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Research Article

# Antibiotic Susceptibility and Molecular Identification of Antibiotic Resistance Genes of Staphylococci Isolated from Bovine Mastitis in Algeria

Radhwane SAIDI [1] Zafer CANTEKİN [2] Djamel KHELEF [3]
Yaşar ERGÜN [4] Hasan SOLMAZ [5] Rachid KAIDI [6]

[1] Department of Agronomy, Telidji Amar University, BP 37G, Ghardaïa Road, 03000 Laghouat, Laboratory of Biotechnology Related to Animal Breeding, University Saad Dahleb, Blida, ALGERIA
[2] Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Microbiology, Tayfur Sokmen Campus, TR-31000 Hatay - TURKEY
[3] Higher National Veterinary School of Algiers, BP 161 Hacène Badi El Harrach, Algiers, ALGERIA
[4] Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Tayfur Sokmen Campus, TR-31000 Hatay - TURKEY
[5] Yüzüncü Yıl University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, TR-65100 Campus, Van - TURKEY
[6] Institute of Veterinary Sciences, Laboratory of Biotechnology Related to Animal Breeding, University Saad Dahleb, BP: 270, Soumaa road, Blida, ALGERIA

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## Abstract

The study aimed to investigate the phenotypic and genotypic identification of *in vitro* antimicrobial susceptibility of 21 Staphylococci (10 *Staphylococcus aureus* and 11 Coagulase Negative Staphylococci) isolated from bovine mastitis to 12 antimicrobial drugs frequently using in veterinary medicine in Algeria. Isolates of staphylococci from bovine mastitis were tested for antibiotics with disc-diffusion method according to the National Committee for Clinical Laboratory Standards guidelines in the Mueller-Hinton agar, and resistant genes *mecA, blaZ, aac-aph, ermA, ermC, tetK* and *tetM* were detected by PCR. Staphylococci isolates showed high resistance to penicillin (95.23%), oxacillin (80.95%), clindamycine (80.95%), and erythromycin (76.19%) but, no resistance in all these strains was detected for gentamicin. Among 21 isolates of Staphylococci, 20 were found to be methicillin and multidrug resistant. Multidrug resistant strains exhibited several antibiogram patterns (antibiotic I to XIII). The distribution of antibiotic-resistant genes was *mecA* (100%) and *tetM* (100) followed by *blaZ* (42.85%). In the present study, the significant determination was the high prevalence of methicillin-resistant Staphylococci, which were resistant to multiple antibiotics. The finding of methicillin-resistant staphylococci from bovine mastitis is the first report in Algeria and revealed the status of resistant isolates in herd that might be helpful in treatment, controlling of resistant strains and for deciding culling of cows.

***Keywords:*** *Antimicrobial susceptibility, Bovine mastitis, Methicillin-resistant staphylococci, Resistance genes*

# Cezayir'de İnek Mastitislerinden İzole Edilen Stafilokokların Antibiyotik Direncinin Fenotipik ve Moleküler Yöntemlerle Belirlenmesi

## Özet

Bu çalışma, Cezayir'de süt sığırlarındaki mastitis vakalarından izole edilen 21 stafilokok (10 *Staphylococcus aureus* ve 11 Koagulaz Negatif Stafilokok) suşunun Cezayir'de veteriner sahada sıklıkla kullanılan 12 antibiyotiğe karşı *in vitro* fenotipik ve genotipik direncinin belirlenmesi amacıyla yapıldı. Stafilokok izolatları disk difüzyon yöntemiyle test edildi. *mecA, blaZ, aac-aph, ermA, ermC, tetK* ve *tetM* direnç genleri ise PCR ile araştırıldı. Stafilokok izolatları penisilin (%95.23), oksasilin (%80.95), klindamisin (%80.95) ve eritromisine (%76.19) karşı yüksek oranda dirençli bulundu. 21 stafilokok izolatından 20 tanesinin metisilin dirençli ve çoklu antibiyotik direncine sahip olduğu belirlendi. Çoklu antibiyotik direncine sahip suşların bir çok antibiyotiğe karşı direnç paterni belirlendi antibiyotik I-XIII). Antibiyotik direnç genlerinin oranları ise *mecA* (%100), *tetM* (%100), *blaZ* (%42.85) şeklinde gerçekleşti. Bu çalışmada çoklu antibiyotik direncine de sahip metisilin dirençli stafilokokların yüksek prevalansı dikkat çekici idi. Bu çalışma Cezayir'de sığır mastitislerinden izole edilen stafilokoklarda metisilin direncini ortaya koyan ilk çalışmadır. Bu direncin ortaya konulması sürü bazında hastalığın tedavi, kontrol ve mastitis nedeniyle sürüden ayrılacak hayvanlar için karar verilmesine yardımcı olabilir.

***Anahtar sözcükler:*** *Antibiyotik direnci, Sığır mastitisi, Metisilin dirençli Stafilokoklar, Direnç genleri*

**İletişim (Correspondence)**
+213 0560519937 Fax: +213 025438078
saidi.radhwane@yahoo.fr

# INTRODUCTION

Mastitis or the inflammation of mammary gland has been recognized as a complex and the most costly disease in dairy herds [1,2]. It imposes serious economic losses for the farmers and the dairy industry [3-5]. Among the various pathogens isolated as causative agents of bovine mastitis, *Staphylococcus aureus* is a predominant etiological agent of both subclinical and clinical forms of mastitis [6-8]. Coagulase Negative Staphylococci (CNS) have traditionally been considered as minor pathogens but, during the last years, their importance has clearly increased and they have become the predominant pathogens isolated from subclinical mastitis in several countries [9-12]. These bacteria can cause mainly subclinical mastitis [6], but some authors reported high percentage of clinical cases evoked by CNS [13,14]. The disease is the most frequent reason for the use of antimicrobial agents on dairy farms [15]. In numerous locations worldwide, cure rates of staphylococci infections are poor after antibiotic treatment [16-18]. In addition, multi-antimicrobial resistance was often seen in staphylococci [19]. In fact, the main reason of low efficacy of antibiotic treatment of staphylococcal mastitis is among others the resistance of bacteria. On the other hand, during the past decade, bacteria that cause human diseases have developed resistance to many of the antibiotics commonly used for treatment. Furthermore, the number and proportion of MRS (Methicillin-Resistant Staphylococci) infections in different countries has increased. Similar results were reported for many countries in the world [6,20-30] but, in Algeria a little information was available on diversity of bovine staphylococcal mastitis isolates and their anti-bacterial resistance, because this problem is not well investigated before. Furthermore, there is dearth of information on MRS from food products including milk in Algeria.

The aim of the present study was to identify and determine the *in vitro* activity of 12 different antimicrobial drugs against staphylococci isolated from bovine mastitis and identify the antibiotic resistance genes by PCR.

# MATERIAL and METHODS

### *Sample Collection and Microbiological Analysis*

The antibiotic susceptibility test was carried out on 21 staphylococcal strains isolated from bovine mastitis during the years 2011-2013 in Algeria. Before sampling the teat ends were cleaned with alcohol swabs and allowed to dry. The first few streams were discarded and then 5 ml of secretion was collected in sterile tubes. Samples were immediately transported to laboratory by cooled container. Bacteriological examinations were performed according to the commonly accepted principles [31]. Briefly, from each milk sample, 0.1 ml was plated on Columbia Agar medium (Merck, Germany), containing 5% sheep blood, and incubated at 37°C for 48 h. The isolates were identified by conventional methods, including Gram staining, colony morphology, haemolysis, catalase and coagulase tests and anaerobic fermentation of mannitol. All the tests were performed as described by Koneman et al.[32]. The identification of the isolates was confirmed subsequently by PCR. To make PCR, all isolates were stored at -20°C in trypticase soy broth containing 20% of glycerol. Prior to the testing, the isolates were twice serially cultured on columbia agar medium, containing 5% of sheep blood, for 24 h at 37°C under aerobic conditions.

### *Antibiotic Susceptibility Test*

Ten colonies from the Columbia blood agar medium, incubated at 37°C for 18 h, were suspended in 2 ml of sterile saline to a density approximately equal to McFarland Opacity Standard No. 0.5. A dry cotton wool swab was placed in the suspension and excess liquid was expressed against the inside of the tube. The bacterial suspension was inoculated onto Mueller-Hinton agar (Merck, Germany) with the swab in such a way that the whole surface of the agar was covered. The antibiotic disks, containing the antibiotics were dispensed on the surface of the medium and incubated aerobically at 37°C for 18 h.

Antimicrobial sensitivity was tested by the disk diffusion method on Mueller Hinton Agar and performed according to National Committee for Clinical Laboratory Standards guidelines (NCCLS) [33]. The following antibacterial agents were used: penicillin G (P) (6 µg, Oxoid), cefoxitin (FOX) (30 µg, Oxoid), amoxicillin + clavulanic acid (AMC) (10 µg, Oxoid), enrofloxacin (ENR) (5 µg, Oxoid), vancomycin (VA) (30 µg, Oxoid), trimethoprim-sulfamethoxazole (SXT) (25 µg, Oxoid), clindamycin (CM) (2 µg, Oxoid), gentamicin (GM) (10 µg, Oxoid), tetracycline (TE) (30 µg, Oxoid), neomycin (N) (30 µg, Oxoid), and erythromycin (E) (15 µg, Oxoid). The results were recorded as resistant, intermediate or susceptible by the measurement of the inhibition zone diameter according to the interpretive standards of NCCLS [33]. All identified Staphylococci isolates were tested for phenotypic methicillin resistance by antibiotic disc diffusion susceptibility test with 1 µg oxacillin (OX) (Oxoid) and 30 µg cefoxitin discs. *S. aureus* ATCC 25923 strain was used as positive control. Resistance of Staphylococci isolates to three or more classes of antibiotics was considered as multidrug-resistance [34].

### *Genomic DNA Extraction*

For nucleic acid isolation, isolates were activated on tripticase soya agar (bioMérieux, France). After overnight incubation at 37°C, single colony for each strain was resuspended on 500 µl of sterile phosphate buffer saline (PBS) (pH: 7.2). Bacterial cells were harvested by centrifugation at 3.000 × g for 10 min, the cell pellet was re-suspended in 350 µl TE buffer [10 mM tris chloride, 1 mM EDTA (pH 8.0)] with 100 µg of lysostaphin (Sigma, USA) per ml, and incubated at 37°C for 1 h. Each tube was vortexed

once every 15 min. Then, 350 µl 10% SDS with 100 µg of proteinase- K (Vivantis Technologies, Malaysia) per ml, and incubated at 37°C for 2 h. Each tube was vortexed once every 15 min. The phenol/chloroform extraction method was used for nucleic acid extraction according to Sambrook and Russel [35].The DNA precipitate was dissolved in 100 µl of TE buffer [10 mM Tris chloride-1 mM EDTA (pH 8.0)], and stored at -20°C until processing.

### *PCR Analysis*

Simplex PCR technique was used of each gene. Properties of primers used in this study are reported in *Table 1*.

All amplification reactions were prepared in a 25 µl volume containing: 10 mM Tris/ HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 200 mM each dNTPs, 10 pmol oligonucleotide primer, 1 U Taq polymerase and 2 µl template DNA.

A pre-PCR step at 95°C for 5 min was applied. A total of 35 cycles were run at the following conditions: denaturation at 95°C for 30 sec, annealing (primer specific temperatures at *Table 1*) for 60 sec, and extension at 72°C for 60 sec. The reaction was achieved with a final extension at 72°C for 7 min. PCR products were checked using 1.5% agarose gel with 0.125 mg/l ethidium bromide. Only clear, unambiguous and reproducible bands were recorded.

### *Legal Permission-Ethics Committee Details*

The name of institute approves the necessary ethical commission report: Laboratory of biotechnology related to animal breeding, University Saad Dahleb, Blida, Algeria.

The serial number of the approval in the material and methods section: MSRV

### *Statistical Analysis*

The results of the phenotypic analyses of the various strains were expressed as frequencies or probabilities of observing a positive result for each performed test in a given bacterium.

Differences in frequencies of *in vitro* resistance to antimicrobials as β-lactams were determined by Pearson's chi-square test to study the possible relationship between β-lactam resistance genes and others and phenotypic resistance to antimicrobials. Value of $P<0.05$ was considered significant.

## RESULTS

The antimicrobial susceptibility results of isolates of Staphylococci are summarized in *Table 2, 3,* and *4,* respectively.

The sensitivity and resistance of the isolated strains were different depending on the antibiotic tested. Regarding to the agar diffusion test, a total of 17 isolates have shown to have a methicillin resistant phenotype (i.e., resistance to oxacillin and cefoxitin). Staphylococci were resistant mostly to penicillin (95.23%), clindamycin (80.95%), vancomycine (76.19%), erythromycin (76.19%) and Amoxicillin + Clavulanic Acid (66.67%). Whereas strains were most sensitive to neomycin and gentamycin (100%), in average. The next effective antibiotics were

***Table 1.*** *Primers and properties used in the study*
***Tablo 1.*** *Çalışmada kullanılan primerler ve özellikleri*

| Gene | Primer Name | Primer Sequences | Annealingng Temperatur (°C) | Amplicon Size (bp) | Reference |
|---|---|---|---|---|---|
| *Staphylococcus* spp. | Staph294-318 | 5′-GCCGGTGGAGTAACCTTTTAGGAGC-3′ | 55 | 106 bp | [36] |
| | Staph 1522-1540 | 5′-AGGAGGTGATCCAACCGCA-3′ | | | |
| *S. aureus* | Sau 327 | 5′-GGA CGA CAT TAG ACG AAT CA-3′ | 64 | 1318 bp | [37] |
| | Sau 1645 | 5′-CGG GCA CCT ATT TTC TAT CT-3′ | | | |
| Oxacillin/ Penicillin | mecA1 | 5′-CCTAGTAAAGCTCCGGAA-3′ | 54 | 314 bp | [38] |
| | mecA2 | 5′-CTAGTCCATTCGGTCCA-3′ | | | |
| Penicillin | blaZ1 | 5′-ACTTCAACACCTGCTGCTTTC-3′ | 56 | 173 bp | [39] |
| | blaZ2 | 5′-TGACCACTTTTATCAGCAACC-3′ | | | |
| Gentamicin | aacA-aphD 1 | 5′-TAA TCC AAG AGC AAT AAG GGC-3′ | 54 | 227 bp | [40] |
| | aacA-aphD 2 | 5′-GCC ACA CTA TCA TAA CCA CTA-3′ | | | |
| *Erythromycin* | ermA 1 | 5′-AAG CGG TAA ACC CCT CTG A-3′ | 54 | 190 bp | [40] |
| | ermA 2 | 5′-TTC GCA AAT CCC TTC TCA AC-3′ | | | |
| *Erythromycin* | ermC 1 | 5′-AAT CGT CAA TTC CTG CAT GT-3′ | 54 | 299 bp | [40] |
| | ermC 2 | 5′-TAA TCG TGG AAT ACG GGT TTG-3′ | | | |
| Oxytetracyclin | tetK 1 | 5′-GTA GCG ACA ATA GGT AAT AGT-3′ | 54 | 360 bp | [40] |
| | tetK 2 | 5′-GTA GTG ACA ATA AAC CTC CTA-3′ | | | |
| Oxytetracyclin | tetM 1 | 5′-AGT GGA GCG ATT ACA GAA-3′ | 54 | 158 bp | [40] |
| | tetM 2 | 5′-CAT ATG TCC TGG CGT GTC TA-3′ | | | |

***Table 2.*** *Susceptibility to various antibiotics of staphylococci strains isolated from bovine mastitis*
***Tablo 2.*** *Sığır mastitislerinden izole edilen Stafilokokların çeşitli antibiyotiklere karşı duyarlılıkları*

| Antibiotics | Total Profile Break Points | Staphylococci (n= 21 isolates: 10 *S. aureus* and 11 CNS) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sensitive | | Resistance | | Intermediate Sensitive | |
| | | Number | % | Number | % | Number | % |
| P | ≤ 28-29≥ | 1 | 04.76 | 20 | 95.23 | 0 | 0 |
| OX | ≤10-13 ≥ | 4 | 19.04 | 17 | 80.95 | 0 | 0 |
| FOX | ≤24-25≥ | 7 | 33.33 | 10 | 47.61 | 4 | 19.04 |
| AMC | ≤ 19-20≥ | 7 | 33.33 | 14 | 66.67 | 0 | 0 |
| ENR | ≤16-23≥ | 19 | 90.47 | 2 | 09.52 | 0 | 0 |
| VA | ≥15 | 5 | 23.80 | 16 | 76.19 | 0 | 0 |
| SXT | ≤10-16 ≥ | 17 | 80.95 | 1 | 04.76 | 3 | 14.28 |
| CM | ≤14-17≥ | 4 | 19.04 | 17 | 80.95 | 0 | 0 |
| GM | ≤12-15≥ | 21 | 100 | 0 | 0 | 0 | 0 |
| TE | ≤14-19 ≥ | 13 | 61.90 | 8 | 38.09 | 0 | 0 |
| N | ≤ 13-18≥ | 20 | 95.23 | 1 | 04.76 | 0 | 0 |
| E | ≤ 13-23≥ | 2 | 09.52 | 16 | 76.19 | 3 | 14.28 |

***Table 3.*** *Antibiotic resistance patterns of 21 staphylococci isolates*
***Tablo 3.*** *21 adet Stafilokok izolatının antibiyotik direnç profilleri*

| Pattern | Resistance Profile 1 | Resistant Isolates | |
|---|---|---|---|
| | | Number | % |
| 1 | P, TE | 1 | 04.76 |
| 2 | ENR, TE | 1 | 04.76 |
| 3 | P, OX, AMC, VA, CM, TE | 2 | 09.52 |
| 4 | P, OX, FOX, AMC, VA, CM, E | 7 | 33.34 |
| 5 | P, OX, FOX, AMC, VA, CM, E | 1 | 04.76 |
| 6 | P, OX, FOX, AMC, ENR, VA, CM, TE, E | 1 | 04.76 |
| 7 | P, OX, TE | 1 | 04.76 |
| 8 | P, VA, SXT, CM, E | 1 | 04.76 |
| 9 | P, OX, VA, CM, TE, N, E | 1 | 04.76 |
| 10 | P, OX, CM, E | 1 | 04.76 |
| 11 | P, OX, FOX, AMC, VA, CM | 1 | 04.76 |
| 12 | P, OX, AMC, VA, CM, E | 1 | 04.76 |
| 13 | P, OX, AMC, VA, CM, TE, E | 1 | 04.76 |
| 14 | P | 1 | 04.76 |

enrofloxacin (90.47%), Trimethoprim-Sulfamethoxazole (80.95%) and tetracycline (61.90%) according to *in vitro* tests. Antibiogram results for the isolates were classified according to pattern of resistance (types I to IX) *(Table 3)*. The most frequent pattern of resistance was type VII, which was found in 10 isolates. Antibiogram pattern IX represents resistance to nine of the drugs that are most commonly used for treatment of mastitis in Algeria.

PCR assay was made to determine whether the multidrug resistant MRS isolates from Algeria locations were genetically clustered *(Table 4)*. This table has shown in party the phenomenon of multiple resistances to three or more antibiotics, none being resistant to only one or two antibiotics. The percentage of multiple resistance strains was 87.71% (18/21).

Antimicrobial susceptibility testing reported a high resistance of Staphylococci strains to antimicrobial agents which was confirmed by PCR by the presence of *mecA* gene. Our results shows presence of *mecA* gene for all Staphylococci strains which were phenotypically resistant to cefoxitin and/or oxacillin.

In spite of the presence of some phenotypic resistance against erythromycin, no genotypic resistance genes were detected following researching *erm (C)* and *erm (A)*. The *blaZ* gene was detected in 42.85% (9/21) of isolates.

Results presented in *Fig. 1* show the presence of *mecA* gene from extracted DNA of all *S. aureus* and CNS strains tested; this result confirmed the antibiogram results for susceptibility to methicillin.

## DISCUSSION

Detection of the antibiotic resistance is important for controlling and treatment of the bacterial disease. The present study was carried out to investigate the antimicrobial susceptibilities and resistance genes in staphylococci isolated from lactating cows with clinical or subclinical mastitis cases in Algeria.

The rate of penicillin resistance (100%) detected in this study is much higher than those reported in other countries such as Korea (52.9%), Switzerland (31%), Finland (32%), USA (22.1%) [41] and Brazil [42]. Strains isolated from mastitis cases were resistant to some antibiotics commonly used in treatment of cow mastitis in Algeria. Almost the same results were reported by other authors [28,29], especially on resistance to penicillin and oxacillin. The existence of antibiotic-resistant in a selective area might be due the frequent and continuing use of the same antimicrobials [18]. In fact, the antibiotics such as penicillin-G, oxacillin, streptomycin, ampicillin, amoxycillin, cloxacillin

***Table 4.*** *Correlation between phenotypic antibiotic resistance and PCR results*
***Tablo 4.*** *Fenotipik antibiyotik direnci ile PCR sonuçları arasındaki bağlantı*

| Strains | Resistance Phenotype | Presence of Fragment | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | BlaZ | mecA | aacA-aphD | Erm (A) | Erm (C) | tetK | TetM |
| 1 | P, TE | - | + | - | - | - | - | + |
| 2 | P, AMC, VA, CM, TE, E | - | + | - | - | - | - | + |
| 3 | OX, P, FOX, AMC, VA, CM, E | - | + | - | - | - | - | + |
| 4 | OX, P, FOX, AMC, VA, CM, E | - | + | - | - | - | - | + |
| 5 | OX P, FOX, AMC, VA, CM | - | + | - | - | - | - | + |
| 6 | OX, P, FOX, AMC, ENRO, VA, CM, TE, E | + | + | - | - | - | - | + |
| 7 | OX, P, FOX, AMC, VA, CM, E | - | + | - | - | - | - | + |
| 8 | OX, P, FOX, AMC, VA, CM, E | + | + | - | - | - | - | + |
| 9 | OX, P, TE | + | + | - | - | - | - | + |
| 10 | OX, P, VA, SXT, CM, E | - | + | - | - | - | - | + |
| 11 | P, VA, CM, TE, N, E | - | + | - | - | - | - | + |
| 12 | OX, P, FOX, AMC, VA, CM, TE, E | - | + | - | - | - | - | + |
| 13 | OX, P | + | + | - | - | - | - | + |
| 14 | P, CM, E | - | + | - | - | - | - | + |
| 15 | OX, P, FOX, AMC, VA, CM | + | + | - | - | - | - | + |
| 16 | OX, P, AMC, VA, CM, E | - | + | - | - | - | - | + |
| 17 | OX, P, AMC, VA, CM, TE, E | + | + | - | - | - | - | + |
| 18 | OX, P, AMC, VA, CM, E | + | + | - | - | - | - | + |
| 19 | OX, P, FOX, AMC, VA, CM, E | + | + | - | - | - | - | + |
| 20 | OX, P, FOX, AMC, VA, CM, E | + | + | - | - | - | - | + |
| 21 | OX, ENR, TE, E | - | + | - | - | - | - | + |

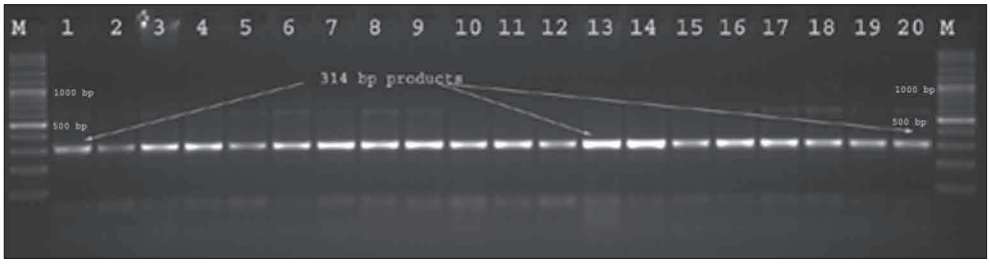


**Fig 1.** Agarose gel electrophoresis of *mecA* gene targeted PCR. **M:** Marker, 100 bp plus (brand). **Lines 1-35:** Amplified products of *mecA* specific PCR (Bands 314 bp)

**Şekil 1.** *MecA* gen hedefli PCR'nin agaroz jel elektroforezi. **M:** Marker, 100 bp plus (brand). **Kuyucuklar 1-35:** *MecA* spesifik PCR amplifiye ürünleri (314 bp)

and tetracycline are frequently used in veterinary clinics of herd selected for this study.

In the study, incidence of vancomycin-resistant isolates of *S. aureus* was observed with frequency of 76.2%. Elsewhere, none of the studies have been reported the vancomycin-resistant *S. aureus* in bovine mastitis. On the other hand, vancomycin-resistant enterococci have been reported in cattle mastitis [43].

Staphylococci examined in this study were more resistant to antibiotics than bacteria isolated earlier in Algeria [44]. But, the *in vitro* resistance to antibiotics of bacteria isolated in the same farm can change from one year to the next one [27].

The majority of authors have noted the increase in the resistance to antibiotics of staphylococci isolated from mastitis [6,19-30]. Therefore, it seems that our results are in accordance with them. The most important factor affecting the cure rates from clinical Staphylococcal mastitis was the capacity of the strain to produce β-lactamase [16]. Apart from this, Watts and Salmon [26] highlight the need to identify methicillin resistant *S. aureus* (MRSA) accurately, because these strains are resistant to all compounds currently approved for treatment of bovine mastitis. On the contrary, de Oliveira et al.[45] found that overall level of resistance was generally low to all antimicrobial agents that are currently commercially available to treat bovine mastitis.

In literature, a few studies have reported the occurrence of MRSA from bovine mastitis and proportion of resisting isolates was low [18,46,47]. In this study, 100% of isolates of *S. aureus* and CNS were methicillin-resistant. The isolates appeared to demonstrate privileged expression of *mecA* genes or production of methicillinase or appear to overproducing beta-lactamase [18,47]. The phenotypic expression of resistance could vary due to growth conditions or might be limitations in detection in microbiological methods [48]. Because of prolonged treatments with same antibiotics frequently is noticed the emergence of resistant variants of bacterial strains. In addition, it is well reported that emergence of drug resistance is the consequence of the inappropriate use of antimicrobials [49].

Analyzing the antibiograms' results of staphylococci strains it was observed that in the isolates was revealed the phenomenon of multiple resistances to three or more antibiotics, none being resistant to only one or two antibiotics. In the present study, the percentage of multiple resistance strains was 87.71% (18/21). Our finding that 18 of the isolates tested were multidrug resistant MRS isolates indicates that antibiotic resistance has emerged in dairy cattle in Algeria. In literature, multiple antimicrobial resistance is defined as resistance to three [34], four or more antimicrobials [50]. Furthermore, Waage et al.[51] reported that *S. aureus* has developed multidrug resistance with a wide variation from herd to herd. Memon et al.[52] reported that 100% of *S. aureus* isolates were multidrug resistant. While, comparatively a less percentage (52%) of isolates were also reported as multi drug resistant in Ethiopia [53]. Bardiau et al.[54] reported that all isolates were resistant to at least three antibiotics, and two-thirds of the strains (58%) were resistant to at least six antibiotics. As multiple antibiotic resistance, high resistance rates presenting was observed in study conducted by Tel et al.[55]. This is in accordance with our study. Using various antibiotics can create selection pressure, ultimately resulting in the development of antibiotic resistance [56].

Methicillin resistance was found in high percentage of *S. aureus* and CNS isolates (100%) which is greater than reported in Korea and India [18,44]. Furthermore, there was *mecA* gene in all isolates. The *S. aureus* exhibit resistance to methicillin was first reported in 1960, by the time MRSA gradually developed multiple resistances and became a source of causing serious nosocomial infections, worldwide [57].

In mastitis, most studies report a low prevalence of MRSA [46,58] but in Belgium, an unpredictably high prevalence of mastitis-associated MRSA was reported [59]. Besides to their resistance to all types of beta-lactam antibiotics, MRSA strains illustrate resistance to other antimicrobial agents, also used for treatment and prevention of mastitis [60]. Susceptibility tests showed here a wide variety of nine different resistance patterns among the isolates. In this study, all isolates except one were resistant to at least three antibiotics, and two-thirds of the isolates were resistant to at least six of the twelve antibiotics. In addition to oxacillin, all of our MRS isolates were resistant to penicillin and tetracycline. This result is in accordance with other studies [59,61]. However, these strains showed more resistance to erythromycin and clindamycin than previously reported [59,61,62]. Furthermore, this high resistance rate to some antibiotics can be explained by their frequent use in veterinary practice in Algeria. Resistance against beta-lactams and presence of *blaZ* gene in our isolates is in agreement with the result of Green and Bradley [63]. But Haveri et al.[64] found that the *blaZ* gene detected in majority of isolates and *mecA* was not found in any isolates, these findings are consistent with previous report. Detection of genes of old generation antibiotics (gentamycin and tetracycline) is also necessary. Also, a number of isolates revealed *tetK* and *tetM* genes and results are in concurrence with phenotypic observations. All isolates were found sensitive to gentamycin phenotypically and also negative to *aac-aph* gene. Following researching *aac-aph* gene from isolates which 100% phenotypically sensitive to gentamycin, no *aac-aph* gene was found. So, the *aacA-D* gene has been reported less prevalent in the mastitis isolates and results of the present study are in disagreement with a previous report [42]. Furthermore, occurrence of tetracycline-resistant genes among the bovine staphylococci isolates has been previously observed [65]. Tetracycline resistance encoding gene *tetM* was present in 100% of isolates which is higher than previously detected in china by Gao et al.[66]. The proportion of the tetracycline genes is almost similar with the previous reports [65]. Li et al.[67] reported that there is a common use of penicillin, tetracycline and erythromycin for the treatment of mastitis in China. Moreover, 76.19% of isolates were phenotypically resistant to erythromycin but all of them were negative for *ermA* and *ermC* genes. This finding is in disagreement with those from in northern China [66]. Acquisition of resistance in *S. aureus* isolates attributed to mutation in gene or due to exchange of genetic material between organisms, since resistance genes carrying mobile genetic elements of *S. aureus* have exceedingly been explored [68].

These results indicate that, these isolates are resistant at high rates to the beta-lactam antibiotics which are intensively used in the control and treatment of mastitis without any antibiogram test in Algeria. The results of present study showed some similarities with previous studies to the same antibiotics [47,69]. The presence of antibiotic-resistant genes and similar antibiotic-resistant patterns among isolates of staphylococci indicates possible diffusing or spreading of isolates between animals.

Almost of staphylococcal strains tested had an increased susceptibility to neomycin and gentamicin (100%) and all of them are resistant to penicillin (100%). The data on antimicrobial susceptibility can help determine the choice

of empirical initial treatment. Our investigations revealed high prevalence of different antibiotic-resistant genes (especially *mecA* and *tetM*) among isolates.

This study reports high prevalence of MRS isolates with having *mecA* gene. That is to say a significant observation was prevalence of methicillin-resistant isolates in the herd. These findings can be considered in designing strategic plans for treatment, prevention and control of Staphylococcal mastitis in Algeria. Occurrence of such isolates, among the mastitis cases needs attention of veterinarians and managers of herds. The findings might be helpful to control, transfer or dissemination of pathogenic strains, segregation of cows for reduction of mastitis and can be applied for treatment policies and antimicrobials strategies. The present study demonstrated that the existence of alarming level of resistance of frequently isolated mastitis agents to commonly used against antimicrobial agents in the farms in Algeria. Consequently, it is very important to implement a systematic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of mastitis. The increasing occurrence of MRSA and MR-CNS should be under consideration from the point of view of antibiotic selection for mastitis treatment and prevention, especially if the possibility exists of the resistance transfer in or between bacteria. The results of this study can be used as a baseline for further investigations.

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