Estimation of the performance of two real-time polymerase chain reaction assays for detection of *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae* in pooled milk samples in a field study

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ABSTRACT

The early detection of major mastitis pathogens is crucial for the udder health management of dairy herds. Testing of pooled milk samples, either individual test-day cow samples (TDCS) or aseptically collected pre-milk quarter samples (PMQS) may provide an easy to use and cost-effective group level screening tool. Therefore, the aim of this study was (1) to evaluate the sensitivity (Se) and specificity (Sp) of 2 commercial multiplex real-time PCR test kits applied to pooled milk samples using a Bayesian latent class analysis and (2) to estimate the probability of detection in relation to the pool size and the number of cows positively tested by bacteriological culture (BC) within a pool. Pools of 10, 20 and 50 cows were assembled from 1,912 test-day samples and 7,336 PMQS collected from a total of 2,045 cows from 2 commercial dairy farms. Two commercial quantitative real-time PCR kits were applied to detect *Staphylococcus aureus*, *Streptococcus* agalactiae, and *Streptococcus dysgalactiae* in the pooled samples, and a BC was applied to PMQS yielding a cumulative pool result. A pool was considered BC-positive if it contained at least one BC-positive PMQS. Pathogens were more frequently detected in the PMQS pools than in the TDCS pools. Pools of 10 cows showed the highest probability of detection irrespective of sample type or type of PCR kit compared with larger pool sizes. Estimation with a Bayesian latent class analysis resulted in a median Se in PMQS pools of 10 cows for *Staph. aureus* of 63.3% for PCR kit I, 78.1% for PCR kit II, and 95.5% for BC; the Sp values were 97.0%, 97.6%, and 89.1%, respectively. The estimated median Se for *Strep.* species for PCR kits ranged between 77.5 and 85.6% and for BC between 73.7% and 79.2%; the median Sp values ranged between 93.6 and 99.2% for PCR kits, and between 96.9% and 97.4% for BC. In addition, the probability of detection increased with an increasing number of BC-positive cows per pool. To achieve a probability of detection of 90%, the estimated number of positive cows in PMQS pools of 10 cows for kit I was 4.1 for *Staph. aureus*, 1.5 for *Strep. agalactiae*, and 1.3 for *Strep. dysgalactiae*; for the equivalent TDCS pools and pathogens, 6.9, 1.9, and 2.0 positive cows were required, respectively. For Kit II and PMQS pools, the number of positive cows required was 2.8 for *Staph. aureus*, 1.4 for *Strep. agalactiae*, and 1.2 for *Strep. dysgalactiae*; for the equivalent TDCS pools and pathogens, 5.3, 1.8, and 2.0 positive cows were required, respectively. In conclusion, the type of samples used for pooling, the pool size and the number of infected cows per pool determine the probability of detecting an infection with major mastitis pathogens within a pool by PCR testing.

Key words: Bayesian latent class analysis, major mastitis pathogens, pooled test-day milk samples, probability of detection

INTRODUCTION

Excellent udder health and associated good quality of raw milk are crucial both for high-quality dairy products and health and welfare on dairy farms (Petersson-Wolfe et al., 2018), as well as economics (Hogeveen et al., 2011). The economic loss of mastitis is estimated to be EUR 124 per cow per year (Hogeveen et al., 2019). Therefore, monitoring of milk quality is necessary for the individual cow and at the herd level for adequate control of mastitis. Traditionally, in nonrobotic systems, this is realized by the manual examination of foremilk and supported by the close human-cow contact these systems entail. In farms with automatic milking systems (**AMS**), daily manual control of the condition of the milk, the udder and the cow by pre-milking is not

conducted. Instead, a selection of different parameters (e.g., milk yield, milk flow rate, electrical conductivity of milk, and estimation of SCC) are recorded within the AMS and interpreted to provide an indication of impaired udder health or reduced marketability of the milk (Zucali et al., 2021). Space within the milking station of an AMS and external access to the udder is limited, thus making the manual collection of sterile pre-milk quarter samples (**PMQS**) difficult. However, bacteriological culture (**BC**) of such samples is the standard method for diagnosing the causative agent of mastitis (Zucali et al., 2021), knowledge of which is important for effective mastitis management and therapy at both the individual and herd level (Ruegg, 2017). Therefore, farms with AMS need monitoring systems which will allow early detection of mastitis pathogens and provide diagnostic test results on a regular basis to detect dominant organisms and shifts in the prevalence of mastitis pathogens in the herd.

Testing milk samples by applying molecular methods instead of cultural methods would allow the use of non-sterile milk samples, due to the targeted detection of specific gene segments. Additionally, molecular methods can detect low shedding cycles of pathogens due to subclinical infections or the presence of substances or cells inhibiting the growth of bacteria in a culture (Phuektes et al., 2001). However, the relevance of the detection of DNA fragments from nonviable pathogens via molecular methods remains unclear. Regarding the sensitivity (**Se**) of the diagnostic test, (multiplex) real-time PCR showed comparable results to BC of PMQS (Phuektes et al., 2001; Koskinen et al., 2010; Spittel and Hoedemaker, 2012). For *Streptococcus agalactiae*, a study reported increased Se for PCR (73.9%–96.2%) compared with BC (25.7%–72.1%) in PMQS depending on the cycle threshold value (**Ct-value**; Mahmmod et al., 2013b). In addition, Sp varied between 96.8 and 97.2 for PCR and 98.9% and 99.7% for BC. To facilitate cost-effective use in commercial dairy herds, a useful approach could be real-time PCR applied to pooled milk samples or bulk milk samples, resulting in a recommendation for the monitoring of the pathogens at herd level (Phuektes et al., 2003; Mweu et al., 2012; Syring et al., 2012; Soltau et al., 2017). In herds enrolled in dairy herd improvement programs, test-day cow samples (**TDCS**) are collected as a composite sample of each quarter for milk recording on a regular basis (e.g., monthly). Regarding *Staphylococcus aureus*, a previous study compared multiplex real-time PCR of TDCS and BC of PMQS using latent class analysis. The authors reported no significant differences in the Se and Sp for PCR and for BC (Cederlöf et al., 2012). In a further study, BC of PMQS was used as a reference method to estimate within-herd prevalence thresholds

of PCR in bulk milk samples testing for major mastitis pathogens in a different study, resulting in an overall diagnostic Se of 83.3% for *Staph. aureus* and 87.5% for *Streptococcus dysgalactiae* (Soltau et al., 2017). The aim of this study was (1) to evaluate the Se and Sp of 2 commercially available multiplex real-time PCR test kits applied to pooled milk samples using a Bayesian latent class analysis and (2) to estimate the probability of detection by PCR in pooled milk samples in relation to the number of BC-positive cows within a pool and the pool size.

MATERIALS AND METHODS

Study Population and Sampling

Two commercial dairy farms situated in Thuringia, a federal state of Germany, both comprising mainly German Holstein dairy cows, were included in the study. Both participated in the Animal Health Program of the Thuringian Animal Health Service. The program involves whole-herd BC testing of PMQS of all lactating cows for mastitis pathogens at regular intervals. In 2018, the prevalence of IMI caused by *Staph. aureus*, *Strep. agalactiae*, or *Strep. dysgalactiae* of all milk samples examined via BC in our laboratory was 15.62% in farm A and 19.15% in farm B. This is comparable to the average for all farms in Thuringia sending milk samples, which is 16.62%. In addition, the prevalence of IMI from all mastitis pathogens was lower both in farm A (34.21%) and in farm B (37.37%) compared with the average for all farms (43.93%). The farms were also enrolled in dairy herd improvement programs and collected TDCS for milk recording at monthly intervals.

The Thuringian State Office for Consumer Protection, which is the authority responsible for research ethics approval in Thuringia, approved the project and granted a formal waiver of the need for animal-use approval because the study was part of the official Cattle Health Monitoring Program in Thuringian Cattle Herds (2684–04–15-TSK-21–103). Every effort was made to minimize discomfort during milk sampling.

In the spring of 2018, herd sampling for the collection of PMQS was conducted in both farms and all lactating cows were included. This resulted in the sampling of 928 of 1,046 cows in total at farm A and 984 of 1,099 cows in total at farm B. Cows at 1 to 5 DIM (n = 17) and cows with clinical mastitis (n = 37), including cows being medicinally treated until the end of the withdrawal period, were excluded from the study. Only clinically healthy cows were included in the study. Both farms kept their cows in free stalls and operated a rotary milking parlor. Aseptic PMQS of each cow were collected for BC during the daily milking routine

within 12 h at each farm. In accordance with to the "Guidelines for Aseptic Collection of Milk Samples and for Isolation and Identification of Mastitis Pathogens" of the German Veterinary Association (DVG, 2009), after pre-milking and cleaning of the teats and especially the tips, an alcoholic disinfection of the teat tip using hygienic wipes was performed. Each sample (8–10 mL) was hand-collected into a sterile tube containing boric acid as a preservative agent. After collection, all samples were immediately cooled and transported to the laboratory of the Animal Health Service, Thuringian Animal Diseases Fund of the State of Thuringia in Jena, within 48 h. The laboratory is an accredited veterinary test laboratory under license of the German Accreditation Body according to the quality standards of the German and European Standard DIN EN ISO 17025.

By scheduling the test-day for milk recording the day after PMQS sampling in the respective month, TDCS were collected within 24 to 48 h after PMQS. Using mobile milk meters, one TDCS consisting of a composite of milk from all 4 quarters was collected automatically from each cow at milking in accordance with the regulations for dairy herd improvement milk recordings. The samples (40–50 mL) were milked into a sterile tube containing bronopol as a preservative agent. All samples were transported to the laboratory of the Qnetics GmbH in Jena, which performs the laboratory tests for milk contents on behalf of the Thuringian Cattle Breeders Association.

Laboratory Testing

BC of PMQS was performed according to the Guidelines of the German Veterinary Association (DVG, 2009, 2018). The PMQS were examined via cultivation on esculin blood agar plates modified with 7% sheep blood (Thermo Fisher Diagnostic GmbH, Hennigsdorf) and on Sabouraud glucose agar (Thermo Fisher Diagnostic GmbH, Hennigsdorf) for 48 h. Using a glass bar, 0.01 mL of each milk sample was spread on each plate. To increase the Se of BC in regard to *Strep. agalactiae*, and *Strep. dysgalactiae*, PMQS were enriched in glucose bouillon for 24 h and subsequently cultivated on additional blood agar plates for 24 h. After each incubation period, an evaluation of colony morphology and hemolysis took place. For differentiation of streptococci and staphylococci, a catalase test was used. *Staph. aureus* was diagnosed by plasma coagulase test with rabbit plasma (Remel, Lenexa). Streptococci were differentiated using esculin conversion, Lancefield classification via antigen test and Christie, Atkins, Munch-Peterson reaction. When one or more colonies of *Staph. aureus*, *Strep. agalactiae*, or *Strep. dysgalactiae* in pure or mixed culture were found either in the primary culture or in

the culture from the enrichment broth, the culture was considered positive. The detection limit was approximately 100 colony-forming units (**cfu**) per mL because about 0.01 mL of milk was inoculated. After the first evaluation after 24 h of incubation, the examiner assessed the degree of contamination flora on the plates as a "cleanness" level. Milk samples were considered to be contaminated if they contained 3 or more pathogens. Both pathogens were considered for the study if one sample was detected with 2 mastitis pathogens.

After BC, PMQS were pooled for each cow (2 mL per quarter), yielding pooled pre-milk cow samples and BC results at cow level. Samples from cows with 3 lactating quarters were treated identically. Following that, pooled pre-milk cow samples and TDCS were processed in the same way, yielding pools from 10 cows containing 0.75 mL from each cow. Pools from 20 cows were created by assembling 2.5 mL each from 2 10-cow pools, and pools from 50 cows contained 1.0 mL each from 5 10-cow pools (Figure 1). A pool of PMQS contained milk from the same cows as the TDCS pools. The PMQS pools were considered BC-positive if they contained at least one BC-positive PMQS. Extraction of DNA from pooled samples was performed once using a DNeasy® Mastitis Mini kit (Qiagen, Hilden, Germany). Subsequently, the commercial multiplex bactotype HP3 real-time PCR kit (**kit I**, Indical bioscience, Leipzig, Germany) and the multiplex Mastit 4 real-time PCR kit (**Kit II**, DNA Diagnostic, Risskov, Denmark) were applied to identify *Staph. aureus*, *Strep. agalactiae*, and *Strep. dysgalactiae* in PMQS and TDCS pools. According to the manufacturer's specifications, kit I considered samples pathogen-positive at a Ct-value ≤40 and Kit II at a Ct-value ≤37.

Due to the high risk of environmental contamination, especially in TDCS, *Strep. uberis* and minor mastitis pathogens were excluded from the study.

Statistical Data Analysis

Data recording and editing were performed using a Microsoft Excel spreadsheet (Microsoft Corporation). All further statistical analyses including descriptive statistics and prevalence computation were made with R version 3.6.2 (2019–12–12; RCoreTeam, 2019).

Estimation of Test Performance. For the estimation of the test performance of BC and PCR we used a latent class model implemented in Just Another Gibbs Sampler and executed in R using the package *runjags* (version 2.2.1–7; Plummer, 2003). We aimed at detecting PMQS from infected cattle, which are represented by the latent class. Therefore, 10 variables each, including the Se and Sp of all 3 test methods, the prevalence of both populations, and the covariances in
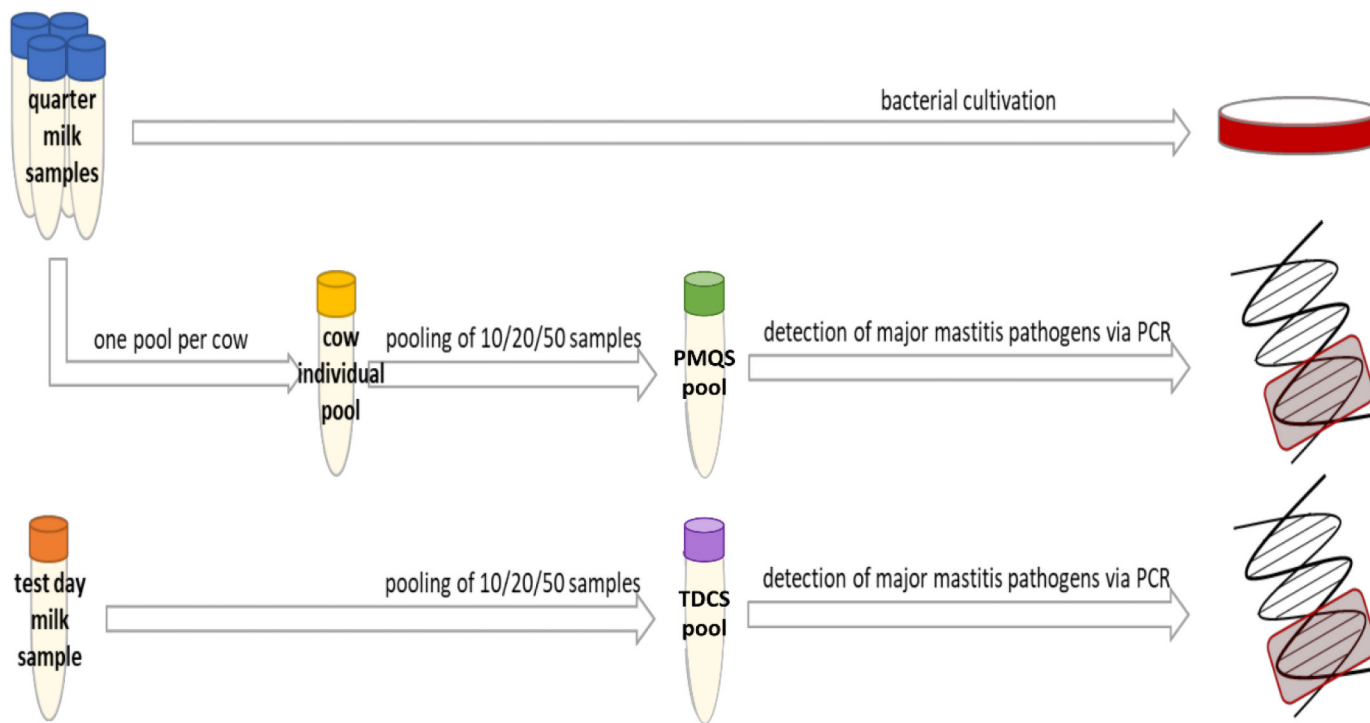
Figure 1. Experimental design of the study to compare the diagnostic sensitivity and specificity of 2 commercial multiplex real-time PCR test kits applied on pooled milk samples of different sample types and BC applied on individual quarter milk samples yielding in a cumulative pool result. PMQS = pre-milk quarter samples for bacteriological culture; TDCS = test-day cow samples.

Se and Sp of PCR kits, were estimated separately for each pathogen, yielding 3 separate Bayesian latent class models. For identifiability of the model, 2 populations were generated with differing prevalence via random sampling. In the model, we assumed that the results of each of the PCR tests were conditionally dependent but conditionally independent of BC, because of the different methodology detecting DNA fragments versus viable bacteria. In addition, a median Se of 0.798 (CI 0.409–0.980) and a median Sp of 0.962 (CI 0.857–0.996) were added as weakly informative priors both for all test methods as well as all tested mastitis pathogens. Two chains were run in parallel for 100,000 iterations in total, excluding a burn-in period of 5,000 samples. Model convergence was ensured by monitoring trace plots, effective sample size and potential scale reduction factor (cut-off 1.05). Additionally, the Se and Sp of PCR Kits I and II were calculated using BC as a reference method.

Estimation of Probability of Detection Using Pooled Samples. To predict the probability of detection depending on 2 independent variables, the number of BC-positive cow-level milk samples (integer variable) and the size of the pooled milk samples (grouping variable), logistic regression models using the maximum likelihood method were fitted. Therefore, for each pathogen, 4 different models (for each PCR kit and each sample type) were calculated in relation to pool size, respecting all assumptions: independence of errors, linearity in the logit, absence of multicollinearity and lack of strongly influential outliers (Stoltzfus, 2011). Subsequently, probabilities of detection were compared formally concerning sample type, number of cows contributing to the pool, number of BC-positive cows per pool and PCR kit. Logistic regressions including their graphical presentation were performed using the packages *lme4* (version 1.1–2) and *ggiraph* (version 0.7.0).

A statistical significance level of $\alpha = 0.05$ was used.

RESULTS

A total of 7,336 aseptically collected PMQS and 1,912 cow-level TDCS were included in the analysis. The number of cows with 3 lactating quarters was 312. Using bacterial culture on PMQS resulted in 176 (9.4%) *Staph. aureus*-positive, 34 (1.7%) *Strep. agalactiae*-positive, and 35 (1.7%) *Strep. dysgalactiae*-positive PMQS. On average, 27.8% of the 3 diagnosed pathogens were only detected after enrichment in glucose bouillon. Two pathogens were detected in 7 PMQS and 3 PMQS were contaminated. On the individual cow level, this corresponded to 158 (8.3%) cows being posi-

tive for *Staph. aureus*, 31 (1.6%) for *Strep. agalactiae*, and 32 (1.7%) for *Strep. dysgalactiae*. Pooling of cow-level samples was undertaken separately for both herds leading to 192 pools of 10, 97 pools of 20 and 39 pools of 50 individual cows of each sample type. A pool was considered BC-positive if it contained at least one BC-positive PMQS or cow sample. If a PMQS was positive for 2 pathogens, both results were considered. For pools of 10, this resulted in 92 (47.9%), 29 (15.1%), and 32 (16.7%) BC-positive pools for *Staph. aureus*, *Strep. agalactiae*, and *Strep. dysgalactiae*, respectively. The numbers of BC-positive pools of 20 were 71 (73.2%), 27 (27.8%), and 29 (29.9%) for *Staph. aureus*, *Strep. agalactiae*, and *Strep. dysgalactiae*, respectively. For pools of 50, the number of BC-positive pools were 38 (97.4%), 19 (48.7%), and 23 (59.0%) for *Staph. aureus*, *Strep. agalactiae*, and *Strep. dysgalactiae*, respectively.

Estimation of Test Performance

PCR using kit I detected *Staph. aureus* in 96 PMQS and 89 TDCS pools, *Strep. agalactiae* in 47 PMQS and 26 TDCS pools, and *Strep. dysgalactiae* in 78 PMQS and 57 TDCS pools (Table 1). Using PCR kit II, *Staph. aureus* was identified in 109 PMQS and 92 TDCS pools, *Strep. agalactiae* in 57 PMQS and 35 TDCS pools, and *Strep. dysgalactiae* in 66 PMQS and 45 TDCS pools. In 2 cases, PCR was not evaluable for one pathogen; therefore, only results of the other pathogens were accepted (Table 1). For both PCR kits, pathogens were detected more frequently in the PMQS pools than in the TDCS pools. The numerical distribution of PMQS and TDCS pools of size 10 that were positive or negative when tested with PCR kit I or Kit II in comparison to the number of BC-positive cows per pool is shown in Table 2.

BC of quarter milk samples resulted in a high number of pathogen-positive samples, leading to a higher number of pools of size 10 including BC-positive samples than any PCR kit detected, regardless of pool size, sample type or pathogen. There is only one exception: from the total of 192 tested PMQS pools of size 10, PCR kit I identified *Strep. dysgalactiae* in 42 (21.9%) and PCR kit II in 27 (18.8%), whereas 32 (16.7%) pools were BC-positive. Regarding test performance for *Staph. aureus* in PMQS pools of 10 cows, the estimation based on a Bayesian latent class analysis comparing BC and PCR resulted in a better Se for BC and a better Sp for PCR (Table 3). Estimated values for median Se for *Strep. agalactiae* and *Strep. dysgalactiae* in PMQS pools of size 10 were similar for BC and PCR. In addition, Sp values for *Strep.* species were similar for BC and PCR (Figure 2 and 3). All models reached convergence as the potential scale reduction factor varied between 1.000 and 1.001. The models were identifiable due to the positive number of degrees of freedom and the additional influence of the priors.

When using BC as a reference method, the calculation of the Se of PCR resulted in decreased values for both kits and all pathogens compared with the estimation via Bayesian latent class analysis (Supplemental Material S1, https://doi.org/10.6084/m9.figshare.23584959.v1; Klassen, 2023). However, calculation of the Sp of PCR using BC as a reference method gave results similar to values estimated via Bayesian latent class analysis for both kits and concerning all pathogens.

Estimation of Probability of Detection Using Pooled Samples

The results show an increasing probability of detection with an increasing number of BC-positive cows per pool. However, there is one exception: the detection of *Staph. aureus* with PCR kit II in pooled TDCS of pool size 50. The regression model predicted that the probability of detection with PCR would increase more rapidly for smaller pool sizes than for larger pool sizes and would increase more rapidly for PMQS pools than for TDCS pools (Figures 4–6).

The estimated number of positive cows within a sample to achieve a given level of probability of detection varied between the pathogens, whether the pool comprised PMQS or TDCS and also with the size of the pool (Table 4).

For PCR kit I, to achieve a probability of detection of 90% in a TDCS pool of 10 cows, 6.9 BC-positive cows for *Staph. aureus* were required, whereas only 1.9 and 2.0 BC-positive cows with *Strep. agalactiae* and *Strep. dysgalactiae*, respectively, were required for the pool to test PCR-positive to these pathogens. The equivalent numbers of BC-positive cows required for a pool of PMQS to test positive with a probability of detection of 90% were 4.1, 1.5 and 1.3 to detect *Staph. aureus*, *Strep. agalactiae*, and *Strep. dysgalactiae*, respectively. By means of PCR kit II, the estimated numbers of BC-positive cows per pool were slightly decreased for pool sizes of 10 and 50 and slightly increased for pools of 20 (Table 4).

DISCUSSION

One objective of this study was to estimate the probability of detection by PCR in pooled milk samples in relation to the number of BC-positive cows within a pool. We showed that for all pathogens involved in this study, the number of BC-positive cows within a pool of TDCS had to be higher than those in a pool of PMQS, regardless of pool size or PCR kit (Table

Table 1. Numbers of positive and negative test results applying PCR kit I and II on pools of pre-milk quarter samples and test-day cow samples[1]

Pathogen	Pool size	PCR	Status	PMQS[2]		TDCS[3]	
				BC (+)	BC (−)	BC (+)	BC (−)
Staphylococcus aureus	10	Kit I[4]	(+)	48	2	36	8
			(−)	44	98	56	92
		Kit II[4]	(+)	59	2	34	8
			(−)	33	98	58	92
	20	Kit I	(+)	28	1	23	5[5]
			(−)	43	25	48	20[5]
		Kit II	(+)	19	4	24	10
			(−)	52	22	47	16
	50	Kit I	(+)	17	0	16	0
			(−)	21	1	22	1
		Kit II	(+)	26	0	17	1
			(−)	12	1	21	0
Streptococcus agalactiae	10	Kit I	(+)	18	7	10	3
			(−)	11	156	19	160
		Kit II	(+)	21	10	13	2
			(−)	8	153	16	161
	20	Kit I	(+)	8	3	4	2
			(−)	19	67	23	68
		Kit II	(+)	3	6[5]	3	11
			(−)	24	63[5]	24	59
	50	Kit I	(+)	8	2	4	1
			(−)	11	18	15	19
		Kit II	(+)	14	4	4	2
			(−)	5	16	15	18
Streptococcus dysgalactiae	10	Kit I	(+)	25	17	15	12
			(−)	7	143	17	148
		Kit II	(+)	25	11	12	6
			(−)	7	149	20	154
	20	Kit I	(+)	13	5	12	5
			(−)	16	63	17	63
		Kit II	(+)	6	4	10	13
			(−)	23	64	19	55
	50	Kit I	(+)	15	2	8	3
			(−)	8	14	15	13
		Kit II	(+)	18	2	4	0
			(−)	5	14	19	16

[1]Total number of pools was 192 pools of pool size 10, 97 pools of size 20 and 39 pools of size 50.
[2]PMQS = pre-milk quarter samples for bacteriological culture.
[3]TDCS = test-day cow samples.
[4]Kit I = bactotype HP3 (indical bioscience); Kit II = Mastit 4 (DNA Diagnostic).
[5]PCR was not evaluable in one pool for this pathogen.

4). The strength of this field study is the large number of samples, consisting of 7,336 PMQS from 1,912 cows and the corresponding TDCS from each cow. To our knowledge, there are no studies considering pooled TDCS for early detection of major mastitis pathogens using PCR. For *Staph. aureus* in pools of 10 cows, our study's Se of PCR, being 63.3% for kit I and 78.1% for Kit II in pooled PMQS (Figure 2), was lower than in a study using bulk milk samples with a Se of 83.3% (Soltau et al., 2017). The Sp values in our study were 97.0% and 97.6%, respectively (Figure 3), being higher than in the bulk milk study of 66.7%. In the study by Soltau et al. (2017), *Staph. aureus* prevalence was 12.8%, being greater than in our study, in which 8.3% of all cows in the study were BC-positive. For *Strep.*

agalactiae, in pools of 10 cows we estimated average Se values of 77.5% for PCR kit I and 84.1% for PCR kit II and average Sp values of 99.2% and 97.6%, respectively. As the number of positive TDCS pools detected by PCR was less than the number of positive PMQS pools detected by PCR, regardless of PCR kit type (Table 1), we suggest applying PCR to PMQS pools rather than TDCS pools for early detection of all 3 pathogens.

The diagnostic performance of PCR in bulk milk samples for *Strep. agalactiae* is reported as 95.2% Se and 98.8% Sp (Mweu et al., 2012) or 78.8% Se and 77.8% Sp (Soltau et al., 2017). In this case, the prevalence of *Strep. agalactiae* in the study by Soltau et al. (2017), being 1.1% cows per herd, is slightly lower but comparable with the portion of BC-positive cows of

Table 2. Numbers of positive and negative test results applying PCR kit I and II on pools of pre-milk quarter samples and test-day cow samples depending on the number of BC-positive cows per pool; 192 pools of 10 cows

Pathogen	Sample[1]	PCR[2]	Status	Pool					
				0	1	2	3	4	5
Staphylococcus aureus	PMQS	Kit I	(−)	98	27	10	4	2	1
			(+)	2	25	13	7	1	2
		Kit II	(−)	98	21	9	2	1	0
			(+)	2	31	14	9	2	3
	TDCS	Kit I	(−)	92	35	11	5	2	3
			(+)	8	17	12	6	1	0
		Kit II	(−)	92	37	15	4	1	1
			(+)	8	15	8	7	2	2
Streptococcus agalactiae	PMQS	Kit I	(−)	156	11	0			
			(+)	7	16	2			
		Kit II	(−)	153	8	0			
			(+)	10	19	2			
	TDCS	Kit I	(−)	160	19	0			
			(+)	3	8	2			
		Kit II	(−)	161	15	1			
			(+)	2	12	1			
Streptococcus dysgalactiae	PMQS	Kit I	(−)	143	7				
			(+)	17	25				
		Kit II	(−)	149	7				
			(+)	11	25				
	TDCS	Kit I	(−)	148	17				
			(+)	12	15				
		Kit II	(−)	154	20				
			(+)	6	12				

[1]PMQS = pre-milk quarter samples for bacteriological culture; TDCS = test-day cow samples.
[2]Kit I = bactotype HP3 (indical bioscience); Kit II = Mastit 4 (DNA Diagnostic).

our study, which was 1.6%. Other studies report test performances of PCR in individual TDCS as 93% with a wide confidence interval of 60% to 99% (Cederlöf et al., 2012) or 73.9% to 96.2% (Mahmmod et al., 2013b) for *Strep. agalactiae.* In our study, the probability of detection of PCR kits in TDCS pools was lower than in PMQS pools. Some of these studies tried to increase the Se of PCR by using dilutions of DNA extracts to minimize possible inhibitors (Steele et al., 2017) or by using enrichment of samples before DNA extraction (Hiitiö et al., 2015). In our study, BC was performed for PMQS and additionally for broth-enriched PMQS. Of the 3 pathogens, 27.8% of the findings came from BC of enriched samples. For PCR, we did not use enriched pooled PMQS and TDCS to avoid enrichment of PCR inhibitors.

Influence of Study Herds

Our objective was to evaluate the diagnostic performance of PCR tests on pooled field samples for the early detection of major mastitis pathogens. Thus, the herds enrolled in our study had good udder health and a low IMI prevalence, and cows with clinical mastitis or which were medicated due to mastitis were excluded from the study. Hence, the prevalence of pathogens detected by BC was low. Other field studies chose

herds after pre-selecting for the occurrence of certain pathogens in bulk milk (Mahmmod et al., 2013a,b) or sampled cows with clinical mastitis (Koskinen et al., 2010; Steele et al., 2017) for the estimation of Se and Sp of PCR in milk samples. When estimating the Se and Sp of a test approach, the amount of the pathogen in the sample is crucial. A test will perform better in a panel of samples from clinically diseased animals containing a high number of pathogens than in a panel of samples from animals in a subclinical stage of disease containing a low number of pathogens, described as patient spectrum in a review from Leeflang et al. (2013). As shown by other studies, there is a positive association between bacterial density in pools and pool probability of detection (Wichert et al., 2021). Therefore, the system Se of a diagnostic approach is expected to be lower in low-prevalence herds.

A previous meta-analysis of 174 studies analyzing the prevalence of major mastitis pathogens globally reported a prevalence of 25% (CI 21–29%) for *Staph. aureus* worldwide and a prevalence of 23% for *Staph.* species in general in Europe. There was a worldwide prevalence of 9% (CI 7–12%) for *Strep. agalactiae* and 6% (CI 5–8%) for *Strep. dysgalactiae*, and a prevalence of 12% (CI 8–18%) for *Strep.* species in general in Europe (Krishnamoorthy et al., 2021), which is an indication of the good udder health of our study herds.

Table 3. Sensitivity and specificity in pre-milk quarter samples for bacteriological culture pools of 10 cows estimated by means of a Bayesian latent class analysis

Item[1]	Variable[2]	Staphylococcus aureus				Streptococcus agalactiae				Streptococcus dysgalactiae			
		Median	CI	effSS[3]	psrf[4]	Median	CI	effSS	psrf	Median	CI	effSS	psrf
BC	Se	0.955	0.878 to 0.999	6,580	1.000	0.737	0.577 to 0.892	10,405	1.000	0.792	0.625 to 0.948	16,976	1.000
	Sp	0.891	0.793 to 0.991	8,094	1.000	0.969	0.937 to 0.997	11,991	1.000	0.974	0.943 to 0.999	14,077	1.000
PCR kit I	Se	0.633	0.492 to 0.792	9,174	1.000	0.775	0.614 to 0.918	7,274	1.000	0.855	0.731 to 0.969	7,493	1.001
	Sp	0.970	0.934 to 0.997	7,927	1.000	0.992	0.975 to 1.000	9,156	1.000	0.936	0.896 to 0.970	17,119	1.000
PCR kit II	Se	0.781	0.629 to 0.943	8,426	1.000	0.841	0.687 to 0.980	4,171	1.001	0.856	0.728 to 0.971	8,983	1.000
	Sp	0.976	0.944 to 1.000	8,430	1.000	0.976	0.950 to 0.996	16,626	1.000	0.971	0.942 to 0.993	13,569	1.000
Prevalence	P1	0.304	0.222 to 0.397	13,490	1.000	0.095	0.052 to 0.149	14,249	1.000	0.133	0.079 to 0.192	16,962	1.000
	P2	0.685	0.463 to 0.888	9,115	1.000	0.660	0.448 to 0.856	14,835	1.000	0.436	0.275 to 0.602	20,000	1.000
PCR	Se	0.008	−0.016 to 0.028	7,165	1.001	0.023	−0.007 to 0.062	4,510	1.001	0.016	−0.020 to 0.064	9,593	1.000
covariance	Sp	0.008	−0.001 to 0.031	6,609	1.000	0.003	0.000 to 0.010	6,114	1.000	0.007	−0.001 to 0.019	4,917	1.000

[1]BC = bacteriological culture; PCR kit I = bactotype HP3 (indical bioscience); PCR kit II = Mastit 4 (DNA Diagnostic).
[2]Se = sensitivity; Sp = specificity; P = population.
[3]effSS = effective sample size.
[4]psrf = potential scale reduction factor.

In our study, the amount of BC-positive cow samples per pool reflects the bacterial density in PMQS and TDCS pools (Table 2). Considering the number of pathogen-positive cows per pool necessary to achieve a probability of detection of 90% in pools of 10 cows, herds would require a prevalence of 50% to 70% for *Staph. aureus* and approximately 20% for *Strep. agalactiae* and *Strep. dysgalactiae* (Table 4). To be able to detect herds with lower prevalence of a pathogen, frequently repeated testing could be helpful. For herd-level monitoring aiming at early detection of risks to udder health, a high Se of the diagnostic approach is essential, especially when working with pooled samples. Furthermore, for detecting subclinical infections, a high Se is crucial due to the relatively low bacterial load. We consider the probability of detection of applying PCR to pooled TDCS as too low; thus, the use of this approach for early detection of major mastitis pathogens is challenging. There might be other issues for this approach (e.g., monitoring the prevalence of certain pathogens or eradication programs). Therefore, further studies are necessary.

Influence of Sampling

PMQS as collected for BC usually consist of cistern milk with 2 or 3 first jets of milk from each teat. For most pathogens, the highest concentration will be excreted with this first milk. In contrast, TDCS are continuously sampled during the whole milking process. Therefore, the amount and the composition of mastitis pathogens in the milk are expected to be different in these samples. This may explain at least in part the lower probability of detection of PCR applied to pools of TDCS. Furthermore, the incalculable number of positive cows per pool required to test positive with PCR (Table 4) occurred almost exclusively for TDCS pools which underlines the differences in the sample type.

The findings of this study must also be seen in light of its limitations. First, the influence of the different preservatives added to the sampling tubes, namely boric acid in PMQS tubes and bronopol in TDCS tubes, is not quantifiable in our study. Additionally, PCR for individual PMQS and TDCS is needed. Both preservatives are added to milk samples to prevent bacterial proliferation and sample spoilage. Previous studies showed an influence of bronopol on total cfu in the sample (Seškēna and Jankevica, 2007) and on St*aph. aureus* in particular (Botaro et al., 2013). Other studies comparing quantitative real-time PCR in TDCS and BC in PMQS did not mention a possible influence of bronopol (Cederlöf et al., 2012; Mahmmod et al., 2013a). Nonetheless, the influence of either bronopol or
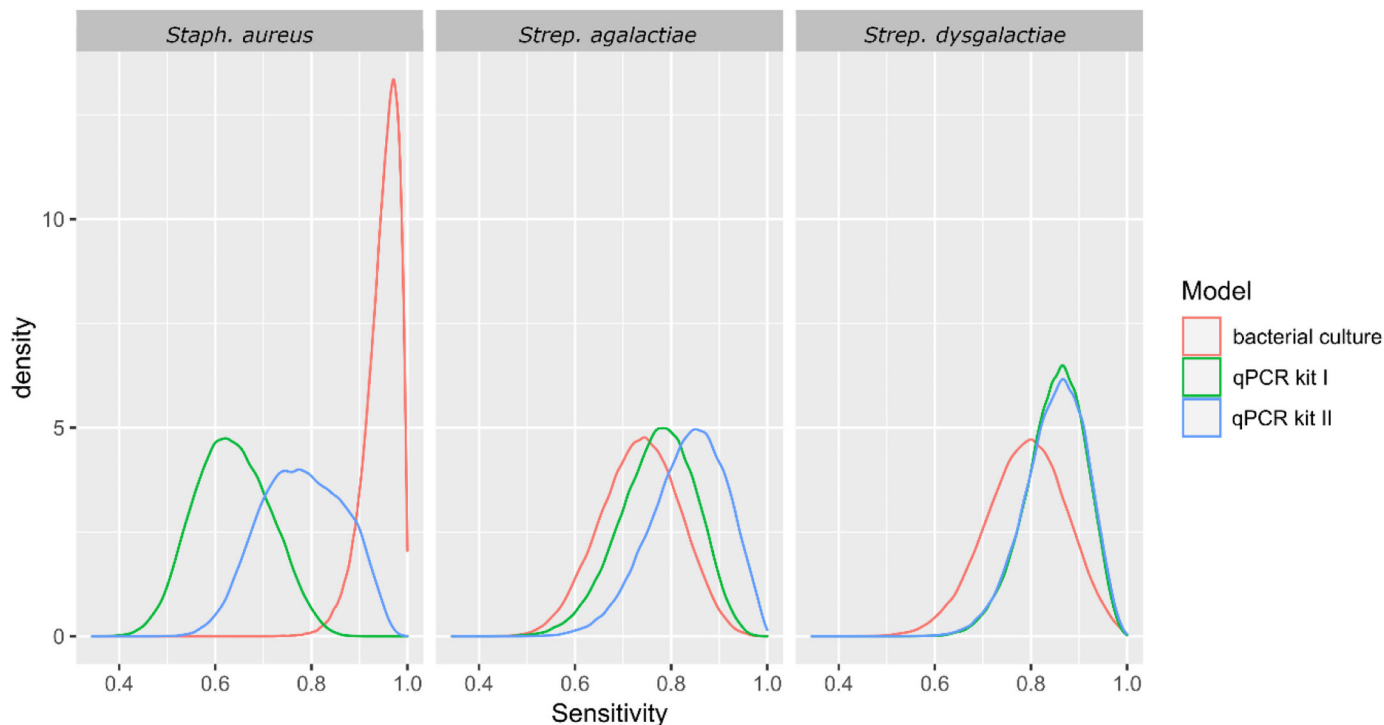
Figure 2. Density plots of sensitivity of bacterial culture, PCR kit I and PCR kit II for *Staphylococcus aureus*, *Streptococcus agalactaie* and *Streptococcus dysgalactiae* in pooled PMQS of 10 cows estimated by means of a Bayesian latent class analysis. PCR kit I = bactotype HP3; PCR kit II = Mastit 4; PMQS = pre-milk quarter samples for bacteriological culture.

boric acid on real-time PCR performance needs further investigation.

Although the use of TDCS for diagnostic purposes may be attractive because of easier sampling and availability on a regular basis, this kind of sampling has additional limitations. The TDCS are not aseptically collected and not intended to be examined for the detection of specific pathogens, having an incalculable level of bacterial contamination that may interfere with the amplification due to unknown PCR inhibitors. Moreover, special attention should be paid to DNA carryover in TDCS in the milking parlor and during implemented tests for dairy herd improvement routines in the lab (Løvendahl and Bjerring, 2006; Mahmmod et al., 2017). We must be aware of all these limitations when using TDCS for either individual or herd-level diagnosis.

The second limitation of our study concerns the time span between collecting PMQS and TDCS. Under practical circumstances in a commercial dairy farm, 24 to 48h was the minimum time we had to accept for organizing a careful sampling within the milking parlor. Considering the high proportion of chronic infections and long-lasting subclinical infections with major mastitis pathogens (e.g., *Staph. aureus*, *Strep. agalactaie*) and udder health in general, the time gap of 2 d may

be negligible for herd-level diagnosis in pooled milk samples as pursued here, but not for individual cases. Studies had reported a dynamic shedding pattern for *Staph. aureus* (Sears et al., 1990) and *Strep. agalactaie* (Svennesen et al., 2019) which could lead to a day-to-day difference in the shedding of those pathogens. In our study, that could have caused an underestimation of PCR performance in TDCS pools.

Influence of Statistical Methods

Cederlöf et al. (2012) estimated Se and Sp using a latent class analysis. They reported a Se for PCR for *Staph. aureus* in individual TDCS of 93.0%. This is much higher than the estimated Se in our study when using Bayesian latent class analysis for the estimation of PCR test performance in pooled PMQS, the values of which were 63.3% and 78.1%. (Table 3). Due to the fact that we detected far fewer positive TDCS pools than PMQS pools (Table 1), we conclude that PCR test performance in pooled TDCS is decreased. The difference relative to the test performance of Cederlöf et al. (2012) might be due to the difference of individual and pooled milk samples. In addition, the difference could be due to using a Bayesian latent class analysis with having the opportunity to add prior information
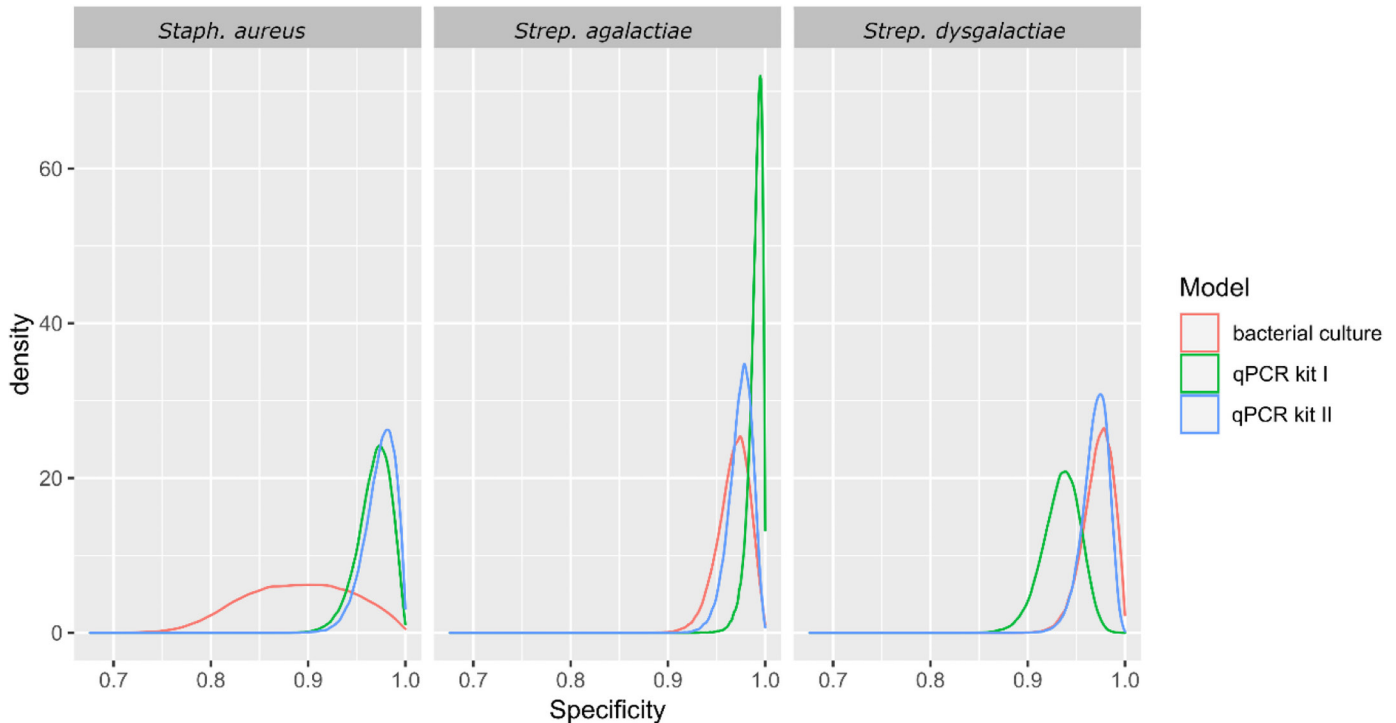
Figure 3. Density plots of specificity of bacterial culture, PCR kit I and PCR kit II for *Staphylococcus aureus*, *Streptococcus agalactaie* and *Streptococcus dysgalactiae* in pooled PMQS of 10 cows estimated by means of a Bayesian latent class analysis. PCR kit I = bactotype HP3; PCR kit II = Mastit 4; PMQS = pre-milk quarter samples for bacteriological culture.

about expected Se, Sp, prevalence and covariances, rather than using a classical latent class analysis. In our analysis, we used noninformative priors for prevalence and enabled a possible covariance between PCR kits. Additionally, we added weakly informative priors for Se and Sp, which were the same for all 3 tests and pathogens. However, a cumulative test result—as with our study, in which the BC result was cumulated from 40 individual BC results for pool size 10—could lead to an increased Se relative to the one we considered for our analysis priors. Therefore, this could have led to an underestimation of the Se of BC. Nevertheless, we chose weakly informative priors for Se and Sp, which were the same for all 3 test methods, because there is very little published data for the Se and Sp of PCR or BC applied to pooled milk samples.

Influence of Different Test Targets

The apparently lower Se of PCR for pooled samples for *Staph. aureus* may be due to dilution effects in pooled milk samples diminishing the concentration of pathogens' genetic material to below the PCR kit's limit of detection. Considering *Staph. aureus*, the literature reports a limit of detection of 1,000 to 10,000 gene copies for a conventional PCR (Chandrashekhar

et al., 2015) or 40 cfu/mL for a quantitative real-time PCR (Boss et al., 2011). In pools of 10 cows, the bacterial density is diluted by a factor of 40 for one infected quarter in a pool of milk samples from those cows compared with a single PMQS. According to the literature, misclassification when using BC occurs in 9% to 37% of cases in laboratories performing routine mastitis diagnostics (Pitkälä et al., 2005) where BC contamination is identified as the main cause. For our study, we assume a much lower rate of misclassification due to both careful sampling and the high laboratory standards.

A crucial difference in using BC or PCR is the reporting of sample results being positive for multiple pathogens. In routine mastitis bacteriology, milk samples with 3 or more pathogens growing on the plate are considered contaminated. Contaminated samples are common in routine diagnosis, mostly due to lack of care when collecting the sample. Excluding those results for BC would affect both Se and Sp. In contrast, PCR detects both viable and nonviable bacteria even if a variety of bacteria suggestive of contamination is detected in the respective sample. The clinical relevance of the presence of nonviable bacteria remains unclear (Schukken et al., 2010). Due to good farm management practices regarding milking and udder
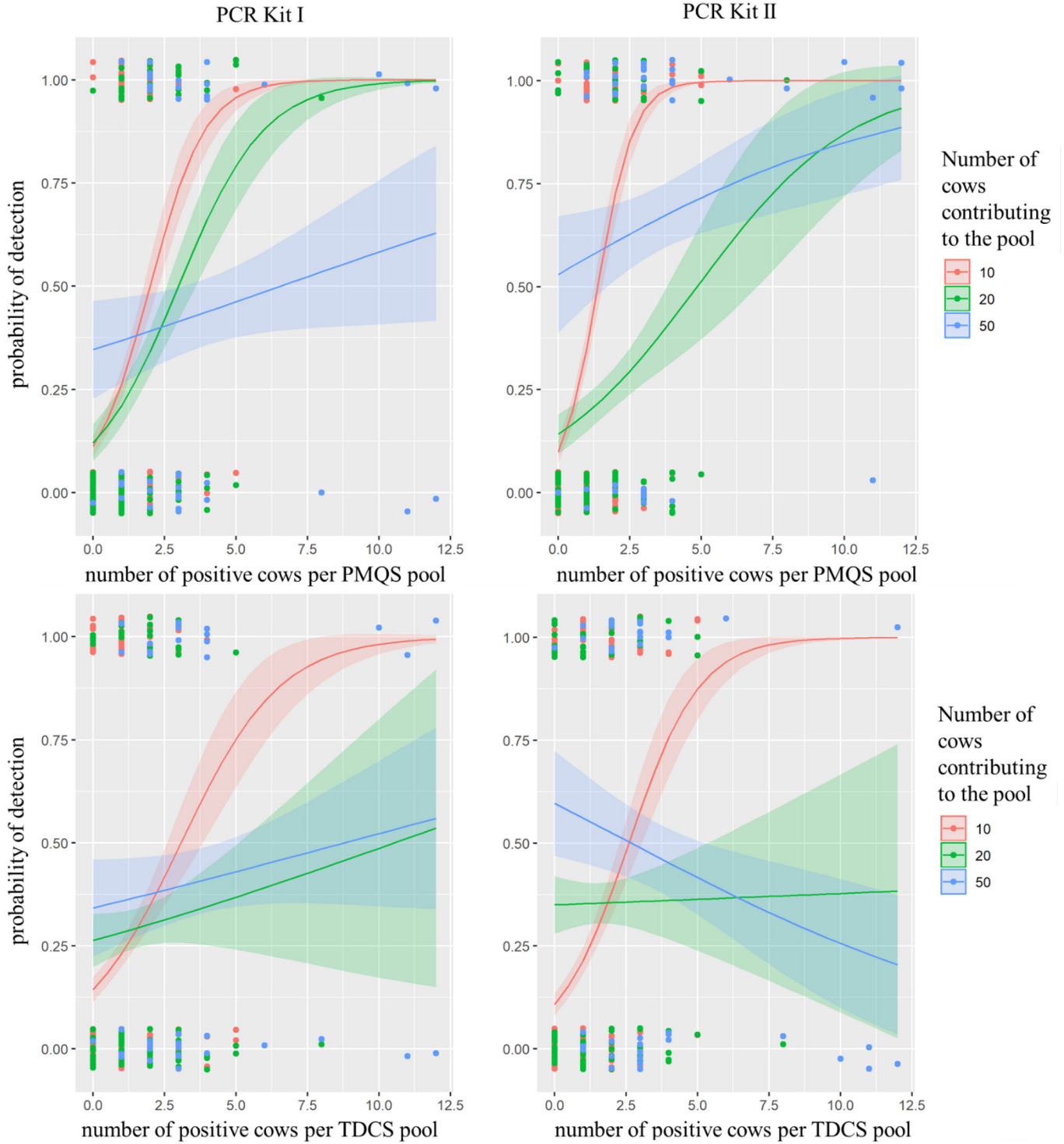
Figure 4. Probability of detection of *Staphylococcus aureus* via PCR in pooled PMQS and TDCS with confidence interval (shaded area). PCR kit I = bactotype HP3; PCR kit II – Mastit 4; PMQS = pre-milk quarter samples for bacteriological culture; TDCS = test-day cow sample.
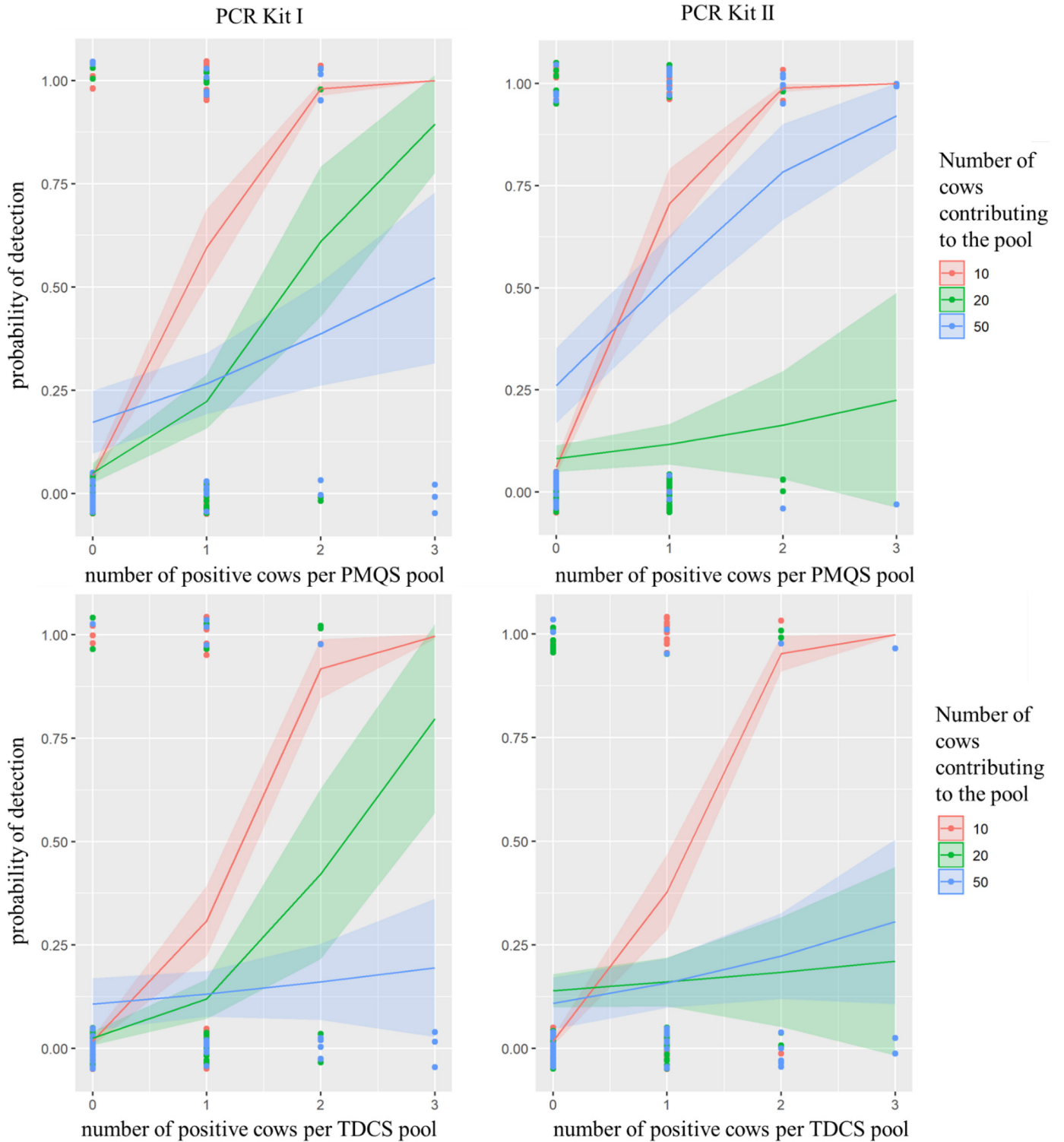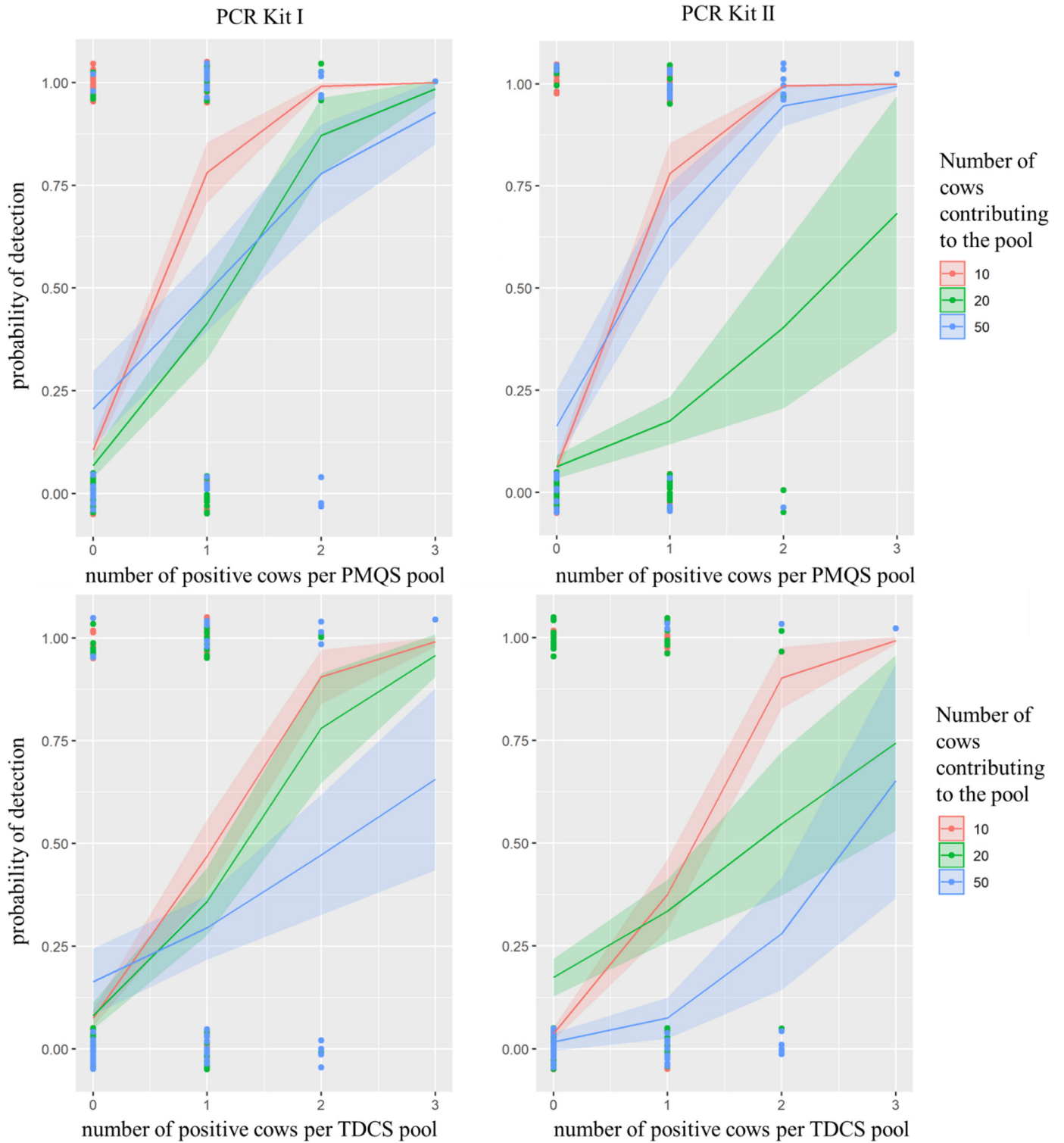
Figure 5. Probability of detection of *Streptococcus agalactiae* via PCR in pooled PMQS and TDCS with confidence interval (shaded area). PCR kit I = bactotype HP3; PCR kit II = Mastit 4; PMQS = pre-milk quarter samples for bacteriological culture; TDCS = test-day cow sample.

Figure 6. Probability of detection of *Streptococcus dysgalactiae* via PCR in pooled PMQS and TDCS with confidence interval (shaded area). PCR kit I = bactotype HP3; PCR kit II = Mastit 4; PMQS = pre-milk quarter samples for bacteriological culture; TDCS = test-day cow sample.

Table 4. Estimated numbers of positive cows per pool of 10, 20, and 50 cows to achieve a diagnostic sensitivity of 50%, 75%, 90%, or 95% using 2 different PCR kits

Pathogen	Sample type[1]	Pool size	PCR kit I[2]				PCR kit II[3]			
			50%	75%	90%	95%	50%	75%	90%	95%
Staphylococcus aureus	TDCS	10	3.1	5.0	6.9	8.2	2.6	4.0	5.3	6.2
		20	10.5	IC[4]	IC	IC	IC[4]	IC	IC	IC
		50	8.8	23.7	38.5	48.6	2.7	IC	IC	IC
	PMQS	10	2.0	3.1	4.1	4.8	1.4	2.1	2.8	3.2
		20	3.0	4.6	6.3	7.4	4.9	7.8	10.8	12.8
		50	6.5	17.9	29.2	36.9	IC	6.1	12.9	17.6
Streptococcus agalactiae	TDCS	10	1.3	1.6	1.9	2.2	1.1	1.5	1.8	2.0
		20	2.2	2.8	3.5	3.9	10.9	17.5	IC	IC
		50	9.0	13.7	18.3	21.5	4.9	7.5	10.1	11.8
	PMQS	10	0.9	1.2	1.5	1.7	0.8	1.1	1.4	1.6
		20	1.7	2.4	3.0	3.5	6.1	8.9	11.7	13.6
		50	2.8	4.8	6.8	8.2	0.9	1.8	2.8	3.4
Streptococcus dygalactiae	TDCS	10	1.1	1.5	2.0	2.3	1.2	1.6	2.0	2.3
		20	1.3	1.9	2.5	2.9	1.8	3.0	4.3	5.1
		50	2.1	3.6	5.0	6.0	2.6	3.3	4.0	4.5
	PMQS	10	0.6	0.9	1.3	1.5	0.7	1.0	1.2	1.4
		20	1.2	1.6	2.1	2.5	2.3	3.3	4.2	4.9
		50	1.0	1.9	2.7	3.3	0.7	1.2	1.7	2.0

[1]TDCS = test-day cow samples; PMQS = pre-milk quarter samples for bacteriological culture.

[2]PCR kit I = bactotype HP3 (indical bioscience).

[3]PCR kit II = Mastit 4 (DNA Diagnostic).

[4]IC = incalculable, when estimated number of positive cows exceeded the number of cows contributing to the pool.

health and careful sampling of PMQS as undertaken in our study, we observed only a very low proportion of samples with contamination or multiple pathogens present.

Influence of the PCR Kit

Using a different PCR kit did not have a significant influence on the study results regarding Se. Most studies on diagnostic Se of commercial mastitis PCR kits used only one kit (Koskinen et al., 2010; Spittel and Hoedemaker, 2012; Syring et al., 2012). Studies applying different kits to the same samples were not available. Comparing the test characteristics of different kits analyzed in different studies is affected by several variables, such as reference method, reference panel or strain of pathogen. By using both methods (PCR kits) on the same samples and comparing them with an independent method (BC), our study provides a valid indication that the influence of the type of PCR kit on the test outcome is limited.

Furthermore, the threshold for interpretation of Ct-values influences the test result. In our study, we used the Ct-value thresholds as set by the manufacturers for individual samples; this resulted in a similar interpretation of the test results when the tests were applied to pooled samples. As known from previous studies, increasing the threshold for the interpretation of the Ct-value increases Se but decreases Sp (Cederlöf et al.,

2012). Previous studies also recommended increasing the Ct-value threshold when applying PCR kits to pooled milk samples or bulk milk samples (Mweu et al., 2012). For this purpose, a slightly reduced Sp is a minor problem that can be handled by confirmation testing.

In general, for early detection of major mastitis pathogens at the herd level, we consider the multiplex real-time PCR testing of pools of PMQS impractical because of the additional time and resources needed for pooling compared with BC of PMQS and because of the reduced diagnostic performance of the testing approach for *Staph. aureus*. Furthermore, the achievable diagnostic results refer to a group of cows and not the respective individuals that should be treated or managed to control the infection. To our best knowledge, this is the first study analyzing the test performance of multiplex real-time PCR on pooled TDCS with a known number of positive cows contributing to the pool. We found quantitative PCR diagnostics applied to pools of TDCS were of limited benefit in pools of 50 cows. Testing of pools comprising 10 or 20 cows resulted in an acceptable probability of detection. In our opinion, however, it is a limited fit for the early detection of *Staph. aureus* at the herd level because at least 4 BC-positive cows had to be involved in the pool to achieve an acceptable probability of detection. Regarding *Strep. agalactiae* and *Strep. dysgalactiae*, 2 cows had to test BC-positive to achieve a probability of detection of 90%. We consider this inadequate for early

detection in large dairy herds but useful for the detection of infected herds, preferably as the first-line test in *Strep. agalactiae* eradication programs using the results in combination with bulk milk SCC. Furthermore, it is useful for the surveillance of major mastitis pathogens in herds with AMS when samples are gained regularly.

CONCLUSIONS

The number of infected cows and the pool size determine the probability of detecting an infection with major mastitis pathogens at the herd level by PCR testing of pooled milk samples. For *Streptococcus* species, an acceptable probability of detection was achieved when at least 2 infected cows were included in a pool of 10, regardless of sample type. For *Staph. aureus*, a higher number of infected cows was required. The probability of detection achieved by testing TDCS pools is lower compared with the Se results achieved by testing PMQS pools. The estimated Se and Sp for *Streptococcus* species were similar for PCR and BC, but for *Staph. aureus* the BC resulted in a better Se, and PCR in a better Sp.

ACKNOWLEDGMENTS

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