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Original Article

Molecular Detection and Phylogenetic Analysis of *Pseudomonas aeruginosa* Isolated from Some Infected and Healthy Ruminants in Basrah, Iraq

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Abstract

Although *P. aeruginosa* is an environmental organism, it is infrequently found on the skin, mucous membranes, and in the feces of some healthy animals (wild, companion, or farm animals). *P. aeruginosa* produces a variety of toxins and enzymes which promote tissue invasion and damage. *P. aeruginosa* demonstrated resistance to several antimicrobial agents. It is of significant importance in both animal and human medicine. The present study aimed to isolate and diagnose *P. aeruginosa* isolates from some ruminants, cow and sheep, from different regions of Basrah, Iraq. A total of 200 samples were taken from infected and healthy ruminants, as well as the environment surrounding the animal in Basrah, Iraq. The identification of *Pseudomonas aeruginosa* was performed by conventional and molecular methods using the 16SrRNA gene and aroE gene by polymerase chain reaction (PCR). The recorded data pointed out that *P. aeruginosa* was successfully isolated from infected animals (cows and sheep) with total percentages of 46% and 22%, respectively. These percentages were obtained at 8% and 4% from healthy cows and sheep, respectively. The percentages of isolation of the environment surrounding cows and sheep were 40% and 32%, respectively. A higher percentage of infection was observed in the eye, skin, and wound swabs of cows. Healthy cows and sheep gave only three isolates of *P. aeruginosa*, while the environmental swabs recorded 18 isolates. Bacterial isolates were identified by culture methods and Vitek- 2. To confirm the diagnosis more accurately at the level of the species, the molecular confirmation was performed by PCR amplification of genus and species with 16S rRNA gene sequences. The results pointed out that all 10 selected isolates gave positive results, and the gene size was ≈ 1500 bp. New strains were recorded in GenBank/NCBI, and the phylogenetic tree was constructed. The isolates fall in three clads. Molecular confirmation of other isolates in this study (42 isolates) was carried out by PCR amplification of aroE gene. All PCR products of these isolates were amplified≈ 495 pb on agarose gel electrophoresis.

Keywords: a*roE* gene, Cow, *Pseudomonas aerugenosa*, Sheep, 16SrDNA

1. Introduction

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 animals) (1). *P. 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. *P. aeruginosa* demonstrates resistance to several antimicrobial agents. It is usually susceptible to the aminoglycosides, semisynthetic penicillins, such as piperacillin and ticarcillin, third- and fourth-generation cephalosporins (ceftazidime and cefepime, respectively), carbapenems (except ertapenem), and the fluoroquinolones (1). *P. aeruginosa* has significant

importance in both human and animal medicine. Multiple studies have concentrated on clinical isolates of *P. aeruginosa* strains that isolated from humans; nonetheless, less attention has been paid to animal (2) and environmental strains (3, 4). There are no specific *P. aeruginosa* strains associated with specific animals, diseases, or habitats (5).

The enzootic or epizootic outbreaks of mastitis in ruminants, bovine, and small ruminants are rooted in several causes. One of the bacteria involved in this occasion would be *P. aeruginosa*. Ruminant mastitis is of great importance to animal scientists and veterinarians. Global human population growth has increased the demand for animal proteins, especially milk of small ruminants. Consequently, animal scientists and veterinarians are striving to prevent some deleterious effects of bacterial infections which lead to a decrease in milk production. The intoxications via milk, cheese, and yogurt in the case of mastitis caused by *P. aeruginosa* create drastic health problems for consumers. *P. aeruginosa* infections occur as clinical/subclinical intramammary infections in the post-partum period and sometimes during drying-off.

In order to prove the seriousness of the presence of P. aeruginosa in the animal environment, as well as on healthy and infected animal organs where these bacteria are not expected to exist, the present study aimed to isolate and diagnose *P. aeruginosa* isolates from some ruminants, cow and sheep, from different regions of Basrah, Iraq, using Vitek system and molecular diagnosis performed by *16S rRNA* gene and one of the Multilocus Sequence genes (*aroE* gene). The *aroE* gene which is one of the Multilocus sequence typing (MLST) is a housekeeping gene-encoded metabolic enzyme called shikimate dehydrogenase.

2. Materials and Methods

2.1. Sample Collection

The study included 200 swab samples which were divided into 100 samples from infected animals taken from the eyes, nose, mouth, ear, skin, and wounds, 50 samples from healthy animals for both cows and sheep, as well as 50 samples from the environment, including water, food, and soil which were collected by sterile transport media swabs. The samples were gathered from scattered local farms or owners from different areas of Basrah, Iraq, from March 2020 to March 2021.

2.2. Culture and Identification

The specimens were directly inoculated onto brain heart infusion broth and incubated at 37°C for 24 h. The culture was inoculated on the MacConky agar (HiMedia/India) plates to distinguish between the ferment and non-ferment lactose bacteria. The culture was also inoculated in blood sheep agars (Oxoid/ England), and both were incubated at 37°C for 24 h. The suspected single colonies were Gram stained, purified onto *Pseudomonas* Chromogenic Agar (Condalab/Spain), and incubated at a temperature of 35±2°C for 24-48 h. The pure colonies (Magenta colonies) were stored at 4°C. The *P. aeruginosa* isolates were identified according to biochemical tests recommended by Mac Faddin (6). After the confirmation of *P. aeruginosa* by biochemical tests, the isolates were sent to the Al-bayan laboratory to perform the bacterial identification by using the VITEK2 automated system (BiomerieuxMarcy l'Etoile, France).

2.3. Genomic DNA Extraction

The DNA extraction from the samples was performed using the gSYNCTMDNA Extrication Kit (Geneaid, Taiwan) according to company instruction. Following that, the extracted DNA was checked by Nanodrop spectrophotometer (NanoDrop® Technologies, Thermo Fisher Scientific, Basingstoke, UK) by measuring the absorbance at 260 nm, then stored in a refrigerator at -20°C until performing polymerase chain reaction (PCR).

2.4. Molecular Confirmation by *16S rRNA* Detection of Some *P. aeruginosa* Isolates

The amplification of the *16S rRNA* was performed by PCR using universal primers and PCR conditions described by Eden, Schmidt (7). The oligonucleotide primers which were used have 1500 bp and their sequence are:

F8-27 (5'-AGAGTTTGATCCTGGCTCAG-3')

R1510-1492(5'-GGTTACCTTGTTACGACTT-3')

AccuPower PCR PreMix Kit (Bioneer, Korea) contained all the components of the PCR, and the process was carried out according to company instructions (Table 1). The master mix components of the PCR were placed in standard PCR Pre Mixtubes containing all other elements needed for PCR reaction, then put in PCR Thermocycler (Labnet, USA). The products were visualized under UV light after being stained with ethidium bromide.

Table 1. Polymerase chain reaction program for *16S rRNA* gene

Genes	Step	Temperature	Time	Cycle
16S rRNA	Initial denaturation	95.0 C°	5 min	1
	Denaturation	95.0 C°	30 sec	30
	Annealing	55.0 C°	45 sec	
	Extension	72.0 C°	1.5 min	
	Final Extension	72.0C°	10 min	1

2.5. Molecular Confirmation by *aroE* Gene

A total of 42 *P. aeruginosa* isolates were identified via MLST of the nucleotide sequences of the gene encoding the *aroE* (shikimate dehydrogenase), utilizing the following primer (8):

aroE-F ATGTCACCGTGCCGTTCAAG

aroE-R TGAAGGCAGTCGGTTCCTTG

PCR conditions for all the primers are presented7 in table 2.

Table 2. Program of conventional polymerase chain reaction for *aroE* gene

Genes	Step	Temperature	Time	Cycle
aro E gene	Initial denaturation	96.0 C°	1 min	1
	Denaturation	96.0 C°	1 min	30
	Annealing	55.0 C°	1 min	
	Extension	72.0 C°	1 min	
	Final Extension	72.0C°	10 min	1

2.6. Sequencing

The products of amplification for *16S rRNA* genes obtained from conventional PCR were sent for sequencing using automated DNA sequence by soul university Corporation, Korea. A phylogenetic tree was constructed by the maximum-likelihood algorithm MEGA- X.

3. Results

3.1. Culture and Identification

The *P. aeruginosa* isolated from samples produces circular mucoid smooth colonies with emits sweat grape odor on nutrient agar and MacConkey agar. It has been observed to make β-hemolysis on blood agar (Figure 1A) and produce Magenta colonies on Pseudomonas Chromogenic Agar (Figure 1B). All *Pseudomonas* isolates from different sources showed positive catalase and oxidase. The result of Kligler's Iron Agar of *P. aeruginosa* was as follows: Alkaline /no change (red) No H2S. No Gas. The VITEK2 test showed 52 samples with a 90% probability of *p. aeruginosa*. A total of 52 (26%) bacterial isolates demonstrated typical characteristics of *P. aeruginosa.*

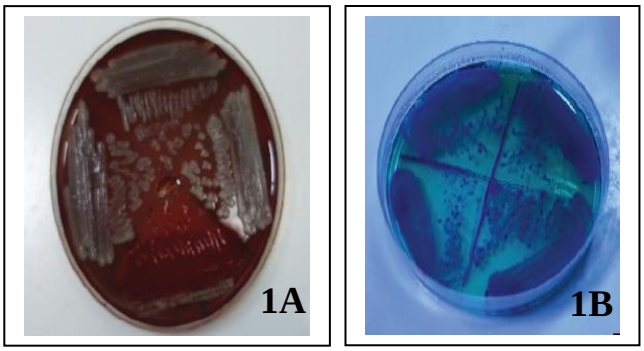

Figure 1. *P. aeruginosa* showing hemolysis on blood agar (A), and growing on *Pseudomonas* Chromogenic Agar (B)

The results also demonstrated that 38% of cow samples were positive for *P. aeruginosa*, while sheep samples illustrated only 24% (Table 3). The healthy animals showed less availability of *P. aeruginosa* (6%), while the surrounding environment illustrated 36% *P. aeruginosa* (Table 4).

3.2. Molecular Confirmation by *16S rRNA*

The amplification of the *16S rRNA* gene of the isolated bacteria illustrated a product size of ≈ 1,500 bp., (Figure 2).

3.3. Molecular Confirmation by *aroE* Gene

The amplification of the *aroE* gene for 42 bacterial isolates yielded a positive result, and the gene size was≈ 495 bp (Figure 3).

Table 3. Distribution of *P. aeruginosa* isolates from unhealthy animal samples

Animal	Ear		Nose		Skin & wound		Eye		Mouth		Total positive (%)
	N	+	N	+	N	+	N	+	N	+	
cow=50	8	4	6	2	17	6	20	7	1	0	19 (38)
sheep=50	6	0	10	2	15	6	18	4	0	0	12 (24)
Total	14	4	16	4	32	12	38	11	1	0	31 (31)

Note: (+) positive *P. aeruginosa* isolates, (N) number of samples.

Table 4. The number of *P. aeruginosa* isolates from healthy animals and environmental samples

Animal	Healthy animal		Environmental Sample*	
	N	+	N	+
cow	25	2	25	10
sheep	25	1	25	8
Total (%)	50	3(6)	50	18(36)

* Food, water, soil

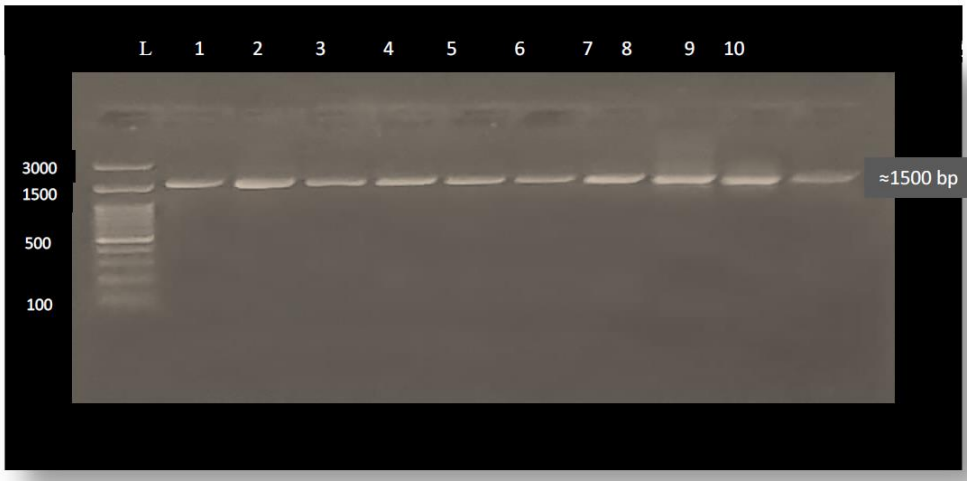

Figure 2. Gel electrophoresis of *Pseudomonas aeruginosa 16S rRNA* with product size ≈1500pb Lane (L): DNA ladder (100-3000bp), Lanes (1-10): *16S rRNA* gene.

Figure 3. Agarose electrophoresis of PCR-amplified for *aroE* gene of *P. aeruginosa* isolates. Lane L: DNA marker. Lanes (1-42) *aroE* gene ≈ 495 bp. Lane C: Negative control

3.4. DNA Sequencing

Nucleotides sequencing data of *16S rRNA* gene for 10 isolates were given positive results of *Pseudomonas aeruginosa*; thereafter, the 10 isolates were recorded in GenBank/NCBI (Table 5).

Table 5. Accession number of *16S rRNA* gene sequence

Number of isolates	Name of isolates in GenBank	Accession number	Length (bp)
1	*P. aeruginosa* strain TBBV1	MZ451962	1238
2	*P. aeruginosa* strain TBBV2	MZ451963	809
3	*P. aeruginosa* strain TBBV3	MZ451964	894
4	*P. aeruginosa* strain TBBV4	MZ451957	938
5	*P. aeruginosa* strain TBBV5	MZ451965	964
6	*P. aeruginosa* strain TBBV6	MZ451966	974
7	*P. aeruginosa* strain TBBV7	MZ451968	858
8	*P. aeruginosa* strain TBBV8	MZ452345	1123
9	*P. aeruginosa* strain TBBV9	MZ451967	560
10	*P. aeruginosa* strain TBBV10	MZ452344	1082

3.5. Phylogenetic Tree Analysis

The phylogenetic tree of *P. aeruginosa* isolates pointed to a relationship between different *P. aeruginosa* isolates in the present study and eight strains available in GenBank as demonstrated in figure 4.

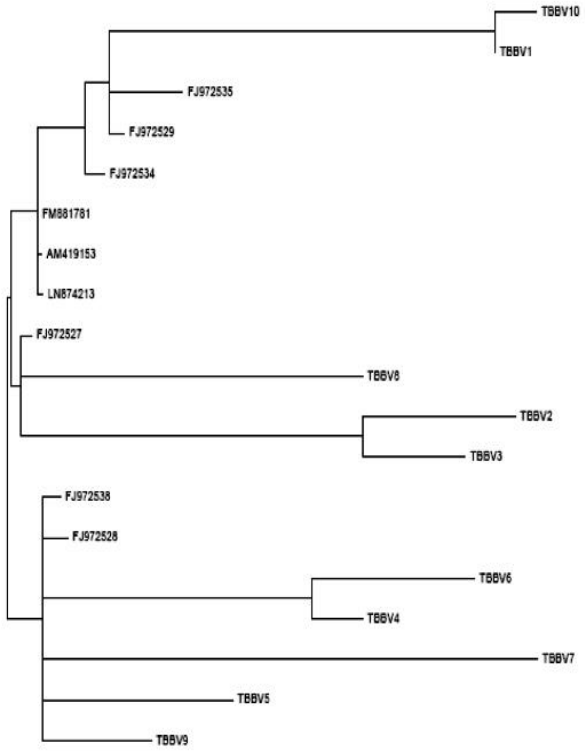

Figure 4. Rooted Neighbour Joining phylogenetic tree constructed from concatenated sequences of 1500 bp for each strain derived from an alignment of *16S rRNA* gene sequences then produced from analysis conducted in MEGA X program. This N-J tree shows the distribution and phylogenetic relationships between 10 isolated *Pseudomonas aeruginosa* in this study and 8 reference strains.

4. Discussion

Although numerous studies have been conducted in Iraq, especially in Basrah (9-12), they were not comprehensive for the regions and types of samples, especially from infected and healthy ruminants, as well as0 their environment. The present study focused on bacteria isolated from some ruminants, including cows and sheep, from different regions in Basrah Governorate and organs of the animal body. It was not previously addressed despite the lack of local studies and its economic importance.

The majority of the isolates showed β-hemolysis on blood agar, while others isolates were non-hemolysis. All isolates grew on MacConkey agar but gave a pale-colored colony owing to an inability to ferment lactose sugar. In agreement with the result of other studies (13), all the isolates were catalase and oxidase-positive and did not produce green pigmentation on the nutrient agar plate. Suspected *P. aeruginosa* Chromogenic agar medium is considered a selective medium for this bacterium. It is easily distinguishable due to the magenta colony color and the color of the medium that change from green to blue-green. The rest of the bacteria are inhibited, and in case of growing, they grow as colorless colonies (14). The isolates of positive pseudomonas were identified using the VITEK2 system. The result showed that 87% of samples were identified as *P. aeruginosa*.

P. aeruginosa was successfully isolated from eyes (7/20), nose (2/10), mouth (1/7), ear (3/8), skin (6/17), and wounds swabs from infected cows, as well as eyes (4/18), nose (2/6), mouth (0/4), ear (1/6), skin (6/15), and wounds swabs from infected sheep, with total percentages of 38% and 24%, respectively, (2/25) and(1/25)from healthy cows and sheep, respectively, as well as (10/25) and (8/25) from the environment of cows and sheep, respectively. A higher percentage of infection was found in the cow. Eyes, skin, and wounds swabs were the highest sample given *P. aeruginosa*. It can be ascribed to the fact that these areas of the animal body are the most susceptible to mechanical injury and contaminants from the surrounding environment. The aforementioned results are in line with the findings of other studies (15-17). Healthy cows and sheep gave only three isolates of *P. aeruginosa*, while environmental swab recorded 18 isolates and this was expected since *P. aeruginosa* is an environmental infection. It is found in soil, water, feed, and farm equipment.

After the identification of bacteria with culture and Vitek-2, it was necessary to confirm the diagnosis more accurately at the level of the species, and for all samples under the study, molecular confirmation was performed by PCR amplification of genus and species with *16S rRNA* gene sequences containing hypervariable regions that can provide species-specific signature sequences which are useful for bacterial identification (18). The current study identified *P. aeruginosa*-specific signature sequences with a universal primer. The PCR assays were used to test 10 isolates, including 5 from clinical, 4 environments, and 1 from a healthy sample.

The phylogenetic tree demonstrated two main branches from the root, one main branch includes isolates TBBV4, TBBV5, TBBV6, TBBV7, and TBBV9 which were closely related to the reference strain *P. aeruginosa* strain 57 *16S rRNA* and strain K2 *16S rRNA*. The second sub-branch gave two sub-branches, one sub-branch consists of three isolates TBBV8, TBBV2, and TBBV3 which were closely associated with the reference strain *P. aeruginosa* strain CJM. The other sub-branch contains two sub-branches, one sub-branch includes only three reference strains *P. aeruginosa* strains of KSG, DSIO, C16S which were not related to other isolates, and another sub-branch gave two sub-branches, one sub-branch includes only one reference strains *P. aeruginosa* strain NO6 which was not related to other isolates, and another sub-branch has two isolates TBBV1 and TBBV10 closely associated with one sub-branch including two reference strains *P. aeruginosa* strain C-1 and NO1.

Molecular confirmation of 42 isolates was performed by PCR amplification of genus and species with *aroE* gene. All PCR products of these isolates were

amplified≈ 495 bp on agarose electrophoresis. The MLST is a typing used on several conserved housekeeping genes, namely *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE* (19). As a result, the molecular confirmation by *aroE* gene in medical microbiology is a rapid and cheap alternative to phenotypic methods of bacterial identification. Although several studies have been carried out on other types of bacteria isolated from animals (20-26), there is a paucity of research on the genetic diversity of *P. aeruginosa* in this area.

Authors' Contribution

Study concept and design: T. M. K. A.
Acquisition of data: B. A. A.
Analysis and interpretation of data: T. M. K. A.
Drafting of the manuscript: T. M. K. A.
Critical revision of the manuscript for important intellectual content: B. A. A.
Statistical analysis: B. A. A.
Administrative, technical, and material support: T. M. K. A.

Ethics

Ethical approval for the study was obtained from the Ethics Committee of the University of Basrah, Basrah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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