ORIGINAL ARTICLE BACTERIOLOGY

Species differentiation within the *Staphylococcus intermedius* group using a refined MALDI-TOF MS database

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Abstract

Among coagulase-positive staphylococci of animal origin, the members of the *Staphylococcus intermedius*-group (SIG: *S. intermedius*, *Staphylococcus pseudintermedius* and *Staphylococcus delphini*) are important opportunistic pathogens in different animal hosts and occasionally in humans. However, the unambiguous species diagnosis of SIG is often challenging. Therefore, matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) -based SIG-identification with Bruker Microflex LT in combination with BIOTYPER 3.0 software (Bruker Daltonics, Bremen, Germany) was evaluated using (i) the original database content and (ii) the database after extension with distinct hierarchical clustered reference spectra for 60 SIG. A convenience sample comprising 200 isolates was used to compare both database performances. As a result, 17 isolates initially diagnosed as *S. intermedius* with the current content of the Bruker database were identified as *S. pseudintermedius* by applying the in-house reference spectra extended version. Furthermore, a significant improvement (average rise of log score value: 0.24) of the SIG identification score values was achieved, emphasizing that further sequence-based refinement of the Bruker database content allows improvement of MALDI-TOF MS-based identification.

Keywords: BIOTYPER, coagulase positive staphylococci, matrix-assisted laser desorption ionization—time of flight mass spectrometry, microflex, *Staphylococcus intermedius*-group, *Staphylococcus pseudintermedius*
Original Submission: 20 February 2014; **Revised Submission:** 28 April 2014; **Accepted:** 2 May 2014
Editor: G. Greub
Article published online: 8 May 2014
Clin Microbiol Infect 2014; **20**: 1007–1015
10.1111/1469-0691.12662

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Introduction

Coagulase-positive staphylococci (CPS) are considered as typical opportunistic pathogens and capable of causing a wide range of different purulent and toxin-mediated diseases in humans and animals. While *Staphylococcus aureus* is the predominant CPS species associated with staphylococcal diseases in humans, *Staphylococcus pseudintermedius* seems to have a similar importance for dogs [1,2]. Further, a rise in reports on methicillin-resistant *S. pseudintermedius* (MRSP), which were frequently associated with multidrug resistance, is a major concern in veterinary medicine, highlighting the need for reliable and fast identification of these bacteria [1,3–7]. In recent years, gene-based approaches have shown that CPS phenotypically identified as *Staphylococcus intermedius* belong to three closely related but distinct species, namely *S. intermedius*, *Staphylococcus delphini* and *S. pseudintermedius*, which represent the *Staphylococcus intermedius*-group (SIG) [8]. These results suggest that most canine isolates previously identified as *S. intermedius* should have been classified as *S. pseudintermedius* [9]. At present, classical biochemical differentiation between the members of the SIG is at the least complex and may result in unreliable or insufficient species identification [10]. A reliable method to discriminate *S. pseudintermedius* from other

SIG members and *S. aureus* by demonstrating a certain *Mbo*I restriction site of the housekeeping gene *pta* was published in 2009 [11]. However, this approach is time-consuming and not suitable for 'high throughput' laboratories.

Other methods, such as intact protein profiling through matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) have been used to demonstrate the possibility of rapid differentiation of SIG [12]. This was achieved using a commercial software tool, Axima-Assurance-Shimadzu/SARAMIS-AnagnosTec (now renamed as VITEK MS) (bioMérieux, Nürtingen, Germany). However, the Bruker Biotyper database has not been evaluated for differentiation of the SIG yet. Our aim was to refine the Biotyper database by including additional SIG strains, which were identified by sequence-based methods [8]. In order to test the reliability and advantage of the additional database content, 200 putative SIG isolates formerly diagnosed by biochemical methods obtained from various sources and hosts were investigated by MALDI-TOF MS and the resulting spectra were analysed twice: First using the current Bruker database content and afterwards with the refined in-house database.

Materials and Methods

Selection of CPS strains for the MALDI-TOF MS spectra library

To build up a reliable reference database for spectra associated with the different members of the SIG, *S. pseudintermedius* ($n = 43$, including 20 MRSP), *S. intermedius* ($n = 5$) and *S. delphini* ($n = 12$) that originated from different sources (see Table 1) were initially selected. Unambiguous species identity of each strain was demonstrated by partial sequencing of the genes *pta*, *cpn*60, *tuf* and 16S rRNA according to previously published methods [8] (Table 1). The obtained sequences were concatenated and based on the corresponding alignment a clustering was performed using unweighted pair group method with arithmetic mean with Geneious R7 (Biomatters, Auckland, New Zealand).

Other CPS such as *S. aureus* ($n = 8$) and *Staphylococcus schleiferi* ssp. *coagulans* ($n = 2$) were included as control strains. Species verification of *S. aureus* was demonstrated by PCR [13] and *S. schleiferi* ssp. *coagulans* was verified by sequencing 1100 bp of 16S rRNA gene [14].

Protein extraction and MALDI-TOF MS measurements

The protein extraction as well as the MALDI-TOF MS measurements on a Microflex LT instrument using FlexControl 3.0 software (Bruker Daltonics, Leipzig, Germany) were carried out as described in Murugaiyan *et al.* [15], with minor modifications. In brief, each SIG isolate was cultured on Müller Hinton agar with 5% sheep blood (Becton Dickinson, Heidelberg, Germany) and extracted using a standard formic acid/acetonitrile procedure. The samples were spotted on the 'MicroScout Target' plate (Bruker Daltonics, Bremen, Germany) using saturated α-cyano-4-hydroxycinnamic acid matrix solution. Measurements were carried out in a range of 2000–20 000 m/z with the following instrument parameters: ion source 1 at 20 kV, ion source 2 at 16.3 kV, lens at 7 kV, extraction delay time of 200 ns, initial laser power of 30%, maximum laser power of 40%, and laser attenuation offset of 38% (range, 30%).

Spectral analysis and reference spectra

A total of 27 spectra were acquired for each investigated isolate (60 SIG and ten other CPS), evaluated and the reference spectra were created using Biotyper 3.0 software (Bruker Daltonics) as described recently by Murugaiyan, *et al.*, [15]. These created references were then assigned to their respective nodes on the taxonomy tree of the Bruker Biotyper database using a library of 4613 entries (January 2014).

Cluster analysis and SIG discrimination

The discriminative power for SIG was evaluated by computing the MALDI-TOF MS reference spectra dendrogram based on elucidation distance measurement and the average-linkage algorithm settings of the Biotyper 3.0 software. The resulting dendrogram was compared with the phylogenetic tree obtained from the sequences of pta, cpn60, tuf and 16S rRNA gene.

Evaluation of the SIG database using 200 staphylococcal isolates

A convenience sample consisting of 193 clinical CPS strains and seven strains from the culture collection of the University of Göteborg (CCUG; see Supporting information, Table S1) represents a broad range of clinical infection sites, hosts and geographic origins since the year 2000. The clinical strains were previously diagnosed as described by Bannerman and Peacock [16] or by using the automated Vitek®2 system (bioMérieux, Nürtingen, Germany) following the manufacturer's instructions.

Overnight cultures were directly transferred on a MALDI-TOF MS target plate, overlaid with saturated α-cyano-4-hydroxycinnamic acid, air dried and subjected to MALDI-TOF MS identification. The identification was carried out following the recommendations of the Bruker system, with identification log (score) values ranging from 0 to 3. A result associated with score values from 0 to 1.699 indicates 'no reliable identification' (NRI); score values from 1.7 to 1.999 indicate a 'probable

TABLE 1. List of well-defined staphylococcal strains included for the creation of a main spectral library for improvement of the distinction among the members of the *Staphylococcus intermedius* group

S. No	Staphylococcal species	ID	Host	*mecA*[a]	Origin	Reference
1	*Staphylococcus pseudintermedius*[b]	BFI38202	Dog	pos.	France	[6]
2	*S. pseudintermedius*[b]	BFI38203	Cat	pos.	France	[6]
3	*S. pseudintermedius*[b]	CCUG32915	Dog	neg.	Sweden	±
4	*S. pseudintermedius*[b]	IMT10762	Dog	pos.	Germany	[6]
5	*S. pseudintermedius*[b]	IMT11384	Dog	pos.	Germany	[6]
6	*S. pseudintermedius*[b]	IMT11897	Dog	pos.	Germany	[6]
7	*S. pseudintermedius*[b]	IMT12531	Dog	pos.	Germany	[6]
8	*S. pseudintermedius*[b]	IMT12559	Dog	pos.	Germany	[6]
9	*S. pseudintermedius*[b]	IMT14052	Horse	pos.	Germany	[6]
10	*S. pseudintermedius*[b]	IMT14529	Dog	pos.	Germany	[6]
11	*S. pseudintermedius*[b]	IMT14532	Dog	pos.	Germany	[6]
12	*S. pseudintermedius*[b]	IMT17831	Human	neg.	Germany	#
13	*S. pseudintermedius*[b]	IMT17931	Human	neg.	Germany	#
14	*S. pseudintermedius*[b]	IMT17932	Human	pos.	Germany	#
15	*S. pseudintermedius*[b]	IMT18786	Dog	neg.	Germany	[7]
16	*S. pseudintermedius*[b]	IMT18789	Dog	neg.	Germany	[7]
17	*S. pseudintermedius*[b]	IMT18796	Dog	neg.	Germany	[7]
18	*S. pseudintermedius*[b]	IMT18797	Human	neg.	Germany	[7]
19	*S. pseudintermedius*[b]	IMT18798	Dog	neg.	Germany	[7]
20	*S. pseudintermedius*[b]	IMT18799	Dog	neg.	Germany	[7]
21	*S. pseudintermedius*[b]	IMT18802	Dog	neg.	Germany	[7]
22	*S. pseudintermedius*[b]	IMT18807	Dog	neg.	Germany	[7]
23	*S. pseudintermedius*[b]	IMT18810	Dog	neg.	Germany	[7]
24	*S. pseudintermedius*[b]	IMT18827	Dog	neg.	Germany	[7]
25	*S. pseudintermedius*[b]	IMT18828	Human	neg.	Germany	[7]
26	*S. pseudintermedius*[b]	IMT18875	Dog	neg.	Germany	[7]
27	*S. pseudintermedius*[b]	IMT18878	Human	neg.	Germany	[7]
28	*S. pseudintermedius*[b]	IMT18880	Dog	neg.	Germany	[7]
29	*S. pseudintermedius*[b]	IMT18883	Human	neg.	Germany	[7]
30	*S. pseudintermedius*[b]	IMT18884	Dog	neg.	Germany	[7]
31	*S. pseudintermedius*[b]	IMT18885	Human	pos.	Germany	[7]
32	*S. pseudintermedius*[b]	IMT18889	Dog	neg.	Germany	[7]
33	*S. pseudintermedius*[b]	IMT18914	Human	neg.	Germany	[7]
34	*S. pseudintermedius*[b]	IMT18915	Dog	neg.	Germany	[7]
35	*S. pseudintermedius*[b]	IMT21837	Dog	neg.	Germany	[7]
36	*S. pseudintermedius*[b]	VB969052	Dog	pos.	Austria	[6]
37	*S. pseudintermedius*[b]	VB969149	Dog	pos.	Germany	[6]
38	*S. pseudintermedius*[b]	VB971162	Cat	pos.	Italy	[6]
39	*S. pseudintermedius*[b]	VB971904	Dog	pos.	the Netherlands	[6]
40	*S. pseudintermedius*[b]	VB973750.1	Dog	pos.	Luxembourg	[6]
41	*S. pseudintermedius*[b]	VB974532	Cat	pos.	Germany	[6]
42	*S. pseudintermedius*[b]	VB976346.3	Dog	pos.	the Netherlands	[6]
43	*S. pseudintermedius*[b]	VB976771.2	Dog	pos.	Italy	[6]
44	*Staphylococcus delphini*[b]	BFI36161.2	Horse	neg.	France	#
45	*S. delphini*[b]	CCUG30107	Dolphin	neg.	Italy	±
46	*S. delphini*[b]	CCUG38984	Unknown	neg.	Unknown	±
47	*S. delphini*[b]	CCUG51769	Mink	neg.	Czech Republic	±
48	*S. delphini*[b]	VB 978908.2	Horse	neg.	Germany	#
49	*S. delphini*[b]	VB958847	Horse	neg.	Germany	#
50	*S. delphini*[b]	VB959563	Horse	neg.	the Netherlands	#
51	*S. delphini*[b]	VB961094	Horse	neg.	Germany	#
52	*S. delphini*[b]	VB969765	Horse	neg.	Germany	#
53	*S. delphini*[b]	VB969967.1	Horse	neg.	Germany	#
54	*S. delphini*[b]	VB972946	Pigeon	neg.	Germany	#
55	*S. delphini*[b]	VB975519	Horse	neg.	Germany	#
56	*Staphylococcus intermedius*[b]	NCTC 11048	Pigeon	neg.	Czech Republic	[8]
57	*S. intermedius*[b]	P-2A	Pigeon	neg.	Japan	[8]
58	*S. intermedius*[b]	P-45A	Pigeon	neg.	Japan	[8]
59	*S. intermedius*[b]	P-53	Pigeon	neg.	Japan	[8]
60	*S. intermedius*[b]	VB963312	Pigeon	neg.	Germany	#
61	*Staphylococcus aureus*[a]	IMT29002	Cat	pos.	Germany	#
62	*S. aureus*[a]	IMT29172	Dog	neg.	Germany	#
63	*S. aureus*[a]	IMT29325	Horse	neg.	Germany	#
64	*S. aureus*[a]	NCTC 8325	Lab. strain	neg.	Unknown	#
65	*S. aureus*[a]	VB 973156.2	Seal	pos.	the Netherlands	#
66	*S. aureus*[a]	VB 978536	Dolphin	pos.	Italy	#
67	*S. aureus*[a]	VB 978537	Dolphin	pos.	Italy	#
68	*S. aureus*[a]	VB965944.1	Pig	neg.	Germany	#
69	*Staphylococcus schleiferi* ssp. *coagulans*[c]	CCUG37248	Dog	neg.	Japan	±
70	*S. schleiferi* ssp. *coagulans*[c]	VB993188	Dog	neg.	Finland	#

CCUG, Culture Collection, University of Göteborg; pos., positive; neg.: negative; #, present study; ±, CCUG/present study.
[a]PCR according to Merlino *et al.* 2002 [13].
[b]Partial sequencing of *pta*, *cpn*60, 16S rRNA, *tuf* according to Bannoehr *et al.* 2007 [8].
[c]16S rRNA sequencing; primers according to Turner *et al.* (1999) [14].

genus identification' (PGI); score values from 2.0 to 2.299 indicate a 'secure genus identification and probable species identification' (PSI), and score values from 2.300 to 3.000 indicate a 'highly probable species identification' (SI).

The score values of each isolate were recorded twice: (i) using the original Bruker database and (ii) after supplementation of 60 SIG reference spectra. The resulting differences between the score values from measurements (i) and (ii) were

visualized and statistically analysed using IBM SPSS 21 (Heidelberg, Germany) and the non-parametric Wilcoxon test (paired data). Results were considered significant at $p < 0.05$.

Results

This study was initiated to evaluate and refine the BIOTYPER 3.0 reference database (Bruker Daltonics) with additional spectra specific for each of the species composing the SIG. To create a reliable reference spectra library containing the most reproducible peaks, 27 spectra were acquired for each individual strain. The FLEXCONTROL software was used to display differences in peak pattern and Fig. 1 shows representative examples of the raw fingerprint spectra obtained for *S. intermedius*, *S. pseudintermedius* and *S. delphini*. The spectra patterns of strains belonging to the same species displayed a similar peak pattern, while differences within the peak intensities occurred among the technical replicates.

Identification rates for the Bruker BIOTYPER 3.0 database before and after additional SIG reference spectra supplementation

The score values achieved for the 200 clinical isolates before (i) and after (ii) the inclusion of additional reference spectra for the distinct SIG members of the Bruker BIOTYPER database 3.0 content are presented in Table 2 and the Supporting information, Table S1. Efficacy of approach (ii) was shown by a graphical representation (Fig. 2), wherein the average score value appeared to be improved by 0.237. Notably, score values for seven isolates identified as *S. delphini* remained unchanged (Table S1). Furthermore, the p-value ($p < 0.001$) indicated a significant discriminatory improvement for procedure (ii).

Within the first approach, three isolates were identified at the SI (1.5%) level and 170 (85%) at the PSI level, while 25 isolates showed score values only associated with the baseline PGI level. For two isolates no reliable identification (NRI) was achieved. Notably, *S. intermedius* appeared to be the second-best hit for 29 (22 on PSI and seven on PGI level) out of 162 isolates whose first-best hit was *S. pseudintermedius*. Following inclusion of the new in-house reference spectra (ii), only seven isolates (3.5%) were identified at the baseline PGI and none on the NRI level, while 36 (18%) were identified at the PSI and 175 (78.5%) at the SI level. In addition, 17 isolates that were formerly identified (approach (i)) as *S. intermedius* on the levels of either PGI ($n = 4$) or PSI ($n = 13$) were assigned as *S. pseudintermedius* (five on PSI and 12 on SI level) in the second measurement (ii).

Dendrograms

The discriminatory abilities of the supplemented protein signature profile library of the BIOTYPER 3.0 for the individual SIG members is shown by a tree based on MALDI-TOF MS results (Fig. 3a). The *S. aureus* and *S. schleiferi* ssp. *coagulans* strains that were used as out-groups cluster clearly apart from the closely related but distinct members of the SIG. The

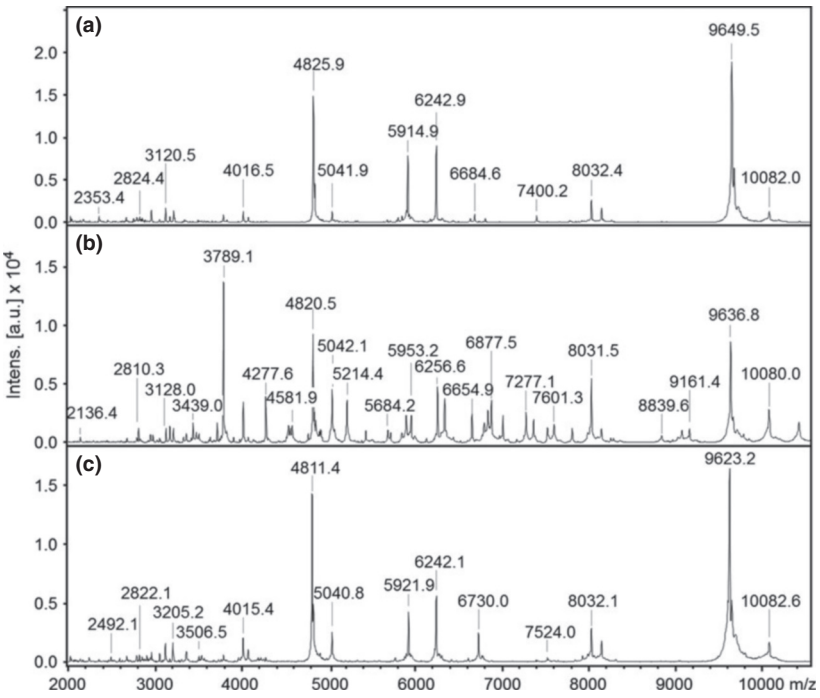

FIG. 1. A zoomed view (range from 2000 to 10 500 m/z) of representative matrix-assisted laser desorption ionization—time of flight mass spectra obtained for the distinct members of the *Staphylococcus intermedius* group (SIG): *Staphylococcus delphini* (a), *Staphylococcus intermedius* (b) and *Staphylococcus pseudintermedius* (c).

TABLE 2. Score values and identification matches obtained for 200 clinical *Staphylococcus intermedius* group isolates before (i) and after (ii) augmentation of in-house reference spectra with Bruker BIOTYPER database 3.0.

Species	Matching with Bruker database				Matching after inclusion of in-house references			
	n	Cut-off log score value			*n*	Cut-off log score value		
		1.7–1.99	2.0–2.29	2.3–3.0		1.7–1.99	2.0–2.29	2.3–3.0
Staphylococcus delphini	19 (9.5%)	4 (2%)	12 (6%)	3 (1.5%)	19 (9.5)	4 (2%)	8 (4%)	7 (3.5%)
Staphylococcus intermedius	17 (8.5%)	4 (2%)	13 (6.5%)	0	0	0	0	0
Staphylococcus pseudintermedius	162 (81%)	17 (8.5%)	145 (72.5%)	0	181 (90.5%)	3 (1.5%)	28 (14%)	150 (75%)
Not identified	2 (1%)				0			
Total	200	25 (12.5%)	170 (85%)	3 (1.5%)	200	7 (3.5%)	36 (18%)	157 (78.5%)

A result associated with score values from 0 to 1.699 indicates 'no reliable identification' (NRI); score values from 1.700 to 1.999 indicate a 'probable genus identification' (PGI); score values from 2.000 to 2.299 indicate a 'secure genus identification and probable species identification' (PSI), and score values from 2.300 to 3.000 indicate a 'highly probable species identification' (SI)

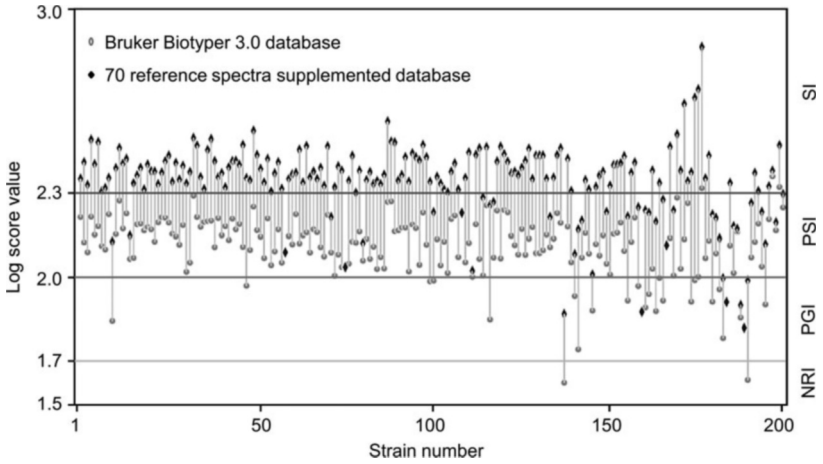

FIG. 2. Graphical representation of the score values obtained for 200 *Staphylococcus intermedius* group (SIG) isolates before and after refinement (ii) of the BIOTYPER 3.0 database with reference spectra for 60 SIG together with ten other coagulase-positive staphylococci (= 70 spectra). Circles indicate the score values achieved by applying the manufacturer's version of the database (approach (i), see Materials and Methods for further explanation) and the diamonds represent the score values reached in the second measurement (ii), associated with a rise of the average value score of 0.24.

clustering within the SIG clearly differentiates *S. intermedius*, *S. delphini* and *S. pseudintermedius*.

The sequence-based clustering shows a similar result (Fig. 3b). All SIG members are uniquely assigned to one group according to the species to which they belong.

Discussion

Supplementation of the original BIOTYPER 3.0 spectra library with distinct reference spectra for *S. intermedius*, *S. pseudintermedius* and *S. delphini* created during this study was evaluated using 200 SIG representing a broad spectrum of different hosts, years, geographic origins and infected or colonized body sites. In contrast to the limited performance of the original database content (see Results), the improvement of the reference spectra library allowed rapid species identification for 78.5% of the investigated samples at the best identification level (SI), while a further 36 isolates (18%) were identified at the second best (PSI) and only seven isolates (3.5%) were assigned at a baseline PGI level. A highly significant p-value ($p < 0.001$) also confirms the discriminatory power for differentiation among SIG after supplementation of the original database. This outcome underscores that the improvement of the reference spectra database could be achieved by including SIG strains associated with an unambiguous species identity originating from various geographical origins, hosts and body sites.

In contrast to the study results by Decristophoris *et al.*, score values obtained for all 19 *S. delphini* isolates (including seven strains with unchanged score results) did not indicate a high heterogeneity within the clinical strains investigated here [12]. However, the average improvement of score values for the *S. delphini* strains was 0.120 and therefore slightly below

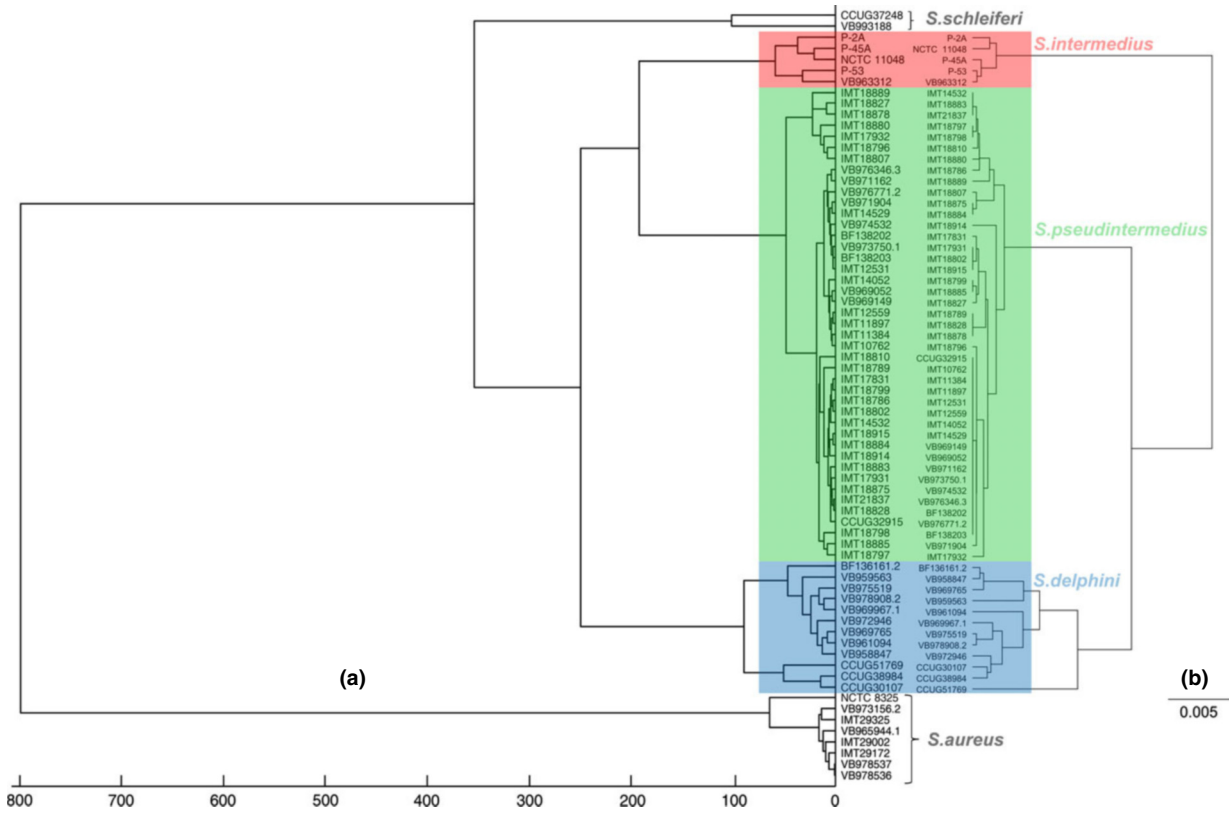

FIG. 3. Comparison of (a) score-oriented dendrogram of matrix-assisted laser desorption ionization—time of flight mass spectral profiles generated using BIOTYPER 3.0 software and (b) unweighted pair group method with arithmetic mean based tree calculated from the distances of the sequences of the four housekeeping genes *pta*, *cpn*60, *tuf* and 16S rRNA.

the overall improvement of 0.237. Moreover, 17 isolates that were initially identified as *S. intermedius* (= best match) using the original BIOTYPER 3.0 spectra library were clearly recognized as *S. pseudintermedius* by use of the supplemented data base. Consequently, a higher average score value improvement for the distinction between *S. intermedius* and *S. pseudintermedius* seems to be reasonable.

In total, 60 methicillin-resistant (n = 20) and methicilin-susceptible (n = 40) SIG strains representing a broad spectrum of different hosts and geographical origins that includes well-characterized European isolates from earlier studies published by our group [6,7] were chosen to set up the reference spectra library (Table 1). However, this study might be limited to some degree by the relatively small *S. intermedius* sample number available from different sources for MALDI-TOF MS and sequence data evaluation.

At present, only a limited number of methods for accurate and rapid differentiation among staphylococci of the intermedius-group (SIG) are available [1]. In recent years, MALDI-TOF MS-based microbial species identification emerged as a rapid method of choice and has been used in identification of a variety of *Staphylococcus* species [17–20]. The reclassification of the *Listeria* CAMP test strain *S. aureus* ATCC 49444 as *S. pseudintermedius* highlights the possibility of identification confusion among CPS, including *S. aureus* [7]. Further, the advent of modern gene-based molecular techniques resulted in revision of the taxonomy of many bacterial species, including the closely related members of the SIG [8,21, 22]. Recently, one of two frequently used commercial software tools, SARAMIS™ (Spectral Archive and Microbial Identification System, AnagnosTec GmbH, Zossen, Germany), was used to demonstrate the possibility of MALDI-TOF MS-based rapid discrimination among the species composing the SIG [12]. While *Staphylococcus* species identification has been evaluated with both frequently used software tools, SARAMIS™ and BIOTYPER (Bruker Daltonics) [23], the capacity of the BIOTYPER 3.0 software for differentiation among SIG has not been demonstrated. Furthermore, due to the fact that CPS originally identified as '*S. intermedius*' turned out to be '*S. pseudintermedius*' in the recent past [8,10,22], the aim of this study was to create additional and reliable reference spectra using well-characterized SIG. In recent years, some reports on severe clinical infections with *S. pseudintermedius* or other SIG (including multidrug-resistant MRSP) in humans were recorded

and recently reviewed [24]. The lack of CPS differentiation from specimens of human origin may lead to underestimation of the real prevalence and incidence of *S. pseudintermedius* (and MRSP) in the human population [25]. Hence, unambiguous and fast identification of SIG by use of MALDI-TOF MS is a powerful tool not only for daily laboratory routine but also to investigate the impact and zoonotic behaviour of *S. pseudintermedius* in the future.

Acknowledgements

We would like to thank Marina Runge from fu:stat (Freie Universität Berlin) for excellent statistical assistance. We thank Prof. Keiichi Hiramatsu for providing *S. intermedius* strains. Szilvia Vincze and Torsten Semmler were supported by the Federal Ministry of Education and Research (MedVet-Staph, Grant no. 01KI1014F) and (FBI-Zoo, Grant no. 01KI1012A).

Transparency Declaration

The authors declare that there is no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Detailed information on individual strain background and score values together with the best and second-best identification matches for 200 clinical *Staphylococcus intermedius* group (SIG) -isolates before (i, left side) and after (ii, right side) augmentation of in-house reference spectra with Bruker database BIOTYPER 3.0. A result associated with score values from 0 to 1.699 (red coded) indicates 'no reliable identification' (*NRI); score values from 1.700 to 1.999 (yellow shaded) indicate a 'probable genus identification' (PGI); score values from 2.000 to 2.299 (light green coded) indicate a 'secure genus identification and probable species identification' (PSI), and score values from 2.300 to 3.000 (intense green coded) indicate a 'highly probable species identification' (SI).

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