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Department of Biology

Cultural, Molecular and Immunological Study on Mycoplasmal Urogenital Infection in Women

A Thesis

Submitted to the Council of the College of Science, University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy in Science in Biology

By

Noor Jasim Mohammed Jabbar

(B.Sc./ microbiology / 2012)

(M.Sc./ microbiology / 2019)

Supervised by

Prof. Dr. Azhar Omran Lateef Abood Al-Thahab

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نور جاسم محمد جبار

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بإشراف

ا.د. أزهار عمران لطيف عبود الذهب

١٤٤٤هـ ٢٠٢٢م

(قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا
عَلَّمْتَنَا ۖ إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ ۝)

صَدَقَ اللهُ العَلِيُّ العَظيم

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Dedications

To.. my supporter dear "**father**" who taught me tender without waiting ..To whom I carry his name with all pride …

To.. the big heart, the great lady and the a successful source …

To the smile of life and the secret of existence …

My mother

To the eyes and heartbeat ……

My brother & Sister

To the second family ……

My friends

To you … dear reader

I dedicate this work

Noor 2022

الخلاصة

المايكوبلازما كائنات حية بطيئة النمو تفتقر الى جدار خلوي و تعزل في الغالب من المسالك البولية التناسلية . على الرغم من ان العديد من انواع المايكوبلازما تعتبر فلورا طبيعة ، الا ان بعض الانواع تسبب امراضا خطيرة في الجهاز التناسلي .

تهدف الدراسة الحالية الى تحديد العلاقة بين العدوى الميكروبية شديدة الحساسية (انواع المايكوبلازما و اليوريابلازما) و بعض المعايير المناعية لعدوى المسالك البولية التناسلية لدى النساء المتزوجات فقط (حوامل - غير حوامل) و لتحقيق هذا الهدف و للحصول على نتائج موثقة ، تم تطبيق تقنيات هي طريقة الزرع و فحص تفاعل سلسلة البلمرة ، للتحقق في مسببات الامراض الميكروبية .

شملت هذه الدراسة جمع ٢٢٣ عينه (١٢٣ من النساء الحوامل و ١٠٠ من النساء غير حوامل)، (ادرار + دم) من مرضى المسالك البولية التناسلية (حسب التشخيص المختبري الاولي من قبل وحده الطفيليات لجميع المرضى قيد الدراسة) ، التي وصلت الى مستشفى الولادة و الاطفال في بابل والعيادات الخاصة في محافظة بابل، خلال ستة اشهر (من كانون الثاني الى حزيران ، ٢٠٢١). العينات التي جمعت من المرضى تشمل كمية من مصل الدم (للاختبارات المناعية) و كمية من الادار قسم الى انبوبين ، الاول للفحص البكتيري و الانبوب الثاني للاختبارات المناعية . بالاضافة ال ٢٠ عينة (ادار و مصل الدم) جمعت من النساء المتزوجات السليمات كمجموعة سيطرة (١٠ حوامل + ١٠ غير حوامل) . تم توزيع عينات المرضى الى ثلاث مجموعات حسب الفئات العمرية (٢٠-٢٩) سنة ١٠٣ (٤٦,١٩%) مريض ، فقط ٣٦ حالة موجبة للزرع المايكوبلازما (٢٦حامل و ١٠ غير حامل)، (٣٠-٣٩) سنة ٧٢ (٣٢,٢٩%) مريض ، فقط ٢١ حالة موجبة للزرع المايكوبلازما (١٢ حامل و ٩ غير حامل) و (٤٠-٤٩) سنة ٤٨ (٢١,٥٢%) مريض ، فقط ١٣ حالة موجبة للزرع المايكوبلازما (٢ حامل و ١١ غير حامل) .

تم تحديد العزلات وفقاً لأوساط زرعيه عادية للتحقق في مسببات الامراض الشائعة و اوساط زرعيه أغنائية للتحقق في مسببات الامراض بطيئة النمو وثم التأكيد بواسطة تفاعل سلسلة البلمره .

عزلت و شخصت بكتريا (*Mycoplasma genitalium* , *Mycoplasma hominis* and *Ureaplasma urealyticum*) على اوساط اغنائية انتقائية زرعية (MAU- medium) فضلا" عن بعض الاختبارات الكيموحيوية و تفاعل سلسلة البلمرة (PCR assay) , كما اختبرت الحساسية الدوائية للمضادات الحيوية تجاه تلك العينات .

أظهرت النتائج ان نسبة عزل بكتريا المايكوبلازما لـ ١٢٣ عينه من النساء الحوامل كانت ٤٠ (٣٢,٥%) شملت *U. urealyticum* , *M. hominis* , *M. genitalium,* ونمو مختلط ،

٢٥(٢٠,٣%) و ٢(١,٦) و ٩(٧,٣%) و ٤(٣,٣%) ، على التوالي . فيما كانت نسبة العزل لغير الحوامل ٣٠(٣٠%) شملت *U. urealyticum , M. hominis , M. genitalium* و نمو مختلط ، ١٩(١٩%) ، ٣(٣%) ، ٤(٤%) ،٤(٤%) ، على التوالي . كانت النسبة المئوية في الريف اعلى عن الحضر في كل من النساء الحوامل و النساء غير الحوامل حيث بلغت ٤٠/٢٨ (٢٢,٧٦%) و ٣٠/١٦ (١٦%) ، على التوالي .

كان أجمالي معدل الاصابة بالمسالك البولية التناسلية التي تسببها *M. genitalium* اعلى معنويا ٧٠/٤٤ (١٩,٧٣%) ، بينما معدل الاصابة بـ *M. hominis , U. urealyticum* و نمو مختلط و التي شكلت ٧٠/١٣ (٥,٨٣%) ، ٧٠/٥(٢,٢٤%) و ٧٠/٨ (٣,٥٩%) ، على التوالي .

علاوة على ذلك ، أشارت النتائج الى ان الاصابة بـالمايكوبلازما التناسلية كانت مرتبطة مع العمر حيث ان الفئة العمرية (٢٠-٢٩) نسبتها ٣٦(١٦,١٤%) اعلى نسبة ايجابية للزرع ، تلتها الفئة العمرية (٣٠-٣٩) نسبتها ٢١(٩,٤٢%) و اخيرا الفئة العمرية (٤٠-٤٩) نسبتها ١٣(٥,٨٣%) .

فيما يتعلق باستخدام اختبار PCR ، أوضحت النتائج ان ٧٠/٣٧ (١٦,٥٩%) ، ٧٠/٢٠ (٨,٩٧%) و ٧٠/١٣ (٥,٨٣%) ، من العينات المختبرة كانت ايجابية لـ *M. genitalium* ، *M. hominis* و *U. urealyticum* ، على التوالي .

تم فحص النتائج الحالية لمجموعة النيوكليوتيدات و تأكيدها بأستخدام جين *16S rRNA* معلومات قواعد بيانات تسلسل *M.gentialium* المسجلة في البنك الجيني . أظهرت النتائج ان تحديد التسلسل يتراوح من ٧٢% الى ٨٤% تم الحصول عليها لـ (٧) عزلات من اصل (١١) عزلة محاذاة مع عزلات مرجعية متقاعدة في قاعدة بيانات البنك في المركز الوطني للمعلومات التكنولوجيا الحيوية . ارتبط التسلسل المحلي لـ *M.genitalium* رقم ٦ ارتباطا وثيقا بالتسلسل MZ379495.1 لـ *Mycoplasma genitalium* strain MIMQ1 (العراق) وبالقرب من تسلسل MZ379496.1 لـ *Mycoplasma genitalium* strain MIMQ2 (العراق) و تسلسلNR 026155.1 لـ *Mycoplasma genitalium* strain G37 ATCCL43967.2 .

شجرة النشوء و التطور للتسلسل الجزئي عملت باستخدام ٧ عزلات من هذه الدراسة مع تسلسلات مرجعية مماثلة في البنك الجيني للعثور على درجات تحديد الجينات و التشابه و مقارنتها بعزلاتنا .

تم استخدم اختبار الحساسة لجميع العزلات ضد ٨ من المضادات الحيوية ، و اظهرت النتائج في هذه الدراسة كانت عزلات *M.genitalium* ، *M.hominis* و *U. urealyticum* حساسة تماما (١٠٠%) لمضادات Ofloxacin ، Doxycycline و Clindamycin .

كما كانت جمع عزلات *M. hominis* و *U. urealyticum* حساسة تماما (١٠٠%) لـ Ciprofloxacin ، لكن عزلات *M. genitalium* كانت حساسة بنسبة (٧٧,٣%) لـ Ciprofloxacin .

أظهرت النتائج ان ١٠٠% من عزلات الـ *U. urealyticum* مقاومة لـ Erythromycin . من ناحية اخرى ، كانت عزلات *M. hominis* مقاومة تماما (١٠٠%) لـ Erythromycin and Azithromycin .

نتائج الفحص المناعي لبعض المعايير المناعية اجريت باستخدام تقنية الاليزا (ELISA) لقياس تركيز انترلوكين ١٨ و TLR-6 و انترفيرون بيتا جهازيا و موضعيا .

انترلوكين ١٨ هو سايتوكين له خصائص محفزة للالتهابات . قياس تركيز انترلوكين ١٨ لإدرار المرضى (١٣,٣٩±٣,٣) اظهر وجود زيادة معنوية مقارنة بالسيطرة (١٠,٦٣±٢,١) ، بينما قياس تركيز انترلوكين ١٨ في مصل المرضى (١٢,٠١±٩,٨) تبين عدم وجود فرق معنوي مقارنة بمجموعة السيطرة (٩,٢٢±٢,٤) .

من ناحية اخرى TLR-6 هو بروتين متغاير له خصائص ذاتية للالتهابات . قياس TLR-6 لإدرار المرضى (٢,٠٨±٠,٣) لوحظ وجود فرق معنوي مقارنة بالسيطرة (١,٧٠±٠,٤)، بينما قياس مستقبل شبيه بالرسم ٦ في مصل المرضى (٢,٢١±١,٤) لم يظهر زيادة معنوية مقارنة مع مجموعة السيطرة (١,٨٠±٠,٥) .

كذلك انترفيرون بيتا هو سايتوكين له خصائص محفزة للالتهابات و مضادة لالتهابات . قياس تركيز انترفيرون بيتا لإدرار المرضى (٢٦٩,٨٩±٦٦,١) اظهر وجود زيادة معنوية مقارنة بالسيطرة (٢١٠,٢٢±٦٩,٣) ، و قياس تركيز انترفيرون بيتا في مصل المرضى (٢٣٠,٥٦±٩١,٥) تبين وجود فرق معنوي مقارنة بالسيطرة (١٧٤,٣٨±٣٦,٢) .

في ضوء النتائج التي قدمتها الدراسة نستنتج ان تقنية الـ PCR هي الطريقة الاكثر فعالية و دقة و مفيدة للكشف عن انواع المايكوبلازما و اليوريابلازما المسببة لالتهابات البولية التناسلية عند النساء المتزوجات ، وايضا اثبتت تأثر الجهاز المناعي للمرضى نتيجة للإصابات البكتيرية موضعيا و جهازيا .

Summary

Mycoplasmas are fastidious slow growing organisms lacking a cell wall and mostly isolated from genitourinary tracts. Although many species of mycoplasmas regard as normal flora, but some species causes serious gential disease .

The present study was aimed to determine the relationships between the fastidious bacterial infection (*Mycoplasma* species and *Ureaplasma* species) and some of immunological parameters for urinary genital tract infections (UGTIs) for married women only (pregnant – non-pregnant) . For purpose, and to obtain reliable results, the following techniques were used; Conventional culture method and polymerase chain reaction (PCR) .

The study includes (223) patients (123 pregnant and 100 non-pregnant), urine and blood specimens were collected from each one with urinary genital tract infections (UGTIs) (according to clinically diagnosed by specialist physician and the initial laboratory diagnosis by Parasitological unit for all the patients under study) , arrived at the Maternity and Children Hospital in Babylon and Private Clinics in Babylon province, during 6 months (from January to June , 2021). The specimens were generally collected from patients amount of serum (for immunological tests) and urine divided into two tubes, the first is used for a bacteriological examination and the second tube is subjected to immunological tests. In addition 20 (urine and serum) specimens collected from (Health married women) as control (10 pregnant and 10 non-pregnant). The patients specimens were distributed into three groups according to the age (20-29) years old, 103 (46.19%) patients, only 36 case positive culture for genital mycoplasmas (26 pregnant and 10 non-pregnant) , (30-39) years old, 72 (32.29%) patients, only 21 case positive culture for genital mycoplasmas (12 pregnant and 9 non-pregnant) and (40-49) years old, 48 (21.52%) patients, only 13 case positive culture for genital mycoplasmas (2 pregnant and 11 non-pregnant) .

The isolates were identified according to conventional culture media to investigate the common microbial pathogen and to investigate the fastidious microbial pathogen by used appropriate culture media and then confirmed by polymerase chain reaction (PCR) .

Mycoplasma genitalium, *Mycoplasma hominis* and *Ureaplasma urealyticum* were isolated and identified using appropriate culturing on selective modified arginine urea medium (MAU- medium), in addition to the diagnostic biochemical tests, and PCR assay. The antibiotic susceptibility of *M. genitalium, M. hominis* and *U. urealyticum* isolates were done.

The results revealed that the percentage of mycoplasmas isolates among 123 pregnant women specimens was 40(32.5%) including *M. genitalium M. hominis* , *U. urealyticum*, and mixed growth , 25(20.3%) , 2(1.6%), 9(7.3%) and 4(3.3%) respectively. On the other hands the number of isolation among the 30 non-pregnant women was 30(30%) low in relative to the pregnant women, since *M. genitalium* accounted for 19 (19%) isolate, *M. hominis* for 3(3%), *U. urealyticum* for 4(4%) , and mixed growth for 4(4%) isolates. The rural percentage was high than urban in both pregnant and non-pregnant as 28/40(22.76%) and 16/30(16%), respectively .

The total rate of (UGTIs) caused by *M. genitalium* was significantly higher 44/70(19.73%) while the rate of infection caused by *U. urealyticum* , *M.hominis* and mixed growth which formed 13/70(5.83%) , 5/70(2.24%) and 8/70(3.59), respectively.

Furthermore, the results indicated the frequency of infection by genital mycoplasmas was significant associated with age, since the age group (20-29) in 36(16.14%) recovered the highest positive for culture, followed by patients with age group (30-39) in 21(9.42%) and finally the age (40-49) in 13(5.83%).

Regarding the use of PCR assay , the results revealed that 37/70(16.59%), 20/70 (8.97%) and 13/70 (5.83%) of tested samples were positive for *M. genitalium* , *M. hominis* and *U. urealyticum*, respectively.

The present results of nucleotide sets were checked and confirmed by using 16S rRNA gene of *Mycoplasma genitalium* sequences databases information recorded in GenBank. The results showed sequencing identify ranged from 72% to 84% obtained only (7) local *Mycoplasma genitalium* isolates from (11) isolates alignment with reference isolates retired from NCBI- BLAST analysis. The local sequence of *Mycoplasma genitalium* No.6 was closely related to the sequence MZ379495.1 *Mycoplasma genitalium* strain MIMQ1(Iraq) and near to the sequence of MZ379496.1 *Mycoplasma genitalium* strain MIMQ2 (Iraq) and NR 026155.1 *Mycoplasma genitalium* strain G37 ATCCL43967.2 .

Phylogenetic tree partial sequence constructed using 7 isolates from this study along with similar referring reference nucleotide sequences (GenBank) to find identify and similarity score degrees of gene and compared with present study isolates.

The used of susceptibility test for all isolates against 8 antibiotics. In this study *M. genitalium*, *M. hominis* and *U. urealyticum* isolates, were completely (100%) susceptible to Ofloxacin, Doxycycline and Clindamycin . Also all *M. hominis* and *U. urealyticum* isolates were completely (100%) susceptibility to Ciprofloxacin, but *M. genitalium* isolates were sensitive (77.3%) to Ciprofloxacin .

The results revealed that 100% of the *U. urealyticum* isolates was resistant for Erythromycin . On the other hand, *M. hominis* isolates were completely (100%) resistant to Erythromycin and Azithromycin .

The results of the immunological examination of some immunological parameters done using ELISA for measurement IL-18, TLR-6 and IFN-β concentration systemic and locally .

IL-18 is a cytokine that has pro-inflammatory properties . The mean of IL-18 urine concentration in patients (13.39±3.3 pg/ml) was significant increase $p < 0.05$ compared with the control (10.63±2.1 pg/ml), while show the mean IL-18 serum concentration in patients (12.01±9.8 pg/ml) was no significant difference compared with the control group (9.22±2.4 pg/ml) .

TLR-6 is protein heterodimer that has auto-inflammatory properties . The mean of TLR-6 urine concentration in patients (2.08±0.3 pg/ml) was significant increased $p < 0.05$ compared with the control group (1.70±0.4 pg/ml), while the mean of TLR-6 serum concentration in patients (2.21±1.4 pg/ml) was no significant increase compared with the control group (1.80±0.5 pg/ml) .

IFN-β is a cytokine that has pro-inflammatory and anti-inflammatory properties . The mean of IFN-β urine concentration in patients (269.89±66.1 pg/ml) was significant increase $p < 0.05$ compared with the control (210.22±69.3 pg/ml), also the mean of IFN-β serum concentration in patients (230.56±91.5 pg/ml) was significant increase $p < 0.05$ compared with the control group (174.38±36.2 pg/ml) .

In light of the results presented by the study, this study concluded that the PCR assay is the most efficient, accurate, and useful method for detecting the types of *Mycoplasma* spp. and *Ureaplasma* spp. that cause urogenital infections in married women, and the study also prove that the immune system of patients are affect locally and systemically by bacterial infection (genital mycoplasmas) .

Certification

I certify that the preparation of this thesis entitled **(Cultural, Molecular and Immunological Study on Mycoplasmal Urogenital Infection in Women)** was made by **(Noor Jasim Mohammed Jabbar)** under my supervision at University of Babylon , College of Science , Department of Biology, as a partial fulfillment of the requirement for the degree of doctor of philosophy of Science in Biology .

Supervisor

Professor

Dr. Azhar Omran Lateef Abood

Department of Biology

College of Science

University of Babylon

/ / 2022

In view of the available recommendation, I forward this thesis for debate by the examining committee.

Professor

Dr. Adi Jassim Abd Al-Rezzaq

Head of Biology Department

College of Science

University of Babylon

/ / 2022

Examination committee

We, the examiner committee, certify that we have read the thesis entitled **(Cultural, Molecular and Immunological Study on Mycoplasmal Urogenital Infection in Women)** and have examined the student (**Noor Jasim Mohammed Jabbar)** in its contents, and that in our opinion it is accepted as a thesis for degree of Doctorate Philosophy of Science in Biology with **(Excellent)** estimation.

Signature:…….
Prof. Dr. Huda Hadi Al-Hasnawy
College of Medicine / University of Babylon
(Chairman)

Signature:……...
Prof. Dr. Abeer Thaher Naji Al-Hasnawi
College of Medicine
University of Karbal
(Member)

Signature:……..…
Prof. Dr. Adil Abaed Hassoni
Technical College
Al-Mussaib
(Member)

Signature:……...
Assist. Prof. Dr. Lubna Abd Muttalib
Enviromental Research and Study Center
University of Babylon
(Member)

Signature:……..…
Assist. Prof. Dr. Sura I. A. Jabuk
College of Sciences
University of Babylon
(Member)

Signature:……..
Prof. Dr. Azhar Omran Lateef Abood
College of Sciences / University of Babylon
(Member and Supervisor)

Approved for the College Committee of Graduate Studies

Signature:……...
Prof. Dr. Mohammed Mansour Kadhum Al-Khafaji
Dean of College of Sciences
University of Babylon

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List of Abbreviations

Symbol	Description
ATP	Adenosine triphosphate
$BaCl_2$	Barium chloride
BHIB	Brain heart infusion broth
BLAST	Basic local alignment search tool
BT LAB	Bioassay technology laboratory
BV	Bacterial vaginosis
CARDS TX	Community-acquired respiratory distress syndrome toxin
CDSs	Coding DNA sequences
CFT	Complement fixation test
DC	Dentritic cells
DNA	Deoxyribonucleic acid
DW	Distilled water
EC	Epithelial cells
ELISA	Enzyme linked immunosorbent assay
EMB	Eosin methylene blue agar
GIT	Growth inhibition test
H_2O_2	Hydrogen Peroxide
H_2S	Hydrogen sulfide
H_2SO_4	Sulfuric acid
HCL	Hydrochloric acid
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
Hrs.	Hours
IFNs	Interferons
IFN-β	Interferon beta

IFT	Immunofluorescence test
IgA	Immunoglobulin A
IHA	Indirect haemagglutination
IL	Interleukin
IL-18	Interleukin - 18
IL-1rα	Interleukin-1 receptor antagonist
IL-1β	Interleukin-1beta
K_2HPO_4	Dipotassium hydrogen
KOH	Potassium hydroxide
LPS	Lipopolysaccharide
LSD	Less Significant Difference
MAU	Modified arginine urea
MDCS	Monophasic-diphasic culture setup
MLS	Macrolides, lincosamides, streptogramines
MEGA	Molecular evolutionary genetics analysis
MG	*Mycoplasma genitalium*
$MgSO_4$	Magnesium sulfate
MHA	Muller hinton agar
MIT	Metabolic inhibition test
MT	Mycoplasmacidal test
NaCL	Sodium chloride
NCBI	National center for biotechnology information
NETs	Neutrophil extracellular traps
NGU	Nongonococcal urethritis
NH_3	Ammonia
NJ	Neighbor joining
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PID	Pelvic inflammatory disease
PPLO	Pleuropneumonia-like organisms
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SC	Small colony
SD	Standard Deviation
SPSS	Statistical program social science
STDS	Sexually transmitted disease syndromes
STDs	Sexually transmitted diseases
TBE	Tris-borate-EDTA buffer
TFN- γ	Interferon gamma
TFN-α	Interferon alpha

Th1	T helper cell type 1
TLR-6	Toll-like receptors-6
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
UGTIs	Urinary genital tract infections
UTI	Urinary tract infection
UV	Ultraviolet

Units of Measurement

Abbreviation	Key
bp	Base pair
CFU	Colony-forming unit
ºC	Degree celsius
dl	Deciliter
gm	Gram
µg	Microgram
IU	International unit
KDa	Kilo dalton
L	Liter
Mg	Miligram
ml	Mililiter
mM	Millimeter
NF-κB	Nuclear factor kappa B
nm	Nanometre
pg	Picogram
pmol/µL	Picomole/microliter
rpm	Revolutions per minute
µL	Microliter
µm	Micromolar

1 . Introduction and Literatures Review

1.1: Introduction

Mycoplasmas are fastidious slow growing organisms lacking a cell wall , which are not detected on routinely used media and mostly isolated from genitourinary tract (Metwally *et al.*, 2014). They are constructed of only three organelles: plasma membranes, ribosomes, and prokaryotic chromosome (Distelhorst *et al.*, 2017; Razin, 2018). Mycoplasmas constitute a large group of microorganisms, but only some, *Mycoplasma* spp. and *Ureaplasma* spp. are pathogenic for humans. They mainly inhabit the mucous membranes of the respiratory tract and genitourinary system (Jafar *et al*., 2010).

Mycoplasmas harbor a variety of virulence factors that enable them to overcome numerous barriers of entry into the host; using accessory proteins, *Mycoplasma* adhesions can bind to the receptors or extracellular matrix of the host cell. During proliferation, successfully surviving mycoplasmas generate numerous metabolites, including hydrogen peroxide, ammonia and hydrogen sulfide; or secrete various exotoxins, such as community-acquired respiratory distress syndrome toxin, and hemolysins; and express various pathogenic enzymes, all of which have potent toxic effects on host cells. Furthermore, some inherent components of mycoplasmas, such as lipids, membrane lipoproteins, and even mycoplasma-generated superantigens, can exert a significant pathogenic impact on the host cells or the immune system (Yiwen *et al.,* 2021) .

Mycoplasmas may also be a component of the commensal flora of the genitourinary tract mucosa and may be found in the majority of sexually active humans . The adverse effects of genital mycoplasmas on outcomes

of pregnancy include ectopic pregnancy, preterm birth and chorioamnionitis, postpartum endometritis, salpingitis, low birth weight and late miscarriage (Taylor-Robinson and Lamont, 2011) . *Mycoplasma* and *Ureaplasma* species are well-known human pathogens responsible for a broad array of inflammatory conditions involving the respiratory and urogenital tracts of neonates, children, and adults (Waites *et al.*, 2012) .

Three species have been isolated from the mucosal surfaces of the genitourinary tract: *Mycoplasma hominis* (*M. hominis*), *Ureaplasma urealyticum* (*U. urealyticum*) and the recently discovered *Mycoplasma genitalium* (*M. genitalium*) . They are commonly referred to as "genital mycoplasmas", as the infection occurs via sexual contact (Moridi *et al.*, 2020) . These *Mycoplasma* species are frequently detected in sexually active asymptomatic young men and women. However, *U. urealyticum* and various *Mycoplasma* are thought to induce a wide spectrum of pathological conditions in both men and women, including unexplained chronic lower urinary tract symptoms, nongonococcal urethritis, pelvic inflammatory disease, chronic prostatitis, and preterm labor and idiopathic abortion through infections in the urogenital organs , *M. hominis* , *M. genitalium* and *U. urealyticum* are recognized agents of genital infections in adults as well as neonates. (Ljubin-Sternak and Meštrović ,2014). Different detection techniques of mycoplasmal infections have been developed, each one of them has its advantages and limitations with respect to cost, time, reliability, specificity, and sensitivity. According to the laboratory's infrastructure, the most common methods include : (İ- culture-based test for isolation, detection, identification, antimicrobial susceptibility profile ; İİ-antigen detection,

mycoplasmal-specific serologic responses ; and İİİ-Polymerase Chain Reaction) (Flores-Medina *et al.,* 2012). Although, culture has been the gold standard for diagnosis. However, cultivation of these microorganisms can prove challenging because they are fastidious and may require weeks for growth but can be diagnosed by improved media and molecular techniques (Yamazaki *et al*., 2012 ; Mobed *et al.,* 2019).

The antimicrobial susceptibility of the two most common genital mycoplasmas, *U. urealyticum* and *M. hominis*, is of interest because they have been recognized as pathogens in infections of the newborn, arthritis and particularly sexually transmitted diseases (STDs) such as urethritis and pelvic inflammatory disease (Pónyai *et al.,* 2013). These organisms naturally resistant to *B*-lactam antibiotics and since resistance to traditional drugs, such as tetracycline, used for treating these infections is widely known for both *U. urealyticum* and *M. hominis*, alternative antimicrobial agents should be evaluated (Longdoh *et al.*, 2018).

Cytokines mediate inflammatory responses, are important in intercellular communication, and play a multifaceted role in the reproductive physiology of men and women. These potent polypeptides are released from inflammatory cells in response to a wide variety of signals, frequently initiated by infection or injury, and usually act, in a network of other cytokines, locally in an autocrine or paracrine fashion but also have systemic effects. Excessive production or actions of cytokines can lead to pathologic consequences (Eggert-Kruse *et al*., 2007). The effect of IL-18 initiates by binding to receptors on the surface of target cells and thus initiates the neutralization of intracellular antigenic peptides such as lipopolysaccharides (LPS), after being stimulated by IL-18 (Liu *et al.,* 2010) . Innate Immune cells, such as

neutrophils, macrophages, and natural killer cells, not only have the capacity to recognize pathogen-related molecular patterns (PAMPs) of *Mycoplasma* via toll-like receptors , but also can kill these microorganisms (Qin *et al.,* 2019). Type-I interferons (IFNs) play a critical role in protecting the host against microbial infection . The IFNs adopt a common alpha-helical structure and bind to the same cell surface complex consisting of IFNAR1 and IFNAR2 receptor chains . IFN binding to the IFNARs induces the phosphorylation of JAK1 and TYK2 kinases, and the subsequent induction of multiple signaling programs that allow the host to combat diverse pathogens (Harris *et al.,* 2020) .

Aim of the study

Due to the existence of high abortion, infertility and difficulty to identify the causative agent of UGTIs the real, so this study was planned. The present study was aim to determine the relationships between the fastidious microbial infection (*Mycoplasma* spp. and *Ureaplasma* spp.) and some of immunological parameters for chronic UGTIs and according to our knowledge's clinical studies on the organisms and their role in colonization of human urogenital *Mycoplasma* in Iraqi population are rare especially those concerned with molecular diagnosis, the achievement of this aim by the following objectives :-

1- Diagnostic bacterial species in urine of patients with complicated urinary-genital tract infections (UGTIs) , including culture , biochemical test , conventional PCR and sequencing .
2- Antibiotics susceptibility test for bacteria isolated .
3- Concentration measurement for some immunological parameters such as IL-18 , TLR-6 and IFN-β (local and systemic immunity) in patients and control .

1.2: Literatures Review

Initial data about Mollicutes were rather confusing since terms as viruses, L-forms, or Pleuropneumonia-like organisms(PPLO) were used to describe these organisms. The first Mollicute, later acknowledged as *Mycoplasma mycoides* subsp. *mycoides* small colony (SC), was isolated and described in 1898, but it took another few decades before other animal mycoplasmas were found (Mishra, 2004).

Mycoplasmas are unique types of bacteria. They are the smallest free living organism known on the planet able to multiply autonomously (Naher *et al.*, 2014). Since mycoplasmas lack a cell wall, they can assume a variety of shapes and are therefore challenging to distinguish. The tip of the filamentous has an attachment organelle. On agar, there are colonies that resemble fried eggs. They are so challenging to grow in the lab and frequently ignored as disease-causing pathogens. (Pascual *et al,* 2010*;* Mavedzenger *et al*., 2012). Moreover, they are completely dependent on host sterols and wide range of biosynthetic precursors (amino acids, nucleotides and fatty acids) (Yiwen *et al*., 2021).

The lack of atypical cell wall containing peptidoglycan renders these organisms insensitive to cell wall-active antimicrobial agents, such as penicillins and cephalosporins (Jafar *et al*.,2010; Miahi *et al*.,2011).

Mycoplasmas have adapted to wide varity of hosts and can colonize many other animals and plants. The colonizing organisms are host specific in human, since they colonize mainly the upper respiratory tract and the genitourinary tract causing atypical pneumonia, pyelonephritis, pelvic inflammatory disease, abortion, infertility, postpartum fever, bacterial vaginosis, neonatal bacteremia, meningitis and abscesses (Haggerty *et al*., 2009; Niebla, 2011).

They usually reside extracellular and rarely penetrate the submucosa, except in the case of immunosuppression or instrumentation, since they invade the bloodstream and disseminate to numerous organs and tissues. Some species also occure as intracellular pathogens (Waites *et al*., 2005). *Mycoplasma* have very little DNA of its own ,but are capable of using DNA from the host cell leading to malfunction cell or can cause DNA mutation of host cell (Bébéar *et al.,* 2002).

Mycoplasmas attach to host cells with a tiny arm coated in protein which attaches to the protein coating of host cells. For this, antibiotics which are classified as "protein synthesis inhibitors" like tetracycline are often used against *Mycoplasma* infections, since these antibiotics block this attachment (Jafar *et al*., 2010).Once adherence to the host cells, the *Mycoplasma* can completely mimic or copy the protein of the host cell. This can cause the immune system to begin attacking the body own cells (Bébéar *et al.,* 2002). When *Mycoplasma* attaches to a host cell, it generates and releases hydrogen peroxide and superoxide radicals which cause oxidative stress and damage to the surrounding tissues (Jenkins *et al*., 2008).

At that time, mycoplasmas were called because the microbe had been shown to cause bovine pleuropneumonia. The term *Mycoplasma* (Greek: mykes - fungus and plasma - formed) was first used to describe the (PPLO) in the 1950s. This designation was initially intended to describe the growth form of *M. mycoides*, but the term soon gained widespread usage and was applied to all (PPLO) of human and animal origin identified at that time (Vilei and Frey, 2010).

Mycoplasma refers to a genus of bacteria that lack a cell wall, consequently, they are unaffected by many common antibiotics such as penicillin or other beta-lactam antibiotics that target cell wall synthesis. They

can be parasitic or saprotrophic. Several species are pathogenic in humans, including *M. pneumoniae*, which is an important cause of atypical pneumonia and other respiratory disorders, and *M. genitalium* is believed to be involved in pelvic inflammatory diseases. Mycoplasmas are the smallest living cells yet discovered can survive (Ryan and Ray, 2004).

1.2.1: Historical background of *Mycoplasma*

1.2.1.1: Phylogeny and taxonomy

Nocard and Roux isolated the first *mycoplasma* specimen in 1898.The infectious bovine peripnemonia's cause, *Mycoplasma mycoides* spp. *mycoides*, was discovered (Hayflick and Chanock, 1965; Al-Aubaidi *et al*.,1972).

The first report of a *Mycoplasma* to be recovered directly from a human and associated with a pathological condition occurred in 1937, when Dienes and Edsall isolated an organism which was probably the one known now as *Mycoplasma hominis* from a Bartholin's gland abscess. In 1944 Eaton described an isolation of *M. pneumoniae* from the sputum of a patient with primary pneumonia (Sleha *et al*., 2013).

Over subsequent years, several other human mycoplasmal species were described. In 1954, Shepard provided the first description of T-strain mycoplasmas, later known as ureaplasmas, when he was able to cultivate them in vitro from the urethras of men with nongonococcal urethritis (Xioa *et al*., 2010). When it was ultimately established that mycoplasmas were incapable of forming cell walls under any circumstances, making them distinct microorganisms among the prokaryotes, PPLO were entirely distinguished from bacterial L- forms in the 1960s. (Trachtenberg, 1998).

M. pneumoniae was culturing in 1962, the causative agent of primary atypical pneumonia, in a cellular medium, which provided solid evidence that this agent was not virus due to affectivity of antibiotics against it. The first

species to be isolated is *M. mycoides* subsp. *mycoides* causing disease in cattle herds at that time. Members of *Mollicutes* such as *Mycoplasma* species and *Acholeplasma* species, are distinguished phenotypically from other bacteria by their minute size (diameters ranging from 0.3 μm to 0.8 μm) and totally lack of a cell wall. The genome size of *Mycoplasmas* ranged 580- 1350 kb, about one-sixth that of *Escherichia coli*, with a low G+C content of 23-40%. They are parasites or commensal organisms of humans and animals. Most human-associated and animal associated *Mycoplasmas* adhere to host cells. Only a few species are recognized to be able to internalize into host cells (Razin *et al*., 1998).

Later in 1981 *M .genitallium* was isolated from swab being collected from men with nongonococcal urethritis (NGU). It has been suspected in pelvic inflammatory disease. The discovery of *M. genitalium* strains on 1988 in human nasopharyngeal throat specimens, where they frequently mixed with strains of *M. pneumoniae*, since there are closely related similarities between these species. Although both species retain the ability to synthesize proteins via translation, neither has the capability of synthesizing amino acids, the building blocks of proteins. Thus, all amino acids must be obtained from the host via transport across the cell inner membrane. The absence of important biosynthetic genes is believed to be a hallmark of genome decay (Björnelius *et al*., 2000; Wood, 2001). Moreover, two species of *Mycoplasma* represented by *M. fermentans* and *M. pentrans* were found in HIV-seropositive patients (Cordova *et al*., 2000; Bruder *et al*., 2005).

Initially, due to their unknown nature and relationships with other organisms, while being minute in size and not being qualified as bacteria they were considered as viruses for years. However, many years later with further discovery mycoplasmas were confused with the L-forms, which are bacteria

that have lost their cell walls either completely or partially. Nevertheless, in 1950s and 1960s this confusion came to end when first genomic analysis data through DNA hybridization were obtained. This analysis ruled out any relationship of mycoplasmas to the L-forms (Waites *et al*., 2005).

1.2.1.2: Structural composition of mycoplasmas

The lack of cell wall convey some unique properties of mycoplasmas such as sensitivity to osmotic shock and detergents, resistance to penicillin, and formation of odd fried-egg shaped colonies(Lee *et al*., 2010). They have no genes involved in amino acid biosynthesis and only a few genes involved in the biosynthesis of cofactors as vitamins (Guimaraes *et al*., 2011). Most mycoplasmas cannot synthesise any fatty acids and some even incorporate exogeneous phospholipids together with cholesterol in their cell membrane. Also the genes involved in the biosynthesis of nucleotides are very limited (Razin, 2006).

Their mode of replication is not different from that of prokaryotes dividing by binary fission. Mycoplasmas are known to consist of just plasma membrane which makes them good models for membrane studies. Due to this reason the availability of these membranes in pure state have enabled in their chemical, enzymatic and antigenic characterization. The membrane mostly consists of 60% to 70% of proteins and rest 20% to 30% of lipid (Razin and Hayflick 2010).

Mycoplasmas are classified in the class (Mollicutes) which mean (soft skin) due to their lacking a rigid bacterial cell wall (Razin, 2006). The Mollicutes are Eubacteria that are thought to have evolved from *Lacobacilli*, *Streptococci*, *Bacilli*, and *Closteridium* through regressive evolution and genome reduction to form the tiniest and most basic free-living and self-replicating cells. In general, the way of life is parasitic. The Mollicutes are

distinguished structurally by the absence of a cell wall and the existence of an internal cytoskeleton (Wolf *et al.,* 2004).

1.2.2: Genomic structure and organization

The *Mycoplasma* genome is a circular, double-strand DNA molecule found in prokaryotes. It boasts the world's tiniest genome of any self-reproducing prokaryote. Therefore, there are few genes; the number of genes in some mycoplasmas is estimated to be less than 500, or around one-sixth of the number of genes in *Escherichia coli*. As a result, mycoplasmas only create a small number of cell proteins and lack a variety of enzymatic activities and metabolic pathways. Their nutritional requirements are also complex, and they live a parasitic lifestyle. Because mycoplasmas rely on their hosts for a variety of nutrients, laboratory cultivation is difficult (Mardassi *et al.,* 2007).

The *Mycoplasma* genome is distinguished by a low guanine-plus-cytosine concentration and the use of adenine and uracil codons . The use of the universal stop codon UGA as a tryptophan codon is particularly intriguing. Mycoplasmal RNA polymerase resistance to rifampicin is another feature that distinguishes mycoplasmas from other bacteria . Apart from rifampicin resistance, mycoplasmas are sensitive to other antibiotics that inhibit protein synthesis on bacterial ribosomes, such as tetracyclines and chloramphenicol (Razin, 2012).

1.2.2.1: Genomic structure of *Mycoplasma hominis*

The genome of *M. hominis* is a single 965,445-bp circular chromosome with a G+C content of 27.1 % and an A-T composition of 72.9 % . It comprises putative coding DNA sequences (CDSs), with a gene density of 89.8% and 14 pseudogenes discovered . There are 537 DNA coding sequences, 345 of which have been determined to have a function, and 40 RNA genes (Pereyre *et al.,* 2009).

It has been determined that *M. hominis* holds two duplicates of *rRNA* genes (Pereyre *et al*., 2002). *M. hominis* most likely underwent horizontal gene transfer and gained genes from *Ureaplasma parvum* which have aided in the bacterium's arginine hydrolysis energy yielding pathway. This has most likely occurred due to both *M. hominis* occupying the urogenital region of humans (Pereyre *et al.*, 2009).

1.2.2.2: Genomic structure of *Mycoplasma genitalium*

M. genitalium (MG) is a fastidious , slow growing and sexually transmitted diseases (STDs) pathogen. This species was first cultured from the urethral exudates of 2 of 13 men with nongonococcal urethritis in 1981. The organism's small size (approximately 0.6 × 0.3 μm), ability to pass through 0.3-μm filters, absence of a cell wall, resistance to penicillin, fried-egg appearance of colonies on soft agar plates, and inability to revert to cell wall–containing bacteria, collectively confirmed their identification as mycoplasmas (McGowin and Totten, 2017).

M. genitalium and *M. pneumoniae* have been proven to have closely linked sister species genomes, and *M. genitalium* has the shortest known genome for a cell capable of autonomous replication (Mirnejad *et al.*, 2011).

1.2.2.3: Genomic structure of *Ureaplasma Urealyticum*

U. urealyticum has a double-stranded DNA circular chromosome of 751,719 bp. This genome is unique because it is smaller than any other sequenced microbial genome, except for *M. genitalium*, a closely related human mucosal pathogen. It also has a relatively low G+C content (25.5%), making it more A+T rich than any other microbial genome that has been sequenced so far. It contains 613 protein-coding genes and 39 genes that code for RNAs (rRNAs, tRNAs, ribonuclease) (Zimmerman *et al*., 2011). Although *U. urealyticum* is similar to the mycoplasmas, its evolutionary divergence is

evident from analysis of gene order. There are 76 proteins coded for by *U. urealyticum* that have a function and/or cellular location that is not found in two different *Mycoplasma* genomes (*M. genitalium* and *M. pneumoniae*) (Waites *et al*., 2012).

Most of these genes are involved in ATP production via urea hydrolysis (something unique to ureaplasmas) and in iron acquisition. It is speculated that the phenotype of *U. urealyticum* is the result of its unique set of genes for energy production and respiration . *U. urealyticum* produces a major surface antigen (gene MBA) that is believed to help the organism evade the host's immune system . MBA has at least 5 other paralogs dispersed throughout the genome (Zimmerman *et al*., 2011).

1.2.3: Pathogenesis

Although it is believed that mycoplasmas remain attached to the surface of epithelial cells as extracellular organisms (Kornspan *et al*., 2015), some mycoplasmas have evolved mechanisms for entering host cells. These organisms have invasive properties enabling theme to localize in the cytoplasm and perinuclear regions (Qin *et al*., 2019). Intracellular localization has been reported for several species including *M. genitalium, M. hominis, M. penetrans* as well as *M. pneumoniae* .This localization may protect the *Mycoplasma* against host defense and contribute to disease chronicity (Chenog *et al*., 2011).

M. pneumoniae attaches to sialoglycoprotein or sialoglycolipid receptor on the tracheal epithelium via protein adhesion on the attachment organelle . The major adhesion is a 170-kilodalton (kda) protein, named P1. Moreover MgPa, P120 are the major adhesion proteins of *M. genitalium* and *M. hominis* .These organelles play a role in cell motility and pathogenicity due to highly antigenicity (Mardassi *et al*.,2007).

Mycoplasma genitalium is increasingly appreciated as a common cause of sexually transmitted disease syndromes (STDS), including urethritis in men and cervicitis , endometritis , pelvic inflammatory disease , and possibly preterm birth , tubal factor infertility and ectopic pregnancy in women (McGowin *et al.*, 2017).

Adhering to the epithelial linings of the respiratory and urogenital tracts is firm enough to prevent the elimination of the parasites by mucous secretions or urine. The intimate association between the adhering mycoplasmas and their host cells provides an environment in which local concentration of toxic metabolites excreted by the parasite build up and cause tissue damage. Moreover, because mycoplasmas lack cell walls, fusion between the membranes of the parasite and host can occurs. Membrane fusion would alter the composition and permeability of the host cell membrane and enable the introduction of the parasite hydrolytic enzymes into the host cell, events cause serious damage (Bébéar *et al.*, 2002).

Toxins are rarely found in mycoplasmas. The end products of *Mycoplasma* metabolism were responsible for tissue damage. Hydrogen peroxide (H_2O_2) the end product of respiration in mycoplasmas, has been implicated as a major pathogenic factor ever since it responsible for the lysis of erythrocytes by mycoplasmas. The mycoplasmas must adhere closely enough to the host cell surface to maintain a toxic, concentration of H_2O_2 sufficient to cause direct damage, such as lipid peroxidation of cell membrane. The accumulation of malonyldialdehyde, an oxidation product of membrane lipids. Moreover, *Mycoplasma* inhibits host cell catalase by excreting superoxide radicals (O_2). This would be expected to further increase the accumulation of H_2O_2 at the site of parasite-host cell contact (Lloyd *et al*., 2021).

However, some *Mycoplasma* belonging to the normal flora and can exist in the oral cavity and gut without causing diseases . *M. salivarium* and *M. orale* reside as normal flora in 80-90% of human body (Rivera-Tapia and Rodríguez-Preval, 2006).

Members of the genus *Mycoplasma* vary widely in their pathogenicity. About 56% of gastric carcinoma were reported to be caused by *Mycoplasma* , (Nascimento Araujo *et al*., 2021). Moreover , Chronic fatigue syndrome ,Crohns colitis, Type 1 diabetes mellitus ,Multiple sclerosis ,Parkinsons disease, Wegeners disease and collagen vascular dieseases such as Rheumatoid arthritis and Alzheimers disease are also reported to be caused by *Mycoplasma*, as shown in Table (1-1) (Namiki *et al*., 2009).

Table (1-1):Common Pathogenic Species of *Mycoplasma*(Razin *et al*., 1998)

No.	Pathogen	Implicated disease
1.	*Mycoplasma hominis*	PID, infertility, NGU , vaginosis , cervicitis , amnionitis.
2.	*Mycoplasma genitalium*	Arthritis, chronic NGU, PID, other urogenital infections, infertility.
3.	*Ureaplasma urealyticum*	NGU,PID, urogenital infection, infertility , vaginosis.
4.	*Mycoplasma penetrans*	Urogenital infection and autoimmune disorders.
5.	*Mycoplasma pirum*	Urogenital infections

1.2.4: *Mycoplasma* and *Ureaplasma* diseases in human

Mycoplasma and *Ureaplasma* spp., lead to the disease of the joints and respiratory tract with bactermic dissemination, particulary in persons with antibody deficiencies. *Ureaplasma* spp. are the most common etiologies of infectious arthritis in person who have hypogammaglobulineama (Ghaed'a Jassim and Kadhim, 2015).

Patients with genitourinary tract infections will notice symptoms like difficulty urinating, painful urination, and unpleasant discharges from the genitourinary tract, they may also feel sore around the pelvic region and can develop fevers, swollen lymph nodes, and surface lesions, depending on the cause of the infection. Some examples of genitourinary tract infections include prostatitis, gonorrhea, pelvic inflammatory disease, trichomoniasis, and candidiasis (Lane and Takhar, 2011).

Mycoplasma spp. are hide deep within tissues. They depend on host cells for nutrients. They compete with the host cells for nutrient which can interfere with host cell function without killing the host cell (Nascimento *et al*., 2002). They colonize mucosal surface of the respiratory and urogenital tracts. Most species reside extracellular, but some like *M.pneumoniae ,M.genitalium* , *M.penetrans* may localize and survive within the cells (Roachford *et al.*, 2019). Only *Mycoplasma pneumoniae, M. hominis, U.urealyticum, M.genitalium* are clearly associated with human diseases (Lee *et al*., 2010 ; Vandepitte *et al*., 2012).

However, *Ureaplama urealyticum* and *Mycoplasma hominis* belong to the normal commensal flora of the genital tract of sexually active healthy women, with colonization rates reach up to 80% in certain area of the world (De Souza Santos *et al*., 2020). *U. urealyticum* can be found in the cervix or vagina of 40-80% of asymptomatic women , *M. hominis* in 20-50% and *M. genitalium* in

0-5% (Taylor-Robinson, 2017). Mycoplasmas are also isolated from the lower urogenital tract of healthy adults, men and women .Colonization varies in relation with several parameters including age, race, hormonal status and the lifetime number of sexual partners, and is greater among women, especially during pregnancy (Redelinghuys, 2014).

1.2.4.1: Urogenital infections

Mycoplasmas are often isolated from the genitourinary tract, especially in sexually active people (Schlicht *et al.,* 2004). The ascent of *Mycoplasma* from the lower genitourinary tract is probably a more likely route for infection of the kidney. These organisms' frequency is strongly linked to socioeconomic status, poverty, and a large number of sexual partners. The menstrual cycle, pregnancy, and the use of vaginal contraception, as well as bacterial and protozoan infections (co-infections), encourage mycoplasmas and ureaplasmas colonization of the genital tract (Açıkgöz *et al.*, 2007 ; Lee *et al*., 2010).

In men *Ureaplasma* spp. and *M. genitalium* cause non gonococcal urethritis (NGU) (Vandepitte *et al*., 2012). Ondondo *et al*. (2010), detected *U. urealyticum* in 26% in patients with non gonococal urethritis(NGU). *M. genitalium* strongly associated with (NGU) as described by several studies in Denmark and Sweden (Björnelius *et al*., 2000).

On the other hand, mycoplasmas, together with other organisms such as *Gardnerella vaginalis* , *Mobiluncus* spp. , *Prevotella* spp. and anaerobes such as *Bacteroide* spp. , proliferate bacterial vaginosis (BV) in women . *M. hominis* was discovered in two-thirds of women with BV, but not in healthy women, discovered evidence associating *M. hominis* to pelvic inflammatory illness and salpingitis (Amabebe and Anumba, 2022).

Some other studies found that atypical organisms, such as mycoplasmas associated with urinary tract infection (UTI) especially in women with over

active bladder symptoms (Lee *et al*., 2010). These organisms considered as opportunists that cause invasive infections in susceptible population *Ureaplasma* spp. have possible role in development of kidney stone .This pathology is related to the urease activity of *Ureaplasma* spp. with crystallization of struvite and calcium phosphate in urine (Kokkayil and Dhawan , 2015). *U. urealyticum* and *M. hominis* has been reported to cause a small number of acute pyelonephritis in immunocompetent patients (Gerber *et al.*, 2018).

1.2.4.2: Reproduction disorders and infection during pregnancy

The female reproductive system is comprised of the vagina, cervix, uterus, uterine (fallopian) tubes and ovaries , as shown in Figure (1-1).

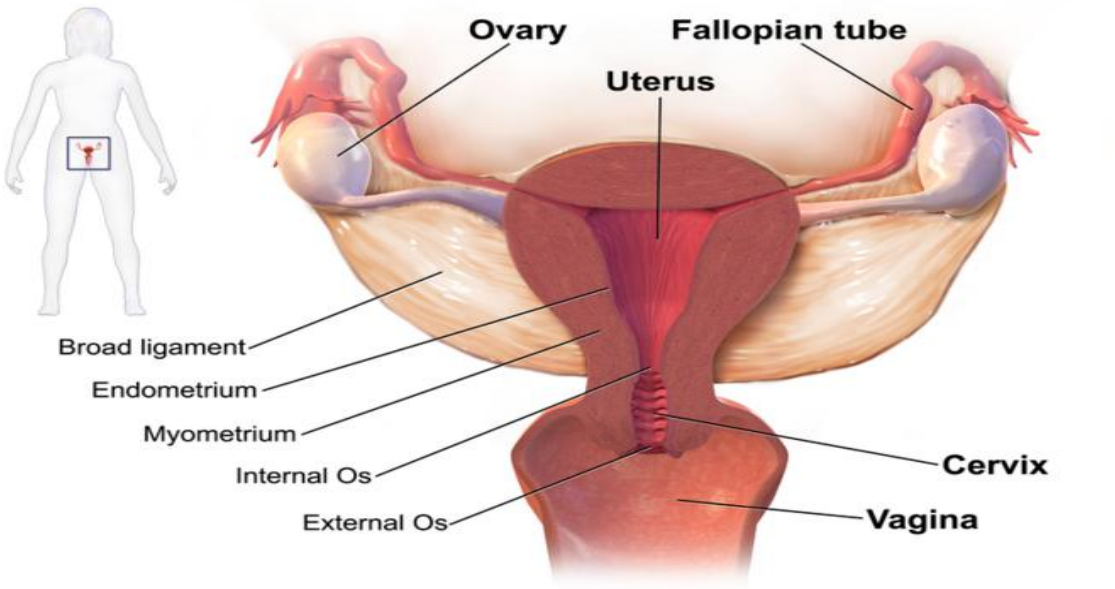


Figure (1-1): Reproductive System in Women

Female genital tract is a suitable place for growth of many microorganisms. Some of these are *Chlamydia trachomatis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Gardnerella vaginalis*, *Listeria monocytogenes* and *Neisseria gonorrheae* upon localization and colonization at the appropriate anatomical

site may cause various pathological disorders like cervicitis, vaginitis, vaginosis, urethritis, endometritis, salpingitis, bartholinitis. The pathological disorders may lead to pelvesitis, ectopic pregnancy, abortion and infertility and cervical dysplasia (Chung *et al.*, 2012).

1.2.4.2.1: Infertility

Mycoplasmas seem to play a very minor role in human infertility (Mihai *et al*.,2011). Genital tract infection and inflammation have been associated to 8-35% of male infertility, worldwide (Zeyad *et al.*, 2017).

Ureaplasma spp. have been reported to decrease sperm motility ,alter the spermatozoa morphology ,increased appoptosis in spermatogenesis, impairment of semen parameters, less stable chromatin , DNA denaturation in spermatozoa and increase the risk of orchitis, epididymitis and prostitis (Peerayeh *et al*., 2008). *Ureaplasma urealyticum* can adhere to the sperm membrane ,thereby potentially causing gamete dysfunction and enhance the adverse effects of superoxide and hydrogen peroxide produced by the organism, with subsequent spermatozoan hyper production of reactive oxygen species(ROS) (Potts *et al*.,2000). The ROS induce lipid peroxidation ,which reduces membrane fluidity and sperm fertilization capability, and may be the mechanism by which *U.urealyticum* impairs sperm function (Sandlow ,2004).

In a recent work, genital *Ureaplasma* spp. (*U. urealyticum* and *U. parvum*) and *Mycoplasma* spp. (*M. hominis* and *M. genitalium*) are an etiological role in female infertility, show that lower genital tract infection with genital mycoplasma may be associated with the pathology of female infertility (Tantengco *et al.*, 2021). *M. genitalium* is also described as independent risk factor in the development of an inflammatory process leading to scarring of the uterine tube in women and thereby causing infertility (Brunham *et al.*, 2015 ; Tsevat *et al.*, 2017) .

1.2.4.2.2:Abortion, still birth, preterm delivery and chorioamnionitis

Infection with *U. urealyticum*, *M. hominis* and *Chlamydia trachomatis* are considered as the etiological agents of abortion since they frequently produce asymptomatic infection and are not identified by routine microbiological techniques (Larsen and Hwang, 2010). Many workers studied the role of *U. urealyticum*, *U. parvum* and *M. hominis* in spontaneous abortion and they showed that there is a higher incidence of colonization during pregnancy among women who had spontaneous abortion than those who had successful pregnancies (Oliveira *et al*., 2020).

Commonly found in the genital and urinary tracts of adults, *M. hominis* and *U. urealyticum* are known as the genital mycoplasmas. These organisms can cause urethritis and contribute to vaginitis in women. They have been associated with sexually transmitted diseases (STDs) and with chronic infections in people with weakened immune systems (WHO, 2012 ; Al-Sweih *et al.*, 2012). Up to 50 percent of sexually active women are colonized with *U. urealyticum*, which can spread to newborn babies during delivery. In premature infants, *U. urealyticum* may contribute to pneumonia and other infections, as well as to chronic lung disease (Duke, 2005).

Moreover, these microorganisms play an important role in chorioamnionitis, still birth and preterm delivery (Novy *et al*., 2009 ; Lee *et al.*, 2016). *U. urealyticum* are the microorganisms frequently isolated from aminiotic fluid or placentae in women who deliver preterm between 23 and 32 weeks gestation. It also initiate a sequence of pathologic events due to release of arachidonic acids from amniotic membranes lead to production of prostaglandins, which can trigger premature labor, induce the production of inflammatory cytokines (tumor necrosis factor-, interleukin-6), and to

stimulate the release of nitric oxide by alveolar macrophage (Li *et al*., 2000) . The extensive release of these cytokines may contribute to the inflammatory response and to the pathology of chronic lung disease in very –low –birth weight premature infants. Respiratory tract colonization of premature infants associated with pneumonia, bronchopulmonary dysplasia and chronic lung diseases (Waites *et al*., 2005). *U. urealyticum* induce apoptosis in human lung epithelial cells and macrophages (Li *et al*., 2002 ; Torres-Morquecho *et al.*, 2010).

M. hominis has been isolated from placenta less frequently but may also linked to fetal bacteremia, neonatal pneumonia, and central nervous system infections (Baum and Edwards, 2017).

Mycoplasmas may be responsible for triggering autoimmune responses, first, during their intracellular replication and release from host cells. Mycoplasmas can capture antigen from the host cell surface and incorporate them into their cell membrane. This can lead to immune responses against these antigens and possibly autoimmune reactions. Second, mycoplasmal antigens can mimic host antigens and trigger immune responses against these antigens with resulting cross reactivity against host antigens. Third, they can cause apoptosis of host cells with subsequent release of normal host antigens (Niclson *et al*., 2000).

Both *Mycoplasma* and *Ureaplasma* transmitted by direct contact between hosts (venerealy through genital-to-genital or oral-to-genital contact), vertically from mother to offspring (either at birth or in utero), or by nosocomial acquisition through transplanted tissues. Respiratory infections caused primarily by *M. pneumoniae* are usually transmitted through respiratory aerosols (Brown *et al*., 2014).

1.2.4.3: Bacterial vaginosis (B.V.)

It is a common lower genital tract infection that may lead to pelvic inflammatory disease (PID), subsequent infertility and preterm birth, and may increase susceptibility to sexually transmitted disease (Ona *et al.*, 2016).

Bacterial vaginosis is a disturbance in the balance of the normal vaginal flora characterized by reduced number of Lactobacilli, a higher pH and 100 folds increased numbers of potential pathogens including *U. urealyticum* , *M. hominis*, *Gardnerella vaginals*, *Bacteroides*, Group B streptococci, anaerobes *peptostreptococcus*. Therefore, the presence of large number of Lactobacilli and low pH are important mechanism to protect against the growth of potential pathogenic organisms (Haggety *et al*., 2009 ; Onderdonk *et al.*, 2016).

Mycoplasma genitalium is able establish chronic urogenital infections by (a) expression of two antigenic proteins associated with attachment (MgpB and MgpC variants) with different amino acid sequences, and (b) phase variation, during which *Mycoplasma* lose the ability to adhere to cultured cells and instead acquires the ability to bind to red blood cells (hemadsorption) . Human genital were susceptible and immunologically responsive to *M. genitalium* infection that likely induced cellular immune responses. Although macrophage phagocytosis was an effective method for *M. genitalium* killing, intracellular localization within vaginal and cervical may provide *M. genitalium* a survival niche and protection from cellular immune responses thereby facilitating the establishment and maintenance of reproductive tract infection (Benedetti *et al.*, 2020) . About (50%) of women B.V. are asymptomatic. If symptoms do occur, the most common is thin, watery, malodors, non itchy discharge. The criteria used to diagnosis B.V. are: pH > 4.5, the presence of thin watery discharge, and fishy odor (with 10%) KOH (potassium hydroxide) (Hainer and Gibson, 2011).

1.2.5: Surface variations

The mycoplasmas cell surface is unique among prokaryotes and is highly evolved to accommodate the life style of these divers' organisms during interaction with their respective hosts. *Mycoplasma* cell devoid of lipopolysaccharides but contain large amounts of lipoproteins. High frequency variation of lipoproteins seems to be common to all mycoplasmas and result in changing mosaic of antigenic structure at the bacterial cell surface. This is thought to help these microorganisms to evade host immune surveillance and hence cause disease (Greenwood *et al.*, 2012). Particularly in the absence of a cell wall, several adaptive features must be in place to support the survival of these organisms during transmission and residence in varied niches, both extracellular and in some cases within host cells (David Sibley, 2011).

Mycoplasmas are replete with systems providing variation in the expression and structure of specific gene products, including several examples affecting the critical cell surface. Acquisition and selection such system may have played a critical evolutionary role that allowed host adaptation despite genomic reduction (Yogev *et al*., 2002).

One variation of these bacteria is the presence of small region of bases (oligonucleotide) called "hot spot" provide favorite targets to frequent insertion or deletions of nucleotide in order to switch genes ON and OFF (Millar *et al.,* 2007). These organisms may loss or gain of nucleotide during DNA replication by process termed slipped strand mispairing (Citti *et al*., 2005).

M. hominis appears to be equipped with a genetic system that allows it in vivo to alter its surface exposed, membrane –associated, antigenic repertoire. Three surface membrane proteins, P120, (*Lmp1* and *Lmp2,* and *Vaa*), whose products undergo genetic variability could account for the ability of this

microorganism to circumvent the host immune system (Mardassi *et al.,* 2007). *U. urealyticum* also undergo phase variation by which the organism may evade host immune responses (Zimmerman *et al*., 2011).

The highly antigenic P120 gene displays a hyper-variable region due to accumulation of mutations, while *Lmp1* and *Lmp2* genes show size variation and could be expressed as a chimearic protein . The *Vaa* gene product, which is involved in cell adherence, displays both size variation and frame shift mutation to create variant products (Mardassi *et al*., 2007).

1.2.6: Virulence factors

Human pathogenic mycoplasmas have been reported to possess many virulence factors, each of which was found to play an essential role in pathogenesis of these organisms. In general virulence factors of *Mycoplasma* spp. can be grouped as; attachment organelles, phospholipase enzymes, immunoglobulin A protease, and nuclease:

1- Attachment organelles: Because cytadherence is a necessary first step for infection of susceptible mucosal surfaces, these proteins P1in *M. pneumoniae* induce production a vigorous antibody response against the P1 adhesin (Williams, 2018).

2- Phospholipase enzymes: These enzymes hydrolyze phospholipids with the release of arachidonic acid. Three types of phospholipase enzymes are produced by *U. urealyticum* (A1,A2 and C) that are localized in the plasma membrane, therfore *U. urealyticum* is associated with amnionitis , and perinatal morbidity and mortality (spontaneous abortion, prematurity, still birth). It has been postulated that infection of the women genital tract may initiate a sequence of pathologic events related to phospholipase production (Novy *et al*., 2009).

U. urealyticum induces production of inflammatory cytokines, tumor necrosis factor (TNF), interleukin-6, and stimulates the release of nitric oxide (Li *et al*., 2000). The extensive release of these cytokines may contribute to the inflammatory response and to the pathology of chronic lung disease seen in very-low-birth weight and premature infants, therefore *U. urealyticum* induce apoptosis in human lung epithelial cells and macrophages (Li *et al*., 2002). This organism can infect neonate lung and involve in the chronic impairment of lung tissues both directly and indirectly by the production of inflammatory cytokines (Li, 2001).

Marai *et al*., (2004) determined that the antiphospholipid antibody syndrome is considered as autoimmune disease which causes recurrent pregnancy loss characterized by elevated titers of antiphospholipid antibody (Bayoumi, 2006).

3- Immunoglobulin A (IgA) protease: This enzyme produced mainly by *M. hominis* and *U. urealyticum* and it facilitates mucosal invasion by hydrolyzing mucosal IgA (Thurman *et al*., 2010).

4- Nucleases: These enzymes degrade nucleic acid of host cells to generate precursors for synthesis of their own nucleic acids . These nucleases could alter the nucleic acid metabolism of the host cells parasitized by Mycoplasmas. *M. fermentans* and *M. pentrans* produce the most potent nuclease activity (Bendjennat *et al*.,1997; Yiwen *et al.*, 2021).

Virulence factors in mycoplasmas include invasiveness, toxin-like substances, exotoxins, pathogenic enzymes, and some membrane components. Invasiveness refers to the ability of mycoplasmas to break through the host's defense function and to settle, reproduce, and spread in vivo; it is mediated by various factors that comprise adhesins and accessory proteins, capsular polysaccharides, invasive enzymes, and

biofilms. Toxin-like substances primarily include metabolites generated during the process of proliferation, such as H_2O_2, NH_3, and H_2S. Certain mycoplasmas can also secrete some exotoxins including community-acquired respiratory distress syndrome toxin (CARDS TX) and hemolysins, as well as express various pathogenic enzymes, such as lipolytic enzymes, peptidases, phosphatases, ecto-ATPases, cytotoxic nucleases and nucleotidases, which are considered important pathogenic factors for mycoplasmas. In addition, some inherent molecules of the cell membrane such as lipids, membrane lipoproteins, and the superantigens produced by *M. arthritidis*, may also have a significant pathogenic effect on the host cells or the immune system, as shown in Figure (1-2) (Yiwen *et al.*, 2021).

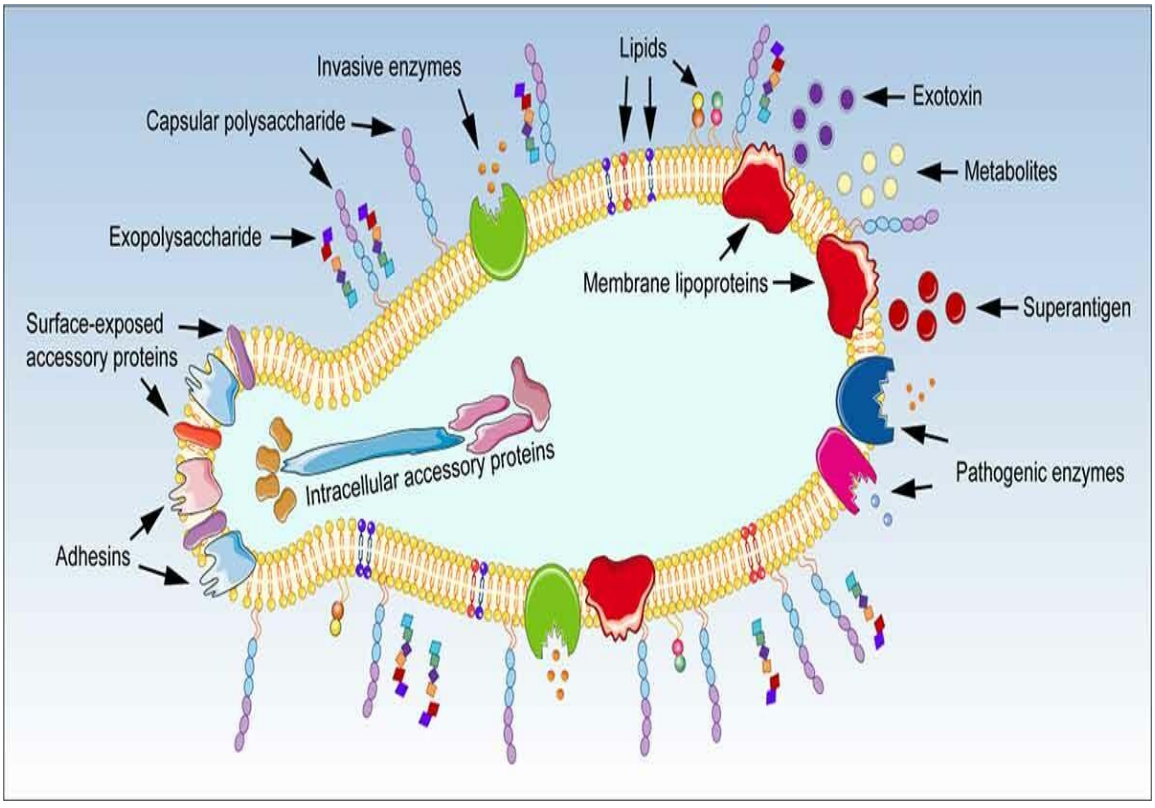


Figure (1-2): Schematic Diagram of Virulence Factors in Mycoplasmas (Yiwen *et al.*, 2021)

1.2.7: Routine laboratory diagnostic approaches

1.2.7.1: Cultural and biochemical test

Direct culture is the most common way to reliably detect *Mycoplasma*, but there is no single medium formulation is adequate for all Mollicutes species due to their different nutritional requirements (Cheong *et al*., 2011).

Previously most mycoplasmas were cultured on media composed in part of cell extracts and animal sera (Kraybill and Crawford, 1965). Shepard and Lunceford (1976), used differential agar medium for isolation of *U. urealyticum* and the basal medium was first prepared and contained trypticase soy broth and urea. Putrescine added to medium enabled better growth of *U. urealyticum* (Razin *et al*., 1977).

Antibacterial such as penicillin are used, thallous acetate are used also therefore, special or complex media are required for isolation of *Mycoplasma* spp. . Transport media are also developed. *Mycoplasma* of genital tracts being of three groups on the basis of the substrate they use: glucose metabolizers, arginine metabolizers and urea hydrolizers (Biernat-Sudolska *et al*., 2006).

Mycoplasmas were cultured in different media but the most *Mycoplasma* was grown in PPLO broth or agar supplemented with horse or other serum. Barber and Fabricant (1962), reported that mycoplasmas were isolated from cattles, pigs, sheeps, rats and dogs, used PPLO enrichment broth and agar supplemented with horse or swine serum. Al-Aubaidi and Fabricant (1968), used horse serum, fetal calf thymus (DNA) for cultivation of *Mycoplasma* from animals. Sasaki and Kihara (1987), were used egg yolk as a source of cholesterol for cultivation of *Mycoplasma*.

Arginine broth and arginine agar used for the isolation of the *M. hominis* and *U. urealyticum* from urogenital tract of women (Simihairi, 1990 ; Naher and Said, 2013). While Al-Bahli (1993), isolated urogenital tract mycoplasmas

from women by used U9B medium. Later a group of culture media were modiefied for isolation and identification of *Mycoplasma* and *Ureaplasma*; SP4 medium (Razin and Tully, 1995), AE10 medium was used for isolation genital *Mycoplasma* from infertile couples (Kareem, 1997), Monophasic-diphasic culture setup medium (MDCS) was used for isolation of *M. pneumoniae* from respiratory tract (Al-Ghizawi, 2001), moreover Al-Mossawi (2005) and Al-Ghizawi and Kadhim (2015), used (MDCS) for isolation genital *Mycoplasma* and *Mycoplasma* spp. from synovial fluid respectively. Many investigators have tried to find other materials to replace horse serum in the growth medium. They have used egg yolks extracted with organic solvents or with distilled water for cultivation of mycoplasmas (Sasaki *et al*., 1983). Penicilline, ampicillin, or cefoperazone and thallium acetate were also added to *Mycoplasma* culture medium to inhibit the growth of other bacterial types (Atlas, 2006).

As it has been mentioned above, mycoplasmas are highly adapted to their host, which provide most of its nutritional requirements for their growth. For this reason, there are only a few biochemical properties that can be investigated in the diagnostic laboratory (Govender, 2010). Biochemical tests allow grouping but not identification of the genital *Mycoplasma* isolates (Mirnejad *et al*., 2011).

M. hominis grows well in SP 4 broth or SP 4 agar supplemented with arginine, but it will also grow on A 8 agar and in 10 B broth (Waites *et al*., 2012). *Mycoplasma* organisms share a typical colonial morphology, the 'fried-egg' colony, which is made up of a central zone, embedded in the agar and a peripheral zone on the agar surface. This character of the colony is regarded as unique for these organisms and serves as one of the most

important criteria in distinguishing them from other bacteria, as shown in Figure (1-3) (Brown,1960).

Although culture is considered the reference standard for detection of Mycoplasmas it is expensive and requires specialized media and expertise that are not widely available outside of larger medical centers or *Mycoplasma* research or reference laboratories (She *et al*., 2010).

Confirmed culture results can usually be available within 2 to 5 days, exclusive of specimen transport time and shipment if an offsite reference laboratory is used. Ureaplasmas can be positively identified to genus level by their colonial morphology and urease production, In contrast, mycoplasmal species such as *M. hominis* that produce fried-egg colonies on A8 or SP4 agar may be presumptively identified based on growth rates, hydrolysis of arginine, and body site of origin, but definitive species identification requires additional tests (Waites *et al*., 2012).

Many types of modified media, including various broths, agars, and biphasic systems, have been employed. This system is consisting of an arginine agar plate, an arginine biphasic medium, a urea agar plate, and urea broth medium, provided a satisfactory means of isolating both *U. urealyticum* and *M. hominis*. However, Kundsin *et al*.,(1978) used an A7 differential agar plate and also has used Boston broth, a modified urea-containing medium, for isolation of *U. urealyticum*.

Al-Mossawi (2005) and Al-Ghizawi and Kadhim (2010) used Monophasic-Diphasic Culture Setup (MDCS) for isolation genital Mycoplasmas, *Mycoplasma* spp. from synovial fluid. While AL-Ghizawi and Ghanem (2014), used MDCS for isolation of some *Mycoplasma* spp. from Urinary tract infection in Basrah City. Al-Azawi, (2012) used modified culture media

(IH broth) and (IH agar) for the isolation of *Mycoplsma* Urogenital infection from women in AL-Diwanyia city.

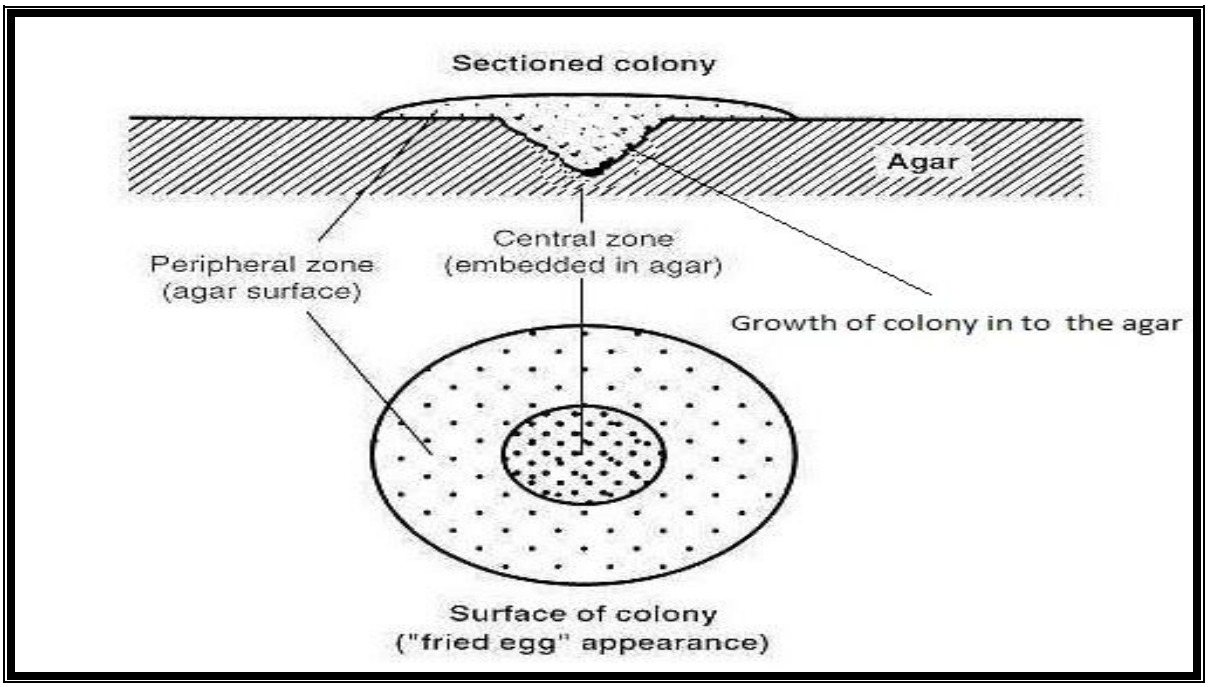


Figure (1-3) : Section of *Mycoplasma* Colony Morphology (Brown,1960)

1.2.7.2: Conventional PCR assays

Molecular assays have developed for detection and specific identification of *M. hominis* and *Ureaplasma* spp. In different specimen such as genital tract specimens, amniotic fluids, and respiratory tract specimens from newborns (Yoon *et al*., 2003). PCR assays for *M. hominis* have used specific *16S rRNA* sequences as the target for amplification and detection; whereas similar assays for *U. urealyticum* have used specific *16S rRNA* sequence, or urease gene sequence for detection and identification of this organism (Seifoleslami *et al*., 2015).

To confirm species identity for mycoplasmas growing on agar, additional procedures (e.g. a PCR assay) must be performed because there are no phenotypic tests that can distinguish them (Naher and Said 2013).

Conventional PCR assays for *M. hominis* have mainly used *16S rRNA* as a gene target (Cunningham *et al*., 2013). Because some heterogeneity has been reported in the *16S rRNA* gene of *M. hominis* other targets including *gap*, *fstY*, and *yidC*, have been developed (Férandon *et al*., 2011).

PCR assays are important to detect and identify the individual *Ureaplasma* species for research purposes. Gelbased conventional PCR assays targeted sequences of *16S rRNA* and *16S rRNA* to *23S rRNA* intergenic spacer regions, the urease gene, and *mba*, (Kong and Gilbert, 2004). Yoshida *et al*., (2003), described a conventional PCR assay applied to urine specimens of patients with urethritis targeting *16S rRNA* genes of *M. genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum.*

Nested and multiplex PCR were also proposed for detecting *Mycoplasma* spp. (McIver *et al.,* 2009). Cervical samples of 200 infertile women were evaluated by Multiplex PCR to detect *M. hominis*, *M. genitalium*, *U. urealyticum* and *Chlamydia trachomatis* (Sadeqi *et al*., 2022).

Real time PCR assays have been shown to be extremely useful for the simultaneous detection and biovar discrimination of mycoplasmas and ureaplasmas in clinical specimens (Choe *et al.,* 2013). Cao *et al.* (2007), have developed two Real –Time PCR assays for quantitative detection of *U. parvum* and *U. urealyticum.* Moreover, this quantitative PCR allowes to confirm the role of *M. hominis* in the patients with severe extragenital infections such as brain abscess, pneumonia, mediastinitis, pericarditis, peritonitis and pyelonephritis both in immunosuppressed and in immunocompetent individuals (Pascual *et al*.,2010).

1.2.7.3: Serological test such as cytokines and their role in mycoplasmas genital tract infections

Several techniques have been proposed for detection of antibodies against bacterial antigens. These techniques are : Growth inhibition test (GIT), Indirect haemagglutination test (IHA), Metabolic inhibition test(MIT), Mycoplasmacidal test (MT) ,Immunofluorescence test (IFT), Enzyme-linked immunoassay sorbent assay (ELISA), and Complement fixation test (CFT) (Clausen *et al*., 2001).

The most common test, used for diagnosis of genital mycoplasmas is the complement fixation test (Kumar *et al*., 2014).The sensitivity of this assay depends on whether the first serum sample is collected early or late after the onset of illness and on the availability of paired sera collected with an interval of 2-3 weeks. Metabolic inhibition test for *M. hominis* and *U. urealyticum,* is based on inhibition of the alkalinization of the medium that follows the production of ammonia due to the hydrolysis either of arginine or urea (La Vignera *et al.*, 2014).

Mycoplasmacidal test in the presence of antibody and complement, *Mycoplasma* spp. are rapidly killed by lysis (Lai *et al.*, 1990; Afriat *et al.*, 2013), while Immunofluorescence test is generally used in the direct or indirect method. This test has been recommended as a useful test for classification of species of *Mycoplasma* (Vervloet *et al.,* 2007).

The use of ELISA for detection of antibodies is one of the most sensitive and convenient methods for measuring antigens and antibodies, it can be performed in short time and without using radiolabelled materials, multiwell microtitre plates used as the solid phase are easy to handle and wash, and when used with automated readers and multiple well washers allow a large number of samples to be assayed (Meager *et al*., 1987).

Mycoplasmas can activate macrophage monocyte, leading to expression and secretion of the major proinflammatory cytokines like TNF-α , IL-1β, IL-6, IL-8, IL-12, IL-16, Interferon-γ (Taylor-Robsion and Lamount , 2011). Toll-like receptors(TLRs) on immune and epithelial cells recognize pathogen-associated molecular pattern (PAMPs), invariant structures of microorganisms, and activate an innate immune response, TLR-2 is involved in the recognition of PAMPs on mycoplasmas(Kim *et al*., 2004).

There are many studies which show the interactions of mycoplasmas with various cell types *in vitro* and *in vivo* and their implication as causative factors, cofactors or opportunistic agents in human diseases . As a result, mycoplasmal infections have a chronic and persisting nature and the host is unable to suppress these infections through immune and non-immune responses (Nicolson, 2019).

Mycoplasmas exhibit host and tissue specificities. As such, they are able to enter the host cells, multiply, and survive within phagocyte and non-phagocyte cells for a long period of time showing slow growth rates. They develop mechanisms of mimicry showing antigenic variability and the ability to suppress the host's immune responses (Rottem, 2003).

Mycoplasmas are a large group of prokaryotes which is believed to be originated from Gram-positive bacteria via degenerative evolution, and mainly capable of causing a wide range of human and animal infections. Although innate immunity and adaptive immunity play crucial roles in preventing *Mycoplasma* infection, immune response that develops after infection fails to completely eliminate this bacterium under certain circumstances. Thus, it is reasonable to speculate that mycoplasmas employ some mechanisms to deal with coercion of host defense system, which can be divided into four aspects: (**i**) Molecular mimicry and antigenic variation on the surface of the bacteria to

evade the immune surveillance; (**ii**) Overcoming the immune effector molecules assaults: Induction of detoxified enzymes to degradation of reactive oxygen species; Expression of nucleases to degrade the neutrophil extracellular traps to avoid killing by neutrophil; Capture and cleavage of immunoglobulins to evade humoral immune response; (**iii**) Persistent survival: Invading into the host cell to escape the immune damage; Formation of a biofilm to establish a persistent infection; (**iv**) Modulation of the immune system to down-regulate the intensity of immune response (Qin *et al.* , 2019).

Mycoplasmas have been shown to affect the immune system using both in *vitro* and in *vivo* model systems, for example, they can activate macrophages, T cells, natural killer cells, and complement; stimulate the proliferation of B and T cells; and induce the expression of major histocompatibility complex class I and II molecules. It has also been shown that some mycoplasmas can induce the gene expression and production of cytokines such as IL-1, IL-2, IL-4, IL-6, IFNs, TNF, and granulocyte- macrophage colony-stimulating factor (Zhang *et al*., 2000).

Ureaplasma urealyticum vaginal colonization in pregnant women has been related to elevated vaginal concentrations of interleukin-1 receptor antagonist (IL-1rα) . IL-1rα is an anti-inflammatory cytokine and a natural competitive inhibitor of the pro-inflammatory cytokine, interleukin-1β (IL-1β) (Ryckman *et al*., 2008). *Mycoplasma genitalium* causes urogenital infections, adhesion to epithelial cells promotes acute inflammation via triggering of innate immune sensors expressed on the cells' surface. Activation of pro-inflammatory signals ultimately results in recruitment of leucocytes to the infection site. The recombinant C-terminal portion of the immunogenic protein MG309 (rMG309c) activates NF-κB via TLR2/6 in genital epithelial cells (EC), which

in turn secreted pro-inflammatory cytokines, including interleukin-6 (IL-6) and IL-8 (Benedetti *et al.,* 2020).

Anti-microbial cell-mediated immune defenses are initiated by IL-1β production. *M. hominis* is associated with a pro-inflammatory immune response, i.e. elevated vaginal IL-1β concentration, in the vagina of healthy, asymptomatic women suggests that the enhanced proliferation of this *Mycoplasma* in conjunction with other infections results in increased vaginal inflammation (Doh *et al*., 2004).

1.2.8: Antimycoplasmal agents

Unlike conventional bacteria, *Mycoplasma* does not have a rigid cell wall, hence, they are fully resistant to antibiotics that directly act on the cell wall synthesis, such as B-lactam antibiotic (Mihai *et al.*,2011).In addition, Mollicutes are generally resistant to other groups of antibiotics represented by polymyxin, rifampin, and the sulfonamide (Jafar *et al.*,2010).

The tetracycline has always been in the forefront of antibiotics usage, particulary for genital tract infections. The quinolones also have the advantage of exhibiting some cidal activity. Fluoroquinolons are also attractive choice for treating genitourinary tract *Ureaplasma* infection (Mihai *et al.,* 2011).

Mycoplasmas are difficult to eradicate from human or animal hosts by antibiotics treatment because of the resistance to the antibiotics, or it lacks cidal activity, or there is invasion of eukaryotic cells by some mycoplasmas (Taylor-Robinson and Bebear,1997). Mycoplasmas and ureaplasmas lacked peptidoglycan. They are not affected by beta-lactams or vancomycin. Also they are not susceptible to sulfonamides or trimethoprim because they do not synthesize folic acid (Kenny and Cartwright, 2001). However, they are generally susceptible to certain antibiotics that interfere with protein synthesis, such as tetracyclines. While ureaplasmas are generally susceptible to

macrolides, they are resistant to lincosamides except in high concentrations. *M. hominis*, in contrast, is naturally resistant to erythromycin *in vitro*, but susceptible to 16-membered macrolides (josamycin and miocamycin) and lincomycin (Waites *et al* ., 2005).

Treatment options for pregnant women are limited due to the potential tetratogenic and harmful effects associated with the use of some antimicrobials during pregnancy. Even fewer options are available for the treatment of intra-amniotic ureaplasma infections, as ureaplasmas are inherently resistant to the beta-lactam and glycopeptide antimicrobials (due to the lack of cell wall), and also demonstrate resistance to trimethoprim, sulfonamides and rifampicin (Kenny and Cartwright, 2001). Antimicrobials that are potentially active against ureaplasmas include the tetracyclines, fluoroquinolones and macrolides (Waites *et al.* 2009). However, not all of these antibiotics are appropriate to administer during pregnancy.

Mycoplasmas are recognized now to play a more important role in human infections than was previously thought. Their slow-growing, non-culturable nature enables them to establish chronic infections, resist the effects of antibiotics, and protect the organisms against immune system reactivity (Mamedaliyeva *et al*., 2011).

Genitourinary tract infections are infections that develop along a patient's urinary or genital tract, or both, in some cases. They are very common, especially among sexually active people and those with certain risk factors like a history of genitourinary anomalies(Blanchard and Bébéar, 2002).Treatment usually involves medication to kill the infectious organism along with proper hydration to keep the urinary tract clear. Some people have a much higher risk of genitourinary tract infections. Women, older patients, patients who use catheters, and people who are sexually active are at increased risk, as are those

with a history of blockages in the urinary tract (Nicolle, 2002). Fungi, viruses, and bacteria can all cause genitourinary tract infections, and some infections are very difficult to treat due to drug resistant organisms(Nicolle, 2008).

The most widely used antibiotics for treatment of mycoplasmas infections include tetracycline, MLS group and fluoroquinolones (Mihai *et al*., 2011). Some antibiotis share the advantages of being potentially active against the bacteria which could be associated with mycoplasmas in respiratory and urogenital tract infections. They share also the ability to reach high intracellular concentrations where mycoplasmas could localize. Only fluoroquinolones and ketolide have shown mycoplasmicidal qualities. Phenicols and aminoglycosides are kept for the treatment of special cases (Bébéar *et al.*, 2011) .

These types of protein inhibiting antibiotics will stop the protein adhesion of the *Mycoplasma* to host cells but not directly kill the *Mycoplasma* itself (Niclson *et al*., 1998; Jafar *et al*., 2010). Although, erythromycin remains the drug of choice for the treatment of *U. urealyticum* (Redelinghuys *et al*., 2014). The antimicrobial susceptibility profile of *M. genitalium* appears to be similar to that of *M .pneumoniae* in that isolates of this species are susceptible to tetracycline and a variety of macrolide (Sethi *et al.*, 2017).

The presence of the organisms in the urogenital tract with or without attendant symptoms following treatment with these agents may be directly related to the dosage and duration of treatment. According to the treatment guide lines for STDs, azithromycin for treatment of urethritis is administered in a single 1-gram dose (Bissessor *et al.*, 2015).

1.2.9: Immune response to genital mycoplasmas

Mycoplasmas are the smallest and simplest self-replicating organisms. Due to the lack of a rigid cell wall, this bacterium is only bound by an outer structure including capsule, adhesive structure and adhesion-related proteins, as well as a unit membrane . Although the basic structure is simpler than the common Gram-negative bacteria, there is a complex cross-talk between *Mycoplasma* and the host immune system involving *Mycoplasma* induced non-specific and specific immune responses. Similar to other microorganisms, at the beginning of infection, the innate immune response, consisting mainly of innate immune molecules and innate immune cells, is non-specific but plays a critical role in the defense against this microbe (Qin *et al.,* 2019) .

Innate Immune cells, such as neutrophils, macrophages, and natural killer cells, not only have the capacity to recognize pathogen-associated molecular patterns (PAMPs) of *Mycoplasma* via toll-like receptors, but also can kill these microorganisms. First of all, phagocytizing neutrophils and monocytes/macrophages inevitably yields oxidative bursts that are elicited by bacterial infections, leading to the release of reactive oxygen species (ROS). ROS are essential participants of various innate immune cell responses against microorganisms, including oxidative radicals such as superoxide, hydroxyl radicals, H_2O_2 and organic hydro-peroxides (Nguyen *et al.,* 2017) .

Mycoplasmas still have the ability to propagate and survive within the host for a long period of time after invading an appropriate host. To maintain their survival and persistent infection, it is very likely that *Mycoplasma* have evolved rather sophisticated mechanisms to resist to coercion by the host immune system , in addition to molecular mimicry and antigenic variation, which was first described and is widely accepted, we will also present other novel strategies that *Mycoplasma* have evolved, including defense against

oxidative stress, degradation of neutrophil extracellular traps (NETs), capture and cleavage of immunoglobulins, cell invasion, the formation of biofilms and negative regulation of the immune response (Hoelzle *et al.,* 2020) .

As early as 2010, a classic review reported that most *Mycoplasma* species could generate antigenic variation, including *Mycoplasma genitalium, M. penetrans, M. hominis, M. hyorhinis, M. gallisepticum, M. capricolum subsp. capricolum, M. pulmonis, M. bovis, M. agalactiae, M. synoviae, Ureaplasma parvum, and U. urealyticum* , this phenomenon provides these wall-less pathogens with a means to escape the host immune response and to modulate surface accessibility by masking and unmasking stably expressed components that are essential in host interaction and survival (Citti *et al.,* 2010) .

Mycoplasma spp. depending on the host cells to achieved their biological activities, thus mycoplasmas have excellent ability to acclimatized to stay alive on exposed surfaces host tissues , avoiding host defenses by several means. Whether attached to the surface of eukaryotic cells or upon invasion, some mycoplasmas interfere and alter cellular pathways of the host cell, both at the regulation and/or functional level .To protect itself from such detrimental consequences, the host organism engages upon infection a series of responses that involves a number of signaling pathways, eventually resulting in the activation of both innate and acquired immunity, which elicit processes stimulating acute and chronic inflammation, respectively. In turn mycoplasmas developed mechanisms to escape immune control, in such a way that they are able to colonize mucosal surfaces and invade different areas of the body (Kumar *et al.,* 2011) .

1.2.9.1: Cytokines

Cytokines are key reactive and modulator molecules participating in innate and adaptive immune response, such as the recruitment and activation of

immune cells and the induction of initial cell differentiation. The complement system not only constitutes part of the innate immune system but also is one of the important tactics by which antibodies exert their immune effects. In the late stage of infection, the adaptive immune response, whose specific participant cells and molecules are different types of T/B lymphocytes and antibodies, promoting further elimination of the invading pathogen (Freeley *et al.,* 2016) .

Interleukin (IL)-18 is a pro-inflammatory cytokine, originally termed interferon (IFN)-gamma-inducing factor, which promotes T-helper type 1 (Th1) cytokine responses (Jensen, 2004) . IL-18 is a cytokine that belongs to the IL-1 super family and is produced by macrophages and other cells. IL-18 works by binding to the interleukin-18 receptor, and together with IL-12 it induces cell-mediated immunity following infection with microbial products like lipopolysaccharide (LPS). After stimulation with IL-18, natural killer (NK) cells and certain T cells release another important cytokine called interferon-γ (IFN-γ) or type II interferon that plays an important role in activating the macrophages or other cells, apart from its physiological role, IL-18 is also able to induce severe inflammatory reactions, which suggests its role in certain inflammatory disorders (Liu *et al.*, 2010) .

The interferons, the group known as type I IFN specifically comprises a family of cytokines that include multiple (>11) IFN-α genes and a single IFN-β gene. Other IFNs include type II IFNs (IFN-γ), which has integral immune function and type III IFNs (IFN-λ), an antiviral cytokine family (Young and Bream, 2007 ; Donnelly and Kotenko , 2010) . It is now known that type I IFNs (IFN-α and IFN-β) are the major contributors in innate immunity serving as the first line of defense against viruses. They are more than just anti-virals as they play a major role in linking innate to adaptive immunity. Type I IFNs

are produced in response to number of viral and bacterial infections in multiple cell types including lymphocytes, macrophages, endothelial cells and fibroblasts. Leukocytes are a major source of IFN-α, with plasmacytoid dentritic cells (DC) as major producers (Santini *et al.*, 2002) . Fibroblast/epithelial cells are major producers of IFN-β, though most cell types can make the cytokine upon stimulation. Both IFN-α and IFN-β signal through a common receptor IFNAR that consists of IFNAR1 and IFNAR2 chains. Binding of the cytokines to the receptor leads to the activation of JAK-STAT signaling pathway, which results in direct antiviral effects of type I IFNs and expression of interferon-inducible genes (Seo and Hahm , 2010) .

1.2.9.2: The role of TLRs in genital mycoplasmas

Mycoplasma spp. and *Ureaplasma* spp. were able to stimulate the innate immune responses via different types of TLRs, including TLR2/6 and TLR1/10 (neutrophil activation and secretion of mucosal antibody of IgA) (Behzadi *et al.,* 2019) . As shown in Figure (1-4) .

TLR-2 and TLR-6 heterodimers were critical for identifying significant microbial causal agents of urinary tract infections, including as *Candida albicans, Staphylococcus* spp., *Streptococcus* spp., *Mycoplasma* spp., and *Ureaplasma* spp. . TLRs recognizes and interacted with a wide range of microorganisms in Gram-positive bacteria, including lipopeptides, peptidoglycan, and lipoteichoic acid, as well as mycoplasmas and Mycobacteria possess lipoproteins. TLR4 is a signaling receptor, on the other hand for lipopolysaccharide (LPS) (Benedetti *et al.,* 2020) .

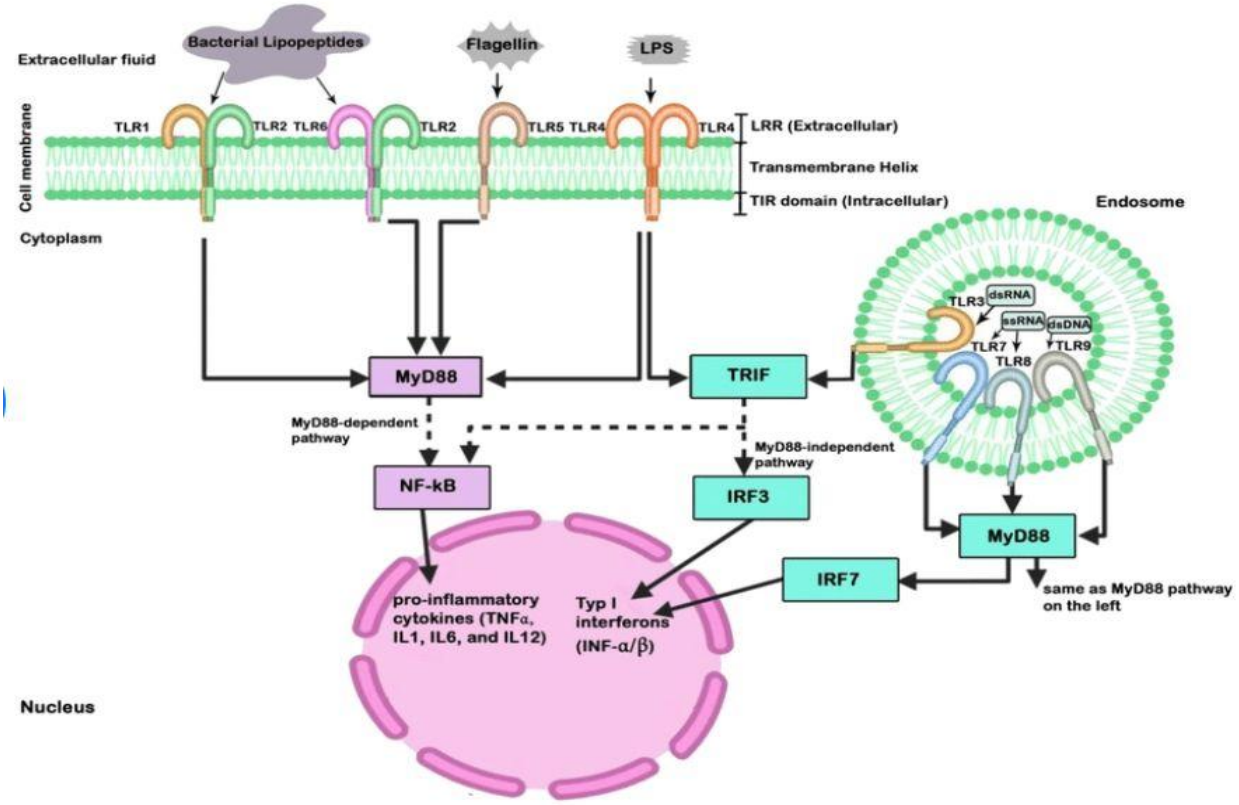


Figure (1-4) : Schematic Diagram of Toll – like Receptors (TLRs) Signaling Pathways (Ashayeri Ahmadabad *et al*., 2020)

2. Materials and Methods

2.1: Materials

2.1.1: Laboratory equipment and instruments

The laboratory equipment and instruments used in the present study are listed in Table (2-1).

Table (2-1): Laboratory Equipment and Instruments

No.	Laboratory Equipment and Instruments	Manufacturer/Origin
1.	Anaerobic Jar	BBL (England)
2.	Autoclave	Hirayama (Japan)
3.	Bench centrifuge	Memmert (Germany)
4.	Benzen burner	Amal (Turkey)
5.	Compound light microscope	Zeiss (Germany)
6.	Digital camera	Sony (Japan)
7.	Disposable (Pteri Dish ,Syringe, Plane and gel tube) and glassware	Citro (China)
8.	Distiller	Ogawa (Japan)
9.	DNA extraction tube 100µl , PCR tube 50µl	Capp (Germany)
10.	Electric sensitive balance	Denver (USA)
11.	ELISA reader , ELISA washer	BioTeck (USA)
12.	Eppendrof centrifuge	Hettich (Germany)
13.	Eppendrof tube	Eppendrof (Germany)
14.	Filter paper	(USA)
15.	Gel electrophoresis system	Cleaver Scientific (UK)
16.	Hood	LabTech (Korea)
17.	Hot plate with magnetic stirrer	Heidolph (Germany)
18.	Incubator, Oven	Memmert (Germany)
19.	Inoculating loop	Memmert (Germany)
20.	Micropipette (0.5-10µl , 5-50µl , 100-1000µl)	Capp (Denmark)

21.	Microwave	Panasonic (Japan)
22.	Millipore filters (0.22 µm , 0.25µm ,0.45µm)	Sigma (USA)
23.	NanoDrop	Implen (Germany)
24.	Paraflim	BDH (England)
25.	PCR Thermal cycler	Techne (UK)
26.	pH-meter	WTW (Germany)
27.	Refrigerator	Concord (Lebanon)
28.	UV – Vis spectrophotometer	Analytic Jena (Germany)
29.	Vortex mixer	Gemmy (Taiwan)
30.	Water bath , Oven , Incubator	Memmert (Germany)

2.1.2: Biological and chemical materials

The biological and chemical materials used in this study are listed in Table (2-2).

Table (2-2): Biological and Chemical Materials

No.	Biological and Chemical Materials	Manufacturer(Origin)
1.	Agar - agar	Difco (USA)
2.	Agarose , TBE buffer	Condalab (Spain)
3.	Ampecillin	Middle east pharmaceutical (Jorden)
4.	Ceftriaxone	
5.	Crystale violet , Iodine , Safranine , Ethanol (96%)	CDH (India)
6.	Fluocanazol	Middle east pharmaceutical (Jorden)
7.	Glucose	Fluka (Germany)
8.	Glycerol	Merck (England)
9.	Horse serum	PAA (Austria)
10.	Hydrogen peroxide	BDH (UK)
11.	L- Arginine	BDH (England)
12.	L- cystine - HCL	
13.	Loading dye(bromophenolblue)	Promega (USA)

14.	McFarland's standard solution	Biomerieux (France)
15.	NaCl	BDH (UK)
16.	Nuclease free water	Bioneer (Korea)
17.	Nystatin	Middle east pharmaceutical (Jorden)
18.	Penicillin-G	Striles (India)
19.	Phenol red	BDH (England)
20.	Potassium chloride solution	Himedia (India)
21.	Sodium chloride	
22.	Tetramethyl-P-phenylenediamine dihydrochloride (Oxidase test)	
23.	Urea	Sigma (USA)
24.	Yeast extract	Difco (USA)

2.1.3: Commercial kits

The commercial kits used in the present study are illustrated in Table (2-3).

Table (2-3): Commercial Kits

No.	Type of Kit	Company(Origin)
1.	DNA extraction kit	Favorgen (Taiwan)
2.	DNA ladder	Bioner (Korea)
3.	Human IFN-β , TLR-6 and IL-18 (ELISA Kit)	BT LAB (China)
4.	Master mix	Bioner (Korea)
5.	Primers	Bioner (Korea)

2.1.4: Culture media

According to Forbes *et al*. (2007) and MacFaddin (2000) culture media used in this study are listed in Table (2-4) . All media were prepared according to the manufacturer's (Himedia (India)) and 5% fresh human blood was added to blood agar base after sterilization to prepare blood agar .

Table (2-4): Culture Media and Purpose of Use

No.	Culture Media	Purpose of Use
1.	**Blood agar base**	Detection of hemolysin production
2.	**Brain heart infusion broth**	It was used in preservation of bacteria
3.	**Eosin methylene blue agar**	Selective and differential of members of Enterobacteriacea
4.	**MacConkey agar**	Isolation and identification of members of Enterobacteriacea
5.	**Mannitol salt agar**	Isolation of *Staphylococci* and differentiation of *Staphylococcus aureus*
6.	**Muller- Hinton agar**	Antibiotic susceptibility
7.	**Nutrient agar , Nutrient broth**	General purpose medium
8.	**Pleuropneumoniae like organism (PPLO broth base) , (PPLO agar base)**	Detection of mycoplasmas

2.1.5: Antibiotics discs

The antibiotics used in this study are listed in Table (2-5) .

Table (2-5): Antibiotic Discs

Antibiotics Group	Antibiotics Disc	Action Effect	Symbol	Potency (µg)	Manufacturer/ Origin
Lincosamides	Clindamycin	Protein synthesis inhibitors	CLD	150	Bioanalyse (Turkey)
Macrolide	Erythromycin		E	15	
	Azithromycin		AZM	15	
	Clarithromycin		CLA	150	
Tetracyclines	Tetracycline		TE	30	
	Doxycycline		DO	100	
Fluoroquinolones	Ciprofloxacin	DNA synthesis inhibitors	CIP	5	
	OFloxacin		OFX	200	

2.1.6: Diagnostic kits for detection of genital mycoplasmas

2.1.6.1: Favor Prep® genomic DNA mini kits was used for DNA extraction /(Favorgen , Taiwan)

DNA extraction kit and it's composed reagent as in Table (2- 6) .

Table (2-6): Compositions of Favor Prep® Genomic DNA Mini Kits

No.	Compositions of Favor Prep® kits	Basic Components
1.	FABG Mini Columns	300 Columns
2.	Collection tube (2ml)	600 tube
3.	FATG Buffer	75 ml
4.	FAGB Buffer	100 ml
5.	W1 Buffer	130 ml
6.	W2 Buffer	50 ml
7.	Elution Buffer	75 ml
8.	Proteinase K	6 ml
9.	Ethanol (96-100%)	200 ml

2.1.7: Polymerase chain reaction (PCR) primer pairs

The primer pairs used in this study were listed in Table (2-7) .

Table (2-7): Primer Pairs

Organism	Primer Name	5'-sequence-3'	Amplicon Size (bp)	Refrence
M. hominis	H1 F	CAATGGCTAATGCCGGATACGC	270	(Nassar *et al.* , 2021)
	H2 R	GGTACCGTCAGTCTGCAAT		
U. urealyticum	U5 F	CAATCTGCTCGTGAAGTATTAC	429	
	U4 R	ACGACGTCCATAAGCAAC		
M. genitalium	MgPa F	AAGTGGAGCGATCATTACTAAC	495	
	MgPa R	CCGTTGTTATCATACCTTCTGA		

16S rRNA	1492R	GGTTACCTTGTTACGACTT GGYTACCTTGTTACGACTT	1479	(Loy *et al.*, 2002)
	27F	AGAGTTTGATCCTGGCTCAG AGAGTTTGATYMTGGCTCAG		

2.2: Methods

2.2.1: Preparation of growth supplements and solutions

2.2.1.1: Horse serum

A 500 ml vial of horse serum was added for enrichment media (Modified arginine urea medium).

2.2.1.2: Yeast-extract broth

Yeast extract was prepared by adding 25 gm of active baker's yeast to 1 liter of D.W at 45 ℃ . After mixing well, the mixture was boiled for 30 minutes, and allowed the yeast cells to settle and clarified by centrifugation for 30 minutes at 1000 rpm. The supernatant was filtered by using filter vacuum pump. The mixture was autoclaved for 121° C, 15 pounds. The sterile yeast extract was dispensed aseptically in 10 ml sterile screw-caped bottles and stored at -20^{o}C (Collee *et al*., 1996).

2.2.1.3: Arginine solution (30%)

It was prepared by dissolving 30 gm of L-arginine in a small volume of D.W, and completed up to 100ml D.W, the mixture was sterilized by millipore filter paper, and stored at 4℃. It was used for supplemention of bacterial growth (Razin and Tully, 1983).

2.2.1.4: Cysteine solution (2%)

This solution was prepared by dissolving 2 gm of L-cystine-HCl in small volume of D.W, and completed up to 100 ml D.W, it was sterilized by millipore filter paper and stored at 4℃. It was used as bacterial growth supplement (Razin and Tully, 1983).

2.2.1.5: Urea solution (10%)

It was prepared by adding 10gm of urea to a small volume of D.W, and completed up to 100 ml D.W, then sterilized by millipore filter paper 0.22µm and stored at 4°C. It was used as bacterial growth supplement (Razin and Tully, 1983).

2.2.1.6: DNA solution (0.2%)

It was prepared by dissolving 0.2 gm of deoxy-ribonucleate (calf-thymus) in small volume of D.W, and completed up to 100 ml D.W, sterilized by millipore filter paper 0.22µm and stored at 4°C (Edward , 1971).

2.2.1.7: Putrescine-dihydrochloride solution (0.2%)

It was prepared by dissolving 0.2 gm of putrescine-dihydrochloride in small volume of D.W, and completed up to 100 ml D.W, sterilize by millipore filter paper 0.22µm and stored at 4°C (Sambrook *et al*.,1989).

2.2.1.8: Dipotassium hydrogen (K_2HPO_4) solution

It was prepared by dissolving 2.5 gm of K_2HPO_4 in 100 ml D.W, sterilized by millipore filter paper 0.22µm and stored at 4ºC (Collee *et al.*, 1996).

2.2.1.9: Phenol red solution (0.4%)

It was prepared by dissolving 0.4 gm of phenol red in small volume of D.W, and completed up to 100 ml D.W, sterilized by millipore filter paper 0.22µm and stored at 4°C (Downes and Ito, 2001).

2.2.1.10: Ethidium bromide solution

It was prepared by dissolving 0.05 gm of ethidium bromide in 10ml distilled water and stored in a dark reagent bottle according to the method of (Sambrook *et al*.,1989).

2.2.1.11: Bacterial inhibitors

2.2.1.11.1: Crystalline ceftriaxone

It was prepared by adding 0.5 gm of crystalline ceftriaxone to 5 ml of D. W, sterillized by millipore filter paper 0.45 µm and used immediately after preparation. It was used as bacterial growth inhibitor (Razin and Tully,1983).

2.2.1.11.2: Nystatin solution

It was prepared by dissolving 1gm of nystatine (after crashed) to 10 ml of D. W, sterile by millipore filter paper 0.45 µm and used as fungal growth inhibitor (Razin and Tully,1983).

2.2.1.11.3: Floucanazol solution

This solution was prepared by dissolving 0.5 gm of floucanazol in 5 ml of D. W, then sterilized by millipore filter paper 0.22µm (Razin and Tully,1983) .

2.2.1.12: Normal saline solution

It was prepared by dissolving (8.5gm) of NaCl in a small volume of distilled water, and then completed to (1000ml), pH fixed at (7.2) and sterilized in autoclave at (121^{o}C) for (15) minutes, then kept at (4^{o}C) . Sterial ready to use normal saline were used for all assays and experiments that need to use it (MacFaddin, 2000).

2.2.1.13: McFarland's turbidity standard (0.5)

The fresh 0.5 McFarland's standard (1.5×10^{8} CFU/ml) was prepared by adding 0.5 ml of 1.175% barium chloride [$BaCl_2$. $2H_2O$: H_2O (w/v, 1.175g: 98.825 ml)] to 1% sulfuric acid [H_2SO_4: H_2O (v/v, 1 ml:99 ml)] in order to obtain a barium sulfate precipitate . The solution was used to visually compare the turbidity of a suspension of bacteria with the turbidity of the 0.5 McFarland's standard. The McFarland's standard tubes were sealed with parafilm to prevent evaporation and stored in the dark for up to 6 months at room temperature . The accuracy of a prepared 0.5 McFarland's standard was checked by using a spectrophotometer. The optical density was measured at 625 nm that should be between 0.08 and 0.1 (Murray *et al.* ,2003) .

2.2.2: Reagents

2.2.2.1: Oxidase reagent

It was prepared directly by dissolving (1gm) of tetramethyl-paraphenylene diamine dihydrechloride in 10ml of distilled water and stored in a dark container. It was used to detect the ability of bacteria to produce oxidase enzyme . A small

portion of the bacterial colonies was spread on the filter paper contains oxidase reagent by a wooden stick , when the color of smear turned to purple , the oxidase test was positive . The test depends on the presence of certain bacterial oxidase that would catalyze the transport of electrons between electron donors in bacteria and a redox dye (tetramethyl-p-phenylene-diamine dihydrechloride (Forbes *et al.*, 2007).

2.2.2.2: Catalase reagent

The reagent was prepared by dissolving (3ml) of H_2O_2 in (100ml) of distilled water (3%) and stored in dark container . It was used to recognize bacterial capability to produce catalase enzyme (Forbes *et al.*, 2007).

2.3:Patients

2.3.1:Specimens collection

A total of 223 specimens were collected (blood + urine) (123 pregnant and 100 non-pregnant married women) were selected from patients laboratory diagnosed with UGTIs , who attended the Teaching Hospital of Maternity and children Hospital and Private Clinics in Babylon province, during 6 months from January to June 2021. The ages of those patients ranged from 20 –49 year .

The blood samples (serum) and part of urine were saved both of them at – 20 C^{o} until systemic and innate immunological study were performed and the other part of urine had been inoculated on different bacterial culture media (PPLO , MAU- broth and agar) ,MacConkey and Blood agar) incubated aerobically at 37^{o}C for 24-72 hrs .

2.3.2:Control group

Twenty cases (pregnant and non-pregnant married women , 10 cases for each) (Heath women), their age ranged from 20 to 49 years old , subject to ELISA test, and compare the results with other groups for patients .

2.3.3: Ethical approval

1- The study was done and the cases were collected after getting the agreement of the patients (verbal acceptance).

2- Approval of Babylon Science College Ethical committee .

3- Before starting the study , permission were taken from Babylon health presidency.

2.3.4: The Study Schematic

The samples were proceed according to study schematic shown in Figure (2-1).

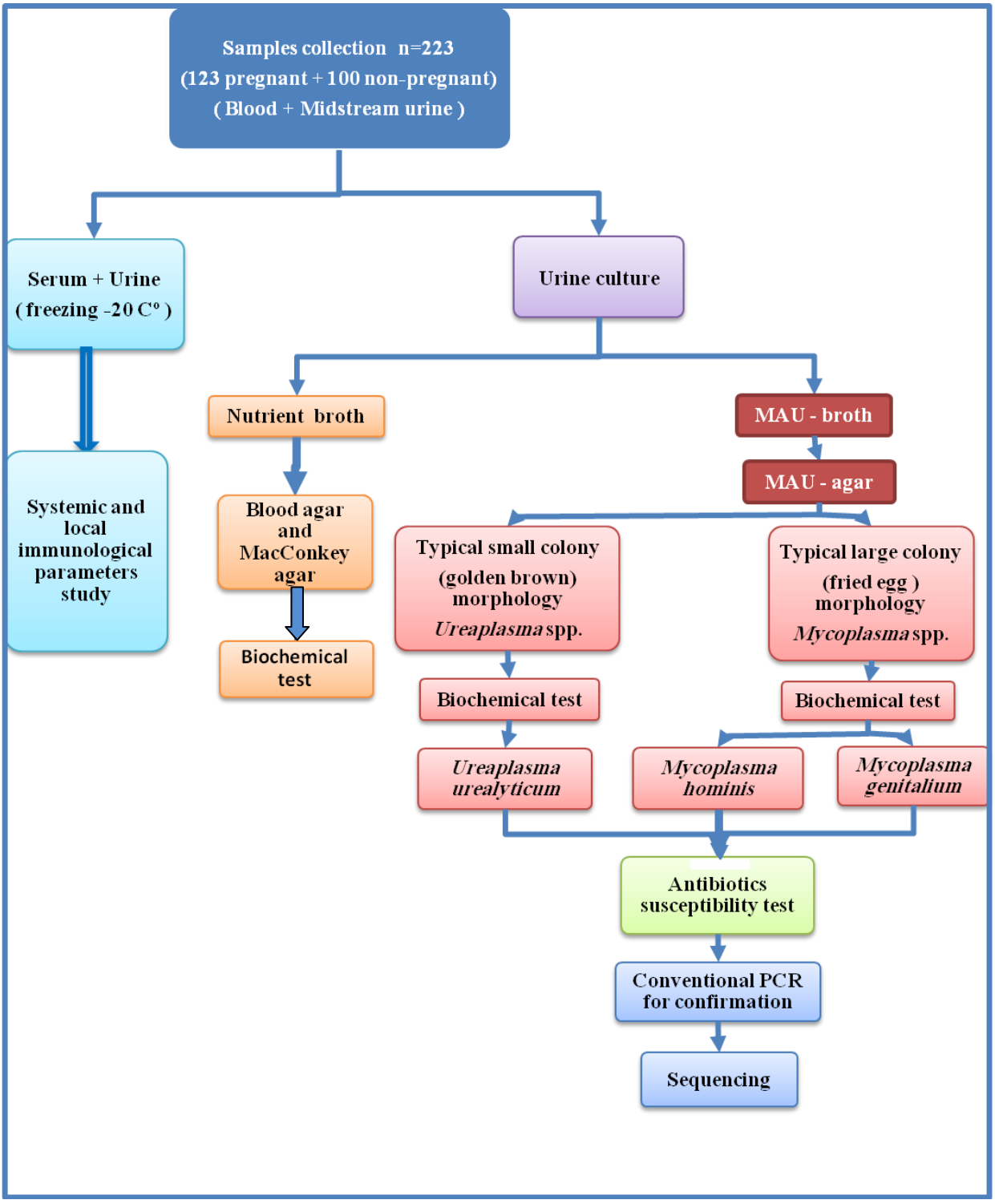


Figure (2-1): Scheme of Study Design

2.4: Preparation of Culture Media

Culture media were prepared according to the instructions of the manufacturer company and serialized by autoclaving in (1 bar) at (121^{o}C) for (15) minutes.

2.4.1: Preparation of culture media(modified arginine urea medium)

it was prepared according to the modified method of Al-Azawiy (2013) .

2.4.1.1: Modified arginine –urea broth (MAU-broth)

This medium was composed of the following components:

Yeast extract (powder)	1 gm
Soya bean casein digest broth	1 gm
pH=6.0	

The first two materials were added to the PPLO broth base (3.625 gm) to (70 ml D.W) and boiling in a water bath at 100℃ for 1 hour, autoclaved, cooled to 57℃, then the following supplements were added, to it (in ml):

Horse serum (unheated) or egg white	10
Yeast extract (25%)	5
L –arginine-HCl (30%)	1
L- cystein-HCl (2%)	1
Urea stock solution (10%)	1
DNA (0.2%)	1
Putrecine - dihydrochloride	1
Ceftriaxone	2
Nystatine + fluocanazole	2
Phenol red (0.4%)	0.5
Crystal violet	5
Glucose	1

After mixing well, it was distributed into tubes, each tube contained 2 ml.

2.4.1.2: Modified arginine – urea agar (MAU-agar)

This medium was composed of the following components:

PPLO broth base	3.625 gm
Soya bean casein digest broth	2 gm
$MgSO_4 . H_2O$	0.031 gm
Yeast extract (powder)	2 gm
Agar –agar	3 gm

These materials were mixed well to (70 ml) D.W. and heated to 100°C in water bath for 1 hours, then autoclaved, the medium was cooled to 57°C, pH (6.0) then supplemented with the following supplements (in ml) :

Horse serum (unheated) or egg white	20
Yeast extract (25%)	2
Urea stock solution (10%)	2
L- cystine-Hcl (2%)	2
L- arginine-Hcl (30%)	2
Glutamine	2
K_2HPO_4 solution	2
DNA (0.2%)	2
Putrecine- dihydrochloride	2
Ceftriaxone	4
Nystatine + Fluocanazole	4
Phenol red (0.4%)	1
Crystal violet	5
Glucose	2

The components were mixed well then poured in petridish .

2.4.2: MacConkey agar medium

MacConkey agar medium was used for the primary isolation of most Gram-negative bacteria and to differentiate lactose fermenters from non-lactose fermenters (MacFaddin, 2000).

2.4.3: Nutrient agar medium

It was used for cultivation of the bacterial isolates when it was necessary (MacFaddin, 2000).

2.4.4: Nutrient broth

This medium was used to grow and preserve the bacterial isolates (MacFaddin, 2000).

2.4.5: Blood agar medium

Blood agar medium was prepared by dissolving (40gm) blood agar base in (1000ml) distal water. It was autoclaved at (121^{o}C) for (15) minute, and then cooled to (50^{o}C). (5%) of fresh human blood was added. This medium was used to cultivate bacterial isolation and to determine the ability of bacteria to hemolysis blood cell (MacFaddin, 2000).

2.4.6: Muller-Hinton agar

Muller-Hinton agar medium was used to antibiotic susceptibility test (MacFaddin, 2000).

2.4.7: Brain heart infusion broth with 5% glycerol

This medium was prepared by adding (5ml) of glycerol to (95ml) of BHI broth before autoclaving. It was used in preservation of bacteria (MacFaddin, 2000).

2.4.8: Mannitol salt agar medium

This media has been used as a selective media for the isolation of *Staphylococci* and differentiation of *Staphylococcus aureus* (MacFaddin, 2000).

2.4.9: Eosin methylene blue (EMB) agar

Culture media were prepared according to the instructions of the manufacturer for diagnostic lactose fermenting bacteria especially *E. coli* (Murray *et al.*, 2003).

2.5:Laboratory Diagnosis

2.5.1: Study group of mycoplasmas isolate

Out of 123 (40) isolates from pregnant and out of 100 (30) isolates from non-pregnant of mycoplasmas were used to determine the sensitivity and specificity among three assays (culture, biochemical test and conventional PCR).

2.5.2: Biochemical test for identification of mycoplasmas

The biochemical tests were performed according to identification and classification of references including :

2.5.2.1: Arginine deaminase

The liquid Mycoplasmas medium was used with (1% w/v) L. arginine and 0.002% phenol red adjusted to pH 7, the positive test is indicated by changing the pale orange color to pink (Rechnitzer *et al.*, 2013).

2.5.2.2: Urea hydrolysis test

A basal broth containing tryptic soy broth (0.75 gm), monopotassium phosphate (0.02g) and 0.5g of sodium chloride was dissolved in (90 ml) D.W. pH was adjusted to 5.5 with 2N HCl and then autoclaved at 121°C for 15 min. After that, the solution was cooled to 50-60°C, and the following filter sterilization supplements were added aseptically: horse serum (4ml), urea solution 10% (0.5ml), phenol red 1%(0.1 ml), Pencillin G 200000 I.U/ml(0.5ml), and the pH was adjust to 6.0 the broth medium was distributed in to sterile screw-capped bottles. The positive result was detected by changing color from yellow to pink with clear supernatant indicates urease activity (Shepard and Luncefored, 1976).

2.5.2.3: Glucose fermentation

The liquid mycoplasmas medium was used for this test. It contained 10 ml of glucose (10%) and 0.2 ml phenol red (0.4%). The pH was adjusted at 7.6. The positive test was indicated by changing the color from pale orange to yellow (Razin and Tully, 1983).

2.5.2.4: Tetrazolium reduction medium

The liquid Mycoplasms medium was used for this test. It contained 2 ml of tetrazolium solution (10%) and 0.2 ml phenol red (0.4%). The pH was adjusted at 7.8. The positive test was indicated by changing the color from yellow to red (Laurence, 1983).

2.5.2.5: Oxidase test

The test was used to detect the presence of oxidase enzyme in bacteria that catalyze electrons transport among electron donors of the bacteria and a redox dye (tetramethyl-paraphenylene diamine dihydrechloride) . A piece of filter paper was placed into sterile petri dish and moisture with several drops of the freshly prepared oxidase reagent, then a small portion of the colony to be tested was picked up by wooden stick and rubbed on the filter paper. Changing the color to blue or deep purple color within (10) seconds indicates for a positive result (Forbes *et al.* , 2007).

2.5.2.6: Catalase test

Catalase is an enzyme that catalyses the release of oxygen from hydrogen peroxide . A colony of organisms is transferred by sterile wooden stick to the surface of a clean, dry glass slide and one drop of (3%) H_2O_2 is added to it. The formation of gas bubbles indicated the positive result (Forbes *et al.* , 2007).

2.6: Hemolysin Production

Hemolysin production was carried out by inoculating of blood agar medium with bacterial isolate at (37^oC) for (24-72 hrs.), an appearance of clear zone around the colonies referred to complete hemolysis (β-hemolysis) or greenish zone around the colonies referred to partial hemolysis (α-hemolysis), while no changing referred to non-hemolysis (γ-hemolysis) (DeBoy *et al*., 1980).

2.7: Colonial Morphology and Microscopic Examination

2.7.1: Culture of specimens

The specimens under test were inoculated in MAU-broth medium for *(M.hominis ,M. genitalium* and *U.urealyticum*) and incubated at 37°C aerobically

for 24-72 hours (Naher *et al.* , 2014) as indicated in Figure (2-1), with daily checking for growth of *Ureaplasma urealyticum* and 3-5 days for *Mycoplasma hominis* and *M. genitalium* . When the broth became an alkaline (arginine and urea changes) a small inoculum was spreaded on agar and incubated at 37°C aerobically or facultative anaerobically in candle jar with small wet cotton to provide a little moisture. Incubation was for 3-5 days (Razin and Tully, 1983 ; Benedetti *et al.*, 2019) .

2.7.2:Purified colonies of *Mycoplasma*

Because *Mycoplasma* colonies grow within the agar, it cannot be removed easily with the loop, so a block of agar contained colonies was cut and inverted on the surface of a fresh *Mycoplasma* agar plate (this step was repeated for 2-3 times) to provide pure colonies. A single colony was picked from each primary positive culture, and foreworded for its identification depending on their diagnostic characteristics represented by morphological properties (colony size, shape, and color). Colonies were investigated directly by dissection microscope, since the colonies of *M. hominis* and *M. genitalium* characterized as fried-egg appearance, while the colonies of *U. urealyticum* appear granular colonies and dark brown color due to accumulation of manganese oxide inside and outside the colony.

2.8: Determination of Antibiotics Susceptibility Test

2.8.1: Antibacterial susceptibility test

The *in vitro* susceptibility was performed, a pure culture of previously identified bacterial isolate to 8 antimicrobial agents were determined via disk diffusion method . Activation of isolates were performed using nutrient broth for (18) hrs. at (37^{o}C) and compared with (0.5 McFarland standard tube 1.5×10^{8} CFU/ml), then spread on MAU-agar and then use Muller Hinton Agar (MHA) with a sterile cotton swab and left to dry. A antibiotic discs were placed on the surface of the medium at evenly spaced intervals with flamed forceps and incubated for 24-72 hrs. at (37^{o}C) and then inhibition zones were measured using a ruler and compared with the zones of inhibition determined by (Lee *et al.*, 2016 ; Tadongfack *et al.*, 2020 ; CLSI, 2017, M43).

2.9: Molecular Detection of Genital Mycoplasmas

2.9.1: Preparation of template DNA using Favor Prep® genomic DNA kits from culture cells

Procedure of extracting DNA from *Mycoplasma* culture was done by using **Favor Prep® genomic DNA kits** as the following the manufacturer's protocol :

- The *Mycoplasma* pellets were harvested via centrifuged at (14000 rpm) for 20 min.
- The supernatant was discarded and Mycoplasmas pellet was resuspended in 200 µl Buffer FATG, and then incubated for 5 min at room temperature .
- Protinase K 20 µl was added and mixed by vortexing, and incubated for 30 min at 56°C until the tissue was completely lysed in shaking water path.
- The mixture was centrifuged in 1.5 ml eppendorf tube to remove drops from the inside of the lid.
- The buffer FABG (200 µl) was added to the sample , mixed by vortexing for 15 second and incubated at 70°C for 10 min until the sample lysate is clear , then centrifuged in 1.5 ml microcenterifuge tube to remove drops from the inside of the lid.
- Ethanol 96% (200 µl) was added to the sample , and mixed by vortexing for 15 second , after mixing, centrifuged in 1.5 ml microcenterfuge tube to remove drops from the inside of the lid.
- Carefully, the mixture from step 6 (including the precipitate) was applied to the FABG Mini spin column (in a 2ml collection tube) without wetting the rim. the cap was closed, and centrifuged at (14000 rpm) for 5 min, FABG Mini spin column was placed in a clean 2 ml collection tube and tube containing filtrate was discarded.
- Carefully, FABG Mini column was opened and W1 Buffer (400 µl) was added without wetting the rim, the cap was closed and centrifuged at (14000 rpm) for 1 min, the FABG Mini column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded.

- Carefully, the FABG Mini column was opened and W2 Buffer (600 µl) was added without wetting the rim, the cap was closed and centrifuged at full speed(14000 rpm) for 1 min.
- The FABG Mini column was transferred to a new 2 ml column collection tube to remove any residual ethanol solution and the old collection tube was discarded with the filtrate, centrifuged at full speed for 3 min.
- The dry FABG Mini column was placed in a clean 1.5 ml microcenterifuge tube and the old collection tube was discarded with the filtrate, carefully the FABG Mini column was opened and (100 µl) Elution Buffer or TE was added, incubated at room temperature for 3-5 min and then centrifuged at (14000 rpm) for 1 min to elute the DNA.
- The purity of eluted genomic DNA was measured by nanodrop (Optical Density at 260/280nm) , then stored at($^{-}20^{o}$ C) until use .

2.9.2: Agarose gel electrophoresis

Agarose sheet was prepared by dissolving agarose powder in 1X TBE buffer . The amount of agarose which can be dissolved depending upon the purpose in which agarose sheet used . 0.7% agarose sheet used for visualizstion the DNA after extraction while (1.5 – 2 %**)** agarose sheet visualization of PCR product (amplicon) .

Ethidium bromide stock solution concentration was 10 mg/ml . Only 5µl of ethidium bromide stock solution were added to 100 ml of melting agarose gel final concentration 0.5 µg/ml (Green and Sambrook, 2012) .

2.9.3: Primer pairs preparation

All primer pairs used in this study were dissolved using TE Buffer **,** (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA-Na2 . Firstly the primer stock tube prepared and then the working solution would prepared from primer stock tube .

Acoording to the instruction provided by primer manufacturer (Bioneer/Korea) the TE buffer were added to get 100 picomole/microliter concentration of primer

stock solution . The working solution prepared from stock by dilution with TE buffer to get 10 picomole/microliter .

2.9.4: Reaction mixture

Amplification of DNA was carried out in a final volume of 25 µl reaction mixture as mentioned in Table (2-8) .

Table (2-8): Content of Reaction Mixture

No.	Contents of Reaction Mixture	Volume
1.	Master mix	12.5µl
2.	Forward primer (10pmol/µl)	1.5µl
3.	Reverse primer (10pmol/µl)	1.5µl
4.	DNA template	3µl
5.	Nuclease free water	6.5µl
Total volume		25µl

2.9.5: Polymerase chain reaction (PCR)

Conventional PCR was used to amplify the target DNA using specific primer pairs, as shown in Table (2-7) . It include three consecutive steps that repeated for specific number of cycles to get PCR product (amplicon) which can be finally visualized after agarose gel electrophoresis . The thermal cycling conditions mentioned in the Table (2-9) .

2.9.6: PCR clean up

This was done according to the manufacturer's (Favorgen Taiwan) .

2.9.7: Sequencing of PCR product

Twenty microliters of clean up *Mycoplasma* spp. and *U. urealyticum* purify PCR product were send to Macrogene/ Korea for Sanger sequencing . After trimming of each sequence, the results of the trimmed sequence were blasted in NCBI to check the similarities and differences among *Mycoplasma* spp. and *U. urealyticum* product of different genital mycoplasmas isolates .

Table (2-9): PCR thermal cycling conditions

Primer name+ gene	Initial denaturation		Denaturation		Annealing		Extension		No. of cycles	Final extension	
	Temp (ºC)	Time	Temp (ºC)	Time	Temp (ºC)	Time	Temp (ºC)	Time		Temp (ºC)	Time
H1 F H2 R	95	2m.	95	30s.	58	30s.	72	40s.	30	72	5m.
U5 F U4 R	95	2m.	95	30s.	55	30s.	72	50s.	30	72	5m.
MgPa F MgPa R	95	2m.	95	30s.	56	30s.	72	50s.	30	72	5m.
16S rRNA	94	5m.	94	1m.	53	45s.	72	2m.	35	72	7m.

2.10: Immunological Study

2.10.1: Blood samples

By using disposable syringe, 3 ml of blood was withdrawn from vein puncture of the patients and control group . Blood samples were placed in test tubes without anticoagulant and it was left at room temperature till being clotted , and then was centrifuged at 2500 r.p.m for 5 min , after centrifugation the serum was separated from the blood using sterilized pasture pipette , then the serum was divided into (0.5ml) and placed in sterilized eppendroff tube and stored at freezing (-20 ^{o}C), till testing time.

2.10.2: Urine samples

Urine samples were placed in container tubes , and then was centrifuged at 2500 r.p.m for 5 min , after centrifugation the urine was separated into supernatants and sediment , then collect supernatants without sediment and placed in sterilized eppendroff tube and stored at freezing (-20 ^{o}C), till testing time .

2.10.3: Immunological parameters

2.10.3.1: Determination of certain interleukins level

There were three interleukins enrolled in the present study , the assay principle and methods were done according to manual protocol of BT LAB (Bioassay Technology Laboratory Company) ELISA kit .The kits contain the basic components required for the development of sandwich ELISA to measure and recombinant human interleukin. BT LAB were designed for the analysis of serum and urine supernatants. Calculation of the result and interpretation were done according to specific standard curve of each interleukins, such (IL-18 , TLR-6 and IFN-β). Choosing of IL-18 protocol and principle as example for these interleukins measurement by such company.

2.10.3.1.1: IL-18 –Enzyme Linked Immunosorbent Assay (ELISA) kit

2.10.3.1.1.1: Principle of test

According to manual protocol (Bioassay Technology Laboratory Shanghai China) company Human IL-18 kit was based on standard sandwich enzyme-linked immune assay technology . The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human IL-18 antibody. Samples were added to the micro ELISA plate wells and combined with the specific antibody, and then a biotinylated Human IL-18 antibody were added and binds to IL-18 in the samples. Then Streptavidin-Horseradish Peroxidase (HRP) were added and binds to Biotinylated IL-18 antibody to each micro plate well (96) and incubated. After incubation unbound Streptavidin-HRP was washed away during a washing step . The substrate solution is added to each well and color develops in proportion to amount of human IL-18 . Only those wells that contain Human IL-18, biotinylated detection antibody and streptavidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction was terminated by the addition of acidic stop solution and the color turns to yellow, the optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm within 10 minutes after adding the stop solution . The OD value is proportional to the concentration of Human

IL-18. The concentration of Human IL-18 was measured by comparing the OD of the samples to the standard curve Figure (2-2) for IL-18, Figure (2-3) for TLR-6 and Figure (2-4) for IFN-β .

2.10.3.1.1.2: Assay procedure

According to information that provided with kits that is described as following:

1. Prepared all reagents, standard solution and samples as instructed bring all reagents to room temperature before use. The assay is performed at room temperature.

2. Determining the number of strips required for the assay. Inserted the strips in the frames for use. The unused strips should be stored at 2-8˚C.

3. A volume (50) microleter added standard to standard well. Not, don't added antibody to standard well because the standard solution contains biotinylated antibody.

4. A volume (40) microleter added sample to sample wells and then 10 µL of anti-IL-18 antibody to sample wells, then a volume 50 µL streptavidin-HRP added to sample wells and standard wells (not blank control well). Mixing well then cover the plate with a sealer and incubate 60 min at 37 ˚C.

5. Removed the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35ml wash buffer for 30 seconds to 1 min for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate on to paper towels or other absorbent material.

6. A volume (50) microleter substrate solution A added to each well and then 50 µL substrate solution B added to each well and incubate plate covered with a new sealer for 10 min at 37 ˚C in the dark.

7. A volume (50) microleter stop solution added to each wells, the blue color will be changed into yellow immediately.

8. Determined the optical density (OD value) of each well immediately using a micro plate reader set to 450 nm (nanometer) within 10 min after adding the stop

solution. Optical density (OD) for each sample was plotted curve to calculate the sample concentration .

2.10.3.1.1.3: Standard curves of IL-18 , TLR-6 and IFN-β

Calculation of result of IL-18 , TLR-6 and IFN-β was measured in present study according to standard curves shown in Figures (2-2) , (2-3) and (2-4) .

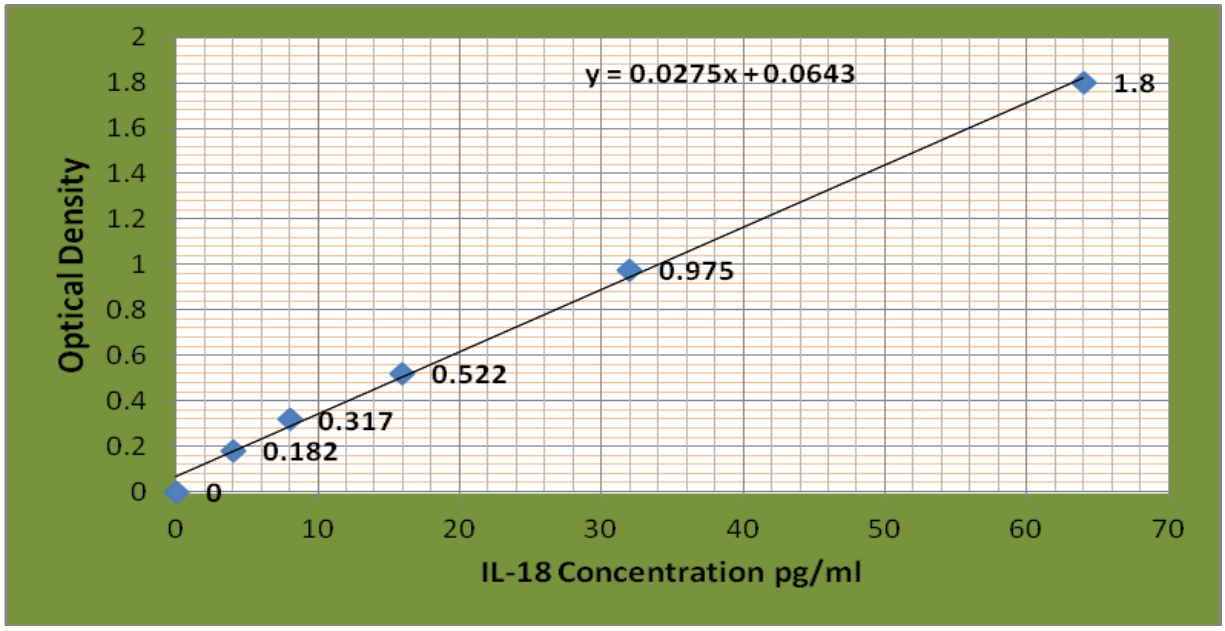


Figure (2-2): Standard Curve of IL-18

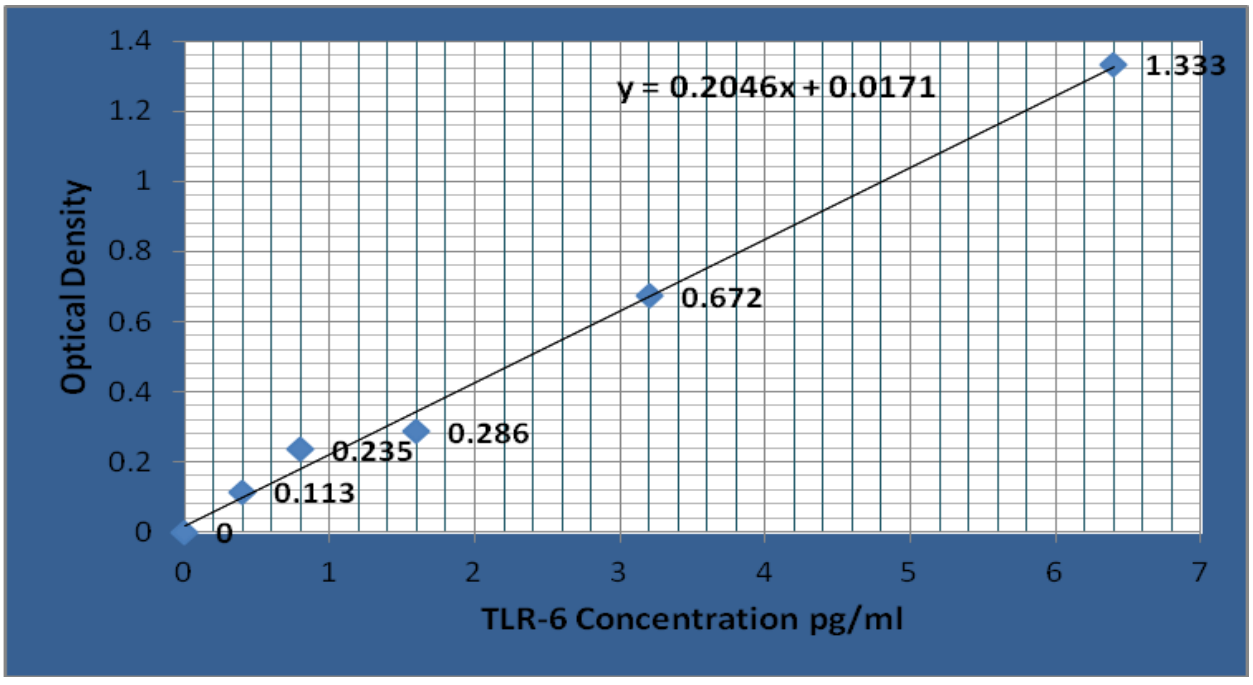


Figure (2-3): Standard Curve of TLR-6

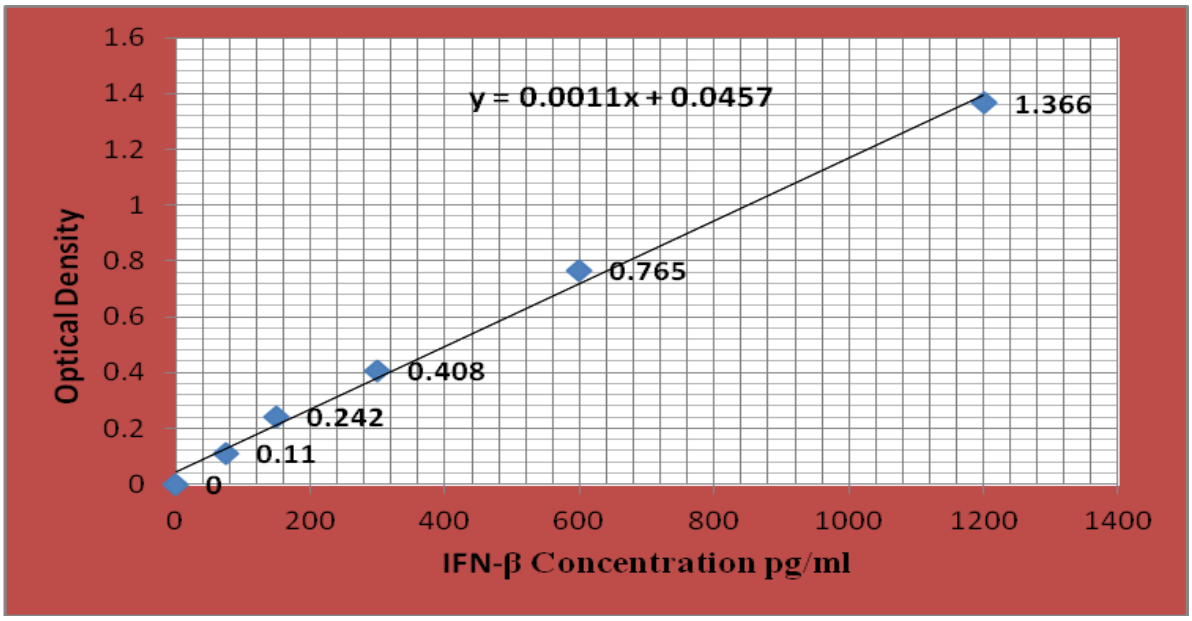


Figure (2-4): Standard Curve of IFN-β

2.11: Statistical Analysis

Data were processed and analyzed with one way ANOVA using statistical program social science (SPSS version 23) and the results were expressed as (Mean ± S.D) . P-values below 0.05 were considered to be statistically significant, and differences between means were assessed by T- test and LSD test as well as correlation with different parameters were done for cytokines and other studied parameters (Morgan *et al.* , 2019) .

2.12:Biosafety and Hazard Material Disposing

Biosafety aspects followed during the work include disposing of all swabs , Petri dishes and all contaminated supplies by autoclaving and then incineration . All benches cleaned with alcohol before and after the work .

3. Results and Discussion

3.1: Study Population

A total number of 223 specimens were collected from all (blood + urine) (123 pregnant and 100 non-pregnant married women and checked up for colonization with *M.hominis*, *M. genitalium* and *U.urealyticum*) were selected from patients diagnosed by laboratory with urinary genital tract infections (UGTIs), who attended the Teaching Hospital of Maternity and children Hospital and Private Clinics in Babylon province, during 6 months from January to June , 2021. Twenty samples as control samples which were apparently (Health women) age marched groups for each 10 pregnant and 10 non-pregnant (married women).

The pregnant women percentage compared with the non-pregnant women in UGTIs as in the patients were distributed as 123 pregnant women percentage (55.16%) and 100 non-pregnant women percentage (44.84%) , non significant association regarding pregnant and non-pregnant (*p=0.124*) . The urban percentage was different than rural in UGTIs as in the patients were distributed as 83 urban percentage (37.22%) and 140 rural percentage (62.78%), there were a highly significant association regarding rural residency (*p= 0.0001*), as illustrated in Table (3-1) .

Table (3-1): Occurrence and Residency Percentage of Women Groups

Women and residency groups	No.	(%)	*p-value*
Pregnant	**123**	**55.16%**	**0.124**
Non-pregnant	**100**	**44.84%**	
Urban	**83**	**37.22%**	**0.0001****
Rural	**140**	**62.78%**	

The results of the current study was differed from the result of Peretz *et al.*, (2020), who found that a sub-grouping by residential setting, demonstrated that 91 (42.7%) women lived in urban settings, and 122 (57.3%) lived in rural settings. Among women living in urban settings, 10 (11%) were found positive, while women living in rural settings, 8 (6.6%) were found positive, with no significant association between carriage rates and settlement type ($P = 0.25$). Present study results agree with the results of the Zhang *et al,* (2021), who found that the women who had an education level of undergraduate degree or above had a decreased risk of *Mycoplasma* isolation, where explained an independent risk factor for *Mycoplasma* isolation was a history of gynecological diseases, while a bachelor's degree, master's degree, or above were protective factors against *Mycoplasma* isolation. A total number of (223) patients (married women only) were distributed into three age groups (20-29) year 103 (46.19%) patients , (30 – 39) year 72 (32.29%) patients , (40 – 49) year 48 (21.52%) patients. Among the women who aged (20-29) settings, 36 (16.14%) were found positive mycoplasmas , and women aged in (30-39) setting, 21 (9.42%) were found positive mycoplasmas, while for the women aged in (40-49) setting, 13 (5.83%) were found positive mycoplasmas, as shown in Table (3-2).

Table (3-2): Subjects Characteristic According to Age and Culture Positive and Negative Mycoplasmas

Age (year)	No.	(%)	No. Positive *Mycoplasma*	(%)	No. Negative *Mycoplasma*	(%)	*p-value*
20-29	103	46.19%	36	16.14%	67	30.04%	0.0001**
30-39	72	32.29%	21	9.42%	51	22.87%	
40-49	48	21.52%	13	5.83%	35	15.70%	
Total	223	100%	70	31.39%	153	68.61%	

In this study, it was found that the age group (20-29), 36 (16.14%), showed the most infection with UGTIs . This study was similar to the results of Peretz *et al.* (2020), who found the age group (18-29) 12 (10.5%) more than the age group (30-39) 7 (7.5%) . Also current study agreed with the results of the Zhang *et al.* (2021), who found that the women aged 30–39 years or with a history of pregnancy or gynecological diseases had an increased risk for *Mycoplasma* isolation, while women who were postmenopausal had a decreased risk of *Mycoplasma* isolation .

Out of 123 (40) pregnant women were distributed into three groups according to the age , 20-29 years 26 (21.1%) patients , 30 – 39 years 12 (9.8%) patients , 40 – 49 years 2 (1.6%) patients . While out of 100 (30) non-pregnant women were distributed into three groups according to the age , 20-29 years 10 (10%) patients , 30-39 years 9 (9%) patients , 40-49 years 11 (11%) patients as shown in Figure (3-1) , (3-2) and Table (3-3).

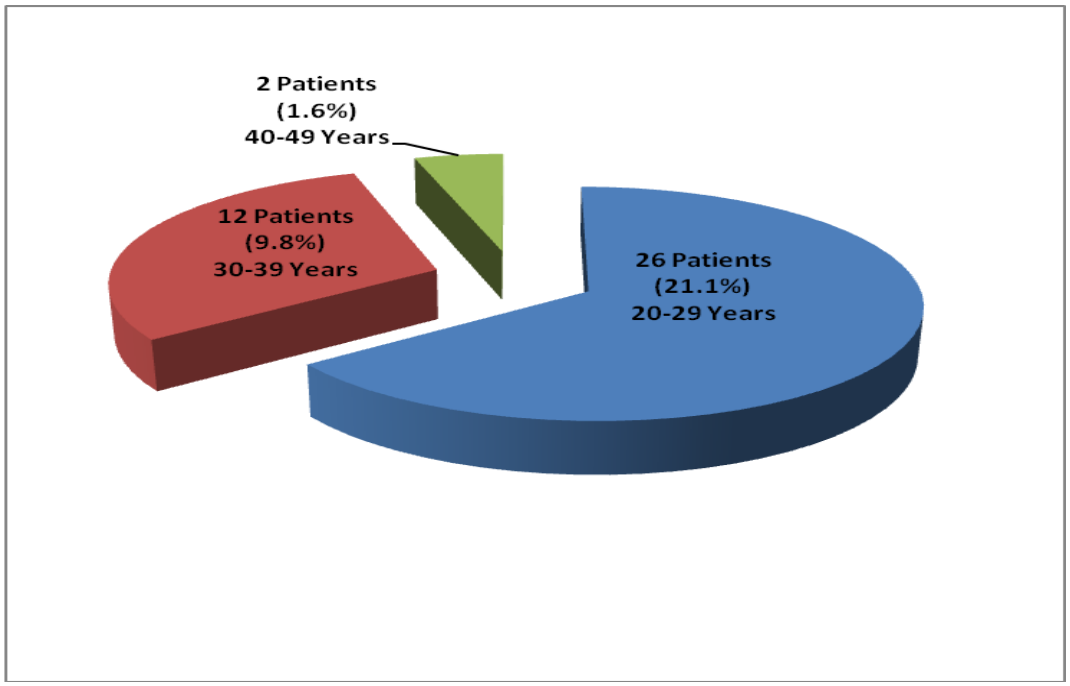


Figure (3-1): Positive Results Distribution According to Age Groups in Pregnant Women

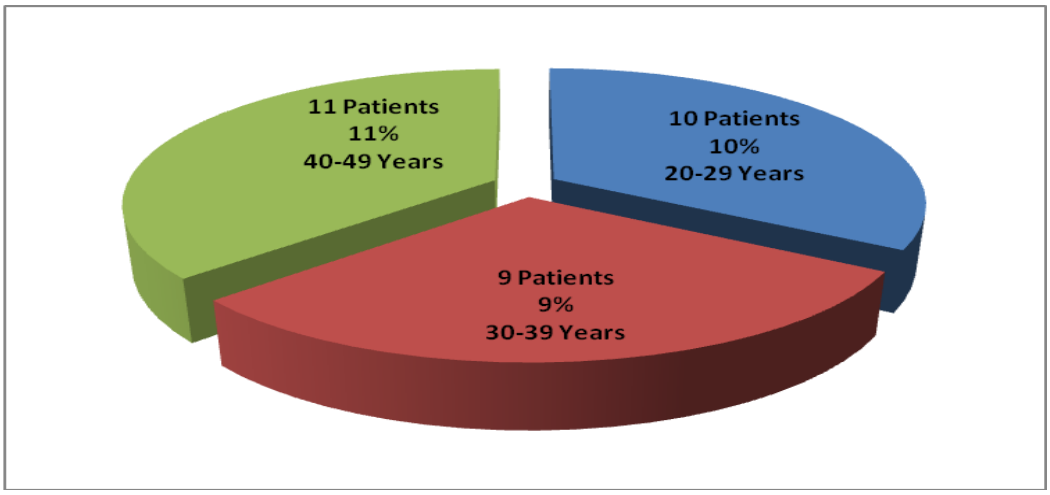


Figure (3-2): Positive Results Distribution According to Age Groups in Non-pregnant Women

Table (3-3): Age Groups Distribution of Pregnant and Non-pregnant Women

No.	Age	Pregnant women	Non-pregnant women	Total
1	20 - 29	26(21.1%)	10(10%)	36(16.14%)
2	30 - 39	12(9.8%)	9(9%)	21(9.42%)
3	40 - 49	2(1.6%)	11(11%)	13(5.83%)
	Total	40(32.5%)	30(30%)	70(31.39%)

The present results showed that the subjects of age range (20-29 and 30-39) year old represent a high rate (16.14%) and (9.42%) , respectively. This can be attributed to the sexual activity among this group since there is an increased in estrogen hormone produced from female genital tract leading to change the vaginal environment which is regarded as a factor for infection. It has become increasingly clear that sex hormones regulate susceptibility to sexually transmitted infections through direct and indirect mechanisms involving inflammation and immune responses (Wessels *et al.*, 2018) .

3.2:Laboratory Identification of *M. hominis* , M. genitalium and *U.urealyticum*

3.2.1: Colonial morphology and biochemical tests

In this study *M.hominis* , *M. genitalium* and *U.urealyticum* isolates were identified by investigating colonial morphology on MAU-medium where colonies resambles as fried- egg were definitive and indicative characteristic for the presence *M.hominis* and *M. genitalium* , while colonies with tiny , granular and brown indicated for the presence of *U.urealyticum* as shown in Figure (3-3) and (3-4) , under X40 dissecting microscope .

The appearance of *M. genitalium* and *M.hominis* variables takes on a characteristic fried-egg aspect because the organisms pentrate deeply into the agar in the central region of the colony Figure (3-3-A) . While, the dark golden-brown or rich deep-brown colonies due to magnesium oxide which accumulate as a result hydrolysis of urea by urease produced by *U. urealyticum,* liberates hydroxyl group from water, and these hydroxyl moieties oxide magnesium sulfate to magnesium oxide, causing the deposition of golden brown precipitate in the colonies themselves and near it Figure (3-3-B) .

Also, *M. genitalium,* different from *M. hominis* stained with Gram stain, appears a purple colonies unlike *M. hominis* that were white colonies, as shown in Figure (3-4) . Many researchers failed in isolation *Mycoplasma* spp. from different specimens on coventional media (Rodríguez *et al.*, 2011).

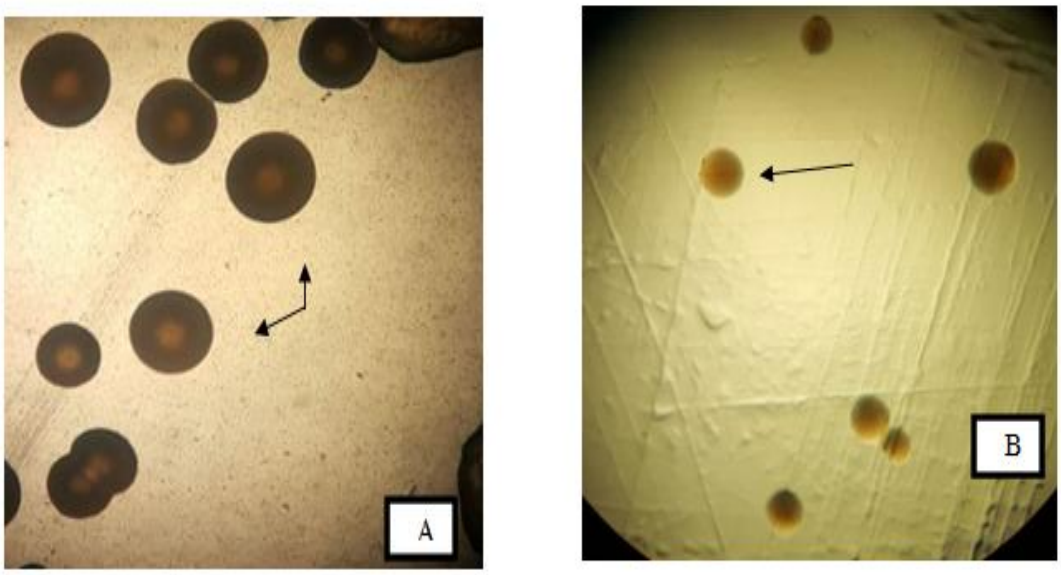


Figure (3-3): : Colonial Morphology of Genital Mycoplasmas Grow on MAU – Medium Under 40X Dissecting Microscope

A- Fried Egg Colony of *Mycoplasma* spp.

B- Tiny Brown Colony of *Ureaplasma urealyticum*

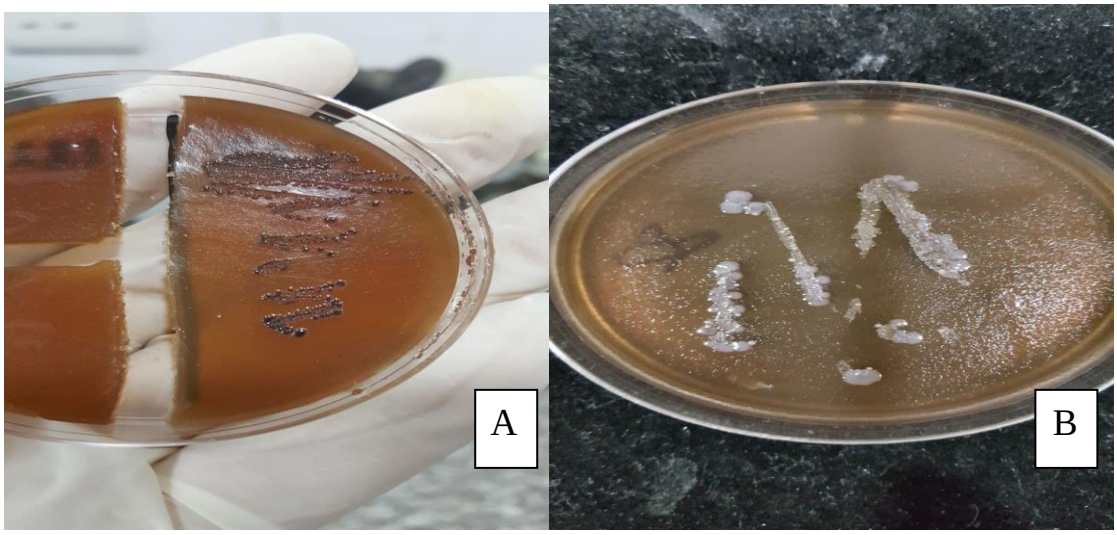


Figure (3-4): Colonial Morphology of *Mycoplasma* spp. Grow on MAU–Medium

A- *M. genitalium* Purple Colony Do Gram Stain

B- *M. hominis* White Colony Do Not Gram Stain

Based on the biochemical test used in this study, all *M.hominis* isolates revealed positive arginine hydrolysis and negative for glucose fermentation, tetrazolium reduction and urea hydrolysis. In contrast, *M. genitalium* isolates were positive for glucose fermentation and negative for arginine hydrolysis, tetrazolium reduction and urea hydrolysis . While *U.urealyticum* isolates reveled positive urea hydrolysis and negative glucose fermentation, arginine hydrolysis and tetrazolium reduction , as shown in Table (3-4).

Table (3-4): Morphological and Biochemical Characteristics of *M.hominis, M. genitalium* and *U.urealyticum* Isolates

Mycoplasma spp.	Colony morphology	Arginine hydrolysis	Glucose fermentation	Tetrazolium reduction	Urea hydrolysis
M. hominis	**Fried-egg**	+	-	-	-
M. genitalium	**Fried-egg**	-	+	-	-
U. urealyticum	**Granular**	-	-	-	+

These characteristic were compared with standard characteristic of *Mycoplasma* spp. recommended by Collee *et al*. (1996), Bébéar *et al.* (2002), Khalil *et al.* (2017).

3.3: Isolation and Occurrence of *M. hominis* , *M. genitalium* and *U. urealyticum* using MAU-medium

Out of the 123 pregnant women being included in this study, 40 of them gave positive culture, only 2(1.6%), 25(20.3%), 9(7.3%) and 4(3.3%) were positive for *M. hominis* , *M. genitalium* , *U.urealyticum* and the mixed culture of them, respectively.

Out of the 100 non-pregnant women, 30 patients gave the positive culture, only 3 (3%) , 19(19%) ,4(4%) and 4(4%) were positive for

M. hominis, *M. genitalium* , *U.urealyticum* and for the mixed culture, respectively, as shown in Figure (3-5) .

The isolation rate of mycoplasmas in pregnant women was different than non-pregnant women, distributed 40 pregnant women (32.5%) and 30 non-pregnant women (30%) as illustrated in Figure (3-6) and (3-7) . In this study, it was shown that the pregnant women were more colonized than non-pregnant women .

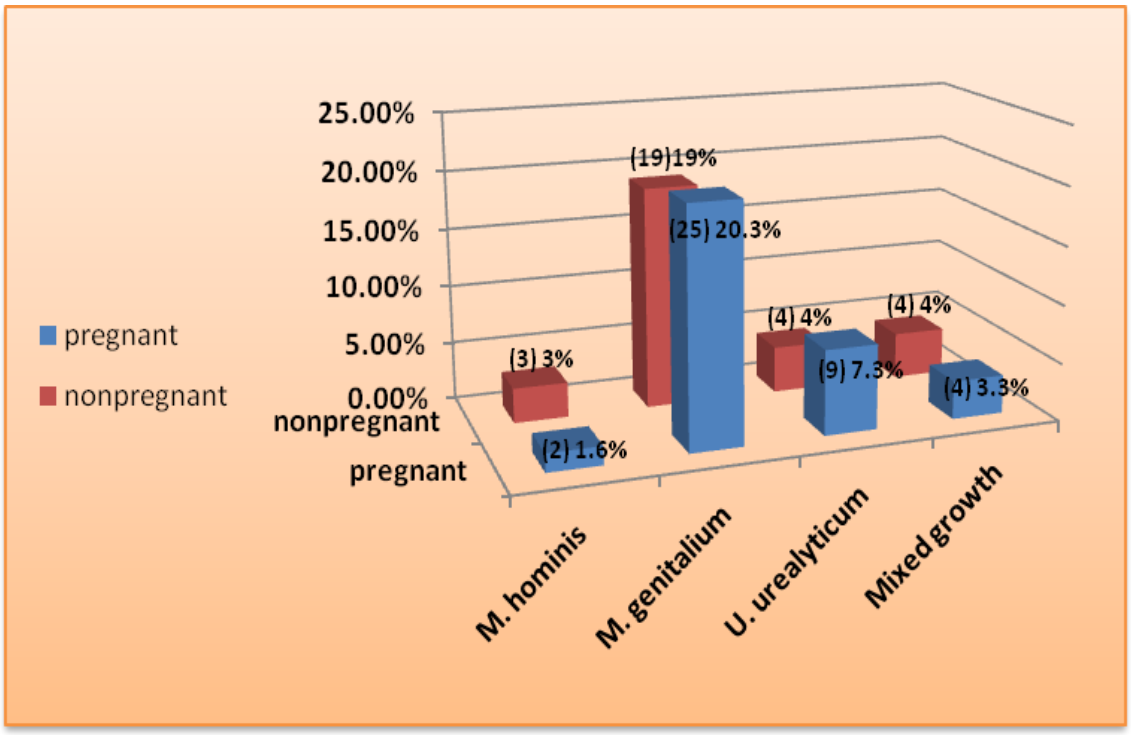


Figure (3-5): Isolation Rate of *Mycoplasma hominis* , *M. genitalium* and *Ureaplasma urealyticum* Using Modified Arginine Urea-Medium in Pregnant and Non-pregnant

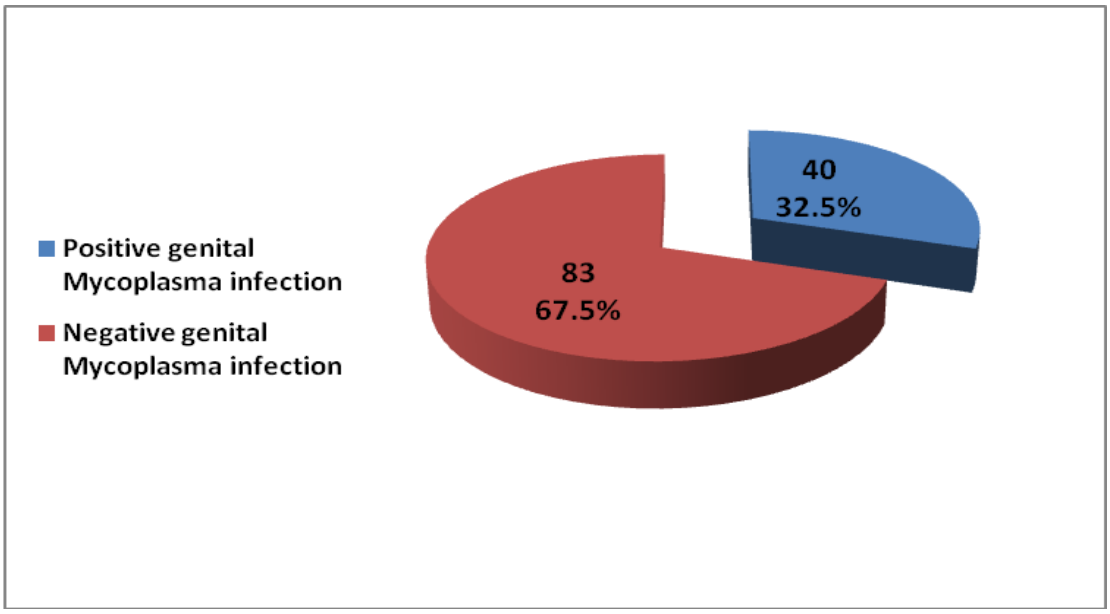


Figure (3-6) : Percentage of Isolation of Genital Mycoplasmas for Pregnant Group

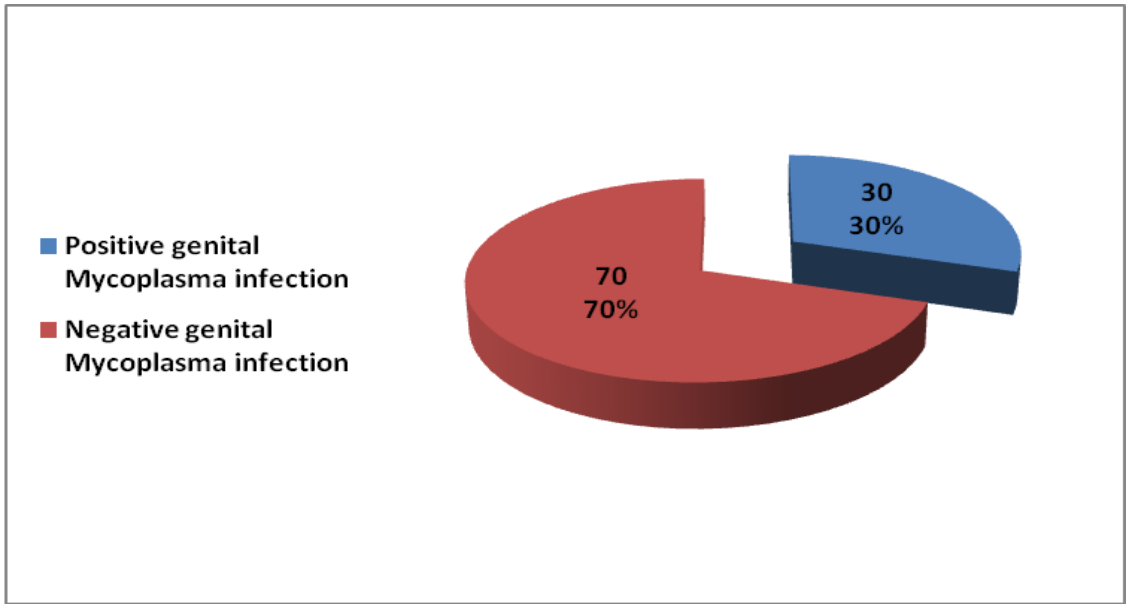


Figure (3-7): Percentage of Isolation of Genital Mycoplasmas for Non-pregnant Group

From a total of (223) patients, urine and blood samples were collected. Twenty patients as control. From 223 samples (70) (31.39%) were given positive genital mycoplasmas culture , whereas (153) (68.61%) cases showed negative genital mycoplasmas culture even after 5 days . The positive genital mycoplasmas infection were divided to *M. genitalium* the most common 44

(19.73%) followed by *U. urealyticum* 13 (5.83%) and *M. hominis* 5 (2.24%) , while mixed growth (*M. homins* + *U. urealyticum*) 5 (2.24%), (*M. genitalium* + *U. urealyticum*) 2 (0.90%) and finally (*M. homins* + *M. genitalium*) 1 (0.45%) as shown in Table (3-5) .

Table (3-5): Culture Isolation Percentage of UGTIs for Patients (Both Pregnant and Non-pregnant Groups)

Culture Mycoplasmas	No.	(%)	*p-value*
Negative mycoplasmas	**153**	**68.61%**	**0.0001****
Positive mycoplasmas	**70**	**31.39%**	
M. genitalium	**44**	**19.73%**	
U. urealyticum	**13**	**5.83%**	
M. hominis	**5**	**2.24%**	
Mix (*M. homins*+*U. urealyticum*)	**5**	**2.24%**	
Mix(*M.genitalium*+*U. urealyticum*)	**2**	**0.90%**	
Mix(*M. homins* +*M. genitalium*)	**1**	**0.45%**	

In this study 70 out of 223 (31.39%) were positive for mycoplasmas, while 153 (68.61%) represent negative mycoplasmas . In present study *M. genitalium* accounted for 44/70 (19.73%) of the cases. This result agree with previous work in which *M. genitalium* was found to be the most common *Mycoplasma* isolate obtained from sexually transmitted disease (STD) (Getman *et al.*, 2016) . Also, considered *Mycoplasma genitalium* was the most important bacterial agent who was isolated from patients with genital infections was in Kerman (Moghadam *et al.*, 2014) . In present study *U. urealyticum* was identified in 13/70 (5.83%) of positive *Ureaplasma* spp. ,

the same observation was previously described in a study in which *U. urealyticum* was identified as many as 40% to 80% of healthy adult women may harbor ureaplasmas in their cervix or vagina (Waites *et al.*, 2012) .While *M. hominis* was found 5/70 (2.24%) of positive *Mycoplasma* spp.. The results of this study are similar with the results of the Kasprzykowska *et al.* (2018), who indicated that the prevalence of *Ureaplasma spp.* in women (14.4%) is higher than *M. hominis* in women (0.2%) with urogenital tract infection in Poland . Also similar with the results of the Moosavian *et al.*(2019), who detected that *U. urealyticum* is (28%) and *M. hominis* is (10%) in semen specimens of infertile men by PCR and isolated 22% of *U. urealyticum* and 2% of *M. hominis* in the same samples by culture. The positive rates of *M. genitalium*, *M. hominis* and *U. urealyticum* are controversial and diverse in the world (Moridi *et al.,* 2020) .

Culture method is a traditional technique that has been widely used for detection of mycoplasmas (Shipitsyna *et al*., 2010). These techniques can be highly specific for detection of some mycoplasmal infections, but they are relatively less sensitive because of many difficulties in culturing of *Mycoplasma* and *Ureaplasma* agents (Waites *et al.*, 2003).

In this study, the culturing method of Al-Azawi, (2012) was used. He developed MAU-medium as enrichment, differential and selective medium for *M. hominis* and *U. urealyticum*. It resolved the important problems of culture. These media contain some enrichment and supplement materials as putericine dihydrochloride and $MgSO_4.H_2O$ that enhanced identified *U.urealyticum* on the basis characteristic morphology and arginine utilization for *M. hominis* (Abdul-Wahab *et al*., 2014). Also used crystal violet and glucose identified for *M. genitalium* based on the source (Patricia, 2021) .

3.3.1:Occurrence of other bacterial species with genital mycoplasmas isolated from pregnant and non-pregnant women

Out of the 123 (40) pregnant women patients being included in this study, 11(8.94%) and 28(22.76%), were positive mixed culture of genital mycoplasmas with other pathogen of Gram-positive bacteria and Gram-negative bacteria, respectively. Only 1(0.81%) for *Mycoplasma* spp. pure growth . Gram-positive bacteria found in 63 (51.21%) and Gram-negative found in 20 (16.26%).

Out of the 100 (30) non-pregnant women patients being included in this study, 10(10%) and 20(20%), were positive mixed culture of genital mycoplasmas with other pathogen for Gram positive bacteria and Gram Negative bacteria, respectively. Gram-positive found in 45 (45%) and Gram-negative found in 25 (25%), as shown in Figure (3-8) and (3-9).

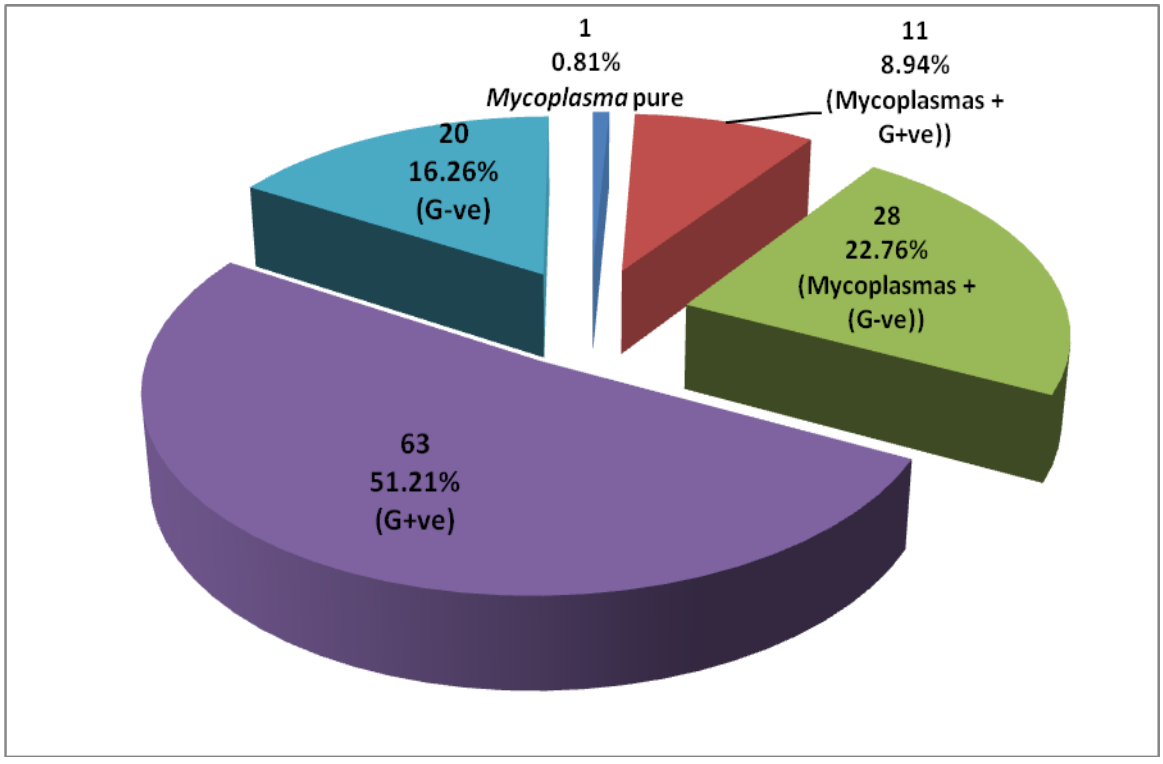


Figure (3-8): Number of Isolation of Genital Mycoplasmas for Pregnant Group with Other Pathogens

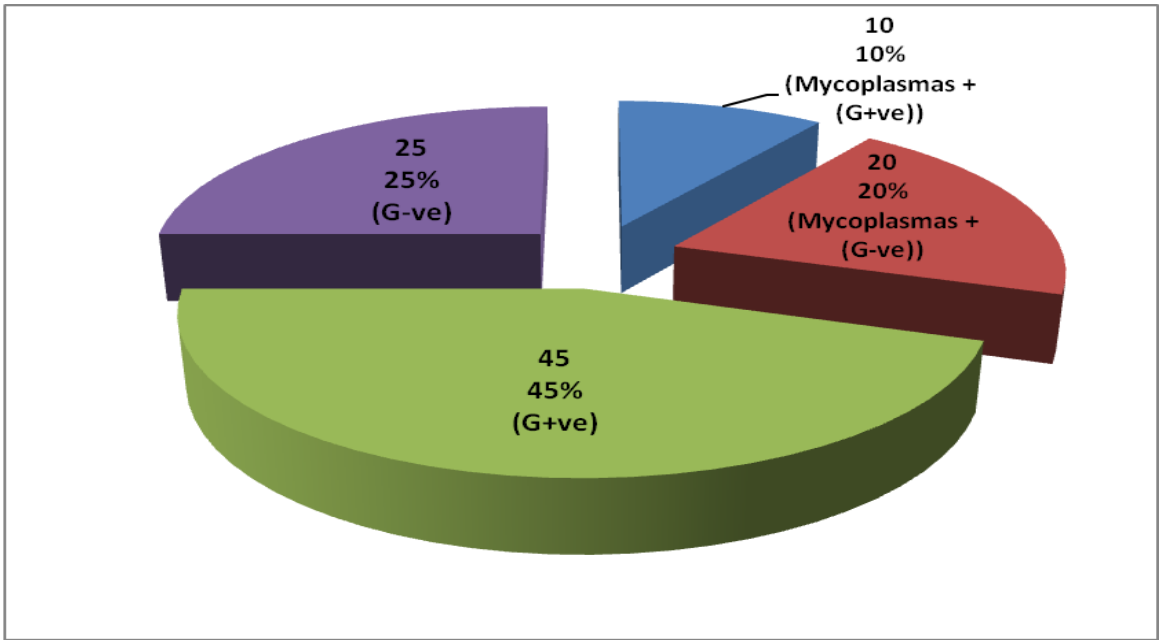


Figure (3-9): Number of Isolation of Genital Mycoplasmas for Non-pregnant Group with Other Pathogens

In this study, it appears that the genital mycoplasmas growth with other pathogen with UGTIs . The result of this study agrees with the result of the Hosny *et al.* (2017), who found that *Mycoplasma hominis* infection with Gram-negative bacilli . Current results are similar with the results of Liu *et al.* (2022), who found that depletion of Gram-positive and Gram-negative bacteria promoted *Mycoplasma* spp. infection .

3.4: Distribution of Mycoplasmas Infections

3.4.1:Distribution of mycoplasmas infections using culture method according to the ages and residency statements in pregnant women

In the group of pregnant women, the features of the infection with the mycoplasmas (Table 3-6) is as follows :- *M. genitalium* was found to be more frequent 25(20.3%) compared with *U. urealyticum* ,*M. hominis* and mixed growth which accounted 9(7.3%), 2(1.6%) and 4(3.3%) respectively . Infections with mycoplasmas were more common in the age group (20-29)

years, 26(21.1%). Individuals of the rural residency are more prone for mycoplasmas infection, 28(22.76%).

Table (3-6): Distribution of *Mycoplasma* SPP. and *Ureaplasma urealyticum* According to Age Group and Residency for Pregnant Women

Age group (yrs)	*M. hominis*	*M. genitalium*	*U. urealyticum*	Mixed growth	Total isolate	%
20 - 29	1/2	18/25	5/9	2/4	26/40	21.1
30 - 39	1/2	6/25	3/9	2/4	12/40	9.8
40 – 49	---	1/25	1/9	---	2/40	1.6
Total	2 (1.6%)	25 (20.3%)	9 (7.3%)	4 (3.3%)	40	32.5%
Urban	1/2	8/25	3/9	---	12/40	9.75
Rural	1/2	17/25	6/9	4/4	28/40	22.76
Total	2 (1.6%)	25 (20.3%)	9 (7.3%)	4 (3.3%)	40	32.5%

The effects of different socioeconomical factors were significant for mycoplasmas in pregnant women rural 22.76% and Urban 9.75% . This results were similar with Kosambiya *et al*. (2009), who founded that there is a significant difference of bacterial vaginosis as 25% among rural women and 24% among urban women.

3.4.2:Distribution of mycoplasmas infections using culture method according to the ages and residency statements in non-pregnant women

In the group of non-pregnant women, the features of the infection with the mycoplasmas (Table 3-7) is as follows :- *M. genitalium* was found to be more frequent 19(19%) compared with *U. urealyticum* ,*M. hominis* and mixed growth which accounted 4(4%), 3(3%) and 4(4%) respectively.

Infections with mycoplasmas were more common in the age group (40-49) years, 11(11%). Individuals of the rural residency are more prone for mycoplasmas infection, 16(16%).

Table (3-7): Distribution of *Mycoplasma* spp. and *Ureaplasma urealyticum* According to Age and Residency for Non-pregnant Group

Age group (yrs)	*M. hominis*	*M. genitalium*	*U. urealyticum*	Mixed growth	Total isolate	%
20 - 29	2/3	7/19	1/4	---	10/30	10
30 - 39	---	7/19	1/4	1/4	9/30	9
40 – 49	1/3	5/19	2/4	3/4	11/30	11
Total	3 (3%)	19 (19%)	4 (4%)	4 (4%)	30	30%
Urban	1/3	11/19	2/4	---	14/30	14
Rural	2/3	8/19	2/4	4/4	16/30	16
Total	3 (3%)	19 (19%)	4 (4%)	4 (4%)	30	30%

The highest prevalence of *M. genitalium* ,*M.hominis* and *U.urealyticum* was seen in pregnant women in (20-29) years age group (Table 3-5). In comparing present finding in culture with those of many previous studies, which have reported between 27% of women to be colonized with *U. urealyticum* but colonization rates with *M. hominis* of less 5%, matched their findings (Keane *et al*., 2000; Bayraktar *et al*., 2010).

The frequency of infection with *M. genitalium* was significantly higher in both pregnant and non-pregnant and *U. urealyticum* only in pregnant women than *M. hominis* . The high rate of *M. genitalium* and *U.urealyticum* infection detected in this study suggested that this agent is widespread among pregnant and non-pregnant, and this is consistent with other studies (Lee *et al*., 2013).

However it is generally difficult to determine whether these agents cause colonization or infection. Since the incidence of infection is affected by some factors, such as: menstrual cycle, bacterial and protozoan infection (co-infections), and socio-economic conditions like poverty, and multiple sexual partners (Demba *et al*., 2005).

3.5:Antibiotic Susceptibility Pattern for Mycoplasmas

Some antibiotics were used to show the effect on genital mycoplasmas types isolated from urinary genital tract infected women . Disc diffusion method was used in studying the susceptibility of genital mycoplasmas to antibiotics, as shown in Figure (3-10 , 3-11 and 3-12) . It has been found that there was clear variation in resistance and most isolates showed resistance and sensitivity to one or more of these antibiotics.

The isolates of *M. genitalium* were completely (100%) sensitive to ofloxacin, doxycycline and clindamycin. While susceptibilities to other antimicrobial agents varied mainly in the range of sensitive or resistance, as shown in Figure (3-10).

All the isolates of *U. urealyticum* were sensitive to ciprofloxacin, doxycycline, clindamycin and ofloxacin as (100%) . Out of (69.2%) were sensitive to tetracycline, while all the isolates were highly resistance (100%) to erythromycin, but clarithromycin and azithromycin as (69.2%) and (92.3%) respectively, as shown in Figure (3-11) .

The isolates of *M. hominis* were completely (100%) sensitive to ciprofloxacin , doxycycline, clindamycin and ofloxacin, but highly resistant (100%) to erythromycin and azithromycin, as shown in Figure (3-12) . The majority of *M. hominis* isolates were sensitive to tetracycline as (60%) and the isolates were resistant to clarithromycin as (80%) .

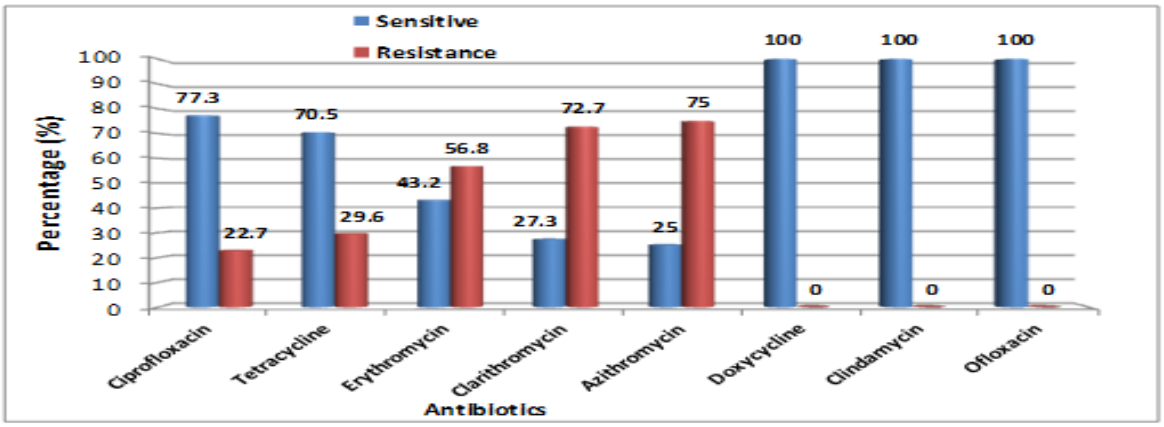


Figure (3-10): Antibiotics Susceptibility Pattern Among *M. genitalium* Isolates

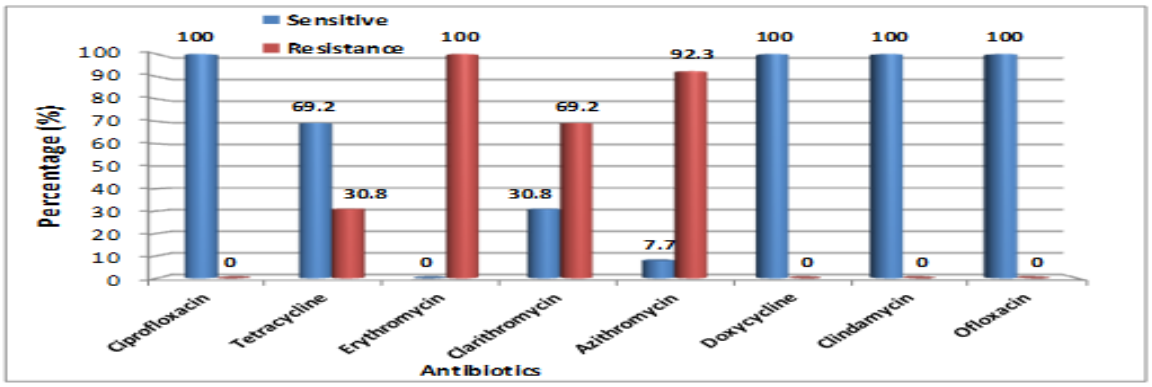


Figure (3-11): Antibiotics Susceptibility Pattern Among *U. urealyticum* Isolates

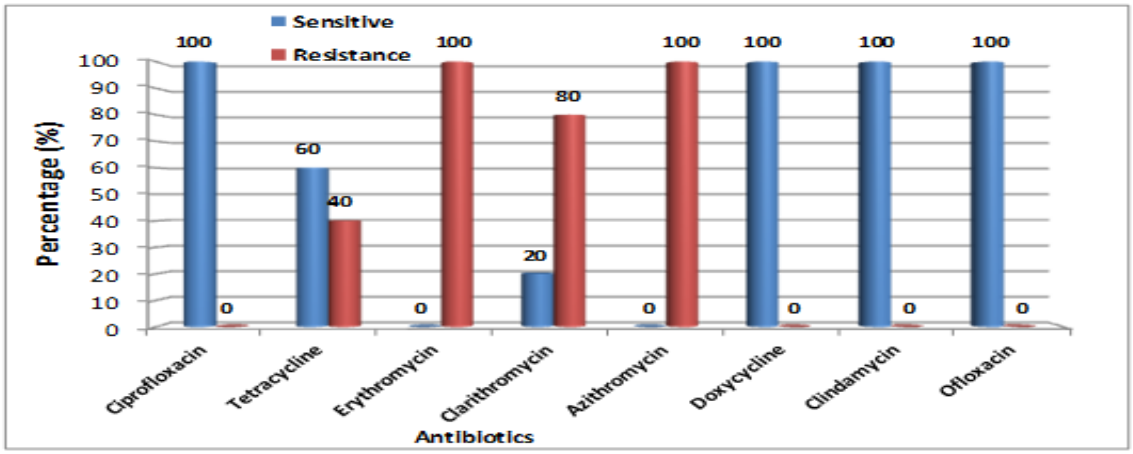


Figure (3-12): Antibiotics Susceptibility pattern Among *M. hominis* Isolates

The current study revealed that genital mycoplasmas were highly sensitive to fluoroquinolones (ciprofloxacin, doxycycline and ofloxacin), followed by lincosamides (clindamycin) . However, the isolates were less sensitive (Intermediate) to tetracycline and resistant to macrolide . These results are in agreement with previous studies in which it was found that macrolides, tetracyclines, and fluoroquinolones eliminate mycoplasmas efficiently both in vivo and in vitro (Zhang *et al.*, 2021) .

It is important to emphasize that most antibiotics that are successfully used in treating mycoplasmal infections have a static effect on the organisms. The greatest cidal activity is exhibited perhaps by the new fluoroquinolones for example ofloxacin and ciprofloxacin (Scheld, 2003).

Resistance of *M. hominis* to tetracycline (Bygdeman and mardh, 1983), probably assumes more importance because of the widespread use of these drugs for genital tract infections. Tetracycline resistance in clinical isolates of *Ureaplasma* spp. and *M. hominis* have been reported in Singapore (Ngan *et al*., 2004), France (Degrange *et al.,* 2008) and United Kingdom (Beeton *et al*., 2009). *U. urealyticum* isolates may also become resistant to tetracyclines for the same reason, the tetM gene encodes a protein also binds to ribosomes (Beeton *et al.,* 2009 ; Kokkayil and Dhawan, 2015).

The present study found that the macrolide were not active against *M. hominis*, *U. urealyticum*, also *M. genitalium* isolate due to intrinsic resistant for it (Fagundo-Sierra *et al*., 2006). As interaction sites in many bacteria are mainly located in the peptidyltransferase region within domain V of the 23 rRNA alterate target site . The DNA sequences in this region of the intrinsically erythromycin-resistant species of *M. hominis* were compared to the DNA sequences of *23 rRNA* from erythromycin-susceptible *M. pneumoniae* . *M. hominis* showed a G-to-A transition at position 2057, while in *M. pneumoniae,* the guanine at position 2057 was conserved, moreover, the *M. hominis* harbored an additional C-to-U transition at position 2610, this

two positions have been found mutated in macrolide resistant bacteria (Pereyre *et al*., 2002).

M. hominis is intrinsically-resistant to erythromycin and azithromycin, *U. urealyticum* are intrinsically-resistant to erythromycin . Antibiotics which are classified as "protein synthesis inhibitors" like tetracycline are often used against *Mycoplasma* infections, since these antibiotics block this attachment (Jafar *et al*., 2010). Tetracycline is also assessed as there have been reports of low-level resistance. Although frequency/rate of resistance tends to vary according to country and levels of antibiotic exposure, while clarithromycin is assessed as there have been reports of high-level resistance . Ofloxacin, clindamycin , doxycycline and ciprofloxacin are reported to be effective against *M. hominis*, *U. urealyticum* and *M. genitalium* (Kasprzykowska *et al.*, 2018).

Despite the availability of susceptible antibiotics to the genital *Mycoplasma* species isolated throughout the 5 years, there has been a continuous oscillation of high prevalence of these pathogens, with the highest prevalence of 80.0% observed in 2017. This is probably due to sexual promiscuity, practice of unprotected sexual intercourse and non-treatment of sexual partners (Tadongfack *et al.*, 2020) .

3.6: Molecular detection of *M. hominis*, *U. urealyticum* and *M. genitalium* by polymerase chain reaction (PCR)

The DNA amplification was accomplished by the Thermo-cycler apparatus under the optimal conditions using specific primers as mentioned in the Tables (2-9) . In the present study, about (70) positive mycoplasmas isolates in culture, out of 44(19.73%) *M. genitalium* positive in culture only 37 (16.59%) isolates were selected for extracted the DNA, amplification by PCR, and *U. urealyticum* all positive in culture 13(5.83%) isolates were selected diagnosis by PCR, while *M. hominis* increase number by PCR

diagnosis from 5 (2.24%) isolates positive in culture to 20 (8.97%) were selected, as shown in Table (3-8) . The selected isolates were taken from clinical cases urinary genital tract infections (UGTIs) .

Table (3-8): Number and Percentage of Positive Genital Mycoplasmas in PCR Versus Culture

Organisms	No. of positive samples by culture	No. of positive samples by PCR for this species	No. of negative samples by PCR for this species
M. genitalium	**44(19.73%)**	**37(16.59%)**	**7(3.14%)**
M. hominis	**5(2.24%)**	**20(8.97%)**	**---**
U. urealyticum	**13(5.83%)**	**13(5.83%)**	**---**
Mix (*M. hominis*+ *U. urealyticum*)	**5(2.24%)**	**----**	**5(2.24%)**
Mix (*M.genitalium*+ *U. urealyticum*)	**2(0.90%)**	**----**	**2(0.90%)**
Mix (*M. hominis* + *M. genitalium*)	**1(0.45%)**	**----**	**1(0.45%)**

In this study PCR was used for detection of *M. genitalium* and *M. hominis* using specific primers targeting a highly conserved region of 16S ribosomal RNA gene . The positive samples showed amplification products of 495 bp bands and 270 bp bands, respectively . In addition, PCR was used also for specific detection of *U. urealyticum*, where the positive samples showed amplification products of 429 bp bands, as showed in Figure (3-13, 3-14 and 3-15) . This result was supported by other works in which PCR was investigated as a means of diagnosing *M. pneumoniae* infections (Buck *et al.*, 1992) . The target DNA sequence was a 375 bp segment of the P1 virulence protein. This DNA segment was amplified from pure cultures of *M. pneumoniae* but not in other species of *Mycoplasma*, *Acholeplasma*, or

Ureaplasma that were tested . The negative PCR results for *Mycoplasma* spp., may be due to its variable presentation, *Mycoplasma* spp. often has a broad differential diagnosis (Lanao *et al.*, 2021) .

Although cultivation is regarded as the gold standard in identifying *Mycoplasma* and *Ureaplasma* infections, greater attention is being given to these organisms in diagnostic microbiology, largely as a result of improved methods for their laboratory detection, made possible by powerful molecular based techniques that can be used for primary detection in clinical specimens. For slow-growing species, such as *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, molecular based detection is the only practical means for rapid microbiological diagnosis (Waites *et al.*, 2012) .

Clinical presentation of *Mycoplasma* spp. is variable and diagnosis confirmation is a challenge to even the most experienced clinicians (Tharwat *et al.*, 2010). Bacterial culture requires specialized media and is time consuming (up to 5 days). Due to the vast reduction in time in comparison with culture, PCR has been used increasingly for *Mycoplasma* spp. and *Ureaplasma* spp. detection (Nour *et al.*, 2005 ; Ross and Jensen , 2006) .

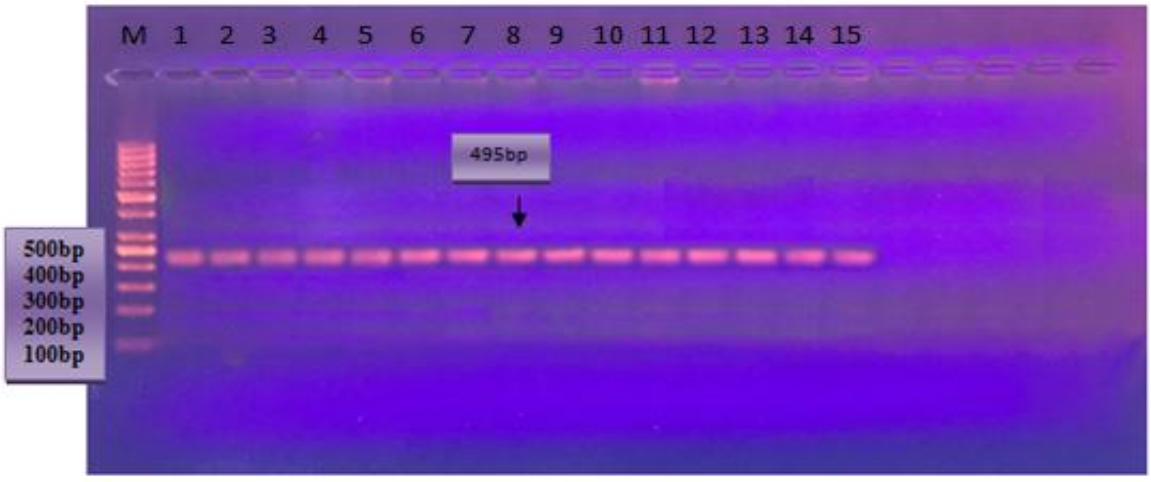


Figure (3-13) : Agarose Gel Electrophoresis Stained with Ethidium Bromide Showing PCR Amplification Product with (495bp) Primers for *M. genitalium* ; Lane M Represent DNA Marker Size (100bp). Lane (1-15) Represent Some of Positive *M. genitalium* Isolates

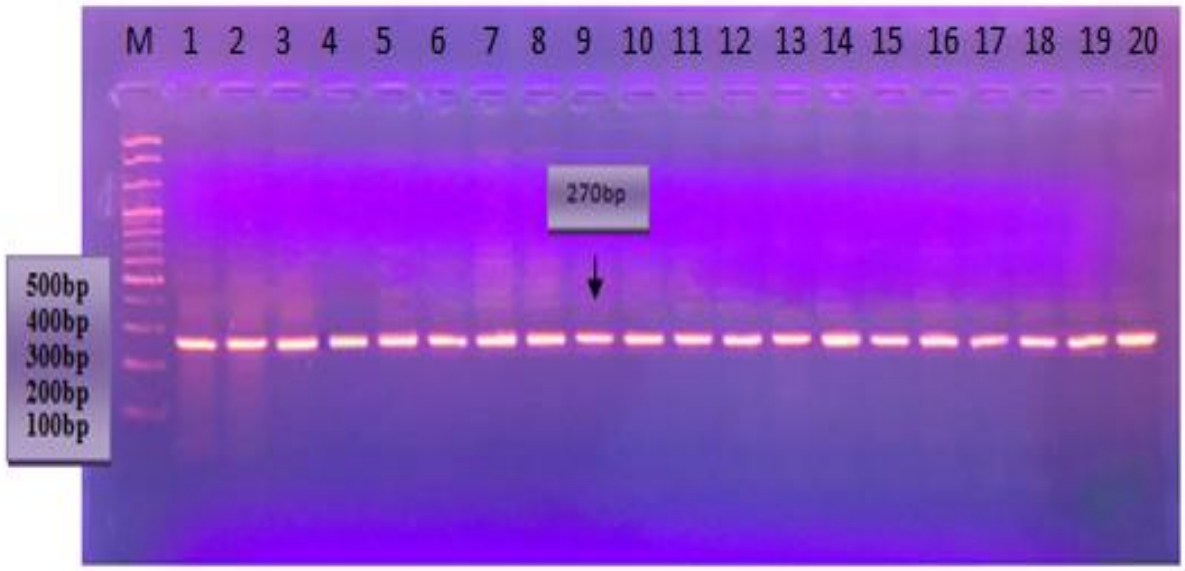


Figure (3-14) : Agarose Gel Electrophoresis Stained with Ethidium Bromide Showing PCR Amplification Product with (270bp) Primers for *M. hominis* ; Lane M Represent DNA Marker Size (100bp). Lane (1-20) Represent Some of Positive *M. hominis* Isolates

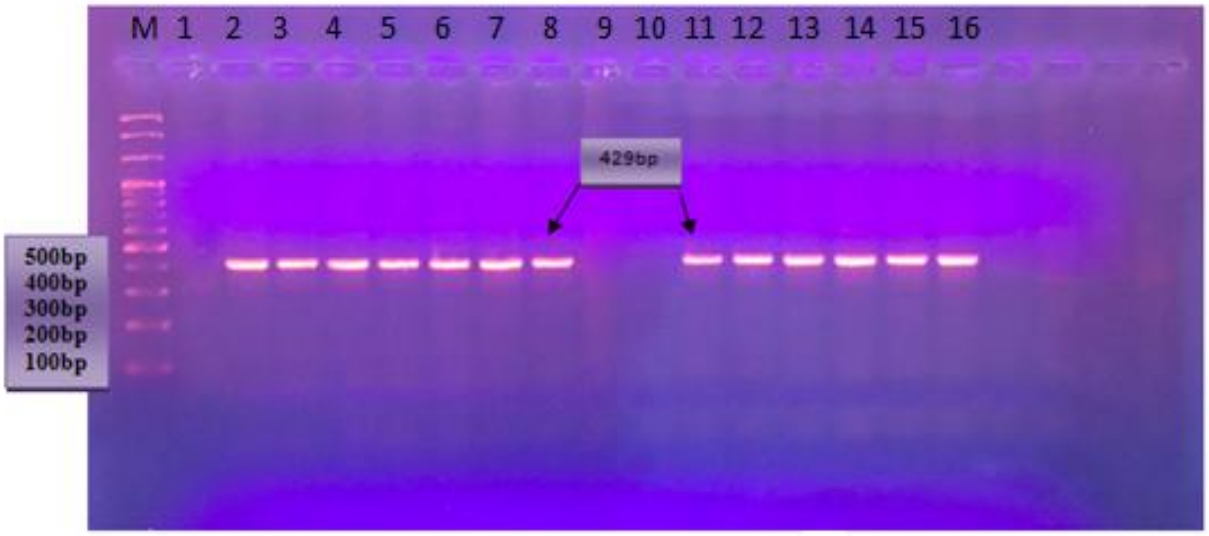


Figure (3-15) : Agarose Gel Electrophoresis Stained with Ethidium Bromide Showing PCR Amplification Product with (429bp) Primers for *U. urealyticum* ; Lane M Represent DNA Marker Size (100bp). Lane (2-8) and (11-16) Represent Some of Positive *U. urealyticum* Isolates

3.6.1: DNA amplification of *16S rRNA* gene

The result of the PCR reaction revealed that the presence of a single band (1479 bp) of the target sequence for *16S rRNA* gene. Choses twelve isolates (4 for each species genital mycoplasmas), one isolates lost not seen during electrophoresis (No. 12), while eleven isolates (1-11) gave positive results as in Figure (3-16).

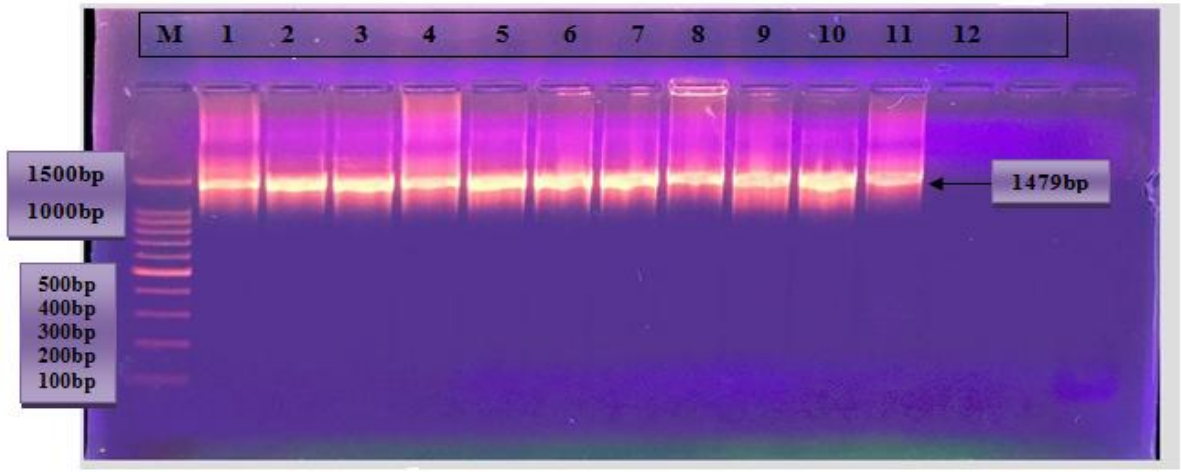


Figure (3-16) : Gel Electrophoresis of a 1479 bp Specific for Detecting *16S rRNA* Gene . PCR Products were Separated by Electrophoresis in an 2% Agarose gel, at 100 Volt for 60 min.

3.6.2: DNA Sequencing of *16S rRNA*

To obtain a trimmed sequence, each data sequence was trimmed from beginning to ending, according to normal waves. When compared to NCBI-Blast, it was found that this sequence has a good level of identity to other global sequence data. The waves produced by scanning the sequences indicate the strong and weak regions of the sequences, which were then trimmed, resulting in increased identity with global sequences at NCBI-Blasting.

The results of nucleotide sets were checked and confirmed by using (NCBI) – Basic Local Alignment Search Tool (BLAST analysis)- nucleotide blast-Search a nucleotide database using a nucleotide query online, which was a perfect program and gave the exact results of identity percentage with other world strains. Sequence alignment must be performed by using *16S*

rRNA gene of *Mycoplasma genitalium* sequences databases information recorded in GenBank to find identity and similarity score degrees of gene and compared with present study isolates. The results showed identify ranged from 72% to 84 %, Figure (3-17 to 3-23) and Table (3-9) , moderate query cover, max score with other world local strains of *Mycoplasma genitalium*.

Score	Expect	Identities	Gaps	Strand
141 bits(156)	3e-34	124/152(82%)	2/152(1%)	Plus/Plus

```
Query  262   GCAAATCTCTTAAAGCTTCTCTCAGTTCTGATTGCAGGCTGCAACTCGCCTGCATGAAGC  321
Sbjct  1255  GCAAATCTGA-AAAGTTGGTCTCAGTTCGGATTGAGGGCTGCAATTCGTCCTCATGAAGC  1313

Query  322   CGGAATCGCTAGTAATCGAGGATCAGC-ACGCCGCGGTGAATACGTTCCCGGGCCTTGTA  380
Sbjct  1314  TGGAATCACTAGTAATCGCGAATCAGCTATGTCGCGGTGAATACGTTCTCGGGTCTTGTA  1373

Query  381   CACACCGCCCGTCACACCACGAGAGTTTGTAA  412
Sbjct  1374  CACACCGCCCGTCAAACTATGAAAGCTGGTAA  1405
```

Figure (3-17): Basic Local Alignment of *Mycoplasma genitalium 16S rRNA* Gene Isolate No.1 with High Similarity NCBI-BLAST *Mycoplasma genitalium* Strain G-37 16S ribosomal RNA Partial Sequence (NR_074611.1)

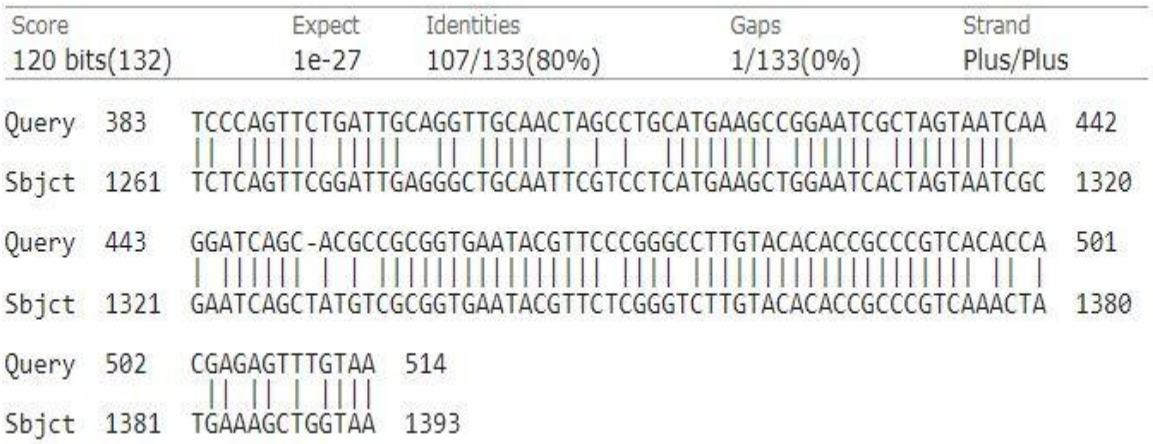
Score	Expect	Identities	Gaps	Strand
134 bits(148)	4e-32	115/141(82%)	1/141(0%)	Plus/Plus

```
Query  185   AAAGCTTCTCTCAGTTCGGATTGCAGGTTGCAACTTGCCTGCATGAAGCTGGAATCGCTT  244
Sbjct  1255  AAAGTTGGTCTCAGTTCGGATTGAGGGCTGCAATTCGTCCTCATGAAGCTGGAATCACTA  1314

Query  245   GTAATCGAGGATCAGC-ACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG  303
Sbjct  1315  GTAATCGCGAATCAGCTATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCG  1374

Query  304   TCACACCACGAGAGTTTGTAA  324
Sbjct  1375  TCAAACTATGAAAGCTGGTAA  1395
```

Figure (3-18): Basic Local Alignment of *Mycoplasma genitalium 16S rRNA* Gene Isolate No.2 with High Similarity NCBI-BLAST *Mycoplasma genitalium* Strain M2288 16S ribosomal RNA Partial Sequence (AY466443.1)



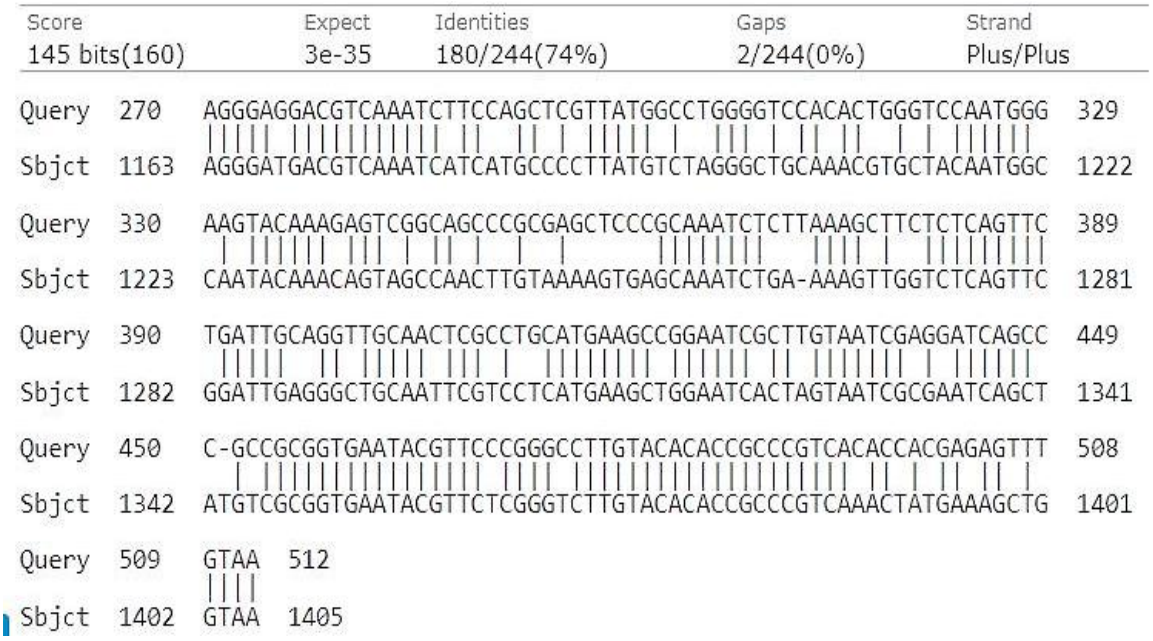
Score	Expect	Identities	Gaps	Strand
120 bits(132)	1e-27	107/133(80%)	1/133(0%)	Plus/Plus

```
Query  383   TCCCAGTTCTGATTGCAGGTTGCAACTAGCCTGCATGAAGCCGGAATCGCTAGTAATCAA  442
Sbjct  1261  TCTCAGTTCGGATTGAGGGCTGCAATTCGTCCTCATGAAGCTGGAATCACTAGTAATCGC  1320

Query  443   GGATCAGC-ACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA  501
Sbjct  1321  GAATCAGCTATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAACTA  1380

Query  502   CGAGAGTTTGTAA  514
Sbjct  1381  TGAAAGCTGGTAA  1393
```

Figure (3-19): Basic Local Alignment of *Mycoplasma genitalium 16S rRNA* Gene Isolate No.3 with High Similarity NCBI-BLAST *Mycoplasma genitalium* Strain G-37 16S ribosomal RNA Partial Sequence (NR_026155.1)



Score	Expect	Identities	Gaps	Strand
145 bits(160)	3e-35	180/244(74%)	2/244(0%)	Plus/Plus

```
Query  270   AGGGAGGACGTCAAATCTTCCAGCTCGTTATGGCCTGGGGTCCACACTGGGTCCAATGGG  329
Sbjct  1163  AGGGATGACGTCAAATCATCATGCCCCTTATGTCTAGGGCTGCAAACGTGCTACAATGGC  1222

Query  330   AAGTACAAAGAGTCGGCAGCCCGCGAGCTCCCGCAAATCTCTTAAAGCTTCTCTCAGTTC  389
Sbjct  1223  CAATACAAACAGTAGCCAACTTGTAAAAGTGAGCAAATCTGA-AAAGTTGGTCTCAGTTC  1281

Query  390   TGATTGCAGGTTGCAACTCGCCTGCATGAAGCCGGAATCGCTTGTAATCGAGGATCAGCC  449
Sbjct  1282  GGATTGAGGGCTGCAATTCGTCCTCATGAAGCTGGAATCACTAGTAATCGCGAATCAGCT  1341

Query  450   C-GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTT  508
Sbjct  1342  ATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAACTATGAAAGCTG  1401

Query  509   GTAA  512
Sbjct  1402  GTAA  1405
```

Figure (3-20): Basic Local Alignment of *Mycoplasma genitalium 16S rRNA* Gene Isolate No.4 with High Similarity NCBI-BLAST *Mycoplasma genitalium* Strain G-37 16S ribosomal RNA Partial Sequence (NR_074611.1)

Score	Expect	Identities	Gaps	Strand
81.5 bits(89)	1e-15	99/134(74%)	1/134(0%)	Plus/Plus

```
Query  379   GTCTCAGTTCTGATTGCAGGTGGCAACTAACCTGCATGACGGTGGAATCGCTTGTAATCA  438
             ||||||||||| |||||  ||  |||| |   |  ||||| | ||||||| || ||||||
Sbjct  1260  GTCTCAGTTCGGATTGAGGGCTGCAATTCGTCCTCATGAAGCTGGAATCACTAGTAATCG  1319

Query  439   AGGATCATCCCCCTC-CGATGAGTACGTTCCCGGGCCTTGTACACACCGCCCGTCACTCC  497
             | ||||  |    || || ||| ||||||| |||| ||||||||||||||||||||  |
Sbjct  1320  CGAATCAGCTATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCAAACT  1379

Query  498   ACGAGAGTTTGTAA  511
             | || || | ||||
Sbjct  1380  ATGAAAGCTGGTAA  1393
```

Figure (3-21): Basic Local Alignment of *Mycoplasma genitalium 16S rRNA* Gene Isolate No.5 with High Similarity NCBI-BLAST *Mycoplasma genitalium* Strain G-37 16S ribosomal RNA Partial Sequence (NR_026155.1)

Score	Expect	Identities	Gaps	Strand
466 bits(516)	1e-131	560/756(74%)	39/756(5%)	Plus/Plus

```
Query  14    CGGCTAACTACGTGCCAGCAGTCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTA  73
             || ||||||| ||||||||||||||||||||||  |||  ||||||||||  |||| |||
Sbjct  416   CGACTAACTATGTGCCAGCAGTCGCGGTAATACATAGGTCGCAAGCGTTATCCGGATTTA  475

Query  74    CTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAAC  133
              ||||||||||||   |||||||||  ||  |||||  | |||  |||  |    ||| |||
Sbjct  476   TTGGGCGTAAAGCAAGCGCAGGCGGATTGAAAAGTCTGGTGTTAAAGGCAGCTGCTTAAC  535

Query  134   CTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGCGGTAGAATTCCAGG  193
                 | | ||||||  ||||||  |||  ||  ||||  | ||||  | |   |  |||||  ||  |
Sbjct  536   AGTTGTA-TGCATTGGAAACTATCAGTCTAGAGTGTGGTAGGGAGTTTTGGAATTTCATG  594

Query  194   TGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA  253
             || |||||||||||||||||  ||  ||  |||||  |||  ||||||||||||     |   |
Sbjct  595   TGGAGCGGTGAAATGCGTAGATATATGAAGGAACACCAGTGGCGAAGGCGAAAACAAAGG  654

Query  254   CAAAGACTGACGCTCAGGTGCGAAAGCGTGGTGAGCAAACAGGATTAGATACCCTGGTAG  313
             | |  |||||||||  |||   |||||  ||||  |||||||  ||||||||||||||  ||||
Sbjct  655   CCATTACTGACGCTTAGGCTTGAAAGTGTGGGGAGCAAATAGGATTAGATACCCTAGTAG  714

Query  314   TCCACGCCGTAAACGAT-----GTCGAT-TTGGAGGTTGTGCCCTTGAGGCGTGGCTTCC  367
             |||||  ||||||||||       | | | ||||    |  |||||  |  ||
Sbjct  715   TCCACACCGTAAACGATAGATACTAGCTGTCGGAG--CGATCCCTTCGGTAGT-------  765

Query  368   GGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA-T  426
             | || |||| | |||| |    ||||||||  |||||   ||||||  | |||||||||
Sbjct  766   GAAGTTAACACATTAAGTATCTCGCCTGGGTAGTACATTCGCAAGAATGAAACTCAAACG  825
```

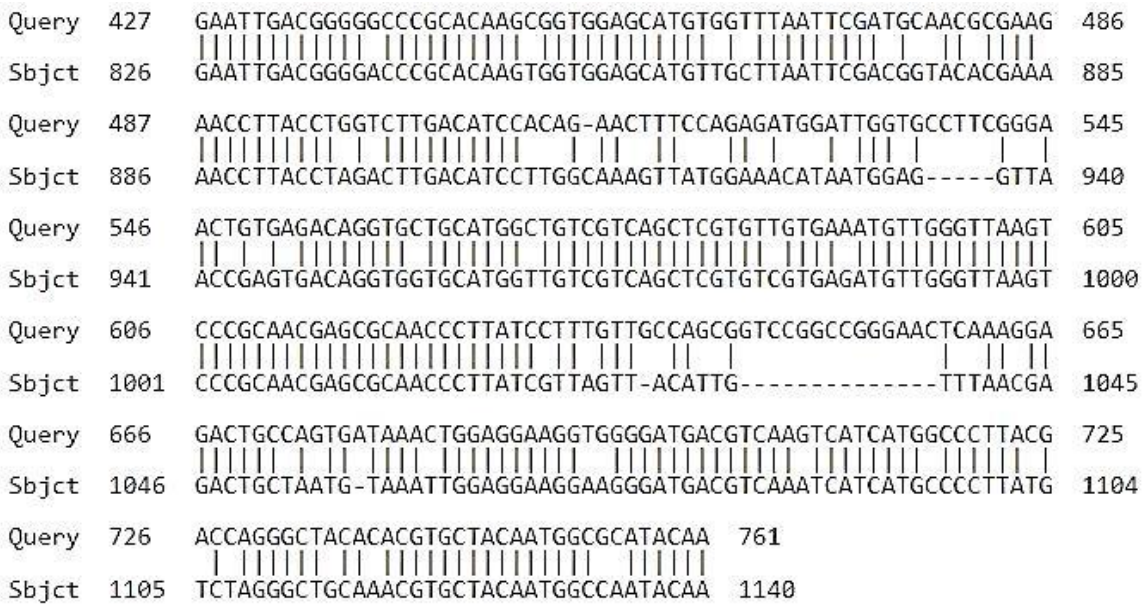


Figure (3-22): Basic Local Alignment of *Mycoplasma genitalium 16S rRNA* Gene Isolate No.6 with High Similarity NCBI-BLAST *Mycoplasma genitalium* Strain MIMQ1 16S ribosomal RNA Partial Sequence (MZ379495.1)

Score	Expect	Identities	Gaps	Strand
120 bits(132)	3e-28	97/115(84%)	2/115(1%)	Plus/Plus

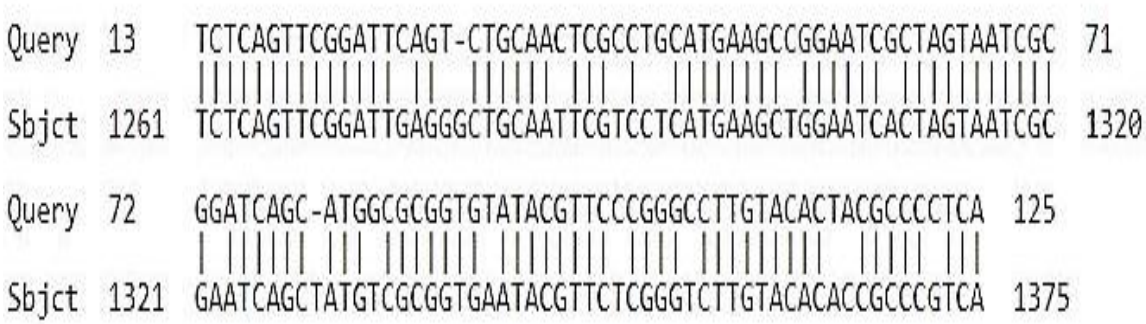


Figure (3-23): Basic Local Alignment of *Mycoplasma genitalium 16S rRNA* Gene Isolate No.7 with High Similarity NCBI-BLAST *Mycoplasma genitalium* Strain G-37 16S ribosomal RNA Partial Sequence (NR_026155.1)

Table (3-9) : Alignment Results of Seven Local *Mycoplasma genitalium* Isolates with Reference Isolates Retired from NCBI

Local Isolate	Reference of the isolate with highest percentage similarity(%)		
	Accession No.	Similarity (%)	Country
M. genitalium No.1	NR_074611.1	82 %	U.S.A
M. genitalium No.2	AY466443.1	82 %	Denmark
M. genitalium No.3	NR_026155.1	80 %	U.S.A
M. genitalium No.4	NR_074611.1	74 %	U.S.A
M. genitalium No.5	NR_026155.1	74 %	U.S.A
M. genitalium No.6	MZ379495.1	74 %	Iraq
M. genitalium No.7	NR_026155.1	84 %	U.S.A

3.6.3: Phylogenetic analysis of local and world strains

The phylogenetic tree was drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree . The dataset was cleansed of positions with gaps or missing data (Complete deletion option). MEGA X 10.2.4 is used to perform phylogenetic analysis .

There were 9 global taxa about *16S rRNA* gene were downloaded from NCBI and submitted with 7 local sequences to Mega X 10.2.4 software to obtain the Figure (3-24) .

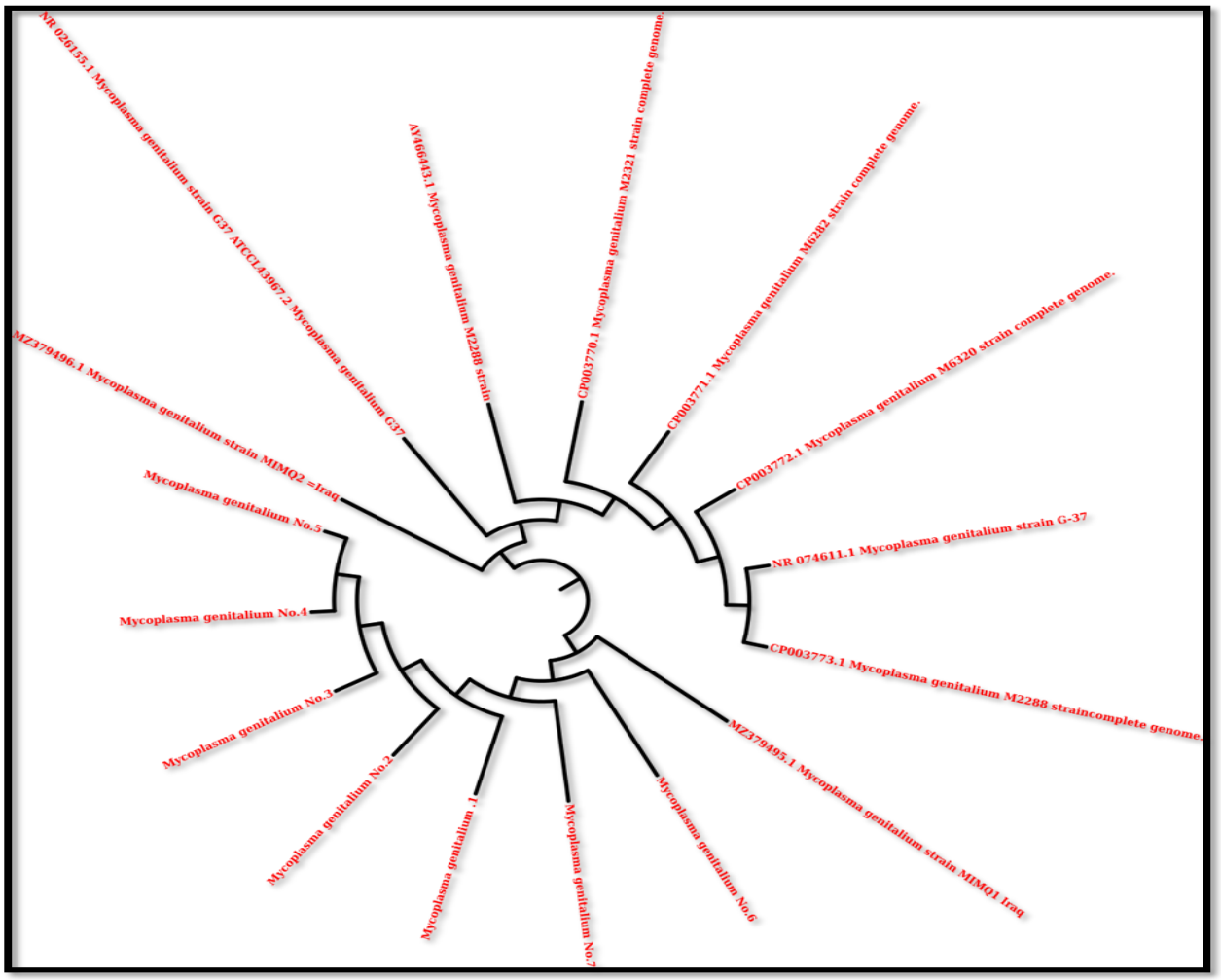


Figure (3-24) : Phylogenic Tree of *16S rRNA* Gene Partial Sequences of Local and Global Sequences Using Neighbor Joining Bootstrap 500 Tree Figure. Evolutionary Relationships of 16 Taxa 1-7 Represent No. of Local Isolates

The Molecular Evolutionary Genetics Analysis (MEGA) program is a desktop software that allows you to compare homologous gene sequences from various species or multigene families, with a focus on inferring evolutionary links and patterns of DNA and protein evolution. MEGA features a number of useful tools for assembling sequence data sets from files or web-based repositories, as well as tools for visualizing the results in the form of interactive phylogenetic trees and evolutionary distance matrices (Kumar *et al.*, 2008). The first stage in the analysis was to align all of the sequences in this study with other world-wide references using MEGA X

10.2.4 's (Clustal W) program step. This program showed a high degree of similarity with all world sequences, including the sequences used in this study. These (Clustal W) results were significant since they were directly utilized in the phylogenetic tree design.

The Neighbor-Joining (NJ) approach, which is a simplified version of the minimal evolution (ME) method, is used in this study to determine the close relationship between world and local sequences. Because it does not need the assumption of a constant rate of evolution, the NJ method yields an unrooted tree. An out group taxon is needed to find the root (Saitou and Nei, 1987; Rzhetsky and Nei, 1992).

In *16S rRNA* gene phylogeny we submitted 16 sequences, 7 sequences belong to local sequences and 9 sequences belong to global sequences obtained by downloading from NCBI they submitted to a MEGA X 10.2.4 software program for obtaining phylogenic relationship among local and global sequences, after submitting these sequences to MEGA X 10.2.4 at the first time we found alignment by Clustal W, then use NJ method at bootstrap 500 we found a group of sequences contain 8 sequences grouped together which contain 7 local isolates (No.1 to No.7). The local sequence of *Mycoplasma genitalium* No.6 was closely related to the sequence MZ379495.1 *Mycoplasma genitalium* strain MIMQ1(Iraq) and near to the sequence of MZ379496.1 *Mycoplasma genitalium* strain MIMQ2 (Iraq) and NR 026155.1 *Mycoplasma genitalium* strain G37 ATCCL43967.2. These Results indicate that *16S rRNA* gene sequence comparison seems to be an appropriate method for inferring genetic relationships within the *Mycoplasma genitalium* species strains on a molecular basis. Phylogenic relationship among local and world strains provide high information about origin and genetic evolution of local isolates.

The most common method for identifying bacteria or constructing bacterial phylogenetic relationships is *16S rRNA* gene sequence analysis. *16S rRNA*

gene sequence information has an expanding role in the identification of bacteria in clinical or public health settings. However, the data also clearly showed that it was not foolproof and applicable in each and every situation (Wang *et al*., 2007) .

The *16S rRNA* gene sequences have been the most often used housekeeping genetic marker in the study of bacterial phylogeny and taxonomy for a variety of reasons. The *16S rRNA* gene is found in almost all bacteria, often as part of a multigene family or operon. The *16S rRNA* gene's function has not changed over time, implying that random sequence changes are a more accurate measure of time (evolution); and the *16S rRNA* gene (1,500bp) is large enough for informatics purposes (Janda *et al.,* 2001). Bacterial analysis by *16S rRNA* has become popular because these sections of RNA are conserved and easy to sequence (Fukushima *et al*., 2002).

The 1.5-kilobase *16S rRNA* gene sequence has recently been considered and widely used in bacterial taxonomy because it includes a high conservation region that has variable regions between species (Kox *et al*., 1995). The fact that the *16S rRNA* gene can be sequenced readily is also significant. Bacterial identification can be improved by integrating molecular phylogeny with traditional approaches such as morphological, physiological, and biochemical features (Zhaolan *et al*., 2003; Li *et al*., 2006; Ma *et al*., 2008). Molecular-based approaches have considerably enhanced the capacity to detect and identify mycoplasmas and ureaplasmas in clinical specimens during the last two decades. Allowing for a greater understanding of the illnesses they may cause and more quick and accurate diagnosis. An amplified DNA detection method is simple, quick, and combines maximum sensitivity with good specificity, according to this and other studies (Amirmozafari *et al*., 2009). The future of diagnostic Mycoplasmology and epidemiological research rests with molecular-based technology.

3.7: Immunological Parameters

3.7.1: Determination of cytokines levels

The IL-18 , TLR-6 and IFN-β cytokines were measured in patients urine and serum against control (Health women) by ELISA technique depending on principle of manufactured company (BT , China) .

3.7.1.1: Concentration of IL-18 in urine and serum of UGTIs patients for mycoplasmas and control

As illustrated in Table (3-10), the mean of IL-18 urine concentration in patients (13.39±3.3 pg/ml) which was significant increased $p < 0.05$ compared with the control (10.63±2.1 pg/ml), while the mean of IL-18 serum concentration in patients (12.01±9.8 pg/ml) has no significant different compared with the control group (9.22±2.4 pg/ml) .

Table (3-10): Urine and Serum IL-18 Concentration of Mycoplasmas Patients and Control

IL-18 pg/ml	No.	Mean ± S.D.	*P-Value*
Patients (urine)	**70**	**13.39±3.3**	**0.000****
Control	**20**	**10.63±2.1**	
Patients (serum)	**70**	**12.01±9.8**	**0.215**
Control	**20**	**9.22±2.4**	

IL-18 has been shown to promote production of interferon gamma and perforin in a variety of cells types, both of which are important for host defense against infection (Bellora *et al.,* 2012 ; Serti *et al.,* 2014) .The IL-18 in urine, is elevated in patients with genital mycoplasmas infection, may be due to the humoral immune activity which induce secretion IL-18 as defense response of immune system against pathogens, the IL-18 in serum not

elevated in patients may be cause local inflammation cytokine rises locally and need time until it turns into a systemic. This depends on the strength and type of the microbe, these findings correspond with preceding studies of Zalinger *et al.* (2017), who stated that interferon (IFN)-g–inducing factor was previously termed interleukin IL–18, (Interferon gamma production by splenic, but not liver cells), the IL-18-dependent defect of interferon gamma in the serum could be due to poor production by cells from all organs, or by cells from specific sites of infection. The cytokines secreted following inflammasome activation, which includes IL-1 and IL-18, regulate cells of both the innate and adaptive immune system, guiding the subsequent immune responses. In the absence of IL-18, had elevated infections and poor survival, and this protective effect of IL-18 was found to be due to promotion of interferon gamma production in αβ T cells.

The level of IL-18 urine was in *M. genitalium* (14.14±3.4 pg/ml), *U. urealyticum* (13.40±3.3 pg/ml), *M. hominis* (14.91±3.4 pg/ml) and mix growth (13.04±2.8 pg/ml) has a significant increase $P < 0.05$ compared with control (10.63±2.1 pg/ml) . However level IL-18 serum concentration was in *M. genitalium* (13.24±2.2 pg/ml), *U. urealyticum* (10.10±2.0 pg/ml), *M. hominis* (10.24±2.1 pg/ml) and mix growth (9.47±1.3 pg/ml) compared with control (9.22±2.4 pg/ml) was no significant increased in patients with UGTIs genital mycoplasmas, can be observed in Table (3-11).

Table (3-11): Urine and Serum IL-18 Concentration of Patients and Control According to *Mycoplasma* spp. and *Ureaplasma* spp.

Diagnosis	IL-18 urine Mean±S.D.	IL-18 serum Mean±S.D.	IL-18 urine *P-Value*	IL-18 serum *P-Value*
M. genitalium	**14.14±3.4**	**13.24±2.2**	**0.002****	**0.430**
U. urealyticum	**13.40±3.3**	**10.10±2.0**		
M. hominis	**14.91±3.4**	**10.24±2.1**		
Mix growth	**13.04±2.8**	**9.47±1.3**		
Control	**10.63±2.1**	**9.22±2.4**		

These results show that the level IL-18 urine concentration were elevated to various degrees in genital mycoplasmas , and there is no significant increase at level IL-18 serum concentration may be due to local inflammation cytokine increase locally and requires time to turn into a systemic, these results were agree with the study of Dinarello and Giamila (2003), who mentioned that IL-18 increases the expression of adhesion molecules ICAM-1 Kohka *et al.* (1998), VCAM-1 Morel *et al.* (2001) and Vidal-Vanaclocha *et al.* (2000), which facilitate the emigration of neutrophils and lymphocytes in containing a nidus of infection. Emigration of neutrophils from the vascular compartment into the tissue spaces is also a primary process in inflammatory diseases. In that regard, IL-18 as an IFN-g–inducing factor serves a key role in controlling infections .

3.7.1.2: Concentration of TLR-6 in urine and serum of UGTIs patients for mycoplasmas and control

As illustrated in Table (3-12), the mean of TLR-6 urine concentration in patients (2.08±0.3 pg/ml) significantly increased (0.000) compared with the control (1.70±0.4 pg/ml), while the mean of TLR-6 serum concentration in patients (2.21±1.4 pg/ml) was not significantly increase compared with the control group (1.80±0.5 pg/ml) .

Table (3-12): Urine and Serum TLR-6 Concentration of Mycoplasmas Patients and Control

TLR-6 pg/ml	No.	Mean ± S.D.	*P-Value*
Patients (urine)	**70**	**2.08±0.3**	**0.000****
Control	**20**	**1.70±0.4**	
Patients (serum)	**70**	**2.21±1.4**	**0.208**
Control	**20**	**1.80±0.5**	

This study agrees with the study of Behzadi *et al.* (2019), show that the relationship between microbial causative agents of urinary-genital tract infections (UGTIs) and Toll like-receptors (TLRs) , *Mycoplasma* spp. and *Ureaplasma* spp. was able to stimulate the innate immune responses via different types of TLRs, including TLR2/6 and TLR1/10 (neutrophil activation and secretion of mucosal antibody of IgA). In current study found that there is a significant difference in urine TLR-6 concentration between patients infected with mycolasmas and control group when *P-value (0.000**)*, this could indicate the activation of a signaling systems that will aid in the eradication of germs from the UGTIs as mycoplasmas . This study could TLRs may have a role in the genesis of UGTIs in female , according to

an increasing body of evidence, also agree with the studies of Behzadi and Behzadi (2016) and Spencer *et al.* (2014), which infered that the TLR2-TLR6 heterodimers are able to identify the 2 kDa mycoplasmal MΦ activating lipoproteins (MALP), the recognition of mycoplasmas target ligands may lead to release of proinflammatory cytokines. The TLR2-TLR6 heterodimers play a key role for detecting important microbial causative agents of UTIs such as *Mycoplasma* spp., *Ureaplasma* spp., *Staphylococcus* spp. and *Streptococcus* spp. .

Bacterial LPs bind TLRs 1, 2, 4, and 6 and, the first lipopeptide expressed in mycoplasmas demonstrated to bind TLRs was the macrophage-activating lipopeptide-2 (MALP-2) of *Mycoplasma* spp.. Subsequently, triacylated or diacylated lipopeptides were shown to bind heterodimers of TLR 1/2 or TLR 2/6, respectively (Peltier *et al.,* 2007 ; Shimizu *et al.,* 2007) . TLR-2 and TLR-6 heterodimers were critical for identifying significant microbial causal agents of urinary tract infections, including as *Candida albicans, Staphylococcus* spp., *Streptococcus* spp., *Mycoplasma* spp., and *Ureaplasma* spp.. TLRs was recognized and interacted with a wide range of microorganisms in Gram-positive bacteria, including lipopeptides, peptidoglycan, and lipoteichoic acid, as well as mycoplasmas and *Mycobacteria* possess lipoproteins. TLR4 is a signaling receptor, on the other hand for lipopolysaccharide (LPS) (Benedetti *et al.,* 2020) .

The level of TLR-6 urine concentration with diagnosis in *M. genitalium* (2.12±0.4 pg/ml), *U. urealyticum* (2.00±0.2 pg/ml), *M. hominis* (2.14±0.4 pg/ml) and mix growth (1.92±0.2 pg/ml) has a significant increase (0.004) compared with control (1.70±0.4 pg/ml) . However level TLR-6 serum concentration was in *M. genitalium* (2.20±1.5 pg/ml), *U. urealyticum* (2.39±1.5 pg/ml), *M. hominis* (1.83±0.3 pg/ml) and mix growth (2.21±1.2 pg/ml) compared with control (1.80±0.5 pg/ml) was no significant increased

in patients with UGTIs genital mycoplasmas, can be observed in Table (3-13) .

Table (3-13): Urine and Serum TLR-6 Concentration of Patients and Control According to *Mycoplasma* spp. and *Ureaplasma* spp.

Diagnosis	TLR-6 urine Mean±S.D.	TLR-6 serum Mean±S.D.	TLR-6 urine *P-Value*	TLR-6 serum *P-Value*
M. genitalium	**2.12±0.4**	**2.20±1.5**	**0.004****	**0.692**
U. urealyticum	**2.00±0.2**	**2.39±1.5**		
M. hominis	**2.14±0.4**	**1.83±0.3**		
Mix growth	**1.92±0.2**	**2.21±1.2**		
Control	**1.70±0.4**	**1.80±0.5**		

These results show that the level TLR-6 urine concentration was elevated to various degrees in genital mycoplasmas , and there is no significant increase at level TLR-6 serum concentration. This may be due to local inflammation increase locally and requires time to turn into a systemic. These results was similar to the study of Benedetti *et al.* (2020), who mentioned that TLR-6 was elevated in urine of the patients with UTIs genital mycoplasmas , is the characteristic of T-helper 2 responses, whose mentioned that elevation of pro-inflammatory TLR-6 marker of UTIs genital mycoplasmas and may be useful in differentiating *Mycoplasma* spp. and *Ureaplasma* spp..

Mycoplasma genitalium is a leading pathogen of nongonoccocal chlamydia-negative urethritis, which has been implicated directly in numerous other genitourinary and extragenitourinary tract pathologies. The pathogenesis of infection is attributed in part to excessive immune responses.

M. genitalium derived lipid-associated membrane proteins (LAMPs) are a mixture of bacterial lipoproteins, exposed at the surface of *Mycoplasma*, that are potent inducers of the host innate immune system. However, the interaction of *M. genitalium* derived LAMPs as pathogenic agents with Toll-like receptors (TLRs) and the signaling pathways are responsible for active inflammation and NF-kappaB activation have not been fully elucidated (He *et al*., 2014).

3.7.1.3: Concentration of IFN-β in urine and serum of UGTIs patients for mycoplasmas and control

As illustrated in Table (3-14), the mean of IFN-β urine concentration in patients (269.89±66.1 pg/ml) was significantly increased to (0.001) compared with the control (210.22±69.3 pg/ml). Also the mean of IFN-β serum concentration in patients (230.56±91.5 pg/ml) was significantly increase (0.009) compared with the control group (174.38±36.2 pg/ml) .

Table (3-14): Urine and Serum IFN-β Concentration of Mycoplasmas Patients and Control

IFN-β pg/ml	No.	Mean ± S.D.	*P-Value*
Patients (urine)	**70**	**269.89±66.1**	**0.001****
Control	**20**	**210.22±69.3**	
Patients (serum)	**70**	**230.56±91.5**	**0.009****
Control	**20**	**174.38±36.2**	

IFN-β is also induced during bacterial infection, following recognition of bacterial ligands by the host viral and DNA sensors. However, the function of IFN-β during bacterial infection is variable and sometimes detrimental to the host . Type I IFNs (IFN-α and IFN-β) are either favorable or detrimental

to the host during bacterial infection depending on their effect on, **i-** Pathogen killing , **ii-** Enhancing or inhibiting the Th1-type response depending on tissue site or kinetics of immune response, and/or **iii-** Inducing an apoptotic response. Overall, the type I IFN-dependent immune response and outcome is largely pathogen and tissue-dependent (Nagarajan, 2011) . Present study show the concentration of IFN-β in urine and serum for patients genital mycoplasmas are higher than that in the control . This study agree with Solis *et al.* (2011) and Sen *et al.* (2010), who show a multitude of intracellular pathways can lead to type I IFN induction following viral/bacterial infection. It is likely that the major driving force in evolution for existence of several redundant pathways that induce type I IFN, the existence of a vast array of receptors and pathways underscores the important role of type I IFN in immune defense.

The level of IFN-β urine concentration with diagnosis in *M. genitalium* (277.98±64.2 pg/ml), *U. urealyticum* (253.37±74.9 pg/ml), *M. hominis* (292.25±81.6 pg/ml) and mix growth (238.28±44.4 pg/ml) has a significant increase (0.005) compared with control (210.22±69.3 pg/ml) . Also level IFN-β serum concentration was in *M. genitalium* (221.86±16.5 pg/ml), *U. urealyticum* (237.79±61.2 pg/ml), *M. hominis* (228.04±10.6 pg/ml) and mix growth (268.21±64.2 pg/ml) compared with control (174.38±36.2 pg/ml) was no significant increased (0.06) in patients with UGTIs genital mycoplasmas, can be observed in Table (3-15) .

Table (3-15): Urine and Serum IFN-β Concentration of Patients and Control According to *Mycoplasma* spp. and *Ureaplasma* spp.

Diagnosis	IFN-β urine Mean±S.D.	IFN-β serum Mean±S.D.	IFN-β urine *P-Value*	IFN-β serum *P-Value*
M. genitalium	**277.98±64.2**	**221.86±16.5**	**0.005****	**0.06**
U. urealyticum	**253.37±74.9**	**237.79±61.2**		
M. hominis	**292.25±81.6**	**228.04±10.6**		
Mix growth	**238.28±44.4**	**268.21±64.2**		
Control	**210.22±69.3**	**174.38±36.2**		

These results show that the level IFN-β urine concentration was elevated to various degrees in genital mycoplasmas , and there is no significant increase at level IFN-β serum concentration . These results agree with Guiton *et al.* (2010), who mentioned that monocytes, macrophages, and neutrophils are all critical components of the host innate immune response to UTI, as for Schiwon *et al.* (2014) and Duell *et al.* (2012), who mentioned that during UTI, macrophages and uroepithelial cells produce proinflammatory cytokines and chemokines that attract neutrophils to the site of infection and regulate antibacterial defenses, including interleukin-8 (IL-8), CCL2 (MCP-1), CCL5 (RANTES), tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), IFN-β, IL-1β, IL-6, IL-10, and IL-17. Type I IFN signaling during bacterial infections is dependent on many factors including whether the infecting bacterium is intracellular or extracellular, as different signaling pathways are activated (Armbruster *et al.*, 2018) .

3.8: Correlation among Immune Markers in Patients Group for UGTIs Genital Mycoplasmas

According to the results of the correlation immune markers in all the patients groups and as shown in Table (3-16), IL-18 (urine) had a highly significant positive correlation with TLR-6 (urine) and IFN-β (urine). IL-18 (serum) also had a highly significant positive correlation with TLR-6 (serum) and a significant positive correlation with IFN-β (serum). TLR-6 (urine) showed a highly significant positive correlation with IFN-β (urine). TLR-6 (serum) also showed a highly significant positive correlation with IFN-β (serum) .

Table (3-16): Correlation Among Immunity Parameters for UGTIs Genital Mycoplasmas Patients

Correlation	IL-18 urine	IL-18 serum	TLR-6 urine	TLR-6 serum	IFN-β urine	IFN-β serum
IL-18 urine	1					
IL-18 serum	0.033	1				
TLR-6 urine	0.491**	0.019	1			
TLR-6 serum	0.003	0.783**	- 0.030	1		
IFN-β urine	0.479**	0.012	0.535**	- 0.008	1	
IFN-β serum	- 0.006	0.259*	0.001	0.323**	0.066	1

** Correlation is significant at the 0.01 level
* Correlation is significant at the 0.05 level

The present study is unique in that it directly compares the expression of these cytokines in pregnant women versus non-pregnant women, all with genital mycoplasmas. Interleukin-18 (IL-18) is a potent pro-inflammatory cytokine involved in host defense against infections and regulates the innate and acquired immune response. IL-18 is produced by both hematopoietic and non-hematopoietic cells, including monocytes, macrophages,

keratinocytes and mesenchymal cell. IL-18 could potentially induce inflammatory and cytotoxic immune cell activities leading to autoimmunity (Ihim *et al.*, 2022). Toll-like receptors (TLRs) with a pattern recognition receptor function play a critical role in early innate recognition and in the inflammatory responses by the host defense against invading microbes, all TLRs, except TLR5, can target *Mycoplasma* spp. because the bacterium contains the ligands for the innate immunity receptors. TLRs in activation of the immune response by lipoproteins from *M. genitalium* and their active components responsible for NF-κB activation (Shimizu *et al.*, 2008) .

The results of the correlation immune markers in the pregnant group showed in Table (3-17), IL-18 (urine) showed a significant positive correlation with TLR-6 (urine) and a significant negative correlation with IFN-β (urine). IL-18 (serum) showed a significant positive correlation with IFN-β (serum). TLR-6 (urine) showed a highly significant positive correlation with IFN-β (urine) .

Table (3-17): Correlation Among Immunity Parameters for UGTIs Genital Mycoplasmas Pregnant Women

Correlation	IL-18 urine	IL-18 serum	TLR-6 urine	TLR-6 serum	IFN-β urine	IFN-β serum
IL-18 urine	**1**					
IL-18 serum	**0.215**	**1**				
TLR-6 urine	**0.341***	**-0.225**	**1**			
TLR-6 serum	**-0.069**	**0.013**	**- 0.186**	**1**		
IFN-β urine	**0.245**	**-0.045**	**0.451****	**- 0.151**	**1**	
IFN-β serum	**-0.380***	**0.394***	**-0.210**	**0.103**	**0.010**	**1**

** Correlation is significant at the 0.01 level
* Correlation is significant at the 0.05 level

Mycoplasma infection is associated with a weak immune function in its host as spontaneous abortion, premature delivery, premature rupture of membranes, and pregnancy complications can all occur at increased rates in cases of *Mycoplasma* and bacterial infections (Cao *et al.*, 2018) .

According to the results of the correlation immune markers in the non-pregnant group, shown in Table (3-18), IL-18 (urine) showed a highly significant positive correlation with TLR-6 (urine) and IFN-β (urine) . IL-18 (serum) showed a highly significant positive correlation with TLR-6 (serum) . TLR-6 (urine) showed a highly significant positive correlation with IFN-β (urine) .

Table (3-18): Correlation Among in Immunity Parameters for UGTIs Genital Mycoplasmas Non-pregnant Women

Correlation	IL-18 urine	IL-18 serum	TLR-6 urine	TLR-6 serum	IFN-β urine	IFN-β serum
IL-18 urine	1					
IL-18 serum	- 0.122	1				
TLR-6 urine	0.588**	-0.047	1			
TLR-6 serum	- 0.105	0.903**	- 0.022	1		
IFN-β urine	0.602**	-0.118	0.538**	- 0.034	1	
IFN-β serum	0.006	0.207	-0.002	0.347	-0.038	1

** Correlation is significant at the 0.01 level
* Correlation is significant at the 0.05 level

The results of this study similar with the results of Beigi *et al.* (2007), who found that lower-genital-tract immunity in response to infectious challenge, as assessed on the basis of endocervical cytokine expression, is influenced by pregnancy; in response to the same microbiologic stimuli (i.e., bacterial vaginosis) pregnant women produced an endocervical cytokine response that

was ~2-fold higher than that produced by non-pregnant women. Moreover, the heightened immune reactivity in the lower genital tract during pregnancy contradicts the paradigm of pregnancy as an immune-compromised state and suggests that immunity may be compartment specific. Given the pivotal role that lower-genital-tract immunity plays in reproductive health, continued investigation is warranted, to further delineate the factors mediating response to infectious challenge.

During and immediately after pregnancy, there are a significant number of physiological changes in the mother and offspring. Pregnant women have an increased risk of developing cardiorespiratory, hematological, immunological, and kidney-related diseases, because of the physiological adaptations that they must endure for the delivery and development of their offspring. These physiological changes explain the increased susceptibility and risk of developing diseases promoted by certain intracellular pathogens, often these microorganisms are difficult to diagnose and treat, and any delay in either their diagnosis and/or treatment can lead to difficultly and potentially life-threatening situations for the mother, the fetus, or the neonate (Ferreira *et al.*, 2022) .

Conclusions

1- Clinical presentation and culture of *Mycoplasma* spp. is variable and diagnosis confirmation is a challenge to even the most experienced clinicians and laboratory.

2- Culture methods and biochemical tests were found to be specific for detection of viable *Mycoplasma* spp. and *U. urealyticum*. However , it is proved to be time consuming, but bacterial culture is generally considered as the gold standard detection method of Mycoplasmosis. It is a heterogenous bacterial type over time .

3- Doxycycline,Clindamycin, Ofloxacin and Ciprofloxacin were more potent against *Mycoplasma genitalium, Mycoplasma hominis* and *Ureaplasma urealyticum*.

4- The present study according to immunological parameters indicated that the patients with UGTIs due to mycoplasmas had significantly increasing TLR-6 and IL-18 than control group in urine only, while IFN-β significantly increasing in urine and serum .

5- Molecular based approaches have considerably enhanced the capacity to detect and identify mycoplasmas and ureaplasmas in clinical specimens that provide the required rapid , combines maximum sensitivity with good specificity and significant strategies.The results sequencing obtained from (11) isolates only (7) local *Mycoplasma genitalium* isolates alignment with reference isolates retired from NCBI – BLAST analysis.

Recommendations

1- It is necessary to use immunological diagnostic methods such as antibody agglutination test to detect antigen released from bacteria in the urine before starting culture .

2- Studying other cytokines for UGTIs and its role in the diagnosis of infection with mycoplasmas is recommended.

3- Further investigations are needed to definitively differentiate between a direct effect of these microorganisms on vaginal cytokine levels or whether *M. genitalium* , *M. hominis* and/or *U. urealyticum* colonization is increased under conditions when immunity in the vaginal milieu is altered due to the presence of other infectious agents or non-infectious factors.

4- Take of appropriate antibiotics for urogenital infections (not random).

5- Cloning and expression some pathogenic genes and identification of mutant isolates with their relative virulence genes.

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