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H. Hasman, A. Moodley, L. Guardabassi, M. Stegger, R.L. Skov, F.M. Aarestrup

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Authors: H. Hasman, A. Moodley, L. Guardabassi, M. Stegger, R.L. Skov, F.M. Aarestrup

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

H. Hasman[a*], A. Moodley[b], L. Guardabassi[b], M. Stegger[c], R. L. Skov[c] and F. M. Aarestrup[a].

[a]Technical University of Denmark, National Food Institute, Bülowsvej 27, 1790 Copenhagen V., Denmark, [b]Department of Disease Biology, Faculty of Life Sciences, University of Copenhagen, Stigbøjlen 4, Frederiksberg C 1870, Denmark, [c]Statens Serum Institut, National Center for Antimicrobials and Infection Control, Artillerivej 5 (B.47/204), 2300 Copenhagen S, Denmark.

*Corresponding author:
Henrik Hasman
Phone: (+45) 3588 6347
Fax: (+45) 3588 6341
Email: hhas@food.dtu.dk

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**Abstract**

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

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## Introduction

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

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

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

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

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

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## Material and methods

### Bacterial isolates

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

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

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**_spa_ typing**

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

**MLST typing**

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

**PFGE**

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

**PCR**

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



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**Results**

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

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

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

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

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

**Discussion**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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**Conflict of interest**

None to declare

**Role of the funding source**

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

**Ethics**



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None to declare.

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

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| Source | *spa* type | *Spa* 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 | CC50 |
| | 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 | r07r23r02r12r23r02r02r34 | 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 | CC5 |
| | 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 |

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