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.

**KEYWORDS:** Antibiotic resistance; Raw milk; *Staphylococcus aureus*; Staphylococcus aureus (MRSA)

**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; Claeyts 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 24 h 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).

| Primer   | Size (bp) | Sequence                        | Target Gene | Amplicon |
|----------|-----------|---------------------------------|-------------|----------|
| 16S rRNA F |           | GTAGTGGCAAGCGTTACC              | 16S rRNA    | 228      |
| 16S rRNA R |           | CGCACATCAGCGTCAG                |             |          |
| Nuc F    |           | GCCATGTGGTGGATACG             | Nuc         | 279      |
| Nuc R    |           | AGCCAGCCTTGCAGGAACTAAAGC       |             |          |

**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 primer 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), tetracycline (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 that 3% of *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)                    |
| Gentamicin                         | 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; Nhatave 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.
REFERENCES

Adjalne-Kaouche, S., R. Benhacine, F. Ghozlane, and A. Mati. (2014). Nutritional and hygienic quality of raw milk in the mid-northern region of Algeria: Correlations and risk factors. Scientific World Journal, 131593.

Al-Ashmawy, M.A., Sallam, K.I., Abdi-Elghany, S.M., Elhadidy, M. and Tamura T. (2016) Prevalence, molecular characterization, and antimicrobial susceptibility of methicillin-resistant Staphylococcus aureus isolated from milk and dairy products. Foodborne Pathogens and Disease., 13(3): 156–162.

Ammari, H. (2011) Standardisation de L’antibiogramme à L’échelle Nationale (Médecine Humaine et Vétérinaire). 6th éd. Réseau Algérien de la Surveillance de la Résistance Des Bactéries Aux Antibiotiques, Algérie. p45-46.

Argudín, M.A.; Mendoza, M.C. ; Rodicio, M.R. (2010). Food Poisoning and Staphylococcus aureus Enterotoxins. Toxins, 2, 1751–1773.

Basanisi, M. G., G. La Bella, G. Nobili, I. Franconieri, and G. La Salandra. (2017). Genotyping of methicillin-resistant Staphylococcus aureus (MRSA) isolated from milk and dairy products in South Italy. Food Microbiology, 62:141–146.

Bharathy, S., Gunaseelan, L., Porteen, K. and Bojiraj, M. (2015) Prevalence of Staphylococcus aureus in raw milk: Can it be a potential public health threat. International Journal of Advanced Research., 3(2): 801-806.

Chaalal, W., N. Chaalal, N. Bourafa, M. Kihal, S. M. Diene, and J. M.Rolain.(2018). Characterization of Staphylococcus aureus isolated from food products in Western Algeria. Foodborne Pathogens and Disease., 15:353–360.

Claeys, W.L.; Cardoen, S.; Daube, G.; De Block, J.; Dewettinck, K.; Dierick, K.; De Zutter, L.; Huygebaert, A.; Imberechts,H.; Thianne, P.(2013). Raw or Heated Cow Milk Consumption: Review of Risks and Benefits. Food Control, 31, 251–262 [CrossRef].

CLSI (Clinical and Laboratory Standard Institute). (2018). Performance Standards for Antimicrobial Susceptibility Testing. 28th ed. Suppl. M100. CLSI, Wayne, PA.

CLSI. (2015) Performance Standards for Antimicrobial Susceptibility Testing; Twenty-fifth Informational Supplement. CLSI document M100-S25. CLSI, Wayne, PA.

Cosgrove, S.E. (2006). The relationship between antimicrobial resistance and patient outcomes: Mortality, length of hospital stay, and health care costs. Clinical Infectious Diseases,42, S82–S89.

CSLI. (2015). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-fifth Informational Supplement. CLSI document M100-S25. CLSI, Wayne, PA.

Derks, M., L. M. A. van de Ven, T. van Werven, W. D. J. Kremer,and H. Hogeveen. (2012). The perception of veterinary herd health management by Dutch dairy farmers and its current status in the Netherlands: A survey. Preventive veterinary medicine. 104:207–215.

Doidge, C., E. Ferguson, F. Lovatt, and J. Kaler. (2021). Understanding farmers’ naturalistic decision making around prophylactic antibiotic use in lambs using a grounded theory and natural language processing approach. Preventive veterinary medicine. 186:105226.

Getahun, K.; Kelay, B.; Bekana, M.; Lobago, F. (2008). Bovine Mastitis and Antibiotic Resistance Patterns in Selalle Smallholder Dairy Farms, Central Ethiopia. Tropical Animal
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Health and Production. 40, 261–268. [CrossRef]

Gücükoğlu, A.; Onur Kevenk, T.; Uyanik, T.; Çadirci, Ö.; Terzi, G.; Alişarli, M. (2012). Detection of enterotoxigenic Staphylococcus aureus in raw milk and dairy products by multiplex PCR. Journal of food science, 77, M620–M623.

Hsueh, P.R.; Chen, W.H.; Luh, K.T. (2005). Relationships between antimicrobial use and antimicrobial resistance in Gram-negative bacteria causing nosocomial infections from 1991–2003 at a university hospital in Taiwan. International Journal of Antimicrobial Agents, 26, 463–472.

ISO. (1999) Microbiology of Food and Animal Feeding Stuffs-horizontal Method for the Enumeration of Coagulase-positive Staphylococci (Staphylococcus aureus and Other Species): Part 1: Technique Using Baird-parker Agar Medium. ISO, Geneva, Switzerland.

Jamali, H., M. Paydar, B. Radmenhr, S. Ismail, and A. Dadrasnia. (2015). Prevalence and antimicrobial resistance of Staphylococcus aureus isolated from raw milk and dairy products. Food Control, 54:383–388.

Kummel, J., B. Stessl, M. Gonano, G. Walcher, O. Bereuter, M. Fricker, T. Grunert, M. Wagner, and M. Ehling-Schulz. (2016). Staphylococcus aureus entrance into the dairy chain: Tracking S. aureus from dairy cow to cheese. Frontiers in Microbiology. 7:1603.

Laxminarayan, R., A. Duse, C. Wattal, A. K. M. Zaidi, H. F. L. Wertheim, N. Sumpradit, E. Vlieghe, G. L. Hara, I. M. Gould, H. Goossens, C. Greko, A. D. So, M. Bigdeli, G. Tomson, W. Woodhouse, E. Ombaka, A. Q. Peralta, F. N. Qamar, F. Mir, S. Kariuki, Z. A. Bhutta, A. Coates, R. Bergstrom, G. D. Wright, E. D. Brown, and O. Cars. (2013). Antibiotic resistance—The need for global solutions. The Lancet Infectious. Diseases. 13:1057–1098. https://doi.org/10.1016/S1473-3099(13)70318-9.

Le Loir, Y.; Baron, F.; Gautier, M. (2003). Staphylococcus aureus and Food Poisoning. Genetics and Molecular Research, 2, 63–76.

Løvseth, A.; Loncarevic, S.; Berdal, K. (2004). Modified multiplex PCR method for detection of pyrogenic exotoxin genes in staphylococcal isolates. Journal of Clinical Microbiology, 42, 3869–3872.

Maes, N.; Magdalena, J.; Rottiers, S.; de Gheldre, Y., Struelens, M. (2002). Evaluation of a triplex PCR assay to discriminate Staphylococcus aureus from coagulase negative staphylococci and determine methicillin resistance from blood cultures. Journal of Clinical Microbiology, 40, 1514–1517.

McMillan, K., Moore, S.C, McAuley, C.M2, Fegan, N and Fox, E.M. (2016). Characterization of Staphylococcus aureus isolates from raw milk sources in Victoria, Australia. BMC Microbiology, 16:169 DOI 10.1186/s12866-016-0789-1.

Moreno-Grúa, E., Pérez-Fuentes, S., Muñoz-Silvestre, A., Viana, D., Fernández-Ros, A.B., Sanz-Tejero, C., Corpas, J.M. and Selva, L. (2018). Characterization of livestock-associated methicillin-resistant Staphylococcus aureus isolates obtained from commercial rabbitries located in the Iberian Peninsula. Frontiers in. Microbiology, 9: 1812.

Muyiwa Ajoke A., Olubukola Oluranti B. and Collins Njie A. (2015). Detection of Antibiotic Resistant Staphylococcus aureus from Milk: A Public Health Implication. International journal of environmental research and public health, 12, 10254-10275; doi:10.3390/ijerph120910254
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Nhatsave, N.; Garrine, M.; Messa, A., Jr.; Massinga, A.J.; Cossa, A.; Vaz, R.; Ombi, A.; Zimba, T.F.; Alfredo, H.; Mandomando, I.; et al. (2021). Molecular Characterization of *Staphylococcus aureus* isolated from Raw Milk Samples of Dairy Cows in Manhiça District, Southern Mozambique. *Microorganisms*, 9,1684. https://doi.org/10.3390/microorganisms9081684

Papadopoulos, P., T. Papadopoulos, A. S. Angelidis, E. Boukouvala, A. Zdragas, A. Papa, C. Hadjichristodoulou, and D. Sergelidis. (2018). Prevalence of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) along the production chain of dairy products in north-western Greece. *Food Microbiology*, 69:43–50.

Papadopoulos, P.; Papadopoulos, T.; Angelidis, A.S.; Kotzamanidis, C.; Zdragas, A.; Papa, A.; Filioussis, G.; Sergelidis, D. (2019). Prevalence, Antimicrobial Susceptibility and Characterization of *Staphylococcus aureus* and Methicillin Resistant *Staphylococcus aureus* Isolated from Dairy Industries in North-Central and North-Eastern Greece. *International journal of food microbiology*, 291, 35–41.

Poizat, A., F. Bonnet-Beauprand, A. Rault, C. Fourichon, and N. Bareille. (2017). Antibiotic use by farmers to control mastitis as influenced by health advice and dairy farming systems. *Preventive Veterinary Medicine*, 146:61–72.

Silbergeld, E.K.; Graham, J.; Price, L.B. (2008). Industrial food animal production, antimicrobial resistance, and human health. *Annual Review of Public Health*, 29, 151–169.

Tamendjari S, Bouzebda FA, Chaib L, Aggad H, Ramdani M, Bouzebda Z. (2021) Antibiotic resistance of *Staphylococcus aureus* isolated from raw cow and goat milk produced in the Tiaret and Souk Ahras areas of Algeria, *Veterinary World*, 14(7): 1929-1934.

Tang, K. L., N. P. Caffrey, D. B. Nóbrega, S. C. Cork, P. E. Ronksley, H. W. Barkema, A. J. Polachek, H. Ganshorn, N. Sharma, J. D. Kellner, and W. A. Ghali. (2017). Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: A systematic review and meta-analysis. *Lancet Planet. Health*, 1: e316–e327. https://doi.org/10.1016/S2542-5196(17)30141-9.

Titouche, Y., Hakem, A., Houali, K., Meheut, T., Vingadassalon, N., Ruiz-Ripa, L., Salmi, D., Chergui, A., Chenouf, N. and Hennekinne, J. (2019) Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) ST8 in raw milk and traditional dairy products in the Tizi Ouzou area of Algeria. *Journal of Dairy Sciences.*, 102(8): 6876-6884.

WHO (World Health Organization). (2015). Global Action Plan on Antimicrobial Resistance. WHO.