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**Molecular characterisation of *Staphylococcus aureus* strains isolated from small and large ruminants reveals a host rather than tissue specificity**

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## Abstract

*Staphylococcus aureus* is an important pathogen in domestic ruminants. The main objective of this study was to determine the similarity of epidemiologically unrelated *S. aureus* isolates from bovine, ovine, and caprine hosts regardless the locus of isolation (nares and udder). By pulsed-field gel electrophoresis, 7 major pulsotypes were identified among 153 isolates recovered from 12 different regions of France as well as from Brazil, the USA and Belgium. Typing of the accessory gene regulator (*agr*) and capsular (*cap*) serotype was carried out on all the isolates and revealed the predominance of *agr*I and III and of *cap*8 regardless the ruminant host species. Screening for MRSA was carried out by disk diffusion and revealed a prevalence of only 3.2% of MRSA among the strains tested. These results suggest the existence of a host rather than tissue specificity among *S. aureus* isolates colonising the ruminant species and suggest a limited transmission of those isolates between large (bovine) and small (ovine-caprine) ruminants. The *agr* class and *cap* types correlated with pulsotype clusters rather than with a specific host species. Antimicrobial resistance appears not to have contributed to the predominance of any given genotypes, and MRSA prevalence appears very low in ruminant isolates.

**Keywords:** *Staphylococcus aureus*, Ruminant, host specificity, mastitis, PFGE

## 1. Introduction

*S. aureus* is one of the most frequent etiologic agents of mastitis in bovines, ovines and caprines, rendering livestock unable to adequately produce milk, which results in heavy economic losses for the dairy industry (Seegers et al., 2003). Studies characterising ruminant isolates of *S. aureus* are scarce compared to the much more abundant data on human isolates. Devriese et al. first described phenotypic differences between *S. aureus* strains isolated from humans and other animal hosts including bovines and ovines, and proposed a scheme for the distinction of several host biotypes (Devriese and Oeding, 1976). Differences between host biotypes are also reflected at the genotypic level as determined by macro-restriction analysis of the chromosome (Hennekinne et al., 2003). Due to the specificity of host-pathogen interactions needed to produce mastitis, it has been postulated that the nature of the virulon and the regulation of its expression are determining factors when it comes to the ability of a strain to produce mastitis (Vautor et al., 2008). The recent release of the complete genome sequence of *S. aureus* ET-3, a bovine isolate, provides new insight into the genomic basis of a putative host adaptation and the existence of host specific genetic traits in *S. aureus* isolated from bovine hosts (Herron-Olson et al., 2007). We recently showed that diversification of the *S. aureus* core genome correlated with host origin in ruminants (Ben Zakour et al., 2008). However, in most studies, the panel of ruminant *S. aureus* strains comprised mostly strains isolated from cases of mastitis. The mammary gland tissues present characteristics such that some authors hypothesized that the specific traits found to be common in bovine strains were related to a tissue- rather than to host- specificity (van Leeuwen et al., 2005). In the present study, we characterised several *S. aureus* isolates obtained from different sites of colonisation / infection, from ruminants in different geographic regions, by PFGE, and further characterised the strains as to their *agr* group and capsular polysaccharide genotype. The aims of this characterisation were to verify whether the strains groups reflected a host- or tissue-

adaptation, whether there is a predisposition of certain *cap* or *agr* types to colonise or infect certain ruminant hosts.

## 2. Material and Methods

### 2.1. Bacterial strains.

In order to minimise bias when evaluating clonality by PFGE, we chose to include in the study strains from different geographic and temporal backgrounds. A total of 153 *S. aureus* isolates were either chosen from existing collections (n=114) or were collected specifically for this study (n=39). Isolates from bovine (65 strains), ovine (57), and caprine (31) hosts were collected between 1964 and 2006 in France (12 different regions, n=117), and Brazil (2 different regions, n=30). The panel of strains included also isolates from Belgium (n=5) and the USA (n=1). Strains chosen from existing collections were confirmed to be *S. aureus* by a specific PCR test (Baron et al., 2004). The strains collected in this study were isolated from the udder (mastitic milk) and nares of bovine, ovine, and caprine hosts as described previously (Vautor et al., 2003). Samples were first grown on selective Baird-Parker medium. Species identification was carried out on coagulase positive staphylococci using previously described PCR tests (Baron et al., 2004; Morot-Bizot et al., 2004). Details of the sampling used in this study (locus of isolation and host) are given in Table 1.

**2.2. PFGE.** Pulsed-Field Gel Electrophoresis for the characterisation of the strain lineage was carried out according to previously described protocols (Prevost et al., 1992b). The band profiles obtained for each strain were analysed using the Bionumerics software, version 2.0 (Applied-Maths, Belgium).

**2.3. Determination of the *agr* group.** Classification of strains into *agr* interference groups was carried out by PCR according to Gilot *et al.* (Gilot and van Leeuwen, 2004), which

involves a forward primer common to all *agr* groups and four primers, each one specific to each *agr* group.

**2.4. Determination of the capsular polysaccharide type.** The capsular polysaccharide type was determined by means of PCR with primers directed to sequences of the staphylococcal capsular polysaccharide gene (*cap*) specific to either the type 5 (primers Cap5k1 and Cap5k2) or type 8 (primers Cap8k1 and Cap8k2) alleles, as described by Verdier et al. (Verdier et al., 2007). Each strain was submitted to a reaction using either the Cap5k1 and Cap5k2 or the Cap8k1 and Cap8k2 primer pair in independent reactions.

**2.5. Determination of the susceptibility of strains to oxacillin.** Susceptibilities of strains to oxacillin were evaluated by means of the Kirby-Bauer disk diffusion method as described previously (CLSI, 2007).

## **2.6. Statistical analyses.**

A  $\chi^2$  test was used to determine the significance of occurrence of genes in a host specific group by use of Statgraphics version 5.1. The nominal P value for statistical significance was 0.05.

## **3. Results and discussion**

### **3.1. *S. aureus* strains grouped with regard to their host- but not tissue- origin.**

Macrorestriction profile analysis using PFGE is one of the most discriminative methods for *S. aureus* when compared to other genotyping methods (Kuhn et al., 2007; Prevost et al., 1992a; van Belkum et al., 1994). All the strains studied here grouped up into 7 clusters (A to G – figure 1) when analyzed by PFGE considering a similarity cut-off of 48 %. Each cluster was significantly correlated ( $P < 0.05$ ) to the subfamily of the host (bovine for large ruminants or

ovine-caprine for small ruminants) from which the strains were isolated. Three clusters were majorly composed of bovine isolates: 42 out of 45 strains (93 %) are bovine isolates in cluster B; 7/10 strains (70 %) in cluster C; and 7/13 strains (54 %) in cluster E. The four other clusters comprised a great majority of small ruminants isolates: 24 out of 24 strains (100 %) are ovine-caprine isolates in cluster A; 43/49 strains (88%) in cluster D; 7/9 strains (78 %) in cluster F; and 3/3 strains (100 %) in cluster G. Strains isolated from small ruminants could not be distinguished as to whether they were ovine or caprine based on their pulsotype, as they were evenly distributed along clusters A, D, F, and G. Strains isolated from ovine and caprine hosts presented a similarity of up to 100 %, whereas no bovine strain presented a 100% similarity to a small ruminant strain. Previous studies based on CGH analysis of *S. aureus* isolates including a few bovine strains suggested the existence of tissue- rather than host-specific genetic traits (van Leeuwen et al., 2005). However, in these studies, only a few bovine mastitis isolates were analyzed amongst a large panel of human strains. Our results are in contrast to another work showing that there is little or no host preference among *S. aureus* presenting different genotypes (Mork et al., 2005), in which isolates were obtained from milk collected from cases of clinical and subclinical mastitis in ewes kept for meat production. In the present study, we included strains isolated from the nares and udder of various ruminant hosts and results showed that they were evenly distributed amongst each of the clusters (Figure 1). Thus *S. aureus* strains clearly grouped up into clusters which correlated to the type of host they were isolated from. In contrast, there was no significant correlation between clusters and the locus of isolation, geographic origin or date of isolation, as strains from the same geographic origin and similar dates of isolation were found to be spread among different clusters. Our results are in accordance with previous studies which reported that host biotypes, as determined according to Devriese's scheme, are reflected by PFGE profiles (Hennekinne et al., 2003). A recent study by Sung et al. (Sung et al., 2008) which evaluated the relatedness of strains of human and non-human (ruminants and equines) origin also found



that bovine strains tend to cluster into a few specific clonal clusters, which are different to predominantly human clusters. In this study, small ruminant strains were grouped into a bovine and equine cluster (ST771), however, the limited number of small ruminant strains analysed (n=4) make it impossible to conclude on the specificity of small ruminant strains. Lineages might be adapted to colonise a certain type of host independent of the site of colonisation/infection. The fact that there was significant correlation between the sub-family of the animal host and lineage, in spite of the strains having been isolated from very different geographical locations (all major regions of France, plus isolates from Brazil, Belgium, and the USA) and in different years (from 1964 to 2006), strongly indicates that a certain strain has a penchant for the type of host it is successful in living on. Our study is as of yet the most comprehensive study involving strains isolated from small ruminants. It is important to realise that bovine strains are different to ovine and caprine strains, for this must bear an impact on the adoption of strategies for the prevention of mastitis in small or large ruminants.

**3.2. Determination of *agr* group.** The ability of *S. aureus*, as a species, to endure in different niches, playing different roles in the relationship with an animal host is testament to either the regulation of the expression of a panoply of accessory genes, or a variability of strains within the species, with lineages presenting a collection of genes specializing in a particular *modus vivendi* (Ben Zakour et al., 2008; Fitzgerald and Musser, 2001). The expression of the accessory genes in *S. aureus* is under the control of a series of systems that interact with each other to form a network. Of all these systems, the accessory gene regulator (*agr*), a two-component quorum-sensing system has arguably been the most studied in *S. aureus*. Four interference classes related to genetic polymorphisms in the *agr* locus have thus far been described, namely, *agr* groups I, II, III and IV (Jarraud et al., 2002a; Ji et al., 1995). So far, *agr* variability has been only rarely studied in isolates obtained from ruminants (Gilot and van Leeuwen, 2004; Reinoso et al., 2008; Vautor et al., 2008).

In this study, altogether, the most prevalent *agr* group found was *agr* I (n=91, 59.5%), followed by *agr* III (n=40, 26.1%) and *agr* II (n=21, 13.7%). Only a single strain bearing an *agr* IV polymorphism was found (0.6%) and corresponded to a bovine isolate. If stratified by host type (small or large ruminant), within bovines, the prevalence of *agr* groups I, II, III and IV were 54.7%, 17.2%, 26.6% and 1.6%, respectively, whereas within small ruminants, their prevalence was 62.9%, 11.2%, 25.8% and 0%. The ratios of the prevalences of each *agr* group are approximately the same when considering only small or only large ruminants. This suggests that *agr* type does not play a role in host specificity. When considering discrete genotype clusters, it appears that, for a given host, some genotypes have a high prevalence of *agr* I whereas for some others *agr* III is clearly prevalent. It has been previously shown that *S. aureus* strains belonging to *agr* group I have a greater ability of invading epithelial cells and persist in the mammary gland, respectively, suggesting that they are better at causing clinical or subclinical mastitis than strains of other *agr* groups (Buzzola et al., 2007). Indeed, this same study found a disproportionately high prevalence (88%) of *agr* type I strains causing mastitis in bovines. Our study shows that *agr* group distribution among ruminant isolates correlated with genotype cluster rather than with a given host. Of note, the distribution of *agr* groups was tissue-independent since isolates from mastitis milk, udder skin or nares were found in each cluster. This observation is in agreement with Jarraud et al. (Jarraud et al., 2002b), who demonstrated a relationship between the genetic background of the strains and the *agr* allele group. We previously reported the prevalence of *agr* III (46% vs 44% for *agr* I) in small ruminant isolates and a correlation between geographical region and predominance of a given *agr* group was hypothesized to explain predominance in some lineages (Vautor et al., 2008). Here, strains originating from various geographical regions grouped within the same clusters and belonged to the same *agr* group. This suggests that the link between *agr* allele groups and a given region is likely due to the predominance of a given lineage within the region considered.

**3.3. Determination of the capsular polysaccharide type.** The *cap* operon responsible for the biosynthesis of capsular polysaccharides (CP) expressed on the surface of *S. aureus* belongs to the virulon (O’Riordan and Lee, 2004). There are 11 serotypes of CP, however, the majority of strains isolated from humans express either CP type 5 or type 8, for which PCR a test has been developed (O’Riordan and Lee, 2004; Verdier et al., 2007). In contrast, a variable prevalence of different CP types has been observed in strains isolated from ruminants from geographically different regions of the world (Guidry et al., 1997; Poutrel et al., 1988; Sordelli et al., 2000) and the actual role of CP in mastitis is questioned (Tuchscher et al., 2005). Here, strains were CP typed using a PCR test enabling differentiation between CP type 5 and 8. The prevalence of the types of CP (5, 8 or non-typeable) for each lineage can be seen in Figure 1. Altogether, the capsular type 8 was predominant in this study and accounted for 65.4 % of the panel. CP type 5 and the non-typeable CP types accounted for 30.7 % and 3.9 % respectively. When considering the host species, CP type 8 was clearly predominant in small ruminants, with an overall prevalence of 83.1 % in ovine-caprine isolates. In contrast, CP type 5 was slightly predominant in bovine isolates (56.3 %) and the prevalence of one type over the other seemed dependent of the genotype cluster. The bovine clusters B and C were indeed majorly CP type 5. These results are in accordance with previous studies on CP typing of *S. aureus* mastitis isolates showing that most bovine isolates were CP type 5 whereas ovine and caprine isolates were CP type 8 (Guidry et al., 1997; Poutrel et al., 1988). The low prevalence of non-typeable strains in this study contrasted with previous work where up to 76.5% of bovine mastitis strains (Sompolinsky et al., 1985) were found to be non-typeable. However, in this latter study only 17 strains were typed and the geographical region of isolation is not mentioned although this criterion, as shown above, is of great importance and may, in this case, bias the results.

### 3.4. MRSA screening.

MRSA strains present a are much more clonal distribution than MSSA (Enright et al., 2002). Thus, our initial worry was that if we had a high prevalence of MRSA amongst our strains, this might pose a bias to our results, for MRSA strains would tend to cluster up. Nevertheless, only 5 strains (3.2%; 3 bovine strains and 2 caprine strains; dispatched in 5 different PFGE clusters) presented resistance to oxacillin, an antibiotic used for the detection of Methicilin Resistant *S. aureus* (MRSA). Our findings contrast with recent studies in which MRSA was found in the nares of 39% and 10.9% of pigs and horses tested, respectively (de Neeling et al., 2007; Van den Eede A. et al., 2008). Together with other studies (Juhász-Kaszanyitzky et al., 2007), our results confirmed that MRSA prevalence is still low in ruminants.

### 4. Conclusions

*S. aureus* strains are phenotypically and genomically variable. Pulsotyping of ruminant isolates showed that *S. aureus* strains clustered with regard to their host origin (bovine and ovine-caprine) and regardless the locus of colonization or infection. Accessory gene regulator polymorphism and the type (or presence) of capsular polysaccharide are facets of strain-to-strain difference. The prevalence of each *agr* group and *cap* type within each lineage varied with the lineage, however, when analysed within the context of each sub-family of host (small or large ruminant), the prevalences of each *agr* group or *cap* type are about the same either in bovines, or ovines/caprines. This hints at the hypothesis that adaptation to a host is not a result of the regulation of a set of accessory genes, but rather the presence/absence or allelic variation of certain genes. The fact that stains found in small ruminants are different from those found in bovines is important enough to warrant the research and development of different immunoprophylactic products against mastitis in small and large ruminants. The low prevalence of resistance to antibiotics found in ruminants suggests that domesticated livestock is not a reservoir of genes which provide resistance to antimicrobial drugs.

In conclusion, this study shows that the nature of *S. aureus* strains differs between large and small ruminants and suggests the existence of a host rather than tissue specificity. Further studies are being pursued for the determination of the molecular nature of this host specificity and strategies for mastitis prevention in small or large ruminants should take account of these differences.

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372 **Table 1** – *S. aureus* isolates used in this study.

Sample site	No. of isolates		
	Bovine	Caprine	Ovine
nares	14	5	39
udder	51	26	18
Total	65	31	57

373

374



## Figure Legend

**Figure 1. PFGE genotyping of the 153 *S. aureus* strains isolated from ruminants and identification of their *agr*, *cap* group.** I, Dendrogram depicting the PFGE macrorestriction analysis of the chromosome and presenting the percentage of genetic similarity between the 153 strains. The unweighted-pair group method using average linkages and a Dice coefficient (with a tolerance limit of 1%) were used to build the dendrogram. A dashed line indicates the cut-off value (48%) chosen to determine the 7 clusters indicated A to G. II, Host of origin of the strains (black, bovine isolates; white, small ruminant isolates). III, site of isolation of the strains belonging to each of the 7 PFGE clusters identified (light grey, nares; dark grey, udder). IV, distribution of *agr* groups within each of the 7 PFGE clusters (white, *agr* I, light grey, *agr* II, dark grey, *agr* III, black, *agr* IV). V, distribution of *cap* groups in the 7 clusters (light grey, *cap*5, dark grey, *cap*8; white, non-typeable). The last row gives the overall proportions for site of isolation, *agr* and *cap* groups within the entire panel of strains.

