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Journal of University of Kerbala

Research Article

Isolation and Identification of Pseudomonas aeruginosa common among microorganisms causing ear infections in Karbala Governorate

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Article Info

Article history:

Received 17 - 3 -2024

Received in revised form 21-4 -2024

Accepted 5 -5 -2024

Available online 30-6-2024

Keywords: Pseudomonas aeruginosa, ear infections ,PCR, virulence factors

Abstract

To detect the prevalence of *Pseudomonas aeruginosa* from different stages of ear infections and to study some of the virulence factors possessed by this bacterium, 110 swabs from ear infections were collected and cultured on blood agar, MacConkey agar and cetrimide agar plates media. The plates were incubated for 24 to 48 hours at 37°C, and growth was determined using these media. The microbial isolates were differentiated using a variety of biochemical techniques. The common isolates identified using standard bacteriological methods namely morphology, colony characteristics and biochemical tests. The results showed that 30.93% (30/97) were *Pseudomonas aeruginosa*, with other 69.07% (67/97) isolates belonging to various microbial genera. *Pseudomonas aeruginosa* was Gram-negative, showed hemolysis upon blood agar, and were motile. Biochemical assays revealed that each isolate were positive for catalase, oxidase, citrate, and indole. The results were confirmed using VITEK 2 system and the polymerase chain reaction (PCR) to investigate the 16srRNA gene. PCR results revealed that 100% of the isolates possessed this gene with a size of 956 base pairs. *Pseudomonas aeruginosa* was also investigated for some virulence factors, the results indicated that all isolates 100% produced hemolysin, whereas 76.66% (23/30) were positive for protease qualitative assay, 80% (24/30) of the isolates produced pyocyanin pigment.

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Peer review under responsibility of Iraqi Academic Scientific Journal and University of Kerbala.

Introduction

The ear is a complex sensory organ. It is divided into three parts, the outer ear, the middle ear and the inner ear, the mechanism of hearing involves the transmission of auditory signals from the outer ear to the middle ear, then to the inner ear, the auditory nerve, and finally to the central nervous system, where that are interpreted by the brain [1] .

There are many lines of defense in the ear, most notably the presence of Cerumen, produced by sebaceous and ceruminous glands in the outer ear canal, serves as a protective barrier against infections and helps maintain ear health, while its antibacterial and antifungal properties are debated, cerumen also acts as a physical shield for the eardrum, making routine cleaning unnecessary unless symptoms like ear pain or hearing loss occur [2].

Hearing loss is one of the leading causes of long-term disability in humans. More than 360 million people worldwide suffer from this disability[3]. The middle ear is the most vulnerable part of the ear because it is open through the auditory canal that connects it to the pharynx, which ensures the entry of air into it, achieving neutral pressure on the tympanic membrane, so infectious agents coming from the nasal and laryngeal tracts reach upwards through the Eustachian tube. Thus, other diseases contribute to the onset of the disease, such as the Cold Common, Sinusitis and Throat Sore[4].

Microbial agents have the ability to infect the skin, cartilage, periosteum, ear canal, eardrum and mastoid cavities, and according to many studies, *P. aeruginosa* is the main cause of ear infections and other bacterial pathogens such as *Staphylococcus aureus*, *Proteus mirabilis* and *Streptococcus spp*. Fungi and viruses also contribute to the occurrence of sepsis, as well as other factors such as age, gender, genetic factors, and climate [5, 6].

P. aeruginosa poses a potential threat to individuals worldwide because of its ubiquitous presence, severe virulence and substantial resistance to several medications. *P. aeruginosa* pathogenicity, which is associated with both chronic and acute infections, is connected to many factors related to virulence and associated secretion systems, such as the capacity to produce biofilm, pili, flagella, alginate, pyocyanin, proteases, hemolysin and other toxins [7].

This study aimed to the identification of *P.aeruginosa* among microorganisms causing ear infections and the detection of some of the virulence factors related to the pathogenesis of this bacteria.

Material and Methods

Collection of specimen

An overall sample of 110 was obtained from patients with ear infections who were referred to the Ear, Nose, and Throat Consultant at Imam Hussein Medical City and private clinics in different areas of the Holy Governorate of Karbala. Their ages ranged from 1 year to 75 years, for the period from August 2023 to October 2023. Samples were collected using sterile cotton swabs and gently rotated over the affected area. The swabs were then transferred with the transport medium to the microbiology laboratory at Imam Hussein Medical City for isolation and diagnosis.

Isolation and diagnosis of microorganisms

The swabs were cultured on blood agar, MacConkey agar and cetrimide agar (Oxoid-England), then the plates were incubated at 37 ˚C for 24 to 48 hours for growth, microscopic and biochemical analyses.

The microorganisms isolated from ear infections were differentiated and the prevalent species diagnosed based on cultural, microscopic characteristics , biochemical tests, vitek 2 system and PCR.

Cultural Characteristics

The phenotypic characteristics of bacterial isolates were studied which include colony shape, color, size, edges, texture and odor [8].

Microscopic examination

Using the sterile loop, a part of the young colony was transferred and mixed with a drop of distilled water on the surface of a clean glass slide for microscopic examination of the growing bacterial cells. It was then spread across the slide surface and dried before being heat fixed. They were then stained with gram stain and viewed under a microscope to observe the morphology of the bacterial cells and their reaction with the gram stain [9].

Biochemical tests

The following biochemical assays were performed on the isolated colonies:

Catalase test

On a sterile slide, a single, pure colony of each bacterial isolate was picked up from culture media, single drop 3% hydrogen peroxide (BDH-England) was added and mixed. The presence of gaseous bubbles indicated the positive result [10].

Oxidase test

A few drops of tetramethyl phenylenediamine dihydrochloride solution (BDH-England) was added to a filter paper and using a sterilized wooden stick, then a loop full of the bacteria was taken from MacConkey agar and spread out on the filter paper. The positive result indicated by the appearance of violet or purple color within ten seconds [11].

The indole test

Test tubes with 5 ml of peptone water were inoculated with a loop full of fresh cultures of every suspected isolate, and the tubes were incubated for 24 hours at 37°C. After growth, drops of Kovacs reagent (Oxoid-England) were added. A red ring appeared on the medium surface, indicated the positive result [12].

Methyl red test

After adding 2-3 colonies of each isolate to 5 ml of MR-VP broth (Oxoid-England) in test tubes, the inoculated tubes incubated for 24 hours at 37°C. Five drops of methyl red reagent were added after growth. A positive result indicated by the brilliant red color appearance [13].

Voges-Proskauer test

After 24 hours of incubation at 37°C in 5 ml of MR-VP broth, bacterial culture was combined with 0.1 ml of potassium hydroxide and 0.6 ml of alpha-naphthol (BDH-England), then the mixture was stirred for 30 seconds. A positive result indicated by the color change from pink to red [14].

UreaseTest

A fresh culture of each isolate was added to test tubes containing 5 ml of urea medium (Oxoid- England), and the tubes were then incubated for 24 hours at 37°C. The medium color changing from yellow to pink signified a positive result [15].

Citrate Utilization test

The Simmons citrate (Oxoid- England) slants was inoculated by streaking the surface with a few isolated colonies and stabbing the loop into the bottom of the media, followed by incubation for 24 hours at 37ºC. A positive result detected by changing the medium color from green to blue [16].

Motility Test

On nutrient agar medium (Himedia, India) tubes, bacterial colonies were stabbed in a straight line and cultured for 24 hours at 37°C. Around the line, cultural bacteria would spread indicating the positive result [17].

Diagnosis of bacteria using vitek 2 system

The prevalent bacterial isolate in this study were diagnosed using the Vitek 2 system (Biomerieux- France) according to the guidance of the manufactures.

Molecular identification by 16s ribosomal RNA gene

According to the method described by [18], DNA was extracted using the Genomic DNA extraction kit (Microgen-Korea). PCR reactions were carried out by specific primers purchased from Macrogen, Korea (shown in Table 1)

using PCR thermal cycler (Biobase-China) at a volume of 25 μL ,The master mix included 10 μL of Taq master mix kit (Bioneer-Korea), 2 μL of each forward and reverse primers at 10 ρmol/μL concentrations, 3 μL of target DNA and 8 μL of nuclease-free water. The PCR reaction conditions involved initial denaturation of templet DNA for 3 minutes at 95°C in the first cycle, followed by 33 cycles of denaturing template DNA for 30 seconds at 94°C, primers annealing with templet DNA for 33 seconds at 62°C, initial extension of 55 second at 72°C, Lastly, one cycle of 5 minutes at 72°C was needed to complete the final extension of DNA strands. Electrophoresis on a 1% agarose gel (with ethidium bromide (Bioneer-Korea)) at 50 volts for 1 hour revealed the formation of PCR products by exposing the gel to UV light.

Table (1) The primers used to diagnose *P. aeruginosa* by the polymerase chain reaction (PCR).

Primer	Primer sequence 5′ → 3′	Product size (bp)	Annealing Tem.	References
Forward Primer Reverse Primer	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	62°C	[19]

Phenotypic screening for some virulence factors

Pyocyanin production test

The ability of the isolated bacterial pathogen to produce pyocyanine was examined by cultivating these isolates in different cultural conditions using the streaking plate method.

Young colonies of the microorganisms under research incubated at 37°C for 24-48 hours [20] [21].

Protease production test

Skim milk agar medium (Himedia, India) was used to detect protease production of the bacterial isolates where 4 wells drilled in the milk culture medium using a 5 millimeters diameter cork borer(Kotterm, Germany). The agar discs were then removed, and 0.1 ml of the bacterial culture broth was taken using a micropipette (Afco-Dipo, Jordan) and placed in the well. Thereafter, the plates were incubated at 37°C for 18-24 hours [22].

Investigation of hemolysin-producing isolates

The isolates were streaked on a blood agar medium (containing 5% human blood) and incubated at 37°C for 24 hours, after growth, the zone around the colony was investigated [23].

Results

Diagnosis of bacterial isolates

The 110 ear swabs collected from ear infections 97 isolates were obtained, 87.63% (85/97) had bacterial growth and 12.37% (12/97) had fungal growth. 10.31% (10/97) isolates of the latter were *Candida spp*. And 2.06% (2/97) were *Aspergillus spp*.

The Gram-negative bacteria were more prevalent than gram-positive bacteria accounting for 69.07% (67/97) and 18.56% (18/97) Gram-positive bacterial isolates of *Staphylococcus ssp.* of the total microorganism isolates. the most predominant gram-negative was *pseudomonas* ssp. Comprising 36.08% (35/97) of the total microorganism isolates. the next common gram-negative bacteria were *Escherichia coli* , *Klebsiella spp*., *Proteus spp*., *Burkholderia ssp*.,11.34%, 10.3%,7.21%,2.06% and1.03% each of *Bordetella spp*. and *Stenotrophomonas ssp*. respectively.

The three dominant bacterial species identified in this research depended on 47 biochemical tests via Vitek 2 compact system were 30.93% (30/97) isolates of *P. aeruginosa*, 3.09% (3/97) *Pseudomonas putida*, and 2.06% (2/97) of *Pseudomonas fluorescens*. These results are illustrated in Figure (1).

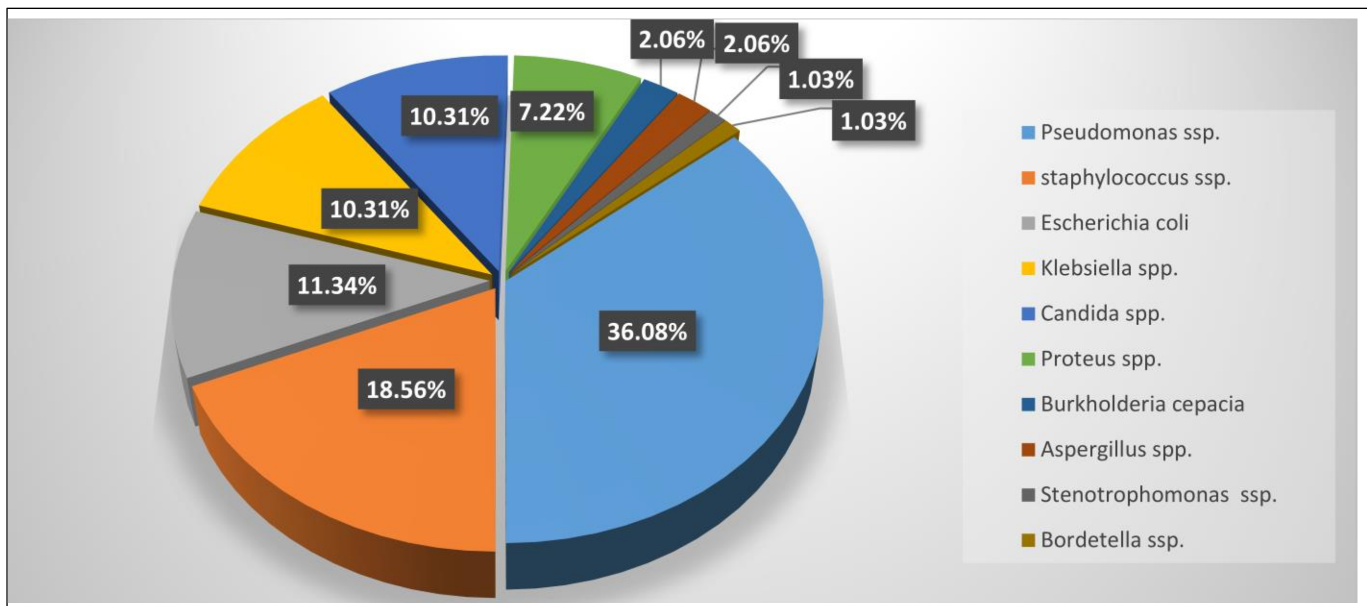

Figure (1) distribution of microorganisms in ear infections

The identification results, demonstrated that *P. aeruginosa* has the dominant role in ear infections, Colonies of *P. aeruginosa* differ in shape, some are round, smooth, and pale yellow on MacConkey agar due to their inability to ferment lactose, some are green and have a grape-like odor. Some large, mucoid colonies appear on the blood agar, which is an enriched medium, and the ability of the bacteria to degrade blood on this medium was determined and most of the isolates appeared to be beta hemolytic, with a transparent zone around the colony. Microscopic examination of bacterial smears stained with Gram stain showed that the bacteria were susceptible to this dye in the form of single bacilli or short chains. One colony from each isolate was selected and transferred to cetrimide agar. The colonies that grew on this medium were characterized by their regular round shape and the bluish-green color of the bacteria due to the presence of cetrimide, which inhibits the growth of all types of bacteria except *Pseudomonas spp*. These types of bacteria were distinguished by studying their growth at 4 and 42 ˚C [24]. The bacteria were grown on a solid cultural medium and the process was repeated for each isolate, firstly they were grown at 4˚C, then at 42˚C. The results showed that 30 isolates grew at 42 ˚C, and this is a characteristic of *P. aeruginosa.* In addition, Chemical tests showed that the isolates were strongly positive for catalase, oxidase, citrate, indole, and KOH. On the other hand, the isolates were negative for methyl red, Voges Proskauer and urease as shown in table (2).

Table (2) Biochemical tests for *P. aeruginosa*

No.	Test	Result
1-	Gram stain	-
2-	KOH	+
3-	Oxidase	+
4-	Catalase	+
5-	Indole	+
6-	methyl red	-
7-	Voges Proskauer	-
8-	Citrate utilization	+
9-	Growth at 42 ˚C	+
10-	Motility	+
11-	Hemolysis	+
12-	Urease	-

In the molecular identification results. PCR assays employing 16SrRNA primer pair specific for *P. aeruginosa* produced PCR products of the predicted size (956 base pair). The assays were conducted by testing 30 isolates of *P. aeruginosa* identified by the conventional methods. The PCR results are summarized in figure 2 and 3.

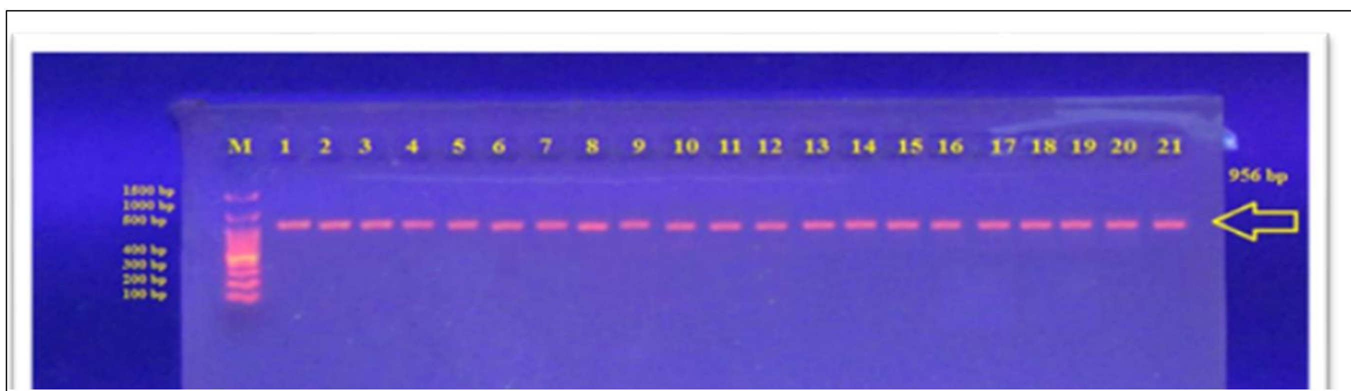

Figure (2) Electrophoresis of PCR products of S1-S22 *P. aeruginosa* isolated from ear infections using the 16srRNA gene-specific primer (956bp) in 1% agarose gel at 50V for 1 hour.

Figure (3) Electrophoresis of PCR products of S22-S30 *P. aeruginosa* isolated from ear infections using the 16srRNA gene-specific primer (956bp) in 1% agarose gel at 50V for 1 hour.

P. aeruginosa was also investigated for some virulence factors, the results showed that all isolates possessed hemolysin100%, while 76.66% (23/30) gave a positive result on the production of Protease as it showed a transparent zone around the growing colonies as a result of the degradation of casein an evidence of protease production, the isolates also had the ability to produce Pyocyanin pigment 80% (24/30) on cetrimide agar medium. This was followed by the production of the dye on Muller Hinton medium, where 20 isolates were able to produce 66.67% of the blue-green pyocyanin dye, while the production rate was 40% (12/30) on the nutrient agar medium. The lowest number was recorded on the MacConkey agar medium with 26.67% (8/30) and as shown in Figure (4).

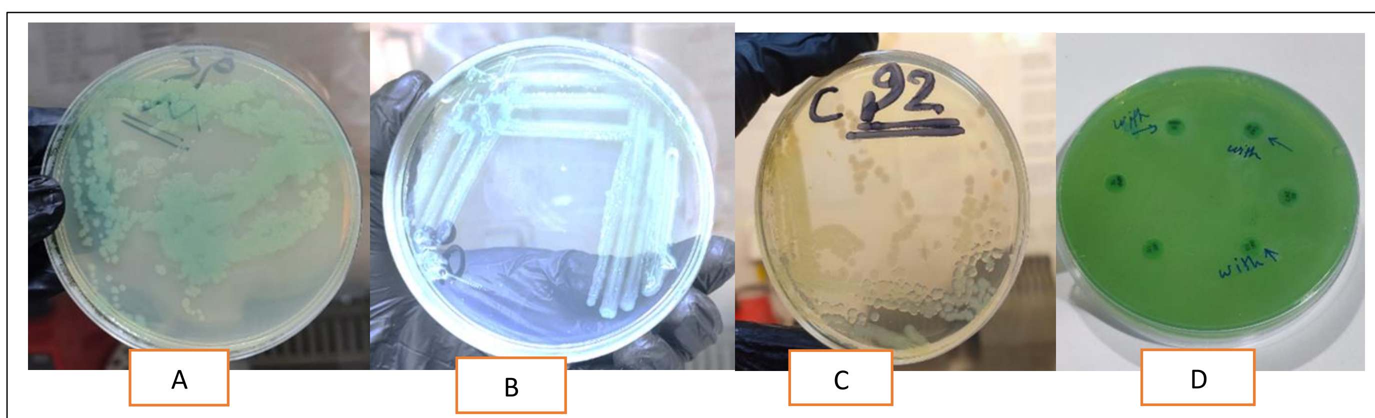

Figure (4): Production of pyocyanin pigment by *P. aeruginosa* on different media (A) Muller-Hinton agar medium (B) cetrimide agar (C) Nutrient agar medium (D) MacConkey agar medium.

Discussion

P. aeruginosa is an opportunistic pathogen that may infect almost all bodily tissues. It has an extremely large genome, which helps it survive in varied habitats. It also contains a variety of gene regulatory functions that aid in adaptability to new environmental situations. As a result, timely and precise identification of *P. aeruginosa* from culture samples is extremely critical. However, identification of this species may be difficult due to significant phenotypic diversity among isolates and the presence of several closely related species [25] To confirm the species identification, the PCR technique was utilized in this investigation to detect the presence of the diagnostic gene 16srRNA, which is an essential gene that is a fixed portion of the genetic code that distinguishes *P. aeruginosa* from other *Pseudomonas spp*. [26, 27].

When investigating some bacterial virulence factors, 75% of the bacterial isolates were able to produce the enzyme protease, which is one of the crucial factors for the virulence of this bacterium, These results were compared with [28] and [29] ,the researchers isolated *P. aeruginosa* from different sources and found that 74.1% of the isolates produced protease, This enzyme plays an important role in degrading structural protein and facilitating bacterial adhesion leading to the progression of infection [30].

All isolates exhibited the hemolytic toxin, and the results were consistent with [31], which reported a production percentage of 100% for hemolysis, it is known to be important for bacterial pathogenicity in *P. aeruginosa* because it breaks down cells and promotes bacterial spread. It is also regarded as one of the enzymes that are toxic to eukaryotic cells.

When detecting pyocyanin production, a majority of the isolates showed the ability to produce pyocyanin pigment. and Pyocyanin is an important virulence factor for bacteria and acts as a carrier for the iron element as it obtains complex iron and binds tightly to it and works to transfer dissolved iron from the environment to the cell when iron deficiency occurs, another study in 2015 showed that the production of pyocyanin is influenced by carbon and nitrogen sources in the growth medium, in addition to pH, aeration and incubation period [32]. Pyocyanin-producing strains were found to be more resistant to several antibiotics than non-producing strains [33].

Conclusion

The study revealed that more than two third of the ear infection samples were bacterial culture positive, which suggests that bacterial ear infections is one of the health problems in the research area. The study also determined that *P. aeruginosa* is a prevalent bacterium in ear infections, primarily due to its possession of various virulence factors. These factors include haemolysin production, protease production, and the ability to produce pyocyanin pigment, which plays a significant role in its pathogenicity. A further investigation for more virulence factors is advisable.

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