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REGULAR ARTICLES

A 3-year long study of *Staphylococcus aureus* isolates from subclinical mastitis in three Azawak zebu herds at the Sahelian experimental farm of Toukounous, Niger

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Abstract *Staphylococcus* (*S.*) *aureus* is one of the most important pathogens causing bovine mastitis. The aim of the present work was to follow in three herds and during the 3 years the clonality of *S. aureus* isolated from California Mastitis Test (CMT)-positive cows at the experimental station of Toukounous (Niger) by (i) comparing their pulsed field gel electrophoresis (PFGE) fingerprints, (ii) identifying their virulotypes by PCR amplification and (iii) assessing the production of capsule and the formation of biofilm. The 88 *S. aureus* isolates belonged to 14 different pulsotypes, 3 of them being predominant: A (30 %), D (27 %), B (15 %). A and B pulsotypes had the highest profile similarity coefficient (94 %), while others had similarity coefficients under 60 %. Seventy-five *S. aureus* isolates were further studied for their virulotypes, capsular antigens and biofilm production. Most surface factor-, leukocidin- and haemolysin-, but not the enterotoxin-encoding genes were detected in the majority (>75 %) of the isolates and were evenly distributed between the A, B and D pulsotype isolates. The majority of the 72 *S. aureus* positive with the *cap5H* or *cap8H* PCR produced the CP5 (82 %) or the CP8 (88 %) capsular antigen, respectively. Biofilm production by the 57 *icaA*-positive isolates was strong for 8 isolates, moderate for 31 isolates but weak for 18 isolates, implying that the *icaA* gene may not be expressed in vitro by one third of the positive isolates. Similar to other studies, those results confirm that a restricted number of *S. aureus* clones circulate within the three herds at Toukounous and that their specific virulence-associated properties must still be further studied.

Keywords Azawak zebu · Mastitis · *Staphylococcus aureus* · Pulsed field gel electrophoresis · Virulotyping · PCR

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Introduction

Livestock farming is the second most important economic activity after crop production in the Sahel region. The specific objective of the Sahelian experimental station of Toukounous (SEST) located 220 km northeast of Niamey in Niger is the selection and the diffusion of the Azawak zebu breed with improved production levels, especially dairy production. Azawak zebu breed is indeed amongst the best dairy cattle in West Africa, with a mean daily production of 7 to 8 l in good standard management practice (Issa et al. 2014).

Still, like all dairy cattle worldwide, Azawak zebu cows suffer from mammary gland infections that impair the level of milk production. Unfortunately, publications on mastitis in Niger are scarce and surveys are highly needed to improve the knowledge on their aetiology and epidemiology (Issa et al. 2013, 2014). During such a first survey at the SEST in 2009

(Issa et al. 2013), 55 bacterial isolates from 104 cows positive at the California Mastitis Test (CMT) were identified, despite absence of evidence of clinical mastitis, with half of them (51 %) belonging to the genus *Staphylococcus* (*S.*) and 42 % to the species *Staphylococcus aureus*.

S. aureus is indeed one of the most important contagious mastitis-causing bacterial pathogens worldwide and is responsible for clinical and subclinical as well as chronic and acute mastitis (Radostits et al. 2010; Zadoks et al. 2011; Keefe 2012). *S. aureus* produces several putative virulence factors contributing to its pathogenicity: protein adhesins, named "Microbial Surface Components Recognizing Adhesive Matrix Molecules" (or MSCRAMMs) interacting with host extracellular components; polysaccharide intercellular adhesins involved in the production of biofilm; other surface antigens intervening in the evasion of the innate and acquired immune responses (capsules, protein A) and secreted toxins causing cellular and tissue damages, such as haemolysins, leukocidins and enterotoxins (Foster and Höök 1998; Dinges et al. 2000; Srinivasan et al. 2006; Nishifuji et al. 2008; Ote et al. 2011; Gogoi-Tiwari et al. 2015).

Besides the virulotyping, a standard discriminatory technique is the pulsed field gel electrophoresis (PFGE), or pulsotyping (Hallin et al. 2007). The comparison of the PFGE profiles or pulsotypes of *S. aureus* isolates is very effective and highly discriminative to characterizing the genetic diversity of *S. aureus* during outbreaks of mastitis in herds and to identifying the contamination source and transmission route (Zadoks et al. 2011).

The purpose of this work was to repeat and complete during the 3 years (from 2010 to 2012) the 2009 survey by isolating *S. aureus* from CMT-positive Azawak zebu cows at the SEST and by comparing their pulsotypes, virulotypes and ability to produce a capsule and to form a biofilm.

Materials and methods

Study design and *S. aureus* identification

At the SEST, the cows are divided into three herds according to their age and level of milk production: "primiparous" cows, "non-elite" cows (<1400 kg milk/lactation) and "elite" cows (>1400 kg milk/lactation). Due to scarcity of resources, the milkers apply no hygienic or antisepsis measures during the milking process that is performed manually twice daily (Issa et al. 2014).

Between 2010 and 2012, the Californian Mastitis Test (CMT) was performed on all lactating cows of the herds: a quarter was considered positive when the score was (++), (+++) or (++++); negative when the score was (−) and doubtful when the score was (+). The CMT-positive cows were sampled for bacteriological analysis and milk samples were stored at −20 °C in 10 % (*v*/*v*) glycerol until further use (Issa et al. 2013). The procedure was repeated three times on the same cows at 2-week intervals in 2010 (Table 1).

Isolated colonies were pre-identified at the Laboratory of Bacteriology of the National Public Health School of Niamey. A loopful of each milk sample was inoculated onto Columbia sheep blood agar (BioMérieux). Staphylococci were suspected on the basis of colony shape, Gram staining, bacterial cell shape and catalase production and were subsequently frozen at −20 °C in glycerol.

Each year, all suspect isolates were air shipped to Belgium in polystyrene boxes (import permits no. CONT/IEC/FRT/589554 in 2010, CONT/IEC/FRT/813231 in 2011 and CONT/IEC/FRT/1016090 in 2012). As soon as they arrived at the Laboratory of Bacteriology of the Veterinary Faculty of the University of Liège, the bacteria were grown on Columbia sheep blood agar, to check purity (BioMérieux) and on selective Mannitol salt phenol red agar (Merck) to check growth and mannitol fermentation. After confirmation of the initial results obtained in Niamey, mannitol-fermenting colonies

Table 1 Number of CMT-positive cows and of *S. aureus* identified per year and per herd

Year	No. of CMT-positive cows/no. of cows tested			No. of *S. aureus*-positive cows/no. of cows sampled		
	Primiparous cows	Non-elite cows	Elite cows	Primiparous cows	Non-elite cows	Elite cows
2010-t0	19/39	20/40	25/65	1[a]/19	5/20	3/25
2010-t+2w	ND	ND	ND	7/19	5/20	7/25
2010-t+4w	ND	ND	ND	7/19	4/20	6/25
2010-t+6w	ND	ND	ND	6/19	6[a]/20	9/25
2011	ND	24/48	19/44	ND	4/24	4/19
2012	ND	19/29	32/51	ND	4/19	8/32

2010-t0=first of four sampling times in 2010; 2010-t+2w=second sampling 2 weeks later; etc.

ND not done

[a] One primiparous and one non-elite cows with two *S. aureus* isolates from two CMT-positive quarters

were picked up and tested by the BD BBL® coagulase test with rabbit plasma (Becton Dickinson).

The final identification was performed on mannitol-fermenting coagulase-positive colonies using API-STAPH® (BioMérieux). After confirmation of their identity, *S. aureus* isolates were frozen at −80 °C in glycerol until further typing.

Pulsotyping

All *S. aureus* isolates were compared by PFGE at once at the end of the study according to the procedure described by Bardiau et al. (2014). In brief, the whole genome DNA was extracted from lysostaphin pre-treated bacterial cells embedded in 1.8 % certified low-melt agarose (Bio-Rad). After enzymatic digestion with *Sma*I restriction enzyme (Sigma-Aldrich), the DNA fragments were separated in a 1 % pulsed field-certified agarose (Bio-Rad) gel using a CHEF MAPPER (Bio-Rad) at 6.0 V/cm for 21 h, with pulsed times ranging from 5 to 60 s, an angle of 120° and a linear ramp factor. The dendrogram was prepared by the Unweighted Pair Group Method using arithmetic average Algorithm (UPGMA) using Biogene Software (Vilber Lourmat) as described by Bardiau et al. (2013).

Virulotyping

Each year, the *S. aureus* isolates were tested by PCR as described by Ote et al. (2011) for the presence of genes coding for MSCRAMMs (*clfA*, *clfB*, *fnbA*, *cna*, *ebpS*, *sdrC*), biofilm formation (*icaA*), capsular antigens (*cap5H*, *cap8H*), protein A (*spa*), haemolysins (*hla*, *hlb*, *hld*, *hlgAC*), leukocidins (*lukD*, *lukM*, *lukF-PV*, *lukS-PV*) and enterotoxins (*sea*, *seb*, *sec4*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*).

Capsular serotyping

The existence of 11 capsule serotypes is reported with two of them, CP5 and CP8, being most frequent in mastitis-associated *S. aureus* (O'Riordan and Lee 2004). Capsular serotyping was performed on all isolates at once by ELISA assays with specific monoclonal and polyclonal antibodies detecting the CP5 and CP8 antigens (Bardiau et al. 2014). Optical density (OD) values were compared to those obtained with *S. aureus* reference strains, namely the human CP5-positive strain Lowenstein (ATCC 49521) and the human CP8-positive strain Wright (ATCC 49525).

Biofilm production

Biofilm production was assayed for all isolates at once in microtitre plates using safranin staining (Bardiau et al. 2014). The quantitative classification of biofilm production was based on OD values at 490 nm using a microplate reader (Bio-Rad). Isolates are classified into non-producers and weak, moderate or strong producers.

Results

S. aureus identification and pulsotyping

From a total of 158 CMT-positive cows that were sampled, 88 *S. aureus* were identified: 68 in 2010, 8 in 2011 and 12 in 2012. Thirty-seven *S. aureus* were isolated from cows of the elite herd, 29 from cows of the non-elite herd and 22 from cows of the primiparous herd (Table 1).

The 88 *S. aureus* isolates exhibited 14 different PFGE fingerprints arbitrarily designated by a capital letter (Table 2). A and B pulsotypes had the highest profile similarity coefficient (94 %), while the other pulsotypes had similarity coefficients ranging between ca. 10 and 60 % (Fig. 1). A, D and B pulsotypes were the most frequent: 26 isolates (30 %), 24 isolates (27 %) and 13 isolates (15 %), respectively (Table 2). The remaining 11 pulsotypes were identified in one to five isolates each.

A and B pulsotypes were identified during the 3 years and D pulsotype in 2010 and 2012, while most other pulsotypes were restricted to the year 2010 (Table 2). A and D pulsotype isolates were equally distributed in the three herds whereas B pulsotype isolates were almost restricted to the non-elite and elite herds (12/13 isolates), as were the other 6 pulsotypes identified in more than one isolate (Table 2). In 2010, 12 of the 14 pulsotypes were identified during at least one of the four sampling events: 7 in the primiparous, 5 in the non-elite and 7 in the elite herds. But only A pulsotype was identified in the three herds at the four sampling events.

Twenty-one cows were *S. aureus* positive more than once between 2010 and 2012 (Table 3). *S. aureus* isolated during two consecutive years belonged to different pulsotypes; nevertheless, in 2010, the same pulsotype was identified at least twice in eight of the 17 cows with more than one positive sample (Table 3). Assuming those isolates had high clonal relationships, only the first ones were further studied, making the total of isolates tested for their virulotypes, capsular antigens and biofilm production to 75.

PCR virulotyping

Apart from the *cap5H* and *cap8H* genes, all surface factor-encoding genes were detected in the majority (>75 %) of the 75 *S. aureus* (Table 4) and three of them were not equally distributed between the A, B and D pulsotype isolates: the *cna* gene (85 % of the isolates) was detected in only two thirds of the B pulsotype isolates and the *spa* and *icaA* genes (77 and 76 % of the isolates, respectively) in 30 to 40 % of the D pulsotype isolates, respectively. The *cap5H* gene (52 % of

Table 2 Number of pulsotypes per year and per herd

PFGE profile	Number of isolates	Year			Herd		
	n=88 (100 %)	2010[a]	2011	2012	Primiparous cows[a]	Non-elite cows	Elite cows
A	26 (30 %)	24	1	1	9[b]	9[b]	8
D	24 (27 %)	20		4	8	9[b]	7
B	13 (15 %)	6	2	5	1	5	7
N	5 (6 %)	1	4		1	3	1
Z	4 (5 %)	4				1	3
W	3 (3 %)	3					3
Q	3 (3 %)	3					3
P	3 (3 %)	3					3
G	2 (2 %)	1		1		2	
F	1 (1 %)			1			1
O	1 (1 %)	1			1		
R	1 (1 %)		1				1
T	1 (1 %)	1			1		
M	1 (1 %)	1			1		

[a] Primiparous cows were sampled only in 2010

[b] One primiparous and one non-elite cows with two *S. aureus* isolates from two CMT-positive quarters

the isolates) was highly associated with A and B pulsotypes and the *cap8H* gene (44 % of the isolates) with D pulsotype. Three isolates (4 %) tested negative with both *cap5H* and *cap8H* PCR.

The *lukD*, *lukM*, *hla*, *hlb*, *hld* and *hlgAC* toxin-encoding genes were also detected in the majority of the 75 *S. aureus* (>80 %) and, with the exception of the *hlgAC* gene, were equally distributed within the A, B and D pulsotype isolates (Table 4). Conversely, the *lukF-PV* and *lukS-PV* genes were present in only 30 to 40 % of the isolates, respectively, and were more frequently identified in D, than in A and B pulsotype isolates.

Although 51 *S. aureus* (68 %) tested positive for at least one enterotoxin-encoding gene, only the *seg* gene was detected in a majority of the 75 isolates (60 %). The *seb* and *sei* genes were identified in 35 to 40 % of the isolates, respectively, but the other genes were detected in none to 15 % of the isolates (Table 4). The *seg*, *sei* and *seb* enteroxin-encoding genes were all highly associated with D pulsotype (>90 %), compared to A and B pulsotypes (0 to 50 %) (Table 4).

Capsular antigen ELISA and biofilm formation

Of the 39 isolates genotyped as *cap5H*, 32 (82 %) were CP5 ELISA positive, and of the 33 isolates genotyped as *cap8H*, 29 (88 %) were CP8 ELISA positive (Table 5). Irrespective of the identity of the capsular antigen the highest correlation was with D pulsotype isolates (100 %), followed by B (85 %) and A (68 %) pulsotype isolates.

All 75 *S. aureus* isolates produced biofilm to some extent: 32 (43 %), 35 (47 %) and 8 (11 %) isolates were weak, moderate and strong biofilm producers, respectively (Table 6). All 8 strong and 89 % of the moderate producer isolates (31/35) harboured the *icaA* gene while 56 % of the weak producer

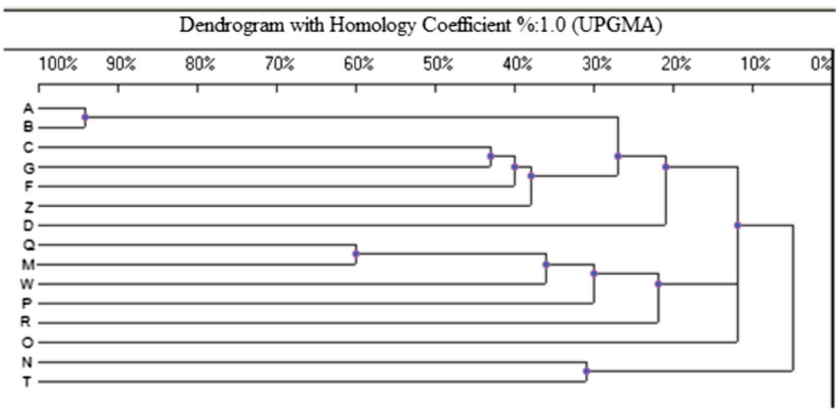

Fig. 1 Dendrogram of the *S. aureus* isolates derived from the PFGE profiles. The pulsotype C isolate was identified in 2009 (Issa et al. 2013), but not between 2010 and 2012

Table 3 Pulsotypes distribution of *S. aureus* isolates from 21 cows with >1 positive sample over the 3 years

Herds	Cow	2010 t0*	2010 t+2w	2010 t+4w	2010 t+6w	2011	2012
Elite	56/11		Q		W	A	
	60/11			Q			F
	61/11		*Z*	*Z*		R	D
	63/11	A	D	Q		N	B
	65/11			*P*	*P*		B
	66/11		*A*		*A*	B	
	68/11			D	A		B
	70/11				D		A
	71/11		Z		D		B
	76/11				W		D
Non-elite	79/11	*A*	*A*	*A*	*A*	B	G
	86/11	*D*	*D*		*A-D*[a]	N	D
	94/11	B		G		N	D
	96/11			A		N	B
	98/11	*D*	*D*				
Primiparous	35/11		D		A	nd	nd
	38/11		*D*	*D*		nd	nd
	40/11	*A-A*[a]	M	*A*	*A*	nd	nd
	43/11		A		D	nd	nd
	50/11			A	D	nd	nd
	52/11			D	O	nd	nd

Cows with more than one isolate in 2010 belonging to the same pulsotype are in italics

nd not done

[a] Two *S. aureus* colonies were isolated from two CMT-positive quarters

isolates (18/32) also tested positive. The majority of A and B pulsotype isolates not only harboured the *icaA* gene (95 and 85 %, respectively) but also were moderate to strong biofilm producers (89 and 69 %, respectively). In contrast, only 10 % of D pulsotype isolates were moderate and none were strong biofilm producers though 40 % tested positive for the *icaA* gene (Table 6).

Discussion

Though the 88 *S. aureus* isolated between 2010 and 2012 belong to 14 different pulsotypes, 3 of them (A, B, D) group together 72 % and 5 of them (A, B, D, N, Z), 83 % of the isolates, confirming, like in other studies, that a restricted number of *S. aureus* clones are present within a herd or a farm (Hata et al. 2006; Aires-de-Sousa et al. 2007; Rabello et al. 2007; Anderson et al. 2012). Nevertheless, apart from the A and B pulsotypes (>90 % similarity), the other pulsotypes are not closely related and represent distinct lineages (Fig. 1), emphasizing different contamination origins and/or ways. Although some influence of the differences in the sampling methods and in the bacteriological analysis cannot be ruled out, this diversity and distribution of pulsotypes may indeed be explained by different management practices at the SEST. The absence of hand and udder washing and disinfection during the milking process certainly represents the most important problem, but other practices, such as the occasional exchange of the milkers between the herds, the mixing of all pregnant cows in the same maternity building and the introduction of recently purchased cows, also represent opportunities for new strains to be introduced and transferred between cows (Middleton et al. 2002; Sommerhauser et al. 2003; De Vliegher et al. 2012).

The majority of pulsotypes are identified during the four sampling events in 2010, but only A and B pulsotypes are present in the three herds from 2010 to 2012, the latter being isolated only once from the primiparous cows (Table 2). Already in 2009, A, B and D pulsotypes had been identified, as had C pulsotype (Issa et al. 2013) which is absent in this study, and B pulsotype was restricted to the non-elite and elite herds (Table 2). Time (over years) diversity and distribution of the pulsotypes is also illustrated by the results obtained for the 21 cows followed with more than one positive milk samples during this survey (Table 3), though in 2010, isolates belonging to the same pulsotype were identified more than once from eight of these cows. To avoid redundancy, only the first isolate was kept for further typing, making the total of isolates tested to 75.

Even if pulsotypes can evolve as consequences of point mutations at the height of the restriction sites and of genetic rearrangement such as deletion, insertion and/or inversion of DNA fragments, the predominance of a limited number of pulsotypes suggests that those isolates have a greater capacity to infect the mammary gland, to persist over time within the mammary gland and/or to spread between cows (Rabello et al. 2007) and, therefore, privileged association(s) with specific virulence factors.

However, according to the PCR results, the virulotypes do not greatly differ between pulsotypes (Table 4). The MSCRAMM-, capsule-, haemolysin-, leukocidin (except *lukS-PV* and *lukF-PV)*-, and one enterotoxin (*seg*)-encoding genes are present in a majority of isolates, while the other genes are detected in ca. less than one third of them, similarly to other published results. The most noticeable difference is the much higher prevalence of the *lukS-PV* (39 %) and *lukF-PV* (31 %) genes coding for the Panton-Valentine leukocidin (PVL) compared to the results of other studies in Europe and Africa (Rainard et al. 2003; Fueyo et al. 2005; Karahan et al. 2009; Ikawaty et al. 2010; Kadja et al. 2010; Ote et al. 2011; Issa et al. 2013). Since the PVL toxin is strongly associated with human *S. aureus* isolates causing skin and soft-tissue infections (Shallcross et al. 2013), the *lukS-PV*- and/or *lukF-PV*-positive isolates in Toukounous might therefore be of human rather than of cattle origin, transferred during the manual milking process.

In addition, different genes are unevenly distributed between the most frequent three pulsotypes (Table 4): the *spa*,

Table 4 Relation between the positive PCR reactions of the 75 *S. aureus* isolates and their pulsotypes

Target genes coding for …	% PCR-positive isolates[a]	Pulsotypes													
		A *n*=19	D *n*=20	B *n*=13	N *n*=5	Z *n*=3	W *n*=3	Q *n*=3	P *n*=2	G *n*=2	F *n*=1	O *n*=1	R *n*=1	T *n*=1	M *n*=1
… surface adhesins and antigens															
clfB	96	19	19	13	5	3	3	3	2	2	1	0	0	1	1
fnbA	93	18	19	12	5	3	3	3	1	2	1	1	0	1	1
clfa	92	19	20	13	4	1	3	3	1	2	1	0	0	1	1
ebpS	92	19	19	12	3	3	3	3	2	1	1	1	0	1	1
sdrC	88	19	19	12	5	0	3	3	1	1	1	0	0	1	1
cna	85	18	19	9	3	1	3	3	2	2	1	0	1	1	1
spa	77	19	6	13	4	3	3	3	1	2	1	1	0	1	1
icaA	76	18	8	11	5	3	3	3	1	2	1	0	0	1	1
cap5H	52	17	2	12	2	0	3	0	1	1	0	1	0	0	0
cap8H	44	2	17	1	3	3	0	3	0	1	1	0	0	1	1
… leukocidins and haemolysins															
hlb	97	19	20	13	5	3	3	3	2	2	1	0	0	1	1
hld	93	18	20	11	5	3	3	3	1	2	1	0	1	1	1
lukM	93	17	20	13	5	3	3	3	1	1	1	0	1	1	1
hla	87	17	18	10	5	3	3	3	1	2	1	0	0	1	1
lukD	85	17	19	12	2	3	3	3	2	1	1	0	0	0	1
hlgAC	81	14	17	12	5	2	3	3	1	1	1	0	0	1	1
lukS-PV	39	1	13	6	1	1	3	0	1	1	1	0	0	1	0
lukF-PV	31	2	12	1	1	0	3	0	1	2	0	0	0	1	0
… enterotoxins															
seg	60	6	20	7	0	2	3	3	1	1	1	0	0	1	0
sei	39	0	18	4	0	0	3	0	1	1	1	0	0	1	0
seb	35	0	19	4	0	0	0	0	0	1	1	0	0	1	0
sec4	15	4	2	0	0	0	1	1	0	1	1	0	0	1	0
seh	7	0	0	0	0	1	1	1	0	1	0	0	1	0	0
sea	5	0	0	0	0	0	0	3	0	0	0	0	0	0	1
sed	4	0	0	1	0	0	1	0	0	0	1	0	0	0	0
sej	4	1	1	0	0	0	0	0	0	1	0	0	0	0	0
see	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Total % of the 75 *S. aureus* that tested positive with the PCR for that gene

Table 5 Comparison of the results of the PCR for the *cap5H* and *cap8H* genes and of the ELISA for the production of CP5 and CP8 capsular polysaccharides

ELISA-positive isolates *n*=75		Pulsotypes													
		A 19[a]	D 20	B 13	N 5	Z 3	W 3	Q 3	P 2	G 2	F 1	O 1	R 1	T 1	M 1
CP5 antigen	32[b]	13	2	10	1	0	3	0	1	1	0	1	0	0	0
	(39)[c]	(17)[c]	(2)	(12)	(2)	(0)	(3)	(0)	(1)	(1)	(0)	(1)	(0)	(0)	(0)
CP8 antigen	29[b]	0	17	1	3	3	0	3	0	0	1	0	0	0	1
	(33) [c]	(2)	(17)	(1)	(3)	(3)	(0)	(3)	(0)	(1)	(1)	(0)	(0)	(1)	(1)

[a] Number of isolates belonging to the pulsotype (Table 2)

[b] Number of isolates producing the CP5 or CP8 capsular antigens

[c] Number of *cap5H* or *cap8H* gene PCR-positive isolates (Table 5)

Table 6 Comparison of the pulsotypes with the results of the biofilm production and of the PCR for the *icaA* gene

Production of a biofilm *n*=75		Pulsotypes													
		A 19[a]	D 20	B 13	N 5	Z 3	W 3	Q 3	P 2	G 2	F 1	O 1	R 1	T 1	M 1
Weak	32[b]	2	18	4	3	1		1	1		1	1			
	(18)[c]	(2)[c]	(8)	(2)	(3)	(1)		(1)	(0)		(1)	(0)			
Moderate	35[b]	15	2	8	1	2	1	2		1			1	1	1
	(31)	(14)	(0)	(8)	(1)	(2)	(1)	(2)		(1)			(0)	(1)	(1)
Strong	8[b]	2		1	1		2		1	1					
	(8)	(2)		(1)	(1)		(2)		(1)	(1)					

[a] Number of isolates belonging to the pulsotype (Table 2)

[b] Number of isolates producing weak, moderate or high levels of a biofilm

[c] Number of *icaA* gene PCR-positive isolates (Table 5)

icaA and *cap5H* genes are detected more frequently in A and B pulsotype isolates; the *cap8H*, *lukS-PV*, *lukF-PV*, *seg*, *sei* and *seb* genes, in D pulsotype isolates. Still, to our knowledge, none of these results can actually explain, from the one hand, the higher prevalence of these three pulsotypes, or, from the other hand, the difference of their isolation rates between years and herds. Indeed if some of the hundreds of the *spa* gene variants and if the *seg* and *sei* genes are more frequently present in *S. aureus* associated with persistent infections and subclinical mastitis (Haveri et al. 2007; Mitra et al. 2013; Spa Server Database 2015), no pulsotype association has been described to our knowledge.

As already reported, the vast majority of the 75 *S. aureus* (96 %) test positive for the *cap5H* or *cap8H* capsule-encoding genes and are not at random distributed between the different pulsotypes (Ikawaty et al. 2010), but the reason for this is unknown. The prevalence of isolates actually expressing CP5 and CP8 (85 %) is high and similar for either antigen (Table 5) in contrast to other studies (Camussone et al. 2012; Bardiau et al. 2014). Several parameters can indeed influence the expression of CP5 and CP8 capsular antigens, including the *S. aureus* growth conditions, the duration of the culture and, of course, mutations in the encoding genes (Poutrel et al. 1995; Herbert et al. 2001; Cocchiaro et al. 2006).

Finally, the frequency of the i*caA* gene in *S. aureus* isolated from bovine mastitis can be highly different between studies: 15 % (Darwish and Asfour 2013), 58 % (Chavhan et al. 2012), 86 % (Ote et al. 2011), 98 % (Castelani et al. 2015) vs. 76 % in the present study. Biofilm production by the 57 *icaA*-positive isolates was strong for 8 isolates (belonging to different pulsotypes), moderate for 31 isolates (including most A and B pulsotype isolates) but weak for 18 isolates (including all 8 D pulsotype isolates) (Table 6), emphasizing not only that the *icaA* gene may not be expressed by one third of the positive isolates in this in vitro assay but also that this lack of expression could be related to the pulsotype.

In conclusion, *S. aureus* isolates collected between 2010 and 2012 can be divided into 14 pulsotypes of which three are predominant. The virulotyping identified some differences between them but none could explain the differences observed in their distribution between herds and years, nor their actual association with only subclinical mastitis. Additional long-term surveys focusing on the isolation and typing of *S. aureus* from the hands of milkers and from non-milk sites (dairy cow teat skin, teat canals, skin lesion and environment of the cows and of the milkers) will help to better understand the epidemiology and the virulence of the different pulsotypes of *S. aureus*-associated mastitis at Toukounous. The results of such surveys will also help to set up effective education programmes for milkers and farmers about the application of hygienic and antiseptic procedures throughout the dairy production chain (Castelani et al. 2013), not only at the SEST but also in urban and peri-urban cattle farms in Niger (Bada-Alambedji et al. 2005; Harouna et al. 2009).

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Conflict of interest The authors declare that they have no competing interests.

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