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Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal

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Abstract

The recent outbreak of Q fever in The Netherlands warned European health authorities of the need of studying *Coxiella burnetii*. In Portugal, little is known about *C. burnetii* infection in animals. A cross-sectional study was designed to investigate the exposure to *C. burnetii* in sheep and goats in the Central region of Portugal, estimating the herd and individual prevalence. A serosurvey was conducted in a two levels random sampling of 89 herds and 460 animals. Individual blood samples were collected from animals older than 6 months, and specific antibodies anti-*C. burnetii* were detected by ELISA testing. Results showed a global herd prevalence of 32.6% (95% CI: 23.1 to 42.1%). Herd prevalence was higher in mixed herds (38.5%; 95% CI: 12 to 65%) and in sheep herds (37.5%; 95% CI: 21 to 54%) than in goat herds (28.8%; 95% CI: 17 to 41%). Global individual prevalence was estimated at 9.6% (95% CI: 6.9 to 12.2%), and it was higher in goats (10.4%; 95% CI: 7.8 to 13%) than in sheep (8.6%; 95% CI: 5.8 to 11.4%). Sample positive percentages (S/P) ranged from 41.5 to 185.9%. S/P percent higher than 100 was found in 18.2% (8/44) of sera from distinct herds. Positive results were significantly associated with goats, older animals and larger herds. These results revealed the presence of *C. burnetii* in small ruminants evidencing their potential role in the infection cycle.

Keywords: *Coxiella burnetii*, epidemiology, seroprevalence, zoonosis.

Introduction

Q fever is a zoonotic infection caused by *Coxiella burnetii*, an obligate intracellular bacterium. It was described in Australia in 1937 for the first time (Maurin and Raoult, 1999). Currently, this disease presents a worldwide distribution, affecting a wide range of domestic and wildlife animals (Arricau-Bouvery and Rodolakis, 2005, Rousset et al., 2010).

The clinical signs of Q fever are not pathognomonic neither in humans nor in animals. This lack of specificity is the first major obstacle to its diagnosis (Arricau-Bouvery and Rodolakis, 2005, Angelakis and Raoult, 2010). In humans, acute Q fever can be asymptomatic or it can manifest as a nonspecific flu-like illness. Complications associated with pneumonia or hepatitis requiring hospitalization may be observed in about 2% of patients. Chronic Q fever may appear as an endocarditis, an osteoarticular infection, a chronic hepatitis or as a chronic pneumonia in patients with predisposing factors and/or inappropriate antibiotherapy. The infection during pregnancy may lead to abortion (Maurin and Raoult, 1999, ECDC, 2010). Also, cases of chronic fatigue syndrome have been described infrequently following *C. burnetii* infection (Angelaskis and Raoult, 2010, van Asseldonk *et al.*, 2013). In animals, Q fever is mainly reported in livestock ruminants and occurs, usually, as an asymptomatic infection (Woldehiwet, 2004, Arricau-Bouvery and Rodolakis, 2005, Rousset et al., 2010). In small ruminants, abortions, premature delivery, delivery of weak offspring and stillbirth are reported (Rodolakis, 2006). In cattle, clinical signs of Q fever can be less obvious than in small ruminants. However, a very recent study demonstrated that abortion and irregular repeat breeding are important risk indicators in cattle dairy herds (Saegerman et al., 2013). Also, an association of Q fever with metritis and infertility has been suggested (To et al., 1998, Woldehiwet, 2004, EFSA, 2010).

Epidemiological studies have demonstrated a relationship between the infection in humans and ruminants (Gilsdorf et al., 2008, Schimmer et al., 2010, van den Brom et al., 2012). However, the Q fever prevalence and incidence are not well known, and have been underestimated for many years (EFSA, 2010). Shedding of bacteria occurs by secretions and excreta from infected animals, namely vaginal secretions, milk, faeces and urine. During birthing and/or abortion the bacterium is excreted massively in genital secretions, placenta and fetal fluids (Berri et al., 2001, Berri et al., 2002, Berri et al., 2005, Arricau-Bouvery et al., 2003, Guatteo et al., 2006). Because of the existence of fecal shedders and the high resistance of *C. burnetii*, bedding material must be considered as a source of infection (Rodolakis, 2006, Guatteo et al., 2007, Rousset et al., 2010).

Recently, the European Commission (EC) formulated concerns about the increase number of human Q fever cases associated with small ruminant herds, in urban or residential areas, in Europe (Panaiotov et al., 2009, Medic et al., 2005, Porten et al., 2006, Gilsdorf et al., 2008). In the Netherlands, 4108 acute human cases were notified between 2007 and 2011 (van Loenhout et al., 2012). Typing of bacteria by multiple-locus variable number tandem repeat analysis (MLVA) showed a genetic similarity of isolates recovered from human and animal samples, indicating a relationship between human cases and the occurrence of infection in ruminant herds (Klaassen et al., 2009, van der Hoek et al., 2010, Roest et al. 2011a, Roest et al. 2011b).

Following the EC's demand to assess the risk for humans and animals associated with Q fever, the European Food Safety Authority (EFSA), in a scientific opinion, highlighted the considerable uncertainty that still exists in the understanding of *C. burnetii* infection in domestic ruminant populations and the knowledge of its prevalence (EFSA, 2010).

In Portugal, Q fever is a notifiable disease since 1999, and the average number of notifications is 0,10 cases per 10^5 inhabitants. However, these data might be underestimated. Between 2004 and 2005, 32 cases were diagnosed in the Centre for Vectors and Infectious Diseases at the National Health Institute but only 12 were notified, clearly suggesting an under-notification (Santos et al., 2007). Despite the zoonotic pattern of Q fever, the information about the occurrence of infection in animals is scarce. A few studies demonstrated the presence of bacteria in clinical samples from zoo animals and from ruminants (Clemente et al., 2008, Clemente et al., 2009). Also, our previous results on screening bulk tank milk indicated the presence of *C. burnetii* in ruminant herds originated from different regions (Anastácio et al., 2012). A genotypic diversity among *C. burnetii* isolates from animals and human clinical samples was shown (Santos et al., 2012). These studies were based on a limited number of samples obtained from clinical cases. They highlighted the need of epidemiological study of *C. burnetii* in other geographical regions, increasing the number of samples randomly sampled. In this context, the present study aimed to understand the current status of small ruminants to the exposure of *C. burnetii* in the Center of Portugal. A cross-sectional study was designed to estimate the herd and the individual apparent prevalence of specific antibodies anti-*C. burnetii*.

Material and Methods

Study design and sampling approach

A cross-sectional survey was carried out during the 4th trimester of 2011 in small ruminant herds from the central region of Portugal.

The number of herds used in the study was calculated taken into account the regional census (N=1527 small ruminant's herds), obtained from the Official Regional Veterinary Services. The sample size calculation was performed using the program WinEpiscope version 2,0 based on the formula $n=[t^2 P_{esp} (1-P_{esp})]/d^2$, considering n the required size sample, t the student value for a 95% confidence level (1,96), P_{esp} the expected prevalence and d the desired absolute precision. Taking into account that the study population (N) was small (n/N > 5%), the required sample size was adjusted by the formula $n_{adj}=(N*n)/(N+n)$ (Thrusfield, 1995).

It was considered an expected herd prevalence of 57% (Fernandes, 2008) a desired absolute precision of 10%, and a 95% confidence interval, resulting in an estimated sample of 89 herds. The list of total herds was used for a simple random sampling, using the program Microsoft Excel®.

In each herd, the sample size was calculated to detect the presence of infection using the WinEpiscope version 2,0 based on the formula $n=[1-(1-p_1)^{1/d}][N-d/2]+1$ in which n is the required sample size, N is the population size, d is the minimal number of affected animals in the population and $p1$ is the probability of finding at least one case in the sample (Thrusfield, 1995). For this purpose, the herd size was considered, the expected proportion of seropositive animals was established in 15% (Guatteo et al., 2011) and a 95% confidence level was considered. On farms sized ≤ 10 animals, samples were taken from all the animals. The list of animals in each herd was used for a simple random sampling using the program Microsoft Excel®.

Blood samples were collected from selected animals simultaneously undergoing statutory routine brucellosis testing (animals aged > 6 months), by the veterinary practitioner group, in charge of the Official Sanitary Campaign. Individual apparent prevalence was calculated globally considering the total amount of samples. The serum

obtained by centrifugation of blood samples was stored at -20ºC until serological testing. A questionnaire was filled up on the surveyed herds by interviewing farmers, during sample collection.

Serological analyses

Sera were tested for the presence of specific antibodies anti-*Coxiella burnetii* using an indirect commercial ELISA kit, LSIVET Ruminant Milk/Serum Q Fever® (LSI, France). Optical density (OD) values were measured at 450 nm. Sample/positive percentages (S/P percent) were calculated by the adjustment with the negative control, using the formula (OD sample – OD negative) / (OD positive – OD negative) x 100. The resulting S/P percent were divided in different classes, according to manufacturer's instructions: negative (Neg; S/P per cent $\leq$ 40), low positive (LP; 40 < S/P $\leq$ 100), positive (Pos; 100 < S/P $\leq$ 200), high positive (HP; 200 < S/P $\leq$ 300) and very high positive (VHP; S/P > 300).

Statistical analysis

For statistical analysis purposes, it was considered the herd size (continuous), species in the herds (categorical nominal: sheep, goats or mixed herds) or species individually (categorical nominal: sheep/goats), productive system (categorical nominal: intensive, extensive, semi-extensive), age (continuous), geographic distribution (categorical nominal: counties), co-habitation with other species (categorical nominal: yes/no), and reports of reproductive disorders within the previous year (ie, at least one of the following disorders: abortion, premature delivery, infertility, metritis and/or placentary retention) (categorical nominal: presence/absence).

The response variables were the S/P percent (continuous) obtained in each individual serum by ELISA testing and its categorization in positive or negative (categorical nominal: positive/negative). So, herds were categorized as positive or negative, according to the results obtained for individual serum. A herd was considered positive when at least one serum showed a positive result to ELISA testing. The apparent prevalence of anti-*C. burnetii* antibodies was calculated at herd and at individual level. Statistical uncertainty was assessed by calculating the 95% confidence interval for each of the proportions according to the expression S.E. 95% C.I. = $1.96\ [p\ (1 - p)\ /n]^{1/2}$ (Thrusfield, 1995) and using WinEpiscope version 2.0.

Statistical analyses were performed using SAS (version 9.1.2). Simple logistic regression test was performed to assess individually the main factors associated with *C. burnetii* seropositivity at herd and individual level. After evaluating these factors with significant influence ($p< 0.05$) on positive results, a multiple logistic regression analysis was conducted to assess the joint relationship between several independent factors and *C. burnetii* seropositivity. Also, a multiple logistic regression analysis was used to evaluate the combined effect of multiple variables in S/P percent (continuous) ($p< 0.05$).

Results

Descriptive analysis

Of all 1527 eligible herds, 89 (5.8%) were selected to this study. The mean herd size was 6.7 animals (SD=11.305, range 1-104) and 46 herds (51.7%) had less than 4 animals. Goat herds were predominant (n=52, 58.4%) followed by sheep herds (n=24, 27%) and mixed herds (n=13, 14.6%). It was also observed a predominance of meat

producing herds (n=79, 88.8%), a semi-extensive grazing system (n=89, 100%) and herd localization at the county of Coimbra (n=58, 65.2%). In these herds, 460 animals were sampled (mean age 45.6 months (SD=29.9, range 9-167).

Table 1 summarizes the descriptive characteristics and seroprevalence results of the ELISA test in herds. Global prevalence in herds was estimated on 32.6% (CI 95%: 23.1 to 42.1%). Herd prevalence was higher in mixed herds 38.5% (95% CI: 12 to 65%) and in sheep herds 37.5% (CI 95%: 21 to 54%) than in goat herds 28.8% (95% CI: 17 to 41%). Geographic distribution of positive herds showed a frequency of 32.8% (19/58) in Coimbra, 42.8% (7/16) in Vila Nova de Poiares, 30% (3/10) in Miranda do Corvo, 0% (0/1) in Lousã and 0% (0/4) in Penacova.

Co-habitation with other species was observed in 64% (57/89) of herds, and a positive result was obtained in 36.8% (21/57). Pets (dogs and/or cats), alone or together with farm animals, were reported in 47.4% (27/57) of herds, amongst which 37% (10/27) showed a positive result.

The occurrence of previous reproductive disorders was reported in 6.7% (6/89) of herds particularly abortion in 2.2% (2/89) and infertility in 4.5% (4/89).

In 27.6% (8/29) of positive herds at least one serum presented a high S/P per cent (> 100), and in 31% (9/29) more than one serum was classified as positive (S/P per cent > 40).

Table 2 shows the descriptive statistic of results at individual level. Global individual seroprevalence was estimated on 9.6% (CI 95%: 6.9 to 12.2%), but considering the ruminants species, seroprevalence was estimated on 10.4% (CI 95%: 7.8 to 13%) in goats and 8.6% (CI 95%: 5.8 to 11.4 %) in sheep. Mean age of positive animals was 50 months (SD 28.4, range 14-135), and 44 months for the negative animals (SD 30.2,

range 9-167). S/P per cent ranged from 41.5 to 185.9 (mean 75.6, SD 34.07), and 18.2% (8/44) of samples were classified as positive ($100 < S/P \leq 200$), all of them from different herds.

Univariable analysis

Individual factors were tested to find associations with positive results in herds and in animals individually. The variable production system was not included as the reference. Categories were inexistent as almost all the herds had a semi-extensive grazing system. Univariable analysis identified three factors with significant effect on *C. burnetii* seropositivity at herd or animal levels. At the herd level, only the herd size evidenced an association with seropositivity ($p< 0.01$), using the logistic regression test (Figure 1). Indeed, it was observed that all the herds with more than 14 (6.8%) animals were classified as positive. Individually, the logistic regression test evidenced an association between the increase of animal's age and seropositivity ($p< 0.01$). Also, it was observed that the probability of having a positive result is higher in goats than in sheep ($p< 0.05$), using the same statistic model.

Multivariable analysis

A multivariable model was performed to test simultaneously variables found to be associated in univariable analysis. A multiple logistic regression test confirmed that species and age were both associated with positive results ($p< 0.05$) (Figure 2).

Also, a linear regression model tested the effect of multiple variables in S/P per cent. The age of the animal was the only factor evidencing an influence with S/P per cent ($p<0.01$).

Discussion

Q fever is recognized as zoonotic disease worldwide with multiple animals acting as *C. burnetii* reservoirs. The present study was designed as an approach to evaluate the exposure of small ruminants to *C. burnetii* in the center of Portugal. A commercial ELISA test was used to detect IgG anti-*C. burnetii* (phase I and phase II).

Our results indicate a global herd prevalence of 32.6%, higher in mixed herds (38.4%) and in sheep herds (37.5%) than in goat herds (28.8%). These data are similar to those reported in sheep herds from Sardinia, Italy (38%) (Masala et al., 2004). However, higher values of seroprevalence (74%) were reported in sheep herds from Spain (Ruiz-Fons et al., 2010) and Turkey (83%) (Kennerman et al., 2010), while in Germany, sheep herd seroprevalence was shown to be lower (28%) (Hilbert et al., 2012). According the data from goat herds in other European countries, a higher seroprevalence was reported compared to this study, namely in The Netherlands (43.1%) (Schimmer et al., 2011), in Northern Ireland (42.9%) (McCaughey et al., 2010), in Spain (45%) (Ruiz-Fons et al., 2010) and in Sardinia, Italy (47%) (Masala et al., 2004). Among these studies, only Ruis-Fonz et al. (2010) and Schimmer et al. (2011) performed the serologic test with the same commercial ELISA used in our study. Overall, it can be suggested that the herd prevalence in our study was lower than the range of herd prevalence described in other European countries.

The global individual seroprevalence was 9.6%. Goats were significantly related with seropositivity at animal level ($p < 0.05$). Indeed, individual seroprevalence was slightly higher (10.4%) in goats than in sheep (8.6%). These results are similar to those obtained in other European seroprevalence studies such as Spain, Ireland, Greece and Sardinia, Italy, in which values ranged from 6.5% and 13% (Ruiz-Fons et al., 2010; McCaughey

et al., 2010; Pape et al., 2009; Masala et al., 2004). A higher individual seroprevalence (17.2%) was reported in The Netherlands, in 2008, during the Q fever epidemic outbreak (van den Brom et al., 2012).

The increase of the age of the animal was associated with seropositive results ($p < 0.01$). This is consistent with the report from Schimmer and collaborators, in The Netherlands, where they also found an increase of seroprevalence with age (Schimmer et al., 2011). This finding suggests the occurrence of horizontal transmission among animals and the maintenance of infection within adult populations (Garcia-Perez et al., 2009, Ruiz-Fons et al., 2010, Astobiza et al., 2012). It may be explained by the increase rate of contagion as a consequence of a higher probability of contact during lifetime (Ruiz-Fons et al., 2010). Furthermore, an IgG based antibody test was used, thus possibly evidencing past exposure to *C. burnetii* (McCaughey et al., 2010). The presence of such antibodies cannot be associated exclusively to a current infection, since animals can remain seropositive for years after the acute infection have been resolved (McQuiston et al., 2002). The high mean age of animals in our study (3.9 years) might be related to regional cultural habits and the traditional consumption of meat from older animals. Indeed, most of the sampled animals came from meat production herds in a semi-extensive grazing system.

The long-time contact with *C. burnetii* in the surveyed herds together with the random selection of sampled herds and animals can explain the lack of association found between reproductive disorders and seropositivity (Garcia-Perez et al., 2009, Ruiz-Fons et al., 2010, Astobiza et al., 2012). However, the presence of an asymptomatic infection in herds cannot be excluded. In fact, our previous results showed the presence of specific antibodies (Anastácio et al., 2012) and DNA of *C. burnetii*, detected by qPCR (unpublished data), in bulk milk tank from dairy ruminant farms with reports of

reproductive disorders. Indeed, an association between reproductive disorders and *C. burnetii* prevalence in ruminants has been reported in some studies (Cabassi et al., 2006, Garcia Perez et al., 2009).

Despite the significant association between goats and positive results ($p<0.05$), from an individual perspective, it was found a lower herd prevalence in goats than in sheep, which is in agreement with data from a study conducted in Northern Spain (Ruiz-Fons et al. 2010). The higher individual prevalence together with the lower herd prevalence may suggest that the within-herd prevalence is high in goats. Nevertheless, this could not be assessed in this study because sample size calculation in herds aimed the detection of infection, not the estimation of within-herd prevalence. Moreover, differences of prevalence between sheep and goats cannot be explained by different sampling periods in relation to the lambing season. Sample collection occurred in early pregnancy in both species and the reproductive cycle is similar among both species in this region.

The herd size was associated to seropositive results ($p< 0.01$), thus the probability of a positive result increases with the number of animals per herd. Other studies in goats (Schimmer et al., 2011; Schimmer et al., 2012) and in cattle (McCaughey et al., 2010) support our findings. The increased risk of introduction and/or transmission of pathogens in a large population is probably related with the increased number lambing females at lambing season (Woldehiwet, 2004) and by other management factors like larger amounts of feed, animal supply and a higher number of professionals working at or visiting the farm (Schimmer et al., 2011). Therefore, larger herds are more prone to acquire and develop Q fever, and the number of animals must be considered a risk factor to *C. burnetii* dissemination.

In conclusion, this study confirms the presence of specific anti-*C. burnetii* antibodies in goats and sheeps in Portugal. To our knowledge, this is the first seroprevalence survey performed in small ruminants in this country. To clarify the infection status in these herds, namely the presence of an active infection, the shedding of bacteria must be assessed. Also, a better elucidation of the epidemiology of Q fever in Portugal requires the inclusion of other animal species from a large geographical area.

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Conflict of interest statement

The authors declare no conflict of interests.

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1 Table 1. Descriptive characteristics and seroprevalence results in sheep and goat herds

Variable	Frequency (n)	Seroprevalence (%)	[a]CI 95%
Selected	89	32.6	23.2-42.1
Herd size			
≤ 10	80	28.8	19.1-38.5
> 10	9	66.7	36-97.4
Herd species			
Goat	52	28.8	16.7-40.1
Sheep	24	37.5	18.3-56.7
Mixed	13	38.4	12.2-64.8
Type of production			
Meat	79	34.2	24.1-45.8
Milk	3	33.3	1.8-87.5
Mixed	7	14.3	1-58
Productive system			
Intensive	0	0	na[b]
Extensive	0	0	na[b]
Semi-extensive	89	32.6	23.2-42.1
County			
Coimbra	58	32.8	21.4-46.5
Miranda do Corvo	10	30	8.1-64.6
Lousã	1	0	na[b]
Pencova	4	0	na[b]
Vila Nova de Poiares	16	43.8	20.8-69.5
Cohabitation with other species			
Yes	61	34.4	23-47.8
No	28	28.5	14-48.9
Cohabitant species			
Pets	7	28.6	5.1-69.7
Farm animals	30	36.7	20.6-56.1
Pets and farm animals	20	40	20-63.6
Reproductive disorders			

Yes	6	33.3	6-75.9
No	83	32.5	22.9-43.8

[a] Confidence interval (range within which is reasonably confident to find the real prevalence)

[b] not aplicable

Table 2: Descriptive statistics of *C. burnetii* antibodies in sheep and goats individually.

Test Category	Nr of animals	Mean age of animals (months)	Apparent prevalence	95% CI[a] (p)	Range of S/P[b]	Mean S/P value
Positive	44	50	0,096	[0,07;0,12]	41,5 - 185,9	75,6
Negative	416	44	0,904	[0,88;0,93]	0-38,82	4,2

[a] Confidence interval (range within which is reasonably confident to find the real prevalence)

[b] S/P - Sample positive percentage

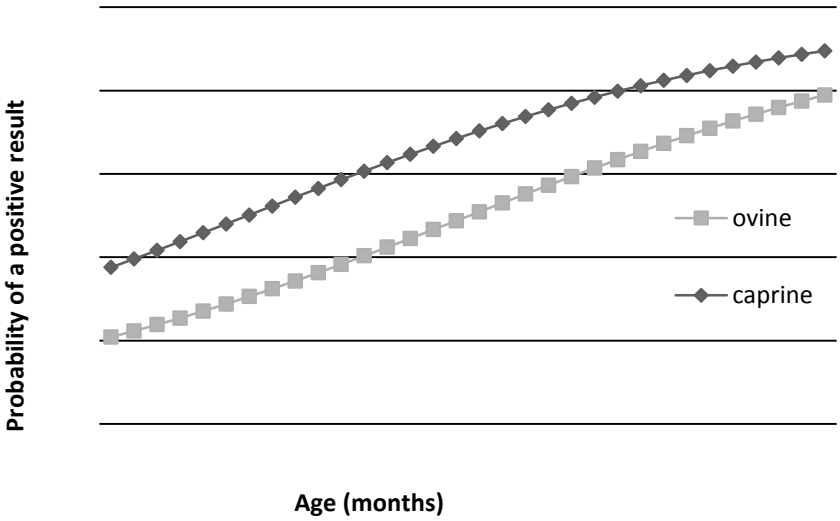

Figure 2: The probability of positive results for *C. burnetii* antibodies increases with age, in each month, by species using a logistic regression model (Ovine: Intercept= -1.5037±0.3204; Caprine: Intercept= -0.6783±0.3552; β_1= 0.0214±0.0084).

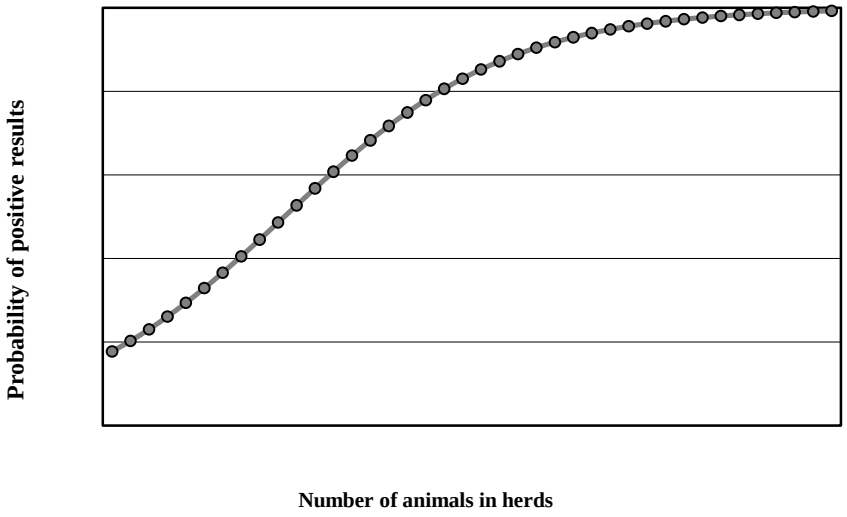

Figure 1. The probability of a positive result for *C. burnetii* antibodies increases with the number of animals in herds (Intercept= -1.7009±0.4459; β_1= 0.1644±0.0668).