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Short communication: Comparing real-time PCR and bacteriological cultures for *Streptococcus agalactiae* and *Staphylococcus aureus* in bulk-tank milk samples

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ABSTRACT

For more than 30 yr, a control plan for *Streptococcus agalactiae* and *Staphylococcus aureus* has been carried out in more than 1,500 dairy herds of the province of Brescia (northern Italy). From 2010 to 2011, the apparent prevalence of *Strep. agalactiae* has been relatively stable around 10%, but the apparent prevalence of *Staph. aureus* has been greater than 40% with an increasing trend. The aim of this paper was to estimate and compare the diagnostic accuracy of 3 assays for the detection of *Strep. agalactiae* and *Staph. aureus* in bulk-tank milk samples (BTMS) in field conditions. The assays were a qualitative and a quantitative bacteriological culture (BC) for each pathogen and a homemade multiplex real-time PCR (rt-PCR). Because a gold standard was not available, the sensitivities (Se) and specificities (Sp) were evaluated using a Bayesian latent class approach. In 2012 we collected one BTMS from 165 dairy herds that were found positive for *Strep. agalactiae* in the previous 2-yr campaigns of eradication plan. In most cases, BTMS collected in these herds were positive for *Staph. aureus* as well, confirming the wide spread of this pathogen. At the same time we also collected composite milk samples from all the 8,624 lactating cows to evaluate the within-herd prevalence of *Strep. agalactiae*. *Streptococcus agalactiae* samples were cultured using a selective medium Tallium Kristalviolette Tossin, whereas for *Staph. aureus*, we used Baird Parker modified medium with added Rabbit Plasma Fibrinogen ISO-Formulation. In parallel, BTMS were tested using the rt-PCR. Regarding *Strep. agalactiae*, the posterior median of Se and Sp of the 2 BC was similar [qualitative BC: Se = 98%, posterior credible interval (95%PCI): 94–100%, and Sp = 99%, 95%PCI: 96–100%; quantitative BC: Se = 99%, 95%PCI: 96–100%, and Sp = 99%, 95%PCI: 95–100%] and higher than those of the rt-PCR (at 40 cycle threshold, Se = 92%, 95%PCI: 85–97%; Sp = 94%, 95%PCI: 88–98%). Also in case of *Staph. aureus*, the posterior medians of BC were generally higher than those of rt-PCR. In fact, although the Se of BC was slightly lower (rt-PCR at 40 cycle threshold, median Se = 99%, 95%PCI: 97–100%, and qualitative BC, median Se = 94%, 95%PCI: 87–99%), the Sp was much higher (rt-PCR at 40 cycle threshold, median Sp = 67%, 95%PCI: 38–97%; qualitative BC, median Sp = 95%; 95%PCI: 76–100%). Our study confirms that BC and rt-PCR are reliable diagnostic tools to detect *Strep. agalactiae* and *Staph. aureus*, and rt-PCR results should be confirmed by BC carried out on BTMS and possibly on composite milk samples.

Key words: bulk-tank milk sample, bacteriological culture, real-time PCR

Short Communication

Streptococcus agalactiae and *Staphylococcus aureus* are a cause of IMI and represent a significant economic problem because of milk production losses (Keefe et al., 1997; Schukken et al., 2009). The primary reservoir of these pathogens is the infected udder, from which the infection spreads between quarters and among cows during the milking process (Eberhart et al., 1987). Infections are typically subclinical and chronic but sometimes are characterized by clinical episodes (Smith and Hogan, 1995).

For more than 30 yr, a control plan for *Strep. agalactiae* IMI has been carried out on bulk-tank milk samples (**BTMS**) in dairy herds of the province of Brescia (Lombardy region, northern Italy). The province of Brescia extends over 4,770 km^2, and the dairy cow population is almost 161,000 in about 1,500 herds. The annual milk production is 120,000 t, which accounts for some 10% of the national production.

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Streptococcus agalactiae is a β-hemolytic gram-positive *Streptococcus*, and it is a highly contagious obligate parasite of the bovine mammary gland. For this reason eradication is achievable by means of milking hygiene practices, suitable dry-cow therapy, culling of refractory cases, and the application of biosecurity measures. In the last 2 yr, the apparent herd prevalence of *Strep. agalactiae* (calculated as the proportion of herds where at least one BTMS was positive for *Strep. agalactiae* out of the total number of herds) has been low and stable in the province of Brescia. In 2010 the apparent herd prevalence was 8.6% [8.6% = (113/1,315) × 100; 95% CI: 7.1–10.1%], and in 2011 it was 10.6% [10.6% = (155/1456) × 100; 95% CI: 9–12.2%].

Staphylococcus aureus is a facultative anaerobic gram-positive *Staphylococcus* bacterium of the mammary gland, which is commonly found also on skin surfaces, nares, vulva (Keefe et al., 1997; Capurro et al., 2010) as well as in the environment. For these reasons, good milking hygiene practices are essential as control measures. In the province of Brescia, apparent herd prevalence of *Staph. aureus* (calculated as the proportion of herds where at least one BTMS was positive for *Staph. aureus* out of the total number of herds) was 34.1% [34.1% = (434/1273) × 100; 95% CI: 31.5–36.7%] in 2010, whereas in 2011 it increased to 45.1% [45.1% = (624/1383) × 100; 95% CI: 31.5–36.7%].

In 2012 we carried out the present study to estimate and compare the sensitivity (**Se**) and specificity (**Sp**) of bacteriological cultures (**BC**) and a multiplex real-time PCR (**rt-PCR**) for *Strep. agalactiae* and *Staph. aureus* in BTMS. The BC were a qualitative bacteriological culture (**BC1_aga** for *Strep. agalactiae* and **BC1_au** for *Staph. aureus*) and a quantitative bacteriological culture (**BC2_aga** for *Strep. agalactiae* and **BC2_au** for *Staph. aureus*). Because a gold-standard assay was not available, we made use of a 2 latent class approach in a Bayesian framework to estimate the diagnostic accuracies in field conditions.

The study population comprised 165 dairy herds that were found positive on BMTS for *Strep. agalactiae* during the campaigns 2010 and 2011 of the annual control plan. In most cases, BTMS collected in these herds were positive for *Staph. aureus* as well, confirming the wide spread of this pathogen. Herd size ranged from 3 to 443 lactating cows per herd, for a total of 8,624 lactating cows. The most common system was free stalls with milking parlor, whereas tie stalls were less common.

From April to June 2012, we collected one BTMS from each herd. At the same time, 8,624 composite milk samples (**CMS**) were collected from all the lactating cows within those herds and tested for *Strep. agalactiae* with BC1_aga to estimate the within-herd prevalence and complete the eradication of *Strep. agalactiae* in the province.

Bulk-tank milk samples were directly collected by the milk-truck driver according to the following procedure. The milk was agitated in the bulk tank for 15 min before collection, and then an automatic collector was used to sample 120 mL of milk into a sterilized plastic container. Samples were immediately stored on ice, transported under refrigeration to the laboratory, and maintained at 4 ± 2°C until the analysis. Bacterial analysis was always performed within 24 h of collection.

For BC1_aga, 10 μL of milk was spread in Tallium Kristalviolette Tossin agar (Hauge and Ellingsen, 1953) and incubated for 18 to 24 h at 37°C. The blue-violet colonies with β-hemolytic activity and without esculin-halo were identified as colonies of *Strep. agalactiae*. A sample was considered positive for *Strep. agalactiae* when one or more colonies were found on plate.

For BC1_au, 10 μL of milk was spread in Baird Parker modified medium with added Rabbit Plasma Fibrinogen ISO-Formulation and incubated first for 48 ± 6 h at 37°C and then for a further 24 ± 6 h at 4°C. The black-gray colonies with a whitish area of precipitation plus an opaque zone of coagulation and with or without a transparent area of clarification (BP+ or BP−, respectively) were identified as colonies of *Staph. aureus*. The isolates were checked for coagulase production and identified using API-20 STAPH (bioMérieux, Marcy l'Etoile, France). A sample was considered positive for *Staph. aureus* when one or more colonies were found on plate.

For BC2_aga, 100 μL of milk was pipetted into the center of a Tallium Kristalviolette Tossin agar plate and then spread on the surface using a sterilized bent glass rod. Samples were plated in parallel and then incubated for 18 to 24 h at 37°C. Each sample was analyzed without dilution and in serial dilutions (1:10) up to the 10^{-7} dilution. Colonies were identified and counted at the end of incubation. A sample was considered positive for *Strep. agalactiae* when one or more colonies were counted on the plate.

For BC2_au, 100 μL of milk was pipetted into the center of a Baird Parker modified medium with added Rabbit Plasma Fibrinogen ISO-Formulation agar plate and then spread on the surface using a sterilized bent glass rod. Samples were plated in parallel and incubated first for 48 ± 6 h at 37°C and then for a further 24 ± 6 h at 4°C. Each sample was analyzed without dilution and in serial dilutions (1:10) up to the 10^{-7} dilution. Finally, colonies were identified and counted. A sample was considered positive for *Staph. aureus* when one or more colonies were counted on a plate.

The rt-PCR was developed according to the following protocol. The BTMS were enriched in tryptic soy

broth at a ratio of 1:2 and then incubated for 24 ± 2 h at 37°C. The DNA extraction was made following the instructions of the QIAamp Cador Pathogen kit (Qiagen, Hilden, Germany) using 400 μL of the enrichment so obtained. The primers and probes, with target gene for regulation of D-alanyl-lipoteichoic acid biosynthesis, sensor histidine kinase (*dltS*) of *Strep. agalactiae* and target gene surface protein A (*spa*) of *Staph. aureus*, were created according to the guidelines provided by Chiba (Chiba et al., 2009). The PCR was performed using the QuantiTect Virus Master Mix kit (Qiagen) and the DNA extraction Control kit (Bioline, London, UK). The DNA amplification procedure was carried out as follows: 5 min of denaturation at 95°C, 50 steps of denaturation at 96°C for 15 s and finally annealing and extension at 60°C for 45 s. The cutoff was selected using spiked samples during the development, optimization, and standardization of the assay. A higher cutoff was then arbitrarily selected by the researches to evaluate the potential change of rt-PCR accuracy in case of lower DNA content in BTMS. For *Strep. agalactiae* the cutoffs were 40 cycle threshold (**Ct**) and 50 Ct, whereas for *Staph. aureus*, they were 38 Ct and 50 Ct.

To estimate the diagnostic accuracy of the 3 assays, we used the Bayesian 2 latent class model proposed by Branscum (Branscum et al., 2005). In our study, the 2 latent class definitions were that of infected herd (for *Strep. agalactiae* or *Staph. aureus*) and that of infection-free herd. Because the 2 BC were based on a common biological phenomenon, we considered the possibility that those test results could be conditionally dependent (Gardner et al., 2000). As suggested by Dendukuri and Joseph (2001), we modeled this condition in terms of 2 pairwise covariances, one for the dependence between the results of BC in infected herds and one for that in infection-free herds.

To ensure a condition for model identifiability, the number of degrees of freedom of the model must be greater than the number of parameters to be estimated. The term "unconstrained parameters" refers to those parameters for which neither informative priors nor stochastic constraints are defined in the model. In our study, we had 10 parameters; that is, 3 Se, 3 Sp, 2 prevalences, and the 2 pairwise covariances among the BC. We modeled the 2 pairwise covariances as a uniform distribution on the interval (0, upper bound) to force them to be positive (Dendukuri et al., 2009). In this way we remained with 8 unconstrained parameters (the 3 Se, 3 Sp, and the 2 prevalences). However, according to the equation $q = M(2k - 1)$ (Jones et al., 2010), where q is the number of degrees of freedom, M the number of populations, and k the number of tests, we still had only 7 degrees of freedom available.

To increase the number of degrees of freedom, we divided our sample in 2 subpopulations for which we assumed different herd prevalence (Hui and Walter, 1980). For this purpose, we chose the threshold of 20 or less cows in lactation on the farm at sampling on the basis of expertise provided by the Italian Reference Centre for Bovine Milk Quality in Brescia. After this step, the total number of degrees of freedom increased to 14, exceeding the number of unconstrained parameters (8) that were modeled using noninformative β distributions.

For both the pathogens, we ran the same model with and without the pairwise covariances, to verify whether a simpler model could fit the data equally well. The deviance information criterion value (**DIC**; Spiegelhalter et al., 2002) and the Bayesian *P*-value (Gelman et al., 1995) were used to select the best model and evaluate goodness of fit.

Eventually, the selected models for *Strep. agalactiae* and *Staph. aureus* were evaluated using 2 Ct for the PCR, as stated previously. Models were run using 2 independent chains with different initial values for 410,000 iterations, with burn-in of 10,000 iterations and a thin factor equal to 50. This gave a total of 16,000 samples from the posterior distributions that were used for inference. Model convergence was evaluated according to the guidelines of Toft (Toft et al., 2007) as well as 4 convergence diagnostics available in the R package BOA (Bayesian Output Analysis, ver. 1.1.7–2, R Core Team, Vienna, Austria), namely the Geweke, Gelman and Rubin, Heidelberger and Welch, and Raftery and Lewis diagnostics. Models were implemented in WinBUGS 1.4.3 software (MRC Biostatistics Unit, Cambridge, UK 1996–2012).

A total of 165 BTMS were available for *Strep. agalactiae*, whereas only 157 were available for *Staph. aureus* because 8 BTMS were contaminated and excluded from the analysis. A total of 8,624 individual milk samples were collected from all the dairy cows in lactation and tested for *Strep. agalactiae* using BC1_aga. For 10 herds it was not possible to track down the results on CMS, and so they were excluded by further analysis. For the remaining 155 herds, Table 1 reports the number of infected herds where at least one CMS was positive for *Strep. agalactiae* and the number of infected herds where the apparent within-herd prevalence was equal or higher than 10, 20, and 30%, respectively, in the 2 subpopulations. Table 2 reports the cross-classification of test results for *Strep. agalactiae* and *Staph. aureus* in the 2 subpopulations at the 2 prespecified Ct.

For *Strep. agalactiae*, the difference in the DIC value between the model with the covariances and the model without the covariances was 1 at 40 Ct and 1.7 at 50

Table 1. Results of BC1_aga (qualitative bacteriological culture for *Streptococcus agalactiae*) on composite milk samples (CMS)

Subpopulation	Number of herds	Between-herd prevalence[1] (%)	Number of infected herds	Number of infected herds by within-herd prevalence[2]		
				≥10%	≥20%	≥30%
Small-size herds[3]	54	59.3	32	31	28	21
Large-size herds[4]	101	54.5	55	39	26	14
Total	155	56.1	87	70	54	35

[1]Herds where at least one CMS was positive for *Strep. agalactiae*.

[2]Number of infected herds with prevalence equal or higher than 10, 20, and 30%, respectively, according to test results on CMS.

[3]Small-size herds: 20 or less lactating cows.

[4]Large-size herds: 21 or more lactating cows.

Table 2. Test results of qualitative bacteriological culture (BC1), quantitative bacteriological culture (BC2), and real-time PCR (rt-PCR) for *Streptococcus agalactiae* (aga) and *Staphylococcus aureus* (au) at different cycle thresholds (Ct; data reported as in Branscum et al., 2005)

Item		Subpopulation 1 rt-PCR		Subpopulation 2 rt-PCR	
		+	−	+	−
Strep. agalactiae					
rt-PCR at 40 Ct					
BC1_aga	BC2_aga				
+	+	32	3	44	4
+	−	0	0	0	0
−	+	0	0	1	0
−	−	2	20	2	57
Total		57		108	
rt-PCR at 50 Ct					
BC1_aga	BC2_aga				
+	+	35	0	48	0
+	−	0	0	0	0
−	+	0	0	1	0
−	−	3	19	3	56
Total		57		108	
Staph. aureus					
rt-PCR at 38 Ct					
BC1_au	BC2_au				
+	+	46	0	81	0
+	−	3	0	0	0
−	+	0	0	1	0
−	−	4	0	9	13
Total		53		104	
rt-PCR at 50 Ct					
BC1_au	BC2_au				
+	+	46	0	81	0
+	−	3	0	0	0
−	+	0	0	1	0
−	−	4	0	10	12
Total		53		104	

Ct, whereas for *Staph. aureus* the difference was 3.9 at 38 Ct and 4.4 at 50 Ct. Given that a rule of thumb suggests that a difference greater than 3 in DIC value should be regarded as important (Spiegelhalter et al., 2002), we selected the model without covariances for *Strep. agalactiae* and the model with the covariances for *Staph. aureus*. Table 3 reports the posterior estimates of mean, median, and 95% posterior credible interval (**95%PCI**) of the relevant parameters (Se, Sp, prevalence, and, when suitable, the covariances) as well as goodness-of-fit statistics (DIC and Bayesian *P*-value). Visual inspection of trace plots suggests that convergence was arguably reached for all the parameters in the models. The Monte Carlo error was largely

Table 3. Herd prevalence in the 2 subpopulations, estimates of sensitivity (Se) and specificity (Sp) of the 3 assays, and goodness-of-fit statistics at the 2 prespecified cutoffs [cycle threshold (Ct)] for *Streptococcus agalactiae* (aga) and *Staphylococcus aureus* (au)

Variable[1]	Median	Mean	95%PCI
Strep. agalactiae—model without the covariance terms			
rt-PCR at 40 Ct			
Prevalence subpopulation 1	0.61	0.61	0.48–0.73
Prevalence subpopulation 2	0.45	0.45	0.36–0.55
Se BC1_aga	0.98	0.98	0.94–1.00
Se BC2_aga	0.99	0.99	0.96–1.00
Se PCR	0.92	0.92	0.85–0.97
Sp BC1_aga	0.99	0.99	0.96–1.00
Sp BC2_aga	0.99	0.99	0.95–1.00
Sp PCR	0.94	0.94	0.88–0.98
Bayes *P*-value	0.66		
DIC	40.2		
rt-PCR at 50 Ct			
Prevalence subpopulation 1	0.61	0.61	0.48–0.73
Prevalence subpopulation 2	0.45	0.45	0.36–0.55
Se BC1_aga	0.98	0.98	0.94–1.00
Se BC2_aga	0.99	0.99	0.96–1.00
Se PCR	0.99	0.99	0.96–1.00
Sp BC1_aga	0.99	0.99	0.96–1.00
Sp BC2_aga	0.99	0.99	0.95–1.00
Sp PCR	0.92	0.91	0.85–0.96
Bayes *P*-value	0.60		
DIC	35.4		
Staph. aureus—model with the covariance terms			
rt-PCR at 38 Ct			
Prevalence subpopulation 1	0.97	0.96	0.86–1.00
Prevalence subpopulation 2	0.82	0.82	0.72–0.90
Se BC1_au	0.94	0.94	0.87–0.99
Se BC2_au	0.92	0.92	0.85–0.98
Se PCR	0.99	0.99	0.97–1.00
Sp BC1_au	0.95	0.93	0.76–1.00
Sp BC2_au	0.93	0.92	0.74–1.00
Sp PCR	0.67	0.68	0.38–0.97
Covariance in infected herds	0.05	0.05	
Covariance in not-infected herds	0.01	0.03	
Bayes *P*-value	0.34		
DIC	39.5		
rt-PCR at 50 Ct			
Prevalence subpopulation 1	0.97	0.96	0.86–1.00
Prevalence subpopulation 2	0.82	0.82	0.72–0.91
Se BC1_au	0.94	0.93	0.86–0.99
Se BC2_au	0.92	0.92	0.85–0.98
Se PCR	0.99	0.99	0.97–1.00
Sp BC1_au	0.94	0.93	0.75–1.00
Sp BC2A_au	0.93	0.91	0.73–1.00
Sp PCR	0.63	0.64	0.34–0.96
Covariance in infected herds	0.05	0.05	
Covariance in not-infected herds	0.02	0.03	
Bayes *P*-value	0.34		
DIC	39.3		

[1]rt-PCR: real-time PCR; BC1: qualitative bacteriological culture; BC2: quantitative bacteriological culture; 95%PCI: 95% posterior credible interval; subpopulation 1: 20 or less lactating cows on the farm at sampling; subpopulation 2: 21 or more lactating cows on the farm at sampling; DIC: deviance information criterion.

lower than 5% of the sample standard deviation for all the parameters. Also results of the 4 convergence diagnostic tests did not give evidence of convergence failure, except in the case of the Geweke diagnostics for the Se of BC1_aga (*Strep. agalactiae*) when the PCR was set at 40 Ct, and the prevalence of *Staph. aureus* in the first subpopulation at 38 Ct.

We used a latent class model to estimate the ability of 3 assays to classify dairy herds in infected or not infected according to the contamination of BTMS. However, the definition of "infected herd" is not unique, because it can go through many intermediate states. For instance, a herd can be defined "highly infected" when many cows are shedding high number of viable bacteria with milk, but it can be also defined "lightly infected" when cows just pass nonviable bacteria or DNA fragments (Mahmmod et al., 2012).

In the case of highly infected herds (that is, many cows shedding high numbers of viable bacteria in BTMS), BC may be superior in accuracy to PCR assays (Mweu et al., 2012). Results of our study (Table 3) suggest that the BC for *Staph. aureus* and *Strep. agalactiae* could be superior to rt-PCR. We believe that the main type of infection being present in our study population was that of "highly infected herd" for *Strep. agalactiae* and *Staph. aureus*. This hypothesis is supported by historical data and epidemiological considerations. First, we included in the study herds that have had a history of IMI and where positive BTMS for *Staph. aureus* and *Strep. agalactiae* had been found in the previous years. Second, results of *Strep. agalactiae* presence in CMS collected in parallel suggested a high level of infection for *Strep. agalactiae* within the herds. In fact, assuming that the condition of high infection of a herd could be associated to a within-herd prevalence of 20% or more, almost all of the infected small size herds (28 out of 32) and about half of the large-size herds (26 out of 55) of the study population were in this condition (Table 1). Finally, when we increased the Ct, the estimated herd prevalences and the diagnostic accuracy of the assays (rt-PCR + BC) did not change substantially (Table 3). Conversely, if accuracies (or herd prevalences) had instead changed, this would have meant that the assays were targeting different types of infection in the study population, for instance from highly infected herds at the lowest Ct to a combination of heavily and slightly infected herds increasing the Ct (Mweu et al., 2012).

Regarding the statistical model, the final model for *Strep. agalactiae* did not include the covariances, whereas that for *Staph. aureus* did. The inclusion of the covariances in the model for *Staph. aureus* did not change the diagnostic accuracy of the assays, except for the Sp of the PCR, that significantly increased at both Ct. Finally, the posterior distribution of the covariance in infected herds was bimodal, with one mode at zero and one at a very low positive value, whereas the covariance in infection-free herds was unimodal, with the mode approximately at zero. This suggests that, even if the model for *Staph. aureus* accounted for the dependence between the test results of the BC, this was weak.

The posterior medians of Se and Sp of rt-PCR for *Strep. agalactiae* were generally lower than that of the BC, regardless the cutoff (Table 3). The corresponding 95%PCI of the 3 assays overlapped. We point out that overlapping 95%PCI is a condition for which it is not necessarily true that estimates are not significantly different. Then, as previously mentioned, BC could be really superior to rt-PCR in highly infected herds.

The accuracy of rt-PCR was slightly lower than the accuracy of a similar PCR reported in a recent study (Mweu et al., 2012). In that study, the estimated herd prevalences were extremely low, and this indicates a very different study population; as diagnostic test properties may change with disease prevalence (Leeflang et al., 2009), this may explain such difference. Also the precision of our estimates was lower than that reported by Mweu et al. (2012), but this could be due to the small sample size and to the small difference between the prevalences of the 2 subpopulations in our study (Toft et al., 2005).

Regarding *Staph. aureus*, the median value of the Se of the rt-PCR was higher than that of the BC, but that of the Sp was much lower, regardless the cutoff (Table 3). Also in this case, the 95%PCI overlapped. The 95%PCI of the Sp of the rt-PCR was particularly large. This could be due to the small sample size (157 herds) but also to the high prevalence in the 2 subpopulations. These conditions probably did not provide sufficient data on healthy herds to reduce uncertainty.

In conclusion, our study confirms that BC and rt-PCR are suitable to detect *Strep. agalactiae* and *Staph. aureus* in heavily infected herds. The rt-PCR with Ct values higher than 40 for *Strep. agalactiae* and 38 for *Staph. aureus* could represent an appealing alternative because of speed and ease of use, and results should be always confirmed by bacteriological culture carried out on BTMS and possibly on CMS.

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