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Identification of the Tetracycline Resistance Gene, *tet*(O), in *Streptococcus pneumoniae*

CAROL A. WIDDOWSON,[1] KEITH P. KLUGMAN,[1]* AND DAVID HANSLO[2]

MRC/SAIMR/WITS Pneumococcal Diseases Research Unit, South African Institute for Medical Research, Hillbrow, Johannesburg 2001,[1] and Department of Medical Microbiology, University of Cape Town, and Red Cross Childrens Hospital, Rondebosch 7700,[2] South Africa

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Five isolates of *Streptococcus pneumoniae* resistant to tetracycline but lacking *tet*(M) were studied. The tetracycline resistance gene, *tet*(O), was detected for the first time in the pneumococcus. The gene was amplified and sequenced and found to share 99% nucleotide sequence identity and 99, 99, and 98% deduced amino acid sequence identity with the *tet*(O) resistance genes of *Streptococcus mutans*, *Campylobacter coli*, and *Campylobacter jejuni*, respectively.

Streptococcus pneumoniae is the major cause of bacterial meningitis, pneumonia, and otitis media (5). Thus far, the only gene described for tetracycline resistance in *S. pneumoniae* is *tet*(M) (1), which codes for a ribosomal protection mechanism and is located on the conjugative transposons Tn*1545* (2) and Tn*5251* (9).

The *tet*(O) gene is a ribosomal protection gene sharing 75% sequence identify with *tet*(M) (4). It was originally described in the gram-negative *Campylobacter* species *C. coli* (8) and *C. jejuni* (11). It has since been described in *Streptococcus mutans* (3), *S. milleri*, group B *Streptococcus* species, and *Enterococcus faecalis* (12), but it has not previously been reported in *S. pneumoniae.*

Bacterial strains and media. 122 clinical strains of *S. pneumoniae* were obtained from patients throughout South Africa. Resistance phenotypes were established by using antibiotic disk assays (tetracycline, 30 μg per disc; erythromycin, 15 μg per disc; clindamycin, 2 μg per disc; rifampicin, 5 μg per disc; oxacillin, 1 μg per disc; chloramphenicol, 30 μg per disc) on 5% horse blood agar. MICs were determined by using a Steers multipoint inoculator. All *S. pneumoniae* strains were grown on 5% horse blood agar at 37°C under aerobic conditions.

DNA methods. DNA was extracted from *S. pneumoniae* strains by using a lysis solution consisting of 0.1% sodium deoxycholate as described by Paton and coworkers (6) with the exception that plate cultures were used rather than broth cultures. Extracted DNA was resuspended in citrate saline buffer (0.1 M sodium citrate, 0.85% NaCl) and stored at −70°C for further use.

Hybridizations. Dot blots were performed by using DNA from tetracycline-resistant strains, with susceptible strains used as negative controls. Probes were kindly supplied by P. Courvalin (Pasteur Institute, Paris, France) (Table 1). These were subsequently labelled with digoxygenin by random primed labelling (DIG DNA Labeling and Detection Kit; Boehringer Mannheim). Hybridization was performed overnight at 42°C. The nylon membranes were subsequently washed twice at room temperature in 2× SSC (0.3 M NaCl and 0.03 M sodium citrate, pH 7)–0.1% sodium dodecyl sulfate (SDS) and twice for 15 min at 68°C in 0.1× SSC–0.1% SDS. The fluorescent dye CSPD ($C_{18}H_{20}ClO_7PNa_2$) (Boehringer Mannheim) was used to elicit chemiluminescence, and the blots were exposed to X-ray film for 2 h.

DNA from 95 of the 100 tetracycline-resistant pneumococci hybridized with the *tet*(M) probe. None of the tetracycline-susceptible strains hybridized with these probes. The five tetracycline-resistant strains that did not hybridize with the *tet*(M) probes (SA40300, SA4054, SA31906, SA34660, and SA98289) were further probed for *tet*(O), *tet*(S), *tet*(K) and *tet*(L). All five strains hybridized with the *tet*(O) probe. DNA from 91 *tet*(M)-containing pneumococci were subsequently probed for *tet*(O), and all were found to be negative for this gene.

PCR. PCR primers were designed from the previously described *tet*(O) gene in *C. coli* (8). The forward primer consisted

TABLE 1. Origin of probes

Gene to be probed	Probe	*Escherichia coli* strain	Plasmid
tet(M)	850-bp *Cla*I-*Hin*dIII intragenic fragment of *tet*(M)	HB101	pUC8
tet(L)	310-bp *Cla*I-*Hpa*II intragenic fragment of *tet*(L)	HB101	pUC18
tet(O)	1.458-kbp *Hin*dIII-*Nde*I intragenic fragment of *tet*(O)	HB101	pUC19
tet(S)	900-bp *Eco*RI-*Bgl*II intragenic fragment of *tet*(S)	JM83	pUC19
tet(K)	870-bp *Hin*cII intragenic fragment of *tet*(K)	HB101	pUC8

* Corresponding author. Mailing address: SAIMR, PO Box 1038, Johannesburg 2000, South Africa. Phone: 27 11 489 9010. Fax: 27 11 489 9012.

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                                                                                      80
SP MKIINLGILA HVDAGKTTLT ESLLYTSGAI AEPGSVDKGT TRTDTMNLER QRGITIQTAV TSFQWEDVKV NIIDTPGHMD
SM .......... .......... .......... ..P....K.. .....M.... .......... .......... ..........
CC .......... .......... .......... ..P....K.. .....I.... .......... .......... ..........
CJ .......... .......... .......... ..L....E.. .....M.... .......... .......... ..........

                                                                                     160
SP FLAEVYRSLS VLDGAVLLVS AKDGIQAQTR ILFHALQTMK IPTIFFINKI DQEGIDLPMV YQEMKAKLSS EIIVKQKVGQ
SM .......... .......... .......... .......T.. .......... .......... .R........ ..........
CC .......... .......... .......... .......I.. .......... .......... .Q........ ..........
CJ .......... .......... .......... .......I.. .......... .......... .R........ ..........

                                                                                     240
SP HPHINVTDND DMEQWDAVIM GNDELLEKYM SGKPFKMSEL EQEENRRFQN GTLFPVYHGS AKNNLGIRQL IEVIASKFYS
SM .......... ......T... .......... .......... .......... .......... ......I... ..........
CC .......... ......A... .......... .......... .......... .......... ......I... ..........
CJ .......... ......A... .......... .......... .......... .......... ......T... ..........

                                                                                     320
SP STPEGQSELC GQVFKIEYSE KRRRFVYVRI YSGTLHLRDV IKISEKEKIK ITEMCVPTNG ELYSSDTACS GDIVILPNDV
SM .......... .......... .......... .......... .R........ ....C..... .......... ..........
CC .......... .......... .......... .......... .R........ ....C..... .......... ..........
CJ .......... .......... .......... .......... .R........ ....Y..... .......... ..........

                                                                                     400
SP LQLNSMLGNE ILLPQRKFIE NPLPMLQTTI AVKKSEQREI LLGALTEISD GDPLLKYYVD TTTHEIILSF LGNVQMEVIC
SM .....I.... .......... .....L.... .......... .......... G......... .......... ..........
CC .....I.... .......... .....L.... .......... .......... G......... .......... ..........
CJ .....I.... .......... .....I.... .......... .......... C......... .......... ..........

                                                                                     480
SP AILEEKYHVE AEIKEPTVIY MERPLRKAEY TIHIEVPPNP FWASVGLSIE PLPIGSGVQY ESRVSLGYLN QSFQNAVMEG
SM .......... .......... .......... .......... .......... ..PI...... .......... ..........
CC .......... .......... .......... .......... .......... ..LL...... .......... ..........
CJ .......... .......... .......... .......... .......... ..PI...... .......... ..........

                                                                                     560
SP VLYGCEQGLY GWKVTDCKIC FEYGLYYSPV STPADFRLLS PIVLEQALKK AGTELLEPYL HFEIYAPQEY LSRAYHDAPR
SM .......... .......... .......... .......... .......... .......... .......... ..........
CC .......... .......... .......... .......... .......... .......... .......... ..........
CJ .......... .......... .......... .......... .......... .......... .......... ..........

                                                                                   639
SP YCADIVSTQV KNDEVILKGE IPARCIQEYR NDLTYFTNGQ GVCLTELKGY QPAIGKFICQ PRRPNSRIDK VRHMFHKLA"
SM .........I .......... .......... N...Y..... .......... .......... .......... ..........
CC .........I .......... .......... N...N..... .......... .......... .......... ..........
CJ .........I .......... .......... T...Y..... .......... .......... .......... .....TS"
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FIG. 1. Comparison of deduced amino acid sequences from *S. pneumoniae* (SP), *S. mutans* (SM) (3), *C. coli* (CC) (8), and *C. jejuni* (CJ) (11). Amino acids that share identity with all ribosome protection proteins and Ef-G are boldfaced and underlined. Dots represent amino acid identity with *tet*(O) from *S. pneumoniae*. The position of the last amino acid on each line is labelled above the line.

of the following sequence: 5′-TGCGGCAAGGTATTCTTA AAT (base pair position 483 to 503) and the sequence of the reverse primer was 5′-ATTTTATATGACTTTTGCAAGCTG (base pair position 2651 to 2674). Amplification reactions were performed in volumes of 50 μl containing 50 mM KCl; 10 mM Tris-HCl (pH 9.0); 4 mM $MgCl_2$; 0.1% Triton X-100; 200 μM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim); 1 μM each primer; 750 ng of pneumococcal chromosomal DNA; and 1 U of *Taq* DNA polymerase (Promega Corporation, Madison, Wis.) overlaid with mineral oil (Sigma). Amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 1 cycle of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 2 min, and extension at 72°C for 2 min, followed by 1 cycle of extension at 72°C for 10 min. Amplification products were run through 1% agarose gels and detected by staining with ethidium bromide. Appropriate bands were extracted from the gels and purified by using the Quiaquick gel extraction kit (QUIAGEN).

A band of approximately 2 kb was produced when the DNA of each of the five strains that hybridized with *tet*(O) was amplified by using the above primers.

Strain relatedness. Arbitrary primed PCR was performed in order to detect the relatedness between the *tet*(O)-positive strains by using the probes P38 (5′-CGGTGGCGAA) and PTB (5′-CCTGCGAGCGTAGGCGTCGG). The reaction mixture was as for the above PCR except that 4 μM of each primer and 25 ng of template DNA were used in each 50-μl reaction volume. The same cycling parameters were used, except that denaturation was at 93°C and primer annealing was at 35°C for 1 min. Banding patterns for each strain were observed by running the PCR products through 2% agarose gels at 10 V/cm. DNA in the gels was stained with ethidium bromide and viewed on a transilluminator.

The banding patterns resulting from P38 were identical in all five strains, and those produced by PTB showed a variance of only one band in one of the strains. These data indicate that the strains are clonally related. In addition, the strains were all of serotype 14. All five strains were isolated from patients in Cape Town between 1987 and 1989. Four of the strains were from children in the Red Cross Childrens Hospital. It is unlikely however, that contact in the community occurred, and none of the patients were simultaneously present in the hospital.

Nucleotide sequence analysis. The DNA sequence of the amplification product obtained from SA40300 was determined by the dideoxy chain termination method (7) using cycle sequencing. The double-stranded PCR product was sequenced from both the 3′ and the 5′ ends by using the *fmol* cycle sequencing kit (Promega). The above PCR primers were initially used for the sequencing. Primers were subsequently synthesized as needed. DNA was labelled with α-^{35}S. The cycling parameters used were as recommended by the manufacturer.

The *tet*(O) gene from SA40300 exhibited 99% sequence identity with the *tet*(O) genes from *S. mutans* (3), *C. coli* (8), and *C. jejuni* (11). Of the nine entirely new base substitutions, only three resulted in amino acid changes (Fig. 1). None of these changes occurred in regions of consensus among all ribosomal protection proteins and elongation factor G (Ef-G) (Fig. 1) (10). The predicted amino acid sequence shares 99% identity with that from *S. mutans* (3) and *C. coli* (8) and 98% identity with that from *C. jejuni* (11), indicating a recent common origin for all of the genes.

Our data show that the *tet*(O) gene originally found in *Campylobacter* species also confers tetracycline resistance in the pneumococcus. Further studies are required to determine whether this resistance determinant occurs in strains outside South Africa.

EMBL nucleotide sequence database accession number. The sequence determined here was deposited in the EMBL database with the number Y07780.

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