Molecular detection and phylogenetic tree assay of Pseudomonus aeruginosa isolated from otitis cases of cats and humans, Iraq

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KEYWORDS

Pseudomonus aeruginosa, Otitis externa, Polymerase chain reaction, Phylogenetic tree

ABSTRACT

The aim of this study was toward isolation and identification of P. aeruginosa from cats with otitis externa in Basra city Using culture media, Biochemical tests and Polymerase chain reaction. Pseudomonas aeruginosa was isolated from 46 isolates that involved 32 cases out of 75 of feline otitis externa (21.3%) and in 14 (9.3%) from 75 human samples .the clinical signs of otitis externa were head shaking, scratching, excessive ear wax, malodor, pain during palpation, alopecia and pus and/or blood. Culture was done on Pseudomonas chrome agar revealed positive samples with production of greenish-blue pigmentation by the bacteria .polymerase chain reaction revealed that isolates were Pseudomonas aeruginosa using specific primers for P. aeruginosa identification 16S rRNA and the result was positive for the 15 isolates. a phylogenetic tree analysis of P. aeruginosa was performed using the 16S rRNA gene sequence analysis which illustrated the phylogenetic relationship between P. aeruginosa strains isolated from cats and other related genera. In conclusion, this study confirmed the higher prevalence of P. aeruginosa in otitis externa infection of cats when compared to human. The using of molecular assay and 16S rRNA gene contributed effectively in detection of P. aeruginosa. Therefore, this study suggests the application of molecular techniques in identification of different microorganisms. Additionally, moreover studies are necessary to detect the prevalence of P. aeruginosa in different infections in animals as well as human.

1. Introduction

Otitis externa is an inflammatory disease of the external ear canal, including the ear pinna. Otitis externa may be acute or chronic (persistent or recurrent otitis lasting for 3 months or longer). Changes that occur in the external ear canal in response to chronic inflammation may include glandular hyperplasia, glandular dilation, epithelial hyperplasia, and hyperkeratosis. These changes usually result in increased cerumen production along the external ear canal, which contributes to increase in local humidity and pH of the external ear canal, thus predisposing the ear to secondary infection (Quim *et al.*, 1996). *Pseudomonas aeruginosa* of the Pseudomonadaceae family classified as an opportunistic pathogen, which causes disease infrequently in normal hosts but is a major cause of infection in patients with underlying or immunocompromising conditions. Quinn *et al.* (2005) mentioned that *P. aeruginosa* can continue in severe environmental conditions and shows essential resistance to a various antimicrobial agents. Among the pathogenic microorganisms that cause human infections, *P. aeruginosa* remains one of the most common agents of outbreaks in hospitals worldwide (Saitou and Nei, 1987). This pathogen is the most common species in the Pseudomonas genus, comprising 144 species, 25% of which are associated with human illnesses (Felsenstein, 1985).

P*seudomonas aeruginosa* is the most common cause of infections (both within the genus and among Gram-negative bacteria in humans and animals (Pachori *et al.*, 2019). This pathogen is abundant in wide variety of environments and can colonize and infect livestock and companion animals. *P. aeruginosa* can cause mastitis in dairy cows and multiple diseases in sheep and goat including ovine mastitis, respiratory manifestations, urogenital disorders, gastrointestinal illness, sinusitis and osteomyelitis (Saha *et al.*, 2008). P*seudomonas aeruginosa* is an opportunistic pathogen and infection is preceded by a breach in host defenses, such as breaks in the skin. This organism produces a variety of toxins and enzymes which promote tissue invasion and damage. Although *P. aeruginosa* is an environmental organism, it is also infrequently found on the skin, mucous membranes, and in the feces of some healthy animals wild, companion, or farm animal (Broglia *et al.*, 2020).

2. Methodology

Identification of Pseudomonus

The specimens were directly inoculated onto brain heart infusion broth and incubated at 37ºC for 24 hrs., then inoculated in the MacConkey agar plates and incubated at 37 °C for 24 hrs to distinguish between the ferment and non-ferment lactose bacteria also the isolates were cultured on *Pseudomonas* chrome agar media. The colonies from essential cultures were purified by subculture onto nutrient agar and incubated at 37ºC for 24 hrs.

Biochemical tests

1- Gram's staining

All isolates were stained with Gram's staining and examined by a light microscope to notice Gram's reaction, shape, and cell arrangements

2- Catalase test

A small amount of pure growth was transferred with a wooden stick into clean slide, and then a drop of catalase reagent (hydrogen peroxide 3%) was added. The evolution of gas bubbles indicates a positive result (MacFaddin, 2000).

3- Oxidase Production

A disc of filter paper was saturated with a little freshly made (1 %) solution of oxidase reagent (tetramethyl p-phenylene-diamine dihydrochloride), then a colony was picked up with a sterile wooden stick and smeared over the saturated filter paper. A positive result was indicated when an intense deep-purple color appeared within 5-10 sec (MacFaddin, 2000).

4-Citrate tests

Heavy inoculums were lined above the superficial slope of Triple sugar iron agar and stabbed into the button, incubated aerobically at 37 °C for 24 hrs. Interpretation of the consequences was improved by the change of color from green to blue (MacFaddin, 2000).

5-Growing on Triple sugar Iron Agar

Heavy inoculums were lined above the superficial slope of Triple sugar iron agar and stabbed into the button, incubated aerobically at 37 °C for 24 hrs. Interpretation of the consequences was improved by the change of color at surface and button, with or without H2S production as follows: Slant /Button Color Alkaline/Acid Red /Yellow Acid /Acid Yellow/Yellow Alkaline/Alkaline Red/Red H2S Black precipitation Gas Bubbles (MacFaddin, 2000).

Molecular identification

After DNAs extraction, PCR was established by adding the reaction mixture to a PCR tube (Table 1). The 20 µL PCR reaction mixture was made up according to (Table2). The PCR amplification settings were as follows in (Table 2) (Miyoshi et al. 2005). The PCR result bands were observed and photographed on an agarose gel electrophoresis at a location of about 1500 bp in relation to a DNA ladder (Promega, USA) Amplification was performed in thermal cycles depend on reaction protocol (Table 3) Samples were loaded on a 100bp as a DNA marker. To visualize the bands, the gel was run on agarose gel electrophoresis and observed under a UV transmission illuminator.

Table 1. The *16S rRNA* gene using specific primer pairs

Primes Name	Sequences	Product size	Reference
16S rRNA-F	(5'-AGAGTTTGATCCTGGCTCAG-3')	1500 bp	Al-Tememe and Abbas (2022)
16S rRNA-F	R, 5'-GGTTACCTTGTTACGACTT-3'		

Table 2: Components of PCR reaction mixture

No.	Reagent	Volume
1-	Master mix Pioneer	-
2-	DNA template	3 µl
3-	Forward primer	1 µl
4-	Reverse primer	1 µl
5-	Nuclease free water	15 µl
Total reaction volume	Total reaction volume	20 µl

Table 3: Steps and Conditions of PCR cycle or primers

Steps	Temperature	Time	*Cycles*
Initial denaturation	96C	5:00 min.	1
Denaturation	96C	5:00 min.	
Annealing	55C	1:00 min.	35
Extension	70 C	1:00 min.	1
Final extension	70 C	10:00 min.	
Hold	4 C		

The PCR products were detected by using agarose. The agarose gel was prepared according to (Sambrook *et al.,* 2004).

Five µl of PCR products was produced to each well. The lid was then placed on the gel tank and the electrical currents were connected for around 45 min with 50 volts. The gel was then removed carefully from the tray and examined under an UV transilluminator. The size of PCR products was determined by contrast with DNA ladder

The 16S rDNA PCR products were sent to Macrogen Company in South Korea for purification and sequencing. The 16S rDNA gene sequences of bacterial isolates were obtained in raw format, edited using MEGA-X, and compared with nucleotide sequence databases of NCBI using BLAST tool (http://www.ncbi.nlm.nih.gov) to determine sequence homology and identify the bacterial isolates. The phylogenetic tree was generated using MEGA X (20).

Phylogenetic analysis: A neighbor-joining phylogenetic tree was constructed for 15 isolates in comparison to other isolates from the NCBI database that displayed the highest homology to the deposited NCBI *P. aeruginosa* isolates, based on the examined 16Sr RNA nucleic acid sequences. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 27 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1247 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura and Kumar 2021).

3. Result and Discussion

Culture

Colonies of *P. aeruginosa* on MacConkey agar were exhibited as mucoid, non-lactose fermenters, colorless colonies, and the agar color changed from pink to transparent and yellowish or tan. On

Chrome agar, *P. aeroginosa* showed green mucoid colony in this medium. Microscopic examination of typical colonies that showed Gram-negative bacilli was considered *P. aeruginosa*.

Biochemical tests

Specific biochemical tests were used for the detection of *P. aeruginosa* isolates. These biochemical tests were catalase, oxidase, TSI, citrate test. All *Pseudomonas* isolates from different sources showed positive results to Citrate utilization tests in which change the medium from green to blue color. The result of TSI of *P. aeruginosa* was as follows: acidic / change color (yellow), and gas production (Table 4).

Table 4: Biochemical tests of the bacterial isolates

Code of isolate	Gram stain	Catalase test	Oxidase test	Citrate test	TSI
1	-	+	+	+	-
2	-	+	+	+	-
3	-	+	+	+	-
4	-	+	+	+	-
5	-	+	+	+	-
6	-	+	+	+	-
7	-	+	+	+	-
8	-	+	+	-	-
9	-	+	+	-	+
10	-	+	+	-	+
11	-	+	+	-	+
12	-	+	+	-	+
13	-	+	+	-	+

Molecular results

The isolates of *P. aeruginosa* isolates were subjected for PCR assay to detect the presence of *16S rRNA* gene (Figure 1). The results of PCR amplification of *16S rRNA* gene from *P. aeruginosa* isolates collected showed that all isolates exhibited clear bands of approximately 1500bp, corresponding to the identification of *P. aeruginosa* strains (Figure 2).

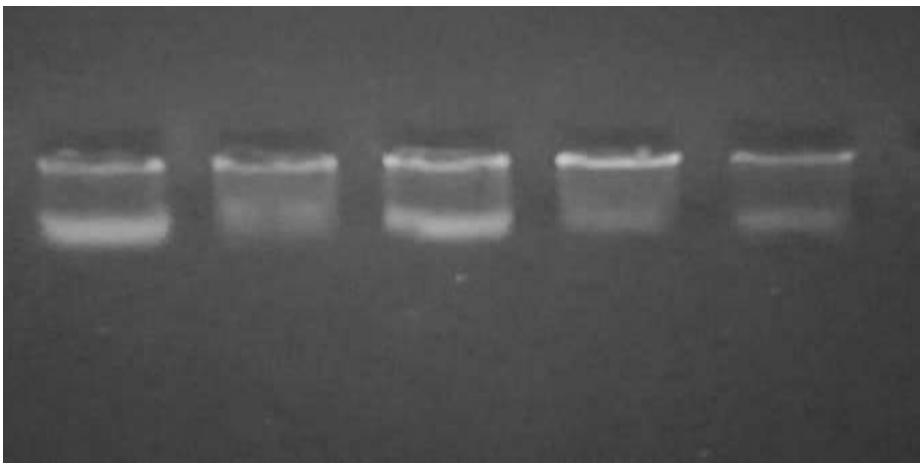

Figure 1: Agarose gel electrophoresis of bacterial genomic DNA

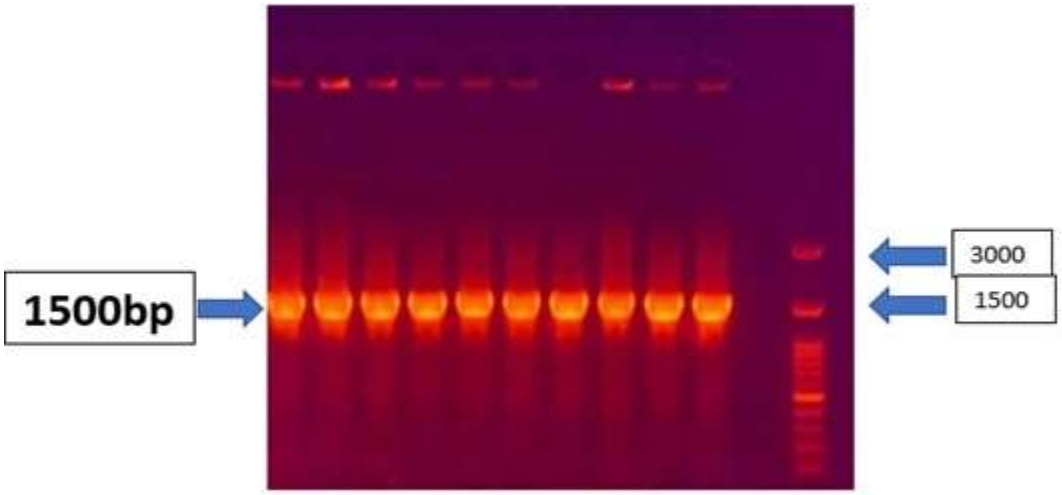

Figure 2: Agarose gel electrophoresis of PCR-amplified *16S rRNA* gene of *P. aeruginosa* isolates. Lane L: DNA ladder. Lanes 1- 10: *16S rRNA* gene 1500bp

Followed sequencing, the 16SRNA gene sequences of the sequenced isolates have been compared to the highly similar DNA sequences in the GenBank database using the BLAST (Basic Local Alignment Search Tool) program in the NCBI database. Based on a homological sequence identity with global isolates at a 99-98% matching rate, fifteen of these were confirmed to be *P. aeruginosa.*

Table 5: The Gene bank database has the accession numbers

No.	sources	species	accession numbers
1	Cat	*P. aeroginosa.*	PQ164087
2	Cat	*P. aeroginosa.*	PQ164088
3	Cat	*P. aeroginosa.*	PQ164089
4	Cat	*P. aeroginosa.*	PQ164090
5	Cat	*P. aeroginosa.*	PQ164091
6	Cat	*P. aeroginosa.*	PQ164092
7	Cat	*P. aeroginosa.*	PQ164093
8	Cat	*P. aeroginosa.*	PQ164094
9	Cat	*P. aeroginosa.*	PQ164095
10	Cat	*P. aeroginosa.*	PQ164096
11	Cat	*P. aeroginosa.*	PQ164097
12	Cat	*P. aeroginosa.*	PQ164098
13	Cat	*P. aeroginosa.*	PQ164099
14	Cat	*P. aeroginosa.*	PQ164100
15	Cat	*P. aeroginosa.*	PQ164101

In addition, a phylogenetic tree analysis of *P. aeruginosa* was performed using the 16S rRNA gene sequence analysis (Figure 2), which illustrated the phylogenetic relationship between *P. aeruginosa* strains isolated from cats and other related genera. The tree was constructed using the neighbor-joining method, and the GenBank ID of each contrast strain is provided (Figure 3).

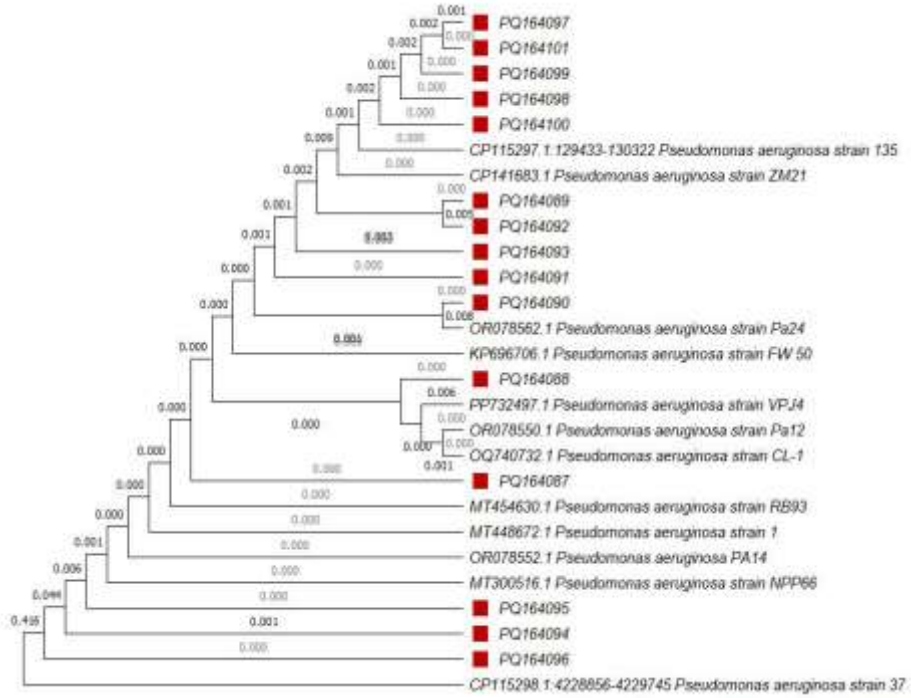

Figure 3: Phylogenetic tree analysis of a: *P. aeroginosa* constructed based on *16S rRNA* gene sequence analysis, showing the phylogenetic relation of strains isolated from cats (Red circles) and closely related *P. aeroginisa* strains from NCBI database. The tree was constructed by the neighbor-joining method.

Discussion

Otitis externa is characterized by a dark pungent discharge from the ear canal and intense pruritis with head shaking, scratching and rubbing of the ears. Damage to the pinna may manifest as a hematoma. The mucosa of the ear canal is painful and swollen (Malayeri *et al.,* 2010). The etiology of this condition is complex. Poor ear conformation, wax retention and immunosuppression are among the factors which may predispose cats to the disease. Predisposing causes should be investigated and eliminated or treated (Broglia *et al.,* 2020). In the present study, brain heart infusion broth was used as an enrichment medium while MacConkey agar and *Pseudomonas* Chrome agar were used for selection and isolation purposes. Bacterial identification was directed based on morphological and biochemical tests that agree with (Abbas *et al.,* 2014).

The result of the current study showed that, from 100 collected samples, only 10 (10%) were characterized as *P. aeruginosa* which distributed in 7(14%) isolates from male and 3 (6%) isolates from female. Samples were planted on the nutrient agar medium to isolate *Pseudomonas spp*. which had the ability to grow on the mentioned medium and produced non-fluorescent bluish pigment which diffuses into the agar (Angell *et al.,* 2006).

The phenotypic diagnosis of this bacterium is very important by recognizing its characteristics as well as its ability to produce many enzymes. Through this study, we demonstrate that these bacteria was capable of decomposing hydrogen peroxide by secreting the enzyme catalase, as hydrogen peroxide is very toxic to bacteria, so the bacteria are convert it to water and O_2. It was also observed through this study that these bacteria have the ability to possess the oxidase enzyme. This confirms that these bacteria use oxygen as a final electron receptor in their metabolic reactions (Forbes *et al.,* 2007). On the other hand, it is clear that these bacteria have the ability to consume citrate as a sole source of carbon and convert it into oxaloacetate, pyruvate and carbon dioxide, which the last reacts with sodium and forms sodium bicarbonate, which converts the medium into the basic form (blue color). It was also

concluded from the results studied in this research that these bacteria have the ability to consume carbohydrates and produce gas in the reactions as described by others (Barrow and Feltham, 3003).

The PCR technique is a highly sensitive and specific technique to amplify a target DNA by using universal or specific primers. The *16S rDNA* gene is commonly used to identify the bacteria and considered the best tool to study the bacterial phylogeny and taxonomy due to its presence in all bacteria, the function of the *16S rDNA* gene has not changed over time and the length of *16S rDNA* gene is suitable, in addition to accurate and reliable analysis that led to detect of new bacterial strains comparing with conventional techniques (Janda and Abbott, 2007; Al-Dhabaan, 2019).

The present study disagrees with the study of Abbas *et al.* (2014) in Iraq who presented that the resistant of *P. aeruginosa* isolates were 100% to Gentamicin, whereas, the study of (Bashir *et al.*, 2014) in Kashmir showed that all isolates (100%) were sensitive to gentamicin . in this study 90% of isolates showed intermediate susceptibility to gentamicin . results discovered for antibiotic susceptibility test revealed that 90% were sensitive to ciprofloxacin and 70% were sensitive to levofloxacin in otherwise 10% were sensitive to amikacin whereas the minor ratio of resistance was 10% to ciprofloxacin.

Major mechanisms of *P. aeruginosa* used to counter antibiotic attack can be classified into intrinsic, acquired and adaptive resistance. The intrinsic resistance of *P. aeruginosa* includes low outer membrane permeability, +expression of efflux pumps that expel antibiotics out of the cell and the production of antibiotic-inactivating enzymes (Bajpai *et al.*, 2019). The acquired resistance of *P .aeruginosa* can be achieved by either horizontal transfer of resistance genes or mutational changes. In addition, adaptive antibiotic resistance of *P. aeruginosa* is a recently characterized mechanism, which includes biofilm-mediated resistance and formation of multidrug tolerant persister cells, and is responsible for recalcitrance and relapse of infections (Pang *et al.*, 2019).

In the current study, *P. aeroginosa* was isolated and confirmed using phenotypic and molecular approaches. Bacterial identification was conducted based on morphological and biochemical tests that agree with Abbas *et al* (Martin *et al.*, 2000). The phenotypic diagnosis of this bacterium is very important in recognizing its characteristics as well as its ability to produce many enzymes. On the other hand, the use of polymerase chain reaction (PCR) allows for fast identification of the causative bacteria using specific primers and sequencing the 16S RNA. This method is a suitable diagnostic test for feline otitis causative bacteria (Saitou and Nei, 1987; Felsenstein, 1985; Tamura et al., 2004). Although these isolates were from the same geographic area, the phylogenic analysis shows some divergences among the isolates inferred from the 16S RNA evolutionary analysis seen in figure 6. We selected seven isolates and deposited them in the NCBI database, which could help track the recurrence of such isolates in different locations.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 15 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1013 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. Although utmost care has been taken to ensure the correctness of the caption, the caption text is provided "as is" without any warranty of any kind. Authors advise the user to carefully check the caption prior to its use for any purpose and report any errors or problems to the authors immediately (www.megasoftware.net). In no event shall the authors and their employers be liable for any damages, including but not limited to special, consequential, or other damages. Authors specifically disclaim all other warranties expressed or implied, including but not limited to the determination of suitability of this caption text for a specific purpose, use, or application.

4. Conclusion and future scope

This study confirmed the higher prevalence of P. aeruginosa in otitis externa infection of cats when compared to human. The using of molecular assay and 16S rRNA gene contributed effectively in detection of P. aeruginosa. Therefore, this study suggests the application of molecular techniques in identification of different microorganisms. Additionally, moreover studies are necessary to detect the prevalence of P. aeruginosa in different infections in animals as well as human.

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