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RESEARCH ARTICLE

Phenotypic and Genotypic Characterization of *Staphylococcus aureus* Strains Associated with Bovine Mastitis and Nasal Carriage of Workers in Contact to Animals in Algeria

Madjid Akkou[1,2*], Kenza Antri[3], Mohamed-Azzedine Bachtarzi[4], Michèle Bes[5,6], Anne Tristan[5,6],Olivier Dauwalder[5,6], Rachid Kaidi[7], Hélène Meugnier[5,6], Mohamed Tazir[4], Jérôme Etienne[5,6], Frédéric Laurent[5,6] and Nadjia Ramdani-Bouguessa[4]

[1]Ecole Nationale Supérieure Vétérinaire, 16000, Alger, Algérie; [2]Institut des Sciences Vétérinaires, Université Blida1, 09000, Blida, Algérie; [3]Département de Biologie Cellulaire et Moléculaire, USTHB, 16000, Alger, Algérie; [4]Service de Microbiologie, CHU Mustapha-Bacha, 16000, Algérie; [5]Inserm U851, IFR 128, CNR des Staphylocoques, Université Lyon 1, 69008, Lyon, France; [6]Centre de biologie Est, Hospices Civils de Lyon, 69500 Bron, France; [7]Laboratoire des Biotechnologies de la Reproduction, Institut Vétérinaire Blida, 09000, Algérie
*Corresponding author: akkou.m@gmail.com

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ABSTRACT

Staphylococcus aureus is a major bovine mastitis pathogen responsible for heavy economic losses in dairy industry. Identification of epidemiological aspects associated with bovine mastitis may be helpful in treatment and management decisions. Due to high concern of zoonotic infections, we describe here, the distribution and antimicrobial susceptibility patterns of *S. aureus* from both cows with mastitis and nasal carriage of workers in contact to animals. Up to 38% workers were nasal carriers. Besides, *S. aureus* was isolated among 74% dairy herds suffering from mastitis, within 29.8% of mastitic quarter-milk samples. The isolates were tested for antimicrobial resistance, *gyr*, *mecA*, *mecC* and *agr* alleles. The gene *gyr* was detected in all *S. aureus* strains, 91 (77.7%) belonged to *agr* specificity group I, 11.9% affiliated to group II, 10 (8.5%) were *agr*III, and 1.7% human derived isolates to the group IV. *agr* I was dominant in both human and animal isolates with 60 and 91%, respectively. Four human isolates harbored *mec*A gene, while no *mec*A or m*ecC* genes were found in bovine derived isolates. Overall, 92% human isolates and 86.5% of cows' derived strains were resistant to penicillin G. The resistance against non beta-lactam antibiotics was considerably greater in human than cows' derived isolates, while different patterns of resistance share the same ecological niches. The high association of penicillin resistance to *S. aureus* in bovine mastitis highlights periodic surveillance of antibiotic resistance in livestock.

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INTRODUCTION

The evolution of antibiotic resistance in *Staphylococcus aureus* strains is a serious cause of concern either for human and veterinary medicine (Antri *et al*., 2011; Wang *et al.,* 2015; Cengiz *et al.,* 2015). *S. aureus* is a persistent resident of the human nose in 20% of the healthy population, and intermittently carried by another 30% (Wertheim *et al.,* 2005). In humans, infections due to methicillin-resistant *S. aureus* (MRSA) are associated with a two-fold higher mortality rate compared to methicillin-susceptible *S. aureus* (MSSA) (Cauda and Garau, 2009). In Algeria, the situation is no better in that, since the frequency of MRSA infections reached 42% in 2007 both in community and hospital settings (Antri *et al.,* 2011). The genetic determinant of methicillin resistance (the *mecA* gene) is located in the staphylococcal cassette chromosome *mec* (*SCCmec*). The *mecA* gene encodes a modified penicillin binding protein (PBP) known as PBP2a, with a low affinity for b-lactams (Kim *et al.,* 2012). In 2011, a new variant (mec_{LGA251}) with 70% identity to *mecA* was described in MRSA isolates (Garcia-Alvarez *et al.,* 2011). The *mecC* (mec_{ALG251}) gene encodes a PBP with 63% identity at the amino acid level with PBP2a, showing more affinity for oxacillin than for cefoxitin (Kim *et al.,* 2012; Mikail and Keskin, 2015).

The increasing evidence that MRSA can be transmitted in both directions, from human to animals and vice versa (Oppliger *et al.,* 2012; Ye *et al.,* 2015) is of special alarm, since *S. aureus* is responsible for about, one-third of clinical and subclinical mastitis in dairy cows (Botrel *et al.,* 2010). The accessory gene regulator (*agr*) was identified as a quo-rum sensing system in *S. aureus*. RNAIII is the major effector of *agr* system. It acts as a small RNA regulating the expression of many virulence factors, including most of those encoding cell-wall-associated and extra-cellular proteins (Thoendel *et al.,* 2010). The main reservoir of *S. aureus* is the infected quarter of udder, and transmission between cows usually occurs during milking process (Fox and Gay, 1993). Multi-resistant phenotypes and MRSA in intra-mammary dissemination have increasingly been described in cows and often produce incurable severe intra-herd infections (Feßler *et al.,* 2010). The present study aimed to describe the antimicrobial resistance patterns and molecular typing of *S. aureus* isolates involved in bovine mastitis and in carriage of workers in close contact to animals.

MATERIALS AND METHODS

Mastitis and milk sampling: Cows' udders were examined by visual inspection and palpation for the presence of any lesion, pain, heat and swelling. Milk from each quarter was withdrawn and checked for any change in color and consistency. California Mastitis Test (CMT) was carried out on lactating cows according to procedures described by Quinn *et al*. (1994) to determine subclinical mastitis. Moreover, milk samples were collected from mastitic quarters according to the National Mastitis Council (NMC, 1990).

Human nasal swabs: After being informed about the importance and scientific benefits of our investigation, cooperative workers provided written informed consent for sampling. Nasal swabs were taken from the two nostrils and transported in the Amies transport medium (Pasteur Institute of Algeria) to laboratory for further procedures. Professional's related data were kept strictly confidential and were served only to the purpose of this study.

Bacteriological examination of samples: From each tube, 0.01mL of milk was inoculated in parallel onto heart and brain broth (BHIB), selective *chromogenic* medium *S. aureus*-select TM (Oxoid, Paris, France), nutritive agar medium supplemented with 5% of blood sheep, and Baird-Parker medium supplemented with 5% of egg yolk and 0.5% tellurite of potassium. In case of a negative primary culture, plates were inoculated from the enriched BHIB. A quarter was considered positive when growth of 100cfu/mL was detected from a sample. Samples yielding over than 2 bacterial species were considered to be contaminated (NMC, 1990). Nasal swabs were enriched overnight at 37°C onto BHIB than inoculated on selective *chromogenic* medium *S. aureus*-select TM and nutritive agar medium supplemented with 5% of blood sheep respectively. All plates were examined after 48h of incubation at 37°C.

***S. aureus* isolation and antimicrobial susceptibility testing:** The suspected colonies were isolated and identified by catalase, *Staphaurex* (*Pastaurex Staph*+, Bio-rad, La-Coquette, France) and coagulase tests. Susceptibility to penicillin, oxacillin, cefoxitin, gentamicin, kanamycin, erythromycin, clindamycin, ofloxacin, teicoplanin, vancomycin, cotrimoxazole, tetracycline, rifampicin, fosfomycin, chloramphenicol and mupirocin for human isolates was determined by the disk diffusion method, as recommended by CLSI (2007). Comité de l'Antibiogramme de la Société Française de Microbiologie interpretative criteria were used for pristinamycin and fusidic acid SFM (2007). Cefoxitin-resistant and/or oxacillin-resistant phenotypes were supposed to be MRSA.

DNA extraction, *mec A, mec C* and *agr* groups typing: Genomic DNA was prepared using DNeasy tissue kit (Qiagen, Courtaboeuf, France), after enzymatic lysis with lysis buffer and lysis enhancer. *gyr* amplification using multiplex PCR with *gyr*-1 5'-AGTACATCGTCGTATAC TATATGG-3' and *gyr*-2 5'-ATCACGTAACAGTTCAA GTGTG-3' primers, was used to confirm the quality of each DNA extract and the absence of PCR inhibitors. The primers: *agr*1 5'GTCACAAGTACTATAAGCTGCGAT-3', *agr*2 5'-TATTACTAATTGAAAAGTGGCCATAG C-3', *agr*3 5'-GTAATGTAATAGCTTGTATAATAATA CC-CAG-3', *agr*4 5'-CGATAATGCCGTAATACCCG-3' and pan *agr* 5'-ATGCACATGGTGCACATGC-3' were used to determine *agr* alleles. In addition, methicillin resistance gene *mec*A and *mec*C were identified by PCR, using *mecA*-1 5'-AAAATCGATGGTAAAGGTTGGC-3, *mec*A-2 5'-AGTTCTGCAGTACCGGATTTGC-3 and $mec_{ALGA251}$ MultiFP 5′-GAAAAAAAGGCTTAGAACGCCT C-3′, $mec_{ALGA251}$ MultiRP 5′-GAAGATCTTTTCCGTT TTCAGC-3′ primers respectively as described elsewhere (Antri *et al.,* 2011; Garcia-Alvarez *et al.,* 2011). Based on the Array Tube platform (CLONDIAG, Jena, Germany), MRSA isolates were genotyped, using the StaphyType test (Alere, Technologies GnbH, Jena, Germany) following the manufacturer's instruction. Genotypes with *mecA* or *mecC* genes were considered MRSA.

Statistical analysis: Chi-square (χ^2) tests, with Yates' continuity correction when needed and nonparametric tests allowing independence analysis between random variables were used. $P<0.05$ was considered statistically significant.

RESULTS

Descriptive data

Cows: Among 31 Algerian mastitis-positive herds, 218 quarter-milk samples were collected from 14 cows with clinical mastitis and 102 cows with CMT-positive subclinical mastitis. *S. aureus* was isolated from 65 samples of 45 cows. One quarter was affected in 32 cows, two in 7 cows, three in 5 and four quarters in one cow. One to six strains were isolated from each *S. aureus*-positive herd. Growth of *S. aureus* was observed on 29.8% milk samples (41.1% clinical cases; 28% subclinical quarters). For two subclinical quarters, two distinct strains of *S. aureus* were found. Thus, 67 isolates were collected from 65 quarters of 45 cows in 23 different herds (Table 1).

Human: Only workers that were directly in contact with animals were included in the study, which resulted in the sampling of 129 persons. Forty-nine (38%) workers were

Table 1: Isolation and identification of *S. aureus* from human and bovine mastitis samples in Algeria

Characteristics	Humans		Animals	
	No.	%	No.	%
Identification				
No. subjects	129	100	218	100
Prevalence	49	37.98	65	29.81
No. of isolates	50	38.75	67	30.73
gyr typing	50	38.75	67	30.73

Table 2: Antimicrobial resistance frequencies of Human and bovine related *S. aureus* isolates.

Antimicrobial agents	Human isolates (n=50)		Animal isolates (n=67)		P value
	No.	%	No.	%	
Penicillin	46	92.0	58	86.5	NS
Oxacillin	4	8.0	0	0.0	
Cefoxitin	4	8.0	0	0.0	
Gentamycin	0	0.0	0	0.0	
Kanamycin	9	18.0	1	1.5	<0.05
Erythromycin	16	32.0	3	4.5	<0.05
Clindamycin	9	18.0	2	3.0	<0.05
Pristinamycin	0	0.0	0	0.0	
Ofloxacin	0	0.0	0	0.0	
Vancomycin	0	0.0	0	0.0	
Teicoplanin	0	0.0	0	0.0	
Chloramphenicol	0	0.0	0	0.0	
Cotrimoxazole	0	0.0	0	0.0	
Fosfomycin	0	0.0	0	0.0	
Tetracycline	21	42.0	10	14.9	<0.05
Fusidic acid	1	2.0	1	1.5	NS
Rifampicin	0	0.0	0	0.0	
Mupirocin	0	0.0	--	--	--

NS, not significant

nasal carriers for *S. aureus*. From these latter, 42 were male and 7 were female, with a mean age of 36.4 (19 to 74) years and, a mean professional activity in close contact to animals of 12.5 (1 to 50) years (Table 1).

Antibiotic susceptibility testing: Four human related strains were found methicillin-resistant (*mecA* positive strains), while the remaining isolates were *mecA* and *mecC* negative strains (Fig.1).

Penicillin resistance was consistently detected and in proportions that did not significantly differ between human (46/50, 92%; including 4 MRSA, P>0.05) and animals (58/67, 86.5%). Thirty-seven isolates (human 24/50, 48%; animal 13/67, 19.4%) were resistant to at least one of the non-β-lactam antibiotics tested; however significant differences (P<0.05) in this respect between species were noted. The differences (Table 2) were observed for resistance to tetracycline, erythromycin, clindamycin and kanamycin (the highest resistance was in human isolates).

Antibiotic susceptibility patterns: Antibiotic resistance determination revealed that 34/117 isolates (29%) were resistant to two or more antibiotics, but in proportion that significantly (P<0.05) differ between human (46%) and animals (16.4%). Of the fifteen characterized patterns of resistance, a high diversity of profiles was detected among the human than bovine derived isolates, while four patterns were isolated either from human and animal samples. All the human MRSA derived isolates showed a *mec* complex A, SCC*mec* IV and resistance to erythromycin, clindamycin and tetracycline. Also, of the 4 MRSA isolates, two were resistant to kanamycin. From the animal methicillin-susceptible isolates, at least two different patterns of resistance were recovered from 9 farms. Moreover multiple patterns were incriminated in 4 udders, while PEN and PEN, ERY, CLI, TET shared the same quarter. Except for the pattern PEN which was detected in 18/23 (78.2%) of infected farms; the others were revealed within a maximum of three herds (Table 3).

***agr* specificity groups:** The representative data of the PCR products for the identification of *agr* specific groups for representative *S. aureus* (Fig. 2). Analysis of *agrD* gene polymorphism showed that all 117 *S. aureus* strains could be assigned to one of the four major *agr* specificity groups. Ninety-one (77.7%) belonged to *agr* specificity group I, 11 (11.9%) belonged to group II, 10 (8.5%) to group III, and 2 (1.7%) human derived isolates to the group IV (Table 4). *agr* specificity group I was constantly detected in proportions that did not differ between human (30/50, 60%) and animals (61/67, 91%). Moreover, *agr* specificity group I was recovered from 22 of the 23 (95.6%) *S. aureus*-positive farms, and co-existed with different *agr* specificity groups in five farms. Different *agr* specificity groups were also recovered from one quarter (PEN, *agr* I and PEN, *agr* II), and from one human nasal swab (PEN, *agr* IV and PEN, ERY, CLI, KAN, TET, *agr* I). In addition, two different patterns of resistance PEN, and PEN, ERY, CLI, TET with *agr* specificity group I co-existed on the same quarter.

DISCUSSION

Staphylococcus aureus is an environmentally very robust bacterium surviving wide extremes of temperature and moisture; it is a major cause of intra-mammary infections in dairy cattle, and highly prevalent in farms with fewer than thirty cows (Kalmus *et al.*, 2011; He *et al.*, 2014; Javed *et al.*, 2015). In this report, *S. aureus* was isolated among 74% of herds (5 to 25 cows) suffering from mastitis. The apparent *S. aureus* herd prevalence reported herein is similar to those reported in a recently published Canadian study (Olde Riekerink *et al.*, 2010), but higher than those reported in the USA and in Mexico (Jayarao *et al.*, 2004; Miranda-Morales *et al.*, 2008). Overall, *S. aureus* is still considered one of the most common etiological agents associated with clinical and subclinical mastitis in lactating cows. It was blamed in 29.8% of bovine mastitis within higher proportion (41%) among the clinical cases in this investigation.

Several Algerian provincial studies showed high frequencies of *S. aureus* in bovine mastitis; it was responsible for 38.9% of bovine clinical and subclinical mastitis in Oran (Benhamed and Kihal, 2013). *Staphylococcus aureus* was also in charge for 30.3% and 40% of subclinical mastitis in eastern and centre provinces respectively (Saidi *et al.*, 2013; Boufaida-Asnoune *et al.*, 2012). However, comparison between results from different prevalence studies must be performed with caution since sample selection and bacterial cultivation techniques may differ.

From human colonization standpoint, prevalence and frequency of *S. aureus* isolation from workers in close contact to animals were relatively high (38% nasal carriers) and similar to the 36% rate reported in Switzerland on farm workers (Sakwinska *et al.*, 2011). Our prevalence however, was higher than that reported in western Algeria on healthy people of general population

Table 3: Antibiotic resistance profiles of *S. aureus* isolated from professionals and bovine mastitis

Resistance profile	*mecA*	*mecC*	Total isolates (n=117)	Human isolates (n=50)	Mastitis isolates (n=67)		
					No.	No. of farms	Farms
PEN, OXA, ERY, CLI, KAN, TET	+	-	2	2	0	0	--
PEN, OXA, ERY, CLI, TET	+	-	2	2	0	0	--
PEN, ERY, CLI, KAN, TET	-	-	3	3	0	0	--
PEN, ERY, CLI, TET	-	-	4	2	2	2	B8, D2
PEN, ERY, KAN, TET	-	-	3	3	0	0	--
PEN, ERY, FU	-	-	1	0	1	1	B2
PEN, ERY, TET	-	-	3	3	0	0	--
PEN, KAN, TET	-	-	1	1	0	0	--
PEN, FU	-	-	1	1	0	0	--
PEN, TET	-	-	12	4	8	5	A5, A11, A12, A16, C1
PEN, ERY	-	-	1	1	0	0	--
PEN	-	-	71	24	47	18	A2, A5, A9, A10, A11, A15, A16, A17, B1, B2, B3, B4, B5, B6, B8, C3, C4, C5
TET	-	-	2	2	0	0	--
KAN	-	-	1	0	1	1	A9
SENSIBLE	-	-	10	2	8	6	A8, A13, A15, B1, B3, B8

[a]PEN, penicillin ; OXA, oxacillin ; ERY, erythromycin; CLI, clindamycin; KAN, kanamycin; TET, tetracycline; FU, Fusidic acid; [b]A, Tizi-Ouzou; B, Boumerdès; C, Khenchela, D, Blida

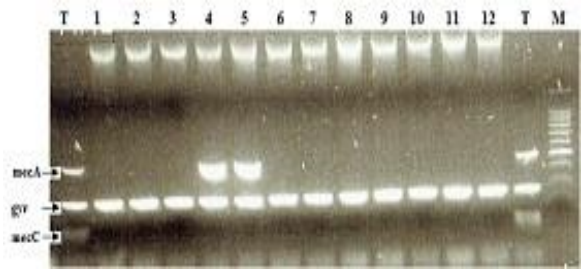

Fig. 1: Agarose gel electrophoresis of PCR triple amplification of *mecA, mecC* and *gyr* genes for professional derived *S. aureus.* Lane M: 100bp DNA ladder. Lane T: positive control with *mec*A (533 bp), *mec*C (138 bp), and *gyr* (280 bp).

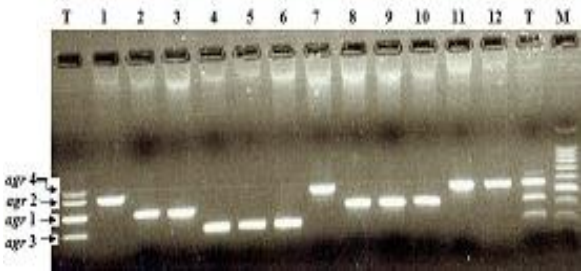

Fig. 2: Analysis of PCR products for the identification of *agr* specific groups from professionals derived *S. aureus*. Lane M: 100 bp DNA ladder. Lane T: positive control with *agr* I (440 bp), *agr* II (550 bp), *agr* III (300 bp), and *agr* IV (650 bp).

Table 4: Frequency of *agr* specificity groups among the *S. aureus* strains tested

agr specificity groups	No. of *agr* specificity groups (%)		No. of *agr* specificity groups (%)	
	Total	Human isolates	Animal isolates	No. Farms
I	91 (77.7)	30 (60)	61 (91.0)	22 (95.6)
II	14 (11.9)	09 (18)	05 (07.4)	05 (21.7)
III	10 (08.5)	09 (18)	01 (01.4)	01 (04.3)
IV	02 (01.7)	02 (04)	00 (00.0)	00 (00.0)
Total	117 (100)	50 (100)	67 (100)	23 (100)

(Ruimy *et al.,* 2009). Variations in these studies related to populations, geographic areas, sampling procedure and methodologies might explain the difference observed.

The levels of antimicrobial resistance among isolates from bovine mastitis, farmers, and veterinarians were relatively different. The only exception was resistance to first-line-treatment penicillin G, which reached 86.5% and 92% in bovine mastitis and workers colonization, respectively. This widespread resistance against penicillin G in Algeria can be attributed to the frequent and long term use of penicillin in therapeutics. The differences in prevalence of penicillin-resistant *S. aureus* in bovine mastitis observed between countries may be due to different management options to control *S. aureus* mastitis but possibly also by different use patterns of antimicrobials for mastitis treatment. Indeed, countries with a policy of prudent use of antibiotics in veterinary practices have shown lower levels of resistance compared to other countries (Kalmus *et al.,* 2011).

Recent reports from Belgium indicate that MRSA was involved in bovine nasal carriage within 19.8% farms, and responsible for Bovine mastitis in 10% of Belgium farms (Nemeghaire *et al.,* 2014). A study from China reported that 15.5% of bovine mastitis isolates were MRSA strains, and 38.2% MRSA strains had observed resistance to more than 8 classes of antibiotics (Wang *et al.,* 2015). In Algeria, increased rates of human MRSA infections were previously reported, both in hospital and community (Antri *et al.,* 2011). Occupational livestock contact showed 5.31 times increased risk of MRSA carriage using no contact as reference (Ye *et al.,* 2015). In this study, only four healthy individuals (3.1%) were colonized by MRSA strains while all mastitis derived isolates were MSSA. No discrepancy was found among data for phenotypic antimicrobial susceptibility test using disk diffusion method and PCR screening for *mec*A and *mec*C genes. Indeed all MRSA isolates showed expression of *mec*A gene; two strains were confirmed later as CC80, *SCCmec* IV, *agr* III, *PVL*+ and two others as CC22, *SCCmec* IV, *agr* I.

With the exception to tetracycline, resistance to other antimicrobials than penicillin G in *S. aureus* isolates was rare (<5%) in strains isolated from cows with mastitis and markedly ($P<0.05$) lower than human isolates (table 2). Resistances genes in *S. aureus* are often plasmid-encoded and disseminate through *S. aureus* populations by horizontal gene transfer mechanisms leading to strains that are more resistant (Werckenthin *et al.,* 2001). While, similar rates of acquisition and loss of *S. aureus* strains were shown in carriers and non carriers' individuals (Miller *et al.* 2014), frequent exposure to the flora of close contacts may lead to mixed-clone *S. aureus* colonization or infection. Since different antimicrobial patterns shared the same ecological niches, the relatively high resistance

to multiple antibiotics of human isolates might constitute a potential important step and risk towards the emergence and spread of more resistant strains in cows. It has been reported that *agr* autoinducer receptor specificity groups may influence host ecology by enhancing or inhibiting the ability of an *S. aureus* isolate to colonize in the presence of resident strains, including other staphylococci (Ji *et al.*, 1997). In this study, *agr* specificity group I was dominant both in human and animal isolates with 60% and 91%, respectively. From bovine mastitis standpoint, different proportions of *agr* specificity groups were reported in previous studies (Kumar *et al.*, 2011; Benhamed and Kihal, 2013). The widespread of *agr* specificity group type I in our study can most likely reflect the contagious aspect of *S. aureus* in bovine mastitis.

At the end of this study, one can assume that *S. aureus* is highly prevalent in bovine dairy herds suffering from mastitis, and in nasal carriage of workers with contact to animals in Algeria. High levels of resistance to penicillin G were noted both in human and mastitic derived isolates. Further concern was the detection of different patterns of resistance on same ecological niches, as well as methicillin resistant *S. aureus* in human.

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Author's contribution: MA, NRB, RK, FL, MB and AT conceived and designed the experiments. MA, KA MB, and HM executed the experiment and analyzed the samples. MA, HM, OD and MB analyzed the data. All authors involved in collecting data and discussing the contents of the manuscript and agreed to publication.

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