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Phenotyping,genotyping and immunological study of otitis media infection

A Thesis

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By

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بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

((قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ))

صدق اللهُ العظيم

(البقرة-الآية٣٢)

CERTIFICATE

We certify that this thesis, entitled (**Phenotyping,genotyping and immunological stady of otitis media infectionis**) was prepared by (**Safa Amer Khalil**) under our supervision at the college of science for women, University of Babylon as a partial requirement for the degree of Master of Science in Biology.

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Dedication

To my beloved parents

I dedicate this thesis

Without them I could not have accomplished this Work

My Teachers & Mentors

The Supporters…

My brother and sisters

Friends & Colleagues

To everyone who helped me in every possible way to make this work see the light

Safa

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Acknowledgment

First of all, I thank GOD for inspiring me with the strength, patience and willingness to perform this work.

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Safa

2023

Summary :

Otitis media (OM) is an inflammation and infective condition of the middle ear mucosa. If the inflammation is accompanied by discharge and perforation in the tympanic membrane.

This study was conducted in the Faculty of Science for women, University of Babylon. 74 clinical samples were collected from patients suffering from otitis media in different age groups, their ages ranged from (1-72) years, and the study period was from October 2022 to January 2023 in the hospitals of Babylon (Hilla Teaching, Imam Al-Sadiq).

The current study was carried out with a number of steps, including the identification of bacteria and fungi associated with otitis media. Bacteria were identified by routine, molecular and antibacterial efficacy tests, fungal isolates were identified by traditional methods and the antifungal efficacy of fungal isolates. The results showed that 62 of the samples gave growth for bacteria and the other samples 12 samples did not show growth for bacteria. Fungi gave 59 positive samples for culture and 15 negative for culture.

The results of gender showed that the percentage among females was (55.5)% higher than among males, as it reached (44.5)% among males. This study revealed that the most affected age among both sexes ranged from (20-40 years). As well as the distribution of patients according to the situation of the living area in the city with a rate of (75.7)% and in the countryside (24.3)%. Those who took antibiotics were recorded in this study (56.8%) of those who did not take (43.2)%. Chronic otitis media was the highest rate among patients.(٦٢.٢)% and acute (37.8)%. Of the clinical samples, *Pseudomonas aeruginosa* bacteria

had the highest presence 24(32.4)% followed by *Staphylococcus aureus* 19 (25.7)%.

The most abundant resistant strain of *Pseudomonas aeruginosa* exhibited varying levels of resistance to seven different antibiotics, with the highest resistance observed against Polymyxin B, Gentamicin, and Ceftriaxone. On the other hand, it showed the highest sensitivity to Imipenem. Conversely, *Staphylococcus aureus* strains displayed the highest resistance to Tetracycline and Amoxicillin, while showing high sensitivity to Levofloxacin.To identify the fungi present, the specimens were cultured on SDA medium. After their growth, they were microscopically examined and CROMAgar medium was used for the diagnosis of *Candida* spp.

The results revealed the predominance of *Aspergillus* spp. isolates, with the highest percentage being A. niger (36.5%), followed by A. flavus (31.1%). Four species of yeast were also isolated and identified from patient samples, with the highest percentage attributed to C. glabrata and C. krusei, accounting for 12.2% and 8.1%, respectively. The second most prevalent yeast species was C. albicans, with a percentage of 4.1% .The most effective antifungal agents in this study were clotrimazole, itraconazole, nystatin, and amphotericin B, which exhibited the highest sensitivity against the fungi.

otitis media (OM) by genotyping 60 specimens30 patients with OM and 30 healthy individuals. This is done to detect genotypic polymorphisms for two genes, MUC5B and IL-1RN, using the variable numbers of tandem repeats (VNTR) region. The results indicate that carriers of allele 2 have an almost doubled risk of developing otitis media.

A genetic polymorphism analysis to IL17A was conducted on 60 specimens of human blood DNA, including 30 patients and 30 healthy individuals. the frequency of risk allele A in both heterozygous (GA) and Homozygous (AA) in SNP rs2275913 based on the Odd Ratio. The results shown lower in genotype GA was OR= 0.57(0.18 - 1.72) with p value 0.3, and the allele frequency was lower in with A allele.

Based on the Elisa method, the level of TNF in the serum was measured in 25 patient samples compared to 10 healthy individuals. The results indicated a difference in tumor necrosis factor-alpha (TNF-a) between the patients and the healthy subjects (272.85+132.21 for patients vs 200.5+36 for the control group). The distribution of serum TNF-a was also analyzed according to bacterial types. The results of the statistical analysis showed a significant increase ($P < 0.05$) in serum TNF-a in pseudomonas aeruginosa bacteria compared to other bacteria. Furthermore, the age group below 20 years had the highest TNF-a level (285.41), followed by the age group above 40 years with a TNF-a level of (264.19), while the low level of TNF-a (107.48) was observed in the age group of 20-40 years. The effect of gender on TNF-a levels was examined, and the statistical analysis results indicated no significant effect ($p > 0.05$) of gender on the levels of TNF-a in both males and females. The statistical analysis showed a significant increase in TNF-a levels in patients with chronic stage compared to acute stage.

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List of Abbreviations

NO.	Items	Meaning
1.	**AB**	Amphotericin –B
2.	**OM**	Otitis Media
3.	**AOM**	Acute Otitis Media
4.	**CSOM**	Chronic Suppurative Otitis Media
5.	**CC**	Clotrimazole
6.	**ENT**	Ear- Noise-Tonsils
7.	**D.W.**	Distilled water

8.	**EDTA**	Ethylene di-amine tetra acetic acid
9.	**DNA**	Deoxyribonucleic acid
10	**TBE**	Tris borate-EDTA buffer
11	**TE**	Tetracyctine
12	**VA**	Vancomycin
13	**AX**	Amoxicillin
14	**LEV**	Levofoxacin
15	**FLU**	Fluconazole
16	**IFI**	Invasive fungal infections
17	**PB**	polymyxin B
18	**IT**	Itraconazole
19	**ITS**	Internal transcribed spacer regions
20	**KT**	Ketoconazole
21	**NS**	Nystatin
22	**PCR**	Polymerase chain reaction
23	**AZM**	Azithromycin
24	**GEN**	Gentamicin
25	**SDA**	Sabouraud Dextrose Agar
26	**TNF**	Tumor necrosis factor
27	**MSA**	Mannitol salt agar
28	**SNPs**	Single nucleotide polymorphisms
29	**MHA**	Mueller-Hinton agar
30	**WHO**	World health organization
31	**IA**	invasive aspergillosis
32	**Nys**	nystatin

Chapter One

Introduction

1.1. Introduction

Otitis media (OM) is defined as an inflammation and infective condition of the middle ear "middle ear mucosa". If the inflammation is associated with discharge and perforation in the tympanic membrane, it results in suppurative OM. It can be acute or chronic. It is one of the most common infectious diseases of the childhood worldwide and may lead to many long-term complications, including conductive and sensorineural hearing loss (Selvakumari *et al*., 2020).

The most common complication is perforation of the tympanic membrane. This is caused by increased pressure produced by fluid accumulation in the middle ear. Patients may describe ear pain, hearing loss, otorrhea, tinnitus, or vertigo (Szmuilowicz & Young, 2019).

Although the disease can affect all age groups, infants and young children are more commonly affected due to the short Eustachian tube that allows easier entry of microorganisms to the nasopharynx. Internationally, the prevalence of ear infection differs depending on the health and economic status. For instance, in the USA and Europe, the prevalence is decreasing due to increased hygiene and awareness, whereas it is increasing in the developing countries (Addas *et al*., 2019).

The majority of these infections is caused by bacteria. The indiscriminate, imprecise, improper, and haphazard use of antibiotics have caused the emergence of multiple resistant strains of bacteria, which can produce both primary and post-operative infections (Kumar& Singh, 2019). Wide-spectrum antibiotics are used in huge amount and in inappropriate way that has triggered difficulties to treat the bacterial isolates which became worldwide wellbeing risk In ear infection high numbers of antibiotic are used to treat bacteria results in the initiation of secondary infections of Brain (Javed *et al*., 2020).

There exist many pathogens that cause inflammation of the middle ear which causes bacterial, viral and fungal, either bacterial infection can be attributed to several bacterial genera such as *Staphylococcus aureus*, *Proteus mirabilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (Kadhim *et al*.,2018).

In recent years fungal infections have gained more attention in medicine due to large number of immunocompromised patients however, these fungi may also yield infections in immunocompetent hosts. *Aspergillus niger* and *Candida* species are the most prevalent fungi in immunocompetent patients (Yahia & Alsayed, 2021).

Otitis media is mostly diagnosed with the use of an otoscope, which is a small handheld medical device with a light source and a magnifying lens, allowing the general practitioner (GP) to get a visual impression of the tympanic membrane (Sundgaard *et al*. 2021).

Mucin glycosylation may impact innate immune responses in the middle ear. Although MUC5B are beneficial antimicrobial defense structures, their rapid clearance from airway surfaces is paramount in preventing inflammatory deleterious effects (Shirai & Preciado, 2019). Proinflammatory cytokines such as tumor necrosis factor (TNF-α) and the interleukins (ILs), (Abdel-Razek *et al*., 2022). Mutations in genes such as CCL3, IL-17. Genetic variants and polymorphisms in several genes, including FNDC1, FUT2, A2ML1, TGIF1, CD44, and IL1-RA variable number tandem repeat (VNTR) allele 2, have been identified as being significantly associated with OM (Geng *et al*.2020).

The Aim of Study

The aim of the present study is to study the spectrum of cases with bacterial and fungal otitis media by following objective:-

1- Isolation and identification the microorganisms associated with otitis media Infection fungi and bacteria.

2- Evaluate the risk factors such as age, gender, presence of chronic disease and duration.

3- Testing the antifungal and antibacterial susceptibility of microorganisms isolates.

4- DNA extraction for blood specimens for each patients and molecular detection of some genetic risk factors such as IL-17 gene by sequencing analysis of PCR products, while IL1-RA and MUC5B by VNTR polymorphism

5- Detection of (TNF) by using ELISA technique.

Chapter Two

Literatures Review

2. Literature Review

2.1. Anatomy of ear

Understanding the pathophysiology of ear disease begins with the normal anatomy of the temporal bone. The temporal bone contains the body's hearing and balance function. The inner ear is composed of the cochlea and vestibular systems. The tympanic cavity is an air-filled middle ear cleft that contains the ossicular chain hearing bones and is bordered laterally by the tympanic membrane (Fig. 2-1).

The facial nerve, chorda tympani nerve, and Jacobson nerve (tympanic branch of the glossopharyngeal) run through this space. The mastoid air cell system exists in continuity with the tympanic cavity and contributes to mucosal gas exchange and regulation of pressure within the middle ear cleft. The tympanic cavity is connected to the nasopharynx by the Eustachian tube, a complex, dynamic canal of mucosa, cartilage, muscles, and bone that allows gas exchange between the nasopharynx and middle ear. Normal gas exchange through the Eustachian tube maintains pressure in the middle ear cleft that is equivalent to atmospheric pressure (760 mm), which enables optimal sound transmission (Emmett *et al*., 2018).

The angle is shallower in children and has been proposed to be a contributing factor to otitis media ،although this angle does not differ between children with and without otitis media (Takasaki *et al* ., 2007). The Eustachian tube grows from approximately 38 mm in children to 44 mm in adults (Tysome & sudhoff ,2018).

microorganisms, occasionally including fungi,within the middle ear. Among these bacteria, Pseudomonas aeruginosa prevails and is deemed clinically consequential due to its adeptness in developing biofilms and capacity for antibiotic resistance. Alongside it Staphylococcus aureus usually cohabits with a similar inflammatory impact, as this bacterium can generateharmful toxins that trigger tissue damage(Khairkar *et al*.,2023).

As the matter of fact ear performs two sensory function :hearing and maintenance of equilibrium balance of the body. Ear can be divided in to three parts Figure (2-1) outer ear, middle ear and inner ear (Poorey, 2002).

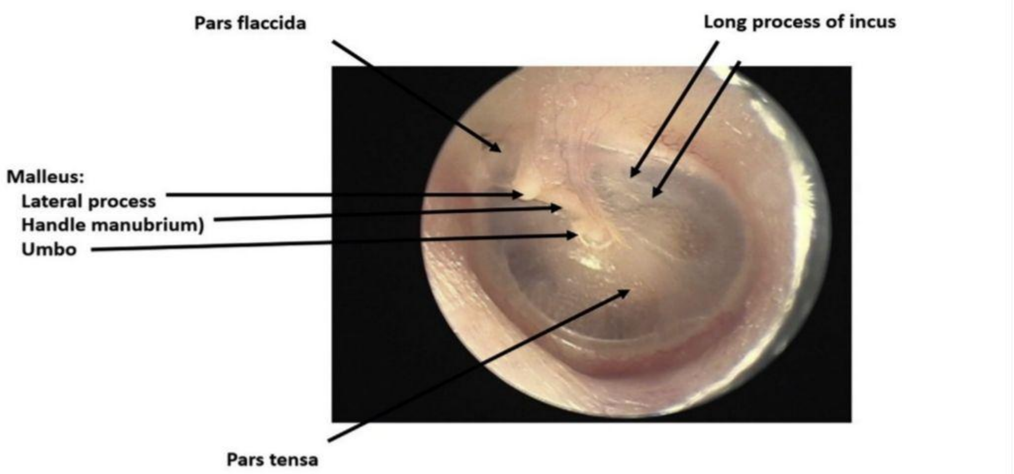


Figure (2-1): Otoscopic view of a normal left tympanic membrane (Sudhoff,2016).

2.1.1. Outer ear

It consists of two parts, pinna and external auditory meatus. The external auditory meatus extends up to the tympanic membrane (ear drum). Pinna and external auditory meatus possess fine hairs and wax secreting sebaceous glands(Vaghela *et al*.,2017).

2.1.2. Middle ear

The middle ear is defined by the TM and the adjacent air-containing chamber that houses the 3 auditory ossicles. Sound waves enter the external ear and are funneled to the middle ear, where the vibrations are then transmitted and amplified via the TM and auditory ossicle to the inner ear. The middle ear chamber additionally communicates with the eustachian tube and the mastoid air spaces(Szmuilowicz & Young, 2019).

2.1.3. Inner ear

Includes the bony labyrinth (otic capsule), which consists of the cochlea, vestibule, semicircular canals, vestibular aqueduct, and membranous labyrinth, which includes the utricle, saccule, endolymphatic sac and duct, as well as the cochlear duct. A fluid known as perilymph fills the space between the osseous labyrinth and membranous labyrinth while the membranous labyrinth is filled with endolymph (Feraco *et a*l .,2021).

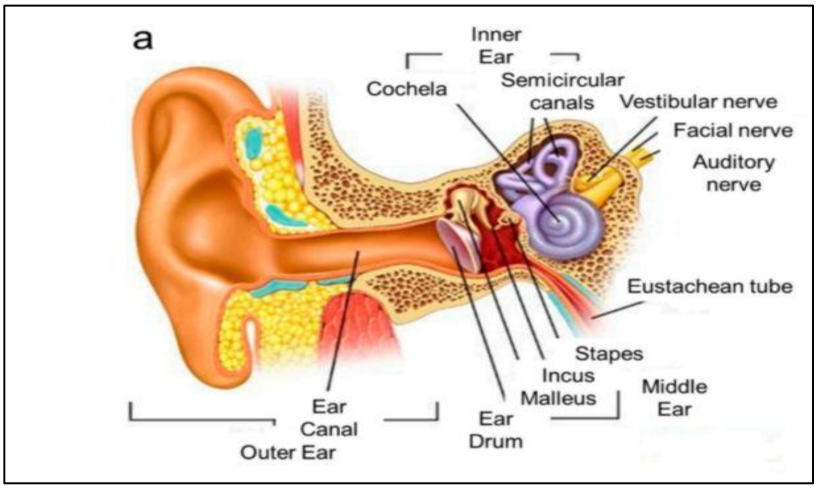


Figure (2-2): Three parts of ear (Prasad *et al*., 2020).

2.2. Ear infection

Microbial agents can infect the middle and external parts of the ear and may involve the skin, cartilage, periosteum, ear canal, and tympanic and mastoid cavities (Argaw-Denboba *et al*., 2016). The infection is usually superficial and extends into the canal to the tympanic membrane (Kurabi *et al*., 2018).

Ear infection is divided to three types according to the site (Barnard, 2017).

2.2.1. Otitis media (OM)

It is a spectrum of infectious and inflammatory diseases that involve the middle ear (Principi & Esposito ,2020). It includes acute otitis media (AOM), otitis media with effusion (OME) is an accumulation of fluid in the middle ear without acute inflammation or infection (Fu *et al*.,2019) and chronic suppurative otitis media (CSOM). AOM is an acute disease, and OME can be both acute and chronic, whereas CSOM is a chronic condition. All of them, particularly AOM and OME, are extremely common in children, particularly the youngest, due to the immaturity of the immune system, the high frequency of upper respiratory tract infections (Marchisio *et al*. 2014).

Although otitis media can occur at any age, it is most commonly seen between the ages of 6 to 24 months (Meherail *et al*., 2019). OM is also a leading cause for medical consultation ،antibiotic prescription, and surgery in childhood (Ciprandi *et al*., 2020).A cute otitis media and is typified by recurrent or persistent otorrhea through a tympanic perforation often with associated thickening of the middle ear mucosa (Shirai & Preciado,2019).

Without proper treatment, suppurative fluid from the middle ear can extend to the adjacent anatomical locations and result in complications such as tympanic membrane (TM) perforation, mastoiditis, labyrinthitis, petrositis, meningitis, brain abscess, hearing loss, and others (Garcia, 2017; Principi & Esposito, 2019).

There exist many pathogens that cause inflammation of the middle ear which causes bacterial, viral and fungal, either bacterial infection can be attributed to several bacterial genera such as *Staphylococcus aureus*, *Proteus mirabilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (Kadhim *etal*.,2018; Dhingra *etal*.,2023). Otitis media include the respiratory syncytial virus (RSV), coronaviruses, influenza viruses, adenoviruses, human metapneumovirus, and picornaviruses (Ubukata *et* *al*., 2019; Protasova *et al*. 2017). Otitis media is a multifactorial disease. Infectious, allergic,involving genetic and environmental factors contribute to otitis media (Lu *et al*.,2023).

2.2.1.1. Acute otitis media (AOM)

Acute otitis media (AOM) is defined as an infection of the middle ear and is the second most common pediatric diagnosis in the emergency department following upper respiratory infections (Danishya & Ashurst, 2022). AOM may also be associated with purulent otorrhea if there is a ruptured tympanic membrane. AOM usually responds promptly to antimicrobial therapy (Limb *et al*., 2017) .

Both bacterial and viral pathogens can cause AOM, (van Dongen *et al*., 2015). it is usually considered to be a bacterial complication of upper respiratory tract viral infection. (Suzuki *et al*., 2020) . This infection of the middle ear cavity is associated with signs and symptoms of rapidly emerging acute inflammation. Ear pain (otalgia) and fever are the most common signs seen in AOM(Jamal *et al*.,2022) . has a major impact on health, given the high number of consultations ،use of antibiotics, related surgery and the need for auditory rehabilitation (Calatayud-Sáez *et al*., 2022).

2.2.1.2. Chronic otitis media (COM)

Chronic otitis media (COM) is an enduring inflammation of the middle ear and mastoid cavity that is presented under several terms chronic suppurative otitis media chronic active mucosal otitis media, chronic oto-mastoiditis and chronic tympanomastoiditis. (Lewis *et al*., 2021).COM is a highly prevalent disease worldwide. Its prevalence in developing countries is as high as 72 cases per 1000 inhabitants.

COM the complications of the disease may lead to death (Ulku *et al*., 2019). COM typically produces conductive hearing loss due to tympanic membrane perforation, (Amali *et al*.,2017). The most common cause of hearing impairment is still COM The hearing loss is due to chronic inflammatory process which is mostly conductive as a result of tympanic membrane perforation and ossicular chain fixation or erosion (Ali *et al*., 2020).

The COM is mainly divided into two types such as a atticoantral and tubotympanic COM. The atticoantral COM comprises of scanty foul smelling discharge with perforated TM involving its margins. Cholesteatoma in addition to the granulations are major related complications (Shakoor *et al*., 2022).

2.2.2. Otitis Externa

It refers to an inflammatory condition of the external auditory canal, with anatomical regions which may stretch distally to the pinna and proximally to the tympanic membrane of the ear (Wiegand *et al*., 2019; Marais, 2015). Acute otitis externa is usually caused by bacteria while fungal involvement is commoner with chronic otitis externa (Allam *et al*.,2020).

The most likely pathogens include *Pseudomonas aeruginosa* and *Staphylococcus* spp, AOE is often a polymicrobial infection. Less often, gram-negative bacteria may be involved. Rarely, AOE may result from a fungal (*Candida* or *Aspergillus*) infection (Wipperman, 2014; Tarazi *et al*., 2012). Otomycosis, also called otitis externa mycotica, is an inflammatory lesion caused by the invasion or the excessive propagation of pathogenic fungi in the external auditory canal (Gu *et al*., 2022).

It is a common disease, accounting for approximately 10–20% of ear canal infections (Kiakojuri *et al*., 2021) Globally, otomycosis is highly prevalent in tropical and subtropical areas with high temperature and high humidity. Otomycosis is often seen in people with antibiotic and steroid use, or who have a weakened immune system or diabetes (Viswanatha & Naseeruddin, 2011 ; Jia *et al*., 2012).

Other risk factors include exposure to contaminated water, frequent ear picking, and chronic otitis media (Jin *et al*., 2015 ; Chen *et al*., 2021). It causes symptoms such as ear itching, aural fullness, otorrhea, otalgia, hearing impairment, and tinnitus.The *Aspergillus* species as *A. niger* and *A. flavus*, followed by the *Candida* species as *C. albicans*, have been reported to be the most common pathogens of otomycosis in the literature (Fang *et al*., 2019; Tasić-Otašević *et al*., 2020).

Otitis externa can be classified as acute (lasts less than 6 weeks) or chronic (lasts more than 3 months). It is also known as swimmer's ear as it often occurs during the summer and in tropical climates and having retained water in the ears increases the risk for it (Medina-Blasini & Sharman, 2022).

The inflammatory response in otitis externa is believed to be caused by a disruption of the normal pH and protective factors within the auditory canal. (Hajioff & Mackeith, 2015; Kaushik *et al*., 2010). And include otalgia ،pruritus, a sense of fullness, drainage ،and occasionally impaired hearing .Signs of inflammation include tender ness or pain with manipulation of the pinna and with pressure on the tragus ،erythema and edema of the external auditory canal, and otorrhea (Long , 2013).

2.2.3. Inner ear infection

The inner ear is sensitive to infections that may produce permanent Sensorineural hearing loss and vestibular dysfunction (Dewyer *et al*., 2018; Prince & Stucken, 2021). One criterion for distinguishing between different inner ear infections concerns the affected structures. From this point of view, there are two main possibilities: labyrinthitis and vestibular neuronitis.

As its name implies, labyrinthitis is an infection located in the membranous labyrinth .This structure is usually affected by bacterial translocation into the inner ear (Bontempo& Shoenberger, 2019). Causingvertigo, nausea, vomiting, tinnitus, and even hearing impairment or hearing loss .The inflammation can result through two different mechanisms. Inflammation can be a secondary manifestation caused by bacterial toxins or host cytokines and inflammatory mediators, producing serous labyrinthitis; or it can be caused directly by the bacteria leading to suppurative labyrinthitis Vestibular neuronitis (or neuritis) is often used as a synonym for labyrinthitis, being usually assimilated as a viral infection rather than bacterial inner ear infections can also be classified depending on their causing pathogen. In this respect, three categories can be distinguished viral, bacterial, and fungal infections (Gheorghe *et al*., 2021).

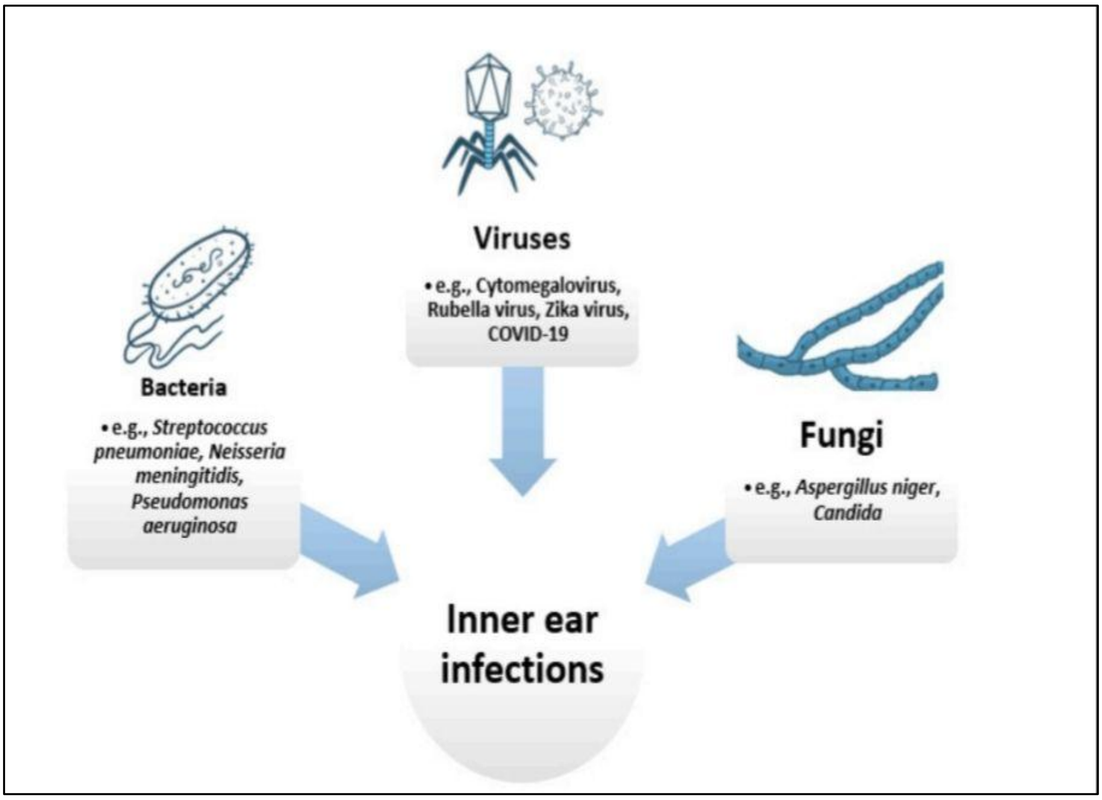


Figure (2-3): Main categories of pathogens causing inner ear infections. Created based on information from literature (Jung *et al* ., 2019).

2.3. Epidemiology

Estimated the average acute otitis media incidence rate at 10.8 new episodes per 100 people per year (Monasta *et al* ., 2012). According to the American Academy of Pediatrics, over 5 million acute otitis media (AOM) cases occur in United States children annually, resulting in >10 million antibiotic prescriptions .About 30 million visits to medical care providers occur due to AOM, resulting in direct and indirect health care costs of about (4-6)$ billion annually(Pichichero *et al*.,2023).Pre-school children in rural India provided population based data from a relatively large sample of 800 children and found the prevalence of OM to be 9.2% (54/587) in children 5 years old (DeAntonio *et al*.,2016).

The World Health Organization (WHO) claimed in 2017 that 330million people suffer chronic Otitis problems (Sikakulya *et al*., 2018). According WHO's report in 1996, Western Pacific countries have a higher prevalence (2.5 to 43)%, accompanied by South East Asia (0.9 to 7.8)%, Africa (0.4 to 4.2)%, South and Central America (3)%, the Eastern Mediterranean (1.4)%, and finally Europe (average prevalence of 0.4)%.

The bigger the perforation of the tympanic membrane, the more likely it is for the patient to grow CSOM (Vikram *et al*., 2008).Community-based epidemiologic studies that examined children in Bangladesh, Nigeria, and Australia reported prevalence of chronic suppurative otitis media (CSOM).

Which was 12%, 2.5% and 15%, respectively. In the Australian aboriginal study 41% had OME, 33% had AOM, and tympanic membrane perfora tions affected 40% of children in their first 18 months of life . Incidence of OM by 6 months was 63% in a cohort of American Indian children (Daly *et al*.,2010).

2.4. Risk factors of otitis media

Risk factors for ear infections still remains a problem of identification and management (Taylor *et al*.,2012). This condition is a leading cause of antibiotic prescription, being an important determinant of preventable hearing loss with negative effects on language, psychosocial, and cognitive development (Gavrilovici *et al*.,2021). The risk factors for ear infections are cultural, socioeconomic, and environmental (such as living in crowded places, living in large families, the duration of breastfeeding, smoking status), genetic (like craniofacial anomalies such as those characteristic in Down syndrome and cleft palate), nutritional, and medical (such as age, gender, race, health status, history of several acute otitis episodes, rhinorrhea, allergic rhinitis, seasonal rhinitis, snoring, upper respiratory tract infections, and adenoid hypertrophy) (Jones *et al*., 2012; Kørvel-Hanquist *et al*., 2018; Hardani *et al*., 2020).Cleaning ear with unsterile material, trauma to the ear all are individual factor in the development of chronic otitis media. Insertion of tympanostomy tube is also a recognized cause of subsequent tympanic membrane perforation (Uddin *et al*.,2021).

2.5. Diagnosis

AOM is diagnosed by otoscopy and can be further assessed using a symptom severity scale. Tympanic membrane perforation associated with CSOM may be diagnosed with otoscopy or otomicroscopy, but may require removal of ear discharge by suctioning for adequate visualization.

Diagnosis and interpretation accuracy of otoscopy is highly variable, largely dependent on physician expertise, and has been shown to range from 40 to 70% (Schilder *et al*., 2016; Merchant *et al*., 2021).

2.6. Bacteria role of otitis media

Bacterial infection is otitis media. Generally caused by microorganisms like *P.aeruginosa, Staph.aureus, P.mirabilis, K.pneumonia and E. coli*, this infection is mainly located in the middle ear (Gheorghe *et al*., 2021). Out of the enumerated pathogens, *P.aeruginosa* is one of the most common bacteria to produce chronic suppurative otitis media and reach perilymph by entering through the round window (Juyal *et al*., 2017).

Moreover, recurring otitis media can destroy ear structures such as small bones, seventh cranial nerve or inner ear, leading to permanent hearing loss (Mohammed & Abdullah, 2020) .

Can also result as a complication and sequela of bacterial infections, such as meningitis (Orman *et al*., 2020). Bacterial nasopharyngeal colonization transition to Middle ear inflammation Viral infection of the nasopharynx alters the environment in this mucosa, modifying host immune function, inducing cytokine activity and inflammatory mediators, and increasing bacterial colonization and adherence to host cells through the upregulation of antigens that act as bacterial receptor sites (Silva & Sillankorva, 2019).

2.7. Pathogenesis of Viral Infection in Otitis Media

Various types of viruses that cause upper respiratory tract infections can induce acute otitis media, including respiratory syncytial virus, rhinovirus, adenovirus, coronavirus (including COVID-19) bocavirus, influenza virus, parainfluenza virus, enterovirus, and human metapneumovirus (Chonmaitree *et al*.,2008 ; Nokso-Koivisto *et al*., 2015; Raad *etal*.,2021).

Viral infection can induce the changes of nasopharyngeal mucosa through modification of host immune function ,promote cytokine activity and proinflammatory mediators and increase the colonization and adherence of bacteria through the upregulation of host cell surface antigens that serve as bacterial receptor sites (Avadhanula *et al* .,2006 ; Patel *et al*.,2009) .

Viral infection promoted the changes in mucus character and disturbed the normal mucociliary clearance in the eustachian tube and nasopharynx.This will lead to eustachian tube dysfunction and promote negative middle ear pressure. Negative middle ear pressure will facilitate the influx of bacteria or viruses into the middle ear (Marom *et al*.,2012) . Independent viral infections can cause acute otitis media However, most acute otitis media occurs after a symptomatic viral upper respiratory tract infection. Where, about 27% of acute otitis media is related to upper respiratory viral infection, especially in those who are under 1 year old (Chonmaitree *et al*.,2015) .

In the case of COVID-19, direct inoculation of SARS-CoV-2 in the middle ear was documented .With the presence of receptors in the middle ear and evidence of COVID-19 inoculation in the middle ear, there is a possibility that COVID-19 can cause further infections in the middle ear. and is situation may then result in the formation of fluid in the middle ear (Mather *et al.*,2021).

Infection in the middle ear can be an infection with or without perforation of the tympanic membrane . fluid produced in the middle ear may contain the virus or part of the COVID-19 virus (Morris *et al* .,2010 ; Venekamp *et al*.,2020).

2.8. Allergy and Otitis Media

A recent review discussed the interaction between allergy and middle ear infection ,by considering the impact of infections in the development of OM. Nevertheless, the role of allergic inflammation was proposed as a promoter of pathogen invasion and increased susceptibility to upper airway infections. Two main mechanisms stand out: Eustachian tube dysfunction and immune dysregulation. The physiological functions of ET are as follows to protect the middle ear from respiratory secretions and internal sound pressure, to drain secretions from the middle ear to the rhinopharynx, to ventilate the middle ear guaranteeing a balance between atmospheric and corporeal pressure, to supply oxygen, and to exchange gases produced into the middle ear (Ciprandi *et al*.,2020) .

Allergic inflammation may affect ET physiology by swelling the mucosa and mucous hyperproduction. On the other hand, allergy may impair the defense mechanisms leading to colonization of pathogens. Different mechanisms are involved in this issue. First, allergy is a type 2 immune reaction(Annunziato *etal*.,2015).

Therefore, allergy is characterized by a T helper 2 polarization and consequent defect of type 1 immune response that is involved in fighting infections by interferon production (An *et al*.,2018) . The final effect is that allergic subjects have more infections than healthy people, both concerning the respiratory and digestive tract (Cirillo *etal*.,2007) .

Avicious circle is established among allergic inflammation, susceptibility to infections, impaired immune defense, and recovery ability. These pathophysiological mechanisms play a role in explaining the potential link between allergy and the AOM recurrence.

Interestingly, the role of bacterial involvement in OM has been documented as live bacteria and biofilms. Moreover, respiratory infections tend to recur in allergic children .As a consequence, allergy could be envisaged as a promoting factor (Ciprandi& Tosca,2021) .

2.9.Antibiotics Resistance in Bacteria

Bacterial resistance is the capability of bacterial cells to prevent antibiotic bacteriostatic or bactericidal effects(Munita & Arias , 2016). The excessive and unintended usage of antibiotics contributes to resistance development in bacteria (Kraemer *et al* ., 2019). Because of the extensive uptake, the evolvement of microorganisms resistant with the time and problems have arisen with these resistant microorganisms for the treatment of certain infections. Nowadays, resistance is determining as a big issue in the path of new drug synthesis ،developing antibiotic resistance is a major public health problem worldwide (Hasan & Al-Harmoosh , 2020) .

The simplest type of resistance is a natural lack of susceptibility, called innate resistance. This is a constant trait of a species, strain, or whole group of bacteria. A given microorganism is insensitive to an antibiotic due to its 'innate' resistance to certain groups of antibiotics. It may be linked to the absence of a receptor for the antibiotic, low affinity ، cell wall impermeability, or enzyme production (Urban-Chmiel *et al*.,2022). Antibiotics of beta-lactam are a wide class of antibiotics ، including Penicillins, Cephalosporins, Monobactams, and Carbapenems (Fernández-Villa *et al*., 2019) Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to most β-lactam antibiotics, including Penicillin, Cephalosporin, and Monobactam Aztreonam. Infections with ESBL-producing organisms have been associated with poor outcomes (Hashemi *et al*., 2018).

Members of the Tetracycline class of antibiotics inhibit bacterial translation by binding to the 30S 70 ribosomal subunit and interfering with delivery of the incoming aminoacyl-tRNA by elongation factor Tu (EF-Tu) during the elongation phase of protein synthesis (Wilson *et* *al*.,2020). The most prevalent tetracycline resistance determinant in clinical isolates of Streptococci ، Staphylococci and Enterococci (Emaneini *et al*., 2013; Shen *et al*., 2018; Tian *et al*., 2019). Vancomycin has been known as the last line of defense line of against gram-positive cocci infection (Guo *et al*.,2020). The resistance of *Staph. aureus* to Vancomycin is increasing daily, causing widespread concern in the medical community (Haseeb *et al*., 2019).

2.10. Pathogenesis of Otomycosis

The external auditory canal is has a hollow cylinder-like shape; it is approximately 2–2.5 cm in length, enclosed by the tympanic membrane on its proximal end. Its other end is unobstructed and directly exposed to the external environment .The inner surface of the canal is lined with skin, which also covers the outer side of the eardrum and continues onto the surface of the auricle. Intact skin acts as a mechanical barrier, preventing the penetration of microorganisms from the external environment (Bojanović *et al*.,2023) .It also contains modified apocrine sweat glands that secrete cerumen, which has hydrophobic properties and prevents water retention in the ear canal. Cerumen forms a protective layer on the skin’s surface and exhibits antimicrobial properties due to its low pH, creating unfavorable conditions for pathogen development (Newlands ,2006) .

The saprophytic nature of these microorganisms can become pathogenic if the balance between bacterial and fungal growth is disrupted, especially if non-specific and specific body defense mechanisms are compromised .Factors contributing to the development of otomycosis are categorized into environmental and hostderived. The warm and humid climate of tropical and subtropical regions is the most significant external risk factor, and its prevalence varies depending on the geographical area (Abdelazeem *et al*.,2015 ; Lee *et al*.,2021) .

Various factors can predispose patients to otomycosis, but the most common are the extensive use of topical antibiotic eardrops for the treatment of otitis media and otitis externa, excessive cerumen secretion, local trauma of the ear canal, use of hearing aids with an occlusive mold, and immunocompromised health status. Otomycosis can also occur secondary to previous bacterial infection of treated with topical antibacterial drugs (Anwar& Gohar,2014 ;Sangare *et al*.,2021).

The most commonly noted species in majority of studies are *A. niger* and *C. albicans* is by far the most common yeast responsible for external auditory canal involvement (Aboutalebian *et al*.,2019; Tasic_Otasevic *et al*.,2020) .

2.11. Antifungal

Antifungal therapy is a critical component of patient management for invasive fungal diseases. Yet, therapeutic choices are limited as only a few drug classes are available to treat systemic disease, and some infecting strains are resistant to one or more drug classes. The ideal antifungal inhibits a fungal-specific essential target not present in human cells to avoid off-target toxicities.

The fungal cell wall is an ideal drug target because its integrity is critical to cell survival and a majority of biosynthetic enzymes and wall components is unique to fungi. Among currently approved antifungal agents and those in clinical development, drugs targeting biosynthetic enzymes of the cell wall show safe and efficacious antifungal properties, which validates the cell wall as a target. (Perlin ,2020)

Invasive fungal infections (IFIs) have a major impact on morbidity and mortality in humans. Many fungal species cause infections with mortality rates exceeding 50%, and IFIs are re sponsible for about one and a half million deaths every year. Especially *Cryptococcus*, *Candida*, *Aspergillus*, to those fungal related deaths (Brown *et al*., 2012) It even seems that this amount is still increasing, despite the current available antifungal medication(Pfaller *et al.*,2006). Indeed, only four molecular classes that target three distinct fungal metabolic pathways were currently used: fluoropyrimidine analogs, polyenes, azoles, and echinocandins. Several other classes, such as morpholines and allylamines are only used as topical agents due to either poor efficacy (Vandeputte *et al*., 2012).

2.11.1. The polyene amphotericin B (AmB)

Polyenes were, after griseofulvin ,the first fungal-specific antibiotics on the market and ever since, more than 200 polyene antifungals have been discovered of which amphotericin B, Nystatin and Natamycin are most commonly used in antifungal therapy(Carolus *et al*.,2020) .

Amphotericin B has a broad spectrum of fungicidal activity, covering yeasts, moulds, and dimorphic fungi; however, its clinical use is hampered by the lack of an oral formula tion, by acute infusion-related toxicity, and by doselimiting acute as well as chronic nephrotoxicity due to the interaction with human cholesterol-containing membranes. Although vari ous lipid-based formulations of AmB (including liposomal AmB and AmB lipid complex) display a more favorable tolerability and toxicity profile, the nephrotoxic side effects and associ ated electrolyte disturbances, albeit diminished, are not fully eliminated (Safdar *et al*.,2010; Carmona & Limper,2017).

Polyene antifungal amphotericin B (AmB) has been used for over 60 years, and remains a valuable clinical treatment for systemic mycoses, due to its broad antifungal activity and low rate of emerging resistance. There is no consensus on how exactly it kills fungal cells but it is certain that AmB and the closely-related nystatin (Nys) can form pores in membranes and have a higher affinity towards ergosterol than cholesterol. Notably, the high nephro- and hemolytic toxicity of polyenes and their low solubility in water have led to efforts to improve their properties Generally, increases in the activity/toxicity ratio were in good agreement with increasing ratios of selective permeabilization of ergosterol- vs. cholesterol-containing membranes (Tevyashova *et al*.,2023).

2.11.2. Azoles

Azoles are commonly used as frst-line agents in the prophy laxis and therapy of invasive fungal infections in patients with hematologic malignancies, autoimmune diseases, and in those undergoing solid organ or stem cell transplantation Often, azoles must be administered for long periods of time (weeks to months), followed by long-term suppressive therapy (Benitez and Carver, 2019).

Chemically, all azoles are weak bases. Itraconazole and posaco_ nazole are verylipophilic and generally not soluble in water Voriconazole is lipophilic and has limited water solubility. In contrast, fluconazole and isavuconazole are more water-soluble. Moreover, the systemic Azoles are substrates and inhibitors of CYP isoforms to various degrees. Some azoles are also substrates and/or inhibitors of drug transporters. (Nivoix *et al*., 2020).

Azoles mainly include two subclasses based on the number of nitrogen atoms in a ring; The first class includes Imidazoles which consist of Miconazole, Oxiconazole, Econazole, Ketoconazole, Tioconazole, and Clotrimazole with two nitrogen atoms in an Azole ring, while another class includes Triazoles such as Fluconazole, Posaconazole, Itraconazole, Terconazole, and Voriconazole which contain three nitrogen atoms in a cyclic ring. Imidazoles mainly used for the mucosal fungal infections while Triazoles administered both for the systemic as well as for the mucosal infections (Sanglard *et al*., 2009) .

2.12.Genes Involved in Inflammatory Responses in OM

During the past decade, considerable progress has been made in our understanding of the fundamental molecular mechanisms underlying the role of innate immunity and inflammatory responses in OM (Kurabi *et al*., 2016).

The innate immune system plays important roles in the initiation of inflammation, clearance of invading pathogens, and recovery in AOM. the innate immune system, plays an important role in the prompt and full recovery of OM .Notably in this context, the innate immune system is involved in maintaining physiochemical barriers through secretion of epithelial-barrier-related substances from the epithelium and effector proteins and cytokines from effector cells (You *et al* .,2022).

Many genes have been identified that are involved in immunity and inflammatory responses in OM, and their functions have been studied in animal models.Pro-inflammatory molecules, such as TNF-a, IL-1b, and C-C motif chemokine ligand 3 (CCL3), play key roles in the recruitment of inflammatory cells into the ear and the activation of these cells for microbial clearance(Leichtle *et al*., 2010; Deniffel *et al*., 2017).It has also been shown that IL-17A levels are significantly upregulated in ME fluid during AOM. Wang et al. reported that IL-17A promotes neutrophil recruitment to the ME cavity and neutrophil apoptosis for bacterial clearance(Wang *et al*.,2014) .

Inflammation in the middle ear during infection is promoted either by increasing the proinflammatory cytokines or suppressing the anti-inflammatory cytokines. Immune cells have been shown to produce inflammatory cytokines like Tumor necrosis factor alpha (TNF-α),interferon gamma(IFNγ), interleukin (Selvakumari *et al*.,2020).

1.MUC5B

(MUCs) are a group of highly glycosylated proteins that are classified into membrane-associated MUC and secretory according to their characteristics (Sun *et al*., 2020). MUC5B mucins being the predominant polymeric mucin glycoproteins in human airwaymucus secretions. Although MUC5B protein has been postulated to be the most abundant mucin in chronic otitis media (Val *et al*., 2015). Mucins are heavily glycosylated proteins that are considered primarily responsible for the gel-like characteristics of mucoid middle ear fluids (Preciado *et al*.,2010) .They are comprised by a heavy carbohydrate content on a core protein backbone consisting of numerous tandem repeats, whose primary amino acid sequence is unique to each mucin (Ali& Pearson,2007) .

Mucins provide important protective functions for the underlying epithelium, including mechanical protection and pathogen eradication through clearance mechanisms and interaction with the host immune system. However, variation in the quantity and character of middle ear secretions, and specifically mucin secretion, are known to be important in the pathophysiologic mechanisms of otitis media as well (Ubell *et al*.,2008)

Mucins are the only component of middle ear effusions responsible for its viscosity, and overproduction of highly viscous mucins contribute to abnormal mucociliary clear ance in the middle ear (ME), which in turn causes pathology, such as chronic otitis media and hearing loss (Ubell *et al*.,2010).

2. Interleukine-1

Interleukine-1 is one of the frst known interleukins involved in several immune responses IL-1 family consists of 11 members: IL-1β, IL-1α, IL-18, IL-33, IL-1F5 to IL-1F10, and IL-1 receptor antagonist (IL-1RA) Overproduction of IL-1β and IL-1α and consequently the up regulation of IL-1R1 has been implicated in numer ous chronic infammatory and auto-immune disorders (Nouri Barkestani *et al*.,2022).

Comparison of the IL- 1 receptor (IL-1RI) bound to IL-1β and IL-1Ra, combined with earlier mutagenesis work, revealed how IL-1Ra can compete with IL-1β for binding its primary receptor yet prevent engagement of the coreceptor IL-1 receptor accessory protein (IL-1RAcP) and, thus, inhibit IL-1 signaling (Fields *et al*., 2019).

The IL-1 signaling pathway is tightly regulated by soluble IL-1R1 as well as soluble and membrane forms of IL-1 receptor 2 (IL-1R2), each of which act as ligand traps or 'decoys'. Additionally, IL-1ra, a secreted anti-inflammatory cytokine, competes with active IL-1 and blocks binding to their common activating receptor, IL-1R1 (Tahtinen *et al*.,2022) .

IL-1 was first described in 1974, originally called leukocytic pyrogen before be ing renamed IL-1α and IL-1β 11 years later .IL-1α mostly exerts its effects local to the producing cell and has been identified in many cell types, including endothelial cells. Both IL-1α and mature IL-1β are potent proinflammatory molecules that activate target cells via the interaction between two membrane-bound receptors, IL-1R1 and IL-1R3(Green *et al*.,2023) .

The number of studies revealed the association of IL-1 gene cluster with a predisposition to certain inflammatory diseases, but only a few had been performed in OM (Patel *et al*,2006).The genetic variant

+3953 (rs1143634) in IL-1β has been suggested to influ ence production of IL-1β protein while the variable number tandem repeat (VNTR) variant (rs2234663) in anti-inflammato ry IL-1 receptor antagonist (IL-1RA, also called IL-1RN) has been proposed to influence expression of IL-1RA protein (Živković *et al*.,2018) .

3. Interleukin-17 A

Interleukin-17 A is a pro-inflammatory cytokine that up regulates a number of cytokines and chemokines, leading to the recruitment of neutrophils to sites of inflammation In terms of infection, IL-17A has been demonstrated to have a protective role against multiple microorganisms, predominantly extracellular bacteria and fungi. IL-17 as pro inflammatory cytokines, in OME. (Yeghaneh Moghaddam *et al*., 2017). . Cytokines are the central molecular regulators of middle ear inflammation and can switch the acute phase of inflammation in the chronic stage and induce molecular-pathological processes(Yang *et al*.,2021).

Among IL-17 family members, IL-17A has been the most strongly implicated in human health and disease. IL-17A is produced from hematopoietic cells, including Th17, CD8+ cytotoxic T cell (Tc17), γδ T cell, natural killer cell, group 3 innate lymphoid cell (ILC3), and “natural” Th17 cell (Furue *et al*., 2020).

(IL-17A) is the signature cytokine of a subset of CD4+ helper T cells known as Th17 cells (McGeachy *et al*., 2019). is the founding member of a novel family of inflammatory cytokines.The IL-17 family consists of six structurally related cytokines IL-17A (IL-17), IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. IL-17A and IL-17F are the most closely related, are coexpressed on linked genes, and are usually coproduced by type 17 cells (Amatya *et al*.,2017).

2.13. Tumor necrosis factor alpha

(TNF-α) is a cytokine that has pleiotropic effects on various cell types. It has been identified as a major regulator of inflammatory responses and is known to be involved in the pathogenesis of some inflammatory and autoimmune diseases (Jang *et al,* 2021). Structurally, TNF-α is a homotrimer protein consisting of 157 amino acids ،mainly generated by activated macrophages, T-lymphocytes, and natural killer cells (Horiuchi *et al*.,2010). It is functionally known to trigger a series of various inflammatory molecules, including other cytokines and chemokines (Jiang *et al*.,2017).

The inflammatory response and tissue remodeling in the middle ear mucosa are both mediated by cytokines. Cytokines can be released from numerous cell types found within the middle ear cleft ،including all types of epithelial, endothelial, and immune cells (Massa *et al*.,2015). It is possible to utilize these cells as biomarkers to detect or track disease progression, and they may also be employed as treatment specific criteria in the clinic (Kany *et al*.,2019) .

Middle ear mucosa contains a variety of cytokines, including proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, interferon (IFN)-γ and IL-6 ؛immunoregulatory cytokines such as IL-2, IL-4, IL-5, IL-10 and transforming growth factor (TGF)-β and granulocyte -macrophage colony-stimulating factor and other mediators such as receptor activator of nuclear factor kappa-B ligand (Pratiwi *et al*.,2022).

Chapter Three

Materials and Methods

3.1.Materials

3.1. 1. Instruments and Equipment

Table (3-1): The equipment used in the implementation of the experiments in this study.

NO.	Equipment	Manufacturing company
1	Autoclave	Hariyama / Korea
2	Benzine burner	Iraq
3	Centrifuge	Labcco / Germany
4	Compound microscope	Olympus / Japan
5	Camera	Sony/ Japan
6	Disposable Gloves	TG Medical/ Malaysia
7	Disposable Syringes	Medico inject/ USA
8	EDTA tubes	Afco-Dispo(Jordan)
9	Electrophoresis meter	Mupid-one / Japan
10	Eppendorf tube	Sigma /England
11	Eppendorf centrifuge	Hettich EBA.20 / Germany
12	Flask (250-500) ml	Oxfords
13	Gel tube	Afco /USA
14	Incubator	Memmert/ Germany
15	Micro pipettes	Gillson instruments / France
16	PCR tube	Bionear/ Japan
17	petri dishes	Sterial (Jordan)
18	Plain tube	Afco-Dispo(Jordan)
19	Sensitive balance	Denver / Swizer land
20	Tips (different volumes)	Afco/ Jordon
22	Thermo cycle	Labnet / USA
23	Transport media Swab	India
24	UV-Trans illuminator	Desktop Gelimage/scope-21/European
25	Vortex mixture	Memmert/ Germany
26	Water path	Gallen kamp / England
27	Water distillatory	Gallenkamp /England

3.1.2. Chemical and Biological Materials

Table (3-2) : Chemicals and biological materials.

No.	Materials	Industrialization
1	Agar	Hi-media/ India
2	Agarose	Shenzhen/China
3	Chloramphenicol	BDH/ England
4	Catalase reagent	BDH / England
5	EDTA	Promega/USA
6	Ethedium bromide	Promega/ USA
7	Ethanol	BDH/ England
8	Extraction kit	Favorgen / Taiwan
9	Formalin	BDH/ England
10	Gram stain kit	AFCO/ Jordan
11	Glycerol	Riedel-Dehaeny /Germany
12	Lacto phenol cotton blue stain	Fluke/Switzerland
13	Ladder	Promega/ USA
14	Master Mix	Intron/ Korea
15	Oxidase reagent	BDH / England
16	Primers	Pioneer/ China
17	Proteinase k	Favorgen / Taiwan

3.1.3. Culture Media

Table (3-3): Culture media used in the Laboratory.

No.	Culture	Manufacturing
1.	Blood agar base	Hi-media /India
2.	MacConkey agar	Hi-media /India
3.	Sabouraud Dextrose Agar	Hi-media /India
5.	CHROMagarCandida	Liofilchem / Italy
6.	Cetrimide agar	Hi-media /India
7.	mannitol agar	Hi-media /India
8.	Brain Heart Infusion Broth	BBL /France
9.	Muller-Hinton agar	Hi-media /India
10.	Eosin Methylene Blue (EMB) agar	Hi-media /India

3.1.4.Antifungal:

Table (3-4): Antifungal used in this study.

Manufacture by Hi-media /India

No.	Antifungal	Abbreviation	Dosage/ disc
1	**AmphotericinB**	**AP**	**100 mcg**
2	**Clotrimazole**	**CC**	**10 mcg**
3	**Fluconazole**	**FLC**	**25 mcg**
4	**Itraconazole**	**IT**	**30 mcg**
5	**Ketoconazole**	**KT**	**10mcg**
6	**Nystatin**	**NS**	**50 mcg**

3.1.5. Antibiotics Disks

Table (3-5): Antibiotics Disks used in this study.

No.	Antibiotic	Abbreviation	Dosage/ disc	Manufacture
1	**Azithromycin**	**AZM**	**15**	LIOFILCHEM/ITALY
2	**Gentamicin**	**CN**	**10**	LIOFILCHEM/ITALY
3	**Tetracyctine**	**TE**	**10**	BIOANALYSE/UK
4	**Vancomycin**	**VA**	**30**	BIOANALYSE/UK
5	**Amoxicillin**	**AX**	**10**	BIOANALYSE/UK
6	**Levofoxacin**	**LEV**	**5**	BIOANALYSE/UK
7	**polymyxin B**	**PB**	**100**	LIOFILCHEM/ITALY
8	**Amikacin**	**AK**	**30**	LIOFILCHEM/ITALY
9	**Imipenem**	**IPM**	**10**	Hi-media /India
10	**Ceftriaxone**	**CRO**	**10**	BIOANALYSE/UK
11	**Cefixime**	**CFM**	**5**	LIOFILCHEM/ITALY

3.1.6. The DNA Extraction Kit

Table (3-6): The Contents of the DNA Extraction Kit (FAVORGEN).

For blood and bacteria

No.	Material	Volume
1.	**FA buffer**	**120 ml**
2.	**FB Buffer**	**65 ml**
3.	**TG1 Buffer**	**45 ml**
4.	**TG2 Buffer**	**30 ml**
5.	**W1 Buffer**	**44 ml**
6.	**Wash Buffer**	**20 ml**
7.	**Elution Buffer**	**15 ml**
8.	**Proteinase K**	**11 mg**

3.1.7. Primers Used in DNA Amplification

Table (3-7): Primers used in this study

Primers	Primer sequence (5' → 3')	Product size
16S RNA	F: AGAGTTTGATCCTGGCTCAG R: CCGTCAATTCCTTTGAGTTT	938 bp
IL- 17A	F:AATCAAGGTACATGACACCAG R:TTAGCCCCAATATAGCTATCTT	684 bp
MUC5B gene/ VNTR*	F: AGTGTGCAGTGACTGGCGAG R: CTAGAGTTGCAGGTGGCAGG	Allele 9 repeats = 692 bp Allele 8 repeats = 633 bp Allele 6 repeats = 515 bp
IL-1RN/ VNTR**	F: CTCAGCAACACTCCTAT R: TCCTGGTCTGCAGGTAA	Allele 1= 410 bp (four repeats) Allele 2= 240 bp (two repeats) Allele 3= 530 bp (five repeats) Allele 4=325 bp (three repeats) Allele 5= 595 bp (six repeats)

***** (Ubell *et al*., 2010).****** (Bashour *et al*., 2013).

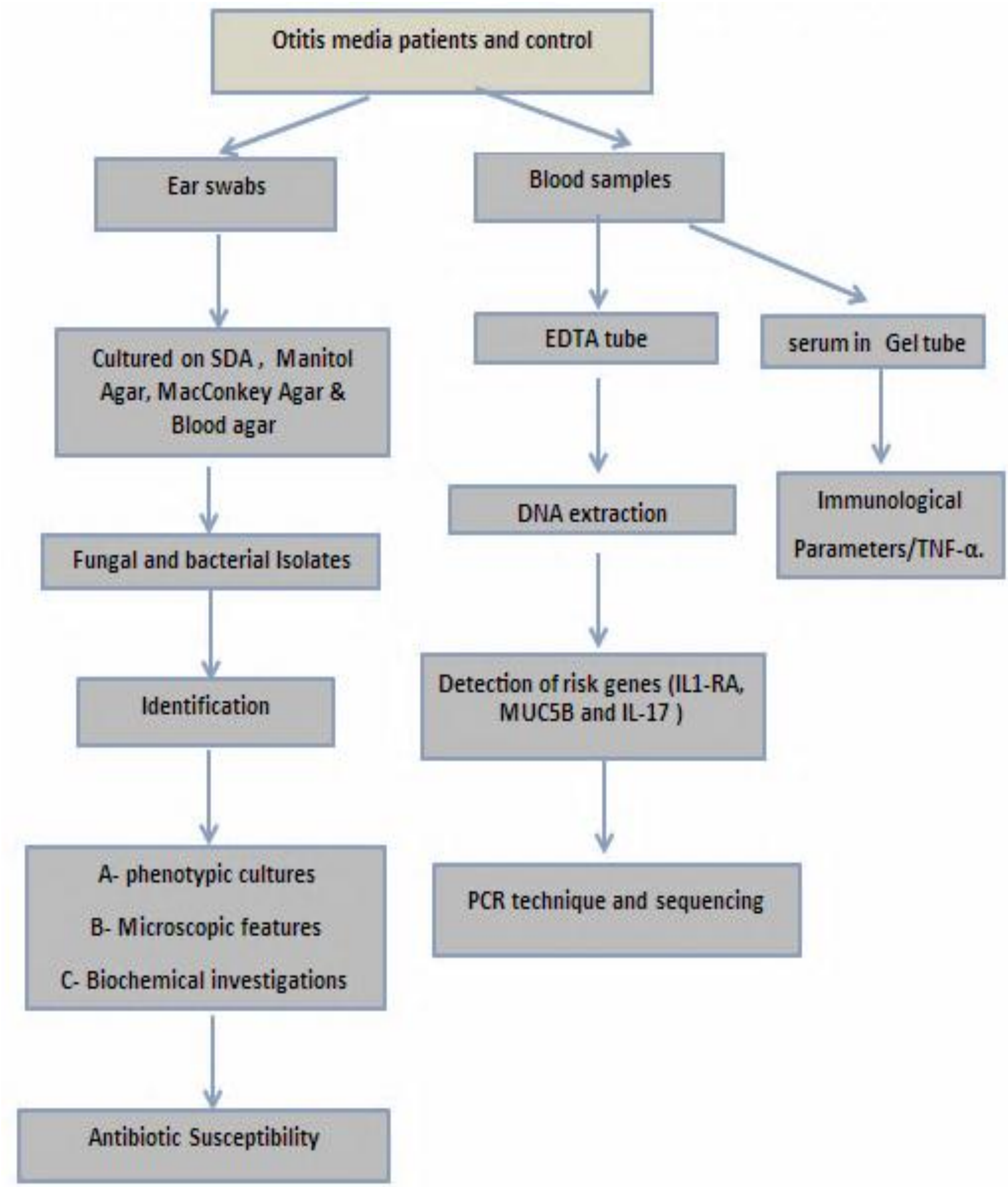
3.1.8. TNF-α ELISA kit

Table (3-8): Contents of Kit(BT LAB) for TNF-α.

No.	Material	Volume
1	Standard solution	0.5ml x1
2	Pre-coated ELISA plate	12 * 4 well strips x1
3	Standard diluent	3ml x1
4	Streptavidin-HRP	3ml x1
5	Stop solution	3ml x1
6	Substrate solution A	3ml x1
7	Substrate solution B	3ml x1
8	Wash buffer	20ml x1
9	Biotinylated Human TNFA antibody	1ml x1
10	User instruction	1
11	Plate sealer	2 pics

3.2. Study Design

According to our proposal, this study was designed as follow:



3.3. Patients and specimens

3.3.1. Collection of specimens

A total of 74 clinical samples in cross section study were collected between October ,2022to January, 2023in main hospitals in Al-Hillah (Alsadiq Hospita, Al-Hillah Teaching Hospital) whose visited Ear-Nose-Throat (ENT) departments, The specimens collected from different ages and taken for both sexes. All study participants perforated tympanic membranes,ear discharge. The specimens were transferred to the laboratory of the college of science for women for the purpose of culturing.

3.3.2.Collection of Blood specimens

Five ml of blood were collected 60 blood samples taken from 30 patients, and 30healthy individuals. We collected 5ml of blood and divided into two tubes: 2ml for EDTA tube which saved -20C$^{\circ}$ in deep freeze for using in molecular study, and 3ml for gel tube to extract serum for TNF kit Elisa.

3.4. Methods

3.4.1. Preparation of Solutions and Stains

3.4.1.1. Lacto phenol cotton blue stain:

Mixing 20 g of phenol nitrate, 20ml of lactic acid and 40ml of glycerol in 20 ml of distilled water should prepare this stain. Then mix well with water bath heating and then add (0.05 g) of a blue cotton stain. This stain is used for staining hyphae and chlamydospores and to distinguish the various microscopic structures (Leck, 1999).

3.4.1.2. Gram stain:

Gram stain was ready- made by AFCO company– Jordan. Four solutions: crystal violates, absolute alcohol, iodine, and safranin all are the components of gram stain (Beveridge, 2011).

3.4.1.3. Tries EDTA buffer (TE buffer):

This buffer is prepared by suspending 90 ml TB buffer in one liter of distilled water, as instructed by the manufacture. Then autoclaved, and kept at room temperature until used for hydration of DNA**.**

3.4.1.4. Ethidium Bromide stain:

It was prepared by dissolving 0.05gm of ethidium bromide in 10ml distilled water and stored in a dark reagent bottle (Sambrook and Rusell, 2001).

3.4.1.5. Agarose Gel:

It was prepared by dissolving agarose (1.5 gm) in 100 ml of TBE buffer (10x), after boiling, leave the solution to cool until 50 Cº, then adding 0.5 µl of ethidium bromide, mixed well and poured into a tray of gel electrophoresis (Green and Sambrook, 2019).

3.4.1.6. Normal saline solution:

Normal saline was produced by adding 100 ml DW to 0.85g of sodium chlorid NaCl until it completely dissolved. The solution was sterilized by autoclaving at 121 Cº and pressure for 15 pounds for min (Freeman and Natanson, 2005).

3.4.1.7. McFarland Standard Solution:

The solution of tube NO. 0.5 was prepared according to (Baron & Finegold 1994) by mixing 0.05 ml of barium chloride with 9.95 ml of concentrated sulfonic acid in which result in turbidity approximately equal to bacterial cells density of 1.5 ×108 cell/ml.

3.4.2. Preparation of culture media

It was prepared in accordance with the manufacturer's instructions and autoclaved at 121C° for 15 minutes to sterilize it.

3.4.2.1. Sabouraud Dextrose Agar medium (SDA)

According to the manufacture's instruction, this medium is prepared by suspending 65 gm of SDA powder in 500 ml of distilled water and homogenized by magnetic stirrer and complete the volume to 1000 ml. 0.05 gm of Chloromenphenicol is added for each to prevent growth of bacteria. Then mixed and sterilized by autoclave. This medium is used for culturing and maintaining the pathogenic fungi and yeast isolates (Odds,1991).

3.4.2.2. Blood agar

This medium was prepared by dissolving blood agar powder (40gm) in one liter of distilled water, then cooled to 50Cº, then add 5% of fresh human blood.This media was used to growth bacterial isolates and help to classify bacteria according to hemolytic appearance(Harley and Prescott, 2002).

3.4.2.3. MacConkey agar

This media was prepared according to the instruction of the industrialized company(5.1 grams of medium was suspended in 100ml of distilled water) and boiling to dissolve the medium completely, and cooled to 50 C º, then mixed well before pouring into sterile petri plates (Carroll et al., 2015). McConkey agar is used to isolate most gram negative bacteria and used to differentiate between lactose fermenter and non-fermenter bacteria (Carroll *et al*., 2015).

3.4. 2.4. Cetrimide agar

It was prepared according to manufacturing company instructions by dissolving 46.7 grams in 1000 ml D.W, heat to boiling to dissolve the medium completely. and add 1 vial of Nalidixic acid selective supplement (FD130). The media were used as a selective media for the isolation of *P. aeruginosa* (Shanthi *et al*., 2010).

3.4.2.5. mannitol agar

Mannitol salt agar was prepared according to the manufacturer instructions, 111 gm was suspended in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving, dispensed in petri dishes and kept at 4Cº until use. The mannitol salt agar plate is inoculated for the isolation of the Staphylococci. This medium was used as a selective medium for isolation of Staphylococci and differentiation of *S. aureus* (MacFaddin, 2000).

3.4.2.6. Brain heart infusion Broth

This medium using to activate, grow and as stock culture for isolates it is prepared by melting 37 gm. of medium in 1L. of distilled water, and adding 20 % glycerin (MacFaddin, 2000).

3.4.2.7. CHROMagar Candida Medium

According to the manufacture's instruction, this medium is prepared by suspending 3 gm of CHROMagar candida powder in 100 ml of distilled water, and then heated until it dissolved completely. This medium is used for preliminary identification of *Candida* spp. (Horvath *et al.*, 2003).

3.4.2.8. Eosin Methylene Blue agar

This media was prepared according the manufacturing company by suspending 35.96 gm in 1000 ml .The media were used as a selective media for the isolation of *Escherichia coli*.(Collee *et al*., 1996).

3.4.2.9. Muller-Hinton Agar

This medium was prepared according to the instruction of supplier company by dissolving 38gm from the medium in 1L of distilled water and sterilization by autoclave at 121º C for 15 min., this medium used an antibiotic sensitivity test (Skov *et al*.,2006).

3.4.3. Sterilization

3.4.3.1. Sterilization by autoclave

All the culture media are sterilized using the autoclave device at a temperature of 121°C and a pressure of 15 lbs. for 20 minutes. All the glassware used in the laboratory sterilized using the oven 150°C one hour as well as some of the tools used in the extraction such as the Eppendorf tube tips and PCR tubes are also sterilized.

3.4.3.2. Sterilization by formalin

Sterilization by formalin is performed by adding 15 ml of formalin into Petri dish and left for(24-48) hours for the purpose of sterilizing the incubator

3.4.3.3. Sterilization by heating and alcohol spirit

Sterilization of the indoor of hood pang surfaces by alcohol while needle, tongs other steel tools is sterilized by heating

3.4.4. Cultivation of Specimens

The otitis ear swabs are cultured by streaking on SDA for fungi and, Blood Agar base، mannitol agar and MacConkey agars for bacteria. All bacterial plates incubated at 37 C° for (24-48) hours hunder aerobic. fungal plates incubated at(28-30) C° for (24-48)hours to yeast isolates and for 7 days to molds isolates.

3.4.5. Bacteria identification

3.4.5.1. Morphological examination

Primary diagnostic tests based on morphological characteristics of bacterial growth on Mannitol salt agar, MacConkey agar and blood agar were studied including colony shape, colony texture, color and edges (Brooks *et al*., 2013; Tille, 2017).

3.4.5.2. Microscopic examination

Gram staining smear is done for all bacterial isolates, have a direct microscopic examination. Morphological characteristics Form (sphere, rod, spiral), Size pseudo groupings (clusters, chains, diplococci), Staining (Gram-positive, Gram-negative) A single colony was selected, stained with Gram stain, and studied with under a light microscope (Jawetz *et al*., 2019). Relevant biochemical tests were performed to each isolate for final identification after staining.

3.4.5.3. Biochemical tests

3.4.5.3.1.Oxidase Test

The test depended on the present specific bacterial oxidase that would stimulate the transport of electrons between electron donors in the bacteria and redox dye (Tetramethyl-P- Phenylene- diamine dihydrochloride) the dye was reduced to a produced deep purple color. A strip of filter paper was saturated with a little freshly made reagent. The colony to be tested was picked up with a sterile wooden stick and smeared above the filter paper. When produced deep purple color through(5-10)sec s indicating a positive result (Forbes *et al*.,2007) **.**

3.4.5.3.2.Catalase test

This test uses for the detection of bacterial catalase enzyme activity. By a wooden stick, an amount of pure growth transferred to a microscope slide then a drop of 3% hydrogen peroxide added on the colony. The appearance of bubbles refers to as catalase positive (Goldman and Lorrence, 2009).

3.4.5.3.3. Coagulase:

This test is used for the detection of the ability of *S. aureus* to produce coagulase enzyme, which is an enzyme that causes clotting in plasma (Nneoma *et al*., 2013). A few colonies were transferred in diluted human plasma (plasma : saline 1 : 5) in a tube . The tube was kept at 37C° and observed for clot after 1 to 4 hours. or, next day according to (Tiwari *et al* ., 2008).

3.4.5.4. Maintenance of Bacterial Isolates

In a medium containing 20% glycerol at -20C°, the bacterial isolates are kept for a long time-at least three months. The medium is made by mixing 8.5 ml of brain heart infusion broth with 1.5 ml of glycerol, then dispensing the mixture into a tiny screw-top vial and autoclaving it to sterilize it. One pure isolated colony of each isolate is added to the medium after chilling, which is then kept after 24 hours of incubation at 37C°. The tubes are then kept in deep freezing at – 20C° (Al-Mayyahi, 2018).

3.4.5.5. Susceptibility Test to Antibacterial Activity

This test performed by Kirby – Bauer method as the following :

1. Using a sterilized inoculating loop, four to five colonies of bacterial isolate were picked up from a fresh pure culture plate and homogenized in 5ml of sterile normal saline until the turbidity was nearly equal to the MacFarland No. 0.5 turbidity level.
2. A swab was used to capture a tiny amount of bacteria from bacterial suspension, and the excess fluid was removed by rotating the swab hard against the inside of the tube above the fluid level to remove the excess fluid.
3. The swab was then streaked onto the dried surface of a Muller hinton agar plate, returning it to the beginning point. This agar plate was then turned completely around and swab a second time. The plate was then rotated another 90 degrees, swabbing it again.
4. Using a sterile forceps, place the selected antimicrobial discs on the surface of the inoculation plate and gently push them into full contact with the agar.
5. The step (4) was repeated with all of the antimicrobial discs in the test, placing them similarly apart.
6. The plates were infected and incubated at(37)C° for (18–24) hours.

Following incubation, using a metric ruler, the diameters of the whole zone of inhibition were observed and quantified in millimeters. When compared to the standard inhibition criteria given by the Clinical and Laboratory Standards Institute, the width of the inhibition zone for each antibiotic disc was translated into terms of sensitive, intermediate, and resistant categories (Wayne, 2017).

3.4.6. Fungi identification

3.4.6.1.Morphological examination

After appearance growth as well as examining colonies of fungi from respect colour, shape and texture (Powdery, Granular, Cottony) as recorded pigments are examined on colony surface, appearance on foundation.

3.4.6.2. Microscopic Examination

Fungi isolates are examined microscopically, the fingerprint of the fungi in the colony is taken by adhesive tape, transparent adhesive tape is used, touches the surface of the fungal colonies and then pastes the tape on a glass slide containing a drop lactophenol cotton blue. Slides examined under magnification 10X, 40X and 100X as described by (Pitt and Samson, 2000; Pitt and Hocking, 2009; Rai, 2016).

3.4.6.3. CHROMagar Test

This test is performed by inoculating CHROM agar Candida medium which is prepared previously from Candida isolate culture grown on SDA for 24 hours, and then incubated at 30˚C for (24-48) hours (Paritpokee *et al*.,2005). CHROM agar test is used for the presumptive identification of *Candida* species by production of different colors on this medium (*C. albicans*= green/ blue green, *C. dubliniensis*= dark green, *C. tropical*= blue, *C. parapsilosi*s= cream white, and *C. krusei*=pink) (Horvath *et al*.,2003).

3.4.6.4. Susceptibility Test to Antifungal Drugs

1. Preparation of Yeast Suspension

Yeast suspension is prepared as mentioned by NCCLS M27-A2 (2002). Fresh yeast colonies are transferred from SDA medium into tube containing 5ml from the normal saline, shake gently. Sporulation was assessed by Hemocytometer according to the equation:

The average number of spores\ml = The average number of spores in five Squares * 25 * 10^4 (Sibounnavong *et al.*,2009).

2.Antifungal Activity Against Growth of Yeasts

The surface of the SDA medium inoculate by using sterile cotton swabs after placing them in the yeast suspension, then remove the excess suspension from the cotton swab by rotating it on the edge of the tube, then it is streaked on the agar with the swab so that the suspension is distributed evenly throughout the agar, leaving the dish for(5- 15) minutes to allow the suspension to be absorbed and until the surface of the agar dries, the of the disks antifungals under study were placed at equal distances on the surface of the agar using sterile forceps and incubated at a(35-37)C°. After 2 days, the inhibition zone by disk deffusion method was measured in mm for each antifungal agent using a metric ruler.

3.4.7. Molecular Study

3.4.7.1. Bacterial DNA extraction:

Using Favrogen DNA extraction kit protocol of human blood to extract Bacterial DNA.

1. Cultivation: bacterial strains were incubated at 37C° on nutrient broth medium 24hours.

2. Sample preparation: Transfer 200ul of bacterial cell suspension for each bacterial strain separately into 1.5 ml microcentrifuge tube. Add 20

of lysozyme enzyme then mix by pipetting. Incubate at 37C° for 15 minutes.

3. A 20 µl of Proteinase K was added to each tube in step 2, mixed well by vortexing. Incubated at 58C° for 30 min, vortex 30 seconds every 5 minutes incubation.

4. Cell lysis: Add 200 µL of FATG buffer then mix by vortex. Incubate at 10C° for 5 minutes, inverting the tube every 2 minutes.

5. FATB buffer 200ul was added to the cells and re-suspend the cells by vortex, Incubate at 70C° for 10 minutes, inverting the tube every 3 minutes.

6. DNA binding: Add 200 µL of absolute ethanol to sample lysate and mix by vortex for 10 second. Place spin column in a 2 ml collection tube. Transfer all of the mixture to the Place spin column. Centrifuge at 13000 rpm for 1 minute. Discard the flow- through, Place spin column back in the 2ml collection tube.

7. Washing: Add 400 µL of W1 buffer to the spin column. Centrifuge at 13000 rpm for 30 seconds then Discard the flow- through. Place spin column back in the 2ml collection tube.

8. Add 600 µL of washing buffer (make sure absolute ethanol was added) to the spin column.Centrifuge at 13000 rpm for 30 seconds then Discard the flow- through. Place spin column back in the 2ml collection tube. Centrifuge at 13000 rpm for 3minutes to dry the column.

9. Elusion: Transfer the dried spin column to clean 1.5 ml microcentrifuge tube. Add 75 µL of pre-heated elusion buffer(in step 3) to center of the column. Waite for 5 min to allow elusion buffer to be completely absorbed. Centrifuge at 13000 rpm for 30 seconds to elute purified DNA.Kept the purified DNA at-20C.

3.4.7.2. DNA Extraction from Blood

Using Favrogen DNA extraction kit protocol of human blood as following steps:

Step 1: RBC Lysis

1. Collect fresh human blood in an anticoagulant-treat collection tube.
2. Transfer up to 300 µl of fresh blood to a 1.5 ml microcentrifuge tube
3. Add 3x the sample volume of RBC Lysis Buffer and mix by inversion. Do not vortex.
4. Incubate the sample mixture at room temperature for 10 min.
5. Centrifuge at 3,000 x g for 5 min. And completely remove the supernatant.
6. Add 100 µl of RBC Lysis Buffer to the pellet and resuspend the cells by pipetting.

Step 2: Cell Lysis

7. Add 200 µl of FABG Buffer to the sample mixture. And mix well by vortexing
8. Incubate the sample mixture at room temperature for 10 min or until the sample mixture is clear. During incubation, invert the tube every 3 min.
9. Preheat required Elution Buffer (for Step 5 DNA Elution) in a 70C°water bath.
10. (Optional Step): If RNA-free genomic DNA is required, add 5 µl of 10 mg/ml RNase A and mix by vortexing. Incubate for 5 min at room temperature.

Step 3: Binding

11. Add 200 µl of ethanol (96~100)% to the sample and vortex for 10 sec. Pipette the sample to mix well if there is any precipitate formed
12. Place a FABG Column to a Collection Tube. Transfer the sample mixture carefully to FABG Column. Centrifuge at speed 14,000 rpm or

18,000 x g for 1 min. Discard the Collection Tube and place the FABG Column to a new Collection Tube.

Step 4: Washing

13. Add 400 µl of W1 Buffer to the FABG Column and centrifuge for 30 sec at speed 14,000 rpm or 18,000 x g. Discard the flow-through and place the FABG Column back to the Collection Tube.

14. Add 600 µl of Wash Buffer to the FABG Column and centrifuge for 30 sec at speed 14,000 rpm or 18,000 x g. Discard the flow-through and place the FABG Column back to the Collection Tube. Make sure that ethanol has been added to Wash Buffer when first open

15. Centrifuge for an additional 3 min at speed 14,000 rpm or 18,000 x g to dry the column.

Step 5: Elution

16. Place the dry FABG Column to a new 1.5 ml microcentrifuge tube.

17. Add 100 µl of Preheated Elution Buffer or TE to the membrane center of FABG Column.

18. Incubate the FAGB Column at 37Cº for 10 min in an incubator

19. Centrifuge for 1 minute at full speed 14,000 rpm or 18,000 x g to elute the DNA .

20. Store the DNA fragment at 4Cº or -20Cº.

3.4.7.3. DNA electrophoresis

1- At first, 100 ml of the T.B.E buffer was placed in a beaker.

2- Then 1 g weight of agarose is added to the buffer.

3- the buffer with the agarose was heated on a hot plate to boiling point so that all of its components were solvent.

4- The agarose mixture was cooled by leaving it between 50-60˚C.

5- Ethidium Bromide dye was added at 0.5 µl to the agarose before solidification of the liquid and mixed it well

6- The comb was put into one of the ends of the agarose gel template.

7- Agarose was poured into the template to prevent the formation bubbles and left to cool at room temperature for 30 minutes.

8- The migration electric basin was filled with the TBE buffer solution so that it rose from the gel surface.

9- The samples were placed in the pits with the addition of the agarose gel loading dye so that the dye was linked to the DNA .

10- The electrophoresis was performed in 70 V to 45 min.

11- The agarose gel was exposed to UV trans illuminator for DNA bands visualized and documented.

3.4.8. PCR Assay:

3.4.8.1. Preparation of Primers for PCR Technique:

The primers were prepared by adding distilled water free of nuclease in the a different volume according to the manufacturing company instructions to obtain a solution of base stock with a concentration of 100 Pico mole / µl, mixed by vortex, then centrifuged for 10sec at 4000 rpm. Then 10 µl of each primer was taken and putt in the micro centrifuge tube with 90 µl of nuclease free distilled water to prepare the working solution.

3.4.8.2. PCR Mixture:

PCR mixture for all primers used in this study was prepared according to theTable (3-9).

Table (3-9): Volumes of chemical materials uses in PCR assay.

No.	Chemical materials	Volumes (µl)
1	Master Mix	5 µl
2	DNA	1µl

3	Forward Primer	1 µl
4	Reveres Primer	1 µl
5	Deionizer D. W	18 µl
6	Total	≈ 26 µl

3.4.8.3. PCR Gel Electrophoresis:

The amplified PCR products were detected by agarose gel electrophoresis which was visualized by staining the Ethidium bromide. The electrophoresis result was detected by using gel documentation system. The positive result was distinguished when the DNA band base pairs of sample equal to the target product size (Bartlett and sterling, 2003). or the size of amplified DNA fragments which were identified by a comparison with molecular size marker DNA (100 - bp DNA ladder).

3.4.8.4. PCR Conditions

The conditions of PCR reaction for all primers used in this study was shown in Table (3-10 and 3-11).

Table (3-10): Show the conditions of PCR for sequencing primers.

16S RNA			
Stages	**Temperature**	**Time**	**Cycles**
Pre denaturation	94C°	3 min	1
Denaturation	94C°	30 sec	30
Annealing	60C°	30 sec	
Extension	72C°	2 min	
Final extension	72C°	5 min	1

Cooling	4C°	∞	
IL- 17A			
Stages	**Temperature**	**Time**	**Cycles**
Pre denaturation	95C°	5 min	1
Denaturation	95C°	30 sec	30
Annealing	56C°	60 sec	
Extension	72C°	60 sec	
Final extension	72C°	5 min	1
Cooling	4C°	∞	

Table (3-11): Show the conditions of PCR for VNTR primers.

MUC5B gene/ VNTR			
Stages	**Temperature**	**Time**	**Cycles**
Pre denaturation	94°C	3 min	1
Denaturation	94°C	30 sec	30
Annealing	64°C	30 sec	
Extension	72°C	2 min	
Final extension	72°C	5 min	1
Cooling	4°C	∞	
IL-1RN/ VNTR			
Stages	**Temperature**	**Time**	**Cycles**
Pre denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	30
Annealing	56°C	60 sec	
Extension	72°C	60 sec	
Final extension	72°C	5 min	1

Cooling	4°C	∞	

3.4.8.5. Detection of PCR Products

1- Detection by Sequencing Analysis

The 60 PCR products (30 patients and 30 control) of sequencing primers (16 sRNA and IL-17A) were directly sequenced by Macrogen Company (Korea). After received the sequencing data for PCR products which are compared with gene bank by using NCBI Blast nucleotide database. Only clear chromatographs obtained from ABI sequence files were further analyzed to ensure that themannotation and variations are not due to PCR or sequencing artifacts.

The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit for multiple alignment sequence based on editor Software version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

2- Detection by electrophoresis

The PCR products of VNTR primers were detected by agarose gel electrophoresis which was visualized by staining the Ethidium bromide. The size of the PCR products of amplified DNA fragments identified by a comparison with the molecular size marker DNA (100-bp DNA ladder).

3.4.9. Serological analyses:

ELISA for Human Tumor Necrosis Factor Alpha,This study was held according to procedure of company's instructions (BT LAB) equipped for TNF-α ELISA kit (AppendixA).

3.4.10. Statistical Analysis

Analysis is carried out using SPSS version 22, numerical data was expressed as mean and standard deviation, qualitative data were expressed as frequency and percentage. independent -sample t test used to compare between two groups, chi-square test used to detect if any relation between ordinal and nominal variable in this study. P value of ≤ 0.05 is considered significant (Iuliano and Franzese,2018).

Chapter Four

Results and Discussion

4. Results and Discussion

4.1. Description of study Demographic

In present study we included 74 patients of both gender ,Age of the patients ranged from (1 to 72) years with signs and symptoms of otitis media.. Table 4.1 show that the year with a majority of them (23%) were seen in (11-20). In relation to gender, more than half (55.5%) of participants were female. Regarding to residence, more than three quarters (75.7%) of participants are urban area.

Table (4-1): Descriptive statistic of demographical.

		Frequency	Percent
Age	1-10	8	10.8
	11-20	**17**	**22.9**
	21-30	12	16.2
	31-40	13	17.6
	41-50	11	14.9
	51-60	6	8.1
	61-More	7	9.5
	Total	74	100
Gender	Male	33	44.5
	Female	**41**	**55.5**
	Total	74	100
Residence	Rural	18	24.3
	Urban	56	75.7
	Total	74	100

The results showed that the prevalence of ear infection among females was higher than males found similar results, (Alenezi *et al* 2017; Yadav, 2019) .But there is no clear explanation whether there is a gender predilection in ear infections (Addas *et al.,*2019) .

Consequently, the results could differ from those found by other studies their research revealed that males were affected most frequently than females (Osazuwa *et al*., 2011). But, there are no genetic and documented anatomical distinctions between males and females. ear-related subjects (Afolabi *et al*.,2012).

This study showed 75.7% patient came from urban area, 24.3% has rural housing. But other study showed that the disease were more common in lack of personal hygiene, poor housing status, illiterate populations. As described by (Biswas *et al*., 2005; Anggraeni *et al*.,2014)

The difference was may be due to the fact that in this study most of the patients came from Particular group of population of urban where their living statuses were better than others. This results were also supported by studies which were carried out in urban population by (Ahmed & Akaiduzzaman, 2000 ; Uddin *et al*.,2021).

Nearly similar rates of middle ear infection were reported by other researchers, who reported that the urban people have a higher incidence of the disease than the rural people (Salih *et al*., 2001 and Mohammad, 2004) and show cause The high incidence of the disease in urban people may be attributed to random and overuse of antibiotics which lead to the production of resistance of the pathogens.

4.2. Descriptive statistic of clinical data

Seventy four samples were studied, and most of the patients had taken antibiotics by (56.8)%, and those who did not take antibiotics (43.2)%. Chronic cases of otitis media accounted for the highest (62.2)% and acute cases(37.8)% Among the patients there was a genetic history (8.1)% and flu (18.9)% , Complications of Covid (4.1) %. Table(4.2) show that the majority of participants (56.8)% take antibiotics. In relation to disease type, (18.9)% are flu while (39.2)% of participants having no disease. Regarding to Disease chronicity, more than half (62.2)%of participants are chronic.

Table (4-2): Descriptive statistic of risk facter.

		Frequency	Percent
Take antibiotics	No	32	43.2
	Yes	42	56.8
	Total	74	100
risk facter	No disease	29	39.2
	DM + otitis externa	10	13.5
	Allergic rhinitis	9	12.2
	Flu	14	18.9
	Genetic	6	8.1
	Complications of Covid	3	4.1
	Swimming	3	4.1
	Total	74	100
Disease chronicity	Chronic	46	62.2
	Acute	28	37.8
	Total	74	100

In another study like our study we found that subjects had family history of recurrent middle ear infection for more than five years either in maternal or paternal side. This gives a clue on genetic association/ predisposition to OM. There are many reports showing association of are on the include ear plugs, and swimming caps and Swimming in chlorinated swimming pools and non-polluted rivers or lakes will also decrease the infection risk (Wang *et al*.,2005).

The entry point of fungal infection is mostly acute or chronic infection of the external ear or middle ear. Long term local administration of anti-inflammatory drugs and steroids, combined with diabetes or immunodeficiency are the main risk factors for invasive aspergillosis (IA) in middle ear (Liu *et al*.,2023) . It is generally believed that mucosal COM arise from an episode of acute otitis media where after rupturing the tympanic membrane fails to heal. Repeated infection occurs in the middle ear from the nasopharynx either by aspiration of nasopharyngeal microbes or due to reflux from the nasopharynx. Repeated infection also occurs by transportation of microbes from the ear canal through the perforation (Poorey *et al*.,2002; Oyeleke *et al*.,2009).

The most prevalent type of otitis media was chronic type .This agrees with Study showed (69.9)% were of chronic type of otitis media and (30.1)% of acute type (Basnet *et al*.,2017). Study reported a significant association of allergy and CSOM in a tropical country, Malaysia (Daud & Rahman, 2019). On the contrary, these outcomes were conflicting with an Iranian study, probably because of the climatic difference (Bakhshaee *et al.*, 2011).

4.3. Isolation and identification

4.3.1. Bacterial isolation

The initial identification of bacterial samples depended on some criteria such as morphological features on culture media, and Gram staining and biochemical testing. The final diagnosis depends on molecular identification. In this study, out of total 74 swabs were isolated from patients with otitis media. From all these specimens, (12) were a negative for bacterial growth appeared, while (62) were positive for bacterial growth appeared.

In this study of different bacterial isolates by many patients with OM is illustrated in Table (4-3). Thus, that 74 clinical specimens isolates of

P. aeruginosa were much more frequent reveal 24(32.4)%, followed by *S. aureus* 19 (25.7)% isolates, while 7(9.5)% *Proteus*, 6 (8.1)% *S. epidermis* and *E. coli* was 5(6.8)%. The rest isolates belong to different bacterial genus such as (3) *klebsiella* (2) *Acidovorax*,(2) *Providencia* and one isolate for *Bacillus smithii* , *P. stutzeri*.

Table (4-3): Distribution of bacterial species isolated from OM patients.

Bacterial type		Frequency	Percent%
	P. aeruginosa	**24**	**32.4**
	S. aureus	**19**	**25.7**
	Proteus	**7**	**9.5**
	S. epidermis	**6**	**8.1**
	E. coli	**5**	**6.8**
	Klebsiella	**3**	**4.1**
	Acidovorax	**2**	**2.7**
	Providencia	**2**	**2.7**
	B. smithii	**1**	**1.4**

	P. stutzeri	**1**	**1.4**

Bacteria can reach the middle ear in otitis media from nasopharynx through the Eustachian tube or from the external ear canal through the non-intact eardrum (Hailu *et al*., 2016). This finding is similar with preceding studies by (Tesfa *et al*.,2020) who have revealed that *P. aeruginosa*, *S. aureus* and *P. mirabilis* are the commonest bacterial pathogens responsible for otitis media.

The result is also in harmony with the study done by (Weckwerth *et al*. 2009) *P. aeruginosa* and *S. aureus* as the most dominant in middle ear infections. Close to the current study results is (Javed *et al*.,2020) of 72 isolates, Gram negative bacteria were 52 (72.2 %) in contrast to Gram positive 20 (27.7 %). So a total of nine bacteria were isolated. The predominant bacteria were *P. aeruginosa* 32 (44.4 %) *S. aureus* 15 (20.83 %) and *K. pneumoniae* 8 (11.11 %), *E.coli* (6.94%) *S. pneumoniae* (4.16%) *Acinetobacter* (4.16%) and *Proteus* (2.77%). But differs from studies in developed countries were *S. pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* predominate (Thornton *et al.,*2011).

Different literatures mentioned S. aureus most common isolates. Variation in study period, climatic and geographic could be the possible reasons for the difference in distribution of the bacteria (Wasihun & Zemene, 2015; Tadesse *et al*.,2019; Getaneh *et al*.,2021).

Otitis media found to be more prevalent during high humidity environment and one of the commonest came of bearing impairment. Most common complain of patient having ear discharge were earache, tinnitus and decrease hearing overall *pseudomonas* appeared to be most common study by (Vaghela *et al*., 2016).

That frequency of *P. aeruginosa* and *Staph. aureus* in our study, virulent nature of both these two organisms and their rapid colonization characteristics also take part to their high rate of recovery (Motayo, *et al*., 2012).

And *P. aeruginosa* reasons for its virulence are attributed to many factors, including the presence of flagella and pili which are essential for the pathogen's motility and adherence (Breidenstein *et al*., 2011).

Virulence factors such is Pilli which helps in the attachment of the pathogen to the epithelial layer of inflamed middle ear. When involved, the pathogen yields proteins degrading enzymes to evade the usual resistance procedure of organ vital to combat infections and this could be associated to its potential of survival in antagonism with other creatures and fight to antibiotics (Seid *et al*.,2013).

Another factor is the presence of enzymes such as proteases and elastases that break down tissue proteins (Kerr & Snelling , 2009). The warm and wet environment of the ear canal and middle ear cavity may be conductive to the growth of *P. aeruginosa* or its spread from one patient to another via medical instruments in outpatient clinics (Lee *et al.*, 2012).

In our study the second most common organism isolated was *S. aureus* in middle ear infections can be attributed to their ubiquitous nature and high carriage of resistant strains in the external auditory canal and upper respiratory tract (Kumar *et al*., 2014).

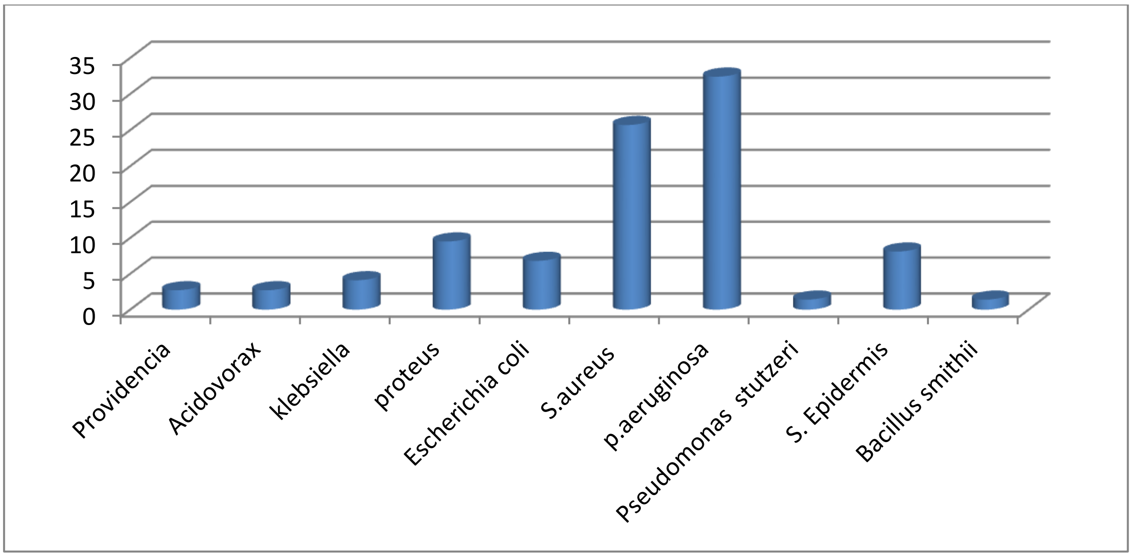


Figure (4- 1): Distribution of bacterial species isolated by percentage.

4.3.2. Fungal isolation

With 74 specimens from patients of otitis medid .59 specimens positive for culture and 15negative The results of fungal cultures are summarized in table (4-4), shows the microbiological results of fungal infection, the majority of microbiological findings was *Aspergillus niger* 27 (36.5%) followed of, *A. flavus* 23 (31.1%), *A. terreus* 16 (21.6%), *Penicilium* sp. 13 (17.6)% and *C.glabrata ,C.krusei* 9(12.2)%.

Table (4-4) : Distribution of Fungi and *Candida* growth isolated from OM patients.

Fungi and candida	Frequency	Percent%
A. niger	**27**	**36.5**
A. flavus	**23**	**31.1**
A. terreus	**16**	**21.6**
Pincillinum sp.	**13**	**17.6**
C. glabrata	**9**	**12.2**
C. krusei	**9**	**12.2**
C. albicans	**6**	**8.1**
C. tropicalis	**3**	**4.1**

In otomycosis, fungi may cause either primary or secondary invasion after tissue abnormality resulting from a primary bacterial infection. Therefore, otomycosis can be observed in mixed bacterial fungal infections (Ismail *et al.*, 2017). *A. niger* is a known cause of otomycosis and it has been reported as the most common etiology of this condition in different studies this similar to our studies (Gharaghani *et al*., 2015; Kamali Sarwestani *et al*.,2018; Sarvestan *et al*.,2022).

Aspergillus niger grows on cerumen, epithelial scales and detritus deep in the external canal. The resulting accumulation of these inflammatory materials along with cerumen and fungal debris result in plug formation, which is extremely significant and usually leads to diminished hearing ability; pruritis, irritation of the surface layer of the external ear itself is a predisposing factor for bacterial colonization. There may be superficial erosion of membranes (Osazuwa *et al*.,2011).

The finding is not in agreement with a lot of reports in which *A. flavus* were introduced as dominant agents of fungal otitis followed by *A. nigar* and *A. fumigates* (Araiza *et al*.,2006).

There are several species of *Candida* spp. isolated in our studies, in compare to other studies have shown that candida species was the mainly responsible for otomycosis, especially in immunocompromised hosts. However infection with candida can be more difficult to detect clinically because of its lake of characteristics appearance like *Aspergillus* which can be present as ottorhea not responding to antimicrobial (Mohammed *et al*.,2020).

Regarding etiology of otomycosis, relevant literature suggests that the most common causative agents are *Aspergillus* spp. and *Candida* spp. These genera are described as dominant agents by the majority of authors both in Europe and globally (García-Agudo *et al*.,2011 ; Jia *et al*.,2012).

and also which agrees with studies in Iraq *A. niger* being the predominant isolate by (Khammas *et al.,* 2010); (Pandey *et al*., 2018) in India also isolated the same *Aspergillus* spp., but different *Candida* spp. (Allam *et al*., 2020) in Egypt also reported a prevalence rate of 95.2% for *Aspergillus* spp.

These findings are consistent with our results, which indicate that the dominance of the *Aspergillus* genus over other species may be due to the widespread occurrence of its spores in all environments and contamination during ear cleaning (Gandhi *et al*., 2016). Some authors noted traditional head coverings in women as main predisposing factor (Aneja *et al*.,2010).

Others indicated a history of swimming in sea or pool due to long-term exposure to moisture and Several conditions have been proposed as predisposing factors for otomycosis, including hot wet clima ،self-imposed abrasion due to the insertion of foreign bodies, use of hearing aids, otic steroids and anti-infectives drops, pH alteration of cerumen, diabetes, and immune defects (Ho *et al*., 2006; Rath *et al*., 2019; Kiakojuri *et al*.,2021).

The negative results of the culture may be due to the patients undergoing treatment before taking the sample, and the effect of the treatment remained during the sample taking, or perhaps due to infection with a type of bacteria or virus, so the result appears negative for fungi (Alarid-Coronel *et al*.,2018).

4.3.3. Identification by CHROMagar medium

Figure (4.2) illustrate that higher percentage of *Candida* spp. are 12.2 % of *C .glabrata* and *C .krusei* , 8.1% of *C. albicans* and 4.1% *C. tropicalis*.

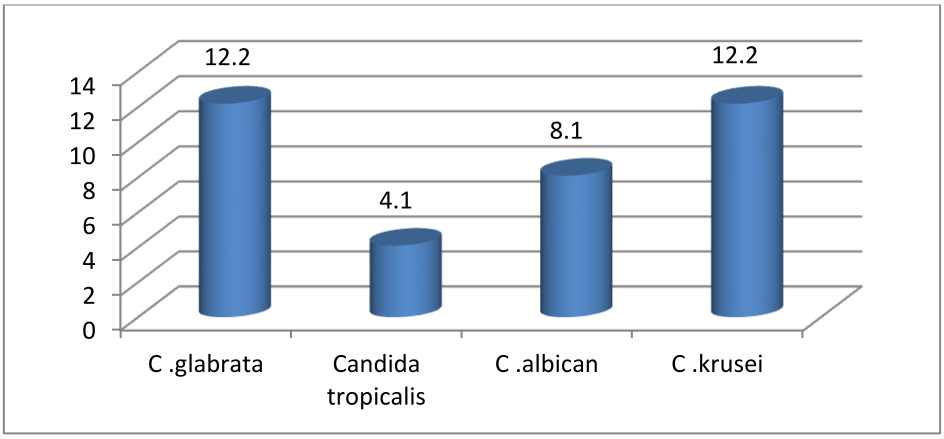


Figure (4- 2) : Distribution of *Candida* spp. by percentage.

Speciation of *Candida* using conventional methods is quite cumbersome and time consuming. Therefore species level identification using CHROMagar. The advantages of Candida agar is that it is easy to prepare, boiling and dispensing in petri plates, It is technically simple and cost effective compared to technically demanding and time consuming conventional methods. It also facilitates identification of two or more different species present in a single clinical sample (Jogender *et al* ., 2020).

Speciation of the *Candida* isolates was done by inoculating it on CHROMagar Candida differential agar. and incubated at 30 c for 24-48 hours aerobically. Species identification was done by the morphology and color of the colony. The results of this test of the colonies produce different color. *C. albicans* colonies was green color , while *C. tropicalis* was metallic blue Figure (4-3), current results are coincided with (Venkatesha, 2020). while *C. krusei* was pink fuzzy, *C. glabrata* was Pink with a darker mauve center this results similar with (Hospenthal *et al.*, 2006; Manikandan *et al.,* 2013; Mulet Bayona *et al* . ,2020).

Candida species is it contains enzymatic substrates that are linked to chromogenic compounds .When a specific enzyme cleaves the substrate, the chromogenic substances produce color. The action of different enzymes produced by yeast species results in color variation which is useful for the presumptive identification of some yeasts (Dharmeswari *et al* ., 2014).

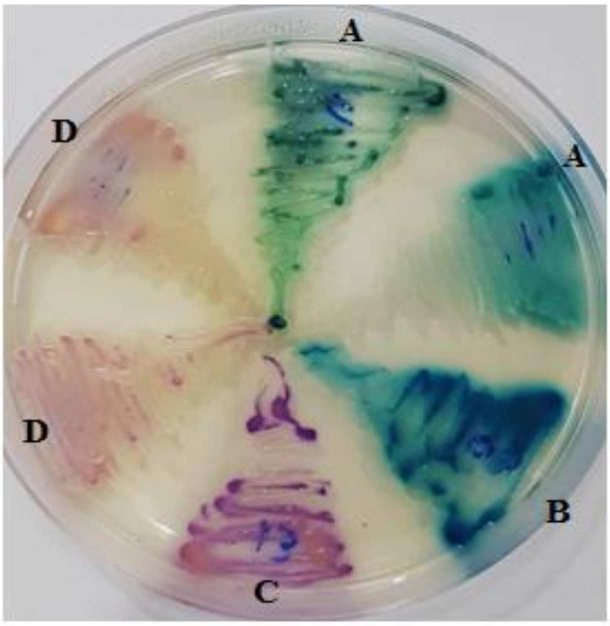


Fig. (4-3): Colonies color of Candida species on CHROMagar medium at 30˚C for 24-48h. A= *C. albicans*, B= *C. tropicalis*, C= *C. glabrata* and D= *C. krusei*.

4.4. Antimicrobial susceptibility testing

Table 4-5 show that all bacterial type sensitive to Imipenem, except with polymyxin B, Gentamicin, and Ceftriaxone that resist to all bacteria. Regarding to Amikacin, (13.6)% are resist and (86.4)% are sensitive. In relation to Azithromycin, (31.8)% are resist and (68.2)% are sensitive. Finally,(76.2)% are resist to Cefixime and (23.8)% are sensitive.

All isolates were resistant to polymyxin B, Gentamicin, and Ceftriaxone, but the isolates showed high sensitivity to imipenem (100)%. *P. aeruginosa* sensitive to, amikacin (81.8)%, Azithromycin

(64.6)% and Cefixime(18.2)%and resistant to Amikacin (18.2)%, Azithromycin(36.4)%, Cefixime (81.2)%.

Furthermore, *Proteus*, *Klebsiella* and *Acidovorax*, exhibited relatively high resistance to Cefixime but *E. coli* resistance (33.3)%, sensitive (66.7)%. Most bacteria sensitive to Amikacin, some isolates show resistant to Azithromycin (*Acidovorax* and *P. stutzeri*).

Table (4-5) : Distribution of Gram negative bacteria in relation to antibiotics sensitivity test.

Antibiotics Sensitivity			Bacterial type						Total
			Acidovorax	***Klebsiella***	***Proteus***	***E. coli***	***p. aeruginosa***	***P. stutzeri***	
Imipenem	R	F	0	0	0	0	0	0	0
		%	0	0	0	0	0	0	0
	S	F	1	1	2	3	11	1	22
		%	100	100	100	100	100	100	100
polymyxin B	R	F	1	1	2	3	11	1	22
		%	100	100	100	100	100	100	100
	S	F	0	0	0	0	0	0	0
		%	0	0	0	0	0	0	0
Gentamicin	R	F	1	1	2	3	11	1	22
		%	100	100	100	100	100	100	100
	S	F	0	0	0	0	0	0	0
		%	0	0	0	0	0	0	0
Ceftriaxone	R	F	1	1	2	3	11	1	22
		%	100	100	100	100	100	100	100
	S	F	0	0	0	0	0	0	0

		%	0	0	0	0	0	0	0

Antibiotics Sensitivity			**Bacterial type**						**Total**
			Acidovorax	***Klebsiella***	***Proteus***	***E. coli***	***p. aeruginosa***	***P. stutzeri***	
Amikacin	R	F	0	0	0	0	2	0	3
		%	0.0	0.0	0.0	0.0	18.2	0.0	13.6
	S	F	1	1	2	3	9	2	19
		%	100	100	100	100	81.8	100	86.4
Azithromycin	R	f	1	0	0	0	4	1	7
		%	100	0.0	0.0	0.0	36.4	100	31.8
	S	f	0	1	2	3	7	0	15
		%	0.0	100	100	100	64.6	0.0	68.2
Cefixime	R	F	1	1	2	1	9	0	16
		%	100	100	100	33.3	81.2	0.0	76.2
	S	F	0	0	0	2	2	1	5
		%	0.0	0.0	0.0	66.7	18.2	100	23.8

Twenty two isolates of different bacterial species that obtained from otitis media a various resistance levels towards 7 antimicrobial agents, Figure (4-4) show the inhibition zones of Gram negative bacteria by different of antibiotics drugs. The results were interpreted according to the recommendation of CLSI (2022). Antibiotics have been pivotal role in treating and preventing common infections, but the laws of evolution and natural selection along with the overuse and misuse have contributed to an alarming increase in antibiotic resistance worldwide (Zhen *et al.*, 2019).

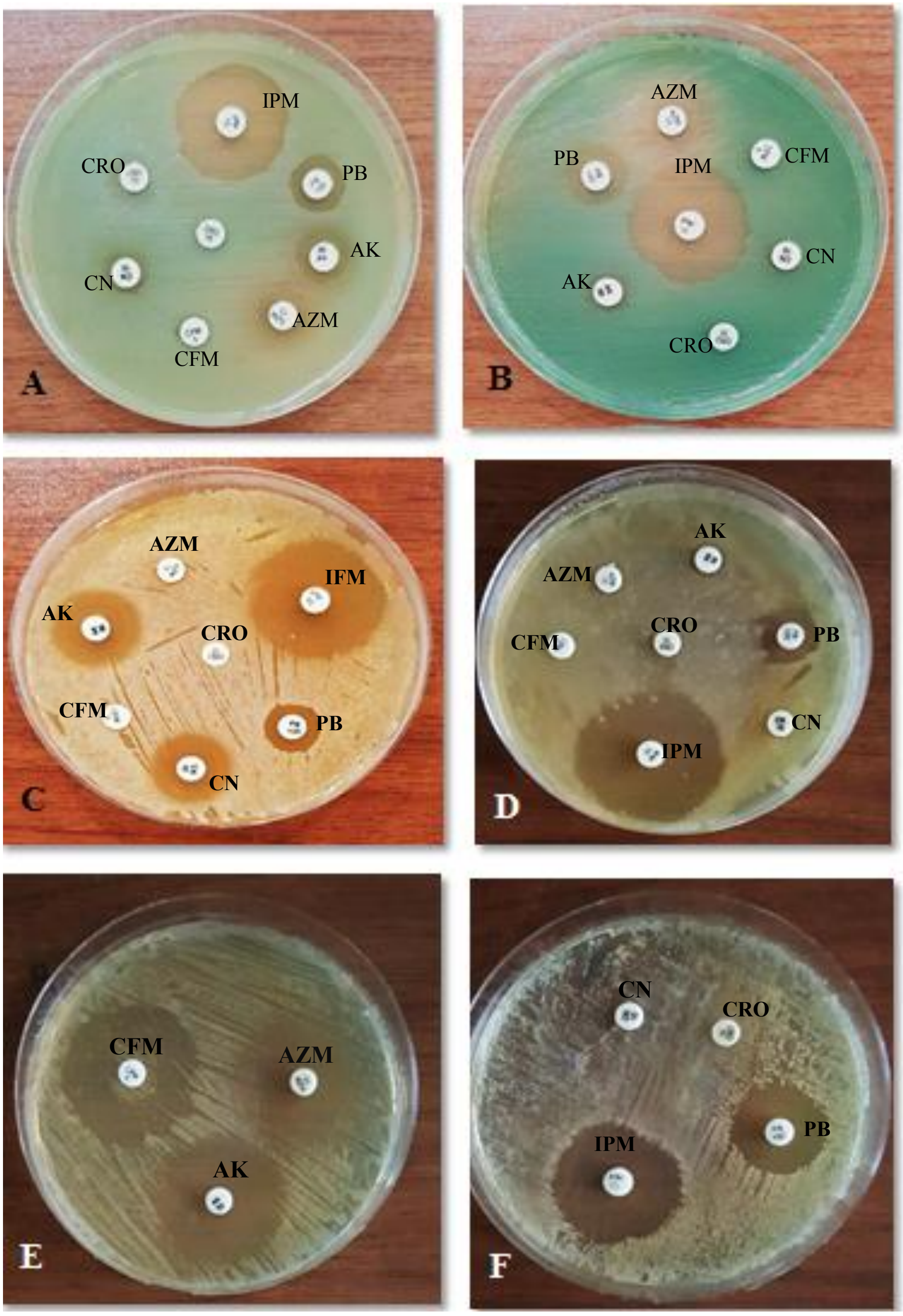


Figure (4-4): the inhibition zones of Gram negative bacteria by different of antibiotics drugs, A and B= *P. aeruginosa*, C= *Klebsiella*, D= *Proteus*, E and F= *E. coli*.

The selective resistance to the different antibiotics tested could be alluded to some obvious reasons, such as; inaccessibility to target site of action, decreased absorption of the active substance through efflux action, and the abuse of antibiotics that are commonly accessible (Bai *et al*., 2017).

Previous studies by (Agrawal *et al*.,2017) Showed most isolated gram negative bacteria highly sensitive to imipenem and amikacin. The result is not consistent with (Wasihun& Zemene,2015) show up *P. aeruginosa* is highly susceptible to Gentamicin. Our result is similar to (Seid *etal*.,2013) *Proteus* shown low levels of resistance to Gentamycin and Ceftriaxone.

The existence of high levels of resistance to beta lactam antibiotics and the bacteria linked with internal ear dysfunctions may be due to absence of study on antibacterial resistance amongst doctors and indulges inaccessibility of inadequate antibiogram minutes, exploitation of medicines and self-use and carelessness on patient part (Javed *et al*., 2020).

In study done by (Raakhee & Unguturu ,2014) *Pseudomonas* showed high sensitivity to Gentamicin (84.61)%, Imipenem (84.61)%. And (Iqbal *et al*., 2011) found Imipenem to be 100% sensitive and Gentamycin only 50%. And our results gave an affinity with the result (Mofatteh *etal*.,2018) against Gram negative bacteria, Imipenem and Amikacin were most effective in our study. These bacteria use a wide variety of mechanisms to resist antimicrobial killing, many of which are carried on mobile genetic elements transmitted to other bacteria (Blair *et al*., 2015)

Table 4-6 show that 90.9% of *S. aureus* are resist to Tetracyctine and 9.1% are sensitive. Regarding to Amoxicillin, all of gram positive bacteria are resist. In relation to Vancomycin, 27.3% of *S.aureus* are resist and 72.7% are sensitive. Concerning to Azithromycin, 63.6% of *S.aureus* are resist and 36.4% are sensitive. In relation to Levofoxacin, all of gram positive bacteria are sensitive. Finally, 27.3 % of *S. aureus* are resist to Gentamicin and 72.7% are sensitive .

Table (4-6): Distribution of Gram positive bacteria in relation to antibiotics sensitivity test.

Antibiotics sensitivity			*S. aureus*
Tetracyctine	R	Count	10
		% within bacteria	90.9
	S	Count	1
		% within bacteria	9.1
Amoxicillin	R	Count	11
		% within bacteria	100
	S	Count	0
		% within bacteria	0
Vancomycin	R	Count	3
		% within bacteria	27.3
	S	Count	8
		% within bacteria	72.7
Azithromycin	R	Count	7
		% within bacteria	63.6
	S	Count	4
		% within bacteria	36.4
Levofoxacin	R	Count	0
		% within bacteria	0
	S	Count	11
		% within bacteria	100

Gentamicin	R	Count	3
		% within bacteria	27.3
	S	Count	8
		% within bacteria	72.7

The most effective antibiotic against *S.aureus* isolated from the ear pus sample was Levofoxacin followed by Gentamycin and Vancomycin (72.7)% The least effective antibiotic was found to be Amoxicillin and Tetracyctine. Figure (4-5) show the inhibition zones of Gram positive bacteria by different of antibiotics drugs.

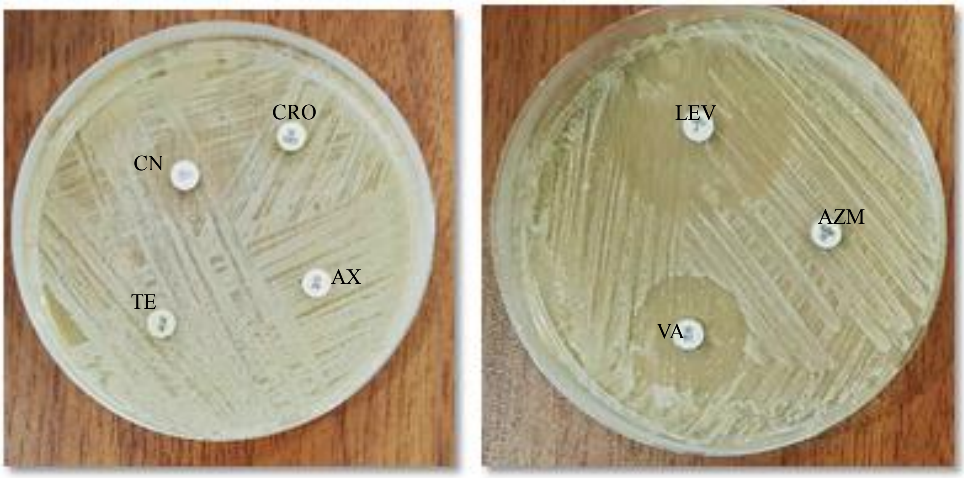


Figure (4-5): *S. aureus* antibacterial susceptibility test

That isolates were highly resistant to most antibiotics. *S. aureus* were 90% resistant to Tetracycline and This result was in line with that of study done in other parts (Osazuwa *et al.*, 2011) and 79 % for Tetracycline was reported (Ferede *et al.* ,2001). In the study carried out by (Kristo & Buljan, 2011) *S. aureus* showed a marked sensitivity to Gentamycin (88.5)%, azithromycin (92.3)%

And result disagrees with (Xu *et al*.,2021) The antibiotics most active against bacteria were Vancomycin (100% killing), Gentamicin (98.1)% followed by Tetracycline (86.8) %, Amoxicillin (80.2)%, Levofloxacin (71.7) %. In the present study, *S. aureus* revealed a high level of resistance to Amoxycillin , Tetracycline which is in agreement with a report from Pakistan (Getachew *etal.*,2009) and Iraq (Al-Marzoqi *et al*.,2013).

This high drug resistance might reflect the degree of misuse of antibiotics, which is a global problem mainly through their purchase without prescription in the local pharmacies and drug stores and through inappropriate prescribing habits and an over-zealous desire to treat every infection (Wasihun& Zemene ,2015)**.**

4.5. Antifungal susceptibility testing

In this study, the antifungal activity are used including Amphotericin-B (AP), Nystatin (NS), Fluconazole (FLC), Ketoconazole (KT), Itraconazole (IT), and Clotrimazole (CC) by using disc diffusion method against growth of *Candida* species under study. Depending on the number of samples studied for each species of yeast.

Table 4-7 show that 100% of all studied fungi and *candida* are sensitive to Clotrimazole, Itraconazole, Amphotericin, and Nystatin. Except with Fluconazole, that revealed some resistance to *C. krusei* and *C. albican* as 83.3% and 75% respectively. In relation to Ketoconazole, 50% are resist to *Candida tropicalis* and *C.krusei* and 75% are resist to *C. glabrata* all tested drugs showed generally good activity against most isolates of yeasts.

Table 4-7: Distribution of *candida* in relation to antifungal sensitivity test.

antifungal sensitivity			*C. tropicalis*	*C. krusei*	*C. albican*	*C. glabrata*
Clotrimazole	S	F (%)	2 (100)	6 (100)	4 (100)	4 (100)
	R	F (%)	0 (0)	0 (0)	0 (0)	0 (0)
Itraconazole	S	F (%)	2 (100)	6 (100)	4 (100)	4 (100)
	R	F (%)	0 (0)	0 (0)	0 (0)	0 (0)
Amphotericin	S	F (%)	2 (100)	6 (100)	4 (100)	4 (100)
	R	F (%)	0 (0)	0 (0)	0 (0)	0 (0)
Nystatin	S	F (%)	2 (100)	6 (100)	4 (100)	4 (100)
	R	F (%)	0 (0)	0 (0)	0 (0)	0 (0)
Fluconazole	S	F (%)	2 (100)	1 (16.7)	1 (16.7)	4 (100)
	R	F (%)	0 (0)	5 (83.3)	3 (75.0)	0 (0)
Ketoconazole	S	F (%)	1 (50)	1 (50)	4 (100)	1 (25)
	R	F (%)	1 (50)	1 (50)	0 (0)	3 (75)

Figure (4-6) show Inhibition zones of *Candida* species by different of antifungal drugs: Amphotericin-B (AP), Nystatin (NS), Fluconazole (FLC), Ketoconazole (KT), Itraconazole (IT), and Clotrimazole (CC). Several antifungal agents, including clotrimazole, Miconazole, Ketoconazole, Itraconazole, Fluconazole, and Nystatin, have been used for the treatment of otomycosis (Chappe *etal*.,2018 ; Ho *etal*.,2006). In

France, nystatin is the only antifungal licensed as a powder or as suspension (in combination with Oxytetracycline, Polymyxin B and Dexamethasone) for local treatment of otomycosis (Chappe *et al*.,2018).

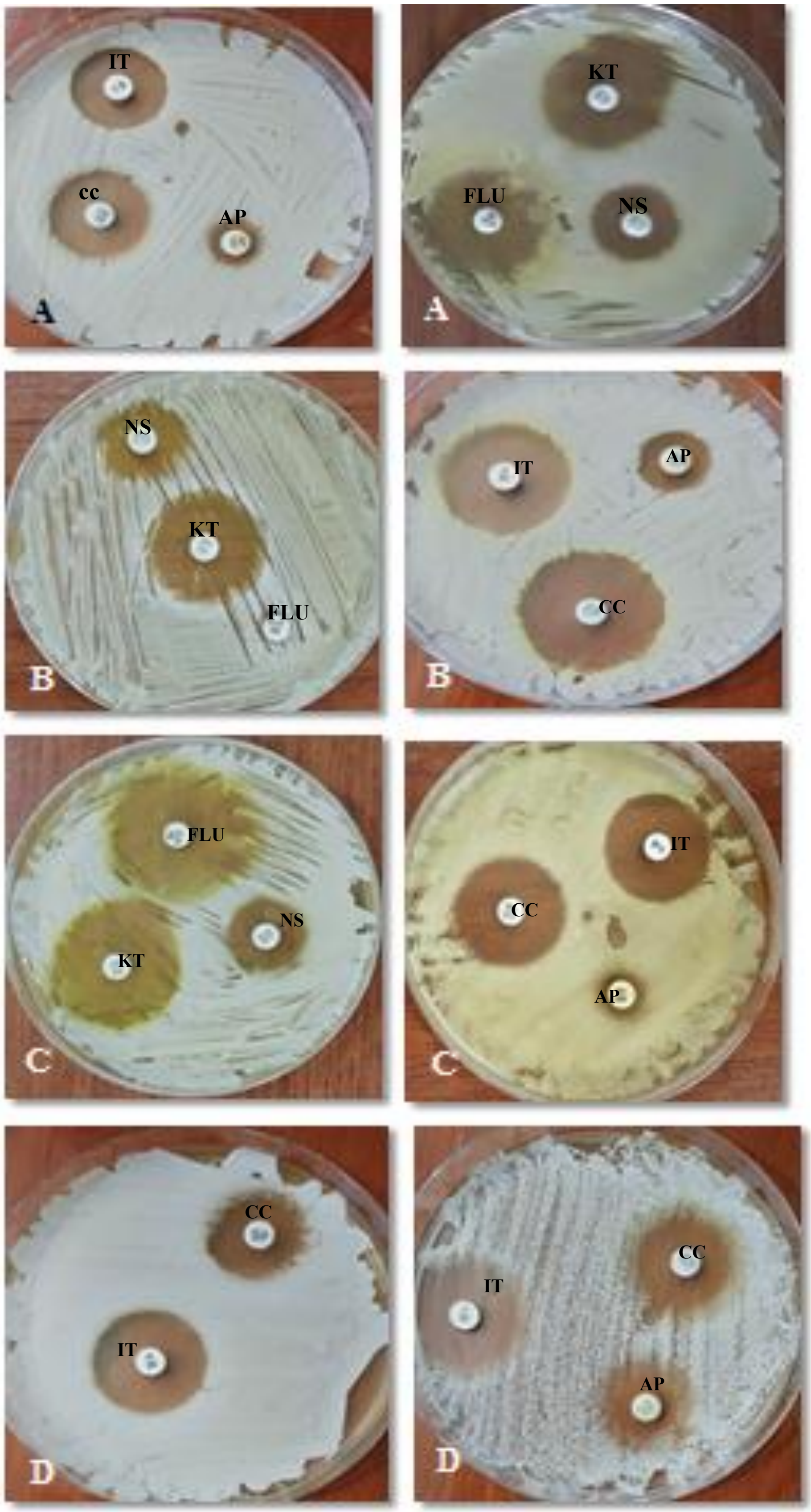


Figure (4-6): Inhibition zones of Candida species by different of antifungal drugs. A= *C. krusei*, B= *C. albicans*, C= *C. glabrata* and D= *C. tropicalis*.

Nystatin had a relatively good effect against *Candida* species in 12 out of 16 cases study by (Ahmed *etal*.,2018) . It is a polyene macrolide antibiotic that inhibits sterol synthesis in cytoplasmic membrane. Many molds and yeasts are sensitive to nystatin including *Candida* species. A major advantage of nystatin is that it is not absorbed from intact skin. Nystatin is not available as an otic solution, but it can be prescribed as cream, ointment, or powder with efficacy rates up to(50–80)% (Munguia& Daniel, 2008) .

Clotrimazole is the most widely used topical Imidazole In the USA, it is considered as the drug of choice for treatment of uncomplicated otomycosis (Sander ,2001) .It displays a high in vitro activity against most yeasts and molds and success rates ranging from 50% to 100% have been reported(Vennewald& Klemm,2010; Mofatteh *etal*.,2018) .

Several studies have reported on the effectiveness of various antifungal treatments both in vivo and in vitro, but a consensus on the most effective therapy remains controversial. Clotrimazole was found to be the most effective in in vitro studies for common fungal organisms by (Anwar& Gohar, 2014) .Other clinical studies have found Clotrimazole to be the most effective in vivo antifungal agent followed by Nystatin (Jackman *et al*.,2005) .

In a study by(Moharram *et al*., 2013) Clotrimazole was the most effective agents, whereas fluconazole and Itraconazole were the least effective. Itraconazole showed an *in vitro* activity against otomycosis agents. In the study by (Mahmoudabadi *etal*., 2015). Anticipate an increase of infections with azole-resistant *Candida albicans* species and Fluconazole-resistant yeasts such as *Candida krusei* (Gunsilius *etal*., 2001). Results by (Gharaghani *etal*.,2020) showed that all *Candida* spp.

(33.3)% isolates were of the tested resistant to both Fluconazole and Itraconazole.

4.6. Molecular study

4.6.1. Molecular diagnosis of Bacteria

4.6.1.1. DNA extraction and PCR assay

Ten isolates of bacteria species of this study were subjected for DNA extraction. The 16S RNA is universal primer pair that targeted the sequences place of the 16S rRNA gene of the bacteria isolates was used to discrimination of bacteria to the species level. The molecular weights of the PCR products for the bacteria isolates under study are 938 bp. Fig. (4-7) shows agarose gel electrophoresis of PCR products for bacteria species.

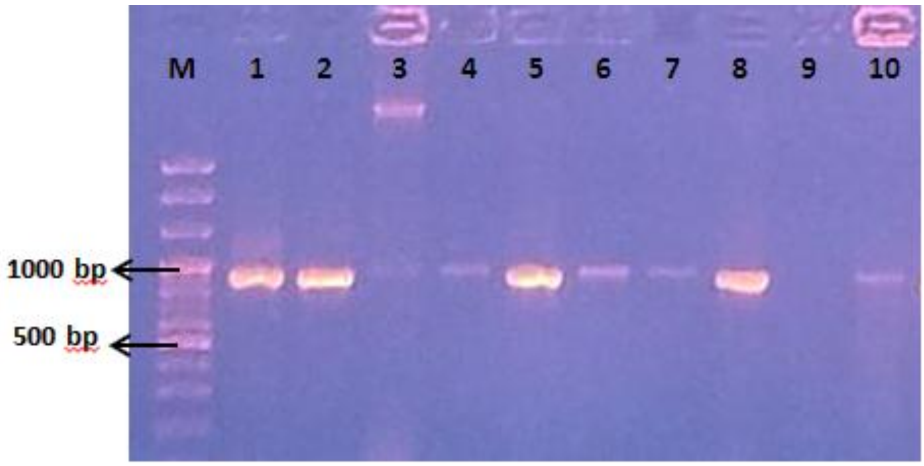


Fig. (4-7): Agarose gel electrophoresis of PCR products (938 bp) for 16S rRNA gene of bacteria species. Lane M= molecular marker 1500 bp.

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) 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); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Janda and Abbott, 2007; Idris *et al*., 2020).

Among the sequence-based microbiome studies, the 16S ribosomal RNA (rRNA) genes have been the most predominantly used molecular marker for bacterial classification, 16S rRNA gene analysis of mock polymicrobial samples indicated that primer sequence optimization is required to avoid preferential detection of particular taxa and to cover a broad range of bacterial species (Park *et al*., 2010; Srinivasan *et al*., 2015; Kai *et al*., 2018).

4.6.1.2. Sequencing analysis

PCR was done for ten bacteria isolates for 16S rRNA gene, Direct sequencing analysis is performed to the 20 µl PCR product of 16S rRNA gene were sent to Macrogen Laboratory in Korea. After obtaining the sequence of the nitrogenous bases of the sent isolates, they are matched with the sequence of reference samples in the gene bank using the NCBI Blast Nucliotide Database to confirm the highest proportion of the genus and species name for each isolates.

Appendix (1) shows the nitrogenous bases sequence for some of the isolates under study and the ratios of their corresponds with the reference isolates in the gene bank. These corresponds between the results of the sequence of the nitrogenous bases of the isolates under study and the sequence of reference specimens in the gene bank showed that the point mutation which represents miss matching and genetic gaps.

Table (4-8) showed a Results of molecular diagnosis for bacteria isolates under study comparison with reference strains in NCBT by Telomorphe name and it is offset by the Anamorphe name. The table showed that the molecular diagnosis using the nitrogenous bases sequence of some specimens were identical to the phenotypic diagnosis using traditional laboratory methods except several isolates, some have not been diagnosed with traditional methods to species level, which are diagnosed by molecular methods.

Table (4-8): Results of molecular diagnosis for bacteria isolates under study comparison with reference strains in NCBT.

No.	Molecular diagnosis		Reference strains
	NCBT strain	Identities	
1.	*E. coli*	99%	KY780340.1
2.	*K. pneumoniae*	99%	MH63827.1
3.	*B. smithii*	98%	OP090256.1
4.	*Providencia* sp.	99%	KY964269.1
5.	*P. stutzeri*	100%	KT380573.1
6.	*Acidovorax* sp.	99%	AB646302.1
7.	*Acidovorax* sp.	99%	MK757665.1
8.	*Enterobacter* sp.	99%	MT020107.1
9.	/		
10.	*P. aeruginosa*	99%	MT113103.1

Alsanie *et al*., (2018) were isolated 30 multidrug-resistant bacterial samples from different hospitals The 16S rDNA genes of all isolates were successfully amplified using PCR, and comprehensive identification results were based on GenBank databases. Analysis revealed nucleotide identities ranging from 76% to 100% based on the consensus sequences of 21 species, namely, *Bacillus cereus*, *B. subtilis, B. tequilensis, Caldimonas manganoxidans*, *Citrobacter freundii*, *Enterococcus faecium*, *Escherichia fergusonii*, *K.pneumoniae*, *Lactobacillus plantarum*, *Lactococcus garvieae*, *Leuconostoc mesenteroides*, *Myristica yunnanensis*, *Pantoea eucrina*, *P. aeruginosa*, *S. aureus*, *S. capitis*, *S. caprae*, *S. epidermidis*, *S. hominis*, *S. petrasii*, and *S. saccharolyticus*. While, Srinivasan *et al*., (2015) they assembled an extensive repository of clinical isolates representing 30 medically important pathogenic species, this strain repository was used to systematically evaluate the ability of 16S rRNA for species level identification, that shows a genus-level concordance rate of 96% and a species-level concordance rate of 87.5%.

4.6.1.3. Phylogenetic tree

The phylogenetic tree was analyzed by using Mega 6 software programe with unweighted pair group method with arithmetic mean (UPGMA) tree type based on sequences data of 16S rRNA gene amplified for 9 isolates. The 16S rDNA gene was sequenced in a this study to determine the evolutionary and phylogenetic relationships among the bacterial isolates. The results of phylogenetic tree analysis for bacteria were observed seven genera (Fig 4-8).

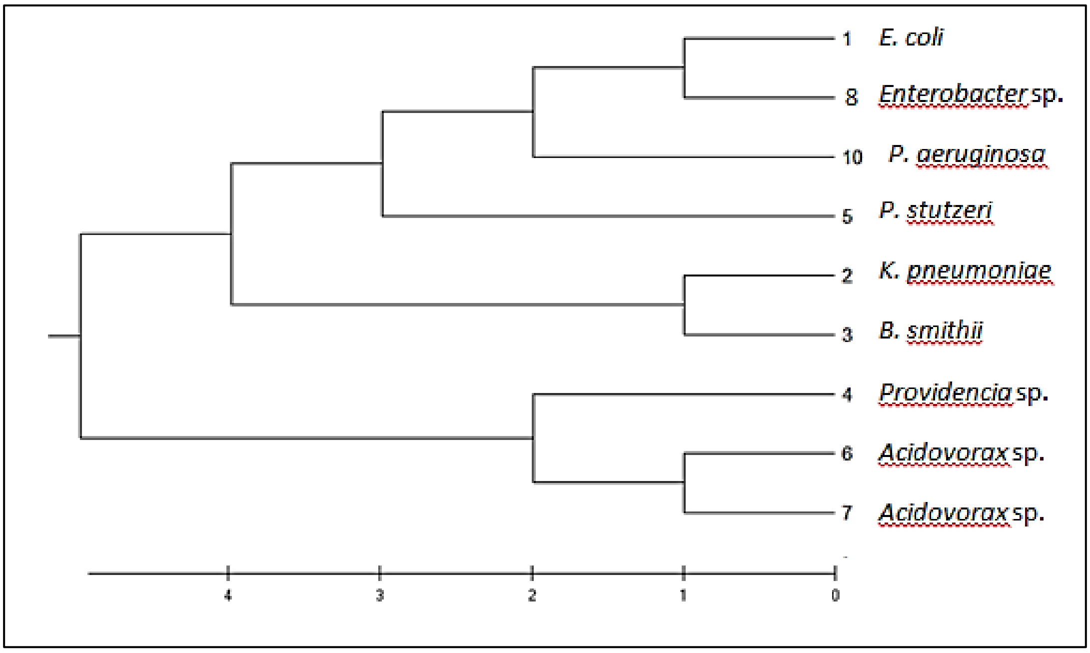


Fig. (4-8): Phylogenetic tree based on 16S rRNA gene sequences for isolates under studies.

By drawing the phylogenetic tree of the 16S rRNA gene sequences for the isolates under study that show a high degree of similarity between species ranging(95- 99)%. The difference in sequence among any clades shows approximately (1-5)%. Un weighted pair group method with arithmetic mean (UPGMA) tree type was a common type of phylogenetic tree to determinate diversity of pathogenic fungi. Therefore, there were many studies that have used this type of tree.

Bacterial phylogenetic analyses are commonly performed to explore the evolutionary relationships among various bacterial species and genera based on their 16S rRNA gene sequences. Results of phylogenetic tree coincided with several recent studies which show a high degrees of similarity and homogeneity in 16S rRNA gene sequences among different of bacterial species. Ibrahim *et al*., (2019).

used 16S ribosomal RNA (16S rRNA) gene sequences to study the relationship between *Klebsiella* spp. isolated from clinical and environmental samples in Iraq. Phylogenetic tree strategies have clearly indicated a relatively close similarity amongst all analysed *Klebsiella* isolates and revealed the intra-species genetic distance between the individual isolates of the *Klebsiella* spp. Nakano *et al*., (2023) phylogenetic trees were performed to distinguish between highly similar species. Moreover, *Escherichia albertii* strains were clearly distinguished from *E. coli* and *Shigella*, despite being closely related to enterohemorrhagic *E. coli* in the phylogenetic tree.

Currently, prokaryotic phylogenetic classification depends on 16S rRNA gene sequences, which are ubiquitously present and highly conserved in bacteria, but species with more than more 99% identity based on 16S rDNA sequencing are rarely classified. In such cases, one or more additional conserved genes are often used as a secondary candidate for indexing during phylogenetic analyses, such as *gyrB* for *Shigella*, *Salmonella*, and *E. coli* (Fukushima *et al*., 2002).

4.6.2. Genotypic polymorphism of *MUC5B* gene

The intronic MUC5B polymorphism was examined by PCR using primers that amplify across the entire variable numbers of tandem repeats (VNTR) region. Agarose gel electrophoresis of PCR products for MUC5B primer of patients with otitis media as well as control persons, that have been shown in (Figure 4-9).

The frequency of the (*MUC5B*) gene polymorphism genotypes and alleles is shown in Table (4-9). In general, the different combination of more than seven known alleles several genotypes for Muc5B VNTR polymorphism but 3 genotypes were found in this study of (*MUC5B*) gene polymorphism in patient with otitis media and control persons.

The frequency of genotypes 6/8, 8/8, and 8/9 was respectively 16.7, 66.6% and 16.7% in Patients, while the frequency of genotypes 6/8, 8/8, and 8/9 was respectively 16.7%, 76.7, and 6.6% in control persons. In both groups (patients and control), the genotypes of (*MUC5B*) gene polymorphism with high frequency was 8/8 (66.6% and 76.7%) respectively.

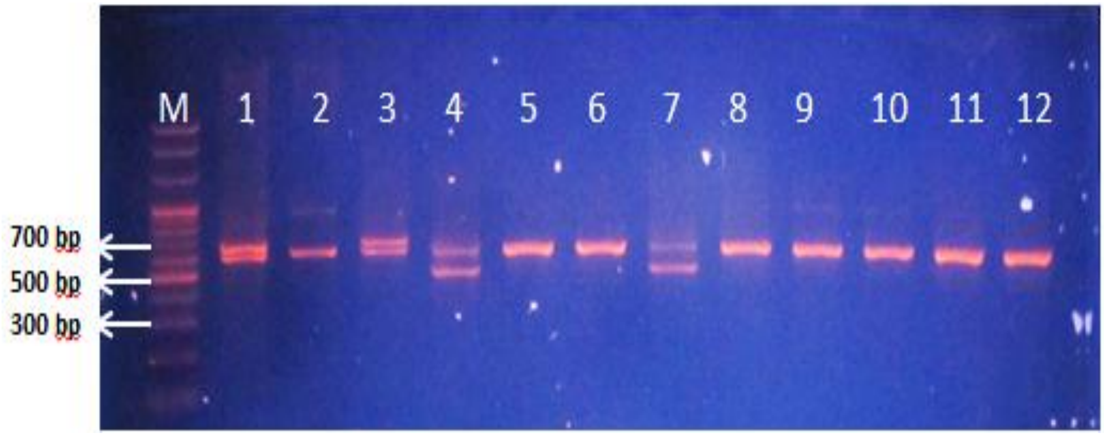


Fig. (4-9): Agarose gel electrophoresis of PCR products for (MUC5B) gene polymorphism of patients (Lane 1-6) and control (7-12). Lane M= molecular marker 100 bp., Lane 1, 3= Heterozygous pattern 1 (Allele 9= 692 bp and Allele 8= 633 bp); Lane 4, 7= Heterozygous pattern 2 (Allele 8= 633 bp and Allele 6= 515 bp); Lane 2, 5, 6, 8- 12= Homozygous (Allele 8= 633 bp).

Therefore, the high allele frequency of allele 8 was 83.4% in patients and 88.3% in control. The other two allele (6 and 9) with frequency 8.3% in patients for tow alleles, while 8.3% and 3.3 respectively in control. Through the results obtained in this study we note the allele 9 of *MUC5B* appears to show a role in susceptibility to otitis media disease Table (4-9), this results indicated that carriers of allele 9 increased risk of evolving otitis media disease. No- significant difference at p≤0.05 between patients and control.

Table (4-9): Comparing genotypes of (*MUC5B*) gene between patients and control groups.

MUC5B Genotype		Genotypes frequency (%)			
		Patients n=30 (%)	Control n= 30 (%)	χ^2 value	Sig.
MUC5B	6/8	5 (16.7)	5 (16.7)	1.495	0.474
	8/8	20 (66.6)	23 (76.7)		
	8/9	5 (16.7)	2 (6.6)		
Allele	Alleles frequency (%)				
	6	5 (8.3)	5 (8.3)	1.373	0.503
	8	50 (83.4)	53 (88.3)		
	9	5 (8.3)	2 (3.3)		

MUC5B are the secreted gel-forming mucins, and this gene is contained within a single 400-kb genomic DNA fragment on chromosome 11 band p15.5 with three other secreted gel-forming mucins, MUC6, MUC2, and MUC5AC. (Pigny *et al*. 1996; Ahn *et al*., 2009). Mucins, the major component of mucus, contain tandemly repeated sequences that differ from one mucin to another. Considerable advances have been made in recent years in our knowledge of mucin genes. The availability of the complete genomic and cDNA sequences of MUC5B, one of the four human mucin genes clustered on chromosome 11, provides an exemplary model for studying the molecular evolution of large mucins (Desseyn *et al*., 2000).

Ahn *et al*., (2009) they identified seven variable number of tandem repeats (VNTRs; minisatellites) from the entire MUC5B region. Six (MUC5BMS1, -MS2, -MS3, -MS4, -MS5, and -MS7) of the seven minisatellites evaluated in this study were novel minisatellites, these minisatellites of MUC5B were analyzed in genomic DNA extracted from controls, patients, and multigenerational families. these minisatellite polymorphisms could be useful as markers for paternity mapping and DNA fingerprinting.

Our results agree with Ubell *et al*., (2010) used polymerase chain reaction to determine the size of *MUC5B* genes between patients with otitis media and control, there were no significant differences between control or study patients, but this is likely related to relatively small patient numbers. Recently, there are several studies findings implicate MUC5B in OME-associated hearing loss and suggest that MUC5B targeted treatment strategies may be beneficial for treatment of OME (Val *et al*., 2015; Samuel *et al*., 2017). Although MUC5AC predominates in the healthy lung, MUC5B is the predominant mucin in cystic fibrosis and chronic obstructive pulmonary disease. Similarly, MUC5B is the predominant mucin in middle ear effusion and mucosa during OME (Kirkham *et al*., 2008; Preciado *et al*., 2010).

4.6.2. Genotypic polymorphism of *IL-1 RN* gene

Polymorphisms were identified by using PCR assay with IL-1 RN VNTR primer couple that targeted the sequences place of the Interleukin-1 receptor antagonist gene (*IL-1RN*). The electrophoresis result for IL-1 RN VNTR primer is coded as follows: (allele 1= 410 bp, allele 2= 240 bp, allele 3= 530 bp, allele 4= 325 bp, and allele 5= 595 bp). Agarose gel electrophoresis of PCR products for IL-1 RN VNTR primer of patients with otitis media as well as control persons, that have been shown in Figure(4-10).

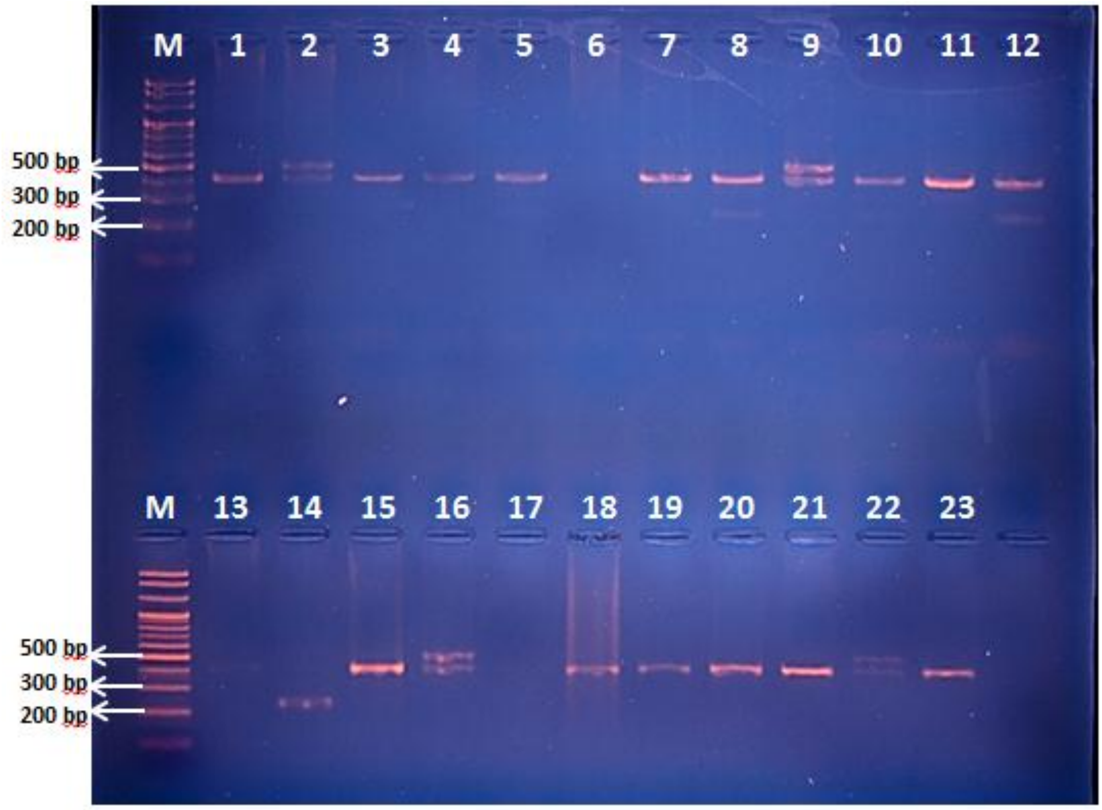


Fig. (4-10): Agarose gel electrophoresis of PCR products for (IL-1RN) gene polymorphism of patients (Lane 1-16) and control (18-23). Lane M= molecular marker 100 bp. Lane 1, 3-7,10,11,13,15,18-21, 23= 1/1 genotype. Lane 2,9,16,22= 1/3 genotype. Lane 8,12= 1/2 genotype. Lane 14= 2/2.

The frequency of the (*IL-1RN*) gene polymorphism genotypes and alleles is shown in Table (4-10). In general, the different combination of the five known alleles gives 15 genotypes for IL-1 RN VNTR polymorphism but 4 genotypes were found in this study of (*IL-1RN*) gene polymorphism in patient with otitis media while there are two genotypes of (*IL-1RN*) gene polymorphism in control persons. The frequency of genotypes 1/1, 1/2, 1/3, and 2/2 was respectively 60.0%, 16.7, 16.7% and 6.6% in Patients, while the frequency of genotypes 1/1 and 1/3 was respectively 83.3% and 16.7% in control persons. In both groups (patients and control), the genotypes of (*IL-1RN*) gene polymorphism with high frequency was 1/1 (60.0% and 83.3%) respectively.

Therefore, the high allele frequency of allele 1 was 76.7% in patients and 91.7% in control, and the low allele frequency of allele 3 was 8.3% in patients and 8.3% in control. While the allele 2 found only in patients with frequency 15.0%. Through the results obtained in this study we note the allele 2 of *IL-RN* appears in control to show a role in susceptibility to otitis media disease (Table 4-9), this results indicated that carriers of allele 2 have an almost double increased risk of evolving otitis media disease.

The individual with 1/2 and 2/2 genotype were significantly prevalent among the otitis media patients 5 (16.7)% and 2 (6.6)%, as compared with healthy control subjects (0%) without significant difference (P value =0.253).

In contrast, the alleles frequency among control when compared with otitis media patients with significant difference (P value =0.027). However, the results for *IL-RN* gene polymorphism have shown that 2 allele was obviously more presented among patients 9 (15.0)%, whereas, the allele 1 were more presented among control subjects 48 (96.0)%, with significant difference, as shown in Table (4-10).

Genes that control the production of cytokines are the first choice candidate genes for COM, same as for a variety of chronic inflammatory and autoimmune human diseases (Hollegaard *et al*., 2006). There are several studies used IL-1 RN VNTR primer as indicator of imperative controller of host immunity. The number of studies revealed the association of IL-1 gene cluster with a predisposition to certain inflammatory diseases, but only a few had been performed in OM (Patel *et al*., 2006). This results agree with Zivkovic *et al*., (2018) they studied the evaluate of the polymorphisms in innate immunity/ inflammation cascade genes from *IL-1 RN* gene association with chronic OM and found that carriers of IL-1RA allele 2 have a favorable association with

COM. The other study revealed that IL-1RN-VNTR 1/2 and 2/L genotypes were more frequent among patients with cutaneous melanoma (43.6 and 45.1)%, respectively compared with healthy controls (Cauci *et al.*, 2019).

Table (4-10): Comparing genotypes of (*IL-1RN*) gene between patients and control groups.

IL-RN Genotype		Genotypes frequency (%)			
		Patients n=30 (%)	Control n= 30 (%)	χ^2 value	Sig.
IL-RN	1/1	18 (60.0)	25 (83.3)	4.079	0.253
	1/2	5 (16.7)	0		
	1/3	5 (16.7)	5 (16.7)		
	2/2	2 (6.6)	0		
Allele	Alleles frequency (%)				
	1	46 (76.7)	55 (91.7)	7.194	0.027*
	2	9 (15.0)	0		
	3	5 (8.3)	5 (8.3)		

*** Significant difference at p≤0.05.**

4.6.3. The Polymorphism of *IL-17A* Gene

The polymorphism of *IL-17A* gene was done for 60 specimens of human blood DNA (30 patients, and 30 healthy people). Site of targeted partial sequence covering the SNP rs2275913 was occurred on Chromosome 6. Figure (4-11) shows the validity of SNP rs2275913. The results shown success the primer pair efficiency to amplification region 52186008 & 52186691as target DNA region of IL17A included SNP :rs2275913 G>A.

The primer pair for the gene of *IL-17A* was successfully amplified the target under amplification. The PCR products of *IL-17A* gene shown 648 bp. Figure (4-12) for patients and Figure (4-13) for control showed agarose gel electrophoresis of PCR products of IL-17A pair primer.

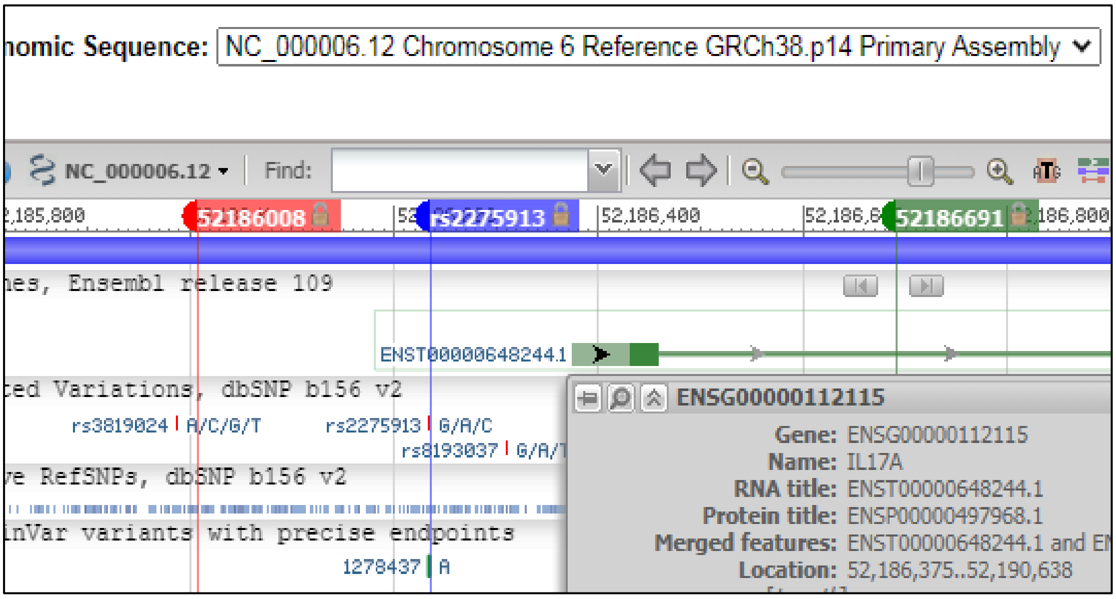


Figure (4-11): The targeted region of partial sequence of IL17A amplified by primer pair F1 covering the SNP rs2275913.

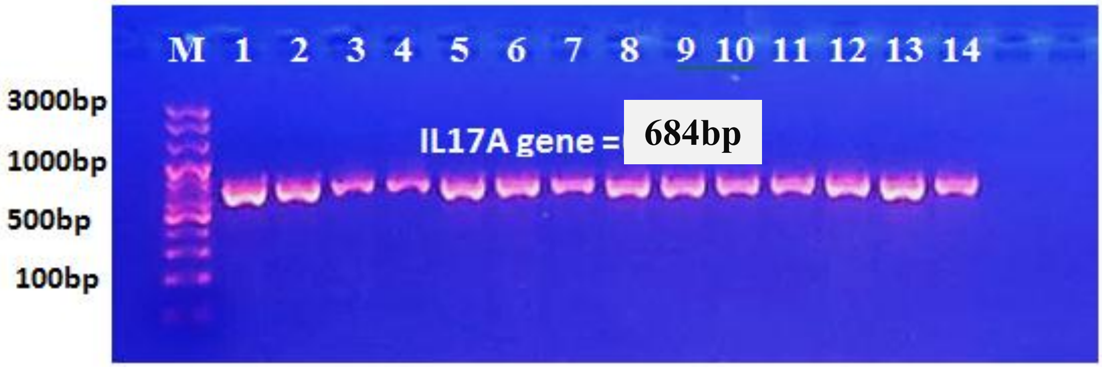


Figure (4-12): Gel electropheresis Profile of PCR products of target of IL10 shown 648bp for 20 PCR product of IL17A of Otitis media. 1-14 patient samples, M=molecular marker first step100bp.

Figure (4-13): Gel electropheresis Profile of PCR products of target of IL10 shown 648bp for 20 PCR product of IL17A of Otites media. 1-13 control samples, M=molecular marker first step100bp.

The result was shown in Figure (4-14) representative amplification the targeted region of partial sequence of IL17A amplified by primer pair F1 and R1 covering the SNP rs2275913. While the Figure (4-15) was shown amplicone sequence region with flanking primers, It also shows the location of SNP of IL17A under study.

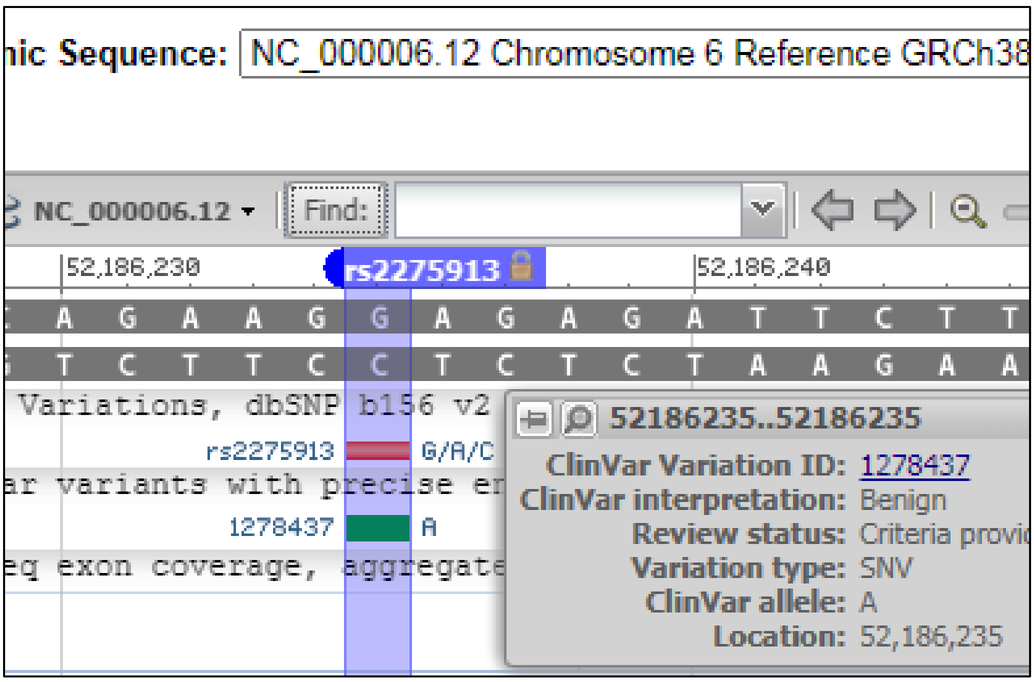


Figure (4-14): Amplification of targeted SNP of IL17A.

```
AATCAAGGTACATGACACCAGaagacctacatgttacttcaaacttttc
ttcctcatgaaccattaaaatagagcataactcttctggcagctgtacat
atgttcataaatacatgatattgacccatagcatagcagctctgctcagc
ttctaacaagtaagaatgaaaagaggacatggtctttaggaacatgaatt
tctgcccttcccattttccttcagaag(G/A)agagattcttctatgacct
cattgggggcggaaattttaaccaaaatggtgtcacccctgaacccactgc
gacacgccacgtaagtgaccacagaaggagaaaagccctataaaagagag
acgatagcgctacattttgtccatctcatagcaggcacaaactcatccatc
cccagttgattggaagaaacaacgatgactcctgggaagacctcattggt
ggtgagtcctgcactaacgtgcgatgctcttgctgatttggaccagatag
tatttctggaccgtgggcatgaaacgctgggttctgactatggagatcca
ggaatactgtatatgtaggataggaaatgaaagctttggtaggtatttaa
gtcattgtgcagcattttcaagaactgatacacagcagtttgaaagataa
gattaaaactgaAAGATAGCTATATTGGGGCTAA
```

Figure (4-15): Amplicone sequence region with flanking primers.

When comparative the chromatograms of SNP rs2275913, based on the facilities of Snap gene software, the wild type was shown black single peak which termed as homozygous wild allele GG, while the heterozygous mutant allele which shown overlapped two peak black and green on to indicated converted wild allele G to A allele, the substitution mutant allele A stead of wild allele G and shown one green peak termed by homozygous mutant allele AA (Figure 4-16).

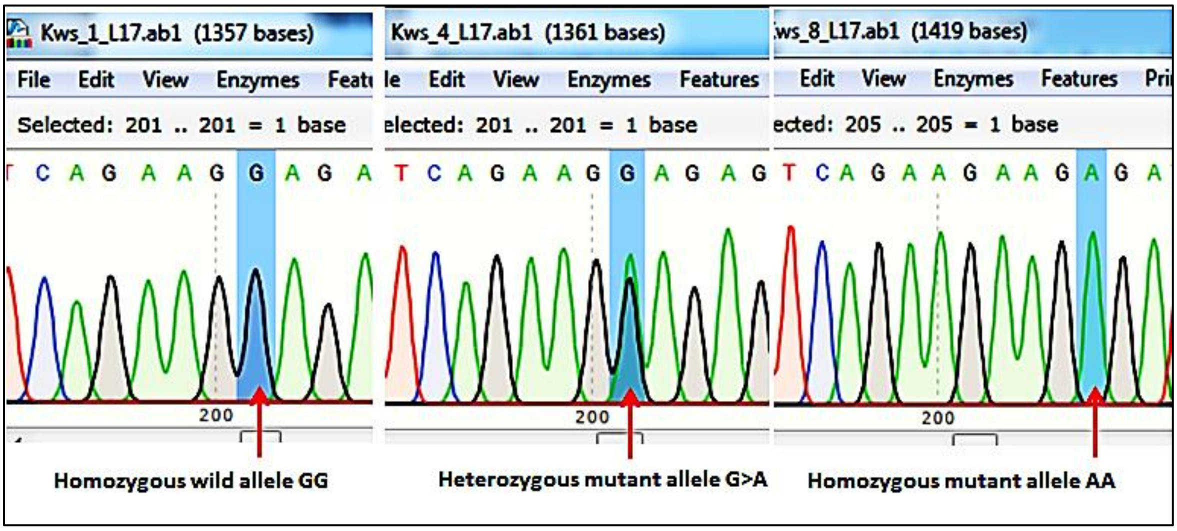


Figure (4-16): The site of Heterozygous allele GA of SNP rs2275913 G>A on IL17A was located on 52186236 chromosome 6,the allele G mutant to allele A, in combination images of NCBI IL17A partial sequence site, Multiple alignment sequence and chromatogram.

The SNP rs2275913 allele and genotype frequencies showed no critical varieties between OTS media patients and control. The subsequent SNP (rs2275913) was seen to have three genotypes (GG, GA and AA) in OT patients and control were noticed. These genotypes were identified with two alleles; G and A. A combination SNP site on chromosome and sites on patient sequence were performed to illustrated SNP validity. The results multiple alignment of chromatograms of patients with Otitis media in Figure (4-17), while of control in Figure (4-18).

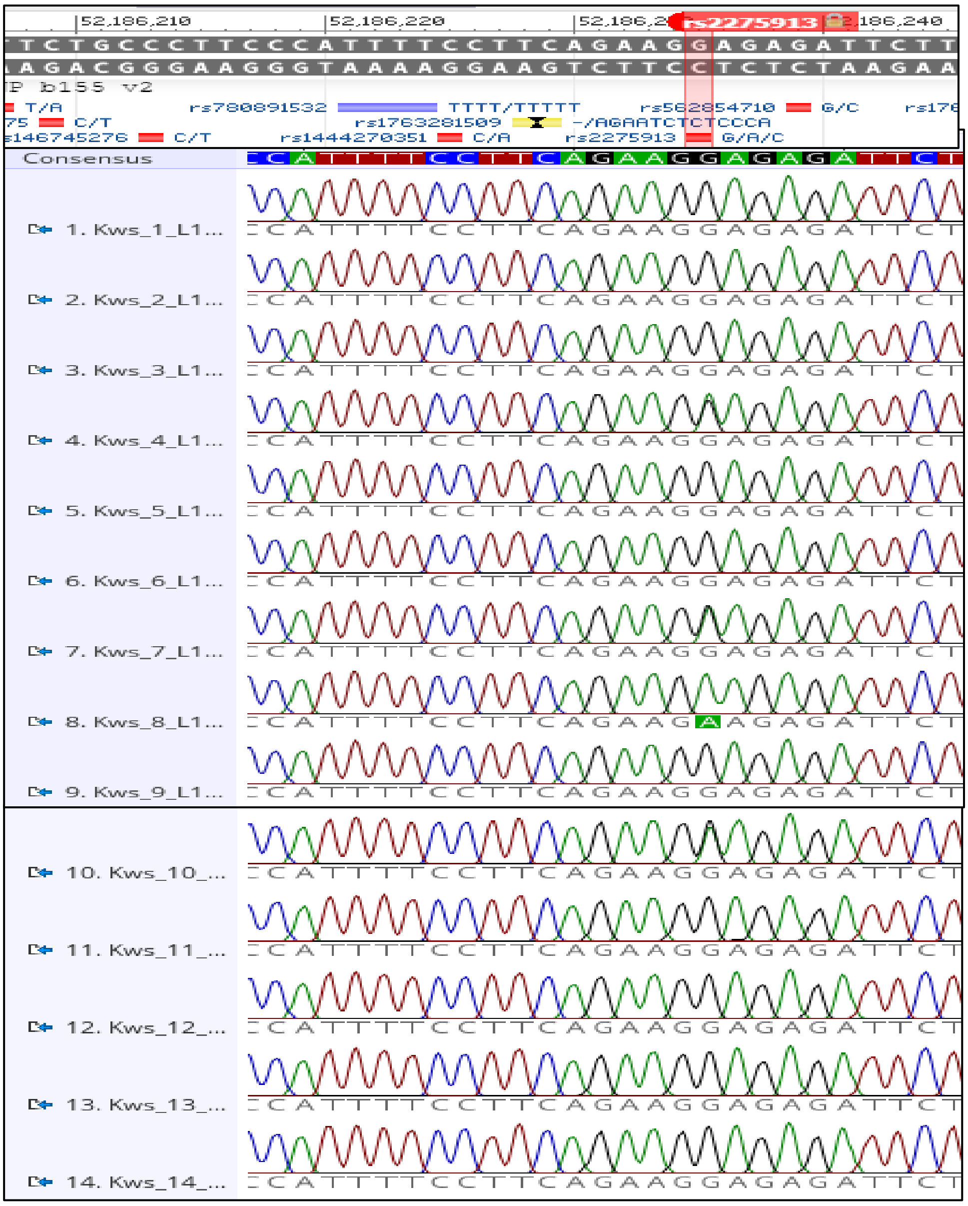


Figure (4-17): The multiple alignment of chromatograms of targeted region of IL17A SNP : rs2275913 G>A 1-14 Patient group. alignment performed by Geneious prime software.

The genotype GA and AA were shown low distribution in patients undergo Otitis media and control group for each, the values of Odd Ratio(OR) were support that A allele in both GA and AA was not far correlated with disease under interest and considered as risk allele. The Odd Ratio was lower in genotype AA OR= 1(0.13-7.6) with no significance p value 1, and the allele frequency was lower in with A allele 11(18.3)% in patient group with low value of OR=**0.67(0.28-1.6)** , p.

value = 0.3, while the allele frequency 15(25)% in control group, This SNP shown low frequent and not been Otitis media disease to be correlated risk allele (Table 4-11).

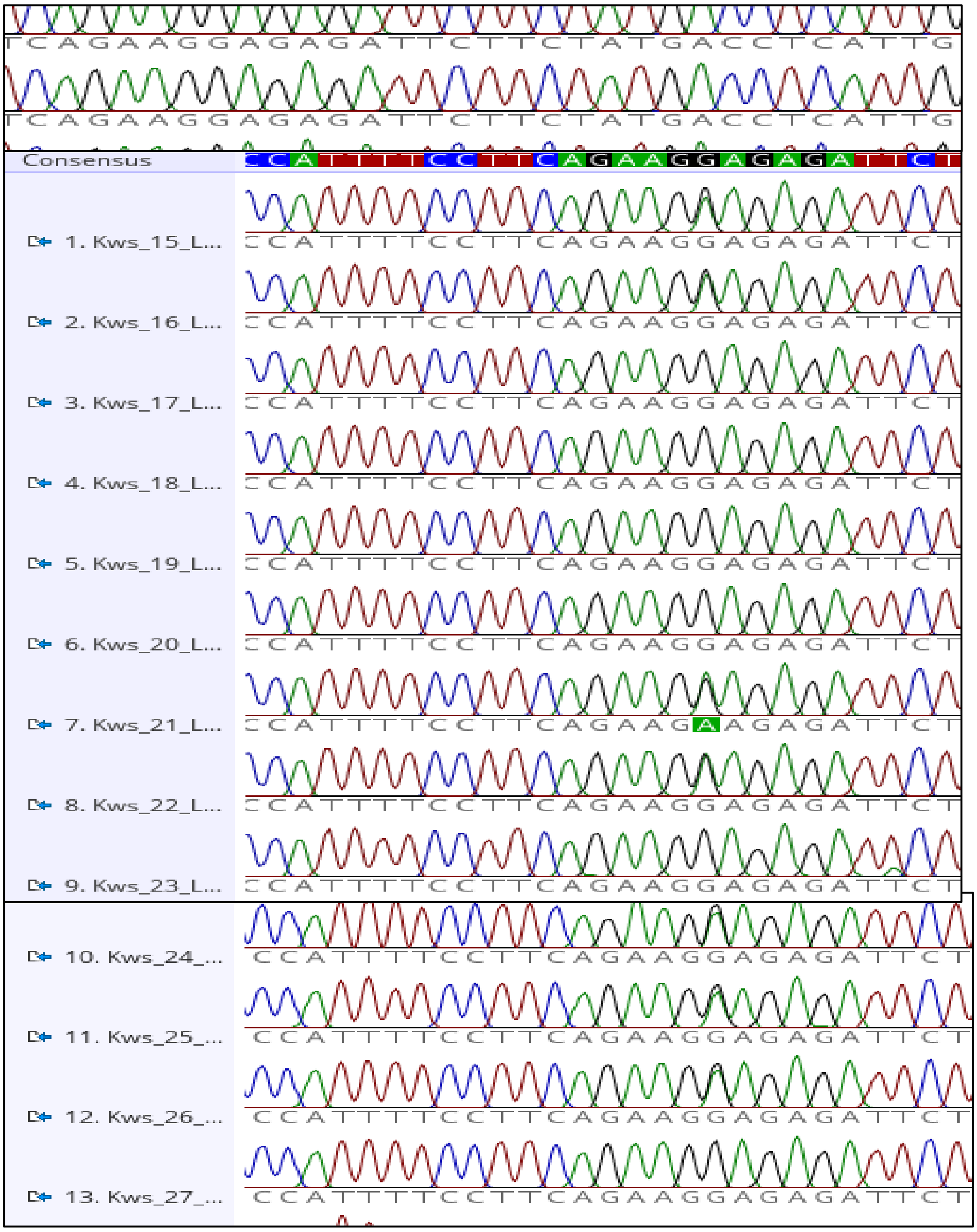


Figure (4-18): The multiple alignment of chromatograms of targeted region of IL17A SNP : rs2275913 G>A 1-13 control group. alignment performed by Geneious prime software.

Genes involved in the inflammatory process are important potential modifiers of individual susceptibility to reduced Otitis function. IL17A is important cytokines involved in inflammation of the human organs. The complex interaction between these more than one proteins makes them interesting potential candidate genes for modulating risk of inflammatory disease.

Table (4-11): Genotypes distribution and allele frequency of wild type allele and mutant allele of SNP: rs2275913 G>A, OR values and p values for Otitis media infection in patients and control groups.

rs2275913 G>A		Case N=30	Control N=30	OR (95%CI)	P-valu
genotypes	GG	21 (70)%	17 (56.7)%	Reference group	
	GA	7 (23.3)%	11 (36.7)%	0.57(0.18 - 1.72	0.3
	AA	2 (6.7)%	2 (6.7)%	**1 (**0.13 - 7.6**)**	1
Allele % Frequenc	G	49(81.7)%	45(75)%	1.48(0.61 - 3.51	0.3
	A	**11** (18.3)%	15(25)%	0.67 **(**0.28-1.6**)**	0.3

This study highlights of Otitis media as important disease, and attempt to evaluate the role of IL17A polymorphism and Otitis media disease. The illness of Otitis media infection was one of the common disease, and the important pathogens that cause a severe disease in immunocompromised patients. The pathology and immune responses associated with the ensuing disease have not been well described by microbial infections.

A current study, highlight on the frequency of risk allele A in both heterozygous (GA) and Homozygous (AA) in SNP rs2275913 based on the Odd Ratio. The results shown lower in genotype GA was OR=

0.57(0.18 - 1.72) with p value 0.3, and the allele frequency was lower in with A allele Table (11).

Many pathogenic and opportunistic microbes were isolated from patients undergo ear infection and considered one of the most impact infections, the isolated bacterial and fungal species were consistent with results of Getaneh *et al*., (2021). isolated and identified *S. aureus* (27.9)%, *Proteus* spps (20.8)%, *Streptococcus* spps (10)%, and *Pseudomonas* spp. (8.92)% were the main isolates. More than 45% of isolates, with 50.9% of Gram-negative and 37.3% of Gram-positive, were multidrug-resistant.

Zhang *et al*.,(2022) concluded that IL-17 plays a key role in the pathogenesis of the OVA-induced OME rat model. IL-17 induced inflammatory responses via the Notch signaling pathway and targeting IL-17 might be an effective approach for OME therapy.

The fungal diseases have emerged as a significant cause of morbidity and mortality Szmuilowicz and Young (2019) Suggesting the need for more effective anti-fungal therapies. IL-17 and Th17 cells are critical for broad immunity to extracellular microbes, and recent studies in both humans and animal models have elucidated the overwhelming role of the Th17/IL-17 axis in protection against superficial candidiasis, caused mainly by *Candida albicans*, which is the primary focus of this review.

Four main genera of fungi, *Candida*, *Cryptococcus*, *Aspergillus* and *Pneumocystis*, cause more than 90% of the mortality due to fungal infections. Several non-*albicans* species of *Candida* (*C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. dubliniensis*) cause disease, yet *C. albicans* is the main causative agent and the best characterized species (Conti *et al*., 2016).

This gene is a member of the IL-17 receptor family, which includes five members (IL-17A-E) and encoded protein is the inflammatory cytokines produced by stimulant T cells. IL-17A tracks by the estuary stimulates the production of inflammatory molecules, chemicals, antimicrobial peptides, and proteins. IL-17A plays a pivotal role in various infectious diseases, inflammatory and autoimmune disorders, and cancer. High levels of this cytokine are associated with several chronic inflammatory diseases (Ramirez-Carrozzi *et al*., 2011).

Similar to IL-17, IL-17C promotes anti-microbial protective responses and barrier maintenance in skin and intestine (Song *et al*., 2011).

Interleukin-17 responses defend against extracellular fungal and bacterial pathogenic species including *Candida, Cryptococcus, Klebsiella and Staphylococcus,* among others. Indeed, genetic defects in the Th17 or IL-17 signaling pathway in humans or in mice lead to severe mucocutaneous *Candida* infections in humans, which points to the particular importance of IL-17 in immunity to fungi (Conti and Gaffen, 2015; Drummond and Lionakis, 2018).

Final finding of this study conduction, the IL17A considered an important interleukin defense and estimation other interleukins for defense, the rs2775913G>A was validated in patients group, but the OR was low than one value to be significant. The justification of this issues may correlated with two cases, first the Otitis media may be not correlated with polymorphism of IL17A but, this think was impossible based on the data of previous researches , which emphasized the pioneer of IL17A in Otitis media infection. The second caused may correlated with limitation of patient sample size may not supported Or values. In general the conducted results highlight on determined the role of polymorphism of IL17A with Otitis media.

4.7. ELISA study for TNF-α

The levels of Tumor Necrosis Factor-Alpha (TNF-α) were evaluated depended on ELISA method, and these levels were compared between two studied groups: patients with otitis media and healthy subjects as a control group. Table (4-12) displays the comparison of TNF-α between otitis media patients and control groups. The results of statistical analysis showed insignificant ($p > 0.05$) difference of TNF-α means between patients and control subjects (272.85 ± 132.21 patients VS 200.5 ± 36 control).

Table 4-12: Mean differences of TNF between patients and control groups

Groups	N	Mean	SD	p-value
Patients	25	272.85	132	0.218^{NS}
Control	10	200.5	36	
Statistical test: Independent sample t-test NS: Non-Significant				

Otitis media is one of the most common ear disorders and a critical healthcare problem. Moreover, chronic Otitis media is one of the most significant causes of acquired and preventable hearing loss (World Health Organization, 2004). Though the pathogenesis of Otitis media is not fully understood, it is currently believed that the etiology is multifactorial, with the innate immune system and its response to bacterial infections playing a crucial role.

A high probability of chronic Otitis media occurs in the absence of central components of the immunological pathways, such as TLR, NLR, MyD88 and TNFα (Leichtle *et al*., 2011). Hence, these pathways are of central importance in the normal recovery from Otitis media and in the pathogenesis of chronic Otitis media.

Although the results of the current study did not record a significant difference between patients and control regarding TNF-α, it showed an increased level in patients compared to healthy subjects. The reason for the absence of significant differences may be due to the small size of the sample and the difference in the quality of the kit used to assess TNF-α levels

A previous studies reported a significant upregulated in OM compared to healthy (Leichtle *et al.,* 2022). TNF-α is well known as a key regulator not for only inflammation but also apoptosis pathways (Aggarwal, 2003; Ebmeyer *et al*., 2011). Therefore, this topic needs further extensive studies to reinforce the importance of TNF-α in otitis media.

4.7.1. The relationship between TNF and bacterial types

The distribution of serum TNF-α according to bacterial types illustrates in Table (4-13). The results of statistical analysis showed a significant (P≤0.05) increase in serum TNF-α in *p. aeruginosa* bacteria compared to other bacteria.

Table 4-13: Mean differences of TNF in relation to bacterial types

Bacterial types	Mean ± SD of TNF	p-value
Klebsiella	106.57± 0.00 [b]	0.00*
Proteus	137.14± 0.00 [b]	
E. coli	109.32± 8.23 [b]	
S.aureus	115.50±45.65 [b]	
p.aeruginosa	511.26± 289 [a]	
Statistical test: ANOVA *(P≤0.05)		

In fact, gram-positive or gram-negative bacteria can drive leukocytes to create distinct patterns of pro-inflammatory cytokines and chemokines (Kaur & Pichichero, 2015). According to the finding of present study, *pseudomonas aeruginosa* infection lead to release increased levels of TNF-α as compared with other bacterial types.

Tumor Necrosis Factor-Alpha is necessary for the extravasation of polymorphonuclear leukocytes into infected tissue as well as the synthesis of prostaglandins, cytokines, neutrophil activation, eosinophil activation, and macrophage activation. Current finding is similar to that of (Al-Husseini *et al.*, 2020) conclude from the results of the current study that the infection with Pseudomonas aeruginosa can elevate inflammation in most organs of the body by induce over expression of TNF-α. P. aeruginosa infection increased the number of inflammatory cells and increased the expression of IL-6 and TNF-, which resulted in severe inflammation (Song *et al*., 2017). TNF- is a multifunctional pro-inflammatory cytokine that is released by immunoregulatory systems in response to any damage, inflammation, or infection (Kawakami *et al*., 1998).

As a result, cytokines are important for both innate and adaptive immunity since they help the host's defenses against bacterial invasion of its cells (Hehlgans and Pfeffer, 2005). TNF-a has a significant impact in the development and progression of *P. aeruginosa* septic shock because it directly affects the immune system's homeostasis (Ernandez and Mayadas, 2009).

On the other hand, the results of the current study do not agree with previous studies revealed that TNF-α increased in in cases infected with gram-positive bacteria (Lee *et al*., 2013; Pratiwi *et al*., 2022).

4.7.2. The impact of age on the levels of TNF-α

The impact of age and the TNF-α level displays in Table (4-14), as the table shows the age category Less than 20 years had higher TNF-α level (285.41) and followed by the category More than 40 years TNF-α level (264.19), while the low level of TNF-α was (107.48) in category (20-40) years. Despite this disparity, the results of statistical analysis showed insignificant ($p>0.05$) differences.

Table 4-14: Mean differences of TNF-α in relation to age groups.

Age	Mean of TNF	p-value
Less than 20	285.4175	0.373 (N.S)
20-40	107.4814	
More than 40	264.1914	
Statistical test: ANOVA NS: Non-Significant ($p>0.05$)		

Regarding impact of age on the levels of TNF-α, the result of present study revealed non-significant differences in the levels of TNF-α among age groups.

As the patient ages, it appears that the macrophage produces more TNF-α (Yellon *et al*., 1992). While the current study did not record significant differences in the levels of necrosis factor according to the ages of the study participants, other studies recorded significant differences (Azzam and Elshafie, 2022).

The researchers found that TNF-α did not result in mucin hypersecretion. The downregulation of TNF-α receptors and the creation of mucus gel on the cell surface, which could block TNF-α and TNF-receptor interactions on the cell surface and reduce TNF-α driven mucin hypersecretion, could be the explanation for this lack of response to a high concentration of TNF-α. This may also be the cause of some adult patients' elevated TNF-α levels (Azzam, and Elshafie, 2022).

4.7.3. The impact of gender on the levels of TNF-α

Table (4-15) shows the impact of gender on TNF-α levels. Where the results of the statistical analysis indicate that there is no significant ($p>0.05$) effect of gender on the level of both males and females.

Table 4-15: Mean differences of TNF in relation to gender.

Gender	Mean of TNF	p-value
Male	216.83	0.879[NS]
Female	199.09	
Statistical test: Independent sample t-test NS: Non-Significant (p>0.05)		

It was previously known that immunity is affects by gender, as evidenced by (Pennell *et al*., 2012). Due to their greater immunoglobulin levels and stronger innate and adaptive immune responses than men, women have a higher frequency of autoimmune illnesses and a lower burden of microbiological infections (Butterworth *et al*., 1967; Beeson, 1994; Klein and Flanagan, 2016).

In present study, we did not find any difference in TNF-α between men and women. Nevertheless, circulating levels might not reflect tissue levels of enzymes and peptides, which could explain the current negative result (Omland *et al*., 2008; Semb *et al*., 2009). On the other hand, the current study is not consistent with the results of previous studies that revealed a gender difference in cytokine production.

For instance, in a study that included more than 500 healthy subjects (blood donors), it was found that the production of pro-inflammatory cytokines TNF-α released from monocytes after stimulation was higher in men (Ter Horst et al., 2016). Other investigators also reported that men had a higher production of monocyte-derived IL-1β, IL-6, TNF-α upon stimulation (Asai *et al*., 2001; Bouman *et al*., 2004)

4.7.4. The impact of disease chronicity on the levels of TNF-α

The distribution of TNF-α levels displays in Table (4-16), the result of statistical analysis showed a significant ($p<0.05$) increase of TNF-α levels in patients with chronic phase as compared with acute.

Table 4-16: Mean differences of TNF-α in relation to disease chronicity.

TNF-α	Disease chronicity	NO.	Mean	p-value
	Chronic	18	250.11±157.12	0.04*
	Acute	7	141.72±45.81	

Statistical test: Independent sample t-test
*: -Significant (p>0.05)

Otitis media is characterized as an infection and inflammation of the middle ear mucosa. If the inflammation is associated with discharge and perforation in the tympanic membrane, it results in suppurative Otitis media. It could be acute or chronic (Selvakumari *et al*., 2020). According to studies conducted worldwide, the prevalence of acute otitis media ranges from 2.3% to 20% and chronic otitis media ranges from 4% to 33.3% (Deshmukh, 1998; Berman 1995).

Immune cells have been shown to produce inflammatory cytokines such as TNF-α at different levels in different stages of diseases. TNF-α is a cytokine generated by macrophages in response to stimuli such as bacterial endotoxin and viruses. This cytokine is also produced by mast cells which are a major source (Shendy *et al*., 2021).

In the current study, we assessed the blood levels of TNF-α to determine its roles in the acute and chronic conditions of Otitis media; present finding showed that levels of TNF-α were higher in chronic cases compared with acute. according this findings, present study suggests that TNFα present may be considered as a predictor marker for late stages of otitis media progression.

In this context, it is important to mention that despite the significant results obtained, some studies, such as the study of(Selvakumari *et al*., 2020) reported insignificant differences between acute and chronic cased, they attribute this result to the fact that the role of these immune mediators could be limited or organ specific.

Conclusions

&

Recommendations

Conclusions

1. In all, bacterial otitis is a noteworthy medical issue in the investigation region *P. aeruginosa, S. aureus*, and other Gram-negative bacteria were the overwhelming detaches.

2. The most effective antibiotic for Gram positive isolates was Levofoxacin followed by Gentamycin , Vancomycin and Gram-negative bacteria, Imipenem, Amikacin.

3. The highest percentage of specimens positive for fungal species was *Aspergillus niger*

4.The most effective antifungals were Itraconazole ,Clotrimazole ,Nystatin and Amphotericin-B has the highest percentage of susceptibility against *Candida* spp.

5. our study established an association between polymorphisms in the MUC5B gene and IL-1 RN gene with the severity of otitis media and hearing loss. Testing for these polymorphisms could aid in the early prediction of disease progression and identify patients who might benefit from introducing antagonists into their treatment regimen. This indicates that targeted treatment strategies based on specific genes could hold potential benefits for managing OME.

6. The Otitis media may be not correlated with polymorphism of IL17A but, this think was impossible based on the data of previous researches , which emphasized the pioneer of IL17A in Otitis media infection.

7. TNF consider as remarkable factor to otitis infection,Increased levels of TNF were associated with *P. aeruginosa* as compared with other bacteria.

Recommendations

1. Routine culture and sensitivity test are important and regarded as a required step in the OM treatment because the multi–etiology that lead to empirical treatment is impossible.

2. Further study is needed to explain the roles of other cytokines such as interferon in the middle ear inflammatory responses .

3. Difficulty in diagnosing some yeast isolates, especially those that show a chromatic reaction close to CHROMagar, genotyping of these isolates must be done and the identification of new species and genetic developments among them.

4. Study of Mycotoxins from Fungi Isolated from Otitis Media

5. Study inoculation against influenza, in otitis media

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Appendices

APPENDIX (A)

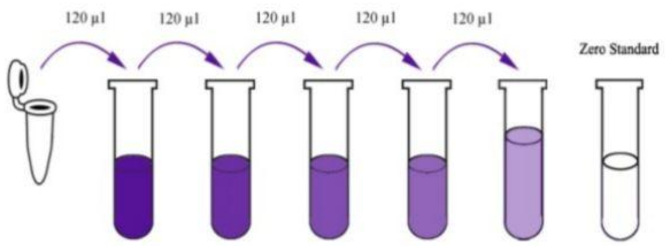
Appendix A

Human Tumor Necrosis Factor Alpha, TNF-A ELISA Kit

Reagent Preparation

1. All reagents should be brought to room temperature before use.

2. Standard Reconstitute the 120ul of the standard (960ng/L) with 120ul of standard diluent to generate a 480ng/Lstandard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (480ng/L) 1:2 with standard diluent to produce 240ng/L, 120ng/L, 60ng/L and 30ng/L solutions. Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:



Standard concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
960ng/L	480ng/L	240ng/L	120ng/L	60ng/L	30ng/L

3.Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

APPENDIX (A)

Assay Procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.

3. Add 50ul standard to standard well. Note: Don't add antibody to standard well because the standard solution contains biotinylated antibody.

4. Add 40ul sample to sample wells and then add 10ul Human TNFA antibody to sample wells, then add 50ul streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material.

6. Add 50ul substrate solution A to each well and then add 50ul substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

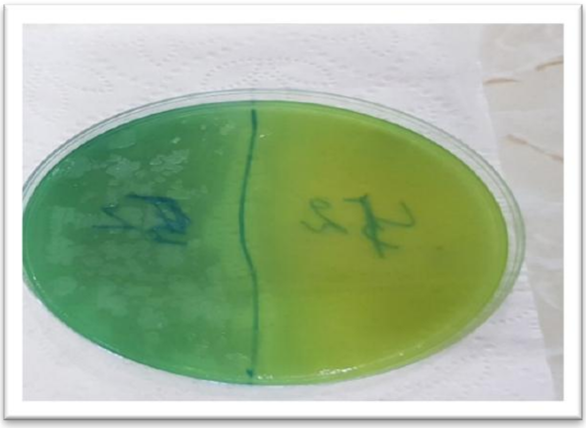
7. Add 50ul Stop Solution to each well, the blue color will change into yellow immediately.

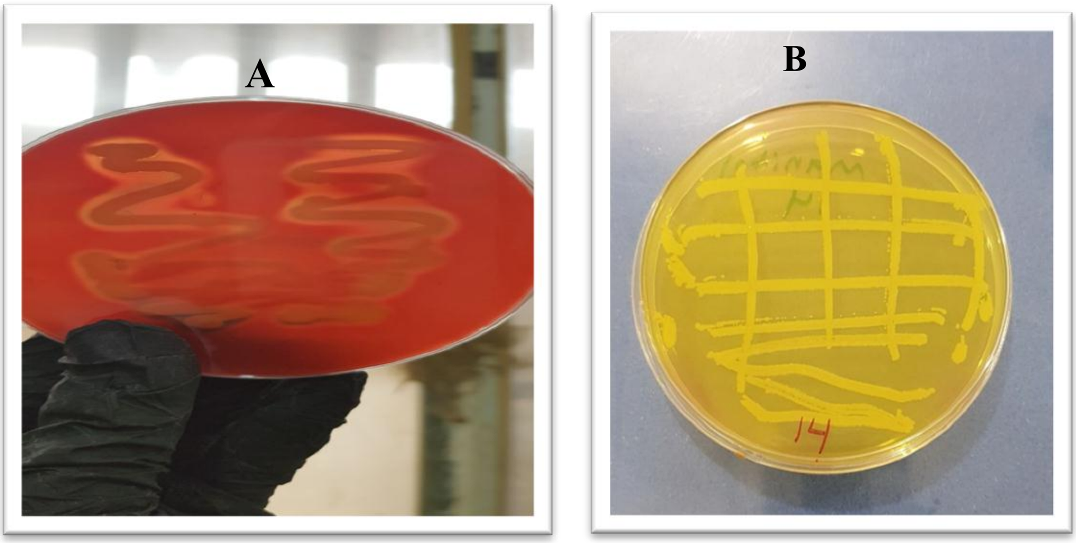
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Appendix B

Appendix B(bacteria)

Growth of Pseudomonas aeruginosa on Cetrimide Agar





S. aureus growth on blood agar that showed β- haemolysis(A), S. aureus growth on mannitol salt agar (B)

Appendix B

Tube Coagulase Test

Of

S. aureus

Appendix C

Appendix C (fungi)

Aspergillus flavus

Aspergillus terreus

Aspergillus niger

Appendix D

Appendix (D): Shows the nitrogenous bases sequences for some of the isolates under study and the ratios of their corresponds with the reference isolates in the gene bank and the sites of genetic gaps (red shapes).

Isolate no. 1

Escherichia coli strain E5 16S ribosomal RNA gene, partial sequence

Sequence ID: KY780340.1 Length: **1211** Number of Matches: **1**

Range 1: 2 to 872 GenBank Graphics ▼ Next Match ▲ P

Score	Expect	Identities	Gaps	Strand
1585 bits(858)	0.0	867/871(99%)	2/871(0%)	Plus/Plus

```
Query  3    GGATTGCGGCAGCTACACATGCAGTCGAACGGTAACAGGAAACAGCTTGCTGTTTTGCTG  62
            ||||||||||||||||||||||||||||||||||||||||| |||||||||| |||||||
Sbjct  2    GGATTGCGGCAGCTACACATGCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTG  61

Query  63   ACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACT  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62   ACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACT  121

Query  123  GGAAACGGTAGCTAATACCGCATAACGTCGC-AGACCAAAGAGGGGGACCTTCGGGCCTC  181
            ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  122  GGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTC  181

Query  182  TTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGC  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182  TTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGC  241

Query  242  GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAG  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242  GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAG  301

Query  302  ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302  ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA  361

Query  362  TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAG  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAG  421

Query  422  TAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG  481
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  TAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG  481

Query  482  CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG  541
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG  541

Query  542  CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC  601
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542  CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC  601

Query  602  TGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT  661
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  662  GCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCT  721
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662  GCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCT  721

Query  722  CAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC  781
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722  CAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC  781

Query  782  GATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCG  841
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782  GATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCG  841

Query  842  ACCGCCTGGGGAGTACGGCCGCAAGGT-AAA  871
            ||||||||||||||||||||||||||| |||
Sbjct  842  ACCGCCTGGGGAGTACGGCCGCAAGGTTAAA  872
```

Appendix D

Isolate no. 2

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

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

Range 1: 2 to 863 GenBank Graphics ▼ Next Match ▲ P

Score	Expect	Identities	Gaps	Strand
1580 bits(855)	0.0	860/862(99%)	2/862(0%)	Plus/Plus

```
Query  11   GCAGCTACACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCG  70
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2    GCAGCTACACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCG  61

Query  71   GACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTA  130
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62   GACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTA  121

Query  131  GCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAG  190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122  GCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAG  181

Query  191  ATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCT  250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182  ATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCT  241

Query  251  AGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG  310
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242  AGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG  301

Query  311  GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT  370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302  GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT  361

Query  371  GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGTTAAGGTTAAT  430
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGTTAAGGTTAAT  421

Query  431  AACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG  490
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  AACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG  481

Query  491  CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCG  550
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCG  541

Query  551  GTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGC  610
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542  GTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGC  601

Query  611  AGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGAT  670
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602  AGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGAT  661

Query  671  CTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAA  730
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662  CTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAA  721

Query  731  AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATT  790
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722  AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATT  781

Query  791  TGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGG  850
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782  TGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGG  841

Query  851  GAGTACGGCCGCA-GGT-AAAA  870
            ||||||||||||| ||| ||||
Sbjct  842  GAGTACGGCCGCAAGGTTAAAA  863
```

Isolate no. 3

Bacillus smithii strain Pb1 16S ribosomal RNA gene, partial sequence

Sequence ID: OP090256.1 Length: 1458 Number of Matches: 1

Range 1: 6 to 889 GenBank Graphics ▼ Next Match ▲

Score	Expect	Identities	Gaps	Strand
1535 bits(831)	0.0	867/884(98%)	4/884(0%)	Plus/Plus

```
Query  5    GGGCGTGTGCTAT-CATGC-AGTCGAGCGTACTTT-GAGAAGCTTGGGTTTTGAGAGTTA  61
            ||||| |||||||  ||||  |||||||| ||||  |||||||||  |||||| |||||
Sbjct  6    GGGCGCGTGCTATACATGCAAGTCGAGCGGACTTTCGAGAAGCTTGCTTTTTGAAAGTTA  65

Query  62   GCGGCGGACGGGTGAGTAACTCGTGGGCAACCTGCCTT-AGGACGGGGATAAGTCCGGGA  120
            |||||||||||||||||||| ||||||||||||||||||  ||||||||||| |||||||
Sbjct  66   GCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTTCAAGACGGGGATAACTCCGGGA  125

Query  121  AACCGGGGCTAATACCGGATAATATGTTCCTTCGCATGAAGGAAGGTTGAAAGGCGGCGC  180
            |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  126  AACCGGGGCTAATACCGGATAATATCTTCCTTCGCATGAAGGAAGGTTGAAAGGCGGCGC  185

Query  181  ACGCTGCCGCTTGCAGATGGGCCCGCGGCGCAGTAGCTAGTTGGTGAGGTAACGGCTCAC  240
            | ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  186  AAGCTGCCGCTTGCAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCAC  245

Query  241  CAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  246  CAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG  305

Query  301  GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  306  GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACG  365

Query  361  GAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTCAGGGAAGA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  366  GAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTCAGGGAAGA  425

Query  421  ACAAGTACCGTTCGAACAGGGCGGTACCTTGACGGTACCTGACCAGAAAGCCACGGCTAA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  426  ACAAGTACCGTTCGAACAGGGCGGTACCTTGACGGTACCTGACCAGAAAGCCACGGCTAA  485

Query  481  CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  486  CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG  545

Query  541  TAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  546  TAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAG  605

Query  601  GGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGCGGAATTCCACGTGTAGCG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  606  GGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGCGGAATTCCACGTGTAGCG  665

Query  661  GTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAAC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  666  GTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAAC  725

Query  721  TGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  726  TGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC  785

Query  781  CGTAAACGATGAGTGCTAAGTGTTAGAGGGCTTCCACCCTTTAGTGCTGCAGCTAACGCA  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  786  CGTAAACGATGAGTGCTAAGTGTTAGAGGGCTTCCACCCTTTAGTGCTGCAGCTAACGCA  845

Query  841  TTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCA  884
            ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  846  TTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCA  889
```

Appendix D

Isolate no. 4

Providencia sp. strain InS-274 16S ribosomal RNA gene, partial sequence

Sequence ID: KY964269.1 Length: 1440 Number of Matches: 1

Range 1: 5 to 876 GenBank Graphics ▼ Next Match ▲

Score	Expect	Identities	Gaps	Strand
1598 bits(865)	0.0	870/872(99%)	2/872(0%)	Plus/Plus

```
Query  10   GGGCGGCGGCTACACATGC-AGTCGAGCGGTAACAGGGGAAGCTTGCTTCTCGCTGACGA  68
            ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  5    GGGCGGCGGCTACACATGCAAGTCGAGCGGTAACAGGGGAAGCTTGCTTCTCGCTGACGA  64

Query  69   GCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAA  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  65   GCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAA  124

Query  129  ACGGTAGCTAATACCGCATAATCTCTCAGGAGCAAAGCAGGGGAACTTCGGTCCTTGCGC  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125  ACGGTAGCTAATACCGCATAATCTCTCAGGAGCAAAGCAGGGGAACTTCGGTCCTTGCGC  184

Query  189  TATCGGATGAACCCATATGGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACG  248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185  TATCGGATGAACCCATATGGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACG  244

Query  249  ATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTC  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245  ATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTC  304

Query  309  CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305  CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC  364

Query  369  GCGTGTATGAAGAAGGCCCTAGGGTTGTAAAGTACTTTCAGTCGGGAGGAAGGCGTTGAT  428
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365  GCGTGTATGAAGAAGGCCCTAGGGTTGTAAAGTACTTTCAGTCGGGAGGAAGGCGTTGAT  424

Query  429  GCTAATATCATCAACGATTGACGTTACCGACAGAAGAAGCACCGGCTAACTCCGTGCCAG  488
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425  GCTAATATCATCAACGATTGACGTTACCGACAGAAGAAGCACCGGCTAACTCCGTGCCAG  484

Query  489  CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG  548
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  485  CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG  544

Query  549  CAGGCGGTTGATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAG  608
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  545  CAGGCGGTTGATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAG  604

Query  609  ACTGGTCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGT  668
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  605  ACTGGTCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGT  664

Query  669  AGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGG  728
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  665  AGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGG  724

Query  729  TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATG  788
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  725  TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATG  784

Query  789  TCGATTTGAAGGTTGTTCCCTTGAGGAGTGGCTTTCGGAGCTAACGCGTTAAATCGACCG  848
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  785  TCGATTTGAAGGTTGTTCCCTTGAGGAGTGGCTTTCGGAGCTAACGCGTTAAATCGACCG  844

Query  849  CCTGGGGAGTACGGCCGCAAGGTTAAAA-TCA  879
            |||||||||||||||||||||||||||| |||
Sbjct  845  CCTGGGGAGTACGGCCGCAAGGTTAAAACTCA  876
```

Appendix D

Isolate no. 5

Pseudomonas stutzeri strain W28 16S ribosomal RNA gene, partial sequence

Sequence ID: KT380573.1 Length: 1432 Number of Matches: 1

Range 1: 2 to 869 GenBank Graphics ▼ Next Match ▲ Previc

Score	Expect	Identities	Gaps	Strand
1604 bits(868)	0.0	868/868(100%)	0/868(0%)	Plus/Plus

```
Query  6    ATGGCGGCAGCTACACATGCAGTCGAGCGGATGAGTGGAGCTTGCTCCATGATTCAGCGG  65
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2    ATGGCGGCAGCTACACATGCAGTCGAGCGGATGAGTGGAGCTTGCTCCATGATTCAGCGG  61

Query  66   CGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGA  125
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62   CGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGA  121

Query  126  ACGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATC  185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122  ACGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATC  181

Query  186  AGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCC  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182  AGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCC  241

Query  246  GTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTAC  305
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242  GTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTAC  301

Query  306  GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGT  365
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302  GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGT  361

Query  366  GTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTA  425
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  GTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTA  421

Query  426  ATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGC  485
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  ATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGC  481

Query  486  CGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGG  545
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  CGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGG  541

Query  546  TGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTG  605
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542  TGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTG  601

Query  606  GCGAGCTAGAGTATGGCAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGAT  665
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602  GCGAGCTAGAGTATGGCAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGAT  661

Query  666  ATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGGCTAATACTGACACTGAGGTGCG  725
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662  ATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGGCTAATACTGACACTGAGGTGCG  721

Query  726  AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA  785
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722  AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA  781

Query  786  CTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCATTAAGTCGACCGCCTG  845
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782  CTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCATTAAGTCGACCGCCTG  841

Query  846  GGGAGTACGGCCGCAAGGTTAAAACTCA  873
            ||||||||||||||||||||||||||||
Sbjct  842  GGGAGTACGGCCGCAAGGTTAAAACTCA  869
```

Appendix D

Isolate no.6

Acidovorax sp. M2 gene for 16S rRNA, partial sequence

Sequence ID: AB646302.1 Length: 1488 Number of Matches: 1

Range 1: 28 to 908 GenBank Graphics ▼ Next Match ▲

Score	Expect	Identities	Gaps	Strand
1572 bits(851)	0.0	872/881(99%)	5/881(0%)	Plus/Plus

```
Query  11   TGGCGGC-GG-T-A-CACATGC-AGTCGAACGGTAACAGGTCTTCGGATGCTGACGAGTG  65
            ||||||| || | | ||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  28   TGGCGGCAGGCTTAACACATGCAAGTCGAACGGTAACAGGTCTTCGGATGCTGACGAGTG  87

Query  66   GCGAACGGGTGAGTAATACATCGGAGCGTGCCCGATCGTGGGGGATAACGAAGCGAAAGC  125
            ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  88   GCGAACGGGTGAGTAATACATCGGAACGTGCCCGATCGTGGGGGATAACGAAGCGAAAGC  147

Query  126  TTTGCTAATACCGCATACGATCTACGGATGAAAGCAGGGGACCGCAAGGCCTTGCGCGAA  185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  148  TTTGCTAATACCGCATACGATCTACGGATGAAAGCAGGGGACCGCAAGGCCTTGCGCGAA  207

Query  186  CGGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAAAAGCTTACCAAGCCGACGATC  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208  CGGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAAAAGCTTACCAAGCCGACGATC  267

Query  246  TGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTA  305
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268  TGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTA  327

Query  306  CGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCG  365
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328  CGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCG  387

Query  366  TGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGACTCTGGTT  425
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388  TGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGACTCTGGTT  447

Query  426  AATACCTGGGGTCCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAG  485
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  448  AATACCTGGGGTCCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAG  507

Query  486  CCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAG  545
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  508  CCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAG  567

Query  546  GCGGTTATATAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACT  605
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  568  GCGGTTATATAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACT  627

Query  606  GTATAGCTAGAGTACGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGA  665
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  628  GTATAGCTAGAGTACGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGA  687

Query  666  TATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGTACTGACGCTCATGCAC  725
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  688  TATGCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGTACTGACGCTCATGCAC  747

Query  726  GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCA  785
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  748  GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCA  807

Query  786  ACTGGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTG  845
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  808  ACTGGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTG  867

Query  846  GGGAGTACGGCCGCAAGGTTGAAACTCAAGAAAATTGACGG  886
            |||||||||||||||||||||||||||||   |||||||||
Sbjct  868  GGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGG  908
```

Isolate no. 7

Acidovorax sp. strain 816OP6 16S ribosomal RNA gene, partial sequence

Sequence ID: MK757665.1 Length: 1431 Number of Matches: 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 5 to 882 GenBank Graphics ▼ Next Match ▲

Score	Expect	Identities	Gaps	Strand
1581 bits(856)	0.0	871/878(99%)	2/878(0%)	Plus/Plus

```
Query  11   TGGCGG[illegible]-[illegible]G[illegible]-TACCATGCAGTCGAACGGTAACAGGTCTTCGGATGCTGACGAGTGGCG  68
Sbjct  5    TGGCGG[illegible]A[illegible]G[illegible]T[illegible]AACATGCAGTCGAACGGTAACAGGTCTTCGGATGCTGACGAGTGGCG  64

Query  69   AACGGGTGAGTAATACATCGGAGCGTGCCCGATCGTGGGGGATAACGAGCGAAAGCTTT  128
Sbjct  65   AACGGGTGAGTAATACATCGGAACGTGCCCGATCGTGGGGGATAACGAAGCGAAAGCTTT  124

Query  129  GCTAATACCGCATACGATCTACGGATGAAAGCAGGGGACCGCAAGGCCTTGCGCGAACGG  188
Sbjct  125  GCTAATACCGCATACGATCTACGGATGAAAGCAGGGGACCGCAAGGCCTTGCGCGAACGG  184

Query  189  AGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAAAAGCTTACCAAGCCGACGATCTGT  248
Sbjct  185  AGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAAAAGCTTACCAAGCCGACGATCTGT  244

Query  249  AGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG  308
Sbjct  245  AGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG  304

Query  309  GAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGC  368
Sbjct  305  GAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGC  364

Query  369  AGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGACTCTGGTTAAT  428
Sbjct  365  AGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGACTCTGGTTAAT  424

Query  429  ACCTGGGGTCCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCG  488
Sbjct  425  ACCTGGGGTCCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCG  484

Query  489  CGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCG  548
Sbjct  485  CGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCG  544

Query  549  GTTATATAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGTA  608
Sbjct  545  GTTATATAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGTA  604

Query  609  TAGCTAGAGTACGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATAT  668
Sbjct  605  TAGCTAGAGTACGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATAT  664

Query  669  GCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGTACTGACGCTCATGCACGAA  728
Sbjct  665  GCGGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGTACTGACGCTCATGCACGAA  724

Query  729  AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACT  788
Sbjct  725  AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACT  784

Query  789  GGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGG  848
Sbjct  785  GGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGG  844

Query  849  AGTACGGCCGCAAGGTTGAAACTCAAGAAAATTGACGG  886
Sbjct  845  AGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGG  882
```

Appendix D

Isolate no. 8

Enterobacter sp. strain YF3 16S ribosomal RNA gene, partial sequence

Sequence ID: MT020107.1 Length: 1448 Number of Matches: 1

Range 1: 6 to 876 GenBank Graphics ▼ Next Match ▲

Score	Expect	Identities	Gaps	Strand
1585 bits(858)	0.0	867/871(99%)	2/871(0%)	Plus/Plus

```
Query  5    TTGGCGGCAGC-ACACATGCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGA  63
            ||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6    TTGGCGGCAGCAACACATGCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGA  65

Query  64   CGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66   CGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG  125

Query  124  GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCT  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126  GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCT  185

Query  184  TGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCG  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186  TGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCG  245

Query  244  ACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGA  303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  246  ACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGA  305

Query  304  CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCAT  363
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  306  CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCAT  365

Query  364  GCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGAT  423
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  366  GCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGAT  425

Query  424  GAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGC  483
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  426  AAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGC  485

Query  484  CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC  543
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  486  CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC  545

Query  544  ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTC  603
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  546  ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTC  605

Query  604  GAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATG  663
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  606  GAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATG  665

Query  664  CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTC  723
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  666  CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTC  725

Query  724  AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG  783
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  726  AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG  785

Query  784  ATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGA  843
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  786  ATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGA  845

Query  844  CCGCCTGGGGAGTACGGCCGCAAG-aaaaa  873
            ||||||||||||||||||||||||  ||||
Sbjct  846  CCGCCTGGGGAGTACGGCCGCAAGTAAAA  876
```

Appendix D

Isolate no. 10

Pseudomonas aeruginosa strain KI6.12 16S ribosomal RNA gene, partial seq

Sequence ID: MT113103.1 **Length:** 1278 **Number of Matches:** 1

Range 1: 1 to 864 GenBank Graphics ▼ Next Match ▲ Pr

Score	Expect	Identities	Gaps	Strand
1578 bits(854)	0.0	861/864(99%)	1/864(0%)	Plus/Plus

```
Query  3    GCTGGGGGCAGCTACACATGCAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCG  62
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GCTGGCGGCAGCTACACATGCAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCG  60

Query  63   GCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACG  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACG  120

Query  123  GGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTAT  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTAT  180

Query  183  CAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATC  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATC  240

Query  243  CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTA  302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTA  300

Query  303  CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCG  362
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCG  360

Query  363  TGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTT  422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTT  420

Query  423  AATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAG  482
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAG  480

Query  483  CCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG  542
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG  540

Query  543  GTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACT  602
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACT  600

Query  603  ACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGA  662
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  ACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGA  660

Query  663  TATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGC  722
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  TATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGC  720

Query  723  GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCG  782
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCG  780

Query  783  ACTAGCCTTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCT  842
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781  ACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCT  840

Query  843  GGGGAGTACGGCCGCAAGGT-AA  865
            |||||||||||||||||||| ||
Sbjct  841  GGGGAGTACGGCCGCAAGGTTAA  864
```

الخلاصة:

التهاب الأذن الوسطى هو التهاب وحالة معدية في الغشاء المخاطي للأذن الوسطى. وقد يكون مصحوبًا بإفرازات وانثقاب في غشاء الطبلة.

أجريت هذه الدراسة في كلية علوم للبنات بجامعة بابل. وقد جمع ٧٤ عينة سريرية من المرضى الذين يعانون من التهاب الأذن الوسطى بمختلف الفئات العمرية تراوحت أعمارهم من 72-1سنة وكانت فترة الدراسة من أكتوبر ٢٠٢٢ إلى يناير ٢٠٢٣ في مستشفيات بابل (الحلة التعليمي، الأمام الصادق) .

وتمت الدراسة الحالية بعدد من الخطوات منها تشخيص البكتيريا والفطريات المرافقة لالتهاب الاذن. شخصت البكتيريا عن طريق الأختبارات الروتينية ، والجزيئية . ودراسة فعالية المضادات البكتيرية، وتم تشخيص العزلات الفطرية بالطرق التقليدية والتعرف على فعالية المضادات الفطرية للعزلات الفطرية. وأظهرت النتائج ان ٦٢ من العينات أعطت نمو للبكتيريا و١٢عينة لم تظهر نمو للبكتيريا .وأعطت الفطريات ٥٩عينة موجبة للزرع و ١٥عينة سالبة للزرع.

أظهرت نتائج المرضى نسبة الإناث كانت (٥٥.٥%)أعلى منها عند الذكور حيث بلغت عند الذكور (٤٤.٥%) وقد كشفت هذه الدراسة أن السن الأكثر تضرراً بين الجنسين تتراوح أعمارهم (٤٠- ٢٠سنة). وكذلك توزع المرضى حسب وضع المنطقة المعيشية في المدينة بنسبة (٧٥.٧%) وفي الريف (٢٤.٣ %) وسجلت هذه الدراسة الذين تناولوا مضاد حيوي (٥٦.٨%) من الذين لم يتناولوا (٤٣.٢%).وقد كان إلتهاب الأذن المزمن هو الأعلى نسبة بين المرضى (٦٢.٢%) والحاد (٣٧.٨%) من العينات السريرية. بكتيريا الزائفة الزنجارية هي الأعلى تواجد ٢٤(٣٢.٤%) تليها المكورات العنقودية الذهبية ١٩ (٢٥.٧%).

بكتيريا الزائفة الزنجارية الأكثر وفرة كشفت عن مستويات مقاومة مختلفة اتجاه ٧ أنواع من المضادات الحيوية كان الأكثر مقاومة polymyxin B و Gentamicin و Ceftriaxoneواكثر حساسية Imipenem بينما أظهرت المكورات الذهبية اعلى مقاومة Tetracyctineو Amoxicillin واظهرت حساسية عالية اتجاه Levofoxacin.

ولتشخيص الفطريات زرعت العينات على وسط SDA للتعرف على الفطريات وبعد نموها تم فحصها مجهريا واستخدم وسط CROMAgar وذلك لاستعماله في تشخيص أنواع *Candida*

spp . وبينت النتائج سيادة جنس *Aspergillus spp* والأنواع التابعة له وكان اعلى نسبة نوع *A . niger* (٣٦.٥%) يتبعه *A.flavus* (٣١.١%).

تم أيضًا عزل أربعة أنواع خميرة من عينات المرضى، وكانت أعلى نسبة تعزى إلى

C. glabrata و*C. krusei*، حيث بلغت ١٢.٢% و ٨.١% على التوالي. ثاني أكثر أنواع الخميرة انتشارا هي *C. albicans* بنسبة ٤.١%

أكثر مضادات الفطريات فاعلية في هذه الدراسة هي كلوتريمازول ، يتراكونازول ونيستاتين و الأمفوتريسين-ب لها أعلى نسبة حساسية ضد المبيضات.

تم دراسة التهاب الأذن الوسطى عن طريق التنميط الجيني لـ ٦٠ عينة، ٣٠ مريضًا مصابًا بالتهاب الأذن الوسطى و٣٠ فردًا سليمًا. يتم ذلك للكشف عن تعدد الأشكال الجيني لجينين، MUC5B و IL-1RN، باستخدام الأعداد المتغيرة لمنطقة التكرارات الترادفية (VNTR). تشير النتائج إلى أن حاملي الأليل ٢ لديهم خطر الإصابة بالتهاب الأذن الوسطى بمقدار الضعف تقريبًا.

تم إجراء تحليل تعدد الأشكال الجيني لـ IL17A على ٦٠ عينة من الحمض النووي لدم الإنسان، بما في ذلك ٣٠ مريضًا و٣٠ فردًا سليمًا. تواتر أليل الخطر A في كل من متغاير الزيجوت (GA) ومتماثل الزيجوت AA في *rs2275913* SNP. النتائج الموضحة أقل في النمط الجيني GA كانت OR= 0.57 (٠.١٨ - ١.٧٢) مع قيمة p 0.3، وكان تردد الأليل أقل مع الأليل A.

واعتمادًا على طريقة ELISA تم قياس مستوى TNF-α في مصل الدم ٢٥ عينة للمرضى مقابل ١٠ أصحاء وبينت النتائج اختلاف TNF-α بين المرضى والأشخاص الأصحاء(٢٧٢.٨٥±١٣٢.٢١ مريض مقابل ٢٠٠.٥± ٣٦ مجموعة السيطرة). وكان توزيع مصل TNF-α وحسب الأنواع البكتيرية. حيث أظهرت نتائج التحليل الإحصائي زيادة معنوية ($P < 0.05$) في مصل TNF-α في بكتيريا *p. aeruginosa* مقارنة بالبكتيريا الأخرى. و أن الفئة العمرية أقل من ٢٠ عامًا كان لديها مستوى TNF-α أعلى (٢٨٥.٤١) وتليها الفئة أكثر من ٤٠ عامًا مستوى TNF-α (٢٦٤.١٩) بينما كان المستوى المنخفض لـ TNF-α (١٠٧.٤٨) في فئة (٢٠-٤٠) سنة تأثير الجنس على مستويات TNF-α. حيث تشير نتائج التحليل الإحصائي إلى عدم وجود تأثير معنوي ($p > 0.05$) للجنس على مستوى كل من الذكور والإناث. وأظهرت

نتيجة التحليل الإحصائي زيادة معنوية في مستويات TNF-α في المرضى الذين يعانون من المرحلة المزمنة مقارنة بالحادة.

وزارة التعليم العالي والبحث العلمي

جامعة بابل/ كلية العلوم للبنات

قسم علوم الحياة

دراسة مظهرية وجزيئية ومناعية لعدوى التهاب الأذن الوسطى

رسالة مقدمة الى

مجلس كلية العلوم للبنات، جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير

في علوم الحياة

من قبل

صفا عامر خليل

(بكالوريوس علوم الحياة/ كلية العلوم للبنات/ جامعة بابل،٢٠١٧)

بإشراف

أ.د. كوثر محمد علي حسن

٢٠٢٣ م **١٤٤٥هـ**