PATHOPROOF MASTITIS PCR ASSAY AND SENSITIVITY VALIDATION OF A NEW TEST KIT FOR RAPID IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS*, *STREPTOCOCCUS AGALACTIAE* AND *MYCOPLASMA BOVIS* FROM BOVINE MILK

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PathoProof Mastitis PCR Assay is a rapid and accurate system for identifying mastitis pathogens from fresh or preserved bovine milk. The system includes: 1) reagent kits; 2) a real-time PCR instrument, i.e. the machine using the reagent kits for analysis of the milk samples; and 3) software for automated interpretation of the results. Rapid and efficient identification of the severe contagious mastitis pathogens *Staphylococcus aureus, Streptococcus agalactiae* and *Mycoplasma bovis* is important for the dairy industry and mastitis management. It is especially useful if these micro-organisms could be screened from DHI and/or bulk tank milk samples. We present an extension of the PathoProof product family to include a new kit ('PathoProof Mastitis Major-3' kit), which simultaneously identifies these contagious pathogens in fresh or preserved milk. The kit does not require any bacterial culturing efforts and it has a total laboratory throughput time of 4 hours. Sensitivity of the PathoProof system has been previously validated and reported for *Staph. aureus* and *Strep. agalactae* (1). We report experiments validating the sensitivity of the new kit for *M. bovis* based on two different approaches: i) detection limit in bacterial genome copy equivalents in PCR reaction; and ii) detection limit in bacterial colony forming units (CFU) in milk originating from cows with clinical mastitis.

Materials and Methods

The new kit (PathoProof Mastitis Major-3) was used for DNA extraction and real-time PCR. The kit identified *Staph. aureus*, *Strep. agalactiae* and *M. bovis*, and an Internal Amplification Control, in a single multiplex PCR reaction. Besides this, all protocol details of the new kit were identical to PathoProof Mastitis Complete-12, which identifies all 11 main mastitis bacteria and the staphylococcal beta-lactamase penicillin resistance gene in four separate PCR reactions.

DNA concentration of an ATCC *M. bovis* strain was converted to genome copy using standard procedures (1). A 10x dilution series of the DNA was prepared in dH2O. The dilutions were calculated to contain 1-0.02 genome copies / µL concentration of *M. bovis* DNA. Each dilution was analyzed with the PathoProof Mastitis Major-3 kit in 24 replicate reactions. The detection limit of the kit was considered to be at the dilution where at least 23 of the 24 replicates yielded positive results, i.e. <5% of the replicates returned a negative result.

All *M. bovis* culturing procedures were carried out according to NMC guidelines. An *M. bovis* strain, isolated from a clinical bovine mastitis case in Ontario Canada, was plated on standard *Mycoplasma* agar and incubated for 7 days at +37°C. Following this, one colony, diagnostic of *M. bovis* based on 'fried egg' morphology, was picked and grown in standard *Mycoplasma* enrichment medium at +37°C for 48h. A 10-fold dilution series (10^{0}-10^{-8}) of the enrichment medium was then performed in peptone water. A total of 100µL of the dilutions were plated on

two replicate plates and incubated at +37°C for 7 days. The PathoProof Mastitis Major-3 kit was used to analyze milk samples from cows with clinical mastitis. Ten milk samples originating from different cows with clinical mastitis, but providing negative PCR results for *M. bovis*, were selected. A 10-fold dilution series (10^{0}-10^{-8}) of the above-mentioned enrichment medium was performed in the 10 mastitis milk samples. All of these mastitis milk samples, spiked with a decreasing amount of *M. bovis*, were analyzed with the kit. *M. bovis* colonies were counted from the culture plates after 4, 5, 6 and 7 days. This information was used to calculate the number of CFUs spiked into each mastitis milk sample. The detection limit of the PCR kit was considered to be at the lowest dilution where all 10 milk samples still yielded positive *M. bovis* results.

Results and Discussion

The detection limit of the PathoProof Mastitis PCR Assay in genome copy equivalents was 1.6 *M. bovis* genomes per PCR reaction. In other words, this copy number of *M. bovis* DNA was detected with >95% probability (at least 23 of the 24 replicate PCR reactions provided positive results). Counting of colonies from the culture plates showed that 100µL the 10^{-3} dilution resulted in an average (across the two replicate plates) of 218 *M. bovis* colonies after 7 days of culturing. Using this information, it was calculated that the spiked undiluted mastitis milk samples contained 2,180,000 CFUs of *M. bovis* per 1mL of milk. The PathoProof Mastitis Major-3 kit yielded positive *M. bovis* results for all 10 mastitis milk samples for the dilutions from 10^{0}-10^{-5}. The 10^{-6} dilution gave a positive result for only one of the 10 samples. It was hence concluded that the detection limit of the kit was the 10^{-5} dilution, which corresponded to 10^{-5} x 2,180,000 CFUs/mL=21.8 *M. bovis* CFUs/mL of mastitic milk. There was a clear association between the cycle threshold (Ct) values of the PathoProof Mastitis Major-3 kit and the amount of *M. bovis* spiked into the milk samples. This demonstrated that the kit was capable of also accurately quantifying *M. bovis* in the milk samples. Furthermore, the Ct values for each dilution formed tight clusters (small standard deviations), indicating that the kit provided similarly robust results for all mastitic milk samples (data not shown).

Both procedures used in this study for estimating the sensitivity of the PathoProof Mastitis Major-3 kit indicated very high sensitivity. Culturing of *M. bovis* yielded only one CFU in only one of the replicate plates following seven days of incubation for the 10^{-5} dilution. Yet, all of the corresponding 10^{-5} milk mastitic milk samples provided positive PCR results, indicating that the 4-hour PathoProof Mastitis Major-3 kit had higher sensitivity in *M. bovis* detection than the 7-day culturing protocol. In conclusion, the PathoProof Mastitis-3 kit is a very sensitive and complete system for 4-hour identification of *M. bovis* from mastitis milk samples. The kit had higher sensitivity than standard, very long, *M. bovis* culturing. The kit will provide tremendous value for the dairy industry and aid in more efficient *M. bovis*, *Staph. aureus* and *Str. agalactiae* mastitis management.

References:

1. Koskinen, M.T., J. Holopainen, L. Salmikivi, H. Lehmusto, S. Niskala and J. Kurkela. 2008. Analytical detection limit of the PathoProof™ Mastitis PCR Assay determined using two different experimental approaches. International Conference on Mastitis Control 2008: From science to practice. Proceedings 183-190.