

## Full Length Research Paper

# Antibiotic resistance trend of *Staphylococcus aureus* isolated between 2010 and 2012 from mastitis cases in Azawak zebu in Niger

Abdoulkarim Issa Ibrahim<sup>1,2\*</sup>, Jean-Noël Duprez<sup>1</sup>, Rianatou Bada-Alambédi<sup>3</sup>, Nassim Moula<sup>4</sup>, Jacques G. Mainil<sup>1</sup> and Marjorie Bardiau<sup>1</sup>

<sup>1</sup>Bacteriology, Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, Sart-Tilman, B43a, B-4000 Liège, Belgium.

<sup>2</sup>Bacteriology, Department of Diagnosis, Epidemiological Investigations and Applied Research, BP 485, Niamey, Niger.

<sup>3</sup>Microbiology, Immunology and Infectious Pathology, Interstate school of Veterinary Science and Medicine, BP 5077, Dakar, Senegal.

<sup>4</sup>Department of Animal Production, Faculty of Veterinary Medicine, University of Liege, Boulevard de Colonster, 20, building B43, 4000 Liege, Belgium.

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The present study was conducted at the Sahelian Experimental Station in Toukounous Niger, on three herds of Azawak zebu breed in order to evaluate antimicrobial susceptibility of a total of 43 *Staphylococcus aureus* isolated from 164 milk samples of cows with subclinical mastitis from 2010 to 2012. The highest frequency of resistance was observed for the  $\beta$ -lactam family: penicillin (46%) followed by oxacillin (16%). Twenty isolates were sensitive to all tested antibiotics, 12 were resistant to one of them and 11 were multi-resistant (2 to 5 antibiotics). The resistance percentage to oxacillin, enrofloxacin and clindamycin varied significantly over the 3 years ( $p < 0.05$ ) but not to the other antibiotics. Moreover, 90% of *S. aureus* isolates resistant to penicillin detected by *in vitro* disk diffusion possessed the *blaZ* gene. In conclusion, the isolates from cases of mastitis at Toukounous are more resistant to the antibiotics frequently used for treatments at the station than to other antibiotics.

**Key words:** Mastitis, *Staphylococcus aureus*, antibiotic resistance, Azawak zebu, Niger.

## INTRODUCTION

Mastitis is the inflammation of the mammary gland most frequently caused by bacterial infection. Bacterial mastitis is a disease causing considerable economic loss in cattle farming. Milk deficits during mastitis vary from 25 to 48%

depending on the intensity of inflammation and the stage of lactation (Gebreyohannes et al., 2009). Treatment (use of antibiotics) costs can be expensive and the incurable cows must be reformed, a preventive method truly efficient

\*Corresponding author. E-mail: karimlebelge@yahoo.fr. Tel: +3243664052, +22796573021. Fax: +3243664261, +22720732147.

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but also very expensive (Radostits et al., 2007). At the Sahelian station in Toukounous (Niger) reformed cows are sold to private farmers for fattening before slaughter.

Several bacterial species can cause bovine mastitis (Radostits et al., 2007). In many countries including Niger, *Staphylococcus aureus* is one of the most frequently isolated species from subclinical and clinical mastitis (Pradeep et al., 2003; Bada et al., 2005; Harouna et al., 2009; Issa et al., 2013). As a consequence of the wide use of antibiotics in dairy cattle farms, several mastitis-causing bacterial species have become resistant to the most frequently used antibiotics (Werckenthin et al., 2001). Nevertheless, antimicrobial therapy still plays an important role in mastitis control by reducing the infection levels and preventing new cases (Rabello et al., 2005).

In Niger, zebu cattle of the Azawak breed offer the best dairy aptitude and since 1975 the Niger government has opted for a policy of development and improvement of the breed. The experimental station in Toukounous was therefore set up with the objective of improving the dairy production by genetic selection and of promoting its distribution to individual farmers across the whole country (MRA, 2002). But, according to the available data in the yearly reports, the extensive use of antibiotics of the  $\beta$ -lactam (amoxicillin, ampicillin), tetracyclin (tetracyclin, oxytetracyclin), aminoglycoside (streptomycin, gentamicin) and sulfonamide families is noticeable, more especially for the treatment of intestinal and mammary gland infections (SSET, 2009). However, the influence of this wide antibiotic use on the level of resistance of mastitis-causing bacterial species is unknown. It is therefore important to study not only the prevalence of the different antibiotic resistance profiles of the most frequent mastitis-causing bacterial species, but also their evolution in time to foresee the efficiency of the treatments in the future.

Therefore the objective of this survey was to follow the evolution of antibiotic resistance of the *S. aureus* isolates during a three year period of time (2010-2012) from cases of mastitis in Azawak zebu at the Sahelian experimental station of Toukounous, Niger.

## MATERIALS AND METHODS

### Bacterial isolation and identification

A total of 164 milk samples were collected between 2010 and 2012 from cows of the three Azawak zebu herds (elites, non-elites and primiparous) of the Toukounous station with subclinical mastitis. The bacteriological analysis of milk samples and the identification of *S. aureus* were performed as described previously (Issa et al., 2013).

### Antibiotic susceptibility testing and resistance profiles

The susceptibility of the *S. aureus* isolates to seven antibiotics was determined by the disc diffusion method on Mueller-Hinton agar plates (Becton Dickinson, Belgium) as described by Bauer et al. (1966). Isolates were tested with discs with tetracyclin (30 UI),

penicillin G (10 UI), gentamicin (10 UI), trimethoprim-sulfamethoxazole (1.25  $\mu$ g / 23.75  $\mu$ g), enrofloxacin (5  $\mu$ g), clindamycin (2 UI) and oxacillin (5  $\mu$ g) (Becton Dickinson, Belgium). The diameter in mm of the inhibition zone of each tested antibiotic was compared with the recommendations of the French Committee Guidelines for susceptibility testing (Comité de l'Antibiogramme vétérinaire de la Société Française de Microbiologie, 2010) to determine the susceptibility/resistance profile of each isolate. The data were processed in a spreadsheet program (Excel for Windows, Microsoft).

### PCR characterization

All the isolates were tested for the presence of *blaZ* gene (coding for  $\beta$ -lactamase) by polymerase chain reaction (PCR) with forward primer 5'-TAA GAG ATT TGC CTA TGC TT-3' and reverse primer 5'-TTA AAG TCT TAC CGA AAG CAG-3' (Olsen et al., 2006). DNA extraction was carried out using the ChargeSwitch DNA Mini Bacteria Kit (Invitrogen, USA) according to the manufacturer's instructions for staphylococci. The PCR reaction mixture contained: 1U of *Taq* DNA polymerase (New England Biolabs, USA), 2.5  $\mu$ l of 2 mM deoxynucleoside triphosphates (Eurogentec, Belgium), 2.5  $\mu$ l of 10X ThermoPol Reaction Buffer, 1  $\mu$ l of each primer (10  $\mu$ M) and 1.5  $\mu$ l of a DNA template in a total volume of 25  $\mu$ l. The PCR reactions were performed using a Mastercycler® (Eppendorf, France). The PCR-amplified DNA fragment of 519 bp was isolated by electrophoresis in a 1.5% agarose gel and photographed under UV light after ethidium bromide staining.

### Statistical analysis

To compare the evolution of the percentages of resistant *S. aureus* to the different antibiotics over the years (2010, 2011 and 2012), the Fisher's Exact Test was performed (SAS, 2001). Differences were considered significant at  $p < 0.05$ .

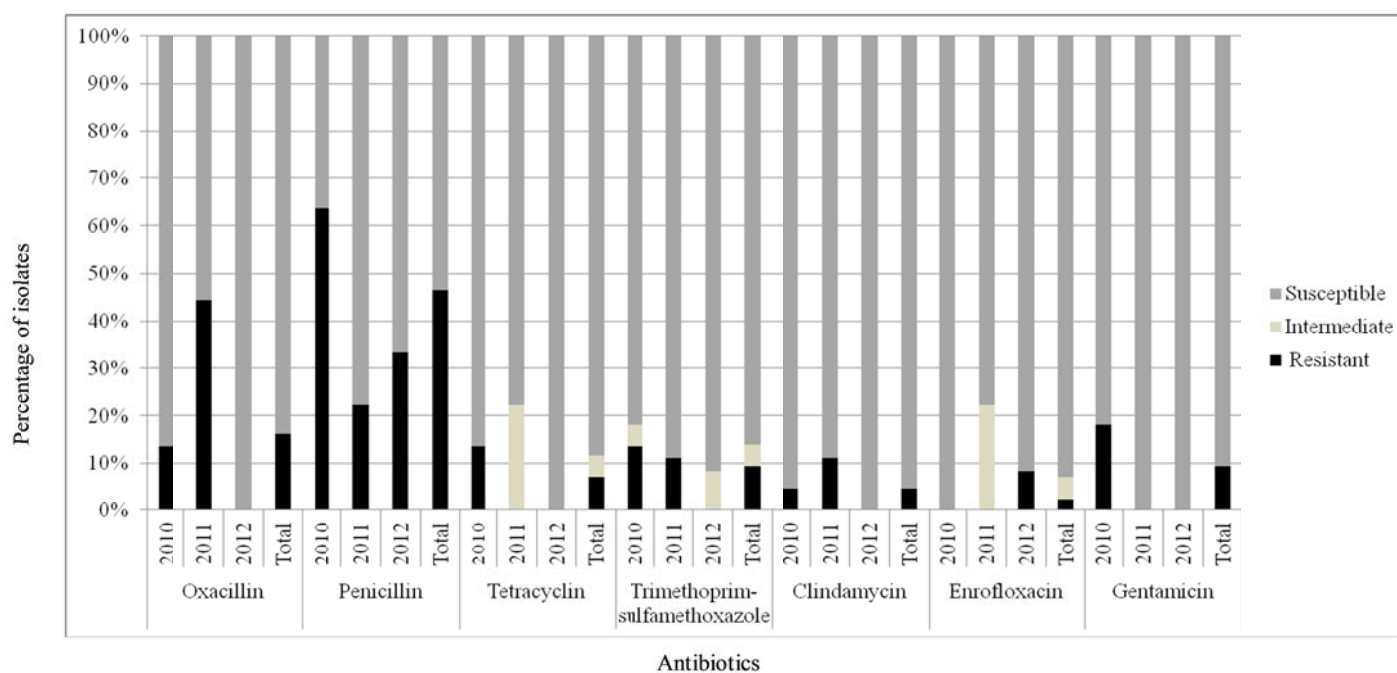
## RESULTS AND DISCUSSION

A total of 43 *S. aureus* isolates were recovered from the 164 milk samples: 22 isolates in 2010, 9 in 2011 and 12 in 2012, with 7 isolates from primiparous cows, 21 from elite cows and 15 from non-elites cows (Table 1). Twenty isolates (46%) were sensitive to all antibiotics tested, but 23 *S. aureus* isolates (54%) were resistant to at least one antibiotic tested: 14 in 2010 (33%), 5 in 2011 (12%) and 4 in 2012 (9%) ( $p < 0.05$ ).

The average resistance rates over the three years differ according to the antibiotic. As many as 20 of the 43 *S. aureus* isolates (46%) were resistant to penicillin (Figure 1); much less isolates were resistant to oxacillin (16%), gentamicin (9%), trimethoprim-sulfamethoxazole (9%), tetracyclin (7%), clindamycin (5%) and enrofloxacin (2%). The annual resistance rates in 2010 (22 isolates), 2011 (9 isolates) and 2012 (12 isolates) were the following: penicillin (64, 22 and 33%), oxacillin (14, 44 and 0%), gentamicin (4, 0 and 0%), trimethoprim-sulfamethoxazole (14, 11 and 0%) tetracyclin (14, 0 and 0%), clindamycin (4, 11 and 0%) and enrofloxacin (0, 0 and 8%). The Fisher's Exact Test confirmed a significant difference ( $p < 0.05$ ) in the resistance percentages to oxacillin, enrofloxacin and clindamycin over the 3 years but not to the other antibiotics. All but three of the 23 resistant

**Table 1.** Number of *S. aureus* isolated at Toukounous according to the herd and to the year.

Herd	2010	2011	2012	Total
	No of sampled animal / No of isolated <i>S. aureus</i>			
Elite	25/9	19/4	32/8	76/21
Non-elite	20/7	24/4	19/4	63/15
Primiparous	19/6	6/1	0/0	25/7
Total	64/22	49/9	51/12	164/43

**Figure 1.** Antibiotic resistance profiles of the 43 *S. aureus* isolates in 2010, 2011 and 2012.

isolates were resistant to penicillin and 11 of them were multidrug resistant (Table 2): six isolates were resistant to two antibiotics, four to three antibiotics and one to 5 antibiotics. These results are probably related to the frequent use of  $\beta$ -lactams in the treatment of any suspected bacterial infection at the station followed by tetracyclins alone or in combination with aminoglycosides in second intention.

The average resistance rates in Toukounous are comparable, sometimes lower, to those obtained in previous studies performed in different African countries and in Pakistan. For instance, 64, 56, 54 and 100% of the *S. aureus* isolates respectively in Tunisia, Pakistan, Senegal and Uganda, are resistant to penicillin (Ben Hassen et al., 2002; Arshad et al., 2006; Kadja et al., 2010; Kateete et al., 2013). The results are also similar for oxacillin and tetracyclin. Oxacillin resistance rate is 7.6, 10 and 15% respectively in Nigeria, Senegal and Morocco (Bendahou

et al., 2008; Kadja et al., 2010; Suleiman et al., 2012); whereas tetracyclin resistance rate is 13 and 15% in two previous studies in Niger in 2007 and 2009 (Harouna et al., 2009; Issa et al., 2013). Finally, 25% of the isolates of this study are multiresistant (resistance to at least two antibiotics), a result also comparable to the 23% reported by Zanette et al. (2010), but lower than the 62% reported by Meideiros et al. (2009), both in Brazil.

Thirteen cows could be followed between 2010 and 2012 to assess the individual antibiotic resistance evolution (Table 3). The antibiotic resistance profiles of the *S. aureus* isolated from the same cow differed from one year to another suggesting that the isolates are not clonal and that reinfection of the udder occurred during the three years. The Pulsed Field Gel Electrophoresis profiles (manuscript in preparation) confirm the hypothesis that isolates from the same cow are indeed not clonal.

Of the 20 isolates of *S. aureus* resistant to the penicillin

**Table 2.** Resistance profiles of the 23 *S. aureus* isolates resistant to at least one antibiotic.

No. of antimicrobials	Resistance profile					No. of isolates		
1	Pen					9		
	Oxa					2		
	Clin					1		
2	Pen		Oxa			2		
	Pen		Gen			2		
	Pen		Tet			1		
	Pen		Eno			1		
3	Pen		Oxa		Tet	1		
	Pen		Oxa		Tsu	1		
	Pen		Tet		Tsu	1		
	Pen		Tsu		Gen	1		
5	Pen		Oxa		Gen	Tsu	Clin	1
Total						23		

Pen = Penicillin, Oxa = oxacillin, Tet = tetracyclin, Tsu = trimethoprim-sulfamethoxazole, Enro = enrofloxacin, Clin = clindamycin, Gen = gentamicin.

**Table 3.** Antibiotic resistance profiles of the *S. aureus* isolates from 13 cows over three years (2010 to 2012).

Cow identification/ year of isolation	Antibiotic resistance profile		
	2010	2011	2012
56/11	Pen, Oxa, Tsu, Clin, Gen	S	0
61/11	0	Pen, Oxa, Tsu	Pen
63/11	0	Clin	S
65/11 and 68/11	S	0	S
66/11	S	Oxa	0
70/11	Pen, Gen	0	S
71/11	Pen	0	S
76/11	Pen, Tet, Tsu	0	S
79/11	S	S	S
86/11	Pen	Pen, Oxa	Pen, Enro
94/11	0	0	Oxa
96/11	S	Oxa	S

Pen = penicillin, Oxa = oxacillin, Tet = tetracyclin, Tsu = trimethoprim-sulfamethoxazole, Enro = enrofloxacin, Clin = clindamycin, Gen = gentamicin, S = sensitive to all tested antimicrobials, 0 = no *S. aureus* isolated from milk sample.

by the *in vitro* disc diffusion test, 18 (90%) tested positive by PCR for the *blaZ* gene suggesting that the production of  $\beta$ -lactamase by most penicillin-resistant *S. aureus* of this study is encoded by the *blaZ* gene (Zscheck and Murray, 1993).

In conclusion, our study provides recent data on the distribution of antimicrobial resistance at the pilot farm of Toukounous. The massive resort to  $\beta$ -lactam antibiotics to treat any condition is most probably responsible for the high rate of resistance observed at the farm. Besides the resistance problem, it has also been reported that intra-

mammary infection remained significantly more often chronic if it was caused by *blaZ*-positive than *blaZ*-negative isolates of *S. aureus* (Taponen et al., 2003). It is therefore an absolute necessity to be more cautious and strict in the application of treatments with antibiotics, particularly in the use of the  $\beta$ -lactams.

#### Conflict of Interests

The authors have not declared any conflict of interests.

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