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A NOVL DETECTION OF BACTERIAL COMMUNITY IN VAGINA BY METAGENOMIC AND MOLECULAR DETECTION OF A NEW LOCAL STRAIN OF MYCOPLASMA GENITALIUM

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Abstract
Metagenomic is a powerful genomic tool that allows the genomic analysis of microorganisms present in various environments. It enables the study of genetic information, including sequences and genomes of cultivable and non-culturable microorganisms that constitute the vast majority of organisms in many environments. In this study, 30 samples of vaginal swabs were taken by a specialized gynecologist using a sterile cotton and speculum swabs from women in Mosul city visiting Al-Khansa Teaching Hospital and Al-Batoul Hospital, and other outpatient clinics. Samples were collected from women who use Intrauterine Device (IUD) and have not used antibiotics, from the ages between 25-40 years and to determine the microbial environment by using metagenomic analysis The results of Mitogenomics NGS showed a variation in the number of bacteria present in the environment of the vagina, as it was found that there are 14 different types of bacteria, some of which are considered normal flora or pathogenic, including Mycoplasma. PCR was also used to detect the presence of Mycoplasma by targeting a specific fragment from the 16SRNA gene. The results showed that 7 samples of vaginal swabs gave a positive result indicating a presence ratio of 23.3% for the presence of Mycoplasma. Our results indicate that the presence of IUD in women is often accompanied by genital tract infections, depending on personal hygiene. Our results show that molecular detection of Mycoplasma via specific primers in PCR is efficient in the direct detection in vaginal swabs.
Keywords: metagenomic, Antibiotics, Genital tract infections, PCR, Mycoplasma genitalium

Introduction

Mycoplasmas are the smallest free-living, self-replicating organisms. They are widespread in nature as parasites of humans, mammals, reptiles, fish, arthropods and plants. Mycoplasma genitalium is considered the smallest self-replicating cell, with a genome consisting of 580,074 bp, and is theorized to approximate the essential complement of genes necessary to sustain life. M. genitalium was first isolated in 1981 from human male urethral cultures and identified as a causative agent of nongonococcal urethritis. Since that time numerous reports have linked M. genitalium to additional genitourinary symptoms, including endometritis, salpingitis, cervicitis, and pelvic inflammatory disease as well as a range of other pathologies such as arthritis, pneumonia, AIDS progression, chronic fatigue, and autoimmune disorders, Mycoplasmas are capable of invading human target cells and persisting and replicating for extended periods intracellularly (Gnanadurai & Fifer(2020)). Mycoplasma genitalium is

Member of genital mycoplasmas, which is emerging as an important causative agent of sexually transmitted infections both in males and females. The advent of polymerase chain reaction and other molecular methods have made studies on M. genitalium more feasible, which is otherwise a difficult organism to isolate. Besides Chlamydia trachomatis, M. genitalium is now an important and established cause of non gonococcal urethritis (NGU) in men. Multiple studies have also shown a positive association of M. genitalium with mucopurulent cervicitis and vaginal discharge in females as well. The evidences for M. genitalium pelvic inflammatory diseases (PID) and infertility are quite convincing and indicate that this organism has potential to cause ascending infection. Lack of clear association with M. genitalium has been reported for bacterial vaginosis and adverse pregnancy outcomes Although there are no guidelines available regarding treatment, macrolide group of antimicrobials appear to be more effective than tetracyclines. The lack of a cell wall makes M. genitalium intrinsically resistant to antibiotics acting at this level, such as beta-lactams, The present review provides an overview of the epidemiology, pathogenesis, clinical presentation and management of sexually transmitted infections due to M. genitalium (Zhang et al.,2023).

Mycoplasma genitalium belongs to the class Mollicutes and is the smallest prokaryote capable of independent replication. It was originally isolated from the urethras of two men with non-gonococcal urethritis (NGU). M. genitalium lacks a cell wall and has a characteristic pear/fask shape with a terminal tip organelle. This organelle enables M. genitalium to glide along and adhere to moist/mucous surfaces, including host cells. M. genitalium has minimal metabolism, and when compared to the other genital mycoplasmas, has the ability to metabolise glucose (Roshina Gnanadurai & Helen Fifer,2020).

Mycoplasma genitalium is a globally important sexually transmitted pathogen. Men infected with M. genitalium frequently present with dysuria, while women may present with or without urogenital symptoms. In some populations, M. genitalium is significantly associated with HIV-1 infection, and is also an etiological agent in pelvic inflammatory disease. (Van et al.,2020).

Mycoplasma genitalium can be detected using PCR-based methods. Several studies have developed and validated PCR assays for the detection and quantification of MG, These assays utilize different targets, such as the mgpB gene, the 23S rRNA gene, and the parC gene. Some assays also allow for the simultaneous detection of macrolide and fluoroquinolone resistance-associated mutations in MG . These PCR assays have been used to detect MG in various sample types, including clinical isolates and DNA extracts from laboratory-cultured strains and clinical samples. Overall, PCR-based methods provide a reliable and sensitive approach for the detection and quantification of MG, as well as the identification of antimicrobial resistance-associated mutations (Cheng et al.,2023).

Mycoplasma genitalium-specific primers have been developed in several studies. Hu Jin et al. designed primers and a probe that can specifically bind to the mycoplasma genitalium outer membrane protein B gene and the human beta-globin gene, resulting in improved detection sensitivity and specificity (Hu et al.,2020)

Diagnosis method using Metagenomics is a powerful genomic tool that allows genomic analysis of microorganisms found in different environments. It enables the study of genetic information, including sequences and genomes of cultivable and non-culturable microorganisms, which constitute the vast majority of organisms in many environments. Metagenomics has greatly advanced studies of microbial ecology, evolution, and diversity over the past years, providing insights into the physiology, ecology, and interactions of environmental microorganisms. By directly accessing the genetic content of entire microbial communities, genetics has facilitated the discovery of novel genes, gene products and metabolic pathways, contributing to the development of better culture strategies and (Nnanake et al.,2023) enhancing our understanding of bioremediation processes Metagenomics plays a critical role in understanding Mycoplasma biology and evolution and in identifying potential drug targets against Mycoplasma genitalium, a sexually transmitted pathogen that causes urethritis and pelvic inflammatory disease. Studies have used bioinformatics tools to analyze hypothetical proteins, and model protein structures (Mohammad et al.,2022). Application: metagenomics is a versatile branch of science, having two basic approaches: (A) taxonomic application (sequence-based analysis) and (B) Functional application (Function driven analysis) or a c ombination of both, depending on the requirement of the objective (Navgire et al.,2022).

Due to the importance of investigating the vaginal microbial community, the research aimed to uncover it using metagenomics.

Materials and methods:

Collection of clinical specimens:

Between July and November 2023, approximately 30 samples (vaginal swab) were collected from women in the city of Mosul/Iraq visiting Al-Khansa and Al-Batoul hospitals in addition to outpatient clinics. The age group was between 25-40 years, and those who suffer from vaginal infections, wear an IUD, and did not administer antibiotics.

The swabs were taken using sterile cotton swabs with the help of a specialist gynecologist. Speculum was used to take the swabs in order to reduce contamination with natural flora. Samples were placed in 2mL normal saline and placed on a cool pack and transported directly to the laboratory to perform the molecular detection.

Detection oF bacteria metagenomic next-generation sequencing (NGS):

Metagenomics involves the use of advanced tools and techniques to analyze complex microbial communities. Various approaches have been proposed to enhance this process. One method involves using the Metagenomic Assessment Tool Analyzer (META) to simulate the data and evaluate different sequencing platforms and analysis algorithms, providing insights into resource usage and performance. Additionally, machine learning techniques have been applied to improve feature selection in metagenomic data, with a focus on interpretability and performance improvement. Another innovative method involves variable importance scoring and Neyman-Pearson detection to identify key metagenomic features, leading to improved taxonomic results and stability in subsequent clinical trials. Furthermore, the effect of ancient DNA damage on taxonomic assignment has been studied in metagenomics, highlighting the The importance of choosing the right classification software tailored to specific research questions (Pellegrini & Thomas, 2023). After performing the DNA extraction process, To understand and study the microbial community in the vagina, One of the samples was sent from women suffering from vaginal infections as a result of the presence of an IUD to ZYMO RESEARCH EUROPE, where the samples were processed, analyzed, and the quality of the sample was assessed, and the sample was prepared for sequencing using an NGS device. Following are the fundamental steps involved in a metagenomics experiment: sample collection This is the very first and essential step to begin a metagenomics project where the particular sample to be examined is selected and used for DNA isolation.

extraction of DNA

DNA extraction also used in polymerase chain reaction: Genomic DNA was extracted directly from samples (swabs) using a genomic DNA isolation kit provided by Geneaid. Steps were followed as recommended by the manufacturer. The concentration and purity of genomic DNA were measured and the DNA was then stored at −20°C until further used. Firstly 0.9 microliters of cultured bacterial cells was add into 1.5 ml Eppendorff tubes and then place them in centrifuge at 16,000 rpm for 1 minute to sediment the bacteria and get rid of the supernatant, then 180 µl of GT buffer was add, then the tube placed in the electrophoresis. Twenty µl of Proteinase K was add to the tube containing the sediment, then incubate the tubes at a temperature of 60°C for 10 minutes, the tubes are stirred every 3 minutes. Two hundred µl of GB buffer was add to the tubes containing the sample and mixed well for 10 minutes ,then incubated at 70°C for 10 minutes .Two hundred µl of 100% ethanol, was mix well in the stirring device, and then placed the mixture in the vortex stirring device, where the mixture is then placed in the filter containing the special column and then centrifuged at 16,000 rpm for 2 minutes, the floating part was removed, then the filter containing the column was replaced with a new column. Four hundred µL was added to the mixture of W1 solution, then centrifuge at 16,000 rpm for 30 seconds and discard the clear solution. Then 600 µl of W2 solution was

added, then centrifuged and the solution was discarded while the filter was kept. Centrifugation was repeated for 3 minutes at 16,000 rpm, then 100 µL from the standard elution buffer was added to the extracted DNA and stored at -20°C. Nanodrops was used to measure the concentration and purity of DNA (Abdulrazzaq et al.,2022).

Targeted Library Preparation: Bacterial 16S ribosomal RNA gene targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA). In most cases, the bacterial 16S primers amplified the V3-V4 region of the 16S rRNA gene. These primers have been custom-designed by Zymo Research to provide the best coverage of the 16S gene while maintaining high sensitivity. Fungal ITS gene targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit with custom ITS2 primers substituted for 16S primers. The sequencing library was prepared using an innovative library preparation process in which PCR reactions were performed in real-time PCR machines to control cycles and therefore limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned with the Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA), then quantified with TapeStation®(Agilent Technologies, Santa Clara, CA) and Qubit® (Thermo Fisher Scientific, Waltham, WA).

Control Samples: The ZymoBIOMICS® Microbial Community Standard (Zymo Research, Irvine, CA) was used as a positive control for each DNA extraction, if performed. The ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA) was used as a positive control for each targeted library preparation. Negative controls (i.e. blank extraction control, blank library preparation control) were included to assess the level of bioburden carried by the wet-lab process.

Sequencing: The final library was sequenced on Illumina® Nextseq™ with a P1 reagent kit (600 cycles). The sequencing was performed with 30% PhiX spike-in.

Bioinformatics Analysis: Unique amplicon sequences variants were inferred from raw reads using the DADA2 pipeline (Callahan et al, 2016). Potential sequencing errors and chimeric sequences were also removed with the Dada2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database, a 16S database that is internally designed and curated, as reference. Composition visualization, alpha-diversity, and beta-diversity analyses were performed with Qiime v.1.9.1 (Caporaso et al., 2010). If applicable, taxonomy that have significant abundance among different groups were identified by LEfSe (Segata et al., 2011) using default settings. Other analyses such as heatmaps, Taxa2ASV Deomposer, and PCoA plots were performed with internal scripts.

Absolute Abundance Quantification*: If performed, a quantitative real-time PCR was set up with a standard curve. The standard curve was made with plasmid DNA containing one copy of the 16S gene and one copy of the fungal ITS2 region prepared in 10-fold serial dilutions. The primers used were the same as those used in Targeted Library Preparation. The equation generated by the plasmid DNA standard curve was used to calculate the number of gene copies in the reaction for each sample. The PCR input volume was used to calculate the number of gene copies per microliter in each DNA sample. The resulting values are shown in the gene_copies column of the absolute abundance results table.

The number of genome copies per microliter DNA sample (genome_copies) was calculated by dividing the gene copy number by an assumed number of gene copies per genome. The value used for 16S copies per genome is 4. The value used for ITS copies per genome is 200. The amount of DNA per microliter DNA sample (DNA_ng) was calculated using an assumed genome size of 4.64 x 106 bp, the genome size of Escherichia coli, for 16S samples, or an assumed genome size of 1.20 x 107 bp, the genome size of Saccharomyces cerevisiae, for ITS samples. This calculation is shown below:

Calculated Total DNA = Calculated Total Genome Copies × Assumed Genome Size (4.64 × 106 bp) × Average Molecular Weight of a DNA bp (660 g/mole/bp) ÷ Avogadros Number (6.022 x 1023/mole)

*Please note that absolute abundance quantification is performed only for 16S and ITS projects.

Polymerase chain reaction:

The primers were prepared according to the manufacturer's instructions by dissolving each primer in nuclease-free water to obtain a stock solution of 100µM according to the manufacture, 10µM forward and reverse primers were prepared from the stock solution and used in PCR experiments (Khaleel et al.,2023).

Table1: Primers for PCR deduced from M.genitalium 16SRNA gene sequence (Jensen et al., 2003).

Primer Name	Primer sequence 5'-3'	Product size (bp)
MG16-45F	TAC ATG CAA GTC GAT CGG AAG TAG C	477bp
MG16-181R	ACC CTT GCA GGT CCT TTC AAC TTT A	

PCR was performed in a 20 µl reaction using GoTaq G2 Green Master Mix provided by Promega (USA). Specific Primers used by Jensen *et al*. (2003) [3] were used for PCR: primers MG16-45F (forward; 5′- TAC ATG CAA GTC GAT CGG AAG TAG C -3′) and MG16-181R (reverse; 5′- ACC CTT GCA GGT CCT TTC AAC TTT A - 3′). A 477bp fragment of the *M.genitalium* 16SRNA gene was amplified using this primer set. The final concentration of primers used in the reaction was (1 µM each) and the total amount of template DNA (100 ng) were added as recommended by the manufacturer. The PCR program for the oligonucleotide primers sequences *M.genitalium* 16SRNA gene was set as follows: initial denaturation at 95°C for 1 minutes followed by 1 cycles of amplification including a denaturation step at 95°C for 30 seconds, annealing at 58°C for 30 sec and extension at 72 °C for 1 min. The final extension step was set at 72 °C. PCR products were separated on a 1% agarose gel and stained with Midori Green Advance DNA stain. A 100 bp DNA marker (New England Biolabs, UK) was used as a molecular weight marker.

Use of PCR Master mix by Promega, USA, according to the instructions provided by the company. Each tube contained the basic components of the PCR, which are Taq DNA polymerase and a mixture of nitrogenous bases d TTP, d ATP, d CTP, d GTP. It also contains Tris-Hcl, d Gcl. MgCl, thus adding only the

DNA, primers, and free nuclease water to the PCR tube to perform the PCR. Table (3) shows the total volume of the PCR mixture, which is 20 µL of DNA amplification of bacterial isolates using a thermal cycler. The PCR mixture was prepared in PCR tubes :5 microliters of DNA, 2 microliters of primers, and 10 microliters of PCR Master mix. The volume was completed to 20 µL using nuclease-free water. The tubes were closed and transferred to a thermal cycler device to perform the polymerase chain reaction under optimal thermal conditions as shown in table 2.

Table2: Optimal conditions for conducting the PCR reaction for the 16SRNA gene primers for *M.genitalium*

Stage	Tem.	Time	Number of cycles
Denaturation	95	1min	1
Annealing	58	30sec	30
Extention	72	1min	1

After the device stopped, the tubes were removed and the PCR products were run on1.5% agarose gels to determine the size of the amplified products. Their molecular sizes were compared to the DNA ladder with known molecular weights.

Table 3: contents and volumes of the reaction mixture.

Contents of the reaction mixture	Volume in microliters
PCR Master mix	10
Forward Primer	1
Reverse Primer	1
DNA template	5
Nuclease-free water	3
Total volume	20

Agarose gel was prepared at a concentration of 1.5% by dissolving 1.5 g of agarose in 100 ml of TAE buffer, then heated using a microwave for a few minutes while ensuring continuous stirring to ensure that the agarose completely dissolved, agarose was then cooled to 45-50 °C, and 3µL of red safe stain was added and poured in the appropriate tray, the comb was placed inside it from one end to form the wells. The gel was poured gently from one side, avoiding the formation of bubbles, then left to harden for 30 minutes, the comb was removed and samples were placed in each well while the first well contained 5µL of the 100bp DNA Ladder. 10 µL from the amplified PCR product was mixed well and added it to the following wells. Gel was covered completely with TAE buffer prior to loading the samples (Abdulrazzaq *et al.,*2022).

The electric current used was 80-100V for 45-60 minutes. After the transfer was completed, the gel was placed inside the UV-transilluminator to see the DNA bands and to determine their sizes by comparing them with the DNA ladder used. A camera was used to photograph the gel inside a dark room.

DNA sequencing:

DNA sequencing were determined to detect the identity of the urease gene sequence of the bacterial isolates. It was performed at Psomagene sequencing company (Maryland/ USA). The results of the polymerase chain reaction (PCR) were sent for 3 samples for the 16SRNA gene belonging to *M.genitalium* isolates. The DNA sequences of the isolates were compared with global isolates through the NCBI-Gene bank website.

Results and Discussion:

Vaginal swabs were collected from 30 women who use IUDs, the results showed that 7/30 (23.3%) samples of vaginal swabs were positive for growth of *M.genitalium*. According to many studies, IUD users have an increased chance of developing cervical infection caused by *M.genitalium* (Vanja *et al.,*2011). A cross-sectional study performed by Li-Ya *et al.,*(2016) in Anhui Province in China also showed that the use of (IUD), male condoms, female sterilization, the rhythm method, and oral contraceptives (OC) was associated with respiratory tract infections (RTIs). The analysis showed that the use of IUD is a risk factor for cervicitis and bacterial vaginosis (BV). IUDs are popular contraceptive choices for women however, the possible risk of PID associated with the use of an IUD has been a long-lasting important concern throughout the world. The connection between the development of any upper genital tract infection immediately after the insertion of the IUD, or due to the extended duration of IUD in place has been studied extensively during the past decades, but controversial results were obtained (Hubacher *et al.,*2013).Although higher rates of PID immediately after insertion of IUDs has been noted in previous studies, some recent data allow for a finer analysis of this relation (Viberga *et al.,*2005*;* Hubacher,(2014)).There is limited evidence regarding the association between a history of sexually transmitted infections (STIs) and the risk of *mycoplasma* infection or IUD expulsion. Study in 2019 of (Mahesh *et al.,*2020)found that *M.genitalium* infection was significantly associated with factors such as IUD use, number of sexual partners, and age (<35 years). In our study samples were collected from women who use IUDs and do not use antibiotics using vaginal swabs to detect *mycoplasma*. Studies have shown that *mycoplasma* can be detected using dry swabs without the need for transport media or liquid culture(Maxime *et al.,*2019). The accuracy of detection using dry swabs has been compared to other methods such as eSwab® and liquid culture, and the results have shown that dry swabs are effective in detecting *mycoplasma*(Xiang *et al.,*2021). Therefore, sterile dry swabs can be a reliable and convenient method for detecting *mycoplasma* infections and Using a speculum, as the potential risks and benefits of using a speculum to take vaginal swabs vary based on the design and use of the speculum. Some potential benefits include improved vision and access to the cervix, allowing for more accurate diagnosis and treatment (Kelly *et al.,*2021). In addition, adding a magnifier to the endoscope can enhance the monitoring of health and disease conditions in the body(Ying *et al.,*2012). On the other hand, there are potential risks associated with using an endoscope. Current standard laparoscopy is not specialized enough to be used for all populations, such as obese patients, resulting in discomfort and inadequate care(Richard *et al.,*2012). DNA extraction from *mycoplasma* was performed in several studies (Khaleel *et al.,*2023). DNA relatedness among different sero-groups and species of *Mycoplasma genitalium* was examined through genetic analysis in various studies, Furthermore, Maria Fookes et al. sequenced *M. genitalium* genomes, highlighting recombination events and identifying genomic regions associated with antibiotic resistance, These studies collectively contribute to understanding the genetic diversity and antibiotic

resistance mechanisms within *M. genitalium* populations (Maria *et al.,*2017).Described a simple technique for culturing and isolating DNA from *mycoplasma genitalium*(Blaylock *et al.,*2004). In our study after the DNA was extracted, the NanoDrop was used to measure the concentration and purity, purity appeared to be 1.8-1.9. Nanodrops can be used to measure the concentration and purity of DNA. One method involves using a nanopore sensor, where a DNA solution is inputted into a vessel with nanopore membranes. Voltage is applied to electrodes, allowing the DNA to pass through the nanopores and move. The current of each ion passing through the nanopore membranes is measured, and the DNA concentration is obtained from these measurements (Abdulrazzaq *et al.,*2022).

To determine the microbial environment by using metagenomic analysis and the result sample (vaginal swab) that was sent was as shown in Figure 3, which represents the percentages of bacteria. Metagenomic sequencing is a target-independent approach that offers a more comprehensive view of the pathogenic agents and provides a detection of common and unexpected pathogens in samples(Chen *et al.,*2022). The results of Mitogenomics NGS showed a variation in the number of bacteria present in the environment of the vagina, as it was found that there are 14 different types of bacteria, some of which are considered normal flora or pathogenic, including Mycoplasma, which causes vaginal infections as a result of the presence of the IUD, as the IUD has biological membranes around it that cause vaginal secretions, sometimes unpleasant odors.It was found that *Stenotrophomonas* in the rate of 92.7% ,it causes urinary tract infection (UTI) as stated in studies Said et al.,(2024).Followed by *Lactobacillus* in the rate 5.2% *Lactobacilli* usually do not cause disease, but They are normally found vagina as stated in studies Ahmad et al.,(2008). *Gardnerella* in the rate of 1.0% ,*Gardnerella* is the genus of bacteria most commonly and most abundantly represented during Bacterial vaginosis (BV) as stated in studies Dillard et al.,(2023). *Atopobium* , *Prevotella*, *Mycoplasma* , *Ureaplasma* and *Sneathia* , respectively (0.4% , 0.2%, 0.1% , 0.0% and 0.0%) where it was linked to Bacterial vaginosis (BV) and pelvic inflammatory disease (PID) and infertility, Studies using cultivation independent nucleic acid amplification of DNA from vaginal swabs, have identified novel bacteria associated with BV(Taylor *et al.,*2018). One type of urinary tract infection is vaginitis caused by bacteria *Proteus* , *Aerococcus* (0.0%) as described by the researcher Sh. Yihia et al (2005). *Porphyromonas* (0.0%) are common inhabitants of the vaginal microbiome, but their presence has been linked to adverse health outcomes for women, including bacterial vaginosis and preterm birth as stated in studies Lithgow et al.,(2021). *Dialister* (0.0%) is mainly isolated from gynecological tract samples although has been detected in vaginal samples Therefore, *Dialister* may be associated with gynecological infections as proven Kitagawa et al.,(2024). *Veillonella* (0.0%) are Gram-negative opportunistic pathogens present in the reproductive tracts of mammals. An abnormal increase in *Veillonella* relative abundance in the body is closely associated with urinary tract infections, and many other diseases as mentioned in Ding et al.,(2024). *Peptostreptococcus* (0.0%)is a gram-positive anaerobic coccus (GPAC) found in the gastrointestinal and vaginal microbiota as described Legaria et al.,(2021). The Gram-positive anaerobic commensal *Finegoldia* (0.0%) colonizes the skin and other non-sterile body surfaces, and is an important opportunistic pathogen as stated in studies Neumann et al., (2020).Kiessling et al.(2008)found that *Anaerococcus prevoti* or *Anaerococcus vaginalis* (0.0%) had higher prevalence among infertile patients that might influence fertility potential. *Gemella*(0.0%) a Gram-positive coccus facultative anaerobe, is part of the normal flora of the mucous membranes of the oropharynx, upper respiratory, gastrointestinal, and female genital tracts. However, this species can also cause serious infection as he mentioned it Miyoshi et al.,(2017). *Chlamydia*(0.0%) is a sexually transmitted infectious disease caused by the bacterium *Chlamydia trachomatis* ,it is the most commonly reported bacterial infection, Globally it is the most common sexually transmitted infection n females, the cervix is the anatomic site that is most commonly infected. This can manifest as cervicitis, urethritis, pelvic inflammatory disease, perihepatitis, or proctitis. Chlamydial infections in women, especially if untreated, increase the risk of infertility and ectopic pregnancy as stated in studies Mohseni et al.,(2024). *Paenibacillus* (0.0%)are known to infect various organisms and occasionally present as opportunistic infections in humans as described Grady et al.,(2016). *Senegalimassilia, Howardella* and Lachnospiraceae, Bradyrhizobiaceae(0.0%) are normal human flora in vagina and characterize the gut microbiota as described by the researcher Ning et al.,(2022). Where 11 types of bacteria appeared that were gram negative, 10 types that were positive for gram stain, and two types that did not take gram stain due to the lack of a cell wall, mycoplasma and ureaplasma.

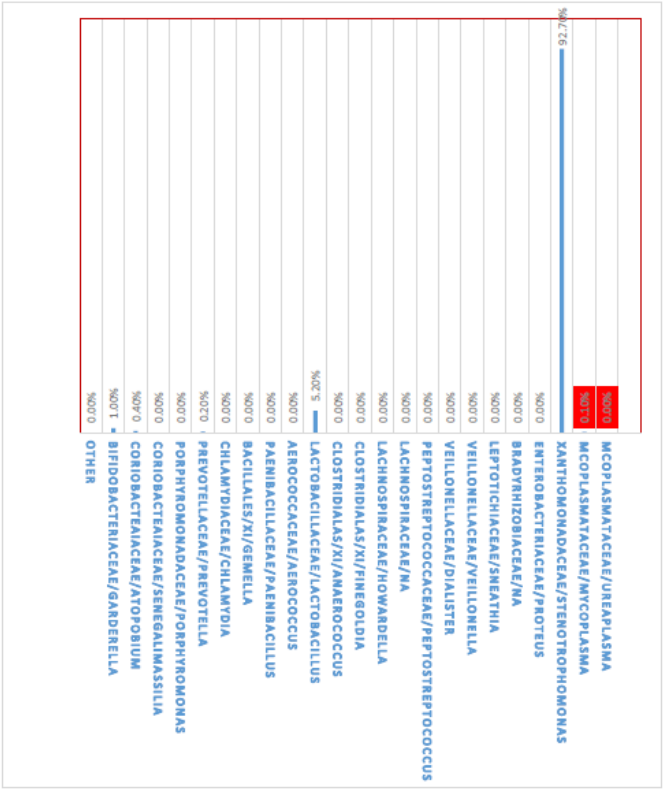


Figure (3): represents the percentages of bacteria in metagenomic (NGS)

PCR was performed to detect the a 550bp fragment of the *16SRNA* gene specifically found in *M.genitalium*, products were run on agarose gels by electrophoresis. The results showed that

the product size of the amplified gene was identical as expected (550bp). PCR product of the amplified fragments are shown in the figures (1). The size of the bands were compared to the 100bp DNA ladder.

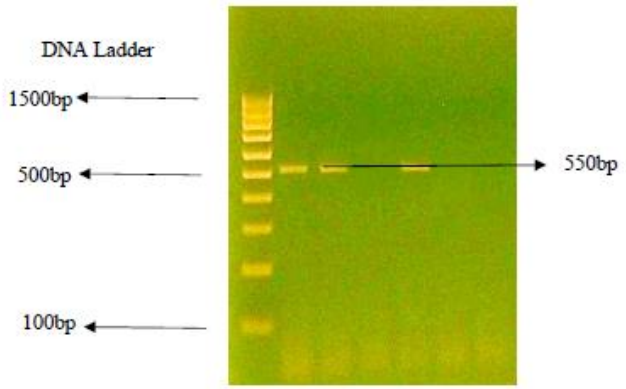
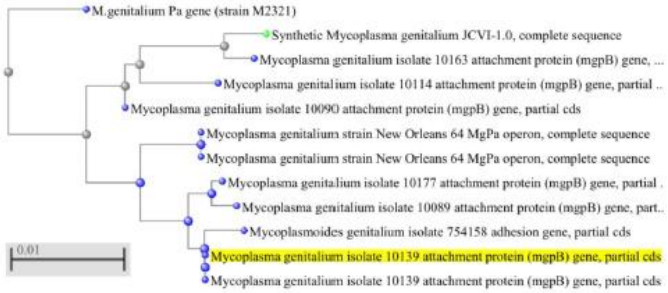


Figure (1): PCR product of the gene band size 426 bp. The product was electrophoresis on 1.5% agarose at7 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder(100bp)

After analyzing the results of the polymerase chain reaction, the DNA sequences of the local isolates were compared with the global isolates through the NCBI-Gene bank website It showed complete conformity with global isolates, as well as using some Bioinformatics programs. Figure(2) represent the tree of evolution and convergence:



Figure(2) represent the tree of evolution and convergence *M.genitalium*.

Mycoplasma genitalium DNA extraction is crucial for various applications. Additional studies were hampered by difficulties in culturing the organism and further detection only occurred once PCR was developed (Qian&Zhou (2022); Waites *et al.,*2023). Additionally, qPCR methods targeting genes like mgpB have been implemented for sensitive detection and quantification of *M. genitalium*, showing high specificity and efficiency (Jennifer *et al.,*2023). DNA extraction from clinical samples has enabled the detection of *M. genitalium* in populations, with prevalence rates reported in different regions like Nairobi, Kenya (Brian *et al.,*2014). Furthermore, genetic profiling through molecular typing has allowed the differentiation of persistent and recurrent infections, aiding in the identification of macrolide-resistant strains and emphasizing the importance of tests of cure and contact tracing for effective control of *M. genitalium* infections (Robinson *et al.,*2019). These advancements in DNA extraction and analysis techniques play a crucial role in understanding and managing *M. genitalium* infections.

Data on *M. genitalium* infections are limited in comparison to those for other *Mycoplasma* species largely due to its more recent discovery, extremely slow growth (generation time of about 16 h), and demanding cultivation requirements(Waites *et al.,*2023).

The results of the current study converged with the study of researcher Moridi and his group (2020) by 19.58%. They also agreed with researcher Ahmed and his group (2023), while the results differed with researcher Robinson and his group (2019) in Nairobi, Kenya, in the polymerase reaction that targeted a gene *16S rRNA*.

Mycoplasma genitalium in clinical samples can be rapidly, sensitively and accurately detected by using the specific primer pair disclosed by the invention, so that the primer pair has great significance in the early diagnosis, timely treatment and effective control of reproductive tract infection caused by *Mycoplasma genitalium* (K. *et al.,*2023).

There are 12 known serotypes of *Mycoplasma* species listed in the NCBI database(Liébana ,(2022)).Health experts say that a bacterium called "*Mycoplasma genitalium*," known as "MG," may turn into the "superbug of the future" unless people are more careful and vigilant about it(Barik *et al.,*2023).

In addition, *M. hominis*, *U. urealyticum, M. genitalium* have also been reported to be associated with some cases of pelvic inflammatory diseases (PID),where *Mycoplasma genitalium* can cause pelvic inflammatory disease, leading to infertility (Jianwei *et al.,*2023). It is a significant risk factor for female reproductive health issues, including pelvic inflammation. How the frequency of the use of IUD and the bacteria, which may form a biofilm on it, influences the development of upper genital tract infection, is still a question?

Conclusions:

Metagenomics plays a crucial role in diagnosing vaginal infections by revolutionizing traditional diagnostic methods. It enables a comprehensive analysis of the vaginal microbiota, aiding in the identification of microbial populations and their functions. Metagenomic techniques, such as high-throughput sequencing, provide a deeper understanding of the microbial diversity in the vaginal ecosystem, By comparing results from traditional methods like culture and microscopic evaluation with metagenomics analysis, a more accurate diagnosis of conditions like bacterial vaginosis (BV) can be achieved. Metagenomics also helps in identifying novel pathogens, antibiotic-resistant phenotypes, and virulence genes, enhancing clinical diagnosis and outbreak investigations. Overall, metagenomics offers a promising approach to early detection, better treatment, and improved women's health outcomes in the context of vaginal infections. Our study confirms the hypothesis that IUDs, independent of the type or duration of use can increase the possibility of an up-spreading gynecological infection. It is a therapeutic challenge to manage these cases because of the mixed bacterial biofilm flora found on the implant's surface.

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