Republic of Iraq

Ministry of Higher Education and Scientific Research

University of Babylon

College of Sciences for Women

Department of Biology

Identification of Vaginal Bacteria Infection with Spontaneous Abortion and Evaluation of Peripheral Blood Levels of Natural Killer Cells

A Thesis

Submitted to the Council of the College of Science for Women/University of Babylon in Partial Fulfillment of Requirements for the Degree of Master in Biology

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2023 A.D **1445 A.H**

بِسمِ ٱللَّهِ ٱلرَّحمَٰنِ ٱلرَّحِيمِ

(و إِذْ قَالَ مُوسَىٰ لِفَتَاهُ لَا أَبْرَحُ حَتَّىٰ أَبْلُغَ

مَجْمَعَ ٱلْبَحْرَيْنِ أَوْ أَمْضِيَ حُقُبًا)

صَدَقَ اللهُ العَلِيُّ العَظِيم

«سورة الكهف: ٦٠»

Dedication

To the tender hands, the passionate hearts, and the warm embraces, to my soul and comfort, and to ***my beloved parents*** If it weren't for your prayers for me and your satisfaction with me, I wouldn't be here.

To whom God chose for me as a partner and lover and made affection and mercy between us, ***my beloved husband***, my friend, and my support, I have not forgotten your support for me and your concern for him as if you were my father.

To my eyeballs and the core of my heart, to those whom I waited so long for until my life blossomed with them, ***my beautiful twins, Abdullah and Fatima***, forgive me if I neglect one day during my studies.

To ***the sisters of my soul***, the ladies of my council, and the consorts of my childhood, to whom my heart yearns for always

To ***my three young brothers,*** *to* the candles and pillars of the house and its bright side.

To ***my husband's family***, my brothers and sisters. Thank you for believing in me and supporting me with the sincerity of your feelings and kind words.

To ***myself***, I tell her, " You were up to the challenge".

Enas 

Acknowledgment

My thanks and praise be to ***Allah***, my Lord and Creator, who destined me to complete this path with success and facilitation and guided me to his path. And to his ***Noble Prophet***, who lit our lives with his religion, And to our Imam, ***the owner of the Era and the time***, upon whom our hopes depend, so that he may fill our lives with justice and equity.

With my sincere thanks and appreciation to my dear supervisor, ***Dr. Oruba Albermani***, Her good manners and humility She did not skimp on me in time, effort, or information. I'm telling you, you're the liberality code. My thanks to the second supervisor in the research, ***Dr. Suha Witwit***, for her sincerity, hard work, and assistance in collecting samples.

I also will not forget the gratitude of **all *the people who allowed me to take the specimens***, along with my wishes for their recovery, as well as ***the staff*** who began taking the samples in the hospitals that I visited in the past period.

I would like to express my gratitude to the College of Science for Women, represented by ***the Dean***, Prof. Dr. Abeer Fauzi Al-Rubaye, *the* ***Head of the Department*** **of Biology**, Dr. Ekhlass Al-Shareefi, and *all* teaching staff for their great care and follow-up with the students.

I thank my best friend, ***Shahd Jamal***, for her authentic stance and generosity with me.

Enas ✍

Summary

A spontaneous abortion (SA) is a loss of the foetus that occurs frequently before 24 weeks of pregnancy, resulting in a severe condition. Depending on the age and health of the woman, between 10 and 50 % of pregnancies are run out with SA.

A history of bacterial vaginosis may enhance a patient's risk of developing this illness. Natural killer (NK) cells are being investigated as a potential contributor to the immunological tolerance imbalance that may cause many cases of unexplained Spontaneous Abortion (USA). Premature labour and spontaneous abortions may happen when the female host's reproductive system experiences an increase in the number of *Mycoplasma hominis* bacteria.

In this study, 50 high vaginal swab (HVS) specimens and 50 samples of blood were collected from women with SA; 50 HVS specimens and 30 samples of blood from women with healthy pregnancy outcomes were collected from Babylon Teaching Hospital for Maternity and Children and from Al-Imam Al-Sadiq Teaching Hospital in Hilla City during the period from October 2022 to December 2022. The age range of the cases as well as the controls was from 17 to 45 years.

This study was divided into two parts: the first part focused on the diagnosis of vaginal microbiota, which depends on the culturing of HVS on different culture media under aerobic and anaerobic conditions.

Initially bacterial identification by conventional bacteriological techniques relied on the phenotypic characteristics of colonies as identified via manual biochemical tests and Gram staining. The diagnosis was also verified genetically through the isolation of bacterial genomic DNA (for 20

bacterial isolates) amplification of the 16S rRNA Loci gene via polymerase chain reaction (PCR), and sequencing.

The second part of the study include collecting blood to obtain serum to assess peripheral natural killer (pNK) cell levels and diagnose *Mycoplasma hominis* infection immunologically using the ELISA method.

Results from this study showed that there is a prevalence of blood group O+ in women with SA. Also, it shows the dominance of the age group of 26–35 years for both pregnant and aborted women.

The current study results show that there is a difference in bacterial genera in women with SA compared with healthy women, and it was noted that embroilment of *Enterococcus faecalis* occurred in most cases of SA with an estimated percentage of 56% (28/50), thus defeating *Escherichia coli* by 32% (16/50) and 4% (2/50) for *Klebsiella pneumonia* and 4% (2/50) for *Enterococcus gallinarum.* In this study, some very rare bacteria species were identified, including *Acinetobacter junii* at 2% (1/50) and *Corynebacterium coyleae* at 2% (1/50), While the percentage of bacteria associated with healthy women was: 30% (15/50) for *E. faecalis*, 26% (13/50) for *E. coli*, 18% (9/50) for *K. pneumonia*, 24% (12/50) for *Staphylococcus epidermidis*, and 2% (1/50) for *Metabacillus niabensis* (This bacterium was diagnosed for the first time in Iraq as well as the rest of the world in a clinical sample).

The molecular identification of bacterial isolates with 16S rRNA gene sequencing detected the high percentage of *Enterococcus faecalis* in SA, this lead to the fact that *Enterococcus faecalis* strains possess an arsenal of virulence factors. Bacterial adhesion and pathogenicity are both boosted by aggregation substances, which act as virulence factors. *asa1* PCR amplicons were employed in this research since this gene has been shown to be linked to

pathogenicity in purified samples of typical *Enterococcus faecalis isolates.* The result showed that the percentage of bacteria positive for this gene was 85% in patient samples.

All the bacterial isolates sequences were compared with known sequences in NCBI Database by using BLAST analysis and the sequences were registered in the NCBI GenBank database and their identification code obtained.

Depending on the data that appeared in the work there is a discrepancy in the concentrations of pNK between patients and controls. The concentrations mean and standard deviation (Std) of peripheral NK in patients was (107.339 ± 46.647), while in controls was (80.229 ± 23.737), respectively. This result suggests that the pNK in women with SA may be of the NK-cytotoxic (NKC) type.

In addition, The study examined the effect of *Mycoplasma hominis* in patients. The concentration of *"Mycoplasma hominis*" in patients means and the Std. (0.39830 ± 0.21166), was not significant compared with the control (0.32497 ± 0.130461).

The current study linked the relationship between *Mycoplasma hominis* and the immunologic status of SA patients. A finding was showed that there is an inverse relationship between the concentrations of NK cells and the concentrations of *M. hominis* in the patient, as the higher the concentrations of NK cells, the lower the concentration of *Mycoplasma*, and vice versa. This bacterial strain is an obligate intracellular organism that lives in a woman's vagina but does not cause illness in healthy people. However, in people with weakened immune systems, encouraged to proliferate and can lead to conditions like pelvic inflammatory disease (PID).

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List of Abbreviation

Abbreviation	Meaning
µl	Microliter
16S rRNA	16 Small sub-unit ribosomal Ribo Nucleic Acid
A	Adenine
Agr	Accessory gene regulator
AIDS	Acquired Immuno-Deficiency Syndrome
ANOVA	Analysis of Variance
ASA1	Aggregation substance
AV	Aerobic Vaginitis
BHI	Brian Heart Infusion
bp	Base pair
BT LAB	Bioassay Technology Laboratory
BV	Bacterial Vaginosis
C	Cytosine
CCL	CC chemokine
CD	Cluster of Differentiation
CFU	Colony-Forming Unit
Ct	Chlamydia trachomatis
CX3CL	Chemokine receptor
cyl	Cytolysin
D.W	Distilled water
dATP	Deoxyadenosine Triphosphate
DB	Databases

DC	Dendritic Cells
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
DNA	Deoxy ribo Nucleic Acid
dNK	decidual Natural Killer
DTNPs	Deoxynucleoside triphosphates
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMB	Eosin- Methylene Blue
EPS	Extracellular Polymeric Substances
EVT	Extra Villous Trophoblast
FATG	FavroPrep Tissue Genomic DNA
G	Guanine
Gel	Gelatinase
gm.	Gram
H2O2	Hydrogen Peroxide
HDN	Haemolytic Disease of the Newborn
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigens.
HMW	High Molecular Weight
HRP	Horseradish Peroxidase enzyme
HVS	High Vaginal Swabs
Hyl	Hyaluronidase
IFN	Interferon

IL	Inter Leukin
IUGR	Intra-Uterine Growth Restriction
IVF	In Vitro Fertilization
KCl	Potassium Chloride
KILs	Killer Immunoglobulin-Like receptors
LBW	Low-Birth-Weight
MgCl2	Magnesium Chloride
MHC-I	Major Histocompatibility Complex class I
Ml	Milliliter
MRS	deMan Rogosa Sharpe
MSA	Mannitole Salt Agar
NCBI BLASTNn	National Center for Biotechnology Information –Basic local Alignment Search Tool.
NCRs	Natural Cytotoxicity Receptors
NGS	Next-Generation Sequencing
NK cell	Natural Killer cell
NKC	Natural Killer Cytotoxity
NKT	Natural Killer T cell
NLD	Non-Lactobacillus Dominant
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
PID	Pelvic Inflammatory Diseases
pNK	peripheral Natural Killer

PROM	Preterm Rupture Of Membranes
PTD	Preterm Delivery
PTL	Preterm Labor
RIF	Recurrent Implantation Failure
RPL	Recurrent Pregnancy Loss
RPM	Revolutions Per Minute
RSA	Recurrent Spontaneous Abortion
RT	Reproductive Tract
SA	Spontaneous Abortion
SD	Standard Deviation
SGA	Small for Gestational Age
SPSS	Statistical Package for Social Sciences
T	Thymine
Taq polymerase	Thermus aquaticus Polymerase
TBE	Tris-Borate-EDTA
TE	Tris EDTA
TE-buffer	Tris- EDTA Buffer
TGF	Transforming Growth Factor
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
TMB	Tetra methyl benzidine
TNF	Tumor Necrosis Factor
uNK	uterine Natural Killer

URSA	Unexplained Recurrent Spontaneous Abortion
UTIC	Urinary Tract Infection Chromogenic
UV	Ultra Violate
VEGF	Vascular Endothelial Growth Factor
VMB	Vaginal Microbiota

Chapter One
Introduction

1.1. Introduction

The vaginal microbiome (VMB) refers to the community of bacteria found in the vaginal canal of a human being. As the first line of defense against hazardous bacteria, vaginal flora also helps maintain a healthy dynamic balance and mutual restriction of reliance.

As a woman ages, her vaginal flora changes, and her body's abnormalities might have an impact. Age, pregnancy, hormone-driven instability, sexual relationships, probiotics, antibiotics, and other drugs can all alter the composition of the vaginal microbiota, potentially leading to an imbalance (Juliana *et al*., 2021).

The microbiome of a healthy female genital tract is dominated by *Lactobacillus* species, which produce lactic acid which can be toxic to or even lethal for many different types of bacteria and hydrogen peroxide to keep the pH low and ward off infections (Diop *et al*., 2019 ; Abdool Karim *et al*., 2019; Juliana *et al*., 2021).

Emotional stress may decrease lactobacilli abundance in the vaginal microbiome and increase inflammation, undermining a healthy vaginal state associated with an abundance of *Lactobacillus* and leading to a condition known as bacterial vaginosis (BV) (Abdool Karim *et al*., 2019). Overgrowth of (facultative) anaerobes such as *Gardnerella*, *Atopobium*, and *Prevotella* species contributes to a dysbiotic vaginal state, which is characterized as a persistent shift from a low-diversity, Lactobacilli-dominated VMB. Preterm birth, increased susceptibility to sexually transmitted diseases, and spontaneous abortion are just a few of the negative pregnancy outcomes linked to vaginal dysbioses (Moosa *et al*., 2020).

Both the mother and the fetus are at risk from spontaneous abortion (SA), making it one of the primary pregnancy diseases. Fetal loss occurs often in women with SA, typically before 24 weeks of pregnancy (Adib-Rad *et al*., 2019; Dehkordi *et al*., 2020).

Depending on the age and health of the woman, between 10 and 50 percent of pregnancies ended with SA. Chromosomes, genetics, endocrine gland dissection, placental abnormalities, infections, weakened immune systems, thrombosis, and environmental factors are all contributors to SA (Zhao *et al*., 2021).

The 16S rRNA gene sequencing-based approach is useful in resolving ambiguous results from culture and biochemical tests to identify pathogens from patient bio samples, and it also helps eliminate potential culture-related biases in pathogen identification (Muhamad Rizal *et al*., 2020).

The universal primers can amplify the nine hyper variable segments (V1–V9) of the 16S rRNA gene because they are bordered by highly conserved sequences. The variable areas enable taxonomic classification. To distinguish between two species, a cutoff of 98.65 percentage points of similarity in their 16S rRNA gene sequences has been found. Associated with the efficiency of controlling infectious diseases, this "gold standard" method for the detection and identification of infectious bacteria can cut the time required for analysis from days to hours (Santos *et al*., 2022).

"*Enterococcus faecalis, Klebsiella pneumonia, Escherichia coli*, and *Mycoplasma hominis* " are the most important vaginal microbiota that can become apportioned in appropriate conditions (Inaba *et al*., 2019).

Aggregate substances encoded by the *asa1* gene are considered to be the most essential virulence factors in *Enterococcus faecalis*, causing

disease and antibiotic resistance, because they carry out the process of cohesion and adhesion between bacterial cells and tissue surfaces, generating the biofilm.

One of the most remarkable aspects of reproductive biology is the ability of a healthy woman with a fully functional immune system to carry a baby until birth without undergoing immunological rejection. Immunoglobulin, cytokines, hormones, and other endometrial factors are all part of the local and systemic immunological responses that affect this process. For implantation to occur and a pregnancy to develop, all of these factors must be in balance (Fu *et al*., 2021).

A lack of immunological tolerance, a condition in which the natural killer cell NK may play a key role, is thought to be the fundamental reason for SA's unexplained origin (Guerrero *et al*., 2020; Rougang *et al*., 2023).

NK cells can undergo a phenotypic switch from non-cytotoxic to cytotoxic in order to participate in immune defense when exposed to pathogens in the uterus during pregnancy. Preterm birth, spontaneous abortion, uterine malignancy, and implantation failure can all occur when the maternal-fetal interface is compromised due to premature NK cell activation in late pregnancy (Wang *et al*., 2018; Zhang and Wei, 2021).

Currently, infertile patients' peripheral NK cells are used as a marker to decide when to begin treatment, (Toth *et al*., 2019; Dons’ koi *et al*., 2022).

1.2. Aim of the Study

The aim of the present study is to evaluate whether there are differences in the vaginal microbiome of women who miscarry compared to those who have normal pregnancy outcomes, and to investigate the natural killer cell risk related to spontaneous abortion.

1.3. The Objectives

1. Characterization of bacterial vaginosis in addition to the normal flora (vaginal microbiota) by the following methods:

- The culture-dependent
- Serology
- Amplification of the 16S rRNA gene at species level and sequencing

2. Evaluation of the serum level of natural killer cells by using the ELISA technique.

Chapter Two
Literature Review

2. Literature Review

2.1. Vaginal Microbiota

Protecting against urogenital infections, the vaginal microbial population ("microbiota") is an important part of women's reproductive health. Maintaining a dynamic equilibrium and mutual restriction of reliance is aided by the vaginal flora, which acts as the first line of defense against imported harmful microorganisms (Bayigga *et al*., 2019).

Age, Dietary requirements, sexual behaviors, use of medicines like probiotics and antibiotics and hormonal movements during ovulation or pregnancy are just a few of the many variables that might affect VMB composition (Diop *et al*., 2019 ; Juliana *et al*., 2020).

By producing substances including bacteriocins, hydrogen peroxide, and biosurfactants, healthy vaginal microbiota might influence immune responses to invading pathogens and contribute to host immunity. Changes in the vaginal microbiota's composition and function have been linked to an increased risk of infection (Diop *et al*., 2019). Increased production of inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor, for instance, could interfere with the innate mucosal barrier function after an infection with Atopobium vaginae. Women's vaginal microbiota contains bacteria that are essential for their health and the maintenance of equilibrium (Haahr *et al*., 2019 ; Bayigga *et al*., 2019; Fan *et al*., 2020).

New research suggests that vaginal dysbacteriosis may be linked to gynaecological cancer, gestational diabetes, adverse pregnancy outcomes, and preterm delivery, despite the fact that these microbial communities are dominated by *Lactobacillus* and are relatively simple compared with the gut microbiome (Sun *et al*., 2022).

2.2. Vaginal Microbiota during Pregnancy

It is unknown what type of vaginal state is related to an *L. iners*-dominated VMB, despite the fact that most *Lactobacillus* species are linked to a healthy vaginal state, low proinflammatory cytokine production, and positive birth outcomes. Although *L. iners* is commonly thought of as a harmless vaginal symbiont, it can occasionally act as an opportunistic pathogen. And whereas *L. iners* can live in harmony with such aerobic microorganisms, *L. crispatus* has been shown to prevent colonisation by anaerobe bacteria such as *G. vaginalis* (Diop *et al*., 2019).

Having said that, the presence of facultative anaerobic bacteria in the vaginal microbiota has been detected in symptom-free women; therefore, it is not yet known if this microbial composition should always be classified as an unhealthy vaginal microbiota state. There is now known to be a difference between the gestational age of a species and its stability (Gupta *et al*., 2020).

Studies have shown that the vaginal microbiota is more stable in the first trimester of pregnancy and that this trend continues throughout the remainder of the pregnancy (Al-Nasiry *et al*., 2020; Gupta *et al*., 2020).

The vaginal pH is maintained at a comfortable level for the host by lactobacilli, which are particularly adapted to the vaginal environment. A low pH acts as a barrier because it inhibits the growth of many invading microorganisms. In addition to lactic acid, lactobacilli produce bacteriocins, which are proteinaceous chemicals that kill bacteria by increasing the permeability of their target cell's outer membrane. *Lactobacillus* also generates hydrogen peroxide (H2O2), which acts as a protective component (Serrano *et al*., 2019). Since H2O2 generation is promoted in aerobic conditions, but the vaginal environment is often

anaerobic, its importance is unclear. Since the specifics are still unclear, H2O2 could serve as an equivalent indicator for an unknown biological marker (Muzny *et al*., 2020).

In addition to producing byproducts, lactobacilli also stimulate our innate immune system in a specific species-specific way when it comes into contact with any gram-negative attack by increasing production of IL-23, which preferentially activates the Th17 pathway (Elovitz *et al*., 2019 ; Chee *et al*., 2020).

Pregnancies that ended in natural delivery had a more stable microbiome than those that ended in pre-term birth PTB, which showed an increase in variety and a decrease in microbiome stability in the first trimester. Alpha diversity was highest in the first trimester, indicating that alterations to the microbiome are particularly important during the first few months of pregnancy (Serrano *et al*., 2019).

Number of researches found no changes in microbiome composition during the course of pregnancy, in contrast to the findings of other investigations. It was observed that by the end of the second trimester, the pregnant microbiome had converged toward the well-established Lactobacilli-predominant microbiome, regardless of whether it had begun with a complex or simple microbiome (Dominguez-Bello, 2019).

2.3. Spontaneous Abortion

The most prevalent form of pregnancy loss occurs in the first trimester (up to 12+6 weeks of gestation). Women who got abortion were assessed for life-threatening conditions and given information and support about the various treatment choices available to them, including expectant, medical, and surgical approaches (Zhao *et al*., 2021). Infection , genetics, anatomy, thrombosis, endocrinology, immunology and external factors are all

potential causes of spontaneous abortion in women (Alves and Rapp, 2022).

These women also tend to have higher NK cell counts and autoantibody levels, both of which reduce blood flow to the uterus in the early stages of pregnancy. As a result, autoimmunity plays a pathogenic role in triggering spontaneous abortion in certain women (Obais and Alsultany, 2021). Some recurrent early-to-middle-pregnancy abortions can be due to the activation of endometrial immune cells by inflammatory substances, which in turn can lead to the invasion of trophoblast cells by the mother's immune system (Fan *et al*., 2020).

2.3.1. The Forms of Spontaneous Abortion

There are numerous names and classifications for SA.

1. When all fetal tissue has been removed, it is considered a complete abortion. The trophoplast, chronic villi, gestational sac, yolc sac, and fetal pole (embryo) are all results of conception; the fetus, umbilical cord, placenta, amniotic fluid, and amniotic membrane develop later in pregnancy.

2. When an abortion is incomplete, only a tiny fraction of the fetus makes it through the cervical canal and into the outside world.
3. In a missed abortion, the embryo or fetus dies but is not expelled because uterine contractions were insufficient.

4. A threatened abortion is characterized by the symptomatic, 'threatening' evacuation of the products of conception, but with a closed cervical os and a viable embryo or fetus.

5. An open cervical os, signifying the 'inevitable' transit of the conception products, distinguishes an inevitable abortion from a threatening abortion.

6. Having three or more consecutive miscarriages is considered recurrent abortion (Alves, 2020 ; Huss, 2021).

7. When residual products of conception become infected—as could occur in a situation of non-sterile induced abortion—the woman faces a high risk of infection spreading throughout her body (septicaemia) and potentially losing her life. Septic abortion can only be diagnosed by doing a histological examination of the fetus and placenta or by analyzing an isolated culture or genome to discover infectious organisms after the fact (Fan *et al*., 2020).

8. When the gestational sac develops normally but the embryonic part of the pregnancy is missing or stops growing extremely early, this condition is known as an empty sac. Blighted ovum and anembryonic pregnancy are additional names for this disease (Huss, 2021).

2.3.2.Etiology

The estimated SA occurrence is between 12 and 15% of clinical pregnancies, while this number varies widely depending on the community under observation. Almost 80% of SAs happen in the first trimester (the first 12 weeks of pregnancy), while only 10% happen in the second.

Not all of the factors that influence SA have been explained. Approximately 50 % of all occurrences of SA can be traced back to genetic disorders. Ethnic background, stress, workplace and chemical exposures,

and lifestyle factors such as being overweight, smoking, and drinking have all been linked to an increased risk of SA (Irina *et al*., 2018).

Anatomical, endocrine/hormonal, and autoimmune/immunological problems, as well as masculine variables, have also been linked to SA. Notably, the cause of up to half of SA cases is unknown. Because of this, can classify them as idiopaths (Zhao *et al*., 2021).

Chromosome changes and/or aberrant chromosome counts in the fetus are thought to be responsible for early pregnancy loss in approximately half of all cases. The most common risk factors are a mother's advanced age and a history of miscarrying a baby at a young age. For instance, although only 9–17% of pregnant women experience miscarriage between the ages of 20 and 30, the rate rises to 80% for mothers over the age of 45. The use of tobacco products, cannabis, and cocaine is also associated with an increased risk (Miyaji *et al*., 2019).

Infections like cervicitis, vaginitis, HIV infection, syphilis, and malaria are all common risk factors. Lastly, there is evidence that structural uterine abnormalities such as congenital malformations, leiomyomas, and intrauterine adhesions raise the probability of a spontaneous miscarriage (Tognon *et al*., 2020). All these reasons are shown in Figure 2-1.

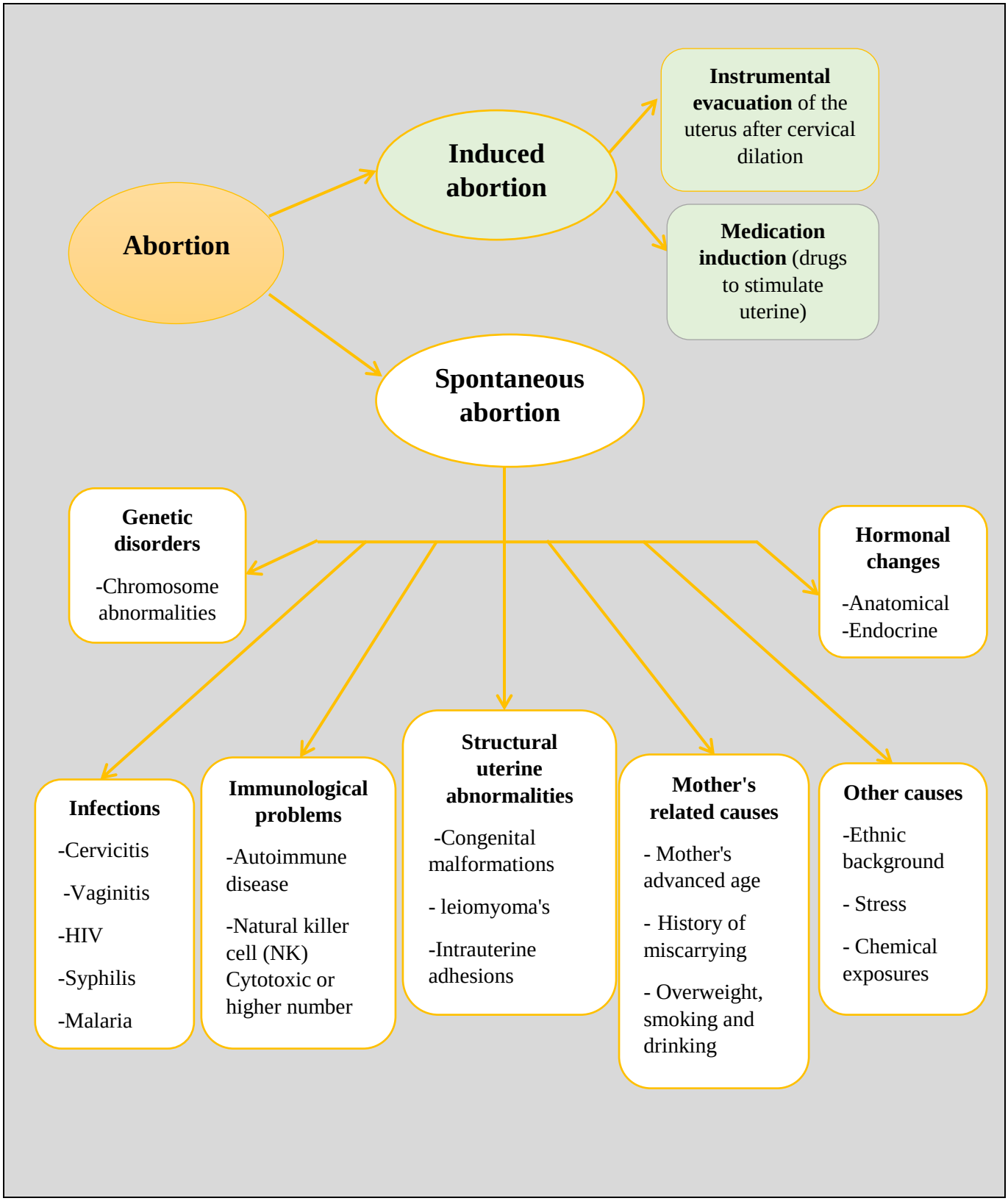


Figure (2-1): Type and Causes of Abortion

2.4. Vaginal Microbiota and Spontaneous Abortion

The human body's urogenital tract is just one area where "microbiota" communities of microorganisms reside. While the bacterial composition of the vaginal microbiota has been discovered and is partially associated with obstetric outcomes, more in-depth study and controversy are being conducted on the uterine microbiota, which was previously assumed to be a sterile environment. Characterizing the microbial communities in the reproductive tract (RT) of asymptomatic infertile women may help researchers locate a specific RT microbiome associated with implantation failure (Riganelli *et al*., 2020).

The discovery of separate microbial communities in the vagina, cervical canal, uterus, fallopian tube, and peritoneal fluid disproved the initial dogma that a healthy uterine cavity was sterile and that the presence of microbes was a sign of pathology. The uterine microbiome may play a role in female fertility, health, and disease, according to recent research. It is now understood that the uterus has its own unique microbial community. The uterine microbiota is a controversial topic due to the lack of consensus on its composition and role (Fu *et al*., 2020) .

Vaginal microbiota appeared to be a "non-lactobacillus dominant" (NLD) environment in women who had implant failure, despite the fact that our cohorts were too small to reliably attain statistical and biological significance (Kyono *et al*., 2019).

In addition, a gram-positive and aerobic bacterium now linked to urinary tract infections and the cause of infections in immunocompromised patients, in the group with failed implantation, suggesting that this bacterium may serve as a bacterial biomarker of implant failure (Buzzaccarini *et al*., 2020) .

The presence of bacteria in the uterine cavity was often thought to be dangerous for both the mother and the developing baby, but recent research suggests that the amniotic fluid, the uterus, and the placenta may actually host a distinct microbiota (Riganelli *et al*., 2020; Fu *et al*., 2020; Buzzaccarini *et al*., 2020).

2.5. The Alteration of Microbiota in Reproductive Tract of Women with Spontaneous Abortion

Patients with SA have a unique microbial population in their genital tract lavage fluid, including the cervix and uterine cavity. Microbiota composition varied significantly between the lower vaginal tract and the uterine cavity, as well as between endometrial tissue and uterine lavage fluid, in the SA group (Theis *et al*., 2020).

Possible risk factors for SA include variations in cytokine levels, which have been linked to changes in the uterine microbiome. Lack of these bacteria may be connected to infertility and poor pregnancy outcomes following in vitro fertilization (IVF) and a healthy vaginal microbiome dominated by *Lactobacillus* can help prevent infections (Bilibio *et al*., 2020).

Embryo implantation failure and infertility have both been linked to an abnormality in the endometrial microbiome. The types of bacteria that have been discovered in the vaginal secretions of women with RSA have been the subject of several studies. Two species, *Atopobium* and *Prevotella*, have significantly higher abundances (Fan *et al*., 2020 ; Jiao *et al*., 2022).

There is a lack of data about the significance of alterations in the microbiota of the upper and lower female reproductive tracts in RSA patients (Liu *et al*., 2022).

Bacillus spp. were also much less common in the RSA group's uterine lavage fluid. Different important bacteria were involved in pathogenic processes in the vagina, the cervix, and the uterine cavity. This highlights the need for the development of therapeutic strategies in clinical settings to control microbiome anomalies in the female reproductive system. More thorough clinical trials with larger sample numbers are needed to clarify specific therapeutic treatment options (Rimmer *et al*., 2021).

2.6. Bacterial Vaginosis and Aerobic Vaginitis

2.6.1. Bacterial Vaginosis and Aerobic Vaginitis associated with Spontaneous Abortion

One of the most common vaginal conditions associated with unusual changes in the vaginal microbiome (VMB) is bacterial vaginosis (BV), also known as vaginal dysbiosis, which is microbiologically characterized by a lesser abundance of *Lactobacillus* species and/or an overgrowth of anaerobic bacteria (Owens *et al*., 2020).

Bacterial vaginosis affects anywhere from 5.8% to 19.3% of pregnant women and up to 29% of women in the United States (Redelinghuys *et al*., 2020 .

Due to its association with negative reproductive health outcomes like pelvic inflammatory disease, miscarriage, and preterm birth (Zhao *et al*., 2021), BV is a serious public health problem for women of reproductive age, their kids, and their partners (Haahr *et al*., 2019; Owens *et al*., 2020).

There is no statistically significant link between BV and RSA, despite the fact that it is commonly seen in women who have a spontaneous abortion in the second trimesters of pregnancy (P 0.05) (Fan *et al*., 2020).

Aerobic vaginitis (AV) is another vaginal dysbiotic condition characterized by an abnormal VMB composed mostly of commensal aerobic microorganisms of intestinal origin, typically *Escherichia coli*, *Staphylococcus aureus*, coagulase-negative staphylococci such as *Staphylococcus epidermidis*, group B *Streptococcus* (*Streptococcus agalactiae*), and *Enterococcus faecalis*. AV affects between 4 and 8% of pregnant women and between 5 and 10.5% of symptomatic non-pregnant women. Preterm prelabor rupture of membranes PROM, chorioamnionitis, and preterm delivery PTD have all been linked to AV. AV has also been linked to spontaneous miscarriage (Owens *et al*., 2020 ; Juliana *et al*., 2020).

VMB dysbiosis (here defined as not dominated by lactobacilli) has been linked to a number of negative pregnancy outcomes including infection after termination of pregnancy, early and late miscarriage, histological chorioamnionitis, postpartum endometritis, premature membrane rupture, and preterm birth (Juliana *et al*., 2020).

Abortions can be caused by infections in the vaginal or urinary tract. Microorganisms entering the amniotic fluid from the lower genital tract (vagina or cervix) appear to be the most common source of intra-amniotic infections. Alterations in the vaginal microecosystem are associated with progressive infections in the uterus (Owens *et al*., 2020 ; Juliana *et al*., 2020).

Deficiencies in vaginal flora can allow for the transmission of infections to the uterus, where they can activate chemokines and provoke a local immunological response. As a result, the local immune system faces microcirculation disruptions, which will ultimately result in RSA.

Improving early SA diagnosis and therapy may get theoretical guidance from focusing on abnormal expression of vaginal flora (Fan *et al*., 2020).

2.6.2. Bacterial Vaginosis During Pregnancy

Studies of pregnant women with bacterial vaginosis show an unexpected twofold or greater increased risk for serious complications, such as premature birth, low-birth-weight (LBW) infants, preterm rupture of membrane (PROM), amniotic fluid infection, chorioamnionitis, and post-cesarean and post-partum endometritis. Despite evidence linking bacterial vaginosis to an increased risk of severe issues, many medical practitioners view the condition as more of a nuisance than a serious risk to the mother and her unborn child (Jin, 2020).

Since low birth weight (LBW) can come from intrauterine growth restriction (IUGR), small for gestational age (SGA), or preterm birth, interpreting BV's impact on fetal and neonatal growth is challenging didn't find any evidence linking BV and LBW pregnancies, but they did find that babies born to moms who carried the virus were smaller than average for their gestational age (Owens *et al*., 2020).

Women who are pregnant and have been diagnosed with bacterial vaginosis should get treatment. The requirement as well as the possible benefit of therapy for asymptomatic pregnant women remain less clear. Despite this, more than half of all women who have bacterial vaginosis show no signs of infection in the lower vaginal tract. Treatment may be warranted for pregnant women despite the lack of symptoms due to the increased risk of significant consequences (Javed *et al*., 2021).

The risk of having a baby born prematurely is decreased when pregnant women receive treatment for bacterial vaginosis. Pregnant women

with bacterial vaginosis and a high risk of preterm delivery (a prior preterm birth or a low prepregnancy weight of less than 50 kg) have been the subject of multiple studies. Testing for and treating bacterial vaginosis during pregnancy may or may not help prevent premature birth. Women suffering symptoms of bacterial vaginosis, whether pregnant or not, are typically treated with an antibiotic drug, either orally or topically given to the vaginal area (Jin, 2020).

Clinical symptoms of inflammation, such as discomfort or redness of the vaginal mucosa, are absent in BV, and there is no rise in circulating leukocytes, generation of interleukin (IL)-8, or levels of IL-1 and IL-6. On the other hand, it is theorized that PROM and PTB occur more frequently in women who have a genetic susceptibility for pathological inflammatory responses to BV. The expression of tumor necrosis factor alpha (TNF- α) in women, for instance, can be triggered by BV (Owens *et al*., 2020).

2.7. 16S r RNA Gene, Its Role in Diagnosis of Bacteria

Intragenomic variation analysis of 16S rRNA genes is a novel method for investigating ribosomal constraints on rRNA genes, and the degree of variation is a crucial factor to take into account when estimating the diversity of a complex microbiome as part of the recently launched human microbiome project (Alibrandi *et al*., 2020).

Deposition of entire unambiguous nucleotide sequences into public or private databases and attaching the correct "label" to each sequence are essential for the effectiveness of 16S rRNA gene sequencing as a tool in microbial identification. Many nucleotide sequences that were deposited into public databases several years ago were of doubtful quality. Because of this, it would be impossible to use this technology alone for identifying

species of aeromonads, as intragenomic heterogeneity of the 16S rRNA gene exists among aeromonads (Dueholm *et al*., 2020) .

According to the aforementioned findings, it is highly probable that any microbiological identifications based on 16S rRNA distance scores of >1% are unsuitable for use in a diagnostic or public health reference laboratory suggested a number of changes to the current criteria for using 16S rRNA gene sequencing for bacterial identification (Yang *et al.*, 2020).

Appropriate application of such technology necessitates the establishment of norms analogous to those established for DNA-DNA hybridization. Species identification using 16S rRNA gene sequencing is still a new occurrence in most clinical laboratories; therefore, it is expected that such standards will continue to develop over time. In addition, future use of microarray-based technologies with targets based on 16S or other housekeeping genes may provide a considerably more sensitive and accurate basis for molecular species identification (Peterson *et al*., 2021).

The 16S rRNA gene sequence has been employed more than any other housekeeping genetic marker in the study of bacterial phylogeny and taxonomy for a variety of reasons. The 16S rRNA gene (1,500 bp) is large enough for informatics purposes because of; its presence in almost all bacteria, often existing as a multigene family, or operons; the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); mutation rate is slow; and the gene's abundance (Alibrandi *et al*., 2020).

The "gold standard" for establishing the correct taxonomic grouping of a strain with indeterminate characteristics or for proposing a new species is DNA-DNA hybridization. The genetic definition of a species can be

quantified using DNA-DNA reassociation kinetics, i.e., (i) ca. 70% DNA-DNA relatedness (Dueholm *et al*., 2021).

2.8. Aerobic Vaginitis Caused by *Enterococcus faecalis*

Enterococci are common gastrointestinal bacteria found in both humans and animals. Nosocomial infections, however, have recently been linked to vancomycin-resistant enterococci (Abdel-Gawad *et al*., 2021).

Aggregation substance *asa1*, gelatinase, cytolysin, enterococcal surface protein, and most recently hyaluronidase is just a few of the virulence factors described in enterococci.

Different *E. faecalis* strains' capacity to subvert host defenses and spread disease is due in part to their accumulation of virulence factors. Fimbriae (which aid bacterial adherence and invasion), iron-acquisition systems (which permit bacterial survival in the iron-limited environment of the urinary tract), flagella and toxins (which promote bacterial dissemination), and capsule (which enhances an infection's capacity to cause disease) are all examples of virulence factors that are located in virulence genes (Yalew *et al*., 2022).

Non-pathogenic strains can acquire additional virulence factors from accessory DNA because virulence genes are placed on transmissible genetic elements (plasmids) and/or the chromosome. Substances that provide significant pathogenicity to bacteria tend to aggregate among these virulence factors. *E. faecalis* exhibited a statistically significant prevalence of 86.51% for the virulence factor (*asa1*), which plays a crucial role in pathogenesis among clinical strains of enterococci (Kao and Kline, 2019).

According to a recent study (Kiruthiga *et al*., 2020), Plasmid-encoded *asa1* is a pheromone-inducible protein that promotes aggregation of

Enterococcus, which in turn facilitates the conjugative transfer of sex pheromone gene-containing plasmids and increases virulence (adherence to renal tubular cells, heart endocardial cells, and internalization by epithelial cells) and immune evasion (Inaba *et al*., 2019).

The most crucial role in aggregation formation is played by the chemicals known as extracellular polymeric substances (EPS), which are released by bacteria and photosynthetic microorganisms and increase adhesion between cells (Papadopoulos *et al*., 2020).

Different microbial strains and laboratory conditions result in distinct EPS due to the influence of numerous parameters, including nutrition availability, light population density, the presence of cations (primarily Ca2+ and Mg2+), temperature, and pH (Kao and Kline, 2019).

2.9. *Mycoplasma hominies* Infection

The *Mycoplasma* genus encompasses the smallest known members of the class Mollicutes. *Mycoplasma hominis* is a bacterium that lives in the urogenital system and is commonly associated with pelvic inflammatory diseases PID as well as postpartum and neonatal infections (Xiang and Lu, 2019).

In humans, infections caused by *M. hominis* outside of the genitourinary system uncommon.

To survive, they act as exterior parasites on the cells of humans, animals, birds, insects, and plants. Some organisms are able to live in their natural habitats in both land and sea environments. Seventeen species of human mycoplasmas have been identified since the first *Mycoplasma* was isolated from a human in 1937 from a Bartholin's gland abscess by Dienes and Edsall (Nuradilova *et al*., 2021).

Mycoplasma hominis, *Mycoplasma genitalium*, and *Ureaplasma urealyticum* are all examples of genital mycoplasmas, which are distinct sexually transmitted bacterial pathogens that can cause asymptomatic, long-term, and chronic infection in the genitourinary tract and hence pose a hazard to public health. Direct sexual contact, vertical transmission from mother to child, and tissue transplantation are all potential routes of transmission (Moridi *et al*., 2020).

Acute urethritis, bacterial vaginosis, pelvic inflammatory disease, and tubal infertility are only a few of the diseases that *M. hominis* has been linked to *Mycoplasma* or *Ureaplasma* infection, even if asymptomatic, may cause adverse pregnancy outcomes by stimulating pro-inflammatory immunological responses in the endometrium (Galyamina *et al*., 2022).

Some theories propose that strains that colonize the vagina but fail to invade may lack or have a defective form of a gene or genes important in amniotic cavity invasion (Paira *et al*., 2021).

A genetic identification of bacteria that can invade the amniotic cavity and induce preterm labor could lead to earlier detection and advances in medical treatment, making this study clinically significant. Understanding the involvement of this bacterium species in premature delivery may be aided by locating the genes responsible for invasion of the amniotic cavity (Moridi *et al*., 2020).

2.9.1. Properties of *Mycoplasma hominis*

The elusive and slow-growing nature of *M. hominis* made it difficult to identify it as a pathogen. The following explanations are possible:

M. hominis lacks a cell wall but instead has a 3-layer sterol membrane. Because of this, Mycoplasma spp. are often missed in clinical specimens because they cannot be identified with standard Gram staining.

Subcutaneous fluid and colony smears stained with gram stain and Wright-Giemsa showed no bacterial morphology in this case (Yacoub *et al*., 2021).

M. hominis takes a while to grow into tiny colonies on the media commonly used in the laboratory, and since a timely diagnosis is less likely because of the need for an extended incubation period, detecting it on plates can be difficult. Furthermore, conventional biochemical techniques might not be able to properly identify it (Paira *et al*., 2021).

In cases of suspected bacteremia, it can be challenging to detect the growth of *M. hominis* in standard blood culture bottle solutions because of the presence of polyanethol sulfonate, an anticoagulant. Instead, special methods for growth through automatic detection systems are required, and false-negative results are likely to be obtained (Galyamina *et al*., 2022).

In general, post-operative *M. hominis* infections are difficult to diagnose using conventional microbiological techniques. The likelihood of postoperative *Mycoplasma* infection may be under-diagnosed or reported due to the high urethral carriage rate of *M. hominis* (healthy individuals) and the prevalence of catheterization as a surgical technique (Xiang and Lu, 2019).

2.9.2. Survival Strategies of *Mycoplasma*

Mycoplasmas cause disease by first attaching to the epithelial mucosa membrane of the host cells, then colonizing the area, and finally causing necrotic death of the sub mucosal tissue. The severity of this pathogenesis is determined by the *Mycoplasma* species' capacity for tissue attachment and invasion. To this end, mycoplasmas have developed a specialized organelle called a cytoadherence tip (Galyamina *et al*., 2022).

However, surface-membrane lipoproteins can maintain the virulence of *Mycoplasma* species that lack the specific structure. Mucus membrane sulfatides and sialoglycoconjugates are targets for the lipoprotein ligands.

Though poorly characterized, the host-mycoplasma connection appears to be underpinned by a wide range of complicated molecular processes that, when activated, set off cytoadherence, immunomodulation, and pathogenicity (Paira *et al*., 2021).

Mycoplasmas, which have highly redundant minimum genomes, are able to adapt, persist, and live successfully in a wide variety of environments by evading and modulating the host's humoral immune response. Examining the host-mycoplasma interaction at the proteogenomic level is also necessary because of the connections between mycoplasma persistence and chronic inflammatory disorders in humans (Moridi *et al*., 2020).

2.10. The Immune System

The ability of a healthy woman with a fully functional immune system to carry a baby to term without immunological rejection is one of the most astounding elements of reproductive biology. This process is affected by immunoglobulins, cytokines, hormones, and other endometrial variables as well as local and systemic immunological responses. For implantation and later pregnancy to occur, a synergy of these elements is essential (Fu *et al*., 2021). The physiological immune responses that comprise the three different inflammatory stages of human pregnancy rely heavily on recognition of fetal antigens (Fuhler, 2020).

Implantation and early placentation are related with the first trimester. Endometrial erosion caused by the blastocyst is accompanied by a rise in pro-inflammatory Type 1 helper T cell (Th1) signaling and a local infiltration of natural killer cells (NK), macrophages (Mac), and dendritic cells (DC) (Kwak-Kim *et al*., 2022).

In the second stage, the equilibrium between Th1 and Th2 type signals is restored, resulting in a brief period of immunological homeostasis. This happens again during the second trimester and for the majority of the third. The cycle ends with a return to a pro-inflammatory microenvironment during the birthing process. During pregnancy in eutherian mammals, these three stages occur in sequence (Timothy *et al*., 2022).

In particular, Th2 cytokines [including interleukin (IL)-4, IL-10, and IL-13] are linked to a successful pregnancy, while Th1 cytokine responses [including interferon (IFN)-, and tumour necrosis factor (TNF)-] are more common in spontaneous miscarriage (Fuhler, 2020) .

Tolerance is a key component of an effective immune response, which is essential for fetal survival. When immune cells are partially dysfunctional and aberrant cytokine release occurs, the immunological tolerance of the maternal-fetal interface is broken, and a spontaneous abortion occurs. Infection of the fetus or embryo is responsible for 10%-15% of abortions (Fan *et al*., 2020).

2.11. Natural Killer Cell

Natural killer (NK) cells are a vital component of both innate and adaptive immunity because they are essential cytotoxic cells. NK cells target and destroy cancer cells while leaving healthy ones alone. Furthermore, once activated, NK cells generate a variety of cytokines and chemokines that might enhance the body's natural defenses against tumors (Wu *et al*., 2017). NK cells detect major histocompatibility complex (MHC)-I molecules on target cells by combining with activating and inhibitory receptors to form killer immunoglobulin-like receptors (KIRs) (Kwak-Kim *et al*., 2022).

NK cells are beneficial to humans because they regulate adaptive immune responses. Early on in the course of an infection or damage, as well as in the presence of tumors and viruses, NK cells are upregulated and eliminate cancer cells and infected viral cells without the need for stimulation or immunity (Fan *et al*., 2020 ; Fu *et al*., 2021).

Multiple cytokines, including interleukin (IL-3, IL-10, IL-12 and IL-18) influence NK cell formation and functional maturation (Wu *et al*., 2017 ; Wang *et al*., 2018). While transforming growth factor (TGF) and IL-15 largely govern regulatory NK cells that reside in the uterus, IL-15, IL-18, and IL-21 can maintain a cytotoxic state of NK cells in the peripheral blood to combat infections (Guerrero *et al*., 2020).

Transduction of target cell signals to NK cells is complicated and requires a shift in the ratio of activating to inhibitory signals. Multiple types of inhibitory and activating receptors are encoded in the germ line and control NK cell activity (Wang *et al*., 2018; Mahajan *et al*., 2022).

Two significant NK subsets (Cluster of Differentiation) are CD56 bright CD16 and CD56 dim CD16 (Ietta *et al*., 2018). These two subsets are very functionally distinct from each other. Instead of participating in cytotoxic activities, CD56bright NK cells regulate the immune response strategy by the release of cytokines, whereas CD56dim cells release substantially more perforin and granzyme, causing strong cytolysis and expression of Killer-Cell Immunoglobulin-Like Receptor KIRs (Fan *et al*., 2020; Rougang *et al*., 2023; Salazar *et al*., 2023).

Pregnancy-related NK cells are frequently CD56-bright (Wang *et al*., 2018).

However, NK cells and macrophages make up the bulk of the immune cells at the placenta-fetal interface. These cells' known functions include stimulating trophoblast recruitment, modifying the spiral artery and regulating local immunological activity (Obais and Alsultany, 2021; Fu *et al*., 2021).

NK cells play crucial roles throughout pregnancy and are attracted and stimulated by ovarian hormones. NK cells produce up to 50–70% of decidua lymphocytes during the first trimester (Zhang & Wei 2021 ; Salazar *et al*., 2023).

Uterine natural killer (uNK) cells, which are part of the innate immune system, make up roughly 70% of total lymphocytes and play an essential role in the development and maintenance of the pregnancy. In order to shed some light on the connection between uNK cells and RSA (Fan *et al*., 2020 ; Von Woon *et al*., 2022). uNK cells are latent in the endometrium even when it is not pregnant. Progesterone, in particular, affects the number of these cells in the mother's body. The endometrium changes into the gestational decidua after conception (Elagab *et al*., 2022).

uNK are involved in placentation, a process that begins after embryo implantation and facilitates the passage of nutrients and oxygen between mother and child by remodeling the spiral arteries (Von Woon *et al*., 2022; Fu *et al*., 2021).

Decidual natural killer (dNK) cells differ from peripheral-blood NK cells in that they are less cytolytic and release cytokines and chemokines that promote trophoblast attack, tissue remodeling, fetal growth, and placentation Decidualization is aided by the presence of an abundance of NK cells at sites of implantation (El-Badawy *et al*., 2020 ; Fu *et al*., 2021).

Also dNK cells play an active role in SA remodeling in human illness. Patients with pre-eclampsia and intra-uterine growth restriction (IUGR), which are linked to insufficient remodeling of SAs and restricted trophoblast invasion in the decidua, have been shown to have fewer dNK cells. Additionally, NK can help the fetus grow and develop, and a lack of growth factors causes growth restriction in the progeny by causing incorrect bone development (Wang *et al*., 2018, Donskoi *et al*., 2022 ; Rougang *et al*., 2023).

During the first three months of pregnancy, dNK cells, which are found near the trophoblast cells and the spiral arteries, are abundant. Then, during the second trimester, their numbers begin to decrease, and by the time of delivery, they are completely gone (Mahajan *et al*., 2022 ; Crespo *et al*., 2022).

In addition to pNK cells, "tissue-resident NK" (trNK) cells are also present in peripheral tissues in humans, including the liver, lungs, skin, and uterus. A subset of CD56-bright NK cells forms the majority of trNK cells. When compared to pNK cells and trNK cells, decidual NK (dNK) cells are a specialized subset of trNK cell present in endometrial decidual tissue. These cells exhibit many distinctive morphological and functional traits (Zhang and Wei 2021 ; Salazar *et al*., 2023).

When exposed to pathogens during pregnancy in the uterus, NK cells can also change their identity to become cytotoxic and perform immunological defense. Premature NK cell activation during late gestation can result in preterm labor, recurrent spontaneous abortion, uterine cancer, and recurrent implantation failure by breaking down the maternal-fetal interface's tolerance (Wang *et al*., 2018 ; Zhang and Wei 2021).

Patients experiencing infertility are increasingly having their peripheral NK cells tested as a diagnostic tool to direct the start of treatment. However, there is increasing confirmation that uNK cells develop from pNK cells that have been transported to the uterus and activated there. NK cell evaluation has yet to be validated as a diagnostic tool for female infertility, or RSA; hence, more study is required (Salazar *et al*., 2022 ; Elagab *et al*., 2022).

However, it is believed that a deficiency of immunological tolerance, which is a state in which NK cells may play a significant role, is the primary cause of seemingly unexplainable SA (Guerrero *et al*., 2020 ; Rougang *et al*., 2023). The initial report by Aoki suggested that elevated pNK activity could lead to RSA (Wang *et al*., 2018).

Currently, pNK cells are employed as an indicator for determining the initiation of therapy for individuals with infertility (Toth *et al*., 2019 ; Dons'koi *et al*., 2022).

Chapter Four
Results and Discussion

4. Results and Discussion

4.1. Distribution of Age Group for Aborted and Healthy Women

The ages of the aborted and healthy women ranged between 16 and 45 years. The highest rate of SA was in the age group of 26–35 years (46%).

According to the results of the current study, there was no significant effect of age group on abortion rates. $P \leq 0.05$ compared with healthy women, as shown in Table (4-1) and Figure (4-1).

Table (4-1): Age Distribution of Study Subject

Sample type	Age group (year)				*P value* (P ≤ 0.05).
	16-25	26-35	36-45	Total	
Patients	15 (30 %)	23 (46%)	12(24 %)	50	.836^{NS}
Controls	8 (26.7 %)	13 (43.3%)	9 (30%)	30	.837^{NS}
Total	23(28.75%)	36 (45%)	21(26.25 %)	80 (100%)	.588^{NS}
NS: Non-significant difference					

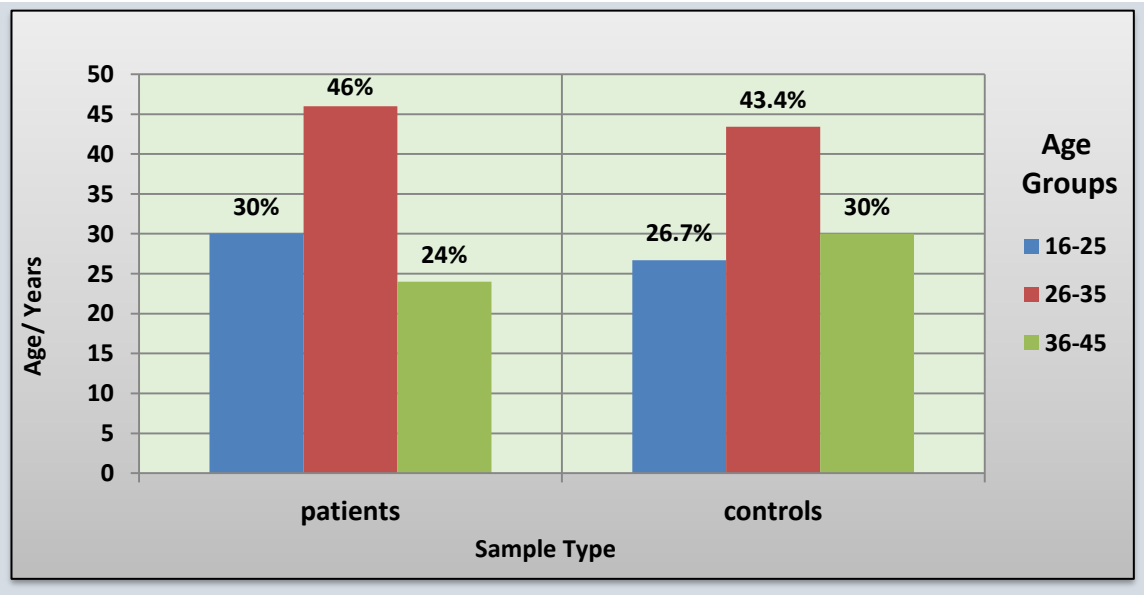


Figure (4-1): Distribution of Cases According to Age Group

This scheme shows the increase in the number of women who got abortion in the age group of 26-35 years, this is due to the reproductive age of women increases at this category.

In a study similar to this one, the majority of the patients with RSA were between 26 and 35 years old (56%) (Anyanwu and Titilope, 2021).

Both parents' ages play a role, with those aged 35 and up having a higher probability of their child having a negative pregnancy outcome (Kuon *et al*., 2017).

4.3. Distribution of Blood Group for Aborted and Healthy Women

The ABO system was another criteria included in this study. Women with SA tend to have a higher prevalence of blood type O+ (42%), as seen in Table (4-2) and Figure (4-2).

Table (4-2): Blood Group Distribution of Study Subject

Sample type	Blood Group				
	A+	B+	AB+	O+	O-
Patients N0=50	10 (20%)	15 (30%)	2 (4%)	21 (42%)	2 (4%)
Controls N0=30	6 (20%)	11 (36.7%)	3 (10%)	9 (30%)	1 (3%)

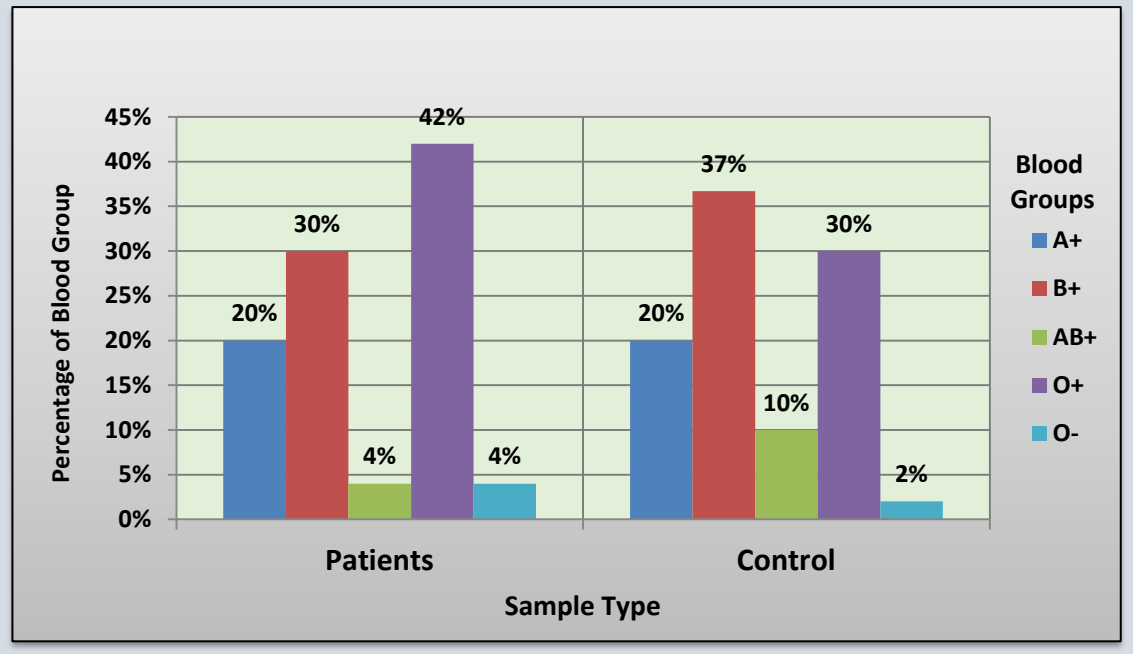


Figure (4-2): Distribution of Cases According to Blood Group

In consistent research, the prevalence of O-type mothers who experience a spontaneous abortion is greater than in the overall population of British Columbia (44.5%), if maternal-fetal ABO incompatibility does play a role in this event (Hassanzadeh *et al*., 2012),.

The ABO blood group frequencies was calculated among Ethiopia's expectant mothers. Women in those situations have adverse pregnancy outcomes were most type O blood group, accounting for 39.9% of all the ABO system (Ayenew, 2021).

4.3. Bacterial Isolation and Identification

The present study has been done on 100 High vaginal swabs (HVS) from women only (50 patients with SA and 50 with a healthy pregnancy outcome). The patients and controls were all between the ages of 17 and 45 years. These specimens were taken from women who attended the Babylon Teaching Hospital for Maternity and Children and the Imam Sadiq Teaching Hospital in Babylon, Iraq.

The primary diagnosis of bacterial isolates, which depended on the color changes in UTIC agar in addition to microscopic and morphological characterization, was supported by a genetic study using 16S rRNA PCR and sequencing. A specified number of bacteria that were frequently detected was chosen, therefore the total number of samples sent to the sequencing was 20 (14 from patients, 6 from controls) as shown in Table 4-3.

Table 4-3: Number and Type of Bacteria that PCR and Sequencing were performed

Expected Sample	NO. of Expected Samples	Confirmed Bacteria After Sequenced	No. of confirmed Bacteria
Enterococcus faecalis	7	*Enterococcus faecalis*	6
		Enterococcus gallinarum	1
Escherichia coli/ Proteus	5	*Escherichia coli*	5
Klebsiella pneumonia	3	*Klebsiella pneumonia*	3
Staphylococcus epidermidis	2	*Staphylococcus epidermidis*	2
Ambiguous bacteria	3	*Acinetobacter junii*	1
		Corynebacterium coyleae	1
		Metabacillus niabensis	1

The current study results show a difference in bacterial genera in women with SA compared with healthy women, and it was noted that embroilment of *Enterococcus faecalis occurred* in most cases of SA with an estimated percentage of 56% (28/50), thus defeating *Escherichia coli* by 32% (16/50) and 4% (2/50) for *Klebsiella pneumonia* and 4% (2/50) for

Enterococcus gallinarum. In this study, very rare bacteria species were identified, including *Acinetobacter junii* at 2% (1/50) and *Corynebacterium coyleae* at 2% (1/50), While the percentage of bacteria associated with healthy women was: 30% (15/50) for *E. faecalis*, 26% (13/50) for *E. coli*, 18% (9/50) for *K. pneumonia*, *24*% (12/50) for *Staphylococcus epidermidis*, and 2% (1/50) for *Metabacillus niabensis* as shown Figure (4-3).

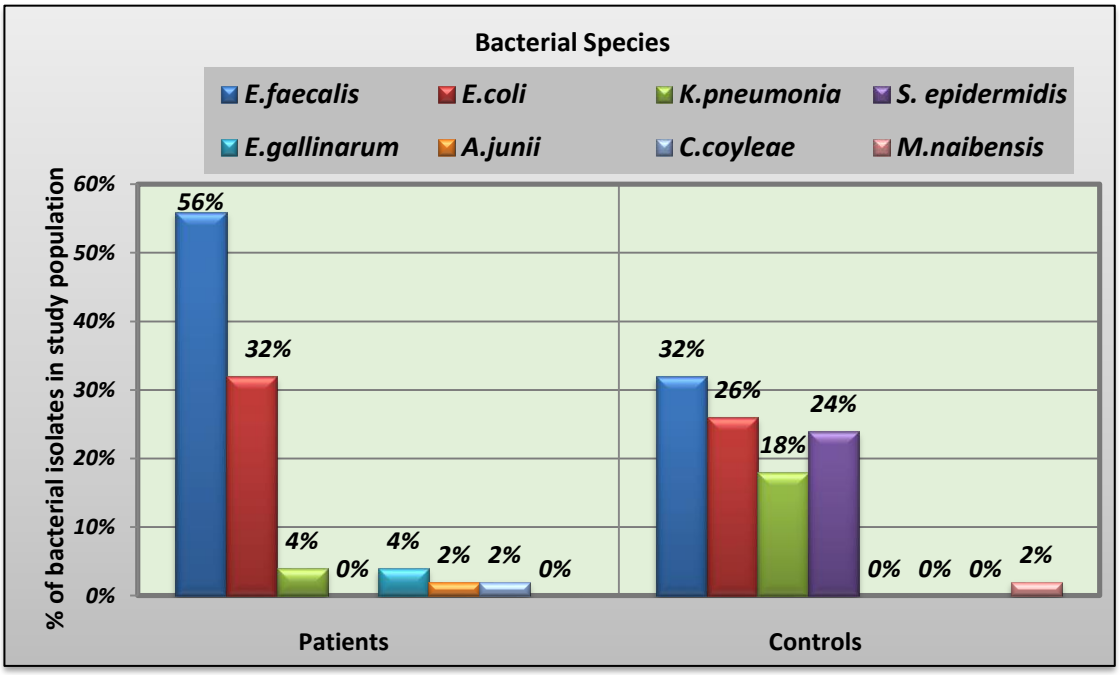


Figure (4-3): Type of Bacterial Isolates in SA Women and Study Control.

During pregnancy, particularly in the second trimester, the presence of pathogenic bacteria in the vagina can cause severe inflammation, which can ultimately lead to the rupture of the pregnancy sac and subsequent pain and bleeding. Infections with bacteria including *Enterococcus faecalis*, *Escherichia coli*, and *Klebsiella pneumonia* have been related to SA the most often. Women who have BV and become pregnant early are more likely to lose the pregnancy (Inaba *et al*., 2019; Jiao *et al*., 2022).

Amniotic fluid is typically sterile and free of microorganisms. Nonetheless, the amniotic cavity can be invaded by germs in a number of ways; the most typical route is up the mother's genitalia. Bacteria in the amniotic cavity, which can be detected by molecular microbiologic methods or culturing, may cause a local inflammatory reaction. Intra-amniotic infection describes this situation. Preterm birth, the development of acute histopathological chorioamnionitis and funisitis, and an embryonic inflammatory response are all adverse pregnancy outcomes that have been linked to intra amniotic infection. (Jung *et al*., 2021).

According to the molecular findings of a recent study, *Enterococcus faecalis* superior to all other species of bacteria identified in this research. When the swabs were cultured in the media, the number of Enterococcus bacterial colonies was found to be larger and more dense in patients and few and scattered in control samples. Perhaps the number and aggressiveness of these bacteria contributed to the abortion. Some strains of enterococci have gained in importance as infection-causing agents in addition to their well-known pathogenic potential, making *Enterococcus faecalis* an increasingly common opportunistic pathogen due to rising antibiotic resistance (Kao and Kline, 2019).

Due to its superior adaption to environments with abundant nutrients, low oxygen levels, and a complex habitat, *E. faecalis* is able to persist in crucial conditions with insufficient nutrition provision and an elevated pH level ranging up to 11.5. For enterococci to cause disease in a host, they must first be able to colonize the host's tissues, then outwit the host's non-specific and immune defenses, and last cause infection (Alghamdi and Shakir, 2020).

Evidence from adhesion studies shows that enterococci can colonize host tissues by adhering to epithelial cells of the gastrointestinal tract, urinary tract, and heart (Sangeetha, 2014).

About 32%, *E. faecalis* is the most commonly isolated pathogen in patients with aerobic vaginosis. As a result of their harmful actions, aerobic bacteria like *E. faecalis* have been linked to spontaneous abortions (Sangeetha, 2014; Redelinghuys *et al*., 2020 ; Jahić and Cerovac, 2022).

Another bacterial species recovered from women with SA was *Escherichia coli*. Aerobic vaginitis often has *E. coli* as a source of infection. Some enterobacteria have been shown to function as uropathogens and be associated with cases of bacterial vaginosis because of their close proximity to the "anorectal/vaginal" zone (Mohammed and Ibrahim, 2022).

Women with a history of UTI have higher levels of *E. coli* colonization in their vaginal introitus (>105 CFU/mL), highlighting the importance of the vaginal milieu in the pathogenesis of recurrent UTI (Dominoni *et al*., 2023).

Both bacterial vaginosis (BV) and aerobic vaginosis (AV) are vaginal dysbioses characterized by a reduction in lactobacilli and associated with chorioamnionitis, preterm delivery, spontaneous abortion, and low birth weight (Nunez *et al*., 2023). A local study by al Juber and Hammoudi (2014) found that 14.8% of *K. pneumonia* and *Klebsiella spp*. were implicated in both AV and BV (Torrone *et al*., 2018; Yalew *et al*., 2022).

Similar research has found that pregnant women with gestational diabetes have a greater capacity to become infected during their pregnancies. *Enterococcus gallinarum* accounted for 23 (28.39%) of the germs isolated from healthy women and women with genital tract

infections. The risks of pathogenic bacterial infection are discussed, as are the effects of vaginal infections on such birth complications as premature birth, membrane rupture, and placental infections (Al-Wandawy *et al*., 2020 ; Şahin and Temtek, 2022).

Since it is unusual to identify *Corynebacterium coyleae* in connection with newborn illnesses, it was believed that this bacterium represented commensal strains and was therefore found. *Corynebacterium coyleae* is a member of the symbiotic microbiota of the urinary tract, mucosal membranes, genital tract, skin, etc. (Akwuobu *et al*., 2023).

The infectious potential of this species continues to be researched. In 1997, it was first discovered by Funke *et al*. using blood cultures from six individuals who had episodes of fever with no clear cause. *C. coyleae* has also been used to treat cases of verified sepsis, probable sepsis, a soft-tissue infection, suspected post-transfusion bacteremia, neonatal bacteremia, burn injuries, pleural fluid samples, abscess formation, and ulceration. Current disagreement surrounds its formal pathogen status (Sokol-Leszczynska *et al*., 2019).

Gram-positive rods that are non-spore-forming, non-acid fast, straight to slightly curved, and often concave at the ends, but can also be club-shaped or ellipsoidal, and arranged in angular or columnar patterns. The exact details of this species are not yet revealed. It is unclear how or why certain species of *Corynebacterium* play a role in the aetiology of human diseases (Barberis *et al*., 2018; Akwuobu *et al*., 2023).

There is clearly a lack of differentiation between colonization and an infectious condition. Although *Corynebacterium coyleae* is rarely isolated, it continues to be considered a pathogen because it can cause complicated

urinary tract infections in women. However, there is little published data from clinically relevant samples (Barberis *et al*., 2018).

Acinetobacter junii, likewise considered a rare species and presumed to be an uncommon form of human-infected bacteria, was recovered from SA women in a similar study to the one presented here. A microscopic examination of a gram-stained smear revealed the presence of small gram-negative cocci. The bacterium *Acinetobacter* can be found virtually anywhere on earth, food, water, garbage, and soil are just some of the places where it can be extracted. It is important to note that, while *Acinetobacter* is typically a non-pathogenic organism, it can cause fatal infections in critically ill people (Yang *et al*., 2019).

It is found on the skin of roughly 25% of healthy people; however, it only rarely acts. In a retrospective study, colonization of the gastrointestinal system was found in both infants and adults (77% of 73 cases). In humans, *Acinetobacter* can be found in the microflora of the mouth, nose, and genitalia. If Acinetobacter is allowed to colonize the genitourinary tract, it could potentially spread to other organs, including the lungs. This behaviour is known as "translocation" (Sarshar *et al*., 2021).

A polysaccharide capsule is found on some *Acinetobacter* strains, so it is usually an indicator of a high level of pathogenicity. Bacteria are protected from the host cell's immune system by the capsule, which is made of polysaccharides and polypeptides (Yang *et al*., 2019). It also facilitates bacterial attachment to surfaces, both living and nonliving. In addition, the polysaccharide capsule stops microorganisms from drying up. Colonies from an encapsulated strain of Acinetobacter are mucoid and lack pigmentation (Sarshar *et al*., 2021).

Related research indicated that *A. junii* was the second most prevalent microbe detected (n = 14, out of a total of 79) in amniotic fluid samples taken from women who did not have intra-amniotic inflammation (Jung *et al*., 2021).

In addition, healthy pregnant women (controls) are often found to have the commensal bacterium *Staphylococcus epidermidis*. Once assumed to be harmless, *S. epidermidis* is now recognized as a major opportunistic pathogen. It keeps things under control, improves the skin's immune system, and prevents disease-causing opportunistic bacteria by means of colonization resistance (Severn and Horswill, 2023).

Staphylococcus epidermidis has been discovered as one of the most important species in this category. This gram-positive, facultatively anaerobic, non-spore-forming, non-motile, catalase-positive, coagulase-negative bacterium frequently causes nosocomial and hospital-acquired infections. *S. epidermidis* strains isolated from human clinical infections carry virulence features from accessory gene regulator (Agr) classes I, II, and III (Noshak *et al*., 2023).

Using clinical samples, specifically from the vagina, this study identified *Metabacillus niabensis* for the first time anywhere in the world, including in Iraq. The isolated *Metabacillus* species employed in this work will aid in further studies of this genus and will increase its taxonomic diversity (Kangale *et al*., 2021).

M. niabensis is an aerobic, gram-negative, motile bacterium that thrives at temperatures between 15 and 40 ℃. In addition to being catalase, beta-galactosidase, and oxidase-positive, the major fatty acid found in *Metabacillus niabensis* is 12-methyl-tetradecanoic acid (Da Costa *et al*., 2022).

Molecular methods utilized for the study of *Bacillus* have led to important new discoveries in the genus's taxonomy. Numerous species have recently been reclassified, entering new genera, leaving almost only *Bacillus* cereus group species in the genus *Bacillus*. Only by sequencing the 16S rRNA gene can these closely related species be distinguished phylogenetically from one another (Hwang *et al*., 2022).

Even though *Bacillus* and related genera have a cell wall characteristic of Gram-positive bacteria, they can stain as Gram-positive (in early cultures), Gram-variable (between Gram-positive and Gram-negative), or Gram-negative. *Bacillus* and related genera may develop spores that are resistant to high and low temperatures and common sanitizers, allowing them to survive in a wide range of circumstances for long periods of time (Reichart *et al*., 2023).

Traditional methods of identifying *Bacillus* and related taxa are extremely challenging due to the high degree of resemblance between closely related species on morphological, biochemical, and genetic levels. Apolyphasic strategies, which combine phenotypic and genotypic techniques, allow for the discovery of novel species and a more precise analysis of the taxonomic and evolutionary relationships among the members of this group (Kangale *et al*., 2021).

Some of these methods of identification include databases (DB) that are more narrowly focused on medicinally essential bacteria than on the microbiota associated with drugs. These organisms are often ecological pioneers, but their metabolic patterns are not always represented in the DB due to the wide variation of their physiologies and nutritional needs (Da Costa *et al*., 2022).

4.4. The Diagnostic Characteristic of Vaginal Microbiota

Bacterial isolates' principal diagnoses, as determined by color changes on UTIC agar in addition to microscopic examination, morphological characterization, and biochemical tests, are tabulated below in Table (4-4).

Table (4-4): Diagnostic Properties of Bacterial Isolates in This Research

Bacteria sp.	Gram stain	Catalase /Oxidase	Hemolytic reaction on Blood agar	Culture Properties under Microscope and in Different Agar Media
Enterococcus faecalis	Gram positive	Negative/Negative	β-hemolysis	Round or ovoid cells that have formed pairs or chains . UTIC/ green or a blue – turquoise.
Escherichia Coli	Gram negative	Positive/ Negative	hemolysis-α, β, γ	UTIC/ Pink colonies, rods shape. EMB/ green metallic sheen. MacConkey/ take pink color
Klebsiella pneumonia	Gram negative	Positive/ Negative	γ-hemolysis	UTIC /Colonies have mucoid form and a dark blue to purple. EMB/ pink color. MacConkey/ pink, mucoid larger size than *E. coli*

Staphylococcus epidermidis	Gram positive	Positive/ Negative	γ-hemolysis	Its colorless to yellow colonies on UTIC, typically shaped like a bunch of grapes.
Enterococcus gallinarum	Gram positive	Negative/Negative	β-hemolysis	Round or ovoid cells clustered in pairs or chains; colonies on UTIC are a vibrant turquoise.
Acinetobacter junii	Gram negative	Positive/Negative	γ-hemolysis	It forms colonies that are soft, sometimes mucoid, pale yellow to greyish white, and of the cocco-bacillary pattern.
Corynebacterium coyleae	Gram positive	Positive/Negative	β-hemolysis	They cluster together in tiny, granular grey colonies that have opaque centers, are curved, and have smooth edges.
Metabacillus niabensis	Gram negative	Positive/Positive	α hemolysis	round, with a moist top and clear edges; UTIC agar showed white to yellow colors.

It must provide adequate nutrition for bacterial cultures, especially those cultivated on blood agar. Hemolysis due to bacterial growth is one of blood agar's principal uses, and it can be utilized for organism identification. Hemolytic extracellular enzymes produced by pathogenic organisms are most commonly cultured on blood agar (Russell *et al*., 2006 and Zhao *et al*., 2023).

The basic components of UTI chromogenic media, such as peptone mixture, tryptophan, chromogenic substrate, and growth factors (Jiao *et al*., 2022). The most common uropathogens were species of *Escherichia coli, Klebsiella*, *Enterococcus*, and *Eneterobacter*. Even in polymicrobial cultures, the distinct colony colors produced by the bacteria's individual enzymes degrading the chromogenic substrate were useful for a preliminary identification (Maganga, 2019).

The use of chromogenic agar media is standard practice for isolating and identifying *E. coli*, *Staphylococcus* and *Enterococci*. This primary screening medium is easy to use, so it can cut down on routine work and identification tests (Mishra *et al*., 2020).

MacConkey media; due to bile salts and crystal violet, this medium (which is selective) favors gram-negative bacteria and inhibits gram-positive bacteria. An acid-sensitive characteristic, and lactose, a sugar, may be present in this differential medium to differentiate gram-negative bacteria. Colonies of lactose-fermenting organisms appear to be pink. After lactose fermentation, the pH lowers because of the presence of acidic byproducts, and the indicator changes color to pink as in *E. coli* and *Klebsiella* with large, mucus pink color. In non-lactose fermenters, Absence of lactose fermentation results in no change in pH which generate colonies from uncolored bacterial cells (Supriatin *et al*., 2021).

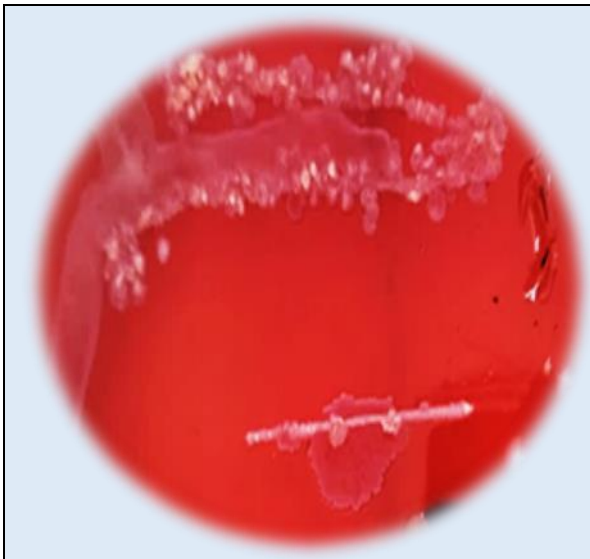
Eosin methylene blue (EMB) agar is also a selective and differential medium made of peptone, lactose, sucrose, and dyes. EMB agar promotes gram-negative bacteria. Methylene blue inhibits most gram-positive bacteria. pH-sensitive eosin turns black in acidic situations. In EMB agar

media, lactose and sucrose provide energy. The medium's carbohydrates stimulate gram-negative bacteria. The ability of enteric bacteria to break down lactose and sucrose on EMB agar can help identify them. Lactose-fermenting gram-negative bacteria (mainly enteric) acidify the medium, causing the dyes to form a dark purple complex with a green metallic sheen, as in *E. coli*, but *Klebsiella* colonies look pink (Gazel *et al*., 2019).

Mannitole Salt Agar (MSA) medium distinguishes *Staphylococcus* sp. that ferment Mannitole. Agar color change indicates mannitole fermentation. Standard MSA medium uses phenol red, which turns yellow in acid pH. This medium isolates, counts, and differentiates pathogenic staphylococci. Salt-tolerant *Enterococcus* spp. can also grow on MSA (Virgianti, and Suhartati, 2020).

De Man, Rogosa and Sharpe (MRS) agar is a laboratory medium designed to rapidly multiply *Lactobacillus*. Sodium acetate inhibits most competitor microorganisms. Clinics rarely employ this test since milky acid bacteria rarely cause disease (Taye *et al*., 2021).

Lactobacillus was not isolated from the HVS cultured on media from patients and controls, since the patient may have taken antibiotics that reduced the number of *Lactobacillus* in the vagina. Endogenous hormones like progesterone and estrogen have an impact on the *Lactobacillus* population in the vagina of pregnant women. These hormones cause the concentration of *Lactobacillus* to change and fall as the gestational period nears delivery (Kindinger *et al*., 2017).

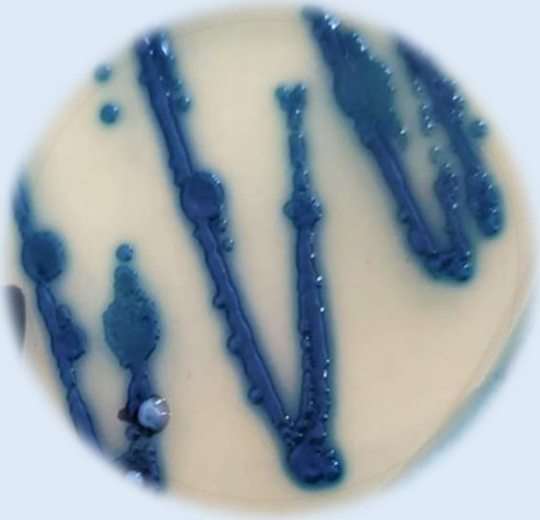


A. Acinetobacter *junii*

In Blood Agar

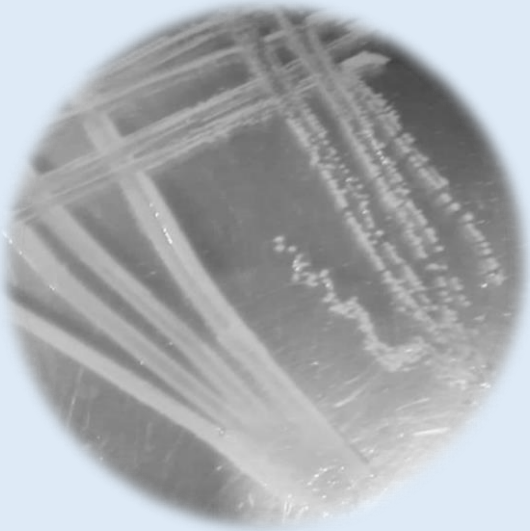
B. Metabacillus *niabensis*

In Blood Agar



C. *Enterococcus gallinarum*

In UTI Agar



D. *Corynebacterium coyleae*

In Nutrient Agar

Figure (4-4): Colonies of A Unique Isolate Growing in Different Type of Agars.

4.5. Genotyping Assay

4.5.1. Agarose Gel Electrophoresis for Bacterial Genomic DNA

The genomic DNA were extracted by Favrogene Kit and used as template for 16S rRNA and *asa1*primers, each genomic DNA samples was checked to evaluate the efficiency of extraction method by agarose gel electrophoresis, at concentration 1%, as shown in Figure (4-5).

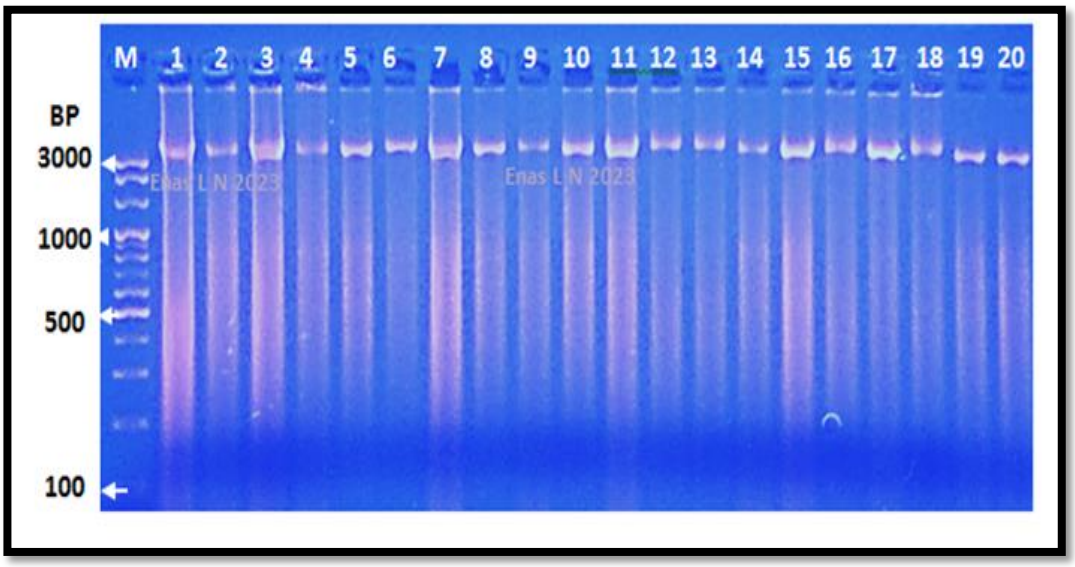


Figure (4-5): 1% Agarose Gel Electrophoresis of Bacterial DNA at 100 Volt for 45 min. PCR Product Visualized Under U.V Light at 280nm. After staining with Ethidium Bromide, M= Molecular Marker (100-3000bp), 1-20 Bacterial Isolates.

The extraction of these samples shown high quality of DNA appeared in bright bands.

4.5.2. The Amplification of 16S rRNA Gene by PCR

The local sample was included by using a locus-specific primer (16S rRNA) to generate amplicons of approximately 938 base pairs in length. The 938 bp region of the 16S rRNA gene was amplified for diagnosis of bacteria by PCR using the isolated bacterial genomic DNA as a template.

Each ribosomal amplicon was checked to make sure it had distinct bands before it was sent for sequencing, as shown in Figure (4-6).

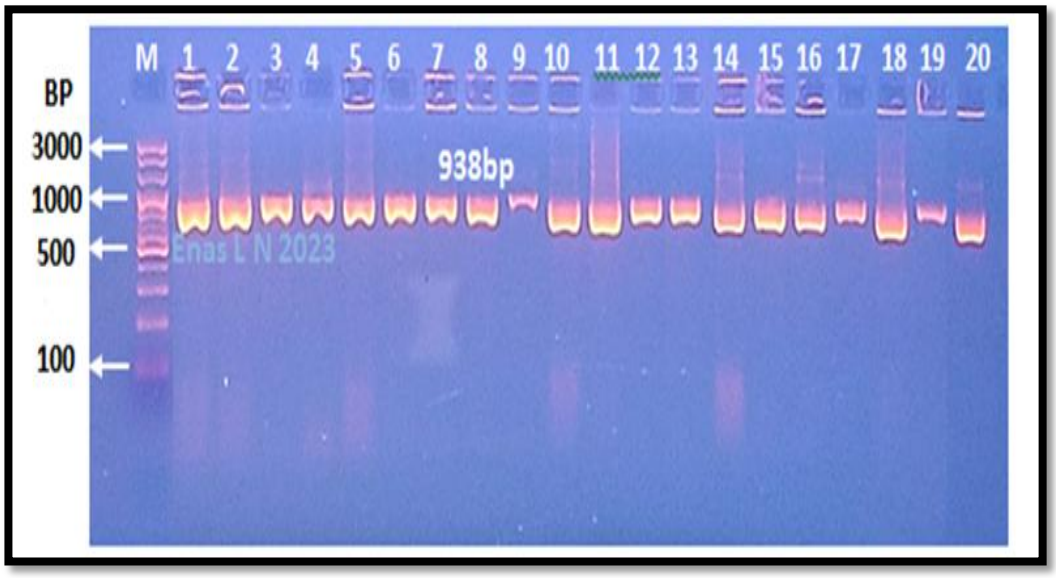


Figure (4-6): Gel Electrophoresis for PCR Products of 16S rRNA Gene (938 bp.) for (1-20) with DNA Ladder 100 bp. (M), Visualized Under U.V light at 280nm. After Staining with Ethidium Bromide, on Agarose Gel 1.5% in 70 Volt, 1 hour.

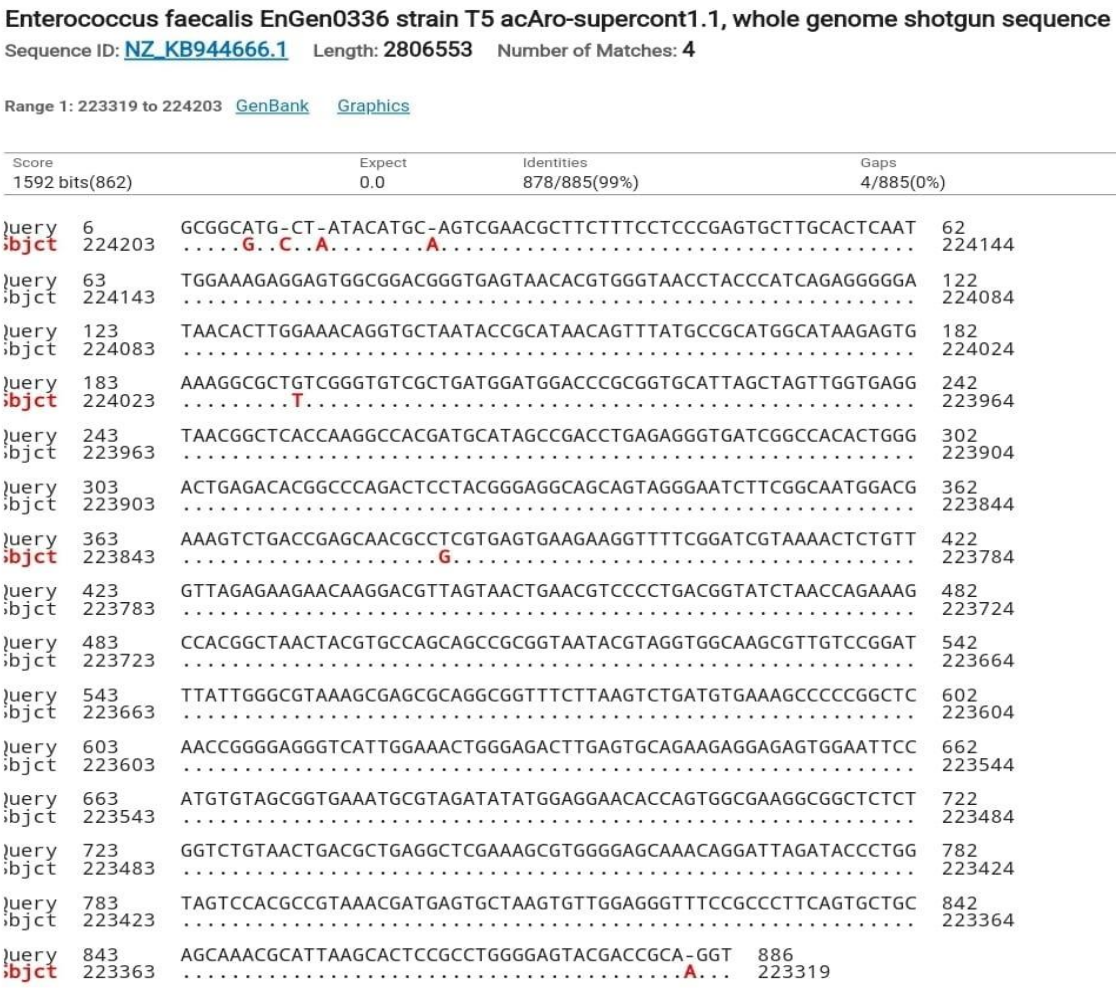
4.5.3. 16S rRNA Gene Sequencing Results

The confirmed identification of the amplified products was shown by the sequencing reactions using NCBI Blast. In regards to the 938-bp PCR amplicons of the ribosomal gene, the sequenced samples and their targets showed complete sequences of similarities, according to analysis by the NCBI BLASTNn search engine.

Nucleotide composition analyses were compared to those derived using the more standard alignment-based method **(***Kiruthiga et al*., 2020).

4.5.3.1. DNA Sequencing for *Enterococcus faecalis*

The sequencing result of *E. faecalis*, which has 99% identity with the subject of (*Enterococcus faecalis* EnGen0336 strain T5 acAro-supercont1.1) in NCBI under Accession number (NZ_KB944666.1), showed 1 transition (A/G) and 2 transversion (G/T and T/G) when compared Query with Subject, as shown in Figures 4–7. Query: represent DNA of the samples, while Subject: represent DNA of the NCBI database.



Enterococcus faecalis EnGen0336 strain T5 acAro-supercont1.1, whole genome shotgun sequence

Sequence ID: NZ_KB944666.1 Length: 2806553 Number of Matches: 4

Range 1: 223319 to 224203 GenBank Graphics

Score	Expect	Identities	Gaps
1592 bits(862)	0.0	878/885(99%)	4/885(0%)

```
Query  6       GCGGCATG-CT-ATACATGC-AGTCGAACGCTTCTTTCCTCCCGAGTGCTTGCACTCAAT  62
Sbjct  224203  .....G..C..A........A.....................................  224144

Query  63      TGGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGGGGA  122
Sbjct  224143  ............................................................  224084

Query  123     TAACACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTG  182
Sbjct  224083  ............................................................  224024

Query  183     AAAGGCGCTGTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGG  242
Sbjct  224023  .........T..................................................  223964

Query  243     TAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGG  302
Sbjct  223963  ............................................................  223904

Query  303     ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACG  362
Sbjct  223903  ............................................................  223844

Query  363     AAAGTCTGACCGAGCAACGCCTCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT  422
Sbjct  223843  ....................G.....................................  223784

Query  423     GTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACCAGAAAG  482
Sbjct  223783  ............................................................  223724

Query  483     CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAT  542
Sbjct  223723  ............................................................  223664

Query  543     TTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC  602
Sbjct  223663  ............................................................  223604

Query  603     AACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCC  662
Sbjct  223603  ............................................................  223544

Query  663     ATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCT  722
Sbjct  223543  ............................................................  223484

Query  723     GGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG  782
Sbjct  223483  ............................................................  223424

Query  783     TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGC  842
Sbjct  223423  ............................................................  223364

Query  843     AGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCA-GGT  886
Sbjct  223363  ....................................A...  223319
```

Figure (4-7): Alignment Analysis of *E. faecalis* with Gene Bank at NCBI

The other *E. faecalis* isolates compared to subject of NCBI in the same method.

4.5.3.2. DNA Sequencing for *Escherichia coli*

The sequencing result of *E. coli* which having 99 % identity with the subject of (*Escherichia coli* O157:H7 str. Sakai DNA) in NCBI under Accession number (NC_002695.2), there were only 1 transition C/T, when compared Query with Subject, as shown in Figure (4-8).

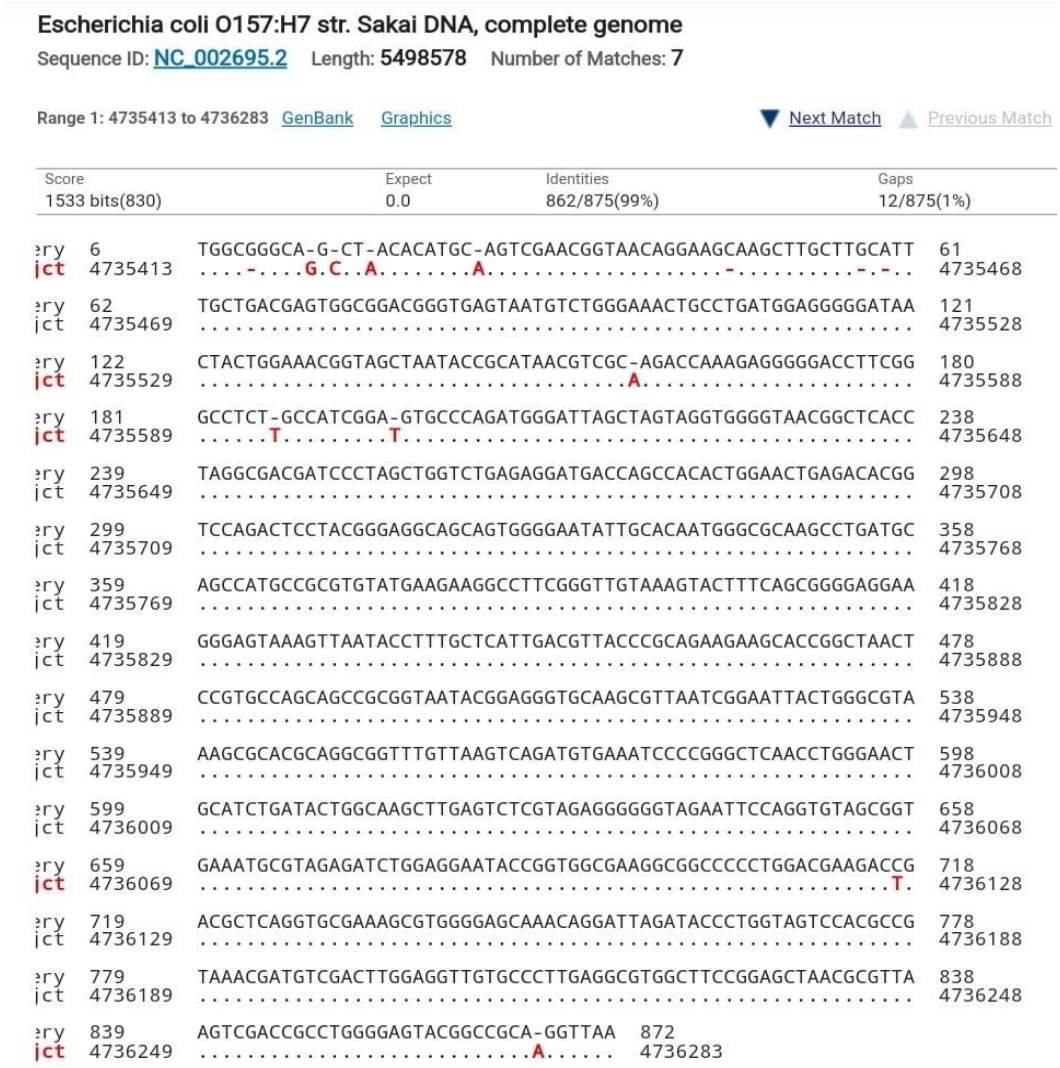


Figure (4-8): Alignment Analysis of *E. coli* with Gene Bank at NCBI

The other *E. coli* isolates compared to subject of NCBI in the same method**.**

4.5.3.3. DNA Sequencing for *Corynebacterium coyleae*

The sequencing result of *C. coyleae*, which has 98% identity with the subject of (*Corynebacterium coyleae* strain DSM 44184) in NCBI under Accession Number (NZ_FNRUO1000002.1), showed 3 transitions (C/T) and 3 transversion (G/T, G/T, and T/G) when compared Query with Subject, as shown in Figure 4-9. The ID of this query sample is OQ920556.1, after it was registered in the GenBank.

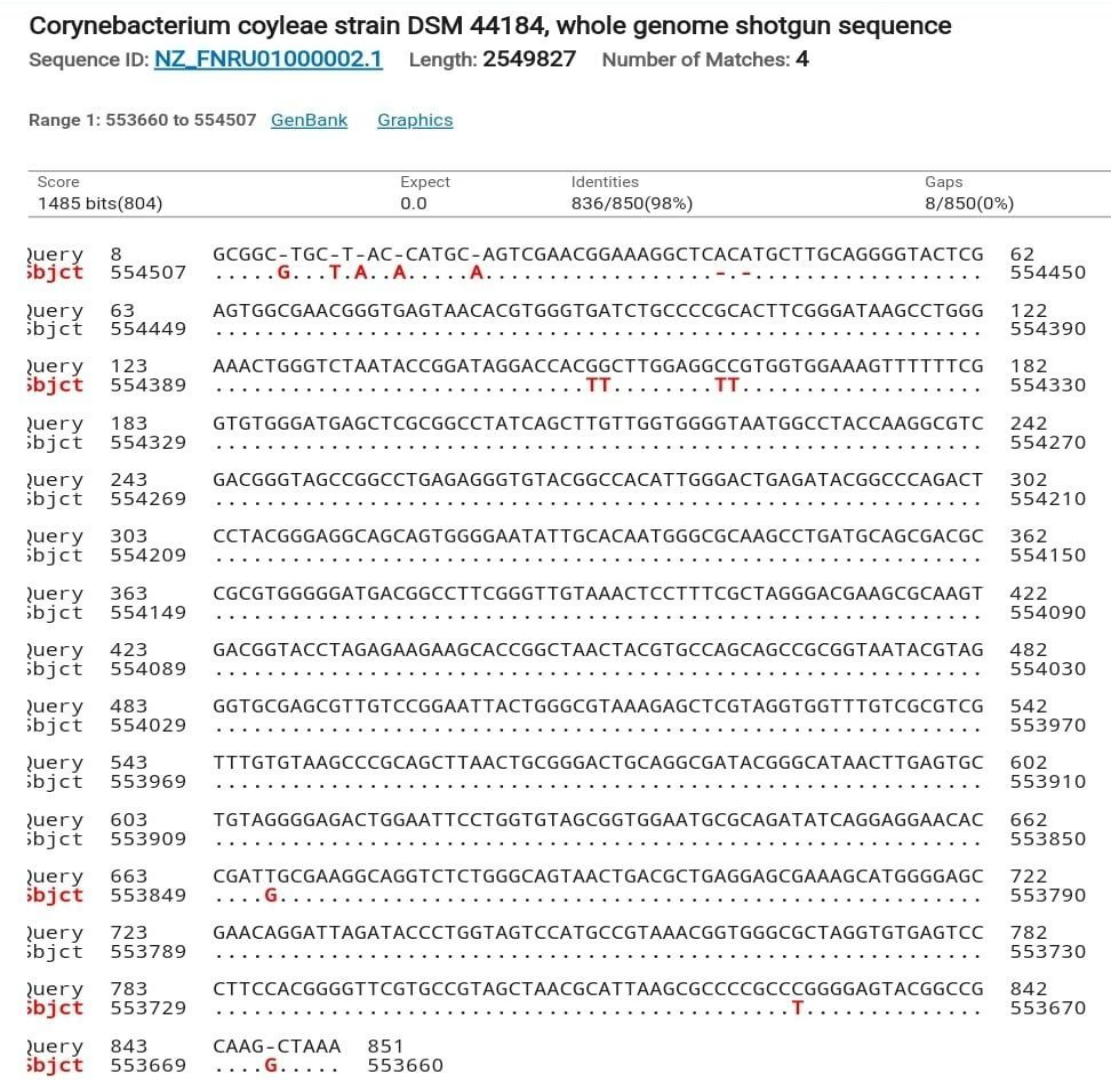


Figure (4-9): Alignment Analysis of *C. coyleae* with Gene Bank at NCBI

4.5.3.4. DNA sequencing of *Enterococcus gallinarum*

The sequencing result of *E. gallinarum*, which has 99% identity with the subject of (*Enterococcus gallinarum* strain ST4 16S ribosomal RNA gene) in NCBI under Accession Number (MK894862.1), showed 1 transition (T/C) and 1 transversion (T/A) when compared Query with Subject, as shown in Figures 4–10.

Enterococcus gallinarum strain ST4 16S ribosomal RNA gene, partial sequence

Sequence ID: MK894862.1 Length: 1457 Number of Matches: 1

Range 1: 1 to 886 GenBank Graphics

Score	Expect	Identities	Gaps
1613 bits(873)	0.0	882/886(99%)	2/886(0%)

```
Query  6    GTGCGCTGCTATACATGC-AGTCGAACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAA  64
Sbjct  1    ......A..........A.........................................  60

Query  65   AGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATA  124
Sbjct  61   ............................................................  120

Query  125  ACACTTGGAAACAGGTGCTAATACCGTATAACACTATTTTCCGCATGGAAGAAAGTTGAA  184
Sbjct  121  ............................................................  180

Query  185  AGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAAC  244
Sbjct  181  ............................................................  240

Query  245  GGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG  304
Sbjct  241  ............................................................  300

Query  305  AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAG  364
Sbjct  301  ............................................................  360

Query  365  TCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTA  424
Sbjct  361  ............................................................  420

Query  425  GAGAAGAACAAGGATGAGAGTAGAACGTTCATCCCTTGACGGTATCTAACCAGAAAGCCA  484
Sbjct  421  ............................................................  480

Query  485  CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTA  544
Sbjct  481  ............................................................  540

Query  545  TTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAAC  604
Sbjct  541  ............................................................  600

Query  605  CGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATG  664
Sbjct  601  ............................................................  660

Query  665  TGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGT  724
Sbjct  661  ............................................................  720

Query  725  CTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG  784
Sbjct  721  ............................................................  780

Query  785  TCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCTCTTCAGTGCTGCAGC  844
Sbjct  781  ..........................................C.................  840

Query  845  AAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGT-GAA  889
Sbjct  841  ..........................................T...  886
```

Figure (4-10): Alignment Analysis of *E. gallinarum* with Gene Bank at NCBI

4.5.3.5. DNA Sequencing for *Acinetobacter junii*

The sequencing result of *A. junii*, which has 99% identity with the subject of Acinetobacter junii strain H230116-022-K21-Ar-10-16s.ab1 16S ribosomal RNA gene) in NCBI under Accession Number (OQ920557.1), showed no transition or transversion when comparing Query with Subject, as shown in Figure 4-11.

Acinetobacter junii strain H230116-022_K21_Ar_10_16s.ab1 16S ribosomal RNA gene, partial

Sequence ID: OQ920557.1 Length: 935 Number of Matches: 1

Range 1: 1 to 935 GenBank Graphics

Score	Expect	Identities	Gaps
1722 bits(932)	0.0	935/936(99%)	1/936(0%)

```
Query  1    GGGCATGGCGCAGCTACACATGCAGTCGAGCGGAGATGAGGTGCTTGCACCTTATCTTAG  60
Sbjct  1    ............................................................  60

Query  61   CGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTCCGAAA  120
Sbjct  61   ............................................................  120

Query  121  GGAATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCT  180
Sbjct  121  ............................................................  180

Query  181  AATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGA  240
Sbjct  181  ............................................................  240

Query  241  TCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCC  300
Sbjct  241  ............................................................  300

Query  301  TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCG  360
Sbjct  301  ............................................................  360

Query  361  CGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTGAGA  420
Sbjct  361  ............................................................  420

Query  421  CTAATACTCTTGGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAG  480
Sbjct  421  ............................................................  480

Query  481  CAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCG  540
Sbjct  481  ................-...........................................  539

Query  541  TAGGCGGCTTTTTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGAT  600
Sbjct  540  ............................................................  599

Query  601  ACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGT  660
Sbjct  600  ............................................................  659

Query  661  AGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGG  720
Sbjct  660  ............................................................  719

Query  721  TACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATG  780
Sbjct  720  ............................................................  779

Query  781  TCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCG  840
Sbjct  780  ............................................................  839

Query  841  CCTGGGGAGTACGGTCGCAAGACTAAAACTCAATGTAAATTTTCCCGGAGAAAAATACGA  900
Sbjct  840  ............................................................  899

Query  901  CCGCAGGTGAAAGCAATAATAACTCTGCTCTCGGGA  936
Sbjct  900  .................................  935
```

Figure (4-11): Alignment Analysis of *A. junii* with Gene Bank at NCBI

4.5.3.6. DNA Sequencing for *Klebsiella pneumonia*

The sequencing result of *K. pneumonia* which having 100% compatibility with the subject of (*Klebsiella pneumonia* strain K39 16S ribosomal RNA gene) in NCBI under Accession number (MH638279.1), there were no any variation when compared Query with Subject, as shown in Figure **(**4-12).

Klebsiella pneumoniae strain K39 16S ribosomal RNA gene, partial sequence

Sequence ID: MH638279.1 Length: 1029 Number of Matches: 1

Range 1: 1 to 862 GenBank Graphics

Score	Expect	Identities	Gaps
1592 bits(862)	0.0	862/862(100%)	0/862(0%)

```
Query  9    CGCAGCTACACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGC  68
Sbjct  1    ............................................................  60

Query  69   GGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGT  128
Sbjct  61   ............................................................  120

Query  129  AGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCA  188
Sbjct  121  ............................................................  180

Query  189  GATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCC  248
Sbjct  181  ............................................................  240

Query  249  TAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG  308
Sbjct  241  ............................................................  300

Query  309  GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTG  368
Sbjct  301  ............................................................  360

Query  369  TGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGTTAAGGTTAA  428
Sbjct  361  ............................................................  420

Query  429  TAACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC  488
Sbjct  421  ............................................................  480

Query  489  GCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGC  548
Sbjct  481  ............................................................  540

Query  549  GGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGG  608
Sbjct  541  ............................................................  600

Query  609  CAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA  668
Sbjct  601  ............................................................  660

Query  669  TCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGA  728
Sbjct  661  ............................................................  720

Query  729  AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGAT  788
Sbjct  721  ............................................................  780

Query  789  TTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGG  848
Sbjct  781  ............................................................  840

Query  849  GGAGTACGGCCGCAAGGTTAAA  870
Sbjct  841  .....................  862
```

Figure (4-12): Alignment Analysis of *K. pneumonia* with Gene Bank at NCBI

The other isolate of *Klebsiella pneumonia* compared to subject of NCBI in the same method**.**

4.5.3.7. DNA Sequencing for *Staphylococcus epidermidis*

The sequencing result of *S. epidermidis*, which has 99% compatibility with the subject of (*Staphylococcus epidermidis* strain EH-4 16S ribosomal RNA gene) in NCBI under Accession Number (KF683949.1), showed 2 transversion (C/G and G/C) when compared Query with Subject, as shown in Figure 4-13.

Figure (4-13): Alignment Analysis of *S. epidermidis* with Gene Bank at NCBI

The other isolate of *Staphylococcus epidermidis* compared to subject of NCBI in the same method**.**

4.5.3.8. DNA Sequencing for *Metabacillus niabensis*

The sequencing result of *M. niabensis*, which has 89% compatibility with the subject of (*Metabacillus niabensis* strain LMR748 16S ribosomal RNA gene) in NCBI under Accession number (MW559669.1), there were a large number of transitions and transversions when comparing Query with Subject, as shown in Table 4–4 and Figure 4–14.

Table (4-4):Type and Number of Substitutions in *M. niabensis* Alignment Sequences

Type of Substitutions									
Transversion							Transition		
C/G	C/A	T/A	A/C	A/T	T/G	G/C	A/G	T/C	C/T
27	6	2	2	1	4	2	26	1	2

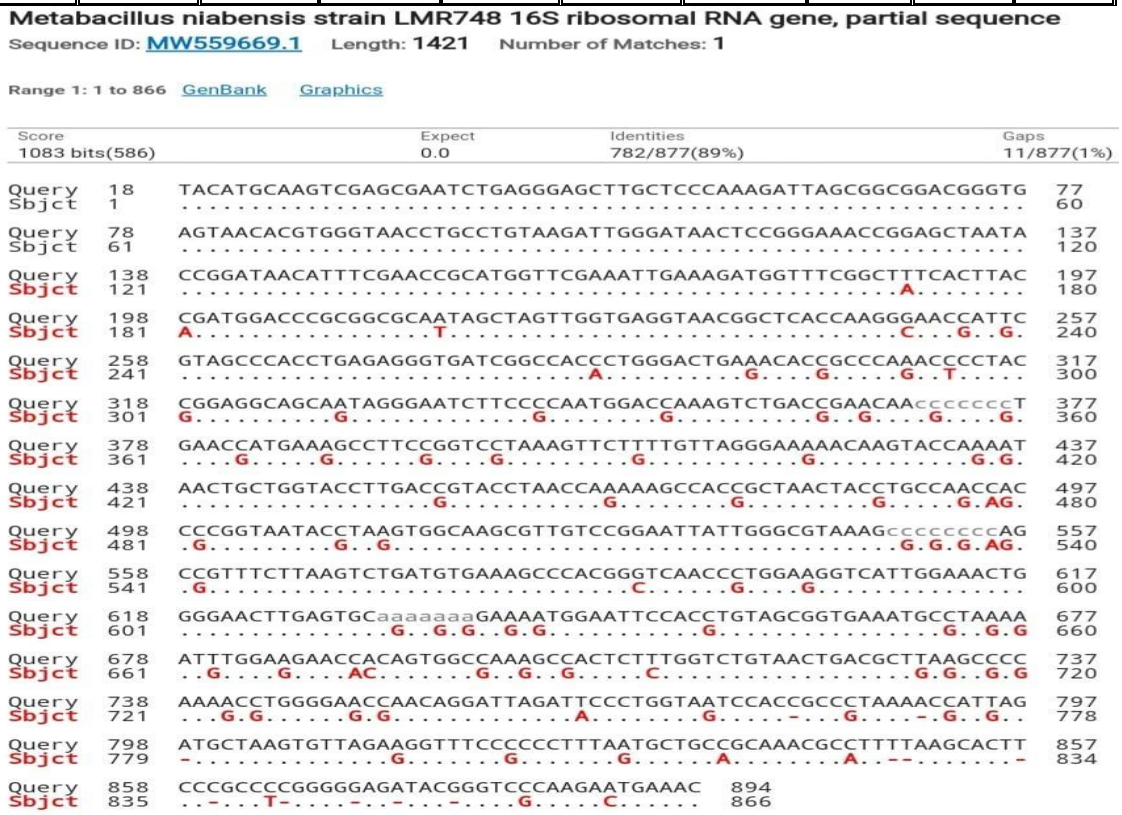


Figure (4-14): Alignment analysis of *M. niabensis* with Gene Bank at NCBI

4.5.4. Detection of *asa1* Gene as a Virulence Gene of *Enterococcus faecalis*

By using a specific primer for the detection of the *asa1* gene in an isolate of *Enterococcus faecalis*, the current study showed that out of 20 isolates from patients, 17 (85%) of them gave positive results for this gene at a replicon size of 375 bp, while in control, out of 10 isolates from this bacterium, 2 (20%) gave positive results when compared with the allelic ladder as shown in Figure 4–15.

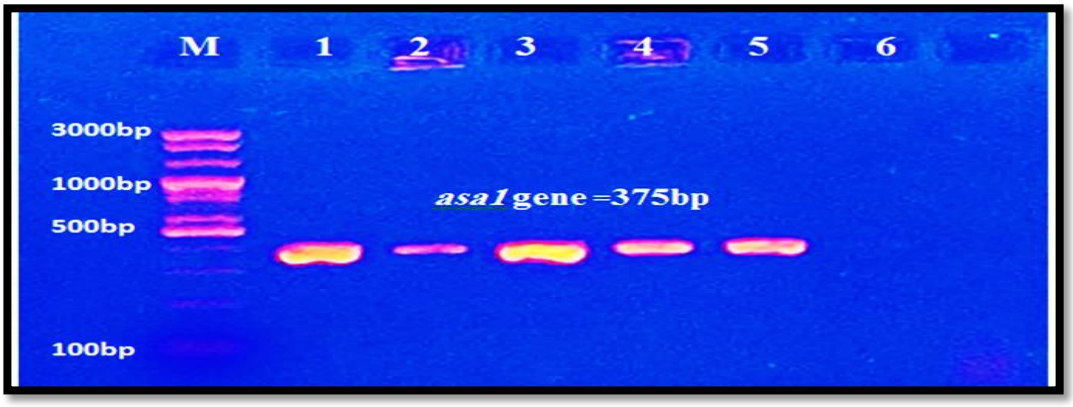


Figure (4-15): Agarose Gel Electrophoresis of PCR Products of Virulence Gene Aggregation Substance (*asa1*) for Isolates of *Enterococcus faecalis*. 1%Agarose, 100 volt/ 40 min

In a closely related study, *E. faecalis asa1* was significantly predominant in 86.51% of samples from individuals with UTIs and pyogenic wound infections (Kiruthiga *et al*., 2020).

This virulence genes are often located on mobile genetic components (plasmids) and/or the chromosomal region, it is possible for non-pathogenic strains to acquire new virulence factors from accessory DNA. Substances that provide extreme pathogenicity on bacteria are clustered among these virulence factors (Negut *et al*., 2018).

Most aggregates are formed by chemicals called extracellular polymeric substances (EPS), which are High molecular weight (HMW) polymers, released by bacteria and photosynthetic microorganisms and primarily responsible for the binding of cells with other particulate materials, such as polysaccharides and proteins, to achieve cohesion and adhesion (Song *et al*., 2019).

The *asa1* gene encodes the aggregating substances that bind bacterial cells to a solid surface, resulting in the formation of a biofilm responsible for bacteria's pathogenicity and resistance to antibiotic (Sehgal, 2020).

Different microbial strains and experimental settings result in distinct EPS (Bayigga *et al*., 2019).

These parameters include nutrition availability, light intensity, the presence of cations (mostly Ca2+ and Mg2+), temperature, and pH. Microorganisms may up their EPS production in response to harsh circumstances as a means of protecting their cells from stress factors (Siddharth *et al*., 2021).

4.6. Serological Assay

4.6.1. Peripheral Natural Killer Cells in Spontaneous Abortion Women and Healthy Women

Results of the current study revealed that there is a discrepancy in the concentrations between different age groups and also between patients and controls especially in the age group 26-35 years, but it doesn't reach the degree of significance as indicated by the statistical analysis P≤005. The concentrations mean and standard deviation (Std) of pNK in patients was (107.339 ± 46.647) nmol/L, while in controls was (80.229 ± 23.737) nmol/L, as shown in Tables (4-6).

Table (4-6): The Concentration of Peripheral Natural Killer Cells in Patients According to Age Group

Age group	Concentration of pNK (nmol/L) Mean ± Std. Deviation		P value ($p \leq 0.05$)
	Patient	Control	
16-25	96.3427 ± 48.8677	86.868 ± 20.8924	0.667^{NS}
26-35	117.4709 ± 52.6364	74.9269 ± 28.99466	0.083^{NS}
36-45	101.6683 ± 36.1114	81.9856 ± 17.5037	0.150^{NS}
Total	107.339 ± 46.647	80.229 ± 23.737	
P value ($p \leq 0.05$)	0.575^{NS}	0.629^{NS}	

NS: Non-significant difference under $p \leq 0.05$ by One way – ANOVA

The results of the study can be interpreted on the basis of the fact that samples of primary spontaneous abortion and not recurrent abortion were obtained, as well as studies that confirmed the increase of pNK cells in the endometrium, so if the sample was a biopsy of the endometrium, it may

have seen that, as well as other studies revealed that the pNK cells present in patients are NK cytotoxicity (NKC), so there was no increase in the percentage or absolute number of pNK cells, indicating that the number is convergent to that in control cases (Kwak-Kim and Gilman-Sachs, 2008; Hou *et al*., 2022).

The biological activity is not always taken seriously when counting pNK or uNK cells. In comparison to the activity of natural killer Cytotoxity (NKC). Over the past two decades, the NKC test by flow cytometry has been developed into a helpful tool in clinical medicine for women with infertility. It is reported to be reproducible and suitable for both clinical and research uses (Rougang, *et al*., 2023).

Zhang and Wei, (2021) confirmed that NKCs were greatly higher in non-pregnant women with a history of recurrent pregnancy loss (RPL) and recurrent implantation failure (RIF) compared to normal controls. RPL, Pre-eclampsia, RIF, unexplained infertility, and other reproductive diseases have all been linked to irregularities in NK cell number and cytotoxicity and also have abnormal pNK subsets which could indicate immune abnormalities at the maternal-fetal interface. These women's immune-phenotypic NK cell features corroborate the shifts in their activity status.

4.6.2. *Mycoplasma hominis* Infection

The SA and healthy women were subjected to evaluation of the concentration of serum Mycoplasma hominis IgM to determine if they were infected with this bacterium or not.

A total of (43/50= 86%) were equivalent, (6/50= 12%) were positive, and about (1/50= 2%) were negative from patients, and (25/30 = 83.3%) were equivalent, (0/30 = 0%) were positive, and (5/30 = 16.6%) were negative from the control group for *Mycoplasma hominis*, whereas positive

≥1.00, negative ≤ 0.10 and equivalent range between ≤ 0.10 to ≥1.00, as shown in Figure 4-16.

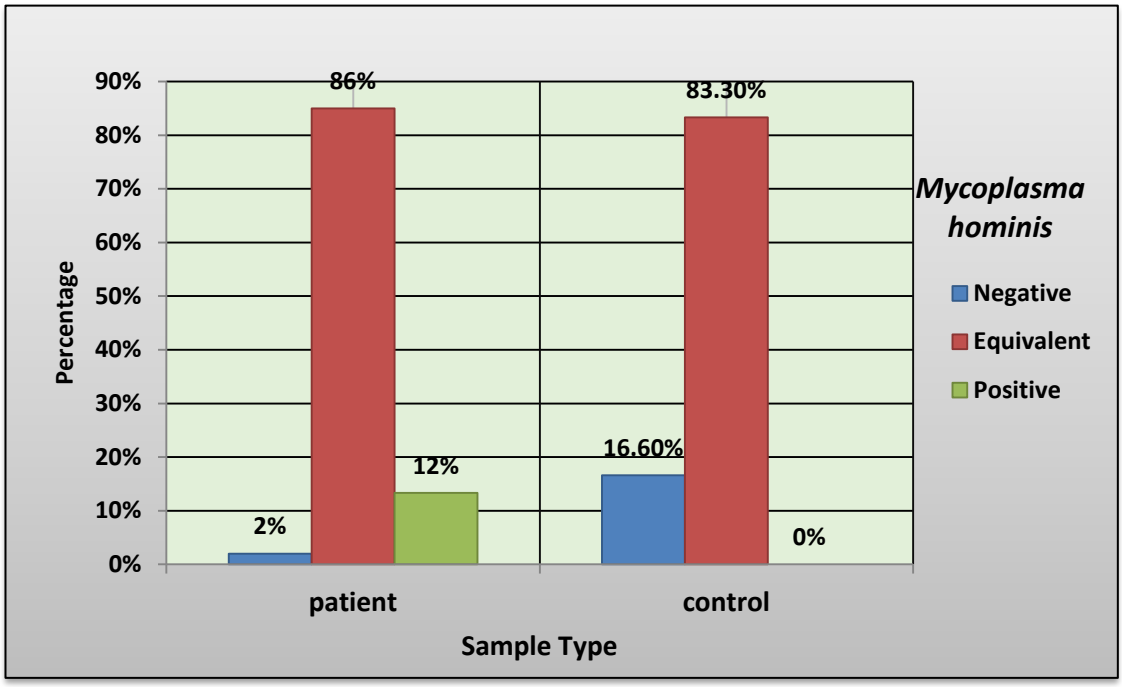


Figure (4-16): Distribution of Study Cases into Positive, Negative and Equivalent, Depending on The Standard Value in *Mycoplasma hominis* Kit.

In similar study, positive "premature rupture of membranes" PPROM cases were found in 12 patients (19.4%) infected with *Mycoplasma hominis* (Fulova *et al*., 2021).

Bacteria of this type are obligate intracellular parasites; they are found in a woman's vagina but do not cause illness in those with a healthy immune system. However, in the presence of a weakened host immune system, this bacterial strain can rapidly proliferate and result in conditions like pelvic inflammatory disease, which affects the ovaries and fallopian tubes. It's also thought to cause the elevated body temperature that comes with abortions and labor (Nuradilova *et al*., 2021).

4.6.3. Spontaneous Abortion Associated with *Mycoplasma hominis* Infection

The statistical analysis of the data indicated that there is a very slight difference in the concentrations between patients and controls, but it doesn't reach the degree of significance P≥005. The concentration of *"Mycoplasma hominis*" in patients means and the Std.(0.39830 ± 0.21166) nmol/L , was not significant compared with the control (0.32497 ± 0.130461) nmol/L, as shown in Table (4-7).

Table (4-7): The Concentration of *Mycoplasma hominis* in Patients and Controls According to Age Group

Age group	Concentration of *M. hominis* (nmol/L) Mean ± Std. Deviation		P value ($p \leq 0.05$)
	Patient	Control	
16-25	0.32167 ± 0.155579	0.28738 ± 0.055374	0.556 ^{NS}
26-35	0.41891 ± 0.084786	0.34492 ± 0.183097	0.540 ^{NS}
36-45	0.45458 ± 0.396070	0.32956 ± 0.081512	0.366 ^{NS}
Total	0.39830 ± 0.21166	0.32497 ± 0.130461	
NS: Non-significant difference under $p \leq 0.05$ by One way – ANOVA			

Similar research indicated that *Mycoplasma hominis*, among bacterial infections, has been most linked to the occurrence of RSA (Yu *et al*., 2022). Women who had experienced a miscarriage were more likely to have been infected with both "*U. urealyticum* and *M. hominis*" (P = 0.04 and P = 0.02 for both pathogens, respectively; (Giakoumelou *et al*., 2016).

During pregnancy, *Mycoplasma hominis* multiplies at an increased rate in the female body, and this has been related to a variety of adverse pregnancy outcomes, including early labour, spontaneous abortion, abnormal uterine bleeding and early abruption of the placenta. The inflammatory process within the uterus has been associated to these disorders (Galyamina *et al*., 2022).

4.6.4. Correlation Between Peripheral Natural Killer Cell and *Mycoplasma hominis* Infection

A finding was made that hadn't been identified in prior immunological assessments, as it showed that there is an inverse relationship between the concentrations of natural killer cells and the concentrations of *Mycoplasma hominis* in the patients, as the higher the concentrations of natural killer cells, the lower the concentration of *Mycoplasma*, and vice versa. as shown in Figure (4-17).

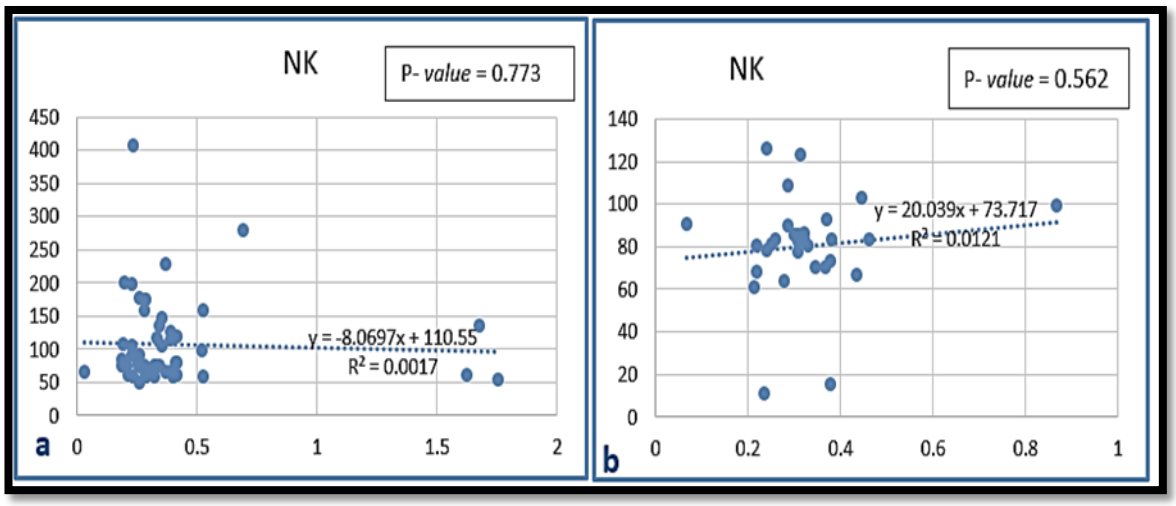


Figure (4-17): Correlation Between NK and *M. hominis* Concentration in Study Population a: Patients, b: Control

Similar studies found that amniotic fluid from women with preterm ruptures of membranes (PROM) had a high inflammation state. Aligning with the human clinical response, *Mycoplasma hominis* is most commonly

isolated from the amniotic fluid and placenta in cases of histologic and clinical chorioamnionitis and in conjunction with spontaneous preterm labor PTL and PROM, as well as elevation of pro-inflammatory cytokines (Fulova *et al*., 2021).

Activated NK cells can function to fend off *Mycoplasma* infections in their early stages. NK cells directly inhibit *Mycoplasma* by secreting interferon (IFN), which can either activate macrophages, which can kill tumor cells infected with obligate and facultative intracellular organisms, or stop *Mycoplasma* from growing, or both (Stewart *et al*., 2019). Alterations in vaginal microbiota, local inflammatory processes, and immunological signs may all play a role in the development of a miscarriage (Kuon *et al*., 2017).

First line of defence against vaginal microbes is provided by a layer of mucosal epithelium and neutrophils, that the most abundant leukocytes in the vaginal tissue (Guerrero *et al*., 2020). Innate immune cells including dendritic cells, macrophages, and NK cells are gathered in by the secretion of cytokines and chemokines which mediates natural killing. Miscarriage can occur if an active infection disrupts the team of immunological members necessary for implantation, placentation, and blood vessel change (Giakoumelou *et al*., 2016).

Other studies conducted on *Mycoplasma*, showed that *Mycoplasmas* are a type of prokaryote that has been linked to human cancer. Mycoplasmas have been linked to a wide variety of serious diseases, including urogenital problems, an infertility, and even AIDS due to the atypical bacteria's ability to cause a low-grade, chronic inflammatory condition during cell infection without that influence the lifespan of cells (Galyamina *et al*., 2022).

However, *Mycoplasma*, which lack a cell wall but are still prokaryotes, have a long, quiet, and deep connection with mammalian cells. This silent, prolonged contact may be a cause for changes in a wide variety of mammalian cellular properties. Researchers have shown great attention in how *Mycoplasma* infection stimulation or suppression affects the expression of specific genes in various cell types. Oncogenes, tumor suppressor genes, proinflammatory mediators and growth factors were all targeted (Yacoub *et al*., 2021).

Conclusions and Recommendations

Conclusions and Recommendations:

1. Conclusions:

The current study reached a set of conclusions, among which:

1. The high percentage of *Enterococcus faecalis* in patients (56%), it leads to the fact that these bacteria strains possess an arsenal of virulence factors that are located in virulence genes and contribute to their ability to cause disease, including *asa1* gene, which is considered the most virulence of *Enterococcus faecalis* genes and its percentage was 85%, which is responsible for biofilm formation.

2. PCR and Sequencing of the (938 bp) target amplicon of the 16S rRNA gene by specific primers used in this study provided an accurate approach for studying bacterial diversity in the vagina of women patients and study controls.

3. The discrepancy in the level of natural killer cells in the blood may be due to bacterial vaginosis and does not constitute an immune risk factor in these cases of the SA Study as depicted in this study.

4. The low infection rate of *Mycoplasma hominis* in SA women indicated a rare involvement of this bacteria in SA cases.

2. Recommendations:

The present study recommends the following:

1. The scope of research can be expanded to include cases of bacterial infection involved in ectopic pregnancy (tubular pregnancy), for example, in which an infection causes a defect in the fallopian tube that prevents the normal movements of the zygote.

2. Introduce real-Time PCR vaginal microbiota detection to detect microorganisms that are slow to grow, difficult to cultivate, or difficult to detect.

3. Enter the flow cytometry assay for evaluation of the level of different types of NK cells in different types of abortion.

4. Using other immunological assays, such as the immunofluorescence test, for the detection of a wide spectrum of microorganisms associated with microbial vaginosis in women.

5. In relation to the immune system, it is recommended to collect uterine biopsy samples from women with spontaneous abortion in order to identify natural killer NK cells and their relationship to spontaneous abortion, or to collect blood samples from women with recurrent spontaneous abortion immediately after abortion in order to prevent the likelihood of a decline in their concentration after days and to see the role of peripheral natural killer PNK cells and the effect of their high concentration.

6. Using next-generation sequencing (NGS) to detect the sequence of the vaginal microbiota.

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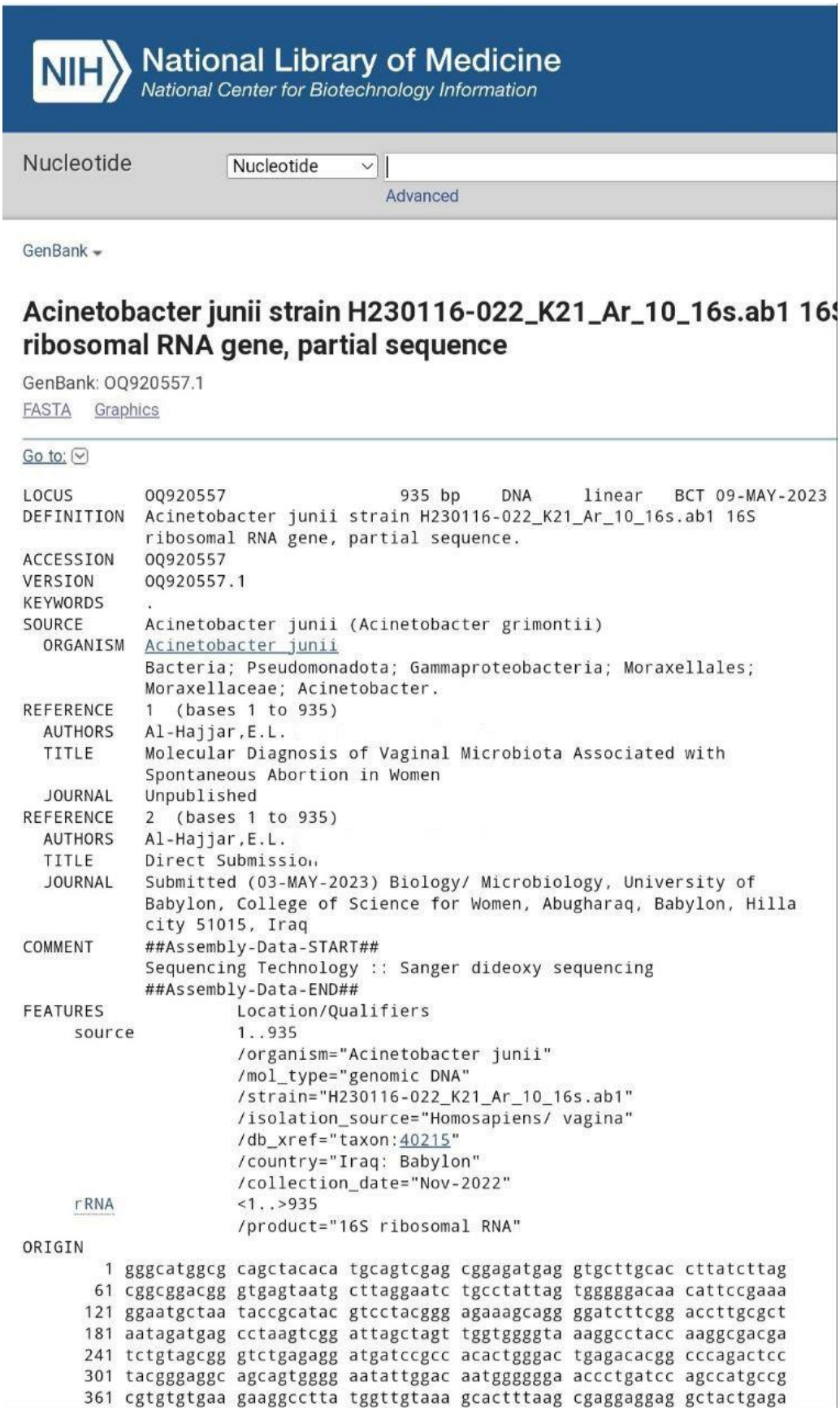
APPENDIX

Appendix (1): Registering isolates in Genebank



National Library of Medicine
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Nucleotide | Nucleotide | Advanced

GenBank

Acinetobacter junii strain H230116-022_K21_Ar_10_16s.ab1 16S ribosomal RNA gene, partial sequence

GenBank: OQ920557.1

FASTA Graphics

Go to:

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  JOURNAL   Unpublished
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  TITLE     Direct Submissio
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National Library of Medicine
National Center for Biotechnology Information

Nucleotide | Nucleotide | Advanced

GenBank | Send to:

Corynebacterium coyleae strain H230116-022_A21_Ar_5_16s.ab1 16S ribosomal RNA gene, partial sequence

GenBank: OQ920556.1

FASTA Graphics

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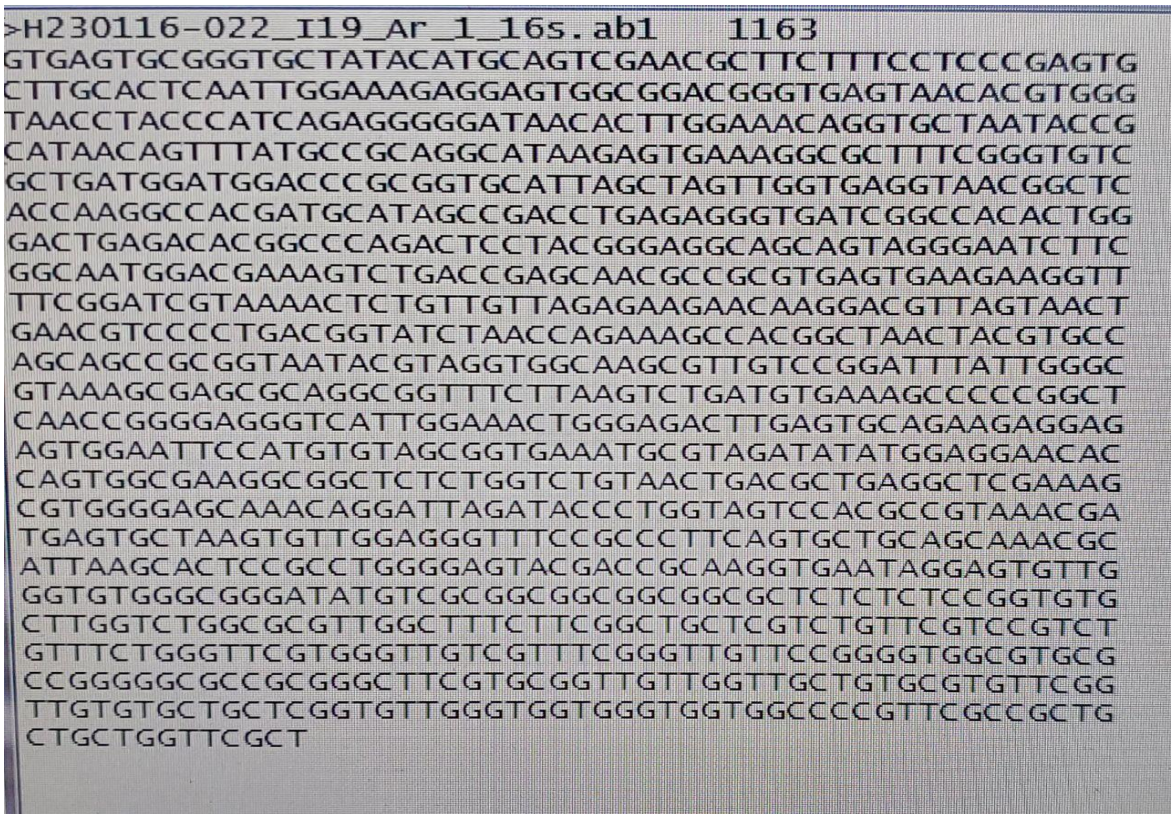
Appendix (2): The Sequences of Bacterial IsolatesUnder Study According to Genebank.



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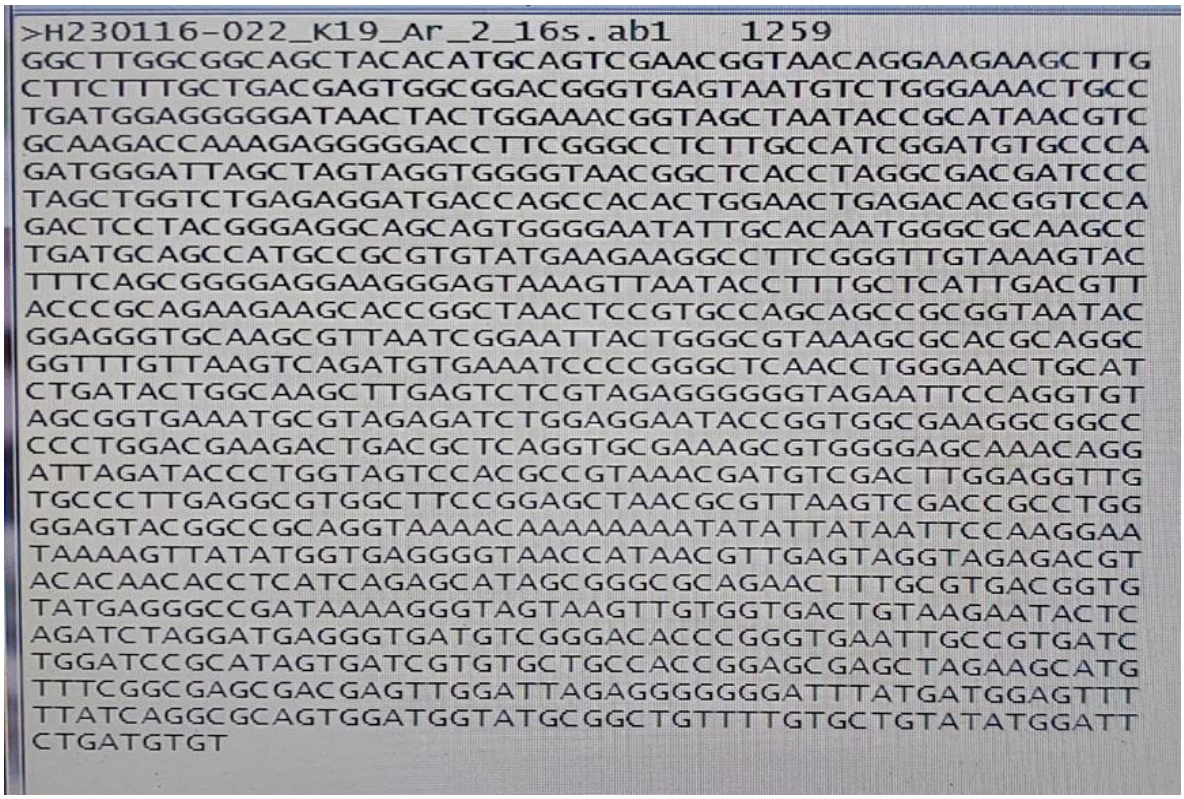

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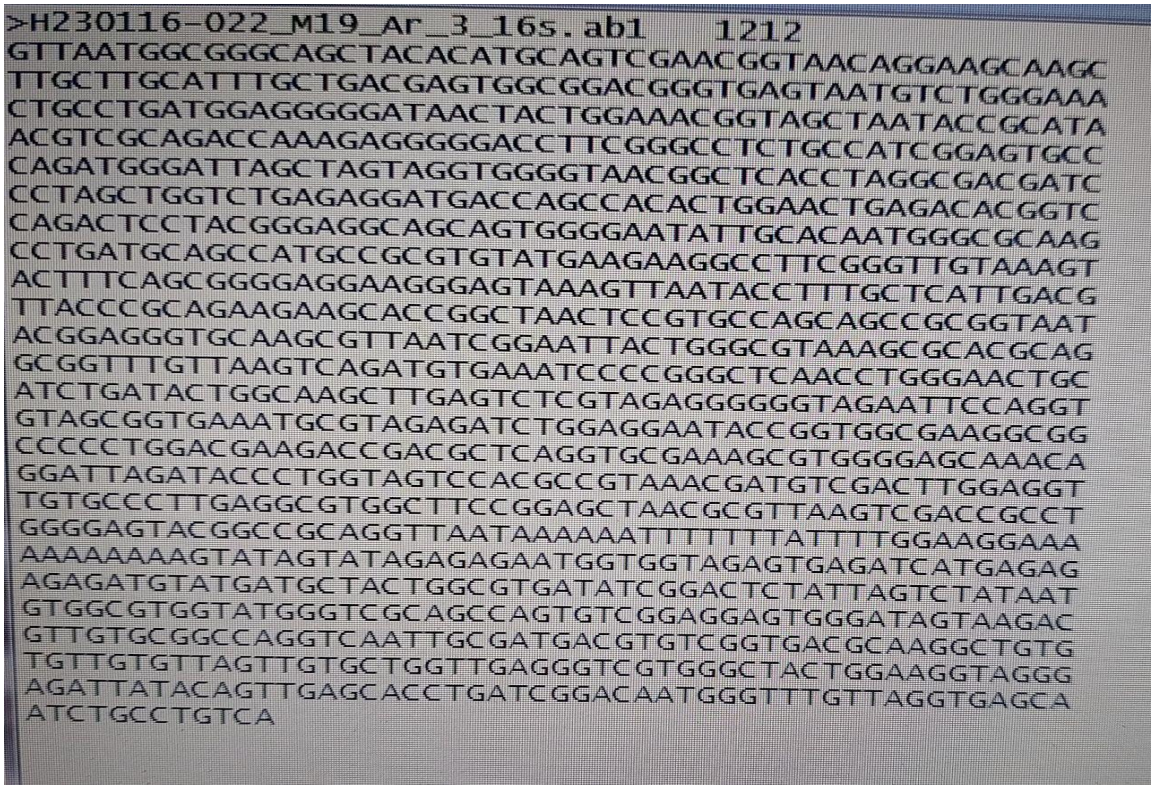

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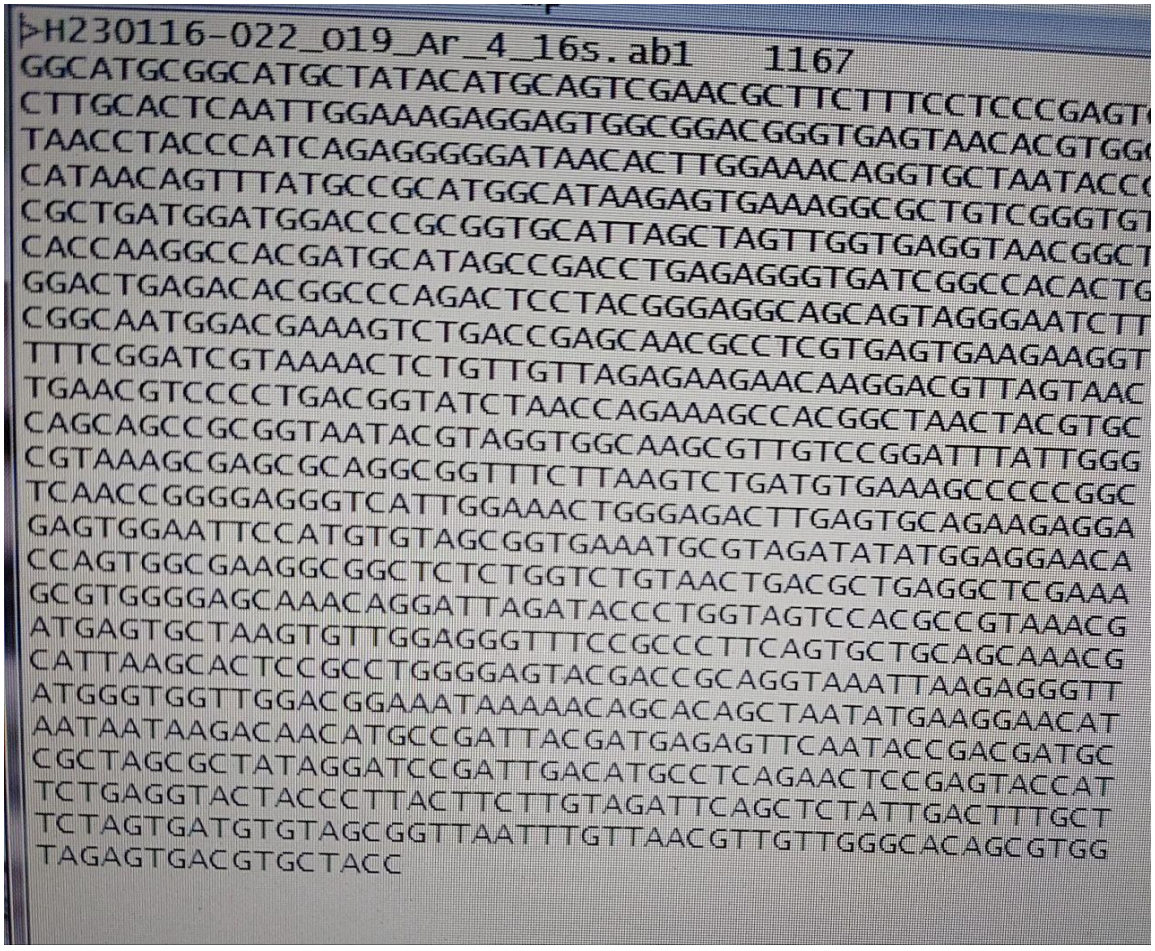

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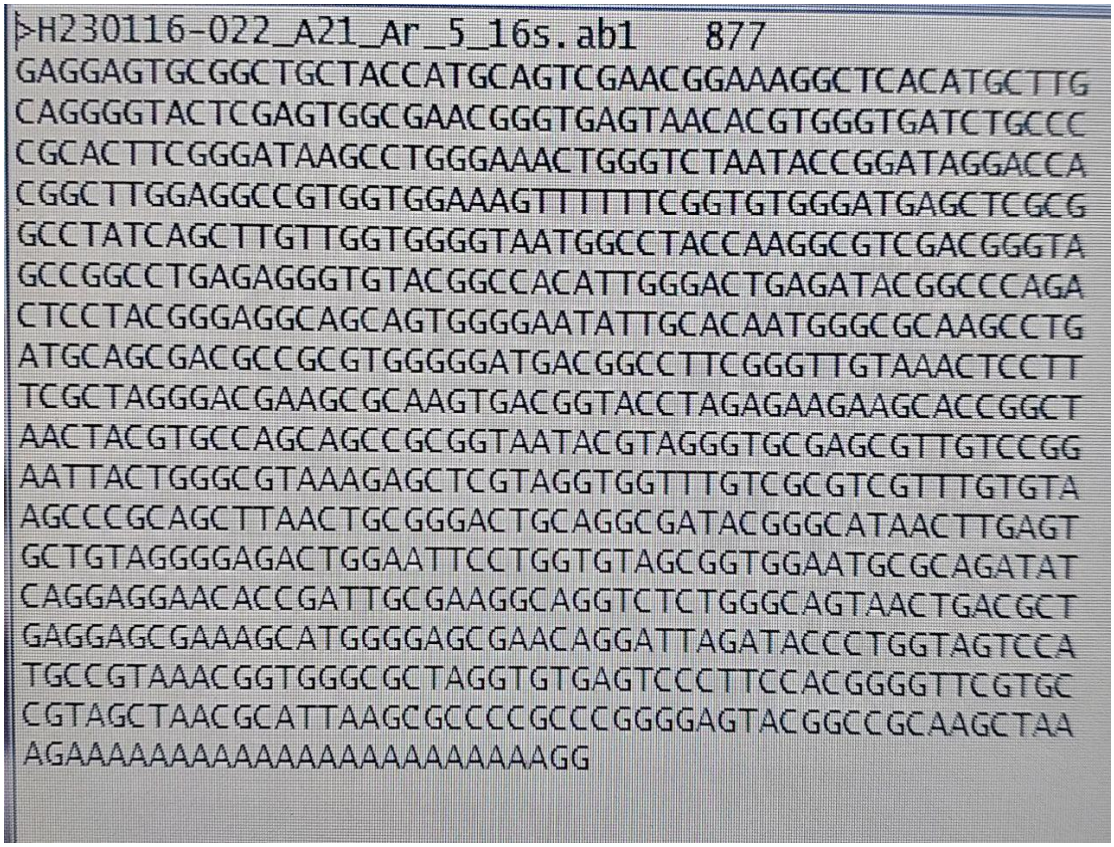
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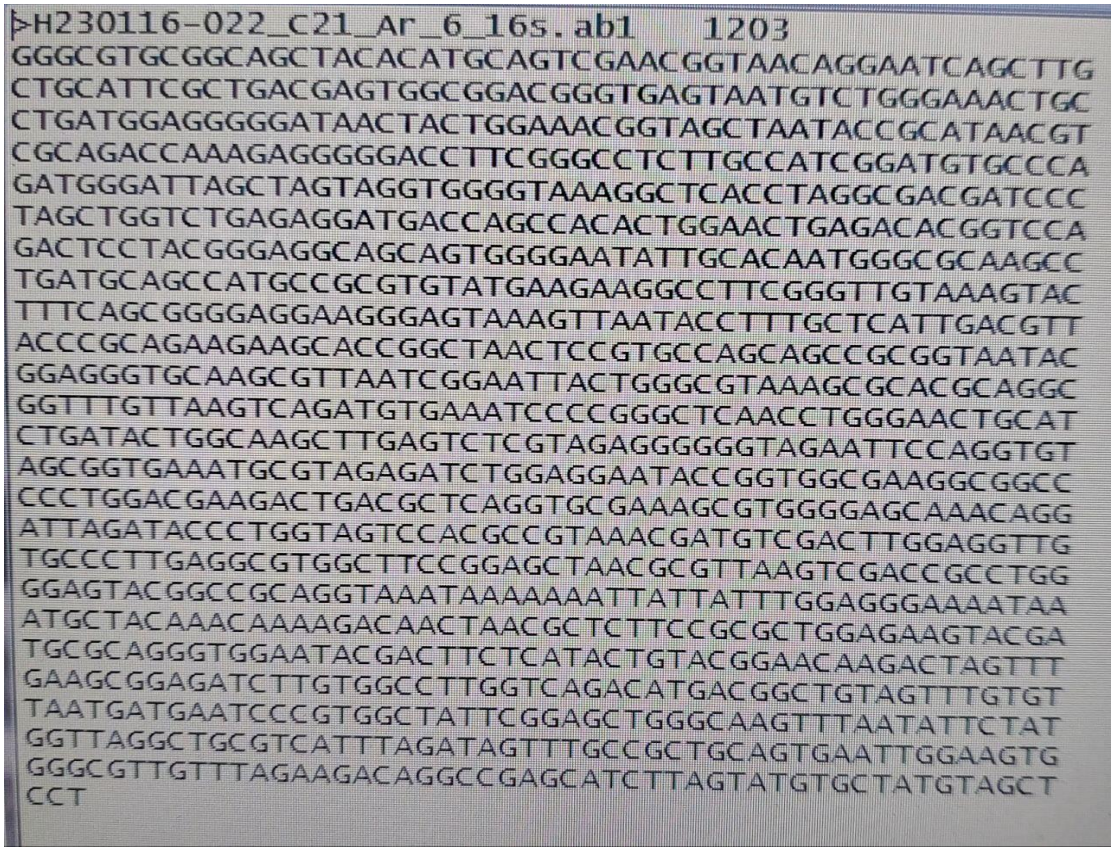
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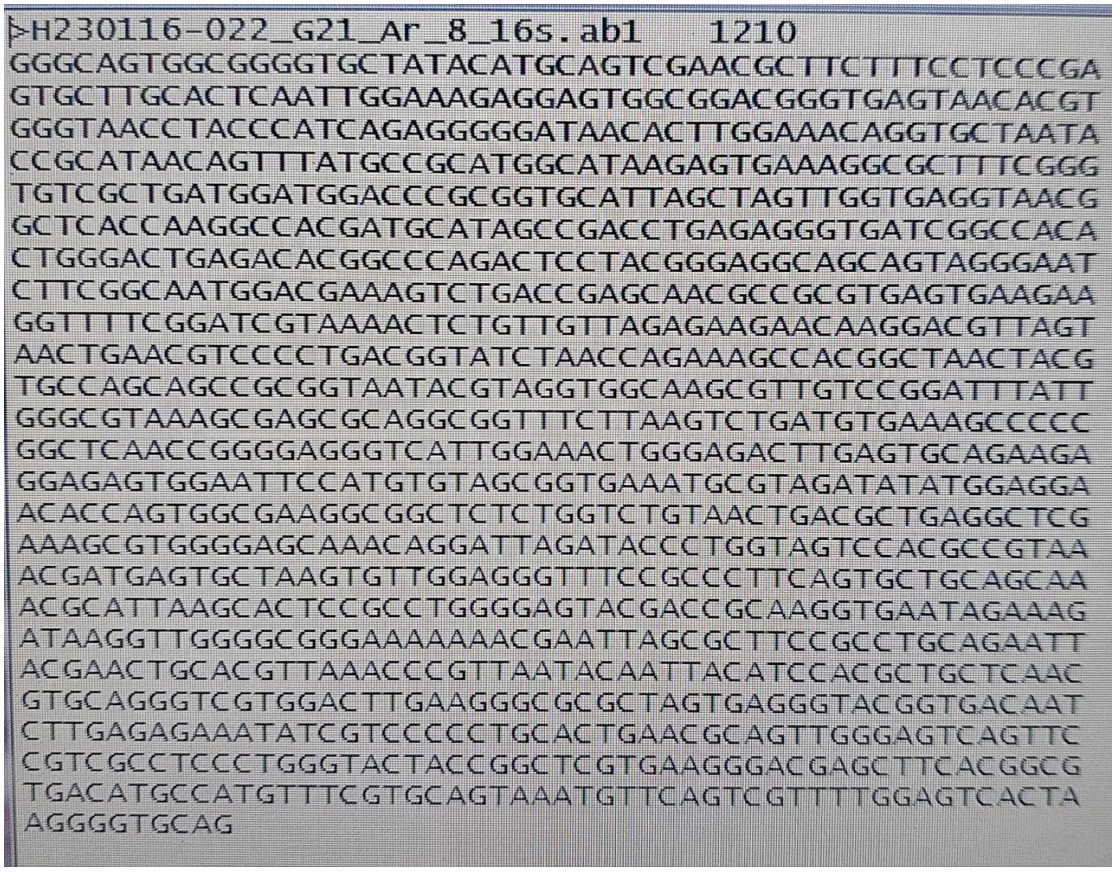

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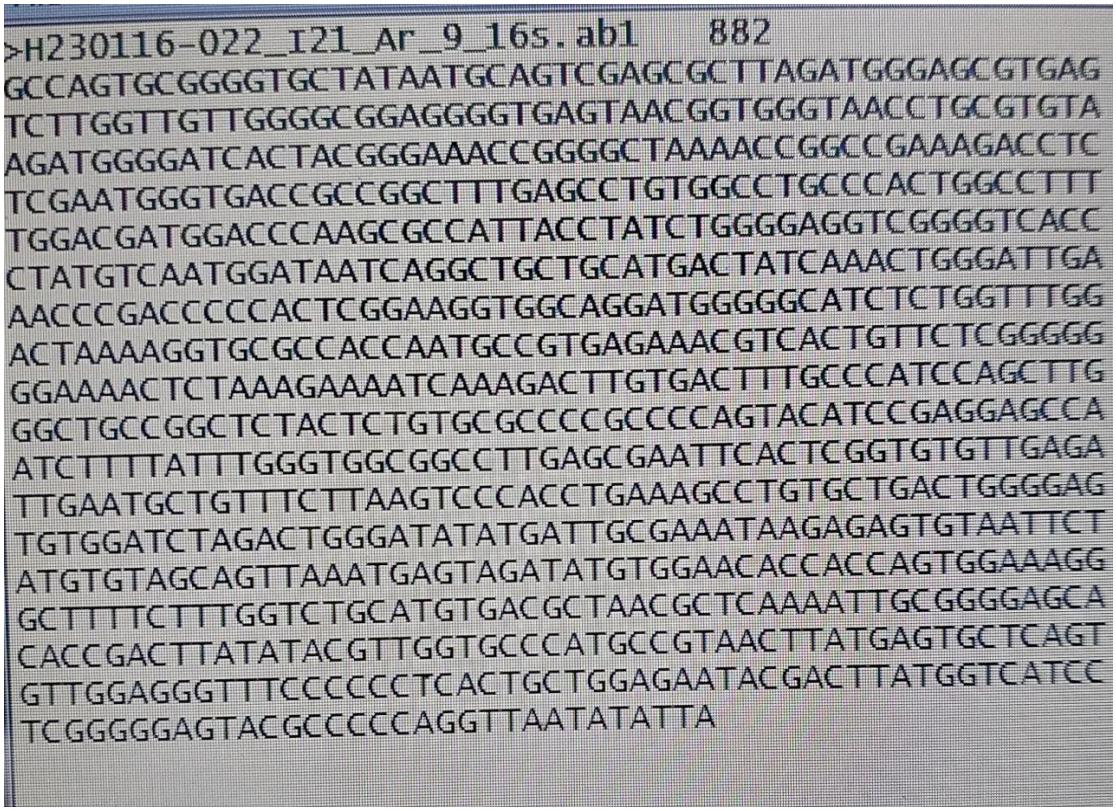

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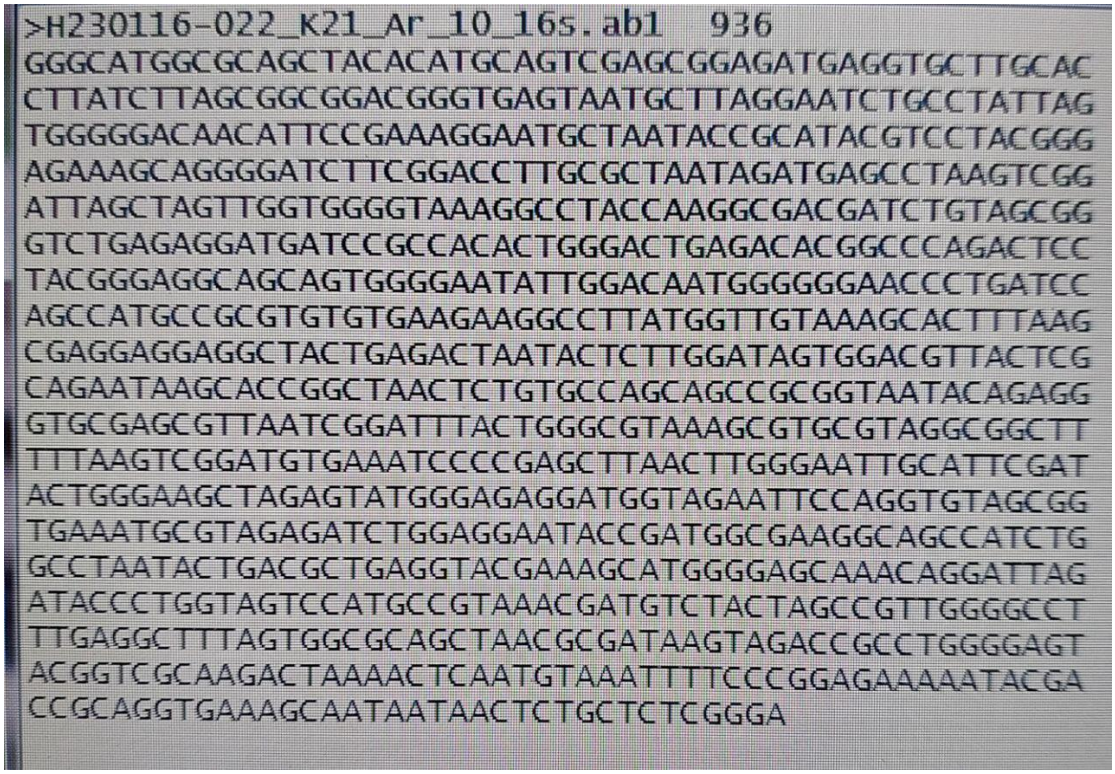

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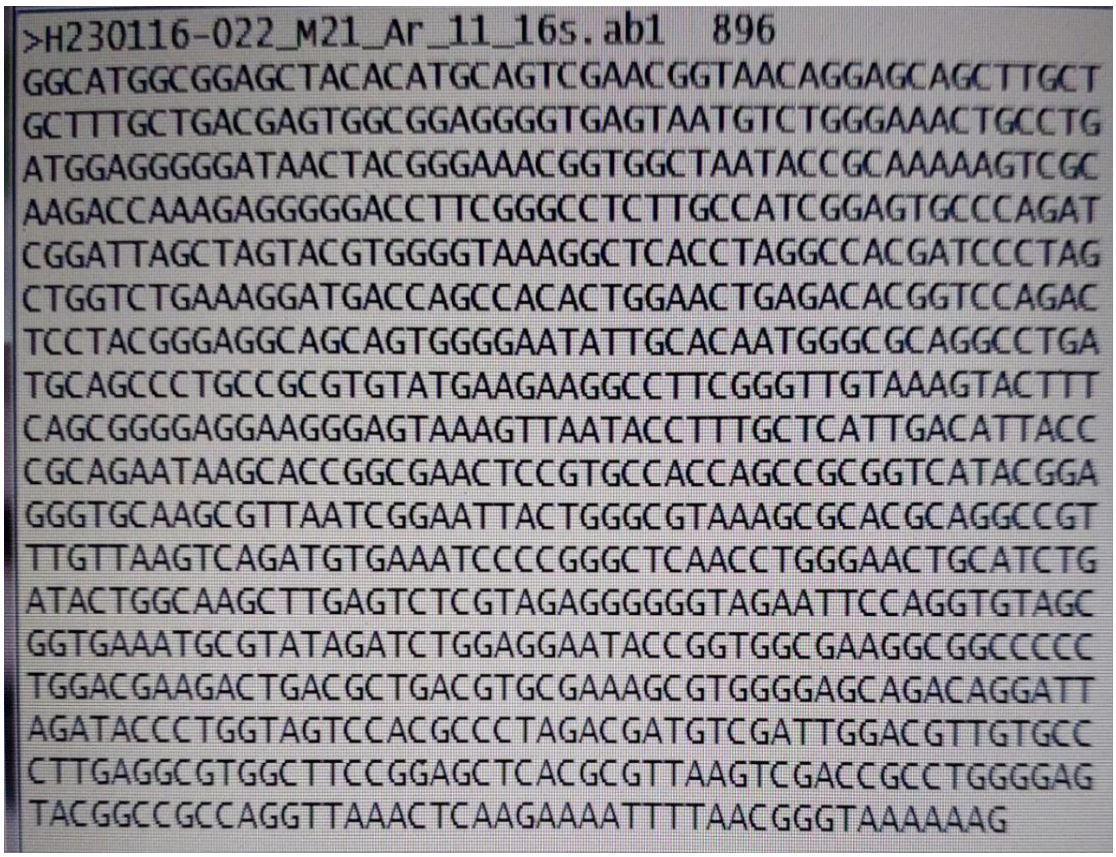

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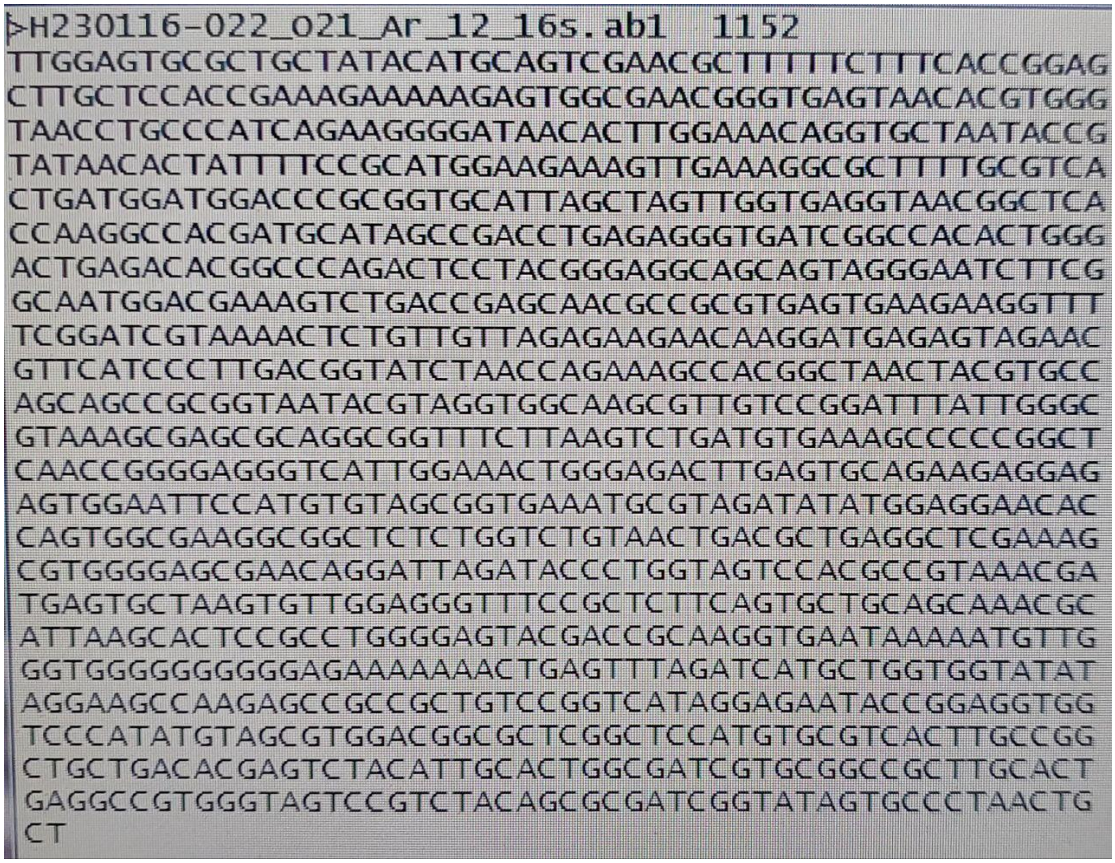
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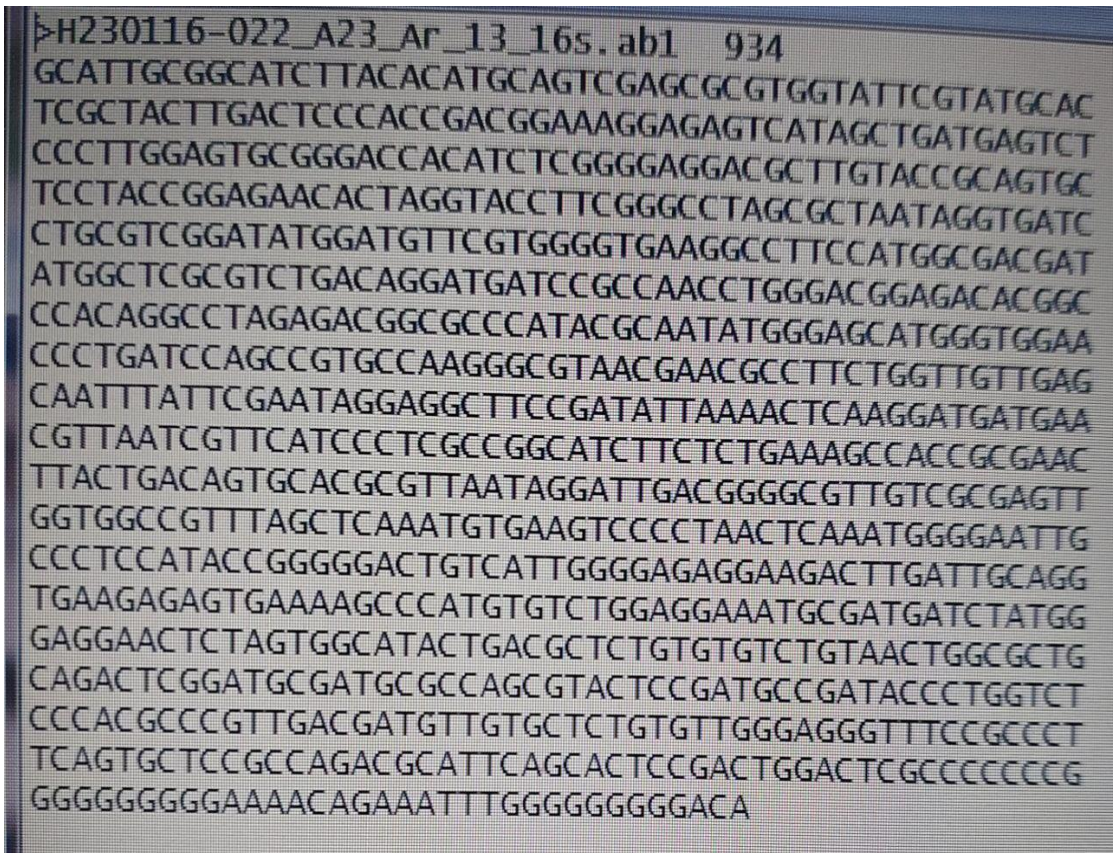
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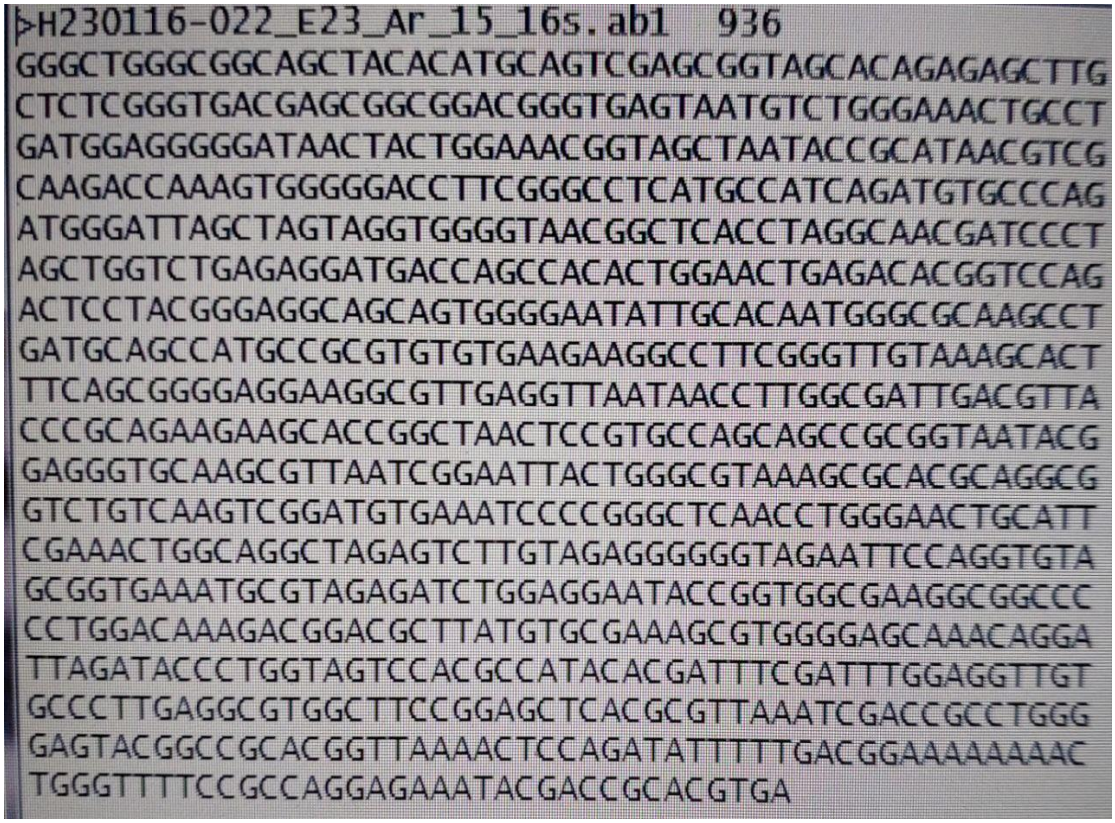
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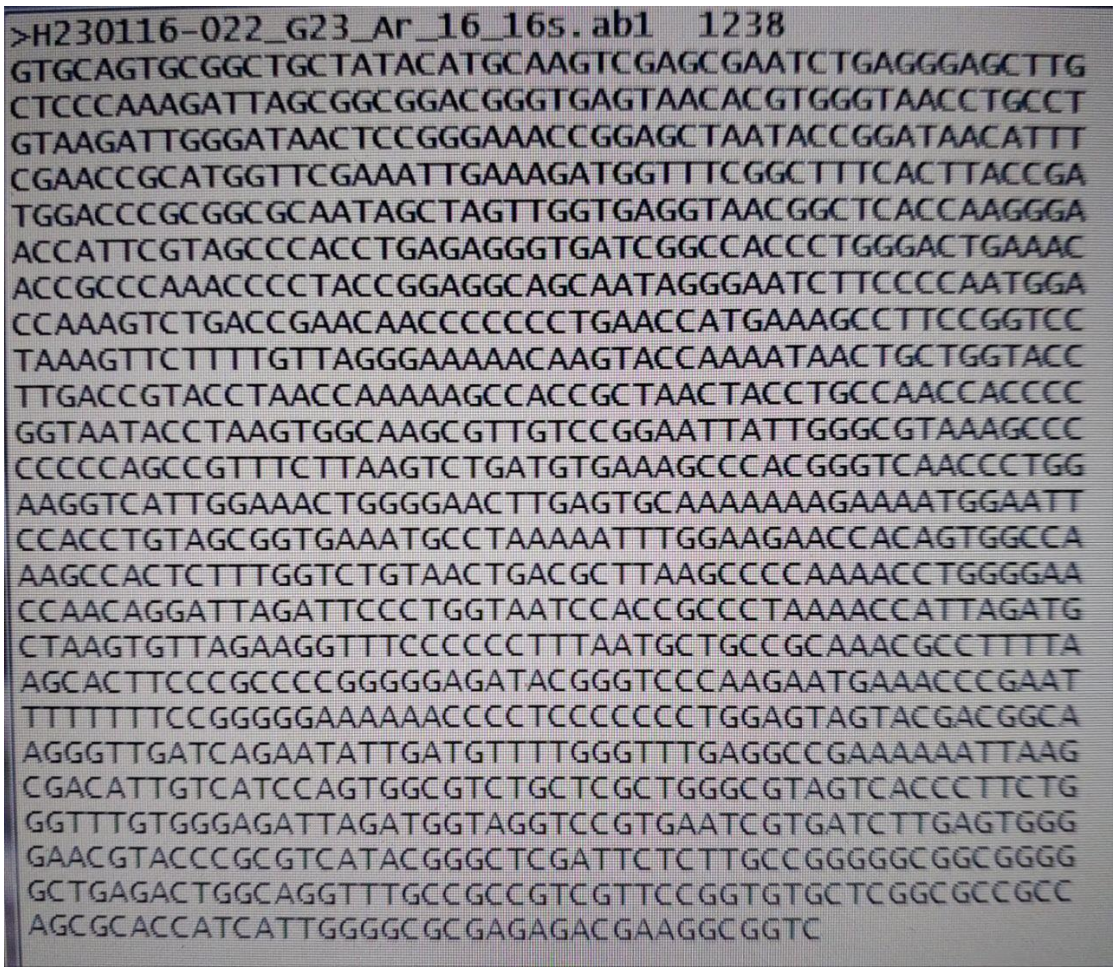
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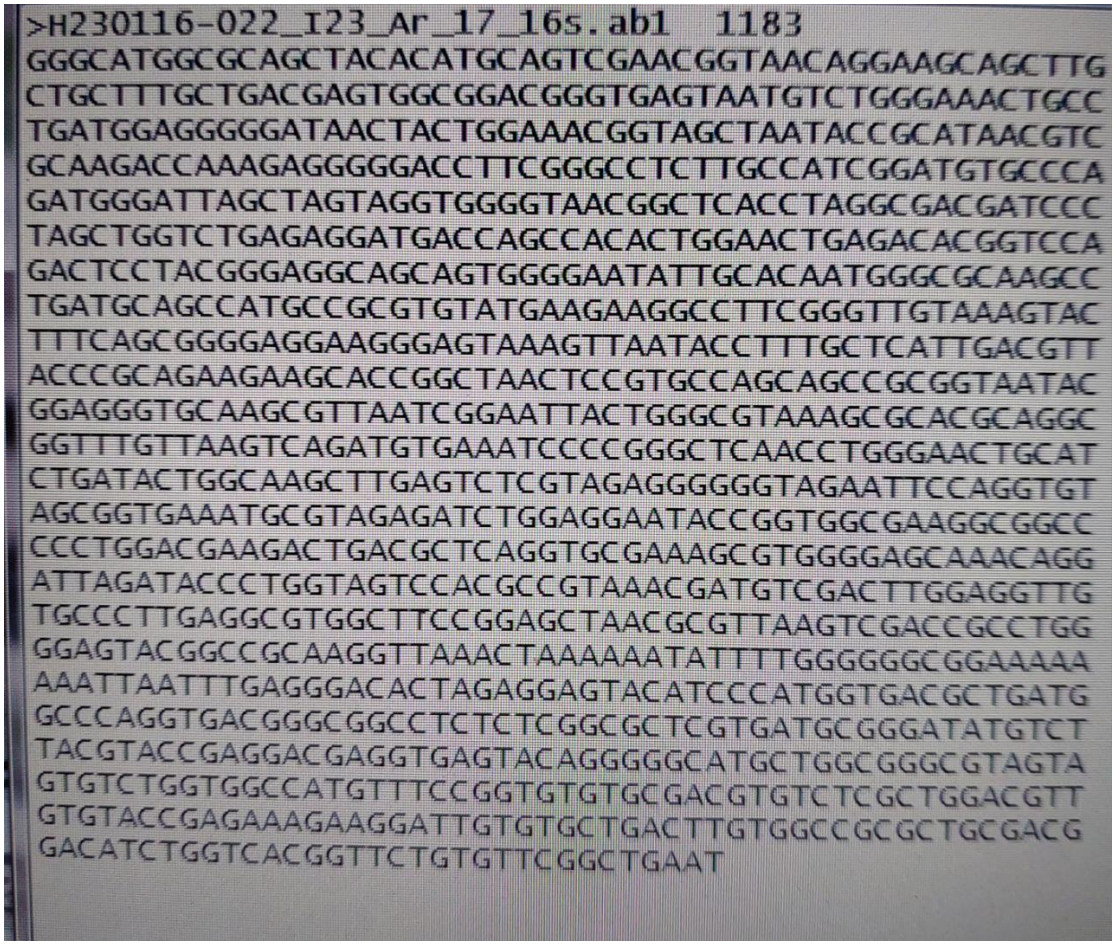
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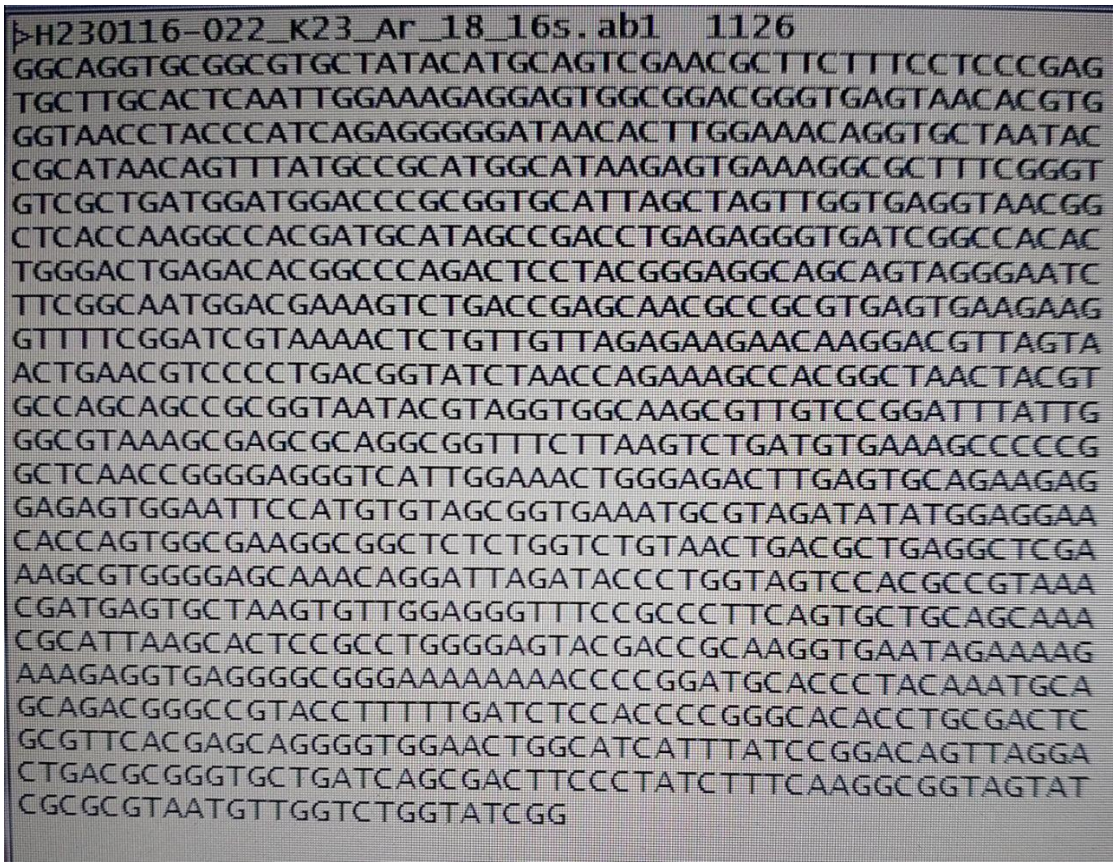
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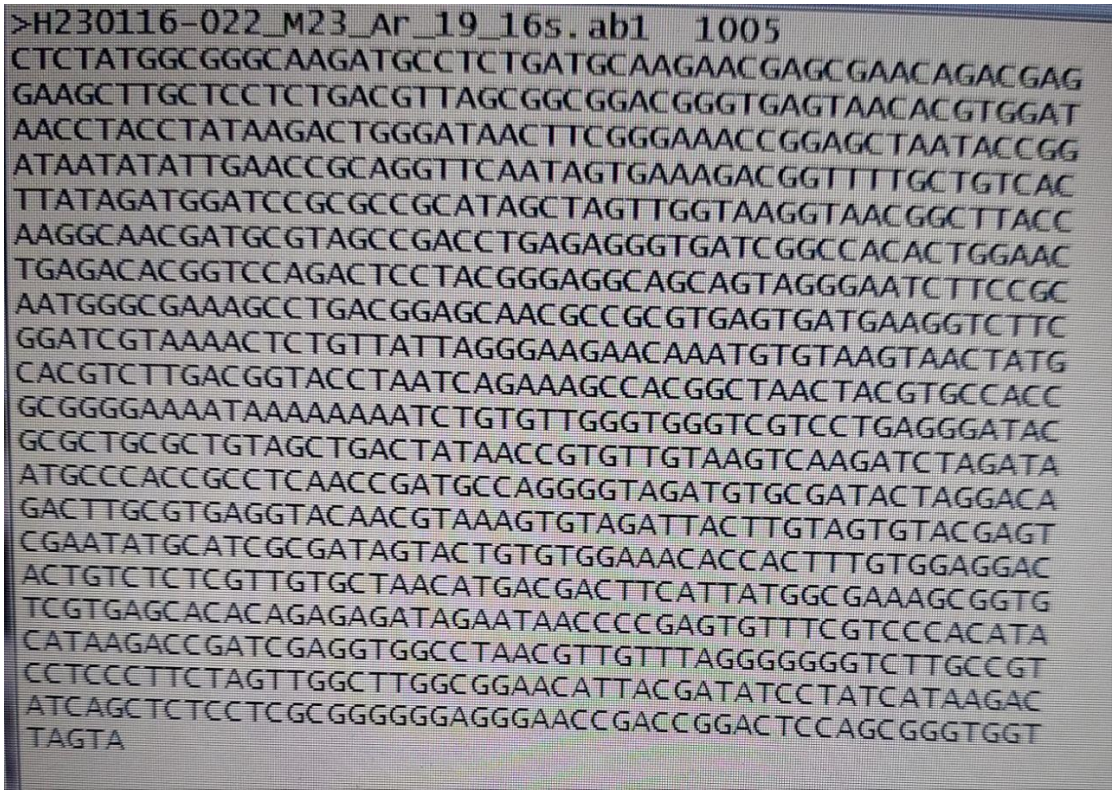

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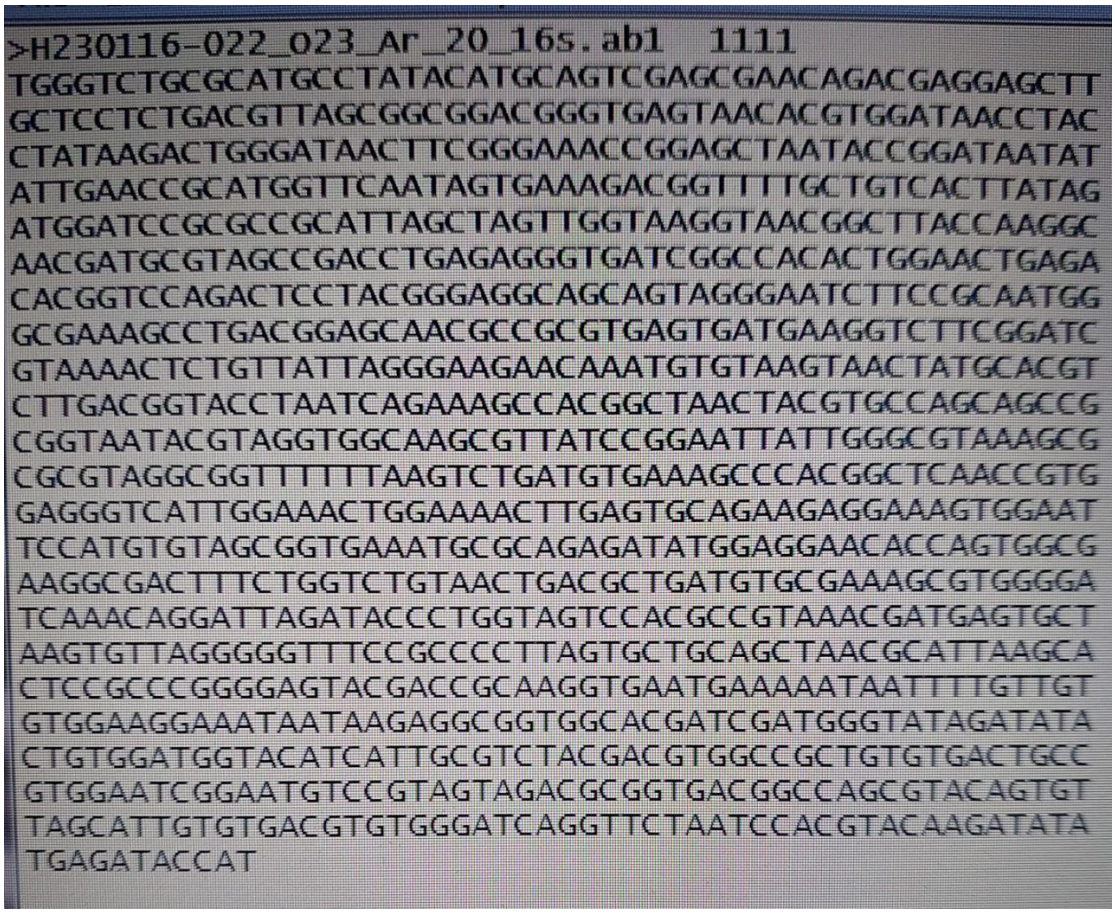

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جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل

كلية العلوم للبنات

قسم علوم الحياة

توصيف البكتريا المهبلية المرتبطة بالإجهاض التلقائي و تقدير مستوى الدم المحيطي للخلايا القاتلة الطبيعية

رسالة

مقدمة الى مجلس كلية العلوم للبنات في جامعة بابل

كجزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل

إيناس لطيف نور الحجار

بكالوريوس علوم حياة، جامعة بغداد، ٢٠١١

بإشراف البروفيسور

أ.د. عروبة كطوف البيرماني

كلية العلوم للبنات/ قسم علوم الحياة

أ.د. سهى جاسم وتوت

كلية الطب/ قسم النسائية و التوليد

جامعة بابل

٢٠٢٣ م

١٤٤٥ ه

الخلاصة:

الإجهاض التلقائي SA هو مرض خطير و يحدث فيه ان المرأة تفقد جنينها عادة قبل الاسبوع الرابع والعشرين من الحمل . تقريبا ١٠% الى ٥٠% من حالات الحمل تنتهي بإجهاض تلقائي لأسباب تتعلق بعمر المرأة وصحتها.

مما يعزز من مخاطر تطور هذا المرض اذا كانت المريضة لها تاريخ من الاصابة بمرض المهبل البكتيري. تتناول العديد من الدراسات الخلايا القاتلة الطبيعية (NK) باعتبارها عاملاً محتملاً مساهماً في اختلال التوازن في التحمل المناعي الذي قد يسبب العديد من حالات الإجهاض التلقائي غير المفسر (USA). وقد تحدث الولادة السابقة لأوانها والإجهاض التلقائي عندما يشهد النظام التناسلي للمرأة المضيف زيادة في عدد بكتيريا *Mycoplasma hominis*.

تم جمع ٥٠ عينة من المسحات المهبلية العالية (HVS) و٥٠ عينة من الدم من النساء المصابات بإجهاض تلقائي. كذلك تم جمع ٥٠ عينة (HVS) و٣٠ عينة من الدم من النساء اللاتي لديهن نتائج حمل صحية من مستشفى بابل التعليمي للولادة والأطفال ومن مستشفى الإمام الصادق التعليمي في مدينة الحلة خلال الفترة من تشرين الأول/أكتوبر ٢٠٢٢ إلى كانون الأول/ ديسمبر ٢٠٢٢. وكانت الفئة العمرية للمرضى فضلاً عن عينات السيطرة تتراوح بين ١٧ و٤٥ سنة.

انقسمت هذه الدراسة إلى جزأين: ركز الجزء الأول على تشخيص الاحياء المجهرية المهبلية، التي تعتمد على زراعة HVS على عدد من الاوساط الزرعية مثل (وسط أكار الدم، (MRS)، واكار الكروموجينك الخاص بأمراض المسالك البولية (UTIC)، واكار المانتول الملحي، اكار الماكونكي واكار ايوسين المثيلين الازرق (EMB)) تحت الظروف الهوائية واللاهوائية.

أعتمد تحديد البكتيريا في البداية باستخدام التقنيات البكتريولوجية التقليدية على الخصائص المظهرية للمستعمرات و الاختبارات الكيميائية الحيوية وصبغة غرام. كما تم التأكد من التشخيص وراثياً من خلال عزل الحمض النووي الجينومي للبكتيريا، وتضخيم قطعة من جين 16S rRNAمن خلال تفاعل سلسلة البوليميريز (PCR)، و تحديد التسلسل النيوكلوتيدي.

واستلزم الجزء الثاني من الدراسة جمع الدم للحصول على المصل لتقييم مستويات الخلايا القاتلة الطبيعية المحيطية وتشخيص العدوى ببكتريا *Mycoplasma hominis* بالطرق المناعية باستخدام تقنية ELISA.

وتبين النتائج المستخلصة من هذه الدراسة تفشي فئة الدم O+ يظهر في النساء المصابات بإجهاض تلقائي. ويبين أيضاً هيمنة الفئة العمرية ٢٦ إلى ٣٥ عاماً على كل من الحوامل والنساء اللاتي أجهضن.

واظهرت نتائج الدراسة الحالية أن هناك فرقاً في الاجناس البكتيرية لدى النساء اللاتي لديهن SA مقارنة بالنساء الأصحاء، ولوحظ تورط بكتيريا *Enterococcus faecalis* في معظم حالات الاجهاض التلقائي حيث تقدر النسبة المئوية ب ٥٦ % (٥٠/٢٨)، وبذلك تتغلب على بكتيريا الإيشيريشية القولونية *Escherichia coli* بنسبة ٣٢% (٥٠/١٦) و٤ % (٥٠/٢) *Klebsiella pneumonia* و٤% (٥٠/٢) *Enterococcus gallinarum*. وفي هذه الدراسة، تم تحديد أنواع نادرة جدا من البكتيريا، بما في ذلك *Acinetobacter junii* بنسبة ٢٪ (٥٠/١) و*Corynebacterium coyleae* بنسبة ٢٪ (٥٠/١)، في حين كانت النسبة المئوية من البكتيريا المرتبطة بعينات السيطرة هي: ٣٠٪ (٥٠/١٥) بالنسبة لـ *E. faecalis*، و٢٦٪ (٥٠/١٣) بالنسبة لـ *E. coli*، و١٨٪ (٥٠/٩) بالنسبة ل *Klebsiella pneumonia* و ٢٤% (٥٠/١٢) كانت نسبة بكتريا *Staphylococcus epidermidis* ٢% (٥٠/١) لبكتريا *Metabacillus niabensis* (والتي تم تشخيصها لأول مره في عينات سرسرية في العراق وكذلك بقية العالم).

كشف التحديد الجزيئي للعزلات البكتيرية والتسلسل الجيني 16S rRNA النسبة المئوية العالية من *Enterococcus faecalis* في SA، وهذا يقود إلى حقيقة أن سلالات *E. faecalis* تمتلك ترسانة من عوامل الضراوة التي تسهم في قدرتها على التغلب على مختلف آليات الدفاع في المضيف وبذلك تسبب المرض. تم استخدام تقنية PCR لتضخيم جين *asa1* في هذا البحث حيث تبين الدراسة الحالية ارتباط الجين بقابلية البكتريا على احداث المرض في العينات التي تم تنقيتها ومعرفة تسلسلها النيوكليوتيدي من عزلات *E. faecalis* النموذجية ويتعزز الالتصاق البكتيري بخلايا المضيف والإمراضية بفعل المواد التجميعية aggregation substances ،التي تمثل عامل الضراوة. . أظهرت النتائج ان نسبة البكتريا الموجبة لهذا الجين في عينات المرضى كانت ٨٥%.

وقد قورنت جميع تسلسلات العزل البكتيري بالتسلسلات المعروفة في قاعدة بيانات NCBI باستخدام تحليل BLAST وتم إيداع التسلسلات في قاعدة بيانات NCBI Gene bank.

اعتماداً على البيانات التي ظهرت في العمل، فان هناك تباين في التراكيز بين المرضى وحالات السيطرة. وأظهر التحليل الإحصائي أن تركيز خلايا NK في المرضى لم يكن ذو اثر واضح مقارنة بحالات السيطرة، المتوسط الحسابي والانحراف المعياري في المرضى (١٠٧.٣٣٩±٤٦.٦٤٧)، بينما في الكونترول (٨٠.٢٢٩±٢٣.٧٣٧) على التوالي. وتوحي هذه النتيجة بأن الخلايا القاتلة الطبيعية المحيطية بنفسها قد لا تتسبب في الاجهاض التلقائي، او ربما تكون من النوع السام NKC.

بالإضافة إلى ذلك، فحصت الدراسة تأثير *Mycoplasma hominis* في المرضى. التحليل الإحصائي للبيانات يشير إلى أن هناك فرق طفيف جدا في التراكيز بين المرضى والكونترول. لم يكن تركيز *Mycoplasma hominis* في المرضى كبيراً بالمقارنة مع حالات السيطرة، المتوسط الحسابي والانحراف المعياري في المرضى (٠.٣٩٨٣±٠.٢١١٦٦)، بينما في الكونترول (٠.٣٢٤٩٧±٠.١٣٠٤٦١)، على التوالي. هذه السلالة البكتيرية هي متطفلة داخل خلوية والتي تستوطن مهبل المرأة ولكنها لا تسبب المرض لدى الأشخاص الأصحاء. في حين ، في الأشخاص الذين يعانون من ضعف الجهاز المناعي، تتشجع هذه البكتريا على التكاثر بصورة كبيرة وبذلك يمكن ان تؤدي إلى حالات مثل مرض الالتهاب الحوضي.

وتحلل الدراسة الحالية تركيبة الكائنات الحية المجهرية المهبلية، ولا سيما المايكوبلازما المهبلية، في مرضى SA وتربطها بالحالة المناعية. حيث أنها أظهرت أن هناك علاقة عكسية بين تركيزات خلايا NK و تراكيز*Mycoplasma hominis* في المرضى، فكلما ارتفعت تراكيز الخلايا القاتلة الطبيعية، كلما انخفض تركيز *Mycoplasma*، والعكس بالعكس.