MOLECULAR CHARACTERIZATION OF *Mycoplasma genitalium* FROM FEMALE SUBJECTS RESIDENT IN BENIN CITY NIGERIA

By

EZEANYA, CHINYERE CHARITY

Registration No: 2015617001F

SUBMITTED TO

DEPARTMENT OF MEDICAL LABORATORY SCIENCE

FACULTY OF HEALTH SCIENCES AND TECHNOLOGY

NNAMDI AZIKIWE UNIVERSITY, NNEWI CAMPUS

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Being a dissertation submitted to the Department of Medical Laboratory Science in partial fulfilment for the Award of Doctor of Philosophy (PhD) degree in Medical Microbiology

MARCH, 2019

Certification

I certify that this research work titled, "Molecular Characterization of *Mycoplasma genitalium* from female subjects resident in Benin City Nigeria," was carried out by Ezeanya, Chinyere Charity with Registration number 2015617001F and that this work has not been submitted to other universities.

_____ _____

Ezeanya, Chinyere Charity Date

Approval Page

This thesis titled "Molecular Characterization of *Mycoplasma genitalium* from female subjects resident in Benin City Nigeria" resulted from research carried out by EZEANYA .C. CHINYERE and we the undersigned have carefully read and approved this work which have not been submitted to other universities as adequate in scope and quality, in partial fulfilment for the award of Doctor of Philosophy degree in Medical Microbiology.

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Dedication

This work is dedicated to God Almighty for His grace towards me and for giving me the courage, strength, faith, sound health and mind to undertake my course of study and for providing me daily inspiration even at the most challenging moments of my career.

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Abstract

Mycoplasma genitalium is a sexually transmitted pathogen of public health concern in reproductive age women. Infections caused by this organism ultimately lead to serious sequelae such as chronic pelvic pain, ectopic pregnancy, infertility and increased risk for HIV transmission. The insidious nature of *M. genitalium* makes it unculturable, thus requiring the use of DNA amplification assays. In describing a novel microbe, the 16S rRNA gene sequence is crucial most especially for many unculturable bacteria. Therefore, the aim of this work was to characterize *M. genitalium* isolated from female subjects resident in Benin City, Nigeria. This study was both population and hospital based, cross-sectional study with 100 participants recruited from among female students of Edo State School of Health Technology and 30 women with gynaecological cases recruited at the Obstetrics and Gynaecology Clinic of Central Hospital, Benin City. The participants were aged 15-39 years and were recruited for the study using simple random sampling method. Ethical approval was obtained from the institutional Research Ethics Committee of Edo State Hospitals' Management Board. Informed consent was also obtained from the participants. Duplicate samples of Endocervical Swabs (ECS) were collected from all the subjects. One set of the ECS were assayed microscopically using Wet Mount, Gram Stain and Giemsa Staining techniques. Evaluation of the second set of ECS for sexually transmitted pathogens (*M. genitalium, M. hominis, U. urealyticum, U. parvum, T. vaginalis and C. trachomatis*) was done using Multiplex Polymerase Chain Reaction with 6 specific primers following DNA extraction. All the samples were assayed for *M. genitalium* DNA using 16SrRNA Gene sequencing method with genus and specie- specific primers while Phylogenetic Analysis was done by Maximum Likelihood method using Tamura-Nei Model with bootstrap on Molecular Evolutionary Genetics Analysis (MEGA) version 7.0. Statistical analysis was done using SPSS version 20. Of the 130 subjects, 36 were positive for *Mycoplasma species*. 16SrRNA gene detection prior to sequencing was positive for *Mycoplasma* genus and negative for *M.genitalium* based on genus and specie - specific primers respectively. The prevalence of the different isolated *Mycoplasma* species were as follows: *M. genitalium* (0.8%), *M. hominis* (7.7%), *M. penetrans* (0.8%), *U. urealyticum* (3.1%) and *U. parvum* (1.5%); however, *M. genitalium* emerged only among the students. The accession numbers were generated for each consensus sequence following submission to GenBank. On the other hand, high number of sexual partners (≥4) (OR=0.18, *P*=0.012), co-infection with other sexually transmitted (ST) pathogens: *T. vaginalis* and *C. trachomatis* (OR=0.51, *P*=0.040) were strongly associated with Mycoplasma infection. Phylogenetic analysis of *M. genitalium* revealed no shared phylogenetic relationship with other species previously reported. Phylogenomics study of *M. species* among the subjects and reference strains revealed that all *M. hominis* from the subjects were found to be of a common ancestral clade and had significant association with two China strains: EU596508 (CV = 81%) and EU596509 (CV = 99%). Despite the relatively low prevalence of *M. genitalium* (0.8%); *M. genitalium* (MG238565) emerged with no shared phylogenetic relationship with others previously reported. This implies that the specie circulating in our environment is relatively unique. High number of sexual partners and co-infection with sexually transmitted pathogens presented as possible risk factors among female subjects. The significant association found between *M. hominis* strains in our study and those of China strains indicates indiscriminate spread of these genital mycoplasmas and when left undetected in the female reproductive tract have the potential of resulting to serious sequelae such as infertility. It is therefore paramount to monitor the spread of circulating genital pathogens among reproductive age women for better containment of infection and prevention of serious sequelae.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Mycoplasma genitalium, an emerging sexually transmitted pathogen was originally isolated in 1981 (Tully *et al.,* 1981). It is known as a cause of male urethritis majorly in nearly 15-20% of non-gonococcal urethritis (NGU), 20-25% of non-chlamydial and almost 30% of persistent or recurrent urethritis (Li *et al*., 2019).

Members of the genera *Mycoplasma* are characterized as small, cell wall-less bacteria exhibiting parasitism relationship with vertebrate hosts in an obligate manner. Infections with these species show a variety of clinical manifestation: asymptomatic states to classical manifestation of chronic inflammatory disease. The prime sites of mycoplasma infections in humans is the respiratory or urogenital tract (Hughes and Saunders, 2018). Accordingly, urogenital mycoplasmosis of humans is typically associated with *M. genitalium, Ureaplasma spp* or a concomitant infection by more than one of these pathogens (Silva *et al*., 2018).

The World Health Organization reports the development of nearly 448 million new cases of sexually transmitted infections (STIs) around the globe annually (WHO, 2011). However, with early diagnosis such infections are treated certainly with slight morbidity and diminished economic burden. *Mycoplasma genitalium,* an emerging cause of STIs has been associated in urogenital infections among men and women globally. The demonstration of the role of *M. genitalium* in male urogenital disease proved to be a significant development in the study of STIs but its role in the female reproductive tract disease remains unclear (McGowin and Anderson-Smits, 2011; Peter *et al*., 2018).

Mycoplasma genitalium demonstrates four major virulence properties which are: adhesion to host epithelium, intracellular localization, antigenic variation and production of enzymes. In females, urogenital infection is promoted by the secretion of the enzyme-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as encoded by the *gap* gene. The enzyme aids attachment of *M. genitalium* to human vaginal and cervical mucin (Aparicio *et al.*, 2018).

The risk factors for *M. genitalium* infection are similar to that of other STIs. Studies have established behavioural risk factors for *M. genitalium* infection: high number of partners, younger age during first intercourse, having a partner with infection symptoms, co-infection with other sexually transmitted pathogens like *Chlamydia trachomatis* (Ronda *et al*., 2018; Buder *et al.*, 2019). Murray *et al.* (2019) demonstrated a regression in the bacterial load of *M. genitalium* to patient symptoms and the success of treatment. Notable, asymptomatic carrier state poses a serious epidemiological threat considering spread to sexual partners and perhaps vertical transmission from mother to new born (Kaida *et al.*, 2018). Furthermore, some studies have reported Mycoplasma infection to have similar prevalence as that of chlamydial and trichomoniasis and more prevalence than gonorrhoea (Pereyre *et al*., 2017; Nolskog *et al.*, 2019).

In studies piloted in Greece, the prevalence of *M. genitalium* infection revealed 5.7% in both asymptomatic and symptomatic women and 5.4% of those with symptoms (Chra *et al.*, 2018). Hamasuna *et al.* (2008) documented the prevalence of *M. genitalium* as 2.8% among female students in Japan. Lillis *et al.* (2018) revealed 13.6% prevalence of *M. genitalium* in young sexually active females (aged ≥18 years) which was relatively high in New Orleans. In sub-Saharan African, reports on genital mycoplasmas have been scarce. In a study on symptomatic women with vaginal discharge and cervicitis in Ghana; Pepin *et al.*, (2005) showed 26.3% prevalence of *M. genitalium*. Further studies by Balkus *et al.*, (2018) in Kenya among sexually active women, the prevalence of *M. genitalium* was 11.3% which was

relatively higher when compared to studies by Kaida *et al*., (2018) in South Africa with reported 9.6% prevalence of *M. genitalium* among women who had genital tract infection. In Nigeria, studies on *Mycoplasma spp* from South West by Agbakoba *et al.*, (2007) and South East by Chukwuka *et al.*, (2013) showed 35.7% and 20% prevalence respectively. In a recent review by Taylor-Robinson (2017), a need for studies using up-to-date sequencing method was emphasized. Recently, amplification of the genome by PCR, followed by determination of the nucleotide sequences and phylogenetic analysis has become a global technique for both classification and identification of etiological agents. Consequently, few characterized *M. genitalium* sequence has emerged from around the world despite its small sized genome (Fookes *et al*., 2017).

To the best of our knowledge, this is the first study in Nigeria on the molecular characterization of *M. genitalium* among female subjects resident in Benin City, Nigeria using up-to-date sequencing techniques. The present study is therefore intended to characterize *M. genitalium* by amplification of the genome followed by determination of the nucleotide sequences and phylogenetic analysis. The subjects studied were screened for other organisms like: *Chlamydia trachomatis*, *M. hominis*, *Trichomonas vaginalis*, *Ureaplasma species* with Nucleic Acid Testing and wet mount testing for *Trichomonas vaginalis* and bacterial vaginosis to ascertain co-infection.

1.2 Statement of Research Problem

The prevalence of *Mycoplasma genitalium* in Nigeria is less documented and access to diagnostic testing is limited. *Mycoplasma genitalium* is a serious public health concern in reproductive age women worldwide. In view that *Mycoplasma* infection is often asymptomatic and with minimal testing rates for *Mycoplasma*, the available data for *Mycoplasma* is likely to underrate the risk of *Mycoplasma* in the general population. An understanding of the associated risk factors will aid in facilitating improved screening

measures for vulnerable groups. It has a wide-range of complications such as chronic pelvic pain (Molenaar *et al*., 2018), ectopic pregnancy, infertility (Adesola *et al*., 2017), increased risk for HIV transmission and have been associated as co-factors in diseases (Adebamowo *et al*., 2017). As an emerging sexually transmitted pathogen causing clinically significant diseases in females, few genomes have been sequenced till date due to the fastidious nature of *M. genitalium* and rare population-level data on prevalence or risk factor (Salado-Rasmussen and Jensen, 2014). Thus, there is lack of phylogenetic framework to provide insights into the nature and diversity of the species. Despite their importance in sexual health, there are only six 16SrRNA sequenced *M. genitalium* to date. They include: the sequence of 1490 bases of the 16SrRNA gene for *M. gentalium* G37 (type strain) and four Danish isolates of *M.genitalium* (Fookes *et al*., 2017).

Among the limited studies on *M. genitalium* in Nigeria, most of them have been conducted in specialized populations such as in clinics. Though these study groups are ideal for studying hypothetically new sexually transmitted pathogens, there is a possibility of overestimate of the prevalence in the general population. Moreover, there have not been documented studies on molecular characterization of *M. genitalium* from Nigeria. Consequently, this study aimed to broaden the understanding of the diversity of *M. genitalium* by sequencing *M. genitalium* from female students and symptomatic patients (aged 15-45 years) using short and variable region involving 280 nucleotide sequence of the 16SrRNA gene sequence. This generated nucleotide sequence aided phylogenetic analysis of the species originated from this study.

1.3 Justification of the Study

Adolescent girls and young women (15 – 39 years) from sub-Saharan Africa are at high risk for sexually transmitted infection (STI) consequently; the incidence of bacterial STIs has shown a significant increase in recent years. To solve an emerging public health problem and ensure that the spread of *M. genitalium* by female subjects; most especially asymptomatics

(Fernández-Huerta *et al*., 2019) is highly significant. The identification of *M. genitalium* and association with possible risk factors is crucial as there are limited strategies to identify individuals at risk of *M. genitalium* in Nigeria. Evolutionary studies have much improved our knowledge on *M. genitalium* history and have highlighted its long co-evolution with humans. Likewise, the phylogenetic relationship between the *M. genitalium* isolated in this study among female subjects resident in Benin City –Edo State School of Health Technology and Central Hospital, Benin City with others previously published is fundamental in the understanding of the nature of this pathogen. Studies have shown that molecular characterization of *M.genitalium* from Nigerian women aids in tracing source of infection, identifying risk factors thus leading to the initiation of policies that combats the spread of infection. Knowledge of circulating pathogen(s) in Nigeria among reproductive age women (15 – 39 years); guides clinicians to administer effective treatment regime thus reducing the disease sequelae (Molenaar *et al*., 2018). The association between *M.genitalium* infection and other genital pathogens (*C. trachomatis, U. parvum, U. urealyticum, T. vaginalis* and other *Mycoplasma species)* and concomitant syndrome (bacterial vaginosis) is of public health importance as it checks the spread of concomitant infection.

1.4 Aim & Specific Objectives of the Study

1.4.1 Aim

The aim of the research was to carry out molecular characterization and phylogenetic analysis of *Mycoplasma genitalium* from female subjects resident in Benin City.

1.4.2 Specific Objectives of the Study

1. To determine the prevalence of *M. genitalium* infections among the female students of Edo State School of Health Technology and female patients attending the Obstetrics and Gynecology clinic in Central Hospital, Benin City.

2. To ascertain co-existence of *M. genitalium* with *M.hominis*, *Chlamydia trachomatis, Trichomonas vaginalis, Ureaplasma parvum* and *U. urealyticum* from cervical specimen among symptomatic and asymptomatic female subjects using Multiplex Polymerase Chain Reaction (mPCR).

3. To determine the possible risk factors associated with infections caused by genital mycoplasmas such as *M. genitalium*.

4. To determine the association between subjects with bacterial vaginosis and isolation of *M. genitalium*.

5. To perform a phylogeny-based analysis of *M. genitalium* among the female subjects and other previously reported *M.genitalium* sequence in GenBank using 16SrRNA sequencing.

6. To determine the genetic relationship between *M. genitalium* and other clinically important bacterial sexually transmitted (ST) pathogens such as *C. trachomatis* using phylogenetic methods.

7. To establish the genetic relatedness between *Mycoplasma spp* isolated from female students and hospital patients with disease sequelae such as infertility in Benin City using Phylogenomics methods.

1.5 Research Questions

To solve an emerging public health problem thus limiting the spread of *M. genitalium* by female subjects; the following research questions are pertinent;

1. What is the prevalence of *M. genitalium* infection among female subjects in Benin City, Nigeria?

2. Does *M. genitalium* infection present as co-infection with other sexually transmitted infection such as *T.vaginalis* and *C. trachomatis* infection among symptomatic and asymptomatic female subjects?
3. What are the possible risk factors associated with *M. genitalium* infection among females?
4. Is there an association between bacterial vaginosis infected females and isolation of *M. genitalium*?
5. Does the 16SrRNA sequence of *M. genitalium* have a genetic relatedness with other 16SrRNA sequence from around the world in the Genbank?
6. Is there a genetic relatedness between *M.genitalium* and other bacterial ST pathogens like *C.trachomatis*?
7. Is there a genetic relationship between *Mycoplasma spp* isolated from the students and patients with the disease sequelae such as infertility?

1.6 Research Hypotheses/Assumptions

1. *Mycoplasma genitalium* infection is not prevalent among female subjects in Benin City, Nigeria.

2. All females with *M.genitalium* infection do not have other genital infections such as *T. vaginalis, C. trachomatis* infection irrespective of the manifestation of the infection.

3. There is no association between the possible risk factors and *M. genitalium* among female subjects resident in Benin City, Nigeria.

4. There is no association between bacterial vaginosis infected females and isolation of *M. genitalium*.

5. The 16SrRNA sequence of *M. genitalium* isolated from female subjects resident in Benin City, Nigeria share phylogenetic relationship with others previously reported.

6. Bacterial sexually transmitted pathogens such as *C. trachomatis* share phylogenetic relationship with *M. genitalium*.

7. Females do not have serious sequelae such as infertility from undetected mycoplasmal infection because of asymptomatic manifestation of the infection.

CHAPTER TWO

LITERATURE REVIEW

2.1. Mycoplasmas

There are well above 150 species in the class of cell wall-less bacteria. With a minimum of 15 of these species originated from human but some others originate from animals and plants (Brown *et al*., 2001; Uuskula and Kohl, 2002). Among humans, four species are of principal significance: *Mycoplasma pneumoniae* (etiologic agent of pneumonia and has been associated with joint and other infections), *Mycoplasma hominis* (somewhat produces postpartum fever and involvement with other bacteria in uterine tube infections has been reported), *Ureaplasma urealyticum* (an etiologic agent of non-gonococcal urethritis in men and found to be associated with lung disease in premature infants of low birth weight) and finally *Mycoplasma genitalium* share close relationship with *M. pneumoniae* (etiologic agent of urethral and other infections). Still, other members of the genus *Mycoplasma* are pathogens of the respiratory and urogenital tracts and joints of animals (Brosh-Nissimov *et al*., 2018).

The smallest sized genome mycoplasma in the genus is *M.genitalium*. It is approximately twice the genome size of certain large viruses. Mycoplasmas have been described as the smallest organisms that are free-living in nature and also self-replicating on laboratory media. (Agbakoba *et al*., 2006a).

They display the following significant characteristics (Brooks *et al*., 2010):

1. The small size; 125-250nm in size
2. They are extremely pleomorphic consequently to the absence of a rigid cell wall, thus bounds by a triple-layered “unit membrane” comprising of sterol. Growth requirement of serum or cholesterol serves to boost sterols content.

3. Intrinsic resistance to antimicrobial agents such as Penicillin which function via inhibition of cell wall synthesis since they lack cell wall structures but they are inhibited antimicrobial that inhibit protein synthesis such as erythromycin.
4. Mycoplasmas possess the ability to replicate on cell-free growth media and it exhibits a distinct colony morphology with the center of the entire colony typically embedded underneath the surface.
5. All mycoplasmas growth are disrupted via specific antibody.
6. Mycoplasmas exhibit a high affinity for mammalian cell membranes.

2.2 Genome

A study on the whole genome sequence of *M.genitalium* was reported in 1995, consequently the second bacterium after *Haemophilus influenza* with the whole-genome fully sequenced (Fraser *et al.*, 1995). It is described as the known smallest sized genome in the genus *Mycoplasma* with size 580 kb. Most mycoplasmas genomes are associated with low guanine plus cytosine (G+C) content (ranging from 24-33% G+C) but *M. genitalium* genome has a relatively greater G+C content of 32% (Fookes *et al.*, 2017). The small sized genome of *M. genitalium* attributed to its adoption into 'The Minimal Genome Project', a study aimed at discovering small sized genetic material required for life sustainability (Pace, 2009).

2.3 Morphology and Identification

2.3.1 Typical Organisms

Mycoplasmas exhibit certain unique properties which makes them impossible to be cultivated with routine bacteriologic methods, such properties includes (Agbakoba *et al*., 2006a):

1. The size of the colonies are typically small.
2. The flexibility and fragility of the Mycoplasma cells
3. Their low affinity for aniline dyes.

Cell morphology varies with individual species, predominant environmental conditions and the phase of the growth. The application of light microscopy divulges pleomorphic organisms which spreads across from spherical, coccoid, coccobacillary, and ring, dome-shaped forms to branching beaded or segmented filaments. There are few species of human origin with specialized structures at one or both ends which contributes to their attachment to the respiratory or genital tract mucosal surfaces, examples includes: *M.pneumoniae, M.genitalium* and *M.penetrans*. Some mycoplasmas have a specialized terminal structure that appears to play a role in attachment. These species reveal a mode of motility in the gliding form in association with an exact terminal organelle. In liquid media, growth is observed in varied ways. Whereas, on solid media growth basically takes place via the formation of protoplasmic masses showing undefined shape subject to simple distortion. The structure reveals different size with a range of 50 to 300nm in diameter. The mode of examination greatly influence the nature of morphology of individual species (Agbakoba *et al*., 2006a).

Their cell is structured in such a manner that it is membraneously restricted with two electron-dense layers stationed apart by a translucent layer. Extra membranous layer are present in few species like: *M.mycoides* subspecies *mycoides* with cell structure comprising of galactan with dense capsular appearance. While others like: *M. gallisepticum* (avian), *M. pneumoniae, M. pulmonis* (murine) and *Spiroplasma citri* have cell structures with projections which serves as sites for attachment by adhesion proteins to eukaryotic cells (Aparicio *et al*., 2018). With close adherence, the organism releases nucleases and other enzymes into the cell. They exhibit broad range of adherence specificity as observed to erythrocytes, tissue culture cells, spermatozoa and eukaryotic cells may be demonstrated with certain mycoplasmas.

The component of their cytoplasm somewhat differs from that of typical bacteria. They lack endoplasmic reticulum or intracellular membranous structures though possess ribosomes and

nuclear material in fibrillary form which is centrally positioned. The ribosomes is similar to that of a typical bacteria with sedimentation coefficient of 70S. The unique feature of the *M. genitalium* which differentiate it significantly from other members of the genus is the size of the genome (580kb). Thus, *M. genitalium* is seemingly the smallest organism able of independent existence with a minimum set of genes (Aaltone *et al*., 2002).

2.3.2 Culture

Pathogenic mycoplasmas with fastidious nature requires growth media supplemented with serum or ascitic fluid, growth factors (yeast extract) and metabolic substrate (glucose or urea). Supplement of culture media is specific to each specie due to diverse properties and substrate requirements. With the universal optimum growth temperature for pathogenic bacteria (37^0C) for 48-96 hours incubation period, growth in broth cultures may not be supported. Nevertheless, giemsa's stains of already centrifuged sediment will reveal the distinctive pleomorphic structures and subculture afterwards on suitable solid culture media support development of minute colonies (Agbakoba *et al*., 2007).

Subsequently, growth on biphasic (broth over agar) and agar medium for an incubation period of 48 to 144 hours in a sealed petri dish (prevention of evaporation) colonies measuring 20-500m are detectable with hand lens. The colonies have distinct morphology which is round, with a granular surface and a dark center classically submerged in the agar. They are sub cultured in a subtle manner which involves cutting out a small square of agar containing one or more colonies and streaking it unto a fresh media plate or submerging it in the case of liquid broth. In microscopy, the bacterial cell is stained via placing a similar square of the bacterial colony on a slide following overlaying of the colony with a cover slip preceding the flooding of the slide with an alcoholic solution of methylene blue and azure and then allowed to evaporate; Specific fluorescent antibody can also be applied for staining (Peeraych and Mirdamadi, 2005).

2.3.3 Colonial morphology

On solid agar, mycoplasmas have growth colonies with distinctive morphology as 'fried egg' form with an opaque central zone growing underneath the agar having a translucent peripheral section on the surface. The fried egg appearance is initiated by the central portion of growth infiltrating downwards into the growth agar with disseminated peripheral growth at the surface.

Another characteristic morphology of the mycoplasma colonies is the variable colony size, for example; some mycoplasmas have approximately 2.0mm in diameter colony size and are readily visualized with the unaided eye whereas ureaplasmas are typically small (15-60µm in diameter) which is consequent of the absence of the peripheral region of growth. However, colony size and form is highly dependent on some factors. These includes:

1. The constituents of the growth medium (Brooks *et al*., 2010).
2. The extent of hydration of the growth medium (Gaydos *et al*., 2019).
3. The agar concentration of the growth medium (Brooks *et al*., 2010).
4. Atmospheric condition (growth requirement) (Murray *et al*., 2017)
5. Time of the culture (Okwoli, 2007).

With the aid of dichotomizing microscope, the Mycoplasma colonies exhibit a typical "fried egg" appearance; demonstrating an opaque central region profound in the agar with a translucent exterior. Furthermore, some *M.hominis* colonies form hole and notch in the marginal regions although some have been described to produce colonies with lacy outlines at the margin. It occasionally extend inwardly in the direction of the colony center. In addition, some others may produce umbonate colonies with shades spreading over the center (Okwoli, 2007).

2.3.4 Growth Characteristics

Mycoplasmas are distinctive in the field of medical microbiology for (Gaydos *et al*., 2019):

1. Their enormously small size.
2. Their ability to grow on composite but cell-free growth media.

Mycoplasmas are filterable (using filters with 450nm pore size) and consequently are similar to *Chlamydiae* and viruses. Nevertheless, opportunistic mycoplasmas thrives on cell-free media consisting of lipoprotein and sterol. Sterol prerequisite for growth and membrane synthesis is exceptional. However, energy source for Mycoplasma and Ureaplasma is glucose and urea respectively (Murray, 2017)

2.3.5 Variation (de Vries, 2019)

The exciting pleomorphic of mycoplasmas has been described as a unique key characteristics

2.4 Reproduction

Mycoplasmas typically reproduce in a manner comparable to other prokaryote which is mainly by binary fission. However, fission via budding is pragmatic among few species. *Mycoplasmas* exhibit a circular genome (double stranded DNA) which in size exists as one-fifth to one-half comparable to that of most bacteria (Fernández-Huerta *et al*., 2019).

The generation time of Mycoplasmas is not comparable to bacteria with a mean generating time of 1-3 hours whereas, for some species is 6-9 hours. As a result, visible growth may result within 1-3 weeks. The best temperature growth requirement for Mycoplasmas has been described as 37^0C. Although, *Spiroplasmas* and *Acholeplasmas* requires a varied temperature range (22-37^0C) (Murray, 2017).

2.5 Pathogenesis

The pathogenesis of *M. pneumoniae* has been reported following extensive study and owing to the close genetic resemblance, definite features in the pathogenesis of *M. pneumoniae* can

be pragmatic to *M. genitalium*. Nevertheless *M. pneumoniae* is principally found in the respiratory tract while *M. genitalium* is chiefly found in the urogenital tract; both organisms have demonstrated cross tissue barriers. *Mycoplasma genitalium* has exhibited affinity for attachment to different cell types such as: erythrocytes, Vero cells, fallopian tube cells, respiratory cells and spermatozoa (Ekiel, 2009).

Mycoplasma genitalium owes its pathogenicity to several virulence factors that have been demonstrated. These include:

1. Adherence to host epithelial cells via the terminal tip organelle with adhesins.
2. Intracellular localization.
3. The release of enzymes.
4. Evasion of the host immune response by antigenic variation (Fernández-Huerta and Espasa, 2019).

Furthermore, *M. genitalium* lipid-associated membrane proteins (LAMPs) demonstrate a significant part in the genito-urinary tract inflammatory reaction (Ekiel, 2009). Even though mycoplasmas are revealed to expel hydrogen peroxide and superoxide metabolites, considerable tissue damage is associated with the host cell responses. The promptness and dominance of cells (including lymphocytes, monocytes, macrophages) via induction of cytokine production (mainly TNF- α, IL-1α, IL-1β, IL-6, IL-8 and IL-10) have substantial role in pathogenesis. Finally, nearly all the mycoplasma cell components serves as super-antigens (Sethi *et al.*, 2012). The Figure 2.1 shows a flow diagram depicting the pathogenesis in *M. genitalium.*

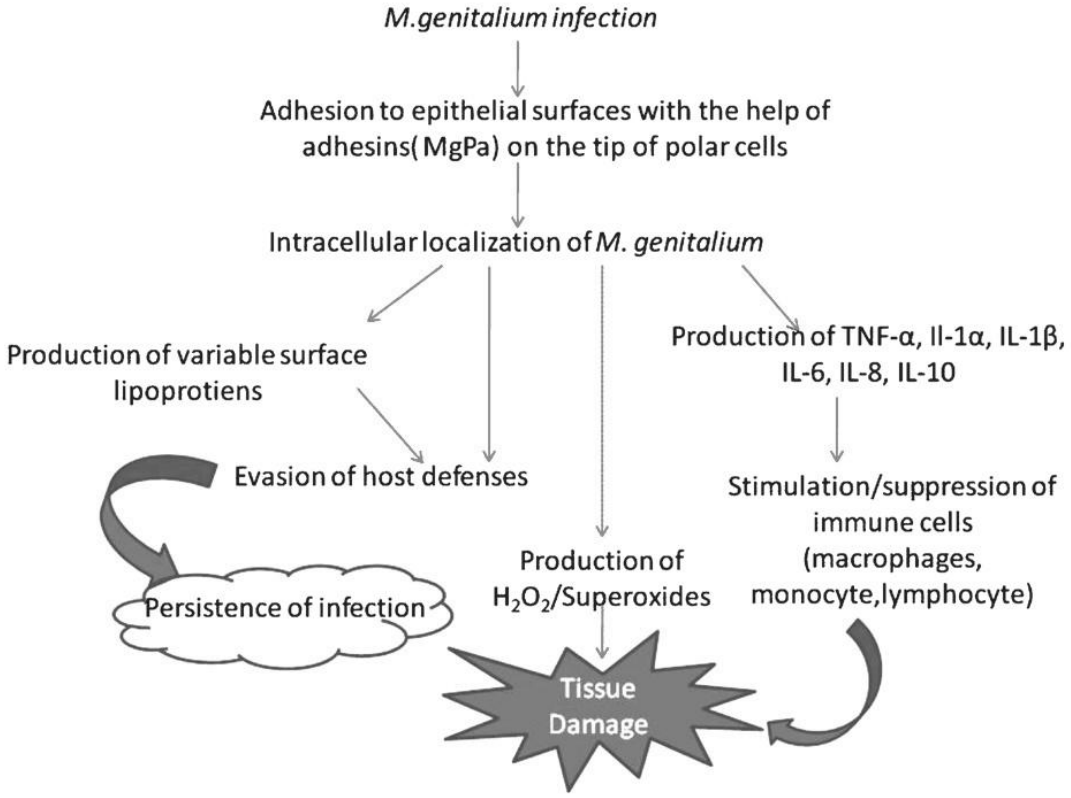

Figure 2.1: An overview of the pathogenic mechanisms of *M. genitalium* (Sethi *et al.*, 2012)

2.6 Mechanisms of Pathogenicity

Various mycoplasma pathogens display filamentous or flask-shaped form and also display conspicuous and specialized polar tip organelles aiding attachment to host cells (Murray *et al*., 2019). The polar tip configurations exhibit certain features which are complex, an interactive proteins network, specialized adhesions and adherence-accessory proteins (Li *et al*., 2019). Reports have shown that Mycoplasmas seldom invade the blood stream and tissues. Instead, they have been described as surface parasites with high affinity for epithelial lining of the respiratory and urogenital tracts of humans and animals. Here, a strong form of adherence is established thus preventing parasites purging by mucus secretion or urine. This is therefore a significant characteristic of Mycoplasmas (Buder *et al*., 2019). A key demonstration of adherence as a mode of pathogenicity is seen in *M.genitalium* adherence to spermatozoa. Studies have reported speculations that genital mycoplasma adhering to spermatozoa affects fertility as well as serves in transporting the organism into the female upper genital tract in fertilization (Piñeiro *et al*., 2019). The ability of pathogenic Mycoplasmas to haemadsorb is a resulting consequent of adherence therefore, pathogenic and non-pathogenic mycoplasmas are differentiated by their tendency to haemadorb (Kufa *et al*., 2019).

The close association among adhering mycoplasmas and its host cells creates a condition in which cell damage results from accumulated toxic metabolites emitted by the organism. One of such toxic metabolites is hydrogen peroxide (pathogenic factor) which has been implicated in close adherence of the organism to the host cell resulting to accumulated concentration high enough to cause direct cell damage (Cedillo-Ramirez *et al*., 2000). Furthermore, superoxide radicals and lipases are also implicated in inflammation and damage to cells as well as tissues (Gracials, 2007). In the case of Ureaplasmas, metabolic end products have also been reflected as a cause of tissue damage (Shibata *et al*., 2002). Among the end-products

expelled by Ureaplasmas, Carbon dioxide and Ammonia which are hydrolysis product of urea have been implicated in improving their growth due to the buffering potential resulting to pH 6.0 to 6.5, an ideal pH for ureaplasma growth as in the case of carbon dioxide (Domingues *et al*., 2003).

Several mycoplasma provoke blast transformation thereby a pathway where immunological cellular response results to tissue damage is formed (Donders *et al*., 2000). Furthermore, pro-inflammatory mediators and chronic inflammation have been implicated in poor pregnancy outcome and severe pulmonary disease among children (McGowin *et al*., 2009). Further studies have implicated substances such as: interleukin 1 (Lis *et al*., 2015) and Amniotic fluid Intercellular Adhesion Molecule-1 (ICAM-1) in pro-inflammatory response induced by ureaplasma infection (Robertson *et al*., 2002). Colonization of genital mycoplasmas in mothers trigger the formation of pro-inflammatory cytokinine such as tumor necrosis factor-alpha (TNF-α) which results to neonatal chronic lung disease (Crouse *et al*., 1998; Kufa *et al*., 2019).

Mycoplasma genitalium and other mycoplasma species such as *M. fermentans* are characterized by their haemolytic activity (Hughes and Saunders, 2018). Apart from these mode of pathogenicity exhibited by genital mycoplasmas, there are others such as: anti-oxidant activity of *M. fermentans* and *M. genitalium* mucin-binding potential to human vaginal-cervical epithelial cells (Lis *et al*., 2015).

2.7 Urogenital Infections in Women

When compared to the number of findings among men, only limited studies on the role of *M. genitalium* among women have been described (McGowin and Anderson-Smits, 2011).

2.7.1 Cervicitis

Limited studies have described the relationship between *M. genitalium* infection and cervicitis. There exist a variation in finding when the results from these studies are compared due to the divergence in the interpretation of cervicitis. Some studies interpret cervicitis as the presence of 10 polymorph nuclear lymphocytes (PMNLs) per high power field (HPF) in a cervical smear whereas other school of thoughts regard 30 PMNLs per HPF (Jensen, 2006). However, Peter *et al.*, (2018) interpreted cervicitis by endocervical discharge and > 20 PMNL/hpf. The study also identified *M. genitalium* in 5 out of 64 females which is comparable to the absence of *M.genitalium* among 80 asymptomatic pregnant women. Another study among 719 American women with filter paper stored cervical secretions revealed that *M. genitalium* was strongly associated with cervicitis in 7% of all subjects, 11% of cervicitis subjects (cervicitis interpreted as > 30 PMNLs/hpf) and in 5% of subjects without cervicitis (Aparicio *et al.,* 2018). Lillis *et al.*, (2018) further proved *M.genitalium* sole role in cervicitis. The attributable risk level was 70%, proposing that among females with cervicitis and habouring *M. genitalium*; 70% of cervicitis cases are linked to *M. genitalium* (Peter *et al*., 2018).

2.7.2 Pelvic inflammatory diseases (PID)

PID is a clinical syndrome characterized by the transition of microbes from the lower to the upper female genital tract. A range of bacterial species have been implicated. This includes bacteria responsible for bacterial vaginosis as well as *C. trachomatis* and *N. gonorrheae*. A linkage between *M. genitalium* and upper genital tract infections is a determinant for establishing the significance of the infection (Nolskog *et al*., 2019). Serology based studies have shown to be contentious. A study by Balkus *et al.*, (2018) in Kenya among 115 women presenting with acute pelvic pain, plasma cell endometritis was studied and *M. genitalium* was detected in 11% of the endocervical swabs comparable to 2% of women without

endometritis as reported by Cohen *et al.,* (2002). Among the subjects, 33% were HIV infected nevertheless, *M. genitalium* was found infrequently among the subjects. Contrary, Irwin *et al*., (2000) study among women with clinically suspected PID; *M. genitalium* was found frequently among HIV infected subjects (19 vs 5%). In a case-control study, Gaydos *et al*. (2019) studied the endocervical swabs of 45 women with clinically diagnosed PID and found *M. genitalium* DNA via PCR in 9 of the subjects (16%) as compared to none of 37 control patients.

2.7.3 Bacterial vaginosis (BV)

Initial studies showed that *M. genitalium* was found among 16.1% of women against 40.4% of women with BV (Lokken *et al*., 2017). Further studies demonstrated dearth of association of *M. genitalium* with BV. Razin (2005) reported that 2 (12%) of 17 women without BV were found to harbor *M.genitalium;* likewise *M. hominis*, *M. genitalium* undoubtedly is associated with BV (Lokken *et al*., 2017).

2.7.4 Adverse pregnancy outcome and infertility

Information presented on *M. genitalium* activity in producing adverse pregnancy condition such as: preterm labour, abortion or still-birth is limited (Rajkumari *et al*., 2015). In a study on preterm delivery, *M. genitalium* was discovered among 4% of mid-trimester vaginal swabs of the 124 women (Tsevat *et al*., 2017). In infertility cases, study by Rajkumari *et al*., (2017) reported a slightly high *M.genitalium* prevalence of 16% in a normal population of 100 infertile women. In another study by Alfarraj and Somily (2017) among infertile women using endocervical swab samples *M. genitalium* was found in 3% of women. Another study on unexplained infertility with normal tubes when examined with additional methods like a whole cell proteins of *M. genitalium* and *M. pneumonia* and recombinant MgPa antigen (Ramazanzadeh *et al*., 2016).

2.8 Risk Factors

The risk factors for *Mycoplasma genitalium* infection is similar to those of other STIs with main emphasis on younger age most especially among females for several reasons. Firstly, younger females have increased susceptibility to infections compared to older females which is attributed to their cervical ectopy. This is as a result of the endocervical columnar epithelial cells which projects to the surface of the vagina consequently resulting to a general increase of the vaginal surface area and number of receptive cells. This in turn promotes the growth of pathogens which colonizes the mucosal surfaces (Kaida *et al*., 2018**).** Secondly, epidemiological studies have shown that younger females have higher tendency to indulge in sexual behaviours, such as unprotected intercourse and multiple sexual partners compared to older females (Kaida *et al*., 2018**).** Douching has been described as a common activity among females which can alter the vaginal microflora thereby remove protective components from the vagina or cervix, consequently promoting the progression of microorganisms from the lower to the upper genital tract aiding susceptibility to infection (Romano *et al*., 2019). Molenaar *et al.,* (2018) studied the sub sample of participants in Wave III of the National Longitudinal Study of Adolescent Health (Add Health) for potential risk factors of *M. genitalium*. Molecular-based (PCR) assay was employed to examine urine of 1714 women and 1218 men within the age 18 to 27 years. *M genitalium* infection was found to be strongly associated with vaginal intercourse irrespective of the number of times (Prevalence Ratio (PR) 22.5, 95% CI 4.3-116.6). Also, multivariate analyses revealed the prevalence of *M.genitalium* increased by 10% with every other vaginal intercourse partner in the past year (PR 1.1 per partner in the past year, 95% CI 1.0-1.2). Besides, *M. genitalium* was found to be more prevalent in individuals that co-habitated with their sexual partner (PR 11.2, 95% CI 3.2-39.5) as well as among Black race subjects (PR 7.2, 95% CI 2.9-17.9) with condom use during last sexual intercourse (PR 3.9, 95% CI 1.3- 11.5) although the frequency and proper

use of the condom was not defined. *Mycoplasma genitalium* was not found to be associated with age, age at first sexual contact, neither correct nor consistent condom use within the past year (Akgul *et al*., 2018).

In a U.S. based study by Kim *et al.* (2018) vaginal swabs from 264 sexually active teenage female aged 14 to 21 years attending inner-city medical center were examined for *M. genitalium* using PCR; molecular-based method. It was revealed that sexual activity within the past 7 days was associated with a 2-fold increase in the odds of *M. genitalium* infection (OR 2.0, 95% CI 1.1-3.2). Following variation for *C. trachomatis* infection, *M. genitalium* infection was found to be associated with demographic variables such as: age. Whereas sexual behaviors such as inconsistent condom use, new sexual partner, or multiple sexual partners were not comparable Kim *et al.* (2018). Similar to former study in U.S., Ronda *et al* (2018) proceeded and studied vaginal samples from 693 female of the same age group as Kim *et al.,* 2018 attending an urban primary health care center. *Mycoplasma genitalium* was detected among all subjects reporting history of vaginal intercourse with exception to one. New sexual partner (OR 1.4, 95% CI 1.2-1.7) was found to be the sexual behaviour independently associated with *M. genitalium* (Ronda *et al.,* 2018).

2.9 Association of *M. genitalium* with Other STDs

Mycoplasma genitalium have been reported by several authors to be associated with other STDs. From Africa with focus on West Africa, Balkus *et al.*, (2018) reported 20% of infection among subjects were linked to *M. genitalium* presenting in co-infections. The incidence of co-infection was further reported as 37.9, 10.6 and 7.6% for gonococcal urethritis, *C. trachomatis* and *Trichomonas vaginalis* infection (Kim *et al.,* 2018). In a study by Amirmozafari *et al.,* (2009) concurrent occurrence of *M. genitalium* and *U. urealyticum* was shown in 1.4% of women with genital infections, while triple infection of *M. genitalium, U. urealyticum* and *M. hominis* was seen in 0.5% of these patients. Thus, demonstrating

simultaneous detection of genital mycoplasmas. Other reports from researchers further demonstrated co-existence of *M. genitalium* with other genital pathogens (Mirnejad *et al*, 2011; Samra *et al*, 2011).

2.10 Laboratory Diagnostic Methods

2.10.1 Clinical Isolation and In vitro Growth

The isolation of the type strain *M.genitalium* strain G-37 was accredited to Tully (Tully, 1991). From the time onwards, there have been reported limited efforts to isolate this clinical strain. The direct cultivation of *M.genitalium* unswervingly from patient specimens is time consuming and laborious involving the following: growing in tissue culture and slow adaptation to cell-free culture. The growth of *M.genitalium* presents as red to yellow color changes resulting from pH shift following production of acid via glucose fermentation in broth culture. In comparison to growth on solid medium, lenient 1% agar and reasonably extended incubation time is required as colonies with the characteristic fried -egg morphology presents subsequently within 2-3 weeks and visualizable using a dissecting microscope. In the case of single–colony- clone, cultures are subjected to filter cloning for a minimum of three times owing to the adherent nature of *M. genitalium* (Ma *et al*., 2010).

2.10.2 Serological Test for *M.genitalium*

M.genitalium shares similar structural properties with *M.pneumoniae* another human pathogen in the *Mycoplasma* genus. Cross-reactivity normally results between these two *Mycoplasma species* in serology assay (Huppert *et al*, 2008).

Consequently, diagnostic methods such as culture and serology are unsuitable for specific diagnosis of *M. genitalium* hence specific identification of infected persons has thereby been entirely dependent on nucleic acid amplification tests (NAATs).

2.11 Molecular Methods for Detection of *Mycoplasma genitalium* In Humans

Mycoplasma genitalium was primarily sequestered from men with urethritis and since then has been a significant source of urethritis and same for female cervicitis and pelvic inflammatory disease (Jensen, 2004). Some genital mycoplasmas are readily detected from healthy adults being commensals in the lower urogenital tract; however, *M. genitalium* is rarely detected among asymptomatic persons. *Mycoplasma genitalium* holds a terminal structure (MgPa adhesion) responsible for attachment to epithelial cells (Hu *et al*., 1987) thus attaches to spermatozoa and erythrocytes and invades epithelial cells with evidence of nuclear localization (Jensen, 2004). A family of repetitive DNA are homologous to the MgPa adhesin gene of *M.genitalium* and contribute to variation in the protein of the MgPa adhesin gene. Sequence divergence and antigenic variation in *M. genitalium* prevents host immune response and optimize adhesion (Peterson *et al*., 1995). *Mycoplasma genitalium* is described as the smallest bacteria due to its 580-kbp genome of *M. genitalium* contains only 485 protein-coding genes.

2.12 Limitations of Culture and Serological Analysis As Diagnostic Tests For Mycoplasmas And Ureaplasmas

2.12.1 Culture

Ureaplasma species are known to grow best in media containing urea producing colonies visible with a stereomicroscope within 1 to 3 days. The appearance of brown granular colonies on culture is indicative for *Ureaplasma* species, but culture alone cannot distinguish between the two species.

Mycoplasma hominis have been reported to thrive best in SP 4 broth or SP 4 agar supplemented with arginine; however, it grows best on A8 agar and in 10B broth (Waites and Taylor-Robinson, 2011). With a stereomicroscope, *M.hominis* colonies appear on agar within 2 to 3 days. Specie confirmation for cultivated mycoplasmas on agar requires a high

throughput procedures (like; PCR assay) which bridges the limitation of speciation. PCR assay; a molecular-based assay is more sensitive than culture method generally without exception of microorganisms such as *M. hominis* and *Ureaplasma* species which are quite less challenging to cultivate as compared to *M.genitalium*. Data from the University of Alabama at Birmingham Diagnostic *Mycoplasma* Laboratory supports this finding by demonstrating the use of real-time PCR to detect ureaplasmal DNA in 52 (39.4%) of 132 specimens against 32 (24.2%) detected by culture (Xiao *et al*., 2010). Despite the fact that culture is termed the "reference method" PCR is ideally suitable for detection of organisms; consequently PCR-positive with culture-negative specimens most likely epitomize true positive. Therefore, PCR is a considered "a gold standard". Notwithstanding, PCR is highly more sensitive and specific for identification of *M. hominis* and *Ureaplasma* species, culture method however maintains the most cost-effective and practical detection method for mycoplasmas. This is one of the key advantages of culture over PCR. Cultures set up could be one at a time but the cheapest form to use the PCR method requires runs of some specimen batches. Conversely, this increases the turnaround time for result. Furthermore, culture method is advantageous since it avails the possibility of undertaking antimicrobial susceptibility testing. In contrast to very sensitive PCR assays. It is most suitable for research studies when turnaround time is not paramount creating an avenue for samples in batches to be examined intended for economical purpose. Irrespective of the different modalities been developed to boost the detection of *M. genitalium* in culture, there remains certain limitations such as: great failure rate and exceptionally slow growth rate. Therefore, culture for *M.genitalium* have been impractical and seldom employed most especially with the advent of molecular-based assay (Waites and Taylor-Robinson, 2011). Confirmatory test for colonies grown on culture media is essential with PCR since several commensal *Mycoplasma species* frequently colonize the human mucosal. Comprehensive approaches for collection of

samples, cultivation, and identification of human mycoplasmas and ureaplasmas *in vitro* via culture-based methods have been further described by other authors (Cumitech, 2001; Waites and Taylor-Robinson, 2011).The use of culture based method for cultivation will solely reply on several factors which includes: facilities and expertise available in routine laboratories and the species under investigation.

2.12.2 Serological analysis

Serological assay an earliest method developed for detection of *Mycoplasma* specifically targeted for *M.pneumoniae*. Serological methods includes: enzyme immunoassays, immunofluorescence, and particle agglutination assays. However in recent times, molecular-based nucleic acid amplification tests (NAATs) have crippled the use of serological tests. Like every laboratory detection methods, there are limitations associated with serological analysis which includes:

1. Necessity for acute and convalescent serum samples requiring simultaneous detection of IgM and IgG aids in confirmation of seroconversion.
2. Distinction of recent infection from past infection is almost impossible.
3. The significant duration (1 to 2 weeks) is required following onset of the infection until detectable antibody develops (Beersma *et al*., 2005).

Adults rarely develop measurable IgM antibodies seemingly due to re-infections cases (Waites and Talkington, 2004). Besides, IgM antibodies are detectable for weeks to months compromising diagnosis of acute infection on a single assay for IgM (Waites and Talkington, 2004). Furthermore, due to recurrent infection, tendency for high seropositivity for IgG can be observed in various apparently healthy adults (Csango *et al*., 2004); therefore, the determination of elevated and declined antibody in acute and convalescent cases is vital.

Immunosuppressed individual or patients are another group of individual with special consideration in serology testing as antibody production are usually limited in few infections

and sometimes not present. Sensitivity and/or specificity are continued challenge with serological assay when compared to PCR (Beersma *et al*., 2005). Thus, there arise the need for serological tests to undergo extensive comparative evaluations and comparison with other diagnostic methods as to establish a confidence interval for their sensitivity and specificity.

Examples of the serological tests available for *M. hominis*, *M. genitalium* and *Ureaplasma species* are enzyme immunoassay, micro-immunofluorescence and metabolism inhibition (Waites and Taylor-Robinson, 2011). However, *Ureaplasma species* and *M. hominis* are known to be abundant and as a result they are readily isolated from healthy people hence compromising interpretation of antibody titers. Currently, there are no standardized serological assays for the genital mycoplasmas.

2.13 Overview of Molecular-Based Tests for Detection of Mollicutes

Due to the limitations associated with culture and serological analysis, nucleic acid amplification tests (NAATs) began to attract increasing attention in mycoplasmology. Still, there are no products of this nature sold commercially in the United States for detection. Meanwhile following the introduction of NAATs in the 1980s; there have been numerous reports which have emerged describing a number of NAATs and their applications for the detection of mycoplasmas and ureaplasmas in clinical specimens (Loens *et al.,* 2010; Fookes *et al*., 2017). These previously described assays allows the detection of particularly fastidious species, such as *M. genitalium*, which are difficult-to-detect (Fookes *et al*., 2017). Nucleic acid amplification tests are advantageous in the identification of microbial colonies grown in culture to species level. Polymerase chain reaction (PCR) has become the most generally accepted NAAT assay for mycoplasma detection. It also serves in the detection of antimicrobial resistance determinants and the determination of the genetic relatedness of clinical isolates. There exist several PCR assays, which are: Conventional PCR, Real-time and Semi-nested PCR. The conventional PCR processes comprises of PCR products using

gels as in gel electrophoresis unlike real-time PCR which identifies and quantifies concurrently with amplification. Furthermore, nested PCR enhances sensitivity via re-amplification of a PCR product using second set of primers (Daxboeck *et al*., 2003). A draw back to the nested PCR method is the increase risk of contamination (Loens *et al*., 2003). Authors have reported certain real-time PCR for detection and characterization of mycoplasmas and ureaplasmas such as: the ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA), the iCycler iQ (Bio-Rad, Hercules, CA), and the Light Cycler 2.0 (Roche Diagnostics, Indianapolis, IN).While, detection system in conventional PCR includes: agarose gel electrophoresis, SYBR Green, TaqMan probes, hybridization probes, molecular beacons, and microchip electrophoresis (Foster City, CA) (Loens *et al.*, 2010).

Real-time PCR using the Roche Light Cycler have been highly recommended over conventional PCR by The UAB Diagnostic *Mycoplasma* Laboratory due to its accuracy, quantitation, and turnaround time. The enhanced specificity of real-time PCR as compared to conventional PCR is majorly due to the application of a third oligonucleotide probe which binds to the target sequence. Labeled probe are advantageous in reducing the likelihood of cross-reaction and detection of undesired amplicons. Secondly, amplicon melting temperature is determined at the end of the assay thus desired PCR product is authenticated (Jensen and Dohn, 2002).

Performing a comparison of conventional PCR and real-time PCR; conventional PCR procedures involving DNA extraction and amplification could last for 2 to 3 days. Whereas, real-time PCR produce instanteous results (Dorigo-Zetsma *et al*., 1999; Nilsson *et al*., 2010). The pros and cons of NAATs, culture, and serological analysis employed in the detection of mycoplasmas and ureaplasmas is shown in Table 2.1.

Table 2.1. Advantages of Molecular-Based Methods Compared with Culture and Serological Analysis for Detection of Mycoplasmas and Ureaplasmas in Humans

Criteria	Molecular-based assays	Culture	Serological analysis
Availability	Not commercially available in the United States, a few PCR kits are available in Europe and Asia. Nonproprietary PCR assays are available in a few US reference laboratories.	Commercially prepared SP 4, 10 B, and An 8 media and test kits for *M. hominis* and *Ureaplasma* species are available. Culture is available in many large hospital and reference laboratories. Additional immunoserological or genotypic tests are required to confirm species identity of large colony mycoplasmas	Commercial qualitative and quantitative antibody assays are available for *M. pneumoniae*. No such assays are available for other *Mycoplasma* or *Ureaplasma* species in the United States.
Cost	Cost of equipment and reagents is significant, and personnel trained in molecular diagnosis are required. Costs are less if equipment, facilities, and personnel can be used for other molecular diagnostic testing.	Media are somewhat expensive to obtain or prepare. Equipment used for general microbiology purposes is usually sufficient. Length of time cultures have to be held for slow-growing Mycoplasmas adds to personnel costs.	Commercial serological kits for *M.pneumoniae* vary in cost. Some assay formats are suitable for testing Single specimens, whereas others are more practical for batches. Cost per test depends on the volume of specimens and equipment requirements.
Turnaround time	Real-time PCR can be Completed in a few hours. Batching specimens and running the assays once or twice each week decrease the costs but delay turnaround time.	*M. hominis* and *Ureaplasma* species can be grown in culture within 1–3 days, whereas *M. pneumoniae* requires from 5 days up to several weeks. *M. genitalium* cannot be reliably grown in culture	Hands-on time varies from a few minutes to a few hours. Acute and convalescent serum sample collection time spans 2–3 weeks.
Analytical sensitivity	High: most assays detect _100 CFU/mL organisms or 100 genome copies.	May detect 100–1000 viable organisms per test	Serological tests do not measure the presence of the microorganism, but instead measure the host immune

			response. Compared with PCR, serological analysis may miss many infected individuals.
Specificity	PCR assays that are carefully validated with targets chosen for diagnostic accuracy and lack of cross-reactivity are specific.	Culture is 100% specific when positive.	Older complement fixation tests had problems distinguishing *M.pneumoniae* from *M. genitalium.* Newer commercial ELISAs do not have this problem.
Specimen Requirements	Organisms do not have to be viable. The same specimen types used for culture can be submitted for PCR assays. Specimens require frozen storage until processing. Formalin-fixed tissue can also be processed by PCR.	Properly collected specimens require appropriate transport media, frozen storage, and shipment to maintain viability.	Serum is the only required specimen type. No special handling or storage, other than refrigeration, is needed

Source: Waites *et al.*, 2012

2.14 Use of Molecular-Based Tests for *M. genitalium* Detection

Most of the early PCR assays for *M.genitalium* targeted several regions of the MgPa operon, but 16SrRNA gene have also been targeted (Eastick *et al*., 2003; Jensen *et al.,* 2003). Reports have described additional molecular-based assays like: transcription-mediated amplification for epidemiological purposes (Hupert *et al*., 2008; Wroblewski *et al*., 2006; Hardick *et al.,* 2006). Quantitative, rapid, real-time PCR have employed MgPa operon, 16S rRNA and *gap* gene encoding glyceraldehyde-3-phosphate dehydrogenase (Yoshida *et al*., 2002; Deguchi *et al*., 2002; Dupin *et al.,* 2003; Jensen *et al*., 2004b; Jurstrand *et al*., 2005; Sventrup *et al*., 2005). Interestingly, not all MgPa target primers demonstrate high sensitivity for all *M. genitalium* strains. Ma *et al.,* (2010) studied the three genes of the MgPa operon for *M. genitalium* (*mgpA, mgpB,* and *mgpC*) and nine repetitive sequences (termed MgPars) distributed in the genome of 15 geographically diverse strains; the operon sequences and all MgPars differed from each other more than from the published G37 operon sequence. It was revealed that one out of the nineteen primers had 19 variable nucleotides and with variation for one of two typing systems was found located in a hyper variable region, thus suggesting the possibility of incorrect results as demonstrated in studies using primers MGS-2 and MgPa-903 (Ma *et al*., 2010).

With the limitation of MgPa, and 98% identity of the 16S rRNA gene for *M. pneumoniae* and *M. genitalium* in prospect, the UAB Diagnostic *Mycoplasma* Laboratory modified the real-time PCR assay already described by Svenstrup *et al*., (2005) for detection of *M. genitalium*. This procedure targets the conserved housekeeping gene *gap* (National Center for Biotechnology Information (NCBI) accession number U39710) in a primer and probe scheme. This target significantly differs from other species, comprising the *gap* homologue in *M. pneumoniae* (72.3% identity) and the genome as a single copy (Svenstrup *et al.,* 2005). Furthermore, PCR based tests is improved to detect mutations in DNA gyrase and/or

topoisomerase IV which mediates fluoroquinolone resistance in *M. genitalium*, thus by-passing culture *in vitro* to establish antimicrobial susceptibilities (Shimada *et al.*, 2010). Gen-Probe (San Diego, CA) established a transcription-mediated amplification real-time PCR assay but available in the United States strictly for research purposes (Mena *et al*., 2009; Schwebke *et al*., 2011). Multiplex PCR-based systems for simultaneous detection of *M. genitalium*, with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and other urogenital mycoplasmas and ureaplasmas, are available as kits in some European countries by various companies, including Bio-Rad (Hercules, CA), Amplex Biosystems (Giessen, Germany), PCR Diagnostics.eu (Bratislava, Slovak Republic), and Seegene, Inc. (Rockville, MD) requiring varied formats and instrument platforms. There are no reports supporting some of these European kits been subjected to large and rigorous clinical trials neither have there been comparative evaluation with already existing assays. The persistent absence of commercial NAATs for detection of *M. genitalium* in the United States has greatly limited interest among physicians in looking for *M.genitalium*. Considering the involvements of *M.genitalium* in certain medical conditions such as urethritis, cervicitis, and pelvic inflammatory disease with the possibility of venereal transmission of disease to other individuals, as studies have reported (Brooks *et al*., 2010). Consequently, in cases where *M. genitalium* is detected in the lower urogenital tract of a symptomatic patient, it is considered medically significant.

The application of precise molecular-based methods with a prompt turnaround time definitely will increase the interest of physicians in *M.genitalium* if they become available most especially in the United States.

2.15 Important Technical Aspects of Molecular-Based Assays Used For Mollicute Detection

2.15.1 Specimen Collection

Proper specimen collection is crucial in all microbiological assays. Specimens for the detection of Mycoplasmas and Ureaplasmas via culture or molecular-based assay are best collected, stored, and processed correctly. Collection tubes with acid citrate dextrose are best suitable for blood samples (Horner *et al*., 2001). Transport media or isotonic solutions such as Phosphate buffer saline (PBS) have been proven to be the best for transporting Mycoplasmas and Ureaplasmas to the laboratory. The use of Dacron or polyester swabs as calcium alginate and cotton swabs can be inhibitory to the growth of Mycoplasmas when swab samples are collected (Waites *et al*., 2001). Broths such as SP 4 broths applicable in culture have demonstrated no negative consequence on the result of real-time PCR assay like; the Roche Light Cycler 2.0 (Roche Applied Science, Penzberg, Germany). However, it is most likely that culture broth could be inhibitory when used with other primers or reaction conditions, or thermocyclers though this is not confirmatory. Still, it is needed to validate that broth or any solution that are not specialized PCR transport buffer lack inhibitory properties prior to transport for PCR assay (Murray, 2017).

2.15.2 DNA Extraction

In DNA extraction process, the lysis stage with proteinase K treatment most often produce clear DNA, otherwise there are inhibitory properties in the sample collected (Blanchard *et al*., 1993). Body fluids (other than blood) and transport systems containing material obtained from swabs are most suitable. However, there are some samples with inhibitory properties such as blood, tissue samples, lower respiratory tract secretions, and subcultures. Such samples may undergo purification process which could be done with commercially available genomic DNA purification kits. Nucleic acid isolation methods could be either automated or

semi-automated which can be employed in sample preparation. There is however no major distinction in the performance of automated extraction systems from manual systems. Both are applicable and are dependent on availability and expertise. Examples of some automated extraction systems include: easyMAG nucleic acid extractor, QIAgen blood mini kit and the NucliSENS miniMAG systems (Loens *et al*., 2008; Waites *et al*., 2012).

2.15.3 PCR Operating Conditions

The typical procedure for real-time PCR involves primer selection and equipment specific requiring the authentication of the methods and instruments. An intensive evaluation of specific assay component; starting from sample type, transport media, extraction methods to final PCR amplification and detection techniques employing specific primers and probes, reaction conditions and specific controls of the assay which ensures techniques are efficient ensuring the elimination of inhibitors (Jensen *et al*., 2016).

2.15.4 Quality Control

The major significance of quality control applied in PCR, is the elimination or reduction of false-positive and false-negative results (De Barbeyrae *et al*., 1993). When comparing both PCR assays: Conventional and Real-time PCR. Real-time PCR are more advantageous than conventional PCR as regards to quality control procedures. In conventional PCR, false-positive results from contamination are mostly associated with the assay (Columbia Encyclopedia, 2001). This does not apply to real time PCR. Quality control checks for limitations which could contribute to false-negative results such as: human errors, occurrence of PCR inhibitors in the sample to be tested, less ideal reagent preparation, reaction conditions, and extraction of unpurified target DNA (Jensen *et al*., 2016). However some of these limitations like inhibitory influences and suboptimal PCR conditions can be checked with the use of a positive control DNA preceding purification. Furthermore, the application

of an internal control in crude sample and treated together for purification and amplification is described as a sure accurate method (Chessbrough, 2000).

2.15.5 Determination of Analytical Sensitivity and Specificity

Serial dilutions of template DNA (genomic or plasmid DNA) can be applied in PCR analytical sensitivity and specificity which is expressed in terms of amount of DNA detected. It is most ideal to validate the analytical specificity of PCR assays applied in human mollicutes (commensal mycoplasma species) and other microorganisms inhabiting a common body site or display a high degree of sequence similarities to the targets (Uphoff *et al*., 2002). To achieve an optimal assay, it is best to validate against various type strains and low-passage isolates. Among Ureaplasmas, the four recognized serovars for *U.parvum* and 10 for *U. urealyticum* ought to be confirmed (Keane *et al*., 2000). In addition, human genomic DNA which are prone to possible inhibitory effects should be considered in validation of the analytical specificity of the PCR assay. Furthermore, the assay replicability can be authenticated by examining one sample several times (Murray, 2017).

2.15.6 PCR Assay Validation

Additional PCR assay validation is required to further authenticate the assay thus, PCR assays on properly characterized micro-organisms which have previously been assayed by other detection methods like: culture and other PCR assays employing different gene targets is ideal (Domingues *et al*., 2002). A practical demonstration of additional PCR assay validation could be seen in the demonstration of comparable molecular results prior to routine PCR-based diagnostic work. Alternatively, a demonstration of the molecular results which exceed the detection efficacy of conventional culture based techniques thereby establishing sensitivity for the assay. For human mycoplasmas and ureaplasmas, there are no comprehensive data for validation of the varied range of PCR assays available for detection (Murray, 2017).

2.16 16S rRNA Gene Sequence Analysis for Identification of Bacteria In Medical Microbiology and Infectious Diseases

The historic technique for identification of microorganisms relied on the comparison of a precise morphologic and phenotypic depiction of type strains or representative strains with the accurate morphologic and phenotypic description of the query isolate. Standards have been established for comparison of query isolates with type strains or representative strains by microbiology authors of several standard references such as *Bergey's Manual of Systematic Bacteriology, Manual of Clinical Microbiology,* Centers for Disease Control and Prevention and the American Type Culture Collection (ATCC). These standard references are published tables with the features of individual bacteria species (Krieg and Holt, 1984; Funke *et al*., 1997; Murray *et al*., 1999). This enables medical microbiologists relate the results of a query microbial strain with group of type strains presented in the standard table. Imperfect match is seldom found and conclusion drawn on the most likely identification. With the advent of numerous schemes and computer programs, identification might differ between laboratories (Sussman *et al*., 1986).

There emerged a new standard for bacteria identification in the 80s. The laboratories of Woese and others demonstrated phylogenetic relationships of bacteria and concluded that all living organisms may perhaps be defined by comparing an invariable region of the genetic code (Woese *et al*., 1985; Woese, 1987). Example of such invariable region in bacteria includes: genes encoding the 5S, 16S (also called the small subunit), and the 23S rRNA. However, the region of the bacterial DNA commonly employed for taxonomic studies is the 16S rRNA gene (Palys *et al*., 1997; Kolbert and Persing, 1999; Garrity and Holt, 2001; Tortoli, 2003; Harmen and Karch, 2004). The 16S rRNA gene is also referred to as "16S rDNA" and the terms have been used interchangeably in literatures. But, the current American Society of Microbiology (ASM) rule suggests "16S rRNA gene" be used as

reference. The 16S rRNA gene can be compared among all microorganisms including: bacteria, archeobacteria and the 18S rRNA gene of eukaryotes.

2.17 Mechanics of the Process

2.17.1 Choice of the 16S rRNA Gene as the Gene to Sequence

Dubnau *et al.,* (1965) was the first to observe the noted conserved nature of the 16S rRNA gene sequence among *Bacillus* spp. However, the adaptation of 16S rRNA gene sequence for bacterial identification and taxonomy purposes preceded studies by Woese who defined revealed some unique features of the gene.

Firstly, is the fact that it acts as a molecular chronometer (Woese, 1987). Secondly, the level of conservation could result in the critical role played in cell function by 16S rRNA. This is different from genes required to encode certain enzymes. When mutations occur in these genes structures are transformed. For instance; if a bacterium lacks the gene encoding enzymes necessary to utilize lactose, alternative pathways can be initiated to produce sugar or protein as an energy source. So, a limited number of genes are highly conserved like the 16S rRNA gene. Even though the frequency of change in the 16S rRNA gene sequence is unknown, it still defines evolutionary relationship and relatedness of organisms (Kimura 1980; Pace 1997; Thorne *et al*., 1998; Harmsen and Karch, 2004). The possible rate of change of 16S rRNA gene differs among organisms (different taxonomic groups could have different rates of change) if present. The rates of mutation could be influenced by certain factors like evolution, and inconsistent throughout the 16S rRNA gene. The term "hot spots" is used to describe larger amounts of mutations (Tortoli, 2003; Ueda *et al*., 2006) and differs among species. 16S rRNA serves as the target for a number of antimicrobial agents. Thus, mutations occurring in the 16S rRNA gene can alter antimicrobial susceptibility to antimicrobial agents and the 16S rRNA gene sequence differentiates phenotypic resistance to antimicrobial agents (Pfister *et al*., 2003a; Pfister *et al*., 2003b). The antimicrobial profile of

microorganisms does not interfere with the application of 16S rRNA gene sequence for bacterial identification and assigning close relationships at the genus and species level in the field of microbiology.

The gene impact the ascribing of linkages of more distantly related organisms seen in the branches (Garrity and Holt, 2001). The 16S rRNA gene sequence of microorganisms is somewhat 1550 bp long and houses both variable and conserved regions. The gene is large in size with appropriate interspecific polymorphisms of 16S rRNA gene thus offering distinctive and statistically valid measurements. Universal primers are mostly employed which are complementary to the conserved regions of the gene, the 540-bp region and at the end of the entire sequence (the sequence of the variable region in between is used for comparative taxonomic study) (Relman, 1999). The common lengths mostly used is the 500 and 1,500 bp sequence but sequences in databases can still be of different sizes. The 16S rRNA gene sequence has been defined for an enormous number of strains. GenBank which is the largest databank of nucleotide sequences till date houses well over 20 million deposited sequences out of which more than 90,000 sequences are of the 16S rRNA gene. This implies that there are readily available 16S rRNA deposited sequences to which unknown strains can be compared to for evolutionary study.

The 16S rRNA gene is universally found among bacteria, and so relationships can be established among all bacteria (Woese *et al*., 1985; Woese 1987). An overview of the 16S rRNA gene sequences permits differentiation among organisms at the genus level cutting-across a large number of major bacteria phyla. Also, classification of strains at species and subspecies level is achievable. However, it could be challenging applying 16S rRNA gene sequencing to understudy several well-known species with same or very similar sequences. Whole sequencing of the 1500-bp length of the 16S rRNA or partial sequencing is dependent on the required comparable information. Sequencing of the entire 1500-bp region is

advantageous in differentiating between particular taxa or strains (Sacchi *et al*., 2002a; Sacchi *et al.,* 2002b).

Secondly, sequencing of the entire 1500-bp sequence are suitable in the description of new species. However, the 500-bp sequence provides adequate disparity for identification and interest, reveals largely the difference between strains since it demonstrates slightly more diversity per kilobase sequence. Kattar *et al*., (2001) established that a 66% variability in the 16S rRNA gene sequence among *Bordetella* species existed in the first 500 bp sequence. An already published literature highlighted the application of MicroSeq database (Applied Biosystems Inc. [ABI], Foster City, Calif.) which normally is based on the 500-bp sequence (Tang *et al*., 1998; Patel *et al*., 2000; Tang *et al*., 2000; Hall *et al*., 2003). However, some other researchers carried out identifications with sequences of about 400 bp (Bosshard *et al*., 2003) and even 200 bp or less (Wilck *et al*., 2001). There have also be revealed that other genomic regions permits examination of the phylogenetic relationships among bacteria. Attempts have been made with whole-genome but pose some difficulties for several reasons like: different sizes of the genome and the application of gene duplication, gene transfer, gene deletion, gene fusion, and gene splitting. Currently, there are fewer than 100 whole genomes to match (Woese *et al.,* 2000; Bansal and Meyer, 2002). Although, the phylogenetic tree following analysis of whole-genomic and the 16SrRNA gene are the same (Bansal and Meyer, 2002). However, other areas of the rRNA gene have also been used for studying evolutionary relationships among bacteria. Roth *et al.,* (1998) revealed that 16S-23S rRNA gene internal transcribed spacer sequences most suitable in distinguishing species that were indistinguishable by 16S rRNA gene sequences following their study among *Mycobacterium spp.*

Other researchers revealed the significance of 23S rRNA sequences in differentiating among *Streptococcus* spp. (Rantakokko *et al*., 2000). Some researchers discovered that an overall

robustness of the method influences the sensitivity of the analysis as the major branching points of the phylogenetic tree display high degree conservation with either the 16S rRNA or 16S-23S rRNA gene sequences (Roth *et al.*, 1998). For some other researchers, the 16SrRNA gene sequence is most suitable for phylogenetic analysis than the 16S-23S rRNA gene region (Song *et al*., 2004). Consequently, 16S-23S rRNA gene region is rarely applied thus very few comparative sequences exist. There are very few exceptions; for mycobacteria, the gene encoding the 65-kDa heat shock protein is highly conserved and thus applied in taxonomic relationships (Pais *et al*., 1997; Tortoli 2003). Despite the usefulness of the 65-kDa heat shock protein sequences in phylogenetic study only a few have been deposited.

On the other hand, application of protein-encoding gene sequence in constructing phylogenetic trees for comparisons discloses deep-rooted taxonomic and evolutionary relationships as demonstrated using the 16S rRNA gene (Garrity and Holt, 2001; Krieg and Garrity,2001). In broad sense, comparison of strains for epidemiological purposes and identification of strains with unique virulence factor; the 16S rRNA gene analysis appears not most suitable to reveal high degree of variation and also the region clearly does not encode virulence factors. However, there are few exceptions, For example; the micro heterogeneity in the 16S rRNA gene sequence found by Sacchi *et al*. (2002) employed in trailing *Neisseria meningitidis* strains. Furthermore, in the case of differentiating species within a particular genus, a more suitable gene than 16S rRNA gene could be employed in such identification of the species. For instance, the citrate synthetase gene in the genera *Bartonella* and *Rickettsia* (; Regnery *et al*., 1992; Fournier *et al*., 2003) demonstrated high uniqueness for each species and therefore has been adopted as an excellent tool in research to differentiate them. Still, no documented gene has shown such wide-use applicability across all the taxonomic groups as the 16S rRNA gene. Therefore, in detailed identification of an unknown organism with no prior knowledge, the 16S rRNA gene sequence is an outstanding and extensively used choice.

The latest edition of the most commonly used and highly recognized as well as respected reference on bacterial taxonomy: *Bergey's Manual of Systematic Bacteriology*, is compiled on the basis of 16S rRNA gene sequence analysis as the mainstay.

2.17.2 Basics of Sequencing

Nucleic acid sequencing methods have undertaken remarkable improvements over the past decade. These rapid improvements have resulted to the feasibility of much smaller laboratory to determine the sequence of millions of base pairs of DNA within a short duration of time. The excellence of sequence data improved with swiftness and technology available. Consequently, it is observed that a public database such as GenBank with 16S rRNA gene sequences deposited in the early 1990s revealing incorrect and indeterminate bases as compared to recently generated sequences. Mostly, there is a high possibility to generate a sequence in less than one and half working days with less than a colony and even directly from a specimen (Tang *et al*., 1998; Celard *et al*., 1999; Cook *et al*., 2003). For the record, there have been several methods to determine DNA sequence.

DNA extraction of Bacterial genomic DNA from intact cells employing standard method (Sambrook *et al*., 1989) or commercial method (e.g., PrepMan DNA extraction reagent; ABI). For PCR amplification of 500 or 1,500 bp of the 16S rRNA gene sequence, the extracted DNA serves as the template DNA. Furthermore, broad-spectrum or universal primers complementary to conserved regions are employed so as to enable amplification from regions from any bacteria. In the same light, genus-specific primers complementary to conserved regions are employed so as to enable amplification from regions from any bacteria within the genus. Later, the PCR products are purified with the aim to remove excess primers and nucleotides. There are several excellent and efficient available commercial kits (e.g., QIAquick PCR purification kit [Zymo Research], QiaQick PCR purification kit [Qiagen] and Microcon-100 Micro concentrator columns [Millipore]). Following this is a process called

cycle sequencing. It is somewhat similar to PCR as it employs DNA but specifically purified products of the first PCR cycle to serve as DNA template. The forward and reverse sequences are both applied as the template in single reactions with just the forward or reverse primer. Although, there exist a distinction between cycle sequencing and PCR; in cycle sequencing no new template is made that is the same template is recycled for the number of cycles programmed which is usually 25 cycles thus the resulting product is a combination of DNA with varying lengths. This is attained by addition of specifically labeled bases called dye terminators along- side with unlabeled bases and are indiscriminately integrated in a second cycle and then the sequence is terminated. Accordingly, fragments of each size are produced. Each of the four added labeled terminator bases possess different fluorescent dye each of them absorbing at a different wavelength and then the terminal base of each fragment is determined by a fluorimeter. The products are further purified to eliminate unincorporated dye terminators with the length of each as determined with the aid of capillary electrophoresis (e.g., ABI PRISM 3100 genetic analyzer with 16 capillaries or ABI PRISM 310 genetic analyzer with 1 capillary) or gel electrophoresis (e.g., the Visible Genetics system). Afterwards, the length and terminal base of each fragment can be visualized and known then the sequence of the bases is determined. Both DNA strands are sequenced separately thus generating both forward and reverse sequences which are complementary. An electropherogram involves the sketching of the recognized separated fragments while elution from the column or while separated in the gel with each base embodied with a different color (edition could be manually or automatically). The process is characterized by a distinct separation of the fragments of various lengths with each base of a 500-bp sequence being determined. In the case of obscurities, it is fixed by visual re-editing of the electropherogram (Lis *et al*., 2015).

Assembling of the resultant DNA sequences is via aligning of the forward and reverse sequences. A consensus sequence is generated and then compared with a database of deposited sequence using available analytic software. There are however few systems that permits the comparisons of only the single forward or reverse sequences. Examples of renowned databases and their website link of 16S rRNA gene sequences are: GenBank (http://www.ncbi.nlm.nih.gov/), the Ribosomal Database Project (RDP-II) (http://rdp.cme.msu.edu/html/), the Ribosomal Database Project European Molecular Biology Laboratory (http://www.ebi.ac.uk/embl/), Smart Gene IDNS (http://www.smartgene.ch), and Ribosomal Differentiation of Medical Microorganisms (RIDOM) (http://www.ridom.com/). The proprietary MicroSeq 500 bacterial database (version 1.4.2) encompasses sequences for 1,434 species or subspecies within 235 genera (Brooks *et al*., 2015).

2.18 Assigning an Identification with 16S rRNA Gene Sequences

2.18.1 Bacterial Identification and Taxonomic Placement Using 16S rRNA Gene Sequences

Consultation with the MicroSeq databases housing over 1,400 organisms for both the 500- and 1,500-bp lengths and 500- and 1,500-bp sequences for 100 organisms by each length to generate dendrograms and determining relationships of species as the same with either length (Petterson *et al*., 2000). In Figure below, the major branches of important bacteria encountered in clinical practice are presented in dendrogram form.

Figure 2.2 below is a dendrogram resulting from 62 genetically widely different strains representing major taxonomic groups or clades of medical interest from the MicroSeq database. *Chlamydia trachomatis* is the out group. The horizontal line at the top indicates the measurement of genetic distance which is 16.105%. The genetic difference between the two clades in the figure below was determined by the two horizontal distances between the species to be evaluated not considering the vertical distance and the whole is compared to the

top horizontal line (Lis *et al*., 2015). For example, *Nocardia asteroides* and *Corynebacterium diphtheriae* differ by almost 10% whereas *Treponema medium* and *Mycoplasma hominis* differ by about 28%. The figures generated by such method is not precise particularly for genera that are not closely related. Computer programs used to generate dendrogram also produce varied data. Nevertheless, they are advantageous for estimating relative relatedness. The scale of the dendrogram and number of missing organisms is observed in *Escherichia coli* representative of the entire *Enterobacteriaceae* clade with above 50 species and *Vibrio cholera;* most closely related organism exemplified in Figure 2.2 below. Finally, inclusive of dendrograms, degree of similarity and dissimilarity as well as whole-gene alignment and concise alignment can be applied in comparison and evaluation of sequences (Ronda *et al*., 2018)**.**

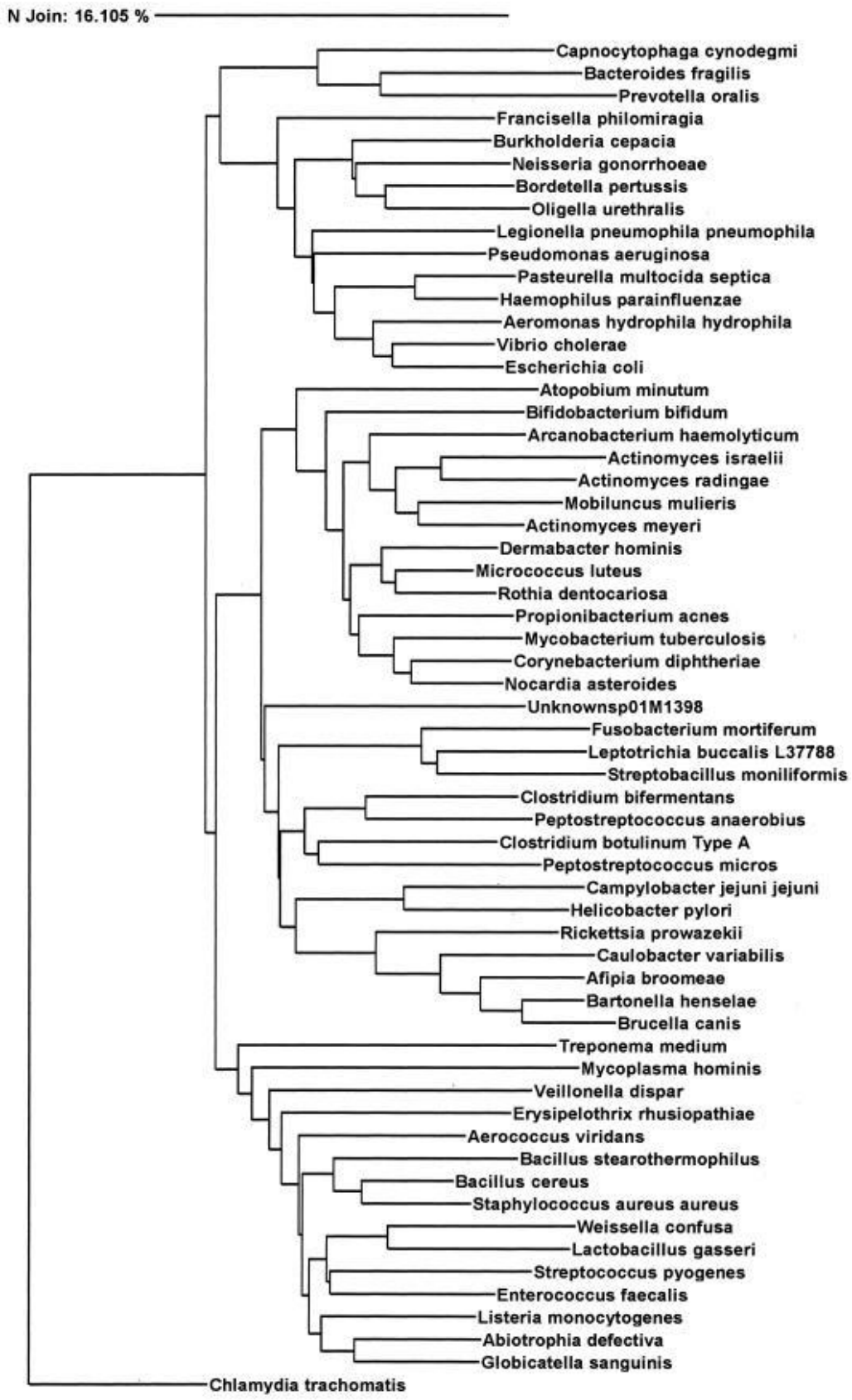

Fig 2.2: Dendrogram showing the genetic relationships of many of the major groups of clinically important organisms based on the 500-bp 16SrRNA gene sequence (Ronda *et al*., 2018)

2.18.2 Dendrograms Generation and Comparison of Sequences

There are several sequence-comparing software packages available for analysis of sequences. Normally, the exclusive software accompanied with the MicroSeq method is used. Examples of some other common software packages includes: PAUP, BLAST, and Phylip (Felsenstein 1981; Felsenstein 1989; Phylogeny Inference Package, University of Washington). BIBI, is a recently developed bioinformatics bacterial identification tool which simplifies and systematizes bacterial identifications using DNA sequence analysis with website link at http://pbil.univ-lyon1.fr/bibi/ (Hamasuna *et al*., 2018). Comparisons are usually shown as dendrograms and linear alignments; however, for short linear alignments, the identical base pairs are not included and only the differences are presented.

The universal methods employed in generating dendrograms are as follows: Maximum Likelihood method, NJ (neighbor-joining) method, UPGMA (unweighted pair group method with arithmetic averages), and WPGMA (weighted pair group method with arithmetic averages) (Fernández-Huerta *et al*., 2019). The methods are similar with the main groups conserved in closely related isolates. Though, in less closely related taxa, the dendrogram relationships are strongly influenced by the program employed.

A second method for comparison is the Concise Alignment. Here, individual differences among the compared sequences are presented. The numbers at the top of the figure represent the base position in the sequence and are best read vertically. In the instance of strain VAMC 5210 differing from strain S7745 at positions 137, 274, and 487; the length of sequence considered and the type of alignment tool greatly affect the comparison of sequences.

2.19 16S rRNA Gene Sequence Analysis Can Identify Non-Culturable Bacteria

In describing a novel microbe, the 16S rRNA gene sequence is an essential part most especially for many non-culturable bacteria it serves as the only taxonomic description (Ronda *et al*., 2018).

The simple technique for deriving a sequence for a noncultured bacterium is best with the aid of universal primers against the 16S rRNA gene region in a PCR step which increases the amount of DNA as the amplicon is sequenced (Silva *et al*., 2018). This is applied in the detection of only one organism. In the case of a mixed culture, such as a clinical specimen from a nonsterile site or an environmental sample the approach is slightly different. Non-recovery of bacterial strains in clinical research results for several reasons. Firstly, prior treatment with antibiotics could result to non-viability. Noteworthy publications on etiological agents of "culture-negative" endocarditis (Brosh-Nissimov *et al*., 2018) identified molecular analysis with broad-spectrum PCR primers complementary to the 16S rRNA gene, sequencing, and database search with diverse softwares. Here, the organisms grown prior to the molecular study (e.g., *Streptococcus salivarius* and *Capnocytophaga canimorsus*) but in these cases collection of samples was done once the patient was being given antibiotics (Chra *et al*., 2018).

Secondly, the organisms is difficult-to-grow in nature. For example; *Bartonella henselae* (the causative agent of bacillary angiomatosis) was identified and associated with the disease via molecular methods (Bradshaw *et al*., 2018). Also, several reports on Whipple's disease are strictly based on PCR amplification and later sequencing of the 16S rRNA genes (Bradshaw *et al*., 2018). This is owing to the fastidious nature of the etiologic agent, *Tropheryma whipplei* which has demanding growth requirements outside the competences of several clinical laboratories. Remarkably, Celard *et al.*, (1999) established *T. whipplei* as the causative agent in two cases of endocarditis without previous evidence of related disease

(Celard *et al*., 1999). Furthermore, Brouqui and Raoult (2001) applied broad-spectrum PCR amplification of the 16SrRNA gene and revealed the most prevalent etiologic agents associated with culture-negative endocarditis were *Bartonella quintana* and *Coxiella burnetii*, both requiring special growth conditions. A portion of the detached valve is regularly frozen at -70°C in the case of unknown etiological agent. In such cases, where pathogens are not subsequently detected by culture, sample valves are subjected to PCR assay with universal primers and sequence analysis is done on the amplified product. Mostly, the organism is identified if the organisms are in significant amount which are high enough to be viewed by electron or light microscopy (Wilck *et al*., 2001). Sequencing have been apparently useful in slow-growing bacteria like: *Mycobacterium spp* with high cost identification, it is advantageous to identify them by direct sequencing of amplified DNA (Balkus *et al*., 2018).

Thirdly, among mats of adherent, diverse, and unknown organisms; periodontal disease and biofilms can be studied (Dewhirst *et al*., 2001). The multiplicity of organisms present in the sub gingival pockets among patients with periodontitis and acute necrotizing ulcerative gingivitis can be studied by amplification of the 16S rRNA gene by means of PCR with a universal forward primer and spirochete-selective reverse primer. Following cloning of the amplified DNA into *Escherichia coli*, the sequenced DNA are compared. However, new genotypes mostly emerge, such as those representative of *Atopobium* species and new genus, *Olsenella* gen. nov. (Dewhirst *et al*., 2001). There have been similar studies on environmental samples resulting to significant novel organisms. Also, medically-important order; *Chlamydiales* has an established number of a few closely related bacteria which occur exclusively in animals and humans. However, employing 16SrRNA sequencing and evolutionary relationship, Horn and Wagner (2001) established a minimum of four novel evolutionary lineages of *Chlamydiales* isolated from environmental sludge. The study thus suggest that a few wastewater treatment plants epitomize reservoirs for a different

assemblage of environmental *Chlamydiae* and hence the environment may be a source of novel organisms with public health significances. In the case of using automatic instruments to detect growth in blood culture bottles occasionally appear positive in the absence of apparent growth. Consequently, the use of direct 16S rRNA gene sequence analysis with universal primers serves as an effective tool detect non-cultured bacteria among apparently false-positive blood cultures (Qian *et al*., 2001). The researchers reported no undetected bacterium as compared to the false-positive blood cultures.

2.20 Molecular Typing

Molecular typing method for *M.genitalium* is majorly based on PCR amplification of a specific genome locus followed by DNA sequencing. Specific methods have included (Jensen *et al*., 1996; Ma *et al*., 2010):

1. Short tandem repeat (STR) analysis of putative lipoprotein gene MG809.
2. Single nucleotide polymorphisms (SNPs) in the rRNA operon.
3. RFLP (restriction fragment length polymorphisms) of the mgpC gene.
4. Sequencing of the mgpB gene.

2.21 Prevalence of *Mycoplasma genitalium* among Women

Following successive initial isolation in 1980 (Tully *et al.,* 1981), a limited number of epidemiologic studies on *M. genitalium* infection commenced significantly due to difficulties in cultivation of the fastidious organism. Approximately 10 years later, the polymerase chain reaction (PCR) was then first employed for detection of *M. genitalium* in patients' specimens (Jensen, 1991) thus aiding more studies on the prevalence and associations with urogenital disease [Table 2.2]. With the aid of such molecular methods, sexual transmission of the organism has been discovered with high concordance rates among sexual partners (Adesola *et al.,* 2017) and reported specifically among infected couples with

concordant *M. genitalium* genotypes (Park *et al*., 2017; Slifirski *et al*., 2017; Wiesenfeld and Manhart, 2017; Seña *et al*., 2018). Furthermore, sexual transmission of *M. genitalium* infection could be incidental following increased prevalence data in partners reporting sexual intercourse and association with number of sex partners (Andersen, 2007). High-risk groups were well-defined using the following parameters:

1. Individuals attending STI clinic.
2. Persons recruited in a study with inclusion criteria including; signs of urogenital disease.
3. Individuals presenting to family planning clinics for termination of pregnancy.
4. Individuals categorized as sex workers.

Whereas, low-risk groups were defined by certain parameters such as:

1. Persons not attending a STI clinic.
2. Persons attending a fertility clinic.
3. Individuals chosen randomly from an apparently healthy population.
4. Females recruited in studies of adverse pregnancy outcomes.

The prevalence data of *M.genitalium* in the general populations of asymptomatic individuals are limited. Adersen *et al*., (2007) described a study in Denmark among 731 men and 931 women aged 21-24 years old who were recruited in an *M.genitalium* population-based survey showed 2.3% and 1.1% prevalence in women and men respectively. In an adolescent population-centered study, Leli *et al* (2018) stated 0.8% and 1.1% prevalence of *M.genitalium* among women and men respectively in urine. In a Japan-based study across some vocational schools, *M.genitalium* was detected in 2.8% and *Chlamydia trachomatis* in

8.8% using first void urine as the specimen (Ljubin-Sternak *et al*., 2017). From Sweden, Bjartling reported *M.genitalium* prevalence of 2.1% among 7598 women (Bjartling, 2009). Lastly, in Nigeria, Chukwuka *et al*., (2013) reported 6% prevalence from asymptomatic adolescent girls in Nnewi presenting high vaginal swabs.

Noteworthy, there have been reports on higher *M.genitalium* prevalence among symptomatic individuals. For example: 4.5% was reported in UK among asymptomatic subjects attending a sexual-health clinic (Mirnejad *et al*., 2011). Furthermore, with the aid of a PCR assay, Mirnejad *et al.,* (2011) described the prevalence of *M.genitalium* as 4.5% from a sexual transmitted disease (STD) clinic in Norway among 7646 women attendees presenting urine and cervical specimens. Also, Rahman (2008) in Bangladesh determined the prevalence of *M.genitalium* as 0.8% among 399 vaginal and cervical swabs collected from clinic attendees with vaginal discharge.

Table 2.2. Comprehensive summary of published studies of women where urogenital *M. genitalium* prevalence was determined using a nucleic acid amplification test (NAAT)

Reference, clinical setting and any specific criteria for enrollment in study	No. tested	MG prevalence (%)	Specimen type for MG detection	Diagnostic method
STUDIES OF HIGH-RISK POPULATIONS				
Anagrius, 2005 (STI clinic, Sweden)	445	6.3	ES and US	PCR
Arraiz, 2008 (Private Gyn clinic; genitourinary signs, Venezuela)	172[1]	7.6[1]	ES	PCR
Baczynska, 2008 (Hospital Ob/Gyn, requesting TOP, Denmark)	102	1.0	ES	RTPCR
Bertille de Barbeyrac, 1993 (STI clinic, France)	55	0	US or ES	Cult/PCR+SB
Bjartling, 2010 (Hospital Ob/Gyn, requesting TOP, Sweden)	2079	2.5	Urine and/or VS, ES	PCR
Blanchard, 1993 (STI clinic or hospital Ob/Gyn controls, cervicitis, USA)	282	1.1	US, ES or AF	PCR
Bradshaw, 2008 (STI clinic, urogenital symptoms, Australia)	313	9.6	FVU or ES	RTPCR
Casin, 2002 (STI clinic, vaginal discharge, France)	170	38.2[2]	ES, VS and/or US	PCR

Cohen, 2002 (STI clinic, pelvic pain >14d, Kenya)	115	8.7	ES or EMB	PCR
Cohen, 2005 (Hospital Ob/Gyn, PID & confirmed salpingitis, Kenya)	123	7.3	ES, EMB, TU	PCR
Cohen, 2007 (Public health clinic, CSW, Kenya)	255	15.7	ES and EMB	PCR
Edberg, 2008 (STI clinic, partner with *M. genitalium*, Sweden)	298	7.7	FVU, ES and/or US	RTPCR
Falk, 2005 (STI clinic or cancer screen controls, Sweden)	520	5.0	FVU and ES	PCR
Gaydos, 2009 (STI clinic, USA)	322	19.3	VS	RTPCR and TMA
Grzesko, 2009 (Hospital Ob/Gyn patients, confirmed infertility, Poland)	74	14.9	ES	PCR
Haggerty, 2006 (ER, Ob/Gyn, STI clinic, or primary health, NGNC EM or cervicitis, USA)	50[3]	14.0[3]	ES and EMB	PCR+SB
Haggerty, 2008 (ER, Ob/Gyn, STI, primary health clinic, endometritis, USA)	586	15.0	ES and EMB	PCR+SB
Hogdahl, 2007 (STI clinic, Sweden)	417	6.5	FVU	RTPCR
Huppert, 2008 (THC or ER, genital signs or high-	331	22.3	VS	TMA

risk behavior, USA)				
Jensen, 1991 (Primary health or STI clinic, Denmark)	74	6.8	ES	PCR+SB
Jurstrand, 2007 (Ob/Gyn, PID or ectopic pregnancy, Sweden)	521[4]	16.1[4]	N/A	Serological
Korte, 2006 (PHC, STI at enrollment, USA)	674[3]	42.0[3]	VS and ES	Cult/PCR+SB
Labbe, 2002 (Hospital Ob/Gyn, pre-term or controls at term, Guinea-Bassau)	1014	6.2	ES	PCR
Lawton, 2008 (Hospital Ob/Gyn, requesting TOP, New Zealand)	300	8.6	VS	RTPCR
Manhart, 2003 (STI clinic, USA)	719	7.0	CS	PCR+SB
Manhart, 2008 (STI clinic, HIV+, 72% CSW, Kenya)	303	17.2	ES	PCR+DIG
Mellenius, 2005 (STI clinic, Sweden)	340	3.8	ES	RTPCR
Moi, 2009 (STI clinic, genital signs or high-risk behavior, Norway)	7646	4.0	FVU and/or ES	RTPCR
Musatovova, 2009 (PHC, non-viral STI at enrollment, USA)	268[3]	16.8[3]	Urine, VS and ES	RTPCR
Palmer, 1991 (STI clinic, UK)	57	19.3	ES, VS and/or US	PCR
Pepin, 2005 (STI clinic, CSW, Benin/Ghana)	826	26.3	ES	PCR

Ross, 2009 (STI clinic, asymptomatic, UK)	138	6.5	ES or VS	RTPCR
Short, 2010 (Pregnant ER patients <22wks gestation, USA)	216	5.6	Urine	RTPCR
Simms, 2003 (STI clinic, Hospital Ob/Gyn, primary health clinic for controls, UK)	82	7.3	ES	RTPCR
Thurman, 2010 (PHC, STI at enrollment, USA)	590[5]	NC[5]	Urine and ES	RTPCR
Tsunoe, 2000 (CSW attending STI clinic or pregnant controls, Japan)	174[6]	12.6[6]	ES	PCR
Total no. of studies of high-risk populations: 37	**18748**	**7.3%**		
STUDIES OF LOW-RISK POPULATIONS				
Andersen, 2007 (In home testing, randomized population screen, Denmark)	921	2.3	VP	RTPCR
Clausen, 2001 (IVF clinic, confirmed infertility, Denmark)	308[4]	13.0[4]	N/A	Serological
Edwards, 2006 (Hospital Ob/Gyn, signs/symptoms of preterm labor, USA)	134	20.2	VL	PCR
Ghebremichael, 2009 (PHC, randomized population screen, Tanzania)	1440	3.3	Urine	RTPCR

Manhart, 2007 (In home testing, randomized population screen, USA)	1714	0.8	FVU	PCR
Oakeshott, 2004 (Primary health or family planning clinic, pregnant, UK)	915	0.7	FVU	PCR
Olsen, 2009 (PHC, healthy married women, Vietnam)	990	0.8	ES	RTPCR
Svenstrup, 2008 (Fertility clinic, confirmed infertility, Denmark)	210	0	ES	RTPCR
Tosh, 2007 (Primary health care clinic, USA)	383	0.8	VS	PCR+DIG
Total no. of studies of low-risk populations: 11	**8434**	**2.0%**		
Total no. of studies of high- and low-risk populations: 48	**27182**	**5.6%**		

STI, sexually transmitted infection; PHC, public health clinic; VS, vaginal swab; ES, endocervical swab; FVU, first void urine; EMB, endometrial biopsy; TU, tubal fluid or biopsy; US, urethral swab; AF, amniotic fluid; CS, cervical secretions; VP, vaginal pipette; RT-PCR, Real-time PCR; PCR+SB, PCR with southern blot confirmation; TMA, transcription mediated amplification; PCR+DIG, PCR with specific digoxigenin probe for confirmation; IVF, in vitro fertilization; NGNC EM, non-gonococcal, non-chlamydial endometritis; TOP, termination of pregnancy; CSW, commercial sex workers; NAAT, nucleic acid amplification test; NC, point prevalence at enrollment not calculable from presented data; VL, vaginal lavage

[1]Excluded patients with CT or human papilloma virus

[2]Prevalence calculated from PCR positive results from any of up to 3 sites sampled at enrollment

[3]Not included in overall prevalence calculation; patient population also represented in another study

[4]Based on serological assay, not included in overall NAAT prevalence calculation

[5]Not included in overall prevalence calculation; point prevalence at enrollment not calculable from presented data

[6]Among only STI clinic attendees

2.22 Treatment/ Current Options

Treatment of symptomatic NGU is concentrated on purge of *Chlamydia trachomatis* (reason for female reproductive morbidity). It is usually established at early appearance in its patient prior to test results aimed to detect specific bacterial etiologic agent. But, in instances of sexually contacted urethritis and cervicitis, laboratory tests are mostly targeted towards *Neisseria gonorrhoeae* and *C. trachomatis*.

There have even evidence of a limited number of countries involved in mundane screening exercise for *M. genitalium.* However, such exercise targets individuals with first-line therapy failure, although samples are also collected at first visit and detection is strictly dependent on the application of in-house nucleic acid amplification tests. Significantly, authenticated and commercially available tests for mundane analytical testing are not available (Manhart, 2014). Although, recent data associating *M. genitalium* with upper genital tract infections and infertility showed uncertainties on the involvement of *M. genitalium* as a pathogen of the

female reproductive tract lingers. And, the lack of an official diagnostic test hindered decisions on testing and treatment practices (Horner *et al*., 2014).

Like other mycoplasmas, *M. genitalium* lacks a cell wall consequently not susceptible to antibiotics targeting cell wall peptidoglycan. Though tetracycline (e.g.; doxycycline) have demonstrated success rate in the treatment of NGU over the past decades, the effectiveness of this antimicrobial class have shown poor and reduced susceptibility (Johannisson *et al.,* 2000; Hamasuna *et al*., 2009). Among the macrolide class of antimicrobial; Azithromycin has been adopted as the drug of choice for NGU and related clinical syndromes for several reasons:

1. Long half-life.
2. Exceptional tissue penetration.
3. Single-dose administration is supported. In several instances, research studies have centered the study of the effectiveness of a single 1 g dose of azithromycin (Gambini *et al*., 2000; Björnelius *et al*., 2008; Jernberg *et al*., 2008; Bradshaw *et al*., 2008; Mena *et al*., 2009; Schwebke *et al.,* 2011; Terada *et al*., 2012; Manhart *et al*., 2013; Terada *et al.,* 2015). Although, studies have also reported the effectiveness of higher doses and extended courses of azithromycin. For example; the comprehensive 1.5 g course, administered as 500 mg on day 1 and following 250 mg daily on days 2–5, or rather less with two 1 g doses given 5–7 days separately (Falk *et al*., 2003; Jernberg *et al*., 2008; Bradshaw *et al*., 2008).

2.23 Clinical Effectiveness of Commonly Used Antimicrobials

Doxycycline

In vitro susceptibility testing suggests that most *M. genitalium* strains demonstrated susceptibility to doxycycline with MIC-50 of 0.25 mg/l and MIC-90 of 1 mg/l among 39 strains studied (Jensen *et al*., 2014). Contrary to clinical experience that primarily revealed

poor efficacy of doxycycline in suppression of *M. genitalium* (Falk *et al*., 2003). A single exemption is an early study where 33 (94 %) of 35 men were *M. genitalium* negative after doxycycline treatment (Gambini *et al*., 2000). Noteworthy, eradication was estimated one week after treatment thus indicative of temporary suppression of the *M. genitalium* load which is consistent with study by Mena *et al.,* (2009) where 47 % of men with early clinical cure after doxycycline demonstrated a subsequent relapse. In precise clinical trials with suitable control, the microbiological cure rate ranged between 22 % and 45 % (Björnelius *et al*., 2008; Mena *et al*., 2009; Schwebke *et al*., 2011; Manhart *et al*., 2013). The inconsistency in result between in vitro and in vivo activity is uncertain however two studies (Björnelius *et al*., 2008 and Anagrius *et al*., 2013) have established the clearance rates in women were somewhat higher compared to men (37 % vs 17 %, and 48 % vs 38 %, respectively).There are postulations of contributing factors such as; lower compliance in men in relation to the 9-day doxycycline regimen applied in these studies. As a result, doxycycline is not suitable to be recommended as first line treatment of *M. genitalium* infection.

Azithromycin

From available documented studies, a considerably high number of *M. genitalium* infected patients have been treated with azithromycin. In susceptibility studies by Renaudin *et al.,* (1992) azithromycin demonstrated high degree of potency. Among STI patients, treatment with 1 g single dose of azithromycin established total eradication of *C. trachomatis* (Martin *et al*., 1992) and this single-dose treatment was easily adopted by STI patients and currently as first line treatment for NGU and *M. genitalium* in several countries. However, in patients infected with *M. pneumoniae* an extended treatment of 500 mg on the first day followed by 250 mg once daily on days two to five (designated as extended azithromycin) demonstrated high effectiveness as erythromycin for ten days (Edouard *et al*., 2017). It was postulated that a slow-growing bacterium such as *M. genitalium* require an extended treatment extended

duration. Subsequent to the reported effect of extended azithromycin on the closely related *M. pneumoniae* (Plantamura *et al*., 2017), it was adopted that the 5-day extended azithromycin regimen as the preferred treatment for *M. genitalium* infections in Denmark, Norway and Sweden. However, a number of other extended regimens were measured including administering 1 g dose on first day so as to act in accordance with treatment guidelines for *C. trachomatis,* and later accompanying with 250 mg on days two to five. This has increased the recognition of extended treatment among doctors treating STIs with no reported adverse influence on the treatment of *M. genitalium*. Nonetheless, eradication of *C. trachomatis* infections with the 5-day extended azithromycin regimen has been standardized (Unemo *et al*., 2015).

For decades, debate has occurred over the optimal dosage of azithromycin (Horner *et al.,* 2014). Fundamentally, no randomized controlled trial has compared azithromycin 1 g single dose with extended azithromycin, but a limited number of observational research with one treatment trial study (Björnelius *et al*., 2008; Jernberg *et al*., 2008; Anagrius *et al*., 2013; Gundevia *et al*., 2015) have incorporated patients treated with both regimens. In all studies, 469 *M. genitalium* infected patients were administered with azithromycin as a 1 g single dose, and 244 were placed on extended azithromycin treatment with microbiological cure rates of 81 and 88 %, respectively ($p = 0.026$). Conversely, a significant number of the patients on the extended azithromycin treatment regime had it as a second line therapy typically after doxycycline.

Quinolones

Quinolones have been employed in the treatment of *M.genitalium*. In vitro susceptibility studies revealed that second generation fluoroquinolones (ciprofloxacin and ofloxacin) is ineffective in eradicating *M. genitalium* and further confirmation in observational studies revealed (Jenniskens *et al.,* 2017 and Lefebvre *et al.,* 2017) an overall cure rate of 59%.

Evidence demonstrated in in vitro data (Hamasuna *et al*., 2009) affirms the third generation quinolone levofloxacin. Conversely, the result from in vito study did not correlate with observational studies which revealed a cure rate of 54% in 82 treated patients (Maeda *et al.*, 2001; Takahashi *et al*., 2011; Terada *et al*., 2012).

Moxifloxacin, a fourth generation quinolone and a second line antimicrobials with first report on efficacy in 2006 (Bradshaw *et al*., 2006). Moxifloxacin is mode of antimicrobial activity is bactericidal with documented cure rate approximately 100 % (Casillas-Vega *et al*., 2016; Pereyre *et al*., 2017). Still, studies have revealed the propensity for hepatotoxicity. Consequently, moxifloxacin was thus limited to second line therapy in countries like Europe (Shimada *et al.*, 2010). As clinical studies advanced, these severe adverse effects of moxifloxacin was revealed to be associated strongly with patients administered with moxifloxacin as monotherapy compared to patients administered with amoxicillin or with doxycycline (Hannan and Woodnutt, 2000; Shimada *et al.*, 2010). Besides the advert of side effects, a steady decreasing cure rate for moxifloxacin has been reported from clinical studies in the Asia-Pacific region. Studies on treatment failure have progressed steadily up to 30 % and with a substantial percentage of the test subjects' strains revealing concurrent macrolide resistance facilitating mutation (Yasuda *et al*., 2005; Couldwell *et al.*, 2013; Bissessor *et al.*, 2015; Gundevia *et al*., 2015). Thus, resistance to moxifloxacin and other fourth generation quinolones initiated by mutations in quinolone resistance determining regions (QRDR) of the parC gene localized in the amino acid positions S83 and D87 (as in *M. genitalium* numbering) (Shimada *et al.*, 2010).

Reports on low mutation rate and treatment failure have been reported from different parts of the world though reports though there are variations in mutation rate. Studies from Europe, reported a low mutation rate in the relevant positions with only 1 (5 %) out of 22 samples from London, UK (Pond *et al*., 2014) with a much lower rate reported in Denmark (Jensen *et*

al., 2014). Reports from the Asia- Pacific region, there are reports on fluoroquinolone mutations with associated treatment failure. In a longitudinal study from an Australian STI clinic, 21(15%) of 143 specimens collected between 2008 and 2011 expressed mutations that were strongly associated with fluoroquinolone resistance (Tagg *et al*., 2013). Likewise, a study from Japan (Kikuchi *et al*., 2014) established parC mutations in 17 (33 %) of 51 specimens collected between 2011 and 2013 demonstrating a vivid progression from 20 % in 2011 to 47 % in 2013. It is noteworthy that results from the in vitro and in vivo studies are consistent with the findings of mutations in the QRDR. The observed progressive trend of parC mutations following reports from Japan was revealed to be triggered by an S83N mutation (Kikuchi *et al*., 2014). However, there are no reports of this type of mutation altering the moxifloxacin MIC of susceptible wild-type G37 *M.genitalium* type-strain (Jensen *et al*., 2014). Accordingly, this justifies the significant 100 % cure-rate of sitafloxacin among 9 *M. genitalium* infected patients harbouring mutations in the QRDR of parC with 8 out of the 9 revealing unrelated S83N mutation.

Other fourth generation quinolones have been understudied in countries like Japan. For example; Gatifloxacin demonstrated cure rate of 90 % among 48 patients (Hamasuna *et al*., 2011).While, Sitafloxacin have undergone five different research studies (Ito *et al*., 2012; Takahashi *et al.,* 2013; Ito *et al*., 2014; Kikuchi *et al*., 2014) demonstrating remarkable cure rates which ranges from 100 % to 85 % but an overall cure rate of 95 % among 105 patients. In in vitro study, sitafloxacin demonstrated outstanding potency with MICs for moxifloxacin-susceptible strains (Hamasuna *et al*., 2009).

Other licensed but less commonly used antibiotics

One major licensed antimicrobial with limited usage is the Pristinamycin; an oral streptogramin antibiotic with bactericidal activity against Gram-positive microbes including methicillin-resistant *Staphylococcus aureus* (MRSA). It demonstrates high activity against

macrolide susceptible *M. genitalium* strains (Renaudin *et al*., 1992). Pristinamycin has been employed for years predominantly in France for the treatment of MRSA although it is registered only within France and some North African countries. In *M. pneumoniae*, there is low tendency for acquisition of antimicrobial resistance than azithromycin in vitro with certain mutants retaining an azithromycin susceptible phenotype (Pereyre *et al*., 2004). Currently, Pristinamycin has been employed extensively as third- line treatment for patients infected with multidrug resistant *M. genitalium* strains. For most of the patients, it was a last resort and the recommended administrative dose of 1 g four times orally a day for 10 days was used.

Antimicrobials under development

Solithromycin is a newly established fluoroketolide (CEM-101) an advance macrolides. It has an effective action against azithromycin susceptible *M. genitalium* strains and considerably against azithromycin resistant strains (Jensen *et al*., 2014). Susceptibility to solithromycin is determined by the mode of macrolide resistance facilitating mutation as in the case of eight strains harbouring mutations in position A2059 with susceptible MICs <2 mg/l, whereas two of the five strains with the A2058G mutation with susceptible MICs (Jensen *et al*., 2014). In a clinical study in Denmark where 60 % of azithromycin resistant strains harboured the A2058G mutation (Salado-Rasmussen *et al.,* 2014), a predicted 65 % cure rate with the use of solithromycin for azithromycin resistant strains was reported (Jensen *et al*., 2014). Recently, single dose solithromycin treatment trial for patients with gonorrhoea is ongoing. In one of the studies, a limited number of concomitant *M. genitalium* infections were eradicated (Hook *et al*., 2015) still, data on the effectiveness most especially clinical efficacy of solithromycin for *M.genitalium* is regulated. Speculations on drug trials aimed at patients with NGU requires extended treatment duration in order to asylum *M. genitalium* infections (Taylor-Robinson, 2014).

Another antimicrobial under development is the Lefamulin; approved in august 2019 (BC-3781) belonging to the pleuromutilin class of antimicrobials (Hamasuna *et al*., 2019). It has been exhaustively applied for years in the veterinary industry to treat infections among pigs and with limited use in poultry on the other hand, this class has no history of human use. Lefamulin has demonstrated high potency in vitro with MIC ≤ 0.06 mg/l against multidrug resistant *M. genitalium* strains (Paukner *et al*., 2014). In veterinary mycoplasmology, mycoplasmas with mutations in position A2058 have been linked with increased MIC levels, but triggered by the presence of other mutations in the 23S rRNA gene, suggestive of a series of process resulting to the acquisition of significant resistance (Li *et al*., 2010). In drug research and trial, Lefamulin has demonstrated an excellent outcome in the phase II trials for skin and soft tissue infections (Prince *et al.,* 2013) consequently, has successfully proceeded into the drug trial stage for community-acquired pneumonia.There have been no report on trial aimed at treatment of STIs or specifically *M. genitalium.*

There have been research report on a new spiropyrimidinetrione AZD0914 (a DNA gyrase inhibitor) tested against azithromycin and quinolone susceptible strains of *M. genitalium* and MIC <1 mg/l for 11 strains (Waites *et al*., 2015). Report on clinical trial has not been documented for this class of compounds. LBM415 fits into a novel antimicrobial class (the peptide-deformylase inhibitors which inhibit bacterial protein synthesis).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

The study was a cross-sectional study where endocervical swab samples were collected from females of reproductive age (15 - 39 years) for the study of *Mycoplasma genitalium* using 16SrRNA gene sequencing methods. The study included both a population and hospital - based setting. The population and hospital- based settings comprised of undergraduate female students and female patients with gynecological cases respectively. Simple random sampling method was used for subject recruitment.

3.2 Study Area

This study was conducted amongst female students at Edo State School of Health Technology and Obstetrics and Gynecology Department, Central Hospital, Benin City, Edo State. Edo State School of Health Technology is a government-owned Tertiary Institution with an estimated student population of ten thousand. Since its establishment in 1964, the Institution has attracted students from different parts of the country due to its high standard of academic excellence. While, Central hospital is a secondary care government owned establishment which serves as a referral center for neighboring states such as Delta State. Both institutions are located at the heart of the city of Benin. The latitude of Benin City, Nigeria is 6.339185, and the longitude is 5.617447. Benin City, Nigeria is located with the coordinates of 6° 20' 21.0660" N and 5° 37' 2.8092" E.

3.3 Sample Size Determination

The sample size for this study was 130 female subjects. This was derived from the formula below. It was estimated that this sample size achieved 0.05 degree accuracy at 95% confidence interval with a prevalence of 8.7% (Hay *et al*, 2015)

$$N = \frac{Z^2Pq}{d^2}$$

N- Minimum sample size required (122 rounded up to 100)

P- Expected prevalence (6%)

q- 1-p

Z- Standard normal deviation at 1.96 at 95% confidence interval

d- Degree accuracy desired at 0.05

Thus $N= (1.96)^2 X 0.087 X (1- 0.087)/ (0.05)^2 = 122$

3.4 Study Population

One hundred female students of the Edo State School of Health Technology, Benin City aged between 15 and 39 years were recruited for this study; in addition, 30 female patients attending an Obstetrics and Gynecology clinic of Central Hospital, Benin City with cases ranging from infertility, symptomatic cervicitis and pelvic inflammatory disease were also recruited for this study. Informed consent were obtained from the females before been recruited into the study. The sampling technique employed was simple random sampling.

3.5 Ethical Consideration and Informed Consent

Informed consent of each participant was obtained prior to sample collection and testing. Ethical approval was also obtained from the Research Ethics Committee Edo State hospitals' management board as well as from the Research Ethics Institutional Review Board of the Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Nnewi Campus.

3.6 Inclusion and Exclusion Criteria

The inclusion criteria for the subjects were as follows:

Age range of 15 to 39 years.

Sexually active females.

Females not on antibiotic therapy within the last three months.

Females who just completed their menstrual period at the time of the study.

Female students admitted by the Institution.

Female patients visiting the Obstetrics and Gynecology clinic of Central Hospital, Benin City with cases ranging from infertility, symptomatic cervicitis and pelvic inflammatory disease.

The exclusion criteria for all subjects included:

Females on antibiotic medication.

Females who self-reported underlying infection such as Human immunodeficiency virus (HIV) infection.

3.7 Questionnaire

A well-structured questionnaire (Appendix 3) was designed for this study to aid as an instrument of data collection. This was used to collect demographic data such as age, marital status and variables such as number of sexual partner(s), age at first intercourse, previous sexually transmitted disease (STD) treatment etc.

3.8 Education of Medical Personnel on the study

In the hospital where ethical approval was obtained, PowerPoint presentation was done in the Obstetrics and Gynecology Department of Central Hospital, Benin City to further enlighten the staff members about the study prior to collection of samples from the studied population. The Head of Department, Consultants, Resident Doctors, House officers and Nurses were in attendance.

3.9 Collection of Sample

Prior to collection of samples from the female subjects, series of health talks were conducted for the participants after the school management had given their approval for the study. Endocervical swabs (ECS) were collected by the clinician who first inserted a non-lubricated sterile disposable plastic speculum into the vagina to expose the cervix, after which ECS was collected by rotating the sterile Dacron swab for 10-30 seconds against the wall of the

endocervical canal without contact with vaginal mucosa. Two swab samples were collected per subject making a total of 260 ECS samples; one for molecular analysis and the second swab for wet mount and other staining procedures. Vaginal swab sample was also collected per subject for pH determination.

One hundred and thirty ECS collected for molecular analysis was inserted into 0.4ml of sterile phosphate buffered saline (Oxoid, UK) and transported to the laboratory at 4^0C in an ice pack. The other 130 ECS collected was assayed immediately.

Subjects were divided into two groups based on presentation of symptoms and absence of symptoms.

3.10 Assay Procedures:

3.10.1 Wet Mount

The same swab was vigorously mixed in saline and then a drop of the saline mix was placed on a glass slide prior to placing a cover slip. The glass slide was viewed under a microscope first with 10X objective noting cellular distribution and obvious cellular and fungal elements. Then finally 40 X objective was used to identify the presence of white and red blood cells, quantity and type of bacteria present, clue cells, motile Trichomonad and yeasts cells.

3.10.2 Whiff Test

Whiff test was performed by adding a drop of 10% Potassium hydroxide to a drop of wet mount fluid. A "fishy" or amine odour which is characteristic of *Trichomonas vaginalis* was considered positive.

3.10.3 Gram's Stain

A smear was made on a clean slide and stained using the Gram staining technique for classification of Bacteria and other cells. The stained smear was examined with oil immersion objective (X100) for the presence of Bacteria, squamous epithelial cells, clue cells and other cells. Bacteria cells that stained purple and red were considered gram positive and gram negative respectively.

3.10.4 Giemsa Stain

A smear was made on a clean slide and stained using the Giemsa staining technique for detailed identification of Trichomonad and Chlamydia inclusion bodies. The stained smear was examined with oil immersion objective (X100) for the presence of Trichomonad, Chlamydia inclusion bodies, Polymorphonuclear leucocytes (PMNLs) and others cells. Polymorphonuclear leucocytes that stained purple in their nuclei and pink in their transparent cytoplasm was considered as neutrophils. Infected epithelial cells with lighter purple inclusion bodies was considered positive for Chlamydia inclusion bodies.

3.10.5 pH Test

The pH of the vaginal secretion was done by placing vaginal swab sample collected from the lateral wall of the vagina on pH paper (Labstar) and the pH value was recorded.

3.10.6 DNA Extraction

The DNA was extracted from the samples in a 1.5ml micro centrifuge tube using QiaQuick DNA mini plus kit as recommended by the manufacturer (Zymo Research, Germany). This procedure included lysis, binding, washing (DNA purification) and elution into DNA elution buffer (provided in the kit). The inoculated PBS was centrifuged and 200µl of the sample was dispensed in a microcentrifuge tube prior to adding 200µl of Biofluid and Cell Buffer and 20µl Proteinase K. Following incubation at 55^0C for 10 minutes, 1 volume of Genomic binding buffer was added to the sample. Spin column technology was used via the use of Zymo-Spin IIC-XL Column which was inserted in a collection tube to wash the sample proceeding series of centrifugation at ≥ 12,000 x g speed for 1 minute with the use of Pre-wash and Wash Buffer. DNA extract was eluted into 50µl of DNA Elution Buffer. DNA extracts were stored at -20^OC until use. The quality and quantity of purified DNA were measured by spectrophotometry (Nano Drop 2000 Spectrophotometer, Thermo scientific, Wilmington, USA) at 260nm wavelength.

3.10.7 Polymerase Chain Reaction (PCR) Primers

Oligonucleotide primers for the PCR were adapted from published nucleotide sequences (Mclver *et al*., 2009), and shown in the table below. Primer sequences were confirmed to be unique for the template sequence by checking for similarity to other known sequences with BLAST software (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 3.1. Oligonucleotide primers for the Multiplex Polymerase chain reaction

Organism	Primer Name/ Sequence (5^1-3^1)	Size of Primer
M. genitalium	**MG SO-2-Bi**	215bp
	CAC CAT CTG TCA CTC TGT TAA CCT C	
	Mgen-P3-Am	
	TGG GAG CGA TCC CTT CGG T	
M. hominis	**MG SO-2-Bi-F**	402bp
	CAC CAT CTG TCA CTC TGT TAA CCT C	
	Mhom-P10-Am	
	GAC ACT AGC AAA CTA GAG TTA G	
C. trachomatis	**CT-O-F**	934bp
	TTG CAA GCT CTG CCT GTG GGG AAT	
	CT-O--R	
	TCA CAT GCG CAG CTC CAG CAA TAG	
U. urealyticum	**UM-S-170-F**	476bp
	GTA TTT GCA ATC TTT ATA TGT TTT CG	
	UM-A-263-R	
	TTT GTT GTT GCG TTT TCT G	
U. parvum	**UM-S-57-F**	326bp
	TAA ATC TTA GTG TTC ATA TTT TTT AC	
	UM-A-222-R	
	GTA AGT GCA GCA TTA AAT TCA ATG	
T. vaginalis	**TricV-1F**	207bp
	CTC AGT TCG CAA AGG CAG TCC TTG A	
	TricV-1R	
	GCT TGG AGA GGA CAT GAA CTT CGG A	

Source: Mclver *et al*., 2009

3.10.8 Primer Mix

Standard quality, desalted and purified primers were synthesized at Inqaba biotechnology, South Africa. All primers stock were normalized to a concentration of 100µM using sterile nuclease-free water (Amresco, UK). A working solution of 25µM primer mix was then prepared in sterile nuclease-free water for each primer (100µM concentration of normalized stock).

3.10.9 PCR Amplification

The extracted genomic DNA was subjected to Multiplex Polymerase Chain Reaction (mPCR). This was done at Lahor Research Laboratory, Benin City. PCR amplification was done using commercial mix '5X multiplex PCR mix' (New England BioLabs) diluted to 1X before use in 25µl containing 1µl of 1µM primer each (11µl), 5µl of master mix, 5µl of genomic DNA and 4µl sterile nuclease-free water to make up 25µl. The 25µl PCR mix of each sample was dispensed in 0.2ml PCR tube and was transferred to a thermal cycler (Gene Amp PCR Systems 9700 Applied Bio system). DNA amplification was carried out using an initial denaturation step at 95^0C for 1 minute followed by a 3-step procedure: denaturation at 95^0C for 20 seconds, annealing at 51^0C for 60 seconds, extension at 68^0C for 60 seconds. This was done for 35 cycles and followed by a final extension at 68^0C for 5 minutes and held at 4^OC.

3.10.10 Agarose Gel Preparation for Electrophoresis

Agarose gel was prepared at a concentration of 1% as follows:

One gram of agarose powder (Ambion, USA) was dissolved by heating in a microwave for 3 minutes after adding 100ml 1XTBE buffer (AppliChem). The agarose solution was cooled to 55^0C in a water bath. Afterwards, 2µl of 100mg/ml ethidium bromide was added, mixed and poured on to the casting tray that had been trapped up appropriately and equipped with suitable comb to form well in plate. The gel was allowed to set at room temperature for 30

minutes. After solidification, the comb was gently removed and the spacer from the open sides was removed.

3.10.11 Detection of PCR Product

For analyses of the PCR amplification, 10µl of the amplified samples was electrophoresed for 30 minutes on a 1% agarose gel made in Tris borate EDTA buffer (pH=8.0-8.5) and ethidium bromide (2µl of 100mg/ml) was added in gel preparation and visualized by Trans-illuminator. Size of band was determined using DNA ladder (New England Bio Labs) of size 100bp. Specimens showing a band of base pair (bp) of 215bp, 402bp, 937bp, 476bp, 326bp and 207bp were considered positive for *M. genitalium*, *M. hominis*, *C. trachomatis*, *U. parvum*, *U. urealyticum* and *T. vaginalis* respectively.

3.10.12 Sequencing and Phylogeny:

This was done at Inqaba Biotechnology, Pretoria, South Africa.

PCR amplification of 16SrRNA gene

The DNA extracted was used as the template for PCR to amplify a segment of the 16S rRNA gene sequence. Genus (*Mycoplasma*) and Specie Specific (*M. genitalium*) primer complementary to conserved regions was used so that the region can be amplified. Primer set: *Mycoplasma* genus specific primers: Forward primer GGG AGC AAA CAG GAT TAG ATA CCC T and Reverse Primer TGC ACC ATC TGT CAC TCT GTT AAC CTC (Metwally *et al*., 2014) and *M. genitalium* specific primer MG 16S- 45F TAC ATG CAA GTC GAT CGG AAG TAG C and MG 16S-447R: AAA CTC CAG CCA TTG CCT GCT AG (Yoshida *et al*., 2002). Primer sequences were confirmed to be unique for the template sequence by checking for similarity to other known sequences with BLAST software (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

PCR amplification was done using OneTaq Quick load 2X master mix with standard buffer (New England BioLabs) in 50µl containing 4µl of 25µM primer, 25µl of master mix, 10µl

of genomic DNA and 11µl sterile nuclease-free water (Amresco, UK) to make up 50µl. The 50µl PCR mix of each sample was dispensed in 0.2ml PCR tube and was transferred to a thermal cycler (2720 Applied Biosystems). DNA amplification was carried out using an initial activation step at 95^0C for 15 minutes followed by a 3-step procedure: denaturation at 94^0C for 45 seconds, annealing at 59^0C for 45 seconds, extension at 72^0C for 60 seconds. This was done for 35 cycles and followed by a final extension at 72^0C for 7 minutes.

For analyses of the PCR amplicon, 10µl of the amplified samples was electrophoresed for 30 minutes on a 1% agarose gel made in Tris borate EDTA buffer (pH=8.0-8.5) and ethidium bromide (2µg of 100mg/ml) was added in gel preparation and visualized by Trans-illuminator. Specimens showing a band of base pair (bp) of 280bp and 475bp were recorded positive for *Mycoplasma* genus and *M. genitalium* respectively.

Purification of PCR product

The PCR products were purified to remove excess primers and nucleotides (impurities) using Sephadex® G-25 powder (Merck, Darmstadt, Germany). Two grams of sephadex powder was dissolved in 100 ml water and a filter plate was used to obtain gel after centrifuging at 2700 rpm for 5 minutes using a micro centrifuge. Amplicons were then poured into wells and filtrates were collected into plates after second series of centrifugation. Impurities were trapped in Sephadex.

Sequencing of 16SrRNA gene

Sequencing of the 16SrRNA gene was done using the same primer set used for amplification performed with the ABI Sequencer and read on an ABI 3130XL genetic analyzer. Purified PCR amplicon was certified fit for sequencing after quality control test was done. Sequences were then submitted to NCBI GenBank using BankIt

(http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) after consensus sequence was generated.

Generation of Consensus Sequence

The bases were edited with BIOEDIT software. Reverse sequence in each case was made to complement the forward sequence by reverse complement then a pair wise alignment was carried out on forward and reverse sequence and the consensus sequence was obtained from the aligned sequence. The consensus sequence was pasted on BLAST (Basic Local Alignment Search Tool) at National Center for Biotechnology Information (NCBI) website to obtain closely related strains. Consensus sequences were saved in Fasta format prior to further analysis.

Analysis of sequence data

The sequence was aligned and compared with reference strains of *Mycoplasma spp* submitted already in GenBank using CLUSTALW2.1 software. This typing method was developed by Hjorth (Hjorth *et al*., 2006).

Phylogenetic Analysis

A phylogenetic analysis was done to determine the evolutionary relationship of strains using Maximum Likelihood method based on the Tamar-kai model with Bootstrap on Molecular evolutionary genetics analysis (MEGA) 7.0 version software (Kumar *et al*., 2016). Phylogenetic analysis was done on sequences obtained from the study with reference sequences and other previously submitted sequence on GenBank from other parts of the world.

3.11 Genetic Relationship between *M. genitalium* and Other Clinically Important Bacterial Sexually Transmitted Pathogens Using Phylogenetic Methods

The genetic relationship of the isolated *M.genitalium* with other clinically important bacterial Sexually Transmitted pathogens (retrieved from nucleotide database; GenBank) was determined using phylogenetic analysis.

3.12 Phylogenetic Study of *Mycoplasma species* isolated from Female Students and Patients

Sequenced *Mycoplasma spp* isolated from both the female students and patients were subjected to phylogenetic analysis with other previously submitted mycoplasmal sequences. The genetic relatedness of all the species was determined.

3.13 Statistical Analysis

The software used was Statistical Package for Social Sciences (SPSS) program - version 20 for data analysis. Number/percentage was used to describe the data obtained. Chi-square test was used for calculating association between two categorical variables. When the expected numbers in the association table were small in the case of 2×2 tables, Fisher's exact test was used. $P<0.05$ was considered statistically significant. A multivariate analysis was done using Logistic Regression Model to identify those variables associated with Mycoplasma and other sexually transmitted infections.

CHAPTER FOUR

RESULT

4.1 Demographic Characteristics

The demographic variables of the 130 subjects in the study population were stratified by age and marital status. The mean age of the 130 subjects was 24.4 years ± 2.66 (15-39 years). The majority of the 130 subjects (87.7%) were unmarried. [Table 4.1].

4.2 Nucleic Acid Based Analysis

Spectrophotometry revealed the concentration and purity of the extracted DNA. The genomic DNA concentration ranged from 0.71ng/µL to > 266ng/µL with purity ratio of 260/280 ranging from 1.8-2.0.

4.2.1 Sequencing

Among the students, PCR amplification of *Mycoplasma* 16SrRNA gene prior to sequencing showed gel electrophoretic positive result for *Mycoplasma spp* [Figure 4.1] and *M.genitalium* negative result [Figure 4.2] and where so, Figure 4.3 shows the gel electrophoretic positive result of *Mycoplasma spp* for the hospital patients.

Following PCR amplification of *Mycoplasma* 16SrRNA gene, thirty six samples were found to be positive prior to sequencing. However, eighteen failed the quality control test for sequencing and thus not sequenced.

The 15 characterized sequence among students subjects with the chromatogram of the characterized sequence showing the result of the sequence run with the arrangement of the nucleotides for *M. hominis, U. urealyticum, M. genitalium, U.parvum* and *M.penetrans* [Figures 4.4-4.8] which were assembled by generating a consensus sequence following pair-wise alignment (optimal Global alignment).

Table 4.1 Demographic characteristics of the overall study population

Characteristics	**Study Population n(%)**
Age (years)	
15-19	9(6.9)
20-24	77(59.2)
25-29	27(20.8)
30-34	12(9.2)
35-39	5(3.8)
Mean ± SD	24.4 ± 2.66
Martial Status	
Married	16(12.3)
Single	114(87.7)

Key:

SD- Standard deviation of mean

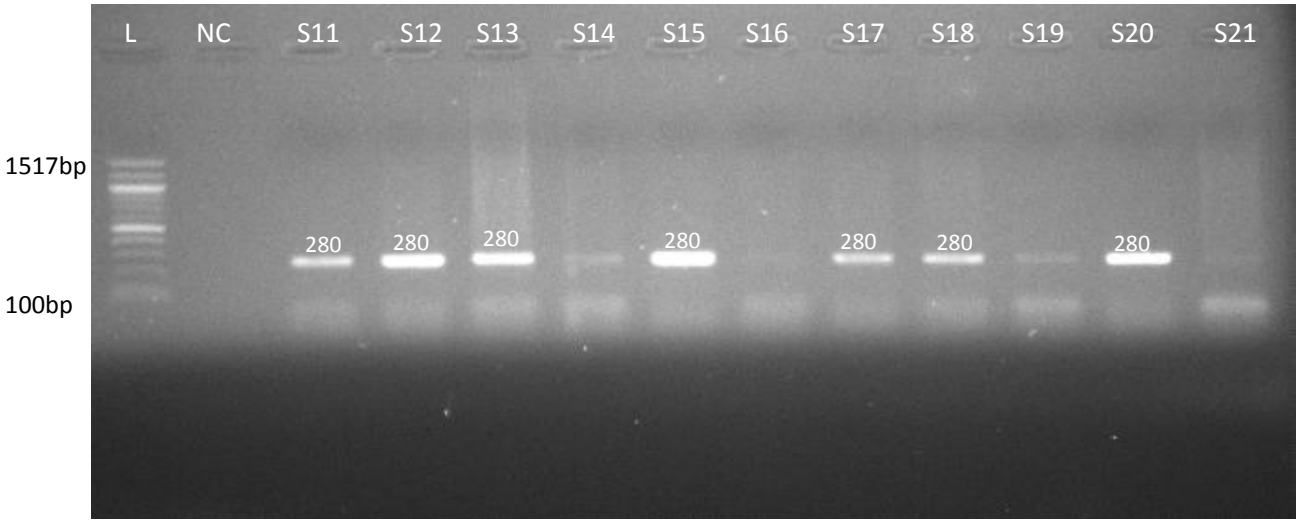

Figure 4.1 Polymerase chain reaction results for *Mycoplasma* genus (students) from ECS analyzed with 1.0% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1517bp DNA ladder (molecular marker). Samples S11, S12, S13, S14, S15, S17, S18, S19 and S20 are positive for *Mycoplasma* genus while samples S16 and S21 are negative for *Mycoplasma* genus. NC is a no DNA template control.

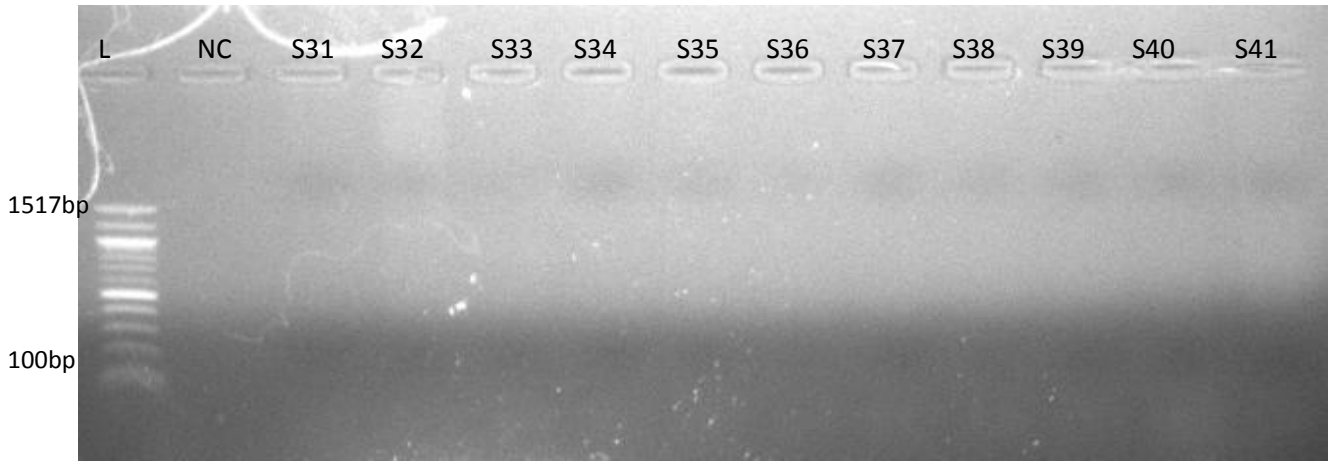

Figure 4.2 Polymerase chain reaction results for *M.genitalium* (students) from ECS analyzed with 1.0% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1517bp DNA (molecular marker). Samples S31, S32, S33, S34, S35, S36, S37, S38, S39, S40 and S41 are negative for *M.genitalium*. NC is a no DNA template control.

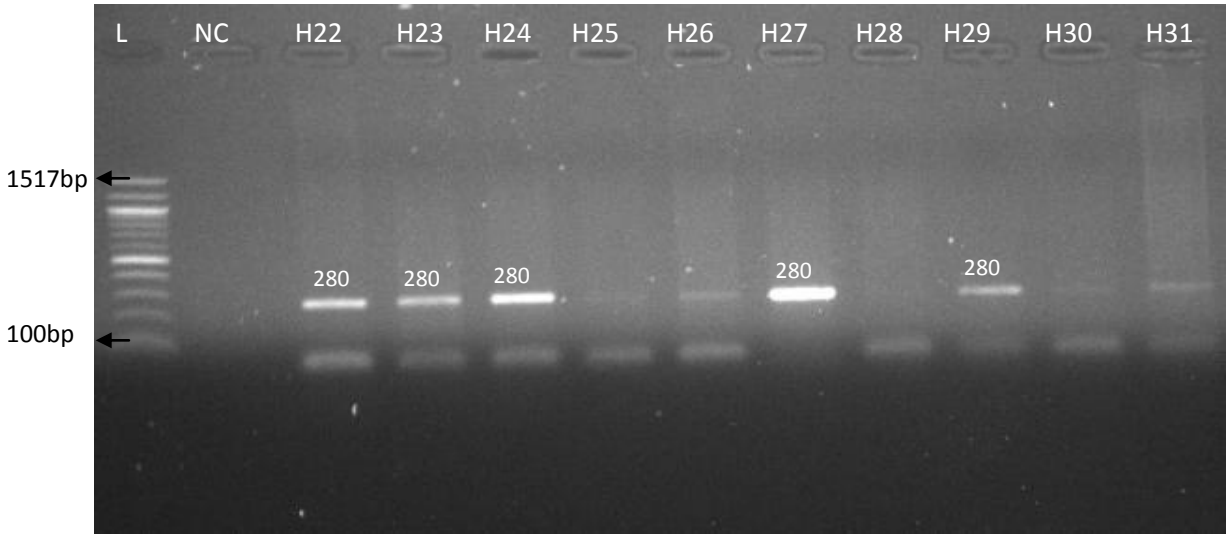

Figure 4.3 Polymerase chain reaction results for *Mycoplasma* genus (hospital patients) from ECS analyzed with 1.0% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1517bp DNA ladder (molecular marker). Samples H22, H23, H24, H26, H27, H29 and H31 were positive for *Mycoplasma* genus while samples H25, H28 and H30 are negative for *Mycoplasma* genus. NC is a no DNA template control.

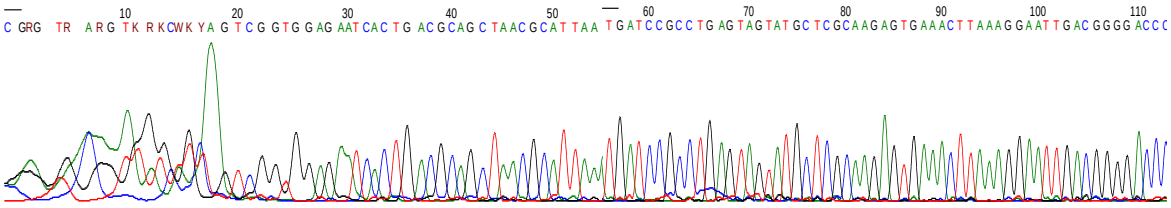

Fig 4.4a *M.genitalium* -16SrRNA-forward sequence chromatogram using MEGA Software version 7

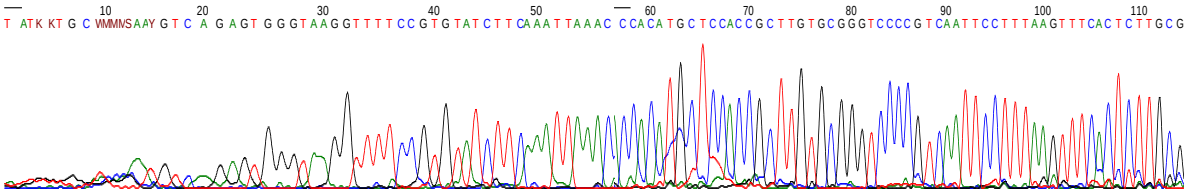

Fig 4.4b *M.genitalium* -16SrRNA-reverse sequence chromatogram using MEGA Software version 7

KEY:

A T C G

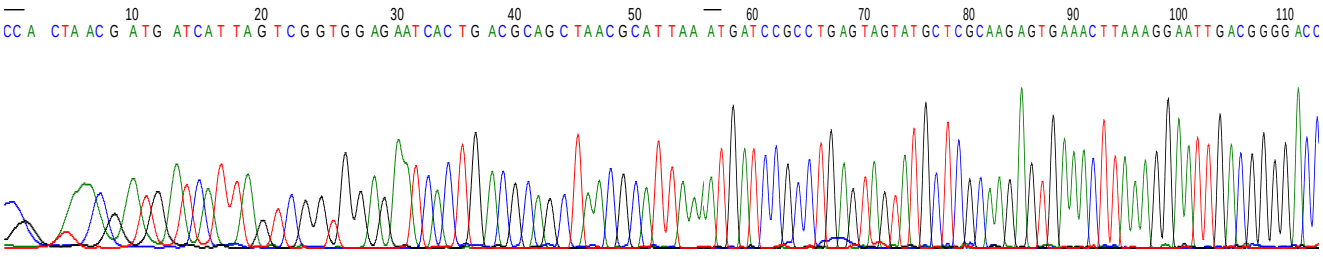

Fig 4.5a *M.hominis* -16SrRNA-forward sequence chromatogram using MEGA Software version 7

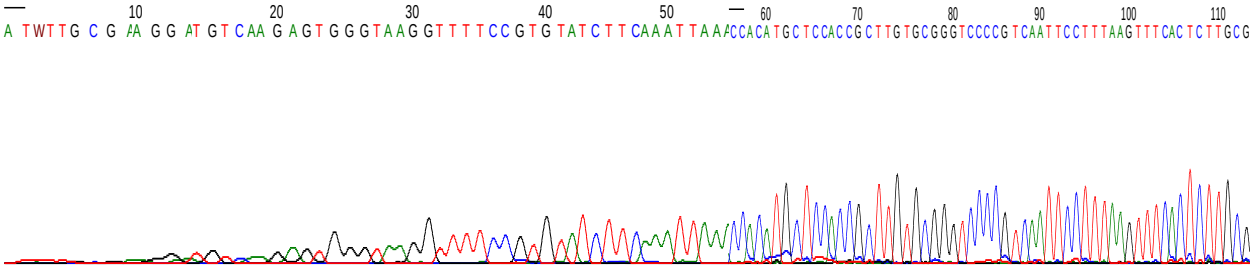

Fig 4.5b *M.hominis* -16SrRNA-reverse sequence chromatogram using MEGA Software version 7

KEY:

A T C G

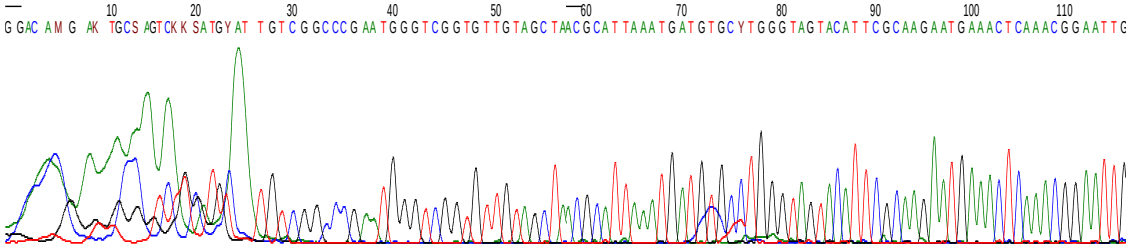

Fig 4.6a *U.urealyticum* -16SrRNA-forward sequence chromatogram using MEGA Software version 7

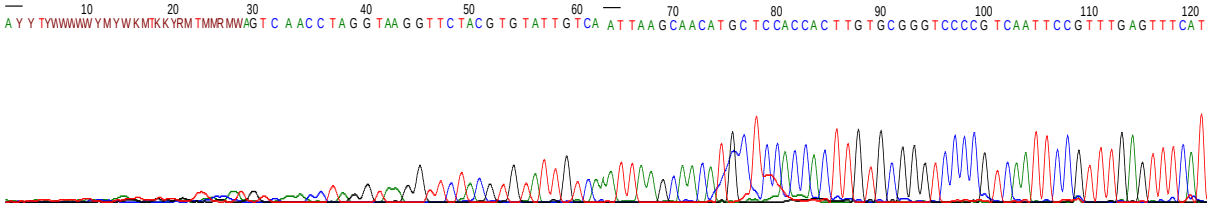

Fig 4.6b *U.urealyticum* -16SrRNA-reverse sequence chromatogram using MEGA Software version 7

KEY:

A T C G

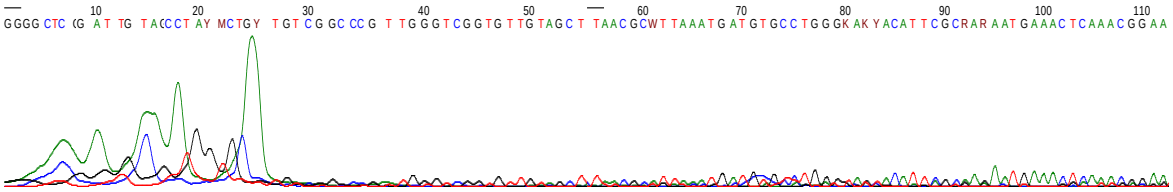

Fig 4.7a *U.parvum* -16SrRNA-forward sequence chromatogram using MEGA Software version 7

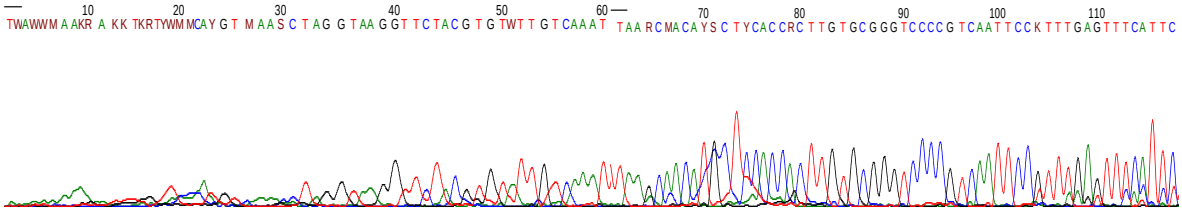

Fig 4.7b *U.parvum* -16SrRNA-reverse sequence chromatogram using MEGA Software version 7

KEY:

A T C G

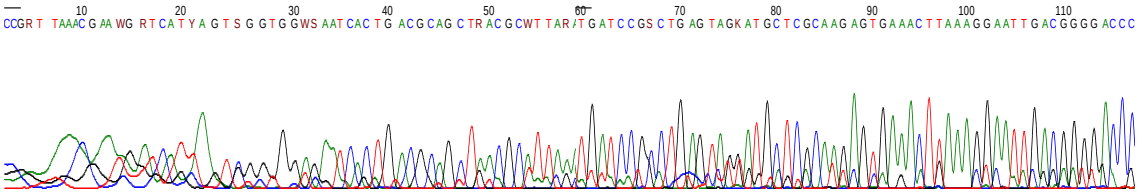

Fig 4.8a *M.penetrans* -16SrRNA-forward sequence chromatogram using MEGA Software version 7

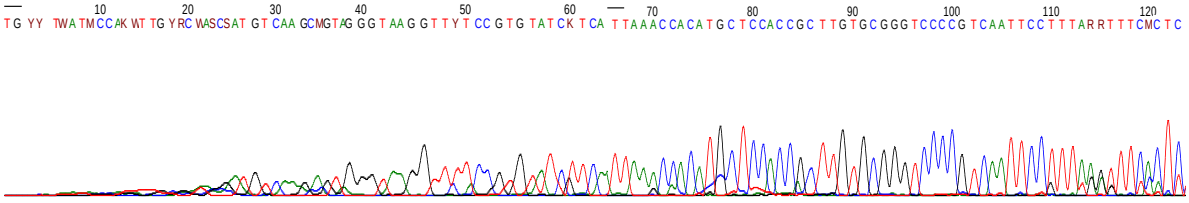

Fig 4.8b *M.penetrans* -16SrRNA-reverse sequence chromatogram using MEGA Software version 7

KEY:

A T C G

The BLAST result of the consensus sequence revealed closely related *Mycoplasma species*: *M.genitalium* (1), *M.hominis* (8), *M.penetrans* (1), *U.urealyticum* (3) and *U.parvum* (2) among the students. The accession numbers (MG238565, MG279045 to MG279052, MG279054 to MG279057, MG279059 and MG279061) was generated for each consensus sequence following submission to GenBank.

The 3 characterized sequences from the patients had a BLAST result showing the predominant *Mycoplasma species*: *M.hominis* (2) and *U.urealyticum* (1). The accession numbers: MG388338 to MG388340 were generated for each consensus sequence following submission to GenBank.

The symptomatic subjects harbouring *Mycoplasma species* all had species strongly associated with watery and smelling vaginal discharge as shown in Figure 4.9. Furthermore, vaginal itching was found to be associated with other species of Mycoplasma except *M.penetrans*. *Mycoplasma penetrans* and *M.genitalium* infection were not found to be associated with burning urination. Similarly, *M.genitalium* and *U.parvum* infection were not found to be associated with pelvic pain. Among the subjects infected with *Mycoplasma spp*, subjects infected with *M.hominis* had the highest number of presented symptoms likewise for *U.urealyticum*. Others presented a maximum of two symptoms except for subjects infected with *U.parvum*.

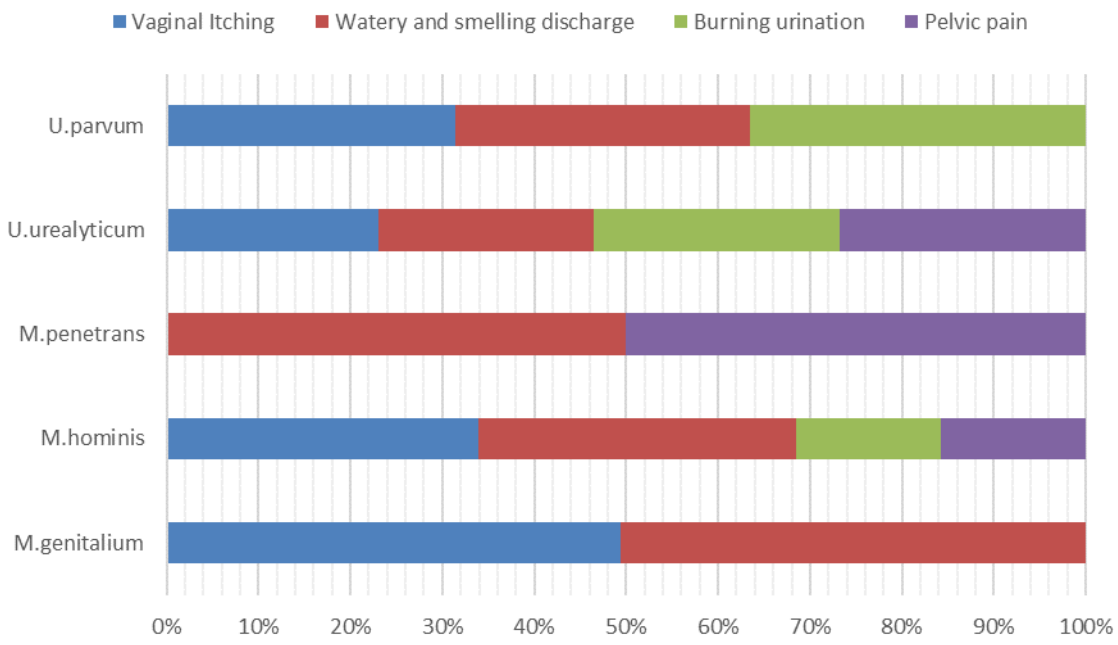

Figure 4.9 The Distribution of symptoms among symptomatic Mycoplasma infected subjects

4.2.2 Six-plex Detection

Following six-plex detection, the prevalence of the organisms: *T. vaginalis, M. hominis, C. trachomatis, U. parvum U. urealyticum* and *M. genitalium* was 65%, 16%, 9.2%, 9.7%, 6.45%, 0% respectively. Multiple colonization had prevalence of 9.67% for two organism colonization and 19.35% for three organism colonization. *T. vaginalis* was associated in all multiple infections. *C. trachomatis, M. hominis, U. parvum* and *U. urealyticum* presented only as concomitant infection [Figures 4.10- 4.13].

The mean age of the subjects presenting with symptoms was 23.1 years against 26.2 years for subjects not presenting with symptoms. Among the symptomatic subjects, the most prevalent symptom was vaginal itching followed by watery and smelling vaginal discharge and then pelvic pain. The least symptoms showed were burning urination, pain during sex and abnormal vaginal bleeding [Figure 4.14].

The proportion of asymptomatic subjects was 77% to that of symptomatic subjects. Subjects who had Chlamydia infection were significantly more likely to have symptoms (P=0.024) contrary to subjects with Bacterial vaginosis, Mycoplasmosis, Concomitant infection and Trichomonasis. Consequently, with exception to Chlamydia infection there was no association with Mycoplasmosis, Bacterial vaginosis, Concomitant infection; likewise Trichomonasis (P-value of 0.281) and symptomatic state [Table 4.2].

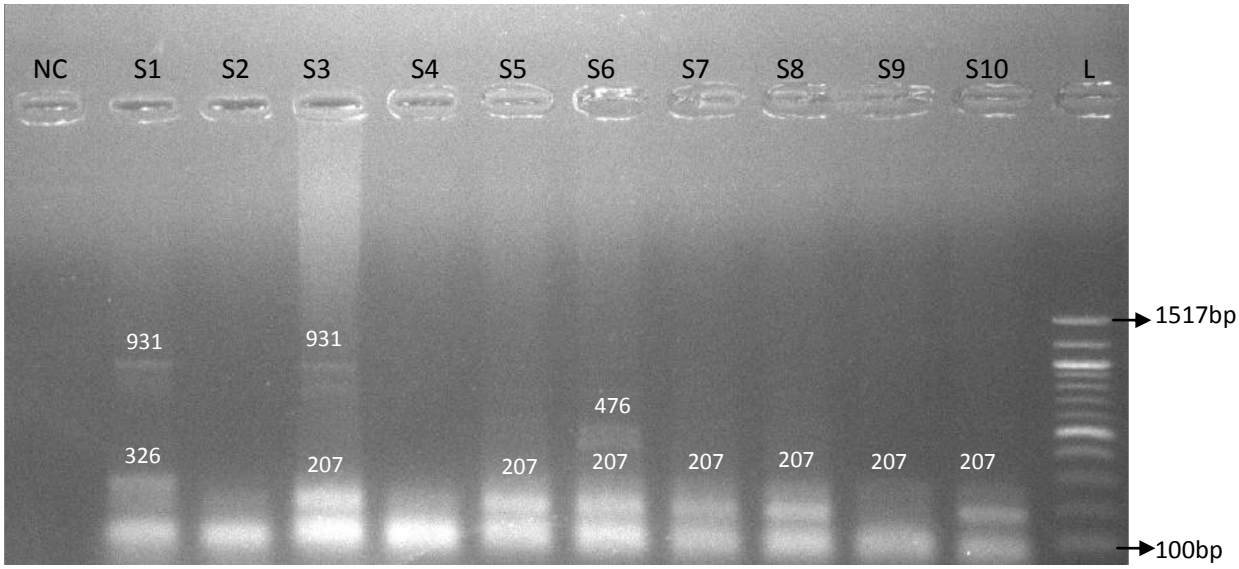

Figure 4.10 Multiplex polymerase chain reaction results for genital isolates from ECS analyzed with 1.0% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1517bp DNA ladder of size 100bp (molecular marker). Samples S1, S3, S5, S6, S7, S8, S9 and S10 are positive for *Trichomonas vaginalis* with bands at 207bp. Sample S1 is positive for *Ureaplasma parvum* with band at 326bp, Sample S6 is positive for *Mycoplasma hominis* and *Ureaplasma urealyticum* with bands at 402bp and 476bp distinctly, samples S1 and S3 are positive for *Chlamydia trachomatis* with band at 931bp. NC is a no DNA template control.

Keys:

Trichomonas vaginalis 207bp

Ureaplasma parvum 326bp

Ureaplasma urealyticum 476bp

Chlamydia trachomatis 931bp

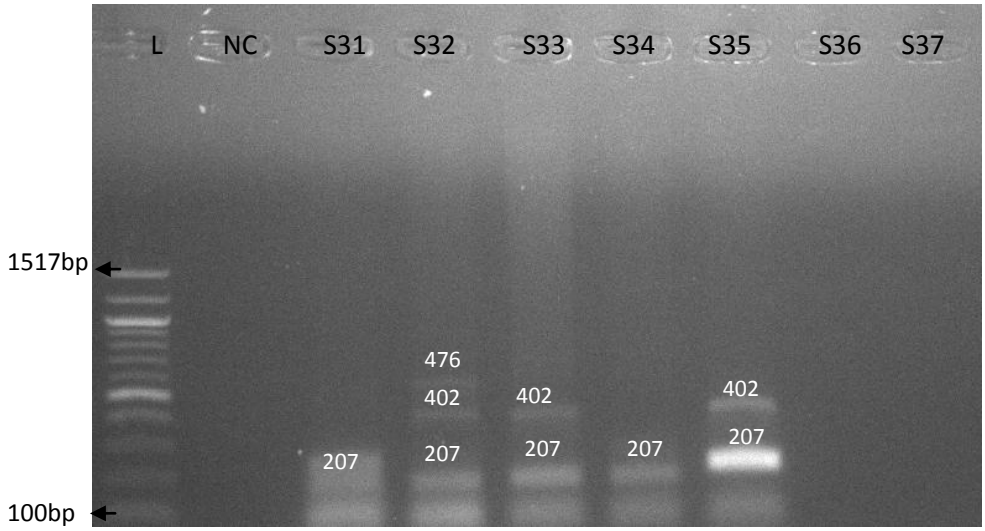

Figure 4.11 Multiplex polymerase chain reaction results for genital isolates from ECS analyzed with 1.0% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1517bp DNA ladder of size 100bp (molecular marker). Samples S31, S32, S33, S34 and S35 are positive for *Trichomonas vaginalis* with bands at 207bp. Samples S32, S33 and S35 are positive for *Mycoplasma hominis* with band at 402bp, sample S32 is positive for *Ureaplasma urealyticum* with band at 476bp while samples S31, S32, S33, S34 and S35 are negative for *Chlamydia trachomatis.* NC is a no DNA template control.

Keys:

Trichomonas vaginalis 207 bp

Mycoplasma hominis 402 bp

Ureaplasma urealyticum 476 bp

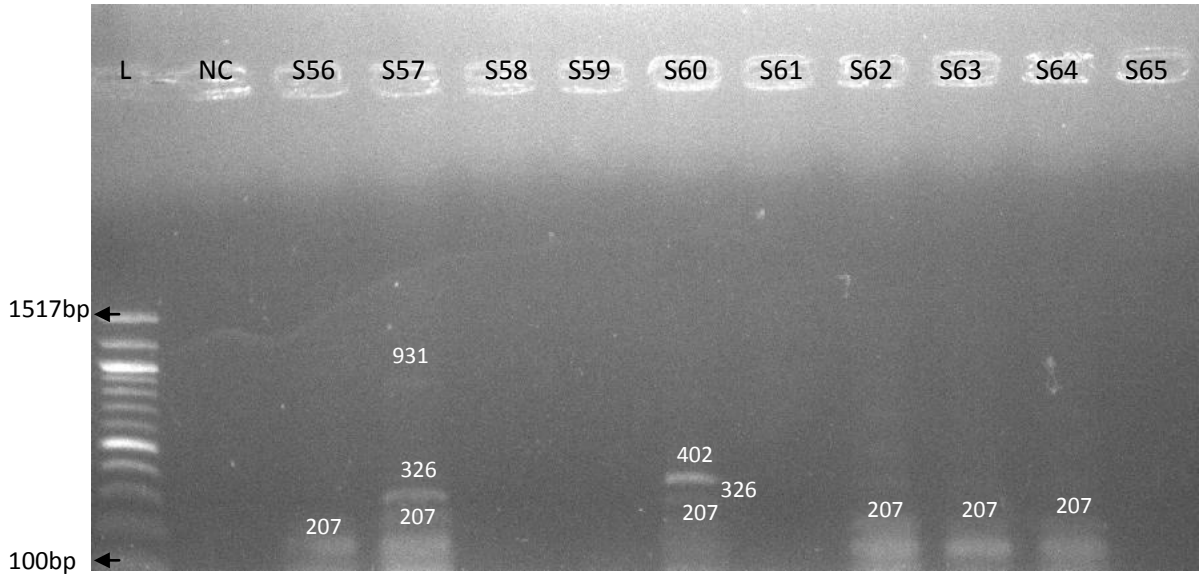

Figure 4.12 Multiplex polymerase chain reaction results for genital isolates from ECS analyzed with 1.0% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1517bp DNA ladder of size 100bp (molecular marker). Samples S56, S57, S60, S62, S63 and S64 are positive for *Trichomonas vaginalis* with bands at 207bp. Samples S57and S60 are positive for *Ureaplasma parvum* with band at 326bp, sample S60 is positive for *Mycoplasma hominis* with band at 402bp while sample S57 is positive for *Chlamydia trachomatis* with band at 931bp. NC is a no DNA template control.

Keys:

Trichomonas vaginalis 207 bp

Ureaplasma parvum 326 bp

Mycoplasma hominis 402 bp

Chlamydia trachomatis 931 bp

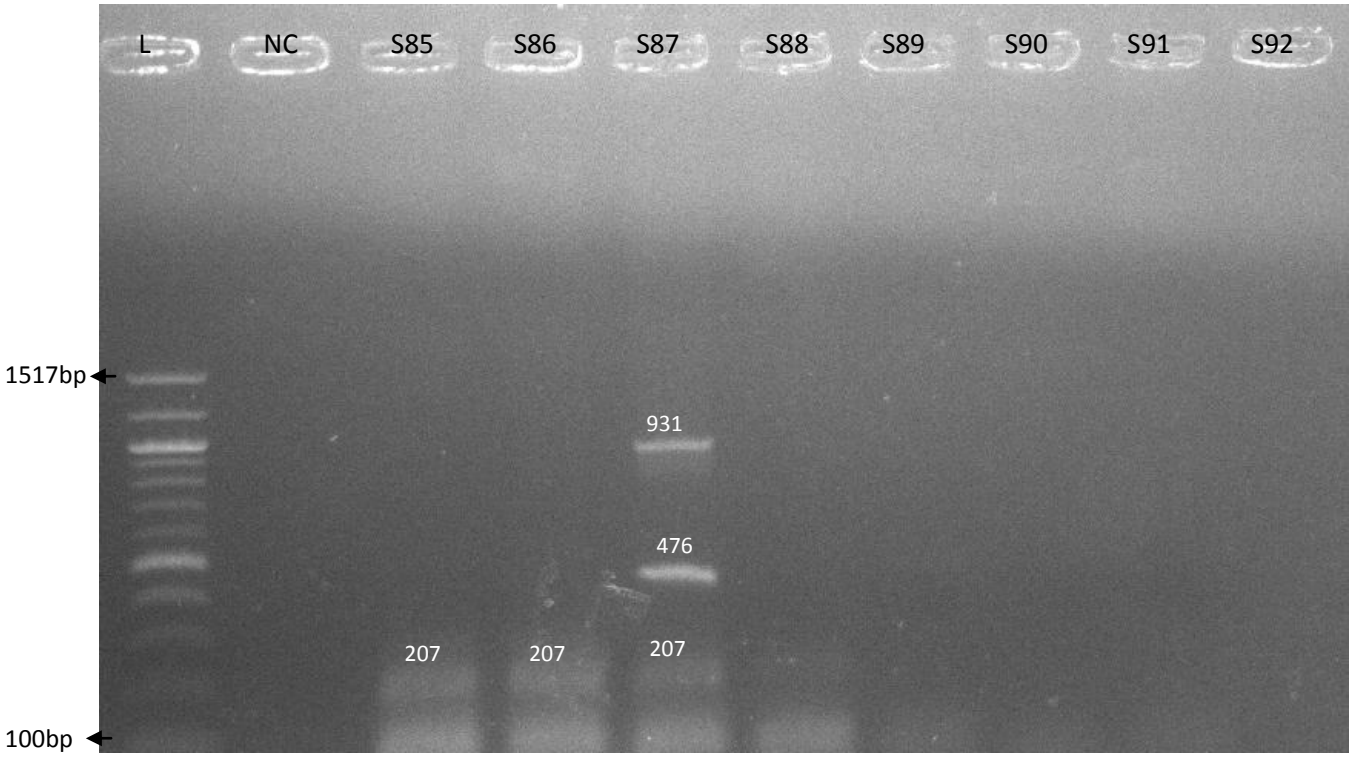

Figure 4.13 Multiplex polymerase chain reaction results for genital isolates from ECS analyzed with 1.0% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1517bp DNA ladder of size 100bp (molecular marker). Samples S85, S86 and S87 are positive for *Trichomonas vaginalis* with bands at 207bp. Samples S87 is positive for *Ureaplasma urealyticum* and *Chlamydia trachomatis* with band at 476bp and 931bp respectively. NC is a no DNA template control.

Keys:

Trichomonas vaginalis 207 bp

Ureaplasma urealyticum 476 bp

Chlamydia trachomatis 931 bp

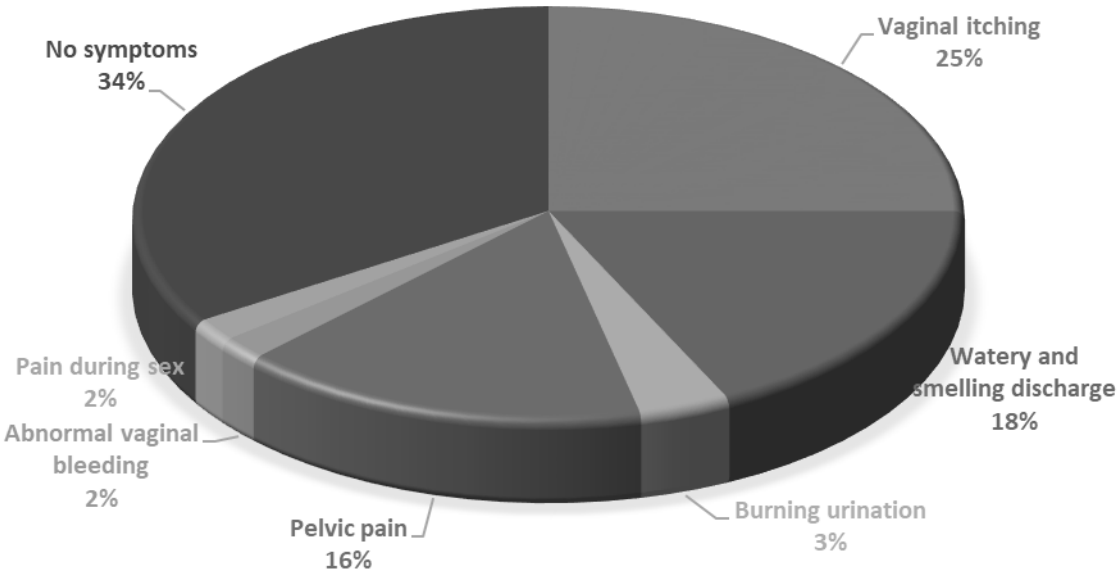

Figure 4.14 Distribution of symptoms of sexually transmitted infection among the study participants

Table 4.2 Association of genital infection with asymptomatic and symptomatic female subjects

Infection (n)	**Asymptomatic n=37(%)**	**Symptomatic n=48(%)**	**p value**
Mycoplasmosis (42)	22(53.4)	20(47.6)	.322
Bacterial vaginosis (60)	23(38.3)	37(61.7)	.102
Trichomonasis (84)	36(42.9)	48(57.1)	.281
Chlamydia (12)	5(41.7)	7(58.3)	.024*
Concomitant (38)	20(51.7)	18(47.4)	.301

*$P<0.05$

Among subjects with Mycoplasma/Ureaplasma infection, logistic regression model showed Mycoplasma/Ureaplasma infection had significant association with higher number of sexual partners (≥4) (OR=0.18, *P*=0.012), co-infection with other sexually transmitted (ST) pathogens (*T.vaginalis, C.trachomatis*) (OR=0.51, *P*=0.040), inconsistent condom use, history of previous STD treatment, bacterial vaginosis and abortion. Hence, the risk factors for Mycoplasma/ Ureaplasma infection among the female subjects encompassed: higher number of sexual partners, co-infection with other ST pathogens, inconsistent condom use, history of previous STD treatment, abortion and bacterial vaginosis. Contrary, consistent, lack of condom use and previous STD treatment for sexual partner were not found to be associated with Mycoplasma/ Ureaplasma infection [Table 4.3].

Table 4.3 Logistic Regression to assess factors associated with Mycoplasma infection

Variables	**OR (95% CI)**	**p value**
Younger age at FI (<18 yrs.) mean (SD)	0.27 (0.03-0.51)	0.101
Higher number of sexual partner (≥4)	0.18 (0.41-0.78)	0.012*
Co-infection with other ST pathogens**	0.51 (0.10-0.73)	0.040*
Consistent condom use	1.30 (0.45-1.87)	0.861
Inconsistent condom use	0.87(0.23-0.91)	0.045*
Non condom use	0.93 (0.73-1.31)	0.622
Abortion	0.57 (0.29-0.99)	0.048*
Previous STD treatment	0.11 (0.05-0.77)	0.020*
STD treatment for partner	3.10 (1.80-6.90)	1.903
Infection with Bacterial vaginosis	0.71(0.53-0.91)	0.041*

***T.vaginalis, C.trachomatis*

**P*<0.05

Key:

FI- First Intercourse

OR-Odds Ratio

CI-Confidence Interval

4.3 Microscopic Examination

Under 40X objective the distribution of clue cells, epithelial cells, bacteria, PMNLs, pseudo hyphae, budding yeast and motile trichomonad was found [Figure 4.15].

Whiff test results were positive for 95 (73%) of the 130 samples. This was indicative for bacterial vaginosis and Trichomonasis.

The Gram stain results were not concordant with the findings obtained with wet mount methods. Variation in the distribution of observed cells was seen. Gram staining showed a lower distribution of cells compared to wet mount. Clue cells had 12% as against 19% found using wet mount [Figure 4.16]. However, bacterial morphology (32%) was well defined unlike in wet mount (13%).

Bacterial Vaginosis was interpreted using Amsel's Diagnostic Criteria supplemented with Gram staining result [Figure 4.17] The occurrence of bacterial vaginosis was found to be 46%. It was found to be associated with symptomatic and asymptomatic state (38%). Among symptomatic subjects, the prevalent symptom was watery and smelling vaginal discharge.

Giemsa stain result was somewhat concordant with that of Gram stain in the distribution of bacteria. Significantly, the distribution of Trichomonad and Chlamydia was found as 21% and 2% respectively [Figure 4.18].

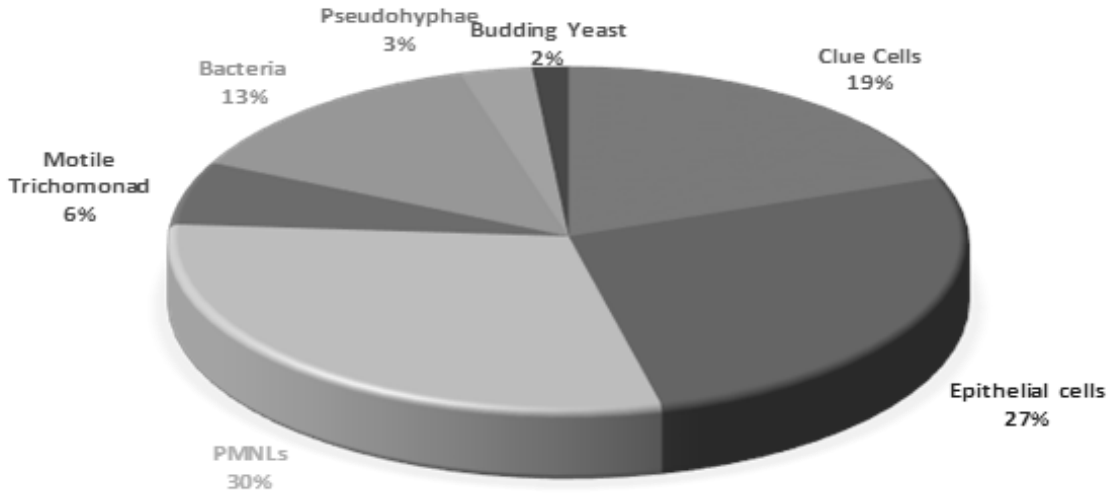

Figure 4.15 Distribution of bacteria, clue cells, epithelial cells, pseudo hyphae, budding yeast, motile trichomonad and PMNLs using wet mount methods.

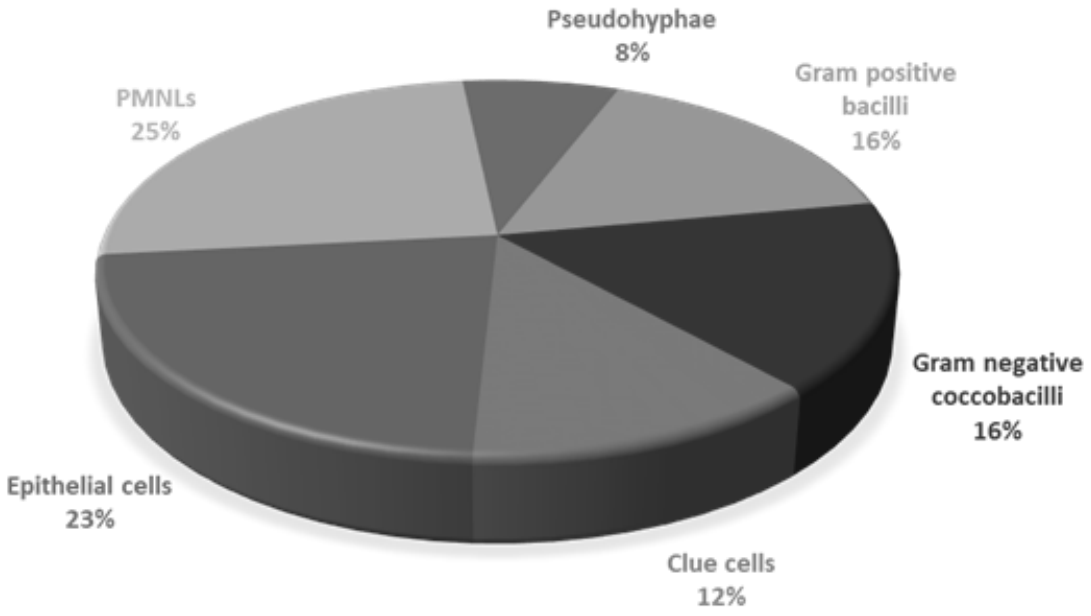

Figure 4.16 Distribution of Gram positive bacteria, Gram negative bacteria, clue cells, epithelial cells, pseudo hyphae using Gram staining methods.

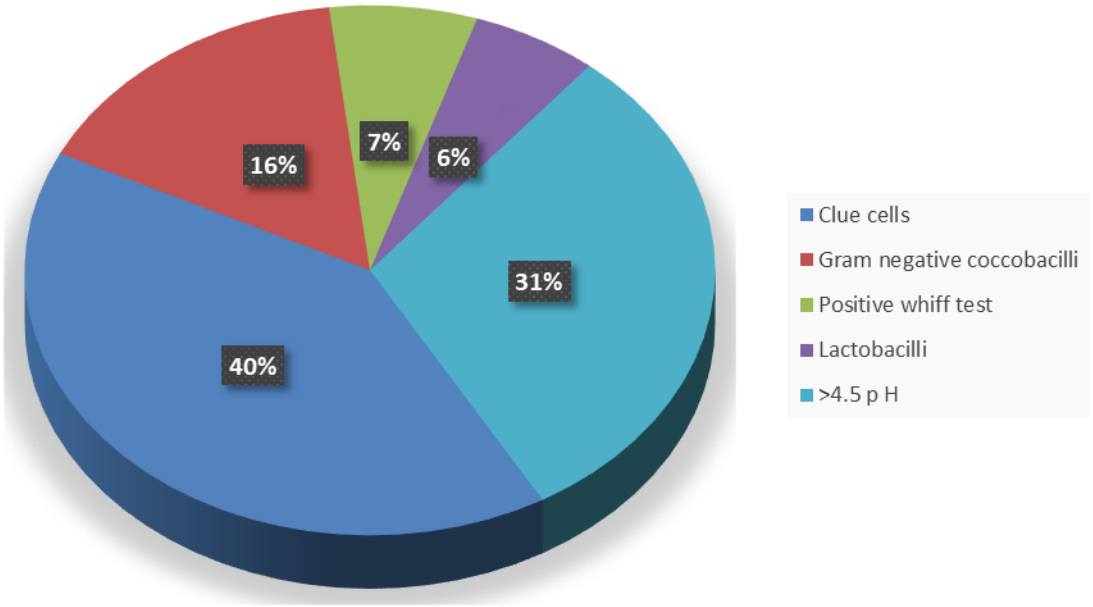

Figure 4.17 Shows diagnosis for bacterial vaginosis using Amsel's Diagnostic Criteria supplemented with Gram staining

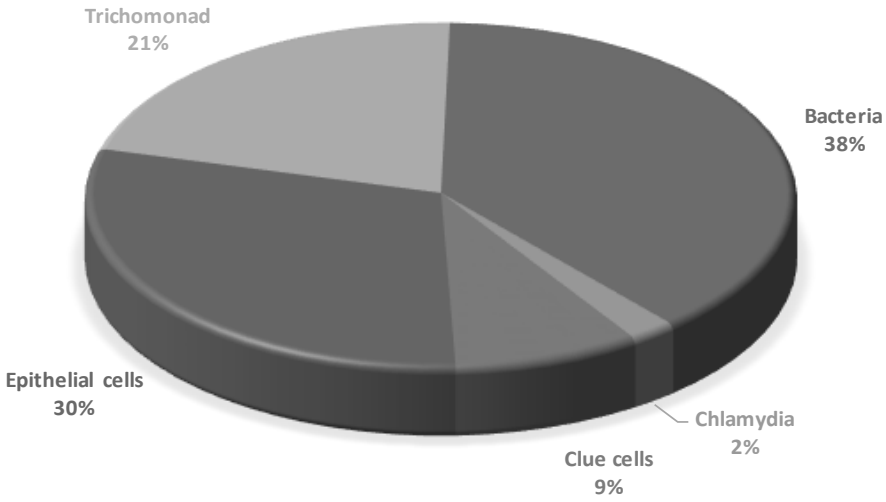

Figure 4.18 Distribution of Chlamydia, Trichomonad, bacteria, clue cells and epithelial cells using Giemsa staining methods.

4.4 Phylogenetic Analysis

Test of phylogeny (Bootstrap method) with maximum likelihood statistical method via Tamura-Nei model resulted to confidence value (CV) following 150 bootstrap replication shown at the branches of the phylogenetic tree.

Phylogenetic analysis of 16SrRNA sequences of the isolated *M.genitalium* from this work bolded with 7 reference strains: two strains of G37, ATCC 33530, ATCC 49895, Strain 1388, Denmark strain and GQ 367563 for *M.genitalium* obtained from Genbank showed that the *M.genitalium* sequence (MG238565) from the students were not found to have any phylogenetic linkage with the reference strains from Japan (AB069813), Denmark (NC000908 and X77334), Iran (GQ367563), G37 (NR074611) [Figure 4.19]

Phylogenetic analysis of 16SrRNA sequences of the isolated *M.hominis* from this work bolded with 6 reference strains: PG21, AF1, Sprott, NBRC 14850, ATCC 23114 strain PG21 and Isolate 27 for *M.hominis* respectively obtained from Genbank showed that all the isolates sequences from the students were of the same clade (CV=91%) and were all linked with China strain (Isolate 27 EU596509) with confidence value at 65%. However, all *M.hominis* showed no phylogenetic linkage to PG21, AF1, Sprott, NBRC 14850, ATCC 23114 strain PG21 with accession numbers NR041881, CP009677, CP011538, NR113679 and JN935871 respectively [Figure 4.20]

Phylogenetic analysis of 16SrRNA sequences of *M.penetrans* from this work bolded with 3 reference strains: GTU-54-6A1 NCBI Reference Sequence NR118664, GTU-54-6A1 and Japan strain for *M.penetrans* obtained from Genbank revealed that the *M.penetrans* sequence MG279046 from the students were not found to be of the same clade or linked to the reference strains from Japan (AB069817), GTU-54-6A1 strains (NR118664 and L10839) [Figure 4.21]

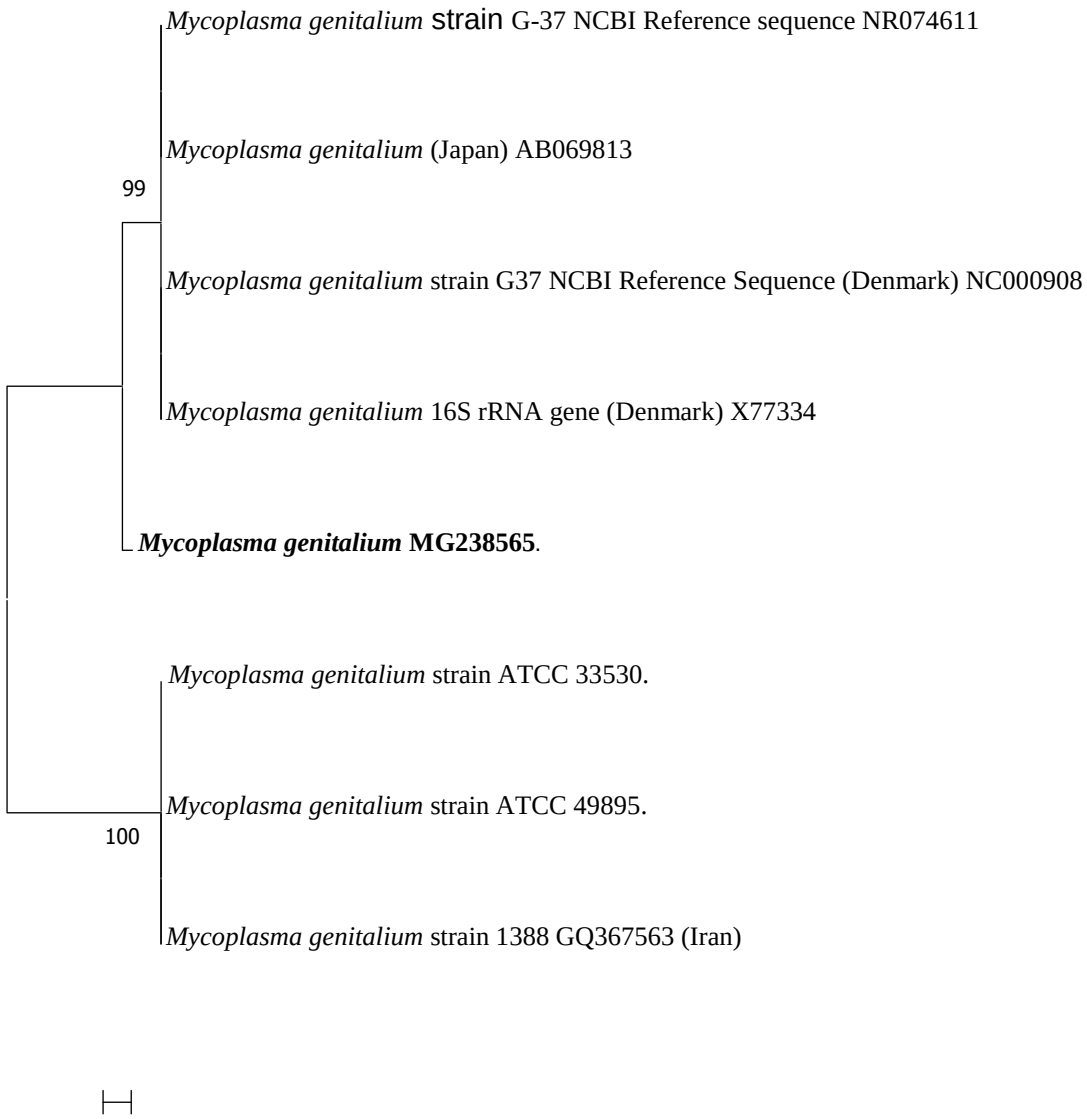

Figure 4.19. Molecular Phylogenetic analysis by Maximum Likelihood method of isolated *Mycoplasma genitalium* from the students and reference strains previously reported

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-280.71) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 81 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

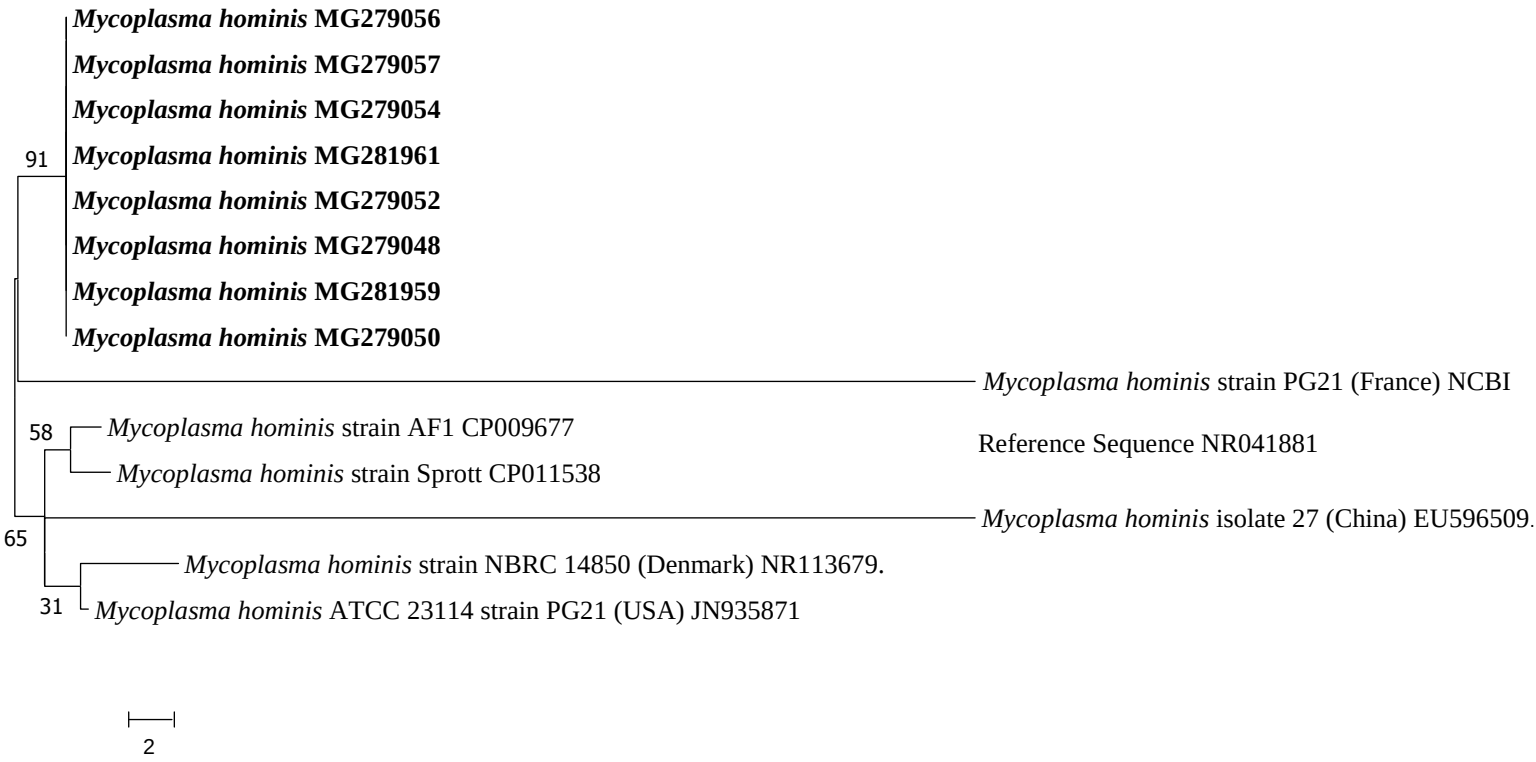

Figure 4.20. Molecular Phylogenetic analysis by Maximum Likelihood method of isolated *Mycoplasma hominis* from the students and reference strains previously reported

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-2230.72) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 233 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

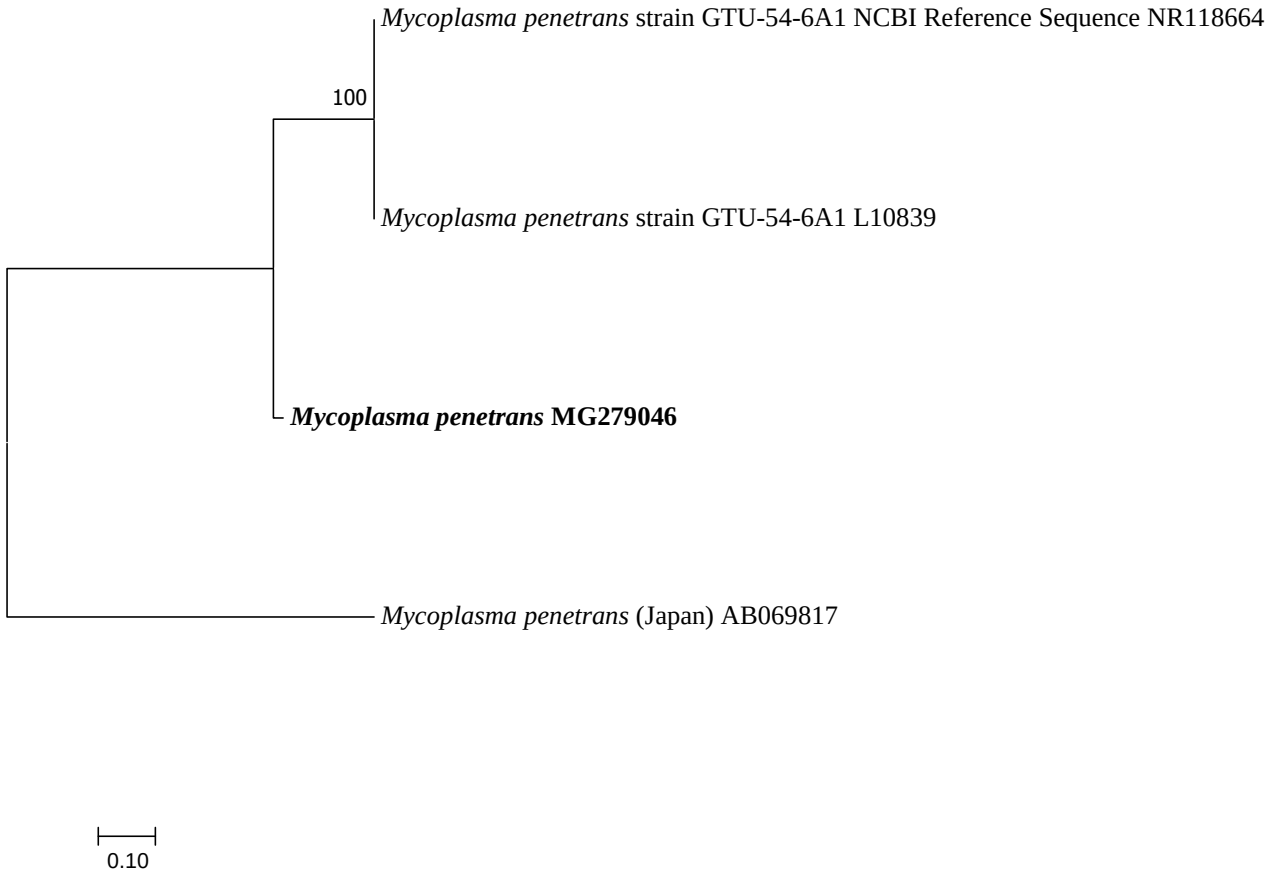

Figure 4.21 Molecular Phylogenetic analysis by Maximum Likelihood method of isolated *Mycoplasma penetrans* from the students and reference strains previously reported

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-707.65) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 222 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

Phylogenetic analysis of 16SrRNA sequences of the isolated *U.urealyticum* from this work bolded with 5 reference strains: ATCC 27618, Canada strain, China strains (Isolate 17 and 18) for *U.urealyticum* obtained from gene bank. Two isolated sequences from the students **(**MG279055 and MG279047) were of the same clade and had a significant association (CV=100%) with the two China strains EU596511 and EU596510. On the other hand, the *U.urealyticum* strain (MG279051) was found to be of the same clade with ATCC 27618 (NR041710) and Canada strain U06096 (CV=99%) [Figure 4.22].

Phylogenetic analysis of 16SrRNA sequences of the isolated *U.parvum* from this work highlighted in yellow with 4 reference strains: ATCC 27815, Serovar 3 strain ATCC 27815, Isolate 14(Australia) and clone ncd1736a05c1 for *U.parvum* obtained from Genbank. All *U.parvum* from the students were found to be of the same clade (CV=99%) and showed a strong linkage with ATCC 27815 (NR074762) and American strain with CV= 90% and 86% respectively [Figure 4.23]

Genetic relatedness of *Mycoplasma spp* and *Ureaplasma spp* isolated from the students as shown in Figure 4.24 revealed a significant clustering (CV=97%) of all *M.hominis*. However, *M.genitalium* and *M.penetrans* had no clade. Among the *Ureaplasma spp*, *U.urealyticum* MG279051 was found not belonging to a common clade with other *Ureaplasma spp* whereas the other *U.urealyticum* strains and *U.parvum* strains had a significant association (CV=96%) belonging to the same clade.

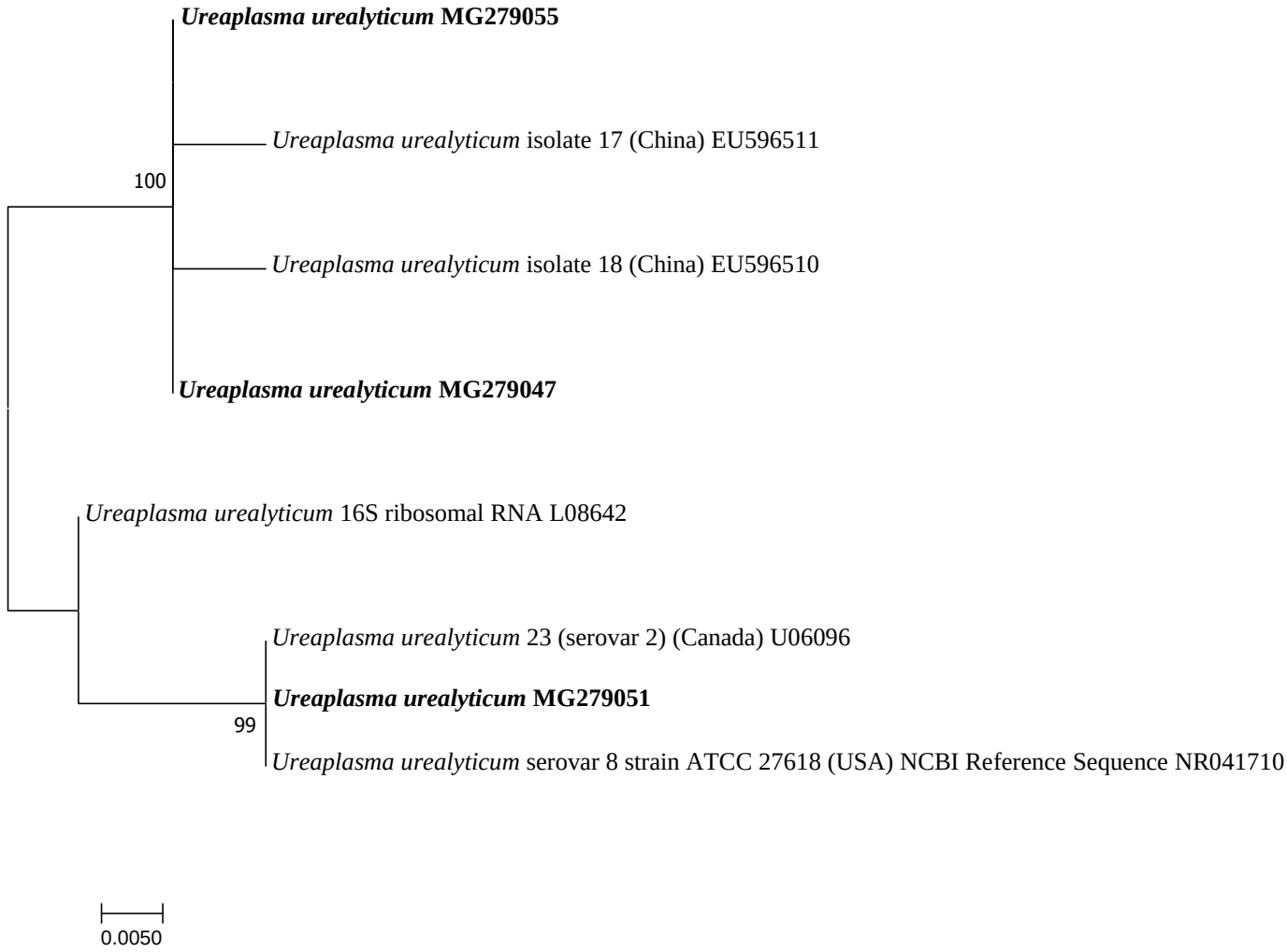

Figure 4.22. Molecular Phylogenetic analysis by Maximum Likelihood method of isolated *Ureaplasma urealyticum* from the students and reference strains previously reported

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-436.28) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 265 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

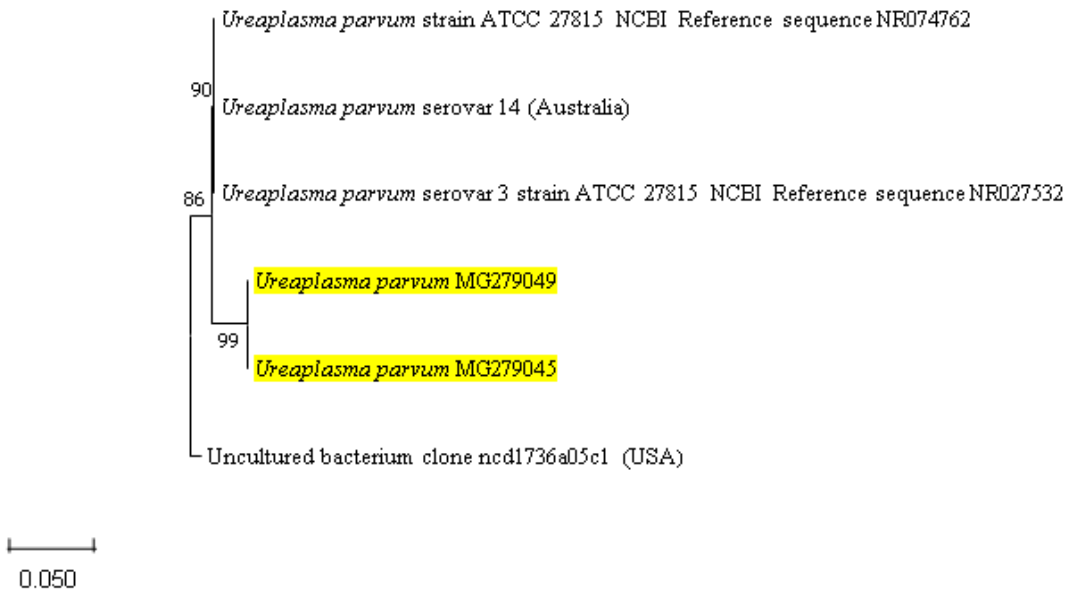

Figure 4.23. Molecular Phylogenetic analysis by Maximum Likelihood method of isolated *Ureaplasma parvum* from the students and reference strains previously reported

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-636.03) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 258 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

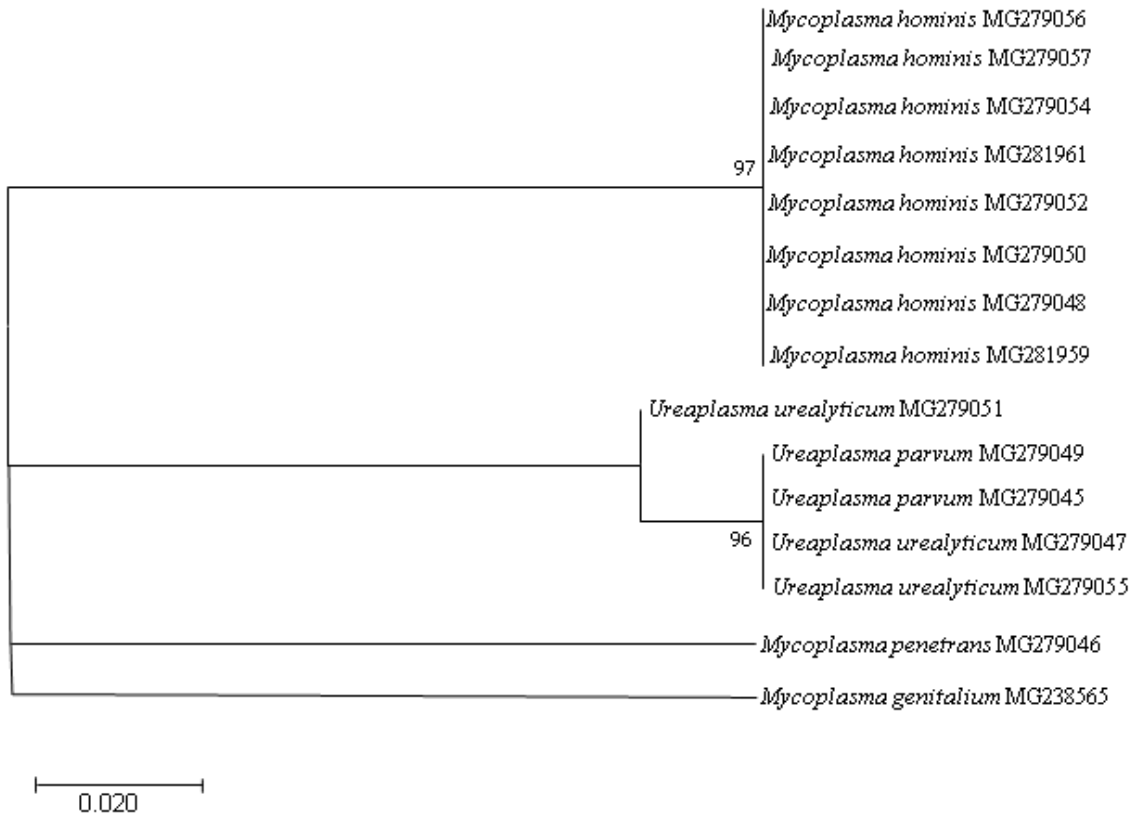

Figure 4.24. Molecular Phylogenetic analysis by Maximum Likelihood method of isolated *Mollicutes* from the students

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-409.15) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 203 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

Evolutionary relationship of *Mycoplasma spp* from this study and other Bacterial ST pathogens obtained from Genbank as shown in Figure 4.25, all the *Mycoplasma spp* (highlighted in yellow) were found to be of a common clade (CV=100%). All the Ureaplasmas (highlighted in yellow) clustered in a clade with 89% confidence value except *U.urealyticum* MG279051. However, all the Ureaplasmas were found to have a strong phylogenetic linkage (CV=98%) with Mycoplasmas. Furthermore, the other bacterial ST pathogens: *Chlamydia trachomatis, Treponema pallidum, Neisseria gonorrhoeae, Haemophilus ducreyi*, *Gardnerella vaginalis* and *Mobiluncus spp* were found to have a strong relationship (CV=93%) with Mycoplasmas. Whereas, *C.trachomatis* and *T.pallidum* had a weak linkage (CV=31%), contrary *N.gonorrhoeae* and *H.durceyi* had a strong linkage (CV=82%) showing they belong to a common clade. Unlike, *Gardnerella vaginalis* and *Mobiluncus spp* which had a strong linkage (CV=71%) they did not belong to the same clade. *Trichomonas vaginalis* was the outgroup organism (parasite).

Phylogenomics study of the isolated organism from the students, patients and reference strains are highlighted in (*), (^) and (′) respectively as seen in Figure 4.26. All *M.hominis* from both the students and the patients were found to be of a common clade having strong phylogenetic linkage (CV=81%) with China strain EU596508 though not of a common clade. *M.hominis* strains from both subjects (students and patients) were found to be strongly associated (CV=99%) with China strain EU596509. On the other hand, *U.urealyticum* MG279051 had a strong linkage (CV=77%) with patient strain: *U.urealyticum* MG388340, though they were not found to have a common clade.

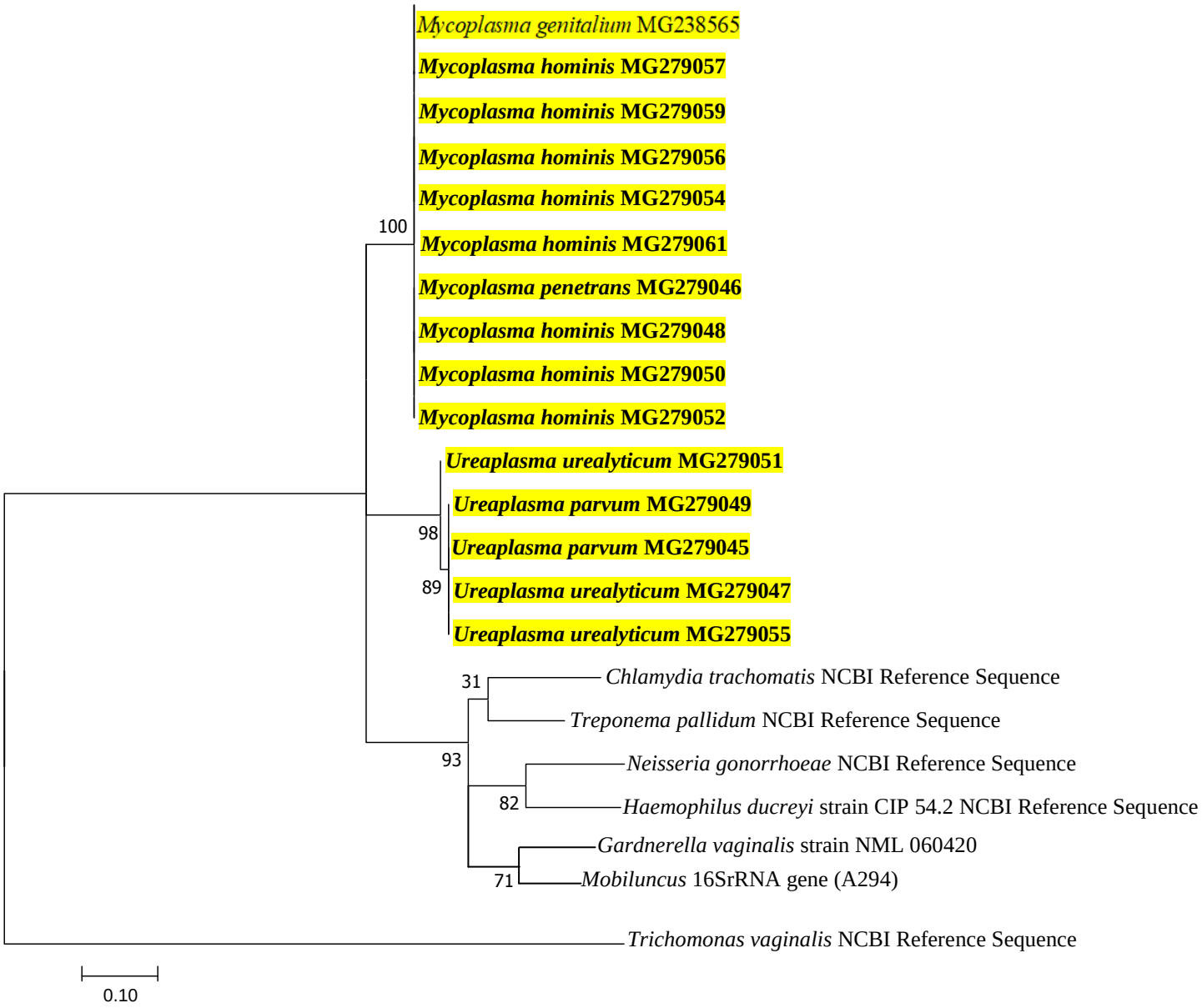

Figure 4.25. Molecular Phylogenetic analysis by Maximum Likelihood method of isolated *Mycoplasma spp*. and other bacterial ST pathogens including agents of bacterial vaginosis

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1140.31) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 191 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016)

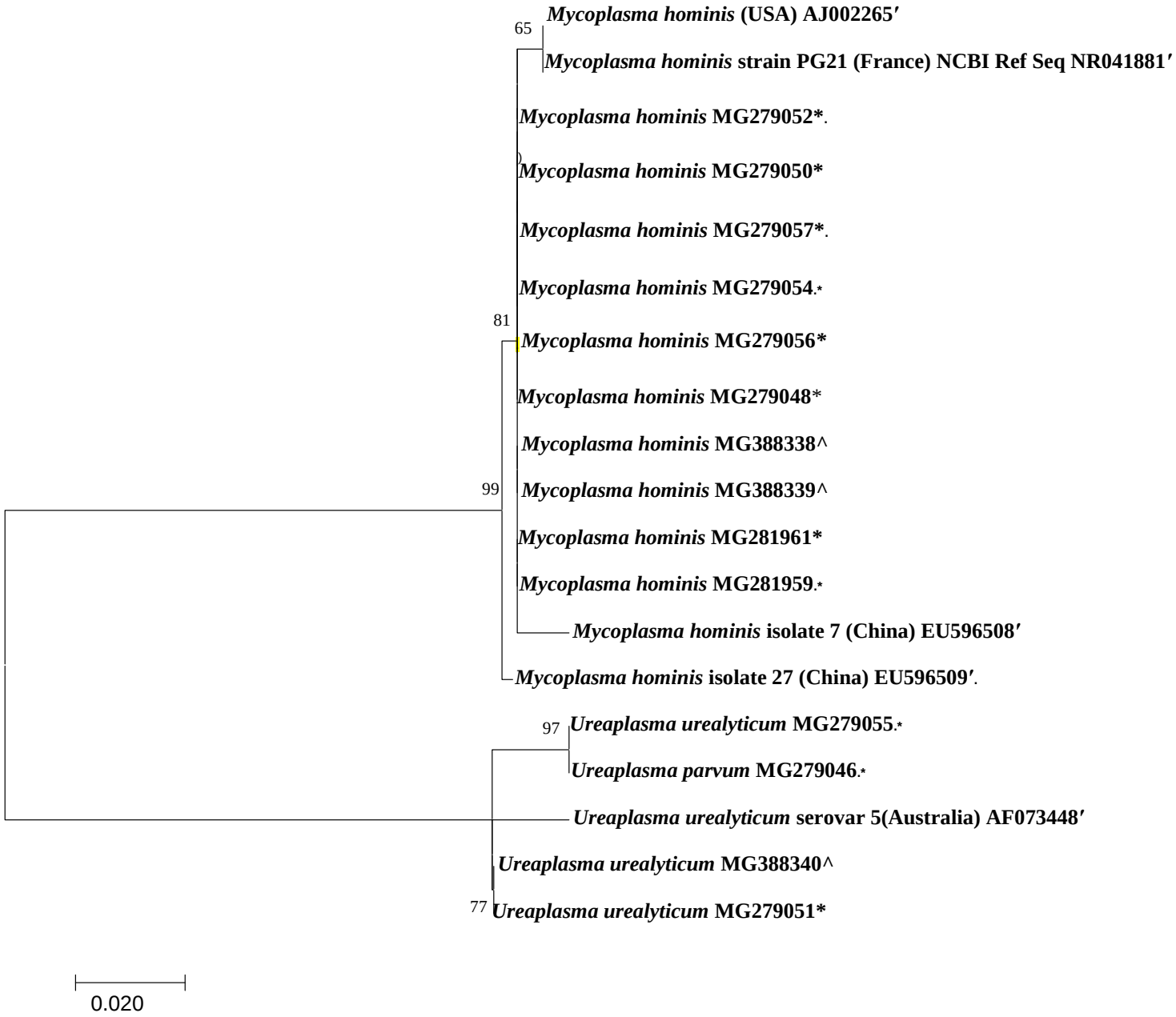

Figure 4.26. Molecular Phylogenetic analysis by Maximum Likelihood method of isolated *Mycoplasma spp*. from students, patients with disease sequelae and reference strains previously reported

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-473.56) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 214 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

CHAPTER FIVE

DISCUSSION

This study revealed 1% prevalence of *M. genitalium* among the female students in a Tertiary Institution. The prevalence was defined based on the sample size of the students. However, very few studies on the prevalence of *M. genitalium* in Tertiary student population have been reported outside Nigeria with paucity of data from Nigeria. This study agreed with a study in Northern Norway by Jensen *et al* (2013) who reported 1% prevalence among 206 female college students. Furthermore, Oakeshott *et al* (2010) reported 3.3% prevalence among 2378 female students from Tertiary Institutions in London. The discrepancy as compared to our study could be as a result of the type of specimen (clinician-collected endocervical swab in our study), age of the subjects and the large sample size. Hamasuna *et al* (2008) reported prevalence of 2.1% among female students from three vocational schools in Miyazaki prefecture, Japan. This study findings contrasts the result from our study considering also the type of the specimen (first voided urine) and the large sample size (298) in their findings. On the otherhand, the prevalence of 0.8% among the overall study population agreed with a recent study by Silva *et al*. (2018) who reported a 0.8% prevalence of *M. genitalium* among Portuguese women of reproductive age (15 – 44 years).

Among the symptomatic subjects, vaginal discharge was found to be associated with *M. genitalium* infection. This is consistent with a recent study by Kufa *et al*., (2018) which demonstrated a significant association between vaginal discharge syndrome and ST pathogens such as *M. genitalium* among South African women under the age of 35 years notwithstanding the high prevalence of bacterial vaginosis in the population. The etiologies of vaginal discharge are enormously varied as microbial or non-microbial, perhaps normal or

abnormal and can be ascribed to infection of other parts of the reproductive tract (Spence and Melville, 2007).

Molecular technology also enables instantaneous detection of more than one microorganism. Multiplex PCR (M-PCR) is an exceptional molecular method as it rapidly detects multiple micro-organisms simultaneously via multiplex function. This study provides the first investigation of six simultaneous sexually transmitted pathogens directly from endocervical swab using M-PCR among female students in Tertiary Institution in Nigeria. The M-PCR revealed vivid result for all the parameters and detected cervical samples with co-infections (two and three- organism colonization with prevalence of 9.67% and 19.35% respectively). *Trichomonas vaginalis* was associated in all multiple infections. This is consistent with findings by Casillas-Vega *et al*. (2016) who reported that female subjects infected with *T. vaginalis* were more likely to have multiple coinfections in Jalisco, Mexico. Further more, *C. trachomatis, M.hominis* and *U. urealyticum* presented only as co-infection. This correlates with studies by Esen *et al*. (2017) who demonstrated that *U. urealyticum* was present in 71.4% of cervical samples among symptomatic females with multiple pathogens.

Trichomonas vaginalis is a protozoan with an extracellular obligatory parasitic existence adapted to the human urogenital tract and responsible for momentous number of sexually transmitted infections worldwide (Fichorova *et al*., 2017). A study in 2016 found *T. vaginalis* at a much lower prevalence (4.5%), but the population selected for the research included only asymptomatic sexually active females using wet mount (Adogo *et al*., 2016). This study revealed that *T. vaginalis* infection was associated with asymptomatic state ($P>0.05$). The association of *T. vaginalis* and *M. hominis* has been described as the first symbiosis involving two obligate human mucosal pathogens which have the ability to invade and infect the same anatomical region with both pathogens capable of producing independent diseases (Fichorova *et al*., 2017). *Trichomonas vaginalis* infection is a well-characterized common parasitic

infection among women. There have been studies from within and outside Nigeria with a lower prevalence (Isiaka-Lawal *et al*., 2014; Mahmoud *et al*., 2015). This can be explained owing to the difference in sensitivity of methods of diagnosis, namely microscopy, culture and molecular based method thus comparable with that of present study. Normally, *T. vaginalis* infection is diagnosed using wet mount (microscopy) and culture as reported in previous studies (Akinbo and Oronsaye, 2017). On the other hand, this present study employed molecular- based methods which could justify the high prevalence (65%); although wet mount (6%) and geimsa staining (21%) was employed in this study.

The second most prevalent sexually transmitted pathogen was *M. hominis* (16%). Knowledge of *M. hominis* is limited generally due to difficulties associated with diagnosis. A recent study by Park *et al*. (2017) reported *M. hominis* prevalence of 17.3% among sexually active Korean females which somewhat correlates with our study with reported 16%. Likewise, another study by Christofolini *et al*., (2012) with reported 11.3% of the subjects having *M. hominis* infection among 106 females using endocervical analyzed with NAAT in Brazil. In contrast, this present study with reported prevalence of 16% for *M. hominis* is inconsistent with a recent findings by Leli *et al*. (2018) with reported 8.6% prevalence. Although, method of detection and sample type was similar for both studies, the number of sample sites could be responsible for the difference in the results. Nucleic acid amplification test (NAAT) has been employed in most studies on *M. hominis* which have proven high recovery rate (Christofolini *et al*., 2012; Mutwally *et al*., 2014).

Chlamydia trachomatis was the third most common sexually transmitted pathogen (9.2%). The proportion of female subjects infected by this pathogen was somewhat similar to another study that showed 9.7% examining the urine of 789 females collectively from Alabama, Mississippi and Geogia (Adelbert *et al*., 2008). The slight difference could be due to the difference in sample size. On the other hand, findings by de Salazar *et al*., 2019 differs from

our study as a 5.2% prevalence was documented for *C. trachomatis* irrespective of the use of multiplex PCR as employed in our study. However, the difference could be attributed to the difference in the sample size. Further more, our result is comparable to other reports within and outside Nigeria with *C. trachomatis* prevalence ranging from 4.9%-29.4% (Imai *et al*., 2010; Ikene *et al*., 2011; Wariso *et al*., 2012; Ella *et al*., 2013; Arinze *et al*., 2014; Burazin *et al*., 2017). These studies employed a common methodology for detection (antibodies and/or antigen detection) unlike in our study where nucleic acid amplification test was explored. Furthermore, other ST pathogens were detected in low prevalence: *U. urealyticum* (6.45%) and *M. genitalium* (0%). Among the asymptomatic subjects, 51.7% were more likely to have had co-infection with other STI and notably, less than that were symptomatic subjects (47.4%) who were found to less likely have had co-infection with other STI as shown in Table 4.2. This does not support the hypothesis that all females with *M.genitalium* infection do not have other genital infections such as *T. vaginalis, C. trachomatis* infection irrespective of the manifestation of the infection.

The risk factors for *M. genitalium* infection are most likely the same for other Mycoplasma genital infection. High number of sexual partners (Oakeshott *et al*., 2010; Adesola *et al*., 2017), co-infection with *C. trachomatis* (Brosh-Nissimov *et al*., 2018) and *T. vaginalis*, inconsistent condom use (Ronda *et al*., 2018), previous STD treatment, abortion (Pereyre *et al*., 2017) and bacterial vaginosis (Oakeshott *et al*., 2010; Seña *et al*., 2018) were found to be risk factors for acquisition of genital mycoplasmas as shown in Table 4.3. The risk factor of co-infection with *C. trachomatis* identified in this study are consistent with those obtained from a recent study from New Orleans by Lillis *et al*. (2018) with reported increased odds of infection with *M. genitalium* by *C. trachomatis* and *T. vaginalis*; likewise another study by Ljubin-Sternak *et al*. (2017) who reported co-infection of *M. genitalium* with *C. trachomatis* among females in the Low-risk population from Croatia.

The other risk factors identified in this study also agreed with a Norway based study by Jensen *et al*. (2013). Interestingly in this study was the finding that asymptomatic state was 50% that of symptomatic state among Mycoplasma-infected subjects. This study correlates with a recent study by Fernández-Huerta *et al* (2019a) who reported *M. genitalium* prevalence among asymptomatic female individuals in Spain. Likewise, another recent study by Kaida *et al*. (2018) with reported high prevalence of asymptomatic *M. genitalium* infection which undermined the syndromic management approach among female adolescents and young adults in South Africa Asymptomatic continues to pose as a serious epidemiological challenge (Manhart *et al.*, 2007; McGowin and Anderson-Smits, 2011). This is indicative that not all infected individuals are symptomatic (Smieszek and White, 2016). This support a number of hypotheses: there is an association between variables (higher number of sexual partners) and *M. genitalium* among female subjects resident in Benin City, Nigeria irrespective of its manifestation and females are likely to have serious sequelae such as infertility from undetected mycoplasmal infection because of asymptomatic manifestation of the infection. In addition to the behavioural risk factors, the transmission dynamics may contribute to the acquisition of the infection.

Bacterial vaginosis is a polymicrobial clinical syndrome resulting from replacement of the normal *Lactobacillus spp* in the vagina with high number of anaerobic bacteria-*Prevotella spp* and *Mobiluncus spp, Gardnerella vaginalis* and fastidious or uncultured anaerobes (Taylor-Robinson, 2017). Bacterial vaginosis was found to be 46% in this study which is slightly similar with recent findings by Lokken *et al*., (2017) with reported 40.6% prevalence of Bacterial vaginosis and association with acquisition of *M. genitalium* among Kenyan women.

At the phylogenetic level, relatedness was established with bootstrap confidence value ≥70% as significant association (Hillis and Bull, 1993). This study shows the first phylogenetic

framework for *Mycoplasma genitalium*, isolated from Nigerian students including isolates from two continents which were reported from 1995 to 2008. The phylogenetic tree separated the understudied species into three main clades, of which Clade A comprised of the 2 genomes sequenced strains of G-37 (Glass *et al*., 2006; Fraser *et al*., 1995). An association of the isolates metadata to the phylogeny revealed no relationship between phylogenetic position and country of origin, in all clades containing isolates from two continents studied: Europe and Asia (Japan and Iran).

Phylogenetic study aids in tracing source of infection. *M. genitalium* was found not to share phylogenetic relationship with *M. genitalium* from other parts of the world. A significant association was found among *M. genitalium* genomes in Clade A and C. Whilst, Clade B comprising of isolated *M. genitalium* from the students had no phylogenetic linkage with other understudied genomes. This implies that the *M. genitalium* circulating in the Nigerian environment is relatively unique. Identification and characterization of *M. genitalium* phylogenetically aids in understanding its distribution and epidemiology.

Apart from *M.genitalium* which was found not to share phylogenetic relationship with other *M.genitalium* from parts of the world, *M. penetrans* and *M.hominis* were also found likewise. However, all *M.hominis* from the students shared a common ancestor; this applied to *U. parvum* and *U. urealyticum* which were however found to be strongly linked with two China strains, American and Canadian strains. This supports indiscriminate spread of sexually transmitted infection.

Following the phylogenetic analysis of all the genital mycoplasmas isolated in this study, all *M.hominis* shared a common ancestor; same for all Ureaplasmas except *U.urealyticum* MG279051. Furthermore, evolutionary relationship of other bacterial sexually transmitted pathogens in this study revealed a strong association (93% confidence value) among genital mycoplasmas and other bacterial sexually transmitted pathogens: *Chlamydia trachomatis,*

Treponema pallidum, Neisseria gonorrhoeae, Haemophilus ducreyi, Gardnerella vaginalis and *Mobiluncus spp.* This is an indication that infection with any genital mycoplasmas could promote infection by the above-listed bacterial sexually transmitted pathogens thus increasing susceptibility for such infections. Therefore, this suggests that Mycoplasma infected individuals are more likely to be infected by the above-listed bacterial sexually transmitted pathogens and *vice versa*. This is consistent with a study on concomitant infection of *M.genitalium, M. hominis, U. urealyticum* and *C.trachomatis* among women with genital tract infection (Saigal *et al*., 2016). In clear view, *Neisseria gonorrhoeae* and *Haemophilus ducreyi* were found to have a significant association (82% confidence value). This implies that a *Neisseria gonorrhoeae* infected individual is more likely to be co-infected by *Haemophilus ducreyi* and *vice versa* (Ison *et al*., 1998).

A better understanding of the disease sequelae is needed for treatment regime and control measures as genital mycoplasmas can persist for months or even years in infected individual (Vandepitte *et al*., 2013). Among the hospital patients with infertility cases (10 in number), *M. hominis* and *U. urealyticum* were found. This was consistent with the study of Sleha *et al.* (2016) who reported an association of *M. homins* and *U. urealyticum* among 88 infertile women. Their results can be comparable though *M. hominis* had higher recovery rate in our study. The difference in sample size could justify the variation in result despite the use of the same sample type (endocervical swab). Also, the result from the study is consistent with study by Baczynska *et al*., (2005) reporting that *M. hominis* can be an infectious cause of infertility. All *M. hominis* from both groups were found to be of a common ancestor and significantly associated to a China strain unlike *U. urealyticum* strains. Phylogenomics study established a significant association among *M. hominis* and *U. urealyticum* strains from both groups with 81% and 77% confidence value respectively. This justifies the fact that these genital mycoplasmas can result to serious sequelae when left undetected and untreated.

However, evidence for the association of genital mycoplasmas with pelvic inflammatory disease and consequently infertility was indicative that a significant proportion of upper tract inflammation maybe attributed to this pathogen (McGowin and Anderson-Smits, 2011). To this best of our knowledge, this is the first study from Nigeria to establish a genetic relatedness among *Mycoplasma species* isolated from the cervix of females in low risk groups (apparently healthy female students) and high risk group (hospital patients) in Nigeria to institute the disease sequelae associated with *Mycoplasma spp* among females.

5.1 Conclusion

This study revealed a relatively low prevalence (1%) of *M. genitalium* among the tertiary female students and 0.8% among the overall study population. *Mycoplasma genitalium*, *M. hominis, U. urealyticum* and *U. parvum* are exclusively human pathogens, largely transmissible through sexual contact; thus, all the mycoplasmas and ureaplasmas isolated in this present study are most probably of human origin acquired via the transmission chain of genital tract infection. The future of genital mycoplasmology lies with molecular- based technology. Based on our prevalence data, screening for *M. genitalium* among reproductive age (15 – 39 years) females in Benin City is crucial.

Multiplex PCR (M-PCR) can be a valuable tool to evaluate the real epidemiology of cervical colonization by pathogens to ascertain co-existence of *M. genitalium* with genital pathogens such as *M.hominis, Chlamydia trachomatis, Trichomonas vaginalis, Ureaplasma parvum* and *U. urealyticum*. Simultaneous detection of genital pathogens should be performed even in population and hospital based studies. *M. genitalium* was not detected using M-PCR, however; detection of *C.trachomatis, M. hominis* and *U. urealyticum*, either alone or together with other pathogens is an important finding, particularly in females.

The risk factors for *M. genitalium* infection are similar to those typical to other sexually transmitted infections. The study revealed high number of sexual partners and co-infection with sexually transmitted pathogens such as *C. trachomatis* and *T.vaginalis* as possible risk factors for acquisition of *Mycoplasma genitalium* among the female subjects. Effective prevention measures for *M. genitalium* infection for reproductive age (15 – 39 years) femlaes should focus on these risk factors.

High rate of prevalent Bacterial vaginosis among the subjects with isolation of *M. genitalium*; suggests the effect of *M. genitalium* screening on female reproductive health outcomes.

The phylogenetic relationship of the isolated *M. genitalium* among the female subjects with other previously reported *M. genitalium* revealed the nature and diversity of isolated *M. genitalium* (MG238565) with no shared phylogenetic linkages with others previously reported. The generated sequence data is essential for developing approaches to trace M. genitalium infection which will better elucidate the role of this organism as a sexually transmitted pathogen.

Genetic relationship between *M. genitalium* and other clinically important bacterial sexually transmitted (ST) pathogens revealed an association as co-factors for other bacterial sexually transmitted pathogens such as *C. trachomatis, Treponema pallidum* and *Neisseria gonorrhoeae*. Routine screening for these pathogens is warranted to prevent and formulate treatment guidelines.

The genetic relatedness of *Mycoplasma spp* from female subjects (students and hospital patients with disease sequelae such as infertility) demonstrated possible association with infertility. The study shows an association of genital mycoplasmas and infertility thus suggests routine screening of this pathogen in patients with infertility as well as to prevent disease sequelae such as infertility. It is therefore paramount to monitor the spread of

circulating species in a general population (low-risk group) not neglecting the hospital population (high-risk group) for better containment of infection.

5.2 Recommendation

Following the findings from the study, the recommendations are thus;

1. Multiplex PCR as a standard diagnostic tool for STI is recommended.
2. The use of highly sensitive and specific molecular diagnostic test is recommended for screening mycoplasmas.
3. Multiplex PCR as a standard diagnostic tool for STI is recommended.
4. Screening exercise of asymptomatic females for *M. genitalum* infection. This could control the infection thereby improving female reproductive health and limiting spread and complications of *M. genitalium* infection within the population.
5. Sex education on the prevention of genital transmissible infections particularly *M.genitalium* infection in a tertiary student population should implemented periodically considering asymptomatic state.
6. Sexually Transmitted Infections (STI) patient management protocol in the country should include strict implementation of treatment for patients' sexual partner(s).
7. Further studies are recommended on longitudinal and quantitative mycoplasma research to:
    i. Reveal more new *M. genitalium* strains among the student population.
    ii. Justify the association of new and higher sexual partners with colonization of the organism.
    iii. Study the sequence variability of the whole 16SrRNA of *M. genitalium* and the likelihood of developing new molecular- based techniques.

iv. Define the prospective influence of *M. genitalium* load on the treatment outcome and presentation of symptoms.

5.3 Contribution to Knowledge

The molecular characterization with phylogenetic framework provided an insight to the nature and diversity of *Mycoplasma genitalium* isolated from female subjects in Nigeria. One unique circulating specie have been identified. New data to the existing data on *Mycoplasma genitalium* infection among females of reproductive age (15 – 39 years) have emerged.

Established phylogenetic linkage between genital mycoplasmas in a general population (student population) and subjects with disease sequelae of mycoplasma infection in a hospital setting.

References

Aaltone, R., Jalava, J., Laurikanen, E., Karkainen, U., Alanen, A. (2002) Cervical *Ureaplasma urealyticum* colonization: comparison of PCR and culture for its detection and association with preterm birth. *Scandinavian Journal of Infectious Diseases* 34: 35-40.

Adebamowo, S.N., Ma, B., Zella, D., Famooto, A., Ravel, J., Adebamowo, C., ACCME Research Group (2017) *Mycoplasma hominis* and *Mycoplasma genitalium* in the vaginal microbiota and persistent high-risk human papillomavirus infection. *Frontiers in Public Health* 5:140-145.

Adelbert, J.B., Simpson, T.T., Chamberlain, W.A. (2008) Chlamydia prevalence among college students: reproductive and public health implications. *Sexually Transmitted Disease* 35(6):529-532.

Adesola, A.T., Oluwasola, T.A.O., Ajani, M.A., Bakare, R.A. (2017) The prevalence of and risk factors for *Mycoplasma genitalium* infection among infertile women in Ibadan: A cross-sectional study. *International Journal of Reproductive BioMedicine* 15(10):613-618.

Adogo, L.Y., Oyewole, E.A., Anyanwu, N.C.J., Omebijie, P.E. (2016) Prevalence and correlates of *Gardnerella vaginalis* and *Trichomonas vaginalis* among female students in Bingham University, Kano, Nigeria. *International Journal of Tropical Disease and Health* 20(3):1-8.

Alfarraj, D.A., Somily, A.M. (2017) Isolation of *Mycoplasma genitalium* from endocervical swabs of infertile women. *Saudi Medical Journal* 38(5):549-552.

Agbakoba, N.R, Adetosoye, A.I and Adewole, I.F. (2006) Biochemical and serological characterization of Mycoplasma strains isolated from the genital tracts of humans in Nigeria. *African Journal of Medicine and Medical Sciences* 35: 125-129.

Agbakoba, N.R., Adetosoye, A.I., Adewole, I.F. (2007) Presence of mycoplasma and ureaplasma species in the vagina of women of reproductive age. *West Africa Journal of Medicine*, 26(1): 28-31.

Akgul, A., Kadioglu, A., Koksal, M.O., Ozmez, A., Agacfidan, A. (2018) Sexually transmitted agents and their association with leucocytospermia in infertility clinic patients. *Andrologia* 50(10):e13127.

Akinbo, F.O. and Oronsaye I.S. (2017) *Trichomonas vaginalis* infection among adolescent girls in some secondary schools in Benin city, Edo State, Nigeria. *African Journal of Clinical and Experimental Microbiology* 18(4):23-28.

Alfarraj, D.A. and Somily, A.M. (2017) Isolation of *Mycoplasma genitalium* from endocervical swabs of infertile women. *Saudi Medical Journal* 38(5):549-552.

Amirmozafari, N., Mirnejad, R., Kazemi, B., Sariri, E., Bojari, M.R., Darkahi, F.D. (2009) Simultaneous detection of genital *Mycoplasma* in women with genital infections by PCR. *Journal of Biological Science 9*: 804-809.

Amsel, R., Totten, P.A., Spiegel, C.A. (1983) Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *American Journal of Medicine* 74:14-22.

Anagrius, C., Lore, B., Jensen, J.S. (2005) *Mycoplasma genitalium*: prevalence, clinical significance, and transmission. *Sexually Transmitted Infections* 81: 458-462.

Anagrius, C., Lore, B., Jensen, J.S. (2013) Treatment of *Mycoplasma genitalium*. Observations from a Swedish STD Clinic. *PLoS ONE* 8:e61481.

Andersen, B., Sokolowski, I., Ostergaard, L., Kjolseth –Moller, J., Olesen, F., Jensen, J.S. (2007) *Mycoplasma genitalium*: prevalence and behavioural risk factors in the general population. *Sexually Transmitted Infections* 83: 237-241.

Aparicio, D., Torres-Puig, S., Ratera, M., Querol, E., Piñol, J., Pich, O.Q., Fita, I. (2018) *Mycoplasma genitalium* adhesin P110 binds sialic-acid human receptors. *Nature Communications* 26: 9(1):4471.

Arinze, A.U., Onyebuchi, N.V., Isreal, J. (2014) Genital *Chlamydia trachomatis* infection among female undergraduate students of University of Port Harcourt, Nigeria. *Nigerian Medical Journal* 55(1):9-13.

Baczynska, A., Svenstruo, F.H., Fedder, J. (2005) The use of enzyme-linked immunosorbent assay for detection of *Mycoplasma hominis* antibiotics in infertile women serum samples. *Human Reproduction* 20:1277-1285.

Balkus, J.E., Manhart, L.E., Jensen, J.S., Anzala, O., Kimani, J., Schwebke, J., Shafi, J, Rivers, C., Kabare, E., McClelland, R.S. (2018) *Mycoplasma genitalium* Infection in Kenyan and US Women. *Sexually Transmitted Diseases* 45(8):514-521.

Bansal, A. K. and Meyer, T.E. (2002) Evolutionary analysis by whole genome comparisons. *Journal of. Bacteriology* 184:2260–2272.

Barradas, D., Jones, C., Carr, J.H. (1992) Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. *Journal of Clinical Microbiology* 30:265–274.

Bebear, C.M., Renaudin, J., Charron, A., Renaudin, H., de Barbeyrac, B., Schaeverbeke, T. (1999) Mutations in the gyrA, parC, and parE genes associated with fluoroquinolone resistance in clinical isolates of *Mycoplasma hominis*. *Antimicrobial Agents and Chemotherapy* 43:954–956.

Bebear, C.M., Renaudin, H., Charron, A., Grusson, D., Legrancois, M, Bebear, C. (2000a) In vitro activity of trovafloxacin compared to those of five antimicrobials against mycoplasmas including *Mycoplasma hominis* and U*reaplasma urealyticum* fluoroquinolone-resistant isolates that have been genetically characterized. *Antimicrobial Agents and Chemotherapy* 44: 2557 - 2560.

Bebear, C.M. Renaudin, H., Bryskier, A and Bebear, C. (2000b) Comparative Activities of Telithromycin (HMR 3647), Levofloxacin, and other Antimicrobial Agents against Human Mycoplasmas. *Antimicrobial Agents and Chemotherapy* 44.7: 1980-1982.

Beersma, M.F., Dirven, K., van Dam, A.P., Templeton, K.E., Claas, E.C., Goossens, H. (2005) Evaluation of 12 commercial tests and the complement fixation test for *Mycoplasma pneumoniae*-specific immunoglobulin G (IgG) and IgM antibodies, with PCR used as the "gold standard." *Journal of Clinical Microbiology* 3:2277–2285.

Bissessor, M., Tabrizi, S.N., Twin, J. (2015) Macrolide resistance and azithromycin failure in a *Mycoplasma genitalium*-infected cohort and response of azithromycin failures to alternative antibiotic regimens. *Clinical Infectious Diseases* 60(8):1228–1236.

Bjartling, C., Osser, S., Perssen, K. (2012) *Mycoplasma genitalium* in cervicitis and Pelvic inflammatory disease among women at gynecologic outpatient service. *American Journal of Obstetrics and Gynecology* 206 (6): 476.

Björnelius, E., Anagrius, C., Bojs, G., Carlberg, H., Johannisson, G., Johansson, E. (2008) Antibiotic treatment of symptomatic *Mycoplasma genitalium* infection in Scandinavia: a controlled clinical trial. *Sexually Transmitted Infections* 84:72–76.

Blanchard, A., Hentschel, J., Duffy, L., Baldus, K., Cassell, G.H. (1993) Detection of *Ureaplasma urealyticum* by polymerase chain reaction in the urogenital tract of adults, in amniotic fluid, and in the respiratory tract of newborns. *Clinical Infectious Diseases* 17(Suppl 1):S148 –S153.

Bradshaw, C.S., Horner, P.J., Jensen, J.S., White, P.J. (2018) Syndromic management of STIs and the threat of untreatable Mycoplasma genitalium. *Lancet Infectious Diseases* 18(3):251-252.

Bradshaw, C.S., Chen M.Y., Fairley C.K. (2008) Persistence of *Mycoplasma genitalium* following azithromycin therapy. *PLoS One* 3(11):e3618.

Bradshaw, C.S., Jensen, J.S., Tabrizi, S.N., Read, T.R., Garland, S.M., Hopkins, C.A. (2006) Azithromycin failure in *Mycoplasma genitalium* urethritis. *Emerging Infectious Diseases* 41:1357–1359.

Brooks, J.P., Edwards, D.J., Harwich, M.D., Rivera, M.C., Fettweis, J.M., Serrano, M.G. (2015) The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiology* 15:66.

Brooks, G.F., Butel, J.S., Carroll, K.C., Morse, S.A., Mietzner, T.A. (2010) Mycoplasmas (mollicutes) and cell-wall defective bacteria. *Medical Microbiology* 25th Edition. Eds. Jawetz, Melnicks and Adelbergs. McGraw-Hill Lange, Connecticut. Pp 313-317.

Brosh-Nissimov, T., Kedem, R., Ophir, N., Shental, O., Keller, N., Amit, S. (2018) Management of sexually transmissible infections in the era of multiplexed molecular diagnostics: a primary care survey. *Sexual Health* 15(4):298-303.

Brotman, R.M., Klebanoff, M.A., Nansel, T.R. (2010) Bacterial vaginosis assessed by gram stain and diminished colonization resistance to incident gonococcal, chlamydial and trichomonal genital infection. *Journal of Infectious Disease* 202:1907-1915.

Brouqui, P., Raoult D. (2001) Endocarditis due to rare and fastidious bacteria. *Clinical Microbiology Review* 14:177–207.

Brown, M. B., Peltier, M., Hillier, M., Crenshaw, B., Reyes, L. (2001) Genital mycoplasmosis in rats: A model for intrauterine infection. *American Journal of Reproductive Immunology* 46: 232 - 237.

Buder, S., Schöfer, H., Meyer, T., Bremer, V., Kohl, P.K., Skaletz-Rorowski, A., Brockmeyer, N. (2019) Bacterial sexually transmitted infections. *Journal der Deutschen Dermatologischen Gesellschaft* 17(3):287-315.

Burazin, J., Bosnjak, Z., Peric, M., Bilic-Kirin, V., Buljan, V. (2017) *Chlamydia trachomatis* urogenital infection and associated risk factors among university students in Croatia. *Central European Journal of Paedetrics* 13(2):130-141.

Carey, A.Y. and Beagly, K.W. (2010) *Chlamydia trachomatis*: a hidden epidemic: effects on female reproduction and options for treatment. *American Journal of Reproductive Medicine* 63(6):360-363.

Casillas-Vega, N., Morfín-Otero, R., García, S., Llaca-Díaz, J., Rodríguez-Noriega, E., Camacho-Ortiz, A., Ayala-Castellanos, L., Mendoza-Olazarán, S., Flores-Treviño, S., Petersen-Morfín, S., Maldonado-Garza, H.J., Bosques-Padilla, F.J., Garza-González, E. (2016) Sexually transmitted pathogens, coinfections and risk factors in patients attending obstetrics and gynecology clinics in Jalisco, Mexico. *Salud Publica de Mexico* 58(4):437-45.

Cedillo-Ramirez, L., Gil, C., Zago, I., Yanez, A., Giono, S. (2000) Association of *Mycoplasma hominis* and *Ureaplasma urealyticum* with some indicators of nonspecific vaginitis. *Review Latino America Microbiology* 42(1): 1 – 6.

Celard, M., Gevigney, G., Mosnier, S., Buttard, P., Benito, Y., Etienne, J., Vandenesch, F. (1999) Polymerase chain reaction analysis for diagnosis of *Tropheryma whippelii* infective endocarditis in two patients with no previous evidence of Whipple's disease. *Clinical Infectious Diseases* 29:1348–1349.

Center for Disease Control and Prevention (2003) Sexually Transmitted Disease Surveillance, Atlanta Ga; US Department of Health and Human services.

Cheesbrough, M. (2000) Microbiological tests. *District Laboratory Practice in Tropical Countries* Part 2. Cambridge University Press. Pp 23.

Cho, C.H., Lee, D.S., Lee, S., Hungs, S., Park, D.C., Lee, M., Kim, T., Cho, Y. (2013) Performance of Anylex TM 11 multiplex real time PCR for the diagnosis of seven sexually transmitted infections: comparison with currently available methods. *International Journal of Infectious Diseases* 17:e1134-e1140.

Chra, P., Papaparaskevas, J., Papadogeorgaki, E., Panos, G., Leontsinidis, M., Arsenis, G., Tsakris, A. (2018) Prevalence of *Mycoplasma genitalium* and other sexually-transmitted pathogens among high-risk individuals in Greece. *Germs* 8(1):12-20.

Christofolini, D.M., Leuzzi, L., Mafra, F.A., Rodat, I., Kayaki, E.A., Bianeo, B., Barbosa, C.P. (2012) Prevalence of cases of *Mycoplasma hominis, M.genitalium, Ureaplasma urealyticum* and *Chlamydia trachomatis* in women with no gynecologic complaints. *Reproductive Medical Biology* 11:201-205.

Chukwuka, C.P., Agbakoba, N.R., Emele, F.E., Oguejiofor, C., Akujobi, C.N., Ezeagwuna, D.A, Onwunzo, M.C. (2013). Prevalence of genital mycoplasmas in the vaginal tracts of adolescents in Nnewi, South Eastern, Nigeria. *World Journal of Medical Sciences* 9(4): 248-253.

Clausen, H.F., Fedder, J., Drasbek, M. (2001) Serological investigation of *Mycoplasma genitalium* in infertile women. *Human Reproduction* 16: 1866-1874.

Cohen, C.R., Manhart, L.E., Bukusi, E.A. (2002) Association between *Mycoplasma genitalium* and acute endometritis. *Lancet* 359:765-766.

Columbia Encyclopedia (2001) Polymerase chain reaction. Columbia Encyclopedia 6th edition. Retrieved 5th March 2015 from http://www.bartley.com/65/po/polychn.html

Cook, V. J., Turenne, C.Y., Wolfe, J., Pauls, R., and Kabani, A. (2003) Conventional methods versus 16S ribosomal DNA sequencing for identification of non-tuberculous mycobacteria: cost analysis. *Journal of Clinical Microbiology* 41:1010–1015.

Couldwell, D.L., Tagg, K.A., Jeoffreys, N.J., Gilbert, G.L. (2013) Failure of moxifloxacin treatment in *Mycoplasma genitalium* infections due to macrolide and fluoroquinolone resistance. *International Journal of STD and AIDS* 24:822–828.

Cox, C., Saxena, N., Watt, A. P., Gannon, C., McKenna, J. P., Fairley, D. J. (2016) The common vaginal commensal bacterium *Ureaplasma parvum* is associated with chorioamnionitis in extreme preterm labor. *Journal of Maternal and Fetal Neonatal Medicine* 29: 3646–3651.

Cristiane, C.T. and Fabiano, L.T. (2011) Towards a genome based taxonomy of Mycoplasmas. *Infection, Genetics and Evolution* 11(7):1798-1804.

Crouse, D.T, English, B.K, Livingston, L., Meals, E.A. (1998) Genital mycoplasmas stimulate tumor necrosis factor – alpha and inducible nitric oxide synthase production from a murine macrophage cell line. *Pediatric Research* 44: 785-790.

Csango, P.A., Pedersen, J.E., Hess, R.D. (2004) Comparison of four *Mycoplasma pneumoniae* IgM, IgG- and IgA-specific enzyme immunoassays in blood donors and patients. *Clinical Microbiology Infections* 10:1094–1098.

Cumitech 34 (2001) Laboratory Diagnosis of Mycoplasma Infections. Edited by Waites K.B., Bebear, C.M, Robertson, J.A., Talkington, D.F., Kenny, G.E. Washington, DC, American Society for Microbiology.

Daxboeck, F., Krause, R., Wenisch, C. (2003) Laboratory diagnosis of *Mycoplasma pneumoniae* infection. *Clinical Microbiology Infection* 9:263–273.

De Barbeyrac, B.C., Berner-Poggi, F.F., Renaudin, H., Dupon, M. and Bebear, C. (1993) Detection of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* in clinical samples by polymerase chain reaction. *Clinical Infectious Disease* 17(Suppl.1): 83-89.

Degrange, S., Renaudin, H., Charron, A., Bebear, C., Bebear, C.N. (2006) Epidemiology and molecular mechanism of tetracycline resistance in *Mycoplasma hominis* and *Ureaplasma* spp.

clinical isolates at Bordeaux, France. Paper presented at the *16th International Organization for Mycoplasmology Congress*, Cambridge, United Kingdom 9th – 14th July.

Deguchi T. and Maeda S. (2002) *Mycoplasma genitalium*: another important pathogen of nongonococcal urethritis. *Journal of Urology* 167:1210-1217.

Deguchi T., Yoshida T., Yokoi S., Ito M., Tamaki M., Ishiko H., Maeda S. (2002) Longitudinal quantitative detection by real-time PCR of *Mycoplasma genitalium* in first-pass urine of men with recurrent nongonococcal urethritis. *Journal of Clinical Microbiology* 40:3854–3856.

Deguchi, T., Maeda, S., Tamaki, M., Yoshida, T., Ishiko, H., Ito, M., (2001) Analysis of the gyrA and parC genes of Mycoplasma genitalium detected in first-pass urine of men with non-gonococcal urethritis before and after fluoroquinolone treatment. *Journal of Antimicrobials and Chemotheraphy.*48:742–744.

De Vries, H.J. (2019) Current challenges in the clinical management of sexually transmitted infections. *Journal of the International AIDS Society.* Suppl 6:e25347.

Dewhirst, F. E., Paster, B.J., Tzellas, N., Coleman, B., Downes, J., Spratt, D.A., Wade, W.G. (2001) Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the family *Coriobacteriaceae:* description of *Olsenella* gen., reclassification of *Lactobacillus uli* as *Olsenella uli* comb. Description of *Olsenella profusa* sp. *International Journal of Systemic. Bacteriology*. 51:1797–1804.

Domingues, D., Tavira, L.T, Duarte, A., Sanca, A., Prieto, E., Exposto, F. (2002) *Ureaplasma urealyticum* biovar determination in women attending a family planning clinic in Guinea-Bissau, using polymerase chain reaction of the multiple-banded antigen. *Journal Clinical Laboratory Analysis* 16 (2):71 – 75.

Domingues, D., Tavora-Tavira L., Duarte, A., Sanca, A., Prieto, E. and Exposto, F. (2003) Genital mycoplasmas in women attending a family planning clinic in Guinea-Bissau and their susceptibility to antimicrobial agents. *Acta Tropicals*. 86(1):19-24.

Donders, G.G., Van-Bulk, B, Candron, J., Londers, L., Verecten, A. and Spitz, B. (2000) Relationship of bacterial Vaginosis and mycoplasmas to the risk of spontaneous abortion. *American Journal of Obstetrics and Gynaecology* 183:431-437.

Dorigo-Zetsma J.W., Zaat S.A., Wertheim-van Dillen P.M., Spanjaard L., Rijntjes J., van Waveren G., Jensen J.S., Angulo A.F., Dankert J. (1999) Comparison of PCR, culture, and serological tests for diagnosis of *Mycoplasma pneumoniae* respiratory tract infection in children. *Journal of Clinical Microbiology*, 37:14–17.

Duffy, L.B., Crabb, D., Scarcey, K., Kempf, M.C. (2000) Comparative potency of gemifloxacin, new quinolones, macrolides, tetracycline and clinidamycin against Mycoplasma spp. *Journal of Antimicrobial Chemotherapy* 45(supplement 1): 29-33.

Dupin, N., Bijaoui, G., Schwarzinger, M., Ernault, P., Gerhardt, P., Jdid, R., Hilab, S., Pantoja, C., Buffet, M., Escande, J.P., Costa, J.M. (2003) Detection and quantification of *Mycoplasma genitalium* in male patients with urethritis. *Clinical Infectious Diseases* 37:602–605.

Eastick, K., Leeming, J.P., Caul, E.O., Horner, P.J., Millar, M.R. (2003) A novel polymerase chain reaction assay to detect *Mycoplasma genitalium*. *Molecular Pathology* 56:25–28.

Edouard, S., Tissot-Dupont, H., Dubourg, G., Bernard, A., Fournier, P.E., Ravaux, I., Stein, A., Raoult, D. (2017) *Mycoplasma genitalium*, an agent of reemerging sexually transmitted infections. *Acta Pathologica Microbiologica et Immunologica Scandinavica* 125(10):916-920.

Ekiel, A., Jozwiak, J., Martirosian, G. (2009) *Mycoplasma genitalium*: a significant urogenital pathogen? *Medical Science Monitor* 15: RA102-106.

Ella, E.F., Shenga, H., Ajoge, H.O. (2013) Prevalence of Chlamydia among female students attending a selected university health services in Zaria, Kaduna State, Nigeria. *The International Journal of England and Science* 2(11):54-57.

Esen, B., Gozalan, A., Sevindi, D.F., Demirbas, A., Onde, U., Erkayran, U., Karakoc, A.E., Hasçiçek, A.M., Ergün, Y., Adiloglu, A.K. (2017) Ureaplasma urealyticum: Presence among Sexually Transmitted Diseases. *Japanese Journal of Infectious Diseases* 70(1):75-79.

Falk, L., Fredlend, H., Jensen, J.S. (2005) Signs and symptoms of urethritis and cervicitis among women with or without *Mycoplasma genitalium* or *Chlamydia trachomatis. Sexually Transmitted Infections* 81:73-78.

Falk, L., Fredlund, H., Jensen, J.S. (2003) Tetracycline treatment does not eradicate *Mycoplasma genitalium*. *Sexually Transmitted Infections* 79(4):318–319.

Felsenstein, J. (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *Molecular Evolution* 17:368–376.

Felsenstein, J. (1989) PHYLIP—phylogeny inference package (version 3.2). *Cladistics* 5:164–166.

Fernández-Huerta, M., Barberá, M.J., Esperalba, J., Fernandez-Naval, C., Vall-Mayans, M., Arando, M., Serra-Pladevall, J., Broto, C., Zarzuela, F., Rando, A., Pumarola, T., Espasa, M. (2019a) Prevalence of *Mycoplasma genitalium* and macrolide resistance among asymptomatic people visiting a point of care service for rapid STI screening: a cross-sectional study. *Sexually Transmitted Infections*. doi: 10.1136/sextrans-2019-054124. [Epub ahead of print].

Fernández-Huerta, M. and Espasa, M. (2019b) *Mycoplasma genitalium* co-infection with *Chlamydia trachomatis* and *Neisseria gonorrhoeae* among asymptomatic patients: the silent wick for macrolide resistance spread. *Sexually Transmitted Infections*. 95(5):391.

Fernández-Huerta, M., Serra-Pladevall, J., Barberá, M.J., Espasa, M. (2019c) *Mycoplasma genitalium* and antibiotic resistance in Spain; the need for an effective response against an emerging problem. *Enfermedades Infecciosas y Microbiología Clínica* 37(2):144-145.

Fethers, K.A., Fairley, C.K., Mortona, A. (2009) Early sexual experiences and risk factors for bacterial vaginosis. *Journal of Infectious Disease* 200:1662-1670.

Fettweis, J.M., Serrano, M.G. Huang, B., Brooks, J.P., Glascock, A.L. (2014) An emerging mycoplasma associated with Trichomonas, vaginal infection and disease. *PLoS One* 9:e110943.

Fichorova, R., Fraga, J., Rappelli, P., Fiori, P.L (2017) The presence of *Trichomonas vaginalis* in the vagina and its symbiosis with Trichomonas virus and Mycoplasma. *Research in Microbiology* 3(5):1-10.

Fookes, M.C., Hadfield, J., Harris, S., Parmar, S., Unemo, M., Jensen, J.S., Thomson, N.R. (2017) Mycoplasma genitalium: whole genome sequence analysis, recombination and population structure. *BMC Genomics* 18(1):993.

Fournier, P. E., Dumler, J.S., Greu,b G., Zhang, J., Wu, Y., Raoul, T. D. (2003) Gene sequence-based criteria for identification of new *Rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. nov. *Journal of Clinical Microbiology* 41:5456–5465.

Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., Bult, C. J., Kerlavage, A. R., Sutton, G. (1995) The minimal gene complement of *Mycoplasma genitalium. Science* 270: 397–403.

Funke, G., Graevenitz A.V., Clarridge III J.E., Bernard K. (1997) Clinical microbiology of coryneform organisms. *Clinical Microbiology Review* 10:125–159.

Gambini, D., Decleva, I., Lupica, L., Ghislanzoni, M., Cusini, M., Alessi, E. (2000) *Mycoplasma genitalium* in males with nongonococcal urethritis: prevalence and clinical efficacy of eradication. *Sexually Transmitted Dis*ease 27(4):226–229.

Garcia, L.S. (2007) Diagnostic Medical Microbiology. *American Society for Microbiology* 13: 471-475.

Garrity, G. M.A and Holt, J.G. (2001). The road map to the manual, *In* G. M. Garrity (ed), Bergey's manual of systematic bacteriology. Springer-Verlag, New York, N.Y. p.119–166.

Gaydos, C.A., Manhart, L.E., Taylor, S.N., Lillis, R.A., Hook, E.W. 3rd, Klausner, J.D., Remillard, C.V., Love, M., McKinney, B., Getman, D.K., AMES Clinical Study Group (2019) Molecular testing for *Mycoplasma genitalium* in the United States; results from the AMES prospective multi-center clinical study. *Journal of Clinical Microbiology* doi: 10.1128/JCM.01125-19. [Epub ahead of print]

Gesink, D.C., Mulvad, G., Montgomery-Anderson, R., Binzer, A., Vernich, L., Frosst, G., Stenz, F., Rink, E., Olsen, O.R., Koch, A., Jensen, S.J. (2012) *International Journal of Circumpolar Health* 71(1): 221-226.

Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M. (2006). Essential genes of a minimal bacterium. *Proceedings of the National Academy of Sciences of the United States of America* 103:425-430.

Gimenes, F., Medina, F.S., Abreu, A.L.P., Irie, M.M.T., Esquic¸ati, I.B., Malagutti, I. N., Vasconcellos, V.R.B., Discacciati, M.G., Bonini, M.G., Maria-Engler, S.S., Consolaro, M.E.L. (2014) Sensitive Simultaneous Detection of Seven Sexually Transmitted Agents in

Semen by Multiplex-PCR and of HPV by Single PCR. *PLoS ONE* 9(6): e98862. doi:10.1371/journal.pone.0098862

Gruson, D., Pereyre, S., Renaudin, H., Charron, A., Bebear, C., Bebear, C.M. (2005) In vitro development of resistance to six and four fluoroquinolones in *Mycoplasma pneumoniae* and *Mycoplasma hominis*, respectively. *Antimicrobial Agents and Chemotherapy* 49:1190–1193.

Grzesko, J., Elias, M., Maczynska, B., Kasprzykowska, U., Tlaczala, M., Goluda M. (2009) Occurrence of *Mycoplasma genitalium* in fertile and infertile women. *Fertil Sterility* 91(6):2376-2380.

Gundevia, Z., Foster, R., Jamil, M.S., McNulty, A. (2015) Positivity at test of cure following first line treatment for genital *Mycoplasma genitalium*: follow-up of a clinical cohort. *Sexually Transmitted Infections* 91:11–13.

Hagiwara, N., Yasuda, M., Maeda, S., Deguchi, T. (2011) In vitro activity of azithromycin against *Mycoplasma genitalium* and its efficacy in the treatment of male *Mycoplasma genitalium*-positive nongonococcal urethritis. *Journal of Infections and Chemotherapy* 17:821–824.

Hall, L., Doerr, K.A., Wohlfiel, L.S., Roberts G.D. (2003) Evaluation of the MicroSeq system for Identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. *Journal of Clinical. Microbiol*ogy 41:1447–1453.

Hamasuna, R., Le, P.T., Kutsuna, S., Furubayashi, K., Matsumoto, M., Ohmagari, N., Fujimoto, N., Matsumoto, T, Jensen, J.S. (2018) Mutations in ParC and GyrA of moxifloxacin-resistant and susceptible *Mycoplasma genitalium* strains. *PLoS One* 13(6):e0198355.

Hamasuna, R., Jensen, J.S., Osada, Y. (2009) Antimicrobial susceptibilities of *Mycoplasma genitalium* strains examined by broth dilution and quantitative PCR. *Antimicrobial Agents and Chemother*apy 53(11):4938–4939.

Hamasuna, R., Imai, H., Tsukino, H., Jensen, J.S., Osada Y. (2008) Prevalence of *Mycoplasma genitalium* among female students in vocational schools in Japan. *Sexually Transmitted Infections 84*: 303-305.

Hamasuna, R., Takahashi, S., Kiyota, H., Yasuda, M., Hayami, H., Arakawa, S., Hardick, J., Giles, J., Hardick, A., Hsieh, Y.H., Quinn, T., Gaydos C. (2006) Performance of the gen-probe transcription-mediated [corrected] amplification research assay compared to that of a multi target real-time PCR for *Mycoplasma genitalium* detection. *Journal of Clinical Microbiology*, 44:1236–1240.

Hannan, P.C. and Woodnutt, G. (2000) In vitro activity of gemifloxacin against human mycoplasmas. *Journal of Antimicrobial Chemotherapy* 45: 367-369.

Harmsen, D. and Karch, H. (2004) 16S rDNA for diagnosing pathogens: a living tree. American Society for Microbiology News 70:19–24.

Hashimoto, O. and Deguchi, T. (2006) Quantitative detection and phylogeny-based identification of Mycoplasmas and Ureaplasmas from human immunodeficiency virus type 1-positive patients. *Journal of Infection and Chemotherapy* 12(1):25-30.

Hay, B., Dubbink, J.H., Ouburg, S., Roy, C., Pereyre, S., van der Eem, L., Morré, S.A., Bébéar, C., Remco, P.H.P. (2015) Prevalence and Macrolide Resistance of *Mycoplasma genitalium* in South African Women. *Sexually Transmitted Diseases* 42(3): 140-142.

Hillis, D. and Bull, J. (1993) An empirical test of bootstrap as a method for assessing confidence in phylogenetic analysis. *System Biology* 42:182-192.

Hjorth, S. V., Bjo¨ rnelius, E., Lidbrink, P., Falk, L., Dohn, B., Berthelsen, L., Ma, L., Martin, D. H., Jensen, J. S. (2006) Sequence-based typing of *Mycoplasma genitalium* reveals sexual transmission. *Journal of Clinical Microbiology* 44: 2078–2083.

Hoffman, R. W., O'Sullivan, F. X., Schafermeyer, K. R., Moore, T. L., Roussell, D., Watson-McKown, R., Kim, M. F., Wise, K. S. (1997) Mycoplasma infection and rheumatoid arthritis:analysis of their relationship using immunoblotting and an ultrasensitive polymerase chain reaction detection method. *Arthritis & Rheumatology* 40:1219–1228.

Hook, E.W. III, Golden, M., Jamieson, B.D., Dixon, P.B., Harbison, H.S., Lowens, S. (2015) A phase 2 trial of oral solithromycin 1200 mg or 1000 mg as single-dose oral therapy for uncomplicated gonorrhea. *Clinical Infectious Diseases* Epub ahead of print

Horn, M., and Wagner, M. (2001) Evidence for additional genus-level diversity of *Chlamydiales* in the environment. *FEMS Microbiology Letter*s. 204:71–74.

Horner, P., Blee, K., Adams, and E. (2014) Time to manage *Mycoplasma genitalium* as an STI: but not with azithromycin 1 g. *Current Opinion in Infectious Disease*s. 27(1): 68–74.

Horner, P., Thomas, B., Gilroy, C.B., Egger, M., Taylor-Robinson, D. (2001) Role of *Mycoplasma genitalium* and *Ureaplasma urealyticum* in Acute and Chronic Non-gonococcal Urethritis. *Clinical Infectious Diseases* 32: 995-1003.

Hughes, G. and Saunders, J. (2018) *Mycoplasma genitalium*: the next sexually transmitted superbug? *British Medical Journal* 29(363): k4376.

Hu, P.C., Schaper, U., Collier, A.M., Clyde, W.A. Jr, Horikawa, M., Huang, Y.S., Barile, M.F. (1987) A *Mycoplasma genitalium* protein resembling and the *Mycoplasma pneumoniae* attachment protein. *Infections and Immunology* 55:1126–1131

Huppert, J.S., Mortensen, J.E., Reed, J.L., Kahn, J.A., Rich, K.D., Hobbs, M.M. (2008) *Mycoplasma genitalium* detected by transcription-mediated amplification is associated with *Chlamydia trachomatis* in adolescent women. *Sexually Transmitted Diseases* 35:250–254.

Ikene, E.C., Ezegwui, H.U., Ikeako, L.C., Agbata, I., Agbata, E. (2011) Seroprevalence of *Chlamydia trachomatis* in Enugu, Nigeria. *Nigerian Journal of Clinical Practice* 14:176-180.

Imai, H., Nakao, H., Shinohara, H., Fujii, Y., Tsukino, H., Hamasuna, R., Osada, Y., Fukushina, K., Inamori, M., Ikenoue, T., Katoh, T. (2010) Population-based study of asymptomatic infection with *Chlamydia trachomatis* female and male students. *International Journal of STD and AIDS* 123:23-32.

Ioannidis, A., Papaioannou, P., Magioikinis, E., Magani, M., Ioannidou, V., Tzanetou, K., Burriel, A.R., Tsironi, M., Chatzipanagoiotou, S. (2017) Detecting the diversity of Mycoplasma and Ureaplasma Endosymbionts hosted by *Trichomonas vaginalis* isolates. *Frontiers in Microbiology* 23:123.

Irwin, K.L., Moorman, A.C., O'Sullivan, M.J., Sperling, R., Koestler, M.E., Soto, I. (2000) Influence of human immunodeficiency virus infection on pelvic inflammatory disease. *Obstetrics and Gynecology* 95: 525-534.

Isiaka-Lawal, S., Nwabuisi, C., Fakeye, C., Saidu, R., Adesina, K.T., Ijaiya, M.A., Jimoh, A.A., Omokanye, L.O. (2014) Pattern of sexually transmitted infection in human: HIV positive women attending antenatal clinics in north central Nigeria. *Sahel Medical Journal* 17(4):145-150.

Ison, C.A., Dillon, J., Tapsall, J.W. (1998) The epidemiology of global antibiotic resistance among *Neisseria gonorrhoae* and *Haemophilus dureyi. The Lancet* 35(1):S8-S11.

Ito, S, Mizutani, K, Seike, K, Sugawara, T, Tsuchiya, T, Yasuda, M. (2014) Prediction of the persistence of *Mycoplasma genitalium* after antimicrobial chemotherapy by quantification of leukocytes in first-void urine from patients with non-gonococcal urethritis. *Journal of Infections and Chemotherapy* 20:298–302.

Ito, S., Yasuda, M., Seike, K., Sugawara, T., Tsuchiya, T., Yokoi, S. (2012) Clinical and microbiological outcomes in treatment of men with non-gonococcal urethritis with a 100-mg twice-daily dose regimen of sitafloxacin. *Journal of Infections and Chemotherapy* 18:414–418.

Jenniskens, M.L., Veerbeek, J.H., Deurloo, K.L., van Hannen, E.J., Thijsen, S.F. (2017) Routine testing of *Mycoplasma genitalium* and *Trichomonas vaginalis. Infectious Diseases* 49(6):461-465.

Jennifer, A., Jones, N.C., Meghan, M. (2013) Global Rates and Prevalence of Urogenital Mycoplasmosis: Assembly of a Dataset from Peer-Reviewed Literature. *Open Journal of Medical Microbiology*, 3, 105-124.

Jensen, J.S., Cusini, M., Gomberg, M., Moi, H. (2016) European guideline on *Mycoplasma genitalium* infections. *Journal of European Academic Dermatology and Venereology* 30:1650-1656.

Jensen, J.S., Fernandes, P., Unemo, M. (2014) In vitro activity of the new fluoroketolide solithromycin (CEM-101) against macrolide-resistant and – susceptible *Mycoplasma genitalium* strains. *Antimicrobial Agents and Chemotherapy*. 58:3151–3156.

Jensen, A.J., Kleveland, C.R., Moghaddam, A., Haaheim, H., Hjelmevoll, S.O., Skogen, V. (2013) *Chlamydia trachomatis*, *Mycoplasma genitalium* and *Ureaplasma urealyticum* among students in northern Norway. *Journal of The European Academy of Dermatology and Venereology* 27(1):e91-e96

Jensen, J.S. (2012) Protocol for the detection of *Mycoplasma genitalium* by PCR from clinical specimens and subsequent detection of macrolide resistance-mediating mutations in regions of the 23SrRNA gene. *Methods in Molecular Biology* 903:129-139.

Jensen, J.S. (2006) *Mycoplasma genitalium* infections: Diagnosis, clinical aspects, and pathogenesis. *Danish Medical Bulletin* 53: 1-27.

Jensen, J.S (2004a) *Mycoplasma genitalium*: the etiological agent of urethritis and other sexually transmitted diseases. *Journal of European Academic Dermatology Venereology* 18:1–11.

Jensen, J.S., Bjornelius, E., Dohn, B., Lidbrink, P. (2004b) Use of TaqMan 5 nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. *Journal of Clinical Microbiology*, 42:683–692

Jensen, J.S., Borre, M.B., Dohn, B. (2003) Detection of *Mycoplasma genitalium* by PCR amplification of the 16S rRNA gene. *Journal of Clinical Microbiology* 41:261–266.

Jensen, S.J. and Dohn, B. (2002) Real-time quantitative PCR for the detection of *Mycoplasma genitalium.* Paper presented at the 14th. International Congress of the International Organization for Mycoplasmology, Vienna, Austria.

Jensen, S.J., Hansen, H.T. and Lind, K. (1996) *Mycoplasma genitalium* strains from the male urethra. *Journal of Clinical Microbiology* 34: 296-291.

Jensen, J.S., Uldum, S.A., Sondergard-Andersen, J., Vuust, J., Lind, K. (1991) Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *Journal of Clinical Microbiology* 29:46-50.

Jernberg, E., Moghaddam, A., Moi, H. (2008) Azithromycin and moxifloxacin for microbiological cure of *Mycoplasma genitalium* infection: an open study. *International Journal of STD and AIDS* 19:676–679.

Jill, E., Clarridge III. (2004) Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. *Clinical Microbiology Reviews* 17(4): 840–862.

Johannisson, G., Enström, Y., Löwhagen, G.B., Nagy, V., Ryberg, K., Seeberg, S. (2000) Occurrence and treatment of *Mycoplasma genitalium* in patients visiting STD clinics in Sweden. *International Journal of STD and AIDS* 11:324–326.

Jurstrand, M., Jensen, J.S., Fredlund, H., Falk, L., Molling, P. (2005) Detection of *Mycoplasma genitalium* in urogenital specimens by real-time PCR and by conventional PCR assay. *Journal of Medical Microbiology* 54:23–29.

Kaida, A., Dietrich, J.J., Laher, F., Beksinska, M., Jaggernath, M., Bardsley, M., Smith, P., Cotton, L., Chitneni, P., Closson, K., Lewis, D.A., Smit, J.A., Ndung'u, T., Brockman, M., Gray, G. (2018) A high burden of asymptomatic genital tract infections undermines the syndromic management approach among adolescents and young adults in South Africa: implications for HIV prevention efforts. *BMC Infectious Diseases*.18(1):499.

Kattar, M. M., Chavez, J.F., Limaye, A.P., Rassoulian-Barrett, S.L., Yarfitz, S.L., Carlson, L.C., Houze,,, Y., Swanzy S., Wood B.L., Cookson, B.T. (2001) Application of 16S rRNA gene sequencing to identify *Bordetella hinzii* as the causative agent of fatal septicemia. *Journal of Clinical. Microbiology* 38:789–794.

Kaye, J.A., Castellsague, J., Bui, C.L., Calingaert, B., McQuay, L.J., Riera-Guardia, N. (2014) Risk of acute liver injury associated with the use of moxifloxacin and other oral antimicrobials: a retrospective, population-based cohort study. *Pharmacotherapy* 34:336–349.

Keane, E.A., Thomas, J., Gilory, B., Renton, A., Taylor-Robinson, D. (2000) The association of *Mycoplasma hominis, Ureaplasma urealyticum* and *Mycoplasma genitalium* with bacterial vaginosis: observations on heterosexual men and their female's partners. *International Journal of STD and AIDS* 11:435-439.

Kikuchi, M., Ito, S., Yasuda, M., Tsuchiya, T., Hatazaki, K., Takanashi, M. (2014) Remarkable increase in fluoroquinolone-resistant Mycoplasma genitalium in Japan. *Journal of Antimicrobial and Chemotherapy* 69(9):2376–2382.

Kim, S.I., Yoon, J.H., Park, D.C., Lee, D.S., Lee, S.J., Choe, H.S., Kim, J.H., Park, T.C., Lee, S.J. (2018) Co-infection Of *Ureaplasma urealyticum* And Human Papilloma Virus In Asymptomatic Sexually Active Individuals. *International Journal of Medical Sciences* 15(9):915-920.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Molecular Evolution* 16:111–120.

Kolbert, C. P. and Persing, D.H. (1999) Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Current Opinion in Microbiology* 2:299– 305.

Korte, J.E., Baseman, J.B., Cagle, M.P., Herrera, C., Piper, J.M. (2006) Cervicitis and genitourinary symptoms in women culture positive for *Mycoplasma genitalium*. *American Journal of Reproduction Immunology* 55:265-275.

Kotrotsiou, T., Exindan, M., Diza, E. (2013) Prevalence and antimicrobial susceptibility of Ureaplasma urealyticum in asymptomatic women in Northern Greece. *Hippokratia* 17:319-321.

Koumans, E. H., Sternberg, M., Bruce C. (2007) The prevalence of bacterial vaginosis in the United States 2001-2004: associations with symptoms, sexual behaviors and reproductive health. *Sexually Transmitted Diseases* 34:864-869.

Krieg, N. R., Garrity, G.M. (2001) On using the manual, *In* G. M. Garrity (ed.), Bergey's manual of systematic bacteriology, 2nd ed. Bergey's Manual Trust. Springer-Verlag, New York, N.Y. Pp. 15–19.

Kufa, T., Gumede, L., Maseko, D.V., Radebe, F., Kularatne, R. (2018) The demographic and clinical profiles of women presenting with vaginal discharge syndrome at primary care facilities in South Africa: Associations with age and implications for management. *South African Medical Journal* 108(10):876-880.

Kumar, S., Stecher, G., Tamura, K. (2016) MEGA 7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33:1870-1874.

Kuppeveld, F. J., van der Logt, J.T., Angulo, A.F., van Zoest,, M.J., Quint W.G., Niesters, H.G., Galama, J.M., Melchers, W.J. (1992) Genus- and species-specific identification of

mycoplasmas by 16S rRNA amplification. *Applied Environmental Microbiology* 58:2606–2615.

Labbe, A.C., Frost, E., Deslandes, S., Mendonca, A.P., Alves, A.C., Pepin, J. (2002) *Mycoplasma genitalium* in early pregnancy and the relationship between its presence and pregnancy in Giunea-Bissau. *Sexually Transmitted Infection* 78:289-291.

Le Roux, M.C., Ramoncha, M.R., Adam, A., Hoosen, A.A. (2010) Etiological agents of urethritis in symptomatic South African men attending a family practice. *International Journal of STD and AIDS 21*: 477-481.

Lefebvre, M., Coutherut, J., Gibaud, S., Biron, C., Chalopin, M., Bernier, C., Raffi, F. (2017) Prevalence of *Mycoplasma genitalium* Infection and Relationship with Symptoms Among Adults Attending a Sexual Health Centre. *Acta Dermato Venereologica* 97(4):543-545.

Leli, C., Mencacci, A., Latino, M.A., Clerici, P., Rassu, M., Perito, S., Castronari, R., Pistoni, E., Luciano, E., De Maria, D., Morazzoni, C., Pascarella, M., Bozza, S., Sensini, A. (2018) Prevalence of cervical colonization by *Ureaplasma parvum, Ureaplasma urealyticum, Mycoplasma hominis* and *Mycoplasma genitalium* in childbearing age women by a commercially available multiplex real-time PCR: An Italian observational multicentre study. *Journal of Microbiology, Immunology and Infection* 51(2):220-225.

Li, Y., Su, X., Le, W., Li, S., Yang, Z., Chaisson, C., Madico, G., Gong, X., Reed, G.W., Wang, B., Rice, P.A. (2019) Mycoplasma genitalium in symptomatic male urethritis: macrolide use is associated with increased resistance. *Clinical Infectious Diseases* doi: 10.1093/cid/ciz294. [Epub ahead of print].

Li, B.B., Shen, J.Z., Cao, X.Y., Wang, Y., Dai, L., Huang, S.Y. (2010) Mutations in 23S rRNA gene associated with decreased susceptibility to tiamulin and valnemulin in *Mycoplasma gallisepticum*. *FEMS Microbiology Letters* 308:144–149.

Lillis, R.A., Martin, D.H., Nsuami, M.J. (2018) *Mycoplasma genitalium* Infections in Women Attending a Sexually Transmitted Disease Clinic in New Orleans. *Clinical Infectious Diseases* doi: 10.1093/cid/ciy922. [Epub ahead of print].

Lillis, R.A., Nsuami, M.J., Myers, L., Martin, D.H. (2011) Utility of urine, vaginal, cervical and rectal specimens for detection of *Mycoplasma genitalium* in women. *Journal of Clinical Microbiology* 49(5):1990-1992.

Lis, R., Rowhani-Rahbar, A., Manhart, L.E. (2015) *Mycoplasma genitalium* infection and female reproductive disease; a meta-analysis. *Clinical Infectious Diseases* 61:418-426.

Ljubin-Sternak, S., Meštrović, T., Kolarić, B., Jarža-Davila, N., Marijan, T., Vraneš, J. (2017) Assessing the Need for Routine Screening for *Mycoplasma genitalium* in the Low-risk Female Population: A Prevalence and Co-infection Study on Women from Croatia. *International Journal of Preventive Medicine* 8:51.

Loens, K., Goossens, H., Ieven, M. (2010) Acute respiratory infection due to *Mycoplasma pneumoniae*: current status of diagnostic methods. *European Journal of Clinical Microbiology and Infectious Diseases* 29:1055–1069.

Loens, K., Ursi, D., Goossens, H., Ieven, M. (2008) Evaluation of the NucliSens miniMAG RNA extraction and real-time NASBA applications for the detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumonia* in throat swabs. *Journal of Microbiological Methods* 72:217–219.

Loens, K., Bergs, K., Ursi, D., Goossens, H., Ieven, M. (2007) Evaluation of NucliSens easyMAG for automated nucleic acid extraction from various clinical specimens. *Journal of Clinical Microbiology* 45:421–425.

Loens, K., Ursi, D., Goossens, H., Ieven, M. (2003) Molecular diagnosis of *Mycoplasma pneumoniae* respiratory tract infections. *Journal of Clinical Microbiology* 41:4915–4923.

Lokken, E.M., Balkus, J.E., Kiarie, J., Hughes, J.P., Jaoko, W., Totten, P.A., McClelland, R.S., Manhart, L.E. (2017) Association of Recent Bacterial Vaginosis With Acquisition of *Mycoplasma genitalium*. *American Journal of Epidemiology* 186(2):194-201.

Ma, L., Jensen, J.S., Mancusa, M., Hamasuna, R., Jia, Q., McGowin, C.L. (2010) Genetic variation in the complete MgPa operon and its repetitive chromosomal elements in clinical strains of *Mycoplasma genitalium. PLoS ONE* 5:e15660.

Ma, L., Taylor, S., Jensen, J. S., Myers, L., Lillis, R., Martin, D. H. (2008) Short tandem repeat sequences in the *Mycoplasma genitalium* genome and their use in a multilocus genotyping system. *BMC Microbiology* 8: 130.

Maeda, S.I., Tamaki, M., Kojima, K., Yoshida, T., Ishiko, H., Yasuda, M., (2001) Association of *Mycoplasma genitalium* persistence in the urethra with recurrence of nongonococcal urethritis. *Sexually Transmitted Diseases* 28:472–476.

Mahmoud, A., Sheriff, N.A., Abdella, R., El-Genedy, A.R., El-Kateb, A.Y. Askalani, A.N.H. (2015) Prevalence of *Trichomonas vaginalis* infection among Egyptian women using culture and latex agglutination: Cross-sectional study. *BMC Women's health* 15:7.

Manhart, L.E. (2014) Editorial commentary: diagnostic and resistance testing for *Mycoplasma genitalium*: what will it take? *Clinical Infectious Dis*eases 59(1): 31–33.

Manhart, L.E., Gillespie, C.W., Lowens, M.S. (2013) Standard treatment regimens for nongonococcal urethritis have similar but declining cure rates: a randomized controlled trial. *Clinical Infectious Diseases* 56(7):934–942.

Manhart, L.E. (2013) *Mycoplasma genitalium*: an emergent sexually transmitted disease? *Infectious Disease Clinics of North America* 27(4):779–792.

Manhart, L.E., Mostad, S.B., Baeten, J.M., Astete, S.G., Mandaliya, K., Totten, P.A. (2008) High *Mycoplasma genitalium* organism burden is associated with shedding of HIV-1 DNA from the cervix. *Journal of Infectious Diseases* 197:733-736.

Manhart, L.E., Holmes, K.K., Hughes, J.P., Houston, L.S.M., Totten, P.A. (2007) *Mycoplasma genitalium* among young adults in the United States: an emerging sexually transmitted infection. *American Journal of Public Health* 97(6): 1118-1125.

Martin, D.H., Zozaya, M., Lillis, R.A., Meyers, L., Nsuami, M.J. (2013) Unique vaginal microbiota that includes an unknown Mycoplasma-like organism is associated with *Trichomonas vaginalis* infection. *Journal of Infectious Diseases* 207:1922-1931.

Martin, D.H., Mroczkowski, T.F., Dalu, Z.A., Mccarty, J., Jones, R.B., Hopkins, S.J. (1992) A controlled trial of a single dose of azithromycin for the treatment of chlamydial urethritis and cervicitis. *New England Journal of Medicine* 327:921–925.

McGowin, C.L. and Anderson-Smits, C. (2011) *Mycoplasma genitalium*: an emerging cause of sexually transmitted disease in women. *PLoS Pathogen* 7: e1001324.

McGowin, C.L., Popov, V.I., Pyles, R.B. (2009) Interacellular *Mycoplasma genitalium* infection of human vaginal and cervical epithelial cells elicits distinct patherns of inflammatory cytokine secretion and provides a possible survival niche against macrophage-mediated killing. *BMC Microbiology* 9:139.

Mclver, C.J., Rismanto, N., Smith, C., Naing, Z.W., Rayner, B., Lusk, J.M., Konecny, P., White, P.A., Rawlinson, W.D. (2009) Multiplex PCR testing detection of higher-than expected rates of cervical Mycoplasma, Ureaplasma and Trichomonas and Viral Agent Infections in Sexually Active Australian Women. *Journal of Clinical Microbiology* 47(5):1358-1363.

Mena, L.A., Mroczkowski, T.F., Nsuami, M., Martin, D.H. (2009) A randomized comparison of azithromycin and doxycycline for the treatment of *Mycoplasma genitalium*-positive urethritis in men. *Clinical Infectious Dis*eases 48(12):1649–1654.

Mena, L.A., Wang, X., Mroczkowski, T.F., Martin, D.M. (2002) *Mycoplasma genitalium* infections in asymptomatic men and men with urethritis attending a STD clinic in New Orleans. *Clinical Infectious Diseases* 35:1167-1173.

Metwally, M.A., Yassin, A.S., Essan, T.M., Hamouda, H.M., Amin, M.A. (2014) Detection, characterization and molecular typing of Human Mycoplasma spp from major hospitals in Cairo, Egypt. *The Scientific World Journal* Article ID 549858, 6 pages.

Min, L., Xiaomei, Z., Huang, K., Qiu, H., Zhang, J., Kang, Y., Wang, C. (2017) Presence of *Chlamydia trachomatis* and *Mycoplasma spp*; but not *Neisseria gonorrohoeae* and *Treponema pallidum* in women undergoing an infertility evaluation: high prevalence of tetracycline resistance gene (tet(M)). *AMB Express* 7:206.

Mirnejad, R., Amirmozafari, N., Kazemi, B. (2011) Simultaneous and rapid differential diagnosis of *Mycoplasma genitalium* and *Ureaplasma urealyticum* based on a polymerase chain reaction-restriction fragment length polymorphism. *Indian Journal of Medical Microbiology* 29: 33-36.

Molenaar, M.C., Singer, M., Ouburg, S. (2018) The two-sided role of the vaginal microbiome in *Chlamydia trachomatis* and *Mycoplasma genitalium* pathogenesis. *Journal of Reproductive Immunology* 130: 11-17.

Moller, B.R., Taylor-Robinson, D., Furr, P. M. (1984) Serological evidence implicating *Mycoplasma genitalium* in pelvic inflammatory disease. *Lancet* 1:1102-1103.

Murray, G.L., Danielewski, J., Bodiyabadu, K., Machalek, D.A., Bradshaw, C.S., Costa, A.M., Birnie, J., Garland, S.M. (2019) Analysis of Infection Loads in *Mycoplasma genitalium* Clinical Specimens by Use of a Commercial Diagnostic Test. *Journal of Clinical Microbiology* 57(9): 233-235.

Murray, P.R., Rosenthal, K.S., Pfaller, M.A. (2017) Medical Microbiology. *Elsevier Health Sciences*.Pp 234 -300.

Musatovova, O. and Baseman, J. B. (2009) Analysis identifying common and distinct sequences among Texas clinical strains of *Mycoplasma genitalium. Journal of Clinical Microbiology* 47: 1469–1475.

Muvunyi, C.M., Dhont N., Verhelst, R. (2011) Evaluation of a new multiplex polymerase chain reaction assay STD Finder for the simulataneous detection of seven sexually transmitted disease pathogens. *Diagnostic Microbiology and Infectious Diseases* 71:29-37.

Nilsson, A.C., Björkman, P., Welinder-Olsson, C., Widell A., Persson K. (2010) Clinical severity of *Mycoplasma pneumoniae* (MP) infection is associated with bacterial load in oropharyngeal secretions but not with MP genotype. *BMC Infectious Diseases* 10:39.

Nolskog, P., Backhaus, E., Nasic, S., Enroth, H. (2018) STI with Mycoplasma genitalium-more common than Chlamydia trachomatis in patients attending youth clinics in Sweden. *European Journal of Clinical Microbiology and Infectious Diseases*. 38(1):81-86.

Nwaguma B., Kalu I., Ezeanyika, L. (2009) Seroprevalence of anti-chlamydia trachomatis IgA antibodies in a Nigerian population. *The Internet Journal of Infectious Diseases* 7:2.

Oakeshott, P., Aghaizu, A., Hay, P., Reid, F., Kerry, S., Antherton, S.I., Taylor-Robinson D., Dohn, B., Jensen, S.J. (2010) Is *Mycoplasma genitalium* in women the "New Chlamydia?" A community-Based prospective cohort study. *Clinical Infectious Diseases* 51(10):1160-1166.

Oakeshott, P., Hay, P., Taylor-Robinson, D., Hay, S., Dohn, B., Kerry, S. (2004) Prevalence of *Mycoplasma genitalium*in early pregnancy and relationship between its presence and pregnancy outcome. *Bjog: An International Journal of Obstetrics and Gynecology* 111: 1464-1467.

Okwoli, N. R. (2007) A Thesis: Prevalence of Mycoplasma and Ureaplasma in women attending Gynaecology clinic at University College Hospital, Ibadan and pathogenicity of *Ureaplasma urealyticum* in mice. Ph.D Thesis University of Ibadan, Ibadan Nigeria.

Ona, S., Molina, R.L., Diouf, K. (2016) *Mycoplasma genitalium*: An Overlooked Sexually Transmitted Pathogen in Women? *Infectious Diseases in Obstetrics and Gynecology* Article ID 4513089, 9 pages.

Pace, H. (2009) Playing God with Synthetic Life? Starting with theminimal genome project. *The Triple Helix Easter*.Pp 8-9.

Pace, N. (1997). A molecular view of microbial diversity and the biosphere. *Science* 276:734–740.

Palmer, H.M., Gilroy, C.B., Claydon, E.J., Taylor-Robinson, D.(1991a) Detection of *Mycoplasma genitalium* in the genitourinary tract of women by the polymerase chain reaction. *International Journal of STD and AIDS 2*: 261-263.

Palmer, H.M., Gilroy, C.B., Furr, P.M, Taylor-Robinson, D. (1991b) Development and evaluation of the polymerase chain reaction to detect *Mycoplasma genitalium. FEMS Microbiology Letters* 61:199-203.

Palys, T., Nakamura, L.K., Cohan, F.M. (1997) Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. *International Journal of Systematic Bacteriology* 47:1145–1156.

Park, J.J., Seo, Y.B., Jeong, S., Lee, J. (2017) Prevalence of and Risk Factors for Sexually Transmitted Infections among Korean Adolescents under Probation. *Journal of Korean Medical Science* 32(11):1771-1778.

Patel, J. B. (2001) 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular Diagnostic*. 6:313–321.

Patel, J. B., Leonard, D.G., Pan, X., Musser, J.M., Berman R.F., Nachamkin, I. (2000) Sequence-based identification of *Mycobacterium* species using the Microseq 500 16S rDNA bacterial identification system. *Journal of Clinical Microbiology* 38:246–251.

Peter, C., Alec, M., Bigoni, J., Toutous-Trellu, L., Yaron, M. (2018) Update on Mycoplasma genitalium among women. *Revue Médicale Suisse* 14(624):1893-1897.

Paukner, S., Gruss, A., Fritsche, T.R., Ivezic-Schoenfeld, Z., Jones, R.N. (2014) In vitro activity of the novel pleuromutilin BC-3781 tested against bacterial pathogens causing sexually transmitted diseases (STD). *Interscience Conference of Antimicrobial Agents and Chemotherapy* (ICAAC 2014): E-1183.

Peerayeh, S.N. and Mirdamadi, R. (2005) Comparison of culture with polymerase chain reaction for detection of *Ureaplasma urealyticum* in endocervical specimens. *Medical Journal of the Islamic Republic of Iran* 19(2): 175-179.

Pepin, J., Labbe, A.C., Khonde, N. (2005) *Mycoplasma genitalium*: an organism commonly associated with cervicitis among West African sex workers. *Sexually Transmitted Infections* 81:67-72.

Pereyre, S., Laurier Nadalié, C., Bébéar, C., Investigator group. (2017) *Mycoplasma genitalium* and *Trichomonas vaginalis* in France: a point prevalence study in people screened for sexually transmitted diseases. *Clinical Microbiology and Infection*. 23(2):122.e1-122.e7.

Pereyre, S., Guyot C., Renaudin, H., Charron, A., Bebear, C., Bebear, C.M. (2004) In vitro selection and characterization of resistance to macrolides and related antibiotics in *Mycoplasma pneumoniae*. *Antimicrobial Agents and Chemotherapy* 48:460–465.

Peterson, B. Tully, J. G. Bolske, G., Johansson, K.E. (2000) Updated phylogenetic description of the *Mycoplasma hominis* cluster based on 16S rDNA sequences. *International Journal of Systemic and Evolutionary Microbiology* 50(1): 291-301.

Peterson, S.N., Bailey, C.C., Jensen, J.S., Borre, M.B., King, E.S., Bott, K.F., Hutchison, C.A. (1995) Characterization of repetitive DNA in the *Mycoplasma genitalium* genome:

possible role in the generation of antigenic variation. *Proceedings of the National Academy of Sciences of the United States of America* 92:11829–11833.

Pfister, P., Hobbie, S., Vicens, Q., Bottger, E.C., Westhof, E. (2003a) The molecular basis for A-site mutations conferring aminoglycoside resistance: relationship between ribosomal susceptibility and X-ray crystal structures. *Chembiochemistry* 4:1078–1088.

Pfister, P., Risch, M., Brodersen, D.E., Bottger, E.C. (2003b) Role of 16SrRNA helix 44 in ribosomal resistance to hygromycin B. *Antimicrobial Agents and Chemotherapy*. 47:1496–1502.

Plantamura, J., Bigaillon, C., Bousquet, A., Delaune, D., Larréché, S., Bugier, S., Mérens, A., Ficko, C. (2017) *Mycoplasma genitalium*: a mycoplasma still underestimated. *Annales de Biologie Clinique* (Paris) 75(2):209-214.

Piñeiro, L., Galán, J.C., Vall-Mayans, M. (2019) Infections caused by *Chlamydia trachomatis* (including lymphogranuloma venereum) and *Mycoplasma genitalium. Enfermedades Infecciosas y Microbiología Clínica* doi: 10.1016/j.eimc.2019.01.014. [Epub ahead of print].

Pond, M.J., Nori, A.V., Witney, A.A., Lopeman, R.C., Butcher, P.D., Sadiq, S.T.(2014) High prevalence of antibiotic-resistant *Mycoplasma genitalium* in nongonococcal urethritis: the need for routine testing and the inadequacy of current treatment options. *Clinical Infectious Diseases* 58:631–637.

Powledge, T.M. (2005) The Polymerase Chain Reaction. Retrieved 5th March 2015 from http://sunsite.berkeley.edu/PCR/whatisPCR.html

Prince, W.T., Ivezic-Schoenfeld, Z., Lell, C., Tack, K.J., Novak, R., Obermayr, F. (2013) Phase II clinical study of BC-3781, a pleuromutilin antibiotic, in treatment of patients with acute bacterial skin and skin structure infections. *Antimicrobial Agents and Chemotherapy* 57:2087–2094.

Qian, Q., Tang, Y., Kolbert, C.P., Torgerson, C.A., Hughes, J.G., Vetter, E.A, Harmsen, W., Montgomery, S.O., Cockerill III, F.R., Persing, D.H. (2001) Direct identification of bacteria from positive blood cultures by amplification and sequencing of the 16S rRNA gene: evaluation of BACTEC 9240 instrument true-positive and false-positive results. *Journal of Clinical Microbiology* 39:3578–3582.

Ramazanzadeh, R., Khodabandehloo, M., Farhadifar, F., Rouhi, S., Ahmadi, A., Menbari, S., Fallahi, F., Mirnejad, R. (2016) A Case-control Study on the Relationship between *Mycoplasma genitalium* Infection in Women with Normal Pregnancy and Spontaneous Abortion using Polymerase Chain Reaction. *Osong Public Health Research and Perspective* 7(5):334-338.

Rantakokko-Jalava, K., Nikkari, S., Jalava, J., Eerola, E., Skurnik, M., Meurman, O., Ruuskanen, O., Alanen, A., Kotilainen, E., Toivanen, P., Kotilainen, P. (2000) Direct amplification of rRNA genes in diagnosis of bacterial infections. *Journal of Clinical Microbiology* 38:32–39.

Rawadi, G., Dujeancourt-Henry, A., Lemercier, B., Roulland-Dussoix, D. (1998) Phylogenetic position of rare human Mycoplasmas: *M.faucium, M.buccale, M.primatum and*

M.spermatophilum based on 16S rRNA gene sequence. *International Journal of Systemic Bacteriology* 48:305-309.

Razin, S. (2005) Mycoplasma. In: Boricello S.P., Murray P.R., Funke G, editors. *Topley& Wilson's microbiology & microbial infections*, 10th ed. London: Hodder Arnold. Pp. 1957-2005.

Relman, D. A. (1999) The search for unrecognized pathogens. *Science* 284:1308–1310.

Renaudin, H., Tully, J.G., Bebear, C. (1992) In-vitro susceptibilities of *Mycoplasma genitalium* to antibiotics. *Antimicrobial Agents and Chemotherapy* 36:870–872.

Ringuet, H., Akoua-Koffi, C., Honore, S., Varnerot, A., Vincent, V., Berche, P., Gaillard, J.L., Pierre-Audigier, C. (1999) *hsp65* sequencing for identification of rapidly growing mycobacteria. *Journal of Clinical Microbiology* 37:852–857.

Romano, S.S., Jensen, J.S., Lowens, M.S., Morgan, J.L., Chambers, L.C., Robinson, T.S., Totten, P.A., Soge, O.O., Golden, M.R., Manhart, L.E. (2019) Long Duration of Asymptomatic Mycoplasma genitalium Infection after Syndromic Treatment for Nongonococcal Urethritis. *Clinical Infectious Diseases*. 69(1):113-120.

Romero, P., Munoz, M., Martinez, M.A., Romero, M.I., Germain, L., Maida, M. (2014) Ureaplasma amd mycoplasma in vaginal samples from prepubertal girls and the reasons for gynecological consultation. *Journal of Pediatrics and Adolescent Gynecology* 27:10-13.

Ronda J, Gaydos CA, Perin J, Tabacco L, Coleman JS, Trent M (2018) Does the Sex Risk Quiz Predict Mycoplasma genitalium Infection in Urban Adolescents and Young Adult Women? *Sexually Transmitted Diseases.* 45(11):728-734.

Roosendaai, R., Walboomers, J.M., Veltman, O.R., Melgers, I., Burger, C., Bleker, O.P., Mac Claren, D.M., Meijer, C.J., Van den Brule, A.J. (1993) Comparison of different primer set for detection of *Chlamydia trachomatis* by Polymerase chain reaction. *Journal of Medical Microbiology* 38(6):426-433.

Roth, A., Fischer, M., Hamid, M.E., Michalke, S., Ludwig, W., Mauch, H. (1998) Differentiation of phylogenetically related slowly growing mycobacteria based on 16S–23S rRNA gene internal transcribed spacer sequences. *Journal of Clinical Microbiology* 36:139–147.

Sacchi, C. T., Whitney, A.M., Mayer, L.W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R.S., Popovic, T. (2002a) Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis*. *Emerging Infectious Diseases* 8:1117–1123.

Sacchi, C. T., Whitney A.M., Reeves M.W., Mayer L.W., Popovic T. (2002b) Sequence diversity of *Neisseria meningitidis* 16S rRNA genes and use of 16S rRNA gene sequencing as a molecular subtyping tool. *Journal of Clinical Microbiology* 40:4520–4527.

Saigal, K., Dhawan, B., Rawre, J., Khanna, N., Chaudhry, R. (2016) Genital Mycoplasma and *Chlamydia trachomatis* infections in patients with genital tract infections attending a tertiary care hospital of North India. *Indian Journal of Pathology and Microbiology* 59(2):194-196.

Salado-Rasmussen, K. and Jensen, J.S. (2014) *Mycoplasma genitalium* testing pattern and macrolide resistance: A Danish nationwide retrospective survey. *Clinical Infectious Diseases* 59:24–30.

Salazar, A., Espadafor, B., Fuentes-López, A., Barrientos-Durán, A., Salvador, L., Álvarez, M., García, F. (2019) Comparison between Aptima Assays (Hologic) and the Allplex STI Essential Assay (Seegene) for the diagnosis of Sexually transmitted infections. *PLoS One* 14(9):e0222439.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. Pp. 489–495.

Samra, Z., Rosenberg, S., Madar-Shapiro, L. (2011) Direct simultaneous detection of 6 sexually transmitted pathogens from clinical specimens by multiplex polymerase chain reaction and auto-capillary electrophoresis. *Diagnostic Microbiology and Infectious Diseases* 70:17-21.

Schönwald, S., Gunjaca, M., Kolacny- Babic, L., Car, V., Gosev, M. (1990) Comparison of azithromycin and erythromycin in the treatment of atypical pneumonias. *Journal of Antimicrobial and Chemotheraphy* 25(Suppl A):123–126.

Schwebke, J.R., Rompalo, A, Taylor, S. (2011) Re-evaluating the treatment of nongonococcal urethritis: emphasizing emerging pathogens – a randomized clinical trial. *Clinical Infectious Di*seases 52(2):163–170.

Schwebke, J.R., Rompalo, A., Taylor, S., Sena, A.C., Martin, D.H., Lopez, L.M., Lensing, S., Lee, J.Y. (2011) Re-evaluating the treatment of nongonococcal urethritis: emphasizing emerging pathogens–a randomized clinical trial. *Clinical Infectious Diseases* 52:163–170.

Seña, A.C., Lee, J.Y., Schwebke, J., Philip, S.S., Wiesenfeld, H.C., Rompalo, A.M., Cook, R.L., Hobbs, M.M. (2018) A Silent Epidemic: The Prevalence, Incidence and Persistence of *Mycoplasma genitalium* among Young, Asymptomatic High-Risk Women in the United States. *Clinical Infectious Diseases* 67(1):73-79.

Sena, A.C., Lensing, S., Rompalo, A., Taylor, S.N., Martin, D.H., Lopez, L.M.(2012) *Chlamydia trachomatis, Mycoplasma genitalium* and *Trichomonas vaginalis* infections in men with nongonococcal urethritis: predictors and persistence after therapy. *Journal of Infectious Diseases* 206:357–365.

Sethi, S., Singh, G., Samanta, P., Sharma, M. (2012) *Mycoplasma genitalium*: An emerging sexually transmitted pathogen. *Indian Journal of Medical Research* 136: 942-955.

Shibata, K., Into, T., Yasuda, M., Hasebe, A., Okada, K. (2002) Cytotoxicity of Mycoplasmal lipoproteins. Paper presented at the 14th. International Congress of the International Organization for Mycoplasmology, Vienna, Austria 7th – 12th July, 2002.

Shimada, Y., Deguchi, T., Nakane, K., Masue, T., Yasuda, M., Yokoi, S., Ito, S., Nakano, M., Ishiko, H. (2010) Emergence of clinical strains of *Mycoplasma genitalium* harbouring alterations in ParC associated with fluoroquinolone resistance. *International Journal of Antimicrobial Agents* 36:255–258.

Silva, J., Cerqueira, F., Teixeira, A.L., Bicho, M.C., Campainha, R., Amorim, J., Medeiros, R. (2018) Genital mycoplasmas and ureaplasmas in cervicovaginal self-collected samples of reproductive-age women: prevalence and risk factors. *International Journal of STD and AIDS*. (10):999-1006.

Simms, I., Eastick, K., Mallinson, H. (2003) Association between *Mycoplasma genitalium, Chlamydia trachomatis* and Pelvic Inflammatory Disease. *Sexually Transmitted Infections* 79:154-156.

Sleha, R., Bostikova, V., Hampl, R., Salavec, M., Halada, P., Stepan, M., Novotna, S., Kukla, R., Slehova, E., Kacerovsky, M., Bostik, P. (2016) Prevalence of *Mycoplasma hominis* and *Ureaplasma urealyticum* in women undergoing an initial infertility evaluation. *Epidemiology Microbiology and Immunology* 65(4):232-237.

Slifirski, J.B., Vodstrcil, L.A., Fairley, C.K., Ong, J.J., Chow, E.P.F., Chen, M.Y., Read, T.R.H., Bradshaw, C.S. (2017) *Mycoplasma genitalium* infection in Adults Reporting Sexual Contact with Infected Partners, Australia, 2008-2016. *Emerging Infectious Diseases* 23(11):1826-1833.

Smieszek, T. and White, P.J. (2016) Apparently-different clearance rates from cohort studies of *Mycoplasma genitalium* are consistent after accounting for incidence of infection, recurrent infection and study design. *PLoS ONE* 11(2):0149087.

Song, J., Lee, S.C., Kang, J.W., Baek, H. J., Suh, J. W. (2004) Phylogenetic analysis of *Streptomyces* spp. isolated from potato scab lesions in Korea on the basis of 16S rRNA gene and 16S-23S rDNA internally transcribed spacer sequences. *International Journal of System Evolutionary Microbiology* 54:203–209.

Spence, D. and Melville, C. (2007) Vaginal discharge. *British Medical Journal* 335:1147-1151.

Sussman, J. I., Baron, E.J.,Tenenbaum, M.J., Kaplan, M.H., Greenspan, J., Facklam, R.R., Tyburski, M.B., Goldman, M.A., Kanzer, B.F., Pizzarello, R.A. (1986) Viridans streptococcal endocarditis: clinical, microbiological, and echocardiographic correlations. *Journal of Infectious Diseases* 154:597–603.

Svenstrup, H.F., Dave, S.S., Cander, C., Grant, P., Moeeis-Jones, S., Kidd, M., Stephenson, J.M. (2014) A cross-sectional study of *Mycoplasma genitalium* infection and correclates in women undergoing population-based screening or clinic based testing for Chlamydia infection in London. *Bristish Medical Journal Open* 4(2):e003947.

Svenstrup, H.F., Jensen, J.S., Bjornelius, E., Lidbrink, P., Birkelund, S., Christiansen, G. (2005) Development of a quantitative real-time PCR assay for detection of *Mycoplasma genitalium*. *Journal of Clinical Microbiology* 43: 3121–3128.

Tagg, K.A., Jeoffreys, N.J., Couldwell, D.L., Donald, J.A., Gilbert, G.L. (2013) Fluoroquinolone and macrolide resistance-associated mutations in Mycoplasma genitalium. *Journal of Clinical Microbiology* 51:2245–2249.

Takahashi, S., Hamasuna, R., Yasuda, M., Ito S, Ito, K., Kawai, S. (2013) Clinical efficacy of sitafloxacin 100 mg twice daily for 7 days for patients with non-gonococcal urethritis. *Journal of Infection and Chemotherapy* 19:941–945.

Takahashi, S., Ichihara, K., Hashimoto, J., Kurimura, Y., Iwasawa, A., Hayashi, K. (2011) Clinical efficacy of levofloxacin 500 mg once daily for 7 days for patients with non-gonococcal urethritis. *Journal of Infection and Chemotherapy* 17:392–396.

Takahashi, S., Takeyama, K., Miyamoto, S., Ichihara, K., Maeda, T., Kunishima, Y. (2006) Detection of *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, and *Ureaplasma parvum* DNAs in urine from asymptomatic healthy young Japanese men. *Journal of Infection and Chemotherapy*12: 269-71.

Tamura, K. and Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10:512-526.

Tang, Y. W., Von Graevenitz A., Waddington A., Hopkins M.G., M.K., Smith, D.H., Li, H., Kolbert, C.P., Montgomery, S.O., Persing, D.H. (2000) Identification of coryneform bacterial isolates by ribosomal DNA sequence analysis. *Journal of Clinical Microbiolology* 38:1676–1678.

Tang, Y. W., Ellis, N.M., Hopkins, M.K., Smith, D.H., Dodge, D.E., Persing, D.H. (1998) Comparison of phenotypic and genotypic technique for identification of unusual aerobic pathogenic gram-negative bacilli. *Journal of Clinical Microbiology* 36:3674–3679.

Taylor, S.N. (2011) Molecular testing for *Trichonomas vaginalis* in women: results from a prospertive US clinical trial. *Journal of Clinical Microbiology* 49:4106-4111.

Taylor-Robinson, D. (2017) Mollicutes in vaginal microbiology: *Mycoplasma hominis, M.genitalium, Ureaplasma urealyticum, U.parvum*. *Research in Microbiology* doi:10.1016/j.resmic.2017.02.009.

Taylor-Robinson, D. (2014) Diagnosis and antimicrobial treatment of *Mycoplasma genitalium* infection: sobering thoughts. *Expert Review of Antimicrobial Infection Therapy* 12:715-722.

Taylor-Robinson, D. and Jensen J.S. (2010) Genital Mycoplasma. In Morse S.A. (eds). Atlas of *Sexually Transmitted Diseases* and AIDS 4th edition. Elsevier.

Taylor-Robinson, D. and Rosenstein, I. J. (2001) Is *Mycoplasma hominis* a vaginal pathogen? *Sexually Transmitted Infection* 77: 302.

Terada, M., Izumi, K., Ohki, E., Yamagishi, Y., Mikamo, H. (2012) Antimicrobial efficacies of several antibiotics against uterine cervicitis caused by *Mycoplasma genitalium*. *Journal of Infection and Chemother*apy 18(3):313–317.

Thorne, J. L., Kishino, H., Painter, I. S. (1998) Estimating the rate of evolution of the rate of molecular evolution. *Molecular Biology and Evolution* 15:1647–1657.

Tortoli, E. (2003) Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clinical Microbiology Review* 16:319–354.

Tosh, A.K., Van Der Pol, B., Fortenberry, J.D., Williams, J.A., Katz ,B.P., Batteiger, B.E. (2007) *Mycoplasma genitalium* among adolescent women and their partners. *Journal of Adolescent Health* 40: 412-417.

Tsevat, D.G., Wiesenfeld, H.C., Parks, C., Peipert, J.F. (2017) Sexually transmitted diseases and infertility. *American Journal of Obstetrics Gynecology* 216(1):1-9.

Tully, J.G., Taylor-Robinson, D., Cole, R.M., Rose, D.L. (1981) A newly discovered mycoplasma in the human urogenital tract. *Lancet* 1:1288-1291.

Tully, J.G., Whitcomb, R.F., Clark, H.F., Williamson, D.L. (1977). Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* 195: 892-894.

Ueda, K., Seki, T., Kudo, T., Yoshida, T., Kataoka, M. (2006) Two distinct mechanisms cause heterogeneity of 16S rRNA. *Journal of Bacteriology*. 181:78–82.

Unemo, M., Endre, K.M., Moi, H. (2015) Five-day azithromycin treatment regimen for *Mycoplasma genitalium* infection also effectively eradicates *Chlamydia trachomatis*. *Acta Dermato-Venereologica*. 95(6):730–732.

Uphoff, C.C. and Drexler, H.G. (2002) Comparative PCR analysis for detection of mycoplasma infections in continuos cell lines. *In Vitro Cell Development Biology* 38: 79-85.

Uuskula, A. and Kohl, K. (2002) Genital Mycoplasmas including *Mycoplasma genitalium*, as sexually transmitted agents. *International Journal of STD AIDS* 13(2): 79 – 85.

Vandepitte, J., Weiss, H. A., Kyakuwa N. (2013) Natural history of *Mycoplasma genitalium* infection in a cohort of female sex workers in Kampala, Uganda. *Sexually Transmitted Diseases* 40(5):422-427.

Waites, K.B., Xiao, L., Paralanov, V., Viscardi, R.M., Glass, J.I. (2012) Molecular methods for the detection of Mycoplasma and Ureaplasma infections in humans. *Journal of Molecular Diagnostics* 14(5):437-450.

Waites, K.B. and Taylor-Robinson D (2011) Mycoplasma and Ureaplasma. Manual of Clinical Microbiology, ed 10. Edited by Versalovic, J., Carroll, K., Funke, G., Jorgensen, J., Landry, M., Warnock, D.W. Washington, DC, ASM Press. Pp 970–985.

Waites, K.B. and Talkington, D.F (2004) *Mycoplasma pneumoniae* and its role as a human pathogen. *Clinical Microbiology Review*17:697–728.

Waites, K.B., Bebear, C.M., Robertson, J.A., Takington, D.F., Kenny, G.E. (2001) Cumitech 34, *Laboratory diagnosis of mycoplasmal infections*. Coordinating ed., F.S. Nolte. American Society for Microbiology, Washington, D.C.

Wariso, K.T., Odigie, J., Eyanu, S. (2012) Prevalence of *Chlamydia trachomatis* infection among female undergraduates of the University of Portharcourt using strand displacement and amplification (SDA) technique. *Nigerian Health Journal* 12(2):35-38.

Washington, C.W., Koneman, W.E., Allen, S.D., Janda, W.M., Procop, G.W., Schreckenberger, P.C, Woods, G.A. (2006) Mycoplasmas and Ureaplasmas. *Konemans color Atlas and Textbook of Diagnostic Microbiology* 6th Edition. Pp1023-1054.

Wiesenfeld, H.C. and Manhart, L.E. (2017) *Mycoplasma genitalium* in Women: Current Knowledge and Research Priorities for This Recently Emerged Pathogen. *Journal of Infectious Diseases* 216(2):S389-S395.

Wilck, M. B., Wu, Y., Howe, J.G., Crouch, J.G., Edberg, S.C. (2001) Endocarditis caused by culture-negative organisms visible by Brown and Brenn staining: utility of PCR and DNA sequencing for diagnosis. *Journal of Clinical Microbiology* 39:2025–2027.

Woese, C. R., Olsen, G.J., Ibba, M., Soll, D. (2000) Comparisons of complete genome sequences allow the most objective and comprehensive descriptions possible of a lineage's evolution. *Molecular Biology Review* 64:202–236.

Woese, C. R. (1987) Bacterial evolution. *Microbiology Review* 51:221–271.

Woese, C. R., Stackebrandt, E., Macke, T.J., Fox, G.E. (1985) A phylogenetic definition of the major eubacterial taxa. *Systematic Applied Microbiology* 6:143–151.

World Health Organization, (2011) Sexually transmitted infections fact sheet. Available from: *http://www.who.int/mediacentre/factsheets/fs110/en/* accessed on August 25, 2016.

Wroblewski, J.K., Manhart, L.E., Dickey, K.A., Hudspeth, M.K., Totten, P.A. (2006) Comparison of transcription-mediated amplification and PCR assay results for various genital specimen types for detection of *Mycoplasma genitalium*. *Journal of Clinical Microbiology* 44:3306–3312.

Xiao, L., Glass, J.I., Paralanov, V., Yooseph, S., Cassell, G.H., Duffy, L.B., Waites, K.B. (2010) Detection and characterization of human Ureaplasma species and serovars by real time PCR. *Journal of Clinical Microbiology* 48:2715–2723.

Yokoi, S., Maeda, S., Kubota, Y., Tamaki, M., Mizutani, K., Yasuda, M. (2007) The role of *Mycoplasma genitalium* and *Ureaplasma urealyticum* biovar 2 in post gonococcal urethritis. *Clinical Infectious Diseases 45*: 866-871.

Yoshida, T., Deguchi, T., Ito, M., Maeda, S., Tamaki, M., Ishiko, H. (2002) Quantitative detection of *Mycoplasma genitalium* from first-pass urine of men with urethritis and asymptomatic men by real-time PCR. *Journal of Clinical Microbiology* 40:1451–1455.

Yoshida, T., Maeda S., Deguchi T., Ishiko H. (2002) Phylogeny-based rapid identification of Mycoplasma and Ureaplasma from urethritis patients. *Journal of Clinical Microbiology* 40:105-110.

Appendix 1: Ethical Approval

EDO STATE HOSPITALS MANAGEMENT BOARD
P. M. B. 1009
BENIN CITY

Our Ref: A732/T/33

Date: December 23th , 2016,

The Medical Director,
Central Hospital,
Benin City.

RE:CHINYERE EZEANYA

This is to inform you that the person named above has been given the ethical approval to do a study titled " DETECTION AND MOLECULAR CHARACTERIZATION OF MYCOPLASMA GENITALIUM FROM FEMALE STUDENTS IN A TERTIARY INSTITUTION IN BENIN-CITY, NIGERIA. "

She is to collaborate with the Head of Obstetrics and Gynaecology Department for the research.

Thanks.

Dr. Chris Obaseki
Chairman Ethical Committee
Hospital Management Board.

Appendix 2: Informed Consent Form

My name is Ezeanya Chinyere, a PhD student of Department of Medical Laboratory Science, Nnamdi Azikiwe Unversity, Awka. I am conducting a research on a topic captioned, "Molecular characterization of *M.genitalium* from female subjects resident in Benin City, Nigeria". The study is designed to benefit the female society as it will mitigate and prevent transmission of genital pathogens as well as add new knowledge. You will be required to answer some questions which might pertain to your health and social life. You will also be required to provide endocervical swab sample which will be obtained by a clinician. Your participation will not alter your personality.

Voluntary nature of Participation: Participation in the study is voluntary. If you choose to participate you are free to withdraw from the study at any stage without penalty. But my hope is that you will participate fully since your view is important to the success of the study. Also the cost of all tests shall be borne by the researcher.

Confidentiality: All information gathered as well as the outcome of the study will be treated with utmost confidentiality and will not be used against you in any form. Data presented from this information will not in any way reveal individual participation.

Response: I have read and understood the above or had someone read and explain the entire study to me.

Also all gray areas have been fully understood. The nature and benefit of the study is clear and hereby consent to participate in it. Thank you for agreeing to participate in this research.

Signature of Participant---------- Date----------

Signature of Interview---------- Date----------

Subject Number----------

Appendix 3: Research Questionnaire

Molecular Characterization of *M.genitalium* from Female Subjects resident in Benin city

Please tick (√) the box:

MARITAL STATUS: SINGLE ☐ MARRIED ☐

AGE: 15-19 ☐ 20-24 ☐ 25-29 ☐ 30-34 ☐ 35-39 ☐

NUMBER OF CHILDREN: NONE ☐ 1-2 ☐ 3-4 ☐ 5 and above ☐

HIV STATUS: POSITIVE ☐ NEGATIVE ☐ Others, please specify_____

RATE YOUR PERSONAL HYGIENE: POOR ☐ GOOD ☐ EXCELLENT ☐

TOILET USE: PRIVATE TOILET ☐ PUBLIC TOILET ☐

AGE AT FIRST INTERCOURSE: _____

PREVIOUS STD TREATMENT: YES ☐ NO ☐ If YES, When? _____

HAVE YOUR PARTNER BEEN TREATED FOR STD BEFORE? YES ☐ NO ☐

If YES, which infection _____

HAVE YOUR PARTNER TRAVELLED ABROAD BEFORE? YES ☐ NO ☐

IF YES, which country _____

NUMBER OF SEXUAL PARTNER SINCE FIRST INTERCOURSE:

NONE ☐ 1-3 ☐ 4-6 ☐ 7-9 ☐ 10 and above ☐

CONDOM USE: ALWAYS USE A CONDOM ☐

SOMETIMES USE A CONDOM ☐

NEVER USE A CONDOM ☐

ABORTION: YES ☐ NO ☐

HOW ENGLIGTENED ARE YOU ABOUT STD? LITTLE KNOWLEDGE ☐

AVERAGE KNOWLEDGE ☐

HIGHLY ENLIGTHENED ☐

Appendix 4: Composition of Reagents and Kit

1. Quick-DNA™ Universal Kit; Miniprep Kit

Proteinase K & Storage Buffer	4x20 mg
BioFluid & Cell Buffer (Red)	45 ml
Solid Tissue Buffer (Blue)*	22 ml
Genomic Binding Buffer	85 ml
DNA Pre-Wash Buffer *	2x50 ml
g-DNA Wash Buffer	200 ml
DNA Elution Buffer	50 ml
Zymo-Spin™ IIC-XL Columns	200 columns
Collection Tubes	400 tubes

* The Solid Tissue Buffer (Blue) and DNA Pre-Wash Buffer may have formed a precipitate. If this is the case, incubate at 37^{o}C to solubilize. DO NOT MICROWAVE.

2. Multiplex PCR 5X Master Mix

Source: An *E. coli* strain that carries the Taq DNA Polymerase gene from *Thermus aquaticus* YT-1.

Reaction Conditions: 1X Multiplex PCR Master Mix, DNA template and primers in a total reaction volume of 25 or 50 µl.

1X Multiplex PCR Master Mix:

- 20 mM Tris-HCl (pH 8.9 @ 25°C)

- 50 mM KCl 30 mM
- NH4Cl 2.5 mM
- MgCl2 100 units/ml Taq DNA Polymerase
- 0.3 mM each dNTP
- 3.2% glycerol
- 0.08% IGEPAL® CA-630
- 0.07% Tween® 20

Unit Definition: One unit is defined as the amount of enzyme that will incorporate 10 nmol of dNTP into acid-insoluble material in 30 minutes at 75°C.

Appendix 5: Preparation of Reagent

1. Phosphate Buffer Saline (Oxoid, UK)

Formula	**gm/litre**
Sodium chloride	8.0
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2

pH 7.3

Preparation

- Dissolve 10 tablets in 1 litre of sterile distilled water (with 2 cycle of autoclaving).
- Autoclave for 10 minutes at 115°C. The solution will be quite free from insoluble matter.

2. 5% Working Solution of Giemsa Stain

Smear of the specimen made on a clean microscopic slide was allowed to air dry.

The air-dried smear was fixed with methanol for 5 - 7 minutes.

Commercially prepared Giemsa stain was diluted 1:20 in buffered water.

The diluted stain was poured on the slide.

The stained smear was left for 30 minutes.

It was rinsed out with buffered water and left to dry.

Oil immersion objective was used for observation.

<u>Preparation of diluted Giemsa stain 1:20</u>

100 mL of buffered water (pH 7.2) was dispensed into a graduated cylinder.

Using a Pasteur pipette, 5 mL of Giemsa stock solution was collected following filteration of stock solution with filter paper.

Giemsa working solution stain was used within 24 hours of preparation.

Appendix 6: Microscopic Examination

Wet Mount Result of the Endocervical Swab from the Students (N=100)

S/N	Lab No.	EC	CC	PMNLs	TV	BY	PH	Bact.	KOH	WHIFF TEST
1.	S1	+++	-	-	-	-	-	+++	Neg	Neg
2.	S2	+	+++	++	-	-	++	+++	Pos	Pos
3.	S3	+++	-	-	-	-	-	+++	Pos	Neg
4.	S4	++	-	-	-	-	-	++	Neg	Neg
5.	S5	++	-	-	-	-	-	++	Neg	Neg
6.	S6	+++	-	-	-	-	-	+++	Neg	Neg
7.	S7	++	+++	+++	++	-	+	-	Pos	Pos
8.	S8	+++	-	-	-	-	-	+++	Neg	Neg
9.	S9	++	-	-	-	-	-	+++	Neg	Neg
10.	S10	-	+++	++	-	-	+++	+	Pos	Pos
11.	S11	+	+++	+++	-	-	+++	+	Pos	Pos
12.	S16	++	+++	+	-	-	-	+	Pos	Pos
13.	S17	-	+++	+++	-	-	-	-	Pos	Pos
14.	S18	+++	-	-	-	-	+	+++	Neg	Neg
15.	S19	+++	-	++	-	-	-	+++	Neg	Neg
16.	S20	-	+++	+++	-	-	-	+	Pos	Pos
17.	S21	+	+++	+++	-	-	-	++	Pos	Pos
18.	S22	+++	-	-	-	-	-	+++	Neg	Neg
19.	S23	-	+++	-	-	++	-	++	Pos	Pos
20.	S24	++	+++	++	-	-	-	+	Neg	Pos

21.	S25	+++	-	-	-	-	-	-	Neg	Neg
22.	S26	-	+++	++	-	-	+++	-	Pos	Pos
23.	S27	-	+++	+++	++	-	-	-	Neg	Pos
24.	S28	-	+++	++	-	-	++	-	Pos	Pos
25.	S29	++	-	+++	-	-	-	-	Neg	Neg
26.	S30	++	-	+++	-	-	-	-	Neg	Neg
27.	S31	+++	-	+	-	-	-	+++	Neg	Neg
28.	S32	++	-	+++	-	-	-	-	Neg	Neg
29.	S33	+++	-	+	-	-	-	+++	Neg	Neg
30.	S35	+	+++	+	-	-	-	-	Neg	Pos
31.	S36	+	+++	-	-	-	-	-	Neg	Pos
32.	S37	+++	-	+	-	-	++	+++	Pos	Neg
33.	S38	+++	-	+	+	-	-	+++	Neg	Neg
34.	S39	+++	-	++	-	-	-	+++	Neg	Neg
35.	S40	+++	-	+++	-	-	-	+++	Neg	Neg
36.	S43	+	+++	+	-	-	-	+	Neg	Pos
37.	S44	+	+++	+	-	-	-	+	Neg	Pos
38.	S45	+++	-	+	-	-	-	+++	Neg	Neg
39.	S46	++	-	++	-	-	-	+++	Neg	Neg
40.	S47	+++	-	++	-	-	-	+++	Neg	Neg
41.	S48	++	-	+	-	-	-	+++	Neg	Neg
42.	S49	+	+++	++	-	-	-	+	Neg	Pos
43.	S50	++	-	+++	++	-	-	-	Neg	Pos
44.	S51	-	+++	+++	-	-	+	+	Pos	Pos

45.	S52	++	-	+++	-	-	-	+++	Neg	Pos
46.	S53	+++	-	+	-	-	-	-	Neg	Neg
47.	S54	++	+++	++	-	-	-	-	Neg	Pos
48.	S55	+	+++	++	-	-	-	-	Neg	Pos
49.	S56	++	+++	-	-	-	+	-	Pos	Pos
50.	S57	-	+++	+++	-	+	-	-	Pos	Pos
51.	S58	-	+++	-	+	-	+	-	Pos	Pos
52.	S59	+++	-	+++	-	-	-	+++	Neg	Pos
53.	S60	++	+++	++	++	-	+	-	Pos	Pos
54.	S61	++	+	+++	+	-	-	++	Neg	Pos
55.	S62	+	+	+++	++	-	-	+	Neg	Pos
56.	S63	+	-	+++	+++	-	-	+	Neg	Pos
57.	S64	+	-	++	+	-	-	-	Neg	Pos
58.	S65	+++	-	+++	++	-	++	-	Pos	Pos
59.	S66	-	+++	-	++	-	-	+	Pos	Pos
60.	S67	-	+++	-	-	++	-	-	Pos	Pos
61.	S68	++	-	++	-	-	-	+++	Neg	Neg
62.	S69	-	++	++	+	-	+	-	Pos	Pos
63.	S70	+++	-	-	-	-	-	++	Neg	Neg
64.	S71	++	-	+++	++	-	-	-	Neg	Pos
65.	S72	-	+++	++	+	+	-	-	Pos	Pos
66.	S73	++	-	++	+++	-	-	+	Neg	Pos
67.	S74	++	-	+++	+	-	-	-	Neg	Pos
68.	S76	+++	-	+++	++	-	-	++	Neg	Pos

69.	S79	-	+++	++	+	-	+	-	Pos	Pos
70.	S80	+++	-	-	-	-	+	+++	Pos	Neg
71.	S81	+++	-	-	-	-	-	-	Neg	Neg
72.	S82	+++	-	-	-	-	-	-	Neg	Neg
73.	S83	-	+++	+	-	+	-	-	Pos	Pos
74.	S84	-	+++	-	-	++	-	++	Pos	Pos
75.	S85	++	+++	++	-	-	-	+	Neg	Pos
76.	S86	+++	-	-	-	-	-	-	Neg	Neg
77.	S87	-	+++	++	-	-	+++	-	Pos	Pos
78.	S88	-	+++	+++	++	-	-	-	Neg	Pos
79.	S89	-	+++	++	-	-	++	-	Pos	Pos
80.	S90	++	-	+++	-	-	-	-	Neg	Neg
81.	S92	++	-	+++	-	-	-	-	Neg	Neg
82.	S93	+++	-	+	-	-	-	+++	Neg	Neg
83.	S95	+++	-	-	-	-	-	+++	Neg	Neg
84.	S96	+	+++	++	-	-	++	+++	Pos	Pos
85.	S97	+++	-	-	-	-	-	+++	Pos	Neg
86.	S98	++	-	-	-	-	-	++	Neg	Neg
87.	S99	++	-	-	-	-	-	++	Neg	Neg
88.	S100	+++	-	-	-	-	-	+++	Neg	Neg
89.	S103	++	+++	+++	++	-	+	-	Pos	Pos
90.	S107	+++	-	+++	-	-	-	+++	Neg	Neg
91.	S108	+	+++	+	-	-	-	+	Neg	Pos
92.	S109	+	+++	+	-	-	-	+	Neg	Pos

93.	S110	+++	-	+	-	-	-	+++	Neg	Neg
94.	S111	++	-	++	-	-	-	+++	Neg	Neg
95.	S112	+++	-	++	-	-	-	+++	Neg	Neg
96.	S113	++	-	+	-	-	-	+++	Neg	Neg
97.	S114	+	+++	++	-	-	-	+	Neg	Pos
98.	S115	++	-	++	-	-	-	+++	Neg	Neg
99.	S116	++	-	+++	-	-	-	+++	Neg	Pos
100.	S118	+++	-	+	-	-	-	-	Neg	Neg

Key:

EC- Epithelial cells

CC- Clue cells

PMNLs- Polymorphonuclear lymphocytes

TV-*Trichomonas vaginalis*

BY-Budding Yeast

PH- Pseudohyphae

KOH-Potassium hydroxide test

Bact.-Bacteria

Neg-Negative

Pos-Positive

Gram Stain Result of the Endocervical Swab from the Students (N=100)

S/N	Lab No.	GPC	GPB	GNCB	GNR	CC	EC	PH	Others (Specify)
1.	S1	-	+++	-	-	-	+++	-	Negative
2.	S2	-	-	+++	-	+++	-	++	Negative
3.	S3	-	++	-	-	-	+++	-	Negative
4.	S4	-	+++	-	-	-	+++	-	Negative
5.	S5	-	+++	-	-	-	++	-	Negative
6.	S6	-	+++	-	-	-	+++	-	Negative
7.	S7	-	+	++	-	+++	+	++	Negative
8.	S8	-	++	-	-	-	++	-	Negative
9.	S9	-	+++	-	-	-	+++	-	Negative
10.	S10	-	-	+++	-	+++		+	Negative
11.	S11	-	-	++	-	+++	+++	++	Negative
12.	S16	-	-	+++	-	+++	+	-	Negative
13.	S17	-	-	++	-	+++	-	-	Negative
14.	S18	-	+++	-	-	-	+++	-	Negative
15.	S19	-	+++	-	-	-	+++	-	Negative
16.	S20	-	-	++	-	+++	-	-	Negative
17.	S21	-	-	+++	-	+++	-	-	Negative
18.	S22	-	+++	-	-	-	+++	-	Negative
19.	S23	-	+	+++	-	++	-	-	Negative
20.	S24	-	-	++	-	+++	+	-	Negative
21.	S25	-	+++	-	-	-	+++	-	Negative

22.	S26	-	-	+++	-	+++	-	++	Negative
23.	S27	-	-	+++	-	++	-	-	TV
24.	S28	-	-	+++	-	+++	-	+	Negative
25.	S29	-	+++	-	-	-	+++	-	Negative
26.	S30	-	+++	-	-	-	+++	-	Negative
27.	S31	-	+++	-	-	-	+++	-	Negative
28.	S32	-	+	+++	-	+++	+	-	Negative
29.	S33	-	++	-	-	-	+++	-	Negative
30.	S35	-	-	+++	-	+++	-	-	Negative
31.	S36	-	-	+++	-	+++	-	-	Negative
32.	S37	-	+++	-	-	-	+++	++	Negative
33.	S38	-	+++	-	-	-	+++	-	Negative
34.	S39	-	+++	-	-	-	+++	-	Negative
35.	S40	-	+++	-	-	-	+++	-	Negative
36.	S43	-	+	++	-	+++	-	-	Negative
37.	S44	-	-	+++	-	+++	+	-	Negative
38.	S45	-	++	-	-	-	+++	-	Negative
39.	S46	-	++	-	-	-	+++	-	Negative
40.	S47	-	+++	-	-	-	+++	++	Negative
41.	S48	-	+++	-	-	-	+++	-	Negative
42.	S49	-	-	++	-	++	+	-	Negative
43.	S50	-	+	-	-	-	++	-	TV
44.	S51	-	-	+++	-	+++	-	-	Negative
45.	S52	-	-	++	-	+++	++	-	TV

46.	S53	-	-	-	-	+++	+	-	TV
47.	S54	-	++	-	-	++	+++	-	TV
48.	S55	-	-	+++	-	+++	+	-	Negative
49.	S56	-	-	+++	-	+++	-	-	Negative
50.	S57	-	-	+++	-	+++	-	-	Negative
51.	S58	-	+++	-	-	-	+++	-	TV
52.	S59	-	+	+++	-	+++	-	-	Negative
53.	S60	-	++	-	-	+	+++	-	Negative
54.	S61	-	+++	-	-	-	+++	-	TV
55.	S62	-	+++	-	-	-	+++	-	TV
56.	S63	-	++	+	-	-	+++	-	Negative
57.	S64	-	+	-	-	-	+++	++	TV
58.	S65	-	-	+++	-	+++	-	-	TV
59.	S66	-		+++	-	+++	-	+	Negative
60.	S67	-	+++	-	-	-	+++	-	Negative
61.	S68	-	-	+++	-	+++	-	-	Negative
62.	S69	-	+++	-	-	-	+++	++	Negative
63.	S70	-	-	-	-	-	+++	-	TV
64.	S71	-	-	++	-	+++	-	-	Negative
65.	S72	-	+++	-	-	-	+++	-	Negative
66.	S73	-	+++	-	-	-	+++	-	TV
67.	S74	-	+++	-	-	-	+++	-	TV
68.	S76	-	-	+++	-	+++	-	-	Negative
69.	S79	-	+++	-	-	-	+++	-	Negative

70.	S80	-	+++	-	-	-	+++	-	Negative
71.	S81	-	+++	-	-	-	+++	-	Negative
72.	S82	-	-	+++	-	+++	-	-	Negative
73.	S83	-	+	+++	-	+++	-	-	Negative
74.	S84	-	++	-	-	+	+++	-	Negative
75.	S85	-	+++	-	-	-	+++	-	TV
76.	S86	-	+++	-	-	-	+++	-	TV
77.	S87	-	++	+	-	-	+++	-	Negative
78.	S88	-	+	-	-	-	+++	++	TV
79.	S89	-	-	+++	-	+++	-	++	Negative
80.	S90	-	-	+++	-	++	-	-	TV
81.	S92	-	-	+++	-	+++	-	+	Negative
82.	S93	-	+++	-	-	-	+++	-	Negative
83.	S95	-	+++	-	-	-	+++	-	Negative
84.	S96	-	+++	-	-	-	+++	-	Negative
85.	S97	-	+	+++	-	+++	+	-	Negative
86.	S98	-	++	-	-	-	+++	-	Negative
87.	S99	-	-	+++	-	+++	-	-	Negative
88.	S100	-	-	+++	-	+++	-	-	Negative
89.	S103	-	+++	-	-	-	+++	++	Negative
90.	S107	-	-	+++	-	+++	-	++	Negative
91.	S108	-	++	-	-	-	+++	-	Negative
92.	S109	-	+++	-	-	-	+++	-	Negative
93.	S110	-	+++	-	-	-	++	-	Negative

94.	S111	-	+++	-	-	-	+++	-	Negative
95.	S112	-	+	++	-	+++	+	++	Negative
96.	S113	-	-	++	-	+++	-	-	Negative
97.	S114	-	-	+++	-	+++	-	-	Negative
98.	S115	-	+++	-	-	-	+++	-	Negative
99.	S116	-	+	+++	-	++	-	-	Negative
100.	S118	-	-	++	-	+++	+	-	Negative

Key:

GPC- Gram positive cocci

GPB-Gram positive bacilli

GNCB-Gram negative coccobacilli

GNR-Gram negative rod

CC-Clue cells

EC-Epithelial cells

PH-Pseudohyphae

TV-*Trichomonas vaginalis*

Giemsa Stain Result of the Endocervical Swab from Students (n=100)

S/N	Lab No.	EC	CC	Bact.	PMNLs	TV	CT	Others (Specify)
1.	S1	+++	-	+++	-	-	-	**Negative**
2.	S2	-	-	-	-	-	++	**Negative**
3.	S3	+	++	+++	-	-	-	**Negative**
4.	S4	+++	-	+++	-	-	-	**Negative**
5.	S5	-	-	-	-	-	++	**Negative**
6.	S6	+++	-	+++	-	-	-	**Negative**
7.	S7	+++	-	+	-	-	-	**Negative**
8.	S8	+++	-	+++	-	-	-	**Negative**
9.	S9	+	+++	+++	-	-	-	**Negative**
10.	S10	+++	-	-	-	-	-	**PMNLs**
11.	S11	+++	-	+++	-	-	-	**PMNLs**
12.	S16	+++	-	+++	-	-	-	**Yeast cell**
13.	S17	++	-	-	-	-	-	**Negative**
14.	S18	+	+++	-	-	-	-	**Negative**
15.	S19	+++	-	+++	-	-	-	**Negative**
16.	S20	-	-	-	-	-	++	**Negative**
17.	S21	+	++	+++	-	-	-	**Negative**
18.	S22	+++	-	+++	-	-	-	**Negative**
19.	S23	-	-	-	-	-	++	**Negative**
20.	S24	+++	-	+++	-	-	-	**Negative**

21.	S25	+++	-	+	-	-	-	**Negative**
22.	S26	+	+++	+++	-	-	-	**Negative**
23.	S27	+++	-	-	-	-	-	**PMNLs**
24.	S28	+++	-	+++	-	-	-	**PMNLs**
25.	S29	+++	-	+++	-	-	-	**Yeast cell**
26.	S30	++	-	-	-	-	-	**Negative**
27.	S31	+	+++	-	-	-	-	**Negative**
28.	S32	-	+++	+++	-	-	-	**Negative**
29.	S33	+	-	-	-	-	++	**Negative**
30.	S35	-	-	-	-	-	++	**Negative**
31.	S36	++	-	-	-	-	++	**Negative**
32.	S37	-	-	-	-	-	++	**Negative**
33.	S38	++	-	-	-	+++	-	**Negative**
34.	S39	+++	-	+++	-	-	-	**Negative**
35.	S40	-	-	-	-	-	++	**Negative**
36.	S43	+	-	-	-	-	++	**Negative**
37.	S44	+	+++	+++	-	-	-	**Negative**
38.	S45	+++	-	-	-	-	-	**PMNLs**
39.	S46	+++	-	+++	-	-	-	**PMNLs**
40.	S47	+++	-	+++	-	-	-	**Yeast cell**
41.	S48	++	-	-	-	-	-	**Negative**
42.	S49	+	+++	-	-	-	-	**Negative**

43.	S50	+++	-	+++	-	-	-	**Negative**
44.	S51	+++	-	+++	-	-	-	**Negative**
45.	S52	+++	-	+++	-	-	-	**Negative**
46.	S53	+++	-	+++	-	-	-	**Negative**
47.	S54	+++	-	+++	-	-	-	**Negative**
48.	S55	+++	-	+++	-	-	-	**Negative**
49.	S56	+	+++	+++	-	-	-	**Negative**
50.	S57	+++	-	+++	-	-	-	**Negative**
51.	S58	+++	-	+++	-	-	-	**Negative**
52.	S59	+++	-	+++	-	-	-	**Negative**
53.	S60	+++	-	+++	-	-	-	**Negative**
54.	S61	+++	-	+++	-	-	-	**Negative**
55.	S62	+++	-	+++	-	-	-	**Negative**
56.	S63	+++	-	+++	-	-	-	**Negative**
57.	S64	+++	-	+++	-	-	-	**Negative**
58.	S65	+++	-	+++	-	-	-	**Negative**
59.	S66	+++	-	+++	-	-	-	**Negative**
60.	S67	+++	-	+++	-	-	-	**Negative**
61.	S68	+++	-	+++	-	-	-	**Negative**
62.	S69	+++	-	+++	-	-	-	**Negative**
63.	S70	+++	-	+++	-	-	-	**Negative**
64.	S71	+++	-	+++	-	-	-	**Negative**
65.	S72	+++	-	+++	-	-	-	**Negative**
66.	S73	+++	-	+++	-	-	-	**Negative**

67.	S74	+++	-	+++	-	-	-	**Negative**
68.	S76	++	-	-	-	+++	-	**Negative**
69.	S79	+++	-	+++	-	-	-	**Negative**
70.	S80	++	-	-	-	+++	-	**Negative**
71.	S81	+++	-	+++	-	-	-	**Negative**
72.	S82	++	-	-	-	+++	-	**Negative**
73.	S83	+++	-	+++	-	-	-	**Negative**
74.	S84	++	-	-	-	+++	-	**Negative**
75.	S85	+++	-	+++	-	-	-	**Negative**
76.	S86	++	-	-	-	+++	-	**Negative**
77.	S87	+++	-	+++	-	-	-	**Negative**
78.	S88	++	-	-	-	+++	-	**Negative**
79.	S89	+++	-	+++	-	-	-	**Negative**
80.	S90	++	-	-	-	+++	-	**Negative**
81.	S92	+++	-	+++	-	-	-	**Negative**
82.	S93	++	-	-	-	+++	-	**Negative**
83.	S95	+++	-	+++	-	-	-	**Negative**
84.	S96	++	-	-	-	+++	-	**Negative**
85.	S97	+++	-	+++	-	-	-	**Negative**
86.	S98	+	-	-	-	-	++	**Negative**
87.	S99	+++	-	+++	-	-	-	**Negative**
88.	S100	++	-	++	-	-	-	**Negative**
89.	S103	-	-	-	-	-	++	**Negative**
90.	S107	++	-	-	-	+++	-	**Negative**

91.	S108	+++	-	+++	-	-	-	**Negative**
92.	S109	++	-	-	-	+++	-	**Negative**
93.	S110	+++	-	+++	-	-	-	**Negative**
94.	S111	+++	-	+++	-	-	-	**PMNLs**
95.	S112	+++	-	+++	-	-	-	**PMNLs**
96.	S113	+++	-	+++	-	-	-	**PMNLs**
97.	S114	++	-	-	-	+++	-	**Negative**
98.	S115	+++	-	+++	-	-	-	**Negative**
99.	S116	+++	-	+++	-	-	-	**PMNLs**
100.	S118	+++	-	+++	-	-	-	**PMNLs**

Key:

EC- Epithelial cells

CC-Clue cells

Bact.-Bacteria

PMNLs- Polymorphonuclear lymphocytes

TV-*Trichomonas vaginalis*

CT- *Chlamydia trachomatis*

Wet Mount Result of the Endocervical Swab from the Hospital Patients (N=30)

S/N	Lab No.	EC	CC	PMNLs	TV	BY	PH	Bact.	KOH	WHIFF TEST
1.	H1	+	+++	+++	-	-	+++	+	Pos	Pos
2.	H2	++	+++	+	-	-	-	+	Pos	Pos
3.	H3	-	+++	+++	-	-	-	-	Pos	Pos
4.	H4	+++	-	-	-	-	+	+++	Neg	Neg
5.	H5	+++	-	++	-	-	-	+++	Neg	Neg
6.	H6	-	+++	++	-	-	++	-	Pos	Pos
7.	H7	++	-	+++	-	-	-	-	Neg	Neg
8.	H8	++	-	+++	-	-	-	-	Neg	Neg
9.	H9	+++	-	+	-	-	-	+++	Neg	Neg
10.	H10	++	-	+++	-	-	-	-	Neg	Neg
11.	H11	+++	-	+	-	-	-	+++	Neg	Neg
12.	H15	+	+++	+	-	-	-	-	Neg	Pos
13.	H16	+	+++	-	-	-	-	-	Neg	Pos
14.	H17	++	-	++	-	-	-	+++	Neg	Neg
15.	H18	+++	-	++	-	-	-	+++	Neg	Neg
16.	H19	++	-	+	-	-	-	+++	Neg	Neg
17.	H20	+	+++	++	-	-	-	+	Neg	Pos
18.	H21	++	-	+++	++	-	-	-	Neg	Pos
19.	H22	-	+++	+++	-	-	+	+	Pos	Pos
20.	H23	+	-	+++	+++	-	-	+	Neg	Pos
21.	H24	+	-	++	+	-	-	-	Neg	Pos

22.	H25	+++	-	+++	++	-	++	-	Pos	Pos
23.	H26	-	+++	-	++	-	-	+	Pos	Pos
24.	H27	-	+++	-	-	++	-	-	Pos	Pos
25.	H28	++	-	++	-	-	-	+++	Neg	Neg
26.	H29	-	++	++	+	-	+	-	Pos	Pos
27.	H30	+++	-	-	-	-	-	++	Neg	Neg
28.	H31	++	-	+++	++	-	-	-	Neg	Pos
29.	H32	++	-	++	-	-	-	+++	Neg	Neg
30.	H33	+++	-	++	-	-	-	+++	Neg	Neg

Key:

EC- Epithelial cells

CC- Clue cells

PMNLs- Polymorphonuclear lymphocytes

TV-*Trichomonas vaginalis*

BY-Budding Yeast

PH- Pseudohyphae

KOH-Potassium hydroxide test

Bact.-Bacteria

Neg-Negative

Pos-Positive

Gram Stain Result of the Endocervical Swab from the Hospital Patients (N=30)

S/N	Lab No.	GPC	GPB	GNCB	GNR	CC	EC	PH	Others (Specify)
1.	H1	-	+++	-	-	-	+++	-	Negative
2.	H2	-	+++	-	-	-	+++	-	Negative
3.	H3	-	-	++	-	+++	-	-	Negative
4.	H4	-	-	+++	-	+++	-	-	Negative
5.	H5	-	+++	-	-	-	+++	-	Negative
6.	H6	-	+	+++	-	++	-	-	Negative
7.	H7	-	++	-	-	-	+++	-	Negative
8.	H8	-	-	+++	-	+++	-	-	Negative
9.	H9	-	-	+++	-	+++	-	-	Negative
10.	H10	-	+++	-	-	-	+++	++	Negative
11.	H11	-	+++	-	-	-	+++	-	Negative
12.	H15	-	+++	-	-	-	+++	-	Negative
13.	H16	-	+++	-	-	-	+++	-	Negative
14.	H17	-	+	++	-	+++	-	-	Negative
15.	H18	-	-	+++	-	+++	+	-	Negative
16.	H19	-	++	-	-	-	+++	-	Negative
17.	H20	-	+++	-	-	-	+++	++	Negative
18.	H21	-	+++	-	-	-	+++	-	Negative
19.	H22	-	-	++	-	++	+	-	Negative
20.	H23	-	+	-	-	-	++	-	TV
21.	H24	-	-	+++	-	+++	-	-	Negative

22.	H25	-	-	++	-	+++	++	-	TV
23.	H26	-	-	-	-	+++	+	-	TV
24.	H27	-	++	-	-	++	+++	-	TV
25.	H28	-	-	+++	-	+++	+	-	Negative
26.	H29	-	-	+++	-	+++	-	-	Negative
27.	H30	-	-	+++	-	+++	-	-	Negative
28.	H31	-	+++	-	-	-	+++	-	TV
29.	H32	-	+	+++	-	+++	-	-	Negative
30.	H33	-	++	-	-	+	+++	-	Negative

Key:

GPC- Gram positive cocci

GPB-Gram positive bacilli

GNCB-Gram negative coccobacilli

GNR-Gram negative rod

CC-Clue cells

EC-Epithelial cells

PH-Pseudohyphae

TV-*Trichomonas vaginalis*

Giemsa Stain Result of the Endocervical Swab from Hospital Patients (n=30)

S/N	Lab No.	EC	CC	Bact.	PMNLs	TV	CT	Others (Specify)
1.	H1	-	-	-	-	-	++	**Negative**
2.	H2	+	-	-	-	-	++	**Negative**
3.	H3	+	+++	+++	-	-	-	**Negative**
4.	H4	+++	-	-	-	-	-	**PMNLs**
5.	H5	+++	-	+++	-	-	-	**PMNLs**
6.	H6	+	+++	+++	-	-	-	**Negative**
7.	H7	+++	-	+++	-	-	-	**Negative**
8.	H8	+++	-	+++	-	-	-	**Negative**
9.	H9	+++	-	+++	-	-	-	**Negative**
10.	H10	+++	-	+++	-	-	-	**Negative**
11.	H11	+++	-	+++	-	-	-	**Negative**
12.	H15	+++	-	+++	-	-	-	**Negative**
13.	H16	+++	-	+++	-	-	-	**Negative**
14.	H17	+++	-	+++	-	-	-	**Negative**
15.	H18	+++	-	+++	-	-	-	**Negative**
16.	H19	+++	-	+++	-	-	-	**Negative**
17.	H20	+++	-	+++	-	-	-	**Negative**
18.	H21	+++	-	+++	-	-	-	**Negative**
19.	H22	+++	-	+++	-	-	-	**Negative**
20.	H23	+++	-	+++	-	-	-	**Negative**
21.	H24	++	-	-	-	+++	-	**Negative**

22.	H25	+++	-	+++	-	-	-	**Negative**
23.	H26	++	-	-	-	+++	-	**Negative**
24.	H27	+++	-	+++	-	-	-	**Negative**
25.	H28	++	-	-	-	+++	-	**Negative**
26.	H29	+++	-	+++	-	-	-	**Negative**
27.	H30	++	-	-	-	+++	-	**Negative**
28.	H31	+++	-	+++	-	-	-	**Negative**
29.	H32	++	-	-	-	+++	-	**Negative**
30.	H33	+++	-	-	-	-	-	**PMNLs**

Key:

EC- Epithelial cells

CC-Clue cells

Bact.-Bacteria

PMNLs- Polymorphonuclear lymphocytes

TV-*Trichomonas vaginalis*

CT- *Chlamydia trachomatis*