High-Level Gentamicin Resistance Mediated by a Tn4001-Like Transposon in Seven Nonclonal Hospital Isolates of *Streptococcus pasteurianus*  

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We report on the first occurrence of high-level gentamicin resistance (MICs ≥ 512 μg/ml) in seven clinical isolates of *Streptococcus pasteurianus* from Hong Kong. These seven isolates were confirmed to be the species *S. pasteurianus* on the basis of nucleotide sequencing of the superoxide dismutase (*sodA*) gene. Epidemiological data as well as the results of pulse-field gel electrophoresis analysis suggested that the seven *S. pasteurianus* isolates did not belong to the same clone. Molecular characterization showed that they carried a chromosomal, transposon-borne resistance gene [aac(6’)/Ie-aph(2’)/Ia] which was known to encode a bifunctional aminoglycoside-modifying enzyme. The genetic arrangement of this transposon was similar to that of Tn4001, a transposon previously recovered from *Staphylococcus aureus* and other gram-positive isolates. Genetic linkage with other resistance elements, such as the *ermB* gene for erythromycin resistance, was not evident. On the basis of these findings, we suggest that routine screening for high-level gentamicin resistance should be recommended for all clinically significant blood culture isolates. This is to avoid the inadvertent use of short-course combination therapy with penicillin and gentamicin, which may lead to the failure of treatment for endocarditis, the selection of drug-resistant *Streptococcus pasteurianus* and other gram-positive organisms, as well as the unnecessary usage of gentamicin, a drug with potential toxicity.

*Streptococcus bovis* is an important cause of bacteremia and endocarditis (21, 22, 26, 27, 31, 33). The association between *S. bovis* bacteremia and malignancy of the colon is well recognized, exceeding 50% in some studies (2, 21, 22, 27, 31). An association between *S. bovis* bacteremia and chronic liver disease has also been noted (12, 38, 44). Recently, this pathogen has increasingly been recognized as an important cause of infective endocarditis, particularly among the elderly. In a recent survey of infective endocarditis cases in France, *S. bovis* accounted for 25% of the 390 cases from whom isolates were collected, while viridans group streptococci were responsible for only 17% of the cases (15). *S. bovis* bacteremia has been associated with higher rates of mortality and cardiac surgery compared with the rates of association for viridans group streptococci (23). The taxonomy of this organism has recently been revised on the basis of DNA homology studies, whole-cell protein analysis, and nucleotide sequencing of the superoxide dismutase (*sodA*) gene. *Streptococcus gallolyticus*, *Streptococcus infantarius*, and *Streptococcus pasteurianus* have been proposed as replacements for *S. bovis* biotype I, *S. bovis* biotype II/1, and *S. bovis* biotype II/2, respectively (9).

Optimal therapy for streptococcal endocarditis requires a combination of penicillin and an aminoglycoside for a synergistic effect (41, 43). The presence of a high-level aminoglycoside resistance phenotype results in the loss of this synergistic effect both in vitro and in experimental animal models (8, 10, 11). High-level resistance to gentamicin in enterococci and streptococci is usually due to the presence of the [aac(6’)/Ie-aph(2’)/Ia] gene, which encodes a bifunctional aminoglycoside-inactivating enzyme with 6’-acetyltransferase and 2’-phosphotransferase activities. The gentamicin resistance determinant is usually plasmid borne in most strains of *Enterococcus faecalis* (17, 29) and *Enterococcus faecium* (42); in addition, it is often found on transposable elements that are structurally related to transposon Tn4001, which was originally recovered from *Staphylococcus aureus* (25). Chromosomally mediated high-level gentamicin resistance (HLGR) has also been reported in several *E. faecalis* strains (32, 37), as well as in one group B streptococcus strain (3, 16) and several *Streptococcus mitis* strains (20).

Although penicillin resistance in *S. bovis* has not yet been reported, isolates resistant to tetracycline and erythromycin are increasingly recognized (24). As for aminoglycoside resistance, high-level resistance to streptomycin in *Streptococcus bovis* has been described (6, 18). However, high-level resistance to gentamicin, the aminoglycoside most commonly used for the treatment of endocarditis nowadays, has not been reported. In the literature, gentamicin MICs ranged from 1 to 32 μg/ml in clinical isolates of *S. bovis* recovered in various clinical settings (10, 30, 36, 40). According to the current CLSI (formerly NCCLS) recommendation, routine screening for HLGR is not necessary for *Streptococcus bovis* (28). In our laboratory, an isolate of *Streptococcus bovis* with HLGR was identified in 1995 upon screening with a 120-μg
genes, which code for various aminoglycoside phosphotransferases, by using the
for the presence of other resistance genes, such as the
quences of all primers and their relative positions and orientations within Tn
. The nucleotide se-
]H9262
g gentamicin disk (Oxoid, Basingstoke,
mined for all
streptomycin, kanamycin, amikacin, minocycline, and netilmicin) were deter-
ccefotaxime, vancomycin, tetracycline, erythromycin, clindamycin, gentamicin,
pital in Hong Kong. The
were further confirmed by nucleotide sequencing of their superoxide dismutase
were found to exhibit HLGR in subsequent antimicrobial susceptibility tests
the isolates as biotype I, II/1, or II/2 (5, 34). The identities of seven isolates which
not in 6.5% NaCl. Their identities were confirmed by using the API 32 Strept
viridans group streptococci by growth on bile-esculin medium and at 45°C but
Czech Republic, May 2004 [V. C. Y. Chow, R. C. Y. Chan,
of Clinical Microbiology and Infectious Diseases, Prague,
12-year period.
prompted us to undertake the present study to assess the preva-
S. pasteurianus
S. bovis
S. PASTEURIANUS
5384
be indicative of the presence of Tn
ermB
ClaI digest of chromosomal DNA with the
PCR amplification of
ermB
5384
in the seven HLGR isolates were in fact part of a larger transposon, Tn
1A). This probe was also used to delineate whether the
within each of the two flanking IS
digest obtained with the restriction enzyme HindIII, which is known to cut once
prepared by PCR amplification with primers 256F and 256R and that covered
element, a 1,102-bp PCR product that was
hybridization experiments was the 469-bp PCR product of primers A62M1F and
was used in the hybridization experiments.
be hybridized by the probe. To probe the possible linkage between the genta-
ting onto a nylon membrane (Amersham) was performed as described by Sam-
purified DNA was digested with the endonuclease HincII or HindIII, and blot-
according to the protocol of Duck et al. (7). Genomic DNA from both gentamicin-
sensitive and -resistant isolates was fragmented with the endonuclease SmaI or
Pulsed-field gel electrophoresis (PFGE) was performed ac-
Southern blotting and DNA hybridization experiments. Total cellular DNA from
each isolate was purified as described previously (13). To determine
whether the auc(6)Ie-aph(2)Ia gene is located in transposon Tn4001, 3 μg
of purified DNA was digested with the endonuclease HindIII or HindIII, and blot-
ting onto a nylon membrane (Amersham) was performed as described by Samb-
brook et al. (35). These two enzymes were chosen because each cuts once in such a
position within the auc(6)Ie-aph(2)Ia gene that one digested fragment could
be hybridized by the probe. To probe the possible linkage between the genta-
micin and erythromycin resistance determinants, a ClaI digest of genomic DNA
was used in the hybridization experiments.
PFGE analysis. Pulsed-field gel electrophoresis (PFGE) was performed ac-
counting with the HindIII and HincII digests of genomic DNA, this probe was
A62M2R, which is an internal fragment of the bifunctional gene. Upon hybrid-
was used to probe the possible linkage between the genta-
micin and erythromycin resistance determinants, a ClaI digest of genomic DNA
was used in the hybridization experiments.
To probe the use for the detection of the auc(6)Ie-aph(2)Ia gene in the hybridization experiments was the 409-bp PCR product of primers A62M1F and
A62M2R, which is an internal fragment of the bifunctional gene. Upon hybrid-
ization with the HindIII and HindII digests of genomic DNA, this probe was expected to produce one labeled band of 2.5 kbp and 2.2 kbp, respectively, if the
auc(6)Ie-aph(2)Ia gene is located in transposon Tn4001; 3 μg of
be hybridized by the probe. To probe the possible linkage between the genta-
micin and erythromycin resistance determinants, a ClaI digest of genomic DNA
was used in the hybridization experiments.

### MATERIALS AND METHODS

**Strain selection.** A total of 57 nonduplicate isolates of *Streptococcus bovis*
recovered from blood cultures between 1993 and 2005 were studied in the
Microbiology Laboratory, Prince of Wales Hospital, a 1,400-bed teaching hos-
pital in Hong Kong. The *S. bovis* isolates were initially identified to the species
level by conventional methods, being differentiated from enterococci and other
viridans group streptococci by growth on bile-esculin medium and at 45°C but
not in 6.5% NaCl. Their identities were confirmed by using the API 32 Strept
system (bioMerieux Inc., Hazelwood, MO), which allowed further delineation of the
isolates as biotype I, II/1, or II/2 (5, 34). The identities of seven isolates which
were found to exhibit HLGR in subsequent antimicrobial susceptibility tests
were further confirmed by nucleotide sequencing of their superoxide dismutase
(sodA) genes (9).

**Antimicrobial susceptibility tests.** The MICs of 12 antibiotics (penicillin,
cefotaxime, vancomycin, tetracycline, erythromycin, clindamycin, gentamicin,
streptomycin, kanamycin, amikacin, minocycline, and neomycin) were deter-
d for all *S. bovis*/S. pasteurianus isolates by the agar dilution method. A
standard disk diffusion test with a 120-μg gentamicin disk (Oxoid, Basingstoke,
United Kingdom) was performed for the screening and detection of HLRG
among the clinical isolates recovered from blood cultures. Both tests were
performed according to the CLSI guidelines (28).

**PCR and nucleotide sequencing.** PCR primers were designed to detect the presence of the auc(6)Ie-aph(2)Ia gene and the IS256 insertion elements in both gentamicin-sensitive and -resistant isolates and to determine their relative positions and orientations compared to those in Tn4001. The nucleotide se-
quencies of all primers and their relative positions and orientations within Tn4001 are listed in Table 1 and Fig. 1A, respectively. The isolates were also investigated for the presence of other resistance genes, such as the aph(2)Ib, -Ic, and -Id genes, which code for various aminoglycoside phosphotransferases, by using the

### Table 1. PCR primers used to detect determinants of gentamicin and erythromycin resistance and analyze their genetic organization in *Streptococcus pasteurianus*

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence</th>
<th>PCR product size (bp)</th>
<th>GenBank nucleotide sequences used in primer design</th>
</tr>
</thead>
<tbody>
<tr>
<td>256RB</td>
<td>5′-GCC GTT CTT ATG GAC CTA CAT-3′</td>
<td>625</td>
<td>M18086</td>
</tr>
<tr>
<td>A62FB</td>
<td>5′-CCA CAA TAA ATT TCT AAT AC-3′</td>
<td>368</td>
<td>M18086</td>
</tr>
<tr>
<td>256RB</td>
<td>5′-GCC GTT CTT ATG GAC CTA CAT-3′</td>
<td>1102</td>
<td>M18086</td>
</tr>
<tr>
<td>256R</td>
<td>5′-GCC GTT CTT ATG GAC CTA CAT-3′</td>
<td>1201</td>
<td>M18086</td>
</tr>
<tr>
<td>A62F</td>
<td>5′-CCA CTA ATT CTT AAT ATA TC-3′</td>
<td>469</td>
<td>M18086</td>
</tr>
<tr>
<td>A62M1F</td>
<td>5′-GCC AGA ACA TGA ATT ACA CCA G-3′</td>
<td>905</td>
<td>AF207840</td>
</tr>
<tr>
<td>A62M2R</td>
<td>5′-CGT TAT AAT CTA AAC CGT GCA-3′</td>
<td>880</td>
<td>U51479</td>
</tr>
<tr>
<td>A2bF</td>
<td>5′-ATG GTC AAC TTG GCT GCT GAG-3′</td>
<td>845</td>
<td>AF016483</td>
</tr>
<tr>
<td>A2bR</td>
<td>5′-TTC CTG CTA AAA TAT AAA CAT CTC TGC T-3′</td>
<td>639</td>
<td>AF242872</td>
</tr>
<tr>
<td>A2cF</td>
<td>5′-TGA CTC AGT TCC CAG AT-3′</td>
<td>368</td>
<td>U51479</td>
</tr>
<tr>
<td>A2dR</td>
<td>5′-AGC ACT GTC TGC CAG ACC AAA-3′</td>
<td>880</td>
<td>U51479</td>
</tr>
<tr>
<td>A2dF</td>
<td>5′-GCC GAG TGA GAA AAG GCA ATA GAT CAG-3′</td>
<td>845</td>
<td>AF016483</td>
</tr>
<tr>
<td>A2dR</td>
<td>5′-ATA CCA ATC TAT ACAA ATC TCC-3′</td>
<td>639</td>
<td>AF242872</td>
</tr>
<tr>
<td>ermBF</td>
<td>5′-GAA AAA GTA CTC AAG CAA ATA-3′</td>
<td>368</td>
<td>AF242872</td>
</tr>
<tr>
<td>ermBR</td>
<td>5′-AGT AAT GGT ACT TAA ATT GTT TAC-3′</td>
<td>905</td>
<td>AF207840</td>
</tr>
</tbody>
</table>

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3.2-kbp fragment if the ermB gene is located in transposon Tn5384 (1). Upon heat denaturation, the probes were labeled with digoxigenin (DIG)-11-dUTP by random priming (DIG DNA labeling kit; Roche GmbH, Penzberg, Germany). All hybridization experiments were performed by using the DIG nucleic acid detection kit (Roche GmbH). The enzyme substrate nitroblue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate allowed visualization of the labeled DNA banding patterns.

RESULTS AND DISCUSSION

Epidemiological analysis and antibiotic susceptibility tests. Antibiotic susceptibility tests revealed that all 57 S. bovis isolates tested were sensitive to penicillin. Seven of the 57 (12%) isolates, which were recovered from six patients, were found to exhibit an HLGR phenotype, with MICs ranging from 512 to 4,096 µg/ml. These seven isolates were all found to belong to the species S. pasteurianus, based on their sodA sequences. Nucleotide sequencing of the sodA gene was not performed with the other 50 isolates, to which we continue to refer as S. bovis. Apart from being gentamicin resistant, the seven isolates were also found to be highly resistant to kanamycin and erythromycin. In addition, all isolates except one were also resistant to tetracycline. Table 2 summarizes the susceptibilities of these seven isolates to a total of 10 antibiotics. Six of the seven HLGR isolates were recovered in 2001 and 2002. These six isolates represented 44% and 29% of the isolates collected in each of the 2 years respectively; the remaining isolate was recovered in 1995. The six patients from whom the seven HLGR isolates were recovered were all aged over 65 years. Five of the patients had underlying diseases such as diabetes mellitus, chronic renal failure, or carcinoma. Among the patients, two were diagnosed with acute cholangitis, one was diagnosed with infective endocarditis, and the other three patients were diagnosed with either secondary peritonitis or primary bacteremia. In two of the patients, Escherichia coli and/or Klebsiella spp. were also recovered from their blood cultures. The dates of isolation for each of the seven HLGR isolates also differed from each other.

PFGE analysis. In order to examine whether the seven HLGR isolates were genetically related to each other, we performed PFGE analysis with these isolates, with the results suggesting that they fell into different pulstype groups. Five of the seven isolates (isolates SB10, SB28, SB30, SB33, SB34, SB40, and SB41) differed from each other by at least three bands when they were tested with each of the two restriction enzymes used in the study (Fig. 2). The other two isolates (isolates SB28 and SB30), which were recovered from the same patient, who had two episodes of infection separated by a 2-month period, had PFGE patterns that differed from each other by less than three bands in assays with each of the two enzymes used for PFGE;
in addition, such patterns were significantly different from those of the other five isolates. The PFGE profiles of the gentamicin-sensitive isolates also revealed a diversity of patterns which also differed significantly from those of the HLGR strains (results not shown). The PFGE results and epidemiological data suggested little clonal relationship among the seven *S. pasteurianus* isolates.

**Detection of resistance genes by PCR.** PCR was performed to detect the *aac(6′)Ie-aph(2′)Ia* gene, which was previously implicated as being responsible for HLGR in *Staphylococcus* and *Enterococcus* species. A primer pair targeting an internal region of the 1,201 nucleotides of the entire *aac(6′)Ie-aph(2′)Ia* gene was used for PCR amplification (Table 1). All seven HLGR *S. pasteurianus* isolates were found to possess the entire resistance gene, as confirmed by nucleotide sequencing of the PCR products. This gene, however, was not detected in any of the gentamicin-sensitive *S. bovis* isolates. Our PCR results also showed that the seven HLGR isolates did not possess other gentamicin resistance genes, such as *aph(2′)Ib, -Ic, and -Id*, which have been reported to confer gentamicin resistance in enterococci (4, 19, 39). On the other hand, all seven HLGR *S. pasteurianus* isolates were also found to harbor the *ermB* gene, the determinant for erythromycin resistance.

**Analysis of genetic arrangement of resistance determinants by PCR and DNA hybridization.** The genetic arrangement of the *aac(6′)Ie-aph(2′)Ia* gene and its possible linkage with transposon Tn4001 were examined by further PCR studies with primer pairs targeting two separate regions spanning the IS256 insertion sequence that contained the transposase gene at one end and either the 5′-terminal or the 3′-terminal region of the *aac(6′)Ie-aph(2′)Ia* gene at the other end (Fig. 1A). The results of PCR assays and subsequent nucleotide sequencing of the PCR products confirmed that in each of the seven HLGR isolates the *aac(6′)Ie-aph(2′)Ia* gene was inversely flanked by the IS256 gene at both the 5′ and the 3′ ends, in the same manner as previously described for Tn4001 (14). In order to determine the genetic location of this transposon, attempts were made to extract plasmids as well as the chromosomal DNA of the resistant strains, followed by restriction enzyme digestion and hybridization studies, by using the PCR products containing partial sequences of the *aac(6′)Ie-aph(2′)Ia* gene or IS256 as probes. As plasmids could not be recovered from the seven resistant isolates, hybridization was performed only with restriction enzyme-digested total DNA, with the results confirming the likelihood that the resistance gene resided in the chromosomes of the resistant isolates and that the sizes and the banding patterns of the detected fragment were compatible with those expected for Tn4001.

Figure 1B shows the results of hybridization studies obtained with HincII and HindIII digests of total DNA of the HLGR strains and the A62M1F-A62M2R PCR product as the probe. While only a single-size band was revealed upon hybridization of the HincII digest, there were bands of two different sizes in the HindIII digest. As this phenomenon suggested the possibility that the 5′ end of Tn4001 in these HLGR isolates may exhibit some form of insertional element, we performed further PCR tests using different combination of primers (Table 1) in a crude attempt to map the region of sequence variation.
in Tn4001. Our results showed that the region between the HindIII site and part of the left-end IS256 fragment of Tn4001, in the vicinity of the 256RB binding site (Fig. 1A) of three HLGR isolates (isolates SB28, SB30, and SB40), was consistently not amplifiable with primers carrying the known IS256 sequences. As these three isolates also exhibited a larger-than-expected fragment upon hybridization of the HindIII digest (Fig. 1B), we hypothesized that such phenomena may be associated with the presence of insertional elements in the left-hand IS256 element of the Tn4001-like transposon of the three isolates mentioned above. Further investigation is required to assess whether such insertion events are associated with a new transposase gene with a possibly altered transposition function.

In addition to the possible genetic variations in the left-hand IS256 element, there were also size differences between the S. pasteurianus strains and that of the positive control strain, ATCC 49532, which may also have been due to the presence of insertional elements or sequence variations that led to alteration of the restriction sites and, hence, the sizes of the detected fragments.

**Examination of possible genetic linkage of gentamicin and erythromycin resistance determinants in HLGR isolates.** As shown in Table 2, the seven HLGR S. pasteurianus isolates were also highly resistant to erythromycin. This prompted us to investigate whether the Tn4001 found in the HLGR S. pasteurianus isolates was in fact part of a larger transposon, namely, Tn5384, which was first identified in *E. faecalis*. Bordering by two inverse repeats of the IS256 insertion elements that are 26 kbp apart, this transposon was found to harbor a Tn4001-like element as well as an erythromycin resistance determinant gene (*ermB*), thereby conferring resistance to both gentamicin and erythromycin in some *E. faecalis* isolates (33, 1). By using *ermB*-specific primers in the PCR, all seven HLGR isolates were found to contain the *ermB* gene (results not shown). Southern blotting and hybridization of Clal-digested total DNA from the HLGR isolates with a DIG-labeled *ermB* gene probe revealed the presence of a 2.5-kbp fragment but not the 3.2-kbp *ermB*-containing fragment, which would have been present in the case of Tn5384 (1). In addition, hybridization of the HindIII digest with the IS256 probe failed to reveal a fragment larger than 7 kbp, which was expected to be present in Tn5384. These results suggest that Tn5384 is not likely to be present in the HLGR isolates; hence, there is not enough evidence to prove that the resistance determinants for gentamicin and erythromycin resistance are genetically linked to each other in the seven *S. pasteurianus* isolates.

**Clinical implications.** This is the first report of HLGR in *S. pasteurianus*. The cluster of HLGR strains in 2001 and 2002 is interesting. The abrupt appearance of the majority of HLGR strains since 2001 could represent the effect of transfer of genes among strains and evolution. The isolates might have acquired the resistance determinant from different sources, possibly involving members of the food chain, through lateral genetic transfer mechanisms such as conjugation, transformation, or transduction. Although we have no data on the usage of gentamicin in 2001 and 2002, we postulated that an abnormal increase in the usage of gentamicin is one way that these drug-resistant isolates had a better chance of being selected in these 2 years.

HLGR in *S. pasteurianus* has significant clinical implications, as well as the American Heart Association and the British Society of Antimicrobial and Chemotherapy recommend a 2-week course of treatment with penicillin and gentamicin for uncomplicated infective endocarditis caused by penicillin-sensitive strains of viridans group streptococci, including *S. pasteurianus* (41, 43). According to the recommendations of the CLSI, routine testing for HLGR is not necessary, an omission that makes the recognition of resistance difficult (28). Infections caused by such strains, which are inadvertently treated with a short course of combination therapy with penicillin plus gentamicin, may have high relapse rates. In addition, the unnecessary use of gentamicin, a drug with a narrow therapeutic range that potentially causes nephrotoxicity and otoxicity, is undesirable.

In view of the data presented here, regular surveillance by antibiotic susceptibility testing, including testing for HLGR, in all clinically significant blood culture isolates of *S. pasteurianus* is warranted. Upon detection of a high prevalence of HLGR, like that which we experienced in our locality during the period of 2001 and 2002, routine screening of *S. pasteurianus* isolates for HLGR is recommended. Although further studies are needed to explore the potential of high-content gentamicin disk testing for the detection of HLGR in *S. pasteurianus*, our preliminary data indicate that high-content gentamicin disk testing may offer the laboratory a convenient and reliable test for the rapid screening of this category of resistant organisms.

**REFERENCES**


