

Original Research Article

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Prevalence of *Staphylococcus aureus* and Antimicrobial Susceptibility Test in Milk and Cheese from Hadman Farm Dairies

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ABSTRACT

A study was undertaken in Alagoas, Brazil, to determine the prevalence, associated risk factors and antimicrobial susceptibility of *Staphylococcus aureus* in milk and cheese from artisan handman dairies. Alleged *S. aureus* isolates (n = 70) were identified out of 100 samples. Most isolates (66%) derived from raw milk and 34% came from cheese. Two hundred (50%) subclinical and 20 (40%) clinical cases out of 250 cross-breed lactating dairy cows examined for mastitis with CMT screening test and clinical examination, were found positive. Current study shows that the prevalence of *Staphylococcus aureus* was significantly associated with mastitis (p<0.05) and revealed that *S. aureus* has 0% susceptibility to penicillin G, followed by tetracycline (15%). However, these randomly selected isolates were found to be totally (100%) susceptible to kanamycin. There should be a regular antimicrobial sensitivity testing, not only in the area under analysis, but also at a national level to select effective antibiotics against *S. aureus* mastitis. Proper prevention and regular antimicrobial sensitivity testing help select effective antibiotics and ultimately reduce the development of resistance towards commonly used antibiotics. Results demonstrate that mastitis caused by *S. aureus* is one of the major issues in milk production and imposes public health hazard in the area under analysis. Every possible control and prevention strategies should be implemented.

Keywords

Staphylococcus aureus,
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Introduction

Around the world, the quality of milk for human consumption has been worrying researchers and authorities, due to its nutritional, economic-social and public health importance, since the use of microbiological quality can lead to and risks to consumer

health. However, most dairy farms in Brazil and in developing countries technology, deficiency in animal health control and hygiene, generating a known product of low quality. Milk is naturally a nutrient-rich culture medium, which makes it susceptible to attack by a large number of microorganisms in the environment, of the animal itself, of the

man and of the utensils used (Barros *et al.*, 2011a,b; Hennekinne and Loir, 2014; Rajic-Savic *et al.*, 2015; Sandes *et al.*, 2016; Santos *et al.*, 2016; Mehli *et al.*, 2017). In fact, in Brazil, the microbiological quality of raw milk produced in several regions of the country is poor, with high concentrations of microorganisms that indicate hygienic-sanitary conditions such as mesophiles and thermotolerant coliforms and *Staphylococcus aureus*. Food of animal origin, especially milk and milk products, associated with outbreaks of food poisoning, public health problem and *S. aureus*, since it is the main etiological agent of bovine mastitis, turns raw milk into a large vehicle of these outbreaks (Peles *et al.*, 2007; Barros *et al.*, 2011a,b; Hennekinne and Loir, 2014; Rajic-Savic *et al.*, 2015; Sandes *et al.*, 2016; Santos *et al.*, 2016).

In staphylococcal food poisoning, symptoms occur between 2 and 4 hours after the ingestion of the preformed toxin in the food and the most common are vomiting, nausea, abdominal pain and diarrhea. The minimum amount of enterotoxin necessary to cause the disease is not known, but the ingestion of, at least less, 1 µg of toxin in 100g of food already induces the appearance of clinical symptoms and this level of toxin is reached when the population of *S. aureus* exceeds 10⁵ per gram and the dose minimum of enterotoxins ingested for the occurrence of these symptoms is lower, of 10 ng (Peles *et al.*, 2007; Even *et al.*, 2010; Barros *et al.*, 2011a,b; Hennekinne and Loir, 2014; Sandes *et al.*, 2016; Santos *et al.*, 2016; Mehli *et al.*, 2017).

Several studies have been undertaken on the quality of bovine bulk milk and on the prevalence of pathogens in cheese, especially in raw milk cheese. Current study presents a holistic approach by sampling CPS and *S. aureus* at different stages in the cheese-manufacturing process and by assessing

results and consequences through (i) the quantification of CPS contamination, (ii) its associated risk factors in the study area and (iii) the assessment of the possible proportion of antibiotic resistance in the *S. aureus* population. The possible risk of *S. aureus* from products derived from artisan cheese farm dairies is discussed.

Materials and Methods

Sampling and California Mastitis Test (CMT)

Current study was performed in the state of Alagoas, northeastern Brazil. Samples were obtained from dairies in the towns of Maceió, Coruripe, Capela, Chã Preta and Quebrangulo, between October 2016 and May 2017. Ten farm dairies producing cheese from bovine milk in the region of Alagoas participated in the project. The farm dairies were asked to provide the following samples: raw milk and cheese milk (pasteurized, if applicable), and further information on the farm, the dairy and production, by filling a questionnaire. Supplementary information was collected through the respective county administrations. The producers were supplied with all the necessary equipments and instructions for sampling. The milk and cheese milk samples were packed in insulated polystyrene boxes with refrigerant gel packs and returned within two days. The box temperature was registered on arrival. Temperature of samples varied between 0°C and 4°C, average 1.6°C and stored at 4°C until analysis the following day. Samples were transported in thermal boxes to the Laboratory of Food Inspection of the Universidade Federal Rural de Pernambuco and analyzed within 24 h.

Each selected lactating cow was screened for mastitis by clinical examinations and California Mastitis Test. Clinical examination of the udder was based on a previously

indicated method. Clinical findings include abnormalities of secretion, abnormalities of udder and teat, and systemic reaction. CMT was performed according to previously established method (Seyouma *et al.*, 2018): approximately 2 ml of milk from each quarter of the udder were placed in each of four shallow cups in the CMT paddle and an equal amount of the reagent was added. A gentle circular motion was applied, at a horizontal plane, for 15 s. Result was based on gel formation, or rather, negative, if no gel was formed, and positive, if there was gel formation in at least one quarter. Gel formation ranged between Trace (T) and +3. Hence, a cow was considered positive for mastitis if one or more quarters were CMT positive, regardless of the microorganisms' isolation.

Quantification of CPS in milk; isolation and identification of *S. aureus* (Mehli *et al.*, 2017)

Milk samples were mixed gently by inverting the sample tubes; ten-fold dilutions of the milk were made with sterile peptone water (1.0g bacteriological peptone and 8.5 g L⁻¹ sodium chloride). Cheese samples were prepared by homogenizing 25 g of cheese and 225 mL of sterile peptone water for 1 min in Stomacher bags (Stomacher® 400 Classic Standard Bag, Seward Limited, Worthing UK). Additional ten-fold dilutions were prepared with sterile peptone water. The appropriate dilutions were spread on Bairde Parker Agar (BP) (CM0275, Oxoid, Basingstoke, UK) and egg yolk tellurite emulsion (SR0047C, Oxoid) was added for the quantification of CPS, following NMKL method 66. The plates were incubated at 37°C for 48 h. Up to five typical colonies from each sample were isolated: black or grey, shining and convex with diameter 1.0 - 1.5 mm after 24 h, and often surrounded by a clear zone. They were further propagated to pure cultures on Brain Heart Infusion Agar (BHIA)

(CM1136, Oxoid) and preserved in Brain Heart Infusion (BHI) (CM1135, Oxoid) containing 20% glycerol bi-distilled 99.5% (VWR chemicals, Leuven, Belgium) at 80°C. All isolates were gram-stained with Gram's Iodine (PL7003, Pro-lab Diagnostics, Bromborough, Wirral, UK) and tested for catalase activity and coagulase production. Further, coagulase positive staphylococcal isolates were streaked on p-agar, supplemented with 7 mg L⁻¹ acriflavine (A8251, Sigma & Aldrich, St. Louis, MO, USA). Positive reaction indicated growth at full length of the streak. Isolates with positive reactions described above were considered to be *S. aureus*.

Testing for antimicrobial susceptibility

Antibiotic susceptibility tests were carried out on selected *S. aureus* isolates and their susceptibility profile was determined with Kirby-Baur disc diffusion method on Mueller-Hinton Agar, following procedures described by (Aqib *et al.*, 2017). Selected isolates from nutrient agar were first cultured in nutrient broth overnight. A suspension of each test isolate was compared to a turbidity equivalent to 0.5 McFarland standard, while each suspension was streaked onto Mueller Hinton Agar (Oxoid, UK) by sterile swab. Oxoid antimicrobial susceptibility disks (Oxoid, Basing Stoke, UK) with their corresponding concentration, Erythromycin (15 µg), Vancomycin (30 µg), Chloramphenicol (30 µg), Tetracycline (30 µg), Kanamycin (30 µg), Sulphamethoxazole (25 µg) and Penicillin (10 units) were positioned on the plates, with a sterile forceps. Inoculated plates were incubated at 37°C for 24 hr. The inhibition zone was then recorded as the diameter of the zone surrounding the individual disc in which bacterial growth was absent. Isolates were thus defined as resistant, intermediate and susceptible, according to the guide lines of XXXXX.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to compare the mean levels of CPS in different groups of milk samples, followed by Tukey's HSD (honest significant difference) test, on log-transformed data with statistical software SPSS Statistics 22 (IBM Corp., Chicago, IL, USA). A significance level of $p \leq 0.05$ was used, and a bacterial count equal to zero was scored as 1 cfu g^{-1} or mL^{-1} .

Results and Discussion

The farm dairies and prevalence of mastitis

Five of the ten invited farm dairies participated in the study. These farms were in the state of Alagoas, northeastern Brazil. Samples were obtained from dairies in the towns of Maceió, Coruripe, Capela, Chã Preta and Quebrangulo, a region of untouched nature, interspersed with small villages and a couple of cities.

Farms A and E (Table 1) are located less than 15 km from each other, whereas Farms B and D are distant more than 30 km from each other. Farm C is more than 40 km from the others. Participating farms provided the same treatment to milk before cheese manufacturing and types of cheese produced. All cheese milk from the farm dairies was pasteurized (Table 1).

Two hundred (50%) subclinical and 20 (40%) clinical cases out of 250 cross-breed lactating dairy cows examined for mastitis by CMT screening test and clinical examination, were positive (Table 2).

CPS in milk and cheese

S. aureus isolates ($n = 70$) were identified from 100 samples. Most isolates derived from

raw milk (66%), while 34% were from cheese. Mean CPS level in raw milk from different farm dairies varied between $4.5 \text{ log cfu mL}^{-1}$ and $6.2 \text{ log cfu mL}^{-1}$ (Table 2). CPS occurred in 22% (A), 82% (B), 37% (C), 50% (D) and 53% (E) of samples. The prevalence of CPS in cheese milk samples from non-pasteurized cheese production was 100%, with mean $1.5 \text{ log cfu mL}^{-1}$ and $3.3 \text{ log cfu mg}^{-1}$ (Table 3). Mean CPS level in cheese made from non-pasteurized milk was not significantly lower than that in raw milk. All isolates were susceptible to the antibiotics included in this study, except penicillin G. Further, *S. aureus* were found to be highly susceptible and resistant to kanamycin (100%) and penicillin G (100%), respectively, as Table 4 shows.

Current analysis revealed (Table 1 and 2) that all raw milk samples contained CPS, with the levels ranging between 4.5 and $6.2 \text{ log cfu mL}^{-1}$, corroborating most studies on CPS in raw milk conducted during the last decade (D'Amico and Donnelly, 2010; Barros *et al.*, 2011a,b; Jakobsen *et al.*, 2011; Walcher *et al.*, 2014; Sandes *et al.*, 2016; Santos *et al.*, 2016; Mehli *et al.*, 2017), with a prevalence ranging between 47.2% and 94.3%.

In corroboration to observations by others researchers (Rosengren *et al.*, 2010; Barros *et al.*, 2011a,b; Sandes *et al.*, 2016; Santos *et al.*, 2016; Mehli *et al.*, 2017) the quality of milk common to human consumption has always been a search of researchers and competent authorities for their nutritional importance, social health and public health, since milk with poor microbiological quality can lead to economic losses and risks to consumer health. In Brazil, a larger dairy farm, low technology, lack of control hygiene, generating a product of low quality. Its rich in nutrients, the size is susceptible to attack by a large number of micro-organisms of the environment, of the animal itself, of man and utensils and equipment used in milking.

Table.1 Information on production retrieved from the questionnaire answered by the participating farm dairies

Farm dairy	A	B	C	D	E
Pasteurized product	No	No	No	No	No
Type of cheese	Soft	Soft	Soft	Soft	Soft
Cheese production (kg year ⁻¹)	200	250	250	330	240
Supplementary milk from other farms	Yes	Yes	Yes	Yes	No
Exchange of ingredients with other producers	Yes	Yes	Yes	Yes	No
Water service provision	No	No	No	No	No
Frequency of mastites	Constantly	Constantly	Constantly	Constantly	Constantly

^a Constantly, ≥ 2 per annum.

Table.2 Prevalence of *Staphylococcus aureus* in sub-clinical and clinical mastitis cases

Type of mastitis	Number of examined animals	Number of isolated <i>S. aureus</i>	Prevalence
Subclinical	200	100	50%
Clinical	50	20	40%
TOTAL	250	120	48%

Table.3 Coagulase positive staphylococci (CPS) in milk, whey and cheese samples from five different farm dairies ^a

Milk treatment	Type of cheese	Farm dairy	Coagulase positive staphylococci (log cfu mL ⁻¹)	
			Raw milk	Cheese Milk
Non-pasteurized	Soft	A	4.5	1.5
	Soft	B	6.2	3.3
	Soft	C	4.8	4.0
	Soft	D	5.5	3.0
	Soft	E	5.3	2.5

^a Data are mean standard deviation (n = 3, in each group). Estimates of CPS per mL milk or per g cheese are pre-formed using log-transformed data, and counts equal to zero were scored as 1 CFU mL⁻¹ or g⁻¹.

Table.4 Summary of antimicrobial susceptibility test

Antimicrobials used plus concentration	Susceptibility		
	Susceptible %	Intermediate %	Resistant %
Erythromycin 15 µg	90	10	0
Vancomycin 30 µg	92.9%	7.1%	0
Chloramphenicol 30 µg	91	9	0
Tetracycline 30 µg	15	36	50
Kanamycin 30 µg	100	0	0
Sulphamethoxazole 25 µg	85	15	0
Penicillin G 10 units	0	0	100

It should be underscored that, at an initial concentration of $3.0 \log \text{ cfu mL}^{-1}$, as reported in current study (Table 1 and 2) and according to Duquenne *et al.*, (2016), growth of *S. aureus* may reach above $5 \log \text{ cfu mL}^{-1}$ during the first 6 h of milk renneting. The levels of *S. aureus* during cheese-making on Farm B may reach levels associated with enterotoxin production, with optimal growth conditions. In fact, generation time for *S. aureus* in milk is reported to be 0.8 h at 25°C (Le Marc *et al.*, 2009).

Current study reveals the prevalence of *Staphylococcus aureus* significantly associated with mastitis ($p < 0.05$). The high frequency of *S. aureus* in cases of mastitis can be explained by the fact that the udder skin is one of the main localization factors of these agents, which facilitates the infection by this microorganism (Rahman *et al.*, 2009; Michelle, 2011; Fitsum, 2016; Seyouma *et al.*, 2018). In addition to this, the higher the parity, or the higher number of times the cow has calved, is associated with lower chance of cure rates, this is due to a much larger mammary gland developed and therefore a much larger volume of tissue or an antibiotic is unable to penetrate and clear of infection (Michelle, 2011).

The literature points out that poor management practices increase susceptibility to mastitis by *Staphylococcus aureus*. In fact, it can be identified by post-partum somatic cell count (SCC), indicating that the animal was exposed to poor environmental condition, or there was inefficiency in the treatment of infections in the dry period. The way the animal is milked is a factor that can significantly predispose to mastitis, if this occurs manually, the milker should have many hygienic care, such as hand washing, cleaning the ceilings, among others; and if milking is performed mechanically the equipment must be strictly septic. Other

aspects are predisposing to mastitis such as: pathogens can be easily transferred from one animal to another if care is not taken in the cleaning (washing and drying) of the ceilings and the hands of the milker or the milking machine; diffusion of the microorganisms between the mammary quarters, through reverse movements of the milk provided by the fluctuations in the vacuum system, besides being able to cause traumas in the teats and the process not to be fulfilled, leaving traces of milk in the teats and with it providing a means of growth bacterial; the roof channel itself if it is not well contracted may lead to the exposure of pathogens (Radostitis *et al.*, 2007; Seyouma *et al.*, 2018)

The antimicrobial susceptibility tests carried out in current analysis revealed low susceptibility of *S. aureus* to the usual antimicrobial agents. The average susceptibility (67.7%) of *S. aureus* to all antimicrobial agents tested in this study corroborated data by Mesele *et al.*, (2010) and Seyouma *et al.*, (2018), but disagreed with other reports (Mekonnen *et al.*, 2005; Amanu *et al.*, 2016). According to Seyouma *et al.*, (2018), divergence among different reports may be explained by difference in resistance level, pathogen strain, antimicrobial used and concentration, plus other related factors that affect the inhibition zone during antimicrobial susceptibility test.

Current study demonstrated zero susceptibility of *S. aureus* to penicillin G, as Seyouma *et al.*, (2018) reported in their study. This fact corroborates findings which reported 0% susceptibility of *S. aureus* to penicillin in Africa (Amanu *et al.*, 2016 and Asmelash *et al.*, 2016), but disagrees with results by Abera *et al.*, (2013). Further, current study has also indicated low susceptibility of *S. aureus* to tetracycline, confirmed by different reports (Abebe *et al.*, 2013; Asmelash *et al.*, 2016; Seyouma *et al.*, 2018). Mean susceptibility of

S. aureus to the other antibiotics in current study reached 92%, corroborating other studies (Amanu *et al.*, 2016; Seyouma *et al.*, 2018).

As a rule, current study demonstrated that susceptibility of *S. aureus* to the usual antimicrobials, such as penicillin G and tetracycline, was very low. A possible justification for this fact would be the development of alarming resistance levels of *S. aureus* due to the regular use of common antibiotics for the treatment of cows. This fact may result in the spread of resistant strains in the area under analysis. Results corroborated reports from earlier studies in other countries (Edward *et al.*, 2002; Jaims *et al.*, 2008; Seyouma *et al.*, 2018) and suggested a possible development of resistance to the prolonged and indiscriminate usage of some antimicrobial agents.

In this case, penicillin G is a great concern since it represents the main antibiotic groups recommended against *Staphylococcal* mastitis infection. Antibiotic resistance is carried on plasmids and transposons that can pass from one *Staphylococcal* species to another (Hulya *et al.*, 2006; Seyouma *et al.*, 2018). The resistance of *S. aureus* to penicillin may be attributed to the production of beta lactamase enzyme that hydrolyzes the beta-lactam ring of penicillin and inactivates it and other closely related antibiotics. It is believed that around 50% of mastitis causing *S. aureus* strains produces beta-lactamase (Green and Bradely, 2004; Seyouma *et al.*, 2018). The low susceptibility of *S. aureus* isolates to this drug is evident.

Besides being a highly relevant human symptom, *S. aureus* is one of the major pathological agents of food poisoning. Its presence in milk and derivatives, such as cheese, the sample unit of current study, elucidated the use of raw material of infected animals (mastitis) or the possibility of contamination by handling agents. Farm dairies are located in close proximity to farms and the habitat of wild animals. Sampling the

production stages from raw milk to cheese contributes towards our understanding of how *S. aureus* contamination pathways occur, and thus enable targeting control measures throughout the cheese-manufacturing process. In addition, based on what has been reported, these findings are extremely worrisome, especially because rates are very close to those required (10^5 CFU g mL⁻¹ - 10^9 CFU g mL⁻¹) for enterotoxigenic strains for the production of enterotoxins in sufficient quantities for the occurrence of outbreaks of staphylococcal food poisoning.

The overall prevalence of *Staphylococcus aureus* in the population in the area under analysis was 48%, respectively 50% and 40% in sub-clinical and clinical cases. Susceptibility of randomly selected isolates were also tested for the usual antimicrobial agents.

However, isolates have very low susceptibility, or rather, 0% to penicillin G; 15% to tetracycline; good susceptibility for other antibiotics. Kanamycin proved to be the most effective drug. In addition to the susceptibility test, results revealed that *S. aureus* was the major problem, causing economic loss in dairy production, with public health hazard in area studied. There should be a regular antimicrobial sensitivity testing, not only in study area but, also at a national level, to select effective antibiotics against *S. aureus* mastitis. Dry cow therapy should be implemented for cows in the dry period. It is actually the most effective method of eliminating existing infections and preventing new infections during the period. Veterinarians should reduce uncontrolled and repeated use of drugs to minimize the development of drug resistance and food safety threat. Kanamycin should be encouraged to treat mastitis caused by *S. aureus*-infected cows in the area analyzed.

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