ISOLATION AND MOLECULAR IDENTIFICATION OF MDR *Pseudomonas aeruginosa* FROM ANIMALS AND PATIENTS IN BASRAH PROVINCE

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ABSTRACT

Throughout the period from October 2018 to February 2019, 278 test samples were collected from animals and human, (55%) animal samples which are distributed to (52.3%) swab samples were from the environment of slaughters and (47.7%) milk samples were from cow and buffalo which collected in sterile containers. The result showed that *Pseudomonas* was found in (44%) samples on pseudomonas agar distributed in (24%) samples from slaughters, (20%) samples from milk. (45%) human samples that are distributed to (48%) swab samples were from diabetic foot patients and (52%) swab samples were from patients suffering from burns in hospitals of Basrah province. The results showed that *Pseudomonas* was found in (56%) samples on pseudomonas agar, (18%) samples from diabetic foot and (38%) samples from patients suffering from burns. 46 isolates were identified using VITEK 2 Kit. 25 samples identified as *Pseudomonas aeruginosa* which presented (54%). Antimicrobial susceptibility testing of 31 *P. aeruginosa* isolates compared to 13 different antibiotics was done by the disk diffusion method. Completely isolates were resistant to as a minimum of 8 antibiotics; they exhibited the form of the multiple resistance to the antibiotics. Thirty-eight samples were tested for *16S rRNA* by conventional PCR assay, 19 from animal sources and 19 from patients' sources. 18 animals and 19 patient samples were demonstrated distinct bands with approximately 618bp corresponding for *P. aeruginosa*.

INTRODUCTION

During the history, an essential cause of morbidity and mortality is the infectious diseases, by the development of the antibiotic history through the twentieth century; there was a growing confidence that the need for infectious disease specialists would all disappear (1). *P. aeruginosa,* like many other nonfermenting gram-negative rods, is a saprophytic organism widespread in nature, particularly in moist environments (water, soil, plants, and

sewage), and endowed with only weak pathogenic potential. However, because of its ability to survive on inert materials and its resistance to most antiseptics and antibiotics, *P. aeruginosa* has become an important and frequent nosocomial pathogen. Indeed, in hospitals, sinks, respiratory therapy equipment, and antiseptic or detergent solutions can act as reservoirs of *P. aeruginosa* (2).

P. aeruginosa is an essence of opportunistic pathogen of human. It causes an infection in urinary tract, dermatitis, infection in soft tissue, bacteremia, respiratory infection, infection of bone and Joint, gastro-intestinal infections, particularly it causes different types of infection such as in people suffer from AIDS, Immunocompomised conditions, burns and cancers and the other diseases. The bacterium causes pneumonia, chronic lung infections, endocarditic and septicemia. From 10.1% of all nosocomial contaminations, this bacterium was the 4th utmost frequently isolated nosocomial pathogen reason (3).

P. aeruginosa has increasing veterinary importance, it is a relevant cause of bovine mastitis, and in diverse animal species it was make various localized infections. The non-clinical strains of *P. aeruginosa* from animals and environment have acquired much less care and rare genetic investigation is identified on bovine strains (4).Raw milk is widely used up from olden times and the need for it is constant all over the world. Milk has a high nutrition importance so is considered a major nutrition for human. It is naturally a respectable medium for growing of microbes. Quality control of raw milk is an essential importance (5).

Defaulting of sanitized condition such as bulk tank unsuitable cleaning, dirty udders, equipment of milking, milk handling technique and inappropriate storing will raise the amount of Gram-positive and Gram-negative bacteria in the bulk tank milk (6). The bacteria in the family Pseudomonadaceae are among the most main spoilage bacteria emerging in chilled raw milk. They are reflected psychrotrophs, growing well at communal refrigeration temperatures (0-15°C). The main psychrotrophic micro flora come across in raw milk are Gram negative rods with *Pseudomonas* spp. (7) which forms at least 50% of all bacteria in milk (8).

P. aeruginosa is a multidrug-resistant common opportunistic pathogen with much importance in medical and veterinary side. This bacteria is a communal causal agent of local infections in humans, and it is a source of severe infections in an immune-compromised patients and causes mortality in patients with cystic fibrosis (CF) (9). The worldwide occurrence of multi-drug resistant bacterial strains in hospitals and communal stays to be a problematic of due scientific anxiety, particularly contaminations caused by *Pseudomonas* species and *P. aeruginosa* in particular. *P. aeruginosa* is an opportunistic pathogen with

intrinsic resistance to various antibiotics and disinfectants including anti-pseudomonal Penicillins, Ceftazidime, Carbapenems, Aminoglycosides and Ciprofloxacin (10).

It has necessary to develop genotype-based characterization systems which are capable of exactly detecting these bacteria in spite of any phenotypic alterations, DNA marker permits rapid identification of species, among DNA markers. The Polymerase Chain Reaction (PCR) is greatly sensitive specific and fast technique which improve the recognition of *P. aeruginosa* especially with using specific primer for *16S rRNA* (11).

Therefore, the aim of this study was detect the presence of *P. aeruginosa* in animal and human subjects using culture, biochemical tests, VITEK and PCR. The other goal is to test the susceptibility of the isolated strains to commonly used antibiotics.

MATERIALS AND METHODS

Collection of specimens

A complete of 278 samples were collected from October 2018 to February 2019. One hundred fifty three (55%) from animals, of which (52.3%) swabs were from the environment of slaughters and (47.7%) samples were from milk of cow and buffalo collected by plastic sterile containers, from different areas in Basrah city (Abu-Sukhair, Slaughter of Basrah). One hundred twenty five (45%) samples were collected by sterile swabs with media. (52%) swabs from patients suffering from burns and (48%) swabs from diabetic foot wounds of both genera from Al- Fayhaa hospital, Basrah province. All specimens were labeled and transported within two hours by a sterile samples collection container to the laboratory then cultured in brain heart infusion broth (BHIB).

Culturing of specimens

The specimens were incubated at 37ºC for 24 hrs. The growth then transferred to MacConkey agar plates and incubated at 37°C for 24 hrs to distinguish between the fermenting and non-fermenting lactose bacteria. The colonies from primary cultures were further purified by re-culture into pseudomonas agar and incubated at 37ºC for 24 hrs.

Identification of *P. aeruginosa*

Preliminary identification of the pure colonies by Gram staining, biochemical tests: catalase test, oxidase test, and kliglar iron agar (12), Then the results were further confirmed with the VITEK 2 Kit.

Antimicrobial susceptibility test

According to NCCLS (13), The antimicrobial sensitivity of the identified isolates was detected by disc diffusion method of 13 different antibiotic with their indicated concentrations: Ampicillin (30µg), Amikacin (30µg), Cefotaxime (30µg), Ciprofloxacin (5µg), Gentamycin (10µg), Ceftazidim (30µg), Imipenem (10µg), Tetracycline (30µg), Trimethoprim (5µg), Meropenem (10µg), Levofloxacin (15µg), Nalidixic acid (30µg), Amoxicillin (30 µg), The Mueller-Hington (MH) agar plates were covered with *P. aeruginosa* growth after standardizing the inoculum turbidity with 0.5 McFarland standards(14). The diameters of inhibition zones were measured after 24 hrs with ruler.

Detection of *P. aeruginosa* by molecular technique

DNA extraction and purification

The DNA of *P. aeruginosa* isolates was extracted and purified according to the company instructions (Geneaid, Lot No. FC26108-G/ Korea).

DNA amplification by PCR:

Amplification of *16S rRNA* gene was conducted using specific primers table 1, achieved on thermocycler instrument (Techne, UK). The total volume of the reaction mixture was 50 µl (Table 2) with the reaction condition (Table 3).

Agarose gel electrophoresis

Agarose gel was prepared according to Sambrook *et al*., (15). The agarose gel was prepared in concentration of (1.5%).

Table 1: Oligonucleotide primer sequence and PCR product size for *16S rRNA* gene amplification in *P. aeruginosa* isolates.

Gene	Primer Sequence	MW (bp)
16S rRNA	F: 5'-GACGGGTGAGTAATGCCTA-3 R: 5'-CACTGGTGTTCCTTCCTATA-3'	618

Table 2: Mixture of PCR (50 µl) for amplification of *16S rRNA* gene in *P. aeruginosa* isolates.

Reagents	Reaction Volume (50 µl)
F Primer	2
R Primer	2
DNA template	10
Nuclease-free water	36
Total	50

Table 3: The conditions of PCR assay for amplification of *16s rRNA* gene in *P. aeruginosa* isolates.

Steps	Temperature (C°)	Time (min)	No. of Cycles
Initial denaturation	95	2	1
Denaturation	95	1	30
Annealing	55	1	
Extension	72	1	
Final extension	72	5	1

RESULTS

In the present study, out of 278 tested samples, (55%) samples from animals, of which (52.3%) were from the environment of slaughters and (47.7%) samples were from milk of cow and buffalo. (45%) swabs from patients, of which (48%) samples were from diabetic foot patients and 65 (52%) samples were from patients suffering from burns in hospitals of Basrah province (Table 4 and 5).

Table 4: Prevalence of *P. aeruginosa* isolated in the animals and environment of slaughter.

Source of samples	No. of Sample	No. of *P. aeruginosa*	(%)
Environment of slaughter	80	12	24
cow's and Buffalo milk	73	10	20
Total	153	22	44
X2=0.053			

No Significant different at p > o.o5

Table 5: Prevalence of *P. aeruginosa* isolated from burns wounds and diabetic foot patients.

Sample Source	No. of Sample	No. of *P. aeruginosa*	(%)
Diabetic foot swab	60	9	18
Burns swab	65	19	38
Total	125	28	56
X2=3.635			

No Significant different at p > o.o5

The present study results showed that from 278 collected samples only (55 %) were non lactose fermenters when samples plated on MacConkey agar distributed in (56.3%) samples from slaughter, (49.3%) milk samples, (70%) samples from burns and (45%) samples from diabetic foot patients. Samples were plated on the selective pseudomonas agar medium to isolate *Pseudomonas spp*., which had the ability to grow on this medium. *P. aeruginosa* produced non fluorescent bluish pigment (pyocyanin) which diffused into the agar. Only 50 (32.5%) samples out of (55%) showed positive result on pseudomonas agar distributed in (24%) samples from slaughters, (20%) samples from milk, (18%) samples from diabetic foot and (38%) samples from burns. Forty-six isolates of these positive pseudomonas were identified using VITEK 2 Kit. The result showed that (54%) samples identified as *P. aeruginosa*, Table (6).

Identification of *P. aeruginosa*

Identification of *P. aeruginosa* was achieved by examining bacterial culture (MacConky agar and Pseudomonas agar), microscopically characteristic (Gram's staining) and their biochemical reactions (catalase, oxidase, kliglar iron tests) (Table 6).

Table 6: Biochemical tests of *P. aeruginosa* isolates from different sources.

Sample type	Oxidase (%)	Catalase (%)	Kliglar iron (%)	VITEK2 (%)
Cow's and buffallo milk	12/12 (100)	12/12 (100)	3/12 (25)	8/16 (50)
Slaughters	12/12 (100)	12/12 (100)	4/12 (33.3)	1/4 (25)
Burns wound	18/18 (100)	18/18 (100)	9/18 (50)	10/16 (62.5)
Diabetic foot wounds	27/27 (100)	27/27 (100)	10/27 (37)	6/10 (60)
Total	100/100	100/100	38/100	54/100
			X2 = 2.086	X2 = 2.068

No significant difference at p>0.05

VITEK2 test showed the highest percentage. (62.5%) of isolates from burn samples, (60%) of isolates from diabetic wounds followed by cow's and buffalo milk (50%), and (25%) from slaughter.

Antibiotic susceptibility testing of *P. aeruginosa* isolated from animals.

This study demonstrated that the results of 13 various antibiotics by disc diffusion method to wards to 31 *P. aeruginosa* isolates, using Kirby-Bauer disk diffusion method that is determined through measuring the diameter of inhibition zones around antibiotic discs affording to CLSI (2007).

The results showed that 100% of *P. aeruginosa* isolates were resistant to Ampicillin, Amoxicillin and Cefotaxime, 97% were resistant to Ceftazidime and Nalidixic acid, 77.4% were resistant to Meropenem, 42% were resistant to each of Ciprofloxacin and Trimethoprim, whereas 87% of *P. aeruginosa* isolates were sensitive to Imipenem, 71% were sensitive to Amikacin, 67.7% were sensitive to Gentamicin, 61% we

re sensitive to Tetracycline, and 58% were sensitive to Levofloxacin, (Figure 1) (Table 7).

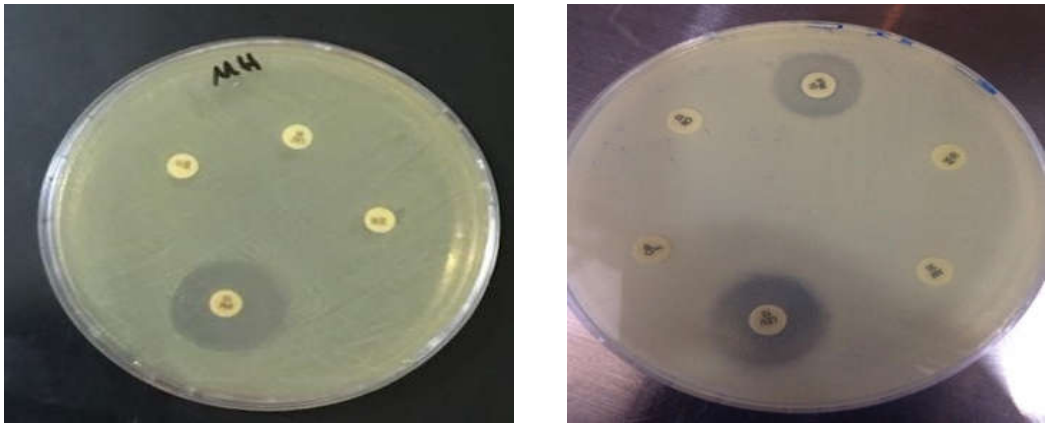

Figure 1: Inhibition zones of some antibiotics against *P. aeruginosa* by disc diffusion method.

Table 7: Antibiotic susceptibility test to 13 antibiotics by disc diffusion test against 31 *P. aeruginosa* isolates.

No.	Antibiotic	Con. (mcg)	No. of isolates		
			R (%)	I (%)	S (%)
1	Amoxicillin (AX)	(30)	31 (100)	-	-
2	Amikacin (AK)	(30)	7 (22.5)	2 (6.5)	22 (71)
3	Ampicillin (AM)	(30)	31 (100)	-	-
4	Ceftazidime (CAZ)	(30)	30 (97)	-	1 (3)
5	Cefotaxime (CTX)	(30)	31 (100)	-	-
6	Ciprofloxacin (CIP)	(5)	13 (42)	4 (13)	14 (45)
7	Gentamicin (CN)	(10)	9 (29)	1 (3.2)	21 (67.7)
8	Imipenem (IPM)	(10)	3 (9.7)	1 (3.2)	27 (87)
9	Levofloxacin (LEV)	(15)	6 (19.4)	7 (22.6)	18 (58)
10	Meropenem (MEM)	(10)	24 (77.4)	-	7 (22.6)
11	Nalidixic acid (NA)	(30)	30 (97)	1 (3)	-
12	Tetracycline (TE)	(30)	8 (25.8)	4 (12.9)	19 (61)
13	Trimethoprim (TMP)	(5)	13 (42)	8 (25.8)	10 (32.3)

***R: Resistant; I: Intermediate; S: Susceptible.**

Molecular identification by PCR assay:

Detection of *16S rRNA* gene in animals.

Nineteen *P. aeruginosa* isolated from animals were subjected to PCR assay to detect *16s rRNA* gene, Eighteen isolates exhibited clear bands of approximately 618bp which corresponding for detection of *P. aeruginosa* strains.

Detection of *16S rRNA* gene in patients.

Nineteen *P. aeruginosa* isolates from patients were subjected to PCR assay to detect *16S rRNA* gene. The results showed that 100% isolates exhibited clear bands of approximately 618bp which corresponding for identification of *P. aeruginosa* strains (Figure. 2).

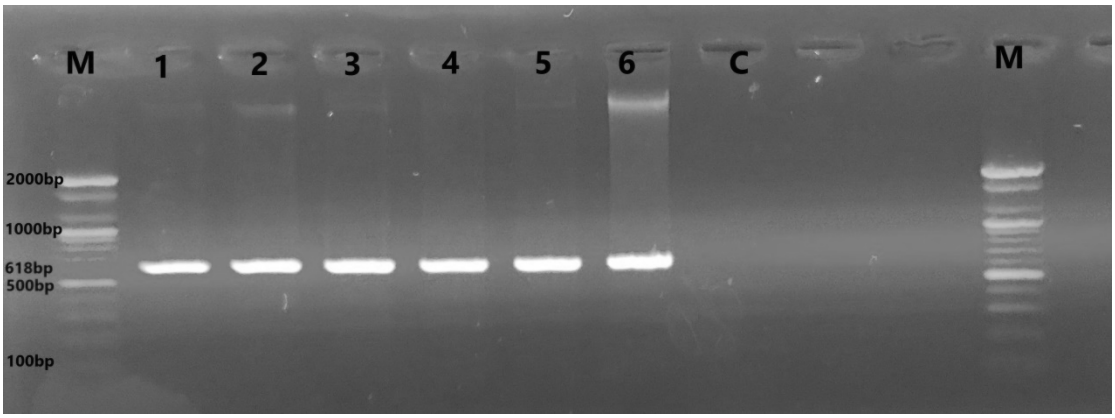

Figure 2: Agarose gel electrophoresis of PCR-amplified *16S rRNA* gene of *P. aeruginosa* isolates. Lane M: DNA marker; Lane 1-6: *16S rRNA* gene (618bp); and Lane C: Negative control.

DISCUSSION

In the present study, Brain heart infusion broth was applied as supplement medium whereas MacConkey agar and pseudomonas medium were applied to detect and isolate *P. aeruginosa*. (55%) *P. aeruginosa* isolates were obtained from 278 from animal samples, of which (52.3%) swabs were from the environment of slaughters and (47.7%) samples were from milk of cow and buffalo collected in sterile containers. (45%) samples from patients, of which (52%) swabs were from patients suffering from burns and (48%) swabs were from diabetic foot patients in hospitals of Basrah province. Moreover, out of 18 *P. aeruginosa* cases, 8.6 % were under Wagner's Grade III ulcer. The percentage of *P. aeruginosa* in the

present study were the topmost infections in comparison with the rate in the study of Hutschinson and McGuckin (16). The uppermost ratio of *P. aeruginosa* was obtained from burn samples (27.7%) This agreed with the study of R'auf (17) who documented the uppermost ratio of *P. aeruginosa* through burn infection then followed by wound samples. This is for the reason that it was the 3rd most-communal pathogen related with infections which acquired in hospitals (18). Regarding the isolation of *Pseudomonas* from burns, the results come in an agreement with the study of Abbas and his colleagues (19) who revealed that *Pseudomonas* spp. gave a positive growth in 71% of burns samples.

The main organisms which cause milk spoilage were *Pseudomonas* species through the production of lipolytic and proteolytic enzymes (20). No difference in smell or appearance between milk contaminated with disease-causing bacteria from non-contaminated milk (21). So, overall of 73(47.7%) samples of raw milk were analyzed for the detection of *Pseudomonas* spp. The number of *Pseudomonas* spp. was 12 isolates. 10(13.6%) isolates were recognized as *P. aeruginosa* by biochemical identification. The result were more than to those described by El- Zubeir and El-Owni (22) in Sudan who isolated *P. aeruginosa* in a percentage of 6.6% from raw milk samples. However, the results showed less prevalence rate of *pseudomonas* compared to that was documented by Jyoti and his assistance (23) in India who showed that *P. aeruginosa* was isolated in a percentage of 11.11% of the raw milk samples and higher than those found by Hussein (24) in Iraq who isolated *P. aeruginosa* in a percentage of 3.7% from raw milk samples. The variance among the current and the earlier studies may be attributed to the technique of sampling, treatment of samples and the kinds of media. Bacterial identification was directed based on morphological and biochemical tests (25).

The present study results revealed high resistance of *P. aeruginosa* isolates against Ampicillin, Amoxicillin, Cefotaxime, Ceftazidime, Ciprofloxacin, Meropenem, Nalidixic acid and Trimethoprim, while they were sensitive to Imipenem, Amikacin, Gentamicin, Tetracycline, and Levofloxacin. These results are in accordance with those of other studies conducted by Fallah and his assistance (26) in Tehran, Abbas and his colleagues (19) in Iraq, but it contradict with the study of Odumosu and his assistance (27) in Nigeria. On the other hand, this study showed the percentage of antibiotic resistance among *P. aeruginosa* isolated from burn patients and altered clinical samples, is also low. It would hence appear that these antibiotics are currently the only antibiotics that are prescribed for burn patients (28). Since *P. aeruginosa* is generally a multidrug resistant organism, this agrees with the results that obtained by NCCLS, (13) who found that 66% of *pseudomonas* strains are resistant to

Cefotaxime However, *Pseudomonas* were high sensitive for Imipenem. Carbapenems are efficient antibiotics against extended-spectrum β-lactamase–producing *P. aeruginosa*. However, there are many potential health risks associated with carbapenem-resistant *P. aeruginosa* (29).

The major issue confronting the treatment of *P. aeruginosa* diseases is the reputation of the pathogen to have a wide array of resistance determinants. *P. aeruginosa* can create resistance to antibiotics either through the expression and/or work of chromosomally encoded mechanisms as a result of mutation or the procurement of resistance genes on portable genetic components (plasmids) (30). The present study results showed increases in the rate of the first and second generation of β-lactam resistance, where these antibiotics now not active in the treatment of *Pseudomonas* spp. infections.

The present study demonstrated that 37 out of 38 *P. aeruginosa* isolated from animals and patients showed accurate bands of approximately 618bp corresponding for identification of *P. aeruginosa* strains. These results are in deal with other studies conducted by (31) in France who found that the *16S rRNA* documents analysis revealed that *P. aeruginosa* sequences were existing in all environments but were most redundant in samples from human and animals. Finally, PCR technique is a perfect molecular technique used for identification in microbiology.

عزل والتشخيص الجزيئي لبكتيريا *Pseudomonas aeruginosa* متعددة المقاومة للمضادات الحيوية المعزولة من الحيوانات والمرضى في محافظة البصرة

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الخلاصة

خلال الفترة من تشرين الاول ٢٠١٨ الى شباط ٢٠١٩ ، جمعت ٢٧٨ عينة من الحيوانات والمرضى، (٥٥ %) عينة من الحيوانات والتي توزعت الى (٥٢,٣%) مسحة من محيط المجزرة و(٤٧,٧%) عينة حليب من الابقار والجاموس جمعت بواسطة الحافظات المعقمة . اوضحت النتائج بأن بكتريا *Pseudomonas* موجودة في (٤٤%) بعد زرعها على وسط Pseudomonas agar موزعة على (٢٤%) عينة من المجزرة و (٢٠%) عينة حليب . (٤٥%) عينة كانت من المرضى مقسمة بين (٤٨%) مسحة كانت من مرضى القدم السكري و (٥٢%) مسحة كانت من مرضى الحروق في مستشفيات محافظة البصرة، اظهرت النتائج أن بكتريا Pseudomonas موجودة في (٥٦%) عينة موزعة في (١٨%) عينة من مرضى القدم السكري و(٣٨%) عينة من مرضى الحروق بعد زرعها على وسط Pseudomonas

agar. شخصت ٤٦ عينة موجبة باستخدام VITEK 2 kit حيث ظهر ٢٥ (٥٤ %) عزلة *P. aeruginosa*. تم اختبار ٣١ عزلة تجاه ١٣ نوع من المضادات الحيوية بطريقة انتشار القرص، جميع العزلات ابدت مقاومة لثمانية منها، اي انها اظهرت نمط المقاومة المتعددة للمضادات الحيوية. تم اختبار ٣٨ عينة للكشف عن وجود ١٦S rRNA بواسطة تقنية تفاعل البلمرة المتسلسل PCR موزعة بين ١٩ عينة من مصدر حيواني و١٩ عينة من الانسان ،حيث اظهرت النتائج حزم واضحة على ٦١٨ قاعدة نيتروجينية موزعة بين ١٩/١٨ عينة موجبة من المصدر الحيواني و١٩/١٩ موجبة لعينات الانسان.

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