Within-herd prevalence thresholds for herd-level detection of mastitis pathogens using multiplex real-time PCR in bulk tank milk samples

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ABSTRACT

The objective of the study was to assess the value of quantitative multiplex real-time PCR examination of bulk tank milk samples for bovine mastitis pathogens as a tool for herd level diagnosis. Using a logistic regression model, this study is aimed at calculating the threshold level of the apparent within-herd prevalence as determined by quarter milk sample cultivation of all lactating cows, thus allowing the detection of a herd positive for a specific pathogen within certain probability levels. A total of 6,335 quarter milk samples were collected and cultured from 1,615 cows on 51 farms in Germany. Bulk tank milk samples were taken from each farm and tested by bacterial culture as well as the commercial PCR assay Mastit 4A (DNA Diagnostic A/S, Risskov, Denmark) identifying *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, and *Streptococcus uberis*. In addition, PCR was performed on pooled herd milk samples containing milk aliquots from all lactating cows in each of the 51 herds. Only 1 out of the 51 herds was found PCR positive for *Streptococcus agalactiae* in bulk tank and pooled herd milk samples, and cultured quarter milk samples. Spearman's rank correlations between the cycle threshold value of bulk tank milk PCR and the apparent within-herd prevalence were calculated in regard to *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*. For these pathogens, significant correlations were found. If 1 bulk tank milk sample per herd was tested, the estimated within-herd prevalence thresholds for 90% probability of detection were 27.6% for *Staphylococcus aureus*, 9.2% for *Streptococcus dysgalactiae*, and 13.8% for *Streptococcus uberis* on the cow level. On the quarter level, the within-herd prevalence had to be at least 32.6% for *Staphylococcus*

aureus, 1.7% for *Streptococcus dysgalactiae*, and 4.3% for *Streptococcus uberis* to detect a herd as positive using a single bulk milk sample. The results indicate that mastitis pathogens in bulk tank milk can be identified by the applied PCR assay. Bulk tank milk examination is not a reliable tool for the identification of the named pathogens by single testing, but might be a valuable monitoring tool when used frequently with repeated testing. Furthermore, this approach could be a useful monitoring tool for detecting new pathogen occurrence in the herd.

Key words: *Staphylococcus aureus*, *Streptococcus*, herd level diagnosis, pooled milk sample

INTRODUCTION

Intramammary infections are the most common diseases in dairy cattle (Gundling et al., 2015) causing large economic losses. In Germany, in 2009, the damage to the national economy caused by mastitis was estimated to be €1.4 billion (DVG, 2012). Mastitis therapy and drying off account for the greatest amount of antibiotics used in dairy farming (DANMAP, 2014). Dairy herd managers consider good udder health vital, not only for economical and animal welfare reasons, but also to minimize antibiotic use to prevent antimicrobial resistance. Knowledge about the mastitis pathogens prevalent in a certain herd is necessary for the control, reduction, and prevention of udder diseases (Krömker and Friedrich, 2011). Reliable diagnostic tools are essential for developing a farm-specific mastitis prevention plan. Bacterial culture (BC) of quarter milk samples is considered the gold standard for mastitis diagnosis, and isolation of the bacteria involved is the prerequisite to perform an antibiotic susceptibility test. Some disadvantages of cultivation are that it is labor intensive and takes a minimum of 24 to 48 h cultivation time (Riffon et al., 2001; Gillespie and Oliver, 2005; Koskinen et al., 2010). A substantial proportion of clinical and subclinical mastitis samples are negative for bacterial growth (Bradley et al., 2007; Taponen et al., 2009). Quantita-

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tive real-time PCR (**qPCR**) technique is an alternative method for examining milk specimens for mastitis pathogens. It provides results within only a few hours, but is more expensive than BC. Polymerase chain reaction shows a higher sensitivity than BC for detecting bacteria in milk samples according to some authors (Taponen et al., 2009; Bexiga et al., 2011; Spittel and Hoedemaker, 2012). On the other hand, qPCR does not differentiate between viable, growth-inhibited, or dead bacteria. Whereas the staphylococcal β -lactamase gene can be detected with qPCR, a BC is still necessary for a full antibiotic susceptibility test.

For herd level monitoring, bulk tank milk (**BTM**) testing is an easy-to-use and cost-saving alternative to individual sample testing. The SCC of the BTM, which is frequently tested by dairy companies at milk collection in some countries, is a prompt, but rough indicator for the prevalence of subclinical mastitis in the herd. Should the SCC in BTM increase, it is not possible to detect the causing pathogen by a single examination of a BTM sample by BC (Ruegg and Reinemann, 2002). Sensitivity for the detection of mastitis pathogens in BTM is known to be low (Godkin and Leslie, 1993; Olde Riekerink et al., 2010; da Costa et al., 2016). Therefore, our hypothesis is that it is advantageous to detect major mastitis pathogens via single or repeated testing of BTM by qPCR. The objective of this study was to identify and describe the relation between the apparent within-herd-prevalence (**WHP_{app}**) and the cycle threshold (**Ct**) values of the qPCR aiming at quantification of the bacteria in BTM, and to calculate a threshold of the **WHP_{app}** that allows the identification of a herd as positive for the relevant pathogen. Besides BTM, we also used pooled herd milk samples as an alternative specimen.

MATERIALS AND METHODS

Dairy Herds

For this study, 51 small and medium-sized farms located in the German Federal States Thuringia (26) and Hesse (25) were selected. Herds of this size were chosen because of a common lack of routine mastitis control programs in such herds, and therefore a screening of BTM for mastitis pathogens could be beneficial. Mean herd size of the study farms was 72 (9–144) cattle. On these farms, quarter milk samples were taken from each lactating cow not excluded from milk delivery. Therefore, cows with signs of clinical mastitis, treated cows, and those within the first 5 d postcalving were not sampled. The average number of cows tested on each farm was 32 (3–61). These cows were kept in multiple housing conditions (free-stall barns with various bed-

dings, tie-stall with or without pasture). Average herd milk yield varied between 4,700 to 11,500 kg/cow per year.

Milk Samples

The samples were collected from October to December 2014 by Animal Health Service of Thuringia veterinarians and the first author. The milk samples were collected during the daily milking routine after udder preparation according to the “Guidelines for aseptic collection of milk samples and for isolation and identification of mastitis pathogens” of the German Veterinary Society (DVG, 2009). After the milking routine was finished, the milk in the bulk tank was stirred for at least 5 min, and 2 BTM samples were taken using sterile disposable syringes. The sample tubes for all types of milk samples contained boric acid as a preservative agent. After collection, all samples were immediately cooled and transported within 48 h to the laboratory of the Animal Health Service, Thuringian Animal Diseases Fund of the State of Thuringia in Jena.

Pooling

At the laboratory, quarter milk samples of each cow were pooled by transferring 0.25 mL per sample to another sterile tube, yielding cow-level milk samples. Furthermore, 2 pooled herd samples were assembled from 0.2 mL of each cow-level sample for each herd. Afterward, the tubes containing pooled herd samples were frozen and maintained at -20°C ($\pm 5^{\circ}\text{C}$) until analysis.

Bacterial Culture

The quarter milk and BTM samples were cultured according to the above mentioned guidelines. Using a glass bar, 0.01 mL of each milk sample was spread on an esculin sheep blood agar plate (Oxoid, Wesel, Germany) prepared with a streak of a β -hemolysin producing *Staphylococcus aureus*. After the process, the glass rod with the adhering residual milk was dipped into a glucose broth (Oxoid) for enrichment. The agar plates and the broths were incubated aerobically at 37°C ($\pm 2^{\circ}\text{C}$). The cultures were examined after 18 to 24 and 42 to 48 h, respectively. The broths were plated out after 18 to 24 h and incubated for 1 d before examination to increase the sensitivity of BC in regard to *Streptococcus agalactiae* and *Staph. aureus*. The colonies grown from primary cultures and the broths were evaluated for morphology, hemolysis, pigmentation, and Gram stain. *Streptococcus* species were differentiated from *Staphylococcus* species via catalase reaction. For identification of *Staph. aureus*, a coagulase test with rabbit plasma

(Remel, Lenexa, KS) was carried out. *Streptococcus* was tested for esculin hydrolysis, Christie, Atkins, and Munch-Peterson (CAMP) reaction, and Lancefield groups. Because of the milk inoculum of about 0.01 mL, the detection limit was about 100 cfu per mL in the sample. For *Staph. aureus*, *Strep. agalactiae*, and *Strep. dysgalactiae*, the culture was considered positive when one or more colonies in pure or mixed culture were found either in the primary culture or in the culture from the enrichment broth. A culture was classified as positive for *Strep. uberis* when at least 10 colonies had grown in the primary culture. As confirmation assays for *Strep. uberis*, cultivation on kanamycin esculin azide agar (Oxoid) as well as an agar diffusion assay with penicillin and rifampicin were performed. Inconclusive results were checked for Lancefield group antigen D via latex agglutination. After taking the required sample material for the BC, the BTM samples were frozen and stored under the same conditions as the pool samples until qPCR analysis was performed. The WHP_{app} of the different mastitis pathogens at the cow level and at the quarter level were calculated from the results of the bacteriological culture of the quarter milk samples.

qPCR

The qPCR testing of the BTM and pooled herd samples was performed according to the manufacturer's protocol (DNA Diagnostic, 2015) in the laboratory of the PCR assay manufacturer, DNA Diagnostic A/S (Risskov, Denmark), by a Thuringian Animal Health Service technician. The samples were tested directly and without any enrichment. The qPCR assay Mastit 4A is a multiplex qPCR capable of detecting DNA of 4 pathogens (*Staph. aureus*, *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis*). With qPCR, a quantitative estimation of DNA in the sample is possible: The fewer cycles necessary to get a fluorescence signal, the greater the amount of the target DNA present in the sample. The obtained result was expressed as a Ct value. A sample was considered positive at Ct ≤ 37.

Statistical Methods

Bacterial culture and qPCR results and descriptive statistics were processed in Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA). For the calculation of sensitivity and specificity, a herd was considered positive for the respective detection method and the tested pathogen if at least one of the 2 BTM samples or pooled herd samples showed a positive result or if the infectious agent was isolated from one of the quarter milk samples. It was classified negative if both BTM samples or pooled herd samples or all quarter milk samples were

tested negative. Further statistical analyses were done using the statistical software package BMDP/Dynamic (Release 8.1; Dixon, 1993) concerning *Staph. aureus*, *Strep. dysgalactiae*, and *Strep. uberis*. As only one herd was positive for *Strep. agalactiae*, herd level sensitivity, specificity, and logistic regression models were not calculated for this pathogen. Statistical significance was designated as $\alpha = 0.05$ for all tests.

Using 2 BTM samples per herd, herd level sensitivity and specificity were calculated for each pathogen (*Staph. aureus*, *Strep. dysgalactiae*, and *Strep. uberis*) and each applied method (BC/PCR). Herd level sensitivity was the proportion of correctly identified positive herds among all positive herds as determined by BC of quarter milk samples. Herd level specificity was the proportion of correctly identified negative herds among all negative herds as determined by BC of quarter milk samples.

Spearman's rank correlation coefficients (r_s) were calculated (program BMDP3D) for analyzing the relationship between the WHP_{app} and the Ct of BTM and pooled herd samples. The relationship between WHP_{app} and herd status (positive/negative) as determined by testing either BTM or pooled herd samples was analyzed by means of separate logistic regression models. These models were calculated using WHP_{app} on either the cow or quarter level as an independent variable. The outcome (dependent variable) was a herd positive or negative for the specific pathogen in BTM samples or pooled herd samples in either single or double sampling. Using the single sampling approach, a randomized selection of 1 of the 2 samples was performed. Considering double sampling, a herd was counted as positive if the infectious agent was detected in either one or both BTM samples or one or both pooled herd samples. This resulted in 8 univariate and univariable logistic models for each pathogen. For the estimation of the threshold of WHP_{app} to detect a herd as positive with a given probability of detection (P_d), these logistic regression models were inverted and asymptotic standard errors of the estimates were calculated. Calculations were performed for $P_d = 0.9$ and $P_d = 0.7$.

RESULTS

Quarter milk samples were taken from 1,615 cows. As 125 quarters were not lactating and therefore not BC tested, a total of 6,335 quarter samples were available for analysis. The BC results and the mean and range of prevalences on the cow and quarter level are given in Table 1. *Staphylococcus aureus* was isolated from 5.0% of the quarters with quarter level WHP_{app} ranging from 0.0 to 38.5%. *Streptococcus uberis* was detected in 2.1% of the quarters with a maximum WHP_{app} of 19.3%.

Table 1. Pathogen findings by bacterial culture regarding the herds, cows, and udder quarters

Item	n	<i>Staphylococcus aureus</i> positive	<i>Streptococcus uberis</i> positive	<i>Streptococcus dysgalactiae</i> positive	<i>Streptococcus agalactiae</i> positive
Herds (no.)	51	36	33	24	1
%		70.6	64.7	47.1	2.0
Cows (no.)	1,615	200	103	47	18
%		12.4	6.4	2.9	1.1
Udder quarters (no.)	6,335	314	135	64	35
%		5.0	2.1	1.0	0.6
Cow-level prevalence (%)		12.8	6.5	2.7	0.6
Range of WHP _{app} ¹		0.0–77.3	0.0–35.7	0.0–17.9	0.0–32.1
Quarter-level prevalence (%)		5.0	2.1	0.9	0.3
Range of WHP _{app}		0.0–38.5	0.0–19.3	0.0–4.5	0.0–15.6

¹Apparent within-herd prevalence.

Streptococcus dysgalactiae (0.9%) exhibited a low quarter level prevalence with a maximum WHP_{app} of 4.5%. Twelve samples were positive for 2 pathogens. Five herds tested negative for all the selected pathogens. Only one out of 51 herds tested positive for *Strep. agalactiae* by BC in quarter milk samples as well as BTM. In this herd, both BTM samples and both pooled herd samples tested positive for *Strep. agalactiae* by qPCR. The prevalence for *Strep. agalactiae* was 32.1% on the cow level and 15.6% on the quarter level. As the pathogen was found in one herd only, it was excluded from further statistical calculations in this study.

The percentage of positive herds for BC of quarter milk and BTM samples as well as qPCR of BTM samples and pooled herd samples are shown in Table 2. Considering all 4 pathogens included in the qPCR, 93 positive PCR results were achieved in the BTM samples. Of the herds classified as positive by PCR of BTM samples, 62.4% showed a positive qPCR result for both tested samples, and 37.6% for only one of the 2 tested samples. Considering pooled herd samples, the Mastit 4 qPCR kit also yielded in 93 positive results, in 75.3% of the herds simultaneously in both samples. When BC was applied on BTM samples, the percentage of positive herds was much lower compared with the other testing method.

Herd level results of BTM samples or pooled herd samples in relation to the herd status as determined

by BC of quarter milk as well as herd level sensitivity and specificity are summarized in Table 3. Some herds classified as positive by BC were determined negative according to the results of qPCR and vice versa. For BC examination of BTM samples, a very low sensitivity (0.0–33.3%) was calculated. Further analysis of BTM BC results were waived because sensitivity was too low for calculating a meaningful correlation as well as estimating a logistic regression model in relation to WHP_{app}.

Using Spearman's rank correlation coefficients, we examined the correlation between the WHP_{app} on the cow level as well as on the quarter level, and the Ct value of the qPCR analysis in either 1 or 2 BTM samples, or 1 or 2 pooled herd samples (Table 4). We found a correlation ($P < 0.001$) for all examined associations with only small deviations between the different pathogens. Spearman's rank correlation coefficients regarding the correlation between the Ct value of a single BTM examination and the WHP_{app} were -0.51 to -0.56 . For pooled herd samples on the cow level, the correlation coefficients of the WHP_{app} were closer to -1 than for the BTM samples. When both pooled herd samples or both BTM samples were tested, the correlations were stronger than when testing only one sample. When analyzing WHP_{app} on the quarter level instead of the cow level, the correlation coefficients were all slightly closer to -1 for all associations. The strongest

Table 2. Total number and percentage of herds ($n = 51$) that tested positive by different methods of examination (bacterial culture of quarter milk samples, quantitative real-time PCR (qPCR) of pooled herd samples, qPCR, and bacterial culture of bulk tank milk samples)

Pathogen	Bacterial culture of quarter milk samples		PCR of pooled herd samples ¹		PCR of bulk tank milk samples ¹		Bacterial culture of bulk tank milk samples ¹	
	Number	%	Number	%	Number	%	Number	%
<i>Staphylococcus aureus</i>	36	70.6	29	56.9	35	68.6	12	23.5
<i>Streptococcus uberis</i>	33	64.7	34	66.7	30	58.8	0	0.0
<i>Streptococcus dysgalactiae</i>	24	47.1	29	56.9	27	52.9	2	3.9
<i>Streptococcus agalactiae</i>	1	2.0	1	2.0	1	2.0	1	2.0

¹A herd was considered as positive if at least 1 of 2 samples showed a positive result.

Table 3. Comparison of the classification of herds by bacterial culture (BC) of quarter milk samples (reference) with the classification by quantitative real-time PCR analysis or culture of 2 bulk tank milk or pooled herd milk samples

Item	<i>Staphylococcus aureus</i>		<i>Streptococcus uberis</i>		<i>Streptococcus dysgalactiae</i>	
	Positive	Negative	Positive	Negative	Positive	Negative
Bulk tank milk BC positive ¹ (no.)	12	0	0	0	2	0
Bulk tank milk BC negative (no.)	24	15	33	18	22	27
Sensitivity (%)		33.3		0.0		8.3
Specificity (%)		100.0		100.0		100.0
Bulk tank milk PCR positive ¹ (no.)	30	5	26	4	21	6
Bulk tank milk PCR negative (no.)	6	10	7	14	3	21
Sensitivity (%)		83.3		78.8		87.5
Specificity (%)		66.7		77.8		77.8

¹A herd was considered as positive if at least 1 of 2 samples showed a positive result.

correlation with r_s of -0.75 was found for *Strep. uberis* WHP_{app} at the quarter level when both pooled herd samples were considered.

Estimated threshold values (Table 5) of the WHP_{app} required to detect a herd as positive for each of the 3 pathogens were calculated using a logistic regression model. The threshold values varied depending on the pathogen, the kind of specimen (BTM or pooled herd sample), and the number of examined samples (single or double sampling). The highest threshold value of the WHP_{app} was calculated for *Staph. aureus* for BTM samples as well as for pooled herd samples for single sampling. The threshold value at a given $P_d = 0.9$ was 27.6% for *Staph. aureus* if 1 BTM sample was tested. In case of double sampling, the estimated threshold value dropped to 17.7%. Lower threshold values were found when pooled herd samples were used. The threshold value for *Staph. aureus* was 18.0% when a single pooled herd sample was tested and 16.1% in case of both pooled herd samples of a herd. Applying $P_d = 0.7$, the threshold value for *Staph. aureus* decreased to 15.5% for a single BTM sample examination. Threshold values for *Staph. aureus* were lower when the calculation

was based on the udder quarter level rather than on the cow level, except for the examination of a single BTM sample, which resulted in a detection limit value of 32.6%. Threshold values for the tested streptococci proved to be lower than for *Staph. aureus*. When only a single BTM sample was examined, the threshold value for *Strep. uberis* at $P_d = 0.9$ was 13.8% at the cow level and 5.3% at the quarter level. When 2 samples were tested, the threshold values were even lower for the BTM samples as well as for the pooled herd samples. The lowest threshold values were found for *Strep. dysgalactiae*. In the case of at least approximately 9.2% of the cows of a herd being infected, the herd could be tested positive at $P_d = 0.9$ testing only 1 BTM specimen.

DISCUSSION

Mastitis control in dairy herds requires the identification of major mastitis pathogens to implement adequate control measures. Testing quarter milk samples or cow level samples is labor intensive and may be expensive depending on the laboratory infrastructure available to

Table 4. Spearman's rank correlation coefficients for the relationship between the apparent within-herd prevalence (WHP_{app}) and cycle threshold values of the quantitative real-time PCR of bulk tank milk and pooled herd samples for the different pathogens¹

Item	<i>Staphylococcus aureus</i>	<i>Streptococcus uberis</i>	<i>Streptococcus dysgalactiae</i>
WHP _{app} (cow level)			
Bulk tank milk (1 sample) ²	-0.55	-0.51	-0.56
Bulk tank milk (both samples)	-0.60	-0.55	-0.68
Pooled herd (1 sample) ³	-0.59	-0.72	-0.57
Pooled herd (both samples)	-0.71	-0.74	-0.65
WHP _{app} (udder quarter level)			
Bulk tank milk (1 sample)	-0.59	-0.55	-0.61
Bulk tank milk (both samples)	-0.66	-0.57	-0.71
Pooled herd (1 sample)	-0.62	-0.74	-0.58
Pooled herd (both samples)	-0.73	-0.75	-0.68

¹P-value for all calculations <0.001 ; number of herds with valid test results = 51.

²One randomly chosen bulk tank milk sample of the 2 samples of a herd.

³One randomly chosen pooled herd sample of the 2 samples of a herd.

Table 5. Estimates of apparent within-herd prevalence (WHP_{app}) threshold value $\pm$ asymptotic SE and approximate 95% CI for classifying a positive herd as positive by quantitative real-time PCR for the different pathogens using 1 or 2 bulk tank milk samples or 1 or 2 pooled herd samples, respectively, at different probabilities of detection¹

P_d (%)	<i>Staphylococcus aureus</i>						<i>Streptococcus uberis</i>						<i>Streptococcus dysgalactiae</i>					
	Bulk tank milk sample		Pooled herd sample		Bulk tank milk sample		Bulk tank milk sample		Pooled herd sample		Bulk tank milk sample		Bulk tank milk sample		Pooled herd sample		Bulk tank milk sample	
	WHP_{app} $\pm$ SE	95% CI	WHP_{app} $\pm$ SE	95% CI	WHP_{app} $\pm$ SE	95% CI	WHP_{app} $\pm$ SE	95% CI	WHP_{app} $\pm$ SE	95% CI	WHP_{app} $\pm$ SE	95% CI	WHP_{app} $\pm$ SE	95% CI	WHP_{app} $\pm$ SE	95% CI	WHP_{app} $\pm$ SE	95% CI
WHP_{app} on cow level																		
1 Sample tested																		
70	15.5 $\pm$ 4.1	7.4–23.6	12.3 $\pm$ 2.2	7.9–16.7	7.7 $\pm$ 1.9	3.9–11.6	4.9 $\pm$ 1.2	2.5–7.3	5.9 $\pm$ 1.4	3.2–8.7	4.8 $\pm$ 1.3	2.2–7.3	5.9 $\pm$ 1.4	3.2–8.7	4.8 $\pm$ 1.3	2.2–7.3	5.9 $\pm$ 1.4	3.2–8.7
90	27.6 $\pm$ 7.8	12.0–43.1	18.0 $\pm$ 3.5	11.0–25.0	13.8 $\pm$ 3.5	6.8–20.9	8.4 $\pm$ 2.0	4.4–12.4	9.2 $\pm$ 2.2	4.7–13.6	8.2 $\pm$ 2.2	3.7–12.7	9.2 $\pm$ 2.2	4.7–13.6	8.2 $\pm$ 2.2	3.7–12.7	9.2 $\pm$ 2.2	4.7–13.6
2 Samples tested																		
70	8.2 $\pm$ 2.7	2.9–13.6	10.4 $\pm$ 2.1	6.2–14.5	6.4 $\pm$ 1.5	3.4–9.4	4.2 $\pm$ 1.2	1.9–6.6	3.1 $\pm$ 0.8	1.5–4.8	3.0 $\pm$ 0.9	1.2–4.8	3.1 $\pm$ 0.8	1.5–4.8	3.0 $\pm$ 0.9	1.2–4.8	3.1 $\pm$ 0.8	1.5–4.8
90	17.7 $\pm$ 5.4	6.9–28.4	16.1 $\pm$ 3.4	9.3–22.9	11.1 $\pm$ 2.6	5.8–16.3	7.9 $\pm$ 2.1	3.8–12.0	5.4 $\pm$ 1.4	2.6–8.3	5.7 $\pm$ 1.6	2.4–9.0	5.4 $\pm$ 1.4	2.6–8.3	5.7 $\pm$ 1.6	2.4–9.0	5.4 $\pm$ 1.4	2.6–8.3
WHP_{app} on quarter level																		
1 Sample tested																		
70	16.1 $\pm$ 9.0	–2.0–34.1	3.9 $\pm$ 0.8	2.2–5.5	2.6 $\pm$ 0.6	1.4–3.8	1.5 $\pm$ 0.4	0.7–2.3	1.1 $\pm$ 0.3	0.6–1.6	1.6 $\pm$ 0.4	0.7–2.4	1.1 $\pm$ 0.3	0.6–1.6	1.6 $\pm$ 0.4	0.7–2.4	1.1 $\pm$ 0.3	0.6–1.6
90	32.6 $\pm$ 19.8	–7.0–72.2	6.2 $\pm$ 1.4	3.4–9.1	4.3 $\pm$ 1.0	2.3–6.4	2.8 $\pm$ 0.7	1.4–4.2	1.7 $\pm$ 0.4	0.9–2.5	2.6 $\pm$ 0.7	1.1–4.1	1.7 $\pm$ 0.4	0.9–2.5	2.6 $\pm$ 0.7	1.1–4.1	1.7 $\pm$ 0.4	0.9–2.5
2 Samples tested																		
70	2.4 $\pm$ 0.8	0.9–4.0	3.1 $\pm$ 0.6	1.9–4.3	1.7 $\pm$ 0.4	0.9–2.5	1.2 $\pm$ 0.3	0.5–1.8	0.9 $\pm$ 0.2	0.4–1.4	0.8 $\pm$ 0.3	0.3–1.4	0.9 $\pm$ 0.2	0.4–1.4	0.8 $\pm$ 0.3	0.3–1.4	0.9 $\pm$ 0.2	0.4–1.4
90	5.0 $\pm$ 1.5	2.0–8.0	4.7 $\pm$ 1.0	2.8–6.6	2.9 $\pm$ 0.7	1.6–4.3	2.1 $\pm$ 0.6	1.0–3.2	1.5 $\pm$ 0.4	0.7–2.4	1.6 $\pm$ 0.5	0.6–2.6	1.5 $\pm$ 0.4	0.7–2.4	1.6 $\pm$ 0.5	0.6–2.6	1.5 $\pm$ 0.4	0.7–2.4

¹The calculation of the WHP_{app} on the cow level and on the quarter level is based on the bacterial culture of quarter milk samples.

²Probability of classifying a positive herd as positive.

the farmer. Therefore, attempts were made to use BTM as specimen to monitor mastitis pathogen occurrence within a herd. The BTM samples were examined via BC in many studies, but only a few have simultaneously tested pathogen prevalence in the cows. Several studies in which quarter milk samples and BTM were examined, showed a low sensitivity (9.2 to 58% for *Staph. aureus*) for BTM BC (Godkin and Leslie, 1990; Bartlett et al., 1991; Wilson and Gonzalez, 1997). This low sensitivity was also confirmed by our study. As a further result, it was ascertained that estimating the number of infected cows by the quantity of the mastitis pathogens present in the BTM was not possible (Ruegg and Reinemann, 2002). The PCR testing of BTM samples was described as an easy and cost-effective herd level screening method for infectious agents such as *Coxiella burnetii* (Rodolakis et al., 2007). Katholm et al. (2012) examined the BTM of 4,258 Danish herds using a commercial real-time PCR and concluded that real-time PCR can be applied in the same way as BC for monitoring BTM samples, and that PCR is particularly useful for bacteria with low prevalence. Other authors stated that PCR results of BTM testing should be confirmed by BC of BTM samples and, if possible, of composite milk samples (Zanardi et al., 2014). Consequently, it is controversial whether this approach is appropriate for herds with a low prevalence of the relevant pathogens.

Therefore, the calculation of a WHP_{app} threshold that allows the identification of a herd as positive is of interest for professional use of this approach. Our study is the first one to estimate these thresholds of the WHP_{app} at the cow level and the quarter level as determined by BC of quarter milk samples. We examined every lactating cow of the herd that was included in milk delivery. In doing this, we ensured that each cow that contributed to the bulk tank was involved in the calculation of WHP_{app} . The WHP_{app} thresholds were calculated for different settings such as single and double sampling of either a BTM sample or a pooled milk sample. We analyzed BTM samples by qPCR and BC. Depending on the pathogen, a sensitivity of 78.8 to 87.5% for qPCR analysis of BTM was calculated using BC of quarter samples as the reference method (Table 3). In BC testing of BTM samples, sensitivity values were much lower (0.0–33.3%). The few herds detected as positive by BC of BTM samples also showed positive in the reference method, the BC of quarter milk samples, resulting in a high specificity of 100%. Because of the low sensitivity, the result regarding specificity must be interpreted with caution. Taking into account the results of other studies (Godkin and Leslie, 1990; Bartlett et al., 1991; Wilson and Gonzalez, 1997), qPCR seems to be a more sensitive method for BTM testing.

In 15 cases we found pathogens in BTM using qPCR that could not be detected in the udder quarters of the herd via BC ($4 \times \textit{Strep. uberis}$, $5 \times \textit{Staph. aureus}$, and $6 \times \textit{Strep. dysgalactiae}$). This reduces the specificity of qPCR. This might be due to contamination of BTM samples, the different specimen used in the reference method or a limited sensitivity of the reference method. First, quantity and composition of the bacteria in BTM are influenced not only by the milk itself but also by several aspects such as the cleanliness of the udder skin, milker's hands, milking equipment, bulk tank, and cooling process (Reinemann et al., 1997). All of the examined pathogens can be found in the cow's environment as well as on the cow's udder surface (Roberson et al., 1994; Wolter et al., 2002; Jørgensen et al., 2016). Second, the BTM does not contain the foremilk of those cows whose milk the farmer considers fit for selling, and for the BC only foremilk was used, as recommended for BC (DVG, 2009). Third, tubes with boric acid as a preservative agent were used for all samples. This could influence the viability of the bacteria in the milk samples and thus also the sensitivity of BC to detect bacterial growth.

We accounted for the selection bias by limiting sampling to cows without clinical mastitis because the selling of milk from cows with clinical mastitis is prohibited in the European Union. Regarding herd level sensitivity in large dairy herds, the dilution effect in large bulk tanks is another major problem of BTM sampling. In low prevalence situations particularly, the number of bacteria in the sample may fall below the detection limit and cause a negative BTM test result.

Using Spearman's rank correlation coefficients, we demonstrated a correlation ($P < 0.001$) between the Ct value of the qPCR and WHP_{app}. Depending on the pathogen, specimen, and number of samplings in our study, correlations ranged between -0.51 and -0.74 on the cow level and between -0.55 and -0.75 on the quarter level. A significant correlation for the prevalence of pathogens on the quarter level for *Strep. agalactiae* and *Strep. dysgalactiae* of 0.65 and 0.36, respectively, was also found in another study (Spohr and Breitenwieser, 2012). Contrary to our results, no significant correlation could be proven for *Staph. aureus* in their study. Our results lead to the hypothesis that it may be possible to detect changes in the WHP_{app} of a specific pathogen by alterations of the corresponding Ct values in BTM. This hypothesis is subject to further research.

Testing a single BTM sample, we calculated cow level WHP_{app} thresholds of approximately 9.2% for *Strep. dysgalactiae*, 13.8% for *Strep. uberis*, and 27.6% for *Staph. aureus* at $P_d = 0.9$. On the quarter level, we found WHP_{app} thresholds of 1.7% for *Strep. dysgalacti-*

ae, 4.3% for *Strep. uberis*, and 32.6% for *Staph. aureus*. These results clearly indicate that a relevant percentage of the cows must be BC positive to get a positive test result in BTM testing. These thresholds were calculated for specific mastitis pathogens in our study and were given for 2 different probabilities of detection. We found remarkable differences in the WHP_{app} threshold between *Staph. aureus* and the streptococci. The higher thresholds for *Staph. aureus* might be caused by either a lower sensitivity of the Mastit 4 qPCR kit for the target *Staph. aureus* compared with streptococci or a lower number of bacteria per BC positive cow or quarter secreted with the milk from 1 infected quarter. Relating to 1 infected cow, the latter might cause a lower amount of DNA copies in the BTM specimen. Further investigation is necessary to determine why the threshold was higher for *Staph. aureus* than for *Strep. uberis* and *Strep. dysgalactiae*.

For all pathogens, the WHP_{app} thresholds, both on cow and quarter levels, were lower if 2 instead of 1 sample was examined. This increase in sensitivity of BTM testing by taking more than 1 sample is consistent with the results of other studies (Godkin and Leslie, 1993; Olde Riekerink et al., 2010; da Costa et al., 2016). Examining 2 instead of just 1 sample lowers the WHP_{app} threshold for the pooled herd samples, too.

In our study, both BTM samples were taken from the content of 1 bulk tank. If the pathogens were homogeneously distributed, the qPCR results of the samples should be similar and the detection limit should not be influenced by double sampling. But in only 62.4% of the herds classified as positive using bulk tank samples, the same pathogen was detected simultaneously in both bulk tank samples. This may be caused by the imperfect distribution of the pathogens in the BTM and the resulting inconsistency caused by the specimen. Therefore, the accuracy of the BTM analysis appears to be limited, independent of the applied method of examination. The "Subclinical Mastitis" expert committee of the German Veterinary Society recommended that a screening of BTM is only useful professionally for the obligatory udder-associated *Strep. agalactiae*, as all of the other pathogens causing IMI could also be found in the environment of the animals (DVG, 2010). However, *Strep. agalactiae* is not considered an obligatory intramammary pathogen anymore, as this pathogen was detected in the environment and in the gastrointestinal tract of dairy cattle (Jørgensen et al., 2016). After a positive BTM result, the infected cows and udder quarters should be identified by individual BC testing of quarter foremilk samples for further decisions regarding mastitis management on the herd level (DVG, 2010). Therefore, the classical tools for diagnos-

ing subclinical and clinical mastitis remain valuable (e.g., for monitoring the presence of certain mastitis pathogens in a herd).

An alternative method for taking bulk tank samples was used in the Danish screening program for *Strep. agalactiae*. The samples were taken during weighing-in the milk from the bulk tank to the truck tank. However, this approach may be prone to cross-contamination between herds if the sampling systems are not disinfected after use (Andersen et al., 2003).

Most studies that evaluated test characteristics of mastitis PCR systems used BC of quarter milk samples as the reference method, although some authors stated that its sensitivity and specificity is limited (Krömker et al., 2008; Paradis et al., 2012). Studies in which quarter milk samples or composite samples were analyzed in parallel with both test methods detected more pathogens by qPCR than by BC (Taponen et al., 2009; Koskinen et al., 2010; Spittel and Hoedemaker, 2012). Spittel and Hoedemaker (2012) compared the results of qPCR examination of quarter milk samples with those of pooled cow level samples. They found reduced sensitivities of 81.3% for *Staph. aureus* and 77.1% for *Strep. uberis* for pooled cow level samples, indicating that the sensitivity of the qPCR is reduced if the pathogens in the specimen were diluted by examining cow level milk samples instead of quarter milk samples.

Beside other factors, the sensitivity of qPCR depends on the choice of the cut-off (Cederlöf et al., 2012). In our study the manufacturer's instructions (DNA Diagnostic, 2015) were used, which recommended a cut-off value of 37 cycles, whereas other studies chose higher cut-off values for the examination of BTM samples (Katholm et al., 2012; Mahmmoud et al., 2013). Using a similar qPCR assay, Bennedsgaard et al. (2016) stated that the Ct value not only depended on the number of colony-forming units determined by BC but also on the pathogen itself. Rattenborg et al. (2015) showed the effect of the applied qPCR assay itself by examining BTM samples with 2 different qPCR assays (PathoProof Mastitis Complete 16 Kit and Mastit 4B). Depending on the target pathogen, they found only low to moderate agreement between the qualitative test results (presence/absence) of the 2 qPCR assays. An alternative approach to consider for qPCR testing is the use of milk samples collected in the framework of a herd improvement test during the milking process representing a proportion of the whole milk output of the cow.

Further research is necessary to evaluate qPCR testing of different milk specimens for mastitis pathogens regarding pool size, sensitivity, and specificity, as well as contamination. We were able to fill a gap of knowledge

regarding the benefit of using qPCR instead of BC in testing bulk milk samples for major mastitis pathogens.

CONCLUSIONS

Applying qPCR on BTM samples allows the detection of herds positive for the examined pathogens when WHP_{app} exceeds a threshold that was calculated in this study. Regarding herd level sensitivity, qPCR outperforms BC of 0.01 mL of BTM. Double testing further increases sensitivity; however, sensitivity is still limited. Testing of BTM using qPCR could be a reliable method for detecting an occurrence of a new pathogen in the herd, but not to confirm that a distinct herd is free from a specific pathogen. Usefulness of qPCR testing applied on several specimens for monitoring changes in prevalence of mastitis pathogens needs to be investigated. Pooled herd samples are an alternative to BTM; for specific pathogens, the use of pooled milk samples may be advantageous compared with BTM samples.

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