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1 ***spa* type distribution in *Staphylococcus aureus* originating from pigs,**
2 **cattle and poultry.**

3
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21 **Abstract**

22 Methicillin resistant *S. aureus* (MRSA) of clonal complex 398 (CC398) is emerging globally
23 among production animals such as cattle, pigs and poultry as well as among humans. However,
24 little is known about the prevalence of CC398 among methicillin sensitive *S. aureus* (MSSA) or
25 the relative clonal distribution of *S. aureus* isolated from these three animal reservoirs. To study
26 this, we have analyzed a random sample of *S. aureus* consisting of 296 epidemiologically
27 unrelated isolates from infections and colonization of pigs, cattle and poultry. These were
28 examined and compared by *spa* and multi-locus sequence typing (MLST) and the result was
29 compared to the most common *spa* types found among human blood isolates. Little overlap in *spa*
30 types was seen between isolates from the three animal reservoirs or between animals and humans.
31 Most of the porcine isolates had the *spa* types t034 (CC398), t1333 (CC30) and t337 (CC9), while
32 the bovine isolates mainly had *spa* types t518 (CC50), t524 (CC97) and t529 (CC151). None of
33 these *spa* types are common among human blood isolates in Denmark. Surprisingly, almost all of
34 the poultry isolates (96%) belonged to CC5 (*spa* types t002 and t306), which is also known to be
35 commonly found among human blood isolates and subsequent pulsed field gel electrophoresis
36 (PFGE) analysis identified indistinguishable PFGE patterns among a poultry isolate and selected
37 human isolates. In conclusion, strains of MSSA CC398 were commonly present in pigs but not
38 present at all in the other reservoirs tested.

39

40 **Introduction**

41 *Staphylococcus aureus* is an opportunistic pathogen and a frequent coloniser of many animal
42 species, including humans. In addition to colonizing various hosts, it can cause a wide range of
43 different infections ranging from dermatitis, pneumonia, septicaemia to osteomyelitis and
44 meningitis in humans and swine as well as bovine mastitis in cattle and bumblefoot disease in
45 poultry (Quinn et al., 2000).

46 It has generally been thought that *S. aureus* from different reservoirs were host-specific (Devriese
47 & Oeding, 1976; Devriese, 1984; Sung et al., 2008). However, recently human carriage and
48 infections has increased in especially pig farmers in many countries by a specific lineage
49 (ST398/CC398) of methicillin-resistant *S. aureus* originating from livestock animals (Huijsdens et
50 al., 2006; Lewis et al., 2008; Smith et al., 2008).

51 A large number of different typing methods, such as MLST, ribotyping, PFGE and Staphylococcal
52 protein A (*spa*) typing have been used in epidemiological investigations of *S. aureus* from humans
53 and several animal species (Blumberg et al., 1992; Melles et al., 2007; Monecke et al., 2007). It is
54 generally agreed that MLST today is the method that gives most reliable information about the
55 phylogenetic clustering of *S. aureus* isolates (Feil et al., 2003; Turner and Feil, 2007). However,
56 the discriminatory power is still relatively low compared to other typing methods such PFGE and
57 *spa* typing. Therefore, the most widely used method today for first line typing and epidemiological
58 studies is *spa* typing (Frenay et al., 1996). This method allows easy and rapid characterisation of
59 isolates and at the same time gives robust evidence for the phylogenetic relationship, since *spa*
60 types are normally associated to specific MLST types (Strommenger et al., 2006).

61 In this study we investigated the clonal diversity, as determined by *spa* and supportive MLST
62 typing, of a random sample of 296 epidemiologically unrelated *S. aureus* isolates from cattle, pigs
63 and poultry in Denmark. Here, we provided evidence at the molecular level of the host-preference
64 of different *S. aureus* lineages and also showed that MSSA ST398 is the predominant *S. aureus*

65 lineage present within the pig population, but was absent among isolates of bovine and poultry
66 origin.

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68 **Material and methods**

69

70 **Bacterial isolates**

71 Clinical *S. aureus* isolates included in this study were randomly sampled from diagnostic
72 submissions sent from all over Denmark for identification and antimicrobial susceptibility testing
73 to either the National Food Institute – DTU or KU-Life between 1993 and 2007. In total 101, 46
74 and 85 clinical isolates from cattle, pigs and poultry, respectively, were analyzed. Colonizing *S.*
75 *aureus* isolates (nasal carriage) were either sampled at individual farms from healthy animals or
76 sampled as part of the DANMAP surveillance program (DANMAP, 2009) in 2007 from healthy
77 animals at the time of slaughter. In total, 11 and 53 isolates from colonization of cattle and pigs,
78 respectively, were included in this study. The criteria used in the strain selection process of both
79 clinical and colonizing isolates excluded duplicate farm isolates in order to minimize sampling
80 bias. All isolates from the National Food Institute – DTU were screened phenotypically for
81 susceptibility towards the 3rd generation cephalosporin ceftiofur as MIC determinations using a
82 commercially prepared, dehydrated panel (Sensitre), while all isolates from KU-LIFE were
83 screened for methicillin resistance using ORSAB screening agar (Oxoid).

84 In Denmark, human *S. aureus* bacteremia isolates (MSSA as well as MRSA) identified at any
85 hospital has to be submitted to the National Staphylococcal laboratory at Statens Serum Institute
86 (SSI) for further analysis. In 2007, a total of 1356 isolates were isolated in Denmark and subjected
87 to *spa* typing as described below. In addition, human MRSA isolates from all hospitals in Denmark
88 have for many years routinely been collected at the National Staphylococcal laboratory at SSI and
89 analyzed by PFGE *Sma*I digestion.

90

91

92

93 ***spa* typing**

94 Amplification of the *spa* repeat region was performed using primers *spa* 1113f (5'-
95 AAAGACGATCCTTCGGTGAGC-3') and *spa* 1514r (5'-CAGCAGTAGTGCCGTTTGCTT-3')
96 with assay conditioned as described by SeqNet (www.SeqNet.org) and using 1 unit of VWR Taq
97 DNA polymerase (VWR International) per reaction. The *spa* types were determined based on the
98 sequencing results using the *spa* plugin included in the Bionumerics v4.6 software (Applied Math).

99

100 **MLST typing**

101 MLST was performed by the method previously described by Enright et al. (Enright et al., 2000)
102 on at least one isolate from all *spa* types with more than two isolates. In case, where isolates with
103 the same *spa* sequence originated from different animal groups, at least one isolate originating
104 from each animal group were analysed. MLST Sequence Types (ST) were assigned through the
105 MLST database (<http://www.mlst.net>). The eBURST algorithm was used to assign individual STs
106 to specific CC types (<http://eburst.mlst.net>).

107

108 **PFGE**

109 PFGE was performed as described previously (Murchan et al., 2003) on selected Danish poultry
110 isolates and Danish human MRSA isolates displaying *spa* type t002.

111

112 **PCR**

113 The presence of the *mecA* gene in suspected methicillin-resistant isolates was tested genotypically
114 using a multiplex PCR directed against the 16S ribosomal RNA, the *mecA* gene and the *nucA* gene
115 as described previously (Maes et al., 2002).

116

117 **Results**

118 A total of 296 *S. aureus* isolates originating from cattle, pigs and poultry in Denmark were
119 included in this study. Among these, 48 different *spa* types were identified. Subsequently, MLST
120 analysis was performed on representative isolates from the predominant (more than two isolates
121 within a *spa* type) of these *spa* types. Based on this, sequence types and clonal complexes (CCs)
122 was inferred through the MLST database (www.mlst.net) using the eBURST algorithm. This
123 resulted in grouping the *spa* types into 12 different clonal complexes (CC's) as well as two
124 singletons (Table 1).

125

126 The porcine isolates (n=99) mainly clustered into three clonal complexes, namely CC398 (39%),
127 CC30 (29%) and CC9 (27%). Of these, only one was found to be *mecA* positive and this isolate
128 belonged to ST398/CC398. This isolate as well as most of the other CC398 isolates belonged to
129 *spa* type t034, thus being the most predominant *spa* type among MSSA isolated from pigs in
130 Denmark. Among the remaining two predominant CC's found among pigs, a single *spa* type in
131 each CC were again dominating, namely t1333 of CC30 and t337 of CC9. Forty-six percent of the
132 porcine isolates originated from infections and 54% from colonisation of the nostrils. There was no
133 evident difference in the *spa* type distribution between isolates from infections and colonisation
134 (Table 1).

135

136 The bovine isolates (n=112) were all methicillin susceptible, and again three major CC's were
137 observed, but here three different clonal lineages CC50 (31%), CC97 (28%) and CC151 (16%)
138 dominated. As with the porcine isolates, each of the three CC's were mainly associated with a
139 specific *spa* type, namely the t518 of CC50, t524 of CC97 and t529 of CC151 (Table 1).
140 Interestingly, several of the remaining isolates belonged to CC9, one of the main porcine CC's.
141 However, different *spa* types dominated in the CC9 isolates from the two animal reservoirs i.e. *spa*

142 types t1419 and t1430 were most dominant among the bovine CC9 isolates and t337 were the
143 dominant porcine type although there was some overlap (marked in bold in Table 1). In particular,
144 isolates with the *spa* type t899 was found in both reservoirs with low prevalence. We therefore
145 tested the three t899 isolates by MLST and found these to belong to ST9, which is within the CC9.
146 Finally, a minor fraction of the bovine isolates belonged to CC131, CC20 and CC8.

147

148 The isolates from the poultry infections (n=85), belonged almost exclusively to CC5 (96%) with
149 the t002 as the far most dominant *spa* type (Table 1). The most frequent *spa* type in poultry (t002)
150 was the seventh most common human *spa* type involved in human *S. aureus* blood infections in
151 Denmark in 2007 (data not shown). To examine the clonal relationship between poultry and human
152 t002 isolates, a subset (n=24) of the poultry isolates were subjected to PFGE using the *SmaI*
153 enzyme and compared to the six most dominant PFGE types identified among the human MRSA
154 isolates in Denmark. In general, a large diversity was seen among the poultry PFGE types, even
155 though some isolates with indistinguishable *SmaI* profiles were seen (data not shown).
156 Interestingly, one of the poultry isolates had a PFGE profile identical to one of the most common
157 human PFGE types, indicating that some poultry and human t002 isolates were in fact closely
158 related.

159

160

161 **Discussion**

162 Recently, several studies have examined the prevalence of MRSA in pigs with special attention to
163 the emerging ST398/CC398 clonal lineage, which was first detected among pigs and has now been
164 isolated from livestock animals worldwide (Armand-Lefevre et al., 2005; Huijsdens et al., 2006;
165 Lewis et al., 2009; Nemati et al., 2008). The initial emergence of this clonal lineage in pigs is
166 somewhat of an enigma as this is not one of the traditional lineages associated with MRSA in

167 human medicine. It could be hypothesized that this lineage was already present among pigs as
168 MSSA and has subsequently emerged as MRSA by acquiring SCCmec cassettes through a yet
169 unknown donor. However, the *spa* type distribution in a larger collection of epidemiologically
170 independent MSSA porcine isolates has to our knowledge not been studied in detail before. A
171 recent study of the clonal distribution of sequence types of isolates from healthy pig farmers
172 (n=44) and pigs (n=14) from France also identified some porcine isolates belonging to CC398,
173 CC9 and CC30 (ST433) in healthy pigs as well as in pig farmers (Armand-Lefevre et al., 2005),
174 but the animal sample size was too small to give an indication of the relative prevalence of the
175 isolates in general. Here we show that ST398/CC398 is a common MLST type in pigs together
176 with two other types. Interestingly, *spa* type t034 of ST398 seemed to be the most dominant type
177 among pigs in Denmark, which is also the predominant *spa* type of Danish MRSA isolates isolated
178 from breeding pig as well as from pigs at the time of slaughter (data not shown) and thus
179 supporting the hypothesis for emergence of MRSA ST398. However, this is difficult to prove
180 unless other typing methods with higher discriminating power can be used to examine the isolates
181 further. Unfortunately, ST398 is untypable by standard PFGE, which could otherwise have been
182 used. As other CC398-related *spa* types such as t011 and t108 seem to be dominant in other
183 European countries (Witte et al. 2007) it would also be interesting to analyse MSSA isolates of
184 porcine origin in these countries to see, if these are correlated with the MRSA *spa* types found in
185 these countries.

186
187 Several studies have examined *spa* types or MLST types of *S. aureus* isolates of bovine origin. A
188 intercontinental MLST study of 258 bovine-associated *S. aureus* isolates from United States, Chile
189 and the United Kingdom identified CC97 to be the far the most dominant clonal complex
190 constituting a total of 87.4% of the tested isolates, however *spa* types were not analysed (Smith et
191 al., 2005). These isolates were sampled from milk, teat skin, milking machines and various other

192 locations but were not sampled from animals with clinical signs of bovine mastitis. Among our
193 bovine sample material, which mostly originate from animals with clinical signs of mastitis (90%),
194 we found 28% of the isolates to belong to CC97, but also found two other dominant clonal
195 complexes (CC50 and CC151), which were only present among few of the intercontinental
196 isolates. An explanation for the more diverse result obtained with our isolates compared to the
197 intercontinental study could be related to this difference in sample material (healthy vs. mastitis
198 origin) or it could be related to geographical variation of the clonal lineages. In support of the
199 latter, bovine mastitis isolates from countries like United States and United Kingdom traditionally
200 have a high prevalence of reduced susceptibility towards penicillin as opposed to Danish isolates,
201 which have lower prevalence of penicillin resistant *S. aureus* (Vintov et al., 2003)

202 A recent study from Switzerland and Germany on 128 bovine mastitis isolates (Monecke et al,
203 2007) identified 20 different subgroups based on microarray analysis and found CC97 and CC151
204 to be the most predominant with other CC's being present in smaller numbers (CC8, CC20,
205 CC398, CC133, CC5 and CC1). Interestingly, most of these CC's were also present among the
206 isolates examined in our study, even though the prevalence of the individual CC's does differ
207 between the two studies.

208 As only a limited number of the bovine isolates included in our study originated from colonisation
209 of the animal nares, it is difficult to evaluate if the same relative distribution of *spa* types was
210 found among isolates from infections and colonisation. However, nothing indicates that this should
211 not be the case, as the *spa* types of isolates associated with colonisations were all found among the
212 mastitis isolates as well (Table 1).

213 Two (MSSA) isolates from pigs and one isolate from cattle had the *spa* type t899. This *spa* type has
214 previously been assigned to both ST398 (van Duijkeren et al., 2008; Wulf et al., 2008) as well as
215 CC9 (Liu et al., 2009). Our three isolates was shown here to belong to CC9 as opposed to the
216 MRSA t899 isolates described previously (Wulf et al, 2008). To verify that isolates with *spa* type

217 t899 in fact can belong to both clonal complexes, we repeated the *spa* and MLST typing of two of
218 the t899 isolates, which were previously reported to belong to ST398 (Wulf et al., 2008). The tests
219 confirmed that the previous isolates had been typed correctly initially and thus were indeed *spa* type
220 t899 and positioned in CC398. A closer examination of the *spa* repeats of *spa* type t899
221 (r07r16r23r02r34) would position this type within the CC9 *spa* types, rather than within the CC398,
222 which bares little similarity to the *spa* repeats of t899 (see Table 1). Therefore, a likely explanation
223 for some t899 isolates to be located in CC398 is horizontal transfer of the *spa* gene from a t899
224 strain into a CC398 strain some point in time rather than horizontal transfer of the seven household
225 genes used to determine the sequence type.

226 Interestingly the *spa* types from poultry isolates were far more clonally conserved than the *spa*
227 types from the two other animal reservoirs. As the poultry isolates used in this study were submitted
228 between 1993 and 2007 from different farms located all across Denmark and thus representing a
229 very diverse sample material, this result indicates that there is a very narrow range of *S. aureus*
230 isolates, which are involved in poultry infections.

231 To our knowledge this is the first time, poultry MSSA isolates has been analysed by *spa* and MLST
232 typing. A recent study of poultry isolates from Belgium (Nemati et al., 2008) found a relatively
233 large proportion (12%) of Belgian poultry isolates to be MRSA ST398 (*spa* types t011 and t567).
234 These were isolated at five out of 39 tested poultry farmers in 2006 whereas no MRSA ST398 was
235 found among similar isolates obtained between 1970 and 1972. However, as only the MRSA
236 isolates were subjected to *spa* typing, this study did not give any information on the *spa* type
237 distribution among the remaining MSSA isolates. Therefore this result can not be used to estimate
238 the clonal diversity of the poultry isolates studied *per se*, but does rather emphasize the recent
239 spread of MRSA ST398 in other livestock reservoirs than pigs.

240 Few studies have investigated the clonal diversity of *S. aureus* among the three animal groups,
241 which we have examined in our study, or compared them to *S. aureus* isolated from humans, but

242 based on the antigenic pattern differences between animal and human isolates, it has previously
243 been suggested that animal and human *S. aureus* isolates does appear to be antigenically different
244 (Oeding, 1960). Similar results have been observed, based on phage typing of mastitis isolates
245 (Vintov et al, 2003). In Denmark, all *S. aureus* isolates (MSSA and MRSA) involved in human
246 blood infections at Danish hospitals collected in 2007 were *spa* typed at the Statens Serum Institute.
247 Only one of the most predominant *spa* types (t034) found among Danish bovine and porcine
248 isolates are located among the top 50 human *spa* types from 2007 (number 46 on the list, consisting
249 of 4 isolates out of 1356 isolates tested), indicating very little overlap between the *spa* types found
250 in humans and in Danish pigs and cattle. In fact, subsequent analysis of these four t034 isolates
251 have revealed that these were isolated from patients with close association with animal production,
252 such as pig farmers and slaughterhouse workers (Lewis et al., 2008)

253 An explanation for the clonal organization of *S. aureus* in the three animal reservoirs is not
254 deducible from the data as it is not known whether it is caused by the fact that there is only limited
255 contact between the different animal species in modern farming or if the different *S. aureus* clonal
256 lineages have specific preferences to each of these three animal species. In the present study,
257 however, at least one clonal lineage seems to contradict this clonal organization, as isolates
258 belonging to CC9 (ST9) were found among both bovine and porcine isolates, even though the
259 relative prevalence of *spa* types within the CC9 isolates varied between the two animal reservoirs.
260 Also, the MRSA CC398 seems to be able to spread rapidly between different animal reservoirs.
261 However, based on the fact that MRSA CC398 was first isolated from pigs as well as the data
262 presented here, where MSSA CC398 are only identified among pig isolates, it could be suggested
263 that the MRSA CC398 initially emerged within the porcine reservoir. As CC398 does not seem to
264 be prevalent among bovine and poultry isolates, the current spread to these animal reservoirs could
265 be driven by a selective force. A likely candidate for this selective force would be one or more
266 antimicrobial agents used in all three reservoirs; however, the exact antimicrobial agent has not

267 been identified yet and will await further investigations. Tetracycline has been suggested
268 previously as most CC398 MRSA isolates appears to be resistant to tetracycline, however,
269 tetracycline susceptibility testing of the MSSA CC398 isolates indentified in this study showed
270 that they were also resistant to tetracycline (data not shown). Therefore this particular
271 antimicrobial agent would not give the MRSA CC398's a selective advantage compared to the
272 other MSSA CC398 isolates present on the pigs.

273 Finally, an explanation for the low level of variation among the *spa* types found in poultry isolates
274 compared to isolates from the two other animal reservoirs could be attributed to the fact that the
275 number of hatcheries in Europe is very limited and therefore most poultry chicks originate from
276 the same hatcheries. The link observed between poultry and human clinical isolates belonging to *S.*
277 *aureus* lineage CC5 t002 will require further investigation to assess possible zoonotic risks
278 associated with poultry farming.

279

280

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284

285 **Conflict of interest**

286 None to declare

287

288 **Role of the funding source**

289 The grant sponsor had no involvement in the any step of this manuscript.

290

291 **Ethics**

292

None to declare.

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383 Table 1: Prevalence data of the *spa* and MLST types among the 296 isolates of porcine, bovine and
384 poultry origin included in this study. *Spa* types, which are present in more than one animal group
385 has been marked in bold.

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Source	<i>spa</i> type	<i>Spa</i> repeats	Number (i/c) ¹	MLST Sequence type	MLST Clonal complex
Pigs	t034	r08r16r02r25r02r25r34r 24r25	32 (14/18)	ST398	CC398
	t571	r08r16r02r25r02r25r34 r25	2 (1/1)	ST398	CC398
	t011	r08r16r02r25 r34 r24r25	1 (1/0)	ST398*	CC398
	t108	r08r16r02r25 r24r25	1 (0/1)	ST398*	CC398
	t1255	r08r16 r34r24r25	1 (0/1)	ST398	CC398
	t1793	r08r16r02r25r02r25r34r24r24r25	1 (0/1)	ST398	CC398
	t2876	r08r16r02r25r02r25r 24r25	1 (1/0)	na	na
	t1333	r15r12r16r34r 02r25r17r24	26 (13/13)	ST433	CC30
	t318	r15r12r16r16r02r16r02r25r17r24	1 (0/1)	ST30*	CC30
	t631	r15r12r16r 17r24	1 (0/1)	na	na
	t2840	r15r12r16r34r02r25r17r24r17r24	1 (1/0)	na	na
	t337	r07r16r23 r23r02r12r23r02r34	16 (9/7)	ST9	CC9
	t1334	r07r16r23 r23r02r34	3 (0/3)	ST9	CC9
	t2462	r07 r23 r23r02r34	3 (0/3)	ST9	CC9
	t899	r07r16r 23r02r34	2 (0/2)	ST9	CC9
	t1430	r07r16r23 r02r12r23r02r34	1 (0/1)	ST9	CC9
	t2498	r07r16r16r23r23r02r12r23r02r34	1 (1/0)	na	na
	t2839	r07r16r23 r23r23r02r34	1 (1/0)	ST9	CC9
	t127	r07r23r21 r16r34r33r13	1 (1/0)	ST1*	CC5
	t1491	r07r23r21r17r13r34r34r16r34r33r13	1 (1/0)	ST1*	CC5
	t230	r08r16r02r16r34	1 (1/0)	ST45*	CC45
	t2678	r03r16r12r21r17r23r13r17r17r17r23r24	1 (1/0)	na	na
	Cattle	t518	r04r20r17r23r24 r20r17r25	25 (25/0)	ST50
t519		r04 r20r17r25	8 (8/0)	ST50	CC50
t3463		r04r20r17r23r31r24r20r17r25	1 (1/0)	na	na
t3465		r04r20r17r17r17r16r20r17r25	1 (1/0)	na	na
t524		r04r17	25 (24/1)	ST71	CC97
t529		r04r34	17 (15/2)	ST151	CC151
t528		r04	1 (1/0)	ST151	CC151
t1403		r03r23r24	7 (6/1)	ST133	CC133
t2873		r04r20r17r31r24	5 (4/1)	ST479	Singleton
t527		r07r23r12r21r17r34r34r34r34r34r33r34	4 (3/1)	ST97	CC97
t267		r07r23r12r21r17r34r 34r34r33r34	1 (1/0)	ST97	CC97
t521		r07r23r12r21r17r34r34 r34r34r33r34	1 (1/0)	ST97	CC97
t1419		r07 r23r02r12r23r02r34	3 (3/0)	ST9	CC9
t1430		r07r16r23r02r12r23r02r34	3 (2/1)	ST9	CC9
t3464		r07r23r02r12r23r02r34	2 (1/1)	na	na
t899		r07 r16r23r02r34	1 (1/0)	ST9	CC9
t2839		r07r16r23r 23r23r02r34	1 (1/0)	na	na
t2875		r07r16r06r20r12r06r02r34	1 (1/0)	ST9	CC9
t008		r11r19r12r21r17r34r24r34r22r25	2 (2/0)	ST8	CC8
t731		r07r06r17r21r34r22r34	1 (1/0)	ST513	CC20
t3046		r03r16r12r21r17r23r13r17r17r17r23r25	1 (1/0)	na	na
t3830		r14r34r17r34r50r17	1 (1/0)	na	na
Poultry		t002	r26r23r17r34r17r20r17r12r17 r16	66 (66/0)	ST5
	t306	r26r23r17r34r17r20r17r12r17r17r16	13 (13/0)	ST5	CC5
	t688	r26r23r17r34r17 r16	1 (1/0)	ST5	CC5
	t2049	r26r23r17 r17r16	1 (1/0)	na	na
	t2308	r26r23r17r34r17r17r20r17r12r17r16	1 (1/0)	na	na
	t213	r07r23r12r21r24r33r22r17	2 (2/0)	ST385	Singleton
	t304	r11r10r21r17r34r24r34r22r25	1 (1/0)	ST8*	CC8

389 ¹. (i/c) = (infections/colonization), na = not available, * = ST type retrieved from the Rindom
390 *spa*Server.