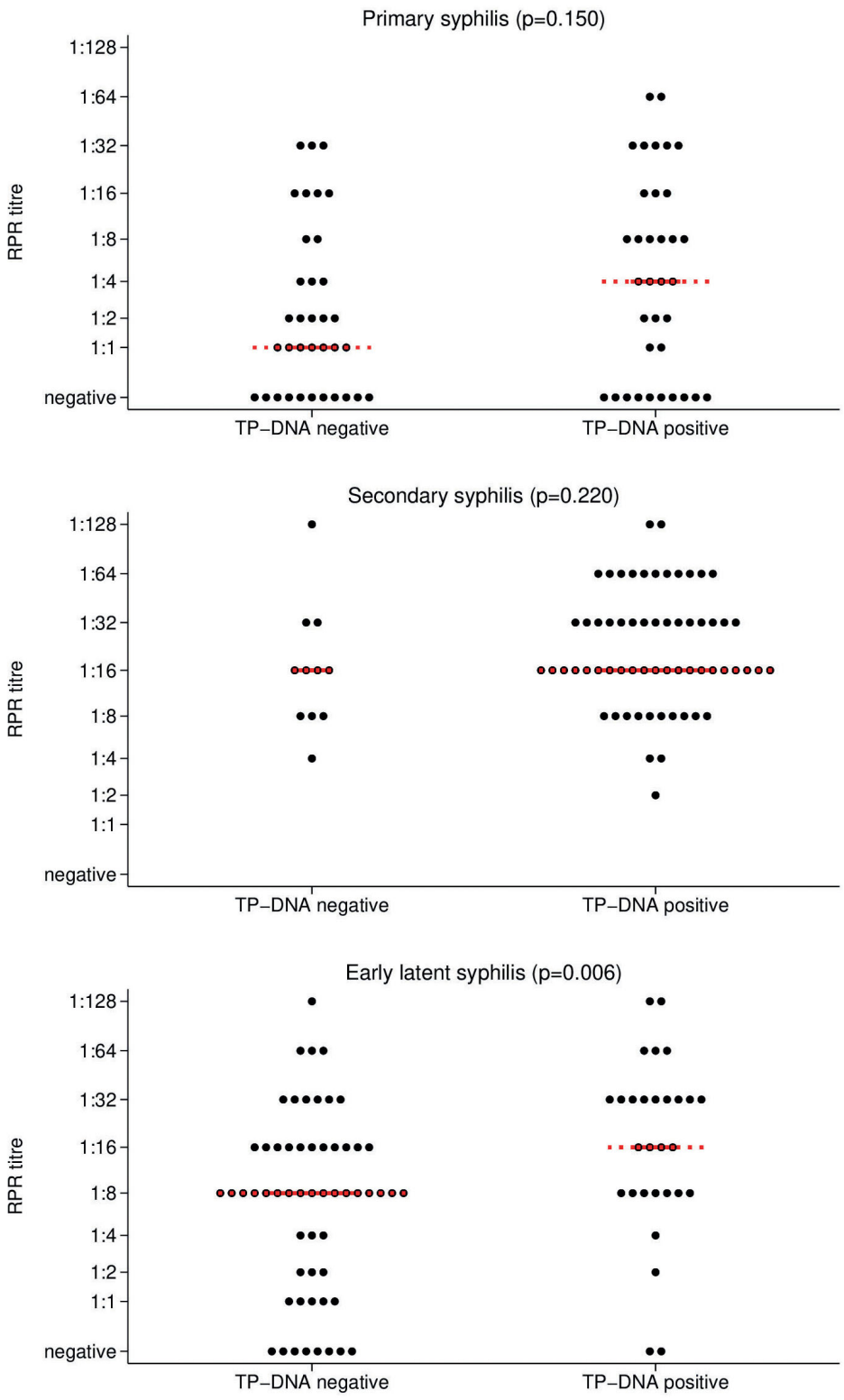


Secondary syphilis

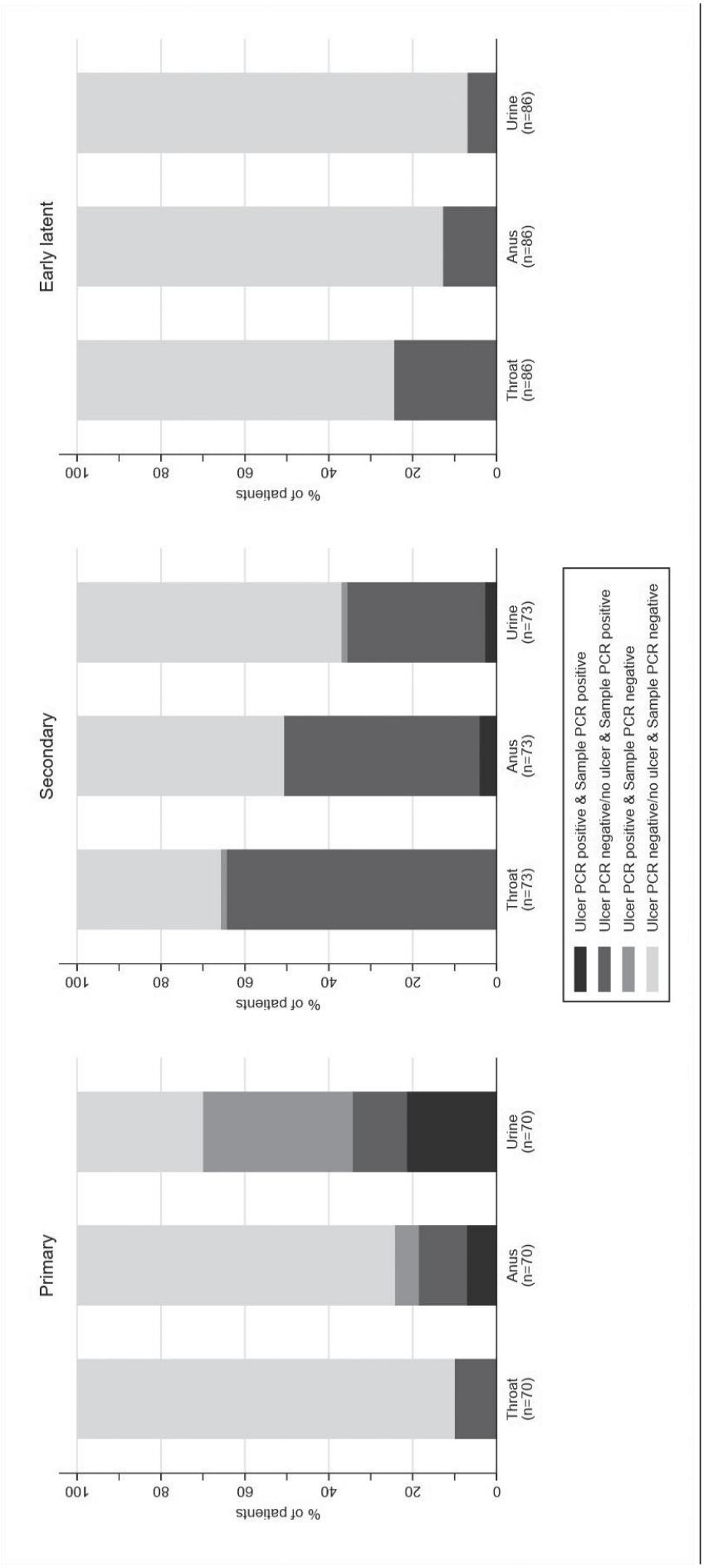


Early latent syphilis

Supplementary Figure 2 – Simultaneous detection of TP-DNA in various sample types ,by syphilis stage, in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019



Supplementary Figure 3 – Non-treponemal antibody titer and the presence of non-lesional TP-DNA in MSM with early syphilis, by syphilis stage, Amsterdam, the Netherlands, November 2018 – December 2019
Red line indicates median



Supplementary Figure 4 – TP-PCR positivity in the presence or absence of ulcers in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

Supplementary Table 1 – Ct values in plasma, throat and anal swabs, and urine, by syphilis stage in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	Peripheral blood	**Throat**	**Anus**	**Urine**
	Median [IQR]	*Median [IQR]*	*Median [IQR]*	*Median [IQR]*
Primary	35 [34-36]	36 [27-37]	30 [25-35]	32 [30-36]
Secondary	36 [35-37]	34 [31-36]	33 [29-36]	34 [33-37]
Early latent	36 [35-37]	34 [31-36]	32 [29-36]	32 [32-35]

Abbreviations: IQR, interquartile range

Supplementary Table 2 – Association between duration of symptoms and TP DNA positivity by syphilis stage per sample type in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	Peripheral blood TP DNA			Throat TP DNA			Anus TP DNA			Urine TP DNA		
	Negative	**Positive**		**Negative**	**Positive**		**Negative**	**Positive**		**Negative**	**Positive**	
	n (%)	*n (%)*	*p-value*[1]	*n (%)*	*n (%)*	*p-value*[1]	*n (%)*	*n (%)*	*p-value*[1]	*n (%)*	*n (%)*	*p-value*[1]
Primary syphilis (n=51)												
Duration of symptoms			1.000			0.111			0.042			0.694
≤2 weeks	42 (98%)	1 (2%)		41 (95%)	2 (5%)		40 (93%)	3 (7%)		30 (70%)	13 (30%)	
>2 weeks	8 (100%)	0 (0%)		6 (75%)	2 (25%)		5 (63%)	3 (38%)		5 (63%)	3 (38%)	
Secondary syphilis (n=40)												
Duration of symptoms			1.000			0.038			0.731			0.451
≤2 weeks	25 (83%)	5 (17%)		11 (37%)	19 (63%)		17 (57%)	13 (43%)		22 (73%)	8 (27%)	
>2 weeks	8 (80%)	2 (20%)		0 (0%)	10 (100%)		5 (50%)	5 (50%)		6 (60%)	4 (40%)	

p-value estimated using the Fisher's exact test

Data missing for: n= 19 patients with primary syphilis and n=33 patients with secondary syphilis

Chapter 3

Timely diagnosis of incubating syphilis infections using *Treponema pallidum* Transcription Mediated Amplification assay

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Submitted manuscript

SUMMARY

The addition of an investigational *Treponema pallidum* Transcription Mediated Amplification assay to routine syphilis diagnostics identified 7% additional syphilis infections among participants of the national pre-exposure prophylaxis program in Amsterdam who are at increased risk of acquiring STI.

ABSTRACT

Syphilis is a complex multistage sexually transmitted infection (STI) caused by the bacterium *Treponema pallidum* subspecies *pallidum* (TP). New diagnostic tools are needed to minimize transmission. This study aimed to assess the additional value of an investigational Transcription Mediated Amplification test for TP (TP-TMA) to routine diagnostics.

Between September 2021 and August 2022, visits of all participants of the national PrEP program at the sexual health center (SHC) in Amsterdam were included. Anal, pharyngeal, vaginal and urine samples collected for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* screening, were additionally tested with the TP-TMA assay based on detection of 23S rRNA of TP.

In total, 9974 SHC visits from 3283 participants were included. There were 191 infectious syphilis cases diagnosed; 26 (14%) primary syphilis, 54 (29%) secondary syphilis and 111 (58%) early latent syphilis. In 79/191 (41%) of these syphilis cases at least one sample was TP-TMA positive. For 16 participants, the positive TP-TMA result was not concordant with routine diagnostics. Of those, two participants were treated for syphilis within a week before the visit. Eight participants were treated for a syphilis notification at the visit or for another STI. Five participants were diagnosed with syphilis in the following visit and one participant was loss to follow-up.

Adding the TP-TMA assay to routine diagnostics, we identified 14/191 (7%) additional syphilis infections among participants of the national PrEP program. The TP-TMA assay is a useful diagnostic tool to increase syphilis case finding and thus limit the transmission of syphilis.

INTRODUCTION

Syphilis is a complex multistage sexually transmitted infection (STI) caused by the bacterium *Treponema pallidum* subspecies *pallidum* (TP). Intensive efforts to decrease the incidence of syphilis have not been successful. In the Netherlands, like in other high-income countries, syphilis rates are highest among men who have sex with men (MSM), accounting for 96% of the syphilis cases in 2021 (1).

Diagnostic tools for syphilis mainly rely on serological methods. However, seroconversion after infection may take up to 90 days (2). In this incubating stage of the infection, the client is infectious with negative serological results, resulting in false negative syphilis diagnosis. As a consequence, undiagnosed and untreated client may become sources of on-going transmission. Some laboratories include PCR testing on syphilis suspected lesions (3, 4). However, in asymptomatic or pre-symptomatic participants, external lesions may not present and internal (e.g. pharynx or anal) lesions may be missed (5, 6). An asymptomatic infection is also increasingly common with subsequent reinfection of *T. pallidum* (7, 8). Infectious syphilis stages include primary, secondary and early latent syphilis. These are the stages in which TP can also be detected using nucleic acid amplification assays, such as a PCR, in non-lesion sites (5, 9-11).

In September 2019 the national pre-exposure prophylaxis (PrEP) program started in Amsterdam (12). This is a program to offer PrEP to individuals, mostly MSM, who are at increased risk of acquiring HIV. Participants are routinely screened for STIs every 3 months at the sexual health center (SHC) in Amsterdam. The main current screening method to diagnose early syphilis is based on serological tests. In addition, an in-house developed PCR targeting the *polA* gene of TP is performed on swab specimens of syphilis suspected lesions and on genital ulcer swabs to diagnose syphilis (13).

Syphilis transmission may be minimized by shortening the time between infection and diagnosis, but new diagnostic tools are needed to achieve this. A research use only (RUO) transcription-mediated amplification (TMA) assay has been developed targeting the 23S rRNA of TP (Hologic, Inc.)(14). Recently, a cross-sectional study from the USA was performed to evaluate the strategy of combining the TP-TMA assay on pharyngeal and anal swab samples with serological assays to improve the diagnostic sensitivity of incubating syphilis and this study showed promising results (14). In addition, the assay has been shown to be analytically sensitive and specific (15).

The aim of this study was to evaluate the additive value of TP-TMA testing of mucosal orifices and urine to current routine diagnostics to improve the detection of incubating syphilis infections in a population of PrEP users with an increased frequency of syphilis infections.

METHODS

Sample selection

Between September 14th 2021 and August 1st 2022, all participants of the national PrEP program at the SHC of Amsterdam who visited the SHC for the three-monthly STI screening were included. Also, in-between visits, scheduled because of symptoms, were included. The ongoing national PrEP program included participants who are at an increased risk of acquiring HIV and who are 16 years or older (12).

Remnant volumes of anal swab, pharyngeal swab, vaginal swab and urine samples, collected in Aptima tubes for *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) screening, were tested in the RUO TP-TMA assay on the Panther system according to the manufacturer's protocol (Hologic, San Diego, US). Participants who had a discrepant positive TP-TMA sample not confirmed with serological, molecular or clinical routine syphilis diagnostics were asked to return to the clinic within 3 to 4 weeks for additional syphilis screening to confirm the syphilis diagnosis.

DNA isolation and in-house PCR

Retrospectively, all samples with a positive TP-TMA test were also tested by PCR. First, DNA was extracted from the Aptima tubes using the MagNA Pure 24, according to the specifications of the manufacturers (Roche Molecular Systems). The in-house validated real-time PCR targeting the *polA* gene was performed to detect TP DNA as previously described (10, 13). All samples were spiked with phocine herpesvirus 1 after DNA extraction and tested as inhibition control. If inhibited, DNA from the sample was re-isolated and retested in parallel with a 1:5 dilution of the extracted DNA. A RotorGene Q thermocycler was used (Qiagen, Hilden, Germany) for the PCR with a standardized fluorescence threshold of 0.04 for determination of cycle threshold (Ct) values.

Molecular and serological tests in routine diagnostics

The treponemal test, chemoluminescence immunoassay (CLIA) (Diasorin) was performed for screening of all participants without a known previous syphilis infection after which two confirmatory tests followed; the non-treponemal quantitative rapid plasma reagin (RPR) flocculation test (RPR-Nosticon II; bioMérieux) and the immunoblot (Inno-LIA; FUJIREBIO). The immunoblot was done if the CLIA index value was between 0.9 and 20, any value below 0.9 was regarded as a negative result (as per manufacturer's protocol). A previous analysis in our department showed that immunoblots were always positive in sera with a CLIA index value >20 (unpublished data). All serological tests were performed according to the specifications of the manufacturers.

Suspected syphilitic ulcers were swabbed using a Copan dry swab and, after DNA extraction, tested for TP DNA using the *polA* targeted PCR as described (13). In addition, dark-field microscopy was performed if there was enough serous fluid in the ulcer.

Clinical syphilis stage diagnoses

Clinical syphilis stages were defined based on presence of clinical symptoms and laboratory tests. Primary syphilis was diagnosed in case of a positive dark-field microscopy test or a positive *polA* targeted TP PCR on an ulcer swab. Secondary syphilis was diagnosed based on typical clinical manifestations, such as a rash with or without lymphadenopathy, the presence of mucosal lesions (e.g. condylomata lata), in combination with a ≥1:4 RPR titer. An early latent syphilis diagnosis was based on a seroconversion of the CLIA, with a positive immunoblot in case of an index value of ≤20 in the CLIA, in an asymptomatic participant. Early latent syphilis was also diagnosed in asymptomatic participants with a RPR ≥1:32 or a 4-fold or higher RPR titer rise. Late latent syphilis was diagnosed in participants without symptoms and no previous syphilis diagnosis and a positive CLIA and a RPR titer <1:32. Primary, secondary and early latent stage syphilis were considered infectious stages.

Data collection and analysis

Participant clinical data was routinely collected at the SHC in Amsterdam. At the end of the study the data was extracted and anonymized by an independent data manager. This included variables on sex, age, ethinicity, education, sexual behavior in the last 6 months, number of sex partners, STI diagnoses, and known syphilis contact.

Ethical statement

This study protocol was reviewed and the need for consent was waived by the Medical Ethical Committee of the Amsterdam University Medical Centers in the Netherlands (W21_331 #21.366). An opt-out system is used at the Public Health Service of Amsterdam to assure that if participants object to having their samples used for research that these are destroyed.

RESULTS

Participant characteristics

Between September 14th 2021 and August 1st 2022, there were 9974 SHC visits from 3283 participants of the national PrEP program. The number of visits for each participant varied, with over two thirds of the participants (69%) visiting the SHC 3 times or more in the 10.5 month study period (**Table 1**). Participant characteristics of the most recent visit are summarized in **Table 1**. The median age of the participants was 34 years. Of the 3283 participants, 3113 (95%) were men, of whom 3005 (97%) were MSM, 162 (5%) were transgender persons, of whom 131 (81%) had sex exclusively with men. There were 8 (<1%) women included, of whom 5 had sex with both men and women. Participants were of diverse ethnicities with 42% being Dutch and one third being from outside of Europe. Two thirds of the participants reported 5 or more sex-partners in the past 6 months.

Table 1 – Demographic characteristics of most recent visit of 3283 participants in the national PrEP program between September 14th 2021 and August 1st 2022 at the Sexual Health Centre of Amsterdam.

		#participants (n=3283)	%participants
Sex			
	Male	3113	94.8%
	Transgender	162	4.9%
	Female	8	0.2%
Sexual orientation			
	MSM	3005	91.5%
	MSMW	107	3.3%
	MSW	1	0.0%
	Transgender M	162	4.9%
	WSM	3	0.1%
	WSMW	5	0.2%
Number of sexpartners in the last 6 months			
	0- 2	473	14.4%
	3- 4	570	17.4%
	5- 9	777	23.7%
	10- 24	937	28.5%
	25- 49	273	8.3%
	≥50	237	7.2%
	Unknown	16	0.5%

Age			
	16-25	471	14.3%
	26-35	1312	40.0%
	36-45	775	23.6%
	46-55	449	13.7%
	>55	276	8.4%
Ethnicity			
	Netherlands	1384	42.2%
	Europe outside of the Netherlands	640	19.5%
	Asia	458	14.0%
	Africa	125	3.8%
	Central and South America	348	10.6%
	Surinam and Dutch Antilles	147	4.5%
	Turkey	73	2.2%
	Other	99	3.0%
	Unknown	9	0.3%
Number of visits per participant			
	1	504	15.4%
	2	518	15.8%
	3	1070	32.6%
	4	870	26.5%
	>5	321	9.8%

MSM: men who have sex with men, MSMW: men who have sex with men and women, MSW: men who have sex with women, WSM: women who have sex with men, WSMW: women who have sex with men and women

Syphilis diagnostics and TP-TMA results

In total 191 infectious syphilis cases were diagnosed during the study period using standard clinic procedures as defined in Methods. There were 26 (14%) primary syphilis, 54 (29%) secondary syphilis and 111 (58%) early latent syphilis diagnoses. In addition, 11 participants were diagnosed with late latent syphilis. TP TMA results were negative in 112/191 (59%) visits in which infectious syphilis was diagnosed (**Table 2**). For 79 of the 191 (41%) visits, at least one sample was positive with the TP-TMA assay in combination with an infectious syphilis diagnosis. In participants with late latent syphilis, none of the samples were TP-TMA positive. The highest proportion of TP-TMA positive samples derived from visits from participants with a secondary syphilis diagnosis (67%) (**Table 2**).

In 95/9974 (1%) visits the TP-TMA was positive in one or more samples. From these 95 visits, 79 (83%) were visits in which the participants were diagnosed with infectious syphilis in the routine diagnostics. The other 16 (17%) visits were from participants who were negative by routine syphilis diagnostics, but had a positive TP-TMA result for one sample (**Table 4**).

Table 2 – Overview of the number of visits by syphilis stage (as diagnosed by routine diagnostics) and by number of TP-TMA positive samples per visit.

Syphilis diagnosis by routine diagnostics	Number of TP-TMA positive samples per visit				TP-TMA positivity rate per syphilis stage
	0	1	2	3	n/N (%)
Primary	20	5	0	1	6/26 (23)
Secondary	18	26	8	2	36/54 (67)
Early latent	74	28	8	1	37/111 (33)
Late latent	11	0	0	0	0/11 (0)
Negative syphilis diagnosis	9756	16	0	0	16/9772 (0.2)

In total, 29742 TP-TMA assays were performed on 9909 anal swabs, 9923 pharyngeal swabs, 9858 urine samples and 52 vaginal swabs from 9974 visits of the 3283 included participants. The TP-TMA assay was positive in 119 samples; 72 (0.7%) anal swabs, 38 (0.4%) pharyngeal swabs and 9 (0.1%) urine samples. None of the vaginal swabs were positive.

Confirmatory TP PCR on TP-TMA positive samples

Of all 119 positive TP-TMA samples, 117 could be retrieved for confirmatory PCR and 98 (82%) were confirmed with a positive TP PCR result (**Table 3**). Anal and pharyngeal swab samples had similar positivity rates in the TP PCR with 58/72 (80%) and 34/38 (89%) of the anal and pharyngeal swabs being positive, respectively. 6/9 (67%) urine samples were confirmed by TP PCR. The mean cycling threshold (Ct) value was 31.3 ranging from 21.6 to 37.9. The 19 PCR negative samples had no signal after 45 PCR cycles. Of the 19 PCR negative samples with a positive TP-TMA result, 5 originated from participants without a syphilis diagnosis (**Table 4**), of whom 3 were notified as a contact of an infectious syphilis case.

Table 3 – Overview of confirmatory PCR results on 119 TP-TMA positive samples from 95 participants

TP-TMA samples	PCR result			
	Positive (%)	Negative (%)	Unavailable (%)	Total
Anus	58 (80)	12 (17)	2 (3)	**72**
Pharyngeal	34 (89)	4 (11)	0	**38**
Urine	6 (67)	3 (33)	0	**9**
Total	**98 (82)**	**19 (16)**	**2 (2)**	**119**

Evaluation of additional syphilis infections detected by TP-TMA

There were 16 participants with a positive TP-TMA result which was not concordant with routine diagnostics (**Table 2** and 3). The TP-TMA results were confirmed by PCR in 10 of these participants. Four of the 16 participants had a seroconversion in the CLIA at the visit at which the TP-TMA samples were obtained (participants 1, 9, 12 and 16, **Table 4**). The confirmatory immunoblot was negative in 3 samples with an index value of ≤20 in the CLIA.

In 5/16 participants, the TP-TMA result was positive and the syphilis diagnosis could be confirmed at a follow-up visit (**Table 4**). One participant presented with an ulcer at follow-up and was diagnosed with primary syphilis (participant 3). Participants 7 and 13 presented with a macular rash on the arms and legs and were diagnosed with secondary syphilis. Participant 12 seroconverted at the follow-up visit and remained asymptomatic and was diagnosed with early latent syphilis. Participant 16 reported in-between treatment for syphilis by another physician and showed a seroconversion in the follow-up visit and was defined as treated syphilis in line with the performed serological assays. No additional data was available on the treatment date of the undetermined syphilis stage.

In 10/16 participants, the TP-TMA result was positive, but the syphilis diagnosis could not be confirmed at a follow-up visit. All 10 had used antibiotics effective against *T. pallidum* shortly before or after the visit at which the TMA samples were obtained. It is therefore likely that the serologic result was decapitated, and a probable syphilis diagnosis can be assumed. Two participants (participants 5 and 9) of whom the anal samples tested positive with the TP-TMA assay visited their general practitioner within a week before the SHC visit and were treated for a syphilis infection. With this prior knowledge, these participants did not receive a syphilis diagnosis. However, participant 9 would have had a syphilis diagnosis based on the seroconversion. Three participants had been treated because of a syphilis notification, as per protocol of the SHC in Amsterdam (**Table 4**). Five other participants were treated for infections with *Chlamydia trachomatis*, *Neisseria gonorrhoeae* or both after the visit during which the TP-TMA samples were collected. None of these 8 participants who received an antibiotic treatment at our clinic got a syphilis diagnosis in their next visit. Data from follow-up visits later than the envisioned 4 weeks were available for most participants.

One participant with a TP-TMA positive result was lost to follow-up, but had a confirmed TP PCR of the TP-TMA positive sample supporting the possible syphilis diagnosis (**Table 4**).

Table 4 – Overview of 16 TP-TMA and routine diagnostic discordant cases among national PrEP participants between the September 14th, 2021 and the August 1st, 2022 at the SHC of Amsterdam, the Netherlands.

Participant	TP-TMA pos samples			Confirmatory TP PCR[a]	Serological results			Co-infections		Official notification	Treatment within the week before or at visit	Clinical follow-up
ID	Anal swab	Pharyngeal swab	Urine sample	Ct value	CLIA	IB	RPR	CT	NG			(# weeks between visit and follow-up)
3	1	0	0	nd	neg		nd	0	0			Pos 1st stage (4)
7	1	0	0	36.33	pp		neg	0	0			Pos 2nd stage (5)
12	0	0	1	34.77	3.91	neg	neg	0	0			Pos early latent stage (3)
13	1	0	0	32.96	neg		nd	0	0			Pos 2nd stage (12)
16	1	0	0	27.48	7.39	neg	neg	0	0			Treated syphilis[d] (3)
2[b]	1	0	0	neg	neg		nd	0	1	TP	CEF, PEN	Neg (12)
5[c]	1	0	0	25.32	pp		neg	0	0		PEN	Neg (12)
9[c]	1	0	0	30.43	67.3	nd	1:32	0	0		PEN	Neg (10)
10	1	0	0	neg	pp		neg	0	0	TP	PEN	Neg (10)
14	1	0	0	neg	pp		neg	0	0	TP	PEN	Neg (5)
1	1	0	0	31.59	1.44	neg	neg	1	0	CT	DOXY	Neg (6)
4	1	0	0	27.64	pp		neg	0	1		CEF	Neg (7)
6	0	1	0	neg	neg		nd	0	1		CEF	Neg (12)
8	1	0	0	neg	pp		neg	1	1		CEF, DOXY	Neg (6)
15	1	0	0	30.23	neg		nd	1	0		DOXY	Neg (7)
11	1	0	0	37.94	neg		nd	0	0			No follow-up

SHC: Sexual Health Centre, CLIA: Chemiluminescence immune assay, IB: immunoblot, pos: positive, pp: previously positive, nd: not done, CT: *Chlamydia Trachomatis*, NG: *Neisseria Gonorrhoeae*, TP: *Treponema pallidum*, CEF: Ceftriaxone, DOXY: Doxycycline, PEN: Benzathinebenzylpenicilline, Green lines indicate participants with a confirmed syphilis diagnosis based on additional routine diagnostics at follow up. Blue lines indicate participants treated with penicillin due to a recent syphilis diagnosis or a syphilis notification. Yellow lines indicate participants with a probable syphilis diagnosis based on the additional TP TMA results, that could not be confirmed due to antibiotic treatment that decapitated syphilis serology. The red line indicates a participant with a possible syphilis diagnosis based on the additional TP TMA results, that could be confirmed due to loss to follow up.

a) Confirmatory TP PCR performed on TP TMA positive samples

b) Notified for a syphilis infection 4 days after visit and treated with penicillin

c) Visited general practitioner before visit and was treated for a presumed syphilis infection

d) Participant seroconverted in between the visits and has been treated for syphilis (undetermined stage), the serological assays at the follow-up visit showed treated syphilis

DISCUSSION

Between September 14th, 2021 and August 1st, 2022, 191 syphilis diagnoses were made in 9974 visits from 3283 participants from the national PrEP program visiting the SHC in Amsterdam, the Netherlands. By adding the TP-TMA assay to the routine diagnostics, 16 additional participants could have had an earlier syphilis diagnosis. However, two of the participants with discrepant results had been treated for a syphilis infection by their general practitioner within a week before the SHC visit. Therefore, a total of 14/191 (7%) additional incubating syphilis infections were identified during the study period using TMA as an adjunct test to routine diagnostics.

In 5/16 (31%) participants with discordant TP-TMA and routine diagnostic results, a diagnosis of clinical syphilis was obtained at a follow-up visit supporting the positive TP-TMA assay result. There were 10/16 (63%) participants with discrepant results who were treated with an antibiotic for either an infection with *Chlamydia trachomatis* or *Neisseria gonorrhoeae*, or for contact with a person infected with *Treponema pallidum* and could not be confirmed for syphilis by routine diagnostics in the following visit. However, in 5 of them the confirmatory TP PCR was also positive. If penicillin is contraindicated, the second choices of antibiotics against syphilis are doxycycline or ceftriaxone (16). These antibiotics were given to 5 participants and were probably effective on TP as well. All participants who had been treated with antibiotics between the visits, had a negative follow-up for syphilis. One participant who had not been treated, had a confirmed TP PCR on the TP-TMA sample and was lost to follow-up. Timely detection of TP is also relevant for clients who were already notified as a contact of an infectious syphilis case and treated at the visit, as contact tracing may be expanded to recent sexual contacts and thereby limit further transmission (17).

The highest proportion of TP-TMA positive samples was found among the participants with secondary syphilis. Previous studies have indicated that the secondary syphilis stage is highly infectious (5, 18). Accordingly, increased shedding of treponemes, and thus increased positivity from molecular testing of mucosal samples, might be expected during this phase of infection. Urine samples were the least often positive in the TP-TMA assay as compared to the anal and pharyngeal swabs. However, even though urine samples were not evaluated by the TP-TMA assay in a prior study (14), other previous studies have, to a lesser extent, found TP in urine samples (9, 19, 20). Interestingly in this study, the use of this specimen resulted in one extra identified syphilis infection in a participant who received an early latent stage syphilis diagnosis in the follow-up visit.

The positive TP-TMA assay results were not always confirmed by PCR, as previously found (14, 15). This may be explained by the probably higher sensitivity of the TP-TMA assay targeting 23S rRNA, of which a large number of copies per bacterium are present, instead of the single

copy *polA* gene that is the target of the PCR. We had no evidence for false-positive results since all positive TP-TMA results in our study that were found in the group of participants without initial diagnosis of syphilis were either confirmed by PCR, seroconverted before the follow-up, or occurred in participants with antibiotic use hampering the serological response.

The large sample size of participants and obtained samples is one of the strengths of this study. In addition, detailed metadata was available from the included population visiting the SHC in Amsterdam. A limitation is that we did not determine the specificity of the TP-TMA assay as we did not test TP-TMA negative samples by PCR. However, an initial verification analysis showed 100% specificity in a small panel of samples (data not shown). Future formal clinical validation studies with this assay should incorporate the measurement of all relevant parameters.

The addition of the TP-TMA assay to conventional diagnostics increased the detection of incubating syphilis infections by 7%. The TP-TMA assay is a diagnostic tool that may therefore have value in complementing current routine syphilis diagnostics and could be used reduce the time between TP infection and treatment by timely detection.

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Data availability statement

Data available upon request.

Funding

Hologic (San Diego, USA) donated the RUO TP-TMA kits. This had no impact on the study design, test results or final data analysis. All other costs were covered by the Public health laboratory, GGD Amsterdam, the Netherlands.

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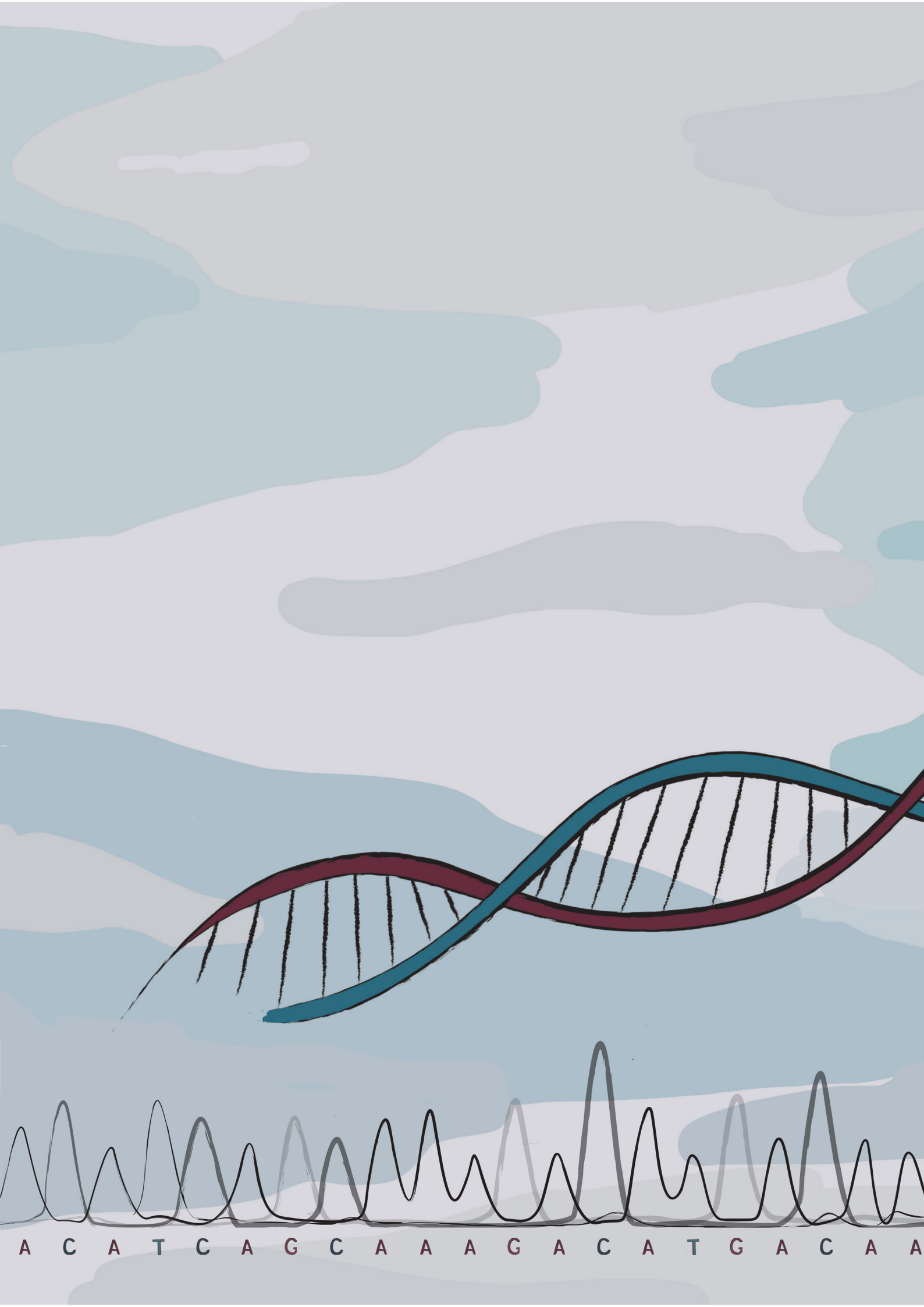
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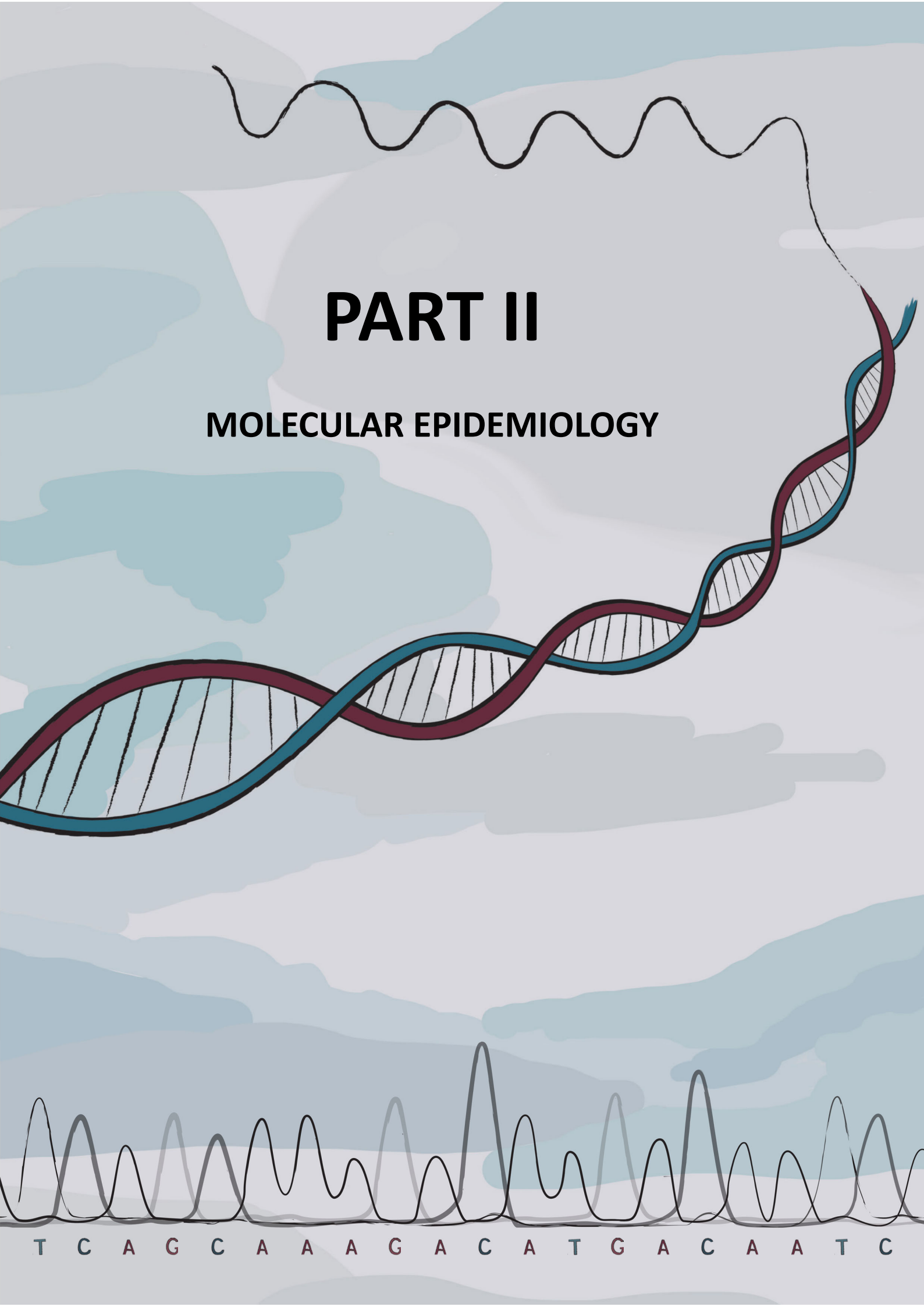
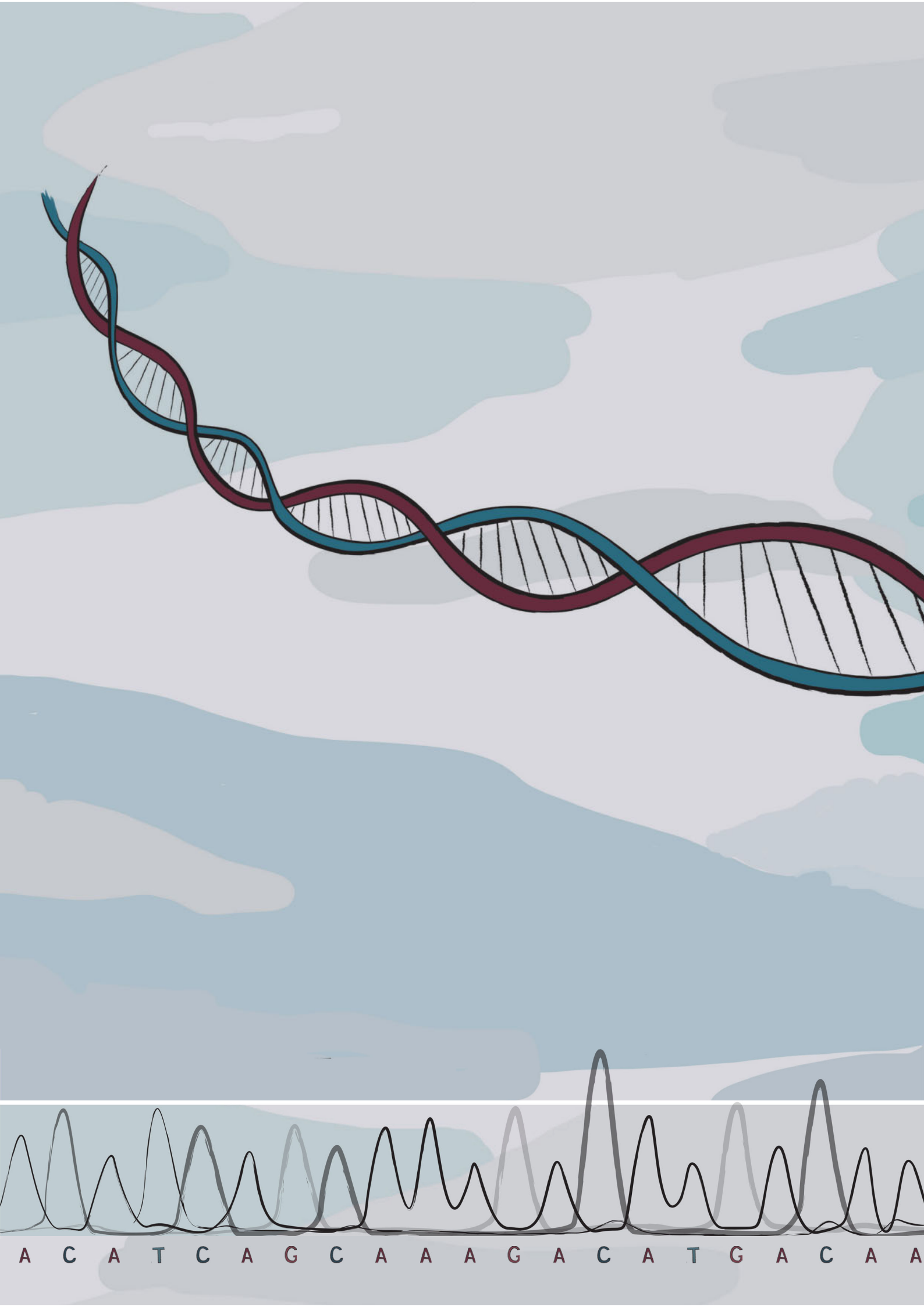


PART II

MOLECULAR EPIDEMIOLOGY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Chapter 4

Molecular diversity of *Treponema pallidum* subspecies *pallidum* isolates in Amsterdam, the Netherlands

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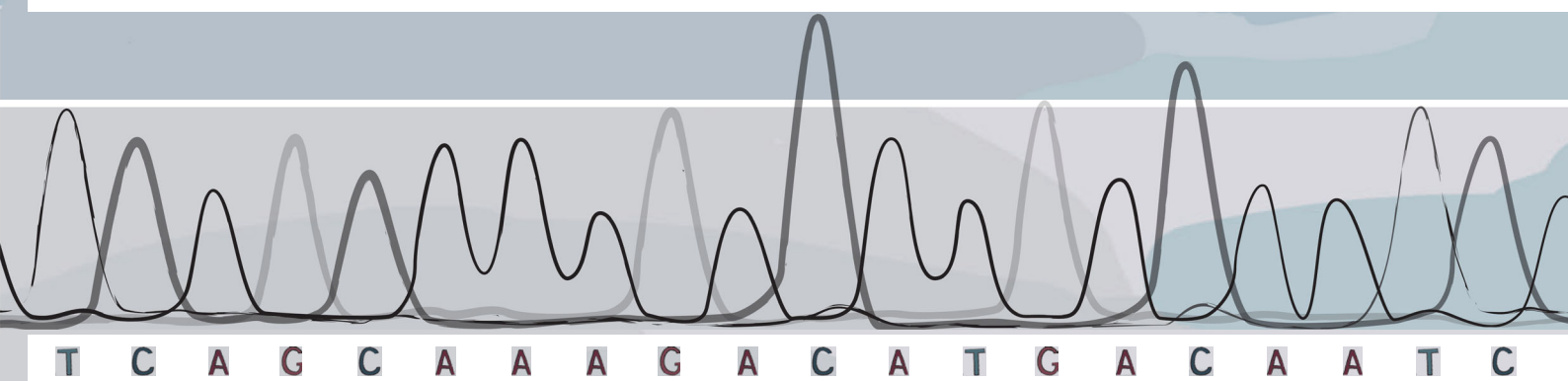

ABSTRACT

Objectives

The prevalence of syphilis, caused by the spirochete *Treponema pallidum* subspecies *pallidum* (TPA), remains high despite the availability of effective antibiotics. In the Netherlands, most syphilis cases are found among men who have sex with men (MSM). We studied the distribution of TPA strain types by molecular characterisation and related this to available characteristics. In addition, resistance to macrolides was assessed.

Methods

TPA DNA was extracted from 136 genital ulcer swab or skin lesions samples deriving from 135 patients diagnosed with syphilis in 2016 and 2017 at the Public Health Service in Amsterdam, the Netherlands. Molecular typing was done according to the enhanced CDC method (E-CDC), in which three genetic regions of the *arp*, *tpr* and *tp0548* genes are analysed by gel electrophoresis of the *arp* and *tpr* regions and by sequence analysis for the *tp0548* region. Part of the 23S rDNA locus was sequenced to determine the presence of macrolide resistance-associated mutations.

Results

Full E-CDC strain types could be determined for 99/136 (73%) DNA samples, which tested positive in a diagnostic PCR targeting the *polA* gene. Types differed within one patient of whom two samples were available. No association was found between the demographic and clinical characteristics and the TPA types. The most prevalent type was 14d/g, found in 23 of the 99 (23%) fully typed samples. Part of the 23S rDNA locus was successfully sequenced for 93/136 (68%) samples and 83 (88%) contained the A2058G mutation. No A2059G mutation was found.

Conclusions

A broad strain distribution was found. Few subtypes were clonally expanded, and most other subtypes were rare. Detection of the most prevalent strain type, 14d/g, is in concordance with other TPA typing studies. The high prevalence of genetic macrolide resistance indicates that azithromycin is not an alternative treatment option.

BACKGROUND

Treponema pallidum subspecies *pallidum* (TPA) is the causative pathogen of the venereal disease, syphilis. Men who have sex with men (MSM) are the most important risk group for contracting syphilis. In 2017, MSM accounted for 95,3% of all syphilis cases in the Netherlands (1).

Molecular epidemiological studies of TPA monitor strain type distribution, and also antimicrobial resistance patterns were studied previously. The genome of TPA is conserved, but also contains highly polymorphic regions useful for typing. Until now the most commonly used typing method for TPA was developed in 1998 (2) and improved in 2010 (3). This method, called the enhanced CDC method (E-CDC), is based on the analysis of three distinct genetic regions; the number of repeats in the acidic repeat protein gene (*arp*), the Mse1 restriction pattern of the *Treponema pallidum* repeat protein (*tpr*) genes (*tprE, tprG,* and *tprJ)* and sequence analysis of the *tp0548* gene (3).

The first line antibiotic for syphilis worldwide is penicillin. Alternatives are ceftriaxone, doxycycline and macrolides. Macrolide resistance among TPA has been widely reported and its prevalence was shown to increase over time (2). Two specific mutations, A2058G and A2059G, in the 23S rRNA genes are associated with macrolide resistance (4).

The objectives of this study were to study the TPA strain distribution and determine the prevalence of macrolide resistance among syphilis cases in Amsterdam, the Netherlands. Furthermore possible associations between demographic and clinical data and strain types were investigated.

MATERIALS AND METHODS

Sample selection and testing

Extracted DNA samples were collected from primary ulcer and skin lesion swabs received at the Public Health Laboratory of the Public Health Service (GGD) in Amsterdam for syphilis diagnostics in 2016 and 2017. All tested positive in the routine diagnostic setting based on a qPCR, targeting the *polA* gene (5). Serologically, a quantitative rapid plasma reagin (RPR) flocculation test (RPR-Nosticon II; bioMérieux) was performed according to the specifications of the manufacturers.

Molecular typing

Arp and *tpr* regions were amplified using a nested PCR. For the *arp* region outer primers were used as described, (2) and inner primers were designed for this study: 5'GCATCTTTGCCGTCCCGTGTGCCT and 5'CGCACGTCCTTTCTGTTCCTCCGGA. The amplified *arp* product was analysed by gel electrophoresis using QIAxcel (Qiagen) or a 2% agarose gel to determine the number of 60 bp repeats. Amplification of the *tprE, tprG and tprJ* genes were performed as described (2). Restriction enzyme *Mse1* digestion pattern of the PCR product was analyzed using a QIAxcel. Amplification of the *tp0548* region was performed as described (3). The amplified *tp0548* product was sequenced using an ABI3130 (Thermofisher). Sequence analyses were performed using Bionumerics version 7.6.2 (Applied Maths).

Macrolide resistance

Part of the 23S rDNA locus was amplified using the following primers; 5'GTACCGCAAACCGACACAG and 5'AGTCAAACCGCCCACCTAC. Sanger sequencing was performed to check for the A2058G and A2059G mutations.

Data analysis

Demographic and clinical data were analysed to investigate associations between typable and non-typable isolates and the presence of macrolide resistance mutations using Fisher's Exact Test, considered significant if $p<0.05$.

Ethical clearing

According to the Dutch Medical Research Act Involving Human Subjects, no additional ethical approval was required for this study (W19_113#19.146).

RESULTS

Gender and age data were available for all 135 included patients with a diagnosis of syphilis, contributing 136 samples (from one patient two ulcer swabs were available). The median age was 42 years (IQR: 33 – 50) and 134/135 (99%) were men. There were 56 samples from 55 patients visiting general practitioners, hospitals or other care providers of whom no additional data were available. The other 80 samples (59%) were collected from patients visiting the sexually transmitted infections (STI) clinic in Amsterdam. From these clients, sexual orientation based on sexual behavior in the past 6 months, HIV status and RPR titer were also known. Most were MSM (74/80, 93%) with four men who have sex with men and women (MSMW) (5%) and only two men who have sex with women (MSW) (3%).

More than one-third (29/80, 36%) of syphilis-positive patients attending the STI clinic were HIV positive. For two persons (2%) the HIV status was unknown. RPR titers were classified as low (1:1 to 1:2), middle (1:4 to 1:16) and high (1:32 to 1:256) and occurred in respectively 20% (16/80), 39% (31/80) and 20% (16/80) in the STI samples. A negative RPR was found for 17/80 (21%) patients.

Molecular typing of the *arp, tpr* and *tp0548* genes resulted in 99/136 (73%) fully typed samples, translating to 25 distinct E-CDC strain types (Figure 1). The remaining 37 samples could only be partially typed. The *arp* genetic region was successfully amplified in 134/136 samples (99%), the *tpr* regions in 111/136 samples (82%) and *tp0548* region in 112/136 (82%) samples. Based on the *tp0548* region of the fully typed samples, 79/99 (80%) were SS14-like and 20/99 (20%) were Nichols-like. The most prevalent type was 14d/g, which was found in 23/99 samples (23%), followed by 14h/g (11%), 14k/g (11%) and 14d/f (9%). Most strain types (14/25, 56%) were found to occur in up to two samples. The patient of whom two ulcer swab samples at the same visit were included differed in *tpr* subtype resulting in types 14k/g and 14d/g.

Macrolide resistance among the different E-CDC strain types is shown in Figure 1A. The 23S rDNA locus was successfully sequenced in 93/136 (68%) samples. The A2058G mutation was found in 83/93 (88%). No A2059G mutation was found.

The HIV status per E-CDC type (Figure 1B) and the RPR titer per E-CDC type (Figure 1C) showed no association with a particular TPA type. In addition, no significant association was found when comparing typable and untypable samples based on RPR titer and HIV status (Supplementary Table 1).

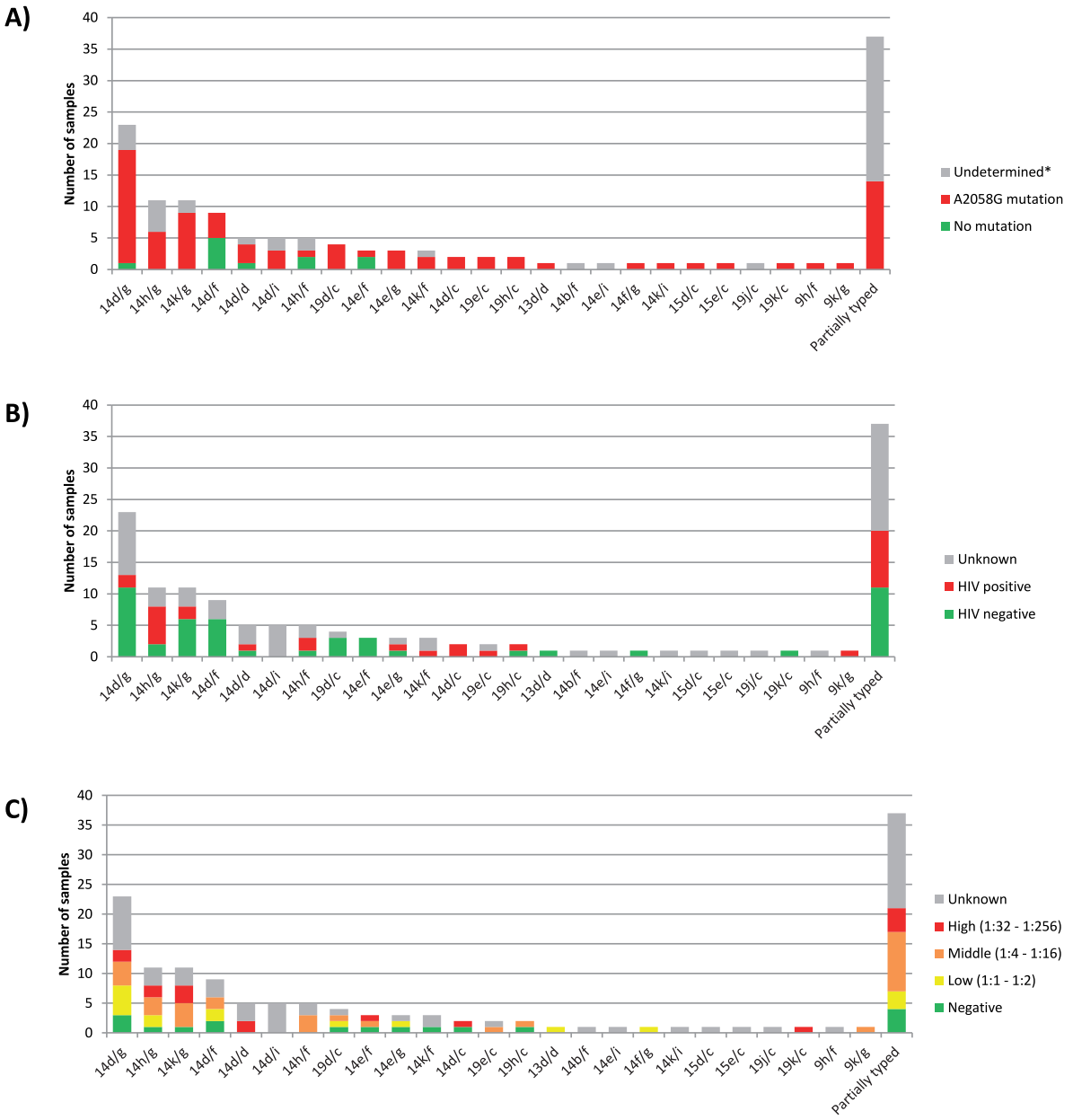

Figure 1 – Overview of the 136 samples sorted by E-CDC strain type (A) including presence (green) or absence (red) of the A2058G mutation. The A2059G mutation was not found. B) E-CDC strain types colored by HIV status; HIV negative (green), HIV positive (red) and unknown (grey). C) E-CDC strain types colored by RPR titer classified as low (1:1 to 1:2, in yellow), middle (1:4 to 1:16, in orange) and high (1:32 – 1:256, in red) and negative (green).
**Part of the 23S rRNA gene was not successfully sequenced. E-CDC, enhanced CDC method; RPR, rapid plasma regain.*

DISCUSSION

A broad strain distribution was found. Few subtypes were clonally expanded, and most other subtypes were rare. This may be related to the wide variety of nationalities residing in Amsterdam. Most syphilis infections occur among MSM in the Netherlands (1). Sexual behavior in the last 6 months was known for patients attending the STI clinic in Amsterdam, and 93% (74/80) of those

with syphilis indeed reported being MSM. This percentage is similar to the percentage of reported MSM syphilis cases in STI clinics in the Netherlands in 2017 (93,5% of 1228 cases), confirming that we studied a representative population at risk for a syphilis infection.

A nested PCR was designed for the *arp* genetic region to increase the quality of the amplified product. The most prevalent strain type in this study was 14d/g, which is also found all over the world (3, 6). Of the 99 fully typed samples 20% belonged to the Nichols-clade, which is relatively high compared to similar studies in Europe (7, 8). A high diversity of TPA types was found in a rather homogeneous setting.

Only 93/136 samples (68%) was successfully sequenced for the 23S rDNA locus. Use of nested PCR for the amplification of the 23S rDNA locus may improve the relatively low rate of successfully sequenced samples. Most samples (83/93, 88%) contained the macrolide resistance associated mutation, A2058G, in agreement with other recent studies (6). Our results further discourage the use of macrolides for the treatment of syphilis. The A2058G mutation was not related to a specific strain type. As suggested by Arora et al. 2016 (8), it is likely that extensive use of macrolides for treatment of diverse bacterial infections has played a significant role in emergence and spread of macrolide resistance in TPA.

It was hypothesized that the bacterial TPA load would be higher in samples derived from HIV infected syphilis patients due to immunodeficiency. In addition, a high RPR titer reflects a high immune response, possibly resulting in a lower bacterial load. Therefore, both RPR and HIV status might have been associated with typability of the samples. However, no association was found in our study. A possible limitation of this study is the absence of TPA DNA bacterial load based on qPCR.

The E-CDC typing method is time consuming and sometimes unreliable especially for *arp* and *tpr* genetic analyses. In this study, the two samples that derived from one patient differed in their *tpr* genetic region. This discrepant finding further enforces previous work suggesting genetic instability for this region. Future molecular TPA typing studies could be improved by using whole genome sequencing (WGS) with a higher resolution (8). Culturing of TPA is possible nowadays, (10) which would facilitate WGS of TPA strains. However, culturing direct patient samples in a routine procedure has not yet been described. A robust and reliable method is multilocus sequence typing, which is possibly best suited for typing purposes (7).

In conclusion, a broad strain distribution was found. Few subtypes were clonally expanded, and most other subtypes were rare. No associations were found between our typing results and the clinical and demographic data. In addition, 88% macrolide resistance shows the importance of discouraging macrolide use to treat syphilis.

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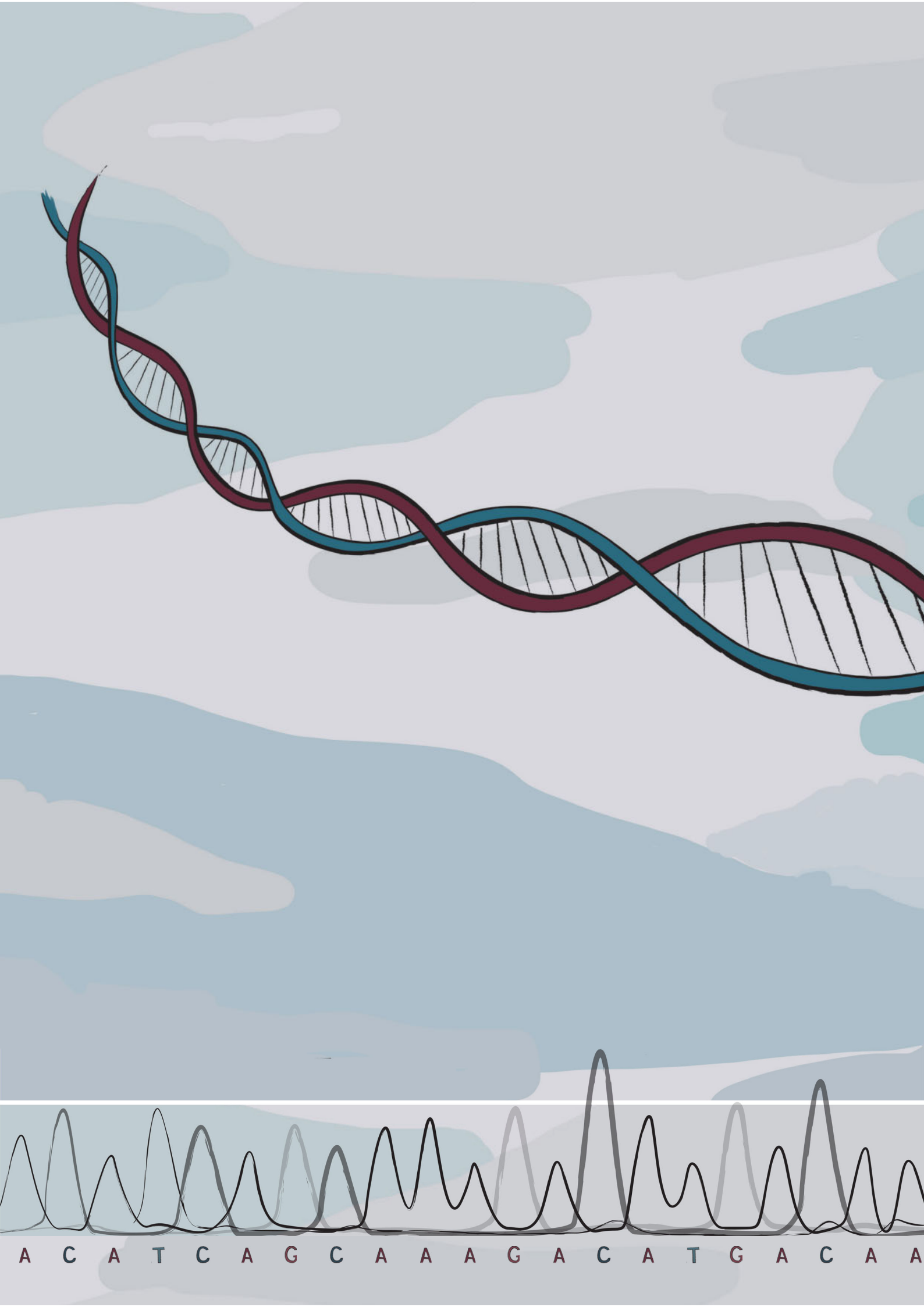
SUPPLEMENTARY DATA

Supplementary Table 1 – Cross tabulation of demographic and clinical factors from 80 patients visiting the STI clinic regarding the ability to obtain a full E-CDC TPA type comparing the typable and untypable isolates. Included factors are HIV status and RPR titer. Fisher's Exact Test was used and a significant association was found if $p<0.05$.

HIV status	HIV positive	9 (31)	20 (69)	0,395
	HIV negative	11 (22,4)	38 (77,6)	
	Unknown	1 (50)	1 (50)	
RPR titer	Negative	4 (25)	13 (76,5)	0,797
	Low (1:1 to 1:2)	3 (18,8)	13 (81,2)	
	Middle (1:4 to 1:16)	10 (32,3)	21 (67,7)	
	High (1:32 to 1:258)	4 (25)	12 (75)	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Chapter 5

Treponema pallidum subspecies *pallidum* intra-patient homogeneity at various body locations in men with infectious syphilis

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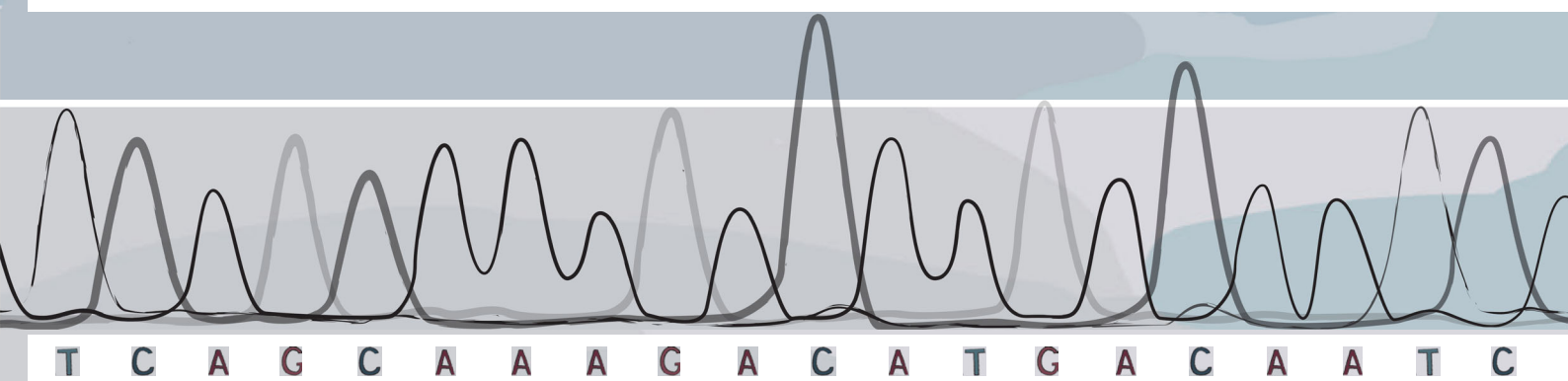

ABSTRACT

Syphilis, caused by *Treponema pallidum* subspecies *pallidum* (TP), is a complex multi-stage infectious disease. Systematic dissemination occurs within a few hours of transmission. We determined the molecular variation of TP at various body locations and peripheral blood within patients in different stages of syphilis to assess the distribution of TP strains at these locations.

We included 162 men who have sex with men (MSM) with syphilis visiting the Sexual Health Center in Amsterdam between 2018 to 2019, who had TP DNA detected in at least one sample type (anal swab, urine sample, peripheral blood, pharyngeal swab and/or ulcer swab). TP DNA was detected in 287 samples using a qPCR targeting the *polA* gene. With multi-locus sequence typing (TP-MLST) based on partial sequence analysis of three genetic regions (*tp0136, tp0548, tp0705*), we characterized all TP DNA positive samples.

Samples could be typed (119/287) from at least one anatomical location or peripheral blood from 93/162 (57%) patients in the following stages: 48 (52%) primary, 35 (38%) secondary and 10 (11%) early latent stage syphilis. The TP-MLST type was identical within each of the 12 patients with typed samples at ≥2 different body locations. The most prevalent TP strains were 1.3.1 (39/93, 42%) and 1.1.1 (17/93, 18%) belonging to the SS14 lineage; 80% (74/93) of the patients carried a SS14 lineage TP strain and 20% (19/93) Nichols lineage. The distribution of TP-MLST types did not differ between patients by syphilis stage.

We found intra-patient TP strain homogeneity and no TP strain variation between anatomical location or syphilis stages. More early latent samples should be typed and added in future studies to investigate this in more detail.

Abstract Importance

Syphilis, caused by *Treponema pallidum* subspecies *pallidum*, is a complex multi-stage infectious disease. Systematic dissemination is known to occur within a few hours of transmission. Despite the effective antibiotic, penicillin, syphilis remains prevalent worldwide. Men who have sex with men are disproportionally affected in high income countries like the Netherlands where 96% of the syphilis cases in 2020 were among this population. The inability to *in vitro* culture *T. pallidum* directly from patient samples limits whole genome sequencing efforts. Fortunately, in 2018 a multi-locus sequence typing technique was developed for *T. pallidum* allowing the monitoring of circulating strains. The significance of our research is in the investigation of *T. pallidum* molecular variation at various body locations and blood within patients in different stages of syphilis in order to assess the distribution of strains at these locations.

INTRODUCTION

Syphilis, caused by the bacterium *Treponema pallidum* subspecies *pallidum* (TP), is a complex multi-stage sexually transmitted disease. Although highly effective treatment is available, the worldwide prevalence of syphilis remains an important public health issue. Like in many high income countries, in the Netherlands men who have sex with men (MSM) are most affected. In 2020, 96% of the syphilis diagnoses are among MSM (1).

Systemic dissemination occurs within a few hours after acquisition (2). Sexual transmission typically occurs during the primary, secondary and early latent stages within the first year of acquisition. During early syphilis stages, syphilis lesions are considered infectious. Yet, recent studies have detected TP DNA at various seemingly non-affected tissues, and in peripheral blood (3, 4). Body locations and fluids that are considered pivotal in sexual transmission of TP, such as genital, anal, and pharyngeal cavities were tested for the presence of TP DNA by PCR. It is unclear whether TP DNA presence at multiple locations is the result of a primary infection at one body site followed by systemic dissemination, or a consequence of multiple sexual exposures at different anatomical locations. Molecular typing of TP may be helpful to elucidate this (5, 6).

Bacterial typing is usually most successful on cultured strains, from which high amounts of specific bacterial DNA can be isolated. Despite recent technological advances, direct *in vitro* culture of TP from patient samples remains unsuccessful (7). Culture independent molecular characterization of TP isolates using the multi-locus sequence typing (MLST) method allows strain identification of TP for comparisons between patients (6), but this technique may also be useful to discriminate TP types within patients.

The aim of this study was to investigate possible intra-patient TP strain variation and to assess possible associations with specific body locations. In addition we aimed to investigate the molecular variation of TP in different stages of syphilis.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Medical Ethics Committee of the Amsterdam University Medical Centers (reference: NL66419.018.18, 2018_236#B2018609).

Sample selection

The Center for Sexual Health (CSH) of the Amsterdam Public Health Service, the Netherlands, is a low-threshold clinic. Clients do not require a referral by a medical doctor, and the consultations are anonymous and free of charge. From November 2018 through December 2019, we invited MSM aged 18 years and older to participate if they: (1) had signs and symptoms suggestive of primary syphilis or secondary stage syphilis; or (2) were diagnosed with early latent or late latent syphilis (8). In total, 293 men were included in the study. After informed consent, we collected an anal swab, urine sample, peripheral blood sample and pharyngeal swab, in addition to routine diagnostic samples, such as swabs from pharyngeal-, anal- or genital ulcer lesions. In this typing study, we included all 287 TP DNA positive samples from 162 participants with at least one TPA DNA positive sample.

For the current analysis TP DNA positive samples were grouped by anatomical locations. Urine samples and genital ulcers were grouped together as “Urogenital”. In a similar fashion pharyngeal swabs and pharyngeal ulcers were grouped as “Pharynx” and anal swabs and anal ulcers were grouped as “Anus”.

DNA extraction and TP detection

DNA extraction was performed using multiple optimized methods for each sample type (4). DNA from anal swab, pharyngeal swab, and ulcer swab samples was extracted using isopropanol precipitation (9) or MagNA Pure 24 (Roche Molecular Systems) according to the specifications of the manufacturers. DNA from urine and peripheral blood samples was extracted within 24 hours of collection. Into each of two Eppendorf tubes, 1ml of urine was transferred . After centrifugation at 14000 rpm for 10 minutes the supernatant was discarded and the pellet resuspended in 200 µl PBS. DNA from resuspended urine pellets was extracted using isopropanol precipitation (9). DNA extraction from 200 µl of peripheral blood was done using the QIAamp Blood Mini Kit following the protocol from the manufacturer (Qiagen, Hilden, Germany).

The in-house validated real-time qPCR targeting the *polA* gene was performed to detect TP DNA as previously described corcerning primers, probes and amplification protocol (10). A RotorGene Q thermocycler was used (Qiagen, Hilden, Germany) with a standardized fluorescence threshold of 0.04 for determination of cycle threshold (Ct) values. The PCR was considered positive with a Ct value <36. For samples with Ct value between 36 and 40, DNA was re-extracted and tested again together with the first DNA extract. When the Ct value was ≤40, the result was positive. Ct values >40 were considered negative. In addition, phocine herpesvirus (PhHV) was used as internal control to exclude PCR inhibition. If a sample was inhibited in the PCR, the sample was re-extracted and tested again simultaneously with a

1:10 dilution of the first DNA extract. Due to frequent PCR inhibition of anal swabs, these samples were tested concomitantly at the 1:10 dilution.

Definition of the clinical syphilis stages

Primary syphilis was defined as the presence of an ulcer with a positive dark-field microscopy test and/or TP DNA detected with the *polA* PCR. A diagnosis of secondary syphilis was based on typical clinical manifestations such as a rash with or without lymphadenopathy, or mucosal lesions such as condylomata lata, and a rapid plasma reagin (RPR) titer ≥1:4. Ulcers may also occur in secondary stage patients. Early latent syphilis was defined as a seroconversion of the chemoluminence immunoassay (CLIA), or a RPR ≥ 1:32, or a 4-fold or higher RPR titer rise in an asymptomatic participant.

Molecular typing of TP

The MLST method for the molecular characterization of TP was used as described (6). This method is based on the partial amplification and sequence analysis of genetic regions: *tp0136, tp0548* and *tp0705*. The allelic profiles consisting of allelic variants per genetic regions, in respective order, constitute the resulting TP-MLST type. Amplification was performed using nested PCR for each genetic region, after which Sanger sequencing was used. Internal primers for genetic region *tp0136* were used if needed. In addition, an extra primer (5'ATCGTTTGTTATGCCGGTTGC) was developed and used, if necessary, for the completion of the 3' region of *tp0705*. The sequence analysis was done using Bionumerics version 7.6.3 (Applied Maths, BioMérieux). Samples with only one or two out of the three regions typed are referred to as partially typed and were excluded from further analyses.

The lineage of the TP strains (SS14 or Nichols) was determined by the analysis of the sequenced genetic regions of *tp0136* and *tp0548* found in the study. Every new allelic variant was aligned to both references, SS14 and Nichols, using MEGA X version 10.1.8 in order to determine its genetic lineage.

All samples that obtained a TP-MLST type were uploaded in the BIGSdb PubMLST TP database (11) (id numbers 967 - 1057) and new allelic variants and new allelic profiles were added to the TP typing scheme with subsequent numbering.

Data analysis

Cycle threshold value differences were tested using the independent sample t-test. Other variables of interest were compared using Fisher's Exact test and Pearson's Chi-square test in SPSS 26.0.0.1 or R 3.6.3 (12). Figures were produced using the packages ggplot2 (13) and VennDiagram (14). To visualize genetic lineages, a phylogenetic tree of the concatenated sequences was generated by first aligning the concatenated sequences using Crustal-Wallis in

MEGA X version 10.1.8 followed by the evolutionary analysis using the bootstrapping maximum-likelihood algorithm and the Tamura-Nei method (15) in MEGA X version 10.1.8 (16).

RESULTS

Patient characteristics

We included 162 patients, of whom the majority (99%) had exclusively sex with men (Table 1) and almost one third (31%) were living with HIV. In 24 of the 162 patients (15%), who tested positive for TP DNA in at least one body location or peripheral blood, the RPR was negative. Of these, 22 patients were diagnosed with primary syphilis and 2 with early latent syphilis determined by a seroconversion of the CLIA.

Sample characteristics

From the 162 included patients we collected 287 TP DNA positive samples (Supplementary Table 1). Among these samples were 73 ulcers of which 16 were ulcers found at the anal site, 55 at the urogenital site and 2 at the pharyngeal site. TP DNA was detected most frequently at the anal site, peripheral blood and the pharyngeal site among the patients with secondary syphilis compared to the other syphilis stages. Samples from the urogenital site were most frequently positive among patients with primary syphilis. There was only one peripheral blood sample that could be fully typed. Over two thirds of the patients with secondary or early latent syphilis had a positive TP DNA sample at the pharynx (Table 2).

In 9/162 (6%) patients TP DNA was detected in samples from all sites including peripheral blood (Figure 1A). Also, a third (53/162) of the patients were positive only at the urogenital site (Figure 1A).

TP typing at the distinct anatomical locations and peripheral blood

TP-MLST types were successfully obtained for 119/287 (42%) samples (Supplementary Table 1), which were grouped to 107/251 (43%) anatomical locations and peripheral blood samples (Table 2) and derived from 93 patients (Figure 1B). Among these 93 patients, 48 (52%) patients had primary syphilis, 35 (38%) secondary syphilis and 10 (11%) early latent syphilis. In 12 patients, two or more locations were successfully typed (Figure 1B). From all but one of these patients a TP-MLST type was obtained from the pharyngeal site in combinations with samples from other sites (11/12, 92%). Samples from the urogenital site were most often successfully typed, followed by samples from the pharynx.

Table 1 – Socio-demographic, behavioural, and clinical characteristics of men who have sex with men (N=162) included in this study in Amsterdam, the Netherlands, 2018-2019.

Age	
<35 years	58 (36%)
35 – 44 years	48 (30%)
≥45 years	56 (35%)
Education	
Primary/secondary	35 (22%)
College/university	106 (65%)
Unknown	21 (13%)
Gender of sex partners	
Men	160 (99%)
Men and women	2 (1%)
No. of sex partners (6mo)[1]	
Median (IQR)	6 (4-15)
<5	46 (28%)
5-9	45 (28%)
10-14	24 (15%)
≥15	44 (27%)
Unknown	3 (2%)
HIV status	
Negative	112 (69%)
Positive	50 (31%)
cART use if HIV positive	
No	3 (6%)
Yes	47 (94%)
Most recent CD4 count (cells/µl)	
<350	0 (0%)
350-499	2 (4%)
≥500	33 (66%)
Unknown	15 (30%)
RPR titre	
Median [IQR]	16 (2-32)
Negative[2]	24 (15%)
1:1-1:4	30 (19%)
1:8-1:16	56 (34%)
1:32-1:128	52(32%)

Abbreviations: IQR, interquartile range; HIV, human immunodeficiency virus; cART, combination antiretroviral therapy; RPR, Rapid Plasma Reagin test.

1. Number of sexpartners in the 6 months before the consultation
2. Of these 24 patients, 22 had primary syphilis with an ulcer and two without symptoms, but with a seroconversion of the chemoluminence immunoassay.

Table 2 – An overview of TP DNA detected and typed at the different anatomical locations and peripheral blood from the 162 included patients between 2018- 2019 at the SHC in Amsterdam, the Netherlands by syphilis stage.

Anatomical location and blood	**Primary syphilis** N = 69 patients		**Secondary syphilis** N = 64 patients		**Early latent syphilis** N = 29 patients		**Total** N = 162 patients	
	TP DNA detected	Typed	TP DNA detected	Typed	TP DNA detected	Typed	TP DNA detected	Typed
	n	n	n	n	n	n	n	n
Anus	16 (23%)	9 (13%)	37 (58%)	7 (11%)	11 (38%)	1 (3%)	**64** (40%)	**17** (11%)
Urogenital	55 (80%)	38 (55%)	28 (44%)	12 (19%)	6 (21%)	2 (7%)	**89** (55%)	**52** (32%)
Peripheral blood	2 (3%)	0 (0%)	15 (23%)	1 (2%)	5 (17%)	0 (0%)	**22** (14%)	**1** (1%)
Pharynx	7 (10%)	4 (6%)	48 (75%)	24 (38%)	21 (72%)	9 (31%)	**76** (47%)	**37** (23%)

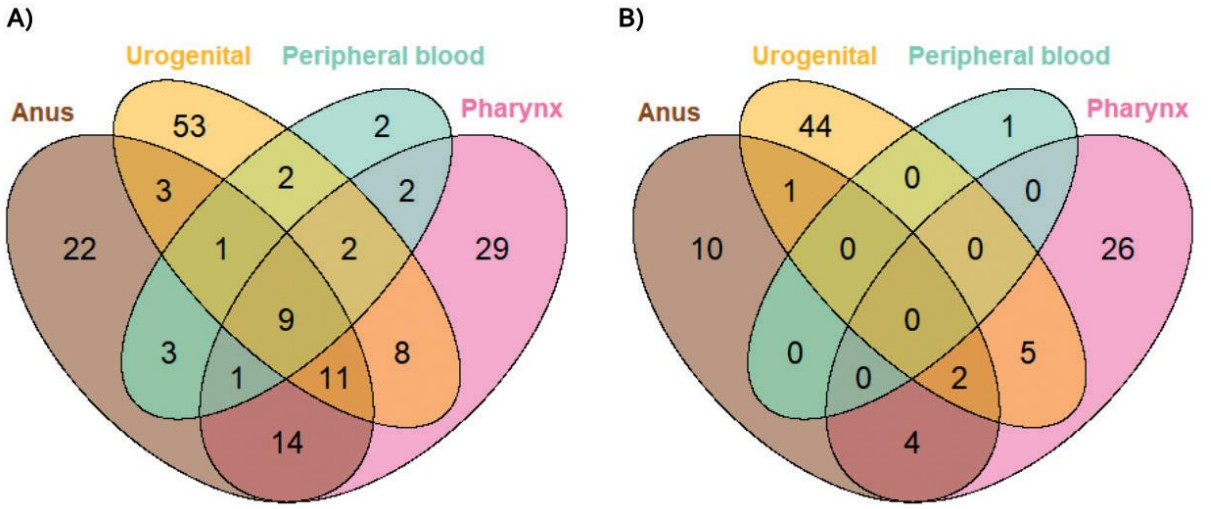

Figure 1 – Venn Diagrams showing the number of patients with (A) TP DNA detected by anatomical location and peripheral blood and (B) where a TP type was obtained in 93 patients with at least one typed sample.

The cycle threshold (Ct) value of the initial *polA* TP DNA PCR was significantly lower in samples that were successfully typed (Supplementary Figure 1a). However, the distribution of Ct values overlapped substantially between the typeable and non-typeable samples. In addition, the Ct value was found to vary greatly by sample type (Supplementary Figure 1b).

TP strain distribution per syphilis stage

The 107 distinct anatomical locations and peripheral blood samples containing typed samples from 93 patients resulted in 14 different allelic profiles (Table 3). Most prevalent allelic profiles were 1.3.1 and 1.1.1 occurring in 39 (42%) and 17 (18%) patients, respectively. Eight of the 14 allelic profiles (57%) were each found in only one patient. Nine distinct allelic profiles were found among the 48 patients with primary syphilis, 10 among patients with secondary syphilis and 5 among patients with early latent syphilis. The distribution of TP-MLST types did not differ between patients by syphilis stage (P = 0.31, Fisher's Exact test).

Table 3 – TP strain distribution per syphilis stage among the 93 typed patients with at least one typed sample out of the 162 included patients between 2018- 2019 at the SHC in Amsterdam, the Netherlands.

	Allelic profiles by syphilis stage				
Allelic profiles	**Primary**	**Secondary**	**Early latent**	**Total (%)**	**Lineage**
1.3.1	25	11	3	**39 (42)**	SS14
1.1.1	9	5	3	**17 (18)**	SS14
9.7.3	3	7	1	**11 (12)**	Nichols
1.1.8	3	3	2	**8 (9)**	SS14
3.2.3	4	3	0	**7 (8)**	Nichols
1.1.9	1	2	0	**3 (3)**	SS14
1.64*.1	1	0	0	**1 (1)**	SS14
1.52.1	1	0	0	**1 (1)**	SS14
1.17.9	1	0	0	**1 (1)**	SS14
1.43.1	0	0	1	**1 (1)**	SS14
1.66*.1	0	1	0	**1 (1)**	SS14
1.65*.1	0	1	0	**1 (1)**	SS14
30*.3.1	0	1	0	**1 (1)**	SS14
29*.7.3	0	1	0	**1 (1)**	Nichols
Total	**48**	**35**	**10**	**93**	

*New allelic variants found in this study.

The overall typing success per genetic region was 148/287 (52%) for *tp0136*, 144/287 (50%) for *tp0548* and 160/287 (56%) for *tp0705*. Besides the 119 fully typed samples, 32/287 (11%) samples were typed for two genetic regions and 59/287 (21%) for only one genetic region (Supplementary Table 2). 77/287 (27%) samples were not successfully typed for any of the regions. We found six new allelic variants of which two for genetic region *tp0136* and five for genetic region *tp0548*. This resulted in five new allelic profiles as one new allelic variant was part of a partially typed sample. One of the new allelic variants for *tp0136*, in the profile 29.7.3, belongs to the Nichols lineage (Figure 3). All other new allelic profiles belong to the SS14 lineage. The new allelic variants and profiles were uploaded in the pubMLST database (11) and were subsequently numbered (*tp0136*: 29, 30 and *tp0548*: 63, 64, 65, 66).

In Figure 2, the diversity of concatenated sequences is shown from the allelic profiles found in this study (an alignment of 2584 base pairs (bp)) and the distinction between the SS14 and Nichols lineages. The three types belonging to the Nichols lineage were found in 19/93 (20%) patients. Strains from neither the SS14 nor the Nichols lineage were found to be overrepresented in patients with a specific syphilis stage (Supplementary table 3A). In addition, no differences were found in the number of Nichols or SS14 lineages per age group, HIV status or the number of sites in which TP DNA was detected within the 93 patients (Supplementary table 3B, 3C, 3D) Single-nucleotide polymorphism (SNP) level variation was found within these lineages.

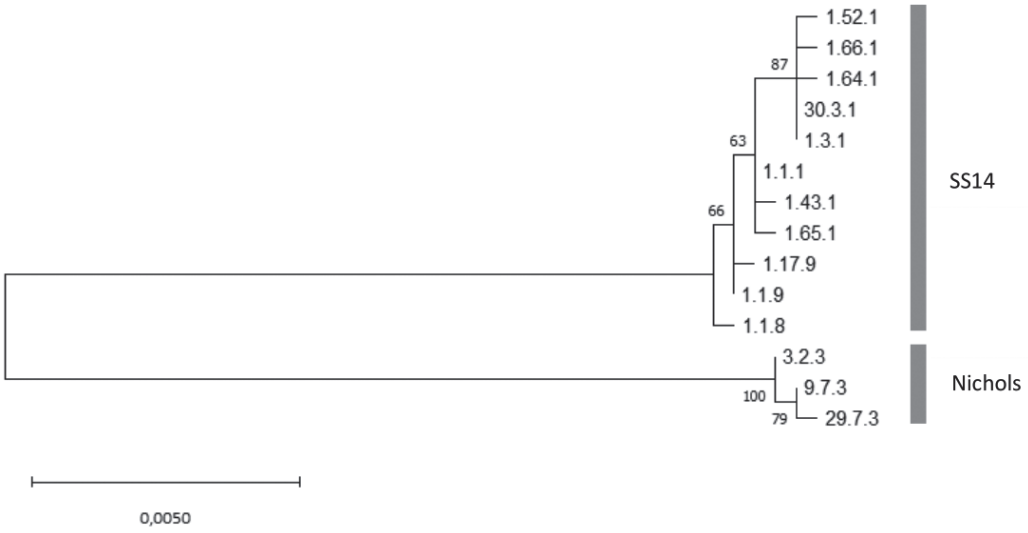

Figure 2 – Phylogenetic tree of the concatenated sequences (MUSCLE alignment of 2584 base pairs) of all allelic profiles found in this study using the maximum likelihood method and Tamura-Nei model with 1000 bootstraps.

In patients with successfully typed samples from multiple different body locations, allelic profiles were identical within each patient (Table 4). None of these patients had a peripheral blood sample that was successfully typed. All patients had a positive RPR titer and, similar to the overall included population, 4 of these 12 patients (33%) were living with HIV. 5/12 (42%) patients reported having had fewer than 5 sex partners in the past 6 months (Table 4).

Table 4 – Overview of patients (n = 12) with 2 or more obtained TP-MLST types at different anatomical locations among the 162 included patients between 2018- 2019 at the SHC in Amsterdam, the Netherlands.

		Demographic and clinical characteristics			Obtained TPA strain per body site (x)[1]				
Patient nr.	**Syphilis stage**	**No. of sexpartners (6mo)[2]**	**HIV status**	**RPR titer**	**Anus**	**Urogenital**	**Pharynx**	**Allelic profile**	**Lineage**
1	Primary	1	Negative	32	x		x	1.1.1	SS14
2	Primary	12	Negative	8		x	x	1.3.1	SS14
3	Primary	10	Positive	16	x		x	9.7.3	Nichols
4	Secondary	2	Positive	8	x		x	1.1.1	SS14
5	Secondary	2	Positive	32	x	x		1.1.9	SS14
6	Secondary	10	Negative	16		x	x	1.3.1	SS14
7	Secondary	7	Positive	32	x		x	1.66*.1	SS14
8	Secondary	5	Negative	16	x	x	x	9.7.3	Nichols
9	Secondary	5	Negative	16	x	x	x	9.7.3	Nichols
10	Secondary	5	Negative	32		x	x	9.7.3	Nichols
11	Early latent	3	Negative	32		x	x	1.1.1	SS14
12	Early latent	2	Negative	8		x	x	1.3.1	SS14

**New allelic variant*

1: There were no peripheral blood samples typed in these 12 patients.

2: In the 6 months before consultation.

DISCUSSION

This is the first study that applied MLST to TP DNA positive samples collected at different anatomical locations and from peripheral blood from patients in different syphilis stages. We found no TP strain variance within a patient. This study focused on the early syphilis stages, primary, secondary and early latent, as we did not detect TP DNA in samples from participants with late stage syphilis or treated syphilis. TP strains were not associated with a specific syphilis stage.

The intra-patient TP strain homogeneity suggests that the TP DNA detected at the different body locations is due to haematogenous dissemination rather than sexual transmission from multiple partners. Direct transmission of multiple different body locations during the same sex act could also explain the intra-patient homogeneity. However, previous studies that included molecular typing techniques on multiple samples of TP isolates within a patient showed identical types in blood samples and swab samples supporting the suggestion of haematogenous dissemination (17, 18). In addition, due to the clonal nature of TP the possibility of infection from multiple partners with the same allelic profile, such as the highly prevalent strain, 1.3.1, also exists. More in depth typing methods such as whole genome sequencing might reveal more polymorphism for intra-patient TP strains.

The significant difference between cycle threshold values of successfully typed samples versus not (fully) typed samples was expected as Ct values are associated with the bacterial load in the sample, and thus influence amplification success of each genetic locus. We could successfully type 119/287 (43%) samples in this study. This is a relatively low percentage compared to other studies on TP using this MLST (6, 19-23), but in these studies mainly ulcer swabs were included, whereas we also included many samples from unaffected tissues and peripheral blood samples, many with high Ct values. The number of multiple typed anatomical locations within patients was therefore limited. Due to the higher TP DNA load in swab samples from ulcers, a higher proportion of patients with primary syphilis had at least one sample successfully typed.

The TP strain diversity was similar to that in previous TP-MLST studies among MSM in Amsterdam, the Netherlands (19). The most prevalent allelic profiles were 1.3.1 (42%) and 1.1.1 (18%), which have been found in all TP-MLST studies (11). With current knowledge, the unique TP strains within countries (6, 19-21, 23) seem to suggest local transmission besides international mixing as was also found in this study with 8/14 (57%) allelic profiles occurring only once. The 20% prevalence of TP strains belonging to the Nichols lineage in this study is similar to previous studies in Amsterdam (19), Japan (24), and Argentina (25), and is comparable to a recent large-scale genomic study of samples from 23 countries (26).

However, an older large study on 970 publicly available sequences described that 93.5% belonged to the SS14 lineage (27) and some countries showed <10% circulating Nichols strains: such as in the Czech Republic (20), France (21) and Switzerland (6). No associations were found between TP strains and syphilis stage (Supplementary Table 3).

Our study has a number of limitations. The molecular method with the highest resolution for the analysis of molecular variation within a patient or between syphilis stages would be whole genome sequencing (WGS). However, current TP WGS efforts using target enrichment of pathogen reads show that TP bacterial loads are crucial and samples with lower bacterial loads, like the majority from this study, would be unsuccessful using WGS (27-29). The Enhanced Centers for Disease Control and Prevention Typing method (ECDCT) is a different typing method for TP combining the analysis of tandem repeats, restriction fragment length patterns and sequence analysis (5). This ECDCT method was previously found to contain genetically unstable loci (17, 30). However, recently this technique was found to remain stable and distinguish typing within different TP-MLST types and vice versa (18). After WGS, the TP MLST provides high resolution with a discriminatory power of 30.8% in comparison to WGS (6).

Also, we had a low number of typed samples from the early latent syphilis stage, because of the lower TP DNA positivity in patients with this stage. Nonetheless, we were able to type samples in 10 patients with early latent syphilis, of whom two had a type that was obtained from both the urogenital site and the pharyngeal site.

The monitoring of molecular changes keeps us updated on the circulating TP strains among populations at higher risk of a syphilis infection. With help of the TP pubMLST database (11) the knowledge on genetic information of TP strains is expanded internationally. In this study we found intra-patient TP strain homogeneity and no TP strain variation between anatomical locations or syphilis stages, although more early latent samples and more within patient samples should be typed in future studies to investigate this in more detail.

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SUPPLEMENTARY DATA

Supplementary Table 1 – Overview of TP DNA positive samples deriving from patients with infectious syphilis and their proportions within each syphilis stage, overall and the number of samples of which a TP type was obtained.

Type of sample	**Primary syphilis** 69 patients (%)	**Secondary syphilis** 64 patients (%)	**Early latent syphilis** 29 patients (%)	**Total #typed / Total #samples (%)**
Anal swab	13 (19)	37 (58)	11 (38)	**10 / 61** (16)
Urine	24 (35)	26 (41)	6 (21)	**23 / 56** (41)
Peripheral blood	2 (3)	15 (23)	5 (17)	**1 / 22** (5)
Pharyngeal swab	7 (10)	47 (73)	21 (72)	**35 / 75** (47)
Ulcer swab	63* (91)	10 (16)	0 (0)	**50 / 73** (68)
Total	109	135	43	**119 / 287** (42)

**Remaining 6 patients with primary syphilis did not have an ulcer swab available for the study.*

Supplementary Table 2 – Overview of all (partial) allelic profiles of samples per body locations of 182 patients with at least one allelic variant. X refers to an undetermined allelic variant. "Unknown" lineages lack typing information of the tp0136 and the tp0548 locus.

Allelic profiles	Lineage	Anus	Urogenital	Peripheral blood	Pharynx	Total
1.3.1	SS14	8	25		9	42
1.1.1	SS14	1	9		9	19
9.7.3	Nichols	2	5	1	8	16
1.1.8	SS14	2	3		3	8
3.2.3	Nichols		4		3	7
1.1.9	SS14		2		1	3
1.66.1	SS14	1			1	2
1.64.1	SS14		1			1
1.52.1	SS14		1			1
1.17.9	SS14		1			1
1.43.1	SS14				1	1
1.65.1	SS14				1	1
30.3.1	SS14				1	1
29.7.3	Nichols		1			1
1.X.1	SS14	3	3		3	9
X.3.1	SS14	2	1		2	5
X.1.1	SS14	1	1	1		3
1.3.X	SS14		1	1		2
1.X.8	SS14			1	1	2
X.1.8	SS14	1				1
X.7.3	Nichols	1				1
29.X.3	Nichols			1		1
1.X.9	SS14				1	1
9.X.3	Nichols		1			1
X.2.3	Nichols		1			1
X.63.1	SS14		1			1
6.X.1	SS14				1	1
X.X.1	Unknown	1		3	4	8
X.1.X	SS14	1	2	2	2	7
1.X.X	SS14	3	2		1	6
X.X.9	Unknown			1	1	2
9.X.X	Nichols			1		1
19.X.X	SS14				1	1
29.X.X	Nichols				1	1
X.66.X	SS14		1			1
X.7.X	Nichols				1	1
X.X.8	Unknown				1	1
X.X.3	Unknown	1				1

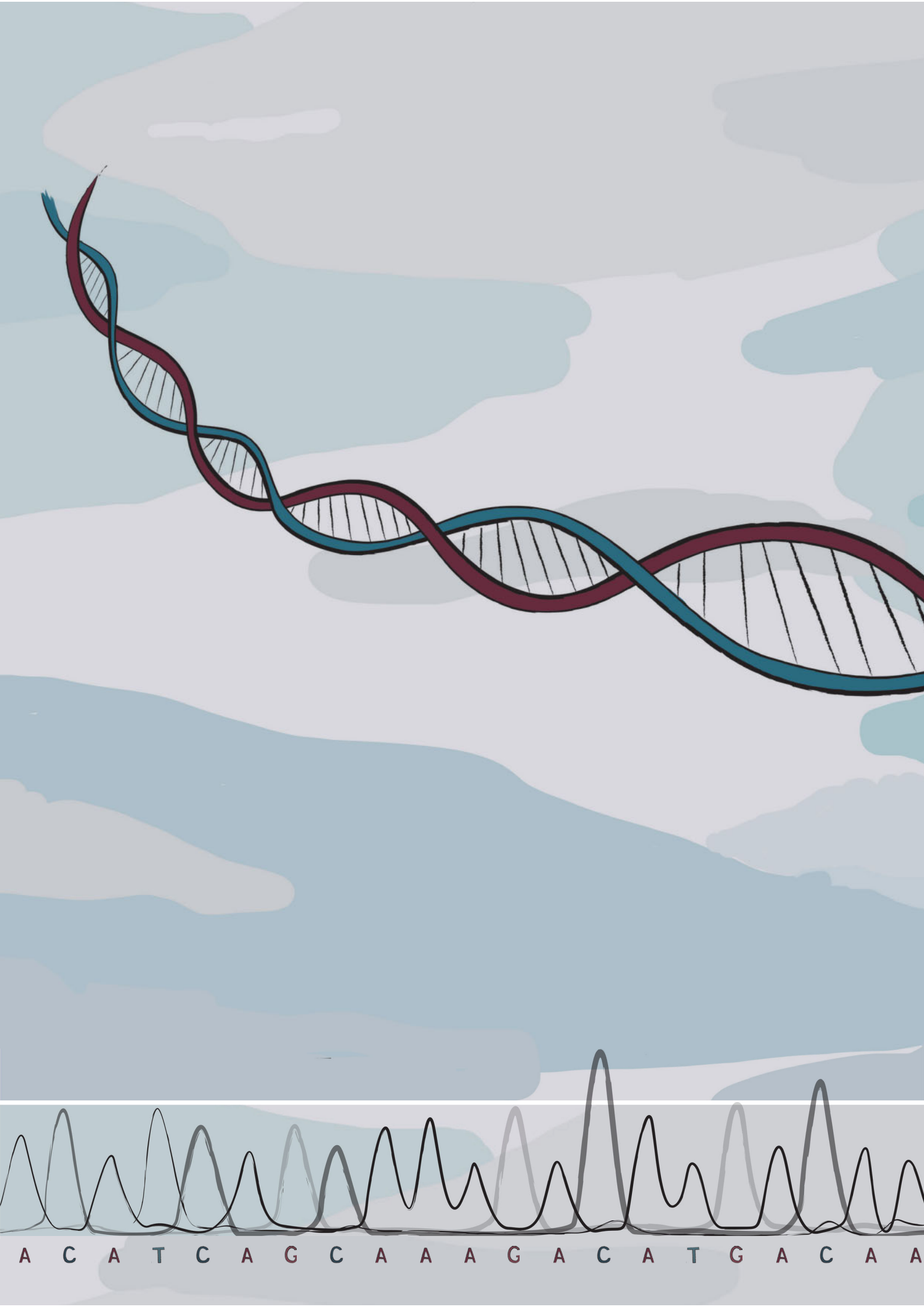
Supplementary Table 3 – Chi-squared or Fisher's Exact Tests of distribution of lineages (Nichols and SS14) by (A) syphilis stage, (B) age, (C) HIV status and (D) number of TP DNA detected at different sites of the 93 patients with at least one TP type.

A)		**Lineage (N = 93 patients)**		
		Nichols n (%)	SS14 n (%)	**Total**
Syphilis stage	Primary	7 (15)	41 (85)	48
	Secondary	11 (31)	24 (69)	35
	Early latent	1 (10)	9 (90)	10
	Total	19 (20)	74 (80)	93
Fisher's Exact Test	*0.13*			

B)		Nichols n (%)	SS14 n (%)	**Total**
Age (in years)	≤34	10 (26)	28 (74)	38
	35-44	4 (15)	23 (85)	27
	45-54	3 (20)	12 (80)	15
	55+	2 (15)	11 (85)	13
	Total	19 (20)	74 (80)	93
Fisher's Exact Test	*0.72*			

C)		Nichols n (%)	SS14 n (%)	**Total**
HIV status	Negative	4 (15)	23 (85)	27
	Positive	15 (23)	51 (77)	66
	Total	19 (20)	74 (80)	93
Pearson's χ^2 test	*0.56*			

D)	#anatomical locations	Nichols n (%)	SS14 n (%)	**Total**
TP DNA detected	1	9 (47)	48 (64)	57
	2	6 (31)	10 (14)	16
	3	2 (11)	10 (14)	12
	4	2 (11)	6 (8)	8
	Total	19 (20)	74 (80)	93
Fisher's Exact Test	*0.26*			


A C A T C A G C A A A G A C A T G A C A A

Chapter 6

Treponema pallidum strains among women and men who have sex with women in Amsterdam, the Netherlands and Antwerp, Belgium between 2014 – 2020

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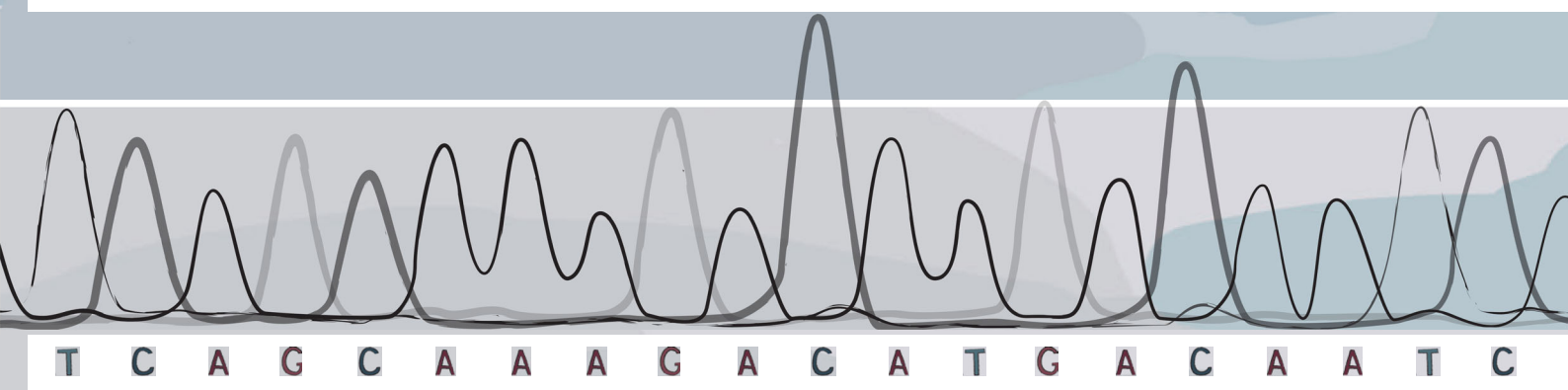

SUMMARY

A different *Treponema pallidum* strain distribution was found among samples derived from men who have sex with women and women from this study as compared to the same population worldwide.

ABSTRACT

The *Treponema pallidum* strain distribution among men who have sex with women were similar to the strain distribution men who have sex with men (MSM). The most prevalent strains and percentage of strains belonging to the Nichols lineage are similar to previous studies in Amsterdam focusing on MSM.

Treponema pallidum subspecies *pallidum* (TPA), causing syphilis, is found globally. The circulating TPA strains have mainly been investigated in men who have sex with men (MSM), a disproportionally affected population in high-income countries, and showed to be geographically similar regarding the major strains (1). To gain insight in the genetic diversity of TPA strains several molecular typing schemes for TPA have been developed in the last two decades. In 2018, a TPA multi-locus sequence typing scheme (MLST) (2) was set up, and soon after the TPA pubMLST database was implemented to record all detected TPA allelic profiles worldwide (3). Limited information is available on the prevalence and variation among women, and men who have sex with women (MSW). We aimed to identify TPA strains within MSW and women and compare them to the TPA strain distribution among MSM.

Between 2014 and 2020, 41 TPA PCR positive samples were included; 32 from the Public health laboratory (PHL) of Amsterdam, the Netherlands (16 women and 16 men) and 9 from the Belgian National Reference Centre for STIs (NRC-STI) in Antwerp, Belgium (1 woman and 8 men). Patients had either visited a sexual health centre (SHC) or a general physician for routine surveillance or symptomatic screening.

TPA was characterized using the TPA multi-locus sequence typing (MLST) method based on the partial amplification and sequence analysis of three genetic regions; *tp0136, tp0548* and *tp0705* (2). In addition, the 23S rRNA loci were checked for macrolide resistance associated mutations (MRAM). All complete allelic profiles (AP) and available patient data were added to the TPA pubMLST database (ID numbers 745, 780, 788 and 1651 – 1677).

We compared our data to TPA typed isolates from women and men whose sexual orientation was known from the TPA pubMLST database (extracted on July 5th 2022) (3).

Possible associations between clinical characteristics and AP were tested using the Chi-squared Test or Fisher's exact test with a statistical significance set at $p \leq 0.05$ and Simpson's diversity index was calculated for comparison within the sample set of the study using RStudio (v1.2.5033).

The median age of the 41 included patients was 36 years old (interquartile range of 25 – 48). From 23/41 (56%) included patients the HIV status was known, with 4 patients living with HIV and 19 without. Sample material was known for 19 samples with 16 ulcer swabs, 2 biopsies and 1 serum (Supplementary Table 1).

Table 1 – Allelic profiles found among the 30 fully-typed *Treponema pallidum* subsp. *pallidum* samples from women, men who have sex with women between 2014 and 2020 in Amsterdam, the Netherlands and Antwerp, Belgium.

Allelic profile	Frequency N (%)	Lineage	23S rRNA mutation (n)	Sex of patients (n)
1.3.1	12# (40)	SS14	A2058G (11) Undetermined (1)	Men (8) Women (4)
1.1.1	5 (17)	SS14	A2058G (3) A2059G (1) No mutation (1)	Men (1) Women (4)
1.43.1	3 (10)	SS14	A2058G (3)	Men (1) Women (2)
3.2.3	3 (10)	Nichols	A2058G (1) Undetermined (2)	Men (3)
1.1.3	2 (7)	SS14	A2059G (1) No mutation (1)	Men (1) Women (1)
9.7.3	2 (7)	Nichols	A2058G (2)	Men (2)
1.1.10	1 (3)	SS14	A2058G (1)	Women (1)
19.3.1	1 (3)	SS14	Undetermined (1)	Men (1)
1.1.8	1 (3)	SS14	No mutation (1)	Men (1)

Including all 3 typed samples from the Belgian National Reference Centre for STIs in Antwerp, Belgium.

A total of 30/41 (73%) samples, derived from 12 women and 18 MSW, could be typed for all three loci. Nine distinct AP were found (Table 1). Samples taken from tissue (biopsy) or blood serum failed to be typed. There were 3/30 (10%) typed samples that originated from Antwerp and all had the same AP, being 1.3.1. The most prevalent AP were 1.3.1 (12/30, 40%) followed by 1.1.1 (17%). AP 3.2.3 and 9.7.3, pertaining to 5 samples (17%), belonged to the Nichols lineage and were found only in men (Table 1).

The presence of a MRAM could be determined in 30/41 samples of which 90% contained a MRAM, 26 of these samples were fully typed (Table 1) and 4 were partially typed (not shown). 23/26 (88%) of the fully typed samples contained MRAM (Table 1). The A2058G mutation was present in samples from both MSW and women. However, the A2059G mutation or the absence of a mutation was only found in MSW.

Similar AP diversity was found in samples from men and women; 8 AP were found in 18/24 (75%) samples derived from men, resulting in a Simpson's diversity index of 0.79, compared to 5 AP from 12/17 (70%) samples among women resulting in a diversity index of 0.80.

The export from the TPA BIGSdb PubMLST database of fully typed samples from women, and men whose sexual orientation was recorded contained 501 typed isolates derived from 55 women (11%) and 446 men (89%). Of these, 76% (379/501) isolates were derived from MSM and 24% (122/501) from women and MSW. The most prevalent AP in the pubMLST database

among women and MSW was 1.1.8 (51/122, 42% records), followed by 1.3.1 (30/122, 25% records). However, in this study AP 1.1.8 was only found in one MSW (Table 1).

No associations between sex and AP were found (p = 0.208). Additionally, no associations were found between HIV status, age, ethnicity and AP (respectively p = 0.096, p = 0.885 and p = 0.420).

This study shows an overall distribution of TPA strains among women and MSW in Amsterdam and Antwerp similar to the distribution found among MSM in Amsterdam (4). The lineage distribution in this study was 83% SS14 and 17% Nichols. Samples with types belonging to the Nichols lineage were found only in samples from MSW. Similar distributions were found in previous studies conducted in The Netherlands, mostly among MSM (4, 5) and worldwide (1, 6).

The most prevalent allelic profile in this study was 1.3.1 occurring in 40% of the samples from MSW as well as in women in the two cities from our study. This was consistent with the data from the TPA pubMLST database. However, the most prevalent strain among women, being 1.1.8, according to the TPA pubMLST database, was only found once in the present study in a sample derived from a MSW. Also, the MSW in our study showed more similarities with the MSM group from the pubMLST database with regards to heterogeneity of strains and a higher percentage of Nichols lineage. This may suggest that MSW, although denying sex with men, contracted their syphilis strain from other men. This is a common finding associated with the stigma associated with homosexuality (7).

Interestingly, a Japanese study discovered a higher prevalence of macrolide resistance mutations in MSW (97%) than in MSM (38%) (8).The 90% MRAM rate among this population is higher than in samples from 2006 – 2018 among mostly MSM in Amsterdam, where 81% of the samples contained a MRAM (5). However, a temporal increase of MRAM has been reported before in TPA isolates (5, 9). MRAM was present in all 7 successfully determined samples from Antwerp, in concordance with a previous study (10).

The small sample size is an important limitation of this study. As a result, the power to detect significant differences between populations is limited. Another limitation is that it was not possible to obtain complete or even partially typed allelic profiles from blood or from tissue samples. This finding has been observed by others as well, supporting that ulcer swabs, which are known to have the highest bacterial load, are the best source for molecular typing (11, 12).

Although this study population is too small to make clear statements about circulating TPA strains in relation to different sexual preferences, further research is warranted to gain a

better understanding of the transmission patterns of TPA within specific populations and help public health surveillance. A better understanding of the transmission patterns of TPA within specific populations could be used to inform policy makers on who should best be targeted for preventive measures.

Ethical clearing

The need for informed consent was waived by the Medical Ethical Committee of the Amsterdam University Medical Centres in the Netherlands (W20_336 # 20.373).

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Competing Interests Statement

None declared**.**

Contributorship Statement

HZ, HV and SB planned the study. HZ, HV and IB provided the study samples. HZ and FJ conducted the study and analyzed the data. HZ and FJ drafted the manuscript. All authors critically reviewed the manuscript.

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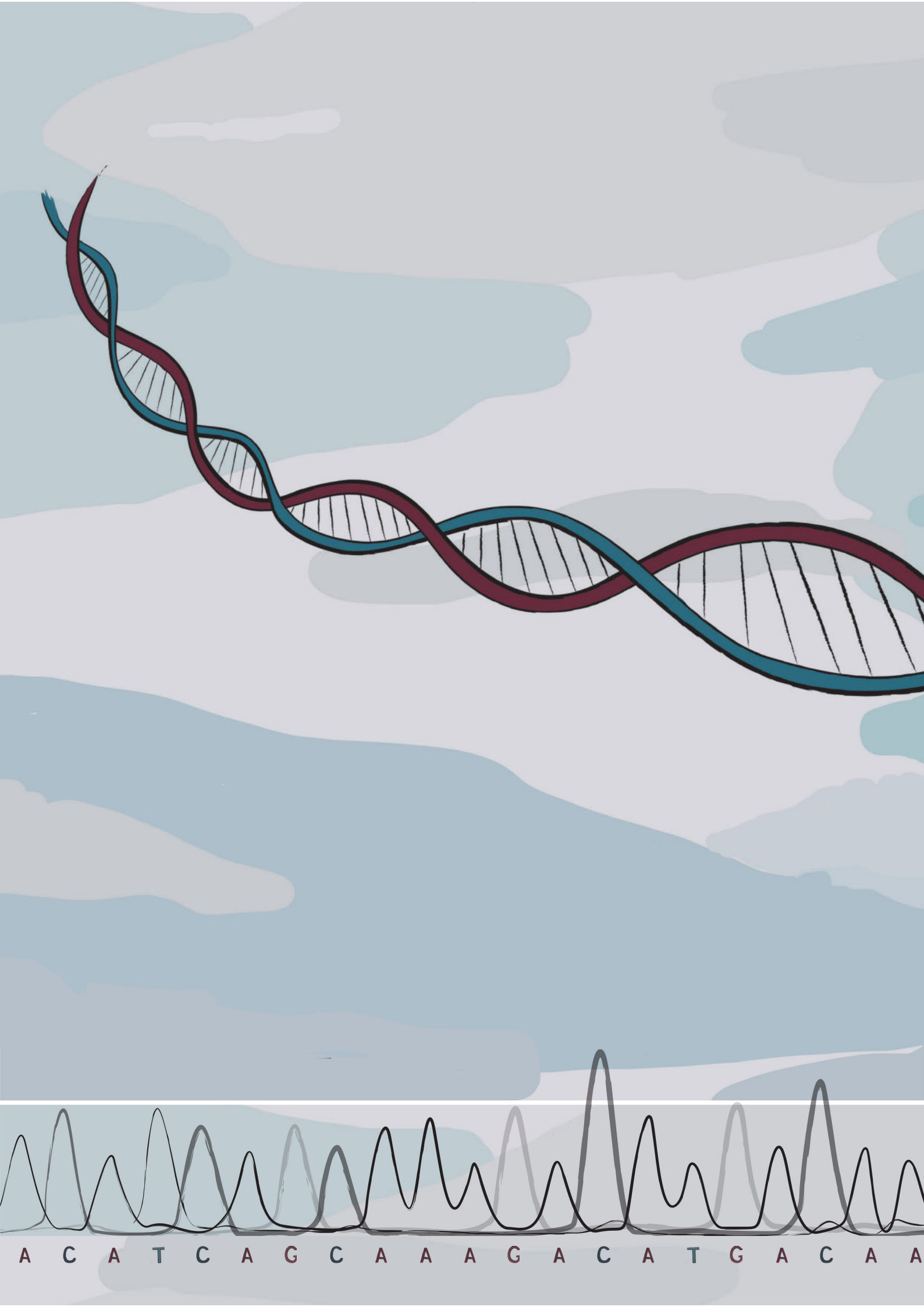
SUPPLEMENTARY DATA

Supplementary Table 1 – Clinical and demographic characteristics of the 41 included women, and men who have sex with women (MSW) in Amsterdam and Antwerp between 2014 and 2020.

Characteristics	Patients (n = 41)
Sexual Health Centre	
Amsterdam	32
Antwerp	9
Sex and sexual orientation, n (%)	
Men who have sex with men	24 (59)
Women	17 (41)
Age, median (IQR)	
	36 (25-48)
HIV status, n (%)	
Positive	4 (10)
Negative	19 (46)
Unknown	18 (44)
Ethnicity, n (%)	
Netherlands	10 (25)
Surinam	4 (10)
Turkey	1 (2)
Other European countries	3 (7)
Sub-Saharan Africa	2 (5)
Asia	1 (2)
Unknown	20 (49)
Sample type (Amsterdam, N = 32), n (%)	
Ulcus swab	8 (25)
Biopsy	1 (3)
Blood serum	1 (3)
Unknown	22 (69)
Sample type (Antwerp, N = 9), n (%)	
Ulcus swab	8 (89)
Biopsy of thyroid gland	1 (11)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Chapter 7

No bejel among Surinamese, Antillean and Dutch syphilis diagnosed patients in Amsterdam between 2006 – 2018 evidenced by multi-locus sequence typing of *Treponema pallidum* isolates

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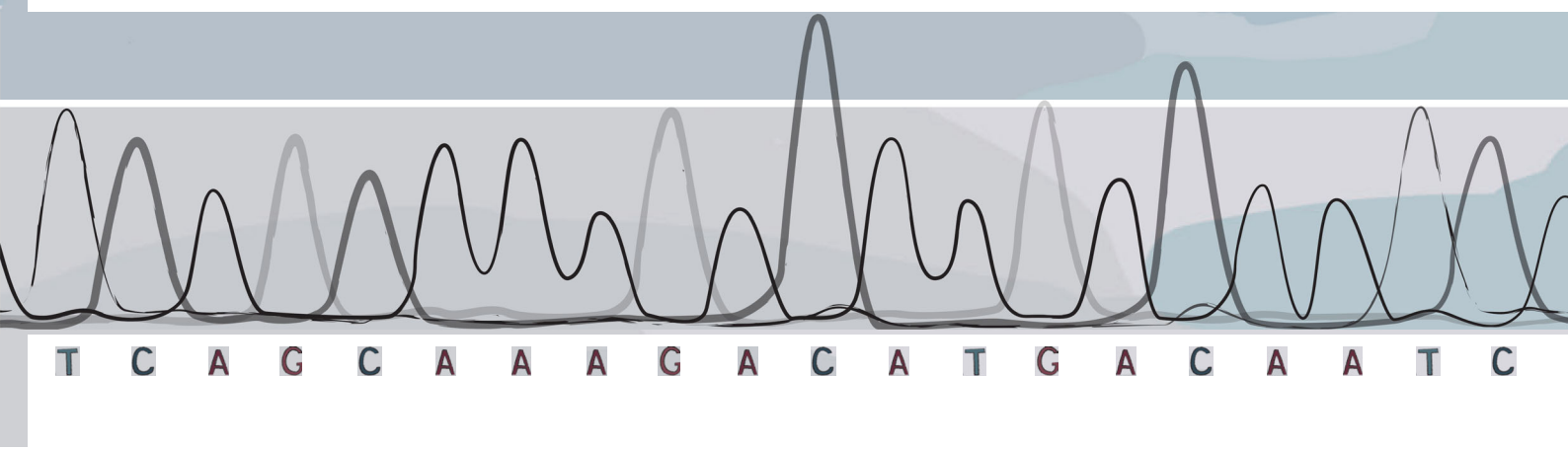

ABSTRACT

Background

Treponema pallidum subspecies *pallidum* (TPA) and subsp. *endemicum* (TEN) are the causative agents of syphilis and bejel, respectively. TEN shows similar clinical manifestations and is morphologically and serologically indistinguishable from TPA. Recently, bejel was found outside of its assumed endemic areas. Using molecular typing we aimed to discover bejel and characterize circulating TPA types among syphilis cases with Surinamese, Antillean and Dutch ethnicity in Amsterdam.

Methods

DNA was extracted from 137 ulcer swabs, which tested positive in the in-house diagnostic PCR targeting the *polA* gene. Samples were collected between 2006 and 2018 from Surinamese, Antillean and Dutch patients attending the Amsterdam STI clinic. Multilocus sequence typing was performed by partial sequence analysis of the *tp0136*, *tp0548* and *tp0705* genes. In addition, the 23S rRNA loci were analyzed for A2058G and A2059G macrolide resistance mutations.

Results

We found 17 distinct allelic profiles in 103/137 (75%) fully typed samples, which were all TPA and none TEN. Of the strains, 82.5% were SS14-like and 17.5% Nichols-like. The prevalence of Nichols-like strains found in this study is relatively high compared to nearby countries. The most prevalent types were 1.3.1 (42%) and 1.1.1 (19%), in concordance with similar TPA typing studies. The majority of the TPA types found were unique per country. New allelic types (7) and profiles (10) were found. The successfully sequenced 23S rRNA loci from 123/137 (90%) samples showed the presence of 79% A2058G and 2% A2059G mutations.

Conclusions

No TEN was found in the samples from different ethnicities residing in Amsterdam, the Netherlands, so no misdiagnoses occurred. Bejel has thus not (yet) spread as a sexually transmitted disease in the Netherlands. The strain diversity found in this study reflects the local male STI clinic population which is a diverse, mixed group.

INTRODUCTION

The spirochetes of the *Treponema* genus consists of different species and subspecies causing syphilis, bejel, yaws and pinta infections. *Treponema pallidum* subspecies *pallidum* (TPA) is the causative pathogen of syphilis, a world-wide prevalent venereal disease. In 2017, there were 33,189 syphilis cases reported in 28 EU/EEA Member States giving an incidence rate of 7.1 cases per 100 000 population [1]. The increasing rates of syphilis cases is mainly driven by behavioral factors and testing strategies by focusing on the risk-group of men who have sex with men (MSM), who accounted for 96% of the 1,224 syphilis cases in the Netherlands in 2018 [2].

Bejel is caused by *T. pallidum* subsp*. endemicum* (TEN) and was, until recently, thought to be non-venereal [3-5]. Bejel shows similar clinical manifestations and is morphologically and serologically indistinguishable from TPA [6]. In 2016, a TEN isolate was identified in France [7]. A recently developed multilocus sequence typing (MLST) scheme [8] enables differentiation between treponemal subspecies as well as the distinction between the two major genetic clades within TPA, Nichols and SS14, and provides strain types within these clades. In 2018, this MLST method was used in Cuba [6] and Japan [9] to retrospectively investigate treponemal subspecies in samples of patients that were diagnosed with syphilis. Interestingly, both studies found cases of bejel, caused by TEN, which strongly suggested sexual transmission of this disease and showed bejel cases outside of the known endemic areas, Sahelian Africa and Saudi Arabia [4].

This study aimed to discover TEN strains causing bejel among syphilis cases from patients with Surinamese or Antillean ethnicity assuming possible importation of bejel from their country of origin. We hypothesized that if bejel was also misdiagnosed in Amsterdam, as was the case in Cuba among patients with syphilis [6], we would more likely find TEN in Dutch patients with a Surinamese or Antillean ethnicity, as these countries are geographically close to Cuba (Fig 1), than in patients with a Dutch ethnicity. Dutch patients were also included to investigate the presence of bejel in Amsterdam.

In addition, molecular characterization data was used to increase the epidemiological knowledge of the strain types and investigate possible associations between allelic types, profiles and patient's clinical and demographical data.

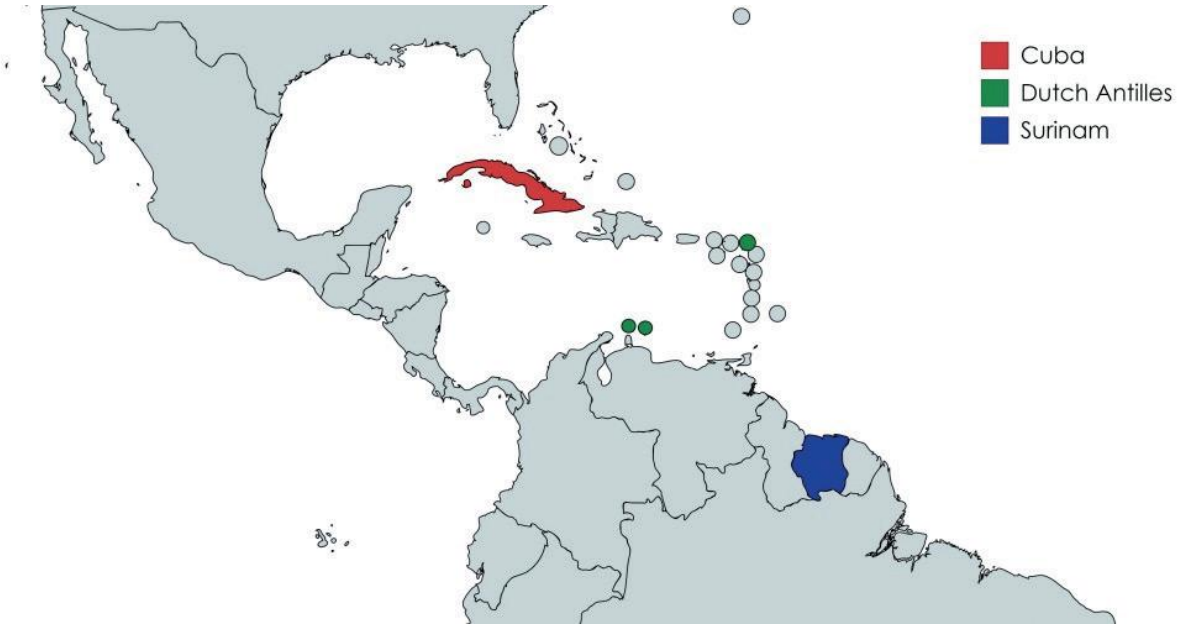

Figure 1 – Map showing geographical location of Cuba (red), the Dutch Antilles (green) and Surinam (blue). This map was constructed using matchart.net.

METHODS

Sample selection and preparation

Based on a positive *polA* PCR on genital ulcer swabs [10] 137 samples were retrospectively selected from patients with a Surinamese, Antillean or Dutch nationality visiting the STI clinic in Amsterdam between 2006 and 2018. Within this time frame all available samples from syphilis diagnosed Surinamese and Antillean patients were included in the study. For a more representative and equal spread of samples the number of Dutch samples were randomly selected per calendar year to match the number of samples from Antillean and Surinamese patients.

If the volume of the DNA isolate was insufficient, DNA from the original patient sample, which was stored at -20^{o}C, was re-extracted using isopropanol precipitation method [11]. Demographic and clinical data was collected for all patients.

Defining clinical stages of syphilis

The primary stage of syphilis is defined as an oro-, ano-genital ulcerative disease with a positive dark field microscopy and/or with a positive ulcer swab PCR result. Criteria for secondary syphilis are a rash with or without lymphadenopathy, or mucosal lesions such as condylomata lata, and an RPR ≥ 1:4. Ulcers may also occur in patients presenting with a rash with or without lymphadenopathy, or mucosal lesions. These patients are diagnosed with

secondary syphilis. All samples from this study are ulcer swabs and were derived from both (primary and secondary) clinical syphilis stages.

Serological testing

Serologically, until 2013 a *Treponema pallidum* particle agglutination (TPPA) assay was performed on all samples. After 2013, the enzyme immunoassay (EIA) for the detection of IgM antibodies to *Treponema pallidum* was introduced and used on all samples unless the patient had a syphilis infection before. In addition, quantitative rapid plasma reagin (RPR) flocculation test (RPR-Nosticon II; bioMérieux) was performed according to the specifications of the manufacturers.

Molecular typing method

Molecular characterization of the samples was performed using the recently developed MLST method by Grillová et al. 2018 [8]. This MLST method is based on the partial amplification and sequence analysis of three chromosomal loci; *tp0136, tp0548* and *tp0705*. Also both 23S rRNA genes were partly sequenced to check for the A2058G and A2059G mutations associated with macrolide resistance. The partial amplification and sequence analysis using Sanger sequencing was performed as described [8, 12]. Sequence analyses were performed using Bionumerics version 7.6.3 (Applied Maths, BioMérieux). All allelic variants and allelic profiles were added to the PubMLST BIGSdb database of TPA [13]. New allelic variants and profiles were given subsequent numbers adding to the TPA database. Typed isolates were uploaded when 2 or more allelic variants were identified.

Genetic clade and subspecies distinction

Clade determination (SS14-like or Nichols-like) was possible with the partly sequenced regions of *tp0136* and *tp0548*. Every new allelic variant was compared to both references in order to determine its genetic group. To visualize subspecies and genetic clades a phylogenetic tree of the concatenated sequences was generated with the bootstrapping maximum-likelihood algorithm and the Tamura-Nei method in MEGA6.06 [14].

Data analysis

Allelic profiles, demographic and clinical data were tested for possible associations with Fisher's Exact Tests and, where possible, Pearson's Chi-square Tests between patients with fully typable and not (fully) typable samples using IBM SPSS Statistics (version 21.0.0.2). A $p<0.05$ was considered significant.

Ethical clearing

This study was reviewed, and the need for consent was waived by our Institutional Review Board, which is the Medical Ethical Committee of the Amsterdam University Medical Centers

in the Netherlands. According to the Dutch Medical Research Act Involving Human Subjects on use of retrospective diagnostic material no additional ethical approval was required for this study (W19_113#19.146). An opt-out system is used at the Public Health Service of Amsterdam to assure that if patients object to having their samples used for research that these are destroyed. Only anonymized patient data were used as provided by an independent datamanager. No samples from patients under the age of 18 years old were included.

RESULTS

Isolated DNA samples were available from 137 ulcer swabs and derived from 24 Antillean patients, 46 Surinamese patients and 67 Dutch patients. Patient characteristics were collected for all 137 patients and are shown in Table 1. Dutch patients had a higher median age, 46 years with an interquartile range (IQR) of 38-51, compared to 37 years (IQR 29-45) in Surinamese and 35 years (IQR 31-41) in Antillean patients. Only 67% of the Surinamese patients were MSM based on their sexual behavior in the past 6 months. This is much lower compared to the 91% and 92% among the Dutch and Antillean patients. The HIV status among all ethnicities was similar with 40% HIV positive Dutch patients, 39% Surinamese and 46% Antillean.

From the 137 DNA samples 103 (75%) were successfully amplified and analyzed for all typing loci. This resulted in 17 distinct allelic profiles (Fig 2). Of these samples, 85 (82.5%) were SS14-like and 18 (17.5%) Nichols-like strains. The most common allelic profile found was 1.3.1 occurring in 42/99 (42%) isolates. None of the samples in this study were TEN.

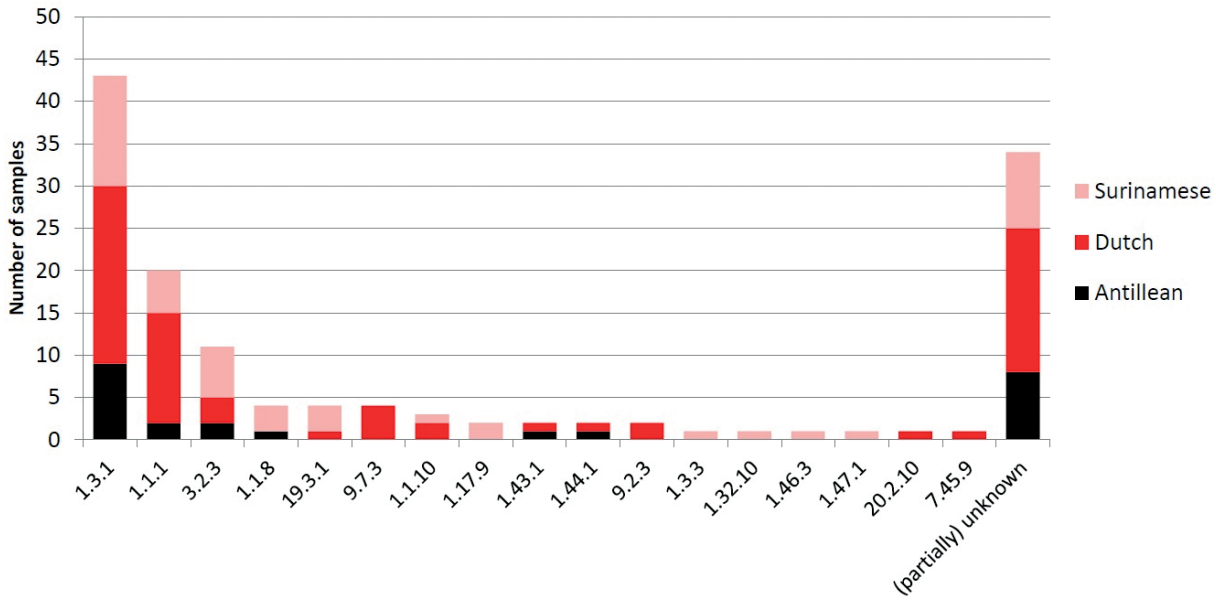

Figure 2 – An overview of allelic profiles colored by ethnicity. Molecular typing of 137 *Treponema pallidum* subspecies *pallidum* isolates from Amsterdam.

Table 1 – Demographic and clinical characteristics of all 137 patients.

Clinical characteristics of patients	**(n = 137)**
General	
Ethnicity	
Antillean	24
Surinamese	46
Dutch	67
Median age (IQR)	41 (34- 48)
Sex (%)	
Male	136 (99.3)
Female	1 (0.7)
Sexual behavior (%)	
MSM	114 (83.2)
MWMW	11 (8.0)
MSW	11 (8.0)
WSM	1 (0.7)
HIV status (%)	
Positive	56 (40.9)
Negative	75 (54.7)
Unknown	6 (4.4)
Serology	
RPR (%)	
High (1:32 ≤)	43 (31.4)
Middle (1:4- 1:16)	42 (30.7)
Low (1:1- 1:2)	26 (19.0)
Negative	24 (17.5)
Unknown	2 (1.5)
TPPA/EIA (%)	
Positive	102 (74.5)
Negative	4 (2.9)
Not tested	26 (18.9)
Unknown	5 (3.7)
Syphilis stage	
Primary syphilis (%)	108 (78.8)
Secondary syphilis (%)	29 (21.2)

MSM, men who have sex with men; MSMW, men who have sex with men and women; MSW, men who have sex with women; WSM, women who have sex with men.

In addition, a total of 6 allelic variants were found for locus *tp0136,* 11 for *tp0548* and 5 for *tp0705.* Of these, 7 were new allelic variants, 2 for the *tp0136* locus (numbers 19 and 20) and 5 for the *tp0548* locus (numbers 43-47). In total, 10 new allelic profiles were found with new and known allelic variants giving rise to a total of 10 new ST (numbered 56-65, Table 2).

Table 2 – Allelic profiles identified from the 103 fully typed samples in this study.

Sequence type	Allelic profile	23S rDNA (no. of samples)	Genetic group	Frequency
1	1.3.1	R8(41)/X(2)	SS14-like	43
2	1.1.1	S(11)/R8(7)/R9(2)	SS14-like	20
6	3.2.3	R(11)	Nichols-like	11
3	1.1.8	S(2)/R(2)	SS14-like	4
56*	19*.3.1	R8(4)	SS14-like	4
26	9.7.3	S(1)/R8(3)	Nichols-like	4
19	1.1.10	S(3)	SS14-like	3
28	1.17.9	R8(2)	SS14-like	2
57*	1.43*.1	R8(2)	SS14-like	2
58*	1.44*.1	R8(2)	SS14-like	2
60*	9.2.3	S(2)	Nichols-like	2
61*	1.3.3	R8(1)	SS14-like	1
62*	1.32.10	S(1)	SS14-like	1
63*	1.46*.3	S(1)	SS14-like	1
64*	1.47*.1	R8(1)	SS14-like	1
65*	20*.2.10	S(1)	Nichols-like	1
59*	7.45*.9	R8(1)	SS14-like	1

*New sequence types and allelic variants were added to the BIGSdb database for *Treponema pallidum* subspecies pallidum [13].

All allelic profiles found in this study were visualized in a phylogenetic tree using the concatenated sequences (Fig 3). Their genetic diversity within the two major genetic clades, SS14 and Nichols, is clearly shown with 13 SS14-like allelic profiles and 4 Nichols-like allelic profiles.

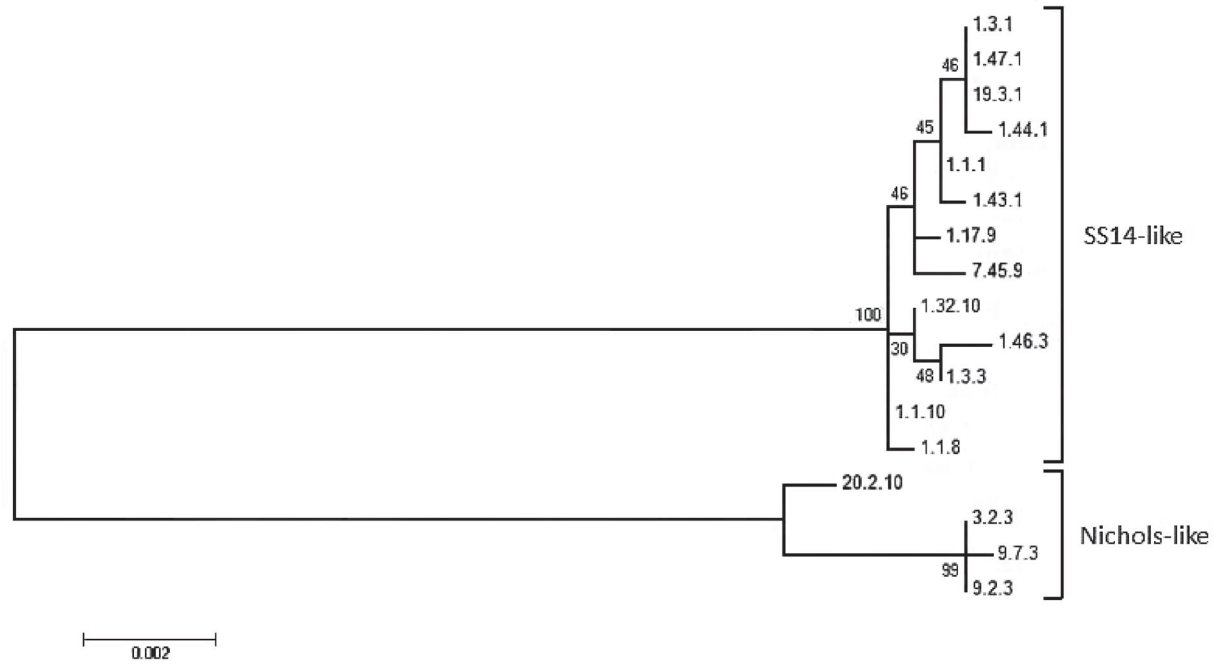

Figure 3 – Phylogenic tree (unrooted) of the 17 allelic profiles. The Maximum Likelihood tree was constructed using the Timura-Nei method in MEGA6.06 with 1000 bootstraps.

There was a significant difference in the typability of ulcer swab samples from patients with primary syphilis, of which the isolates were more often fully typed, as compared to patients with secondary syphilis (S1c). In addition, secondary syphilis patients have a significantly higher RPR titer than primary patients (unpublished data) and all fully typed secondary syphilis isolates (n = 18) contained SS14-like TPA strains (S2). No associations were found between the TPA types and ethnicity (Fig 2). Also, no significant differences were found based on the patient's HIV status, syphilitic stage or RPR titer of the typable isolates versus the non-typable isolates (S1).

There were 123/137 (90%) isolates successfully sequenced for the relevant parts of the 23S rRNA genes and 81% of all isolates contained one of the macrolide resistance mutations, 79% contained the A2058G mutation and 2% the A2059G mutation. None of the isolates carried both mutations. Both of the samples containing the A2059G mutation had allelic profile 1.1.1. The prevalence of macrolide resistance causing mutations in the 23S rRNA genes showed an increased trend over time from 53% in 2007 to 79% in 2017 (Fig 4).

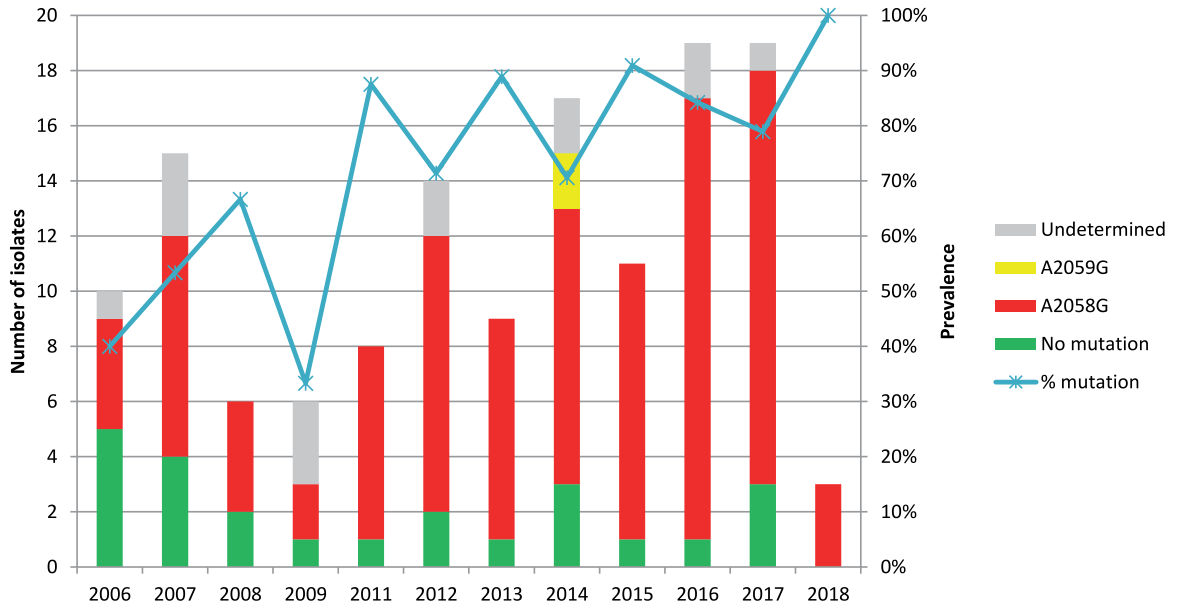

Figure 4 – Macrolide resistance causing mutations over time among the included isolates in Amsterdam between 2006 and 2018.

DISCUSSION

No TEN was found suggesting that bejel has not (yet) spread as a sexually transmitted disease to the Netherlands. By including Surinamese and Antillean patients who visited the Amsterdam STI clinic we aimed to increase the likelihood of finding strains from that geographical location which is close to Cuba where bejel was found within syphilis diagnosed patients [6]. An important limitation of this study is that we only had samples available from persons who reside in the Netherlands, probably all in the Amsterdam region. In addition, no data was available on the location of the acquisition of infection nor the ethnicity of the partners of these patients. Among these patients in Amsterdam with an Antillean or Surinamese ethnicity we did not see TEN infections, but it is still possible that bejel occurs in their countries of origin.

The most prevalent allelic profile found in this study was the SS14-like strain, 1.3.1 (42%), followed by 1.1.1 (19%) in concordance with similar TPA MLST studies [8, 12, 15, 16]. Until now, these two types have been found in every TPA MLST study [13], while all geographic locations (Czech Republic [16], Switzerland [8], France [12], Cuba [15], and the Netherlands) from which the samples were derived also have less common and unique allelic profiles suggesting the combination of global mixing populations and more local mixing of the hosts (S3). To investigate possible bias in the typable versus not (fully) typable samples these groups were compared based on ethnicity, RPR titer, HIV status and syphilis stage. Of these

variables, the syphilis stage of the patient showed a significant effect on the typability of the sample (S1c). Ulcer swab samples from patients with primary stage syphilis were significantly more likely to be fully typed than samples from secondary stage syphilis patients. A possible explanation may be that ulcers from primary syphilis patients contain more serous fluid as compared to the mostly dried and older ulcers present in secondary syphilis patients. Not surprisingly, patients with a secondary syphilis infection have a significant higher RPR titer than primary syphilis patients.

The use of the recently developed MLST for TPA strains allowed molecular characterization and subspecies determination with a high resolution for 103/137 (75%) of the selected samples. Obtaining a full type was more challenging for the older samples. Similar studies using this sequence based typing method obtained full types for up to 94% [15]. A previous molecular characterization study using the enhanced CDC method on isolates from Amsterdam showed a similar percentage of fully typed samples and a comparable typing distribution [17]. However, the enhanced CDC method combines partial sequence analysis of *tp0548* with the analysis of a restriction fragment length pattern of *tpr* genes and the number of acidic repeat proteins of 60bp each making it a complicated and occasionally unstable method [17] for TPA typing.

The genetic clade distribution found in this study were 83% SS14-like strains and 17% Nichols-like strains. This Nichols-like prevalence is more comparable to Argentina, Peru and Taiwan than to the countries closer to the Netherlands like France, Denmark, Ireland, the UK and the Czech Republic [18]. Worldwide only 117/1989 (5.9%) clinical samples were classified as Nichols-like [19]. This relatively high ratio of Nichols-like strains versus SS14-like strains was not explained by ethnicity as 2/16 (13%) fully typed samples from Antillean patients, 6/37 (16%) samples from Surinamese patients and 10/50 (20%) samples from Dutch patients, contained Nichols-like strains. Interestingly, all fully typed isolates from secondary stage syphilis patients contained TPA strains belonging to the SS14 clade (S2), whereas an association was found between secondary stage syphilis and Nichols-like strains in a previous study [12].

Seven new allelic variants and 10 new ST were found adding to the knowledge of TPA strain diversity. All allelic variants and ST were added to the pubMLST BIGSdb database of TPA which was recently published for the surveillance and epidemiology of syphilis [13].

Furthermore, the successfully sequenced part of the 23S rRNA genes from 123/137 (90%) samples showed the presence of A2058G and A2059G mutations, 79% and 2% respectively. When analyzing the samples over time an increase in macrolide resistant mutations was seen from 53% in 2007 to 79% in 2017. The samples were not selected to investigate this as the

distribution is not ideal, but the upward trend is significant and supports findings in earlier studies focusing on this topic [20].

The strain diversity found in this study reflects the local male STI clinic population which is a diverse, mixed group. Future studies should collect samples from the specific country of interest as ethnicity is not enough to investigate the epidemiology of bejel. Molecular characterization of the TPA bacteria remains important for network analyses and uncovering pathogenic associations with certain genetic variants.

Funding

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SUPPLEMENTARY DATA

Supplementary Table 1 (ST1) – Fisher's Exact and Pearson Chi-squared Tests for ethnicity, RPR titer, syphilis stage and HIV status versus typability of the samples.
S1a) Table. Pearson Chi-squared test for ethnicity and typability of samples. S1b) Table. Fisher's Exact test for RPR titer and typability of samples. S1c) Table. Pearson Chi-squared test for syphilis stage and typability of samples. S1d) Table. Fisher's Exact test for HIV status and typability of samples.

A)		Fully typed		**Total**
Ethnicity		No	Yes	
	Antillean	8	16	24
	Dutch	17	50	67
	Surinamese	9	37	46
Total		34	103	137
	Pearson Chi-Square	*p = 0.444*		

B)		Fully typed		**Total**
VDRL titer		No	Yes	
	high	9	34	43
	low	5	21	26
	middle	12	30	42
	negative	7	17	24
	unknown	1	1	2
Total		34	103	137
	Fisher's Exact Test	*p = 0.651*		

C)		Fully typed		**Total**
Syphilis stage		No	Yes	
	Primary stage	22	86	108
	Secondary stage	12	17	29
Total		34	103	137
	Pearson Chi-Square	*p = 0.02*		

D)		Fully typed		**Total**
HIV status		No	Yes	
	negative	16	59	75
	positive	16	40	56
	unknown	2	4	6
Total		34	103	137
	Fisher's Exact Test	*p = 0.572*		

Supplementary Table 2 (ST2) – Fisher's Exact test for syphilis stage versus genetic *Treponema pallidum* subspecies *pallidum* clade.

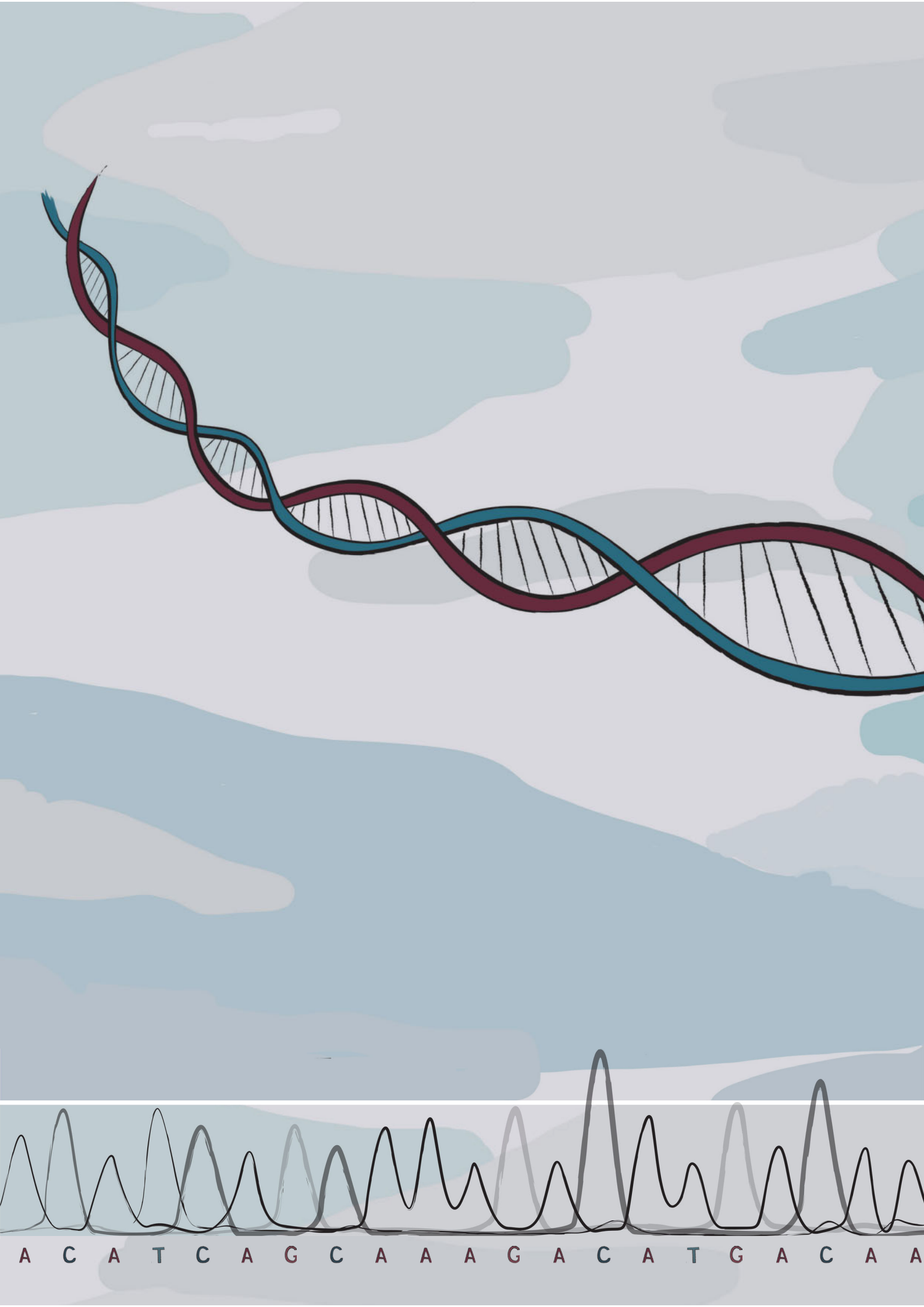
		TPA genetic clade			**Total**
Syphilis stage		Unknown	Nichols-like	SS14-like	
	Primary stage	22	18	68	108
	Secondary stage	12	0	17	29
Total		34	18	85	137
	Fisher's Exact Test	*p = 0.006*			

Supplementary Table 3 (ST3) – Overview of full MLST *Treponema pallidum* subspecies *pallidum* types found in the public database [13].

	Country							**Total**
type	Australia	Cuba	Czech Republic	France	Portugal	Switzer-land	The Netherlands	
1.1.1	0	1	19	18	7	9	20	**74**
1.1.10	0	1	0	0	0	0	3	**4**
1.1.11	0	0	0	1	0	0	0	**1**
1.1.13	0	0	0	1	0	0	0	**1**
1.1.16	0	0	1	0	0	0	0	**1**
1.1.3	0	0	5	0	0	2	0	**7**
1.1.8	0	0	31	5	0	0	4	**40**
1.1.9	0	0	0	1	0	0	0	**1**
1.11.8	0	0	0	3	0	0	0	**3**
1.17.9	0	0	0	2	0	0	2	**4**
1.18.1	0	0	0	1	0	0	0	**1**
1.19.1	0	0	0	1	0	0	0	**1**
1.22.12	0	0	0	1	0	0	0	**1**
1.23.1	0	0	0	2	0	0	0	**2**
1.26.1	0	0	17	0	0	0	0	**17**
1.28.1	0	0	1	0	0	0	0	**1**
1.29.1	0	0	1	0	0	0	0	**1**
1.3.1	0	66	60	80	13	17	43	**279**
1.3.3	0	0	0	0	0	0	1	**1**
1.3.5	0	0	0	0	0	1	0	**1**
1.3.7	0	0	0	0	0	1	0	**1**
1.31.1	0	0	1	0	0	0	0	**1**
1.32.1	0	0	1	0	0	0	0	**1**
1.32.10	0	0	0	0	0	0	1	**1**
1.36.1	0	0	4	0	0	0	0	**4**
1.4.1	0	0	1	0	0	3	0	**4**
1.43.1	0	0	0	0	0	0	2	**2**
1.44.1	0	0	0	0	0	0	2	**2**
1.46.3	0	0	0	0	0	0	1	**1**
1.47.1	0	0	0	0	0	0	1	**1**
1.5.1	0	0	0	0	0	2	0	**2**
1.8.1	0	0	0	0	0	1	0	**1**
1.9.1	0	0	0	0	0	1	0	**1**
13.1.1	0	0	0	0	0	1	0	**1**

Supplementary Table 3 (ST3) – Continued

14.3.1	0	0	0	1	0	0	0	**1**
15.7.3	0	5	0	0	0	0	0	**5**
16.3.1	0	1	0	0	0	0	0	**1**
17.1.1	0	0	1	0	0	0	0	**1**
18.1.1	0	0	1	0	0	0	0	**1**
19.3.1	0	0	0	0	0	0	4	**4**
2.1.2	0	0	0	1	0	2	0	**3**
20.2.10	0	0	0	0	0	0	1	**1**
3.2.3	0	0	0	1	0	2	11	**14**
4.1.1	0	0	1	0	0	0	0	**1**
4.3.1	0	0	0	1	0	0	0	**1**
5.3.8	0	0	0	1	0	0	0	**1**
6.3.1	0	0	0	1	0	0	0	**1**
7.1.9	0	0	0	0	5	0	0	**5**
7.45.9	0	0	0	0	0	0	1	**1**
9.14.3	1	0	0	0	0	0	0	**1**
9.2.3	0	0	0	0	0	0	2	**2**
9.20.3	0	0	0	2	0	0	0	**2**
9.24.8	0	2	0	0	0	0	0	**2**
9.25.3	0	1	0	0	0	0	0	**1**
9.7.3	0	0	3	7	0	0	4	**14**
Total	**1**	**77**	**148**	**131**	**25**	**42**	**103**	**527**


A C A T C A G C A A A G A C A T G A C A A

Chapter 8

Increased clonality among *Neisseria gonorrhoeae* isolates during the COVID-19 pandemic in Amsterdam, the Netherlands

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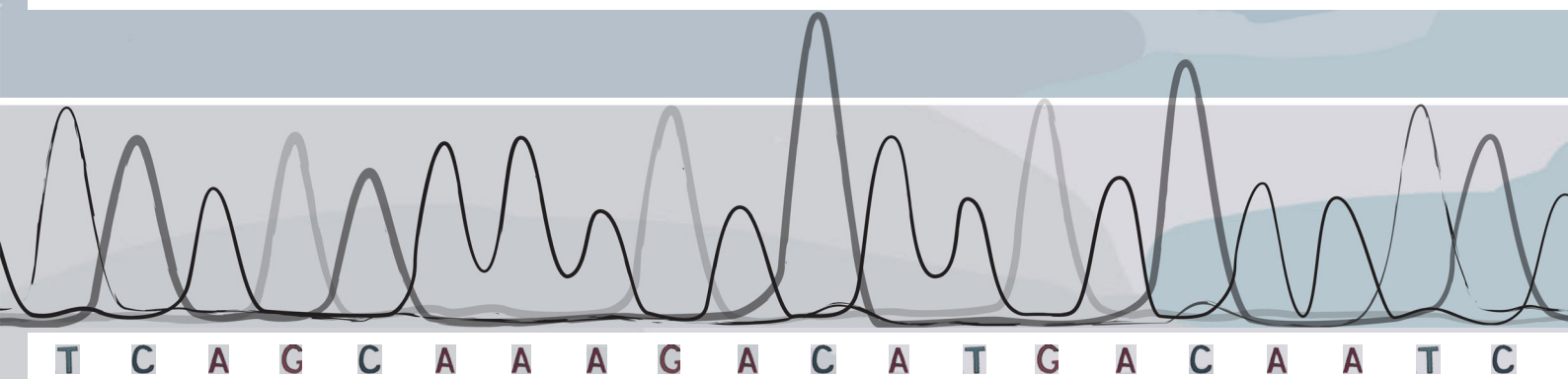

ABSTRACT

Distancing measures during the COVID-19 lockdown led to a temporary decrease of casual sex partners among clients of the Centre for Sexual Health (CSH) of Amsterdam, the Netherlands. We investigated the effect of this change on the genotypic and phenotypic distribution of *Neisseria gonorrhoeae* (*Ng*) isolates from CSH patients. From each *Ng*-positive patient we sequenced one isolate, resulting in 322 isolates which constituted two groups: 181 isolates cultured from January 15th-February 29th 2020 (before the first lockdown) and 141 cultured from May 15th-June 30th 2020 (during the first lockdown). Patient characteristics showed significantly more symptomatic patients and significantly less reported sex partners during the lockdown. Phenotypic data showed an increase in low-level azithromycin resistance and ceftriaxone susceptibility during the lockdown, and this remained after the study period. The diversity in sequence types (STs) slightly decreased during the lockdown. A shift occurred from ST 8156 being predominant before lockdown to ST 9362 during lockdown and a remarkably low median SNP distance of 17 SNPs was found between ST 9362 isolates obtained during lockdown. These findings reflect restricted travel and the change in sexual behaviour of CSH clients during the lockdown, with a potentially increased local transmission of the ST 9362 strain during this period, which led to genotypic and phenotypic changes in the *Ng* population. This shows that public health measures have far-reaching consequences and should be considered in the surveillance of other infectious diseases.

Impact statement

Neisseria gonorrhoeae (Ng) is a worldwide prevalent sexually transmitted infection. The molecular epidemiology of this bacterium is closely monitored providing information on circulation genotypes associated with antimicrobial resistance over time. However, major events like the COVID-19 pandemic causes behavioural changes in patient populations and impact infectious diseases. By analysing patients who visited the Centre of Sexual Health in Amsterdam before (January – February) and during (May – June) the first lockdown in 2020 in the Netherlands, we found a shift in the genotypic and phenotypic distribution of *Ng* and the emergence of a highly clonal *Ng* strain, suggesting local transmission. This supports previous research, that found reduced numbers of sex with casual partners among this population during the lockdown. This work shows that public health measures have an impact on the epidemiology of other infectious diseases, which should be taken into consideration for public health surveillance.

Data summary

Raw sequence data are available in the European Nucleotide Archive under project number PRJEB55899 and individual accession numbers can be found in Table S1. Assemblies are available in the PubMLST Neisseria database (https://pubmlst.org/organisms/neisseria-spp). PubMLST identifiers and additional metadata can also be found in Table S1. The bioinformatic pipeline used in this study can be found on Github (https://github.com/jolindadekorne/Diversity-In-Neisseria-Gonorrhoeae-during-Lockdown).

INTRODUCTION

The COVID-19 pandemic has had an extraordinary global impact on public health (1). Also, the epidemiology of sexually transmitted infections (STIs) has been impacted by the COVID-19 pandemic (2). Gonorrhoea, caused by the sexually transmitted pathogen *Neisseria gonorrhoeae (Ng),* is one of the most prevalent bacterial STIs worldwide and its prevalence is globally on the rise (3). This rising trend has also been reported in Europe, including the Netherlands, where before the pandemic the prevalence of gonorrhoea and other bacterial STIs increased, especially among men who have sex with men (MSM) (4-6). However, interpreting the number of STI cases during the COVID-19 pandemic has been challenging, since social measures and large scale COVID-19 testing temporarily restricted STI testing at Centres for Sexual Health (CSH) (4, 7).

In the Netherlands, efforts to reduce and contain the transmission of SARS-CoV-2 started with gradual measures since the first notified infection on February 27th, 2020. The first lockdown with additional measures was put into place on March 16th 2020, from then all events were cancelled, gatherings of three and more persons were discouraged and all stores (except grocery stores), schools and leisure areas were closed (Table S2). In general, 1.5 meters of physical distance was enforced both inside and outside, and no more than three adult visitors were allowed in a household per day. International travel was restricted to essential travel only and also nationally, people were encouraged to stay at home. From mid-May onwards, schools, stores, museums, bars and restaurants were gradually reopened with restrictions. However, during this time, any form of travel and especially international travel remained highly reduced (8). The healthcare at the CSH in Amsterdam was also impacted by the restrictions of the lockdown (Table S2). Between March 23rd and June 1st routine HIV/STI testing was halted for asymptomatic clients unless urgent PrEP prescription was needed (9).

The perceived risk of SARS-CoV-2 infection and public health measures were found to be associated with a change in sexual behaviour (10, 11). A recent study from Bilsen et al. (2021) found that distancing measures during lockdown led to a temporary decrease in casual sex partners and a relatively low STI positivity rate among clients of the CSH of Amsterdam (9). Besides the influence of this behavioural change on number of STI cases, it could also have had an influence on STI epidemiology due to changes in transmission networks. In this study, we aimed to investigate the influence of the change in sexual behaviour of CSH clients during the first lockdown in Amsterdam on the genotypic and phenotypic distribution of *Ng* isolates from CSH patients.

MATERIALS AND METHODS

Isolate selection

Two study periods of equal duration were selected before and during the lockdown based on similarity of patient groups regarding sex and sexual orientation. The study periods were also chosen based on the number of *Ng* isolates obtained during each period, because during the first eight weeks of the lockdown, access to the CSH was highly restricted which subsequently strongly reduced the number of gonorrhoea cases. *Ng* isolates from all culture-positive patients who visited the CSH between January 15th and February 29th and between May 15th and June 30th in 2020 were included. The period January 15th - February 29th 2020 will be referred to as "before lockdown" and the period May 15th - June 30th 2020 will be referred to as "during lockdown" throughout this study. According to our routine clinical practice, *Ng*-positive patients always got treated with a single intramuscular dose of 1 gram ceftriaxone, irrespective of having symptoms. Therefore, patients who had *Ng* both before and during lockdown were included in both sets of patient groups, since these can be considered as two separate infection episodes. From each *Ng*-positive patient, one isolate was selected with prioritisation of the rectal isolate in case multiple isolates were obtained from a single patient. To prevent SARS-CoV-2 transmission at the CSH, the sampling policy no longer included pharyngeal sampling during the lockdown. Therefore, pharyngeal *Ng* isolates obtained before the lockdown were not included in this study.

Phenotypic analysis

At the Public Health Laboratory, minimum inhibitory concentrations (MICs) for azithromycin and ceftriaxone are routinely determined for all *Ng* isolates obtained from CSH patients, using E-tests according to manufacturer's instruction (bioMerieux SA). The clinical breakpoint used in this study for ceftriaxone resistance was MIC > 0.125 mg/L and for azithromycin MIC ≥ 1 mg/L (ECOFF), according to EUCAST guidelines v12.

To determine whether phenotypic trends observed during the study period were already seen before the study period or still present in the months afterwards, azithromycin and ceftriaxone MIC distributions of anogenital isolates obtained 2.5 months before (15 August – 30 September 2019) and 2.5 months after the study period (15 September – 1 November 2020) were compared to MICs of isolates obtained during the study period. The interval of 2.5 months was chosen because the study periods before- and during lockdown also had an interval of 2.5 months. Pharyngeal isolates were excluded from the phenotypic data because pharyngeal isolates were not included during the study period.

Isolate preparation and WGS squencing

All selected samples were taken from the -80°C freezer and grown overnight on chocolate blood agar plates. DNA was extracted from pure cultures in DNA/RNA shield buffer using the ZymoBIOMICS TM Magbead DNA kit (ZYMO RESEARCH). The Nextera XT DNA Library Preparation kit with Integrated DNA Technologies for Illumina DNA/RNA Unique Dual Indexes (Illumina) was used for library preparation. WGS was performed by 150 base-pairs (bp) paired-end sequencing using Illumina NovaSeq 6000 at the sequencing facility BaseClear B.V. (L457; NEN-EN-ISO/IEC 17025).

Quality control and assembly

Raw reads were filtered and trimmed using fastp v0.20.1 (12). The mean coverage depth and the percentage of covered reference genome bases were calculated with the SAMtools package v1.15 by first mapping the reads to reference genome FA1090 (NC_002946.1) with BWA-MEM2 v2.1 (13). Genomes were assembled using the 'isolate' option in SPAdes v.3.15.3 (14). QUAST v5.0.2 was used to assess the quality of the assembly and to identify assemblies with aberrant assembly lengths or GC content, which could be indications of read contamination (15). For these isolates, reads not belonging to *Ng* (taxid:485) were identified and filtered out with Kraken2 v2.1.1 (16). Filtered reads were assembled again and quality was again assessed.

Typing and determination of SNP distances

All assemblies were annotated automatically by uploading them to the PubMLST Neisseria database. Multi-locus sequence types (MLST) were extracted and novel MLSTs were assigned novel STs (17). Snippy v.4.6.0 (https://github.com/tseemann/snippy) was used for variant calling, by first mapping reads to reference genome FA1090 followed by identifying SNPs between the isolate and reference genome. Only SNPs with a minimum base quality of 13, a minimum read coverage of 10x and a read concordance of 90% were reported (default settings). The 'core' option of Snippy was used to create a core genome alignment of all isolates. To determine genetic diversity between isolates of the *Ng* population before or during the lockdown with maximimum resolution, SNP distances were determined in a pairwise manner between all possible combinations of isolates with identical STs without prior recombination filtering using snp-dists v.0.7.0 (https://github.com/tseemann/snp-dists). In addition, recombination filtering was applied and a recombination filtered core SNP tree was created using Gubbins v2.4.1. Isolate pairs that differed <10 recombination filtered SNPs were identified, as this is the threshold previously defined for identical isolates that are putatively transmitted between individuals (18, 19). Transmission clusters were defined as networks of isolates that differ <10 recombination filtered SNPs. The bioinformatic pipeline was managed using Snakemake v7.2.1 (20).

Statistical analyses

Sociodemographic and clinical data were extracted from electronic patient files. Chi square or Fisher's exact tests were used to compare participant characteristics from before and during lockdown and to analyse its associations with the most prevalent ST during the lockdown. A p-value ≤0.05 was deemed significant. Diversity in MLSTs was assessed by calculating the Simpson's diversity index, using the R package abdiv. SNP distances between groups were compared with the Wilcoxon rank test, for which a p-value ≤0.001 was deemed significant. Data was analysed using RStudio (version 1.2.5033).

Ethics statement

All clients of the CSH in Amsterdam were informed of the "opt-out" system regarding research on remnants of patient material. All data were pseudonymised before analysis.

RESULTS

Patient characteristics

A total of 322 *Ng* isolates were included in the study, consisting of 181 isolates before lockdown and 141 during lockdown. Demographic characteristics of the patient groups before (181 patients) and during (141 patients) lockdown were comparable regarding sex and sexual orientation, as expected since study periods were chosen based on similarity of these characteristics. Five patients were *Ng*-positive both before and during lockdown and therefore included in both groups. The patient groups significantly differed in number of sex partners (p=0.009) and symptomatology (p=0.006) (Table 1). During the lockdown, patients reported fewer sex partners in the past six months and a higher proportion of patients was symptomatic, compared to before the lockdown.

Table 1 – Clinical and demographic data of patients before (January 15th – February 29th) and during (May 15th – June 30th) the lockdown in 2020.

	Before lockdown N = 181 (%)	**During lockdown N = 141 (%)**	**OR/ P-value**
Sex			0.966
Male	171 (94)	133 (94)	
Female	10 (6)	8 (6)	
Age			0.421
<25	34 (19)	36 (26)	
25-34	79 (44)	62 (44)	
35-44	47 (26)	30 (21)	
≥45	21 (12)	13 (9)	
Anatomical location			0.850[a]
Cervix	3 (2)	3 (2)	
Anus	117 (65)	87 (62)	
Urethra	54 (30)	47 (33)	
Vagina	7 (4)	4 (3)	
Country of origin			0.697[a]
The Netherlands	93 (51)	65 (46)	
Suriname + Dutch Antilles	17 (9)	19 (13)	
Europe + Turkey	28 (15)	28 (20)	
Middle + South America	20 (11)	11 (8)	
Asia	14 (8)	10 (7)	
Africa	7 (4)	5 (4)	
Other	2 (1)	2 (1)	
Unknown	0 (0)	2 (1)	
Sexual orientation			0.707[a]
MSM	158 (86)	122 (87)	
MSW	11 (6)	11 (8)	
Female	10 (6)	8 (6)	
Transgender	2 (1)	0 (0)	
Sex work			
Yes	15 (8)	11 (8)	0.859
No	166 (92)	130 (92)	
Number of sex partners in last six months			0.009*
0-1	6 (3)	10 (7)	
2-4	38 (21)	47 (33)	
5-9	46 (25)	37 (26)	
10-19	32 (18)	24 (17)	
20-49	43 (24)	16 (11)	
≥50	15 (8)	7 (5)	
Symptomaticity			0.006*
Symptomatic	48 (27)	58 (41)	
Asymptomatic	133 (73)	84 (59)	

[a] Fisher's Exact Test
* $p \leq 0.05$

Distribution of STs changed during lockdown

All 322 isolates were typed according to the ST scheme. Diversity in STs slightly decreased during the lockdown, from a Simpson's diversity index of 0.94 before lockdown to 0.92 during lockdown. The number of different STs identified was 34 before lockdown and 30 during lockdown (Figure 1). The majority of the types were found less than five times, which was the case in 22/34 (65%) STs before lockdown and 21/30 (70%) during lockdown. Before lockdown, a variety of STs was prevalent among the population, with ST 8156 predominantly found in 22/181 (12%) of the isolates. During lockdown, ST 8156 prevalence was reduced to 13/141 (9%), whereas ST 9362 became predominant. ST 9362 went from being present in 3/181 (2%) of the isolates before the lockdown to 29/141 (21%) during the lockdown (Figure 1). ST 9362 was significantly associated with MSM (Table S3). A decline was also found for ST 7827, which went from being present in 10/181 (6%) isolates before the lockdown to 3/141 (2%) isolates during the lockdown. The low prevalent STs 1587, 1599, 7363, 9363, 7827 and 10314 were no longer found more than five times during the lockdown, whereas STs 7359, 9362 and 15183 were found less than five times before the lockdown and their prevalence increased during lockdown.

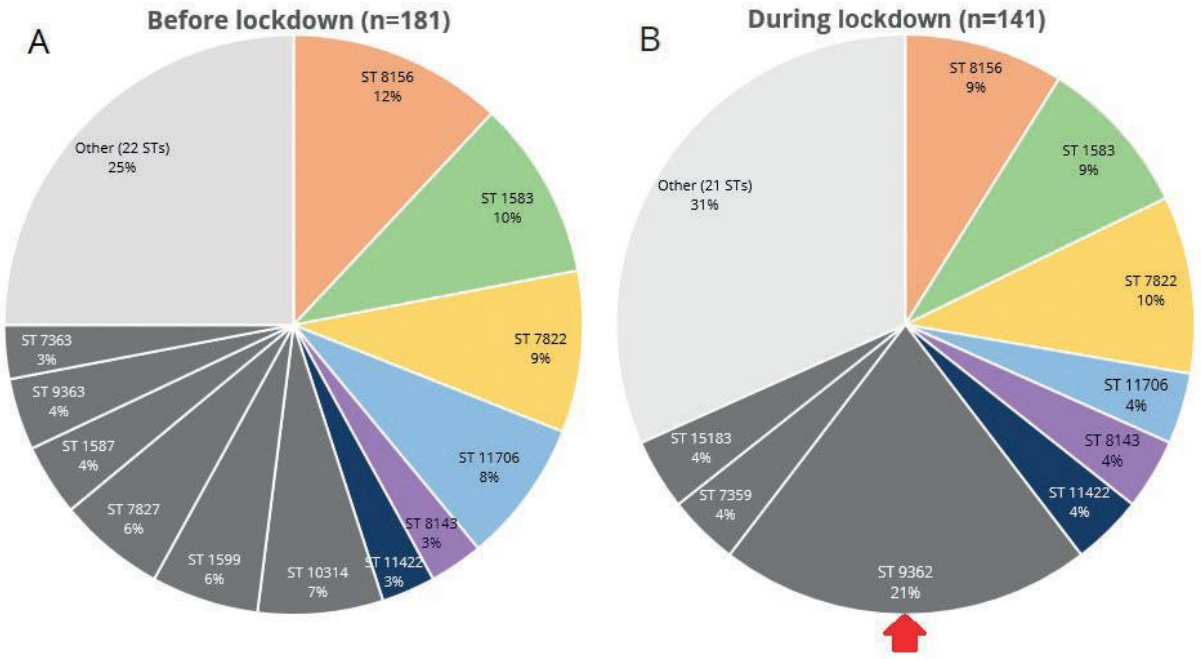

Figure 1 – ST distribution before (A) and during (B) lockdown. Prevalence are shown for STs that were found >5 times in the isolates before- or during lockdown. STs that were found <5 times were categorised as 'Other'. Coloured STs were found >5 times before- and during the lockdown, whereas grey STs are only found >5 times in one of the periods. The arrow indicates ST 9362, which was the predominant ST during lockdown

SNP distances between isolates with identical STs significantly decreased during lockdown

Pairwise SNP distances were determined between isolates obtained before- and during lockdown. SNP distances significantly decreased between isolates with identical STs during lockdown, with median SNP distances of 503 before lockdown and 98 during lockdown (Figure 2A). When further categorizing on ST, a remarkably low median SNP distance of 17 SNPs was found between ST 9362 isolates obtained during the lockdown, despite the high number of ST 9362 isolates found in that period (Figure 2B and 2C). Low SNP distances were also found between ST 15183 isolates (median 29 SNPs), however, this ST was found only six times during the lockdown. Remarkably, diverse SNP distances were found between isolates with STs 7363, 7822, 7827 and 8143 (Figure 2C). Using recombination filtered SNP distances, potential transmission clusters were defined which consisted of networks of isolates that differed <10 filtered SNPs. The percentage of isolates that belonged to a cluster was significantly higher during lockdown (75%; 106/141) compared to before lockdown (63%; 114/181) (p=0.020, Chi-square test). A total of 53 clusters were identified, of which 17 clusters contained more than 3 isolates (Figure 3). The largest cluster contained 28 isolates with ST 9362, of which 96% (27/28) was obtained during the lockdown.

Association between genotype and phenotype

The majority of isolates within a ST had azithromycin and ceftriaxone MICs within a range of 2 dilutions (Figure 4A, 4B, 4C), showing an association between genotype and phenotype. Azithromycin resistance (MIC ≥ 1 mg/L) was predominantly found in isolates belonging to the STs 9362, 9363 and 11422. Mosaicism in the *mtrR* gene and/or the mtrCDE operon were found to be the resistance determinants in these three STs (Figure 4D). Azithromycin resistance-associated 23S rRNA mutations were not found. The highest ceftriaxone MICs were found for ST 7827 isolates, which predominantly carried the *penA* A501V mutation. Isolates belonging to STs 8156 and 9362 were highly susceptible to ceftriaxone. No ceftriaxone resistant isolates were identified in this study.

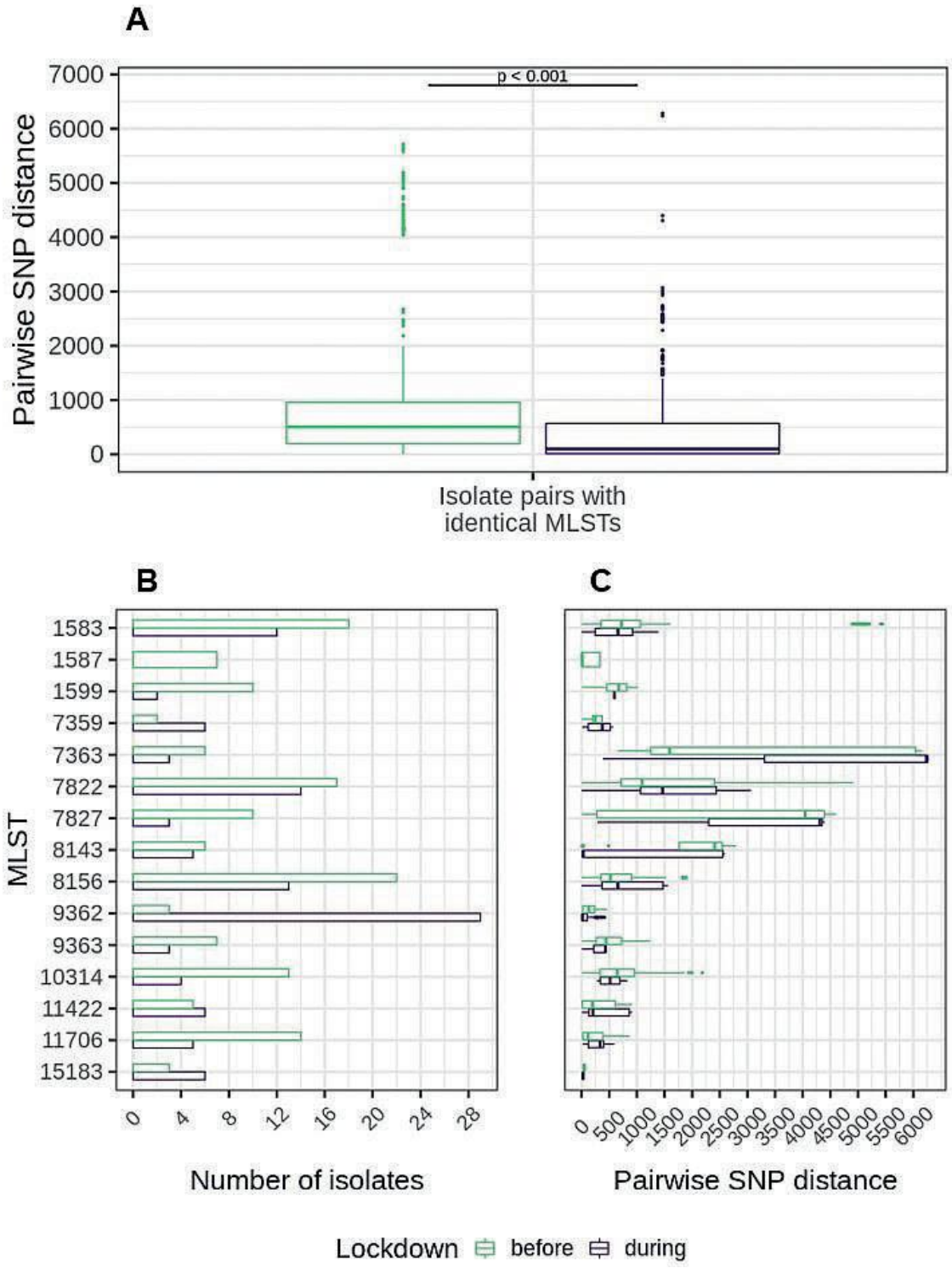

Figure 2 – Pairwise SNP distances between isolates obtained before- or during lockdown. A. Boxplots of pairwise SNP distances between isolates with identical STs (901 pairwise comparisons before lockdown, 742 during lockdown). B. Number of isolates per ST before- or during the lockdown. STs found <5 times were not shown. C. Boxplots of pairwise SNP distances between isolates within ST groups before or during lockdown.

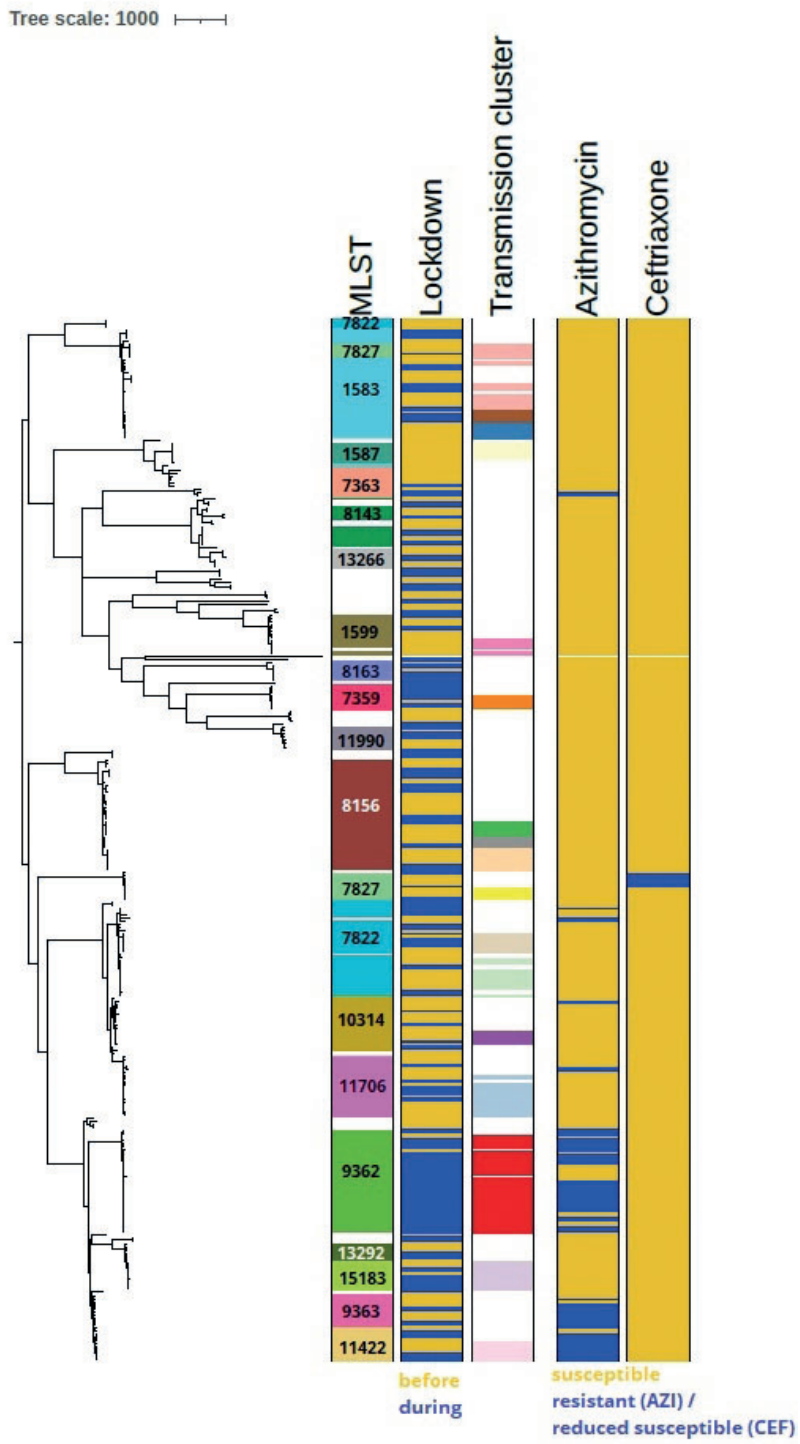

Figure 3 – Mid-point rooted core-SNP phylogenetic tree containing all 322 isolates isolated before and during lockdown with corresponding metadata. SNPs were determined by aligning to reference genome FA1090. Transmission clusters were defined as networks of isolates with <10 recombination filtered SNPs between them.

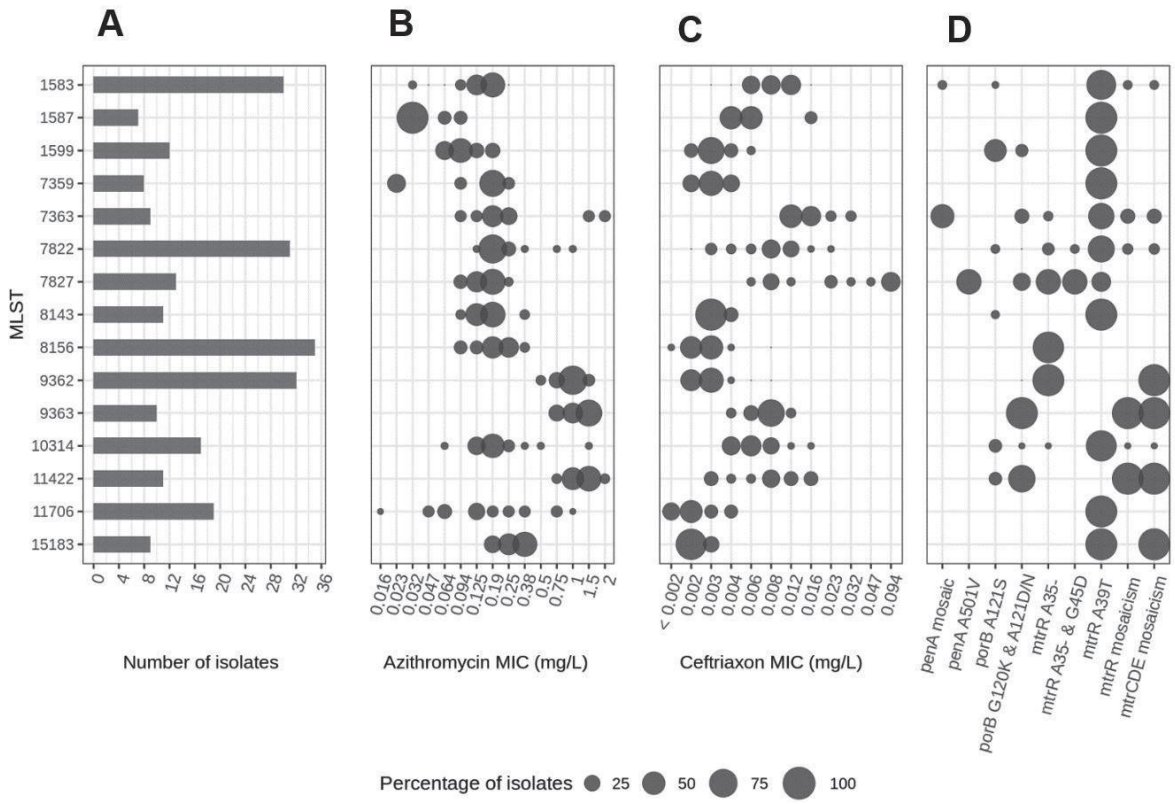

Figure 4 – Distribution of phenotypes and resistance determinants among STs. A. Total number of isolates per ST (numbers before- and during lockdown were taken together). B. Prevalence of azithromycin MICs per ST. C. Prevalence of ceftriaxone MICs per ST. D. Prevalence of azithromycin- and ceftriaxone resistance determinants in *penA, porB, mtrR* genes and the mtrCDE operon per ST. Only STs with >5 isolates are shown.

Increased azithromycin resistance and ceftriaxone susceptibility during the lockdown

The proportion of isolates with low-level azithromycin resistance and ceftriaxone susceptibility increased during the lockdown (Figure 5A and B). To determine whether this increasing trend was already seen before the study period or was still present in the months afterwards, we compared MIC distribution of isolates before and during the lockdown with MIC data regarding 163 anogenital isolates cultured 2.5 months before our study period and 167 anogenital isolates cultured 2.5 months after our study period. The azithromycin MIC distribution showed that isolates with high-level azithromycin resistance (MIC >4 mg/L) already disappeared before the study period (Figure 5A). However, the proportion of low-level resistant isolates (MIC 1-4 mg/L) strongly increased during the lockdown and the high prevalence remained 2.5 months after the study period. An opposite trend was observed for ceftriaxone MICs, with an increase in ceftriaxone susceptible isolates and a decrease in intermediate susceptible (MIC 0.012-0.064 mg/L) and reduced susceptible (MIC >0.064 mg/L) isolates over time. Highly susceptible isolates were predominant during lockdown. Reduced susceptible isolates were no longer found after the lockdown (Figure 5B).

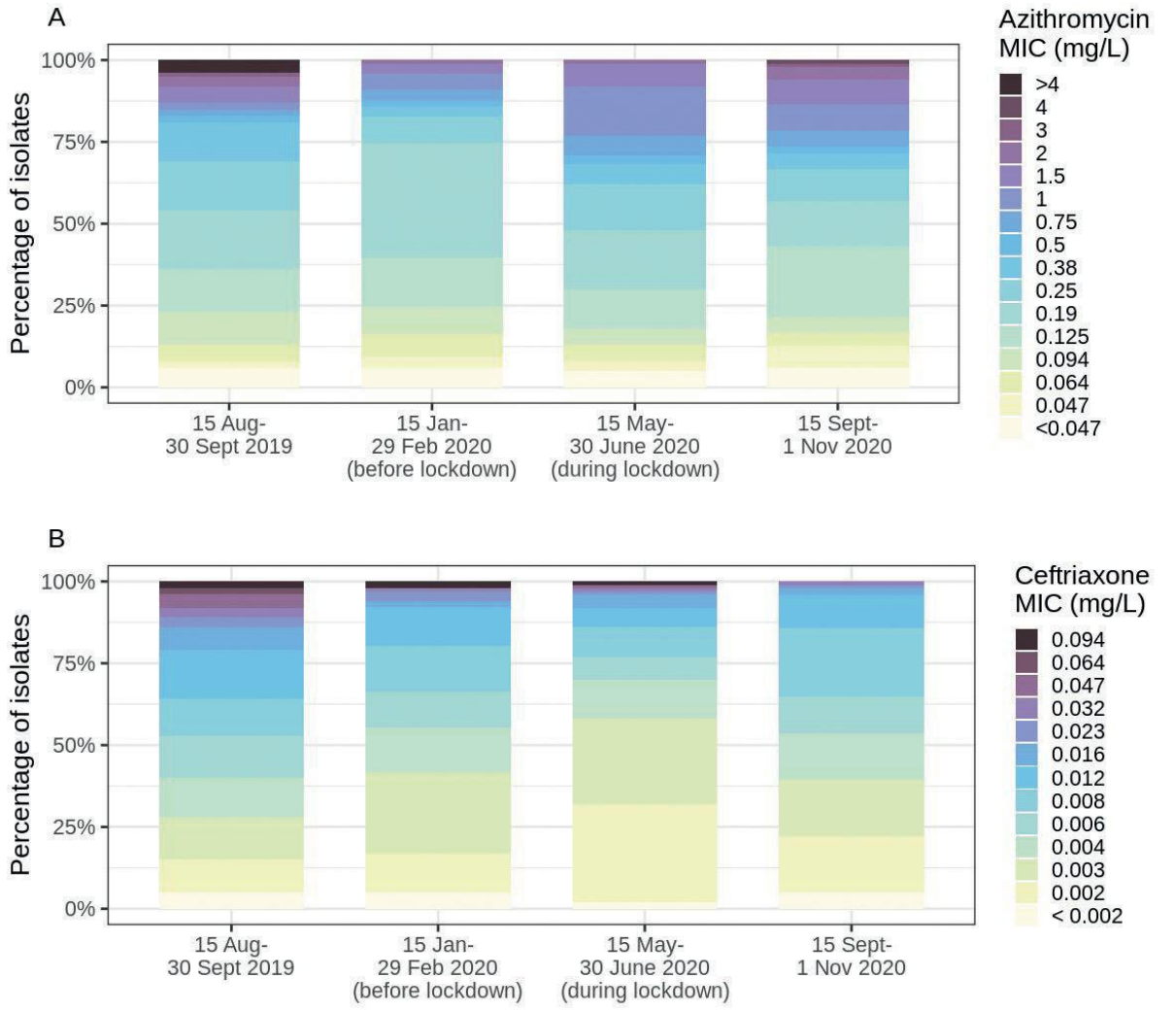

Figure 5 – Distribution of azithromycin (A) and ceftriaxone (B) MICs before, during and after the study periods with intervals of 2.5 months.

DISCUSSION

In this study we investigated whether changed sexual behaviour of CSH clients during the first lockdown in Amsterdam, the Netherlands, had led to changes in genotypic and phenotypic distribution within the *Ng* population. A change was observed in ST distribution with the largest shift for ST 9362, which went from a prevalence of 2% before- to 21% during the lockdown and was exclusively present among MSM. When comparing the ST distribution found in our study to the one found in 2018 in Amsterdam as part of the European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP), the ST distribution before lockdown was much more similar to the one in 2018 than the ST distribution found during the lockdown (Figure S1) (21). Most STs that were prevalent in the period before the lockdown were already prevalent in 2018 and no isolates from the Netherlands in the 2018 collection belonged to ST

9362. Therefore, the rapid emergence of ST 9362 does not just reflect a natural trend in ST patterns but may be a result of the COVID-19 lockdown and distance measures. In addition, the limited genetic diversity found among the ST 9362 isolates during the lockdown also suggested extensive local transmission of this strain during the lockdown period. This could be explained by the restricted national- and international travel, which logically reduced introduction of *Ng* isolates from sexual networks outside of Amsterdam and the Netherlands. In addition, the significantly reduced number of casual sex partners during the lockdown, as described by Van Bilsen et al (9), may have resulted in a more concentrated sexual network leading to this local transmission. The behavioural changes were only temporary (22), whereas phenotypic data showed that the prevalence of low-level azithromycin resistance stayed high after the study period (Figure 5A), suggesting that ST 9362 remained prevalent. Additional genotypic data is needed to confirm whether easing of measures has led to a decrease of this ST over time or whether this ST is still predominant in Amsterdam.

ST 9362 was associated with low-level azithromycin resistance and ceftriaxone susceptibility and its emergence therefore drove the observed shift in phenotypic data, with increased low-level resistance to azithromycin and increased susceptibility to ceftriaxone during the lockdown. This is further enhanced by the decline in STs associated with azithromycin susceptibility during the lockdown, such as STs 1587, 1599 and 10314 (Figure 2B, 4). The phenotypic shift towards increased low-level azithromycin resistance in *Ng* that has been observed in the Netherlands over the years 2019 and 2020 has also been observed in other European countries and worldwide (21, 23). European genomic surveillance, which formed part of the Euro-GASP, described that this phenotypic change was caused by the emergence of STs 9363 and 11422, both carrying mosaic *mtrR* and *mtrD* genes which have been associated with azithromycin resistance (24). ST 9363 became the predominant MLST in Europe in 2018 (21). Interestingly, in our study we found ST 9362 being mainly responsible for the increased azithromycin MICs instead, which only carried the mosaic *mtrD* gene and not the *mtrR* mosaic gene. However, we might have missed ST 9363 isolates because our study did not include pharyngeal swabs, and ST 9363 was associated with pharyngeal *Ng* infections in MSM (21). This could have led to a bias in the MLST distribution that we observed in our study population, however it would only enforce the observed phenotypic shift towards increased azithromycin MICs.

The increased ceftriaxone susceptibility observed in this study was reflected by a decline in ST 7827, which emerged among the Dutch *Ng* population between 2017 and 2019, and was associated with ceftriaxone reduced susceptibility (25). The trend towards increased ceftriaxone susceptibility observed in this study was also supported by European surveillance data (21).

During the lockdown, more symptomatic patients were seen at the CSH, which may be have been a temporary effect from the prioritisation of symptomatic patients during the strict lockdown, due to the reduced capacity for STI testing. To our knowledge, no association has been found between having symptoms and *Ng* genotype, thus we do not expect a major effect of this change in patient population on the results of this study. Although we cannot exclude that the composition of the patient population influenced the *Ng* epidemiology, we tried to minimise this effect by selecting study periods based on number of isolates that were obtained at the CSH and the similarity of patient populations regarding sex and sexual orientation. Only few isolates were obtained in the first months of the lockdown, due to precautions taken at the CSH, therefore this period was not chosen. For these reasons, we selected May and June as period 'during lockdown' instead of the first months of the lockdown (second half of March and April), in which the number of obtained *Ng* isolates was strongly reduced and the patient population was much less representative. Since we included isolates from all of the patients who visited the CSH during the study periods, we did not introduce any selection bias in our study.

This study did not include pharyngeal isolates because pharyngeal swabs were no longer taken during lockdown to prevent SARS-CoV-2 transmission. Treatment failures often occurred with pharyngeal *Ng* infections (26). Pharyngeal *Ng* infections are also important because of the possibility for AMR development due to possible exchange of genetic material between *Neisseria* species at the oropharyngeal site (27). The Euro-GASP study indeed found an association between pharyngeal isolates from MSM and low-level resistance to azithromycin (21). The lack of pharyngeal isolates in this study could therefore have led to missed isolates with elevated MICs.

In conclusion, a major change in genotypic and phenotypic distribution was identified during the first COVID-19 lockdown among *Ng* isolates from CSH patients in Amsterdam. The phenotypic shift towards increased azithromycin resistance and increased ceftriaxone susceptibility is in line with European surveillance data, however, the lockdown might have enhanced these trends as it has led to expansion of the ST 9362 strain with low-level azithromycin resistance and high ceftriaxone susceptibility. The low SNP distances between ST 9362 isolates suggested local transmission of this strain in Amsterdam, reflecting restricted travel and the previously identified change in sexual behaviour and subsequent more local STI transmission networks during the lockdown (9). This shows that public health measures also have consequences for the epidemiology of other infectious diseases which should be taken in consideration for public health surveillance.

Ethics statement

Clients of the SHC Amsterdam were informed of the "opt-out" system regarding research on remnants of patient material. All data were pseudonymized before analysis. The need for informed consent was waived by the Medical Ethical Committee of the Amsterdam University Medical Centres in the Netherlands (W22_372 # 22.443).

Conflict of interest

The authors declare that there are no conflicts of interest.

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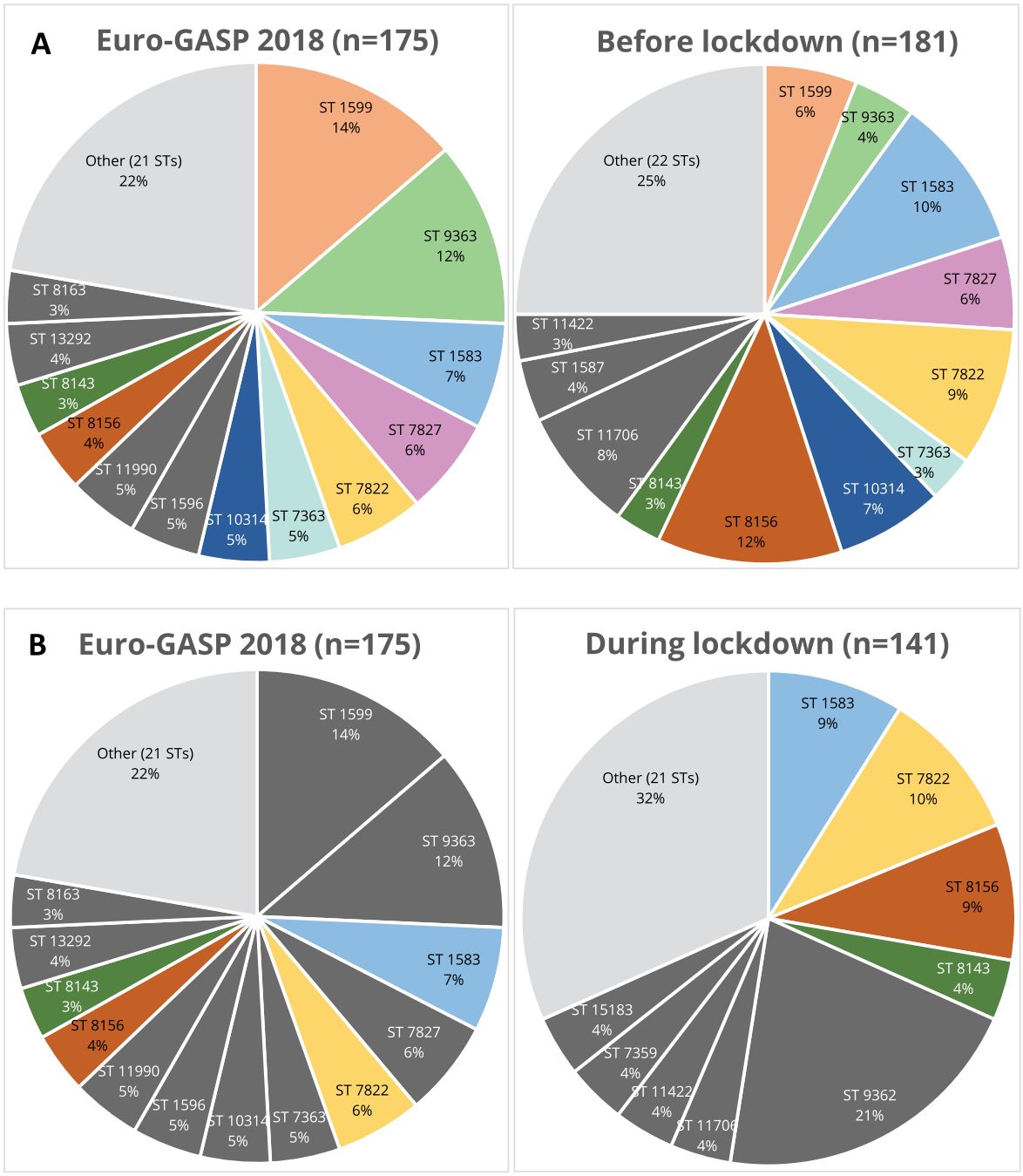
SUPPLEMENTARY DATA



Supplementary figure 1 – ST distribution of isolates from Amsterdam in the Euro-GASP 2018 collection compared to the ST distribution of isolates obtained before (A) and during (B) lockdown. Prevalence are shown for STs that occurred >5 times in the isolates before- or during lockdown. STs that occurred <5 times were categorized as 'Other'. Coloured STs were found > 5 times in both periods that were compared, whereas grey STs are only found >5 times in one of the periods.

Supplementary Table 1 – Metadata of all isolates included in this study, including individual accession numbers, patient data, phenotypic data and STs.
This table (17 columns and 323 rows) is available on Figshare (https://tinyurl.com/mtpjhuh7).

Supplementary Table 2 – Timeline of the main COVID-19 events in the Netherlands in the study period of January – July 2020.

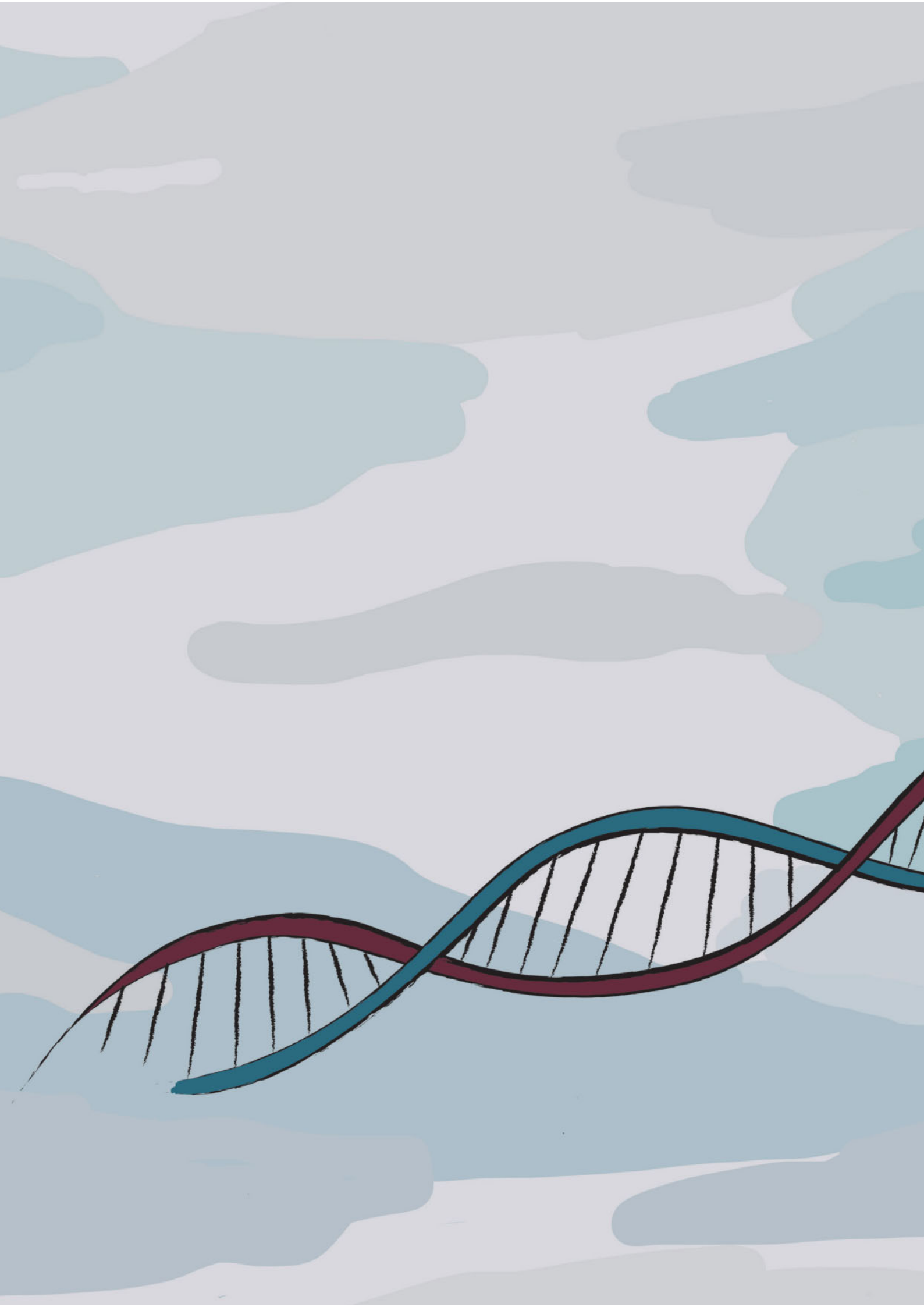
Date (DD/MM)* 2020	Measures*
27/02	First notified Sars-CoV-2 infection in the Netherlands
11/03	WHO declares COVID-19 to pandemic
12/03	Events with more than 100 people were cancelled, advice to work from home
15/03	Sport clubs, sex clubs, coffeeshops, bars and restaurants closed
16/03	First lockdown with additional measures: work from home, 1.5 meter distance, schools closed
17/03	Advice to not travel unless urgent, no flights to specific areas “at risk” (continuously updated travel restrictions)
18/03	EU closed borders for non-essential travel, face masks at work
23/03	“Intelligent lockdown” with reminder measures and additional measures: maximum of 3 people visiting a household per day with 1.5m distance, no groups larger than 2 people outside, all events cancelled, all contact jobs closed (for example hairdresser, beauty specialists), ‘non-essential’ shops, casino’s closed
Healthcare CSH Amsterdam	*No routine HIV/STI testing for asymptomatic clients unless urgent PrEP prescription needed*
09/04	Travelers from “high risk” areas 14 days in quarantine at home
11/05	Elementary school, libraries open and fitness outside allowed with 1.5 meter, contact jobs can start again
01/06	Easing measures: cinema’s, theatres, bars and restaurants open with maximum 30 people who reserved a timeslot and keep 1.5m distance, Secondary school opens with distancing measures, obligatory face masks on public transport
Healthcare CSH Amsterdam	*Open for all clients*
15/06	Easing travel restrictions between EU borders, extension closure of EU outside borders
01/07	Sex workers can start again, sport clubs are opened with 1.5m distance, bars, restaurants and indoor events are allowed to welcome up to 100 people with 1.5m distance, outdoor events up to 250 people with 1.5m distance

CSH: Centre for Sexual Health
* Data from the Dutch government website (https://www.rijksoverheid.nl/onderwerpen/coronavirus-tijdlijn)

Supplementary Table 3 – Statistical association of ST 9362 with epidemiological characteristics of patients from the CSH in Amsterdam before and during the COVID-19 lockdown.

	Isolates with STs other than ST 9362 (N = 290)	**ST 9362 isolates (N = 32)**	**P - value**
Sex			0.235
Male	272	32	
Female	18	0	
Age			0.880
<25	63	7	
25-34	127	14	
35-44	68	9	
>=45	32	2	
Country of origin			0.516
The Netherlands	141	17	
Suriname + Dutch Antilles	35	1	
Europe + Turkey	51	5	
M-S America	28	3	
Asia	19	5	
Africa	11	1	
Other	4	0	
Unknown	1	0	
Sexual orientation			0.021*
MSM + Transgender	250	32	
MSW + Female	40	0	
HIV status			0.503
Negative	214	27	
Positive	55	4	
Unknown	21	1	
Sex work			0.492
Yes	25	1	
No	265	31	
Number of sex partners in last six months			0.592
0-1	14	2	
2-4	80	5	
5-9	72	11	
10-19	49	7	
20-49	55	5	
>=50	20	2	
Symptomaticity			0.442
Symptomatic	97	8	
Asymptomatic	193	24	

* $p \leq 0.05$



Chapter 9

General Discussion

This thesis focused on the molecular diagnostics (part I) and epidemiology (part II) of *T. pallidum*. Part I described the possibility to detect *T. pallidum* in various body locations in different early syphilis stages and evaluated the added value of a research-use only *T. pallidum* Transcription Mediated Amplification (TMA) assay in current routine syphilis diagnostics. In part II the application of multiple typing techniques are described for different research questions. Also, antibiotic resistance is a recurrent topic emphasizing the need for surveillance and alternative treatment against STIs. I will dedicate a part of this discussion on the importance and the future of molecular typing for *T. pallidum* and other pathogens.

The presence of *T. pallidum* in multiple anatomical locations

T. pallidum can be found in multiple anatomical locations in different infectious syphilis stages (**chapter 2** and **3**). Our findings contribute to the understanding of the natural disease progression of syphilis. The hematogenous dissemination of *T. pallidum* is reflected by *T. pallidum* DNA detection in peripheral blood in the early infectious syphilis stages (**chapter 2**, (1). This is not surprising, based on the multi-stage nature of this disease with distinct signs and symptoms (2, 3). *T. pallidum* DNA was most frequently found in multiple anatomical locations in patients with secondary syphilis, which is in concordance with previous studies reflecting its infectiousness (**chapter 2,** (4-6).

T. pallidum DNA was also present in mucosal tissue and body fluids of patients with early latent syphilis (**chapters 2** and **3,** (6, 7). In **chapter 2,** *T. pallidum* DNA was found in 29/86 (34%) patients with early latent syphilis similar to the *T. pallidum* TMA positivity in 37/111 (33%) patients with early latent syphilis in **chapter 3**. The detected *T. pallidum* DNA in these patients suggests that this pathogen resides in the anal, oral and urethral mucosa and is potentially sexually transmissible. A recent Australian study with a similar population in the study, Towns et al. 2021, found *T. pallidum* DNA in 4/53 (8%) of patients with early latent syphilis who seroconverted less than 1 year ago (personal communication, (6). The higher positivity rate in **chapters 2** and **3** is due to *T. pallidum* DNA or rRNA detection in anal and especially pharyngeal swabs. However sampling in the studies differed, in **chapter 2** and **3** pharyngeal swabs were collected while in Towns et al. oral rinses and swabs from the mucosa in the whole oral cavity were collected making comparisons difficult (6). Another difference with Towns et al. 2021 is the staging definition of early latent syphilis. In Australia, early latent syphilis is defined as having positive serology, without symptoms of primary or secondary syphilis within 2 years of infection (6, 8), whereas the European Centre for Disease Prevention and Control (ECDC) and CDC of the United States (US) defines early latent syphilis when acquired less than 1 year ago (9). A number of the patients diagnosed with early latent syphilis in the study by Towns et al. may have acquired syphilis more than 1 year ago. Relapses of secondary syphilis symptoms are known to occur in 25% of patients with early latent syphilis, of which the vast majority occurs within the first year after syphilis acquisition (10). This supports the classification of this syphilis stage to belong to the infectious syphilis stages together with primary and secondary stage syphilis (2).

The duration of transmissibility of *T. pallidum* after initial infection remains ambiguous. Late latent stage syphilis requires a prolonged treatment with penicillin and partner notification is not performed (11, 12). Stage definition is important for appropriate treatment and partner notifications as well as the international usability of epidemiological data. The World Health Organization (WHO) has the same definition of early latent syphilis as in Australia, being a generally asymptomatic infection acquired less than 2 years ago (11). The ECDC defines

syphilis acquired less than 1 year ago as infectious; this includes the primary, secondary and early latent syphilis stages (9). Interestingly, the 2021 treatment guidelines of the CDC of the US states that latent syphilis is not infectious as "Sexual transmission of *T. pallidum* is thought to occur only when mucocutaneous syphilitic lesions are present" (page 41 of STIs Treatment Guidelines, 2021 (13). It is difficult to say where in the natural course of the syphilis infection patients with early latent stage syphilis are. These patients might not yet have developed clinical manifestations characteristic for primary or secondary syphilis. However, our findings of *T. pallidum* DNA and rRNA in anal, oral and urethral mucosa in the absence of lesions suggest likely infectiousness and cast doubt on this claim by the CDC (**chapters 2** and **3** (6, 7). The absence of *T. pallidum* DNA in patients with late latent syphilis also confirms the notion that late latent syphilis is considered not sexually transmissible (**chapters 2** and **3**). Interestingly, a study by Tantalo et al. from the United States of America (USA), found *T. pallidum* DNA in an oropharyngeal swab and whole blood sample from one patient with late latent (4). Future studies should focus on gaining insight on *T. pallidum* transmissibility in the latent stages.

T. pallidum TMA assay in routine syphilis diagnostics

There is a diagnostic gap between *T. pallidum* infection and clinical signs and symptoms as well as serological reactivity. The formation of a syphilitic ulcer takes approximately 3 weeks, ranging from 10 to 90 days (12, 14). Serological response usually occurs 1 to 2 weeks after onset of the primary lesion (2). Treponemal assays, like the chemiluminescence assay (CLIA), become positive first after infection, but cannot distinguish an active infection from a previously treated or persisting infection (14, 15). In populations with a high incidence of STI, previous syphilis infections are common (16). With non-treponemal assays, like the rapid plasma regain (RPR) assay, a new infection can be identified, but these assays are less sensitive in the early stage of syphilis (17).

To fill this diagnostic gap PCRs, targeting the *polA* or *tpp47* gene of *T. pallidum*, were developed and proved to be an important addition to the serological assay for syphilis diagnostics (18-23). Most diagnostic PCRs require the presence of syphilis lesions for the detection of *T. pallidum* DNA. However, these lesions may be absent due to their location, for example anal or pharyngeal, or due to an asymptomatic infection (**chapters 2** and **3** (6, 15). Asymptomatic infections are increasingly common with subsequent reinfection of *T. pallidum* (16, 24). Repeated syphilis infections are more likely in men who have sex with men (MSM) living with human immunodeficiency virus (HIV) (25, 26). In incubating syphilis during which lesions and serological response are absent, a different diagnostic assay may allow a timely diagnosis. **Chapter 3** describes the use of an additive *T. pallidum* TMA assay on multiple anatomical locations in a screening setting of populations at increased risk of acquiring syphilis and found 7% additional syphilis infections. Whether the few timely syphilis diagnoses justify the costs

of the implementation of this assay remains unclear. The relatively simple integration of this assay into routine syphilis diagnostics would increase syphilis case finding. This increase might be greater in a laboratory setting without diagnostic syphilis PCR for syphilis suspected lesions.

The low sensitivity of samples taken from each individual anatomical location (pharynx, anus, urine and peripheral blood) makes them not suited as a replacement of syphilis diagnostics that includes a Nucleic Acid Amplification Test (NAAT) on ulcer swabs (**chapters 2** and **3**). However, the use of an additive *T. pallidum* TMA assay on multiple anatomical locations identifies 7% additional syphilis infections and may therefore be used to increase syphilis case finding (**chapter 3,** (7). Other innovative efforts are on its way to improve current syphilis diagnostics by for example combining modern technologies such as CRISPR-LwCas13a to a real time PCR or using different sample specimen for diagnostics (1, 5, 27). Further research is warranted into other diagnostic tools that may also be used in smaller diagnostic settings with less resources.

Molecular epidemiology of *T. pallidum*: lineages

Molecular typing is a powerful tool to monitor the spread and diversity of pathogens and to get insights into the population dynamics. Discriminating lineages and strains of pathogens allows the investigation of possible associations between certain lineages or specific strains and genetic markers for antibiotic resistance or increased virulence (14). Except for a few reports, finding specific *T. pallidum* strains associated with neurosyphilis, no *T. pallidum* lineages or strains have been associated with altered pathogenesis (28-30).

In 2010, a molecular typing study described the observation of two lineages of *T. pallidum* strains clustering separately, one containing the Nichols and DAL-1 strains and the other lineage containing the SS14 and Mexico A strains (31). The two lineages were supported by later studies after more sequencing was performed and sequence data was made publicly available (32, 33). The SS14 lineage was estimated to originate from 1964, after which from 2000 on, a rapid clonal expansion occurred (33). This rapid expansion was suggested by Beale et al. to be due to a bottleneck that occurred between late 1990s and early 2000s and may possibly be related to the increase use of pre-exposure prophylaxis (PrEP) against HIV (34). Strains belonging to the SS14 lineage are generally more homogenous than strains from the Nichols lineage. Worldwide, the circulating *T. pallidum* strains predominantly belong to the SS14 lineage (33-35). Strains from each lineage can be further subdivided into sublineages (33, 34, 36). Genomic comparisons could not find a difference in genes that would show an advantageous fitness of strains or lineages (34).

The distinction between the two major lineages can also be done based on the sequence analysis of the *tp0136* or *tp0548* genes (37). This distinction is part of the MLST analysis which many studies have implemented and reported (37-40). Also, in **chapter 5, 6** and **7** this typing scheme was used and found that 80-83% of the circulating *T. pallidum isolates* was SS14 and 17-20% belonged to the Nichols lineage among the population with syphilis visiting the SHC in Amsterdam, the Netherlands. This is similar to previous studies performed in Japan (41) and Argentina (42), and is comparable to a recent large-scale genomic study of samples from 23 countries (34). However, a previous large study including 970 publicly available sequences described that 93.5% belonged to the SS14 lineage (33) and some studies from European countries reported less than 10% circulating Nichols strains, such as in the Czech Republic (43), France (44) and Switzerland (37).

Molecular epidemiology of *T. pallidum*: strains

Distinction between *T. pallidum* lineages alone is insufficient for monitoring of circulating *T. pallidum*. Molecular typing methods can distinguish strains within *T. pallidum* lineages and increase the resolution for studies on transmission and sexual networks. To our knowledge, the studies described in **chapters 4, 5, 6** and **7** are the first and only reports on the molecular diversity of *T. pallidum* in the Netherlands.

The majority of studies on molecular diversity of *T. pallidum* find that the most prevalent strains in those studies are similar. Other strains are found less frequently (**chapters 4, 5, 6,** and **7**). In **chapter 4** the first *T. pallidum* molecular typing study of the circulating strains among patients in Amsterdam in 2017 is described. The applied enhanced CDC method distinguished *T. pallidum* strains by analyzing the number of 60 basepair repeats in the acidic repeat protein gene (*arp*), the restriction pattern of the *T. pallidum* repeat protein (*tpr*) genes (*tprE, tprG* and *tprJ*) and partial sequence analysis of the *tp0548* gene. In total, 25 strains among 99 typed isolates were identified, with type 14d/g being the predominant strain. Many European reports have also shown a high prevalence of this strain and type 14d, which is the predecessor strain recognized prior to the addition of the partial sequence analysis of *tp0548* (45, 46). The 14d/g type was found in 23% of the included isolates in Amsterdam (**chapter 4**), which is similar to the 14d/g prevalence in Barcelona (27%)(46). However, this is a lower percentage than reported in other studies with 41% in Italy (45), 48% in Australia (47) and 69% in France (48). Type 14d/f was predominant in studies from North America and in China and was found in 9% of *T. pallidum* isolates in Amsterdam (**chapter 4,** (14). The unique *T. pallidum* strains within countries (37, 43, 44, 49, 50) seem to suggest local transmission as well as international mixing. This was also found in the studies in Amsterdam described in **chapters 4, 5, 6** and **7** with, respectively, 11/25 (44%), 8/14 (57%), 3/9 (33%), 6/17 (35%) allelic profiles occurring only once.

Using the *T. pallidum* MLST method, the allelic profile 1.3.1 corresponds to the "g" type of *tp0548* in the enhanced CDC method. The most prevalent *T. pallidum* strains in Switzerland (37), France (44), Spain (46) and Czech Republic (43) and other countries (40) have allelic profiles 1.3.1 and 1.1.1, which both belong to the SS14 lineage. This was also the case in circulating *T. pallidum* within Amsterdam regardless of the host population. In these studies allelic profiles 1.3.1 and 1.1.1 were prevalent in, respectively, 42% and 18% (**chapter 5**), 40% and 17% (**chapter 6**), and 43% and 20% (**chapter 7**), of the typed isolates. Our findings provide insight on the molecular variation of circulating *T. pallidum* in Amsterdam, the Netherlands and therefore contribute to the available data for global analyses on the epidemiology of syphilis.

T. pallidum diversity within and between patients

Molecular typing methods can potentially uncover tissue tropism and distinguish between a simultaneous infection with multiple strains or a disseminated infection within a patient. In addition, reinfection or persistent infection may be distinguished by molecular techniques in a patient at different time points. When using the MLST method for *T. pallidum* isolates from different anatomical locations in patients in different syphilis stages, no strain variation was found (**chapter 5**). This intra-patient *T. pallidum* strain homogeneity suggests haematogenous dissemination rather than multiple separate and simultaneous infections from multiple partners. However, when infection occurs with one of the more prevalent strains such as 1.3.1 and 1.1.1, simultaneous infection by sexual transmission with multiple partners cannot be excluded. Molecular methods with the highest resolution like whole genome sequencing (WGS) should preferably be performed when trying to uncover transmission within sexual networks. However, the diversity in the core genome of *T. pallidum* isolates in a well sampled setting is zero making transmission dynamics nearly impossible to investigate (34).

Patient information on ethnicity has been taken into account and included in the analysis in the molecular typing studies of **chapters 4** and **7**. Regardless of ethnicity, *T. pallidum* strain distribution remained similar. Interestingly, the analysis of circulating *T. pallidum* strains among women and men who have sex with women (MSW) also showed a similar distribution to the ones already found among the visitors of the SHC in Amsterdam (**chapter 6**). This may reflect a diverse and mixed sexual network in Amsterdam or could be explained by stigma against MSM (51). MSW, although denying sex with men, may have contracted their syphilis strain from other men. Studies in Japan have found distinct circulating *T. pallidum* strains among women and MSW as compared to MSM (41, 52, 53).

The challenges of molecular typing of *T. pallidum*

A low bacterial load is a major challenge for sequencing *T. pallidum*, especially for WGS. This is why the majority of the samples from sequencing studies were derived from genital ulcers or from cultured isolates propagated in rabbits. This challenge was evident in all molecular typing studies of *T. pallidum* in this thesis, but is also mentioned in genomic studies from others (**chapters 4, 5, 6** and **7**, (54, 55). We aimed to perform *T. pallidum* WGS from different anatomical non-lesional sites as described in **chapter 2** in collaboration with a group with experience in this technique, but were so far not successful. It makes it especially difficult to investigate *T. pallidum* in later stages and in different anatomical locations (**chapter 5**). *T. pallidum* DNA has been found in urine in relatively higher loads (reflected by lower cycling threshold (Ct) values in the PCR) than in whole blood or plasma samples and may therefore be an alternative option when lesions are absent (**chapter 5**, (56).

Subspecies determination

Even though pathogenic treponemes are thought to be distinguishable by clinical, epidemiological and geographical characteristics (3), studies from Cuba, Japan and Australia reported the presence of *Treponema pallidum* subspecies *endemicum* (*T. pallidum endemicum*) causing bejel among patients who were diagnosed with syphilis (53, 57-60). Serological assays and routine syphilis PCRs targeting the *polA* or *tpp47* genes do not distinguish between these treponemes (61). Besides the misdiagnosis, bejel instead of syphilis, the presence of bejel outside of its endemic geographic area requires further investigation. However, no *T. pallidum endemicum* was found at the Sexual Health Centre (SHC) of Amsterdam among retrospectively selected patients diagnosed with syphilis between 2006 and 2018 with a Surinamese, Dutch Antillean or Dutch ethnicity (**chapter 7**). For this analysis all patients between 2006 and 2018 were included and based on those numbers a similar number of Dutch patients were randomly selected per year.

Bejel may be less frequent than found in the studies from Cuba and Japan (53, 58). A recent genotyping study on *T. pallidum* in Cuba did not find new *T. pallidum* subspecies *endemicum* (62). A study from Australia reported 1% of their samples from syphilis diagnosed patients to contain either *T. pallidum endemicum* or *pertenue* (60). Other recent molecular typing studies did not find other subspecies than *Treponema pallidum* subspecies *pallidum* among the patients with a syphilis diagnosis (41). However, increasing mobility of people around the world will likely have an effect on currently endemic infectious diseases as well as the endemic treponematoses, *endemicum* and *pertenue*. Future molecular epidemiological studies on *T. pallidum* should therefore continue to report the presence of these cases outside of their endemic areas.

Antibiotic resistance in *T. pallidum*

Historically, patients with syphilis with an allergy to penicillin were recommended to be treated with macrolides, which is an antibiotic that affects protein synthesis in the bacterial ribosome (63, 64). Azithromycin (belonging to the macrolides) was found to be effective in treating syphilis infections, but increasing reports describing azithromycin treatment failure of patients with syphilis eventually led to the discouragement of the use of this antibiotic (33, 65, 66). The first reported *T. pallidum* macrolide resistant strain was SS14 (the original Street strain 14), which was isolated by rabbit inoculation in 1977 (14, 64). Its high resistance to macrolides was found to be associated to a mutation from adenine to guanine at the 2058 location in the 23S rRNA gene corresponding to the positioning in the *Escherichia coli* 23S rRNA gene (A2058G) (67). In 2009, a novel A2059G mutation associated with macrolide resistance (MRAM) was reported in the Czech Republic (68). Macrolides are used to treat another STI causing pathogen, *Chlamydia trachomatis* (69).

Genetic markers that translate into phenotypical antibiotic resistance can be monitored with the use of molecular typing methods. A recent genomic study revealed evidence of MRAM evolving independently in multiple sublineages, especially of SS14, most likely due to antibiotic pressure (55). Allelic profile 1.3.1 belonging to the SS14 lineage has been associated with the presence of MRAM (44). Among the population visiting the SHC in Amsterdam, 81 – 88% of the included isolates were genotypically macrolide resistant (**chapter 4, 6** and **7**). The lowest prevalence in our studies was found among isolates originating from 2006 to 2018 and showed an increasing trend of MRAM over the years (**chapter 7**). The MRAM prevalence among circulating *T. pallidum* isolates in Amsterdam is similar to other countries, like Australia (36, 70), Italy (45), and Czech Republic (43). Increasing macrolide resistance has been found in circulating *T. pallidum* strains in other studies as well (55, 71, 72). A study from South Africa only detected MRAM in *T. pallidum* in 2013, after which its prevalence grew to 71% in 2018 (71). Some studies reported 100% MRAM among the included isolates including in Europe and Asia (41, 73). Increased macrolide resistance in *T. pallidum* is associated with macrolide consumption causing antibiotic pressure (74).

There is no evidence yet that suggests a reduced penicillin susceptibility of *T. pallidum*, but amino acid variations in the penicillin binding proteins (PBP) are found in genomic assays (41, 55). Monitoring antibiotic resistant mutations is important. Besides macrolide resistance, *T. pallidum* surveillance studies should also include known penicillin associated mutations and tetracycline resistant mutations in their analysis (45). Doxycycline and ceftriaxone are the recommended alternative treatment options for a syphilis infection (11, 64). Further research on alternative treatment is on-going, a preclinical trial positively evaluated the efficacy of linezolid (75). A future research target may be to identify mutations that affect the sensitivity of these agents.

An important option for prevention of a syphilis infection is the development of a vaccine (76, 77). Several studies are ongoing to develop such a vaccine but so far they have not been very successful. For vaccine development, multiple outer membrane proteins are potential vaccine targets, one of them being *tprK,* which has been shown to play an important role in the immune evasive nature of *T. pallidum* (76, 78). A previous study found variation of the *tprK* genes within a patient making it unsuitable to use in molecular epidemiological studies (79).

In vitro culture of *T. pallidum*

In vitro culturing is used to elucidate phenotypic characteristics of pathogens including studies on their physiology and measuring minimum inhibitory concentrations (MIC) against antibiotics (80). In addition, *in vitro* culture is used to study host-pathogen interactions and immunology and avoids the use of laboratory animals, reducing costs and allowing for large-scale experimental analyses to be performed (81). *In vitro* culturing is a successful and broadly used method to grow a pathogen of interest and thus obtaining high loads of specific DNA suitable for genomic studies. Previous propagation of *T. pallidum* was accomplished by inoculation of rabbit testes. However, the auxotrophic *T. pallidum* was uncultivable in the long-term until a culture system was published by Edmondson et al in 2018 (81). The developed culture system combined the knowledge gained from extensive previous studies by Fieldsteel et al in which the essential rabbit epithelial cells and a microaerophilic condition was already described (82). The culture system focused on a Nichols strain, which was isolated from the nervous system in 1914 and since then propagated in rabbit testes (83). Refinements of the long-term *in vitro* culturing method are described to restrict the complex co-culture system to the bare essentials that allowed *T. pallidum* to grow continuously (84). Some research groups, for instance the group of Giacani in Seattle, US, and Śmajs in Brno, Czech Republic (personal communication), have replicated the culture system (85). This also includes our group at the Amsterdam University Medical Center, however, unfortunately no long-term consistent success was achieved.

Ongoing experiments in Amsterdam aim to culture *T. pallidum* directly from patient material. So far, we succeeded in finding viable spirochetes up until 3 weeks after inoculation in 3 out of 4 specimens collected directly from genital ulcers. Unfortunately, regardless of preventative antibiotics, phosphomycin and amphotericin B, and targeted antibiotics against contaminants, other bacteria took over the *in vitro* cultures. This caused inhibited growth of *T. pallidum* and no visible spirochetes using dark-field microscopy after 4 weeks in the culture system.
So far, the complexity of the co-culture system with the Sf1Ep cells and the fragility of *T. pallidum* has hindered the next step of being able to *in vitro* culture *T. pallidum* directly from a clinical sample. The sensitivity of *T. pallidum* in the *in vitro* culture is the antipode of its infectiousness in the untreated human host in which it may survive for decades (15). The Nichols strain showed a significantly shorter generation time in the *in vitro* culture system than the currently predominant strains belonging to the SS14 lineage (84). However, the

majority of circulating *T. pallidum* strains belong to the SS14 lineage, as found in molecular typing studies (**chapters 4, 5, 6** and **7**, (33, 34, 36, 40, 43). Adjustments may be necessary to optimize the culture system to the modern circulating *T. pallidum* strains. In our sampling experiments, the aspiration with needles was unsuccessful as the serous fluid from a genital ulcer was too viscous. A recent report suggested the use of a syringe to aspirate the serous fluid (86). This sampling method may reduce the contaminants in the culture. Future efforts will probably enable researchers to establish a successful *T. pallidum in vitro* culture directly from patient material.

Impact of major events on molecular diversity of *Neisseria gonorrhoeae*

Molecular techniques can be used to investigate of the impact of major events such as a pandemic on the transmission and diversity of infectious pathogens. In **chapter 8**, a major genotypic shift was found among *Neisseria gonorrhoeae* strains from before (January 15th – February 29th) versus during (May 15th and June 30th) the first COVID-19 lockdown in 2020 in the Netherlands. Using MLST, the predominant sequence type (ST) 8156 before the lockdown got replaced by ST 9362, which went from a prevalence of 2% to 21%, becoming the predominant type, during the lockdown. In addition, a phenotypic shift was observed towards low-level azithromycin resistance and ceftriaxone susceptibility. ST 9362 isolates contain genetic markers associated with azithromycin resistance and ceftriaxone susceptibility. The increase in low-level resistance against azithromycin in *N. gonorrhoeae* has been found to be a trend in the last years in the Netherlands and other countries worldwide (87-89). The limited genetic diversity found among the ST 9362 isolates during the lockdown also suggested a rapid clonal expansion, possibly by local transmission of this strain during the lockdown period. The restriction measures as a response to the pandemic limited national- and international travel, which likely reduced the importation of *N. gonorrhoeae* strains to Amsterdam and to the Netherlands. Also, a significant reduction in number of casual sex partners were reported during the lockdown among the clients of the SHC in Amsterdam, as described by Van Bilsen et al (90). This suggests a more concentrated sexual network leading to this local transmission. Future studies should monitor *N. gonorrhoeae* to confirm whether the genotypic and phenotypic shift found in this study had a lasting effect. In addition, the effect of public health measures on the epidemiology of infectious diseases should be taken into consideration for surveillance.

The future of molecular typing

Molecular typing is increasingly used to screen for genotypic antibiotic resistance by sequencing genetic determinants that have been associated with phenotypic antibiotic resistance. At present, this is less relevant in routine diagnostics and treatment of *T. pallidum*. However, genotypic antibiotic resistance determination has an important role in

the treatment of many other infectious pathogens like, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Staphylococcus aureus* and many more (88, 91-94).

The genome of *T. pallidum* is highly conserved, which is also seen in the molecular typing studies in this thesis studying different host populations and degrees of variable genetic regions of the *T. pallidum* genome (**chapters 4**, **5**, **6** and **7**). Thanks to the increased number of published full genomes and to technical innovations, more precise molecular typing methods have been developed. The partial sequence analysis of *tp0548* proved to be significantly correlated with identified *T. pallidum* subgroups (36). The concatenated sequences based on the combination of partial *tp0136, tp0548* and *tp0705* genetic regions provides over 30% resolution compared to whole genome data (37, 40). While we move towards genome wide analyses with the highest resolution, there is still practical use for molecular typing methods such as MLST and the enhanced CDC typing method for basic surveillance and geographical mapping of *T. pallidum*. These molecular techniques are more widely accessible as they are easier to perform and less expensive. This is especially useful for countries from which obtaining *T. pallidum* samples is more challenging and resources are limited.

The eradication of syphilis has been a goal on the action plan agenda of public health organizations for years, but remains unsuccessful (69, 95). Insights into molecular diagnostics and epidemiology aid this goal. The focus is, and must be, on the detection of the pathogen in disproportionally burdened populations (95-97). The WHO incorporates and emphasizes the importance of sexual health in their latest guidelines on STI management (69). Improving overall sexual health in combination with STI campaigns, early diagnosis and treatment and surveillance will reinforce potential effects on STI control. With the use of appropriate molecular methods, future studies should encourage (inter)national collaborations to gain a better geographical overview and understanding of the complex *T. pallidum* spirochete.

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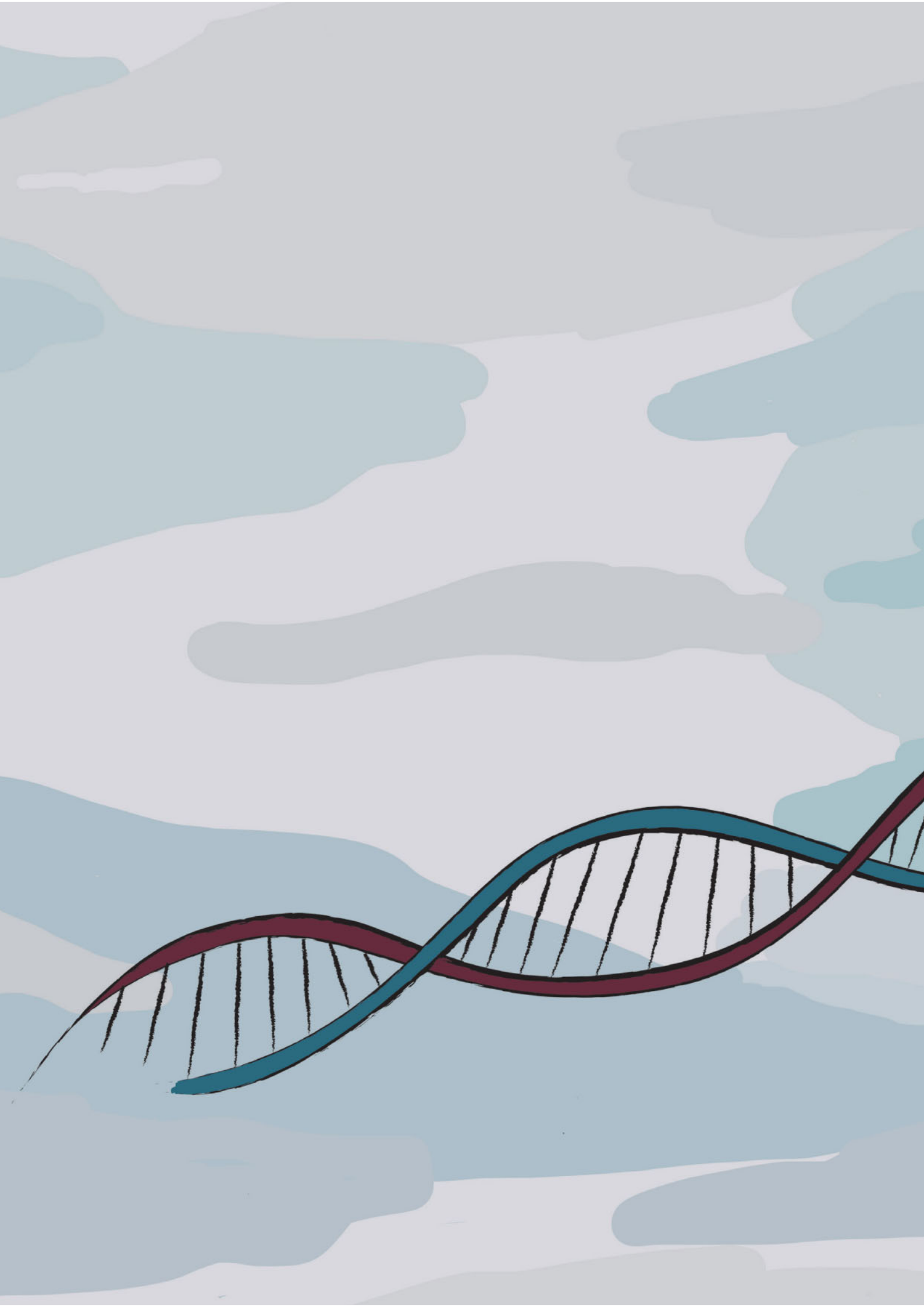

Appendix

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SUMMARY

Treponema pallidum subspecies *pallidum* (*T. pallidum*) is the causative pathogen of the sexually transmitted infection (STI), syphilis. As a stealthy spirochete it infects its obligate human host causing a complex multistage infection. This thesis is comprised of two parts; part I contains studies focused on investigating the presence and possible diagnostical opportunity for *T. pallidum* in different disease stages. Part II contributes to the knowledge on molecular variation of *T. pallidum* with the investigation of its diversity in different populations in Amsterdam. In addition, part II contains the investigation of the impact of the first coronavirus disease 2019 (COVID-19) COVID-19 lockdown on *Neisseria gonorrhoeae* genetic diversity in Amsterdam.

Chapter 1 introduces *T. pallidum* ranging from its history and discovery to its detection and surveillance. It describes syphilis diagnostics and its challenges from the initial infection to the fast dissemination and disease progression to other stages. Molecular typing techniques, used to investigate molecular variation of pathogens, are described with a focus on the developed methods to study the molecular variation of *T. pallidum*.

PART I: MOLECULAR DIAGNOSTICS

T. pallidum DNA detection in various anatomical locations

T. pallidum multiplies at the site of infection and disseminates systemically within hours of transmission. In **chapter 2**, we evaluated the presence of *T. pallidum* DNA in peripheral blood, oropharynx, ano-rectum and urine to elucidate transmissibility during infectious syphilis stages. DNA of *T. pallidum* was frequently detected in oral and anal mucosa and in urine of men with infectious syphilis, even in the absence of lesions. *T. pallidum* DNA was detected in at least one study sample for 35/70 (50%) participants with primary syphilis, 62/73 (85%) with secondary syphilis and 29/86 (34%) with early latent syphilis. *T. pallidum* was not found in participants with late latent syphilis, which supports the notion that this stage is not considered infectious. The presence of *T. pallidum* on multiple sites in patients with infectious syphilis emphasizes the view of syphilis having a high transmission rate and being contagious in these early stages even in the absence of symptoms.

Addition of a *T. pallidum* TMA assay to routine diagnostics

Syphilis diagnostics is complex and requires a combination of assays as well as expertise. Even with this combination, an infection with *T. pallidum* may go undetected. In **chapter 3** the additional value of a new research use only *T. pallidum* Transcription Mediated Amplification assay (TP TMA) on mucosal orifices was evaluated in a screening setting among clients with

an increased risk of acquiring HIV and STI. Between September 2021 and August 2022, 9974 visits were included at the sexual health centre (SHC) in Amsterdam from 3283 participants of the national pre-exposure prophylaxis (PrEP) program. Among these participants, 191 syphilis infections were diagnosed within the study period based on routine diagnostics. In total, 119 samples from 95 visits tested positive in the TP-TMA assay, of which 16 samples from 16 participants were discrepant positive in the TP-TMA as compared to the routine diagnostics. However, two of the participants with discrepant results had been treated for a syphilis infection by their general practitioner within a week before the SHC visit. Therefore, a total of 14/191 (7%) additional incubating syphilis infections were identified during the study period using TMA as an adjunct test to routine diagnostics.

PART II: MOLECULAR EPIDEMIOLOGY

The second part of the thesis contains studies using molecular typing methods to map molecular variation of *T. pallidum* and *N. gonorrhoeae*. Molecular typing methods can be used to differentiate *T. pallidum* strains in order to investigate sexual networks and get more insight into the transmission of syphilis infections. To our knowledge, the studies described in **chapters 4, 5, 6** and **7** are the first and only reports on the molecular diversity of *T. pallidum* in the Netherlands. **Chapters 4, 5, 6** and **7** revealed a similar distribution of distinct *T. pallidum* strains. Type 14d/g using the enhanced CDC method and allelic profiles 1.3.1 and 1.1.1 using the multi-locus sequence typing (MLST) method were predominant. Apart from the predominant strains of *T. pallidum*, many singletons or strains occurring twice were found. The Nichols and SS14 lineages were present among *T. pallidum* from each included population in approximately a 1:4 ratio, respectively. This is in concordance with a recent large scale genomic study, Beale et al. 2021, who included samples of 23 countries. Interestingly, the prevalence of *T. pallidum* strains belonging to the Nichols lineage is relatively high in the Netherlands compared to the Nichols prevalence in other individual European reports. Phylogenetic trees generated from concatenated sequences from the multi-locus sequence typing (MLST) method visualized the genetic variation of *T. pallidum* between the strains that were found (**chapters 5** and **7**). Partial sequences were shown to correlate well with *T. pallidum* lineages and sub-clusters from genome analyses.

Enhanced CDC method for molecular typing of *T. pallidum*

To determine the molecular diversity of *T. pallidum* in Amsterdam, in chapter 4, *T. pallidum* PCR positive genital ulcer and skin swab isolates were included between 2016 and 2017, which were derived from syphilis routine diagnostics at the Public Health Laboratory (PHL) of Amsterdam. The enhanced centers for Disease Control and Prevention (CDC) method was used, which is based on the number of 60 base pair tandem repeats in the *arp* gene, the

restriction fragment length polymorphism analysis of the *T. pallidum* repeat subfamily II genes (*tprE, tprG* and *tprJ*) and partial sequencing analysis of the *tp0548* gene. This combination of molecular techniques allowed the isolates to be typed and resulted in a broad distribution with a few types occurring in the majority of the isolates and most types occurring in only one or two isolates. No associations were found between clinical or demographic data and the strain types. In addition, the analysed 23S rRNA genes in this study showed a high rate of macrolide associated resistance mutations (MRAM).

T. pallidum strain homogeneity within patients

T. pallidum can be found in multiple anatomical locations within patients in different infectious syphilis stages. Possible tissue tropism of *T. pallidum* strains and intra-patient molecular variation were investigated in **chapter 5**. Multi-locus sequence typing (MLST) was performed in all *T. pallidum* PCR positive samples including ulcer swab samples taken during routine diagnostics from participants in the study described in **chapter 2.** This resulted in typed isolates from a total of 107 anatomical sites derived from 93 participants. Among the 93 participants in whom at least one sample could be typed, 48 (52%) had primary syphilis, 35 (38%) had secondary syphilis and 10 (11%) had early latent syphilis. The cycling threshold value of the PCR was found to be significantly lower, reflecting a higher bacterial load, in the samples that were successfully typed. *T. pallidum* was found to have the same type in the 12 participants who had multiple typed samples from different body locations. There was no strain variation between anatomical site nor an association between certain types and syphilis stage.

Molecular typing of *T. pallidum* in different populations

Chapter 6 describes the *T. pallidum* molecular variation among a small sample set of women and men who have sex with women (MSW) in Amsterdam, the Netherlands and Antwerp, Belgium. The strain distribution was similar as the distribution found among MSM in previous studies in Amsterdam. Interestingly, the strain distribution from *T. pallidum* among MSW in our study differed slightly from the included samples from women. MSW shared more similarities with MSM based on the extracted data from the pubMLST database. This suggests an overlap in sexual networks in which some MSW may have had sex with men. Stigma on sexual behaviour may have played a role for these men in reporting sex with women versus sex with men.

T. pallidum subspecies *endemicum* prevalence in Amsterdam

Chapter 7 describes the molecular variation among clients of the SHC of Amsterdam with different ethnicities. *T. pallidum* isolates from all Surinamese, Dutch Antillean and the same number of Dutch clients per year between 2006 and 2018 were selected. Previous studies from Cuba and Japan showed the presence of *T. pallidum* subspecies *endemicum*, the

causative agent of bejel, among clients who were diagnosed with syphilis. The Netherlands has a colonial history with Surinam and the Dutch Antilles which is why a lot of people who originated from these countries and live in the Netherlands. It was hypothesized that due to their ethnicities their sexual networks might expand to their countries of origin, which are geographically close to Cuba, and were possibly exposed to *T. pallidum* subspecies *endemicum*. However, no *endemicum* subspecies were found among the selected clients suggesting that bejel is not present in the Netherlands and may also not be present in Surinam or the Dutch Antilles. However, future studies will need to be performed with *T. pallidum* samples directly from these countries to state this with more certainty. Recent genomic studies found none or very few *endemicum* subspecies isolates among the *T. pallidum* isolates included in analyses which confirms that the presence of *endemicum* might be less than previously suggested.

Antimicrobial resistance surveillance in *T. pallidum* by sequence analysis

Penicillin has effectively been used as a treatment against syphilis for 80 years. Antibiotic resistance is a public health threat, but fortunately no resistance or reduced susceptibility has been described in *T. pallidum.* Azithromycin used to be an effective alternative treatment of syphilis, but treatment failure and MRAM were found. Surveillance of these mutations in the 23S rRNA loci of *T. pallidum* show increasing prevalence. This temporal increase was also found in **chapter 7** between 2006 and 2018 among isolates from Amsterdam. MRAMs were present in more than 80% of the *T. pallidum* isolates from the studies described in **chapters 4, 6** and **7**. This increasing prevalence of macrolide resistance in *T. pallidum* has resulted in the adjustment of public health treatment guidelines, discouraging the use of azithromycin for the treatment of syphilis in patients.

Neisseria gonorrhoeae genetic diversity around first COVID-19 lockdown

Neisseria gonorrhoeae causes another worldwide highly prevalent bacterial STI, gonorrhoea. Circulating genotypes of this bacterium that are associated with antimicrobial resistance are closely monitored through genomic surveillance studies by public health organizations. The COVID-19 pandemic has had a big impact on other infectious diseases. Like many infectious diseases, STIs rely on humans being in contact with each other. This was reduced by restriction measures as a response to the pandemic. Among clients of the SHC in Amsterdam a reduced number of casual sex partners were reported during the first lockdown in the Netherlands in 2020. In **chapter 8**, the genetic and phenotypic diversity of *N. gonorrhoeae* in Amsterdam is described before (January 15th – February 29th) and during (May 15th and June 30th) the first COVID-19 lockdown in 2020. An shift was observed from *N. gonorrhoeae* sequence type (ST) 8156 to ST 9362, which became predominant during the lockdown. Single nucleotide polymorphism (SNP) differences were remarkably low with a median of 17 SNPs distance between ST 9362 isolates during the lockdown suggesting rapid clonal expansion, possibly by local transmission. Additionally, the phenotypic shift towards azithromycin resistance

and increased ceftriaxone susceptibility are in concordance with the emergence of ST 9362 containing genetic markers for azithromycin resistance and lacking mutations associated with ceftriaxone resistance. This chapter shows that the impact of public health measures are noticeable in the epidemiology of infectious diseases, which may have significant subsequent consequences and should be taken into consideration for surveillance.

General discussion

Chapter 9 provides an overview of the findings of the included chapters and future recommendations are made in context of current literature and advancements in the field. Both parts of the thesis; Molecular Diagnostics and Molecular Epidemiology, are discussed separately. Future studies should expand (inter)national collaborations to obtain a better geographical overview and understanding of *T. pallidum* diversity using appropriate molecular methods and by sharing data.

NEDERLANDSE SAMENVATTING

Treponema pallidum subspecies *pallidum* (*T. pallidum*) is een bacterie die de veel voorkomende seksueel overdraagbare aandoening (soa), syfilis, veroorzaakt. Deze spiraalvormige bacterie infecteert zijn menselijke gastheer en veroorzaakt een complexe ziekte die zonder behandeling meerdere stadia heeft. Dit proefschrift bestaat uit twee delen. In het eerste deel worden studies beschreven die gericht zijn op de aanwezigheid van *T. pallidum* in het menselijke lichaam en de mogelijkheden om diagnostiek in te zetten voor het vaststellen van de verschillende ziektestadia. Deel II draagt bij aan de kennis over moleculaire variatie van de *T. pallidum* bacterie door onderzoek naar de diversiteit in verschillende bevolkingsgroepen in Amsterdam. Daarnaast bevat deel II ook een studie naar de invloed van de eerste coronavirus 2019 (COVID-19) lockdown in Amsterdam op de genetische diversiteit van een andere belangrijke soa bacterie, *Neisseria gonorrhoeae*, die gonorroe veroorzaakt.

In **hoofdstuk 1** wordt *T. pallidum* geïntroduceerd, vanaf de historie en ontdekking tot de detectie en het monitoren van de genetische variatie van de bacterie. De syfilis diagnostiek en de uitdagingen hiervan worden beschreven vanaf het moment van infectie tot de verspreiding in het lichaam en ziekteverloop naar andere stadia. Moleculaire typeringstechnieken worden gebruikt om de moleculaire variatie van ziekteverwekkers te onderzoeken. De moleculaire typeringstechnieken die gebruikt worden voor het bestuderen van *T. pallidum* worden beschreven.

DEEL I: MOLECULAIRE DIAGNOSTIEK

T. pallidum DNA detectie op verschillende anatomische locaties

T. pallidum vermenigvuldigt zich eerst op de plaats van infectie en verspreidt zich binnen enkele uren na overdracht via het bloed door het hele lichaam. In **hoofdstuk 2** evalueerden we de aanwezigheid van *T. pallidum* DNA in perifeer bloed, keel, anus en urine om de besmettelijkheid tijdens de verschillende syfilis stadia op te helderen. DNA van *T. pallidum* werd vaak gevonden in slijmvliezen van de mond en de anus en in urine van mannen met syfilis, zelfs bij afwezigheid van wondjes. Het DNA van *T. pallidum* werd gevonden in ten minste één monster bij 35/70 (50%) studiedeelnemers met primaire syfilis, bij 62/73 (85%) met secundaire syfilis en bij 29/86 (34%) met vroeg latente syfilis. *T. pallidum* DNA werd niet gevonden bij deelnemers met laat latente syfilis, wat de gedachte ondersteunt dat besmettelijkheid in dit stadium onwaarschijnlijk is. De aanwezigheid van *T. pallidum* DNA op meerdere plaatsen bij patiënten met infectieuze syfilis (primair, secundair en vroeg latente syfilis) benadrukt het idee dat syfilis in deze vroege stadia zeer besmettelijk is, zelfs als er geen duidelijke symptomen zijn.

Toevoeging van een nieuwe *T. pallidum* TMA test aan routine diagnostiek

De syfilis diagnostiek is complex en vereist een combinatie van laboratorium testen en medische expertise. Ook met deze combinatie kan een infectie met *T. pallidum* onopgemerkt blijven. In **hoofdstuk 3** werd de toegevoegde waarde van een nieuwe moleculaire test, de *T. pallidum* Transcription Mediated Amplification test (TP-TMA), op slijmvlies en urine monsters geëvalueerd. Dit werd uitgevoerd onder deelnemers van het landelijke pre-exposure profylaxe (PrEP) programma die een verhoogd risico hebben op het krijgen van een HIV infectie of andere soa en op bezoek kwamen bij het Centrum voor Seksuele Gezondheid (CSG) in Amsterdam. Tussen september 2021 en augustus 2022 zijn er 9974 bezoeken van 3283 deelnemers geweest bij het CSG waarvan de afgenomen monsters voor het testen op *Chlamydia trachomatis* en *Neisseria gonorrhoeae* ook getest werden met de TP-TMA test. Onder deze deelnemers werden er, op basis van routinematige diagnostiek, 191 syfilis diagnoses vastgesteld binnen de onderzoeksperiode. In totaal waren 119 monsters van 95 bezoeken van deelnemers positief in de TP-TMA test, waarvan er 16 monsters van 16 deelnemers afwijkend positief waren in de TP-TMA in vergelijking met de routinematige diagnostiek. Twee van de deelnemers met afwijkende resultaten waren echter binnen een week voor het bezoek aan het CSG door hun huisarts behandeld voor syfilis en zijn daarom geen gemiste diagnoses. Met de toevoeging van de TP-TMA test aan de routine diagnostiek konden er 14/191 (7%) extra syfilis infecties worden vastgesteld.

DEEL II: MOLECULAIRE EPIDEMIOLOGIE

In het tweede deel van het proefschrift worden studies beschreven die gebruik maken van moleculaire typeringsmethoden om de genetische variatie van *T. pallidum* en *N. gonorrhoeae* in kaart te brengen. Verschillende moleculaire typeringsmethoden kunnen worden gebruikt om *T. pallidum* te onderscheiden in verschillende stammen om seksuele netwerken te onderzoeken en om meer inzicht te krijgen in de overdracht van syfilis. Voor zover wij weten, zijn de studies beschreven in de **hoofdstukken 4, 5, 6** en **7** de eerste en enige studies over de moleculaire diversiteit van *T. pallidum* in Nederland. **Hoofdstukken 4, 5, 6** en **7** onthulden een vergelijkbare verdeling van verschillende *T. pallidum* stammen voor verschillende bevolkingsgroepen. Type 14d/g, zoals vastgesteld met behulp van de verbeterde Centra voor Ziektebestrijding en preventie (CDC) methode, en types 1.3.1 en 1.1.1, zoals vastgesteld met de multi-locus sequentietyperingsmethode (MLST), waren in de meerderheid aanwezig. Afgezien van de meest frequent aanwezige stammen, werden veel *T. pallidum* stammen in slechts één of twee cliënten gevonden. Er zijn twee genetische clusters van *T. pallidum*, namelijk Nichols en SS14. Deze clusters waren aanwezig in een verhouding van ongeveer 1:4 in Amsterdam. Dit is in overeenstemming met een recente grootschalige *T. pallidum* typering studie, Beale et al. 2021, waarin het volledige genoom van monsters zijn geanalyseerd uit 23

landen. De prevalentie van *T. pallidum* stammen die behoren tot de Nichols cluster is relatief hoog in Nederland in vergelijking met andere Europese landen. Fylogenetische bomen (een boom om de verwantschap tussen stammen te zien) werden gegenereerd in **hoofdstukken 5** en **7** met gebruik van aaneengeschakelde sequenties van de MLST methode. Deze fylogenetische bomen visualiseerden de genetische variatie van *T. pallidum* stammen tussen de gevonden stammen (**hoofdstukken 5** en **7**). De gekoppelde MLST sequenties bleken goed te correleren met *T. pallidum* clusters en subclusters zoals die zijn beschreven in de literatuur door middel van volledige genoomanalyses.

Verbeterde CDC-methode voor moleculaire typering van *T. pallidum*

Om de moleculaire diversiteit van *T. pallidum* in Amsterdam te bepalen, werden in **hoofdstuk 4** *T. pallidum* PCR positieve monsters van genitale wondjes en huiduitstrijken bestudeerd die waren verzameld tussen 2016 en 2017. Deze waren afkomstig van de routine syfilis diagnostiek op het Streeklaboratorium van de GGD Amsterdam. De verbeterde CDC typeringsmethode werd gebruikt. Deze is gebaseerd op het aantal herhalingen van een 60 basenparen fragment in het *arp* gen, de variant analyse van de fragment lengte van de genen van de *tpr* genen (*tprE*, *tprG* en *tprJ*) en de analyse van de gedeeltelijke sequentiebepaling van het *tp0548* gen. Deze combinatie van moleculaire technieken maakte het mogelijk de monsters te typeren. Dit liet een grote variatie van *T. pallidum* stammen zien. Enkele *T. pallidum* stammen kwamen heel vaak voor en een grote hoeveelheid andere types kwamen slechts in één of twee cliënten voor. Er werden geen verbanden gevonden tussen klinische of demografische gegevens van cliënten en de verschillende *T. pallidum* types. Er is daarnaast gekeken naar macrolide-geassocieerde resistentiemutaties (MRAM). Dit zijn mutaties in het 23SrRNA gen die samenhangen met resistentie tegen het antibioticum, azitromycine, behorend tot de macroliden antibiotica klasse. In de geanalyseerde 23S rRNA genen in deze studie vonden we in deze mutaties in 88% van de *T. pallidum* monsters.

T. pallidum homogeniteit binnen patiënten

T. pallidum kan op meerdere locaties in het lichaam worden gevonden bij patiënten in verschillende syfilis ziektestadia. Het zou mogelijk kunnen zijn dat bepaalde *T. pallidum* stammen een voorkeur hebben voor bepaalde lichaamslocaties (ook wel weefseltropisme genoemd). In **hoofdstuk 5** is dit en de moleculaire variatie van *T. pallidum* binnen een patiënt onderzocht. MLST werd uitgevoerd op alle *T. pallidum* PCR positieve monsters van de studie beschreven in **hoofdstuk 2**. Naast deze studiemonsters werden ook de uitstrijken van eventuele genitale zweren of wondjes tijdens de routine syfilis diagnostiek van deelnemers getypeerd. Dit resulteerde in getypeerde *T. pallidum* stammen afkomstig van 107 lichaamslocaties van 93 deelnemers. Van de 93 deelnemers bij wie ten minste één monster kon worden getypeerd, waren er 48 (52%) met primaire syfilis, 35 (38%) met secundaire syfilis en 10 (11%) met vroeg latente syfilis. De monsters die met succes werden getypeerd

hadden significant vaker een hoog positief signaal in de *T. pallidum* PCR, wat een indicatie is van de aanwezigheid van veel *T. pallidum* bacteriën. Voor de 12 deelnemers met meerdere getypeerde monsters van verschillende lichaamslocaties bleek steeds hetzelfde *T. pallidum* type aanwezig te zijn. Er was dus geen genetische variatie van *T. pallidum* types voor de verschillende lichaamslocaties. Ook was er geen verband gevonden tussen bepaalde typen en het syfilisstadium.

Moleculaire typering van *T. pallidum* in verschillende populaties

In **Hoofdstuk 6** wordt de moleculaire variatie van *T. pallidum* beschreven in een kleine groep vrouwen en mannen die seks hebben met vrouwen (MSW) in Amsterdam, Nederland en in Antwerpen, België. De verdeling van *T. pallidum* stammen was vergelijkbaar met de verdeling gevonden onder mannen die seks hebben met mannen (MSM) in eerdere studies die we uitvoerden in Amsterdam (in **hoofdstukken 4, 5** en **7**). Interessant genoeg verschilde de verdeling van *T. pallidum* stammen onder MSW in onze studie wel enigszins van de bestudeerde monsters van vrouwen. De *T. pallidum* stammen bij MSW kwamen namelijk meer overeen met die van de (inter)nationaal gepubliceerde stammen van MSM die opgenomen zijn in de publieke MLST database. Dit suggereert een overlap in seksuele netwerken waarin sommige mannen die naar eigen zeggen seks hadden met alleen vrouwen ook mogelijk seks hebben gehad met mannen. Stigma op seksueel gedrag kan een rol hebben gespeeld voor deze mannen bij het rapporteren van seks met vrouwen versus seks met mannen.

T. pallidum subspecies *endemicum* in Amsterdam

In **Hoofdstuk 7** wordt de moleculaire variatie beschreven van *T. pallidum* stammen onder cliënten met verschillende etniciteiten die het CSG Amsterdam bezochten. *T. pallidum* PCR positieve routine diagnostiek restmaterialen van cliënten met Surinaamse, Nederlands-Antilliaanse en Nederlandse ethiciteit tussen 2006 en 2018 werden voor deze studie teruggezocht. In eerdere studies uit Cuba en Japan was namelijk gerapporteerd dat onder cliënten bij wie syfilis werd vastgesteld in werkelijkheid de aanwezigheid van een andere *Treponema* soort was vastgesteld, namelijk *T. pallidum* subspecies *endemicum*, de veroorzaker van bejel. Nederland heeft een koloniale geschiedenis met Suriname en de Nederlandse Antillen en daarom wonen er veel mensen uit deze landen in Nederland. Het doel van de studie was om te onderzoeken of bij deze Surinaamse en Nederlands-Antilliaanse cliënten met een syfilis diagnose, het mogelijk zou zijn dat zij ook werden blootgesteld aan *T. pallidum* subspecies *endemicum* via seksuele netwerken met hun landen van herkomst, die geografisch dicht bij Cuba liggen. Door middel van MLST is de *endemicum* subspecies te onderscheiden van *pallidum*. Er werden echter geen *endemicum* subspecies gevonden onder de onderzochte cliënten, wat suggereert dat bejel waarschijnlijk niet aanwezig is in Nederland en mogelijk ook niet in Suriname of de Nederlandse Antillen. Echter zullen er studies uitgevoerd moeten worden met *T. pallidum* monsters rechtstreeks van deze landen

om dit met meer zekerheid te kunnen zeggen. Recente andere studies vonden ook geen of zeer weinig *endemicum* subspecies in de onderzochte materialen, wat bevestigt dat de aanwezigheid van *endemicum* mogelijk minder is dan eerder werd gesuggereerd.

Monitoring van antimicrobiële resistentie in *T. pallidum*

Penicilline wordt al 80 jaar effectief gebruikt als behandeling tegen syfilis. Antibioticaresistentie vormt een bedreiging voor de volksgezondheid, maar gelukkig is er vooralsnog geen resistentie of verminderde gevoeligheid bekend voor penicilline bij gebruik tegen een syfilis infectie. Azitromycine was vroeger een effectieve alternatieve behandeling van syfilis, maar er werd therapie falen gevonden die geassocieerd werden met mutaties in het 23S rRNA loci van *T. pallidum*. Veel studies laten zien dat MRAM in *T. pallidum* stammen steeds vaker voorkomt. Deze toename werd tussen 2006 en 2018 ook gevonden in Amsterdam en is beschreven in **hoofdstuk 7.** MRAM waren aanwezig in meer dan 80% van de *T. pallidum* isolaten uit de onderzoeken beschreven in de **hoofdstukken 4, 6** en **7**. Deze toenemende aanwezigheid van macrolideresistentie in *T. pallidum* heeft geleid tot een aanpassing van behandelrichtlijnen voor de volksgezondheid, waardoor het gebruik van azitromycine voor de behandeling van syfilis bij patiënten wordt afgeraden.

Genetische variatie van *Neisseria gonorrhoeae* voor en tijdens de eerste COVID-19-lockdown

Neisseria gonorrhoeae veroorzaakt een andere wereldwijd veel voorkomende bacteriële soa, namelijk gonorroe. Circulerende genotypen van deze bacterie die in verband worden gebracht met antimicrobiële resistentie worden nauwlettend gevolgd door middel van monitoring studies door volksgezondheidsorganisaties. De COVID-19-pandemie heeft een grote invloed gehad op vrijwel alle infectieziekten. Net als veel andere infectieziekten zijn soa's afhankelijk van contact tussen mensen. Dit menselijke contact werd verminderd door beperkende maatregelen, zoals de anderhalve meter regel en uiteraard de lockdown, als reactie op de pandemie. Onder de cliënten van het CSG in Amsterdam werd een verminderd aantal losse sekspartners gemeld tijdens de eerste lockdown in Nederland in 2020. In **hoofdstuk 8** is de genetische diversiteit van *N. gonorrhoeae* in Amsterdam beschreven van de periodes vóór (15 januari – 29 februari) en tijdens (15 mei- 30 juni) de eerste COVID-19 lockdown in 2020. Er werd een verschuiving waargenomen van *N. gonorrhoeae* sequentietype (ST) 8156 naar ST 9362, die tijdens de lockdown het vaakst voorkwamen. Er waren opmerkelijk weinig verschillen in de volledige genoom sequenties tussen de bestudeerde monsters met ST 9362 tijdens de lockdown. Deze bevinding duidt op klonale verspreiding binnen Amsterdam, mogelijk door lokale overdracht. Ook was er een fenotypische verschuiving naar azitromycine resistentie en verhoogde ceftriaxon (meest gebruikte antibioticum tegen gonorroe) gevoeligheid. Dit was in overeenstemming met de opkomst van ST 9362 dat genetische markers voor azitromycine resistentie bevat en mutaties mist die geassocieerd zijn met ceftriaxonresistentie. **Hoofdstuk 8**

laat zien dat de invloed van volksgezondheidsmaatregelen merkbaar is in de epidemiologie van soa infectieziekten en waarmee rekening moet worden gehouden bij het uitvoeren van monitoring studies.

Algemene discussie

Hoofdstuk 9 geeft een overzicht van de bevindingen van alle opgenomen hoofdstukken in het proefschrift. Ook worden er toekomstige aanbevelingen gedaan in de context van de huidige literatuur en vorderingen in met name het syfilis veld. Beide delen van het proefschrift, Moleculaire Diagnostiek en Moleculaire Epidemiologie, worden afzonderlijk besproken. Voor toekomstige studies zouden (inter)nationale samenwerkingen uitgebreid moeten worden om een beter geografisch overzicht en begrip te krijgen van de diversiteit van *T. pallidum* met behulp van geschikte moleculaire methoden en gegevens te delen in publiek toegankelijk databases.

ABOUT THE AUTHOR

Hélène was born on the 17th of January 1993 in Darmstadt, Germany and grew up in the municipality of Katwijk in the Netherlands. After high school (Rijnlands Lyceum, Oegstgeest), she started a Bachelor in Biology at the Vrije Universiteit in Amsterdam and moved to Amsterdam. Her first encounter with research in the field of infectious diseases was during her Bacherlor internship titled "Improved diagnostics of *vancomycin resistant enterococcus* by PCR on direct patient samples" at the Public Health Laboratory (PHL) of Amsterdam in 2014. After the internship, she worked at the administrative department of the PHL where she registered and distributed the incoming samples. After two months, she worked as a research assistant at the molecular biology laboratory within the PHL where she helped with ongoing research. In 2015, she continued her studies with a Biology Master, specialized in Microbial Biotechnology and Health, at the University of Leiden.

During the master she got more interested in infectious diseases and so her scientific research interest grew. To pursue this interest, she started a PhD in 2018 focusing on molecular epidemiology of *Treponema pallidum* at the PHL of Amsterdam. In the last half of 2018, she was awarded a travel grant and had the opportunity to spend 2 months at the laboratory lead by David Šmajs at Department of Biology of the Masaryk University in Brno, Czech Republic. There, she learned the developed typing method for *T. pallidum* and applied it to her selection of samples from the Netherlands. After returning to the PHL in Amsterdam, she implemented this technique in the laboratory and used it in following research. Through collaborations, she also got to work on other pathogens causing sexually transmitted infections; *Mycoplasma genitalium, Chlamydia trachomatis* and *Neisseria gonorrhoeae.* Most of the conducted research during her PhD is presented in this thesis. Her research resulted in several (co-) authored publications in peer-reviewed journals and numerous presentations at (inter) national conferences.

LIST OF PUBLICATIONS

Year **In this thesis**

2020 **Zondag HCA,** Cornelissen AR, van Dam AP, Bruisten SM. (2020) Molecular diversity of *Treponema pallidum* subspecies pallidum isolates in Amsterdam, the Netherlands. *Sex Transm Infect.* 96(3):223-226.

2020 **Zondag HCA,** Bruisten SM, Vrbová E, Šmajs D. (2020) No bejel among *Treponema pallidum* isolates diagnosed as syphilis from Surinamese, Antillean and Dutch patients in Amsterdam, the Netherlands. *PLoS One*. 15(3):e0230288.

2022 **Zondag HCA*,** Nieuwenburg SA*, Himschoot M, van Dam AP, Schim van der Loeff MF, de Vries HJC, Bruisten SM. (2022) *Treponema pallidum* Subspecies *Pallidum* Intrapatient Homogeneity at Various Body Locations in Men with Infectious Syphilis. *Microbiol Spectr.* 10(4):e0248221.

2022 Nieuwenburg SA*, **Zondag HCA***, Bruisten SM, Jongen VW, Schim van der Loeff MF, van Dam AP, de Vries HJC. (2022) Detection of Treponema pallidum DNA During Early Syphilis Stages in Peripheral Blood, Oropharynx, Ano-Rectum and Urine as a Proxy for Transmissibility. *Clin Infect Dis.* 75(6):1054-1062.

2023 **Zondag HCA,** (2023) *Treponema pallidum* strains among women and men who have sex with women in Amsterdam, the Netherlands and Antwerp, Belgium between 2014 – 2020. *Sex Transm Dis. 50(6):10.1097*

2023 **Zondag HCA***, de Korne-Elenbaas J*, de Vries HJC, Bruisten SM, van Dam AP. (2023). Increased clonality among *Neisseria gonorrhoeae* isolates during the COVID-19 pandemic in Amsterdam, the Netherlands. *Microb Genom. 9(4); 000975.*

2023 **Zondag HCA,** van Dam AP, Bosch J, Getman D, Nenninger A., de Vries HJC, Bruisten SM (2023) Timely diagnosis of incubating syphilis infections using *Treponema pallidum* Transcription Mediated Amplification assay. *Submitted.*

*Shared first authorship

Other publications

2022 Braam JF*, **Zondag HCA***, van Dam AP, de Vries HJC, Vergunst C, Hetem D, Schim van der Loeff MF, Bruisten SM. (2022) Prevalence of fluoroquinolone resistance-associated mutations in *Mycoplasma genitalium* among clients of two sexual health centres in the Netherlands: a cross-sectional study. *BMJ Open* 12:e066368.

2022 Schim van der Loeff MF, **Zondag H**, Bruisten S, Jongen VW. (2022) Does Previous Syphilis Alter the Course of Subsequent Episodes of Syphilis? *Clin Infect Dis.* 28;74(8):1503.

2022 Dukers-Muijrers NHTM, Schim van der Loeff M, Wolffs P, Bruisten SM, Götz HM, Heijman T, **Zondag H**, Lucchesi M, De Vries H, Hoebe CJPA. (2022) Incident urogenital and anorectal Chlamydia trachomatis in women: the role of sexual exposure and autoinoculation: a multicentre observational study (FemCure). *Sex Transm Infect*. 98(6):427-437.

2023 Vrbová E., **Zondag HCA**, Bruisten SM, Šmajs D. Low prevalence of Arg753Gln polymorphism of Toll-like receptor 2 in syphilis-infected and control population in The Netherlands: heterozygotes have higher rate of self-reported sexual contacts but the same syphilis positivity rate. *Manuscript in preparation*

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LIST OF CONTRIBUTING AUTHORS

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Dept. : Department

PHS: Public Health Service

AUTHOR'S CONTRIBUTIONS

Chapter 2

Nieuwenburg SA*, **Zondag HCA***, Bruisten SM, Jongen VW, Schim van der Loeff MF, van Dam AP, de Vries HJC. (2022) Detection of Treponema pallidum DNA During Early Syphilis Stages in Peripheral Blood, Oropharynx, Ano-Rectum and Urine as a Proxy for Transmissibility. *Clin Infect Dis.* 75(6):1054-1062.

SN, SB, AD and HV conceived and designed study; SN, HZ, HV contributed to data collection; SN, VJ, HZ, MS conducted analysis; SN, HZ, SB, MS, AD, HV drafted and revised manuscript. All authors read and approved the final version. *SN and HZ are shared first authors.

Chapter 3

Zondag HCA, van Dam AP, Bosch J, Getman D, Nenninger A., de Vries HJC, Bruisten SM (2023) Timely diagnosis of incubating syphilis infections using *Treponema pallidum* Transcription Mediated Amplification assay. *Submitted.*

HZ, AD, DG, AN and SB conceived and designed study; HZ, JB contributed to data collection; HZ, AD conducted analysis; HZ, AD, SB, DG, AN, HV drafted and revised manuscript. All authors read and approved the final version.

Chapter 4

Zondag HCA, Cornelissen AR, van Dam AP, Bruisten SM. (2020) Molecular diversity of *Treponema pallidum* subspecies pallidum isolates in Amsterdam, the Netherlands. *Sex Transm Infect.* 96(3):223-226.

SB and AD conceived and designed study; HZ, AC contributed to data collection; HZ conducted analysis; HZ, AD, SB, AC drafted and revised manuscript. All authors read and approved the final version.

Chapter 5

Zondag HCA*, Nieuwenburg SA*, Himschoot M, van Dam AP, Schim van der Loeff MF, de Vries HJC, Bruisten SM. (2022) *Treponema pallidum* Subspecies *Pallidum* Intrapatient Homogeneity at Various Body Locations in Men with Infectious Syphilis. *Microbiol Spectr.* 10(4):e0248221.

HZ, SN, SB, AD and HV conceived and designed study; SN, HZ, MH contributed to data collection; HZ, MH conducted analysis; HZ, SN, SB, MS, AD, HV drafted and revised manuscript. All authors read and approved the final version. *SN and HZ are shared first authors.

Chapter 6

Zondag HCA, Zwezerijnen-Jiwa FH, De Baetselier I, Bruisten SM (2023) *Treponema pallidum* strains among women and men who have sex with women in Amsterdam, the Netherlands and Antwerp, Belgium between 2014 – 2020. *Sexually Transmitted Diseases 2023 Feb 8; 50(6):10.1097.*

HZ and SB conceived and designed study; HZ, FJ, IB contributed to data collection; HZ, FJ conducted analysis; HZ, FJ, IB, SB drafted and revised manuscript. All authors read and approved the final version.

Chapter 7

Zondag HCA, Bruisten SM, Vrbová E, Šmajs D. (2020) No bejel among *Treponema pallidum* isolates diagnosed as syphilis from Surinamese, Antillean and Dutch patients in Amsterdam, the Netherlands. *PLoS One*. 15(3):e0230288.

HZ, DS and SB conceived and designed study; HZ and EV contributed to data collection; HZ conducted analysis; HZ, SB, DS drafted and revised manuscript. All authors read and approved the final version.

Chapter 8

Zondag HCA*, de Korne-Elenbaas J*, de Vries HJC, Bruisten SM, van Dam AP. (2023). Increased clonality among *Neisseria gonorrhoeae* isolates during the COVID-19 pandemic in Amsterdam, the Netherlands. *Microbial Genomics. 9(4); 000975.*

HZ, JK and AD conceived and designed study; HZ, JK contributed to data collection; HZ, JK conducted analysis; HZ, JK, AD, SB, HV drafted and revised manuscript; All authors read and approved the final version. *HZ and JK are shared first authors.

*Shared first authorship

PHD PORTFOLIO

	PhD training	
Year	**Courses**	**Workload (ECTS)**
2018	Infectious Diseases, AMC Graduate School	1.3
2018	Bioinformatics Sequence Analysis, AMC Graduate School	1.1
2019	Computing in R, AMC Graduate School	0.4
2019	Genetic Epidemiology, AMC Graduate School	1.1
2019	Practical Biostatistics, AMC Graduate School	1.3
2020	Bacterial Genomes: Antimicrobial Resistance in Bacterial Pathogens (online), Wellcome Genome Campus Advanced Courses and Scientific Conferences	0.4
2020	Bacterial Genomes II: Accessing and Analysing Microbial Genome Data Using Artemis (online), Wellcome Genome Campus Advanced Courses and Scientific Conferences	0.4
2020-2021	Weekly/monthly COVID-19 science update	1.0
2018-2023	Weekly PhD educational hour (Journal Club, Peer education, Epidemiology education), Department of Infectious Diseases, Public Health Service of Amsterdam	17.5
2022	Effectieve Persoonlijke Communicatie, De Baak, Driebergen	1.1
	Seminars, workshops and master classes	
2018	GGD Onderzoeksdag 2018, 2019, 2021 (virtual), 2023	0.8
2018	Symposium: Infectieuze Diarree, wat moet je er nou mee? Public Health Service Amsterdam	0.1
2019	SOA & HIV expert meeting, RIVM, Bilthoven	0.2
2020	New look PubMLST showcase event, virtual	0.1
2022	Special Interest Groep Bioinformatics in Medical Microbiology meeting, Nederlandse Vereniging voor Medische Microbiologie (NVMM), Utrecht	0.2
2021	Symposium: Monsterlijke Microben, mooi maar moeilijk! Public Health Service Amsterdam	0.1
2021	Genomics Workshop (online), Data Carpentry Genomics	0.2
2022	KNVM & NVMM Scientific Spring Meeting, Arnhem, Netherlands	0.2
2022	Monthly seminar of Bioinformatics group, AMC	0.1
2022	IUSTI-Europe Webinar 7- Unfolding the secrets of *Treponema pallidum*	0.2
2018-2023	Monthly seminar of the Public Health Laboratory, Public Health Service of Amsterdam	1.5
	Presentations	
2018	Molecular typing and macrolide resistance of *Treponema pallidum pallidum* in Amsterdam, the Netherlands (oral), IUSTI 2018, Dublin, Ireland	0.5
2019	Dynamiek van syfilis infecties onder Amsterdammers, Symposium Public Health Laboratory, Amsterdam	0.5
2019	No bejel among Surinamese, Antillean and Dutch syphilis diagnosed patients in Amsterdam between 2006 – 2018 evidenced by multi-locus sequencing of *Treponema pallidum* isolates (poster), STI & HIV 2019 World Congress, Vancouver, Canada	0.5
2020	Moleculaire epidemiologie door typering van *Treponema pallidum* in Amsterdam (oral), Werkgroep Moleculaire Diagnostiek van Infectieziekten (WMDI), virtual	0.5

Year	Presentations *continued*	Workload (ECTS)
2021	Frequent detection of *Treponema pallidum* subspecies *pallidum* in different body locations and intra-patient homogeneity in patients with early syphilis (poster), KNVM & NVMM Scientific Spring Meeting, Arnhem, Netherlands	0.5
2021	Improving syphilis screening by early detection using *Treponema pallidum* Transcription Mediated Amplification assay (oral), STI&HIV 2021 World Congress, Amsterdam, Netherlands, virtual	0.5
2021	*Treponema pallidum* intra-patient homogeneity between various body locations in patients with infectious syphilis (oral), STI&HIV 2021 World Congress, Amsterdam, Netherlands, virtual	0.5
2021	*Treponema pallidum* intra-patient homogeneity between various body locations in patients with infectious syphilis (oral pitch), Talkshow: The Syphilis Epidemic, STI&HIV 2021 World Congress, Amsterdam, Netherlands	0.5
2021	*Treponema pallidum* intra-patient homogeneity between various body locations in patients with infectious syphilis (oral), Seminar of the Laboratory of Experimental Biology, Amsterdam UMC, Netherlands	0.5
2022	*Treponema pallidum* strains among heterosexual men and women in Amsterdam and Antwerp, 2014 – 2020 (poster), KNVM & NVMM Scientific Spring Meeting, Arnhem, Netherlands	0.5
2022	Moleculaire epidemiologie van *Neisseria gonorrhoeae* tijdens de eerste lockdown in Amsterdam (oral), SOA & HIV Expert Meeting 2022, RIVM, Bilthoven	0.5
2022	Improving syphilis screening by early detection using *Treponema pallidum* Transcription Mediated Amplification assay (oral), ECCMID 2022, Lisbon, Portugal	0.5
2022	Molecular epidemiology of *Treponema pallidum* subspecies *pallidum* by multi-locus sequence typing (oral), IUSTI-Europe Webinar, virtual	0.5
2022	Improving syphilis screening by early detection using *Treponema pallidum* Transcription Mediated Amplification assay (oral), Hologic Innovation Forum, Brussels (presented by Dr. Sylvia Bruisten)	0.5
2022	Potential improvement of syphilis screening by early detection using *Treponema pallidum* TMA assay (oral), IUSTI-Europe Congress, Tbilisi, Georgia, virtual	0.5
2022	Increased clonality among *Neisseria gonorrhoeae* isolates during the COVID-19 pandemic in Amsterdam (poster), IMMEM XIII, Bath, United Kingdom	0.5
	(Inter)national conferences	
2018	NVMM KNVM Scientific Spring Meeting	
2018	International Union against STI (IUSTI) World Congress, Dublin, Ireland	0.8
2019	European Congress of Microbiology and Infectious Diseases (ECCMID), Amsterdam, The Netherlands	1.1
2019	STI & HIV 2019 World Congress, Vancouver, Canada	0.8
2020	IUSTI-Europe, virtual	0.5
2021	KNVM & NVMM Scientific Spring Meeting, virtual	0.6
2021	STI & HIV World Congress, Amsterdam, the Netherlands, virtual	0.5
2022	ECCMID, Lisbon, Portugal	1.1
2022	13th International Meeting on Microbial Epidemiological Markers (IMMEM XIII), Bath, United Kingdom	1.1

Year	Supervising	Workload (ECTS)
2019	Belle Toussaint, Extracurricular Assisting in Research, Bsc student Clinical Science, VU Amsterdam Topic: Detection of *Treponema pallidum* in various anatomical locations	1.0
2020 - 2021	Nassima All Abbass, Internship, student ROC Midden Nederland, final internship for Biological medical analist Topic: MGMRAM isolatie validatie MagNA Pure 24	2.0
2021	Florine Jiwa, Internship, Msc student Medicine, VU Amsterdam Topic: Typing of Treponema pallidum strains among heterosexual men and women in Amsterdam, The Netherlands and Antwerp, Belgium between 2014 – 2020	1.0
2022	Eliska Vrbová, PhD student, Masaryk University, Brno, Czech Republic Topic: Low prevalence of Arg753Gln polymorphism of Toll-like receptor 2 in syphilis-infected and control population in The Netherlands: heterozygotes have higher rate of self-reported sexual contacts but the same syphilis positivity rate	0.5

	Parameters of Esteem: Grants and Funding
2018	**Research and Travel grant** (FEMS-GO-2018-117) from the federation of European microbiological societies (FEMS) in 2018: **H.C.A. Zondag**, S.M. Bruisten, E. Vrbová, D. Smajs (2018) No bejel among Treponema pallidum isolates diagnosed as syphilis from Surinamese, Antillean and Dutch patients in Amsterdam, the Netherlands
2020	TP-TMA kits provided by Hologic

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