

## High Prevalence of Inducible Erythromycin Resistance among *Streptococcus bovis* Isolates in Taiwan

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Susceptibilities to 13 antimicrobial agents were determined by measurement of MICs for 60 isolates of *Streptococcus bovis* from blood cultures. Thirty-eight isolates (63.3%) had high-level resistance to erythromycin (MICs,  $\geq 128$   $\mu\text{g/ml}$ ). Among the 38 erythromycin-resistant strains, 21 isolates (55%) had inducible resistance to macrolides-lincosamides-streptogramin B (iMLS isolates) and 17 (45%) had constitutive resistance to macrolides-lincosamides-streptogramin B (cMLS isolates). Tetracycline resistance was also found among all of the erythromycin-resistant strains. None of the strains displayed resistance to penicillin, chloramphenicol, or vancomycin. Detection of erythromycin resistance genes by PCR and sequencing indicated that all 17 cMLS isolates were positive for the *ermB* gene and that 7 of 21 iMLS isolates carried the *ermB* gene and the remaining 14 iMLS isolates carried the *ermT* gene. Sequence analysis of amplified partial *ermB* fragments (594 bp) from *S. bovis* isolates revealed a 99.8% nucleotide identity and a 100% amino acid homology compared with the sequences from gene banks. The sequences of amplified fragments with primers targeted for *ermC* were shown to be very similar to that of *ermGT* (*ermT*) from *Lactobacillus reuteri* (98.5% nucleotide identity). This is the first report to describe the detection of the *ermT* class of erythromycin resistance determinants in *S. bovis*. The high rate of inducible erythromycin resistance among *S. bovis* isolates in Taiwan was not reported before. The iMLS *S. bovis* isolates were shown to be heterogeneous by randomly amplified polymorphic DNA analysis. These results indicate that the prevalence of inducible erythromycin resistance in *S. bovis* in Taiwan is very high and that most of the resistant strains carry the *ermT* or the *ermB* gene.

*Streptococcus bovis* is a group D streptococcus frequently found as part of the commensal bowel flora in humans and animals. This organism is recognized as a cause of endocarditis in elderly people and an uncommon cause of septicemia and meningitis in newborn infants (2, 10, 19). Bacteremia due to *S. bovis* has been reported to be associated mostly with underlying colonic neoplasms and to a lesser extent with gastrointestinal tract and oropharyngeal carcinoma (2). Clinical isolates of *S. bovis* are usually susceptible to penicillin. However, macrolides and related drugs have been suggested as alternatives for treatment of streptococcal infections when the patient is allergic to penicillin.

High rates of erythromycin resistance have been recognized among streptococci in Taiwan since the mid-1990s (3, 12, 34, 35). Recent studies found erythromycin resistance in 23.5 to 81.3% of streptococci in Taiwan, depending on the species (12, 34, 35). Two major mechanisms account for erythromycin resistance in many gram-positive bacteria: target site modification and active efflux (16, 26, 30). Target site modification, generally known as macrolide-lincosamide-streptogramin B (MLS) resistance, is mediated by Erm methylases, which methylate 23S rRNA and induce ribosome modification. Expression of MLS resistance in streptococci can be either constitutive or inducible (11, 16, 27). Macrolide efflux, which is effected by a membrane protein encoded by the *mefA* or the *mefE* gene, has

been reported in group A streptococci, *Streptococcus pneumoniae*, and other streptococci (1, 4, 22, 32).

The objectives of the present study were to determine the incidence and patterns of antimicrobial resistance among *S. bovis* isolates that cause significant infections and to classify the phenotypes and genotypes of erythromycin-resistant strains.

### MATERIALS AND METHODS

**Bacterial strains.** A total of 60 nonredundant isolates of *S. bovis* isolated from cultures of blood from distinct patients between 1996 and 2000 were collected from the Bacteriology Laboratory, National Taiwan University Hospital, a 2,000-bed teaching hospital in northern Taiwan. The *S. bovis* isolates were identified to the species level by conventional methods as well as with a commercial identification system, the API 20 Strep system (bioMérieux Vitek, Inc., Hazelwood, Mo.). The *S. bovis* isolates were differentiated from enterococci and other viridans group streptococci by growth on bile esculin medium and at 45°C but not in 6.5% NaCl or at 10°C and by urease negativity (6). With the API system, the *S. bovis* isolates were further identified as biotype I, II/1, or II/2 (5, 6).

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing of the isolates was performed by a standard agar dilution method according to the guidelines established by the National Committee for Clinical Laboratory Standards (20). The isolates were grown on Trypticase soy agar plates supplemented with 5% sheep blood at 37°C. Bacterial inocula were prepared by suspending the grown bacteria in normal saline and adjusting the suspension to a 0.5 McFarland standard. By using a Steers replicator, an organism density of  $10^4$  CFU/spot was inoculated onto Mueller-Hinton agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood with various concentrations of antimicrobial agents, and the plate was then incubated aerobically at 35°C for 24 h.

The following antimicrobial agents were obtained as standard reference powders of known potency for laboratory use: penicillin, erythromycin, clindamycin, tetracycline, chloramphenicol, gentamicin, and vancomycin, from Sigma Chemical Co. (St. Louis, Mo.); cefotaxime, from Hoechst AG (Frankfurt, Germany); imipenem, from Merck Sharp & Dohme (West Point, Pa.); clarithromycin, from Abbott Laboratories (North Chicago, Ill.); quinupristin-dalfopristin, from Rhone-Poulenc Rorer (Amstelveen, The Netherlands); ciprofloxacin, from

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TABLE 1. Susceptibilities of 60 clinical isolates of *S. bovis* to various antimicrobial agents

| Antimicrobial agent       | MIC ( $\mu\text{g/ml}$ )  |             |             | % Resistant                        |
|---------------------------|---------------------------|-------------|-------------|------------------------------------|
|                           | Range                     | 50%         | 90%         |                                    |
| Penicillin                | $\leq 0.03$ –0.12         | 0.06        | 0.12        | 0                                  |
| Imipenem                  | $\leq 0.03$ – $\leq 0.03$ | $\leq 0.03$ | $\leq 0.03$ | 0                                  |
| Cefotaxime                | $\leq 0.03$ –0.5          | $\leq 0.03$ | 0.25        | 0                                  |
| Erythromycin              | 0.03–>512                 | >512        | >512        | 63.3                               |
| Clarithromycin            | $\leq 0.03$ –>512         | 256         | 512         | 63.3                               |
| Clindamycin               | 0.03–256                  | 0.06        | 256         | 28.3, <sup>a</sup> 35 <sup>b</sup> |
| Quinupristin-dalfopristin | 1–16                      | 4           | 8           | NA <sup>c</sup>                    |
| Tetracycline              | 0.5–64                    | 64          | 64          | 75                                 |
| Chloramphenicol           | 1–2                       | 2           | 2           | 0                                  |
| Gentamicin                | 1–32                      | 2           | 4           | NA                                 |
| Ciprofloxacin             | 1–4                       | 2           | 4           | NA                                 |
| Vancomycin                | 0.25                      | 0.25        | 0.25        | 0                                  |
| Teicoplanin               | 0.06–0.5                  | 0.25        | 0.5         | 0                                  |

<sup>a</sup> For cMLS isolates.

<sup>b</sup> For iMLS isolates.

<sup>c</sup> NA, no interpretive NCCLS breakpoints are available.

Bayer Corporation (West Haven, Conn.); and teicoplanin, from Marion Merrill Dow (Kansas City, Mo.). *Staphylococcus aureus* ATCC 29213 and *S. pneumoniae* ATCC 49619 were used as control organisms in each batch.

**Double-disk test.** The conventional double-disk induction test was performed to test the erythromycin resistance phenotypes (28). Erythromycin resistance was classified on the basis of the double-disk test with erythromycin and clindamycin. The disks were placed 15 to 20 mm apart on Mueller-Hinton agar (Becton Dickinson Microbiology Systems) supplemented with 5% sheep blood, and the plates were incubated aerobically at 35°C for 18 h. Blunting of the clindamycin inhibition zone proximal to the erythromycin disk indicated an inducible type of MLS resistance (iMLS), and resistance to both erythromycin and clindamycin indicated a constitutive type of resistance (cMLS). Susceptibility to clindamycin with no blunting indicated the M phenotype (30).

**Detection of erythromycin resistance genes.** DNA samples from *S. bovis* isolates were prepared with a DNA isolation kit (Puregene; Gentra Systems, Inc., Minneapolis, Minn.), according to the manufacturer's instructions. The DNAs of the erythromycin-resistant isolates were amplified with primers specific for the *ermA*, *ermB*, *ermC*, *ermTR*, and *mef* genes (29, 31). Amplification reactions were performed in volumes of 50  $\mu\text{l}$  containing 1 $\times$  PCR buffer, each deoxynucleoside triphosphate at a concentration of 0.2  $\mu\text{M}$ , 2 mM  $\text{MgCl}_2$ , 1 pmol of each primer, and 1 U of *Taq* polymerase. PCR products were resolved by electrophoresis on 1.5% agarose gels. The expected amplicons were 640 bp for *ermA*, *ermB*, and *ermC*; 530 bp for *ermTR*; and 348 bp for *mef*. Another pair of primers that targeted *ermC* was also used (17). The tetracycline resistance genes of *tet(M)* and the *int* gene were also examined by PCR (9, 21).

**RAPD analysis.** The preparation of the isolates for randomly amplified polymorphic DNA (RAPD) analysis, the DNA for which was generated by arbitrarily primed PCR, was performed as described previously (13). The following two arbitrary oligonucleotide primers were used: primer OP-H2 (5'-TCGGACGTG A-3') and primer OP-H7 (5'-CTGCATCGTG-3') (Operon Technologies, Inc., Alameda, Calif.). The amplification products were electrophoresed in a 1.2% agarose gel. Isolates which differed by two or more major bands were considered to have different patterns.

## RESULTS

**Biotype, antimicrobial susceptibility patterns, and prevalence of erythromycin resistance.** With the API 20 Strep system, the *S. bovis* isolates were divided into three subtypes. Among the 60 isolates tested, 4 biotype I, 3 biotype II/1, and 53 biotype II/2 isolates were identified. The four biotype I isolates and the three biotype II/1 isolates were all susceptible to erythromycin. The results of testing of the susceptibilities to 13 antibiotics obtained by the agar dilution method are shown in Table 1. The *S. bovis* isolates studied were all susceptible to penicillin, cefotaxime, imipenem, chloramphenicol, vancomycin, and teicoplanin. However, of the 60 *S. bovis* isolates tested,

38 (63%) isolates were highly resistant to two macrolides tested, erythromycin and clarithromycin. The MIC ranges, the MICs at which 50% of isolates are inhibited ( $\text{MIC}_{50}$ s), and the  $\text{MIC}_{90}$ s of these two macrolides were similar. The  $\text{MIC}_{50}$ s of erythromycin and clarithromycin were >512 and 256  $\mu\text{g/ml}$ , respectively, and the  $\text{MIC}_{90}$ s of erythromycin and clarithromycin were  $\geq 512$   $\mu\text{g/ml}$ . The  $\text{MIC}_{50}$  of clindamycin was 0.06  $\mu\text{g/ml}$ , which was much lower than those of erythromycin and clarithromycin. The activity of quinupristin-dalfopristin was not high, with an  $\text{MIC}_{50}$  of 4  $\mu\text{g/ml}$  and an  $\text{MIC}_{90}$  of 8  $\mu\text{g/ml}$ . The rate of tetracycline resistance was 75%.

**Resistance phenotypes.** Since the incidence of erythromycin resistance was high in the *S. bovis* isolates tested, the double-disk test was performed to determine the resistance phenotypes. Two phenotypes were observed among the 38 erythromycin-resistant isolates. Twenty-one strains (55%) presented an inducible phenotype (iMLS isolates) with typical blunting of the clindamycin zone of inhibition, and 17 (45%) exhibited the constitutive phenotype (cMLS isolates). None of the strains displayed the M phenotype.

**Erythromycin resistance determinants.** To understand the mechanism of erythromycin resistance, macrolide resistance genes were detected by PCR and sequencing. Amplification with the primers specific for *ermB* revealed that 17 cMLS isolates and 7 iMLS isolates contained this gene. Sequence analysis of amplified partial *ermB* fragments (594 bp, not including the primers) from four randomly selected resistant isolates revealed 99.8% identity (at the DNA level) and 100% amino acid homology with published sequences of ErmB from *Streptococcus* or *Enterococcus*. None of the isolates contained the *ermA*, *ermC*, *ermTR*, or *mef* gene. However, amplification with one pair of primers that initially targeted *ermC* (17) was positive for 14 of 21 iMLS isolates. These amplification products were subsequently sequenced but were found to be more similar to *ermGT* from *Lactobacillus reuteri* (98.5% of nucleotides identical) than to *ermC* (77.5% identity).

Since all the erythromycin-resistant isolates were also resistant to tetracycline, tests for the detection of *tet(M)* and *int* genes were also performed. The results revealed that all the erythromycin-resistant isolates contained *tet(M)* and *int* genes.

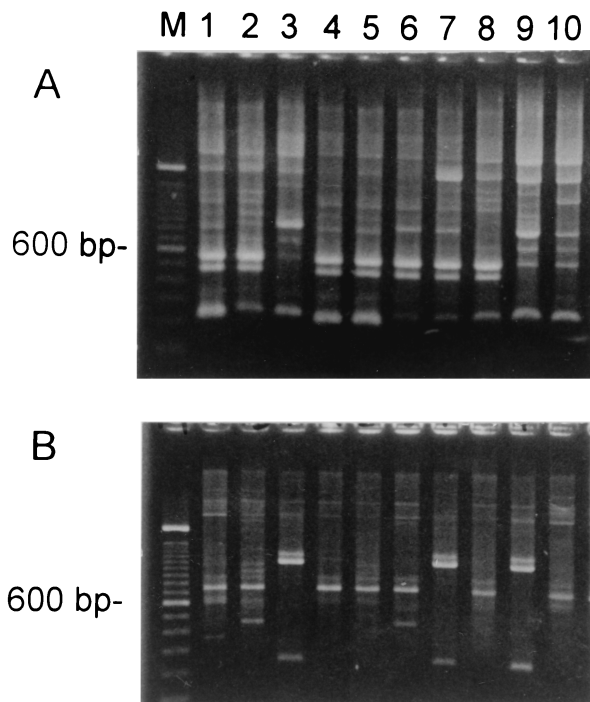


FIG. 1. RAPD patterns of *S. bovis* isolates obtained with two primers. Lane M, molecular size marker (100-bp ladder; Gibco BRL, Gaithersburg, Md.). (A) Primer OP-H2; (B) primer OP-H7. The isolates in lanes 4 and 5 were considered to have the same RAPD patterns. The isolates in the other lanes were considered different.

**RAPD patterns.** To understand whether the high prevalence of inducible erythromycin resistance of *S. bovis* was due to clonal spread, RAPD analysis was performed with 21 iMLS isolates. The results are partially shown in Fig. 1. The isolates in lanes 4 and 5 were considered to have the same RAPD patterns. The isolates in the other lanes were considered different. Eighteen RAPD patterns were identified with two different primers. These results indicate that these isolates correspond to a heterogeneous population rather than the expansion of a single clone.

## DISCUSSION

Introduction of erythromycin into clinical practice was rapidly followed by resistance to MLS antibiotics. A high incidence of erythromycin-resistant streptococci in Taiwan has been recognized since the mid-1990s. In the present study, the incidence of resistance to erythromycin among the *S. bovis* isolates tested was also very high. Only 36.7% of isolates were fully erythromycin susceptible, and the remaining isolates (63.3%) had high-level resistance to erythromycin. The rate of resistance to erythromycin among *S. bovis* isolates in Taiwan was similar to those for other streptococci. Therefore, it is of concern that *S. bovis*, like other streptococci, exhibits a high level of resistance to macrolides, making them of limited use for empirical therapy. *S. bovis* isolates were found to be more resistant to erythromycin and clindamycin than other streptococcal species in Denmark (25). The susceptibility to quinupristin-dalfopristin was also tested in the present study. For clinical use, quinupristin-dalfopristin may be an alternative in patients with endocarditis due to streptococci. However, the

MICs of quinupristin-dalfopristin for the *S. bovis* isolates were shown to be high. A study of antimicrobial susceptibility among viridans group streptococci in The Netherlands in 1997 revealed that the activity of quinupristin-dalfopristin is somewhat dependent on the species, with *S. bovis* being the least susceptible (18). Although vancomycin-resistant *S. bovis* isolates have been reported (23), our results indicated that all isolates remained susceptible to vancomycin and teicoplanin.

In the present study, the prevalence of the iMLS phenotype among *S. bovis* isolates was much higher than that among other streptococci in Taiwan. The iMLS phenotype was not common in other streptococcal species in Taiwan. For example, the incidence of the iMLS phenotype was only 3.1% among *Streptococcus pyogenes* isolates (36). The incidence of iMLS phenotypes in other streptococci was also very low (22). Our *S. bovis* isolates were predominantly biotype II/2. This is in agreement with a recent report from Clarridge et al. (5). Among our 60 isolates, only 4 biotype I isolates were recovered, and these 4 biotype I isolates were all susceptible to erythromycin. Since all 38 erythromycin-resistant isolates were biotype II/2, more data are needed to obtain a final conclusion as to whether isolates of biotype II/2 are more resistant. The RAPD patterns of isolates with the iMLS phenotype suggest that these isolates correspond to a heterogeneous population rather than the expansion of a single clone. Since the RAPD analysis results were preliminary, pulsed-field gel electrophoresis might be further performed for epidemiological study.

This is the first report characterizing the *ermT* class of erythromycin resistance determinants in *S. bovis*. The *ermA*, *ermC*, *ermTR*, and *mef* genes were not detected in any of our *S. bovis* isolates. Detection of erythromycin resistance genes showed that target site modification mediated by the *ermB* or *ermT* gene was the most common mechanism responsible for erythromycin resistance in *S. bovis* isolates in Taiwan. Although the PCR result obtained with one pair of *ermC*-specific primers was positive, the sequence data revealed that the sequence of the amplification product was more similar to that of *ermT*. The nucleotides of the amplified fragment from *S. bovis* obtained with primers based on the *ermC* sequence were shown to be 98.5% identical to those of *ermGT* from *L. reuteri* (33). Recently, Roberts et al. (26) suggested a new nomenclature for naming of the MLS genes and proposed the use of a single letter rather than the two-letter designations presently used in the literature. The *ermT* includes *ermGT*, and *ermA* includes *ermTR*. Differences in the prevalence of *erm* gene classes in streptococci have been reported. The *ermTR* (*ermA*) gene has been found to be predominant among iMLS group A streptococcus isolates in Finland (100%) (14) and Canada (100%) (8). The *ermTR* gene has also been found in group A streptococcus isolates in Taiwan, although there were only four iMLS isolates (36). This finding indicates that the distribution of resistance genes varies among species.

Since the erythromycin-resistant isolates were also revealed to be resistant to tetracycline, the isolates were tested for the presence of the *tet(M)* and *int* genes by PCR. The association of *tet(M)* with the integrase gene was found in all but one of the tetracycline-resistant *S. bovis* strains. Many of the *erm* genes are associated with conjugative or nonconjugative transposons. Several *tet(M)*-containing conjugative transposons have been described in streptococci (including *S. bovis*), en-

terococci, and staphylococci (7, 21). It has been reported that Tn916 and Tn1545 are associated with resistance to tetracycline as well as resistance to other drugs (9). In the present study, *tet(M)* and the integrase gene were detected in most *ermB*- or *ermT*-containing strains. However, more work will be necessary to determine the nature of the resistance element, which possibly contains a transposon, on which the integrase gene is located.

High rates of erythromycin resistance among streptococci and other organisms in Taiwan have been reported previously (3, 34, 35). Because *S. bovis* isolates are part of the normal flora of humans, they are challenged by every antibiotic treatment, and selection of highly resistant strains may occur. Although *S. bovis* displayed some phenotypic resemblance to other viridans group streptococci (6), the phylogeny determined by analysis of several genes showed that *S. bovis* has a unique trait among streptococcal species (15, 24). The presence of *ermB* and *ermT* in *S. bovis* suggests that interactions between streptococci and other organisms might exist. Although erythromycin is not widely used for the treatment of *S. bovis* infections, the significance of the high prevalence of macrolide resistance in *S. bovis* isolates cannot be ignored.

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