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Short note

Shedding routes of *Coxiella burnetii* in dairy cows: implications for detection and control

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Abstract – Reliable detection of *Coxiella burnetii* shedders is a critical point for the control of the spread of this bacterium among animals and from animals to humans. *Coxiella burnetii* is shed by ruminants mainly by birth products (placenta, birth fluids), but may also be shed by vaginal mucus, milk, and faeces, urine and semen. However, the informative value of these types of samples to identify shedders under field conditions is unknown. Our aim was then to describe the responses obtained using a real-time PCR technique applied to milk, vaginal mucus and faeces samples taken from 242 dairy cows in commercial dairy herds known to be naturally infected with *Coxiella burnetii*, and to assess their putative associations. Positive results were found in all types of tested samples even in faeces. No predominant shedding route was identified. Among the shedder cows, 65.4% were detected as shedders by only one route. By contrast, cows with positive results for all three samples were scarce (less than 7%). Testing a cow based on only one type of biological sample may lead to misclassify it with regards to its shedding of *Coxiella burnetii* and thereby underestimate the risk of bacterial spread within a herd.

dairy cows / *Coxiella burnetii* / shedding routes / real-time PCR / Q fever

1. INTRODUCTION

Q fever is a widespread zoonosis that is caused by an obligate intracellular bacteria, *Coxiella burnetii* [3, 5, 6, 20]. This disease, described for the first time among abattoir workers in Australia [14], is now recognised as being worldwide endemic [25, 27] except in New-Zealand [19]. Ruminants (sheep, goats and cattle) and pets, namely dogs and cats are the main sources of human infection [3, 5, 16, 21].

Detection of shedders of *Coxiella burnetii* is one of the critical points for the control of its spread among animals and from animals to humans [3]. Serological tests (complement fixation, immunofluorescence, enzyme linked immunosorbent assays) which are classically used in routine diagnosis and large-scale epidemiological studies to detect antibody-carriers against *Coxiella burnetii*, demonstrate the previous exposure to the pathogen and not the current shedding of the pathogen [25]. Moreover, a lack of sensitivity in these

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techniques (i.e. existence of seronegative shedders) has already been reported [8,17]. In that context, only techniques allowing the direct identification of *Coxiella* shedders appear to be informative for assessing the actual risk of transmission of the infection. Bacterioscopic examination (with a Stamp-Machiavello coloration) of stained smears, which is used routinely for the detection of *Coxiella burnetii* in the placenta of aborted cows, has poor specificity and sensitivity [3, 20]. Moreover, bacteriological isolation requires confined level-3 laboratories and cannot be performed for mass-screening. In the last few years, conventional polymerase chain reaction (PCR) has become a very useful method for the detection of *Coxiella burnetii* DNA in several biological samples taken from sheep and goats [7, 26, 28, 31]. In dairy cattle, this technique has been evaluated for the detection of *Coxiella burnetii* only in milk [24, 34]. The main limitation of conventional PCR techniques is that they do not provide any quantifiable approach of the bacteriological burden in the tested samples, whereas this assessment is crucial to evaluating the risk of zoonosis.

Real-time PCR techniques are currently being developed with the aim of providing this quantifiable information. As a matter of fact, Real-time PCR has been used for the evaluation of *Coxiella burnetii* antibiotic susceptibilities [13]. In addition, the real-time PCR technique allows one to scale a priori the importance of sources of bacterium with regards to the risk of transmission of *Coxiella burnetii* among animals and from animals to humans. Finally, on the contrary to conventional PCR, real-time PCR can be automated, leading to both a lower risk of sample contamination and a time gain, allowing its use in large scale studies.

From the literature, *Coxiella burnetii* is shed by Ruminants mainly by birth products (placenta, birth fluids), but may also be shed by ruminants via the vaginal mucus [9, 12, 15], milk [1, 7, 15, 24, 34], faeces [2, 7], urine [18] and semen [22]. However, the informative value of these different types of biological samples to identify shedders in field conditions remains unknown. Whether or not cows shed concomitantly through several routes has never been evaluated, even though this information is crucial to identifying the sources of infection and then to estimating the risk of bacterial spread within a herd.

Therefore, the present study was aimed at assessing the distribution of responses obtained using the real-time PCR technique applied to milk, vaginal mucus and faeces samples taken from cows in commercial dairy herds known to be naturally infected with *Coxiella burnetii*, and at describing their putative associations.

2. MATERIALS AND METHODS

2.1. Study sample

The studied herds were selected from among those included in a control programme performed by the "Union Bretonne des Groupements de Défense Sanitaire" in Brittany (western France) regarding cattle abortions. In each monitored herd, 10 cows including the aborted cow and its herdmates were sampled for serological testing for *Coxiella burnetii*, Bovine Viral Diarrhoea Virus, *Chlamydia* spp., *Neospora caninum, Leptospira* spp. In addition, a bacterioscopic examination (after Stamp-Machiavello coloration) was performed on the placenta sample of the aborted cow(s) to detect the presence of *Coxiella burnetii* and/or *Chlamydia* spp.

To be included in the present study, herds had to fulfil the following requirements: a positive stamp coloration of less than 2 months when visiting and sampling the herd; and/or more than 20% of antibody-positive cows for *Coxiella burnetii* assessed less than 6 months before visiting and sampling the herd. In order

to maximise the probability of detecting and then sampling shedder cows, the cows sampled were those which had aborted and had a positive stamp coloration from the control programme described above, cows found seropositive from the control programme described above and cows calving in the last 45 days before the sampling day, under the assumption that the calving period is at a higher risk for *Coxiella* shedding [7, 23, 29, 30].

Consequently, a total of 242 cows, among which 46 had aborted, were sampled in 31 herds.

2.2. Collection of samples

For a given cow, raw milk was collected in a sterile container. In order to minimise the risk of contamination during the collection process, teats were washed first with clean water to remove dirt. Then, each teat end was scrubbed with teat wipes impregnated with ethanol and chlorhexidine digluconate. Lastly, milk was collected from the 4 teats after elimination of the first streams. Faeces were collected using a sterile boxing glove for rectal examination in sterile containers. A vaginal swab was obtained after vulva disinfection with chlorhexidine solution. All the samples were stored at +4 °C during transport. Before being sent to the laboratory, 1 mL of raw milk mixed with bronopol (2-brono-2-nitro-1,3-propanediol) was prepared in order to be tested using the real-time PCR assay. The vaginal swab was extensively washed with 1 mL of sterile physiologic serum, and one cryotube of 0.5 mL was prepared to be tested using the real-time PCR assay. All the samples were conditioned and sent on the sampling day. During postal routing (less than 48 h), the samples were maintained at +4 °C. All the analyses were performed blind, that is, the laboratory had no information regarding the connection (identification number) among milk, faeces and vaginal mucus samples.

2.3. Real-time PCR assay

Each sample was tested using the commercial kit (targeting the repetitive transposon-like region of *Coxiella burnetii*), LSI Taqvet *Coxiella burnetii*® (Laboratoire Service International, 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 UR INRA IASP, Nouzilly, France). DNA from all types of samples were extracted using the QIAmp DNA mini kit® (Qiagen S.A., Courtaboeuf Cedex, 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. For faeces, 1 g of original sample was weighed and mixed by vortexing for 30 s with 4 mL of DNase RNase free water. Then, 400 μL was taken. A last centrifugation step at 6000 *g* for 1 min was performed and 200 μL of supernatants were used to perform DNA extraction according to the manufacturer's instructions as for 200 μL of milk or vaginal mucus. All PCR assays were performed on ABIPRISM® sequence Detection System 7000 (Applied Biosystems; Applera France S.A., Courtaboeuf Cedex, France). For positive samples (having a typical amplification curve), the results are given in Ct (cycle threshold) values. Only the samples presenting a typical amplification curve with a Ct below 40 were considered positive.

2.4. Responses obtained with the real time PCR assay

A sample of milk, faeces or vaginal mucus was considered positive in the case of

Coxiella burnetii DNA detection. In addition, a cow was defined as being a shedder if at least one out of the three collected samples was positive. The number of positive versus negative samples and shedder or not shedder cows was assessed, as well as the distribution of Ct values obtained from each type of the samples. Whether or not the likelihood of being a shedder increased with previous occurrence of abortion was assessed using the chi-square test. In addition, distribution of Ct values was compared in aborted and non-aborted cows using the ANOVA test. Lastly, the relative proportion of positive-tested cows as defined by their shedding routes was calculated.

3. RESULTS

A total of 726 real time PCR were performed: 242 of each type of sample (milk, faeces, vaginal swabs) were tested. The proportion of positive samples of milk, faeces and vaginal mucus were 24.4, 20.7 and 19.0% respectively (Tab. I). The apparent proportion of shedder cows (i.e. positive at least in one out of the three collected samples) was 45.5%. This proportion did not differ significantly between previously aborted cows (21 out of 46) and non-aborted cows (89 out of 196).

Distributions of Ct values were quite similar regardless of the type of sample (Tab. II). The Ct values obtained did not differ significantly between previously aborted and non-aborted cows.

The number of cows detected to be shedders by one, two or the three routes (milk, faeces or vaginal mucus) is displayed in Table III. Among the shedder cows, 65.4% were detected as shedders by only one route. When shedding by two routes, the positive samples were faeces and vaginal mucus for 14.6% of shedder cows. Cows detected as shedders concomitantly by the three studied routes were scarce (6.4%).

4. DISCUSSION

The aim of this study was to investigate the informative value of three types of biological samples (milk, vaginal mucus and faeces) to identify *Coxiella burnetii* shedders in naturally-infected herds using the real-time PCR technique. To our knowledge, this technique has never been used for the detection of *Coxiella* shedding by dairy cows.

Whatever the type of biological sample, positive results were found using the assay under study. *Coxiella burnetii* has already been detected using different PCR methods in the placenta and milk samples of infected dairy cows [24, 26, 34]. But, to our knowledge, this study is the first report of direct identification of *Coxiella burnetii* by PCR in the faeces of naturally-infected dairy cows. Faecal material is known to contain several inhibitors of Taq polymerase [10, 33] but several methods allowing their inactivation are now available [7,31–33], leading to the improvement of detectability by PCR techniques.

From Table III, no predominant shedding route was identified. In addition, cows with only one positive sample out of the 3 collected accounted for more than 65% of the shedders. By contrast, cows with positive results for all three samples were scarce (less than 7%) (Tab. III). Given the selection criteria (cows taken mainly in the peripartum period in herds with cases of abortions), which aimed at maximising their probability of shedding, the distribution of cows according to their likely concomitant shedding routes (Tab. III) cannot be extrapolated for the entire population of cows infected by *Coxiella burnetii*. *Coxiella burnetii* DNA was detected in both previously aborted and non-aborted cows, demonstrating the existence of shedder cows with no clinical signs which may contribute to the bacterium spread within and between herds.

Table I. Distribution of positive and negative samples.

Real time PCR assay result	Type of sample			Cow status[a]
	Milk	Faeces	Vaginal mucus	
Positive	59	50	46	110
Negative	183	192	196	132
Total	242	242	242	242

[a] Cow status: a cow was considered positive if at least one sample out of the 3 was positive.

Table II. Distribution of Ct values in milk, faeces and vaginal mucus.

Type of sample	Population of cows	Minimum	1st quartile	Median	3rd quartile	Maximum	Mean	*N*
Milk	All cows	25.90	33.59	35.32	38.00	39.69	35.05	59
	Aborted cows	32.18	34.43	35.74	37.33	39.69	35.77	10
	Non aborted cows	25.90	33.59	35.12	38.00	39.58	34.90	49
Faeces	All cows	20.64	33.73	35.96	37.97	39.87	35.11	50
	Aborted cows	30.35	34.40	35.15	35.42	38.75	34.93	9
	Non aborted cows	20.64	33.73	36.73	38.11	39.87	35.15	41
Vaginal	All cows	20.65	33.00	35.24	37.26	39.70	34.54	46
mucus	Aborted cows	20.87	32.30	33.59	35.75	38.62	32.85	8
	Non aborted cows	20.65	33.15	35.51	37.42	39.70	34.90	38

Table III. Relative proportions of positive cows as defined by their shedding routes.

Shedding route			Positive cows (%) (N = 110)
Milk	Faeces	Vaginal mucus	
+	+	+	6.4
+	–	+	10.0
–	+	+	14.6
+	+	–	3.6
–	–	+	10.9
–	+	–	20.9
+	–	–	33.6

+ Detection of *Coxiella* DNA in the considered sample using real-time PCR.
– No detection of *Coxiella* DNA in the considered sample using real-time PCR.

Thus, testing a cow based on only one type of biological sample may lead to misclassify it with regards to its shedding of *Coxiella burnetii* and thereby underestimate the risk of bacterial spread within a herd or contagion to humans.

Animals and humans become infected mainly through inhalation of contaminated aerosols produced from shedder animals and the contaminated environment [3, 20]. The similar proportion of positive faeces and vaginal mucus samples (Tab. I), as

well as the similar bacterial burden (interpreted from Ct values) in these specimens (Tab. II) suggest that control measures should pay attention to these two sources of *Coxiella burnetii* transmission. Control measures in infected herds may consist first in preventing infection in susceptible animals and in reducing *Coxiella* shedding in infected cows. Antibiotics (e.g. tetracyclines) are recognised to reduce the incidence of abortions but do not prevent *Coxiella burnetii* shedding [25, 35]. Recent experimental results obtained in vaccinated and then challenged dairy goats [4] using a phase I vaccine demonstrated a drastic reduction of *Coxiella burnetii* shedding. However, to our knowledge, the efficiency of this vaccine to prevent *Coxiella burnetii* shedding and to reduce the shed bacterial burden in infected cows has never been assessed in naturally-infected dairy cows. Control measures in contaminated environments mainly deal with precautions around parturition (calving box with cleaning and disinfection after each calving, destruction of the placenta and foetus) [3, 12, 20], decontamination of bedding material with, for instance, calcium cyanamide 0.4% as described for goat manure [2]. In addition, spreading manure on the pasture when the wind blows should be avoided [11].

All together, these control measures could limit the risk of transmission among animals and from animals to humans. However, owing to the existence of apparently healthy (i.e. non-aborted) cows shedding *Coxiella burnetii* in various specimens, as well as the high infectivity of *Coxiella* [18], the exposed workers (especially farmers, veterinarians and abattoir workers) must be informed about the risks and clinical signs relative to human Q fever, in order to allow for early detection of the disease.

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REFERENCES

[1] Adesiyun A.A., Jagun A.G., Kwaga J.K., Tekdek L.B., Shedding of *Coxiella burnetii* in milk by Nigerian dairy and dual purposes cows, Int. J. Zoonoses 12 (1985) 1–5.

[2] Arricau-Bouvery N., Souriau A., Moutoussamy A., Ladenise K., Rodolakis A., Étude de l'excrétion de *Coxiella burnetii* dans un modèle expérimental caprin et décontamination des lisiers par la cyanamide calcique, Renc. Rech. Ruminants 8 (2001) 153–156.

[3] Arricau-Bouvery N., Rodolakis A., Is Q fever an emerging or re-emerging zoonosis? Vet. Res. 36 (2005) 327–350.

[4] Arricau-Bouvery N., Souriau A., Bodier C., Dufour P., Rousset E., Rodolakis A., Effect of vaccination with phase I and phase II *Coxiella burnetii* vaccines in pregnant goats, Vaccine 23 (2005) 4392–4402.

[5] Baca O.G., Paretsky D., Q fever and *Coxiella burnetii*: a model for host-parasite interactions, Microbiol. Rev. 47 (1983) 127–49.

[6] Behymer D., Riemann H., *Coxiella burnetii* infection (Q fever), J. Am. Vet. Med. Assoc. 194 (1989) 764–767.

[7] Berri M., Laroucau K., Rodolakis A., The detection of *Coxiella burnetii* from ovine genital swabs, milk and fecal samples by the use of a single touchdown polymerase chain reaction, Vet. Microbiol. 72 (2000) 285–293.

[8] Berri M., Souriau A., Crosby M., Crochet D., Lechopier P., Rodolakis A., Relationships between the shedding of *Coxiella burnetii*, clinical signs and serological responses of 34 sheep, Vet. Rec. 148 (2001) 502–505.

[9] Berri M., Souriau A., Crosby M., Rodolakis A., Shedding of *Coxiella burnetii* in ewes in two pregnancies following an episode of *Coxiella* abortion in a sheep flock, Vet. Microbiol. 85 (2002) 55–60.

[10] Berri M., Arricau-Bouvery N., Rodolakis A., PCR-based detection of *Coxiella burnetii* from clinical samples, in: Sachse K., Frey J. (Eds.), Methods in molecular biology, Humana Press Inc., Totowa, NJ, 2003, pp. 153–161.

[11] Berri M., Rousset E., Champion J.L., Arricau-Bouvery N., Russo P., Pepin M., Rodolakis A., Ovine manure used a garden fertiliser as a suspected source of human Q fever, Vet. Rec. 153 (2004) 269–270.

[12] Bildfell R.J., Thomson G.W., Haines D.M., McEwen B.J., Smart N., *Coxiella burnetii* infection is associated with placentitis in cases of bovine abortion, J. Vet. Diagn. Invest. 12 (2000) 419–425.

[13] Brennan R.E., Samuel J.E., Evaluation of *Coxiella burnetii* antibiotic susceptibilities by real-time PCR assay, J. Clin. Microbiol. 41 (2003) 1869–74.

[14] Derrick E.H., "Q" fever, new fever entity: clinical features, diagnosis and laboratory investigation, Med. J. Aust. 2 (1937) 281–299.

[15] Durand M.P., L'excrétion lactée et placentaire de *Coxiella burnetii*, agent de la fièvre Q chez la vache. Importance et prévention, Bull. Acad. Nat. Med. 177 (1993) 935–945.

[16] Fontaine M., Giauffret A., Russot P., Durand M., Importance des troupeaux ovins dans l'épidémiologie de la fièvre Q, Med. Mal. Infect. 8 (1975) 445–449.

[17] Hassig M., Lubsen J., Relationship between abortions and seroprevalences to selected infectious agents in dairy cows, J. Vet. Med. B 45 (1998) 435–441.

[18] Heinzen R.A., Hackstadt T., Samuel J.E., Developmental biology of *Coxiella burnetii*, Trends Microbiol. 7 (1999) 149–154.

[19] Hilbink F., Penrose M., Kovacova E., Kazar J., Q fever is absent from New Zealand, Int. J. Epidemiol. 22 (1993) 945–949.

[20] Kazar J., *Coxiella burnetii* Infection, Ann. NY Acad. Sci. 1063 (2005) 105–114.

[21] Komiya T., Sadamasu K., Toriniwa H., Kato K., Arashima Y., Fukushi H., Epidemiological survey on the route of *Coxiella burnetii* infection in an animal hospital, J. Infect. Chemother. 9 (2003) 151–155.

[22] Kruszewska D., Tylewska-Wierzbanowska S., Isolation of *Coxiella burnetii* from bull semen, Res. Vet. Sci. 62 (1997) 299–300.

[23] Lange S., Sollner H., Dittmar H., Hofmann J., Lange A., Q fever antibody titer follow up study in cattle with special reference to pregnancy, Berl. Munch. Tierarztl. Wochenschr. 105 (1992) 260–263.

[24] Lorenz H., Jager C., Willems H., Baljer G., PCR detection of *Coxiella burnetii* from different clinical specimens, especially bovine milk, on the basis of DNA preparation with a silica matrix, Appl. Environ. Microbiol. 64 (1998) 4234–4237.

[25] Maurin M., Raoult D., Q fever, Clin. Microbiol. Rev. 12 (1999) 518–553.

[26] Muramatsu Y., Maruyama M., Yanase T., Ueno H., Morita C., Improved method for preparation of samples for the polymerase chain reaction for detection of *Coxiella burnetii* in milk using immunomagnetic separation, Vet. Microbiol. 51 (1996) 179–185.

[27] Norlander L., Q fever epidemiology and pathogenesis, Microbes Infect. 2 (2000) 417–424.

[28] Öngör H., Cetinkaya B., Karahan M., Nuri Acik M., Bulut H., Muz A., Detection of *Coxiella burnetii* by immunomagnetic separation-PCR in the milk of sheep in turkey, Vet. Rec. 154 (2004) 570–572.

[29] Plommet M., Capponi M., Gestin J., Renoux G., Fièvre Q expérimentale des bovins, Ann. Rech. Vet. 4 (1973) 325–346.

[30] To H., Htwe K.K., Kako N., Kim H.J., Yamaguchi T., Fukushi H., Hirai K., Prevalence of *Coxiella burnetii* infection in dairy cattle with reproductive disorders, J. Vet. Med. Sci. 60 (1998) 859–861.

[31] Uwatoko K., Sunairi M., Yamamoto A., Nakajima M., Yamaura K., Rapid and efficient method to eliminate substances inhibitory to the polymerase chain reaction from animal fecal samples, Vet. Microbiol. 52 (1996) 73–79.

[32] Widjojoatmodjo M.N., Fluit A.C., Trensma R., Verdonk G.P.H.T., Verhoef J., The magnetic immunopolymerase chain reaction assay for direct detection of *Salmonella* in fecal samples, J. Clin. Microbiol. 30 (1992) 3195–3199.

[33] Wild J., Eiden J., Yolken R., Removal of inhibitory substances from human fecal specimens for detection of group A rotavirus by reverse transcriptase and polymerase chain reaction, J. Clin. Microbiol. 28 (1990) 1300–1307.

[34] Willems H., Thiele D., Frölich-Ritter R., Krauss H., Detection of *Coxiella burnetii* in cow's milk using the polymerase chain reaction (PCR), J. Vet. Med. B 41 (1994) 580–587.

[35] Woernle H., Limouzin C., Muler K., Durand M.P., La fièvre Q bovine – effets de la vaccination et de l'antibiothérapie sur l'évolution clinique et l'excrétion de *Coxiella* dans le lait et les sécrétions utérines, Bull. Acad. Vet. Fr. 58 (1985) 91–100.