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Antimicrobial Susceptibility Profiling of *Staphylococcus aureus* Isolates from Milk

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Authors' contributions

This work was carried out in collaboration between all authors. Author WC conducted the study. Author HA designed the study and served as principal supervisor. Author KZ searched for the literatures and participated in manuscript writing. Authors NS and MK wrote the protocols. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This study investigated the occurrence of *Staphylococcus aureus* in milk and its sensitivity to twenty antibiotics.
Study Design: The research was laboratory-based investigation.
Place and Duration of the Study: The study was carried out at the Laboratory of Hygiene and animal pathology, University of Tiaret, Algeria, between September 2012 and May 2013.
Methodology: Thirty eight milk specimens were collected from cattle and examined to estimate the prevalence of *Staphylococcus aureus*. The sensitivity of the isolates to twenty (20) antibiotics was evaluated and the presence of methicillin resistant *Staphylococcus aureus* (MRSA) was also determined.
S. aureus was characterized using standard microbiological methods and confirmation was done using the API Staph Identification System. Antibiotic sensitivity of isolates was evaluated by means

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of agar diffusion technique while the minimum inhibitory concentration (MIC) was established using broth dilution technique for oxacillin, E-test for tetracyclin, and chloramphenicol respectively.
Results: 55.26% of analyzed samples were contaminated with *S. aureus*. 100% of Methicillin Sensitive *S. aureus* were resistant to nalidixic acid, 70% to bacitracin, 65% to spiramycin, and 45% to penicillin and fosfomycin. There was no resistance to vancomycin, chloramphenicol, gentamicin and pristamycin among isolates.
A total of 76% of the isolated strains were found to be resistant to at least 4 antibiotics.
One Methicillin Resistant *S. aureus* strain (4.76%) was detected and showed multiple drug resistance. This resistance was crossed with all beta lactams and its resistance profile to macrolides was constitutive (MLSB const) while aminoglycosides phenotype was ANT (4') (4'').
Conclusion: A high prevalence of *S. aureus* with multiple drug resistance was established. Improved food safety measures are thus necessary to prevent transmission and spread of antimicrobial resistance by these pathogens.

Keywords: Staphylococcus aureus; milk; antimicrobial resistance; MRSA.

1. INTRODUCTION

Together with *S. pseudointermedius* and *S. hyicus*, *S. aureus* is one of the three major pathogenic *Staphylococcus* species affecting animals [1].

An important impediment to the control of *S. aureus* infections is its tendency to gain resistance to almost all classes of antimicrobial agents which it is subjected to [2]. Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are a world health problem causing the gravity of the diseases which they can cause. Until recently, infections with MRSA were mainly confined to hospital environments [3].

The increasing prevalence of MRSA multi-drug resistant strains which limits the therapeutic options available for the management of MRSA associated infections has become a worrisome issue worldwide [4].

In cows, *S. aureus* plays a significant role as a major cause of mastitis [1] and most studies on MRSA in cattle deal with identification of MRSA from cases of mastitis.

In Algeria, occurrence of MRSA among isolated staphylococcus is increasing: 4.5% in 2002, 33.2% in 2004, and 45% in 2006 and 52% in 2011 [5,6].

Regarding MRSA infections at the community level, colonization and infections of domestic animals are of particular interest because of potential sharing with humans with regard to a mutual dissemination. There is increasing evidence of MRSA in food animals [7], creating concern about MRSA contamination of food. However, it remains unclear if the animals are the source of the contamination, or if it is the food handlers who are responsible for the contamination [8].

Transmission of MRSA by consumption of food products has not been investigated thoroughly [9], although *S. aureus* may be often detected in food and may be involved in food-borne diseases [10,11].

Therefore, the determination of susceptibility or resistance of strains to antibiotics is very important from a clinical and economic point of view. Moreover, the public health significance of this issue should be of concern because of the danger posed by antibiotics therapy of infectious diseases in animals. Sufficient and valid data are an indispensable component in the assessment of a possible health risk related to MRSA-contaminated food animals, especially meats and bovine milk. This paper reports the results on the occurrence of MRSA strains isolated from milk produced in Algeria.

2. METHODOLOGY

2.1 Study Area

This study was carried out in Tiaret area, Western Algeria, characterized by a semi-arid climate. Milk samples were collected from five farms located between 10 Km to 30 Km from the city. Herd size varied from 4 cows to 20 cows. Thirty eight (38) milk specimens from cattle were collected and immediately transported to the laboratory in a refrigerated box (4-8℃) and processed usually at the same day of collection.

2.2 Staphylococci Isolation

10 ml of each sample were transferred to flasks containing 90 mL of Peptone Water and then plated onto Baird Parker Agar (Merck, USA) with Egg Yolk Tellurite Emulsion (Pasteur Institute, Algiers) according to ISO 6888-1 [12]. The plates were incubated under aerobic conditions at 37℃ for 24-48 h. From each positive sample, 5 typical *S. aureus* colonies (black colonies surrounded by 2-5 mm clear zones) were transferred onto Mannitol Salt agar (Fluka, Spain) for further purification. Typical colonies-yellow colonies showing Mannitol fermentation were cultured in Brain Heart Infusion broth (Fluka, India) for 24 h at 37℃ and tested using standard microbiological procedures such as Gram staining, catalase and oxidase reactions, coagulase by test tube technique and TDNase. Strains were also streaked on blood agar plates to test hemolytic activity. Identification of *S. aureus* was confirmed with biochemical test API STAPH (bioMérieux, Marcy l'Etoile, France). After identification, strains were stored at -20℃ in Brain Heart Infusion Broth with glycerol (50% v/v).

2.3 Biotyping

Biotyping was carried out according to the simplified scheme [13] which uses four discriminative tests: The production of staphylokinase and β-haemolysin, the coagulation of bovine plasma within 6 h and the type of growth on crystal violet agar.

2.4 Antimicrobial Susceptibility

All *S. aureus* isolates were screened for methicillin-resistance using disc diffusion. This was performed on Mueller-Hinton agar plates (Fluka, India) as per the Clinical Laboratory Standards Institute (CLSI) guidelines [14] using 1 µg oxacillin and 30 µg cefoxitin discs (Bioanalyse, UK). A zone diameter ≤ 10mm for oxacillin and ≤ 21 mm for cefoxitin were classified as resistant. The MIC of oxacillin was determined by an agar dilution method in accordance with NCCLS recommendations [15] on Mueller-Hinton agar containing 4% NaCl and oxacillin at concentrations ranging from 0.016 to 16 µg/ml for *S. aureus.*

Furthermore, the antibiotic susceptibility pattern of *S. aureus* strains was determined by disc diffusion method for penicillin G (10 UI), gentamicin (10 µg), tobramycin (10 µg), kanamycin (30 µg), amikacin (30 µg), erythromycin (15 µg), spiramycin (10 µg), lincomycin (10 µg), pristinamycin (15 µg), vancomycin (30 µg), ofloxacin (5 µg), tetracyclin (30 µg), chloramphenicol (30 µg), fosfomycin (50 µg), fusidic acid (10 µg), bacitracin (8 µg), nalidixic acid (30 µg), noviobicin (30 µg) (Bioanalyse, UK). Chloramphenicol, erythromycin and tetracycline susceptibility was determined by the E-test according to the manufacturer's guidelines (Biomerieux, France).

The diameter of the zone of inhibition produced by each antibiotic disc was measured, recorded and the isolates were classified as "resistant", "intermediate" and "sensitive" based on the standard interpretative chart updated according to the current NCCLS standard [16].

Quality control was performed before every antibiogram on reference strains: A methicillin-susceptible *S. aureus* strain (ATCC 25923) and a MRSA strain (ATCC 4330). Multi-resistance was defined by resistance of the strain to at least three antibiotic agents [17].

2.5 Detection of β- lactamase Production

All isolates that showed resistance to penicillin were tested for β-lactamase activity by Clover Leaf Technique according to the method described by Parvathi and Appalaraju [18].

3. RESULTS

3.1 Prevalence of Methicillin Resistant *Staphylococcus aureus* Strain (MRSA)

A total of 21 (55.26%) *S. aureus* strains were isolated from the 38 milk samples. Of the 21 *S. aureus* isolates, one (4.76%) was found to be methicillin-resistant (MIC≥16 µg/ml). Therefore the overall MRSA prevalence was 1/38 (2.63%).

The single MRSA strain was also resistant to most antibiotics (spiramycin, lincomycin (constitutive type) also to bacitracin, oflaxacin, nalidixic acid). Specifically, this strain was shown to have the capacity to produce β lactamase to erythromycin with an MIC of > 256 µg/ml and tetracyclin with an MIC of 24 µg/ml. Our MRSA expresses two phenotypes of resistance to aminoglycosides involving two inactivating enzymes; *aph (3 ')-III*, which confers resistance to kanamycin and amikacin (phenotype *K*) and *ant (4') (4")* which confers resistance to

kanamycin, amikacin and tobramycin (phenotype *KT*).

The isolate was susceptible to vancomycin, noviobiocin, pristinamycin, fusidic acid and gentamycin. Chloramphenicol was also active with weak MIC (0.016 µg/ml).

3.2 Methicillin Sensitive *Staphylococcus aureus* (MSSA) Strains

Resistance to nalidixic acid was 100% followed by bacitracin (70%), spiramicin (65%), penicillin and Fosfomycin (45%). The 20 studied strains were sensitive to oxacillin.

4. DISCUSSION

The contamination rate of *S. aureus* (55.26%) was higher than that observed in the survey previously done in Italy which revealed total prevalence of 17% in milk and dairy products [19]. However, other studies report higher rates of contaminations: 66.7% in raw cow milk in Brazil [20]: 75% in bulk milk in Norway [21]; 71.8% in various food items in Portugal [22]. Then we can state animal-derived food act as reservoirs of MRSA and can transmit such pathogenic agent to human being [10].

S. aureus is very frequently isolated from cases of bovine mastitis all over the world [20]. In Algeria, we estimate this organism to be responsible for 40% of all bovine mastitis in the country [23].

In this study, of 21 *S. aureus* strains, only 1 (4.7%] was methicillin resistant (MRSA) which is higher to that stated by López [24] (3% in meat and milk) and Normanno et al. [3] (0.4% in milk and cheese).

However, in the present research, the ecological origins of the MRSA isolates were traced by using the simplified biotyping scheme of Devriese et al. [13]. The MRSA strain belonged to the bovine biovar. In an Italian survey of 1634 foodstuff samples 6 (0.4%) MRSA strains were isolated from bovine milk and cheese. This suggests that ruminants may act as reservoirs of MRSA strains [9].

4.1 Methicillin Resistant *Staphylococcus aureus* Strain *(*MRSA) Resistance

Gentamicin and vancomycin showed activity against MRSA strain, as reported in Spain [25].

However the monitoring of the sensibility of the MRSA towards glycopeptides remains compulsory cause emergence of resistant strains to vancomycin in several countries but also in Algerian hospital environment [4].

Table 1. Resistance of MSSA isolates (N=20)

Antibiotic disc	Abreviation	Resistant n (%)	Intermediate
Amikacin	An	0	
Bacitracin	B	14 (70 %)	
Cefoxitin	Fox	0	1
Chloramphenicol	C	0	
Erythromycin	E	3 (5%)	
Fosfomycin	FF	9 (45%)	
Fusidic acid	FA	1 (1%)	
Gentamicin	Gm	0	1
Kanamicin	K	0	1
Lincomycin	L	1	
Nalidixic Acid	NA	20 (100%)	
Noviobiocin	Nov	0	
Ofloxacin	Ofx	0	
Oxacillin	Ox	0	
Penicillin G	P	9 (45%)	
Pristinamycin	PT	0	
Spiramycin	SP	13 (65%)	
Tetracyclin	TE	1	
Tobramycin	Tm	0	
Vancomycin	VA	0	

Chloramphenicol, noviobiocin and pristinamycin exhibit a good activity on MRSA as reported in Algeria and Belgium with a sensitivity of 100% [26,27] and 99% in Morocco [28].

Resistance of MRSA strain to macrolides is of MLS_B phenotype which means also crossed resistance to macrolides, lincosamides and streptogramines B by ribosomic RNA 23S methylation. This phenotype also confers resistance to erythromycin, spiramycin and lincomycin. However, in Tunisia MRSA strains in sheep express MLSB inducible phenotype [29] while in Italia MRSA strains isolated from food of animal origin express sensible phenotype [3].

In hospital environment, the most part of MRSA isolated in Algeria and Morocco are MLS_B inducible [26,4] whereas in Belgium, Greece and Korea phenotype MLSB constitutive is dominant [30]. All these observations indicate that the incidence of both phenotypes of resistance MLS_B(c) and MLS_B (i) to *S. aureus* varies according to the geographical regions.

MRSA strain express 2résistance phenotypes to aminoglycosides involving 2 inactivating enzymes [31].Isolated strain is resistant to kanamycin-amikacin-tobramycin; phenotype KT due to production of enzyme ANT (4') (4'').

4.2 Resistance of Methicillin Sensitive *Staphylococcus aureus* (MSSA) Strains

As the isolates showed MIC values very low to chloramphenicol (MIC: 0.032 to 0.12 µg/ml), therefore, this molecule is suitable for the treatment of serious infections due to these bacteria. This encourages continuous monitoring of the sensitivity of *S. aureus* to chloramphenicol.

A high rate (70%) of resistance to bacitracin was detected among *S. aureus* strains (MSSA).This antibiotic is among the most widely used in both human medicine and additive. Although the European Commission has recently banned the use of bacitracin as a food additive [32], the appearance of high resistance in our samples can be attributed to the addition of this antibiotic to food.

MSSA isolated in this study show a resistance (45%) vis-a-vis of penicillin G. This result is lower than those reported on different foods [22,32] with rates of 53.8%, 46.4%, 70.0% respectively. While Rebiahi et al. [4] reported resistance of 87.2% among hospital strains in Algeria. This can be explained by the fact that the penicillin is one of the antibiotics most commonly used for the treatment of infections in humans and animals.

S. aureus resistance rate to tetracycline was 5% (MIC = 24 µg / ml) while in Portugal, Pereira et al. [22] reported a very low resistance rates (0.7%) among *S. aureus* isolated from different foodstuffs.

In Italy, Normanno et al. [19] showed that among 125 strains of *S. aureus* collected from meat and dairy products, 23.2% revealed resistance to tetracycline when in Brazil the resistance was (24.7%) for isolates collected from cheese and cow's raw milk [20].

Similar to our study, André et al. [20] in Brazil and Yesim Can and Haluk Çelik [33] in Turkey showed that all *S. aureus* strains isolated from samples of cheese and raw milk were sensitive to vancomycin and gentamicin while Pereira et al. [22] reported resistance to gentamicin and chloramphenicol 2% and 1.4% respectively.

The methicillin-sensitive strains (MSSA) have multiple resistances. The most common resistance pattern is noted for B.NA.FF/NA.FF (5 strains), followed by phenotype P.B.NA.E (3 strains).

5. CONCLUSION AND RECOMMENDATIONS

The present study highlighted that milk raw of animal origin contaminated with MRSA in Algeria may constitute a health hazard to consumers, in spite of the low frequency of isolation presently. An effective strategy for ensuring food safety aimed at preventing the emergence or spread of MRSA through contamination of raw foods is imperative. The need for improved hygiene practices during food processing, distribution and consumption of finished products cannot be over emphasized if this goal is to be achieved.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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