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Validation of Real-Time PCR and Bacteriological Culture for Identification of *Streptococcus agalactiae* and *Staphylococcus aureus* in Milk and on Teat Skin in Herds with Automatic Milking System

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Introduction

Based on the annual Danish bulk tank surveillance program for *Streptococcus agalactiae (S. agalactiae)* herd-level prevalence is 7 % and the majority of herds are *Staphylococcus aureus (S. aureus)* positive. Intramammary infections (IMI) with these pathogens result in financial losses for the farmer and impaired milk quality. Teat skin may be an important reservoir in automatic milking systems (AMS). Valid diagnostic tests are key for correct diagnosis and treatment.

The objective of this field study was to estimate the sensitivity (Se) and specificity (Sp) for the real-time PCR assay 'Mastit4' against bacteriological culture (BC) for the identification of *S. agalactiae* and *S. aureus* in milk and on teat skin.

Materials and Methods

Selection criteria 8 AMS herds

- ≥ 3 milking robots
- Positive status for *S. agalactiae* in bulk tank milk (PCR)
- January 2017: status confirmed by three BTM samples (PCR Ct<32)

Selection of cows and quarters

Part A (first visit):

- 30-40 cows > 200,000 cells/mL at last milk recording (5-33 days before visit) were randomly selected
- All four quarters tested with BC, right hind (RH) quarters tested with both BC and PCR

Part B (1-3 weeks later):

- Resampling ≤ 20 positive quarters (Ct < 40, CFU ≥ 1 colony) per herd by following prioritized criteria:
  - Teat skin sample positive from *S. agalactiae* by PCR or BC > milk sample positive from *S. agalactiae* by PCR or BC > Teat skin sample positive from *S. aureus* by PCR or BC > Milk sample positive from *S. aureus* by PCR or BC

Sampling protocol

Part A:

- Teat skin sample: Teat cleaned with dry paper towel. Modified wet-dry method with rayon swabs and 2 mL ¼ Ringer's solution, 360 degrees rotation
- Milk sample: Aseptic, PCR swab immersed at the lab the day after

Part B:

- Teat skin sample: As in part A but alternate order of BC (Wet-dry method) and PCR swab directly on teat skin
- Milk sample: Aseptic, PCR swab immersed on the farm

Laboratory protocol

Teat skin samples:

- 100 µl on modified Edward's medium (part A+B), calf blood agar (A+B), and SA media (A)

Milk samples:

- 10 µl on a quarter of a plate of modified Edward's medium (A+B), calf blood agar (A+B), and SA media (A)
- Incubated aerobically at 37°C. Reading after 24h (A) and 48h (A+B)

Results

Data set

- Part A: Milk and teat skin samples of 289 RH quarters (17 RH discarded) for further analysis
- Part B: 159 quarters (1 per cow) selected, complete data of 132 milk and teat skin samples for further analysis

Results from statistical analyses

- BC CFU & PCR Ct-values: Box-and-whisker plots, Spearman Rank Correlation Coefficient
- Agreement PCR & BC: Kappa & McNemar
- Sensitivity & specificity: Bayesian latent class analysis

Visit type	Pathogen	Sample type	Ct ≤ 37 and CFU ≥ 1		Ct ≤ 32 and CFU ≥ 1	
			McNemar (P)	Kappa coeff. (95 % CI)	McNemar (P)	Kappa coeff. (95 % CI)
Part A	*S. agalactiae*	Teat skin	0.000	0.031 [0-0.091]	0.000	0.041 [0-0.119]
		Milk	0.000	0.61 [0.461-0.757]	0.000	0.65 [0.500-0.795]
	S. aureus	Teat skin	0.012	0.13 [0-0.265]	0.243	-0.011 [-0.124-0.101]
		Milk	0.096	0.72 [0.577-0.864]	0.752	0.76 [0.623-0.905]
Part B	*S. agalactiae*	Teat skin	0.000	0.0084 [0-0.025]	0.000	0.011 [0-0.032]
		Milk	0.027	0.74 [0.611-0.872]	0.070	0.78 [0.652-0.901]
	S. aureus	Teat skin	0.000	0.086 [0.017-0.155]	0.000	0.24 [0.055-0.425]
		Milk	0.001	0.58 [0.376-0.787]	0.023	0.71 [0.516-0.911]

McNemar test and Kappa Comparison of polymerase chain reaction (PCR) and bacteriological culture (BC) for two different cycle threshold (Ct) value cutoffs (≤ 37 and ≤ 32) and colony forming units (CFU) ≥ 1 using McNemar test and kappa. Confidence intervals (CI) for kappa coefficients (coeff.) are shown in brackets. Moderate agreement: 0.4 < kappa ≤ 0.6, good agreement: 0.6 < kappa ≤ 0.8. McNemar p-values (P) are shown, the probability of being tested positive is significantly different for the two methods if P ≤ 0.05.

Sample type	Visit type	Pathogen	Test estimates (median in %)			
			Se_{PCR}	Se_{BC}	Sp_{PCR}	Sp_{BC}
Milk	Part A	*S. agalactiae*	96.4 [82.0-99.9]	82.4 [43.6-99.3]	93.4 [89.2-99.2]	99.7 [98.5-99.9]
		S. aureus	87.6 [68.2-98.8]	74.0 [51.8-95.6]	98.2 [95.1-99.9]	99.4 [97.5-99.9]
	Part B	*S. agalactiae*	95.4 [81.7-99.8]	88.6 [65.7-99.5]	91.4 [83.6-98.6]	97.9 [93.2-99.9]
		S. aureus	93.2 [68.3-99.7]	57.7 [31.1-88.7]	94.7 [87.8-99.5]	99.4 [96.6-99.9]

Results from Latent Class Analysis Estimates and 95 % posterior credibility intervals (PCR) (in brackets) of sensitivity (Se_{PCR}) and specificity (Sp_{PCR}) for Mastit4 qPCR Assay and sensitivity (Se_{BC}) and specificity (Sp_{BC}) for bacteriological culture for diagnosis of intramammary infections (IMI) with *S. agalactiae* and *S. aureus,* determined with latent class analysis (LCA) at cycle threshold (Ct) value cutoff ≤ 37 and colony forming units (CFU) ≥ 1. In part B, PCR swabs were immersed in milk samples within 5 minutes after collection.

- The agreement between BC and PCR for the detection of *S. aureus* and *S. agalactiae* on teat skin was poor, but good for milk samples.
- The sensitivity of PCR was higher than the sensitivity of BC for *S. aureus* and *S. agalactiae* in milk samples in both part A and B.

Take Home Message

The number of positive teat skin samples with BC were low compared to PCR, especially for *S. agalactiae,* indicating that the two methods do not measure the same disease condition. Further studies are needed before introducing PCR teat skin swabs as diagnostic tool in herd health management. PCR swabs seem to be a more sensitive method for diagnosing IMI with *S. agalactiae* or *S. aureus* compared to BC.

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