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Bayesian estimation of test characteristics of real-time PCR, bacteriological culture and California mastitis test for diagnosis of intramammary infections with *Staphylococcus aureus* in dairy cattle at routine milk recordings

Yasser S. Mahmmod[a,b,*], Nils Toft[c], Jørgen Katholm[d], Carsten Grønbæk[e], Ilka C. Klaas[a]

[a] *HERD: Centre for Herd-oriented Education, Research and Development, Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, DK-1870 Frederiksberg C, Copenhagen, Denmark*
[b] *Infectious Diseases, Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig City 44511, Sharkia Province, Egypt*
[c] *Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, DK-1870 Frederiksberg C, Copenhagen, Denmark*
[d] *Knowledge Centre for Agriculture, Cattle, Agro Food Park 15, Skejby, DK-8200 Aarhus N, Denmark*
[e] *Eurofins Steins Laboratory, DK-7500 Holstebro, Denmark*

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ABSTRACT

Danish farmers can order a real-time PCR mastitis diagnostic test on routinely taken cow-level samples from milk recordings. Validation of its performance in comparison to conventional mastitis diagnostics under field conditions is essential for efficient control of intramammary infections (IMI) with *Staphylococcus aureus* (*S. aureus*). Therefore, the objective of this study was to estimate the sensitivity (Se) and specificity (Sp) of real-time PCR, bacterial culture (BC) and California mastitis test (CMT) for the diagnosis of the naturally occurring IMI with *S. aureus* in routinely collected milk samples using latent class analysis (LCA) to avoid the assumption of a perfect reference test. Using systematic random sampling, a total of 609 lactating dairy cows were selected from 6 dairy herds with bulk tank milk PCR cycle threshold (Ct) value ≤39 for *S. aureus*. At routine milk recordings, automatically obtained cow-level (composite) milk samples were analyzed by PCR and at the same milking, 2436 quarter milk samples were collected aseptically for BC and CMT. Results showed that 140 cows (23%) were positive for *S. aureus* IMI by BC while 170 cows (28%) were positive by PCR. Estimates of Se and Sp for PCR were higher than test estimates of BC and CMT. Se_{CMT} was higher than Se_{BC} however, Sp_{BC} was higher than Sp_{CMT}. Se_{PCR} was 91%, while Se_{BC} was 53%, and Se_{CMT} was 61%. Sp_{PCR} was 99%, while Sp_{BC} was 89%, and Sp_{CMT} was 65%.

In conclusion, PCR has a higher performance than the conventional diagnostic tests (BC and CMT) suggesting its usefulness as a routine test for accurate diagnosis of *S. aureus* IMI from dairy cows at routine milk recordings. The use of LCA provided estimates of the test characteristics for two currently diagnostic tests (BC, CMT) and a novel technique (real-time PCR) for diagnosing *S. aureus* IMI under field conditions at routine milk recordings in Denmark.

* Corresponding author at: HERD: Centre for Herd-oriented Education, Research and Development, Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, DK-1870 Frederiksberg C, Copenhagen, Denmark. Tel.: +45 35 33 30 56; fax: +45 35 33 30 22.
E-mail addresses: yasser@sund.ku.dk, yasser_saad_eldien@yahoo.com (Y.S. Mahmmod).

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1. Introduction

Staphylococcus aureus (*S. aureus*) is a major cause of intramammary infections (IMI) leading to damage of udder tissue and clinical mastitis episodes with subsequent high economic losses (Barkema et al., 2006; Reyher et al., 2012). Halasa et al. (2009) concluded that subclinical IMI with *S. aureus* was associated with high economic losses of an average of €1219 total annual net costs for a herd of 100 dairy cows. Sørensen et al. (2010) reported that clinical *S. aureus* IMI was associated with the highest economic losses of €570 per case, in comparison to economic losses caused by other investigated mastitis pathogens. Hence, reducing the incidence of *S. aureus* IMI in dairy cows represents a corner stone for economically sound udder health management. However, the reduction of the incidence of *S. aureus* IMI will definitely require an accurate diagnostic test. Therefore, diagnostic techniques with high performance are a necessity for efficient biosecurity and control of *S. aureus* IMI.

The control of *S. aureus* is contingent on accurate diagnosis of IMI, and bacteriological culture (BC) of milk samples is the most common carried out diagnostics of *S. aureus* IMI. The sensitivity (Se) of BC for detection of *S. aureus* IMI from single quarter milk samples has previously been estimated to 75% (Sears et al., 1990), to 62% (Sanford et al., 2006), and a range 44–90% based on 12 different definitions of IMI (Dohoo et al., 2011). The Se can be increased to 94–99%, if two or three consecutive samples are collected over a period of time (Sears et al., 1990; Buelow et al., 1996; Anderson and Pritchard, 2012), however, such requirements are cumbersome and cost prohibitive in field studies and day-to-day mastitis diagnostics. *S. aureus* bacteria are shedded intermittently (Sears et al., 1990; Anderson and Pritchard, 2012), so that the numbers of *S. aureus* bacterial numbers in milk may be high for a while, followed by a period with much lower to undetectable numbers. As a consequence not all the bacterial cells of *S. aureus* that are present in an infected mammary gland will be present in single milk sample (Sol et al., 2002; Ghorbanpoor et al., 2007). Hence, BC may not be completely satisfactory for the diagnosis of *S. aureus* IMI.

The California mastitis test (CMT) has been accepted as a quick, cheap, and simple cow-side test for screening for subclinical IMI under field conditions (Schalm and Noorlander, 1957). Sanford et al. (2006) found that the Se and Sp of CMT for identifying the major pathogens including *S. aureus* were 79% and 46%, respectively, while they were 66.7% and 54.8%, respectively in a study by Sargeant et al. (2001). The CMT can be used to point out potentially subclinically infected cows for follow-up diagnostics with BC or PCR.

A commercially available multiplex real-time PCR technique such as the PathoProof™ Mastitis PCR Assay (Thermo Fisher Scientific, Vantaa, Finland), is a faster and accurate alternative to BC (Taponen et al., 2009; Koskinen et al., 2009). The assay has previously shown a higher Se and specificity (Sp) than BC in isolates originating from clinical bovine mastitis (Koskinen et al., 2009) and subclinical IMI with *Streptococcus agalactiae* (*S. agalactiae*) (Mahmmod et al., 2013a). The assay is performed directly on raw or preserved milk samples and takes about 3–4 h from DNA extraction until results are available.

Since 2010, Danish farmers have been able to order cow-level PCR tests (PathoProof™ Mastitis PCR assay) automatically as part of routine milk testing (Katholm, 2010). Relatively few published studies have evaluated the accuracy of different PCR techniques based on comparison to imperfect reference tests (Ahmadi et al., 2010; Friendship et al., 2010). On quarter-level, Paradis et al. (2012) estimated the diagnostic accuracy of a multiplex real-time PCR and BC for subclinical IMI with *S. aureus* using Bayesian analysis. The authors estimated the Se of BC of 66% and 83% based on the IMI definition of 10 cfu and 1 cfu per 0.01 ml, while the Se of PCR was 95% and 88%, respectively. In both tests Sp was ≥99%. Cederlöf et al. (2012) reported Se and Sp of 93% and 95% for PCR tests on routinely taken samples from milk recording, while it was 83% and 97% for BC on sterile quarter foremilk samples, respectively. In that study, all milk samples used for BC were frozen prior to analysis. Freezing was found to increase the likelihood for obtaining *S. aureus* in the milk sample and improve the Se of BC (Villanueva et al., 1991; Sol et al., 2002). To the best of our knowledge, PCR tests have not been evaluated on cow level against BC and CMT from fresh milk samples obtained as part of routine milk recording from all cows within the herd.

When neither diagnostic test can be regarded as perfect test, latent class analysis (LCA) (Hui and Walter, 1980) can be an alternative procedure, which does not assume that the accuracy of either the reference test or the test under evaluation is known (or that a perfect reference test for subject classification is available). By using LCA, the disease status exists but is not known; it is latent (Toft et al., 2007). Using LCA Sanford et al. (2006) estimated the Se and Sp of CMT and BC for detection of IMI at dry-off and demonstrated that CMT could be a valuable tool to identify cows with IMI for selective dry cow treatments in herds with low prevalence of major pathogens. However, studies estimating the Se and Sp of PCR from routinely collected test-day samples against CMT and BC for *S. aureus* from randomly selected cows in different stages of lactation have not yet been published. The objective of this study was to estimate the Se and Sp of real-time PCR assay, BC and CMT for the diagnosis of the naturally occurring IMI with *S. aureus* in routinely collected milk samples using LCA to avoid the assumption of a perfect reference test.

2. Materials and methods

2.1. Study population

Six dairy herds with Danish Holstein cows were selected from 34 Danish dairy herds participating in a project investigating relations between bulk tank PCR and the prevalence of *S. agalactiae* and *S. aureus* in the herds (Mahmmod et al., 2013a). To be eligible for inclusion in the present study, herds had to have a conventional milking parlour and a bulk tank milk (BTM) PCR Ct value ≤39 for *S. agalactiae* and *S. aureus* at the annual PCR testing in October 2010. The cut-off was chosen according to Katholm et al. (2012), as it is a common practice for bulk tank