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Sources of Contamination of Bovine Milk and Raw Milk Cheese by *Staphylococcus aureus* Using Variable Number of Tandem Repeat Analysis

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Abstract

Milk and dairy products are frequently implicated in food-borne infections caused by *Staphylococcus aureus* and infected animals may contaminate bulk milk. In addition, human handlers, milking equipment, the environment, the udder and the teat skin of dairy animals are possible sources of bulk milk contamination.

The main objective of this study was to identify and prioritize the sources of *S. aureus* contamination of bulk milk and raw milk cheese, and secondly to investigate the diversity of strains involved in bovine mastitis, and test the controversial hypothesis of cross-infection between humans and animals.

Four farms manufacturing cheese from raw milk with a total of 135 dairy cows were selected. Bacteriological analyses were performed on quarter milk samples, swabs of udders and teats, the milking machine, bulk milk, cheeses, swabs taken from staff members' hands and nasal cavities. Typing of *S. aureus* isolates was carried out using the Multiple Locus Variable Tandem Repeat Analysis [MLVA] including five genes [*clfA, clfB coa, fnb* and *SAV1078*] combined with the investigation of the presence of staphylokinase gene [*sak*].

A total of 537 isolates were genotyped. The genotyping results confirmed that most intramammary infections in each farm were due to a prevalent genotype. The majority of genotypes present on the teat skin were also isolated from quarter milk samples. These isolates are the main sources of the contamination of bulk milk and cheese. The identity of certain genotypes characterized in both humans and animals was sometimes associated with the presence of the *sak* gene and suggests the existence of cross-contamination and also the occasional involvement of human handlers in the contamination of milk and cheeses.

Keywords: *S. aureus*; MLVA typing; Mastitis; Contamination; Bulk milk; Cheese

Introduction

Staphylococcus aureus is an important cause of declared foodborne, and milk and dairy products are among the foods most often implicated [1]. Moreover *S. aureus* is a major pathogen responsible for severe nosocomial and community-associated infections of humans. Livestock strains may have the capacity to colonize humans and humans represent an important source of new pathogenic strains affecting livestock [2-6]. Controlling milk and cheese contamination is an economic and health stake for all raw milk productions.

A decrease in *S. aureus* contamination of raw milk cheese can only be achieved by reducing S. aureus in the raw milk and by limiting contamination throughout the process of cheese manufacturing. Identifying sources of contamination in dairy herds/cows is key to the development of targeted, appropriate control measures.

Many sources of milk and cheese contamination by *S. aureus* exist including housing materials, milking equipment, bovine teat skin and humans. Dairy animals and more specifically infected mammary glands are commonly accepted as the main cause of contamination [7-10]. However, when mastitis control measures are implemented and the milk from *S. aureus* infected cows is excluded, new infections and contamination of the bulk milk and cheese will continue to occur as eradication is difficult to achieve. This means that other sources of contamination exist, either on the dairy farm or in dairy processing plants.

Analysis of variable numbers of tandem repeat of loci [MLVA] is relevant to discriminate large number of *S. aureus* isolates from dairy surroundings [9-11] thereby providing better possibility of control. Moreover typing of isolates could help further evaluate the diversity of strains involved in mastitis in a given herd in relation with their sensitivity to antibiotics and thus test the hypothesis often put forward of a prevalent strain leading to a predominant sensitivity [or resistance] profile.

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The main objective of this study was to identify and prioritize the sources of contamination by *S. aureus* in bulk milk and cheeses produced from raw milk by using MLVA. The secondary objectives were as follows: (a) Investigate the diversity of isolates involved in mastitis for a given herd. (b) Test the hypothesis that a single isolate is present in the quarter in the case of mastitis. (c) Test the currently controversial hypothesis of cross-infection between humans and animals.

Materials and Methods

Herd selection

Four dairy farms, A, B, C and D, with 37, 26, 40 and 32 cows, respectively, producing rennet type raw milk cheeses, were selected on the basis of monthly counts of *S. aureus* in cheese, demonstrating a significant and persistent contamination except for herd D (Table 1).

Herd	No. of controls	No. of S. aureus (x10^3)/g of cheese			
		<100	100 to 500	500 to 1500	>1500
A	9	11.1%	33.3%	33.3%	22.2%
B	8	12.5%	50%	25%	12.5%
C	6	33.3%	33.3%	33.3%	-
D	6	83.2%	16.8%	-	-

Table 1: Levels of contamination by *S.aureus* in cheese samples in the period preceding the start of the study in the selected herds.

Type of samples and procedures for the collection

Quarter milk samples: Aseptic foremilk samples (approximately 2 ml) of all quarters from all cows present in the herd were collected by the Comite Interprofessionnel du Fromage staff in sterile vials, on one occasion for each farm.

Smear of udders and teats: Random survey on approximately 30% of cows in each herd, repeated twice (not necessarily the same animals), using 10% sterile wipes soaked with peptone water and after a summary cleaning with a wet wipe without antiseptic or soap.

Milking equipment: In the beginning of milking, suction of approximately 5 to 8 liters of UHT milk by one of the teacup clusters with circulation throughout the entire milking circuit and sampling of 15 ml in a sterile vial.

Bulk milks and cheeses: Samples were taken according to the methods already used in existing controls for ground Tomme cheese, a step corresponding to the peak of staphylococcal growth within 24 h.

Human: Swabs from the palms and nostrils were performed just before milking of the first cow for each of the milkers and just before the decurdling and intervention in the cheese factory for the processor(s).

The number of samplings according to the source is shown in Table 2. All samples were collected in sterile bottles and immediately refrigerated(4°C to 10°C), (quarter milk samples, nostrils, hands and skin teat swabs, milk mixtures and cheeses) and sent to the Laboratoire Departemental Veterinaire (Marcy- l'Etoile) where they were frozen (-25°C) until analysis.

Herd	Teat	Bulk Milk	Cheese	Human[a]
A	9	2	1	Nose 5 Hands 5
B	21	3	3	Nose 5 Hands 5
C	23	3	3	Nose 4 Hands 5
D	20	1	2	Nose 5 Hands 5

Table 2: Origin and number of samples taken on study farms. [a]Samples taken from the farmer, the manufacturer and the sampler.

Bacteriological analysis

All samples were inoculated onto solid medium selective NA (nalidixic acid) and then Baird Parker RPFA agar (Rabbit Plasma Fibrinogen, bioMerieux, Marcy-l'Etoile)) on which staphylococci colonies appear black, shiny, convex, surrounded by a clear halo, which actually characterizes the production of clumping factor.

BHI broths (Brain Heart Infusion, bioMerieux) were used when the agar cultures of quarter milk samples were negative, likewise for first line teat smear wipes.

The colonies were subcultured on TSA (Trypticase Soy Agar) and an investigation for free coagulase was performed (rabbit plasma and bioMerieux PW-T peptone) on colonies selected for the rest of the study.

Selection, conservation and genotyping of *S. aureus* isolates

The number of colonies to be transplanted for each type of sample for genotyping was equal to the square root of the number of colonies present on the selective primary isolation medium with a maximum of 10. Each colony was transplanted in deep TSA agar to be preserved.

The genotyping method used was the MLVA (Multilocus Variable Number of Tandem Repeat Analysis) method for the following five genes, *clfA*, *clfB*, *coa*, *fnb* and *SAV1078* as previously reported [11] and used for genotyping of strains isolated on goat cheese farms [10].

Briefly, this technique takes advantage of the existence of short nucleotide sequences repeated in tandem and of variable number depending on the strain, which makes it possible to discriminate them based on the migration of the bands obtained after amplification by PCR and electrophoresis. For each herd, genotypes defined by identical electrophoretic profiles obtained for the selected genes containing specific repeated sequences were given arbitrary numbers according to their recording in the study.

In addition, the presence of the *sak* gene encoding staphylokinase was investigated [11] as a potential marker of strains of human origin.

Results and Discussion

Bacteriological analysis

Although several species of staphylococci, in particular *S. hyicus* and *S. intermedius* can produce free coagulase, a criterion for identification of coagulase-positive staphylococci, the simultaneous presence of *coa* and *clf* genes that encode free coagulase and clumping factor, respectively used in the present study, confirmed that the isolates studied belonged to the *S. aureus* species.

Three herds, A, B, and C, had a high prevalence of intramammary infection, 37.8%, 33.7% and 39.4% of quarters, respectively and represented approximately 2/3 of the cows (Table 3). This high prevalence could be related to the high contamination of cheeses and suggests, even in the absence of typing results, that intramammary infections were an important source of contamination. This was supported by the low prevalence of mammary infections by *S. aureus* observed in herd D and associated with a relatively low contamination of the cheeses.

Herd				
	A	B	C	D
No. of cows	37	26	40	32
No. of infected cows	25	16	29	2
	67.5%	61.5%	72.5%	6.3%
No. of cows according to the No. of infected quarters				
1 quarter	8	4	10	2
2 quarters	6	6	7	0
3 quarters	8	5	9	0
4 quarters	3	1	3	0

Table 3: Prevalence and distribution of *S. aureus* mastitis infections in herds.

Genotyping of different isolates

A total of 537 isolates were genotyped, 272 of which belonged to herd A, 80 to herd B, 127 to herd C and 58 to herd D (Table 4).

	Herd A	Herd B	Herd C	Herd D
Quarter milk samples	Genotype 1 (241) Genotype 2 (1) Genotype3 (6) Genotype 4 (2)	Genotype7 (1) Genotype 8 (5) Genotype 9 (7) Genotype 12 (1) Genotype 13 (1) Genotype 14 (10) Genotype 15 (6) Genotype 16 (1) Genotype 17 (1)	Genotype 18 (51)	Genotype 22 (5) Genotype 23 (5)
Skin of teats	Genotype 1 (1)	Genotype 14 (4) Genotype 15 (4) Genotype 11 (1) Genotype 17 (1)	Genotype 18 (32) Genotype 19 (1)	Genotype 22 (6) Genotype 24 (5) Genotype 25 (4) Genotype 26 (6)
Milking machine			Genotype 18 (4)	
Human nasal cavities	Genotype 1 (2) Genotype 5 (1) Genotype 6 (2)	Genotype 13 (1) Genotype 17 (1)	Genotype 20 (5) Genotype 21 (5)	Genotype 27 (1) Genotype 28 (1) Genotype 29 (1) Genotype 31 (1) Genotype 32 (10) Genotype 30 (1)
Human hands		Genotype 9 (2)	Genotype 18 (1)	Genotype 24 (1) Genotype 33 (1)

Bulk milk	Genotype 1 (1)	Genotype 1 (2) Genotype 9 (2) Genotype 14 (2) Genotype 7 (4) Genotype 15 (2)	Genotype 18 (14)	Genotype 34 (1)
Cheese	Genotype 1 (14) Genotype 3 (1)	Genotype 8 (1) Genotype 1 (7) Genotype 9 (4) Genotype 15 (1) Genotype 11 (5) Genotype 10 (1) Genotype 12 (2)	Genotype 18 (14)	Genotype 22 (3) Genotype 35 (1) Genotype 36 (5)
TOTAL	272	80	127	58

Table 4: MLVA types of *S. aureus* identified by electrophoretic profiles and number of isolates per genotype.

A lack of signal was sometimes observed for certain genes used for the MLVA, which is likely to affect the discriminatory power of genotyping, which, as a result was only based on four genes. This phenomenon may result from modification of the genome of the isolate subjected to genotyping at the location of the attachment of one of the two primers used in the PCR.

It should be noted that within the same herd certain genotypes (MLVA profiles) differ only by a single gene or the absence of a signal for a gene. This was the case for 3 profiles in herd A, 4 in herd B and 8 in herd D. This suggests that, provided that there was no bias resulting from the implementation of typing, these quite similar strains belonging to these quite similar genotypes could be from a common "ancestor" having undergone mutations. However, when the differences between two genotypes relate to small differences in the size of the resulting fragments observed on agarose gel, these can be interpreted as the result of deletions affecting a few base pairs. The data may suggest the existence of variants of the same genotype rather than two different genotypes. This was observed in particular for isolates of the quarter milk samples of herds A and B. In this respect, the comparison of antimicrobial susceptibility profiles of such isolates could be of interest, especially if they prove different and/or reveal relatively unusual resistance to certain antibiotics.

Only one sample from the milking machine (herd C) led to the identification of an isolate of *S. aureus* , for which the MLVA type was also identified from isolates present in the quarter milk sample, on the teat skin, in the bulk milk, cheese and on hands of the breeder. This can be interpreted as evidence of effective cleaning procedures implemented by farmers in herds A, B and D leading to an absence of biofilm, without excluding the effect of dilution of the sample, limiting the likelihood of isolation of the pathogen.

Isolates from quarter milk samples

The comprehensive review of the profiles Table 4 shows that the diversity of genotypes of isolates from quarter milk samples was associated with the genotype diversity of bulk milk and cheese isolates. Thus, in herd B, 9 different profiles were characterized in quarter milk samples and 7 in cheeses, compared to one respectively for herd C. This relationship is confirmed by the fact that for all herds, certain genotypes present in cheese and bulk milk were also present in quarter milk samples. This indicates that *S. aureus* intramammary infections were a significant source of bulk milk and cheese contamination as has already been reported after typing of isolates by pulsed field gel electrophoresis (PFGE) [8,12,13].

MLVA typing results confirmed that S. aureus intramammary infections of dairy ruminants were mostly due to a prevalent strain circulating in the herd as previously reported [7-9]. Thus, a single genotype was identified in herd C, 2 in herd D and out of 250 isolates in herd A, 241 belonged to the same genotype (Table 4). It is recognized that *S. aureus* is a pathogen essentially located in the mammary gland,therefore the prevalence of a genotype in a herd in which the infection rate is also high suggests that some hygiene recommendations/milking techniques are not followed and/or are ineffective. Relying on the fact that there is a predominant strain, sometimes it is recommended to investigate possible antibiotic resistance from a single isolate and generalize the results for the choice of treatments to all of the herds *S. aureus* isolates. In so far as resistance factors are often on mobile elements (plasmids), while genotyping techniques are mostly based on the analysis of the bacterial chromosome, it is possible that isolates belonging to the same genotype have different antibiotic sensitivity profiles. Moroni et al. reported no genetic variability among the strains isolated from udder half of goats although isolates did vary in susceptibility to various antimicrobial agents [9].

Herd B differs from the 3 others since 9 different MLVA profiles were characterized for the 51 isolates of quarter milk samples and, in the absence of information this important diversity leads us to reflect on how this herd was managed. For example, are new animals frequently introduced into the herd? This diversity could account for cases of treatment failure in certain herds and more generally for the difficulty in controlling *S. aureus* contamination of milk and cheeses.

The genotype predominantly present in herd A (genotype 1) was also identified in herd B (genotype 8). It was previously reported that isolates with the same PFGE pattern were detected on geographically distant dairies [13]. This could be explained by the presence of a common source of the contamination. This hypothesis should be explored because some isolates had the *sak* gene suggesting an identical human origin.

In one case, two different genotypes (genotypes 1 and 5) were identified in milk from the same quarter as a cow in herd B. The same observation about the existence of two different pulsotypes present in the milk of a single quarter has also been reported [14]. In this case, we cannot rule out the accidental contamination of the sample at the time of sampling. To confirm the possible coexistence of two different genotypes in the same quarter, a new collection of quarter milk samples would have been necessary. The confirmation of such a case, probably rare, may explain some treatment failures and promote the exchange of resistance factors between genotypes.

Teat skin isolates

According to Zadoks et al. [15] the *S. aureus* pulsotypes present on the teat skin of cows are different from those identified from quarter milk samples and teat skin is not an important reservoir for bovine intramammary infections. However, in our study, for all herds, with a few exceptions (in particular herd D), all of the genotypes identified on the teat skin were also present in the quarter milk samples, the bulk milk and cheeses (Table 4). This is in keeping with the importance of teat disinfection to control mastitis and the contamination of bulk milk and cheese. However, despite the practice of pre-teat dipping in herds A, B, C and post-teat dipping in the 4 herds, we found that the teats were contaminated between milking sessions. It is always difficult when evaluating the effectiveness of the teat dipping to determine what is attributable to the practice or to the products or to the manner in which the teat dipping is applied. It has been reported that the conventional cleaning of udders with wipes was very effective in eliminating *S. aureus* present on the teats as opposed to pre-teat dipping [16] and that post-teat dipping also had limited effectiveness [17]. The only herd not practicing pre-dipping of teats (herd D) had the lowest rate of cows infected with *S. aureus*.

Bulk milk isolates

The diversity of genotypes present in bulk milk is a little less than that observed from quarter milk samples (Tables 4 and 5), probably due to a dilution effect that reduces the probabilities of strain isolation. With the exception of herd D, all genotypes present in bulk milk were also present in quarter milk samples.

Isolates No.	Profiles No.	Cow		Human	
		Quarter	Teat	Nose	Hands
28	8	6/8	4/8	1/8	2/8

Table 5: Origin of the 28 *S. aureus* isolates and distribution of the number of MLVA profiles identical to those identified in the bulk milk.

Subject to the limitations of the procedure for analysis of bulk milk indicated above, the results suggest that there is very little contamination by *S. aureus* strains of extra-mammary origin. The main sources of contamination of milk were quarter milk samples and teat skins, while humans (nasal cavities: herd A; hands: herds B and C) were occasionally a source of contamination if we consider that *sak* gene is a marker for human isolates.

Cheese isolates

Similar interpretations to those made for bulk milk can be transposed to the contamination of cheese (Table 6): the main sources were quarter milk samples and teat skin of cows, as previously reported by Jorgensen et al. [8] and occasionally humans (nasal cavities: herd A; hands: herds B and C). It should be noted that in herds A, B and C, the same staff does the milking and also makes the cheese, which may explain the presence of identical genotypes. For herd D, for which the staff doing the milking is different from those making the cheeses, the same genotype present in the quarter milk sample and teat skin was also present in cheese, but no genotype identified in the humans was present. In herds A, B and C, the same genotype was identified in two samples of cheese made carried out 9 days, 22 days and 42 days apart, respectively, demonstrating the existence of a persistent contamination by the same *S. aureus* isolate.

For all of the genotypes identified in the products, we noted, by comparison with the three other herds, particularities for herd D which was also the one with the lowest prevalence of infection: no genotype present in humans was found in bulk milk and cheese, suggesting more hygienic conditions for milk collection and processing.

Isolates	Profiles	Bulk milk	Cow		Human	
			Quarter	Teat	Nose	Hands
59	13	4/13	5/13	5/13	1/13	2/13

Table 6: Origin of 59 *S. aureus* isolates and distribution of 13 MLVA profiles identical to those identified in the cheese.

Isolates of human origin

In general, the nasal cavities of staff involved in the breeding were found to be much more "rich" in terms of different profiles than the hands (Table 4), which can be explained by cleaning hands before milking as called for in the protocol. No *S. aureus* genotype identified in humans was identified in cows in herd D.

In herds A, B and C, *S. aureus* isolates of potential human origin [*sak*+] present in the cow came from both the nostrils and hands of personnel usually present on these farms, breeder and milker (Table 7). More surprisingly, in herd B, two genotypes [13,17] present in the nasal cavities of the sampler, external to the farm, were also present in the quarter milk sample, one of which was also found on the teat skin. In the latter case, the sampler was most likely the origin of the contamination of the samples. The question was also raised for herd A in which the same MLVA profile (genotype 1) was identified both in the nose of the milker, the quarter milk sample, the skin teat, the bulk milk and cheese.

In what may seem surprising, isolates belonging to the same genotype either possessed or did not possess the *sak* gene. This is explained by the fact that the gene is carried by a prophage, which is a mobile element that may be transferred or lost by the bacterial cell.

However, we cannot rule out that this involves different variants or isolates that the typing method used here could not distinguish.

Out of a total of 29 isolates with the *sak* gene, 18 were isolated from human sources (Table 7), which represents 62.1% of all human isolates. Thirteen different MLVA profiles were characterized in the nasal cavities and the *sak* gene was demonstrated in eight of them.

Origin	Herd			
	A	B	C	D
Quarter milk sample	1 a	1 ab	-	-
Skin of teats		1 b	-	5
Human hands				1(F)
Human nasal cavities	1 a (S), 2(F)	1 b (S)	5(S), 5(F)	3(S)
Bulk milk		2 a	-	-
Cheese		1 a		

Table 7: Number and origin of *S. aureus* isolates possessing the sak gene. S=sampler; F= farmer. Identical letters in each column correspond to the existence of identical isolate profiles.

This high prevalence confirms that this gene is probably a good potential marker of strains of human origin because it is less common in animal *S. aureus* isolates [18,19].

The identity of the MLVA profiles found in humans and animals added to the fact that human isolates with the *sak* gene were also present in animals strongly suggests that cross-contamination between humans and animals exists. In their study Sakwinska et al. [4] reported that more one third of mastitis isolates belonged to CC8, a clonal complex well known to be of human carriage and infection. They suggest the emergence of a new bovine-adapted genotype due to a recent host shift from human to cow concurrent with a loss of ability to colonize humans.

Conclusion

The MLVA typing method used in this study appears to be sufficiently discriminating to characterize different *S. aureus* genotypes and, therefore, identification of the source of contamination in dairy products. It is much simpler to implement than the technique currently recognized as the gold standard, the pulsed-field gel electrophoresis (PFGE). For all that, its current cost prohibits PFG E being used routinely. In our view, MLVA is advantageous when the conventional methods of contamination control, milking hygiene and/or treatment and/or reforms are ineffective in solving problems on a farm.

The results presented show that the diversity of MLVA profiles for quarter milk isolates varies from herd to herd, low for herds A, C and D, and high for herd B (Table 4). The limited number of herds included in this study did not clarify whether this diversity of genotypes, which may have implications for the effectiveness of treatment with antibiotics, can be recorded generally. Other authors have also reported the prevalence of a genotype in a given herd [14,18]. Moreover, this diversity of genotypes is sometimes relative, with the profiles only differing in a single gene. Additional research would be required to determine if the observation is the result of a deletion in the "ancestor" strain or variants of a given genotype.

The high prevalence of the *sak* gene in isolates from the nasal cavities and hands of farm staff confirms that it can actually be considered a potential marker of strains of human origin.

In our study, only 2 strains isolated from quarter milk sample had the *sak* gene, a result that is in agreement with that of Monecke et al. [18] who noted the absence of this gene in 90% of bovine mastitis isolates, whereas the gene is present in 50% of goat isolates [10]. Among the non-exclusive possible explanations for this difference are the lack of pre-teat dipping and cleaning of the udder in goats and the absence of significant environmental flora, which can compete with *S. aureus.*

MLVA profiles with identical isolates possessing or not possessing the *sak* gene have been identified in humans and animals, suggesting the existence of cross-contamination. These cross-infections between cows, humans and dairy products emphasize the importance of milking hygiene as well as the precautions to take during collection of milk samples destined for bacteriological examination.

Although constituting the main source of contamination of bulk milk and cheese, our results show that sources other than quarter milk may exist, particularly, although marginal, staff working on the farm. This goes against the results of Hata et al. [19] who concluded that pulsotypes isolates found in bulk milk are always different from those isolated from humans, but confirms the identity of certain strains of mastitis and human strains reported by other authors [4,18-20]. These differences can be explained by the fact that isolates submitted to genotyping are taken randomly both in terms of the "time" of the collection and the selection of colonies on the primary isolation medium. The results are more or less representative of genotypes actually present on farms. Therefore, in order to limit the impact of this bias, we repeated the samples after a relatively short period for some sources.

Controlling the contamination of cheeses by controlling mastitis and teat contamination in some cases may not be sufficient. Other sources during the manufacturing process may also be involved. The MLVA genotyping method used in this study could therefore be useful to identify them.

The identification of sources of contamination by highly discriminatory genotyping methods of strains of *S. aureus* such as the MLVA method used here, offer better possibilities of control in a situation of failure. This method also evaluates the diversity of strains involved in mastitis for a given herd in relation to their sensitivity to antibiotics, and therefore tests the often advanced hypothesis of a predominant strain leading to a dominant sensitivity or resistance profile, or to evaluate the possible emergence of methi-R strains of human origin.

Among the other possible future “benefits" from the culture collection of *S. aureus* carried out in this work, in addition to antibiotic susceptibility profiles, are phylogenetic studies and studies relating to sensitivity to antiseptics and the characterization of genes encoding enterotoxins.

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