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Diagnostic value of composite milk sample vs single quarter milk sample for the detection of *Staphylococcus aureus* intra-mammary infections in cattle

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is one of the most important causes of mastitis in dairy cattle. Control and eradication programs of *S. aureus* intra-mammary infections (IMI) are based on different factors included the correct detection and management of the infected cows. The present study aimed at evaluating the efficacy of composite milk samples (CMS) analysis, compared to quarter milk samples (QMS) analysis, for the bacteriological detection of *S. aureus* intra-mammary infections. During 2016, 661 CMS (hygienically collected) and 2644 QMS (aseptically collected) were obtained from 661 cows in 5 herds. All the samples were submitted to *S. aureus* bacteriological culture and somatic cell count (SCC) analysis.

QMS bacteriological analysis on blood agar plates was able to detect 236 cows excreting *S. aureus*, while the bacteriological analysis of CMS, using selective agar, identified 229 positive cows. The concordance was 95% with an excellent Cohen's κ (0.89). Relative sensitivity and specificity of CMS vs QMS, considered as the reference test, were 91.5% ± 2.1 and 96.9% ± 1.3 (CI 95%), respectively. In addition, the relative sensitivity of CMS improved as the number of infected quarters per cow and the number of colony forming units (cfu) per sample increased.

The predictive value of CMS results was better when paired with SCC data, in particular CMS showed better negative predictive value when SCC was < 200,000 cells/mL and better positive predictive value when SCC was > 200,000 cells/mL. The probability for a cow to be *S. aureus* positive was 56.4% in case of SCC > 200,000 cells/mL, while it was 18.6% in case of SCC < 200,000 cells/mL. The average SCC in CMS was significantly higher in positive cows and the value rose as the number of infected quarters per cow increased.

Given the intermittent excretion of *S. aureus* in milk from dairy cows, it could be more advantageous to carry out several serial CMS, rather than few QMS, being CMS an easier to collect and less expensive milk sampling method. Thus, bacteriological examination of CMS, combined with SCC data of the same sample, could be extremely useful for the success of *S. aureus* IMI control plans, because repeated CMS are easier to be performed and could be more easily proposed to the farmers.

Abbreviations: IMI, intra-mammary infection; CMS, composite milk sample; QMS, quarter milk sample; SCC, somatic cell count; *S. aureus*, *Staphylococcus aureus*; CFU, colony forming units; RPF, Baird-Parker agar with Rabbit Plasma Fibrinogen; ASE, Blood Agar with Esculin; GLMs, generalized linear models; GLMMs, generalized linear mixed models; LMs, linear models; LMMs, linear mixed models; Se, sensitivity; Sp, specificity

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1. Introduction

Staphylococcus aureus (*S. aureus*) is the major cause of contagious mastitis in dairy farms worldwide, with a serious impact on production and consequently on the profitability of the farms. At farm level, the control and the eradication of *S. aureus* infection are based on the application of good milking practices, but, in the majority of the cases, identification and culling of infected cows must be applied for a successful control of the disease. For this purpose, different diagnostic methods, such as the culture of individual cow milk samples or PCR on individual or pooled milk samples, can be used (Sartori et al., 2017). The method of choice (considered as the gold standard) is still represented by the sterile sampling of single mammary quarters (QMS), followed by a non-selective examination on blood agar plates. For the best results, QMS examination should be repeated up to 3 times during 3 weeks, because the excretion of *S. aureus* by some cows with intramammary infection (IMI) is low or intermittent (Andersen et al., 2010). Based on QMS test, a mammary quarter is considered infected (IMI) when the pathogen is isolated from at least 1 sample with more than 1000 cfu/mL or when 2 out of the 3 consecutive weekly samples are culture-positive (Andersen et al., 2010). However, due to time and costs needed for QMS, a sample of composite milk from all 4 cow quarters (CMS) is often preferred and collected by practitioners. CMS can be taken sterilely or simply hygienically, after dipping and cleaning of the teats. When sample collection is done in a non-sterile way, CMS must be analysed on a selective medium (Reyher and Dohoo, 2011). Several selective culture media are available, based on the recognition of *S. aureus* coagulase activity or on the presence of specific chromogens (Reyher and Dohoo, 2011; Graber et al., 2013). This approach represents a valid alternative method in diagnosing IMI caused by *S. aureus*, since cultural positivity of CMS shows a good agreement with QMS bacteriological results, especially when more than one mammary quarter is infected (Buelow et al., 1996; Reyher and Dohoo, 2011). In addition, recent papers showed a good reliability of single QMS, taken once, compared to the gold standard (i.e. several single QMS, taken in a given time) with Sensitivity (Se) and Specificity (Sp) respectively of 90.4% and 99.8%. (Lam et al., 1996; Reyher and Dohoo, 2011).

Somatic cell count (SCC) is a well-known indicator of IMI and the practical cut-off used to distinguish between infected and uninfected quarters is commonly considered 200,000 cells/mL for QMS (Schukken et al., 2003). However, when using CMS, the SCC seems to have a limited predictive value (Se of 31–54%) in case of *S. aureus* infection (Buelow et al., 1996).

The aim of this study was to investigate whether CMS analysis (i.e. the combination of CMS bacteriological results and SCC data) can be used to detect *S. aureus* IMI during specific control plans. For this purpose, the concordance between CMS and QMS bacteriological analysis was evaluated, taking into account the results of the two tests, the number of infected quarters per cow and the level of *S. aureus* excretion obtained from QMS and SCC results from CMS.

2. Materials and methods

2.1. Milk samples

Milk samples were collected from June to November 2016, from 661 cows reared in 5 dairy farms, ranging from 37 to 239 lactating cows and located in Northern Italy. From all the cows, the following samples were taken in sequence: first of all, CMS (n = 661) were obtained after a simple teat pre-dipping and drying; immediately after, each teat was carefully disinfected and QMS (n = 2644) were sterilely collected (NMC, 1999). The milk samples were promptly stored at 4 °C, sent to the laboratory and analysed within 16–18 hours from the collection.

2.2. Bacteriological examination

CMS were cultured on Baird-Parker agar with Rabbit Plasma Fibrinogen (RPF), by inoculating 20 µL of the sample on half agar plate. QMS were cultured on Blood Agar with Esculin (ASE) and on RPF, by inoculating 10 µL of the sample on plates divided in 4 sectors. The plates were incubated at 37 °C and observed at 24 and 48 h of incubation in order to identify the typical *S. aureus* colonies (black with opaque coagulation halo on RPF and beta-haemolytic on ASE). Doubtful colonies, poorly or non-haemolytic on ASE or with reduced halo on RPF, were verified by tube coagulase test.

Positive cultures were defined according to the presence of *S. aureus* colonies in pure or mixed culture. QMS positive cultures were semi-quantitatively (growth classes) evaluated according to NMC guidelines, slightly modified: 1+, scarce growth, (1–10 cfu); 2+, moderate growth, (11–50 cfu); 3+, high growth (50–100 cfu); 4+, very high growth (> 100 cfu) (NMC, 1999).

2.3. Somatic cell count

After bacteriological seeding, sodium azide (0.024 g/100 mL) was added to the samples and the analysis for determining SCC were performed within 48 h using Fossomatic 5000® (FOSS, Denmark).

2.4. Statistical analysis

The results obtained from CMS and QMS bacteriological tests were compared by calculating the concordance and the Cohen's kappa coefficient (κ) for the data of each dairy farm separately and for the five farms together. The existence of statistically significant differences in Cohen-κ coefficients between farms were estimated through permutation tests with 100,000 iterations. According to Reyher and Dohoo (2011), single QMS was used as the reference method, thus relative Se and Sp were determined in relation to an imperfect test for the practical purpose of using CMS instead of QMS. Sensitivity (Se) and Specificity (Sp) of CMS, with QMS as the reference, were evaluated, using generalized linear models (GLMs) with binomial error distribution and a logit link (logistic regression). Specifically, separate analyses were carried out for QMS-positive samples (estimation of Se) and QMS-negative samples (estimation of Sp), using the CMS outcomes as response variable (Dohoo et al., 2011a, 2011b). In a similar way, the positive (PPV) and negative (NPV) predictive values of CMS analyses were estimated using logistic regressions with QMS outcomes as response variables. Specifically, separate analyses were carried out alternatively for CMS-positive (estimation of PPV) and CMS-negative (estimation of NPV) samples (Dohoo et al., 2011a, 2011b). Confidence intervals were estimated using profile likelihood methods (Venables and Ripley, 2002).

In addition, GLMs were implemented to assess whether the Se of CMS (representing the response variable) is affected by different explanatory variables (i.e. predictors). The explanatory variables used in the analysis were: the farm of origin, the number of infected mammary quarters per cow obtained from QMS, the levels of somatic cell count (SCC) obtained from CMS, the mean of SCCs from the four quarters obtained from QMS, the mean of the semi-quantitative bacteriological counts of QMS positive cultures, the number of the lactations, and the number of days in lactation. Following Dohoo et al. (2011b), the levels of SCC found in QMS and CMS were defined as categorical variables: SCC above or below 200,000 cells/mL. Specifically, the response variable (Se) was modelled for dependence on the explanatory variables using a forward stepwise selection procedure with log-likelihood ratio test to define the model providing the better predictions, by using < 0.05 as the inclusion criterion and > 0.10 as the exclusion criterion (Venables and Ripley, 2002).

GLMs were also used to provide estimates of the differences in PPV and NPV of CMS as a function of the level of SCC found in CMS and

Table 1
Concordance between composite milk sample (CMS) and quarter milk sample (QMS) results in the five investigated dairy farms. Concordance and Cohen's κ were calculated for each farm and for the entire set of samples.

Farm	Milking Cows (n)	CMS positive cows (%)		QMS positive cows (%)		Concordance (%)	κ of Cohen
1	239	100	(41.8)	97	(40.6)	93.7	0.87
2	131	25	(19.1)	34	(26.0)	93.1	0.80
3	205	56	(27.3)	54	(26.3)	97.1	0.93
4	37	15	(40.5)	17	(45.9)	94.6	0.89
5	49	33	(67.3)	34	(69.4)	98.0	0.95
tot	661	229	(34.6)	236	(35.7)	95.0	0.89

Table 2
Forward stepwise model selection for sensitivity (Se) in CMS from GLMs. Comparisons made through log-likelihood ratio test. The best models for *m* variables are shown, with the log-likelihood (loglik), number of parameters (*k*) and p-value (*p*) of the comparison with the *m*-1 variables best model.

Response variable	Model with *m* variables	loglik	*k*	*p*
Se in CMS	~ 1[a]	−68.49	1	–
	~ Semiq. in QMS[b]	−60.14	2	$4.4*10^{-5}$
	~ Semiq. in QMS + Pve Quart in QMS[c]	−53.86	4	0.0018
	~ Semiq. in QMS + Pve Quart in QMS + SCC in QMS[d]	−52.03	5	0.057

[a] Null model.
[b] Semiq. in QMS: mean of the semi-quantitative bacteriological counts of QMS positive cultures.
[c] Pve Quart in QMS: the number of infected mammary quarters per cow obtained from QMS.
[d] SCC in QMS: mean SCCs from the four quarters obtained from QMS.

defined as categorical variable: SCC above or below 200,000 cells/mL, as in Dohoo et al. (2011b).

Finally, linear models (LMs) were used to assess whether the (log-transformed) SCC derived from CMS were related to different fixed effects; specifically: (i) the results (positive or negative) of the bacteriological selective cultures, and (ii) the number of infected mammary quarters (resulted from QMS). Significance of the predictors was evaluated through F-tests. Analyses were performed using packages "stats", "MASS", and "lmtest" of the statistical software R (R Core Team, 2016).

3. Results

The apparent prevalence of infected cows estimated through QMS cultures in the five different farms ranged between 26.0% and 69.4%. QMS identified 236 cows (35.7%) positive to *S. aureus*, while CMS revealed a total of 229 (34.6%) positive cows. In the five different investigated farms, the concordance of the 2 tests ranged between 93% and 98%, while the Cohen-κ varied between 0.80 and 0.95. There were no statistically significant differences in Cohen-κ coefficients between farms. The analysis of the concordance between the 2 tests on all the examined samples (661) resulted 95.0% (confidence interval [CI] 95%: 93.3–96.7%) and the Cohen-κ 0.89 (CI95%: 0.87-0.91). The prevalence of infected cows obtained from CMS and QMS and the levels of concordance between the two methods, estimated for each farm, are shown in Table 1.

Table 4
Efficacy of CMS bacteriological analysis to identify *S. aureus* positive cows. Estimates of sensitivity (Se) of CMS test in relation to the number of *S. aureus* positive quarters obtained from QMS.

Number of *S. aureus* positive quarters (QMS)	N. of QMS Pve cows	CMS results		Se (CI95%)
		Pve	Nve	
0	0	13	412	–
1	121	103	18	85.1 (78.1–90.7)
2	61	60	1	98.4 (93.0–99.9)
3 - 4	54	53	1	98.1 (92.1–99.9)

QMS = quarter milk sample; CMS = composite milk sample; Pve = Positive; Nve = Negative; CI = confidence interval.

The estimates (obtained through GLMs) of Se and Sp for CMS were 91.5% (CI95%: 87.5–94.6%) and 97.0% (CI95%: 95.1–98.3%), respectively. The estimates of PPV and NPV for CMS were 94.3% (CI95%: 90.8–96.8%) and 95.4% (CI95%: 93.2–97.1%), respectively.

The statistical analysis of the variables affecting the level of sensitivity (Se) in CMS revealed that the number of infected mammary quarters per cow obtained from QMS and the semi-quantitative bacteriological counts of QMS positive cultures were the only predictors included in the best model following forward stepwise selection (see Table 2). Further insights showed that the semi-quantitative bacteriological counts of QMS positive cultures strongly affects the Se in CMS when only one mammary quarter per cow is infected (n = 121, p = 0.00036), while it did not show any significant effect on Se in CMS when the number of infected mammary quarters is larger than one (n = 115, p = 0.26). Specifically, Table 3 showed that, the Se of CMS test, found in case of cows with only one positive quarter at QMS, significantly increased as a function of the semi-quantitative bacteriological count of the positive cultures ($p < 0.001$). In detail, the results obtained in the analysis showed increasing values of Se, which ranged from 58.1%, when QMS positive quarter excretion was classified as" 1+", to > 90%, when QMS positive quarter excretion was classified as" 2+" or higher. Furthermore, the estimate of the performance of CMS, evaluated as a function of the number of infected mammary quarters obtained from QMS, displayed significantly lower level of Se in animals with one quarter positive to *S. aureus* (85.1%) than in animals with more than one positive quarter, with Se ranging from 98.1% to 98.4% ($p < 0.001$, see Table 4).

The predictive values of CMS were evaluated in association with the results of the SCC classes obtained from the same CMS. The estimates of NPV and PPV with SCC < 200,000 cells/mL were 98.6% (CI95% 96.8–99.6%) and 89.0% (80.6–94.8%), respectively. The marginal estimates of NPV and PPV with SCC < 200,000 cells/mL were 98.5% (CI95% 96.1–99.6%) and 90.8 (CI95% 79.9–99.9%), respectively.

Table 3
Efficacy of CMS bacteriological analysis for the identification of *S. aureus* positive cows. Estimates of CMS sensitivity (Se) in relation to the semi-quantitative *S. aureus* presence in QMS, in case of cows with only one *S. aureus* positive quarter.

Semi-quantitative QMS bacteriological analysis (growth class)	N. of cows with only 1 Pve quarter at QMS	CMS results		Se (CI95%)
		Pve	Nve	
1–10 colonies (1+)	31	18	13	58.1 (40.6–74.3)
11–50 colonies (2+)	35	34	1	97.0 (87.3–99.8)
51–100 colonies (3+)	35	33	2	94.6 (84.2–99.1)
> 100 colonies (4+)	20	18	2	90.0 (72.2–98.3)

QMS = quarter milk sample; CMS = composite milk sample; Pve = Positive; Nve = Negative, CI = confidence interval.

Table 5
Results of *S. aureus* bacteriological examination: number of positive quarters obtained from QMS vs somatic cell count (SCC) in CMS (mean and CI95%).

Number of *S. aureus* positive quarters (QMS)	N. of cows	Log_{10} SCC CMS (CI95%)
1	103	5.45 (5.36–5.55)
2	60	5.60 (5.46–5.73)
3–4	53	5.83 (5.69–5.97)

QMS = quarter milk sample; CMS = composite milk sample; CI = confidence interval.

The estimates of NPV and PPV with SCC > 200,000 cells/mL were 88.6% (CI95% 82.6–93.1%) and 96.8% (93.2–98.8%), respectively. The marginal estimates of NPV and PPV with SCC > 200,000 cells/mL were 88.2% (CI95% 77.4–94.3%) and 97.1 (91.8–99.1%), respectively. Moreover, the estimate of NPV showed a better value when SCC was < 200,000 cells/mL, than when SCC was > 200,000 cells/mL, and this difference was significant ($p < 0.001$).

In addition, the estimate of PPV showed a better value when SCC was > 200,000 cells/mL, than when SCC was < 200,000 cells/mL, and also this difference was significant ($p = 0.0235$).

Moreover, the estimate of the probability that a cow with SCC < 200,000 cells/mL tested positive to *S. aureus* at CMS was 18.6% (CI95%: 14.9–22.8%); while, in case of SCC > 200,000 cells/mL, the probability of testing positive to CMS rose to 56.4% (CI95%: 50.7–62.0%), and the difference was significant ($p < 0.001$). The average SCC in CMS was significantly higher in positive cows through QMS (5.58 Log_{10}, CI95%: 5.49–5.66) than in negative ones (4.45 Log_{10}, CI95%: 4.89–5.01, $p < 0.001$). In addition, the level of SCC in cows positive to CMS rose from 5.45 to 5.83 Log_{10} with the increasing of the number of infected quarters per cow at QMS (Table 5); the estimate of SCC in CMS increased with the number of infected quarters per cow ($p < 0.001$).

4. Discussion

The usefulness of CMS has previously been described for contagious pathogens of IMI, such *S. aureus*, for practical approach in control programs (Buelow et al., 1996; Lam et al., 1996; Reyher and Dohoo, 2011). In this study, we compared the results of *S. aureus* bacteriological examination of milk, obtained from QMS and from CMS. Cohen's κ was found to be good or very good for all the farms (Cohen's $\kappa \geq 0.80$) (Kwiecien et al., 2011). In the present study, single QMS was considered as the reference method, as in Reyher and Dohoo (2011), thus the calculated relative Se and Sp of CMS were determined in relation to an imperfect test (single QMS) and with the practical purpose of using CMS instead of QMS. Data showed a promising level of relative Se of CMS test compared to QMS, considered as the reference test (Se 91.5%; Sp 97.0%). Other studies reported a moderate Se (63%) and high Sp (98%), but the Se increased at 96% for high-shedding cows (Lam et al., 1996) and Reyher and Dohoo (2011) reported a Se of 77.1% (CI95% = 73.3–80.5%) and a Sp of 99.6% (CI95% = 99.4–99.8%). In case of CMS taken in 3 subsequent days, but pooled together and cultured once, Se and Sp increased up to 100% (CI95% = 76–100% for Se; CI95% = 90–100% for Sp) (Buelow et al., 1996).

False-positive results (positive CMS vs negative QMS) could be interpreted as the consequence of a contamination of milk samples by *S. aureus* from the teat surface due to the non-sterile sampling (Buelow et al., 1996). On the contrary, false-negative results (CMS negative vs QMS positive) could be most likely attributed to *S. aureus* low excreting infected quarters, or to the dilution effect of the milk from the healthy mammary quarters. In other studies, false-negative results have been reported to occur in case of lower volume of milk (10 μL) used for the bacterial culture (Buelow et al., 1996; Lam et al., 1996; Reyher and Dohoo, 2011).

Previous investigations showed that the predictive value (relative Se) of CMS vs QMS, considering the number of infected quarters per cow, increased from 58%, when only one quarter was infected, to 89% when all the quarters were *S. aureus* positive (Lam et al., 1996). Other authors reported results that ranged from 72.7%, for one infected quarter, to 92.6% in case of more than three infected quarters (Reyher and Dohoo, 2011). Our study showed higher test performances than those mentioned before, since the relative Se ranged from 85.1%, for one infected quarter, to 98%, for 3 or more infected quarters. This could be partially explained by the larger amount of milk that was used for the inoculum (20 μL) of CMS (Barkema et al., 2006).

In the present study, the level of *S. aureus* excretion from the positive quarters was also considered. The relative Se of the CMS was strongly influenced by the number of colonies detected in the QMS. When the relative Se of CMS was evaluated on cows with only one positive quarter, the lowest performance (58.1%) was obtained for the growth class 1+ (from 1 to 10 cfu), since the majority of negative CMS corresponded to weakly positive QMS. These false negative results should be carefully evaluated, because several researchers consider positive only samples exhibiting more than 10 cfu (approximately 1000 cfu/mL) (Andersen et al., 2010). Conversely, when larger amounts of *S. aureus* were excreted by the infected quarter (growth classes 2+, 3+, 4+ - > 10 cfu), the relative Se of the CMS was good or very good (> 90%).

SCC in CMS showed a significant difference between positive and negative samples ($p < 0.001$), in particular when SCC of CMS was > 200,000 cells/mL (56.4% probability of true infection), confirming the pathogenicity of *S. aureus* in subclinical mastitis as widely known. In addition, our analysis highlighted the increasing of SCC values in CMS with the increase of the number of infected quarters per cow. In this regard, it's important to remember that cows with high SCC and more than one *S. aureus* infected quarters have a lower cure rate when the therapy is applied during lactation (Sol et al., 1997).

False negative bacteriological results of CMS (CMS negative / QMS positive) involved samples that showed high SCC, while false positives CMS (QMS positive / QMS negative) were characterized by low SCC values.

In conclusion, this work demonstrated that the majority of the false negative results obtained from CMS bacteriological analysis was most likely a consequence of the dilution effect (Lam et al., 1996; Reyher and Dohoo, 2011). False positives were very few and the main cause was probably the environmental contamination (Reyher and Dohoo, 2011). This study evaluated the results obtained using CMS from a single sampling, but it should be considered that repeated sampling is crucial for *S. aureus* control and eradication programmes, due to intermittent or low-grade shedding of the pathogen (Barkema et al., 2006). In this view, it could be more advantageous to carry out several serial CMS, rather than few QMS. Finally, the bacteriological examination of hygienically collected CMS can be extremely useful, being easier to perform than the sterile QMS, less expensive and consequently more easily to be proposed to the farmers for repeated sampling; in addition, it can be highly informative, especially when combined with SCC data of the same samples.

Declarations of interest

None.

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