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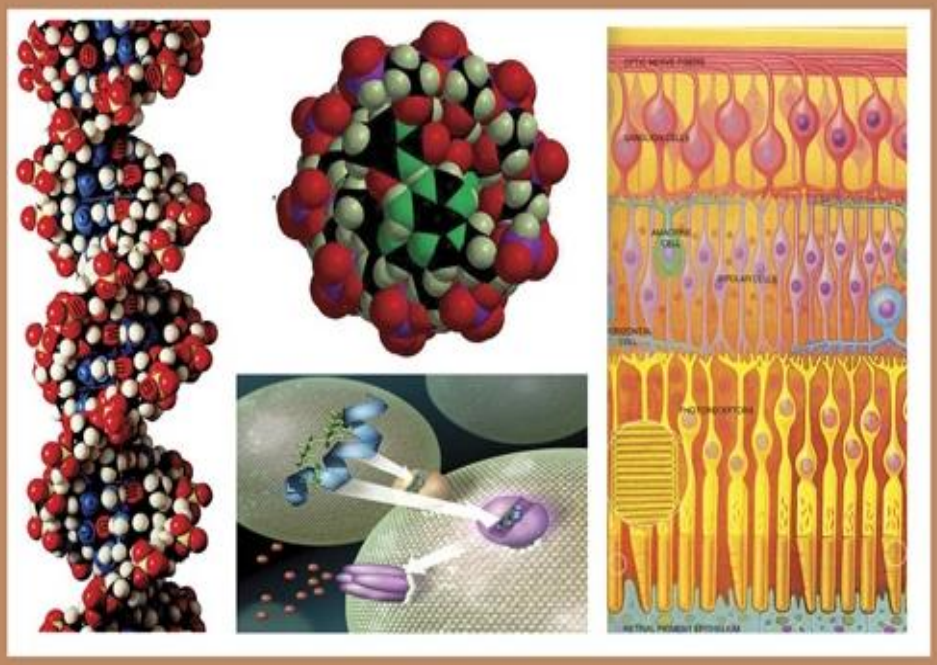

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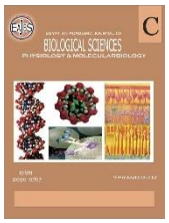

Antibiotic Resistance of *Staphylococcus Aureus* Isolated from Raw Cow Milk In Northwest Algeria

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ABSTRACT

The contamination of raw milk by S. aureus remains an important issue in dairy food production. The objectives of this study were to determine the prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw cow milk in Northwest Algeria. A total of 81 samples of raw milk were collected from (shops and farms. *S. aureus* was detected in 15 samples (18,5%). 25 S. *aureus* strains were identified of which 5 were positive for methicillin-resistant *S. aureus and 9 were* multiple antibiotic resistance (MAR). The *S. aureus* isolates showed resistance to penicillin G (92%), followed by tetracycline (72%), ofloxacin (24%), cefoxitin (24%), oxacillin (20%), Sulfamethoxazole/trimethoprim (8%) lincomycin, Tobramycin, Lincomycin, Fosfomycin and Fusidic acid (4%). the MIC determination against OXA of these strains varied from 35 to 190 (µg/mL)**.** The results indicated that raw cow milk samples contaminated by S. aureus can be a potential risk to public health.

INTRODUCTION

Antimicrobial resistance is an important health problem worldwide (Cosgrove, 2006). The development of resistance, both in human and animal bacterial pathogens, has been ascribed to the extensive therapeutic use of antimicrobials or their use as growth promoters in animal feed production (Hsueh et al., 2005; Silbergeld et al.,2008). The use of antimicrobials for both humans and animals has caused a rising global challenge of antimicrobial resistance (AMR; Laxminarayan *et al.,* 2013; Tang *et al.,* 2017). Reducing antimicrobial use (AMU) in dairy farming is therefore one area of importance (WHO, 2015). Staphylococcal foodborne disease (SFD) is one of the most common foodborne diseases and a major concern in public health programs worldwide SFD results from the consumption of contaminated foods by Staphylococcus aureus enterotoxins (SEs) that are resistant to heat treatment (Le Loir *et al.*,2003; Claeys *et al.*,2013). The foods that have been frequently implicated in SFD are meat and meat products, poultry and egg products and milk and dairy products (Argudín *et al.*,2010).Raw milk has been frequently implicated in staphylococcal food poisoning. The potential risk of raw milk contaminated by Staphylococcus aureus (S. aureus) in Algeria is still not well documented.

The treatment of infections caused by S. aureus has been challenged by the emergence of multidrug-resistant (MDR) strains, including methicillin-resistant S. aureus (MRSA) (Papadopoulos *et al.*,2019), as the result of indiscriminate use of antibiotics both in human and veterinary medicine (Løvseth *et al.,* 2004; Getahun *et al.*,2008).

This study aims to investigate S. aureus contamination in raw cow milk in the Northwest region of Algeria. For this purpose, we analyzed the prevalence, contamination levels and antibiotic susceptibility profiles of the S. aureus isolates obtained from the North West region of Algeria.

MATERIALS AND METHODS

A total of 81 milk samples of raw cow, milk was collected from (shops and farms) in the four districts of West Algeria. All samples were collected aseptically in sterile boxes, transferred to the laboratory with ice packs, samples were analysed for *S. aureus* within 4 h of collection.

Isolation and Identification of *S. aureus*:

To isolate *S. aureus*, 10 mL of raw milk of each sample was added to 90 mL of buffered peptone water and homogenized. The resulting suspensions were diluted 1:10 in Baird−Parker base (Conda Pronadisa, Spain), supplemented with egg yolk and potassium tellurite. Incubation of the plates was carried out at 37°C for 24h to 48 h ISO. (1999). The putative colonies were tested for catalase, coagulase rabbit plasma and the Pastorex Staph Plus assay (Bio-Rad, Marnes-la-Coquette, France) are considered as *S. aureus* strains, the isolates that responded positively to the three tests. All tests were performed according to standard guidelines and *S. aureus* (ATCC 25923) was used as a positive control in each test protocol.

Molecular Identification:

DNA extraction the isolates were retrieved from the storage and cultured on blood agar plates and incubated overnight at 37°C for 24 h, before DNA extraction. The extraction protocol used was as instructed by the manufacturer (http: // www. bio-rad .com/ webroot/ web/ pdf/lsr/ literature/ LIT544 .pdf).

The quality and quantity of the extracted genomic DNA were determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). and diluted to a working concentration of 50 ng/µL.

Identification of Staphylococci Isolates by PCR amplification of the 16S rRNA

We performed a specific PCR assay that amplifies the 16S rRNA gene sequence specific for staphylococci according to (Løvseth *et al.*, 2004). Primer sequences used are shown in Table 1. The PCR products were stored at 4 °C and later separated by agarose gel electrophoresis (Løvseth *et al.*, 2004).

Table 1. Oligonucleotide primers used for molecular identification

Primer Size (bp)	Sequence	Target Gene	Amplicon
16S rRNA F	GTAGGTGGCAAGCGTTACC	16S rRNA	228
16S rRNA R	CGCACATCAGCGTCAG		
Nuc F	GCGATTGATGGTGGATACGGT	Nuc	279
Nuc R	AGCCAAGCCTTGACGAACTAAAGC		

Specific PCR for the Identification of *Staphylococcus aureus*:

The *S. aureus* isolates were confirmed using a specific PCR that targeted the thermonuclear (*nuc*) gene-specific for *S. aureus (*Maes *et al.*,2002) using primers sequences listed in Table 1.

The reference strains S. aureus strains ATCC 25923TM, and Staphylococcus epidermidis ATCC 12228TM were included as positive and negative controls, respectively. The DNA of confirmed S. aureus isolates was stored at −20 °C.

Determination of the Antibiotic Resistance Profiles of *S. aureus* Isolates:

Antimicrobial susceptibility of strains was determined by the disk diffusion method on Mueller− Hinton agar (Conda, Pronadisa, Spain) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018). And the

recommendations of the Algerian Antimicrobial Resistance Network (Ammari, 2011). The antibiotics disks used were from Liofilchem (Roeseto, Italy), and types and concentrations (µg) unless otherwise specified): penicillin G (10 IU), cefoxitin (30), gentamicin (10), amikacin (30), tobramycin (10), neomycin (30), tetracycline (30), spiramycin (100), lincomycin (15), ofloxacin (15), chloramphenicol (30), trimethoprim/ sulfamethoxazole (1.25/23.75), fosfomycin (50), fusidic acid (10), and novobiocin (30). The reference strains S. *aureus* ATCC 25923 were used in susceptibility testing as a control.

At the time of data collection, the mean C-reactive protein (CRP) was 56.78 mg/L. Therefore, there was no significant minimal inhibitory concentration (MIC) of oxacillin MIC determination was detected according to the guidelines of the U.S. Clinical and Laboratory Standards Institute (CLSI.,2015). The bacteria suspension (adjusted to 0.5 McFarland turbidity standard) was inoculated on the OXA salt screen agar (Mueller–Hinton agar containing 4% NaCl and 6 µg/mL OXA). Plates were incubated at 37°C for 24 h, and any growth on the plate was regarded as methicillin resistance. Two *S. aureus* reference strains, ATCC 25923 and ATCC 43300 were used as negative and positive controls, respectively.

MRSA Detection:

MRSA was detected by oxacillin (OXA) agar screen, cefoxitin disk diffusion and the minimal inhibitory concentrations determination of oxacillin (MIC).

RESULTS

Detection of Staphylococcus Species in Milk Samples:

A total of 81 raw cow milk samples were collected and screened for the presence of *Staphylococcus* species. 15 were contaminated. The frequency of *S. aureus* contamination varied by the sampling point: it was higher in raw milk from farms samples (66%) than in shops samples (33%).

A total of 25 isolates obtained from the contaminated milk samples were screened for the characteristics of *S. aureus* All the isolates were Gram-positive and catalase-positive.

Antibiotic Resistance of *S. aureus:*

The resistance profile of isolates to the tested antimicrobial agents is presented in Table 2. Resistance to penicillin was the most common (95%), followed by tetracycline (72%). However, we observed Nine *S. aureus* isolates (36%) were resistant to at least 3 different classes of antibiotics. We observed 4 phenotypes of multidrug resistance (Table 3) and 5 MRSA strains. These strains were confirmed by the OXA agar test and the MIC determination against OXA.

MRSA Strain Detection:

We identified five MRSA strains (cefoxitin/oxacillin resistant), and the MIC determination against OXA of these strains varied from 35 to 190 (µg/mL).

Table 2: The number and percentages of *S. aureus* isolate resistant to different antibiotics.

Antibiotics	% (N°) of resistant strains
Penicillin G	92 (23)
Cefoxitin	24 (6)
Oxacillin	20 (5)
Gentamic	0 (0)
Amikacin	0 (0)
Spiramycin	4 (1)
Sulfamethoxazole/trimethoprim	8 (2)
Neomycin	4 (1)
Tobramycin	4 (1)
Fusidic acid	4 (1)
Lincomycin	4 (1)
Tetracycline	72 (18)
Chloramphenicol	0 (0)
Ofloxacin	24 (6)
Fosfomycin	4 (1)
Novobiocin	0 (0)

Table 3: Phenotypic antibiotic resistance for *S. aureus* isolates

Antibiotic-phenotypes	No. of isolates with phenotype
P-OX-FOX-OFX-TE-TOB	5
P-TE-N-FC	2
P-FOS-TOB	1
P-SP-TE	1

P = penicillin ;FC = fusidic acid; TE = tetracycline ; OX = oxacillin;SP = spiramycin ; N = neomycin; OFX = ofloxacin ; FOX = cefoxitin ; TOB = tobramycin FOS = fosfomycin; OFX = ofloxacin

DISCUSSION

The objective of this study has been to isolate and identify *S. aureus* from raw milk obtained from shops and farms around the North-West of Algeria. According to many previous studies (Adjalne-Kaouche *et al.* (2014); Chaalal *et al.* (2018); Tamendjari *et al.* (2021) these authors reported an *S. aureus* contamination rate higher than 32.6% in raw milk. In the present study, the overall presence of the pathogen was 18,5 % among samples screened. However, a high prevalence of *S. aureus* has been reported in other countries (Jamali *et al.,* 2015; Bharathy *et al.,*2015; Al-Ashmawy et al 2016; Papadopoulos *et al.,* 2018; Nhatsave *et al.,* 2021). The prevalence of *S. aureus* can vary according to hygienic conditions (McMillan *et al.*,2016). Cows with mastitis represented the major source of *S. aureus* contamination of milk and dairy products (Kummel *et al.,* 2016).

Traditionally, bacteria species are identified using phenotypic traits based on results obtained from biochemical tests. Despite the fact that biochemical protocols and assays are constantly being refined, results obtained from these tests are usually not very reliable and are time-consuming (Gücükoğlu *et al* .,2012; Muyiwa Ajoke *et al* .,2015).

In this study, the amplification of *Staphylococcus* genus-specific 16S rRNA gene fragment and the thermo nuclease gene (*nuc*) specific for *S. aureus*, were used for positive identification of *S. aureus* strains.

The highest resistance rates were for penicillin G (92%), followed by tetracycline (72%), ofloxacin (24%), cefoxitin (24%), oxacillin (20%), Sulfamethoxazole/ trimethoprim (8%) lincomycin, Tobramycin, Lincomycin, Fosfomycin and Fusidic acid (4%). Similar to our study, high rates of resistance to penicillin and tetracycline were previously reported among S. aureus isolated from raw milk samples and no isolate was sensitive to all antibiotics (Derks *et al.,* 2012; Poizat *et al.,* 2017; Doidge *et al.,* 2021; Titouche *et al.*,2019; Tamendjari *et al.*,2021). In our study, Six MRSA strains (6%) were isolated from 81 raw milk samples and 36% of strain isolates were multi-drug resistant. The MRSA contamination rate differed between studies can be related to attributes such as sample source, geographic origin, the sensitivity of identification methods, and sample quantity (Basanisi *et al.,* 2017; Papadopoulos *et al.,* 2018; Titouche *et al.*,2019; Tamendjari et al.,2021).in our study, the MIC ranged from 35 to 190**(**µg/mL) similar to (Moreno-Grúa *et al.,* 2018) and lower than **(**Tamendjari *et al.*,2021). The use of antibiotics in dairy farms is currently known to be one of the major factors responsible for the emergence of drug-resistant bacteria worldwide. Ampicillin and tetracycline are mostly used on dairy cattle farms.

CONCLUSION

Staphylococcus aureus (*S. aureus*) is one of the most common pathogens that cause mastitis in dairy cows. The results of this study indicate a presence of (*S. aureus*) in raw milk samples with positive strains for methicillin-resistant and multiple antibiotic resistance (MAR). The emergence of methicillin-resistant *S. aureus* (MRSA) constitutes a serious public health concern due to its ability to colonize and infect humans and animals.

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