Evaluation of two herd-level diagnostic tests for *Streptococcus agalactiae* using a latent class approach

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**A R T I C L E   I N F O**

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**A B S T R A C T**

*Streptococcus agalactiae* mastitis persists as a significant economic problem for the dairy industry in many countries. In Denmark, the annual surveillance programme for this mastitis pathogen initially based only on bacteriological culture of bulk tank milk (BTM) samples, has recently incorporated the use of the real-time PathoProof Mastitis PCR assay with the goal of improving detection of infected herds. The objective of our study was to estimate the herd sensitivity (Se) and specificity (Sp) of both tests of BTM samples using latent class models in a Bayesian analysis while evaluating the effect of herd-level covariates on the Se and Sp of the tests. BTM samples were collected from all 4258 Danish dairy herds in 2009 and screened for the presence of *S. agalactiae* using both tests. The highest Se of PCR was realized at a cycle threshold (Ct) cut-off value of 40. At this cut-off, the Se of the PCR was significantly higher (95.2; 95% posterior credibility interval [PCI] [88.2; 99.8]) than that of bacteriological culture (68.0; 95% PCI [55.1; 90.0]). However, culture had higher Sp (99.7; 95% PCI [99.3; 100.0]) compared to PCR (98.8; 95% PCI [97.2; 99.9]). The accuracy of the tests was unaffected by the herd-level covariates. We propose that screenings of BTM samples for *S. agalactiae* be based on the PCR assay with Ct readings of <40 considered as positive. However, for higher Ct values, confirmation of PCR test positive herds by bacteriological culture is advisable especially when the between-herd prevalence of *S. agalactiae* is low.

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1. Introduction

*Streptococcus agalactiae* is a highly contagious obligate pathogen of the bovine mammary gland which often causes subclinical mastitis in dairy cattle with attendant economic losses to the industry (Keefe, 1997b). The success of any control programme is largely dependent on the accuracy of screening tests which should limit the misclassification of test subjects (Christensen and Gardner, 2000). Since the inception of the Danish *S. agalactiae* control programme, herd screenings have been based on culture of bulk tank milk (BTM) (Andersen et al., 2003). Unlike environmental bacteria, such as the other streptococci, culture of the BTM for *S. agalactiae* has been justified by the obligatory nature of the pathogen whose finding in the pooled milk reliably indicates infection of the udder (Hogan and Smith, 1992). However, the herd sensitivity (Se) of bacteriological culture has been shown to be low and highly variable (Keefe, 1997b), often dependent on the protocol employed and the degree of bacterial shedding from infected cows, which in turn is related to the stage of infection (Gonzalez et al., 1986).

Recently, a novel, rapid, real-time polymerase chain reaction (PCR) assay, the PathoProof Mastitis PCR (Finnzymes Oy, Espoo, Finland), has become commercially available. This assay has been considered as holding more
promise than the conventional bacteriological culture given its capability in detecting both growth-inhibited and nonviable bacteria, and thus possesses an inherent potential for use in routine bovine mastitis testing programmes (Koskinen et al., 2009).

Although the performance of both BTM bacteriological culture and the PathoProof Mastitis PCR (at quarter-level) has been evaluated (Bartlett et al., 1991; Koskinen et al., 2009), this has only been based on imperfect reference standards which are subject to information and/or selection bias, and may therefore result in under or overestimation of the accuracy of the index test(s). In situations where neither a reasonable reference standard, nor a test(s) with known Se and specificity (Sp) is existent, latent class models provide an invaluable option for the simultaneous estimation of Se and Sp of 2 or more tests without any assumption about the underlying true disease status of each subject. These models can be fit using maximum-likelihood procedures or Bayesian inference (Enoe et al., 2000). Three assumptions need to be considered when evaluating diagnostic tests using latent class models: (1) two or more populations with different prevalences are required, (2) the Se and Sp of the diagnostic tests should be the same across the populations, and (3) the tests should be conditionally independent given the disease status (Hui and Walter, 1980). Point 1 can be addressed by choosing stratifiers, which are independent of factors affecting Se and Sp (Nielsen et al., 2002). Point 2 requires that the “disease definition” or target condition be constant across populations. Constancy of the target condition is challenged when using quantitative tests such as real-time PCR, because the test response may be affected by differences in bacterial load, which affects the probability of test-positivity. A proposed solution has been use of latent class receiver-operating characteristics (ROC) curves (Wang et al., 2007). However, ROC analysis effectively forces the target condition to be constant, and thus masks differences in bacterial load which might result from specific covariates affecting the load. Therefore, use of quantitative tests requires that tests be evaluated at a defined cut-off based on the purpose of testing.

There is mounting evidence suggesting that Se and Sp of diagnostic tests vary within populations of herds (Greiner and Gardner, 2000). Gonzales et al. (1986) found that the Se of culture of BTM varied based on the within-herd prevalence of S. agalactiae. This prevalence is also expected to differ between large and small herds, organic versus conventional herds and between farms with and without automatic milking systems (AMS). Availability of such covariate information affords opportunities for the calculation of stratum–specific estimates of Se and Sp in non-homogeneous populations.

This study therefore aimed at estimating the herd Se and Sp of bacteriological culture and the PCR test using latent class models in a Bayesian analysis, while evaluating the effect of herd-level covariates on the Se and Sp of both tests. Furthermore, the effect of changing the target condition was explored. The findings from this study will be central to improving detection of infected herds to which control measures can be applied.

2. Materials and methods

2.1. Sample collection

BTM samples derived from all 4258 Danish dairy herds were collected between the 20th of October 2009 and the 6th of January 2010 through the mandatory milk quality surveillance scheme. Information on geographical location of the herds, use of AMS, herd size and the type of production (organic or conventional), were obtained from the Danish Cattle Database. Sampling was conducted by the BTM truck drivers during milk collection, after which samples were stored on ice. Within 24 h, they were sent to Eurofins Laboratory, Holstebro, Denmark, for processing. At the laboratory, samples were screened by bacteriological culture and the PathoProof Mastitis PCR.

2.2. Target condition

The target condition in this study was a BTM sample containing S. agalactiae or parts of it. Thus, any concentration of bacteria in the sample was considered a case, irrespective of whether the tests detected the bacteria or not.

2.3. Bacteriological culture

Each BTM sample was cultured following the National Mastitis Council standards. A 120 µl of milk inoculum was mixed with 9 ml of selective agar in a Petri dish containing 5% sterile calf blood, 1% wt/vol aesculin supplied with neomycin sulphate and Polymyxin B, sodium fusidate and Staphylococcus aureus β-toxin. This mixture was incubated for 18–24 h at 37 °C. Any colonies showing β-haemolytic activity were counted on each plate following which one of the colonies was selected and recultured on 5% bovine blood agar with the S. aureus β-toxin to elicit the characteristic CAMP reaction. Isolates that were positive in the CAMP as well as in a Lancefield group B latex agglutination test were identified as S. agalactiae.

2.4. PathoProof mastitis PCR

The PCR reactions were run using reagents and protocol instructions as described in the PathoProof Mastitis PCR manual (Finnzymes Oy). Briefly, 350 µl of milk was used as the starting volume for DNA extraction. The extraction protocol involved an enzymatic lysis step disrupting the somatic cells present in mastitic milk, a centrifugation step, an additional lysis step involving the disruption of the bacterial cell walls and a magnetic bead-based DNA purification and elution step. Cycle threshold (Ct) values were recorded for each sample. Notably, the assay's thermal cycling protocol involved 40 cycles; generally, the higher the CFUs in the milk the lower the resulting Ct value. For statistical analysis, 5 different Ct cut-off values of the PCR test were selected i.e. <31, <33, <35, <37 and <40. The cut-off value yielding the highest Se of PCR was selected for subsequent analyses.
2.5. Population classification

Data were aggregated by geographical location into 4 populations of herds with different densities comprising: the eastern Danish islands: Bornholm, Zealand and Funen (population 1); South Jutland (population 2); Mid-Jutland (population 3) and North Jutland (population 4). These populations were assumed to have different between-herd prevalences and hence formed the basis for the estimation of the herd Se and Sp of both tests.

2.6. Statistical model

We assumed that the true between-herd prevalence of *S. agalactiae* differed across the 4 regions. Additionally, constancy of Se and Sp of each of the 2 diagnostic tests was assumed across the populations. But in order to allow for separate estimates between large and small herds, organic versus conventional herds and farms with and without AMS, each population was stratified by each of the aforementioned herd-level covariates. Both tests were assumed to be conditionally independent given the herd infection status since they employ different techniques to detect the agent (Branscum et al., 2005). Therefore, detection of bacteria by either of the tests conditional on the infection status would be a function of their respective sensitivities. This issue is further elucidated in the discussion.

Counts ($O_{ij}$) of the different test combinations e.g. POS/POS, POS/NEG, were assumed to follow a multinomial distribution: $O_{ij} | Se_k, Sp_k, P_k \sim \text{multinomial} (Pro_b, n_k)$ for population $k$, test $i$ in stratum $j$. $Pro_b$ is a vector of probabilities of observing the individual combinations of test results for the $k$th population. The probabilities are specified using the Se and Sp of the tests and the prevalence (P) of each population:

$$\text{Prob}_k = \left( \frac{Pr(T_1^1 T_2^2)}{Pr(T_1^1 T_2^2)} \right) = \left( \frac{Pr(T_1^1 T_2^2 \mid D^+)^{Pr(D^+)} \cdot Pr(T_1^1 T_2^2 \mid D^-)^{Pr(D^-)}}{Pr(T_1^1 T_2^2 \mid D^+)^{Pr(D^+)} \cdot Pr(T_1^1 T_2^2 \mid D^-)^{Pr(D^-)}} \right)$$

where $Se_{ij}$ and $Sp_{ij}$ are the Se of bacteriological culture and PCR respectively in stratum $j$. The same applies for Sp.

Therefore, for each stratifier the resulting 8 populations giving a total of 24 degrees of freedom were sufficient to estimate 16 parameters (stratum-specific Se and Sp estimates for each of the tests and 8 prevalence estimates corresponding to the 8 populations). A Bayesian model implemented in OpenBUGS version 3.2.1 rev 781 (Thomas et al., 2006) was used to estimate the test parameters and population prevalences. Non-informative priors (beta(1, 1)) were used to fit the models since no prior information for the considered target condition was available.

We ran 20,000 iterations of the models with the first 10,000 discarded as the burn-in phase. Convergence of the Markov Chain Monte Carlo (MCMC) chain was assessed by visual inspection of the time-series plots of selected variables as well as the Gelman-Rubin diagnostic plots using two sample chains with different initial values.

Hypotheses for the differences between stratum-specific test parameter estimates were evaluated based on a Bayesian posterior probability (POPR), the frequentist $P$-value analog. Additionally, a separate model ignoring differences in test characteristics between stratifiers was constructed. The resulting nested models were compared using the Deviance Information Criterion statistic (DIC) (the smaller the value the better the fit). We also computed the Differential Positive Rate (DPR), which indicates the cut-off value at which Se and Sp are maximized simultaneously, as: $DPR = (Se + Sp) - 100$.

3. Results

The median herd size of 120 lactating cows (range 1-1227) formed the basis for classifying herds into the small and large categories. Table 1 displays the cross-tabulated counts of the dichotomous outcome of the two tests. There were 530 (12.4%), 1332 (31.3%), 1406 (33.0%) and 990 (23.3%) herds included in populations 1, 2, 3 and 4, respectively. Of the farms, 22.3% had AMS and 9.3% were organic.

The stratum-specific estimates of Se and Sp of both tests at one of the PCR cut-off values (<40, which corresponds to the highest Se of PCR) are displayed in Table 2. There were no demonstrable significant differences between the stratum-specific estimates as indicated by the POPR value. Furthermore, the DIC estimate for the null model (Table 3) i.e. the model ignoring differences in test estimates
Table 2
The Deviance Information Criterion (DIC), stratum-specific estimates of sensitivity and specificity of bulk tank bacteriological culture and PCR tests and a significance value for the difference between the stratum estimates at PCR cut-off <40.

<table>
<thead>
<tr>
<th>Test parameter</th>
<th>Herd size</th>
<th>POPR&lt;sup&gt;d&lt;/sup&gt; value</th>
<th>DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small (95% PCI&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>Large (95% PCI)</td>
<td></td>
</tr>
<tr>
<td>Sp&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>99.8 (99.5; 100.0)</td>
<td>99.5 (99.0; 100.0)</td>
<td>0.80</td>
</tr>
<tr>
<td>Se&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>63.4 (44.9; 91.6)</td>
<td>73.9 (58.4; 96.5)</td>
<td>0.25</td>
</tr>
<tr>
<td>Sp&lt;sub&gt;PCR&lt;/sub&gt;</td>
<td>98.8 (97.4; 99.9)</td>
<td>92.4 (96.1; 99.9)</td>
<td>0.61</td>
</tr>
<tr>
<td>Se&lt;sub&gt;PCR&lt;/sub&gt;</td>
<td>94.5 (84.9; 99.8)</td>
<td>94.0 (86.0; 99.7)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Table 3
The differential positive rate (DPR), DIC and pooled estimates of sensitivity and specificity of bulk tank bacteriological culture and PCR tests at various PCR cut-off values.

<table>
<thead>
<tr>
<th>PCR cut-off value</th>
<th>Test parameter</th>
<th>Estimate (95% PCI)</th>
<th>DPR&lt;sub&gt;CUL&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DPR&lt;sub&gt;PCR&lt;/sub&gt;</th>
<th>DIC</th>
</tr>
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<td>99.7 (99.3; 100.0)</td>
<td>67.6</td>
<td>94.0</td>
<td>71.0</td>
</tr>
<tr>
<td></td>
<td>Se&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>68.0 (55.1; 90.0)</td>
<td>70.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sp&lt;sub&gt;PCR&lt;/sub&gt;</td>
<td>98.8 (97.2; 99.9)</td>
<td>70.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Se&lt;sub&gt;PCR&lt;/sub&gt;</td>
<td>95.2 (88.2; 99.8)</td>
<td>70.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sp&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>96.9 (99.1; 100.0)</td>
<td>76.0</td>
<td>88.9</td>
<td>70.7</td>
</tr>
<tr>
<td>&lt;37</td>
<td>Sp&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>99.6 (99.1; 100.0)</td>
<td>76.0</td>
<td>88.9</td>
<td>70.7</td>
</tr>
<tr>
<td></td>
<td>Se&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>76.3 (61.0; 97.3)</td>
<td>70.0</td>
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</tr>
<tr>
<td></td>
<td>Sp&lt;sub&gt;PCR&lt;/sub&gt;</td>
<td>98.9 (97.7; 99.9)</td>
<td>70.0</td>
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</tr>
<tr>
<td></td>
<td>Se&lt;sub&gt;PCR&lt;/sub&gt;</td>
<td>90.0 (80.7; 99.2)</td>
<td>70.0</td>
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<tr>
<td></td>
<td>Sp&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>80.1 (66.3; 97.9)</td>
<td>70.0</td>
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<tr>
<td>&lt;35</td>
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<td>79.6</td>
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<td>88.8 (77.8; 99.3)</td>
<td>70.0</td>
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<td></td>
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<tr>
<td></td>
<td>Sp&lt;sub&gt;PCR&lt;/sub&gt;</td>
<td>99.2 (58.2; 100.0)</td>
<td>70.0</td>
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<tr>
<td></td>
<td>Se&lt;sub&gt;PCR&lt;/sub&gt;</td>
<td>88.8 (77.8; 99.3)</td>
<td>70.0</td>
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</tr>
<tr>
<td></td>
<td>Sp&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>83.6 (71.3; 98.1)</td>
<td>70.0</td>
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</tr>
<tr>
<td>&lt;33</td>
<td>Sp&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>99.2 (98.5; 99.9)</td>
<td>82.8</td>
<td>86.0</td>
<td>69.3</td>
</tr>
<tr>
<td></td>
<td>Se&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>99.0 (97.9; 99.9)</td>
<td>82.8</td>
<td>86.0</td>
<td>69.3</td>
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<tr>
<td></td>
<td>Sp&lt;sub&gt;PCR&lt;/sub&gt;</td>
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<td>&lt;31</td>
<td>Sp&lt;sub&gt;CUL&lt;/sub&gt;</td>
<td>99.0 (97.9; 99.9)</td>
<td>82.8</td>
<td>86.0</td>
<td>69.3</td>
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<td></td>
<td>Se&lt;sub&gt;CUL&lt;/sub&gt;</td>
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<td>99.7 (99.3; 100.0)</td>
<td>82.8</td>
<td>86.0</td>
<td>69.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> DPR: (Se + Sp) – 100.

between stratifiers, was considerably smaller than for the full model clearly in support of the simpler one. Thus, the results from the simpler model were used for subsequent analyses.

The Se of bacteriological culture decreased with increasing PCR cut-off values (Table 3). At a cut-off value of <40, the Se and Sp values of the PCR test were maximized (Se, 95.2; Sp, 98.8; DPR = 94). On the other hand, at the same cut-off, the culture test estimates were minimized (Se, 68.0; Sp, 99.7; DPR = 67.6).

In Fig. 1, the population-specific between-herd prevalences are plotted against each of the PCR cut-off values. For each population, the prevalence increased with increasing Ct values. At the cut-off value of <40, the posterior mean prevalences were 4.3%, 6.3%, 6.4% and 8.4% for populations 1, 2, 3 and 4, respectively.
4. Discussion

We have used a Bayesian framework to estimate the Se and Sp of both BTM bacteriological culture and PCR tests at the highest cut-off value of the PCR. To our knowledge, this is the first study to evaluate the performance of the PathoProof Mastitis PCR as a herd test. The analysis demonstrated that the PCR assay had higher Se but lower Sp than bacteriological culture. Although a PCR Ct value of 37 has been recommended as an appropriate cut-off for scoring reactions either positive or negative (Koskinen et al., 2009), this study has shown that a Ct value of <40 affords estimates of Se and Sp of the real-time PCR test yielding the highest DPR. A test with a high Se and reasonable Sp is desirable in a control programme in order to ensure that as many of the truly infected herds are detected with only a tiny fraction of those truly uninfected being misclassified as positive.

Even though the Sp of culture of S. agalactiae from BTM has been shown to be consistently high, its Se has been highly variable amongst published studies with estimates ranging from 20.5% (Godkin, 1989) to 78% (Keefe, 1997a). In those studies, estimation of culture test characteristics was based on the assumption of an existing perfect reference standard. In order to minimize bias in the test estimates while concomitantly ensuring internal as well as external validity (generalisability), we have employed latent class analysis of data derived from all the dairy herds in Denmark.

Greiner and Gardner (2000), contend that in non-homogeneous populations stratum-specific estimates of Se and Sp have greater diagnostic utility than crude (pooled) estimates. Although we allowed Se and Sp of both bacteriological culture and PCR to vary between strata, the resulting estimates were not statistically significantly different from each other (though some differences might exist). Thus, pooled estimates were computed. This implies that within a specific population, for either of the tests its ability to detect the herd infection status is the same regardless of the size of the individual herds, their production type or AMS status.

The between-herd prevalence of S. agalactiae was higher with higher cut-off values of PCR. This phenomenon can be explained as follows: at the lowest Ct value (<31), primarily herds with high colony forming units (CFUs) in their BTM would be included in the target condition. As the cut-off is further raised, an increasing number of herds with low CFUs would be added to the existing pool of herds with the target condition such that at the highest cut-off (<40), the target condition would constitute the entire spectrum of infection i.e. from the very heavily infected herds (high CFUs in BTM) to the very lightly infected (low CFUs in BTM), however, with a preponderance of lightly infected herds. This changing target condition has implications on the interpretation of Se and Sp estimates of both tests. Bacteriological culture Se was highest at the lowest cut-off (<31) whereas the PCR Se was lowest at the same cut-off value. This suggests that culture is superior to the PCR assay in detecting heavily infected herds. However, the probability of misclassifying non-infected herds is lower for PCR given its higher Sp at this cut-off. At the highest cut-off, which comprises mainly of lightly infected herds, the PCR assay outperforms culture at their detection. Nevertheless, considering the PCR lower Sp, it becomes necessary to confirm its positives by bacteriological culture. Andersen et al. (2003) propose that with declining pathogen concentrations in the BTM, it might be necessary to increase the amount of milk cultured in order to improve the Se of culture. The findings from this study therefore illustrate that the usefulness of either of these tests is dependent on the target condition (level of CFU in BTM) under consideration.

The uncertainty associated with the Se estimates of both tests is a reflection of the varying number of truly infected herds for each assay at different cut-off values that are used in the tests’ Se estimation. At the highest cut-off value of the PCR assay (<40), bacteriological culture had the largest uncertainty around its Se estimate whereas PCR had the smallest. At the lowest cut-off (<31), the reverse was true. Toft et al. (2005) demonstrated that the precision of the estimates of Se and Sp increased with greater difference in the prevalences amongst the populations studied. As earlier mentioned, the uncertainty associated with the Se estimates of both tests at different cut-offs was a consequence of the changing target condition.

The assumption of conditional independence of both tests is supported by two key arguments: (1) a difference in their detection techniques (bacteriological culture relies on the isolation of the pathogen whereas PCR targets the pathogen’s DNA) and (2) the fact that the PathoProof Mastitis PCR assay is performed directly from raw milk, without the need for bacteriological culturing, unlike previous PCR-based mastitis tests (Koskinen et al., 2009). Therefore, amongst known infected herds with S. agalactiae, the probability of a positive result to PCR will be the same in BTM samples that test negative to bacteriological culture as it will be in those that test positive to culture. A similar interpretation applies to non-infected herds. Consequently, if the true herd infection status is known,

![Fig. 1. A plot of the posterior mean prevalence estimates for the four populations at different PCR cut-off values.](image-url)
knowing one test result will not change our belief of the result of the other test and as such, the test results can be considered as conditionally independent given the infection status of the herd.

As previously noted, screening of BTM samples for S. agalactiae is justified by its obligatory nature such that its presence in BTM is indicative of infected udder quarters (Hogan and Smith, 1992). In light of this fact, the tests’ estimates obtained in this study should be considered as applicable to only S. agalactiae and thus not by any means extendable to other mastitis pathogens, which apart from the udder may have an environmental source. For these pathogens, environmental contamination of BTM would lead to false positive test results that would bias Sp estimates of the index tests. For this reason, quarter-level testing may arguably be the only reliable way to determine the infection status of the herd (Cousins, 1972). As a means to validate the belief that the number of S. agalactiae in milk is a function of the number of infected quarters shedding the organism (Keefe, 1997b), BTM PCR Ct values could be assessed against the within-herd prevalence of the pathogen. For a herd in which this prevalence is high, its corresponding Ct value would be expected to be low. However, this computation was not possible in the present study owing to the lack of within-herd prevalence data.

5. Conclusion

Using latent class analysis we have estimated the Se and Sp of both the conventional bacteriological culture and the real-time PCR assay. The real-time PCR has been shown to have a higher Se but lower Sp than the culture test. Consequently, screenings of bulk tank milk samples for S. agalactiae should rely on the PCR assay with Ct readings of <40 considered as positive. However, for higher Ct values, confirmation of PCR test positive herds by bacteriological culture is advisable especially when the between-herd prevalence of S. agalactiae is low.

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