Multiplex PCR for identification of six clinically relevant streptococci

Rujirat Hatrongjit,1 Yukihiro Akeda,2 Shigeyuki Hamada,3 Marcelo Gottschalk4 and Anusak Kerdsin5,*

Abstract

Purpose. The aim of this study was to develop a multiplex PCR (mPCR) for simultaneous detection (single reaction) of six clinically relevant streptococcal species: Streptococcus pneumoniae, Streptococcus suis, Streptococcus gallolyticus subsp. gallolyticus, S. gallolyticus subsp. pasteurianus, Streptococcus intermedius and Streptococcus anginosus/constellatus.

Methods. mPCR with primers specific for S. pneumoniae (lytA), S. suis (recN), S. galleyticus subsp. galleyticus (IanB), S. galleyticus subsp. pasteurianus (SGPB0680 cell wall surface protein), S. intermedius (ily) and S. anginosus/constellatus (moA) was employed with 37 reference bacterial strains and 442 clinical streptococcal isolates collected from seven tertiary hospitals in north-east Thailand. Results from this mPCR were compared to those obtained with the API 20 Strep and conventional biochemical tests.

Results. The six clinically relevant streptococcal species gave the expected amplification products of 229, 362, 531, 723, 819 and 978 bp for S. pneumoniae, S. galleyticus subsp. galleyticus, S. galleyticus subsp. pasteurianus, S. suis, S. intermedius and S. anginosus/S. constellatus, respectively. Non-specific reactions were not observed with the other bacterial species tested. For the 442 clinical streptococci, this mPCR assay confirmed the identity of the species in accordance with results obtained with the API 20 Strep and conventional biochemical tests.

Conclusion. This mPCR can be applied to the rapid identification of pure cultures of these six streptococci. The test was shown to be rapid, simple and reliable for the identification of these streptococci at the species level. This assay should be useful for laboratory identification and surveillance of human infections by these bacterial species.

INTRODUCTION

Streptococci are part of the normal flora and are pathogenic in humans and animals. They possess variable pathogenic potential, being responsible for meningitis, pneumonia, endocarditis, arthritis, abscesses and septicaemia. For diagnostic purposes, it is important to differentiate Streptococcus pneumoniae from other viridans group streptococci (α, γ or small-zone β-haemolytic) [1]. Identification of Streptococcus suis is also important since it is a zoonotic pathogen that causes invasive infections in humans: such infections have received increasing attention worldwide [2]. Phenotypically, S. suis resembles the viridans streptococcal species Streptococcus gordonii, Streptococcus sanguinis and Streptococcus parasanguinis, and may therefore be misidentified in many human diagnostic laboratories [3].

The Streptococcus bovis/Streptococcus equinus complex (SBSEC) comprises a variety of species and subspecies including Streptococcus galleyticus subsp. galleyticus (S. bovis biotype I), S. galleyticus subsp. pasteurianus (S. bovis biotype II/2), S. galleyticus subsp. macedonicus, Streptococcus infantarius subsp. infantarius (S. bovis biotype II/1), S. infantarius subsp. coli (S. bovis biotype II/I), S. bovis, S. equinus and Streptococcus lactolyticus [4, 5]. Of these, S. galleyticus is considered an emerging cause of infectious endocarditis, bacteremia and meningitis in humans, and cases have been reported in the USA, Scotland, Spain, Australia, Japan, Korea and Thailand [6]. Misidentification has been reported due to the biochemical diversity of SBSEC members [7].

The Streptococcus anginosus group is considered a part of the viridans group of streptococci and is composed of three
distinct species: S. anginosus (comprises subsp. anginosus and subsp. whileyi), Streptococcus intermedius and Streptococcus constellatus (comprises subsp. pharyngis, subsp. constellatus and subsp. viborgensis) [8, 9]. The S. anginosus group has been associated with multiple types of infections such as pharyngitis, bacteraemia, endocarditis, and soft tissue infections of the neck, brain, lungs and liver [9]. In addition, S. anginosus was reported to be more likely to be involved in polymicrobial infections while S. intermedius was more commonly associated with haematogenous spread and deep-seated infections and S. constellatus was more often associated with superficial infections [10]. Due to the lack of reliable biochemical profile differences, isolates of the S. anginosus group are rarely routinely identified to the species level by conventional biochemical tests in clinical laboratories [8, 9]. However, the S. anginosus group can be identified to the species level by using matrix-assisted laser desorption ionization time-of-flight MS, which provides accurate species-level identification [11].

Data from the laboratory surveillance system of the National Institute of Health (Thai-NIH), a reference laboratory in Thailand, revealed that S. pneumoniae, S. suis and S. galloiticus are the most frequent isolates of the non-β-haemolytic streptococcal group sent from many hospitals in Thailand to be confirmed at Thai-NIH (Annual reports, 2011–2015 in Thai language). Non-β-haemolytic and β-haemolytic S. anginosus group members are also frequently sent for confirmation to the Thai-NIH (Annual reports, 2011–2015 in Thai language). Identification of these streptococci using conventional biochemical tests is laborious, time-consuming and expensive (for some reagents), and may lead to misidentification. Commercial identification kits provide an alternative that is quick and easy; however, they are too expensive for many laboratories in developing countries. The need for a rapid, simple and reliable method for the identification of streptococci at the species level may be developed based on molecular methods. Therefore, the PCR method would be an attractive alternative for the identification of streptococci, due to its rapid analytical capacity and low cost. Herein, we describe a multiplex PCR (mPCR) used to identify six medically relevant streptococci, S. suis, S. pneumoniae, S. galloiticus subsp. galloiticus, S. galloiticus subsp. pasteurianus, S. intermedius and S. anginosus/S. constellatus, recovered from various clinical specimens of several diseases for diagnostic, treatment and epidemiological or outbreak investigations.

**METHODS**

**Bacterial strains**

Six reference streptococcal species, S. suis CCUG 7984, S. pneumoniae ATCC 33400, S. galloiticus subsp. galloiticus ATCC 43143, S. galloiticus subsp. pasteurianus ATCC 43144, S. intermedius ATCC 27335 and S. anginosus ATCC 33397 (a representative of the S. anginosus group) were used as positive controls for the mPCR in this study. In addition, S. constellatus subsp. pharyngis CCUG 46377 and S. constellatus subsp. constellatus ATCC 27823, members of the S. anginosus group, were also included in this study to evaluate the primers of the S. anginosus group used in the mPCR (Table 1). Collectively, 442 streptococcal isolates collected from sterile sites in seven tertiary hospitals in northeast Thailand between 2014 and 2015 were used to validate the mPCR (Table 2).

We also included reference strains of other bacterial species in this study to evaluate possible non-specific reactions. These strains were Streptococcus pyogenes SF370, Streptococcus agalactiae ATCC 13813, Streptococcus dysgalactiae subsp. equisimilis CCUG 36637, S. dysgalactiae subsp. dysgalactiae ATCC 43078, Streptococcus porcinus ATCC 43138, S. anginosus ATCC 33137, Streptococcus oralis ATCC 35037, Streptococcus mitis ATCC 6249, Streptococcus sanguinis ATCC 10556, Streptococcus gordonii ATCC 10558, Streptococcus mutans ATCC 25175, Streptococcus infantarius subsp. infantarius ATCC BAA-102, S. infantarius subsp. coli ATCC 27960, Enterococcus faecalis ATCC 29212, Enterococcus faecium ATCC 10541, Lactococcus plantarum.

### Table 1. Primers and target genes used in the mPCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
<th>Size (bp)</th>
<th>Gene</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ssuis-F</td>
<td>CTAAAGACCGTTATCAGACAACCT</td>
<td>723</td>
<td>recN</td>
<td>S. suis</td>
<td>This study</td>
</tr>
<tr>
<td>Ssuis-R</td>
<td>ATCGAACCTGGAAGACGGCTTCT</td>
<td>362</td>
<td>tanB</td>
<td>S. galloiticus subsp. galloiticus</td>
<td>This study</td>
</tr>
<tr>
<td>Sgallo-F</td>
<td>TGGTCAAGCTCAGCACAATT</td>
<td>331</td>
<td>SGPB0680 cell wall surface protein</td>
<td>S. galloiticus subsp. pasteurianus</td>
<td></td>
</tr>
<tr>
<td>Sgallo-R</td>
<td>TACACAAGCCGACGGTTCT</td>
<td>978</td>
<td>moaC</td>
<td>S. anginosus and S. constellatus</td>
<td>This study</td>
</tr>
<tr>
<td>Spasteu-F</td>
<td>GTTACGCGTTGTTCCGGTTG</td>
<td>819</td>
<td>ify</td>
<td>S. intermedius</td>
<td>[13]</td>
</tr>
<tr>
<td>Spasteu-R</td>
<td>GCTTTGAATCCGCTGCTTCTT</td>
<td>229</td>
<td>lytA</td>
<td>S. pneumoniae</td>
<td>[20]</td>
</tr>
<tr>
<td>ILY-4DFw</td>
<td>CTCACCCCTAATCTGTTGAG</td>
<td>723</td>
<td>S. suis</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>ILY-wholeC BW</td>
<td>CGACTCACTTAGGGGATCACGTGG</td>
<td>978</td>
<td>moaC</td>
<td>S. anginosus and S. constellatus</td>
<td>This study</td>
</tr>
<tr>
<td>lytA-F</td>
<td>CGGACTACGCCCTTATATC</td>
<td>819</td>
<td>ify</td>
<td>S. intermedius</td>
<td>[13]</td>
</tr>
<tr>
<td>lytA-R</td>
<td>GTTTCAATCGCAGCCGTTT</td>
<td>229</td>
<td>lytA</td>
<td>S. pneumoniae</td>
<td>[20]</td>
</tr>
</tbody>
</table>
sizes of the PCR products were determined by comparison of the bands with a molecular size standard (GeneRuler 100 bp Plus DNA ladder; Thermo Fisher Scientific).

The limit of detection of the mPCR was then evaluated. Each of the six reference species was diluted using 10-fold serial dilutions from an original concentration of OD$_{600}$ of 0.6, which was equivalent to $10^8$ c.f.u. ml$^{-1}$. Genomic DNA was prepared from 1 ml of each dilution using the QiAamp DNA Mini Kit (Qiagen). Each dilution was also plated onto sheep blood agar for quantification of colony-forming units to determine the minimum number required for the mPCRs.

RESULTS AND DISCUSSION

We developed this mPCR to identify six medically relevant streptococci, *S. suis*, *S. pneumoniae*, *S. galloyticus* subsp. *galloyticus*, *S. galloyticus* subsp. *pasteurianus*, *S. intermedius* and *S. anginosus*/*S. constellatus*, that are clinically important and have the potential to cause invasive infections and diseases, such as community-acquired pneumonia, meningitis, sepsis, septic-shock-like syndrome, infective endocarditis, gallbladder or liver parenchymal disease, and coincident colorectal carcinoma [12]. Many such invasive infections are of considerable public health concern; for example, pneumococcal disease causes over 800 000 deaths in children (<5 years old) worldwide annually, [12]. Additionally, these streptococci are difficult to identify by using biochemical tests compared to $\beta$-haemolytic streptococci such as *S. pyogenes* or *S. agalactiae*. The mPCR in this study can provide an alternative identification method for isolated colonies which are suspected to be non-$\beta$-haemolytic or small-colony-forming $\beta$-haemolytic streptococci (*S. anginosus* group suspected), recovered from various clinical specimens of several diseases for diagnostic, treatment and epidemiological investigation.

As shown in Fig. 1, the six streptococcal species were all positive, based on amplification of the 723 bp of *recN* for
Of the 442 clinical streptococci isolates collected from seven tertiary hospitals in north-east Thailand, this mPCR assay confirmed, in accordance with the biochemical tests, 38 isolates of S. suis, 62 isolates of S. pneumoniae, 45 isolates of S. gallolyticus subsp. pasteurianus, two isolates of S. gallolyticus subsp. galolyticus, one isolate of S. intermedius and 15 isolates of S. anginosus/S. constellatus (Table 2). In addition, the API 20 Strep identified the S. anginosus group as S. anginosus (n=9) and S. constellatus subsp. constellatus (n=6) (Table 2). However, 279 isolates were not detected using this mPCR, in agreement with biochemical identification that revealed S. pyogenes (n=49), S. agalactiae (n=171), S. canis (n=1), S. dysgalactiae subsp. equisimilis (n=17), the S. salivarius group (n=4), the S. mitis group (n=27) and the S. sanguinis group (n=10) (Table 2). This suggests that our mPCR is promising for use regarding the identification of these six medically relevant streptococci.

The fact that S. gallolyticus was the second most common Streptococcus found in this study after S. pneumoniae indicates the importance of this species in human infections in north-east Thailand. S. gallolyticus has also been reported to be predominant in northern Thailand and misidentifications between S. gallolyticus and S. suis have been reported [6]. Since we used a specific primer for S. suis (recN), strains of both subspecies of S. gallolyticus and S. infantarius (SBSEC) gave a negative PCR. This suggests that our mPCR has high specificity towards the bacterial species targeted by the genes and that the PCR conditions are optimal.

As shown in Table 2, the use of conventional biochemical tests failed to identify some of the streptococci when compared with the API 20 Strep and mPCR. In particular, the conventional biochemical tests could not differentiate between S. suis and the S. sanguinis group nor between the viridans streptococcal species, such as the S. mitis group, S. salivarius group, S. sanguinis group and S. anginosus group. Although the API 20 Strep could identify most streptococci included in this study to the species level, some bacterial species needed additional complementary biochemical tests to confirm them as belonging to the S. anginosus group or S. pneumoniae. For example, for the latter, evaluation of...
optochin susceptibility and bile solubility was required. Both tests required at least an additional 18–24 h to obtain results, while our mPCR combined the annealing and extension reactions into a single step that can shorten the whole PCR time by about 50 min; therefore, the mPCR process (DNA extraction + mPCR + gel electrophoresis) only needed 3 h and correctly identified these species. Although this mPCR method is less time-consuming, a drawback is that it does not differentiate S. anginosus/S. constellatus at the species level. However, Takao et al. [13] recently reported a PCR to identify species of the S. anginosus group which may be used in association with this mPCR method.

To the best of our knowledge, there is currently no report of an mPCR to detect or identify several species of these clinically relevant streptococci, such as that developed in this study. Most of the described PCRs detect or identify only single species or streptococcal groups: this is the case for S. suis [14, 15], S. gallolyticus [16], SBSEC [17, 18], S. pneumoniae [19, 20] and the S. anginosus group [13, 21].

The mPCR described herein can be applied to the identification of these six clinically relevant streptococci from pure culture. It was shown to be rapid (3 h for the process overall), simple and reliable for the identification of these six streptococci at the species level (Table 2). It can be easily used in some tertiary hospitals, university hospitals and reference laboratories where rapid diagnostic or epidemiological investigation is required. However, an improved mPCR should be developed to cover other clinically important streptococcal species, such as S. dysgalactiae, S. pseudopneumoniae, S. infantarius (SBSEC) and viridans streptococci (e.g. S. mitis, S. salivarius, S. gordoni, S. sanguinis and S. oralis), as well as to differentiate between S. constellatus (three subspecies) and S. anginosus in the S. anginosus group. Although a limitation of our mPCR is that it was developed to be used with pure cultures, an extension of this protocol for direct use with specimens would be very useful if developed and validated in the near future.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The ethics committees of the seven tertiary hospitals approved this study. Patient data were anonymized and clinical data were not collected; therefore, individual patient consent was not required.

References


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