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Short communication

Viable *Coxiella burnetii* in hard cheeses made with unpasteurized milk

Jesús F. Barandika[a], Raquel Alvarez-Alonso[a], Isabel Jado[b], Ana Hurtado[a], Ana L. García-Pérez[a,*]

[a] NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario, Derio, Bizkaia, Spain
[b] Instituto de Salud Carlos III - Centro Nacional de Microbiología, Majadahonda, Madrid, Spain

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ABSTRACT

Q fever is a bacterial zoonosis caused by *Coxiella burnetii* whose main reservoir are small ruminants. Infected animals shed the bacteria into the environment through the products of abortion as well as through feces, urine, and milk. Susceptible people are mainly infected by the inhalation of contaminated aerosols, while food-borne infection is unclear. High prevalence of *C. burnetii* DNA in cheeses from cattle, sheep or goat has been reported, but studies on viability of *C. burnetii* in hard cheeses are scarce. In this study, 67 sheep handicraft hard cheeses of different geographic origins made with unpasteurized milk were analyzed for the presence of *C. burnetii* DNA. To investigate viability of *C. burnetii* in cheese, 5 cheeses were selected among the 20 that tested DNA positive. Presence of viable *C. burnetii* was demonstrated in one cheese by experimental inoculation in BALB/c mice and culture in Vero cells. To further investigate the effect of cheese ripening in *C. burnetii* viability, another 12 cheeses elaborated in the same farm and season, and ripened for between 2.0 and 10.1 months were investigated. Results showed presence of *C. burnetii* DNA in all of them and viable *C. burnetii* in 5, indicating that *C. burnetii* can remain viable after at least 8 months of ripening in hard cheeses made with unpasteurized milk under the acid pH (4.96–5.41) and low water activity (0.9065–0.9533) conditions observed.

1. Introduction

Coxiella burnetii is a zoonotic bacterium worldwide distributed that causes Q fever in animals and humans. Susceptible people in contact with infected livestock can become infected by the inhalation of aerosols contaminated with the bacteria. Domestic ruminants are considered the main reservoirs of the infection, and infected animals release large amounts of bacteria into the environment after abortion or normal parturition, especially through placenta and fetal fluids, but also through milk, feces, urine, etc. This means that, after a Q fever outbreak, the lambing period represents the time of greatest risk of infection for humans. Milk is the main route of *Coxiella* shedding in cattle (Rodolakis et al., 2007), and excretion remains active at least throughout two lactation periods (Piñero et al., 2014). In addition, studies carried out worldwide (Astobiza et al., 2012; Kim et al., 2005; Muskens et al., 2011) have shown that 51.4%–94.3% of bovine farms have *C. burnetii* milk shedders. In small ruminants, feces are the main route of excretion but *C. burnetii* shedding through milk also occurs and can last up to several weeks (Alvarez-Alonso et al., 2018; Astobiza et al., 2010). However, the risk associated with the ingestion of raw milk products from farms affected by Q fever is unclear (EFSA, 2010; Eldin et al., 2017). Several studies confirmed the viability of *C. burnetii* in raw milk obtained from cattle (Loftis et al., 2010; To et al., 1998), but pasteurization temperatures have been shown to destroy the bacteria (Maurin and Raoult, 1999). However, unpasteurized milk and milk products elaborated with raw milk are consumed in many parts of the world (Loftis et al., 2010). In Europe, several types of cheese with high quality and gastronomic value are made with unpasteurized raw milk. Several dairy sheep breeds are reared in European Mediterranean countries, and ewes are milked for several months for cheese production. A number of studies have shown a high prevalence of *C. burnetii* DNA in cheeses from sheep (27%–32%) (Capuano et al., 2012; Eldin et al., 2013; Galiero et al., 2016), goats (51%) (Eldin et al., 2013) or cattle (15%–82%) (Capuano et al., 2012; Eldin et al., 2013; Hirai et al., 2011). In general, prevalence of *C. burnetii* DNA in handicraft cheeses is reported to be lower than in non-handicraft cheeses (made with a milk mixture from different farms) (Galiero et al., 2016). First data on *C. burnetii* viability in milk, butter and cheese were obtained from experiments carried out in the 1940s and 1950s and, *C. burnetii* viability was only detected in cottage-type cheese after 42 days (reviewed by Gale et al., 2015). However, in two recent viability tests (Eldin et al., 2013; Hirai et al., 2011), *C. burnetii* detected in cheeses were not viable. In one of these studies (Eldin et al., 2013) that combined experiments in animal model and cell-line culture assays, mice inoculated with cheese

* Corresponding author.
E-mail address: agarcia@neiker.eus (A.L. García-Pérez).

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homogenates were sacrificed at 7-days post-inoculation (p.i.) and culture of their spleens in Vero cell line produced negative results. Conversely, similar viability studies carried out on environmental samples (dust, manure) showed multiplication of *C. burnetii* in mice tissues 9–21 days p.i. (Alvarez-Alonso et al., 2018; Berri et al., 2003; Kersh et al., 2010). These results suggest that experimental infection protocols need to be adapted to the type of sample and the bacterial load and, that animals might need to be kept alive for longer periods to successfully achieve *C. burnetii* growth in mice tissues. The objectives of this study were: i) to investigate presence of *C. burnetii* in handicraft sheep hard cheeses made with raw milk bought at supermarkets from different geographic areas of Spain, and ii) to study its viability in a selection of positive cheeses (Stage I) and assess the effect of different ripening periods on viability (Stage II).

2. Material and methods

2.1. Sample collection and detection of C. burnetii DNA

A total of 67 handicraft sheep hard cheeses with > 2 months ripening, made with unpasteurized milk, and from different geographical origins were bought in Spanish supermarkets. If available, date of fabrication was recorded to calculate ripening. Before DNA extraction, cheese sample preparation followed a protocol already published (Hirai et al., 2011) with some modifications. Briefly, 10 g of cheese were incubated with 15 mL phosphate buffered saline (PBS) at 56 °C for 30 min. Then, the sample was homogenized in a Stomacher for 1 min and placed into 50 mL screw cap tubes. Tubes were centrifuged at 900 ×*g* for 20 min at 4 °C. The aqueous layer was preserved, and the pellet and the fat layer were washed with 10 mL of PBS and incubated at 45 °C for 20 min. Tubes were vortexed, centrifuged at 900 ×*g* for 20 min at 4 °C, and the aqueous layer was collected and mixed with the one previously preserved. The mixture was centrifuged at 11,000 ×*g* for 30 min at 4 °C, the aqueous layer was discarded and the remaining pellet was resuspended in 300 μL of PBS. DNA was then extracted using QIAmp DNA mini kit (Qiagen Hilden, Germany), and presence of *C. burnetii* was analyzed by real-time PCR targeting the IS*1111* gene (Schets et al., 2013). A commercial internal amplification control (IAC) (TaqMan® Exogenous Internal Positive Control, ThermoFisher Scientific, USA) was included in the assay to monitor for PCR inhibitors. Cheese samples were considered real time PCR-positive when producing a $C_T \leq 35$.

2.2. Viability studies

Viability studies were carried out in BSL3 building facilities, and consisted on experimental inoculations in 6 week-old BALB/c male mice combined with culture in Vero cells. Permissions were obtained from the Ethical & Animal Welfare Committee (Bizkaiko Foru Aldundia, document 3/2017, Reg. 15.328, 22/02/2017). Cheese homogenates were prepared following the above mentioned procedure but without the incubations at 56 °C and 45 °C to preserve the viability of the possible bacteria present in the cheese samples. Quantification of *C. burnetii* genome equivalents (GE) in each homogenate was carried out by quantitative real time PCR (qPCR) using 5 μL of DNA (in triplicates) and specific primers and a probe targeting the IS*1111* gene as described elsewhere (Schets et al., 2013). In each qPCR run, a standard curve was generated using 10-fold serial dilutions of a known concentration of Nine Mile (RSA439) phase II strain of *C. burnetii* DNA. After quantification, aliquots of 500 μL were prepared from each homogenate of cheese, containing approximately 10^3–10^4 *C. burnetii* GE. These aliquots were inoculated intraperitoneally in 4–6 mice each; a homogenate of cheese positive to the presence of *C. burnetii* DNA in real-time PCR (C_T 24.1) was pasteurized and 500 μL used as negative control, inoculating intraperitoneally mice ($n = 6$). Mice were euthanized on days 7, 14 and 21 p.i., and the spleens were removed. The level of splenomegaly was determined from the ratio of the spleen weight to the body weight. Half of the spleen from each mouse was processed for DNA extraction and real time PCR amplification as fully detailed elsewhere (Alvarez-Alonso et al., 2018). Positive samples were subjected to qPCR to quantify the number of *C. burnetii* GE detected in spleen in order to compare it with the number of GE inoculated. For qPCR-positive samples, the second half of the spleen was homogenized with 700 μL DMEM medium and 2% FBS in a TissueLyser. A hundred microliters of each homogenate were placed on shell vials (SV) containing Vero cells [African green monkey epithelial cells VERO C1008 (Vero 76, clone E6, Vero E6 ATCC® CRL-1586™)], as fully detailed elsewhere (Alvarez-Alonso et al., 2018). Briefly, after harvesting *C. burnetii* from SV on day 6 p.i., three passages of 1,000 μL of harvested cells were transferred at weekly intervals into T25 culture flasks containing a Vero layer. At day 6 p.i. and before each passage, 200 μL were collected for DNA extraction and qPCR, following procedures described above. Cultures with unchanged or increased GE during the procedure were considered to be positive. Uninfected control cells were kept close to infected cells to rule out possible cross-contaminations.

2.3. Physico-chemical analyses

Two hundred grams of cheeses included in the study of viability were submitted to AENOR laboratory (Miguel Yuste 12, Madrid, Spain) for physico-chemical analyses. These analyses included pH determination by potentiometry, and water activity (a_w) by hygrometry. Before the analyses cheeses had been kept at −80 °C.

2.4. Statistical analysis

SAS Enterprise Guide 7.1 software was used for statistical analyses. To evaluate the degree of splenomegaly, the mean spleen weights in positive vs. negative mice were compared by non-parametric Wilcoxon two-sample test. Student's *t*-test was used to check differences in pH or water activity in cheeses with viable *Coxiella* compared to negative cheeses. Probability values < 0.050 ($P < 0.050$) were considered significant.

3. Results

C. burnetii DNA was detected in 29.9% (20 out of 67) of the sheep hard cheeses elaborated with raw milk. A selection of 5 DNA positive cheeses with 2.0–3.1 months of ripening, were included in the study of *C. burnetii* viability using mice model and cell line culture (Stage I). Selected cheeses, with C_T values in real-time PCR ranging between 24.1 and 32.7, were homogenized and inoculated in 6 weeks-old BALB/c mice (4–6 replicates per cheese homogenate). The spleen of one mouse inoculated with a 3.1 months ripened cheese was PCR-positive at 14 day p.i. (Table 1). *C. burnetii* GEs recovered from the spleen of this mouse were higher than the GE inoculated, suggesting that *C. burnetii* was viable and bacteria multiplied in vivo. Culture in Vero cells of the homogenate of the spleen of this mouse was also positive, and bacterial loads (GE) recovered at each passage are shown in Table 1. No *C. burnetii* DNA was detected in the spleen of mice inoculated with the remaining 4 cheese homogenates.

To further investigate the effect of cheese ripening in *C. burnetii* viability, another 12 cheeses elaborated in the same farm and season as the positive cheese, with ripening ranging between 2.2 and 10.1 months, were investigated (Stage II). Results showed presence of *C. burnetii* DNA in all 12 cheeses with C_T values in real-time PCR ranging between 23.5 and 30.8. Inoculation of 6 week-old BALB/c male mice resulted in viable *C. burnetii* being recovered 21 days p.i. from the spleens of 6 mice inoculated with homogenates of 5 different cheeses (Table 1). Cultures in Vero cells of homogenates of spleens from the 6 real-time PCR positive mice resulted in growth in all cases but one. Thus, *C. burnetii* GEs recovered through the different passages in Vero

Table 1
Results of the viability study of *C. burnetii* in unpasteurized cheeses using BALB/c mice and culture in Vero cell lines.

Cheese selection					Experimental infection - mice BALB/c model					Culture in cell lines (Vero E6) (GE[d]/ml)					
Cheese	Ripening (months)	pH	a_w[a]	C_T real-time PCR	No. of mice/cheese and day of sacrifice p.i.[b]			No. of positive mice (day p.i.)	No. of GE[c] in spleen	Inoculated	Day 6 p.i.	1st passage	2nd passage	3th passage	Viable *C. burnetii*
					+7 p.i.	+14 p.i.	+21 p.i.								
Stage I															
1	2	5.24	0.9688	24.4	–	2	2	0	0	NA	NA	NA	NA	NA	No
2	2.9	4.95	0.9539	26.4	2	2	2	0	0	NA	NA	NA	NA	NA	No
3	3.1	5.04	0.9533	24.1	2	2	2	1 (+14)	5.8×10^5	2.8×10^4	4.3×10^5	9.1×10^4	2.1×10^5	0	Yes
4	2	5.02	0.9680	32.7	2	2	2	0	0	NA	NA	NA	NA	NA	No
5	2.3	5.13	0.9542	31.4	2	2	2	0	0	NA	NA	NA	NA	NA	No
Neg. control	3.1	5.04	0.9533	24.1	2	2	2	0	0	NA	NA	NA	NA	NA	No
Stage II															
6	2.2	4.56	0.9573	27.1	2	2	2	0	0	NA	NA	NA	NA	NA	No
7	2.7	4.96	0.9508	26.8	2	2	2	1 (+21)	7.8×10^5	9.4×10^4	2.6×10^5	2.2×10^5	7.3×10^5	5.2×10^5	Yes
8	3.1	5.00	0.9484	28.9	2	2	2	1 (+21)	1.4×10^6	3.1×10^5	1.3×10^6	1.6×10^6	3.9×10^6	2.1×10^6	Yes
9	3.6	5.00	0.9407	30.4	2	2	2	0	0	NA	NA	NA	NA	NA	No
10	4.5	5.02	0.9236	30.8	2	2	2	0	0	NA	NA	NA	NA	NA	No
11	4.8	ND	ND	30.3	2	2	2	1 (+21)	1.3×10^{10}	1.3×10^8	3.5×10^8	4.6×10^8	1.3×10^9	1.1×10^9	Yes
12	5.1	ND	ND	28.1	2	2	2	0	0	NA	NA	NA	NA	NA	No
13	5.3	5.16	0.9344	28.0	2	2	2	1 (+21)	2.3×10^3	1.2×10^2	0	0	0	0	No
14	8.7	5.41	0.9065	25.7	–	2	2	2 (+21)	2.3×10^6	4.8×10^4	1.5×10^5	1.7×10^5	1.3×10^5	0	Yes
									1.3×10^9	1.0×10^7	4.9×10^7	5.5×10^7	4.6×10^7	5.0×10^7	Yes
15	8.9	5.42	0.9074	25.5	–	2	2	0	0	NA	NA	NA	NA	NA	No
16	10.1	5.25	0.9101	23.5	–	2	2	0	0	NA	NA	NA	NA	NA	No
17	10.1	4.56	0.9040	28.8	–	2	2	0	0	NA	NA	NA	NA	NA	No
Neg. control	3.1	5.04	0.9533	24.1	2	2	2	0	0	NA	NA	NA	NA	NA	No

[a] a_w, water activity.
[b] p.i., post-infection.
[c] GE, genome equivalents of *C. burnetii* determined by qPCR targeting IS*1111*.
[d] GE, genome equivalents inoculated in Shell vial, and recovered at following passages in Vero cells; NA, non-applicable, only qPCR-positive spleens were cultured in Vero cells;
ND, non-determined, insufficient amount of cheese available for analysis (< 200 g)

cells were higher than the GEs inoculated in shell vials in homogenates of spleens from 5 mice. Spleen of one mouse with *C. burnetii* GEs below 1×10^4 did not show growth in cell cultures. The bacterial loads (GE) recovered at each passage are shown in Table 1. Spleens from negative control mice were also negative. No relevant splenomegaly was detected in positive mice ($P = 0.0737$). Cheeses with viable *C. burnetii* (6 of 13 cheeses from one sheep farm, 1 at Stage I and 5 at Stage II) covered a wide range of ripening (2.7–8.7 months) and bacterial loads expressed as C_T values in real-time PCR (C_T 24.1–30.3) (Table 1).

Table 1 shows the pH and a_w values of 15 of the 17 cheeses included in the viability study. No clear trend in the evolution of pH throughout the ripening process was observed. On the contrary, a decrease in a_w was observed along ripening. In the cheeses with viable *Coxiella* pH ranged between 4.96 and 5.41 (mean 5.11 ± 0.18), and did not statistically differ from pH of the negative cheeses which ranged between 4.56 and 5.42 (5.05 ± 0.23) ($P = 0.6915$). Similarly, cheeses with viable *C. burnetii* had a_w between 0.9065 and 0.9533 (mean 0.9387 ± 0.02) and differences with respect a_w of cheeses without viable *Coxiella* (range between 0.9040 and 0.9688; mean 0.9388 ± 0.03) were not significant ($P = 0.6352$).

4. Discussion

C. burnetii is a Gram negative intracellular bacterium that in its life cycle passes through two phases, the large cell variant and the small cell variant. The latter is highly resistant to physical and chemical stresses, and allows *Coxiella* to survive for months in the environment or in different types of matrices such 7 to 10 months in wool, one month in fresh meat and > 40 months in fresh milk (Eldin et al., 2017). Although viability of *C. burnetii* in milk has been widely demonstrated (Loftis et al., 2010; To et al., 1998), viability in cheese is uncertain. Whereas backdated studies (1940s–1950s) demonstrated viability in cottage-cheese (reviewed by Gale et al., 2015), more recent investigations were unable to demonstrate *C. burnetii* viability in cheese manufactured with unpasteurized milk (Eldin et al., 2013; Hirai et al., 2011). However, only 5 and 7 cheeses were analyzed in those studies, respectively. Here, *C. burnetii* real time PCR-positive cheeses, with high-moderate bacterial loads (C_T 23.5–32.7) and 2.0–10.1 months ripening were analyzed for viability studies. Intraperitoneal inoculation of these cheese homogenates in BALB/c mice and culture in Vero cells of the spleens of mice euthanized at 7, 14 and 21 days p.i. showed that *Coxiella* remains viable in raw milk cheeses for almost 9 months of ripening. *C. burnetii* was viable in 6 of 17 cheeses tested and positivity was revealed in mice at 14 and 21 days p.i., but not at 7 days p.i. Similarly, authors who failed to demonstrate viability in cheese, sacrificed mice at 7 days p.i. (Eldin et al., 2013). According to the results reported herein, ripening periods of up to 9 months did not seem to efficiently kill *C. burnetii* present in raw milk. The acid pH (4.9–5.5) reached during the hard cheese elaboration process (Gale et al., 2015), apparently allowed *C. burnetii* survival. In agreement with this, the pH values obtained in this study in cheeses with viable *Coxiella* were within the above mentioned range (4.96–5.41), confirming that acid pH allows *Coxiella* to remain viable along ripening. The water activity trend observed in this study along ripening was in agreement with values reported for Spanish sheep hard cheeses, which decreased from 0.9880 (on ripening day 1) to 0.9130 (on ripening day 240) (Etayo et al., 2006). In this study, a_w ranged between 0.9688 (on ripening day 60) and 0.9040 values (on ripening day 300). It is noticeable that *Coxiella* viability was not affected by low a_w since viable *Coxiella* was detected in cheeses with a_w values as low as 0.9065.

It is difficult to explain why 4 of the 5 cheeses initially tested showed negative results and lack of growth in mice tissues despite having similar C_T values and ripening times. One hypothesis is that cheeses would have a different proportion of viable/non-viable bacteria, both undistinguishable by PCR. Also, bacteria growth would had been unnoticed if multiplication in mice tissues occurred outside the pre-established sacrifice days (7, 14 and 21 p.i.). Moreover, the limited number of animals that can be used in experimental studies (here 2 mice per day of sacrifice and cheese), reduces chances of isolation.

Although these results would confirm exposure of cheese-consumers to *C. burnetii*, no clear evidences of human oral infection by consumption of infected unpasteurized milk and milk products have been found (EFSA, 2010; Eldin et al., 2017; Gale et al., 2015). Some studies have shown high seroprevalences and clinical disease in people consuming raw milk (Benson et al., 1963; Eldin et al., 2017), but it is difficult to ascertain if patients that reported consumption of raw milk products were previously exposed to aerosol inhalation of *C. burnetii*. In fact, most Q fever cases in humans occur after inhalation of *C. burnetii*-infected aerosols in livestock-associated environments around the parturition period (EFSA, 2010; Eldin et al., 2017; Maurin and Raoult, 1999). Evidences suggest that the oral route is of minor relevance in Q fever acquisition. A recent risk assessment study concluded that the risk of infection by the consumption of unpasteurized milk would be relatively low in comparison with the aerial route, but not negligible (Gale et al., 2015). In spite of the high prevalence of livestock farms positive to *C. burnetii* in bulk-tank milk (Astobiza et al., 2012; Kim et al., 2005) and high prevalence of *C. burnetii* DNA in cheeses (Capuano et al., 2012; Eldin et al., 2013; Galiero et al., 2016; Hirai et al., 2011), even higher is the bacterial load found in placentas (millions of *C. burnetii* per gram of placental tissue) (EFSA, 2010) and expelled to the environment by infected animals. This situation results in a high environmental contamination that leads to high risk of Q fever outbreaks. In addition, human infection by the digestive route does not seem to be as efficient as infection by inhalation of contaminated air, probably due to the small number of macrophages, which are the major targets for *Coxiella* multiplication, in the digestive tract in comparison to those found in lung tissues (Gale et al., 2015).

In summary, the prevalence of *C. burnetii* DNA estimates reported here in sheep cheeses are within the ranges obtained in other similar European studies (Capuano et al., 2012; Eldin et al., 2013; Galiero et al., 2016), in agreement with the worldwide distribution of *C. burnetii* infection in domestic ruminants. While the airborne route is the main route for *C. burnetii* infection, contribution of dairy products to Q fever epidemiology should not be neglected particularly after demonstrating the survival of the bacterium in hard cheese along the ripening period. Consequently, efforts should be made to implement control methods based in vaccination and biosafety implementation in order to progressively decrease infection levels in dairy farms and obtain *Coxiella*-free milk and milk products. This study has been carried out with sheep cheeses made with unpasteurized milk, but the results would be applicable to all hard cheeses made with raw milk regardless of the ruminant species.

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Declaration of competing interest

The authors declare that they have no competing interests.

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