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Within-herd prevalence thresholds for herd-level detection of mastitis pathogens using multiplex real-time PCR in bulk tank milk samples

J. B. Soltau,* E. Einax,* K. Klengel,* J. Katholm,† K. Failing,‡ A. Wehrend,§[1] and K. Donat*[1,2]
*Animal Health Service, Thuringian Animal Diseases Fund, Jena, 07745, Thuringia, Germany
†DNA-Diagnostic, Risskov, 8240, Denmark
‡Unit for Biomathematics and Data Processing, Veterinary Faculty of the Justus-Liebig-University, Giessen, 35392, Germany
§Clinic of Obstetrics, Gynecology and Andrology for Small and Large Animals, Justus-Liebig-University, Giessen, 35392, Hesse, Germany

ABSTRACT

The objective of the study was to assess the value of quantitative multiplex real-time PCR examination of bulk tank milk samples for bovine mastitis pathogens as a tool for herd level diagnosis. Using a logistic regression model, this study is aimed at calculating the threshold level of the apparent within-herd prevalence as determined by quarter milk sample cultivation of all lactating cows, thus allowing the detection of a herd positive for a specific pathogen within certain probability levels. A total of 6,335 quarter milk samples were collected and cultured from 1,615 cows on 51 farms in Germany. Bulk tank milk samples were taken from each farm and tested by bacterial culture as well as the commercial PCR assay Mastit 4A (DNA Diagnostic A/S, Risskov, Denmark) identifying *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, and *Streptococcus uberis*. In addition, PCR was performed on pooled herd milk samples containing milk aliquots from all lactating cows in each of the 51 herds. Only 1 out of the 51 herds was found PCR positive for *Streptococcus agalactiae* in bulk tank and pooled herd milk samples, and cultured quarter milk samples. Spearman's rank correlations between the cycle threshold value of bulk tank milk PCR and the apparent within-herd prevalence were calculated in regard to *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*. For these pathogens, significant correlations were found. If 1 bulk tank milk sample per herd was tested, the estimated within-herd prevalence thresholds for 90% probability of detection were 27.6% for *Staphylococcus aureus*, 9.2% for *Streptococcus dysgalactiae*, and 13.8% for *Streptococcus uberis* on the cow level. On the quarter level, the within-herd prevalence had to be at least 32.6% for *Staphylococcus aureus*, 1.7% for *Streptococcus dysgalactiae*, and 4.3% for *Streptococcus uberis* to detect a herd as positive using a single bulk milk sample. The results indicate that mastitis pathogens in bulk tank milk can be identified by the applied PCR assay. Bulk tank milk examination is not a reliable tool for the identification of the named pathogens by single testing, but might be a valuable monitoring tool when used frequently with repeated testing. Furthermore, this approach could be a useful monitoring tool for detecting new pathogen occurrence in the herd.

Key words: *Staphylococcus aureus*, *Streptococcus*, herd level diagnosis, pooled milk sample

INTRODUCTION

Intramammary infections are the most common diseases in dairy cattle (Gundling et al., 2015) causing large economic losses. In Germany, in 2009, the damage to the national economy caused by mastitis was estimated to be €1.4 billion (DVG, 2012). Mastitis therapy and drying off account for the greatest amount of antibiotics used in dairy farming (DANMAP, 2014). Dairy herd managers consider good udder health vital, not only for economical and animal welfare reasons, but also to minimize antibiotic use to prevent antimicrobial resistance. Knowledge about the mastitis pathogens prevalent in a certain herd is necessary for the control, reduction, and prevention of udder diseases (Krömker and Friedrich, 2011). Reliable diagnostic tools are essential for developing a farm-specific mastitis prevention plan. Bacterial culture (**BC**) of quarter milk samples is considered the gold standard for mastitis diagnosis, and isolation of the bacteria involved is the prerequisite to perform an antibiotic susceptibility test. Some disadvantages of cultivation are that it is labor intensive and takes a minimum of 24 to 48 h cultivation time (Riffon et al., 2001; Gillespie and Oliver, 2005; Koskinen et al., 2010). A substantial proportion of clinical and subclinical mastitis samples are negative for bacterial growth (Bradley et al., 2007; Taponen et al., 2009). Quantita-

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[1]Contributed equally as senior authors.
[2]Corresponding author: kdonat@thueringertierseuchenkasse.de

tive real-time PCR (**qPCR**) technique is an alternative method for examining milk specimens for mastitis pathogens. It provides results within only a few hours, but is more expensive than BC. Polymerase chain reaction shows a higher sensitivity than BC for detecting bacteria in milk samples according to some authors (Taponen et al., 2009; Bexiga et al., 2011; Spittel and Hoedemaker, 2012). On the other hand, qPCR does not differentiate between viable, growth-inhibited, or dead bacteria. Whereas the staphylococcal β-lactamase gene can be detected with qPCR, a BC is still necessary for a full antibiotic susceptibility test.

For herd level monitoring, bulk tank milk (**BTM**) testing is an easy-to-use and cost-saving alternative to individual sample testing. The SCC of the BTM, which is frequently tested by dairy companies at milk collection in some countries, is a prompt, but rough indicator for the prevalence of subclinical mastitis in the herd. Should the SCC in BTM increase, it is not possible to detect the causing pathogen by a single examination of a BTM sample by BC (Ruegg and Reinemann, 2002). Sensitivity for the detection of mastitis pathogens in BTM is known to be low (Godkin and Leslie, 1993; Olde Riekerink et al., 2010; da Costa et al., 2016). Therefore, our hypothesis is that it is advantageous to detect major mastitis pathogens via single or repeated testing of BTM by qPCR. The objective of this study was to identify and describe the relation between the apparent within-herd-prevalence ($\mathbf{WHP_{app}}$) and the cycle threshold (**Ct**) values of the qPCR aiming at quantification of the bacteria in BTM, and to calculate a threshold of the WHP_{app} that allows the identification of a herd as positive for the relevant pathogen. Besides BTM, we also used pooled herd milk samples as an alternative specimen.

MATERIALS AND METHODS

Dairy Herds

For this study, 51 small and medium-sized farms located in the German Federal States Thuringia (26) and Hesse (25) were selected. Herds of this size were chosen because of a common lack of routine mastitis control programs in such herds, and therefore a screening of BTM for mastitis pathogens could be beneficial. Mean herd size of the study farms was 72 (9–144) cattle. On these farms, quarter milk samples were taken from each lactating cow not excluded from milk delivery. Therefore, cows with signs of clinical mastitis, treated cows, and those within the first 5 d postcalving were not sampled. The average number of cows tested on each farm was 32 (3–61). These cows were kept in multiple housing conditions (free-stall barns with various beddings, tie-stall with or without pasture). Average herd milk yield varied between 4,700 to 11,500 kg/cow per year.

Milk Samples

The samples were collected from October to December 2014 by Animal Health Service of Thuringia veterinarians and the first author. The milk samples were collected during the daily milking routine after udder preparation according to the "Guidelines for aseptic collection of milk samples and for isolation and identification of mastitis pathogens" of the German Veterinary Society (DVG, 2009). After the milking routine was finished, the milk in the bulk tank was stirred for at least 5 min, and 2 BTM samples were taken using sterile disposable syringes. The sample tubes for all types of milk samples contained boric acid as a preservative agent. After collection, all samples were immediately cooled and transported within 48 h to the laboratory of the Animal Health Service, Thuringian Animal Diseases Fund of the State of Thuringia in Jena.

Pooling

At the laboratory, quarter milk samples of each cow were pooled by transferring 0.25 mL per sample to another sterile tube, yielding cow-level milk samples. Furthermore, 2 pooled herd samples were assembled from 0.2 mL of each cow-level sample for each herd. Afterward, the tubes containing pooled herd samples were frozen and maintained at −20°C (±5°C) until analysis.

Bacterial Culture

The quarter milk and BTM samples were cultured according to the above mentioned guidelines. Using a glass bar, 0.01 mL of each milk sample was spread on an esculin sheep blood agar plate (Oxoid, Wesel, Germany) prepared with a streak of a β-hemolysin producing *Staphylococcus aureus*. After the process, the glass rod with the adhering residual milk was dipped into a glucose broth (Oxoid) for enrichment. The agar plates and the broths were incubated aerobically at 37°C (±2°C). The cultures were examined after 18 to 24 and 42 to 48 h, respectively. The broths were plated out after 18 to 24 h and incubated for 1 d before examination to increase the sensitivity of BC in regard to *Streptococcus agalactiae* and *Staph. aureus*. The colonies grown from primary cultures and the broths were evaluated for morphology, hemolysis, pigmentation, and Gram stain *Streptococcus* species were differentiated from *Staphylococcus* species via catalase reaction. For identification of *Staph. aureus*, a coagulase test with rabbit plasma