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Detection of *Exo* A Gene in *Pseudomonas aeruginosa* from Human and Dogs Using Polymerase Chain Reaction

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Abstract: *Pseudomonas aeruginosa is the most frequent etiological agent of otitis externa. In this study polymerase chain reaction was used to investigate the genotypic properties and genetic relationship between P. aeruginosa obtained from human and dogs with otitis externa in Iraq. Seventeen (17%)P. aeruginosawere isolated from 100 ear swabs of human and six isolate (6%) from 100 ear swabs of dogs by conventional methods (culture and biochemical tests). A total of 23 P. aeruginosa isolate from human and dog were subjected to PCR to detect the Exo A gene by amplifying a 396-bp region using the same primer. Results showed that, six(26 %) and two (8.6 %) isolates from human and dogs give positive product for exo A gene respectively. in conclusion, there is a genetic relationship between P. aeruginosa isolated from human and dogs in Iraq.*

Key words: *Pseudomonas aeruginosa, otitis externa, PCR, exo A gene*.

I. Introduction

P. aeruginosa is gram-negative, non spore-forming, motile, aerobic bacterium. It is found in moist, warm environments and can often be isolated from soil, water, sewage, and occasionally from human skin [1]. P. aeruginosa is an opportunistic pathogen capable of infecting both human and animals [2], and are responsible for about 13 % of eye, ear, nose and throat infections [3].It is the most frequent etiology of otitis externa[4]. Otitis externa is an acute or chronic inflammation of the external auditory canal, auricle, or both [5]. It is a common disease that can be found in all ages. It's characterized by symptoms such as ear discharge, ear canal swelling, pain, periauricular cellulitis, and fever[6].*P. aeruginosa* can causes otitis externa in canines [7].It has not been isolated from healthy canine ears and when present, can result in inflammation and ulceration within the external ear canal [8,9].While [10] and [11] reported 1% percentage of *P. aeruginosa* was isolated in healthy dogs. Clinical signs in dogs of *Pseudomonas* otitis can usually be recognized, such as unilateral or bilateral ear damage, including head shaking, scratching or rubbing the ear, namely the development of an aggressive response to palpation canal. Vertical portion of the canal can be obstructed due to moderate or severe skin hyperplasia, and a greenish- yellow, purulent and stinks discharge [12].*P. aeruginosa* produces many extra cellular products, proteases (Elastase, Las A protease, protease IV, and alkaline protease), toxins (exotoxin A (ETA) and exoenzyme S) and hemolysins (phospholipase and rhamno lipid [13,14].The highly toxic ETA is produced by the majority of *P. aeruginosa* strains and can inhibit eukaryotic protein biosynthesis at the level of polypeptide chain elongation factor 2 [15,16]. More than 90% of *P. aeruginosa* produce exotoxin A [17]. Exotoxin A (ExoA, toxA) is a 66 kD A protein acts as a major virulence factor of P. aeruginosa, analogous in action to that of diphtheria toxin[18].The gene encoding Exotoxin A is found in 90-95 % of *P. aeruginosa* [19],whereas other Pseudomonas spp. and GC-rich bacteria did not yield any 396-bp fragment[20].Polymerase chain reaction was became very rapid reliable tool for molecular biology based diagnosis of the variety of infectious disease, because of its speed and versatility[19].Thisstudy was aimedto investigate the genotypic properties and genetic relationship between *P. aeruginosa* obtained from human and dogs with otitis externa**.**

II. Methodology

Collection of samples

A total of 100 ear swabs were collected from clinical cases of patients suffering from otitis externa from Department of ENT(Ear, Nose and Throat) in Al-Emamein Kadhimein Medical City \ Baghdad, During a period from (December / 2013 – April / 2014) and at the same time one hundred ear swabs were collected randomly from different breeds of dogs, with apparently healthy ears, in the several geographical areas in Iraq. The samples were collected in transport media with sterile swab sticks which labeled for source, age, time of collection, date and personal history, or animal history. They were transported in cooler boxes to the Microbiology department\College of Medicine –Al-Nahrain University, for culturing.

Phenotypic identification by culturing

All swabs were streaked immediately on nutrient, MacConkey and blood Agar. A single colony was selected ,streaked and incubated in the selective medium (cetrimide agar), then phenotypic characteristics of *P. aeruginosa* was described according to gram staining and pigment production after incubation at 37 C°, the confirmation of diagnosis was done by conventional biochemical test which include; Oxidase , catalase test, indole production test, methyl red test and voges-proskauer.

Genotypic identification

DNA Extraction

DNA of *P. aeruginosa* isolates was extracted and purified using Genomic DNA Mini Kit (blood/cultured cell) (Geneaid)®.

Primer selection

The primers (Forward and Reverse) used in PCR were specific for *Exo* A gene which chosen according to [21]. The sequence of primer used in this study is given in Table (1).

Table (1): Sequences of primers for *Exo* A gene.

Gene		Nucleotide sequences (5' ⟶ 3')	Products bp
Exo A	F	GACAACGCCCTCAGCATCACCAGC	396
	R	CGCTGGCCCATTCGCTCCAGCGCT	

Amplification

PCR was done by modification of previously described PCR protocol [22] for amplification of *Exo* A gene in a final volume 20µl. The amplification was performed using Green Master Mix 2x **(Bioneer)®,** specific primers for *Exo* A gene (1µl forward and 1µl reverse), 1µl of *P. aeruginosa* DNA extract(each for human and dogs) as a template. Sterile distilled water was used instead of DNA template to ensure absence of contaminants in the reaction preparations as a negative control. The PCR conditions started with thermocycler program showed in table (2).

Table (2): The PCR thermocycler program for Exotoxin A gene

Steps	Temperature	Time	Cycles
Initial Denaturation	95ºC	2 min	
Denaturation	94ºC	1 min	30
Annealing	68 ºC	1 min	
Elongation	72°C	1 min	
Final extension Hold	72°C	7 min	
Soaking	4ºC	Unlimited time	

Agarose gel electrophoresis

PCR products were resolved by horizontal agarose gel electrophoresis according to [23].Ten microleter of the PCR product were electrophoresed on 2% agarose gel supplied with ethidium bromide at final concentration 0.5µg/ml, and using10 µl of 100bp DNA ladder at 5 V/cm. Then visualized under UV light and photographed [24].

III. Results and discussion

Isolation

In current study, out of the 200 samples were collected from human and dogs, the isolation rate was 17 (17%) in human. The results were consistent with percent (20%) from ear infection reported by [25] in Diwaniya teaching hospital and [26] in Egypt, while disagreed with the other study [27] and [28] from ear infections when they recorded higher percentage (28%) and (37.5%) respectively. Also [29] and [30] recorded lower percentage (8.33%) and (1.81%) respectively in Iraq. Whereas in Isfahan, [31] reported that the ear infections occur only in (3%).

In this study the isolation rate was 6 (6%) in dogs that agreed with [32], [33] and [34] when they recorded that the ear infection percentage ranged from (3.1- 9.4%) but disagreed with [35] and [29] they were showed that, the percentage was (26.7%), (13.33%) respectively, from otitis externa in Baghdad. The percentage of *P. aeruginosa* is a variable in the different studies this may be attributed to drug overuse, location of research,

time collection. Moreover geographic climate and hygienic factors may also be correlated with the relative variability of results among different areas [36].

Phenotypic properties

Twenty three (23) isolate were diagnosed successfully as *P. aeruginosa*. All isolates gave positive results for the smears as a gram negative bacteria [1].The greenish-yellow or blue pigment and characteristic odour on cetrimide agar were seen. *P. aeruginosa* reacted positively to catalase and oxidase tests, while it was negative for methyl red, Voges- Proskauer and indole, these results were inagreement with [37,38].

Genotypic properties

PCR results showed that, 8 of 23 *P. aeruginosa* isolates was positive for the *Exo* A gene with amplified size (396 bp) in a percentage (26 %, 6/ 23) in human and (8.6%, 2/23) in dogs (Figure1, Table 3).The result was disagreed with [19] who was showed that, 30/32 isolate of *P. aeruginosa* from otitis externa in patients were harbored *Exo* A gene. Frequent recombination of chromosomal genes between different isolates of *P. aeruginosa* may have led to the genetic diversity of the organism [39]. The mechanisms of genetic exchange, including transformation, transduction, and conjugation, affect *P. aeruginosa* to adapt to changing conditions by acquiring new genetic information [40], also the sequences are absent, or rearranged from isolate to isolate[18] or the gene may not be found in some these isolates[19].

Results of occurrence of *Exo* A gene on chromosomal DNA of *P. aeruginosa* showed significant differences between human & dogs. (Table 3).

(Table 3): Numbers and percentages of occurrence of *Exo*A gene on chromosomal DNA of *P.aeruginosa* in human & dogs.

Total No. of samples	Human					Dog				
	No. of samples	Positive ExoA gene	Percentage (%)	Negative ExoA gene	Percentage (%)	No. of samples	Positive ExoA gene	Percentage (%)	Negative ExoA gene	Percentage (%)
23	17	6	26	11	47.8	6	2	8.6	4	17.4

$\chi 2$ test = 19.3 ($p < 0.05$).

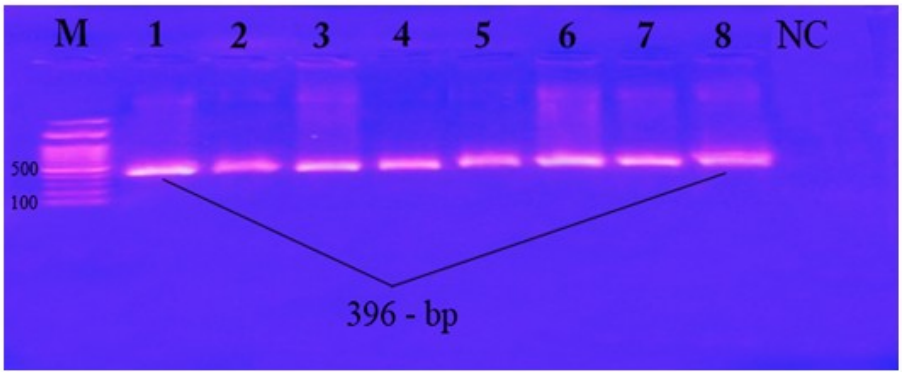

Figure (1): Agarose gel (2 %) electrophoresis of conventional PCR products of *Exo* A gene (396 bp) for *P. aeruginosa* in 1X TBE buffer using 75 V. for approximately 1hrs.; lanes 1,2,3,4,5,6:positive results of human otitic isolates ; lanes 7,8: positive results of dogs otitic isolates ; NC, negative control; M,100bp kappa ladder marker .

IV. Conclusion

The study concluded that there is a genetic relationship between human and dogs isolates was confirmed by conventional biochemical tests and PCR amplification targeting the *Exo* A gene of *P. aeruginosa*.

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