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ORIGINAL ARTICLE

Comparison of quantitative PCR and MALDI-TOF mass spectrometry assays for identification of bacteria in milk samples from cows with subclinical mastitis

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bacteria, cattle, MALDI-TOF MS®, milk samples, multiplex qPCR.

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Abstract

Aims: The objective of this study was to compare qualitatively and quantitatively the results of identification of the bacteria present in milk samples from cows with subclinical mastitis using multiplex qPCR assay and matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS®) after bacteriological growth.

Methods and Results: A total of 182 samples were aseptically collected from 119 cows with high somatic cell counts (>2·10^5 SCC per ml) on 11 farms in Belgium in 2014. The mutiplex qPCR assay was carried out on 350 µl of milk with the PathoProof® Complete-16kit. Ten microlitre of milk was streaked on Columbia blood agar and three selective agar plates. Growing colonies were identified by MALDI-TOF MS. Of the 182 samples, 90 gave positive results with either or both tests for one or two bacterial species/genera. Total qualitative agreement of the bacteria identified was observed in 41 mono- or bi-bacterial samples (46%) and partial agreement in 19 bi-bacterial samples at both or either tests (21%). The results of both tests on those mono- and bi-bacterial samples were not significantly different (McNemar test; $P = 0{\cdot}395$) with a fair agreement (Cohen's kappa test; $k = 0{\cdot}375$; $P = 0{\cdot}055$). Moreover, quantitative correlation between the qPCR intensity and the numbers of growing colonies was observed in half of the 60 samples with qualitative matching results.

Conclusions: Both methods give identical qualitative and quantitative results with approximately a half and a quarter of the mono- and bi-bacterial samples respectively. Several reasons can explain the differences. The multiplex qPCR assay only targets the most important mammary gland pathogens and can detect DNA of bacteria both alive and dead. Conversely, bacteria only grow when alive and the MALDI-TOF MS databases do not include all bovine milk-associated bacterial species yet.

Significance and Impact of the Study: This study further highlights the limitations and complementarity of the genetic and phenotypic tests for the identification of bacteria present in milk samples.

Introduction

In dairy cattle, different bacterial species are responsible for mastitis, whose origin is classically described as contagious or environmental according to the mode of transmission. Contagious mastitis is caused by bacterial species living on the udder skin and/or in the infected udder, such as *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis*. On the other hand, the majority of environmental mastitis is caused by bacterial species present in the intestinal and faecal microbiota, such as *Escherichia coli*, *Klebsiella* sp., *Enterobacter* sp., *Streptococcus uberis* and *Streptococcus dysgalactiae* (El-Sayed *et al.,* 2017). The former bacterial species are more frequently associated with chronic inflammation and subclinical mastitis with high somatic cell counts (SCC) and drop in milk production whereas the latter most often cause acute inflammation and clinical mastitis, coupled with macroscopic alteration of the milk (Gussmann *et al.,* 2019). Nevertheless several other bacterial species of environmental origin can also be associated with subclinical mastitis, such as different non-*aureus* staphylococci, nonclassical streptococci/enterococci or corynebacteria (Reyher *et al.,* 2012; El-Sayed *et al.,* 2017). The consequence of mastitis is high financial costs due to decreased milk production, penalty for high SCC ($>4{\cdot}10^5$ cells per ml), application of different antibiotic treatments and the culling of incurable cases (Yalcin *et al.,* 1999; Halasa *et al.,* 2007; Reyher *et al.,* 2012; Ashraf and Imran, 2018).

The aetiological diagnosis of mastitis is therefore crucial to apply appropriate therapeutic and prophylactic control measures, which are different whether the mastitis is of contagious or environmental origin (Ashraf and Imran, 2018). For many years, most routine laboratories have been using classical bacteriology with biochemical tests (for instance, API® sugar tests) to identify mastitis-associated bacterial species. Nevertheless the database of these biochemical tests was initially developed for human medicine and can therefore lead to species and even genus misidentification when used in veterinary medicine in general and for mastitis diagnosis in cattle in particular (Pinsky *et al.,* 2009; Schabauer *et al.,* 2014). Therefore, nowadays, veterinary diagnostic laboratories favour mass spectrometry (for instance, matrix-assisted laser desorption-ionization–time of flight mass spectrometry or MALDI-TOF MS®) for the identification of bacterial species. The MALDI-TOF MS databases for the different bacterial genera and families in veterinary medicine are indeed progressively validated like recently the database of staphylococcal species associated with bovine mastitis (Cameron *et al.,* 2017, 2018; Mahmmod *et al.,* 2018; Nonnemann *et al.* 2019). However, a delay of 24–72 h, that is not often compatible with the dairy industry, is still necessary to obtain results. Moreover, other bacterial species than the mammary gland pathogens, present in the milk samples as a result of external contamination at the time of sampling, can also be detected.

Today, several dairy routine diagnostic laboratories resort to genetic assays, mainly multiplex quantitative (q) PCR assays, that can be applied directly to milk samples skipping the step of growing the bacteria present in the samples. Though qPCR assay kits detect a limited number of bacterial species, these are the most frequent and pathogenic ones (Koskinen *et al.,* 2010; Cederlöf *et al.,* 2012; El-Sayed *et al.,* 2017).

The aim of this study was therefore to compare qualitatively and quantitatively the qPCR assay using the PathoProof® Complete-16 kit and the MALDI-TOF MS assay after bacterial growth on four agar media to identify the bacterial species present in milk samples from cows with high SCC in 11 herds in Belgium.

Materials and methods

Milk sampling

A total of 182 milk samples were aseptically (washing of the udder, disinfection of the teat and discarding of the first milk) collected in duplicate in sterile vials from 119 cows with more than $2{\cdot}10^5$ SCC per ml and suspicion of subclinical mastitis (one sample corresponds to one quarter), on 11 farms in Wallonia, Belgium, between January and March 2014. These 11 farms were included in a study on the epidemiology of contagious mastitis in Wallonia (MammiScan project, Service Public Wallonie, Division Générale de l'Agriculture RNE) between 2011 and 2015. Within the MammiScan project, 300 farms were randomly chosen out of the *c.* 4000 dairy farms in all five provinces of Wallonia (A.S. Rao and L. Théron, unpublished data). The cows were not receiving any antibiotics at the time of sampling. To minimize bacterial growth, the samples were transported in coolers the same day either to the 'Comité du Lait' or to the Veterinary Faculty, where they were kept in fridges at 4°C and analysed on the following day at the latest.

Multiplex qPCR assay

The DNA extraction and the multiplex qPCR assay with the commercial PathoProof Complete-16kit (Thermo Scientific) were carried out by the 'Comité du Lait' (Battice, Belgium) on 350 µl of each milk sample, according to the manufacturer's instructions (Thermo Scientific, 2015). The PathoProof Complete-16 kit PCR assay detects the presence of DNA of the following mammopathogens: *Staph. aureus, Staph.* sp. including all major

non-*aureus* species, *Strep. agalactiae, Strep. dysgalactiae, Strep. uberis, E. coli, Corynebacterium bovis, Enterococcus* sp., *Klebsiella oxytoca* and *K. pneumoniae, Serratia marcescens, Trueperella* (previously *Arcanobacterium*) *pyogenes* (*T. pyogenes*) and *Peptococcus indolicus*, *M. bovis, Mycoplasma* sp., yeasts and *Prototheca* sp. The 16th PCR detects the staphylococcal β-lactamase-encoding gene (*blaZ*). The results were reported following the protocol described by the supplier (Suisselab AG Aollikofen, 2006; Thermo Scientific, 2015): negative results (−) with cycle threshold (Ct) cut-off values higher than 37; weakly positive results (+) with Ct values between 37 and 30; moderately positive results (++) with Ct values between 30 and 20; highly positive results (+++) with Ct value lower than 20.

Bacteriological culture

Ten microlitre of each second milk sample was inoculated onto Columbia agar supplemented with 5% sheep blood (nonselective medium; BioMérieux, Marcy-l'Etoile, France), modified Chapman's agar (selective for staphylococci; VWR, Leuven, Belgium), Edwards' agar (selective for streptococci and enterococci; Oxoid, Aalst, Belgium) and Mac Conkey's agar (selective for enterobacteria; Merck, Overijse, Belgium) using the EDDY JET device® (LED Techno, Heusden-Zolder, Belgium). After overnight aerobic incubation at 37°C, the different colony types were counted with a cut-off value of 100 colony forming units (CFU) per ml and distributed into three classes: 10^2–10^4 CFU per ml, 10^4–10^6 CFU per ml, $>10^6$ CFU per ml. Each colony type was subsequently subcultured overnight in brain heart infusion broth and stored at −80°C in 50% glycerol until further use. Isolation of mycoplasmas and *Prototheca* was not attempted.

Species identification with MALDI-TOF assay

After thawing, isolates were grown on the same appropriate agar media to confirm the purity of the cultures. Identification of the species level was performed with an Autoflex Biotyper Mass Spectrometer (MALDI-TOF MS, Bruker, Germany) using the direct transfer method and α-cyano-4-hydroxycinnamic acid as matrix, according to the manufacturer's guidelines. In case of no peak detection, the sample was rerun with the extended direct transfer method, using either on-target formic acid treatment or full ethanol-formic acid extraction, according to the manufacturer's guidelines. The spectra were analysed with the MALDI Bio Typer Compass software ver. 4.1. (Bruker Daltonik, Bremen, Germany), which includes a combination of 'mean spectra projections' (MSP) and 'research use only' (RUO) reference databases of 8252 bacterial and fungal species, expanded with 13 MSP covering 8 species of coagulase negative staphylococci as previously described (Cameron *et al.*, 2017). Only the identifications with a score value between 2·00 and 3·00 (green colour) were taken into account.

Statistical analysis

The data were statistically analysed using the software SPSS 25.0 (Statistical Package for Social Sciences, Chicago, IL). The difference and the agreement levels between qPCR and MALDI-TOF MS results were assessed by the McNemar and Cohen's kappa tests, respectively, with the significance threshold of $P = 0{\cdot}05$ (McNemar, 1947; Cohen, 1960; Statistiques biomédicales, 2017).

Results

qPCR PathoProof DNA detection

Of the 182 milk samples, 133 (73%) tested positive (Ct values ≤37) with one of the qPCR reactions for a microbial species or genus, and 32 (18%) with two, three or four qPCR reactions while 17 (9%) samples tested negative (Table 1). Of the 165 qPCR-positive samples, 45 were positive for *Staph. aureus* (27%), 64 for non-*aureus* staphylococci (39%), 43 for streptococci (26%), five for enterococci (3%), 13 for enterobacteria (8%), 12 for corynebacteria (7%), one for *T. pyogenes* (0·6%), 15 for mycoplasmas (9%), one for *Prototheca* (0·6%) and two for yeasts (1%). The *blaZ* gene was detected in 28 samples testing positive for *Staph. aureus* only, in 22 samples testing positive for non-*aureus* staphylococci only, and in six samples testing positive for both *Staph. aureus* and non-*aureus* staphylococci (Table 1).

Classical bacterial growth and MALDI-TOF MS species identification

At the detection limit of 100 CFU per ml, bacterial growth on Columbia blood agar was obtained with 144 (79%) of the 182 milk samples. Nineteen samples grew only on Columbia blood agar, while 98 samples also grew on Chapman's agar, 69 samples also on Edwards' agar and 14 samples also on Mac Conkey's agar. A total of 216 colony types were subcultured and stored at −80°C: one colony type was obtained from 82 (57%) samples, two colony types from 54 (38%) samples, three colony types from six (4%) samples and four colony types from two (1%) samples. Of these 216 isolates, 112 grew on Chapman's agar (putative staphylococci), 68 grew on Edwards' agar (putative streptococci/enterococci) and 17

Table 1 qPCR assay results of the 182 milk samples

Pathoproof® identification	No. (%) milk samples	*blaZ* gene qPCR
Staphylococcus aureus	32 (18)	24
Staphylococcus sp.	39 (21)	12
Streptococcus agalactiae	12 (7)	
Streptococcus dysgalactiae	5 (3)	
Streptococcus uberis	12 (7)	
Enterococcus sp.	4 (2)	
Escherichia coli	5 (3)	
Klebsiella sp.	3 (1·6)	
Mycoplasma bovis	14 (8)	
Mycoplasma sp.	1 (<1)	
Corynebacterium bovis	4 (2)	
Yeasts	2 (1)	
Staph. aureus, *Staph.* sp.	3 (1·6)	2
Staph. aureus, *Strep. agalactiae*	1 (<1)	
Staph. aureus, *Strep. uberis*	2 (1)	2
Staph. aureus, *Enterococcus* sp.	1 (<1)	
Staph. aureus, *E. coli*	1 (<1)	1
Staph. sp., *Strep. agalactiae*	4 (2)	3
Staph. sp., *Strep. uberis*	3 (1·6)	1
Staph. sp., *E. coli*	2 (1)	2
Staph. sp., *C. bovis*	5 (3)	2
C. bovis, *Prototheca* sp.	1 (<1)	
Staph. aureus, *Staph.* sp., *Strep. agalactiae*	3 (1·6)	3
Staph. aureus, *Strep. agalactiae*, *Strep. uberis*	1 (<1)	1
Staph. sp., *Strep. agalactiae*, *C. bovis*	2 (1)	2
Staph. sp., *Strep. uberis*, *E. coli*	1 (<1)	
Staph. aureus, *Staph.* sp., *Strep. agalactiae*, *Strep. dysgalactiae*	1 (<1)	1
Staph. sp., *E. coli*, *Klebsiella* sp., *Trueperella pyogenes*	1 (<1)	
Negative results	17 (9)	
Total	182 (100)	56

grew on Mac Conkey's agar (putative enterobacteria) (Table 2). The 19 isolates growing only on Columbia blood agar were identified as *Bacillus* sp. (10 isolates) and yeasts (9 isolates) by classical tests (not shown) and were not further studied.

The MALDI-TOF MS gave an identification profile for 108 of the 112 putative staphylococci, 65 of the 68 putative streptococci/enterococci and the 17 putative enterobacteria (Table 2). The 108 putative staphylococci identified belonged to 13 species (Table 2a): *Staph. aureus* (60 isolates, 55·5%), 46 non-*aureus* staphylococci (46 isolates, 42·5%) and *Aerococcus viridans* (2 isolates, 2%), while the 65 putative streptococci/enterococci identified belonged to 11 species (Table 2b) of the *Streptococcus* genus (34 isolates, 52%), the *Enterococcus* genus (20 isolates, 31%) and the *Aerococcus* genus (11 isolates, 17%). The 17 putative enterobacteria belonged to four species (Table 2c), including *K. pneumoniae* (5 isolates, 29%) and *E. coli* (3 isolates, 18%).

Comparison between the qPCR assay and the MALDI-TOF identification results

Of the 182 milk samples, 133 gave positive results (73%) and six negative results (3%) with both methods while 32 (18%) were positive with the qPCR assay only and 11 (6%) only gave a positive bacterial growth. Of the 133 samples, 43 gave positive results with either or both tests for more than two bacterial species/genera and were considered as contaminated samples. Therefore only the results of the 54 mono-bacterial samples, nine bi-bacterial samples at both tests and 27 bi-bacterial samples at either test (Table 3; Table S1) were statistically compared.

Qualitative matching results were observed with 36 (67%) of the 54 mono-bacterial samples (Fig. 1): 26 samples with staphylococci (including 20 with *Staph. aureus*), six samples with streptococci, two samples with enterococci, and two samples with yeasts. Total qualitative matching results were also observed with five (55%) of the nine bi-bacterial samples in which two bacterial species/genera were identified by both tests (Fig. 2).

Partial qualitative matching identification results (one identical identification result out of two positive results) were observed with 19 samples: 2 (22%) of the nine bi-bacterial samples at both tests and 17 (63%) of the 27 bi-bacterial samples at either test: five samples with two positive qPCR reactions and one growing bacterial species and 12 samples with two growing bacterial species and one positive qPCR reaction.

Statistically, the McNemar test was performed on 51 samples whose bacterial profile shows at least one positive agreement for both tests (same bacteria identified by both tests in the same samples), while the Cohen's kappa test included all 90 samples mono- and bi-bacterial samples. The McNemar test showed that the qPCR and MALDI-TOF test results were not significantly different ($P = 0{\cdot}395$; $>0{\cdot}05$), while the Cohen's kappa test showed a fair agreement between both methods ($k = 0{\cdot}375$; $P = 0{\cdot}055$).

Moreover quantitative matching was observed for half (31) of those 60 qualitatively matching qPCR reaction and bacterial growth results on the basis of the categories previously defined: +, ++, +++ for the qPCR reactions and 10^2–10^4, 10^4–10^6, $>10^6$ CFU per ml for the bacterial growth (Table 4; Table S2). For the other half, either higher intensity of the qPCR reactions compared to the bacterial growth or higher numbers of CFU per ml than the qPCR reaction intensity were obtained.

Table 2 MALDI-TOF MS® identification results of the colonies growing on Chapman's agar (a), Edwards' agar (b) and Mac Conkey's agar (c) from 144 milk samples

(a) Chapman		(b) Edwards		(c) Mac Conkey	
MALDI-TOF MS identification	No. (%) isolates	MALDI-TOF MS identification	No. (%) isolates	MALDI-TOF MS identification	No. (%) isolates
Aerococcus viridans	2 (2)	*Aerococcus viridans*	11 (16)	*Escherichia coli*	3 (18)
Staphylococcus aureus	60 (54)	*Enterococcus faecalis*	9 (13)	*Klebsiella pneumoniae*	5 (29)
Staphylococcus chromogenes	9 (8)	*Enterococcus faecium*	6 (9)	*Pantoea agglomerans*	8 (47)
Staphylococcus cohnii	3 (3)	*Enterococcus hirae*	5 (7)	*Serratia marcescens*	1 (6)
Staphylococcus epidermidis	3 (3)	*Streptococcus. agalactiae*	13 (19)		
Staphylococcus equorum	2 (2)	*Streptococcus dysgalactiae*	7 (10)		
Staphylococcus haemolyticus	9 (8)	*Streptococcus lutetiensis*	1 (1·5)		
Staphylococcus hominis	1 (<1)	*Streptococcus pluranimalium*	1 (1·5		
Staphylococcus lentus	3 (3)	*Streptococcus salivarius*	1 (1·5)		
Staphylococcus sciuri	10 (9)	*Streptococcus uberis*	10 (15)		
Staphylococcus simulans	1 (<1)	*Streptococcus vestibularis*	1 (1·5)		
Staphylococcus warneri	2 (2)	Not identified	3 (4)		
Staphylococcus xylosus	3 (3)				
Not identified	4 (4)				
Total	112 (100)	Total	68 (100)	Total	17 (100)

Discussion

Of the 182 milk samples, 133 (73%) gave positive and six (3%) negative results with both methods, and 90 of them (49%) were positive for one or two bacterial species/genera with either or both tests. These 90 samples were considered as noncontaminated and further analysed for result agreement. Total or partial qualitative agreement of the species/genus identified was obtained with 60 (67%) of the 90 mono- or bi-bacterial samples. Moreover, quantitative correlation was observed between the qPCR intensity and the bacterial growth rate in ca. half (31) of these 60 samples. Statistical analysis with McNemar and Cohen's kappa tests confirm the absence of any significant difference with a fair agreement between the qPCR and the MALDI-TOF results, similarly to other recently published studies, although these were based on milk samples instead of isolates (Barreiro *et al.,* 2018; Wilson *et al.,* 2019). The reasons for the differences in the qualitative and quantitative results can be several, some dealing with the sampling method, others with the multiplex qPCR assays and still others with the bacterial growth or the MALDI-TOF MS identification.

Although the milk samples were aseptically collected, contamination by bacteria of the teat/udder skin, of the faeces or of the air cannot be totally avoided, as exemplified by the presence of more than two colony types in eight samples and of *Bacillus* sp. in 10 samples. A general rule also is to consider more than two colony types in any sample as a proof of contamination (Hogan *et al.,* 1989; Koskinen *et al.,* 2010) though this assertion may be pondered by the identity of the isolates. Most probably, some non-*aureus* staphylococci, enterococci and enterobacteria, but maybe also some *Staph. aureus* and *Strep. agalactiae* as recently demonstrated (Nyman *et al.,* 2016; Svennesen *et al.,* 2018) especially from polybacterial samples, can therefore be regarded as contaminants. Conversely 38 samples gave no bacterial growth and only one colony type grew from 82 samples, confirming that the sampling method was correctly performed for the majority of the samples.

The question of the presence of bacterial contaminants as mentioned before set aside, different reasons may explain why some samples with negative qPCR results had positive bacterial growth, more particularly:

- **i** the identification range of the PathoProof Complete-16 kit is limited to 15 genus/species, while classical bacteriology is limited only by the growth media and conditions;
- **ii** a technical problem may arise in the qPCR procedure, due to the inadequate homogenization, or incomplete mixing of the sample leading to negative results (Hiitiö *et al.,* 2015), while the bacteria may still grow on agar media;
- **iii** the sampling method, with presampling procedure or insertion of a cannula or directly sampling the udder cistern with a needle for instance, can influence the results by decreasing the number of bacterial species detected, especially by qPCR (Mahmmod *et al.,* 2013a; Hiitiö *et al,* 2016; Friman *et al.,* 2017) compared to classical routine procedures. Nevertheless,

Table 3 Comparison of the identification results of the 90 mono- and bi-bacterial samples by both the qPCR assay and the culture on agar media

	MALDI-TOF MS® identification results of the growing colonies			
Pathoproof® identification[a]	Total matching	Partial matching	No matching	No. Milk samples
Staphylococcus aureus	*Staph. aureus*			20
		Staph. aureus, OS		3
			OS	3
			NI	1
Staphylococcus sp.	*Staph.* sp.			6
		Staph. sp., OS		1
		Staph. sp., NI		1
			OS	6
			NI	2
Streptococcus agalactiae	*Strep. agalactiae*			4
		Strep. agalactiae, OS		2
		Strep. agalactiae, NI		1
Streptococcus dysgalactiae	*Strep. dysgalactiae*			2
		Strep. dysgalactiae, OS		2
Streptococcus uberis		*Strep. uberis*, OS		2
			OS	9
Enterococcus sp.	*Enterococcus* sp.			2
			OS, NI	1
Escherichia coli			OS	2
Klebsiella sp.			OS	2
Yeasts	Yeasts			2
Staph. aureus, *Strep. agalactiae*	*Staph. aureus, Strep. agalactiae*			1
Staph. aureus, *Strep. uberis*	*Staph. aureus, Strep. uberis*			2
Staph. aureus, *Enterococcus* sp.	*Staph. aureus, Enterococcus* sp.			**1**
Staph. aureus, *Staph.* sp.		*Staph. aureus*		3
Staph. sp., *Strep. agalactiae*	*Staph.* sp., *Strep. agalactiae*			1
		Strep. agalactiae		1
			OS	2
Staph. sp., *Strep. uberis*		*Staph.* sp., NI		1
			OS	1
Staph. sp., *Escherichia coli*		*Staph.* sp., *Enterococcus* sp.		1
Staph. sp., *Corynebacterium bovis*		*Staph.* sp.		1
			OS	1
Total milk samples				90

*NI, not identified; OS, other species (details in Table S1). Not tested by MALDI-TOF MS.

this is not the case in this study, since the sampling procedure was identical for both qPCR and classical bacteriological culture.

Conversely, and disregarding the samples with positive qPCR assays for mycoplasmas and *Prototheca* sp. that were not looked for using microbiological culture, the most probable reasons to explain the negative bacteriological results of qPCR-positive samples are:

i the possible need for definition of different Ct values according to the bacterial species/genus. For instance, the Ct values have been assessed and re-evaluated in different studies for diagnosis of *Staph. aureus* and *Strep. agalactiae* mastitis with proposal of minor modifications, especially for diagnosis of subclinical infections (Cederlöf *et al.*, 2012; Mahmmod *et al.*, 2013b, 2017). This was not considered in this study since the protocol recommended by the company was followed, but more studies are clearly needed;

ii the classical bacteriology can only characterize alive bacteria while the qPCR assays can detect the DNA of bacteria whether dead or alive (Taponen *et al.*, 2009);

iii when low bacterial loads are present in the milk samples, the number of colonies can be below the cut-off values (100 CFU per ml in this study) and the samples classified as negative, while the qPCR

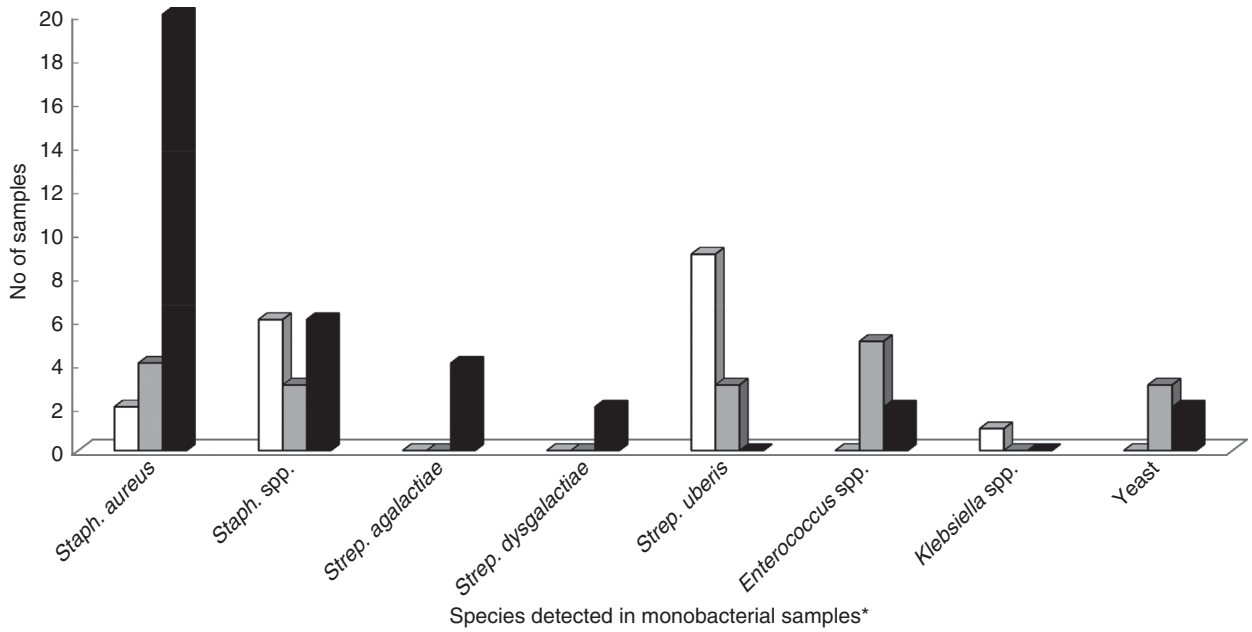

Figure 1 Identification results in 54 mono-bacterial samples using qPCR and the culture on agar media. (□) PP only = positive results with qPCR (Pathoproof®) only; (▨) MDT only = positive results with MALDI-TOF MS® after bacteriological culture only; (■) PP/MDT = positive results with qPCR (Pathoproof) and with MALDI-TOF MS after bacteriological culture. *The total number of samples is higher than 54 because samples with nonmatching results are counted twice.

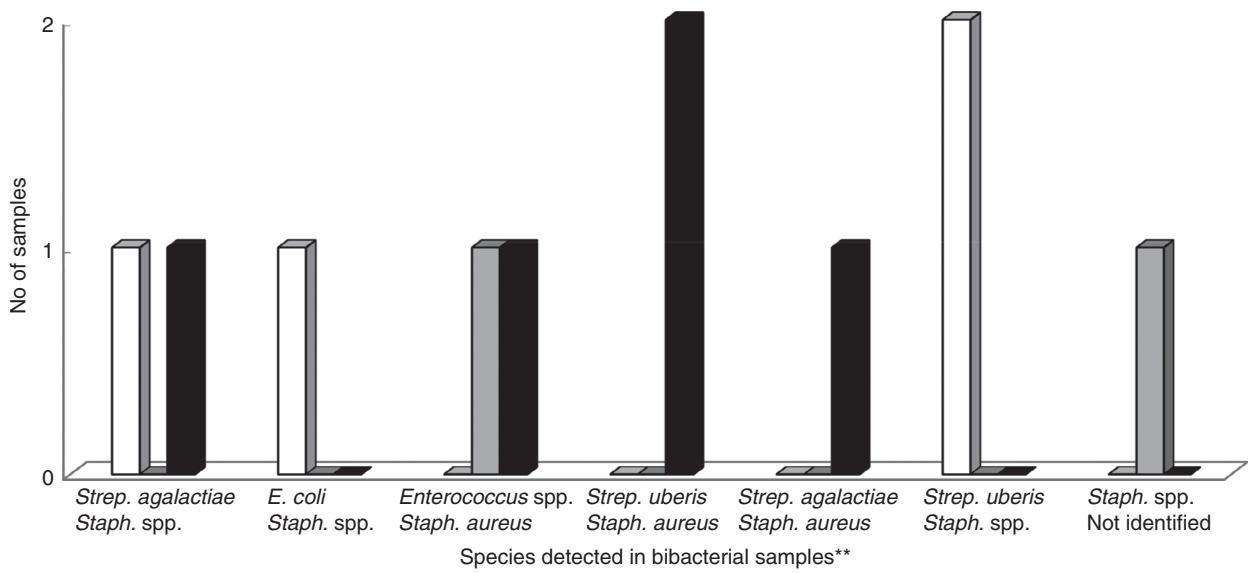

Figure 2 Identification results of nine bi-bacterial samples using qPCR and the culture on agar media (□) PP only = positive results with qPCR (Pathoproof®) only; (▨) MDT only = positive results with MALDI-TOF MS® after bacteriological culture only; (■) PP/MDT = positive results with qPCR (Pathoproof) and with MALDI-TOF MS after bacteriological culture. **The total number of samples is higher than nine because samples with nonmatching results are counted more than once.

assays would probably classify the same sample as positive (Hiitiö *et al.*, 2015);

iv the possible presence of antibiotic residues in the milk interfering with the bacterial growth, but not affecting the qPCR assays (Zadoks *et al.*, 2014). However, this should not be the case in this study since the cows were not receiving any treatment at the time of sampling.

Table 4 Quantitative comparison of the matching positive qPCR assay and culture on agar media results for the 60 milk samples with total or partial qualitative matching identification results

Pathoproof® identification and detection intensity		Bacterial growth (CFU per ml[a]) and MALDI-TOF MS® identification			No. milk samples
+	++	10^2–10^4	10^4–10^6	>10^6	
Staphylococcus aureus		*Staph. aureus*			1
			Staph. aureus		12
	Staph. aureus	*Staph. aureus*			1
			Staph. aureus		6
				Staph. aureus	4
Staph. sp.		*Staph.* sp.			5
	Staph. sp.		*Staph.* sp.		3
	Streptococcus agalactiae		*Strep. agalactiae*		6
				Strep. agalactiae	1
	Strep. dysgalactiae		*Strep. dysgalactiae*		3
				Strep. dysgalactiae	1
Streptococcus uberis			*Strep. uberis*		1
	Strep. uberis			*Strep. uberis*	1
Enterococcus sp.			*Enterococcus* sp.		2
Yeast		Yeast			1
	Yeast	Yeast			1
Staph. sp.	*Strep. agalactiae*		*Strep. agalactiae*		1
			Staph. sp., *Strep. agalactiae*		1
Staph. sp.	*E. coli*	*Staph.* sp.			1
Staph. aureus			*Staph. aureus, Enterococcus* sp.		1
Enterococcus sp.		*Staph. aureus*			1
Staph. aureus	*Staph.* sp.		*Staph. aureus*		1
Staph. aureus	*Strep. uberis*		*Staph. aureus, Strep. uberis*		1
	Staph. aureus, Strep. uberis		*Staph. aureus*	*Strep. uberis*	1
Staph. sp., *Corynebacterium bovis*			*Staph.* sp.		1
Staph. aureus	*Strep. agalactiae*	*Strep. agalactiae*	*Staph. aureus*		1
Staph. sp.	*Strep. uberis*		*Staph.* sp.		1
Total milk samples					60

+: weakly positive results with Ct values between 37 and 30; ++: moderately positive results with Ct values between 30 and 20; none of the 60 samples gave highly positive results (+++) with Ct values lower than 20.
*100 CFU per ml was the detection limit.

It must also not be forgotten that the MALDI-TOF MS databases for the different bacterial species and genera in veterinary medicine are progressively validated and do not incude all bovine mammary gland associated bacterial species yet (Cameron *et al.*, 2018). This may account for the absence of identification of seven isolates by MALDI-TOF MS.

As a conclusion to this comparison study, bacterial culture gives positive results if suitable growth media are used and when the bacteria are alive and present in sufficient amounts. Nevertheless, there is some delay before they can be identified and the antibiotic sensitivity test performed (up to 72 h) and nonpathogenic bacterial contaminants can also grow. Conversely, the multiplex qPCR is a rapid, sensitive and specific test, but targets only the most important mammary gland pathogens, can detect DNA of bacteria both alive and dead, and does not provide the antibiotic sensitivity profile of the pathogens, besides the detection of the *blaZ* gene of staphylococci. In that respect, if 56 staphylococcus-positive samples are also positive for the *blaZ* gene, four staphylocccus isolates also test positive by classical PCR for the *mecA* gene (not shown) coding for methicillin resistance. Therefore, adding a qPCR for the detection of the *mecA* gene would be of value because of their zoonotic potential and risk for public health (Lakhundi and Zhang, 2018). Independently of chosen methodology, human supervision of the crude results is essential to interpret their clinical meaning correctly and avoid misdiagnosis as often as possible (Zadoks *et al.*, 2014), because neither test is perfect to diagnose mammary gland infection (Cederlöf *et al.*, 2012) and because Ct values of qPCR assay should more carefully be chosen according to the health status of the herd/cows and to the associated bacterial species/genus (Mahmmod *et al.*, 2013b).

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Conflict of interest

The authors have no conflict of interest to declare.

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Supporting Information

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

Table S1. Detailed comparison of the identification results of the 182 milk samples with the qPCR assay and the culture on agar media.

Table S2. Detailed quantitative comparison of the 82 qualitatively matching qPCR reactions and bacterial growth from the 77 milk samples with total or partial qualitative matching identification results.