Detection of a Novel Tet M Determinant in Tetracycline-Resistant Neisseria gonorrhoeae *from Uruguay, 1996–1999*

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Background: **Determination of the diversity within the *tet*(M) sequence from *N gonorrhoeae* is a useful epidemiologic tool for monitoring the movement or importation of strains within a geographic region. Only two distinct *tet*(M) genes in clinical gonococcal isolates have been described up to now: the Dutch and the American types.**

Goal: **The study involved surveillance of the *tet*(M) gene types in high-level-tetracycline-resistant gonococcal isolates from Uruguay during the period 1996 to 1999.**

Study Design: **Among 181 gonococcal isolates, those showing MICs ≥16 μg/ml to tetracycline were analyzed for detection and characterization of the *tet*(M) gene by a polymerase chain reaction (PCR) and further *Hpa*II restriction fragment polymorphism methods, respectively. The plasmid content and antibiogram were determined.**

Results: **Twenty-two of 181 isolates (12%) exhibited high levels of resistance to tetracycline (MICs ≥16 μg/ml) and harbored a putative 25.2-Mda plasmid that contained the *tet*(M) gene. A high percentage of isolates (95%; 21/22) presented the Dutch type *tet*(M) gene. One isolate from 1999 revealed a new restriction pattern. Such a pattern had been previously noted in 1991. This new restriction pattern has not been described previously as occurring in isolates of *N gonorrhoeae*. The *tet*(M) amplimer sequence showed 100% identity with a previously described *tet*(M)-carrying plasmid from *N meningitidis*.**

Conclusion: **A new *Hpa*II restriction pattern of the *tet*(M) gene is present in low frequency. The *tet*(M) sequence was different from the gonococcal *tet*(M) sequences already known and not typable with the use of a differential PCR assay. Accordingly, with the genetic diversity already present within the *tet*(M) sequence of *N gonorrhoeae* isolates, we should be aware of the sensitivity of the PCR assays in use for tetracycline-resistant *N gonorrhoeae* detection.**

HIGH-LEVEL TETRACYCLINE RESISTANCE (MIC ≥16 μg/ml) in *Neisseria gonorrhoeae* (TRNG) is mediated by a Tet M determinant carried on a 25.2-MDa conjugative plasmid.[1] These plasmids have been described worldwide since 1983.[2–6]

Various methods have been developed for the detection and typing of the tetracycline resistance genotype in gonococci. Two different restriction endonuclease patterns designated Dutch and American have been described for the 25.2-MDa Tet M–containing plasmid.[4]

Analysis based on polymerase chain reaction (PCR) assay followed by restriction fragment length polymorphism (RFLP) of the *tet*(M) amplimer or based on differential PCR assay detecting the size of the *tet*(M) insert showed two profiles or two insert sizes, respectively.[7–9] Each PCR/RLFP profile and each *tet*(M) insert size corresponded with each of the two plasmid restriction patterns.[7–9] The nucleotide sequences of the *tet*(M) genes from the 25.2-MDa conjugative plasmid types are known and differ significantly.[10] We previously described both the Dutch and the American *tet*(M) types among TRNG isolated between 1991 and 1995 in Uruguay; the Dutch *tet*(M) type was the most frequent type isolated.[5,11]

The purpose of the current study was to continue the surveillance of the *tet*(M) gene types in TRNG isolated in Uruguay up to 1999. We describe a new *tet*(M) gene restriction endonuclease pattern and analyze the sequence of the PCR product.

Methods

Bacterial Isolates

Between 1996 and 1999, a total of 181 *N gonorrhoeae* genital isolates were collected in various health centers of

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the country and sent to our Reference Gonococcus Unit for characterization of their antimicrobial resistance profiles. Colonies that were oxidase-positive, gram-negative cocci were identified as *N gonorrhoeae* on the basis of the presence of a typical gonococcus colony morphology and colony size variation, a strongly positive Superoxyl test, the requirement of cysteine/cystine for growth, and the utilization of glucose but not maltose, sucrose, or lactose.[12–14]

β-Lactamase detection was tested by the chromogenic cephalosporin method (Nitrocefin, Oxoid Co., Basingstoke, UK).

We also included in this study a TRNG β-lactamase-negative isolate from 1991, strain 1453, belonging to the P/IB-1 auxotype/serovar (A/S) class.

The World Health Organization set of strains designated WHO III, WHO V, and WHO VII and strains 85 to 912 TRNG were used as controls for the antimicrobial susceptibility testing. Gonococcal strains GC 1–159, GC 1–182, and GC 1–452 were used as controls for the 3.05-MDa, 3.2-MDa, and 4.4-MDa β-lactamase type plasmids, respectively. All control strains were kindly provided by the Coordinating Center for the Gonococcal Antimicrobial Susceptibility Program (GASP) in the Americas and Caribbean, based at the University of Ottawa, Canada.

Four representative TRNG clones from the period 1991 to 1995 were used as positive controls for both the PCR/RFLP and the differential PCR assays. Three of them, strains 323, 545, and 344, belonging to the O/IA-2, O/IA-10, and P/IA-8 A/S classes, respectively, carried the Dutch *tet*(M) type, and the other one, strain 727, belonging to the P/IB-1 A/S class, carried the American *tet*(M) type.[5,11] A tetracycline-susceptible isolate (strain 30; MIC, 0.25 μg/ml) was used as a non-TRNG control strain.

Antibiotic Susceptibility Testing

MICs were determined by the agar dilution method, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS).[15] Isolates were tested with penicillin G, tetracycline, ceftriaxone, ciprofloxacin, spectinomycin, and azithromycin. Isolates exhibiting MICs ≥16.0 μg/ml of tetracycline were studied for the presence of the *tet*(M) gene. All antimicrobial agents were obtained from Sigma Chemical Co. (St. Louis, MO), with the exception of azithromycin, which was obtained from Urufarma (Montevideo, Uruguay).

Plasmid Detection

Plasmid DNA was extracted by the alkaline lysing procedure of Birnboim and Doly.[16] The plasmid content was determined by electrophoresis on 0.8% agarose gels, stained with ethidium bromide (0.5 μg/ml) and photographed under long-wave ultraviolet transillumination with a Kodak Digital Science DC120 camera. The image was analyzed with 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY).

Polymerase Chain Reaction for tet*(M) and Restriction Endonuclease Analysis*

Polymerase chain reaction analysis was performed with a Perkin Elmer DNA thermal cycler 2400, with the previously described[8] tetA and tetB primers synthesized on CyberSyn (Lenni, PA). A reaction volume of 100 μl contained 20 to 40 ng of DNA preparation as template, 0.2 mmol/l of each dNTPs (Pharmacia Biotech, Uppsala, Sweden), 0.2 μg of each primer, 1.5 mmol/l $MgCl_2$, 2 U Taq polymerase (Promega Co, Madison, WI), and 1× PCR buffer (Promega). Amplification consisted of 3 minutes at 94 °C and then 30 cycles as follows: 94 °C for 30 seconds, 60 °C for 15 seconds, 72 °C for 5 minutes, and a final extension at 72 °C for 5 minutes. The PCR product was analyzed by electrophoresis of 10 μl in a 1% agarose gel and visualized under UV light by ethidium bromide staining. All amplicons were digested with *Hpa*II (BioLabs) according to the manufacturer's instructions for 2 hours at 37 °C and analyzed on a 3% agarose gel. The Dutch type amplicon's predicted size is 765 bp, and the sizes of the expected *Hpa*II restriction fragments are 529, 145, and 91 bp. The predicted size of the American type amplicon is 765 bp and the sizes of the expected *Hpa*II restriction fragments would be 357, 263, and 145 bp. pGEM Marker (Promega) and SmartLadder 200 bp (Eurogentec Bel S.A., Seraing, Belgium) were used as molecular weight markers.

Differential PCR Assay to Detect tet*(M)–Containing Plasmids*

To detect *tet*(M)-containing plasmids, the method of Xía et al was used.[9] This assay can distinguish the two types of 25.2-MDa plasmid on the basis of differences in the size of the *tet*(M) insertion and was designated to amplify from within the *tet*(M) gene through the downstream region insertion in the 24.5-MDa plasmid. As specified, primers RM4 and G1 were used and were synthesized at the University of Ottawa Biotechnology Research Institute. The predicted size of the amplicons for the Dutch and American *tet*(M) types are 700 bp and 1600 bp, respectively.

Sequencing PCR Product

The *tet*(M) amplified product of isolate CM9925 was purified with the Wizard PCR Preps DNA Purification System protocol supplied by the manufacturer (Promega). Automated DNA sequencing of both strands of DNA was performed in an ABI Prism 377 instrument at the Centro Técnico de Análisis Genéticos (CTAG) of the Facultad de Ciencias, Montevideo, Uruguay, with use of oligonucleotide primer tetA or tetB.

TABLE 1. Distribution of TRNG and PP/TRNG Isolates Per Year, 1996–1999

Year of Isolation	No. of *N gonorrhoeae* Isolates (%)		
	Total	TRNG	PP/TRNG
1996	41	2 (5)	4 (10)
1997	56	—	6 (11)
1998	47	4 (9)	—
1999	37	5 (13)	1 (3)
Total	181	11 (6)	11 (6)

TRNG = tetracycline-resistant *Neisseria gonorrhoeae*; PP = penicillinase producing.

The two sequences were aligned and compared with all accessible sequences in databases with the BLAST server at NCBI (National Center of Biotechnology Information). Alignments were performed and corrected with Clustal W and Seq-pup software. A matrix distance was constructed with DNADIST (Jukes & Cantor algorithm) and NEIGHBOR-JOINING programs contained in the PHYLIP.

Results

Antibiotic Susceptibility Testing

Twenty-two of 181 isolates (12%) exhibited high levels of resistance to tetracycline, with MICs ≥16 μg/ml. These isolates harbored a putative 25.2-Mda plasmid and a cryptic plasmid of 2.6 MDa (data not shown). All the TRNG isolates were susceptible to ceftriaxone (MIC range, 0.002–0.016 μg/ml), ciprofloxacin (MIC range, 0.004–0.032 μg/ml), azithromycin (MIC range, 0.063–0.25 μg/ml), and spectinomycin (MIC range, 8.0–16.0 μg/ml). Eleven of the 22 TRNG isolates were also penicillinase-producing (PP), carrying the Toronto-type β-lactamase plasmid of 3.05 MDa. The PP/TRNG population widely predominated during 1996 to 1997 (10/12), while a shift toward a TRNG β-lactamase-negative population was noted during 1998 to 1999 (9/10) (Table 1).

Polymerase Chain Reaction for tet*(M) and Restriction Endonuclease Analysis*

Amplicons of 765 bp were obtained for all TRNG isolates with primers tetA and tetB (Fig. 1). After digestion of the amplicons with *Hpa*II, 21 isolates showed the Dutch restriction pattern and one isolate showed a pattern with only two fragments of approximately 500 and 250 bp (Fig. 2). This third gonococcal restriction pattern, designated the Uruguayan pattern, was obtained from strain CM9925, isolated in 1999, and was identical to the pattern obtained with isolate 1453 from 1991 (Márquez, Borthagaray, and Dillon, unpublished data). The characteristics of the two TRNG isolates that exhibited the novel *tet*(M) *Hpa*II restriction pattern are shown in Table 2. One of the two strains exhibiting a new *tet*(M) *Hpa*II restriction pattern, strain 1453, was subjected to the differential PCR assay with a different set of primers. TRNG control strains known to carry the small *tet*(M) insert and the large *tet*(M) insert showed an amplicon of 700 bp and 1600 bp, respectively. No amplification product was seen in strain 1453.

Sequencing PCR Product

The GenBank-published *tet*(M) DNA sequence of *Neisseria meningitidis* (X75073), *Ureaplasma urealyticum* (UO8812), *Staphylococcus aureus* (M21136), *Streptococcus faecalis tet*(M) 916 (X56353), and *Gardnerella vaginalis* (U58985,U58986) also anneals with tetA and tetB primers, yielding a predictable fragment size amplimer of 765 bp with a single restriction *Hpa*II site, a pattern indistinguishable from the Uruguayan pattern.

The amplicon showing the novel restriction endonuclease pattern from strain CM9925 was sequenced (Fig. 3). The GenBank accession number for the *tet*(M) gene sequence from CM9925 strain is AF362991. The nucleotide sequence was aligned with the most closely related *tet*(M) genes, and a matrix distance of 618 bp was constructed that revealed a DNA sequence showing sequence similarity of 100% with *N meningitidis* (X75073), 99.8% with *U urealyticum*

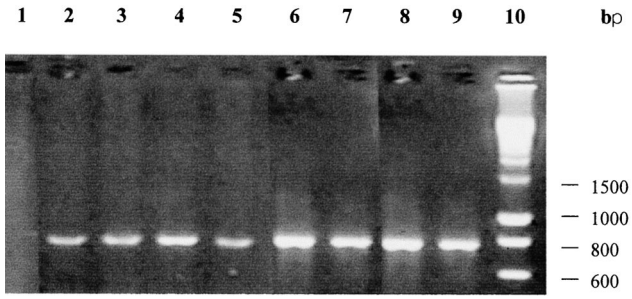

Fig. 1. Analysis of amplification products by agarose gel electrophoresis and ethidium-bromide staining. Lane 1, non–tetracycline-resistant *Neisseria gonorrhoeae* (TRNG) control strain; lane 2, TRNG Dutch control strain; TRNG isolates from 1996: lane 3, 326, lane 4, 545; TRNG isolates from 1997: lane 5, 9730, lane 6, 9735; TRNG isolates from 1998: lane 7, 9820; TRNG isolates from 1999: lane 8, CM9925, lane 9, 9907; lane 10, SmartLadder (Eurogentec Bel S.A.) molecular weight marker, from bottom to top, 600 bp, 800 bp, 1000 bp, 1500 bp.

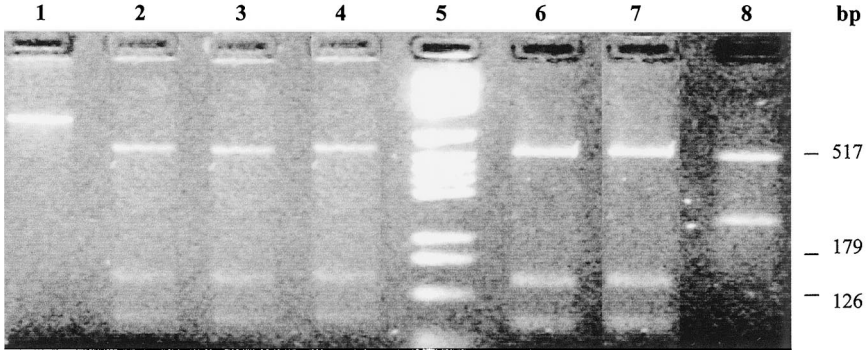

Fig. 2. Restriction enzyme digest patterns of *tet*(M) PCR products using *Hpa*II. Lane 1, undigested *tet*(M) amplicon; tetracycline-resistant *Neisseria gonorrhoeae* (TRNG) isolates with Dutch pattern: lanes 2, 326, lane 3, 545, lane 4, 9730, lane 6, 9820, lane 7, 9907; TRNG isolate with Uruguayan pattern: lane 8, CM9925; lane 5, pGEM (Promega) Marker. Numbers at right belong to pGEM in bp.

(UO8812), 99.7% with *S aureus* (M21136), 99.3% with *Enterococcus faecalis tet*(M) Tn*916* (X56353), 97.1% with *G vaginalis* (U58985), 96.8% with *G vaginalis* (U58985), 96.3% with *Streptococcus pneumoniae* (X900939), 95.4% with American type plasmid pOZ101 (L12241), and 94.1% with Dutch type plasmid pOZ100 (L12242).

The sequenced DNA comprises the region where the two *Hpa*II restriction sites are present in plasmid pOZ101. Only one *Hpa*II restriction site was present in the sequence analyzed, which was consistent with the two restriction fragments obtained for the Uruguayan pattern.

Discussion

The frequency of isolation of TRNG in Uruguay during the period 1996 to 1999 rose to 12%, from the 4% reported during the period 1991 to 1994.[5]

Although tetracyclines are not recommended in the Uruguayan guidelines for the treatment of uncomplicated gonococcal infection since 1996, they are used for the treatment of chlamydial infection.[17] Thus, the increase in the percentage of TRNG isolates is probably due to the continual selective pressure exerted by the simultaneous treatment with tetracycline. The analysis during 1996 to 1999 of TRNG isolates by PCR/RFLP assay to determine the subtype of the *tet*(M) gene present in each isolate revealed a high percentage (95%; 21/22) presenting the Dutch restriction pattern. We previously demonstrated the Dutch *tet*(M) type to be overall the most prevalent type among the clones found in Uruguay during 1991 to 1995.[5,11]

We describe a third *Hpa*II restriction pattern of the *tet*(M) gene, not previously described as occurring in gonococci. We recommend the use of the common name "Uruguayan Tet M determinant" for these novel TetM plasmid types.

Only one other isolate with this *tet*(M) type that we observed previously (unpublished results) has been characterized.

The lack of surveillance and strain recollection limited the monitoring of this new *tet*(M) gene type before 1990. Since such an isolate was discovered only 1 year after our surveillance studies were initiated, it could have been present in Uruguay at the same time the other gonococcal *tet*(M) gene types were being described in 1985 in other geographic areas.[4] We confirmed a new *tet*(M) DNA sequence within the gonococcus species. We found striking differences between this sequence and the ones from the Dutch and the American *tet*(M) gene, and the sequence was identical to the DNA sequence of a *tet*(M) gene (X75073) carried in a 25.2-MDa conjugative plasmid of an oropharyngeal *N meningitidis*.[10,18]

TABLE 2. Characteristics of TRNG Isolates Containing the Uruguayan *tet*(M) Type

Strain No.	Isolation date	A/S Class*	β-lactamase†	MIC (mg/l)‡						Plasmid Content (MDa)	*tet*(M) Type by Method§	
				T	P	Cro	Cip	Az	S		A	B
1453	07/91	Pro/IB-1	–	16	0.5	0.008	0.125	ND	16	2.6, 25.2	Uruguayan	Nontypable
CM9925	09/99	ND	–	16	0.5	0.016	0.032	0.25	16	2.6, 25.2	Uruguayan	ND

*Pro = proline-requiring.
†– = negative.
‡T = tetracycline; P = penicillin; Cro = ceftriaxone; Cip = ciprofloxacin; Az = azithromycin; S = spectinomycin.
§A = PCR method described by Ison *et al*, 1993; B = PCR method described by Xía *et al*, 1995.
ND = not determined. A/S = auxotype/serovar; TRNG = tetracycline-resistant *Neisseria gonorrhoeae*.

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CM9925          (1)      ATCAATAAGATTGACCAAANTGGAATTGATTTATCAACGGTTT
pOZ105        (606)      ------------------A----------------------
pOZ100        (606)      ------------------A----------------------
pOZ101        (606)      ------------------A----------------------

CM9925        ATCAGGATATTAAAGAGAAACTTTCTGCCGAAATTGTAATCAAACAGAGGGTAGAACTGT
pOZ105        ------------------------------------------------------------
pOZ100        ------------------------------------------------------------
pOZ101        ------------------------------------------------------------

CM9925        ATCCTAATATGTGTGTGACGAACTTTACCGAATCTGAACAATGGGATACGGTAATAGAGG
pOZ105        ------------------------------------------------------------
pOZ100        ------------------------------------------------------------
pOZ101        ---------------------------------------------T---------A-

CM9925        GAAACGATGACCTTTTAGAGAAATATATGTCCGGTAAATCATTAGAAGCATTGGAACTCG
pOZ105        ------------------------------------------------------------
pOZ100        ------------------------------------------------------------
pOZ101        ----T---T-------G----------C---T--G----T---G--------A-------

CM9925        AACAAGAGGAAAGCATAAGATTTCATAATTGTTCCCTGTTCCCTGTTTATCACGGAAGTG
pOZ105        ------------------------------------------------------------
pOZ100        -------------------------G--------T---------C---------------
pOZ101        ------------------------------------------------------------

CM9925        CAAAAAACAATATAGGGATTGATAACCTTATAGAAGTGATTACGAATAAATTTTATTCAT
pOZ105        ------------------------------------------------------------
pOZ100        ------GT-----------------------------T-----T----------------
pOZ101        ------------------------------------------------------------

CM9925        CAACACATCGAGGTCCGTCTGAACTTTGCGGAAATGTTTTCAAAATTGAATATACAAAAA
pOZ105        ------------------------------------------------------------
pOZ100        ----------------------------------A-------------G---T-GG---
pOZ101        ---------------A------------------A-------------G---T-GG---

CM9925        AAAGACAACGTCTTGCATATATACGCCTTTATAGTGGAGTACTACATTTACGAGATTCGG
pOZ105        ------------------------------------------------------------
pOZ100        -------G-----------------T-----------C-----G-----G----------
pOZ101        -------G-----------------T-----------C-----G-----G------C---

CM9925        TTAGAGTATCAGAAAAAGAAAAAATAAAAGTTACAGAAATGTATACTTCAATAAATGGTG
pOZ105        ------------------------------------------------------------
pOZ100        -----A----G-----G------------A------------------------------
pOZ101        -----A----G-----G------------A------------------------------

CM9925        AATTATGTAAGATTGATAGAGCTTATTCTGGAGAAATTGTTATTTTGCAAAATGAGT
pOZ105        ------------------------------------------------------------
pOZ100        ----------A-------AG--------C--G-----------------G-------
pOZ101        ----------A--C----AG--------C--G-----------------G-------

CM9925        TTTTGAAGTTAAATAGTGTTCTTGGAGATACAAAACTA     618
pOZ105        ----------------------------------       1223
pOZ100        ----------------------------------G---   1223
pOZ101        ----------------------------------G---   1223
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Fig. 3. The nucleotide sequence of the *tet*(M) gene from *Neisseria gonorrhoeae* CM9925. Differences in the sequences from *N meningitidis* plasmid pOZ105 and gonococcal plasmids pOZ100 and pOZ101 are shown beneath (Gascoyne-Binzi et al, 1994; X75073, Gascoyne-Binzi et al, 1993; L12241 and L12242). Broken lines indicate DNA sequence homology. Numbers in brackets correspond to the aligned region for each *tet*(M) gene.

The *tet*(M) gene is usually carried by conjugative transposons, which do not confer immunity to the acquisition of further Tn*916*-like elements, therefore allowing them to recombine and generate hybrid elements showing *tet*(M) genes under mosaic structure.[19] The hybrid elements would be created in different genera, and once integrated in the progenitor 24.5-MDa plasmid the insertion site is fixed.

We believe that there may exist various unknown *tet*(M) subtypes in conjugative transposons and that genetic events similar to those that generated the already known Dutch and American *tet*(M)-containing plasmid have given rise to this new *tet*(M) plasmid as well as others not yet detected.

Strain 1453, showing the novel restriction endonuclease pattern, was analyzed with the differential PCR assay, which amplifies the downstream insertion site of the transposon with primers RM4 and G1.[9] All the 25.2-MDa plasmids examined until now were shown to be typable with the use of the above set of primers, supporting the hypothesis that the insertion site of the Tet M transposon was similar or identical for all the 25.2-MDa plasmids studied.[9,20] Hence, the lack of amplification with strain 1453 may indicate this new *tet*(M) gene subtype is inserted in a different location for its 25.2-Mda containing plasmid. Therefore, the sensitivities of various PCR techniques for TRNG detection should undergo reevaluation as long as new *tet*(M) subtypes emerge. This is the first description of a third *tet*(M) subtype in *N gonorrhoeae*, probably inserted in a different plasmid region than the other two *tet*(M) subtypes. There is a need to further study the entire *tet*(M) gene and its harboring plasmid in order to establish its possible origin and to determine the typability of the different methods in use.

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