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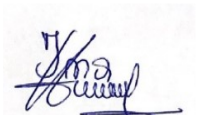
DECLARATION

I hereby declare that I have authored this thesis independently, that I have not used other than the declared sources / resources and that I have explicitly marked all material which have been quoted either literally or by content from the used sources.

07.03.2022 

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ABSTRACT IN ENGLISH

Genital mycoplasmas are known as sexually transmitted agents, frequently isolated from the female genital tract. *Trichomonas vaginalis* causes trichomoniasis, the most recurrent sexually transmitted infection (STI) worldwide. Symbiosis between *Mycoplasma* species and *T. vaginalis* have been described and linked to numerous reproductive morbidities. Cross-sectional studies are based on investigating the association of risk factors and disease as well as estimating the incidence of the latter. In the Outpatients Centre for Infectious Venero-dermatological Diseases (OCD) in Vienna, patients are routinely screened for STIs. The main aim of this two-phase retrospective study was to assess the prevalence of genital *Mycoplasma* species in swab specimens obtained from female patients attending the OCD. In total, 582 samples from female patients and an additional 20 *T. vaginalis* cultured isolates were analysed by culture, molecular and microscopic methods. In 178 (30%) of the collected samples, distinct *Mycoplasma* species were detected. *Mycoplasma hominis* was the most prevalent mollicute found in 18.5% and 25% of the specimen in Phase I and II of the study, respectively. *Ureaplasma* species counted for the second most prevalent group, the two serovars *U. urealyticum* and *U. parvum* were detected individually. Taken together, they were found in 5.6% and 7.6% of the samples. Lastly, *M. genitalium* occurred in 2.3% of the specimens in phase II. The 16S rDNA sequence of the recently newly described species, *Candidatus* Mycoplasma girerdii was obtained for the first time in Austria, in a sample also positive for the protozoan *T. vaginalis*. Additionally, molecular analyses of the cultivated *T. vaginalis* strains confirmed the symbiotic relationship with *M. hominis* in two out of 20 samples. The presence of *Ca*. M. girerdii was confirmed only directly in vaginal discharge and not in pure cultures of the parasite *T. vaginalis*. Altogether, this study demonstrates the presence of genital *Mycoplasma* infections in sexually active women of reproductive age, detected by new diagnostic assays, thus enabling better screening of patients.

ABSTRACT IN GERMAN

Genitale Mycoplasmen sind sexuell übertragbare Erreger und werden häufig aus dem weiblichen Genitaltrakt isoliert. *Trichomonas vaginalis* ist der Errerger der Trichomoniasis, der weltweit am häufigsten auftretenden sexuell übertragbaren Infektion (STI). Symbiosen zwischen *Mycoplasma*-Arten und *T. vaginalis* wurden beschrieben und mit zahlreichen reproduktiven Erkrankungen in Verbindung gebracht. In Querschnittsstudien wird der Zusammenhang zwischen Risikofaktoren und Krankheit untersucht und die Inzidenz der Krankheit geschätzt. Im Ambulatorium für Pilzerkrankungen sowie Erkrankungen durch Protozoen und Neisserien (OCD) in Wien werden die Patienten routinemäßig auf STIs untersucht. Das Hauptziel der vorliegenden zweiphasigen, retrospektiven Studie war es, die Prävalenz genitaler *Mycoplasma*-Spezies in Abstrich-Proben von Patientinnen des OCD zu bestimmen. Insgesamt wurden 582 Proben von Patientinnen und zusätzlich 20 kultivierte *T. vaginalis*-Isolate mittels Kultur, molekularer und mikroskopischer Methoden analysiert. In 178 (30 %) der gesammelten Proben wurden *Mycoplasma*-Arten nachgewiesen. *Mycoplasma hominis* war in Phase I und II der Studie mit 18,5 % bzw. 25 % der Proben die am häufigsten vorkommende Art. Jeder Serovar der *Ureaplasma*-Arten, welche die zweithäufigste Gruppe waren, wurde einzeln nachgewiesen. Insgesamt waren sie in 5,6 % bzw. 7,6 % der Proben zu finden. *M. genitalium* kam in Phase II bei 2,3 % der Proben vor. Die 16S rDNA-Sequenz einer kürzlich neu beschriebenen Spezies, *Candidatus* Mycoplasma girerdii, wurde zum ersten Mal in Österreich in einer Probe gefunden, die auch positiv für den Protozoen *T. vaginalis* war. Zusätzlich bestätigten molekulare Analysen der kultivierten *T. Vaginalis*-Stämme, die symbiotische Beziehung mit *M. hominis* in zwei von 20 Proben. Das Vorhandensein von *Ca*. M. girerdii wurde nur direkt im Vaginalausfluss und nicht in Reinkulturen des Parasiten *T. vaginalis* bestätigt. Insgesamt zeigt diese Studie das Vorhandensein von genitalen Mycoplasmen-Infektionen bei sexuell aktiven Frauen im reproduktiven Alter. Neue diagnostische Tests nachgewiesen ermöglichen ein besseres Screening der Patienten.

1 INTRODUCTION

1.1 General Introduction

Sexually transmitted diseases (STDs) are a major burden on the health care systems worldwide. According to the WHO (2018) more than one million sexually transmitted infections (STIs) are accounted globally every day. In a single year the number of people who acquire syphilis, gonorrhoea, trichomoniasis and chlamydia infections surpasses 370 million (Rowley *et al.*, 2019). Furthermore, over 500 million people were infected with herpes simplex virus (HSV2), whereas human papilloma virus (HPV) infections in women reached 290 million worldwide (Looker *et al.*, 2015; WHO, 2018).

Trichomoniasis is the most common, curable, non-viral genitourinary STI in the world. It is caused by the non-invasive mucosal protist *Trichomonas vaginalis* (*WHO, 2011*; Harp and Chowdhury, 2011). More than 160 million *T. vaginalis* infections are registered annually (McClelland, 2008; Kissinger, 2015b). Although trichomoniasis can be treated, the current challenge concerns the high rate of asymptomatic infections in men and women (Ryan et al., 2011). It is estimated that around 85% of women with T. vaginalis are asymptomatic (Sutton *et al.*, 2007) and one third of them will become symptomatic within 6 months (Petrin *et al.*, 1998) In men, probably more than 70% of infections are asymptomatic (Seña *et al.*, 2007). Among women, symptoms range from a relatively asymptomatic state, to vulvar irritation, abdominal pain, severe inflammation and complications including pelvic inflammatory disease (PID), sterility and increased risk of cervical cancer (Yarlett *et al.*, 1996; Petrin *et al.*, 1998). Although *T. vaginalis* infection is mainly asymptomatic in men, when manifested it can cause urethritis (Holmes *et al.*, 1975), can lead to a more aggressive form of prostate cancer (Sutcliffe *et al.*, 2006, 2012) as well as infertility (Tuttle et al., 1977). Moreover, trichomoniasis has been linked to an elevated infection risk with the human immunodeficiency virus (HIV), aided by local accumulation of immune cells to the virus (Laga *et al.*, 1993; Buvé *et al.*, 2001; Kalichman,et al., 2011; Kissinger and Adamski, 2013).

Mycoplasmas are wall-less bacteria which inhabit the genitourinary tract (Waites and Taylor-Robinson, 2015). Some of the most prevalent genital mycoplasmas are *Mycoplasma hominis*, *Mycoplasma genitalium* and *Ureaplasma urealyticum*. Numerous reports on these organisms have been conducted, nevertheless not much progress in researching mycoplasmas was being made, until molecular methods were established for larger scale use (Larsen and Hwang, 2010). The latter enabled scientists to obtain information regarding their pathogenicity and involvement as disease co-factors (Larsen and Hwang, 2010; Taylor-Robinson, 2017). An early study from McCormack *et al.*, in 1972 asserted that colonization with Ureaplasmas and *M. hominis* starts after puberty. With the number of sexual partners, the bacterial colonization in the vagina or the cervix increases (Taylor-Robinson and McCormack, 1980). Overall, women

are more susceptible to colonization by genital mycoplasmas then men (Taylor-Robinson, 2017). Ureaplasmas are identified in 40-80% of asymptomatic, sexually active women, *M. hominis* in 20-50% and *M. genitalium* is detected in 1-5% of women screened for genital infections (Taylor-Robinson D, 2010). Published data support the role of genital mycoplasmas as etiological agents of bacterial vaginosis (BV), cervicitis and adverse pregnancy outcomes (Taylor-Robinson and Furr, 1997; Uusküla and Kohl, 2002). The clinical implication of mycoplasma infections in humans is rather suggestive of damaging host's immune responses and suppressing inflammation, thus establishing a chronic infection (Razin et al., 1998).

1.1.1 A brief history of the Mollicutes

The term *Mycoplasma* also described in "Etymology of the Term *Mycoplasma*" (Krass, Gardner and Ma, 1973) dates back to 1889. *Mycoplasma* is the best documented genus in the class of mollicutes (Edward and Freundt, 1967) with more than 170 identified species isolated from vertebrates, plants and arthropods ('International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes', 1995). Mycoplasmas are the smallest free-living prokaryotic organisms. They differ phenotypically from other bacteria due to their lack of a cell wall (Freundt,' *et al.*, 1984; Razin, Yogev and Naot, 1998). The first *Mycoplasma* to be cultivated was that of a bovine strain in 1898 (*Le microbe de la peripneumonie by NOCARD, Edmond & ROUX, Pierre 1898*). The unique characteristics of *Mycoplasma* caused disagreement in regards to their taxonomic status. In the 1930s, mycoplasmas were recognized as bacteria and were later considered bacterial L-forms which had lost their cell wall. Finally, through genomic analysis in the 1960s, mycoplasmas were determined as a well-established group separate from other bacteria (Bak *et al.*, 1968; Leth Bak *et al.*, 1969). The term *Mycoplasma,* μύκης, mykes (fungus) and πλάσμα, plasma (formed) from the Greek was first used in the late 1880s (B. Frank: Ueber die Pilzsymbiose der Leguminosen', in 1889). The Mollicutes class is comprised of 8 genera: *Anaeroplasma, Asteroleplasma, Spiroplasma, Mycoplasma Mesoplasma, Ureaplasma,* and *Entomoplasma* ('International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes', 1995; Freundt,' *et al.*, 1984).

Morowitz and Wallace (1973) analysed the life cycle of *Mycoplasma* and determined the simplicity of the organisms as being a predecessor of bacteria with a peptidoglycan cell wall. Once rRNA sequencing data were established as a phylogenetic estimate, it was proven that mycoplasmas were the outcome of a degenerative evolution from gram-positive bacteria, which is partly owed to their parasitic nature (Woese, Maniloff and Zablen, 1980). From an evolutionary point of view, according to Razin in *Molecular Biology and Pathogenicity of Mycoplasmas (2002*) ancestral mycoplasmas arose from the *Streptoccocus* phylogenetic branch nearly 600 million years ago.

To sum up, mycoplasmas are now clearly defined as part of the eubacteria group and related to gram-positive bacteria phylogenetically while preserving the unique features as the smallest free-living prokaryotes (Razin and Hayflick, 2010).

1.2 Mycoplasma hominis

The first association of mycoplasmas with humans was reported in 1937. The organisms were isolated from a Bartholin's gland abscess and it is probable that it was *Mycoplasma hominis* (Dienes and Edsall, 1937). Four distinct serotypes of human origin have been described (S Nicol, ff Edward, 1953), namely: *M. hominis* Type 1 (referred to as *M. hominis*), *M. hominis* Type 2, *M. fermentans* and *M. salivarium* (Edward and Freundt, 1956).

Table 1. Scientific classification of *M. hominis* (S Nicol, 1953; Chanock *et al.*, 1963; Esward and Freundt, 1967).

Scientific classification	
Domain:	Bacteria
Phylum:	Tenericutes
Class:	Mollicutes
Order:	Mycoplasmatales
Family:	Mycoplasmataceae
Genus	*Mycoplasma*
Species:	*Mycoplama hominis*

Mycoplasmas have a preference for epithelial surfaces, e.g. in the mouth, the respiratory and the urogenital tract (Whitcomb, 1989; Razin and Hayflick, 2010), and exhibit organ or tissue specificity. For instance, *Mycoplasma genitalium* is found in the urogenital tract, whereas *Mycoplasma pneumoniae* is primarily found in the respiratory tract. Nonetheless, there are exceptions and the opposite can be true as these *Mycoplasma* species have also been isolated elsewhere in the body (Goulet *et al.*, 1995). Increasing numbers of genital mycoplasmas i.e. *Mycoplasma hominis* and *Ureaplasma urealyticum* have been collected from atypical sites in the body of patients suffering from AIDS, or receiving immunosuppressive medications as a preventive measure before an organ transplantation (Meyer and Clough, 1993; Gass *et al.*, 1996). In Table 2, sixteen species of *Mycoplasma* isolated from humans are listed. Only a few species have a significant medical relevance e.g.: *M. pneumonia, M. hominis*, *U. urealyticum* and *M. genitalium*.

Table 2. Mycoplasmas isolated from humans and their primary site of colonization (Waites et al, 2005; Waites *et al.*, 2012).

Microorganism	**Site of colonization**
Mycoplasma pneumoniae	Respiratory tract
Mycoplasma primatum	Respiratory and genitourinary tract
Mycoplasma orale	Oral cavity
Mycoplasma penetrans	Genitourinary tract
Mycoplasma fermentans	Genitourinary tract
Mycoplasma amphoriforme	Respiratory tract
Mycoplasma genitalium	Genitourinary tract
Mycoplasma hominis	Genitourinary tract
Candidatus Mycoplasma girerdii	Genitourinary tract
Ureaplasma urealyticum	Genitourinary tract
Ureaplasma parvum	Genitourinary l tract
Mycoplasma buccale	Respiratory tract
Mycoplasma lipophilum	Respiratory tract
Mycoplasma faucium	Respiratory tract

Mycoplasma hominis inhabits the lower genital tract as a commensal with a minimal genome size of 665,445 bp and a 27.1% G+C content. Due to its small size, light microscopy cannot visualize morphological patterns of *Mycoplasma* species and this is achieved by electron microscopy (Maniloff, 1969). The standard procedure for detection of *M. hominis* is culture in specific media. Characteristic for *M. hominis* are the so called “fried egg” colonies when grown on A8 or SP4 agar. A reliable identification of species must include additional tests including immunoblotting with monoclonal antibodies, conventional PCR and sequencing (Waites, Katz and Schelonka, 2005).

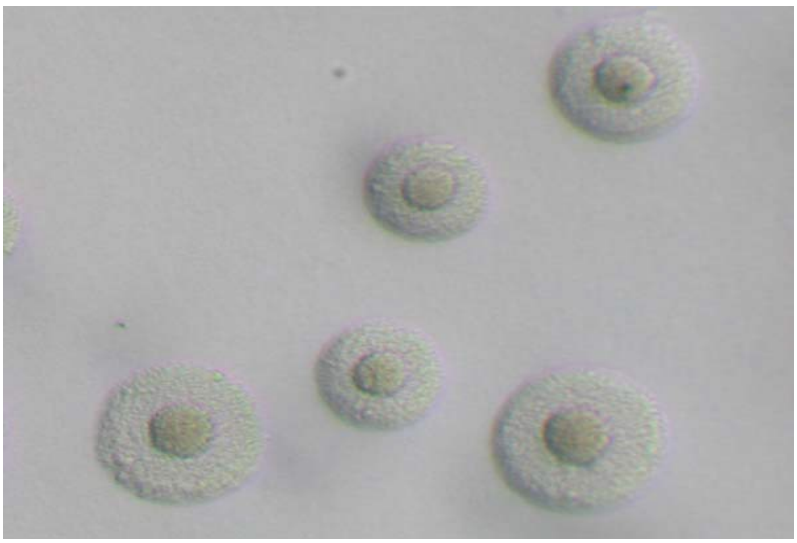


Figure 1. *Mycoplasma hominis* colonies exhibiting fried-egg appearance. The organism was grown on A8 agar for 72 hours and observed under a stereomicroscope (Waites, Katz and Schelonka, 2005).

1.2.1 Genome size and base composition

Leth Bak *et al.*, (1969) were the first to estimate the genome size of various *Mycoplasma* species. The data obtained through the renaturation kinetics method divided the Mollicutes into two classes, which coincides with their taxonomic classification. Application of Pulsed Field Gel Electrophoresis (PFGE) provided an accurate estimation of the genome size (Pyle *et al.*, 1988; Neimark and Lange, 1990; Ladefoged and Christiansen, 1992). Genomes of *Mycoplasma* species vary in their size from 580 kb counting for the smallest genome of *M. genitalium* to more than 2200 kb in same genus (Razin, 1992). Genomes of several *M. hominis* strains, namely *M.hominis*- PG21, 132, 4195, 93, 7488 have a similar range to that of *M. genitalium* ranging between 696 to 825 kb (Ladefoged and Christiansen, 1992).

More data on the genomes of *Mycoplasma* spp. became available with the establishment of molecular biology methods. One of the first data obtained was the (G+C) content, which is also used for phylogeny classification (Ladefoged, 2000). A noticeable characteristic of mycoplasmas is the high A+T content, with up to 80-82 % (SA and G, 1991; Ladefoged and Christiansen, 1994). Therefore, the G+C content is low, specifically 28-29% in the *M. hominis* genome and 48% in the 16s rRNA (Neimark, 1970; Mygind, Birkelund and Christiansen, 1998).

1.2.2 Detection and quantification methods for *M. hominis*

Several laboratory-based methods are available for the detection of *M. hominis* in clinical samples. Culture is the primary and the most economical means of detection used for the bacterium. *M. hominis* grows relatively fast (within two to three days) in specialized media; a stereomicroscope can be used to observe colonies. There are no tests available for

phenotypical differentiation among *Mycoplasma* species. Therefore, if needed, additional testing via PCR is conducted. Culture is often preferred over molecular methods due to the possibility of performing additional tests such as susceptibility to antimicrobial agents.

Tests conducted for *M. hominis* comprise enzyme immunoassay (EIA), metabolism inhibition and micro-immunofluorescence. Different isolates have high antigenic variation and heterogeneity, which makes the tests difficult to develop and to standardise for diagnostic use. The enzyme-linked immunosorbent assay (ELISA) has been performed to measure the Immunoglobulin G (IgG) antibody response to *M. hominis* infection, with a mix of membrane proteins from two *M. hominis* strains used as the antigen (Baczynska *et al.*, 2005). Molecular detection is performed mainly by PCR, which has been progressively used due to its high sensitivity and a reduced work-flow to attain results (Skov Jensen *et al.*, 1989). The 16s rRNA is the main target gene used in traditional PCR assays. This gene is highly conserved and also allows for detection of *Ureaplasma* spp. Real-time quantitative PCR assays are a high throughput and sensitive method when compared to conventional PCR as it requires less time to obtain results and no subsequent electrophoretic analysis of PCR products is needed (Waltes *et al.,* 2008).

MLVA, short for Multiple Locus Variable-Number Tandem-repeat Analysis is a molecular typing method relying on the variation in the copy number of repeated sequences at different loci along a chromosome. This method has been used frequently for distinct microbial species including *M. hominis.* Férandon *et al.*, (2013) analysed more than 200 urogenital samples using MLVA and confirmed the heterogeneity of *M. hominis* among isolates.

1.2.3 Virulence factors: Cytoadhesin proteins as a strategy for phenotypic switching

Mycoplasmas in humans are often reported as commensals of the normal flora e.g. *M. hominis* in the urogenital tract. Several mechanisms drive the affinity of *Mycoplasma* species when it comes to their persistent affinity for epithelial surfaces of the respiratory or urogenital tract. Mycoplasmal adhesins have been described as the main factor for infectivity. P1, P30 and MgPa, have been described as adhesins for *M. genitalium* and *M. penumoniae* (Razin and Jacobs, 1992; Razin, Yogev and Naot, 1998).

A majority of *Mycoplasma* species, including *M. hominis*, have developed strategies for a better uptake by host cells which include phenotypic plasticity as well as altering their shape to enter the host cells. *M. hominis* on the other hand lacks the attachment adhesines, and contains lipid-modified membrane proteins such as OppA, P60, P80 secretin and variable adherence-associated (Vaa) proteins instead (Henrich et al., 1993). These proteins have dual functionality and a high antigenic variability, thus making them fit for binding a higher number of host cell

receptors (Henrich *et al.*, 1996; Razin, Yogev and Naot, 1998). In addition, the infective agent will better circumvent and adjust to receptors of the immune system and niches within the host prior to colonization (Hopfe, Hoffmann and Henrich, 2004). The variable adherence-associated (Vaa) antigen of *M. hominis* (encoded by the gene with the same name), acts as a mediator for the interaction between M*ycoplasma* and its host cell. This surface lipoprotein adhesion is seemingly controlled at the translational level. There is a direct correlation between high-frequency phase variation in expression and the ability of *M. hominis* to adhere to cultured human cells (Zhang and Wise, 1997). As a response, elevated amount of collagen and laminin for internalisation has been observed on the membrane of the host cell during *M. hominis* infection (Olson and Gilbert, 1993; Kitzerow, Hadding and Henrich, 1999).

1.2.4 Association of *M. hominis* in genitourinary tract diseases

M. hominis has been detected in 30-60 % of women who have and women who lack signs of urogenital infection (Mårdh, 1983). Higher infection rates are observed among younger women, lower income status and multiple sexual partners (Mårdh, 1983; Waites et al., 2005). A variety of conditions including postpartum endometritis, bacterial vaginosis (BV) osteoarthritis, pyelonephritis, pelvic inflammatory disease (PID), bacteremia and meningitis have been associated with *M. hominis* (Waites and Taylor-Robinson, 2015). The organism and Ureaplasma spp. inhabit the genitourinary tract of heathy adult and are transmitted vertically and venerally (Waites 2004; Waites et al., 2005)

BV, previously known as '*Gardnerella* vaginitis', is a syndrome characterized by depletion of the normal *Lactobacillus* population. As a result, it is accompanied by an overgrowth of vaginal anaerobes and loss of the vaginal acidity (Hay, 2005). Overall, half of BV registered cases are either asymptomatic or women exhibit only mild symptoms. The concentration of certain bacteria including *Prevotella* spp. (*Bacteroides*), *Gardnerella vaginalis*, *M. hominis* and *Mobiluncus* spp. is significantly elevated (Hay, 2005; Waites, Katz and Schelonka, 2005). *M. hominis* and antibodies for the bacterium are found frequently in the vagina of approximately 60% of women diagnosed with BV. The mechanism as to how *M. hominis* contributes to BV pathology is yet to be understood (Hay, 2005). PID is diagnosed by laparoscopy. A frequent isolate from the lower genital tract of female patients is *M. hominis*. One fourth of women with PID develop a notably high antibody response against *M. hominis* (Mårdh, 1983).

1.3 *Ureaplasma* species

1.3.1 *Ureaplasma urealyticum* history of discovery

U. urealyticum, a human pathogen, was first isolated in the 1950s from men with or without non-gonococcal urethritis (NGU) (*Shepard MC.*, 1954). A decade later, these mycoplasmas were further characterised and unique features such as hydrolysing of urea to produce adenosine triphosphate (ATP) were identified. This property facilitated the detection of these organisms by a test which inhibited their metabolism. The first attempt to detect antibodies against them was performed by Feizi and Taylor-Robinson in 1967. The observations and data collected led to a unanimous decision of classifying the organisms into a new genus: *Ureaplasma* and the species *U. urealyticum*. This species is currently divided into 14 serovars and two biovars, namely *U. urealyticum* (biovar 1) *U. parvum* and *U. urealyticum* (biovar 2) (Kong *et al.*, 2000). U. urealyticum has the second smallest genome among prokaryotes, after *M. genitalium* (Glass *et al.*, 2000).

1.3.2 Detection and morphology

Ureaplasma urealyticum was divided into two species in 2002, *U. urealyticum* and *U. parvum* (Robertson *et al.*, 2002). These two species have each distinct genotypic and phenotypic features, but differ most importantly in their pathogenicity. *U. parvum* is considered a commensal organism (Wetmore *et al.*, 2011) and is nevertheless detected more frequently in populations of patients (Waites, Katz and Schelonka, 2005). Ureaplasmas attach to epithelial surfaces of the urogenital tracts of adults, causing multiple morbidities including preterm delivery (PTD), urethritis in both men and women, postpartum endometritis, pneumonia, abscesses, meningitis et cetera (Waites et al., 2005).

First observations of *U. urealyticum* by electron microscopy, showed fried egg morphology, similar to that of other organisms in the Mycoplasmataceae family (Rottem, Pfendt and Hayflick, 1971; Black, Birch-Andersen and Freundt, 1972). In 1976, a new modified medium for the identification of *Ureaplasma* spp. was reported by Shepard and Lunceford. The colonies were described to resemble dark granules and have golden to deep-brown colours (Shepard and Lunceford, 1976).

Detection and identification of *Ureaplasma* species is based on culture methods or amplification of target genes. Conventional PCR assays have targeted urease genes or sequences of 16S rRNA, though real-time PCR assays targeted mainly the urease genes (Robertson *et al.*, 1993; Teng *et al.*, 1995; Kong *et al.*, 2000).

1.3.3 Association of *U. urealyticum* and *U. parvum* with genital complications in women

Ureaplasma spp. reside in the urogenital tract of healthy men and women (Kokkayil and Dhawan, 2015; Shah, 2017). Asymptomatic carriage of *U. urealyticum* is particularly higher in women, ranging between 40-80% compared to 20-50% in men (Deguchi *et al.*, 2004; Takahashi *et al.*, 2006; Yoshida *et al.*, 2007). One report found that *U. urealyticum* is less prevalent in young adolescents or children and the colonization of the genitourinary tract correlates with sexual activity and sex (Foy *et al.*, 1975; Shah, 2017). Vertical transmission of infection is observed in half of the mothers infected with *U. urealyticum* (Sanchez and Regan, 1987, 1990). Given the high rate of asymptomatic infections, a relationship between *U. urealyticum* and symptomatic manifestations has not been determined (Shah, 2017). There is accumulating proof that unlike *U. urealyticum, Ureaplasma parvum* is not associated with urethritis in men (Deguchi *et al.*, 2004; Takahashi *et al.*, 2006; Povlsen *et al.*, 2014). In pregnant women, the U. urealyticum has been associated with stillbirth, spontaneous abortion and preterm delivery. In preterm infants, invasive infections have been reported (Larsen and Hwang, 2010; Shah, 2017). In a study conducted in the Czech Republic in women with Preterm Premature Rupture of Membranes (PPROM), nearly 70% tested positive for cervix colonization by *U. urealyticum* (Kacerovský et al., 2009). Numerous studies on women who reported adverse pregnancy complications, confirmed a high incidence of abnormal bacterial flora and colonization by *U. urealyticum* and less by *M. hominis* (Kapatais-Zoumbos, Chandler and Barile, 1985; Kataoka *et al.*, 2006; Lee *et al.*, 2009; Pickler *et al.*, 2010). A study on women of childbearing age, testing for genital *Mycolasma* infections, identified *U. parvum* to be the most prevalent mollicute (Leli *et al.*, 2018). A common finding was reported by an australian study on sexually active women, confirming that *U. parvum* was the most commonly isolated mollicute (McIver *et al.*, 2009). The study by Kataoka *et al.* from 2006 detected *U. parvum* in higher frequencies in women in the early weeks of gestation. The authors conferred an association between the mollicute and early preterm birth and late abortion (Kataoka *et al.*, 2006). The clinical implication of *Ureaplasma* spp. in the genitourinary tract of females remains unclear, partly due to lack larger scale studies (Larsen and Hwang, 2010; Marovt *et al.*, 2015).

1.3.4 Antimicrobial susceptibility of *M. hominis* and *U. urealyticum* or spp.

Ureaplasmas and Mycoplasmas are intrinsically resistant to all beta-lactams, rifampicin and sulphonamides, due to the lack of a rigid cell wall (Taylor-Robinson and Bébéar, 1997; Waites et al., 2005). Resistance to macrolides varies according to species, with *M. hominis* being

susceptible to clindamycin and resistant to erythromycin and macrolides (Furneri *et al.*, 2000; Waites and Xiao, 2015). Resistance to fluoroquinolones and tetracyclines is more frequent among genital mycoplasmas and rarely observed in *M. pneumoniae* (Waites, Katz and Schelonka, 2005; Xie and Zhang, 2006). Described mechanisms of acquired resistance for mycoplasmas are acquisition of a transposon or chromosomal mutations (Li *et al.*, 2012; Waites and Xiao, 2015). In tetracycline resistant *Ureaplasma* spp. and *M. hominis* the presence of the *tet*(M) gene, is associated with resistance to all tetracyclines. Detection of resistant strains is conducted by in vitro susceptibility tests, or by PCR amplification of the *tet*(M) gene (Waites et al., 2005; Waites and Xiao, 2015). The prevalence of resistance to tetracycline, differs according to the population's exposure to the antibiotic. In the USA, a higher resistance to tetracycline among *Ureaplasma* spp. has been reported compared to studies from Europe (Waites et al., 2005; Dégrange *et al.*, 2008). A study on antibacterial resistance in genital mycoplasmas in young women reporting a urinary tract infection (UTI), revealed low antibiotic resistance levels (Valentine-King and Brown, 2017). Fluoroquinolones (a class of antibiotics) have been used extensively for treatment over the past few years, which as a consequence has led to reported infections by fluoroquinolone-resistant *M. hominis* and *Ureaplasma* spp (Xie and Zhang, 2006). In vitro susceptibility testing is advised particularly for *M. hominis* and *Ureaplasma* spp. which grow well in cell culture, unlike the slow-growing strains *M. genitalium* and *M. pneumoniae* (Shimada *et al.*, 2010).

1.4 M. genitalium

1.4.1 History of discovery

Examinations of smears obtained from 13 male patients diagnosed with NGU back in the 1980s, revealed the presence of microorganisms that appeared similar to spiroplasmas (D. Taylor-Robinson, 1995). Instead of spiroplasmas, two new strains of the class *Mycoplasma* were isolated (G-37 and M-30) from the specimen (Tully *et al.*, 1981). These strains were found to be related to *M. pneumoniae* but serologically different from all other known mycoplasmas. These findings resulted in the proposal that they be considered as belonging to a new species, *M. genitalium* (D. Taylor-Robinson, 1995; Taylor-Robinson and Jensen, 2011).

1.4.2 Morphology and genomic features

This bacterium has a flask, or bottle- shaped morphology, different from every other species of pathogenic *Mycoplasma* (Tully, Taylor Robinson and Rose, 1983).

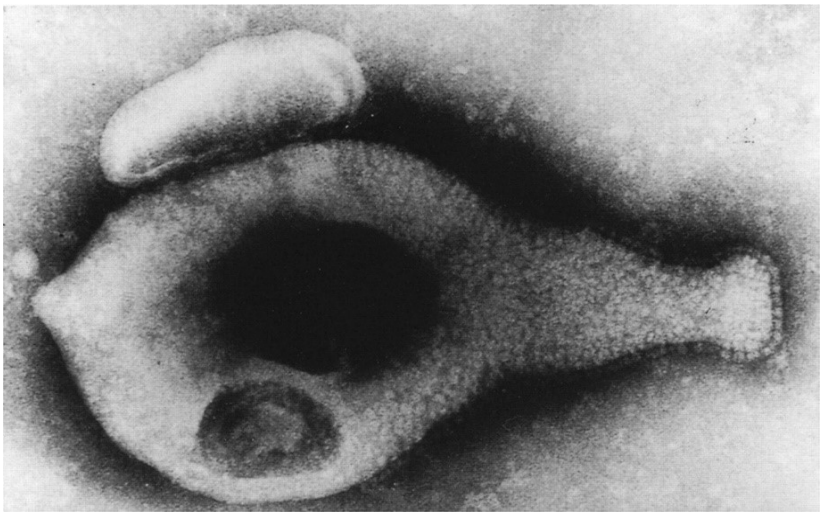


Figure 2. Electron micrograph of *M. genitalium* (original magnification x120,000) (Tully, Taylor Robinson and Rose, 1983).

The genome of *M. genitalium* is only 580kb in size, codes for approximately 500 genes and is regarded as the smallest organism capable of self-replication (*Taylor-Robinson, Jensen and Gilroy,* 2000). These characteristics gave rise to the minimal cell genome concept, where only the necessary structural elements and metabolic pathways are enclosed (Maniloff, 1996). Needless to say that *M. genitalium* evolved to have a parasitic way of life, receiving thus all the nutrients it cannot synthesise from the host (Taylor-Robinson and Jensen, 2011). The primary site of infection of *M. genitalium* is shown in Table 2. Other features include metabolism of glucose (unlike *M. hominis* or Ureaplasmas), a G+C content of 32% and very slow growth (Tully *et al.*, 1981; Tully, Taylor Robinson and Rose, 1983).

M. genitalium and *M. pneumonia*e have been shown to have many similarities. Nevertheless, these two mycoplasmas are genomically very distinct**.** The small genome size of *M. genitalium* facilitated the sequencing of the entire genome via whole genome shotgun sequencing (Fraser *et al.*, 1995). *M. genitalium* is an organism that hardly grows in culture, unlike other mycoplasmas which have a small genome. A dependent relationship on extrinsic supply has been reported (Fraser *et al.*, 1995; Himmelreich *et al.*, 1997; Razin, Yogev and Naot, 1998). The tip-like structure of the flask-shaped morphology in *M. genitalium* provides gliding motility and aids attachment to host cells (Sethi *et al.*, 2012).

1.4.3 Detection of *M. genitalium*

The flask-shaped morphology of *M. genitalium* was confirmed by electron microscopy (Kirchhoff *et al.*, 1984). As mentioned beforehand, detection of *M. genitalium* in culture takes time and is not always successful (Fraser *et al.*, 1995). In the early 1990s, a more sensitive approach for the detection of *M. genitalium* was developed; PCR targeting of different gene fragments, namely the MgPa gene (HM *et al.*, 1991; Jensen *et al.*, 1991). Nucleic acid amplification tests (NAATs) are highly sensitive methods crucial for *M. genitalium* detection, owed to the fact that in many patients, low levels of mycoplasmas are present (Jensen *et al.*, 2004c). Commercial PCR assays are accessible yet thus most laboratories use different methods depending on the type of specimen obtained from patients (Ross and Jensen, 2006). An earlier study (Jensen *et al.*, 2004a) showed that obtaining two or more specimens (e.g.: cervical swab and urine specimen) in women, increased sensitivity and specificity of diagnosis.

1.4.4 Association of *M. genitalium* in genitourinary tract disease.

M. genitalium infection in men and women can be asymptomatic. In a survey from the UK, more than half of female participants (56.2%) and almost all men (94.4%) did not report symptoms (Sonnenberg *et al.*, 2015). Also in a student cohort study (Oakeshott *et al.*, 2010), female participants who tested positive for *M. genitalium* infection did not exhibit symptoms such as pelvic pain or abnormal vaginal discharge. These data confirmed the hypothesis of older studies, claiming that *M. genitalium* infection in women is less prevalent compared to *C. trachomatis*. Many studies have confirmed an infection of *M. genitalium* in women with urethritis which attend STD centres (Anagrius, Loré and Jensen, 2005; Falk, Fredlund and Jensen, 2005; Moi, Reinton and Moghaddam, 2009). Presence of this organism in the urogenital track of women has been early detected by PCR, namely in two studies conducted in London and Copenhagen with prevalence of 20% and 6.7%, respectively (Jensen *et al.*, 1991; Palmer *et al.*, 1991). On average, 7 - 20% of women attending STI clinics and receiving treatment for such infections, resulted positive for a *M. genitalium* infection. *M. genitalium* has been identified in women showing signs of genital tract infections, or in women whose partners tested positive (Ross and Jensen, 2006). In contrast to *M. hominis*, *M. genitalium* is not associated with bacterial vaginosis (BV) (Palmer *et al.*, 1991 Taylor-Robinson, 1996), but rather with urethritis and cervicitis in women (Manhart *et al.*, 2003; Falk, Fredlund and Jensen, 2005).

Multi-locus sequence typing analysis was used in a study for sexual transmission and compared 31 couples undergoing *M. genitalium* infection with 74 unrelated patients. 87% (27/31) of the couples had a matching genotype unlike the group of unrelated patients (Ma *et al.*, 2008). The same trend was observed in a previous study which analysed the data from 19 couples jointly infected, were the same sequence types were found in the specimens of the

partners (Hjorth *et al.*, 2006). These two studies confirm the hypothesis that *M. genitalium* is a sexually transmitted infection (Gnanadurai and Fifer, 2020).

PID is defined as a complex disease that has multiple causative agents. Specimens of the upper genital tract are necessary for detection and in their absence, NAATs of pathogenic infections are a means of alternate diagnostics (Taylor-Robinson and Jensen, 2011). Up to 60% of women showing signs of genital tract infection and testing positive for *M. genitalium* after cervical specimen examination, also had positive detections from endometrial biopsy specimens (Haggerty *et al.*, 2008). Evidence that *M. genitalium* is a causative agent of PID, is the adherence to fallopian tube mucosal epithelial cells, observed in organ culture (Collier, A. M., et al. 1991). All data associating *M. genitalium* to pelvic inflammatory disease is rather inconclusive due to lack of studies showing a temporal relation between infection and the disease (Ross and Jensen, 2006), preferably in people already diagnosed with PID.

1.4.5 Antimicrobial Treatment, Susceptibility

One of the first antibiotics used to treat NGU in men was tetracycline (Waites, 2007). It has failed to eliminate *M. genitalium* from the urethra of male patients (Horner *et al.*, 2001; Maeda *et al.*, 2001; Falk, Fredlund and Jensen, 2003) and as a consequence a chronic infection persisted. In women, both doxycycline and cefoxitin did not have activity against endometrial infection with *M. genitalium* (Haggerty *et al.*, 2008). Macrolides, particularly azithromycin when prescribed, eliminated *M. genitalium* infection in up to 85% of male patients with urethritis (Björnelius *et al.*, 2008). It has been reported that some *M. genitalium* strains have mutated and have become resistant to azithromycin (Jensen *et al.*, 2008). A new quinolone, moxifloxacin has been effective in eradicating *M. genitalium* infection and presents a promising treatment alternative (Hamasuna, Jensen and Osada, 2009).

1.5 A new *Mycoplasma*-like species detected in the vaginal microbiota

Martin *et al.* (2013), performed a study in New Orleans at the STI centre on the role of *M. genitalium* in endocervicitis. 400 vaginal swab specimens (four swabs per patient) were obtained and analysed. Roughly 122 Operational taxonomic units (OTUs) were identified in this study and their respective prevalence in specimens positive and negative for *T. vaginalis* infection was compared. A *Mycoplasma* strain given the name “Mnola” was particularly prevalent in specimens positive for *T. vaginalis* infection. 16s rRNA gene sequences of Mnola reads were obtained and showed 85% similar identity to *M. genitalium* and 78% to *M. hominis*, (Martin *et al.*, 2013).

A year later, Fettweis *et al.*, (2014) investigated microbiome profiles based on the 16s rRNA gene for 1,361 vaginal specimens collected from women attending an STI centre as well as additional samples obtained from a laboratory and delivery unit. The authors reported that 96% (49/51) female carriers of *T. vaginalis*, exhibited a higher association with ‘Mnola’ compared to other vaginal microorganisms. 85% of women who carried Mnola were *T. vaginalis* positive. The previously described organism, now named *Candidatus* Mycoplasma girerdii was strongly associated with both genotypes of *T. vaginalis*. It is hypothesized that a dependent relationship exists between the two organisms (Fettweis, Serrano, *et al.*, 2014).

1.6 *Trichomonas vaginalis* discovery and morphology

T. vaginalis is a flagellated parasitic extracellular protozoan that infects the urogenital tract of humans. It was first identified in Paris, by Alfred François Donné in 1836 from leukorrhea of a women who had symptoms of vaginal irritation (Harp and Chowdhury, 2011). It was Donné himself who would later establish the microorganism as a protozoan found in the urogenital tract of women (Diamantis, Magiorkinis and Androutsos, 2009).

This pathogen has a variable shape and size; depending on the conditions exposed to. On average, the parasite has a length of 10 μm and a width of 7 μm (Petrin *et al.*, 1998). *T. vaginalis* has four anterior located flagella and a fifth flagellum that joins the undulating membrane, a large nucleus and an axostyle that divides the parasite longitudinally. All flagella are involved and contribute to the twitching motility of T. vaginalis (Petrin *et al.*, 1998; Benchimol, 2004).

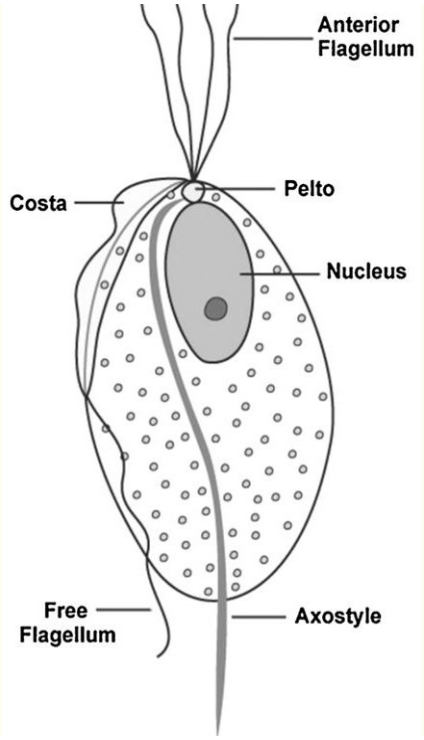


Figure 3. *T. vaginalis* fine structure at the trophozoite stage (Harp and Chowdhury, 2011).

A pseudocyst stage of *T. vaginalis* was observed under unfavourable conditions, lacking cell wall formation and internalized flagella. Although formation of pseudocyst is to date considered a degenerate state, it was found in culture under natural conditions and capable of division (Pereira-Neves, Ribeiro and Benchimol, 2003).

The infective, motile form of *T. vaginalis* is the trophozoite. The organism has a typical piriform shape and recurrently amoeboid shape (Harp and Chowdhury, 2011). *T. vaginalis* is an obligate parasite, able to phagocytose bacteria, epithelial cells in the vaginal tract and red blood cells (Petrin *et al.*, 1998). Upon *in vivo* contact with epithelial cells from the cervix, vagina, prostate and proteins of the extracellular matrix (ECM), *T. vaginalis* can alter its shape, thus increasing the contact surface (Harp and Chowdhury, 2011; Fiori *et al.*, 2016). The amoeboid form, first reported in the early 1960s (Honiberg and King, 1964), is nowadays known as the most virulent form of *T. vaginalis*.

Table 3. Classification of *T. vaginalis* (*Donné, 1836*; Aquino et al., 2020).

Scientific classification	
Kingdom:	Protozoa
Phylum:	Parabasalia
Class:	Trichomonadea
Order:	Trichomonadida
Family:	Trichomonadidae
Genus:	*Trichomonas*
Species:	*T. vaginalis*

1.5.1 Detection of *T. vaginalis*

Clinical manifestations of trichomoniasis resemble the symptoms of other urogenital infections such as with *C. trachomatis* and *N. gonorrhoeae*, which makes diagnosis of the disease based on symptoms only, not reliable (Petrin *et al.*, 1998). Laboratory diagnoses are necessary for an accurate detection of *T. vaginalis* infection. The pathogen was first detected in 1837 by wet mount microscopy. This detection method is used worldwide, has minimal cost and results are obtained immediately. However, low sensitivity was reported, especially in samples of male patients (Huppert *et al.*, 2005; Hobbs and Seña, 2013; Poole and McClelland, 2013). Various culture media for *T. vaginalis* have been established over the years, and the one used most frequently is the Diamond's (TYM) medium and modified variants (Diamond, 1957; Stary A. *et al.*, 2002). Liquid medium cultures ought to be incubated at 37°C and examined daily. Three to five days is the period of examination to observe growth for cultures from women and men, respectively.

NAATs like PCR and transcription-mediated amplification (TMA) are widely used nowadays in *T. vaginalis* diagnostics (Hobbs *et al.*, 2006). These diagnostics methods are highly sensitive and up to 100% specific (Chapin and Andrea, 2014). Sterile swabs are used to examine vaginal, urethral and endocervical regions. This method is reliable in asymptomatic cases of both men and women, thus being a useful testing tool in routine STD screening (Poole and McClelland, 2013; Van Gerwen and Muzny, 2019). Rapid diagnostic tests are non-culture based methods, with sensitivity rates between 50-100%. Two important aspects are the non-requirement of additional equipment and no highly trained staff is needed to carry out the tests. Currently, no such tests are available for diagnosis in male patients (Huppert *et al.*, 2005; Hobbs and Seña, 2013; Chapin and Andrea, 2014).

1.5.2 *T. vaginalis* pathogenicity

T. vaginalis infects primarily the squamous epithelium in the male urethra and prostate and the female lower genital tract (Kissinger, 2015a). It is a persistent pathogen, able to cause dysbiosis of the urogenital microflora of women, tissue damage and increase inflammation levels of the host (Mercer and Johnson, 2018). *T. vaginalis* is almost always transmitted through sexual intercourse (Edwards *et al.*, 2016). Non-sexual transmission has been discussed for many years and data presented has been inconclusive. Vertical transmission has been reported in several case studies, causing the infant respiratory and urogenital infections (Carter and Whithaus, 2008; Schwandt, Williams and Beigi, 2008; Costello *et al.*, 2017). In order for the protozoan to initiate infection, it has to adhere to genital epithelial cells with the help of surface adhesins. The latter are responsible for cytoadherence which concomitantly with cytotoxicity lyse mucosal cells and secrete numerous proteases (Edwards *et al.*, 2016). In the recent years, a higher number of factors which directly aid adherence in *T. vaginalis* have been identified (Leitsch, 2016).

1.5.3 The symbiotic relationship between *T. vaginalis* and *M. hominis*.

A symbiotic relationship between the protozoan *T. vaginalis* and *M. hominis* has been documented (Taylor-Robinson *et al.*, 1998; Rappelli *et al.*, 2001). The first observation of the potential symbiosis was made by Nielsen and dates back to 1975. *M. hominis* can enter, reside and even proliferate within trichomonad cells, confirming thus a closer cooperation than originally thought (Rappelli *et al.*, 2001; Dessì *et al.*, 2005). Both *T. vaginalis* and *M. hominis* metabolize arginine in anaerobic conditions and as the main source of energy respectively (Yarlett *et al.*, 1996). Co-cultures of *T. vaginalis* infected with *M. hominis* showed increased metabolism of arginine, suggesting a mechanism in place that enables their association (Morada *et al.*, 2010). Infected trichomonad cells were shown to produce higher ATP levels per cell *in vitro* and were highly haemolytic when compared to *M. hominis*-free trichomonads (Margarita *et al.*, 2016). This association between these two species might have a direct effect on evading the host's immune response. Two studies from the same research group have documented that the pro-inflammatory monocytes have an upregulated response to *T. vaginalis* when the mollicute is present. On the other hand, symbiosis with the protozoan is beneficial for *M. hominis* being protected from environmental conditions and from subsequent antibiotic treatments (Dessi *et al.*, 2006; Morada *et al.*, 2010).

Metronidazole and tinidazole are approved antibiotics for *T. vaginalis* treatment (Hager *et al.*, 1980). It is suggested that a co-infection with the bacterium *M. hominis* increases metronidazole resistance of the protist (Rappelli *et al.*, 2001). Several studies have backed up this hypothesis and a correlation between *M. hominis* infection and resistance has been shown (Xiao *et al.*, 2006; Butler, Augostini and Secor, 2010; da Luz Becker *et al.*, 2015). Fürnkranz *et al.* (2018) reported that *M. hominis* had a direct impact on the downregulation of genes of *T. vaginalis*, important for metronidazole susceptibility. A strain-dependent tolerance to the antibiotic, being higher in artificially infected *T. vaginalis* strains was observed (Fürnkranz, Henrich and Walochnik, 2018a).

1.5.4 Antimicrobial Treatment

Metronidazole (MTZ) is a drug used for over five decades against trichomoniasis and found to be very effective (Nanda *et al.*, 2006; Wendel and Workowski, 2007). Once administered, MTZ cell entry occurs through diffusion (Muller and Lindmark, 1976); resulting in hindering motility, cell division and subsequent cell death (Nielsen, 1976).

The standard trichomoniasis treatment according to the World Health Organization (WHO) and the United States Center for Disease Control and Prevention (CDC), is 2gm single dose of MTZ or Tinidazole (TNZ) given orally. The alternative treatment regimen is 400-500 mg MTZ, twice a day for seven days (*Trichomoniasis - STI Treatment Guidelines*, 2021; Kissinger and Muzny, 2019; Workowski *et al.*, 2021). The patient together with their sexual partner should be treated simultaneously (Underhill R. A., Peck J. E., 1974; Hager *et al.*, 1980). Failure of trichomoniasis treatment is common and the main cause is resistance of *T. vaginalis* to the drug (Lumsden *et al.*, 1988). The increasing resistance was first reported in 1962, followed by an estimation by the Centre for Disease Control and prevention (CDC) that 5% of all *T. vaginalis* isolates from patients had a certain resistance to MTZ (Narcisi and Secor, 1996). TNZ is a 5-nitrimidazole also used to treat trichomoniasis and has a longer half-life compared to metronidazole, thus prescribed for a shorter time span (Wood and Monro, 1975). Recurrent cases, previously treated with metronidazole require either higher doses of the latter or a tinidazole course of treatment (Nielsen, 1976; Narcisi and Secor, 1996). According to the CDC (*Trichomoniasis - STI Treatment Guidelines*, 2021), women who have received treatment for *T. vaginalis* infection and are sexually active, should get a follow-up test after three months.

1.7 Aims of the study

In the Outpatients Centre for Diagnosis of Venero-dermatological infections in Vienna (OCD), patients are routinely screened for *T. vaginalis* and *Mycoplasma* spp. by culture methods (Stary et al., 2002) and by PCR.

This study solely focused on obtaining vaginal specimens from female patients. The main aim was to conduct molecular based analyses of each sample, thus assessing the prevalence of genital *Mycoplasma* infections, namely *Mycoplasma hominis*, *Candidatus* Mycoplasma girerdii, *Mycoplasma genitalium*, *Ureaplasma parvum* and *U. urealyticum*.

In parallel, isolates of *T. vaginalis* collected at the OCD were cultured at the Institute for Specific Prophylaxis and Tropical Medicine (ISPTM) with the aim to characterize the isolates by molecular methods as well as to screen the specimen positive for *T. vaginalis* for the presence of mycoplasmal symbionts and determine whether symbionts are harbored intracellularly.

The third aim was to screen vaginal specimens and isolates of *T. vaginalis* collected from patients attending the OCD with published *Ca*. M. girerdii primers (Fettweis *et al.*, 2014) to determine whether these primers could be used for diagnostic purposes.

2 MATERIALS AND METHODS

2.1 Study setting / Collection of Clinical Specimens

A total of 581 clinical specimens from adult women, namely 319 samples in April (Phase 1) and 262 samples in September 2021 (Phase 2) were collected. The women enrolled into this study were patients attending the Outpatients Centre for Diagnosis of Venero- Dermatological Diseases (OCD) in Vienna. Vaginal swabs were obtained during routine pelvic examinations. Every sample was anonymized. As the study was retrospective, no patients' consent was necessary. The Ethics Committee of Medical University of Vienna approved the study (EK No: 1965/2017).

2.2 DNA isolation from vaginal swab specimens

Vaginal swab material was transferred into small collection tubes containing 2 ml PBS at the OCD and then transported to the ISPTM for further use of which 1 ml was pipetted into a 1.5 ml micro test tube and used for DNA extraction.

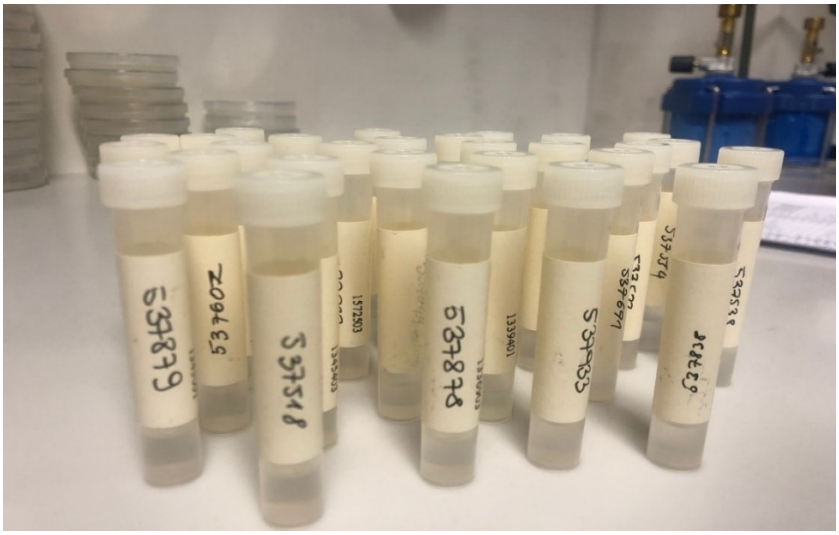


Figure 4. Vaginal swab specimens of patients transferred into the collection tubes.

The QIAamp ® DNA Mini Kit 250 (QIAGEN, Hilden, Germany) was used for isolation. To the micro test tube 30 µL Proteinase K was added, mixed by vortexing, centrifuged at 6000 x g for 2 minutes and incubated at 56°C in a thermomixer for 10 minutes. The mixture was centrifuged for 1 min at 6000 x g. The supernatant was discarded and 400 µL 1x PBS and 400 µL AL were added to the micro test tubes containing the cell sediment, followed again by pulse vortexing and incubation at 56°C in a thermomixer for 10 minutes. Next, 700 µL of the mixture were transferred onto a spin column placed in a 2 mL collection tube and centrifuged for 1min at

6000 x g. This centrifugation step was repeated by replacing the 2 mL collection tube and applying the rest of the mixture to the spin column. Thereafter, two washing steps were performed by adding 500 µL AW1 & AW2 wash buffers and centrifuging at 6000 x g and 20,000 x g for 1min and 3 minutes respectively. Subsequently AE Elution buffer was added, and the suspension was centrifuged at 6000 x g resulting in eluted DNA in a final volume of 100 µL. The DNA eluate was stored at -20°C.

Schematic depiction of the workflow.

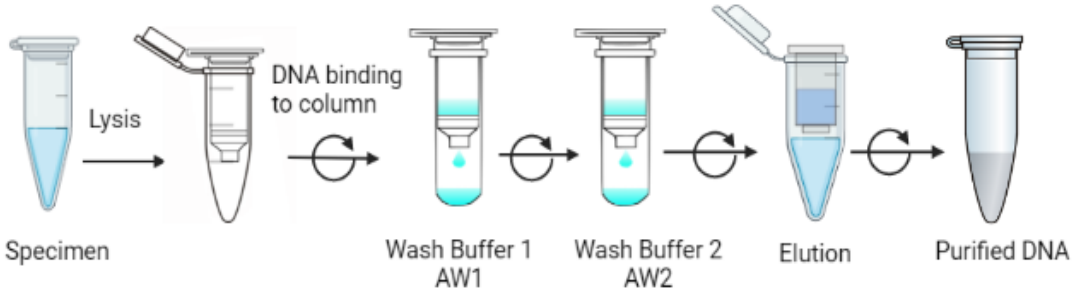


Figure 5. DNA purification procedure, consisting of five steps. The image is an adaptation of the DNA extraction scheme by QIAamp ®.

2.3 Specific mollicute primers

Different sets of published primers were used for amplification and sequencing analysis during the study, i.e. the GPO-1 and MGSO set of primers (Tab.4) according to the designs by Kuppeveld *et al.* (1993) Furthermore, a set of primers specific for *M. genitalium* (Tab.5; Jensen *et al.*, 1991) were used.

Table 4. Primers for *Mycoplasma* spp. / *Ureaplasma* spp. targeting the Mycoplasma genus-specific16S rRNA gene (Kuppeveld *et al.*, 1993).

Primer	Sequence (5' - 3')	Product (bp)
GPO-1	ACTCCTACGGGAGGCAGCAGTA	
MGSO	TGCACCATCTGTCACTCTGTTAACCTC	717

Table 5. Primers for *M. genitalium* targeting the MgPa gene (Jensen *et al.*, 1991).

Primer	Sequence (5' - 3')	Product (bp)
MgPa1	AGTTGATGAAACCTTAACCCCTTGG	
MgPa3	CCGTTGAGG GGT TTT CCATTTTTGC	282

2.3.1 Specific primers for *Candidatus* Mycoplasma girerdii

Six sets of specific primers for *Candidatus* Mycoplasma girerdii (Tab. 6; Fettweis *et al.*, 2014) were used to determine whether these sets of primers are suitable for diagnostic purposes.

Table 6. List of *Ca*. M. girerdii primers (Fettweis *et al.*, 2014).

Primer Name	Sequence (5'-3')	Product (bp)
M1_Contig2-231-7_F	TGGTTCAATGTGAGAGCAATC	
M1_Contig2-231-7_R	TGACTTCAAAATCTTCTTCACTGT	453
M1-Contig4-1409-10_F	AATTCGATCAGCCATTCTATTTGG	
M1-Contig4-1409-10_R	GATTCTTGGTGGGAACTTACAG	625
M1-10-X-6_F	TTTGTGCAATGTATTCGCCCTATG	
M1-10-X-6_R	ATGGAAGCCACTTTCCTTTTGATG	1093
M1-10-X-6_Internal_F1	CAATATACAGGAACATTTACTCGATAC	
M1-10-X-6_Internal_R1	GGAACTCAATCATAAGTTTGATTTC	539
M1_Contig1409-10_F2	GCTGTAAGTTCCCACCAAG	
M1_Contig1409-10_R	CAAAGCAATGCATACAAGTGAG	616

2.4 Polymerase chain reaction (PCR)

The amplification of target genes was carried out following the PCR protocol by Mullis and Faloona (1987). In brief, PCR amplification was set up in a reaction volume of 50 µL containing 2.5 mM $MgCl_2$, 10x reaction Buffer B, 1.25 units of DNA polymerase, 1.6 µM dNTPs, 10 µM primers, 1-3 µl DNA and finally autoclaved sterile H_2O was added.

Table 7. Typical PCR reaction set-up.

Components and Concentration	**Volume**
H_2O	11 µL
Reaction Buffer B 10x	5 µL
MgCl2 2.5 mM	5 µL
dNTPs 1.6 µM	1 µL
Primer 1 10 µM	5 µL
Primer 2 10 µM	5 µL
DNA polymerase 1.25 units	0,25 µL
Master Mix	**32.25 µL**
DNA	1-3 µL
H_2O fill up to	**50 µL**

PCR conditions used for *Mycoplasma* and *Ureaplasma* spp. were as follows: 95 °C for 15 min, followed by 35 cycles of 95 °C for 1 min (denaturation), annealing at 56 °C for 2 min, elongation at 72 °C for 3 min and the final extension for 7 min at 72 °C.

2.5 Agarose-gel electrophoresis and band purification

2% Agarose gels containing 1x TAE Buffer (0.04 M Trisacetate & 0.001 M EDTA) and agarose (Sigma-Aldrich, St. Louis, MO, USA) were used to separate the PCR products. To visualize the DNA, 25 µL or 50 µL GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) were added for 50 mL and 100 mL gels, respectively.

Each sample was prepared by adding 5 µL of Gel Loading Buffer (dye) (Sigma-Aldrich, St. Louis, MO, USA) and 25 µL of the PCR product, to a final volume of 30 µL and then loaded onto the gel. To determine the size of DNA fragments that were amplified, 25 µL of the DirectLoad™ 50 bp DNA Step Ladder marker (Sigma-Aldrich, St. Louis, MO, USA) was loaded on the first row. The gel was then subjected to electrophoresis and was run at a current of 300 mA and 130 V tension.

The Gel DocTM XR+ Imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to analyse the bands, which were subsequently cut out from the gel and purified using the Gel Purification Kit, Illustra™ GFXTM PCR DNA (GE Healthcare, Buckinghamshire, UK), following strictly the manufacturer's instructions. Finally, bands were eluted to a final volume of 20-50 µL as deemed necessary and stored at -20°C.

2.6 Sanger sequencing

The sequencing PCR reaction contained 1-3 µL of PCR product, 2 µL AB Mix (BigDye™ Terminator v1.1 Cycle Sequencing RR-100, Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), 1 µL Sequencing Buffer (BigDye™ Terminator v1.1, v.3.1, Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), 2 µL of either forward or reverse Primer pair and sterile H_2O to a final volume of 10 µL.

Table 8. Reaction setup of sequencing PCR.

Sequencing PCR	
AB Mix	2 µL
PCR Product	1-3 µL
Primer	2 µL
Sequencing Buffer (AB Big Day Terminator)	1 µL
Sterile water	Fill up to **10 µL**

The conditions followed: 96°C for 30 sec. then 30 cycles for 96°C for 5 sec (denaturation), followed by 50°C for 5 sec (annealing) and finally 60°C for 4 min (elongation).

The PCR products were then transferred into a micro test tube containing 1 µL Sodium acetate, and mixed with 40 µL 100% ethanol, incubated on ice for 17 minutes and later centrifuged for 30 minutes at 12,000 xg at 4°C. The supernatant was then discarded and 90 µL of 70% ethanol was added and subjected to centrifugation for 10 minutes at the conditions previously stated. After the second centrifugation step, ethanol was removed from each sample and 20 µL Hi-DI™ Formamide was added followed by an incubation for 4 minutes at 94°C and a second incubation at -20°C. Samples were loaded to sequencing tubes and subjected to sanger sequencing.

The procedure was performed using the Applied Biosystems SeqStudio Genetic Analyzer (Thermo Fischer Scientific, Waltham, MA, USA). Sequences from the forward and reverse strands were obtained and the Prabi Software was used to generate contigs of sequences for each sample. To obtain sequence identity, the sequences were compared to available sequences in GenBank using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequence alignment (Figure 16, Results), was edited in GeneDoc 2.7.0 (*Nicholas* 1997) and for identification of variable and conserved regions MEGAX was used (Kumar *et al.*, 2018). The sequences downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) were used as reference and were aligned with Clustal (Larkin *et al.*, 2007).

2.7 *Trichomonas vaginalis* samples from pure cultures

In this study, 20 *Trichomonas vaginalis* samples also obtained from patients through collection of swab specimens, were included for *T. vaginalis* culture. In short, vaginal swabs were smeared onto *T. vaginalis* specific agar plates (Stary et al., 2002) - cultivated for 5 days at 37°C and examined microscopically for the presence of *T. vaginalis* at the Outpatients Centre. Positive samples were then transferred into liquid TYM Medium (Trypticase-Pepton Medium) and cultured at 37°C micro-aerobically.

2.7.1 Growth and selective media for *T. vaginalis*

T. vaginalis positive samples were cultured in modified Diamond's TYM medium (Diamond L. S. 1957) (Table 9) containing: Trypticase peptone (BD, 211921), Yeast extract (Merck KGaA 8013-01-2), Maltose (Merck KGaA 6363-53-7), L-Ascorbic Acid (A0278 Sigma-Aldrich, St. Louis, MO, USA), Fetal calf serum (FCS) Horse serum (1133C Sigma-Aldrich, St. Louis, MO, USA), MEM Vitamin Solution (100X) (M6895 Sigma-Aldrich, St. Louis, MO, USA), Antibiotic Antimycotic Solution (100X), stabilized (contained Antibiotics: Amphotericin B, Streptomycin, Penicillin) (A5955 Sigma-Aldrich, St. Louis, MO, USA) and Cysteine (Sigma-Aldrich, St. Louis, MO, USA).

Table 9. List of reagents used in the preparation of TYM medium.

Reagents	Volume
Trypticase peptone	20 g
Yeast extract	10 g
Maltose	5 g
L-Ascorbic Acid	0.2 g
MEM Vit. Solution	30 mL
FCS Horse serum	100 mL
AA Antibiotics and antifungal agent	10 mL
Cysteine	1 g
Demineralized Water	860 mL

Aliquots were frozen and subsequently thawed when needed. Chemicals were dissolved in a 1L conical flask in 860 mL of demineralized water. The flask was placed on a magnetic stirrer until the components were dissolved and the fluid got a yellowish colour. The pH was adjusted to 6.0 with 20% hydrochloric acid (HCl). The medium was sterilized under the hood using Filtropur BT 25, Bottle top filters (volume 250mL), PES, 0.2 µm (Sarstedt AG & Co. KG) with the help of a vacuum pump. The medium was either stored at 4°C or frozen at -20°C and was warmed up to 37°C before use.

Figure 6. *T. vaginalis* strains cultured micro-aerobically in selective TYM medium, in 35mL tissue culture flasks.

In phase II of the study, Modified Columbia Agar (MCA) was used for the isolation of *T. vaginalis* from clinical samples at the Outpatients Centre (Stary et al., 2002). MCA medium containing Columbia Blood Agar Base (Oxoid Ltd, Basingstoke, Hampshire, England), KH_2PO_4 0,4 g (Merck KGaA 64721, Darmstadt, Germany), K_2HPO_4 0,2 g (Merck KGaA 64721, Darmstadt, Germany), Cysteine 0,4 g (Sigma-Aldrich, St. Louis, MO, USA), Demineralized Water 350 mL, HCl 0,5 mL was dissolved, sterilized by autoclaving for 20 minutes at 121°. The solution was let to cool and upon reaching 50°C it was mixed with FCS Horse serum 50 mL (Sigma-Aldrich, St. Louis, MO, USA), Antibiotic Antimycotic Solution (100X), stabilized (contained Antibiotics: Amphotericin B, Streptomycin, Penicillin) 4 mL (A5955 Sigma-Aldrich, St. Louis, MO, USA), Nystatin solution (10,000 U/ml; Biochrom KG, Berlin, Germany), Chloramphenicol (50mg/mL in EtOH) (Carl-Roth GmBH + Co KG Karlsruhe DE), Dextrose 4 g (Sigma-Aldrich, St. Louis, MO, USA) and Maltose 4 g (Sigma-Aldrich, St. Louis, MO, USA). 10-15 mL of the mixture was poured onto 4/6-cm-diameter petri dishes (Stary et al., 2002). Plates were stored at 4°C and were allowed to reach room temperature before use.

2.7.2 DNA isolation from *Trichomonas vaginalis* culture

DNA extraction from *Trichomonas vaginalis* liquid culture was performed as follows: One tissue culture flask (35ml culture) was centrifugd at 600 x g for 10 minutes. The supernatant was discarded and the pellet was resupended in 20 mL sterile 1X phosphate buffered saline (PBS) followed by a second centrifugation step at 600 x g for 10 minutes. The supernatant was discarded again and the cell sediment was resuspended in 20 µL PBS. DNA isolation was

performed using the QIAamp ® DNA Mini Kit 250 (QIAGEN, Hilden, Germany). The subsequent DNA isolation steps have been described above in (DNA isolation from vaginal swab specimens).

2.7.3 Specific primers and PCR reaction set-up for *T. vaginalis*

Table 10. Primers targeting *T. vaginalis*-specific repeat DNA fragment (Pillay *et al.*, 2007).

Name	Sequence (5'-3')	Product (bp)
TVK-3	ATTGTCGAACATTGGTCTTACCCTC	
TVK-7	TCTGTGCCGTCTTCAAGTAGC	261

PCR reaction set-up shown in (Table 7). The following PCR conditions for *T. vaginalis* were used: 95 °C for 15 min, followed by 35 cycles of 95 °C for 1 min (denaturation), (annealing) at 52 °C for 1:30 min and (elongation) at 72°C for 2 min and finally the extension for 10 min at 72°C.

2.8 Microscopic Analyses

2.8.1 Detection of *Mycoplasma hominis* and *Ureaplasma urealyticum*

Vaginal swabs obtained from patients during check-up were smeared into plates containing A7-Mycoplasma agar. Upon observation of grown colonies of *M. hominis* and *U. urealyticum* on *Ureaplasma* spp. / *Mycoplasma* spp. plates (bioMérieux, Marcy-l'Etoile, France), the organisms were microscopically examined, using an Olympus Bx41 microscope (Olympus Europa SE & Co. KG).

2.8.2 Microscopic analysis of *T. vaginalis*

Microscopic observations were made by identifying motile organisms after 48-72 hours. Thereafter, the microscopic analysis of *T. vaginalis* was conducted. 30-40 µL of the culture were placed onto a microscopic slide, covered with cover glass and examined at a magnification of 1000x using a Nikon Eclipse E800 microscope (Nikon Instruments Inc., USA).

3 RESULTS

3.1 Microscopic analysis

In Figures 7 and 8 colonies of *Ureaplasma* spp. and *Mycoplasma hominis* are shown. Morphological features of respective microorganism's colony were observed.

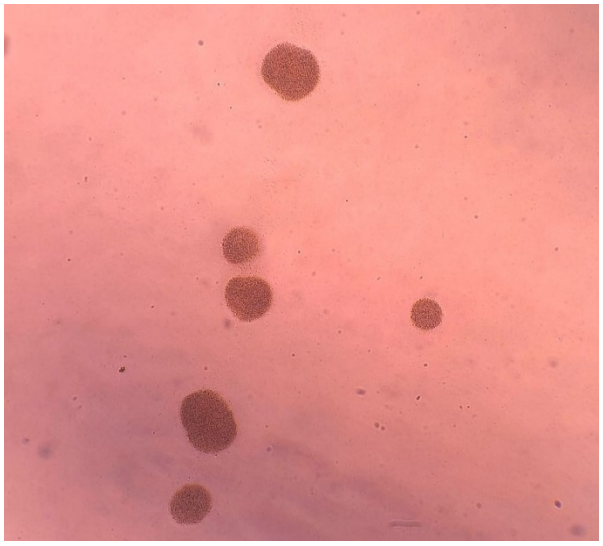


Figure 7. *Ureplasma* spp. colonies exhibiting dark granulated appearance. The photo was taken under 1000x Magnification using an Olympus Bx41 microscope.

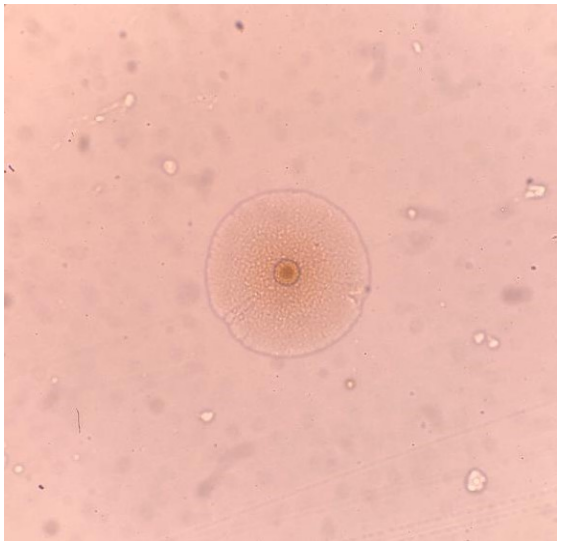


Figure 8. *Mycoplasma hominis* colony exhibiting a fried-egg appearance. The photo was taken using an Olympus Bx41 microscope. Magnification: 1000x.

In Figure 9, *T. vaginalis* in direct microscopy is shown. The strain, originally obtained from a patient testing positive for *T. vaginalis* at the OCD, was cultured in selective TYM Medium at the ISPTM.

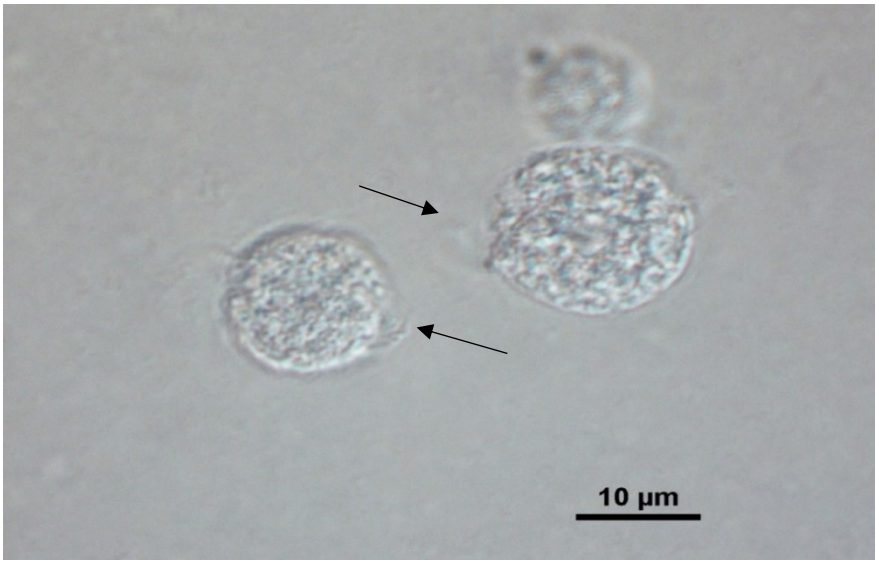


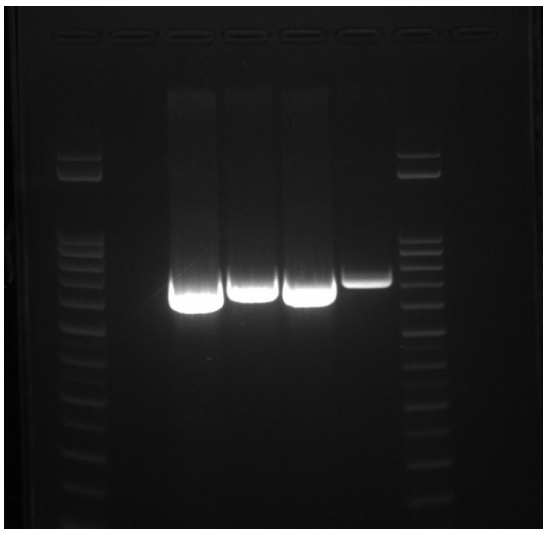
Figure 9.*Trichomonas vaginalis* from a cultured strain. Arrows indicate the flagella. Magnification 1000x.

3.2 Molecular analysis

3.2.1 PCR Results

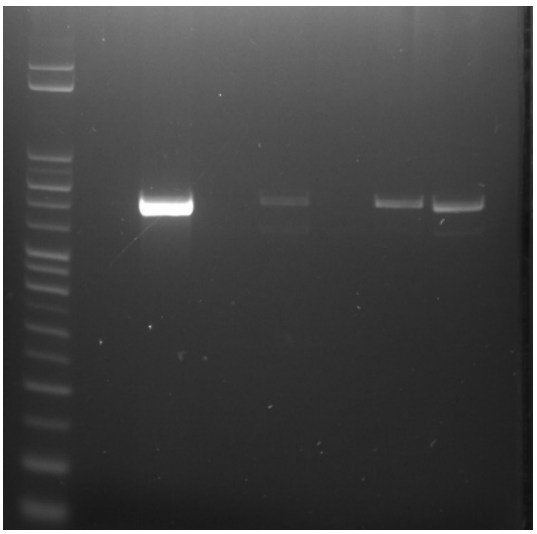
Conventional PCRs with specific *Mycoplasma* spp. primers were run for all 582 swab specimens obtained. *T. vaginalis* (TV) culture isolates as well as *T. vaginalis* positive specimens were analyzed by PCR with TV primers. Visible gel bands of PCR products were excised from the electrophoretic gel for subsequent DNA sequencing.

In figures 9 and 10 two examples of PCR amplification are shown. In figure 7 only DNA samples of *Mycoplasma* spp. were used to confirm the accuracy of the 16S rDNA primes. In figure 9 and 10, the first amplified samples of patients of Phase 1 of the study are included.



M - Mh Mh2 Uu Up M

Figure 10. *Mycoplasma* / *Ureaplasma* spp. PCR results with 16S rDNA primers. Only *Mycoplasma* spp DNA, (positive controls) were used as samples, namely Mh 1 & 2= *M. hominis*, Uu= *U. urealyticum* and Up= *U. parvum*; M=marker, - = negative control.



M - + 1 2 3 4 5

Figure 11. PCR amplification with MGSO and GPO-1 (16S rDNA) primers. Each slot is labelled with the sample number. M=marker, - = negative control, + = positive control.

The PCR results of the collected samples (swab specimens) amplified with 16S rDNA *Mycoplasma* spp., *T. vaginalis* and *M. genitalium* specific primers shown in figures 12, 13 and 14. Successfully amplified samples were obtained at the correct length according to the respective target genes. Bands of sample 1 (Figure 12) in the upper and lower part of the gel represent the successful amplification of *Ca*. M. girerdii 16S rRNA gene and *T. vaginalis* gene. Bands of samples 3 and 5 represent the amplification of *M. hominis* and *T. vaginalis* in the upper and lower part of the gel, respectively.

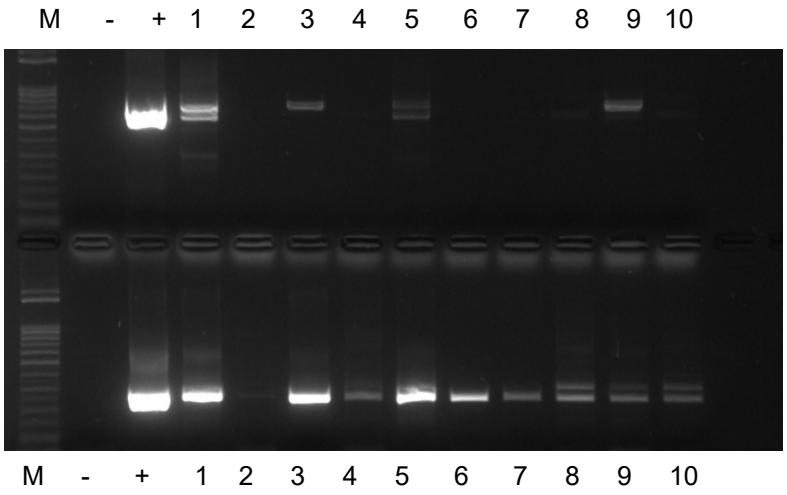


Figure 12. 10 samples were amplified with *Mycoplasma* spp. 16S rDNA and *T. vaginalis* primers. The PCR products are shown respectively in the upper part and lower of the gel. Both sets of slots are labelled with the sample numbers. Sample 1, 3 and 5 are positive for both *T. vaginalis* and *Mycoplasma* spp. M=marker, - = negative control, + = positive control.

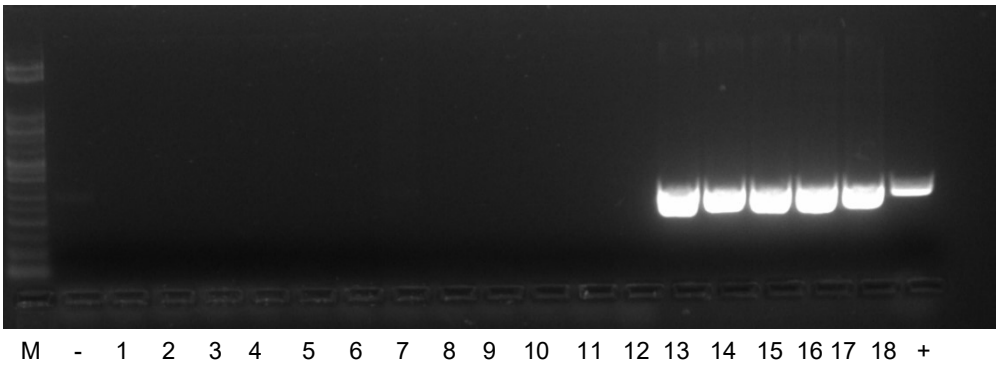


Figure 13. *M. genitalium* PCR results. Each slot is labelled with the sample numbers. M=marker, - = negative control, + = positive control.

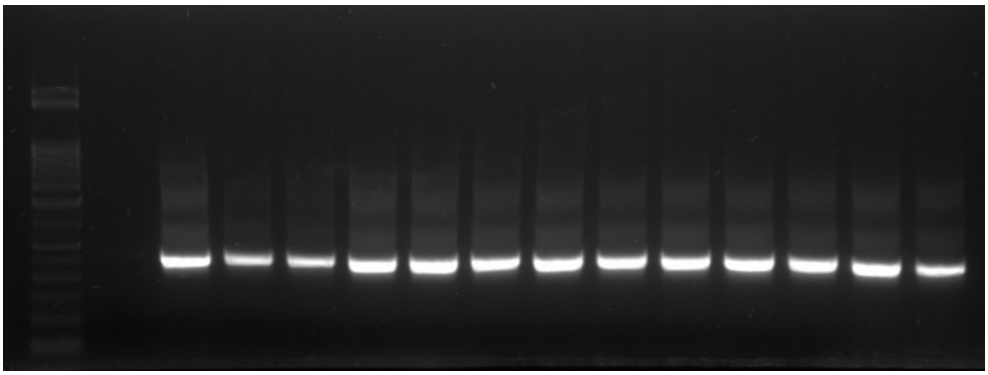


Figure 14. *T. vaginalis* PCR results of TV isolates. Successful amplification by PCR of *T. vaginalis* isolates of interest with TVK-3 and TVK-7 primers (Pillay *et al.*, 2007). All 12 analyzed samples were positive for *T. vaginalis* targeted gene, hence amplicons of the correct sizes were obtained Wells are labelled according to the samples numbers. M=marker, - = negative control, + = positive control.

Amplification with five sets of *Ca*. M. girerdii primers of respectively the TV isolates and swab specimens was unsuccessful.

3.3 Data collection and characteristics of vaginal swab specimens

3.3.1 Phase I of the study

From April to May 2021, vaginal swab specimens were obtained from 319 female patients attending the OCD. In the first phase of the study, one vaginal swab was obtained from each female patient for molecular analysis. The vast majority of patients attending the Clinic reported genital tract infection symptoms, discharge or pain. In parallel, examinations of the samples at the Outpatients Centre detected *Candida* spp., *Mycoplasma genitalium, Ureaplasma* spp*., Chlamydia trachomatis, Neisseria gonorrhoea*, *Gardnerella vaginalis* and *Prevotella* spp. The Amine Test was also performed for each specimen and if positive, together with an elevated vaginal pH as well as presence of clue cells in the microscopic examination, would be suggestive of BV.

Table 11. Characteristics of specimens in the first study phase.

Total number of female patients	**319**
Available data on pH and amine tests	312
Age (mean age)	18–84 (37)
Positive for mollicutes	86/319 (27%)
Genital Candidiasis	38/319 (12%)
Positive Amine Tests	8 (2.5%)

Table 12. Summary of data from patients diagnosed with BV.

BV	**MH**	**Mean Age**
8	2 (25%)	29 (20–45)

3.3.1.1 Prevalence of genital mycoplasmas in swab specimens

Genital mycoplasmas were detected in 86 samples by PCR and subsequent sequencing. *M. hominis* (MH) was detected in 59 (18.5%), whereas uncultured bacteria (according to Genbank) were detected in 8 (2.5%) of the samples. Ureaplasmas were the second most abundant group with 16 (5%) *U. parvum* and 2 (0.6%) *U. urealyticum* detected. *Ca*. M. girerdii sequence was detected in one vaginal swab. Due to collection of a single swab specimen per patient, detection was confirmed only by PCR and not by culture. No *M. genitalium* was detected by PCR with specific primers nor with the primers for the *Mycoplasma* spp. 16S rDNA gene in the first phase of sampling.

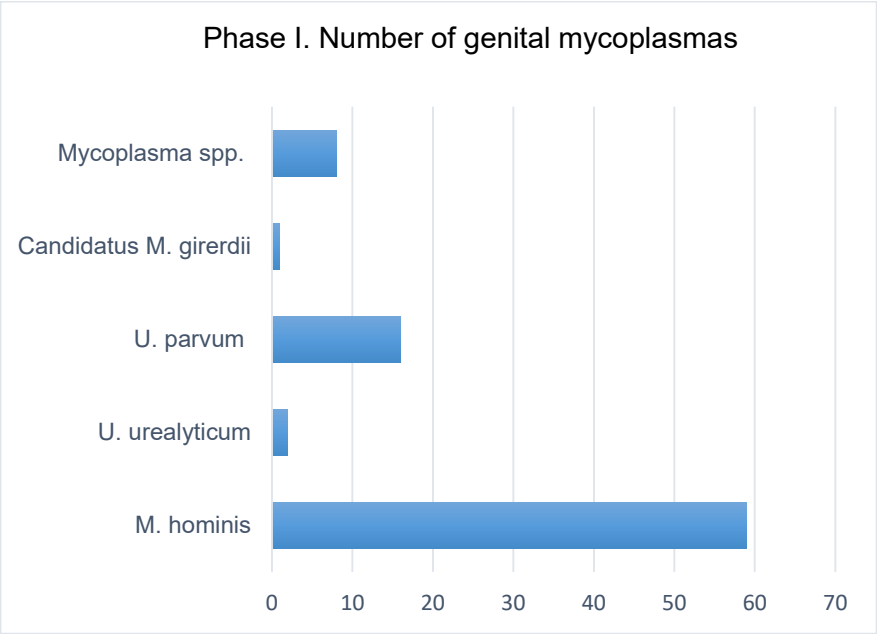
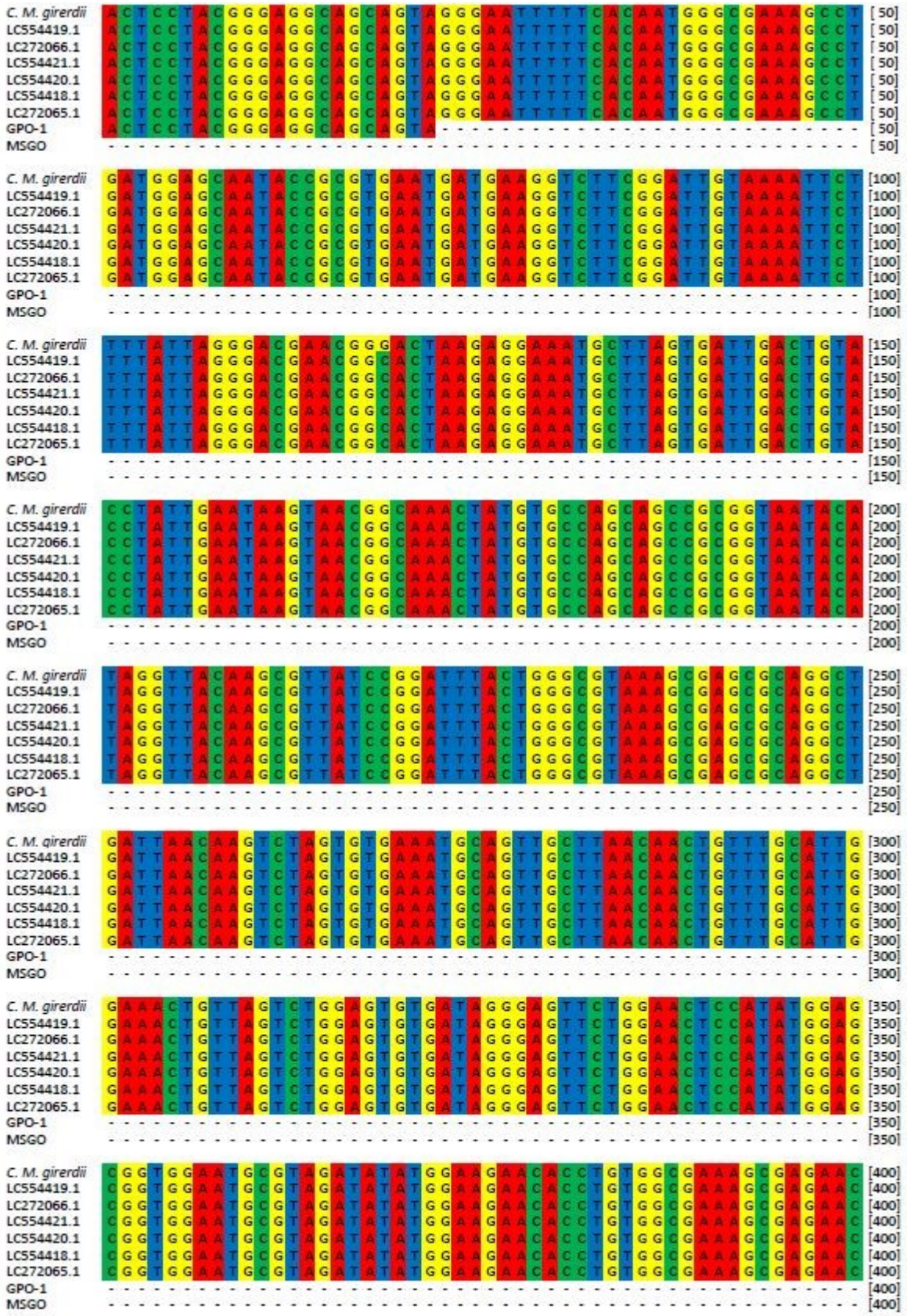


Figure 15. Number of *M. hominis, U. parvum*, *U. urealyicum*, *Candidatus* Mycoplasma girerdii and uncultured bacteria in vaginal swabs of female patients in the first sampling phase attending the OCD.

3.3.1.2 First *Candidatus* M. girerdii sequence obtained from a sample also positive for *Trichomonas vaginalis*.

T. vaginalis infection was detected in three specimens by culture and PCR at the OCD and by PCR at the ISPTM in parallel. In two specimens positive for *T. vaginalis* also *M. hominis* was detected. One specimen was positive for *T. vaginalis* and *Ca*. M. girerdii.

3.3.1.3 Sequence alignment of *Ca*. M. girerdii



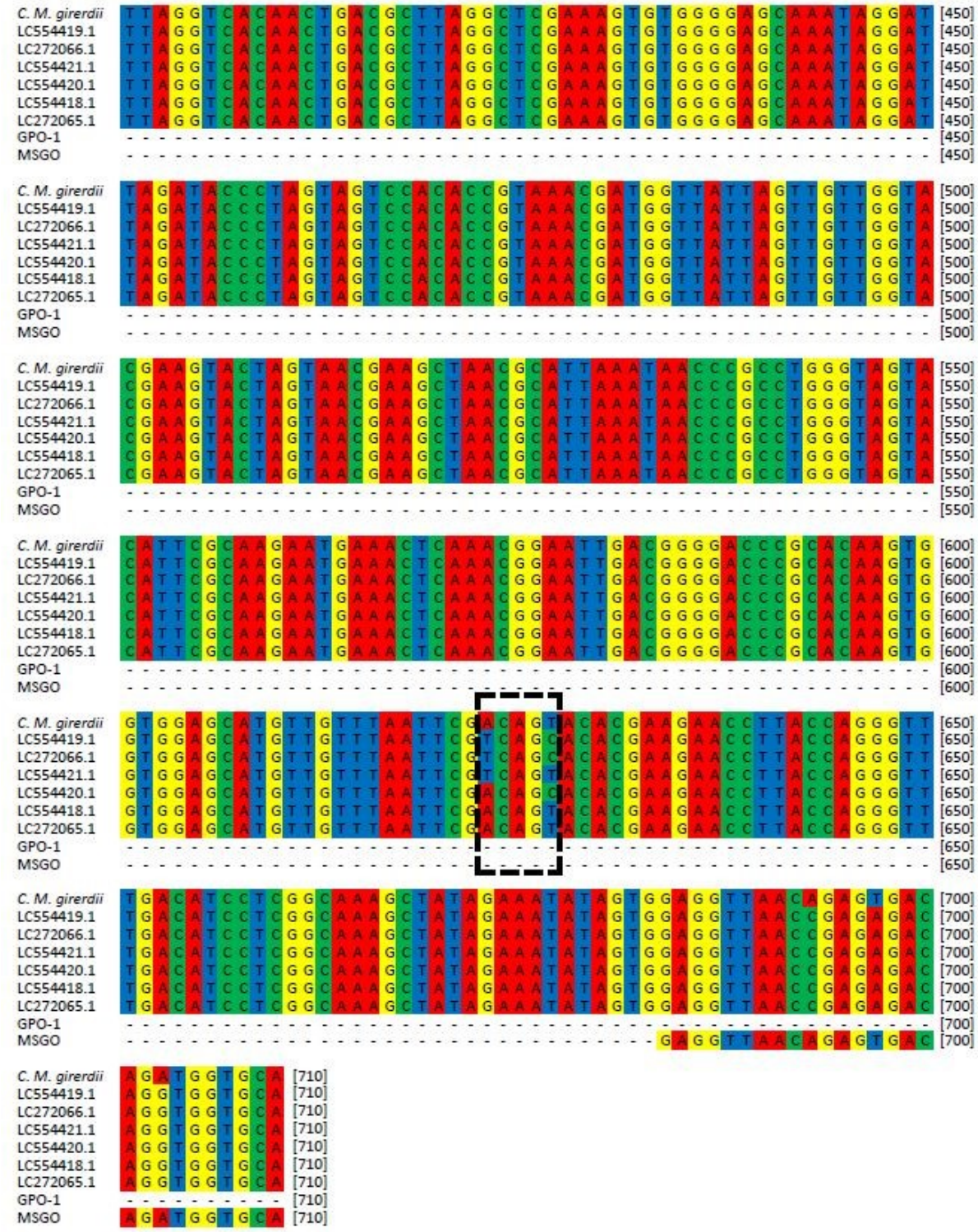


Figure 16. Sequence alignment of *Ca*. M. girerdii (first row) with reference 16S rDNA sequences from GenBank (rows 2-7). Lastly, (rows 8-9) GPO-1 and MGSO the forward and reverse primers respectively. The black frame with dashed lines marks the region where the *Ca*. M. girerdii sequence (depicted in the first row) and the GenBank sequences show the highest variability.

3.3.1.4 Data collection in the phase II of the study

The second phase of sampling took place in September 2021 and 263 samples were collected. Two swab specimens per patient were collected in the second sampling phase, smeared on the agar plates for detection of *T. vaginalis* and subjected to molecular analyses.

Table 13. Characteristics of specimens in the second study phase.

Total number of women patients	**263**
Available data on pH and amine tests	238
Age (mean age)	18–93 (35)
Positive for mollicutes	86/263 (33%)
Genital Candidiasis	64/263 (24%)
Positive Amine Test	27/263 (10 %)

Table 14. Summary of data from patients diagnosed with BV.

BV	**MH**	**Mean Age**
18	4 (22%)	32 (21–48)

3.3.1.5 Prevalence of mycoplasmas in swab specimens

Genital mycoplasmas were detected in 92 (35%) samples. *M. hominis* (MH) was detected in 66 (25%), whereas *M. genitalium* was detected in 6 (2.3%) of the samples. *U. parvum* and *U. urealyticum* were detected in 6 (2.3%) and 14 (5.3%) samples respectively. *Ca*. M. girerdii was not detected in the vaginal swabs by PCR in the second sampling phase. *T. vaginalis* infection was detected in a single sample and it was confirmed by culture and by PCR by collection of two swab specimen per sample used to culture the samples in specific medium for *T. vaginalis* and for DNA extraction and subsequent PCR. *M. hominis* was also detected in the sample positive for TV only by PCR. No *Mycoplasma* spp. were detected by PCR amplification in the *T. vaginalis* sample grown in culture. *M. genitalium* was detected by PCR in six specimens with *M. genitalium* specific primers not with the 16S rDNA primers. In three of the samples, *M. hominis* was also detected.

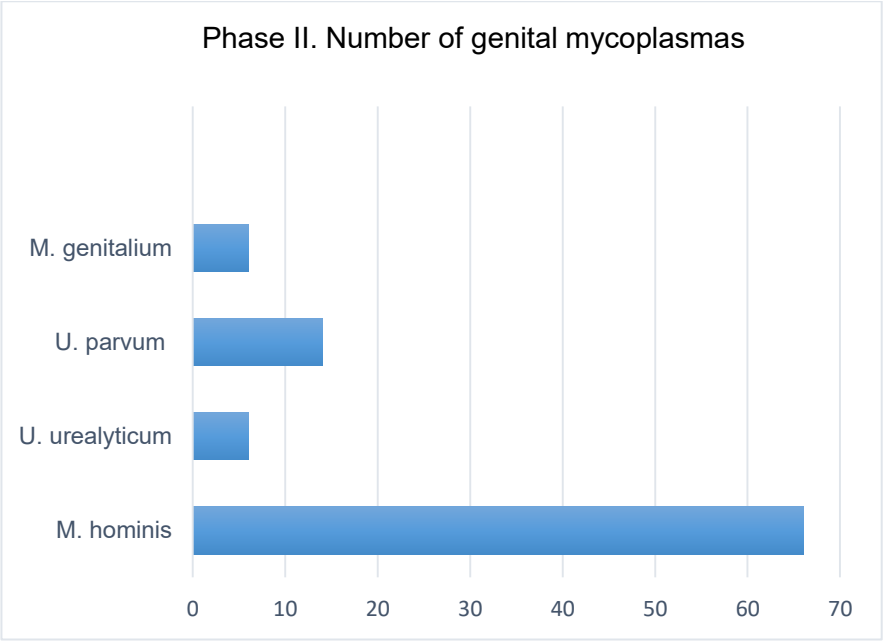


Figure 17. Detection of *M. hominis*, *U. urealyticum*, *U. parvum*, & *M. genitalium*, in vaginal swabs obtained in over a two-week period from women attending the OCD.

3.4 Detection of *M. hominis* and *Ca*. M. girerdii in cultured TV isolates

All in all, 20 cultured *T. vaginalis* isolates were analysed by PCR for the detection of *Mycoplasma* species. Two samples harboured *M. hominis* intracellularly, confirmed after amplification with the 16S rDNA primers. *Ca*. M. girerdii was not detected neither with the genus specific *Mycoplasma* primers, nor with the circularization primers of the unculturable bacterium (Fettweis, Paul Brooks, *et al.*, 2014). The attempt to amplify amplify *Ca*. M. girerdii from the positive sample with several circularization primers was unsuccessful.

4 DISCUSSION

In this study, DNA of *Ca*. Mycoplasma girerdii was detected and amplified from a genital swab sample and the 16S rDNA sequence was obtained. To our knowledge, this finding represents the first molecular data on this bacterium to date in Austria. Furthermore, molecular diagnosis of the vaginal specimens revealed the presence of genital mycoplasmas and *T. vaginalis*. The swab specimens analyzed revealed a high prevalence of *M. hominis* and *U. parvum*, *U. ureayticum* and lastly *M genitalium*. Moreover, the isolates of *T. vaginalis* that were investigated revealed prevalence of co-infection with *M. hominis* and no evidence of co-infection with *Ca*. M. girerdii.

4.1 Prevalence of genital mycoplasmas in vaginal swab specimens

In this retrospective study, a total 582 swab specimens were investigated using molecular approaches including DNA isolation and amplification with specific mollicute primers, of which 178 were successfully amplified and sequenced. The prevalence of genital mycoplasmas was 27% and 34.9% in the respective phases of the study. The *Mycoplasma* spp. primers used to amplify the isolated DNA from each sample targeted the highly conserved 16s rRNA gene, allowing for identification at the species level (Kuppeveld *et al.*, 1993). Ribosomal RNA molecules are present in high copy numbers independent of gene expression and were therefore used as feasible PCR targets (Gray *et al*., 1984; Kuppeveld *et al.*, 1993). Sequencing analysis revealed that *M. hominis* was the most common mollicute in both phases of study followed by *U. parvum* (UP). The latter is rarely differentiated from *U. urealyticum* (UU) in laboratory settings that detect such species mainly by culture, which oftentimes leads to false diagnosis. *U. urealyticum* was the third most prevalent mollicute in each study phase.

M. hominis infections detected in this study (18.5% and 25%) were significantly more prevalent than infections with *Ureaplasma* species (UP and UU) taken together, 7.6% and 5.6%. Women positive for *M. hominis* infection (mean age in phase I 35.52 and phase II 34.83) were mostly of reproductive age. An early study by McCormack *et al.* (1972) confirmed a correlation between sexual activity and colonization with genital mycoplasmas among participants. Although the mean age is not an indicator of the hormonal state, an association between the latter and the isolation of genital mycoplasmas in the female genitourinary tract has been described by Iwasaka *et al.* (1986). In that study, the occurrence of *U. urealyticum* was low among women who were sexually inactive. The highest incidence of genital mycoplasmas was observed in pregnant women, followed by sexually active non-pregnant women (Iwasaka *et al.* 1986). Several studies with female patients conducted in Asia have reported that the prevalence of ureaplasmas was higher than that of *M. hominis*. Lee and Yang (2020) reported that the

prevalence of *Ureaplasma* spp. and *M. hominis* among women attending an Outpatient Center in South Korea was 30.8% and 1.2% respectively. The data is supported by an earlier Korean study by Joo Kweon *et al.* (2016), which found *Ureaplasma* spp. to be the most prevalent organism among genital mycoplasmas in women. In China, similar values were reported by Wang *et al.* (2016). Studies conducted in European countries show a varying prevalence of *Ureaplasma* spp. and *M. hominis* mostly among female patients. Nevertheless, data confirms that *Ureaplasma* spp. are isolated more often. This is further supported by studies from Baka *et al.* (2009) and Leli *et al.* (2018) in Italy and Greece, which reported a corresponding prevalence of 52.9% and 41.9% for *Ureaplasma* spp. and 3.3% and 5% for *M. hominis.* In Poland, a retrospective study by Kasprzykowska *et al.* (2018) revealed that the prevalence of *Ureaplasma* spp. in women is nearly five times higher than *M. hominis*. Another study on the same mollicutes conducted in Hungary by Pónyai *et al.* (2013), reported a lower prevalence of *Ureaplasma* spp. (8.3%). In contrast to the current study, genital mycoplasmal infections were mainly confirmed by culture, using culture rapid kits or the Mycoplasma IST 2 kit (Choe *et al.*, 2013). In an intersectional study of female volunteers conducted in Porto (Portugal), PCR analysis identified *U. parvum* and *U. urealyticum* as the most prevalent mollicutes, followed by *M. hominis* and *M. genitalium* (Silva *et al.*, 2018). Another study on the prevalence of genital *Mycoplasma* and *C. trachomatis* in Brazil by Christofolini *et al.* (2012) identified *M. hominis* via PCR to be the most abundant species (11.3%) in female participants. A recent report of a cross-sectional study of nearly 1200 Tanzanian women undergoing screening for cervical cancer corroborated that *M. hominis* and *Ureaplasma* spp. were more common in patients who had previously been diagnosed with a STI or HIV (Klein *et al.*, 2020). In a smaller cohort of women infected with high risk Human Papilloma Virus (hrHPV), continual infections with *M. hominis* and *M. genitalium* were confirmed after consecutive tests had been performed. *M. hominis* was strongly correlated with hrHPV, approximately 30% of the women continued to test positive for infection with the mollicute (Adebamowo *et al.*, 2017).

Although the findings in the current study do not fully align with the data from other studies related to prevalence, a similar distribution of infections was reported. The findings of this study can be confirmed for various reasons. The sampling was performed by physicians in an OCD meaning that -no self-collected samples were investigated-, and were then safely transported to and stored in the laboratory at the ISPTM. Molecular analyses were subsequently performed under sterile conditions.

The infection rate of female patients diagnosed with Bacterial Vaginosis (BV) was 2.5% in Phase I and 10% in Phase II. In all patients, elevated levels of *Prevotella* spp. and *G. vaginalis* were detected, whereas *M. hominis* was detected in 25% and 22% of the total BV confirmed cases in the respective study phases. In Central and Western Europe, namely Denmark, Poland and Norway small cohort studies shared common findings, where the overall

prevalence of BV was less than 20% (Bjørnerem *et al.*, 1997; Nilsson *et al.*, 1997; Kaźmierczak, Wnek and Kamiński, 2004). High BV prevalence of 68.3% was registered in Mozambique (the Sub-Saharan Africa) among young women attending a planned parenthood clinic (*Newman et al.,* 2004), whereas lower BV prevalence was confirmed in Western Africa (Walraven *et al.*, 2001). A study in the United States conducted over the span of four years revealed the prevalence of BV in women under the age of 50 years old to be 29.2% (Koumans *et al.*, 2007). Although *M. hominis* alone is not able to instigate BV, its presence correlates with depletion of *Lactobacillus* species (Mardh *et al.*, 1997; Margolis and Fredricks, 2015). In a study by Cox *et al.* (2016) in Northern Ireland (UK), high *M. hominis* and *G. vaginalis* co-infection rates were detected whereas the same was not observed for *M. genitalium* or *Ureaplasma* spp. Here, one patient positive for BV and *M hominis* was also infected with *M. genitalium*. Nevertheless, for *M. genitalium*, a direct association with BV has not been confirmed and is at best circumstantial (Keane *et al.*, 2000; Lawton *et al.*, 2008). However, in a longitudinal study on female sex workers, it was concluded that BV may enhance susceptibility to *M. genitalium* infection (Lokken *et al.*, 2017). At the OCD, BV cases were microbiologically diagnosed based on the Amsel criteria (Amsel *et al.*, 1983). A new PCR-based diagnostic approach includes the amplification of Bacterial Vaginosis Associated Bacteria-2 (BVAB2) (Fredricks *et al.*, 2007), with almost 100% sensitivity and more than 90% specificity compared to the Nugent criteria which relies on Gram-stain interpretation (Nugent et al. 1991). The current study provides information on the correlation of BV prevalence, bacteria indicative of the condition and emphasizes the need for additional diagnostic methods to explain the underlying risk factors.

M. genitalium was detected with the species-specific MgPa gene primers, exclusively in phase II of the study. The mollicute is considered the etiological agent of endocervicitis and is overall less prevalent in women (Ross and Jensen, 2006). The prevalence and mean age of women who screened positive for *M. genitalium* was 2.3% and 41.6 years respectively. Previous studies have reported that *M. genitalium* is sexually transmissible with a 63% assessed risk of infection for patients whose partners are positive for *M. genitalium* (Manhart and Kay, 2010). Global infection rates of *M. genitalium* range from 0.8 to 7% in young women and are predominantly asymptomatic (Ross and Jensen, 2006; Tosh *et al.*, 2007). In Reunion Island (France), a very recent study by Begnis *et al.* (2021) identified more women testing positive for *M. genitalium* than men. Another study in Bordeaux (France) revealed that women had a lower infection rate with the mollicute (Pereyre *et al.*, 2017). In a prospective study, during screening of young patients for STIs, Peuchant *et al.*, (2015) found that the highest *M. genitalium* prevalence was observed in patients younger than 25 years of age. A nationwide study in Denmark confirmed that the majority of patients testing positive for *M. genitalium* and receiving treatment at STI clinics were men, counting for a two-fold increase in prevalence (10.3%) compared to that observed in women (4%) (Salado-Rasmussen and Jensen, 2014). In 2016, Getman *et al.* tested specimen of male and female participants for *M. genitalium* and other STIs

in multiple clinics throughout the United States. Prevalence of *M. genitalium* infection among females was 16.1%, and the highest rate of infection was detected in young patients who reported symptoms (Getman *et al.*, 2016). Similar findings regarding single infections and co-infections have been reported in studies conducted in Europe (Jensen *et al.*, 2004b), Asia (Nakashima *et al.*, 2014; Zheng *et al.*, 2014) and Africa (Pépin *et al.*, 2005; Vandepitte *et al.*, 2014). Although the data in this study differ from those previously reported, the lower incidence of infections caused by *M. genitalium* can be attributed to the study participants with a higher mean age. Epidemiological studies have been conducted targeting high risk groups such as men with NGU, revealing an estimated prevalence of 10-35% (Jensen *et al.*, 2016). In the USA, low risk populations of women were found to have a low prevalence of 2% in *M. genitalium* infections. A higher prevalence of 7.3 % was found for women from high risk populations (Andersen *et al.*, 2007). Meta-data studies focusing on the worldwide incidence of *M. genitalium* from 1991 onwards, in men and women aged above 13 years (Baumann *et al.*, 2018; Cina *et al.*, 2019) confirmed the trend and correlation between low prevalence in high developed countries 1.3 %, and higher prevalence of infections in less developed countries 3.9 %.

In a 2018 statement from the European STI Guidelines Editorial Board, the routine testing or treatment of asymptomatic patients for *U. parvum*, *U. urealyticum* and *M. hominis* was not recommended *(*Horner *et al.,* 2018). *M. hominis* and *U. urealyticum* are found in asymptomatic and symptomatic patients, particularly those with associated respectively with Bacterial Vaginosis (BV) and urethritis in men (Taylor-Robinson, 2017). *U. parvum*, on the other hand, does not present a risk factor for disease development (Beeton, *et al.*, 2019). The introduction of multiplex PCR assays has facilitated the detection of non-viral STI agents also allowing for *Ureaplasma* spp. and *M. hominis* identification (Fernández *et al.*, 2016; Del Prete *et al.*, 2017). However, often these assays have unsatisfactory specificity and sensitivity, which can lead to inaccurate interpretation of the etiological status of the three mycoplasmas. In the current study, a well-established molecular approach was used for screening.

In most women, the occurrence of *M. hominis* or other genital mollicutes does not result in discomfort or typical STI symptoms. These microorganisms are not considered as highly pathogenic (Garland and Kelly, 1996) and thus not solely responsible for diseases (Waites and Taylor-Robinson, 2015). Mycoplasmas are usually seen as commensals of the genitourinary tract, particularly if without symptoms. During pregnancy, due to hormonal changes, very high levels of mycoplasmas (*M. hominis* or *Ureaplasma* spp) can be detected. Here, we suggest that if an infection with genital mycoplasmas is suspected, examinations should be conducted in laboratory facilities. The testing and treatment of symptomatic patients with a high load of *M. hominis* and *U. urealyticum*, also exhibiting symptoms such as adverse pregnancy outcomes, pelvic inflammatory disease (PID) or non-gonococcal urethritis (in men) is encouraged (Horner *et al.*, 2018). *M. genitalium* is considered a causative agent of STIs, strongly associated with

urethritis and weakly associated with endometritis and infertility (Skov Jensen, 2004). Jensen *et al.* (2016) and Workowski *et al.* (2021) emphasize the importance of *M. genitalium* diagnosis by nucleic acid amplification tests (NAAT) and subsequent macrolide resistance assay.

It is noteworthy that based on this retrospective study the expected overall prevalence of genital *Mycoplasma* would be seemingly higher for *M. hominis* and *Ureaplasma* spp. due to higher rates of infection carriage in women. In order for examinations and diagnosis to be accurate, single sampling of specimens is no longer advised (Taylor-Robinson, 2017). Furthermore, it is suggested that microbiological studies should focus more on quantitative assessments of infectious agents (Rosenstein *et al.*, 1996). The new guidelines emphasize the need for more modern detection methods, better treatments, and that greater focus should be put on the most prevalent pathologies. However, early data and on-going research on genital mycoplasmas should not be overlooked. For a better understanding of a patient's vaginal health, the use of the latest diagnostic techniques may improve diagnosis and treatment in women who are prone to developing imbalances or infections (Fettweis *et al.*, 2012). Targeting the 16S rRNA gene, as in the current study, is advantageous due to its early implementation in microbiome research (Fettweis *et al.*, 2012), enabling a strain-level diagnosis.

4.2 First molecular data of *Candidatus* Mycoplasma girerdii in Austria

The sequence of a *Ca*. Mycoplasma girerdii strain (712 bp long) was obtained in phase I of the study from a patient attending the Centre for STD diagnosis. The BLASTN analysis revealed that the sequence identity compared to reference sequences in the GenBank was 100% with 99% query coverage. Interestingly, in the same specimen, the presence of *T. vaginalis* was molecularly confirmed. The DNA isolated from the swab specimen was successfully amplified and sequenced with the primers of the 16S ribosomal RNA gene of mycolasmas (Kuppeveld *et al.*, 1993). In Phase II of the study no samples were positive for *Ca*. M. girerdii. To the best of my knowledge, this is the first DNA sequence of this species in Austria.

The first molecular data of *Ca*. M. girerdii (previously named 'Mnola') was reported by Martin *et al.* (2013). That study revealed two main groups of vaginal samples infected with *T. vaginalis*. *M. hominis* and 'Mnola' were highly abundant in the first and second group respectively (Martin *et al.*, 2013). Thereafter, a 99% identical 16S rDNA sequence was reported by (Costello *et al.*, 2013). Fettweis *et al.*, (2014) confirmed a dependent relationship between *Ca*. M. girerdii and *T. vaginalis* in female patients. In the current study, co-infections with *Mycoplasma* species for all three samples positive for *T. vaginalis* were confirmed, however intracellular presence of *M. hominis* and *Ca*. M. girerdii was not proven. Ioannidis *et al.* (2017) confirmed *Ca*. M. girerdii to be the most prevalent species among *Mycoplasma* species in *T. vaginalis*-positive samples. In

accordance to the previous assertion by Martin *et al.* (2013) and Allen-Daniels *et al.* (2015), this study also reported that *Ca*. M. girerdii can be detected in association with *T. vaginalis* in vaginal specimens but not in pure protozoan cultures. The association between the protozoan and *Mycoplasma* species may be determined by similar metabolic strategies and the ability to incite host inflammatory responses (Fettweis *et al.*, 2014). Costello *et al.* (2017) provided the first proof of *Ca*. M. girerdii growth in vivo, in the oral cavity of an infant also positive for *T. vaginalis*. That was the first instance of a co-infection confirmed outside of the vaginal microbiota (Costello *et al.*, 2017). High prevalence of *M. hominis* and *Ca*. M. girerdii in young women positive for *T. vaginalis* infection was confirmed by Xu *et al.* (2021). These common findings confirm the strong correlation between the species, but do not confer a symbiotic relationship (Martin *et al.*, 2013; Fettweis, Serrano, *et al.*, 2014).

4.3 Assessment of *Mycoplasma* species in *T. vaginalis* cultures

In this study, one-fifth of the *T. vaginalis* samples grown in culture harbored *M. hominis* intracellularly. In comparison to this finding, the presence of *Mycoplasma* species of vaginal swabs from patients was confirmed in all four *T. vaginalis* positive samples. In Italy, a strong association between the mollicute and the protozoan was found in 78.6% of all positive samples for *T. vaginalis* (Diaz *et al.*, 2010). A clinical study in the Netherlands similarly revealed that *M. hominis* was present in 79% of the *T. vaginalis* confirmed isolates (van Belkum *et al.*, 2001), whereas in the United States a lower prevalence of *M. hominis* infected *T. vaginalis* (20%) was reported (Butler, Augostini and Secor, 2010). The two microorganisms can separately establish long-term infections in the genitourinary tract (Taylor-Robinson *et al.*, 1998); however, it has been corroborated that the pathogenicity of the protozoan and the mollicute is greatly affected by their symbiotic relationship (Dessì *et al.*, 2019). Xiao *et al.* (2006) suggested that *M. hominis* symbiosis with *T. vaginalis* may confer better resistance against metronidazole *in vitro*. Fürnkranz et al. (2018b) found in a similar study, that *T. vaginalis* strains infected with *M. hominis in vitro*, exhibited a two-fold increase in minimal inhibitory concentration (MIC) to the antibiotic. Therefore, it can be inferred that an *in vitro* influence on drug susceptibility of the protozoon is feasible. However, the sensitivity to metronidazole was not assessed in the present study. The unsuccessful amplification of cultured *T. vaginalis* strains and the *Mycoplasma*-positive specimens with the specific circularization primers of *Candidatus* Mycoplasma girerdii (Fettweis *et al.*, 2014), proved that the targeted genome regions are less conserved compared to the 16s or 18s ribosomal RNA genes.

The mean age of women who tested positive for *T. vaginalis* infections was 54 (Phase I) and 59 (Phase II). Furthermore, elevated values of vaginal pH were detected, ranging between 5 - 5.3, which is typical for patients during trichomoniasis or BV. In a 2009 study, Peterman *et al.*

reported that female participants were found to be infected with *T. vaginalis,* during increments in which they claimed to not have engaged in sexual activities. Therefore, the authors hypothesized that the infection with the protozoan was not reported or not detected by previous testing. Additionally, women aged between 25-40 years old were found to be more prone to infection (Peterman *et al.*, 2009). In a later study by Stemmer *et al.* (2018), it was similarly found that the *T. vaginalis* infections occurred in women of perimenopause age (47-53). The overall low *T. vaginalis* prevalence in the current study (0.7%), is not suggestive of reduced systematic screening for trichomoniasis. On the contrary, epidemiological data have shown high rates of asymptomatic infections, especially in women that can go untreated for years (Peterman *et al.*, 2006, 2009; Sutton *et al.*, 2007). Delayed diagnosis increases the rate of infection with the human immunodeficiency viruses (HIV) and other sexually transmitted diseases. In the new Trichomoniasis - STI Treatment Guidelines, by the CDC (2021), the CDC states that diagnostic tests should be carried out for women who are seeking care for vaginal shedding in STI clinics or correctional institutions.

5 CONCLUSIONS

In conclusion, this study gives the first molecular data for *Ca*. M. girerdii in Austria. The reported findings of this still uncultivated *Mycoplasma* species are still very recent, and the knowledge regarding its metabolic strategies and pathogenic potential are limited. Additionally, a comparably high prevalence of infections with genital mycoplasmas was revealed, resulting in *M. hominis* and *U. parvum* being the most prevalent species. The symbiotic relationship between *M. hominis* and the protozoan *T. vaginalis,* previously described, was confirmed intracellularly. Co-infections were also present in all four *T. vaginalis* positive swab specimens, which proves how intricate the interaction between these distinct species is. Taken together, this study underlines the importance of implementing new and advanced diagnostic techniques, thus enabling better screening of patients and better understanding of women's urogenital health. Future studies will be necessary for elucidating the role of *Mycoplasma* species and *T. vaginalis* in sexually transmitted diseases.

6 BIBLIOGRAPHY

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