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BRIEF REPORT

Short term micro-evolution and PCR-detection of methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398

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Abstract Micro-evolutionary analysis of 70 ST398 isolates by pulsed-field gel electrophoresis (PFGE) using *Cfr*9I revealed three sub-clones with abundant inter- and intra-sub-clone heterogeneity in *spa*- and SCC*mec*-types. In addition, we developed two specific PCRs for the detection of *Staphylococcus aureus* sequence type 398 (ST 398) isolates with 100% specificity and high sensitivity.

Staphylococcus aureus strains of ST398 are becoming a worldwide threat. ST398 strains are found in human carriers and patients, domestic animals but also in meat products for human consumption [1]. At first it was considered that dissemination of ST398 strains was restricted to animals, especially pigs, and humans working with them only. Recently however, several studies reported the more promiscuous transmission of ST398 among humans [2–4]. Notably, in China ST398 strains are now causing hospital-acquired infections [5]. Since ST398 strains belong to a single multilocus sequence typing (MLST) class, are PFGE non-typeable using *Sma*I, and have related *spa*-types, little is known about their micro-epidemiology.

In total, 70 ST398 *S. aureus* isolates were included in this study, 50 of which were previously *spa*-typed and analyzed using amplified fragment length polymorphism (AFLP) [6]. Isolates were of clinical origin, but carriage isolates from pigs and humans were also included. The 20 additional ST398 isolates included in our study comprised of seven clinical horse isolates, nine carriage pig isolates obtained from the Veterinary Medical Diagnostic Centre in Utrecht, one strain isolated from the nares of a pig in a slaughterhouse, a cow mastitis isolate and Hong Kong Chinese ST398 strains T-235 and T-252 (courtesy of Dr. M. Ip [7], Prince of Wales Hospital, The Chinese University of Hong Kong). For all new ST398 isolates the presence of *mec*A and the SCC*mec* type was defined by polymerase chain reaction (PCR) [8]. All additional ST398 isolates except the cow isolate were methicillin-resistant *S. aureus* (MRSA). *Spa*-types were determined as described previously [6]. The non-ST398 strains, MRSA of human origin COL (SCC*mec* I), Mu50 (SCC*mec* II), N315 (SCC*mec* II), BK2464 (SCC*mec* II), ANS46 (SCC*mec* III), HDE288 (SCC*mec* IV), MW2 (SCC*mec* IVa) and WIS (SCC*mec* V), three MRSA strains isolated from horses and four pig MSSA isolates were included as controls. *Spa*-typing of these seven non-ST398 animal strains was performed as mentioned above. Finally from our AFLP database [9] 48 randomly chosen representatives of the different AFLP-(sub) clusters were included for comparative reasons.

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Table 1 Primers used in this study and ST398 specific polymerase chain reaction (PCR) analyses

Primer set	Sequence	Annealing temperature	Fragment length (bp)	ST398 *n*=70 (%)	Non ST398 *n*=63 (%)
A04F A04R	TCATTGCTTGGCGTGTAGGT TATCAACAGCCGGTGACAAC	58°C	317	70 (100)	63 (100)
A07F A07R	GATCCCAGAATACTTAAATA TGACCGTAATCTTGTAAATA	50°C	197	70 (100)	0 (0)
C01F C01R	CATTCATCACACGTATATTC GGTGATTATTCATGGTTAAG	52°C	140	70 (100)	0 (0)
B04F B04R	GGCAAGATGGCTGGTCACAA CTGAGAAACTGCGGGTGCAA	60°C	107	69 (99)	2 (3)
A10F A10R	CTAGGCCTGGTTTAATAATA CAAGTTTCATCGTTTACTTC	52°C	133	40 (57)	2 (3)

PFGE of all 70 ST398 isolates and ten non ST398 strains (6 MRSA [3 human and 3 horse isolates] and 4 MSSA strains [all pig isolates]) was performed and analyzed using previously described methods [10] except for the use of *Cfr*9I (a neoschizomer of *Sma*I) to digest the chromosomal DNA. Banding patterns were interpreted by two independent persons using GelCompar software (Applied Maths NV, Sint-Martens-Latem, Belgium).

In an approach to develop an ST398 specific PCR, four ST398-specific DNA sequences previously obtained from AFLP analyses were studied [6]. Using the genomic sequence of S0385 (ST398) [11] we found that fragments A07, A10 and C01 have a 100% match with the DNA sequence of S0385, but not with any of the other 13 sequenced *S. aureus* strains present in the database (www.ncbi.nlm.nih.gov/blast). Besides a 100% identity with S0385, also B04 showed a 100% match with the 3′end of SAB0857 and the 5′end of SAB0858 of the bovine strain RF122 [12]. PCR primers were developed for A07, A10, C01, B04 and a positive control A04 (an AFLP fragment available in all *S. aureus* strains). PCRs were performed using the following protocol: 1 min at 94°C, 1 min at the specific annealing temperature and 1 min at 72°C; this sequence was repeated 25 times (Table 1).

When performing PCRs with primer sets A07 and C01 all 70 ST398 isolates were positive, but all 63 non-ST398 isolates were negative (Table 1). Primer set A10 was positive in only 57% of all ST398 isolates, while B04 was positive in 99% of the ST398 isolates but also in 3% of the non-ST398 isolates.

As expected, all *spa*-types of the ST398 isolates were related though among the pig and horse isolates of the additional collection three relatively obscure *spa*-types were identified: t899, t1939 and t2123 (Table 2). In the non-ST398 isolates unrelated *spa*-types were found more frequently. The three MRSA isolates from horses had *spa*-type t064, two pig isolates displayed t337, and two pig isolates were characterized as t3427. The *Cfr*9I and *Sma*I digestion patterns of non-ST398 strains were identical (data not shown). The previous AFLP analyses alike [6], PFGE data clearly show that ST398 isolates form a distinct lineage (Fig. 1). However, on the basis of our results the ST398 stains can be divided into three different clusters: A, B, and C, with A being most abundant. In clusters A and B, six and two sub clusters can be determined, respectively. In isolates from cluster C and the sub clusters of A and B, in more or in lesser extent, a dominant *spa*-type was found. Associations were also found between SCC*mec* type or its absence and PFGE patterns. The most prominent examples

Table 2 *spa*-types of the ST398 isolates in this study

spa-type	Repeats									
t2123	08			25						
t1255	08	16					34		24	25
t567	08		02	25					24	25
t108	08	16	02	25					24	25
t1254	106[a]	16	02	25			34		24	25
t011	08	16	02	25			34		24	25
t571	08	16	02	25	02	25	34			25
t034	08	16	02	25	02	25	34		24	25
t898	08	16	02	25	02	25	34	34	24	25
t899	07[a]	16		23[b]	02		34			
t1939	07[a]			23[b]	02		34			

[a] Repeat differing in one base from repeat 8

[b] Repeat differing in two bases from repeat 25

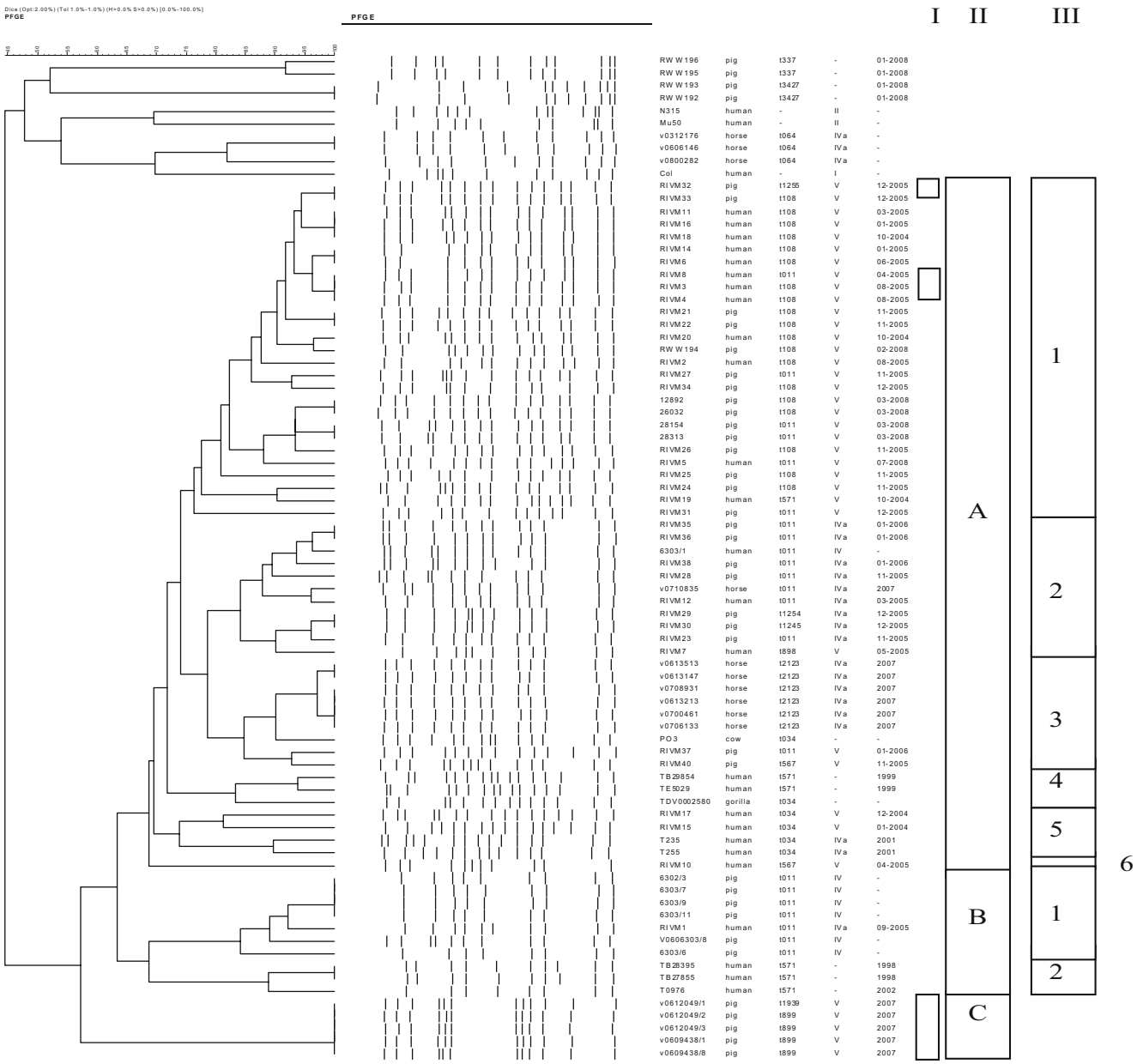

Fig. 1 Dendrogram of the PFGE data from ten non ST398 and 70 ST398 isolates. Next to the dendrogram, the PFGE of *Cfr*9I macrorestriction fragments, strain name, host, *spa*-type, SCC*mec*-type, isolation date and PFGE-type are given. The boxes indicated with I, II and III represent isolates with a similar PFGE banding pattern but with different *spa*-types, PFGE clusters, and PFGE sub clusters, respectively

are cluster C and sub cluster A1 harboring only SCC*mec* V and sub cluster B2 isolates being negative for SCC*mec* (Fig. 1). The sub division of sub cluster A1 is presumably the result of the presence of a *Cfr*9I restriction site in SCC*mec* V. In case of cluster C this is probably of lesser consequence for the digestion patterns are very different from clusters A and B. These data indicate that isolates from PFGE-cluster A belong to a successful ST398 clade, that can either diversify rapidly or are members of older clones that diversified more slowly. In the different branches of this cluster different SCC*mec* were introduced, so did repeat deletions, duplication and/or base modifications in the variable region of *spa*. This degree of variation might be the result of a random process or differences due to selective immunological pressure in the different hosts from which PFGE-cluster A isolates were obtained. This raises the question whether *spa*-typing can be used for monitoring the epidemiology of ST398 isolates. Although a predominant *spa*-type was found in PFGE clusters C and the different sub clusters of A and B, in several isolates with an identical PFGE profile *spa*-types were different (Fig. 1). Another problem is the fact that two *spa*-types, t011 and t571, were found in both PFGE-clusters

A and B. So, in these lineages either *spa* did not diversify or this is the result of homoplasia. On the other hand, all isolates from the distinct PFGE-cluster C (isolated from pigs at the same farm) carried one of two obscure but close related ST398 *spa*-types. This indicates that *spa*-types can predict genetic background in ST398. Finally, no clear relation between PFGE clusters and hosts was found.

In conclusion, we developed two ST398-specific PCRs for detection of *S. aureus* ST398. Such rapid, powerful and convenient diagnostic tools are important in clinical settings but also for monitoring colonization of individuals involved in pig farming or other forms of livestock handling. Also for those involved in quality control of food products these tests are an important commodity. PFGE using *Cfr*9I can be used to type ST398 isolates; this identifies three major genotypes. Although among PFGE-cluster C and the different sub clusters A and B dominant *spa*-types were found, *spa* typing is not a robust indicator of genetic background in ST398, in particular for t011 and t571. Based on our data we feel that the evolution of the ST398 genome as a whole proceeds at a different pace than the ST398 *spa* gene does.

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