PCR-RFLP assay for species and subspecies differentiation of the *Streptococcus bovis* group based on *groESL* sequences

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The sequence diversity of *groESL* genes among *Streptococcus bovis* group isolates was analysed, including five reference strains and 36 clinical isolates. Phylogenetic analysis of the *groES* and *groEL* sequences showed that the isolates that belonged to the same species or subspecies usually clustered together. The intergenic spacer region between *groES* and *groEL* was variable in size (67–342 bp) and sequence and appeared to be a unique marker for species or subspecies determination. Sequence similarities of the *groESL* genes among species and subspecies ranged from 84.2 to 99.0% in *groES*, and from 88.0 to 99.0% in *groEL*. Based on the sequences determined, a *Streptococcus bovis* group-specific PCR assay was developed, which may provide an alternative means of distinguishing the *bovis* group from other viridans streptococci. Restriction digestion of the amplicon with *AcI*I further differentiated the species and subspecies.

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INTRODUCTION

The '*Streptococcus bovis*' group is a large bacterial complex that includes various species and subspecies isolated from humans or animals. In humans, members of the group can cause bacteraemia and endocarditis in elderly people and may sometimes cause septicaemia and meningitis in newborns (Bochud *et al.*, 1994; Gerber *et al.*, 2006; Kupferwasser *et al.*, 1998; Tripodi *et al.*, 2004). The group is also known to be associated with underlying gastrointestinal malignancy (Ellmerich *et al.*, 2000). In previous

studies, the authors found that biotype I (*Streptococcus gallolyticus*) was highly associated with infective endocarditis and colonic cancer, whereas biotype II was associated with primary bacteraemia (Jean *et al.*, 2004; Ruoff *et al.*, 1989). However, the association of species or subspecies and clinical significance is still not clear and sometimes even controversial. To better understand the relationship between pathogens and disease, it is important to identify the species or subspecies correctly.

The taxonomy/nomenclature of members of the *Streptococcus bovis* group is still evolving. Farrow *et al.* (1984) identified six different DNA groups in the *Streptococcus bovis* group using total DNA–DNA hybridization assays. Other investigators further separated the *Streptococcus bovis* group into five species (*S. bovis*–*S. equinus*, *S. gallolyticus*, *S. infantarius*, *S. pasteurianus* and *S. lutetiensis*) (Facklam, 2002; Osawa *et al.*, 1995; Poyart *et al.*, 2002; Schlegel *et al.*, 2000). According to their biochemical characteristics, *Streptococcus bovis* strains isolated from humans were divided into three biotypes,

Abbreviation: ITS, intergenic spacer.

The GenBank/EMBL/DDBJ accession numbers for the *groESL* gene sequences reported in this study are given in Supplementary Table S1 in JMM Online.

Tables showing the GenBank/EMBL/DDBJ accession numbers for the *groES* and *groEL* gene sequences of strains used in this study and the nucleotide lengths and sequences of *groES*–*groEL* spacer regions are available as supplementary material with the online version of this paper.

biotype I (mannitol-positive), II/1 (mannitol-negative and β -glucuronidase-negative) and II/2 (mannitol-negative and β -glucuronidase-positive) (Clarridge *et al.*, 2001; Coykendall, 1989). The human isolates of the *Streptococcus bovis* group have been reclassified as *S. gallolyticus*, *S. infantarius* and *S. pasteurianus* for biotypes I, II/1 and II/2, respectively (Poyart *et al.*, 2002; Schlegel *et al.*, 2000). Schlegel *et al.* (2003) suggested that biotypes I and II/2 and *Streptococcus gallolyticus* represent a single species and proposed the name *Streptococcus gallolyticus* subsp. *pasteurianus* for *Streptococcus bovis* biotype II/2. *Streptococcus bovis* biotype II/1 was described as *Streptococcus infantarius*, with two subspecies, *Streptococcus infantarius* subsp. *infantarius* (starch hydrolysis-positive, aesculin hydrolysis-variable and β -glucosidase-variable) and *Streptococcus infantarius* subsp. *coli* (reclassified as *Streptococcus lutetiensis*) (starch hydrolysis-variable, aesculin hydrolysis-positive and β -glucosidase-positive) (Facklam, 2002; Schlegel *et al.*, 2000; Poyart *et al.*, 2002). Although these new names have been proposed, most clinical laboratories have not yet accepted the nomenclature. Currently, the identification of the *Streptococcus bovis* group in clinical laboratories depends mostly on conventional methods and/or rapid identification systems. Misidentification has been reported due to the diversity of the biochemical characteristics of members of the group (Gavin *et al.*, 2002). Molecular-based identification using the *sodA* gene, encoding manganese-dependent superoxide dismutase, has also been used recently for species identification and to distinguish the *Streptococcus bovis* group (Poyart *et al.*, 1998, 2002; Sasaki *et al.*, 2004). The sequence of the 16S rRNA gene has also been used for phylogenetic analysis (Schlegel *et al.*, 2000, 2003), but it is difficult to differentiate species or subspecies using PCR only.

The *groESL* genes (also known as *cpn10/60* or *hsp10/60*), which encode 10 kDa (GroES) and 60 kDa (GroEL) heat-shock proteins, are ubiquitous and evolutionarily highly conserved among bacteria (Hemmingsen *et al.*, 1988). Amplification of the partial *cpn60* (or *groEL*) gene segment has been used in the identification of bacteria such as *Staphylococcus* (Goh *et al.*, 1996), *Enterococcus* (Teng *et al.*, 2001b; Tsai *et al.*, 2005), *Streptococcus* (Goh *et al.*, 1998; Hung *et al.*, 2005; Teng *et al.*, 2002), *Ehrlichia* species (Sumner *et al.*, 2000), *Bartonella* (Marston *et al.*, 1999), *Mycobacterium* (Rastogi *et al.*, 1999) and rickettsiae (Lee *et al.*, 2003). We have previously determined the *groESL* sequence of *Streptococcus bovis* biotype I (Teng *et al.*, 2002). In this study, we determined the *groESL* sequences of other species and subspecies of the *Streptococcus bovis* group and analysed the differences between them. Based on these sequences, we have developed a *Streptococcus bovis* group-specific PCR. Restriction digestion of the amplicon with *AclI* further differentiated the species and subspecies.

METHODS

Bacterial strains. Five reference strains and 36 clinical isolates were used in this study. The reference strains were obtained from the American Type Culture Collection (ATCC) and included

Streptococcus gallolyticus ATCC 9809 (biotype I), *Streptococcus gallolyticus* subsp. *gallolyticus* ATCC 43143 (biotype I), *Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102^T (biotype II/1), *Streptococcus infantarius* subsp. *coli* ATCC BAA-103 (biotype II/1) and *Streptococcus gallolyticus* subsp. *pasteurianus* ATCC 43144 (biotype II/2). Twenty-nine of the 36 clinical isolates were collected between 2000 and 2003 in the Bacteriology Laboratory, National Taiwan University Hospital (NTUH), a 2000-bed teaching hospital in northern Taiwan, and the other seven were obtained from the National Cheng Kung University Hospital (NCKUH), a teaching hospital in southern Taiwan. All strains were recovered from blood cultures. The clinical strains were identified to species level and distinguished as *Streptococcus gallolyticus* ($n=12$), *Streptococcus infantarius* II/1 ($n=11$) and *Streptococcus gallolyticus* subsp. *pasteurianus* II/2 ($n=13$), by using commercial identification systems [API 20 Strep system (bioMérieux Vitek), Phoenix System or Rapid ID 32 STREP system (bioMérieux Vitek)]. Strains from NCKUH were also identified by using the ribosomal 16S–23S intergenic spacer (ITS) region sequence (Tung *et al.*, 2007).

PCR amplification and sequencing. For the determination of *groESL* sequences, two sets of PCR were performed. The primers Strep-ES-UP (5'-GACTATTTCTGACCAAGTGAT-3', located upstream of *groES*) and Strep-EL-120-100 (5'-CTCAAGAACACRTTRCGDCC-3', where R is A or G and D is A or T or G), located in the 5'-region of *groEL*, and which have been described previously (Teng *et al.*, 2002), were used to amplify a fragment containing the entire *groES* gene, ITS region and the short 5'-end region of *groEL*. The primers bovis-1F (5'-TCAGGACATTTTGCCACTTCTT-3', corresponding to positions 684–705 of the *groEL* gene) and Gor1907-1927 (5'-YTACATCATNCCNCCCATCAT-3', where Y is C or T and N is A, T, C or G), corresponding to positions 1623–1603 of the *groEL* gene, were used to amplify a partial *groEL* gene. PCR was carried out using a DNA thermal cycler (MJ Research) with 30 cycles of denaturation (94 °C, 30 s), annealing (50 °C, 30 s) and extension (72 °C, 1 min), followed by a final extension step (72 °C, 10 min). The PCR products were purified and subsequently sequenced using a sequencing system (model ABI PRISM 3100; Applied Biosystems) with a Taq BigDye-Deoxy Terminator cycle sequencing kit (Applied Biosystems), according to the instructions of the manufacturer.

Phylogenetic analysis. The phylogenetic relationships among species were analysed by using the neighbour-joining method in the MEGA2 (molecular evolutionary genetic analysis) analytical package (Kumar *et al.*, 2001). For the neighbour-joining analysis, the distance between the sequences was calculated using Kimura's two-parameter model. Levels of similarity among species were determined. Bootstrap values were obtained for 500 randomly generated trees.

Intraspecies and interspecies variation. The intraspecies variation of *groES* genes, ITS region and partial *groEL* genes and the interspecies variation of *groES* and *groEL* genes were evaluated among the clinical isolates. The strains were subjected to PCR amplification of *groES* and ITS region with primers Strep-ES-UP and Strep-EL-120-100. PCR with primers bovis-1F and Gor1907-1927 was performed to amplify partial *groEL* genes. The amplified PCR products were subsequently sequenced. DNA and deduced amino acid sequences were aligned using Gene-Works software (IntelliGenetics).

***Streptococcus bovis* group-specific PCR.** Based on the sequence obtained, we developed a *Streptococcus bovis* group-specific PCR. The forward primer ES5-29F (5'-TAAACCHTTAGGHAHCGWRTBG-3'), together with the reverse primer, *Streptococcus bovis* EL1265R (5'-CAAGTTCAAGTTCAGCAACTTTTG-3'), were derived to amplify a target region with an amplicon size that ranged from 1635 to 1910 bp, depending on the species. PCR was performed with 35 cycles of denaturation (94 °C, 30 s), annealing (54 °C, 30 s) and extension

(72 °C, 2 min), followed by a final extension step (72 °C, 10 min). The PCR products were analysed by using agarose gel electrophoresis (1.5 % agarose; FMC BioProducts), and then stained with ethidium bromide and photographed under UV light. A visible band with a size of 1.6–1.9 kb was considered to be a positive reaction.

PCR-RFLP for species and subspecies differentiation. After PCR, a restriction analysis was performed to further differentiate between species and subspecies. The amplification product was digested with the restriction enzyme *AclI* (New England BioLabs). After incubation, the DNA fragments were subjected to gel electrophoresis (FMC BioProducts), stained with ethidium bromide and photographed under UV light.

RESULTS AND DISCUSSION

Nucleotide sequences of *groESL* genes in reference strains

The nucleotide sequences of full-length *groES* and partial *groEL* genes were determined for five reference strains. The sequences showed that the *groES* genes were all 285 bp in length. The similarities between the *groES* sequences ranged from 84.2 % (between *Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102^T and both *Streptococcus gallolyticus* ATCC 9809 and *Streptococcus gallolyticus* subsp. *pasteurianus* ATCC 43144) to 99.0 % (between *Streptococcus gallolyticus* subsp. *pasteurianus* ATCC 43144 and *Streptococcus gallolyticus* ATCC 9809) at the nucleotide sequence level (Table 1). Pairwise sequence similarity of partial *groEL* (nucleotide positions 883–1572) among species and subspecies tested with reference strains ranged from 88.0 % (between *Streptococcus gallolyticus* ATCC 9809 and *Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102^T) to 98.8 % (between *Streptococcus gallolyticus* ATCC 9809 and *Streptococcus gallolyticus* subsp. *pasteurianus* ATCC 43144) (Table 1).

The length of the ITS region between *groES* and *groEL* was also found to be species and subspecies specific. The ITS regions in two *Streptococcus gallolyticus* reference strains, *Streptococcus gallolyticus* ATCC 9809 and *Streptococcus gallolyticus* subsp. *gallolyticus* ATCC 43143, were of the

same length (67 bp), which was 2 bp less than that (69 bp) in *Streptococcus gallolyticus* subsp. *pasteurianus* ATCC 43144. The size of the ITS regions in *Streptococcus infantarius* and two subspecies of *Streptococcus infantarius* (*Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102^T and *Streptococcus infantarius* subsp. *coli* ATCC BAA-103) was the same (342 bp), but the sequences differed in 6 nucleotides (see Supplementary Table S2 available in JMM Online).

Intraspecies similarities in clinical isolates

To evaluate the general applicability of the *groESL* sequence to species and subspecies identification, the *groES* and partial *groEL* sequences and the ITS regions of 36 clinical isolates were tested to identify intraspecies similarities. Comparison of these sequences from clinical isolates with reference strains for each species or subspecies are shown in Table 2. The intraspecies identity of *groES* was slightly higher than that for *groEL* sequences (Table 2). The ITS sequences from the *Streptococcus gallolyticus* clinical isolates were the most divergent, ranging from 95.5 to 100 %.

The ITS region between *groES* and *groEL* appeared to be another useful marker for species or subspecies differentiation. The lengths of the ITS regions of the clinical isolates were 67 (*Streptococcus gallolyticus*), 68 or 69 bp (*Streptococcus gallolyticus* subsp. *pasteurianus*) and 342 bp (*Streptococcus infantarius*), respectively. However, minor sequence variation was observed.

We found that the *groES* or *groEL* sequences provided more discrimination than the 16S rRNA gene for the differentiation of species and subspecies within the *Streptococcus bovis* group. The difference in the sequences of the 16S rRNA gene between *Streptococcus gallolyticus* and *Streptococcus gallolyticus* subsp. *pasteurianus* is only 0.3 %, whereas the differences in *groES* and *groEL* were more than 1.0 %. The higher interspecies divergence will make the differentiation easier. In addition to the low intraspecies variation of *groESL* sequences among the *Streptococcus bovis* group, these findings are attractive for species identification among this group.

Table 1. *groES* and *groEL* sequence similarities among reference strains

Data in the upper right portion indicate *groES* gene sequence similarity and data in the lower left portion indicate *groEL* gene sequence similarity. The numbers in the column headings represent the species numbered in the leftmost column.

Species	Biotype	Sequence similarity (%)				
		1	2	3	4	5
1. <i>Streptococcus gallolyticus</i> ATCC 9809	I	—	99.7	84.2	84.6	99.0
2. <i>Streptococcus gallolyticus</i> subsp. <i>gallolyticus</i> ATCC 43143	I	99.7	—	84.6	84.9	99.0
3. <i>Streptococcus infantarius</i> subsp. <i>infantarius</i> ATCC BAA-102 ^T	II/1	88.0	88.3	—	99.7	84.2
4. <i>Streptococcus infantarius</i> subsp. <i>coli</i> ATCC BAA-103	II/1	88.7	89.0	97.1	—	84.6
5. <i>Streptococcus gallolyticus</i> subsp. <i>pasteurianus</i> ATCC 43144	II/2	98.8	99.0	88.0	88.8	—

Table 2. Intraspecies variation of *groES*, *groEL* and ITS regions of the clinical isolates

Species	Biotype (no. of isolates)	<i>groES</i> (full-length, 285 bp)		ITS region		Partial <i>groEL</i> (nt positions 883–1572)	
		No. of different nucleotides	Percentage identity	No. of different nucleotides	Percentage identity	No. of different nucleotides	Percentage identity
<i>Streptococcus gallolyticus</i>	I (12)	0–4	98.6–100	0–3	95.5–100	0–4	99.4–100
<i>Streptococcus infantarius</i>	II/1 (11)	0–1	99.7–100	0–7	98.0–100	1–23	96.7–99.9
<i>Streptococcus gallolyticus</i> subsp. <i>pasteurianus</i>	II/2 (13)	0–2	99.3–100	0–1	98.6–100	0–5	99.3–100

Phylogenetic relationships

Unrooted phylogenetic trees constructed from full-length *groES* and partial *groEL* (nucleotide positions 883–1572) from the 36 clinical isolates and five reference strains are presented in Fig. 1. The phylogenetic analysis revealed that the nucleotide sequences of *groES* from 41 tested strains were divided into three major clusters, which were consistent with species and subspecies. The results of the phylogenetic analysis of the *groEL* gene were similar to those for the *groES* gene. The clinical isolates of biotype II/1 all clustered with *Streptococcus infantarius* subsp. *coli* ATCC BAA-103. In agreement with the 16S rRNA gene analysis, phylogenetic analysis of *groES* or *groEL* revealed that *Streptococcus gallolyticus* subsp. *pasteurianus* was more closely related to *Streptococcus gallolyticus* than to *Streptococcus infantarius*.

Streptococcus bovis group-specific PCR

Based on the sequences determined in this study, a pair of primers, ES5-29F and *Streptococcus bovis* EL1265R, was designed for *Streptococcus bovis* group-specific PCR. The specificity of the primers was tested with four reference strains and clinical isolates of the *Streptococcus bovis* group and 19 reference strains of other common Gram-positive bacteria, including *Streptococcus criceti* E49 (serotype a), *Streptococcus rattus* EA1 (serotype b), *Streptococcus mutans* GS-5 (serotype c), *Streptococcus sobrinus* B13 (serotype d), *Streptococcus mutans* MT730R (serotype e), *Streptococcus mutans* OME175 (serotype f), *Streptococcus sobrinus* 6715 (serotype g), *Streptococcus sobrinus* MFe28 (h), *Streptococcus pyogenes* ATCC 19615, *Streptococcus anginosus* ATCC 33397^T, *Streptococcus gordonii* ATCC 10558^T, *Streptococcus mitis* ATCC 49456, *Streptococcus constellatus* subsp. *constellatus* ATCC 27823^T, *Streptococcus sanguinis* ATCC 10556^T, *Streptococcus oralis* ATCC 35037^T, *Streptococcus agalactiae* ATCC 13813^T, *Enterococcus faecium* ATCC 35667, *Enterococcus casseliflavus* ATCC 25788^T and *Gemella haemolysans* ATCC 10379^T. Four reference strains of the *Streptococcus bovis* group generated amplicons of the size expected: 1635 bp for *Streptococcus gallolyticus*, 1637 bp for *Streptococcus gallolyticus* subsp. *pasteurianus* and 1910 bp for *Streptococcus infantarius* (Fig. 2a). None of the other bacterial species tested generated products of similar size (data not shown). Amplification using clinical isolates, including the *Streptococcus bovis* group and other bacteria, gave the same

results as the corresponding reference strains (part of the data are shown in Fig. 2a).

The *Streptococcus bovis* group-specific PCR detected all species and subspecies of the *Streptococcus bovis* group isolated from humans and could distinguish members of the *Streptococcus bovis* group from other bacteria, whereas PCR developed by other investigators usually resulted in the detection of only one or two species or subspecies of the *Streptococcus bovis* group. For example, the *sodA* gene has been reported previously to be an alternative target for *Streptococcus gallolyticus* (biotype I and II/2)-specific PCR (Sasaki *et al.*, 2004).

PCR-RFLP for differentiation of species and subspecies

To further differentiate between species and subspecies, an *AclI* restriction enzyme analysis was performed (Fig. 2b). The PCR-RFLP patterns from different species and subspecies were distinguishable (Fig. 2b). Using this assay, *Streptococcus gallolyticus* and *Streptococcus gallolyticus* subsp. *pasteurianus* were clearly separated. All clinical isolates of *Streptococcus gallolyticus* and *Streptococcus gallolyticus* subsp. *pasteurianus* tested also yielded identical patterns to those of the corresponding reference strains. Two reference strains representing two subspecies of *Streptococcus infantarius*, *Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102^T and *Streptococcus infantarius* subsp. *coli* ATCC BAA-103, generated similar but different PCR-RFLP patterns (Fig. 2b).

We checked the restriction patterns shown in the gel in Fig. 2b with the sizes estimated from the sequence data. According to the sequence data, the PCR products of *Streptococcus gallolyticus* were expected to be digested into five fragments (495, 363, 299, 286 and 171 bp) using the restriction enzyme *AclI*. Among these fragments, the 299 and 286 bp fragments are too close in size and could not be discriminated by gel electrophoresis; therefore, only four bands can be seen in the gel. The restriction fragments of the amplicon from *Streptococcus gallolyticus* subsp. *pasteurianus* showed the expected sizes (667, 495, 286 and 171 bp). Five restriction fragments of the amplicon were expected (733, 702, 286, 161 and 10 bp) for *Streptococcus infantarius* subsp. *infantarius* and four fragments (733, 702,

Fig. 1. Phylogenetic relationships among *Streptococcus bovis* group isolates (including reference strains and clinical isolates) based on the nucleotide sequences of (a) *groES* and (b) *groEL* genes. The phylogenetic trees were generated by using the neighbour-joining method in the MEGA2 package. Numbers at nodes are confidence levels expressed as percentages of occurrence in 500 bootstrapped resamplings. Scale bars indicate the evolutionary distance between sequences determined by measuring the lengths of the horizontal lines connecting two organisms. GenBank accession numbers for the gene sequences used are available in Supplementary Table S1 in JMM Online.

447 and 10 bp) for *Streptococcus infantarius* subsp. *coli*. As the 10 bp fragment was too small to be seen using electrophoresis, only three fragments were shown in *Streptococcus infantarius* subsp. *coli*. The PCR-RFLP patterns obtained for all clinical isolates of biotype II/1 were identical to that of *Streptococcus infantarius* subsp. *coli* ATCC BAA-103.

Differentiation of species and subspecies within the *Streptococcus bovis* group is important clinically. The association of species and subspecies with types of infection and

underlying diseases has been reported previously (Jean *et al.*, 2004; Ruoff *et al.*, 1989). In many reports, *Streptococcus gallolyticus* was more commonly associated with colorectal carcinoma than other species and subspecies (Ruoff *et al.*, 1989), whereas *Streptococcus gallolyticus* subsp. *pasteurianus* was more frequently associated with neonatal meningitis. Clarridge *et al.* (2001) reported that biotype II/2 (*Streptococcus gallolyticus* subsp. *pasteurianus*) formed a separate genospecies and was the most common in adult males. This hypothesis has been confirmed by additional taxonomic studies conducted

Fig. 2. *Streptococcus bovis* group-specific PCR and RFLP analysis of the *groESL* genes among species and subspecies of the *Streptococcus bovis* group. (a) *Streptococcus bovis* group-specific PCR. (b) Restriction fragments of amplified products after *AclI* digestion. Lanes: M, 100 bp DNA ladder; 1–4, *Streptococcus gallolyticus* (biotype I) (strains ATCC 9809, NTUH 7051, NTUH 6222 and NTUH 8995); 5, *Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102^T (biotype II/1); 6, *Streptococcus infantarius* subsp. *coli* ATCC BAA-103 (biotype II/1); 7–9, clinical isolates of *Streptococcus infantarius* (biotypes II/1) (strains NTUH 4936, NTUH 195 and NTUH 2639); 10–13, *Streptococcus gallolyticus* subsp. *pasteurianus* (biotype II/2) (strains ATCC 43144, NTUH 3004, NTUH 1443 and NTUH 7499); 14, negative control.

by other teams (Poyart *et al.* 2002; Schlegel *et al.* 2003). In Taiwan, *Streptococcus bovis* biotype I has been correlated with infective endocarditis (Jean *et al.*, 2004). In addition to the pathogenesis, different species and subspecies may display distinct antimicrobial patterns. We have reported that the prevalence of inducible erythromycin resistance among *Streptococcus gallolyticus* subsp. *pasteurianus* was higher than that of other species and subspecies (Teng *et al.*, 2001a).

One limitation of the present study is that only human isolates were included. Avian isolates may show different geno- or phenotypes (Chadfield *et al.*, 2007). In summary, based on the *groESL* sequences determined, we have developed a *Streptococcus bovis* group-specific PCR assay that may provide an alternative way of distinguishing the *Streptococcus bovis* group from other viridans streptococci. Restriction digestion of the amplicon with *AclI* improved the differentiation between the predominant human species or subspecies of the *Streptococcus bovis* group.

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