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Published in:
Journal of Antimicrobial Chemotherapy

DOI:
10.1093/jac/dkp214

Publication date:
2009

Document version
Early version, also known as pre-print

Diversity of the Tetracycline Resistance Gene *tet*(M) and Identification of Tn*916*- and Tn*5801*-like (Tn*6014*) Transposons in *Staphylococcus aureus* from Human and Animals

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Short running title: *tet*(M) in *S. aureus*

Keywords: *tetA*(M), horizontal gene transfer, conjugative transposons, mobile elements

Abstract

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

***Methods:* In total 205 tetracycline resistant isolates were screened for *tet*(M) by PCR. *tet*(M) were sequenced and compared to *tet*(M) deposited with GenBank. Based on phylogenetic analysis isolates were screened for Tn*916*- and Tn*5801*-like *xis*/*int* genes and transposons were confirmed by linking PCR. *spa* typing was performed and selected isolates were used as donors in a filter mating experiment.**

***Results:* Forty-one isolates (21.3 %, 60.7 %, 2.6 % and 4.4 % of the human, pig, poultry and cattle isolates, respectively) were *tet*(M) positive. *tet*(M) was located on Tn*5801*-like and Tn*916*-like transposons in humans and on a specific Tn*916*-like element in animals. Human isolates were of different *spa* types (t034, t008, t037, t051, t065, t078, t318 and t964) corresponding to different clonal complexes (CC398, CC8, CC25 and CC30). Animal isolates were of *spa* type t034, t011 or t0571 corresponding to CC398. *tet*(M) sequence types correlated with CC types. Tn*916*-like and Tn*5801*-like (Tn*6014*) transposons were able to transfer to *S. aureus* recipients.**

***Conclusion: S. aureus* of human origin contained diverse *tet*(M) located on Tn*916*- and Tn*5801*-like (Tn*6014*) transposons and *S. aureus* of animal origin contained Tn*916*-like *tet*(M) genes. This suggest that conjugative transposition play an important role in the evolution and horizontal spread of *tet*(M) in *S. aureus*. This is the first study showing horizontal transfer of Tn*5801* (Tn*6014*).**

Introduction

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

tet(M) together with *tet*(K) are the most common genes conferring tetracycline resistance in *S. aureus*.[5-8] *tet*(M) is widely distributed among both Gram-positive and Gram-negative bacteria and it has be found in 59 genera.[3, 9] This is probably due to the association of *tet*(M) with integrative and conjugative transposons, facilitating horizontal transfer.[10] Particularly in Gram-positive streptococci and enterococci, *tet*(M) has been found associated with Tn*916*/*1545*-like conjugative transposons which form the basis of a family of conjugative transposons that have an extremely broad host range.[11, 12] *tet*(M) associated with Tn*916* was originally identified in *Enterococcus faecalis* DS16 and Tn*1545* was identified in *Streptococcus pneumoniae*.[11, 13] Recently *tet*(M) was identified on a putative transposon Tn*5801* in *S. aureus* Mu50. [14] Tn*5801* contains many open reading frames similar to Tn*916*, but differs by using an integrase (*int*) different from the excisionase/integrase (*xis*/*int*) present in Tn*916* (Figure 1).[14, 15] Other conjugative transposons, like Tn*5397* and CW459*tet*(M) have been found to harbour *tet*(M) in *Clostridium difficile* and *Clostridium perfringens*.[16, 17] In addition, *tet*(M) have also been found on different plasmids.[2]

A limited number of studies concerning the diversity of the *tet*(M) gene have been performed.[18-22] In streptococci and enterococci *tet*(M) has been found to be diverse and mainly present on Tn*916*/Tn*1545* like conjugative transposons whereas two different allele types of *tet*(M) from

Lactobacillus has been found to be located mainly on plasmids.[19-22] Recently, Agersø *et al.* (2006) showed correlation between diversity of the *tet*(M) DNA sequence and their presence on Tn*916,* Tn*5397* or plasmids in enterococci from different sources in Denmark.[21]

To our knowledge no one has studied the diversity of *tet*(M) and its association with mobile elements in *S. aureus*. A former study found only one out of thirty-four *S. aureus* strains to carry *tet*(M) on Tn*916*/Tn*1545*-like transposons.[23] The aim of this study was to analyse sequence diversity of *tet*(M) in *S. aureus* from human and different animals mainly from Denmark, and thereby determine mobile elements associated with *tet*(M) in *S. aureus*.

Material and Methods

Strains

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

Of the 111 animal isolates (Table 1), 39 poultry[25], 27 pig and 2 lamb isolates were diagnostic submissions to either The National Veterinary Institute or to The National Food Institute, Technical University of Denmark. One pig isolate (9b) was obtained from a healthy pig in 2007. All animal isolates were tested for susceptibility to tetracycline, penicillin, streptomycin, erythromycin, ciprofloxacin, spectinomycin, tiamulin, trimethoprin, ceftiofur, chloramphenicol, florfenicol and

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

Screening and sequencing of *tet*(M)

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

Detection of Tn*916*-like and Tn*5801*-like transposons

The presence of the *int* genes specific for Tn*5801* was detected by PCR using primers 1811 and 1812 (product size 722 bp) and DNA Taq polymerase from Ampliqon. Tn*916*-like *xis* genes were detected by PCR as previously described.[29] Tn*916-xis* screening PCR products from human isolates 8797 and 5377 were sequenced using amplification primers (327-328) and the Tn*5801-int* screening product from human isolate 1680 was sequenced with amplification primers (1811-1812). Primers are listed in Table 3.

Long PCR linking *tet*(M) to Tn*916*-like *xis* or Tn*5801*-like *int* was performed with Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Finland) using condition as recommended by the manufacturer. Primers 328 and 804 (Table 3) were used for long PCRs linking *tet*(M) with Tn*916*-like *xis*. PCR conditions were 30 sec at 98°C followed by 30 cycles of 10 sec at 98°C, 30 sec at 51°C, 85 sec at 72°C and a final extension for 10 min at 72°C. Primers 709 and 1812 (Table 3) were used for long PCR linking Tn*5801*-like *int* with *tet*(M) using conditions of initial denaturation for 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

extension for 10 min at 72 °C. The *tet*(M)-Tn*5801*-like *int* product from of human isolate 1680 was sequenced with primers 1812 and 1835-1840 (GenBank submission no. EU918655).

In all PCRs the reference strains *E. faecalis* DS16 [30] and *S. aureus* Mu50[14] were used as positive control for the presence of Tn*916*- and Tn*5801*- like transposons, respectively. As negative control the reference strain containing the other transposon was used.

Phylogenetic analysis

GenBank was searched for full length *tet*(M) genes based on the definition that *tet*(M) genes share ≥80% similarity on the amino acid level.[2] Fifty two unique gene sequences were selected to represent different species from different hosts. A Neighbor Joining (NJ) tree based on a multiple alignment of 41 sequences obtained in this study and 52 *tet*(M) genes from GenBank (1920 bp) was constructed in Clustal X [31] and visualized by MEGA 3.1.[32] The tree was rooted with the *tet*(O) gene (GenBank/EMBL/DDBJ accession nr. Y07780) as outgroup. Another tree based on the 450 bp region downstream of *tet*(M) was constructed in the same way. Sequences were compared pairwise with the EMBOSS program water used for local alignments.[33 34]

spa and MLST typing

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

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

CC types were deduced from the *spa* types by using information available from the Ridom Spa Server and from MLSTnet.[37 38] In cases where the CC type could not be deduced from the *spa* type

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

Filter mating

Filter mating experiments were performed as described previously[40] using nine human isolates (1591, 1680, 21995, 34148, 34168, 34801, 35366, 4520, 8797) and five animal isolates (7413532-2, 7611472-1, 9877324-3, USA42, 9b) as donors and the two *S. aureus* recipients, R1 (8794RF)[41] and R2 (RN4220RF).[42] The detection limit of transconjugants and the rates of spontaneous mutations were calculated for each of the mating experiment. In all experiments the donors tend to grow faster than the recipient, therefore the transfer rates and the detection limits were calculated as transconjugants per recipient. Transconjugants were selected on brain heart infusion agar plates (Becton, Dickinson and Company, USA), supplemented with 8 mg/L of tetracycline, 12.5 mg/L of rifampicin and 12.5 mg/L of fusidic acid. The numbers of donors and recipient were counted on brain heart infusion agar plates supplemented with 8 mg/L of tetracycline or 12.5 mg/L of rifampicin and 12.5 mg/L of fusidic acid, respectively. Transconjugants were further verified by *spa* typing and screened for *tet*(M) by PCR. Long PCR linking *tet*(M) to Tn*916*-like *xis* or Tn*5801*-like *int* verified that *tet*(M) was present on either Tn*916*- or Tn*5801*-like transposons in the transconjugants.

Results

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

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

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

Sequencing of *tet*(M)

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

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

The result of the phylogenetic analysis is shown in Figure 3. The sequences fell into three groups. All staphylococci sequences including the 41 *tet*(M) from *S. aureus*, Tn*5801 tet*(M) from *S. aureus* Mu50 (BA000017) and Tn*916 tet*(M) from *E. faecalis* DS16 (U09422) fell into group II.[21] Tn*5397 tet*(M) from *C. difficile* (AF333235) and two similar Tn*5397*-like *tet*(M) from *E. faecium* were contained in group I and Tn*1545 tet*(M) from *E. faecalis* (X04388) and other composite transposons (Tn*2009* and Tn*5251*) fell into group III.

Based on similarity, the 41 *tet*(M) genes from *S. aureus* (consisting of 6 sequence types) were divided into three subgroups within group II (Figure 3). Subgroups 1 and 2 were identical or highly related (98.8-100% similarity at DNA level) to *tet*(M)-Tn*916* from *E. faecalis* DS16, however subgroup 2 formed an individual branch supported with a bootstrap of 100%. Sequences of subgroup 3 were identical to *tet*(M) Tn*5801* from *S. aureus* Mu50 (BA000017). This indicates that *tet*(M) of subgroups 1 and 2 were located on Tn*916*-like transposons and that *tet*(M) sequences of subgroup 3 were located on the putative transposon Tn*5801*. This was further supported by a phylogenetic tree based on the 450 bp region downstream of *tet*(M) that divided the sequences into

two groups (data not shown). One group was identical or highly related to the downstream region of Tn*916* (99.8-100%) and the second group was identical to the downstream region of Tn*5801*.

S. aureus tet(M) genes are located on Tn*916*-like and Tn*5801*-like transposons

The presence of *tet*(M) on Tn*916*-like transposons in subgroups 1 and 2 and on the putative transposon Tn*5801* in subgroup 3 (Figure 3) was confirmed by PCR. All isolates from subgroup 1 and 2 were positive for Tn*916-xis* and negative for Tn*5801-int* and all isolates from subgroup 3 were positive for Tn*5801-int* and negative for Tn*916-xis* (data not shown). Two *xis* and one *int* PCR screening products from the human isolates 8797, 5377 and 1680 representing subgroup 1, 2 and 3 respectively were sequenced. Both *xis* sequences were 100% identical to the corresponding *xis* sequence from Tn*916* in *E. faecalis* DS16 and the *int* sequence were 100% identical to the corresponding *int* sequence from Tn*5801* in *S. aureus* Mu50.

For all isolates with *tet*(M), linking PCR confirmed that the Tn*916-xis* and Tn*5801-int* genes detected in the PCR screen were actually located in the same element as *tet*(M) (Figure 4A). The DNA sequence of *tet*(M)-*int* (GenBank accession no. EU918655) from human isolate 1680 (subgroup 3) had 99.9% similarity with the corresponding sequence in Tn*5801* (BA000017). Thus *tet*(M) of subgroups 1 and 2 are located on Tn*916*-like elements and *tet*(M) of subgroup 3 is located on Tn*5801*-like elements.

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

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

In order to compare how the different *tet*(M) genes may have been disseminated within and between different CC types of *S. aureus*, the *tet*(M) sequences were grouped according to their CC type (Figure 5). Figure 5 shows a clear correlation between different *tet*(M) sequence types and different CC types of *S. aureus*. Moreover, *tet*(M) of subgroup 3 was identical to *tet*(M)-Tn*5801* from *S. aureus* Mu50 belonging to CC5, indicating horizontal transfer of Tn*5801* between CC5 and CC8 (Figure 5C).

Horizontal transfer of Tn*916*- and Tn*5801-like (*Tn*6014)* transposons

To test whether the identified Tn*916*-like and Tn*5801*-like transposons were functional conjugative transposons, filter mating experiments with 14 selected isolates as donors and two *S. aureus* recipients were performed. The detection limits for the mating experiments were between $2.7*10^{-10}$ to $1.5*10^{-9}$ transconjugants/recipient except for mating with donor 1591 and recipient R1 (8794) where the detection limit was $2.5*10^{-8}$ transconjugants/recipient. Spontaneous mutations to rifampicillin and fudisic acid were observed only for donor 8797 ($1.4\text{-}1.9*10^{-8}$) and donor 34801 ($0.9\text{-}8.5 *10^{-10}$). Transconjugants conferring resistance to tetracycline, but not containing *tet*(M) were observed only in matings with donor 9877324-3 to both recipients ($1*10^{-7}$ and 9*10-^{9} transconjugants/recipient, respectively).

Tn*916*-like *tet*(M) from the human isolates 34801 and 35366 from subgroup 1 were able to transfer to R1 (8794RF) at transfer rates of $1*10^{-9}$ transconjugants/recipient and $3*10^{-8}$ transconjugants/recipient, respectively. Transfer from isolate 35366 into R2 (RN4220RF) was also observed ($1*10^{-9}$ transconjugants/recipient). Tn*5801*-like *tet*(M) of the human isolate 1680 from subgroup 3 was able to transfer to R2 (RN4220RF) with a transfer rate of $1*10^{-9}$ transconjugants/recipient (Figure 4B and 4C). This transfer was in addition to *spa* typing verified by a PFGE analysis showing the recipient R2 and the transconjugant (1680R2_4) to have the same PFGE pattern distinct of the donor (1680) after *Sma*I digestion (data not shown). The Tn*5801*-like

element from human isolate 1680 was therefore registered as a novel conjugative transposon, Tn*6014* in the Transposon Nomenclature Database from the UCL Eastman Dental Institute, London (http://www.ucl.ac.uk/eastman/tn/).[44]

Discussion

The screening of tetracycline resistant *S. aureus* isolates showed the highest prevalence of *tet*(M) among the tetracycline resistant pig isolates (60.7 %) and the lowest in tetracycline resistant poultry (2.6 %) and bovine-mastitis (4.4 %) isolates. All animal isolates belonged to CC398 that has recently emerged as a Methicillin-resistant clone in the Netherlands and other countries including Denmark.[45-47] Beside the animal isolates four of the twenty one human isolates also belonged to CC398 and contained the same Tn*916*-like *tet*(M) gene as all the animal isolates. CC398 isolates are usually tetracycline resistant and a recent study detected *tet*(M) in all methicillin-resistant *S. aureus* (MRSA) CC398 studied from human and companion animals in Germany and Austria.[43, 45, 47] This suggests that a Tn*916*-like *tet*(M) was integrated and adapted early in the evolution of the clone and may be disseminated vertically within CC398.

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

Sequence analysis divided the sequenced *tet*(M) into three subgroups corresponding to two different transposons. *tet*(M) of subgroup 1 and 2 were located on Tn*916*-like transposons and subgroup 3 was located on Tn*5801*-like transposons. Subgroup 1 contained human isolates from different *spa* types (and phage types) and all animal isolates from the time span 1959-2007. The sequence variations of *tet*(M) within subgroup 1 correlated with different CC types of *S. aureus* (see

Figure 5A) which supports the general idea that *S. aureus* of different lineages are not very good at sharing DNA. Subgroup 2 formed an individual branch with five identical *tet*(M) sequences, all from human isolates of phage type 94/96 isolated between 1970-1992. All were shown to have the same *spa* type (t078) belonging to CC25. Thus *tet*(M) of subgroup 2 appears to have been integrated in this clone over 30 years ago without changing. PCR mapping of the elements in subgroup 2 were of expected size (data not shown), indicating that all the ORFs necessary to conjugate were present. Whether or not this new Tn*916*-like transposon is functional is however not clear. Subgroup 3 consisted of seven isolates from 1957-2000 with identical *tet*(M), different phage types and *spa* types all belonging to CC8. Comparing the isolates within this group shows a correlation between *spa* type and resistance pattern (see Table 2). The three isolates from *spa* type t037 (all from 2000) were resistant to the same seven antimicrobial agents including Methicillin and were suspected to be from the same outbreak. The two other *spa* types in this group, t008 and t051 (1957-1963) are clonally related and are only resistant to 3 and 5 agents respectively, the latter was Methicillin-resistant. These differences may be time dependent or reflect resistance profiles in different sub-clones of CC8.

As shown by the phylogenetic tree in Figure 3, *S. aureus tet*(M) sequences belonging to subgroups 1A, 1C and 3 were identical to *tet*(M) of *Streptococcus agalactiae* (AAJQ1000009), Tn*916* -*tet*(M) from *E. faecalis* DS16 (U09422) and to *tet*(M) found in *Streptococcus agalactiae* COH1 (NZ_AAJR01000021), respectively. This indicates horizontal transfer of *tet*(M) from subgroup 1 and subgroup 3 between *S. aureus* and other Gram-positive species of enterococci and streptococci. Previously horizontal transfer of Tn*916*-like *tet*(M) from *Bacillus cereus* group into *S. aureus* has been shown.[29] Clustering of CC *spa* types versus *tet*(M) sequences type suggested that horizontal transfer of Tn*5801* between different CC types of *S. aureus* has occurred.

The filter mating experiment showed that the Tn*916*-like element from subgroup 1A (CC398) could be transferred into both recipient stains (8794RF, CC121 and RN4220RF, CC8) whereas the Tn*916*-like element from subgroup1D (CC5) was only transferred into one of the recipient strains (8794RF, CC121). The Tn*5801*-like element, Tn*6014* from subgroup 3 (CC8) was transferred into the other recipient strain (RN4220RF, CC8). The new *tet*(M)-like elements of subgroup 2 did not transfer into any of the recipients. Although, both recipients are known to be very good in taking up foreign DNA, transconjugants were obtained with very low frequencies. Recently the restriction-modification system, *Sau*1 was suggested to control horizontal gene transfer between *S. aureus* of different lineages.[48] RN4220RF was shown to have a mutation in this system making it able to take up DNA from different CC types. However, in our study RN4220RF only received *tet*(M) from two of the 14 tested donors which indicates that other factors may also play a role or that transfer occurs at rates below our detection limit.

Staphylococci *tet*(M) were only located in part of the phylogenetic tree associated with the well characterized conjugative transposon Tn*916* and with Tn*5801* described in *S. aureus* Mu50 and Mu3[14, 49] (group II, Figure 3). *tet*(M) from other Gram-positive bacteria were distributed in the whole tree and were besides Tn*916*-like elements associated with Tn*5397* (group I) and/or composite transposons like Tn*1545*, Tn*2009* and/or Tn*5251* (group III). Moreover, *tet*(M) from *E. faecium* (DQ223243 and DQ223244) has also been found on plasmids (group I).[21] Thus *tet*(M) from *S. aureus* appear to be less diverse than *tet*(M) from other Gram-positive bacteria.

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

to be the case for putative transposon CW459*tet*(M) from *Clostridium peringens*.[16] In this transposon *tet*(M) is highly related to Tn*916*-*tet*(M) whereas the rest of the transposon sequence is more related to Tn*5801*.[15]

In conclusion, we have used the diversity of the *tet*(M) gene to determine associated mobile elements in *S. aureus* from human and different animal origin. *S. aureus* of human origin was shown to contain diverse *tet*(M) genes located on Tn*916*-like and Tn*5801*-like conjugative transposons that corresponded with different CC types. *S. aureus* of different animal origin contained one specific type of *tet*(M) located on Tn*916*-like elements, all belonging to CC type 398. This is the first report showing that a Tn*5801*-like element, Tn*6014* can transfer between *S. aureus* isolates.

Acknowledgements

We want to thank Maria Louise Johannsen, Jacob Dyring Jensen and Hanne Mordhorst for excellent technical assistance. Part of this work was presented as a poster at the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 2007 and at the 1st International ASM meeting on Antimicrobial Resistance in Zoonotic bacteria and foodborne pathogens, 2008.

Funding

This study was founded by a grant from the The Danish Research Council for Technology and Production Sciences (274-05-0117).

Transparency declarations

None to declare

Reference

1. Aarestrup FM, Schwarz S. Antimicrobial Resistance in Staphylococci and Streptococci of Animal Origin. In: Aarestrup FM, ed. *Antimicrobial Resistance in Bacterial of Animal Origin*. Washington: ASM, 2006; 187-212.

2. Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001; **65**: 232-60.

3. Roberts MC. Update on acquired tetracycline resistance genes. *FEMS Microbiol Lett* 2005; **245**: 195-203.

4. Ridenhour MB, Fletcher HM, Mortensen JE, et al. A novel tetracycline-resistant determinant, *tet*(U), is encoded on the plasmid pKq10 in *Enterococcus faecium*. *Plasmid* 1996; **35**: 71-80.

5. Bismuth R, Zilhao R, Sakamoto H, et al. Gene heterogeneity for tetracycline resistance in *Staphylococcus* spp. *Antimicrob Agents Chemother* 1990; **34**: 1611-4.

6. Schmitz FJ, Krey A, Sadurski R, et al. Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. *J Antimicrob Chemother* 2001; **47**: 239-40.

7. Warsa UC, Nonoyama M, Ida T, et al. Detection of *tet*(K) and *tet*(M) in *Staphylococcus aureus* of Asian countries by the polymerase chain reaction. *J Antibiot (Tokyo)* 1996; **49**: 1127-32.

8. Schwarz S, Roberts MC, Werckenthin C, et al. Tetracycline resistance in *Staphylococcus* spp. from domestic animals. *Vet Microbiol* 1998; **63**: 217-27.

9. Distribution of *tet*(M) in Gram-possitive and Gram-negative bacteria. http://faculty.washington.edu/marilynr/ (23 September 2008, date last accessed).

10. Burrus V, Pavlovic G, Decaris B, et al. Conjugative transposons: the tip of the iceberg. *Mol Microbiol* 2002; **46**: 601-10.

11. Rice LB. Tn*916* family conjugative transposons and dissemination of antimicrobial resistance determinants. *Antimicrob Agents Chemother* 1998; **42**: 1871-7.

12. Clewell DB, Francia MV. Conjugation in Gram-positive bacteria. In: Funnell BE, Phillips G.J.E., eds. *Plasmid Biology*. Washington DC: American Society for Microbiology, 2004; 227-56.

13. Courvalin P, Carlier C. Tn*1545*: a conjugative shuttle transposon. *Mol Gen Genet* 1987; **206**: 259-64.

14. Kuroda M, Ohta T, Uchiyama I, et al. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 2001; **357**: 1225-40.

15. Ito T, Okuma K, Ma XX, et al. Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. *Drug Resist Updat* 2003; **6**: 41-52.

16. Roberts AP, Johanesen PA, Lyras D, et al. Comparison of Tn*5397* from *Clostridium difficile*, Tn*916* from *Enterococcus faecalis* and the CW459*tet*(M) element from *Clostridium perfringens* shows that they have similar conjugation regions but different insertion and excision modules. *Microbiology* 2001; **147**: 1243-51.

17. Lyras D, Rood JI. Genetic organization and distribution of tetracycline resistance determinants in *Clostridium perfringens*. *Antimicrob Agents Chemother* 1996; **40**: 2500-4.

18. Oggioni MR, Dowson CG, Smith JM, et al. The tetracycline resistance gene *tet*(M) exhibits mosaic structure. *Plasmid* 1996; **35**: 156-63.

19. Doherty N, Trzcinski K, Pickerill P, et al. Genetic diversity of the *tet*(M) gene in tetracycline-resistant clonal lineages of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 2000; **44**: 2979-84.

20. Huys G, D'Haene K, Collard JM, et al. Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. *Appl Environ Microbiol* 2004; **70**: 1555-62.

21. Agersø Y, Pedersen AG, Aarestrup FM. Identification of Tn*5397*-like and Tn*916*-like transposons and diversity of the tetracycline resistance gene *tet*(M) in enterococci from humans, pigs and poultry. *J Antimicrob Chemother* 2006; **57**: 832-9.

22. Gevers D, Danielsen M, Huys G, et al. Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. *Appl Environ Microbiol* 2003; **69**: 1270-5.

23. Poyart-Salmeron C, Trieu-Cuot P, Carlier C, et al. Nucleotide sequences specific for Tn*1545*-like conjugative transposons in pneumococci and staphylococci resistant to tetracycline. *Antimicrob Agents Chemother* 1991; **35**: 1657-60.

24. Aarestrup FM, Wegener HC, Rosdahl VT, et al. Staphylococcal and other bacterial species associated with intramammary infections in Danish dairy herds. *Acta Vet Scand* 1995; **36**: 475-87.

25. Aarestrup FM, Agersø Y, Ahrens P, et al. Antimicrobial susceptibility and presence of resistance genes in staphylococci from poultry. *Vet Microbiol* 2000; **74**: 353-64.

26. Multiplex PCR for the detection of the *mec*A gene. http://www.crl-ar.eu/data/images/meca-pcr_protocol%2006.02.08.pdf (24 September 2008, date last accessed).

27. Aarestrup FM, Agersø Y, Gerner-Smidt P, et al. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn Microbiol Infect Dis* 2000; **37**: 127-37.

28. Macrogen. http://www.macrogen.com/eng/sequencing/sequence_main.jsp (23 September 2008, date last accessed).

29. Agersø Y, Jensen LB, Givskov M, et al. The identification of a tetracycline resistance gene *tet*(M), on a Tn*916*-like transposon, in the *Bacillus cereus* group. *FEMS Microbiol Lett* 2002; **214**: 251-6.

30. Clewell DB. Movable genetic elements and antibiotic resistance in enterococci. *Eur J Clin Microbiol Infect Dis* 1990; **9**: 90-102.

31. Thompson JD, Gibson TJ, Plewniak F, et al. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; **25**: 4876-82.

32. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 2004; **5**: 150-63.

33. Rice P, Longden I, Bleasby A. EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* 2000; **16**: 276-7.

34. EMBOSS Pairwise Alignment Algorithms. http://www.ebi.ac.uk/emboss/align/ (23 September 2008, date last accessed).

35. *spa* typing. http://www.seqnet.org/pdf/Ridom_spa_sequencing.pdf (23 September 2008, date last accessed).

36. MLST typing. http://saureus.mlst.net/misc/info.asp#experimental (23 September 2008, date last accessed).

37. Ridom SpaServer. http://spaserver2.ridom.de/mlst.shtml (23 September 2008, date last accessed).

38. *Staphylococcus aureus* MLST database. http://saureus.mlst.net/ (23 September 2008, date last accessed).

39. eBURST. http://eburst.mlst.net/ (28 September 2008, date last accessed).

40. Hammerum AM, Jensen LB, Aarestrup FM. Detection of the *satA* gene and transferability of virginiamycin resistance in *Enterococcus faecium* from food-animals. *FEMS Microbiol Lett* 1998; **168**: 145-51.

41. Stobberingh EEWKC. Restriction-deficient mutants of *Staphylococcus aureus*. *J Gen Microbiol* 1977; **99**: 359-67.

42. Kreiswirth BN, Lofdahl S, Betley MJ, et al. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* 1983; **305**: 709-12.

43. Wulf MW, Sørum M, van NA, et al. Prevalence of methicillin-resistant *Staphylococcus aureus* among veterinarians: an international study. *Clin Microbiol Infect* 2008; **14**: 29-34.

44. Roberts AP, Chandler M, Courvalin P, et al. Revised nomenclature for transposable genetic elements. *Plasmid* 2008; **60**: 167-73.

45. Witte W, Strommenger B, Stanek C, et al. Methicillin-resistant *Staphylococcus aureus* ST398 in humans and animals, Central Europe. *Emerg Infect Dis* 2007; **13**: 255-8.

46. Huijsdens XW, van Dijke BJ, Spalburg E, et al. Community-acquired MRSA and pig-farming. *Ann Clin Microbiol Antimicrob* 2006; **5**: 26.

47. Guardabassi L, Stegger M, Skov R. Retrospective detection of methicillin resistant and susceptible *Staphylococcus aureus* ST398 in Danish slaughter pigs. *Vet Microbiol* 2007; **122**: 384-6.

48. Waldron DE, Lindsay JA. Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. *J Bacteriol* 2006; **188**: 5578-85.

49. Hiramatsu K, Aritaka N, Hanaki H, et al. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* 1997; **350**: 1670-3.

50. Clewell DB, Flannagan SE, Jaworski DD. Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposons. *Trends Microbiol* 1995; **3**: 229-36.

51. Vintov J, Aarestrup FM, Zinn CE, et al. Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. *Vet Microbiol* 2003; **95**: 133-47.

Legends to Figures

Figure 1: Comparison of the 18 kb conjugative transposon Tn916 (U09422) and the 25 kb putative transposon Tn*5801* (BA000017/NC002758). Besides *tet*(M) (dark grey arrows) they both consist of 3 structural domains containing genes associated with conjugation, regulation (light grey arrows) and excision/integration (dotted arrows).[11, 15, 50] Both elements contain an integrase (*int*) gene, however these *int* genes are very different. In addition, Tn*916* also contains an annotated excisionase (*xis*) gene (black arrow). Tn*5801* contains several open reading frames (white arrows) whose functions are unknown. *sav415* may however encode a transposase.[15] The relation between open reading frames of Tn*916* and Tn*5801* are shown in percent identity on nucleotide level calculated with the EMBOSS program water.[34]

Figure 2.: Amplification and sequencing strategy for *tet*(M). Top: The *tet*(M) gene sequence including a downstream region. Primers used for amplification and sequencing are illustrated with arrows. Bottom: Two different sequencing strategies using different combination of primers. Strategy 1: Sequences from 7 human isolates (213, 229, 1680, 1742, 33597, 34148, 34168) were obtained. Strategy 2a: 3 human, 8 pig, 1 lamb and 1 bovine isolate (22034, 35414, 35679, 9b, 7215311, 7311242, 7413093-4, 7413714-1, 7512986-1, 7611472.1, 7611995-1, 7612628-4, sw356).Strategy 2b: 10 human, 9 pig, 1 turkey and 1 bovine isolate (617, 1591, 4520, 4865, 5331, 5377, 8797, 21995, 35801, 35366, 7215190-1, 7312330-1, 7412791, 7413532-2, 7413727, 74140355-2, 7512166-1, 7611280, 7711730-1, 9877324-3, usa42).

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

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

Figure 4: PCR products linking *tet*(M) to Tn*916*-like *xis* and Tn*5801*-like *int* genes with the expected size of 2835 bp and 4820 bp respectively. **A:** A representative from every *tet*(M) type from different origins are shown. Lane 1: 9877324-3 (turkey), 2: USA42 (cattle), 3: 7611472-1 (lamb), 4: 9b (pig), 5: 21995 (human), 6: 617 (human), 7: 8797 (human), 8: 34801 (human), 9: 4520 (human), 10: positive control (*E. faecalis* DS16), 11: negative control (*S. aureus* Mu50), 12: 1680 (human), 13: positive control (*S. aureus* Mu50), 14: negative control (*E. faecalis* DS16). M: Gene Ruler 1Kb ladder from Fermentas. **B**: Transconjugants (TC), donors (D) and recipients showing horizontal transfer of Tn*916*-like transposons. Lane 1: 34801_1_R1 (TC), 2: 34801 (D), 3: 35366_1_R1 (TC), 4: 35366_1_R2, 5: 35366 (D), 6: R1 (8794RF), 7: R2 (RN4220RF), 8: positive control (*E. faecalis* DS16), 9: negative control (*S. aureus* Mu50). **C:** TC, D and recipient showing horizontal transfer of the Tn*5801*-like transposon Tn*6014*. Lane 1: 1680R2_4 (TC), 2: 1680 (D), 3: R2 (RN4220RF), 4: positive control (*S. aureus* Mu50), 5: negative control (*E. faecalis* DS16).

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

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

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

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

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

Strain	Source/year	Other resistance phenotypes	Mobile element carrying *tet*(M)	*spa* type/CC types	GenBank accession number
213	Human/1957	Pen^R, Str^R	Tn*5801*-like	t008/CC8	EU918651
229	Human/1957	Pen^R, Str^R	Tn*5801*-like	t008/CC8	EU918652
1680	Human/1963	Pen^R, Str^R, Ery^R, Met^R	Tn*5801*-like (Tn*6014*)	t051/CC8	EU918655
1742	Human/1963	Pen^R, Str^R	Tn*5801*-like	t008/CC8	EU918656
33597[b]	Human/2000	Pen^R, Str^R,Gen^R, Ery^R, Met^R, Cip^R	Tn*5801*-like	t037/CC8	EU918664
34148[b]	Human/2000	Pen^R, Str^R,Gen^R, Ery^R, Met^R, Cip^R	Tn*5801*-like	t037/CC8	EU918665
34168[b]	Human/2000	Pen^R, Str^R,Gen^R, Ery^R, Met^R, Cip^R	Tn*5801*-like	t037/CC8	EU918666
4520	Human/1970	Pen^R	Tn*916*-like	t078/CC25	EU918657
4865	Human/1970	Pen^R	Tn*916*-like	t078/CC25	EU918658
5331	Human/1971	Pen^R	Tn*916*-like	t078/CC25	EU918659
5377	Human/1971	Pen^R	Tn*916*-like	t078/CC25	EU918660
22034	Human/1992	Pen^R, Ery^R, Cip^R	Tn*916*-like	t078/CC25	EU918663
34801	Human/2001	Pen^R, Fus^R	Tn*916*-like	t688/CC5[a]	EU918667
8797	Human/1977	Pen^R	Tn*916*-like	t065/CC45	EU918661
617	Human/1959	Pen^R	Tn*916*-like	t318/CC30	EU918653
1591	Human/1962	Pen^R	Tn*916*-like	t964/CC30[a]	EU918654
21995	Human/1992	Pen^R	Tn*916*-like	t034/CC398	EU918662
35366	Human/2001	Pen^R	Tn*916*-like	t034/CC398	EU918668
35414	Human/2001		Tn*916*-like	t034/CC398	EU918669
35679	Human/2001	Pen^R	Tn*916*-like	t034/CC398	EU918670
9877324-3	Turkey/1998	Pen^R	Tn*916*-like	t034/CC398	EU918671
USA42	Cattle	Pen^R, Str^R, Ery^R, Chl^R, Sul^R	Tn*916*-like	t034/CC398	EU918673
Sw356	Cattle	Str^R, Spt^R, Tmp^R	Tn*916*-like	t034/CC398	EU918672
7611472-1	Lamb/2004	Pen^R, Cip^R	Tn*916*-like	t034/CC398	EU918687
9b	Pig/2007	Pen^R, Str^R, Ery^R, Spt^R, Tmp^R,Cef^R, Met^R	Tn*916*-like	t034/CC398[a]	EU918691
7215190-1	Pig/2000	Pen^R, Str^R, Tmp^R	Tn*916*-like	t034/CC398	EU918674
7215311-1	Pig/2000	Pen^R, Str^R	Tn*916*-like	t034/CC398	EU918675
7311242-1	Pig/2001	Ery^R, Str^R, $Spec^R$	Tn*916*-like	t034/CC398	EU918676
7312330-1	Pig/2001	Pen^R, Str^R, $Spec^R$	Tn*916*-like	t034/CC398	EU918677
7412791-1	Pig/2002	Pen^R, Str^R, Tmp^R	Tn*916*-like	t034/CC398	EU918678
7413093-4	Pig/2002	Pen^R, Ery^R, Tia^R, Tmp^R	Tn*916*-like	t034/CC398	EU918679
7413532-2	Pig/2002	Pen^R	Tn*916*-like	t011/CC398	EU918680
7413714-1	Pig/2002	Pen^R, Tmp^R	Tn*916*-like	t034/CC398	EU918681
7413727-1	Pig/2002	Pen^R, Ery^R	Tn*916*-like	t034/CC398	EU918682
7414035-2	Pig/2002	Pen^R, Str^R, Ery^R	Tn*916*-like	t034/CC398	EU918683
7512166-1	Pig/2003	Pen^R, Str^R, Ery^R, Tmp^R	Tn*916*-like	t034/CC398	EU918684
7512986-1	Pig/2003	Ery^R, Spt^R	Tn*916*-like	t034/CC398	EU918685
7611280-5	Pig/2004	Pen^R, Tmp^R	Tn*916*-like	t011/CC398	EU918686
7611995-1	Pig/2004	Pen^R, Str^R, Ery^R, Spt^R, Tia^R, Tmp^R	Tn*916*-like	t034/CC398	EU918688
7612628-4	Pig/2004	Ery^R, Spt^R	Tn*916*-like	t571/CC398	EU918689
7711730-1	Pig/2005	Pen^R, Spt^R, Tia^R, Tmp^R	Tn*916*-like	t034/CC398	EU918690

526 [a] : Determined by MLST
527 [b] : Isolates suspected to be from the same outbreak.
528 Pen^R: penicillin, Str^R: streptomycin, Gen^R: gentamicin, Ery^R: erythromycin, Met^R: methicilin, Cip^R: ciprofloxacin,
529 Chl^R:chloramphenicol, Spt^R: spectinomycin, Sul^R: sulphamethoxazo, Tia^R: tiamulin, Tmp^R: trimethoprin, Cef^R: Ceftifur,
530 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'-GTCCATACGTTCCTAAAGTCGTC-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'-CATGAAGGAGTGTAAAGAATGA-3'	This study
1837 (F2R)	5'-GTGTCTTATACCATGGAAGGA-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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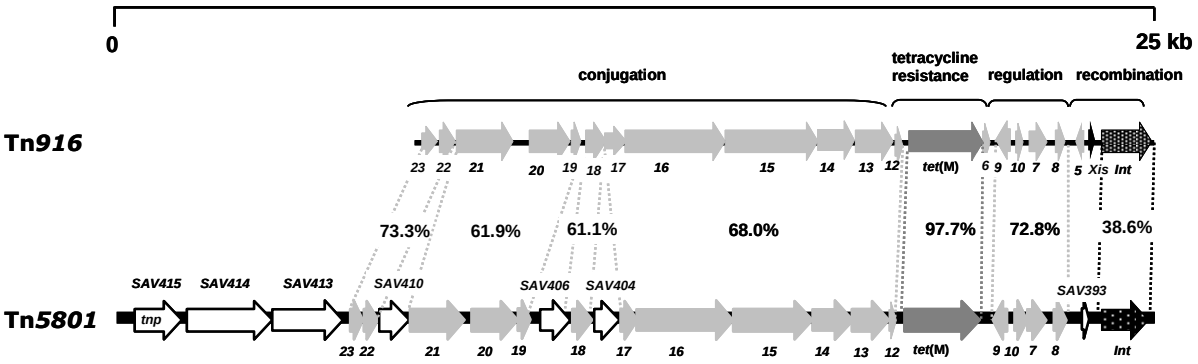
Figure 1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61.1%
68.0%
97.7%
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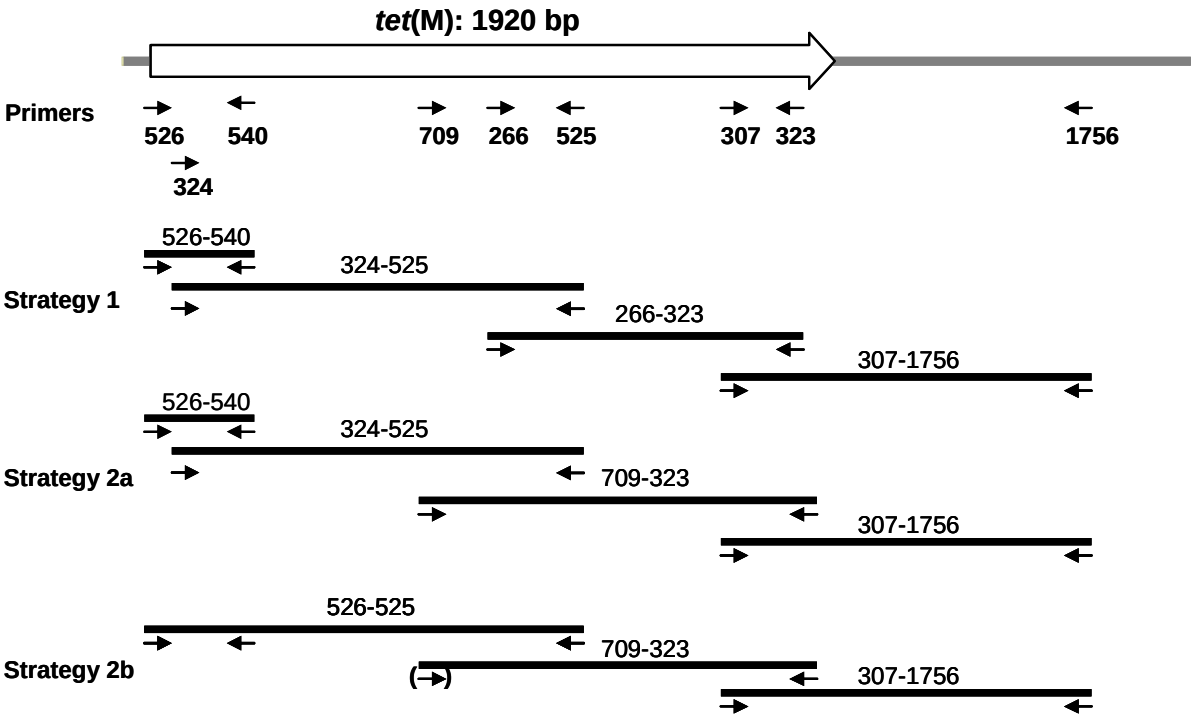
563 Figure 2



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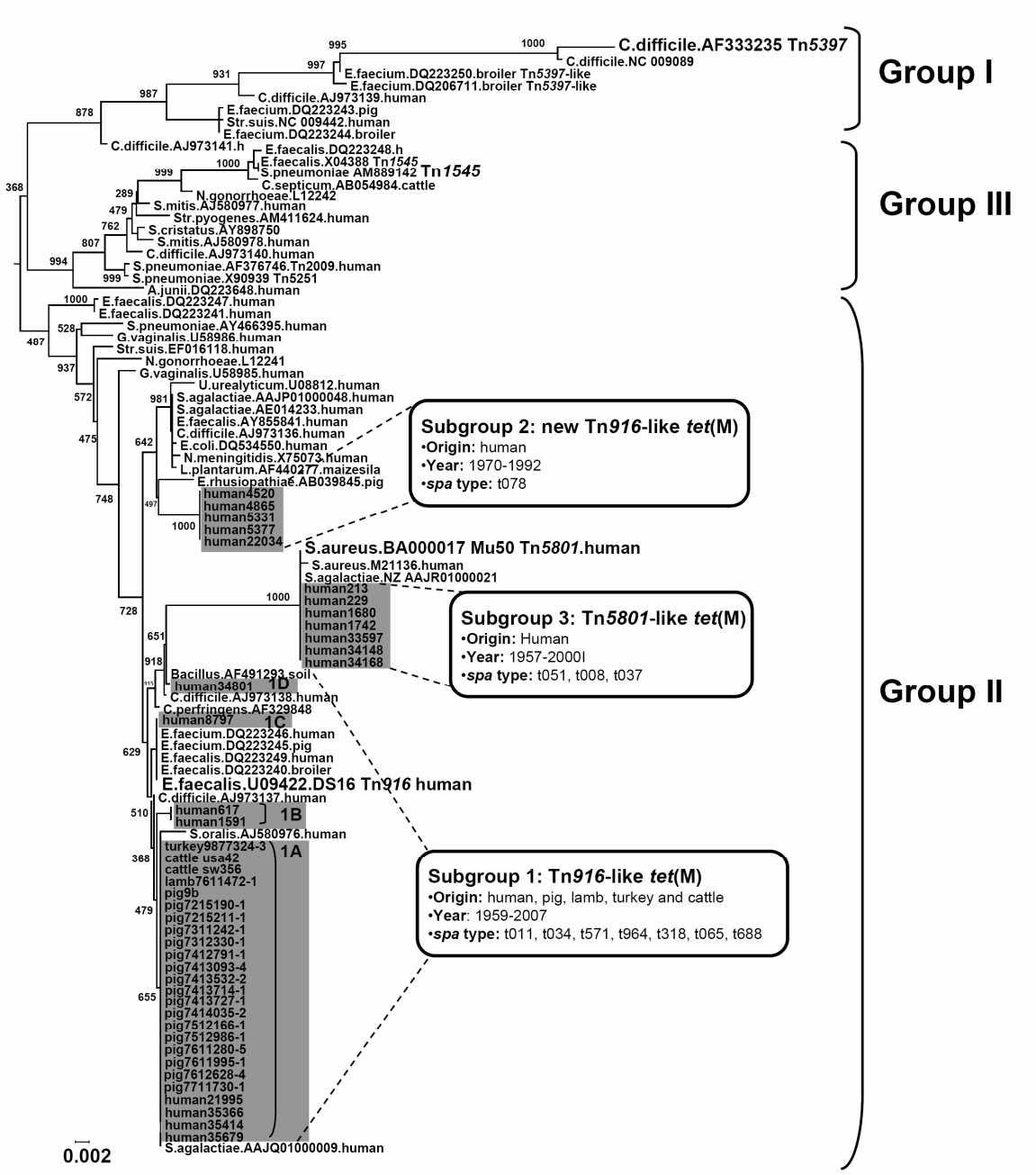

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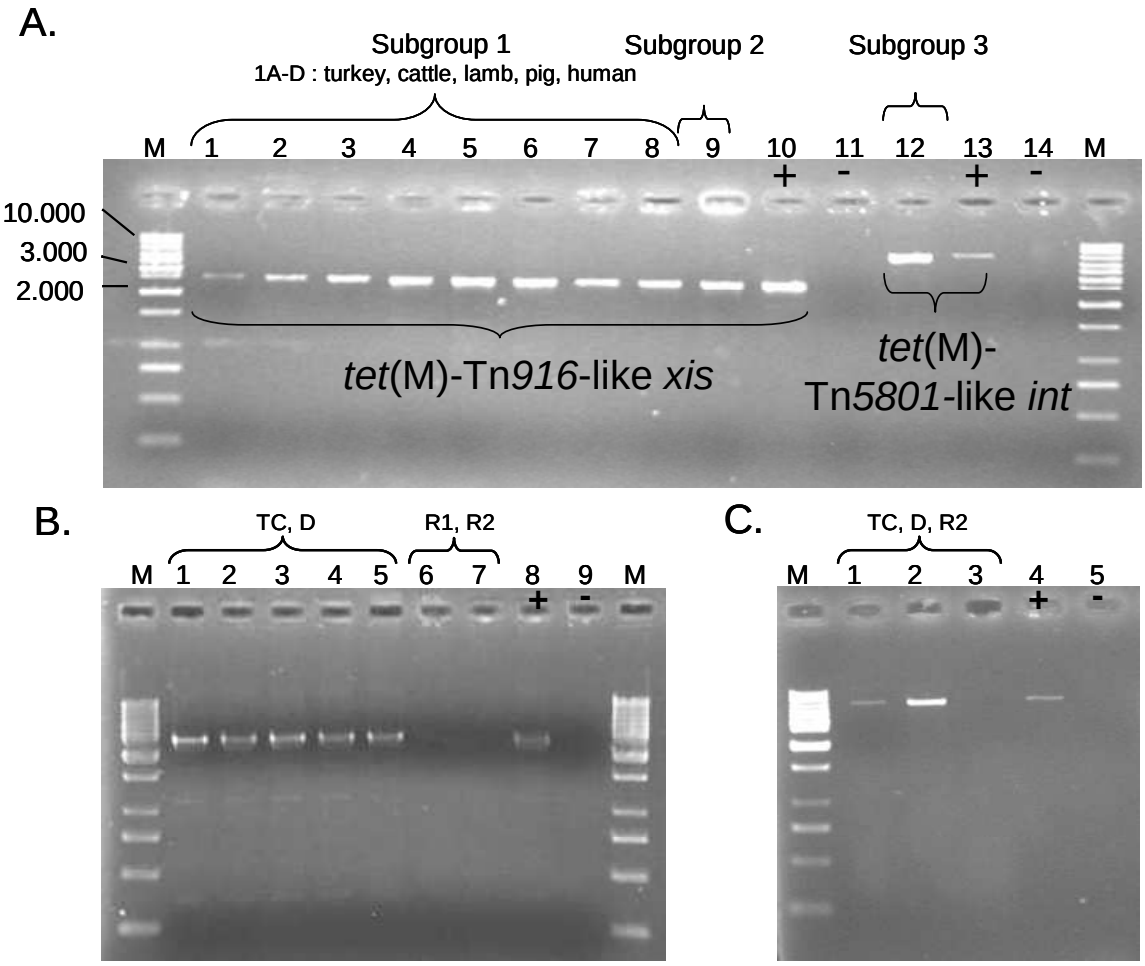

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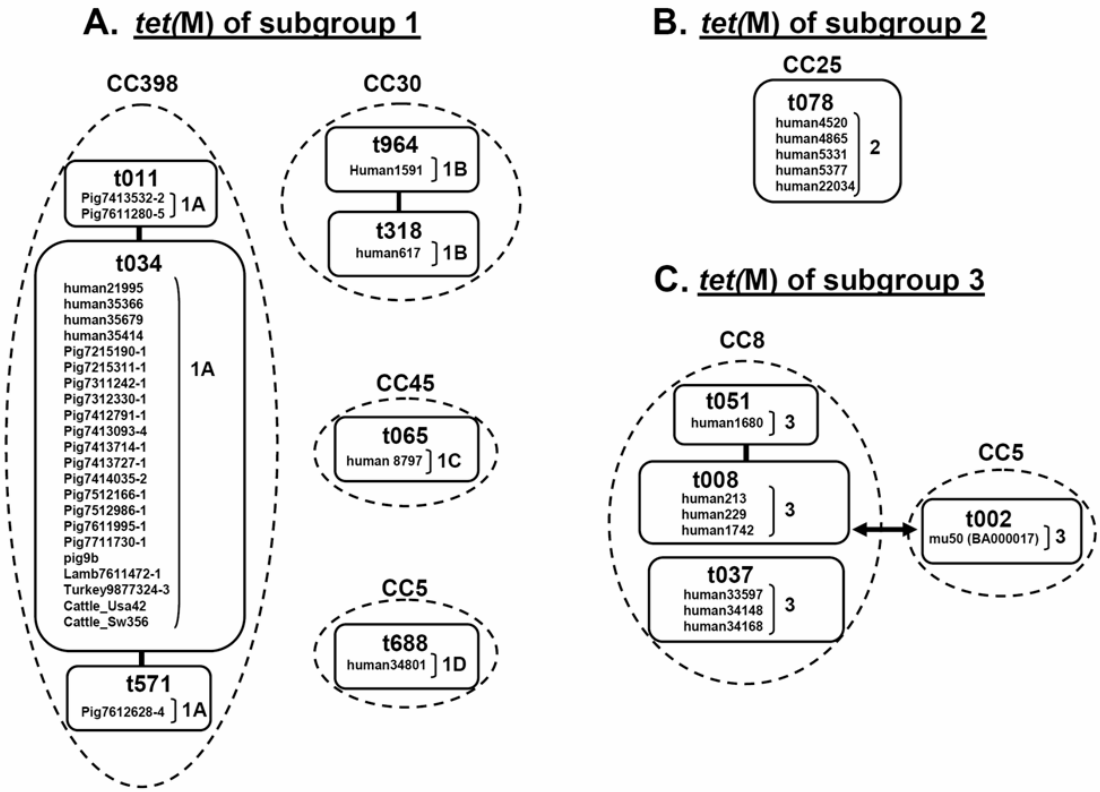
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1C
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human34801
1D
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CC25
t078
human4520
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human5331
human5377
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2
C. tet(M) of subgroup 3
CC8
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