



Journal of the Hellenic Veterinary Medical Society

Vol 74, No 1 (2023)



To cite this article:

Turkyilmaz, S., & Ocak, F. (2023). Comparative study on genotypic properties of Pseudomonas aeruginosa isolated from subclinical mastitis in Türkiye. *Journal of the Hellenic Veterinary Medical Society*, *74*(1), 5387–5402. https://doi.org/10.12681/jhvms.29590 (Original work published April 12, 2023)

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

F. Ocak¹, S. Turkyilmaz^{2,*}

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

²Department 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 aeru*ginosa 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 (χ^2) test. Ten virulence genes (*las*], *las*B, *rhl*, *rhl*R, rhlAB, plcH, plcN, ppyR, exoT) were found in all isolates whilst another virulence gene (aprA) 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 exoU 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 Date of initial submission: 09-02-2022 Date of acceptance: 27-06-2022

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 pilA 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 algD, algU algL 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 (plcH, plcN) 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 toxA gene, which is responsible for the invasion and regional tissue damage of the bacteria (Woods and Iglewski, 1984). The pvdA 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 phzI, phzII, phzM, phzS genes (Denning et al., 1996). The toxins of *P. aeruginosa* released by the type III secretion system (T3SS) are: exoS (invazin), exoT, exoY and exoU (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 phzS gene, which allows bacteria to survive tissue damage under anaerobic conditions. The third is the toxA 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. pslA and pelA 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 ppyR 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 (InstaGeneTm 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

Fable 1. Primers used in this stud	y
------------------------------------	---

$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Virulenece gene/Gene Group	Target Gene	Sequence (5'-3')	Product Length (bp)	T _m	Referenece	Result (%)
spp. CACTGGTGTTCCTTCCTTAA 01.5 55.3 al. 2004) Lof RNA P aerogiono GGGGATCTCGGACGACCTGA 956 6.10 22.9.8) Inlegrase Incl CCTCGCACGATGTC 280 57.2 190 Inlegrase Incl CCTCGCACGATGTCTTCCC 231 470 etal.2001 0.00.9 Inlegrase Incl ACTGGGTGGCGCAAGGAGTG 58.4 etal.2001 0.00.9 Protease QS IssAF GGAAGGAAGGTCC 1075 52.8 (F2rel) 101.9 32 (100.0) Ado induce synthesi potain IssAF GGAAGGAAGCCCAAGGACCTTGCCGGAC 24 64.4 201.9 32 (100.0) Ramenyl Insal activator protain IssBF AGGTGGCAAGAACCTTGGAGGAACCTGGAG 13 5 32 (100.0) Synthesion Sector <i>inla</i> RF AAGTGGGAAGGAACCTGGAG 13 55.5 32 (100.0) Ado induce synthesion IssRF AAGTGGAAGGACACTGGAGGAACTGGAGGAGAGTA 53.9 32 (100.0) 32 (100.0) Synthesion Sector <i>inla</i> RF AAGTGGGCAGGAAGACTGGAGGAACTGGGAGGAGTA </td <td>16S rRNA</td> <td>Pseudomonas</td> <td>GACGGGTGAGTAATGCCTA</td> <td>618</td> <td>56.7</td> <td>(Spilker et</td> <td>34 (10.4)</td>	16S rRNA	Pseudomonas	GACGGGTGAGTAATGCCTA	618	56.7	(Spilker et	34 (10.4)
168 rRNA P. aeruginan GGGGATCTTCGGACCTCA 956 61.0 32 (9.8) Integrase Int1 CCTCCGGACGATCACCC 280 57.2 (Rass ct al., 72.19) Integrase Int2 TTATTGCTGGACGATCACCC 233 51.6 (Gubbin) 4(12.5) Integrase Int2 TTATTGCTGGACATCACC 233 51.6 (Gubbin) 4(12.5) Integrase Int2 TTATTGCTGGACGTGCGGTC 233 51.6 (Gubbin) 4(12.5) Integrase Int3 TOTTCTTGTATGGCAGGTGC 600 53.4 0(0.0) Integrase Int3 GGAATGCAGTGCGCGCG 284 64.4 2014) 22 (100.0) Ato indecer synthesis protein IasBR TGCGTGCACGAGTGGAGG 295 5.7 23 (100.0) 23 (100.0) QS Inscriptional activator protein IasR AGTGGGAAATGGGAGTGGAGG 130 5.5 32 (100.0) Qs Inscriptional regulatorQS IasR AGTGGGAAGTGGAGG 130 5.5 32 (100.0) Qs Inskriptional regulat		spp.	CACTGGTGTTCCTTCCTATA	018	55.3	al., 2004)	
$ \begin{array}{c} \mbox{trans} & \mbox{trans} $	16S rRNA	P aeruginosa	GGGGGATCTTCGGACCTCA	956	61.0		32 (9.8)
$ \begin{array}{l} \mbox{Integrase} & Int \\ \mbox{TCCCCCCACGACCACCCCC} 280 & 57.2 & [199] \\ \mbox{TCCCCCCCCCCCACCACGACC} 280 & 57.2 & [199] \\ \mbox{TCCCCCCCCCCCCCCCCC} 280 & 51.6 & (1601stain) & 4(12.5) \\ \mbox{TCCCCCCCCCCCCCCCC} 280 & 55.8 & (1601stain) & 4(12.5) \\ \mbox{Integrave} & Int3 & TGTTCTTGTTGGGGGGGGGGGGGGGGGGGGGGGGGGG$		1. uer aginosa	TCCTTAGAGTGCCCACCCG	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	61.0		
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Integrase	Int1	CCTCCCGCACGATGATC	280	57.2	(Bass et al.,	7 (21.9)
$ \begin{array}{l} \mbox{Integrase} & m2 & 1.02 & 1.000 m 1000 m 2 (12.3) & 1.03 & $	-		TATTCCTCCCATTACCC	222	51.6	(Goldstein	4 (12.5)
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$	Integrase	Int2	ACGGCTACCCTCTGTTATC	233	57.3	(001031011)	4 (12.3)
$ \begin{array}{l} \mbox{Integrave} & \mbox$			AGTGGGTGGCGAATGAGTG	600	59.5	et al., 2001)	0 (0.0)
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Integrase	Int3	TGTTCTTGTATCGGCAGGTG	000	58.4		0 (010)
$ \begin{array}{llllllllllllllllllllllllllllllllllll$	Proto and IOS	lasAF	GCAGCACAAAAGATCCC	1075	52.8	(Fazeli and	17 (53.1)
	Protease/QS	lasAR	GAAATGCAGGTGCGGTC	10/5	55.2	Momtaz,	
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Flastase/OS	lasBF	GGAATGAACGAAGCGTTCTCCGAC	284	64.4	2014)	32 (100.0)
Auto inducer synthesis proteinlast RCCTGCTCAAGTGTTCAAGG29556.732 (100.0)QSmained activator protein/last RAAGTGGAAAAATGCAAGTGCAAG13065.932 (100.0)Acyl-homoserine-lactonerhll FTTCATCCTCCTTTAGTCTTCCC15558.4et al., 2014)32 (100.0)Acyl-homoserine-lactonerhll FTTCCATCGATTCGAGAGGC13355.532 (100.0)Arguinase/QSrhll BFTTCCATCGATTCTCCAGAGAGC15157.932 (100.0)QSrhl ABFTTCATGGAAATATCATGCACACCC15157.932 (100.0)QSrhl ABFTTCATGGAATATCATGCACACCC15157.60 (0.0)QSrhl ABFTTCATGGAATATCATGCACACC57.932 (100.0)Alkaline protesseaprAFGTCCACCCGGGGGGGAGACAGATA99396.60 (0.0)Descine/Tagglin proteinfiCCFGGCAGCCCGGGGGGAGACACA70.957.621 (65.6)Protein/Tagglin proteinfiCCFGGCAGCCGCGGGCGGAGCCAGCC60.141.4(Razi and 22 (100.0)Proslein/Tagglin proteinfiCCFGGCAGCCGCGGCGGAGCCGAGCC60.141.4(Razi and 22 (100.0)Proslein/Tagglin proteinfiCCFGCCAGCGCGCGGCGGAGCCGAGCC60.141.4(Aiayi et al. 2014)32 (100.0)Proslein/Tagglin proteinfiCCFGCCAGCGCGCGGCGGAGCCGAGCC60.541.4(Aiayi et al. 2014)32 (100.0)Proslein/Tagglin proteinpiclARCCCGTGGGCGCGGCGGAGCCGAGCC60.540.440.00.3ProspholipasepiclAR <td>Elastase QB</td> <td>lasBR</td> <td>TGGCGTCGACGAACACCTCG</td> <td>204</td> <td>63.5</td> <td></td> <td></td>	Elastase QB	lasBR	TGGCGTCGACGAACACCTCG	204	63.5		
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	Auto inducer synthesis protein/	lasIF	CGTGCTCAAGTGTTCAAGG	295	56.7		32 (100.0)
$ \begin{array}{llllllllllllllllllllllllllllllllllll$	QS	lasIR	TACAGICGGAAAAGCCCAG		56.7		22 (100 0)
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	Iranscriptional activator protein/	lasRF		130	55.9 62.0	(Sabhamyal	32 (100.0)
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	QS A gul homogoring lastons	USKK white	TTCATCCTCCTTTACTCTTCCC		58.4	(Sabharwai	32 (100.0)
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	synthase/OS	rh/IR		155	56.7	et al., 2014)	32 (100.0)
$ \begin{array}{llllllllllllllllllllllllllllllllllll$	synthase, QD	<i>rh/</i> RF	TGCATTTTATCGATCAGGGC		55.5		32 (100.0)
$ \begin{array}{llllllllllllllllllllllllllllllllllll$	Transcriptional regulator/QS	<i>rh/</i> RR	CACTTCCTTTTCCAGGACG	133	56.7		52 (10010)
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Rhamnosyl transferase/	rhlABF	TCATGGAATTGTCACAACCGC	1.51	57.9		32 (100.0)
	QS	<i>rhl</i> ABR	ATACGGCAAAATCATGGCAAC	151	55.9		
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Alkaline metalloproteinase/	aprAF	GTCGACCAGGCGGCGGAGCAGATA	003	69.6	-	0 (0.0)
$ \begin{array}{l lllllllllllllllllllllllllllllllllll$	Alkaline protease	aprAR	GCCGAGGCCGCCGTAGAGGATGTC	995	71.3	_	
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Flagellar filament structural	<i>fli</i> CF	GGCAGCTGGTTNGCCTG	Type A: 1.02 kb	57.6		21 (65.6)
$ \begin{array}{llllllllllllllllllllllllllllllllllll$	protein/Flagella protein	fliCR	GGCCTGCAGATCNCCAA	Type B: 1.250 kb	55.2	(T. 1)	22 (100.0)
$\begin{array}{llllllllllllllllllllllllllllllllllll$	Haemolytic phospholipase C/	<i>plc</i> HF	GCACGIGGICAICCIGAIGC	608	61.4	(Fazeli and	32 (100.0)
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	Phospholipase	plcHK plcNF			61.0 66.1	Momtaz,	32 (100.0)
$\begin{array}{ c c c c c c c c c c c c c c c c c c c$	Phospholipase	<i>pleNR</i>	TEGETGTEGAGEAGGTEGAAC	481	63.7	2014)	32 (100.0)
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Exotoxin A/Toxin	torAF	CTGCGCGGGTCTATGTGCC		63.1	-	15 (46.8)
	Exotoxin'r Foxin	toxAR	GATGCTGGACGGGTCGAG	270	60.5		15 (10.0)
Pyoverdin biosynthetic pathway $pvdAR$ TTCAGGTGCTGGTACAGG128156.0Exoenzyme S/T3SSexoSRGCGAGGTCAGCAGAGTACG11861.4(Ajayi et al., 29 (90.6)Phospholipase/T3SSexoUFCCGTTGTGGTGCCACTGGGAGGC11861.42003)Phospholipase/T3SSexoUFCCGATTCTATGGCACCGACTGCG13458.832 (100.0)Exopolysaccharide productionexoVFCCGATTCTATGGCAGCACCGC13461.42 (6.2)protein/T3SSexoVFGCCCTTGATGCACTCGACCGC161.42 (6.2)Exoenzyme T/T3SSexoTFAATCGCCGTCCAACTGCGCG61.431(96.8)exoorpphzIFCATCAGCTTAGCAATCCC39253.7(Fazeli and 31(96.8)operonphzIFCGCAATGGCGATTGTCTGTCGGG54.690.6)operonphzIRCGCATTGACGATATGGAAC103656.02014)29 (90.6)operonphzIRCGCATGACGATATGGAAC103656.02014)29 (90.6)operonphzIRCGCATGACGATATGGAAC103656.02014)29 (90.6)Phenazine operonphzIRATGCGCGTTCCAACTGGACAG87561.424(75.0)Phenazine operonphzSRACAACCTGACCGACACG61.424(75.0)Phenazine operonphzSRACAACCTGAACCGCACCCC175261.424(75.0)Phenazine operonphzSRACAACCTGAACCGCACCCCC175261.424(75.0)Phenazine operonphzSRACAACCTGAACCGCACCCCC60.351.73 (9.3)protein/Fimbrial prot	L-ornitin N5-oksijenaz/	pvdAF	GACTCAGGCAACTGCAAC	1201	56.0	-	8 (25.0)
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	Pyoverdin biosynthetic pathway	pvdAR	TTCAGGTGCTGGTACAGG	1281	56.0		
$ \begin{array}{c} \mbox{Exotil} \mbox{in} \mbo$	Excenzyme S/T3SS	exoSF	GCGAGGTCAGCAGAGTATCG	118	61.4	(Ajayi et al.,	29 (90.6)
Phospholipase/T3SSexoUFCCGTITGTGTGTGCGCCGTTGAAG13458.832 (100.0)Exopolysaccharide productionexoYFCCGAGATGTTCACCGACTCGC13461.42 (6.2)Exopolysaccharide productionexoYFCGGATT CTATGGCAGGGAGG28961.42 (6.2)protein/T3SSexoTRTGTTCGCCAGAGTACGCATGCG15261.42 (6.2)Exoenzyme T/T3SSexoTRTGTTCGCCGAGGTACTGCTC15261.431 (96.8)operonphzIFCATCAGCTTAGCAATCCC39253.7(Fazeli and 56.02016.8)operonphzIIRCGGAGAAACTTTTCCCTC39253.7MomtazPhenazine operon II/PhenazinephzIIFGCCAAGGTTTGTTGTCGG103656.02014)29 (90.6)operonphzphzIRCGGAGAGCGGACGGACGGACG56.02014)29 (90.6)operonphzphz/RFATGGAGGGGATCGACGG56.02014)29 (90.6)operonphz/RFATGGAGGGGATCGACGG61.424 (75.0)Flavin-dependent hydroxylase /phzSFTCGCCATGACCGATACGCTC175261.424 (75.0)Phenazine operonphz/RFACAACCTGAGCGTCCACGGG65.08 (25.0)7Phenazine operonphz/RFTCGCAACGACTCCACGGCGC56.08 (25.0)Phenazine operonphz/RFTCGCACTGACGACGCGC61.424 (75.0)Phenazine operonphz/RFACAACCTGAGCGTCCCCC175261.424 (75.0)Phenazine operonphz/RFTCGCAACGACTCCACGGCGCCCC56.08	Extenzyme 5/1555	exoSR	TTCGGCGTCACTGTGGATGC	110	61.4	2003)	
Let LexoURCCAGATGTTCACCGACTCGC61.4Exopolysacharide productionexoYFCGGATTCTATGGCAGGGAGG28961.42 (6.2)protein/T3SSexoYRGCCCTTGATGCACTCGACCA28961.431 (96.8)Exoenzyme T/T3SSexoTFAATCGCCGTCCAACTGCATGCC15261.431 (96.8)Phenazine operon l/PhenazinephzIFCATCAGCTTAGCAATCCC39253.7(Fazeli and 31 (96.8)operonphzIRCGCAATGACGATTGTGTCGG103656.0201429 (90.6)operonphzIRCGCATGACGATATGGACA103656.0201429 (90.6)operonphzIRATGGAGAGAGCGGGATCGACAG87561.424 (75.0)Phenazine operonphzMRATGGAGAGCGGGATCGACAG87561.424 (75.0)Phenazine operonphzMRATGGAGAGCGGACGCGCTC61.424 (75.0)Phenazine operonphzMRATGGAGCGATACGGCTC61.424 (75.0)Phenazine operonphzMRATGGAGCATCGACGATACGGTC61.424 (75.0)Phenazine operonphzMRATGGAGCATCGACGATCGGCTC61.424 (75.0)Phenazine operonphzMRTTGACCTGAGCGATACGGTC61.424 (75.0)Phenazine operonphzMRACGACCTCACCCAGCGCTCC61.424 (75.0)Phenazine operonphzMRTTGACCTGAGCAACGCGTC61.424 (75.0)Piontini protein/Fimbrial proteinpi/BRTTGACCTGAGCGATACGCTC61.424 (75.0)Piotein/Fimbrial proteinpi/BRCTCCTACCTCAGCGCAACTCG60.0 </td <td>Phospholipase/T3SS</td> <td>exoUF</td> <td>CCGTTGTGGTGCCGTTGAAG</td> <td>134</td> <td>58.8</td> <td></td> <td>32 (100.0)</td>	Phospholipase/T3SS	exoUF	CCGTTGTGGTGCCGTTGAAG	134	58.8		32 (100.0)
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $		exoUR	CCAGATGTTCACCGACTCGC		61.4		2 ((2)
protein 7535exoTRGCCCTTCAACTGCACTGCAC01.4Exoenzyme T/T3SSexoTRTGTTCGCCGCACACTGCACGCG64.031(96.8)operonphzIRCATCAGCTTAGCAATGCC39253.7(Fazeli and 56.031(96.8)operonphzIRCGGAGAAACTTTCCCTC39253.7MomtazPhenazine operon II/PhenazinephzIIRGCCAATGGAAGCTTGTTGTCGG103656.02014)29 (90.6)operonphzIIRCGCATTGACGATATGGAAC103654.67Phenazine operon II/Phenazine operonphzMRATGGAGAGCGGGATCGACAG87561.424(75.0)transferas/Phenazine operonphzMRATGGCGGGTTCCCATCGGCAG61.424(75.0)Finansferas/Phenazine operonphzSFTCGCCATGACCGATACGCTC175261.424(75.0)Finabrial protein/Fimbrial proteinpilAFACAGCATCCAACTGAGCGGGG66.08 (25.0)Fimbrial protein/Fimbrial proteinpilAFTCGAACTGAGGGGAACATCG56.08 (25.0)protein/Fimbrial proteinpilAFTCCGAACTGAGCAGCGGGGGGGGGGGGGGGGGGGGGGGG	Exopolysaccharide production	exo Y F		289	61.4 61.4		2 (6.2)
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	protein/1555	eroTE	A ATCGCCGTCCA ACTGCATGCG		64.0		31(96.8)
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Exoenzyme T/T3SS	eroTR	TGTTCGCCGAGGTACTGCTC	152	61.4		51(90.0)
operon $phzIR$ CGGAGAAACTTTTCCCTC 392 53.7 MomtazPhenazine operon $phzIIF$ GCCAAGGTTTGTTGTCGG 1036 56.0 2014) 29 (90.6)Operon $phzIIR$ CGCATTGACGATATGGAAC 1036 54.6 54.6 Phenazine specific methyl $phzMF$ ATGGAGAGGGGGATCGACAG 875 61.4 $24(75.0)$ Flavin-dependent hydroxylase / $phzSF$ TCGCCATGACCGATACGCTC 1752 61.4 $24(75.0)$ Phenazine operon $phzSR$ ACAACCTGAGCCAGCCTTCC 1752 61.4 $24(75.0)$ Phenazine operon $phzSR$ ACAACCTGAGCCAGCCTTCC 1752 61.4 $24(75.0)$ Phenazine operon $phzSR$ ACAACCTGAGCCAGCCTCC 1675 56.0 $8(25.0)$ Phenazine operon $phzSR$ ACAACCTGAGGAGTGAGCGGGG 1675 56.0 $8(25.0)$ Primbrial protein/Fimbrial protein $piIAR$ TTGACTTCCTCCAGCGGG 408 53.7 $3(9.3)$ protein/Fimbrial protein $piIBR$ CTTTCGGAGTGAACATCG 60.3 et al., $6(18.7)$ Biofilm formation protein/ $psIAR$ TGTGTAGCCGAGCGCACTCCT 656 61.4 $32(100.0)$ Biofilm formation protein/ $peIAR$ CGCATTCGCCGCACTCCAG 60.5 $-$ Pyoverdin operon editor/ $ppyRR$ ACGCAGAGCCTCCCAACCG 60.5 $-$ Pyoverdin operon editor/ $ppyRR$ ACGCAGAGCCTCCCAACCG 61.4 $32(100.0)$ Alginate formation $algUF$ CCGCTTGCCGCAGAGGAGTG 292 61.4 $29(90.$	Phenazine operon I/Phenazine	phzIF	CATCAGCTTAGCAATCCC	202	53.7	(Fazeli and	31(96.8)
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	operon	phzIR	CGGAGAAACTTTTCCCTC	392	53.7	Momtaz	· · /
operon phz IIRCGCATTGACGATATGGAAC105054.6Phenazine specific methyl phz MFATGGAGAGCGGGATCGACAG875 61.4 24(75.0)transferase/Phenazine operon phz MRATGCCGGGTTTCCATCGGCAG 875 61.4 24(75.0)Flavin-dependent hydroxylase / phz SFTCGCCATGACCGATCAGCGTC 1752 61.4 24(75.0)Phenazine operon phz SRACAACCTGAGCCAGCCTTCC 1752 61.4 24(75.0)Fimbrial protein/Fimbrial protein pil AFACAGCATCCAACTGAGCG 61.4 24(75.0)Type IV fimbrial biogenesis pil BRTTGACTTCCTCCAGGGTG 1675 56.0 8 (25.0)protein/Fimbrial protein pil BRCTTTCGGAGTGAACATCG 408 53.7 3 (9.3)protein/Fimbrial protein pil BRCTTTCGGAGTGAACATCG 60.3 et al.,Biofilm formation protein/ psl ARTGTTGTAGCCGTAGCGTTCTG 666 61.4 (GhadaksazBiofilm formation protein/ pel AFCATACCTTCAGCCATCCGTTCTC 786 62.7 2015) 2 (6.2)Biofilm operon editor/ ppy RFACGCAGTCCCAACCG 61.4 32 (100.0) 7 Transmembrane protein ppy RFCGTGTGACCGCAGAGGCGC 61.4 29 (90.6)Alginate synthesis gene L/ alg UFCGGCTCCCAGCGCCAACAGA 59.8 $-$ Alginate formation alg UFCGCGCTCACGCCCACCC 550 64.4 29 (90.6)Alginate formation alg DFAGAAGTCGCAACGCAACCC 550 64.4 29	Phenazine operon II/Phenazine	phzIIF	GCCAAGGTTTGTTGTCGG	1036	56.0	2014)	29 (90.6)
Phenazine specific methyl phz MFATGGAGAGCGGGATCGACAG ATGCGGGTTTCCATCGGCAG 875 61.4 $24(75.0)$ transferase/Phenazine operon phz MRATGCGGGTTTCCATCGGCAG ACGCATCGACCGATACGCTC 61.4 $24(75.0)$ Phenazine operon phz SRACAACCTGAGCCATCAGCGCTC pilAR 1752 61.4 $24(75.0)$ Fimbrial protein/Fimbrial protein $pilAF$ ACAGCATCCAACTGAGCCAGCCTTCC pilAR 1675 56.0 $8(25.0)$ Type IV fimbrial biogenesis $pilBF$ TCGAACTGATGATGATGATGGTGG pilAR 408 53.7 $3(9.3)$ protein/Fimbrial protein $pilBR$ CTTTCGGAGTGAACATCG CTTCGGAGTGAACATCG 61.4 (Ghadaksaz 6 (18.7)Biofilm formation protein/ $pslAF$ TCCCTCACCTCAGCAGCAAGCC GCATCCGCCGCACTCAG 61.4 (Ghadaksaz 6 (18.7)Biofilm $pslAR$ TGTTGTAGCCGTAGCGTTCTCT GCAACTCAGCCGTCCTCTC 66.0 $et al.,$ Biofilm $pelAF$ CGCATTCGCCGCACTCAG CGCATCCGCCGCATCTCG 61.4 $32(100.0)$ Transmembrane protein $ppyRR$ ACAGCAGACCTCCCAACCG 61.4 $29(90.6)$ Alginate formation $algUR$ TCAGGCTTCTCGCAACAAAGG 59.8 $-$ Alginate formation $algUR$ TCAGGCTTCCCGACAGCGC 61.4 $29(90.6)$ Alginate formation $algDF$ AGAAGTCCGAACGCAACCC 550 64.4 $29(90.6)$ Alginate formation $algDR$ CGCATCAACGAACCCCACCC 550 64.4 $29(90.6)$	operon	phzIIR	CGCATTGACGATATGGAAC	1050	54.6		
transferase/Phenazine operon phz MRATGCGGGTTTCCATCGGCAG61.4Flavin-dependent hydroxylase / phz SFTCGCCATGACCGATACGGTC175261.424 (75.0)Phenazine operon phz SRACAACCTGAGCCAGCCTTCC175261.424 (75.0)Fimbrial protein/Fimbrial protein pil /AFACAGCATCCAACTGAGCG167556.08 (25.0)Type IV fimbrial biogenesis pi/RF TCGAACTGATGATCGTGG167556.08 (25.0)protein/Fimbrial protein pi/RF TCGAACTGATGATCGTGG40853.73 (9.3)protein/Fimbrial protein pi/RF TCCTACCTCAGCAGCAGCAGC65661.4(Ghadaksaz6 (18.7)Biofilm formation protein/ ps/AF TCCCTACCTCAGCCAGCAGCAGC65661.4(Ghadaksaz6 (18.7)Biofilm ps/AR TGTTGTAGCCGTAGCGTTCTG78660.3et al.,	Phenazine specific methyl	phzMF	ATGGAGAGCGGGGATCGACAG	875	61.4		24(75.0)
Plavin-dependent hydroxylase / phz SFICGCCATGACCCGATACGGTC175261.424 (/5.0)Phenazine operon phz SRACAACCTGAGCCAGCCTTCC175261.424 (/5.0)Fimbrial protein/Fimbrial protein $pilA$ FACAGCATCCAACTGAGCG167556.08 (25.0)Type IV fimbrial biogenesis $pilB$ FTCGAACTGATGATCGTGG167556.08 (25.0)protein /Fimbrial protein $pilB$ RCTTTCGGAGTGAACATCG40853.73 (9.3)Biofilm formation protein $pslA$ FTCCCTACCTCAGCAGCAAGC65661.4(Ghadaksaz6 (18.7)Biofilm formation protein $pslA$ RTGTTGTAGCCGTAGCGTTCTG65660.3et al.,	transferase/Phenazine operon	<i>phz</i> MR	ATGCGGGTTTCCATCGGCAG		61.4		24 (75.0)
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Playin-dependent hydroxylase /	phzSF		1752	61.4		24 (75.0)
Finbrial protein/Fimbrial protein $plrAr$ FICACCATECARCEACE167550.06 (25.0)Type IV fimbrial biogenesis $pilBF$ TTGACTTCCTCCAGGCTG167556.03 (9.3)protein /Fimbrial protein $pilBR$ CTTTCGGAGTGAACATCG40853.73 (9.3)Biofilm formation protein/ $pslAF$ TCCCTACCTCAGCAGCAAGC65661.4(Ghadaksaz6 (18.7)Biofilm formation protein/ $pelAF$ CATACCTTCAGCCGCAGTCGTTCTC78662.72015)2 (6.2)Biofilm $pelAR$ CGCATTCGCCGCACTCAG60.5	Phenazine operon	phzSK pilAF			56.0	-	8 (25 0)
Type IV fimbrial biogenesis protein / Fimbrial proteinpi/IR pi/IR pi/IR 	Fimbrial protein/Fimbrial protein	nilAR	TTGACTTCCTCCAGGCTG	1675	56.0		0 (25.0)
Jack Protein <i>pilBR</i> CTTTCGGAGTGAACATCG40853.7Biofilm formation protein/ <i>pslAF</i> TCCCTACCTCAGCAGCAGCAGC65661.4(Ghadaksaz6 (18.7)Biofilm <i>pslAR</i> TGTTGTAGCCGTAGCGTTCTG65660.3et al.,	Type IV fimbrial biogenesis	pilBF	TCGAACTGATGATCGTGG	100	53.7		3 (9.3)
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	protein /Fimbrial protein	pilBR	CTTTCGGAGTGAACATCG	408	53.7		- ()
Biofilm $pslAR$ TGTTGTAGCCGTAGCGTTTCTG 050 60.3 et al.,Biofilm formation protein/ $pelAF$ CATACCTTCAGCCATCCGTTCTC 786 60.3 et al.,Biofilm $pelAR$ CGCATTCGCCGCACTCAG 786 60.5 60.5 Pyoverdin operon editor/ $ppyRF$ CGTGATCGCCGCCTATTTCC 160 61.4 $32 (100.0)$ Transmembrane protein $ppyRR$ ACAGCAGACCTCCCAACCG 160 61.4 $32 (100.0)$ Alginate synthesis gene U/ $algUF$ CGATGTGACCGCAGAGGATG 292 61.4 $29 (90.6)$ Alginate formation $algLF$ CCGCTCGCAGATCAAGGACATC 432 61.4 $29 (90.6)$ Alginate formation $algLR$ TCGCTCACCGCCCAGTCG 61.8 $-$ Alginate synthesis gene D/ $algDF$ AGAAGTCCGAACGCCACACC 550 64.4 $29 (90.6)$ Alginate formation $algDF$ CGCATCAACGAACCGCAGCC 550 64.4 $29 (90.6)$ Alginate formation $algDR$ CGCATCAACGAACCGAGCATC 62.8 $29 (90.6)$	Biofilm formation protein/	pslAF	TCCCTACCTCAGCAGCAAGC	656	61.4	(Ghadaksaz	6 (18.7)
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Biofilm	<i>psl</i> AR	TGTTGTAGCCGTAGCGTTTCTG	050	60.3	et al.,	
BiofilmpelARCGCATTCGCCGCACTCAG10060.5Pyoverdin operon editor/ppyRFCGTGATCGCCGCCTATTTCC16061.432 (100.0)Transmembrane proteinppyRRACAGCAGACCTCCCAACCG16061.061.0Alginate synthesis gene U/algUFCGATGTGACCGCCAGAGGATG29261.429 (90.6)Alginate formationalgURTCAGGCTTCTCGCAACAAAAGG59.8	Biofilm formation protein/	pelAF	CATACCTTCAGCCATCCGTTCTTC	786	62.7	2015)	2 (6.2)
Pyoverdin operon editor/ppyRFCGTGATCGCCGCCTATTTCC61.432 (100.0)Transmembrane proteinppyRRACAGCAGACCTCCCAACCG16061.0	Biofilm	pelAR	CGCATTCGCCGCACTCAG	,00	60.5		
Transmembrane protein ppyRR ACAGCAGACCTCCCAACCG 61.0 Alginate synthesis gene U/ algUF CGATGTGACCGCAGAGGATG 292 61.4 29 (90.6) Alginate formation algUR TCAGGCTTCTCGCAACAAAGG 59.8	Pyoverdin operon editor/	ppyRF	CGTGATCGCCGCCTATTTCC	160	61.4		32 (100.0)
Alginate synthesis gene D/algOFCGATGTOACCCCAAGGGATG29201.429 (90.6)Alginate formationalgURTCAGGCTTCTCGCAACAAAGG59.8	Iransmembrane protein	ppyKK alaUE	ACAGCAGACUICCCAACCG	202	61.0	_	20 (00 6)
Alginate formation algOR FCAGGCTFCGCGAGACAAAGG 57.6 Alginate synthesis gene L/ algLR CCGCTCGCAGATCAAGGACATC 432 61.4 29 (90.6) Alginate synthesis gene D/ algDF AGGAAGTCCGAACGCCACACCC 550 64.4 29 (90.6) Alginate formation algDR CGCATCAACGAACCGAGCATC 62.8	Alginate formation	algUr algUR		272	50 Q		29 (90.0)
Alginate formationalgLRTCGCTCACCGCCCAGTCG61.8Alginate formationalgDFAGAAGTCCGAACGCCACCC55064.429 (90.6)Alginate formationalgDRCGCATCAACGAACCGAGCATC62.8	Alginate synthesis gene I /	aloLF	CCGCTCGCAGATCAAGGACATC	432	61 4		29 (90.6)
Alginate synthesis gene D/algDFAGAAGTCCGAACGCCACACC55064.429 (90.6)Alginate formationalgDRCGCATCAACGAACCGAGCATC62.8	Alginate formation	algLR	TCGCTCACCGCCCAGTCG	152	61.8		27 (70.0)
Alginate formation algDR CGCATCAACGAACCGAGCATC 62.8	Alginate synthesis gene D/	algDF	AGAAGTCCGAACGCCACACC	550	64.4		29 (90.6)
	Alginate formation	algDR	CGCATCAACGAACCGAGCATC		62.8		

J HELLENIC VET MED SOC 2023, 74 (1) ПЕКЕ 2023, 74 (1)

used as negative control in PCR.

PCR, for each sample was carried out on a volume of 30 μ l, final concentration was 10x Taq enzyme buffer solution 1x, 25 mM MgCl₂ 2 mM, 10 mM dNTP 0.2 mM, 100 pmol primer (for each) 0.4 pmol, 5 U Taq DNA polymerase 1.5 U (Fermentas, Massachusetts, USA), 3 μ l 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 (intII, lasA, phzI, pilB, phzII), 55°C (intI, lasI, lasR, rhIR, rhIAB, pvdA, fliC, pilA), 57°C (PA-GS, PA-SS, intIII, exoU, algU), 60°C (exoY, plcH, phzM, phzS, toxA, pslA, pelA, ppyR, algL), 61°C (exoS, algD), 63°C (lasB, plcN), 68°C (aprA) 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 (χ 2) 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 $\chi 2$ 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 grapelike 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, *phzl*I, *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%), pvdA and pilA (8/32=25.0%), pslA (6/32=18.7%), pilB (3/32=9.3%), exoU and pelA (2/32=6.2%), while there was no detection for aprA 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.).

Tabl	e 2. Virulence gene profiles of P. aeruginosa isolates.			
No	Vigulance Construe	Gene	Isolate	Total
INO	virulence Genolype	number	number	(%)
1	*lasA	11	1	1 (3.1)
2	*exoS, exoY, phzl, phzll	14	2	2 (6.2)
3	*exoS, exoY, phzl, phzll, pslA, algU, algL, algD, toxA	19	1	
4	*exoS, exoY, phzl, phzll, pilB, algU, algL, algD, toxA	19	1	5 (15.6)
5	*exoS, exoY, lasA, phzl, phzll, algU, algL, algD, toxA	19	3	
6	*exoS, exoY, phzl, phzll, phzM, phzS, , algU, algL, algD, fliC	20	3	1 (3.1)
7	*exoS, exoY, phzl, phzll, phzM, phzS, , algU, algL, algD, toxA, fliC	21	2	
8	*exoS, exoY, lasA, phzl, phzll, phzM, phzS, , algU, algL, algD, fliC	21	4	8 (25.0)
9	*exoU, exoY, lasA, phzl, phzM, phzS, , algU, algL, algD, pvdA, fliC	21	2	
10	*exoS, exoY, lasA, phzl, phzll, phzM, phzS, pslA, , algU, algL, algD, fliC	22	2	
11	*exoS, exoY, phzl, phzll, phzM, phzS, pilA, toxA, algU, algL, algD, pvdA	22	1	
12	*exoS, exoY, lasA, phzl, phzll, phzM, phzS, algU, algL, algD, toxA, fliC	22	1	7 (21.9)
13	*exoS, exoY, lasA, phzl, phzll, phzM, phzS, pelA, algU, algL, algD, fliC	22	1	
14	*exoS, exoY, lasA, phzl, phzll, phzM, phzS, pilA, , algU, algL, algD, pvdA	22	2	
15	*exoS, exoY, lasA, phzl, phzll, phzM, phzS, algU, algL, algD, pvdA, toxA, fliC	23	1	3 (9.4)
16	* exoS, exoY, phzl, phzll, phzM, phzS, pilA, pilB, , algU, algL, algD, toxA, fliC	23	2	
17	* exoS, exoY, phzl, phzll, phzM, phzS, pilA, pslA, pelA, , algU, algL, algD, toxA, fliC	24	1	3 (9.4)
18	* eros eros nhzl nhzll nhzM nhzs nila nsla algi algi algi nyda tora flic	24	2	

*: Virulence genes found in all isolates: exoT, lasI, lasR, rhll, rhlR, lasB, rhlAB, plcH plcN, ppyR.



Figure 2. Gel electrophoresis image of *P. aeruginosa* virulence genes 1. *exoS* (118 bp) 2. *exoU* (134 bp) 3. *exoT* (152 bp) 4. *exoY* (289 bp) 5. *lasI* (295 bp) 6. *lasR* (130 bp) 7. *rhII* (155 bp) 8. *rhIR* (133 bp) 9. *lasA* (1075 bp) 10. *lasB* (284 bp) 11. *rhIAB* (151 bp) 12. *plcH* (608 bp) 13. *plcN* (481 bp) 14. *pvdA* (1281 bp) 15. *toxA* (270 bp) 16. *phzI* (392 bp) 17. *rhIII* (1036 bp) 18. *phzM* (875 bp) 19. *phzS* (1752 bp) 20. *fliCA*(1020 bp) 21. *fliCB* (1250 bp) 22. *pilA* (1675 bp) 23. *pilB* (408 bp) 14. *psIA* (656 bp) 25. *pelA* (786 bp) 16. *ppyR*(160 bp) 27. *algU* (292 bp) 28. *algL* (432 bp) 29. *algD* (550 bp) gene positive isolates 12. Negative Control (DNA-free master mix) M: 100 bp DNA ladder.

J HELLENIC VET MED SOC 2023, 74 (1) ПЕКЕ 2023, 74 (1)







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 *exoU* genes. There was no significant relationship with other virulence genes (Table 6).

Iable 3. Antimicrobial agents and results.												
Group	Antimicrobial agent	Disc content	≥S	≤R	Reference	R (%)	S (%)					
Beta Lactam	Ampicillin (AMP)	10 µg	14	11	15	32 (100.0)	0 (0.0)					
Penicillins	Penicillin (PEN)	10 U	22	11	15	32 (100.0)	0 (0.0)					
	Gentamycin (GEN)	10 µg	15	12	14	2 (6.2)	30 (93.7)					
Aminoglyco-sides	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/	1 25/23 75ug	16	10	16	32(100.0)	0(0,0)					
Sunonannaes	Sulfametaxazole (TMS)	1.25/25.75µg	10	10	10	52 (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-	Cefoperazone (SPZ)	75 μg	21	15	16	20 (62.5)	2 (6.2)					
rins	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-	Ciprofloxacin (CIP)	5 µg	21	15	14	5 (15.6)	26 (81.2)					
lones	Enrofloxcin (ENR)	5 µg	21	16	16	8 (25.0)	9 (28.1)					

TIL 3



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 resistant	nce status of isolates with a	nd without int genes		
Antibiotic	Integron + (n=11)	Integron – (n=21)	Р	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 g	genes			
Gen	Íntegron + (n=11)	Íntegron – (n=21)	Р	χ2		
<i>las</i> A (+)	4	13	0.160	1 801		
lasA (-)	7	8	0.109	1.071		
<i>exo</i> S (+)	9	20	0.216	1 530		
exoS (-)	2	1	0.210	1.550		
<i>exo</i> U (+)	2	0	0.044	4 073		
<i>exo</i> U (-)	9	21	0.044	1.075		
<i>exo</i> Y (+)	11	20	0.462	0 541		
<i>exo</i> Y (-)	0	1	0.402	0.541		
phzl(+)	11	20	0.462	0 541		
<i>phz</i> l (-)	0	1	0.402	0.541		
phzll (+)	9	20	0.216	1 530		
phzll (-)	2	1	0.210	1.550		
phzM(+)	7	17	0.283	1 154		
<i>phz</i> M (-)	4	4	0.205	1.1.0 1		
phzS(+)	7	17	0.283	1.154		
phzS (-)	4	4	0.200			
<i>pil</i> A (+)	2	6	0.519	0.416		
pilA (-)	9	15	01019	01110		
<i>pil</i> B (+)	0	3	0.188	1.734		
pilB (-)	11	18				
<i>psl</i> A (+)	3	6	0.938	0.006		
pslA(-)	8	15				
pelA(+)	0	2	0.290	1.117		
pelA (-)		19				
<i>alg</i> D, algU, alg L (+)	9	20	0.216	1.530		
algD, $algU$, $alg L$ (-)	2					
$\int \mathcal{U}(T)$	/	14	0.864	0.029		
file (-)	4	2				
i o x A (+)	0	У 2	0.170	1.886		
IOXA (-)	5	2				
$pvaA(\tau)$	4	4	0.283	1.154		
<i>pvaA</i> (-)	1	Γ'		-		

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

netilmicin, cefoperazone and ciprofloxacin; *pelA* with netilmicin and ciprofloxacin; *algD*, *algU*, *algL* with amikacin; aztreonam with *toxA*; *pvdA* with enrofloxacin (Table 7).

Table 7.	Relatio	onship	betwee	en viru	lence g	genes i	solates	and re	sistanc	e to ar	ntimicr	obial a	gents.											
Gene		G	N	A	MI	N	ЕТ	T	ET	SI	PZ	I	ЛI	AZO CIP				E	NR					
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R					
lasA	-	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					
	Р	0.9	927	0.9	986	0.1	120	0.0	0.055		117	0.2	228	0.2	295	<u>0.015</u>		0.015 0.07						
exoS	-	3	0	0	3	3	0	0	2	0	2	2	1	1	0	3	0	1	0					
	+	27	2	4	4 23		2	0	19	2	18	26	3	17	2	23	5	8	7					
	Р	0.6	539	0.6	583	0.6	539	0.9	968	0.8	396	0.2	252	0.5	531	0.6	583	0.6	525					
<i>exo</i> 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					
	Р		-	0.7	782	0.7	706	0.9	968	0.5	527	0.	09	0.8	388	0.7	/82	0.0)65					
exoY	-	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					
	Р	0.7	793	0.8	388	0.7	793	0.1	60	0.3	321	0.7	701	0.4	123	0.8	888	0.2	267					
phzl	_	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					
	Р	0.7	793	0.8	388	0.7	793	0.1	60	0.3	321	0.7	701	0.4	123	0.8	888	0.2	267					
<i>phz</i> ll	_	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					
	Р	0.6	539	0.6	583	0.6	539	0.9	968	0.8	396	0.2	252	0.5	531	0.6	583	0.5	567					
<i>phz</i> 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					
	Р	0.3	399	0.3	359	0.3	399	0.8	330	0.670		0.217		0.095		0.611		0.766						
phzS	-	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	0.3	399	0.3	359	0.3	399	0.8	330	0.6	0.670		217	0.411		0.6	511	0.7	766					
pilA	-	22	2	4	19	23	1	0	15	2	15	20	4	14	1	20	4	8	6					
	+	8	0	0	7	7		0	6	0	5	8	0	4	1	6	1	1	2					
	Р	0.3	399	0.3	359	0.3	399	0.5	519	0.670 0.217		17 0.690		0.210		0.477								
pilB	-	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					
	Р	0.6	539	0.1	22	<u>0.0</u>	<u>)42</u>	0.2	216	<u>0.(</u>	<u>)26</u>	0.4	192	0.5	531	<u>0.0</u>	006	0.8	381					
pslA	-	25	1	3	21	25	1	0	17	1	17	23	3	14	1	22	3	9	6					
	+	5		1	5	5		0	4	1	1 3		1 3		1 3			5 1	4		4	2	0	2
7.4	P	0.2	242	0.7	133	0.2	242	0.9	10	0.4	+/8	0.7	/32	0.3	520	0.3	086	0.4	235					
pelA	-	28	2	4	24	30	0	0	19	2	18	26	4	16	2	0	29	8	8					
	+ D	2		0	2		2		2	0	2	2	0	2		2								
	P	0.7	00	0.7	1	2		0.2	290	0.3	$\frac{527}{2}$	0.3	1 1	0.4	+30	<u>2</u>	<u> </u>	0.0) 3 /					
algD	-	3	0	0	1	3		0	2	0	<u> </u>	2		2	0	2	1	2						
	+ 	21	20	4	23	21	2		19	2	18	26	5	10	2	24	4		<u> /</u>					
al-TT	Р	0.0	039	<u><u> </u></u>	<u>134</u>	0.0	000	0.9	08	0.8	090	0.2	1	0.8	0/	0.652		0.1	185					
aigu	-	3	0	0	1	3			2	0	10	2		2		2		2						
	+ 	27	2	4	23	27	2		19	2	18	26	5	16	$\frac{2}{2}$	24	4		1.02					
	Р	0.6	039	<u> </u>	104	0.6	039	0.9	80%	0.8	590		1	0.8	50/	0.6	1	0.1	183					
algL	-	3	0	0	1	3			2	0	10	2		2		2		2						
	+ 	27	2	4	23	27	2	0	19	2	18	26	5	16	2	24	4	/	1.02					
	P	0.6	59	<u> </u>	134	0.6	539	0.968		0.968 0.89		0.896 0.252		0.867		0.6	52	0.183						

fliC	-	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
	Р	0.6	531	0.0)52	0.3	313	0.864		0.551 0.482		82	0.544		0.743		0.977		
toxA	-	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
	Р	0.1	20	0.1	132	0.9	927	0.108		0.2	289	0.3	349	<u>0.(</u>	<u>)32</u>	0.1	30	0.1	75
pvdA	-	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 0.39		399	0.133		0.2	0.231 1		1 0.690		0.798		0.0)24					

-: Isolates carrying / not carrying all of the virulence genes (*lasB*, *lasI*, *lasR*, *rhl*, *rhlR*, *rhlAB*, *plcH*, *plcN*, *exoT*, *ppyR*, *aprA*) 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 (exoU), ADP-ribosylating enzymes (exoS and exoT), and adenyl cyclase (exoY). exoU kills a variety of eukaryotic cells through its phospholipase activity. exoT and exoY play a role in the adhesion, phagocytosis and systemic spread of bacterial cells together with exoS (Vance et al., 2005). exoS and exoU are more important than exoT and exoY 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 exoS, exoT, and exoY genes. Cytotoxic strains that cause inflammation and tissue necrosis with their cytotoxic effects typically have genes for exoU, exoT, and exoY (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 (exoU - / exoS +), while only 8.2% of isolates were cytotoxic / non-invasive strains (exoU + / exoS); interestingly, they reported 6 isolates identified with both exoU and exoS (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 (exoS +, exoU-), 2 of them were cytotoxic (exoU +, exoS-); There were no cytotoxic (exoU +) or invasive (exoS-) strains. exoT and exoY 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 exoU 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 exoA gene. This toxin is responsible for an invasion and regional tissue damage of the bacteria (Woods et al., 1983). Neamah (2017) reported that the toxA gene was detected in 100% of strains while the exoS gene was in 75% of P. aeruginosa isolates isolated from the milk of cows with mastitis. Banerjee et al. (2017) detected the toxA gene at a rate of 63.2% and the exoS 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 toxA and exoS 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 (algD, algU, algL) 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 *fliC*, toxA and phzS genes are the most frequently detected genes in P. aeruginosa isolates isolated from the diabetic foot; reported the absence of the aprA gene. Similarly, in this study, aprA was not detected in the isolates. However, all isolates had ten virulence genes (lasI, lasR, *rhl*, *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 exoU 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.

ACKNOWLEDGEMENTS

This manuscript was supported by Aydin Adnan Menderes University Scientific Research Projects Unit (Project Number: VTF-17004) and the authors would like to thank Prof. Dr. Bulent Bozdogan (Aydin Adnan Menderes University, Medical Faculty, Department of Medical Microbiology, Aydin, Türkiye) and Merve Engin KURT (Ege University, Faculty of Letters, Department of Translation and Interpreting Studies, Izmir, Türkiye) for help and supports.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

- Aggarwal S (2008) Techniques in Molecular Biology. Lucknow: International Book Distributing CO. Chapter 1 (Nucleic Acid Extraction), Page: 21.
- Ahmed AM and Shimamoto T (2011) Molecular characterization of antimicrobial resistance in Gram-negative bacteria isolated from bovine mastitis in Egypt. Microbiol. Immunol. 55: 318–327.
- Ajayi T, Allmond LR, Sawa T and Wiener-Kronish JP (2003) Single-nucleotide-polymorphism mapping of the *Pseudomonas aeruginosa* type III secretion toxins for development of a diagnostic multiplex PCR system. J. Clin. Microbiol. 41 (8): 3526-3531.
- Ama A, El-Shafii SS, Amo AE and El-dayim ZAA (2016) Be detection of multidrug resistance genes in *Pseudomonas aeruginosa* isolated from bovine mastitic milk. JDVAR 3 (2): 43-49.
- Banerjee S, Batabyal K, Joardar SN, Isore DP, Dey S, Samanta I and Samanta TK (2017) Mice pathology study of *toxA* and *exoS* genes bearing *Pseudomonas aeruginosa* isolated from bovine sub-clinical mastitis in West Begal with their Antibiogram. Indian J. Anim. Res. 52 (8): 1-6.
- Bass L, Liebert CA, Lee MD, Summers AO, White DG, Thayer SG and Maurer JJ (1999) Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. Antimicrob. Agents Chemother. 43: 2925–2929.
- Chengappa MM (1990) Antimicrobial Agents and Susceptibility Testing. In Carter GR, Cole JR. (Eds.) Diagnostic Procedures in Veterinary Bacteriology and Mycology. 5th edition, Academic Press Inc., San Diego, California.
- CLSI (2012) Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S22. Wayne, PA: Clinical and Laboratory Standards Institute.
- Denning GM and Iyer SS, Reszka KJ, O'Malley Y, Rasmussen GT and Britigan BE (2003) Phenazine-1-carboxylic acid, a secondary metabolite of *Pseudomonas aeruginosa*, alters expression of immunomodulatory proteins by human airway epithelial cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 285: L584–L592.
- Engel J and Balachandran P (2009) Role of *Pseudomonas aeruginosa* type III effectors in disease. Curr. Opin. Microbiol. 12: 61–66.
- Ertugrul BM, Oryasin E, Lipsky BA, Willke A and Bozdogan B (2018) Virulence genes *fliC*, *tox*A and *phz*S are common among *Pseudomo-nas aeruginosa* isolates from diabetic foot infections. Infect. Dis. 50 (4): 273–279.
- Faghri J, Nouri S, Jalalifar S, Zalipoor M and Halaji M (2018) Investigation of antimicrobial susceptibility, class I and II integrons among *Pseudomonas aeruginosa* isolates from hospitalized patients in Isfahan, Iran. BMC Res. Notes 11: 806.
- Fazeli N and Momtaz H (2014) Virulence gene profiles of multidrug-resistant *Pseudomonas aeruginosa* isolated from Iranian hospital infections. Iran Red. Crescent Med. J. 16 (10): e15722.2.
- Fleiszig S, Wiener-Kronish JP, Miyazaki H, Vallas V, Mostov KE, Kanada D, Sawa T, Yen T and Frank DW (1997) *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. Infect. Immun. 65, 579–586.
- Fouad Z (2011) Antimicrobial Disk Diffusion Zone Interpretation Guide. https://www.researchgate.net/publication/315844044. DOI: 10.13140/RG.2.2.13801.70240.
- Gasink LB, Fishman NO, Weiner MG, Nachamkin I and Bilker WB (2006) Fluoroquinolone-resistant *Pseudomonas aeruginosa*: assessment of risk factors and clinical impact. Am. J. Med. 119 (6): 526e19-25.
- Ghadaksaz A, Fooladi AAI, Hosseini HM and Amin M (2015) The prevalence of some *Pseudomonas* virulence genes related to biofilm formation and alginate production among clinical isolates. J. Appl. Biomed. 13: 61-68.
- Goldstein C, Lee MD, Sanchez S, Hudson C, Phillips B, Register B, Grady M, Liebert, C, Summers AO, White DG and Maurer JJ (2001) Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents Chemother. 45: 723-726.

- Goli HR, Nahaei MR, Rezaee MA, Hasani A, Kafl HS and Aghazadeh, M (2017) Prevalence and molecular characterization of class 1 integrons among clinical isolates of *Pseudomonas aeruginosa* in Northwest of Iran. Mol. Gen. Microbiol. Virol. 32:109–11.
- Greenberg EP (1997) Quorum sensing in Gram-negative bacteria. ASM News 63: 371-377.
- Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M and Parsek MR (2001) Alginate overproduction affects *Pseudomonas* aeruginosa biofilm structure and function. J. Bacteriol. 183: 5395– 5401.
- Howe TR and Iglewski BH (1984) Isolation and characterization of alkaline protease-deficient mutants of *Pseudomonas aeruginosa* in vitro and in a mouse eye model. Infect. Immun. 43: 1058-1063.
- Kibebew K (2017) Bovine mastitis: A review of causes and epidemiological point of view. J.B.A.H. 7 (2): 1-14.
- Kipnis E, Sawa T and Wiener-Kronish J (2006) Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. Med. Mal. Infect. 36:78-91.
- Kirk JH and Bartlett PC Nonclinical *Pseudomonas aeruginosa* mastitis in a dairy herd. J. Am. Vet. Med. Assoc. 184: 671–673, 1984.
- Kouchaksaraei FM, Shahandashti EF, Molana Z, Kouchaksaraei MM, Asgharpour F, Mojtahedi A and Rajabnia R (2012) Molecular detection of integron genes and pattern of antibiotic resistance in *Pseudomonas aeruginosa* strains isolated from intensive care unit, Shahid Beheshti Hospital, North of Iran. IJMCM 1 (4): 2009-2016.
- Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbart S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT and Monnet DL (2012) Multidrug-resistant, extensively drug resistant and pandrug resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18: 268–281.
- Mazel D (2006) Integrons: agents of bacterial evolution. Nat. Rev. Microbiol. 4: 608–620.
- McIntyre-Smith A, Schneiderman J and Zhou K (2010) Alginate does not appear to be essential for biofilm production by PAO1 *Pseudomonas* aeruginosa. JEMI 14: 63–68.
- Meyer JM, Neely A, Stintzi A, Georges C and Holder IA (1996) Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. Infect. Immun. 64: 518–523.
- Mokhtari AR, Salehi ZT, Amini K and Shahcheraghi F (2016) Isolation *Pseudomonas aeruginosa* bacteria and genes integron class 1 of subclinical mastitis in dairy cows in Tehran. Veterinary Researches Biological Products 29 (2): 37-44.
- Neamah AA (2017) Molecular detection of virulence factor genes in *Pseudomonas aeruginosa* isolated from human and animals in Diwaniya province. Kufa J. Vet. Med. Sci. 8 (1): 218-230.
- Ozturk D, Sahan Yapıcıer O, Sababoglu E, Kaya M, Pehlivanoglu F and Turutoglu H (2019) Antibiotic resistance of Gram negative bacteria isolated from bovine mastitis. Van Vet. J. 30(2): 85-89.
- Park SJ, Kim AK, So YI, Park HY, Li XH, Yeom DH, Lee MN, Lee BL and Lee JH (2014) Protease IV, a quorum sensing-dependent protease of *Pseudomonas aeruginosa* modulates insect innate immunity. Mol. Microbiol. 94(6): 1298-1314.
- Quinn PJ, Markey BK, Leonard FC, FitzPatrick ES, Fanning S and Hartigan PJ (2011) Veterinary Microbiology and Microbial Disease. Second Edition, Blackwell Science Ltd, Oxford, UK.
- Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, Sawa T, Frank DW and Wiener-Kronish JP (2001) Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. J. Infect. Dis. 183 (12): 1767–1774.
- Sabharwal N, Dhall S, Chhibber S and Harjai K (2014) Moleculer detection of virulence genes as markers in *Pseudomonas aeruginosa* isolated from urinary tract infections. IJMEG 5 (3): 125-134.
- Sahin C and Erbas G (2015) Antibiotic susceptibilities and isolation of Pseudomonas aeruginosa from clinical mastitis in cattle. Etlik J. Vet.

Microbiol. 26 (1): 16-20.

- Selezska L, Wagner G, Gramer N, Siebert B, Gudowius P, Morales G, Kohler T, Van Delden C, Weinel C and Slickers P (2007) Population structure of *Pseudomonas aeruginosa*. PNAS USA, 104: 8101–8106.
- Spilker T, Coenye T, Vandamme P and LiPuma JJ (2004) PCR-Based for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystik fibrosis patients. J. Clin. Microbiol. 42 (5): 2074-2079.
- Szmolka A, Cramer N and Nagy B (2012) Comparative genomic analysis of bovine, environmental and human strains of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 335: 113–122.
- Tel OY, Keskin O, Zonturlu AK and Arserim KNB (2009) Subclinical mastitis prevalance and determination of the antibiotics susceptibility in Sanliurfa Region. Firat Uni. J. Health Sci. 23 (2): 101-106.
- Vance RE, Rietsch A and Mekalanos JJ (2005) Role of the type III secreted exoenzymes S, T, and Y in systemic spread of *Pseudomonas* aeruginosa PAO1 in vivo. Infect. Immun. 1706–1713.
- Wiehlmann L, Wagner G, Gramer N, Siebert B, Gudowius P, Morales G, Kohler T, Van Delden C, Weinel C and Slickers P (2007) Population structure of *Pseudomonas aeruginosa*. PNAS USA, 104: 8101–6.
- Wiener-Kronish JP, Sakuma T, Kudoh I, Pittet JF, Frank D, Dobbs L, Vasil ML, and Matthay MA (1993) Alveolar epithelial injury and pleural empyema in acute *P. aeruginosa* pneumonia in anesthetized rabbits. J. Appl. Physiol. 75 (4): 1661–1669.
- Woese CR (1987) Bacterial evolution. Microbiol. Rev. 51: 221-271.
- Woods DE and Iglewski BH (1983) Toxins of *Pseudomonas aeruginosa*: new perspectives. Rev. Infect. Dis. 5 (Suppl 4): S715-722.