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Research Article

Evaluating the antibacterial and biofilm activity of *Pseudomonas aeruginosa* isolated from dog's wound infections

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Abstract: This study aimed to evaluate the antibacterial and biofilm activity of *P. aeruginosa* isolated from dog wound infections. One hundred samples were collected from February to December 2022 including 28 male and 72 female. *Pseudomonas aeruginosa* isolates were identified by cultural characteristics, biochemical tests. Thirty *P.aeruginosa* isolates (30%) were identified. The biofilm producing ability of *P. aeruginosa* isolates was evaluated by using pre-sterilized 96 well polystyrene microtiter plates. The results showed that out of 20 *P. aeruginosa*, 1 (5%) was non-adherent and weak, 18 (90%) strong biofilms. The susceptibility test of *P. aeruginosa* isolates toward 10 different antibiotics were carried out by Kirby-Bauer method. The results revealed that all isolates were resistant to Ciprofloxacin (CIP), Cefotaxime (TX) and Gentamycin (CN) 43.33%,follow by 46.66% resistant to Levofloxacin (LEV), 63.33% to Ceftraxone (CRO), 50% to Azithrofomycin(AZM), 96.66% *P. aeruginosa* to Ampicillin (AM) and Erythromycin (E), 26.66% to Ceftaziaime (AZ), and 6.66% to Amikacin (AK). The results of the current study showed that the majority of isolates with high resistance to antibiotics had the ability to form biofilms.

Keywords: *Pseudomonas aeruginos*a, Antibacterial, Biofilm activity.
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Introduction

Pseudomonas aeruginosa is a gram-negative bacteria, saprophytic and opportunistic pathogen infecting both humans and animals (Alhazmi 2015). In animals, it causes mastitis, metritis, pneumonia, dermatitis, and enteritis (Quinn et al. 1998). *Pseudomonas aeruginosa* has been reported to have high levels of resistance to commonly used antimicrobial agents such as penicillins, tetracyclines (Vingopoulou et al. 2018). It possesses many virulence factors such as enzymes that degrade effector molecules of the immune system and elements essential to the host's cellular and tissue structure (Marques 2015). Antibiotics are commonly used as therapy and to control microbial infections in humans and animals. However, the widespread use of an antibiotic may trigger the rise of antibiotic resistance (Kempf et al. 2015; Hayati et al. 2019). The increase of multidrug resistance in gram-negative bacteria is now a serious challenge (Exner et al. 2017). Cases of multidrug resistance (MDR) have been reported in *P. aeruginosa* isolates against more than three types of antibiotics (Hayati et al. 2019). Notably, the prevalence of antibiotic resistance is increasing among Enterobacteriacea, including *P. aeruginosa* strains isolated from animals (Wu et al. 2019) due to the extensive use of antibiotics in humans, veterinary medicine, and agricultural practice during the last few decades. Nosocomial infections caused by *P. aeruginosa* have been a health concern, mostly due to the high resistance to certain antibiotics (Rosenthal et al.

Table 1. Antibiotic discs used in the present study.

No.	Antibiotics discs	Code	Disc potency (µg/disc)	Company	Origin
1	Levofloxacin	LEV	5	Bioanalyse	Turkey
2	Cefotaxime	TX	30	CONDA	Spain
3	Ceftraxone	CRO	10	CONDA	Spain
4	Azithrofomycin	AZM	15	Bioanalyse	Turkey
5	Ciprofloxacin	CIP	30	Bioanalyse	Turkey
6	Gentamycin	CN	10	Bioanalyse	Turkey
7	Ampicillin	AM	25	Bioanalyse	Turkey
8	Ceftaziaime	AZ	30	CONDA	Spain
9	Erythromycin	E	10	Bioanalyse	Turkey
10	Amikacin	AK	10	Bioanalyse	Turkey

2016). Biofilm is an important player in *P. aeruginosa* drug resistance, because the dense extracellular matrix of biofilms reduces the efficacy of detergents and antibiotics (Oliver et al. 2015). The formation of biofilm is induced and regulated by numerous genes and environmental factors of which three are the most important, including many genes that are actively involved in the biofilm development and dispersal (Shaomin et al. 2019). Biofilm growth is a typical characteristic of bacteria that allows for improved survival under adverse conditions such as low nutritional levels or the presence of antimicrobial agents (Schroeder et al. 2017). *Pseudomonas aeruginosa* biofilm matrix primarily encompasses polysaccharides, extracellular DNA (eDNA), proteins, and lipids. The three exopolysaccharides of Psl, Pel and alginate are tremendously involved in surface attachment, formation and the stability of biofilm architecture (Oluyombo et al. 2019). Therefore, this study aimed to evaluate the antibacterial and biofilm activity of *P. aeruginosa* isolated from dog wound infections.

Materials and methods

This study was conducted in Baghdad Province and the samples were collected from February to December 2022 from Al-Shifa clinic, Mila, dr. Shefa Badrana, Adhamiya, and Shabaad veterinary clinics in the Adhamiya area, and Al-Farah and dr. Muhannad veterinary clinics in Al-Bayaa. This study included a total of 100 (28 male and 72 female) of dog's wound infection. The samples were collected and transported immediately for bacterial culturing, antibiotic susceptibility and biofilm formation of *P. aeruginosa* to Baghdad University and Al- Nahrain University laboratories.

Isolation: After transporting to the laboratory, the swabs were streaked on MacConkey agar, blood agar, and nutrient agar, and incubated at 37°C for 24 hours. After incubation, samples were subcultured on *Pseudomonas* chromogenic agar.

Identification:

A. Colonial morphology: Colonies of the bacterial isolates that culture on blood agar, MacConkey agar and nutrient agar, and *Pseudomonas* chromogenic agar were described according to their shape, color, diameter, and odor (Branson 1972).

B. Microscopical examination: It was performed by transferring one isolated colony to a microscope slide, fixed well and stained with Gram stain. Gram reaction, the shape of the cell, and arrangement were observed (Exner et al. 2017).

C. Biochemical tests: Oxidase test, Catalase test, Urease test, protease enzyme production on skim milk agar, Lactose fermentation on MacConkey, and Growth on Nutrient agar at 42˚C were examined (Davis et al. 1990).

Antibiotic susceptibility test: Ten antibiotics (Table 1) were selected to use in the present study based on the Clinical and Laboratory Standards Institute (CLSI 2022). According to the agar disc diffusion method (Bauer et al. 1966), colony was taken by a sterile loop from a culture grown for 24-hour on nutrient agar medium and placed in a plain tube

Table 2. Evaluation of biofilm formation by microtiter plate method.

Optical density (OD)	Adherence
OD ≤ ODc	Non–adherent
2ODc ≥ OD > ODc	Weak
4ODc ≥ OD > 2ODc	Moderate
OD >4ODc	Strong
Cut off value (ODc) = average OD of negative control + (3 *Standard Deviation).	

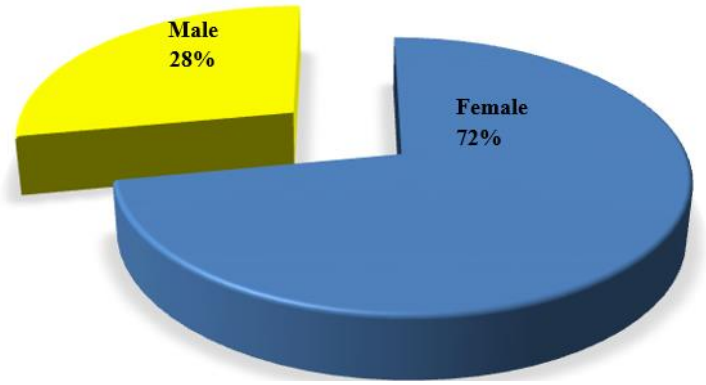


Fig.1. Percentage of *Pseudomonas aeruginosa* isolated from male and female dog's wound infections.

containing 5ml of the brain heart infusion broth to make the bacterial suspension and compared with a turbidity of McFarland standards solution. The bacterial suspension was taken and spread by sterile cotton swab onto the Muller Hinton agar medium, and the dishes were left to dry in the incubator without turning for 5min.

Antibiotics disc concentrations were spread using 10 antibiotics in the dish and were placed using sterile forceps with alcohol and flam, with a distance of 24mm between one disc to another. Then they gently pressed on the surface of the disc to ensure contact with the surface of the agar. The dishes were incubated upside down at 37°C for 24 hours. The inhibition diameters were determined as the sensitive, intermediate and resistant bacteria depending on the inhibition zones (CLSI 2022).

Biofilm formation assay for *P. aeruginosa* isolates: A total of 20 clinical isolates of *P. aeruginosa* were screened for their ability to form biofilm via the microtitration plate's method (Zhang et al. 2016) with some modifications as follows. *Pseudomonas aeruginosa* isolated from fresh agar plates were inoculated in 5mL of brain heart infusion (BHI) with 2% sucrose, and incubated for 24 h at 37ºC (Mathur et al. 2006). Twenty microliters of bacterial suspension from each isolate (equivalent to 0.5 Mcfarland standard) was added and used to inoculate microtiter wells containing 180µl from the brain heart infusion broth to each well of the microplate, and incubated at 37°C for 24 hours. After incubation, the plate was washed three times with normal saline to eliminate non-adherent cells. To fix the adhered cells, 200µl of 99% methanol per well was added for 15min. The plate was dried for 30min at room temperature. Then, 200µl of 1% crystal violet was added for 15min. After eliminating the dye solution and washing it with sterile distilled water, the attached dye was solubilized with 96% ethanol, and the optical density was read in a micro-titer plate reader at 630nm. The biofilm formation was evaluated as described in Table 2.

Results and Discussion

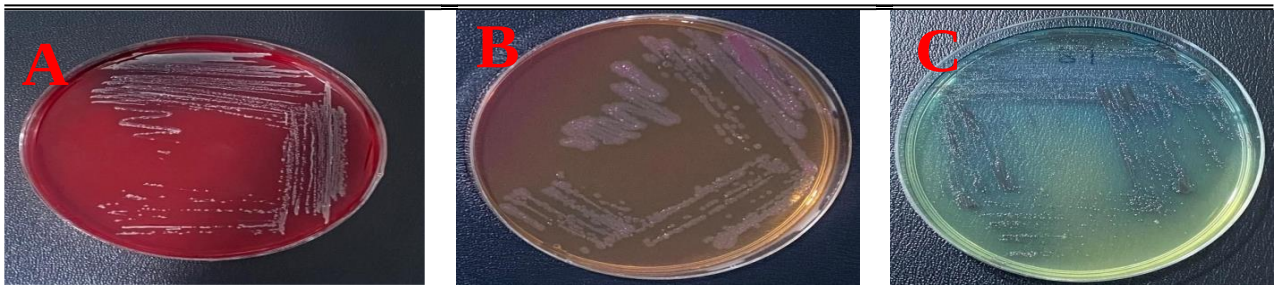
Isolation and identification of *P. aeruginosa*: This study included a total of 100 samples (28 male and 72 female) from dog's wound infections (Fig. 1). Only 30 samples were gave positive culture that

Table 3. Antibiotic susceptibility tests to select the most resistant isolate.

Antibiotic discs	Code	Disc potency (µg/disc)	*Pseudomonas isolates*									
			1	2	3	4	5	6	7	8	9	10
Levofloxacin	LEV	5 µg	R	R	15	R	R	R	18	R	R	R
Cefotaxime	TX	30 µg	R	R	R	R	R	R	R	R	R	14
Ceftraxone	CRO	10 µg	R	R	R	R	R	R	R	R	R	R
Azithrofomycin	AZM	15 µg	R	R	R	R	R	R	R	R	R	R
Ciprofloxacin	CIP	30µg	R	R	20	R	16	R	20	R	R	R
Gentamycin	CN	10 µg	R	R	R	R	R	R	15	R	R	R
Ampicillin	AM	25 µg	R	R	R	R	R	R	R	R	R	R
Ceftaziaime	AZ	30 µg	14	R	R	R	12	10	R	12	R	13
Erythromycin	E	10 µg	R	R	R	R	R	R	R	R	R	R
Amikacin	AK	10 µg	16	15	20	15	17	15	R	15	14	10
Antibiotic discs	Code	Disc potency (µg/disc)	*Pseudomonas isolates*									
			11	12	13	14	15	16	17	18	19	20
Levofloxacin	LEV	5 µg	R	R	R	R	R	24	25	23	30	24
Cefotaxime	TX	30 µg	R	15	R	20	R	33	18	14	45	24
Ceftraxone	CRO	10 µg	R	R	R	R	R	20	R	R	42	28
Azithrofomycin	AZM	15 µg	R	R	R	R	R	26	28	17	16	25
Ciprofloxacin	CIP	30µg	R	R	R	R	R	25	26	25	33	29
Gentamycin	CN	10 µg	R	R	R	16	R	4	20	16	30	23
Ampicillin	AM	25 µg	R	R	R	R	R	R	R	R	34	R
Ceftaziaime	AZ	30 µg	R	12	R	15	15	23	14	16	35	10
Erythromycin	E	10 µg	R	R	R	R	R	R	R	R	10	R
Amikacin	AK	10 µg	15	18	R	16	17	20	18	19	24	29
Antibiotic discs	Code	Disc potency (µg/disc)	*Pseudomonas isolates*									
			21	22	23	24	25	26	27	28	29	30
Levofloxacin	LEV	5 µg	20	18	19	19	20	26	22	22	20	R
Cefotaxime	TX	30 µg	23	24	24	22	26	13	11	R	23	18
Ceftraxone	CRO	10 µg	27	25	19	14	32	R	18	R	21	17
Azithrofomycin	AZM	15 µg	18	19	17	17	20	25	26	20	13	13
Ciprofloxacin	CIP	30µg	24	25	24	24	25	28	26	24	24	R
Gentamycin	CN	10 µg	16	16	15	19	13	14	11	12	18	14
Ampicillin	AM	25 µg	R	R	R	R	R	R	R	R	R	R
Ceftaziaime	AZ	30 µg	10	10	14	11	11	R	10	13	14	15
Erythromycin	E	10 µg	R	R	R	R	R	R	R	R	R	R
Amikacin	AK	10 µg	18	18	18	19	20	18	17	19	21	18

identified using morphological characteristics and biochemical tests. On blood agar, they developed as large flat bacterial colonies with a grape-like odour and after 24 hours of incubation the majority of bacterial isolates produced hemolysis (B). *Pseudomonas aeruginosa* was identified on MacConkey agar as tiny round convex colonies with a pale yellowish color (lactose non-fermenting). *Pseudomonas* chromogenic agar was employed and it is easily identified in this media by the green colony medium colour, which changes from pale or neutral to pale green (Alfred, 2005). Figures 2-5 show the growth of *P. aeruginosa* isolates on MacConkey agar, *Pseudomonas* chromogenic agar and blood agar. *Pseudomonas aeruginosa* will appear as reddish/pink rods. This indicates that they are Gram-negative bacteria showing that they are unable to retain the primary stain (crystal violet).

Antibiotic susceptibility of *P. aeruginosa*: The results of antibiotic susceptibility of *P. aeruginosa* showed that 43.33% of isolates were resistant to Ciprofloxacin (CIP), Cefotaxime (TX) and Gentamycin (CN), followed by 46.66% to Levofloxacin (LEV), 63.33% to Ceftraxone (CRO), 50% to Azithrofomycin (AZM), 96.66% *P* to Ampicillin (AM) and Erythromycin (E), 26.66% to Ceftaziaime (AZ), and 6.66 % to Amikacin (AK) (Figs. 6-7). The current study found that *P. aeruginosa* is developing resistance to routinely used antibiotics as a result of overuse of medicines. Mahdi et al. (2019) mentioned that in *P. aeruginosa*, the highest resistance are found to Ampicillin



P. aeruginosa on Blood agar

P. aeruginosa on MacConkey agar

P. aeruginosa on *Pseudomonas* chromogenic agar

Fig.2. Growth of *Pseudomonas aeruginosa* isolates A: Blood agar, B: MacConkey agar, and C: *Pseudomonas* chromogenic agar.

Fig.3. Urease enzyme production.

Fig.4. Protease enzyme production on skim milk agar.

Fig.5. Growth on Nutrient agar at 42˚C.

(81.1%), then moderate resistance to Ciprofloxacin (56.7%), and the lowest level to Amikacin (6.66%) which agreed with the results of the current study. While another study disagrees with our results regarding the level resist to Ciprofloxacin (14%), and Amikacin (2%) (Mahdi et al. 2018).

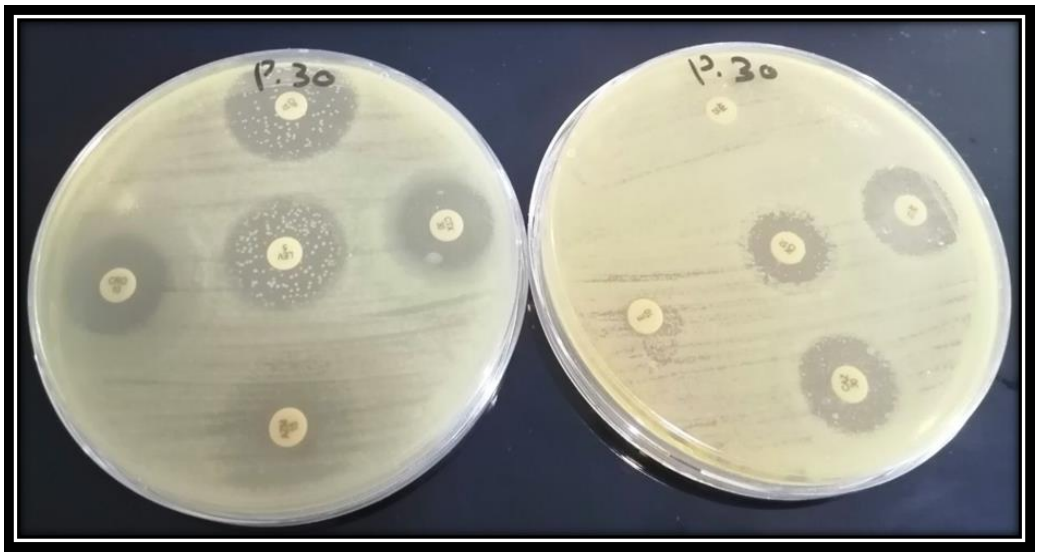


Fig.6. Zone appearance of antibiotic susceptibility test on MHA.

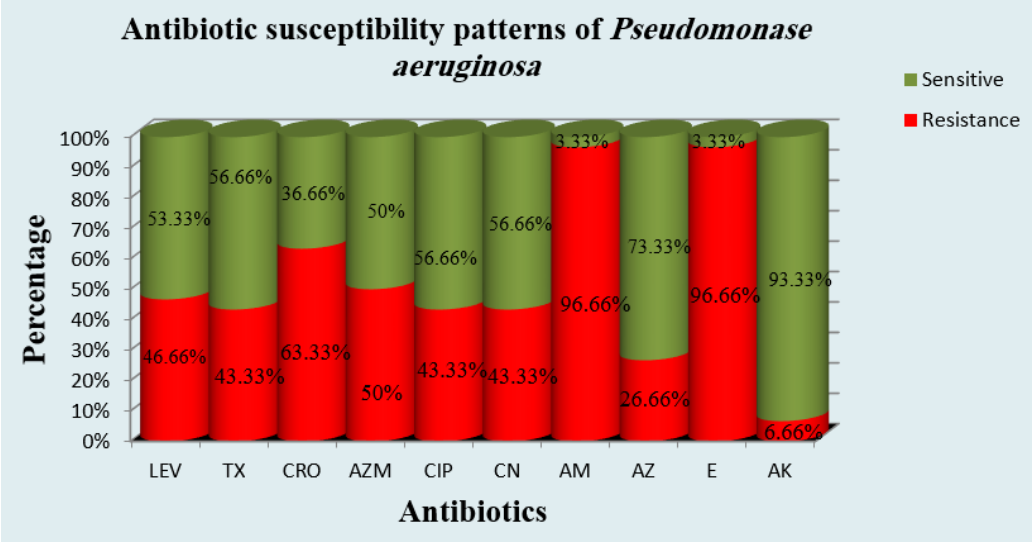


Fig.7. Antibiotic susceptibility of *P. aeruginosa* isolates for 10 antibiotics. Abbreviations: Levofloxacin (LEV), Cefotaxime (TX), Ceftraxone (CRO), Azithrofomycin (AZM), Ciprofloxacin (CIP), Gentamycin (CN), Ampicillin (AM), Ceftaziaime (AZ), Erythromycin (E), and Amikacin (AK).

Biofilm formation by *P. aeruginosa*: In this study, the ability of *P. aeruginosa* biofilm-producing isolates was evaluated using pre-sterilized 96-well polystyrene microtiter plates (Azeredo et al. 2017) (Fig. 8). The absorbance values represented the intensity of the biofilm thickness that formed by the studied isolates on the surface of the microtiter well. The results were categorized into four groups viz. non-adherent, weak, moderate, and strong based on limits. (Table 3), the present study declared that out of 20 *P. aeruginosa*, one isolate formed a weak biofilm, one isolate formed a moderate biofilm, whereas 18 isolates formed a strong biofilm.

The biofilm intensity based on estimated cutoff value of *P. aeruginosa* isolates summarized in Table 4. This result is an agreement with other studies regarding *P. aeruginosa* biofilm formation. Mahdi et al. (2018) stated that the majority of *P. aeruginosa* isolates (70%) were moderate biofilm producers. Mahdi (2021) revealed that out of 16 *P. aeruginosa* isolates, 12-formed weak biofilm; while only four isolates developed a mild biofilm. Mahdi et al. (2019)

Table 4. Evaluation of biofilm formation by microtiter plate method.

No.	Optical density (OD)	Optical density control (ODC)	OD ≤ ODc Non–adherent Number of isolates 0 Percentage 0%	2ODc ≥ OD > ODc Weak Number of isolates 1 Percentage 5%	4ODc ≥ OD > 2ODc Moderate Number of isolates 1 Percentage 5%	OD >4ODc Strong Number of isolates 18 Percentage 90%
1	0.255		0.255≤0.047	0.094 ≥0.255>0.047	0.188≥0.255>0.094	0.255>0.188
2	0.380		0.380≤0.047	0.094 ≥0.380>0.047	0.188≥0.380>0.094	0.380>0.188
3	0.358		0.358≤0.047	0.094 ≥0.358>0.047	0.188≥0.358>0.094	0.358>0.188
4	0.33		0.33≤0.047	0.094 ≥0.33>0.047	0.188≥0.33>0.094	0.33>0.188
5	0.46		0.46≤0.047	0.094 ≥0.46>0.047	0.188≥0.46>0.094	0.46>0.188
6	0.316		0.316≤0.047	0.094 ≥0.316>0.047	0.188≥0.316>0.094	0.316>0.188
7	0.589		0.589≤0.047	0.094 ≥0.589>0.047	0.188≥0.589>0.094	0.589>0.188
8	0.466		0.466≤0.047	0.094 ≥0.466>0.047	0.188≥0.466>0.094	0.466>0.188
9	0.266		0.266≤0.047	0.094 ≥0.266>0.047	0.188≥0.266>0.094	0.266>0.188
10	0.369		0.369≤0.047	0.094 ≥0.369>0.047	0.188≥0.369>0.094	0.369>0.188
11	0.203		0.203≤0.047	0.094 ≥0.203>0.047	0.188≥0.203>0.094	0.203>0.188
12	0.235		0.235≤0.047	0.094 ≥0.235>0.047	0.188≥0.235>0.094	0.235>0.188
13	0.261		0.261≤0.047	0.094 ≥0.261>0.047	0.188≥0.261>0.094	0.261>0.188
14	0.452		0.452≤0.047	0.094 ≥0.452>0.047	0.188≥0.452>0.094	0.452>0.188
15	0.307		0.307≤0.047	0.094 ≥0.307>0.047	0.188≥0.307>0.094	0.307>0.188
16	0.407		0.407≤0.047	0.094 ≥0.407>0.047	0.188≥0.407>0.094	0.407>0.188
17	0.210	0.047	0.210≤0.047	0.094 ≥0.210>0.047	0.188≥0.210>0.094	0.210>0.188
18	0.483		0.483≤0.047	0.094 ≥0.483>0.047	0.188≥0.483>0.094	0.483>0.188
19	0.494		0.494≤0.047	0.094 ≥0.494>0.047	0.188≥0.494>0.094	0.494>0.188
20	0.422		0.422≤0.047	0.094 ≥0.422>0.047	0.188≥0.422>0.094	0.422>0.188

Cut off value = 0.047 (defined as the Mean of control OD630 plus 3 Standard deviation).

Fig.8. Biofilm formation of *P. aeruginosa* isolates.

also revealed that only 16% of *P. aeruginosa* isolates were strong biofilm producers; while 51% and 32% of the isolates were moderate and weak producers, respectively. The results of the current study showed that the majority of isolates with high resistance to antibiotics had the ability to form biofilms (Alwan 2020). The results of the current study showed that the majority of isolates have high resistance to antibiotics with the ability to form biofilms.

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Table 5. Relationship between antibiotic susceptibility and biofilm formation of *Pseudomonas aeruginosa* isolated from dog's wound infections.

Samples	Antibiotic susceptibility	Biofilm formation
1	Resistance	0.255
2	Resistance	0.380
3	Resistance	0.358
4	Resistance	0.33
5	Resistance	0.46
6	Resistance	0.316
7	Resistance	0.589
8	Resistance	0.466
9	Resistance	0.266
10	Resistance	0.369
11	Resistance	0.203
12	Resistance	0.235
13	Resistance	0.261
14	Resistance	0.452
15	Resistance	0.307
16	Sensitive	0.407
17	Sensitive	0.210
18	Sensitive	0.483
19	Sensitive	0.494
20	Sensitive	0.422

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