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Evaluation of Three Bacterial Identification Systems for Species Identification of Bacteria Isolated from Bovine Mastitis and Bulk Tank Milk Samples

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Abstract

A study was conducted to evaluate Sensititre® Automated Reading and Incubation System 2x System (ARIS), API® (API), and Bruker MALDI-TOF MS (MALDI) bacterial species identification systems using 132 diverse bacterial isolates from bovine milk samples and bulk tank milk received at the Penn State Animal Diagnostic Laboratory. The results were compared with 16S rRNA gene sequence analysis, which served as the reference method for species identification. The ARIS, API, and MALDI identified 0%, 40%, and 33.4% of species classified as Gram-positive rod isolates belonging to genera *Arthrobacter*, *Bacillus*, *Brachybacterium*, *Brevibacterium*, and *Corynebacterium*, respectively. It was observed that 76.5%, 93.9%, and 96.9% of catalase-negative, Gram-positive cocci ($n=33$; *Aerococcus*, *Enterococcus*, *Lactococcus*, *Streptococcus*) were correctly identified to the species level by ARIS, API, and MALDI, respectively, while 33.4%, 84.5%, and 97.7% of catalase-positive, Gram-positive cocci ($n=45$; *Kocuria*, *Staphylococcus*) were correctly identified to their species by ARIS, API, and MALDI, respectively. A total of 48 isolates (*Acinetobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Pantoea*, *Pasteurella*, *Providencia*, *Pseduomonas*, *Serratia*) of Gram-negative bacteria were examined, of which 85.4%, 93.7%, and 95.8% of the isolates were correctly identified to the species level by ARIS, API, and MALDI, respectively. In our laboratory, the MALDI had the least costs associated with consumables and reagents compared to ARIS, API, and 16S rRNA identification methods. Identification of bacterial species was accomplished in <2 h using MALDI and 24 h for ARIS, API, and 16S rRNA identification systems.

Keywords: bacterial species identification, milk

Introduction

Bovine mastitis remains the most frequent and costly disease in the dairy industry that results in major economic losses, including reduction in milk production and milk quality, therapeutic interventions, premature culling, loss of antibiotic-contaminated milk, and issues with animal welfare (Hogeveen *et al.*, 2011; Geary *et al.*, 2012; Heikkila *et al.*, 2012). Isolation of mastitis pathogens from milk of infected cows is still the gold standard for diagnosis of mastitis (NMC, 1999).

Bacterial species identification in many veterinary diagnostic laboratories is performed using conventional biochemical tests, or using rapid, semiautomated or automated variations of classic methods (Garcia-Garrote *et al.*, 2000; Chapin and Musgnug, 2004; Wragg *et al.*, 2014). However, these methods can be labor-intensive, time-consuming, requiring at least 24 h for identification, and more importantly these identification systems rely on a phenotype database (van Veen *et al.*, 2010; Wyder *et al.*, 2011; El-Bouri *et al.*, 2012). Some of these databases comprise a small repertoire of species and therefore the results can be subjected to variability and misinterpretation (Jayarao *et al.*, 1991; Bosshard *et al.*, 2006; Vithanage *et al.*, 2014).

Molecular methods, such as real-time PCR, DNA sequence analysis, and microarray analysis can also be used to identify bacterial species. These methods are rapid and have high

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sensitivity and specificity; however, they are only able to identify a few specific microorganisms in a single assay and are difficult to multiplex (Friedrichs *et al.*, 2007). Often 16S rRNA gene sequencing is used to identify bacterial species and has been used as the reference method to compare other identification systems (Wickhorst *et al.*, 2016). Although 16S rRNA gene sequencing removes subjectivity in the determination of the species involved, it is still expensive, time-consuming, and technically demanding (Lau *et al.*, 2014).

Matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently emerged as an alternate technique for identification of bacterial and fungal pathogens and is now routinely used in human clinical laboratories (Santos *et al.*, 2011; Yaman *et al.*, 2012; Lau *et al.*, 2014). The MALDI-TOF MS system has been described as rapid, accurate, easy-to-use, and inexpensive for a wide range of bacterial identification (Krishnamurthy and Ross, 1996; van Veen *et al.*, 2010; Lau *et al.*, 2014).

Previous studies on identifying mastitis pathogens using MALDI-TOF MS have largely focused on comparing MALDI-TOF MS to 16S rRNA (Wickhorst *et al.*, 2016), PCR-restriction fragment length polymorphism (Tomazi *et al.*, 2014), Fourier transform infrared spectroscopy (Schabauer *et al.*, 2014), or biochemical methods, and species-specific PCR (Werner *et al.*, 2012). Although these studies do provide valuable data, they do not compare other rapid identifications systems used in veterinary diagnostic laboratories to MALDI-TOF MS identification system as it relates to correct identification of bacterial species, time, and cost of the supplies and reagents for performing the species identification.

The objective of this study was to compare Sensititre® Automated Reading and Incubation System 2x System (ARIS), a specific group of API® systems (API), and MALDI-TOF MS Bruker Biotyper (MALDI) using 16S rRNA gene sequence analysis as a reference method to determine the correct identification of bacterial species, time required for identification, and the cost of supplies and reagents incurred for identifying bacteria isolated from bovine mastitis and bulk tank milk.

Materials and Methods

Bacterial isolates and culturing

A total 68 milk samples (51 pooled quarter milk samples and 17 bulk tank milk samples) from 11 dairy herds were included for the study. One to three colonies per sample from tryptic soy agar (TSA) plate with 5% sheep blood (Remel, Inc., Lenexa, KS) were subcultured on TSA plate with 5% sheep blood (Remel, Inc.) and incubated aerobically for 24–48 h at 37°C. A collection of 139 isolates were examined for hemolysis on blood agar, Gram reaction, morphology, catalase reaction, and lactose fermentation, and subjected to 16S rRNA gene sequencing method for species identification.

A total of 132 isolates had >90% DNA sequence match and were identified as the same species on two of the three databases, while seven isolates belonging to *Prototheca* spp. ($n=2$), *Nocardia* spp. ($n=1$), and Yeast spp. ($n=4$) were not included in the study. The 132 isolates were categorized as follows: (1) Gram-positive rods ($n=6$), (2) Gram-positive, catalase-negative cocci ($n=33$), (3) Gram-positive, catalase-positive cocci ($n=45$), and (4) Gram-negative bacteria ($n=48$). The isolates were inoculated into 5 mL brain heart infusion (BHI) agar tube (BD Diagnostics, Sparks, MD) and grown aerobically for 48 h at 37°C. The tubes with growth were layered with 0.5 mL of sterile mineral oil and sealed with parafilm over the screw top lid. These tubes served as stock cultures throughout the study. Stock cultures were streaked on TSA plate with 5% sheep blood (Remel, Inc.) and reconfirmed by Gram stain and morphology. A single colony was inoculated into 5 mL of BHI broth (BD Diagnostics) and incubated overnight aerobically at 37°C. A loopful of culture was streaked onto three TSA plates with 5% sheep blood. A single plate was used for each species identification system using one of the three methods (ARIS, API, or MALDI).

16S rRNA sequence-based identification system

One to two colonies from a TSA plate with 5% sheep blood were inoculated into 1 mL of BHI broth in a 1.7 mL microcentrifuge tube and grown overnight at 37°C. A boiled prep method was used for DNA extraction. The bacteria were harvested by centrifugation at 5000 rpm for 5 min. The supernatant was decanted and 200 μL of nuclease-free water was added to the pellet. This mixture was then heated in a water bath for 10 min at 100°C. The bacterial suspension was then vortexed for 30 s to disrupt the cell membranes. Cell debris was precipitated by centrifugation at 6000 rpm for 5 min. The supernatant containing the DNA was then collected and placed into a new tube. All DNA was stored at −20°C until needed for PCR. The PCR was performed in 50 μL reactions on PTC-200 DNA Engine Peltier Thermal Cycler (MJ Research, Inc., Waltham, MA).

Two universal 16S rRNA primer sets described by Relman *et al.* (1992) were used in this study. The sequences of the forward and reverse primers were as follows: p8FPL-p806R (834 bp product) F 5′ GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG 3′, R 5′ GCG GAT CCG CGG CCG CGG ACT ACC AGG GTA TCT AAT 3′ and p515FPL-p13B (904 bp product) F 5′ GCG GAT CCT CTA GAC TGC AGT GCC AGC AGC CGC GGT AA 3′, R 5′ CGG GAT CCC AGG CCC GGG AAC GTA TTC AC 3′. Reactions consisted of 22.1 μL of water, 4 μL of each primer pair at 0.4 μM, 0.5 μL of DNTPs at 0.1 μM, 0.4 μL of *Taq* Polymerase (Promega, Madison, WI) at 2 U per reaction, 4 μL of $MgCl_2$ at 2 mM, 5 μL of 1 × Taq Polymerase Buffer (100 mM Tris HCl; 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), and 10 μL of DNA template.

Reactions were run with the following thermocycling conditions: 94°C for 2 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; then, 72°C for 10 min and 4°C holding. To visualize the amplicon, products were run on a 2% agarose gel for 45 min at 180 V using PCR Markers as the molecular weight standards (Promega). Any reactions yielding a positive result were then purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). Purified products were sent to Penn State Genomics Core Facility (University Park, PA) according to their preparation instructions.

To ensure proper identification, the PCR products (834 and 904-bp) were bidirectionally sequenced and the sequences were then identified using three different databases including the following: (1) NCBI BLAST (https://blast.ncbi.nlm.nih.gov/), (2) University of Hong Kong Department of Microbiology database of 16S rRNA sequences of medically relevant bacteria (www.microbiology.hku.hk/16SpathDB/identifyBy16S_U.php), and (3) EZ Biocloud (www.ezbiocloud.net/eztaxon). DNA

sequences with ≥90% match and identified as the same species on two of the three databases were used for evaluating the three identification systems.

Sensititre ARIS 2x identification system

All 132 isolates were either run on Gram-negative identification or Gram-positive Sensititre ARIS 2x identification plates according to manufacturer's instructions (Thermofisher, Inc., Waltham, MA). Plates were automatically read after 18 or 24 h and identified by the Sensititre SWIN software (Thermofisher, Inc.). The level of identification of the isolate was interpreted as follows: (1) excellent, (2) good, (3) acceptable, (4) low selectivity, (5) Group ID, (6) Reincubate, and (7) No ID probable. The final interpretation of the result was at the discretion of the microbiologist. In our study, isolates identified as excellent (>99% ID), good (≥95% ID), and acceptable (≥90% ID) were presumed as correct identification by the ARIS system. Isolates with a <90% ID were repeated that included all of the steps previously taken for species identification of the isolate.

API identification kits

Group-specific API (API Coryne; API 20 Strep; API Staph and API 20E) identification kits were used to identify bacterial species as described by the manufacturer (BioMérieux, Inc., Hazelwood, Saint Louis, MO). The API strips were examined after 24 h of incubation at 37°C and bacterial species were determined using online Apiweb™ database v3.0 (https://apiweb.biomerieux.com). Isolates were classified into one of the three groups: (1) identification at species level, (2) identification at genus level, and (3) no identification (i.e., low discrimination). Isolate identification at the species level was divided into four subcategories: (1) excellent species identification (≥99.9% ID); (2) very good species identification (≥95.0% ID); (3) good species identification (≥90.0% ID); and (4) acceptable species identification (≥80.0% ID). Isolates with a <80% ID were repeated that included all of the steps previously taken for species identification of the isolate.

Bruker MALDI-TOF MS identification system

Bacterial isolates were grown overnight aerobically on TSA plate with 5% sheep blood (Remel, Inc.) at 37°C. The tube extraction method described by Rodríguez-Sánchez *et al.* (2014) was used in our study. A single large colony or two to four small colonies were transferred to an Eppendorf tube containing 300 μL of sterile distilled water. Bacteria were inactivated by the addition of 900 μL of ethanol. The mixture was thoroughly vortexed and centrifuged at 13,000 rpm for 2 min. All of the ethanol was pipetted out and the tube was dried at room temperature for 10 min. To this tube, 50 μL of 70% formic acid was added and vortexed thoroughly and allowed to stand for about 5 min. This was followed by the addition of 50 μL of 100% acetonitrile and the contents were centrifuged for 2 min. A 1 μL volume of the supernatant was transferred onto the steel target and the spot was allowed to dry at room temperature.

The dried spot was overlaid with 1 μL of matrix solution, consisting of α-cyano-4-hydroxy-cinnamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid. A bacterial test standard provided by the manufacturer was included in every run for calibration purposes. The MALDI-TOF MS was performed in a Bruker Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), operated in the linear mode and equipped with a 337-nm nitrogen laser using FlexControl 3.3 software (Bruker Daltonics). The mass spectra were collected within the mass range of 2000 to 20,000 *m/z*. Two hundred forty laser shots were accumulated to generate each spectrum. Spectra were analyzed with MALDI Biotyper 2.0 software (Bruker Daltonics). For each sample, the automatic analysis generated a peak list, which was used to match a reference library by the integrated pattern-matching algorithm.

The result was given by means of a log score with a maximum value of 3.0. Score values higher than 1.7 were considered reliable for genus identification, and scores higher than 2.0 were considered probable for species identification. Isolates with a score <2.0 were repeated, which included all of the steps previously taken for species identification of the isolate.

Cost and time analysis

The cost incurred for species identification using each of the four methods was determined by accounting for materials and supplies required for the analysis and reported as cost per test. The cost of labor, instruments and their maintenance, and software were not included in determining the cost of the test.

The cost for supplies and reagents was calculated, which included the cost for the following: (1) TSA plate with 5% sheep blood, (2) Gram stain, (3) catalase test, (4) oxidase test, (5) lactose fermentation, (6) cost involved in 16S rRNA-based identification, including DNA extraction, PCR primers, PCR reagent mix, PCR product visualization, purification of PCR products, and sequencing of PCR product on a 96-well plate run at the Penn State Genomics Core Facility, (7) Sensititre ARIS 2x Gram-positive and Gram-negative plates, (8) API strips (API Coryne, API 20 Strep, API Staph, API 20 E) and detection reagents, and (9) Bruker MALDI-TOF MS reagents, including pipette tips, Eppendorf tubes, premixed organic solvent solution, HCCA matrix, and BTS control. An isolated colony on secondary TSA 5% sheep blood agar plate (Remel, Inc.) was used for the study.

The time required for performing Gram's stain (all isolates), catalase test (for Gram-positive cocci), and oxidase or lactose fermentation (Gram-negative bacteria) was not taken into consideration. The time (in hours) required for species identification was determined based on the incubation or run time specified for the kit or the ID system. In addition, the time needed for interpretation of the result using their respective databases was also included to determine the total time required for species identification.

Data analysis

The ARIS, API, and MALDI identification systems were compared to species identified by 16S rRNA sequence-based identification method, which in our study served as the reference method for species identification. The organisms identified by ARIS were then examined for their listing in the Sensititre Gram-positive identification database of veterinary origin and Gram-negative identification combined human and veterinary database (Sensititre SWIN software; Thermofisher, Inc.). Bacterial species identified by API and MALDI were then examined for their listing in the API

Table 1. Genus and Species Identification of Gram-Positive Rods

16S rRNA gene sequence-based identification		*Aris® 2x Sensititre identification system*		*API® Coryne identification system*		*Bruker Biotyper 2.0 MALDI-TOF identification system*	
Species[ab]	*% ID*	*Species*	*% ID*	*Species*	*% ID*	*Species*	*MALDI score*[c]
Arthrobacter mysorens (1)[a]	100	*Streptococcus pneumoniae* (1)	100	*Arthrobacter* spp.	24	*Arthrobacter arilaitensis* (1)	1.99[c]
Bacillus cereus (1)	100	*Bacillus thuringiensis* (1)	99	Not tested	—	*B. cereus* (1)	2.19
Brachybacterium rhamnosum (1)[ab]	99	*Streptococcus dysgalactiae* (1)	100	No identification (1)	—	*Brachybacterium faecium* (1)	1.47[c]
Brevibacterium epidermis (1)[ab]	100	*Micrococcus luteus* (1)	100	*Brevibacterium* spp. (1)	31	*Brevibacterium linens* (1)	1.36
Corynebacterium glucuronolyticum (2)[a]	97–99	*Corynebacterium* spp. (2)	—	*Corynebacterium glucuronolyticum* (2)	100	*C. glucuronolyticum* (2)	1.78–2.39
Isolates identified correctly to genus level, total (%)		3 (50)		2 (40)		4 (66.7)	
Isolates identified correctly to species level, total (%)	—	0 (0)	—	2 (40)	—	2 (33.4)	—

[a]Species not listed in Aris 2x Sensititre Gram-positive veterinary origin database.
[b]Species not listed in Bruker Biotyper library.
[c]MALDI score between 1.700 and 1.999 is suggestive of probable genus identification and a score of <1.699 is indicative of unreliable identification.

Table 2. Genus and Species Identification of Catalase-Negative, Gram-Positive Cocci

16S rRNA gene sequence-based identification		*Aris 2x Sensititre identification system*		*API 20 Strep identification system*		*Bruker Biotyper 2.0 MALDI-TOF identification system*	
Species[ab]	*% ID*	*Species*	*% ID*	*Species*	*% ID*	*Species*	*MALDI score*
Aerococcus viridans (4)	96–98	*A. viridans* (4)	95–96	*A. viridans 3* (4)	95–100	*A. viridans* (4)	2.02–2.24
Enterococcus avium (2)	90–92	*E. avium* (2)	96–100	*E. avium* (2)	95–100	*E. avium* (1)	2.19
						Enterococcus gilvis (1)	2.25
Enterococcus faecalis (3)	99–100	*E. faecalis* (3)	100	*E. faecalis* (3)	96–100	*E. faecalis* (3)	2.42–2.51
Enterococcus faecium (4)	90–99	*E. faecium* (4)	96–100	*E. faecium* (4)	98–99	*E. faecium* (4)	2.31–2.49
Enterococcus saccharolyticus (2)[ab]	96–95	*Streptococcus mutans* (2)	100	*E. avium* (1)	99	*E. saccharolyticus* (2)	2.23–2.30
				A. viridans 1 (1)	100		
Lactococcus garvieae (2)[ab]	100	No identification (2)	—	*E. faecalis* (2)	100	*L. garvieae* (2)	2.15–2.20
Lactococcus lactis (2)[a]	99	*Enterococcus sp.* (1)	85	*L. lactis* (2)	97–99	*L. lactis* (2)	2.39–2.51
		Streptococcus pyogenes (1)	100				
Streptococcus dysgalactiae (6)	99–100	*S. dysgalactiae* (5)	100	*S. dysgalactiae* (6)	99–100	*S. dysgalactiae* (6)	2.09–2.31
		Streptococcus group-G (1)	45				
Streptococcus gallolyticus (1)[a]	100	*Streptococcus bovis*	89	*S. bovis* I	99	*S. gallolyticus*	2.48
Streptococcus uberis (7)	100	*S. uberis* (7)	100	*S. uberis* (7)	100	*S. uberis* (7)	2.21–2.39
Isolates identified correctly to genus level, total (%)	—	29 (87.8)	—	32 (96.9)		33 (100)	—
Isolates identified correctly to species level, total (%)	—	25 (75.6)	—	31 (93.9)	—	32 (96.9)	—

[a]Species not listed in Aris 2x Sensititre Gram-positive veterinary origin database.
[b]Not listed in API 20 Strep database.

identification database (www.biomerieux-diagnostics.com/sites/clinic/files/9308960-002-gb-b-apiweb-booklet.pdf) and the clinical application Bruker Biotyper database (http://spectra.folkhalsomyndigheten.se/spectra/database/searchBruker.action), respectively. The results of this study are presented as percent species correctly identified compared to the 16S rRNA sequence-based identification system.

Results

Six Gram-positive rods belonging to five different species were evaluated using the three identification systems. The ARIS system correctly identified three of the six (50%) isolates to genus level, while none of the six isolates were identified correctly to the species level. A review of the Sensititre ARIS Gram-positive identification database showed that four of the five species tested were not listed in the database. The API (API Coryne) identification system identified correctly four of the five isolates to genus and species level (80%), although the API system identified *Arthrobacter mysorens* as *Arthrobacter* spp. (24% probability of identification) and *Brevibacterium epidermidis* as *Brevibacterium* spp. (31% ID), the Apiweb database v3.0 (https://apiweb.biomerieux.com) classified the isolates as low discrimination/no identification, and hence in our study were considered as no identification.

The appropriate API system to identify *Bacillus* species was not used in our study. The MALDI identification system identified correctly four of the six isolates to genus level (66.7%), while two of the six isolates were correctly identified to species level (33.4%). One isolate of *Corynebacterium glucuronolyticum* had a low MALDI score and therefore was acceptable only to the genus level (MALDI score of 1.78) (Table 1).

A total of 33 catalase-negative, Gram-positive cocci were examined, of which ARIS correctly identified 29 (87.8%) and 25 (75.6%) of the isolates correctly to the genus and species level, respectively. ARIS system misidentified two isolates each of *Enterococcus saccharolyticus* as *Streptococcus mutans*, and *Lactococcus garvieae*, while two isolates of *Lactococcus lactis*, one of which was misidentified as *Enterococcus* spp. (85% ID) and the other as *Streptococcus pyogenes.* Of the six isolates of *Streptococcus dysgalactiae*, one isolate of *S. dysgalactiae* was misidentified as *Streptococcus* group G, (45% ID). *Streptococcus gallolyticus* was misidentified as *Streptococcus bovis*, (89% ID). Four of the 10 species were not listed in the ARIS® Gram-positive identification database (Table 2).

The API Strep identification system correctly identified 32 (96.9%) and 31 (93.9%) of the isolates to the genus and species level, respectively. The system misidentified two isolates of *E. saccharolyticus* as *Enterococcus avium* and *Aerococcus viridans*, respectively. *E. saccharolyticus* is not included in the API Strep database, but is listed under Rapid ID 32 Strep identification system. Similarly, *Lactococcus gravieae* is not listed in the API Strep database and was misidentified as *Enterococcus faecalis.* The MALDI correctly identified 33 (100%) and 32 (96.9%) of the isolates to the genus and species level, respectively. One isolate of *E. avium* was misidentified as *Streptococcus gilvis* (Table 2).

The ARIS system correctly identified 40 (88.9%) and 15 (33.4%) of catalase-positive, Gram-positive cocci isolates to the genus and species level, respectively. The ARIS systems correctly identified five isolates of *Staphylococcus aureus*, two isolates of *Staphylococcus epidermidis*, and three isolates of *Staphylococcus xylosus*. Eight of the 16 species are not listed in the Sensititre ARIS Gram-positive identification database (Table 3). The API Staph identification system correctly identified 44(97.7%) and 38 (84.5%) of the isolates to the genus and species level, respectively. Four of the 16 species were not listed under API Staph database (Table 3). The MALDI system correctly identified all 45 (100%) and 44 (97.7%) of the isolates both to the genus and species level, respectively. One isolate of *Staphylococcus chromogenes* was misidentified as *Staphylococcus piscifermentans* (Table 3).

A total of 48 Gram-negative bacterial isolates were speciated using the three identification systems and results were compared to the 16S rRNA gene sequencing technique, which was served as the reference method. The ARIS system correctly identified 44 (91.6%) and 41 (85.4%) of the isolates to the genus and species level, respectively. Five of the 13 isolates of *Klebsiella pneumoniae* were misidentified by the system. The API 20E identification system correctly identified 47 (97.9%) and 45 (93.7%) of the isolates to the genus and species level, respectively. One isolate each of *K. pneumoniae*, *Pseudomonas koreensis*, *and Serratia proteamaculans* was misidentified, while one isolate of *Pantoea agglomerans* was identified to genus level (Table 4). The MALDI system correctly identified 47 (97.9%) and 46 (95.8%) of the isolates to the genus and species level, respectively. One isolate each of *Enterobacter aerogenes* and *S. proteamaculans* was misidentified as *K. pneumoniae* and *S. liquefaciens*, respectively (Table 4).

The cost (supplies, reagents, and consumables) for species identification, the time taken for identification (primary isolate on blood agar to identification), and the percent correct identification of the species tested are presented in Table 5. The cost of performing a species identification using 16S rRNA, ARIS, and MALDI was $7.80, $5.95, and $2.00, respectively. Species identification using the API system ranged from $9.90 to $16.50 depending on the type of system used for bacterial identification. Identification of bacteria with the exception of MALDI was accomplished in 24 h, while the MALDI required <2 h for identifying the bacterial species. MALDI identification system had the highest percentage (93.9%) of correctly identified bacterial species followed by API (87.8%), and ARIS (61.6%) identification systems (Table 5).

Discussion

Although the bacterial isolation and identification techniques used in a veterinary diagnostic laboratory are similar to the techniques used in human diagnostic laboratories, some veterinary pathogens require unique cultivation or identification procedures. Several studies have shown that commercial identification systems provide rapid, correct identification of human pathogens (Holmes *et al.*, 1994; Bosshard *et al.*, 2006). However, the accuracy of these systems with veterinary pathogens varies widely depending on the bacterial species and the host animal from which they were isolated (Watts and Yancey, 1994). The focus of our study was to evaluate the Bruker MALDI-TOF MS system and compare it with two widely used bacterial identification systems in veterinary laboratories, including ARIS and the API bacterial identification systems, using the 16S rRNA gene sequence analysis as the reference method.

Table 3. Genus and Species Identification of Catalase-Positive, Gram-Positive Cocci

16S rRNA gene sequence-based identification		*Aris 2x Sensititre identification system*		*API Staph identification system*		*Bruker Biotyper 2.0 MALDI-TOF identification system*	
Species	*% ID*	*Species*	*% ID*	*Species*	*% ID*	*Species*	*MALDI score*
Kocuria rhizophila (1)[ab]	100	*Staphylococcus hyicus* (1)	57	*Micrococcus* spp. (1)	90	*K. rhizophila* (1)	2.33
Staphylococcus arlettae (1)[ab]		*S. aureus (1)*	100	*S. lentus* (1)	96	*S. arlettae* (1)	2.02
Staphylococcus aureus (6)	99–100	*S. aureus* (5)	95–100	*S. aureus* (6)	87–98	*S. aureus* (6)	2.21–2.51
		Staphylococcus intermedius (1)	78				
Staphylococcus capitis (2)[a]	99–100	*Staphylococcus epidermidis* (1)	100	*S. capitis* (1)	99	*S. capitis* (2)	2.13–2.29
		Corynebacterium sp. (1)	85	*S. epidermidis* (1)	98		
Staphylococcus chromogenes (11)[a]	95–100	No identification (4)	—	*S. chromogenes* (10)	99	*S. chromogenes* (10)	2.21–2.46
		S. hyicus (1)	100				
		Staphylococcus schleiferi (5)	94–100				
		Bacillus cereus (1)	100	*S. caprae* (1)	100	*Staphylococcus piscifermentans* (1)	2.08
Staphylococcus cohnii (2)[a]	99–100	*Staphylococcus warneri* (1)	81	*S. cohnii* (2)	100	*S. cohnii* (2)	2.22–2.13
		Staphylococcus saprophyticus (1)	64				
S. epidermidis (2)	100	*S. epidermidis* (2)	100	*S. epidermidis* (2)	95–97	*S. epidermidis* (2)	2.08–2.17
Staphylococcus equorum (2)[ab]	99–100	*Staphylococcus xylosus* (2)	100	*S. xylosus* (1)	100	*S. equorum* (2)	2.02–2.13
			100	*S. lentus* (1)	100		
Staphylococcus haemolyticus (3)	99–100	*S. haemolyticus* (1)	100	*S. haemolyticus* (2)	93	*S. haemolyticus* (3)	2.15–2.72
		S. warneri (2)	100	*S. warneri* (1)	97		
Staphylococcus hyicus (2)	100	*S. hyicus* (2)	100	*S. hyicus* (2)	86–97	*S. hyicus* (2)	2.05–2.10
Staphylococcus piscifermentans (1)[ab]	99	No identification (1)	—	*S. caprae* (1)	99	*S. piscifermentans* (1)	2.14
S. saprophyticus (1)	100	*S. saprophyticus* (1)	97	*S. saprophyticus* (1)	85	*S. saprophyticus* (1)	2.38
Staphylococcus sciuri (1)[a]	100	*S. aureus* (1)	100	*S. sciuri* (1)	96	*S. sciuri* (1)	2.05
Staphylococcus simulans (5)[a]	98–100	*S. hyicus* (1)	100	*S. simulans* (5)	98–99	*S. simulans* (5)	2.06–2.33
		S. xylosus (1)	44				
		S. schleiferi (1)	97				
		S. intermedius (2)	86–95				
S. warneri (1)	99	*S. warneri* (1)	100	*S. warneri* (1)	95	*S. warneri* (1)	2.05
S. xylosus (4)	99–100	*S. xylosus* (3)	97–100	*S. xylosus* (4)	100	*S. xylosus* (4)	2.02–2.18
		S. warneri (1)	88				
Isolates identified correctly to genus level, total (%)		40 (88.9)		44 (97.7)		45 (100)	
Isolates identified correctly to species level, total (%)		15 (33.4)		38 (84.5)		44 (97.7)	

[a]Species not listed in Aris 2x Sensititre Gram-positive veterinary origin database.
[b]Species not listed in API Staph database.

Table 4. Genus and Species Identification Methods for Gram-Negative Bacteria

16S rRNA gene sequence-based identification		*Aris 2x Sensititre identification system*		*API 20E identification system*		*Bruker Biotyper 2.0 MALDI-TOF identification system*	
Species	*% ID*	*Species*	*% ID*	*Species*	*% ID*	*Species*	*MALDI Score*[a]
Acinetobacter baumannii (1)	97	*A. baumannii* (1)	84	*A. baumannii* (1)	99	*A. baumannii* (1)	1.90[a]
Citrobacter koseri (1)	99	*C. koseri* (1)	100	*C. koseri* (1)	98	*C. koseri* (1)	2.45
Enterobacter aerogenes (1)	94	*Enterobacter aerogenes* (1)	95	*E. aerogenes* (1)	96	*K. pneumoniae* (1)	2.45
Enterobacter cloacae (4)	91–98	*E. cloacae* (3)	77–100	*E. cloacae* (4)	94–99	*E. cloacae* (4)	2.17–2.39
		Kluyvera ascorbata (1)	96				
Escherichia coli (5)	97–100	*E. coli* (5)	100	*E. coli* (5)	79–100	*E. coli* (5)	2.34–2.61
Klebsiella oxytoca (2)	97–100	*K. oxytoca* (2)	100	*K. oxytoca* (2)	97–98	*K. oxytoca* (2)	2.22–2.30
Klebsiella pneumoniae (13)	92–99	*K. pneumoniae* (8)	86–100	*K. pneumoniae* (12)	78–98	*K. pneumoniae* (13)	2.26–2.52
		Raoultella planticola (1)	91				
		Klebsiella group 47 (2)	100				
		E. coli (1)	100				
		Serratia marcescens (1)	90	*S. liquefaciens* (1)	78		
Pantoea agglomerans (1)[b]	99	*P. agglomerans* (1)	98	*Pantoea spp 3* (1)	100	*P. agglomerans* (1)	2.05
Pastuerella multocida (2)	99–100	*P. multocida* (2)	100	*P. multocida* (2)	96	*P. multocida* (2)	2.25–2.49
Proteus mirabilis (1)	90	*P. mirabilis* (1)	100	*P. mirabilis* (1)	100	*P. mirabilis* (1)	2.45
Providencia stuartii (1)	99	*P. stuartii* (1)	100	*P. stuartii* (1)	98	*P. stuartii* (1)	2.50
Pseudomonas aeruginosa (3)	97–99	*P. aeruginosa* (3)	77–100	*P. aeruginosa* (3)	94–100	*P. aeruginosa* (3)	2.37–2.54
Pseudomonas koreensis (1)[bc]	100	*P. aeruginosa* (1)	91	*P. fluorescens* (1)	98	*Pseudomonas koreensis* (1)	2.25
S. marcescens (10)	92–99	*S. marcescens* (10)	86–100	*S. marcescens* (10)	72–100	*S. marcescens* (10)	2.12–2.52
Serratia proteamaculans (1)[ab]	98	*S. proteamaculans* (1)	100	*S. marcescens* (1)	84	*S. liquefaciens* (1)	2.34
Serratia rubidaea (1)	99	*S. rubidaea* (1)	100	*S. rubidaea* (1)	100	*S. rubidaea* (1)	2.49
Isolates identified correctly to genus level, total (%)		44 (91.6)	—	47 (97.9)	—	47 (97.9)	—
Isolates identified correctly to species level, total (%)		41 (85.4)		45 (93.7)		46 (95.8)	—

[a]MALDI score between 1.700 and 1.999 is suggestive of probable genus identification.
[b]Species not listed in API 20E database.
[c]Species not listed in Aris 2x Sensititre Gram-negative database.

TABLE 5. COST AND TIME COMPARISON FOR BACTERIAL IDENTIFICATION SYSTEMS

Identification system	*Cost per isolate ($)*	*Time required for identification (h)*	*% Gram-positive rods*	*% catalase-negative, Gram-positive cocci*	*% catalase-positive, Gram-positive cocci*	*% Gram-negative bacteria*	*All species (n = 132)* n (%)
				% Correct ID (genus/species)			
16S rRNA gene sequence analysis	7.80	24	—	—	—	—	—
ARIS	5.95	24	50/0	87.8/75.6	88.9/33.4	91.6/85.4	80 (61.6)
API			—	—	—	—	
API 20E system	10.35	24	—			97.9/93.7	116 (87.8)
API Coryne	16.50	24	40/40	—	—	—	
API Staph	9.90	24	—	—	97.7/84.5	—	
API Strep	11.25	24	—	96.9/93.9	—	—	
MALDI	2.00	<2	66.7/50	100/96.9	100/97.7	97.9/95.8	124 (93.9)

In our study, the ARIS system correctly identified 61.6% of the isolates to the species level. It was observed that 0%, 75.6%, 33.4%, and 85.4% of Gram-positive rods, catalase-negative, Gram-positive cocci, catalase-positive, Gram-positive cocci, and Gram-negative rods were correctly identified to the species level, respectively. This could be primarily due to limited database, especially for Gram-positive bacteria. With an expanded database, the ARIS system could improve its accuracy of identification.

The API system correctly identified 87.8% of our samples to the species level. It was observed that 40%, 93.9%, 84.5%, and 93.7% of Gram-positive rods, catalase-negative, Gram-positive cocci, catalase-positive, Gram-positive-cocci, and Gram-negative rods, respectively, were correctly identified to the species level. We observed that API systems are valuable when only a few isolates need to be identified; the process of preparing API strips can be done in less than an hour. However, identification of a microorganism is limited to the database of the API identification system being used. For instance, species that were not listed in the API Strep database were listed in alternate identification systems, such as Rapid ID 32 Strep.

When more than one API system can be used for identification, this can be a challenge in a diagnostic laboratory to keep up with the inventory, taking into consideration the cost and expiration date on the identification system. The API identification systems required at least 24 h of incubation, and depending on the type of API identification system used, the cost could vary (Table 5). It was also noted on some occasions that the interpretation of the biochemical tests was subjective, which could lead to misidentification of the organism.

The ARIS system along with the API 20E identification system was evaluated for identification of *Enterobacteriaceae* obtained from three different hospital laboratories and it was observed that 94.6% and 91.1% of the isolates were correctly identified by ARIS and API 20E identification systems, respectively (Staneck *et al.*, 1983). A similar study reported that 83% of *Enterobacteriaceae* isolates ($n = 251$) were correctly identified to the species level by both API 20E and the ARIS system and both the systems poorly discriminated between species of the genera *Klebsiella*, *Enterobacter*, and *Serratia* (Barr *et al.*, 1989).

The Sensititre ARIS Gram-positive identification plates and API Staph strips were evaluated previously for identification of clinically relevant coagulase-negative staphylococci (Garza-Gonzalez *et al.*, 2010) and it was observed that the ARIS and API Staph identification systems correctly identified 68.9% and 91% of the isolates, respectively. Based on these findings, it was concluded that the ARIS system provided moderate to less reliability for identification of most commonly identified coagulase-negative staphylococci and other less commonly occurring species (Garza-Gonzalez *et al.*, 2010).

The API Staph-Trac system, a manual biochemical kit, identified only 66.1% of staphylococci isolated from bovine mammary glands. The discrepancy was attributed to misidentification of *Staphylococcus hyicus* and the inability of the system to differentiate *Staphylococcus intermedius* from *S. aureus (*Watts and Nickerson, 1986*)*. A similar study examined 14 staphylococcal species of bovine origin using API Staph Trac System and observed that 80.8% of the isolates were correctly identified compared to conventional identification methods (Matthews *et al.*, 1990). The API Staph-Trac

system incorrectly identified strains of *S. chromogenes*, *Staphylococcus gallinarium*, *Staphylococcus hemolyticus*, *S. hyicus*, *Staphylococcus simulans*, and *Staphylococcus warneri* (Matthews *et al.*, 1990). In our study, the API Staph correctly identified 80% of the isolates, and *S. chromogenes* and *S. hemolyticus* were incorrectly identified in our study.

The API 20 Strep system (previously also known as API Rapid Strep System) has been a widely evaluated system with veterinary isolates. A study conducted in France by Poutrel and Ryniewicz (1984) showed that the API Rapid Strep system identified 71.4% of 84 isolates. They stated that *Streptococcus uberis* and *S. bovis* isolates were incorrectly identified and recommended that the database should be updated for identification of streptococcal mastitis pathogens. A study conducted in the United States showed that API Rapid Strep system correctly identified 88.4% of 199 streptococci isolated from bovine mastitis (Watts, 1989).

This study also stated that the system was unable to identify strains of *S. bovis*, but correctly identified 96.2% of *S. uberis* strains. A similar study showed that the API Rapid Strep system had an overall accuracy level of 96.5%, of which 95.0% of the *S. uberis* and 100.0% of the *S. bovis* strains were correctly identified (Jayarao *et al.*, 1991). In this study, 93.9% of the catalase-negative, Gram-positive cocci were correctly identified by API 20 Strep system, and these findings are in agreement with previously reported findings (Watts, 1989; Jayarao *et al.*, 1991).

There are only a few reports that have evaluated the API 20E system using veterinary isolates. Studies done in the early 1980s showed that the API 20E system correctly identified 80% and 97.9% of isolates of Gram-negative nonfermenters and *Enterobacteriaceae* from clinical specimens of animal origin, respectively (Devenish and Barnum, 1980, 1982). In this study, API 20E correctly identified 93.7% of Gram-negative bacteria isolated from mastitis and bulk tank milk samples.

The MALDI method successfully identified 93.9% isolates. The MALDI system allowed identification of bacterial isolates in <2 h, while the ARIS and the group of specific API identification systems required 24 h. Other advantages observed with MALDI were that the target plates are reusable and require very few other reagents and materials. This makes the system more cost-effective when the initial start-up cost of the unit is not considered. In addition, the Bruker Biotyper database is extensive and robust with over 3000 unique entries (El-Bouri *et al.*, 2012).

The MALDI-TOF MS system was shown to be a more reliable identification method for microorganisms from milk and provided cheaper and faster results (Barreiro *et al.*, 2010). A previous study that examined MALDI-TOF used a combination of phenotypic microbiological tests and multiplex PCR assays for identification of streptococci and related bacteria isolated from bovine intramammary infections. The study concluded that MALDI-TOF MS was a fast method, although identification of *S. dysgalactiae* at the subspecies level was variable (Raemy *et al.*, 2013).

A total of 180 isolates of *Corynebacterium* spp. were examined using MALDI-TOF MS and 16S rRNA gene sequencing (Goncalves *et al.*, 2014). The MALDI-TOF MS methodology correctly identified 89.4% of the isolates at the species level, while the 16S rRNA gene sequencing at the species level identified 86.7% of the isolates (Goncalves *et al.*, 2014). It was concluded that the MALDI-TOF MS analysis could serve as an alternative method to 16S rRNA gene sequencing method for identification of *Corynebacterium* spp. isolated from dairy cow intramammary infections.

Streptococcus spp. isolates from cases of bovine mastitis were evaluated using MALDI-TOF MS and compared to Fourier-transform infrared spectroscopy (Schabauer *et al.*, 2014). The MALDI-TOF MS system had an overall accuracy of 95.2%. The study determined that the Fourier transform infrared spectroscopy was slightly superior to MALDI-TOF MS, however, both techniques could be used as alternate methods to conventional microbiologic methods as these two methods provided high accuracy at low operating costs following acquisition of the instruments (Schabauer *et al.*, 2014).

Vithanage *et al.* (2014) observed that the 16S rRNA gene sequencing (100.0%) was the most reliable method for identification of Gram-negative bacilli milk spoilage bacteria followed by Biolog® (86.8%), MALDI-TOF MS (63.2%), API 20E (60.5%), and Microbact® (57.5%), while for Gram-positive bacilli, the study again found that 16S rRNA gene sequencing (100.0%) was the most reliable method followed by Biolog (85.0%), MALDI-TOF MS (95.0%), and API 20E (90.0%). It was inferred that manual biochemical kits such as API and Microbact, automated biochemical system such as Biolog, and small protein detection systems such as MALDI-TOF MS had limited reference profiles of milk spoilage bacteria and therefore could have reduced their accuracy in bacterial identification compared to 16S rRNA gene sequencing

The MALDI-TOF MS system correctly identified 95.4% of coagulase-negative staphylococci isolated from bovine intramammary infections and was found to be a reliable alternate method for differentiating coagulase-negative staphylococci (Tomazi *et al.*, 2014). Our study supports previous studies investigating the value of MALDI in identifying bacteria from milk and bovine mastitis.

In summary, all three identification systems had low accuracy of identification (0–50%) for Gram-positive rods. This could be primarily due to limited database, especially for Gram-positive bacteria. With an expanded database, the system could improve its accuracy of identification. For the other three groups of bacteria (catalase +/−, Gram-positive cocci, and Gram-negative bacteria), MALDI provided an equivalent or slightly better identification than the API systems. The significant gain was in time (<2 h) and lower cost ($2.00), compared to ARIS and API identification systems. The repertoire of bacteria in the Sensititre ARIS x2 database is limited and needs to be significantly expanded to improve its accuracy of identification. The API identification systems provided correct identification of bacterial isolates, however, the cost and time required for identification were high. In conclusion, MALDI is a time-efficient and cost-effective method for identifying bacteria isolated from bulk tank milk and cases of bovine mastitis

Disclosure Statement

No competing financial interests exist.

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