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Genotyping of *Staphylococcus aureus* Isolated from Bovine Clinical Mastitis by Pulsed-Field Gel Electrophoresis (PFGE)

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Abstract: Thirty-two strains of *Staphylococcus aureus* have been collected in the setting of an epidemiological investigation in Rhone-Alps region in France (collaboration AFSSA-ENV Lyon) from samples of milk of districts of thirty-two cows affected by clinical mastitis, belonging to 17 herds, on one period of 5 months (January 2007 to May 2007). This set of withdrawals has been achieved in 4 veterinary clienteles situated in Rhone-Alps. Twenty five strains of *Staphylococcus aureus* were the object of identification phenotypic and a genetic characterization. Epidemiological scorers have been taken, the profiles of resistance to the antibiotics, the profiles of Pulsed Field Gel Electrophoresis (PFGE). The one has been chosen here because of its excellent power of discrimination. A sensitivity of 89% of the isolates to all tested antibiotics has been determined by the method of disk by diffusion on agar Mueller Hinton (MH) (Sanofi Diagnosis Pasteur or Bio-Rad). The frequencies of resistance to the Penicillin to the ampicillin to the oxacillin to the Cefquinome to the Cefalotin and to the Amoxicillin + AC clavulanique is respectively 20, 08, 08, 08, 08 and 04%, those to the trimethoprime more the sulfamethoxazole and the Sulfamethoxypyridazine are 08 and 24%. No resistance of the strains of *Staphylococcus aureus* to the oxytetracycline to the Doxycycline to the erythromycin to the Spiramycin to the Lincomycin to the Gentamicin and to the Enrofloxacine. The molecular typage by Pulsed-Field Gel Electrophoresis (PFGE), after digestion of the chromosomal DNA of the isolates with the SmaI endonuclease restriction, revealed 09 different genetic profils (A-I). The two main pulsotypes A and B represented 58% together and have been found in 64% of studied exploitations. So the strains of *Staphylococcus aureus* are in majority of the genotype A and B. They belonged to the predominant genotypes and could have a certain predilection to cause some mastitis among the dairy cow. In spite of the heterogeneity of the some, the results of the PFGE are in favor of a dissemination of a small number limited of predominant genotypes.

Key words: *Staphylococcus aureus*, Pulsed-Field Gel Electrophoresis (PFGE), clinical mastitis, bovine, genotyping, epidemiology

INTRODUCTION

The mastitis is always a topic of major preoccupation for the world of dairy industry. They generate important economic losses for the breeder and for dairy industry. This economic importance is the subject of various assessments of the losses that are, to the cost of the antibiotic treatment, longevity of the animals (Caraviello *et al*., 2005), milk eliminated during the treatment, to the penalties imposed by dairy industry, reduction of the price of the bad bacteriological quality milk, falls of production, cost of reform them precocious and genetic potential loss (Barbano *et al*., 1991). The *Staphylococcus aureus* is one of the most important etiologic agents of the mastitis of the Ruminants (Katsuda *et al*., 2005; Mork *et al*., 2005; Aires-De-Sousa *et al*., 2007). It has been implied in these infections, with an active frequencto 44% of the cases of clinical mastitis (Sargeant *et al*., 1998; Waage *et al*., 1999; Sabour *et al*., 2004; Peles *et al*., 2007). At the bovines the prevalence of clinical mastitis due to *Staphylococcus aureus* fluctuates from 5-50% in different countries (Aires-De-Sousa *et al*., 2007). The organism is responsible of about 30-40% of all cases of mastitis (Asperger and Zagerl, 2003).

In spite of the use of a variety of antibiotics, a therapy seems to be very often inefficient (Annemuller *et al*., 1999). It can be of the to its resistance in the outside environment, on the animals, his/her/its resistance to the antibiotics that can reach him and its contagiousness. The main objectives of this survey were to contribute to a better understanding of the epidemiology of *Staphylococcus aureus* in the dairy raisings of the Mounts of Lyon and their sensitivities to

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the antimicrobials. The Pulsed Field Gel Electrophoresis (PFGE) that is considered like a technique of reference because of its excellent power of discrimination and its reproducibility (Vautor *et al*., 2003; Goñi *et al*., 2004; Anderson and Lyman, 2006; Anderson *et al*., 2006; Jorgensen *et al*., 2005; Middleton *et al*., 2005; Rabello *et al*., 2005; Laplana *et al*., 2007) has been used. However, its use is limited because it is technically demanding, heavy, long and expensive; require the material and facilities, several days of analysis and a qualified staff (Gilbert *et al*., 2006). A second technique the PCR-RFLP can be foreseen in a second research to epidemiological ends to the source of the strains and to the information born of the field.

MATERIALS AND METHODS

Strains of *Staphylococcus aureus*: The strains of *Staphylococcus aureus* have been collected in the setting of an epidemiological investigation in Rhone-Alps region in France (collaboration AFSSA-ENV Lyon) from samples of milk of cows affected by clinical mastitis, before administration of all antibiotic treatment. The withdrawals have been sent to the laboratory, under table setting of the cold weather. This set of withdrawals has been achieved in 4 veterinary clienteles: C, D, F and M (Table 1).

The number of the strains contains: the number of raising, the clinical mastitis (C) and the number of the withdrawal. The strains of a same raising are (28C/02 and 28C/01), (50C/01, 50C/02 and 50C/03), (71C/01 and 71C/04) and (275C/01 and 275C/02).

Isolation and identification of the species of *Staphylococcus aureus*: After isolation and identification, the strains have been kept in BHI middle (Bio-Merieux) to -20°C during the time of the study.

Study of the sensitivity to the antibiotics: The study of the sensitivity to the antibiotics has been achieved by the study disk test by diffusion on agar middle or antibiogram, according to the technique of the committee of the antibiogram of the Microbiology Company French (CA-SFM) (Soussy, 2002).

The antibiogram have been achieved on agar Mueller Hinton (MH (Sanofi Diagnosis Pasteur or Bio-Rad) according to the recommendations of the CA-SFM (Soussy, 2002). Fifteen antibiotics have been tested of the Penicillin (Pen, 6 μg), of the Ampicillin (Amp, 10 μg), of the Oxacillin (Oxa, 5 μg), of the Cefquinome (Cq, 30 μg), of the Cefalotin (Cf, 30 μg), of the Amoxicillin more of the acidic clavulanique (AmC, 25 μg+), of the Oxytetracycline (Oxt, 30 UI), of the Doxycycline (Dot, 30 UI), of the Erythromycin (Ery, 15 UI), of the spiramycin (Spi, 100 μg, 333 UIS), of lincomycin (Lin, 15 μg), of the Sulfamethoxypyridazine (Sss, 200 μg) of the Gentamycine (Gen, 15 μg), to the Trimethoprime sulfamethoxazole (Tsu, 1.25 μg + 23.75 μg) and finally of the Enrofloxacine (Enr, 5 μg) (Sanofi Diagnosis Pasteur or BioRad). The interpretation has been made according to the recommendations of it (CA-SFM) (Soussy, 2002).

Table 1: Distribution and origin of the 25 strains of *Staphylococcus aureus* studied

No. of the srains	Clientele
12C/02	D
16C/02	D
27C/01	D
28C/02	D
28C/01	D
36C/02	D
50C/01	F
50C/02	F
71C/01	F
77C/01	F
71C/04	F
50C/03	F
58C/02	F
97C/01	F
168C/01	M
275C/01	M
275C/02	M
161C/03	M
188C/01	M
86C/01	C
93C/01	C
118C/01	C
96C/01	C
81C/01	C
126C/01	C

Genomic typing by pulsed-field gel electrophoresis: The chromosomal DNA has been prepared according to the protocol describes by Talon *et al*. (1996). With some modifications. Of the small blocks (2.5 blocks/strain) of agarose (Low Melting. Bio-Rad) to 1% have been prepared while mixing (v v^{-1}) 150 μL of the bacterial suspension in 150 μL of agarose. After solidification of the blocks, these have been immersed in 1.5 mL of lysis buffer (0.5 M EDTA pH = 8, Sarcosyl) 1%, containing 30 μL of lysostaphin (Sigma- Aldrich) to 5 mg mL^{-1} and 15 mg of lysozyme (Sigma-Aldrich) and incubated during 1 h to 37°C. The Plugs have been hatched then during the overnight at 50°C in 1.5 mL of lysis buffer (0.5M EDTA pH = 8, Sarcosyl 1%), with 250 μL of k proteinase k to 1% (Eurebio). The Plugs have been rinsed 4 times then, during 1 h in 3 mL of lysis buffer TE (10 m M Tris (pH 8), 1 mM EDTA, 1000 mL Distilled water) and then conserved to + 4°C. The DNA has been digested with 30 U of SmaI

enzymes (Promega), to 24°C. The blocks of agarose have been rinsed then in 500 µL of tampon TE (10 mM Tris (pH 8), 1 mM EDTA, 1000 mL Distilled water). The fragments of DNA were separated by electrophoresis in a frost of agarose to 1% in buffer TBE 0.5X (Tris-Borate-EDTA) (Bio-Rad) dilute to the 1/20 in the distilled water of a containing solution: Tris Base 0.89 M; boric acid 0.89 M; EDTA 20 mM; Distilled water 1000 mL) in apparatus CHEF DRIII (Bio-Rad). It is driven in the following conditions: running 6 V cm^{-1} of agarose, time of migration 20 h, temperature 14°C, initial pulse time 5S, final pulse time 35S, the angle of deviation 120°. A scorer of size of molecular weight (λ-ladder Biorad) and the stump of reference (No. ATCC: 29213) has been used like norms. After electrophoresis the DNA is revealed under UV light after coloration 10 min in the BET (Ethidium Bromide) and fading 20 min. The number and the sizes of the fragments of DNA separated by electrophoresis have been valued and the proximity of the strains has been interpreted according to Tenover *et al*. (1996) criteria.

RESULTS AND DISCUSSION

Profiles of the resistance to the antibiotics: Eighty nine percent of the strains of *Staphylococcus aureus* were sensitive to all tested antibiotics. The frequencies of resistance to the Penicillin to the ampicillin to the oxacillin to the Cefquinome to the Cefalotin and to the Amoxicilline + AC clavulanique are respectively 20, 08, 08, 08, 08 and 04%, those to the trimethoprim more the sulfamethoxazole and the Sulfamethoxypyridazine are 08 and 24%. No resistance of the strains of *Staphylococcus aureus*; to the oxytetracycline to the Doxycycline to the erythromycin to the Spiramycin to the Lincomycin to the Gentamicin and to the Enrofloxacin (Table 2).

Genetic typing by Pulsed Field Gel Electrophoresis (PFGE) restriction profiles: The 22 isolates of *Staphylococcus aureus* tested were distributed in nine different pulsotypes (A-I). The profiles have been designated according to the criteria of Tenover *et al*. (1996). Four profiles contained more of strains and the five that remain were constituted of unique strains. The B pulsotypes contained the biggest number, either nine isolates (lanes: 2, 4, 7, 10, 18, 19, 20, 21 and 26) follow-up by the pulsotype A that contained four isolates (lanes: 3, 17, 27 and 28) by C and D with two isolats each (lanes: 6 and 29) and (lanes: 5 and 24) and finally the E pulsotypes (line: 12), F (lane: 14), G (lane: 13), H (lane: 25) and I (lane: 11) that contained each a isolated (Fig. 1 and 2) Pulsotype B: (lanes: 2, 4, 7, 10, 18, 19, 20, 21 and 26); Pulsotype A: (lanes: 3, 17, 27 and 28); Pulsotype C: (lanes: 6 and 29); Pulsotype D: (lanes: 5 and 24); Pulsotype E: (lane: 12); Pulsotype F: (lane: 14); Pulsotype G: (lane: 13); Pulsotype H: (lane: 25); Pulsotype I: (lane: 11).

One of the main pulsotypes, B has been identified in nine strains, either (40%) found in the four studied clienteles: C, D, F and M. The profile A include 4 strains (18%), it is also present in the four clienteles. The genotypes A and B represent thirteen strains together is (58%) are the predominant genotypes being in 64% of the exploitations. So the strains of *Staphylococcus aureus* are the majority of the genotype A and B. In spite of the heterogeneity of the some, the results of the pulsed field gel electrophoresis are in favor of a predominant dissemination of a small number of genotypes (Table 3).

Table 2: Frequency of resistance of 25 strains of *Staphylococcus aureus* to 15 different antibiotics tested by the method of diffusion in agar

Antibiotics	No. of *S. aureus*	Resistance (%)
Penicillin	05/25	20
Ampicilline	02/25	08
Oxacillin	02/25	08
Cefquinome	02/25	08
Cefalotin	02/25	08
Amoxicillin + Ac clavulanique	01/25	04
Oxytetracycline	00/25	0.0
Doxycycline	00/25	0.0
Erythromycin	00/25	0.0
Spiramycin	00/25	0.0
Lincomycin	00/25	0.0
Sulfaméthoxypyridazine	06/25	24
Gentamicin	00/25	0.0
Trimethoprim sulfamethoxazole	02/25	08
Enrofloxacin	00/25	0.0

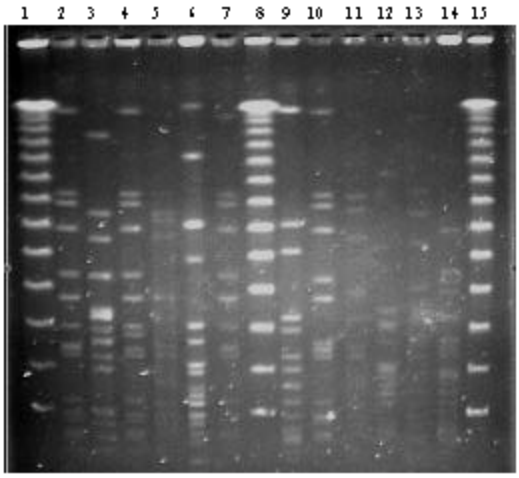

Fig. 1: Presentation of the profiles of PFGE of the strains of *Staphylococcus aureus* after digestion by the SmaI endonuclease. Lanes; 1, 8 and 15: Scorer of size of molecular weight. 48, 5-1000 kb (λ-ladder Bio-Rad). Lane 9: Strains of reference ((No ATCC: 29213). Lanes 2, 3, 4, 5, 6, 7, 10, 11, 12, 13 and 14: Strains of *Staphylococcus aureus*

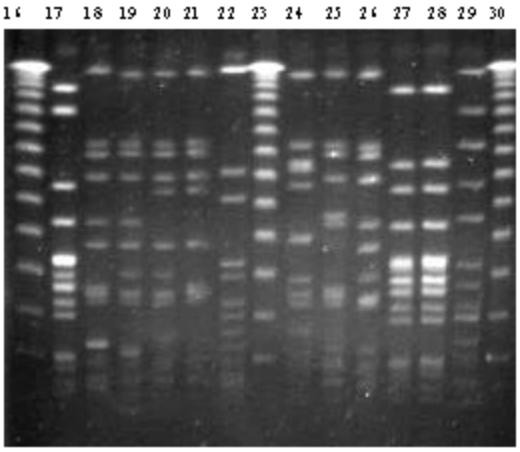

Fig. 2: Presentation of the profiles of PFGE of the strains of *Staphylococcus aureus* after digestion by the SmaI endonuclease restriction. Lanes; 16, 23 and 30: Scorer of size of molecular weight. I ladder (λ-ladder Bio-Rad). Lane 22: Strains of reference (No. ATCC: 29213). Lanes; 17, 18, 19, 20, 21, 24, 25, 26, 27, 28 and 29: Strains of *Staphylococcus aureus*

Table 3: Distribution of the profiles of the pulsed field gel electrophoresis of 22 isolates of *Staphylococcus aureus* of bovines reached of clinical mastitis

Clientele	Lane of the strains	No. of the strains	Profile PFGE
D	17	12C/02	A
	18	16C/02	B
	19	27C/01	B
	20	28C/02	B
	21	28C/01	B
F	2	50C/01	B
	4	50C/03	B
	3	50C/02	A
	5	71C/01	D
	11	77C/01	I
	6	71C/04	C
	10	58C/02	B
C	12	81C/01	E
	13	86C/01	G
	7	93C/01	B
	14	118C/01	F
	28	96C/01	A
	29	126C/01	C
M	24	188C/01	D
	25	168C/01	H
	26	275C/01	B
	27	275C/02	A

The resistance to the antibiotics: This study made to come out again, of the very elevated frequencies of sensitivity. About 89% of the strains of *Staphylococcus aureus* were sensitive to all antibiotics tested. The resistance to the G penicillin concern 20% of the strains of *Staphylococcus aureus*, the one to the oxacillin and to the ampicilline 08% each, to the Amoxicillin + AC clavulanique only 04%. The frequencies of resistance to the Cefquinome and to the Cefalotin are 08%, those to the triméthoprime more the sulfamethoxazole and the Sulfamethoxypyridazine are respectively, 08 and 24%. A rate of 11% of the remaining isolats was resistant to all tested antibiotics. This big sensitivity to all antimicrobials is generally observed among the isolats of *Staphylococcus aureus*, at the bovines (Lange *et al*., 1999; Werckenthin *et al*., 2001). Of the sensitivity frequencies of (75.5%) in Canada (86%) in USA, (91%) in Allemage and 94% in Norway, respectively, have been returned (Anderson *et al*., 2006; Mork *et al*., 2005; Sabour *et al*., 2004; Stephan *et al*., 2001). Of the slightly lower rates 67 and 44.9% have been signaled (Aires-De-Sousa *et al*., 2007; Lange *et al*., 1999; Rabello *et al*., 2005). However, a global decrease of resistance to the antibiotics has been recorded. This observation has also been confirmed by the results of the studies on a big scale carrying on a very elevated number of isolats, what could be due to two important changes: the generalization in the use of the Cloxacillin and the treatment of the dried cows (Werckenthin *et al*., 2001; Sabour *et al*., 2004).

The elevated frequencies notably for the Penicillins, the Sulfamethoxypyridazine and the Cephalosporin have been observed. For the Penicillin, rates of (38 and 35%) have been reported, respectively (Grinberg *et al*., 2005; Erskine *et al*., 2004). According to geographical distribution of the populations, it has been suggested that the resistant strains selection, occurs following the use rep eated of an antibiotic (Werckenthin *et al*., 2001; Sabour *et al*., 2004). These data underline the necessity of a politics on the use discriminating of the antimicrobials in the dairy production. In the treatment of the infected animals, it is important to determine the phenotype of resistance and to avoid the selection of resistant strains. The resistance to the Methicillin of the strains of *Staphylococcus aureus* (MRSA) is among the most menacing bacteria, implied in the nosocomials infections. They are signaled more and more in the veterinary medicine. The SARM are classified by their capacity to resist against the Penicillin, the oxacillin, the Ampicillin, the Cefalotin and the Sulfamides (Middleton *et al*., 2005). This functionality being conferred by the gene of resistance mecA that their permit to become resistant, against all penicillins and the Cephalosporins (Werckenthin *et al*., 2001).

Pulsed Field Gel Electrophoresis (PFGE): In the world of *Staphylococcus aureus*, clinical mastitis accountable officer at the bovines, PFGE has been used a lot. This system of genotyping has been chosen because of its big power of discrimination and its excellent power of reproducibility (Joo *et al*., 2001; Vautor *et al*., 2003;

Goni *et al*., 2004; Anderson and Lyman, 2006; Anderson *et al*., 2006; Jorgensen *et al*., 2005; Middleton *et al*., 2005; Rabello *et al*., 2005; Laplana *et al*., 2007). However, its use is limited because it is technically too demanding, heavy, long and expensive; require the material and facilities, several days of analysis and a qualified staff (Gilbert *et al*., 2006).

Several studies were already about the comparison of this technique with other techniques of genetic characterization (Hennekinne *et al*., 2003; Villard *et al*., 2003; Hata *et al*., 2006). The PFGE proved to have the best power of discrimination. It is also better the adapted to solve the relations clonales (Murrun *et al*., 2005; Vancraeynest *et al*., 2006; Aires-De-Sousa *et al*., 2007). It is capable to mark all strains. The enzyme of SmaI restriction is the more used and generate an easy profile to read 13-17 fragments of 20-750 kb (Tenover *et al*., 1996; Faria *et al*., 2008).

The 22 isolates of *Staphylococcus aureus* tested were distributed in 09 different pulsotypes (A-I). The two main pulsotypes A and B represent together (54%). They have been found in the four studied clienteles: C, D, F and M, so the strains of *Staphylococcus aureus* are in majority of the genotype A and B. In spite of the heterogeneity of the some, the results of the pulsed field gel electrophoresis are in favor of a dissemination of a small number limited of predominant genotypes. Several epidemiological studies disassembled the existence of a number limited of genotype or clone predominating (Goni *et al*., 2004; Sabour *et al*., 2004; Mork *et al*., 2005; Anderson *et al*., 2006; Vancraeynest *et al*., 2006; Aires-De-Sousa *et al*., 2007; Peles *et al*., 2007). Two pulsotypes that represented 63 strains together is (72, 2%), have been found and that have been charged of case of mastitis at the bovines in Brazil (Cabral *et al*., 2004; Rabello *et al*., 2005). The same observations have been returned in Norway (Mork *et al*., 2005). Of the strains isolated from various cash of hosts (sheep, goats and cows) were genetically near the some of the other. To Canada, of the genotypes bound, respectively closely with rates of 61, 8 and 60%, a large geographical distribution have frequently been found among the isolats of bovines (Sabour *et al*., 2004; Randy *et al*., 2006). A research of the dairy exploitations of sheep in France, proved that the more part of the cases of mastitis to *Staphylococcus aureus* has been provoked closely by some genotypes linked but that have been distributed widely (Vautor *et al*., 2003, 2005).

The results demonstrate that although, several variants of genotypes of *Staphylococcus aureus* are present, only some predominate and that are responsible for bovine mastitis in the region. Their main reservoir, seem to be the infected udder, it is a strain to mammary reservoir, it survives, increase and persist essentially in the breast and on skin of milk them, in particular in the infected districts. The cows to chronic infections, constitute the main source of infection of the healthy animals, colonize skin of their milk their breast then (Jorgensen *et al*., 2005). The transmission between the cows, in lactation, essentially makes it self during the milking.

CONCLUSION

The genotypic study of *Staphylococcus aureus* by the methods described in the present study permitted a better characterization and discrimination of this bacterium. They could serve to develop measures of control, while founding on a better comprehension of the epidemiology of the infection. The Pulsed Field Gel Electrophoresis (PFGE) is an interesting technical but doesn't permit to individualize the strains. It could be interesting to add another molecular technique and we could go further as returning in raisings to see what animals had these strains and what is their origin (local birth, purchase, contacts with the other animals of another raising, pension, ready) to make a real epidemiological study about it.

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