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Evaluation of DNA preparation methods combined with different PCR-based assays for *Coxiella burnetii* detection in milk



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SUMMARY

Introduction - *Coxiella burnetii* (Cb) is the causative agent of Q fever, a zoonosis that occurs worldwide. Due to health concerns, unpasteurized cow's milk and a number of dairy products produced by unpasteurized milk may contain virulent Cb. PCR method is commonly employed for sensitive, specific and rapid test for Cb detection in biological samples including milk.
Aim - In this study, six DNA purification methods for recovering Cb DNA from experimentally contaminated cow's milk were evaluated, together with three PCR-based assays targeting the *IS 1111* Cb-repeated element.
Materials and methods - For DNA extraction, the *cetyltrimethylammonium bromide* method was implemented and the following commercial kits were used: *QIAamp DNA Mini kit*; *DNeasy Mericon Food kit*; *NucliSENS miniMAG*; *NucleoSpin Food*; *Wizard Genomic DNA Purification Kit*. The three assays considered were standard PCR, TaqMan real-time PCR and SYBR Green combined with the evaluation of the melting temperature of the amplicon.
Results and discussion - The best extraction methods, *QIAamp DNA Mini kit*, *DNeasy Mericon Food kit* and *NucleoSpin Food,* combined with the TaqMan real-time PCR assay, allowed us to detect the presence of 5 Cb cells per µL of milk.
Conclusion - The analysis of bulk milk seems to be a suitable means of monitoring the Q fever health condition in cows' herds, as long as efficient extraction methods and sensitive amplification assays are used.

KEY WORDS

Coxiella burnetii; cow milk; DNA extraction methods; PCR-based assays.

INTRODUCTION

Coxiella burnetii (Cb), the etiological agent of Q fever, is a Gram-negative intracellular obligate bacterium affecting humans and a variety of domestic and wild animals, mainly ruminants[1,2]. In humans, Q fever occurs as either an acute or chronic illness characterized by myalgia, severe headache, endocarditis, hepatitis and premature delivery or abortion in pregnant woman[3]. Infected animals excrete Cb in their urine, feces, and birth products as well as in milk[2]. Inhalation of contaminated aerosols or dust is the main source of infection. However, given that Cb is excreted through the udder, the consumption of contaminated raw milk[4] or of dairy products has also been proposed as a source of human infection[1,5].
Isolation of Cb is not performed for routine diagnosis in veterinary medicine because it is difficult, time consuming and requires confined level L3 laboratories due to the zoonotic nature of the agent. Therefore standard detection of infected herds and the agent etiology is based on serological or molecular assays. However, the evaluation of ELISA or CF performance as a tool for Q fever diagnosis showed that domestic ruminants shed Cb in biological samples including milk despite being seronegative[6,7,8]. Thus, serological tests are not useful for determining which animals represent a current risk for Q fever transmission. In addition, the longtime circulating antibodies, as well as the use of vaccines, hampered the use of serological tests for the evaluation of Q fever prevalence in ruminant's herd. It is critical to establish and to improve diagnosis procedures like DNA amplification for testing tissue and fluid samples like milk to assess the absence of Cb shedding and to minimize the potential risks of Q fever transmission.
The development of highly sensitive and specific molecular assays has prompted the routine use of PCR based analysis to assess the health status of the herds towards many pathogens, including Cb[9,10], and ensure the safety of food possible sources of Q fever infection. In our previous investigation[5], contamination of Cb in Italian cheeses was assessed by means PCR-based assays. In this study, a direct association between prevalence and milk of different species of ruminants used for the production was highlighted. However, the importance of verify the efficiency of different methods of analysis and asses the best combination of extraction methods and DNA amplification assays, emerged as significant.
In this study, the efficiency of six methods of extracting Cb DNA from cow's milk in combination with three molecular assays for DNA amplification, all targeting the Cb *IS 1111* repeated element, was evaluated. Our aim was to investigate different combinations of extraction methods/PCR-based assays in order to find the best diagnostic protocol for detecting Cb in milk.

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MATERIALS AND METHODS

Sample preparation

A total of two liters of milk were collected from a herd in which no abortions or reproductive disorders, and no positivity to immunological assays for Q fever diagnosis (Checkit Q fever, IDEXX Laboratories, Westbrook, ME, USA) had been recorded. Taking care to prevent any contamination, milk samples were collected from 10 cows, which were negative to ELISA assays toward Cb antibodies. Milk was transported to the laboratory under refrigeration (4°C) and analyzed by means TaqMan real-time PCR[1] for Cb DNA detection. Because milk was negative to the assay, a tenfold dilution series were prepared by using Cb genomic DNA stock solution (100 pg/µL) provided by the UMR ISP1282 of INRA Val de Loire Research Center (Nouzilly, France). The number of Cb cells in the DNA stock solution (~$5x10^4$ Cb Ge/µL) was determined on the basis of the Cb genome size[11,12], the average weight of a nucleotide (http://www.basic.northwestern.edu/biotools/oligocalc.html) and the DNA concentration of the stock solution (100 pg/µL).

DNA extraction

The following five commercial DNA extraction kits were used according to the manufacturers' instructions: *QIAamp DNA Mini kit* (method **A**) and the *DNeasy Mericon Food kit* (method **B**) (Qiagen, Hilden, Germany), *NucliSENS miniMAG* with Nuclisens Magnetic Extraction Reagents (method **C**) (bioMérieux, Florence, Italy), *NucleoSpin Food* (method **D**) (Macherey-Nagel, Düren, Germany) and the *Wizard Genomic DNA Purification* Kit (method **E**) (Promega, Madison, USA). In addition, the *cetyltrimethylammonium bromide* (CTAB) method, a protocol based on CTAB and chloroform purification (method **F**) was carried out. Briefly, 0,5 mL of milk was mixed with .0,8 mL of preheated (65° C) CTAB lysis buffer (CTAB 20 g/L, Tris-HCl 0.1 M, EDTA 20 mM, NaCl 1,4 M), incubated at 65°C for 30 min, then centrifuged at 12,000 g for 5 min at 4°C. The supernatant was collected and mixed in a 2 ml test-tube with Proteinase K (0,1 mg/mL) and incubated at 65 °C for 30 min. After a centrifugation step at 12,000 g during 10 min at 4°C, the upper aqueous phase was mixed with 0.8 mL of chloroform. This step was repeated once again and 0.500 mL of supernatant were then treated with 1 mL of CTAB precipitation solution (CTAB 5 g/L; NaCl 0,04 M). After overnight incubation, the solution was centrifuged and the pellet was suspended in a solution of NaCl 1,2 M: chloroform (1:1). After rapid centrifugation at 12,000 g, 350 µL of supernatant were mixed with 210 µl isopropanol (Sigma-Aldrich, Saint Louis, USA) and incubated overnight at 4°C. The obtained supernatant was discarded and the pellet was washed three times with 1 mL of 70% (v/v) ethanol. The pellet dried was dissolved in DNase/RNase free water (the volume of water depend on the visible quantity of pellet in the tube). For all six extraction methods, the processing times were recorded and the cost of a single extraction was considered. The quality of the purified DNA was assessed by means a Biophotometer (Eppendorf, Hamburg, Germany).

DNA amplification assays

Detection of the Cb *IS 1111* element was performed by means the following three assays: a standard PCR (PCR), as already reported[1]; TaqMan real-time PCR[1,5] and SYBR Green combined with the melting temperature (Tm) evaluation of the amplicons[5]. Briefly, the TaqMan real-time PCR assay was performed in a 20 µL reaction volume containing 0,9 µM of primers, 0,2 µM of probe (Table 1), 10 µL of the TaqMan Universal Master mix (Life Technologies, Foster City, USA), and DNA (80 ng/reaction). The SYBR Green test reaction was carried out in a total of 25 µL containing 80 ng of DNA, 0,125 µM each of the forward and reverse primers (Table 1), 12,5 µL of IQ SYBR Green supermix (Biorad, Hercules, CA, USA), and to quote volume of nuclease-free water (Promega, Madison-WI, USA). The amplicon melting curve was generated by means of the following thermal profile: 30 s at 61°C, 30 s at 95°C. The PCR reaction was performed on 80 ng of DNA in a total volume of 25 µL. The final reaction mixture contained 2 µM of each primer (Table 1) and 0,5 U of Taq DNA polymerase (Promega, Madison, USA). The PCR thermal program for the standard PCR was used as previously described[6]. TaqMan and SYBR Green assays were performed on a Stratagene MX 3005 P instrument (Agilent Technologies, Santa Clara, USA), while the PCR reaction was carried out on a Mastercycler Nexus (Eppendorf, Hamburg, Germany). PCR amplified products were analyzed using electrophoresis on 1,5% (w/v) agarose gel stained with SYBR safe (Life Tecnologies, Foster City, USA). For all assays, the negative control used was DNase-RNase-free water and non contaminated milk. Each milk dilution was analyzed in triplicate and the limit of detection (LOD) of each molecular assay was taken as the highest dilution, which gives a positive result with at least two tests.

The analytical sensitivity of the assays was determined by spiking DNase-RNase-free water with serial 10- fold dilutions of Cb genomic DNA stock solution. As in the LOD evaluation, the assays were performed in triplicate for each dilution.

RESULTS

Extraction methods evaluation

The six extraction methods considered in this study yielded unequal results. Indeed, the concentration and DNA purity,

Table 1 - Primers and probe sequences.

Assay	Primers (5'-3')	Probe(5'-3')	Tm (°C)	Amplicon (bp)
PCR	F-tatgtatccaccgtagccagtc R-cccaacaacacctccttattc			687
SYBR Green	F-gtaacgatgcgcaggcgat R-ccaccgcttcgctcgcta		88.1 ± 0.3	243
TaqMan	F-gggtaaaacggtggaacaaca R-acaacccccgaatctcattg	FAM-aacgatcgcgtatctttaacagcgcttg-TAMRA		101

estimated by measuring the 260 nm absorbance and 260 nm/280 nm absorbance ratio (AR), respectively, were often discordant (Table 2).

DNA amplification assay sensitivity

The analytical sensitivity of the three assays was evaluated by spiking DNase-RNase-free water with serial dilutions of Cb stock solution. The obtained results are shown on Table 3. TaqMan real-time PCR assay showed the highest performance (0,5 CbGe/µL), followed by SyBr Green/TM (5 CbGe/µL) and by PCR (50 CbGe/µL).

The standard curve drawn using the mean threshold cycle (*Ct*) obtained in the assays for analytical sensitivity determination showed a good linearity of response for TaqMan real-time (R2 = 0,996) as well as for SYBR Green (R2 = 0,991).

DNA recovery and PCR-based assays sensitivity evaluation

The performance of the three molecular assays carried out on experimentally contaminated milk differed according to the extraction method that was used (Table 4).

Overall, the extraction method **B** allows to obtain the best performance. Indeed, the detection limit of the three assays performed on the purified DNA by this method ranged from 5 CbGe/µL (TaqMan PCR and SyBr Green/TM) to $5x10^2$ CbGe/µL (PCR). Methods **A** and **D** showed the same efficiency than method **B** when combined to TaqMan (5 CbGe/µL) and PCR ($5x10^2$ CbGe/µL), but a reduction in Cb detection efficiency was obtained when the purified DNA using these three methods was tested with SYBR Green assay (50 CbGe/µL). However, the three other extraction methods (**C**, **E** and **F**) displayed lower efficiency of Cb DNA detection (Table 4). In fact, method **C** displayed a sensitivity reduction in combination to TaqMan (50 CbGe/µL) and PCR ($5x10^3$ CbGe/µL). Meanwhile method **F** exhibited the same results than method **C** combined to TaqMan and PCR assays, but a sensitivity reduction when combined to SYBR Green ($5x10^2$ CbGe/µL). Finally, method **E** was the less sensitive with $5x10^2$ CbGe/µL detection limit in combination with TaqMan and SYBR Green assay and a sensitivity of $5x10^3$ CbGe/µL in combination with standard PCR.

Table 2 - DNA concentration, efficiency, costs and run time of the extraction methods evaluated.

Methods	Concentration (ng/µL)	Ratio 260/280	Run time (h)	Cost (€)
A	18.5	1.78	4	5.02
B	6.9	1.66	4	4.98
C	2.9	1.26	3	7.0
D	10.8	1.65	4	2.75
E	26.6	1.13	~18	1.91
F	7.1	1.27	~48	2.5

Table 3 - Analytical sensitivity of the assays.

	Coxiella burnetii genome equivalent/µL				
	500	50	5	0.5	0.05
TaqMan Real Time	+++	+++	+++	++-	—-
SYBR Green	+++	+++	++-	—-	—-
PCR	+++	+++	+—	—-	—-

+ positive assay; - negative assay.

Table 4 - Limit of detection of the three molecular assays considered in relation to the extraction methods evaluated, expressed as Cb genome equivalent/µL. The analysis were performed in triplicate, mean values are reported.

Extraction methods	TaqMan Real-Time	SYBR Green/Tm	PCR
A	5	50	$5x10^2$
B	5	5	$5x10^2$
C	50	50	$5x10^3$
D	5	50	$5x10^2$
E	$5x10^2$	$5x10^2$	$5x10^3$
F	50	$5x10^2$	$5x10^3$

DISCUSSION

DNA amplification-based assays are commonly used for Q fever research and diagnosis purposes, owing to their notable sensitivity and specificity. Based on our knowledge, this is the first study based on experimentally contaminated milk with known amounts of Cb to evaluate the efficiency of the methods of detection. Our study highlights the importance of properly combining PCR amplification assays and DNA extraction methods in order to improve the analysis sensitivity and to enable bulk milk to be used in epidemiological surveys to assess the health status of cattle herds and dairy products safety. Comparison of the methods evaluated in this study revealed different degrees of efficiency in DNA recovering as well as in the sensitivity of the methods for target gene amplification. The six extraction methods tested displayed different degrees of efficiency in purifying DNA and removing PCR inhibitors that might interfere with the molecular assays (Table 2). Extraction methods **A** and **D** displayed the best combination of both DNA concentration and purity, while method **B** exhibited good purity of a low amount of yielded DNA. Although the sensitivity of the molecular assays relied on DNA extraction methods, the TaqMan real-time and SYBR/TM assays showed an overall better performance (from 5 to $5x10^2$ CbGe/µL) than PCR (from $5x10^2$ to $5x10^3$ CbGe/µL). The lowest LOD value (5 CbGe/µL) was obtained when the TaqMan real-time assay was performed on DNA purified using methods **A**, **B** and **D**. The SYBR Green/TM assay displayed a LOD value of 5 CbGe/µL only in combination with extraction method **B**. In all other cases, the SYBR Green/TM assay allowed to obtain a LOD ranged from 50 to $5x10^2$ CbGe/µL, while the LOD of PCR was generally higher (from $5x10^2$ to $5x10^3$ CbGe/µL). The higher sensitivity of the TaqMan assay was confirmed by comparative evaluation of the analytical sensitivity of the three assays (Table 3). Indeed, the sensitivity of TaqMan PCR was 10 times higher than SYBR Green, which was, in turn, 10 times more sensitive than PCR. While the different degrees of sensitivity of the molecular assays evaluated in this study depends on their features, it is also conceivable, as suggested[11] that sensitivity may be also influenced by the size of the am-

plicon. Because some methods of nucleic acid extraction can cause DNA fragmentation, it is hypothesized that the small-size DNA sequences as PCR-based assays targets, could yield better performance than large DNA (Table 1). This is particularly likely with regard to PCR, whose amplicon is about 6 and 3 times larger than those of TaqMan and SYBR Green, respectively.

The costs and run-times of the six DNA analyzed extraction methods displayed a direct relationship: lower cost/higher execution time. Methods **E** and **F** were the most time-consuming, while method **C** displayed the highest cost per extraction (Table 2). Furthermore, method **C** requires the purchase of equipment. In the general evaluation of the six extraction methods, the use of toxic chemicals must also be taken into account in addition to efficiency, cost and running procedure time. This consideration highlights the lesser manageability of method **F**, which uses chloroform and needs a fume hood.

Based on the outcomes of our study, the TaqMan assay performed on DNA purified using methods **A**, **B** or **D** proved to be the more effective combinations for Cb DNA detection in milk sample. Milk has been identified as a major Cb excretion route, that can be suitable to use to monitor herd exposure to Cb infection based on the bulk milk analysis[4;6;10;13]. The results comparison of assays carried out on both individual and bulk milk tank samples has led to some inconsistency. Indeed, PCR analysis may prove to be positive on individual milk samples and, at the same time, negative on bulk milk if the level of Cb contamination of the bulk milk is low[10] or the presence of a few animals with high bacterial excretion through milk could give a overestimation of the problem[14]. Discrepancies have also been reported with regard to the results of immunological and molecular assays performed on milk samples[15]. This should not deter us from sampling bulk milk to assess the health of herds with respect to Q fever, but it should steer us to choose the most appropriate assays on the basis of the size of the herd, the number and the age of lactating animals, or reports of reproductive disorders.

Bulk milk is an appropriate biological material for detection of Cb-infected herds[9;15]. Based on the assessment of the present study, the quantitative analysis of bulk milk by real-time PCR allows a more accurate evaluation than conventional PCR of the infection status at the herd level.

In conclusion, this study highlights the varying sensitivity level of molecular assays for the detection of Cb in bovine milk and, at the same time, reveals that the choice of an inadequate method of DNA extraction can affect the sensitivity of the selected assay. The sensitivity of TaqMan real-time PCR suggests its use on bulk milk from large herds, while PCR and SYBR Green assays are better suited to the analysis of individual samples or small groups of animals.

Although milk is the most frequent shedding route in cows affected by Q fever[9], Cb may be shed by other routes, such as vaginal mucus, feces, urine, placenta, or birth fluids[2]. Thus, a single bulk milk sample analysis can lead to misclassification of the health status of herds. It is therefore appropriate to plan multiple analyses during the year.

Future investigations will be done to assess the risk of Q fever infection related to the consumption of milk or dairy products and to assess animal health at herd level with regard to Cb.

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