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IFAT and ELISA phase I/phase II as tools for the identification of Q fever chronic milk shedders in cattle

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ABSTRACT

Q fever is a widespread zoonotic disease caused by *Coxiella burnetii*. In cattle the bacterial shedding can persist without symptoms for several months and the shedders identification is a critical issue in the control of the infection at herd level. Following the example of the human protocols for the assessment of Q fever infection status, the aim of this study was the evaluation of the antibody response dynamics to phase I and phase II antigens in *C. burnetii* shedder dairy cows by means of a phase-specific serology, to verify the suitability of the investigated tools in recognising milk shedders. A total of 99 cows were monitored during time and classified on the basis of serological and PCR results in five groups identifying different shedding patterns. The 297 sera collected in three sampling times were tested by means of ELISA IgG for differential phase I and phase II antibodies detection, while a selection of 107 sera were tested by means of phase specific IgM and IgG IFAT. Both ELISA IgG and IFAT IgG highlighted a low reactivity in non-shedder seropositive animals compared to chronic milk shedder animals. ELISA IgG seemed to perform better than IFAT IgG–IgM, showing significant serological differences among groups that allowed recognising specific serological group patterns, in particular for chronic and occasional milk shedders. These results supported the hypothesis that an animal classification based on phase patterns is reasonable, although it needs to be further investigated.

1. Introduction

Q fever is a widespread zoonotic disease caused by *Coxiella burnetii*, an obligate intracellular bacterium with a wide range of hosts. In domestic ruminants, which represent the major source of human infection, the disease is frequently subclinical, but late abortions, stillbirths and reproductive disorders can occur (Arricau-Bouvery and Rodolakis, 2005). Shedding of bacterium into the environment mainly occurs during parturition or abortion, but infected animals can also shed bacteria in milk, urine, faeces and vaginal mucus (Rodolakis, 2009). The shedding can last for variable time depending on species and excretion route. Infected cows can persistently shed bacteria in milk for several months without symptoms, while sporadic or intermittent shedding can occur in faeces

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or vaginal mucus (Guatteo et al., 2007); the faecal and vaginal excretion has been debated especially in goats as possibly due to environmental contamination (Roest et al., 2012).

Phase variation, similar to the smooth to rough transition of Enterobacteriaceae, is a significant characteristic of *C. burnetii*. Phase I is the virulent form that can be isolated from acutely infected animals, while phase II is the avirulent one that appears after several passages in cell culture (Raoult and Parola, 2007). The phase transition induces a detectable immunological response that in human medicine allows the differentiation between acute and chronic statuses: high titres of IgG to phase II antigens and lower titres of IgG to phase I antigens are associated to an acute stage of infection, while in chronic Q fever, the IgG titres to phase I and phase II antigens may both be high (Angelakis and Raoult, 2010). Phase II IgM increases before phase II IgG and allows the identification of the early stage of infection.

In veterinary medicine the commercially available immunological assays do not allow individual identification of animals that shed *C. burnetii*, although the identification of the stage of infection associated to the excretion patterns is a critical issue for the control of Q fever at herd level, considering that the presence of chronic shedders is reported in ruminants with or without clinical signs (Guatteo et al., 2007). The EFSA Opinion in 2010 (Sidi-Boumedine et al., 2010) underlined the need of a better awareness concerning the pathogenesis of Q fever in domestic animals, the infection kinetics and shedding patterns and emphasised the need for an improved diagnosis based on phase I and phase II antibodies detection.

The aim of this study was to evaluate in dairy cattle the antibodies response dynamics to phase I and phase II antigens in chronic and occasional milk shedders of *C. burnetii*, using a commercial experimental kit ELISA IgG for the differential detection of IgG anti-*Coxiella* phase I and phase II and an IFAT IgG–IgM kit targeted to the human diagnosis, experimentally modified for the detection of anti-*Coxiella* phase I and phase II antibodies in cattle.

2. Materials and methods

2.1. Background

The samples used in the study originated from animals farmed in four herds with confirmed Q fever cases. The herds were selected among those participating in the Veneto Regional Programme for surveillance and diagnosis of abortions, for which the laboratory analyses are routinely performed at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Legnaro, Italy.

The Regional Programme includes direct diagnoses on placenta and/or aborted foetus (BVDV, SBV, *Chlamydia* spp., *C. burnetii*, *Neospora caninum*, broad-spectrum microbiological tests) and serological tests on the aborting cow (IBRV, BVDV, *Neospora caninum, Brucella abortus/melitensis*, *Chlamydia* sp., *C. burnetii*). The Veneto region is free from Bluetongue and Brucellosis.

2.2. Herds selection and description

The four herds were selected according to the following criteria:

- At least one *C. burnetii* positive PCR result on an aborted foetus during the last 12 months;
- Herd size with an average of at least 100 cows milked;
- No other confirmed causes of abortion;
- No vaccination against *C. burnetii*.

These criteria were established because in the herds with PCR-positive abortions there is a high probability to detect shedder cows in milk, and milk excretion of *C. burnetii* could last up to 32 months (Angelakis and Raoult, 2010). Furthermore a herd size of at least 100 lactating cows allows obtaining an acceptable number of milk shedder cows, considering that the percentage of milk shedders is estimated around 14% (Guatteo et al., 2007).

- Herd 1: 215 cattle; breed Brown Swiss, an average of 107 milking cows, freestalls housing and a tandem milking parlour. During the last year, three cases of abortion with *C. burnetii* positive PCR were found in the farm. Poor fertility performances were reported.
- Herd 2: 200 cattle; breed Holstein, an average of 100 milking cows, freestalls housing and a herringbone milking parlour. During the last year, two cases of abortion with *C. burnetii* positive PCR were found in the farm. No specific problems on fertility were reported.
- Herd 3: 230 cattle; breed Holstein, an average of 100 milking cows, freestalls housing and a herringbone milking parlour. During the last year, one case of abortion with *C. burnetii* positive PCR was found in the farm. Poor fertility performances were reported.
- Herd 4: 606 cattle; breed Holstein, an average of 294 milking cows, freestalls housing and a herringbone milking parlour. During the last year, two cases of abortion with *C. burnetii* positive PCR were found in the farm. Poor fertility performances were reported.

2.3. Herds monitoring during time

At the first sampling (S0) all the lactating cows were screened on individual milk by means of real time PCR and on sera for total IgG anti-*Coxiella* by means of a commercial ELISA in order to assess their initial status concerning *C. burnetii* infection. The animals were then classified into four groups:

- Positive to PCR in milk, seropositive (ELISA+_PCR+);
- Positive to PCR in milk, seronegative (ELISA−_PCR+);
- Negative to PCR in milk, seropositive (ELISA+_PCR−);
- Negative to PCR in milk, seronegative (ELISA−_PCR−).

To follow the evolution of the shedding patterns in the groups during time, an average of 10 animals for each group were initially selected in each herd and monitored by collecting individual milk and blood samples every 2 months for three further times (S1, S2, S3). Some groups, such as ELISA−_PCR+ and ELISA+_PCR+, were scarcely represented, so a number <10 has been accepted.

Table 1
Animals selected to be monitored for *C. burnetii* infection in four diary herds during time for each group. The four groups were defined on the basis of screening on individual milk (real time PCR) and sera (IgG anti-*Coxiella* by means of a commercial ELISA).

	ELISA-_PCR−	ELISA-_PCR+	ELISA+_PCR+	ELISA+_PCR−	Tot
Herd 1	10	2	7	9	28
Herd 2	9	5	4	9	27
Herd 3	8	1	6	10	25
Herd 4	9	2	0	8	19
Tot	36	10	17	36	99

For some cows it was not possible to collect the complete set of samples (dry period, or culling), so from the total number of 139 animals, only 99 of them were included in the study (Table 1).

Stool samples were collected, together with the individual milk samples, to better assess the shedder status of each animal during time, but stool PCR results did not affect the study results and therefore were not included in the animals' classification. The limited relevance of faecal excretion in this species is already reported in other studies (Guatteo et al., 2007)

2.4. Samples collection

Blood samples were taken from the coccygeal vein into a 10 ml vacuum tube (Vacutainer, Becton Dickinson, Franklin Lakes, USA), stored in a refrigerated bag, brought to the laboratory and centrifuged at 2500 × *g* for 10 min within 12 h. Serum was then removed, split in two aliquots and stored at −20 °C.

Individual milk samples were aseptically collected after disinfection of the teat ends and discarding the first streams of milk according to the National Mastitis Council guidelines (1999) for mastitis milk sampling and stored at −20 °C.

2.5. Study design and samples selection for phase I/phase II evaluation

In order to evaluate the antibody response against phase I/phase II antigens as a tool for estimating the risk of *C. burnetii* milk shedding and in absence of an experimental infection, we classified the cows retrospectively on the basis of serological results on serum and molecular results on milk. The animals were classified on the basis of PCR results on milk as infected (at least one positive PCR result during the study) or non infected (no positive PCR results). With the 396 serum samples (99 subjects, four times) selected from the four herds at S0, S1, S2 and S3 we set up the following groups of animals:

- NI− (non infected, seronegative, not shedder, $n = 26$): always seronegative, never PCR positive on milk;
- NI+ (non infected, seropositive, not shedder, $n = 29$): always seropositive, never PCR positive on milk;
- CS (infected, seropositive chronic shedder, $n = 12$): always seropositive, always PCR positive on milk;
- OS+ (infected, seropositive, occasional shedder, $n = 20$): always seropositive, PCR positive on milk at just one sampling, or at two samplings but not consecutive;
- OS− (infected, seronegative, occasional shedder, $n = 12$): always seronegative, PCR positive on milk at just one sampling, or at two not consecutive samplings.

No seroconversions were demonstrated during the observation period.

From the four samplings, just S0, S1 and S2 (total of 297 samples) were considered for further study. S3 was excluded because we could not follow the evolution of the PCR and serological status after this time.

All the 297 sera were tested with an ELISA kit allowing the differential detection of IgG to phase I and phase II antigens (Table 2a).

With regard to the IFAT testing for the detection of IgG and IgM to phase I and phase II antigens, we followed the same classification criteria as the one considered for ELISA IgG, but reducing the number of tested samples due to the high costs of the commercial kit. We included about one sample for each selected animal; in some cases more than one sample per animal has been considered in order to create homogeneous groups. The total number of selected samples corresponded to 107 sera (Table 2b).

2.6. Analytical methods

Preliminary tests and herd selection: DNA from milk and stool samples was extracted with "QIAmp® DNA mini kit" (Qiagen) and tested with a commercial real time PCR kit (ADIAVET® COX REALTIME) for *C. burnetii.*

Blood samples were tested by means of a commercial ELISA kit, LSIVET Ruminants milk/serum Q-Fever (LSI, Lissieu, FR) to detect total anti-*Coxiella* IgG. The assay, which uses a cocktail of both antigen phases (I and II) to detect total anti-*C. burnetii* immunoglobulins G (IgG), was performed following the manufacturer's instructions.

ELISA phase I/phase II: blood samples collected at S0, S1 and S2 and selected for the study were analysed with an experimental ELISA kit, Chekit Q fever, (IDEXX Laboratories, Bern, CH). This kit is an indirect ELISA for the detection

Table 2a
Serum samples distribution among groups and herds tested by means of Q fever phase I/phase II ELISA IgG*.

	NI−	NI+	CS	OS+	OS−	Tot
Herd 1	24	27	6	21	6	84
Herd 2	27	15	6	18	15	81
Herd 3	15	21	21	12	6	75
Herd 4	12	24	3	9	9	57
Tot	78	87	36	60	36	297

Table 2b
Serum samples distribution among groups and herds tested by means of Q fever phase I/phase II IFAT IgG–IgM*.

	NI−	NI+	CS	OS+	OS−	Tot
Herd 1	5	9	8	1	6	29
Herd 2	5	5	5	6	6	27
Herd 3	5	4	9	2	2	22
Herd 4	5	5	7	3	9	29
Tot	20	23	29	12	23	107

* NI− = non infected, seronegative, not shedder; NI+ = non infected, seropositive, not shedder animals; CS = infected, seropositive chronic shedder animals; OS+ = infected, seropositive, occasional shedder animals; OS− = infected, seronegative, occasional shedder.

of phase I and phase II anti-*C. burnetii* immunoglobulins (IgG).

The kit had been produced by IDEXX with two different plates coated separately with PhI and PhII antigens. Antigens, their concentration and coating conditions were the same as for CHEKIT Q-fever, and the same negative and positive controls were used. The test was performed according to manufacturer's instructions, as already applied by other Authors (Böttcher et al., 2011; Sting et al., 2013). The results were expressed as S/p ratio, calculated using the following formula: $[S/p = (OD_{sample} - OD_{neg})/(OD_{pos} - OD_{neg})]$. According to manufacturer's instructions, for each antibody phase, samples were classified as positive when S/p was ≥ 0.4.

IFAT (Immunofluorescent antibodies test): a selection of 107 sera were tested with two commercial micro-immunofluorescent antibody tests (Q Fever IgG IFA and Q Fever IgM IFA, Focus Diagnostics Inc., Cypress, USA) for human blood test use. The diagnostic procedure was modified following the OIE indications (OIE, 2010) by replacing the anti-human antibodies conjugate of the two tests with an antibovine IgG conjugate (Sigma–Aldrich, Saint Louis, USA) and an antibovine IgM conjugate (Abcam, Cambridge, UK) respectively. The conjugates were diluted following the manufactured instructions. The cut-off serum dilution was stated at 1:16 as stated for the human protocol. Furthermore, positive control sera for IgG were replaced using bovine sera that tested positive to both ELISA IgG and CFT method, while positive control sera for IgM were replaced with bovine sera coming from cattle sampled in the acute phase of infection. For this purpose only positive sera taken from cattle that showed a seroconversion in the following 3–4 weeks were used. All the other steps of the procedures were performed following the manufacturer's instructions. A similar protocol has been used by Rousset et al. (2007) to adapt another commercial IFA kit for the diagnosis on goat.

2.7. Data analysis (ELISA IgG phase I/II and IFAT IgG–IgM phase I/II)

The analysis was limited to seropositive samples (groups OS+, CS, NI+), both for ELISA ($n = 183$) and IFAT ($n = 64$).

The S/p values obtained by the ELISA test phase I/II were examined by means of a mixed effects linear model. Group, phase and sampling time were included in the model as fixed effects; the interactions among these three factors were added in the full model and, if not significant to Type III tests of fixed effects, were further removed using a backward elimination process. Given that we observed different patterns of S/p values for the samples over the time, random sample-specific time slopes were included in the model; furthermore, the S/p values of the two phases tend to differ by sample, suggesting a random sample-specific intercept. Additionally, the sampling time was added in the model as repeated measure of the phase nested within a sample; the unstructured correlation structure was used to model the residuals. The results were expressed as Least-Squares Means (LS-Means) and Standard Errors (StdErr) of S/p values. The Akaike Information Criterion (AIC) and the residual diagnostics were used to evaluate the goodness of fit of the model.

Considering the IFAT, the non-parametric Kruskal–Wallis test was adopted to analyse the distribution of antibodies titres among the three groups (NI+, CS, OS+), after having evaluated the homogeneity of variance by means of the Siegel–Tukey test; the phase was used to stratify the analyses. Afterwards, the two-sample Wilcoxon–Mann–Whitney test was performed for the pairwise comparisons of the groups.

All statistical analyses were performed using SAS v9.3.

3. Results

The following results refer to seropositive samples belonging to groups CS, NI+ and OS+. With regard to NI− and OS−, both ELISA IgG phase I/II and IFAT IgG–IgM confirmed the negative results obtained with the ELISA IgG kit (LSI) used for the screening analyses, so they were not included in the statistical analysis.

With regard to the distributions of S/p values of the ELISA IgG test phase I/II, the results of the mixed model are reported in Table 3. Significant differences were observed among groups (p-value <0.001); in particular, the NI+ group showed significantly lower S/p values (Ls-mean 0.649) than both CS (Ls-mean 1.311) and OS+ (Ls-mean 1.422) groups. No differences were observed among sampling times (p-value = 0.081). The group-phase interaction resulted significant (p-value = 0.003), highlighting different trends between phase I and phase II of the three groups. For samples belonging to OS+ group, the S/p values of phase I resulted significantly higher than S/p values of phase II (p-value < 0.001). Conversely, no differences were observed between the two phases for NI+ (p-value = 0.578) and CS (p-value = 0.529) groups. The interaction between sampling time and phase highlighted a different trend for the two phases (p-value = 0.014); in particular, looking at LS-means, phase I showed broader variation during time compared to phase II which resulted more stable.

The serological analyses performed with the IFAT provided the following results:

- Sera positive for IgG vs. phase I: 71
- Sera positive for IgG vs. phase II: 62
- Sera positive for IgM vs. phase I: 5
- Sera positive for IgM vs. phase II: 1

Table 3
A total of 183 cow sera tested positive with ELISA phase I/phase II IgG and effect of group (CS, NI+, OS+), sampling time and phase on S/p values were analysed by means of a mixed effects linear model. Results were expressed as Least-Squares Means (LS-Means) and Standard Errors (S.E.) of *S/p* values. (CS = infected, seropositive chronic shedder animals; NI+ = non infected, seropositive, not shedder animals; OS+ = infected, seropositive, occasional shedder animals).

	Effects			*S/p* values			
	Group	Sampling time	Phase	LS-mean	S.E.	*F* value*	*p* value*
Group	CS			1.311	0.154	14.21	<0.001
	NI+			0.649	0.101		
	OS+			1.422	0.119		
Sampling time		1		1.103	0.087	2.58	0.081
		2		1.069	0.080		
		3		1.210	0.081		
Phase		1	1	1.244	0.093	4.22	0.044
		2	2	1.012	0.093		
Group × phase	CS		1	1.366	0.191	6.58	0.003
	CS		2	1.257	0.191		
	NI+		1	0.593	0.126		
	NI+		2	0.706	0.126		
	OS+		1	1.773	0.149		
	OS+		2	1.072	0.149		
Sampling time × phase		1	1	1.207	0.109	4.61	0.014
		1	2	1.000	0.109		
		2	1	1.134	0.098		
		2	2	1.005	0.098		
		3	1	1.391	0.101		
		3	2	1.030	0.101		

*The *F*-value and the *p*-value are the results of the Type III analysis of fixed effects.

All the samples positive to ELISA IgG LSI were confirmed as IFAT IgG positive, at least for phase I or phase II, with titres ranging from 1:16 to ≥1:4096. Only six samples were positive to IgM. Due to the limited number of positive samples, IgM results were not included in the statistical analysis.

Table 4 shows the distribution of antibodies titres of the IFAT test for IgG in each group, stratified by phase. The statistical analyses highlighted an overall significant difference among groups in both phases (phase I: *p*-value = 0.001, phase II: *p*-value <0.001), mainly due to the comparison between NI+ and CS groups. The CS group showed a median titre significantly higher than the NI+ group (*p*-value <0.001 for both phases): 256 and 32 versus 4096 and 1024 were the median titres in phase I and phase II for NI+ and CS groups, respectively. No differences were observed comparing the median titre of OS+ versus CS and of NI+ versus OS+, with regards to both phases; nevertheless, the OS+ group showed a significantly higher variability than NI+ group (phase I: *p*-value = 0.003; phase II: *p*-value = 0.040). Independently from the groups, the two phases resulted significantly different (*p*-value < 0.001), showing higher titres in phase I compared to phase II.

4. Discussion

The identification of shedders has been suggested as one possible strategy to control Q fever in animals. This strategy relies on a "test and cull" approach, to facilitate the identification and culling of infected animals that are excreting *C. burnetii*, thereby reducing the overall prevalence of infection in a herd (EFSA, 2010). Since individual diagnosis based on commercially available serological tools is not achievable in cattle, a combination of methods that includes serology and agent detection by PCR is usually suggested as the proper procedure (Sidi-Boumedine et al., 2010; Niemczuk et al., 2014). In human medicine, good results in term of individual diagnosis had been achieved by the application of a phase I/II serology. Different trend in IgM and IgG phase-antibodies levels are recognisable and associated to acute and chronic infection statuses, making serology the tool of choice for human

Table 4
Distribution of antibodies titres of Q fever IFAT IgG test, stratified by phase and group (CS = infected, seropositive chronic shedder animals; NI+ = non infected, seropositive, not shedder animals; OS+ = infected, seropositive, occasional shedder animals). Groups providing negative IFAT results at the 1:16 screening cut off in both phase I and II were not included.

Phase	Group	Antibodies titres distribution					
		N	Min	25th Pctl	Median	75th Pctl	Max
1	CS	29	8	1024	4096	4096	4096
	NI+	23	32	128	256	1024	4096
	OS+	12	8	68	2048	4096	4096
2	CS	29	8	128	1024	2048	4096
	NI+	23	8	32	32	256	1024
	OS+	12	8	12	128	640	4096

diagnosis and IFAT the gold standard method (Fournier et al., 1998).

Following the example of human protocol, phase-specific ELISA IgG and IFAT IgG–IgM were performed on cattle sera and compared to verify if phase patterns and reactivity could help in the cattle chronic milk shedders identification, providing encouraging data.

The phase-specific modified CHEKIT Q-Fever has been reported to be a valuable diagnostic tool in cattle (Böttcher et al., 2011) and goats (Sting et al., 2013), showing association between phase II positivity and *C. burnetii* shedding in the acute stage of infection. As far as the Authors know, no data are available about the use of a phase specific IFAT detecting IgM and IgG in cattle. An "in-house" phase specific IgM and IgG indirect ELISA was found useful in understanding the dynamics of immunological response and identifying the infection status in goats (Roest et al., 2013) as it is in human diagnostics, suggesting that IgM investigation could have a significant application combined with IgG in ruminants. To test IgM patterns in cattle, the use of a commercially available IFAT adapted to cattle serum was an easy and practical solution.

The tests analysis revealed that ELISA IgG seemed to perform better than IFAT IgG–IgM, showing significant serological differences among investigated groups, which allowed recognising specific group patterns.

The CS group showed high reactivity in both phases in ELISA IgG, suggesting that a cow with high phase I and II values is likely to be a chronic milk shedder. A persistently high seropositivity in chronic shedder cows had been reported by means of traditional ELISA using a combination of phase I and phase II antigens (Guatteo et al., 2007, 2012), but with a positive predictive value around 50% (Guatteo et al., 2007); the other 50% included non or sporadic/intermittent shedders. The phase specific ELISA IgG provided a further indication among high seropositive samples: the finding of significantly higher S/p values of phase I than S/p of phase II in OS+ made this group recognisable from the CS, even if the mean values of the two phases were observed to not differ between OS+ and CS. This result suggested the need of a combined phase I and II diagnosis to differentiate CS, which is more relevant from an epidemiological point of view as chronic milk shedders mainly maintain bacterial circulation. The occasionally shedder animals are a common finding in cattle (Guatteo et al., 2007) and goats (Arricau-Bouvery et al., 2003; Berri et al., 2007; Rousset et al., 2009), but their role and epidemiological significance are still controversial, since the pathogenesis and shedding patterns of Q fever in ruminants are still not completely clarified.

The meaning of OS+ pattern was of difficult interpretation: phase I positive and phase II negative animals were rarely found by Böttcher et al. (2011) in multiparous cows by means of phase-specific modified CHEKIT Q-Fever. This status was reported to be sometime subsequent to a phase I and II positive pattern, followed by a negative serological evolution in time. OS+ pattern could be a possible evolution of the infection status in cows that have built up an efficient cellular immunity as reported in the cited study for phase I positive and phase II negative animals. Anyway, in Böttcher et al. (2011) a low or undetectable level of antibodies was reported. The common dominance of phase II on phase I in the acute Q fever infection in goats (Sting et al., 2013) and cattle (Böttcher et al., 2011) supported the association of OS+ to a late stage of infection, but a pick of phase I IgG was also detected in experimentally infected goats 4 weeks before parturition and consequent *C. burnetii* shedding (Roest et al., 2013).

The difference in group-phase interaction between CS and OS+ was not found by means of IFAT IgG.

ELISA IgG and IFAT IgG were in agreement in detecting low reactivity in NI+ compared to shedder animals, suggesting that cows showing low reactivity values in both phases are unlikely to be shedders, as it was previously reported (Guatteo et al., 2007). These results supported the hypothesis that an animal classification based on phase patterns seems to be reasonable, although it needs to be further investigated.

During the period of observation, the time of sampling did not significantly influence results in ELISA IgG (p-value = 0.081). This lack of difference among sampling times justifies the choice of testing about one sample per animal in IFAT IgG–IgM.

Referring to interaction between sampling time and phase, no specific trends were observed concerning phase I values fluctuation during the study; a longer period of observations could clarify this finding and eventually allow recognising a specific trend.

The limited number of positive IFAT IgM samples in at least one phase and the absence of specific study about IgM trends in cattle during *C. burnetii* infection did not allow analysing data and taking any conclusion. IgM antibodies are known to be the first immunological response in acute stage of infection. Phase II IgM were demonstrated to rise significantly early after *C. burnetii* infection with a strong response in mice (Andoh et al., 2007), humans (Maurin and Raoult, 1999), and goats (Roest et al., 2013). The four investigated herds were identified as infected but the time of infection was unknown, so an endemic presence of bacterium circulation was probable and consistent with a low number of new acute infections. This conclusion is supported by the fact that the reactivity of phase I was likely to be higher or at least equal to phase II in both ELISA IgG and IFAT IgG–IgM for the all investigated groups. To better assess the suitability of the phase specific serological tests for the detection of chronic milk shedders, a study for which the time of infection is known for each sample would be advisable, but several aspects, like suitable premises and biosecurity risks, make an experimentally infection a very difficult study to be realised with cattle. Further investigations could verify the strength of the above statements starting from a serological screening on a given population by means of phase specific serology, followed by classification into groups and confirmation tests based on molecular methods. The availability of an individual serological test able to identify chronic milk shedder animals may represent a practical and useful tool for a rapid diagnosis and control of Q fever in dairy cow herds. Considering the other investigated groups, a point to be clarified should be what kind of epidemiological relevance they really have and, in particular, if one of these phase patterns could be related to a high risk of

becoming a chronic shedder. The lack of available current tests that allow recognising high-risk animals has been underlined in goats by Authors (Hogerwerf et al., 2014), for which animals that will shed large amount of bacteria cannot be recognised before parturition. The issue has posed considerable limitation to the "test and cull approach" in goats. This topic is poorly investigated in cattle, but the awareness on the dynamics of phase specific response and its diagnostic application in a field setting could help in this sense. The practical approach to the infection control could be different for goats and cattle: due to different shedding patterns, in goats it is an overriding concern to identify the high-risk animals before parturition. In cattle, for which the milk shedding can last for long time and has not been clearly associated to a zoonotic risk, this objective could not be considered as a priority and the starting point of an epidemiological control could be not necessarily linked to the pre-partum period.

5. Conclusion

This study, focused on the evaluation of the suitability of phase specific serology in detecting *C. burnetii* chronic milk shedders in cattle, showed significant results in terms of recognisable serological patterns in shedder/non-shedder animals, in particular by means of ELISA IgG that allows a further discrimination between chronic and occasional milk shedders. The serological patterns were recognisable at group level, while an individual classification seemed to be premature, due to an often low, but present, intra-group variability. The mentioned findings will be the starting point to investigate the dynamics of this variability for the assessment of an individual diagnosis based on phase specific serology.

Conflict of interest

None.

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