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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 resistome, but *fos*A genes in all isolates, and different combinations of mutations in *gyr*A, *par*C, *opr*D, 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 gözlendi. 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 rezistomun, *gyrA*, *parC*, *oprD*, efflux pump ve *ampC* gen ekspresyonunun regulasyonunda 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, Virulens, 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 [1]. *P. aeruginosa* is one of the most common nosocomial pathogens worldwide, causing life-threatening nosocomial infections especially among immunocompromised and critically ill patients [1,2]. 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) [3]. *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 [2].

The frequency of other mastitis pathogens differs between herds, geographic locations, and whether or not mastitis control programs are implemented [4]. 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 [5,6]. 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 [7]. Currently, β-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 [8-11]. Although resistance rates to quinolones (enrofloxacin and danofloxacin) and third-generation cephalosporins classified as critically important antimicrobials by the World Health Organization (WHO) [12] 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 [13].

P. aeruginosa-related mastitis cases were mostly related to outbreaks [14-16]. Ohnishi et al.[17] 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.[18] isolated 116 *P. aeruginosa* without any bacteria from 35 625 raw milk samples collected from nine provinces in Korea. Ibrahim et al.[19] 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 [20]. 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 [21]. The integrons were transferred between species by hitchhiking with other MGEs [22].

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 [23]. Some of the virulence factors are coordinated by a cell density recognition mechanism called Quorum Sensing (QS) [24]. 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 [25,26].

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

ß-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 (*alg*U, *alg*D, and *alg*L), oxidative stress (*phz*I, *phz*II, *phz*M, and *phz*S), quorum sensing (QS)/regulation (*las*A, *las*B, *las*I, *las*R, *rhl*AB, *rhl*I, and *rhl*R), T3SS (*exo*S, *exo*U, *exo*T, and *exo*Y), biofilm (*psl*A, *pel*A, and *ppy*), alkaline protease (*aprA*) and flagellin (*fli*C), exotoxin A (*exoA*), pilin (*pil*A) and type IV pili (*pil*B), phospholipases C (*plc*H, *plc*N), L-ornithine N5-oxygenase, and class I, II, and III integrons [30-35]. Primers used for the detection of virulence and integron genes genus 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 (*Pseudomonas* 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	CACTGGTGTTCCTTCCTATA			
16S rRNA (*P. aeruginosa*)	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	
	PA-SS-R	TCCTTAGAGTGCCCACCCG			
Biofilm	*psl*AF	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	[28]
	*psl*AR	TGTTGTAGCCGTAGCGTTTCTG			
	*pel*AF	CATACCTTCAGCCATCCGTTCTTC		786	
	*pel*AR	CGCATTCGCCGCACTCAG			
	*ppy*RF	CGTGATCG CCGCCTATTTCC		160	
	*ppy*RR	ACAGCAGACCTCCCAACCG			
Alginate	*alg*UF	CGATGTGACCGCAGAGGATG		292	
	*alg*UR	TCAGGCTTCTCGCAACAAAGG			
	*alg*LF	CCGCTCGCAGATCAAGGACATC		432	
	*alg*LR	TCGCTCACCGCCCAGTCG			
	*alg*DF	AGAAGTCCGAACGCCACACC		550	
	*alg*DR	CGCATCAACGAACCGAGCATC			
Type III Secretion System	*exo*SF	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]
	*exo*SR	TTCGGCGTCACTGTGGATGC			
	*exo*UF	CCGTTGTGGTGCCGTTGAAG		134	
	*exo*UR	CCAGATGTTCACCGACTCGC			
	*exo*YF	CGGATT CTATGGCAGGGAGG		289	
	*exo*YR	GCCCTTGATGCACTCGACCA			
	*exo*TF	AATCGCCGTCCAACTGCATGCG		152	
	*exo*TR	TGTTCGCCGAGGTACTGCTC			

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	*las*A-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]
	*las*A-R	GAAATGCAGGTGCGGTC			
	*las*B-F	GGAATGAACGAAGCGTTCTCCGAC		284	
	*las*B-R	TGGCGTCGACGAACACCTCG			
	*las*I-F	CGTGCTCAAGTGTTCAAGG	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]
	*las*I-R	TACAGTCGGAAAAGCCCAG			
	*las*R-F	AAGTGGAAAATTGGAGTGGAG		130	
	*las*R-R	GTAGTTGCCGACGACGATGAAG			
	*rhl*I-F	TTCATCCTCCTTTAGTCTTCCC		155	
	*rhl*I-R	TTCCAGCGATTCAGAGAGC			
	*rhl*R-F	TGCATTTTATCGATCAGGGC		133	
	*rhl*R-R	CACTTCCTTTTCCAGGACG			
	*rhl*AB-F	TCATGGAATTGTCACAACCGC		151	
	*rhl*AB-R	ATACGGCAAAATCATGGCAAC			
Alkaline metalloproteinase	*aprAF*	GTCGACCAGGCGGCGGAGCAGATA	95°C 2 min; 30 cycles x [95°C 40 sec, 65°C 1 min, 72°C 2 min]; 72°C 10 min	993	
	aprAR	GCCGAGGCCGCCGTAGAGGATGTC			
Initial colonization factor flagellin	*fli*CF	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)	
	*fli*CR	GGCCTGCAGATCNCCAA			
Major pilin	*pil*AF	ACAGCATCCAACTGAGCG	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]
	*pil*AR	TTGACTTCCTCCAGGCTG			
Type IV fimbrial biogenesis protein	*pil*BF	TCGAACTGATGATCGTGG		408	
	*pil*BR	CTTTCGGAGTGAACATCG			
Phenazine operon I	*phz*IF	CATCAGCTTAGCAATCCC		392	
	*phz*IR	CGGAGAAACTTTTCCCTC			
Phenazine operon II	*phz*IIF	GCCAAGGTTTGTTGTCGG		1036	
	*phz*IIR	CGCATTGACGATATGGAAC			
Phenazines	*phz*MF	ATGGAGAGCGGGATCGACAG		875	
	*phz*MR	ATGCGGGTTTCCATCGGCAG			
	*phz*SF	TCGCCATGACCGATACGCTC		1752	
	*phz*SR	ACAACCTGAGCCAGCCTTCC			
Hemolytic phospholipase C	*plc*HF	GCACGTGGTCATCCTGATGC	94°C 3 min; 30 cycles x [94°C 30 sec, 58°C 30 sec, 72°C 1 min]; 72°C 7 min	608	
	*plc*HR	TCCGTAGGCGTCGACGTAC			
Non-hemolytic phospholipase C	*plc*NF	TCCGTTATCGCAACCAGCCCTACG		481	
	*plc*NR	TCGCTGTCGAGCAGGTCGAAC			
Exotoxin A	*tox*AF	CTGCGCGGGTCTATGTGCC		270	
	*tox*AR	GATGCTGGACGGGTCGAG			
L-ornithine N5-oxygenase	*pvd*AF	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	1281	
	*pvd*AR	TTCAGGTGCTGGTACAGG			
Integron	*int*1F	CCTCCCGCACGATGATC	95°C 5 min; 30 cycles × [95°C 15 sec, 56°C 15 sec, 72°C 1 min]; 72°C 7 min	280	[33]
	*int*1R	TCCACGCATCGTCAGGC			
	*int*2F	TTATTGCTGGGATTAGGC	95°C 5 min; 30 cycles x [95°C 15 sec, 50°C 15 sec, 72°C 1 min]; 72°C 7 min	233	[32]
	*int*2R	ACGGCTACCCTCTGTTATC			
	*int*3F	AGTGGGTGGCGAATGAGTG		600	
	*int*3R	TGTTCTTGTATCGGCAGGTG			

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/tseemann/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 slico,* 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).*

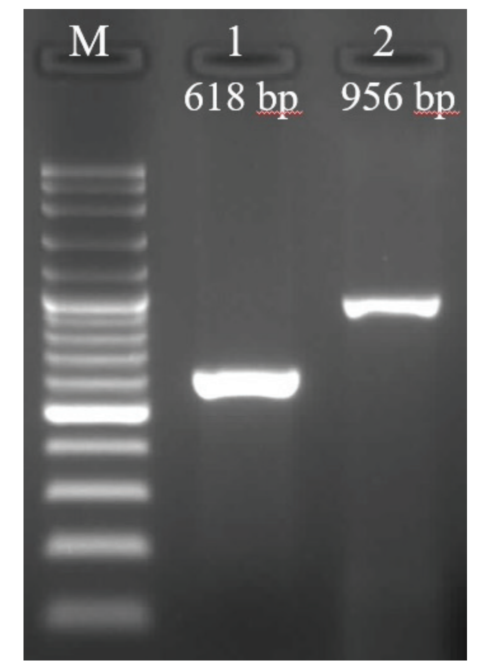

Fig 1. Agarose gel electrophoresis image of amplified products of the PCRs. Lane M: 100 bp plus molecular marker, Lane 1: 618 bp size amplified product of 16S rRNA *Pseudomonas* spp., Lane 2: 956 bp size amplified product of 16S rRNA *Pseudomonas aeruginosa*

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[a]
ADU_VET_ST3[b]	CIP
ADU_VET_ST5[b]	CIP
ADU_VET_ST6	CIP
ADU_VET_ST11	CIP
ADU_VET_ST22[c]	MER, IPM, CIP
ADU_VET_ST24	CN, TOB, AK, CIP, CAZ
ADU_VET_ST30[c]	MER, IPM
ADU_VET_ST32	CN, CIP
HARRAN_VET_OK7	CIP
HARRAN_VET_OK11	CIP
HARRAN_VET_OK12	CIP
HARRAN_VET_OK28	CIP

[a] ***MER:*** *meropenem,* ***IMP:*** *imipenem,* ***CIP:*** *ciprofloxacin,* ***CN:*** *gentamicin,* ***TOB:*** *tobramycin,* ***AK:*** *amikacin,* ***CAZ:*** *ceftazidime;* [b] *These two isolates had meropenem and imipenem MIC of ≤2 µg/mL,* [c] *These two isolates had meropenem and imipenem MIC of ≥16 µg/mL*

ADU_VET_ST5) were found to be susceptible to both meropenem and imipenem by E-test (MIC values of ≤2 μg/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 ≥16 μg/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 *exo*U and/or *exo*S genes, including non-cytotoxic/invasive strain *(exo*U-/*exo*S+, 18.2%*)* and cytotoxic/non-invasive strain *(exo*U+/*exo*S-, 6.8%*)*. The non-cytotoxic/non-invasive strain genotype *(exo*U-/*exo*S-*)* was detected in 33 (75%) isolates, of which 30 (68.2%) isolates carried both *exo*Y and *exo*T, and 3 (6.8%) carried only *exo*T 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 *phz*I$^+$/*phz*II$^+$/*phz*M$^+$/*phz*S$^+$ (16/44, 36.4%) and *phz*I$^+$/*phz*II$^+$/*phz*M$^+$ (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, *pel*A and *ppy*R genes were detected in 16 isolates, *psl*A and *pel*A in three isolates, and *pel*A in three isolates. Fimbria genes were only presented in 34.1% (15) of the isolates, of which 12 isolates carried *pil*A and 3 had both *pil*A and *pil*B. Of the investigated toxin gene, the *exo*A and *pvd*A genes were detected in 17 and 13 isolates, respectively. The investigated phospholipase genes, *plc*H, and *plc*N were simultaneously detected in 34 isolates, and *plc*N 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	*exoS, exoY, exoT*	8 (18.2)
	exoU, exoY, exoT	3 (6.8)
	exoY, exoT	30 (68.2)
	exoT	3 (6.8)
QS/regulation	*lasA, lasB, lasI, lasR, rhlI, rhlR, rhlAB*	30 (68.2)
	lasB, lasI, lasR, rhlI, rhlR, rhlAB	8 (18.2)
	lasA, lasB, lasI, lasR, rhlI, rhlAB	3 (6.8)
	lasA, lasB, lasI, rhlI, rhlR, rhlAB	1 (2.3)
	lasB, lasI, rhlI, rhlR, rhlAB	2 /4.5)
Adhesin	*algU, algL, algD*	44 (100)
Oxidative stres	*phzI, phzII, phzM, phzS*	16 (36.4)
	phzI, phzM, phzS	15 (34.1)
	phzI, phzM	11 (25)
	phzI, phzII, phzM	1 (2.3)
	phzM, phzS	1 (2.3)
Biofilm related genes	*pslA, pelA, ppyR*	22 (50)
	pelA, ppyR	16 (36.4)
	pslA, pelA	3 (6.8)
	pelA	3 (6.8)
Exotoxin A	*exoA*	17 (38.6)
L-ornithine N5-oxygenase	*pvdA*	13 (29.5)
Pilus	*pilA, pilB*	3 (6.8)
	pilA	12 (27.3)
Phospholipase	*plcH, plcN*	34 (77.3)
	plcN	10 (22.7)
Initial colonization factor flagellin	*fliC type A*	24 (54.5)
	fliC type B	18 (40.9)

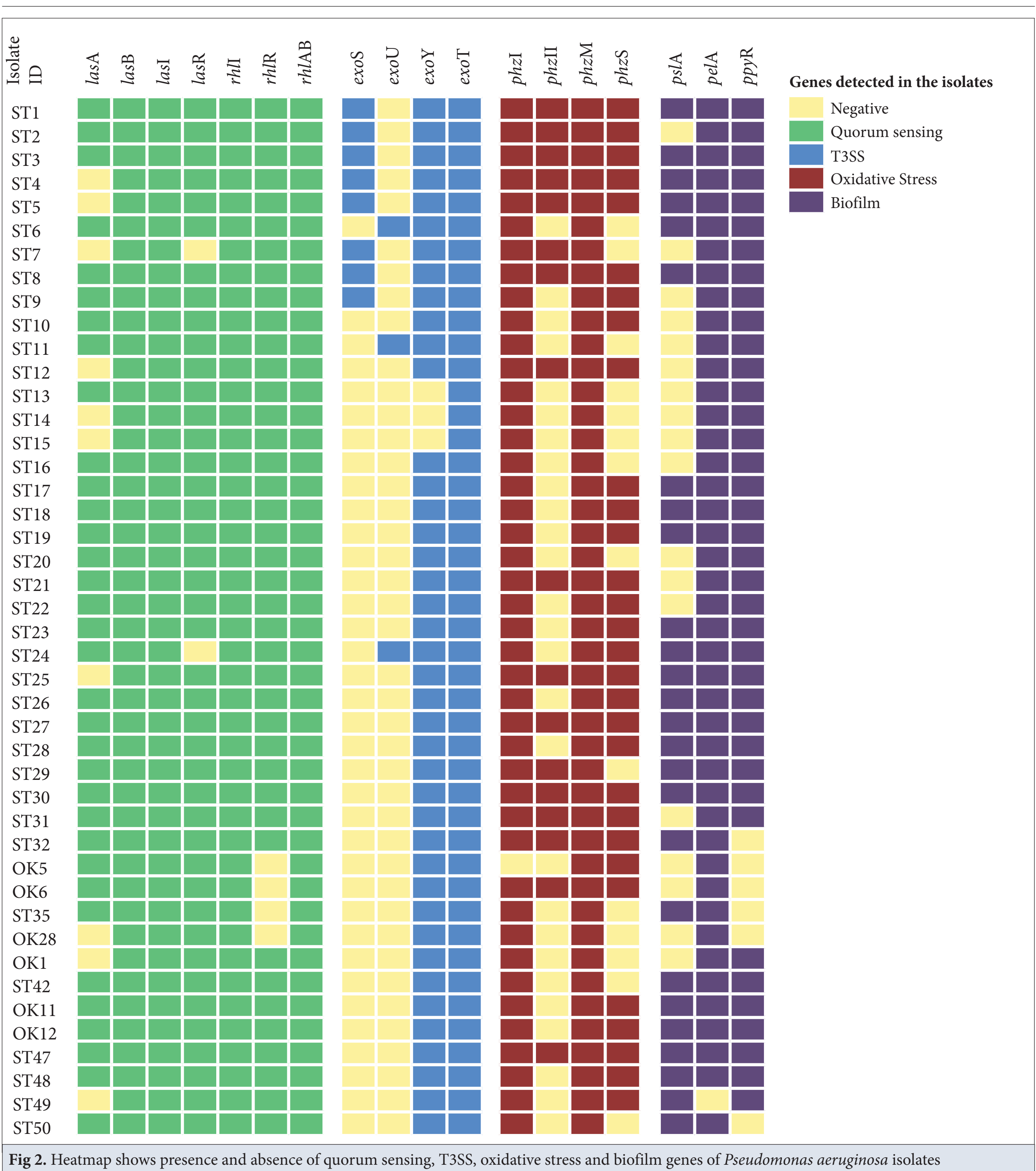

Fig 2. Heatmap shows presence and absence of quorum sensing, T3SS, oxidative stress and biofilm genes of *Pseudomonas aeruginosa* isolates

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±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

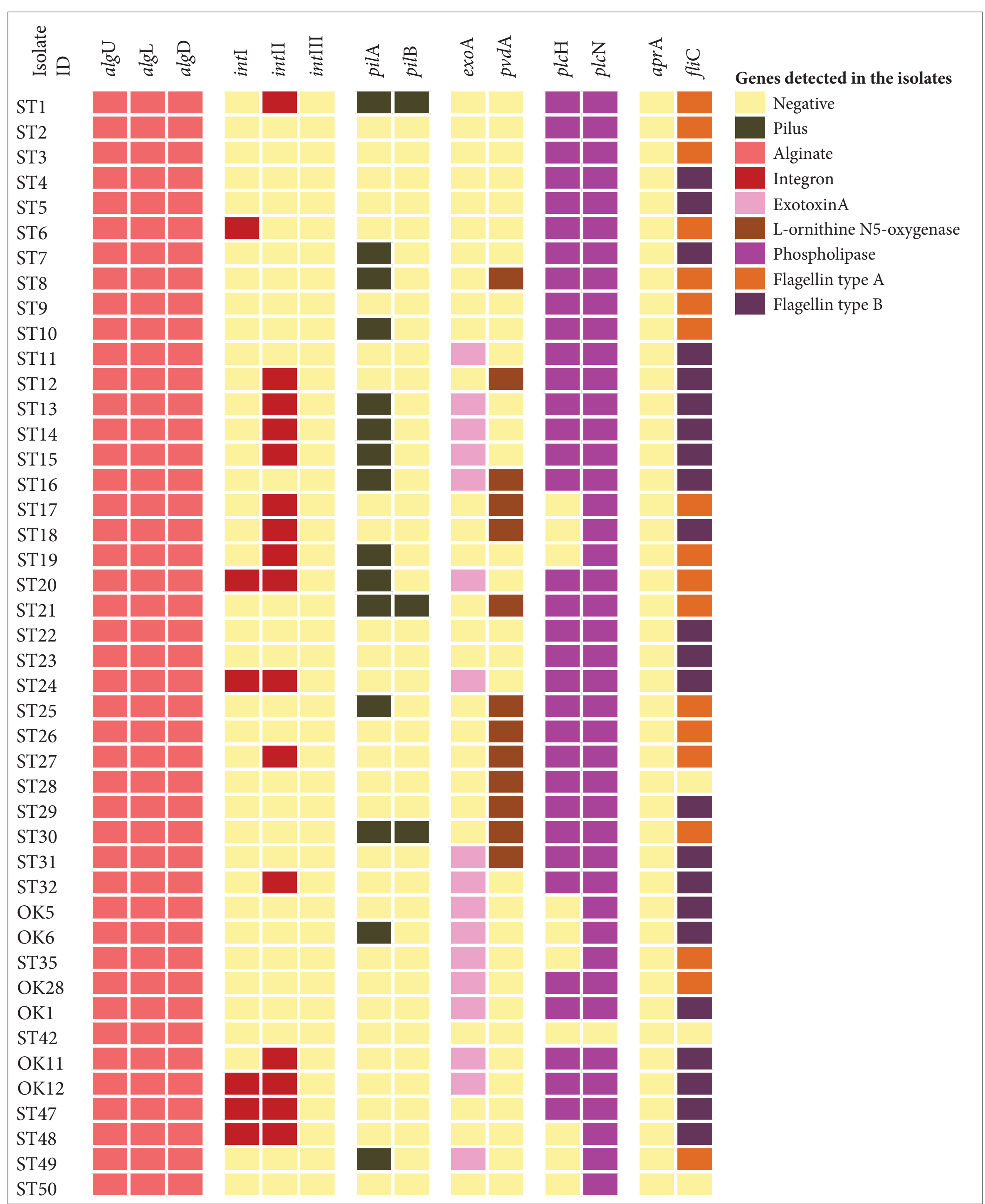

Fig 3. Heatmap shows presence and absence of pilus, alginate, virulence, integron, exotoxinA, L-ornithine N5-oxygenase, phospholipase, flagellin type A and B genes of *Pseudomonas aeruginosa* isolates

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 Slico 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*$_{\text{PAO}}$, *bla*$_{\text{PDC-374}}$, *bla*$_{\text{OXA-488}}$, *bla*$_{\text{OXA-396}}$, *bla*$_{\text{OXA-494}}$, *bla*$_{\text{GES-1}}$, *bla*$_{\text{OXA-904}}$, *bla*$_{\text{PDC-55}}$, and *bla*$_{\text{OXA-50}}$), aminoglycosides *(aph(3')-IIb*, *aad*A6, *aac(6')-Ib4*, and *aph(3')-XV)*, phenicols *(cat*B7 and *flo*R2),

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 (×)	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_{50} (bp)	781 852	272 482	355 003	431 554	29 306	469 602
N_{75} (bp)	362 714	185 975	224 305	239 081	13 490	306 699
L_{50} (bp)	4	7	7	4	71	5
L_{75} (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[a]/ST865	O5/ST591
Resistance phenotype	CIP	CIP	CIP, CN, TOB, AK, CAZ	MER, IPM	CIP, CN	CIP
Antimicrobial resistance genes	$bla_{\text{PDC-374}}$, $bla_{\text{OXA-50}}$, bla_{PAO}, *aph(3')-IIb*, *crp*P, *cat*B7, *fos*A	$bla_{\text{PDC-374}}$, $bla_{\text{OXA-488}}$, bla_{PAO}, *aph(3')-IIb*, *aad*A6, *crp*P, *cat*B7, *sul*1, *fos*A, *qac*E	$bla_{\text{PDC-374}}$, $bla_{\text{OXA-488}}$, $bla_{\text{GES-1}}$, bla_{PAO}, *aph(3')-IIb*, *aad*A6, *aac(6')-Ib4*, *aph(3')-XV*, *aac(6')-Ib-cr*, *cat*B7, *flo*R2, *tet*G, *fos*A, *qac*E	$bla_{\text{PDC-374}}$, $bla_{\text{OXA-494}}$, $bla_{\text{OXA-396}}$, bla_{PAO}, *aph(3')-IIb*, *cat*B7, *fos*A	$bla_{\text{OXA-904}}$, $bla_{\text{OXA-396}}$, *aph(3')-IIb*, *cat*B7, *fos*A	$bla_{\text{OXA-494}}$, $bla_{\text{PDC-55}}$, bla_{PAO}, *aph(3')-IIb*, *cat*B7, *fos*A
Amino acid substitutions[b]						
AmpC regulators						
*amp*R	M288R, G283E	M288R, G283E	M288R, G283E			M288R
*amp*D	D183T, P162L, G148A	G148A	G148A		D183T	G148A
*amp*Dh2		A239V, A196T				
*amp*Dh3		A208V	A208V	A219T		A219T
QRDR						
*gyr*A	T83I	T83I	T83I		N652T, N87T	T83I
gyrB				S466F		
*par*C			S87L		P595L	S87L
*par*E	E459V	D533E, S457R	D533E			
Efflux pumps regulatory genes						
MexAB-OprM regulators						
*mex*R						V126E
*nal*C	G71E	G71E, E153Q, S209R	G71E, E153Q, S209R	G71E	G71E	G71E, S209R
*nal*D		A162E				
MexCD-OprJ regulator						
*nfx*B				R21H, D56G, V134L		
MexEF-OprN regulators						
MexS						
MexT	P60S, Q80fs, F172I	P60S, Q80fs, F172I	P60S, Q80fs, F172I	P60S, Q80fs, R164H, F172I	F172Ile	P60S, Q80fs, F172I
MexXY-OprA regulator						
MexZ						
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 (Δnt 402/fs)		D43N, S57E, E202Q, I210A, E230L, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G

[b] ***ND:*** *not detected;* [a] *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-β-lactamase genes (bla_{IMP}, bla_{NDM}, bla_{VIM}, and bla_{GES}). Additionally, the *qac*E gene conferring resistance to quaternary ammonium compounds (QACs) was also detected. Different amino acid substitutions in *gyr*A, *par*C, *opr*D, efflux pump, and AmpC β-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_{VIM} gene.

Carbapenems are a class of ß-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 *mex*AB and *amp*C) 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 *opr*D 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 *nal*C and *nfx*B (involved in the regulation of efflux pumps) and *amp*C 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 *gyr*A, *gyr*B, *par*C, and *par*E [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 *nal*C and *nfx*B 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 *crp*P genes responsible for decreased susceptibility to ciprofloxacin were detected in two isolates. *crp*P (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 *crp*P (variant crpP-2) was reported in two carbapenemase-producing *P. aeruginosa* isolates in a tertiary hospital in Türkiye [52].

Integrons 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]. Integrons have been reported to be closely related to MDR, especially class I integrons, and are widely distributed in clinical *P. aeruginosa* isolates [53]. The class I and II integrons 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 *las*I and *rhl*I 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 *exo*U and/or *exo*S genes, including the invasive (*exo*U-/*exo*S+, 18.2%) and cytotoxic (*exo*U+/*exo*S-, 6.8%) strains. In contrast, higher rates of *exo*S or *exo*U were reported by Park et al.[18], who found that 82.7% of the isolates had *exo*U and/or *exo*S genes. The researchers also suggested that *P. aeruginosa* isolates having either *exo*S or *exo*U 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 *exo*S and *exo*U and 2 isolates had only *exo*S and one isolate *exo*U. Horna et al.[55] investigated the presence of the *exo*U+/*exo*S+ genotype in 189 *P. aeruginosa* clinical isolates and found that all isolates had *exo*S, *exoT* and *exo*Y genes, but 22.8% (43/189) of the isolates carried *exo*U. The authors stated that *exo*U was significantly associated MDR as well as with higher level quinolone resistance. Indeed, *exo*U 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 *exo*U 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

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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.

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