Investigation of Antimicrobial Resistance, Biofilm Production, Biofilm Associated Virulence Genes and Integron Genes of *Pseudomonas aeroginosa* Isolates Obtained from Animal Clinical Samples

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ABSTRACT

The results of antimicrobial treatment in *Pseudomonas aeroginosa* infections may depend not only on the antibiotic susceptibility of the etiologic agents, but also on the biofilm production and integron carrying capabilities of the bacteria. This study aimed to examine the relationship between antimicrobial resistance and biofilm production, biofilm related virulence genes, integron genes carried by *P. aeroginosa* isolates obtained from different animal clinical samples. A total of 67 *P. aeroginosa* isolates obtained from bovine mastitis, canine otitis and dermatitis cases were used as material. Bacterial identification was carried out using conventional methods. Qualitative (Congo Red Agar Test (CRA)) and quantitative (Microplate Test (MP)) methods were used to determine the phenotypic biofilm production capacity of the isolates. Polymerase chain reaction (PCR) was used to confirm the genus and species level identification of the isolates, and to identify biofilm-associated virulence genes and integron genes. The resistance patterns of the isolates against 12 antibiotics belonging to 6 antimicrobial families were examined using the disk diffusion method. Isolates resistant to at least three drug classes were defined as multi-drug resistant (MDR). The Chi-Square ($\chi 2$) test was used to compare the relationship between the MDR capacity of the isolates and biofilm formation, the prevalence of biofilm-associated virulence genes and the prevalence of integron genes. It was determined that 41.8% of the isolates as by qualitative method and 64.2% by the quantitative method were biofilm producers. The genes responsible for biofilm formation, *ppy*R, *psl*A and *pel*A, were detected in 94.0%, 83.6% and 65.7% of the isolates, respectively. 23.9% of the isolates carried the integron gene. Piperacillin tazobactam and ceftolozane tazobactam were found to be the most effective drugs against *P. aeruginosa* isolates studied. 28.4% of the isolates were MDR. As a result of this study, it was determined that MP was more effective than CRA in determining biofilm production phenotypically. While there was no significant relationship between MDR capacities with phenotypically biofilm formation, the prevalence of *ppy*R and *psl*A virulence genes, the relationship between the prevalence of *pel*A virulence gene and the presence of integron genes, was significant.

Key words: *Pseudomonas aeruginosa*; Multiple Antibiotic Resistance; Biofilm; Integron.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen that is commonly found in soil, water, sewage and mammalian intestines, often causing infections in immuno-compromised individuals (1, 2). This opportunistic pathogen is gaining increasing importance as a causative agent of many diseases in animals, including otitis media and pyoderma in dogs and mastitis in cows (3-6). While *P. aeruginosa* is

naturally resistant to many antimicrobial agents, it also has the capacity to rapidly develop resistance to many drugs during treatment. Therefore, the treatment of infections caused by *P. aeruginosa* is difficult due to both naturally occurring (intrinsic) and acquired (acquired) resistance in these bacteria (1, 2).

To survive, microorganisms have developed, various strategies such as slime production and development of biofilms to colonize and cause infections. A bacterial biofilm is a bacterial community embedded in a self-produced matrix (i.e. slime) (7). It is known that *P. aeruginosa* has the ability to form biofilms and that there are three important genes (*psl*A, *pela*, *ppy*R) that encode exopolysaccharides that play a role in biofilm formation (8, 9). The *psl*A and *pel*A genes play an important role in the formation of the carbohydrate-rich structure of the biofilm matrix. Mutations in these two genes cause a deficiency in the bacteria's ability to form biofilms. However, following the inactivation of the *ppy*R gene (the putative transmembrane protein), the *psl* operon is suppressed (8, 9).

Genetic elements such as plasmids, transposons and integrons play an important role in increasing the antibiotic resistance. Integrons cannot move; they contain integrase (*int*) genes that allow the insertion of antimicrobial resistance gene cassettes between highly conserved nucleotide sequences. Integrons can capture exogenous gene cassettes. Therefore, they play an important role in the horizontal spread of antibiotic resistance genes among bacteria. Based on the differences in the amino acid sequences of the encoded integrases, integrons have been divided into five classes. The class 1 integron with the widest variety of gene cassettes is the most clinically important and has a wide distribution among resistant strains (10).

P. aeruginosa can infect both animals and humans (1, 2). Therefore, it attracts the attention of both medical doctors and veterinarians. Although virulence genes (11) and integron genes of *P. aeruginosa* have been well researched in human medicine in Turkey (12), there are no studies in veterinary medicine on this subject. However, there are a limited number of studies investigating the antibiotic resistance status of *P. aeroginosa* isolates obtained from animal clinical samples (13, 14).

The success of antibacterial treatment in *P. aeroginosa* isolates may be related to the biofilm formation and integron bearing of the agent. In this study, it was aimed to examine the relationship between multiple antimicrobial resistance capacity and biofilm production, biofilm related virulence genes (*ppy*R, *psl*A and *pel*A) and integron genes (*int*1, *int*2, *int*3) carrying the status of *P. aeroginosa* isolates obtained from different animal clinical samples (canine otitis/pyoderma, bovine mastitis milk). It is suggested that as a result of this study, susceptibility patterns to antibiotics commonly used in clinics for the treatment of *P. aeruginosa* infections in our region will be determined. Thus, the findings are prososed both as an empirical antimicrobial drug selection guide and to contribute to antimicrobial resistance surveillance programs in animal health.

MATERIAL AND METHOD

Bacterial Isolates

In this study, 67 *Pseudomonas* isolates obtained from animal clinical samples (12 canine pyodermas, 17 canine otitis externa, 38 clinical/subclinical mastitis bovine milk samples) over a period of approximately one year (January-December 2020) were used as material. The *Pseudomonas* isolates were identified by colony morphology and Gram staining and standard biochemical tests (production of pigments, indole, oxidase, catalase, growth at 42°C, hemolysis, motility) (2).

Phenotypic characterization of biofilm production

For the qualitative biofilm production test Congo Red Agar (CRA) method was used (15). In this test, Congo red dye was used as a pH indicator showing black coloration in the pH ranges from 3.0 to 5.2. The isolates were incubated at 37°C for 24-48 hours after inoculation on Congo red agar. As a result of incubation, isolates that formed dry crystallized black colonies were designated as slime positive, and strains that formed red or pink colonies were evaluated as slime negative. The test was repeated three times for each isolate. *P. aeruginosa* ATCC 27853 strain was used as positive control.

For quantitative biofilm production analysis (Microtiter plate Test) (MP) a modification of the method described by Merritt *et al.* (16) was used. The test was performed using Mueller Hinton Broth (MHB) (Oxoid CM0405, UK) with the addition of glucose at a concentration of 5 g/L. *P. aeruginosa* isolates were incubated in BHI broth (Oxoid CM 1135, UK) at 37°C for 24 hours. After incubation, planktonic

bacteria were removed by washing with distilled water. All wells were stained with 125 ml of 0.2% (w/v) crystal violet solution for 10 minutes at room temperature. The washing process was repeated twice. 200 ml of 95% ethanol was added and incubated for 10-15 minutes at room temperature. 125 ml of each well was transferred to a new microtiter plate. Finally, the optical density (OD) of each sample was measured at 570 nm with a spectrophotometer (BioTek ELx808 Absorbance Plate Reader, USA). OD was calculated. Isolates with an optical density higher than 0.2 were considered as biofilm-forming isolates (17). The test was repeated three times for each isolate. *P. aeruginosa* ATCC 27853 (USA) strain was used as positive control and MHB with glucose was used as negative control (8).

DNA extraction, purity and quantity checks

DNA extraction from isolates was performed using a commercial genomic DNA extraction kit (Fermentas, Massachusetts, USA) as recommended by the manufacturer. DNA purity and quantity controls were performed. The OD260 / OD280 ratio of 1.6-2.0 indicated the DNA purity (18).

Polymerase Chain Reaction (PCR)

Genus and species-level identifications of isolates phenotypically determined to be *Pseudomonas* spp. were confirmed by PCR using 16S rDNA-based PCR-specific primers (PA-GS and PA-SS), which provided fast, simple and reliable identification of bacteria. *P. aeroginosa* ATCC 27853 strain was used as a positive control and *E. coli* ATCC 25922 strain was used as a negative control in PCR. In the study, the presence of integron in *P. aeroginosa* isolates was determined by PCR amplification of integrase specific parts of *int* genes (*int*1, *int*2, *int*3). The presence of biofilm-related virulence genes (*psl*A, *pel*A, *ppy*R) of *P. aeruginosa* was determined using specific primer pairs for each gene (Table 1).

PCR, for each sample was carried out on a volume of 50 µl, final concentration was 10x Taq enzyme buffer solution 1x, 25 mM $MgCl_2$ 2 mM, 10 mM dNTP 0.2 mM, 100 ρmol primer (for each) 0.4 ρmol, 5 U Taq DNA polymerase 1.5 U (Fermentas, Massachusetts, USA), 3 µl of each DNA. The prepared tubes were loaded in the thermal cycler (Boeco, Hamburg, Germany).

The DNA was amplified using the following protocol: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (95°C for 10 s), annealing for 15 s [52°C (*int*II), 55°C (*int*I), 57°C (PA-GS, PA-SS, *int*III), 60°C (*psl*A, *pel*A, *ppy*R) 30 sn] and extension (72°C for 1 min), with a single final extension for 7 min at 72°C. On electrophoresis, a 2% agarose gel stained with Safe View (100 ml/6 µl) (ABM, Richmond, Canada) was used and the gel was exposed to 100 volts for 45 min. After electrophoresis, the gel was placed in the chamber of the transilluminator device that was connected to the computer and photographed under UV light (Vilbert Lourmat, Collegien, France). When the amplified product formed a band of the expected size (Table 1), it was assumed to carry the gene examined.

Antimicrobial susceptibility test

In this study, 12 antibiotics (Oxoid, UK) belonging to 6 commonly used antimicrobial families were used (Table 2). The test was performed on Muller-Hinton agar (Oxoid CM CM0337, UK) using the Kirby Bauer disk diffusion technique with the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2017). For this, MH agar plates were incubated at 35±2°C for 16-18 hours. *P. aeruginosa* ATCC 27853 strain was used for quality control.

Multiple antibiotic resistance (MAR) index

The MAR for each isolate was determined by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested. A MAR index higher than 0.2 is said to be an indicator of isolates originating from an environment where antibiotics are frequently used (23).

Multiple antibiotic resistance (MDR) and multiple antibiotic susceptibility (MDS)

Isolates resistant to at least three drug classes from various antimicrobial classes were evaluated as multi-antibiotic resistant (MDR) and isolates resistant to two or fewer antimicrobial classes were evaluated as multi-antibiotic susceptible (MDS) (24).

Statistical analysis

SPSS (Statistical Package for Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) package program was used for statistical analysis of the data obtained. Pearson Chi-square ($\chi 2$) test was used to compare frequency data. The statistical significance level was defined as $P<0.05$ at the 95% confidence interval.

Table 1. All primers used in this study.

Virulenece gene /Gene Group	Target Gene	Sequence (5'-3')	Amplicon (bp)	T_m	Reference	Result (%)
16S rRNA	*Pseudomonas* spp.	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618	56.7 55.3	(19)	67 (100.0)
16S rRNA	*P. aeruginosa*	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	61.0 61.0	(19)	67 (100.0)
Integrase	*Int*1	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	57.2 57.2	(20)	11 (16.4)
Integrase	*Int*2	TTATTGCTGGGATTAGGC ACGGCTACCCTCTGTTATC	233	51.6 57.3	(21)	5 (7.5)
Integrase	*Int*3	AGTGGGTGGCGAATGAGTG TGTTCTTGTATCGGCAGGTG	600	59.5 58.4	(21)	0 (0.0)
Biofilm formation protein/Biofilm	*psl*A	TCCCTACCTCAGCAGCAAGC TGTTGTAGCCGTAGCGTTTCTG	656	61.4 60.3	(9)	56 (83.6)
Biofilm formation protein/Biofilm	*pel*A	CATACCTTCAGCCATCCGTTCTTC CGCATTCGCCGCACTCAG	786	62.7 60.5	(9)	44 (65.7)
Pyoverdine operon editor/ Transmembrane protein	*ppy*R	CGTGATCGCCGCCTATTTCC ACAGCAGACCTCCCAACCG	160	61.4 61.0	(9)	63 (94.0)

Table 2. Antimicrobial susceptibility and resistance pattern of *P. aeruginosa* isolates.

Antimicrobial Classes/ Antibiotic (Symbol)	Disk Content (µg)	Zone Diameter (mm)		*P. aeruginosa* (n =67)	
		S	R	S (%)	R (%)
Penicillins:					
Piperacillin (P)	100	≥21	≤14	32 (47.8)	28 (41.8)
β Lactam/ β Lactamase Inhibitor Combination:					
Piperacillin-tazobactam (PT)	100/10	≥21	≤14	57 (85.0)	8 (11.9)
Ceftolozane-tazobactam (CT)	30/10	≥21	≤16	57 (85.0)	4 (6.0)
Cephem:					
Ceftazidime (CZ)	30	≥18	≤14	35 (52.2)	21 (31.3)
Cefepime (CP)	30	≥18	≤14	28 (41.8)	30 (44.8)
Carbapenem:					
Imipenem (IM)	10	≥19	≤15	54 (80.6)	5 (7.5)
Meropenem (MP)	10	≥19	≤15	54 (80.6)	6 (8.9)
Aminoglycosides:					
Amikacin (AMI)	30	≥17	≤14	52 (77.6)	12 (17.9)
Gentamicin (GEN)	10	≥15	≤12	54 (80.6)	9 (13.4)
Tobramycin (TOB)	10	≥15	≤12	56 (83.6)	8 (11.9)
Fluoroquinolones:					
Ciprofloxacin (CIP)	5	≥21	≤15	54 (80.6)	10 (14.9)
Levofloxacin (LEV)	5	≥17	≤13	52 (77.6)	13 (19.4)

S: Susceptible, R: Resistant

Chi-Square ($\chi 2$) test was used to calculate the association between multiple antibiotic resistance capacity of the isolates and i) phenotypically biofilm formation (Congo Red Agar and Microplate Method) ii) prevalence of biofilm-associated virulence genes (*ppy*R, *ps*lA and *pel*A), iii) prevalence of integron genes (*int*1 and *int*2).

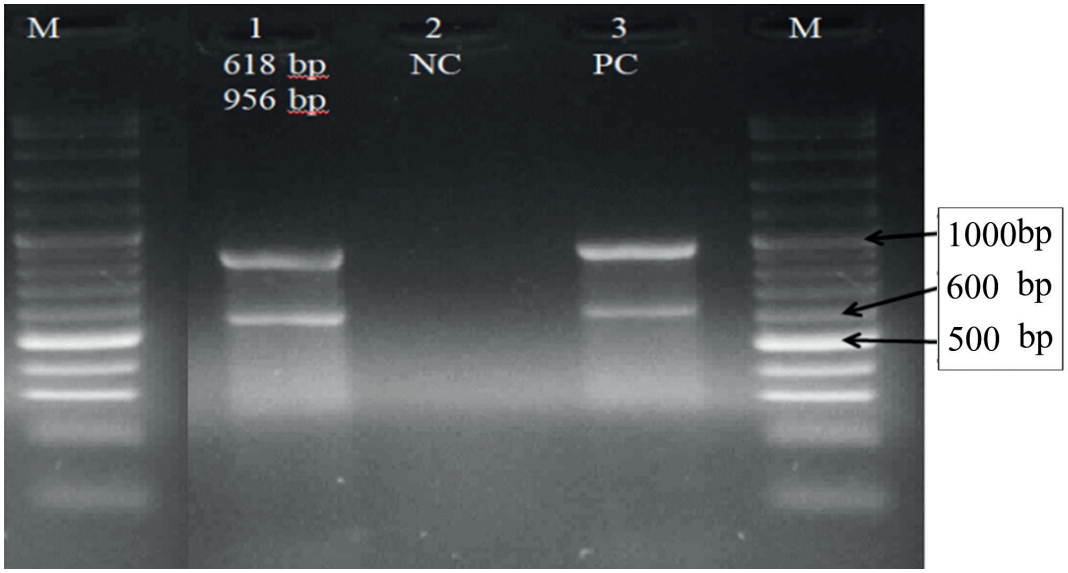

Figure 1. Gel electrophoresis image of *Pseudomonas* isolates 1. *P. aeroginosa* (618 and 956 bp) field isolate 2. NC: Negative control (*E. coli* ATCC 25922) 3. PC: Positive Control (*P. aeroginosa* ATCC 27853) M: Marker (100 bp DNA Ladder).

RESULTS

Bacteria isolation

Sixty-seven isolates which were Gram-negative, oxidase +, catalase +, haemolysed, pigment forming, fruity grape-like odor detected, lactose negative colony forming on MacConkey agar, grown at 42°C were identified as *Pseudomonas* spp. (2).

Biofilm formation

The CRA technique showed that 41.8% of the isolates were considered biofilm producers, while the MP showed that 64.2% of the isolates were biofilm producers.

While 22.4% of the isolates were determined to be biofilm-forming by both methods; it was observed that 16.4% did not form biofilms with both methods. However, while 41.8% of the isolates gave positive results only with the MP method; 19.4% gave positive results only with the CRA technique. It was concluded that quantitative technique (MP) was more efficient/effective than the qualitative technique (CRA) in detecting biofilm production of isolates.

PCR

Genotypic identification

As a result of the study, it was determined that all of the isolates producing 618 bp and 956 bp products with multiplex PCR, were confirmed to be genotypically *P. aeruginosa* (Figure 1).

The virulence gene, integron and antibiotic resistance profiles of 67 identified *P. aeroginosa* were analysed.

Biofilm-related virulence genes

*ppy*R, *psl*A and *pel*A, genes responsible for biofilm formation, were detected in 94.0%, 83.6%, and 65.7% of the isolates, respectively (Table 1, Figure 2.)

Integron genes

While 23.9% of the isolates carry integrons [only 16.4% of them were class 1 and only 7.5% were class 2]; 76.1% did not carry an integron (Table 1, Figure 3.).

Antimicrobial susceptibility test

The percentage of strains susceptible or resistant to each antibiotic is presented in Table 1. The most effective antibiotics against isolates were piperacillin-tazobactam and ceftolozane-tazobactam (85.0% susceptibility rate). Almost all *P. aeruginosa* isolates included in this study were resistant to cefepime (94.8%, 30/67), piperacillin (41.8%, 28/67), ceftazidime (31.3%, 21/67), levofloxacin (19.4%, 13/67), amikacin (17.9%, 93/101), ciprofloxacin (14.9%, 10/67), gentamicin (13.4%, 9/67), tobramycin and piperacillin-

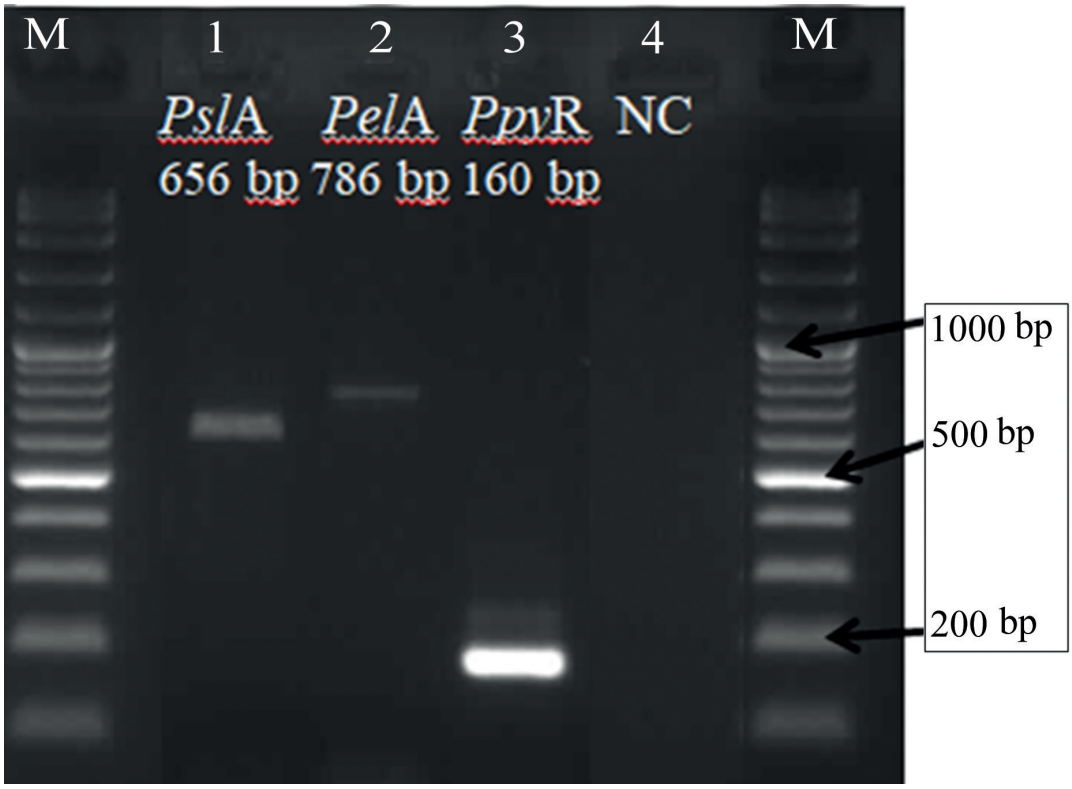

Figure 2. Agarose gel electrophoresis (2%) of virulence gene PCR products. **1.** *psl*A (656 bp) **2.** *pel*A (786 bp) **3.** *ppy*R(160 bp) **4.** NC: Negative Control (*E. coli* ATCC 25922) **M:** 100 bp DNA ladder.

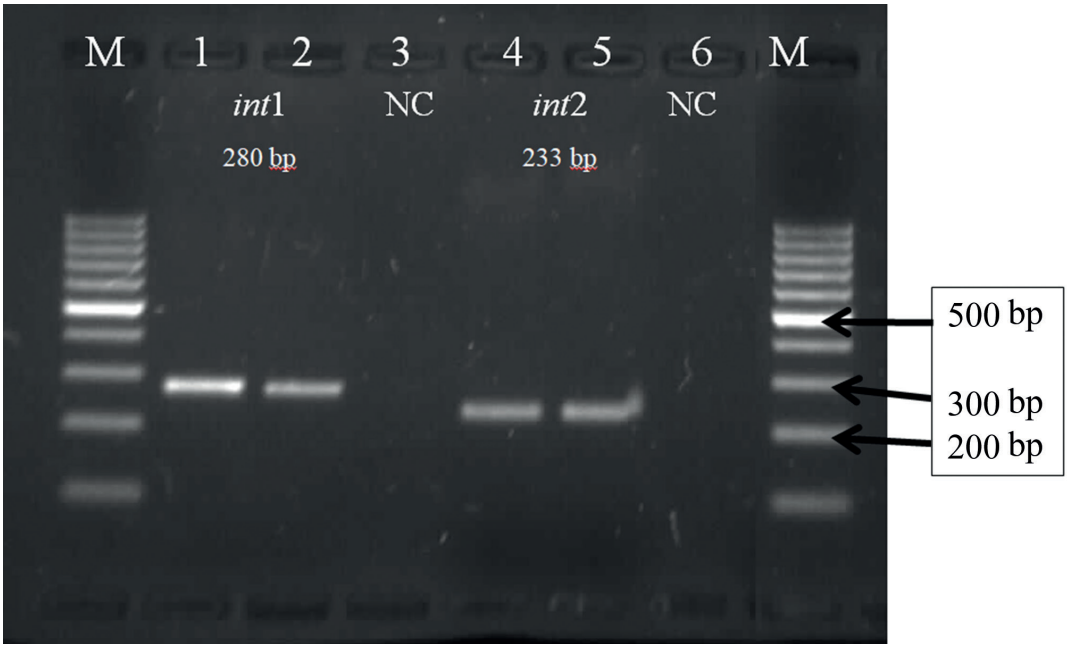

Figure 3. Agarose gel electrophoresis (2%) of integron gene PCR products. **1.2.** *Int*1 gene positive *P. aeroginosa* isolates (280 bp) **4.5.** *int*2 gene positive *P. aeroginosa* isolate (233 bp) **3.6.** NC: Negative Control (master mix without DNA) **M:** (Marker) 100 bp DNA ladder.

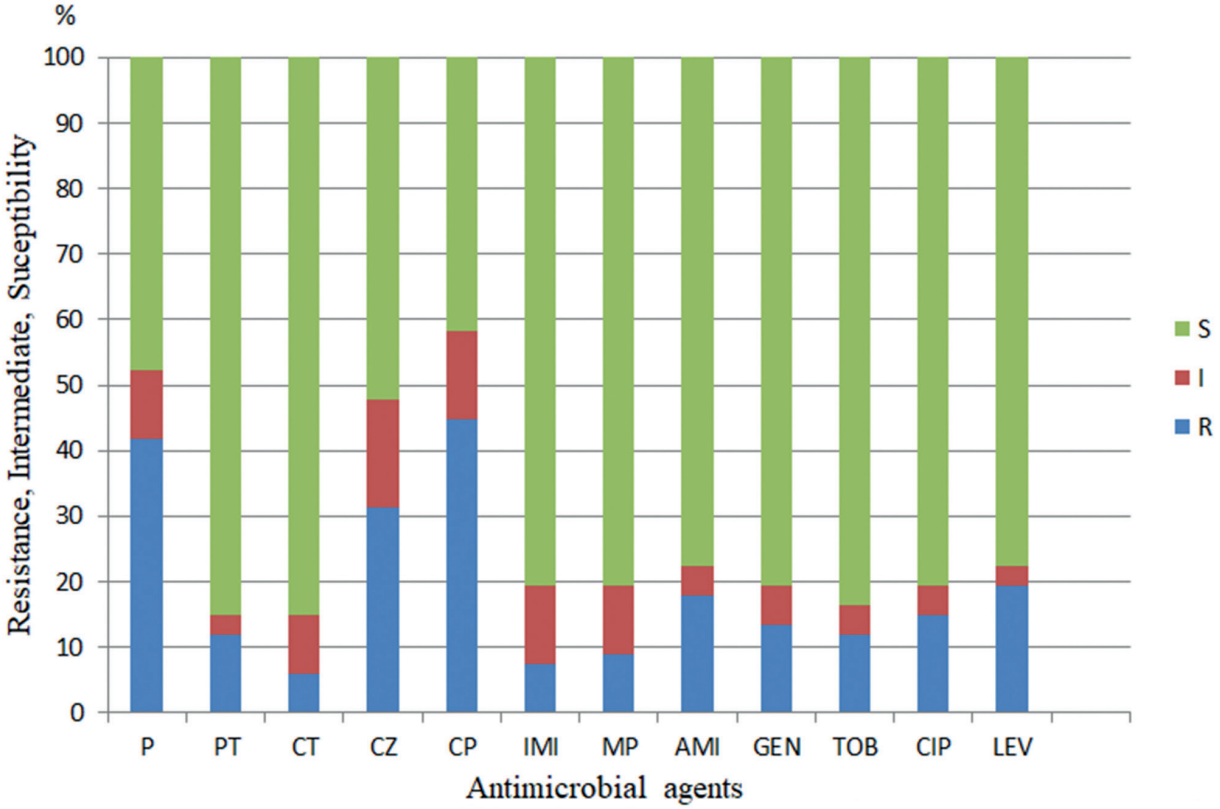

Figure 4. Antimicrobial susceptibility and resistance profiles of *P. aeruginosa* isolates. P: Piperacillin, PT: Piperacillin-tazobactam, CT: Ceftolozane-tazobactam, CZ: Ceftazidime, CP: Cefepime, IP: Imipenam, MP: Meropenem, AMI: Amıkacin S: Sensitive, R: Resistant, I: Intermediate.

tazobactam (11.9%, 8/67). However, lower levels of resistance were observed to meropenem (8.9%, 6/67), imipenem (7.5%, 5/67), and ceftolozane-tazobactam (6.0%, 4/67), tobramycin (12%, 12/96) and gentamicin (18%, 29/154) (Table 1). Figure 4, shows the graphical presentation of antibiotic susceptibility and resistance profiles of *P. aeruginosa* isolates in which show that cefepime and piperacillin were more resistant to *P. aeruginosa*. Furthermore, piperacillin tazobactam and ceftolozane-tazobactam were more sensitive than all other drugs used.

MAR index

MAR index of *P. aeruginosa* isolates showed 41.8% had MAR index of 0.2 and above (Table 4). However, two of these clinic samples, showed the highest MDR 6/12 antibiotics tested (MAR 0.5).

MDR ve MDS

MDR was observed in 28.4% and MDS in 71.6% of the P. aeruginosa isolates obtained (Table 5). In this study, the highest proportion of MDR P. aeruginosa isolates (50.1%) was observed in canine pyoderma samples, followed by mastitis-infected bovine milk (26.3%) and canine otitis externa (17.6) samples.

The origins of isolates with multiple antibiotic resistance, biofilm production phenotypically, biofilm-related virulence genes and aggregate presentation of integron genes are shown in Table 6.

Statistical analysis

The relationship between multi-antibiotic resistance and biofilm production, the prevalence of biofilm-associated virulence genes and the prevalence of integron genes are presented in Table 7.

There was no significant relationship between the multi-antibiotic resistance capacity of the isolates and the phenotypical biofilm formation, prevalence of *ppy*R and *psl*A virulence genes. However, the relationship between the prevalence of the *pel*A virulence gene and the integron genes was significant.

Table 3. Comparison of CRA test and MP test methods used in the determination of biofilm production of isolates.

	CRA (+) (%)	CRA (-) (%)	Total (%)
MP (+) (%)	15 (22.4)	28 (41.8)	43 (64.2)
MP (-) (%)	13 (19.4)	11 (16.4)	24 (35.8)
	28 (41.8)	39 (58.2)	67 (100)

Table 4. MAR index of *P. aeruginosa* isolates.

No of isolates (%)	MAR index	Result (%)
8 (11.9)	0	39 (58.2)
14 (20.9)	0.08	
17 (25.4)	0.16	
12 (17.9)	0.25	
12 (17.9)	0.33	28 (41.8)
2 (3.0)	0.42	
2 (3.0)	0.50	

Table 5. MDR and MDS status of *P. aeruginosa* isolates.

No of isolates (%)	Number of Resistant Antibimicrobial Families	MDR (%) or MDS (%)
2 (3.0)	4	MDR (28.4)
17 (25.4)	3	
20 (29.8)	2	MDS (71.6)
20 (29.8)	1	
8 (12.0)	0	

Table 6. Origins of isolates with multiple antibiotic resistance, phenotypically biofilm production, biofilm-related virulence genes and integron genes.

Isolate No (Origin)	MP Test Results	Biofilm-related virulence genes			Integron Genes
		ppyR	*psl*A	*pel*A	
1 (Bovine Mastitis)	–	+	+	–	–
2 (Bovine Mastitis)	+	+	–	–	–
3 (Bovine Mastitis)	+	+	+	–	+
4 (Bovine Mastitis)	+	+	+	+	+
5 (Bovine Mastitis)	+	+	–	–	–
6 (Bovine Mastitis)	+	+	+	–	–
7 (Bovine Mastitis)	+	+	+	+	+
8 (Bovine Mastitis)	+	+	+	+	–
9 (Bovine Mastitis)	–	+	–	–	–
10 (Bovine Mastitis)	+	+	+	+	–
11 (Canine otitis)	+	+	+	+	–
12 (Canine otitis)	–	+	+	–	+
13 (Canine otitis)	+	+	+	+	+
14 (Canine pyoderma)	–	–	–	–	–
15 (Canine pyoderma)	+	+	–	–	–
16 (Canine pyoderma)	+	+	+	–	+
17 (Canine pyoderma)	+	–	–	–	–
18 (Canine pyoderma)	+	+	+	–	+
19 (Canine pyoderma)	+	+	+	–	+

DISCUSSION

The treatment of *P. aeruginosa* infections remains a major challenge today. Antibiotic resistance in *P. aeruginosa* is multifactorial in that it can occur through congenital or adaptive mechanisms. The diversity of antibiotic resistance mechanisms contributes to the development of multidrug-resistant strains, which renders conventional antibiotics used in the treatment of *P. aeruginosa* infections ineffective (25).

Biofilm formation is an important virulence factor that plays a role in the escape of bacteria from host defence. Quantitative and qualitative testing to investigate the *in vitro* biofilm forming abilities of pathogens is inexpensive, fairly simple, and rapid. Although not as common as quantitative MP, many studies use the CRA test. The CRA method was developed by Freeman *et al.* (15) in 1989 for coagulase-negative staphylococci. The CRA test is a qualitative method to predict whether staphylococcal isolates can form biofilms i*n vitro* and the specific mechanism is not fully known (26).

There are few studies evaluating the effectiveness of the CRA test for *P. aeruginosa* isolated from animal clinical specimens (27). Most of the studies use the MP method for *in vitro* examination of biofilm formation. In MP test, bacteria are grown on polystyrene microplates. The wells of the microplate are emptied and washed at different times. Thus, the remaining biofilm biomass can be stained with crystal violet and quantified. The crystal violet stain is used to measure the total biomass in this system because the stain binds to negatively charged molecules, which means both to bacteria and exopolysaccharides (16). In previous studies, it has been reported that the MP test was a more effective method than the CRA method in detecting biofilm formation, as in this study (26, 28). The MP technique is accepted as the standard method for demonstrating biofilm formation (26, 28, 29). Accordingly, it can be stated that 64.2% of the isolates in this study were biofilm producers. Lima *et al.* (2017) (26) and Kunwar *et al.* (2021) (28), found the biofilm forming abili-

Table 7. The relationship between multi-antibiotic resistance and biofilm production, prevalence of biofilm-associated virulence genes and prevalence of integron genes.

		MDR (+)	MDR (-)	P	χ2
Biofilm production	**CRA (+)**	5	25		
	CRA (-)	14	23	0.064	3.65
	MP (+)	15	28		
	MP (-)	4	20	0.159	2.52
Biofilm gens	***ppy*R (+)**	18	45		
	***ppy*R (-)**	1	3	1	0.02
	***psl*A (+)**	15	41		
	***psl*A (-)**	4	7	0.492	0.42
	***pel*A (+)**	16	28		
	***pel*A (-)**	3	20	0.05	4.04*
Integron gens	***int* (+)**	8	8		
	***int* (-)**	11	40	0.05	4.85*

* Statistically significant results.

ties of clinical *P. aeroginosa* isolates were 75.0% and 25.0%, respectively. Differences in these results may be due to differences in the sites from where the clinical isolates were taken. In this study, the relationship between biofilm formation (using two methods) and MDR capacity was analyzed. Of 19 isolates with MDR, 26.3% (5 isolates) by CRA test were biofilm producers and 78.9% (15 isolates) by MP test. There are always question marks on the relationship between MDR properties and biofilm production (26). Concerning this issue, Lima *et al.* (2017) did not perform any statistical analysis due to the small number of samples (26). In this study, similar to a study conducted in Iran (28), no statistically significant relationship was found between the MDR capacity of the isolates and biofilm production phenotypically. This result was considered to be due to the low number of isolates and/or the fact that the effectiveness of antibiotics in the biofilm was not investigated.

In this study, *ppy*R, *psl*A and *pel*A genes responsible for biofilm formation were detected in 94.0%, 83.6% and 65.7% of the isolates, respectively. It has been reported in previous studies that the *ppy*R gene may be a conserved gene due to its high prevalence and activity in virulence gene expression among clinical isolates (8). Like *psl*, *pel* is an essential matrix component of the biofilm and plays a role in the initiation of surface binding as well as maintaining biofilm integrity. *Pel* is a cationic polysaccharide polymer (30). *Pel* is responsible for the pellicle biofilm formed at the air-liquid interface in broth culture (31). *Psl* and *pel* synthesis is strain specific and can be altered in response to surrounding conditions (32). The prevalence of *ppy*R gene was found to be 94.0% in this study. High prevalence of this gene has been reported in other studies (8, 11). Of the isolates with MDR were carrying 94.7% *ppy*R, 78.9% *psl*A and 84.2% *pel*A genes. However, together with the high prevalence of *ppy*R and *psl*A genes in our study; only the difference in *pel*A gene distribution was significant for biofilm formation. Therefore, the results of this study are consistent with other studies in which the *pel*A gene was considered a biofilm formation marker for *P. aeruginosa* isolates (8).

The multiple resistance mechanism of *P. aeruginosa* is extremely complex. Transpose elements (transposons, plasmids, and also integrons) that can transfer resistance genes between bacteria are held responsible for the increased capacity of multiple antibiotic resistance (1, 25). In a study conducted in Turkey in 2013, the presence of integron 1 gene in *P. aeroginosa* isolates obtained from human clinical samples was reported as 4.8%; class 2 integron could not be detected (12). Studies conducted in recent years have reported that class 2 integron positive rates among *P. aeruginosa* isolates have been increasing over the years. Xu *et al.* found that class 2 integron positive rates increased from 8.0% to 60.8% over the five-year study period (33). In this study, while 42.1% of MDR 19 isolates carried integrons, 16.7% of MDS 48 isolates carried integron genes. High integron positivity in MDR isolates has also been reported in other studies (12, 34). This finding is similar to the results of other studies reporting that the acquisition of resistance genes is not random and the transfer of integron-bearing elements plays a dominant role in the development of MDR (35,36). Based on these results, it can be said that integrons may play a role in the possible transmission of resistance genes to clinical *P. aeruginosa* isolates.

Infections caused by antibiotic resistant isolates are a concern, as they are associated with high mortality, secondary infections and increased treatment costs (37). *P. aroginosa* prefer moist environments, are resistant to physical environments, need very little nutrients to reproduce, are resistant

to many antiseptics and antibiotics, and can grow even in distilled water, making it easier for this microorganism to live in outdoor environments (1,2). *P. aeruginosa*, through their enzymes are resistant to anti-staphylococcal penicillins, sulbactam-ampicillin, amoxycillin-clavulanate, first, second and some third generation cephalosporins, trimethoprim-sulfamethaxazole and nalidixic acid (37).

Carbapenems are the newest group of beta-lactam antibiotics. It is the first antibiotic group used in multiresistant Gram-negative bacterial infections due to its wide spectrum of action in human medicine, resistance to extended spectrum beta lactamase enzymes, and its ability to rapidly pass through the bacterial membrane (2, 24). In this study, imipenem resistance was 7.5%, and meropenem resistance was 8.9%. According to our current information, although imipenem resistance has not been reported in *P. aeroginosa* isolates from the milk of cattle with mastitis in Turkey; it has been reported in 2011 with a rate of 11.8% in Gram-negative bacteria of animal origin (*Enterobacter cloacae, Citrobacter freundii, Proteus mirabilis,* and *Pseudomonas stutzeri*) in Egypt (38).

Fluoroquinolones are broad spectrum bactericidal drugs and resistance to these drugs develops rapidly, however, they are effective drugs against many Gram-negative bacilli, including *P. aeruginosa* (24). Among *P. aeruginosa* isolates, it has been reported that fluoroquinolone resistance has increased at an alarming rate due to its widespread use (24). Ciprofloxacin is one of the antibiotics effective against pseudomonas. (39). In this study, it was determined that isolates of animal origin in Turkey have started to develop resistance against fluoroquinolones like carbapenems. Similar results have been reported in different studies (39, 40).

In this study, the three antibiotics to which *P. aerogisa* isolates were most resistant were: cefepime (44.8%), piperacillin (41.8%) and ceftazidim (31.3%). High levels of piperacillin resistance (86%) in *P. aeroginosa* isolates of animal origin were reported in South Africa (39) and ceftazidime resistance (30%) in Colombia (40). In addition, lower levels of resistance were observed to aminoglycosides (17.9% amikacin, 13.4% gentamicin, 11.9% tobramycin), which act by binding to 16S rRNA, inhibiting protein synthesis and disrupting bacterial cell membrane integrity (41). This finding is similar to studies in Colombia (39) and South Africa (40). However, a higher level of aminoglycoside resistance (65% tobramycin, 70% amikacin, 71% gentamicin) has been reported among the of *P. aeruginosa* isolates obtained from canine clinical cases in Brazil (42). In this study, piperacillin tazobactam and ceftolozane-tazobactam were found to be the most effective antibiotics against *P. aeruginosa*. This was followed by tobramycin, gentamycin and carbapenems, respectively.

The MAR index is considered a good risk assessment tool. A MAR index value of 0.20 was used to distinguish between low and high contamination risks (23). Of the 67 isolates tested in this study, 41.8% had MAR index values higher than 0.2, suggesting high antibiotic use and high selective pressure. Meng *et al.* reported that 59.3% of the *P. aeroginosa* isolates obtained from raw milk had a MAR index higher than 0.2 (43). In another study, MAR index values of 19 *P. aeruginosa* isolated from raw milk were reported to vary between 0.33-1.0 (44). MAR values are considered important, as they are indicative of the degree of exposure to the antibiotics used.

The presence of populations of MDR strains among clinical isolates is a cause of concern for physicians practicing empirical therapy because of their rate increases over the years. In this study, 28.1% of the isolates were determined as MDR. However, it is interesting that there are three studies conducted in Iran in different years: MDR was reported as 30.1% (45) in 2010, 45.3% (46) in 2013, and 58.6% in 2015 (8). Therefore, it is important to continuously monitor antimicrobial susceptibility profiles of *P. aeroginosa* isolates and to investigate the prevalence of MDR *P. aeruginosa* isolates.

In this study, samples taken from different cases such as bovine milk, canine otitis and canine pyoderma were examined. There were a total of 19 isolates, 10 of which were multi-antibiotic resistant, of bovine mastitis, 3 of canine otitis, and 6 of canine pyoderma origin. When we evaluate the virulence gene carrying status of these isolates according to the origin of the isolates: while the *ppy*R gene was detected in all isolates of milk and otitis media it was present in 66.7% of the samples isolated from canine pyoderma. While the *psl*A gene was found in 70% of milk samples and 100% of otitis samples it was absent in any of the pyoderma samples. The *pel*A gene was present in 40% of milk samples with mastitis and in 66.6% of samples with otitis, but it was not present in any of the isolates obtained from dogs with pyoderma. However, 30.0% of multi-antibiotic resistant cattle isolates, 66.7% of otitis isolates and 50.0% of pyoderma isolates carried at least one integron gene (Table 6). All of the multi-drug resistant isolates carrying the integron gene had the biofilm genes

*ppy*R and *psl*A. Considering these results, it is considered that the samples coming from different species and in some cases from different environments (intra-mammary, skin or ear) may have an effect on the results. Perhaps bacteria can express different genes depending on the environment. Of course, it is necessary to carry out studies using more isolates in order to evaluate this situation statistically.

In conclusion, most of the antibiotics used in our study seem to be suitable for the treatment of *P. aeruginosa* infections. However, we found moderate antimicrobial resistance among *P. aeruginosa* isolates obtained from animal clinical specimens with 28.4% of the isolates having MDR capacity. The *pel*A virulence gene and *int* genes appeaered to play an important role in multidrug resistance.

An important consideration in investigating the genetic basis of multiple antibiotic resistances in *P. aeruginosa* infections is the gene cassettes associated with integrons. The roles of these cassettes in the acquisition and expression of resistance genes should be examined in future studies. Biofilm forming properties of isolates obtained from cow milk with mastitis have been investigated *in vitro* in current research conducted in the veterinary field. In this study, we examined the antibiotic resistance, biofilm formation and integron gene carrying properties of *Pseudomonas* isolates that we obtained from different animal origins. However, *in vitro* observations of biofilm formation cannot easily be compared with the *in vivo* situation. *In vivo* studies are required to reveal the true role of biofilm formation in the pathogenesis of infections.

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