A Problem of Species Determination Using Housekeeping Genes in the *Streptococcus anginosus* Group

TOMONORI MIZUTANI^{*1)}, SHIGEKAZU IGUCHI^{*1)}, KEIICHI HIRAMATSU^{*1) 2)}, KEN KIKUCHI^{*3)}

^{*1)} Department of Infection Control Science, Juntendo University Faculty of Medicine, Tokyo, Japan, ^{*2)} Department of Microbiology, Juntendo University Faculty of Medicine, Tokyo, Japan, ^{*3)} Department of Infectious Diseases, Tokyo Women's Medical University, Tokyo, Japan

For identification of three species categorized in *Streptococcus anginosus* group (SAG), genotyping employing multiplex PCR for detection of species-specific genes has been widely used. We wondered whether possession of the specific genes correlated to differentiation of housekeeping genes, with an attempt to obtain more information on genetic background of clinical isolates of SAG strains and to evaluate reliability of the multiplex PCR. We therefore determined nucleotide sequences of some housekeeping genes of 52 SAG isolates from healthy volunteer to investigate whether the sequences phylogenetically corresponded to the identification by the multiplex PCR. The phylogenetic trees drawn with all of the sequences of 16S rRNA, *sodA*, *groEL* and *rpoB* were found to clearly correlate to species identified by the multiplex PCR. Interestingly, phylogenetic tree drawn only with single housekeeping gene often did not match with the species identified by the multiplex PCR. Our analysis implies even housekeeping genes can have their sequence varieties as independent manner from presence of species-specific genes in SAG strains, and the choice of housekeeping genes for the phylogenetic analyses may result in failure of the species identification.

Key words: *Streptococcus anginosus* group, phylogenetic tree, housekeeping gene, *rpoB*

Introduction

The taxonomy of species in the *Streptococcus anginosus* group (SAG) has changed many times since Guthof first identified “*Streptococcus milleri*” for SAG. These species are sometimes misidentified as other pyrogenic streptococci such as *Streptococcus pyogenes* or *Streptococcus dysgalactiae* because several SAG strains exhibit β -hemolysis or Lancefield A, C, or G serotypes¹⁾. At present, SAG is composed of three different species with five subspecies as follows: *Streptococcus anginosus* (SA), including *S. anginosus* subsp. *anginosus* and *S. anginosus* subsp. *whileyi*; *Streptococcus constellatus* (SC), including *S. constellatus* subsp. *constellatus*, *S. constellatus* subsp. *pharyngis*, and *S. constellatus* subsp. *viborgensis*; and *Streptococcus intermedius* (SI). These species comprise one phylogenetic cluster in the genus *Streptococcus*²⁾.

SAG is recognized as a part of the indigenous flora of the oral cavity, upper respiratory tract, gastrointestinal tract, and genitourinary tract. SAG strains have been recognized as important pathogens because of their role in purulent infections such as deep abscesses and pulmonary exacerbations in patients with cystic fibrosis^{3) 4)}. Furthermore, *S. anginosus* is frequently isolated from several cancer tissues as the possible carcinogen⁵⁾, and *S. intermedius* is one of the possible causative agents of primary biliary cirrhosis⁶⁾. Regarding their specific virulence, although it is important to differentiate the species from each other, it is extremely difficult because the phenotypic and genotypic characteristics of SAG species are closely related.

Consequently, accurate, rapid, simple, and economical methods to differentiate SAG species are required for epidemiological, ecological, and pathological analyses. The polymerase chain reaction

Corresponding author: Ken Kikuchi

Department of Infectious Diseases, Tokyo Women's Medical University

8-1 Kawadacho, Shinjuku-ku, Tokyo 162-8666, Japan

TEL: +81-3-3353-8112 (ext. 38311) FAX: +81-3-5269-7003 E-mail: kikuchi.ken@twmu.ac.jp

[Received May 18, 2015] [Accepted Oct. 19, 2015]

(PCR) method described by Takao is one solution⁷⁾. This system, which is composed of two PCR steps, namely SAG-specific PCR and species-specific multiplex PCR, is simple, reliable, and accurate. Since whole-genome analyses for SAG strains became available, the target genes using this species-specific PCR (intermedilysin or hyaluronate lyase genes) remain valid⁸⁾. Although the usefulness and effectiveness of the multiplex PCR were shown as above, a concern still remains: since the pathogenicity-related genes are often acquired horizontally⁹⁾, possession of such genes as hyaluronate lyase and intermedilysin of SAG may not be always related to the species' genetic backgrounds. By contrast, direct sequencing methods using housekeeping genes such as the 16S rRNA gene, *sodA*, *groEL*, *rpoB*, and *tuf* have been generalized for taxonomic analysis among the streptococcal species¹⁰⁾.

Regardless of the usefulness of genotyping methods using housekeeping genes such as the 16S rRNA gene, incorrect phylogenetic classifications are occasionally obtained for some bacterial species such as SAG strains¹¹⁾. One of the reasons is considered to be the low discriminative power of 16S rRNA for closely related species such as SAG strains.

On the other hand, the phylogenetic analysis using the housekeeping genes should nevertheless reflect differentiation pathways of each SAG strain. We therefore wondered why such misidentification occurs upon using sequences of the housekeeping genes, and this question promoted us to determine the nucleotide sequences of several housekeeping genes of clinical isolates of SAG strains in comparison with their phylogenetic classifications by the multiplex PCR. We also expected to check whether the so-called 'housekeeping genes' were statically retained in a species.

In this study, we found that concatenated phylogenetic tree drawn with sequences of 16S rRNA, *sodA*, *groEL* and *rpoB* genes perfectly matched with classification by multiplex PCR, indicating that both sequencing of the housekeeping genes and the multiplex PCR for species-specific genes are reliable methods for determination of SAG species. In addition, we also noticed that a phylogenetic tree drawn only with sequences of *groEL* and *rpoB* genes for RNA polymerase subunit beta was

somehow inconsistent with the result determined by the multiplex PCR. Our analysis revealed that there seem mosaic structures in the *rpoB* genes among SAG strains, which have been presumably generated by multiple times of intra- and inter-species crossovers in the genes, explaining the inconsistency between the *rpoB* genes sequences and the classification by the multiplex PCR.

Methods

1. Bacterial isolates and culture conditions

We used 52 non-duplicate clinical SAG isolates and four type and reference strains (*S. anginosus* ATCC 33397^T, *S. constellatus* subsp. *constellatus* ATCC 27823^T, *S. constellatus* subsp. *pharyngis* CCUG 46377, and *S. intermedius* NCDO 2227^T). All the strains were stored at -80°C until use and were then cultured anaerobically (AnaeroPack, Mitsubishi Chemical, Tokyo, Japan) overnight on trypticase soy agar II with 5% sheep blood (Nippon Beckton Dickinson, Fukushima, Japan) at 37°C .

2. DNA extraction and species identification by multiplex PCR

A single colony was suspended to a McFarland 2.0 standard in 100 ml of Tris-EDTA buffer with 320 U of achromopeptidase (Wako Chemical, Osaka, Japan), and the suspension was incubated at 55°C for 15 min. After centrifugation at $15,000 \times g$ for 5 min, the supernatant was used as the DNA extract for PCR. Clinical strains were identified using SAG group-specific and species-specific PCR systems described by Takao *et al*⁷⁾.

3. PCR primers and analyses

Oligonucleotide primers used to amplify fragments of the 16S rRNA gene, *rpoB*, *groEL*, and *sodA* were designed on the basis of conserved sequences identified by aligning the relevant sequences of *Streptococcus* species obtained from the GenBank nucleotide database. The primers are listed in Table-1. All PCR mixtures (50 ml) contained 1.25 U of Ex Taq polymerase (TaKaRa, Shiga, Japan), 0.2 mM of dNTPs, 25 pmol of each primer, and 2 ml of a 200-fold dilution of the preparation of the aforementioned template DNA. Amplification was performed with the following parameters: an initial denaturing step at 95°C for 10 min, 30 cycles of denaturing 95°C for 30 s, primer annealing at 62°C

for 1 min, and extension at 72 °C for 1–4 min depending on the product size, and a final extension step at 72 °C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis after staining with ethidium bromide.

4. Sequencing of the 16S rRNA gene, *sodA*, *groEL*, and *rpoB*

Amplicons of four housekeeping genes, including partial sequences of *rpoB* genes used in Figure-4 analyses, were obtained by PCR using primers

listed in the Table-1 (partial sequences of *rpoB* genes used in Figure-4 analyses are also obtained by using corresponding primer sets listed in Table-1). The amplicons were purified using Wizard minicolumns (Promega, Madison, WI). The gene fragments were sequenced on both strands using the same primers and the Thermo Sequence Dye Terminator cycle sequencing premix kit (Amersham Bioscience AB, Uppsala, Sweden) according to the manufacturer's instructions. The following program was used: 30 cycles

Table-1 Primers for amplifying and sequencing housekeeping genes

Target	Name of primers	Usage	Primer sequence (5' to 3')	Product length
16S rRNA	16Sr27-M13UnivF	Amplification	GTTGTAAAACGACGGCCAGTAGAGTTTGATCCTGGCTCAG	~1500 bp
	16Sr1492-M13UnivR	Amplification	CACAGGAAACAGCTATGACCGGTACCTTGTTACGACTT	
	800F	sequencing	ATTAGATACCCTGGTA	
	800R	sequencing	CTACCAGGGTATCTAAT	
<i>sodA</i>	SAGsodA2-M13UnivF	Amplification	GTTGTAAAACGACGGCCAGTARCAAATCTGGTTTATTATTGG	981 bp
	SAGsodA2-M13UnivR2	Amplification	CACAGGAAACAGCTATGACCTTTRGGACGAAAGGTRAATC	
	sodA-310F	sequencing	CTTGAAGCAACWTTTGGTTCATTTG	
	sodA-376R	sequencing	AGCCAAGCCCARCCTGAACC	
<i>rpoB</i>	Int-rpoB-M13F	Amplification	TGTAAAACGACGGCCAGTTTGGCAGGACATGAAGTTCAATAC	3567 bp
	Int-rpoB-M13R	Amplification	CAGGAAACAGCTATGACCTTATTCTTTTCCTTCAGCTTCAAAAAGC	
	rpoB-583F	sequencing	CTTGTTCTGTCRCTTGTTT	
	rpoB-583R	sequencing	AAACCAAGYGCACGAACAAG	
	rpoB-1210F	sequencing	GATATTGACCACTTGGGAAATCGTCGGATTTCGTG	
	rpoB-1210R	sequencing	CACGAATCCGACGATTTCCCAAGTGGTCAATATC	
	rpoB-1597F	sequencing	AATAACTTGTCTTCTTATGG	
	rpoB-1597R	sequencing	CCATAAGAAGACAAGTTATT	
	rpoB-2116F	sequencing	TATCATATTACGAAATTCGCCG	
	rpoB-2116R	sequencing	CGGCGGAATTTTCGTAATATGATA	
	rpoB-2659F	sequencing	AAAATCTTTACTCGTGCGAA	
	rpoB-2659R	sequencing	TTCGCACAGTAAAGATTTT	
	rpoB-3199F	sequencing	GGTGGACAACGTTTTGGAGA	
	rpoB-3199R	sequencing	TCTCCAAAACGTTGTCCACC	
<i>groEL</i>	Int-groEL-M13F	Amplification	TGTAAAACGACGGCCAGTATGGCAAAAGATATTAAATTTTCAG	1662 bp
	Int-groEL-M13R	Amplification	CAGGAAACAGCTATGACCTTACATCATCCGCCCATCATGC	
	groEL-498F	sequencing	AGTTGGCAACGACGGTGTCACTACTATT	
	groEL-498R	sequencing	AATAGTGATGACACCGTCGTTGCCAACT	
	groEL-1080F	sequencing	AGAAAACTACAAGAACGTTTGGCG	
	groEL-1080R	sequencing	CGCCAAACGTTCTTGTAGTTTTTCT	
Universal sequence	M13UnivF	sequencing	GTTGTAAAACGACGGCCAGT	
	M13UnivR	sequencing	CACAGGAAACAGCTATGACC	
	M13F	sequencing	TGTAAAACGACGGCCAGT	
	M13R	sequencing	CAGGAAACAGCTATGACC	

of denaturation at 96 °C for 30 s, primer annealing at 45 °C for 15 s, and extension at 60 °C for 4 min. The products of the sequencing reaction were examined with an automatic DNA sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems, Foster City, CA).

5. Phylogenetic analyses of the 16S rRNA gene, *sodA*, *groEL*, and *rpoB*

Clustal X software downloaded from <http://www.ebi.ac.uk> was used to align the sequences for each gene separately and as concatenated sequences in the order 16S rRNA gene, *sodA*, *groEL*, and *rpoB*. Attempting to establish simpler method, we also tested phylogenetic analysis using only one out of the four genes listed above. Phylogenetic analysis by the neighbor-joining algorithm was performed using MEGA version 3. Missing sequences as a result of a lack of a PCR amplification product for genes that may be missing or present in a significantly different sequence variant were dealt with as gaps of the same length. The corresponding parameter of the neighbor-joining algorithm was set at "pairwise deletion." The model used was "nucleotide: Kimura two-parameter."

6. Criteria for identification based on the sequences of the housekeeping genes

Unequivocal clustering with type and reference strains of SAG in the unrooted phylogenetic tree based on sequences of the housekeeping genes was taken as evidence of conclusive identification of that species. Deviations from this identification based on phylogenetic analysis of single gene loci were recorded.

Results

1. Identification of clinical isolates of SAG strains by multiplex PCR and concatenated sequence analysis based on multiple housekeeping genes

In order to assess consistency between results of the multiplex PCR employed for species determination of SAG and sequences of their housekeeping genes, we obtained 52 independent isolates of SAG strains from healthy volunteer in National Center for Global Health and Medicine Kohnodai hospital, and performed the multiplex PCR as well as determination of nucleotide sequences of 16S rRNA gene, *sodA*, *groEL*, and *rpoB* genes. The multiplex PCR is

designed to detect genes for penicillin-binding protein 2b, hyaluronate lyase, intermedilysin or 16S rRNA. The combination of appeared bands corresponding to individual genes in the gel electrophoresis presumably determines species⁷⁾. On the other hand, the nucleotide sequences of each housekeeping gene was simply combined as a single text and then used for drawing a phylogenetic tree (we designated this procedure as concatenated sequence analysis) as shown in Figure-1.

The analysis indicated that the results obtained by the multiplex PCR for determination of the SAG species perfectly corresponded to the phylogenetic tree by the concatenated sequence analysis using

Figure-1 Phylogenetic tree based on concatenated sequences

A phylogenetic tree drawn based on concatenated sequences using four housekeeping genes from clinical isolates of 52 *Streptococcus anginosus* group (SAG) strains together with their type strains completely corresponds to their identification determined by multiplex polymerase chain reaction (PCR). The tree was constructed using the neighbor-joining method. Strains A1-20 were identified as *Streptococcus anginosus* (SA) by multiplex PCR, strains I1-15 were identified as *S. intermedius* (SI), and C1-16 were identified as *S. constellatus* (SC). The type strains were ATCC 33397T for *S. anginosus*, ATCC 27335T for *S. intermedius*, ATCC 27823T for *S. constellatus*, subsp. *constellatus*, and CCUG 46377 for *S. constellatus*, subsp. *pharyngis*.

the housekeeping genes: the strains belonging to three groups of branches derived from the root of the phylogenetic tree were identified as either *Streptococcus anginosus* (SA, upper part of Figure-1), *S. constellatus* (SC, middle) or *S. intermedius* (SI, bottom) by the multiplex PCR. This result indicated that the multiplex PCR is effective for identification of SAG species.

2. Possession of species-specific genes is not always consistent with sequences of housekeeping genes among SAG strains

As described above, employment of 16S rRNA gene, *sodA*, *groEL*, and *rpoB* genes altogether successfully classified the SAG strains. If the number of genes used for the analysis could be reduced, this would help us to establish simpler method. We therefore decided to draw the phylogenetic tree with one of the four genes.

When a phylogenetic tree was drawn based on the *groEL* sequences, the branching pattern showed good consistency with species determination by the multiplex PCR, although a few exceptions was seen as shown in Figure-2. This tendency was also seen with 16S rRNA and *sodA* genes (data not shown). These data and the result shown in Figure-1 suggested that overall genomes of SAG strains showed consistency with the carriage of species-specific genes detectable with the multiplex PCR, however, the consistency was not always the case when focused onto single housekeeping gene. In addition, we noticed that the discrepancy is conspicuous when a phylogenetic tree is drawn with *rpoB* sequence (Figure-3). Three *S. anginosus* strains, or A4, A6 and A20, were grouped under a branch of *S. constellatus* strains (Figure-3A) when whole *rpoB* sequences were employed for the phylogenetic analysis, clearly indicating that even a gene indispensable for keeping integrity of a cell can be altered regardless of the presence or absence of species-specific genes. In other words, the genomic backgrounds of a species including so-called housekeeping genes may not be static, but can be altered dynamically. The sequence diversity and subsequent deviation from the species determination by the multiplex PCR became more conspicuous when the sequences between 2401st and 3000th of 3567 base pairs of *rpoB* structural genes from its 5'-termini (Figure-3B) were used for the phyloge-

Figure-2 Phylogenetic tree based on *groEL* sequences
A phylogenetic tree drawn only with *groEL* sequences of the clinical isolates mostly matches with their species identified by the multiplex PCR.

netic tree: among strains identified as *S. anginosus* by the multiplex PCR, A2, A3, A4, A5, A6 and A17 were clustered into *S. constellatus* branch of the tree, and A13 was grouped into *S. intermedius* branch. To our surprise, even ATCC 27335T that is distributed as *S. intermedius* type strain was seen under branch of *S. anginosus*.

Taken together, both multiplex PCR for detecting species-specific genes and concatenated sequence analysis using multiple housekeeping genes are effective for determination of species, however, employing only one housekeeping gene for identifying SAG species should be avoided due to unexpected sequence varieties occurring in such genes.

Figure-3 Phylogenetic tree based on *rpoB* sequences

Phylogenetic trees drawn with whole and 3'-portion of *rpoB* sequences of the clinical isolates show inconsistency with their species identification by the multiplex PCR. A. A tree drawn with whole *rpoB* sequences of the clinical SAG strains. Three *S. anginosus* strains are grouped under a branch of *S. constellatus* strains. B. A tree drawn with 2401st to 3000th nucleotides from the start codon of *rpoB* structural genes. Out of seven strains that have been identified as *S. anginosus*, six are clustered into *S. constellatus* branch of the tree, and one is grouped into *S. intermedius* branch. *S. intermedius* type strain ATCC 27335T is seen under branch of *S. anginosus*.

Discussion

There is no doubt that species-identification of clinically isolated bacteria is very important to establish therapeutic strategy of infectious diseases. For this purpose, many procedures have been used depending on target bacteria and/or their pathogenic factors. Multiplex PCR for detecting species-specific genes provides species determination with reliable accuracy and easiness, whereas concatenated sequence analysis of housekeeping genes is

considered as not only accurate but also informative method for tracing evolutionary pathways. However, the reliability of these procedures should be carefully assessed in some species, such as SAG, that has not widely known as pathogens until recent years. Therefore, the information of such species determined by multiplex PCR or concatenated sequence analyses are less available than that of other well-known bacteria. In that sense, the methods identifying SAG species still requires evaluation on the accuracy of species determination, which promoted

Figure-4 Mosaic structure in *rpoB*

Mosaic structures are seen in *rpoB* genes of SAG strains. The DNA sequences of *rpoB* genes are divided into 600 bp segments (the division of the 3'-end consists of 3001st to 3567th) and compared them with those of three type or reference strains. The values in % in the table parts indicate difference (defined as 1 minus identity) to reference or type strains at the corresponding segments. 'A', 'C' and 'T' shown in boxes in the figures represent the closest type or reference strains of *S. anginosus* (ATCC 33397T), *S. constellatus* subsp. *constellatus* (ATCC 27823T), or *S. intermedius* (ATCC 27335T), respectively, at the corresponding division. A. A case of strain A-17 (identified as *S. anginosus* by the multiplex PCR). The 1201st to 1867th and 2401st to 3000th of the *rpoB* gene is rather closest to *S. constellatus* subsp. *constellatus*. The latter part is completely identical to that of *S. constellatus* subsp. *constellatus*. B. A case of strain A-4 (identified as *S. anginosus* by the multiplex PCR). The 5'- half is the most similar to *S. constellatus* subsp. *constellatus*. C, D. A cases of strains A-6 and A-20 show the similar tendency as strain A-4.

us to investigate on relationship between the results obtained by the multiplex PCR and concatenated sequence analyses of the housekeeping genes. As we expected, the results of phylogenetic analysis based on concatenated sequence analysis of four housekeeping genes (16S rRNA, *sodA*, *groEL*, and *rpoB* genes) excellently corresponded to the species identification by multiplex PCR as we indicated above. However, we also noticed that the phylogenetic analyses employing single housekeeping gene or only parts the housekeeping genes showed discrepancy to results of the multiplex PCR, indicating that indiscretion upon choice of housekeeping genes for phylogenetic analyses may lead to misidentification of SAG species.

Nevertheless, it is surprising that inconsistency of species determination was often seen between sequences of housekeeping genes and presence of species-specific genes. This means that sequence alteration often occurs even in some housekeeping genes that are indispensable for bacterial growth and therefore generally considered to be stable. Our analysis revealed that the *rpoB* gene, encoding for an indispensable RNA polymerase subunit, also had sequence variety that might cause misidentification of species when phylogenetic relationship was analyzed with the sequences. We further analyzed to see whether the sequence diversity seen in *rpoB* gene occurred over whole gene or concentrated to limited domain of the gene. We then divided the whole gene into 600 bp segments and compared them with those of three type or reference strains (Figure-4, values indicated in % are difference to reference or type strains at the corresponding segments). To our surprise, this analysis illuminated a 'mosaic structure' in *rpoB* gene. For instance, a fraction of the *rpoB* sequence between 2401th and 3000th of strain A-17, which had been identified as *S. anginosus* by multiplex PCR, was completely identical to that of *S. constellatus* subsp. *constellatus* type strain. In addition, a fraction of other strains (A-4, A-6, and A-20) that had been identified as *S. anginosus* by multiplex PCR were similar to *S. constellatus* subsp. *constellatus* rather than *S. anginosus*. The reason why the mosaic structure is unknown, however, the fact that the *rpoB* genes are not closely located to DNA recombinases suggests that the structures are considered to be presumably consequence of multiple times of

inter- and intra-species homologous recombination rather than horizontal transfer of the gene.

Thus, our analyses strongly suggest that even sequences for housekeeping genes often alter with independent manner from presence of species-specific genes. The instability of the housekeeping genes should therefore be noted upon identification of species, and at the same time, this fact gave us a question about appropriateness of current way of bacterial species definition.

Conflicts of interest

All the contributing authors declare no conflicts of interest.

Acknowledgments

This study was supported by JSPS KAKENHI Grant Number 24590536, a Grant-in-Aid (S0991013) from the Ministry of Education, Culture, Sport, Science, and Technology, Japan (MEXT) for the Foundation of Strategic Research Projects in Private Universities and a Grant from the Strategic Research Base Development Program for Private Universities from MEXT 2010-2014 (S1001024). We are indebted to Dr. Tadashi Baba for his support in writing and reviewing this paper, in addition to designing concept of the manuscript.

References

- 1) Whiley RA, Fraser H, Hardie JM, Beighton D: Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strains within the "Streptococcus milleri group". J Clin Microbiol, 1990; 28: 1497-1501.
- 2) Jensen A, Hoshino T, Kilian M: Taxonomy of the Anginosus group of the genus *Streptococcus* and description of *Streptococcus anginosus* subsp. *whileyi* subsp. nov. and *Streptococcus constellatus* subsp. *viborgensis* subsp. nov. Int J Syst Evol Microbiol, 2013; 63: 2506-2519.
- 3) Giuliano S, Rubini G, Conte A, et al: Streptococcus anginosus group disseminated infection: case report and review of literature. Infez Med, 2012; 20: 145-154.
- 4) Olson AB, Sibley CD, Schmidt L, Wilcox MA, Surette MG, Corbett CR: Development of real-time PCR assays for detection of the *Streptococcus milleri* group from cystic fibrosis clinical specimens by targeting the *cpn60* and 16S rRNA genes. J Clin Microbiol, 2010; 48: 1150-1160.
- 5) Sasaki H, Ishizuka T, Muto M, et al: Presence of *Streptococcus anginosus* DNA in esophageal cancer,

- dysplasia of esophagus, and gastric cancer. *Cancer Res*, 1998; 58: 2991–2995.
- 6) Haruta I, Kikuchi K, Hashimoto E, *et al*: Long-term bacterial exposure can trigger nonsuppurative destructive cholangitis associated with multifocal epithelial inflammation. *Lab Invest*, 2010; 90: 577–588.
 - 7) Takao A, Nagamune H, Maeda N: Identification of the anginosus group within the genus *Streptococcus* using polymerase chain reaction. *FEMS Microbiol Lett*, 2004; 233: 83–89.
 - 8) Olson AB, Kent H, Sibley CD, *et al*: Phylogenetic relationship and virulence inference of *Streptococcus Anginosus* Group: curated annotation and whole-genome comparative analysis support distinct species designation. *BMC Genomics*, 2013; 14: 895.
 - 9) Baba T, Takeuchi F, Kuroda M: Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet*, 2002; 359: 1819–1827.
 - 10) Summanen PH, Rowlinson MC, Wooton J, Finegold SM: Evaluation of genotypic and phenotypic methods for differentiation of the members of the *Anginosus* group streptococci. *Eur J Clin Microbiol Infect Dis*, 2009; 28: 1123–1128.
 - 11) Ferrándiz MJ, Ardanuy C, Liñares J, *et al*: New mutations and horizontal transfer of *rpoB* among rifampin-resistant *Streptococcus pneumoniae* from four Spanish hospitals. *Antimicrob Agents Chemother*, 2005; 49: 2237–2245.