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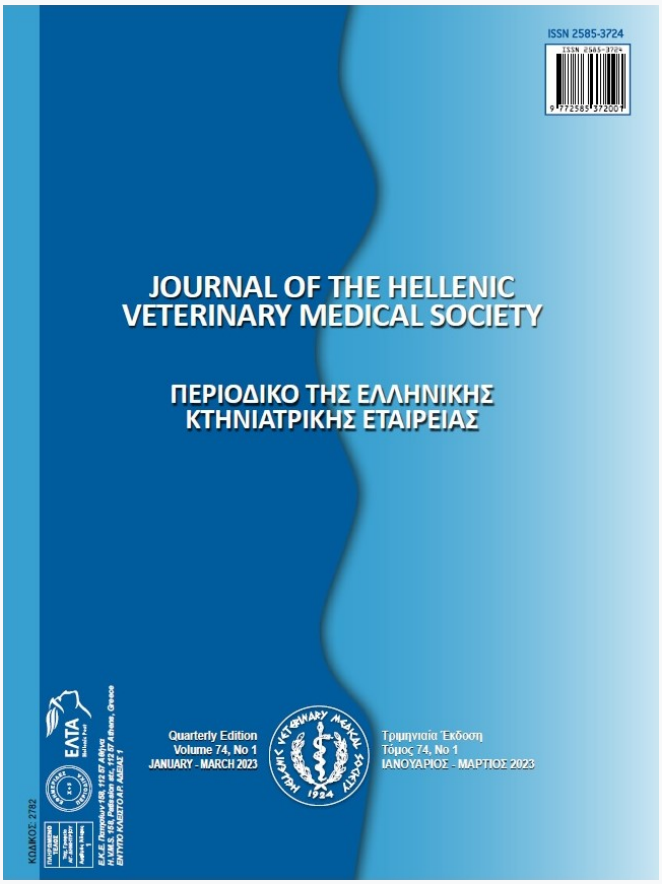

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S Turkyilmaz, F Ocak

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Research article
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Comparative study on genotypic properties of *Pseudomonas aeruginosa* isolated from subclinical mastitis in Türkiye

F. Ocak[1], S. Turkyilmaz[2,*]

[1]*Department of Biology, Basic and Industrial Microbiology, Manisa Celal Bayar University, Manisa, Türkiye*

[2]*Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydin, Türkiye*

ABSTRACT: The results of antibacterial treatment in mastitis may be related not only to the antibiotic sensitivity of the etiological factors but also to their abilities to carry integron and virulence genes. Integrons are mobile DNA elements that can capture and carry genes, particularly those responsible for antibiotic resistance. *Pseudomonas aeruginosa* has numberless virulence factors which are contributed to bacterial invasion and toxicity. This study aims to investigate antibiotic resistance, integron and virulence gene profiles of *P. aeruginosa* isolates obtained from cow milk with subclinical mastitis; further, was to evaluate the relationship between the antimicrobial resistance of bacteria with the integron and virulence gene carrying. The material of the study consists of 32 (9.8%) *P. aeruginosa* isolates obtained from 326 subclinical mastitis milk samples. After the bacterial identification by classical conventional methods, polymerase chain reaction (PCR) was carried out to confirm the genus and species of the isolates and to determine the integron and virulence gene profiles. Antimicrobial resistance was determined by the disk diffusion method using fifteen antibiotics belonging to eleven antimicrobial families. The relationship between the presence of integron and virulence genes associated with antibiotic resistance, further the relationship between the presence of virulence genes and antimicrobial resistance was calculated with the Chi-Square ($\chi 2$) test. Ten virulence genes (*las*I, *las*R, *las*B, *rhl*I, *rhl*R, *rhl*AB, *plc*H, *plc*N, *ppy*R, *exo*T) were found in all isolates whilst another virulence gene (*apr*A) was not present in any isolate. It was found that 34.4% of the isolates carried any integron gene. The results showed that the relationship is important between the presence of *int* genes and gentamicin, amikacin, tetracycline, cefoperazone, imipenem, aztreonam, ciprofloxacin, enrofloxacin resistance and *exo*U virulence gene presence. Also; there were important associations between resistance to certain antibiotics and the presence of *P. aeruginosa* virulence genes. All isolates obtained in this study showed multiple antibiotic resistance (MDR). In these cases, showing the presence of integron and some virulence genes could play a prominent role in the resistance of *P. aeruginosa* isolates to antimicrobial drugs. This study may be important as it is the first study to show the presence of antibiotic resistance, integron and virulence genes together in subclinical mastitis cow's milk isolates of *P. aeruginosa* in Türkiye. The presence of antibiotic-resistant *P. aeruginosa* strains in cattle farms may also pose a public health risk, as these bacteria can transmit their resistance genes to humans through food consumption.

Key words: Antibiotic resistance, Cow milk, Integron gene, *P. aeruginosa*, Virulence gene.

Corresponding Author:
Dr. S. Turkyilmaz, Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Isikli, Aydin-Türkiye.
E-mail address: sturkyilmaz@adu.edu.tr

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INTRODUCTION

Pseudomonas aeruginosa is a well-known opportunistic pathogen that causes nosocomial infection in humans (Quinn et al., 2011). *P. aeruginosa* are common in dairy cows because they require very little nutrients to grow and reproduce. *P. aeruginosa* infections in cattle mostly result in mastitis and associated with this may be systemic infections develop (Kirk et al., 2011).

It has been proven that the pathogenicity of the bacteria is due to virulence factors contributing to bacterial invasion and toxicity (Kipnis et al., 2006). The structure is responsible for the movement of *P. aeruginosa* in the form of swimming as flagella. The flagella protein flagellin is encoded by the *fli*C gene. Fimbriae are short and filamentous surface structures of the bacteria, which are responsible for twitching movement in *P. aeruginosa* and help to spread and colonize rapidly in the airways. The *pil*A gene is the colonization factor and *pil*B gene is responsible for providing energy (Kipnis et al., 2006). Alginate is a mucoid exopolysaccharide composed of repeating polymers of mannuronic and glucuronic acid. Alginate plays a role in the adhesion of bacteria on the host epithelium. The *alg*D, *alg*U *alg*L genes are also responsible for alginate production (Kipnis et al., 2006). The production of many extracellular virulence factors in *P. aeruginosa* is controlled by the quorum sensing (QS) mechanism that allows communication between bacteria. There are two QS systems in *P. aeruginosa*; *las* and *rhl* systems (Greenberg, 1997). The alkaline protease is a fibrinolysis-efficient metalloprotease of *P. aeruginosa* and this enzyme is encoded by the *apr* gene. Although the role of alkaline protease is not fully known in tissue invasion and systemic infections, it has been shown to have an important role in the pathogenesis of corneal infections (Howe and Iglewski, 1984). Phospholipases play a role in the pathogenesis of acute lung injury by targeting phospholipids, which are a component of the eukaryotic cell membrane. There are two types of phospholipase C (*plc*H, *plc*N) that differ in their haemolytic activity (Wiener-Kronish et al., 1993). One of the most of the infectious agents' *P. aeruginosa,* secretes an exotoxin A encoded by the *tox*A gene, which is responsible for the invasion and regional tissue damage of the bacteria (Woods and Iglewski, 1984). The *pvd*A gene in *P. aeruginosa* encodes the L-ornithine N5-oxygenase enzyme and catalyzes an important step in the pyoverdin biosynthetic pathway. Piyoverdin is a siderophore, which provides iron support for the metabolism of *P. aeruginosa* by binding the surrounding iron. It also regulates the production of exotoxin A and plays a role in virulence by regulating its production (Meyer et al., 1996). Pyocyanin is the blue pigment metabolite of *P. aeruginosa* and its related phenazine operon contains *phz*I, *phz*II, *phz*M, *phz*S genes (Denning et al., 1996). The toxins of *P. aeruginosa* released by the type III secretion system (T3SS) are: *exo*S (invazin), *exo*T, *exo*Y and *exo*U (cytotoxin) (Kipnis et al., 2006). Ertugrul et al. (2018) reported that the presence of three virulence genes were significantly higher in *P. aeruginosa* isolates obtained from diabetic foot infections. The first of these genes is the *fli*C gene, which plays a role in tissue adhesion. The second is the *phz*S gene, which allows bacteria to survive tissue damage under anaerobic conditions. The third is the *tox*A gene, which plays a role in cell death. Finding new molecules that can inhibit the synthesis of these genes may be useful to investigate whether they can provide new therapeutic strategies for the treatment of diabetic foot infections.

The ability of *P. aeruginosa* to form biofilms is an important feature. Biofilm affects the antibiotic resistance of bacterial cells. Biofilms facilitate the colonization of pathogens in the mammary glands. *psl*A and *pel*A genes play role in the formation of the carbohydrate-rich structure of the biofilm. It has been reported that biofilm formation showed a decrease when *psl* operon was suppressed after the inactivation of the *ppy*R gene (Ghadaksaz et al., 2006). Long term and widespread antibiotic usage may result in the development of antibiotic resistance among bacteria (Mazel, 2006). Studies have shown that such isolates are resistant to important antibiotic groups and also develop resistance to more than three antimicrobial families over time, becoming multiple antibiotic resistant (MDR) isolates (Magiorakos et al., 2012). Mobile genetic elements such as plasmids, transposons and integrons play an important role in terms of the high level of antibiotic resistance. Although four important integron classes have been reported so far, class 1 and class 2 integrons are frequently seen in Gram negative bacteria. Integrons can capture exogenous gene cassettes, thus enabling the expression of genes in these cassettes plays an important role in the horizontal spread of antibiotic resistance genes (Mazel, 2006).

Since *P. aeruginosa* usually has multiple antibiotic resistance, it causes infections that are difficult

to treat (Kipnis et al., 2006; Kirk et al., 2011). For this, this bacterium has the attention of both medical and veterinarians. Although a few studies are related to the presence of *P. aeruginosa* in milk samples taken from dairy cows with mastitis in Türkiye, a recent study has focused on the monitoring of antibiotic resistance in *P. aeruginosa* isolated from milk samples of cows with mastitis (Sahin and Erbas, 2015). Nothing is known concerning *P. aeruginosa* isolated from milk samples of cows with mastitis in Türkiye, the presence of integrons and virulence factor production abilities. This study aims to investigate the antibiotic resistance, integron and virulence gene profiles of *P. aeruginosa* isolates obtained from subclinical mastitic cow milk and to determine the contribution of the presence of integron and virulence gene to antimicrobial resistance in these microorganisms.

MATERIALS AND METHODS

Study material

In this study, 1651 milk samples from 422 cows belonging to 13 farms in Aydin province were examined between January and December 2019. Samples were taken into sterile tubes aseptically from animals that have not been treated with antibiotics for at least two weeks. Several milk samples ranging between 16 and 41 were taken from each dairy farm. Milking machines were used in all enterprises. The cows' ages varied between 3 and 11years and the numbers of cows were between 22 and 52 in each farm.

Clinical examination and sample collection

California Mastitis Test (CMT) was used to detect subclinical mastitis. The procedures and interpretations have been described previously (Quinn et al., 2011). The CMT results were scored based on gel formation. Negative (0), weak positive (1), distinct positive (2) and strong positive. Positive cows were defined as having at least one quarter with a CMT score of >1.

A total of one milk sample was taken from each cow, from the udder lobe with the highest CMT positivity under aseptic conditions. While the milk was being taken, after cleaning the nipples with 70% alcohol, the first few streams of milk were discarded. Approximately 5 ml of milk sample was taken to sterile tubes and brought to the laboratory under the cold chain on the same day. Milk samples were stored at -20°C until the bacteriological identification process was completed.

Isolation and identification

Milk samples were centrifuged at 3500 rpm for 5 minutes and the supernatant was discarded. The residue was vortexed. A loopful of the sample suspension was streaked onto *Pseudomonas* F agar (Merck 1.10989, Germany), Blood agar (Merck 1.10886, Germany) and MacConkey agar (Merck 1.05465, Germany) and incubated at 37°C for 24-48 h aerobically. Biochemical tests (oxidase, catalase, urease, citrate, motility, VP, MR) were performed after phenotypic identification of suspicious colonies with Gram staining. Isolation of *Pseudomonas* spp. was performed using standard bacteriological methods (Quinn et al., 2011).

Antibiotic susceptibility tests

The antimicrobial resistance of the isolates was investigated by the standard disk diffusion method (CLSI, 2012). The *P. aeruginosa* isolates were characterized for their resistance to 15 antibiotics belonging to eleven different antibiotic families. Zone diameters of susceptibility testing results were categorized as sensitive (S), intermediate (I), or resistant (R) and evaluated as previously reported (CLSI, 2012; Chengappa, 1990; Fouad, 2011). The antibiotics tested were purchased from Oxoid, Hampshire UK. *P. aeruginosa* ATCC 27853 was used as the quality control strain. Multiple drug resistance (MDR) was defined as resistance to three or more antimicrobial classes (Magiorakos et al., 2012).

DNA extraction, purity and quantity controls

DNA extraction from *Pseudomonas* spp. was performed as recommended by the manufacturer using a commercial genomic DNA extraction kit (InstaGene™ Martix, Biorad, Dubai). DNA purity and quantity controls were also performed. The OD260/OD280 ratio of 1.6-2.0 indicated sufficiently DNA purity (Aggarwal et al., 2012).

Polymerase chain reaction (PCR)

Pseudomonas spp. genus and species identification of isolates identified as being were verified by PCR with 16S rRNA-based PCR specific primers that enable the differentiation and identification of *P. aeruginosa* genetically and reliably among other closely related *Pseudomonas* species. Target genes, sequences, product lengths, melting temperature (Tm), references of primers used in this study and the results were shown in Table 1.

P. aeruginosa ATCC 27853 strains were used as positive control and *E. coli* ATCC 25922 strains were

Table 1. Primers used in this study.

Virulenece gene/Gene Group	Target Gene	Sequence (5'-3')	Product Length (bp)	T_m	Referenece	Result (%)
16S rRNA	P*seudomonas* spp.	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618	56.7 55.3	(Spilker et al., 2004)	34 (10.4)
16S rRNA	*P. aeruginosa*	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	61.0 61.0		32 (9.8)
Integrase	*Int*1	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	57.2 57.2	(Bass et al., 199)	7 (21.9)
Integrase	*Int*2	TTATTGCTGGGATTAGGC ACGGCTACCCTCTGTTATC	233	51.6 57.3	(Goldstein et al., 2001)	4 (12.5)
Integrase	*Int*3	AGTGGGTGGCGAATGAGTG TGTTCTTGTATCGGCAGGTG	600	59.5 58.4		0 (0.0)
Protease/QS	*las*AF *las*AR	GCAGCACAAAAGATCCC GAAATGCAGGTGCGGTC	1075	52.8 55.2	(Fazeli and Momtaz, 2014)	17 (53.1)
Elastase/QS	*las*BF *las*BR	GGAATGAACGAAGCGTTCTCCGAC TGGCGTCGACGAACACCTCG	284	64.4 63.5		32 (100.0)
Auto inducer synthesis protein/ QS	*las*IF *las*IR	CGTGCTCAAGTGTTCAAGG TACAGTCGGAAAAGCCCAG	295	56.7 56.7		32 (100.0)
Transcriptional activator protein/ QS	*las*RF *las*RR	AAGTGGAAAATTGGAGTGGAG GTAGTTGCCGACGACGATGAAG	130	55.9 62.0	(Sabharwal et al., 2014)	32 (100.0)
Acyl-homoserine-lactone synthase/QS	*rhl*IF *rhl*IR	TTCATCCTCCTTTAGTCTTCCC TTCCAGCGATTCAGAGAGC	155	58.4 56.7		32 (100.0)
Transcriptional regulator/QS	*rhl*RF *rhl*RR	TGCATTTTATCGATCAGGGC CACTTCCTTTTCCAGGACG	133	55.5 56.7		32 (100.0)
Rhamnosyl transferase/ QS	*rhl*ABF *rhl*ABR	TCATGGAATTGTCACAACCGC ATACGGCAAAATCATGGCAAC	151	57.9 55.9		32 (100.0)
Alkaline metalloproteinase/ Alkaline protease	*apr*AF *apr*AR	GTCGACCAGGCGGCGGAGCAGATA GCCGAGGCCGCCGTAGAGGATGTC	993	69.6 71.3		0 (0.0)
Flagellar filament structural protein/Flagella protein	*fli*CF *fli*CR	GGCAGCTGGTTNGCCTG GGCCTGCAGATCNCCAA	Type A: 1.02 kb Type B: 1.250 kb	57.6 55.2		21 (65.6)
Haemolytic phospholipase C/ Phospholipase	*plc*HF *plc*HR	GCACGTGGTCATCCTGATGC TCCGTAGGCGTCGACGTAC	608	61.4 61.0	(Fazeli and Momtaz, 2014)	32 (100.0)
Nonhaemolyticphospholipase C/ Phospholipase	*plc*NF *plc*NR	TCCGTTATCGCAACCAGCCCTACG TCGCTGTCGAGCAGGTCGAAC	481	66.1 63.7		32 (100.0)
Exotoxin A/Toxin	*tox*AF *tox*AR	CTGCGCGGGTCTATGTGCC GATGCTGGACGGGTCGAG	270	63.1 60.5		15 (46.8)
L-ornitin N5-oksijenaz/ Pyoverdin biosynthetic pathway	*pvd*AF *pvd*AR	GACTCAGGCAACTGCAAC TTCAGGTGCTGGTACAGG	1281	56.0 56.0		8 (25.0)
Exoenzyme S/T3SS	*exo*SF *exo*SR	GCGAGGTCAGCAGAGTATCG TTCGGCGTCACTGTGGATGC	118	61.4 61.4	(Ajayi et al., 2003)	29 (90.6)
Phospholipase/T3SS	*exo*UF *exo*UR	CCGTTGTGGTGCCGTTGAAG CCAGATGTTCACCGACTCGC	134	58.8 61.4		32 (100.0)
Exopolysaccharide production protein/T3SS	*exo*YF *exo*YR	CGGATT CTATGGCAGGGAGG GCCCTTGATGCACTCGACCA	289	61.4 61.4		2 (6.2)
Exoenzyme T/T3SS	*exo*TF *exo*TR	AATCGCCGTCCAACTGCATGCG TGTTCGCCGAGGTACTGCTC	152	64.0 61.4		31(96.8)
Phenazine operon I/Phenazine operon	*phz*IF *phz*IR	CATCAGCTTAGCAATCCC CGGAGAAACTTTTCCCTC	392	53.7 53.7	(Fazeli and Momtaz 2014)	31(96.8)
Phenazine operon II/Phenazine operon	*phz*IIF *phz*IIR	GCCAAGGTTTGTTGTCGG CGCATTGACGATATGGAAC	1036	56.0 54.6		29 (90.6)
Phenazine specific methyl transferase/Phenazine operon	*phz*MF *phz*MR	ATGGAGAGCGGGATCGACAG ATGCGGGTTTCCATCGGCAG	875	61.4 61.4		24(75.0)
Flavin-dependent hydroxylase / Phenazine operon	*phz*SF *phz*SR	TCGCCATGACCGATACGCTC ACAACCTGAGCCAGCCTTCC	1752	61.4 61.4		24 (75.0)
Fimbrial protein/Fimbrial protein	*pil*AF *pil*AR	ACAGCATCCAACTGAGCG TTGACTTCCTCCAGGCTG	1675	56.0 56.0		8 (25.0)
Type IV fimbrial biogenesis protein /Fimbrial protein	*pil*BF *pil*BR	TCGAACTGATGATCGTGG CTTTCGGAGTGAACATCG	408	53.7 53.7		3 (9.3)
Biofilm formation protein/ Biofilm	*psl*AF *psl*AR	TCCCTACCTCAGCAGCAAGC TGTTGTAGCCGTAGCGTTTCTG	656	61.4 60.3	(Ghadaksaz et al., 2015)	6 (18.7)
Biofilm formation protein/ Biofilm	*pel*AF *pel*AR	CATACCTTCAGCCATCCGTTCTTC CGCATTCGCCGCACTCAG	786	62.7 60.5		2 (6.2)
Pyoverdin operon editor/ Transmembrane protein	*ppy*RF *ppy*RR	CGTGATCGCCGCCTATTTCC ACAGCAGACCTCCCAACCG	160	61.4 61.0		32 (100.0)
Alginate synthesis gene U/ Alginate formation	*alg*UF *alg*UR	CGATGTGACCGCAGAGGATG TCAGGCTTCTCGCAACAAAGG	292	61.4 59.8		29 (90.6)
Alginate synthesis gene L/ Alginate formation	*alg*LF *algL*R	CCGCTCGCAGATCAAGGACATC TCGCTCACCGCCCAGTCG	432	61.4 61.8		29 (90.6)
Alginate synthesis gene D/ Alginate formation	*alg*DF *alg*DR	AGAAGTCCGAACGCCACACC CGCATCAACGAACCGAGCATC	550	64.4 62.8		29 (90.6)

used as negative control in PCR.

PCR, for each sample was carried out on a volume of 30 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 thermalcycler (Boeco, Hamburg, Germany).

The DNA was amplified using the following protocol: an 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, *las*A, *phz*I, *pil*B, *phz*II), 55°C (*int*I, *las*I, *las*R, *rhI*R, *rhI*AB, *pvd*A, *fli*C, *pil*A), 57°C (PA-GS, PA-SS, *int*III, *exo*U, *alg*U), 60°C (*exo*Y, *plc*H, *phz*M, *phz*S, *tox*A, *psl*A, *pel*A, *ppy*R, *alg*L), 61°C (*exo*S, *alg*D), 63°C (*las*B, *plc*N), 68°C (*apr*A) 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 which 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.

Statistical analysis

SPSS (Statistical Package for Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) package program was used for the statistical analysis of the data obtained. Pearson Chi-square (χ2) test was used to compare frequency data. The results were evaluated within a 95% confidence interval. The P value less than 0.05 ($p<0.05$) was considered statistically significant. With χ2 test: i. of the isolates with and without integron-related *int* genes *antibiotic resistance, **prevalence of virulence-related genes and ii. to compare the relationship between virulence genes and resistance to antimicrobial agents in *P. aeruginosa* isolates.

RESULTS

Clinical examination

In this study, 422 cows were analyzed. A milk sample with subclinical mastitis belonging to the udder lobe with the highest CMT score was taken from each cow. Therefore, a total of 326 (77.2%) samples were included in the study.

Isolation and identification

Thirty-four colonies were detected with yellow colour on *Pseudomonas* F agar, β-haemolytic, greenish on blood agar, lactose negative mucoid, and grape-like fruity odor on MacConkey agar were detected. A total of 34 (34/326=10.4) isolates determined to be Gram negative, motile, oxidase +, catalase +, citrate +, urease +, VP - and MR - were evaluated as *Pseudomonas* spp.

Genotypic identification

Genotypic identification of *P. aeruginosa* isolates by multiplex PCR using genus and species-specific primers. Following PCR, only 618 bp long product was obtained in 2 (2/326=0.6%) isolates (*Pseudomonas* spp.); both 618 bp and 956 bp long product were obtained in 32 (32%326=9.8%) isolates. These isolates were genotypically confirmed to be *P. aeruginosa* (Figure 1.).

The virulence gene, integron and antibiotic resis-

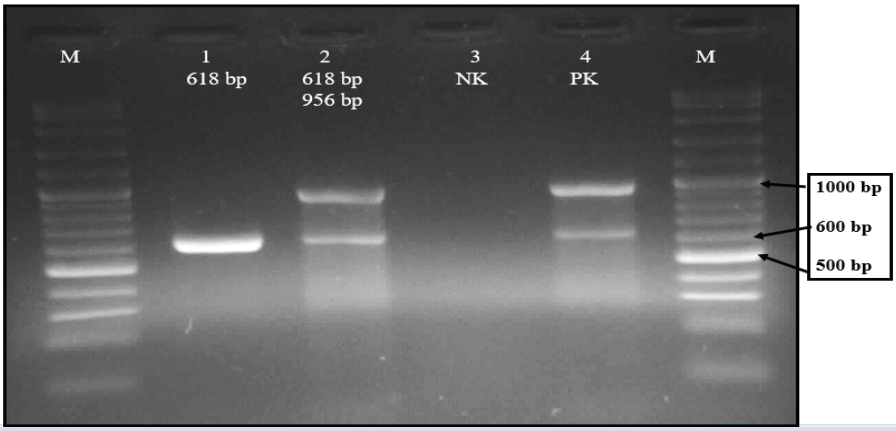

Figure 1. Gel electrophoresis image of *Pseudomonas* isolates 1. *Pseudomonas* spp. (618 bp) 2. *P. aeruginosa* (618 and 956 bp) field isolate 3. *E. coli* ATCC 25922 (Negative control) 4. Positive Control (*P. aeruginosa* ATCC 27853 strain) M: Marker (100 bp DNA Ladder).

tance profiles of 32 *P. aeruginosa* isolates identified were analysed.

Detection of virulence factors

As a result of the detection of 29 virulence genes in a total of 32 *P. aeruginosa* isolates: (Figure 2.): In all isolates, we found ten virulence genes: *las*B, *las*I, *las*R, *rhl*I, *rhl*R, *rhl*AB, *plc*H, *plc*N, *exo*T, *ppy*R. The most prevalent virulence genes were *exo*Y, *phz*I (31/32=96.8%), *exo*S, *phz*II, *alg*D, *alg*U, *alg*L (29/32=90.6%), *phz*M, *phz*S (24/32=75.0%), *fli*C (21/32=65.6%), *las*A (17/32=53.1%), *tox*A (15/32=46.8%), *pvd*A and *pil*A (8/32=25.0%), *psl*A (6/32=18.7%), *pil*B (3/32=9.3%), *exo*U and *pel*A (2/32=6.2%), while there was no detection for *apr*A in any of the isolates with the primers used (Figure 3.). In total, 32 *P. aeruginosa* isolates had 18 virulence genotypes (Table 2.)

Presence of integron

While 34.4% (11/32) of the isolates carry integrons [21.9% (7/32) of them are only class 1, 12.5% (4/32) is only class 2]; 65.6% (21/322) did not carry an integron (Figure 4.).

Table 2. Virulence gene profiles of P. aeruginosa isolates.

No	Virulence Genotype	Gene number	Isolate number	Total (%)
1	**las*A	11	1	1 (3.1)
2	**exo*S, *exo*Y, *phz*I, *phz*II	14	2	2 (6.2)
3	**exo*S, *exo*Y, *phz*I, *phz*II, *psl*A, *alg*U, *alg*L, *alg*D, *tox*A	19	1	
4	**exo*S, *exo*Y, *phz*I, *phz*II, *pil*B, *alg*U, *alg*L, *alg*D, *tox*A	19	1	5 (15.6)
5	**exo*S, *exo*Y, *las*A, *phz*I, *phz*II, *alg*U, *alg*L, *alg*D, *tox*A	19	3	
6	**exo*S, *exo*Y, *phz*I, *phz*II, *phz*M, *phz*S, , *alg*U, *alg*L, *alg*D, *fli*C	20	3	1 (3.1)
7	**exo*S, *exo*Y, *phz*I, *phz*II, *phz*M, *phz*S, , *alg*U, *alg*L, *alg*D, *tox*A, *fli*C	21	2	
8	**exo*S, *exo*Y, *las*A, *phz*I, *phz*II, *phz*M, *phz*S, , *alg*U, *alg*L, *alg*D, *fli*C	21	4	8 (25.0)
9	**exo*U, *exo*Y, *las*A, *phz*I, *phz*M, *phz*S, , *alg*U, *alg*L, *alg*D, *pvd*A, *fli*C	21	2	
10	**exo*S, *exo*Y, *las*A, *phz*I, *phz*II, *phz*M, *phz*S, *psl*A, , *alg*U, *alg*L, *alg*D, *fli*C	22	2	
11	**exo*S, *exo*Y, *phz*I, *phz*II, *phz*M, *phz*S, *pil*A, *tox*A, *alg*U, *alg*L, *alg*D, *pvd*A	22	1	
12	**exo*S, *exo*Y, *las*A, *phz*I, *phz*II, *phz*M, *phz*S, *alg*U, *alg*L, *alg*D, *tox*A, *fli*C	22	1	7 (21.9)
13	**exo*S, *exo*Y, *las*A, *phz*I, *phz*II, *phz*M, *phz*S, *pel*A, *alg*U, *alg*L, *alg*D, *fli*C	22	1	
14	**exo*S, *exo*Y, *las*A, *phz*I, *phz*II, *phz*M, *phz*S, *pil*A, , *alg*U, *alg*L, *alg*D, *pvd*A	22	2	
15	**exo*S, *exo*Y, *las*A, *phz*I, *phz*II, *phz*M, *phz*S, *alg*U, *alg*L, *alg*D, *pvd*A, *tox*A, *fli*C	23	1	3 (9.4)
16	** exo*S, *exo*Y, *phz*I, *phz*II, *phz*M, *phz*S, *pil*A, *pil*B, , *alg*U, *alg*L, *alg*D, *tox*A, *fli*C	23	2	
17	** exo*S, *exo*Y, *phz*I, *phz*II, *phz*M, *phz*S, *pil*A, *psl*A, *pel*A, , *alg*U, *alg*L, *alg*D, *tox*A, *fli*C	24	1	3 (9.4)
18	** exo*S, *exo*Y, *phz*I, *phz*II, *phz*M, *phz*S, *pil*A, *psl*A, *alg*U, *alg*L, *alg*D, *pvd*A, *tox*A, *fli*C	24	2	

*: Virulence genes found in all isolates: *exo*T, *las*I, *las*R, *rhl*I, *rhl*R, *las*B, *rhl*AB, *plc*H *plc*N, *ppy*R.

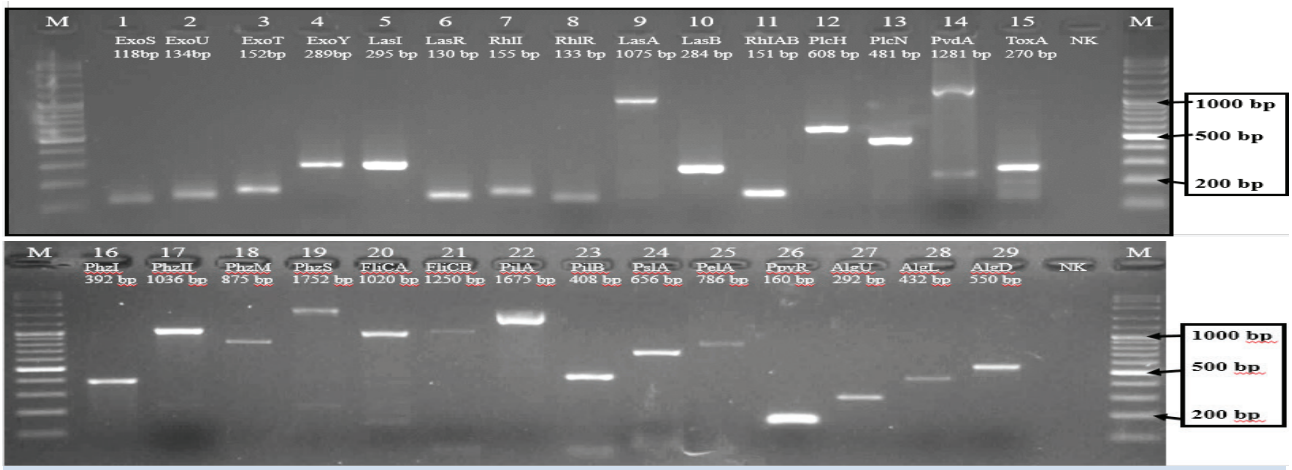

Figure 2. Gel electrophoresis image of *P. aeruginosa* virulence genes 1. *exo*S (118 bp) 2. *exo*U (134 bp) 3. *exo*T (152 bp) 4. *exo*Y (289 bp) 5. *las*I (295 bp) 6. *las*R (130 bp) 7. *rhl*I (155 bp) 8. *rhl*R (133 bp) 9. *las*A (1075 bp) 10. *lasB* (284 bp) 11. *rhl*AB (151 bp) 12. *plc*H (608 bp) 13. *plc*N (481 bp) 14. *pvd*A (1281 bp) 15. *tox*A (270 bp) 16. *phz*I (392 bp) 17. *rhl*II (1036 bp) 18. *phz*M (875 bp) 19. *phz*S (1752 bp) 20. *fliC*A(1020 bp) 21. *fliC*B (1250 bp) 22. *pil*A (1675 bp) 23. *pil*B (408 bp) 14. *psl*A (656 bp) 25. *pel*A (786 bp) 16. *ppy*R(160 bp) 27. *alg*U (292 bp) 28. *alg*L (432 bp) 29. *alg*D (550 bp) gene positive isolates 12. Negative Control (DNA-free master mix) M: 100 bp DNA ladder.

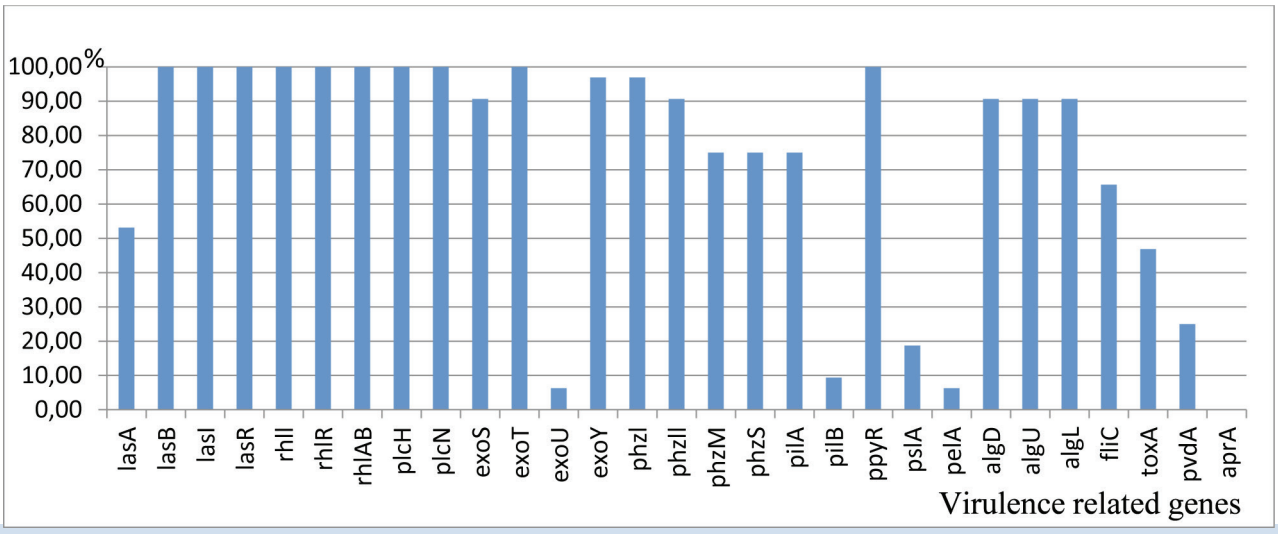

Figure 3. Virulence gene distributions carried by the isolates.

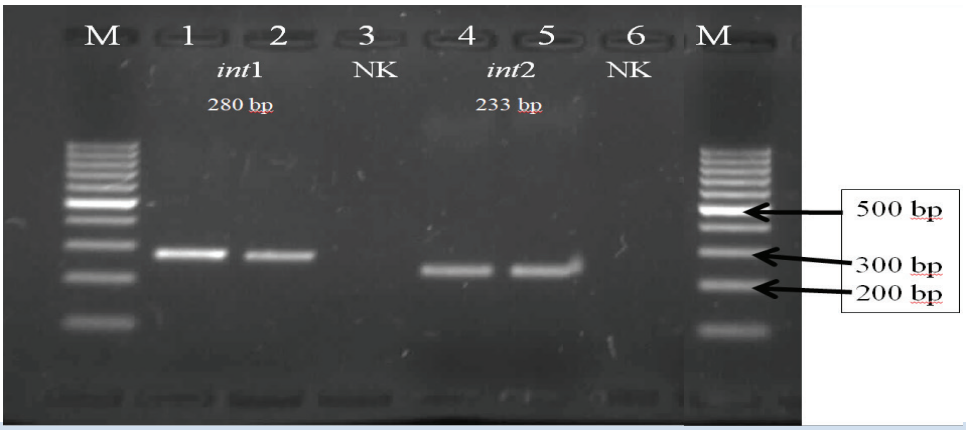

Figure 4. The image of integron classes carried by *P. aeruginosa* isolates on agarose gel. 1. Positive Control (*int*1 positive sequenced field isolate, 280 bp) 2. *Int*1 gene positive *P. aeruginosa* isolate 3. Negative Control (master mix without DNA) 4. Positive Control (*int*2 positive sequenced field isolate, 233 bp) 5. *int*2 gene positive *P. aeruginosa* isolate 6. Negative Control (master mix without DNA) M: 100 bp molecular marker.

Antibiotic resistance

The resistance profiles to fifteen antibiotics from eleven different antimicrobial families were studied. All of the isolates (100.0%) were resistant to ampicillin, penicillin, trimethoprim-sulfamethoxazole, erythromycin, and cefotaxime. Out of 32 *P. aeruginosa* isolates, 26 (81.2%) were found as resistant to amikacin, 21 (65.6%) to tetracycline, 20 (62.5%) to cefoperazone, 8 (25.0%) to enrofloxacin, 5 (15.6%) to ciprofloxacin, 4 (12.5%) to imipenem, 2 (6.2%) to gentamicin, netilmicin and aztreonam (Figure 5.). All isolates obtained in this study showed multiple antibiotic resistance (MDR). Antimicrobial agents used in the study, disc contents, evaluation criteria, references and antibiogram results were given in Table 3.

While all the isolates had multiple antibiotic resistance (Table 3.); in total, there were 13 antibiotic resistance phenotypes (Table 4.).

Statistical analysis

Relationship between *int* genes and antibiotic resistance: It was found that the relationship between the presence of integron genes and gentamicin, amikacin, tetracycline, cefoperazone, imipenem, aztreonam, ciprofloxacin, enrofloxacin resistance was found to be important (Table 5).

Relationship between *int* genes and virulence genes: A significant relationship was found between the presence of integron genes and the presence of *exo*U genes. There was no significant relationship with other virulence genes (Table 6).

Table 3. Antimicrobial agents and results.

Group	Antimicrobial agent	Disc content	≥S	≤R	Reference	R (%)	S (%)
Beta Lactam Penicillins	Ampicillin (AMP)	10 µg	14	11	15	32 (100.0)	0 (0.0)
	Penicillin (PEN)	10 U	22	11	15	32 (100.0)	0 (0.0)
Aminoglyco-sides	Gentamycin (GEN)	10 µg	15	12	14	2 (6.2)	30 (93.7)
	Amikacin (AMI)	30 µg	17	14	14	26 (81.2)	4 (12.5)
	Netilmicin (NET)	30 µg	15	12	14	2 (6.2)	30 (93.7)
Sulfonamides	Trimethoprim/ Sulfametaxazole (TMS)	1.25/23.75µg	16	10	16	32 (100.0)	0 (0.0)
Macrolides	Erythromycin (ERI)	15 µg	18	13	15	32 (100.0)	0 (0.0)
Tetracyclines	Tetracycline (TET)	30 µg	19	14	15	21 (65.6)	0 (0.0)
Lipopeptides	Colistin (COL)	10 µg	11	10	14	0 (0.0)	32 (100.0)
Cephalospo-rins	Cefoperazone (SPZ)	75 µg	21	15	16	20 (62.5)	2 (6.2)
	Cefotaxime (STS)	30 µg	23	14	16	32 (100.0)	0 (0.0)
Carbapenems	Imipenem (IMI)	10 µg	19	15	14	4 (12.5)	28 (87.5)
Monobactams	Aztreonam (AZO)	30 µg	22	15	14	2 (6.2)	18 (56.2)
Fluoroquino-lones	Ciprofloxacin (CIP)	5 µg	21	15	14	5 (15.6)	26 (81.2)
	Enrofloxcin (ENR)	5 µg	21	16	16	8 (25.0)	9 (28.1)

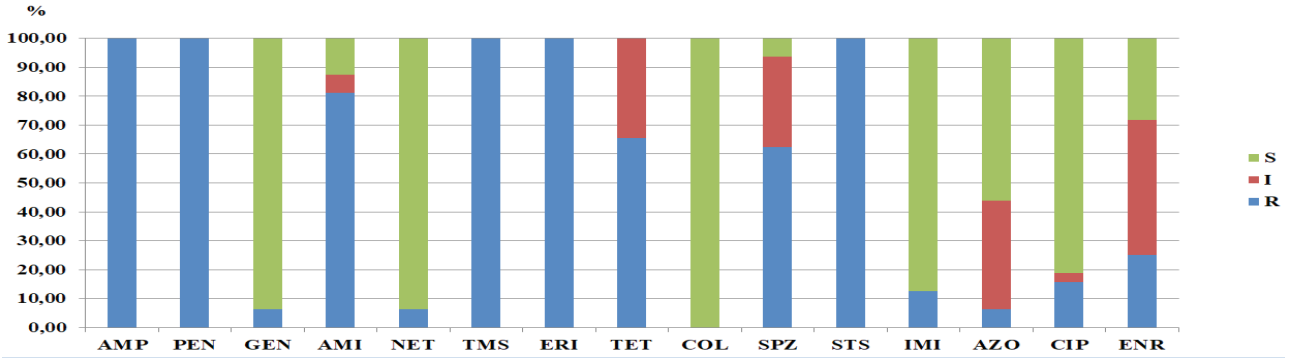

Figure 5. Antibiotic resistance rates of isolates.

Table 4. Antibiotic resistance phenotypes of isolates

No	Number of Resistant Antibiotics	Isolate Number (%)	Total isolate number (%)
1	*(5)	5 (15.6)	5 (15.6)
2	*AMI (6)	3 (9.3)	
3	*NET (6)	1 (3.1)	4(12.4)
4	*AMI, ENR (7)	2 (6.2)	
5	*AMI, TET (7)	1 (3.1)	3 (9.3)
6	*AMI, TET, SPZ (8)	13 (40.6)	13 (40.6)
7	*AMI, TET, SPZ, IMI (9)	1 (3.1)	1 (3.1)
8	*AMI, TET, SPZ, IMI, ENR (10)	1 (3.1)	1 (3.1)
9	*AMI, TET, SPZ, AZO, CIP, ENRO (11)	1 (3.1)	
10	*GEN, AMI, TET, SPZ, CIP, ENR (11)	1 (3.1)	3 (9.3)
11	*AMI, TET, SPZ, IMI, CIP, ENRO (11)	1 (3.1)	
12	*GEN, AMI, TET, SPZ, IMI, CIP, ENRO (12)	1 (3.1)	
13	*AMI, NET, TET, SPZ, AZO, CIP, ENRO (12)	1 (3.1)	2 (6.2)

*: All isolates are resistant to AMP, PEN, TMS, ERI, STS.

Table 5. Antibiotic resistance status of isolates with and without *int* genes

Antibiotic	Integron + (n=11)	Integron – (n=21)	P	χ2
Gentamicin (R)	2	0	0.044	4.073
Gentamicin (S)	9	21		
Amikacin (R)	11	15	0.049	3.868
Amikacin (S)	0	6		
Netilmicin (R)	1	1	0.631	0.231
Netilmicin (S)	10	20		
Tetracycline (R)	11	10	0.003	8.780
Tetracycline S	0	11		
Cefoperazone (R)	11	9	0.002	10.057
Cefoperazone (S)	0	12		
Imipenem (R)	4	0	0.003	8.237
Imipenem (S)	7	21		
Aztreonam (R)	2	0	0.044	4.073
Aztreonam (S)	9	21		
Ciprofloxacin (R)	5	0	0.001	11.313
Ciprofloxacin (S)	6	21		
Enrofloxacin (R)	6	2	0.005	7.804
Enrofloxacin (S)	5	19		

-: Isolates that were resistant/sensitive to all antibiotics (ampicillin, penicillin, trimethoprim-sulfamethoxazole, erythromycin, cefotaxime, colistin) were excluded.

Table 6. The prevalence of virulence related genes in isolates with and without *int* genes

Gen	İntegron + (n=11)	İntegron – (n=21)	P	χ2
***las*A (+)**	4	13	0.169	1.891
lasA (-)	7	8		
***exo*S (+)**	9	20	0.216	1.530
***exo*S (-)**	2	1		
***exo*U (+)**	2	0	0.044	4.073
***exo*U (-)**	9	21		
***exo*Y (+)**	11	20	0.462	0.541
***exo*Y (-)**	0	1		
***phz*I (+)**	11	20	0.462	0.541
***phz*I (-)**	0	1		
***phz*II (+)**	9	20	0.216	1.530
***phz*II (-)**	2	1		
***phz*M (+)**	7	17	0.283	1.154
***phz*M (-)**	4	4		
***phz*S (+)**	7	17	0.283	1.154
***phz*S (-)**	4	4		
***pil*A (+)**	2	6	0.519	0.416
***pil*A (-)**	9	15		
***pil*B (+)**	0	3	0.188	1.734
***pil*B (-)**	11	18		
***psl*A (+)**	3	6	0.938	0.006
***psl*A (-)**	8	15		
***pel*A (+)**	0	2	0.290	1.117
***pel*A (-)**	11	19		
***alg*D, algU, alg L (+)**	9	20	0.216	1.530
***alg*D, algU, alg L (-)**	2	1		
***fli*C (+)**	7	14	0.864	0.029
***fli*C (-)**	4	7		
***tox*A (+)**	6	9	0.170	1.886
***tox*A (-)**	5	2		
***pvd*A (+)**	4	4	0.283	1.154
***pvd*A (-)**	7	17		

-: Isolates carrying / not carrying all of the virulence genes (*las*B, *las*I, *las*R, *rhl*I, *rhl*R, *rhl*AB, *plc*H, *plc*N, *exo*T, *ppy*R, *apr*A) were excluded from the test.

Relationship between virulence genes and resistance to antimicrobial agents: The present results also showed important associations between resistance to certain antibiotics and the presence of *P. aeruginosa* virulence genes: *las*A with ciprofloxacin; *pil*B with netilmicin, cefoperazone and ciprofloxacin; *pel*A with netilmicin and ciprofloxacin; *alg*D, *alg*U, *alg*L with amikacin; aztreonam with *tox*A; *pvd*A with enrofloxacin (Table 7).

Table 7. Relationship between virulence genes isolates and resistance to antimicrobial agents.

Gene		GN		AMI		NET		TET		SPZ		IMI		AZO		CIP		ENR	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
***las*A**	-	14	1	2	12	13	2	0	12	1	12	12	3	8	2	9	5	5	6
	+	16	1	2	14	17	0	0	8	1	8	16	1	10	0	17	0	4	2
	P	0.927		0.986		0.120		0.055		0.117		0.228		0.295		**0.015**		0.072	
***exo*S**	-	3	0	0	3	3	0	0	2	0	2	2	1	1	0	3	0	1	0
	+	27	2	4	23	27	2	0	19	2	18	26	3	17	2	23	5	8	7
	P	0.639		0.683		0.639		0.968		0.896		0.252		0.531		0.683		0.625	
***exo*U**	-	30	0	4	24	28	2	0	2	2	18	27	3	17	2	24	5	7	8
	+	2	0	0	2	2	0	0	19	0	2	1	1	1	0	2	0	2	0
	P	-		0.782		0.706		0.968		0.527		0.09		0.888		0.782		0.065	
***exo*Y**	-	1	0	0	1	1	0	0	0	0	2	1	0	0	0	1	0	1	0
	+	29	2	4	25	29	2	0	21	2	20	27	4	18	2	25	5	8	8
	P	0.793		0.888		0.793		0.160		0.321		0.701		0.423		0.888		0.267	
***phz*I**	-	1	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0
	+	29	2	4	25	29	2	0	21	2	20	27	4	18	2	25	5	8	8
	P	0.793		0.888		0.793		0.160		0.321		0.701		0.423		0.888		0.267	
***phz*II**	-	3	0	0	3	3	0	0	2	0	2	2	1	1	0	3	0	1	0
	+	27	2	4	23	27	2	0	19	2	18	26	3	17	2	23	5	8	8
	P	0.639		0.683		0.639		0.968		0.896		0.252		0.531		0.683		0.567	
***phz*M**	-	7	1	0	7	8	0	0	5	0	5	6	2	6	0	6	2	3	2
	+	23	1	4	19	22	2	0	16	2	15	22	2	12	2	20	3	12	6
	P	0.399		0.359		0.399		0.830		0.670		0.217		0.095		0.611		0.766	
***phz*S**	-	7	1	0	7	8	0	0	5	0	7	6	2	6	0	6	2	3	2
	+	23	1	4	19	22	2	0	16	2	15	22	2	12	2	20	3	6	6
	P	0.399		0.359		0.399		0.830		0.670		0.217		0.411		0.611		0.766	
***pil*A**	-	22	2	4	19	23	1	0	15	2	15	20	4	14	1	20	4	8	6
	+	8	0	0	7	7	1	0	6	0	5	8	0	4	1	6	1	1	2
	P	0.399		0.359		0.399		0.519		0.670		0.217		0.690		0.210		0.477	
***pil*B**	-	27	2	4	24	28	1	0	20	2	20	25	4	17	2	24	5	8	7
	+	3	0	0	2	2	1	0	1	0	0	3	0	1	0	2	0	1	1
	P	0.639		0.122		**0.042**		0.216		**0.026**		0.492		0.531		**0.006**		0.881	
***psl*A**	-	25	1	3	21	25	1	0	17	1	17	23	3	14	1	22	3	9	6
	+	5	1	1	5	5	1	0	4	1	3	5	1	4	1	4	2	0	2
	P	0.242		0.753		0.242		0.952		0.478		0.732		0.320		0.386		0.235	
***pel*A**	-	28	2	4	24	30	0	0	19	2	18	26	4	16	2	0	29	8	8
	+	2	0	0	2	0	2	0	2	0	2	2	0	2	0	2	0	1	0
	P	0.706		0.782		**32**		0.290		0.527		0.581		0.436		**32**		0.637	
***alg*D**	-	3	0	0	1	3	0	0	2	0	2	2	1	2	0	2	1	2	1
	+	27	2	4	25	27	2	0	19	2	18	26	3	16	2	24	4	7	7
	P	0.639		**0.034**		0.639		0.968		0.896		0.252		0.867		0.652		0.183	
***alg*U**	-	3	0	0	1	3	0	0	2	0	2	2	1	2	0	2	1	2	1
	+	27	2	4	25	27	2	0	19	2	18	26	3	16	2	24	4	7	7
	P	0.639		**0.034**		0.639		0.968		0.896		0.252		0.867		0.652		0.183	
***alg*L**	-	3	0	0	1	3	0	0	2	0	2	2	1	2	0	2	1	2	1
	+	27	2	4	25	27	2	0	19	2	18	26	3	16	2	24	4	7	7
	P	0.639		**0.034**		0.639		0.968		0.896		0.252		0.867		0.652		0.183	

***fli*C**	-	10	1	0	9	11	0	0	7	0	7	9	2	7	0	9	2	3	3
	+	20	1	4	17	19	2	0	14	2	13	19	2	11	2	17	3	6	5
	P	0.631		0.052		0.313		0.864		0.551		0.482		0.544		0.743		0.977	
***tox*A**	-	17	0	4	12	16	1	0	9	2	9	14	3	6	0	16	1	6	2
	+	13	2	0	14	14	1	0	12	0	11	14	1	12	2	10	4	3	6
	P	0.120		0.132		0.927		0.108		0.289		0.349		**0.032**		0.130		0.175	
***pvd*A**	-	22	2	4	19	23	1	0	14	2	17	21	3	14	1	19	4	9	7
	+	8	0	0	7	7	1	0	7	0	3	7	1	4	1	7	1	0	1
	P	0.399		0.359		0.399		0.133		0.231		1		0.690		0.798		**0.024**	

-: Isolates carrying / not carrying all of the virulence genes (*las*B, *las*I, *las*R, *rhl*I, *rhl*R, *rhl*AB, *plc*H, *plc*N, *exo*T, *ppy*R, *apr*A) were excluded from the test.

DISCUSSION

The 16S rRNA sequence is used as the "gold standard" to determine the phylogeny of bacterial species (Woese, 1987). Spilker et al. (2004) designed 16S rRNA-based primers that enable *P. aeruginosa* to be identified quickly, simply and reliably and to be distinguished among other closely related *Pseudomonas* species. In this study, 32 of 34 *Pseudomonas* isolates were identified molecularly as *P. aeruginosa* using these primers. In the study, the isolation rate of *P. aeruginosa* from bovine milk with subclinical mastitis was found to be 9.8%. While few studies have reported the isolation of *P. aeruginosa* from milk samples with subclinical mastitis in our country, the isolation rate has varied between 1.1% and 7.9% (Sahin and Erbas, 2015; Tel et al., 2009; Ozturk et al., 2019). In the previous studies, conducted in the world, the isolation rate has been reported as 6.5% (Banerjee et al., 2017) in India, 26.6% (Neamah et al., 2017) in Iraq, and 34% in Iran (Mokhtari et al., 2016). These differences in isolation rates may be due to regional variations and differences between isolation methods. *Pseudomonas* spp. are environmental mastitis agents. It is abundant in wet grounds and can survive for a long time. In addition to taking hygienic measures in farms, applying appropriate milking procedures such as using udder disinfectants before milking and drying teats will reduce the incidence of new cases (Quinn et al., 2011, Kibebew 2017).

In recent years, the emergence and spread of MDR bacteria have resulted in great challenges to the clinical treatment of bacterial infections (Magiorakos et al., 2012). In this study, all isolates were found resistant to ampicillin, penicillin, trimethoprim- sulfamethaxazole, erythromycin and cefotaxime. Therefore, all isolates were assessed to be MDR. Beta-lactams, penicillins, sulfonamides, macrolides, and cephalosporins are commonly used to treat mastitis in Türkiye. This high MDR rate was not surprising, when added to all of these, the structural its natural resistance properties (to penicillins, ampicillin, cephalosporins, trimethoprim-sulfamethaxazole) of *P. aeruginosa* and non-moderate of antibiotics. Antibiotic susceptibility test results showed that the isolates have high-level resistance to amikacin, tetracycline, cefoperazone (62.5-81.2%) and low-level resistance (6.2-25.0%) to enrofloxacin, ciprofloxacin, imipenem, gentamicin, netilmicin, aztreonam. Due to the differences in the antimicrobial treatments applied, the antibiotic resistance phenotypes of bacteria isolated from subclinical bovine mastitis may differ among countries or even regions. Large differences have been observed in terms of antibiotic resistance against bacteria isolated from mastitis milk samples in different countries. Thus, this study could be a guide to be understood the importance of antibiotic susceptibility testing and periodic surveillance of antibiotic susceptibility of mastitis-causing bacteria.

All isolates examined in this study were found susceptible to colistin. Colistin is a lipopeptide antibiotic used as a "last resort" when other antibiotic treatments are unsuccessful in cases where various super bacterial infections are seen (Magiorakos et al., 2012). In a study conducted in our country in 2015, it was reported that colistin resistance was not found, as in this study (Sahin and Erbas, 2015). However, a study conducted in Egypt in 2016 showed that all isolates were resistant to colistin on the contrary (Ama et al., 2016).

Fluoroquinolones are broad-spectrum bactericidal drugs and resistance to these drugs is rapidly developing. However, they are effective drugs against many Gram-negative bacilli, including *P. aeruginosa* (Magiorakos et al., 2012). Among *P. aeruginosa* isolates, it has been reported that fluoroquinolone resistance has increased at an alarming rate due to its widespread use (Gasink et al., 2006). However, the low number

of fluoroquinolone-resistant isolates in this study is promising since these antimicrobials can still be used in *Pseudomonas* mastitis in the farms where the material was taken.

Carbapenems constitute the newest group of beta lactam antibiotics. They are among the first antibiotic groups used in multi-resistant Gram-negative bacterial infections due to their resistance to extended-spectrum beta lactamase enzymes and their ability to pass through the bacterial membrane rapidly (Magiorakos et al., 2012). They are also effective on many bacteria in terms of human medicine (Magiorakos et al., 2012). In the study, imipenem resistance was found with a value of 12.5%. According to our current information, isolated from mastitic in dairy cattle in Türkiye *P. aeruginosa* isolates although not reported imipenem resistance, in Egypt years ago in Gram-negative bacteria (*Enterobacter cloacae, Citrobacter freundii, Proteus mirabilis, Pseudomonas stutzeri*) has been reported as 11.8% (Ahmeds et al., 2011).

Resistance of mastitis pathogens to antimicrobial drugs in dairy cows is a common issue worldwide. Many studies have shown antibacterial susceptibility patterns of bacteria isolated from mastitis (Ama et al., 2016; Oztürk et al., 2011; Sahin and Erbas, Tel et al., 2009). To our knowledge, *P. aeruginosa* isolated from mastitis in dairy cattle has not been reported as imipenem resistant in Türkiye. However, there are very few studies examining integron (Mokhtari et al., 2016) or virulence genes carried by the bacteria (Banerjee et al., 2017; Neamah et al., 2017).

P. aeruginosa with many virulence factors affects the outcome of infections. However, when the studies investigating the virulence genes of *P. aeruginosa* isolates isolated from the milk of cattle with mastitis are examined; as in our study, no study was found in which all virulence genes were examined collectively. Type III secretion system is a virulence factor that affects somatic cell numbers by damaging udder epithelial cells in intra-mammary infections (Roy-Burman et al., 2001). However, only a few studies have investigated the type III secretion system of *P. aeruginosa* isolated from bovine milk (Park et al., 2014; Szmolka et al., 2012). Type III secretion system proteins include cytotoxin (*exo*U), ADP-ribosylating enzymes (*exo*S and *exo*T), and adenyl cyclase (*exo*Y). *exo*U kills a variety of eukaryotic cells through its phospholipase activity. *exo*T and *exo*Y play a role in the adhesion, phagocytosis and systemic spread of bacterial cells together with *exo*S (Vance et al., 2005). *exo*S and *exo*U are more important than *exo*T and *exo*Y in bacterial pathogenesis (Engel et al., 2009). For example, invasive strains that can cross the epithelial barrier and spread the *P. aeruginosa* infection to tissues by means of *exo*S, *exo*T, and *exo*Y genes. Cytotoxic strains that cause inflammation and tissue necrosis with their cytotoxic effects typically have genes for *exo*U, *exo*T, and *exo*Y (Fleiszig et al., 1997). In studies on human, environmental and bovine isolates of *P. aeruginosa* in Korea, 99.2% of the isolates were reported to have at least 1–4 virulence genes related to TTSS (Selezska et al., 2007; Szmolka et al., 2012; Wiehlmann et al., 2007). Park et al. (2014) found that 68.8% of 122 *P. aeruginosa* isolates were non-cytotoxic / invasive strains (*exo*U– / exoS +), while only 8.2% of isolates were cytotoxic / non-invasive strains (*exo*U + / *exo*S–); interestingly, they reported 6 isolates identified with both *exo*U and *exo*S (Park et al., 2014). This is a remarkable finding because exoU and exoS were not detected together in human and environmental isolates (Wiehlmann et al., 2007; Selezska et al., 2007). In this study, 29 of 32 *P. aeruginosa* isolates were invasive (*exo*S +, *exo*U–), 2 of them were cytotoxic (*exo*U +, *exo*S–); There were no cytotoxic (*exo*U +) or invasive (*exo*S–) strains. *exo*T and *exo*Y were detected in both invasive and cytotoxic strains. It is known that both cytotoxic and invasive strains can cause inflammation and necrosis of infected epithelial cells (Fleiszig et al., 1997). In addition, in this study, it was determined that the relationship between the presence of integron genes and cytotoxic isolates containing only *exo*U virulence gene was found to be important.

The pathogenicity of *P. aeruginosa* is regulated by enzymes (e.g., elastase and protease) released outside the cell (Kipnis et al., 2006; Woods et al., 1983). Most *P. aeruginosa* isolates that cause infection synthesize exotoxin A, encoded by the *exo*A gene. This toxin is responsible for an invasion and regional tissue damage of the bacteria (Woods et al., 1983). Neamah (2017) reported that the *tox*A gene was detected in 100% of strains while the *exo*S gene was in 75% of *P. aeruginosa* isolates isolated from the milk of cows with mastitis. Banerjee et al. (2017) detected the *tox*A gene at a rate of 63.2% and the *exo*S gene at a rate of 36.8%. Similar to these studies (Banerjee et al., 2017; Neamah et al., 2017), many studies including virulence factors such as *tox*A and *exo*S have generally been reported at high rates and have supported the data of our study.

Alginate is a linear polymer consisting of a C-5 epimer of 1-4-linked saccharide β-D mannuronic acid and α-L-guluronic acid (Ghadaksaz et al., 2015; McIntyre-Smithet et al., 2010). Three genes (*alg*D, *alg*U, *alg*L) from three alginate biosynthesis are available (Ghadaksaz et al., 2015). Alginate production reduces macrophage phagocytosis and complement activation (Ghadaksaz et al., 2015; McIntyre-Smithet et al., 2010). In addition, it has been shown that excessive alginate production may lead to the development of resistance to some antibiotics (Hentzer et al., 2001). In this study, 90.6% of the isolates were multi-antibiotic resistant isolates with alginate synthesis genes. However, although there are studies reporting that alginate has a fundamental role in biofilm structure (McIntyre-Smithet et al., 2010); there are also studies reporting that alginate production ability and the frequency of alginate genes are not significantly different between biofilm-forming and non-biofilm-forming isolates (Ghadaksaz et al., 2015).

The alkaline protease is a fibrinolysis-effective metalloprotease of *P. aeruginosa*. The 49-kDa enzyme is encoded by the *apr* gene. It has been shown that in the early stage of acute lung injury, the dissolution of dense fibrin formed in the alveoli with alkaline protease leads to the progression of the infection (Kipnis et al., 2006). Although the role of alkaline protease in tissue invasion and systemic infections is not fully known, it could be important in the pathogenesis of corneal infections (Howe et al., 2006). Ertugrul et al. (2018) reported that although *fli*C, *tox*A and *phz*S genes are the most frequently detected genes in *P. aeruginosa* isolates isolated from the diabetic foot; reported the absence of the *apr*A gene. Similarly, in this study, *apr*A was not detected in the isolates. However, all isolates had ten virulence genes (*las*I, *las*R, *rhl*I, *rhl*R, *las*B, *rhl*AB, *plc*H *plc*N, *ppy*R *exo*T). The difference in virulence genes detected in the studies may be due to the different origins of the isolates.

Ten virulence genes (*las*I, *las*R, *rhl*I, *rhl*R, *las*B, *rhl*AB, *plc*H *plc*N, *ppy*R *exo*T) were detected in the isolates obtained from this study, while some virulence genes (*exo*S, *exo*T, *phz*I, *phz*II, *phz*M, *phz*S, *alg*U, *alg*L, *alg*D) were seen high percentages (75%-96.8%). Evaluating the results in terms of gene groups, most of the genes involved in the formation of QS, phospholipase, T3SS, phenazine operon, biofilm and alginate formation were detected in the isolates obtained in this study. Although the virulence genes were seen at high rates in the isolated strains; the pathogenicity of the isolate also depended on the expression of virulence factors. In future studies, it could be examined whether these virulence genes are expressed phenotypically or not.

Antibiotic resistance in bacteria can be acquired by mobile genetic elements such as a plasmid, transposon, and insertion sequences. Integrons are genetic structures found in some transposons or plasmids and they are responsible for the acquisition of new genes. Integrons also play an important role in the emergence and spread of multi-drug resistant strains, especially in a very short time. (Mazel et al., 2006). In the investigation of the genetic basis of *P. aeruginosa*'s multi-drug resistance, integron related gene cassettes could be recommended for further steps. Among the five classes of integrons discovered, class 1 integrons are the most important group with regards to antibiotic resistance genes to be transferred (Mazel et al., 2006). In this study, 21.9% of the *P. aeruginosa* isolates were categorized as class 1 while 12.5% of them were in class 2 integrons and none of the isolates carried both classes of integron genes together. It was reported that in Iran, *P. aeruginosa* isolates obtained from mastitic milk of cattle carried 1.9% class 1 integrons (Mokhtari et al., 2006). However, the rate of integron class 1 genes was higher in clinical isolates (27.5%-55.5%) (Goli et al., 2017; Faghri et al., 2018). Similar to our findings, the prevalence of the class 2 gene in isolates of *P. aeruginosa* was variable, which was generally found to be lower than the class 1 integron (Faghri et al., 2018).

In this study, 34.4% of the isolates were with integron genes. The correlation between the presence of integron genes and resistance to some antibiotics (gentamicin, amikacin, tetracycline, cefoperazone, imipenem, aztreonam, ciprofloxacin, enrofloxacin) was significant. A similar significant relationship between the presence of integrons and antibiotic resistance (gentamicin, ticarcillin, imipenem, amikacin, cefotaxime and ofloxacin) has been observed in other studies (Kouchaksaraei et al., 2012). Similarly, in this study, a significant relationship was found between the presence of integron genes and the presence of *exo*U virulence gene. In addition to that, the present results have shown an important interaction between resistance to certain antibiotics and the presence of *P. aeruginosa* virulence genes. Overall, these results may explain the potential of pathogens with these virulence genes to attenuate antibiotic activity by suppressing host immune cells.

CONCLUSIONS

With this study, multiple antibiotic resistance in *P. aeruginosa* obtained from subclinical mastitis isolates in Türkiye was examined firstly in terms of integron presence and virulence genes. However, in this study, we couldn't investigate the genetic mechanisms of resistance, we were able to examine the antibiotic resistance phenotypically in isolates. In future, it could be investigated whether the selective pressure in different geographical regions causes changes in the integron gene content by determining the antibiotic resistance genes in the isolates containing integron. However, integron-related gene cassettes should be examined to investigate the genetic basis of *P. aeruginosa* multi-drug resistance.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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