

## RESEARCH ARTICLE

## Molecular Characterization of *Pseudomonas aeruginosa* Isolated From Clinical Bovine Mastitis Cases

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**Abstract:** This study aimed to investigate the presence and distribution of virulence determinants and their antimicrobial susceptibilities of 44 *Pseudomonas aeruginosa* isolates obtained from clinical bovine mastitis cases. In addition, selected 6 *P. aeruginosa* isolates were further characterized using whole-genome sequencing (WGS). Based on the presence of T3SS-related genes, 25% of the isolates were found to carry *exoU* and/or *exoS* genes belonging to invasive (*exoU*-/*exoS*+, 18.2%) and cytotoxic (*exoU*+/*exoS*-, 6.8%) strains. But, none of the isolates carried both *exoU* and *exoS* genes. In terms of other virulence genes examined, various virulence gene profiles were observed among the isolates. The majority of the isolates (72.7%) were susceptible to all tested antimicrobials. Resistance rates to ciprofloxacin and carbapenems (imipenem and meropenem) were determined as 25% and 4.5%, respectively. WGS analysis indicated the presence of different resistance genes in all isolates, and different combinations of mutations in *gyrA*, *parC*, *oprD*, efflux pump, and genes playing a role in the regulation of *ampC* gene expression. Different sequence types (STs) and serotypes were found in representative isolates with the occurrence of the O11-ST235 clone, which is a worldwide multidrug-resistant high-risk clone representing a serious public health threat. The findings of this study provide valuable information on *P. aeruginosa* isolated from clinical bovine mastitis cases and current antimicrobial resistance levels and virulence determinants.

**Keywords:** Antimicrobial resistance, Clinical bovine mastitis, *Pseudomonas aeruginosa*, Virulence, Whole-genome sequencing

## Klinik İnek Mastitis Vakalarından İzole Edilen *Pseudomonas aeruginosa* Suşlarının Moleküler Karakterizasyonu

**Öz:** Bu çalışmada klinik sığır mastitis vakalarından izole edilen 44 *Pseudomonas aeruginosa* izolatının virülans genlerinin varlığı ve dağılımı ile antimikrobiyal duyarlılıklarının araştırılması amaçlandı. Ayrıca, seçilen 6 *P. aeruginosa* izolatının tüm genom dizileme (WGS) ile taslak genomları elde edilerek daha detaylı karakterizasyonları yapıldı. T3SS ilişkili genlerin varlığına dayalı olarak, izolatların %25'inin invaziv (*exoU*-/*exoS*+, %18.2) ve sitotoksik (*exoU*+/*exoS*-, %6.8) suşlara ait *exoU* ve/veya *exoS* genleri taşıdığı belirlendi. Ancak izolatların hiçbirinde *exoU* ve *exoS* genleri birlikte tespit edilmedi. İncelenen diğer virülans genleri açısından ise izolatlar arasında çeşitli virülans gen profilleri gözlemlendi. İzolatların çoğu (%72.7) incelenen tüm antimikrobiyallere duyarlı bulundu. Siprofloksasin ve karbapenemlere (imipenem ve meropenem) direnç oranları sırasıyla %25 ve %4.5 olarak belirlendi. WGS analizi tüm izolatlarda farklı rezistans genleri, *gyrA*, *parC*, *oprD*, efflux pump ve *ampC* gen ekspresyonunun regülasyonunda rol oynayan genlerde farklı mutasyon kombinasyonlarının varlığını gösterdi. Seçilen izolatlarda ciddi bir halk sağlığı tehdidi oluşturan ve dünya çapında çoklu ilaç dirençli yüksek riskli O11-ST235 klonu dahil farklı sekans tipleri (ST) ve serotipleri bulundu. Bu çalışmanın bulguları, klinik sığır mastitis vakalarından izole edilen *P. aeruginosa* suşlarının mevcut antimikrobiyal direnç düzeyleri ve virülans determinantları hakkında değerli bilgiler sunmaktadır.

**Anahtar sözcükler:** Antimikrobiyal direnç, Klinik inek mastitis, *Pseudomonas aeruginosa*, Virülans, Tüm genom dizileme

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## INTRODUCTION

As a versatile and adaptable opportunistic pathogen, *Pseudomonas aeruginosa* inhabits diverse ecological niches including environments, plants, and mammals, due to having a relatively large genome (5-7 Mb) encoding diverse metabolic pathways and defense mechanisms that enable the bacteria to cope with these hostile conditions<sup>[1]</sup>. *P. aeruginosa* is one of the most common nosocomial pathogens worldwide, causing life-threatening nosocomial infections especially among immunocompromised and critically ill patients<sup>[1,2]</sup>. Therefore, this pathogen was included in the ESKAPE pathogens group (*Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Enterobacter* species) by World Health Organization (WHO)<sup>[3]</sup>. *P. aeruginosa* has also veterinary importance, and causes different clinical manifestations in livestock and companion animals, and clinical mastitis is one of the most encountered infections in dairy cows<sup>[2]</sup>.

The frequency of other mastitis pathogens differs between herds, geographic locations, and whether or not mastitis control programs are implemented<sup>[4]</sup>. The overuse and misuse of antimicrobials for the prevention and treatment of mastitis increased antimicrobial resistance (AMR) in bacteria implicated, and increasingly undermined the sustainable use of antimicrobials<sup>[5,6]</sup>. The presence of such strains in dairy animals also implies a potential risk for their transfer to humans through the food supply chain, direct animal contact, or through environmental routes<sup>[7]</sup>. Currently,  $\beta$ -lactams combined with beta-lactamase inhibitors (amoxicillin-clavulanic acid) and fluoroquinolones are the most frequently used antimicrobial classes used for intramammary infusion in Türkiye. In addition, in recent years, last generation cephalosporins have been used in the treatment of dry mastitis in cows. The studies conducted in Türkiye are based on specifically selected animal populations and represent estimates of AMR in specific geographic regions at a given time, but they do not provide an overall picture of the situation. In addition, the proportion of AMR in bovine mastitis pathogens indicated differences in AMR proportions according to mastitis pathogens, regions and populations. The highest proportions of resistance were observed for penicillins and tetracyclines due to their long-term use in Türkiye<sup>[8-11]</sup>. Although resistance rates to quinolones (enrofloxacin and danofloxacin) and third-generation cephalosporins classified as critically important antimicrobials by the World Health Organization (WHO)<sup>[12]</sup> are not very high, in a recent study, higher rates of resistance against enrofloxacin and ceftiofur were observed in various bacteria species isolated from clinical bovine mastitis cases<sup>[13]</sup>.

*P. aeruginosa*-related mastitis cases were mostly related to outbreaks<sup>[14-16]</sup>. Ohnishi et al.<sup>[17]</sup> isolated 116 *P. aeruginosa*

strains from milk samples obtained from 115 cows affected by clinical mastitis in 89 dairy herds in Japan. Park et al.<sup>[18]</sup> isolated 116 *P. aeruginosa* without any bacteria from 35 625 raw milk samples collected from nine provinces in Korea. Ibrahim et al.<sup>[19]</sup> isolated 34 *P. aeruginosa* from 100 milk samples collected from cows with clinical mastitis in Egypt. In addition to being intrinsically resistant to many antimicrobials, *P. aeruginosa* can acquire new resistance mechanisms, resulting in the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains, which are resistant to fluoroquinolones, carbapenems, and aminoglycosides. Acquired resistance occurs either by chromosomal mutations on target sites or by the acquisition of resistance genes via horizontal transfer<sup>[20]</sup>. Another mobile genetic element (MGE) that plays an important role in the acquisition and transfer of antimicrobial resistance is integrons. These elements can capture, integrate, and express gene cassettes conferring resistance to different classes of antimicrobials<sup>[21]</sup>. The integrons were transferred between species by hitchhiking with other MGEs<sup>[22]</sup>.

The pathogenesis of *P. aeruginosa* infections is mainly associated with a large arsenal of both cell-associated (lectins, flagella, pili, lipopolysaccharide, alginate/biofilm) and extracellular (hemolysins, proteases, cytotoxin, pyocyanin, siderophores, exotoxin A, exoenzyme S, exoenzyme U, etc.) virulence factors<sup>[23]</sup>. Some of the virulence factors are coordinated by a cell density recognition mechanism called Quorum Sensing (QS)<sup>[24]</sup>. The type III secretion system (T3SS) is among the major virulence factors in *P. aeruginosa*. The T3SS is an injectisome that delivers its four exotoxins (ExoS, ExoT, ExoU, and ExoY), which have different functions and action mechanisms, into the cytoplasm of target eukaryotes<sup>[25,26]</sup>.

This study aimed to investigate the current status of antimicrobial resistance among *P. aeruginosa* isolates from clinical bovine mastitis cases and their virulence characteristics, to further characterize the representative isolates by whole genome sequencing (WGS).

## MATERIAL AND METHODS

### Bacterial Strains

The study was conducted on 226 dairy farms located in two provinces (Aydın and Şanlıurfa) between 2017-2021. Throughout the study period, 12964 lactating cows were examined and 822 cows were found to have clinical mastitis manifestations such as swollen udder quarters, entirely swollen udders, abnormal milk secretion, and loss of appetite. A total of 1546 milk samples were collected aseptically from the infected quarters and inoculated onto Blood Agar (Merck, Germany) supplemented with 5% defibrinated sheep blood, and incubated at 35°C for 24-48 h. The isolates were identified by classical biochemical

methods (Triple Sugar Iron and oxidase reactions), and species identification was carried out using MALDI-TOF MS (Bruker Daltonics, Billerica, MA, United States) and 16S rRNA based PCR amplification of *Pseudomonas* spp. and *P. aeruginosa* [27].

### Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2021) [28]. Eleven anti-pseudomonal agents were used: gentamicin (CN, 10 µg), tobramycin (TOB, 10 µg), amikacin (AK, 30 µg), piperacillin/tazobactam (TZP, 100/10 µg), aztreonam (ATM, 30 µg), meropenem (MEM, 10 µg), imipenem (IPM, 10 µg), ciprofloxacin (CIP, 5 µg), cefepime (FEB, 30 µg), piperacillin (PIP, 100 µg), and ceftazidime (CAZ, 30 µg). The *P. aeruginosa* ATCC 27853 was used as a quality control strain. Following the screening of carbapenem resistance using disc diffusion method, MICs values of carbapenem resistant isolates were determined using E-test strips (Bionalyse, Ankara, Türkiye). The isolates that have acquired resistance to at least one antimicrobial in three or more antimicrobial classes were defined as multi-drug resistant (MDR) [29].

### DNA Extraction

Genomic DNA was extracted from overnight culture using Qiagen DNeasy Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The isolated DNA was stored at -20°C until use.

### Detection of Virulence and Integron Genes

β-hemolytic, greenish on blood agar, lactose negative mucoid, and grape-like fruity odor on MacConkey agar were detected. A total of 44 isolates determined to be Gram negative, motile, oxidase +, catalase +, citrate +, urease +, VP - and MR - were evaluated as *Pseudomonas* spp. The presence of the following virulence and integron genes was investigated by polymerase chain reaction (PCR) among *P. aeruginosa* isolates: adhesion (*algU*, *algD*, and *algL*), oxidative stress (*phzI*, *phzII*, *phzM*, and *phzS*), quorum sensing (QS)/regulation (*lasA*, *lasB*, *lasI*, *lasR*, *rhlAB*, *rhlI*, and *rhlR*), T3SS (*exoS*, *exoU*, *exoT*, and *exoY*), biofilm (*pslA*, *pelA*, and *ppy*), alkaline protease (*aprA*) and flagellin (*fliC*), exotoxin A (*exoA*), pilin (*pilA*) and type IV pili (*pilB*), phospholipases C (*plcH*, *plcN*), L-ornithine N5-oxygenase, and class I, II, and III integrons [30-35]. Primers used for the detection of virulence and integron genes and species determination are compiled in [Table 1](#).

**Table 1.** Primers used for the detection of virulence genes, integron types, genus and species determination

Target	Primer	Sequence (5'→3')	PCR Conditions	Product Size (bp)	Reference
16S rRNA ( <i>Pseudomonas</i> spp.)	PA-GS-F	GACGGGTGAGTAATGCCTA	95°C 2 min; 25 cycles x [94°C 20 sec, 54°C 20 sec, 72°C 40 sec]; 72°C 1 min	618	[25]
	PA-GS-R	CACTGGTGTTCCTTCTCATA			
16S rRNA ( <i>P. aeruginosa</i> )	PA-SS-F	GGGGGATCTTCGGACCTCA	95°C 2 min; 25 cycles x [94°C 20 sec, 58°C 20 sec, 72°C 40 sec]; 72°C 1 min	956	[28]
	PA-SS-R	TCCTTAGAGTGCCACCCG			
Biofilm	<i>pslA</i> F	TCCCTACCTCAGCAGCAAGC	95°C 5 min; 35 cycles x [94°C 1 min, 60°C 1 min, 72°C 1 min]; 72°C 5 min	656	[29]
	<i>pslA</i> R	TGTTGTAGCCGTAGCGTTTCTG		786	
	<i>pelA</i> F	CATACCTCAGCCATCCGTCTTC		160	
	<i>pelA</i> R	CGCATTCGCCGCACTCAG		292	
	<i>ppy</i> RF	CGTGATCG CCGCTATTTC		432	
	<i>ppy</i> RR	ACAGCAGACCTCCCAACCG		550	
Alginate	<i>algU</i> F	CGATGTGACCCGAGGATG	94°C 2 min; 36 cycles x [94°C 30 sec, 58°C 30 sec, 68°C 1 min]; 68°C 7 min	118	[29]
	<i>algU</i> R	TCAGGCTTCTCGCAACAAAGG		134	
	<i>algL</i> F	CCGCTCGCAGATCAAGGACATC		289	
	<i>algL</i> R	TCGCTCACC GCCAGTCG		152	
	<i>algD</i> F	AGAAGTCCGAACGCCACACC			
	<i>algD</i> R	CGCATCAACGAACCGAGCATC			
Type III Secretion System	<i>exoS</i> F	GCGAGGTCAGCAGAGTATCG	94°C 2 min; 36 cycles x [94°C 30 sec, 58°C 30 sec, 68°C 1 min]; 68°C 7 min	118	[29]
	<i>exoS</i> R	TTCGGCGTCACTGTGGATGC		134	
	<i>exoU</i> F	CCGTTGTGGTGCCGTTGAAG		289	
	<i>exoU</i> R	CCAGATGTTACCGACTCGC		152	
	<i>exoY</i> F	CGGATT CTATGGCAGGGAGG			
	<i>exoY</i> R	GCCCTTGATGCACTCGACCA			
	<i>exoT</i> F	AATCGCCGTCCAACCTGCATGCG			
<i>exoT</i> R	TGTTCCGCCGAGGTACTGCTC				

Table 1. Primers used for the detection of virulence genes, integron types, genus and species determination (continued)							
Target	Primer	Sequence (5'→3')	PCR Conditions	Product Size (bp)	Reference		
Quorum sensing genes	<i>lasA</i> -F	GCAGCACAAAAGATCCC	94°C 3 min; 30 cycles x [94°C 30 sec, 55°C 30 sec, 72°C 1.5 min]; 72°C 5 min	1075	[30]		
	<i>lasA</i> -R	GAAATGCAGGTGCGGTC		284			
	<i>lasB</i> -F	GGAATGAACGAAGCGTTCTCCGAC					
	<i>lasB</i> -R	TGGCGTCGACGAACACCTCG					
	<i>lasI</i> -F	CGTGCTCAAGTGTTC AAGG	95°C 2 min; 30 cycles x [95°C 40 sec, 60°C 1 min, 72°C 2 min]; 72°C 10 min	295	[31]		
	<i>lasI</i> -R	TACAGTCGGAAAAGCCAG		130			
	<i>lasR</i> -F	AAGTGGA AATGGAGTGGAG		155			
	<i>lasR</i> -R	GTAGTTGCCGACGACGATGAAG		133			
	<i>rhlI</i> -F	TTCATCCTCCTTTAGTCTTCCC		151			
	<i>rhlI</i> -R	TTCCAGCGATT CAGAGAGC					
	<i>rhlR</i> -F	TGCATTTTATCGATCAGGGC					
	<i>rhlR</i> -R	CACTTCCTTTTCCAGGACG					
	<i>rhlAB</i> -F	TCATGGAATTGTCACAACCGC					
	<i>rhlAB</i> -R	ATACGGCAAATCATGGCAAC					
Alkaline metalloproteinase	<i>aprAF</i>	GTTCGACCAGCGCGGAGCAGATA	95°C 2 min; 30 cycles x [95°C 40 sec, 65°C 1 min, 72°C 2 min]; 72°C 10 min	993			
	<i>aprAR</i>	GCCGAGGCCGCGTAGAGGATGTC					
Initial colonization factor flagellin	<i>fliCF</i>	GGCAGCTGGTTNGCCTG	95°C 2 min; 30 cycles x [95°C 40 sec, 55°C 1 min, 72°C 2 min]; 72°C 10 min	1.02 kb (Type A) 1.25 kb (Type B)			
	<i>fliCR</i>	GGCCTGCAGATCNCCAA					
Major pilin	<i>pilAF</i>	ACAGCATCCA AACTGAGCG	94°C 3 min; 30 cycles x [94°C 30 sec, 55°C 1 min, 72°C 1.5 min]; 72°C 5 min	1675	[30]		
	<i>pilAR</i>	TTGACTTCCTCCAGGCTG		408			
Type IV fimbrial biogenesis protein	<i>pilBF</i>	TCGAACTGATGATCGTGG		392			
	<i>pilBR</i>	CTTTCGGAGTGAACATCG		1036			
Phenazine operon I	<i>phzIF</i>	CATCAGCTTAGCAATCCC		875			
	<i>phzIR</i>	CGGAGAAACTTTCCCTC		1752			
Phenazine operon II	<i>phzIIF</i>	GCCAAGGTTTGTGTGTCGG		608			
	<i>phzIIR</i>	CGCATTGACGATATGGAAC					
Phenazines	<i>phzMF</i>	ATGGAGAGCGGGATCGACAG		94°C 3 min; 30 cycles x [94°C 30 sec, 58°C 30 sec, 72°C 1 min]; 72°C 7 min		481	
	<i>phzMR</i>	ATGCGGGTTTCCATCGGCAG					
	<i>phzSF</i>	TCGCCATGACCGATA CGCTC					
	<i>phzSR</i>	ACAACCTGAGCCAGCCTTCC					
Hemolytic phospholipase C	<i>plcHF</i>	GCACGTGGTCATCCTGATGC		94°C 3 min; 30 cycles x [94°C 30 sec, 55°C 1 min, 72°C 1.5 min]; 72°C 5 min		270	
	<i>plcHR</i>	TCCGTAGGCGTCGACGTAC					
Non-hemolytic phospholipase C	<i>plcNF</i>	TCCGTTATCGCAACCAGCCCTACG	94°C 3 min; 30 cycles x [94°C 30 sec, 55°C 1 min, 72°C 1.5 min]; 72°C 5 min	1281			
	<i>plcNR</i>	TCGCTGTCGAGCAGGTCTGAAC					
Exotoxin A	<i>toxAF</i>	CTGCGCGGTCTATGTGCC	94°C 3 min; 30 cycles x [94°C 30 sec, 55°C 1 min, 72°C 1.5 min]; 72°C 5 min	280	[33]		
	<i>toxAR</i>	GATGCTGGACGGGTCGAG					
L-ornithine N5-oxygenase	<i>pvdAF</i>	GACTCAGGCAACTGCAAC	94°C 3 min; 30 cycles x [94°C 30 sec, 55°C 1 min, 72°C 1.5 min]; 72°C 5 min	233	[32]		
	<i>pvdAR</i>	TTCAGGTGCTGGTACAGG					
Integron	<i>int1F</i>	CCTCCCGCAGATGATC	95°C 5 min; 30 cycles x [95°C 15 sec, 56°C 15 sec, 72°C 1 min]; 72°C 7 min	600	[32]		
	<i>int1R</i>	TCCACGCATCGTCAGGC					
	<i>int2F</i>	TTATTGCTGGGATTAGGC					
	<i>int2R</i>	ACGGCTACCCTCTGTTATC					
	<i>int3F</i>	AGTGGGTGGCGAATGAGTG					
	<i>int3R</i>	TGTTCTGTATCGGCAGGTG					

## Whole-Genome Sequencing

For whole-genome sequencing, 6 isolates were selected based on antimicrobial resistance phenotype and isolation site. The genomic DNA of selected 6 *P. aeruginosa* strains was extracted using a MagAttract HMW DNA extraction kit (Qiagen, Hilden, Germany). Genomic DNA was quantified on a Qubit 2.0 fluorometer using the dsDNA BR assay kit (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). The sequencing libraries of the bacterial genomes were prepared using The Illumina Nextera XT DNA library preparation kit (Illumina Inc, San Diego, CA, USA). The paired-end (2x150 bp) sequencing run was performed on the NovaSeq platform (Illumina Inc., San Diego, CA).

## Quality Control, Trimming, Assembling and Annotation

The raw sequenced reads were checked for quality using FastQC v.0.11.5 [36]. After trimming for low-quality reads and adapter regions using Trimmomatic v.0.36 [37], *de novo* genome assembly was performed using the SPAdes algorithm (version 3.1.14) [38], and contigs less than 200 bp were filtered out using BBmap 38.06 (<https://github.com/BioInfoTools/BBMap>). Assembly metrics were calculated using QUAST v.5.0.0 [39]. Gene predictions and annotations were performed using the National Center for Biotechnology Information's (NCBI) Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) [40].

## Identification of The Resistome

The detection of the acquired antimicrobial resistance genes (ARGs) was conducted using the Center for Genomic Epidemiology (CGE) (<http://www.genomicepidemiology.org/>) tools and ABRicate v.0.8.13 (<https://github.com/tsemann/abricate>) with the default settings. In addition, mutations involved in fluoroquinolone and carbapenem resistance were also analyzed using SNIPPY software (v4.4.3) (<https://github.com/tsemann/snippy>), mapping all assemblies against the *P. aeruginosa* PAO1 reference genome (GenBank Accession No. NC\_002516.2).

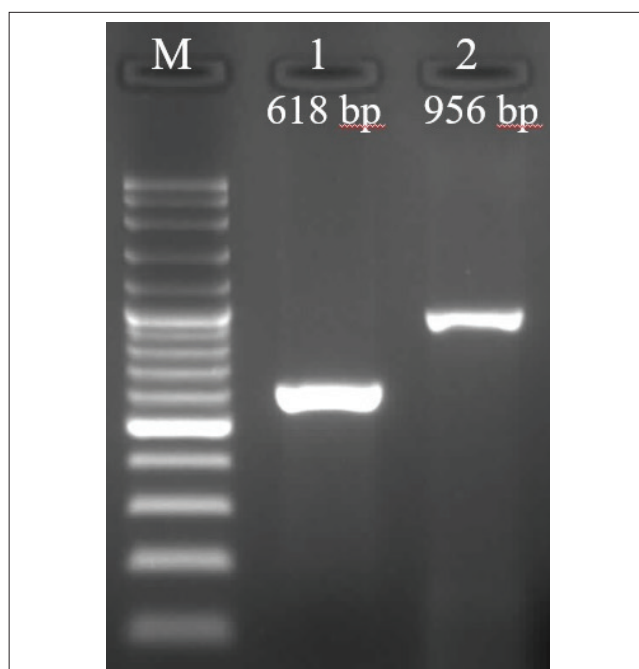
## Multi-Locus Sequence Typing and Serotyping

Serotypes of the isolates were determined using the *P. aeruginosa* serotypes (PAst) script available on CGE [41]. *In silico*, multi-locus sequence typing (MLSTs) was performed using the MLST 2.0 server (<http://www.cge.cbs.dtu.dk/services/MLST/>).

# RESULTS

## Isolation and Identification

Fourty four (2.84%) *P. aeruginosa* were isolated and identified from 1546 milk samples examined (Fig. 1).



**Fig 1.** Agarose gel electrophoresis image of amplified products of the PCRs. Lane M: 100 bp plus molecular marker, Lane 1: 618 bp size amplified product of 16S rRNA *Pseudomonas* spp., Lane 2: 956 bp size amplified product of 16S rRNA *Pseudomonas aeruginosa*

## Antimicrobial Susceptibility

Antimicrobial resistance phenotypes of *P. aeruginosa* are given in Table 2. The test results revealed that most of the isolates (n=32) were susceptible to all tested antimicrobials. Only 12 (27.3%) isolates were resistant to at least one of the antimicrobials tested. One isolate displayed the MDR phenotype. Of the meropenem and imipenem resistant four *P. aeruginosa* isolates by disc diffusion method, two isolates (ADU\_VET\_ST3 and

**Table 2.** Antimicrobial resistance phenotypes of *Pseudomonas aeruginosa* isolated from bovine clinical mastitis cases

Isolate ID	Resistance Phenotype <sup>a</sup>
ADU_VET_ST3 <sup>b</sup>	CIP
ADU_VET_ST5 <sup>b</sup>	CIP
ADU_VET_ST6	CIP
ADU_VET_ST11	CIP
ADU_VET_ST22 <sup>c</sup>	MER, IMP, CIP
ADU_VET_ST24	CN, TOB, AK, CIP, CAZ
ADU_VET_ST30 <sup>c</sup>	MER, IMP
ADU_VET_ST32	CN, CIP
HARRAN_VET_OK7	CIP
HARRAN_VET_OK11	CIP
HARRAN_VET_OK12	CIP
HARRAN_VET_OK28	CIP

<sup>a</sup>MER: meropenem, IMP: imipenem, CIP: ciprofloxacin, CN: gentamicin, TOB: tobramycin, AK: amikacin, CAZ: ceftazidime; <sup>b</sup>These two isolates had meropenem and imipenem MIC of  $\leq 2$   $\mu\text{g}/\text{mL}$ ; <sup>c</sup>These two isolates had meropenem and imipenem MIC of  $\geq 16$   $\mu\text{g}/\text{mL}$

ADU\_VET\_ST5) were found to be susceptible to both meropenem and imipenem by E-test (MIC values of  $\leq 2$   $\mu\text{g}/\text{mL}$ ), other two isolates (ADU\_VET\_ST22 and ADU\_VET\_ST30) were also found to be resistant to both meropenem and imipenem by E-test (MIC values of  $\geq 16$   $\mu\text{g}/\text{mL}$ ). Antimicrobial resistance rates for ciprofloxacin, gentamicin, tobramycin, amikacin and ceftazidime were recorded as 22.7% (10), 4.5% (2), 2.3% (1), 2.3% (1) and 2.3% (1), respectively.

### Detection of Virulence Genes

Several virulence-associated genes with various combinations were detected in all isolates (Table 3, Fig. 2, Fig. 3). Of the examined 44 *P. aeruginosa* isolates, all carried at least one of the four T3SS-related genes. The eleven isolates (25%) harboured *exoU* and/or *exoS* genes, including non-cytotoxic/invasive strain (*exoU*-/*exoS*+, 18.2%) and cytotoxic/non-invasive strain (*exoU*+/*exoS*-, 6.8%). The non-cytotoxic/non-invasive strain genotype (*exoU*-/*exoS*-) was detected in 33 (75%) isolates, of which 30 (68.2%) isolates carried both *exoY* and *exoT*, and 3 (6.8%) carried only *exoT* gene.

The distribution of QS genes revealed the presence of five different gene combinations among the isolates. The thirty (68.2%) isolates were positive for all investigated QS genes.

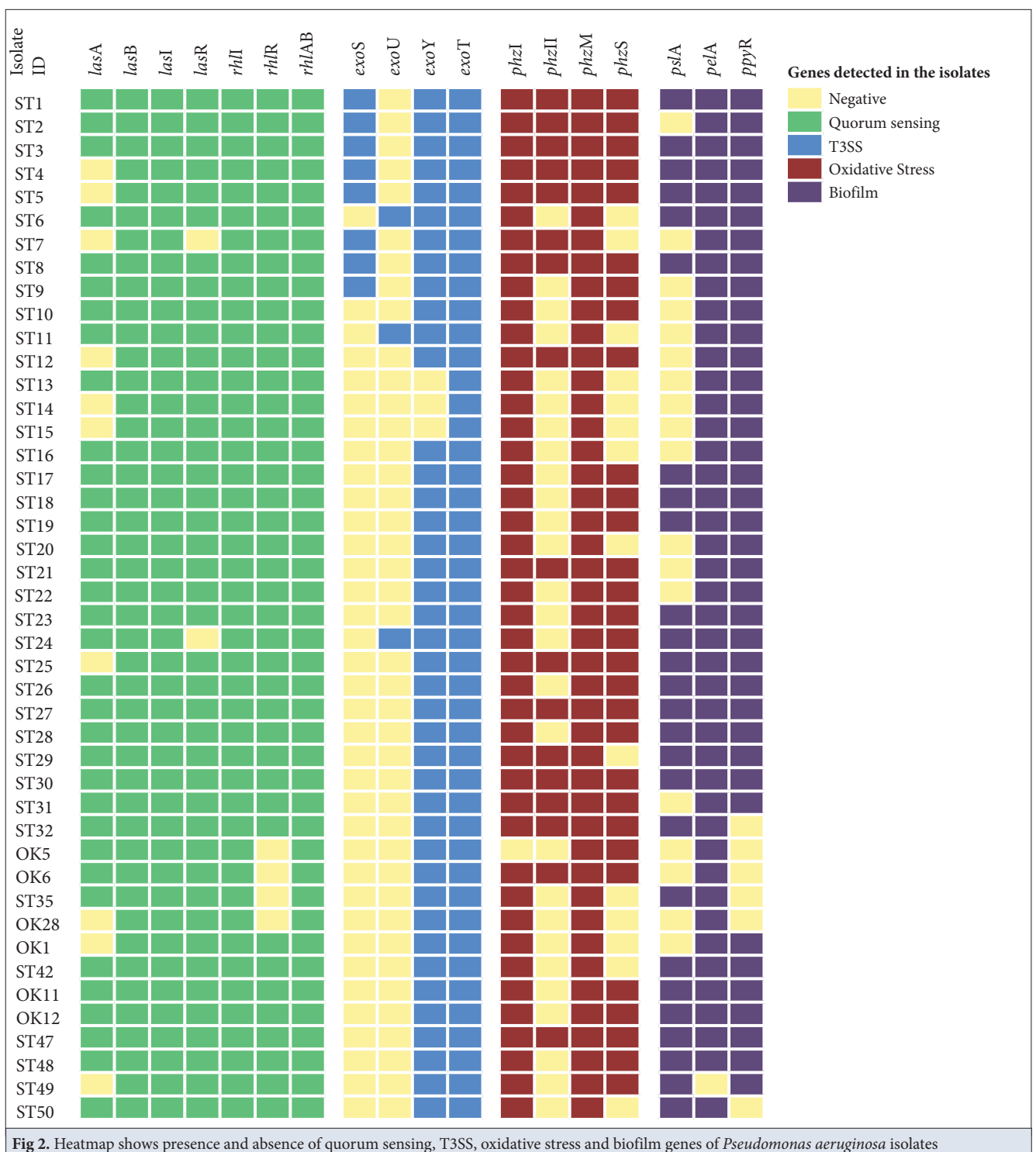
Oxidative stress-related phenazine genes were observed among isolates being *phzI*+/*phzII*+/*phzM*+/*phzS*+ (16/44, 36.4%) and *phzI*+/*phzII*+/*phzM*+ (15/44, 34.1%) as most common gene combination. While all isolates were positive for alginate genes, half of the isolates were positive for all examined biofilm-related genes. Of biofilm-related genes, *pelA* and *ppyR* genes were detected in 16 isolates, *pslA* and *pelA* in three isolates, and *pelA* in three isolates. Fimbria genes were only presented in 34.1% (15) of the isolates, of which 12 isolates carried *pilA* and 3 had both *pilA* and *pilB*. Of the investigated toxin gene, the *exoA* and *pvdA* genes were detected in 17 and 13 isolates, respectively. The investigated phospholipase genes, *plcH*, and *plcN* were simultaneously detected in 34 isolates, and *plcN* was detected alone in 10 isolates.

### Detection of Integrons

Eighteen (40.9%) isolates were found to be integron positive. Class II integron was detected in eleven (25%)

**Table 3.** Virulence genes detected in *Pseudomonas aeruginosa* isolated from clinical bovine mastitis cases

Related Function	Gene or Gene Combination	Number of the Isolates (%)
T3SS	<i>exoS, exoY, exoT</i>	8 (18.2)
	<i>exoU, exoY, exoT</i>	3 (6.8)
	<i>exoY, exoT</i>	30 (68.2)
	<i>exoT</i>	3 (6.8)
QS/regulation	<i>lasA, lasB, lasI, lasR, rhII, rhIR, rhIAB</i>	30 (68.2)
	<i>lasB, lasI, lasR, rhII, rhIR, rhIAB</i>	8 (18.2)
	<i>lasA, lasB, lasI, lasR, rhII, rhIAB</i>	3 (6.8)
	<i>lasA, lasB, lasI, rhII, rhIR, rhIAB</i>	1 (2.3)
	<i>lasB, lasI, rhII, rhIR, rhIAB</i>	2 (4.5)
Adhesin	<i>algU, algL, algD</i>	44 (100)
Oxidative stress	<i>phzI, phzII, phzM, phzS</i>	16 (36.4)
	<i>phzI, phzM, phzS</i>	15 (34.1)
	<i>phzI, phzM</i>	11 (25)
	<i>phzI, phzII, phzM</i>	1 (2.3)
	<i>phzM, phzS</i>	1 (2.3)
Biofilm related genes	<i>pslA, pelA, ppyR</i>	22 (50)
	<i>pelA, ppyR</i>	16 (36.4)
	<i>pslA, pelA</i>	3 (6.8)
	<i>pelA</i>	3 (6.8)
Exotoxin A	<i>exoA</i>	17 (38.6)
L-ornithine N5-oxygenase	<i>pvdA</i>	13 (29.5)
Pilus	<i>pilA, pilB</i>	3 (6.8)
	<i>pilA</i>	12 (27.3)
Phospholipase	<i>plcH, plcN</i>	34 (77.3)
	<i>plcN</i>	10 (22.7)
Initial colonization factor flagellin	<i>fliC type A</i>	24 (54.5)
	<i>fliC type B</i>	18 (40.9)



isolates, class I integron in one (2.3%) isolate, and both class I and II integron in 6 (13.6%) isolates. Whereas no isolate carried class III integron.

### Sequencing Statistics

The median length of genome assembly of the isolates was 6.78 Mbp with an average GC% of  $66.07 \pm 0.176$ . The average  $N_{50}$  of the assembled contigs was 3.93 Mbp.

Detailed sequence statistics and genome features are summarized in [Table 4](#).

### Serotypes and Multi-Locus Sequence Types

While five *P. aeruginosa* isolates were serotyped, one isolate (ADU\_VET\_ST32) was found to be non-typeable by this approach due to the absence of a single definitive hit to the gene encoding the entire O-specific antigen (OSA). Four

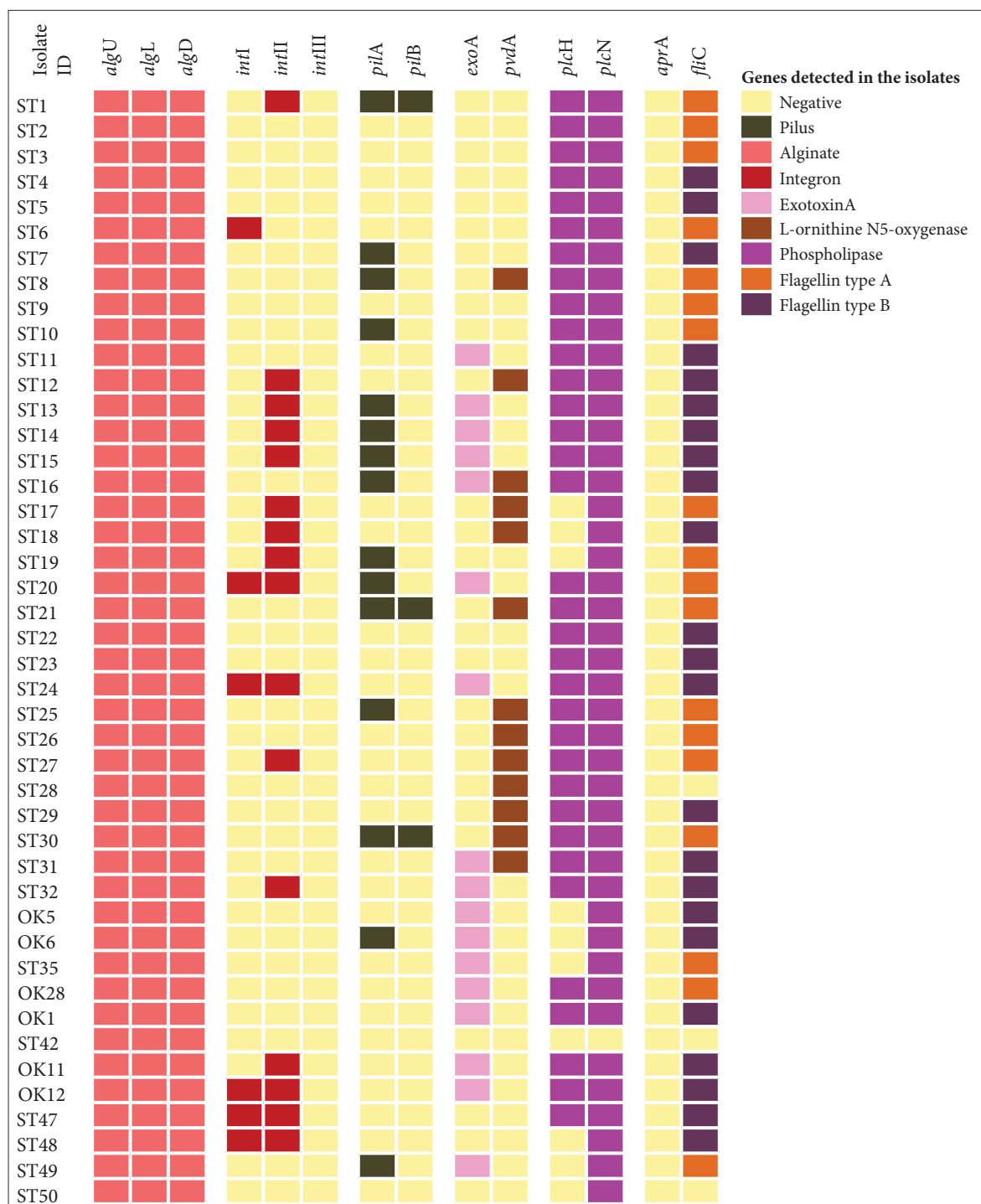


Fig 3. Heatmap shows presence and absence of pilus, alginate, virulence, integron, exotoxinA, L-ornithine N5-oxygenase, phospholipase, flagellin type A and B genes of *Pseudomonas aeruginosa* isolates

different serotypes were detected among the five isolates, i.e., O5 (HARRAN\_VET\_OK28), O6 (ADU\_VET\_ST3), O7 (ADU\_VET\_ST30), and O11 (ADU\_VET\_ST11, ADU\_VET\_ST24). The isolates were assigned to the respective sequence types (STs) with ST3246 (ADU\_VET\_ST3), ST235 (ADU\_VET\_ST11), ST235 (ADU\_VET\_ST24), ST1247 (ADU\_VET\_ST30), ST865 (ADU\_VET\_ST32), and ST591 (HARRAN\_VET\_OK28).

### In Silico Detection of Acquired and Mutation Mediated Resistance

Based on the WGS analyses, 20 different types of acquired resistance genes found against various classes of antimicrobials including beta-lactams ( $bla_{PAO}$ ,  $bla_{PDC-374}$ ,  $bla_{OXA-488}$ ,  $bla_{OXA-396}$ ,  $bla_{OXA-494}$ ,  $bla_{GES-1}$ ,  $bla_{OXA-904}$ ,  $bla_{PDC-55}$ , and  $bla_{OXA-50}$ ), aminoglycosides ( $aph(3')-Iib$ ,  $aadA6$ ,  $aac(6')-Ib4$ , and  $aph(3')-XV$ ), phenicol ( $catB7$  and  $floR2$ ),



**Table 4.** The sequence statistics and genome features of whole-genome sequencing analysis

Characteristic	Data for Isolate					
	ADU_VET_ST3	ADU_VET_ST11	ADU_VET_ST24	ADU_VET_ST30	ADU_VET_ST32	HARRAN_VET_OK28
Genome coverage (x)	83	83	83	83	83	83
No. of the contigs	89	310	362	168	4973	121
Total length (bp)	6 776 179	6 866 621	6 797 270	6 368 118	6 790 887	6 749 145
Largest contig	1 344 433	907 132	700 981	1 225 473	94 170	1 340 126
GC content (%)	65.9	65.99	66.04	66.41	66.02	66.04
N <sub>50</sub> (bp)	781 852	272 482	355 003	431 554	29 306	469 602
N <sub>75</sub> (bp)	362 714	185 975	224 305	239 081	13 490	306 699
L <sub>50</sub> (bp)	4	7	7	4	71	5
L <sub>75</sub> (bp)	7	15	13	9	158	9
GenBank accession no	JAKGDU000000000	JAKGDT000000000	JAKGDS000000000	JAKGDR000000000	JAKGDQ000000000	JAKGDP000000000
BioSample no.	SAMN25050669	SAMN25050670	SAMN25050671	SAMN25050672	SAMN25050673	SAMN25050674

**Table 5.** Genetic characteristics of *Pseudomonas* isolates

Characteristics	ADU_VET_ST3	ADU_VET_ST11	ADU_VET_ST24	ADU_VET_ST30	ADU_VET_ST32	HARRAN_VET_OK28
ST Serotype/Type	O6/ST3246	O11/ST235	O11/ST235	O7/ST1247	ND <sup>a</sup> ST865	O5/ST591
Resistance phenotype	CIP	CIP	CIP, CN, TOB, AK, CAZ	MER, IPM	CIP, CN	CIP
Antimicrobial resistance genes	<i>bla</i> <sub>PDC-374</sub> , <i>bla</i> <sub>OXA-50</sub> , <i>bla</i> <sub>PAO</sub> , <i>aph(3')-IIb</i> , <i>crpP</i> , <i>catB7</i> , <i>fosA</i>	<i>bla</i> <sub>PDC-374</sub> , <i>bla</i> <sub>OXA-488</sub> , <i>bla</i> <sub>PAO</sub> , <i>aph(3')-IIb</i> , <i>aadA6</i> , <i>crpP</i> , <i>catB7</i> , <i>sul1</i> , <i>fosA</i> , <i>qacE</i>	<i>bla</i> <sub>PDC-374</sub> , <i>bla</i> <sub>OXA-488</sub> , <i>bla</i> <sub>GES-1</sub> , <i>bla</i> <sub>PAO</sub> , <i>aph(3')-IIb</i> , <i>aadA6</i> , <i>aac(6)-Ib4</i> , <i>aph(3')-XV</i> , <i>aac(6)-Ib-cr</i> , <i>catB7</i> , <i>floR2</i> , <i>tetG</i> , <i>fosA</i> , <i>qacE</i>	<i>bla</i> <sub>PDC-374</sub> , <i>bla</i> <sub>OXA-494</sub> , <i>bla</i> <sub>OXA-396</sub> , <i>bla</i> <sub>PAO</sub> , <i>aph(3')-IIb</i> , <i>catB7</i> , <i>fosA</i>	<i>bla</i> <sub>OXA-904</sub> , <i>bla</i> <sub>OXA-396</sub> , <i>aph(3')-IIb</i> , <i>catB7</i> , <i>fosA</i>	<i>bla</i> <sub>OXA-494</sub> , <i>bla</i> <sub>PDC-55</sub> , <i>bla</i> <sub>PAO</sub> , <i>aph(3')-IIb</i> , <i>catB7</i> , <i>fosA</i>
<b>Amino acid substitutions<sup>b</sup></b>						
AmpC regulators						
<i>ampR</i>	M288R, G283E	M288R, G283E	M288R, G283E			M288R
<i>ampD</i>	D183T, P162L, G148A	G148A	G148A		D183T	G148A
<i>ampDh2</i>		A239V, A196T				
<i>ampDh3</i>		A208V	A208V	A219T		A219T
QRDR						
<i>gyrA</i>	T83I	T83I	T83I		N652T, N87T	T83I
<i>gyrB</i>				S466F		
<i>parC</i>			S87L		P595L	S87L
<i>parE</i>	E459V	D533E, S457R	D533E			
<b>Efflux pumps regulatory genes</b>						
MexAB-OprM regulators						
<i>mexR</i>						V126E
<i>nalC</i>	G71E	G71E, E153Q, S209R	G71E, E153Q, S209R	G71E	G71E	G71E, S209R
<i>nalD</i>		A162E				
MexCD-OprJ regulator						
<i>nfxB</i>				R21H, D56G, V134L		
MexEF-OprN regulators						
<i>MexS</i>						
<i>MexT</i>	P60S, Q80fs, F172I	P60S, Q80fs, F172I	P60S, Q80fs, F172I	P60S, Q80fs, R164H, F172I	F172Ile	P60S, Q80fs, F172I
MexXY-OprA regulator						
<i>MexZ</i>						
OprD mutations	D43N, S57Glu, E202Q, I210A, E230L, S240T, N262, A267S, A281G, L296Q, Q301E, R310G	T103S, L115T, P185Q, F170L, V189T, R310E, A315G, G425A	T103S, L115T, Q185G, F170L, V189T, R310E, A315G, G425A	Frameshift ( $\Delta$ nt 402/fs)		D43N, S57E, E202Q, I210A, E230L, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G

<sup>b</sup> ND: not detected; <sup>a</sup> Amino acid substitution compared to the sequences of *Pseudomonas aeruginosa* strain PAO1 (GenBank accession number NC\_002516.29)

sulphonamides (*sul1*), tetracycline (*tetG*), fosfomycin (*fosA*), and quinolones (*crpC* and *aac(6')-Ib-cr*) in the study strains. Carbapenem-resistant isolates did not harbor metallo- $\beta$ -lactamase genes (*bla<sub>IMP</sub>*, *bla<sub>NDM</sub>*, *bla<sub>VIM</sub>*, and *bla<sub>GES</sub>*). Additionally, the *qacE* gene conferring resistance to quaternary ammonium compounds (QACs) was also detected. Different amino acid substitutions in *gyrA*, *parC*, *oprD*, efflux pump, and AmpC  $\beta$ -lactamase regulatory genes were identified among representative isolates (Table 5).

### Data Availability

The genomic sequences for these isolates are available at NCBI under the BioProject number PRJNA798228.

## DISCUSSION

In this study, 72.7% of the isolates were susceptible to all tested antimicrobials, and resistance rates for aminoglycosides and cephalosporins were less than 4.5%, and for carbapenems and fluoroquinolones were 9.1% and 25%, respectively. In a previous study conducted in Japan [17], no resistance for this class of antimicrobials was reported, another study carried out in South Korea [18], resistance rates for meropenem and ciprofloxacin were reported as 4.9% and 0.8%, respectively. In Egypt, Ibrahim et al. [19] found that 11.76% of 34 *P. aeruginosa* from clinical mastitis were resistant to meropenem, and 3 of these isolates were positive for the *bla<sub>VIM</sub>* gene.

Carbapenems are a class of  $\beta$ -lactam antibiotic with broad-spectrum activity used as a last resort for treating infections caused by multidrug-resistant bacteria. Carbapenems are not used in veterinary medicine in Türkiye. The presence of carbapenem-resistant isolates among the isolates might be attributed to environmental contamination rather than the selective pressure exerted by these antimicrobials. Resistance to carbapenems in *P. aeruginosa* is mainly due to a contribution of several genetic mechanisms including horizontally acquired carbapenemases, low permeability or loss of *oprD*, overexpression of efflux pumps (mainly MexAB-OprM and MexXY-OprM), and overexpression of intrinsic AmpC beta-lactamases [42]. Previous studies demonstrated that *oprD* mutation together with other intrinsic mechanisms (overexpression of *mexAB* and *ampC*) might play an important role in carbapenem resistance [43,44]. Although amino acid substitutions in OprD have not been well elucidated in carbapenem resistance development, *oprD*-mediated carbapenem resistance can be achieved by the downregulation of *oprD* expression or the inactivation of this porin through a frameshift by the insertion/deletion mutation and/or a premature stop codon [44]. In this study, carbapenem-resistant isolate (ADU\_VET\_ST30) had frameshift mutation together with *nalC* and *nfxB* (involved in the regulation of efflux pumps) and

*ampC* regulator genes (Table 5). Similar observations were also reported by Hayashi et al. [45]. However, further molecular studies are needed to elucidate evolutionary mechanisms for carbapenem resistance in *P. aeruginosa* isolates.

Fluoroquinolones are one of the most prescribed antimicrobials to treat various bacterial infections in both human and veterinary medicine in Türkiye. Misuse and overuse of this class of antimicrobials have contributed to the selection and spread of resistant bacteria species isolated from animals and humans. Fluoroquinolone resistance rates against some bovine mastitis pathogens were reported as 23.17% in *E. coli*, 41.67% in *P. aeruginosa*, and 29.63% in *K. pneumonia* in Burdur province [46]; 25% in *S. aureus*, 26.32% in *E. coli*, 25% in *P. aeruginosa* and 100% *K. pneumonia* in Bursa province [13]; 3.7% in *S. aureus*, 4.5% in CoNS, and 36.5% in *E. coli* in Kars province [8]. The differences in fluoroquinolone resistance rates could be explained by the usage rates of this group of antimicrobials.

Major resistance mechanisms of fluoroquinolones in *P. aeruginosa* isolates are associated with mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* [47]. Consistent with previous studies, analysis of WGS data revealed that the main mechanism of resistance in ciprofloxacin-resistant isolates was related to mutations in gyrase and topoisomerase encoding genes, especially DNA gyrase II (*gyrA*) and topoisomerase IV (*parC*) [48-50]. Other resistance mechanisms, such as decreased intracellular accumulation regulated by the *nalC* and *nfxB* genes, are also important in clinical resistance to fluoroquinolones in *P. aeruginosa* but were mostly found in strains with high-level resistance together with mutations in DNA replicating enzymes [47]. In addition, horizontally transferred *aac(6')-Ib-cr* and *crpP* genes responsible for decreased susceptibility to ciprofloxacin were detected in two isolates. *crpP* (plasmid-encoded ciprofloxacin resistance protein) is the novel resistance determinant, which was recently described in Mexico within the plasmid named pUM505 in *P. aeruginosa*, conferring resistance to decreased susceptibility to ciprofloxacin through the enzymatic phosphorylation of the antibiotic [51]. Recently, the presence of *crpP* (variant *crpP*-2) was reported in two carbapenemase-producing *P. aeruginosa* isolates in a tertiary hospital in Türkiye [52].

Integrations are genetic elements that have the ability to capture, exchange, and express resistance genes called gene cassettes, and hence, they play a key role in the horizontal transmission of resistance genes [21]. Integrations have been reported to be closely related to MDR, especially class I integrations, and are widely distributed in clinical *P. aeruginosa* isolates [53]. The class I and II integrations were only observed in 5 of twelve resistant isolates. However,

it is not possible to ascertain an association between integron carriage and resistance phenotype due to the not known genetic context of class I and II integrons. Further studies are therefore needed to elucidate the genetic context of integrons.

*P. aeruginosa* possesses a large and variable arsenal of virulence factors that contribute to its pathogenesis. Most of *P. aeruginosa* virulence factors are regulated by a cell density-dependent mechanism called Quorum Sensing (QS) [23]. In this study, all isolates harbored various combinations of QS system genes, including *lasI* and *rhlI* genes, which are responsible for the autoinducers (PAI-1 and PAI-2) biosynthesis and upregulation of virulence genes.

Based on T3SS related genes, 25% of the isolates were found to harbour *exoU* and/or *exoS* genes, including the invasive (*exoU*-/*exoS*+, 18.2%) and cytotoxic (*exoU*+/*exoS*-, 6.8%) strains. In contrast, higher rates of *exoS* or *exoU* were reported by Park et al. [18], who found that 82.7% of the isolates had *exoU* and/or *exoS* genes. The researchers also suggested that *P. aeruginosa* isolates having either *exoS* or *exoU* genes should be considered as pathogenic for dairy cattle. Similarly, Tartor et al. [54] examined 8 *P. aeruginosa* isolates from clinical and subclinical milk samples and found that 5 isolates carried both *exoS* and *exoU* and 2 isolates had only *exoS* and one isolate *exoU*. Horna et al. [55] investigated the presence of the *exoU*+/*exoS*+ genotype in 189 *P. aeruginosa* clinical isolates and found that all isolates had *exoS*, *exoT* and *exoY* genes, but 22.8% (43/189) of the isolates carried *exoU*. The authors stated that *exoU* was significantly associated MDR as well as with higher level quinolone resistance. Indeed, *exoU* carrying 3 isolates had ciprofloxacin-resistant phenotype; and one of these isolates had also MDR phenotype.

Epidemiological studies have revealed that some sequence types (ST111, ST175, ST235, ST244, and ST395) of *P. aeruginosa* are distributed worldwide and are frequently associated with outbreaks [56]. Of these clones, ST235 (serotype O11) has been reported to be the predominant globally high-risk clone with MDR profiles [57]. It has been also reported that ST235 is highly associated with *exoU* and infections caused by this clone have a highly unfavorable prognosis than infections with other strains [58]. Detection of the ST235/O11 serotype associated with *exoU* in two *P. aeruginosa* isolates from clinical bovine mastitis suggests that mastitis pathogens may have adverse public health implications.

In conclusion, this is the first comprehensive study to date to determine the presence and distribution of virulence traits in *P. aeruginosa* isolated from clinical bovine mastitis. The findings of the study also indicate a low rate of resistance to tested antimicrobials and the presence of high-risk clone ST235 among the isolates.

Comprehensive studies are needed to better understand the molecular epidemiology of *P. aeruginosa* isolates at the human-animal-environment interface and to assess their clinical implications on humans and animals. In addition, considering the importance of virulence determinants, anti-virulence strategies should be developed to combat infections caused by *P. aeruginosa*.

#### Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author (Ö. Aslantaş) on reasonable request.

#### Ethical Statement

The study does not require ethical approval from Animal Experiments Local Ethics Committee

#### Funding Support

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#### Conflict of Interest

The authors declared that there is no conflict of interest related to this study.

#### Author Contributions

ÖA, ST, OK and KB planned, designed, and supervised the research procedure, ÖA, ST and AGY performed all microbiological and molecular experiments, ÖA and KB performed bioinformatic analyses, and ÖA wrote the manuscript. All authors have read and approved the manuscript.

## REFERENCES

1. Moradali MF, Ghods S, Rehm BH: *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. *Front Cell Infect Microbiol*, 7:39, 2017. DOI: 10.3389/fcimb.2017.00039
2. Haenni M, Hocquet D, Ponsin C, Cholley P, Guyeux C, Madec JY, Bertrand X: Population structure and antimicrobial susceptibility of *Pseudomonas aeruginosa* from animal infections in France. *BMC Vet Res*, 11:9, 2015. DOI: 10.1186/s12917-015-0324-x
3. Mulani MS, Kamble EE, Kumkar SN, Tawre MS, Pardesi KR: Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A review. *Front Microbiol*, 10:539, 2019. DOI: 10.3389/fmicb.2019.00539
4. Amer S, Gálvez FLA, Fukuda Y, Tada C, Jimenez IL, Valle WFM, Nakai Y: Prevalence and etiology of mastitis in dairy cattle in El Oro Province, Ecuador. *J Vet Med Sci*, 80 (6): 861-868, 2018. DOI: 10.1292/jvms.17-0504
5. El-Sayed A, Kamel M: Bovine mastitis prevention and control in the post-antibiotic era. *Trop Anim Health Prod*, 53 (2): 236, 2021. DOI: 10.1007/s11250-021-02680-9
6. Kurt S, Eski F, Mis L, Ayzazoglu Demir P: Evaluation of oxidative stress, immune system and mineral concentrations in milk and serum of cows with clinical and subclinical mastitis naturally infected by *Staphylococcus aureus*. *Kafkas Univ Vet Fak Derg*, 27 (6): 755-762, 2021. DOI: 10.9775/kvfd.2021.26281
7. Lhermie G, Gröhn YT, Raboisson D: Addressing antimicrobial resistance: An overview of priority actions to prevent suboptimal antimicrobial use in food-animal production. *Front Microbiol*, 7:2114, 2016. DOI: 10.3389/fmicb.2016.02114
8. Gülmez Sağlam A, Otlı S, Coşkun MR, Çelik E, Büyük F, Şahin M: Prevalence of subclinical mastitis in cows, isolation of agents and determination of their antibiotic susceptibility. *Eurasian J Vet Sci*, 34, 92-98, 2018. DOI: 10.15312/EurasianJVetSci.2018.188

9. Macun HC, Yağcı İP, Ünal N, Kalender H, Sakarya E, Yıldırım M: Agent isolation and antibiotic resistance in dairy cows with subclinical mastitis in Kırıkkale. *J Fac Vet Med Univ Erciyes*, 8, 83-89, 2011.
10. Tel OY, Keskin O, Zonturlu AK, Arserim NB: Subclinical mastitis prevalence and determination of the antibiotics susceptibility in Şanlıurfa region. *Fırat Univ Vet J Health Sci*, 23, 101-106, 2012.
11. Sarıçam İnce S, Baştan A, Salar S, Dikmeoğlu E, Oğuz T, Akan M: Detection of bacterial isolation and antimicrobial resistance profiles in goat mastitis. *Kafkas Univ Vet Fak Derg*, 27 (6): 741-747, 2021. DOI: 10.9775/kvfd.2021.26239
12. WHO: Worldwide country situation analysis: Response to antimicrobial resistance.2015. [https://apps.who.int/iris/bitstream/handle/10665/163468/9789241564946\\_eng.pdf](https://apps.who.int/iris/bitstream/handle/10665/163468/9789241564946_eng.pdf); Accessed: June 16, 2022.
13. Ardıçlı Ö, Kahya Demirbilek S, Çarlı KT: Pathogens isolated from bovine clinical mastitis and their antimicrobial resistance. *Med Weter*, 78 (1): 19-24, 2022. DOI: 10.21521/mw.6606
14. Daly M, Power E, Björkroth J, Sheehan P, O'Connell A, Colgan M, Korkeala H, Fanning S: Molecular analysis of *Pseudomonas aeruginosa*: Epidemiological investigation of mastitis outbreaks in Irish dairy herds. *Appl Environ Microbiol*, 65 (6): 2723-2729, 1999. DOI: 10.1128/aem.65.6.2723-2729.1999
15. Petridou EJ, Fragkou IA, Lafi SQ, Giadinis ND: Outbreak of *Pseudomonas aeruginosa* mastitis in a dairy cow herd in northern Greece and its control with an autogenous vaccine. *Pol J Vet Sci*, 24 (2): 303-305, 2021. DOI: 10.24425/pjvs.2021.137666
16. Sela S, Hammer-Muntz O, Krifucks O, Pinto R, Weisblit L, Leitner G: Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* strains isolated from mastitis outbreaks in dairy herds. *J Dairy Res*, 74 (4): 425-429, 2007. DOI: 10.1017/s0022029907002610
17. Ohnishi M, Sawada T, Hirose K, Sato R, Hayashimoto M, Hata E, Yonezawa C, Kato H: Antimicrobial susceptibilities and bacteriological characteristics of bovine *Pseudomonas aeruginosa* and *Serratia marcescens* isolates from mastitis. *Vet Microbiol*, 154 (1-2): 202-207, 2011. DOI: 10.1016/j.vetmic.2011.06.023
18. Park H, Hong M, Hwang S, Park Y, Kwon K, Yoon J, Shin S, Kim J, Park Y: Characterisation of *Pseudomonas aeruginosa* related to bovine mastitis. *Acta Vet Hung*, 62 (1): 1-12, 2014. DOI: 10.1556/AVet.2013.054
19. Ibrahim NA, El-Metwally Farag VM, Abd-El-Moaty AM, Atwa SM: Resistant gene of *Pseudomonas aeruginosa* in mastitic cattle with reference to some biochemical and immunological parameters. *World Vet J*, 7, 5-13, 2017. DOI: 10.5455/wvj.20170287
20. Pang Z, Raudonis R, Glick BR, Lin TJ, Cheng Z: Antibiotic resistance in *Pseudomonas aeruginosa*: Mechanisms and alternative therapeutic strategies. *Biotechnol Adv*, 37 (1): 177-192, 2019. DOI: 10.1016/j.biotechadv.2018.11.013
21. Gillings MR: Integrons: Past, present, and future. *Microbiol Mol Biol Rev*, 78 (2): 257-277, 2014. DOI: 10.1128/mmbr.00056-13
22. Domingues S, da Silva GJ, Nielsen KM: Integrons: Vehicles and pathways for horizontal dissemination in bacteria. *Mob Genet Elements*, 2 (5): 211-223, 2012. DOI: 10.4161/mge.22967
23. Jurado-Martín I, Sainz-Mejías M, McClean S: *Pseudomonas aeruginosa*: An audacious pathogen with an adaptable arsenal of virulence factors. *Int J Mol Sci*, 22 (6):3128, 2021. DOI: 10.3390/ijms22063128
24. Lee J, Zhang L: The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell*, 6 (1): 26-41, 2015. DOI: 10.1007/s12328-014-0100-x
25. Horna G, Ruiz J: Type 3 secretion system of *Pseudomonas aeruginosa*. *Microbiol Res*, 246:126719, 2021. DOI: 10.1016/j.micres.2021.126719
26. Sauvage S, Hardouin J: Exoproteomics for better understanding *Pseudomonas aeruginosa* virulence. *Toxins (Basel)*, 12 (9):571, 2020. DOI: 10.3390/toxins12090571
27. Spilker T, Coenye T, Vandamme P, LiPuma JJ: PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. *J Clin Microbiol*, 42 (5): 2074-2079, 2004. DOI: 10.1128/jcm.42.5.2074-2079.2004
28. CLSI: Performance standards for antimicrobial susceptibility testing. CLSI document: M100-32. Wayne, PA; 2022.
29. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL: Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect*, 18 (3): 268-281, 2012. DOI: 10.1111/j.1469-0691.2011.03570.x
30. Ghadaksaz A, Fooladi AAI, Hosseini HM, Amin M: The prevalence of some *Pseudomonas* virulence genes related to biofilm formation and alginate production among clinical isolates. *J Appl Biomed*, 13 (1): 61-68, 2015. DOI: 10.1016/j.jab.2014.05.002
31. Ajayi T, Allmond LR, Sawa T, Wiener-Kronish JP: 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, 2003. DOI: 10.1128/JCM.41.8.3526-3531.2003
32. Fazeli N, Momtaz H: Virulence gene profiles of multidrug-resistant *Pseudomonas aeruginosa* isolated from Iranian hospital infections. *Iran Red Crescent Med J*, 16 (10):e15722, 2014. DOI: 10.5812/ircmj.15722
33. Sabharwal N, Dhali S, Chhibber S, Harjai K: Molecular detection of virulence genes as markers in *Pseudomonas aeruginosa* isolated from urinary tract infections. *Int J Mol Epidemiol Genet*, 5 (3): 125-134, 2014.
34. Goldstein C, Lee MD, Sanchez S, Hudson C, Phillips B, Register B, Grady M, Liebert C, Summers AO, White DG, Maurer JJ: Incidence of class 1 and 2 integrase in clinical and commensal bacteria from livestock, companion animals, and exotics. *Antimicrob Agents Chemother*, 45 (3): 723-726, 2001. DOI: 10.1128/aac.45.3.723-726.2001
35. Bass L, Liebert CA, Lee MD, Summers AO, White DG, Thayer SG, Maurer JJ: Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. *Antimicrob Agents Chemother*, 43 (12): 2925-2929, 1999. DOI: 10.1128/aac.43.12.2925
36. Andrews, S. FastQC: A quality control tool for high throughput sequence data. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>; Accessed: January 12, 2022.
37. Bolger AM, Lohse M, Usadel B: Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30 (15): 2114-2120, 2014. DOI: 10.1093/bioinformatics/btu170
38. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA: SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol*, 19 (5): 455-477, 2012. DOI: 10.1089/cmb.2012.0021
39. Gurevich A, Saveliev V, Vyahhi N, Tesler G: QUILT: quality assessment tool for genome assemblies. *Bioinformatics*, 29 (8): 1072-1075, 2013. DOI: 10.1093/bioinformatics/btt086
40. Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, Lomsadze A, Pruitt KD, Borodovsky M, Ostell J: NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res*, 44 (14): 6614-6624, 2016. DOI: 10.1093/nar/gkw569
41. Thrane SW, Taylor VL, Lund O, Lam JS, Jelsbak L: Application of whole-genome sequencing data for O-specific antigen analysis and in silico serotyping of *Pseudomonas aeruginosa* isolates. *J Clin Microbiol*, 54 (7): 1782-1788, 2016. DOI: 10.1128/jcm.00349-16
42. Wolter DJ, Lister PD: Mechanisms of  $\beta$ -lactam resistance among *Pseudomonas aeruginosa*. *Curr Pharm Des*, 19 (2): 209-222, 2013.
43. Wołkowicz T, Patzer JA, Kamińska W, Gierczyński R, Dzierżanowska D: Distribution of carbapenem resistance mechanisms in *Pseudomonas aeruginosa* isolates among hospitalised children in Poland: Characterisation of two novel insertion sequences disrupting the oprD gene. *J Glob Antimicrob Resist*, 7, 119-125, 2016. DOI: 10.1016/j.jgar.2016.08.007
44. Shu JC, Kuo AJ, Su LH, Liu TP, Lee MH, Su IN, Wu TL: Development of carbapenem resistance in *Pseudomonas aeruginosa* is associated with OprD polymorphisms, particularly the amino acid substitution at codon 170. *J Antimicrob Chemother*, 72 (9): 2489-2495, 2017. DOI: 10.1093/jac/dkx158

45. Hayashi W, Izumi K, Yoshida S, Takizawa S, Sakaguchi K, Iyori K, Minoshima KI, Takano S, Kitagawa M, Nagano Y, Nagano N: Antimicrobial resistance and type III secretion system virulotypes of *Pseudomonas aeruginosa* isolates from dogs and cats in primary veterinary hospitals in Japan: Identification of the international high-risk clone sequence type 235. *Microbiol Spectr*, 9 (2):e0040821, 2021. DOI: 10.1128/Spectrum.00408-21
46. Öztürk D, Yapıcıer ÖŞ, Şababoğlu E, Kaya M, Pehlivanoglu F, Türütoglu H: The antibiotic resistance of Gram negative bacteria isolated from bovine mastitis. *Van Vet J*, 30, 85-89, 2019.
47. Rehman A, Patrick WM, Lamont IL: Mechanisms of ciprofloxacin resistance in *Pseudomonas aeruginosa*: New approaches to an old problem. *J Med Microbiol*, 68 (1): 1-10, 2019. DOI: 10.1099/jmm.0.000873
48. Harada K, Arima S, Niina A, Kataoka Y, Takahashi T: Characterization of *Pseudomonas aeruginosa* isolates from dogs and cats in Japan: Current status of antimicrobial resistance and prevailing resistance mechanisms. *Microbiol Immunol*, 56 (2): 123-127, 2012. DOI: 10.1111/j.1348-0421.2011.00416.x
49. Nouri R, Ahangarzadeh Rezaee M, Hasani A, Aghazadeh M, Asgharzadeh M: The role of *gyrA* and *parC* mutations in fluoroquinolones-resistant *Pseudomonas aeruginosa* isolates from Iran. *Braz J Microbiol*, 47 (4): 925-930, 2016. DOI: 10.1016/j.bjm.2016.07.016
50. Vingopoulou EI, Delis GA, Batzias GC, Kaltsogianni F, Koutinas A, Kristo I, Pournaras S, Saridomichelakis MN, Siarkou VI: Prevalence and mechanisms of resistance to fluoroquinolones in *Pseudomonas aeruginosa* and *Escherichia coli* isolates recovered from dogs suffering from otitis in Greece. *Vet Microbiol*, 213, 102-107, 2018. DOI: 10.1016/j.vetmic.2017.11.024
51. Chávez-Jacobo VM, Hernández-Ramírez KC, Romo-Rodríguez P, Pérez-Gallardo RV, Campos-García J, Gutiérrez-Corona JE, García-Merinos JP, Meza-Carmen V, Silva-Sánchez J, Ramírez-Díaz MI: CrpP is a novel ciprofloxacin-modifying enzyme encoded by the *Pseudomonas aeruginosa* pUM505 plasmid. *Antimicrob Agents Chemother*. 62 (6):e02629-17, 2018. DOI: 10.1128/AAC.02629-17
52. Çekin ZK, Dabos L, Malkoçoğlu G, Fortineau N, Bayraktar B, Iorga BI, Naas T, Aktaş E: Carbapenemase-producing *Pseudomonas aeruginosa* isolates from Turkey: First report of *P. aeruginosa* high-risk clones with VIM-5- and IMP-7-type carbapenemases in a tertiary hospital. *Diagn Microbiol Infect Dis*, 99 (1): 115174, 2020. DOI: 10.1016/j.diagmicrobio.2020.115174
53. Liu M, Ma J, Jia W, Li W: Antimicrobial resistance and molecular characterization of gene cassettes from class 1 integrons in *Pseudomonas aeruginosa* strains. *Microb Drug Resist*, 26 (6): 670-676, 2020. DOI: 10.1089/mdr.2019.0406
54. Tartor YH, Gharieb RMA, Abd El-Aziz NK, El Damaty HM, Enany S, Khalifa E, Attia ASA, Abdellatif SS, Ramadan H: Virulence determinants and plasmid-mediated colistin resistance *mcr* genes in Gram-negative bacteria isolated from bovine milk. *Front Cell Infect Microbiol*, 11:761417, 2021. DOI: 10.3389/fcimb.2021.761417
55. Horna G, Amaro C, Palacios A, Guerra H, Ruiz J: High frequency of the *exoU*+/*exoS*+ genotype associated with multidrug-resistant “high-risk clones” of *Pseudomonas aeruginosa* clinical isolates from Peruvian hospitals. *Sci Rep*, 9 (1):10874, 2019. DOI: 10.1038/s41598-019-47303-4
56. Miyoshi-Akiyama T, Tada T, Ohmagari N, Viet Hung N, Tharavichitkul P, Pokhrel BM, Gniadkowski M, Shimojima M, Kirikae T: Emergence and spread of epidemic multidrug-resistant *Pseudomonas aeruginosa*. *Genome Biol Evol*, 9 (12): 3238-3245, 2017. DOI: 10.1093/gbe/evx243
57. Nageeb W, Amin DH, Mohammedsaleh ZM, Makharita RR: Novel molecular markers linked to *Pseudomonas aeruginosa* epidemic high-risk clones. *Antibiotics (Basel)*, 10 (1):35, 2021. DOI: 10.3390/antibiotics10010035
58. Treepong P, Kos VN, Guyeux C, Blanc DS, Bertrand X, Valot B, Hocquet D: Global emergence of the widespread *Pseudomonas aeruginosa* ST235 clone. *Clin Microbiol Infect*, 24 (3): 258-266, 2018. DOI: 10.1016/j.cmi.2017.06.018

