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Identification of *Staphylococcus intermedius* Group by MALDI-TOF MS☆

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ABSTRACT

The *Staphylococcus intermedius* Group includes *S. intermedius*, *S. pseudintermedius* and *S. delphini*, coagulase-positive bacteria commonly isolated from animals. The identification of organisms belonging to this group is presently carried out using molecular methods. This study assessed the suitability of MALDI-TOF MS for their identification. 69 strains of different biological and geographic origins, identified by partial *hsp60* gene sequencing as *S. intermedius* ($n = 15$), *S. pseudintermedius* ($n = 32$) and *S. delphini* ($n = 22$), were analyzed by MALDI-TOF MS. The estimated sensitivity, specificity and efficiency were calculated. In addition we computed the agreement between the outcome of MALDI-TOF MS identification and partial *hsp60* gene sequencing. The sensitivity of MALDI-TOF MS was higher for *S. intermedius* [0.95 (95% CI: 0.68–0.99)], than for *S. pseudintermedius* [0.78 (95% CI: 0.60–0.90)] and *S. delphini* [0.64 (95% CI: 0.41–0.83)], whereas the specificity was 1 for *S. intermedius* and *S. delphini* and 0.97 (95% CI: 0.86–0.99) for *S. pseudintermedius*. The Cohen's kappa coefficient indicated almost perfect agreement between MALDI-TOF MS and *hsp60* gene sequencing for the identification of *S. intermedius* [0.96 (95% CI: 0.87–1.04)], and substantial agreement for *S. delphini* and *S. pseudintermedius* [0.70 (95% CI: 0.52–0.89) and 0.76 (95% CI: 0.616–0.92), respectively]. The overall efficiency of the proteomic identification ranged between 0.88 (95% CI: 0.78–0.95) for *S. pseudintermedius* and *S. delphini* and 0.99 (95% CI: 0.92–0.99) for *S. intermedius*. MALDI-TOF MS is thus a valuable and reliable tool for the rapid and accurate identification of bacteria belonging to the *S. intermedius* Group.

Introduction

The *Staphylococcus intermedius* Group (SIG) includes *S. intermedius*, *S. pseudintermedius* and *S. delphini*. The denomination SIG was first used because the three species were indistinguishable by biochemical and morphological characters [10,22]. SIG are the most common coagulase-positive staphylococci (CPS) isolated from animals, in which they may act as opportunistic pathogens and cause a variety of infections such as otitis externa, pyoderma, abscesses, reproductive tract infections, mastitis, and wound infections [21]. The identification of bacteria belonging to SIG is problematic. Phenotypic identification is unreliable, no commercial kits are available, and molecular identification is so far the only reliable tool [23]. SIG strains share many phenotypic characteristics with *Staphylococcus aureus* and this further complicates their identification [3]. *S. pseudintermedius*, and not *S. intermedius* as previously thought, is the most common CPS species isolated from cats and dogs [9]. Therefore, from a phylogenetic point of view, *S. pseudintermedius* is not a new emerging species among dogs, but rather a misidentified biotype of *S. intermedius* [15]. In veterinary medicine, failure in treatments against staphylococcal infections might stem from inadequate species identification as for example in the case of methicillin-resistant CPS isolates, for which the MIC breakpoints of oxacillin differ with species [4,19]. Thus, a reliable and accurate method allowing a fast identification of staphylococci belonging to SIG is needed.

Many methods used to identify CPS were developed before the description of *S. pseudintermedius* in 2005 [20]. Recently, Sasaki et al. [23], provided a first reliable molecular phylogenetic analysis and species identification based on partial *hsp60* gene sequences. Other genes already shown to be useful for the identification of staphylococcal species, e.g. the partial r*poB* gene [17], might also be used for the identification of SIG species, but their adequacy in identifying SIG species has not yet been proven.

Abbreviations: SIG, *Staphylococcus intermedius* Group; MALDI-TOF MS, matrix assisted laser desorption ionization-time of flight mass spectrometry; SARAMIS™, Spectral Archive and Microbial Identification System.

☆ *hsp60* sequence data are accessible under the numbers FR731134–FR731159.

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In the last decade matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been increasingly used for the identification of microorganisms because of its ease of use, the extremely small amount of sample needed and the possibility of simultaneous detection of analytes without previous isolation of bacterial strains [5]. MALDI-TOF MS was shown to be highly accurate for bacterial classification and identification even in samples with low abundances and mixed flora [8]. The technique produces a fingerprint spectrum of peptides and proteins of the analyzed microorganisms that allows an accurate identification of the bacterial species. In contrast to molecular biology, MALDI-TOF MS is a taxonomic tool with no direct phylogenetic component, being at least partly independent of the genomic features of the analyzed bacteria [18]. This technique was already successfully applied to the identification of different staphylococcal isolates, both CPS and coagulase-negative species (CNS) [7,11].

Our study aimed to assess the suitability of MALDI-TOF MS for the identification of members of the SIG complex. We calculated the estimated sensitivity, specificity and efficiency as well as the percentage of agreement in the identification of MALDI-TOF MS as compared to the sequencing of partial *hsp60* gene for the identification of strains belonging to the species *S. intermedius*, *S. pseudintermedius* and *S. delphini*.

Methods

Analyzed strains

We analyzed 69 strains belonging to the *S. intermedius* Group (SIG) and identified them by sequencing of the partial *hsp60* gene as *S. intermedius* ($n = 15$), *S. pseudintermedius* ($n = 32$) and *S. delphini* ($n = 22$). The strains had different biological and geographic origins (Table 1). We included one reference strain and one type strain (**T**) each for *S. intermedius* (LMG19136, LMG13351-**T**) and *S. pseudintermedius* (LMG22221, LMG22219-**T**) from BCCM/LMG and the type strain for *S. delphini* (CCUG 30107-**T**) from the CCUG, Sweden. The BCCM/LMG reference strain LMG19136 was identified as *S. pseudintermedius* by *hsp60* gene sequencing. All strains were stored in 7% skimmed milk at −80 °C.

DNA extraction and genetic analysis

Pure cultures were grown on blood agar at 37 °C for 24 h and genomic DNA was extracted using the InstaGene™ kit (Bio-Rad, Cat. No. 732-6030) according to the manufacturer's instructions. Genetic analyses were performed using the partial heat shock protein (*hsp60*) gene sequences. The PCR mixture consisted of 20 μl of InstaGene DNA extract, 25 μl Taq PCR Master-Mix (Cat. No. 201445), 2 μl filtered (0.2 μm) and sterilized H_2O, 1.5 μl of a 10 μM primer forward and 1.5 μl of a 10 μM primer reverse solution. The positive control consisted of 20 μl of DNA extracted from the MRSA strain ATCC43300.

The primers for the amplification of the partial *hsp60* gene sequence were Staph H279 (nucleotide sequence 5′-GAATTCG-AIIIIGCIGGIGA(TC)GGIACIACIAC-3′) and Staph H280 (nucleotide sequence 5′-CGCGGGATCC(TC)(TG)I(TC)(TG)ITCICC(AG)AAICCI-GGIGC(TC)TT-3′), which allowed the amplification of a 600 bp DNA fragment [14,16]. The PCR thermal cycling conditions were 3 min at 95 °C for 1 cycle, followed by 40 cycles of 1 min at 94 °C, 2 min at 37 °C, and 5 min at 72 °C. The last cycle was performed at 72 °C and lasted 10 min [16]. DNA amplified fragments were stained on 0.8% agar gel with GelRed (Biotium, Cat. No. 41003). DNA purification was performed using NucleoSpin® (Cat. No. 740609.250) according to the instructions for direct purification of PCR products. We quantified the amplified and purified DNA fragments before the sequencing reaction using the software NANO DROP® ND-1000.

Sequencing reactions were carried out using Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with a 15 μl total volume composed of 3 μl Big Dye® Terminator, 1.5 μl Big Dye® buffer, 2.4 μl primer 1 μM, 7.1 μl H_2O and 1 μl DNA (∼20 ng/μl) sample. Primers for the sequencing of the partial *hsp60* gene were the same as those used for the PCR [16]. The thermal cycling conditions were 1 min at 96 °C for 1 cycle, followed by 25 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C. Sequence reactions were purified on a 0.025 μm membrane filter in a Tris-EDTA buffer solution pH 8 before sequencing with HiDi™ Formamide (Applied Biosystems, P/N: 4311320) on an ABI Prism™ 310-Genetic Analyzer (Perkin Elmer Instrument, Applied Biosystems).

Proteomic analysis

Pure cultures were grown on blood agar at 37 °C for 24 h. All samples were processed with a MALDI-TOF MS Axima Confidence™ spectrometer (Shimadzu-Biotech Corp., Kyoto, Japan) in positive linear mode ($m/z = 2000–20{,}000$). A small amount of a colony of each pure culture was transferred to a FlexiMass™ target well using a disposable loop, overlaid with 0.5 μl of 2,5-dihydroxybenzoic acid matrix solution (DHB; 10 mg/ml in acetonitrile/0.1% trifluoroacetic acid 1:1) and air-dehydrated within 1–2 min at 24–27 °C.

The reference strain *Escherichia coli* K12 (GM48 genotype) was used as a standard for calibration and as reference measurement for quality control. Sample information such as medium and grown conditions was imported into the software Shimadzu Biotech Launchpad™, v.2.8 (Shimadzu-Biotech Corp., Kyoto, Japan). Protein mass profiles were obtained with detection in the linear positive mode at a laser frequency of 50 Hz and within a mass range from 2000 to 20,000 Da. Acceleration voltage was 20 kV, and the extraction delay time was 200 ns. A minimum of 20 laser shots per sample was used to generate each ion spectrum. For each bacterial sample, 50 protein mass fingerprints were averaged and processed. Spectra were analyzed using SARAMIS™ (Spectral Archive and Microbial Identification System, AnagnosTec GmbH) at default settings. We created the reference spectra (SuperSpectra) on the basis of the most discriminating peaks for a given species and for each species we selected an amount of mass to charge ratios (m/z) that were genus specific, i.e. they were present in all SIG strains. Species specific peaks had to be present only in a given species. Mass to charge ratios that were species specific were given a larger relevance, as described in the SARAMIS™ user manual. Dendrograms were based on the peak patterns of all analyzed strains submitted to single-link clustering analysis using SARAMIS™ (0.08% error, range from m/z 2000 to 20,000).

Data analysis

Genetic data were analyzed using the software ABI Prism™ 310 Collection Genetic Analyzer (Applied Biosystems). Multiple alignments were performed using the BioNumerics software v.6.01 (Applied Maths). The modular microorganism identification system AnagnosTec AXIMA@SARAMIS was used to archive and evaluate MALDI-TOF MS data. SARAMIS™ was also used to construct dendrograms to show relationships among the strains.

We calculated the estimated sensitivity and specificity and the 95% confidence intervals (CI) compared to a constructed perfect standard [12], corresponding to the identification by partial *hsp60* gene sequences. We calculated the estimated sensitivity and specificity separately for the three species *S. intermedius*, *S. pseudintermedius* and *S. delphini*, defining a positive identification by MALDI-TOF MS when the identification confidence was ≥90%. The Cohen's kappa coefficient was also computed [1].

Table 1
Description of the investigated strains, name (**T**: type strain), source, biological origin, geographic origin (B: Belgium, CH: Switzerland, CZ: Czech Republic, DK: Denmark, F: France, I: Italy, J: Japan, PL: Poland, S: Spain, UK: United Kingdom, USA: United States of America) and *hsp60* identification.

Strain	Source	Biological origin	Geographic origin	*hsp60* identification
8086	Horse	N.D.	UK	*S. delphini*
8485	Horse	N.D.	UK	*S. delphini*
9106	Horse	N.D.	UK	*S. delphini*
AV8047	Pigeon	N.D.	J	*S. delphini*
AV8051	Pigeon	N.D.	J	*S. delphini*
AV8061	Pigeon	N.D.	J	*S. intermedius*
AV8063	Pigeon	N.D.	J	*S. intermedius*
AV8081	Pigeon	N.D.	F	*S. intermedius*
CCUG 30107-**T**	Dolphin	Purulent skin lesion	I	*S. delphini*
E021	Dog	Skin (pyoderma)	USA	*S. pseudintermedius*
G1	Badger	N.D.	S	*S. delphini*
Gi1	Dog	Skin (pyoderma)	Europe	*S. pseudintermedius*
h4A	Domestic horse	Nares	J	*S. delphini*
h6C	Domestic horse	Nares	J	*S. delphini*
h-9D	Domestic horse	Nares	J	*S. delphini*
HT20030674	Camel	N.D.	F	*S. delphini*
HT20030676	Camel	N.D.	F	*S. delphini*
HT20030677	Camel	N.D.	F	*S. delphini*
HT20030679	Camel	N.D.	F	*S. delphini*
HT20030680	Camel	N.D.	F	*S. delphini*
I0005	Dog	Ear	CH	*S. pseudintermedius*
I0008	Dog	Nares	CH	*S. pseudintermedius*
I0010	Dog	Nose	CH	*S. pseudintermedius*
I0048	Dog	Nares	CH	*S. pseudintermedius*
I0049	Dog	Nares	CH	*S. pseudintermedius*
I0057	Dog	Nares	CH	*S. pseudintermedius*
I0065	Dog	Nares	CH	*S. pseudintermedius*
I0073	Dog	Ear	CH	*S. pseudintermedius*
I0075	Dog	Nares	CH	*S. pseudintermedius*
KM241	Dog	Ear (otitis externa)	CH	*S. pseudintermedius*
KM337	Dog	Ear (otitis externa)	CH	*S. pseudintermedius*
KM1087	Dog	Vaginal mucosa (vaginitis)	CH	*S. pseudintermedius*
KM1250	Dog	Infected wound	CH	*S. pseudintermedius*
KM1381	Dog	Fistula after surgery	CH	*S. pseudintermedius*
KM1591	Dog	Pyoderma	CH	*S. pseudintermedius*
LMG13351-**T**	Pigeon	Nares	CZ	*S. intermedius*
LMG19136	Dog	Skin	B	*S. pseudintermedius*
LMG22219-**T**	Cat	Lung tissue	B	*S. pseudintermedius*
LMG22221	Dog	Ear (otitis)	B	*S. pseudintermedius*
M0612	Dog	Skin (pyoderma)	USA	*S. pseudintermedius*
M1	Mink	N.D.	DK	*S. delphini*
M86	Mink	N.D.	DK	*S. delphini*
NVAU02012	Dog	Wound pus from skin	J	*S. pseudintermedius*
NVAU02031	Dog	Wound pus from skin	J	*S. pseudintermedius*
NVAU02083	Cat	Wound pus from skin	J	*S. pseudintermedius*
P2A	Wild pigeon	Nares	J	*S. intermedius*
P4A	Wild pigeon	Nares	J	*S. intermedius*
P6A	Wild pigeon	Nares	J	*S. intermedius*
P9B	Wild pigeon	Nares	J	*S. intermedius*
P26	Domestic pigeon	Nares	J	*S. delphini*
P27B	Domestic pigeon	Nares	J	*S. delphini*
P30A	Domestic pigeon	Nares	J	*S. delphini*
P45A	Wild pigeon	Nares	J	*S. intermedius*
P46A	Wild pigeon	Nares	J	*S. intermedius*
P50	Wild pigeon	Nares	J	*S. delphini*
P52B	Wild pigeon	Nares	J	*S. intermedius*
P53	Wild pigeon	Nares	J	*S. intermedius*
P54A	Wild pigeon	Nares	J	*S. intermedius*
P69A	Wild pigeon	Nares	J	*S. intermedius*
P66A	Wild pigeon	Nares	J	*S. intermedius*
PL1	Dog	Ear (otitis)	PL	*S. pseudintermedius*
PL2	Dog	Skin of head	PL	*S. pseudintermedius*
PL3	Dog	Nose	PL	*S. pseudintermedius*
PL5	Dog	Skin (dermatosis)	PL	*S. pseudintermedius*
PL6	Dog	Skin	PL	*S. pseudintermedius*
RPC05C0284	Human	N.D.	F	*S. pseudintermedius*
S61H7	Horse	Skin (inflammation)	DK	*S. delphini*
SD1071	Dog	Nares	CH	*S. pseudintermedius*
TW6698	Human	Wound pus	J	*S. pseudintermedius*

Results

The estimated sensitivity of MALDI-TOF MS for the identification of *S. intermedius* was 0.95 (95% CI: 0.68–0.99), of *S. pseudintermedius* 0.78 (95% CI: 0.60–0.90), and of *S. delphini* 0.64 (95% CI: 0.41–0.83); the estimated specificity was 1.00 for *S. intermedius* and *S. delphini* and 0.97 (95% CI: 0.86–0.99) for *S. pseudintermedius*. The efficiency of identification was 0.99 (0.92–0.99) for *S. intermedius* and 0.88 (95% CI: 0.78–0.95) for *S. pseudintermedius* and *S. delphini*. The Cohen's kappa was 0.96 (95% CI: 0.87–1.04) for *S. intermedius*, 0.76 (95% CI: 0.62–0.92) for *S. pseudintermedius* and 0.70 (95% CI: 0.52–0.89) for *S. delphini*.

Spectra with specific peaks for the type strains of the three investigated species are shown in Fig. 1. Spectra of strains belonging to the same species displayed a high level of similarity; within the same species, however, some variation in the pattern composition and the measured relative intensities were observed (e.g. *S. intermedius*, Fig. 2). We created 2 new SuperSpectra for the identification of *S. intermedius*, 3 for *S. pseudintermedius* and 2 for *S. delphini*. The selected mass to charge ratios ranged from 2002.8 *m*/*z* to 19,883.7 *m*/*z* (error 0.08%), with an average of 21.14 ± 1.46 (SD) different mass to charge ratios used for the creation of each SuperSpectra. Details are reported in Table 2.

Within the strains identified as *S. pseudintermedius* with the created SuperSpectra 65.6% (21/32) strains were correctly identified with a confidence of 99.9%, 12.5% (4/32) with a confidence between 99.8% and 90%, and 21.9% (7/32) with a confidence lower than 90%. For *S. intermedius* 93.3% (14/15) of the strains were correctly identified with a confidence of 99.9% and 6.7% (1/15) with a confidence lower than 90%. For *S. delphini*, 31.8% (7/22) of the strains were correctly identified with a confidence of 99.9%, 31.8% (7/22) with a confidence between 99.8% and 90%, and 36.4% (8/22) with a confidence lower than 90%.

The dendrogram showed two main clusters, one including all *S. intermedius* and the other *S. pseudintermedius* and *S. delphini*. The latter two where closer in the dendrogram but all the strains identified as *S. delphini* formed a cluster distinct from all *S. pseudintermedius* isolates (Fig. 3). All 7 strains identified as *S. pseudintermedius* with a confidence lower than 90% were included in the cluster of *S. pseudintermedius*. Isolate P2A, one of the two

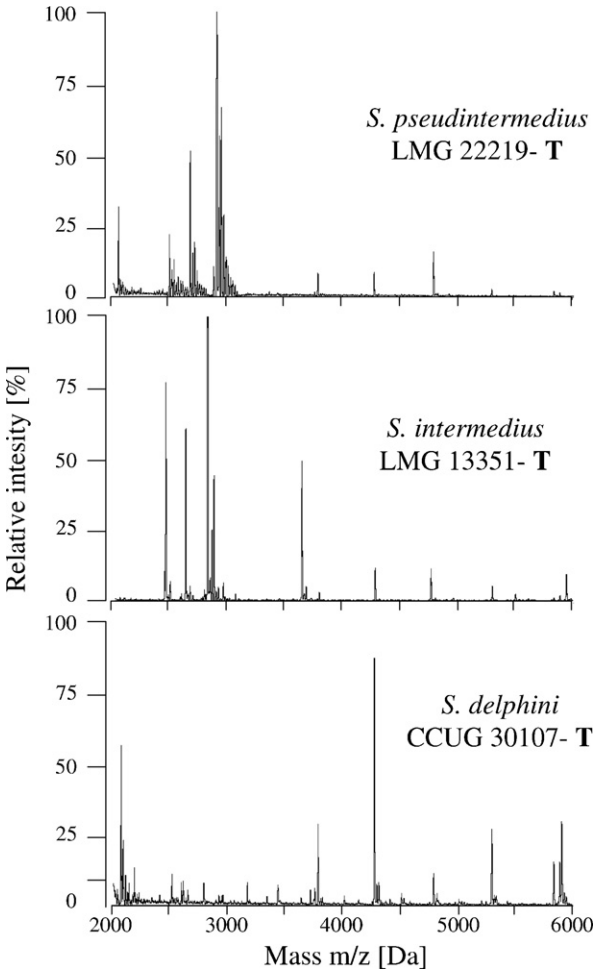

Fig. 1. Representative spectra of the type strains *S. pseudintermedius* LMG 22219-**T**, *S. intermedius* LMG 13351-**T** and *S. delphini* CCUG 30107-**T** with relative intensity [%] of the protein profile peaks [*m*/*z*] ranging between 2000 and 6000 Da. **T**: type strain.

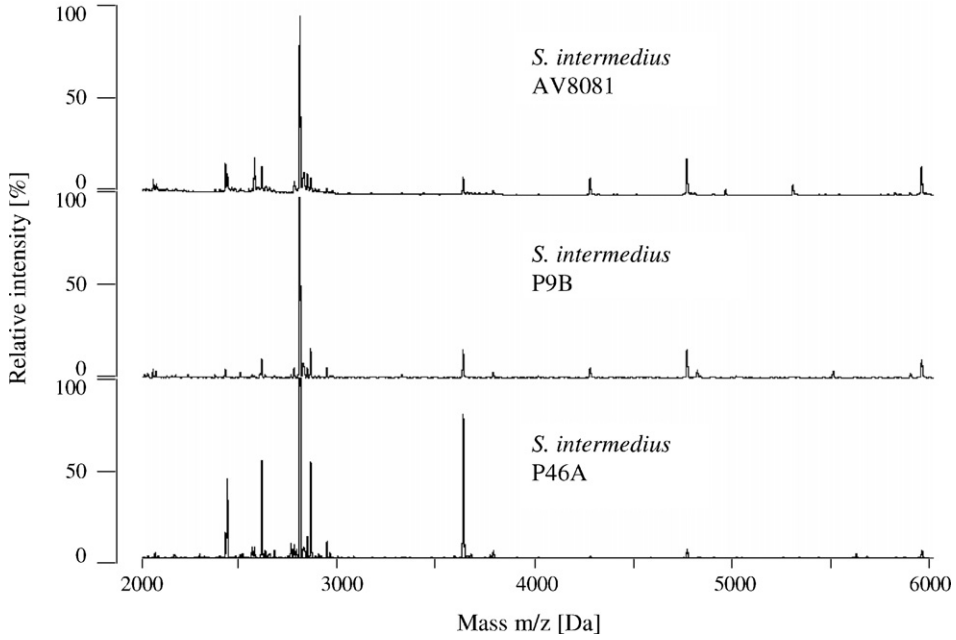

Fig. 2. Spectra of different *S. intermedius* strains with relative intensity [%] of the protein profile peaks [*m*/*z*] ranging between 2000 and 6000 Da.

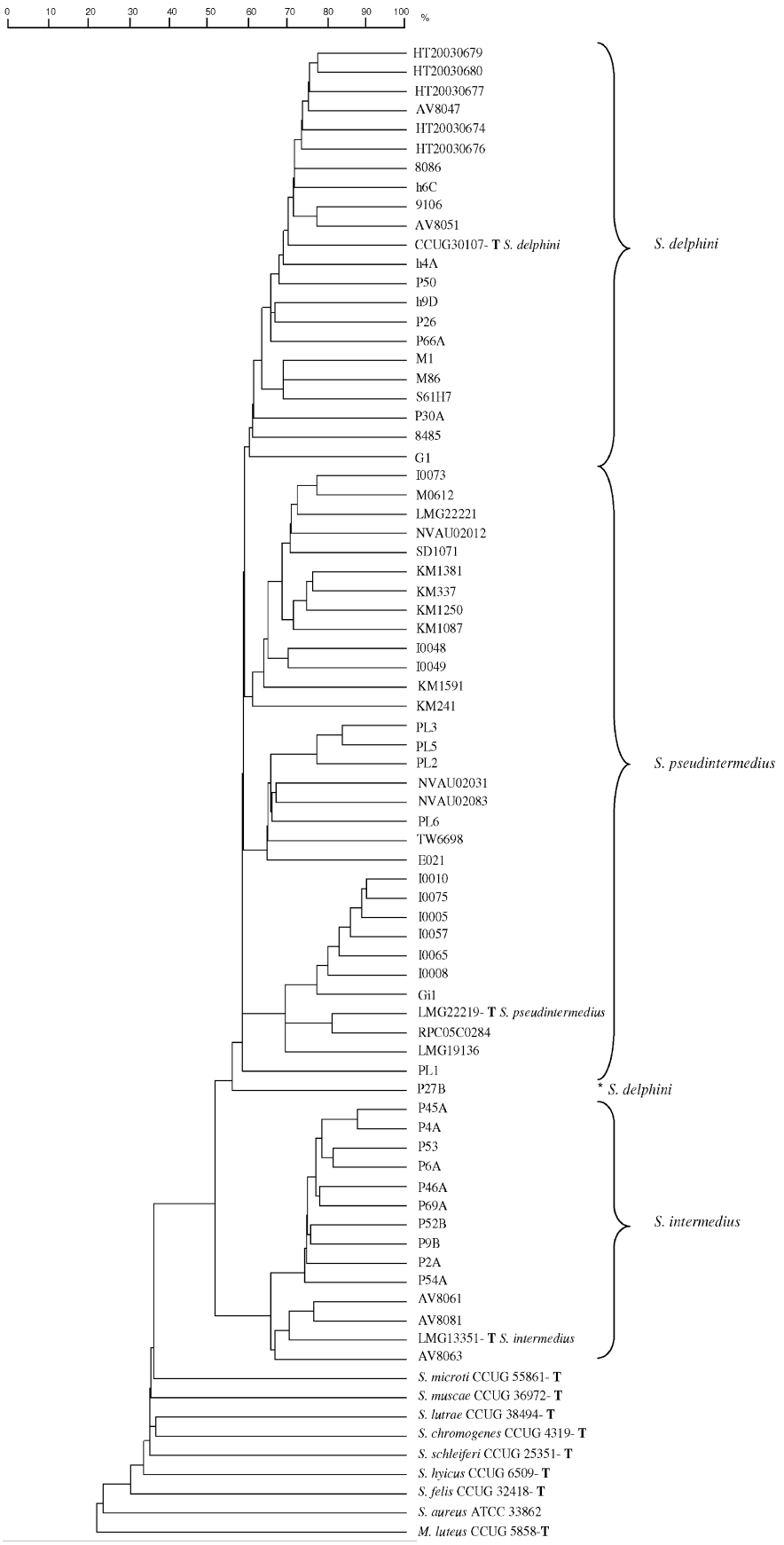

Fig. 3. Dendrogram resulting from single-link clustering analysis (SARAMIS™ database software) of MALDI-TOF MS on *Staphylococcus intermedius* Group strains. Error 0.08%; range of *m*/*z* from 2000 to 20,000 Da. **T**: type strain.

Table 2
Mass to charge ratios (m/z) used for the creation of the different SuperSpectra. Error 0.08%.

SuperSpectra_Staphylococcus_intermedius_ICM_13may 2009				
2226.1	2304.1	2580.6	2634.7	2810.6
2827.1	2849.0	2866.0	2904.1	2947.3
2963.5	3638.1	3676.3	3790.9	4771.5
5021.3	5627.5	5959.9	6663.0	7602.2
8012.7				
SuperSpectra_Staphylococcus_intermedius_ICM_12may 2009				
2024.8	2036.0	2042.0	2067.8	2091.4
2226.4	2304.3	2327.2	2569.0	2634.6
2651.0	2681.7	2849.1	2865.9	3084.0
3195.6	3654.2	4282.1	5022.1	6662.9
SuperSpectra_Staphylococcus_delphini_ICM_12may 2009				
2024.8	2042.1	2068.1	2363.8	2495.0
2538.5	3416.6	4146.5	4185.4	4624.6
5790.7	6057.1	6619.7	8076.4	8620.7
9955.9	10,006.3	10,843.5	14,809.2	19,883.7
SuperSpectra_Staphylococcus_delphini_ICM_27may 2009				
2041.6	2362.9	2433.9	2593.2	2609.2
2689.2	2922.0	3342.0	3438.1	4829.0
5849.8	5920.5	5945.6	6123.0	6248.6
6717.5	7299.7	8066.4	9039.5	9084.3
9679.0				
SuperSpectra_Staphylococcus_pseudintermedius_ICM_12may 2009				
2137.0	2188.8	2510.6	2697.1	2713.0
2732.2	2917.3	3785.1	3820.8	4183.9
4275.3	5302.4	5845.4	6343.9	6731.2
6751.1	6767.5	7443.3	9035.7	9626.9
SuperSpectra_Staphylococcus_pseudintermedius_ICM_14may 2009				
2132.7	2163.4	2180.5	2239.0	2357.8
2379.9	2489.2	2527.7	2541.8	2564.5
2587.6	2669.8	2845.7	2910.7	3361.1
3431.8	3757.1	3784.6	4165.3	4275.5
7205.0	8479.7			
SuperSpectra_Staphylococcus_pseudintermedius_ICM_14may 2009-2				
2002.8	2005.4	2006.6	2010.8	2066.5
2083.0	2120.2	2471.3	2493.8	2516.2
2553.6	2567.7	2570.2	2589.3	2610.6
2674.7	2693.0	2751.4	2779.2	2876.4
2917.4	6732.3	6767.2	7545.8	

identified as *S. intermedius* with a confidence lower than 90%, was included in the *S. intermedius* and isolate P66A in the *S. delphini* cluster. All strains identified as *S. delphini* with a confidence lower than 90%, with the exception of isolate P27B, belonged to the *S. delphini* cluster (Fig. 3).

Discussion

MALDI-TOF MS can be used to reliably identify bacterial species belonging to SIG. The estimated sensitivity of MALDI-TOF in the identification of the SIG species was higher for *S. intermedius* than for *S. pseudintermedius* and *S. delphini*, whereas the estimated specificity was 1 for *S. intermedius* and *S. delphini* and 0.97 for *S. pseudintermedius*. The Cohen's kappa coefficient indicated almost perfect agreement between MALDI-TOF MS and *hsp60* gene sequencing in the identification of *S. intermedius* and substantial agreement for *S. delphini* and *S. pseudintermedius*. The overall efficiency of the proteomic identification was quite high and ranged between 88% and 99% for *S. pseudintermedius*–*S. delphini* and *S. intermedius*, respectively.

We based the choice of the constructed standard used in this study, the *hsp60* gene, for the calculation of the estimated specificity, sensitivity, efficacy and agreement on the results of the work carried out by Sasaki et al. [23]. The choice of another constructed standard (e.g. the *nuc*, *gap*, or *sodA* genes [13,23]) might have led to slightly different agreement values between the two identification methods, but overall the identification with MALDI-TOF MS showed to be robust enough to allow the creation of reliable SuperSpectra.

In the last year new genetic tests have been described for the identification of CPS species. For example, the PCR-RFLP method based on *pta* gene allows accurate differentiation of *S. pseudintermedius* from the other SIG species and also from other important human and animal pathogenic staphylococcal species such as *Staphylococcus schleiferi* and *S. aureus* [2]. This approach, however, does not allow conclusive identification of other SIG species. Recently, Blaiotta et al. have described the same methodological approach but based on another housekeeping gene, the *kat* gene, which allows unambiguous identification of CPS, including *S. pseudintermedius* and *S. delphini* [6]. A multiplex-PCR method based on the *nuc* gene was also shown to reliably identify CPS [24]. All these methods are based on the analyses of genetic components of the investigated bacteria and thus need the classic approach of culture of the organism followed by DNA extraction, PCR amplification and gel staining for the detection of amplified fragments. This genetic approach allows reliably identification of the investigated species but it is time-consuming if compared with the MALDI-TOF MS proteomic approach which shows equivalent efficiency.

MALDI-TOF MS allows rapid and accurate identification of SIG bacteria within 24 h, provided a reliable comparison database is available. This means that for each group of microorganisms careful phylogenetic characterization of a sufficient number of geographically and genetically diverse isolates of the species under consideration is needed before they can be used to construct SuperSpectra. In fact, SuperSpectra for *S. intermedius* previously present in the database led to erroneous identification of strains in the present study. This relates most probably to an insufficient characterization of the strains used for the creation of these SuperSpectra. It is therefore crucial to use only fingerprints of strains well characterized by phylogenetic studies (e.g. by analysis of at least two different genes) for the creation of SuperSpectra. The SuperSpectra for *S. pseudintermedius*, *S. intermedius* and *S. delphini* described in Table 2 have been constructed according to these criteria and have been shown to be highly reliable.

The created SuperSpectra were able to identify only 31.8% of the *S. delphini* strains with a confidence greater than 99%. This reflects the high heterogeneity within the *S. delphini* group which has been already described in a molecular study suggesting the presence of a new species within the *S. delphini* group and dividing this species in two groups, A and B [23]. In particular the strain P27B was identified as *S. delphini* by partial *hsp60* gene sequence and by MALDI-TOF with a confidence of 79.5%; in the dendrogram resulting from the MALDI-TOF analysis, however, it was not included in the cluster of *S. delphini* strains but was closer to the *S. pseudintermedius* strains. The position of strain P27B, known to belong to *S. delphini* group B [24], might be explained by the fact that strains of group B are more closely related to those of *S. pseudintermedius* than to *S. delphini* group A [23].

This study has provided evidence of the validity and usefulness of MALDI-TOF MS for a rapid, comparatively cheap and reliable identification of bacterial isolates belonging to the *S. intermedius* Group. Additional work with MALDI-TOF MS, coupled with corresponding phylogenetic analyses, may allow better insight in the ongoing speciation within *S. delphini* as well as the geographic validation of the newly created SuperSpectra for *S. pseudintermedius* and *S. intermedius* with a larger number of collected strains.

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References

[1] Attermann, J. (2003) On the agreement between two diagnostic methods with binary outcomes. Scand. J. Clin. Lab. Invest. 63, 525–528.
[2] Bannoehr, J., Franco, A., Iurescia, M., Battisti, A., Fitzgerald, J.R. (2009) Molecular diagnostic identification of *Staphylococcus pseudintermedius*. J. Clin. Microbiol. 47, 469–471.
[3] Baron, F., Cochet, M.F., Pellerin, J.L., Ben Zakour, N., Lebon, A., Navarro, A., Proudy, I., Le Loir, Y., Gautier, M. (2004) Development of a PCR test to differentiate between *Staphylococcus aureus* and *Staphylococcus intermedius*. J. Food Prot. 67, 2302–2305.
[4] Bemis, D.A., Jones, R.D., Hiatt, L.E., Ofori, E.D., Rohrbach, B.W., Frank, L.A., Kania, S.A. (2006) Comparison of tests to detect oxacillin resistance in *Staphylococcus intermedius*, *Staphylococcus schleiferi*, and *Staphylococcus aureus* isolates from canine hosts. J. Clin. Microbiol. 44, 3374–3376.
[5] Bizzini, A., Greub, G. (2010) MALDI-TOF MS, a revolution in clinical microbial identification. Clin. Microbiol. Infect. 16, 1614–1619.
[6] Blaiotta, G., Fusco, V., Ercolini, D., Pepe, O., Coppola, S. (2010) Diversity of Staphylococcus species strains based on partial kat (catalase) gene sequences and design of a PCR-restriction fragment length polymorphism assay for identification and differentiation of coagulase-positive species (*S. aureus*, *S. delphini*, *S. hyicus*, *S. intermedius*, *S. pseudintermedius*, and *S. schleiferi* subsp. coagulans). J. Clin. Microbiol. 48, 192–201.
[7] Carbonnelle, E., Beretti, J.L., Cottyn, S., Quesne, G., Berche, P., Nassif, X., Ferroni, A. (2007) Rapid identification of Staphylococci isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. 45, 2156–2161.
[8] Claydon, M., Davey, S., Edwards-Jones, V., Gordon, D. (1996) The rapid identification of intact microorganisms using mass spectrometry. Nat. Biotechnol. 14, 1584–1586.
[9] Devriese, L.A., Hermans, K., Baele, M., Haesebrouck, F. (2009) *Staphylococcus pseudintermedius* versus *Staphylococcus intermedius*. Vet. Microbiol. 133, 206–207.
[10] Devriese, L.A., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., Decostere, A. (2005) *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. Int. J. Syst. Evol. Microbiol. 55, 1569–1573.
[11] Dubois, D., Leyssene, D., Chacornac, J.P., Kostrzewa, M., Schmit, P.O., Talon, R., Bonnet, R., Delmas, J. (2010) Identification of a variety of Staphylococcus species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. 48, 941–945.
[12] FDA. (2007) Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests.
[13] Ghebremedhin, B., Layer, F., Konig, W., Konig, B. (2008) Genetic classification and distinguishing of Staphylococcus species based on different partial gap, 16S rRNA, hsp60, rpoB, sodA, and tuf gene sequences. J. Clin. Microbiol. 46, 1019–1025.
[14] Goh, S., Potter, S., Wood, J., Hemmingsen, S., Reynolds, R., Chow, A. (1996) HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. J. Clin. Microbiol. 34, 818–823.
[15] Hesselbarth, J., Schwarz, S. (1995) Comparative ribotyping of *Staphylococcus intermedius* from dogs, pigeons, horses and mink. Vet. Microbiol. 45, 11–17.
[16] Kwok, A.Y.C., Su, S.C., Reynolds, R.P., Bay, S.J., Av-Gay, Y., Dovichi, N.J., Chow, A.W. (1999) Species identification and phylogenetic relationships based on partial HSP60 gene sequences within the genus Staphylococcus. Int. J. Syst. Evol. Microbiol. 49, 1181–1192.
[17] Mellmann, A., Becker, K., von Eiff, C., Keckevoet, U., Schumann, P., Harmsen, D. (2006) Sequencing and staphylococci identification. Emerg. Infect. Dis. 12, 333–336.
[18] Petersen, C.E., Valentine, N.B., Wahl, K.L. (2009) Characterization of microorganisms by MALDI mass spectrometry. Methods Mol. Biol. 492, 367–379.
[19] Pottumarthy, S., Schapiro, J.M., Prentice, J.L., Houze, Y.B., Swanzy, S.R., Fang, F.C., Cookson, B.T. (2004) Clinical isolates of *Staphylococcus intermedius* masquerading as methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. 42, 5881–5884.
[20] Roberson, J.R., Fox, L.K., Hancock, D.D., Besser, T.E. (1992) Evaluation of methods for differentiation of coagulase-positive staphylococci. J. Clin. Microbiol. 30, 3217–3219.
[21] Ruscher, C. (2009) Prevalence of Methicillin-resistant *Staphylococcus pseudintermedius* isolated from clinical samples of companion animals and equidaes. Vet. Microbiol. 136, 197–201.
[22] Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S., Hiramatsu, K. (2007) Methicillin-resistant *Staphylococcus pseudintermedius* in a veterinary teaching hospital. J. Clin. Microbiol. 45, 1118–1125.
[23] Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S., Hiramatsu, K. (2007) Reclassification of phenotypically identified *Staphylococcus intermedius* strains. J. Clin. Microbiol. 45, 2770–2778.
[24] Sasaki, T., Tsubakishita, S., Tanaka, Y., Sakusabe, A., Ohtsuka, M., Hirotaki, S., Kawakami, T., Fukata, T., Hiramatsu, K. (2010) Multiplex-PCR method for species identification of coagulase-positive staphylococci. J. Clin. Microbiol. 48, 765–769.