

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

Eric Vautor, Joshua Cockfield, Caroline Le Marechal, Yves Le Loir, Marlène Chevalier, D. Robinson, Richard Thiery, Jodi Lindsay

► **To cite this version:**

Eric Vautor, Joshua Cockfield, Caroline Le Marechal, Yves Le Loir, Marlène Chevalier, et al.. Difference in virulence between *Staphylococcus aureus* isolates causing gangrenous mastitis versus subclinical mastitis in a dairy sheep flock.. *Veterinary Research*, BioMed Central, 2009, 40 (6), pp.56. <10.1051/vetres/2009039>. <hal-00409357>

HAL Id: hal-00409357

<https://hal-anses.archives-ouvertes.fr/hal-00409357>

Submitted on 16 Nov 2009

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Eric VAUTOR^{1*}, Joshua COCKFIELD², Caroline LE MARECHAL³, Yves LE LOIR³,
Marlène CHEVALIER¹, D. Ashley ROBINSON⁴, Richard THIERY¹, Jodi LINDSAY²

¹ Agence Française de Sécurité Sanitaire des Aliments (AFSSA), Unité Pathologie des Ruminants, 105 routes des Chappes, 06902 Sophia-Antipolis, France

² Centre of Infection, Department of Cellular and Molecular Medicine, St George's, University of London, Cranmer Terrace, London, SW17 0RE, United Kingdom

³ INRA, UMR1253 STLO, 85 rue de Saint Briec, 35042 Rennes, France

⁴ Department of Microbiology and Immunology, New York Medical College, Valhalla, 10595 NY, USA

* Corresponding author: e.vautor@afssa.fr

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-17¹) and also ArrayExpress (Accession No. A-BUGS-17). Fully annotated
131 microarray data have been deposited in BμG@Sbase (accession number E-BUGS-76²) 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.

¹ <http://bugs.sgul.ac.uk/A-BUGS-17>

² <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 sequenced³. 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 Software⁴. Finally, a multiplex PCR assay was used for the detection of prophages in the

³ <http://www.spa.ridom.de>

⁴ <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 potential⁵ [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.

⁵ 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 β), 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_{ssl}, 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.

350

351

352

353 **ACKNOWLEDGEMENTS**

354 We acknowledge G. Rossi for excellent technical assistance. We acknowledge H. Meugnier
355 (INSERM U851, Centre National de Référence des Staphylocoques, Lyon, France) for the
356 *spa* typing and clustering. We also acknowledge BμG@S (the Bacterial Microarray Group at
357 St George's, University of London) for supply of the microarray and advice and The
358 Wellcome Trust for funding BμG@S under its Functional Genomics Resources Initiative. The
359 support of the Réseau National GénopoleTM and the CNRS (Centre National de la Recherche
360 Scientifique) are greatly appreciated and we thank Dr P. Barbry. Caroline Le Marechal was
361 recipient of a Ph.D. fellowship from Institut National de la Recherche Agronomique (INRA)
362 and Agence Française de Sécurité Sanitaire des Aliments (AFSSA). Part of this work was
363 supported by INRA-AFSSA transversality project (IMISa project).

364

365 **REFERENCES**

- 366 [1] Aguiar-Alves F., Medeiros F., Fernandes O., Gudziki Pereira R.M., Perdreau-
367 Remington F., Riley L.W., New *Staphylococcus aureus* genotyping method based on
368 exotoxin (*set*) genes, J. Clin. Microbiol. (2006) 44:2728-2732.
- 369 [2] Amorena B., García de Jalón J.A., Baselga R., Duchá J., Latre M.V., Ferrer L.M., et
370 al., Infection of rabbit mammary glands with ovine mastitis bacterial strains, J. Comp.
371 Pathol. (1991) 104:289-302.
- 372 [3] Bergonier D., de Cremoux R., Rupp R., Lagriffoul G., Berthelot X., Mastitis of dairy
373 small ruminants, Vet. Res. (2003) 34:689-716.
- 374 [4] Bor A., Winkler M., Gootwine E., Non-clinical intramammary infection in lactating
375 ewes and its association with clinical mastitis, Br. Vet. J. (1989) 145:178-184.
- 376 [5] Brussow H., Hendrix R.W., Phage genomics: small is beautiful, Cell (2002) 108:13-
377 16.
- 378 [6] Callon C., Gilbert F.B., De Cremoux R., Montel M.C., Application of variable number
379 of tandem repeat analysis to determine the origin of *S. aureus* contamination from
380 milk to cheese in goat cheese farms, Food Control (2008) 19:143-150.
- 381 [7] de Haas C.J., Veldkamp K.E., Peschel A., Weerkamp F., Van Wamel W.J., Heezius
382 E.C., et al., Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial anti-
383 inflammatory agent, J. Exp. Med. (2004) 199:687-695.
- 384 [8] Edgeworth J.D., Yadegarfar G., Pathak S., Batra R., Cockfield J., Wyncoll D., et al.,
385 An outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA)-ST 239
386 associated with a high rate of bacteremia, Clin. Infect. Dis. (2007) 44:493-501.

- 387 [9] Endo Y., Yamada T., Matsunaga K., Hayakawa Y., Kaidoh T., Takeuchi S., Phage
388 conversion of exfoliative toxin A in *Staphylococcus aureus* isolated from cows with
389 mastitis, *Vet. Microbiol.* (2003) 96:81-90.
- 390 [10] Gilbert F.B., Fromageau A., Gelineau L., Poutrel B., Differentiation of bovine
391 *Staphylococcus aureus* isolates by use of polymorphic tandem repeat typing, *Vet.*
392 *Microbiol.* (2006) 117:297-303.
- 393 [11] Goerke C., Papenberg S., Dasbach S., Dietz K., Ziebach R., Kahl B.C., Wolz C.,
394 Increased frequency of genomic alterations in *Staphylococcus aureus* during chronic
395 infection is in part due to phage mobilization, *J. Infect. Dis.* (2004) 189:724-734.
- 396 [12] Goerke C., Wolz C., Regulatory and genomic plasticity of *Staphylococcus aureus*
397 during persistent colonization and infection, *Int. J. Med. Microbiol.* (2004) 294:195-
398 202.
- 399 [13] Haveri M., Taponen S., Vuopio-Varkila J., Salmenlinna S., Pyörälä S., Bacterial
400 genotype affects the manifestation and persistence of bovine *Staphylococcus aureus*
401 intramammary infection, *J. Clin. Microbiol.* (2005) 43: 959-961.
- 402 [14] Herron-Olson L., Fitzgerald J.R., Musser J.M., Kapur V., Molecular correlates of host
403 specialization in *Staphylococcus aureus*, *PLoS ONE* (2007) 2:e1120.
- 404 [15] Iandolo J.J., Worrell V., Groicher K.H., Qian Y., Tian R., Kenton S., et al.,
405 Comparative analysis of the genomes of the temperate bacteriophages phi 11 phi 12
406 and phi 13 of *Staphylococcus aureus* 8325, *Gene* (2002) 289:109-118.
- 407 [16] Kuhn G., Francioli P., Blanc D.S., Evidence for clonal evolution among highly
408 polymorphic genes in methicillin-resistant *Staphylococcus aureus*, *J. Bacteriol.* (2006)
409 188:169-178.

- 410 [17] Lina G., Bohach G.A., Nair S.P., Hiramatsu K., Jouvin-Marche E., Mariuzza R.,
411 Standard nomenclature for the superantigens expressed by *Staphylococcus*, *J. Infect.*
412 *Dis.* (2004) 189:2334-2336.
- 413 [18] Lindsay J.A., Moore C.E., Day N.P., Peacock S.J., Witney A.A., Stabler R.A., et al.,
414 Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus*
415 has a unique combination of surface-associated and regulatory genes, *J. Bacteriol.*
416 (2006) 188:669-676.
- 417 [19] Lindsay J.A., *S. aureus* evolution: lineages and mobile genetic elements (MGE), in:
418 Lindsay J.A. (Ed.), *Staphylococcus: Molecular Genetics*, Caister Academic Press,
419 Norfolk UK, 2008, pp. 45-69.
- 420 [20] Meulemans L., Hermans K., Duchateau L., Haesebrouck F., High and low virulence
421 *Staphylococcus aureus* strains in a rabbit skin infection model, *Vet. Microbiol.* (2007)
422 125:333-340.
- 423 [21] Moore P.C.L., Lindsay J.A., Genetic variation among hospital isolates of methicillin-
424 sensitive *Staphylococcus aureus*: evidence for horizontal transfer of virulence genes,
425 *J. Clin. Microbiol.* (2001) 39: 2760-2767.
- 426 [22] O'Neill E., Pozzi C., Houston P., Humphreys H., Robinson D.A., Loughman A., et al.,
427 A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-
428 binding proteins, FnBPA and FnBPB, *J. Bacteriol.* (2008) 190: 3835-3850.
- 429 [23] Pantucek R., Doskar J., Ruzickova V., Kasperek P., Oracova E., Kvardova V.,
430 Rosypal S., Identification of bacteriophage types and their carriage in *Staphylococcus*
431 *aureus*, *Arch. Virol.* (2004) 149:1689-1703.

- 432 [24] Pichon C., Felden B., Small RNA genes expressed from *Staphylococcus aureus*
433 genomic and pathogenicity islands with specific expression among pathogenic strains,
434 Proc. Natl. Acad. Sci. USA (2005) 102:14249-14254.
- 435 [25] Popowicz G.M., Dubin G., Stec-Niemczyk J., Czarny A., Dubin A., Potempa J., Holak
436 T.A., Functional and structural characterization of Spl proteases from *Staphylococcus*
437 *aureus*, J. Mol. Biol. (2006) 358:270-279.
- 438 [26] Robinson D.A., Enright M.C., Evolutionary models of emergence of methicillin-
439 resistant *Staphylococcus aureus*, Antimicrob. Agents Chemother. (2003) 47:3926-
440 3934.
- 441 [27] Rodriguez-Calleja J.M., Garcia-Lopez I., Santos J.A., Otero A., Garcia-Lopez M.,
442 Molecular and phenotypic typing of *Staphylococcus aureus* isolates from rabbit meat,
443 Res. Microbiol. (2006) 157:496-502.
- 444 [28] Rooijackers S.H., Ruyken M., Roos A., Daha M.R., Presanis J.S., Sim R.B., Immun
445 invasion by a staphylococcal complement inhibitor that acts on C3 convertases, Nat.
446 Immunol. (2005) 920-927.
- 447 [29] Rozen S., Skaletsky H., Primer3 on the WWW for general users and for biologist
448 programmers, Methods Mol. Biol. (2000) 132:365-386.
- 449 [30] Sabat A., Melles D.C., Martirosian G., Grundmann H., van Belkum A., Hryniewicz
450 W., Distribution of the serine-aspartate repeat protein-encoding *sdr* genes among
451 nasal-carriage and invasive *Staphylococcus aureus* strains, J. Clin. Microbiol. (2006)
452 44:1135-1138.

- 453 [31] Straub J.A., Hertel C., Hammes W.P., A 23S rDNA-targeted polymerase chain reaction-
454 based system for detection of *Staphylococcus aureus* in meat starter cultures and dairy
455 products, J. Food Prot. (1999) 62:1150-1156.
- 456 [32] Sung J.M., Lloyd D.H., Lindsay J.A., *Staphylococcus aureus* host specificity:
457 comparative genomics of human versus animal isolates by multi-strain microarray,
458 Microbiology (2008) 154:1949-1959.
- 459 [33] Tenover F.C., McDougal L.K. Goering R.V., Killgore G., Projan S.J., Patel J.B.,
460 Dunman P.M., Characterization of a strain of community-associated methicillin-
461 resistant *Staphylococcus aureus* widely disseminated in the United States, J. Clin.
462 Microbiol. (2006) 44: 108–118.
- 463 [34] Vancraeynest D., Hermans K., Haesebrouck F., Genotypic and phenotypic screening
464 of high and low virulence *Staphylococcus aureus* isolates from rabbits for biofilm
465 formation and MSCRAMMs, Vet. Microbiol. (2004) 103:241-247.
- 466 [35] Vautor E., Abadie G., Guibert J.M., Huard C., Pepin M., Genotyping of
467 *Staphylococcus aureus* isolated from various sites on farms with dairy sheep using
468 pulsed-field gel electrophoresis, Vet. Microbiol. (2003) 96:69-79.
- 469 [36] Vautor E., Abadie G., Guibert J.M., Chevalier N., Pepin M., Nasal carriage of
470 *Staphylococcus aureus* in dairy sheep, Vet. Microbiol. (2005) 106:235-239.
- 471 [37] Vautor E., Magnone V., Rios G., Le Brigand K., Bergonier D., Lina G., et al., Genetic
472 differences among *Staphylococcus aureus* isolates from dairy ruminant species: a
473 single-dye DNA microarray approach, Vet. Microbiol. (2008) doi:
474 10.1016/j.vetmic.2008.06.006 .

475

- 476 [38] Wagner P.L., Waldor M.K., Bacteriophage control of bacterial virulence, *Infect.*
477 *Immun.* (2002) 70:3985-3993.
- 478 [39] Winter A., Mastitis in ewes, *In Practice* (2001) 23:160-163.
- 479 [40] Witney A.A., Marsden G.L., Holden M.T., Stabler R.A., Husain S.E., Vass J.K., et al.,
480 Design Validation, and Application of a Seven-Strain *Staphylococcus aureus* PCR
481 Product Microarray for Comparative Genomics, *Appl. Environ. Microbiol.* (2005)
482 71:7504-7514.
- 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

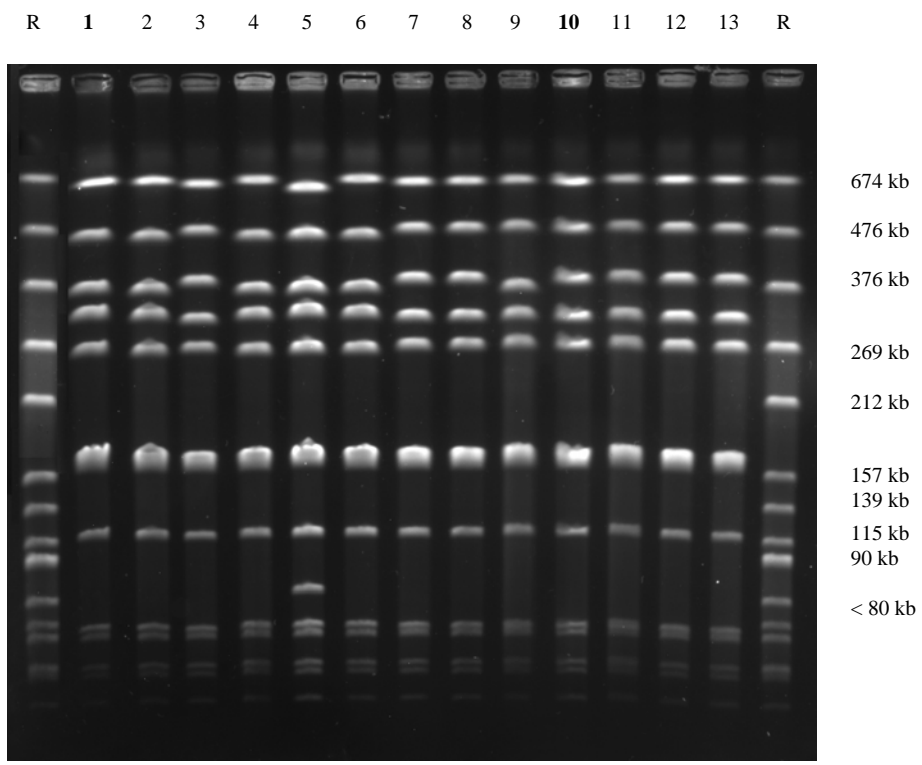
499 **Supplementary data**-DNA array oligonucleotide 65-mer (Excel document)

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> ^a | + | + | + | + | + | - | - | + | + | - | - | - |
| <i>SplE</i> | - | - | - | - | - | + | + | - | - | + | + | + |
| SAS0897 | + | + | + | + | + | - | - | + | + | + | - | + |
| SAR1558 | + | + | + | + | + | - | - | + | + | - | - | - |
| SAR0940 | + | + | + | + | + | - | - | + | + | + | - | + |
| SAR2100 | + | + | + | + | + | - | - | + | + | - | - | - |
| SACOL0343 | + | + | + | + | + | - | - | + | + | + | + | - |
| <i>Spa</i> types | 3568 | 3568 | 3568 | 3568 | 3568 | 524 | 524 | 3568 | 3568 | 524 | 524 | 524 |
| <i>Spa Clonal Complex</i> | 1773 | 1773 | 1773 | 1773 | 1773 | 1773 | 1773 | 1773 | 1773 | 1773 | 1773 | 1773 |
| RAPD types ^b | R | R2 | R | R | R | R1 | R1 | R3 | R2 | R1 | R1 | R1 |
| PFGE types ^c | 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) ^d | B | F | E | D | B | A | A | C | B | A | A | A |
| Prophages ^e | 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 ^f | 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 ^g | 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.

^a [16]; ^b [27]: at least one band of difference for each RAPD types; ^c [35] the PFGE profiles were named, OV, OV', OV'', OV'''; ^d [6, 10]: the letters correspond to a unique pattern made by the combination of the variable number-number tandem repeats of each gene; ^e [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). ^f [1]: Single Nucleotide Polymorphism (SNP). The same exotoxin sequence type had an identical numerical number, N.D.: not done; ^g [26]: unique sequences defined alleles and unique series of alleles defined a sequence type (named I or II).