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Evaluation of PCR electrospray-ionization mass spectrometry for rapid molecular diagnosis of bovine mastitis

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ABSTRACT

Bovine mastitis, an inflammatory disease of the mammary gland, is one of the most costly diseases affecting the dairy industry. The treatment and prevention of this disease is linked heavily to the use of antibiotics in agriculture and early detection of the primary pathogen is essential to control the disease. Milk samples (n = 67) from cows suffering from mastitis were analyzed for the presence of pathogens using PCR electrospray-ionization mass spectrometry (PCR/ESI-MS) and were compared with standard culture diagnostic methods. Concurrent identification of the primary mastitis pathogens was obtained for 64% of the tested milk samples, whereas divergent results were obtained for 27% of the samples. The PCR/ESI-MS failed to identify some of the primary pathogens in 18% of the samples, but identified other pathogens as well as microorganisms in samples that were negative by culture. The PCR/ESI-MS identified bacteria to the species level as well as yeasts and molds in samples that contained a mixed bacterial culture (9%). The sensitivity of the PCR/ESI-MS for the most common pathogens ranged from 57.1 to 100% and the specificity ranged from 69.8 to 100% using culture as gold standard. The PCR/ESI-MS also revealed the presence of the methicillin-resistant gene *mecA* in 16.2% of the milk samples, which correlated with the simultaneous detection of staphylococci including *Staphylococcus aureus*. We demonstrated that PCR/ESI-MS, a more rapid diagnostic platform compared with bacterial culture, has the significant potential to serve as an important screening method in the diagnosis of bovine clinical mastitis and has the capacity to be used in infection control programs for both subclinical and clinical disease.

Key words: milk, detection, dairy cow, method

INTRODUCTION

Bovine mastitis, an inflammatory and infectious disorder of the udder tissue in cows, often occurs in response to bacterial invasion, and less frequently to invasion by molds, yeasts, *Prototheca*, and viruses (Wellenberg et al., 2002; Rakesh et al., 2006; Möller et al., 2007; Zadoks et al., 2011). The economic effect of bovine mastitis is significant, and in some cases nearly 10% of total milk production is unusable (Seegers et al., 2003; Halasa et al., 2007; Hogeveen et al., 2011). The diagnosis of clinical bovine mastitis is based on the abnormal appearance of milk, visible and or palpatory changes in the udder, and elevated SCC (Ruegg, 2003). Subclinical infections, however, are economically more problematic and clinical signs are not obvious, as the milk appears normal despite an increased SCC. Microbiological examination of the milk is necessary to determine the cause of the infection and allow veterinarians to use appropriate therapeutic measures. To date, cultures of the pathogens and microscopy remain the most common approaches to identify the pathogens in milk. However, the samples may contain a large variety of microorganisms, making the identification of the primary pathogens very difficult. Additionally, mastitis may be caused by slow-growing bacteria, such as *Mycoplasma* spp., which require special growth medium, thus delaying diagnosis. To overcome these problems, PCR-based methods have been developed; they are frequently restricted to a limited number of pathogens, however (Viguier et al., 2009; Ajitkumar et al., 2012).

Multiple PCR followed by electrospray-ionization mass spectrometry (**PCR/ESI-MS**) has been developed to rapidly detect nearly all known pathogens,

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including those causing bovine mastitis, as well as some important antibiotic-resistant genes. This technology consists of DNA amplification by PCR of specific regions of ribosomal and conserved house-keeping genes, as well as some antibiotic-resistant genes, which are electrosprayed into a time-of-flight MS for molecular weight measurement. The mass of each amplicon is translated into base composition for organism and antibiotic-resistant gene identification (see Ecker et al., 2008, and Wolk et al., 2012, for reviews of the methodology, flow scheme, and laboratory application). This technology has also found applications in human clinical diagnostics (Arciola et al., 2011; Wolk et al., 2012). The objective of this pilot study was to assess whether PCR/ESI-MS can be a useful technology for the rapid identification of bovine mastitis-causing pathogens compared with standard milk cultures. Earlier and more precise detection of the pathogens would help to identify risk factors for bovine mastitis and aid in the therapeutic strategy, as well as in the development of appropriate screening and control programs.

MATERIALS AND METHODS

Milk Sampling

Milk samples (10 mL) were prospectively collected aseptically from cows affected by clinical (changes in secretion or changes in the consistency of the mammary gland) and subclinical mastitis (SCC > 150,000 cells/mL) and from healthy cows (SCC < 150,000 cells/mL) in Switzerland. Cows were selected according to their last individual SCC test day as measured using a Fossomatic 500 cell counter (Foss, Hillerød, Denmark), and a California Mastitis Test was performed after forestripping as described previously (Barnum and Newbould, 1961). A threshold of ≥800,000 to 5,000,000 cells/mL, providing a sensitivity of 72% and a specificity of 64% for detecting IMI, was used (Ruegg 2003). Eight milliliters of milk were used directly for bacteriological analysis and the remaining 2 mL were shipped overseas at room temperature without preservatives for subsequent DNA extraction and PCR/ESI-MS analyses.

Bacteria Isolation and Identification

Milk samples were centrifuged at 590 × *g* for 10 min at room temperature. Cultivating for the common milk pathogens, one loop-full (10 μL) of the resulting pellet and supernatant was plated directly onto tryptone soy agar plates containing 5% sheep blood (**TSA-SB**; BD, Franklin Lakes, NJ) and onto selective bromothymol-blue lactose (Brolac; bioMérieux SA, Marcy-l'Étoile, France) agar plates. The TSA-SB plates were incubated with 5% CO_2 for 24 h for the detection of microaerophilic bacteria, such as *Histophilus somni*, and for another 24 h at 37°C under aerobic conditions to support the growth of strict aerobic bacteria. Brolac agar plates were incubated under aerobic conditions at 37°C for 24 to 48 h for the detection of lactose-positive or negative bacteria and the specific growth of yeast. Additionally, samples suspected of containing *Aspergillus* spp. were cultivated on Sabouraud Agar (Oxoid, Basingstoke, UK); those suspected of containing *Mycoplasma bovis* were cultivated on specific Mycoplasma agar (Oxoid) at 37°C for 2 to 5 d.

Staphylococcus aureus was identified on TSA-SB agar plates based on the production of an α- and β-(double) hemolysis. α-Hemolytic *Staph. aureus* were identified using chromogenic agars SA select (Bio-Rad, Hercules, CA) and *Staph. aureus* ID agar (bioMérieux). Other species of staphylococci (non-*aureus Staphylococcus* spp., including CNS) were not further identified. Streptococci, enterococcoci, and lactococci were identified using a biochemical scheme described previously (Guélat-Brechbuehl et al., 2010). *Enterobacteriaceae* (e.g., *Escherichia coli*) were identified by microscopy, lactose fermentation, and indole production assays. *Corynebacterium bovis*, *Arcanobacterium pyogenes*, yeasts, and *Prothoteca* were identified by microscopy or phenotypic reactions using Vitek Compact 2 (bioMérieux).

Anaerobic bacteria were identified by direct microscopy of the milk pellet and strict anaerobic growth on TSA-SB. *Aspergillus* spp. were identified by microscopic morphology and by specific coloration appearance on Sabouraud agar. *Mycoplasma bovis* was identified by PCR (Subramaniam et al., 1998). Plates grown with a layer or with more than 3 microorganisms displaying different morphology were considered as containing a mixture of bacteria, which were not further identified by culture. A method of semiquantification [few (>30 colonies), moderate (30–100), or many (>100)] was used to report the relative numbers of bacteria present in the milk samples (Washington, 1996).

PCR/ESI-MS

The DNA for PCR/ESI-MS was extracted from 1 mL of each milk samples using a method that combines bead-beating cell lysis with a magnetic-bead base extraction (Abbott, Des Plaines, IL). Briefly, the milk was mixed with proteinase K, and 20% SDS solution was mixed with the extraction control in a tube containing 1.5 g of 0.2-mm yttrium-stabilized zirconium oxide beads. The mixture was then homogenized in a tissue homogenizer (Precellys 24, Bioamerica Inc., Miami, FL) at 6,200 rpm for 90 s three times, with 5-s intervals between events. Each homogenized lysate was incubated at 56°C

for 15 min and then centrifuged for 3 min at 16,000 × *g* in a bench-top microcentrifuge. Next, DNA from the lysate was isolated using a magnetic particle processor (Kingfisher Flex, Thermo Scientific, Waltham, MA). The lysate was transferred to a 24 deep-well plate along with lysis buffer and magnetic particles. Each lysate mixture was incubated for 16.5 min in the lysis buffer at 56°C. Specimens were then washed once in wash buffer 1, and 3 times in wash buffer 2 (1-min incubation for each wash step). The magnetic beads were then dried for 3 min at 65°C, and nucleic acids were eluted into 250 μL of DNA/RNA-free water by incubating the magnetic particles at 65°C for 3 min. The PCR/ESI-MS analyses were performed on the PLEX-ID using the PLEX-ID BAC Detection assay (Cat. No. 05N13–62, Abbott) for the detection and identification of more than 3,400 species of bacteria, 40 species of *Candida*, and 4 antibiotic-resistant markers (*mecA*, *vanA*, *vanB*, and bla_{KPC}) direct from the sample. All groups of bacteria, including intracellular organisms such as Mycoplasma, Chlamydia, and Rickettsia and hard to culture or nonculturable organisms, could be detected by this assay. Analysis was performed using the software version 2.6.052 (Ibis Biosciences; Ecker et al., 2008). The PLEX-ID BAC Detection assay has a DNA calibrant in each reaction, which allows for semiquantitative analysis (Hofstadler et al., 2005). By comparing the relative intensity of the target DNA to that of the calibrant, the relative concentration of target DNA initially present is determined (Ecker et al., 2008).

The PLEX-ID BAC assay uses signal thresholds (cutoffs) designed to limit reporting of irreproducible detections. Cutoffs are applied to 2 measurements. The first, termed the level, is an indication of the amount of the amplicon present in the sample reported as genome equivalents (**GE**) per well. This is calculated with reference to the internal calibrant and has been described previously (Hofstadler et al., 2005). The linear range for reporting these levels is between 0.1× and 10× the levels of internal controls in the assay, which, in the case of the fungal assay, represents a working range of ~2 GE/well to 200 GE/well. The second is the quality score (Q-score), which represents a relative measure of the strength of the data supporting identification. The Q-Score is a rating between 0 (low) and 1 (high), based on several parameters. Parameters include an indicator of how well the hypothesized organisms, as a group, represent the observed data; an indicator of how significant the contribution of a single organism is to the solution; the fraction of missed detections, which represents the percentage of primers for a detected organism that should have produced known base count compositions, but did not; and, finally, the percentage of primers for a detected organism for which no known data exists within the PLEX-ID system. The Q-score cutoffs are designed to prevent reporting (positive identification) of specific organisms when the information obtained is not sufficient to confidently resolve the organism's identity. For the PLEX-ID BAC assay, a Q-score $\geq$ 0.85 is considered a reportable result. The specificity and sensitivity of the PCR/ESI-MS method was determined using culture as gold standard.

RESULTS

A total of 67 milk samples originating from 21 cows with clinical mastitis, 34 cows with subclinical mastitis, and 12 healthy cows were analyzed using both standard bacteriology and PCR/ESI-MS analysis. Overall, a 64.1% agreement was observed between the 2 methods, with results concurrent with both methods for 43 milk samples (Table 1, samples 1–43). Among them, PCR/ESI-MS and cultures generated the exactly same results for 18 milk samples (Table 1, samples 1–18). In the other 24 milk samples with concurrent results, PCR/ESI-MS identified additional microorganisms in 18 milk samples compared with culture (Table 1, samples 19–36), whereas culture revealed additional microorganisms in only 3 milk samples compared with PCR/ESI-MS (Table 1, samples 37–39). Besides a concurrent identification of 1 type of microorganism in 4 milk samples, PCR/ESI-MS and cultures both identified additional but different organisms (Table 1, samples 40–43). Both methods revealed the presence of CNS in 12 milk samples: *Streptococcus uberis* (7 samples), *Streptococcus dysgalactiae* (1 sample), *Staph. aureus* (4 samples), *C. bovis* (3 samples), *Mycoplasma* spp. (2 samples), *A. pyogenes* (3 samples), *Enterococcus* spp. (4 samples), *Enterobacteriaceae* (2 samples), as well as the absence of detectable microorganisms in 4 samples (Table 1, samples 15–18). Specific identification of the pathogens could not be made by culture or by PCR/ESI-MS for 2 milk samples, but both methods identified a mixture of different bacteria in one sample and a mixture of anaerobic bacteria in the other (Table 1, samples 10 and 14). Otherwise, PCR/ESI-MS allowed identification of bacteria to the species level, as well as yeast and fungi.

Indeed, in 6 additional samples containing a mixture of bacteria (8.8%) consisting of more than 3 different microorganisms, which are routinely not further identified individually, PCR/ESI-MS allowed identification of CNS (*Staphylococcus sciuri*, *Staphylococcus vitulinus*, *Staphylococcus xylosus*, *Staphylococcus equorum*) in all samples and also yeasts (*Candida albicans*), molds (*Penicillium marneffei*), *Pseudomonas* spp., *Acineto-*

Table 1. List of samples that generated concurrent results when analyzed with standard culture identification and PCR electrospray-ionization mass spectrometry (PCR/ESI-MS)

					PCR/ESI-MS				
Sample no.	Milk no.	Mastitis	Standard identification	Number of colonies	Genomes/well[1]	Q-score[2]	Identification	Agreement	Broader identification
1	M0439	Clinical	*Staphylococcus aureus*	<30	166	1	*Staph. aureus*[3]	Yes	None
2	M0671	Subclinical	*Staph. aureus*	>100	220	1	*Staph. aureus*[3]	Yes	None
3	M0478	Clinical	*Streptococcus uberis*	>100	852	0.87	*Strep. uberis*[3]	Yes	None
4	M0479	Clinical	*Strep. uberis*	>100	64	0.97	*Strep. uberis*[3]	Yes	None
5	JM0501	Clinical	*Strep. uberis*	30–100	149	1	*Strep. uberis*[3]	Yes	None
6	M2716	Subclinical	*Strep. uberis*	>100	226	1	*Strep. uberis*[3]	Yes	None
7	M0625	Control	*Corynebacterium bovis*	30–100	297	1	*C. bovis*[3]	Yes	None
8	M2745	Clinical	*Mycoplasma* spp.				*Mycoplasma* spp.[3]	Yes	None
9	M2744	Clinical	*Arcanobacterium pyogenes*	>100	7	0.92	*Arcanobacterium* spp.[3]	Yes	None
			Mycoplasma spp.		1,344	0.92	*Mycoplasma* spp.[3]		
10	M2727	Subclinical	*A. pyogenes*	>100	19	0.96	*Arcanobacterium* spp.[3]	Yes	None
			Mix of anaerobes	>100			Firmicutes (anaerobes)		
11	M2746	Subclinical	*Enterococcus* spp.	30–100	74	0.99	*Enterococcus faecalis*[3]	Yes	None
12	M2741	Subclinical	CNS	<30	60	0.99	*Staphylococcus xylosus*[3]	Yes	PCR/ESI-MS
13	M2786	Subclinical	CNS	30–100	153	1	*Staph. xylosus*[3]	Yes	PCR/ESI-MS
					7	1	*mecA*		
14	M0472	Clinical	Mix of bacteria	<30	NA[4]	NA	Unidentified complex mixture	Yes	None
15	M0667	Subclinical	No growth		NA	NA	Negative	Yes	None
16	M0680	Subclinical	No growth		NA	NA	Negative	Yes	None
17	M0682	Control	No growth		NA	NA	Negative	Yes	None
18	M0689	Subclinical	No growth		NA	NA	Negative	Yes	None
19	M0421	Subclinical	CNS	<30	79	1	*Staphylococcus saprophyticus*[3]	Yes	PCR/ESI-MS
					28	0.87	*Gemella hemolysans*[5]		
					99	1	*Candida parapsilosis*[3]		
					175	1	*mecA*		
20	M0424	Subclinical	CNS	>100	96	1	*Staphylococcus.simulans*[3]	Yes	PCR/ESI-MS
					1,038	0.91	*Staphylococcus sciuri/Staphylococcus vitulinus*[3]		
					41	0.95	*C. bovis*		
21	M0481	Subclinical	CNS	<30	120	0.96	*Staphylococcus warneri/Staphylococcus haemolyticus*	Yes	PCR/ESI-MS
			C. bovis	<30	135	0.98	*C. bovis*[3]		
					16	0.97	*Phaeosphaeria nodorum/Phaeosphaeria* sp. Sn 48–1[5]		
22	M0624	Control	CNS	<30	290	1	*Staph. xylosus*	Yes	PCR/ESI-MS
					52	0.89	*Pseudomonas syringae*[5]		
23	M2778	Subclinical	CNS	<30	338	0.99	*Staph. xylosus*[3]	Yes	PCR/ESI-MS
					186	1	*mecA*		
							Streptococcus spp.[3]		
							Unknown fungus[5]		
24	M0626	Control	CNS	<30	135	1	*Staph. xylosus*	Yes	PCR/ESI-MS
			Mix of bacteria	<30	34	0.88	*Pseudomonas. fluorescens*		
					13	0.86	*Pseudomonas. entomophila/Pseudomonas. putida*		
25	M0426	Clinical	*Staph. sciuri*	<30	265	1	*Staph. sciuri/Staph. vitulinus*[3]	Yes	PCR/ESI-MS
			Mix of bacteria	<30	164	0.86	*Enterococcus faecium*[3]		
					51	0.95	*Candida famata*[3]		
					193	1	*mecA*		
26	M2750	Subclinical	*Staph. aureus*	30–100	88	0.99	*Staph. aureus*[3]	Yes	PCR/ESI-MS
							Additional firmicutes		
27	M0463	Clinical	*Strep. uberis*	<30	287	0.97	*Strep. uberis*[3]	Yes	PCR/ESI-MS
					23	0.86	*Staphylococcus* spp.[3]		
28	M2810	Subclinical	*Strep. uberis*	<30	26	0.92	*Strep. uberis*[3]	Yes	PCR/ESI-MS

Continued

Table 1 (Continued). List of samples that generated concurrent results when analyzed with standard culture identification and PCR electrospray-ionization mass spectrometry (PCR/ESI-MS)

					PCR/ESI-MS				
Sample no.	Milk no.	Mastitis	Standard identification	Number of colonies	Genomes/well[1]	Q-score[2]	Identification	Agreement	Broader identification
					38	0.89	*Pseudomonas* spp.[5]		
							Enterobacteriaceae[5]		
							Unknown fungus[5]		
29	M2687	Subclinical	*Strep. uberis*	30–100	120	0.93	*Strep. uberis*[3]	Yes	PCR/ESI-MS
					55	0.99	*Staph. xylosus*[3]		
30	M2783	Subclinical	*Streptococcus dysgalactiae*	>100	1,672	0.90	*Strep. dysgalactiae*[3]	Yes	PCR/ESI-MS
					53	0.97	*Staph. xylosus*[3]		
					4	0.90	*Staph. aureus*[3]		
31	M0464	Subclinical	Yeasts	>100	124	0.99	*Candida tropicalis*[3]	Yes	PCR/ESI-MS
					208	1	*Staph. xylosus*[3]		
32	M0480	Clinical	*A. pyogenes*	>100	80	0.91	*A. pyogenes*[3]	Yes	PCR/ESI-MS
					65	1	*Staph. simulans*[3]		
33	M2736	Subclinical	*C. bovis*	<30	16	0.97	*C. bovis*[3]	Yes	PCR/ESI-MS
					17	0.94	*Leuconostoc pseudomesenteroides*[5]		
34	M2740	Clinical	*Enterococcus* sp.	<30	26	0.97	*Enteroc. faecalis*[3]	Yes	PCR/ESI-MS
					10	0.94	*Lactococcus lactis*[5]		
35	M2777	Control	*Enterococcus* sp.	>100	108	0.99	*Lc. lactis*[5]	Yes	PCR/ESI-MS
			CNS	>100			*Staph. xylosus*[3]		
					147	1	*mecA*		
							Unknown fungus[5]		
36	M2781	Clinical	*Escherichia coli*	<30	7	0.97	*E. coli*[3]	Yes	PCR/ESI-MS
					8	0.92	*C. bovis*[3]		
							Streptococcus spp.[3]		
37	M0664	Control	CNS	<30	1,509	0.92	*Staph. sciuri/Staph. vitulinus*[3]	Yes	Culture
			C. bovis	30–100					
38	M0465	Clinical	*Strep. uberis*	<30	357	0.99	*Strep. uberis*[3]	Yes	Culture
			Staph. aureus	<30					
39	M0669	Subclinical	*Strep. uberis*	<30	183	0.99	*Strep. uberis*[3]	Yes	Culture
			CNS	<30					
40	M0423	Clinical	*Staph. aureus*	>100	212	0.99	*Staph. aureus*[3]	Yes	Not definable
			Bacillus cereus	30–100	12	0.9	*Enterobacter cancerogenus/Enterobacter cloacae* complex[5]		
					198	1	*mecA*		
41	M9217	Subclinical	*Enteroc. faecalis*	>100	131	0.99	*Enteroc. faecalis*[3]	Yes	Not definable
			C. bovis	<30	38	0.91	*Pseudomonas* spp.[5]		
42	M0668	Subclinical	CNS	<30	>1,000	0.92	*Staph. sciuri/Staph. vitulinus*[3]	Yes	Not definable
			C. bovis	<30	113	0.97	*Staph. xylosus*[3]		
					137	1	*Penicillium marneffei*[5]		
					178	1	*mecA*		
43	M2737	Control	*Enterobacteriaceae*	>100	89	0.98	*Shigella sonnei/E. coli*[5]	Yes	Not definable
			Staph. aureus	30–100	19	0.87	*Enterob. cancerogenus/Enterob. cloacae* complex[5]		
					105	0.95	*Le. mesenteroides*[5]		

[1]Genome equivalents per well is an indication of the amount of the amplicon present in the sample.

[2]Q-score (quality score) ≥0.85 is considered a reportable result (see Materials and Methods for details).

[3]Organisms known as mastitis pathogens.

[4]NA = not applicable.

[5]Organisms likely environmental in context with anamnesis (control, subclinical or clinical mastitis);

bacter spp. *Chryseobacterium indologenes*, and enterococci (*Enterococcus saccharolyticus* and *Enterococcus faecalis*; Table 2, samples 44–49). However, PCR/ESI-MS failed to identify the complete flora in such highly contaminated samples. For those samples, PCR/ESI-MS identified only up to 2 different microorganisms (Table 2, samples 45–49), except for 1 sample where more than 3 microorganisms (*Staph. xylosus*, *Enteroc. faecalis*, *Acinetobacter johnsonii*, and *C. albicans*) were identified using PCR/ESI-MS (Table 2, sample 44).

Divergent results between the culture and the PCR/ESI-MS methods were obtained for 18 milk samples (27.9%; Table 3). In this instance, PCR/ESI-MS failed to identify one of the primary pathogens in 17.9% of the samples, but it identified microorganisms in samples that were negative by culture (9%) as well as other pathogens (3%). In one sample, PCR/ESI-MS identified *Pseudomonas* spp. instead of *C. bovis* (Table 3, sample 50). The PCR/ESI-MS only identified the facultative anaerobic bacteria *Enterococcus* spp. and *Psychrobacter* spp. in 1 milk sample that also contained a mix of strictly anaerobic bacteria (Table 3, sample 51). The PCR/ESI-MS failed to identify yeasts in 1 sample, but revealed the presence of *Moraxella*/*Acinetobacter* spp. and *Staph. xylosus* instead (Table 3, sample 52). The PCR/ESI-MS identified *Lactococcus lactis* in 2 milk samples, and did not detect the primary mastitis pathogens *Staph. aureus* and *Strep. uberis* in these samples (Table 3, samples 53 and 54). In 2 samples containing *Strep. uberis*, PCR/ESI-MS revealed the presence of *Leuconostoc mesenteroides* and fungi in 1 sample, and *Aspergillus amstelodami*, *Staph. aureus*, and unknown bacteria in the other sample (Table 3, samples 55 and 56). In 5 samples, PCR/ESI-MS remained negative, whereas bacteria such as CNS, *E. coli*, *Strep. dysgalactiae*, and yeasts could be cultivated on the agar plates (Table 3, samples 58–61). On the other hand, PCR/ESI-MS identified fungi as well as bacteria (*Staph. xylosus*, *Staph. haemolyticus*, *Staph. vitulinus*, *Janthinobacterium lividum*, *Erwinia tasmaniensis*/*rhapontici*) in 6 milk samples that remained negative on agar plates (Table 3, samples 62–67).

The *mecA* gene was detected in 11 samples (16.4%) and was always associated with the presence of staphylococci. In one sample, *mecA* was associated with the presence of *Staph. aureus* (Table 1, sample 40), otherwise it was linked to CNS (Table 1, samples 13, 19, 23, 25, 35, and 42; Table 2, samples 44, 45, and 46; Table 3, sample 67).

The relative quantity of microorganisms growing on the culture media and the genomic quantification obtained by PCR/ESI-MS differed from sample to sample and no significant quantitative association could be made between both methods (Tables 1–3). Overall,

Table 2. Identification of microorganisms from milk samples containing more than 3 different microorganisms using PCR electrospray-ionization mass spectrometry (PCR/ESI-MS)

Sample no.	Milk no.	Mastitis	Standard identification	Number of colonies	PCR/ESI-MS			Agreement	Broader identification
					Genomes/well[1]	Q-score[2]	Identification		
44	M0466	Control	Mix of bacteria	<30	132	1	*Staphylococcus xylosus*[3]	Yes	PCR/ESI-MS
					172	1	*Candida albicans*[3]		
					83	0.96	*Acinetobacter johnsonii*[4]		
					38	0.91	*Enterococcus faecalis*[4]		
					117	1	*mecA*		
45	M0670	Subclinical	Mix of bacteria	30–100	115	1	*Staph. xylosus*[3]	Yes	PCR/ESI-MS
					267	1	*mecA*		
					215	1	*Enteroc. faecalis*[3]		
46	M0467	Subclinical	Mix of bacteria	<30	143	1	*Staph. xylosus*[3]	Yes	PCR/ESI-MS
					136	1	*Penicillium marneffei*[4]		
					87	1	*mecA*		
47	M0563	Clinical	Mix of bacteria	<30	225	1	*Staph. xylosus*[3]	No	Not definable
48	M0665	Control	Mix of bacteria	<30	68	0.94	*Staphylococcus sciuri*/*Staphylococcus vitulinus*[3]	Yes	PCR/ESI-MS
					541	0.85	*Enterococcus saccharolyticus*[4]		
49	M0666	Control	Mix of bacteria	>100	90	0.95	*Chryseobacterium indologenes*[4]	Yes	
					7	0.91	*Staphylococcus equorum*[3]		PCR/ESI-MS

[1]Genome equivalents per well is an indication of the amount of the amplicon present in the sample.
[2]Q-score (quality score) ≥0.85 is considered a reportable result (see Materials and Methods for details).
[3]Organisms known as mastitis pathogens.
[4]Organisms likely environmental in context with anamnesis (control, subclinical, or clinical mastitis).

Table 3. List of results showing divergence between the culture method and PCR electrospray-ionization mass spectrometry (PCR/ESI-MS)

Sample No.	Milk no.	Mastitis	Standard identification	Number of colonies	PCR/ESI-MS GE/well[1]	PCR/ESI-MS Q-score[2]	PCR/ESI-MS Identification	Agreement	Broader identification
50	M0420	Subclinical	*C. bovis*	30–100	101	0.94	*Pseudomonas fluorescens*[3]	No	Not definable
					33	0.91	*Pseudomonas stutzeri*[3]		
					23	0.89	*Enterobacter aerogenes*[3]		
51	M2743	Subclinical	Mix of anaerobes	30–100			*Enterococcus* species[4]	No	Not definable
					25	0.8694	*Psychrobacter* spp.[3]		
52	M2747	Subclinical	Yeasts		47	0.90	*Moraxella/Acinetobacter* spp.[3]	No	Not definable
					20	0.98	*Staphylococcus xylosus*[4]		
53	M0425	Control	*Staph. aureus*	<30	153	1	*Lactococcus lactis*[3]	No	Culture
			Mix of bacteria	<30					
54	M0427	Subclinical	*Strep. uberis*	<30	109	1	*Lc. lactis*[3]	No	Culture
			C. bovis	<30					
55	M2735	Subclinical	*Strep. uberis*	30–100	21	0.99	*Leuconostoc mesenteroides*[3]	No	Not definable
							Unknown fungus[3]		
56	M2787	Clinical	*Strep. uberis*	>100	3	0.96	*Aspergillus amstelodami*[3]	No	Not definable
							Staph. aureus[4]		
							Unknown bacteria		
57	M0477	Clinical	CNS	<30	NA[5]	NA	Negative	No	Culture
58	M0422	Subclinical	CNS	<30	NA	NA	Negative	No	Culture
59	M2715	Subclinical	Yeasts	>100			Negative	No	Culture
60	M2721	Clinical	*E. coli*	>100			Negative	No	Culture
61	M2739	Clinical	*Strep. dysgalactiae*	>100			Negative	No	Culture
62	M0462	Clinical	No growth		237	1	*Staph. xylosus*[4]	No	PCR/ESI-MS
63	M0681	Subclinical	No growth		49	0.9	*Erwinia tasmaniensis/rhapontici*[3]	No	PCR/ESI-MS
					127	1	*Staph. xylosus*[4]		
64	M2707	Clinical	No growth		811	0.88	*Janthinobacterium lividum*[3]	No	PCR/ESI-MS
65	M2748	Subclinical	No growth				Unknown fungus[3]	No	PCR/ESI-MS
66	M2779	Subclinical	No growth		151	0.99	*Staphylococcus haemolyticus*[4]	No	PCR/ESI-MS
							Unknown fungus[3]		PCR/ESI-MS
67	M0627	Control	No growth		153	1	*Staphylococcus vitulinus*[4]	No	PCR/ESI-MS
					198	1	*mecA*		

[1]GE/well = genome equivalents per well; an indication of the amount of the amplicon present in the sample.

[2]Q-score (quality score) ≥0.85 is considered a reportable result (see Materials and Methods for details).

[3]Organisms likely environmental in context with anamnesis (control, subclinical, or clinical mastitis).

[4]Organisms known as mastitis pathogens.

[5]NA = not applicable.

PCR/ESI-MS showed a sensitivity and specificity of 56.8 and 59.5% compared with cultures. The specificity and sensitivity increased when the detection of the pathogenic bacteria was considered individually; with 57.1% sensitivity and 96.6% specificity for *Staph. aureus*, 71.4% and 96.2% for streptococci, 80.0% and 69.8% for CNS, 37.5% and 96.6% for *C. bovis*, 75% and 93.7% for enterococci, 33.3% and 94.4% for yeasts, 66.7% and 95.3% for *Enterobacteriaceae*, and 100% sensitivity and specificity for *M. bovis* and *A. pyogenes*.

DISCUSSION

This is the first study that evaluates the potential of PCR/ESI-MS for veterinary microbiology diagnosis. In particular, bovine mastitis represents one of the more challenging diagnostic tasks due to the large heterogeneity of the microorganisms that may cause the disease and the permanent evolution of the disease through microbial adaptation (Bradley, 2002).

Our study demonstrates that PCR/ESI-MS appears to be a promising tool for the identification of the mastitis pathogens directly from milk using a kit developed for microbiological diagnosis in human medicine (Ecker et al., 2008). The predominant pathogen detected by PCR/ESI-MS in each sample was consistent with standard milk culture results in two-thirds of the examined samples. The PCR/ESI-MS also allowed an estimate of the relative abundance of microorganisms present in the milk samples, which may be helpful for the interpretation of the results, particularly if heterogeneity exists among the types of microorganisms present in the milk. However, the relative number of genomic copies of DNA that were amplified did not always correspond to the relative number of microorganisms estimated from the cultures, likely due to the different measurement techniques of the 2 semiquantitative methods. It should also be noted that the milk samples were sent overseas and remained room temperature for at least 1 wk, which may have allowed some contaminants to overgrow or lead to degradation of DNA from the isolates. Further validations using milk preservatives or sample refrigeration for transport are necessary before this technology can be used for diagnostic purposes.

The PCR/ESI-MS identified some of the bacteria present in highly contaminated milk, which contained more than 3 different microorganisms. However, this technology failed to identify some of the primary mastitis pathogens in 17.9% of the samples which may also be related to the prolonged transport time. Negative results of up to 12% were also reported for other PCR-based assays compared with culture (Koskinen et al., 2010). Otherwise, primary pathogens, such as *Strep. uberis*, *Staph. aureus*, *Staphylococcus* spp., *A. pyogenes*, and *Mycoplasma* spp., were identified. For those organisms, the sensitivity of the PCR/ESI-MS ranged from 57.1 to 100% and the specificity ranged from 69.8 to 100% using culture as gold standard. Similar ranges were obtained when PCR-based molecular methods were compared with culture (Paradis et al., 2012; Spittel and Hoedemaker, 2012). However, molecular methods have been shown to higher detection potential than culture (Koskinen et al., 2009; Koskinen et al., 2010). We also show that the rapid detection of barely cultivatable and slow-growing mastitis pathogens, such as *M. bovis*, by PCR/ESI-MS represents a major advantage for veterinarians in preventing the spread of the disease within respective herds (Aebi et al., 2012). Indeed, the overall workflow for PCR/ESI-MS from receipt of a sample in the laboratory to providing organism identification requires approximately 8 h (Wolk et al., 2012). Additionally, PCR/ESI-MS detected the presence of fungi, yeasts, and antibiotic-resistant genes. For example, *Janthinobacterium lividum*, an organism known to possess THIN-B, a metallo β-lactamase of class B3, was detected by PCR/ESI-MS (Docquier et al., 2004). The PCR/ESI-MS also detected the *mecA* gene, indicating the presence of methicillin-resistant staphylococci including methicillin-resistant *Staph. aureus* in bovine mastitis milk. The presence of these staphylococci in milk is a growing problem in veterinary medicine not only limiting antimicrobial treatment options, but also representing a burden for public health (Walther and Perreten, 2007; Vanderhaeghen et al., 2010; Holmes and Zadoks, 2011; Zadoks et al., 2011). Methicillin-resistant *Staph. aureus* present in milk may easily be transferred to humans through the consumption of raw milk or cheese (Perreten et al., 1998; Normanno et al., 2007). In this regard, PCR/ESI-MS could also find application for the direct determination of microbiological quality of bulk tank, raw milk to be used for raw milk products.

CONCLUSIONS

Our study demonstrates that PCR/ESI-MS possesses the potential to be a robust method used to screen for pathogens in the etiology of bovine mastitis. Even if the cost for analysis of a single sample on the PCR/ESI-MS system would be as high as ~$50 to $100 per sample (quoted by Wolk et al., 2012), PCR/ESI-MS has the significant advantage that it can be adapted to different settings and organisms (bacteria, mycobacteria, fungi, parasites, and viruses). The ability to perform a broad-range analysis on a variety of organism types without the need for culture or prior knowledge of the target may make the PCR/ESI-MS a more financially

feasible option than it appears at first glance. The broad range of bacterial and fungal DNA signatures detected by PCR/ESI-MS indicates that milk may also be a reservoir of genetic elements that are important in establishing the microbial flora of an individual, which may have consequences for therapy and public health. Accomplishing these identifications in less than 6 h, as compared with 24 to 72 h or longer for standard microbiological analyses, has significant implications for the dairy industry and veterinary medicine in the diagnosis of subclinical and clinical disease.

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