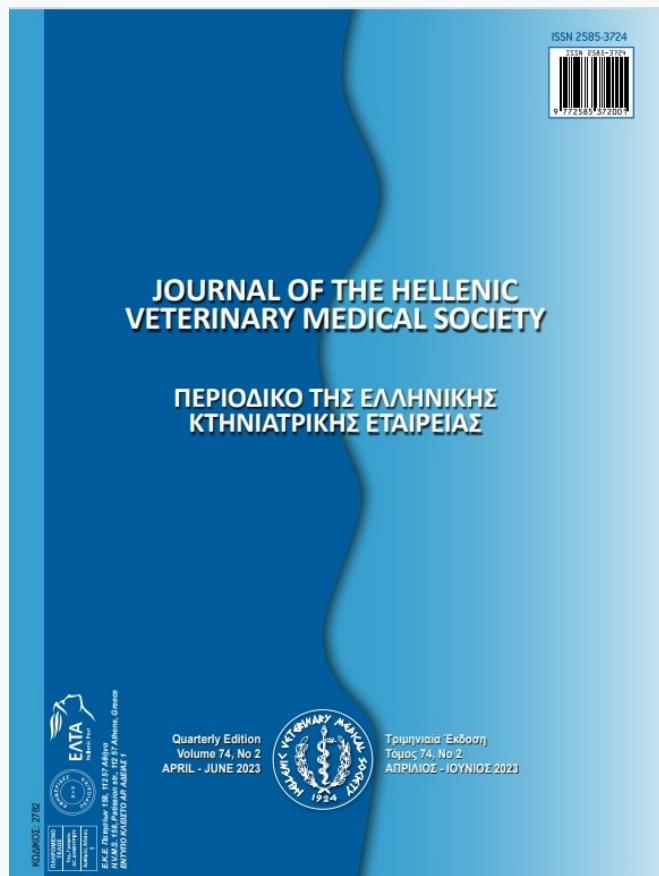


Journal of the Hellenic Veterinary Medical Society

Vol 74, No 3 (2023)



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doi: [10.12681/jhvms.30641](https://doi.org/10.12681/jhvms.30641)

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To cite this article:

Ocak, F., & Turkyilmaz, S. (2023). Investigation of genetic relatedness, antimicrobial resistance, biofilm formation, biofilm-related virulence genes and integron-related genes of *Stenotrophomonas maltophilia* isolates obtained from bovine milk samples with mastitis: Characteristics of *Stenotrophomonas maltophilia* isolates. *Journal of the Hellenic Veterinary Medical Society*, 74(3), 5979–5992. <https://doi.org/10.12681/jhvms.30641> (Original work published October 18, 2023)

Investigation of genetic relatedness, antimicrobial resistance, biofilm formation, biofilm-related virulence genes and integron-related genes of *Stenotrophomonas maltophilia* isolates obtained from bovine milk samples with mastitis

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ABSTRACT: Treatment of infections caused by the opportunistic pathogen *Stenotrophomonas maltophilia* is complicated by the bacterium's ability to produce biofilms and high antibiotic resistance. This study aimed to investigate the prevalence of genetic relatedness, antimicrobial resistance, biofilm formation, biofilm-related genes with virulence and integron-related genes among isolates of *S. maltophilia* recovered from bovine milk with subclinical mastitis. In this study, bacterial identification was performed using conventional methods. The *smeT* gene-based Polymerase Chain Reaction (PCR) was used for bacterial identification. PCR was also used to detect virulence and integron-related genes, too. The quantitative Microplate Test (MP) method was used to determine the phenotypic biofilm production capacity of the isolates. The resistance patterns of the isolates against nine antibiotics belonging to nine antimicrobial families were examined using the disk diffusion method. Isolates resistant to at least three drug classes from various antimicrobial drug classes were defined as multi-drug resistant (MDR). The genetic linkage of *S. maltophilia* isolates was investigated by Enterobacterial Repetitive Intragenic Consensus (ERIC) PCR. Chi-square (χ^2) test was used to reveal statistical difference between MDR and integron-related gene prevalances as well as biofilm formation capacity of isolates and biofilm-related virulence genes. In the study, a total of 312 milk samples with subclinical mastitis were taken from 27 farms. Ten isolates from five farms were phenotypically and genotypically identified as *S. maltophilia*. All isolates were resistant to cefepime and imipenem. 80% of the isolates carried at least one of the integron-related genes and 70% were MDR. The phenotypically biofilm-forming capacity identified in isolates was detected at 80%. The prevalence of the studied virulence genes was *rpfF* 60%, *rmlA* 70%, *spgM* and *smf1* 80%. There was no significant relationship between the biofilm-forming capacity of the isolates and the prevalence of biofilm-related virulence genes and MDR with integron-related genes. In the UPGMA analysis performed, a total of five genotypes were found, two single and three multiple according to 18% similarity coefficient. The presence of same isolates on the same farm and closely related isolates on different farms may suggest a clonal spread. ERIC-PCR can be useful in identifying *S. maltophilia* isolates with epidemic potential. *S. maltophilia* isolates were detected simply and quickly, using PCR based on the *smeT* gene, from bovine milk samples for the first time in Türkiye.

Keywords: Antibiotic Resistance, Bovine Mastitis, Biofilm, ERIC-PCR, *Stenotrophomonas maltophilia*.

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Date of initial submission: 27-06-2022
Date of acceptance: 13-10-2022

INTRODUCTION

Stenotrophomonas maltophilia is a Gram-negative, aerobic bacillus and increasingly important pathogen in the medical field due to its alarmingly high rates of drug resistance despite its low virulence (Falagas *et al.*, 2009). This bacterium has a significant mortality rate compared to other nosocomial infections because it causes serious complications (Gozel *et al.*, 2015). It has been reported that the mortality rate in *S. maltophilia* infections can be high (up to 37.5%) and therefore clinicians should not underestimate the clinical significance of *S. maltophilia* infections (Falagas *et al.*, 2009). *S. maltophilia* can be isolated from humid environments, plants, animals, food, etc. and it can cause infections in both children and adults. Transmission to susceptible individuals occurs through direct contact with the source of the bacterium (Schable *et al.*, 1991).

Formerly, there were doubts about the pathogenicity of *S. maltophilia*. However, it is not considered a harmless colonizer today, it is known to be an important nosocomial pathogen in human medicine (Falagas *et al.*, 2009; Gozel *et al.*, 2015). While the bacterium is known to cause serious infections in humans (pneumonia, bacteremia, sepsis, endocarditis, meningitis, bone, joint infections, eye infections, etc.) (Brooke, 2014), it has increasing importance in animal health. *S. maltophilia* has been isolated from lymphadenitis in goats (Johnson *et al.* 2003), arthritis (Muir *et al.*, 2007) and urinary tract infections in dogs (Kralova-Kovarikova *et al.* 2012), respiratory systems in horses, dogs and cats (Abbassi *et al.*, 2009, Ucan *et al.* 2019), pyogranulomatous hepatitis in buffalo (Petridou *et al.*, 2010) and mastitis in bovines (Ohnishi *et al.*, 2012).

The role of animals in human *S. maltophilia* infections is not fully understood, but in a study conducted in France, it was determined that *S. maltophilia* strains from animals share common phylogenetic features with some human strains (Jayol *et al.*, 2018). In the study, it is emphasized that although some genogroups are reported to be related only to animal strains, it is of particular interest that various genetic backgrounds are shared by human and animal strains (Jayol *et al.*, 2018). This situation necessitates examining the characteristics of animal isolates.

The SmeDEF pump was the first multidrug efflux pump described in *S. maltophilia*. This pump contributes to *S. maltophilia* intrinsic resistance to quinolones, tetracyclines, macrolides, chloramphenicol and

novobiocin (Alonso and Martinez, 2000). Expression of the SmeDEF pump is down-regulated by *smeT*, a tetracycline repressor family of transcriptional regulators, and can be used for PCR identification of *S. maltophilia* as the *smeT* gene is species-specific (Zhang *et al.*, 2001). For the first time, PCR technique based on the *smeT* gene was used in cheese samples to detect the presence of *S. maltophilia* by Okuno *et al.* (Okuno *et al.*, 2018).

S. maltophilia exhibits an exceptionally high intrinsic resistance to treatment with many antibiotics (lactams, aminoglycosides, quinolones, macrolides, trimethoprim/sulfamethoxazole, tetracyclines, phenicols, and polymyxins) (Brooke, 2014). The increase in *S. maltophilia* infections may be primarily the result of inadequate treatment with antibiotics, which may be further complicated by biofilm formation (Falagas *et al.*, 2009; Zhang *et al.*, 2001). The biofilm-forming ability of *S. maltophilia* is known as an important virulence trait. *S. maltophilia* biofilm is a bacterial community embedded in a self-generated polymeric matrix from a mixture of polysaccharides, proteins, nucleic acids and lipids attached to a surface. Biofilm-forming isolates are up to 1000 times more resistant to antimicrobial agents (Olsen, 2015). The presence of virulence genes and increased prevalence of resistance in antibacterial therapy may contribute to the pathogenicity of *S. maltophilia*. The *spgM* gene is a homologue of the *algC* gene responsible for alginate biosynthesis in *Pseudomonas aeruginosa*. The *rpfF* gene regulates virulence expression such as motility, extracellular proteases, lipopolysaccharide and biofilm production (Fouhy *et al.*, 2007). Also, the presence of either *spgM* or *rpfF* genes is required for biofilm formation, but the presence of both leads to stronger biofilm production (Madi *et al.*, 2016). The *rmlA* gene encodes glucose-1-phosphate thymidyl transfers. This gene is responsible for activities such as lipopolysaccharide /exopolysaccharide biosynthesis, motility, attachment and biofilm. It has a high prevalence in *S. maltophilia* strains (65.2-97.7%) (Zhuo *et al.*, 2014; Madi *et al.* 2016; Bostanghadiri *et al.*, 2019). Mutations in the *rmlA* and *rpfF* genes have been reported to result in reduced biofilm formation (Fouhy *et al.*, 2007). The *S. maltophilia* fimbriae (*smf1*) gene is responsible for the surface adherence of bacteria and early stages of biofilm formation and agglutination in species-specific red blood cells. It was reported that fimbria encoding the *smf1* gene was detected in 23% of clinical *S. maltophilia* strains and 42% of environmental strains (Gallo *et al.*, 2016).

Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences are repetitive palindromes of 127 bp in size that occur in multiple copies on bacterial genomes. ERIC-PCR analysis is a PCR-based genotyping system based on variations in the location of ERIC sequences in the bacterial genome (Wilson and Sharp, 2006). Although originally used in members of the *Enterobacteriaceae* family, it now allows molecular typing of many different bacteria. In several studies, antibiotic resistance (Nam *et al.*, 2009) and its genetic association (Jayol *et al.*, 2018) were investigated in *S. maltophilia* isolates from bovine milk with mastitis. However, there are limited studies on the role of *S. maltophilia* in bovine mastitis. Treatment of infections caused by the opportunistic pathogen *S. maltophilia* is complicated by the bacterium's ability to produce biofilms and high antibiotic resistance. This study aimed to investigate the antimicrobial resistance, biofilm formation, integron-related genes and biofilm-related genes with virulence (such as *spgM*, *rmlA*, *rpfF* and *smf1*), and genetic relatedness among isolates of *S. maltophilia* recovered from bovine subclinical mastitis.

MATERIAL AND METHODS

Bacterial isolates

California Mastitis Test (CMT; Bavivet CMT Liquid, Kruuse®) was applied after udder cleaning in dairy farms visited for mastitis screening. The procedures and interpretations were performed previously (Quinn *et al.*, 2011). Approximately 5-10 ml milk sample taken from a single mammary lobe with the highest CMT score was sent to the laboratory under aseptic conditions. Bovines that were not treated with antibiotics for at least three weeks were used. CMT positive milk samples were collected from 312 dairy cattle in 27 farms. Milking machines were used in all enterprises. The cows' ages varied between 3 and 11 years and the numbers of cows were between 9 and 35 on each farm.

Isolation and identification

Milk samples were centrifuged at 3500 rpm for 5 min and the supernatant was discarded. The sediment was vortexed and a loopful was inoculated onto blood agar supplemented with 7% defibrinated sheep blood (Merck 1.10886, Germany) and MacConkey agar (Merck 1.05465, Germany) and incubated overnight on *aerobic conditions* at 37°C. The isolates were identified by colony morphology and Gram staining and standard biochemical tests (oxidase, catalase, in-

dole, motility, hemolysis, nitrate reduction, hydrogen sulfide, and fermentation of sugars in TSI agar) (Murray *et al.*, 2007). All bacterial isolates were stored at -20°C in brain heart infusion broth (Merck 1.10493, Germany) supplemented with 20% glycerol.

Phenotypic determination of biofilm production

A modification of the method described previously was used for the quantitative determination of biofilm production (Stepanovic *et al.*, 2000). The test was performed using Mueller Hinton Broth (MHB) (Oxoid CM0405, UK) with the addition of glucose at a concentration of 5 g/L. On the first day, the isolates were inoculated in MHB and incubated for 24 hours at 37°C. Then each of the isolate densities was adjusted to 0.5 McFarland. On the second day, the cultures were then diluted in 1:40 ratio in 1000 µl MHB with glucose. Then 200 µl suspension was inoculated into the wells of a flat-bottomed polystyrene 96-well plate. Microtitre plates were incubated at 37°C for 48 hours and wells were subsequently washed three times with sterile PBS (pH: 7.2). Adherent biofilms were fixed for 60 min at 37°C, stained for 30 min at room temperature with 200 µl of 0.1% crystal violet then rinsed in still water and dried at 37°C. Biofilms were resolubilized with 200 µl of the solution containing 96% ethanol and acetone in a ratio of 4:1 for 15 min. Absorbance reading was conducted at 595 nm using a microplate photometer (BioTek ELx808 Absorbance Plate Reader, USA). The optical density cut-off value (ODc) is the sum of the average OD of the negative control and three times the standard deviation of the negative control. Classification of strains was performed according to the following criteria: no biofilm producer (NB) ($OD \leq ODc$), weak biofilm producer (WB) ($ODc < OD \leq 2 \times ODc$), moderate biofilm producer (MB) ($2 \times ODc < OD \leq 4 \times ODc$) and strong biofilm producer (SB) ($4 \times ODc < OD$). *Staphylococcus aureus* 25923 was used as positive control and the negative control was sterile MHB media.

PCR

DNA extraction, purity and quantity control: In this study, DNA extraction was performed by the sonication method as previously reported (Maniatis and Sambrook, 1989). For this purpose, isolates were passaged from stock cultures to blood agar and incubated at 37°C for 24 hours. A colony was taken from this bacterial culture and transferred to 5 ml Nutrient Broth (NB) (Merck 1.05443, Germany). Thereafter NB was incubated at 37°C for 18-24 hours. The broth was centrifuged at 13500 rpm for 5 min. The supernatant was discarded. The residue was diluted with 200 µl PBS in an eppendorf tube ($\sim 10^8$ /ml). The suspension was sonicated at 40 Hz for 10 minutes then cen-

trifuged at 13500 rpm for 5 min. Three microliters of supernatant were used as template DNA in each PCR reaction. DNA purity and quantity controls were also performed. The ratio of OD260/OD280 was between 1.6-2.0 indicating that the DNA was pure (Aggarwal, 2008). Then, DNA was electrophoresed on 1% agarose gel and the presence of DNA bands in the UV transilluminator was investigated.

Primers: Firstly, the bacterial presence and DNA extraction was confirmed by amplification of the 16S rRNA gene. In the PCR performed using 16S rRNA universal primers, *Escherichia coli* ATCC 25922 strain was used as positive control, and mastermix without DNA was used as negative control. Sequence analysis of one of the amplicons was performed to identify the bacteria. Later, species-level identifications of isolates phenotypically determined to be *Stenotrophomonas* species were confirmed by PCR using *smeT* gene as species-specific primers. The sequenced field strain of *S. maltophilia* was used as a positive control and the *E. coli* ATCC 25922 strain was used as a negative control in PCR. The presence of integron and biofilm-related virulence genes (*spgM*, *rmlA*, *rpfF*, *smf1*) of isolates was determined by PCR amplification using specific primer pairs for each gene. ERIC-PCR method, which is a molecular typing method, was performed using ERIC1 and

ERIC2 primers to determine the genetic diversity and clonal relationship between isolates (Versalovic *et al.*, 1991) (Table 1.).

To amplify the genes, 50 µL of reaction mixture was made containing 2 mM MgCl₂, 0.4 mM of each of the four dNTPs, 0.1 mM oligonucleotide primers, 1.5 U Taq polymerase (Fermentas, Massachusetts, USA) and 20 ng template DNA. The prepared tubes were loaded in the thermal cycler (Boeco, Hamburg, Germany). The DNA was amplified using the following protocol: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (95°C for 30 s), annealing for 30 s [52°C (*int2*), 54°C (*spgM*, *rmlA*, *rpfF*), 56°C (*int1*, *int3*, 16S rRNA, *smeT*), 60°C (*smf1*)] and extension (72°C for 1 min), with a single final extension for 7 min at 72°C. On electrophoresis, a 1.5% agarose gel stained with Safe View (100 ml/6 µl) (ABM, Richmond, Canada) was used and the gel was exposed to 100 volts for 45 min.

After electrophoresis, the gel was placed in the chamber of the transilluminator device which was connected to the computer and photographed under UV light (Vilbert Lourmat, Collegien, France). When the amplified product formed a band of the expected size (Table 1.), it was assumed to carry the gene examined.

Table 1. All primers used in this study.

Primers	Target Gene	Sequence (5'-3')	Amplicon (bp)	T _m	Reference	Result (%)
Universal	16S rRNA	AGAGTTTGATCCTGGCTCAG		58.4	(Edwards <i>et al.</i> , 1989, Zheng <i>et al.</i> , 1996)	12
		GACGGGCGGTGTGTACAA	1371	58.4		(100)
<i>S. maltophilia</i> <i>sme1</i>		GCATGATCTCCATSGTYTTG		56.4	(Okuno <i>et al.</i> , 2018)	10
		GGCACTTCAAGAACAAAGAGC	192	58.4		(83)
Integron	<i>int1</i>	CCTCCCGCACGATGATC		57.2	(Bass <i>et al.</i> , 1999)	5
		TCCACGCATCGTCAGGC	280	57.2		(50)
	<i>int2</i>	TTATTGCTGGGATTAGGC		51.6		7
		ACGGCTACCCTCTGTTATC	233	57.3	(Goldstein <i>et al.</i> , 2001)	(70)
	<i>int3</i>	AGTGGGTGGCGAATGAGTG		59.5		0
		TGTTCTTGTATCGGCAGGTG	600	58.4		(0.0)
Biofilm formation protein/ Biofilm	<i>spgM</i>	GCTTCATCGAGGGCTACTACC		63.3		8
		ATGCACGATCTGCCGC	80	54.8		(80)
	<i>rmlA</i>	GCAAGGTCATCGACCTGG		54.8	(Pompilio <i>et al.</i> , 2011)	7
		TTGCCGTC GTAGAAAGTACAGG	82	61.3		(70)
	<i>rpfF</i>	CTGGTCGACATCGTGGTG		58.4		6
		TGATCCGCATCATTCATGC	151	56.4		(60)
	<i>smf1</i>	GGAAGGTATGTCCGAGTCCG		62.5	(Nicoletti <i>et al.</i> , 2011)	8
		GCGGGTACGGCTACGATCAGTT	674	65.9		(80)
ERIC	ERIC1	ATGTAAGCTCCTGGGGATTCAC	Variable	62.1	(Versalovic <i>et al.</i> , 1991)	
	ERIC2	AAGTAAGTGACTGGGTGAGCG		64.0		

ERIC-PCR: The PCR reaction consisted of an initial denaturation phase at 95°C for 5 min, 30 cycles at 95°C for 1 min, 37°C for 1 min and 72°C for 3 min, and a final extension phase at 72°C for 10 min. In this study, we used master mix without DNA as a negative control. Bio-1D++ software (Vilber Lourmat, Collégien, France) were used to estimate molecular size, genetic relations were detected and a dendrogram was drawn. The appearance of the gels was digitized, and a PC assisted examination of genomic fingerprints was made with the Bio-Gene software programme (Version 11.02, Vilber Lourmat, France). Similarity matrices of the complete densitometric curves of the gel tracks were calculated using the Dice coefficient. Cluster analysis of similarity matrices was made by the UPGMA algorithm. Dendograms were obtained by analysis of the gel images with the Pyelph 1.4. software (Pavel and Vasile, 2012). All the amplification products were inspected by electrophoresis on 1.7% agarose gel at 90 V for 1.5 hours.

Sequence Analysis: The DNA fragments were visualized by UV after electrophoresis. Samples with the expected size (1371 bp) of the amplified DNA were purified using the GeneJet Gel Purification Kit (ThermoScientific, United States) according to the manufacturer's instructions. After the purification process, the amplicons were sent to Macrogen Europe (Amsterdam, The Netherlands). Sequence analysis was done using ABI Primse sequencing system. Sequences were compared using the Nucleotide-nucleotide BLAST (blastn) program available at the gene bank (www.ncbi.nlm.nih.gov) and the species of strain and homologies were determined.

Antimicrobial susceptibility test

For each *S. maltophilia* isolate confirmed by the PCR, the antimicrobial resistance against nine antibiotics (levofloxacin, trimethoprim/sulfamethoxazole, piperacillin/tazobactam, cefepime, aztreonam, imipenem, gentamicin, chloramphenicol, tetracycline) (Oxoid, Hampshire, United Kingdom) belonging to nine different antibiotic families was tested by the disk diffusion method (Table 2.). A bacterial suspension of 0.5 McFarland standard turbidity was first prepared using a 24 h culture. A sterile cotton swab was dipped into the bacterial suspension, and the swab was pressed and twisted against the inner surface of the test tube to remove excess fluid. The swab was streaked across a Mueller-Hinton agar (MHA) (Oxoid, Hampshire, United Kingdom) surface in a zigzag manner. The MHA plate was turned 45° clockwise and streaked again using the same swab, and this step was repeated one more time so that the swab had been streaked across the agar a total of three times. The antibiotic discs were placed onto the agar using a pair of sterile forceps. Antibiotics disks were placed onto the same *S. maltophilia* inoculated MHA plate, and the plates were incubated at 37°C for 18-20 h. Zone diameters of susceptibility testing results were categorized as sensitive (S), intermediate (I), or resistant (R) and evaluated as previously reported (CLSI, 2020). Due to the lack of CLSI breakpoint values of many antibiotics for *S. maltophilia*, the breakpoint values for the close-related *P. aeruginosa* and *Enterobacteriaceae* were used instead. The specific breakpoint values were given in Table 2. *E. coli* ATCC 25922 (Oxoid, Hampshire United Kingdom) was used as the quality control microorganism.

Table 2. Antimicrobial susceptibility and resistance pattern of *S. maltophilia* isolates.

Antimicrobial Classes/ Antibiotic (Abbreviation)	Disk Content (µg)	Zone Diameter (mm)		<i>S. maltophilia</i> (n=10)		Evaluation criteria
		≥S	≤R	S (%)	R (%)	
Phenolics (Chloramphenicol, CHL)	30	18	12	9 (90)	1 (10)	<i>Enterobacteriales</i>
Tetracyclines (Tetracycline, TET)	30	15	11	7 (70)	1 (10)	
Fluoroquinones (Levofloxacin, LVF)	5	17	13	9 (90)	1 (10)	<i>S. maltophilia</i>
Folate Pathway Antagonists (Trimethoprim/Sulfamethoxazole, TS)	1.25/23.75	16	10	8 (80)	1 (10)	
B Lactam Combination Agents (Piperacillin/Tazobactam, PT)	100/10	21	14	7 (70)	1 (10)	<i>P. aeruginosa</i>
Aminoglycosides (Gentamicin, GEN)	10	15	12	7 (70)	3 (30)	
Monobactams (Aztreonam, AZT)	30	22	15	1 (10)	7 (70)	
Cephems (Cefepime, CFP)	30	18	14	0 (0)	10 (100)	
Carbapenem (Imipenem, IMI)	10	19	15	0 (0)	10 (100)	

Multiple antibiotic resistance (MDR) and multiple antibiotic resistance index (MAR)

Multiple drug resistance was defined as resistance to three or more antimicrobial classes (Magiorakos *et al.*, 2012). The MAR for each isolate was determined by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested (Krumpernam *et al.*, 1983).

Statistical analysis

SPSS (Statistical Package for Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) package program was used for statistical analysis of the data obtained. Pearson Chi-square (χ^2) test (the Fisher exact test) was used to compare frequency data. A p-value of <0.05 was considered statistically significant at the 95% confidence interval. The χ^2 test was used to reveal statistical difference between MDR and integron-related gene prevalances as well as biofilm formation capacity of isolates and biofilm-related virulence genes.

RESULTS

Biochemical tests and phenotypic identification

In this study, 12 *Stenotrophomonas* spp. isolates were obtained from subclinical mastitis. Biochemical test results of isolated *Stenotrophomonas* spp.: catalase, motility, glucose fermentation were positive; and, oxidase, indole, hemolysis, hydrogen sulfide, urea, lactose, sucrose fermentation, gas production were negative.

Sequence analysis

Firstly, bacterial presence and DNA extraction were confirmed with amplification of the 16S rRNA gene. After PCR, one of the amplicons obtained using 16S universal primers was sequenced. As a result of the sequence analysis, the similarity rate of this isolate with *S. maltophilia* was determined as 96.2% and this isolate was used as a positive control in species-specific PCR as it showed high homology with *S. maltophilia* (Figure 1.).

Genotypic identification

Phenotypically, 12 isolates were identified as *Stenotrophomonas* spp., ten of these isolates were confirmed to be genotypically *S. maltophilia* (Figure 2.).

The following characteristics of ten *S. maltophilia* isolates were examined:

Biofilm formation

Among the isolates examined, while two (20%) isolates did not form biofilms; eight (80%) were able to produce biofilm: Three were SB producers, whereas four and one were MB and WB producers, respectively. Biofilm assay for *S. maltophilia* isolates using the MP method is shown in Figure 3.

Biofilm-related virulence genes

The frequency of biofilm-related genes among the *S. maltophilia* isolates was generally high: *rpfF*, *rmlA*, *spgM* and *smf1* were 60%, 70%, 80% and 80%, respectively (Table 1., Table 4., Figure 4.).

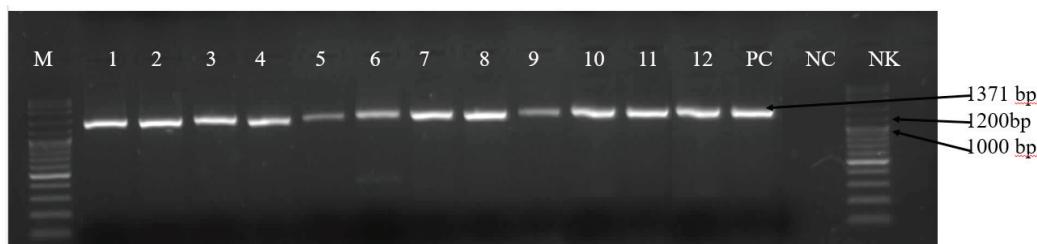


Figure 1. PCR performed by using 16S rRNA universal primers. M: Marker (100 bp DNA Ladder, Fermentas) 1-12: PCR performed by using isolated microorganism's DNA. PC: Positive Control (*E. coli* ATCC 25922) NC: Negative Control (Mastermix without DNA).

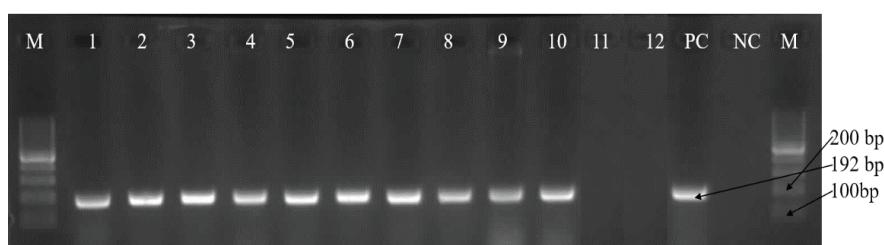


Figure 2. Gel electrophoresis image of *S. maltophilia* isolates 1-10. *S. maltophilia* (192 bp) positive field isolate 11-12. *S. maltophilia* negative field isolates PC: *S. maltophilia* sequenced field strain NC: *E. coli* ATCC 25922 3. M: 100 bp DNA Ladder, (Fermentas, USA).

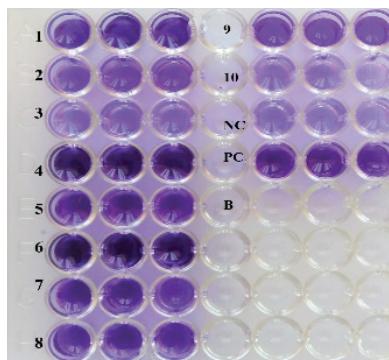


Figure 3. Biofilm assay for *S. maltophilia* isolates using 96-well microtiter plate method. B: Blank, Wells 1, 4, 6: SB producer isolates, Wells 5, 7, 8, 9: MB producer isolates, Well 2: WB producer isolates. Wells 3, 10: NB producer isolates NC: Sterile MHB, PC: *S. aureus* 25923.

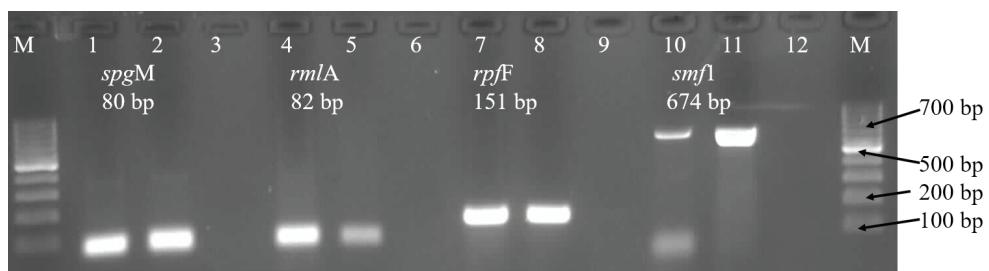


Figure 4. Agarose gel electrophoresis of biofilm-related virulence gene PCR products. 1, 2. *spgM* (80 bp) 4, 5. *rmlA* (82 bp) 7, 8. *rpfF* (151 bp) 10, 11. *smf1*: 674 bp 3, 6, 9, 12. NC: Mastermix without DNA M: 100 bp DNA Ladder (Fermentas, USA).

Integron-related genes

Integron-related gene was detected in 80.0% of isolates. One isolate carried only class 1 and three isolates carried only class 2 integron gene. Four isolates contained both class 1 and 2 integron-related genes together. Two isolates did not carry class 1 and class 2 integron-related genes. The class III integron gene could not be detected. (Table 1., Table 4., Figure 5.).

ERIC-PCR

Ten *S. maltophilia* were isolated and identified from 10 bovines on five farms. As a result of electrophoresis of PCR products, at least one and at most eight bands were detected in the range of 100 to 3000

bp., which is the marker size.

In the analysis performed, a total of five genotypes were found, two single (B and D) and three multiple (A, C and E) according to an 18% similarity coefficient. It was determined that isolate 3 with isolate 4 and isolate 1 with isolate 5 obtained from the same farm in A genotype were identical. Isolate 7 with isolate 9 in the C genotype and isolate 2 with isolate 8 in the E genotype were closely related isolates obtained from different farms. However, the two isolates (6, 10) obtained from different farms (2, 5) were unrelated isolates with different genotypes (D and B) (Table 4., Figure 6.).

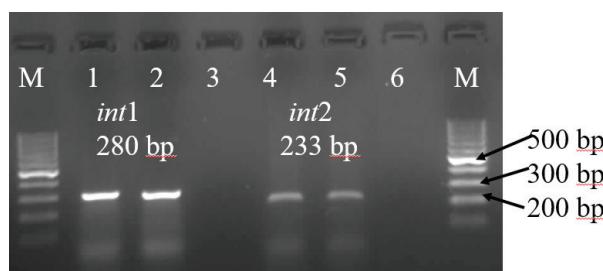


Figure 5. Agarose gel electrophoresis of integron-related gene PCR products. 1, 2. *int1* gene positive *S. maltophilia* isolates (280 bp) 4, 5. *int2* gene positive *S. maltophilia* isolate (233 bp) 3, 6. NC: Master mix without DNA M: 100 bp DNA Ladder (Fermentas, USA).

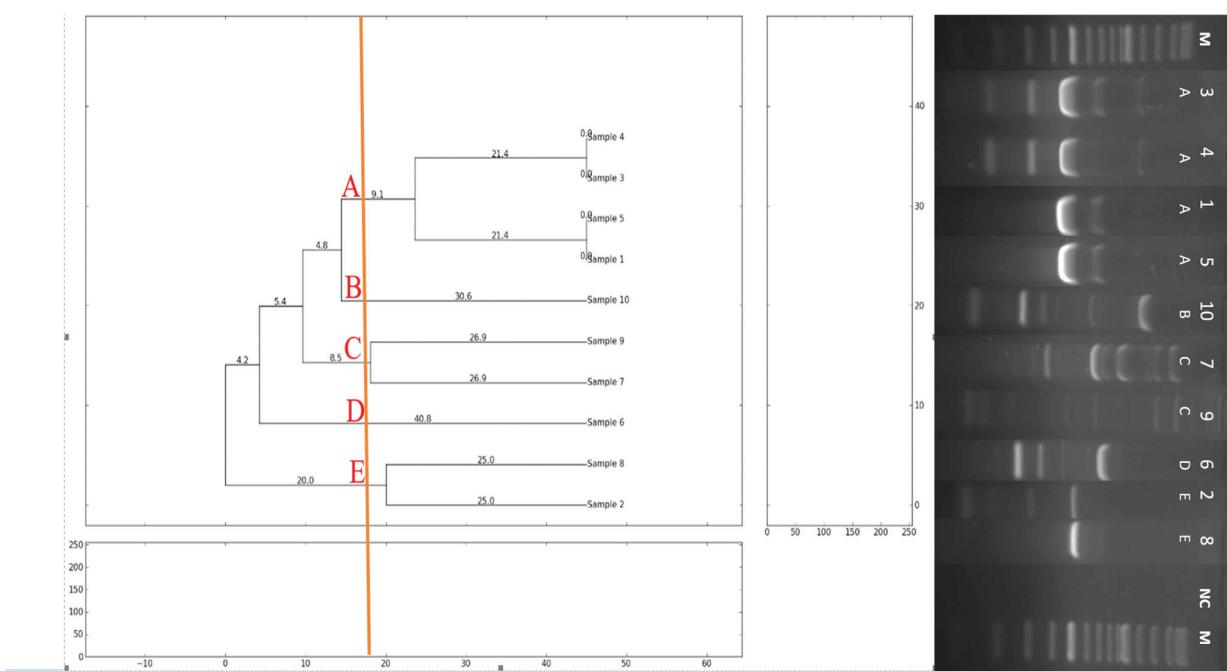


Figure 6. Dendrogram of ERIC-PCR profiles of ten *S. maltophilia* isolates. In the UPGMA analysis performed in the PyElph 1.4 program, a total of five genotypes were found, two single (B and D) and three multiple (A, C and E) according to an 18% similarity coefficient. It was determined in the A genotype isolated from the same farm were the identical strains that sample 3 with sample 4 and sample 1 with sample 5.

Antimicrobial susceptibility test

The percentage of strains susceptible or resistant to each antibiotic is presented in Table 2. All isolates were resistant to imipenem and cefepime. The rates of resistance to other antimicrobial drugs were: aztreonam 70%, gentamicin 30%, chloramphenicol, levofloxacin, trimethoprim/sulfamethoxazole, piperacillin/tazobactam and tetracycline 10%. One *S. maltophilia* isolate was resistant to all antibiotics used (Figure 7.). The most effective antibiotics against isolates are chloramphenicol, and levofloxacin (90% susceptibility rate).

Antimicrobial susceptibility and resistance profiles of *S. maltophilia* isolates were shown in Figure 8.

MAR index and MDR

MAR index of all *S. maltophilia* isolates was found above 0.2. One of these samples showed resistance to all antibiotics used (MAR 1.0). While one isolate is a pan-drug resistant (PDR); six isolates were found to have multiple antibiotic resistance. Three of the ten isolates were resistant to antibiotics of two antimicrobial families, so they were not multi-antibiotic resistant (NMDR) (Table 3).

Results of antibiotic resistance, resistance phenotype, phenotypically biofilm production, biofilm-related virulence genes, integron-related genes, ERIC type of ten *S. maltophilia* isolates are shown in Table 4.



Figure 7. Disk diffusion test of isolate resistant to all antibiotics.

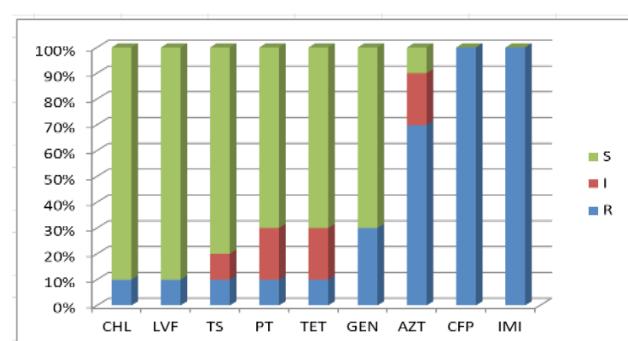


Figure 8. Antimicrobial susceptibility and resistance profiles of *S. maltophilia* isolates.

Statistical analysis

The relationship between biofilm production (BP) and the prevalence of biofilm-related virulence genes, resistance phenotype and integron-related genes is shown in Table 5.

There was no significant relationship between the biofilm-forming capacity of the isolates and the prevalence of biofilm-related virulence genes, MDR and integron-related genes.

DISCUSSION

S. maltophilia is a well known opportunistic bacteria. It can be isolated from a wide variety of sources, including animals and foods of animal origin. It has gained importance with its high rate of isolation from hospital-acquired infections in recent years (Falagas *et al* 2009). Although it is a pathogen with low virulence, it can be isolated from a wide variety of infections in people with weakened immune systems due

Table 3. MAR index and resistance phenotype of *S. maltophilia* isolates.

Number of isolate (%)	Number of resistant antibiotic	MAR index	Resistance Phenotype
3 (30 %)	2	0.2	NMDR
4 (40 %)	3	0.3	MDR
2 (20 %)	4	0.4	MDR
1 (10 %)	9	1.0	MDR (PDR)

Table 4. All study results by farms.

	Antibiotic resistance					R phenotype	Biofilm			Integron		ERIC type	Farm	
	CHL, LVF TS, PT, TET	GEN	AZT	CFP	IMI		MP	rpf F	rml A	spg M	smf1	int 1	int 2	
1	-	-	R	R	R	MDR	SB	+	+	+	+	+	+	A 1
2	-	-	-	R	R	NMDR	WB	-	+	+	-	-	+	E 1
3	-	-	R	R	R	MDR	NB	-	-	+	-	-	-	A 1
4	R	R	R	R	R	MDR	SB	+	+	+	+	+	+	A 1
5	-	-	R	R	R	MDR	MB	+	+	-	+	+	+	A 1
6	-	-	-	R	R	NMDR	SB	+	-	+	+	+	-	D 2
7	-	-	-	R	R	NMDR	MB	-	+	+	+	-	+	C 3
8	-	R	R	R	R	MDR	MB	+	-	+	+	-	+	E 4
9	-	R	R	R	R	MDR	MB	-	+	+	+	-	-	C 4
10	-	-	R	R	R	MDR	NB	+	+	-	+	+	+	B 5

Table 5. The relationship between biofilm production and the prevalence of biofilm-related virulence genes, resistance phenotype and integron-related genes.

		Biofilm Production		P	χ^2
		BP (+)	BP (-)		
Biofilm-related virulence genes	<i>spgM</i> (+)	7	1	1.3	0.4
	<i>spgM</i> (-)	1	1		
	<i>rmlA</i> (+)	6	1	0.4	1.0
	<i>rmlA</i> (-)	2	1		
	<i>rpfF</i> (+)	5	1	0.1	1.0
	<i>rpfF</i> (-)	3	1		
	<i>smf1</i> (+)	7	1	1.3	0.4
	<i>smf1</i> (-)	1	1		
Integron-related genes	Resistance Phenotype				
	MDR (+)	MDR (-)			
	<i>int1</i> (+)	3	2	1.0	0.4
	<i>int1</i> (-)	4	1		
	<i>Int2</i> (+)	5	2	1.0	0.0
	<i>Int2</i> (-)	2	1		

to its ability to form biofilms, and its high multi-drug resistance and high availability in the environment (Falagas *et al.* 2009). While there is a probability of transmission from animal products, there is no direct evidence to show its zoonotic potential. However, it was determined that *S. maltophilia* strains from animals share common phylogenetic features with some human strains (Jayol *et al.*, 2018). In this study, ten isolates obtained from cow milk samples with mastitis were characterized for the first time in Türkiye and some interesting findings were obtained.

S. maltophilia has been rarely reported in animals. Therefore, studies of *S. maltophilia* from animal origin are very few compared to studies of human origin (Abbasi *et al.*, 2009; Kralova-Kovarikova *et al.*, 2012; Ohnishi *et al.* 2012). It is still an overlooked pathogen in veterinary medicine (Ohnishi *et al.*, 2012). Although *S. maltophilia* was not generally considered a primary pathogen in veterinary medicine in previous years, nowadays it has gained importance due to its presence in the natural environment and its resistance to many antibiotics. The presence of *S. maltophilia* of animal origin (in the upper respiratory tract of cattle, sheep and horses and their environment) was first reported in Türkiye in 2011 (Celikel, 2012). With this study, it has been shown that *S. maltophilia* can be isolated from animals, especially respiratory system infections, as a primary or secondary pathogen in Türkiye. In a study conducted in 2019, antibiotic resistance was investigated in *S. maltophilia* isolates isolated from horses (Ucan, 2019). To our current knowledge, there is no study examining the characteristics of *S. maltophilia* isolates obtained from bovine mastitis.

The role of *S. maltophilia* in bovine mastitis remains unclear because there are few studies on this subject in the world (Ohnishi *et al.*, 2012). *S. maltophilia* is usually identified by classical conventional methods, but sometimes these tests cannot adequately distinguish these bacteria from other Gram-negative bacteria. Due to the variable oxidase reaction of *S. maltophilia*, it is often confused with other Gram-negative bacteria and misdiagnosed. There are selective media developed for the conventional isolation of *S. maltophilia* in the world (Kerr *et al.*, 1996). However, these media cannot be used in routine diagnostic laboratories due to their high cost. As a result, other non-fermentative Gram-negative bacteria (eg *Pseudomonas* spp., *Shigella* spp.) are incorrectly identified in laboratories instead of *S. maltophilia*. This may re-

sult in an underdiagnosis of *S. maltophilia* infections.

One of the most important reasons why biochemical identification of *S. maltophilia* isolates is difficult is the variability of the results of important biochemical tests for example oxidase, motility, and hemolysis. In our study, all isolates were oxidase negative. While some researchers reported that 20% of their isolates were oxidase-positive (Carmody *et al.*, 2011; Amoli *et al.*, 2017), some researchers reported that they found *S. maltophilia* oxidase negative, similar to the one in this study (Murray *et al.*, 2007). Denton and Kerr (1998) tested the motility of *S. maltophilia* strains in their study; they reported that motility was variable at 37°C and the rate of motile strains was 16-85%. In this study, all of our isolates were motile. One of the phenotypic features observed in this study was hemolysis. Only two of the 10 strains obtained showed little hemolytic activity. Thus, the variability of phenotypic characters such as oxidase, hemolysis and motility in various strains of *S. maltophilia* isolates was also confirmed in this study, similar to other studies (Denton and Kerr, 1998; Murray *et al.*, 2007; Carmody *et al.*, 2011).

In this study, the 16S rRNA gene was used to identify bacteria by sequence analysis. Thus, at the same time, the presence of bacteria and DNA extraction were confirmed and a positive control isolate was provided for use in PCR. However, the *smeT* gene was studied by conventional PCR for the definitive and accurate diagnosis of the bacterium. Bacterial identification was carried out by culture, biochemical tests and finally molecular methods. Two of the twelve specimens identified as *S. maltophilia* by culture methods and biochemical tests were not confirmed by molecular method, suggesting that there may be issues with false positives with the culture method.

Conventional PCR is highly sensitive and can be routinely used to detect Gram-negative bacteria such as *S. maltophilia*. Accurate identification in a short time is very useful for controlling the disease. PCR using the 16S rRNA gene as the target to differentiate *S. maltophilia* has low specificity due to the significant genetic similarity among other non-fermentative Gram-negative bacilli (Stephanie and Locosque, 2013). The *smeT* gene is species-specific and is a viable alternative to the 23S rRNA and *smeD* genes for the identification of *S. maltophilia* (Okuno *et al.*, 2018). Therefore, a primer pair designed specifically for *S. maltophilia* targeting the *smeT* gene by Okuno *et al.* (2018) was used to identify the isolates in the

study. Since the primers used in this study were designed by targeting only the *smeT* gene, it is a good method to PCR identification of *S. maltophilia* due to its specificity.

Treatment of infections caused by *S. maltophilia* is a problem for clinicians because the bacteria are resistant to a wide variety of antimicrobial drugs (Brooke, 2014). *S. maltophilia* is intrinsically resistant to many antibiotics. Treatment of *S. maltophilia* infections in humans is usually with antibiotics not available in veterinary medicine, such as imipenem or vancomycin (Falagas *et al.*, 2009; Gozel *et al.*, 2015). Studies conducted with the aim of determining the resistance rates of bacteria against antibiotics help physicians to choose appropriate antimicrobial drugs when starting empirical antimicrobial therapy. It has been shown that *S. maltophilia* isolates from humans mostly have multidrug resistance (Falagas *et al.*, 2009). In this study, all isolates were resistant to carbapenems (imipenem) and cephems (cefepime). Similarly, previous studies showed that the rates of resistance to imipenem and cefepime in *S. maltophilia* were 100% and 67.4%, respectively (Azimi *et al.*, 2020). Furthermore, our results showed a sensitivity rate of 90.0% against chloramphenicol and levofloxacin, and 80% for trimethoprim/sulfamethoxazole. It has been shown that the sensitivity of *S. maltophilia* to levofloxacin and trimethoprim/sulfamethoxazole is similarly high in Iran (95.3%, 97.7%) (Bostanghadiri *et al.*, 2019). The findings suggest that such antibiotics serve as effective agents for the treatment of *S. maltophilia* infections. Overall, this study reveals a low antibiotic resistance in *S. maltophilia* isolates to antibiotics (chloramphenicol, tetracycline, levofloxacin, trimethoprim/sulfamethoxazole, and piperacillin/tazobactam) other than imipenem, cefepime, and aztreonam. The differences in antibiotic resistance rates suggest that this is due to regional strain distribution and the primary use of different antibiotics in different regions. For this reason, each farm should follow its antimicrobial resistance rates and an empirical treatment policy should be determined according to the resistance status of each farm. However, the monitoring of the antibiotic resistance trends is imperative, both geographically and over time.

Data on biofilm formation by clinical *S. maltophilia* isolates of animal origin are very limited. Biofilms are known to play a role in many chronic and persistent infections (Brooke, 2014). Among our isolates examined, while two (20%) isolates did not form

biofilms; eight (80%) were able to produce biofilm: Three were SB producers, whereas four and one were MB and WB producers, respectively. Similarly, *S. maltophilia* isolates in Iran were categorized as weak, medium and no biofilm producers at 27.4%, 38.4%, 29.9% and 4.3% rates (Bostanghadiri *et al.*, 2019). In a study conducted in Brazil, it was shown that isolates were weak (3%), medium (45%) or strong (48%) biofilm producers (Gallo *et al.*, 2016).

To reveal the relationship between phenotypically biofilm formation and biofilm genes, different results have been obtained in previous studies: The data obtained in a study by Pompilio *et al* (2011) revealed that the presence of *spgM* significantly supports strong biofilm formation. In some studies, it has been reported that biofilm formation is significantly associated with the presence of *rpfF* and *spgM* genes (Zhuo *et al.*, 2014; Madi *et al.*, 2016). In another study, it was reported that the presence of *spgM*, *rpfF* and *rmlA* genes significantly increased biofilm production in isolates. In a recent study, the presence of *rpfF* and *smf1*, but the absence of *spgM*, was associated with biofilm formation (Azimi *et al.*, 2020). In this study, the importance of *rpfF* in biofilm formation is emphasized (Azimi *et al.*, 2020). The isolates obtained in this study carried high rates of biofilm-related virulence genes (*rpfF*, *rmlA*, *spgM* and *smf1* were 60%, 70%, 80% and 80%). However, we could not detect a significant relationship between phenotypic biofilm formation and the prevalence of biofilm-related virulence genes. The very small number of isolates included in our study is the most important deficiency of this study. Studies with more isolates will shed a better light on this issue.

Integrons are conserved DNA sequences that can efficiently receive and transfer resistance genes between bacteria and are often found on mobile genetic elements. It is accepted that integrons are one of the important mechanisms in the transfer of resistance genes (Akrami *et al.*, 2019). Five mobile integron classes have been defined so far. Integron class 1, class 2, and class 3 are related to the distribution of multiple antibiotic resistance phenotypes. The most frequently detected integron classes from clinical isolates are class I and class 2 integrons (Akrami *et al.*, 2019). In a study conducted in Mexico, 80.0% of *S. maltophilia* strains carried the class 1, 40.0% class 2 and 6.7% class 3 integron (Cruz-Córdova *et al.*, 2020). In this study, 10% of our isolates carry only class 1, 30% only class 2, 40% carried both class 1

and class 2 integron genes together. However, 20% did not carry any integron-related genes. There is no data about class 3 integrons in our country yet. However, in recent years, it has been reported that 6.7% of *S. maltophilia* isolates obtained from tertiary care hospitals in Mexico carry class 3 integrons (Cruz-Córdova *et al.*, 2020). In addition, there was no significant relationship between the prevalence of MDR and integron-related genes of the isolates. This suggests that antibiotic resistance genes in multidrug-resistant *S. maltophilia* isolates are probably carried on other elements such as transposons or plasmids. The transfer of resistance markers by integrons is only one factor that may contribute to the increase of multiresistant bacteria (Bass *et al.*, 1999).

It has been reported that *S. maltophilia* isolates obtained from different patients in hospitals are mostly different strains, do not spread easily among humans, and most epidemic isolates are unrelated to each other (Sader *et al.*, 1994). Examining the clonal spread of isolates from outbreaks can be very useful in determining whether the bacteria are the same or different. In a study done in Japan, over seven months 11 out of 13 isolates from nine cows in a herd exhibited a closely related ERIC2 type (A). The remaining two isolates from two cows from the other two herds displayed two different types of ERIC2 (B and C). This study showed that closely related *S. maltophilia* isolates played a role in the herd outbreak to some extent (Ohnishi *et al.*, 2012). In our study, two different genotypes (B and D) were obtained from two farms. The genetic diversity among the isolates within the different farms might be due to insertions, deletions or point mutations, which could lead to the observed variation in ERIC profiles. This situation suggests that *S. maltophilia* follows an opportunistic spread in epidemic formation and the epidemic formation course may be slow. However, the presence of same or clonally related genotypes on the same or different farms may suggest clonal spread of an epidemic strain and that *S. maltophilia* may play a role in the herd outbreak.

In this study, the MAR index of all *S. maltophilia* isolates was found to be above 0.2. It is known that a MAR index higher than 0.2 is an indicator of isolates originating from an environment where antibiotics are frequently used (Magiorakos *et al.*, 2012). One of the most important problems encountered on farms is the indiscriminate use of antibiotics without an antibiogram test. Similarly, this may be one of the pos-

sible reasons for the high multi-antibiotic resistance rate and high MAR index.

It is also known that *S. maltophilia* adheres easily to plastic surfaces (Olsen, 2015). Therefore, when *S. maltophilia* is detected on a farm, any equipment used can become contaminated. Unfortunately, it was not possible to analyze environmental samples on farms from which *S. maltophilia* was isolated and therefore the exact source could not be determined. Quantitative real-time PCR could not be performed to evaluate the expression levels of biofilm-related genes.

CONCLUSION

In this study, bacterial isolation was carried out by both conventional and molecular methods. We think that PCR is useful and practical to confirm biochemical test results. We determined that our isolates formed very high levels of biofilms and carried biofilm-related virulence genes and integron-related genes. The presence of the same isolates in the same farm and closely related isolates in different farms may suggest that transmission from cow to cow has occurred and that there may be a clonal spread. In this study, the MAR index of all isolates was found to be above 0.2, an indication that the isolates were obtained from an environment where antibiotics are frequently used. The most effective antibiotics against our isolates were chloramphenicol, and levofloxacin. While an isolate is resistant to all antibiotics used; seven isolates were detected to have multiple antibiotic resistance. As a result of the statistical analysis, there was no significant relationship between the biofilm forming capacity of the isolates and the prevalence of biofilm-related virulence genes, and between integron-related genes and MDR. In order to better understand this issue, more comprehensive studies using more isolates are needed. *S. maltophilia* could be considered in the etiology of mastitis. For an effective treatment, it is important to carry out antibiogram tests as well as the correct isolation of the agent. By adapting molecular typing methods to epidemiology and revealing the clonal relationships between bacteria in detail, information about the scope, source and reservoir of diseases can be obtained. In the light of this information, effective strategies can be developed in the fight against the disease. To control diseases in the livestock sector in our country, such studies should be continued and national databases should be established.

ACKNOWLEDGEMENTS

This manuscript was supported by Aydin Adnan Menderes University Scientific Research Projects Unit (ADU BAP Project Number: VTF-21011) and

the authors would like to thank to Mehmet ÖZTÜRK (Bioeksen R&D Technologies, İstanbul, Türkiye) for his helps and supports.

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