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Abstract

Differentiation of microorganisms by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a fast growing application in several fields of microbiology. The comparison of mass-spectra of a microorganism under investigation with spectra in a given database results in a hit list, ranking the best matching spectra. For this approach, commercial databases contain several thousand spectra from microorganisms of a broad taxonomic variety. Until today, the focus of these databases is on microorganisms from clinical microbiology. For application of the technique in the context of food control or animal health, a formal validation procedure must be created for each important target organism. This is especially important for parameters, which were created by the user to supplement the commercial database.

This study describes a workflow and documentation of a validation procedure for MALDI-TOF MS, based predominantly on reliably identified field isolates, representing the main organisms of interest. As an example for a relevant group of microorganisms from a specific environment, the validation of MALDI-TOF MS identification of staphylococcal species, mainly isolated from raw milk, is presented. The results show the reliability obtained with the initial commercial version of Bruker's MALDI Biotyper (Version 3.1.66, BT 5,989) in comparison with the results obtained with a database version extended by our own additional entries: using the commercial database version 165 (74.3 %) of 222 *Staphylococcaceae* isolates from 29 species were identified correctly. Only one isolate was incorrectly assigned to *Staphylococcus (S.) aureus* but actually belonged to the just recently described coagulase-positive species *S. argenteus*. For the remaining 56 isolates a species decision was not achieved.

The extension of the commercial database by 22 own entries, including one for *S. argenteus*, resulted in 94.6 % correct identifications. False differentiation results were not obtained with the extended database, while for 5.4 % of the isolates a concluding species decision could not be achieved. For *S. aureus*, the diagnostically most relevant species, a 100 % match rate was obtained with the commercial and the extended database.

A selection of database entries made for this study can be obtained by exchange via the MALDI-user platform MALDI-UP (http://maldi-up.ua-bw.de).

Introduction

For governmental food control and animal disease monitoring methods for microbiological analysis have to comply with high standards for reliability and reproducibility. Therefore, great efforts are made to define the standards for relevant methods. The validation of methods in order to comply with the formal requirements of standardization is a very time consuming process. While modern, rapidly evolving methods are usually quickly adopted within the scientific community, the demand for a formal validation process is one reason why such methods are only slowly finding their way into official laboratories. Standards for the differentiation of bacteria and broadly accepted methods (e.g. method collection according to § 64 German food and feed law, DIN EN ISO standards) usually include cultural methods and biochemical tests (incl. commercialized systems), and to some extend specialized PCR applications [Makarewicz et al., 2015]. However, spectroscopic methods, like matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

[Holland et al., 1996; Claydon et al., 1996; Lay, 2001; Zimmermann, 2015; Singhal et al., 2015; Cassagne et al., 2016], Fourier transform infrared spectroscopy (FT-IR), or Raman-spectroscopy are not part of these official methods yet, although they were successfully introduced in several fields of microbiology more than ten years ago [Maquelin et al., 2002; Wenning and Scherer, 2013; Wulf et al., 2012; Münchberg et al., 2015].

Spectroscopic methods for differentiation of microorganisms are based on iteratively developed databases, resulting in subsequent versions with accumulated numbers of database entries. The same circumstances apply to methods of molecular biology used for species identification, which are based on the evaluation of specific gene segments of high phylogenetic and taxonomic significance (e.g. parts of 16S rRNA gene, or *rpo*B gene [Woese & Fox, 1977; Böttger, 1989; Dahllöf et al., 2000]). These parts of the genome are sequenced and compared to reference gene sequences, available in public or commercial databases [Benson et al., 2015; Kim et al., 2012]. In contrast to genetic methods, mass spectrometry and vibrational-spectroscopy do not show the genotype but the phenotype. The individual spectra mirror characteristic features of the biochemical composition of the cell in a complex pattern. Like fingerprints, the resulting spectroscopic information is compared with spectra of reference isolates [Helm et al., 1991; Münchberg et al., 2015].

The validation of database-linked methods is complex. In spectroscopy, the long-term comparability of results is dependent on a high degree of standardization, e.g. regarding sample preparation, spectrometer type, or hardware settings. When these technical requirements are met, the quality of the database (the number of available spectra and isolates, the reliability of the designation of isolates used for reference, and the environment of potentially conspicuous species) is crucial for the result and the interpretation thereof [Clark, 2013; Lasch et al., 2015; Singhal et al., 2015].

One solution to handle these multi-factorial influences is the validation of a static database version that forms a systematic unity with the spectrometer device [Pranada et al., 2016]. Such a validation was for example the basis for a large comparative study on 7,068 clinically relevant bacteria that the U.S. Food and Drug Administration (FDA) carried out in a review (and subsequent approval) of the Vitek® MS system (bioMérieux, Durham, N.C., USA) [Anonymous, 2013]. In the same year, Bruker obtained the in vitro diagnostic status for an instrument-database combination for the clinical market in the US [Pranada et al., 2016]. Any modification of such a formally validated complete system needs considerable efforts. Hence, such a validation mode binds the user to the manufacturer of the MALDI-TOF MS system and its fixed (and certified)

database. For economic reasons, this concept leads to long intervals between validated database updates by the manufacturer. In this case an independent integration of own database entries to improve or allow for user specific applications, is complicated.

Hence, approaches in which existing device-database-combinations can be validated in a modular manner, step by step for each field of use, appear more flexible and therefore more attractive. To do so, the respective environment of the validated parameter set has to be defined. The various factors that influence misidentifications must be considered. Moreover, the desired range of validation should be adapted to the actually available resources. Fundamentally, by using self-made database entries, the user takes full responsibility for the functionality and results of the system [Pranada et al., 2016].

One way to facilitate personal input into the database is the consistent use of a well-stocked isolate collection, representing the own fields of work. Many microbiological laboratories have isolate collections e.g. consisting of reference strains for quality control, research isolates from projects with a different focus, temporary storage of isolates for transfer to a national reference laboratory, or isolates from animals for subsequent vaccination projects. Therefore, the reliability of the designation of the isolates stored can be diverse. Before such isolates can be used for validation of a new MALDI-TOF MS parameter, it is necessary to review the species assignment of field isolates in a standardized fashion.

Here we describe the validation of single species parameters, using Bruker's MALDI Biotyper (database version V3.3.1.0, update from 11/2015) with 5,989 deposited entries (hereinafter "BT 5,989"). This commercial database represents a flexible system component, which is updated by the manufacturer in irregular intervals. To fill gaps, we added our own database entries, made from strains from public strain collections or reliably identified isolates from our own isolate collection.

To show an in-house validation procedure, we chose the genus *Staphylococcus* as an example, based on the following considerations:

- *S. aureus* is a major causative agent of food-borne disease, which has also been demonstrated by current cases of well described outbreaks in the state of Baden-Wuerttemberg [Fetsch et al., 2014; Johler et al., 2013].
- The monitoring of this bacterial pathogen as coagulase-positive *Staphylococcus* (CoPS) in several milk based products is demanded by European regulations for foodstuffs [COMMISSION REGULATION (EC) No 2073/2005].

- *Staphylococcus* species are the cause of several clinical human infections ranging from skin and soft tissue infections, pneumonia and endocarditis to lethal septicemia. Hospital acquired infections with *Staphylococcus* species are a major public health concern [Antonanzas et al., 2015].
- *S. aureus* and other staphylococci represent a serious veterinary health problem as a causative agent of animal disease, especially mastitis of cows [Pyörälä and Taponen, 2009, Spohr et al., 2011; Friedrich et al., 2011; Tomazi et al., 2014].
- Some other *Staphylococcus* species are the causes of animal diseases (*S. pseudintermedius*: otitis externa of dogs [Murugaiyan et al., 2014], *S. hyicus*: exudative epidermitis of piglets [Lämmler, 1990]).
- *S. carnosus* is used as a starter culture in meat fermentation [Janssens et al., 2012].
- In many official diagnostic methods, the resolution of the differentiation of *Staphylococcaceae* stops at the sum parameter CoPS or coagulase negative staphylococci (CoNS), due to limited resources [Anonymous, 2009].

For validation the identification rates for a given set of *Staphylococcaceae* isolates (n = 222) were determined for 29 species, using the commercial MALDI Biotyper version BT 5,989. The identification was compared with the outcome using an extended database, including 22 additional entries.

The database extension and the validation are transferable between several laboratories with similar equipment, standard operating protocol and area of interest.

Materials and Methods

Isolate collection

A total of 222 isolates from the *Staphylococcacae* family were used for this study (Table 3a). The majority of isolates were obtained by standard procedures from food and diagnostic milk samples and had been isolated as coagulase positive staphylococci (CoPS, in particular *S. aureus*) or coagulase negative staphylococci (CoNS) according to the guidelines of the DVG [Anonymous, 2009]. Additionally, 90 isolates from milk samples and other sources (pet animals, life stock animals, food, humans) were made available from other institutions: State Health Office Baden-Wuerttemberg, Stuttgart (LGA); Friedrich-Loeffler-Institut – Institute of Farm Animal Genetics, Neustadt-Mariensee (FLI-ING); Vet Med Labor GmbH, IDEXX Laboratories, Ludwigsburg (IDEXX); Technische Universität München – Department of Microbiology at the Central Institute

for Food and Nutrition Research (TUM); University of Zurich – Institute for Food Safety and Hygiene, Switzerland (UZH), including one recently characterized food-borne coagulase-negative *S. aureus* strain [Johler et al., 2013] (Appendix A). Seventeen copies of *Staphylococcus* strains from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany) were used in this study as a reference (Appendix A). Altogether, 85 isolates of CoPS species and 127 CoNS species were included (Appendix A).

From the closely related taxonomic family, ten isolates of *Macrococcus caseolyticus*, obtained from milk samples, were included as a control. Other genera of the *Staphylococcacae* family, described within the last twenty years, were mainly isolated from saline and marine environments (*Jeotgalicoccus, Salinicoccus, Nosocomiicoccus, Aliicoccus*) [Amoozegar et al., 2014]. Hence, these genera were not considered to be relevant in the current examination context of food and animal associated microorganisms, and were consequently not included in this study.

Storage and cultivation of isolates

All strains were stored at -70 °C on ceramic beads in cryo-vials (Protect©, Transia, Ober-Mörlen, Germany) before analysis. Isolates were streaked directly onto sheep blood agar (SBA, Oxoid, Wesel, Germany) for cultivation under aerobic conditions. Bacterial isolates were cultivated at 37 °C for 24 +/- 4 hours on SBA under aerobic conditions prior to preparation for MALDI-TOF MS analysis.

Isolation and phenotypic characterization of Staphylococcaceae

Depending on the sample material, different isolation procedures were used routinely: Isolation from milk samples was performed according to the guidance for isolation and identification of mastitis pathogens [Anonymous, 2009; cf. Spohr et al., 2011]. In cases of uncertainty a latex slide agglutination test (Staphytect Plus®, Oxoid, Wesel, Germany) was carried out, to detect the clumping factor (bound coagulase) present on the bacterial cell surface. Alternatively we have used a chromogenic agar specific for *S. aureus* (CHROMagar Staph aureus, Becton Dickinson). For detection of CoPS in food samples the official method collection according to § 64 of the German Food and Feed Law was applied with slight modifications [Anonymous 2004, L00.00-55]. Isolates were further differentiated and identified as *S. aureus* by detection of DNase/staphylococcal thermonuclease [Anonymous 1988, L01.00.-33].

Seven *S. aureus* isolates from human origin were made available from the LGA, Stuttgart. The phenotypic and genotypic confirmation and further characterization of several isolates from human origin was performed by the German Reference Centre for staphylococci and enterococci (NRC for staphylococci) at the Robert-Koch-Institute (RKI, Wernigerode branch). Confirmation of several isolates from foodstuff was performed at the National Reference Laboratory for coagulase-positive staphylococci including *S. aureus* (NRL-Staph) at the Federal Institute for Risk Assessment (BfR, Berlin) as described by Fetsch et al. (2014).

Phenotypic characterization was performed by standard microbiological procedures. Biochemical reaction profiles of the API Staph32ID (bioMérieux, Nürtingen, Germany) were interpreted following the manufacturer's instructions, using apiweb (Vers. 3.0).

The taxonomically closely related species *S. hyicus* and *S. chromogenes* were distinguished by testing for colony pigmentation. Additionally, isolates were tested for hyaluronidase according to Carter and Chengappa (1991) using a mucoid *Pasteurella multocida* (CVUAS 6611; Soike et al., 2011). Non-pigmented *S. hyicus* were hyaluronidase-positive, while pigmented *S. chromogenes* were negative [Devriese et al., 1978].

Amplification and sequencing of the gene for 16S rRNA and the *rpoB* gene for the RNA polymerase beta subunit were carried out as described in detail by Contzen et al. (2011). The obtained 16S rDNA and *rpoB* sequences were analyzed using BLASTN [Altschul et al., 1990; http://www.ncbi.nlm.nih.gov]. Additionally 16S rDNA sequences were compared with the EzTaxon server for an assignment on species level [Kim et al., 2012; http://www.ezbiocloud.net/eztaxon].

Classes of reliability of designation of isolates

Based on the available information about taxonomically relevant characteristics of a single strain, the quality of denomination of every isolate was classified in one of five classes (0–4; see Table 1). Classes 3 and 4 represent reliably named isolates with more extensive genetic and phenotypic information.

TABLE 1. Reliability classes of isolate designation

Reliability Class of Designation	Description
0	Unknown isolate, sample
1	Isolate with basic information from a standardized differentiation e.g. obtained with a method according to the guidelines of DVG [Anonymous, 2009], to the collection according to § 64 of the German food and feed law or other international or national norms, resulting in a group parameter (e.g. *CoPS, Salmonella* sp., presumptive *Bacillus cereus*)
2	Isolate denomination confirmed by additional phenotypic and biochemical test results (e.g. *Staphylococcus* with detailed biochemical profile). The classification of the genus is assumed to be confirmed on this level.
3	Isolate with additional genetic and/or specific phenotypic information. Denomination on species level is reliable. For example: - copies of reference isolates from public strain collections (e.g. DSMZ, ATCC) - isolate with confirmation to a species by reliable comparison result of the 16S-rDNA, *sod*A, *gyr*B or *rpo*B sequence, or other species-specific genetic sequence information - isolates with information about species-specific genetic results (e.g. positive detection of the *ail* gene for *Yersinia enterocolitica,* or the *ces* gene for emetic *Bacillus cereus*)
4	Isolate, reliably named on species level, with additional detailed typing information (e.g. *S. aureus* with *spa* typing information).

The designation reliability for every isolate is given in Appendix B, and summarized for each species in Table 3a.

Sample preparation for MALDI-TOF MS

For sample preparation the following protocols were used, as recommended by the manufacturer (Bruker) [Anonymous, 2012; cf. Pranada et al., 2016]:

Direct transfer sample preparation (DT)

Cells from an isolate were transferred with the tip of an autoclaved tooth pick onto the sample zone of a trifluoroacetic acid-cleaned 96 position steel target (Bruker). After drying, the thin film of microorganisms was covered with 1 µl of a 10 g/L solution of α-cyano-4-hydroxycinnamic acid (IVD Matrix HCCA-portioned, Bruker, or HCCA, Sigma-Aldrich, Seelze, Germany) in a solvent mixture of acetonitrile (50 %), water (47.5 %), and trifluoroacetic acid (2.5 %) (all Sigma-Aldrich). After the sample spots were dried at room temperature, the homogeneous preparation was used

directly for MALDI-TOF MS in accordance with the manufacturer's instructions (Bruker). Each sample was prepared in duplicate.

Enhanced direct transfer sample preparation (eDT)

To enhance the presentation of proteins, the direct transfer sample method was modified according to Matsuda et al. (2012): The dried film of microorganisms was mixed with 1 µl of formic acid (70 %, Sigma-Aldrich), dried at room temperature, and covered with HCCA.

Extraction sample preparation (EFEx)

The ethanol/formic acid extraction sample preparation increases the efficiency of protein extraction. The extraction preparation according to the manufacturers protocol was used, if the DT or/and the eDT-preparation results for an isolate had a log(score) of < 2.0. The EFEx-preparation was applied for the spectra that were used to create own database entries in accordance with the manufacturer's instructions (Bruker):

Up to 10 mg of bacterial material were suspended thoroughly in 300 µl deionized water. After adding 900 µl of ethanol (99.5 %, Sigma-Aldrich) and mixing, cells were centrifuged at 14,000 rpm (i.e. 20817 x g, Centrifuge 5417R, Eppendorf, Hamburg, Germany) for 2 minutes. After removal of the liquid phase the pellet was dried for five minutes at room temperature. 30 µl of 70 % formic acid were added to the pellet and mixed thoroughly by pipetting to lyse bacterial cells. An equal volume of acetonitrile was added to the tube and mixed. The cell debris was centrifuged at 14,000 rpm for 2 minutes. One µl of the supernatant was placed in the sample zone of a steel target, and dried at room temperature. The loaded sample spot was immediately covered with HCCA matrix solution to prevent oxidation reactions. After drying at room temperature the homogeneous preparation was used directly for MALDI-TOF mass spectrometry.

MALDI-TOF mass spectrometry

The acquisition of MALDI-TOF mass spectra was performed with a Microflex LT mass spectrometer (Bruker Corporation, Bremen, Germany), controlled by the manufacturer's software flexControl 3.4 (3.4.135.0). The analysis of spectra was carried out using the Biotyper software (version 3.0.66) and by comparison with the database version V3.3.1.0, containing 5,989 entries (BT 5,989, Bruker). As proposed by the manufacturer the default parameter settings of the spectrometer were used (positive linear

mode, laser frequency 60 Hz, ion source 1: 20 kV, ion source 2: 18 kV) to get spectra in the mass range from 2,000 to 20,000 Da [cf. Schulthess et al., 2013].

For mass calibration the Bruker IVD bacterial test standard (BTS, Bruker) was used according to the manufacturer's instructions. This standard shows a typical *Escherichia coli* DH5α peptide and protein profile plus additional proteins for adjustment of the used mass range.

Commercial database

The MALDI Biotyper Version V3.3.1.0 (BT 5,989) includes Biotyper main spectra projections (MSP) of 38 species from the genus *Staphylococcus*, and *Macrococcus caseolyticus* as the sole representative of the genus *Macrococcus* (cf. Table 3b).

Own database entries

In addition to the commercial database, own entries were prepared according to the guidance and training manual of the manufacturer: Briefly, a freshly grown pure culture of the isolate was prepared in triplicate using the EFEx protocol. Each extract was spotted on 8 spots, to create 24 raw spectra according to the manufacturer's instructions. Subsequently, spectra were checked for flat lines, outliers and peculiarities. After selection and editing by flexAnalysis, calculation of main spectral projections (MSP) using the Biotyper module V3.0 was performed on the basis of at least 20 spectra. One MSP mirrors mass and intensity of up to 70 of the largest mass-signals of the organism. This reduced dataset was deposited as a specific database-entry in form of a btmsp-file. With Biotyper V3.0, these MSPs can easily be exported and imported, allowing for a fast exchange between different laboratories. As an example, in this study we integrated external MSP's from four *S. pseudintermedius* isolates, compiled by Ivonne Stamm (Vet Med Labor GmbH, IDEXX-Labratories Ludwigsburg, Germany).

Identification criteria

In the workflow for identifying microorganisms, the use of the Biotyper software results in a hit-list, ranking the best matching MSPs in descending order, expressed in terms of a log-score value [cf. Pranada et al., 2016]. Identification criteria used in our analysis, outlined by the manufacturer, were as follows: combined with species consistency of the first hits, a score of ≥ 2.000 indicated species level identification, a score

of 1.700 to 1.999 indicated identification at the genus level, and a score of < 1.700 was interpreted as no identification. Additional information is indicated by letters, “A” for species consistency, “B” for genus consistency or “C” for genus inconsistency or no reliable match with the current database.

In the validation process three interpretations of a score-based results for a defined isolate are possible: “correct”, “incorrect” or “without decision”. Table 2a) and table 2b) show the interpretation of examples for the species decisions “*Staphylococcus aureus”* and *“*no *Staphylococcus aureus”*, respectively, in detail.

TABLE 2a). Exemplary interpretation of Biotyper-results concerning *Staphylococcus aureus (S au),* consisting of hit-list and corresponding score-values. This interpretation is the basis for the determination of the *true positive rate* (sensitivity). (*S ep = S. epidermidis*, *S hy = S. hyicus*, *M ca = Macrococcus caseolyticus*, ? = without decision)

Expected result for validated isolate	BT-result score class	BT-result First Hit	BT-result further Hit within score class	BT-result consistency classifier	Result on species level	Interpretation as *S au* pos	Evaluation on species level
S au	≥ 2.0	*S au*	*S au*	A	*S au*	yes	correct
S au	≥ 2.0	*S ep*	*S ep*	A	*S ep*	no	incorrect / false negative
S au	≥ 2.0	*M ca*	*M ca*	A	*M ca*	no	incorrect / false negative
S au	≥ 2.0	*S ep*	*S hy*	B	?, but non *S au*	no	incorrect / false negative
S au	≥ 2.0	*S ep*	*M ca*	C	?, but non *S au*	no	incorrect / false negative
S au	≥ 1.7	*S au*	*S ep*	B	?	?	without decision
S au	≥ 1.7	*S ep*	*S au*	B	?	?	without decision
S au	1.7 – 2.0	*S au*	*S au*	B	?	?	without decision
S au	1.7 – 2.0	*S ep*	*S ep*	B	?	?	without decision
S au	1.7 – 2.0	*M ca*	*M ca*	B	*?, but non *S*	no	incorrect / false negative
any	< 1.7	any	any	C	?	?	without decision

**S. aureus* isolate, interpreted on genus-level as “*Macrococcus*“. Therefore this result is interpreted as a false negative.

TABLE 2b). Exemplary interpretation of Biotyper-results concerning the no-*Staphylococcus aureus* (*Non S au*) decision, as a basis for the determination of the *true negative rate* (specificity).

Expected result for validated isolate	BT-result score class	BT-result First Hit	BT-result further Hit within score class	BT-result consistency classifier	Result on species level	Interpretation as *S au* pos	Evaluation on species level for *S au*
Non S au	≥ 2.0	*Non S au*	*Non S au*	A	*Non S au*	no	correct*
Non S au	≥ 1.7	*Non S*	*Non S*	B	*Non S*	no	correct*
S ep	≥ 2.0	*S au*	*S au*	A	*S au*	yes	incorrect / false positive
S ep	≥ 1.7	*S au*	*any S*	B	?	?	without decision
S ep	≥ 1.7	*any S*	*S au*	B	?	?	without decision
S ep	1.7 – 2.0	*S ep*	*S ep*	B	?	?	without decision
M ca	≥ 2.0	*S au*	*S au*	A	*S au*	yes	incorrect / false positive
M ca	≥ 1.7	*S au*	any	B	?	?	without decision
M ca	≥ 1.7	any	*S au*	B	?	?	without decision
any	< 1.7	any	any	C	?	?	without decision

*For the decision "no *Staphylococcus aureus*" it is irrelevant, if the designation is correct for the investigated species.

TABLE 3a). Number and level of reliability of designation of *Staphylococcus* (*S.*) and *Macrococcus* (*M.*) isolates used for validation.

*Species subsumed as CoPS in this study.

TABLE 3b). Number of database (db) entries in the used Biotyper version and number of own db entries, used for extension.

** Entries made available by I. Stamm (IDEXX).

a) Isolates used for validation					b) species-specific db-entries	
Species	total	number according to reliability classes of designation			Bruker's Biotyper BT 5,989	own db entries
		2	3	4		
***S. agnetis** *	3		3		0	1
***S. argenteus** *	1		1		0	1
S. arlettae	5	2	3		3	2
***S. aureus** *	51	1	16	34	14	
S. auricularis	0				6	
S. capitis	2		2		7	
S. caprae	2		2		8	
S. carnosus	2	1	1		3	
S. chromogenes	17	11	6		1	
S. cohnii	4	1	3		6	2
S. condimenti	0				2	
S. delphini	0				3	
S. epidermidis	10	1	9		10	
S. equorum	4	2	2		4	
S. felis	2	1	1		8	
S. fleurettii	0				1	
S. haemolyticus	20	9	11		12	1
S. hominis	1		1		7	

a) Isolates used for validation					b) species-specific db-entries	
Species	total	number according to reliability classes of designation			Bruker's Biotyper BT 5,989	own db entries
		2	3	4		
***S. hyicus* ***	18	17	1		2	
***S. intermedius* ***	0				2	
S. kloossii	0				4	
S. lentus	1		1		2	1
S. lugdunensis	0				7	
S. lutrae	0				6	
S. microti	1		1		0	1
S. muscae	0				1	
S. nepalensis	0				2	
S. pasteuri	6		6		8	
S. pettenkoferi	0				6	
S. piscifermentans	0				2	
***S. pseudintermedius* ***	11	6	1	4	5	4 **
S. saccharolyticus	0				5	
S. saprophyticus	5	4	1		10	1
S. schleiferi	1		1		7	
***S. schweitzeri* ***	1		1		0	1
S. sciuri	6	3	3		4	1
S. simiae	0				4	
S. simulans	12	10	2		9	
S. succinus	2		2		2	
S. vitulinus	2		2		6	3
S. warneri	7	3	4		6	
S. xylosus	15	7	8		4	2
M. caseolyticus	10		10		7	1
Sum	222				206	22

If not explicitly denoted, for validation measurements sample preparation was performed using the direct transfer method (DT) or the extended direct transfer method (eDT). The extraction method (EFEx) was chosen only if the primary classification for validation was not successful (sc < 2.0) and for the preparation of samples for own database entries (Figure 1).

Bruker's MALDI Biotyper (BT)

The used version of the database (Biotyper Version V3.3.1.0) includes 5,989 entries (BT 5,989), thereof 206 main spectra projections (MSP's) from 38 species of genus *Staphylococcus* (see Table 3b). There are no database entries available for *S. agnetis, S. argenteus, S. microti* and *S. schweitzeri*.

With the commercial database version some isolates of designation classes 3 or 4 could not be assigned successfully, regardless of the sample preparation (DT, eDT, EFEx). 22 of these isolates was used for preparation of own database entries using the extraction protocol (EFEx). Thus, own records were integrated for *S. agnetis, S. argenteus, S. microti*, *and S. schweitzeri,* where the commercial database lacked any entry, as well as for *S. lentus*, *S. haemolyticus*, *S. sciuri*, *S. vitulinus*, *S. xylosus*, and *Macrococcus caseolyticus* to complement the given isolate variance of the database. For *S. pseudintermedius* four entries made by I. Stamm (IDEXX) were imported. The results obtained using the original BT 5,989 database and the extended version are listed in Table 4.

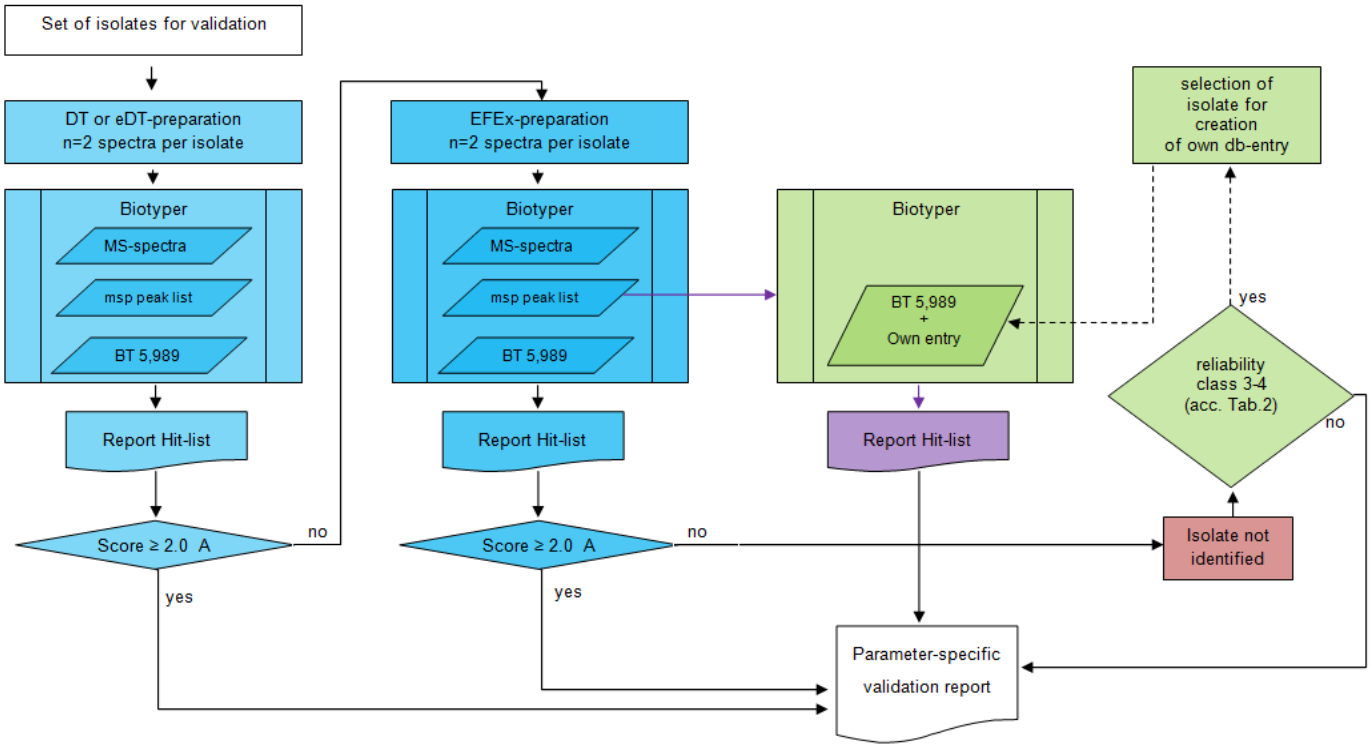

FIGURE 1. Workflow for the validation of a specific parameter by MALDI-TOF MS, using individual isolates as samples. The right column shows the steps for selecting and integrating isolates for own database entries of the MALDI Biotyper database

Results

The results (score-value and qualifier) of the assignment by MALDI-TOF MS for every specific isolate are given in Table 4 for the Biotyper version including 5,989 entries and the extended database. As an exception *S. warneri* and *S. pasteuri,* which are not differentiable by sequencing of the 16S rRNA gene and by the Bruker Maldi Biotyper, were recorded as a group. All other species were identified and evaluated specifically.

In summary, on the genus level, 93.4 % of *Staphylococcus* were identified correctly with the commercial database version. Through the targeted extension of the database, the percentage of assignment to the correct genus increased to 100 %.

On the species level, 74.3 % of the *Staphylococcaceae* isolate set of this study were identified correctly with the commercial BT version, while in 25.2 % no species decision was obtained (Table 4). Of the 222 isolates, only one false positive result was observed (0.5 %): *S. argenteus*, a recently described CoPS, was assigned as *S. aureus* (Appendix B) [comp. Tong et al., 2015]. The addition of 22 own database-entries, including hitherto not identified species or isolates (like *S. argenteus*), resulted in an increase of the true positive rate from 74.3 % to 94.6 % for the species-decision. Thus, the mean score value of all isolates was increased from 2.111 to 2.227.

All 51 *S. aureus* isolates from food, animal or human sources where already identified correctly with the current commercial BT-database. This was also the case for twelve further species, including *S. epidermidis*, *S. hyicus* and *S. chromogenes*. For these species, additional database entries were not required. For the species, already represented in the commercial database version, the integration of additional entries was noticeably effective for *M. caseolyticus* (increase of the mean score value (sc) from 1.811 to 2.258, and improvement of identification rate from 10 % to 100 %; n = 10), *S. cohnii* (1.550 to 2.469; 0 % to 100 %, n = 4), *S. haemolyticus* (1.952 to 2.193; 55 % to 95 %, n = 20), and *S. pseudintermedius* (1.988 to 2.177; 27.3 % to 100 %, n = 11). For the species not yet included and assigned in the commercial database, the addition of these entries resulted in the identification of *S. agnetis*, *S. microti, and S. schweitzeri.*

The only species in this study with a conflict in the result was *S. argenteus,* which is identified as *S. aureus* by the MALDI Biotyper database (sc 2.117, A). Consequently the false positive rate for *S. aureus* is 0.5 % in the set of 222 *Staphylococcaceae*. On the basis of the first hit (sc 2.370) for *S. argenteus* and a score value of 2.05 (*S. aureus*) for the second hit, no clear species decision was obtained by the extendend database version. The two species with score > 2.0 were considered as species inconsistency “B”. Thus, the new entry for *S. argenteus* does not result in a definite species identification but prevents an incorrect, false positive result for *S. aureus*.

The definition of CoPS and CoNS-species via MALDI-TOF MS does not mirror the positive or negative coagulase reaction [Anonymous, 2009]. Therefore, in this study relevant isolates were grouped using the usual

assignment of the respective species to the CoPS-*species* (*S. aureus, S. agnetis, S. argenteus, S. hyicus, S. pseudintermedius, S. schweizeri*) or CoNS-*species* (*S. capitis, S. cohnii*, *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. homnis*, *S. saprophyticus*, *S. schleiferi*, *S. warneri*, *S. xylosus*), respectively, supplemented by the recently described species [Anonymous, 2009; Tong et al., 2015; Taponen et al., 2012]. Consequently, the coagulase-negative *S. aureus* strain MSSA 129 [Johler et al., 2012] is correctly identified as *S. aureus* on the species level, independent of the coagulase reaction. In summary, within CoPS-species 85.9 % were identified correctly with the Biotyper 5,989, and 100 % with the extended database. With the commercial database 72.4 % and with the extended version 92.1 % of the CoNS-species were assigned correctly, respectively. For the respective remaining isolates no false identification was obtained. Both, the Biotyper and the extended database version, left the result open in these cases. In the common workflow of identification, such isolates were candidates for further differentiation methods (biochemistry, sequencing etc.).

All species-specific evaluation results were summarized in Table 4.

TABLE 4. Results of the assignment of 222 *Staphylococcacae* by MALDI-TOF MS: Biotyper database BT 5,989 versus the extended database.

species	n isolates of species	n of versus-species	Biotyper Database BT 5,989								Biotyper Database BT 5,989 with addition of 22 own entries							
			Score mean	Score std. deviation	True positive rate (sensitivity) [%]	False negative rate [%]	without decision rate [%]	True negative rate (specifity) vs. species [%]	False positive rate vs. species [%]	without decision rate [%]	Score mean	Score std. deviation	True positive rate (sensitivity) [%]	False negative rate [%]	without decision rate [%]	True negative rate (specifity) vs. species [%]	False positive rate vs. species [%]	without decision rate [%]
M. caseolyticus (M ca)	10	212	1.811	0.155	10	0	90	93.4	0	6.6	2.258	0.133	100.0	0	0	100	0	0
S. agnetis (S at)	3	219	1.952	0.038	0 *	0	100	78.5	0	21.5	2.195	0.279	66.7	0	33.3	95.4	0	4.6
S. argenteus (S ar)	1	221	2.117		0 *	100	0	77.4	0	22.6	2.370		0	0	100	95.0	0	5.0
S. arlettae (S ar)	5	217	1.504	0.305	0	0	100	79.3	0	20.7	2.224	0.204	80.0	0	20	95.4	0	4.6
S. aureus (S au)	51	171	2.381	0.087	100	0	0	70.2	0.6	29.2	2.381	0.087	100	0	0	93.0	0	7.0
S. capitis (S ca)	2	220	2.226	0.032	100	0	0	77.3	0	22.7	2.226	0.032	100	0	0	95.5	0	4.5
S. caprae (S cp)	2	220	2.111	0.071	100	0	0	77.3	0	22.7	2.111	0.071	100	0	0	95.5	0	4.5
S. carnosus (S cs)	2	220	2.211	0.037	100	0	0	77.3	0	22.7	2.211	0.037	100	0	0	95.5	0	4.5
S. chromogenes (S ch)	17	205	2.321	0.101	100	0	0	75.6	0	244	2.321	0.101	100	0	0	94.6	0	5.4
S. cohnii (S co)	4	218	1.550	0.233	0	0	100	78.9	0	21.1	2.469	0.123	100	0	0	95.0	0	5.0
S. epidermidis (S ep)	10	212	2.226	0.077	100	0	0	76.4	0	23.6	2.226	0.077	100	0	0	94.8	0	5.2
S. equorum (S eq)	4	218	2.140	0.056	100	0	0	77.1	0	22.9	2.140	0.056	100	0	0	95.0	0	5.0
S. felis (S fe)	2	212	2.126	0.006	100	0	0	77.3	0	22.7	2.126	0.006	100	0	0	95.0	0	5.0
S. haemolyticus (S ha)	20	202	1.952	0.207	55.0	0	45.0	79.7	0	20.3	2.193	0.147	95.0	0	5.0	95.0	0	5.0
S. hominis (S ho)	1	221	2.133		100	0	0	77.4	0	22.6	2.133		100	0	0	95.0	0	5.0
S. hyicus (S hy)	18	204	2.085	0.059	100	0	0	75.5	0	24.5	2.085	0.059	100	0	0	94.6	0	5.4
S. lentus (S le)	1	221	1.463		0	0	100	77.8	0	22.2	2.405		100	0	0	95.0	0	5.0
S. microti (S mi)	1	221	1.361		0 *	0	100	77.8	0	22.2	2.477		100	0	0	95.0	0	5.0
S. pasteuri/ S. warneri (S pa / S wa)	13	209	2.058	0.078	76.9	0	23.1	77.5	0	22.5	2.058	0.078	76.9	0	23.1	96.1	0	3.8
S. pseudintermedius (S ps)	11	211	1.988	0.117	27.3	0	72.7	80.1	0	19.9	2.177	0.106	100	0	0	94.8	0	5.2

species	n isolates of species	n of versus-species	Biotyper Database BT 5,989								Biotyper Database BT 5,989 with addition of 22 own entries							
			Score mean	Score std. deviation	True positive rate (sensitivity) [%]	False negative rate [%]	without decision rate [%]	True negative rate (specifity) vs. species [%]	False positive rate vs. species [%]	without decision rate [%]	Score mean	Score std. deviation	True positive rate (sensitivity) [%]	False negative rate [%]	without decision rate [%]	True negative rate (specifity) vs. species [%]	False positive rate vs. species [%]	without decision rate [%]
S. saprophyticus (S sa)	5	217	2.004	0.434	80	0	20	77.4	0	22.6	2.164	0.095	100	0	0	94.9	0	5.1
S. schleiferi (S sl)	1	221	2.153		100	0	0	77.4	0	22.6	2.153		100	0	0	95.0	0	5.0
S. schweitzeri (S sw)	1	221	1.727		0 *	0	100	77.8	0	22.2	2.470		100	0	0	95.0	0	5.0
S. sciuri (S sc)	6	216	1.946	0.128	33.3	0	66.7	78.7	0	21.3	2.058	0.116	66.7	0	33.3	95.8	0	4.2
S. simulans (S si)	12	210	2.204	0.084	100	0	0	76.2	0	23.8	2.204	0.084	100	0	0	94.8	0	5.2
S. succinus (S su)	2	220	2.094	0.050	100	0	0	77.3	0	22.7	2.094	0.050	100	0	0	95.0	0	5.0
S. vitulinus (S vi)	2	220	1.851	0.394	50	0	50	77.8	0	22.2	2.244	0.028	100	0	0	95.0	0	5.0
S. xylosus (S xy)	15	207	1.982	0.210	60	0	40	78.7	0	21.3	2.052	0.117	80	0	20	96.1	0	3.9
all	222		2.111	0.264	74,3	0.5	25.6				2.227	0.158	94.6	0	5.4			
***Staphylococcus* genus**	212	10	2.125	0.260	93.4	0	6.6	70	0	30	2.226	0.159	100	0	0	100	0	0
CoPS-species**	85	137#	2.241	0.197	85.9	0	14.1	72.3	0	27.7	2.286	0.157	100	0	0	92.7	0	7.3
CoNS-species	127	95	2.047	0.269	72.4	0	27.6	84.2	0	15.8	2.185	0.147	92.1	0	7.9	100	0	0

* *S. agnetis*, *S. argenteus*, *S. microti* and *S. schweitzeri* entries are not available in the Bruker database 5,989 version.

** CoPS species: *S. aureus*, *S. hyicus*, and the *S. intermedius* group (including *S. pseudintermedius*) (according to DVG-guidance [Anonymous, 2009]), extended for S. argenteus, S. schweizeri and *S. agnetis* [Taponen et al., 2012; Tong et al., 2015].

Negative control group for CoPS: CoNS and *M. caseolyticus*, comparative-group for CoNS: CoPS and *M. caseolyticus*.

Discussion

In microbiology, MALDI-TOF MS systems, like the MALDI Biotyper (Bruker) or the VITEK® MS (bioMérieux) are used for qualitative identifications. The systems have been commercially available on a large scale for several years. In addition to the classic area of clinical microbiology, they are applied in adjacent fields like veterinary diagnostics or food microbiology. Especially in the latter field, commercial databases still show gaps, as they are oriented towards the main market of clinical microbiology. Therefore, it is necessary for the manufacturer to supplement the database [Clark et al., 2013; Zhu et al., 2015; Randall et al., 2015] or own efforts must be made by the user to close diagnostically relevant gaps, even for *Staphylococcacae* [e.g. Murugaiyan et al., 2014; Król et al., 2016, Pranada et al., 2016].

For official acceptance of results it is necessary to validate the used analytic method for every parameter in the intended scope of application. Such a validation has to fulfill certain formal criteria, which depend on the type of parameter [Anonymous, 2005]. For quantitative methods, statements regarding the limit of detection, the limit of quantification, variation coefficients, recovery-rates and so on, have to be documented. For qualitative microbiological parameters, like a species assignment, primarily data for sensitivity (true positive rate) in the provided sample material and for specificity (true negative rate) have to be attested. Furthermore, information for interlaboratory and intralaboratory precision should be given, if applicable.

In contrast to fixed qualitative chemical or physical parameters, the definition of a biological parameter like an identifiable species is more variable over time. Changes in taxonomical classification of microorganisms can occur (e.g. 1916: *Micrococcus caseolyticus* > 1982: *S. caseolyticus* > 1998: *Macrococcus caseolyticus* [Kloos et al., 1998]). Several species or subspecies classes show soft transitions into each other, in particular in case of close relatives (e.g. the members of the *Bacillus cereus* group [Guinebretière et al., 2008]).

Thus, to be up to date, databases for fingerprint methods, like MALDI-TOF MS, FT-IR or gene sequencing, have to be maintained continuously. Besides the development in taxonomy (like newly described species or recently divided or merged species), rare isolates, not included in the spectrometry database, require constant editing of the database entries. Using MALDI-TOF MS as a spectrometric fingerprint technique, an iterative adaption of the databases and the subsequent validation is necessary to mirror the growing experience and knowledge.

In principle the problem can be solved in several ways:

- By using a specific fixed MALDI-TOF MS database version, a validation of this version for the own field of application can be done [Moon et al., 2013; Anonymous, 2013; Zhu et al., 2015].
- Definition of adjustable species-specific cut off values, based on own experience, divergent from the general recommendation of the manufacturer to get higher allocation rates in routine identifications [Schulthess et al., 2013; Richter et al., 2012; Szabados et al., 2012].
- Extension of the commercial database with user-made entries, built from well-known isolates, from in-house or external sources [Murugaiyan et al., 2014; Rau et al., 2016]. This can be combined with the manufacturers decision values (see “identification criteria” in Materials and Methods), or with appropriate own species-specific values [Pranada et al., 2016].

In all cases a documented validation procedure should support transparency of the differentiation decision of every parameter.

For MALDI-TOF MS validation presupposes a comparable hardware type, software-system and compatible database version. In this setting a site-independent validation can be carried out. Thus, validation reports of single parameters, created in an accredited laboratory, can be mutually exchanged to other locations, when defined procedures are used. In our working-group consisting of five Baden-Wuerttemberg state laboratories (CVUA Freiburg, CVUA Karlsruhe, CVUA Sigmaringen, CVUA Stuttgart, STUA-DZ Aulendorf), the same Biotyper hard- and software versions are used in a comparative workflow in food control and animal health microbiology. A standard operation procedure (SOP) is formally adapted and integrated into the QA systems of each member of the working-group.

For user extension of the database, the members of the working-group follow the same validation criteria for assignment of the isolates, intended for database enhancement (Table 2). They use the same protocols, following the manufacturer instructions. In this study four additional external database entries were made available from I. Stamm (IDEXX). These MSPs were integrated to demonstrate the ease of data exchange between Biotyper-users (Table 3b) [cf. Rau et al., 2016].

An absolute statement about the reliability of a version of a MALDI-TOF MS database would only be possible in a synopsis of independent results of all species. This is difficult because of the wide range of bacteria, applications and the large variation in outcome statements. Easier to validate is a selective view on single species, or on clear taxonomic or thematic groups [e.g. Clark et al., 2013; Lasch et al., 2015]. For

representation of a more comprehensive validation with own database extensions, in this study the group of *Staphylococcaceae* was selected, as established in the introduction. The approach in this case was not directed at achieving the highest possible score values for the single isolate, but to get the highest proportion of isolates with a clear species decision. Unlike the standard recommendations of the manufacturer, a species decision was accepted, when the score was ≥ 2.0 and the consistency classifier was “A” (Tables 2a and 2b) [cf. Pranada et al., 2016]. In routine laboratories, the DT or eDT sample preparation is preferred to the somewhat more complicated EFEx or other specialized preparations [e.g. Lasch et al., 2008], generally resulting in slightly lower score values. The validation presented here, corresponds to the routine procedure actually used.

Validation in general

Previous general validation studies of MALDI-TOF MS for species identification of bacteria demonstrated from > 65 % to nearly 100 % correct identified isolates on species level, regardless of the equipment platform and gold standard used [e.g. Carbonelle et al., 2012: 83.4 % vs. 65.9 %; Zhu et al., 2015: 81.9 %; Wenning et al., 2014: 88 %; Mellmann et al., 2008: 85.9 %; Dupont et al., 2010: 93.2 %]. Correspondingly, these studies showed a high reliability of the identification of the genus of bacteria in general with > 80 % of correct identifications [Carbonelle et al., 2012].

In these studies, the MALDI-TOF MS technique was tested on microorganisms selected from several foci: the respective field of work (clinical microbiology [Clark et al., 2013; Zhu et al., 2015; Carbonelle et al., 2012], veterinary microbiology [Randall et al., 2015; Wudy et al., 2012], food microbiology [Wenning et al., 2014]), a special taxonomic or phenotypically defined group like CoNS [Dupont et al., 2010; Delport et al., 2015], or a group of highly pathogenic bacteria [Lasch et al., 2015].

Staphylococcus validation

Studies concentrated on staphylococci, showed high rates of correct assignment by MALDI-TOF MS. At species level Moon et al. (2013) obtained 97.2 % correctly identified staphylococci with the Vitek® MS System, with an isolate collection equivalent (13 species, 218 isolates) to the one used in this study. Recently Zhu et al. (2015) demonstrated a correct species decision for 81.9 % *Staphylococcus* isolates (n = 216,

19 species), using the Biotyper (BT 5,627, V3.0). In a comparable Canadian study, 100 % of 485 CoNS were identified correctly with the MALDI Biotyper [Delport et al., 2015]. Unfortunately the database version used was not published by the latter authors. The result of our study emphasize these high assignment ratios, by showing 74.3 % correctly identified staphylococci on species level with the commercial version BT 5,989, and 94.6 % with the extended database (Table 4).

The selected workflow for validation and the specific extension of the MALDI-TOF MS database is adapted to the resources of a routine laboratory. The gradually increasing complexity in sample preparation (DT, eDT, EFEx), with subsequent evaluation of the species with the commercial database version (BT 5,989), at acceptance of a score > 2.0 for the species decision, leads to a minimized workload (Figure 1). Only if unsatisfactory results were obtained for relevant, reliably designated isolates (see Appendix B), efforts were undertaken to build an own database entry.

Our results for several relevant members of the genus *Staphylococcus* will be discussed hereafter in detail:

The undoubtedly most important CoPS-species with pathogenic potential for humans and animals is ***S. aureus***, whether as a food-borne pathogen, or as the causative agent of infections. The interest in *S. aureus* was intensified since resistance to critical antimicrobial agents were observed in MRSA-strains [Fetsch et al., 2013; Johler et al., 2013; Antonanzas et al., 2015; Friedrich et al., 2011; Wendlandt et al., 2015].

For our set of 51 *S. aureus* isolates we obtained score-values of 2.381 (+/- 0.087) calculated according to the Biotyper evaluation, a value not influenced by the extended database. This score-value is already described by others for this species. Lasch et al. (2014) reported average score values of 2.36 (+/- 0.09) for a collection of 59 *S. aureus*, heterogenous in sampling year, country of origin and source (animal or human), focused on subspecies differentiation with an adapted protocol of sample preparation. Others also obtained similar values for *S. aureus* with the standard sample preparations (DT, EFEx) [e.g. Richter et al., 2012; Clark et al., 2013].

For ***S. argenteus*** and ***S. schweitzeri***, two recently described staphylococci, uncertainties in the assignment by MALDI-TOF MS were reported by Tong et al. (2015). These representatives of CoPS are genetically and phenotypically closely related to *S. aureus*. In MALDI-TOF MS examination both staphylococci were identified as *S. aureus* with the former Bruker-database version 2.1 (score values > 2.0, without species

conflict (classifier A)) [Tong et al., 2015]. In our study the identification of *S. argenteus* with the commercial database version BT 5,989 resulted in a false positive assignment as *S. aureus*, while *S. schweizeri* was not identified on species level (see Table 4).

S. chromogenes, ***S. hyicus*** and the recently described ***S. agnetis***, are species linked to animal diseases [Lämmler 1990; Taponen et al., 2012]. In the Biotyper version BT 5,989, one *S. chromogenes*, and two *S. hyicus* entries were provided for reference, but no entry for *S. agnetis* (Table 3b). Consequently in our study the type strain DSM 23656^T and two further isolates of *S. agnetis* showed score values below 2.0, and thus were identified only on the genus level, by showing *S. hyicus* as the first hit. With the additional entry for DSM 23656^T the three *S. agnetis* were identified correctly (Appendix B).

Differentiation of representatives of the *S. intermedius*-group (*S. intermedius*, ***S. pseudintermedius***, *S. delphini*) is challenging, in classical microbiology, in gene amplification methods, or in MALDI-TOF MS [Zhu et al., 2015]. Murugaiyan et al. (2014) solved the problem by creating a comprehensive private collection of database spectra. In the present study, the integration of four external database entries (from I. Stamm, IDEXX) increased the correct assignment for eleven *S. pseudintermedius* isolates from dogs and a cat significantly from 27.3 % (BT 5,989) to 100 % (extended version). With the update of version BT 5,627 to BT 5,989, a single interfering entry for *S. intermedius* 08_STAINT MVO was deleted by the manufacturer. Thus the assignment-ratio was decreased from formerly 54.5 % to 27.3 % for our set of 11 isolates. However, by the addition of the four external entries the assignment-ratio could be improved to 100 % (Table 4).

The relatedness of some reported species is too close for an unequivocal differentiation by routine methods. ***S. warneri*** and ***S. pasteuri*** could not be differentiated by 16S rDNA sequencing [cf. Schulthess et al., 2013]. Likewise, the separation of these two genetically closely related species by MALDI-TOF MS was not successful [Zhu et al., 2015]. Especially the differentiation of *S. warneri* seems to be difficult [Moon et al., 2013; Zhu et al., 2015]. We decided to define a combined group for *S. warneri* and *S. pasteuri*, and have evaluated this group accordingly.

Score values < 2.0 were achieved with the Biotyper system for ***S. cohnii*** isolates repeatedly [Delport et al., 2015: n = 49, eDT, score value 1.654 – 1.804; Zhu et al., 2015: n = 13, score value < 2.0]. Also in our study, score values below 1.8 were received for *S. cohnii* using the commercial database version BT 5,989. As a result of the extension of the database

with two own reference entries, all four *S. cohnii* were identified correctly with score values > 2.0. (Appendix B). Therefore, the most likely reason for the low assignation of this species is, that the reference entries in the used commercial database version (entries n = 6; BT 5,989, Table 3b) do not mirror the complete variability of the species.

Impact of new entries

As shown by the examples above, gaps in the database used can be rapidly closed by a few own entries. The possibility to improve the commercial version with own database additions has been tested by others with good results [Schulthess et al., 2013; Schulthess et al., 2014; Murugaiyan et al., 2014; Król et al., 2016], whereas further groups have requested the manufacturers perform these updates [Mellmann et al., 2008; Clark et al., 2013; Zhu et al., 2015; Randall et al., 2015].

When a user is capable of generating own database extensions, principles and standards for creating and validating these own database entries have to be established. In particular, protocols should contain at least minimum information concerning reliability of designation, scope, origin of isolates, and quality of results. Especially for own extensions, a validation statement should be provided for every relevant parameter (Appendix C), to avoid conflict with the commercial database version used in routine [Pranada et al., 2016].

In our study the preparation of own database entries was performed according to the instructions of the manufacturer by trained personnel. The database entries were linked with all obtainable information about the isolate, origin, and on spectra quality. Further details for individual entries made in this study are compiled in the MALDI-UP catalog (http://maldi-up.ua-bw.de/) [Rau et al., 2016].

S. argenteus and *S. schweitzeri* were examples for the fast integration of new database entries, as a reaction to changes in taxonomy [Tong et al., 2015]. In the case of *S. argenteus*, the new entry prevents a false positive result for *S. aureus* (Table 4).

S. microti, not identified with the former MALDI Biotyper version with 4,727 entries, could easily be identified with the user made specific additional reference [Król et al., 2016]. In our study a single isolate from raw milk (CVUAS 6077) is also not identified by a newer commercial Biotyper version (BT 5,989), but is assigned successfully by the extended database (Table 4).

In smaller laboratories only a limited selection of isolates, which are suitable for database entries, may be available. To overcome the limit of inhouse database-extensions, the first steps for an exchange with external institutions were carried out successfully. Therefore, we use the recently developed online catalog MALDI-UP for documentation and exchange of information about high quality user created entries [Rau et al., 2016; http://maldi-up.ua-bw.de].

After integration of the own database extensions, the species-specific results show the strength of differentiation by MALDI-TOF MS for the discussed *Staphylococcaceae* species (Table 4). This applies even more to the diagnostic groups, summarized as CoPS and CoNS.

However, the MALDI-TOF MS result is not combined with information about the coagulase reaction, which is understood as a sign for pathogenicity in mastitis. Hence, it is not possible to completely mirror the empiric groups of CoPS and CoNS, using MALDI-TOF MS. This is for example, due to the known mixed designation of *S. hyicus*-isolates, which can be coagulase-positive or -negative [Anonymous, 2009], and to *S. agnetis*, a recently described coagulase-variable species from bovine mild mastitis [Taponen et al., 2012]. Recently, coagulase negative variants of *S. aureus* were described [Johler et al., 2014].

Of the species designated as CoPS (*S. aureus, S. pseudintermedius, S. hyicus, S. agnetis, S. argenteus, S. schweitzeri*), 85.9 % were identified correctly with the BT 5,989. The value could be increased significantly to 100 % with the extended database, now suitable for routine use (Table 4). Thus, the use of MALDI-TOF MS results in an enhanced differentiation of common CoPS- and CoNS-species, without increasing analysis duration.

In governmental laboratories, every relevant single microbiological parameter should be confirmed by a concrete validation statement, completely documented and therefore transparent, according to the formal criteria of accreditation. Validations targeting specific questions of concern, that focus on each relevant microbiological parameter (such as "*S. aureus* yes or no") separately in the typical background (such as "CoPS and CoNS from milk"), are better suited than a sum validation of a spectrometry-system (instrument and used database version) [e.g. Anonymous, 2013; Schulthess et al., 2014; Randall et al., 2015]. The experts can thereby correctly classify and evaluate results presented by the MALDI-system. In the working group of Baden-Wuerttemberg, five state institutions collaborate closely for database extensions and joint validations, using the same MALDI system and extended database version. Validation-statements will be mutually exchanged and are used at

every site in connection with the database version mentioned (Appendix C).

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APPENDIX A. Bacterial stains used in this study.

(T) = type strain; CVUAS = Chemisches und Veterinäruntersuchungsamt Stuttgart; DSMZ = German Collection of Microorganisms and Cell Culture, Braunschweig; LGA = State Health Office Baden-Wuerttemberg, Stuttgart; TUM = Technische Universität München.

species	isolate	isolated from	reference/source
Micrococcus caseolyticus	CVUAS 121	boiler meat	
	CVUAS 3051	contamination of submitted isolate	
	CVUAS 4448	bovine, raw milk	
	CVUAS 4454	bovine, raw milk	
	CVUAS 4456	bovine, raw milk	
	CVUAS 4503	bovine, raw milk	
	CVUAS 4506	bovine, raw milk	
	CVUAS 4510	bovine, raw milk	
	CVUAS 4513	bovine, raw milk	
	CVUAS 4636	bovine, raw milk	
S. agnetis	DSM 23656 (T)	bovine, raw milk, mastitis	DSMZ, [Taponen et al., 2012]
	CVUAS 5687	bovine	
	TUM 7385	bovine, raw milk	M. Wenning
S. argenteus	DSM 28299	human	DSMZ [Tong et al., 2015]
S. arlettae	coa031	bovine, raw milk	FLI-ING
	CVUAS 3762	bovine, raw milk	
	DSM 20672 (T)	skin of poultry	M. Wenning
	KNS 35	bovine, raw milk	FLI-ING
	TUM MG88	bovine, raw milk	M. Wenning
S. aureus	ATCC 29213	human, wound	
	DSM 11729	human, blood	DSMZ
	DSM 1104	human, clinical isolate	DSMZ
	DSM 20491		DSMZ
	DSM 346		DSMZ
	COL		S. Johler
	RKI4		S. Johler
	S6C		S. Johler
	MSSA 129	bovine, mastitis milk	S. Johler [Johler et al., 2012]
	CVUAS 935	pork meat	
	CVUAS 1083	turkey neck skin	
	CVUAS 1304,2	turkey, dust from barn	
	CVUAS 1305,2	turkey, dust from barn	
	CVUAS 1894	turkey meat	
	CVUAS 2645	turkey meat	
	CVUAS 3072	bovine, raw milk	
	CVUAS 3194	human, nasal swab	LGA
	CVUAS 3195	human, nasal swab	LGA
	CVUAS 3196	human, nasal swab	LGA
	CVUAS 3199	human, nasal swab	LGA
	CVUAS 3204	poultry meat	
	CVUAS 3206	meat; food-borne outbreak	SA_5 [Johler et al., 2013]
	CVUAS 3260	human, stool sample; food-bourne outbreak	LGA; SA_8 [Johler et al., 2013]
	CVUAS 3320	bovine, raw milk	
	CVUAS 3330	human, nasal swab	LGA; SA_2 [Johler et al., 2013]
	CVUAS 3433	human, nasal swab	LGA; Spohr et al., 2011

species	isolate	isolated from	reference/source
	CVUAS 3452,2	potato salad; food-borne outbreak	
	CVUAS 3740,2	turkey, dust from barn	
	CVUAS 3771	bovine, mastitis milk	
	CVUAS 3776	bovine, mastitis milk	
	CVUAS 4645	bovine, mastitis milk	Friedrich et al., 2011
	CVUAS 5756	turkey meat	
	CVUAS 6080	turkey meat	
	CVUAS 6459	bovine, mastitis milk	
	CVUAS 6963	turkey neck skin	
	CVUAS 7112	turkey neck skin	
	CVUAS 7665	bovine, meat	
	CVUAS 7779	minced meat	
	CVUAS 7963,2	ice-creme yoghurt-lemon	5-F [Fetsch et al., 2014]
	CVUAS 7958,2	ice-creme vanilla, food-borne outbreak	1-F [Fetsch et al., 2014]
	CVUAS 8134	bovine, nasal swab	
	CVUAS 8135	bovine, minced meat	
	CVUAS 8312	chicken meat	
	CVUAS 8900	bovine, calf meat	
	CVUAS 9019	human, wound after tatooing	LGA
	CVUAS 9197	turkey meat	
	CVUAS 9373	hygiene swab	
	CVUAS 9388	bovine, mastitis milk	
	CVUAS 9642	bovine, mastitis milk	
	CVUAS 9704	turkey meat	
	CVUAS 10197,2	turkey meat	
S. capitis	DSM 20326 (T)	human skin	M. Wenning
	TUM 5814	bovine, raw milk	M. Wenning
S. caprae	DSM 20608 (T)	goat milk	M. Wenning
	TUM 4073	air	M. Wenning
S. carnosus	CVUAS 1426	bacon	
	CVUAS 4590	salami	
S. chromogenes	5380	bovine, raw milk	FLI-ING
	coa009a	bovine, raw milk	FLI-ING
	coa096	bovine, raw milk	FLI-ING
	coa 051	bovine, raw milk	FLI-ING
	coa121	bovine, raw milk	FLI-ING
	coa143	bovine, raw milk	FLI-ING
	CVUAS 3348	bovine, raw milk	
	CVUAS 3349	bovine, raw milk	
	CVUAS 3706	bovine, raw milk	
	CVUAS 3784	bovine, raw milk	
	KNS 44	bovine, raw milk	FLI-ING
	KNS 60	bovine, raw milk	FLI-ING
	KNS 73	bovine, raw milk	FLI-ING
	MKNS 02	bovine, raw milk	FLI-ING
	MKNS 26	bovine, raw milk	FLI-ING
	TS 084	bovine, raw milk	FLI-ING
	TS 176	bovine, raw milk	FLI-ING
S. cohnii	MKNS 18	bovine, raw milk	FLI-ING
	MKNS 24	bovine, raw milk	FLI-ING
	MG98		M. Wenning
	DSM 20260 (T)		M. Wenning

species	isolate	isolated from	reference/source
S. epidermidis	DSM 20044 (T)	nose	DSMZ
	coa076	bovine, raw milk	FLI-ING
	CVUAS 3134	bovine, raw milk	
	CVUAS 3439	bovine, raw milk	
	CVUAS 3441	bovine, raw milk	
	CVUAS 3560	bovine, raw milk	
	CVUAS 3701	bovine, raw milk	
	CVUAS 3786	bovine, raw milk	
	CVUAS 3787	bovine, raw milk	
	CVUAS 6867	bovine, raw milk	
S. equorum	DSM 20674 (T)	skin of horse	M. Wenning
	TUM MG847	bovine, raw milk	M. Wenning
	4357	bovine, raw milk	FLI-ING
	coa034	bovine, raw milk	FLI-ING
S. felis	CVUAS 8599	cat	
	CVUAS 8203	cat	
S. haemolyticus	coa008a	bovine, raw milk	FLI-ING
	coa015	bovine, raw milk	FLI-ING
	coa022	bovine, raw milk	FLI-ING
	coa095	bovine, raw milk	FLI-ING
	coa098	bovine, raw milk	FLI-ING
	coa127	bovine, raw milk	FLI-ING
	coa142	bovine, raw milk	FLI-ING
	TS062	bovine, raw milk	FLI-ING
	TS171	bovine, raw milk	FLI-ING
	TS301	bovine, raw milk	FLI-ING
	CVUAS 2927	bovine, raw milk	
	CVUAS 3076	bovine, raw milk	
	CVUAS 3443	bovine, raw milk	
	CVUAS 3444	bovine, raw milk	
	CVUAS 3564	bovine, raw milk	
	CVUAS 3693	bovine, raw milk	
	CVUAS 3694	bovine, raw milk	
	CVUAS 3777	bovine, raw milk for consumption	
	CVUAS 4637	bovine, raw milk	
	CVUAS 5860	bovine, raw milk	
S. hominis	CVUAS 789,2	bovine, raw milk	
S. hyicus	coa 179	bovine, raw milk	FLI-ING
	CVUAS 212		
	CVUAS 330		
	CVUAS 593	pig, liver	
	CVUAS 1346	pig	
	CVUAS 1347		
	CVUAS 1525	pig	
	CVUAS 1526	pig	
	CVUAS 1552		
	CVUAS 1553		
	CVUAS 1702	pig, lung	
	CVUAS 1742		
	CVUAS 1917	pig	
	CVUAS 1984	pig, skin	
	CVUAS 2148	pig	
	CVUAS 2152	pig	

species	isolate	isolated from	reference/source
	CVUAS 2838		
	CVUAS 3767	bovine, raw milk	
S. lentus	CVUAS 2968		
S. microti	CVUAS 6077	pig, fetus	
S. pasteuri	CVUAS 3071	bovine, raw milk	
	CVUAS 3086	bovine, raw milk	
	MKNS 30	bovine, raw milk	FLI-ING
	CVUAS 4714	sandwich	
	DSM 10656 (T)	human vomit	M. Wenning
	TUM 5823	bovine, raw milk	M. Wenning
S. pseudintermedius	VB969390	cat, eye	I. Stamm [Murugaiyan et al., 2014]
	VB696149	dog, wound	I. Stamm [Murugaiyan et al., 2014]
	VB971580	dog, wound	I. Stamm
	VB971904	dog, wound	I. Stamm
	CVUAS 2375	dog	
	CVUAS 3688	dog	
	CVUAS 3831	dog	
	CVUAS 3856	dog, liver	
	CVUAS 5457	dog	
	CVUAS 5594	dog, lung	
	CVUAS 8759	dog	
S. saprophyticus	A 436	bovine, raw milk	FLI-ING
	A 446	bovine, raw milk	FLI-ING
	coa045	bovine, raw milk	FLI-ING
	4824	bovine, raw milk	FLI-ING
	CVUAS 503,2	bovine, raw milk	
S. schleiferi	CVUAS 9352	horse	
S. schweitzeri	DSM 28300	monkey	DSMZ [Tong et al., 2015]
S. sciuri	4363	bovine, raw milk	FLI-ING
	A 4778	bovine, raw milk	FLI-ING
	coa145	bovine, raw milk	FLI-ING
	CVUAS 3442	bovine, raw milk	
	CVUAS 3565	bovine, raw milk	
	CVUAS 4650	bovine, raw milk	
S. simulans	202-42	bovine, raw milk	FLI-ING
	4919	bovine, raw milk	FLI-ING
	coa117	bovine, raw milk	FLI-ING
	coa126	bovine, raw milk	FLI-ING
	coa150	bovine, raw milk	FLI-ING
	coa037	bovine, raw milk	FLI-ING
	KNS 27	bovine, raw milk	FLI-ING
	KNS 05	bovine, raw milk	FLI-ING
	MKNS 04	bovine, raw milk	FLI-ING
	MKNS 08	bovine, raw milk	FLI-ING
	DSM 20322 (T)	human skin	M. Wenning
	TUM 7391	food	M. Wenning
S. succinus	CVUAS 761,2	bovine, raw milk	
	CVUAS 2439,2	bovine, raw milk	
S. vitulinus	CVUAS 343,2	bovine, raw milk	
	CVUAS 9937,2	spice-mix	
S. warneri	DSM 20316	human skin	M. Wenning
	TUM 6412	bovine, raw milk	M. Wenning

species	isolate	isolated from	reference/source
	coa083	bovine, raw milk	FLI-ING
	4379	bovine, raw milk	FLI-ING
	4380	bovine, raw milk	FLI-ING
	coa032	bovine, raw milk	FLI-ING
	CVUAS 0687	capuccino-ice cream	
S. xylosus	CVUAS 974,2	bovine, raw milk	
	CVUAS 3440	bovine, raw milk	
	CVUAS 3562	bovine, raw milk	
	CVUAS 3563	bovine, raw milk	
	CVUAS 3566	bovine, raw milk	
	CVUAS 3692	bovine, raw milk	
	CVUAS 3699	bovine, raw milk	
	CVUAS 4732	bovine, raw milk	
	5741	bovine, raw milk	FLI-ING
	6124	bovine, raw milk	FLI-ING
	coa157	bovine, raw milk	FLI-ING
	KNS 61	bovine, raw milk	FLI-ING
	MKNS 06	bovine, raw milk	FLI-ING
	TS 227	bovine, raw milk	FLI-ING
	TS 251	bovine, raw milk	FLI-ING

APPENDIX B. Comparison of the specific results for each isolate used, obtained with the commercial MALDI Biotyper database (BT 5,989) and the version extended with 22 own entries.

Reliability class of designation of the individual isolate according Table 1, on basis of data for [a] API Staph ID32; [b] biochemical tube tests; [c] strain copy from public strain collection; [d] 16S rDNA-sequencing; [e] *rpo*B-sequencing; [f] sequencetype. Additionally to the result of 16S rDNA-sequencing, a xylose-positive reaction was necessary for designation of *S. xylosus* on level 3 to differentiate this species from *S.saprophyticus*. The close related species *S. chromogenes* and *S. hyicus* were separated by the hyaluronidase-reaction. *S. pasteuri* and *S. warneri* were recorded together, without further differentiation.
Score value and classifier of species/resp. genus-consistency (A, B, C) according to the rules/definition of the manufacturer. no id = no identification. Isolate designation of additional own database entries were given in **bold** (n = 22).

species (abbrevation)	isolate	Reliability class of designation	reason	score value of Bruker-DB; consistency-classifier	first hit species	Evaluation on species level correct, incorrect, or without decision	score value of extended DB; consistency-classifier	first hit species with extended database	Evaluation on species level
M. caseolyticus (M ca)									
	CVUAS 121	3	d	2.080; A	*M ca*	correct	2.080; A	*M ca*	correct
	CVUAS 3051	3	d	1.871; B	*no id (M ca)*	without decision	2.188; A	*M ca*	correct
	CVUAS 4448	3	d	1.887; B	*no id (M ca)*	without decision	2.401; A	*M ca*	correct
	CVUAS 4454	3	d	1.760; B	*no id (M ca)*	without decision	2.204; A	*M ca*	correct
	CVUAS 4456	3	d	1.695; C	*no id*	without decision	2.350; A	*M ca*	correct
	CVUAS 4503	3	d	1.816; B	*no id (M ca)*	without decision	2.420; A	*M ca*	correct
	CVUAS 4506	3	d	1.892; B	*no id (M ca)*	without decision	2.334; A	*M ca*	correct
	CVUAS 4510	3	d	1.581; C	*no id*	without decision	2.027; A	*M ca*	correct
	CVUAS 4513	3	d	1.601; C	*no id*	without decision	2.250; A	*M ca*	correct
	CVUAS 4636	3	d	1.931; B	*no id (M ca)*	without decision	2.329; A	*M ca*	correct
S. agnetis (S at)									
	DSM 23656 T	3	c	1.989; B	*no id (S hy)*	without decision	2.501; A	*S at*	correct
	CVUAS 5687	3	d	1.913; B	*no id (S hy)*	without decision	2.130; A	*S at*	correct
	TUM 7385	3	e	1.954; B	*no id (S hy)*	without decision	1.954; B	*no id (S hy)*	without decision
S. argenteus (S ar)									
	DSM 28299	3	c	2.117; A	*S au*	incorrect	2.370; B	*S ar*	without decision next Hit: *S au* score 2.117
S. arlettae (S ar)									
	DSM 20672	3	c	1.962; B	*no id (S ar)*	without decision	2.208; A	*S ar*	correct
	CVUAS 3762	3	d	1.419; C	*no id*	without decision	2.114; A	*S ar*	correct
	coa031	2	a	1.134; C	*no id*	without decision	2.424; A	*S ar*	correct

species (abbrevation)	isolate	Reliability class of designation	reason	score value of Bruker-DB; consistency-classifier	first hit species	Evaluation on species level correct, incorrect, or without decision	score value of extended DB; consistency-classifier	first hit species with extended database	Evaluation on species level
	KNS **35**	2	a	1.598; C	*no id*	without decision	2.424; A	*S ar*	correct
	TUM MG88	3	e	1.406; C	*no id*	without decision	1.951; B	*no id (S ar)*	without decision
S. aureus (S au)									
	ATCC 29213	3	c	2.386; A	*S au*	correct	2.386; A	*S au*	correct
	DSM 11729	3	c	2.209; A	*S au*	correct	2.209; A	*S au*	correct
	DSM 1104	3	c	2.231; A	*S au*	correct	2.231; A	*S au*	correct
	DSM 20491	3	c	2.316; A	*S au*	correct	2.316;A	*S au*	correct
	DSM 346	3	c	2.397; A	*S au*	correct	2.397;A	*S au*	correct
	COL	3	d	2.401; A	*S au*	correct	2.401; A	*S au*	correct
	RKI4	3	d	2.456; A	*S au*	correct	2.456; A	*S au*	correct
	S6C	3	d	2.450; A	*S au*	correct	2.450; A	*S au*	correct
	MSSA 129	4	d	2.266; A	*S au*	correct	2.266; A	*S au*	correct
	CVUAS 935	3	d	2.391; A	*S au*	correct	2.391; A	*S au*	correct
	CVUAS 1083	4	f	2.384; A	*S au*	correct	2.384; A	*S au*	correct
	CVUAS 1304.2	4	f	2.481; A	*S au*	correct	2.481; A	*S au*	correct
	CVUAS 1305.2	4	f	2.042; A	*S au*	correct	2.042; A	*S au*	correct
	CVUAS 1894	4	f	2.382; A	*S au*	correct	2.382; A	*S au*	correct
	CVUAS 2645	4	f	2.301; A	*S au*	correct	2.301; A	*S au*	correct
	CVUAS 3072	4	f	2.369; A	*S au*	correct	2.369; A	*S au*	correct
	CVUAS 3194	4	f	2.460; A	*S au*	correct	2.460; A	*S au*	correct
	CVUAS 3195	4	f	2.450; A	*S au*	correct	2.450; A	*S au*	correct
	CVUAS 3196	4	f	2.456; A	*S au*	correct	2.456; A	*S au*	correct
	CVUAS 3199	4	f	2.432; A	*S au*	correct	2.432; A	*S au*	correct
	CVUAS 3204	4	f	2.450; A	*S au*	correct	2.450; A	*S au*	correct
	CVUAS 3206	4	f	2.486; A	*S au*	correct	2.486; A	*S au*	correct
	CVUAS 3260	4	f	2.469; A	*S au*	correct	2.469; A	*S au*	correct
	CVUAS 3320	4	f	2.398; A	*S au*	correct	2.398; A	*S au*	correct
	CVUAS 3330	4	f	2.462; A	*S au*	correct	2.462; A	*S au*	correct
	CVUAS 3433	4	f	2.445; A	*S au*	correct	2.445; A	*S au*	correct
	CVUAS 3452.2	4	f	2.311; A	*S au*	correct	2.311; A	*S au*	correct
	CVUAS 3740.2	4	f	2.280; A	*S au*	correct	2.280; A	*S au*	correct
	CVUAS 3771	3	d	2.425; A	*S au*	correct	2.425; A	*S au*	correct
	CVUAS 3776	3	d	2.451; A	*S au*	correct	2.451; A	*S au*	correct
	CVUAS 4645	3	d	2.408; A	*S au*	correct	2.408; A	*S au*	correct
	CVUAS 5756	4	f	2.415; A	*S au*	correct	2.415; A	*S au*	correct
	CVUAS 6080	4	f	2.446; A	*S au*	correct	2.446; A	*S au*	correct
	CVUAS 6459	3	d	2.349; A	*S au*	correct	2.349; A	*S au*	correct
	CVUAS 6963	4	f	2.295; A	*S au*	correct	2.295; A	*S au*	correct
	CVUAS 7112	4	f	2.343; A	*S au*	correct	2.343; A	*S au*	correct
	CVUAS 7665	4	f	2.362; A	*S au*	correct	2.362; A	*S au*	correct
	CVUAS 7779	3	d	2.343; A	*S au*	correct	2.343; A	*S au*	correct
	CVUAS 7963.2	3	f	2.320; A	*S au*	correct	2.320; A	*S au*	correct
	CVUAS 7958.2	3	f	2.184; A	*S au*	correct	2.184; A	*S au*	correct

species (abbrevation)	isolate	Reliability class of designation	reason	score value of Bruker-DB; consistency-classifier	first hit species	Evaluation on species level correct, incorrect, or without decision	score value of extended DB; consistency-classifier	first hit species with extended database	Evaluation on species level
	CVUAS 8134	4	F	2.397; A	*S au*	correct	2.397; A	*S au*	correct
	CVUAS 8135	4	F	2.343; A	*S au*	correct	2.343; A	*S au*	correct
	CVUAS 8312	4	F	2.446; A	*S au*	correct	2.446; A	*S au*	correct
	CVUAS 8900	4	F	2.405; A	*S au*	correct	2.405; A	*S au*	correct
	CVUAS 9019	4	f	2.422; A	*S au*	correct	2.422; A	*S au*	correct
	CVUAS 9197	4	f	2.442; A	*S au*	correct	2.442; A	*S au*	correct
	CVUAS 9373	4	f	2.443; A	*S au*	correct	2.443; A	*S au*	correct
	CVUAS 9388	4	f	2.345; A	*S au*	correct	2.345; A	*S au*	correct
	CVUAS 9642	2	b	2.489; A	*S au*	correct	2.489; A	*S au*	correct
	CVUAS 9704	4	f	2.392; A	*S au*	correct	2.392; A	*S au*	correct
	CVUAS 10197.2	4	f	2.390; A	*S au*	correct	2.390; A	*S au*	correct
S. capitis (S ca)									
	DSM 20326	3	c	2.248; A	*S ca*	correct	2.248; A	*S ca*	correct
	TUM 5814	3	e	2.203; A	*S ca*	correct	2.203; A	*S ca*	correct
S. caprae (S cp)									
	DSM 20608	3	c	2.161; A	*S cp*	correct	2.161; A	*S cp*	correct
	TUM 4073	3	e	2.061; A	*S cp*	correct	2.061; A	*S cp*	correct
S. carnosus (S cs)									
	CVUAS 1426	2	a	2.184; A	*S cs*	correct	2.184; A	*S cn*	correct
	CVUAS 4590	3	d	2.237; A	*S cs*	correct	2.237; A	*S cn*	correct
S. chromogenes (S ch)									
	5380	2	b	2.256; A	*S ch*	correct	2.256; A	*S ch*	correct
	coa009a	3	d	2.222; A	*S ch*	correct	2.222; A	*S ch*	correct
	coa051	3	d	2.470; A	*S ch*	correct	2.470; A	*S ch*	correct
	coa096	2	a	2.226; A	*S ch*	correct	2.226; A	*S ch*	correct
	coa121	2	a	2.232; A	*S ch*	correct	2.232; A	*S ch*	correct
	coa143	2	a	2.285; A	*S ch*	correct	2.285; A	*S ch*	correct
	CVUAS 3348	3	d	2.260; A	*S ch*	correct	2.260; A	*S ch*	correct
	CVUAS 3349	2	b	2.446; A	*S ch*	correct	2.446; A	*S ch*	correct
	CVUAS 3784	3	d	2.319; A	*S ch*	correct	2.319; A	*S ch*	correct
	CVUAS 3706	3	d	2.336; A	*S ch*	correct	2.336; A	*S ch*	correct
	KNS 44	2	a	2.420; A	*S ch*	correct	2.420; A	*S ch*	correct
	KNS 60	2	a	2.452; A	*S ch*	correct	2.452; A	*S ch*	correct
	KNS 73	2	a	2.434; A	*S ch*	correct	2.434; A	*S ch*	correct
	MKNS 02	2	a	2.225; A	*S ch*	correct	2.225; A	*S ch*	correct
	MKNS 26	2	a	2.158; A	*S ch*	correct	2.158; A	*S ch*	correct
	TS 084	3	d	2.435; A	*S ch*	correct	2.435; A	*S ch*	correct
	TS 176	2	a	2.278; A	*S ch*	correct	2.278; A	*S ch*	correct
S. cohnii (S co)									
	MKNS 18	2	a	1.726; B	*no id (S co)*	without decision	2.551; A	*S co*	correct

species (abbrevation)	isolate	Reliability class of designation	reason	score value of Bruker-DB; consistency-classifier	first hit species	Evaluation on species level correct, incorrect, or without decision	score value of extended DB; consistency-classifier	first hit species with extended database	Evaluation on species level
	MKNS 24	3	e	1.776; B	*no id (S co)*	without decision	2.597; A	*S co*	correct
	MG98	3	e	1.338; C	*no id*	without decision	2.371; A	*S co*	correct
	DSM 20260	3	c	1.360; C	*no id*	without decision	2.357; A	*S co*	correct
S. epidermidis (S ep)									
	DSM 20044	3	c	2.305; A	*S ep*	correct	2.305; A	*S ep*	correct
	coa076	3	d	2.183; A	*S ep*	correct	2.183; A	*S ep*	correct
	CVUAS 3134	3	d	2.320; A	*S ep*	correct	2.320; A	*S ep*	correct
	CVUAS 3439	3	d	2.204; A	*S ep*	correct	2.204; A	*S ep*	correct
	CVUAS 3441	3	d	2.244; A	*S ep*	correct	2.244; A	*S ep*	correct
	CVUAS 3560	3	d	2.309; A	*S ep*	correct	2.309; A	*S ep*	correct
	CVUAS 3701	3	d	2.240; A	*S ep*	correct	2.240; A	*S ep*	correct
	CVUAS 3786	3	d	2.085; A	*S ep*	correct	2.085; A	*S ep*	correct
	CVUAS 3787	3	d	2.237; A	*S ep*	correct	2.237; A	*S ep*	correct
	CVUAS 6867	2	b	2.133; A	*S ep*	correct	2.133; A	*S ep*	correct
S. equorum (S eq)									
	DSM 20674	3	c	2.108; A	*S eq*	correct	2.108; A	*S eq*	correct
	TUM MG847	3	e	2.083; A	*S eq*	correct	2.083; A	*S eq*	correct
	4357	2	a	2.156; A	*S eq*	correct	2.156; A	*S eq*	correct
	coa034	2	a	2.211; A	*S eq*	correct	2.211; A	*S eq*	correct
S. felis (S fe)									
	CVUAS 8599	3	d	2.130; A	*S fe*	correct	2.130; A	*S fe*	correct
	CVUAS 8203	2	b	2.122; A	*S fe*	correct	2.122; A	*S fe*	correct
S. haemolyticus (S ha)									
	coa008a	2	b	2.154; A	*S ha*	correct	2.154; A	*S ha*	correct
	coa015	2	b	1.801; B	*no id (S ha)*	without decision	2.114; A	*S ha*	correct
	coa022	2	b	1.951; B	*no id (S ha)*	without decision	2.320; A	*S ha*	correct
	coa095	2	b	1.669; B	*no id (S ha)*	without decision	2.278; A	*S ha*	correct
	coa098	2	b	1.956; B	*no id (S ha)*	without decision	2.520; A	*S ha*	correct
	coa127	2	b	1.426; C	*no id*	without decision	1.911; B	*no id*	without decision
	coa142	3	d	2.013; A	*S ha*	correct	2.013; A	*S ha*	correct
	TS062	2	b	1.629; B	*no id (S ha)*	without decision	2.228; A	*S ha*	correct
	TS171	2	b	1.765; B	*no id (S ha)*	without decision	2.321; A	*S ha*	correct
	TS301	2	b	1.884; B	*no id (S ha)*	without decision	2.430; A	*S ha*	correct
	CVUAS 2927	3	d	2.225; A	*S ha*	correct	2.225; A	*S ha*	correct
	CVUAS 3076	3	d	2.011; A	*S ha*	correct	2.011; A	*S ha*	correct
	CVUAS 3443	3	d	2.090; A	*S ha*	correct	2.090; A	*S ha*	correct
	CVUAS 3444	3	d	2.075; A	*S ha*	correct	2.075; A	*S ha*	correct
	CVUAS 3564	3	d	1.980; B	*no id (S ha)*	without decision	2.251; A	*S ha*	correct
	CVUAS 3693	3	d	2.016; A	*S ha*	correct	2.293; A	*S ha*	correct
	CVUAS 3694	3	d	1,923; B	*no id (S ha)*	correct	2.172; A	*S ha*	correct

species (abbrevation)	isolate	Reliability class of designation	reason	score value of Bruker-DB; consistency-classifier	first hit species	Evaluation on species level correct, incorrect, or without decision	score value of extended DB; consistency-classifier	first hit species with extended database	Evaluation on species level
	CVUAS 3777	3	d	2.223; A	*S ha*	correct	2.223; A	*S ha*	correct
	CVUAS 4637	3	d	2.122; A	*S ha*	correct	2.122; A	*S ha*	correct
	CVUAS 5860	3	d	2.117; A	*S ha*	correct	2.117; A	*S ha*	correct
S. hominis (S ho)									
	CVUAS 789.2	3	d	2.133: A	*S ho*	correct	2.133: A	*S ho*	correct
S. hyicus (S hy)									
	coa 179	2	a	2.027; A	*S hy*	correct	2.027; A	*S hy*	correct
	CVUAS 212	2	b	2.030; A	*S hy*	correct	2.030; A	*S hy*	correct
	CVUAS 330	2	b	2.036; A	*S hy*	correct	2.036; A	*S hy*	correct
	CVUAS 593	2	b	2.093; A	*S hy*	correct	2.093; A	*S hy*	correct
	CVUAS 1346	2	b	2.028; A	*S hy*	correct	2.028; A	*S hy*	correct
	CVUAS 1347	2	b	2.203; A	*S hy*	correct	2.203; A	*S hy*	correct
	CVUAS 1525	2	b	2.052; A	*S hy*	correct	2.052; A	*S hy*	correct
	CVUAS 1526	2	b	2.183; A	*S hy*	correct	2.183; A	*S hy*	correct
	CVUAS 1552	2	b	2.078; A	*S hy*	correct	2.078; A	*S hy*	correct
	CVUAS 1553	2	b	2.022; A	*S hy*	correct	2.022; A	*S hy*	correct
	CVUAS 1702	2	b	2.133; A	*S hy*	correct	2.133; A	*S hy*	correct
	CVUAS 1742	2	b	2.126; A	*S hy*	correct	2.126; A	*S hy*	correct
	CVUAS 1917	2	b	2.067; A	*S hy*	correct	2.067; A	*S hy*	correct
	CVUAS 1984	2	b	2.162; A	*S hy*	correct	2.162; A	*S hy*	correct
	CVUAS 2148	2	b	2.124; A	*S hy*	correct	2.124; A	*S hy*	correct
	CVUAS 2152	2	b	2.016; A	*S hy*	correct	2.016; A	*S hy*	correct
	CVUAS 2838	2	b	2.043; A	*S hy*	correct	2.043; A	*S hy*	correct
	CVUAS 3767	3	d	2.114; A	*S hy*	correct	2.114; A	*S hy*	correct
S. lentus (S le)									
	CVUAS 2968	3	d	1.463; C	*no id*	without decision	2.405; A	*S le*	correct
S. microti (S mi)									
	CVUAS 6077	3	d	1.361; C	*no id*	without decision	2.477; A	*S mi*	correct
S. pasteuri (S pa)									
	DSM 10656	3	c	2.020; A	*S pa/S wa*	correct	2.020; A	*S pa/S wa*	correct
	CVUAS 3071	3	d	2.173; A	*S wa*	incorrect	2.173; A	*S wa*	incorrect
	CVUAS 3086	3	d	2.081; A	*S wa*	incorrect	2.081; A	*S wa*	incorrect
	MKNS 30	3	d	1.968; B	*no id (S pa/ S wa)*	without decision	1.968; B	*no id (S pa/ S wa)*	without decision
	CVUAS 4714	3	d	2.058; A	*S wa*	incorrect	2.058; A	*S wa*	incorrect
	TUM 5823	3	e	1.981; B	*no id (S pa)*	without decision	1.981; B	*no id (S pa)*	without decision
S. pseudintermedius (S ps)									
	VB971904	4	f	1.940; B	*no id (S. ps)*	without decision	2.104; A	*S ps*	correct
	VB969390	4	f	1.900; B	*no id (S. ps)*	without decision	2.144; A	*S ps*	correct

species (abbrevation)	isolate	Reliability class of designation	reason	score value of Bruker-DB; consistency-classifier	first hit species	Evaluation on species level correct, incorrect, or without decision	score value of extended DB; consistency-classifier	first hit species with extended database	Evaluation on species level
	VB969149	4	f	1.903; B	*no id (S. ps)*	without decision	2.161; A	*S ps*	correct
	VB971580	4	f	2.065; A	*S ps*	correct	2.343; A	*S ps*	correct
	CVUAS 2375	2	b	1.979; B	*S ps*	correct	2.159; A	*S ps*	correct
	CVUAS 3688	2	b	2.089; A	*S ps*	correct	2.161; A	*S ps*	correct
	CVUAS 3831	2	b	1.998; B	*S ps*	correct	2.150; A	*S ps*	correct
	CVUAS 3856	3	d	2.262; A	*S ps*	correct	2.397; A	*S ps*	correct
	CVUAS 5457	2	b	1.976; B	*S ps*	correct	2.213; A	*S ps*	correct
	CVUAS 5594	2	b	1.913; B	*S ps*	correct	2.061; A	*S ps*	correct
	CVUAS 8759	2	b	1.838; B	*no id (S ps)*	without decision	2.057; A	*S ps*	correct
S. saprophyticus (S sa)									
	A 436	2	b	2.175; A	*S sa*	correct	2.175; A	*S sa*	correct
	A 446	2	b	2.133; A	*S sa*	correct	2.133; A	*S sa*	correct
	coa045	2	b	2.298; A	*S sa*	correct	2.298; A	*S sa*	correct
	4824	2	b	1.235; C	*no id*	without decision	2.034; A	*S sa*	correct
	CVUAS 503.2	3	e	2.181; A	*S sa*	correct	2.181; A	*S sa*	correct
S. schleiferi (S sl)									
	CVUAS 9352	2	d	2.153; A	*S sl*	correct	2.153; A	*S sl*	correct
S. schweitzeri (S sw)									
	DSM 28300	3	c	1.727; B	*no id (S au)*	without decision	2.470; A	*S sw*	correct
S. sciuri (S sc)									
	4363	2	a	1.717; B	*no id (S sc)*	without decision	2.160; A	*S sc*	correct
	A 4778	2	a	1.975; B	*no id (S sc)*	without decision	2.206; A	*S sc*	correct
	coa145	2	a	1.885; B	*no id (S sc)*	without decision	1.885; B	*no id (S sc)*	without decision
	CVUAS 3442	3	d	2.026; A	*S sc*	correct	2.026; A	*S sc*	correct
	CVUAS 3565	3	d	2.070; A	*S sc*	correct	2.070; A	*S sc*	correct
	CVUAS 4650	3	d	2.000; B	*no id (S sc)*	without decision	2.000; B	*no id (S sc)*	without decision
S. simulans (S si)									
	DSM 20322	3	c	2.023; A	*S si*	correct	2.023; A	*S si*	correct
	202-42	2	a	2.134; A	*S si*	correct	2.134; A	*S si*	correct
	4919	2	a	2.263; A	*S si*	correct	2.263; A	*S si*	correct
	coa117	2	a	2.236; A	*S si*	correct	2.236; A	*S si*	correct
	coa126	2	a	2.320; A	*S si*	correct	2.320; A	*S si*	correct
	coa150	2	a	2.158; A	*S si*	correct	2.158; A	*S si*	correct
	coa037	2	a	2.277; A	*S si*	correct	2.277; A	*S si*	correct
	KNS 27	2	a	2.280; A	*S si*	correct	2.280; A	*S si*	correct
	KNS 05	2	a	2.263; A	*S si*	correct	2.263; A	*S si*	correct
	MKNS 04	2	a	2.182; A	*S si*	correct	2.182; A	*S si*	correct
	MKNS 08	2	a	2.142; A	*S si*	correct	2.142; A	*S si*	correct
	TUM 7391	3	e	2.174; A	*S si*	correct	2.174; A	*S si*	correct

species (abbrevation)	isolate	Reliability class of designation	reason	score value of Bruker-DB; consistency-classifier	first hit species	Evaluation on species level correct, incorrect, or without decision	score value of extended DB; consistency-classifier	first hit species with extended database	Evaluation on species level
S. succinus (S su)									
	CVUAS 761.2	3	d	2.058; A	*S su*	correct	2.058; A	*S su*	correct
	CVUAS 2439.2	3	d	2.129; A	*S su*	correct	2.129; A	*S su*	correct
S. vitulinus (S vi)									
	CVUAS 343.2	3	d	2.129; A	*S vi*	correct	2.224; A	*S vi*	correct
	CVUAS 9937.2	3	d	1.572; C	*no id*	without decision	2.264; A	*S vi*	correct
S. warneri (S wa)									
	DSM 20316	3	c	2.164; A	*S wa*	correct	2.164; A	*S wa*	correct
	TUM 6412	3	e	2.125; A	*S wa*	correct	2.125; A	*S wa*	correct
	coa083	3	e	2.080; A	*S wa*	correct	2.080; A	*S wa*	correct
	4379	2	a	1.942; B	*no id (S pa/ S wa)*	without decision	1.942; B	*no id (S pa/ S wa)*	without decision
	4380	2	a	2.150; A	*S wa*	correct	2.150; A	*S wa*	correct
	coa032	2	a	2.011; A	*S wa*	correct	2.011; A	*S wa*	correct
	CVUAS 0687	3	d	2.007; A	*S wa*	correct	2.007; A	*S wa*	correct
S. xylosus (S xy)									
	CVUAS 974.2	3	d*	2.013; A	*S xy*	correct	2.013; A	*S xy*	correct
	CVUAS 3440	3	d*	2.084; A	*S xy*	correct	2.084; A	*S xy*	correct
	CVUAS 3562	3	d*	1.911; B	*S xy*	correct	1.911; B	*S xy*	correct
	CVUAS 3563	3	d*	2.069; A	*S xy*	correct	2.069; A	*S xy*	correct
	CVUAS 3566	3	d*	2.221; A	*S xy*	correct	2.221; A	*S xy*	correct
	CVUAS 3692	3	d*	2.199; A	*S xy*	correct	2.199; A	*S xy*	correct
	CVUAS 3699	3	d*	2.135; A	*S xy*	correct	2.135; A	*S xy*	correct
	CVUAS 4732	3	d*	1.796; B	*no id (S xy)*	without decision	1.796; B	*no id (S xy)*	without decision
	5741	2	b	2.042; A	*S xy*	correct	2.042; A	*S xy*	correct
	6124	2	b	1.372; B	*no id (S xy)*	without decision	2.098; A	*S xy*	correct
	coa157	2	b	1.878; B	*no id (S xy)*	without decision	1.878; B	*no id (S xy)*	without decision
	KNS 61	2	b	1.919. B	*no id (S xy*	without decision	2.006; A	*S xy*	correct
	MKNS 06	2	b	2.117; A	*S xy*	correct	2.117; A	*S. xy*	correct
	TS 227	2	b	2.100; A	*S xy*	correct	2.100; A	*S xy*	correct
	TS 251	2	b	1.880; B	*no id (S xy)*	without decision	2.117; A	*S xy*	correct

APPENDIX C. Protocol for parameter validation, used in the MALDI-working-group of the five state laboratories in Baden-Wuerttemberg. For example the protocol for *S. hyicus* is given [in German].

Validierung des MALDI Biotyper

Gültig ab Freigabe

KNS

Parameter ***Staphylococcus hyicus***

Version 1

erstellt von RAU (CVUAS)

Validierungsset

Anzahl der Isolate des Parameters: 18

Die verfügbaren Isolate stammen überwiegend aus der Untersuchung von Proben vom Schwein. Sta hyicus ist dabei über den bei Sta chromogenes negativen Hyaluronidase-test abgrenzbar.

Anzahl der Vergleichsisolate: 204

Als ggf. verwechselbarer Satz wurden andere Staphylococcaceae ausgewählt, die ebenfalls in den Untersuchungsmatrices vorkommen.

Getestete Bedingungen

Schafsblutagar, 37°C, über Nacht

Direkttransfermethode, eDT

Verwendete Datenbank(en)

Biotyper Version 3.4.66, Datenbank DB 5989

CVUAS-DB, Stand 01.04.2016; 222

Validierungsergebnis

	Validierungsset	
Biotyper Ergebnis	*Staphylococcus hyicus* n = 18	*# Staphylococcus hyicus* n = 204
Staphylococcus hyicus	100% (18)	-
# Staphylococcus hyicus	-	94,6% (193)
fraglich*	-	4,9% (10)

Auswertung analog Tabelle 2a) und Tabelle 2b).

Freigabe

Staphylococcus hyicus

sind hiermit zur Messung mit dem MALDI Biotyper freigegeben.

Validierung durchgeführt am/von: 01.04.2016 RAU

Die unterschriebene Version befindet sich im erstellenden Labor

geprüft durch Laborleiter am/von: 02.04.2016 HiE

diese Validierung ersetzt Version vom: *22.04.2013*

Validierung übernommen am/von/für:

(Andere Institution)

LIMS-Parameter f. CVUAS erstellt am/von: 22.04.2013 RAU

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