Risk of false-negative results when delaying PCR from sampling for Coxiella burnetii detection in dairy cows

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SUMMARY

Reliable detection of Coxiella burnetii shedders is a critical point for the control of the spread of the infection between animals and from animals to humans. PCR is the most suitable tool for the detection of Coxiella burnetii in cattle. In routine diagnosis, the samples are usually sent to laboratories by postal routing. This study aimed at assessing the risk of false-negative results with delaying PCRs from sampling for Coxiella burnetii detection. Thirty six milk and 13 vaginal mucus samples being PCR positive on sampling day were divided in 8 aliquots each: 3 stored at +4°C and 5 frozen at −20°C. Real-time PCR were performed on days (D) 1, 2, 3 (or 5) for samples stored at +4°C and days 3 (or 5), 7, 14, 28, 63 for frozen samples. Regardless of the storage, the proportion of samples which remained positive on the first test day after sampling day decreased by about one third and one half for milk and vaginal mucus respectively. Regardless the storage, samples found to be persistently-positive had a significantly higher estimated titre on D0 than those which became negative after D0. In absence of hypothesis on the expected bacterial burden in a milk or vaginal mucus sample, PCR assay should be performed on sampling day to minimise the risk of obtaining a false-negative result.

Keywords: Dairy Cow, Coxiella burnetii, real-time PCR, detectability, false negative.

Introduction

Q fever is a worldwide zoonosis, caused by Coxiella burnetii and the main reservoir consists in Ruminants [2, 3, 6, 9]. Identification of shedding cows is one of the critical point to control the bacterial spread between cows and from cows to humans [5 - 7]. PCR technique is nowadays the most suitable tool for the detection of Coxiella burnetii in vaginal mucus and milk in ruminants [4, 8, 10, 12]. In routine diagnosis, recommendations are that the biological samples must be conveyed as soon as possible to the laboratory in order to at least perform DNA extraction on the sampling day. Unfortunately, these conditions cannot always be fulfilled. An alternative may consist in sending biological samples to laboratories by postal routing at +4°C. Moreover, within large-scale longitudinal studies aiming at investigating the characteristics of Coxiella burnetii’s shedding in commercial herds, a strategy could be to take biological samples periodically and to perform, if necessary, PCRs retrospectively from the last collected sample, in order to limit the number of analyses and to decrease the induced costs. In both cases, a risk of false-negative responses obtained from samples taken from actually shedder cows. Our aim was then to test the worth of these strategies by assessing the risk of false-negative result with delaying PCRs from sampling for Coxiella burnetii’s detection, when storing samples either at +4°C or under freeze control at –20°C.

Material and Methods

STUDY SAMPLE

A total of 36 raw milk and 13 vaginal mucus samples known to be PCR positive on the sampling day (Day 0) were included in
was extracted using the QIAmp DNA mini kit® (Qiagen S.A., IASP, 37380 Nouzilly, France). DNA from all types of samples were quantified from Ct in comparison to a standard curve obtained from samples spiked with a serial dilution of the bacteria (from External positive control provided). From this sample, 8 aliquots of 1 mL were prepared. The initial milk sample was homogenised between each aliquot. Three vaginal swabs were obtained after vulva disinfection with chlorhexidine solution. Each vaginal swab was extensively washed with 1 mL of PBS (phosphate buffered saline). The 3 mL obtained were divided in 8 aliquots.

Each aliquot was tested using the most widely used real-time PCR assay in French veterinary laboratories, i.e. a commercial kit targeting the repetitive transposon-like region of Coxiella burnetii, LSI Taqvet Coxiella burnetii® (Laboratoire Service International, 3 bis allée la Combe, 69380 Lissieu, France) assay, according to the manufacturer’s instructions. The negative control sample used was DNase RNase free water. The external positive control used was a solution containing $10^5$ Coxiella burnetii/mL (provided by Annie Rodolakis, UR INRA IASP, 37380 Nouzilly, France). DNA from all types of samples was extracted using the QiAmp DNA mini kit® (Qiagen S.A., France) according to the manufacturer’s instructions. For milk and vaginal mucus, extraction was performed directly from 200 µL of raw milk or 200 µL of the obtained vaginal mucus solution. All PCR assays were performed on ABIPRISM® sequence Detection System 7000 (Applied Biosystems; Applera France S.A., 91943 Courtaboeuf Cedex, France). For positive samples (having a typical amplification curve), results were given in Ct (cycle threshold) values. Only the samples presenting a typical amplification curve with a Ct below 40 were considered positive. Estimated titres (in log scale) of Coxiella burnetii were quantified from Ct in comparison to a standard curve obtained from samples spiked with a serial dilution of the bacteria (from External positive control provided). Furthermore, a normalisation for the result of the target gene was performed with an endogenous standard (here GAPDH, specific gene of ruminant cells) was performed (as described by PFAFFL[11]).

DNA extraction and real time PCR assay were performed for samples stored at 4°C on day 1, day 2, day 3 or 5 according to working days of the laboratory; for frozen samples, on day 3 (or 5), day 7, day 14, day 28, day 63.

### Data Analysis

Data description was performed separately for the storage of samples (from day 0 to day 3 when stored at 4°C and from day 0 to day 63 when frozen). Possible differences over time in the proportions of positive samples calculated on each day were first investigated. In a second step, each sample was gathered into kinetic pattern according to the persistency or not of a positive result over time. Estimated titres of Coxiella burnetii obtained on sampling day were described according to the kinetic patterns and the storage to investigate the putative relationships between the initial bacterial burden on sampling day and the likelihood of being found negative afterwards. In a third step, the distribution over time of estimated titres of Coxiella burnetii was displayed for samples which tested persistently-positive over the study period.

### Results

#### Descriptive Results

Median estimated titre of Coxiella burnetii in milk and vaginal mucus samples at sampling day (day 0) were 2.18 and 0.33 respectively (minimum of 0.18 and -0.30 and maximum of 4.59 and 3.63 respectively for milk and vaginal mucus).

Regardless of the storage, the proportion of samples which remained positive on the first test day after sampling day (day 0) decreased by about one third and one half for milk and vaginal mucus respectively (Table I). From day 1 to day 3 (or 5) for samples stored at 4°C, and from day 3 (or 5) to day 63 for frozen samples, these proportions were not significantly different (P > 0.05). The storage had no significant effect (P > 0.05) on the proportion of positive results at day 3 (or 5).

#### Description of Kinetic Patterns

Samples were gathered into three classes depending on their kinetic pattern of responses at successive testing (Table II): the “early negativation” pattern included samples that were found negative from the day after sampling day; the “persistency of positive result” pattern included samples which were positive on all test days; the “intermittent negativation” pattern included samples which were sporadically positive over time. These samples were taken from cows located in naturally infected dairy herds, and exhibiting no clinical sign in relation to Coxiella burnetii infection like abortions or metritis. The 49 samples were divided into 8 aliquots each: 3 aliquots were stored at 4°C, and 5 frozen at -20°C. In each cow, raw milk was collected from the 4 teats after disinfection and elimination of the first streams in a sterile pot containing bromopol (2-bromo-2-nitro-1,3-propanediol).

From this sample, 8 aliquots of 1 mL were prepared. The initial milk sample was homogenised between each aliquot. Three vaginal swabs were obtained after vulva disinfection with chlorhexidine solution. Each vaginal swab was extensively washed with 1 mL of PBS (phosphate buffered saline). The 3 mL obtained were divided in 8 aliquots.

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![Table 1: Number of positive results from sampling day (D0) to day 3 (D3) (or D5) for samples stored at 4°C and from D0 to day 63 (D63) for frozen samples.](image-url)

### Table 1: Number of positive results from sampling day (D0) to day 3 (D3) (or D5) for samples stored at 4°C and from D0 to day 63 (D63) for frozen samples.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Storage at + 4°C</th>
<th>Positive results</th>
<th>Freeze</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0</td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>Milk (n = 36)</td>
<td>36</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>Vaginal mucus (n = 13)</td>
<td>13</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
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negative (once or twice). The distribution of milk and vaginal samples in kinetics patterns according their storage is displayed in Table II. Three samples (1 milk and 2 vaginal mucus), all stored at 4°C, were found negative, only when tested on day 5. Regardless of the storage and the type of sample, estimated titres in *Coxiella burnetii* on sampling day differed significantly (P < 0.05) according to kinetic patterns (Table II). Initial estimated titres (in log) for samples included in the “persistency of positive result” pattern were significantly higher than those obtained in samples included in the two others kinetic patterns.

Estimated titres in *Coxiella burnetii* of samples included in the “persistency of positive result” pattern were not statistically different during the period (P > 0.05) from the estimated titre obtained on sampling day (Table III).

### Discussion

The aim of this study was to assess the risk of false negative when delaying real time PCR from sampling for the detection of *Coxiella burnetii* in milk and vaginal mucus samples taken from naturally shedder cows. On the first test day after sampling, the proportion of samples which remained positive was about 65% and 50% for milk and vaginal mucus respectively (Table I). More than 60% of milk samples remained positive during the experimental time-frame, whereas 25% exhibited an early negativation. These percentages were of about 40% and 30% respectively for vaginal mucus samples (Table II). Thus, there is a high risk of obtaining false-negative responses when delaying the PCR assay from the sampling day.

Questions arise from the existence of a quite large proportion of samples exhibiting intermittently negative results (about 15% and 25% of milk and vaginal mucus samples respectively). Although the PCR technique is recognised as a very sensitive method to detect *Coxiella burnetii* (limit of detectability estimated as less than 10 bacteria per millilitre with conventional PCR) [4, 12] the PCR assay used in this experiment could have failed to detect the very low bacterial burden (initial median titre of less than 10 bacteria by mL)
present in these samples (Table II). Thus, this hypothesis together with the putative heterogeneity of aliquots offered to successive testing could explain sporadic occurrence of negative results. Indeed, the risk of unequal distribution of bacteria in the 8 aliquots is a priori increased when the original sample contained a low bacterial burden. Moreover, the assumption that false-negative responses could have occurred in relation to DNA damage with aging process was here found unlikely. Samples containing a high initial bacterial burden displayed almost steady bacterial burden over time, whatever the storage modality (Table III).

The proportion of initially positive samples which became negative was higher for vaginal mucus than for milk samples. The possible impact of the biological sample per se could not be here investigated, owing to the fact that (i) original vaginal mucus samples exhibited lower titres in Coxiella burnetii than milk samples and (ii) the probability for a biological sample to be persistently positive appears to be associated with the bacterial burden. No short-term (within a 5 day-period) effect of storage (4°C vs. –20°C) on the likelihood for a milk or vaginal mucus sample of remaining positive was here evidenced. Both storage modalities limited the activity of DNases.

All together, these results suggest that the PCR assay (at least the one used in this experiment) should be performed on the sampling day to minimise the risk of obtaining a false-negative PCR result, especially in absence of any hypothesis on the expected burden of bacteria in the collected milk or vaginal mucus sample (case of longitudinal studies aiming at investigating the characteristics of Coxiella burnetii’s shedding). However, in routine practice of abortion diagnostic, for which a high burden of Coxiella burnetii, if present, is a priori expected in parturition product or vaginal mucus [1], the PCR assay can be performed within 3 days after sampling without dramatically increasing the risk of false negative PCR result.

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References