NOTE**Identification of *Staphylococcus* species by 16S–23S rDNA intergenic spacer PCR analysis**

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To investigate whether 16S–23S rDNA (rDNA) spacer region length polymorphisms are suitable for the identification of *Staphylococcus* strains, the 16S–23S rDNA intergenic spacer region lengths of 221 strains belonging to 31 species were studied by using a PCR-based method. Each species presented a specific 16S–23S pattern made of 1–8 fragments ranging from 104 to 771 bp, with the exception of the species *Staphylococcus warneri*, *Staphylococcus caprae* and *Staphylococcus piscifermentans*, which presented larger or smaller fragments. Very few species showed more than one pattern, *Staphylococcus saprophyticus* subsp. *saprophyticus* and *Staphylococcus aureus* being the most heterogeneous species (five different patterns for eight strains). Five clinical strains that could not be identified at the species level by phenotypal tests were finally identified using this method. Discrimination between some species that showed close patterns (*Staphylococcus cohnii*/*Staphylococcus chromogenes*/*Staphylococcus equorum*, *Staphylococcus aureus*/*Staphylococcus intermedius*, *Staphylococcus sciuri*/*Staphylococcus pasteurii*/*Staphylococcus gallinarum*, *Staphylococcus delphini*/*Staphylococcus felis*, *Staphylococcus vitulus*/*Staphylococcus auricularis*) was further achieved after *DraI* digestion of the PCR products. Although it does not allow discrimination of subspecies, the use of 16S–23S spacer region length data determined by PCR-mediated amplification is suitable for the identification of the 31 *Staphylococcus* species tested in this study. The method is rapid, easy and may be a useful tool for the identification of *Staphylococcus* species in the clinical microbiology laboratory.

Keywords: *Staphylococcus*, 16S–23S intergenic spacer PCR analysis, ITS-PCR, identification

Staphylococci remain among the most important pathogens isolated in the clinical microbiology laboratory (13). Phenotypic methods, including biochemical systems (9, 11, 17, 18, 19) and GLC analysis of cellular fatty acids (20) have been evaluated as identification methods for *Staphylococcus* species. However, a large number of isolates are still incorrectly or not identified by these methods, and supplementary tests are often required for complete identification (9, 19). Molecular methods that enable identification of staphylococci to the species level include ribotyping analysis (12), DNA PFGE (16), total genomic DNA–DNA hybridization, and genomic DNA hybridization to labelled probes specific to hypervariable sequences

(5). However, these methods are fairly laborious, the patterns may be difficult to interpret, and inter-laboratory reproducibility and standardization still need improvement. There is therefore a need for accurate genotyping systems that can be easily applied for the identification of *Staphylococcus* species in clinical microbiology laboratories. The genes encoding rRNA are highly conserved in bacteria and other kingdoms. With some exceptions, the rRNA operon is transcribed into one pre-rRNA transcript that contains the following sequences (in the order 5' → 3'): 16S, internal transcribed spacer, tRNA, internal transcribed spacer, 23S, internal transcribed spacer and 5S rRNA. Although the 16S and 23S rRNA genes have been used extensively to study the evolution and differentiation of bacterial species, the sequences of 16S and 23S rRNA genes are very similar from one

Abbreviation: ITS-PCR, intergenic spacer PCR analysis.

species to another within a genus, making it necessary to find other species-specific sequences. The sequence of the intergenic spacer region between these two genes, however, is more variable than the sequence of the genes themselves and has led some authors to propose the study of this 16S–23S spacer for bacterial identification (2, 8). Many bacteria have multiple copies of the rRNA operon per genome which has raised the possibility of using this method for the epidemiological typing of *Burkholderia cepacia* (14) and *Clostridium difficile* (6). The method has also been proposed for the identification and typing of many other bacteria (1, 8), and has been used previously to type *Staphylococcus aureus* isolates (3, 7, 15). In the present study, we tested 16S–23S rDNA intergenic spacer PCR analysis (ITS-PCR) as a tool to identify *Staphylococcus* isolates at the species level.

The study was carried out with 221 *Staphylococcus* strains, comprising 153 human clinical isolates, 30 animal isolates and 38 reference strains (Table 1). The human and animal strains were collected and phenotypically identified as described previously (4) by the Centre National de Référence des Staphylocoques (Lyon, France) between 1985 and 1997. Most of the clinical and animal strains were also identified by ribotyping, and some of them were identified by DNA–DNA hybridization (19). The oligonucleotide primers used for PCR amplification of the 16S–23S intergenic spacer regions have been described previously (10). Primer G1 (5′-GAAGTCGTAACAA-GG-3′) (Eurogentec), which was selected from a highly conserved region in the 16S RNA gene, is located about 30–40 nucleotides upstream from the spacer region. Primer L1 (5′-CAAGGCATCCACCGT-3′) contained the most conserved 23S sequence immediately following the spacer and is located about 20 nucleotides downstream from the spacer boundary. Primers for both the 16S and 23S regions were limited to a length of 15 nucleotides because of variations in sequence beyond these highly conserved regions. Strains were grown for 48 h at 37 °C on trypticase soy agar plates containing 5% horse blood (TSAB) (bioMérieux). A 10 µl loopful of bacteria was then resuspended in 10 ml distilled water. The bacterial suspension was centrifuged for 3 min at 3000 g. One microlitre of the pellet was disrupted by incubation in 55 µl of a 75 µg ml⁻¹ lysostaphin solution (Sigma). This mixture was incubated for 30 min at 37 °C, then 30 min at 95 °C, and finally centrifuged for 10 min at 13000 g. PCR amplification was carried out in a 100 µl (total volume) mixture containing 5 µl of the supernatant and 95 µl of a solution which contained 0.5 µM primer G1, 0.5 µM primer L1, 200 µM each dNTP (Pharmacia Biotech), 10 µl Gene Amp 10× PCR buffer (Perkin-Elmer) and 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer). PCR amplification was performed with an OmniGene TR3 CM220 thermal cycler (Hybaid). After an initial denaturation step of 2 min at 94 °C, a total of 25 cycles of amplification was performed using the following thermal profile: 1 min

at 94 °C (denaturation), 7 min at 47 °C (annealing) and 2 min at 72 °C (elongation). The last cycle was followed by a 7 min extension step at 72 °C. To check on possible contamination, a negative control containing distilled water instead of template DNA was included in each experiment. The 16S–23S rDNA intergenic spacer regions were amplified twice for each *Staphylococcus* strain included in this study. To investigate the reproducibility of the band pattern obtained by PCR amplification, ten independent PCR amplifications were performed for *Staphylococcus capitis* subsp. *capitis* CCM 2734^T, and the amplicons were included on ten different gels. Gel-to-gel variation of PCR fragment migration was assessed by comparing *S. capitis* subsp. *capitis* CCM 2734^T profiles in these gels. The mean size and standard deviation of each fragment were calculated and compared. Genomic stability was tested by passaging single colonies of *S. capitis* subsp. *capitis* CCM 2734^T from TSAB three times a week over 5 weeks. The amplified products were analysed by electrophoresis with a GNA-200 apparatus (Pharmacia Biotech) at a constant 4.5 V cm⁻¹ 4 h in 3% agarose gels (MetaPhor; FMC) containing 2 µg ethidium bromide ml⁻¹ (BioProbe System) in 0.5 × TBE buffer. The PCR products were visualized by UV fluorescence. To calculate the lengths of fragments, DNA Molecular Weight Marker VI (Boehringer Mannheim) was included on each gel every eight lanes. The gels were photographed under UV light with an MP-4 camera (Polaroid) using a Wratten 2A filter with a Polaroid type 55/pn film, and then scanned. The lengths of the PCR products of each strain were calculated automatically by using the RestrictoScan and RestrictoTyper programs (Taxolab, Institut Pasteur, Paris, France). The similarity of the strains was calculated using the Dice coefficient, and hierarchical clustering was performed using the unweighted pair group method with averages (UPGMA). For some groups of species that could not be differentiated because they showed very little variation in their patterns, the amplified products were digested with 20 U *Dra*I or *Hinf*I, as instructed by the manufacturer (Oncor Appligene). The digested PCR products and the amplicons of the same strain without digestion were run side by side on the same gel.

All the strains except *S. aureus* CCM 885^T were typeable. The mean size and the standard deviation of *S. capitis* subsp. *capitis* CCM 2734^T amplicons were calculated from the results of ten separate PCR runs. The standard deviation did not exceed 10 bp, indicating good reproducibility. The level of uncertainty in the calculated sizes of amplification products did not exceed 2.6%. Fifteen subcultures (three per week) of the same strain were amplified to test genomic stability. We did not observe a variation in the number of fragments within the 5 week test period. The standard deviation of fragment size with time did not exceed 4 bp for each of the fragments when run on the same gel. In some cases, weaker and unreproducible amplification products were observed and were not con-

Table 1. Strains used in this study

^{a, b, c, d, e, f}Strains kindly provided by W. E. Kloos, P. E. Varaldo, S. Igimi, N. El Solh, S. Tanasupawat and V. Hajek, respectively. Type strains (^T) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA), the Czechoslovak Collection of Microorganisms (CCM) (Brno, Czech Republic), the Deutsche Sammlung von Mikroorganismen (DSM) (Göttingen, Germany) and the Japan Collection of Microorganisms (JCM) (RIKEN, Saitama, Japan). *S. aureus* CCM 885^T was not typeable by ITS-PCR.

Taxon as determined by ITS-PCR (after restriction if necessary)	No. of strains	Reference strains and <i>Staphylococcus</i> isolates as identified by phenotypical or molecular methods
<i>Staphylococcus arlettae</i>	2	<i>S. arlettae</i> DSM 20672 ^T , N910254
<i>Staphylococcus aureus</i>	9	<i>S. aureus</i> CCM 885 ^T , N950351, N950382, N950448, N950522, N950534, N950565, N950603, N950619
<i>Staphylococcus auricularis</i>	3	<i>S. auricularis</i> ATCC 3753 ^T , N910215, N910217
<i>Staphylococcus capitis</i>	10	<i>S. capitis</i> subsp. <i>capitis</i> CCM 2734 ^T , N880027, N880193, N900125, N910309, N920161, N930202, N930252, N930315, N940269
<i>Staphylococcus capitis</i>	10	<i>S. capitis</i> subsp. <i>ureolyticus</i> ATCC 49326 ^T , ATCC 49325, ATCC 49324, ATCC 49327, N970267*, 3600-4 ^a , 3740-4 ^a , 4147-1 ^a , 5394-1 ^a , 5520-1 ^a
<i>Staphylococcus caprae</i>	3	<i>S. caprae</i> CCM 3573 ^T , N920116†, N920117†
<i>Staphylococcus carnosus</i>	1	<i>S. carnosus</i> DSM 20501 ^T
<i>Staphylococcus caseolyticus</i>	3	<i>S. caseolyticus</i> ATCC 13548 ^T , N880066†, N880071†
<i>Staphylococcus chromogenes</i>	3	<i>S. chromogenes</i> CCM 3387 ^T , N900352†, N930152†
<i>Staphylococcus cohnii</i>	9	<i>S. cohnii</i> subsp. <i>cohnii</i> CCM 2736 ^T , N920077, N870198, N900274, N920270, N930144, N930306, N940210, N940379
<i>Staphylococcus cohnii</i>	8	<i>S. cohnii</i> subsp. <i>urealyticum</i> ATCC 49330 ^T , N920176, N880225, N910033, N910058, N930190, N930253, N950054
<i>Staphylococcus delphini</i>	2	<i>S. delphini</i> DSM 20771 ^T , Nono ^{b†}
<i>Staphylococcus epidermidis</i>	18	<i>S. epidermidis</i> CCM 2124 ^T , N860069, N880212, N930084, N930131, N910102, N910160, N910191, N910231, N910249, N910275, N910280, N910288, N910308, N920102*, N930332*, N940056*, N940057*
<i>Staphylococcus equorum</i>	12	<i>S. equorum</i> DSM 20674 ^T , N880062†, N880063†, N940280†, N940282†, N940284†, N940298†, N940300†, N940301†, N940461†, N940462†, N940465†
<i>Staphylococcus felis</i>	3	<i>S. felis</i> ATCC 49168 ^T , S141 ^c , GD104 ^c
<i>Staphylococcus gallinarum</i>	3	<i>S. gallinarum</i> CCM 3572 ^T , N880497†, N910325†
<i>Staphylococcus haemolyticus</i>	13	<i>S. haemolyticus</i> CCM 2737 ^T , N850435, N870222, N910076, N910248, N910287, N910291, N920160, N920186, N930100, N930168, N930185, N930208
<i>Staphylococcus hominis</i>	10	<i>S. hominis</i> DSM 20328 ^T , N850447, N850456, N930160, N930207, N930305, N930317, N930329, N930341, N940139
<i>Staphylococcus hyicus</i>	3	<i>S. hyicus</i> CCM 2368 ^T , N900350†, N900354†
<i>Staphylococcus intermedius</i>	10	<i>S. intermedius</i> CCM 5739 ^T , N870338, N900260, N900269, N910201, N930300, N940276, N940453, N940457, N950082
<i>Staphylococcus kloosii</i>	1	<i>S. kloosii</i> DSM 20676 ^T
<i>Staphylococcus lentus</i>	3	<i>S. lentus</i> ATCC 29070 ^T , N850542†, N870235†
<i>Staphylococcus lugdunensis</i>	12	<i>S. lugdunensis</i> ATCC 43809 ^T , N880214, N880325, N920143, N930432, N940025, N940084, N940113, N940135, N940164, N950232, N950646
<i>Staphylococcus muscae</i>	1	<i>S. muscae</i> CCM 4175 ^T
<i>Staphylococcus pasteurii</i>	13	<i>S. pasteurii</i> ATCC 51129 ^T , BM 10426 ^d , BM 10507 ^d , N910099, N910242, N910243, N910259, N920288, N930200, N930231, N930248, N930314, N950027
<i>Staphylococcus piscifermentans</i>	3	<i>S. piscifermentans</i> JCM 6057 ^T , SK 14 ^{e†} , SK 16 ^{e†}
<i>Staphylococcus saprophyticus</i>	5	<i>S. saprophyticus</i> subsp. <i>bovis</i> CCM 4410 ^T , KV 19 ^{f†} , KV 20 ^{f†} , KV 30 ^{f†} , KV 56 ^{f†}
<i>Staphylococcus saprophyticus</i>	10	<i>S. saprophyticus</i> subsp. <i>saprophyticus</i> CCM 883 ^T , N850206, N930040, N910122, N910128, N910131, N910132, N920147, N940065, N940472
<i>Staphylococcus schleiferi</i>	3	<i>S. schleiferi</i> subsp. <i>coagulans</i> JCM 7470 ^T , GA64 ^e , N920247
<i>Staphylococcus schleiferi</i>	10	<i>S. schleiferi</i> subsp. <i>schleiferi</i> ATCC 43808 ^T , N860173, N880218, N910093, N910245, N920053, N920068, N920162, N920172, N920260
<i>Staphylococcus sciuri</i>	3	<i>S. sciuri</i> ATCC 29062 ^T , N920212, N860392
<i>Staphylococcus simulans</i>	3	<i>S. simulans</i> ATCC 27848 ^T , N900050, N850124
<i>Staphylococcus vitulus</i>	1	<i>S. vitulus</i> ATCC 51145 ^T
<i>Staphylococcus warneri</i>	9	<i>S. warneri</i> CCM 2730 ^T , N850183, N860227, N890412, N910159, N910224, N920234, N920275, N930014
<i>Staphylococcus xylosus</i>	9	<i>S. xylosus</i> ATCC 29971 ^T , N850267, N930157, N930173, N930189, N930239, N930249, N930408, N930410

* Clinical strains which could not be identified by phenotypical methods.

† Animal strain. The strains without indication, except type strains, are clinical strains.

Fig. 1. Schematic representation of PCR-amplified 16S–23S rDNA spacer region from 220 *Staphylococcus* strains representing 31 species. The number of strains tested is given in parentheses. ^aPattern 2 was presented by *S. saprophyticus* subsp. *saprophyticus* N910222, pattern 1 was presented by the nine other strains of the same species cited in Table 1. ^bPatterns 1, 2, 3, 4 and 5 were presented respectively by *S. aureus* N950351/N950448/N950619/N950603, N950534, N950522, N950382 and N950565. ^cStrains not identified by biochemical characteristics.

sidered when comparing amplifications results. Without enzyme digestion, each species presented a specific PCR pattern made of 1–8 fragments whose sizes ranged from 297 to 771 bp, with the exception of three species: *Staphylococcus warneri* which presented three larger fragments (828, 1146 and 1445 bp), *Staphylococcus caprae* which showed five smaller fragments (148, 185, 205, 227 and 273 bp), and *Staphylococcus piscifermentans* which presented special patterns with only two small fragments (104 and 205 bp) (Fig. 1). *Staphylococcus carnosus* and *Staphylococcus arlettae* also showed special patterns with only one fragment of 314 and 375 bp, respectively. When several strains of the same species were tested, the pattern showed very little variation within a species, with the exception of *S. aureus* and *Staphylococcus saprophyticus* subsp. *saprophyticus* which showed more than one pattern. Five

different patterns were observed for *S. aureus*, the most heterogeneous species. Nevertheless, the *S. aureus* patterns shared a core of common fragments: 569 bp (patterns 1–5), 457 bp (patterns 1, 3, 4, 5) and 437 bp (patterns 1–4) which allowed discrimination from other *Staphylococcus* species (Fig. 1). *S. saprophyticus* subsp. *saprophyticus* strains presented two patterns differing by two large fragments (368 and 360 bp). Pattern 2 was represented by only one strain (Fig. 1). The method did not allow discrimination of subspecies within a species (Fig. 1).

Since the species *Staphylococcus cohnii*/*Staphylococcus chromogenes*/*Staphylococcus equorum*, *S. aureus*/*Staphylococcus intermedius*, *Staphylococcus sciuri*/*Staphylococcus pasteurii*/*Staphylococcus gallinarum*/*Staphylococcus delphini*/*Staphylococcus felis*,

Fig. 2. Schematic representation of PCR-amplified 16S–23S rDNA spacer regions from 73 strains representing 14 *Staphylococcus* species after enzymic restriction with *Dra* I. The number of strains tested is given in parentheses.

Fig. 3. 16S–23S spacer region patterns of staphylococcal species. Lanes: 1, *Staphylococcus* sp. N920102; 2, *Staphylococcus* sp. N930332; 3, *S. epidermidis* CCM 2124^T; 4, *Staphylococcus* sp. N940056; 5, *Staphylococcus* sp. N940057; 6–11, the same strains after enzymic restriction with *Dra*I; 12, DNA size marker (M). The reproducibility of the technique was evaluated by the amplification of *S. capitis* subsp. *capitis* CCM 2734^T; the mean size of the five relevant bands (lane 15) were respectively 496, 388, 349, 337 and 297 bp with standard deviation of 10, 10, 9, 8 and 6 bp, respectively.

Staphylococcus vitulus/*Staphylococcus auricularis* presented a high similarity in the number and the size of fragments, we performed enzymic digestion of the PCR products before electrophoresis. Restriction of PCR products with *Dra*I allowed complete differentiation between all these species and confirmed identification to the species level (Fig. 2). Restriction of PCR products with *Hinf*I also showed a specific pattern for each of the species tested. However, this enzyme generated a greater number of fragments per profile when compared to *Dra*I. Restriction by one or the other enzyme did not improve discrimination of subspecies within a species, as shown for *S. cohnii* subsp. *cohnii* and *S. cohnii* subsp. *urealyticum* with *Dra*I digestion (Fig. 2). Finally, we tested five *Staphylococcus* sp. strains which could not be identified by phenotypical tests. The first strain, N970267, was quickly confirmed to belong to the group of novobiocin-susceptible staphylococci. Although the shape and the pigmentation of the colonies, resistance to fosfomycin, and other biochemical characters made us suspect that it was *S. capitis* subsp. *ureolyticus*, it was impossible to achieve a definite identification, mainly because of the absence of nitrate reductase and the presence of *N*-acetylglucosaminidase and pyrrolidonyl arylamidase. PCR amplification of intergenic spacers of this strain showed the pattern specific for *S. capitis* (Fig. 3), thus confirming our presumptive identification. Four additional strains (N920102, N930332, N940056 and N940057) were not identified by the ID32 STAPH system and other complementary tests (19), and were considered to be biochemical variants. Strains N940056 and N940057 were isolated from blood cultures of the same patient and were difficult to identify because of variations in some tests (D-fructose, lactose and sucrose acidification, and urease activity) when different subculture media or detection methods were used. The other two strains

were atypical because of the absence or presence of fermentations uncommonly encountered in *Staphylococcus epidermidis* isolates. DNA–DNA hybridization and ribotyping demonstrated that these four strains belonged to the species *S. epidermidis*. PCR amplification of intergenic spacers showed the pattern specific for *S. epidermidis* and confirmed these identifications (Fig. 3).

A PCR-based strategy for studying the length polymorphisms of the 16S–23S rDNA spacer regions has proved useful for both epidemiological (6, 14) and taxonomic (2, 10) investigations. In our study, PCR-mediated amplification of the 16S–23S rDNA spacer region using oligonucleotide primers G1 and L1 as described by Jensen *et al.* (10) proved to be an adequate tool for the identification of 220 *Staphylococcus* strains representing 31 species. PCR products were generated by using a unified set of primers and PCR conditions without regard to the origin of the bacterial DNA. Among *Staphylococcus* species, the lengths or nucleotide sequences of the 16S–23S rDNA spacer region, as well as the exact number of rRNA operons per genome, have only been determined with precision for *S. aureus* (7). Consequently, the lengths of amplification products could not be predicted in our study. We showed that the lengths of the 16S–23S rDNA spacer region of *Staphylococcus* species, which varied from 104 to 771 bp, are similar to the lengths described for other genera (8). Despite of similarities in the sizes of 16S–23S spacer amplification products, each of the *Staphylococcus* species examined could be identified by its PCR profile. These results were in agreement with the biochemical and phenotypical methods (4) previously used to identify these strains. The potential usefulness of restriction enzyme analysis with *Dra*I or *Hinf*I has already been reported for *S. aureus* which

presents cleavage sites for both enzymes (7). Both enzymes were tested to improve the discrimination between *Staphylococcus* species which presented a high similarity in the number and the size of 16S–23S spacer fragments. We showed that only restriction of PCR products by *Dra*I permitted the discrimination of all species tested. PCR-mediated amplification of the 16S–23S rDNA spacer region can be considered an additional tool for the identification of atypical *Staphylococcus* strains as demonstrated in this study with the identification of five atypical *Staphylococcus* strains as *S. epidermidis* (four strains) or *S. capitis* (one strain). Our choice of primers proved adequate for *Staphylococcus* species identification. However, Gürtler & Stanisich (8) suggested that, at least from a theoretical standpoint, amplification of all the 16S–23S rDNA spacer regions from a strain is most likely to be successful when using region 2 of the 16S rRNA gene and region 10 of the 23S rRNA gene for primer construction (8). Following their recommendations, we tested primers 2 and 10 on ten *Staphylococcus* species but did not obtain a better discrimination than with primers 3 and 5 (or G1 and L1) (data not shown).

Our method did not allow detection of intraspecies polymorphism among most of *Staphylococcus* species, with the exception of *S. aureus* and, in a limited fashion, *S. saprophyticus* subsp. *saprophyticus*. For *S. aureus*, we observed a high level of heterogeneity (five different PCR patterns for eight strains), confirming the results of previous studies (10, 16). Other authors who used primers 2 and 7 with *S. aureus* strains (3, 15) demonstrated the utility of these primers for studying the molecular epidemiology of *S. aureus*. However, more strains from defined outbreaks and other sources should be compared to confirm the possible application of the study of intergenic spacer length polymorphisms with G1 and L1 primers for the epidemiological typing of *S. aureus* isolates. It may also be possible to improve the separation of bands by the use of other methods such as denaturing gradient gel electrophoresis (DGGE) or single-strand conformation polymorphisms (SSCP); however, these methods are not easy to use in the routine microbiology laboratory.

In conclusion, the study of the length polymorphisms of the 16S–23S rDNA spacer region by PCR-mediated amplification with primers G1 and L1 (10), showed significant promise as a tool to identify *Staphylococcus* strains to the species level. Current on-going studies indicate that this method allows identification of a clinical *Staphylococcus* strain at the species level within 24–48 h of receipt of a pure culture, and that it can be readily and economically implemented, thus enabling routine use in the clinical microbiology laboratory.

Acknowledgements

We thank Dominique Monnet for helpful discussions.

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