New variants of the tet(M) gene in Clostridium difficile clinical isolates harbouring Tn916-like elements

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Objectives: To detect Tn916-like elements in Clostridium difficile clinical isolates from different time periods and to analyse the genetic structure of these elements, in particular the tet(M) region.

Methods: Ninety C. difficile clinical isolates were examined by PCR assays for tet(M) and int, which are markers for the Tn916 family of elements. Positive isolates were typed by PCR-ribotyping, and tetracycline MIC values were evaluated by Etest. The genetic organization of the Tn916 elements was investigated by PCR mapping and hybridization assays. The tet(M) region of eight selected C. difficile isolates was sequenced.

Results: Nineteen isolates were tet(M)/int positive and the majority (12/19) belonged to PCR-ribotype R, previously found to be predominant in clinical strains of more recent isolation. Eleven isolates were tetracycline resistant, three inducibly resistant and five susceptible. Fifty-eight per cent of the C. difficile isolates harboured one Tn916 element and 42% harboured two. Most isolates showed elements with a genetic organization very similar to that of Enterococcus faecalis DS16 Tn916. Sequence analysis highlighted variations in the leader peptide and six tet(M) variants were identified, five of which have never been described before.

Conclusions: C. difficile isolates harbouring Tn916-like elements have mainly been isolated since 1997, suggesting a recent circulation of these elements among C. difficile strains in Italian hospitals. Molecular analysis of these Tn916-like elements showed that they may have different genetic structures and carry new tet(M) alleles.

Keywords: tetracycline resistance, transposons, nosocomial pathogens

Introduction

Clostridium difficile is the most common nosocomial pathogen causing antibiotic-associated diseases. In this bacterium, tetracycline resistance is commonly conferred by the tet(M) gene, which is regulated by transcriptional attenuation. In C. difficile 630, tet(M) was identified on the Tn5397 transposon, an element that differs from Tn916 in that it contains a group II intron and has different integration/excision modules. As elements related to Tn916 have recently been found in some C. difficile clinical isolates, in this study, 90 C. difficile clinical isolates from different time periods were examined for the presence and molecular characteristics of Tn916-like elements.

Materials and methods

C. difficile isolates and control strains

Ninety C. difficile strains isolated from patients in different Italian hospitals, between 1986 and 2001, were examined in this study. All isolates were tested for the presence of tet(M), int and tndX genes. The last two genes were used as markers for Tn916- and Tn5397-related elements, respectively.

Streptococcus pneumoniae PN20 strain was used as a control for the int gene, whereas C. difficile 630 was used as a control for the tndX gene.

DNA extraction and detection of tet, int and tndX genes

Genomic DNA of each C. difficile strain was extracted using a Nucleobond AXG20 Kit (Macherey-Nagel, Düren, Germany)
from 10 mL of an overnight brain heart infusion (BHI) culture.

The primers for tet, int and tndX gene amplification are shown in Table 1. Amplifications for tet(M), int and tndX were carried out as previously described.5,6 PCRs for the other tet gene classes were performed using the following conditions: 5 min at 94°C, followed by 30 cycles of 94°C for 1 min, 50 or 55°C for 1 min and 72°C for 1 min.

Susceptibility testing and PCR-ribotyping

Tetracycline MIC values were determined by the Etest method (AB Biodisk, Solna, Sweden). The breakpoint for tetracycline was 8 mg/L, according to the Clinical and Laboratory Standards Institute (CLSI) criteria. Induction of resistance to tetracycline was performed as previously described.5

PCR-ribotyping was performed with primers complementary to conserved regions of the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene, as previously described.6

Hybridization assays and molecular analysis of tetracycline resistance determinants

tet(M) and int PCR products were purified using the NucleoSpin Extract kit (Macherey-Nagel), and DNA labelling was performed as described in the DIG High Prime DNA labelling and detection kit (Roche Diagnostics, Mannheim, Germany). The genomic DNA of C. difficile isolates was digested with HindIII and transferred to a nylon membrane (Roche Diagnostics) by Southern blotting.

Genetic organization of the Tn916-like elements was characterized by PCR mapping as previously described.5,7 Amplification of the tet(M) region in eight selected isolates was carried out using the set of primers reported in Table 1. PCR reactions were carried out in a final volume of 50 μL with a reaction mixture containing buffer (25 mM Tris–HCl/50 mM KCl/2.0 mM MgCl₂), 200 mM of each deoxynucleoside triphosphate, 50 pmol of each primer and 2.5 U of TaKaRa ExTaq™ (Takara Shuzo Co., Ltd, Japan). Cycling parameters were denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min (depending on the size of the amplified fragments), followed by a final extension step at 72°C for 5 min. PCR products obtained from the tet(M) region were sequenced after purification by the NucleoSpin Extract kit (Macherey-Nagel).

DNA sequencing and analysis

Sequencing of the tet(M) region in the eight selected isolates was carried out using the same primers designed for the PCR assay (Table 1) and the Big Dye Terminator v.1.1 Cycle Sequencing


Results and discussion

Nineteen of the 90 *C. difficile* isolates were positive for both tet(M) and int (data not shown). None of these isolates was positive for the *tndX* gene that was detected in 28% (25/90) of the isolates. All 19 *int* positive isolates were collected from 1997 to 2001. Twelve of the isolates (63%) belonged to PCR-ribotype R, found to be predominant in clinical strains isolated mainly in the years 2000 and 2001.\(^5,6\) The other seven isolates belonged to different PCR-ribotypes. Eleven were resistant to tetracycline (MIC values between 8 and 32 mg/L), three were inducibly resistant to tetracycline (MIC 4 mg/L before induction and MICs between 8 and 12 mg/L after induction) and five were susceptible (MICs between 0.047 and 4 mg/L) (data not shown).

Hybridization assays were performed using the amplified products from tet(M) and int as probes. The signals obtained using the two different probes co-banded (Figure 1). One isolate showed a band at about 14 kb, four isolates at 8.7 kb and six at 6.3 kb, indicating the presence of one element related to Tn916. Eight strains showed two hybridizing bands at about 8.7 and 6.3 kb, respectively, suggesting that two Tn916-like elements can co-exist in the same chromosome. No correlation was found between the number of elements present in the *C. difficile* isolates and the tetracycline resistance phenotype.

Eight of the 19 isolates examined had an element with a genetic organization very similar to that of *Enterococcus faecalis* DS16, whereas three isolates showed a different genetic organization. As already observed,\(^7\) sequence variations were located in the DNA regions containing *orf* 16-15, *orf* 20-17 and *orf* 12-tet(M). When two similar elements were in the same chromosome, PCR mapping could not be used for characterization. None of the examined *C. difficile* isolates contained plasmids (data not shown).

The tet(M) gene and the upstream region of eight selected *C. difficile* strains (cd8, cd11, cd12, cd53, cd60, cd63, cd1911 and cd1920) with different genotypic and phenotypic characteristics were sequenced. Since different classes of the tet gene have been found in clostridia (http://faculty.washington.edu/marilynlr/), the selected strains were also examined for tet(K), tet(Q), tet(W), tetA(P), tet(32) and tet(36) by PCR; the results were negative (data not shown).

Six different tet(M) variants, tet(M)1 to tet(M)6, were identified: cd11, cd12 and cd1911 harboured tet(M)1, cd60 tet(M)2, cd63 tet(M)3, cd53 tet(M)4, cd1920 tet(M)5 and cd8 tet(M)6. Sequence analysis revealed that except for tet(M)1, which was 100% identical to the tet(M) gene found in *Streptococcus agalactiae* 2603V/R (GenBank accession no. AE014233), the other alleles did not show complete identity with other tet(M) genes. The phylogenetic tree obtained by nucleotide sequence comparison suggests that acquisition of these tetracycline resistance determinants from a hypothetical precursor carrying a Tn916-like tet(M) gene is a relatively more recent event compared with the acquisition of the tet(M) gene by *C. difficile* 630 (Figure 2).

tet(M)1, 2, 3 and 4 variants were found in *C. difficile* isolates resistant or inducibly resistant to tetracycline, and tet(M)5 and 6 were found in susceptible isolates. The organization of the element carrying tet(M)3, tet(M)6 and tet(M)1 of cd12 was similar to that of *E. faecalis* DS16. This analysis was not possible in the other two isolates harbouring tet(M)1 for the hypothesized presence of two Tn916-like elements in the chromosome. However, the sequencing of the tet(M) region in these strains revealed that there was no superimposition of different PCR products, suggesting that if two elements were present both elements carried the same allele. tet(M)2, tet(M)4 and tet(M)5 were located on elements that showed nucleotide variations compared with *E. faecalis* DS16.

Analysis of the region upstream of the tet(M) genes showed that tet(M)1, tet(M)3, tet(M)5 and tet(M)6 were preceded by a complete leader peptide sequence, whereas this region was partially deleted in the region upstream of tet(M)2 and tet(M)4 for the loss of 124 bp (data not shown). The residual transcriptional attenuation activity could be maintained in these isolates since the inverted repeats 7 (CCCTT) and 8 (TTCCC), forming the stem-loop structure 7:8 in mRNA, a weaker terminator site, were still present. The leader peptide DNA region of tet(M)3, 5 and 6 had 100% identity with that of Tn916 of *E. faecalis* DS16, whereas that of tet(M)1 had 99% identity with one amino acid conservative mutation (M to I) in position four.

As the sequence of the region upstream of tet(M)5 and 6 does not show variations compared with that of *E. faecalis* DS16 Tn916, the amino acid changes of Tet(M)5 and 6 proteins or a post-transcriptional block may be responsible for the tetracycline susceptibility of the *C. difficile* strains carrying tet(M)5 and tet(M)6.

No differences were observed in the –35 and –10 promoter region of all tet(M) alleles compared with that of the Tn916 of *E. faecalis* DS16, except for two nucleotide changes observed in the –10 promoter region of tet(M)4, from TATTAT to TAAAT, that could have some positive influence on transcription.

This study reports for the first time that one or two Tn916-like elements can be present in *C. difficile* clinical isolates of recent
isolation and that new tet(M) alleles are carried by these elements. Tn916 elements are known for their ability to transfer antibiotic resistance genes and also virulence determinants among bacteria belonging to different ecosystems.8–10 The presence in C. difficile of elements related to Tn916 could play an important role in the acquisition of new characteristics that could increase the pathogenicity of this microorganism. For this reason, further studies are necessary to monitor the spread of these elements in strains circulating in hospital environments.

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Transparency declarations

None to declare.

References


Figure 2. Unrooted neighbour-joining phylogenetic tree obtained from the nucleotide multiple alignment of the six C. difficile tet(M) variants identified in this study and 16 other tet(M) gene sequences available in GenBank. The branch lengths are scaled in proportion to the extent of the change per position as indicated by the scale bar. GenBank accession numbers are in parentheses.

0.1

Streptococcus mitis (AJ580977)
- E. faecalis-Tn1545 (X04388)
- Streptococcus cristatus (AY898750)
- C. difficile cd1920-tet(M)5
- S. pneumoniae (X90939)
- C. difficile 630-Tn5397 (AF333235)
- C. difficile cd53-tet(M)4
- C. difficile cd8-tet(M)6
- Neisseria gonorrhoeae (L12241)
- Gardnerella vaginalis (U58985)
- S. agalactiae (AE014233)
- Neisseria meningitidis (X75073)
- C. difficile cd1911-tet(M)1
- Ureaplasma urealyticum (U08812)
- S. aureus (M21136)
- C. difficile cd63-tet(M)3
- C. perfringens (AF329848)
- C. difficile cd60-tet(M)2
- E. faecalis (X56353)
- Lactococcus lactis (DQ060148)
- E. faecalis-Tn916 (U09422)
- E. faecalis (X92947)

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