A Tn1545-like transposon carries the tet(M) gene in tetracycline resistant strains of *Bacteroides ureolyticus* as well as *Ureaplasma urealyticum* but not *Neisseria gonorrhoeae*

Bertille de Barbeyrac*, Michel Dupon*, Patricia Rodriguez*, Hélène Renaudin* and Christiane Bébear*

"Laboratoire de Bactériologie; ^ Service de Maladies infectieuses et Médecine interne, Hospital Pellegrin, Place Amélie-Raba-Léon, F-33076 Bordeaux cedex, France

The presence of the tet(M) determinant and conjugative transposons related to Tn1545 in urogenital pathogens, *Bacteroides ureolyticus* (10 strains), *Neisseria gonorrhoeae* (37 strains) and *Ureaplasma urealyticum* (81 strains), was studied by PCR analysis and hybridization assay. All tetracycline-resistant strains that hybridized with a probe specific for tet(M) gave the expected fragment when their DNA's were subject to PCR. The tet(M) gene and int-Tn, the gene encoding the protein required for the movement of Tn1545-like transposons, were always found together in tetracycline resistant strains of *B. ureolyticus* and *U. urealyticum*. In contrast, int-Tn was not detected in tet(M)-mediated tetracycline-resistant *N. gonorrhoeae* strains. These data suggest carriage of tet(M) by a Tn1545-like transposon in some tetracycline-resistant *B. ureolyticus*.

**Introduction**

Tetracyclines have limited use in the treatment of sexually transmitted diseases because tetracycline resistance has appeared in many of the bacteria implicated. Of the different strategies that bacteria can use to become resistant to tetracyclines, ribosome protection is probably the most widespread mechanism. Of the three classes of ribosome protection genes (classes M, O, Q), the tet(M) determinant has been found in various bacteria, particularly in urogenital pathogens such as *Neisseria gonorrhoeae*, and genital mycoplasmas (Roberts & Hillier, 1990). The presence of tet(M) related sequences in two tetracycline-resistant strains of *Bacteroides ureolyticus*, a bacteria associated with non-gonococcal urethritis, has recently been detected (de Barbeyrac et al., 1991).

The tet(M) determinant is often associated with conjugative chromosomal elements which code for their own transfer. Members of the transposon Tn1545 family and especially Tn916 carry the tet(M) gene. Excision of this class of transposon requires two proteins designated Xis-Tn and Int-Tn because they are structurally and functionally homologous to the excisionase (Xis) and the integrase (Int) encoded by lambdoid phages (Poyart-Salmeron et al., 1989).

The purpose of this study was to develop a rapid detection assay based on the amplification of DNA sequences using PCR to detect the tet(M) gene and int-Tn, the
gene encoding the integrases of transposon Tn\textsubscript{1545} and Tn\textsubscript{916}. The presence of an integrase gene was demonstrated in \textit{tet(M)}-mediated tetracycline-resistant strains of \textit{B. ureolyticus} and \textit{Ureaplasma urealyticum}. This suggests that a conjugative transposon is present in these bacteria. To our knowledge, this is the first description of the presence of nucleotide sequences related to the conjugative transposon Tn\textsubscript{1545} in the genus \textit{Bacteroides}.

**Materials and methods**

**Bacterial strains and antibiotic sensitivity determination**

A total of ten \textit{B. ureolyticus} strains, 81 \textit{U. urealyticum} strains and 37 \textit{N. gonorrhoeae} strains was examined in this study. The strains of \textit{B. ureolyticus} were selected according to their tetracycline susceptibility as reported in a previous study (de Barbeyrac \textit{et al.}, 1991). Four strains were classified as tetracycline-resistant and two hybridized with the \textit{tet(M)} probe. Six strains were classified as sensitive and did not hybridize with the \textit{tet(M)} probe. The 81 \textit{U. urealyticum} strains included two reference strains (one tetracycline-sensitive serotype 8 T960 strain, and one \textit{tet(M)}-mediated tetracycline-resistant serotype 9 Vancouver strain), and 79 clinical strains. The 37 \textit{N. gonorrhoeae} strains included five reference strains designated A, B, C, D, E, and 32 clinical strains. In addition, two clinical strains, one \textit{tet(O)}-mediated tetracycline resistant \textit{Campylobacter jejuni} strain and one \textit{tet(M)}-mediated tetracycline-resistant \textit{Streptococcus agalactiae} strain were used as negative and positive controls, respectively, in the PCR experiments.

The antibiotic sensitivities of strains of \textit{N. gonorrhoeae} were determined by an agar dilution method (Tjiam \textit{et al.}, 1986) and those of \textit{U. urealyticum} strains were determined by a broth dilution method (Bebear \textit{et al.}, 1985). The following antimicrobial agents were tested: tetracycline hydrochloride (Lederle), doxycycline (Pfizer) and minocycline (Lederle).

**Plasmids used as probes or positive controls**

For \textit{tet(M)}, the plasmid used consisted of pUC8 \Omega 850 bp \textit{HindIII-ClaI} DNA intragenic \textit{tet(M)} fragment of transposon Tn\textsubscript{1545} isolated from a strain of \textit{Streptococcus pneumoniae} (Martin, Trieu-Cuot & Courvalin, 1986). The entire plasmid or the insert was used as a probe in a hybridization assay or as a positive control in a PCR assay. The probe was labelled with $\alpha^{32}\text{P}$ dCTP (ICN Biomedicals, Inc) by nick translation (Sambrook, Fritsch & Maniatis, 1989).

For \textit{int-Tn} of the conjugative transposon, the plasmid consisting of pUC18 \Omega 830 bp \textit{TagI} DNA intragenic fragment of the gene encoding the protein required for movement of Tn\textsubscript{1545} (Poyart-Salmeron \textit{et al.}, 1989) was used as a positive control in a PCR experiment. Both plasmids were kindly provided by P. Courvalin (Institut Pasteur, Paris, France).

**Hybridization procedure**

**Dot blot.** The distribution of the \textit{tet(M)} gene in 16 tetracycline-resistant and three tetracycline-sensitive \textit{U. urealyticum} strains was studied by dot blot hybridization.
Dot blots were prepared from concentrates of *U. urealyticum* as previously described (Brunet et al., 1989).

**Southern blot of plasmid preparations of N. gonorrhoeae strains.** Bacteria harvested from chocolate agar suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8) were centrifuged and the cell pellets were treated with extraction TELT buffer to prepare plasmids, as previously described (de Barbeyrac et al., 1991). Plasmid-enriched preparations were subjected to electrophoresis in (1%) w/v agarose gel, and then transferred to nylon membranes following the method of Southern (Sambrook et al., 1989).

Dot blots and Southern blots were hybridized with the purified 850 bp *tet(M)* fragment as probe. Hybridization and washes were done at 60°C. The filters were autoradiographed using Kodak XAR film.

**PCR experiments**

**Choice of oligonucleotides.** PCR primers, designated tet1 (GAACGTATCCTAATGTG, in position 697–716) and tet2 (GATACTCTAACCGAATCTCG, in position 1054–1073) for the specific amplification of a 377-bp fragment of the *tet(M)* gene were chosen after consulting the published sequence of the *tet(M)* gene of transposon Tn916 (Burdett, 1990, accession number EMBL X56353) and after aligning it with the published sequences of the *tet(M)* gene of transposon Tn1545. Oligonucleotides were chosen from sequences which are not homologous with *tet(O)* gene and diverse translational elongation factors (Ladefoged & Christiansen, 1991; Cousineau et al., 1992). PCR primers, designated Int1 (TGACACTCTGCCAGCTTTAC, in position 758–777) and Int2 (CCATAGGAACTTGACGTTGG, in position 1317–1336), for the specific amplification of a 579-bp fragment of the *int-Tn* gene were chosen after consulting the published sequence of the genes of Tn1545 that are involved in the excision of the element (Poyart-Salmeron et al., 1989, accession number EMBL X61025). Both primer sets were located inside the cloned fragments described above.

Oligonucleotide probes (tet3, GTTCTCTGTTCCCTTTATCATGG and Int3, GCCATCAGACGCTAAAGAATGGGC, in position 854–878 and 894–917, on their gene, respectively), internal to the amplified fragments, were used after Southern blotting to verify the specificity of the assay.

**Treatment of strains.** For *U. urealyticum*, microorganisms cultivated in Shepard medium (10 mL) were submitted to amplification either directly or after removal of culture medium by centrifugation. The pellet was resuspended in distilled water (100 μL), and used directly or cells were lysed with lysis buffer containing 10 mM Tris-HCl pH 8, 1 mM EDTA, 0.1% Triton X-100 and 20 μg/mL of proteinase K at 55°C for 90 min and then at 95°C for 30 min. The influence of culture medium pH on PCR effectiveness was tested by adding the plasmid carrying *tet(M)* to Shepard medium at different pHs. For *N. gonorrhoeae*, *S. agalactiae* and *C. jejuni*, PCR was performed either on purified DNA or on crude DNA obtained by lysing one colony with lysis buffer as described above, with and without lysozyme. Purified DNA was obtained by treatment with lysozyme, SDS, RNase and proteinase K followed by phenol-chloroform extraction (Sambrook et al., 1989). For *B. ureolyticus*, PCR was performed on purified DNA.

**PCR conditions.** For amplification of *tet(M)* or *int-Tn*, samples consisted of 5 μL of either a lysed cell suspension or purified DNA (1 μg/mL). Amplification was performed
in a final volume of 50 μL. The reaction mixture consisted of 1.5 units of Taq DNA polymerase (Promega Corporation, Madison, WI), 200 μM each of dATP, dCTP, dTTP, and dGTP, 1 μM primers tet1-tet2 or Int1-Int2, and 1 x assay buffer supplied with the enzyme by the manufacturer. Samples were denatured at 95°C, and then 35 amplification cycles were performed as follows: 95°C for 1 min, 60°C for 1 min and 72°C for 1 min.

Detection and control of specificity of amplified fragments. The products of PCR were analysed by 1% (w/v) agarose gel electrophoresis and ethidium bromide staining. Samples containing a DNA product of the expected size were subjected to digestion with the restriction enzymes Hpall and AvaII for tet(M) and Alul, Rsal, HinfI and DraI for int-Tn. For both products of amplification, tet(M) and int-Tn, Southern transfer was carried out and the filters were hybridized with their respective 32P radiolabelled internal probes, overnight at 60°C for tet(M) and 65°C for int-Tn.

All PCR tests were carried out with strict precautions to prevent contamination. Negative controls (culture medium, reaction mixture without template) and positive controls (plasmids carrying cloned fragments of tet(M) gene and int-Tn gene) were included at every stage to validate the results.

Results

Tetracycline susceptibility and hybridization patterns of U. urealyticum and N. gonorrhoeae.

All strains showed the same susceptibility profile to the three antibiotics tested. The 81 strains of U. urealyticum tested consisted of one sensitive reference strain and 40 sensitive clinical strains (MIC ≤ 1 mg/L), one resistant reference strain (MIC = 32 mg/L), 37 highly resistant clinical strains (MIC ≥ 16 mg/L) and two clinical strains resistant at a low level (MIC 2: 16 mg/L). The 37 strains of N. gonorrhoeae tested included five reference strains (strains A and B: MIC <, 0.5 mg/L, strains C and E: MIC = 1 mg/L, strain D: MIC = 4 mg/L), and 32 clinical strains. Of these, 24 were sensitive (MIC ≤ 0.2 mg/L), four were found to be highly resistant (MIC ≥ 16 mg/L) and four were resistant at a low level (4–8 mg/L).

In the dot blot, an hybridization signal was obtained for the 16 tetracycline-resistant clinical strains of U. urealyticum (14 highly resistant and two resistant at a low level) but not for the three sensitive strains (data not shown). In the Southern blot, a hybridization signal was seen with the 25.2 MDa plasmid only in the four highly tetracycline-resistant N. gonorrhoeae (TRNG) (data not shown). DNA from the five strains of N. gonorrhoeae with a low level of resistance (four clinical strains and strain D) and the sensitive strains did not hybridize to the probe.

PCR-based detection of tet(M) and int-Tn genes

For U. urealyticum, the more reproducible PCR results were obtained with a pellet of an overnight culture (10 mL) in Shepard medium resuspended in 100 μL of water and then lysed. The PCR assays performed with the plasmid carrying tet(M) in culture medium at different pHs indicated that the quality of the results varied according to pH (data not shown). Removal of the culture medium by centrifugation resolved this problem. For N. gonorrhoeae, the results of PCR using a colony lysed without the
use of lysozyme were identical to those using purified DNA. It therefore seemed unnecessary to extract whole or plasmid DNA, for PCR analysis.

The results of hybridization and PCR experiments are summarized in the Table. There was an excellent correlation between the hybridization results and PCR assays for tet(M). For U. urealyticum, the tetracycline resistance at high and low levels was always associated with the presence of tet(M), whereas only the TRNG that were highly resistant carried the tet(M) gene and gave the expected fragment by PCR (Figure 1). The specificity of the reaction was checked by hybridization with an internal tet(M) probe and by digestion with appropriate restriction enzymes (Figure 2). Of the four tetracycline-resistant strains of B. ureolyticus, the two that gave positive hybridization results with tet(M) probes, also gave PCR products of the appropriate size (Figure 3, at top, lanes 9, 10) while the two strains that did not hybridize with tet(M) probe failed to give PCR products (Figure 3, at top, lanes 7, 8). However, the restriction digestion profiles showed that the amplified products from the B. ureolyticus strains did not possess the Aval restriction site (Figure 2, lanes 6', 7').

Association of tet(M) with the integrase gene int-Tn was found in all strains of U. urealyticum and B. ureolyticus (Figure 1, lanes 3, 4 and Figure 3, lanes 9, 10, at bottom). In contrast, int-Tn was not detected in strains of N. gonorrhoeae (Figure 1, lanes 7, 8, 9, at bottom). The amplification products from B. ureolyticus strains were digested by the four restriction enzymes as expected (Figure 4, lanes 2, 6, 10, 14) and hybridized with the internal int-Tn probe (data not shown). However, the amplified products from U. urealyticum strains were digested by all enzymes except DraI (Figure 4, lane 13) while all amplified products hybridized with the internal probe (data not shown).

Concerning the controls, S. agalactiae gave amplification products with the two sets of primers (Figure 1, lane 10) that possessed the expected restriction sites (Figure 2, lanes 5, 5' and Figure 4, lanes 3, 7, 11, 15). C. jejuni failed to give PCR products (Figure 1, lane 11).
Figure 1. Ethidium bromide-stained gel of PCR products from *U. urealyticum* and *N. gonorrhoeae* DNA with primers for tet(M) and int-Tn. Lanes 1, 2, correspond to tetracycline-sensitive *U. urealyticum*, lanes 3, 4, tetracycline-resistant *U. urealyticum*, lanes 5, 6, tetracycline-sensitive *N. gonorrhoeae*, lanes 7, 8, 9, tetracycline-resistant *N. gonorrhoeae*, lane 10, tetracycline-resistant *S. agalactiae*, lane 11, tetracycline-resistant *C. jejuni* and lane 12, the cloned fragments as positive controls. Lane M: molecular size marker. The PCR was performed with primers for tet(M) (at top) and int-Tn (at bottom). Only the tetracycline-resistant strains (lanes 3, 4, 7, 8, 9), the tetracycline-resistant *S. agalactiae* and the cloned fragment gave the expected fragment with primers for tet(M). The tetracycline-resistant *C. jejuni* was negative. With primers for int-Tn, only the two tetracycline-resistant *U. urealyticum* strains, the tetracycline-resistant *S. agalactiae* and the cloned fragment gave an amplified product.

**Discussion**

The tet(M) gene is common in Gram-positive and Gram-negative pathogens, and mycoplasmas. Dissemination of this resistance gene may reflect the fact that it is carried by broad host-range conjugative transposons such as Tn916 and Tn1545. Our results suggest that the presence of tet(M) in *B. ureolyticus* and *U. urealyticum* is a consequence of the acquisition of Tn1545-like elements. This proposal is based on the association of tet(M) with int-Tn (the gene encoding the protein required for the movements of this class of transposons) in tetracycline-resistant strains.

In this study, a diagnostic protocol for the rapid identification of tetracycline resistance genes related to tet(M) in *U. urealyticum*, *N. gonorrhoeae* and *B. ureolyticus* strains by PCR, using tet(M) specific primers was developed. All the tetracycline-resistant strains that hybridized with the tet(M) probe by dot blot or Southern blot analysis gave the expected product when examined by PCR assay. While the tet(M) gene is the only tetracycline-resistant determinant described in *U. urealyticum* so far and in *N. gonorrhoeae* with high-level resistance, very little is known about the tetracycline resistance in *B. ureolyticus*. The tet(Q) determinant is prevalent among tetracycline-resistant *Bacteroides* spp. Nikolich, Shoemaker & Salyers, (1992) and Aktar & Eley,
PCR-based detection of tet(M) and Tn1545 in urogenital pathogens

Figure 2. Restriction analysis of tet(M) amplified fragment. Lanes 1, 2, 1', 2', correspond to two U urealyticum strains, lanes 3, 4, 3', 4', two N. gonorrhoeae strains, lanes 5, 5', S. agalactiae strain and lanes 6, 7, 6', 7', two B. ureolyticus strains. Lane 8 represents an amplified product before hydrolysis. Lane M: molecular size marker. After HpaII digestion, lanes 1–7, all strains gave two expected fragments (279 and 98 bp). After AvaII digestion, lanes 1'–7', only U. urealyticum and N. gonorrhoeae strains gave two expected fragments (259 and 118 bp). The fragments from B. ureolyticus did not possess the AvaII restriction site.

(1992) confirmed a previous report (de Barbeyrac et al., 1991) of the presence of tet(M) in B. ureolyticus. There may be another tetracycline resistance mechanism in the two tetracycline-resistant B. ureolyticus that did not hybridize to the tet(M) probe and did not give an amplification product.

Concerning the choice of tet(M) primers, sequences were chosen outside zones of homology with the several elongation factor Tu genes because the ribosome protection resistance proteins share considerable amino acid homology with elongation factors (Sanchez-Pescador et al., 1988a) and also with the tet(O) and tet(Q) genes. The tet(O) gene is closely related to tet(M) and tet(Q) is 40% identical to tet(M) and to tet(O). The C. jejuni strain that carries tet(O) and which was used as a control gave no amplification product. The sequences of tet(M) in Tn916 (Sanchez-Pescador et al., 1988b) and in Tn1545 (Martin et al., 1986) are closely related but show some differences as detected by restriction enzyme analysis. The restriction analysis of the 377-bp amplification products showed that in U. urealyticum and N. gonorrhoeae, the tet(M) gene corresponded to the sequence of the tet(M) gene in Tn916 while in B. ureolyticus the AvaII site was lacking and the HpaII site was present. In the sequence of the tet(M) in Tn1545, the two sites are lacking.

A PCR system to detect the int-Tn gene required for excision of the Tn1545 was also developed. The nucleotide sequence of the integrase gene of Tn916 has been determined (Clewell et al., 1991) and is identical to that of Tn1545 except for two nucleotides. In U. urealyticum, this study confirms the previous report (Roberts, 1990) of the association of tet(M) with the conjugative transposon Tn916 in tetracycline-resistant strains. In N. gonorrhoeae, tet(M) does not appear to be associated with an intact conjugative transposon. Swartley et al., (1993) showed that the 25.5 MDa, tet(M)
encoding, conjugative plasmid of *N. gonorrhoeae* contains the remains of a Tn916-like transposon. Deletions have resulted in the loss of the majority of both arms of the original transposon, the integrase and excisionase genes and the broad-host range conjugative domains while the tet(M) determinant has been retained.

By using PCR, we have shown the association of the Tn1545 integrase gene and the tet(M) gene, in *B. ureolyticus*. These findings again raise the question of the precise taxonomic position of *B. ureolyticus*. Sequencing studies of rRNA have suggested that *B. ureolyticus* is not a true *Bacteroides* species, and its proteolytic metabolism and fatty acid components have caused it to be excluded from the genus *Campylobacter* (Vandamme *et al*., 1995). Recently, genes in the tet(Q) operon which are important for self-transfer have been identified. The sequence of an open reading frame on these genes, that are necessary for mobilization, has been determined (Li Shoemaker & Salyers, 1993) and it is not homologous to int-Tn. We concluded therefore that the two tetracycline-resistant *B. ureolyticus* isolates that possess tet(M) also contain the integrase gene of Tn1545 because the int-Tn amplification products hybridized to the internal probe and possessed all expected restriction sites. However, more work will be necessary to determine the nature of the transposon on which the integrase gene is located.

Figure 3. Ethidium bromide-stained gel of PCR products from *B. ureolyticus* with primers for tet(M) and int-Tn. Lanes 1–6, correspond to tetracycline-sensitive strains, lanes 7–10, tetracycline-resistant strains, lane 11, cloned fragment as positive control, and lane 12, negative control. Lane M: molecular size marker. The PCR was performed with primers for tet(M) (at top) and for int-Tn (at bottom). Only the two tetracycline-resistant strains (lanes 9, 10) which hybridized with the tet(M) probe gave the expected fragments with both primers for tet(M) and for int-Tn. The two other tetracycline-resistant strains were not amplified (lanes 7, 8).
PCR-based detection of tet(M) and Tn1545 in urogenital pathogens

Figure 4. Restriction analysis of int-Tn amplified fragments. Lanes 1, 5, 9, 13, correspond to one U. urealyticum strain, lanes 2, 6, 10, 14, one B. ureolyticus strain, lanes 3, 7, 11, 15, one S. agalactiae strain, lanes 4, 8, 12, 16, the cloned fragment digested by the restriction enzymes described on the figure. All amplified products were digested as predicted, except for U. urealyticum whose fragment did not possess a DraI site (lane 13).

References


(Received 8 March 1995; returned 15 May 1995; revised 11 July 1995; accepted 4 August 1995)