Bayesian Estimation of qPCR and Bacterial Culture Accuracy for Detection of Bovine Coagulase-Negative Staphylococci from Milk and Teat Apex at Different Test Cut-off Points

Running headline: **qPCR and Culture for non-aureus *Staphylococci* Diagnosis**

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Abstract

Aim: primarily to estimate the sensitivity (Se) and specificity (Sp) of the commercially available Mastit4 qPCR assay and bacterial culture (BC) for diagnosis of intramammary infections (IMI) and teat apex colonization (TAC) with coagulase-negative staphylococci (CNS) at different cut-offs for qPCR cycle threshold (Ct) values using Bayesian latent class analysis (LCA). A secondary objective was to evaluate two cut-offs of BC for diagnosis of IMI and TAC with CNS.

Methods and Results: we randomly selected 13 to 20 cows with subclinical mastitis from 8 dairy herds. Teat skin samples and aseptically collected foremilk samples were collected from the right hindquarters (n = 149) for BC and qPCR analysis. The Se of qPCR was always higher than BC_{Se} in diagnosis of IMI, however; the Sp of BC was higher than $qPCR_{Sp}$. BC_{Se} and BC_{Sp} showed no substantial difference between the tested BC cut-offs. In contrast to IMI, estimates of BC and qPCR in diagnosing TAC were different. BC_{Se} was higher than $qPCR_{Se}$ at all tested cut-offs, however; $qPCR_{Sp}$ was higher than BC_{Sp}.

Conclusion: the overall performance of qPCR is higher than BC in the diagnosis of IMI however; the performance of BC is better than qPCR in diagnosis of TAC. The qPCR and BC are valid diagnostics for bovine IMI with CNS. Whereas for TAC, both techniques require further investigation to reduce the uncertainty of the true status of the quarter and teat skin.

Significance and Impact of the Study: we reported, for the first time, the diagnostic performance of new mastitis technology (Mastit4 PCR) and culture for detection of CNS in milk and non-milk samples in dairy herds with automatic milking systems. Our findings will improve the interpretation of the test results of culture and qPCR assay and subsequently, will strengthen the control of IMI with CNS in dairy cows.

Keywords: bovine mastitis; non-aureus staphylococci; teat skin colonization; diagnostic test evaluation; sensitivity and specificity; latent class analysis

Introduction

Coagulase-Negative Staphylococci (CNS) are considered the most common cause of intramammary infections (IMI) in dairy cows (Piepers *et al.* 2007; De Visscher *et al.* 2016). In Denmark, Katholm *et al.* (2012) found staphylococci species including *Staphylococcus aureus* in 100% of the bulk tank milk samples from all 4,258 Danish dairy herds in 2009 using a commercial real-time qPCR analysis. Traditionally, CNS are considered as minor IMI pathogens in dairy cows. However, CNS that persist in the mammary gland have a high antimicrobial resistance capacity and moderately increase milk somatic cell count (Nobrega et *al.* 2018; Qu *et al.* 2019). Sørensen *et al.* (2010) estimated the economic losses due to IMI with CNS to be € 380. Additionally, Bexiga *et al.* (2011a) found the average cost to be € 38.7 for antimicrobial treated quarters with CNS subclinical mastitis. Beside its important role in causing IMI, CNS have the ability to colonize extra-mammary habitats such as teat apex and teat canal (Vanderhaeghen *et al.* 2014; Souza *et al.* 2016). Therefore, improving control measures including efficient diagnostic methods for identifying CNS IMI is vital for improving of udder health in dairy herds.

DNA-based molecular diagnostics such as quantitative PCR (qPCR) assays are increasingly implemented as a routine method for mastitis control programs in the last few years in several countries (Nyman *et al.* 2016; Timonen *et al.* 2017; Soltau *et al.* 2017). However, conventional bacterial culture (BC) is still regarded as the gold standard for mastitis diagnosis and implemented in routine diagnostic by most laboratories worldwide (Persson Waller 2013). Despite that, standard BC may show few limitations such as time-consuming, and limited precision in bacterial species identification (Ruegg 2009). Therefore, genotypic identification systems became an acceptable diagnostic tool for most of the mastitis pathogens to overcome misclassification based on phenotypic patterns.

Recent research showed that the different CNS species have different species-specific characteristics in epidemiology and adaptation (Piessens *et al.* 2012). CNS species can be classified according to their different characteristics into udder-adapted, opportunistic and environmental CNS (Supré *et al.*

2011; Piessens *et al.* 2012) or as virulent, protective and of no importance (Wilson *et al.* 2004; Schukken *et al.* 2009). CNS species colonizing teat apex could play a role in relation to IMI, e.g. by being a risk factor or having a protective role. Therefore, it is important to have highly accurate diagnostic tools to identify CNS from teat skin as well as milk samples to identify the distribution of CNS species in extramammary and intramammary sites and subsequently, their potential as causative of IMI (Mahmmod *et al.* 2018a, b). In this regard, the application of molecular techniques would provide sensitive, quantitative and large-scale routine mastitis diagnosis tools to evaluate the effect of colonization on the probability of IMI (Svennesen *et al.* 2019).

The diagnostic accuracy of PCR-based methods has shown high sensitivity (Se) and specificity (Sp) in detection of bacteria in milk, compared to conventional BC for many udder pathogens such as *S. aureus*, *Streptococcus agalactiae* and *Streptococcus uberis* (Cederlof *et al.* 2012; Mahmmod *et al.* 2013a,b; Nyman *et al.* 2016; Steele *et al.* 2017; Holmøy *et al.* 2018). Using latent class analysis (LCA) approach, Nyman *et al.* (2016) estimated the Se and Sp of PCR (PathoProof ^{TM} PCR) and BC for identification of IMI with CNS in Swedish dairy herds, although that PCR test type did not directly measure CNS but all *Staphylococcus* species that then were evaluated against the *S. aureus* content. However, the estimates of the qPCR assay were based on composite, non-aseptically collected milk samples from milk test day (DHI) with the risk of false positive results due to contamination or carry-over effects (Mahmmod *et al.* 2014, 2017). Using the "Gold standard" approach, Dohoo *et al.* (2011) evaluated several definitions for classifying a quarter as having, or not having an IMI with CNS by comparing the results from a single culture to a gold standard diagnosis based on a set of three milk samples. However, a major drawback of using imperfect reference test is that the index test's characteristics are subject to selection and information bias (Dohoo 2014; Haine *et al.* 2018).

In the absence of a reasonable reference test or true gold standard for detection of CNS with known Se and Sp, Bayesian LCA provides an invaluable option for the estimation of Se and Sp of two or more tests without any assumption about the underlying true disease status of each subject (Hui and Walter 1980). To the best of our knowledge, there is no available literature that quantifies the

diagnostic performance of BC and real-time qPCR, for the detection of CNS from aseptically collected quarter milk (indicator for IMI) and teat apex (indicator for teat apex colonization, TAC) in dairy herds with automatic milking systems (AMS). The primary objective of this cross-sectional field study was to estimate the diagnostic Se and Sp of the commercially available Mastit4 qPCR assay and BC for diagnosis of IMI and TAC with CNS at different cut-offs for cycle threshold (Ct) values within a Bayesian framework. A secondary objective was to evaluate two cut-offs of BC based on colony forming units (CFU) for diagnosis of IMI and TAC with CNS.

Materials and Methods

Study population

During the period from February to May 2017, eight dairy herds with Danish Holstein cows were selected for participating in a project to investigate the epidemiology and diagnostics of mastitis pathogens in Danish dairy herds (Svennesen *et al.* 2019). To be eligible for inclusion in the present study, herds had to have AMS with ≥ 3 milking robots and BTM qPCR Ct-value ≤ 32 for *Streptococcus agalactiae.* Between 30 and 40 lactating dairy cows were selected randomly from each herd. These cows were randomly selected among those with a SCC > 200,000 cells/mL at the preceding milk recording, and with no clinical mastitis or antimicrobial treatment four weeks prior to sample collection. From every second sampled cow (odd laboratory running numbers), teat skin swab and aseptic milk samples were taken from right hind quarter for laboratory investigation (Mahmmod *et al.* 2018a).

Quarter samples collection

Each herd was visited once to collect teat swab samples and aseptically collected quarter foremilk samples for BC and qPCR. The farmers were asked to separate the selected cows. Cows were fixed in head lockers during sampling. After cleaning the teats with dry paper towel, the teat swab samples were collected followed by the quarter foremilk samples. Teat swab samples were collected according

to the modified wet-dry method (Paduch *et al.* 2013). Briefly, the first swab (Dakla Pack®) was moistened with ¼ Ringer's solution (Merck, Darmstadt, Germany) and rotated 360° around the teat about 1 cm from the teat canal orifice. The same procedure was carried out with the dry swab. Immediately after sampling, the tips of both swabs were transferred into a tube with 2 mL of sterile Ringer's solution.

Quarter milk samples were collected directly after harvesting the teat swab samples according to National Mastitis Council (NMC 1999) guidelines. Briefly, the teat end was thoroughly disinfected with cotton swabs drenched with ethanol (70%). Individual quarter foremilk samples were then aseptically collected in sterile screw cap plastic tubes. Before sampling procedures, latex gloves were worn and changed after each cow. Tubes containing the teat skin swabs and milk samples were stored at max 5°C until laboratory analysis the following day.

Diagnostic procedures

Phenotypic identification

Bacterial culture of milk samples was conducted in accordance with National Mastitis Council recommendations (NMC 1999). After vortexing, 0.01 mL of the milk sample from each quarter was streaked out with disposable, calibrated bacterial loops on a quarter of a calf blood agar plate. Simultaneously, another 0.01 mL of the same sample was streaked out on chromogenic agar plates selective for staphylococci (SaSelect™, Bio-Rad, Marnes-la-Coquette, France). Bacterial culture of teat swab samples was performed according to the procedures of Paduch *et al.* (2013). Briefly, the teat swab sample was vortexed before removing the swab tips from the tubes. A total of 0.1 mL of a swab solution was inoculated onto the agar plates and was evenly spread with a sterile Drigalski spatula onto the agar surface of calf blood agar plates and SaSelect™ media for each quarter.

All the inoculated plates were labelled with the laboratory running number and quarter and were then incubated aerobically at 37 °C for 48h. All plates were examined for growth of CNS colonies after 24h and 48h. CNS species were identified on blood agar based on the phenotypic characteristics of

158 their colonies including morphology (round, glossy) according to the National Mastitis Council
159 recommendations (NMC 2004) and were confirmed based on their color on the selective media
160 according to the manufacturer's guidelines. The counts (CFU) of CNS species identified on the
161 selective medium "SaSelect™" were recorded at the quarter level for both milk and teat swab
162 samples. Because the inoculum size was standardized, we considered these counts to be approximate
163 counts for choosing the BC cut-offs (Mahmmod et al. 2015). We only regarded quarter milk samples
164 and teat skin swabs having cut-off ≥ 5 CFU of CNS on the selective agar plate (Thorberg *et al.* 2009).
165 Maximum three isolates with different colony colors on the selective agar per sample were regarded
166 for further identification at species level using Matrix-assisted laser desorption/ionization time of
167 flight mass spectrometry (MALDI-TOF) assay (Mahmmod *et al.* 2018b). The bacteria were prepared
168 for mass spectrometry analysis according to a standard extraction protocol using formic acid (Bizzini
169 *et al.* 2010), as recommended by the manufacturer.
170
171 **Genotypic identification (qPCR assay)**
172 Two qPCR swabs (DNA Diagnostic FLOQ Swabs) were immersed in the corresponding milk and
173 teat skin sample immediately after plating. The swabs were shipped to the laboratory of DNA
174 Diagnostic A/S on the same day that BC was performed, for analysis 1 or 2 days later. A commercial
175 real-time qPCR assay (M4BD, DNA Diagnostic A/S, Risskov, Denmark) was used for qPCR analysis
176 to detect bacterial DNA directly from the milk and teat swab samples. According to the manufacturer,
177 the qPCR assay was developed to target CNS species with a detection limit of 10 CFU/mL at cut-off
178 <40, 10.000 CFU/mL at cut-off <27, and 1.000.000 CFU/mL at cut-off <19.5 (Katholm, personal
179 communication). One of the FLOQ Swabs were added directly to the deep well plate and stayed in
180 the deep well for the first 10 min heating step before being removed. This corresponds to around 250
181 µl sample volume added (Timonen *et al.* 2017). Ct-values were reported for all samples. The Ct-value
182 represents the number of qPCR cycles required to reach the set threshold fluorescence signal level.
183 The thermal cycling protocol for the qPCR assay involved 40 cycles for the reaction where the higher

184 the number of bacteria present in the milk sample, the lower the Ct-value obtained with the assay.
185 The assay included negative DNA extraction controls, internal amplification standard (positive qPCR
186 controls), and non-template control. The laboratory personnel were blinded concerning cow identity
187 and result of the corresponding BC.
188
189 **Test categorization**
190 To evaluate the diagnostic performance of BC, we used two BC cut-offs (≥ 5 and ≥ 10 colonies per
191 quarter) for defining the positivity for CNS IMI and TAC. A quarter was defined as positive in milk
192 (= IMI), if at least 5 colonies or 10 colonies of CNS were identified on the selective medium,
193 corresponding to 500 CFU/ml or 1000 CFU/ml, respectively. Likewise, a quarter was defined as
194 positive on teat apex (= TAC) if at least 5 colonies of CNS were identified, corresponding to 50
195 CFU/mL of the teat swab sample solution. To evaluate the diagnostic performance of qPCR, the LCA
196 analysis was run separately for each of the qPCR cut-off values under investigation (i.e. <40, ≤37,
197 ≤34, ≤32 and ≤ 29) against the two BC cut-offs for diagnosis of IMI and TAC.
198
199 **Statistical analysis**
200 Out of 150 eligible cows, one cow was excluded for the reason of having a dry right hind quarter. A
201 total of 149 cows with complete observations for both qPCR and BC tests from milk and teat skin
202 samples from right hind quarters were subjected to the LCA. We followed the guidelines for reporting
203 of diagnostic accuracy in studies that use Bayesian LCA (Kostoulas *et al.* 2017). We used the results
204 of qPCR assay and BC to implement in Bayesian LCA model to estimate the Se and Sp of each test
205 for diagnosing IMI and TAC of CNS in dairy cattle. Essentially, Bayesian LCA models (Branscum
206 *et al.* 2005) are based on the paradigm described by Hui and Walter (1980), which includes three key
207 assumptions; 1) the target population should consist of two or more subpopulations with differing
208 prevalences, 2) there should be a constant Se and Sp of the index tests across the subpopulations, and
209 3) the tests under evaluation should be conditionally independent given the disease status. We divided

our study population into two subpopulations of herds with different densities according to geography/location, because we assumed that geography would not influence Se and Sp. Population 1 refers to herds (H) located in Northern Denmark including H1, H2, H6, H7 and H8, while population 2 refers to herds located in Southern Denmark including H3, H4, and H5. The Hui-Walter 2-test 2-populations model was used to simultaneous estimate Se and Sp of qPCR and BC, as well as the prevalence of the investigated disease in each of the two subpopulations.

The Bayesian model was implemented in OpenBUGS software, version 3.2.3 rev 1012 (Thomas *et al.* 2006) to estimate the test parameters and population prevalence. This software uses a Markov Chain Monte Carlo (MCMC) sampling algorithm to obtain a Monte Carlo sample from the posterior distribution of all model parameters. The first 10.000 samples were discarded as burn-in to allow convergence. The following 20,000 iterations of the model were used for posterior inference. Convergence of the MCMC chain was assessed by visual inspection of the time-series plots of selected variables as well as by inspecting Gelman-Rubin diagnostic plots and autocorrelation plots using three sample chains with different initial values (Toft *et al.* 2007). There is no published evidence of the diagnostic properties for the tests under comparison, when applied to identify, the target condition IMI and TAC with CNS in dairy cows from AMS herds. Thus, we have chosen to use uninformative priors in the shape of uniform distributions on the interval between zero and one, using the Beta (1,1) distribution to fit the model. The posterior distribution of the test properties (Se and Sp for qPCR and BC) and the prevalence in the two subpopulations were reported as the median and corresponding 95% posterior credibility interval (PCI, the Bayesian analog of a confidence interval).

Results

No marked difference was detected in the distribution of CNS at qPCR cut-offs ≤40 and ≤37 therefore, we presented the distribution and results at qPCR cut-offs ≤37, ≤34, ≤32 and ≤29. Results

of cross-tabulated dichotomous outcome of qPCR and BC for detection of CNS at the different cut-offs are displayed in Table 1 (IMI) and Table 2 (TAC). The estimates of posterior median and 95% PCI of true prevalence and Se and Sp of qPCR and BC for detection of CNS at the different cut-offs for two tests are displayed in Table 3 and 4.

As for the target condition "IMI with CNS", $qPCR_{Se}$ was always higher than BC_{Se}, however; BC_{Sp} was higher than $qPCR_{Sp}$ at all tested cut-offs (Table 3). BC estimates were changed by changing the BC cut-off. Estimates of BC_{Se} and BC_{Sp} showed no substantial variations across the high qPCR cut-offs, whereas at cut-off ≤29 an obvious increase in BC_{Se} was prominent, Table 3. BC_{Sp} was decreasing by decreasing qPCR cut-offs, whereas $qPCR_{Sp}$ was increasing. Estimates of BC_{Se} and BC_{Sp} showed no substantial difference between the tested BC cut-offs.

In contrast to IMI condition, estimates of BC and qPCR in diagnosing TAC with CNS were different and characterized by very wide PCIs. BC_{Se} was higher than $qPCR_{Se}$ at all tested cut-offs, however; $qPCR_{Sp}$ was higher than BC_{Sp} (Table 4). At higher qPCR cut-offs (≤37), the difference between $qPCR_{Se}$ and BC_{Se} was lower than the difference between their estimates at low qPCR cut-offs (≤29), (Table 4). For example, at BC cut-off ≥5, the BC_{Se} estimate was 85% and higher than the estimate at cut-off ≥10 (78%), whereas for BC_{Sp}, it was the other way around. BC and qPCR estimates were affected with changing cut-offs of BC and qPCR, indicating that IMI and TAC definition is associated with the chosen cut-off. The range of 95% PCI of the estimates of qPCR and BC for diagnosis of TAC was obviously wider than those ranges for estimates of qPCR and BC for diagnosis of IMI. Moreover, the range of 95% PCI of BC estimates was wider than the 95% PCI range of qPCR estimates for diagnosis of TAC.

Discussion

Estimates of qPCR and BC for IMI

Our findings showed that both tests are equally highly specific for detection of CNS from milk, whereas qPCR has a higher Se than BC at all the tested cut-offs for qPCR and BC. This is consistent

262 with previous studies, which found that PCR shows a higher Se than BC for detecting bacteria in milk
263 samples (Taponen *et al.* 2009; Shome *et al.* 2011; Keane *et al.* 2013).
264 The Se and Sp estimates of qPCR (94% and 77%) are comparable with estimates of Nyman *et al.*
265 (2016), whereas our estimates of BC were higher than their estimates. That variation could be argued
266 by the difference in the study design, inclusion criteria and sample size of study population, definition
267 of IMI with CNS in milk, and sample type for BC and qPCR (composite Vs quarter). In their research,
268 Nyman *et al.* (2016) used cut-off ≤ 37 for qPCR and ≥ 3 CFU/mL for BC to define IMI with CNS.
269 Furthermore, a different PCR test, the PathoProof test kit (Finnzymes Oy, ThermoFischer, Finland)
270 was used, which may have a different DNA purification method and/or identification system for
271 staphylococci species compared to the CNS in the Mastit4 qPCR test. Hiitiö *et al.* (2015) concluded
272 that when using PCR as the only microbiological method for mastitis diagnostics in normally
273 aseptically collected quarter samples, low amounts of minor pathogens such as CNS should be
274 ignored. In our study, on aseptically taken quarter milk samples, we found that Se estimate of qPCR
275 was higher than that of BC. This finding may be applicable to low and high SCC cows, but keeping
276 in mind our selection criteria (cows with high SCC >200.000 cell/ml), the findings could be more
277 relevant for those cows that meet such criterion. Using a conjoint analysis approach, Andersen *et al.*
278 (2010) reported that 20% of the times when CNS were isolated on the test day, the quarter was
279 considered IMI-negative according to the consensus standard rules (i.e., a- the organism of interest
280 was isolated on the test day with 10 colonies or more, and b- the organism of interest was isolated at
281 least twice out of 3 consecutive weekly tests). This finding could have different reasons such as a)
282 culture with staphylococci in quarters with subclinical IMI, often have less than 10 colonies, b) CNS
283 clear spontaneously from the quarter (Ruegg 2009; Taponen and Pyörälä 2009), and c) CNS are not
284 growing/ detected at every sampling time point. The finding of Andersen *et al.* (2010) and subsequent
285 explanations could explain the difference between our study findings (quarter level, cross-sectional)
286 and those of Hiitiö *et al.* (2015) at quarter level and Nyman *et al.* (2016) at cow level. Dohoo *et al.*
287 (2011) evaluated several definitions for classifying a quarter as having, or not having an IMI with

288 CNS by comparing the results from a single bacterial culture to a gold standard diagnosis based on a
289 set of three weekly taken milk samples. Our Sp estimates of BC at the two BC cut-offs (≥5 and ≥10)
290 were 92%, and 94%, while those estimates reported by Dohoo *et al.* (2011) at cut-off definitions ≥2,
291 and ≥10 were all above 99% for those quarters with minimum SCC threshold 200,000 cells/mL,
292 which are the same selection criteria as in the current study. Meanwhile, our Se estimates of BC,
293 regardless the BC cut-offs, were higher than those estimates obtained by Dohoo *et al.* (2011) based
294 on the gold standard approach. This difference could be explained by the use of an imperfect reference
295 test that result in misclassification bias (Toft *et al.* 2005). Using traditional diagnostic test evaluation,
296 Reyher and Dohoo (2011) reported Se (60%) and Sp (83%) estimates for CNS IMI based on
297 composite milk samples at cut-off ≥1 CFU for IMI. Our BC estimates at the tested cut-offs showed
298 higher Se (above 63%) but also higher Sp (above 92%).
299
300 Several factors could explain the variation between our estimates of Se and Sp for BC and qPCR tests
301 for IMI and TAC. For example, time of sampling for BC and qPCR (test day versus early lactation
302 sampling), sampling type (quarter versus composite sampling), and study design could have a
303 substantial impact on the test performance. Additionally, udder health management practices and
304 milking system type (conventional versus automatic) as well as the choice of qPCR Ct-value cut-off,
305 type of PCR, and IMI definitions on BC may have an effect. Previous research demonstrated that
306 management of udder health under conventional milking systems differs from AMS (Dohmen *et al.*
307 2010; Sørensen *et al.* 2016). Cows in AMS can be milked up to 5 times daily leading to frequent
308 exposure of the teat skin to the teat milking preparations and disinfectants, which may affect the teat
309 apex microbiota. Furthermore, application of teat spray in the AMS is imperfect compared to routine
310 teat dipping in conventional system. Hovinen and Pyörälä (2011) showed that CNS was reported the
311 most common cause of IMI in cows milked in AMS. In this study, only cows with elevated SCC were
312 included, which could have increased the probability of CNS detection by qPCR and BC and thus,
313 increased Se estimates compared to Dohoo *et al.* (2011). Svennesen *et al.* (2018) stated that sampling

cows only with SCC > 200,000 cells/mL may have increased the frequency of IMI and the test performance due to an increased chance of a high concentration of bacteria in IMI quarters with an active infection. In line with this conclusion, Condas *et al.* (2017a) reported that *S. chromogenes, S. simulans, S. xylosus, S. haemolyticus, S. epidermidis, S. agnetis, S. arlettae, S. capitis, S. gallinarum, S. sciuri*, and *S. warneri* were more prevalent in high than in low SCC udder quarters.

IMI with CNS are less severe and/ or mild in comparison to contagious pathogens (e.g., *S. aureus* and *Streptococcus agalactiae*). Furthermore, most of research studies consider the presence of a single CFU of contagious mastitis pathogens or major pathogens as an acceptable definition of IMI, whereas the presence of a single or double CFU of CNS may not be considered as an indicator of IMI with CNS, which requires urgent interventions. Therefore, higher cut-offs are reported to define IMI with CNS, such as ≥ 5 CFU/mL for CNS (Thorberg *et al.* 2009; Bexiga *et al.* 2011b) and ≥ 10 CFU/mL (Tomazi *et al.* 2015; Dolder *et al.* 2017; Condas *et al.* 2017b). Additionally, TAC with CNS species increases the risk of false positive samples (Friman *et al.* 2017) and therefore, finding of few CFU in milk can be labelled as "contamination" rather than true IMI and thus, be disregarded. Consequently, higher CFU cut-offs to increase Sp at the cost of lower Se may be preferable in udder health management.

Estimates of qPCR and BC for TAC

Our findings showed that both tests succeeded in detecting CNS from the teat apex. BC showed a higher Se than qPCR, but lower Sp across the tested cut-offs in diagnosis of TAC with CNS. The detectable high Se of BC and low Se of qPCR in detection of CNS from teat skin can be argued by many factors. For instance, a) the inoculum volume submitted for the diagnostic test was 0.1 mL of a swab solution for BC, and around 0.25 mL for the qPCR, but in the extraction and lysis process the test volume is reduced 12 times so this is compared to 0.02 mL, and b) the sampling technique, which seems to influence largely the amount of extracted DNA. To test the latter assumption, a set of 73

CNS isolates from teat skin were preserved in glycerol at – 80°C (data not shown) and identified to species level using MALDI-TOF assay. These isolates were subjected to the same qPCR assay at DNA Diagnostic's laboratory for validation. The results showed that the qPCR assay identifies successfully all the submitted CNS isolates with high accuracy indicating that the reported low Se is probably due to effect of sampling technique. We reported the full description of 15 CNS species isolated from the teat skin samples in Mahmmod *et al.* (2018b) including *S. arlettae, S. capitis, S. chromogenes, S. cohnii, S. epidermidis, S. equorum, S. haemolyticus, S. hominis, S. piscifermentans, S. saprophyticus, S. sciuri, S. succinus, S. vitulinus, S. warneri, and S. xylosus*. Using the same study design, Skjølstrup *et al.* (2018) reported that Se of qPCR on teat skin was 30% for the identification of *S. aureus* compared with 91% for a sampling method where special qPCR swabs "FLOQ swabs" were directly applied on the teat skin for swabbing. FLOQ swabs differ from the ordinary cotton swabs by consisting of short nylon fibers, which do not absorb material but facilitates uptake of fluid while keeping material close to the surface to allow rapid elution. This indicates that sampling technique has an important impact on qPCR test results and that collecting the teat swab samples for qPCR using FLOQ swabs will considerably improve the Se of qPCR. According to the manufacturer, the detection of bacteria isolates directly in water samples is inefficient with the Mastit4 qPCR. We therefore, think that testing of the fluid from the teat swab samples, despite being collected with the FLOQ swab, is still a water sample, which may contain too little cells or other debris that make the bacterial detection from the pellet in the two centrifugation steps of the Mastit4 test inefficient for the water samples. This argument gives a plausible explanation to the obtained low Se of qPCR for TAC with CNS. Previous studies concluded that the sampling technique based on an experimental set-up using needle puncture (Hiitiö *et al.* 2016) or through cannula (Friman *et al.* 2017) has an obvious effect on the results of microbiological methods and PCR-based techniques for bovine mastitis diagnosis. A huge variety of bacterial communities are sharing the teat skin habitat and form the normal microbiota system (Falentin *et al.* 2016). Therefore, identification of CNS species using phenotypic methods from the teat skin is a challenge.

In our recent study, Svennesen *et al.* (2018) reported a different pattern of the Se and Sp estimates for qPCR and BC of *Streptococcus agalactiae* (qPCR: 0.97 and 0.96 – BC: 0.33 and 1.00) and *S. aureus* (qPCR: 0.94 and 0.98 – BC: 0.44 and 0.74) in teat skin samples, respectively. The remarkable variation between our estimates in this study and our previous study (Svennesen *et al.* 2018) could be argued by difference in the target pathogen (contagious versus environmental), and cut-off detection limit for qPCR (Ct value ≤ 37 versus multiple Ct value cut-offs) and BC (single colony versus ≥ 5 colonies).

The Se and Sp of the selective agar (SaSelect™) for staphylococci species was 98% and 99.9% according to the manufacturer manual but could perform differently for teat skin samples than milk. According to the manufacturer's instructions, the majority of microorganisms, other than staphylococci, are inhibited. However, Corynebacterium, bacillus, certain gram-negative rods, and yeasts can sometimes grow on SaSelect™ agar. In this regard, MALDI-TOF identified 6% (25/396) of the submitted teat skin isolates, which were originally identified as CNS on culture as *Bacillus pumilis*, *Aerococcus viridans*, or *Corynebacterium stationis* (Mahmmod *et al.* 2018b). We, therefore, think that some of the colonies identified as CNS on BC could be other bacterial species, which were misidentified as CNS.

Typically, molecular methods such as qPCR can target the CNS species for which they have been designed based on their relevance for udder health, whereas in BC most species able to grow- whether it is pathogenic or commensal - can potentially be identified (Koskinen *et al.* 2010). Although Se of BC was higher than Se of qPCR, it was noted that the range of 95% PCI of the estimates of BC was wider than 95% PCI range of qPCR estimates for diagnosis of TAC, which makes our reliance on qPCR is preferable. Moreover, BC at its best performance, is still a phenotypic identification method that may have some shortcomings such as need for experienced laboratory personnel, and subjectivity despite the standardized procedures (Sears and McCarthy 2003). Additionally, other confirmatory tests (e.g., biochemical identification) are needed for definite identification of colonies are necessary.

Test properties and cut-offs

The Se and Sp estimates of BC within the frame of each BC cut-off were not affected or changed much by changing the qPCR cut-offs confirming that BC is measuring the same target (i.e., viable cells of CNS), unlike the target of qPCR. The same pattern was also noted for qPCR estimates where Se and Sp estimates of qPCR within the frame of each BC cut-off were not proportionally changed by changing the BC cut-offs confirming that qPCR is measuring the same target (i.e., DNA of CNS), apart from the detection target of BC. This comes in agreement with the findings of previous studies (Mahmmod *et al.* 2013a; Soltau *et al.* 2017). One of the main different aspects between the qPCR analysis and BC is that qPCR analysis detects DNA, whereas BC detects only viable bacteria. Thus, the detected DNA by qPCR can come from bacteria that are viable or killed by the immune system, or, in case of TAC, by teat spray applied after milking. Cressier and Bissonnette (2011) added that, unlike BC, qPCR detects bacterial DNA, which means that it is not dependent on the viability of bacteria which creates difficulty when interpreting whether a positive qPCR result is significant. However, the Mastit4 qPCR test is not detecting free DNA in the milk sample. Only DNA inside viable cells or intact dead cells that still house the DNA is centrifuged to a pellet and then later released from the cells in the lysis process.

Model assumptions

When applying LCA to estimate test performance of diagnostic tests, there are model assumptions and conditions to consider. The first implied assumption of LCA model is that the two tests (qPCR and BC) are conditionally independent given disease status. This assumption is fulfilled because qPCR and BC have different biological identification mechanisms. Furthermore, no culturing was involved in the qPCR procedure. The second assumption that the test characteristics (Se and Sp) should be constant across the tested populations was fulfilled as herd geographical location would not affect test characteristics. The final assumption is that prevalence of infection/disease status should differ between populations. We assumed a priori that the apparent prevalences of CNS IMI

418 and/or TAC differs among the eight herds due to different herd management. That assumption was
419 verified, as posterior estimates of prevalences in our study were markedly different (Table 3 and Table
420 4).
421 In conclusion, we estimated the Se and Sp of both the conventional BC and the qPCR assay for
422 diagnosis of IMI and TAC with CNS at different cut-offs for qPCR and BC using latent class analysis
423 approach. The qPCR showed a higher Se than BC across the tested cut-offs and, hence holds better
424 promise for routine use in diagnosis of IMI with CNS. BC showed a higher Se than qPCR but lower
425 Sp across the tested cut-offs in diagnosis of TAC with CNS. However, it appears that BC and qPCR
426 are partially detecting the same CNS from teat skin. Further research may be necessary to investigate
427 the sampling technique for qPCR on teat skin. To conclude, qPCR and BC are valid diagnostics for
428 identification of CNS in milk. However, the overall performance of qPCR was better than BC
429 suggesting its usefulness for diagnosis of IMI with CNS. For detection of CNS in teat skin, BC
430 showed a higher performance than qPCR however, both techniques require further investigation to
431 reduce the uncertainty of the true status of the quarter and teat skin.
432
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440
441 **Declaration of Interests**
442 Our co-author Jørgen Katholm is affiliated with DNA Diagnostic A/S, which provided us with the
443 PCR swabs and ran the qPCR analyses in their laboratory. We confirm that the laboratory personnel

444 was blinded to the sample identification and results of bacterial culture, and that the company had no
445 impact on the data handling or statistical analysis. Therefore, DNA Diagnostic A/S could not bias the
446 contents of this paper.
447
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640

641 Table 1. Cross-tabulated results for combinations of qPCR at different qPCR Ct-values cut-offs, and
642 BC at two CFU cut-offs for diagnosis of IMI with CNS in the right hind quarter from 149 cows in 8
643 Danish dairy herds with AMS stratified based on the location of herd to two subpopulations (Pop 1=
644 north "5 herds" and Pop 2= south "3 herds")

BC cutoff	qPCR cutoff	Population	Test combinations (T1; qPCR and T2; BC) for IMI				Total (%)
			T1+/T2+	T1+/T2-	T1-/T2+	T1-/T2-	
≥ 5 colonies (=500 CFU/ml)	≤ 37	Pop 1 (north)	31	22	4	34	91
		Pop 2 (south)	29	18	2	9	58
	≤ 34	Pop 1 (north)	31	22	4	34	91
		Pop 2 (south)	29	18	2	9	58
	≤ 32	Pop 1 (north)	29	18	6	38	91
		Pop 2 (south)	27	17	4	10	58
	≤ 29	Pop 1 (north)	23	9	12	47	91
		Pop 2 (south)	25	9	6	18	58
	≤ 26	Pop 1 (north)	12	5	23	51	91
		Pop 2 (south)	21	5	10	22	58
≥ 10 colonies (=1000 CFU/ ml)	≤ 37	Pop 1 (north)	25	28	3	35	91
		Pop 2 (south)	27	20	1	10	58
	≤ 34	Pop 1 (north)	25	28	3	35	91
		Pop 2 (south)	27	20	1	10	58
	≤ 32	Pop 1 (north)	24	23	4	40	91
		Pop 2 (south)	25	19	3	11	58
	≤ 29	Pop 1 (north)	19	13	9	50	91
		Pop 2 (south)	24	10	4	20	58
	≤ 26	Pop 1 (north)	11	6	17	57	91
		Pop 2 (south)	20	6	8	24	58

645

646 Table 2. Cross-tabulated results for combinations of qPCR at different qPCR Ct-values cut-offs, and
647 BC at two cut-offs for diagnosis of CNS in teat skin colonization (TAC) in the right hind quarter from
648 149 cows in 8 Danish dairy herds with AMS stratified based on the location of herd to two
649 subpopulations (Pop 1= north "5 herds" and Pop 2= south "3 herds")

BC cutoff	qPCR cutoff	Population	Test combinations (T1; qPCR and T2; BC) for TAC				Total (%)
			T1+/T2+	T1+/T2-	T1-/T2+	T1-/T2-	
≥ 5 colonies (=50 CFU/ml)	≤ 37	Pop 1 (north)	38	9	35	9	91
		Pop 2 (south)	25	0	29	4	58
	≤ 34	Pop 1 (north)	37	8	36	10	91
		Pop 2 (south)	25	0	29	4	58
	≤ 32	Pop 1 (north)	27	4	46	14	91
		Pop 2 (south)	25	0	29	4	58
	≤ 29	Pop 1 (north)	11	1	62	17	91
		Pop 2 (south)	12	0	42	4	58
	≤ 26	Pop 1 (north)	0	1	73	17	91
		Pop 2 (south)	3	0	51	4	58
≥ 10 colonies (=100 CFU/ ml)	≤ 37	Pop 1 (north)	35	12	31	13	91
		Pop 2 (south)	23	2	27	6	58
	≤ 34	Pop 1 (north)	34	11	32	14	91
		Pop 2 (south)	23	2	27	6	58
	≤ 32	Pop 1 (north)	25	6	41	19	91
		Pop 2 (south)	23	2	27	6	58
	≤ 29	Pop 1 (north)	10	2	56	23	91
		Pop 2 (south)	10	2	40	6	58
	≤ 26	Pop 1 (north)	0	1	66	24	91
		Pop 2 (south)	2	1	48	7	58

650

651 Table 3. Posterior median and 95% posterior credibility interval (PCI) of true prevalence of CNS in
652 milk (IMI) in the right hind quarter from 149 cows in 8 Danish dairy herds with AMS diagnosed by
653 qPCR at different qPCR Ct-values cut-offs and BC at two CFU cut-offs
654

BC cutoff	Parameter	Test estimates at different qPCR cut-offs for IMI							
		≤ 37		≤ 34		≤ 32		≤ 29	
		Med	95% PCI	Med	95% PCI	Med	95% PCI	Med	95% PCI
≥ 5 colonies (=500 CFU/ml)	SeqPCR[1]	0.94	0.84-1.00	0.94	0.84-1.00	0.90	0.78-0.99	0.86	0.67-0.99
	SeBC[2]	0.67	0.53-0.90	0.67	0.53-0.90	0.67	0.54-0.88	0.79	0.64-0.97
	SpqPCR[3]	0.77	0.52-0.99	0.77	0.52-0.99	0.83	0.59-0.99	0.90	0.75-1.00
	SpBC[4]	0.92	0.78-1.00	0.92	0.78-1.00	0.90	0.75-0.99	0.85	0.72-0.98
	Prevelance Pop1 (north)	0.51	0.30-0.68	0.51	0.30-0.68	0.48	0.29-0.65	0.35	0.20-0.53
	Prevelance Pop2 (south)	0.79	0.54-0.93	0.79	0.54-0.93	0.78	0.53-0.93	0.61	0.41-0.79
≥ 10 colonies (=1000 CFU/ ml)	SeqPCR	0.96	0.86-1.00	0.96	0.86-1.00	0.91	0.79-0.99	0.89	0.70-0.99
	SeBC	0.63	0.47-0.90	0.63	0.47-0.90	0.62	0.47-0.87	0.76	0.58-0.97
	SpqPCR	0.69	0.47-0.97	0.69	0.47-0.97	0.78	0.55-0.98	0.86	0.71-0.99
	SpBC	0.94	0.82-1.00	0.94	0.82-1.00	0.93	0.80-1.00	0.89	0.78-0.99
	Prevelance Pop1 (north)	0.43	0.24-0.64	0.43	0.24-0.64	0.43	0.23-0.62	0.30	0.15-0.48
	Prevelance Pop2 (south)	0.75	0.48-0.90	0.75	0.48-0.90	0.75	0.48-0.92	0.57	0.37-0.76

655 [1]SeqPCR= sensitivity of qPCR; [2]SeBC= sensitivity of culture; [3]SpqPCR= specificity of qPCR; [4]SpBC=
656 specificity of culture
657

658 Table 4. Posterior median and 95% posterior credibility interval (PCI)) of true prevalence of CNS in
659 teat skin colonization (TAC) in the right hind quarter from 149 cows in 8 Danish dairy herds with
660 AMS diagnosed by qPCR at different qPCR Ct-values cut-offs, and BC at two CFU cut-offs

BC cutof	Parameter	Test estimates at different qPCR cut-offs for TAC							
		≤ 37		≤ 34		≤ 32		≤ 29	
		Med	95% PCI	Med	95% PCI	Med	95% PCI	Med	95% PCI
≥ 5 colonies (=50 CFU/ml)	SeqPCR[1]	0.48	0.15-0.81	0.47	0.14-0.79	0.36	0.04-0.76	0.17	0.01-0.52
	SeBC[2]	0.85	0.27-0.99	0.85	0.25-0.99	0.83	0.24-1.00	0.86	0.29-1.00
	SpqPCR[3]	0.52	0.20-0.85	0.53	0.21-0.86	0.62	0.23-0.95	0.84	0.49-0.99
	SpBC[4]	0.15	0.01-0.72	0.15	0.01-0.75	0.13	0.004-0.75	0.15	0.01-0.69
	Prevelance Pop1 (north)	0.49	0.04-0.96	0.51	0.05-0.95	0.50	0.09-0.91	0.49	0.05-0.95
	Prevelance Pop2 (south)	0.49	0.01-0.99	0.49	0.02-0.99	0.47	0.02-0.98	0.50	0.02-0.98
≥ 10 colonies (=100 CFU/ ml)	SeqPCR	0.48	0.14-0.82	0.47	0.12-0.80	0.36	0.05-0.78	0.17	0.02-0.51
	SeBC	0.77	0.21-0.98	0.78	0.20-0.98	0.76	0.19-0.99	0.78	0.26-0.98
	SpqPCR	0.52	0.18-0.86	0.53	0.20-0.86	0.62	0.21-0.94	0.84	0.47-0.98
	SpBC	0.23	0.02-0.79	0.23	0.02-0.81	0.20	0.01-0.80	0.23	0.02-0.74
	Prevelance Pop1 (north)	0.50	0.04-0.96	0.50	0.04-0.95	0.50	0.07-0.93	0.50	0.04-0.97
	Prevelance Pop2 (south)	0.50	0.02-0.98	0.50	0.02-0.98	0.48	0.03-0.97	0.51	0.02-0.98

661 [1]SeqPCR= sensitivity of qPCR; [2]SeBC= sensitivity of culture; [3]SpqPCR= specificity of qPCR; [4]SpBC=
662 specificity of culture

663