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1 **Diversity of the Tetracycline Resistance Gene *tet(M)* and**
2 **Identification of Tn916- and Tn5801-like (Tn6014) Transposons**
3 **in *Staphylococcus aureus* from Human and Animals**

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12
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23 **Abstract**

24 **Objectives:** To analyse the sequence diversity of the tetracycline resistance gene *tet(M)* in
25 *Staphylococcus aureus* from human and animals and to determine mobile elements associated
26 with *tet(M)* in *S. aureus*.

27 **Methods:** In total 205 tetracycline resistant isolates were screened for *tet(M)* by PCR. *tet(M)*
28 were sequenced and compared to *tet(M)* deposited with GenBank. Based on phylogenetic
29 analysis isolates were screened for Tn916- and Tn5801-like *xis/int* genes and transposons were
30 confirmed by linking PCR. *spa* typing was performed and selected isolates were used as
31 donors in a filter mating experiment.

32 **Results:** Forty-one isolates (21.3 %, 60.7 %, 2.6 % and 4.4 % of the human, pig, poultry and
33 cattle isolates, respectively) were *tet(M)* positive. *tet(M)* was located on Tn5801-like and
34 Tn916-like transposons in humans and on a specific Tn916-like element in animals. Human
35 isolates were of different *spa* types (t034, t008, t037, t051, t065, t078, t318 and t964)
36 corresponding to different clonal complexes (CC398, CC8, CC25 and CC30). Animal isolates
37 were of *spa* type t034, t011 or t0571 corresponding to CC398. *tet(M)* sequence types correlated
38 with CC types. Tn916-like and Tn5801-like (Tn6014) transposons were able to transfer to *S.*
39 *aureus* recipients.

40 **Conclusion:** *S. aureus* of human origin contained diverse *tet(M)* located on Tn916- and
41 Tn5801-like (Tn6014) transposons and *S. aureus* of animal origin contained Tn916-like *tet(M)*
42 genes. This suggest that conjugative transposition play an important role in the evolution and
43 horizontal spread of *tet(M)* in *S. aureus*. This is the first study showing horizontal transfer of
44 Tn5801 (Tn6014).

45

46 **Introduction**

47 *Staphylococcus aureus* is part of the normal flora and a frequent cause of infection in humans and
48 many animal species.¹ *S. aureus* are often resistant to tetracycline and two known mechanisms of
49 tetracycline resistance have been identified among staphylococci. Active efflux is a result of
50 acquisition of the genes *tet(K)*, *tet(L)* or *tet(38)*, mainly located on plasmids. Ribosomal protection
51 is conferred by the genes *tet(M)*, *tet(O)*, *tet(S)* or *tet(W)* that are mainly located on different
52 transposons on the chromosome.^{2,3} In addition *tet(U)* has also been found in staphylococci but the
53 mechanism is not fully understood.^{3,4}

54 *tet(M)* together with *tet(K)* are the most common genes conferring tetracycline resistance in *S.*
55 *aureus*.⁵⁻⁸ *tet(M)* is widely distributed among both Gram-positive and Gram-negative bacteria and it
56 has been found in 59 genera.^{3,9} This is probably due to the association of *tet(M)* with integrative and
57 conjugative transposons, facilitating horizontal transfer.¹⁰ Particularly in Gram-positive streptococci
58 and enterococci, *tet(M)* has been found associated with Tn916/1545-like conjugative transposons
59 which form the basis of a family of conjugative transposons that have an extremely broad host
60 range.^{11,12} *tet(M)* associated with Tn916 was originally identified in *Enterococcus faecalis* DS16
61 and Tn1545 was identified in *Streptococcus pneumoniae*.^{11,13} Recently *tet(M)* was identified on a
62 putative transposon Tn5801 in *S. aureus* Mu50.¹⁴ Tn5801 contains many open reading frames
63 similar to Tn916, but differs by using an integrase (*int*) different from the excisionase/integrase
64 (*xis/int*) present in Tn916 (Figure 1).^{14,15} Other conjugative transposons, like Tn5397 and
65 CW459*tet(M)* have been found to harbour *tet(M)* in *Clostridium difficile* and *Clostridium*
66 *perfringens*.^{16,17} In addition, *tet(M)* have also been found on different plasmids.²

67 A limited number of studies concerning the diversity of the *tet(M)* gene have been performed.¹⁸⁻
68 ²² In streptococci and enterococci *tet(M)* has been found to be diverse and mainly present on
69 Tn916/Tn1545 like conjugative transposons whereas two different allele types of *tet(M)* from

70 *Lactobacillus* has been found to be located mainly on plasmids.¹⁹⁻²² Recently, Agersø *et al.* (2006)
71 showed correlation between diversity of the *tet(M)* DNA sequence and their presence on Tn916,
72 Tn5397 or plasmids in enterococci from different sources in Denmark.²¹

73 To our knowledge no one has studied the diversity of *tet(M)* and its association with mobile
74 elements in *S. aureus*. A former study found only one out of thirty-four *S. aureus* strains to carry
75 *tet(M)* on Tn916/Tn1545-like transposons.²³ The aim of this study was to analyse sequence
76 diversity of *tet(M)* in *S. aureus* from human and different animals mainly from Denmark, and
77 thereby determine mobile elements associated with *tet(M)* in *S. aureus*.

78 **Material and Methods**

79 **Strains**

80 The 205 tetracycline resistant isolates used in this study (Table 1) were identified as *S. aureus* as
81 previously described.²⁴ The 94 human isolates from bacteraemia prospectively collected in
82 Denmark, were selected to represent different phage types and time periods (1957-2002). All
83 human isolates were tested for susceptibility to tetracycline, penicillin, gentamicin, streptomycin,
84 erythromycin and methicillin by tablet diffusion on Danish blood agar as described by the
85 manufacturer (Rosco Neosensitabs, Taastrup). Due to a change in the standard procedure, strains
86 isolated after 1991 were additionally tested for susceptibility to fusidic acid, ciprofloxacin and
87 rifampicin in the same way (Table 2).

88 Of the 111 animal isolates (Table 1), 39 poultry²⁵, 27 pig and 2 lamb isolates were diagnostic
89 submissions to either The National Veterinary Institute or to The National Food Institute, Technical
90 University of Denmark. One pig isolate (9b) was obtained from a healthy pig in 2007. All animal
91 isolates were tested for susceptibility to tetracycline, penicillin, streptomycin, erythromycin,
92 ciprofloxacin, spectinomycin, tiamulin, trimethoprim, ceftiofur, chloramphenicol, florfenicol and

93 sulphamethoxazole by use of sensititre method as described previously (Table 2).²⁵ One pig isolate
94 9b was confirmed to be MRSA by *mecA* PCR.²⁶

95 **Screening and sequencing of *tet(M)***

96 All 205 isolates were screened for *tet(M)* by PCR as described previously (Table 1).²⁷ For all 41
97 *tet(M)* positive isolates three or four overlapping PCR fragments covering *tet(M)* including a
98 downstream region were amplified and used as templates for sequencing. Different sequencing
99 strategies were used in parallel as outlined in Figure 2. DNA Taq polymerase (Ampliqon, Denmark)
100 was used for all PCR amplifications. Sequencing was performed by Macrogen, Korea.²⁸ Primers
101 526, 540, 324, 525, 266, 323, 307, 323, 307 and 1756 were used (Table 3).

102 **Detection of Tn916-like and Tn5801-like transposons**

103 The presence of the *int* genes specific for Tn5801 was detected by PCR using primers 1811 and
104 1812 (product size 722 bp) and DNA Taq polymerase from Ampliqon. Tn916-like *xis* genes were
105 detected by PCR as previously described.²⁹ Tn916-*xis* screening PCR products from human isolates
106 8797 and 5377 were sequenced using amplification primers (327-328) and the Tn5801-*int* screening
107 product from human isolate 1680 was sequenced with amplification primers (1811-1812). Primers
108 are listed in Table 3.

109 Long PCR linking *tet(M)* to Tn916-like *xis* or Tn5801-like *int* was performed with Phusion™ High-
110 Fidelity DNA Polymerase (Finnzymes, Finland) using condition as recommended by the
111 manufacturer. Primers 328 and 804 (Table 3) were used for long PCRs linking *tet(M)* with Tn916-
112 like *xis*. PCR conditions were 30 sec at 98°C followed by 30 cycles of 10 sec at 98°C, 30 sec at
113 51°C, 85 sec at 72°C and a final extension for 10 min at 72°C. Primers 709 and 1812 (Table 3) were
114 used for long PCR linking Tn5801-like *int* with *tet(M)* using conditions of initial denaturation for
115 30 sec at 98°C followed by 30 cycles of 10 sec at 98°C, 30 sec at 61°C, 144 sec at 72°C and a final

116 extension for 10 min at 72 °C. The *tet(M)*-Tn5801-like *int* product from of human isolate 1680 was
117 sequenced with primers 1812 and 1835-1840 (GenBank submission no. EU918655).

118 In all PCRs the reference strains *E. faecalis* DS16³⁰ and *S. aureus* Mu50¹⁴ were used as positive
119 control for the presence of Tn916- and Tn5801- like transposons, respectively. As negative control
120 the reference strain containing the other transposon was used.

121 **Phylogenetic analysis**

122 GenBank was searched for full length *tet(M)* genes based on the definition that *tet(M)* genes share
123 $\geq 80\%$ similarity on the amino acid level.² Fifty two unique gene sequences were selected to
124 represent different species from different hosts. A Neighbor Joining (NJ) tree based on a multiple
125 alignment of 41 sequences obtained in this study and 52 *tet(M)* genes from GenBank (1920 bp) was
126 constructed in Clustal X³¹ and visualized by MEGA 3.1.³² The tree was rooted with the *tet(O)* gene
127 (GenBank/EMBL/DDBJ accession nr. Y07780) as outgroup. Another tree based on the 450 bp
128 region downstream of *tet(M)* was constructed in the same way. Sequences were compared pairwise
129 with the EMBOSS program water used for local alignments.^{33 34}

130 ***spa* and MLST typing**

131 All *tet(M)* positive isolates were *spa* typed (Table 2) using primers and conditions recommended by
132 SeqNet.³⁵ The *spa* types were determined using BioNumerics 4.61 (Applied maths, Sint-Martens-
133 Latem, Belgium). Two human and one pig isolate (1591, 34801 and 9b) were also MLST typed as
134 recommended by MLSTnet.³⁶

135 **Clustering of *tet(M)* verses *spa* types in clonal complexes**

136 CC types were deduced from the *spa* types by using information available from the Ridom Spa
137 Server and from MLSTnet.^{37 38} In cases where the CC type could not be deduced from the *spa* type

138 (isolate 1591, 34801 and 9b), the CC type was determined from the MLST type by using the
139 eBURSTv3 algorithm.³⁹ Related *spa* types within different CC types were revealed by using the
140 Minimum Spanning Tree method in BioNumerics, cut off distance ≤ 3 .

141 **Filter mating**

142 Filter mating experiments were performed as described previously⁴⁰ using nine human isolates
143 (1591, 1680, 21995, 34148, 34168, 34801, 35366, 4520, 8797) and five animal isolates (7413532-2,
144 7611472-1, 9877324-3, USA42, 9b) as donors and the two *S. aureus* recipients, R1 (8794RF)⁴¹ and
145 R2 (RN4220RF).⁴² The detection limit of transconjugants and the rates of spontaneous mutations
146 were calculated for each of the mating experiment. In all experiments the donors tend to grow faster
147 than the recipient, therefore the transfer rates and the detection limits were calculated as
148 transconjugants per recipient. Transconjugants were selected on brain heart infusion agar plates
149 (Becton, Dickinson and Company, USA), supplemented with 8 mg/L of tetracycline, 12.5 mg/L of
150 rifampicin and 12.5 mg/L of fusidic acid. The numbers of donors and recipient were counted on
151 brain heart infusion agar plates supplemented with 8 mg/L of tetracycline or 12.5 mg/L of
152 rifampicin and 12.5 mg/L of fusidic acid, respectively. Transconjugants were further verified by *spa*
153 typing and screened for *tet(M)* by PCR. Long PCR linking *tet(M)* to Tn916-like *xis* or Tn5801-like
154 *int* verified that *tet(M)* was present on either Tn916- or Tn5801-like transposons in the
155 transconjugants.

156 **Results**

157 **Screening *S. aureus* isolates for *tet(M)***

158 Out of 205 tetracycline resistant *S. aureus* isolates, 20 human and 21 animal isolates were shown to
159 be positive for *tet(M)* by PCR (Table 1). The highest prevalence of *tet(M)* was found among

160 isolates from pigs with 60.7 % *tet(M)* positives compared to 21.3% in humans, 4.3% in cattle and
161 2.6% in poultry.

162 **Sequencing of *tet(M)***

163 The *tet(M)* gene including a downstream region from 41 *S. aureus* isolates were sequenced
164 according to the strategies shown in Figure 2. Comparing all 41 *tet(M)* gene sequences (1920 bp)
165 revealed 6 unique sequence types, of which one type was sequenced with strategy 1 and the other
166 five types were sequenced with either strategy 2a or 2b.

167 **Phylogenetic analysis predicts mobile elements associated with *tet(M)***

168 The result of the phylogenetic analysis is shown in Figure 3. The sequences fell into three groups.
169 All staphylococci sequences including the 41 *tet(M)* from *S. aureus*, Tn5801 *tet(M)* from *S. aureus*
170 Mu50 (BA000017) and Tn916 *tet(M)* from *E. faecalis* DS16 (U09422) fell into group II.²¹ Tn5397
171 *tet(M)* from *C. difficile* (AF333235) and two similar Tn5397-like *tet(M)* from *E. faecium* were
172 contained in group I and Tn1545 *tet(M)* from *E. faecalis* (X04388) and other composite transposons
173 (Tn2009 and Tn5251) fell into group III.

174 Based on similarity, the 41 *tet(M)* genes from *S. aureus* (consisting of 6 sequence types) were
175 divided into three subgroups within group II (Figure 3). Subgroups 1 and 2 were identical or highly
176 related (98.8-100% similarity at DNA level) to *tet(M)*-Tn916 from *E. faecalis* DS16, however
177 subgroup 2 formed an individual branch supported with a bootstrap of 100%. Sequences of
178 subgroup 3 were identical to *tet(M)* Tn5801 from *S. aureus* Mu50 (BA000017). This indicates that
179 *tet(M)* of subgroups 1 and 2 were located on Tn916-like transposons and that *tet(M)* sequences of
180 subgroup 3 were located on the putative transposon Tn5801. This was further supported by a
181 phylogenetic tree based on the 450 bp region downstream of *tet(M)* that divided the sequences into

182 two groups (data not shown). One group was identical or highly related to the downstream region of
183 Tn916 (99.8-100%) and the second group was identical to the downstream region of Tn5801.

184 ***S. aureus tet(M)* genes are located on Tn916-like and Tn5801-like transposons**

185 The presence of *tet(M)* on Tn916-like transposons in subgroups 1 and 2 and on the putative
186 transposon Tn5801 in subgroup 3 (Figure 3) was confirmed by PCR. All isolates from subgroup 1
187 and 2 were positive for Tn916-*xis* and negative for Tn5801-*int* and all isolates from subgroup 3
188 were positive for Tn5801-*int* and negative for Tn916-*xis* (data not shown). Two *xis* and one *int* PCR
189 screening products from the human isolates 8797, 5377 and 1680 representing subgroup 1, 2 and 3
190 respectively were sequenced. Both *xis* sequences were 100% identical to the corresponding *xis*
191 sequence from Tn916 in *E. faecalis* DS16 and the *int* sequence were 100% identical to the
192 corresponding *int* sequence from Tn5801 in *S. aureus* Mu50.

193 For all isolates with *tet(M)*, linking PCR confirmed that the Tn916-*xis* and Tn5801-*int* genes
194 detected in the PCR screen were actually located in the same element as *tet(M)* (Figure 4A). The
195 DNA sequence of *tet(M)-int* (GenBank accession no. EU918655) from human isolate 1680
196 (subgroup 3) had 99.9% similarity with the corresponding sequence in Tn5801 (BA000017). Thus
197 *tet(M)* of subgroups 1 and 2 are located on Tn916-like elements and *tet(M)* of subgroup 3 is located
198 on Tn5801-like elements.

199 **Dissemination of *tet(M)* within *S. aureus* of human and animal origin**

200 In Table 2, *spa* types and corresponding CC types are shown. Most animal isolates had *spa* type
201 t034 except two pig isolates of *spa* type t011 and one pig isolate with *spa* type t571, all belonging to
202 CC398.⁴³ The human isolates had different *spa* types: t034 (CC398), t008, t037 and t051 (CC8),
203 t065 (CC45), t078 (CC25), t318 and t964 (CC30) and t668 (CC5). Thus isolates of *spa* type t034
204 (CC398) were found both in different animals and in humans.

205 In order to compare how the different *tet(M)* genes may have been disseminated within and
206 between different CC types of *S. aureus*, the *tet(M)* sequences were grouped according to their CC
207 type (Figure 5). Figure 5 shows a clear correlation between different *tet(M)* sequence types and
208 different CC types of *S. aureus*. Moreover, *tet(M)* of subgroup 3 was identical to *tet(M)*-Tn5801
209 from *S. aureus* Mu50 belonging to CC5, indicating horizontal transfer of Tn5801 between CC5 and
210 CC8 (Figure 5C).

211 **Horizontal transfer of Tn916- and Tn5801-like (Tn6014) transposons**

212 To test whether the identified Tn916-like and Tn5801-like transposons were functional conjugative
213 transposons, filter mating experiments with 14 selected isolates as donors and two *S. aureus*
214 recipients were performed. The detection limits for the mating experiments were between 2.7×10^{-10}
215 to 1.5×10^{-9} transconjugants/recipient except for mating with donor 1591 and recipient R1 (8794)
216 where the detection limit was 2.5×10^{-8} transconjugants/recipient. Spontaneous mutations to
217 rifampicillin and fudisic acid were observed only for donor 8797 ($1.4\text{-}1.9 \times 10^{-8}$) and donor 34801
218 ($0.9\text{-}8.5 \times 10^{-10}$). Transconjugants conferring resistance to tetracycline, but not containing *tet(M)*
219 were observed only in matings with donor 9877324-3 to both recipients (1×10^{-7} and 9×10^{-9}
220 transconjugants/recipient, respectively).

221 Tn916-like *tet(M)* from the human isolates 34801 and 35366 from subgroup 1 were able to transfer
222 to R1 (8794RF) at transfer rates of 1×10^{-9} transconjugants/recipient and 3×10^{-8}
223 transconjugants/recipient, respectively. Transfer from isolate 35366 into R2 (RN4220RF) was also
224 observed (1×10^{-9} transconjugants/recipient). Tn5801-like *tet(M)* of the human isolate 1680 from
225 subgroup 3 was able to transfer to R2 (RN4220RF) with a transfer rate of 1×10^{-9}
226 transconjugants/recipient (Figure 4B and 4C). This transfer was in addition to *spa* typing verified by
227 a PFGE analysis showing the recipient R2 and the transconjugant (1680R2_4) to have the same
228 PFGE pattern distinct of the donor (1680) after *SmaI* digestion (data not shown). The Tn5801-like

229 element from human isolate 1680 was therefore registered as a novel conjugative transposon,
230 Tn6014 in the Transposon Nomenclature Database from the UCL Eastman Dental Institute, London
231 (<http://www.ucl.ac.uk/eastman/tn/>).⁴⁴

232 **Discussion**

233 The screening of tetracycline resistant *S. aureus* isolates showed the highest prevalence of *tet(M)*
234 among the tetracycline resistant pig isolates (60.7 %) and the lowest in tetracycline resistant poultry
235 (2.6 %) and bovine-mastitis (4.4 %) isolates. All animal isolates belonged to CC398 that has
236 recently emerged as a Methicillin-resistant clone in the Netherlands and other countries including
237 Denmark.⁴⁵⁻⁴⁷ Beside the animal isolates four of the twenty one human isolates also belonged to
238 CC398 and contained the same Tn916-like *tet(M)* gene as all the animal isolates. CC398 isolates are
239 usually tetracycline resistant and a recent study detected *tet(M)* in all methicillin-resistant *S. aureus*
240 (MRSA) CC398 studied from human and companion animals in Germany and Austria.^{43, 45, 47} This
241 suggests that a Tn916-like *tet(M)* was integrated and adapted early in the evolution of the clone and
242 may be disseminated vertically within CC398.

243 In our study one of the human CC398 isolates dates back to 1992. The two CC398 isolates from
244 cattle are from the beginning of the 1990s and the turkey CC398 isolates is from 1998. The rest of
245 CC398 were isolated between 2000 and 2008. Thus already in the early 1990s inter-species
246 transmission of CC398 may have occurred. Whether the high occurrence of CC398 among the pig
247 isolates found in this study reflects that pigs are the main reservoir for CC398 *tet(M)* is unknown.

248 Sequence analysis divided the sequenced *tet(M)* into three subgroups corresponding to two
249 different transposons. *tet(M)* of subgroup 1 and 2 were located on Tn916-like transposons and
250 subgroup 3 was located on Tn5801-like transposons. Subgroup 1 contained human isolates from
251 different *spa* types (and phage types) and all animal isolates from the time span 1959-2007. The
252 sequence variations of *tet(M)* within subgroup 1 correlated with different CC types of *S. aureus* (see

253 Figure 5A) which supports the general idea that *S. aureus* of different lineages are not very good at
254 sharing DNA. Subgroup 2 formed an individual branch with five identical *tet(M)* sequences, all
255 from human isolates of phage type 94/96 isolated between 1970-1992. All were shown to have the
256 same *spa* type (t078) belonging to CC25. Thus *tet(M)* of subgroup 2 appears to have been
257 integrated in this clone over 30 years ago without changing. PCR mapping of the elements in
258 subgroup 2 were of expected size (data not shown), indicating that all the ORFs necessary to
259 conjugate were present. Whether or not this new Tn916-like transposon is functional is however not
260 clear. Subgroup 3 consisted of seven isolates from 1957-2000 with identical *tet(M)*, different phage
261 types and *spa* types all belonging to CC8. Comparing the isolates within this group shows a
262 correlation between *spa* type and resistance pattern (see Table 2). The three isolates from *spa* type
263 t037 (all from 2000) were resistant to the same seven antimicrobial agents including Methicillin and
264 were suspected to be from the same outbreak. The two other *spa* types in this group, t008 and t051
265 (1957-1963) are clonally related and are only resistant to 3 and 5 agents respectively, the latter was
266 Methicillin-resistant. These differences may be time dependent or reflect resistance profiles in
267 different sub-clones of CC8.

268 As shown by the phylogenetic tree in Figure 3, *S. aureus tet(M)* sequences belonging to
269 subgroups 1A, 1C and 3 were identical to *tet(M)* of *Streptococcus agalactiae* (AAJQ1000009),
270 Tn916 -*tet(M)* from *E. faecalis* DS16 (U09422) and to *tet(M)* found in *Streptococcus agalactiae*
271 COH1 (NZ_AAJR01000021), respectively. This indicates horizontal transfer of *tet(M)* from
272 subgroup 1 and subgroup 3 between *S. aureus* and other Gram-positive species of enterococci and
273 streptococci. Previously horizontal transfer of Tn916-like *tet(M)* from *Bacillus cereus* group into *S.*
274 *aureus* has been shown.²⁹ Clustering of CC *spa* types versus *tet(M)* sequences type suggested that
275 horizontal transfer of Tn5801 between different CC types of *S. aureus* has occurred.

276 The filter mating experiment showed that the Tn916-like element from subgroup 1A (CC398)
277 could be transferred into both recipient stains (8794RF, CC121 and RN4220RF, CC8) whereas the
278 Tn916-like element from subgroup 1D (CC5) was only transferred into one of the recipient strains
279 (8794RF, CC121). The Tn5801-like element, Tn6014 from subgroup 3 (CC8) was transferred into
280 the other recipient strain (RN4220RF, CC8). The new *tet(M)*-like elements of subgroup 2 did not
281 transfer into any of the recipients. Although, both recipients are known to be very good in taking up
282 foreign DNA, transconjugants were obtained with very low frequencies. Recently the restriction-
283 modification system, *SauI* was suggested to control horizontal gene transfer between *S. aureus* of
284 different lineages.⁴⁸ RN4220RF was shown to have a mutation in this system making it able to take
285 up DNA from different CC types. However, in our study RN4220RF only received *tet(M)* from two
286 of the 14 tested donors which indicates that other factors may also play a role or that transfer occurs
287 at rates below our detection limit.

288 Staphylococci *tet(M)* were only located in part of the phylogenetic tree associated with the well
289 characterized conjugative transposon Tn916 and with Tn5801 described in *S. aureus* Mu50 and
290 Mu3^{14, 49} (group II, Figure 3). *tet(M)* from other Gram-positive bacteria were distributed in the
291 whole tree and were besides Tn916-like elements associated with Tn5397 (group I) and/or
292 composite transposons like Tn1545, Tn2009 and/or Tn5251 (group III). Moreover, *tet(M)* from *E.*
293 *faecium* (DQ223243 and DQ223244) has also been found on plasmids (group I).²¹ Thus *tet(M)* from
294 *S. aureus* appear to be less diverse than *tet(M)* from other Gram-positive bacteria.

295 The predicted mobile elements associated with *tet(M)* in *S. aureus* from different origins were
296 confirmed experimentally by long PCR. The same approach was also used successfully in a
297 previous study of the diversity of *tet(M)* among Enterococci.²¹ Thus in general *tet(M)* appear to be
298 more related to its mobile element than to the host species, however module exchange between
299 transposons and recombination within *tet(M)* may also have occurred.^{10, 21} Module exchange seems

300 to be the case for putative transposon CW459*tet*(M) from *Clostridium peringens*.¹⁶ In this
301 transposon *tet*(M) is highly related to Tn916-*tet*(M) whereas the rest of the transposon sequence is
302 more related to Tn5801.¹⁵

303 In conclusion, we have used the diversity of the *tet*(M) gene to determine associated mobile
304 elements in *S. aureus* from human and different animal origin. *S. aureus* of human origin was
305 shown to contain diverse *tet*(M) genes located on Tn916-like and Tn5801-like conjugative
306 transposons that corresponded with different CC types. *S. aureus* of different animal origin
307 contained one specific type of *tet*(M) located on Tn916-like elements, all belonging to CC type 398.
308 This is the first report showing that a Tn5801-like element, Tn6014 can transfer between *S. aureus*
309 isolates.

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318 **Transparency declarations**

319 None to declare

320 **Reference**

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458 Legends to Figures

459 Figure 1: Comparison of the 18 kb conjugative transposon Tn916 (U09422) and the 25 kb putative
460 transposon Tn5801 (BA000017/NC002758). Besides *tet(M)* (dark grey arrows) they both consist of
461 3 structural domains containing genes associated with conjugation, regulation (light grey arrows)
462 and excision/integration (dotted arrows).^{11, 15, 50} Both elements contain an integrase (*int*) gene,
463 however these *int* genes are very different. In addition, Tn916 also contains an annotated
464 excisionase (*xis*) gene (black arrow). Tn5801 contains several open reading frames (white arrows)
465 whose functions are unknown. *sav415* may however encode a transposase.¹⁵ The relation between
466 open reading frames of Tn916 and Tn5801 are shown in percent identity on nucleotide level
467 calculated with the EMBOSS program water.³⁴

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469 **Figure 2.: Amplification and sequencing strategy for *tet(M)*.** Top: The *tet(M)* gene sequence
470 including a downstream region. Primers used for amplification and sequencing are illustrated with
471 arrows. Bottom: Two different sequencing strategies using different combination of primers.

472 Strategy 1: Sequences from 7 human isolates (213, 229, 1680, 1742, 33597, 34148, 34168) were
473 obtained. Strategy 2a: 3 human, 8 pig, 1 lamb and 1 bovine isolate (22034, 35414, 35679, 9b,
474 7215311, 7311242, 7413093-4, 7413714-1, 7512986-1, 7611472.1, 7611995-1, 7612628-4,
475 sw356). Strategy 2b: 10 human, 9 pig, 1 turkey and 1 bovine isolate (617, 1591, 4520, 4865, 5331,
476 5377, 8797, 21995, 35801, 35366, 7215190-1, 7312330-1, 7412791, 7413532-2, 7413727,
477 74140355-2, 7512166-1, 7611280, 7711730-1, 9877324-3, usa42).

478

479 **Figur 3:** Phylogenetic gene tree of *tet(M)*. Bootstrap values are indicated at branch points (out of
480 1000 generated NJ trees). Group I is supported by a bootstrap value of 87.8%, group II by a

481 bootstrap value of 48.7% at the first branching and 93.7% at the second branching and group 3 by a
482 bootstrap value of 99.4%.

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484 **Figure 4:** PCR products linking *tet(M)* to Tn916-like *xis* and Tn5801-like *int* genes with the
485 expected size of 2835 bp and 4820 bp respectively. **A:** A representative from every *tet(M)* type
486 from different origins are shown. Lane 1: 9877324-3 (turkey), 2: USA42 (cattle), 3: 7611472-1
487 (lamb), 4: 9b (pig), 5: 21995 (human), 6: 617 (human), 7: 8797 (human), 8: 34801 (human), 9: 4520
488 (human), 10: positive control (*E. faecalis* DS16), 11: negative control (*S. aureus* Mu50), 12: 1680
489 (human), 13: positive control (*S. aureus* Mu50), 14: negative control (*E. faecalis* DS16). M: Gene
490 Ruler 1Kb ladder from Fermentas. **B:** Transconjugants (TC), donors (D) and recipients showing
491 horizontal transfer of Tn916-like transposons. Lane 1: 34801_1_R1 (TC), 2: 34801 (D), 3:
492 35366_1_R1 (TC), 4: 35366_1_R2, 5: 35366 (D), 6: R1 (8794RF), 7: R2 (RN4220RF), 8: positive
493 control (*E. faecalis* DS16), 9: negative control (*S. aureus* Mu50). **C:** TC, D and recipient showing
494 horizontal transfer of the Tn5801-like transposon Tn6014. Lane 1: 1680R2_4 (TC), 2: 1680 (D), 3:
495 R2 (RN4220RF), 4: positive control (*S. aureus* Mu50), 5: negative control (*E. faecalis* DS16).

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497 **Figure 5:** CC *spa* type clustering versus different *tet(M)* sequence types in *S. aureus*. Predicted
498 horizontal gene transfer of *tet(M)* is illustrated by an arrow. Related *spa* types are illustrated with a
499 black line: *spa* type t034 is related to t011 by a deletion of two repeats in t011 compared to t034.
500 Furthermore, t034 is related to t571 by a deletion of one repeat in t571 compared to t034. *spa* type
501 t051 is related to t008 by a deletion of one repeat in t008 compared to t501. *spa* types t964 and t318
502 shared 8 out of their 9 or 10 repeats, respectively and one repeat varies by one substitution.

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505 **Table 1:** Origin, source, phage types, country, year and numbers of *S. aureus* isolates screened for
 506 *tet(M)* by PCR and the number found positive for *tet(M)*.

<i>Source</i>	<i>Human^a</i>	<i>Pig</i>	<i>Poultry</i>	<i>Sheep</i>	<i>Cattle^b</i>
<i>Phage types</i>	80 complex, gr1, gr2, gr3, 83A, 94/96, 95, MIX, NI	ND	ND	ND	ND
<i>Country/Region</i>	Denmark	Denmark	Denmark	Denmark	Europe & US
<i>Year</i>	1957-2002	2000-2007	1994-1998	2004-2005	Early 1990s
<i>Isolates</i>	94	28	39	2	42 ⁵¹
<i>tet(M) positive</i>	20 (21.3%)	17 (60.7%)	1 (2.6%)	1 (50%)	2 (4.3%)

507 a: bacteraemia, b: mastitis, ND: Not determined

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525 **Table 2.** *S. aureus* strains with *tet(M)* used in this study

Strain	Source/year	Other resistance phenotypes	Mobile element carrying <i>tet(M)</i>	<i>spa</i> type/CC types	GenBank accession number
213	Human/1957	Pen ^R , Str ^R	Tn5801-like	t008/CC8	EU918651
229	Human/1957	Pen ^R , Str ^R	Tn5801-like	t008/CC8	EU918652
1680	Human/1963	Pen ^R , Str ^R , Ery ^R , Met ^R	Tn5801-like (Tn6014)	t051/CC8	EU918655
1742	Human/1963	Pen ^R , Str ^R	Tn5801-like	t008/CC8	EU918656
33597 ^b	Human/2000	Pen ^R , Str ^R , Gen ^R , Ery ^R , Met ^R , Cip ^R	Tn5801-like	t037/CC8	EU918664
34148 ^b	Human/2000	Pen ^R , Str ^R , Gen ^R , Ery ^R , Met ^R , Cip ^R	Tn5801-like	t037/CC8	EU918665
34168 ^b	Human/2000	Pen ^R , Str ^R , Gen ^R , Ery ^R , Met ^R , Cip ^R	Tn5801-like	t037/CC8	EU918666
4520	Human/1970	Pen ^R	Tn916-like	t078/CC25	EU918657
4865	Human/1970	Pen ^R	Tn916-like	t078/CC25	EU918658
5331	Human/1971	Pen ^R	Tn916-like	t078/CC25	EU918659
5377	Human/1971	Pen ^R	Tn916-like	t078/CC25	EU918660
22034	Human/1992	Pen ^R , Ery ^R , Cip ^R	Tn916-like	t078/CC25	EU918663
34801	Human/2001	Pen ^R , Fus ^R	Tn916-like	t688/CC5 ^a	EU918667
8797	Human/1977	Pen ^R	Tn916-like	t065/CC45	EU918661
617	Human/1959	Pen ^R	Tn916-like	t318/CC30	EU918653
1591	Human/1962	Pen ^R	Tn916-like	t964/CC30 ^a	EU918654
21995	Human/1992	Pen ^R	Tn916-like	t034/CC398	EU918662
35366	Human/2001	Pen ^R	Tn916-like	t034/CC398	EU918668
35414	Human/2001		Tn916-like	t034/CC398	EU918669
35679	Human/2001	Pen ^R	Tn916-like	t034/CC398	EU918670
9877324-3	Turkey/1998	Pen ^R	Tn916-like	t034/CC398	EU918671
USA42	Cattle	Pen ^R , Str ^R , Ery ^R , Chl ^R , Sul ^R	Tn916-like	t034/CC398	EU918673
Sw356	Cattle	Str ^R , Spt ^R , Tmp ^R	Tn916-like	t034/CC398	EU918672
7611472-1	Lamb/2004	Pen ^R , Cip ^R	Tn916-like	t034/CC398	EU918687
9b	Pig/2007	Pen ^R , Str ^R , Ery ^R , Spt ^R , Tmp ^R , Cef ^R , Met ^R	Tn916-like	t034/CC398 ^a	EU918691
7215190-1	Pig/2000	Pen ^R , Str ^R , Tmp ^R	Tn916-like	t034/CC398	EU918674
7215311-1	Pig/2000	Pen ^R , Str ^R	Tn916-like	t034/CC398	EU918675
7311242-1	Pig/2001	Ery ^R , Str ^R , Spec ^R	Tn916-like	t034/CC398	EU918676
7312330-1	Pig/2001	Pen ^R , Str ^R , Spec ^R	Tn916-like	t034/CC398	EU918677
7412791-1	Pig/2002	Pen ^R , Str ^R , Tmp ^R	Tn916-like	t034/CC398	EU918678
7413093-4	Pig/2002	Pen ^R , Ery ^R , Tia ^R , Tmp ^R	Tn916-like	t034/CC398	EU918679
7413532-2	Pig/2002	Pen ^R	Tn916-like	t011/CC398	EU918680
7413714-1	Pig/2002	Pen ^R , Tmp ^R	Tn916-like	t034/CC398	EU918681
7413727-1	Pig/2002	Pen ^R , Ery ^R	Tn916-like	t034/CC398	EU918682
7414035-2	Pig/2002	Pen ^R , Str ^R , Ery ^R	Tn916-like	t034/CC398	EU918683
7512166-1	Pig/2003	Pen ^R , Str ^R , Ery ^R , Tmp ^R	Tn916-like	t034/CC398	EU918684
7512986-1	Pig/2003	Ery ^R , Spt ^R	Tn916-like	t034/CC398	EU918685
7611280-5	Pig/2004	Pen ^R , Tmp ^R	Tn916-like	t011/CC398	EU918686
7611995-1	Pig/2004	Pen ^R , Str ^R , Ery ^R , Spt ^R , Tia ^R , Tmp ^R	Tn916-like	t034/CC398	EU918688
7612628-4	Pig/2004	Ery ^R , Spt ^R	Tn916-like	t571/CC398	EU918689
7711730-1	Pig/2005	Pen ^R , Spt ^R , Tia ^R , Tmp ^R	Tn916-like	t034/CC398	EU918690

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^a : Determined by MLST

^b : Isolates suspected to be from the same outbreak.

Pen^R: penicillin, Str^R: streptomycin, Gen^R: gentamicin, Ery^R: erythromycin, Met^R: methicilin, Cip^R: ciprofloxacin,
Chl^R: chloramphenicol, Spt^R: spectinomycin, Sul^R: sulphamethoxazo, Tia^R: tiamulin, Tmp^R: trimethoprin, Cef^R: Ceftifur,
Fus^R : fusidic acid

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534 **Table 3.** Primers used in this study

Primer number/name	Sequence	Reference
266 (Tet(M)-1)	5'-GTTAAATAGTGTTCTTGGAG-3'	27
267 (Tet(M)-2)	5'-CTAAGATATGGCTCTAACAA-3'	27
327 (Tn916-1)	5'-GCCATGACCTATCTTATA-3'	21
328 (Tn916-2)	5'-CTAGATTGCGTCCAA-3'	29
1811 (intcw459-1)	5'-CCGATATTGAGCCTATTGATGTG-3'	This study
1812 (intcw459-2)	5'-GTCCATACGTTTCCTAAAGTCGTC-3'	This study
709 (TetM sekvens 6)	5'-TCGAGGTCCGTCTGAAC-3'	This study
804 (TET-Down-1)	5'-GTCGTCCAAATAGTCGGATA-3'	This study
323 (TetM-up)	5'-CTGGCAAACAGGTTC-3'	21
324 (TetM-down)	5'-TAGCTCATGTTGATGC-3'	This study
526 (tet M upstream)	5'-TTGAATGGAGGAAAATCAC-3'	21
525 (tet M sekvens-1)	5'-TACTTTCCCTAAGAAAGAAAGT-3'	21
540 (Tet M seq-3)	5'-GCAGAAATCAGTAGAATTGC-3'	21
307 (Revers TetM-2)	5'-TTGTTAGAGCCATATCTTAG-3'	21
1835 (F1F)	5'-CGTGCAAATCTAGGTTATG-3'	This study
1836 (F2F)	5'-CATGAAGGAGTGTAAGAATGA-3'	This study
1837 (F2R)	5'-GTGCTTATACCATGGAAGGA-3'	This study
1838 (F3F)	5'-GAGCCTCTTTAATCGCT-3'	This study
1839 (F3R)	5'-CATATTCGTCTGTCATGC-3'	This study
1840 (F4)	5'-GCTAGTGCTTCCATTAAGGA-3'	This study

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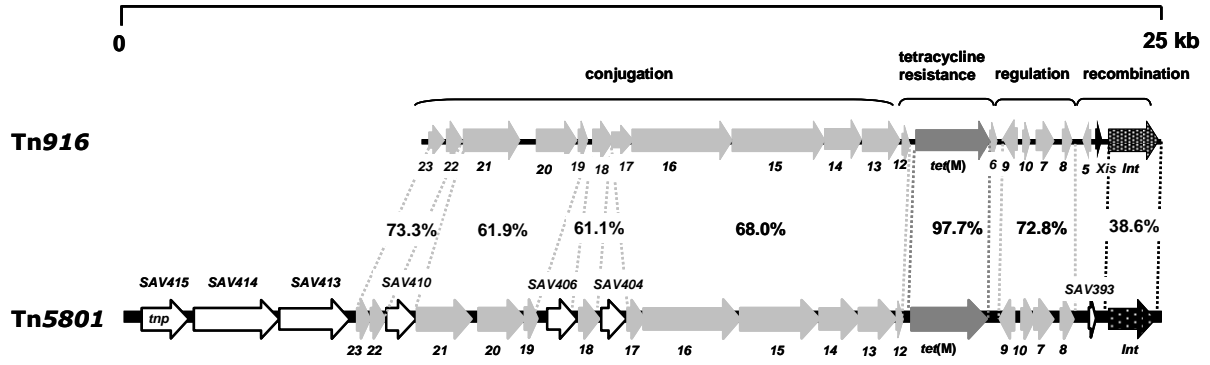
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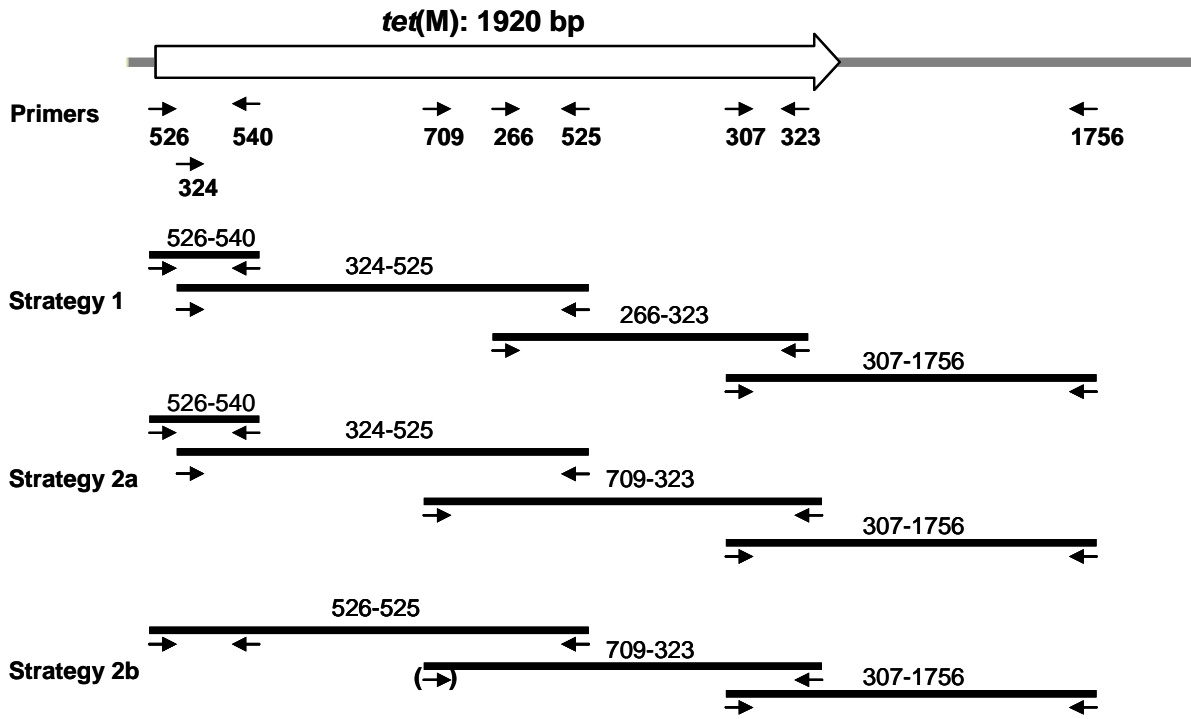
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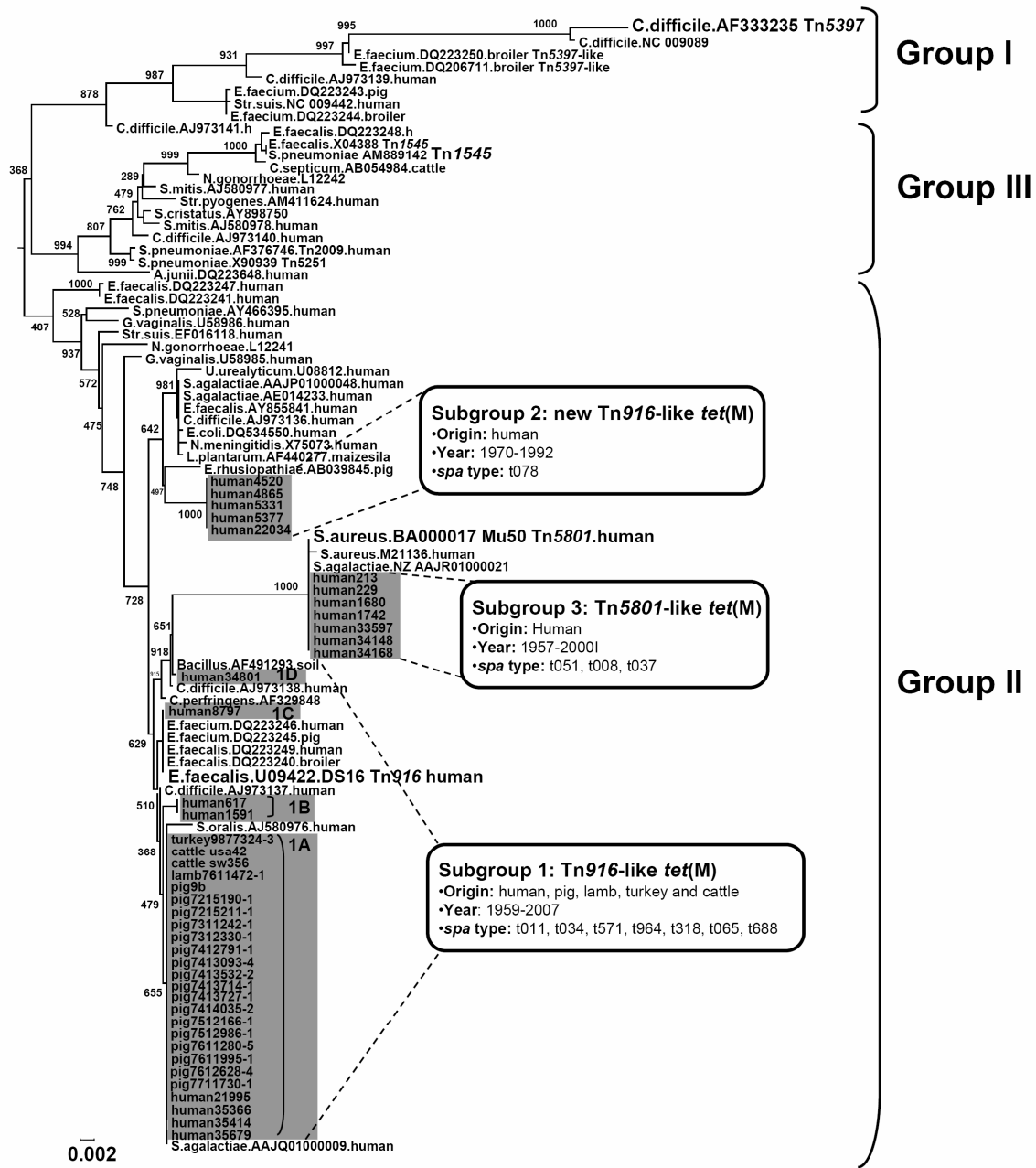
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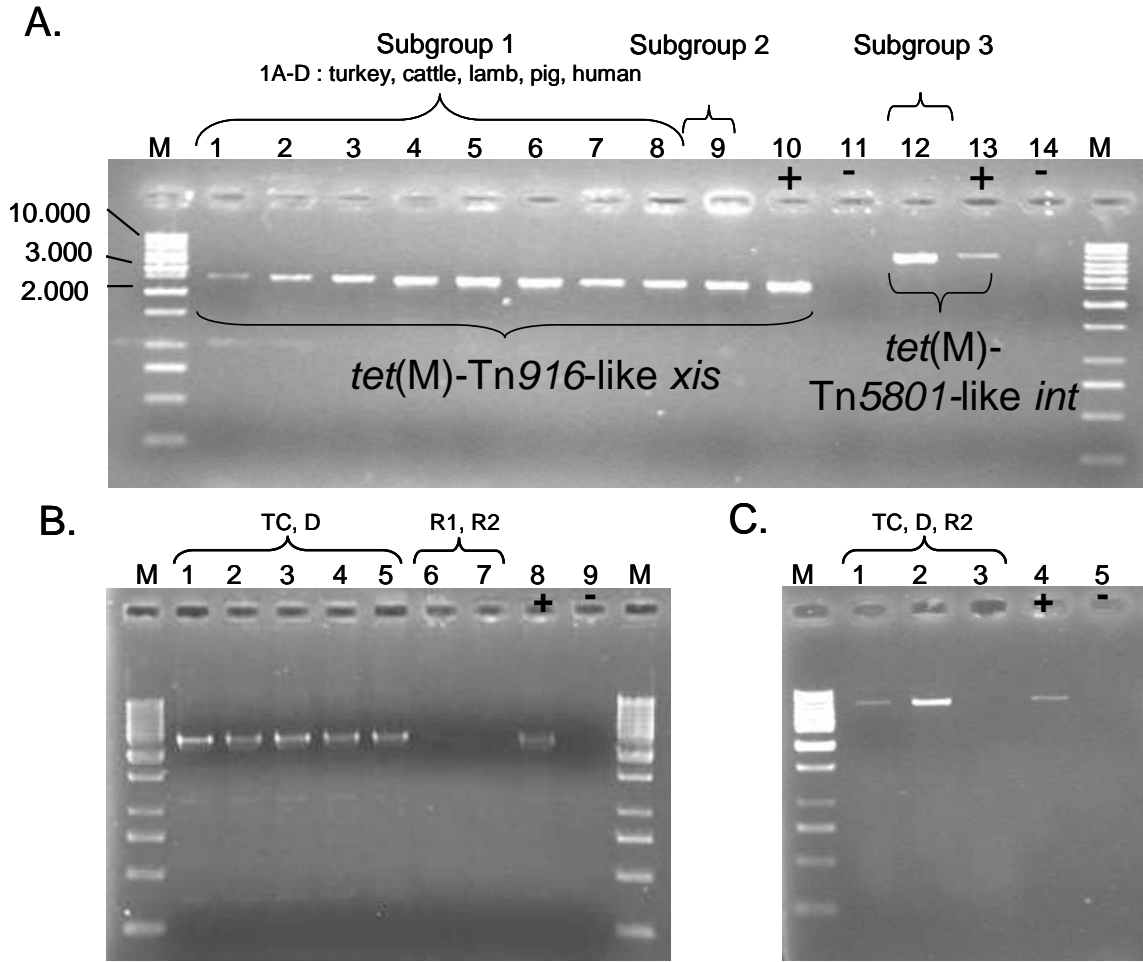
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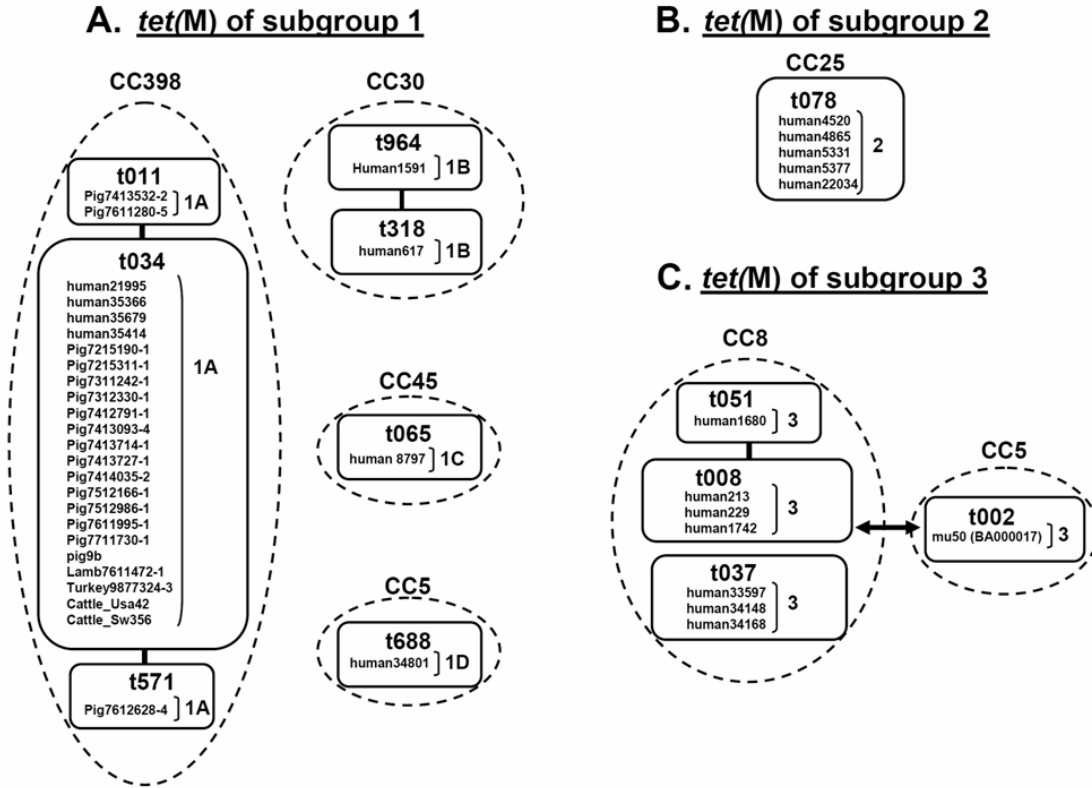
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