Effect of presampling procedures on real-time PCR used for diagnosis of intramammary infections with *Staphylococcus aureus* in dairy cows at routine milk recordings

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ABSTRACT

Aseptic procedures for milk sample collection are considered crucial for bacterial culture to avoid misdiagnosis and subsequently unnecessary treatment or culling. The objective of this field study was to investigate the effect of presampling procedures on the PCR-positivity at cycle threshold value ≤ 37 of real-time PCR assay to detect *Staphylococcus aureus* from composite milk samples at routine milk recordings while accounting for known cow-level risk factors. A total of 1,199 dairy cows from 6 herds with conventional milking parlors were sampled and tested by PCR in 2011. Following the farmers' routine premilking preparations, 624 cows of the 1,199 cows were randomly selected for bacterial culture preceded by presampling procedures. These procedures were: cleaning of udder teats, removing the first streams of milk, and 70% alcohol teat disinfection. Data on parity, somatic cell counts, days in milk, daily milk yield, fat %, and protein % were extracted from the Danish Cattle Database, whereas energy-corrected milk was calculated based on the latter 3. The within-herd prevalence of intramammary infections with *Staph. aureus* was 31%, ranging from 16 to 48% in the 6 herds. Univariable analysis showed that the presampling procedures, somatic cell counts, energy-corrected milk, and days in milk were significantly associated with PCR-positivity, whereas parity was not significant. A multivariable model with herd as random effect showed that presampling procedures decreased the chance of being PCR-positive to 0.75 (95% CI; 0.58–0.97) compared with cows where the presampling procedures were not carried out. In conclusion, presampling procedures decreased the cow's chance of being PCR-positive to *Staph. aureus*. Presampling procedures appeared to improve the specificity of PCR for detection of *Staph. aureus* by reducing false positives through destruction

of *Staph. aureus* bacteria colonizing or contaminating the teat skin, orifice, and canal. Random herd effects accounted for only 8.9%, indicating that the cluster effect due to herd management on the PCR positivity to *Staph. aureus* was virtually negligible.

Key words: presampling procedure, teat disinfection, *Staphylococcus aureus*, dairy cow

INTRODUCTION

Staphylococcus aureus is a leading cause of IMI, representing a major agent of contagious bovine mastitis, which is associated with high economic losses (Barkema et al., 2006; Kirchhofer et al., 2007). Cow-level prevalence of *Staph. aureus* has been reported from clinical and subclinical cases ranging from 3.3 and 5.2% (Bradley et al., 2007) to 37.5 and 62% (Giannechini et al., 2002), respectively. In Denmark, the prevalence of *Staph. aureus* within 20 dairy herds was 10.2% (Aarstrup et al., 1995), whereas it ranged from 24 to 34% with an overall prevalence of 32% within 57 organic and conventional dairy herds (Bennedsgaard et al., 2006). The estimated cost of mastitis in the United States was \$2.1 billion in 2007, where 70 to 80% of loss was due to subclinical *Staph. aureus* IMI (Bar et al., 2008).

A high number of teat skin and teat canal bacteria may increase the chance of getting IMI or of being falsely diagnosed as infected when the cow has no IMI. Green and Bradley (2004) reported that *Staph. aureus* has an ability to survive in the keratin of the teat canal of healthy cows, a substance normally inhibiting bacterial growth. Furthermore, *Staph. aureus* is known to adhere or colonize strongly to the mammary epithelial cells in large numbers (Frost, 1975; Frost et al., 1977; Ditcham et al., 1996). Consequently, teat skin adherence or colonization may increase the risk of false positives, leading to unnecessary culling or overtreatment with antibiotics. Hence, microbial populations of *Staph. aureus* on the teat skin or in the teat canal are common, as reported by Haveri et al. (2008). Therefore, sampling procedures are performed to aseptically obtain milk

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samples for bacterial culture (BC), to ensure that the cultured bacteria originate from an IMI and not from contamination or colonization. Thus, decreasing the microbial population of *Staph. aureus* bacteria on the teat skin or teat canal may reduce the probability of false diagnosis.

Identified cow-level risk factors for IMI with *Staph. aureus* include parity, milk yield, SCC, DIM, teat lesions or callused teats, protein percentage, and fat percentage (Schukken et al., 1991; Sol et al., 2000; Zadoks et al., 2001; Kivariva et al., 2004; Breen et al., 2009; Michel et al., 2011). The diagnostic used in these studies was based on BC of one or several consecutive milk samples. However, BC is not completely satisfactory because its overall diagnostic sensitivity for detection of IMI with *Staph. aureus* from a single quarter milk sample is only 75.5% (Sears et al., 1990). A single negative culture result is usually not proof that a cow is free of *Staph. aureus* infection; testing 2 or 3 milk samples collected on different days may be required for accurate diagnosis of infected quarters and determination of a cow's infection status (Sears et al., 1990; Anderson and Pritchard, 2012). Buelow et al. (1996) concluded that bacteriological culture often is insensitive for identification of *Staph. aureus* IMI based on culturing of 245 quarter milk samples 6 times. Bacterial culture has specific limitations in detection of *Staph. aureus* due to the intermittent shedding with cycling pattern from infected quarters during lactation (Sears et al., 1990; Walker et al., 2011), which requires consecutive samples for accurate diagnosis of infected quarters. *Staphylococcus aureus* may also be shed in amounts too low to be detected in the inoculum sample (Buelow et al., 1996; Lam et al., 1996). Moreover, the presence of residual therapeutic antibiotics in the milk sample may inhibit bacterial growth in vitro (Phuektes et al., 2001).

Reliable and rapid methods for the identification of *Staph. aureus* from milk are crucial for economically sound udder health management. As a faster alternative, PCR has been used for DNA-based direct detection of *Staph. aureus* in milk (Kim et al., 2001; Ahmadi et al., 2010). Recently, PathoProof Mastitis PCR Assay (Thermo Fisher Scientific, Vantaa, Finland), a multiplex real-time PCR technique, was developed (Koskinen et al., 2009; Friendship et al., 2010). This technique is performed directly on raw or preserved milk samples without the need for BC. The assay is characterized by a higher sensitivity and specificity than BC for detection of *Staph. aureus* IMI in cow-level and quarter-level milk samples (Taponen et al., 2009; Koskinen et al., 2009). Since 2010, Danish farmers can order cow-level PCR tests (PathoProof Mastitis PCR assay), which are easy to use, less time-consuming, and automatically obtained during routine milk recordings (Katholm, 2010).

The cow-level milk samples are collected after the milking personnel's usual premilking practices, including cleaning of teats and foremilk. In contrast to sampling for BC, no presampling procedures are carried out, including treatment of the teat ends with 70% alcohol. The objective of this field study was to investigate the effect of presampling procedures on the PCR-positivity at cycle threshold (Ct) value ≤ 37 of real-time PCR assay to detect *Staph. aureus* from composite milk samples at routine milk recordings while accounting for known cow-level risk factors.

MATERIALS AND METHODS

Study Population

Six dairy herds with Danish Holstein cows were selected from 34 Danish dairy herds participating in a project investigating relations between a bulk tank milk (BTM) PCR and the prevalence of *Streptococcus agalactiae* and *Staph. aureus* in the herds (J. Katholm, unpublished data). To be eligible for inclusion in the present study, herds had to have a conventional milking parlor system and BTM PCR Ct value ≤ 39 for *Strep. agalactiae* and *Staph. aureus* at the annual PCR testing in October 2010. The cutoff was chosen according to Katholm et al. (2012), as it is a common practice for bulk tank monitoring of *Strep. agalactiae* in Denmark; 7 herds met the criteria. To confirm that *Strep. agalactiae* was still a problem in the selected herds, monthly PCR BTM tests were taken from January to March 2011. One herd had a Ct value of 40 in each test and was excluded, whereas the remaining 6 herds had Ct values of ≤ 37 in at least 2 out of 3 tests. Information about herd size, sample size per herd, type of milking system, and PCR Ct values from BTM for *Strep. agalactiae* and *Staph. aureus* are given in Table 1. The inclusion criteria of PCR Ct value ≤ 39 was considered to make it highly likely that *Strep. agalactiae* and *Staph. aureus* were present in the study herds.

Sample Collection for Real-Time PCR Testing

Cow-level PCR-samples (composite milk samples) were taken at the routine milk recording between March 28 and May 28, 2011, after the farm personnel had carried out their routine premilking practices. Bronopol-preserved milk samples were taken with the Tru-Test Electronic Milk Meter device (Tru-Test Group Co., Auckland, New Zealand) and delivered to Eurofins Steins Laboratory (Holstebro, Denmark) within 24 h. The composite milk samples were tested using the PathoProof Mastitis PCR assay (Thermo Fisher Scientific). A total of 350 μ L of milk was used as

Table 1. Number of lactating dairy cows examined by real-time PCR and cows that received presampling procedures (PSP) for bacterial culture (BC), type of milking parlor, and cycle threshold (Ct) values of bulk tank milk (BTM) PCR testing in 6 Danish dairy herds for *Staphylococcus aureus* (SA) and *Streptococcus agalactiae* (GBS)

Herd code	Total no. of cows in herd	No. of cows receiving PSP and BC	No. with complete records	Type of milking parlor	Ct value of BTM PCR testing			
					October 2010		March 2011	
					SA	GBS	SA	GBS
H1	146	66	127	Herringbone	34	38.2	31	31
H2	372	172	317	Herringbone	34.6	32.1	31	31
H3	177	75	154	Herringbone	33.3	27	34	28
H4	225	96	193	Side by side	27	31.8	31	32
H5	282	123	251	Rotary	26.9	32	26	32
H6	187	77	157	Herringbone	31	27	32	28
Total	1,389	609	1,199					

a starting volume for DNA extraction. Cycle threshold values were recorded for all samples and targeting DNA of *Staph. aureus*; the Ct value represents the number of PCR cycles required to obtain the threshold level (Koskinen et al., 2009). The assay's thermal cycling protocol involved 40 cycles for the reaction. A clear association between Ct values and the amount of the bacteria was noted; in other words, the higher the amount of bacteria present in the milk sample, the lower the Ct value obtained with the assay (Koskinen et al., 2008). For example, low Ct values are indicative for higher DNA concentrations of *Staph. aureus* in the milk sample and vice versa.

Presampling Procedures for Culture Sampling

Quarter milk samples from 624 cows in the 6 herds were collected for examination by BC and California Mastitis Test. At the same milking, composite milk samples from all milking cows were tested by PCR. For collection of the samples, each herd was visited by 2 trained technicians from the Danish Cattle Federation. For collection of quarter milk samples for bacteriological testing, approximately 50% of the cows in each herd were selected using systematic random sampling for the milking unit as follows: in herringbone or side-by-side parlors, the first, third, and fifth unit on the one side were chosen, and the second, fourth, and sixth, and so on, from the other side. In the herd using rotary parlors, every second milking unit was chosen.

After farm personnel had carried out their routine premilking practices, quarter milk samples were collected before attachment of the milking device from the randomly selected cows with the following procedure according to the National Mastitis Council recommendations (NMC, 1999). First, the ventral surfaces of udders and teats were cleaned using paper towels. Then, the first 2 or 3 streams of foremilk from each quarter were discarded. Each teat apex was then scrubbed for

several seconds with a cotton swab moistened with 70% alcohol until it was thoroughly clean. Finally, about 5 mL of milk was collected aseptically from each quarter of each cow in clean, sterile snap-cap plastic tubes and then placed in ice boxes at 5°C. Ice boxes were delivered to the Eurofins Steins Laboratory within 24 h of collection for bacterial examination according to Buelow et al. (1996). Information on the farmers' milking practices was gathered in a questionnaire.

Statistical Analysis

Data on parity, SCC, DIM, daily milk yield, fat percentage, and protein percentage were extracted from the Danish cattle database and merged with the data of presampling procedures and PCR results for *Staph. aureus*. Energy-corrected milk was calculated based on daily milk yield, fat percentage, and protein percentage, as specified by Sjaunja et al. (1990):

$$\text{ECM} = \text{milk in kg} \times (0.122 \times \text{fat } \%) + (0.077 \times \text{protein } \%) + 0.249.$$

Initial exploratory analysis, including summary statistics of dependent and independent variables, was performed followed by hierarchical multivariable modeling. The outcome variable of interest was "PCR-positivity for *Staph. aureus*" with the explanatory variable of primary interest being presampling procedures while SCC, DIM, ECM, and parity were accounted for in the analysis.

The PCR Ct value was dichotomized based on Ct value ≤ 37 (Bexiga et al., 2011). This cutoff for the Ct value was chosen based on the recommendations of the manufacturer of PathoProof Mastitis PCR Assay (Thermo Fisher Scientific). Furthermore, Koskinen et al. (2009), who thoroughly described the details of PCR protocol and Ct values, suggested the same cut-

off as a demarcation point for differentiation between infected and noninfected samples. The sensitivity and specificity of the PCR at the cutoff for Ct value ≤ 37 has been estimated to 91 and 97%, respectively [Y.S. Mahmmod, N. Toft, J. Katholm, C. Grønbaek (Eurofins Steins Laboratory, DK-7500-Holstebro, Denmark), I.C. Klaas, unpublished data]. Presampling procedures were expressed as a dichotomous variable with 2 levels (applied or not applied). Continuous variables were transformed into categorical variables with at least 3 levels for testing the linearity and obtaining a better description of the data. Test day ECM was categorized into 3 levels: low = <24 ; moderate = ≥ 24 to <33 ; and high = ≥ 33 based on the upper and lower quartiles. Parity was grouped based on the distribution into first parity, second parity, and third or higher parity. For SCC, no discrete individual cow SCC threshold value was observed that could be used to identify all infected and uninfected cows. Therefore, the results were categorized into 3 levels based on previous studies (Dohoo and Meek, 1982; Jones et al., 1984): low SCC $<200,000$ cells/mL; moderate SCC $\geq 200,000$ to $<400,000$ cells/mL; and high SCC $\geq 400,000$ cells/mL. Days in milk was categorized into 3 levels based on the stages of lactation that were regarded as high-risk periods (i.e., cows from calving to 90 DIM and cows with more than 300 DIM).

Statistical analyses were carried out using the “glmer” function of R version 2.14.0 (Bates et al., 2011; R Development Core Team, 2011). A generalized hierarchical logistic mixed model was used to run the analysis, where herd was included as a random effect to account for within-herd clustering. A univariable logistic model was used to test the associations between dependent and independent variables. Variables with P -value ≤ 0.25 in the univariable model were offered to the initial final model. The final multivariable model was generated using a Hosmer-Lemeshow strategy (Hosmer and Lemeshow, 2000) and backward elimination by dropping the least significant variable until we got the final model, which included only variables with P -value ≤ 0.05 . Biologically reasonable 2-way interactions between the remaining significant variables in the final model were investigated.

The proportion of the total variance for IMI with *Staph. aureus* attributable to herds, intraclass correlation coefficient (ICC), was estimated assuming variance at the cow level on the logit scale was $\frac{\pi^2}{3}$, with $\pi = 3.1416$ (Dohoo et al., 2001); $ICC = \sigma_h^2 / \left(\sigma_h^2 + \frac{\pi^2}{3} \right)$, where σ_h^2 is the variance at herd level.

RESULTS

After removing the records of 15 culture-sampled cows with missing PCR results, complete records composed of 1,199 lactating cows were considered for the analysis in the present study (Table 1). Cross tabulation of dependent and independent variables are listed in Tables 2 and 3. The within-herd prevalence of IMI with *Staph. aureus* was 31% based on PCR results and ranged from 16 to 48% for the 6 herds, whereas the within-herd prevalence based on BC was 25% ($n = 151$), ranging from 13 to 33%. The routine milking practices were similar between the 6 herds and can be summarized as follows. The teats were cleaned with individual boiled towels in 5 herds, whereas 1 herd used excelsior wood wool. None of the 6 herds used any premilking teat dips or chemicals, and 5 of the 6 herds used postmilking teat dipping. No specific milking order was used for *Staph. aureus*-infected cows.

The results of univariable and multivariable logistic regressions are listed in Tables 4 and 5, respectively. Univariable analysis showed that the presampling procedures, SCC, ECM, and DIM were significantly associated with PCR-positivity, whereas parity was not. The resulting final model consisted of the significant variables, presampling procedures, and SCC. Presampling procedures decreased the chance of PCR-positive results to 0.75 (95% CI: 0.58–0.97) compared with cows where the presampling procedures were not carried out. No interaction between the tested variables was observed. The random effect of herd was 0.32 and accounted for 8.9% of the total variation.

DISCUSSION

To our knowledge, this is the first study investigating the effect of presampling procedures on the Ct values of PCR assays on composite milk samples from routine milk recording. Our findings demonstrated that presampling procedures, in connection with BC, decrease the cow's chance of having a low Ct value of PCR for *Staph. aureus* IMI.

Presampling Procedures

Teat skin, teat canal, and teat orifices are the key points for introduction and invasion of bacterial cells of *Staph. aureus*, which adhere readily and in large numbers to the ductular epithelial cells of the teats of bovine udder compared with most other bacterial species (Frost, 1975; Frost et al., 1977). Recently, Paduch and Krömker (2011) isolated *Staph. aureus* from 72.2% of 1,358 investigated teat canals of clinically

Table 2. Description and cross tabulation of potential risk factors associated with IMI of *Staphylococcus aureus* diagnosed by real-time PCR assay in 6 Danish dairy herds

Variable	Category	PCR-positivity for <i>Staph. aureus</i> IMI, no. (%)		
		Positive	Negative	Total
Presampling procedures	No: not applied	201 (34.1)	389 (65.9)	590 (49.2)
	Yes: applied	171 (28.1)	438 (71.9)	609 (50.8)
SCC	Low: <200,000 cells/mL	176 (21.3)	650 (78.7)	826 (68.9)
	Moderate: ≥200,000 to <400,000 cells/mL	82 (53.6)	71 (46.4)	153 (12.8)
	High: ≥400,000 cells/mL	114 (51.8)	106 (48.2)	220 (18.3)
ECM	Low: <24 kg	119 (40.5)	175 (59.5)	294 (24.5)
	Moderate: ≥24 to <33 kg	164 (27.6)	430 (72.4)	594 (49.6)
	High: ≥33 kg	89 (28.6)	222 (71.4)	311 (25.9)
Parity	1	195 (31.1)	433 (68.9)	628 (52.4)
	2	86 (35.1)	167 (68.2)	253 (21.1)
	>2	91 (28.6)	227 (71.4)	318 (26.5)
Stage of lactation (DIM)	Early: <90 d	80 (33.6)	158 (66.4)	238 (19.8)
	Mid: ≥90 to <300 d	205 (28.3)	517 (71.7)	722 (60.3)
	Late: ≥300 d	87 (36.4)	152 (63.6)	239 (19.9)

healthy cows. Therefore, presampling procedures can reduce the quantity of *Staph. aureus* that colonizes or contaminates the teat skin and teat orifice (Bramley et al., 1979). Hence, aseptic procedures for milk sample collection for BC are an absolute necessity.

Regardless of the farmers' premilking practices, our finding demonstrated that the presampling procedures as carried out for BC reduced the proportion of cows with positive PCR tests for *Staph. aureus* significantly in the 6 herds. This significant association between performing the presampling procedures and the PCR-positivity to *Staph. aureus* could be the result of alcohol teat disinfection, the physical effect of discarding the first 2 to 3 streams of milk before sample collection, and the 5-mL milk sample taken for BC from each quarter.

Alcohol acts by cell dehydration, disruption of cell membranes, and by causing cell protein coagulation. This destroys the bacterial cells of *Staph. aureus* (Sridhar Rao, 2008; Salvia et al., 2011), which colonize or contaminate the teat orifice or teat skin. Thus, alcohol teat disinfection improves the specificity of PCR for detection of *Staph. aureus* IMI by reducing false positive results that increase the number of positive cases that are not a true infection, but more likely a contamination.

Discarding the first streams of milk from the teat canal, including the amount taken for the BC—which includes more bacteria than the rest of the milk—minimizes chances of sample contamination for culture from bacteria located on the teat end (Bewley et al., 2012). It also reduces the quantity of bacterial cells of *Staph. aureus* that are not adhered to the teat canals (washing/mechanical effect).

Premilking Practices

Intramammary infection with *Staph. aureus* of the bovine udder is the culmination of events beginning with contamination or colonization of the teat orifices and the teat skin with the bacterial cells, entry through the teat canal, and establishment of bacterial growth within milk secretory tissue (Larsen et al., 2000; Zecconi, 2006; Barkema et al., 2009). The farm personnel's premilking practices may have reduced the amount of teat skin bacterial cells of *Staph. aureus* in both groups of cows: those with and without having samples taken for BC.

None of the 6 herds used any premilking teat dips or chemicals with teat disinfecting abilities that could have reduced the amount of *Staph. aureus* on teat skin,

Table 3. Descriptive statistics of variables included in the analysis for estimation of the effect of presampling procedures on real-time PCR used for diagnosis of IMI with *Staphylococcus aureus* from dairy cows at routine milk recordings

Variable	Mean	SD	Minimum (0%)	First quartile (25%)	Median (50%)	Third quartile (75%)	Maximum (100%)
PCR Ct ¹ value	37.7	3.3	23.3	35.7	40.0	40.0	40.0
SCC (×1,000 cells/mL)	335.8	781.9	3.0	42.5	103.0	271.0	9,999
ECM	28.7	7.8	2.0	24.2	28.9	33.4	64.0
Parity	1.9	1.2	1.0	1.0	1.0	3.0	7.0
DIM	211.4	137.7	7.0	107.0	196.0	279.0	1,069

¹Ct = cycle threshold.

Table 4. Univariable random effects logistic regression for risk factors; teat disinfection, stage of lactation (DIM), SCC, parity, and ECM associated with IMI of *Staphylococcus aureus* diagnosed by real-time PCR assay in 6 Danish dairy herds with the variable herd included as a random effect ($P \leq 0.25$)

Variable	Category	β	SE	Odds ratio	95% CI	P-value
Presampling procedures ¹	No: not applied			Ref. ²		0.024
	Yes: applied	-0.28	0.13	0.76	0.59–0.97	
SCC class ¹	Low: <200,000 cells/mL			Ref.		<0.0001
	Moderate: $\geq 200,000$ to <400,000 cells/mL	1.45	0.18	4.27	2.98–6.12	
	High: $\geq 400,000$ cells/mL	1.38	0.16	3.97	2.91–5.44	
ECM ¹	Low: <24			Ref.		<0.001
	Moderate: ≥ 24 to <33	-0.58	0.15	0.56	0.42–0.75	
	High: ≥ 33	-0.53	0.17	0.59	0.42–0.83	
Parity	1			Ref.		0.4
	2	0.13	0.16	1.14	0.84–1.56	
	>2	-0.12	0.15	0.89	0.66–1.20	
Stage of lactation (DIM) ¹	Early: <90 d			Ref.		0.04
	Mid: ≥ 90 to <300 d	-0.25	0.16	0.78	0.57–1.07	
	Late: ≥ 300 d	0.12	0.19	1.13	0.78–1.65	

¹Variables offered to the multivariable model according to Hosmer-Lemeshow strategy.²Ref. = reference value.

the rate of teat canal infections, or the new infection rate with *Staph. aureus* in general. Pankey et al. (1987) compared the efficacy of premilking teat disinfection by 3 different iodophor compounds with good udder preparation against udder preparation alone. They found that premilking teat disinfection significantly reduced the rate of *Staph. aureus* IMI by 54%. Clean boiled towels or wood wool were used in the present study, which may have mechanically reduced the amount of *Staph. aureus* on teat skin. Use of cotton towels for cleaning of teats before milk sampling could efficiently reduce number of bacteria from teat skin. This was demonstrated by Rasmussen et al. (1991), who found that premilking teat preparations using cotton towels for 20 s are effective to reduce bacteria from teat surfaces because of the physical structure of the towel, physical action on the teat surface, and scrubbing of the teat ends.

Handling of cows before taking PCR milk sample at routine milk recordings could influence the probability of the test being positive. However, the significant association of presampling procedures with PCR-positivity for *Staph. aureus* could suggest that the bacteria of *Staph. aureus* on the teat skin, despite postmilking teat dipping or premilking preparation, are either reintro-

duced from different unknown sources or the dipping time or disinfectant type is not sufficiently effective to kill the bacteria well or *Staph. aureus* adheres well to the cells of the upper part of the mammary gland (Frost et al., 1977).

The estimated random herd effects (within-herd ICC) for *Staph. aureus* was 8.9%. This finding is similar to ICC estimates for *Staph. aureus* by Barkema et al. (1997). The within-herd ICC could be explained by differences in herd management systems and hygienic measures (Zadoks et al., 2001). However, the finding of a low estimated ICC may indicate that the cluster effect of herd management on the PCR-positivity for *Staph. aureus* is virtually negligible. This low variation could be explained by similar premilking practices, breeds, conventional milking systems, as well as similar strategies or measures for mastitis control for all the study herds. However, it could also be due to preselection based on BTM PCR results.

In brief, routine premilking cow preparations or practices and postmilking dipping could be important procedures for reduction of the quantity of bacterial cells. However, these procedures are not sufficient to avoid contamination of milk samples collected for PCR testing for *Staph. aureus*. Therefore, it is recommended

Table 5. Final model: Multivariable random effects logistic analysis for risk factors associated with IMI of *Staphylococcus aureus* diagnosed by real-time PCR assay in 6 Danish dairy herds with the variable herd included as a random effect ($P \leq 0.05$)

Variable	Category	β	SE	Odds ratio	95% CI	P-value
Presampling procedures	No: not applied			Ref. ¹		0.03
	Yes: applied	-0.29	0.13	0.75	0.58–0.97	
SCC class	Low: <200,000 cells/mL			Ref.		<0.0001
	Moderate: $\geq 200,000$ to <400,000 cells/mL	1.50	0.18	4.30	3.00–6.18	
	High: $\geq 400,000$ cells/mL	1.38	0.16	3.97	2.90–5.43	

¹Ref. = reference value.

the specified presampling procedures (teat cleaning, discarding the first milk streams, and 70% alcohol teat disinfection) be performed in addition to the other routine milking practices for PCR sampling, as it is recommended for culture-sampling to reduce the number of false positives for PCR results of *Staph. aureus*. Hence, it is feasible, and even preferable, to perform such procedures at routine DHI recording before sampling for PCR testing for *Staph. aureus* IMI detection.

The study design used in this study included only PCR sampling after presampling procedures as part of the milk recording scheme, where minute amounts of milk are collected throughout the entire milking process. This design was chosen to mimic the situation in practice. A study design including both pre- and postsampling procedures for PCR testing might have been preferable, but the above-mentioned conditions could not have been mimicked, whereas milk can only be collected for the entire milking once.

CONCLUSIONS

Our findings demonstrated that presampling procedures, in connection with BC, decrease the cow's chance of having a low Ct value of PCR for *Staph. aureus* IMI. Presampling procedures may improve the specificity of PCR tests taken at routine milk recording for detection of *Staph. aureus* IMI by reducing the number of false positives and it should be performed before PCR sampling.

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