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Accuracy of qPCR and bacterial culture for the diagnosis of bovine intramammary infections and teat skin colonisation with *Streptococcus agalactiae* and *Staphylococcus aureus* using Bayesian analysis

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ABSTRACT

Streptococcus agalactiae (*Strep. agalactiae*) and *Staphylococcus aureus* (*Staph. aureus*) are originally regarded as contagious mastitis pathogens, however, both pathogens have recently been isolated from extramammary and environmental sites, indicating that other sites than the udder might contribute to the spread of these pathogens potentially causing intramammary infections. Diagnostic tools to identify pathogens at extramammary sites are available but still needs to be validated. The objective of this cross-sectional field study was to estimate the diagnostic sensitivity (Se) and specificity (Sp) of the commercially available Mastit4 qPCR assay and bacterial culture (BC) in identifying *Strep. agalactiae* and *Staph. aureus* from milk and teat skin samples. We randomly selected 30–40 cows with high somatic cell counts from eight Danish *Strep. agalactiae*-positive dairy herds with automatic milking systems. Teat skin samples and aseptic milk samples were collected from right rear quarters (n = 287) for BC and PCR analysis. Se and Sp were estimated in a Bayesian latent class analysis. For milk samples, the Se and Sp of qPCR for *Strep. agalactiae* were estimated to 0.97 and 0.99, respectively, whereas the Se and Sp of BC were 0.41 and 1.00, respectively. The Se and Sp of qPCR for *Staph. aureus* were estimated to 0.95 and 0.99, respectively, whereas the Se and Sp of BC were 0.54 and 0.77, respectively. For teat skin samples, the Se and Sp of qPCR for *Strep. agalactiae* were estimated to be 0.97 and 0.96, respectively, whereas the Se and Sp of BC were 0.33 and 1.00, respectively. The Se and Sp of qPCR for *Staph. aureus* were estimated to 0.94 and 0.98, respectively, whereas the Se and Sp of BC were 0.44 and 0.74, respectively. In conclusion, the Se for diagnosing *Strep. agalactiae* and *Staph. aureus* IMI was higher for qPCR than BC, suggesting that qPCR is a valuable method for detecting both pathogens from quarter-level milk samples. The performance of BC in the detection of *Strep. agalactiae* and *Staph. aureus* on teat skin was poor compared to qPCR, indicating that differences in the target condition of the two methods should be considered when implementing them as routine diagnostic tests for detecting teat skin colonisers. The low Se of BC may preclude the use of BC for skin testing, and qPCR is better for this task.

1. Introduction

Intramammary infections (IMI) are a major economic and public health challenge in dairy herds (Keefe, 2012), and the use of antibiotics for mastitis treatment constitutes the majority of the total usage in Danish dairy herds (DANMAP, 2016). Therefore, effective control of mastitis is an important factor in reducing the risk of antimicrobial resistance.

The contagious udder pathogen *Staphylococcus aureus (Staph. aureus)* is widespread in dairy herds, and despite successful control

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efforts to reduce *Streptococcus agalactiae (Strep. agalactiae)* in Scandinavian countries during the 20th century (Katholm et al., 2012; Lyhs et al., 2016), the proportion of positive herds in Denmark and Norway increased throughout the early 21st century (Katholm et al., 2012; Mweu et al., 2012; Radtke et al., 2012). The estimated herd-level prevalence of *Staph. aureus* and *Strep. agalactiae* in Denmark, Germany, Belgium and Canada has been reported at 91% and 7% (Katholm et al., 2012), 90% and 29% (Tenhagen et al., 2006), 86% and 5.3% (Piepers et al., 2007), and 74% and 1.6% (Olde Riekerink et al., 2006), respectively.

Although both bacteria are considered contagious pathogens, environmental reservoirs have been described in the scientific literature (Haveri et al., 2008; Jørgensen et al., 2016). Klaas and Zadoks (2017) added that a faeco-oral transmission cycle may perpetuate and amplify the presence of *Strep. agalactiae* within dairy herds, but the importance of these environmental reservoirs is still being discussed. Furthermore, it has yet to be determined whether it is primarily milk that is contaminating the environment, or if colonisation of extramammary sites also leads to IMI.

Advances in the dairy industry have led to larger herds, and automatic milking systems (AMS) became more frequent in the Nordic countries in the early 21st century (Barkema et al., 2015). Milking hygiene and teat cleaning in AMS differ from the conventional milking system, with more cows per milking unit and no contact with human hands (Hovinen and Pyörälä, 2011; Rodenburg, 2017). This, together with the environmental reservoir of contagious mastitis pathogens, could explain why *Staph. aureus* remains a problem, and why there has been a re-emergence of *Strep. agalactiae* in line with an increase in the proportion of farms using AMS in Denmark (Bennedsgaard and Katholm, 2013).

Accurate diagnostic tests to detect pathogen-specific subclinical mastitis are essential in initiating appropriate control efforts (Barkema et al., 2006; van den Borne et al., 2010), e.g., separating infected from susceptible animals and establishing other measures to reduce the risk of spread within and between herds (Barkema et al., 2009). Bacterial culture (BC) has been considered the reference standard for identifying mastitis pathogens, but studies comparing the sensitivity (Se) and specificity (Sp) of real-time PCR and BC for diagnosing IMI with *Staph. aureus* or *Strep. agalactiae* in composite milk samples have suggested a higher Se for PCR compared to BC (Holmöy et al., 2018; Mahmmod et al., 2013a, 2013b). Furthermore, the bovine teat skin may be an important reservoir for contagious mastitis pathogens, as the presence of bacteria on teat skin has been associated with IMI in the same quarter and bacteria has been found on teat skin in quarters not having IMI, suggesting that colonisation or contamination of teat skin from sources other than milk of the same quarter is likely (da Costa et al., 2014; Svennesen et al., 2018). Furthermore, controlling *Staph. aureus* and *Strep. agalactiae* in large herds without considering the environmental reservoirs may lead to unsuccessful control and eradication. Therefore, PCR tests on teat skin or environmental samples could become a useful tool in controlling *Strep. agalactiae* and *Staph. aureus* mastitis.

The objective of this cross-sectional field study was to estimate the Se and Sp of the commercially available Mastit4 qPCR assay and BC for the identification of *Strep. agalactiae* and *Staph. aureus* in milk and teat skin samples from high somatic cell count (SCC) cows in AMS herds positive for *Strep. agalactiae*. In the absence of a reference test, a Bayesian latent class analysis (LCA) framework was used.

2. Materials and methods

We followed the guidelines for reporting diagnostic accuracy in studies that use Bayesian LCA (Kostoulas et al., 2017).

2.1. Study population

Eight dairy herds with Danish Holstein cows were selected for a project investigating the epidemiology and diagnostics of *Strep. agalactiae, Staph. aureus* and non-aureus staphylococci in Danish AMS herds. Eligible herds had ≥ 3 milking robots and a bulk tank milk PCR cycle threshold (Ct) value ≤ 32 for *Strep. agalactiae*. More herd characteristics are presented in Mahmmod et al. (2018). Samples were collected from the right rear quarters of 30 to 40 lactating dairy cows from each herd. These cows were randomly selected among those with a SCC > 200,000 cells/mL at the preceding milk recording, and with no clinical mastitis or antimicrobial treatment four weeks prior to sample collection.

2.2. Sample collection

The cows selected for sampling were separated by the farmer and restrained in headlocks during sampling. Before sampling, the cows followed their normal milking routine, meaning that the time since last milking varied from 30 min to approximately 12 h. All herds used post-milking teat disinfection in the AMS (Mahmmod et al., 2018).

The teats were cleaned with dry paper towels using at least one for each teat until they were visually clean. The teat skin samples were taken with the modified wet-dry method (Paduch and Kroemker, 2011) using a wet and a dry rayon swab (DaklaPack, Glostrup, Denmark) for each teat. The swabs were rolled 360° around the teat about 1 cm from the teat canal orifice and were then broken into a corresponding tube containing 2 mL of ¼ Ringer's solution (Merck, Darmstadt, Germany).

Quarter-level milk samples were collected directly after the teat skin swab samples, according to the National Mastitis Council guidelines (NMC, 1999). In brief, the teat end was disinfected with cotton swabs soaked in ethanol (70%). Individual quarter foremilk samples were then aseptically collected in sterile screw-cap plastic tubes. Latex gloves were worn and were changed after each cow and sampling procedure. Tubes containing the teat skin and milk samples were stored at a maximum of 5 °C and delivered to the microbiology laboratory within 24 h. All study activities including farm visits, collection of samples and laboratory examination were carried out between February and May 2017.

2.3. Bacterial culture

Milk samples were vortexed for 10 s, and 10 µL was streaked with a disposable calibrated loop onto a quarter of a plate of each a calf blood agar (5% sheep blood), a chromogenic agar selective for staphylococci (SA*Select*, Bio-Rad, Hercules, CA) and a modified Edward's medium (Oxoid, Roskilde, Denmark) supplemented with 5% calf blood and 2% filtrate of a β-toxin producing *Staph. aureus,* prepared as described by Jørgensen et al. (2016).

Teat skin samples were vortexed for 20 s, and the swabs were removed with a sterile pair of tweezers before 100 µL of the sample was inoculated and spread with a Drigalski spatula on a whole plate of calf blood agar, SA*Select* medium and modified Edward's medium. The plates were incubated aerobically at 37 °C for 48 h in total and read after 24 and 48 h. The approximate number of colony forming units (cfu) was determined by colony counting.

Colonies of *Strep. agalactiae* were phenotypically identified on blood agar and modified Edward's medium. Suspected colonies were confirmed as *Strep. agalactiae* using latex agglutination for Lancefield group B (PathoDxtra Strep Grouping Kit, ThermoFisher Scientific, Waltham, MA) or MALDI-TOF (Bruker Biotyper software system, Microflex LT, Bruker Daltonics GmbH, Bremen, Germany). *Staph. aureus* colonies were phenotypically identified on SA*Select* medium according to the manufacturer's guidelines, and on calf blood agar according to the National Mastitis Council recommendations (NMC, 1999). Suspected colony types were confirmed as *Staph. aureus* using MALDI-TOF. For BC, a quarter was defined as positive in milk or teat skin if at least one colony of *Staph. aureus* or *Strep. agalactiae* appeared on any of the used agar plates. This corresponded to a detection limit of 100 cfu/mL for

milk and 10 cfu/mL for teat skin samples.

2.4. qPCR assay

A FLOQswab (COPAN ITALIA spa, Brescia, Italy) was immersed in the original milk and teat skin samples immediately after streaking for BC. The principle of using these swabs were that they would dry out quickly, thus there was no need for cooling under transport. The swabs were shipped to the laboratory of DNA Diagnostic A/S on the same day that BC was performed, for analysis 1 or 2 days later.

The samples were tested using the Mastit4 qPCR assay (DNA Diagnostic, Risskov, Denmark). The personnel at the laboratory were blinded to the samples and results of the BC. Ct values were recorded for each sample, and samples were defined as positive for *Staph. aureus* or *Strep. agalactiae* if the Ct value was $\leq$ 37.

The approximate volume soaked by the FLOQSwab was 220 µl, and after DNA extraction and purification steps, this left 18 µl of the original sample for qPCR analysis, corresponding to 60% of the 3 × 10 µl spread on plates for milk samples and 6% of the 3 × 100 µl inoculated on plates for teat skin samples.

2.5. Statistical analysis

In the absence of a reference standard to classify true cases of IMI and teat skin colonisation with *Strep. agalactiae* and *Staph. aureus*, the test characteristics (Se and Sp) of qPCR and BC were estimated using a Bayesian LCA model (Branscum et al., 2005) based on the paradigm described by Hui and Walter (1980).

The study population was divided into two subpopulations based on robot type used in the herds from which the cows originated. Robot type could be considered a risk factor, thus different herd prevalences were expected. Priors for *Staph. aureus* were used based on the results from Mahmmod et al. (2013a), and we considered these to be informative despite them being based on composite milk samples and the PCR test being from another manufacturer. Priors for *Strep. agalactiae* (Se and Sp of BC and PCR) were based on the results from Holmøy et al. (2018), who used the same PCR test, but composite milk samples. No prior information on the diagnostic performance of BC or qPCR on teat skin samples was available, so non-informative priors and the priors from milk were used. All priors are given in Table 1. For non-informative priors, a Beta(1,1) distribution was used. For informative priors, the particular prior distribution was created based on the median and the 95% posterior credibility interval (PCI) reported in the original publications with the Beta-distributions shown in Table 1.

The model was implemented in the freeware program OpenBUGS, version 3.2.3, rev. 1012 (Thomas et al., 2006). OpenBUGS uses a Markov Chain Monte Carlo (MCMC) sampling algorithm to obtain a Monte Carlo (MC) sample from the posterior distribution. Three chains were run and the first 10,000 MC samples were discarded as a burn-in to allow convergence, and the following 20,000 iterations were used for posterior inference. Convergence of the MCMC chain after the initial burn-in period was assessed by visual inspection of the time-series plots of the chains. Posterior inference was based on median and 95% PCI for the Se and Sp of the two tests, where the PCI was constructed based on the percentiles of the posterior distributions. An example of the Open Bugs code is available in Appendix I.

Table 1
Priors used for analysis: Median and 95% posterior credible interval (PCI) for sensitivity (SeqPCR) and specificity (SpqPCR) estimates for qPCR and sensitivity (SeBC) and specifity (SpBC) estimates for bacterial culture for *Streptococcus agalactiae* (Holmøy et al., 2018) and *Staphylococcus aureus* (Mahmmod et al., 2013a) in composite milk samples.

Pathogen	Parameter	Test estimates		Probability distribution
		Median	95% PCI	
Strep. agalactiae	SeqPCR	0.93	0.78 - 1.00	Beta(16.3; 1.2)
	SeBC	0.39	0.32 - 0.47	Beta(78; 121)
	SpqPCR	0.99	0.95 - 1.00	Beta(94; 1.46)
	SpBC	1.00	0.99 - 1.00	Beta(365; 1.36)
Staph. aureus	SeqPCR	0.91	0.74 - 1.00	Beta(14.3; 1.55)
	SeBC	0.52	0.44 - 0.61	Beta(72; 67)
	SpqPCR	0.99	0.94 - 1.00	Beta(71; 1.35)
	SpBC	0.90	0.86 - 0.94	Beta(138; 114)

3. Results

In total, 287 quarters with complete observations for qPCR and BC from milk and teat skin samples were used for the LCA analysis. Results of cross-tabulation (contingency table) of the dichotomous outcome of qPCR and BC for the detection of *Strep. agalactiae* and *Staph. aureus* from Population 1 (robot type 1) and Population 2 (robot type 2) are displayed in Table 2. Estimates of the posterior median and 95% PCI of Se and Sp of qPCR and BC for the detection of *Strep. agalactiae* and *Staph. aureus* are displayed in Table 3.

For milk, the posterior median Se estimates for *Strep. agalactiae* were 0.97 and 0.41 for qPCR and BC, respectively, with PCI as shown in Table 3. The corresponding Sp estimates were 0.99 and 1.00, using the results from the analysis with informative priors. For *Staph. aureus*, the median Se estimates were 0.95 and 0.54 for qPCR and BC, respectively, with corresponding Sp estimates of 0.99 and 0.77. For teat skin, Se for *Strep. agalactiae* were 0.97 for qPCR and 0.33 for BC, and the corresponding Sp estimates were high (0.96 and 1.00). For *Staph. aureus*, Se estimates were 0.94 and 0.44 for qPCR and BC, respectively, with corresponding Sp estimates of 0.98 and 0.74. The sensitivity analyses demonstrated that most estimates were relatively unaffected by the choice of priors, except for the sensitivity of BC, which appeared to be affected to some extent (Table 3).

4. Discussion

This study estimated the test accuracy of BC and the commercially available Mastit4 qPCR assay using LCA, which does not require a perfect reference test. To our knowledge, this is the first study to evaluate BC and qPCR assays on quarter-level milk and teat skin samples for detection of *Staph. aureus* and *Strep. agalactiae*.

In general, we found a higher Se of qPCR compared to BC across pathogen and sample type. The Sp of BC and qPCR were at the same level for *Strep. agalactiae*, but for *Staph. aureus* the Sp of BC was lower than for qPCR.

The considerable differences in the performance of BC and qPCR is probably due to that the two tests have different target conditions; viable bacteria vs. bacterial DNA, which subsequently could affect the test performance. This particular aspect should be considered for clinical application, interpretation, and future investigation.

4.1. Estimates of qPCR and BC for IMI

The higher Se of qPCR compared to BC is in line with the general perception that qPCR is more sensitive than BC (Holmøy et al., 2018; Mahmmod et al., 2013a, 2013b; Nyman et al., 2016).

In the current study, the Sp of BC and qPCR were comparable for *Strep. agalactiae*, but for *Staph. aureus*, the Sp of BC was lower than for qPCR. Holmøy et al. (2018) also reported similar Sp estimates for BC and qPCR for *Strep. agalactiae*, and Mahmmod et al. (2013a, 2013b) reported lower Sp of BC compared to qPCR for *Staph. aureus*, but also for *Strep. agalactiae*, whereas Nyman et al. (2016) and Cederlöf et al. (2012) reported comparable estimates for the Sp of BC and qPCR for *Staph. aureus*.

These differences could be explained by the different sampling and laboratory procedures. In the current study, we evaluated both qPCR and BC on the same aseptic quarter-level milk sample, whereas previous

Table 2
Cross-tabulated results for combinations of qPCR at a Ct value cut-off ≤ 37 and bacterial culture (BC) at a cut-off ≥ 1 cfu for identification of *Streptococcus agalactiae* and *Staphylococcus aureus* from 287 quarter-level milk and teat skin samples collected from eight Danish dairy herds with AMS, stratified based on robot type (Population 1 = 6 herds, Population 2 = 2 herds).

Sample	Population	Pathogen	Test combinations (T1; qPCR and T2; BC)				Total
			T1 + /T2 +	T1 + /T2-	T1-/T2 +	T1-/T2-	
Milk	Population 1	*Strep. agalactiae*	10	20	0	184	214
	(Robot type 1)	*Staph. aureus*	7	5	1	201	214
	Population 2 (Robot type 2)	*Strep. agalactiae*	9	1	0	63	73
		Staph. aureus	12	5	2	54	73
Teat skin	Population 1	*Strep. agalactiae*	1	47	0	166	214
	(Robot type 1)	*Staph. aureus*	5	35	8	166	214
	Population 2 (Robot type 2)	*Strep. agalactiae*	0	3	0	70	73
		Staph. aureus	2	0	8	63	73

Table 3
Posterior median and 95% posterior credible interval (PCI) of sensitivity (SeqPCR) and specificity (SpqPCR) for Mastit4 qPCR assay at a Ct value cut-off ≤ 37, and sensitivity (SeBC) and specificity (SpBC) for bacterial culture at a cut-off of ≥ 1 cfu of *Streptococcus agalactiae* and *Staphylococcus aureus* in quarter-level milk and teat skin samples from 287 cows in eight Danish dairy herds with AMS.

Sample	Pathogen	Parameter	Test estimates Informative priors[1]		Test estimates Non-informative priors	
			Median	95% PCI	Median	95% PCI
Milk	*Strep. agalactiae*	SeqPCR	0.97	0.88 – 1.00	0.96	0.82 – 1.00
		SeBC	0.41	0.35 – 0.47	0.82	0.44 – 0.99
		SpqPCR	0.99	0.97 – 1.00	0.93	0.89 – 0.99
		SpBC	1.00	0.99 – 1.00	1.00	0.99 – 1.00
	Staph. aureus	SeqPCR	0.95	0.82 – 1.00	0.88	0.68 – 0.99
		SeBC	0.54	0.46 – 0.62	0.74	0.52 – 0.96
		SpqPCR	0.99	0.96 – 1.00	0.98	0.95 – 1.00
		SpBC	0.77	0.73 – 0.81	0.99	0.98 – 1.00
Teat skin	*Strep. agalactiae*	SeqPCR	0.97	0.87 – 1.00	0.23	0.0041 – 0.95
		SeBC	0.33	0.27 - 0.41	0.0092	0.00030 – 0.071
		SpqPCR	0.96	0.89 – 1.00	0.91	0.06 – 1.00
		SpBC	1.00	0.99 – 1.00	0.99	0.93 – 1.00
	Staph. aureus	SeqPCR	0.94	0.80 – 0.99	0.077	0.0022 – 0.90
		SeBC	0.44	0.36 – 0.52	0.087	0.030 – 0.26
		SpqPCR	0.98	0.94 – 1.00	0.82	0.08 – 1.00
		SpBC	0.74	0.70 – 0.78	0.91	0.72 – 0.97

[1] The used priors are shown in Table 1.

studies (Cederlöf et al., 2012; Mahmmod et al., 2013a, 2013b; Nyman et al., 2016) evaluated a different PCR assay (PathoProof Mastitis PCR assay) on non-aseptically collected composite milk samples and compared this to results from BC on aseptic quarter-level milk samples.

Koskinen et al. (2009) estimated the analytical Se and Sp of the PathoProof Mastitis PCR assay at 100% for identifying bacteria from isolates originating from bovine mastitis. Rattenborg et al. (2015) found moderate-to-high agreement between the PathoProof Mastitis PCR assay and Mastit4 qPCR assay for *Strep. agalactiae* in bulk tank milk samples, whereas the agreement for *Staph. aureus* was moderate (Ct value cut-off ≤ 37).

Using different samples for the two tests increases the risk of not having the same concentration of bacteria (bacterial load), and variation in shedding has been demonstrated for both *Staph. aureus* and *Strep. agalactiae* (Thieme and Haasmann, 1978; Sears et al., 1990). Furthermore, non-aseptically collected milk samples may increase the number of false positive samples due to contamination and carryover (Mahmmod et al., 2017), and evaluating BC on quarter-level against PCR on composite milk samples could also decrease Se of PCR due to dilution of the sample (unless all four quarters are infected). Furthermore, the selection of cows may influence the results, especially the prevalence in the investigated population. We selected cows with SCC > 200,000 cells/mL which may not only increase the frequency of IMI, but also the test performance due to an increased chance of a high concentration of bacteria in IMI quarters with an active infection. This could have led to the higher Sp of PCR, where non-viable bacteria have a relatively minor influence compared to a setup with random selected cows.

Our estimates for *Strep. agalactiae* in milk samples fell within the range reported by Mahmmod et al. (2013b) and Holmöy et al. (2018). For *Staph. aureus,* our estimates fell within the range reported by Mahmmod et al. (2013a), while our estimates of Se and Sp for BC were lower (0.54 and 0.77, respectively) than those reported by Cederlöf et al. (2012; 0.83 and 0.97, respectively). This may be explained by the use of results from Mahmmod et al. (2013a) as informative priors, which seem to highly influence the estimates because of less robust estimates due to the relatively small sample size and low number of positive BC test results (Table 2).

4.2. Estimates of qPCR and BC for teat skin colonisation

As there were no priors for teat skin samples available, the current teat skin results may serve as priors for future studies. Like for milk samples, the estimates were highly influenced by the informative priors used, primarily increasing the Se estimates of both BC and qPCR (Table 3).

The Sp estimates were generally high, except the Sp of BC for *Staph. aureus* (informative priors). Using non-informative priors resulted in very low Se of both BC and qPCR. When informative priors were used, the Se increased and the Se of qPCR was significantly higher than the Se

of BC, even though the amount of original sample material for qPCR analysis made up only 6% of that for BC. Previous studies used BC to detect teat skin colonisation with *Staph. aureus* (Haveri et al., 2008; da Costa et al., 2014), but overgrowth with other bacteria could challenge the Se of BC. In contrast, by using BC we ensure that the pathogens detected are potentially capable of colonising the teat skin, whereas qPCR could detect non-viable bacterial cells (Koskinen et al., 2009; Mahmmod et al., 2013b; Holmöy et al., 2018), e.g. contamination or flora of the teat skin inactivated by post-milking teat disinfection. Detection of non-viable bacteria with qPCR and a low Se of BC could explain the lower number of positive teat skin samples detected by BC for both *Staph. aureus* and *Strep. agalactiae* compared to those detected by qPCR (Table 2). However, according to the manufacturer, in the Mastit4 qPCR assay the extraction step includes that the bacteria are centrifuged to a pellet two times before the lysis procedure. This ensures that the test will not detect free DNA, meaning that the test result is only influenced to small extent by DNA from dead bacteria for which the bacteria membrane is still intact. Furthermore, PCR methods that enable distinction between live and dead bacteria have been developed (Nocker et al., 2007) and such method could be applied to environmental and teat skin samples. Result would help ruling out the issue of whether bacteria colonize or merely contaminate teat skin.

Teat skin colonisation has been associated with IMI (Haveri et al., 2008; da Costa et al., 2014; Svennesen et al., 2018), and environmental reservoirs could easily colonise the teat skin (Haveri et al., 2008; Jørgensen et al., 2016). However, the load of bacteria necessary to cause an infection is not known, and the clinical relevance of a low concentration of possibly non-viable bacteria detected by qPCR on teat skin is hard to estimate. Furthermore, as we collected samples from cows at different times since last milking, some of our samples may be more affected by post-milking teat disinfection (cows sampled just after milking), and others more by the environmental reservoir of bacteria (cows laying in cubicles before sampling). It is therefore generally difficult to assess whether it is teat skin colonisation or contamination detected in these types of samples, and the choice of test (high or low Se and Sp) should depend on the goal of the sampling.

A lower Ct value cut-off could increase Sp of the qPCR test by increasing the detection limit and not consider very low concentration of bacteria, e.g. contamination, as positive test results. However, as the Sp of qPCR for teat skin is relatively high [0.96 for *Strep. agalactiae* and 0.98 for *Staph. aureus* (informative priors)], it is unlikely that setting a lower threshold would make a substantial difference.

4.3. Model assumptions

Firstly, variation in prevalence between populations is fundamental to LCA models (Kostoulas et al., 2017; Toft et al., 2005). In this study, we used robot type to create populations with different prevalences, and the assumption was verified, as posterior estimates of prevalence were different (data not shown). Secondly, the test characteristics (Se and Sp) should be constant across the tested populations, which was the case as robot type would not affect test characteristics. Thirdly, there should be independence of tests given the target condition (i.e. the presence of pathogen or parts of the pathogen); this assumption was fulfilled because qPCR and BC have different biological identification mechanisms and no culturing was involved in the qPCR procedure.

5. Conclusions

The Se for diagnosing IMI with *Strep. agalactiae* and *Staph. aureus* was higher using qPCR compared to BC. This suggests that qPCR is a valuable method for detecting both pathogens from quarter-level milk samples. For testing teat skin samples qPCR also has potential due to the higher Se in the detection of *Strep. agalactiae* and *Staph. aureus* and higher Sp for *Staph. aureus*. However, the clinical importance of the findings in teat skin samples with the two different tests must be carefully evaluated, and further studies are required to reduce the uncertainty.

Declaration of interests

Our co-author Jørgen Katholm is affiliated with DNA Diagnostic A/S, which provided us with the PCR swabs and ran the qPCR analyses in their laboratory. We confirm that the laboratory personnel were blinded to the sample identification and results of bacterial culture, and that the company had no impact on the data handling or statistical analysis. Therefore, DNA Diagnostic A/S could not bias the contents of this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prevetmed.2018.10.013.

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