

Rapid identification of *Streptococcus intermedius* by PCR with the *ily* gene as a species marker gene

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Streptococcus intermedius belongs to the anginosus group of streptococci (AGS) and is associated with endogenous infections leading to abscesses in the oral cavity and at deep-seated sites, such as the brain and liver. Two other species, *S. anginosus* and *S. constellatus*, and some presently unnamed taxa, are also classified as AGS. Recently, *S. constellatus* subsp. *pharyngis*, a new subspecies with biochemical characteristics similar to *S. intermedius*, was described with the potential for causing confusion when trying to identify isolates of these two species routinely with commercial identification kits, such as Rapid ID32 Strep and Fluo-Card Milleri. To correctly identify *S. intermedius*, this study attempted to develop an accurate PCR identification system with the *ily* gene as a species marker. This approach relies on amplification of an 819-bp fragment of the *ily* gene and its 3'-flanking region and is shown here to be specific for *S. intermedius* strains among all other streptococcal species. Moreover, this PCR system was applicable in direct rapid PCR with whole bacterial cells and TaKaRa Z-TaqTM (TaKaRa), a highly efficient DNA polymerase, as the template and DNA amplification enzyme, respectively.

Introduction

Anginosus group streptococci (AGS), formerly called '*Streptococcus milleri*', are recognised as a part of normal human oral flora and are opportunist pathogens in endogenous infections [1–8]. AGS are currently classified as *S. anginosus*, *S. constellatus* subsp. *constellatus*, *S. constellatus* subsp. *pharyngis* and *S. intermedius* [9, 10]. *S. intermedius* exhibits a tropism for infections of deep sites such as the brain and liver [11] and is also associated with periodontitis [12]. In spite of the clinical significance of *S. intermedius* reported previously [7, 8, 11, 12], it is difficult for the clinical laboratory to identify *S. intermedius* among AGS isolates because of the paucity of differential biochemical tests [11]. Indeed, it is known that rapid

systems that utilise standard phenotypic tests, such as API20 Strep and Rapid ID32 Strep (bioMérieux) are not satisfactory for the accurate identification of AGS at the species level [13, 14]. Specifically, there is potential confusion between *S. intermedius* and *S. constellatus* subsp. *pharyngis*, which have similar phenotypes. Fluo-Card Milleri (Key Scientific), which is a biotyping kit specifically developed for identification of AGS [15], also misidentifies *S. constellatus* subsp. *pharyngis* because its phenotype is similar to that of *S. intermedius*. Therefore, to identify *S. intermedius* with such commercial kits, some additional tests, for sialidase production, Lancefield grouping and haemolysis on the animal blood agar, are necessary to distinguish it from *S. constellatus* subsp. *pharyngis*. Moreover, although some studies have developed genetic identification methods for AGS [9, 16, 17], more rapid and accurate methods are desirable for the routine laboratory. A previous study reported that a human-specific cytolysin, intermedilysin (ILY), was secreted by a strain of *S. intermedius* UNS46 isolated

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from human liver abscess [18]. It has been suggested recently that ILY is likely to be a pathogenic or triggering factor of significance in inducing deep-seated infections with *S. intermedius* and that the *ily* gene is potentially useful for the identification of *S. intermedius* among AGS [19]. However, to use the *ily* gene as a species marker gene of *S. intermedius* in clinical diagnosis, further examination of the distribution of the *ily* gene and any closely related genes among other human isolates of streptococci is essential. The present study attempted to confirm the appropriateness of the *ily* gene as a species marker, to design an improved *ily* gene-specific PCR primer set that can specifically amplify the *ily* gene and its 3'-flanking region in *S. intermedius* in contrast to other AGS, other species groups of streptococci and some related genera, and to apply the primer set to a rapid direct colony PCR system. The accuracy of this PCR method was compared with the current reference identification method described by Whiley *et al.* [11] and a commercial biochemical test kit (Rapid ID32 Strep).

Materials and methods

Bacterial strains and culture conditions

Type strains of each group within the genus *Streptococcus*: *S. acidominus* (GTC234), *S. agalactiae* (GTC1234), *S. alactolyticus* (GTC432), *S. anginosus* (NCTC10713), *S. bovis* (GTC235), *S. canis* (GTC423), *S. constellatus* subsp. *constellatus* (NCDO2226), *S. constellatus* subsp. *pharyngis* (MM9889a), *S. criceti* (GTC242), *S. cristatus* (GTC631), *S. difficilis* (GTC730), *S. downei* (GTC632), *S. dysgalactiae* subsp. *equisimilis*, (GTC842), *S. dysgalactiae* subsp. *dysgalactiae* (GTC431), *S. equi* subsp. *equi* (GTC269), *S. equi* subsp. *zooepidemicus* (GTC542), *S. equinus* (GTC 246), *S. ferus* (GTC 279), *S. gallolyticus* (GTC 809), *S. gordonii* (GTC 497), *S. hyointestinalis* (GTC 496), *S. hyovaginalis* (GTC 1229), *S. infantis* (GTC 849), *S. iniae* (GTC 244), *S. intermedius* (NCDO2227), *S. intestinalis* (GTC537), *S. macacae* (GTC 538), *S. macedonicus* (GTC 1230), *S. mitis* (GTC 495), *S. mutans* (GTC 218), *S. oralis* (GTC 276), *S. parasanguinis* (GTC 498), *S. parauberis* (GTC 539), *S. peroris* (GTC 848), *S. phocae* (GTC 776), *S. pleomorphus* (GTC 690), *S. pneumoniae* (GTC 261), *S. porcinus* (GTC 543), *S. pyogenes* (GTC 262), *S. rattii* (GTC 245), *S. salivarius* (GTC 215), *S. sanguinis* (GTC 217), *S. sobrinus* (GTC 278), *S. suis* (GTC 430), *S. thermophilus* (GTC 241), *S. thoralensis* (GTC 1231), *S. uberis* (GTC 271) and *S. vestibularis* (GTC 488); and the type strains of related genera: *Staphylococcus aureus* (GTC286), *Staph. epidermidis* (GTC 289), *Enterococcus faecalis* (GTC 228), *E. faecium* (GTC 227), *Lactococcus lactis* (GTC 232) and *Gemella haemolysans* (GTC 219) were used as the species standards. Fifty-five clinical isolates of AGS from human tonsillitis (TW4466, TW4468, TW4469, TW4470, TW4471, TW4472, TW4473, TW4474,

TW4475, TW4476, TW4477, TW4478, TW4479, TW4480, TW4481, TW4482, TW4483, TW4484, TW4485, TW4486, TW4487, TW4488, TW4489, TW4490, TW4491, TW4492, TW4493, TW4494, TW4495, TW4496, TW4497, TW4498, TW4499, TW4500, TW4501, TW4502, TW4504, TW4505, TW4506, TW4507, TW4508, TW4509, TW4510, TW4511, TW4512, TW4513, TW4514, TW4515, TW4516, TW4517, TW4518, TW4519, TW4520, TW4521 and TW4522) and 33 reference strains from various sites [10, 19, 20] including *S. anginosus* (AS/JM4, EF222, KR461, KR687, HW31, NCTC11169, POOLE PHLS457, R84/4972, R88/111 and 153ii), *S. anginosus* group 2 (MAS 624), *S. constellatus* subsp. *constellatus* (F436, NCTC11063, NMH4, NMH6, R87/3795, W277, 47, 1340, 1792b and 3206443), *S. constellatus* subsp. *pharyngis* (I751), *S. intermedius* (AC4720, AM47, A4676a, DP102, GN6146, HARDY-DAVID T1, PC574, PC941, UNS35, UNS38(=38) and UNS46) were also used. TW strains and accompanying Lancefield grouping and Rapid ID32 Strep data were kindly provided by Dr Ken Kikuchi, Tokyo Women's Medical College. Strains were maintained on horse blood 5% v/v agar based on Brain Heart Infusion (BHI) Broth (Difco Laboratories) at 37°C under anaerobic conditions except *S. pneumoniae* and *S. aureus* which were cultured at 37°C under aerobic conditions.

Phenotypic characterisation of AGS

Production of enzymes α -arabinosidase, α -L-fucosidase, β -D-fucosidase, α -D-galactosidase, β -D-galactosidase, α -D-glucosidase, β -D-glucosidase, β -N-acetyl-galactosaminidase, β -N-acetylglucosaminidase and sialidase was determined with fluorogenic substrates as described previously [11]. Haemolysis was observed on horse blood agar.

Preparation of bacterial genomic DNA

Bacterial genomic DNA was prepared as follows. All bacterial cells cultured in 20 ml of BHI broth containing D(+)-glucose 0.5% w/v for 18 h were collected by centrifugation and washed with 0.8 ml of TE buffer, pH 8.0. The washed cells were suspended in 0.1 ml of TE, pH 8.0, and mixed with 10 μ l of lysozyme 50 mg/ml in TE and 3 μ l of RNAase I 10 mg/ml in sterile water. The enzyme reaction was allowed to proceed at 37°C for 30 min, then 0.5 ml of the lysis mixture containing guanidine isothiocyanate 60% w/v, 100 mM EDTA sodium salt (pH 8.0) and N-laurylsarcosine 0.5% w/v was mixed with each reaction mixture and left at room temperature for 10 min to complete the cell lysis. After the addition of 0.25 ml of cold 7.5 M ammonium acetate, the mixture was mixed and placed on ice for 10 min. Subsequently, 0.5 ml of chloroform:isoamylalcohol (24:1) was mixed thoroughly with each reaction mixture. Then, the mixture was centrifuged at 13 000 rpm for 10 min in a microcentrifuge. A 0.7-ml sample of the supernate

was transferred to a new microcentrifuge tube and mixed with 0.378 ml of cold isopropanol. After gently mixing by inverting the tube for 1 min, the mixture was centrifuged at 13 000 rpm for 2 min and the supernate was discarded. Precipitated DNA was resuspended in 0.2 ml of TE. After the addition of 0.5 ml of cold absolute ethanol to the DNA solution, the mixture was placed at -80°C for 15 min, then centrifuged at 13 000 rpm for 3 min. The supernate was removed, the precipitated DNA was air-dried for 15 min, dissolved in 0.1 ml of TE and stored at 4°C until required. The concentration and quality of extracted DNA were checked by agarose gel electrophoresis.

Dot-blot hybridisation

The *ily* gene fragment of 1502 bp was amplified by PCR with primers: ILY-N terminal Fw, 5'-GAAA CACCTACCAAACCAAAAGCAGCTCAA-3', ILY-C end Bw, 5'-GCTTAATCAGTGTTATCTTTCACAAC-3' and plasmid pILYw carrying the whole *ily* gene (Fig. 1). This fragment was labelled with α - ^{32}P -dCTP with a Random Primer DNA Labeling Kit Ver.2 (TaKaRa, Tokyo, Japan), and then used as a probe in the following dot-blot hybridisation system to evaluate the distribution of homologue(s) of the *ily* gene among other streptococci (48 strains) and several species from related genera before developing a molecular approach suitable for use in a routine laboratory. Each extracted bacterial genomic DNA (100 ng) was blotted on a nylon membrane (Gene screen plus; NEN research) pre-equilibrated with $10\times$ SSC with a blotting apparatus (Milliblot-V Jr; Millipore). The blotted membranes were treated with 0.5 M NaOH for 1 min and with $1\times$ SSC containing 0.2 M Tris-HCl (pH 7.5) for 1 min. After the denaturation step, the membranes were washed with $2\times$ SSC twice and dried on filter paper. The blotted membranes were prehybridised at 55°C for 1 h in the prehybridisation solution ($6\times$ SSC containing skimmed milk 5% w/v, sodium azide

0.02% w/v and SDS 1% w/v). Subsequently, the ^{32}P -labelled probe solution (final activity *c.* 5×10^5 cpm/ml) and 0.2 mg of salmon sperm DNA were added to 0.5 ml of distilled water and heat-treated at 95°C for 3 min and immediately chilled to make the working probe solution. After prehybridisation, the working probe solution was added to the prehybridisation solution and hybridisation was allowed to proceed at 55°C (low stringency) or 68°C (high stringency) overnight. Then, the membranes were washed twice in $2\times$ SSC containing SDS 1% at room temperature for 5 min, for 30 min in $0.2\times$ SSC containing SDS 1% at a temperature corresponding to that used for the hybridisation. Air-dried membranes were exposed to X-ray films at -80°C overnight.

PCR with extracted genomic DNA as the template

The PCR primers specific for the *ily* gene used in the present study were as follows: ILY-4D Fw as the forward primer, 5'-CTCACCCCTCAATCATGATGG TGC-3' and ILY-wholeC Bw as the reverse primer, 5'-CGACTCACTATAGGGAGATCAGCATGG-3'. The expected amplicon size was 819 bp (Fig. 1). These primers were synthesised by the manufacturer on the basis of ORF and 3' flanking region of the *ily* gene sequence (DDBJ/EMBL/GenBank nucleotide sequence databases with the accession no. AB029317). PCR with the extracted genomic DNA as a template was performed in a reaction mixture of 50 μl final volume containing 1 μl (150 ng) of each template genomic DNA in TE (pH 8.0), 1 μl of 2 mM each deoxyribonucleotide triphosphate (Perkin-Elmer), *Taq* DNA polymerase (Promega) 1 U, 10 pmol of each primer, 5 μl of 10-fold concentrated PCR buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, glycerol 50% and Triton X-100 1%) and 3 μl of 25 mM MgCl_2 . Amplification was performed in a GeneAmp PCR System 2400 (Perkin-Elmer) programmed for 35 cycles as follows: 1 min of denaturation at 95°C , 1 min of

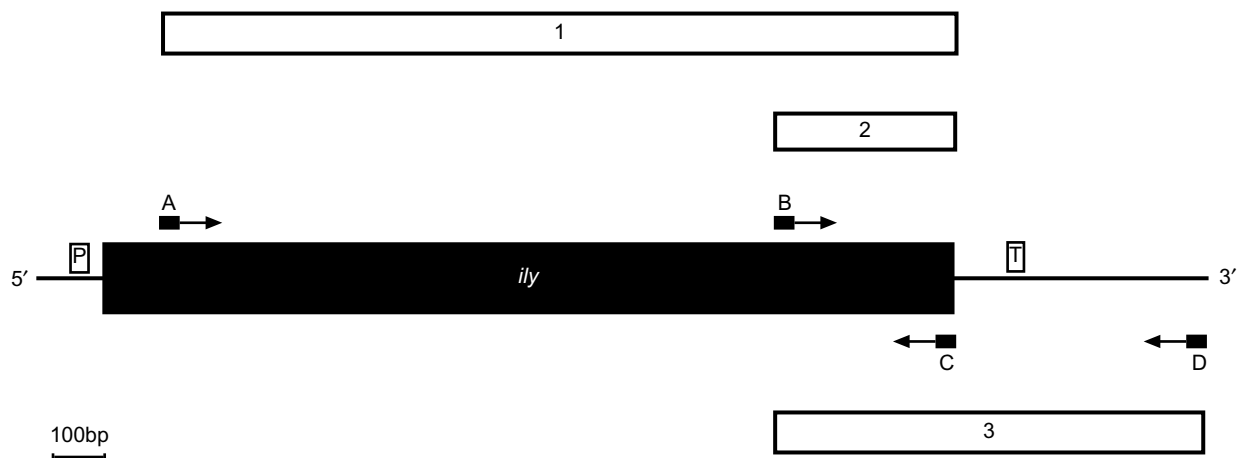


Fig. 1. Schematic representation of the location of primers, probes and *S. intermedius*-specific amplicon described in the present paper. A genomic fragment of 2212 bp of *S. intermedius* including whole *ily* gene (GeneBank/EMBL/DDBJ no. AB029317) is shown. Primers: **A**, ILY-N terminal Fw; **B**, ILY-4D Fw; **C**, ILY-C end Bw; **D**, ILY-wholeC Bw. **1**, the probe for dot-blot hybridisation of genomic DNA; **2**, the probe for Southern blot hybridisation of PCR products; **3**, *S. intermedius*-specific amplicon. Boxed P and T show the location of the promoter region and the terminator region of the *ily* gene, respectively.

primer annealing at 55°C and 2 min of extension at 72°C. The PCR products were analysed by electrophoresis in agarose 2.0% gels in a 0.5× TBE buffer system. AmpliTaq Gold™ (Perkin-Elmer) or *TaKaRa Z-Taq*™ (TaKaRa) DNA polymerase were used for PCR with extracted genomic DNA as a template, as required and detailed below.

Direct colony PCR

Direct detection from a single colony of *S. intermedius* strains was performed by PCR with *Taq* DNA polymerase or *TaKaRa Z-Taq*™. PCR with *Taq* DNA polymerase was performed as described above with a slight modification, i.e., an additional preheating step of a single colony as the template at 95°C for 10 min in the PCR reaction mixture before the PCR cycles. PCR with *TaKaRa Z-Taq*™ was performed in the reaction mixture of 50 µl containing a single colony preheated at 95°C for 10 min as the template, 1 µl of 2 mM each deoxyribonucleotide triphosphate (Perkin-Elmer), *TaKaRa Z-Taq*™ 1 U, 10 pmol of the forward primer, 10 pmol of the backward primer and 5 µl of 10× PCR buffer attached with *TaKaRa Z-Taq*™. The amplification was performed in a GeneAmp PCR System 2400 (Perkin-Elmer) programmed for 30 cycles as follows: 1 s of denaturation at 98°C, 15 s of primer annealing and extension at 68°C. The PCR products were analysed by agarose 2.0% gel electrophoresis in a 0.5× TBE buffer system.

Southern hybridisation

After analysis by agarose gel electrophoresis, the PCR products were transferred to a nylon membrane with a Model 785 Vacuum Blotter (BioRad). Hybridisation was performed as follows. Blotted membrane was treated at 68°C for 1 h in a prehybridisation buffer, i.e., 5× SSC containing skimmed milk 0.25% w/v, N-laurylsarcosine 0.025% w/v, SDS 0.02% w/v and 0.25 mg of salmon sperm DNA denatured by heat treatment (95°C for 5 min followed by rapid chilling on ice). Then, 5 µl of the digoxigenin-labelled *ily* gene fragment denatured by the heat treatment was added to the 5× SSC containing skimmed milk 0.25% w/v, N-laurylsarcosine 0.025% w/v, SDS 0.02% w/v and 0.13 mg of heat-denatured salmon sperm DNA, and allowed to react with the blotted membrane at 68°C overnight. The probe used in this hybridisation was the digoxigenin-labelled *ily* gene fragment of 341 bp amplified by PCR with the primer set of ILY-4D Fw and ILY-Cend Bw shown in Fig. 1. Subsequently, the membrane was washed twice with 2× SSC containing SDS 0.1% at room temperature for 5 min, twice with 0.1× SSC containing SDS 0.1% at 68°C for 5 min and then once with the washing buffer – 0.1 M maleic acid buffer (pH 7.5) containing 0.15 M NaCl and Tween 20 0.3% v/v – at room temperature for 5 min. The washed membrane was dipped into the blocking buffer in a polymer bag containing skimmed milk 1% w/v, sodium

azide 0.004% w/v, 0.08 M maleic acid and 0.12 M NaCl. The membrane sealed in the bag was incubated at room temperature for 30 min. The horseradish peroxidase-labelled anti-digoxigenin antibody (Roche Diagnostics, Tokyo, Japan) was diluted to 75 mU/ml (1 in 10 000 dilution) in the blocking buffer and allowed to react with the blotted membrane at room temperature for 30 min with gentle agitation. Subsequently, the membrane was washed twice with washing buffer at room temperature for 20 min with gentle agitation. Signals on the washed membrane were detected by chemiluminescence with ImmunoStar Reagents (Wako Pure Chemical Industries, Osaka, Japan).

Sensitivity assay of PCR

Serial dilutions of 100–0.1 ng of genomic DNA or (1×10^6) – (1×10^2) cells per PCR reaction of *S. intermedius* type strain (NCDO2227) were prepared to estimate the sensitivity of PCR with *TaKaRa Z-Taq*™ as a DNA polymerase. PCR conditions were the same as described above. The PCR products were analysed by agarose 2.0% gel electrophoresis in a 0.5× TBE buffer system.

Results

Genomic dot-blot hybridisation of the type strains of streptococci with the *ily* gene

Fig. 2 shows a dot-blot hybridisation profile of the type strains (48 strains) of all groups of streptococci and some closely related genera (6 strains) with the *ily* gene fragment as the probe at low stringency. At high stringency, a strong signal was recognised with the three strains of *S. intermedius* (the type strain NCDO2227, strain UNS38 and strain UNS46) and no signal was found with any other type strains (data not shown). As shown in Fig. 2, under low stringency, a very weak signal was also detected with the type strains of *S. anginosus*, *S. constellatus* subsp. *constellatus*, *S. constellatus* subsp. *pharyngis* and *S. infantis*. No (or a very faint) signal was found with other type strains, despite a significant degree of homology (51.7–60.1%) between the *ily* gene and genome-linked streptococcal toxin genes such as pneumolysin (*S. pneumoniae*) and streptolysin O (*S. pyogenes*). Therefore, although it has been shown that *S. canis* and *S. dysgalactiae* subsp. *equisimilis* both contain a gene with high homology to the gene for streptolysin O from *S. pyogenes* (98.2% and 98.6% homology, respectively), it seems that gene(s) with high homology to the *ily* gene are not present among the other streptococci or closely related genera. Moreover, it has also been shown that the *ily* gene is ubiquitous within *S. intermedius* strains [19]. Taken together, these results indicate that specific amplification of the *ily* gene by PCR is a promising method to rapidly and accurately identify *S. intermedius* strains from among other streptococcal strains from clinical sources.

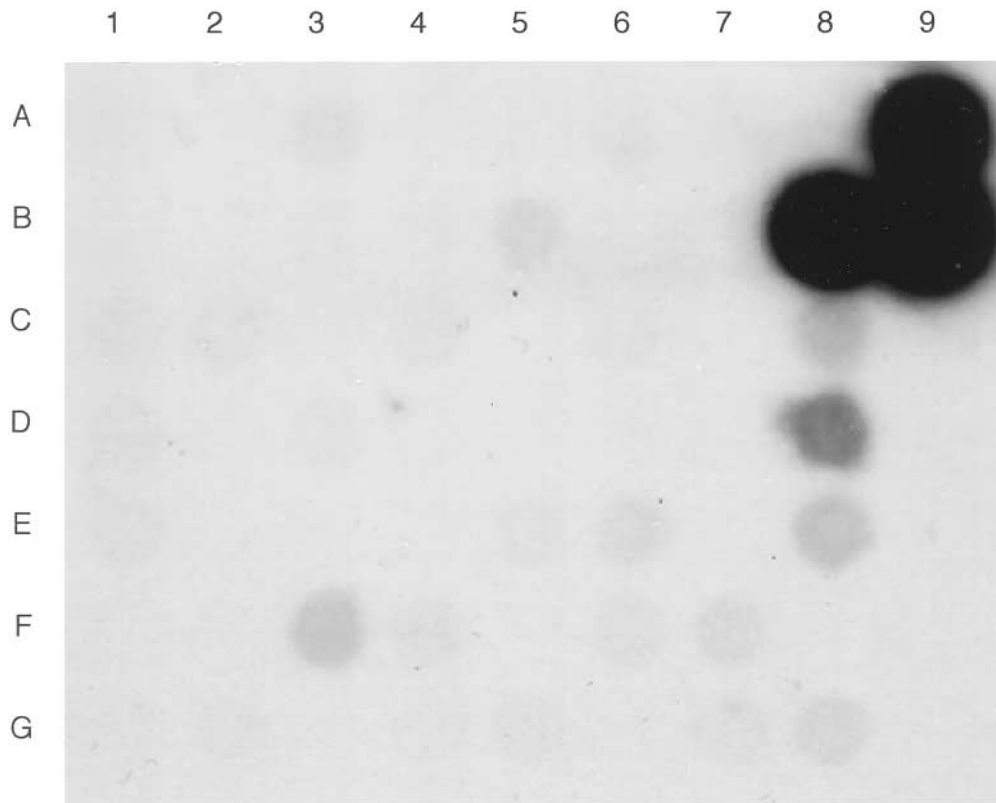


Fig. 2. Dot-blot hybridisation of genomic DNA from the type strains of streptococci and related genera with the *ily* gene probe. A1, *S. acidominimus*; B1, *S. agalactiae*; C1, *S. alactolyticus*; D1, *S. bovis*; E1, *S. canis*; F1, *S. criceti*; G1, *S. cristatus*; A2, *S. difficilis*; B2, *S. downei*; C2, *S. dysgalactiae* subsp. *equisimilis*; D2, *S. dysgalactiae* subsp. *dysgalactiae*; E2, *S. equi* subsp. *equi*; F2, *S. equi* subsp. *zooepidemicus*; G2, *S. equinus*; A3, *S. ferus*; B3, *S. gallolyticus*; C3, *S. gordonii*; D3, *S. hyointestinalis*; E3, *S. hyovaginalis*; F3, *S. infantis*; G3, *S. iniae*; A4, *S. intestinalis*; B4, *S. macacae*; C4, *S. macedonicus*; D4, *S. mitis*; E4, *S. mutans*; F4, *S. oralis*; G4, *S. parasanguinis*; A5, *S. parauberis*; B5, *S. peroris*; C5, *S. phocae*; D5, *S. pleomorphus*; E5, *S. pneumoniae*; F5, *S. porcinus*; G5, *S. pyogenes*; A6, *S. ratti*; B6, *S. salivarius*; C6, *S. sanguinis*; D6, *S. sobrinus*; E6, *S. suis*; F6, *S. thermophilus*; G6, *S. thoraltensis*; A7, *S. uberis*; B7, *S. vestibularis*; C7, *Staph. aureus*; D7, *Staph. epidermidis*; E7, *E. faecalis*; F7, *E. faecium*; G7, *L. lactis*; A8, *G. haemolysans*; B8, *S. intermedius*; C8, *S. anginosus*; D8, *S. constellatus* subsp. *constellatus*; E8, *S. anginosus* group 2 (MAS624); F8, *S. constellatus* subsp. *pharyngis*; G8, *S. constellatus* subsp. *pharyngis* (M5823); A9, *S. intermedius* (UNS38); B9, *S. intermedius* (UNS46). All strains except E8, G8, A9 and B9 are the type strain of each species.

PCR of the *ily* gene fragment with extracted genomic DNA as the template

The study first examined whether *S. intermedius* can be detected specifically by PCR with genomic DNA extracted from streptococci. Genomic DNA from the type strains of streptococcal species (48 strains), species from related genera (6 strains) and from clinical and reference AGS strains including *S. intermedius* derived from various sites (88 strains) were used as templates. Six primer sets were designed and used to test the specificity of the amplification of the *ily* gene by PCR. Five sets were a combination of the primers corresponding to the sequence within the ORF of ILY including the primer set which was used previously to analyse the distribution of the *ily* gene among AGS [19] and the other was a set (ILY-4D Fw and ILY-wholeC Bw) for amplifying the sequence which included the C-terminal side of ORF of ILY and its 3' flanking region (819-bp fragment shown in Fig. 1 as no. 3). Fig. 3 shows the PCR amplification pattern of the *ily* gene fragment with primers ILY-4D Fw and ILY-

wholeC Bw against the streptococcal type strains and other related genera. Only primers ILY-4D Fw and ILY-wholeC Bw gave a clear *S. intermedius*-specific amplicon; the other five primer sets which were expected to amplify a part of the ORF region of ILY showed one or more additional non-specifically amplified band(s) in other groups of streptococci besides the specific amplification of the *ily* gene fragment within *S. intermedius* strains (data not shown). Therefore, primers ILY-4D Fw and ILY-wholeC Bw were selected for the identification of *S. intermedius* by PCR and were used subsequently throughout this study. Furthermore, as shown in Table 1, only *S. intermedius* strains were positive for amplification of the *ily* gene fragment. These results suggest that the present PCR system with the *ily* gene as a species marker gene could provide a clear and rapid method for the unambiguous identification of *S. intermedius* strains.

Subsequently, the study also investigated whether the *ily* gene could be detected in all strains of *S. intermedius* among AGS by the present PCR system

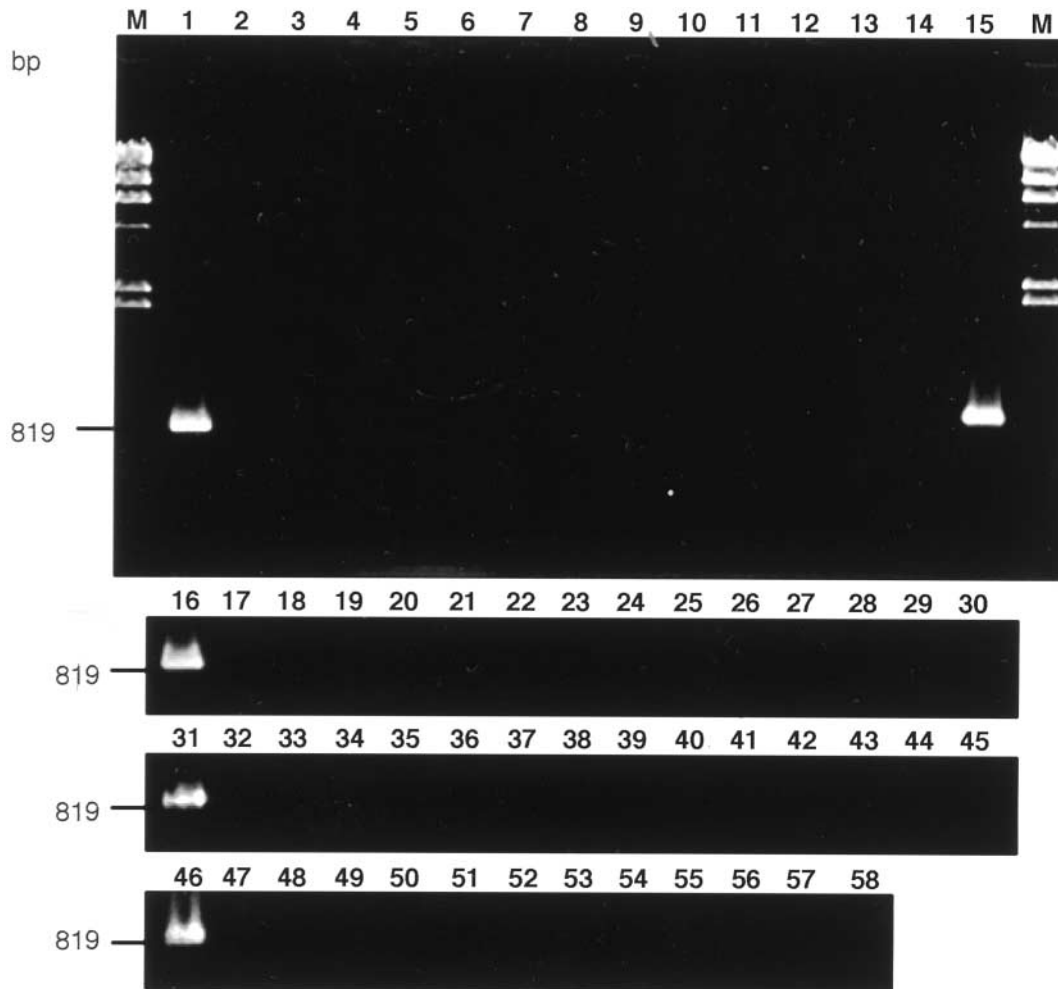


Fig. 3. Agarose gel electrophoresis of PCR products amplified from the genomic DNA of streptococci and related genera with the PCR primer set specific for the *ily* gene. Lanes: **1, 15, 16, 31, 46**, *S. intermedius*; **2**, *S. anginosus*; **3**, *S. constellatus* subsp. *constellatus*; **4**, *S. acidominimus*; **5**, *S. agalactiae*; **6**, *S. alactolyticus*; **7**, *S. bovis*; **8**, *S. canis*; **9**, *S. criceti*; **10**, *S. cristatus*; **11**, *S. difficilis*; **12**, *S. downei*; **13**, *S. dysgalactiae* subsp. *equisimilis*; **14**, *S. dysgalactiae* subsp. *dysgalactiae*; **17**, *S. equi* subsp. *equi*; **18**, *S. equi* subsp. *zooepidemicus*; **19**, *S. equinus*; **20**, *S. ferus*; **21**, *S. gallolyticus*; **22**, *S. gordonii*; **23**, *S. hyointestinalis*; **24**, *S. hyovaginalis*; **25**, *S. infantis*; **26**, *S. iniae*; **27**, *S. intestinalis*; **28**, *S. macacae*; **29**, *S. macedonicus*; **30**, *S. mitis*; **32**, *S. mutans*; **33**, *S. oralis*; **34**, *S. parasanguinis*; **35**, *S. parauberis*; **36**, *S. peroris*; **37**, *S. phocae*; **38**, *S. pleomorphus*; **39**, *S. pneumoniae*; **40**, *S. porcinus*; **41**, *S. pyogenes*; **42**, *S. ratti*; **43**, *S. salivarius*; **44**, *S. sanguinis*; **45**, *S. sobrinus*; **47**, *S. suis*; **48**, *S. thermophilus*; **49**, *S. thoraltensis*; **50**, *S. uberis*; **51**, *S. vestibularis*; **52**, *Staph. aureus*; **53**, *Staph. epidermidis*; **54**, *E. faecalis*; **55**, *E. faecium*; **56**, *L. lactis*; **57**, *G. haemolysans*; **58**, *S. constellatus* subsp. *pharyngis*; **M**, λ DNA *Hind*III cut. All strains are the type strain of each species.

with the extracted genomic DNA of clinical AGS strains (55 strains) derived from human tonsillitis. Recently, several β -haemolytic, Lancefield group C strains with a biotype similar to *S. intermedius* were found to be genetically different from *S. intermedius*, and have been proposed as a new subspecies (*pharyngis*) within *S. constellatus* [10]. These strains are

Table 1. PCR amplification of the *ily* gene fragment in reference strains of AGS

Species (n)	Amplification of 819-bp fragment (% positive)
<i>S. anginosus</i> (11*)	– (0)
<i>S. anginosus</i> group 2 (1)	– (0)
<i>S. constellatus</i> subsp. <i>constellatus</i> (11*)	– (0)
<i>S. constellatus</i> subsp. <i>pharyngis</i> (2*)	– (0)
<i>S. intermedius</i> (12*)	+ (100)

*Including type strain.

sufficiently similar to *S. intermedius* in biochemical test reactions to cause confusion when employing commercial identification test kits. Therefore, the clinical strains examined in this study were re-tested according to the criteria reported by Whiley *et al.* [10] and the result of PCR detection of the *ily* gene fragment was compared with that of the biochemical and immunological identification method which consisted of haemolytic reaction on horse blood agar, Lancefield grouping and glycosidase assays [10, 11] (Table 2). As shown in Table 2, and as expected, the result of the biochemical and immunological identification according to the new criteria differed from those obtained with the Rapid ID32 Strep test kit, especially in the identification of *S. intermedius* and *S. constellatus* subsp. *pharyngis*. There was 58.3% agreement between biochemical and immunological identification methods and Rapid ID32 Strep for *S. intermedius* identification. However, the

Table 2. Comparison of the results of *S. intermedius* identification by three methods

Species by RM (n)	Species by RID (n)	PCR of the <i>ily</i> gene* (n)	Percentage matching of <i>S. intermedius</i> identification between RM and PCR
<i>S. anginosus</i> (16)	<i>S. anginosus</i> (15)	- (15)	...
	<i>S. constellatus</i>	- (1)	...
	subsp. <i>constellatus</i> (1)		
<i>S. constellatus</i> subsp. <i>constellatus</i> (20)	<i>S. constellatus</i>		
	subsp. <i>constellatus</i> (19)	- (19)	...
	<i>S. intermedius</i> (1)	- (1)	...
<i>S. constellatus</i> subsp. <i>pharyngis</i> (9)	<i>S. anginosus</i> (5)	- (5)	...
	<i>S. intermedius</i> (4)	- (4)	...
<i>S. intermedius</i> (9)	<i>S. anginosus</i> (2)	+ (2)	100
	<i>S. intermedius</i> (7)	+ (7)	100
UI (1)	<i>S. anginosus</i> (1)	- (1)	...

RM, the reference identification method [11, 21]; RID, Rapid ID32 Strep; UI, unidentified *Streptococcus* sp.

*The same results were obtained in PCR with whole bacterial cells in colonies as with template DNA.

PCR approach showed 100% agreement with the identification of *S. intermedius* by the recently published [10] identification criteria.

Direct colony PCR of the *ily* gene fragment

To improve the rapidity of identification by this PCR system, the study attempted to use a single bacterial colony as a template. Fig. 4a shows a typical pattern of the detection of *S. intermedius* strains within the reference strains of AGS by direct colony PCR. The expected 819-bp amplicon was detected only in *S. intermedius* strains. Subsequently, Southern hybridisation was used to confirm that the fragments amplified

by PCR were those amplified from the *ily* gene (Fig. 4b). It also confirmed that the identification results shown in Table 2 with 55 clinical isolates were in complete agreement with the direct colony PCR approach. Furthermore, use of a highly efficient DNA polymerase, *TaKaRa Z-Taq*TM, instead of ordinary *Taq* DNA polymerase resulted in a shortened total PCR programme (18 min) to achieve a positive PCR result.

Sensitivity of PCR amplification of the *ily* gene fragment

The sensitivity of the present PCR method was determined by performing PCR with whole cells or

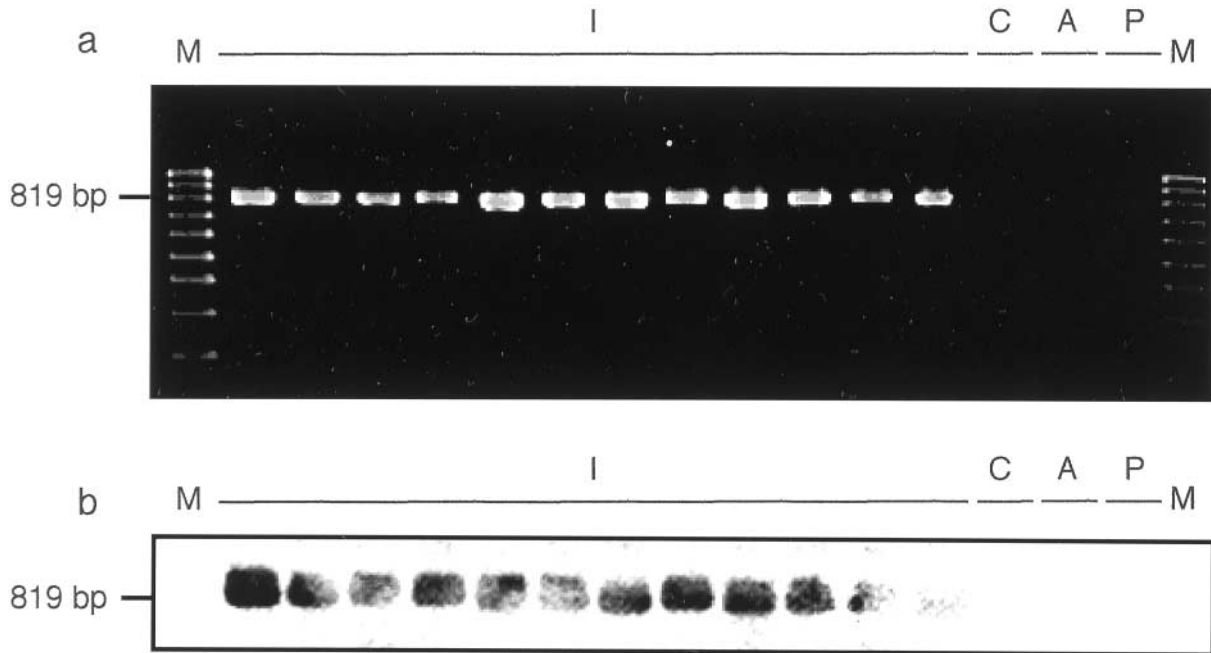


Fig. 4. Agarose gel electrophoresis of PCR products amplified from the colonies of AGS reference strains and Southern hybridisation of the PCR products with the *ily* gene probe. (a) Result of the colony direct PCR with reference strains of AGS. (b) Result of Southern hybridisation of the PCR products shown in (a) with a 341-bp fragment of the *ily* gene. **I**, *S. intermedius* strains: NCDO2227 (the type strain), PC941, AM47, DP102, UNS35, GN6146, PC574, UNS38, UNS46, A4676a, AC4720, HARDY-DAVID T1 from left to right; **C**, the type strain of *S. constellatus* subsp. *constellatus*; **A**, the type strain of *S. anginosus*; **P**, the type strain of *S. constellatus* subsp. *pharyngis*; **M**, 100-bp ladder marker.

extracted genomic DNA from the type strain, *S. intermedius* NCDO2227. The minimum detectable level of extracted genomic DNA by PCR with *TaKaRa Z-Taq* was 0.1 ng DNA (Fig. 5a). When bacterial whole cells were used, 1×10^5 cells was the minimum number required for detection (Fig. 5b).

Discussion

AGS, including *S. intermedius*, are known to be a heterogeneous streptococcal group [21–23]. Recently, an AGS strain formerly described as atypical β -haemolytic, Lancefield group C *S. anginosus* with similar biochemical characteristics to *S. intermedius* was proposed as '*S. constellatus* subsp. *pharyngis*' [10]. This subspecies is associated with pharyngitis and can be confused with *S. intermedius* or *S. anginosus* by commercial identification kits such as Rapid ID32 Strep and Fluo-Card Milleri. Therefore, for accurate identification of *S. intermedius*, the current biochemical and metabolic reactions for identification of AGS in commercial identification kits need to be revised (e.g., by the inclusion of a test for sialidase activity). Thus, this study attempted to develop a simple, rapid and reliable identification method for *S. intermedius* employing PCR. It has confirmed that the *ily* gene can be used as a species marker gene. As shown by dot-blot hybridisation and by PCR amplification of the *ily* gene, the present study demonstrated that neither this gene nor any with significant homology existed outside the species *S. intermedius*, thereby demonstrating its suitability as a reliable species marker for identification on a routine basis. Indeed, the results of

PCR with 33 reference strains and 55 clinical AGS isolates from tonsillitis revealed that identification of *S. intermedius* by this PCR approach was in agreement with the results obtained with the identification scheme of Whiley *et al.* [10, 11]. On the other hand, the percentage agreement with results obtained by a commercial identification kit (Rapid ID32 Strep), was only 58.3%, which was essentially the same value as reported previously [14], again demonstrating the great improvement in identification of this species that this approach represents.

Subsequently, to simplify the entire PCR methodology, the test was developed on the basis of single colony PCR, thus circumventing the need for lengthy and tedious purification of template genomic DNA. With this approach, the results obtained were the same as those obtained with purified template. Furthermore, by making use of a highly efficient DNA polymerase, *TaKaRa Z-Taq*TM, the study succeeded in reducing the programming time for PCR to <20 min. PCR sensitivity with purified genomic DNA as the template was >0.1 ng DNA/PCR reaction. The genome sizes of streptococci such as *S. equi*, *S. mutans*, *S. pneumoniae* and *S. pyogenes* are in the range 1.98–2.3 Mbp [24]. Thus, 0.1 ng of genomic DNA roughly corresponds to the genomic DNA from 4.1×10^4 cells if the genome size of *S. intermedius* is taken to be the average of the four values in the range shown above (i.e., 2.17 Mbp). This estimated cell number of the detection limit was essentially consistent with the experimental number, 1×10^5 cells/PCR reaction if cell lysis by heat treatment was not completed. A single colony on an agar plate containing more than the cell number

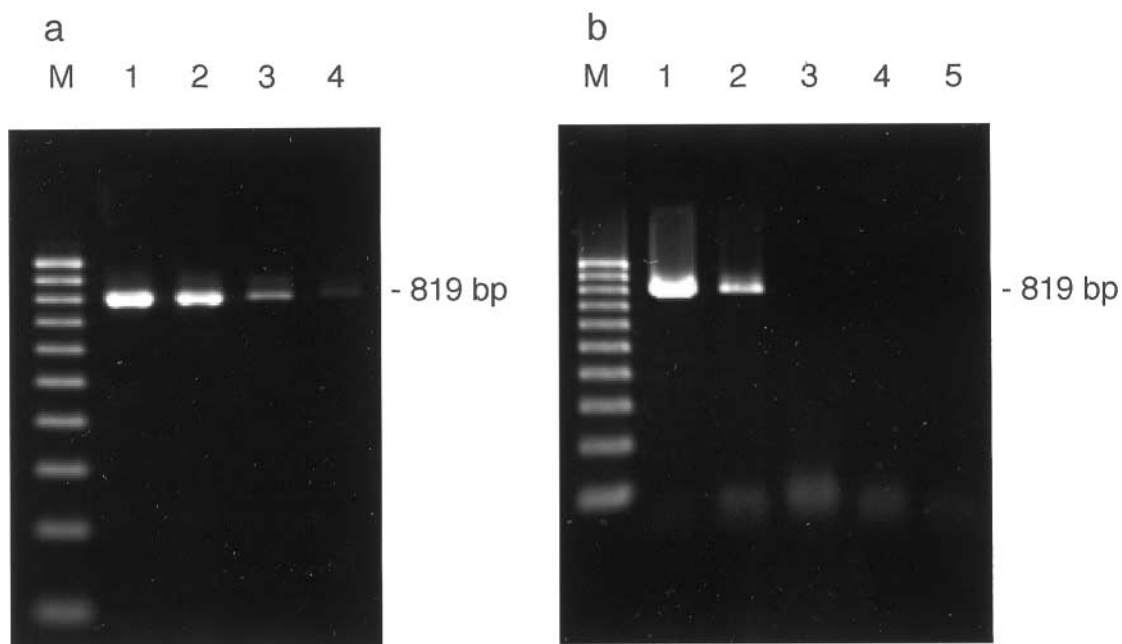


Fig. 5. Sensitivity of the PCR system with *TaKaRa Z-Taq*. (a) PCR with purified genomic DNA as a template. Lanes: 1, 100 ng DNA; 2, 10 ng; 3, 1 ng; 4, 0.1 ng; M, 100-bp ladder marker. (b) PCR with whole bacterial cells as a template. Lanes: 1, 1×10^6 cells; 2, 1×10^5 cells; 3, 1×10^4 cells; 4, 1×10^3 cells; 5, 1×10^2 cells; M, 100-bp ladder marker. The type strain, NCDO2227, was used in these experiments.

required in the present PCR system is sufficient for the detection of the *ily* gene for *S. intermedius* identification.

The present PCR system is superior in rapidity to the standard biochemical and immunological identification method and also superior in accuracy to commercial identification kits such as Rapid ID32 Strep or Fluo-Card Milleri. However, optimisation of sample preparation from the potentially different clinical specimens in which *S. intermedius* might be found may be necessary for the routine application of this PCR method for identification.

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