



Full Length Research Paper

Bacterial mastitis in the Azawak zebu breed at the Sahelian experimental station in Toukounous (Niger): Identification and typing of *Staphylococcus aureus*

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Abstract

Though the government of Niger opted in 1975 for a policy of improving the dairy capability of the Azawak zebu cows, the national level of dairy production satisfies only 50% of population needs. Several reasons may explain this shortage, including the prevalence of mastitis. The purpose of this research was (i) to study the prevalence of mastitis at the Sahelian experimental station in Toukounous; (ii) to identify the bacterial species responsible; and (iii) to type the *Staphylococcus* (*S.*) *aureus* isolates. Two hundred and sixty-five cows were tested using the California Mastitis Test (CMT) and the 112 CMT-positive milk samples (10.6%) from 104 cows (39.2%) were further tested for bacterial growth. Fifty-one of them (45.5%) gave a positive growth: half of the isolated bacteria belonged to the genus *Staphylococcus* and 23 (41.8%), to the species *S. aureus*. The majority of the *S. aureus* isolates belonged to the bovine biotype, formed biofilms, produced Small Colony Variants, grouped into closely related virulotypes, and belonged to two closely related pulsotypes. In conclusion, (i) the prevalence of presumptive mastitis in Toukounous is 40%; (ii) the genus *Staphylococcus* is the most frequent; and (iii) the 23 *S. aureus* isolates are closely related, though not clonal.

Keywords: Niger, Azawak zebu, mastitis, *Staphylococcus aureus*, virulotyping.

INTRODUCTION

In Niger, zebu cattle of the Azawak breed offer the best dairy aptitude with a level of production that varies between 800 and 3000 kg of milk for a lactation period of between 270 and 300 days. Moreover, since 1975, the Niger government has opted for a policy of developing the breeding of the Azawak zebu and of improving its dairy capability (MRA, 2002). Various pilot farms have been set up, such as the Sahelian experimental station in Toukounous (Figure 1), with the objective of carrying out the genetic selection of the Azawak zebu breed and of

distributing these cows to individual farmers across the country.

However, despite the existence of such experimental farms and of almost 3.5 million heads of Azawak zebu cows in 2000, national dairy production in Niger satisfies only 50% of population needs (REPOL, 2001; Vias et al., 2005). Several reasons may explain this shortage, including the genetic background of the Azawak zebu compared to European and American dairy cattle breeds and the infection rates of the mammary gland.

Infection of the mammary gland by microorganisms, generally bacteria, causes inflammation and mastitis. Clinical mastitis is characterized by macroscopic modification of the milk, whereas only high cell counts with no macroscopic change are observed during sub-clinical mastitis. Mastitis can also be subdivided as either contagious or environmental, according to the identity of the bacterial species: contagious mastitis may be caused by bacterial species such as *Staphylococcus aureus*, *Streptococcus agalactiae*, coagulase-negative staphylococci, *Corynebacterium bovis*, *Mycoplasma bovis*, and environmental mastitis by *Escherichia coli*, *Streptococcus uberis*, *Streptococcus dysgalactiae* (Radostis et al., 2007; Quinn et al., 2011).

Mastitis prevalence and mastitis-associated bacterial species can nevertheless vary from country to country and also between farms, mainly as the consequence of differences in livestock management and milking process hygiene (Olde et al., 2008). In Niger, the prevalence of mastitis in the urban and peri-urban livestock of Niamey and of Hamdallaye (Figure 1) is 44% and 42% respectively, and *S. aureus* is the most frequently identified bacterial species (Bada-Alambéji et al., 2005; Harouna et al., 2009). But no survey has ever been performed at the pilot farms, such as the one in Toukounous.

Therefore, the purpose of this study was (i) to estimate the prevalence of mastitis in Azawak zebu cows at the Sahelian experimental station in Toukounous; (ii) to identify the bacterial species responsible; and (iii) to type the *Staphylococcus (S.) aureus* isolates phenotypically and genetically.

MATERIALS AND METHODS

The station in Toukounous

The Sahelian experimental station in Toukounous was created in 1936 by the colonial countries in order to produce meat for exportation to Europe. It was transformed in 1975 by the Niger government into a pilot farm to improve the dairy production of the Azawak zebu breed and to promote its distribution across the whole country (MRA, 2002). The Toukounous station is located 220 km North East of Niamey (Figure 1) and covers a surface area of 4474 hectares divided into several parcels allowing a rational use of pastures (Saidou et al., 2010). The climate is of the Sahelian type, characterized by a rainy season from June to October, with an average of 300 mm of rain per year, and a dry season from November to May.

At the Toukounous station, Azawak zebu cows are divided into three herds according to their level of milk production and age: "elite" cows (>1400 kg milk/lactation), "non-elite" cows (<1400 kg milk/lactation), and "primiparous" cows. Feeding is based on pasture

grazing, which is complemented by the distribution of cottonseed during the dry season. The animals drink water *ad libitum* during the course of the day. The milking process is performed manually twice daily with no general or specific disinfection procedure.

Bacterial milk analyses

Milk samples were collected during the rainy season (September 2009) from the four quarters of 265 lactating cows (120 elite, 74 non-elite, and 71 primiparous) and were submitted to the Californian Mastitis Test (CMT): a quarter was considered positive when the score was (++) , (+++) , or (++++), negative when the score was (-), and doubtful when the score was (+). Only CMT-positive quarters were sampled a second time for bacteriological analysis. Milk samples were stored at -20 °C in 10% (v/v) glycerol until further use.

Bacteriological analysis was performed at the Laboratory of Bacteriology in the National Public Health School of Niamey. After thawing, a loopful of each milk sample was inoculated onto Columbia base agar plates supplemented with 5% sheep blood (BioMérieux, France). Plates were incubated at 37 °C for 24 h to 48 h. Bacterial species were pre-identified on the basis of colony shape, presence of haemolysis, Gram staining, bacterial cell shape, and catalase and peroxidase production.

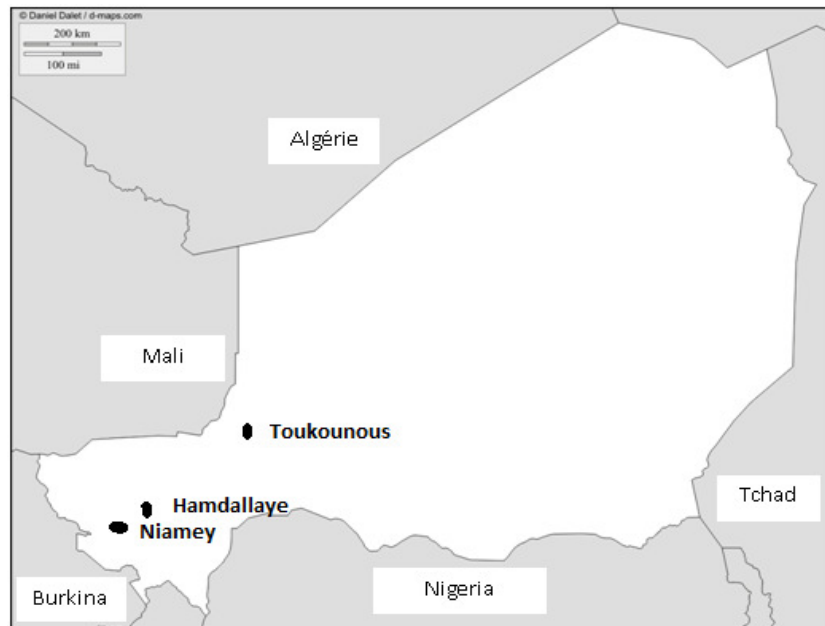
Pre-identified colonies were further identified at the Laboratory of Bacteriology in the Veterinary Faculty of the University of Liège (import permit n° CONT/IEC/FRT/376095). First, the colonies were grown on Columbia blood agar plates and on different selective agar plates: Mannitol salt phenol red agar (Merck, Germany) for staphylococci, Edwards medium modified (Oxoid, England) for streptococci, D-Coccosel agar (BioMérieux, France) for enterococci, and Gassner agar (Merck, Germany) for enterobacteria. Final identification was performed using appropriate API tests (BioMérieux, France), except for bacilli, which were identified as described by Reva and collaborators (Reva et al., 2001). All *Staphylococcus (S.) aureus* isolates were further identified and typed phenotypically and genetically.

S. aureus phenotyping

S. aureus isolates were classified into bovine, human, or poultry biotypes according to the results of the following tests (Devriese, 1984): the production of staphylokinase and of β -haemolysis on Columbia blood agar, the coagulation of bovine plasma and growth on Trypticase Soy Agar plates (Merck, Germany) supplemented with 6 μ g/ml crystal violet (Acros Organics, Belgium).

Biofilm production was assayed in microtitre plates using the safranin staining method (Stepanovic et al.,

Figure 1. Map of Niger showing the locations of the Niamey and Hamdallaye areas and of the Sahelian experimental station in Toukounous



2007). Isolates from frozen stocks were grown in Trypticase Soy Broth (TSB) (Merck, Germany) and incubated at 37 °C under shaking. Overnight cultures were then diluted 1:100 in TSB containing 0.25% glucose (TSB_{glc}) and were transferred into wells of sterile 96-well flat-bottom tissue culture (TC) plates. Non-inoculated TSB_{glc} served as the negative control. The TC plates were subsequently incubated at 37 °C for 24 h. The supernatant was then discarded and the wells were carefully washed twice with sterile Phosphate Buffered Sodium (PBS). The plates were dried, stained with safranin 0.1% (w/v) for 10 min, washed twice with distilled water and dried again at 37 °C. A mixture of 50% ethanol – 50% acetic acid was added to each well and the plates were incubated at room temperature for 15 min. The quantitative classification of biofilm production was based on optical density (OD) values at 490 nm using a microplate reader (Bio-Rad, Belgium). The results were collected from at least two independent experiments in which the biofilm formation of each culture tested was evaluated in triplicate. Isolates were classified into non-producers, weak producers, moderate producers, or strong producers.

In order to study the formation of Small Colony Variants (SCVs), *S. aureus* isolates were first inoculated into Brain Heart Infusion (BHI) broth (Merck, Germany) and incubated at 37 °C overnight. The OD value of each broth culture was determined at 600 nm so that the bacterial concentration could be adjusted to a value of 10⁹ Colony Forming Unit/ml (CFU/ml). Following this, 5.10⁵ CFU/ml were transferred into BHI broth containing

1 µg/ml of gentamicin, and were incubated at 37 °C overnight on a rotary shaker (200 rpm). Each isolate was then plated on Columbia blood agar containing 1 µg/ml of gentamicin and was incubated at 37 °C for 48 h. The production of SCVs was then interpreted as previously described (Atalla et al., 2008).

***S. aureus* virulotyping**

S. aureus isolates were therefore virulotyped by PCR for the presence of genes coding for Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) to colonize the host epithelia and extra-cellular matrix components (*clfA*, *clfB*, *fnbA*, *cna*, *ebpS* and *sdrC*) (Dinges et al., 2000), for capsular and other surface antigens conferring resistance to phagocytosis (*cap5H*, *cap8H*, *spa* and *icaA*) (Hermans et al., 2010; Vasudevan et al., 2003), for exfoliative toxins active on extra-cellular matrix components (*etA*, *etB* and *etD*) (Nishifuji et al., 2008), for haemolysins and leukocidins counteracting the cells of the host's innate immune response (*hla*, *hly*, *hld*, *hlgAC*, *lukD*, *lukM*, *lukF-PV* and *lukS-PV*) (Hermans et al., 2010), and for enterotoxins causing diarrhoea by action on the host enterocytes (*sea*, *seb*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sen*) (Dinges et al., 2000).

DNA extraction was carried out using the ChargeSwitch gDNA Mini Bacteria Kit (Invitrogen, USA) according to the manufacturer's instructions for staphylococci. All PCR reactions were performed with a

Table 1. Prevalence of California Mastitis Test (CMT)-positive cows and samples according to herd

| Herd | No. of tested cows | No. of positive cows (%) | No. of positive samples (%) |
|--------------|--------------------|--------------------------|-----------------------------|
| Elite | 120 | 51 (42.5) | 55 (11.5) |
| Non-elite | 74 | 29 (39.1) | 32 (10.8) |
| Primiparous | 71 | 24 (33.8) | 25 (8.8) |
| Total | 265 | 104 (39.2) | 112 (10.6) |

Mastercycler® (Eppendorf, France) as previously described (Ote et al., 2011). All PCR products were analysed by electrophoresis in 1.5% agarose gel. Gels were then stained with ethidium bromide and photographed under UV light at 254 nm.

S. aureus pulstotyping

The whole genome of the *S. aureus* isolates were compared by Pulsed Field Gel Electrophoresis (PFGE). Lysostaphine pre-treated bacterial cells were embedded in 0.9% certified low-melt agarose (Bio-Rad, Belgium), lysed in a 0.5M EDTA (pH 8) buffer containing 10% N-lauroylsarcosine and 2 mg/ml lysozyme, and treated with 1 mg/ml proteinase K (Sigma-Aldrich, Germany). Genomic DNA was digested with 10U of *Sma*I (Sigma-Aldrich, Germany) according to the manufacturer's instructions. Restricted fragments were separated through a 1% pulsed-field certified agarose gel in 0.5% TBE buffer by using a CHEF MAPPER (Bio-Rad, Belgium). The electrophoresis conditions were 6.0V/cm for 21h, with pulse times ranging from 5 to 60s, an angle of 120° and a linear ramp factor. Bacteriophage lambda DNA (Bio-Rad, Belgium) was used as molecular weight marker.

S. aureus antibiotic susceptibility testing

The susceptibility of the *S. aureus* isolates to antibiotics was determined by the disc diffusion method on Mueller-Hinton agar plates (Becton Dickinson, Belgium) as previously described (Bauer et al., 1966). The interpretation of susceptibility was based on the recommendations of the French Committee Guidelines for susceptibility testing (Soussy, 2010). Isolates were tested for tetracycline (30 UI), penicillin (10 UI), gentamicin (10 UI), trimethoprim-sulfamethoxazole (1.25 µg / 23.75 µg), enrofloxacin (5 µg), clindamycin (2 UI), and oxacillin (5 µg) (Becton Dickinson, Benelux).

Statistical analysis

All data and information were registered and checked

using Microsoft Excel 2003. The data were analysed statistically and compared, in 2 x 2 and 2 x 3 contingency tables, using the χ^2 test and the Fisher's Exact Test, when the χ^2 test was not relevant (P value >0.05), using SAS Software 9.1 (SAS, 2001).

RESULTS

California Mastitis Test (CMT)

Though no clinical mastitis was observed during the study, 112 milk samples from 104 cows tested positive on the CMT: 63 (++) , 40 (+++) and 9 (++++) scores. Ninety-six cows had one positive quarter and eight cows two positive quarters. The prevalence of CMT-positive cows and quarters was higher ($P < 0.05$) in the herds of elite and non-elite cows than in the herd of primiparous cows (Table 1).

Identification of bacterial species

Of the 112 CMT-positive milk samples, 51 (45.5%) gave a positive bacterial culture: 7 samples with a (++++) CMT score (7/9: 77.8%), 16 samples with a (+++) CMT score (16/40: 40.0%) and 28 samples with a (++) CMT score (28/63: 44.4%). The difference in isolation rates according to the CMT score was not significant. In four samples, two bacteria were isolated. Half of the 55 identified isolates belonged to the genus *Staphylococcus* (50.9%), in particular to the species *S. aureus* (41.8%) (Table 2). Other identified species belonged to the family *Enterobacteriaceae* (25.5%) and to the genera *Enterococcus* (12.7%), *Bacillus* (9.1%), and *Acinetobacter* (1.8%) (Table 2). The majority of *Staphylococcus* species were isolated from elite and non-elite cows, while the majority of *Enterobacteriaceae* and *Enterococcus* species were isolated from the primiparous cows; these differences were significant ($P < 0.001$).

S. aureus was isolated from 5 samples with a (++++) score on the CMT and 9 samples with a (+++) or a (++) score, while the majority of the *Enterobacteriaceae* were isolated from samples with a (+++) or a (++) score (Table 3). Nevertheless these differences were not significant.

Table 2. Distribution of the identified bacterial species according to herd

| Species identified | No. of isolates (%) | Herd | | |
|---|---------------------|-------------|-------|-----------|
| | | Primiparous | Elite | Non-elite |
| Genus <i>Staphylococcus</i> | 28 (50.9) | | | |
| <i>Staphylococcus aureus</i> | 23 (41.8) | 2 | 14 | 7 |
| <i>Staphylococcus sciuri</i> | 2 (3.6) | 0 | 1 | 1 |
| <i>Staphylococcus warneri</i> | 1 (1.8) | 1 | 0 | 0 |
| <i>Staphylococcus hyicus</i> | 1 (1.8) | 0 | 1 | 0 |
| <i>Staphylococcus epidermidis</i> | 1 (1.8) | 0 | 1 | 0 |
| Genus <i>Enterococcus</i> | 7 (12.7) | | | |
| <i>Enterococcus faecium</i> | 3 (5.5) | 3 | 0 | 0 |
| <i>Enterococcus faecalis</i> | 3 (5.5) | 2 | 1 | 0 |
| <i>Enterococcus avium</i> | 1 (1.8) | 1 | 0 | 0 |
| Family <i>Enterobacteriaceae</i> | 14 (25.5) | | | |
| <i>Escherichia coli</i> | 1 (1.8) | 1 | 0 | 0 |
| <i>Klebsiella pneumoniae</i> | 3 (5.5) | 3 | 0 | 0 |
| <i>Enterobacter cloacae</i> | 2 (3.6) | 2 | 0 | 0 |
| <i>Enterobacter spp</i> | 3 (5.5) | 3 | 0 | 0 |
| <i>Cronobacter sakazakii</i> | 1 (1.8) | 1 | 0 | 0 |
| <i>Citrobacter freundii</i> | 1 (1.8) | 1 | 0 | 0 |
| <i>Citrobacter farmeri</i> | 1 (1.8) | 1 | 0 | 0 |
| <i>Citrobacter spp</i> | 2 (3.6) | 2 | 0 | 0 |
| Genus <i>Acinetobacter</i> | 1 (1.8) | | | |
| <i>Acinetobacter baumannii</i> | 1 (1.8) | 1 | 0 | 0 |
| Genus <i>Bacillus</i> | 5 (9.1) | | | |
| <i>Bacillus subtilis</i> | 2 (3.6) | 1 | 1 | 0 |
| <i>Bacillus coagulans</i> | 3 (5.5) | 1 | 2 | 0 |

Table 3. Association of CMT scores with identified bacterial species

| CMT scores | No. of samples | No. of positive samples (%) | Bacterial species |
|------------|----------------|-----------------------------|--|
| ++++ | 9 | 7* (77.8) | <i>S. aureus</i> (5), <i>S. warneri</i> (1), <i>E. faecium</i> (1), <i>Enterobacter spp</i> (1) |
| +++ | 40 | 16 (40.0) | <i>S. aureus</i> (9), <i>E. faecium</i> (1), <i>E. avium</i> (1), <i>C. sakazakii</i> (1), <i>C. freundii</i> (1), <i>Citrobacter spp</i> (1), <i>B. subtilis</i> (1), <i>B. coagulans</i> (1) |
| ++ | 63 | 28** (44.4) | <i>S. aureus</i> (9), <i>S. sciuri</i> (2), <i>S. hyicus</i> (1), <i>S. epidermidis</i> (1), <i>E. faecium</i> (1), <i>E. faecalis</i> (3), <i>E. coli</i> (1), <i>K. pneumoniae</i> (3), <i>E. cloacae</i> (2), <i>Enterobacter spp</i> (2), <i>C. farmeri</i> (1), <i>Citrobacter spp</i> (1), <i>A. baumannii</i> (1), <i>B. subtilis</i> (1), <i>B. coagulans</i> (2). |

* One sample with two bacterial isolates

** Three samples with two bacterial isolates

Phenotyping of *S. aureus*

The majority of *S. aureus* isolates (18/23) belonged to the bovine biotype; two isolates belonged to the human biotype and three belonged to a non host-specific biotype. Five isolates were strong producers of biofilms, 12 were moderate producers, 6 were weak producers and no biofilm production was detected for the last

isolate. Moreover, 20 isolates produced SCVs at different levels, while the remaining three did not.

Virulotyping of *S. aureus*

All or most of the *S. aureus* isolates gave amplified fragments with most PCRs for the genes coding for

surface antigens: *clfA* (91%), *clfB* (100%), *fnbA* (100%), *cna* (100%), *ebpS* (100%), *sdrC* (87%), *cap5H* (83%), *spa* (96%), *icaA* (96%); and with several PCRs for the toxin-encoding genes: *etD* (74%), *hla* (96%), *hly* (100%), *hld* (96%), *hlyAC* (74%), *lukD* (96%), *lukM* (96%). Conversely, a minority of isolates tested positive with the PCRs for the following genes: *cap8H* (4%), *lukF-PV* (17%), *lukS-PV* (9%); only one isolate (4%) tested positive for 2 of the 9 enterotoxin-encoding genes (*sej* and *seg*); and none with the PCRs for the *etA* and *etB* genes. Therefore the 23 *S. aureus* isolates could be grouped into 14 virulotypes, with 7 of them (30.4%) belonging to the same virulotype and half of them (52%) to three virulotypes (Table 4). The enterotoxin-encoding gene-positive isolate was also the only one that tested positive with the PCR for the *cap8H* gene and negative with the PCRs for the *spa* and *icaA* genes.

Pulsotyping of *S. aureus*

The 23 isolates showed four distinct pulsotypes (Figure 2) which were named A (n=13), B (n=8), C (n=1) and D (n=1). Pulsotypes A and B differ by only one band. The pulsotype A was recovered from the three herds, the pulsotype B from the elite and non-elite herds, whereas the pulsotypes C and D were recovered respectively from the elites and the primiparous herds (Table 5).

Antibiotic susceptibility/resistance profiles of *S. aureus*

Clinical levels of resistance to gentamicin were detected in five isolates (22%), to tetracycline in three isolates (13%), to penicillin in three isolates (13%), and to clindamycin in one isolate (4%). Conversely, resistance was not detected to enrofloxacin, oxacillin, or trimethoprim-sulfamethoxazole (Figure 2).

DISCUSSION

The prevalence of presumptive mastitis (CMT-positive milk samples) at the Sahelian experimental station in Toukounous (39.2%) is similar to, though lower than, the observations reported by Bada-Alambéji and collaborators also using the CMT test (Bada-Alambéji et al., 2005) and by Harouna and collaborators with reference to somatic cell counts (Harouna et al., 2008), in urban and peri-urban farms in Niger during the rainy season (44% and 52%, respectively). Although not directly relevant to this study, it is essential to take into account the fact that the season can influence CMT test results in Niger, with a prevalence as low as 27% being reported during the dry season (Harouna et al., 2008).

Of the different bacterial species that can be involved

in bovine mastitis, several are isolated from Azawak zebu dairy cows with the highest isolation rate being for staphylococci (50.9%), but an absence of streptococci (Table 2). Since *Streptococcus* represents one of the genera known to be the most likely cause of clinical mastitis (Bradley, 2002), the absence of these bacteria in our results supports the observation that clinical mastitis is not present in Toukounous. Although a few of the bacterial species identified here (Table 2) are also known to be possible causes of clinical mastitis, most of them, including *S. aureus* and coliforms, are associated with sub-clinical mastitis, high CMT scores and elevated cell counts (Momtaz et al., 2010; Sargeant et al., 2001) (Table 3). The high prevalence of staphylococci in the present study can be partly explained by the contagious nature of these bacterial species. This is especially the case for *S. aureus* (Radostits et al., 2007), with transmission from cow to cow being favoured by the lack of basic hygiene procedures during the manual milking process, such as disinfection of the teats and of the hands of the milkers.

Surprisingly all but two *S. aureus* are isolated from elite and non-elite cows, most frequently from milk samples with a (+++++) or a (++++) CMT score (Table 1). This difference between the primiparous herd and the elite and non-elite herds cannot be explained at this stage. However, this observation might be related to the fact that a different set of milkers works with the primiparous herd than with the elite and non-elite herds. An alternative explanation for the difference could be related to the age of the cows (Harouna et al., 2009): elite and non-elite cows are older than primiparous cows and are therefore more often chronically infected with contagious bacterial species such as *S. aureus*.

Phenotypic and genotypic typing of the *S. aureus* isolates was subsequently performed in order to identify their virulence profiles. The majority of the 23 *S. aureus* isolates express properties and/or possess genes (Table 4) of strains able of persisting in the mammary gland and therefore causing chronic mastitis, in agreement with the presence of only chronic mastitis in Toukounous: adherence to extra-cellular matrix components (McDonald, 1989), biofilm and SCV formation (Costerton et al., 1999; Brouillette et al., 2003; Atalla et al., 2008), production of the Spa protein and clumping factors (Matsunaga et al., 1993), of capsular antigens (O'Riordan and Lee, 2004), and of haemolysins and leukotoxins (Rainard et al., 2003; Haveri et al., 2007). Though the results of the phenotypic assays (biotypes, biofilm formation, and SCV formation), of the PCR virulotyping, of the PFGE pulsotyping and of the antibiotic resistance profiles do not support the clonality of all 23 *S. aureus* isolates, several of them are closely related, supporting (though only partly) the hypothesis of transmission from cow to cow by the milkers' hands during the milking process.

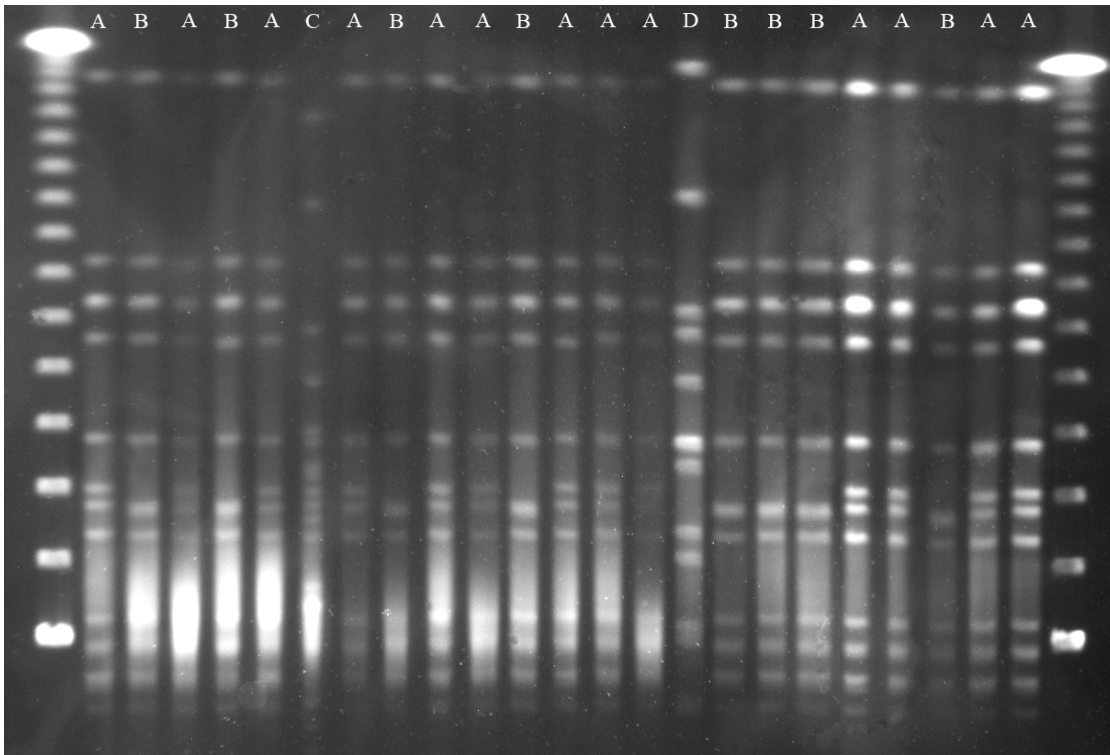
The 14 virulotypes identified (Table 4) also show

Table 4: Virulotypes of the 23 *S. aureus* isolates

| | Genes | | | | | | | | | | | | | | No. of isolates | | | | | |
|---|-------------|-------------|------------|-------------|-------------|-------------|-------------|------------|------------|------------|-------------|-------------|--------------|-------------|-----------------|-------------|----------------|----------------|--------------|--------------|
| | <i>clfA</i> | <i>clfB</i> | <i>cna</i> | <i>fnbA</i> | <i>icaA</i> | <i>epbS</i> | <i>sdrC</i> | <i>spa</i> | <i>etD</i> | <i>hla</i> | <i>hlyB</i> | <i>hlyD</i> | <i>hlyAC</i> | <i>lukD</i> | | <i>lukM</i> | <i>lukF-PV</i> | <i>lukS-PV</i> | <i>cap5H</i> | <i>cap8H</i> |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | 7 |
| + | + | + | + | + | + | + | + | + | + | - | + | + | - | + | - | - | + | - | - | 3 |
| + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | - | - | + | - | - | 2 |
| + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | - | - | + | - | - | 1 |
| + | + | + | - | + | + | - | + | - | + | + | + | + | + | - | + | + | + | - | - | 1 |
| - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + | - | - | 1 |
| - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | - | - | + | - | - | 1 |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | 1 |
| + | + | + | + | + | - | + | - | - | + | + | + | + | + | + | + | + | - | - | + | 1* |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | 1 |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | 1 |
| + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | - | - | - | 1 |
| + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | - | - | + | - | - | 1 |
| + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 1 |

* Isolate positive with the PCRs for the *sej* and *seg* enterotoxin-encoding genes

Figure 2. The A, B, C and D PFGE patterns of *Sma*I-digested DNA of the *S. aureus* isolates.



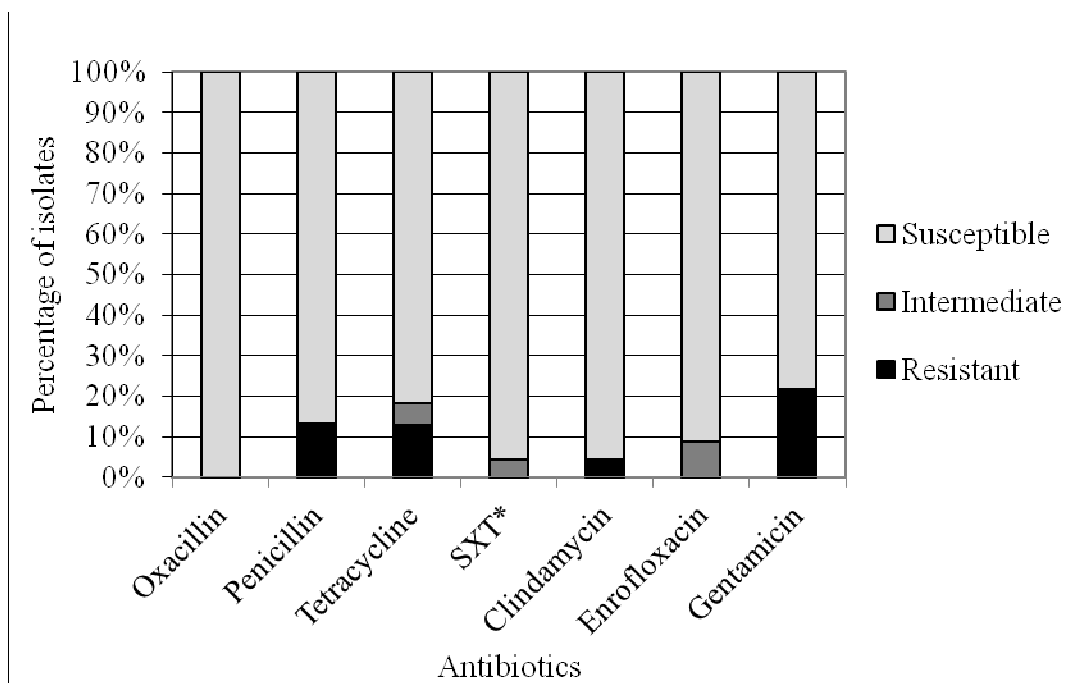
similarities and differences in comparison with the virulotypes identified during other surveys in different countries, emphasizing once more the great diversity of the mastitis-associated *S. aureus* isolates. For instance, the *clfA*, *clfB*, *cna*, *ebpS*, *hla*, *hly*, *lukD*, *lukM*, *spa*, and *ica* genes are widely distributed in our study. To cite only a few recently published surveys in various countries, the *clfA*, *clfB*, *ebpS*, *hla*, *hly*, *lukD*, *lukM* genes have also been detected in the vast majority (more than 95%) of isolates from bovine mastitis in Iran and Belgium (Momtaz et al., 2010; Ote et al., 2011). On the other hand, the *lukD*, *lukM* and *spa* genes are present in only a small number of isolates in Senegal (Kadja et al., 2010), the *cna* gene in one third of the isolates in Italy and Belgium (Zecconi et al., 2006; Ote et al., 2011) and the *icaA* gene in one quarter to two thirds of the isolates in Brazil and Belgium (Coelho et al., 2011; Ote et al., 2011). Conversely, none of the 23 isolates of the present study contains the *etA* and *etB* genes, in agreement with results already published in Finland, Turkey and Belgium (Haveri et al., 2007; Karahan et al., 2009; Ote et al., 2011).

The respective prevalence of the capsular antigen-encoding *cap5H* and *cap8H* genes can also differ greatly between bovine strains of *S. aureus* depending on the geographical source (Sordelli et al., 2000). In our study, the *cap5H* gene is the more frequent (87%) and the *cap8H* gene is present in only one isolate. This result is in agreement with some other published results, showing

that more than two thirds of isolates possess the gene *cap5H* (Poutrel et al., 1988; Baselga et al., 1994; Kadja et al., 2010). However, the same result contrasts with those of Ote et al. (2011), who report that only one-third of isolates harbour the *cap5H* gene, but two-thirds, the *cap8H* gene. Such differences may be related not only to actual geographical variation in the clones of *S. aureus* causing bovine mastitis, but also to the samples of the isolates tested (numbers and/or clinical status of the cows).

Finally, in the present study, only one isolate belonging to a unique virulotype tests positive with PCR for enterotoxin-encoding genes (Table 4), i.e. the *seg* and *sei* genes, that are frequently associated since they are located on one "enterotoxin gene cluster" (*egc*) (Karahan et al., 2009). This result is comparable to those of Harouna and collaborators in Niger (Harouna et al., 2009), who report the absence of genes coding for any enterotoxin. Enterotoxin-encoding genes are actually most frequent in strains causing food toxin-infection in humans (Zschock et al., 2005). Nevertheless, these genes have also been described in some isolates from mastitis in other countries (Rosec and Gigaud, 2002; Zschock et al., 2005; Kadja et al., 2010; Ote et al., 2011) questioning their possible public health hazard.

Our results also indicate that the 23 *S. aureus* isolates show a good level of sensitivity to the seven antibiotics tested (Figure 2), since a clinical level of resistance to gentamicin, tetracycline, trimethoprim-sulfamethoxazol

Figure 3. Prevalence (%) of antibiotic resistance amongst the 23 *S. aureus* isolates

(*) sulfamethoxazole and trimethoprim

Table 5. Distribution of the pulsotypes A, B, C and D (see Figure 3) in the three herds

| Pulsotypes | No. of isolates | Herds | | |
|------------|-----------------|-------------|-------|-----------|
| | | Primiparous | Elite | Non-Elite |
| A | 13/23 | 1 | 7 | 5 |
| B | 8/23 | 0 | 6 | 2 |
| C | 1/13 | 0 | 1 | 0 |
| D | 1/23 | 1 | 0 | 0 |

and/or oxacillin is observed in only one to five isolates. Similar results have been obtained during surveys in African countries (Niger, Kenya and Senegal) (Shitandi and Sternesjo, 2004; Bada-Alambedji et al., 2005; Harouna et al., 2009; Kadja et al., 2010) and in other countries (Turkey and Italy) (Güler et al., 2005; Moroni et al., 2006). However, the level of resistance to β -lactams is usually higher than in our results.

Several other bacterial species apart from *S. aureus* are also identified from the CMT-positive milk samples, and some of these have a (++) or a (+++) CMT score. These species can also be responsible for contagious mastitis (other staphylococci) or cause environmental mastitis (enterococci, enterobacteria). Due to the low number of isolates of each species, a comparison of their phenotypic and genotypic traits could not be performed. The role of the remaining species (acinetobacteria and bacilli) is more difficult to assess and one cannot rule out

the possibility that they are, in fact, contaminants of the milk samples.

Finally, a very interesting feature is that no bacterial growth is obtained from ca. half of the milk samples with high CMT scores, emphasizing once more that elevated cellular concentrations as evidenced by the CMT is not always associated with the presence of bacterial infection (Longo et al., 1994). Indeed, physiological cellular concentrations in milk can vary according to the number of lactations and the stage of lactation (Serieys, 1995). Moreover, the failure to isolate bacteria from CMT-positive milk samples may be associated with the presence of antibiotic residues following treatment of the cows or with the destruction of the bacteria during transport (Bouchot et al., 1985). A further possible cause of this failure to isolate bacteria may be found in the growth conditions used at the National Public Health School of Niamey, which do not allow the isolation of

some mastitis-associated bacteria, such as *Mycoplasma bovis* or anaerobes (Laak and Ruhnke, 1995).

In conclusion, results from the present study at the Sahelian experimental station in Toukounous are similar to those obtained during two studies in urban and peri-urban farms in Niger (Bada-Alamedji et al., 2005; Harouna et al., 2008). It is most likely that the absence of hygienic procedures at the time of milking contributes not only to the original contamination but also to the perpetuation of the infection. It is hoped that wider transversal and longitudinal surveys in the three herds will confirm these data and demonstrate the positive role of prophylactic measures when applied, and that the genetic typing of isolates will clarify the mode of contamination and of transmission of the infection.

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