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Optimization of a genotyping scheme for *Treponema pallidum* subsp. *pallidum* including markers for macrolide resistance

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Abstract

Syphilis, a sexually transmitted disease, is an important public health problem, both in Norway and worldwide. The incidence of syphilis has increased in the recent years, particularly among men who have sex with men (MSM). Infection with the uncultivated spirochaete bacteria *Treponema pallidum* subsp. *pallidum* (TPA), is the cause of syphilis. Syphilis can be diagnosed with antibody test against TPA antigen or by directly identification of TPA DNA with polymerase chain reaction (PCR). Untreated syphilis infection can be divided into different stages that give different symptoms: primary, secondary, latent, and tertiary. No vaccine against TPA has been developed yet. Penicillin is the first choice of antibiotic treatment for syphilis. Azithromycin, a macrolide antibiotic, has shown to be an effective treatment of early syphilis in many cases as well as treating infections with other venereal diseases. However, macrolide antibiotic resistance in TPA has been detected globally.

By molecular characterization, TPA can be divided into two main lineages: Nichols- and SS14-like strains. The combination of molecular genotyping of TPA and macrolide antibiotic resistance is important for understanding the epidemiology of syphilis in Norway, and the surveillance of antibiotic resistance. Thus, a genotyping scheme for TPA including markers for macrolide resistance, need to be established and optimized.

Molecular characterization of TPA was performed by multi-locus sequence typing (MLST) of three loci: TP0136, TP0548, and TP0705. MLST consists of a nested PCR followed by Sanger sequencing of each of the three loci. The allelic profiles and sequence type can be determined by PubMLST. For detection of macrolide resistance in TPA, due to point mutations in position A2058G or A2059G in one or both copies of the 23S rRNA gene, a SimpleProbe real-time PCR assay was developed followed by a melting point analysis. Macrolide resistance in TPA is detected by having a lower melting temperature than the wild-type.

Eighty-one clinical samples were collected from October 2020 to September 2022. Molecular characterization of TPA revealed nine different sequence types, including three novel sequence types. Macrolide resistance was also confirmed among the collected samples.

Sammendrag

Syfilis er en seksuelt overførbar infeksjon, og er et viktig folkehelseproblem både i Norge og i verden. Forekomst av syfilis har økt i de senere årene, spesielt blant menn som har sex med menn (MSM). Bakterien *Treponema pallidum* subspecies *pallidum* (TPA), en spiroket som ikke lar seg dyrke, er årsaken til syfilis. Diagnostisering av syfilis gjøres ved hjelp av antistofftest mot TPA antigen, eller ved direkte identifisering av TPA DNA med polymerasekjedereaksjon (PCR). Ubehandlet syfilisinfeksjon, kan deles inn i ulike stadier som gir forskjellige symptomer: primær, sekundær, latent og tertiær. Det er ikke utviklet noen vaksine mot TPA. Penicillin er førstevalg ved behandling av syfilis. Azitromycin, et makrolidantibiotikum, har også vært anvendt i behandling av syfilis og brukes også i behandling av andre kjønnssykdommer. Resistens mot makrolidantibiotika i TPA har imidlertid blitt detektert globalt.

TPA kan deles inn i to hovedgrupper utfra molekylær subtyping: Nichols- og SS14-stammer. Kombinasjonen av molekylær genotyping av TPA og resistens mot makrolidantibiotika er viktig for å forstå epidemiologien ved syfilis i Norge, og overvåkning av antibiotikaresistens. Genotypiskbestemmelse av TPA, inkludert markører for makrolidresistens, må først optimaliseres og etableres.

Molekylær subtyping av TPA utføres ved multilokus sekvens typing (MLST) av tre loci: TP0136, TP0548 og TP0705. MLST består av en nested PCR etterfulgt av Sanger sekvensering for hvert locus. Allele profiler og sekvenstyper blir bestemt av PubMLST. For deteksjon av makrolidresistente TPA, som skyldes punktmutasjonene A2058G eller A2059G i ett eller begge kopier av 23S rRNA-genet, ble det utviklet en SimpleProbe real-time PCR analyse etterfulgt av en smeltepunktsanalyse. Makrolidresistente TPA har en lavere smeltepunkts-temperatur enn villtype TPA.

Det ble samlet inn 81 pasientprøver fra oktober 2020 til september 2022. Ved molekylær subtyping ble det avdekket ni forskjellige sekvenstyper, deriblant tre nye sekvenstyper. Makrolidresistens kan også bekreftes blant disse prøvene.

List of abbreviations

A		Adenine
bp	-	Base pairs
C	-	Cytosine
CDC	-	Centers for Disease Control and Prevention
Ct	-	Cycle threshold
ddNTP	-	Dideoxynucleotide Triphosphate
dNTP	-	Deoxynucleotide Triphosphate
E. coli	-	*Escherichia coli*
EF primer	-	External forward primer
ER primer	-	External reverse primer
G	-	Guanine
HIV	-	Human Immunodeficiency Virus
IF primer	-	Internal forward primer
IgM/IgG	-	Immunoglobulin M/ Immunoglobulin G
In vitro	-	Meaning “in the glass”. Studies of microorganism are performed in test tube, outside from normal biological context
IR primer	-	Internal reverse primer
LNA	-	Locked Nucleic Acids
MLST	-	Multi-Locus Sequence Typing
MP96	-	MagNA Pure 96
MSIS	-	The Norwegian Surveillance System for Communicable Diseases
MSM	-	Men who have sex with men
NCBI	-	National Center for Biotechnology Information
NIPH	-	Norwegian Institute of Public Health
ORF	-	Open Reading Frame
PCR	-	Polymerase Chain Reaction
PrEP	-	Pre-exposure Prophylaxis
QCMD	-	Quality Control for Molecular Diagnostics
REK	-	Regional Committees for Medical and Health Research Ethics
RPR	-	Rapid Plasma Reagin test

ST	-	Sequence Type
STD	-	Sexually Transmitted Disease
T	-	Thymine
TEN	-	*Treponema pallidum* subsp. *endemicum*
Tm	-	Melting temperature
TPA	-	*Treponema pallidum* subsp. *pallidum*
T. pallidum	-	*Treponema pallidum*
TPE	-	*Treponema pallidum* subsp. *pertenue*
TPPA	-	*Treponema pallidum* particle agglutination test
WHO	-	World Health Organization

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1 Introduction

1.1 History of syphilis

The bacterium *Treponema pallidum* subspecies *pallidum* (TPA), causing syphilis, was first identified in 1905 in lymph nodes by the zoologist Fritz Schaudinn (1871-1906) and the dermatologist Erich Hoffmann (1868-1959). One year later, Landsteiner used the dark-field microscopy method to visualize the TPA, and in 1910, the first serological test was developed by August Wassermann. Using blood samples for the serologic test, made it easy to diagnose syphilis in TPA infected patients as well as in patients in the latent stage of syphilis (Sefton, 2001; Tampa et al., 2014; Thorburn, 1971).

The origin of syphilis is uncertain, but the first descriptions and observations of syphilis in Europe were probably from the Battle of Fornovo in Italy in 1495. Some military doctors had observed several symptoms from the knights and described the disease. Patients had spots all over their bodies and faces, and it could sometimes be itchy. At first it was only a single innocent spot with a bubble that appeared, which later could result in a penetrating wound. The knights would feel intense pain on their arms, legs, and feet, and the doctors had also observed that some people had lost parts of their noses, eyes, feet, and hands (Plagens-Rotman et al., 2021).

Because syphilis has been a disgraceful disease from the very beginning, this disease has many different names in the different countries. Italy, Germany, and United Kingdom named syphilis the “French disease”, while the French called it the “Neapolitan disease”. The Russians named it the “Polish disease” while the Polish called it the “German disease”. Furthermore, Denmark, Portugal, and North Africa named syphilis the “Spanish disease”, and Turkey called it the “Christian disease”. However, Japan named it the “Canton rash” or the “Chinese ulcer”, the Muslims in Northern India blamed the Hindu while the Hindu blamed the Muslims (Rothschild, 2005; Tampa et al., 2014).

Even with all these names, the origin of syphilis is not well known. There are three different hypotheses of how syphilis was spread in Europe: the Evolutionary hypothesis, pre-Columbian hypothesis, and the Columbian hypothesis. The

Evolutionary hypothesis suggests that different *Treponema* subspecies have evolved from a single organism due to changes in the environment. Pinta was probably the first disease in Africa and Asia to evolve into Yaws. Mutation of other existing treponematoses in Europa might define the pre-Columbian hypothesis. At the beginning, the different treponematoses caused a mild disease, but with several mutations, the diseases evolved and changed to the known syphilis. The Columbian hypothesis is the most common one, stating that Columbus brought syphilis back from the New World (North America, South America, and the Caribbean) to the Old World (Europe, Africa, and Asia) when he and his men returned to Europe in 1493. Right after the return of Columbus, the disease spread through Europe and Asia, causing a pandemic known as the "the Great Pox". A study done in 2005 by Rothschild, showed that characteristics of syphilis were absent in samples from pre-Columbian in Europe, Africa, and Asia (Petrarca, 2021; Rothschild, 2005; Sefton, 2001).

Syphilis is named after the shepherd Syphilus, from a Latin poem, who had insulted the God of sun and was therefore punished with a venereal disease. This disease not only infected poor people, but also high-ranking people, making prostitution no longer a tolerant profession although being the oldest profession in the world (Plagens-Rotman et al., 2021).

When syphilis was first discovered, there was no treatment. Approximately 10% of the population of England were infected with syphilis at the end of 19th century. At that time, before any treatment, getting syphilis were like getting a death sentence (Vernon, 2019). Many believed that getting diagnosed with syphilis was a punishment for their sins and therefore they should be isolated (Plagens-Rotman et al., 2021).

Mercury has been used to treat syphilis, as well as other skin disorders, for a long time (Bircher, 2014). As a heavy metal, mercury is very toxic and exist in different forms. Mercury will enter the brain and other organs and leads to malfunctioning of muscles, kidneys, and nerves as well as causing asthma bronchitis and other respiratory problems (Jaishankar et al., 2014). Calomel, Hg_2Cl_2, was directly injected or taken orally, and mercuric chloride, $HgCl_2$, was applied to the skin as ointment. From early 16th century until 1920, syphilis was commonly treated with fumigation. Infected patients were locked in a tent or overheated room and were forced to inhale gas from

mercuric chloride and metallic mercury. It was normal to rub in mercuric ointments several times a day, near a hot source such as a hot fire, which results in sweating patients. These practices could last from weeks to months until the disease was gone (Vernon, 2019; Zuckerman, 2016).

In 1908, the German scientist Paul Ehrlich (1854-1915) received the Nobel Prize in medicine for the discovering of Salvarsan. This drug was called a “Magical bullet” because this drug would specifically bind to the bacteria and kill it, but not destroying the human cell, making it safer to use than mercury. Salvarsan was commonly used as treatment before discovering the antibiotic, penicillin, by Alexander Fleming in 1928. However, penicillin was not used as treatment until 1943 (Aminov, 2010; Sefton, 2001; Tampa et al., 2014).

1.2 *Treponema pallidum*

Treponema pallidum includes different subspecies that can cause several human diseases. On genomic level, the different subspecies are closely related, with a DNA sequence identity up to 99.8%, but the different *T. pallidum* subspecies can cause different diseases (Nechvatal et al., 2014; Smajs et al., 2015). Venereal syphilis, a sexually transmitted disease (STD), is caused by *T. pallidum* subsp. *pallidum* (TPA). Other nonvenereal *T. pallidum* disease such as yaws is caused by *T. pallidum* subsp. *pertenue* (TPE), endemic syphilis is caused by *T. pallidum* subsp. *endemicum* (TEN) and pinta is caused by *Treponema carateum* (Centurion-Lara et al., 2006). These nonvenereal *T. pallidum* diseases are also known as endemic treponematoses (Giacani & Lukehart, 2014). Syphilis is the only one of the diseases, caused by *T. pallidum*, that is transmitted by sexual activity and hence use human as natural host (Radolf et al., 2016). The other *T. pallidum* diseases are usually transmitted through direct contact with an infected person (Tampa et al., 2014).

The different diseases of nonvenereal *T. pallidum* have two different stages: an early and a late stage. Normally, the early stage consists of a lesion at the infected area, and at this stage it is more infectious. The late stage is noninfectious and appears approximately 5 years after infection and mostly affect bone tissues (Giacani & Lukehart, 2014; Perine et al., 1984).

Yaws, caused by TPE, is a tropical disease and primarily occurs in Africa, Central and South America, Southeast Asia, and the Caribbean where the climate is warm and humid. Children between 2-15 years old are predominantly getting infected, especially boys because they are more physically active (Kazadi et al., 2014; Perine et al., 1984). This disease affects skin, soft tissue, and bone and is transmitted by skin-to-skin contact with an infected lesion (Giacani & Lukehart, 2014; Smajs et al., 2012). According to the World Health Organization (WHO), over 100 000 cases of yaws from 13 different countries were reported in 2021, most of them are diagnosed clinically (WHO, 2023c).

Endemic syphilis, also known as bejel caused by TEN, is seen in areas where the climate is dry such as Arabian Peninsula, Southwest Asia, and southern border of the Sahara Desert. Of all the treponematoses, the endemic syphilis is the one closest related to the venereal syphilis. Endemic syphilis is a common infection in children just as yaws. The primary lesion is hard to observe and occurs mostly in the oral mucosa, making the transmission of the disease easier by sharing foods and drinks (Giacani & Lukehart, 2014; Perine et al., 1984).

Pinta is the mildest disease of all the treponematoses. The disease is common in Central and South America. Unlike yaws and endemic syphilis, pinta seems to appear among young adults between the age of 15 and 30 years with chronic skin lesion. The infection is transmitted through lesion-to-skin contact (Giacani & Lukehart, 2014; Perine et al., 1984).

1.2.1 *Treponema pallidum* subsp. *pallidum* (TPA)

The venereal syphilis, caused by TPA, is a global health problem which occurs among people of all ages around the world. Syphilis infections are mainly among sexually active adults unlike the others *T. pallidum* which are endemic and only infect via skin-to-skin contact (Perine et al., 1984). Infection with syphilis does not only occur sexually, but also congenital (from mother-to-child during pregnancy) and by nonsexual contact via direct contact with the infected lesion (Giacani & Lukehart, 2014; Perine et al., 1984). Just like most other bacteria, transmission with TPA through intact skin will not

occur. TPA can enter the body through endothelial cells, mucous membranes, and open wounds (Perine et al., 1984; Thomas et al., 1988).

1.2.2 Biology of TPA

Even though there are minimal genetical differences between the different *T. pallidum* strains, there are several variable regions within the *T. pallidum* genome. By molecular differentiation, TPA can be grouped into two different main lineages, Nichols- or SS14-like strains. The differentiation of the two strains can give information about the TPA evolution (Nechvatal et al., 2014). In 1998 and 2008, the first genomes of Nichols- and SS14-like strains were sequenced respectively. Both lineages are distantly related (Petrosova et al., 2013; Stamm, 2010). TPA has one of the smallest genomes among the prokaryotic organisms with a circular chromosome of 1.14 Mb pairs. The sequence from the Nichols strain showed a total of 1041 predicted open reading frames (ORFs). Most of the ORFs encode several predicted outer membrane proteins and hypothetical proteins as well as novel gene products (Fraser et al., 1998; Nechvatal et al., 2014).

TPA is considered a Gram-negative bacterium and is a cylindric spiral-shaped organism which is a part of the Spirochaetaceae family (see Figure 1.1). The bacterium size ranges from 10 to 20 µm long and 0.1 to 0.2 µm wide (Liu et al., 2010; Peeling et al., 2017). Because of the bacterium size, a dark-field microscopy is needed to visualize the bacterium (Lafond & Lukehart, 2006). The cell envelope of TPA consists of an outer membrane, a thin peptidoglycan layer, and a cytoplasmic membrane. At each pole of the bacterium, there are flagellar filaments and cytoplasmic filaments which are needed for the bacterium to move. The layer of peptidoglycan gives structural stability to the bacterium and the flexibility to move (Liu et al., 2010; Radolf et al., 2016).

Unlike other Gram-negative bacteria, TPA lacks lipopolysaccharide. Instead, it contains other outer membrane proteins which are important for the immune response of the host. One of the outer membrane proteins, also known as the surface-exposed protein, is the TP0136. The *tp0136* gene encodes a lipoprotein signal peptide which binds to human fibronectin, an important part of the extracellular matrix. This protein is expressed during infection with TPA and is recognized by the immune system

(Brinkman et al., 2008; Ke et al., 2015). Another gene is the *tp0548* which hypothetically encode an outer membrane protein (Cox et al., 2010). According to a study done by Fernandez-Naval et al., the *tp0548* gene is reported in TPA and TPE subspecies (Fernandez-Naval et al., 2019). Characterization of the locus TP0548, has also been used to differentiate the two TPA strains, SS14- and Nichols-like strains, and *T. pallidum* subspecies (Gallo Vaulet et al., 2017; Kou et al., 2021; Mikalova et al., 2014). Another gene, which is associated with antibiotic resistance, is the *tp0705* gene encoding a penicillin-binding protein (Liu et al., 2020).

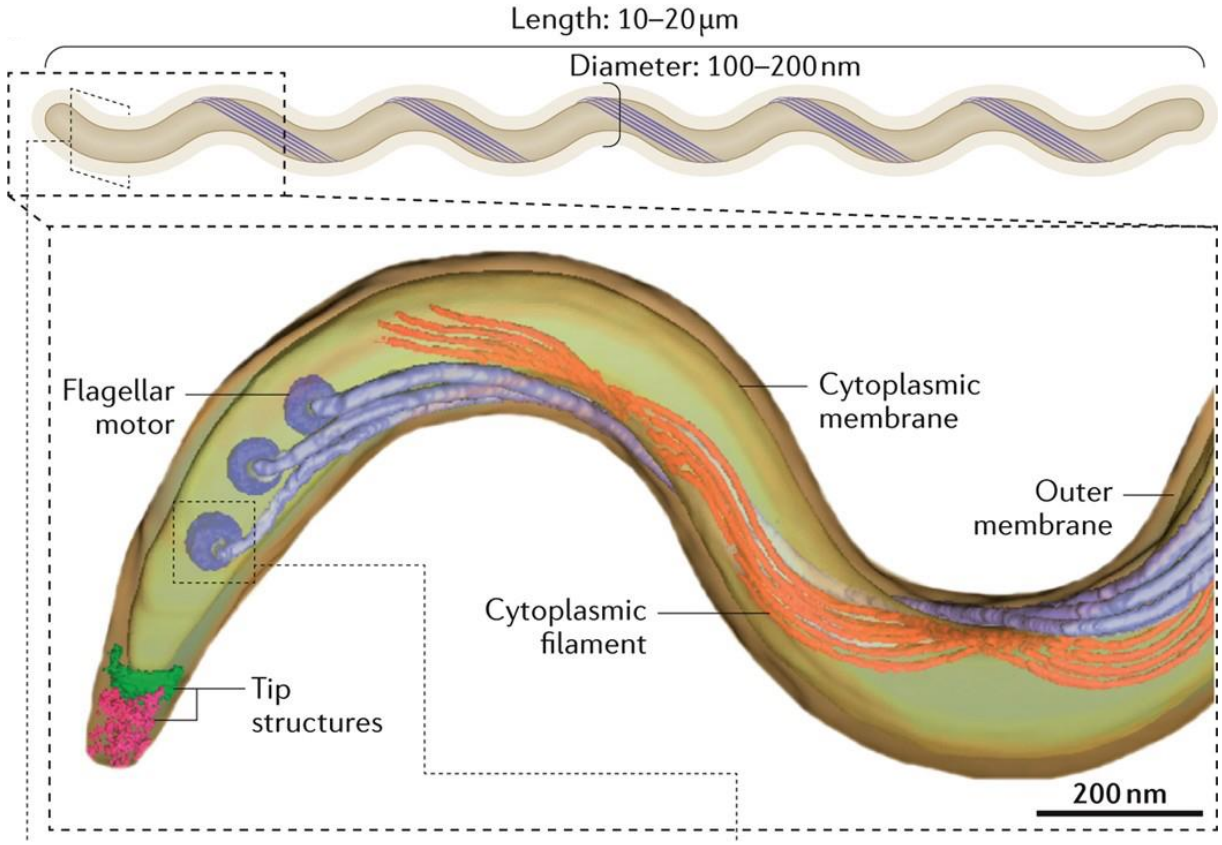


Figure 1.1 The spirochaete bacterium *Treponema pallidum* subsp. *pallidum*. A spiral-shaped organism which is considered a Gram-negative bacterium. The bacterium consists of an outer membrane and a cytoplasmic membrane which gives structural stability to the bacterium, as well as flagellar filaments and cytoplasmic filaments which is important for the movement of the bacterium. The figure is taken from Peeling et al. (2017).

TPA has a slow metabolism, and the time for multiplying in the host is about 30-33 hours (Molini et al., 2016). TPA is very fragile outside the mammalian host cells and will be quickly inactivated by mild heat, cold, dehydration, and disinfectant (Radolf, 1996). Because of these characteristics, the bacterium will not be able to survive outside the mammalian host, hence TPA will usually not be cultured *in vitro* (Lafond & Lukehart, 2006; Peng et al., 2011). In recent years, TPA have been able to be cultured

in vitro but the method is complex (Belkacemi et al., 2019; Edmondson et al., 2018). Molecular typing is therefore the best tool to determine infection with syphilis (Peng et al., 2011).

1.2.3 Disease development

Syphilis is known as the Great imitator or the Great mimicker, making it hard to be diagnosed because the symptoms of the disease can mimic other infections. Syphilis infected individuals will mostly go through different stages of the disease throughout several years if left untreated. The different syphilis stages can be classified into primary, secondary, latent, and tertiary, see Table 1.1 (Peeling et al., 2017).

Table 1.1 Classification of the different stages of syphilis.

Syphilis stages	**Incubation period**	**Symptoms**
Primary syphilis	3 weeks to 90 days	Appearance of chancre and lesions at infection site
Secondary syphilis	4-8 weeks after primary syphilis	Fever, headache, lack of well-being, skin rash, inflammation of mucosal tissues
Latent syphilis	<1 year of infection, early latent >1 year of infection, late latent	Asymptomatic
Tertiary syphilis	Years	Neurosyphilis: headache, fever, facial weakness, blindness Cardiovascular syphilis: aortic aneurysm Gummatous syphilis: skin, bones, and deformation of tissues

Primary syphilis is typically recognized by painless single chancre or multiple and painful lesions on the genitals which appears three weeks to 90 days after infection (French, 2007; Peeling et al., 2017). Due to the appearance of chancre and lesions among syphilis infected individuals in the early stage, the risk of getting co-infection with other disease-causing agents, like the human immunodeficiency virus (HIV), are increased. Four to five weeks later, these chancres and lesions will heal spontaneously without treatment. Most of the TPA will be eliminated by macrophages, but some TPA

will still be able to escape and enter other tissues in the body through blood and lymph which will lead to the next stage of syphilis (Stamm, 2016).

Four to eight weeks after the primary syphilis, or at the same time the chancre and lesions are healed and up to six months later, symptoms of the secondary stage of syphilis will appear. The symptoms might include fever, headache, overall feeling of discomfort and lack of well-being, skin rash on palms and soles, and inflammation of mucosal tissues in the mouth and genitals. The secondary stage might last for several weeks or months (Peeling et al., 2017; Stamm, 2016).

TPA can still enter tissues in the body without causing symptoms or other clinical signs. This stage is called latent and might last many years before entering tertiary stage (Peeling et al., 2017). The latent stage can be divided into early latent stage (within the first year of the infection) and late latent stage (asymptomatic after the first year of infection) (FHI, 2010b). Absence of chancre and lesions will make it difficult to diagnose infected syphilis patients in latent stage, but serologic tests of antibodies to TPA will be detected by blood sampling. While sexually transmission of syphilis is uncommon because of absence of chancre or lesions, congenital syphilis is more common (Ho & Lukehart, 2011).

The last stage of the disease is called tertiary syphilis and the symptoms might appear many years to decades after infection. About 15-40% of untreated syphilis will get to this stage (Peeling et al., 2017). Due to wide use of antibiotics, tertiary syphilis is not very common today compared to pre-antibiotic era. At tertiary stage, TPA can enter the cardiovascular and central nervous systems, skin, bones, eyes, and cause damage to multiple organs. The three main manifestations of tertiary syphilis are neurosyphilis, cardiovascular, and gummatous syphilis (Stamm, 2016).

Neurosyphilis can occur within short time after infection during primary or secondary stage. The symptoms of neurosyphilis include dementia, headache, fever, facial weakness, visual change leading to blurred vision or loss of vision, and loss of hearing (Ho & Lukehart, 2011; Stamm, 2016). According to a study by Division of STD prevention at the Centers for Disease Control and Prevention (CDC), located in the United States of America, and Department of Public Health, located in Los Angeles,

neurosyphilis was frequently diagnosed among HIV-infected individuals compared to non-HIV infected individuals. The progression to neurosyphilis happens faster in HIV-infected individuals. It is therefore important for HIV-infected individuals to routine test for syphilis (Taylor et al., 2008).

Cardiovascular syphilis usually occurs 10-30 years after primary syphilis, affecting the aortic arch and resulting in aortic necrosis causing aortic aneurysm (Peeling et al., 2017).

Gummatous syphilis, destruction of lesion, occurs mostly in skin and bones but can affect any tissues. The lesions can vary in size. This can occur up to twelve years after infection of primary syphilis (French, 2007).

1.2.4 Pathogenesis

TPA is known as a stealth pathogen because of the lack of lipopolysaccharides on the outer membrane of the bacteria which is important for the immune response (Radolf et al., 2016). In comparison, TPA has approximately 100 times fewer outer membrane proteins than *Escherichia coli (E. coli)*. Instead, TPA have other lipoproteins on the outer membrane. It is hard for the immune system to recognize the bacteria because of the few proteins present at the outer membrane. However, these outer membrane proteins are capable to activate macrophages at the infected area (Lafond & Lukehart, 2006; Radolf et al., 2016). The activated macrophages at the infected area will degrade and kill TPA by phagocytosis (Lafond & Lukehart, 2006).

The lack of the protein to control heat shock, regulated by σ32, and an unstable enzyme at normal body temperature, 3-phosphoglycerate mutase, makes the bacterium sensitive to heat and is the reason why metabolism of TPA is slow (Benoit et al., 2001; Lafond & Lukehart, 2006). Even though TPA is a very fragile bacterium, TPA is able to enter and survive in different tissues and organs of the host (Lafond & Lukehart, 2006). When infected with TPA, the bacterium will be attached to the host epithelial cells and extracellular matrix components, mainly to fibronectin and laminin, and starts to multiply leading to primary syphilis. TPA will then be spread in the host through the bloodstream or lymphatics and start multiplying in other tissues leading to

secondary stage of syphilis (Peeling et al., 2017; Radolf, 1996; Radolf et al., 2016). To make it easier for this spiral-shaped bacterium to move and infiltrate the host cells, most of the virulent factors, adhesins, are located on each end of the bacterium. TPA may succeed in surviving in the host cells by not activating the immune system because of its slow metabolism. At latent stage with no symptoms, the metabolism of TPA is probably even slower. The metabolism might increase for unknown reasons, which lead to late symptomatic syphilis (Lafond & Lukehart, 2006).

1.3 Diagnostic tests of syphilis

Serologic analysis or direct identification of TPA are the main methods for diagnosing TPA infection (French, 2007). Direct identification of TPA includes dark-field microscopy, TPA DNA amplification by Polymerase Chain Reaction (PCR), and direct testing for TPA by fluorescently labelled antibodies (Henao-Martinez & Johnson, 2014). While PCR is the best alternative to test for primary syphilis, if there are any form of chancre on the genitals, serologic tests is the main tool to diagnose syphilis as well as screening and to follow up the activity and treatment of the disease (FHI, 2010b; French, 2007; Satyaputra et al., 2021).

Serological tests consist of two different types of tests, treponemal and nontreponemal tests, and will normally be performed on serum. None of the serological tests can distinguish venereal syphilis from other nonvenereal treponematoses due to genetic similarity between the subspecies. For treponemal tests, immunoglobulin M (IgM) and IgG antibodies against *T. pallidum* antigens are being measured. For a normal syphilis screening, both IgM and IgG are being measured using the automated enzyme or chemiluminescence immunoassay. Another treponemal test is the *T. pallidum* particle agglutination test (TPPA), which is a traditional and manual test used as a confirmatory test. The treponemal assay is qualitative, and the antibodies are usually detectable 2-4 weeks after infection. Once TPA antibodies have been detected in a patient, it will remain detectable for life. As a qualitative assay, the results will be reported either as reactive or non-reactive. Treponemal tests is not useful for following up the activity of the disease nor if the treatment has been effective. However, the nontreponemal tests are used for following up the activity of the disease, treatment efficiency as well as reinfection of any *T. pallidum*. Rapid plasma reagin test (RPR) is a nontreponemal test

used for detection of the total antibodies against cardiolipin- and lecithin lipoidal antigens. These antigens are being released in TPA infected patients because the human cells are being damaged. The results of the RPR-test will be reported as titers (Forrestel et al., 2020; Janier et al., 2021; Satyaputra et al., 2021).

A study performed in 2019 by Zhou et al, revealed that the routine TPA PCR has a sensitivity and specificity of 89.1% and 99.1% respectively when detecting early syphilis before any serological response in the patient (Zhou et al., 2019). In secondary syphilis, the sensitivity and specificity were 50% and 100% respectively. According to another study performed in 2012 by Shields et al, co-infected patients with HIV had a delayed serological response to syphilis (Shields et al., 2012). These studies confirmed that PCR is an effective method to detect early syphilis. While PCR is a good method to detect early syphilis, serological tests have a 100% sensitivity in secondary and tertiary syphilis and specificity of 98% and 96% respectively. For primary syphilis, the sensitivity of serological assays is 86% for RPR-test and 88% for TPPA-test. (Forrestel et al., 2020).

Screening of TPA was mandatory for pregnant women in Norway until 1995. Due to the risk of getting congenital syphilis, all pregnant women are still recommended to do the screening at least once during their pregnancy. Groups like men who have sex with men (MSM) and immigrants from countries with high incidence of syphilis, are also getting the opportunity to do the screening for free (FHI, 2010b).

Pre-exposure prophylaxis (PrEP), a medical drug with tenofovir disoproxil fumarate-emtricitabine, is an effective drug to prevent HIV infection. This drug is given to HIV-negative individuals who have a higher risk of getting HIV, such as MSM. Several studies have shown an increase of syphilis cases and other STD among men using the drug, partly because of the lack of using condoms. Therefore, PrEP individuals are recommended to be tested for syphilis and other STD every three months (Beymer et al., 2018; Stoltey & Cohen, 2015; Traeger et al., 2018).

1.4 Antibiotics and antibiotic resistance

Antibiotics have been used as medical drugs against bacterial infection since the 1940s, after the discovering of penicillin by Alexander Fleming in 1928 (Aminov, 2010). It was a revolutionary discovery and has since saved countless of lives (Davies & Davies, 2010). Antibiotics can be divided into different classes which target the bacteria differently. The main purpose of the antibiotic is to inhibit the bacterial growth, or to kill the bacteria. This is done by the different antimicrobial mechanisms such as inhibition of cell wall synthesis, protein synthesis, nucleic acid synthesis, and metabolic pathways as well as altering the bacterial membrane structure (Abushaheen et al., 2020).

Not long after the first discovery of antibiotics, the first observation of antibiotic resistance in bacteria was found (Martinez, 2014). The development of antibiotic resistance was not a surprise as the bacteria followed Darwin's theory of evolution and survival in the environment (Munita & Arias, 2016). The antibiotic resistance is caused by mutations of the bacterial genome or by horizontal gene-transfer (Larsson & Flach, 2022; Martinez, 2014).

According WHO, antibiotic resistance is one of the biggest global health threats today (WHO, 2020). Antibiotic resistance is specially a big concern in low- and middle-income countries because of the overuse of antibiotics, overcrowding hospitals with poor hygiene control, and poor diagnostic opportunities with limited surveillance. The low production costs make it easy to produce most antibiotics and therefore consumption of antibiotics is higher in these countries (Larsson & Flach, 2022).

1.4.1 Macrolide resistance in TPA

Azithromycin, a macrolide antibiotic, has been used as an effective treatment of early syphilis in many cases as well as treating infections with other venereal diseases like *Chlamydia trachomatis* and *Mycoplasma genitalium* (FHI, 2010a; Ghanem et al., 2020; Li et al., 2018; Li & Jiang, 2020). The mechanism of the macrolide antibiotic is to inhibit the bacterial protein synthesis by binding to the 23S rRNA of the 50S ribosomal subunit (Stamm, 2015).

Macrolide resistance in syphilis has occurred because of a point mutation, A→G, in position 2058 or 2059 (A2058G or A2059G) in one or both copies of the 23S rRNA gene. The position numbers correspond to the positions of the same gene in *E. coli* (Smajs et al., 2015; Stamm, 2010). The mutation of either of these two positions give a conformational rearrangement of the 23S rRNA domain, reducing the affinity of the macrolide to bind (Chen et al., 2013).

The complete sequence of TPA Nichols strain performed in 1998, showed that the Nichols-strain is missing the mechanism of horizontal gene transfer which is one of the important mechanisms for acquiring antibiotic resistance. This led to the hypothesis that macrolide resistance arose in SS14-strains due to the mutation in the 23S rRNA gene, and Nichols-strain is sensitive to macrolides because this mutation was not present in the sequenced TPA genome from 1998 (Stamm, 2010).

Macrolide resistance in syphilis was first observed shortly after the introduction of erythromycin in the 1950s. According to a study in the 1990s, Stamm confirmed that TPA SS14-like strains were highly resistant to erythromycin and azithromycin. Since then, resistance to macrolide antibiotics has been detected globally, and macrolide antibiotics are therefore no longer a good choice for treating syphilis (Ghanem et al., 2020; Stamm, 2010).

1.5 Syphilis treatment today

To date, there is no vaccine against TPA in humans (Xu et al., 2021). The only way to prevent infection with TPA through sexual intercourse, is using of condoms or to avoid intercourse with infected persons. Hence testing and treatment of TPA infection are important. The most effective way of syphilis treatment is using an antibiotic cure. The WHO guideline and the European guideline have the same antibiotic treatment procedure, both recommending penicillin treatment of syphilis. Penicillin is the primary antibiotic of use when treating syphilis infection in all four stages (Ghanem et al., 2020; Peeling et al., 2017; Rosana et al., 2022). High concentration of penicillin G benzathine are given intramuscularly in a single dose when treating uncomplicated early syphilis, and weekly doses, a total of three doses, in late latent syphilis. Patients with neurosyphilis are getting penicillin G benzathine intravenously (Ghanem et al., 2020).

The high dosage of penicillin is to make sure that the antibiotic is present during reproduction of the bacteria (Molini et al., 2016).

Penicillin resistance in syphilis has still not been observed, and therefore penicillin is the first choice when treating syphilis. It is still preferred to use desensitize penicillin even though the patient is allergic to penicillin. Doxycycline or ceftriaxone are other antibiotics being used if penicillin cannot be used (Ghanem et al., 2020; Peeling et al., 2017). Due to increase of macrolide resistance in TPA globally, the use of macrolide antibiotics as treatment are not recommended. However, macrolide antibiotics for treating syphilis might be considered, with utmost caution, in certain population if either penicillin or doxycycline cannot be used (Peeling et al., 2017; Stamm, 2015).

1.6 Epidemiology of syphilis

Infection with TPA is an important public health problem in groups like MSM and in endemic low-income countries. According to WHO, 7 million new cases of syphilis were registered in 2020 globally (WHO, 2021). From the year 2016 to 2021, a new strategy in sexually transmitted infections was released by WHO. This strategy includes elimination of congenital syphilis by syphilis screening among all pregnant women, and treatment which might leads to 50 or fewer cases of congenital syphilis per 100 000 live births in 80% of countries, as well as a 90% reduction of syphilis incidence globally. These goals are to be set by 2030 (Seale et al., 2017; WHO, 2016).

Congenital syphilis is due to mother-to-child transmission of TPA during pregnancy, through the placenta, or at child delivery. This is very common in early latent stage during pregnancy in low-income countries, but it is still a risk in primary and secondary syphilis (Kojima & Klausner, 2018; Peeling et al., 2017; Stamm, 2016). Some pregnancies end up in miscarriage or stillbirth, and some children will be born with congenital syphilis. Congenital syphilis might result in growth retardation, skin rash, dental abnormalities, deafness, and neurosyphilis. The risk of getting congenital syphilis can easily be prevented by screening program for pregnant women (French, 2007).

So far, 15 countries have received WHO validation for syphilis elimination of mother-to-child transmission, meaning that there is no longer a public health problem or a threat of getting congenital syphilis. The countries who got validated are Cuba, Thailand, Belarus, Moldova, Anguilla, Montserrat, Bermuda, Cayman Islands, St. Kitts and Nevis, Antigua and Barbuda, Malaysia, Maldives, Sri Lanka, Dominica, and Oman (Elgalib et al., 2023; WHO, 2023b). According to WHO statistics for syphilis transmission from mother-to-child, it has been zero reported cases of congenital syphilis in Norway since 2004. There are no data of syphilis transmission from mother-to-child in Norway before 2004 (WHO, 2023a).

Data on syphilis cases reported from WHO are most likely from high-income countries due to underreporting from low-income countries. Several studies have shown that syphilis cases from low-income countries are from female sex workers and due to mother-to-child infection. In general, most of the syphilis cases reported worldwide are among MSM and sex workers (Kojima & Klausner, 2018; Peeling et al., 2017). Figure 1.2 is showing a world map with the percentage of active syphilis in MSM in the different countries between 2008 and 2018. WHO has no data regarding syphilis cases among MSM in Norway, but Norway has its own surveillance system for reporting all syphilis cases.

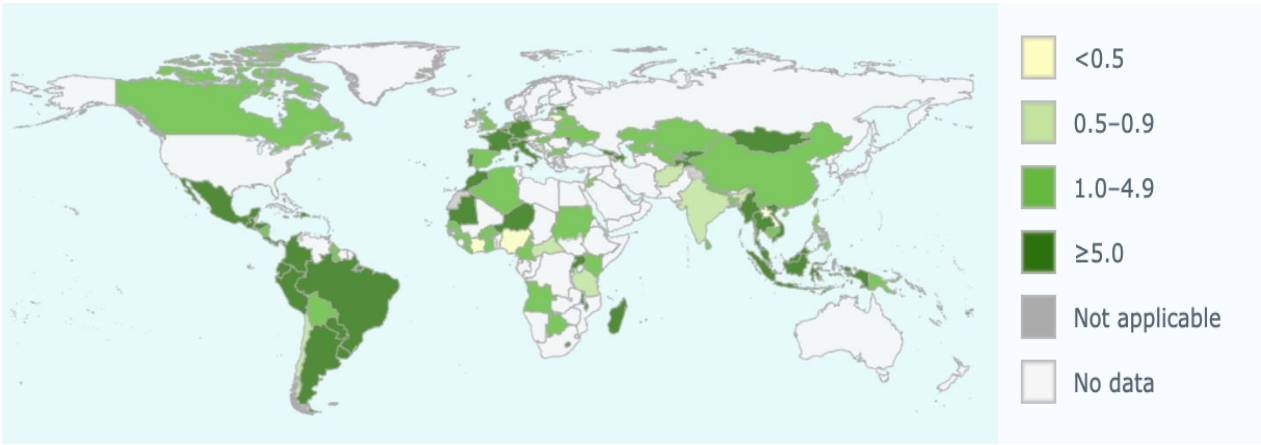


Figure 1.2 World map with the percentage of active syphilis in men who have sex with men (MSM) from 2008 to 2018 (map obtained from the Global Health Observatory, WHO, 2 April 2023, https://gamapserver.who.int/gho/interactive_charts/sti/msm_syphilis/atlas.html).

Several studies are showing that most of the TPA sequences in the world belong to the SS14-like strains. Sequence type ST1 seems to be the most dominant strain in

many countries followed by ST2 and ST3. These three STs belong to SS14-like strains. ST26 is the most common ST among the Nichols-like strains (Grillova et al., 2018; Grillova, Jolley, et al., 2019; Grillova, Oppelt, et al., 2019; Sweeney et al., 2022; Taouk et al., 2022). However, molecular characterization of TPA sequence types is not established globally, therefore there are biases in the sequence type data retrieved.

1.6.1 Syphilis in Norway

All syphilis cases in Norway have been reported anonymously since 1993 to the Norwegian Surveillance System for Communicable Diseases (MSIS), based at Norwegian Institute of Public Health (NIPH) (Olsen, 2013). Syphilis has been reported nominative as a category A disease since 22 March 2019, meaning that patient identification needs to be reported both from the medical microbiological laboratory and from the diagnosing physician to MSIS (FHI, 2012). MSIS is an important tool for NIPH for monitoring and surveillance of various infectious diseases (FHI, 2017).

Despite the availability of free drop-in sexually transmitted infections check and treatment, the prevalence of syphilis has increased (Helsedirektoratet, 2020). Increase of syphilis infected individuals in Norway began in the late 1990, especially among MSM. In the last ten years, infection of syphilis is also being seen in women, but the highest prevalence is still seen in men, see Figure 1.3. The highest prevalence of syphilis cases was in 2020 with a total of 288 syphilis cases reported to MSIS. A total of 163 syphilis cases were reported to MSIS in 2021, and 193 cases were reported in 2022. According to NIPH, a decrease of syphilis infections in 2021 were probably due to Covid-19 pandemic restriction (FHI, 2023).

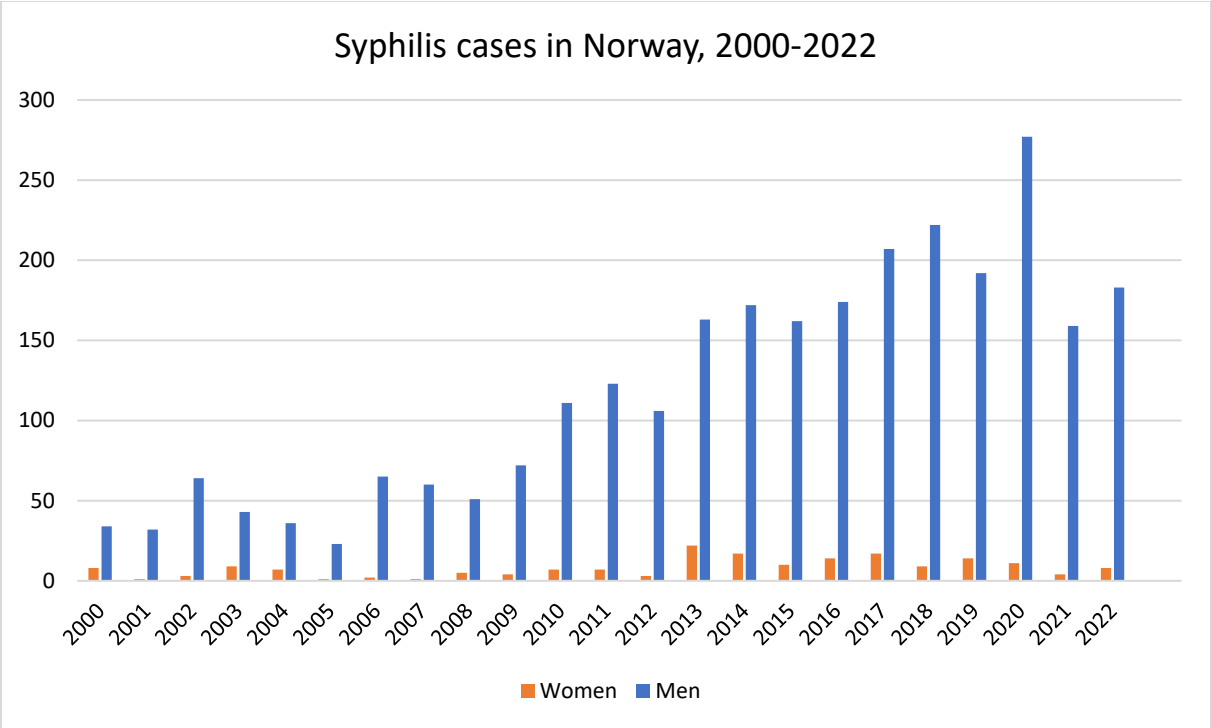


Figure 1.3 Number of syphilis cases in Norway among women and men from 2000-2022 (data obtained from MSIS, 5 January 2023).

1.7 Aim of study

The aim of the study is to establish a molecular typing method for TPA strains in Norway and simultaneously to assess genotypic macrolide resistance. This is an important task for the National reference laboratory for syphilis diagnostics in Norway, located at Oslo University Hospital. As a reference laboratory, it is important to do an epidemiological surveillance of the disease using both clinical information and molecular tools. Thus, characterization of strain diversity, using molecular data, plays a critical role in the epidemiological understanding of the re-emergence of TPA. Molecular typing is required in order to discriminate genetic variants and investigate their potential association with phenotypic features such as pathogenicity and antibiotic resistance. To date there is no established molecular typing of Norwegian strains of TPA, and as a consequence, Norway does not have any overview of genetic variants, antibiotic resistance, or molecular epidemiology of clinical TPA strains.

For this study, a TPA DNA reference strain will be used to optimize nested PCR and sequencing of three loci multi-locus sequence typing (MLST) genotyping analysis, as well as genotypic markers for resistance against macrolide antibiotics. The aim of this

study is to establish a molecular typing scheme to get the first overview of the molecular epidemiology of TPA in Norway, as well as getting the first overview of macrolide resistance in the Norwegian TPA strains.

2 Materials and methods

2.1 Materials

2.1.1 *Treponema pallidum* reference strain

Reference Nichols strain of TPA was used for the optimizing of a melting point analysis, for detection of macrolide resistance in TPA, and the nested PCR, for MLST of TPA. This *Treponema pallidum* DNA control kit is from Vircell Microbiologists (Granada, Spain), see Appendix A (Vircell). This reference locus is named NC_000919.1 and the complete sequence can be found in National Center for Biotechnology Information (NCBI) (NCBI, 2022). The given Treponema DNA control has the allelic profile 10-14-10 for the TP0136, TP0548 and TP0705 respectively, giving the ST20, a Nichols strain. This reference strain is sensitive to macrolide antibiotics.

2.1.2 Clinical samples

Positive syphilis samples from October 2020 to September 2022, were collected for this study. In total, 81 clinical samples from 70 different patients were collected. Most of these samples were genital swabs from MSM patients from the Oslo-area in Norway. All the samples were handled anonymously, and no clinical data from the patients were collected for this study. Therefore, no approval by the Regional committees for medical and health research ethics (REK) was needed.

In addition, one negative control and 7 positive TPA samples from Quality Control for Molecular Diagnostics (QCMD) panel were analyzed. Table 2.1 gives an overview of the clinical samples collected from this period.

Table 2.1 An overview of the different materials collected between October 2020 and September 2022.

	Women	**Men**	**Total**
Genital/wound swabs	2	72	74
Spinal fluid	1	3	4
Other materials*	0	3	3
Total number of samples	3	78	81

*Other materials consist of biopsy, lymph node, and unknown material.

2.1.3 List of instruments and equipment

All instruments used for this master thesis are listed in Table 2.2. General equipment is not listed. Other equipment necessary for the instruments are listed in Appendix B with catalogue number/lot number and manufacturer.

Table 2.2 List of instruments and equipment used for this master thesis. The models are listed in alphabetical order.

Instrument	Model	Manufacturer
PCR plate spinner	521-1648	VWR International AS/Avantor
Centrifuge	5810R	Eppendorf
DNA sequencer	ABI 3130xL Genetic Analyzer	Thermo Fisher Scientific
DNA sequencer	ABI 3500xL Genetic Analyzer	Thermo Fisher Scientific
Centrifuge-mixer	CM-70M-09	ELMI
Automated isolation and purification of nucleic acid	EZ1 Advanced XL	Qiagen
Real-time PCR machine	Light Cycle 480	Roche Diagnostics
Conventional PCR system	MiniAmp Thermal Cycler	Thermo Fisher Scientific
Automated isolation and purification of nucleic acid	MagNA Pure 96	Roche Diagnostics
Microcentrifuge	Pico 21	Thermo Fisher Scientific
Microplate sealer	PlateLoc Thermal Microplate Sealer	Agilent
Capillary electrophoresis	QIAxcel Advanced system	Qiagen
Conventional PCR machine	SimpliAmp Thermal Cycler	Thermo Fisher Scientific
Vortex	Vortex Genie 2 G560	Scientific Industries, Inc.
Vortex	Vortex Mixer 444-2790	VWR International AS/Avantor

2.1.4 Kits

All kits used for this master thesis are listed in Table 2.3.

Table 2.3 List of kits used in this master thesis. The kits are listed in alphabetical order.

Kit	Catalogue number	Manufacturer
Amplirun® *Treponema pallidum* DNA control	MBC109	Vircell Microbiologists
AmpliSens® *Treponema pallidum*-FRT PCR Kit	R-B20-F (RG, iQ)-CE	Ecoli Dx s.r.o
BigDye™ Terminator v1.1 Cycle Sequencing Kit	4337450	Applied Biosystems
EZ1® DSP Virus Kit	62724	Qiagen
MagNA Pure 96 DNA and Viral NA Large Volume Kit	06374891001	Roche Diagnostics
QIAxcel DNA Screening Kit	929004	Qiagen

2.1.5 Primer, probe, and reagent mixtures

The primers and probes used for detection of macrolide resistance in TPA were: TP23S_F, TP23S_S, TP23S_A, TP23S_R, TP23S_P_[A], and TP23S_P_[A]-LNA. These were ordered by TIB Molbiol (Berlin, Germany) who designed the primers and probes based on a publication from Stamm and Bergen (2000).

The external and internal primers used in the nested PCR for TPA MLST were forward- and reverse primers of TP0136, TP0548, and TP0705 (TP0136_EF/IF and TP0136_ER/IR, TP0548_EF/IF and TP0548_ER/IR, TP0705_EF/IF and TP0705_ER/IR). The primer sequences used for this MLST are the same as a publication from Grillova et al. (2018) and was ordered from TIB Molbiol.

All primer- and probe sequences used in this master thesis are listed in Appendix C. The primers and probes used in this study were dissolved to the final molar concentration of 100 µM (primers and probes for the macrolide resistance) and 10 µM (nested PCR primers). The length of the PCR products, in base pairs (bp), for the nested PCR are listed in the Table C.1 (Appendix C) for all three loci.

PrimeSTAR® GXL Premix, developed by Takara Bio, are being used in the nested PCR for the MLST. The Premix is composed of PrimeSTAR GXL DNA polymerase, reaction

buffer, and deoxynucleotide triphosphates (dNTP) mixture (Takara Bio Inc.). In addition, forward- and reverse primers were added to the nested PCR together with the Premix.

2.2 Methods

2.2.1 Preparing of *Treponema pallidum* reference strain

TPA DNA control needed to be prepared before using. The preparation of the TPA DNA control was the same as the routine procedure in the laboratory. When making the stock solution, 100 µl of the Vircell Control Reconstitution solution was used to dissolve the dry matter fraction of the TPA DNA control. To be able to use the stock solution as a TPA DNA control for the analysis, the stock solution needed to be diluted. The stock solution was diluted 1:50 with PCR-water.

2.2.2 DNA extraction

In the laboratory routine diagnostic, the extraction instruments MagNA Pure 96 (MP96) (Roche Diagnostics) and EZ1 Advanced XL (Qiagen) are being used to isolate DNA from swabs and spinal fluid/other materials respectively.

For the venereal routine diagnostic, 500 µl of swab material was being transferred into an MP96 Processing Cartridge. The protocol-settings and the placement of equipment was according to the MagNA Pure 96 System, User Training Guide V4.1 (Roche, 2021). MP96 uses magnetic glass particle technology for the extraction. This instrument can process up to 96 samples, and the time for the extraction is 1.5 hours regardless the number of samples. See Table 2.4 for the MP96 protocol.

While MP96 can handle up to 96 samples, EZ1 are only capable to isolate 14 samples at a time. The advantage is that the extraction time is only 45 minutes from pressing start to the end. See the user manual for the EZ1 protocol and equipment placement (Qiagen, 2015, 2017a). EZ1 are using magnetic-particle technology for the purification. 200 µl of spinal fluid or blood was transferred to a 2 ml ampule. In every extraction, an RNA Carrier was needed for optimal and efficient purification of DNA from the samples, especially in spinal fluid where the DNA concentration might be low (Qiagen, 2015). See Table 2.4 for the EZ1 protocol.

Table 2.4 Overview of the protocols for MagNA Pure 96 (Roche Diagnostics) and EZ1 (Qiagen).

	MagNA Pure 96 protocol	**EZ1 protocol**
Kit name	DNA/Viral NA LV 2.0	EZ1® DSP Virus Kit
Protocol	Pathogen Universal 500 4.0	EZ1 Advanced XL Virus Card v2.0
Volume: sample/elution	500 µl/100 µl	200 µl/90 µl
Internal control	IC2	RNA Carrier

2.2.3 Polymerase chain reaction (PCR)

Conventional PCR is an enzymatic assay and is a well-known method for amplification *in vitro* of specific DNA sequence. The principle of PCR was demonstrated by the Norwegian researcher Kjell Kleppe in 1969 at the Gordon Conference, and he published its practical use in 1971. PCR was further developed for automatization by Kleppe's previous student Kary Mullis in the 1980s by using a thermostable DNA polymerase (Kleppe et al., 1971; Lorenz, 2012; Templeton, 1992). In each PCR-assay, specific reagents are required: forward- and reverse primers, nucleotides (dNTP), DNA polymerase and DNA template. DNA template is the DNA target of interest. The primers are short single-stranded DNA oligos, complementary to the template. The nucleotides consist of the four bases which are the building blocks for DNA: adenine (A), thymine (T), guanine (G), and cytosine (C). DNA polymerase assembles the different nucleotides and synthesize new strands of the DNA complementary to the DNA template (Garibyan & Avashia, 2013).

All the reagents are mixed in a PCR-test tube and placed in a PCR-machine which is a thermal cycler. In this thermal cycler, the test tube is going through different heating and cooling steps to amplify the decided DNA strands. The three different steps are denaturation, annealing and extension. These three steps are usually repeated 25-35 times, cycle, and results in exponential amplification of PCR products. The first step is denaturation which is to separate the double stranded DNA template for subsequent primer annealing. The temperature is usually 94°C to 98°C, and last about 10 seconds to 1 minute. The next step is annealing, making sure that the primers are binding to the DNA template of interest. This step is usually between 52°C and 58°C, and last about 30 seconds. For the last step of the cycle, extension, the DNA polymerase are

binding to the 3'-OH end of the primers that have hybridized to the DNA template. The DNA polymerase will add complementary nucleotides to the 3'-OH end of the primer and in this way synthesize new DNA. The temperature for this step is around 70°C to 80°C, and last about 5 minutes. The temperature, time and number of cycles in the PCR are depend of DNA template and DNA polymerase characteristics (Lorenz, 2012).

2.2.3.1 Real-time PCR

Real-time PCR follows the same technique as the conventional PCR. In real-time PCR, data collecting happens throughout the PCR process. The main method is fluorescent detection system. A probe, which contains a fluorescent molecule, is added to the PCR reagent mixture. It is important that the probe is complementary to a part of the DNA template of interest (Singh & Roy-Chowdhuri, 2016). A positive reaction in a real-time PCR will give a fluorescent signal. The fluorescent signal correlates with the number of PCR cycles it takes to amplify enough DNA. Cycle threshold (Ct) -value is defined as the number of PCR cycles at which the fluorescent signal of the positive reaction crosses the threshold. The Ct-value is inversely proportional to the amount of DNA in the sample; the lower the Ct-value, the greater the amount of template DNA products, and the higher the Ct-value, the lesser the amount of template DNA products (Schmittgen & Livak, 2008).

Detection of TPA in our routine laboratory is by AmpliSens *Treponema pallidum*-FRT PCR kit (Ecoli Dx s.r.o). One PCR-reaction mix includes 10 µl of PCR-mix-1FL *Treponema pallidum*, 5 µl PCR-mix-2-FRT and 0.5 µl Polymerase (TaqF). 10 µl of extracted DNA are added to 15 µl PCR-mix, for a total volume of 25 µl. The TPA PCR-protocol for LightCycler 480 (Roche Diagnostics) is shown in Table 2.5.

Table 2.5 Routine TPA PCR-protocol for LightCycler 480 (Roche Diagnostics).

Step	Temperature	Time (mm: ss)	Number of cycles
Initial denaturation	95°C	15:00	1
Denaturation	95°C	00:05	5
Annealing	60°C	00:20	
Extension	72°C	00:15	
Denaturation	95°C	00:05	40
Annealing	60°C	00:30	
Extension	72°C	00:15	

2.2.3.1.1 Melting point analysis

Examination of macrolide resistance in TPA is based on the real-time PCR assay followed by a melting point analysis. In this case, the melting point analysis is based on different melting temperature (Tm) in macrolide resistant (mutant) and in none-macrolide resistant sequences (wild-type) when binding to a specific sequence probe (Sjøberg, 2013). A melting temperature is defined as the temperature where half of the DNA molecules are double-stranded DNA and half are single-stranded (Khandelwal & Bhyravabhotla, 2010). For the melting point analysis, the LightCycle 480 real-time PCR machine was used. See chapter 2.2.8.1 for the optimization of the macrolide resistance analysis.

2.2.3.1.2 SimpleProbe technology

SimpleProbe probes are used for detection of macrolide resistance in TPA. Therefore, a SimpleProbe PCR assay is needed followed by a melting point analysis to differentiate between samples with macrolide resistance and wild-type. The SimpleProbe technology is used for detection of a single nucleotide (point) mutation, and the probe will only bind to the double stranded DNA. The fluorescent signal will be continuously detected while the probe is binding to the target sequence, however the signal will decrease with increasing temperature as the DNA become single stranded, see Figure 2.1 (Klaassen et al., 2008).

The difference between the SimpleProbe technology, for the melting point analysis, compared to other probe technologies, is that the SimpleProbe technology only needs one single probe. This probe must cover the point mutation site and is only labelled

with one fluorophore. The downside of using the SimpleProbe technology is that it is neither suitable for quantitative analysis nor for multiplex-assays. However, this technology is ideally used for melting point analysis when searching for point mutation, such as for macrolide resistance (Klaassen et al., 2008).

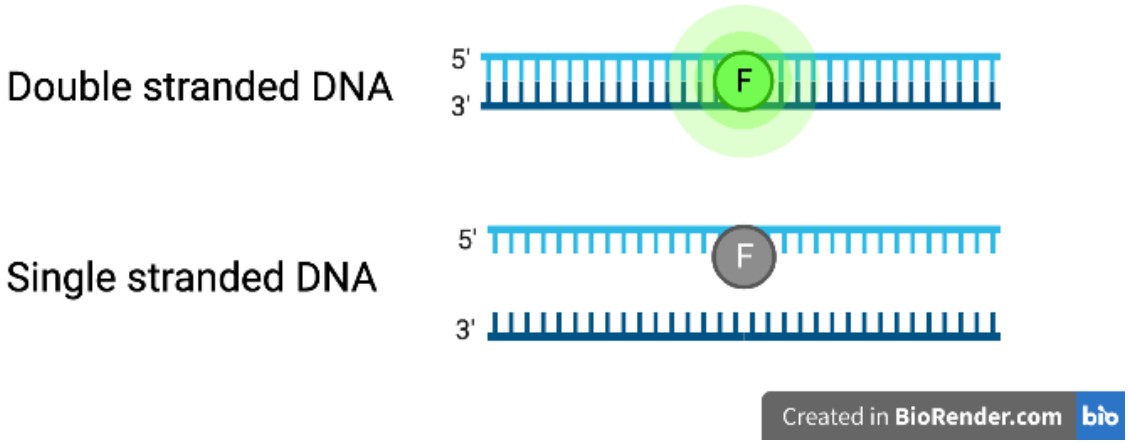


Figure 2.1 An illustration of the SimpleProbe technology. The probe will only give a fluorescent signal when binding to a double stranded DNA. When single stranded, the probe will not give any fluorescent signal. The figure was created in BioRender.com.

2.2.3.2 Nested PCR

Three TPA loci for the MLST (TP0136, TP0548 and TP0705) were amplified in the reference strain and patient samples by nested PCR. Nested PCR is method to increase the sensitivity and specificity of a PCR. This often consist of two separately PCR with separate primer-sets, where the PCR products from the first PCR (PCR1) are being used as DNA template for the second PCR (PCR2). Nested PCR is efficient for amplifying long DNA templates (Green & Sambrook, 2019). The external primers, in the first PCR reaction, result in larger PCR products, and the internal primers, in the second PCR, result in shorter PCR products. An illustration, created in BioRender.com, of the nested PCR method is shown in Figure 2.2. For the optimization of nested PCR, see chapter 2.2.8.2.

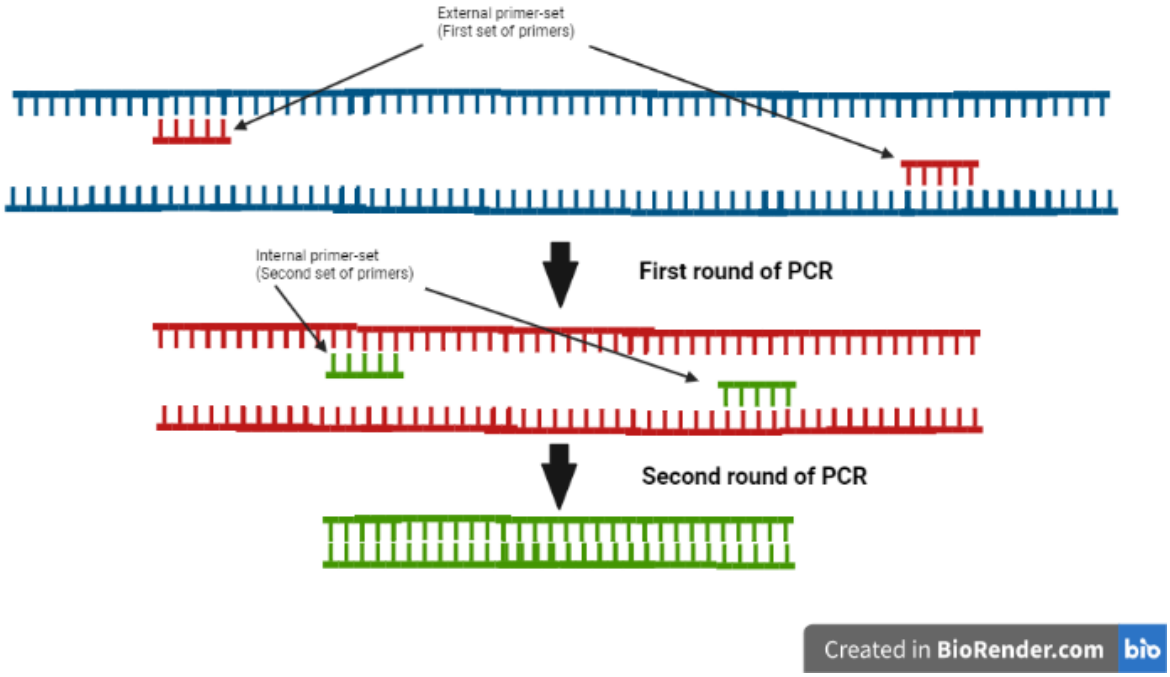


Figure 2.2 The principle of a nested PCR. Two primer-sets, external and internal primers, used for nested PCR to increase the sensitivity and specificity of PCR. For the first round of PCR, external primers are used to amplify a part of a sequence of interest. PCR products from the first round are being used as template for the second round of PCR. Internal primers are used to amplify even more shorter PCR products. The figure was created in BioRender.com.

2.2.3.2.1 Touchdown PCR

A touchdown protocol was performed as the first step of the nested PCR (see Table 2.6). The touchdown protocol was performed accordingly to Grillova et al. (2018), S1 Supplementary methods.

Touchdown PCR is a method for increasing the specificity and sensitivity of a PCR amplification by avoiding amplification of nonspecific sequences. Using higher annealing temperatures in the early steps of the PCR, only specific binding between primer and template will occur. The annealing temperature is usually set 5°C-10°C higher than the Tm of the primers being used. Usually, the touchdown PCR should have 10-15 cycles. For every cycle, the annealing temperature is gradually being decreased. When entering the last cycle of the touchdown protocol, the temperature should be between 2°C to 5°C below the Tm of the primers. After the touchdown protocol, additional 15-20 cycles with a normal annealing temperature for the primers being used, are being performed (Green & Sambrook, 2018; Korbie & Mattick, 2008).

Table 2.6 Touchdown protocol for the first step of nested PCR on MiniAmp™ from Applied Biosystems.

Step		**Temperature**	**Time (mm: ss)**	**Number of cycles**
Initial denaturation		94°C	01:00	1
Touchdown protocol	Denaturation	98°C	00:10	8 (Annealing temperature gradually decreased by 1°C for every cycle)
	Annealing	68°C	00:15	
	Extension	68°C	01:45	
Denaturation		98°C	00:10	35
Annealing		61°C	00:15	
Extension		68°C	01:45	
Final extension		68°C	07:00	1
Hold		4°C	∞	1

2.2.4 Multi-locus sequence typing (MLST)

A nested PCR is needed for the multi-locus sequence typing (MLST) analysis for TPA as well as a sequencing analysis. MLST is a method for characterizing bacterial species by using sequences of different loci of the bacteria to obtain different allele numbers. The combination of the allele numbers will determine an allelic profile or sequence type (Jolley et al., 2018). See Figure 2.3 for the principle of an MLST. The advantage of doing an MLST compared to other typing method is that this method can detect different changes on the DNA level. This method is also a great tool for the epidemiological analysis and for the surveillance of the disease (Ibarz Pavon & Maiden, 2009).

MLST of TPA will only be using three different loci according to Grillova et al. (2018); TP0136, TP0548, and TP0705. A nested PCR was performed for all three loci. The three different loci were sequenced, by Sanger sequencing, and further processed with a data software, Sequencher (see chapter 2.2.7.1). The allelic profiles and sequence types were assigned by PubMLST.

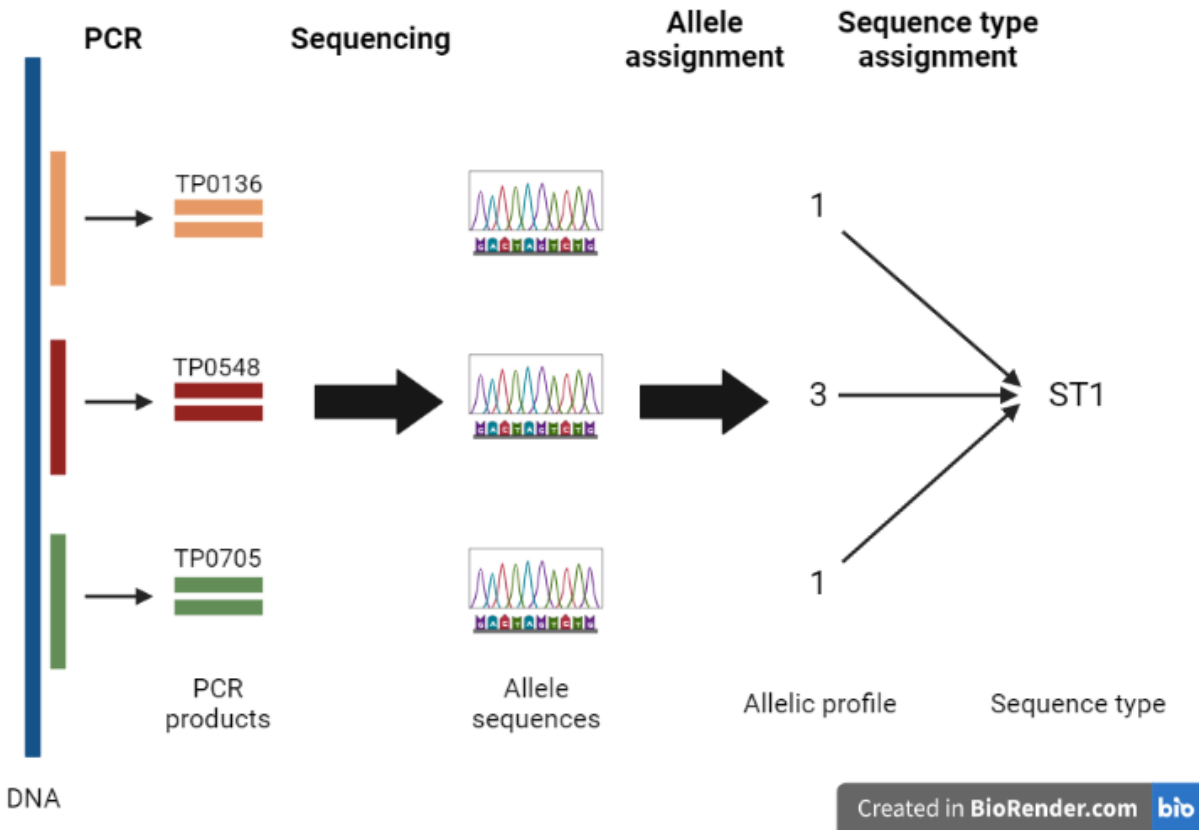


Figure 2.3 The principle of a multi-locus sequence typing scheme. MLST of three TPA loci, TP0136, TP0548, and TP0705 was performed in this study. First, a nested PCR was performed followed by Sanger sequencing of the PCR products. An allelic profile, as well as a sequence type, based on the allele sequences, were assigned by PubMLST. The figure was created in BioRender.com.

The genetic variability is important for the molecular typing of syphilis by MLST (Petrosova et al., 2013). To perform an MLST scheme, using genes with high variability is enough for discriminating the different sequence types from each other and to differentiate between the SS14- and Nichols-like strain. *tp0136* and *tp0548* are genes with high genetical differences. Together with *tp0705*, the discrimination power is high and good for molecular typing. A single nucleotide polymorphism in one of the genes, can change the sequence type entirely (Grillova et al., 2018; Grillova, Jolley, et al., 2019; Nechvatal et al., 2014).

2.2.5 Capillary electrophoresis

To detect PCR products in our conventional nested PCR, a capillary electrophoresis is applied to separate the PCR products according to size, and to visualize them to predict the right PCR product length (Singh & Roy-Chowdhuri, 2016). Capillary electrophoresis by QIAxcel Advanced system (Qiagen) were used to visualize the PCR

products (Qiagen, 2017b). The electrophoresis was performed both after the first and the second PCR of the nested PCR to make sure that the PCR products amplified, had the expected length. For every run, a QX alignment marker is needed to calibrate the migration time variation (Qiagen, 2017b). When the electrophoresis was performed for the nested PCR, QX alignment marker 15 bp/3 kb was used. QX alignment marker 15 bp/600 bp was used when performing electrophoresis for checking the PCR products from the macrolide resistance analysis.

The principle of electrophoresis is to separate the different PCR products from each other, and to detect the size of the product (Srinivas, 2019). According to QIAxcel Advanced User Manual, a small amount of each of the samples are being pushed through a capillary which consist of a gel and ethidium bromide. Ethidium bromide is a fluorescent dye that binds to all double-stranded DNA. It will only fluorescence when bound to DNA making it able to visualize the PCR products (Galindo-Murillo & Cheatham, 2021). Since DNA is negatively charged, the DNA fragment will be wandering towards the positive end in an electric field according to the DNA sizes (Lee et al., 2012).

2.2.6 Sanger sequencing

Both sequencing instruments 3130xl Genetic Analyzer (Applied Biosystems) and 3500xL Dx Genetic Analyzer (Applied Biosystems) was used in this study. The 3130xl Genetic Analyzer is a 16-canals capillary electrophoresis while the 3500xL Genetic Analyzer is 24-canals capillary electrophoresis. Both the sequencing instruments are using the same fluorescent-reading method when analyzing the samples. See the user manuals for the analysis-setup (AppliedBiosystems, 2010, 2012).

Depending on the richness of the PCR products, the PCR products were diluted with PCR-water to final concentration of either 1:50 or 1:100. Before sequencing, a BigDye sequencing reaction was performed with these diluted PCR products. Table 2.7 and Table 2.8 show the sequencing reaction mix components and the BigDye-protocol respectively. The reason for BigDye sequencing reaction is to produce DNA fragments with various length by incorporating terminating nucleotides, dideoxynucleotide triphosphates (ddNTP) (Crossley et al., 2020).

When the BigDye sequencing reaction is done, a cleaning step with BigDye XTerminator Purification Kit is needed to ensure that all unwanted reagents will not be incorporated into the sequencing step. 90 µl of SAM Solution together with 20 µl BigDye XTerminator were added to each well which contains samples. 130 µl water is being added to the rest of the empty wells (in total 2 columns for the 3130xl, and 3 columns for the 3500xL need to contain 130 µl of either samples or water). After covering the PCR plate, the plate needed to be vortexed for 30 minutes using the Vortex-Genie 2 on level 6. After 30 minutes on the vortex, the plate is spun down in 2 minutes at 1000xg. The cover is removed and is being changed to a septa gummy-mat. The PCR plate is then paced on a sequencing stand and is now ready for sequencing.

Table 2.7 BigDye sequencing reaction mix components prepared for one PCR reaction.

BigDye sequencing with ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit	
Reagents	**Amount in each well (µl)**
BigDye Terminator Ready Reaction mix	2
PCR-water	11
5x Sequencing Buffer	3
2.5 µM IF/IR primer*	2
Template	2
In total	20

*The IF/IR primer are separated in each BigDye sequencing reaction mix. The test tube consists of either IF or IR primer.

Table 2.8 BigDye sequencing protocol using SimpliAmp Thermal Cycler from Applied Biosystems.

Step	**Temperature**	**Time (mm: ss)**	**Number of cycles**
Denaturation	96°C	00:10	25
Annealing	50°C	00:05	
Extension	60°C	02:30	
Hold	4°C	∞	1

2.2.7 Data processing

2.2.7.1 Sequencher

Sequencher is a sequence analysis software used to assembly, trimming and manually editing DNA sequences (*Sequencher® version 5.4.6 DNA sequence analysis software*, 2023). The Sanger DNA sequences in this study were processed using this software.

2.2.7.2 PubMLST

PubMLST is a public database for molecular typing and microbial genome diversity (Jolley et al., 2018). After processing the data from Sequencher, the sequence-data were pasted into this database through: Home > Organisms > *Treponema pallidum* > *Treponema pallidum* typing > Sequence query, selecting the locus/scheme: MLST (Grillová). The database will find the allelic number which matches the sequence.

After finding the allelic numbers for all the three loci, the allelic numbers were submitted into: Home > Organisms > *Treponema pallidum* > *Treponema pallidum* typing > Search by locus combinations. After entering the allelic numbers, the database gave us a sequence type, see Figure 2.4. Novel loci sequences were submitted to the PubMLST database to receive an allelic number as well as getting a new sequence type number.

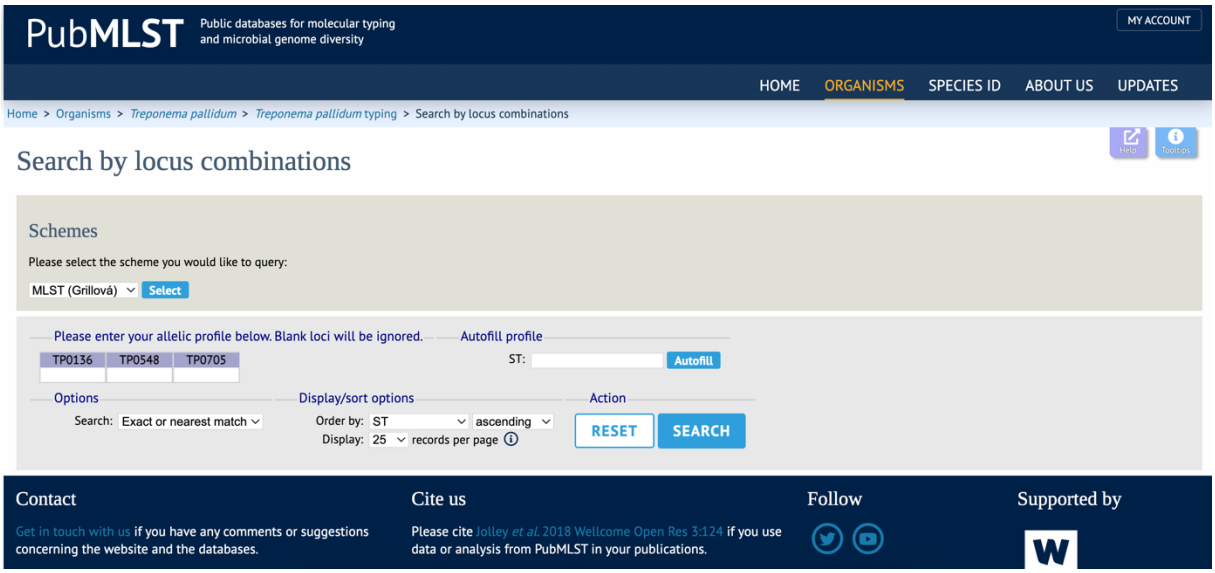


Figure 2.4 PubMLST database. The different allelic numbers for the three loci were submitted into the database to receive a sequence type.

2.2.7.3 PHYLOViZ

A phylogenetic tree was obtained by using the online version of PHYLOViZ software, https://online.phyloviz.net/index (see Figure 2.5). This software will generate an allelic profile and associated epidemiological data by using a sequence-based typing method. PHYLOViZ uses goeBURST algorithm to generate a phylogenetic tree based on the evolutionary relationships between strains. The distance between each sequence type is based on the number of differences between sequences and is calculated using Hamming distance (Francisco et al., 2009; Ribeiro-Goncalves et al., 2016). Fasta-files from all sequence types are uploaded in this software, see Appendix F for all the sequences for the different sequence types. Auxiliary data was also uploaded. This data only contains sequence type and sample numbers, see Appendix E (columns marked with a *). All the data generated, by using this online software, were erased after 24 hours after uploading.

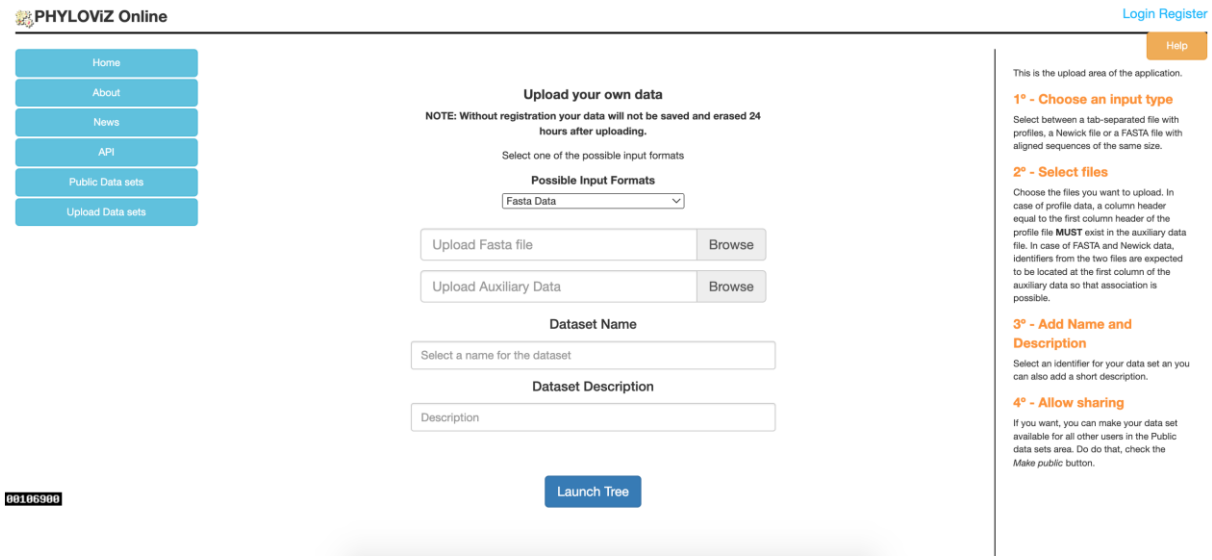


Figure 2.5 PHYLOViZ online software. This software was used to generate a phylogenetic tree.

2.2.8 Optimization of macrolide resistance and nested PCR

2.2.8.1 Optimization of macrolide resistance in TPA

In total, four different PCR-reaction mixes were made with different combinations of primers and probes, to find the optimal combination for the melting point analysis for the macrolide resistance in TPA. See Table 2.9 for the different combinations of the primers and probes (PCR-mix 1-4). Table D.1 and Table D.2, in Appendix D, show the

PCR-reaction mix setups for all the four mixes. For the primer- and probe sequences used for this optimization, see Appendix C, Table C.2.

Table 2.9 Four different PCR-reaction mixes with different combination of four primers and two probes*.

PCR-reaction mix	Primer	Probe
PCR-mix 1	TP23S_F TP23S_A	TP23S_P_[A]
PCR-mix 2	TP23S_F TP23S_A	TP23S_P_[A]_LNA
PCR-mix 3	TP23S_S TP23S_R	TP23S_P_[A]
PCR-mix 4	TP23S_S TP23S_R	TP23S_P_[A]_LNA

*Other reagents needed for the PCR-setup are not included in this Table.

The PCR- and melting point protocol is showed in Table D.3 in Appendix D. A melting step at 75°C was executed for the first PCR attempt. For the final optimization the melting step temperature was increased to 80°C.

2.2.8.2 Optimization of nested PCR

An optimization of the nested PCR was needed for the MLST of TPA. No change of the touchdown protocol, the first step of the nested PCR was needed. Only the reaction mixes for both the first (PCR1) and the second step (PCR2) of the nested PCR, as well as the protocol for the PCR2, needed to be optimized. See Table D.4 and Table D.5 for reaction mix setups for PCR1 and PCR2 respectively, and Table D.6 for the optimization of PCR2 protocol. See Figure 2.6 for the different steps of the optimization. The underlined texts in the figure, are conditions which gave the best results for the optimizing.

Different volume of DNA was applied to each of the TP0136-, TP0548-, and TP0705-PCR-reaction mixes to find the optimum volume of DNA for PCR1. Volume of 1, 2, 5, 10, 12, and 15 µl of DNA were added to the PCR reaction mixes, containing Premix, EF- and ER primer, and PCR-water, to make a total volume of 50 µl (see Table D.4). Capillary electrophoresis was used to visualize the PCR products after each PCR analysis. The best volume of DNA for the first step of the nested PCR was 10 µl.

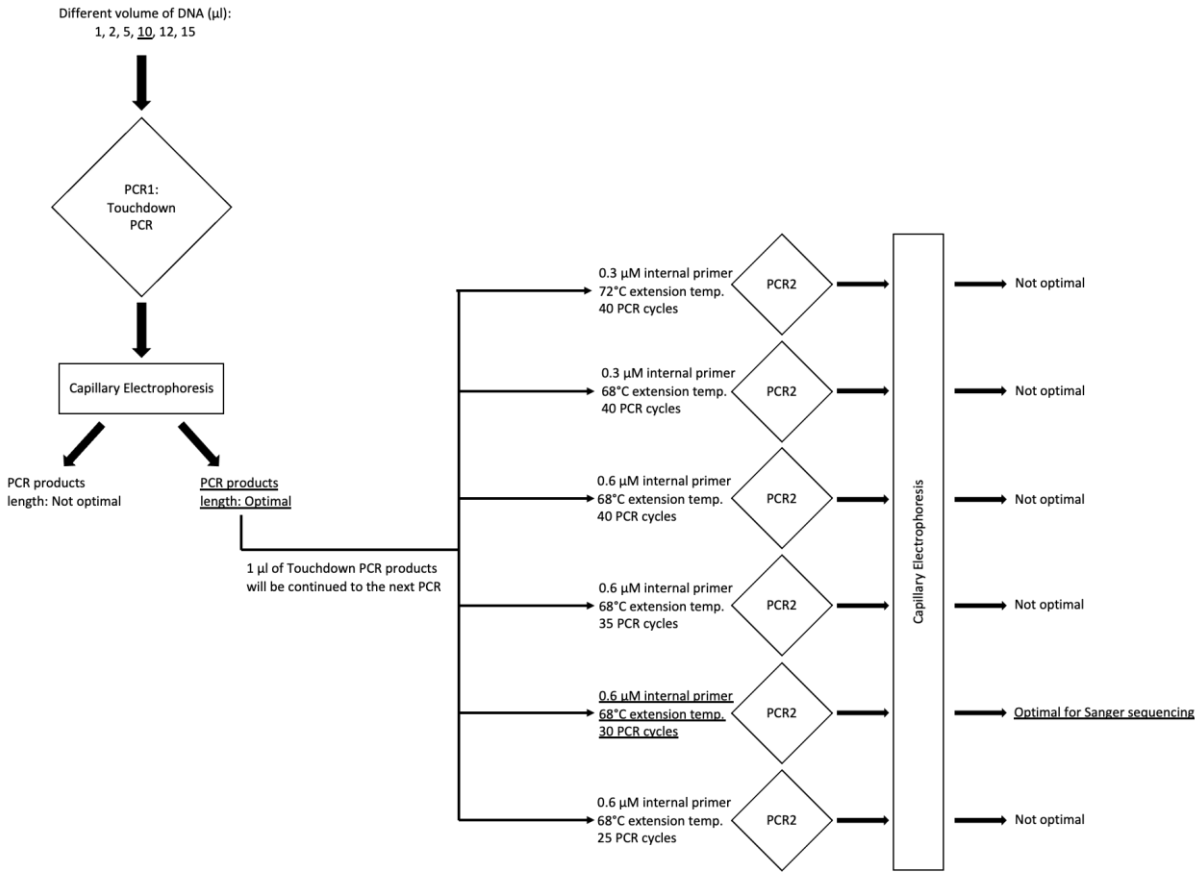


Figure 2.6 A flowchart of the optimization of nested PCR for the TPA MLST. Touchdown PCR (PCR1) was optimized by testing different volume of DNA in the PCR (1, 2, 5, 10, 12, and 15 µl). Capillary electrophoresis was performed to visualize and find the optimum volume of DNA. One µl of touchdown PCR products was used as template for the second PCR (PCR2). Different internal primer concentrations, extension temperatures, and PCR cycles was tested for the optimization of the second PCR. After the electrophoresis, the optimum PCR products was continued to Sanger sequencing. The conditions for best optimization for this study are underlined.

One µl of PCR products from PCR1 were used as DNA template for PCR2 optimization. Different concentrations of TP0136-, TP0548-, and TP0705-IF and -IR primers were tested out, see Table D.5. To optimize the PCR2, small adjustments were done to the PCR2-reaction mix and -protocol (extension temperatures and the number of PCR cycles), see Table D.6. Primer concentrations of 0.3 µM and 0.6 µM, and extension temperatures at 72°C and 68°C were tested out, as well as different number of PCR cycles, 25, 30, 35, and 40, were applied to find the best combination. Capillary electrophoresis was used to visualize the PCR products. For the optimized PCR2, the primer concentration was 0.6 µM, the extension temperature was 68°C, and the number of cycles 30.

3 Results

The data obtained from this study will be the first macrolide resistance and molecular typing data of TPA in Norway as this has not been done previously. Chapter 3.1 and 3.2 present the results from the optimization of TPA macrolide resistance and MLST. The macrolide resistance results, and the sequence types determined by PubMLST will be presented in chapter 3.3–3.5. Appendix E shows a table with all the information gathered and obtained from this study: Ct-value, macrolide resistance, sequence type, allelic profile, and clonal complexes. The results from the electrophoresis for the optimization of nested PCR are showed in Appendix G.

3.1 Optimization of TPA macrolide resistance detection

The four different PCR-reaction mixes for the TPA macrolide resistance analysis were made as described in Table D.1 and Table D.2. LightCycler 480 was used to perform the PCR for macrolide resistance. See chapter 2.2.3.1.1 for the protocol of the analysis. For each of the PCR-reaction mixes, a TPA DNA control and a negative control were tested. For the first setup with only TPA DNA control and negative control, the melting step temperature was set to 75°C. This resulted in two nice melting curves and two half curves, see Figure 3.1. All the flat curves in the figure, represented negative controls. PCR-mix 1 (blue curve) and PCR-mix 3 (grey curve), containing the TP23S_P_[A] probe, showed a Tm approximately at 68°C. For PCR-mix 2 (green curve) and PCR-mix 4 (brown curve), containing the TP23S_P_[A]-LNA probe, showed two half curves but no Tm-value.

For the next setup, the melting step temperature was increased to 80°C. The same reaction mixes were used to test TPA DNA control and negative control. The increase of the melting step temperature resulted in four nice melting curves, see Figure 3.2. All the flat curves represented negative controls. PCR-mix 1 (blue curve) and PCR-mix 3 (grey curve), containing the TP23S_P_[A] probe, had a Tm approximately at 68°C, and PCR-mix 2 (green curve) and PCR-mix 4 (brown curve), containing the TP23S_P_[A]-LNA probe, had a Tm approximately at 76°C.

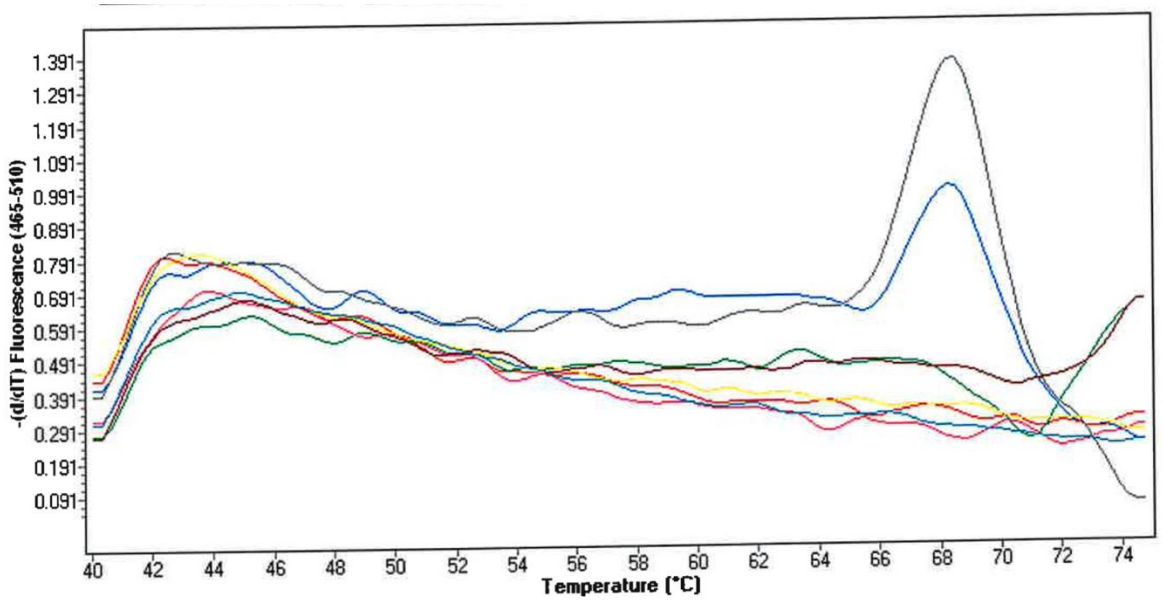


Figure 3.1 Melting point analysis (75°C)for macrolide resistance. The melting step temperature was increased to 75°C, resulted in Tm at 68°C for PCR-mix 1 (blue curve) and PCR-mix 3 (grey curve), and no Tm for PCR-mix 2 (green curve) and PCR-mix 4 (brown curve).

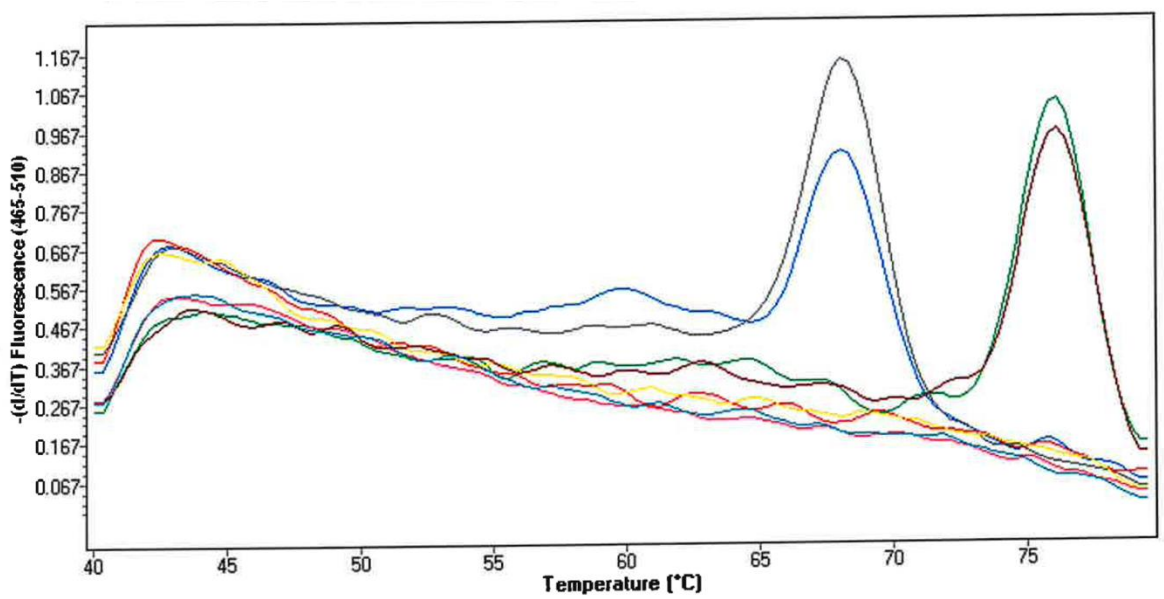


Figure 3.2 Melting point analysis (80°C) for macrolide resistance. The melting step temperature was increased to 80°C, resulted in Tm at 68°C for PCR-mix 1 (blue curve) and PCR-mix 3 (grey curve), and Tm at 76°C for PCR-mix 2 (green curve) and PCR-mix 4 (brown curve).

Regardless of the primer-set used for the macrolide resistance analysis, the two different probes, TP23S_P_[A] and TP23S_P_[A]-LNA, had two different Tm for TPA DNA control. The difference between the Tm between the two probes were 8°C.

Then, 18 clinical samples were picked out and analyzed in parallels with both the PCR-mix 3 and PCR-mix 4, with a melting step temperature at 80°C. The two PCR-mixes contained the same forward and reverse primer, TP23S_S and TP23S_R, but they had different probes, TP23S_P_[A] and TP23S_P_[A]-LNA respectively. The melting point analysis showed two single curves for the TPA DNA control, one curve with a Tm at 68°C (PCR-mix 3), and the other Tm at 76°C (PCR-mix 4). As for the clinical samples, the Tm for all the samples analyzed with PCR-mix 3 was approximately at 64°C, while the same clinical samples analyzed with PCR-mix 4 had Tm approximately at 73°C, see Figure 3.3.

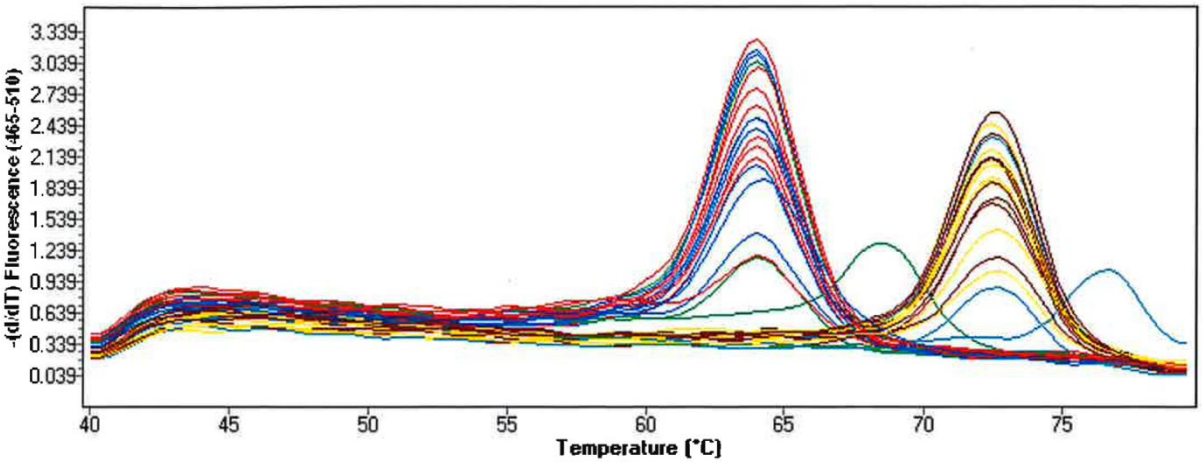


Figure 3.3 Macrolide resistance melting point analysis with clinical samples. Eighteen clinical samples were analyzed in parallel for macrolide resistance with two different PCR-mixes with different probes. Tm at 64°C, with PCR-mix 3, and Tm at 73°C, with PCR-mix 4 are patient samples showing macrolide resistance. Tm at 68°C and 76°C, PCR-mix 3 and PCR-mix 4 respectively, are TPA DNA control which are sensitive to macrolides.

After the melting point analysis, Sanger sequencing was performed from one clinical sample and TPA DNA control to confirm the A→G point mutation of the 23S rRNA gene. A random clinical sample and the TPA DNA control from PCR-mix 3 were sequenced by using the ABI genetic analyzer. Both samples were diluted 1:50 with PCR-water. See chapter 2.2.6 for Sanger sequencing method, and chapter 2.2.8.1 for Sequencher software. Point mutation of A→G was confirmed by Sanger sequencing, see Figure 3.4.

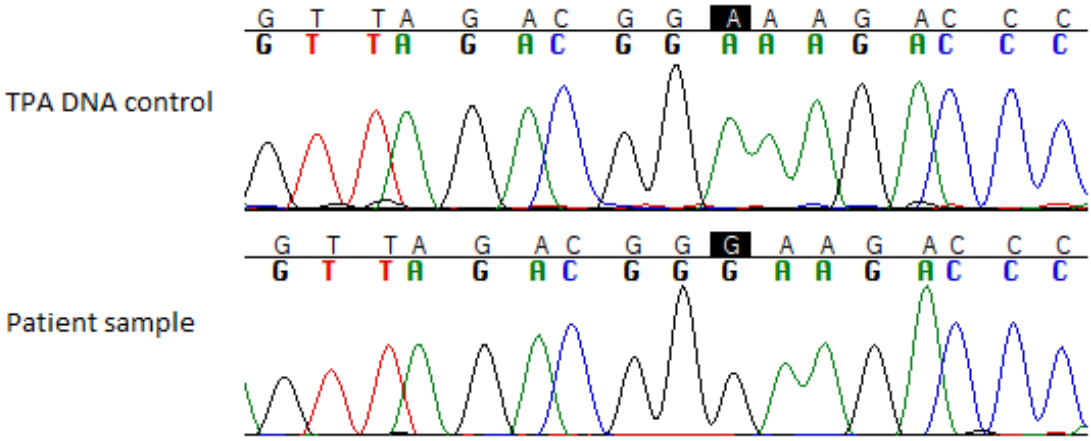


Figure 3.4 Sanger sequencing of TPA DNA control and one patient sample. The sequencing was performed to confirm the A2058G or A2059G mutation of the 23S rRNA gene. Only the relevant part of the sequences is shown. From this dataset, the A→G point mutation was confirmed in the patient sample. The A and G, marked in black, show the point mutation in the patient sample and no mutation in the TPA DNA control.

New TP23S_P_[A] probe was ordered to make a new PCR-mix 3 for analyzing the remaining clinical samples. No results were generated, neither the PCR-curves nor the melting curves were seen. The same PCR-mix 3 was made twice and tested with TPA DNA control to make sure that it was made correctly. There were still no results after the analysis. Capillary electrophoresis was done to see if there were any PCR products present, but no DNA bands were present leading to no generated PCR products.

To check if the probe interfered with the PCR, new PCR-mix 3 was made with no probe present, only with the two primers, TP23S_S and TP23S_R. LightCycler 480 was still used to perform the PCR, but a capillary electrophoresis was needed to check if the PCR products was successfully amplified. However, no PCR products were produced. The TP23S_P_[A] probe was ordered twice, but there were still no positive results. Then, both primers for PCR-mix 3, TP23S_S and TP23S_R, were ordered from TIB Molbiol. With these new primers, new PCR-mix 3 was made with the previously TP23S_P_[A] probes ordered, and TPA DNA control was used to test this new PCR-mix. This resulted in nice PCR curve and melting point curve for the TPA DNA control. By using the new batches of primers, the target sequence was successfully amplified from the remaining samples. Three of the samples showed two curves with different Tm, 64°C and 68°C. The melting point curves from a selection of patient samples is shown in Figure 3.5. The yellow melting point curve with Tm at 68°C is the TPA DNA

control, while the flat yellow curve is the negative control. The blue curves, at 64°C, are clinical samples showing mutations. The red curve, at 64°C and 68°C, is a clinical sample, containing both a wild-type and a mutant.

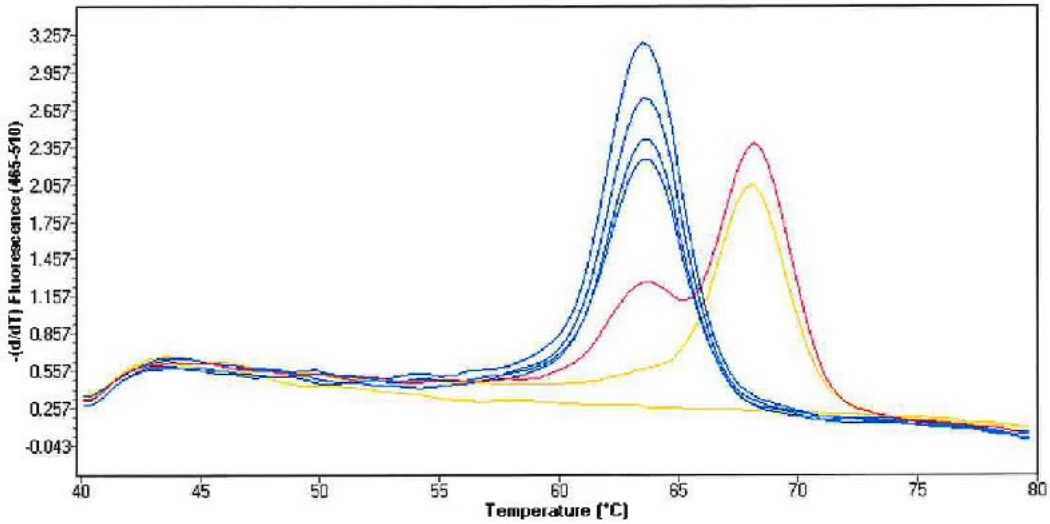


Figure 3.5 A selection of melting point curves from the macrolide resistance analysis. The yellow curve is the TPA DNA control which is a wild-type. The blue curves are patient samples showing mutation in the 23S rRNA gene. The red curve is a patient curve which is both wild-type and showing mutation.

The TPA DNA control, one clinical sample with Tm at 64°C, and one clinical sample with Tm at 64°C and 68°C were sequenced, see Figure 3.6. The first patient sample shows a mutation from A→G (marked in black in the figure), while the second patient sample shows both nucleotides A and G in the same position (the Sequencher software interpreted it as the nucleotide G).

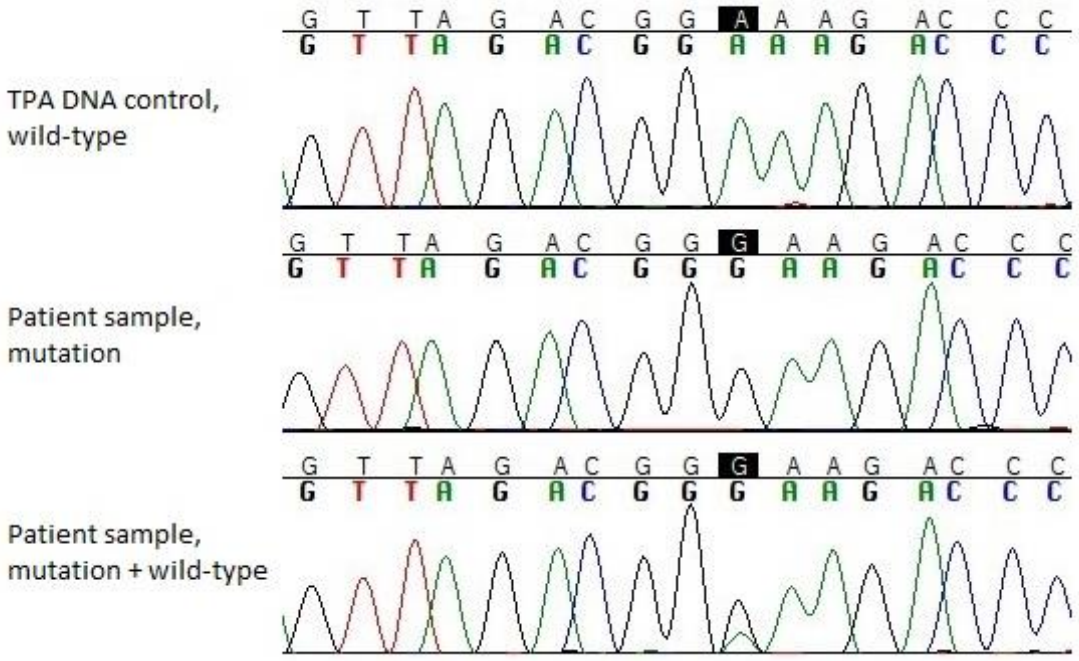


Figure 3.6 Sanger sequencing of the 23S rRNA gene in TPA DNA control and two patient samples. Only the relevant part of the sequences is shown. The positions marked in black, show the position of interest. TPA DNA control shows no mutation. The patient sample in the middle has confirmed the mutation A→G, while the bottom patient sample shows both A and G in the same position.

3.2 Optimization of nested PCR for TPA MLST

In order to use the sequence information of the genes *tp0136*, *tp0548*, and *tp0705* for MLST, successful amplification of these loci is crucial. The following section describes the results from different approaches employed to optimize the reaction conditions for specific amplification of these genes. After every nested PCR performed, all samples were analyzed with QIAxcel electrophoresis to verify successful amplification of PCR products and the desired length. See Table C.1 for the expected PCR product length for loci TP0136, TP0548, and TP0705. Table D.4 to Table D.6 show the ratio between the reaction mixes for the nested PCR as well as the PCR-protocol for the second PCR.

Non-diluted TPA DNA control was used for the first setup, see the results from Figure G.1 to Figure G.11 in Appendix G. For the touchdown (first) PCR (PCR1) of the nested PCR. PCR1 mixtures were made for all three loci, TP0136, TP0548, and TP0705. A volume of 25 µl Premix and 0.3 µM of each external primer-set were added to 5 µl of non-diluted TPA DNA control and 17 µl PCR-water to make a total volume of 50 µl.

The capillary electrophoresis showed that all PCRs produced target-specific amplicons between 1286 and 1500 bp for all three loci, and some minor unspecific amplicons in TP0136, see Figure G.1.

Another PCR1 mixtures were made: 3 µl of the non-diluted TPA DNA control was added to the TP0136-, TP0548-, and TP0705-PCR mixture with the same amount of reaction mix as previous, and 19 µl PCR-water to make a total volume of 50 µl. The electrophoresis results gave distinct bands as in the previous reaction, see Figure G.2.

Both PCR1 setups, with 5 µl and 3 µl of non-diluted TPA DNA, were continued to the second PCR (PCR2). Follow the flowchart, Figure 3.7, for the optimization of PCR2. One µl of PCR1-products was added to each of the TP0136-, TP0548-, and TP0705-PCR2 mixture, consisting of internal primer-set with a primer concentration at 0.3 µM, 25 µl Premix, and 21 µl PCR-water to make a total volume of 50 µl. The extension temperature was 72°C, and the number of cycles was 40 (Figure 3.7, Blue). In general, the electrophoresis results revealed some unspecific amplicons for all three loci, for both 5 µl and 3 µl non-diluted TPA DNA control, see Figure G.3. Locus TP0136, had a lot of amplicons ranging from 700 bp-1614 bp. TP0548 had amplicons ranging from 700 bp-1062 bp. TP0705 were more or less negative.

To optimize primer annealing and the working conditions for DNA polymerase, PCR2 was once again performed as previously for each of the loci, but only with PCR1-products from non-diluted 3 µl TPA DNA control. The reaction mixtures and the PCR-protocol were the same, but the extension temperature was decreased from 72°C to 68°C (Figure 3.7, Red). This resulted in more distinct bands with some unsatisfied PCR-products length. TP0136 produced amplicons between 700 bp-1400 bp. TP0548 has a distinct amplicon just below 400 bp. TP0705 had amplicons at 712 bp, as well as unspecific amplicons at 1194 bp-2027 bp. See Figure G.4.

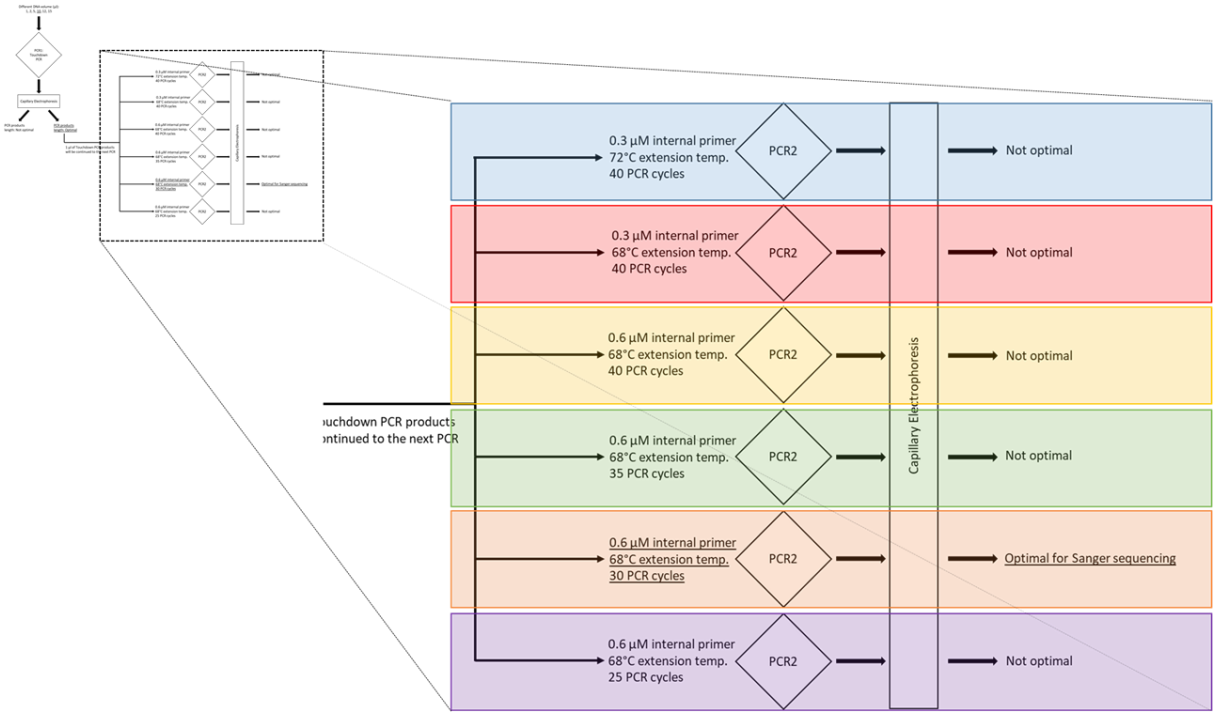


Figure 3.7 Enlargement of the second PCR (PCR2) from the flowchart of the optimization of nested PCR. One µl from the first PCR was continued to PCR2 with different conditions of primer concentrations, extension temperatures, and number of PCR cycles (listed in the figure). **Blue:** With these conditions, the PCR-products size were not optimal for sequencing. **Red:** With these conditions, the PCR-products size were not optimal for sequencing. **Yellow:** With these conditions, the PCR-products size were not optimal for sequencing. **Green:** With these conditions (including 0.3 µM internal primer), the PCR-products size were not optimal for sequencing. **Orange:** With these conditions (including 0.3 µM, 3 µM, and 6 µM internal primers), the PCR-products size were optimal for sequencing. **Purple:** With these conditions (including 0.3 µM internal primer), the PCR-products size were not optimal for sequencing.

A new PCR1 mix was made for each of the loci with the same volume of Premix and external primers, but the amount of non-diluted TPA DNA control was decreased to 2 µl. Electrophoresis resulted in reduced amounts of unspecific amplicons for all three loci, between 1315 bp and 1500 bp, while at the same time producing more of the target sequence. However, there were still some unspecific amplicons for TP0136, see Figure G.5. One µl of this PCR1, for all loci, were continued to PCR2. The same volume of Premix was used (25 µl), but the primer concentration was doubled to 0.6 µM for each of the internal primer-set. The extension temperature was 68°C (Figure 3.7, Yellow). The capillary electrophoresis results from PCR2 showed more unspecific amplicons for all three loci from 695 bp to 2500 bp, see Figure G.6.

A dilution series with 0.5 µl, 1 µl, 1.5 µl and 2 µl of non-diluted TPA DNA control for only locus TP0136, was tested for PCR1 to find the optimized volume of DNA needed.

The primer concentration for the external primers was at 0.3 µM. The capillary electrophoresis results showed nice and clearly amplicons for all for dilutions, about 1700 bp, but the PCR products for 1 µl DNA seemed to be a little shorter than the rest. There were also two unspecific amplicons at 495 bp for 1.5 µl and 2 µl non-diluted DNA control, as well as unspecific amplicons at 971 bp and 2500 bp for all four dilutions, see Figure G.7

One µl from the four different PCR1 products were used as template in the next PCR2-setups. Two different internal primer concentrations, 0.3 µM and 0.6 µM, with an extension temperature at 68°C, and different PCR cycles, 40, 35, 30, and 25 were tested to find the best combination (Figure 3.7, Red, Yellow, Green Orange, Purple). The electrophoresis results can be seen from Figure G.8 to Figure G.11 respectively. The idea was that increasing the primer concentration might increase the efficiency of primer annealing, and different number of PCR cycles could result in different PCR products length. At 40 cycles, the electrophoresis showed distinct PCR-products, from approximately 1400 bp to 2500 bp, for all different primer concentration and the different non-diluted TPA DNA control from PCR1. One µl non-diluted TPA DNA control from PCR1 seemed to have shorter PCR product in PCR2 than the rest of that setup, approximately 1000 bp. At 35 cycles, the electrophoresis was showing almost the same picture as for 40 cycles, with the same PCR-products size. At 30 cycles, the capillary electrophoresis setup showed PCR-products sizes ranged from about 1200 bp to 2300 bp, and the amplicons was less distinct than the setups with 40 and 35 cycles. At 25 cycles, the electrophoresis was still showing several distinct amplicons, but less compared to the setup with 40 cycles. The amplicons were at 1450 bp. However, 1.5 µl TPA DNA control from PCR1 setup, for both 0.3 µM and 0.6 µM internal primers for PCR2, had PCR products at 800 bp.

From this point, TPA DNA control was diluted according to chapter 2.2.1. Locus TP0136 was tested with this diluted TPA DNA control to find the best optimization. A primer concentration at 0.3 µM, of TP0136_EF and TP0136_ER, was mixed with 25 µl Premix and 6 different volumes of TPA DNA control, 5 µl, 2 µl, 1 µl (see Figure G.12) and 15 µl, 12 µl and 10 µl (see Figure G.13). PCR-water was added to the different PCR1 reaction mix to make a total volume of 50 µl. For the TPA DNA control, electrophoresis showed no PCR-products with 1 µl DNA, some PCR-products at 2500

bp with 2 µl DNA and 15 µl DNA, and some PCR-products at 1000 bp and 2500 bp for 5 µl DNA, while electrophoresis showed amplicons with the right size, at 2000 bp, with 10 µl DNA and 12 µl DNA.

One µl TP0136 PCR1-products from 12 µl DNA and 10 µl DNA previously, was continued to PCR2 with 3 µM, 6 µM, 0.3 µM and 0.6 µM internal primers for TP0136, using 30 PCR cycles (Figure 3.7, Orange). The electrophoresis results showed that each well had acceptable amplicons, ranging between 700 bp and 1438 bp, with some unspecific amplicons in the area with large PCR products, see Figure G.14.

Sanger sequencing was performed on TP0136 to ensure that the PCR products from PCR2 were able to be sequenced, that the unspecified PCR products would not interfere, and the allelic number for TP0136 are the same as predicted. PCR2-products from TP0136 10 µl DNA 0.6 µM primer were decided to be sequenced. The TP0136 PCR products were diluted with PCR-water, 1:50 and 1:100, to find the best concentration for sequencing. BigDye PCR reaction mix, and protocol were performed according to Table 2.12 and Table 2.13 respectively. ABI genetic analyzer was used to sequence the TP0136 PCR product. The sequencing results were nice and the allelic number for TP0136 was 10.

Since the sequencing results for TP0136 were promising, the nested PCR was continued for TP0548 and TP0705 with the same concentration of primers as the last run of TP0136. Ten and 12 µl TPA DNA, and 0.3 µM of external primers (EF and ER for TP0548 and TP0705) were added in the separated PCR1 reaction mix, for TP0548 and TP0705, with Premix, external primers, and PCR-water. The electrophoresis results were acceptable, approximately 1500 bp for the locus TP0548 and 1282 bp for TP0705, see Figure G.15. 1 µl of the PCR1-products were continued to PCR2 with a primer concentration at 0.6 µM and 30 PCR cycles. Some unspecified PCR products in the electrophoresis results, for both the locus at 10 µl and 12 µl, but overall, the PCR-products length were satisfied. PCR product sizes were roughly 1200 bp for TP0548 and 900-1000 bp for TP0705, see Figure G.16. Sanger sequencing of these PCR products, on ABI genetic analyzer, gave allelic number 14 and 10 for locus TP0548 and TP0705 respectively. Together with locus TP0136, the TPA DNA control

resulted in ST20, a Nichols-like strain, with allelic number 10-14-10 (TP0136, TP0548, and TP0705 respectively).

The nested PCR, as well as Sanger sequencing, was well performed for all three loci. Therefore, a random patient sample (sample number 9) was chosen to test if the optimization for the TPA DNA control was good enough for analyzing patient samples. Ten µl DNA from the chosen patient sample was added in the PCR1 reaction mix for TP0136, TP0548, and TP0705. PCR1-products from all three loci were continued to PCR2 with 30 cycles with primer concentration at 0.6 µM (Figure 3.7, Orange). The electrophoresis results for PCR1 showed distinct bands with the expected PCR products size for all three loci, as well as multiple unspecific amplicons, see Figure 3.8A. For the PCR2, the capillary electrophoresis results showed amplicons of expected size for all three loci and with much less unspecific amplicons, see Figure 3.8B.

Sanger sequencing of the three loci for this sample resulted in a SS14-like strain, ST1, allelic number 1-3-1 (TP0136, TP0548, and TP0705 respectively). The rest of the patient samples were performed accordingly to the last optimization of the nested PCR.

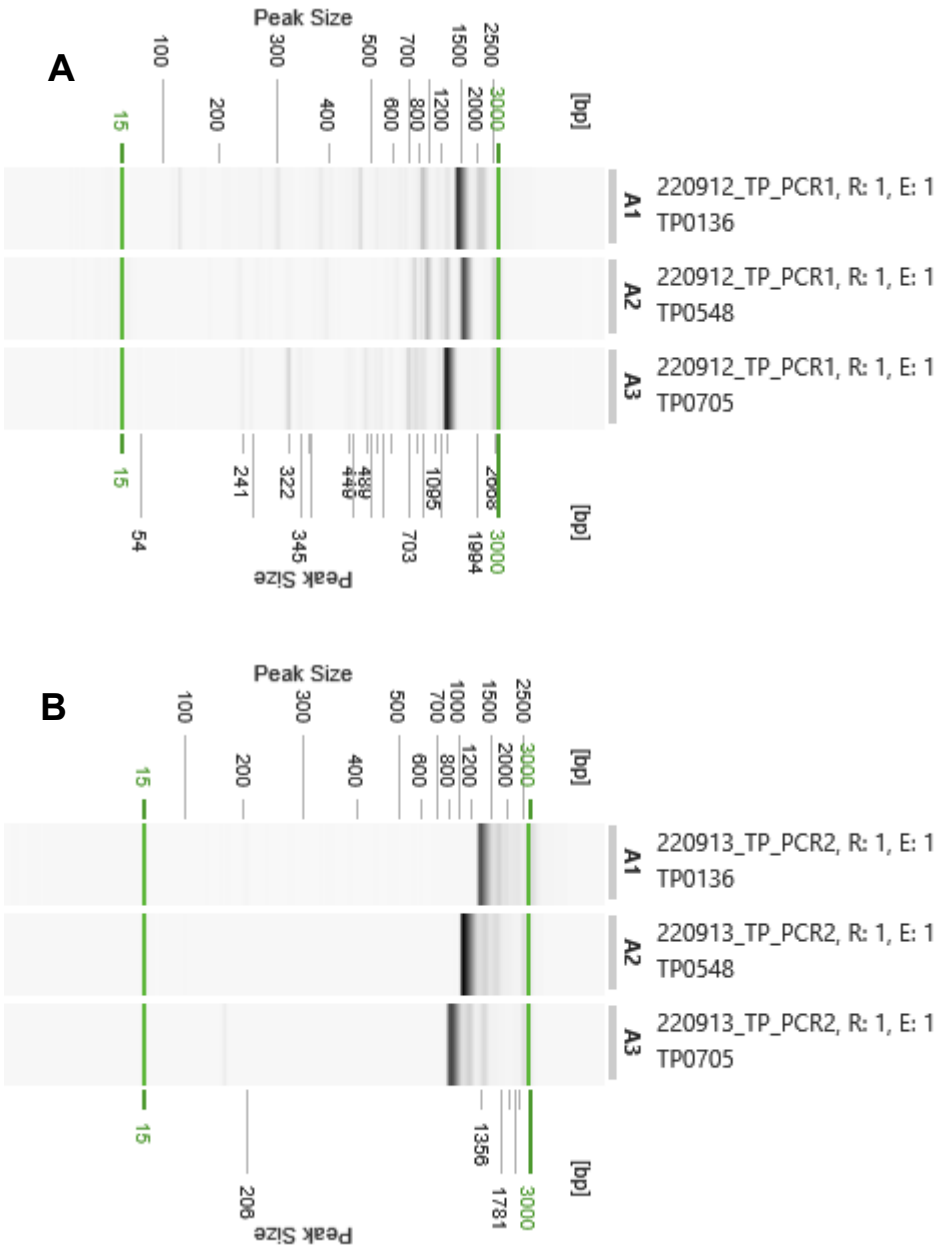


Figure 3.8 Electrophoresis results from the nested PCR **A.** PCR1 of the nested PCR. Patient sample number 9 was tested on TP0136, TP0548, and TP0705 with 10 µl DNA in the reaction mix. **B.** PCR1-products were continued to PCR2 of the nested PCR with 0.6 µM primer and 30 PCR cycles.

3.3 Macrolide resistance in TPA

In total, 78 clinical samples (of 81 collected samples from October 2020 to September 2022) were analyzed for macrolide resistance by melting point analysis. 96% of the samples were conclusive, while three of the samples were inconclusive. Three samples had both the sensitive and the resistant gene to macrolide antibiotics, they were all SS14-like strains (ST1 and ST2). The rest of the samples were resistant to macrolides. The results of macrolide resistance in TPA from this study are presented

in Table 3.1. All the Nichols-like strains determined in our study, are macrolide resistant. In this study, no samples were sensitive to macrolides.

Seven samples from QCMD were also analyzed for macrolide resistance, and they were all proved to be resistant. The results from QCMD are not included in Table 3.1.

Table 3.1 Macrolide resistance results. In total 78 positive syphilis samples were analyzed for macrolide resistance.

	Wild type (Macrolide sensitive)	**Mutant (Macrolide resistant)**	**Both (Macrolide sensitive and resistant)**	**Undetermined**
Number of samples	0	72	3	3

3.4 TPA allelic profiles

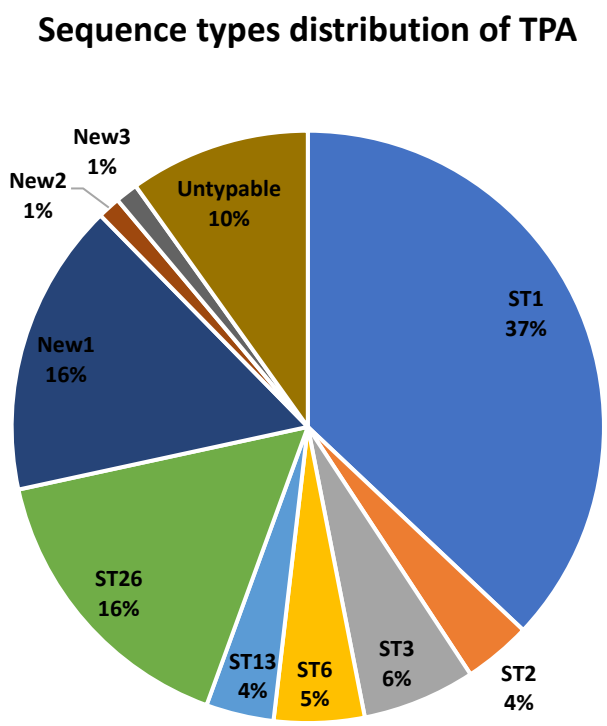
After performing Sanger sequencing of all the samples, TPA allelic profiles were determined by PubMLST. In total, 81 clinical samples from 70 different patients were analyzed. Complete allelic profiles were successfully determined in 90% of the clinical samples. Eight samples were partially typed, but the final sequence types were not typable. The result from this study showed that 82% of the typable samples are SS14-like strains, while 18% are Nichols-like strains. Nine different sequence types were found, including three novel sequence types. Excluding the novel sequences, four sequence types belongs to SS14-like strains and two belongs to Nichols-like strains. Thirty samples were determined to be ST1, which is the most frequent sequence type among the samples from this period. Thirteen samples were determined to be ST26, five samples are ST3, four samples are ST6, and three samples were determined each to be ST2 and ST13. See Figure 3.9 for the sequence type distribution of TPA (left) and the allelic profiles for the different sequence types (right).

One patient with three different samples at two different locations, wound from the throat and anus, had two different sequence types, ST26 and ST1 respectively. The other eight patients with multiple samples had the same sequence type for all locations.

Four new sequences were found, resulting in three novel sequence types. The new sequences were submitted into PubMLST to receive allelic numbers for the allelic

profiles. See Figure 3.9 (right) to see the new allelic numbers obtained from this study (the numbers are underlined). Thirteen of the samples gave a new sequence type, NEW1, and only one sample each seems to be NEW2 and NEW3.

Seven QCMD samples were also analyzed, but only five of them were typable: ST90, ST3, 2 with ST1, and ST75. The results from QCMD are not included in Figure 3.9.



	TP0136	TP0548	TP0705
ST1	1	3	1
ST2	1	1	1
ST3	1	1	8
ST6	3	2	3
ST13	6	3	1
ST26	9	7	3
NEW1	1	80	1
NEW2	36	81	3
NEW3	1	82	1

Figure 3.9 Left: The distribution of the TPA sequence types from 81 analyzed samples collected from October 2020 to September 2022. Nine different sequence types were found. This sector diagram was made by plotting the data from Appendix E into Excel. **Right:** The different allelic profiles from the nine sequence types, are obtained from PubMLST. Underlining numbers are new allelic numbers submitted and received from PubMLST.

3.5 Phylogenetic tree of TPA sequence type

The phylogenetic tree was produced by uploading the fasta-file (Appendix F) and auxiliary data (Appendix E) to the online software of PHYLOViZ, see Figure 3.10. ST1 node have obtained two new branches, leading to sequence type NEW1 and NEW3, while NEW2 is between ST6 and ST26. Because NEW2 is between two Nichols-like strains, NEW2 is assumed to be a Nichols-like strain too. And NEW3 are assumed to be SS14-like strain. The distances between the nodes are relative. The results from QCMD are not included in this phylogenetic tree.

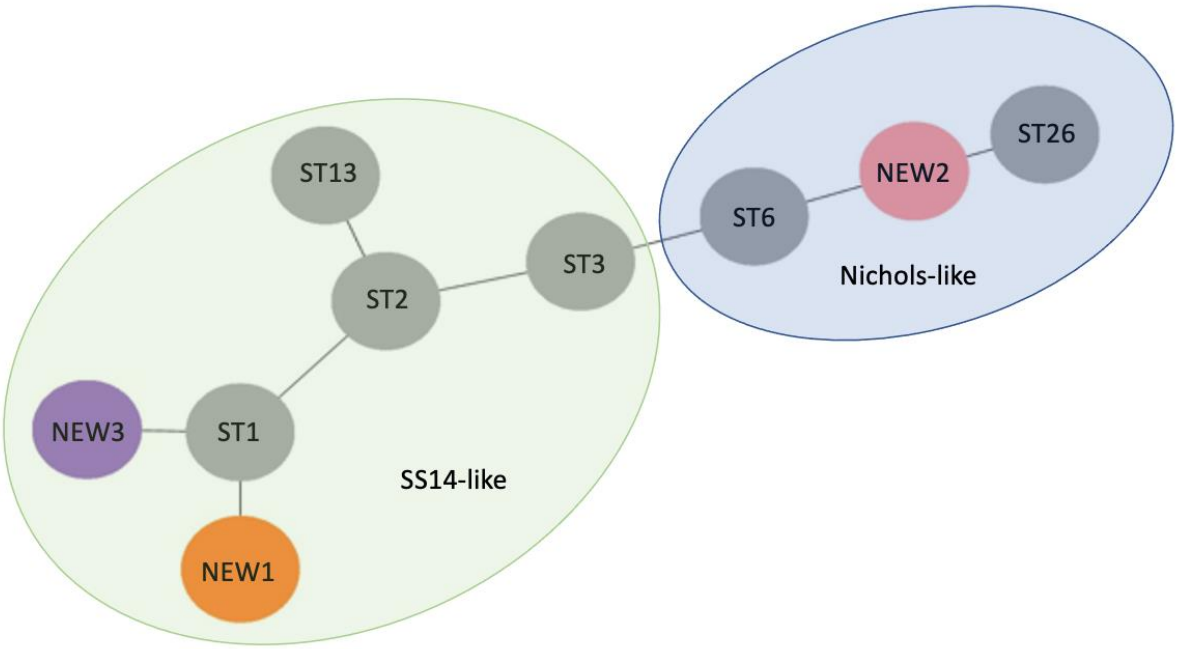


Figure 3.10 Phylogenetic tree of the TPA sequence types. The phylogenetic tree from this study was produced by PHYLOViZ. The tree is showing relative distances of the sequence types. The different subtypes are grouped into SS14-like and Nichols-like strains.

4 Discussion

Molecular characterization by MLST is important for understanding the epidemiology of syphilis. The combination of macrolide antibiotic resistance and the molecular genotyping of TPA is important for the surveillance of antibiotic resistant strains. These data are the first results of molecular characterization and macrolide resistance done in TPA in Norway.

QCMD, located in United Kingdom, are sending an annual syphilis panel to the laboratory for analyzing TPA to make sure that the laboratory is adequate. The QCMD syphilis panel analyzed in this study is from 2021. When asking QCMD for the sequences for the TPA samples, QCMD informed us that these were clinical samples and the access to sequences was unavailable. These QCMD samples were still analyzed, just to see the characterization, and maybe we could learn something, but these data were not included in the results of this study.

4.1 Prevalence of macrolide resistance TPA in Norway

Macrolide resistance is associated with point mutations of either A2058G or A2059G in one or both copies of the 23S rRNA gene (Smajs et al., 2015). As positions for the resistance mutations were known, Sanger sequencing of all the samples was not necessary. However, one sample from the first macrolide resistance analysis was sequenced to ensure that the point mutation was either of the two positions. As well as three patient samples, with double melting points (one at 64°C and one at 68°C), were also sequenced.

Three of the clinical samples were not analyzed for macrolide resistance because there was no more eluate available. Three other samples, which were not detectable for this melting point analysis, had a Ct-value >32 for the TPA PCR. A high Ct-value indicates lower amount of template DNA present which probably caused the inconclusive results. All our clinical samples from this study, which were typable, showed A→G point mutation in either position 2058 or 2059 in the 23S rRNA gene, except from three samples which by Sanger sequencing showed the presence of both the A2058G mutation and a wild-type version of the gene, see Figure 3.6. According to Stamm and Bergen (2000), which this macrolide resistance analysis is based on, the point

mutation, proved by Sanger sequencing, is in position 2058. The sequences of TPA DNA control from this study was compared to the sequences from the article, making it possible to know the position of the mutation. Based on Tm alone, point mutation is proved by changing in Tm, but the position of the mutation is not known based in melting point curves alone.

In this study, macrolide resistance mutations, in the A2058G or A2059G of 23S rRNA gene, are proved to be present in both the SS14- and Nichols-like strains in all the TPA positive samples collected from October 2020 to September 2022. No samples in this study were analyzed to be wild-type, hence no samples were sensitive to macrolide antibiotics. The results from this study of macrolide resistance in TPA, corresponds to what is reported globally (Ghanem et al., 2020; Stamm, 2010). Macrolide resistance has increased over time, however the prevalence of macrolide resistance in TPA differs from country to country (Grillova et al., 2018). See Table 4.1 for an overview of the prevalence of macrolide resistance in TPA in selected different countries. Countries like Argentina, Madagascar, Peru, and Taiwan have little or no macrolide resistance in TPA, however more than 50% of TPA are macrolide resistance in many other countries.

Since TPA lacks genetic elements needed for the mechanism of horizontal gene transfer (see chapter 1.4.1), macrolide resistance has been arisen by chromosomal mutation in the 23S rRNA gene (Stamm, 2010). Different mechanisms have been discussed on how the macrolide resistance, in TPA, have been introduced in certain populations. One alternative mechanism is that 23S rRNA gene in TPA strains, have mutated spontaneously by widespread use of macrolides in the environment. Another alternative is that an already mutated strain was introduced to a wild-type population, and spread the mutation further in the population even in the absence of macrolides in the environment (Grimes et al., 2012; Stamm, 2010). The different mechanisms of how the mutation have been introduced, might explain the variation of macrolide resistance between countries. The high prevalence of macrolide resistance in the Norwegian TPA isolates, might be explained by the use of macrolide antibiotic, azithromycin, for treatment of genital infections, such as *Chlamydia trachomatis* and *Mycoplasma genitalium* (OUS, 2020).

Table 4.1 The prevalence of macrolide resistance in TPA from selected countries.

Country	**Percentage of macrolide resistance, (n) = number of samples tested**	**Reference**
Argentina	14.3% (6/42)	(Morando et al., 2022)
Australia	87% (398/456)	(Taouk et al., 2022)
Belgium	65.5% (19/29)	(Mikalova et al., 2017)
China	97.5% (390/400)	(Xiao et al., 2016)
Cuba	61% (25/41)	(Noda et al., 2016)
Czech Republic	66.7% (46/69)	(Grillova et al., 2014)
Ireland	93.1% (27/29)	(Muldoon et al., 2012)
Madagascar	0% (0/141)	(Van Damme et al., 2009)
Peru	0% (0/10)	(Flores et al., 2016)
Spain, Barcelona	94.7% (89/94)	(Fernandez-Naval et al., 2021)
Taiwan	0% (0/102)	(Wu et al., 2012)
United Kingdom, London	66.6% (12/18)	(Tipple et al., 2011)
United State of America	64.4% (83/129)	(Katz et al., 2010)

The three samples, which showed a mix of A2058G mutation and a wild-type, are associated with macrolide resistance since mutation in position 2058 is present (Smajs et al., 2015). The reason for the presence of both mutation and wild-type in the samples was not studied in this thesis, therefore, the presence of both version of the gene can be discussed based on the different mechanisms of how the mutation has arisen. The presence of both mutation and wild-type versions of the gene in the sample, might be because of the present of one clone or two clones of TPA which have one mutated and one wild-type version of the gene. Another reason for both versions of the gene might be emerging of a macrolide resistant mutant due to treatment with macrolide antibiotics (Grimes et al., 2012).

Since all the samples from this study are showing macrolide resistance, the possibility for using macrolide antibiotic for treating more than one infection at a time will therefore not be considered. Macrolide resistance is being tested for all samples with positive *Mycoplasma genitalium* in the laboratory routine for Unit for Molecular diagnostics, and Virology. The connection between macrolide resistance of TPA and *Mycoplasma*

genitalium has not been studied as the focus for this study is the prevalence of macrolide resistance in TPA alone.

4.2 TPA allelic profiles in Norway

Molecular characterization of TPA was performed by MLST according to Grillova et al. (2018) by identifying three loci: TP0136, TP0548 and TP0705. This MLST method will give the opportunity for studying the epidemiology of the different TPA allelic profiles and give a better understanding of the epidemiology of syphilis and the infections (Grillova et al., 2018). TPA DNA control (reference strain) was being used to optimize the nested PCR and sequencing of the three loci.

All clinical TPA can be divided into SS14- and Nichols-like strain. Globally, 94% of TPA are related to the SS14-like strain and 6% to the Nichols-like strain (Morando et al., 2022). For all typable samples from this study, 82% of the samples were typed to be SS14-like strain, while 18% of the samples were typed to be Nichols-like strain. The eight samples which were untypable, had a Ct-value >32, which means there was small amount of template DNA present. This first study of TPA strains in Norway shows a surprisingly high frequency of Nichols-like strains, with a different distribution of SS14- and Nichols-like strains compared to the global distribution pattern. These results are similar to the results from Argentina, however the macrolide resistance in TPA, from this study, is present in all samples compared to 14.3% in Argentina (Morando et al., 2022).

Nine different sequence types were determined in this study, of which three sequences are new and therefore not determinate by PubMLST database until now. ST1, ST26 and NEW1 are the three most common sequence types obtained, see Figure 3.9. The frequencies of the sequence types (except from the three new sequence types) were compared with the frequencies in other continents, see Table 4.2. This comparison showed that the prevalence of the different sequence types did not correspond to any of the sequence type pattern from any of the continents. However, ST1 seemed to be the most common sequence type in Europe and North America, while ST3 is most common in Asia. The prevalence of ST2 in Norway are the same as in Asia, and the prevalence of ST3 is the same as in Europe. Surprisingly, the prevalence of ST26, a

Nichols-like strain, is much higher than what is found in Europe, North America, and Asia. The results from this study were compared to the data published in PubMLST even though the sequence type data retrieved, are biases as the molecular typing has not been established globally.

Table 4.2 Frequencies of TPA allelic profiles between this study and in other continents. The three novel sequence types revealed from this study are not listed below. The data from other continents are according to the PubMLST database (Grillova, Jolley, et al., 2019; Morando et al., 2022).

Allelic profiles (ST)	**1.3.1 (ST1)**	**1.1.1 (ST2)**	**1.1.8 (ST3)**	**3.2.3 (ST6)**	**6.3.1 (ST13)**	**9.7.3 (ST26)**
Norway (this study)	37%	4%	6%	5%	4%	16%
Europe	48.9%	15.1%	7.7%	2.7%	0.2%	4.4%
North America	60%	9.6%	0.7%	19.6%	2.5%	2.2%
Asia	3.9%	3.9%	75.8%	5.2%	0%	0.8%

Patients with several samples from different locations, were found to have the same sequence type except from one patient, who surprisingly had two completely different TPA sequence types, ST1 and ST26 that belongs to SS14-like strains and Nichols-like strains, respectively. These two samples were from different locations (throat and anus), that could indicate double infection with TPA, possibly from two different persons. Further, these two sequence types are located at opposite ends of the phylogenetic tree, see Figure 3.10.

A phylogenetic tree was produced by PHYLOViZ for demonstrating the relative distances of the nine TPA sequence types obtained from this study, see Figure 3.10. These relative distances show that there is genetic difference between the sequences, but not how big the differences are. Knowing that ST6 and ST26 are Nichols-like strains, makes it easy to group SS14-like and Nichols-like strains. ST1, SS14-like strain, have three branches, where one branch leads to ST2, and the other two branches lead to NEW1 and NEW3 each. NEW1 and NEW3 are closer to ST1 than to other sequences, and therefore these two sequence types are assumed to be SS14-like strains. NEW2 is branching to both ST6 and ST26, which is assumed to be a Nichols-like strain. ST3 and ST6 makes the boundary between the SS14- and Nichols-like strains. The genetic differences between these two sequences are not seen from

this phylogenetic tree. What we know is that these two sequences are related even though they belong to different strains.

The distribution of the different sequence types obtained from this study are the first in Norway. The clinical samples collected for this study are from October 2020 to September 2022 and are restricted to MSM patients from the Oslo-area in Norway. Little to no travel during 2020-2021, due to Covid-19 restrictions, were probably the reason for fewer reported cases (FHI, 2023; Regjeringen, 2020-2022). NIPH predicts that syphilis cases will increase in the next few years as the Covid-19 restrictions will be wound up (FHI, 2023). According to MSIS, an increase of domestic infection of syphilis cases were reported in 2020-2021 during the Covid-19 pandemic, see Appendix H. Limited travel opportunities, restricted group of patients, and dominant domestic infection of syphilis in the pandemic year, explain the poor TPA allelic profiles diversity for this first study. How the allelic profiles would look like if the collected samples were more diverse (from other area in Norway), or in the next few years will be hard to predict. But with an increase of travel activity, and an increase of syphilis infection, we can assume that the allelic profiles for TPA will also be more diverse.

4.2.1 MLST revealed novel allelic profiles

This MLST typing scheme revealed three novel sequence types (NEW1, NEW2, and NEW3) of which one new sequence was found for locus TP0136 and three new sequences were found for locus TP0548. These sequences were submitted into the PubMLST database and new allelic numbers were received. The allelic profiles for NEW1, NEW2, and NEW3 are determined to be 1.80.1, 36.81.3, and 1.82.1 respectively. The frequency of NEW1 was 16% from this study, which makes it to the top three of the most common sequence type (see Figure 3.9). The sequence types for these three new allelic profiles have still not been received.

4.3 Discussion of the optimization

4.3.1 Optimization of TPA Macrolide resistance

Four primers, two forward and two reverse, and two probes were used for the optimization of TPA macrolide resistance. The primers and probes were designed by TIB Molbiol who based the design on a publication from Stamm and Bergen (2000)

who identified a point mutation in the 23S rRNA genes which referred to macrolide resistance.

Four primers, with different combination with the probe, were tested to find the best combination, see Table 2.7 for the different primer and probe combinations. For getting a good melting curve analysis, different concentration of forward and reverse primers was necessary, see Table D.1 and Table D.2 for the concentration of primers (and probe). TIB Molbiol stated that combination with the TP23S_F primer is specific only for TPA as TP23S_S primer can potentially detect other *Treponema* species. Detection of other *T. pallidum* is eliminated as the samples used in this study are identified as TPA in routine diagnostics.

The macrolide resistance analysis is risky because there are two stem-loops proximity to the probe that might weaken the binding of the probe. The SimpleProbe used for the macrolide resistance analysis was designed to be a wild-type specific probe and has a Tm close to the stem-loop. TP23S_P_[A], a standard SimpleProbe with a length up to 30 nucleotides, has a Tm around 69°C. TP23S_P_[A]-LNA, a longer probe of 35 nucleotides has a Tm around 75°C. The LNA (Locked Nucleic Acids) are added to the probe to increase the Tm and therefore increasing the binding to the complementary nucleotide.

Four different PCR-mixtures were made and analyzed to find the best combination of primers and probes. All the four PCR-mixes showed acceptable and similar results. PCR-mix 3, containing TP23S_S and TP23S_R primers and TP23S_P_[A] probe, was chosen for the analysis of clinical samples because the fluorescence of the probe was higher with this combination (see Figure 3.2). To make sure that the TP23_P_[A]-LNA probe would give the same results or better, PCR-mix 4, containing the same primers, was also used to test clinical samples in parallel with PCR-mix 3, see Figure 3.3. The results from PCR-mix 3 and PCR-mix 4 matches with each other, but the melting point curves had higher fluorescens in PCR-mix 3 than PCR-mix 4.

A new batch of PCR-mix 3 was made for analysis of the remaining clinical samples. This mix resulted in no PCR curves or melting point curves. Several PCR-mix 3 was made to make sure that the PCR-mix was made correctly. None of the PCR-mixtures

gave any positive results. As a risky analysis, the first thought was that it was something wrong with the probe, which therefore gave no results. A new TP23S_P_[A] probe was therefore ordered again. New PCR-mixes were made, both with and without the new TP23S_P_[A] probes, trying to identify the error of the PCR reactions. No PCR curves were seen in the PCR-mix containing the probe. The results from the capillary electrophoresis were also negative. PCR-products should be present in the electrophoresis if nothing were wrong with the primers. Because of this outcome, the probe might not be the problem of the assay, but the problem might be the primers. New TP23S_S and TP23S_R primers were ordered to confirm the hypothesis.

Two new PCR-mix 3 was made with the new primers, one with each of the TP23S_P_[A] probe which we though were the problem. Both PCR-mixes with the probes gave good results. The doubt that there was something wrong with the probes was removed when the PCR-mix with the new primers worked well with both the TP23S_P_[A] probes. The statement about a risky analysis, was strengthened after running the last melting point analysis. The reason why the second batch of PCR-mix 3 did not work is not known as we did not investigate the possible errors of the primers or probes. The inability of the probe to bind, due to the formation of stem-loop proximity to the binding site of the probe, might be the reason why PCR assay did not generate any fluorescence signals. However, the PCR assay should have generated some PCR products which should be visualized by the capillary electrophoresis. For that reason, the problem of the assay was assumed to be the primers, as the last batch with the new primers worked well. However, this was only an assumption as the hypothesis was not confirmed other than making new batch of PCR-mix.

PCR-mix 1 and PCR-mix 2, which were made at first for this analysis, were analyzed with TPA DNA control out of curiosity. The results turned out good like the first time, see Figure 3.2 of how the melting curves looked like.

Of experience from the routine diagnostics, resuspended primers in TE-buffer are stable and can usually be stored in the freezer for a long time. In this case, these primers were probably not stable in the freezer. However, primers that were already mixed into the PCR-mixes, and reused after freezing, were stable. This was proven when analyzing PCR-mix 1 and PCR-mix 2. Other reagents added to the mix, such as

PerfeCTa MultiPlex qPCR ToughMix and PCR-water might be the reason why the primers are stable in the PCR-mix and unstable when stored alone. For further macrolide resistance analysis, PCR-mix 3 is already made and is ready to use. If this primer-stability theory is right, the frozen PCR-mix will not cause any problems.

4.3.2 Optimization of nested PCR

The TPA DNA control and the same primer sequences taken from Grillova et al. (2018), were used to optimize the MLST scheme for TPA. The nested PCR for the MLST of the three TPA loci, TP0136, TP0548, and TP0705, was optimized, and then followed by Sanger sequencing. To visualize the PCR products, capillary electrophoresis was applied. The length of the PCR-products, by electrophoresis, is not very exact and might vary with some hundred bp in difference from the true PCR-products length. It was therefore sufficient to check that the length of the PCR-product is around the area that is expected.

For the touchdown protocol of the nested PCR, nothing was changed. Instead of adding all the reagents separately, PrimeSTAR® GXL Premix, developed by Takara Bio, was used. As written in chapter 2.1.5, this Premix is composed of DNA polymerase, reaction buffer, and dNTP mixture. The PrimeSTAR® GXL Premix document recommends using a final concentration of 0.3 µM of each primer as well as 25 µl of the Premix. DNA template is added, and PCR-water is added to the mix to make a volume of 50 µl in total. For the PCR1, only different amount of TPA DNA control was tested out to find the optimal one. The dry matter of TPA DNA control was dissolved with Vircell Control Reconstitution solution as described in Appendix A.

For the first four setups of the nested PCR, the non-diluted TPA DNA control was used. See Figure G.1, Figure G.2, Figure G.5, and Figure G.7 for the electrophoresis results. For PCR1, nothing was wrong when looking at the electrophoresis for the loci TP0136, TP0548, and TP0705. The PCR products from the PCR1 have approximately the same PCR lengths as we wanted no matter the volume of TPA DNA control added (0.5 µl, 1 µl, 1.5 µl, 2 µl, 3 µl, and 5 µl). The electrophoresis showed just a couple of unspecific amplicons but nothing to be aware of. By performing the next PCR, PCR2, these

unspecific amplicons might disappear as this step, using internal primers, will be more specific to what we want to produce of PCR products.

PCR1 products were continued to PCR2, see Figure G.3, Figure G.4, Figure G.6, and Figure G.8 to Figure G.11. Different concentrations of primers were tested out at this step, as well as the number of PCR cycles. The idea was that increasing the primer concentration might increase the efficiency of primer annealing, and different number of PCR cycles could result in different PCR products length. For the first setup of PCR2, the protocol was the same as Grillova et al. (2018): the extension temperature was 72°C, the primer concentration was 0.3 µM, and the number of PCR cycles was 40. Only TP0548 locus gave PCR products with the desired length. The other two loci had either no PCR products or several unspecific products. The Premix document recommends 68°C for the extension temperature. To optimize the Premix function, the extension temperature was therefore decreased to 68°C for the next PCR2 setups. When decreasing the extension temperature, the length of the TP0705 PCR products was better correlated with the expected length of the amplicon. PCR products for TP0548 had the wrong length, and many weak and unspecific TP0136 PCR products. The concentration of internal primers was increased to 0,6 µM to make sure that there were enough primers for the PCR reaction. The increasing of the primer concentration leads to better PCR products for TP0548 and TP0705. There were still a lot of unspecific PCR products in all three loci. For the next four PCR2, the different amount of TPA DNA control added in the PCR1 were tested out with different amount of PCR cycles (40, 35, 30, and 25 cycles), see Figure G.8 to Figure G.11. The primer concentration and the extension temperature were kept to 0,6 µM and 68°C respectively. The amount of PCR products depends on the number of PCR cycles. Increasing of PCR cycles will result in greater amount of PCR products, but too many cycles will often results in more unwanted PCR products (Lorenz, 2012). Therefore, the right amount of PCR cycles is important to generate the desired PCR products. 30 cycles for the PCR2 seemed to be the best as the length of the PCR products generated have desired length compared to 40, 35, and 25 cycles where the PCR products for these cycles generate larger products.

Even though the PCR products generated at 30 cycles gave the desired length of PCR products, the electrophoresis showed a lot of unspecific products. As the TPA DNA

control, used for this optimizing of nested PCR, was the same as for detection of TPA in the routine laboratory, the procedure of how this control is made in routine is followed, see chapter 2.2.1. The control needed to be dissolved in reconstitution solution, as described earlier, but also to be diluted with PCR-water to make the right concentration. The reason why PCR2 generated a lot of PCR products might be because the concentration of TPA DNA was too high.

After diluting the TPA DNA control as described in chapter 2.2.1, PCR1 and PCR2 were once again performed, see Figure G.12 to Figure G.16. Different volume of TPA DNA control was tested for TP0136 for the PCR1, 1 µl, 2 µl, 5 µl, 10 µl, 12 µl, and 15 µl. Only the volume with 10 µl and 12 µl of TPA DNA control added, gave nice results, with the expected PCR-products length, with the electrophoresis. These two PCR products were continued to PCR2. Different concentration of internal primers was tested out with the combination of the two PCR products. It turned out that there was little to no differences when starting with 10 µl or 12 µl of TPA DNA in the first PCR. The nested PCR was then performed for TP0548 and TP0705 to make sure that the results was still satisfying. Generally, there were some more unspecific PCR products after the PCR2 than PCR1, but overall, the results was satisfying. Sanger sequencing was still performed for all three loci, with the expectation that these unspecific products will not interfere as the internal primers are being used and hopefully eliminate it.

The results from the Sanger sequencing turned out good with high quality of the sequences. The Sequencher software was used to trim off the low quality of the ends and editing the sequences in the software if needed. The sequences for all three loci were paste into PubMLST database to obtain an allelic profile.

This optimization of nested PCR turned out good for TPA DNA control. Now, the clinical samples needed to be tested to decide of this optimization can be used, or the nested PCR needed to be adjusted. To minimize the volume of DNA added for the nested PCR, 10 µl of DNA was therefore decided to be used. Generally, the PCR products produced in the nested PCR were good and had the decided length of PCR product. For PCR1, several unspecific PCR products were produced, compared to TPA DNA, but when continued to PCR2, almost all the unspecific products disappeared. For the nested PCR, it seemed that all clinical samples had more unspecified PCR products

after PCR1, but less unspecific products after PCR2. The PCR products, after PCR2, are the most important when sequencing. Therefore, no more optimization was needed.

5 Conclusion

Molecular characterization by MLST is important for understanding the epidemiology of the different TPA allelic profiles and give a better understanding of syphilis epidemiology. The combination of macrolide antibiotic resistance and the molecular genotyping of TPA is important for the surveillance of antibiotic resistant strains. Since these are the first ever study of macrolide resistance and molecular characterization done in TPA in Norway, we do not have any previous data for comparison. Specimen sampling during travel restrictions under Covid-19 pandemic reflects a part of inland syphilis transmission and can become an important starting point for future monitoring.

The primers and probe for the macrolide resistance in TPA, designed by TIB Molbiol, worked well when using the same protocol as for macrolide resistance in *Mycoplasma genitalium*. As well as the three loci MLST, established by Grillova et al. (2018), was easy to be performed. Using PrimeSTAR® GXL Premix by Takara Bio makes it easier as this Premix consists of all the elements needed for the nested PCR except for primers. Using the PubMLST database after Sanger sequencing makes it easy to identify the different loci and obtain the sequence type.

The optimization of macrolide resistance and nested PCR in TPA, reveals the first molecular characterization of TPA in Norway. As a national reference laboratory, the result from this study is the first step for having more understanding of the syphilis epidemiology in Norway. The result from this study showed similarity to situation of TPA in other parts of the world, especially in Northern America and Europe, but less compared to Asia. Molecular typing by MLST, reveals nine different sequence types in Norway including three novels sequence types, and macrolide resistance are proved to be present in all the typable samples.

5.1 Future perspective

Molecular characterization by MLST was in this study done by nested PCR. If this analysis could be shortened down to a single PCR, using only the internal primers, the protocol would be more effective. More time and resources are needed for this potentially new PCR, and new optimization might be needed.

For this study, only syphilis positive samples, collected from October 2020 to September 2022, were analyzed. Most of these samples are from MSM patient located in the Oslo area. For the future, the collection of syphilis positive samples should come from several area in Norway to give a more accurate overview of the molecular subtypes and macrolide resistance of syphilis. Having more clinical information regarding traveling, and how the infection was acquired, might give us a better understanding of the national syphilis epidemiology.

References

Abushaheen, M. A., Muzaheed, Fatani, A. J., Alosaimi, M., Mansy, W., George, M., Acharya, S., Rathod, S., Divakar, D. D., Jhugroo, C., Vellappally, S., Khan, A. A., Shaik, J., & Jhugroo, P. (2020). Antimicrobial resistance, mechanisms and its clinical significance. *Dis Mon*, *66*(6), 100971. https://doi.org/10.1016/j.disamonth.2020.100971

Aminov, R. I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Front Microbiol*, *1*, 134. https://doi.org/10.3389/fmicb.2010.00134

AppliedBiosystems. (2010). *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide*. Applied Biosystems. Retrieved 15 March 2023 from https://tools.thermofisher.com/content/sfs/manuals/4401661.pdf

AppliedBiosystems. (2012). *Applied Biosystems® 3130/3130xl Genetic Analyzers*. Applied Biosystems. Retrieved 15 March 2023 from https://tools.thermofisher.com/content/sfs/manuals/4477796.pdf

Belkacemi, S., Bou Khalil, J., Ominami, Y., Hisada, A., Fontanini, A., Caputo, A., Levasseur, A., Lagier, J. C., Khelaifia, S., & Raoult, D. (2019). Passive Filtration, Rapid Scanning Electron Microscopy, and Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for *Treponema* Culture and Identification from the Oral Cavity. *J Clin Microbiol*, *57*(10). https://doi.org/10.1128/JCM.00517-19

Benoit, S., Posey, J. E., Chenoweth, M. R., & Gherardini, F. C. (2001). *Treponema pallidum* 3-phosphoglycerate mutase is a heat-labile enzyme that may limit the maximum growth temperature for the spirochete. *J Bacteriol*, *183*(16), 4702-4708. https://doi.org/10.1128/JB.183.16.4702-4708.2001

Beymer, M. R., DeVost, M. A., Weiss, R. E., Dierst-Davies, R., Shover, C. L., Landovitz, R. J., Beniasians, C., Talan, A. J., Flynn, R. P., Krysiak, R., McLaughlin, K., & Bolan, R. K. (2018). Does HIV pre-exposure prophylaxis use lead to a higher incidence of sexually transmitted infections? A case-crossover study of men who have sex with men in Los Angeles, California. *Sex Transm Infect*, *94*(6), 457-462. https://doi.org/10.1136/sextrans-2017-053377

Bircher, A. J. (2014). Drug hypersensitivity. *Chem Immunol Allergy*, *100*, 120-131. https://doi.org/10.1159/000358617

Brinkman, M. B., McGill, M. A., Pettersson, J., Rogers, A., Matejkova, P., Smajs, D., Weinstock, G. M., Norris, S. J., & Palzkill, T. (2008). A novel *Treponema pallidum* antigen, TP0136, is an outer membrane protein that binds human fibronectin. *Infect Immun*, *76*(5), 1848-1857. https://doi.org/10.1128/IAI.01424-07

Centurion-Lara, A., Molini, B. J., Godornes, C., Sun, E., Hevner, K., Van Voorhis, W. C., & Lukehart, S. A. (2006). Molecular differentiation of *Treponema pallidum* subspecies. *J Clin Microbiol*, *44*(9), 3377-3380. https://doi.org/10.1128/JCM.00784-06

Chen, C. Y., Chi, K. H., Pillay, A., Nachamkin, E., Su, J. R., & Ballard, R. C. (2013). Detection of the A2058G and A2059G 23S rRNA gene point mutations

associated with azithromycin resistance in *Treponema pallidum* by use of a TaqMan real-time multiplex PCR assay. *J Clin Microbiol*, *51*(3), 908-913. https://doi.org/10.1128/JCM.02770-12

Cox, D. L., Luthra, A., Dunham-Ems, S., Desrosiers, D. C., Salazar, J. C., Caimano, M. J., & Radolf, J. D. (2010). Surface immunolabeling and consensus computational framework to identify candidate rare outer membrane proteins of *Treponema pallidum. Infect Immun*, *78*(12), 5178-5194. https://doi.org/10.1128/IAI.00834-10

Crossley, B. M., Bai, J., Glaser, A., Maes, R., Porter, E., Killian, M. L., Clement, T., & Toohey-Kurth, K. (2020). Guidelines for Sanger sequencing and molecular assay monitoring. *J Vet Diagn Invest*, *32*(6), 767-775. https://doi.org/10.1177/1040638720905833

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev*, *74*(3), 417-433. https://doi.org/10.1128/MMBR.00016-10

Edmondson, D. G., Hu, B., & Norris, S. J. (2018). Long-Term In Vitro Culture of the Syphilis Spirochete *Treponema pallidum* subsp. *pallidum. mBio*, *9*(3). https://doi.org/10.1128/mBio.01153-18

Elgalib, A., Lau, R., Al-Habsi, Z., Shah, S., Al-Rawahi, B., Memish, Z. A., Lee, S. S., & Al-Abri, S. (2023). Elimination of mother-to-child transmission of HIV, syphilis and viral hepatitis B: A call for renewed global focus. *Int J Infect Dis*, *127*, 33-35. https://doi.org/10.1016/j.ijid.2022.11.031

Fernandez-Naval, C., Arando, M., Espasa, M., Anton, A., Fernandez-Huerta, M., Silgado, A., Pinatar, C., Zarzuela, F., Gonzalez-Lopez, J. J., Serra-Pladevall, J., Sulleiro, E., Pumarola, T., Vall-Mayans, M., & Esperalba, J. (2021). Multilocus sequence typing of *Treponema pallidum* subsp. *pallidum* in Barcelona. *Future Microbiol*, *16*, 967-976. https://doi.org/10.2217/fmb-2021-0037

Fernandez-Naval, C., Arando, M., Espasa, M., Anton, A., Gimferrer, L., Pinana, M., Fernandez-Huerta, M., Gonzalez-Lopez, J. J., Serra-Pladevall, J., Pumarola, T., Vall-Mayans, M., & Esperalba, J. (2019). Novel tp0548 Sequence-Type of *Treponema pallidum* Identified in Barcelona, Spain. *Sex Transm Dis*, *46*(5), e50-e52. https://doi.org/10.1097/OLQ.0000000000000947

FHI. (2010a, 3 Janueary 2019). *Mykoplasmainfeksjon, genital*. Folkehelseinstituttet. Retrieved 23 April 2023 from https://www.fhi.no/nettpub/smittevernveilederen//sykdommer-a-a/mykoplasmainfeksjon-genital/

FHI. (2010b, 7 April 2019). *Syfilis - veileder for helsepersonell*. Folkehelseinstituttet. Retrieved 16 January 2023 from https://www.fhi.no/nettpub/smittevernveilederen/sykdommer-a-a/syfilis---veileder-for-helsepersone/

FHI. (2012, 29 June 2022). *Meldingspliktige sykdommer i MSIS*. Retrieved 16 January 2023 from https://www.fhi.no/hn/helseregistre-og-registre/msis/meldingspliktige-sykdommer-i-msis/

FHI. (2017, 24 January 2022). *Om Meldingssystem for smittsomme sykdommer (MSIS)*. Folkehelseinstituttet. Retrieved 16 January 2023 from

https://www.fhi.no/hn/helseregistre-og-registre/msis/meldesystemet-for-smittsomme-sykdommer/
FHI. (2023). *Årsrapport 2022. Overvåkning av seksuelt overførbare infeksjoner*. FHI. Retrieved 27 April 2023 from https://www.fhi.no/contentassets/3e70076e6e704b27843e26cc33c4214e/soi_arsrapport_2022_endelig.pdf
Flores, J. A., Vargas, S. K., Leon, S. R., Perez, D. G., Ramos, L. B., Chow, J., Konda, K. A., Calvo, G. M., Salvatierra, H. J., Klausner, J. D., & Caceres, C. F. (2016). *Treponema pallidum pallidum* Genotypes and Macrolide Resistance Status in Syphilitic Lesions among Patients at 2 Sexually Transmitted Infection Clinics in Lima, Peru. *Sex Transm Dis*, *43*(7), 465-466. https://doi.org/10.1097/OLQ.0000000000000465
Forrestel, A. K., Kovarik, C. L., & Katz, K. A. (2020). Sexually acquired syphilis: Laboratory diagnosis, management, and prevention. *J Am Acad Dermatol*, *82*(1), 17-28. https://doi.org/10.1016/j.jaad.2019.02.074
Francisco, A. P., Bugalho, M., Ramirez, M., & Carrico, J. A. (2009). Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. *BMC Bioinformatics*, *10*, 152. https://doi.org/10.1186/1471-2105-10-152
Fraser, C. M., Norris, S. J., Weinstock, G. M., White, O., Sutton, G. G., Dodson, R., Gwinn, M., Hickey, E. K., Clayton, R., Ketchum, K. A., Sodergren, E., Hardham, J. M., McLeod, M. P., Salzberg, S., Peterson, J., Khalak, H., Richardson, D., Howell, J. K., Chidambaram, M., . . . Venter, J. C. (1998). Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science*, *281*(5375), 375-388. https://doi.org/10.1126/science.281.5375.375
French, P. (2007). Syphilis. *BMJ*, *334*(7585), 143-147. https://doi.org/10.1136/bmj.39085.518148.BE
Galindo-Murillo, R., & Cheatham, T. E. (2021). Ethidium bromide interactions with DNA: an exploration of a classic DNA-ligand complex with unbiased molecular dynamics simulations. *Nucleic Acids Res*, *49*(7), 3735-3747. https://doi.org/10.1093/nar/gkab143
Gallo Vaulet, L., Grillova, L., Mikalova, L., Casco, R., Rodriguez Fermepin, M., Pando, M. A., & Smajs, D. (2017). Molecular typing of *Treponema pallidum* isolates from Buenos Aires, Argentina: Frequent Nichols-like isolates and low levels of macrolide resistance. *PLoS One*, *12*(2), e0172905. https://doi.org/10.1371/journal.pone.0172905
Garibyan, L., & Avashia, N. (2013). Polymerase chain reaction. *J Invest Dermatol*, *133*(3), 1-4. https://doi.org/10.1038/jid.2013.1
Ghanem, K. G., Ram, S., & Rice, P. A. (2020). The Modern Epidemic of Syphilis. *N Engl J Med*, *382*(9), 845-854. https://doi.org/10.1056/NEJMra1901593
Giacani, L., & Lukehart, S. A. (2014). The endemic treponematoses. *Clin Microbiol Rev*, *27*(1), 89-115. https://doi.org/10.1128/CMR.00070-13
Green, M. R., & Sambrook, J. (2018). Touchdown Polymerase Chain Reaction (PCR). *Cold Spring Harb Protoc*, *2018*(5). https://doi.org/10.1101/pdb.prot095133

Green, M. R., & Sambrook, J. (2019). Nested Polymerase Chain Reaction (PCR). *Cold Spring Harb Protoc*, *2019*(2). https://doi.org/10.1101/pdb.prot095182

Grillova, L., Bawa, T., Mikalova, L., Gayet-Ageron, A., Nieselt, K., Strouhal, M., Sednaoui, P., Ferry, T., Cavassini, M., Lautenschlager, S., Dutly, F., Pla-Diaz, M., Krutzen, M., Gonzalez-Candelas, F., Bagheri, H. C., Smajs, D., Arora, N., & Bosshard, P. P. (2018). Molecular characterization of *Treponema pallidum* subsp. *pallidum* in Switzerland and France with a new multilocus sequence typing scheme. *PLoS One*, *13*(7), e0200773. https://doi.org/10.1371/journal.pone.0200773

Grillova, L., Jolley, K., Smajs, D., & Picardeau, M. (2019). A public database for the new MLST scheme for *Treponema pallidum* subsp. *pallidum*: surveillance and epidemiology of the causative agent of syphilis. *PeerJ*, *6*, e6182. https://doi.org/10.7717/peerj.6182

Grillova, L., Oppelt, J., Mikalova, L., Novakova, M., Giacani, L., Niesnerova, A., Noda, A. A., Mechaly, A. E., Pospisilova, P., Cejkova, D., Grange, P. A., Dupin, N., Strnadel, R., Chen, M., Denham, I., Arora, N., Picardeau, M., Weston, C., Forsyth, R. A., & Smajs, D. (2019). Directly Sequenced Genomes of Contemporary Strains of Syphilis Reveal Recombination-Driven Diversity in Genes Encoding Predicted Surface-Exposed Antigens. *Front Microbiol*, *10*, 1691. https://doi.org/10.3389/fmicb.2019.01691

Grillova, L., Petrosova, H., Mikalova, L., Strnadel, R., Dastychova, E., Kuklova, I., Kojanova, M., Kreidlova, M., Vanousova, D., Hercogova, J., Prochazka, P., Zakoucka, H., Krchnakova, A., Vasku, V., & Smajs, D. (2014). Molecular typing of *Treponema pallidum* in the Czech Republic during 2011 to 2013: increased prevalence of identified genotypes and of isolates with macrolide resistance. *J Clin Microbiol*, *52*(10), 3693-3700. https://doi.org/10.1128/JCM.01292-14

Grimes, M., Sahi, S. K., Godornes, B. C., Tantalo, L. C., Roberts, N., Bostick, D., Marra, C. M., & Lukehart, S. A. (2012). Two mutations associated with macrolide resistance in *Treponema pallidum*: increasing prevalence and correlation with molecular strain type in Seattle, Washington. *Sex Transm Dis*, *39*(12), 954-958. https://doi.org/10.1097/OLQ.0b013e31826ae7a8

Helsedirektoratet. (2020, 12 August 2020). *Test deg for kjønnssykdommer gratis*. Retrieved 10 April 2023 from https://www.helsenorge.no/sykdom/kjonnssykdommer/test-deg-for-kjonnssykdommer-gratis/

Henao-Martinez, A. F., & Johnson, S. C. (2014). Diagnostic tests for syphilis: New tests and new algorithms. *Neurol Clin Pract*, *4*(2), 114-122. https://doi.org/10.1212/01.CPJ.0000435752.17621.48

Ho, E. L., & Lukehart, S. A. (2011). Syphilis: using modern approaches to understand an old disease. *J Clin Invest*, *121*(12), 4584-4592. https://doi.org/10.1172/JCI57173

Ibarz Pavon, A. B., & Maiden, M. C. (2009). Multilocus sequence typing. *Methods Mol Biol*, *551*, 129-140. https://doi.org/10.1007/978-1-60327-999-4_11

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. *Interdiscip Toxicol*, *7*(2), 60-72. https://doi.org/10.2478/intox-2014-0009

Janier, M., Unemo, M., Dupin, N., Tiplica, G. S., Potocnik, M., & Patel, R. (2021). 2020 European guideline on the management of syphilis. *J Eur Acad Dermatol Venereol*, *35*(3), 574-588. https://doi.org/10.1111/jdv.16946

Jolley, K. A., Bray, J. E., & Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res*, *3*, 124. https://doi.org/10.12688/wellcomeopenres.14826.1

Katz, K. A., Pillay, A., Ahrens, K., Kohn, R. P., Hermanstyne, K., Bernstein, K. T., Ballard, R. C., & Klausner, J. D. (2010). Molecular epidemiology of syphilis--San Francisco, 2004-2007. *Sex Transm Dis*, *37*(10), 660-663. https://doi.org/10.1097/OLQ.0b013e3181e1a77a

Kazadi, W. M., Asiedu, K. B., Agana, N., & Mitja, O. (2014). Epidemiology of yaws: an update. *Clin Epidemiol*, *6*, 119-128. https://doi.org/10.2147/CLEP.S44553

Ke, W., Molini, B. J., Lukehart, S. A., & Giacani, L. (2015). *Treponema pallidum* subsp. *pallidum* TP0136 protein is heterogeneous among isolates and binds cellular and plasma fibronectin via its NH2-terminal end. *PLoS Negl Trop Dis*, *9*(3), e0003662. https://doi.org/10.1371/journal.pntd.0003662

Khandelwal, G., & Bhyravabhotla, J. (2010). A phenomenological model for predicting melting temperatures of DNA sequences. *PLoS One*, *5*(8), e12433. https://doi.org/10.1371/journal.pone.0012433

Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I., & Khorana, H. G. (1971). Studies on polynucleotides. XCVI. Repair replications of short synthetic DNA's as catalyzed by DNA polymerases. *J Mol Biol*, *56*(2), 341-361. https://doi.org/10.1016/0022-2836(71)90469-4

Klaassen, C. H., van Aarssen, Y. A., & van der Stappen, J. W. (2008). Improved real-time detection of the H63D and S65C mutations associated with hereditary hemochromatosis using a SimpleProbe assay format. *Clin Chem Lab Med*, *46*(7), 985-986. https://doi.org/10.1515/CCLM.2008.197

Kojima, N., & Klausner, J. D. (2018). An Update on the Global Epidemiology of Syphilis. *Curr Epidemiol Rep*, *5*(1), 24-38. https://doi.org/10.1007/s40471-018-0138-z

Korbie, D. J., & Mattick, J. S. (2008). Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nat Protoc*, *3*(9), 1452-1456. https://doi.org/10.1038/nprot.2008.133

Kou, C. X., Wang, Q. Q., Zhang, J. P., Liu, J. Q., Zhang, H. N., & Zhang, R. L. (2021). A Novel *tp0548* Gene Type of *Treponema pallidum* Identified in Nanjing, China: Case Report and Review of Literature. *Sex Transm Dis*, *48*(5), 357-361. https://doi.org/10.1097/OLQ.0000000000001320

Lafond, R. E., & Lukehart, S. A. (2006). Biological basis for syphilis. *Clin Microbiol Rev*, *19*(1), 29-49. https://doi.org/10.1128/CMR.19.1.29-49.2006

Larsson, D. G. J., & Flach, C. F. (2022). Antibiotic resistance in the environment. *Nat Rev Microbiol*, *20*(5), 257-269. https://doi.org/10.1038/s41579-021-00649-x

Lee, P. Y., Costumbrado, J., Hsu, C. Y., & Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. *J Vis Exp*(62). https://doi.org/10.3791/3923

Li, H. Y., Qu, H. Q., Wang, X. M., Zhang, Y. J., & Zhang, F. R. (2018). A meta-analysis of the efficacy of azithromycin and benzathine penicillin in early syphilis. *Tropical Journal of Pharmaceutical Research*, *17*(2), 345-350. https://doi.org/10.4314/tjpr.v17i2.21

Li, Y., & Jiang, G. (2020). Azithromycin vs penicillin G benzathine for early syphilis: A meta-analysis of randomized controlled trials. *Dermatol Ther*, *33*(6), e14025. https://doi.org/10.1111/dth.14025

Liu, D., He, S. M., Zhu, X. Z., Liu, L. L., Lin, L. R., Niu, J. J., & Yang, T. C. (2020). Molecular Characterization Based on MLST and ECDC Typing Schemes and Antibiotic Resistance Analyses of *Treponema pallidum* subsp. *pallidum* in Xiamen, China. *Front Cell Infect Microbiol*, *10*, 618747. https://doi.org/10.3389/fcimb.2020.618747

Liu, J., Howell, J. K., Bradley, S. D., Zheng, Y., Zhou, Z. H., & Norris, S. J. (2010). Cellular architecture of *Treponema pallidum*: novel flagellum, periplasmic cone, and cell envelope as revealed by cryo electron tomography. *J Mol Biol*, *403*(4), 546-561. https://doi.org/10.1016/j.jmb.2010.09.020

Lorenz, T. C. (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *J Vis Exp*(63), e3998. https://doi.org/10.3791/3998

Martinez, J. L. (2014). General principles of antibiotic resistance in bacteria. *Drug Discov Today Technol*, *11*, 33-39. https://doi.org/10.1016/j.ddtec.2014.02.001

Mikalova, L., Grillova, L., Osbak, K., Strouhal, M., Kenyon, C., Crucitti, T., & Smajs, D. (2017). Molecular Typing of Syphilis-Causing Strains Among Human Immunodeficiency Virus-Positive Patients in Antwerp, Belgium. *Sex Transm Dis*, *44*(6), 376-379. https://doi.org/10.1097/OLQ.0000000000000600

Mikalova, L., Strouhal, M., Grillova, L., & Smajs, D. (2014). The molecular typing data of recently identified subtype 11q/j of *Treponema pallidum* subsp. *pallidum* suggest imported case of yaws. *Sex Transm Dis*, *41*(9), 552-553. https://doi.org/10.1097/OLQ.0000000000000165

Molini, B. J., Tantalo, L. C., Sahi, S. K., Rodriguez, V. I., Brandt, S. L., Fernandez, M. C., Godornes, C. B., Marra, C. M., & Lukehart, S. A. (2016). Macrolide Resistance in *Treponema pallidum* Correlates With 23S rDNA Mutations in Recently Isolated Clinical Strains. *Sex Transm Dis*, *43*(9), 579-583. https://doi.org/10.1097/OLQ.0000000000000486

Morando, N., Vrbova, E., Melgar, A., Rabinovich, R. D., Smajs, D., & Pando, M. A. (2022). High frequency of Nichols-like strains and increased levels of macrolide resistance in *Treponema pallidum* in clinical samples from Buenos Aires, Argentina. *Sci Rep*, *12*(1), 16339. https://doi.org/10.1038/s41598-022-20410-5

Muldoon, E. G., Walsh, A., Crowley, B., & Mulcahy, F. (2012). *Treponema pallidum* azithromycin resistance in Dublin, Ireland. *Sex Transm Dis*, *39*(10), 784-786. https://doi.org/10.1097/OLQ.0b013e318269995f

Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiol Spectr*, *4*(2). https://doi.org/10.1128/microbiolspec.VMBF-0016-2015

NCBI. (2022). *Treponema pallidum subsp.pallidum str. Nichols, complete sequence*. NCBI. Retrieved 29 April 2023 from https://www.ncbi.nlm.nih.gov/nuccore/NC_000919.1

Nechvatal, L., Petrosova, H., Grillova, L., Pospisilova, P., Mikalova, L., Strnadel, R., Kuklova, I., Kojanova, M., Kreidlova, M., Vanousova, D., Prochazka, P., Zakoucka, H., Krchnakova, A., & Smajs, D. (2014). Syphilis-causing strains belong to separate SS14-like or Nichols-like groups as defined by multilocus analysis of 19 *Treponema pallidum* strains. *Int J Med Microbiol*, *304*(5-6), 645-653. https://doi.org/10.1016/j.ijmm.2014.04.007

Noda, A. A., Matos, N., Blanco, O., Rodriguez, I., & Stamm, L. V. (2016). First Report of the 23S rRNA Gene A2058G Point Mutation Associated With Macrolide Resistance in *Treponema pallidum* From Syphilis Patients in Cuba. *Sex Transm Dis*, *43*(5), 332-334. https://doi.org/10.1097/OLQ.0000000000000440

Olsen, A. O. (2013, 10 November 2022). *Om syfilis*. Folkehelseinstituttet. Retrieved 5 January 2023 from https://www.fhi.no/sv/smittsomme-sykdommer/seksualitet-og-helse/diag/om-syfilis/

OUS. (2020, 10 January 2023). *Behandling med Azitromax*. Oslo University Hospital. Retrieved 12 May 2023 from https://oslo-universitetssykehus.no/fag-og-forskning/nasjonale-og-regionale-tjenester/nasjonal-kompetansetjeneste-for-seksuelt-overforte-infeksjoner/behandling-med-azitromax

Peeling, R. W., Mabey, D., Kamb, M. L., Chen, X. S., Radolf, J. D., & Benzaken, A. S. (2017). Syphilis. *Nat Rev Dis Primers*, *3*, 17073. https://doi.org/10.1038/nrdp.2017.73

Peng, R. R., Wang, A. L., Li, J., Tucker, J. D., Yin, Y. P., & Chen, X. S. (2011). Molecular typing of *Treponema pallidum*: a systematic review and meta-analysis. *PLoS Negl Trop Dis*, *5*(11), e1273. https://doi.org/10.1371/journal.pntd.0001273

Perine, P. L., Hopkins, D. R., Niemel, P. L. A., St. John, R., Causse, G., Antal, G. M., & World Health, O. (1984). Handbook of endemic treponematoses : yaws, endemic syphilis and pinta / Peter L. Perine and Donald R. Hopkins, Paul L. A. Niemel, Ronald K. St. John, Georges Causse and G. M. Antal. In. Geneva: World Health Organization.

Petrarca, R. (2021). *The Old World and the New World*. Study.com. Retrieved 17 January 2023 from https://study.com/academy/lesson/the-old-world-and-the-new-world.html

Petrosova, H., Pospisilova, P., Strouhal, M., Cejkova, D., Zobanikova, M., Mikalova, L., Sodergren, E., Weinstock, G. M., & Smajs, D. (2013). Resequencing of *Treponema pallidum* ssp. *pallidum* strains Nichols and SS14: correction of sequencing errors resulted in increased separation of syphilis treponeme subclusters. *PLoS One*, *8*(9), e74319. https://doi.org/10.1371/journal.pone.0074319

Plagens-Rotman, K., Jarzabek-Bielecka, G., Merks, P., Kedzia, W., & Czarnecka-Operacz, M. (2021). Syphilis: then and now. *Postepy Dermatol Alergol*, *38*(4), 550-554. https://doi.org/10.5114/ada.2021.108930

Qiagen. (2015). *EZ1® DSP Virus Kit Handbook*. Qiagen. Retrieved 13 March 2023 from https://www.qiagen.com/kr/resources/download.aspx?id=6185be15-b095-4c9c-b1ce-9915300b89bf&lang=en

Qiagen. (2017a). *EZ1 Advanced XL User Manual* Qiagen. Retrieved 13 March 2023 from https://www.qiagen.com/au/resources/download.aspx?id=c9ecd500-147b-4a8e-ae71-3dc86cd3d17a&lang=en

Qiagen. (2017b). *QIAxcel Advanced User Manual*. Qiagen. Retrieved 15 March 2023 from https://www.qiagen.com/us/resources/download.aspx?id=e3edf734-1e5a-4ebf-957e-a35e120d6290&lang=en

Radolf, J. D. (1996). *Treponema*. In th & S. Baron (Eds.), *Medical Microbiology*. https://www.ncbi.nlm.nih.gov/pubmed/21413263

Radolf, J. D., Deka, R. K., Anand, A., Smajs, D., Norgard, M. V., & Yang, X. F. (2016). *Treponema pallidum*, the syphilis spirochete: making a living as a stealth pathogen. *Nat Rev Microbiol*, *14*(12), 744-759. https://doi.org/10.1038/nrmicro.2016.141

Regjeringen. (2020-2022). *Tidslinje: myndighetenes håndtering av koronasituasjonen*. Regjeringen.no. Retrieved 30 April 2023 from https://www.regjeringen.no/no/tema/Koronasituasjonen/tidslinje-koronaviruset/id2692402/

Ribeiro-Goncalves, B., Francisco, A. P., Vaz, C., Ramirez, M., & Carrico, J. A. (2016). PHYLOViZ Online: web-based tool for visualization, phylogenetic inference, analysis and sharing of minimum spanning trees. *Nucleic Acids Res*, *44*(W1), W246-251. https://doi.org/10.1093/nar/gkw359

Roche. (2021). *MagNA Pure 96 System Operator's Manual 4.1 SW 3.2 - Feb 2021-EN*. Roche Diagnostics. Retrieved 13 March 2023 from https://elabdoc-prod.roche.com/eLD/api/downloads/0a5e5533-8f7a-ec11-0f91-005056a71a5d?countryIsoCode=pi

Rosana, Y., Yasmon, A., Indriatmi, W., Effendi, I., Kusumawati, R. L., Rowawi, R., Sudigdoadi, S., Pradini, G. W., Wiraguna, A., Puspawati, N. M. D., Kusumawaty, M., & Massi, M. N. (2022). Detection of A2058G and A2059G on the 23S rRNA Gene by Multiplex Nested PCR to Identify *Treponema pallidum* Resistance to Azithromycin in Indonesia. *Jpn J Infect Dis*, *75*(4), 355-360. https://doi.org/10.7883/yoken.JJID.2021.738

Rothschild, B. M. (2005). History of syphilis. *Clin Infect Dis*, *40*(10), 1454-1463. https://doi.org/10.1086/429626

Satyaputra, F., Hendry, S., Braddick, M., Sivabalan, P., & Norton, R. (2021). The Laboratory Diagnosis of Syphilis. *J Clin Microbiol*, *59*(10), e0010021. https://doi.org/10.1128/JCM.00100-21

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*, *3*(6), 1101-1108. https://doi.org/10.1038/nprot.2008.73

Seale, A., Broutet, N., & Narasimhan, M. (2017). Assessing process, content, and politics in developing the global health sector strategy on sexually transmitted infections 2016-2021: Implementation opportunities for policymakers. *PLoS Med*, *14*(6), e1002330. https://doi.org/10.1371/journal.pmed.1002330

Sefton, A. M. (2001). The Great Pox that was...syphilis. *J Appl Microbiol*, *91*(4), 592-596. https://doi.org/10.1046/j.1365-2672.2001.01494.x

Sequencher® version 5.4.6 DNA sequence analysis software. (2023). Gene Codes Corporation. Retrieved 4 April 2023 from http://www.genecodes.com

Shields, M., Guy, R. J., Jeoffreys, N. J., Finlayson, R. J., & Donovan, B. (2012). A longitudinal evaluation of *Treponema pallidum* PCR testing in early syphilis. *BMC Infect Dis*, *12*, 353. https://doi.org/10.1186/1471-2334-12-353

Singh, C., & Roy-Chowdhuri, S. (2016). Quantitative Real-Time PCR: Recent Advances. *Methods Mol Biol*, *1392*, 161-176. https://doi.org/10.1007/978-1-4939-3360-0_15

Sjøberg, N. O. (2013). *Molekylær genetikk Genteknologi - humant DNA* (5th ed.). Forlaget Vett & Viten.

Smajs, D., Norris, S. J., & Weinstock, G. M. (2012). Genetic diversity in *Treponema pallidum*: implications for pathogenesis, evolution and molecular diagnostics of syphilis and yaws. *Infect Genet Evol*, *12*(2), 191-202. https://doi.org/10.1016/j.meegid.2011.12.001

Smajs, D., Pastekova, L., & Grillova, L. (2015). Macrolide Resistance in the Syphilis Spirochete, *Treponema pallidum* ssp. *pallidum*: Can We Also Expect Macrolide-Resistant Yaws Strains? *Am J Trop Med Hyg*, *93*(4), 678-683. https://doi.org/10.4269/ajtmh.15-0316

Srinivas, P. R. (2019). Introduction to Protein Electrophoresis. *Methods Mol Biol*, *1855*, 23-29. https://doi.org/10.1007/978-1-4939-8793-1_2

Stamm, L. V. (2010). Global challenge of antibiotic-resistant *Treponema pallidum*. *Antimicrob Agents Chemother*, *54*(2), 583-589. https://doi.org/10.1128/AAC.01095-09

Stamm, L. V. (2015). Syphilis: antibiotic treatment and resistance. *Epidemiol Infect*, *143*(8), 1567-1574. https://doi.org/10.1017/S0950268814002830

Stamm, L. V. (2016). Syphilis: Re-emergence of an old foe. *Microb Cell*, *3*(9), 363-370. https://doi.org/10.15698/mic2016.09.523

Stamm, L. V., & Bergen, H. L. (2000). A point mutation associated with bacterial macrolide resistance is present in both 23S rRNA genes of an erythromycin-resistant *Treponema pallidum* clinical isolate. *Antimicrob Agents Chemother*, *44*(3), 806-807. https://doi.org/10.1128/AAC.44.3.806-807.2000

Stoltey, J. E., & Cohen, S. E. (2015). Syphilis transmission: a review of the current evidence. *Sex Health*, *12*(2), 103-109. https://doi.org/10.1071/SH14174

Sweeney, E. L., Lowry, K., Seel, M., Rahimi, F., Langton-Lockton, J., Bletchly, C., Nimmo, G. R., & Whiley, D. M. (2022). Two *Treponema pallidum* strains account for the majority of syphilis infections, including among females, in Queensland, Australia. *Commun Dis Intell (2018)*, *46*. https://doi.org/10.33321/cdi.2022.46.26

Tampa, M., Sarbu, I., Matei, C., Benea, V., & Georgescu, S. R. (2014). Brief history of syphilis. *J Med Life*, *7*(1), 4-10. https://www.ncbi.nlm.nih.gov/pubmed/24653750

Taouk, M. L., Taiaroa, G., Pasricha, S., Herman, S., Chow, E. P. F., Azzatto, F., Zhang, B., Sia, C. M., Duchene, S., Lee, A., Higgins, N., Prestedge, J., Lee, Y. W., Thomson, N. R., Graves, B., Meumann, E., Gunathilake, M., Hocking, J. S., Bradshaw, C. S., . . . Williamson, D. A. (2022). Characterisation of *Treponema pallidum* lineages within the contemporary syphilis outbreak in Australia: a genomic epidemiological analysis. *Lancet Microbe*, *3*(6), e417-e426. https://doi.org/10.1016/S2666-5247(22)00035-0

Taylor, M. M., Aynalem, G., Olea, L. M., He, P., Smith, L. V., & Kerndt, P. R. (2008). A consequence of the syphilis epidemic among men who have sex with men (MSM): neurosyphilis in Los Angeles, 2001-2004. *Sex Transm Dis*, *35*(5), 430-434. https://doi.org/10.1097/OLQ.0b013e3181644b5e

Templeton, N. S. (1992). The polymerase chain reaction. History, methods, and applications. *Diagn Mol Pathol*, *1*(1), 58-72. https://doi.org/10.1097/00019606-199203000-00008

Thomas, D. D., Navab, M., Haake, D. A., Fogelman, A. M., Miller, J. N., & Lovett, M. A. (1988). *Treponema pallidum* invades intercellular junctions of endothelial cell monolayers. *Proc Natl Acad Sci U S A*, *85*(10), 3608-3612. https://doi.org/10.1073/pnas.85.10.3608

Thorburn, A. L. (1971). Fritz Richard Schaudinn, 1871-1906: protozoologist of syphilis. *Br J Vener Dis*, *47*(6), 459-461. https://doi.org/10.1136/sti.47.6.459

Tipple, C., McClure, M. O., & Taylor, G. P. (2011). High prevalence of macrolide resistant *Treponema pallidum* strains in a London centre. *Sex Transm Infect*, *87*(6), 486-488. https://doi.org/10.1136/sextrans-2011-050082

Traeger, M. W., Schroeder, S. E., Wright, E. J., Hellard, M. E., Cornelisse, V. J., Doyle, J. S., & Stoove, M. A. (2018). Effects of Pre-exposure Prophylaxis for the Prevention of Human Immunodeficiency Virus Infection on Sexual Risk Behavior in Men Who Have Sex With Men: A Systematic Review and Meta-analysis. *Clin Infect Dis*, *67*(5), 676-686. https://doi.org/10.1093/cid/ciy182

Van Damme, K., Behets, F., Ravelomanana, N., Godornes, C., Khan, M., Randrianasolo, B., Rabenja, N. L., Lukehart, S., Cohen, M., & Hook, E. (2009). Evaluation of azithromycin resistance in *Treponema pallidum* specimens from Madagascar. *Sex Transm Dis*, *36*(12), 775-776. https://doi.org/10.1097/OLQ.0b013e3181bd11dd

Vernon, G. (2019). Syphilis and Salvarsan. *Br J Gen Pract*, *69*(682), 246. https://doi.org/10.3399/bjgp19X702533

Vircell. *Amplirun® Treponema DNA Control*. Vircell. Retrieved 19 April 2023 from https://en.vircell.com/products/amplirun-treponema-dna-control/

WHO. (2016). *Global health sector strategy on Sexually Transmitted Infections, 2016-2021*. W. H. Organization. apps.who.int/iris/bitstream/handle/10665/246296/WHO-RHR-16.09-eng.pdf;jsessionid=E4D32CCE518F251732D5F81B63B01E48?sequence=1

WHO. (2020, 31 July). *Antibiotic resistance*. World Health Organization. Retrieved 19 March 2023 from https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance

WHO. (2021, 9 July). *New study highlights unacceptably high global prevalence of syphilis among men who have sex with men*. World Health Organization. Retrieved 4 January 2023 from https://www.who.int/news/item/09-07-2021-new-study-highlights-unacceptably-high-global-prevalence-of-syphilis-among-men-who-have-sex-with-men

WHO. (2023a, 3 February). *Congenital syphilis - Data by country*. World Health Organization. Retrieved 2 April 2023 from https://apps.who.int/gho/data/node.main.CONGENITALSYPHSTI?lang=en

WHO. (2023b). *Elimination of mother-to-child transmission of HIV, syphilis and hepatitis B*. World Health Organization. Retrieved 2 April 2023 from https://www.who.int/initiatives/triple-elimination-initiative-of-mother-to-child-transmission-of-hiv-syphilis-and-hepatitis-b

WHO. (2023c, 12 January 2023). *Yaws*. World Health Organization. Retrieved 27 March 2023 from https://www.who.int/new-room/fact-sheets/detail/yaws

Wu, H., Chang, S. Y., Lee, N. Y., Huang, W. C., Wu, B. R., Yang, C. J., Liang, S. H., Lee, C. H., Ko, W. C., Lin, H. H., Chen, Y. H., Liu, W. C., Su, Y. C., Hsieh, C. Y., Wu, P. Y., & Hung, C. C. (2012). Evaluation of macrolide resistance and enhanced molecular typing of *Treponema pallidum* in patients with syphilis in Taiwan: a prospective multicenter study. *J Clin Microbiol*, *50*(7), 2299-2304. https://doi.org/10.1128/JCM.00341-12

Xiao, Y., Liu, S., Liu, Z., Xie, Y., Jiang, C., Xu, M., Zhao, F., Zeng, T., Yu, J., & Wu, Y. (2016). Molecular Subtyping and Surveillance of Resistance Genes In *Treponema pallidum* DNA From Patients With Secondary and Latent Syphilis in Hunan, China. *Sex Transm Dis*, *43*(5), 310-316. https://doi.org/10.1097/OLQ.0000000000000445

Xu, M., Xie, Y., Zheng, K., Luo, H., Tan, M., Zhao, F., Zeng, T., & Wu, Y. (2021). Two Potential Syphilis Vaccine Candidates Inhibit Dissemination of *Treponema pallidum*. *Front Immunol*, *12*, 759474. https://doi.org/10.3389/fimmu.2021.759474

Zhou, C., Zhang, X., Zhang, W., Duan, J., & Zhao, F. (2019). PCR detection for syphilis diagnosis: Status and prospects. *J Clin Lab Anal*, *33*(5), e22890. https://doi.org/10.1002/jcla.22890

Zuckerman, M. K. (2016). More Harm than Healing? Investigating the Iatrogenic Effects of Mercury Treatment on Acquired Syphilis in Post-medieval London. *Open Archaeology*, *2*(1). https://doi.org/doi:10.1515/opar-2016-0003

Appendices

Appendix A – Amplirun® *Treponema pallidum* DNA Control

AMPLIRUN® *TREPONEMA PALLIDUM* DNA CONTROL

Complete genome: NC_000919.1

Kit contents:

1. VIRCELL TPA DNA CONTROL: Nichols strain, 700-2000 copies/µl once reconstituted. DNA quantification has been performed by real-time PCR.
2. VIRCELL CONTROL RECONSTITUTION SOLUTION: 500 µl of molecular biology grade water, DNase, RNase free.

Lot number	21MBC109001-C
Concentration, copies/µl	1100

Preparation of the reagents:

1. Tear the foil pouch containing VIRCELL DNA CONTROL.
2. Centrifuge VIRCELL DNA CONTROL 1 minute at 1000 g.
3. Add 100 µl of VIRCELL CONTROLL RECONSTITUTION SOLUTION and mix until completely reconstituted.
4. Shake with vortex for 30 seconds to dissolve and homogenize completely.

Appendix B – Lists of equipment, and other reagents

Table B.1 List of specific equipment and reagents needed for the different instruments. The equipment and reagents are listed in alphabetical order.

	Catalogue no.	Manufacturer
ABI 3130xl Genetic Analyzers		
Buffer with EDTA (10X), 25 ml	402824	Life Technologies
Buffer/water/water reservoir, 3130	628-0163	Life Technologies
Capillary Array 3130, 16 cap x 50 cm	4315930	Life Technologies
MicroAmp Adhesive film	4306311	Life Technologies
Optical 96-well plates barcoded, 20	4306737	Life Technologies
Plate base 96-well	4317237	Life Technologies
Plate retainer 96-well	4317237	Life Technologies
Plate septa 96-well	4315933	Life Technologies
POP-7 polymer for 3130, 3.5 ml	4363785	Life Technologies
Reservoir septa (20/pk)	4315932	Life Technologies
Seq Buffer 5x for BigDye Term kit, 1 ml	4336697	Life Technologies
UltraPure DNAse/RNAse-Free Distilled Water	GIBCO 10977-035	Life Technologies
ABI 3500xL Dx Genetic Analyzer		
3500xL Dx genetic analyzer 24-capillary array	4404688	Thermo Fisher Scientific
Anode buffer container (ABC) 3500 XL Dx Series	4393925	Thermo Fisher Scientific
Cathode buffer container 3500 Dx	4408258	Thermo Fisher Scientific
Conditioning reagent 3500 XL Dx series	4409543	Thermo Fisher Scientific
Hi-Di formamide 3500Dx series, 5 mL	4404307	Thermo Fisher Scientific
Invitrogen UltraPure distilled water	10977-035	Thermo Fisher Scientific
POP-7™ polymer 3500/3500xL Dx genetic analyzers 384 samples	4393709	Thermo Fisher Scientific
Septa 3500 Dx/3500xL genetic analyzer, 96-well	4410700	Thermo Fisher Scientific
Septa cathode buffer container 3500 Dx/3500xL Dx genetic analyzer	4410716	Thermo Fisher Scientific
Sequencing standard v3.1 3500 Dx series	4404310	Thermo Fisher Scientific
Sequencing standard v1.1 3500 Dx series	4462113	Thermo Fisher Scientific

EZ1 Advanced XL		
EZ1® DSP Virus Kit	62724	Qiagen
Light Cycle 480		
LightCycler® 480 Multiwell Plate 96	05220319001	Roche Diagnostics
LightCycler® 480 Sealing Foil	04729757001	Roche Diagnostics
MagNA Pure 96		
MagNA Pure 96 DNA and Viral NA Large Volume Kit	06374891001	Roche Diagnostics
MagNA Pure 96 Outout Plate	06241611001	Roche Diagnostics
MagNA Pure 96 Processing Cartridge	06241603001	Roche Diagnostics
MagNA Pure 96 System Fluid (External)	06640729001	Roche Diagnostics
MagNA Pure 96 Waste Cover	06541275001	Roche Diagnostics
MagNA Pure Tip 1000µL	06241620001	Roche Diagnostics
MiniAmp Thermal Cycler		
0.2 ml non-skirted 96-well PCR Plate	AB-0600	Thermo Fisher Scientific
QIAxcel Advanced		
QIAxcel DNA Screening Kit	929004	Qiagen
QX Alignment Marker 15 bp/3 kb (1.5 ml)	929522	Qiagen
QX Alignment Marker 15 bp/600 bp (1.5 ml)	929530	Qiagen
QX Nitrogen Cylinder	929705	Qiagen
SimpliAmp Thermal Cycler		
Ultra Clear qPCR Caps, strips of 8	AB-0866	Thermo Fisher Scientific
MicroAmp™ Optical 96-well Reaction plate with barcode	4306737	Applied Biosystems
BigDye™ Terminator v1.1 Cycle Sequencing Kit	4337450	Applied Biosystems

Table B.2 List of other equipment and reagents which are not instrument specific. Pipettes and pipette tips used in this thesis are not listed.

Reagents	Catalogue no./Lot no.	Manufacturer
Amplirun® *Treponema pallidum* DNA Control	MBC109 Lot no.: 21MBC109001-C	Vircell Microbiologists
Micro tube 1.5 ml, PP	72.692.005	Sarstedt
Micro tube 2 ml, PP	72.694.005	Sarstedt
PerfeCTa MultiPlex qPCR ToughMix	733-2323	Quanta Biosciences
UltraPure™ DNase/RNase-Free Distilled Water (PCR-water)	10977035	Invitrogen
TE-buffer pH 8.0 RNase-free	AM9849	Thermo Fisher Scientific

Table C.1 Primers used for nested PCR for the three loci TPA MLST with primer-sequences and the length of the PCR products (Grillova et al., 2018). The primers were ordered from TIB Molbiol.

Locus	External primers (5- 3) (EF/ER)	Length of PCR product	Internal primers (5-3) (IF/IR)	Length of PCR product
TP0136	AACCCGTTAGCGCCCAACAT*	1789 bp	AGTGTCTTCCTCGTCCGTTC*	1206 bp
	TCCCAGCTCAGCCGAATCTC**		CACGTGGTGGTGTCAAACTT**	
TP0548	TGGGGCACTAAACCGGAAGA*	1567 bp	GCGGTCCCTATGATATCGTGT*	1065 bp
	TACGGGCATTTGCGGATAGG**		GAGCCACTTCAGCCCTACTG**	
TP0705	GGTCTATATGCAGCCCTTCTTC*	1181 bp	TGCGGCTTATCCTGATGAATAG*	803 bp
	GCTTGAGAACGATACCGGATAC**		TATTCTGCGGCGTTGGATAG**	

*Forward primer (EF/IF). **Reverse primer (ER/IR).

Table C.2 Primer- and probe-sequences used for macrolide resistance. The melting temperature (Tm) for primers and probes are listed below. These sequences were designed and ordered from TIB Molbiol.

Primer/probe name	Sequence	Tm
TP23S_F*	CGCACGAATGGTGTAACG	56.2°C
TP23S_S*	TGGACACTGTCTCGACGC	55.7°C
TP23S_A**	ACCAAGTTCCAATAGTAAGCTACG	55.4°C
TP23S_R**	CTCTGTCTCCCACCTATACTACACA	54.9°C
TP23S_P_[A]***	CGGTTACCCATAGTXITAGACGGAAAGACCCC- -PH	69.1°C
TP23S_P_[A]-LNA***	CG+GTTAC+CCAT+AGTXITAGACGG+AAAGA+CCCC- -PH	76.4°C

*Forward primer. **Reverse primer. ***Probe.

Appendix D – Optimization of macrolide resistance and nested PCR

Macrolide resistance

The four different PCR-reaction mixes are showed in Table D.1 and Table D.2. The tables show the volume of the different reagents needed for one single sample, however in practice, the mixes were made in batches enough for 25 samples. The PCR- and melting point protocol used for this analysis is showed in Table D.3. For the first attempt of the PCR- and melting point analysis, the melting step temperature was first set to 75°C before ending up with 80°C as the final temperature.

Table D.1 PCR-mix 1 and PCR-mix 2. TPA macrolide-resistant PCR-reaction mixes for one PCR reaction with the primer-set TP23S_F and TP23S_A, and with the two different probes TP23S_P_[A] or TP23S_P_[A]_LNA (PCR-mix 1 and PCR-mix 2 respectively).

PCR-mix 1/PCR-mix 2	Volume (µl)	Stock concentration (µM)	Final concentration (µM)
PerfeCTa MultiPlex qPCR ToughMix	4	5X	1X
TP23S_F	0.02	100	0.1
TP23S_A	0.1	100	0.5
TP23S_P_[A] / TP23S_P_[A]_LNA	0.1	100	0.5
PCR-water	10.78		
DNA	5		
Total volume	20		

Table D.2 PCR-mix 3 and PCR-mix 4. TPA macrolide-resistant PCR-reaction mixes for one PCR reaction with the primer-set TP23S_S and TP23S_R, and with the two different probes TP23S_P_[A] or TP23S_P_[A]_LNA (PCR-mix 3 and PCR-mix 4 respectively).

PCR-mix 3/PCR-mix 4	Volume (µl)	Stock concentration (µM)	Final concentration (µM)
PerfeCTa MultiPlex qPCR ToughMix	4	5X	1X
TP23S_S	0.02	100	0.1
TP23S_R	0.1	100	0.5
TP23S_P_[A] / TP23S_P_[A]_LNA	0.1	100	0.5
PCR-water	10.78		
DNA	5		
Total volume	20		

Table D.3 PCR- and melting point protocol for macrolide resistance in TPA on LightCycler 480 from Roche Diagnostics.

Step		Temperature	Time (mm: ss)	Number of cycles
Initial denaturation		95°C	10:00	1
Denaturation		95°C	00:10	45
Annealing		60°C	00:10	
Extension		72°C	00:15	
Melting step	Denaturation	95°C	00:30	Slow increase in temperature to *75°C /80°C**, 1.05°C/sec
	Continuous acquisition	40°C	02:00	
		40°C – *75°C /80°C**	Continuous	
Final cooling		40°C	00:30	1

*Different temperature was tested in the melting step. **The final temperature we ended up with.

Nested PCR

Table D.4 is showing the volume of the different reagent using for the touchdown PCR. Different volume of DNA (volume of PCR-water had to be adjusted according to the volume of DNA) were tested out. The final volume of DNA using for this PCR is 10 µl, this leads to 12 µl of PCR-water.

Table D.4 The reaction mixes for one PCR reaction for the touchdown PCR, the first step of nested PCR. Different volume of DNA was tested.

	Volume (µl)	Stock concentration (µM)	Final concentration (µM)
Premix	25		
Primer EF*	1.5	10	0.3
Primer ER*	1.5	10	0.3
PCR-water**	21/20/17/12***/10/7		
DNA**	1/2/5/10***/12/15		
Total volume	50		

*See Table B.1 for the different EF/ER primers. **Different volume of DNA was tested out, automatically resulting in different volume of PCR-water. ***The final volume we ended up with.

Table D.5 is showing the volume and concentration of the different reagent using for the second step of the nested PCR. Two different concentration of IF- and IR primers were tested out. The final concentration of the IF- and IR primers for the second step of PCR was 0.6 µM, leading to 18 µl of PCR-water.

Table D.5 The reaction mixes for one PCR reaction for the second step of nested PCR. Different internal primer concentration was tested.

	Volume (µl)	**Stock concentration (µM)**	**Final concentration (µM)**
Premix	25		
Primer IF*/**	1.5/3***	10	0.3/0.6***
Primer IR*/**	1.5/3***	10	0.3/0.6***
PCR-water**	21/18***		
PCR product from the first step of nested PCR	1		
Total volume	50		

*See Table B.1 for the different IF/IR primers. **IF/IR primer was testes out with different concentration and volume, automatically resulting in different volume of PCR-water. ***The final concentration and volume we ended up with.

A change of the second PCR protocol leads to a decrease of the extension temperatures from 72°C to 68°C. Different numbers of cycles were also tested out, ending with 30 cycles for the final PCR protocol, Table D.6.

Table D.6 The second PCR protocol for the nested PCR on MiniAmp™ from Applied Biosystems.

Step	**Temperature**	**Time (mm: ss)**	**Number of cycles***
Initial denaturation	94°C	01:00	1
Denaturation	94°C	00:30	25/30**/35/40
Annealing	50°C	00:30	
Extension	68°C**/72°C	01:45	
Final extension	68°C**/72°C	07:00	1
Hold	4°C	∞	1

*Different number of cycles was tested. **The temperature and number of cycles we ended up with.

Appendix E – Sample information

Table E.1 Samples data with the different Ct-values from routine diagnostic, macrolide resistance, sequence types and allele numbers for TP0136, TP0548, and TP0705 achieved from this master thesis.

Sample number*	**Ct-value** (from the routine diagnostic)	**Macrolide resistance**	**Sequence type (ST)***	**TP0136**	**TP0548**	**TP0705**	**Clonal complex**
1	21.83	Resistance	NEW1	1	80	1	SS14-like
2	25.06	Resistance	ST1	1	3	1	SS14-like
3	31.80	Resistance	ST2	1	1	1	SS14-like
4	26.69	Resistance	ST1	1	3	1	SS14-like
5	26.68	Resistance	ST1	1	3	1	SS14-like
6	29.98	Resistance	NEW1	1	80	1	SS14-like
7	28.35	Resistance	ST13	6	3	1	SS14-like
8	20.20	Resistance	ST2	1	1	1	SS14-like
9	28.26	Resistance	ST1	1	3	1	SS14-like
10	23.83	Resistance	NEW1	1	80	1	SS14-like
11	33.08	Resistance	ST13	6	3	1	SS14-like
12	24.17	Resistance	NEW1	1	80	1	SS14-like
13	31.08	Resistance	NEW1	1	80	1	SS14-like
14[a]	33.18	Resistance	ST26	9	7	3	Nichols-like
15[a]	23.61	Resistance	ST26	9	7	3	Nichols-like
16	28.88	Resistance	NEW1	1	80	1	SS14-like
17	27.12	Resistance	ST26	9	7	3	Nichols-like
18	24.70	Resistance	NEW1	1	80	1	SS14-like
19	27.88	Resistance	NEW1	1	80	1	SS14-like
20	25.54	Resistance	ST1	1	3	1	SS14-like
21[b]	35.00	Resistance	ST1	1	3	1	SS14-like
22[b]	26.12	Resistance	ST1	1	3	1	SS14-like
23[c]	26.46	Resistance	ST26	9	7	3	Nichols-like
24[c]	31.43	Resistance	ST1	1	3	1	SS14-like
25[c]	27.24	Resistance	ST1	1	3	1	SS14-like
26	27.20	Resistance	ST1	1	3	1	SS14-like
27	26.37	Resistance	NEW1	1	80	1	SS14-like
28	33.38	Resistance	NEW2	36	81	3	Nichols-like
29[d]	35.00	Non detecTable	Undetermined	?	?	1	
30[d]	32.85	Non detecTable	Undetermined	?	?	1	
31	29.04	Resistance	ST1	1	3	1	SS14-like
32	29.74	Resistance + sensitive	ST1	1	3	1	SS14-like
33	29.65	Resistance	ST1	1	3	1	SS14-like
34	23.00	Resistance	ST1	1	3	1	SS14-like
35	27.88	Resistance	ST1	1	3	1	SS14-like

36	25.68	Resistance	NEW1	1	80	1	SS14-like
37	27.23	Resistance	ST1	1	3	1	SS14-like
38	30.15	Resistance	ST1	1	3	1	SS14-like
39	29.57	Resistance	ST6	3	2	3	Nichols-like
40	29.84	Resistance	ST26	9	7	3	Nichols-like
41	32.03	Resistance	ST6	3	2	3	Nichols-like
42	24.00	Resistance	ST1	1	3	1	SS14-like
43	19.71	Resistance	ST1	1	3	1	SS14-like
44	25.81	Resistance	ST26	9	7	3	Nichols-like
45	23.68	Resistance	ST1	1	3	1	SS14-like
46	25.05	Resistance + sensitive	ST1	1	3	1	SS14-like
47	26.34	Resistance	NEW3	1	82	1	SS14-like
48	26.05	Resistance	ST6	3	2	3	Nichols-like
49	35.00	Resistance	NEW1	1	80	1	SS14-like
50	28.98	Resistance	ST3	1	1	8	SS14-like
51	32.26	Resistance	ST1	1	3	1	SS14-like
52[e]	21.34	Resistance	NEW1	1	80	1	SS14-like
53[e]	25.75	Resistance	NEW1	1	80	1	SS14-like
54	34.11	Resistance	ST3	1	1	8	SS14-like
55	32.38	Resistance	ST1	1	3	1	SS14-like
56	24.18	Resistance	ST1	1	3	1	SS14-like
57	35.00	Resistance	Undetermined	?	2	3	
58	25.98	Resistance	ST6	3	2	3	Nichols-like
59[f]	34.21	Resistance	ST3	1	1	8	SS14-like
60	29.11	Resistance	ST1	1	3	1	SS14-like
61	25.80	Resistance	ST26	9	7	3	Nichols-like
62	34.55	Resistance	Undetermined	?	1	8	
63[g]	35.00	Resistance	Undetermined	9	?	?	
64[g]	35.00	Non detecTable	Undetermined	9	7	?	
65	28.59	Resistance + sensitive	ST2	1	1	1	SS14-like
66	24.17	Resistance	ST26	9	7	3	Nichols-like
67[h]	21.63	Resistance	ST26	9	7	3	Nichols-like
68[h]	22.01	Resistance	ST26	9	7	3	Nichols-like
69	27.08	Resistance	ST26	9	7	3	Nichols-like
70[i]	19.90	Resistance	ST1	1	3	1	SS14-like
71[i]	19.16	Resistance	ST1	1	3	1	SS14-like
72	32.07	Resistance	ST26	9	7	3	Nichols-like
73	22.98	Resistance	ST1	1	3	1	SS14-like
74	24.02	Resistance	ST1	1	3	1	SS14-like
75[d]	28.57	Resistance	ST1	1	3	1	SS14-like
76	28.93	Resistance	ST26	9	7	3	Nichols-like

77	Positive	Not analyzed	Undetermined	?	?	1	
78	Positive	Resistance	ST13	6	3	1	SS14-like
79[f]	20.50	Resistance	ST3	1	1	8	SS14-like
80	Positive**	Not analyzed	Undetermined	1	?	?	
81	Positive	Not analyzed	ST3	1	1	8	SS14-like
82 (QCMD)	31.16	Resistance	ST90	29	7	3	
83 (QCMD)	31.63	Resistance	ST3	1	1	8	SS14-like
84 (QCMD)	33.62	Resistance	Undetermined	1	?	1	
85 (QCMD)	31.49	Resistance	ST1	1	3	1	SS14-like
86 (QCMD)	34.26	Resistance	Undetermined	?	?	1	
87 (QCMD)	31.56	Resistance	ST1	1	3	1	SS14-like
88 (QCMD)	32.73	Resistance	ST75	1	55	1	SS14-like
89 (TPA control)	31.57	Sensitive	ST20	10	14	10	Nichols-like

The question mark means that the quality from the Sanger sequencing was poor, and the sequences was difficult to read, therefore untypable. The new allele numbers and clonal complexes (based on the phylogenetic tree), obtained from this study, are written in red. *These columns were used to make auxiliary data for the PHYLOViZ software. Sample number from 82-89, and undetermined sequence type were not included to make the auxiliary data. ** 2 out of 4 parallels were positive.

[a] Samples from the same patient.
[b] Samples from the same patient.
[c] Samples from the same patient.
[d] Samples from the same patient.
[e] Samples from the same patient.
[f] Samples from the same patient.
[g] Samples from the same patient.
[h] Samples from the same patient.
[i] Samples from the same patient.

Appendix F – Sequences, fasta file

>ST1
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>ST2
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>ST3
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Appendix G – Electrophoresis, nested PCR optimization results

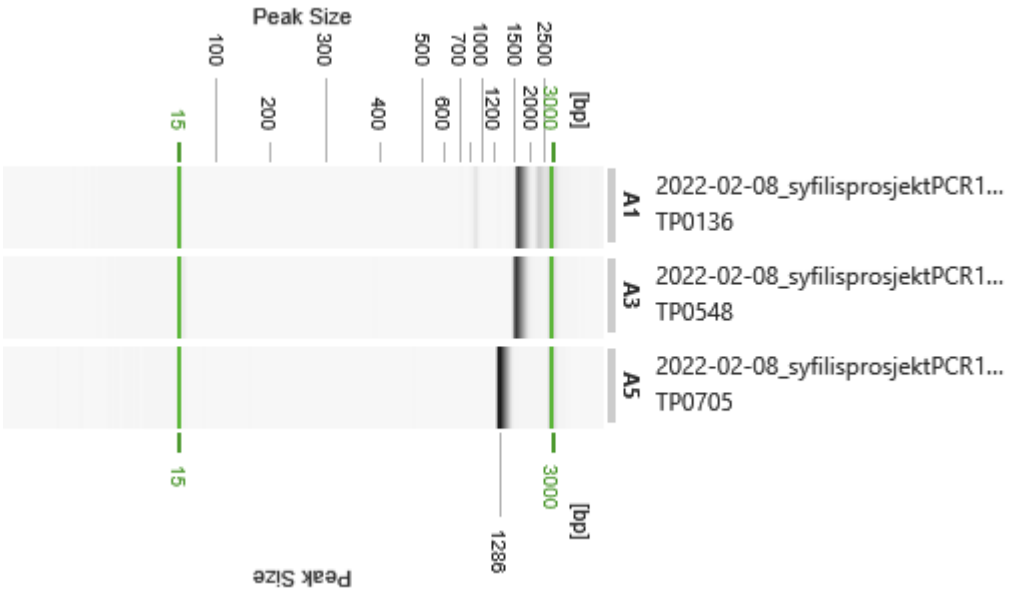

Figure G.1 PCR1 of the nested PCR. Non-diluted 5 µl TPA DNA control in the reaction mix with TP0136, TP0548-, and TP0705 external primers.

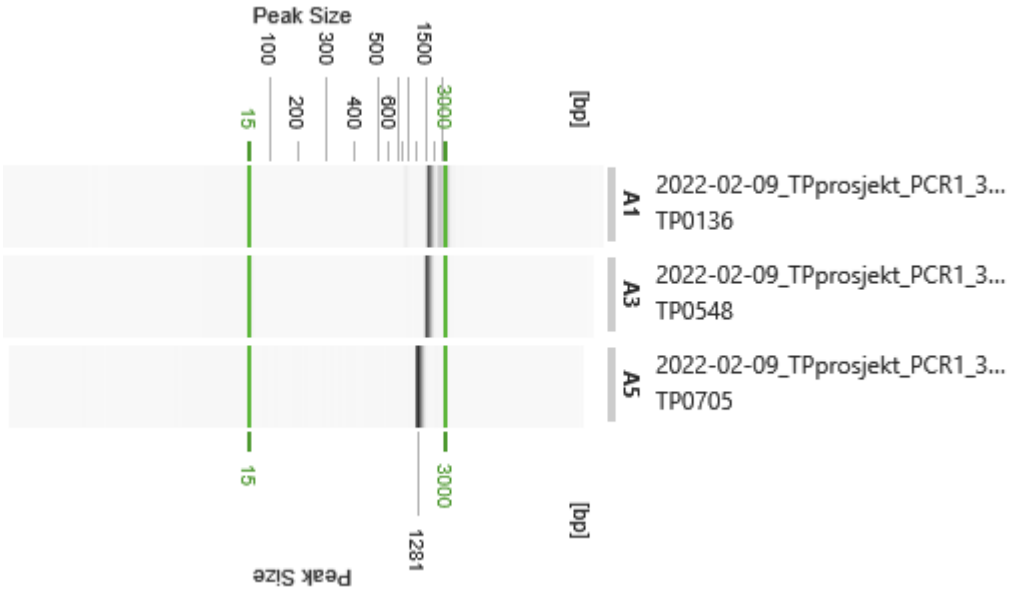

Figure G.2 PCR1 of the nested PCR. Non-diluted 3 µl TPA DNA control in the reaction mix for TP0136-, TP0548-, and TP0705 external primers.

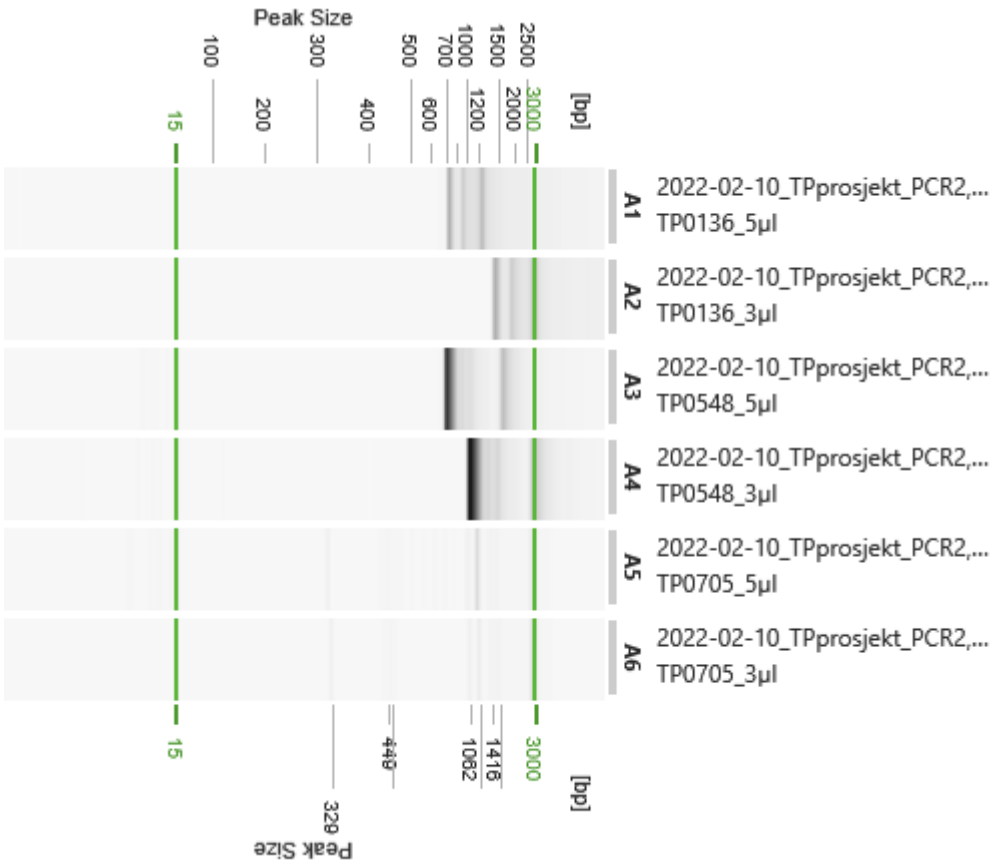

Figure G.3 PCR2 of the nested PCR. 1 µl PCR product from PCR1, Figure G.1 and Figure G.2, in the reaction mix with TP0136-, TP0548-, and TP0705 internal primers. The extension temperature is 72°C, and the primer concentration is 0.3 µM. Number of cycles is 40.

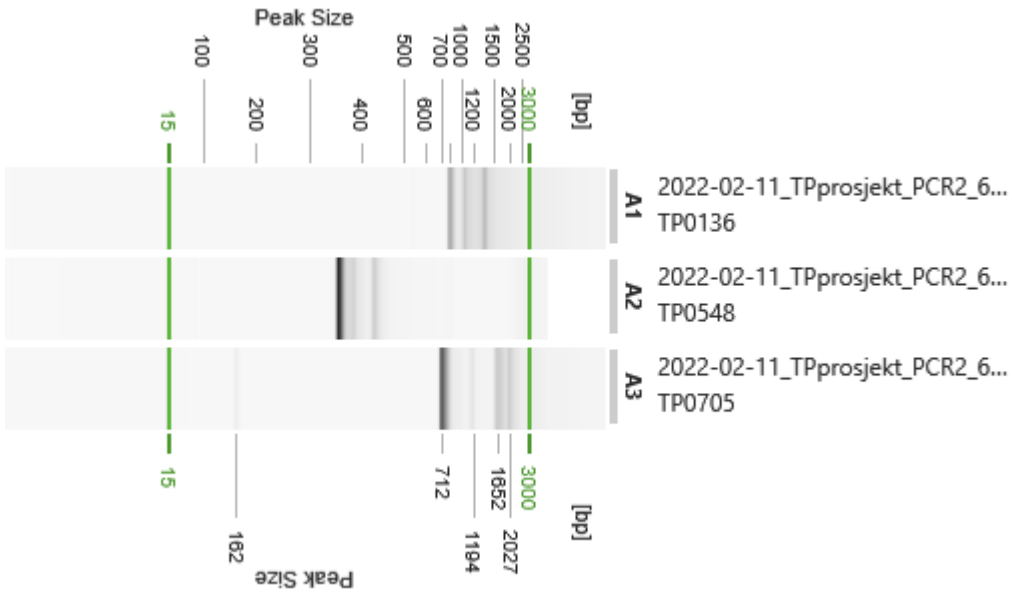

Figure G.4 PCR2 of the nested PCR. 1 µl PCR product from PCR1, Figure 2, is added to TP0136-, TP0548-, and TP0705 internal primers. Same PCR-protocol as in Figure G.3, but changing the extension temperature from 72°C to 68°C.

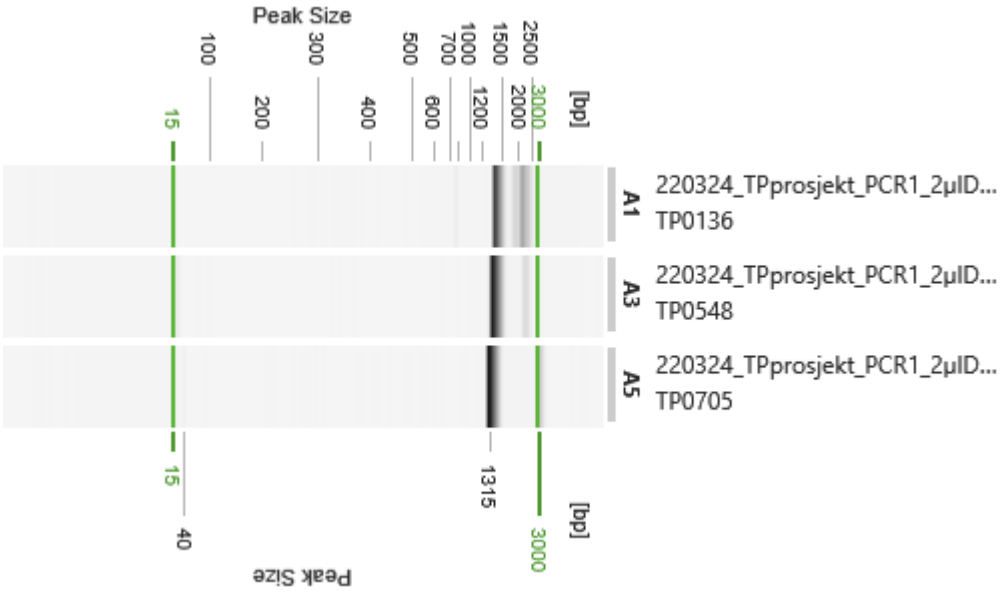

Figure G.5 PCR1 of the nested PCR. Non-diluted 2 µl TPA DNA control is added to each reaction mix with TP0136-, TP0548-, and TP0705 external primer.

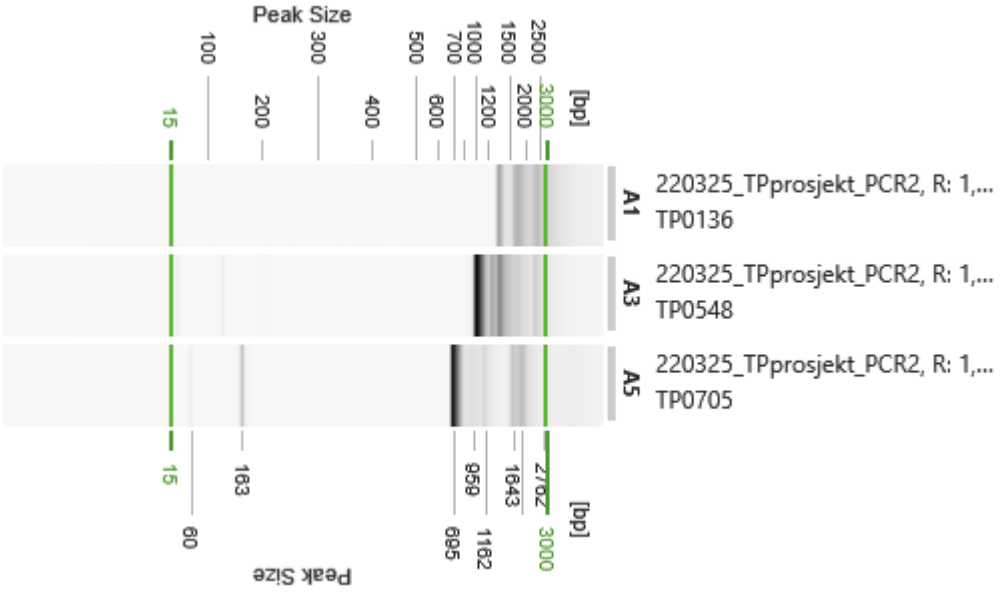

Figure G.6 PCR2 of the nested PCR. All the primer concentration is changed from 0.3 µM to 0.6 µM. The extension temperature is 68°C.

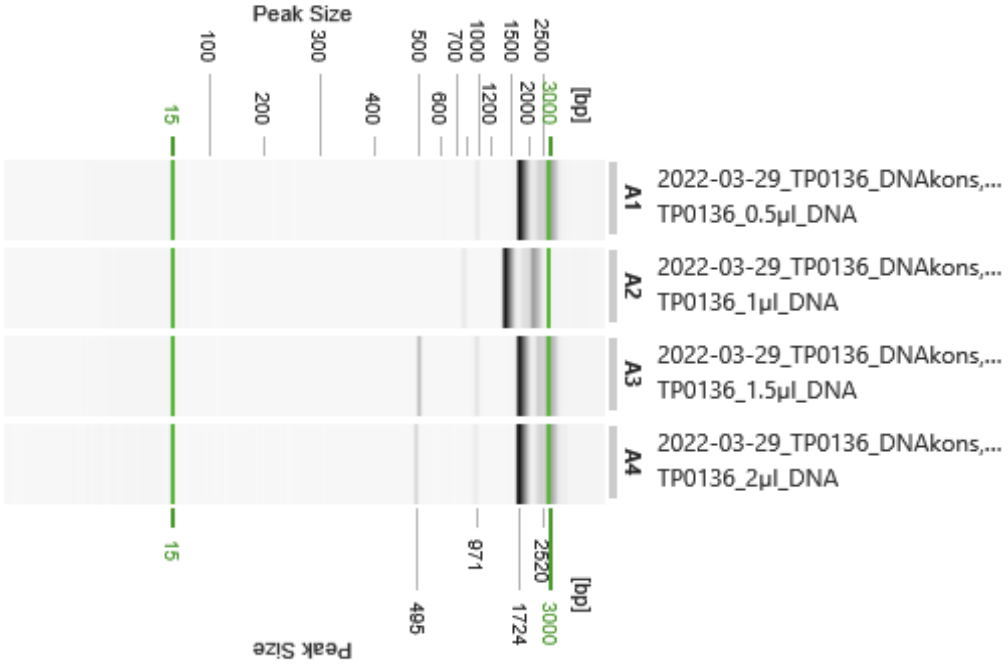

Figure G.7 PCR1 of the nested PCR. Making a dilution series of non-diluted TPA DNA control and tested it with locus TP0136: 0.5 µl, 1 µl, 1.5 µl, and 2 µl.

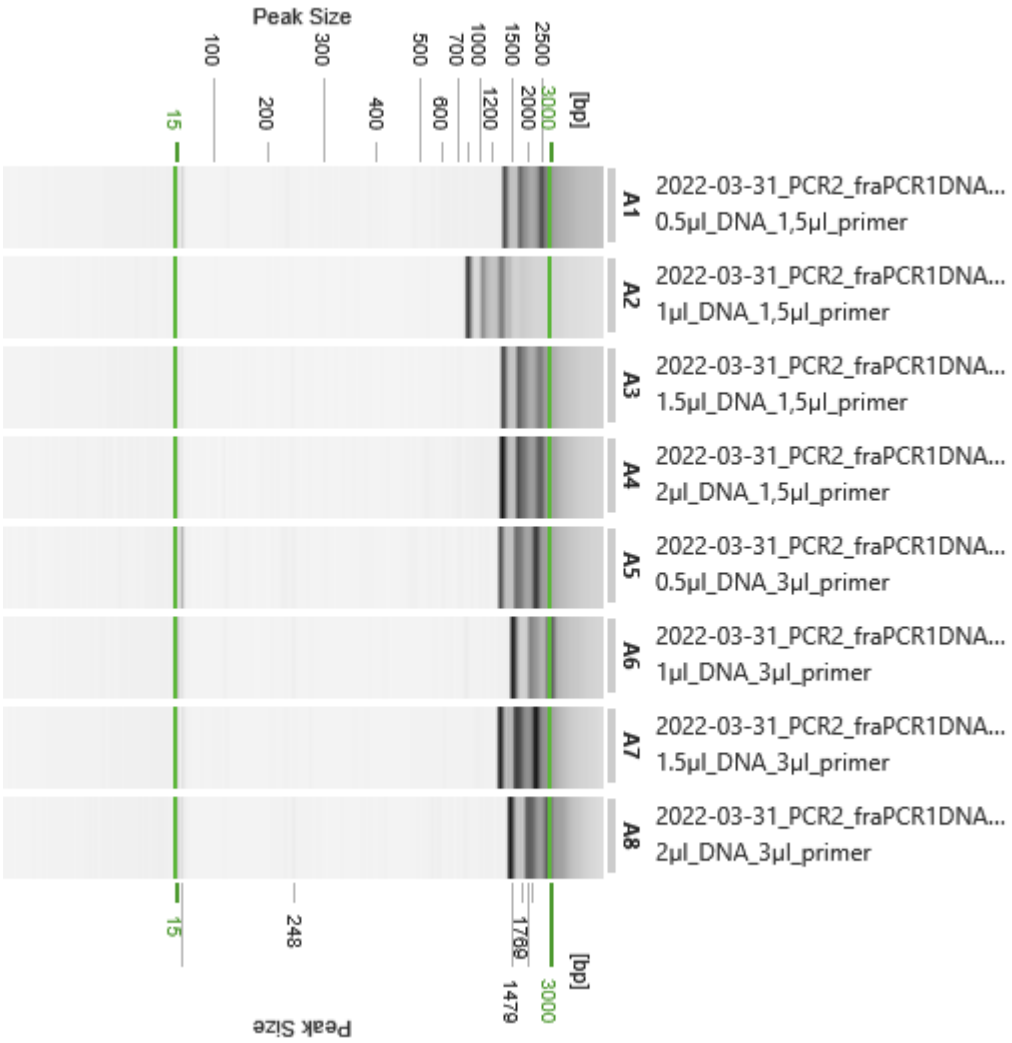

Figure G.8 PCR2 of the nested PCR with PCR products from Figure G.7. Primer concentration for TP0136 is 0.3 µM from A1-A4 and 0.6 µM from A5-A8. The extension temperature is 68°C and the number of cycles is 40.

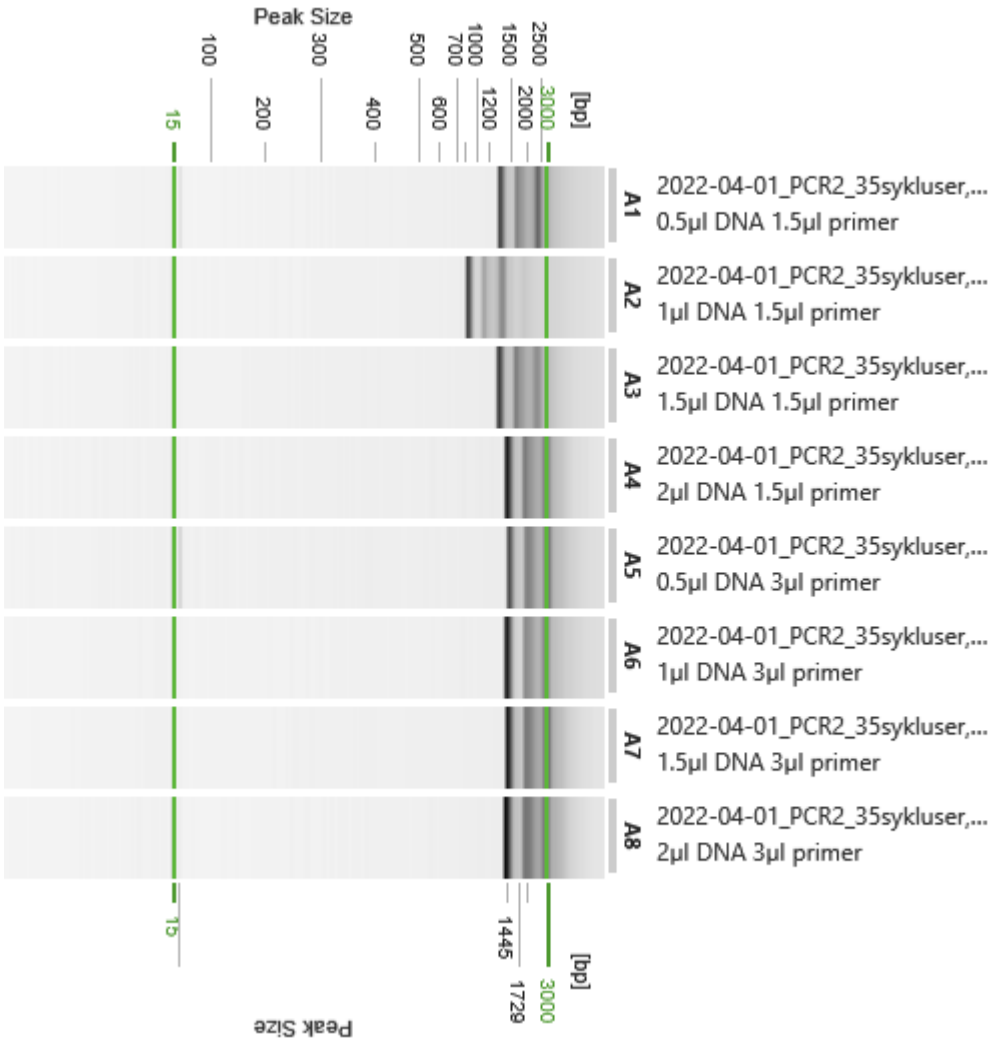

Figure G.9 PCR2 of the nested PCR. The PCR conditions are the as Figure G.8, but the number of cycles is decreased to 35.

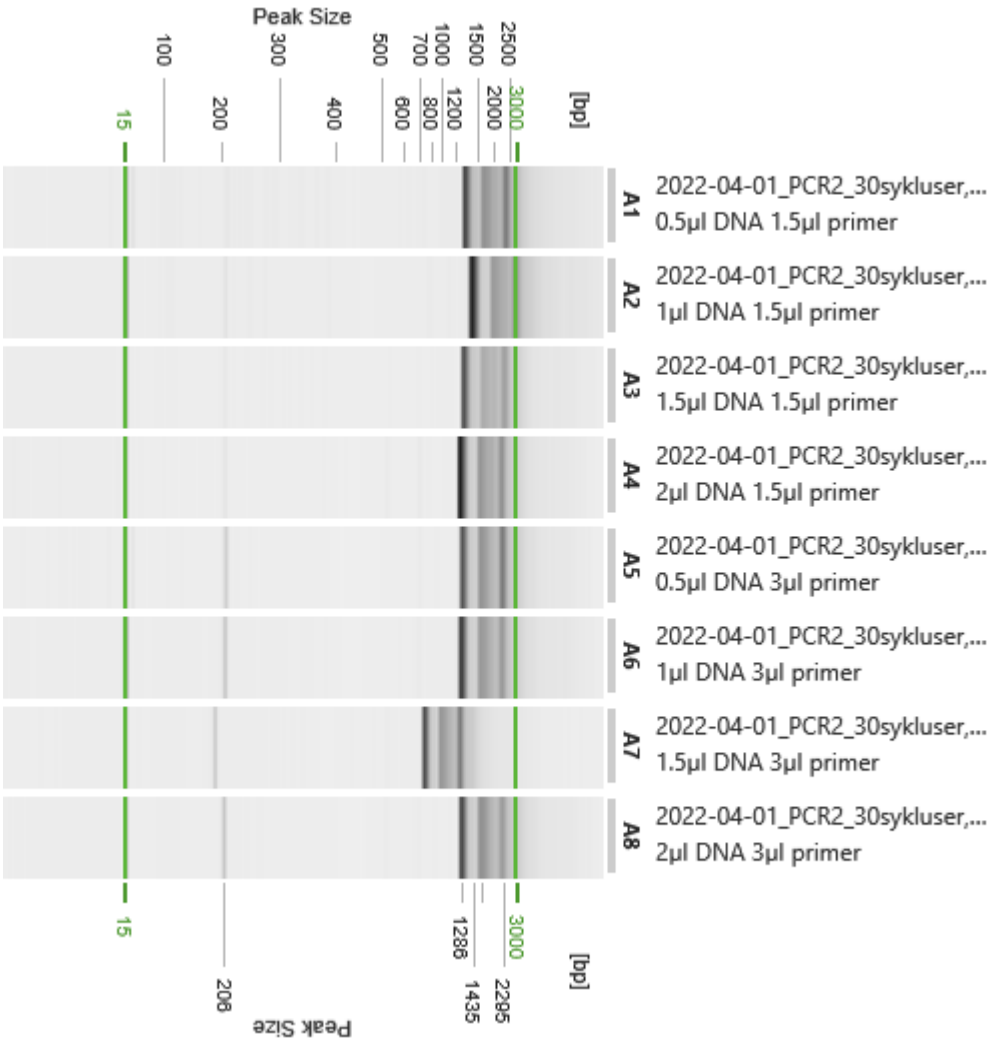

Figure G.10 PCR2 of the nested PCR. The PCR conditions are the same as in Figure G.8, but the number of cycles are changed to 30.

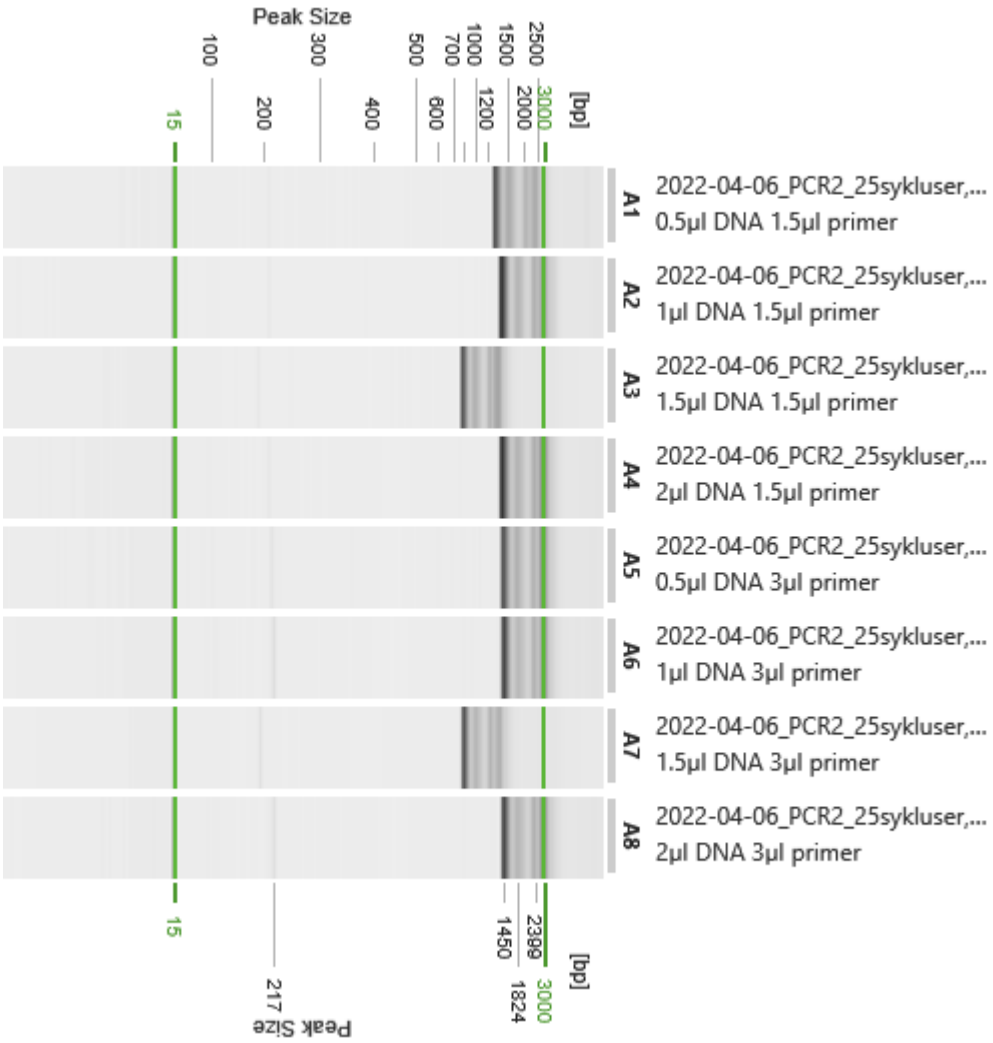

Figure G.11 PCR2 of the nested PCR. the PCR conditions are the same as in Figure G.8, but the number of cycles are changed to 25.

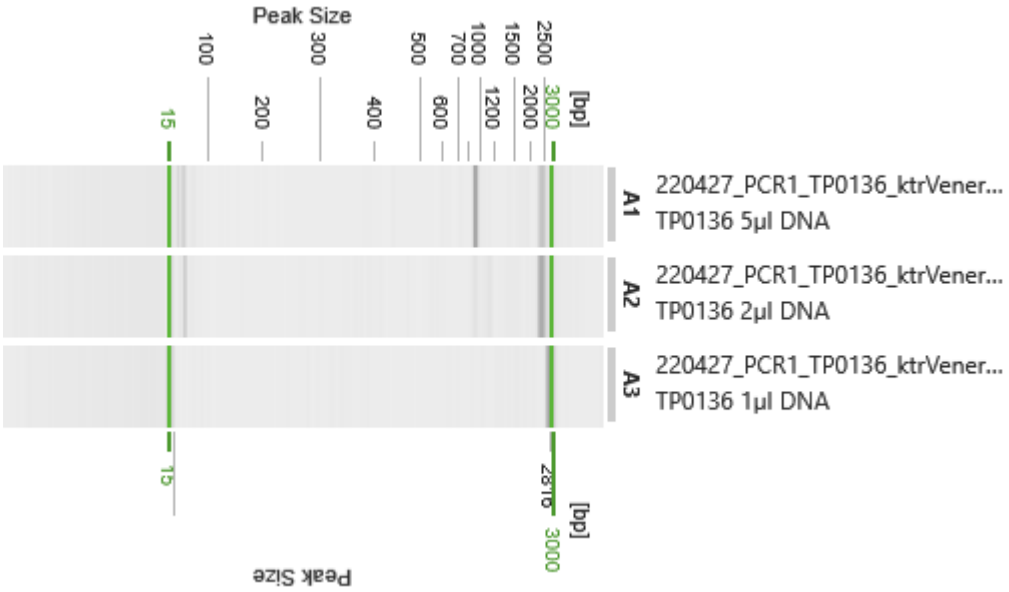

Figure G.12 PCR1 from the nested PCR. Using the diluted TPA DNA control for testing the locus TP0136. Testing different volume of DNA: 5 µl, 2 µl, and 1 µl.

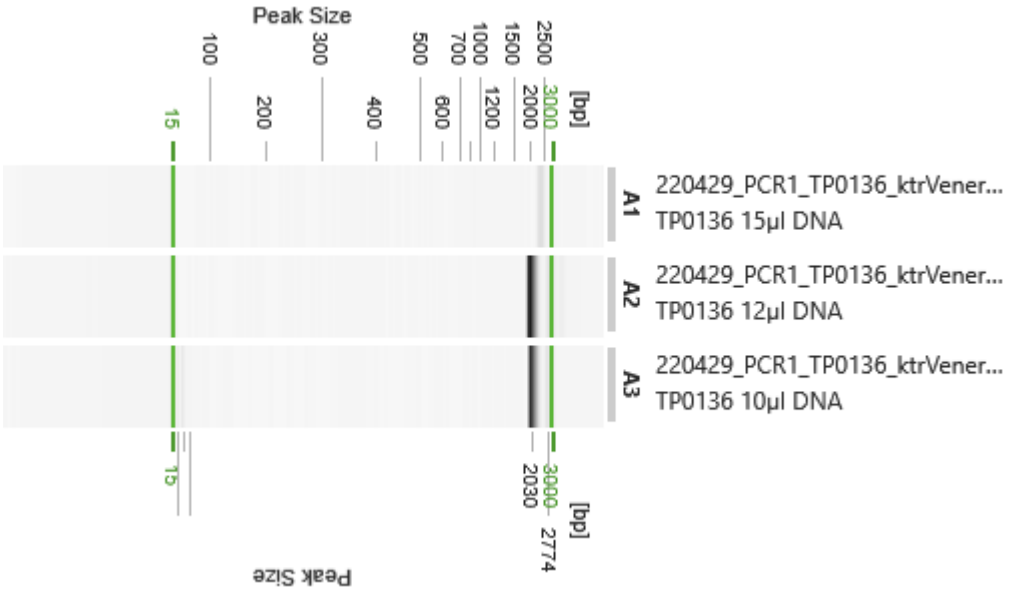

Figure G.13 PCR1 from the nested PCR. Using the diluted TPA DNA control for testing TP0136. Testing different volume of DNA: 10 µl, 12 µl, and 15 µl.

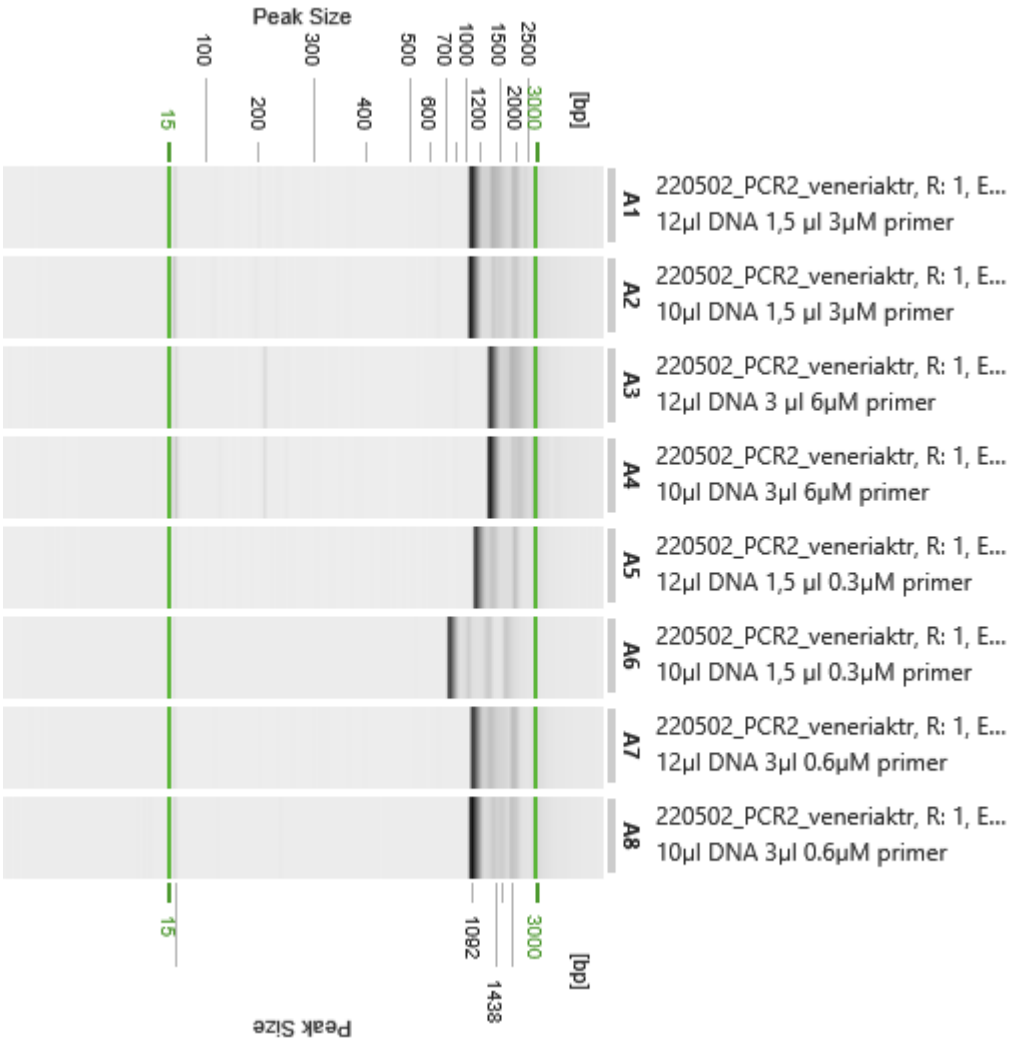

Figure G.14 PCR2 of the nested PCR. 1 µl from 12 µl and 10 µl of the PCR1 (Figure G.13) where continued to the next PCR. A1-A2 were tested with 3 µM internal primers, A3-A4 were tested with 6 µM primers, A5-A6 were tested with 0.3 µM primers, and A7-A8 were tested with 0.6 µM primers. The number of cycles was 30.

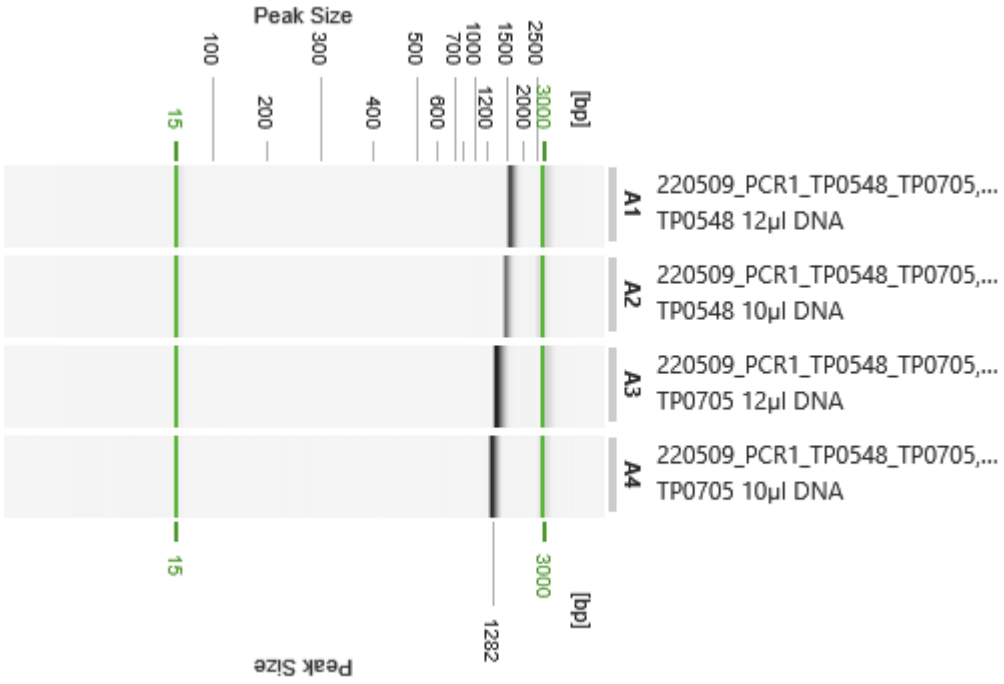

Figure G.15 PCR1 of the nested PCR. Testing TP0548 and TP0705 with 10 µl and 12 µl DNA to see if the results are good.

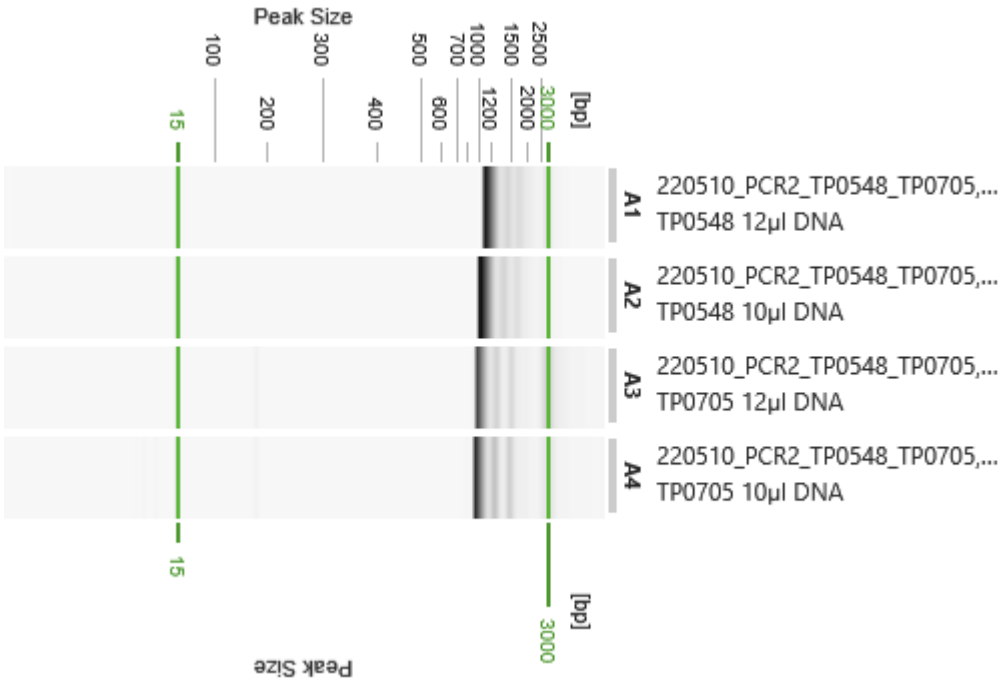

Figure G.16 PCR2 of the nested PCR. Continue Figure G.15 from PCR1 to PCR2.

Appendix H – Domestic and overseas syphilis infection in Norway, 2013-2022

Table H.1 An overview of domestic and overseas syphilis infection in Norway from 2013-2022. These data were obtained from MSIS 30. April 2023.

	2013	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**	**2022**
Domestic infection	147 (79%)	133 (70%)	126 (73%)	127 (68%)	141 (63%)	149 (65%)	128 (62%)	236 (82%)	137 (84%)	124 (64%)
Overseas infection	37	56	45	61	83	81	78	52	26	69
Unknown infection	1	-	1	-	-	1	-	-	-	-
Total	185	189	172	188	224	231	206	288	163	193

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