Identification of *Staphylococcus* spp. by PCR-Restriction Fragment Length Polymorphism of *gap* Gene

JAVIER YUGUEROS,1 ALEJANDRO TEMPRANO,1 MARÍA SÁNCHEZ,1 JOSE MARIA LUENGO,2 AND GERMAÑ NAHARRO1*

Departamento de Sanidad Animal, Microbiología e Immunología1 and Departamento de Bioquímica y Biología Molecular,2 Universidad de León, 24071 León, Spain

Received 2 February 2001/Returned for modification 17 June 2001/Accepted 23 July 2001

Oligonucleotide primers specific for the *Staphylococcus aureus* *gap* gene were previously designed to identify 12 *Staphylococcus* spp. by PCR. In the present study, AluI digestion of PCR-generated products rendered distinctive restriction fragment length polymorphism patterns that allowed 24 *Staphylococcus* spp. to be identified with high specificity.

The staphylococci are considered important human and animal pathogens responsible for causing nosocomial infections (18), bacteremia (3, 13, 15), infective endocarditis (9, 15, 37, 38), cerebrospinal fluid shunt infection (12), subdural empyema (13), vertebral osteomyelitis (2, 4, 16, 38), and urinary tract infection (17). Although *Staphylococcus aureus* is the most clinically significant, other coagulase-negative staphylococci are increasingly recognized as etiologic agents of infections in humans and animals (7, 18, 21, 31, 35).

Several reports have described *Staphylococcus warneri* as the third to the fifth most common coagulase-negative staphylococci species from blood and foreign body infections (6, 13, 22, 32). For this reason, it is very important to isolate and identify the offending species in order to initiate appropriate antibiotic therapy.

*Staphylococcus* spp. have been identified by traditional phenotypic properties, available from different commercial companies (1, 14, 25, 36), and gas-liquid chromatography analysis of cellular fatty acids (33). *Staphylococcus* spp. identification systems have been in use for some isolates, but other isolates are poorly identified by these traditional methods, and supplementary tests are often required for good identification.

Molecular methods such as PCR using different DNA targets have been used successfully for the identification of staphylococci at the species level (8, 11, 20, 24, 39). The use of universal pathway genes and universal function genes whose nucleotide sequences are more conserved in bacteria as DNA targets for PCR amplification is becoming more and more frequent (11, 23, 30).

A useful PCR-based DNA amplification method combined with restriction fragment length polymorphism (RFLP) for the identification of 12 staphylococcal species has been described recently (40). PCR of the *gap* gene, which encodes a 42-kDa transferrin-binding protein located within the cell wall of *S. aureus* and a number of coagulase-negative staphylococci, was used in that study. Tpn is a member of the newly emerging family of multifunctional cell wall-associated glyceraldehyde-3-phosphate dehydrogenases (GAPDH), which catalyze the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and incorporate binding sites for both transferrin and the serine protease plasmin (26, 27, 28, 29). However, this protein is absent from *S. saprophyticus* and *S. warneri* cell wall, and these species are incapable of binding human transferrin (28).

In this study we present an expanded list of staphylococcal species which could be identified (24 species in total) by PCR amplification of the *gap* gene in combination with RFLP, using AluI restriction endonuclease.

All *Staphylococcus* spp. used in this study are detailed in Table 1. Strains were grown on Luria agar or Luria broth supplemented with glucose or yeast extract and incubated at 37°C except for *S. equorum* which was incubated at 30°C.

Chromosomal DNA from *Staphylococcus* strains was extracted following the procedure detailed elsewhere (39), and 5 μl of each sample was used for PCR analysis. Blotting and hybridization were performed by standard procedures, and DNA labeling was carried out by random priming with digoxigenin-dUTP. Hybrids were detected by enzyme immunoassay following the manufacturer’s instructions (Boehringer GmbH, Mannheim, Germany). For digestion of PCR products, a 5-μl sample was used. Restriction endonucleases were purchased from Boehringer GmbH, Mannheim, Germany.

PCR amplification tests were performed using a pair of primers selected on the basis of the *gap* gene nucleotide sequence of *S. aureus* (933 bp long; GenBank accession number AJ133520). A 26-nucleotide forward primer, GF-1 (5'-ATGG TTITGGTAGAATGGTCGTTTA-3'), corresponding to positions 22 to 47 of the *gap* gene, and a 25-nucleotide reverse primer, GR-2 (5'-GACATTTCGTTATCATACCAAGCTG-3'), corresponding to positions 956 to 932 of the previously mentioned gene, were selected. Primers were synthesized by British Bio-Technological Products (Avingdon, England). PCR amplification was carried out with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) by using a PCR kit (Boehringer GmbH) and following the manufacturer’s instructions with some modifications detailed elsewhere (40). The RFLP procedure was carried out by digesting PCR-amplified products with *AluI* endonuclease and analysis by agarose gel electrophoresis as before (40).
The primer pair GF-1 and GR-2, selected on the S. aureus gap gene sequence, successfully primed the synthesis of the expected 933-bp fragment, which represents most of the gap gene sequence, when DNA extracts from 14 species and subspecies of Staphylococcus were tested in this study (Fig. 1A). However, no PCR amplification products were obtained when S. caseolyticus high-molecular-weight DNA was used as a target (Fig. 1A, lane 15). These results agree with the new taxonomic situation of S. caseolyticus, which has recently been reclassified as Macrococcus caseolyticus (19).

A single 933-bp hybridization band was also obtained when PCR products hybridized with the Alu 279-bp internal fragment of the gap gene, which was cloned from the S. cohnii CECT 86 PCR product and digested with AluI endonuclease (data not shown). These results also demonstrate that PCR amplification of the gap gene is highly specific and a very useful tool for rapid identification of Staphylococcus spp. The list of Staphylococcus species that could be identified by gap PCR increased considerably, from 12 to 24.

In order to know whether the 14 Staphylococcus species and subspecies tested in this study could be differentiated, the 933-bp PCR-amplified products of these were AluI digested, and the resulting fragments were separated by MetaPhor agarose gel electrophoresis. A distinctive RFLP pattern was obtained for every species analyzed (Fig. 1B). S. capitis subsp. capitis and S. capitis subsp. urealyticus gave identical RFLP patterns (Fig. 1B, lanes 2 and 3), but S. cohnii subsp. cohnii and S. cohnii subsp. urealyticum gave different RFLP patterns (Fig. 1B, lanes 5 and 6). Collectively, these results show that the gap gene encoding GAPDH could be a very useful target that allows easy identification of at least 24 Staphylococcus species tested so far.

The combination of PCR and RFLP has been shown to be a powerful taxonomic tool for bacterial identification at the species level and used extensively over the last decade (5, 23, 30, 34, 40). This combination procedure of gap PCR and RFLP with AluI has been used previously (40), allowing easy and rapid identification of 12 species of Staphylococcus. In this study, we were able to increase the list of species which can be identified in a precise way to 24. The procedure discriminates between the two subspecies of S. cohnii, although it was not capable of discriminating between S. capitis subsp. capitis and S. capitis subsp. urealyticus.

This work was supported by grants from the Spanish Ministerio de Educación y Cultura (DGICYT AGF98-0187) and European funds (IFD97-1063). J.Y. is a fellowship holder at the University of León.

REFERENCES


TABLE 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>gap PCR amplification</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus arlettae</td>
<td>+</td>
<td>DMSZ 20672 (ATCC 43957)</td>
</tr>
<tr>
<td>Staphylococcus capitis</td>
<td></td>
<td>DMSZ 20326 (ATCC 27840)</td>
</tr>
<tr>
<td>Staphylococcus capitis subsp. capitis</td>
<td>+</td>
<td>DMSZ 6717 (ATCC 49326)</td>
</tr>
<tr>
<td>Staphylococcus chromogenes</td>
<td></td>
<td>DMSZ 20454 (ATCC 43764)</td>
</tr>
<tr>
<td>Staphylococcus cohnii</td>
<td></td>
<td>DMSZ 20260 (ATCC 29974)</td>
</tr>
<tr>
<td>Staphylococcus cohnii subsp. cohnii</td>
<td>+</td>
<td>DMSZ 6718 (ATCC 49930)</td>
</tr>
<tr>
<td>Staphylococcus delphini</td>
<td></td>
<td>DMSZ 20771 (ATCC 49171)</td>
</tr>
<tr>
<td>Staphylococcus equorum</td>
<td></td>
<td>DMSZ 20674 (ATCC 43958)</td>
</tr>
<tr>
<td>Staphylococcus gallinarum</td>
<td></td>
<td>DMSZ 20610 (ATCC 35539)</td>
</tr>
<tr>
<td>Staphylococcus kloosii</td>
<td></td>
<td>DMSZ 20676 (ATCC 43959)</td>
</tr>
<tr>
<td>Staphylococcus lentus</td>
<td></td>
<td>DMSZ 20352 (ATCC 29070)</td>
</tr>
<tr>
<td>Staphylococcus muscae</td>
<td></td>
<td>DMSZ 7068 (ATCC 49910)</td>
</tr>
<tr>
<td>Staphylococcus piscifermentans</td>
<td></td>
<td>DMSZ 7373 (ATCC 51136)</td>
</tr>
<tr>
<td>Staphylococcus pulvereri</td>
<td></td>
<td>DMSZ 9930 (ATCC 51698)</td>
</tr>
<tr>
<td>Staphylococcus caseolyticus</td>
<td></td>
<td>DMSZ 20597 (ATCC 13548)</td>
</tr>
</tbody>
</table>

* DMSZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; ATCC, American Type Culture Collection.

FIG. 1. (A) Agarose gel electrophoresis of PCR amplification products from Staphylococcus reference strains (Table 1) using primers GF-1 and GR-2. Lanes: 1, Staphylococcus arlettae; 2, Staphylococcus capitis subsp. capitis; 3, Staphylococcus capitis subsp. urealyticus; 4, Staphylococcus chromogenes; 5, Staphylococcus cohnii subsp. cohnii; 6, Staphylococcus cohnii subsp. urealyticum; 7, Staphylococcus delphini; 8, Staphylococcus equorum; 9, Staphylococcus gallinarum; 10, Staphylococcus kloosii; 11, Staphylococcus lentus; 12, Staphylococcus muscae; 13, Staphylococcus piscifermentans; 14, Staphylococcus pulvereri; 15, Staphylococcus caseolyticus (Macrococcus caseolyticus); M, DNA molecular mass markers (6X174 phage, HaeIII digested). (B) Agarose gel electrophoresis of the fragments produced by AluI digestion of the 933-bp PCR amplification products from Staphylococcus species. Lanes are the same as in panel A. M, DNA molecular mass markers, 50-bp ladder; bottom band, 50 bp.


