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**Difference in virulence between *Staphylococcus aureus* isolates causing gangrenous mastitis versus subclinical mastitis in a dairy sheep flock**

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**Short title:** *S. aureus* gangrenous mastitis in dairy sheep

22 **Abstract** – *Staphylococcus aureus* mastitis in dairy sheep ranges from subclinical mastitis to  
23 lethal gangrenous mastitis. Neither the *S. aureus* virulence factors nor the host-factors or the  
24 epidemiological events contributing to the different outcomes are known. In a field study in a  
25 dairy sheep farm over 21 months, sixteen natural isolates of *S. aureus* were collected from six  
26 subclinical mastitis cases, one lethal gangrenous mastitis case, nasal carriage from eight ewes,  
27 and one isolate from ambient air in the milking room. A genomic comparison of two strains,  
28 one responsible for subclinical mastitis and one for lethal gangrenous mastitis, was performed  
29 using multi-strain DNA microarrays. Multiple typing techniques (pulsed-field-gel-  
30 electrophoresis, multiple-locus variable-number, single-nucleotide polymorphisms, randomly  
31 amplified polymorphic DNA, *spa* typing and *sas* typing) were used to characterise the  
32 remaining isolates and to follow the persistence of the gangrenous isolate in ewes' nares. Our  
33 results showed that the two strains were genetically closely related and they shared 3 615  
34 identical predicted open reading frames (ORF). However, the gangrenous mastitis isolate  
35 carried variant versions of several genes (*sdrD*, *clfA-B*, *sasA*, *sasB*, *sasD*, *sasI*, and *splE*) and  
36 was missing *fnbB* and a prophage. The typing results showed that this gangrenous strain  
37 emerged after the initial subclinical mastitis screening, but then persisted in the flock in the  
38 nares of four ewes. Although we cannot dismiss the role of host susceptibility in the clinical  
39 events in this flock, our data support the hypothesis that *S. aureus* populations had evolved in  
40 the sheep flock and that *S. aureus* genetic variations could have contributed to enhanced  
41 virulence.

42

43 **subclinical mastitis / gangrenous mastitis / dairy sheep / *Staphylococcus aureus* /**  
44 **microarray**

45

## 46 1. INTRODUCTION

47 Staphylococci are the main aetiological agents of small ruminant intramammary infections  
48 (IMI), and *S. aureus* is the most frequent isolate from clinical IMI cases and coagulase-  
49 negative species are the most frequent in subclinical IMI. The annual incidence of clinical IMI  
50 in dairy sheep is generally lower than 5%, but in a small percentage of herds the incidence  
51 may exceed 30-50% of the animals, causing mortality (gangrenous mastitis) or culling of up  
52 to 70% of the herd [3]. In addition subclinical or hidden *S. aureus* IMI cases occur in 3 to  
53 37% of dairy sheep, and are important for economic, hygienic (dairy products consumers) and  
54 legal reasons in Europe (EU Directives 46/92 and 71/94 defining the bacteriological quality of  
55 milk) [3]. In ewe flocks on farms with small-scale production of raw milk cheese, culling for  
56 *S. aureus* subclinical IMI is not the general rule but when a gangrenous case occurs, the death  
57 of the ewe due to the infection is common, even with emergency antibiotic therapy. If an  
58 animal survives the acute disease, the affected gland becomes necrotic and gradually separates  
59 from the surrounding tissue; in this case, healing can take many months [4, 39]. Why some  
60 cases should remain subclinical while others become gangrenous is not known. Bacterial  
61 features, which can be identified by genetic content, could be responsible [4], or alternatively,  
62 ewe factors might be important.

63 There has been some conflicting data about whether some *S. aureus* strains are more virulent  
64 than others. A large study of 161 human nasal carriage of *S. aureus* in healthy people versus  
65 isolates that caused invasive disease failed to identify genetic markers associated with  
66 infection [18]. In contrast, some human clones of Methicillin-resistant *Staphylococcus aureus*  
67 (MRSA), such as the USA300 and TW isolates, clearly cause unique types of infection [8,  
68 33]. In rabbits, differences in virulence between *S. aureus* strains have clearly been  
69 demonstrated in experimental infection studies as well as in the field [20, 34].

70 In bovine intramammary infection, the virulence of *S. aureus* differs among strains according  
71 to previous studies but no specific virulence factor or combination of factors has been  
72 strongly associated with the severity of mastitis [13].

73 Because most previous studies have focussed on well known virulence factors (e.g.  
74 exoenzymes, toxins, adhesions) to try to explain the difference of *S. aureus* virulence between  
75 subclinical and acute cases of mastitis, the aim of this study was to explore if other genetic  
76 features could be found (e.g. genes evolved in cellular processes, cell-wall synthesis,  
77 transport, intermediary metabolism) that might contribute to virulence differences. This study  
78 was made possible because genetically similar *S. aureus* isolates caused either subclinical  
79 mastitis or gangrenous mastitis within the same flock. Here we used microarray technologies  
80 for the first time to probe the genomes of two isolates. These strains were then compared to  
81 additional isolates over a 21 month period using a variety of typing techniques to investigate  
82 the evolution of *S. aureus* in this flock.

83

## 84 **2. MATERIALS AND METHODS**

### 85 **2.1. Clinical examination and sample collection**

86 The field study was carried out over a twenty-one month period in a dairy sheep farm with  
87 small-scale production of raw milk cheese located in the southeast of France. The sheep were  
88 a crossbreed of the Lacaune breed and the “Rouge du Péone” breed. Three visits were made.  
89 The first was in January 2002 to sample eighty ewes for *S. aureus* subclinical mastitis. The  
90 second was in November 2002 to sample a primipare ewe with *S. aureus* gangrenous mastitis.  
91 This ewe died within 24 h in spite of systemic and intramammary therapies. The last visit to  
92 the farm was made one year later in October 2003 to look for the gangrenous *S. aureus* strain  
93 in the flock after one dry period for the ewes (absence of milk production during five  
94 months). The isolates were recovered from the anterior nares of the ewes and the air of the  
95 milking room. The shepherd stopped the exploitation of the farm at the end of 2003.

96 Bacterial examination of milk samples was performed by streaking 0.1 mL of milk on Baird-  
97 Parker rabbit plasma fibrinogen agar (BPRPFA) medium (AES, Combourg, France).  
98 Moreover, in order to increase the sensitivity of the isolation, 1 mL of milk was incubated for  
99 24 h at 37 °C in 9 mL of Chapman selective broth (AES) prior to streaking 0.1 mL on  
100 BPRPFA medium. Ambient air was sampled by using three plates with BPRPFA medium  
101 exposed to the environment of milking room for 15 min and incubated for 24-48 h at 37 °C.  
102 For the ewes' nares, a swab was rubbed inside each nostril and streaked directly on BPRPFA  
103 medium plates [35, 36]. One randomly chosen coagulase-positive isolate per plate was  
104 confirmed as *S. aureus* by PCR performed on the 23S rDNA gene [31]. On the first visit  
105 (January 2002), 6 out of 80 ewes were found to have subclinical intramammary infection with  
106 *S. aureus* in their milk directly or after growth in selective broth. The six isolates were named  
107 O33, O46, O47, O54, O63 and O64. The second visit (November 2002) was for the isolation  
108 in pure culture of the *S. aureus* responsible of the death of the ewe with a gangrenous mastitis.  
109 This isolate was named O11. The last visit (October 2003) was to look for the *S. aureus* strain  
110 O11 in the nares of the ewes and in the air of the milking parlor. Eight out of 71 ewes were  
111 positive with *S. aureus* in their nares. These isolates were named O193-O200. The ambient air  
112 of the milking room during milking time was sampled and one isolate on the plate with  
113 BPRPFA was randomly selected and named O192.

114

## 115 **2.2. Genomic comparison of the subclinical (O46) and gangrenous (O11) *S. aureus*** 116 **isolates by DNA microarray studies**

117 In the following methods, the isolate O46 (subclinical) was randomly chosen between the 6  
118 isolates responsible of the subclinical mastitis in January 2002. This isolate O46 was  
119 genetically compared with O11 (gangrenous) with the DNA microarrays.

120 A one colour dye DNA microarray was constructed with 188 oligonucleotide probes of 65-  
121 mer, mainly designed from the putative virulence genes of *S. aureus* Mu50. Chromosomal

122 DNA extraction, microarray design, hybridisation and data analysis methods have been  
123 described in detail by Vautor et al. [37]. The results can be found in the supplemental  
124 material. In addition, the two strains were compared using the well-validated, two-colour *S.*  
125 *aureus* seven strain whole genome microarray that has been described previously [40] and  
126 contains 3 623 PCR products representing every predicted open reading frame in the first  
127 seven genome sequencing projects: MRSA252, N315, Mu50, COL, 8325, MW2, MSSA476.  
128 Each strain was co-hybridised with DNA from the reference strain MRSA252, and data were  
129 analysed using GeneSpring 7.2. The array design is available in BμG@Sbase (Accession No.  
130 A-BUGS-17<sup>1</sup>) and also ArrayExpress (Accession No. A-BUGS-17). Fully annotated  
131 microarray data have been deposited in BμG@Sbase (accession number E-BUGS-76<sup>2</sup>) and in  
132 ArrayExpress (accession number E-BUGS-76).

133 Microarray profiles of the strains were compared to a database of previously characterised  
134 human and animal isolates, and clustered using 723 core-variable genes to identify lineages  
135 [18, 32]. The genes which were found “present” in one strain and not in the other using the  
136 DNA microarray were confirmed by PCR. Moreover, all the sixteen isolates were tested by  
137 PCR for the genes found different between O11 and O46 with the DNA microarrays. The  
138 PCR was performed using primers designed with the software Primer3 [29] except for *fnbB*  
139 for which the primers designed by Kuhn et al. were used [16]. Primers are listed in Table I.

140

### 141 **2.3. Typing milk, air and nasal carriage *S. aureus* isolates from the flock**

142 Sixteen *S. aureus* isolates from mastitis milk, air and nares were compared with different  
143 discriminating typing techniques. DNA was extracted with the DNeasy®Tissue kit (Qiagen,  
144 Courtaboeuf, France) according to the manufacturer’s recommendations.

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<sup>1</sup> <http://bugs.sgul.ac.uk/A-BUGS-17>

<sup>2</sup> <http://bugs.sgul.ac.uk/E-BUGS-76>

145 Pulsed-field-gel-electrophoresis (PFGE) of the chromosomal DNA was performed with the  
146 restriction enzyme *Sma*I and subsequent analysis as described by Vautor et al. [36].  
147 Randomly Amplified Polymorphic DNA (RAPD) typing was performed three times for each  
148 isolates [27]. The RAPD pattern with at least one band of difference was considered as one  
149 type and named (R, R1, R2 or R3).  
150 For *spa* typing, the polymorphic X region of the protein A gene (*spa*) was amplified using the  
151 primers *spa*-1113f (5' TAA AGA CGA TCC TTC GGT GAG C 3') and *spa*-1514r (5' CAG  
152 CAG TAG TGC CGT TTG CTT 3') and sequenced<sup>3</sup>. Applying the recently developed  
153 algorithm BURP (Based Upon Repeat Patterns), *spa* types (*spa*-t) were clustered into  
154 different groups with calculated cost between members of a group less than or equal to five.  
155 BURP *spa* clonal complexes (*spa*-CC) were automatically assigned by Ridom Staph Type  
156 software using the code system described on the Ridom SpaServer website.  
157 Multiple-locus variable-number (MLVA) tandem repeats analysis for *sdrD*, *sdrC*, *fnb*, *clfA*,  
158 *clfB*, and SAV1078 genes was performed according to Gilbert et al. [10] and for *coa*  
159 according to Callon et al. [6]. The MLVA profiles for the *S. aureus* strains were determined  
160 by the combination of types of allele found for each gene analysed.  
161 A genotyping method was also used, based on single nucleotide polymorphisms of exotoxin  
162 genes (*ssl*) [1]. The sequences of PCR-amplified internal fragments of three different *ssl*  
163 genes (*ssl2*, *ssl4*, *ssl9*) were compared. These genes encode *S. aureus* superantigen-like  
164 proteins (*ssl*), belong to a family of exotoxins called staphylococcal exotoxins. For coherence  
165 with the literature, these genes, originally named *set2*, *set5* and *set7* by Aguiar-Alves et al.  
166 [1], have been renamed *ssl4* (432 bp), *ssl9* (467bp) and *ssl2* (496bp), respectively [17].  
167 Sequences were compared for single-nucleotide polymorphisms (SNP) using the BioEdit  
168 Software<sup>4</sup>. Finally, a multiplex PCR assay was used for the detection of prophages in the

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<sup>3</sup> <http://www.spa.ridom.de>

<sup>4</sup> <http://bioedit.software.informer.com/>



169 genomes of lysogenic *S. aureus* strains. These PCR results allow the prophages to be  
170 classified into serogroups A, B, Fa, Fb, L or D [23].

171 *S. aureus* surface protein typing (*sas* typing) was implemented using the method described by  
172 Robinson and Enright [26]. Briefly, gene fragments from seven putative or proven surface  
173 protein-encoding loci (*sasA*, *sasB*, *sasD*, *sasE*, *sasF*, *sasH*, *sasI*) were PCR-amplified and  
174 sequenced. Unique nucleotide sequences defined *sas* alleles, and unique series of alleles  
175 defined *sas* sequence types.

176

### 177 **3. RESULTS**

#### 178 **3.1. Genomic comparison of the subclinical (O46) and gangrenous (O11) *S. aureus*** 179 **isolates by DNA microarray studies**

180 Genomic differences between the two strains O11 and O46 are summarized in Table II along  
181 with PCR screening results for the other strains. The core genes (except SAR0940), the core  
182 variable genes (except *sdrD*, *fnbB*, *splE*), and the mobile genetic elements (plasmids,  
183 staphylococcal cassette chromosome, transposons, *S. aureus* pathogenicity islands, but  
184 excepting some bacteriophage genes) hybridize with similar intensity to the genomic DNA of  
185 both strains. PCR confirmed the genetic difference between the strains listed in Table II,  
186 including the presence of SAS0897, SAR1558, SAR2100, SACOL0343 in strain O46 but not  
187 in O11; these genes are typical of *S. aureus* lysogenic bacteriophage (hydrolase, lipoprotein,  
188 repressor, helicase *dnaB*). There were different weak comparative signals in the DNA array  
189 for MW0387 (exotoxin), SAR2036 (CHIPS) and SACOL0886 (enterotoxin K) but these  
190 targets were not confirmed by PCR.

191 The two strains O11 and O46 clustered with isolates of the CC130 lineage from cows (data  
192 not shown). The two strains had in common 3,615 identical predicted open reading frames as  
193 designed from the first seven genome sequencing project, including redundant open reading

194 frames (ORF) printed from multiples strains on the arrays. Moreover, both strains O11 and  
195 O46 had no evidence of free plasmids (data not shown).

196

### 197 **3.2. Typing *S. aureus* isolates from milk, air and nasal carriage in the flock**

198 The PFGE results comparing the sixteen *S. aureus* isolates recovered over twenty one months  
199 are illustrated in Figure 1. Four patterns with more than one band difference were identified  
200 and named OV, OV', OV'' and OV'''. The strain O46 (OV) and the strain O11 (OV') had a  
201 PFGE pattern within three-band difference of each other. The strain O11 and the strains  
202 O193, O194, O195 and O196 had an identical PFGE pattern (OV').

203 Two *spa* types were found: t3568 with the repeats 04-39-17 and t524 with the repeats 04-17.  
204 These two *spa* types, by clustering with BURP (Based Upon Repeat Patterns), belonged to the  
205 *spa*-CC 1773. The other genes found by DNA microarray results in O46 or in O11 (Table II),  
206 distinguishing these two isolates, were used to track by PCR the gangrenous strains in the  
207 ewes' nares.

208 The multiple locus variable-number tandem repeats (MLVA) showed six types named A-F.  
209 With the MLVA, the patterns between O11 and O46 were found different for the genes *sdrD*,  
210 *fnb*, *clfA* and *clfB* (data not shown).

211 The RAPD typing technique showed four types named R-R3. Between the strain O11 (R1)  
212 and the strain O46 (R) three bands of difference were found. The strain O11 had the same  
213 RAPD pattern than O193, O194, O195 and O196.

214 For the *ssl* SNP typing, only *ssl9* showed a difference, which was detected by the nucleotide  
215 replacement of T for G. The detection of prophages by the multiplex PCR showed that the  
216 isolates present on the farm over twenty one months had prophages of serotypes A, B and Fb.  
217 The gangrenous isolate O11 was missing prophage B compared to the subclinical mastitis  
218 isolate O46.

219 The last typing technique used was the highly variable *sas* genes, which was done only for the  
220 strains found identical with all the previous typing techniques (i.e. O11, O193-O196) and  
221 O46. The strain O46 had *sas* type I (*sasA26*, *sasB28*, *sasD20*, *sasE26*, *sasF33*, *sasH36*,  
222 *sasI29*), whereas the strains O11 and O193-O196 had *sas* type II (*sasA34*, *sasB27*, *sasD19*,  
223 *sasE26*, *sasF33*, *sasH36*, *sasI28*). The *sasA* and *sasB* alleles differed at two and one  
224 nucleotide sites, respectively, whereas the *sasD* and *sasI* alleles differed by insertion-deletion  
225 events.

226 In summary, with all the techniques used to discriminate the *S. aureus* isolates, the  
227 gangrenous isolate O11 was found identical to the isolates O193, O194, O195, O196  
228 recovered in the nares of the ewes during the last visit. It was also very closely related to  
229 O192, O199 and O200 isolated in the last visit, varying only in bacteriophage profiles. No  
230 isolate in the first visit was identical to O11. In contrast, the subclinical mastitis isolate O46  
231 was found to be indistinguishable from subclinical mastitis isolates O33, O54 (recovered  
232 during the first visit) and was not found in ewes' nares (the last visit). Some variations in  
233 MLVA and *ssI5* SNP were seen in the remaining isolates from the first and final visits,  
234 although they looked closely related to all of the isolates.

235

#### 236 **4. DISCUSSION**

237 In ewe mastitis it is suspected that *S. aureus* strains have different virulence potential<sup>5</sup> [2], but  
238 the genetic features associated with the different outcomes were unknown. This is the first  
239 comprehensive genomic comparison of an ovine *S. aureus* strain responsible for subclinical  
240 mastitis versus a related strain responsible for a case of acute gangrenous mastitis. We found  
241 genomic differences between these genetically closely related strains but we did not find  
242 differences in genes evolved in cellular processes, cell-wall synthesis, transport, or  
243 intermediary metabolism.

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<sup>5</sup> Bergonier D., personal communication.

244 As defined by Lindsay et al. by microarray [18], the two strains O11 and O46 were closely  
245 related and clustered into the same lineages as two bovine isolates of MLST Clonal Complex  
246 130 [32]. The PFGE and the *spa* typing results confirmed that the two isolates were  
247 genetically closely related (Fig. 1 and Tab. II) [33, 37] although there are allelic variants  
248 between the strains as detected by MLVA, *ssl*/SNP and *sas* genes. The microarray results  
249 found that the gangrenous strain O11 was positive for the *sdrD* gene, which belongs to the  
250 Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM)  
251 family. The human protein that SdrD binds to is not known, but a study on human strains [30]  
252 showed that the strains carrying *sdrD* and *sdrE* had an increased association with bone  
253 infection. In fact, O46 had a variant copy of the *sdrD* gene as determined by the MLVA (data  
254 not shown). One limitation of the microarray studies was that these microarrays were  
255 designed using only genes from seven human *S. aureus* strains [32] so we were unable to  
256 detect animal *S. aureus*-specific genes potentially present in O11 and O46. Moreover these  
257 microarrays do not provide information about the position of genes in the chromosome.

258 Serine protease-like E (*splE*) is one of several highly homologous *spl* genes found on the *S.*  
259 *aureus* genomic island beta (GI $\beta$ ), and is predicted to be a substrate-specific serine proteases  
260 [25]. *splE* was only found in the gangrenous strain O11. The product of this *splE* gene might  
261 participate in the difference in disease manifestation between O11 and O46, as some Spl  
262 proteases possess restricted substrate specificity similar to that of the V8 protease and  
263 epidermolytic toxins [25].

264 Fibrinogen binding protein B (*fnbB*) was missing in the gangrenous O11 compared to O46.  
265 *fnbB* also belongs to the MSCRAMM family, and can bind to host proteins fibrinogen,  
266 fibronectin and elastin, and also play a role in biofilm production [22]. In rabbit flocks with *S.*  
267 *aureus* infections (e.g. pododermatitis, subcutaneous abscesses and mastitis), there are "low  
268 virulence" strains where the infection remains limited to a small number of animal, and "high  
269 virulence" strains which spread throughout the rabbitry [20, 34]. Vancraeynest et al. [34]

270 showed that *fnbB* was less common in high virulence isolates, and is associated with reduced  
271 spread of infection. It is interesting to notice that the O11 gangrenous strain did not have the  
272 *fnbB* compared to O46. Sung et al. [32] have also shown that animal strains of *S. aureus*  
273 generally encode variant types of *fnbB* compared to human *S. aureus* strains.

274 A major genomic difference between the O11 and O46 isolates was the missing prophage B  
275 in the gangrenous isolate. Because only a few genes on the microarray correlated with this  
276 phage, we presume it is relatively unrelated to those in the sequenced genomes.  
277 Bacteriophages are among the most abundant inhabitants of the biosphere, considering that an  
278 environmental sample contains nearly 10-fold more phage particles than prokaryotes [5]. All  
279 sequenced strains of *S. aureus* carry between one and four bacteriophage in their genomes  
280 [19]. The contribution of these prophages to pathogenesis is probably multifactorial. Many  
281 prophage carry putative virulence genes on them. For example, *S. aureus* phages of the phi3  
282 family carrying immune evasion genes (coding for staphylokinase [11], enterotoxins [15],  
283 chemotaxis inhibitory protein CHIPS [7], antiphagocytic protein SCIN [28]), yet integrate  
284 specifically into the beta-haemolysin gene, potentially offsetting the virulence afforded by  
285 carriage of the immune evasion genes. However, these phage are widely distributed in human  
286 isolates, but less common in animal isolates [18, 33], and not found in the ewe isolates of this  
287 study. The integration site of the prophage B is currently unknown as are the rest of the genes  
288 on this putative phage. Bacteriophages are also involved in the horizontal transfer of virulence  
289 genes between isolates, perhaps allowing for adaptation to new environmental conditions  
290 [38], and there is evidence that bacteriophage move during the course of human infections  
291 [12, 21]. A case of phage conversion of exfoliative toxin A in *S. aureus* isolated from cows  
292 with mastitis was documented [9]. The exfoliative toxin is the causative agent of  
293 staphylococcal scalded-skin syndrome in young children. The study suggested the possibility  
294 of horizontal transmission of *eta* gene by temperate bacteriophages among bovine isolates of  
295 *S. aureus* [9]. Under stressful conditions such as the use of antibiotics or UV light, prophage

296 can be induced and cause lysis and death of bacterial populations, potentially decreasing  
297 pathogenic potential of a phage carrying strain. More recent data has suggested that phage  
298 could encode small RNA molecules that control gene regulation in *S. aureus*, and affecting  
299 virulence potential [24]. Therefore the role of bacteriophage in *S. aureus* pathogenesis is  
300 complex, and some phage may actually be a burden to the bacteria and reduce pathogenic  
301 potential.

302 The DNA array technologies used in this study were constructed from human *S. aureus*  
303 strains so it is possible there are differences between O11 and O46 that are not detected by  
304 these microarrays. However, recent studies suggest that animal isolates may only have a  
305 limited number of unique genes [14, 32] so one future direction for this study will be to  
306 implement massively parallel sequencing. The various typing methods used showed that the  
307 isolates O11 and O46 were genetically closely related. The major differences were in the  
308 presence/absence of alleles for *sdrD*, *splE*, *fnbB*, *clfA-B* and the presence of prophage genes  
309 (SAS0897, SAR1558, SAR0940, SAR2100, SACOL0343). Furthermore, the *sas* typing  
310 between O11 and O46 (variations in *sasA*, *sasB*, *sasD*, and *sasI*) indicates minor variation in  
311 additional putative surface products, beside *sdrD*, *fnbB* and *clfA-B*, not revealed by the DNA  
312 microarray technologies. All surface expressed proteins may play a role in binding to specific  
313 host proteins and/or in immune recognition.

314 The sequence-based typing methods, *spa* and *sas* typing, were the easiest methods for  
315 comparing and could be used for short-term epidemiologic studies as well as long-term  
316 epidemiologic or phylogenic studies. The difference in the *spa* repeat between O11 and O46  
317 shows that O11 had a deletion of only one repeat (r39) compared with O46, indicating that  
318 they are closely related and they belong to the *spa* Clonal Complex 1773 (Ridom SpaServer  
319 website) [37]. The sixteen strains belong to the same *spa* clonal complex 1773 underscoring  
320 that the *S. aureus* presence in this flock is probably to link to a common ancestor. The MLVA  
321 typing provided the greatest level of discrimination of the isolates because 6 MLVA different

322 patterns were found, which confirms its utility in epidemiological study in a given herd [10].  
323 PFGE as a band-based, rather than sequence-based, typing method gave subtle differences  
324 between the isolates (Fig. 1) so was not easy to compare in all cases. The *S aureus* exotoxin-  
325 like protein genes (*ssl*) did not exhibit significant allelic variability. Variation between  
326 individual isolates indicated that individual genes or the bacteriophage were capable of  
327 changes in a relatively stable background, suggesting local evolution of *S. aureus* from a  
328 common ancestor over an unknown period of time.

329 Altogether, with all the typing techniques used in this study (PFGE, MLVA, SNP<sub>ssl</sub>, RAPD,  
330 *spa* typing, *sas* typing and by PCR for all the genes found different between O11 and O46)  
331 the strain O11 was found in the nares of four ewes (O193, O194, O195, O196) eleven months  
332 after the gangrenous mastitis case. The ewes's nares are an ecological niche for *S. aureus* and  
333 are important in the epidemiology of mastitis in dairy sheep farms [36]. So with this study we  
334 suggest that this body site could serve for transmission of potentially mastitis gangrenous  
335 strains in dairy sheep flock. The subclinical mastitis genotype was common in the flock, until  
336 a gangrenous genotype emerged and caused a gangrenous disease. Subsequently, the  
337 gangrenous genotype was found in the nares of ewes in the flock. But, we could not assert  
338 that the mastitis gangrenous case was a primary case with a new emerged virulent strain or if  
339 it appear after the nasal carriage had participated to the dissemination of the gangrenous  
340 strains in the flock. Maybe *S. aureus* in this flock has evolved to become more virulent.  
341 Evolution of *S. aureus* is driven by survival of the fittest in different environments, which  
342 include host interaction, antibiotic use, and environmental reservoirs, conditions which may  
343 fluctuate over time [19].

344 Although we cannot dismiss the role of host susceptibility in the clinical events in this flock,  
345 this study could support the hypothesis that different strains of *S. aureus* may have different  
346 virulence potential in ewe mastitis. Surprisingly, absence of a prophage and particular  
347 combinations of putative surface products occurred in the isolate with enhanced virulence. It

348 will be useful to determine whether the evolution of *S. aureus* in this flock may be typical of  
349 *S. aureus* populations in general.

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352

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- 483
- 484

485 **Figure 1.** Representative example of *S. aureus* PFGE pattern of DNA digested by *Sma*I. Lane  
486 R: reference strain of *S. aureus* (strain CIP57.10); 1: O46 (subclinical strain); 2: O64  
487 (sub.); 3: O200 (nares); 4: O54 (sub.); 5: O197 (nares); 6: O198 (nares); 7: O192  
488 (ambient air); 8: O199 (nares); 9: O47 (sub); 10: O11 (gangrenous strain); 11: O194  
489 (nares); 12: O195 (nares); 13: 196 (nares). The subclinical isolates (O46, O47, O64,  
490 O54) had been recovered in January 2002, the gangrenous isolates (O11) had been  
491 recovered in November 2002, the nares isolates (O200, O197, O198, O199, O194,  
492 O195, O196) and the ambient air isolate (O192) had been recovered in October 2003 in  
493 a dairy sheep farm.

494

495 **Table I.** Primers used in the study to confirm the microarray data.

496

497 **Table II.** Typing study of sixteen *S. aureus* isolates in a dairy sheep flock over 21 months.

498

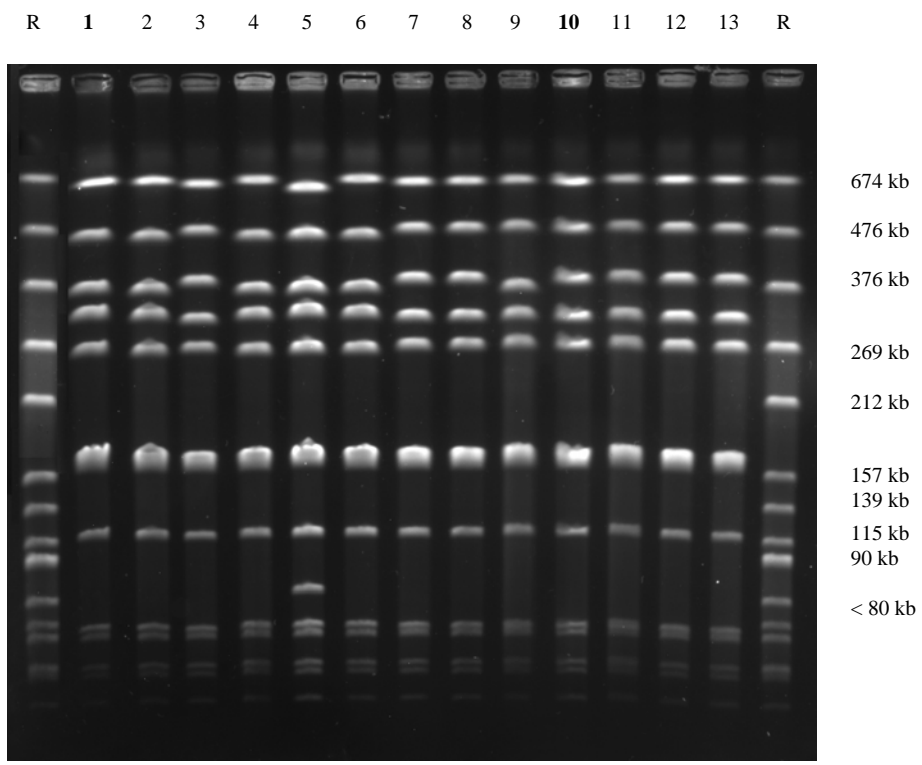
499 **Supplementary data**-DNA array oligonucleotide 65-mer (Excel document)

500

501 Figure 1.

502

503



504



**Table I.**

Gene	Primers used in this study (5'-3') or author's references
<i>SdrD</i> (Mu50 NC_002758)	AACGATTGTACCAGCCCAAG TTTGCAGTCGCAATTGTTTC
<i>FnbB</i>	Khun et al. [16]
<i>SplE</i> (SAR1902)	CAGCCAAAGCCGAACATAAT TATGTGCGCCAATTTCCATA
SAS0897	GAGAACTTGCTGAAGCTATTGGA CCCTCCTTATCAAAATGAGCA
SAR1558	CAAACCAAAAACGCAACAAG CAGGCGAAACGACATACTCA
SAR0940	TTTGCGGACACTGTAGGATG ATTACCCGCTCTCTCACCAA
SAR2100	GCTGATGTTTTTCGAGGTTGG TACACCAGCAGAGACGCAAC
SACOL0343	CAAGCAATGAGGCATTCAGA GTCCGATAGCATTGGTCGTT

Table II.

	O54, O33 (sub.)	O47 (sub.)	O63 (sub.)	O64 (sub.)	O46 (sub.)	O11 (gangrenous)	O193, O194, O195, O196 (nares)	O197 (nares)	O198 (nares)	O200 (nares)	O192 (air)	O199 (nares)
<i>SdrD</i>	-	-	-	-	-	+	+	-	-	+	+	+
<i>FnbB</i> <sup>a</sup>	+	+	+	+	+	-	-	+	+	-	-	-
<i>SplE</i>	-	-	-	-	-	+	+	-	-	+	+	+
SAS0897	+	+	+	+	+	-	-	+	+	+	-	+
SAR1558	+	+	+	+	+	-	-	+	+	-	-	-
SAR0940	+	+	+	+	+	-	-	+	+	+	-	+
SAR2100	+	+	+	+	+	-	-	+	+	-	-	-
SACOL0343	+	+	+	+	+	-	-	+	+	+	+	-
<i>Spa</i> types	3568	3568	3568	3568	3568	524	524	3568	3568	524	524	524
<b><i>Spa Clonal Complex</i></b>	1773	1773	1773	1773	1773	1773	1773	1773	1773	1773	1773	1773
RAPD types <sup>b</sup>	R	R2	R	R	R	R1	R1	R3	R2	R1	R1	R1
PFGE types <sup>c</sup>	OV	OV	OV	OV	OV	OV'	OV'	OV'''	OV	OV''	OV'	OV'
MLVA types ( <i>coa</i> , <i>sdrD</i> , <i>sdrC</i> , <i>fnb</i> , <i>clfA</i> , <i>clfB</i> , SAV 1078) <sup>d</sup>	B	F	E	D	B	A	A	C	B	A	A	A
Prophages <sup>e</sup>	A, B, Fb	A, B, Fb	A, B, Fb	A, B, Fb	A, B, Fb	A, Fb	A, Fb	A, B, Fb	A, B, Fb	A, B, Fb	A, Fb	A, Fb
<i>Ssl9</i> SNP <sup>f</sup>	1	2	2	2	1	2	2	2	2	2	2	2
<i>Ssl4</i> SNP	1	N.D.	N.D.	N.D.	1	1	1	N.D.	1	N.D.	1	1
<i>Ssl2</i> SNP	1	N.D.	N.D.	N.D.	1	1	1	N.D.	1	N.D.	1	1
<i>Sas</i> types <sup>g</sup>	N.D.	N.D.	N.D.	N.D.	I	II	II	N.D.	N.D.	N.D.	N.D.	N.D.

The absence/presence of the genes, *sdrD*, *splE*, SAS0897, SAR0940, SAR1558, SAR2100 and SACOL0343 were evaluated with primers designed in this study.

<sup>a</sup> [16]; <sup>b</sup> [27]: at least one band of difference for each RAPD types; <sup>c</sup> [35] the PFGE profiles were named, OV, OV', OV'', OV'''; <sup>d</sup> [6, 10]: the letters correspond to a unique pattern made by the combination of the variable number-number tandem repeats of each gene; <sup>e</sup> [23]: bacteriophages serogroups.

+ positive with PCR, - negative with PCR; sub.: isolates recovered in a *S. aureus* subclinical mastitis case (January 2002); O192-O200 are the isolates recovered in the last visit to look for the gangrenous strain O11 in the ewes' nares and in the air of the milking room (October 2003). <sup>f</sup> [1]: Single Nucleotide Polymorphism (SNP). The same exotoxin sequence type had an identical numerical number, N.D.: not done; <sup>g</sup> [26]: unique sequences defined alleles and unique series of alleles defined a sequence type (named I or II).