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Performance of a real-time PCR assay in routine bovine mastitis diagnostics compared with in-depth conventional culture

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Reliable identification of the aetiological agent is crucial in mastitis diagnostics. Real-time PCR is a fast, automated tool for detecting the most common udder pathogens directly from milk. In this study aseptically taken quarter milk samples were analysed with a real-time PCR assay (Thermo Scientific PathoProof Mastitis Complete-12 Kit, Thermo Fisher Scientific Ltd.) and by semi-quantitative, in-depth bacteriological culture (BC). The aim of the study was to evaluate the diagnostic performance of the real-time PCR assay in routine use. A total of 294 quarter milk samples from routine mastitis cases were cultured in the national reference laboratory of Finland and examined with real-time PCR. With BC, 251 out of 294 (85·7%) of the milk samples had at least one colony on the plate and 38 samples were considered contaminated. In the PCR mastitis assay, DNA of target species was amplified in 244 samples out of 294 (83·0%). The most common bacterial species detected in the samples, irrespective of the diagnostic method, was the coagulase negative staphylococci (CNS) group (later referred as *Staphylococcus* spp.) followed by *Staphylococcus aureus*. Sensitivity (Se) and specificity (Sp) for the PCR assay to provide a positive Staph. aureus result was 97·0 and 95·8% compared with BC. For *Staphylococcus* spp., the corresponding figures were 86·7 and 75·4%. Our results imply that PCR performed well as a diagnostic tool to detect *Staph. aureus* but may be too nonspecific for *Staphylococcus* spp. in routine use with the current cut-off *Ct* value (37·0). Using PCR as the only microbiological method for mastitis diagnostics, clinical relevance of the results should be carefully considered before further decisions, for instance antimicrobial treatment, especially when minor pathogens with low amount of DNA have been detected. Introducing the concept of contaminated samples should also be considered.

Keywords: Bovine mastitis, diagnostics, real-time PCR, bacteriological culture.

Mastitis is the most common disease of dairy cows (Halasa et al. 2007), mainly caused by intramammary infection (IMI) due to a variety of contagious and environmental pathogens. The importance of reliable identification of the aetiological agent is crucial in mastitis diagnostics. Proper microbiological diagnostics represents the basis for developing efficient mastitis control programmes in herds, as problems due to different pathogens need different approaches. Mastitis is also the major reason for use of antimicrobials for dairy cows. Treatment of mastitis should be based on microbiological diagnosis and when necessary, susceptibility testing in vitro, to avoid inefficient or unnecessary use of antimicrobials. Targeted treatment protocols may increase cure rates, minimise costs, and lower the volumes of antimicrobials used (Lago et al. 2011; Cameron et al. 2014; Steeneveld et al. 2014; Ruegg, 2014).

Bacteriological culture (BC) of an aseptically taken quarter milk sample is the current gold standard for laboratory diagnostics of mastitis causing microorganisms (National Mastitis Council, 2004). Conventional culturing has a few well-known shortcomings such as the rather long time to obtain results (Hogan et al. 1999), need for experienced laboratory personnel, subjectivity despite the standardised procedures (Sears & McCarthy, 2003) and the high numbers of milk samples with no growth (Makovec & Ruegg, 2003; Bradley et al. 2007; Koivula et al. 2007; Olde Riekerink et al. 2008; Persson Waller et al. 2011; Oliveira et al. 2013).

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Development of modern molecular technologies has offered alternative methods for mastitis diagnostics. PCR assays for detection of mastitis pathogens were introduced at the beginning of this century (Phuektes et al. 2001; Gillespie & Oliver, 2005; Studer et al. 2008) and the first commercial real-time multiplex PCR test for quarter-based identification of udder pathogens (Thermo Scientific PathoProof Mastitis PCR Assay, ThermoFisher Scientific Ltd., former Finnzymes, Vantaa, Finland) was globally launched in 2008. This assay was quickly adopted for use in Finland and the laboratories of the largest dairy company switched from conventional culturing to PCR in 2010. The method is easy for a farmer: an aseptically taken quarter milk sample is sent to the laboratory in the milk truck or via mail, and the result is provided electronically on the same or the following day. Currently it is estimated that over 80% of the Finnish quarter milk samples are diagnosed using the PCR method alone.

Relative to culture-based methods, PCR is faster as the results are provided within 4 h, and is more sensitive in detecting common udder pathogens (Koskinen et al. 2010). The number of the target species varies according to the used PCR kit. The results of the assay are expressed in *Ct* (cycle threshold) values, which reflect genome copy numbers of the detected species. In Finland the results are provided with semi-quantitative estimates of the amounts of the bacterial DNA detected (low, intermediate or high). Using a PCR test as the sole diagnostic method in mastitis diagnostics has raised some concerns. All target species detected from the sample in amounts under the set threshold *Ct* value (37·0 for all pathogens) are reported without further interpretation by the laboratory personnel. Compared with conventional bacteriology, where the quality of the samples is assessed by the laboratory staff and samples with growth of more than two species are deemed contaminated (Hogan et al. 1999), interpretation of PCR results has been considered more challenging.

In studies published to date, comparison of real-time PCR and conventional culture has focused on detection capabilities of the PCR assay (Taponen et al. 2009; Koskinen et al. 2010; Bexiga et al. 2011; Keane et al. 2013). Bacteriological culturing has been carried out according to routine procedures, excluding samples with more than two species detected (Spittel & Hoedemaker, 2012; Keane et al. 2013). In the present study quarter milk samples from subclinical and clinical mastitis were analysed with a real-time PCR assay and semi-quantitative, in-depth bacteriological culture carried out in the Finnish national reference laboratory for mastitis. Detection abilities and diagnostic capability of the PCR assay was compared with the in-depth conventional culturing, aiming to evaluate the diagnostic performance of the real-time PCR assay with routine milk samples.

Materials and methods

Milk samples

A total of 294 quarter milk samples were sent to the local laboratory of Valio Ltd. (Lapinlahti, Finland) between October 2010 and January 2011. The samples were collected by farmers and local veterinarians and arrived at the laboratory refrigerated within 48 h from sampling. Milk samples originated mainly from quarters with subclinical or mild clinical mastitis (Laura Kulkas, Valio Ltd., personal communication). Data on the clinical status or milk somatic cell count (SCC) of the cows were not available. From Valio Ltd. the samples were transferred immediately to the national mastitis reference laboratory, Finnish Food Safety Authority Evira (Kuopio, Finland), and were cultured directly on arrival. The samples were refrigerated without preservatives during both transportations. Two aliquots of the samples were deep frozen (−20 °C) immediately after culturing. One aliquot was sent to the laboratory of the Department of Production Animal Medicine (University of Helsinki, Mäntsälä, Finland) for milk N-acetyl-β-D-glucosaminidase (NAGase) activity determination and the other to the laboratory of Thermo Fisher Scientific Ltd. (Vantaa, Finland) for PCR analysis.

Bacterial culture and interpretation of the microbiological results

A total of 0·01 ml of milk was streaked on to a tryptic soy agar plate containing 5% bovine blood (TSA, CASO agar, Merck, Darmstadt, Germany) and spread over the entire 9-cm plate with a conventional loop. Plates were incubated aerobically at 37 °C for 48 h, and inspected after 24 and 48 h. All colonies on the plate were enumerated when possible. Morphologically different colonies were counted and representatives of colonies of a similar type identified using standard identification schemes (Hogan et al. 1999). Enumeration and identification of the bacterial colonies was made also for cultures with ≥3 species, which in routine diagnostics are defined as contaminated (National Mastitis Council, 2004). Colony-forming units exceeding 100 could not be accurately counted and were recorded as 100. Gram staining, catalase test, oxidase test (Pro-Lab Diagnostics, Richmond Hill, ON, Canada), API Staph, API ID32 Strep, API 20 E and API Coryne (bioMérieux, Marcy l'Etoile, France) were used in the identification schemes. For *Staphylococcus* spp., a positive coagulase test (BBL™ Rabbit plasma, BD Diagnostics, USA) was confirmed with API Staph (bioMérieux, Marcy l'Etoile, France). Eosin methylene blue agar (EMB) (Oxoid, Basingstoke, Hampshire, UK) was used for detection of *Escherichia coli* and TSA was supplemented with Tween-80 (0·1%) (Amresco (Solon, OH, US)) to detect lipophilic *Corynebacterium bovis*.

A positive result for bacterial growth for all micro-organisms was detection of at least one CFU per 0·01 ml of milk. *Staphylococcus* spp. results and the species not included in the target panel of the PCR assay were additionally examined with a higher threshold for positive result (5 CFU per 0·01 ml of milk) owing to their minor status as mastitis pathogens.

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Multiplex real-time PCR

The frozen milk aliquots were tested using Thermo Scientific PathoProof™ Mastitis Complete-12 Kit within 1 week of arrival at the laboratory of Thermo Fisher Scientific Ltd. (Vantaa, Finland). The oligos of the Complete-12 Kit are designed to detect DNA of *C. bovis, Enterococcus faecalis/faecium, Esch. coli, Klebsiella oxytoca/pneumoniae, Serratia marcescens, Staph. aureus, Staphylococcus* spp., *Streptococcus agalactiae, Str. dysgalactiae, Str. uberis* and *Peptoniphilus indolicus* and/or *Trueperella pyogenes*. Bacterial targets have been divided into four PCR reactions, each of them detecting three targets and an internal amplification control. Sample volume for the PathoProof Mastitis PCR Assay was 350 µl of which 17·5 µl ended up in the PCR reaction after the DNA extraction procedure. *Enterococcus* spp., *Klebsiella* spp., and *Staphylococcus* spp. were identified to group levels. *Staph. aureus* was separately identified. *Ct* values above 37·0 were reported as negative results. The number of genome copies targeted in PCR was recorded for *Staph. aureus* and *Staphylococcus* spp.

N-acetyl-β-D-glucosaminidase activity in the milk

Milk N-acetyl-β-D-glucosaminidase (NAGase) activity was used as an inflammation indicator of the milk sample. NAGase activity of the milk samples was determined in the laboratory of the Department of Production Animal Medicine (University of Helsinki, Mäntsälä, Finland) using an in-house microplate modification (Hovinen et al. 2014) of the fluorogenic method developed by Mattila & Sandholm (1985). The results were expressed as picomoles of 4-MU/min/µl milk at 25 °C. The maximum detection limit of NAGase activity is 24·49 pmol 4-MU/min/µl milk. The mean NAGase activity of normal milk (SCC < 100 000 cells/ml) is 0·45 pmol 4-MU/min/µl milk (range 0·09–1·04 pmol 4-MU/min/µl milk) (Hovinen et al. 2014). We used the upper range value for normal milk as our definition for mastitic milk (1·04 pmol 4-MU/min/µl milk).

Calculations for specificity and sensitivity of PCR in diagnosing Staph. aureus and Staphylococcus spp.

Specificity (Sp) and sensitivity (Se) of the real-time PCR to detect *Staph. aureus* and *Staphylococcus* spp. was calculated assessing the conventional culture as golden standard method. Agreement to provide a positive diagnosis for these species sample by sample was tested with the κ-coefficient. For the calculations of Se, Sp and κ-coefficient, only culture results containing one or two different species and their corresponding PCR results were included ($n = 256$). In these calculations, a cut-off of one CFU was used for *Staph. aureus* and a cut-off of ≥5 CFU for *Staphylococcus* spp. (Honkanen-Buzalski & Seuna, 1995). In PCR, detection of the target species with a $Ct \leq 37$ was considered as a positive result. For *Staph. aureus* and *Staphylococcus* spp., the correlation of CFUs from BC and genome copy numbers from PCR was also tested. Data were transformed to CFUs and genome copy numbers per ml of milk as follows: Genomic copy number $= 10^{(Ct-x)/y}$, where Ct = cycle threshold value, X = slope of a standard curve and Y = interception of a standard curve. Spearman's rho correlation was used because the data were not normally distributed. The statistical analysis was performed with SPSS Statistics 20 (IBM, Armonk, NY, USA).

Results

Out of the total 294 milk samples, 251 (85·7%) were positive for bacterial growth in the BC when ≥1 CFU was considered positive. In the real-time PCR, 244 samples out of 294 (83·0%) were positive (Table 1). With both methods, the most common finding was *Staphylococcus* spp. (CNS group), followed by *Staph. aureus* (Tables 2 and 3). Assessing culture results of *Staphylococcus* spp. using a threshold of ≥5 CFU, the number of detections decreased from 103 to 58. Similarly, the number of *C. bovis* detections decreased from 35 to 18. Species not included in the target panel of the PCR assay were cultured from 77/294 samples (≥1 CFU), but using the threshold of ≥5 CFU, only 7/294 yielded a positive diagnosis. These diagnoses included two samples with yeast (both > 50 CFU), one with *C. ulcerans* (>100 CFU), one with *Aerococcus* spp. (33 CFU), one with *Str. bovis* (64 CFU), one with *Corynebacterium* spp. (10 CFU) and one with an unidentified coccus (33 CFU). PCR results were negative for five of these samples, while two contained *Staphylococcus* spp. in low amounts. Half (38/77) of the samples with non-target PCR species cultured contained at least two other species, indicating contamination.

Three or more species were cultured from 38/294 (12·9%) samples. *Staphylococcus* spp. was the major finding 23/38 (60·5%) in these samples, which are defined as contaminated in the BC (National Mastitis Council, 2004). Other species commonly cultured from the contaminated samples were *Corynebacterium* spp., *Aerococcus* spp., *Bacillus* spp. and *Str. dysgalactiae* (Table 2). *Staph. aureus* was found in seven samples with three or more species. PCR results of these 38 contaminated samples included DNA of the target pathogens usually in moderate on low amounts (Fig. 1). In the PCR, 24 samples (8·1%) with ≥3 different species were detected, *Staphylococcus* spp. also being the most prevalent finding (Table 3). Other frequently detected species in these multi-species samples were *Str. uberis, Str. dysgalactiae* and *T. pyogenes*. *Staph. aureus* was present in 8 samples (Table 3).

In BC, 52/294 (17·7%) of samples contained two different species. In 30 out of these 52 samples (57·7%) at least the other cultured species was not included in the target panel of the PCR assay. The number of samples with two species was 59/294 (20·0%) in the PCR. Among these 59 samples, only one species was cultured in the BC in 30 (50·8%) samples, four were culture-negative, 18 had growth of two species and the rest (7) three or more species.

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Table 1. The number of bacterial species per sample detected by the conventional bacteriological culture (BC) or real-time PCR assay. Samples ($n = 294$) were aseptically taken quarter milk samples. A positive result in BC was one CFU per 0·01 ml of milk and in PCR detection of bacterial DNA under a cycle threshold value of 37

Species detected per sample	BC		PCR	
	n	%	*n*	%
0	43	14·6	50	17·0
1	161	54·8	161	54·8
2	52	17·7	59	20·1
3†	30	10·2	18	6·1
4†	6	2·0	5	1·7
5†	2	0·7	1	0·3
Total	294	100·0	294	100·0

†Considered contaminated in the conventional bacteriological culture

Table 2. Species isolated in aseptically taken quarter milk samples ($n = 294$) by the conventional bacteriological culture. The total number of isolations of each species (*n*) is presented in the second column. The number of different species detected per sample (including the species in the first column) is also shown. A positive result was defined as detection of ≥1 CFU per 0·01 ml of milk for all microorganisms. Samples containing >5 species were not found

Species	*n*	Total number of different species per sample				
		1	2	3	4	5
Aerococcus spp.	20	3	3	11	3	0
Bacillus spp.	12	2	1	7	2	0
Chryseobacterium spp.	3	0	0	0	2	1
Corynebacterium spp.	35	1	15	14	3	2
C. bovis	21	10	8	3	0	0
C. ulcerans	1	1	0	0	0	0
Enterococcus spp.	10	3	1	4	1	1
Escherichia coli	11	7	2	1	1	0
Enterobacter spp.	1	0	0	1	0	0
Enterococcus casseliflavus	1	1	0	0	0	0
Gemella spp.	1	0	0	0	1	0
Globicatella spp.	2	0	0	2	0	0
Klebsiella spp.	6	4	1	1	0	0
Lactobacillus spp.	1	0	0	1	0	0
Lactococcus spp.	6	1	3	0	1	1
Leuconostoc spp.	1	0	0	1	0	0
Micrococcus spp.	1	0	0	1	0	0
Moraxella spp.	1	0	1	0	0	0
Raoultella spp.	1	0	0	0	1	0
Serratia marcescens	1	0	1	0	0	0
Staph. aureus	73	49	17	6	1	0
Staphylococcus spp.	103	51	29	16	5	2
Str. agalactiae	1	1	0	0	0	0
Str. bovis	5	0	4	1	0	0
Str. dysgalactiae	27	11	7	7	2	0
Str. uberis	19	11	5	1	2	0
Trueperella pyogenes	16	5	5	4	2	0
Unidentified Gram positive coccus	4	0	2	1	1	0
Unidentified rod	2	0	0	2	0	0
Yeast	4	2	1	1	0	0

Species detected by PCR and their minimum, maximum and median *Ct* values are shown in Table 3. In 79/113 (69·9%) of the samples positive for *Staphylococcus* spp. in the PCR, only a low amount of DNA ($Ct > 31·1$) was detected, and none of the samples contained a high amount of DNA ($Ct < 24$). Samples positive for *Staph. aureus* contained mainly high ($Ct < 24$) or intermediate (Ct 24–30) amounts of DNA; in 32/79 (40·5%) of the samples the amount of DNA was low.

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Table 3. Species detected from aseptically taken quarter milk samples ($n = 294$) with real-time PCR. The total number of detections of each species (n) is presented in the second column. The number of different species detected per sample (including the species in the first column) is also shown. Samples containing >5 species were not found. A positive microbiological result was based on amplifying DNA of the target species using a cycle threshold cut-off value of 37·0

		Total number of species per sample					Cycle threshold values		
Species	*n*	1	2	3	4	5	Median	Min.	Max.
Corynebacterium bovis	28	4	17	5	2	0	32·3	26·3	36·9
Enterococcus spp.	7	2	1	2	1	1	30·7	30·7	35·8
Escherichia coli	17	7	6	3	1	0	29·2	23·7	36·6
Klebsiella spp.	6	1	3	1	1	0	33·9	16·1	35·9
Staph. aureus	79	52	19	6	1	1	29·7	17·1	36·7
Staphylococcus spp.	113	60	36	12	5	0	33·1	25·1	37·1
Serratia marcescens	1	1	0	0	0	0	35·0	35·0	35·0
Streptococcus agalactiae	1	0	1	0	0	0	22·7	22·7	22·7
Str. dysgalactiae	39	14	13	9	2	1	28·1	15·3	36·8
Str. uberis	38	13	11	9	4	1	29·2	19·0	36·7
Trueperella pyogenes	29	7	11	7	3	1	31·2	9·2	36·6

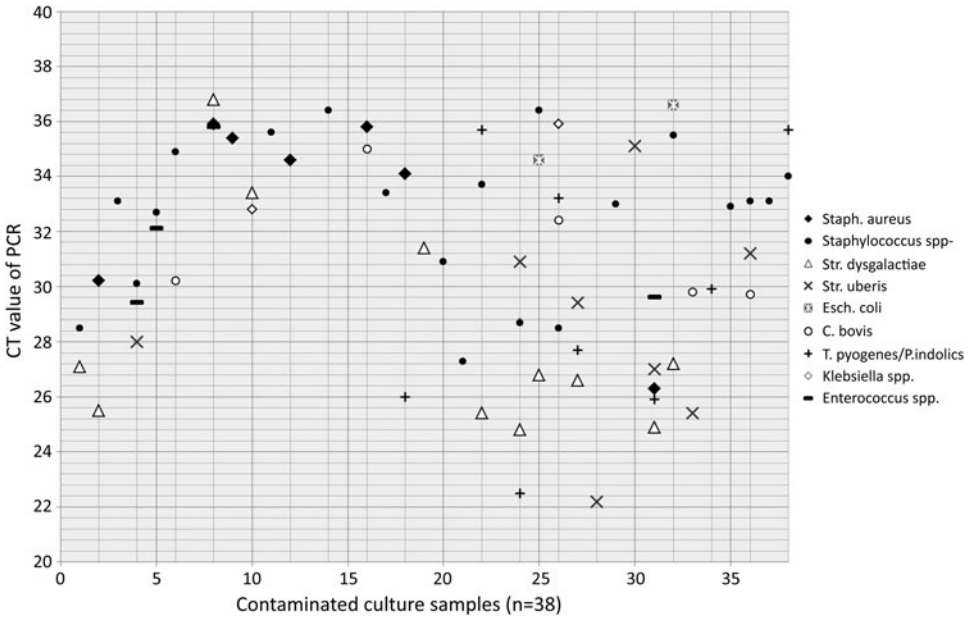

Fig. 1. Real-time PCR results for aseptically taken quarter milk samples in which ≥3 different species were detected in the conventional bacteriological culture (38/294).

Among the 43 culture-negative samples, PCR gave a positive result for 20 samples (46·5%) (Fig. 2). *Staphylococcus* spp. was the most common finding, and *Staph. aureus* was amplified in three samples (*Ct* range 34·1–36·7). All culture-negative samples, except those positive for *Str. dysgalactiae,* contained only low amounts of the target DNA in the PCR and no more than two different species (Fig. 2).

From the total of 50 PCR-negative samples, 27 (54·0%) samples were culture positive (Fig. 3). One of these samples yielded ≥3 species on the plate indicating contamination, 8 samples yielded two species and 18 samples only one species (threshold ≥ 1 CFU). In 13 out of the 27 (48·1%) samples cultured species were not included in the PCR target panel (Fig. 3). *Staphylococcus* spp., *Klebsiella* spp., *Staph. aureus* and *Enterococcus* spp., which belong to the target panel of the PCR assay were isolated in 20 PCR negative samples. *Staphylococcus* spp. was detected in 15/27 (55·6%) samples, but only in one sample in numbers higher than 5 CFU per 0·01 ml of milk. One CFU of *Staph. aureus* was found in one PCR-negative sample.

The mean NAGase activity of all milk samples was 5·79 pmol 4-MU/min/µl milk (range 0·11–24·49 pmol 4-MU/min/µl). Based on the milk NAGase activities, 246/294 (83·7%) samples originated from mastitic quarters (activities over 1.04 pmol 4-MU/min/µl milk). For *Staph. aureus* samples the mean NAGase value was 6·95 pmol 4-MU/

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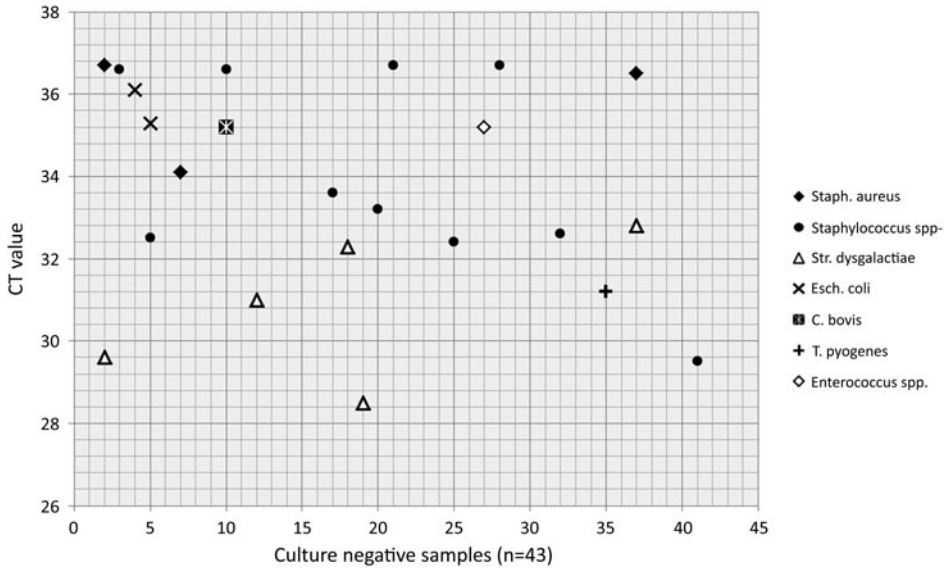

Fig. 2. Real-time PCR results and their *Ct* values for aseptically taken quarter milk samples with no growth in the conventional bacteriological culture (43/294). The cycle threshold cut-off value for a positive PCR result was 37·0.

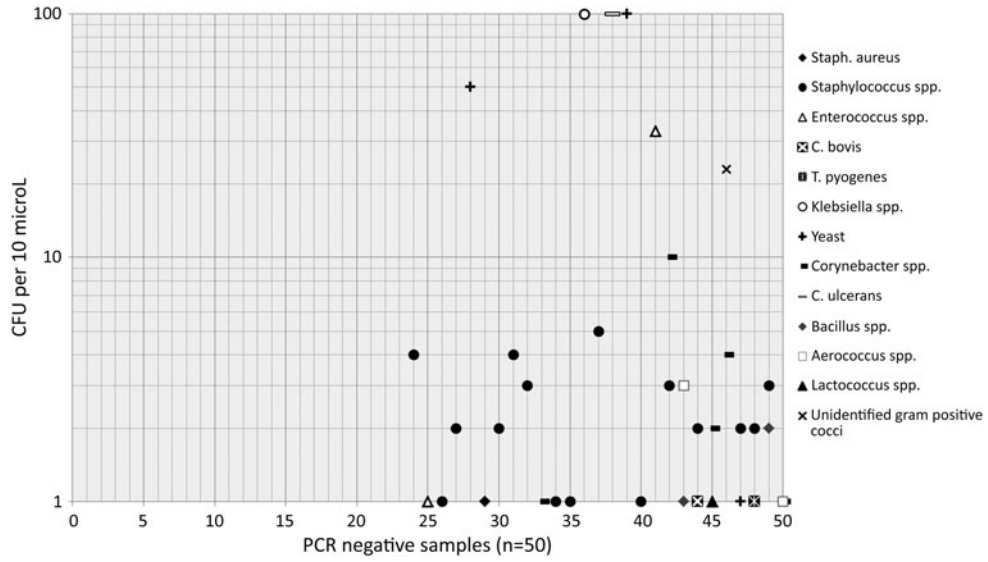

Fig. 3. Results from conventional bacteriological culture for aseptically taken quarter milk samples negative in real-time PCR assay (50/294). A positive result was defined as detection of ≥1 CFU per 0·01 ml of milk for all micro-organisms.

min/µl milk (range 0·11–24·49) and for *Staphylococcus* spp. samples 4·44 pmol 4-MU/min/µl milk (0·12–17·20). Among samples positive for *Staph. aureus*, in 16/73 (21·9%, BC) and in 14/79 (17·7%, PCR) milk NAGase activity remained low. The corresponding figures for *Staphylococcus* spp. were 9/103 (8·7%, BC) and 22/113 (19·5%, PCR).

PCR was compared with BC in detecting *Staph. aureus* and *Staphylococcus* spp. in the milk samples. The total number of samples in these calculations was 256, as contaminated BC samples and the respective PCR samples were excluded. For *Staph. aureus* ($n = 9$ PCR, $n = 3$ BC), Se and Sp were 97·0 and 95·8%, respectively. The positive predictive value (PPV) was 88·9%, negative predictive value (NPV) 98·9% and κ-coefficient between PCR and BC 0·90 ($P < 0·05$). For *Staphylococcus* spp. ($n = 113$ PCR, $n = 58$ BC, threshold ≥5 CFU) the corresponding figures for PCR were Se 86·7%, Sp 75·4%, PPV 42·9%, NPV 96·4% and κ-coefficient 0·44 as compared with BC ($P < 0·05$ for all results).

The number of CFU/ml in BC and the number of genome copies/ml in PCR for *Staph. aureus* and *Staphylococcus* spp. are shown in Fig. 4. Only samples where the species were

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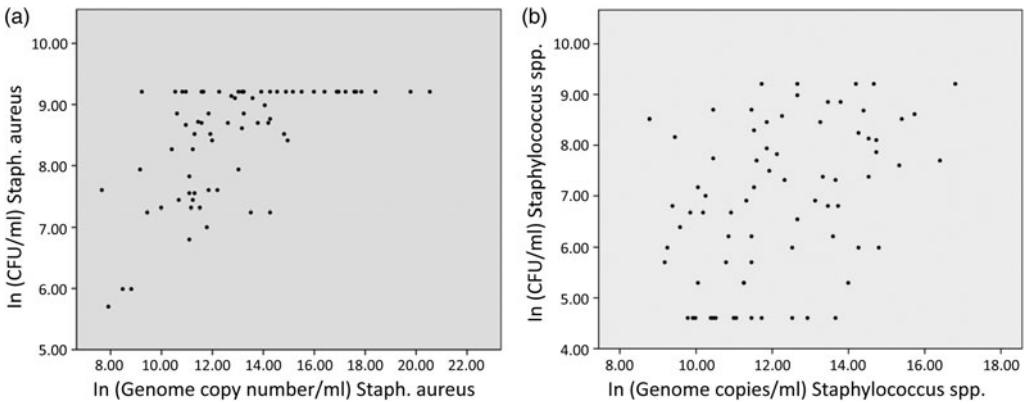

Fig. 4. The number of CFU/ml in the conventional bacteriological culture and the number of genome copies/ml in real-time PCR for samples positive for *Staph. Aureus* (a) and *Staphylococcus* spp. (b) are presented on a logarithmic (ln) scale. Only samples where the target species was detected by both methods are included (*Staph. aureus* $n = 70$, *Staphylococcus* spp. $n = 75$).

detected by both methods are included ($n = 70$ for *Staph. aureus* and $n = 75$ for *Staphylococcus* spp.). Spearman's correlation coefficient for *Staph. aureus* was 0·68 ($P < 0·05$) and for *Staphylococcus* spp. 0·47 ($P < 0·05$). High numbers of CFU (≥100) were detected in 27 samples with *Staph. aureus* and in 6 samples with *Staphylococcus* spp. When the samples with over hundred CFU of *Staph. aureus* were excluded, the correlation coefficient decreased to 0·52 ($P < 0·05$). For *Staphylococcus* spp., the removal of the 6 high-CFU samples decreased the coefficient to 0·32 ($P = 0·007$).

Discussion

In this study, routine quarter milk samples from subclinical and clinical mastitis were analysed with a real-time PCR assay and semi-quantitative, in-depth bacteriological culture carried out in the Finnish national reference laboratory for mastitis. All bacterial colonies grown on the agar plates were quantified if possible and different colonies identified. Detection abilities and diagnostic capability of the PCR assay was compared with the conventional culturing, aiming to evaluate the performance of the real-time PCR assay in routine use. In papers published to date, the focus has been mainly on the detection capabilities of the PCR assay compared with conventional culture carried out in routine mastitis laboratories (Taponen et al. 2009; Koskinen et al. 2010; Bexiga et al. 2011; Keane et al. 2013). In the previous studies routine microbiological methods for mastitis diagnostics have been used, with no quantification of the bacterial colonies and excluding samples with more than two species detected (Spittel & Hoedemaker, 2012; Keane et al. 2013).

Based on the NAGase results, the majority (83·7%) of the milk samples originated from mastitic quarters. In the rest of the samples, no inflammatory reaction was present, and they may have been taken to monitor treatment response or because of some clinical signs in the quarter. Regarding the capability of the tests to detect microbes in the milk, the proportion of positive samples was almost similar in PCR (83·0%) and in BC (85·7%), despite the limitations in the number of targets in the PCR panel. Given the relatively high number of samples (77/294) containing species not included in the target panel of the PCR assay, only 7 (1·1%) yielded a positive microbiological diagnosis using a cut-off of ≥5 CFU for a positive result. According to our results the target panel of the PCR assay was adequate. Yeasts, *Mycoplasma* spp., *Mycoplasma bovis* and *Prototheca* spp. have been included in the more recent version of the PCR assay, which is currently in routine use in Finland.

The number of negative samples was rather similar with PCR and BC, but only about half of these samples were negative with both methods. Of the culture-negative samples, PCR provided a positive result in 46%, which is in line with previous studies (Taponen et al. 2009; Bexiga et al. 2011; Spittel & Hoedemaker, 2012). The majority of these samples yielded minor pathogens, usually *Staphylococcus* spp. or environmental pathogens with a low amount of bacterial DNA. *Staph. aureus* was detected by PCR from three culture-negative samples with low amounts of DNA (Fig. 2). Keane et al. (2013) also found that PCR-positive but culture-negative *Staph. aureus* samples are quite rare. In general, explanations for culture-negative PCR-positive samples could be the inability of the damaged or dead cells to grow, in addition to the presence of inhibitory substances in the milk. A larger volume of milk is analysed with PathoProof Mastitis Assay (17·5 µl) than with BC (10 µl), increasing the sensitivity of the PCR test. The clinical relevance of positive PCR results from samples negative in culture could be questioned. However, when PCR is used as the sole diagnostic test, culture results are not available.

More than half of the 50 PCR-negative samples (54·0%) were positive in BC, and some of them contained target bacteria of the PCR assay, mainly *Staphylococcus* spp. (Fig. 3).

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Staph. aureus was cultured only once from a PCR-negative sample with only one CFU on the plate. Non-target microbes were cultured in 26·0% of the PCR-negative samples. An explanation for samples negative in PCR but positive in BC could be incomplete mixing of the sample before the PCR test i.e. inadequate homogenisation of the sample. In the present study, samples where PCR failed to detect bacterial DNA but which were positive in the BC, the number of CFU was always low, with the exception of species not included in the PCR panel.

The correlation between CFU counts in the BC and genome copy numbers in the PCR was fairly good for *Staph. aureus* (0·68, $P < 0·05$), but for *Staphylococcus* spp. only from moderate to poor (0·47, $P < 0·05$), with considerable variation. For instance, from 14 samples, where PCR indicated a low amount of *Staph. aureus* in the sample (*Ct* 30·1–37·0), over 20 CFU per 0·01 ml were cultured and from 5 samples even ≥100 CFU per 0·01 ml (data not shown). Our results support the previous observations on poor agreement between plate counts and quantification of *Staph. aureus* by PCR (Hein et al. 2001; Studer et al. 2008). Botaro et al. (2013) found that plate counts and genome copy numbers of *Staph. aureus* correlated very well in milk samples artificially inoculated with a single *Staph. aureus* strain, but for samples from naturally infected quarters genome copy numbers of *Staph. aureus* could not be recorded. The ability of PCR to detect DNA from dead or growth-inhibited cells is a well-known cause for variation. Disagreement between plate counts and amount of bacterial DNA amplified by PCR may be related to differences in the growth ability of the strains (Hein et al. 2001). The DNA targets (usually proprietary information) may also be present in more than one copy in the target organisms, making the comparison even more difficult. Comparison between published studies is difficult in general, owing to the different real-time PCR assays and sample sizes used. Regarding *Staphylococcus* spp. (CNS or non-aureus staphylococci), which consists of a group of species, comparison is even more complicated.

The PCR assay appeared to perform as a reliable diagnostic tool for *Staph. aureus*. Using the conventional culture as an acceptable gold standard could be argued, as this approach may underestimate Se and Sp of the PCR assay because of the inaccuracy of the reference test (Koskinen et al. 2010; Cederlöf et al. 2012). In a previous study of Cederlöf et al. (2012) Se of the PCR assay to detect *Staph. aureus*-IMI at drying off was 93% and Sp 95%. They used the same *Ct* cut-off value of 37, but the statistical method of analysing the results differed (Latent Class Analysis). Paradis et al. (2012) used a different multiplex real-time PCR assay, resulting in a median Se of 72 to 79%. In our calculations, a positive result for *Staph. aureus* was based on presence of at least one CFU on the plate (Dohoo et al. 2011). The clinical relevance of such a finding can be debated, as no agreed standards are available, though proposals have been made (Andersen et al. 2010; Dohoo et al. 2011). Based on our results and considering the nature of *Staph. aureus* as a major pathogen, we recommend that also low amounts of *Staph. aureus* DNA amplified ($Ct > 30·1$) in the PCR should be noted when using PCR as the only diagnostic method, presuming that the sample is aseptically taken. The performance of the PCR assay for diagnosing other staphylococci than *Staph. aureus* seems not to be as optimal. In calculations for *Staphylococcus* spp., using at least 5 CFU as a cut-off for positive diagnosis, over 50% of the positive PCR results could be false positives. The majority of these detections contained only small amounts of DNA of *Staphylococcus* spp. We suggest that PCR results of low amounts of *Staphylococcus* spp. should not be automatically interpreted as IMI, but the possibility of contamination should be considered to avoid misdiagnoses and unnecessary antimicrobial treatments or other measures. The cut-off *Ct* value for *Staphylococcus* spp. should probably be adjusted to avoid possible false positive diagnoses in the PCR.

The hygienic quality of milk samples was good as 83·9% of samples examined by BC and 86·6% of samples examined by PCR contained only one or two different species. This indicates that aseptic milk sampling routine has been adapted well by the local veterinarians and farmers. In conventional mastitis diagnostics a sample containing more than two species is considered contaminated (Hogan et al. 1999) and the result is provided to the client as 'mixed growth', with no bacterial species identified in the sample. In contrast, the results of the PCR assay are presented as a list of species with *Ct* values or a semi-quantitative classification of the amount of DNA detected. Wrong interpretation of samples with multiple species may lead to misdiagnoses and even to non-justified antimicrobial treatments. In the previous studies PCR has provided more multi-species results than BC (Bexiga et al. 2011; Spittel & Hoedemaker, 2012; Keane et al. 2013), which is in contrast to our study (BC 12·9% and PCR 8·1%, respectively). This might be due to the in-depth culturing performed in our study as the majority of the contaminated culture samples contained species not included in the PCR target panel and was detected with low CFU numbers. The interpretation of the PCR results was strict: despite the predominance of some pathogens in the PCR results (proportion of some species in the sample >90 or >99%), all detected pathogens were recorded. Detection of more than two bacterial species, especially species of environmental origin and in low amounts of DNA, raises concerns about the hygienic quality of the sample, despite the analysing method. In PCR diagnostics a definition for contaminated sample could be advantageous, especially in cases where a predominant species cannot be identified.

All microbiological tests have their limitations. For all tests, the importance of a proper aseptic technique in milk sampling cannot be over-emphasised. Handling, temperature, transportation and time after sampling can all affect the results despite the method. BC provides results only when organisms are living and can grow on the plate. PCR assays yield microbiological results irrespective of the fitness of the bacteria, detecting DNA also from dead

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bacteria. Consequently, the clinical relevance of some PCR results can be questionable. It must be emphasised that results from any microbiological analysis provide only a microbiological diagnosis and should always be interpreted with all available information, such as clinical signs and history of the cow, and SCC of the milk, when used in decision-making for antimicrobial treatment or other measures. When using PCR as the only microbiological method for mastitis diagnostics, low amounts of minor pathogens, such as *Staphylococcus* spp. should be ignored. Introducing the concept of a contaminated sample to mastitis diagnostics with PCR could be helpful to avoid wrong interpretation of the results.

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