Coxiella burnetii (Q fever) seroprevalence in prey and predators in the United Kingdom: evaluation of infection in wild rodents, foxes and domestic cats using a modified ELISA

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Summary

Coxiella burnetii, the agent of Q fever, is recognised as a worldwide zoonosis with a wide host range and potentially complex reservoir systems. Infected ruminants are the main source of infection for humans, but cats and other mammals, including wild rodents, also represent potential sources of infection. There has been a recent upsurge of reported cases in humans, domestic ruminants and wildlife in many parts of the world, and studies have indicated that wild brown rats may act as true reservoirs for *C. burnetii* and be implicated in outbreaks in livestock and humans. However, investigation of reservoir systems is limited by lack of validated serological tests for wildlife or other non-target species. In this study, serum samples from 796 wild rodents (180 bank voles, 309 field voles, 307 wood mice) 102 wild foxes and 26 domestic cats from three study areas in the UK were tested for the presence of antibodies to *C. burnetii* using a commercial indirect ELISA kit modified for use in multiple wildlife species. Test thresholds were determined for each species in the absence of species-specific reference sera using a bi-modal latent class mixture model to discriminate between positive from negative results. Based on the thresholds determined, seroprevalence in the wild rodents ranged from 15.6% to 19.1% depending on species (overall 17.3%), and was significantly higher in both foxes (41.2%) and cats (61.5%) than in rodents. This is the first report to quantify seroprevalence to *C. burnetii* in bank voles, field voles, wood mice, foxes and cats in the UK, and provides evidence that predator species could act as indicators for the presence of *C. burnetii* in rodents. The study demonstrates that wildlife species could be significant reservoirs of infection for both livestock and humans, and the high seroprevalence in domestic cats highlights the potential zoonotic risk from this species.

Introduction

Coxiella burnetii is an obligate intracellular proteobacterium belonging to the family *Rickettsiaceae* and is the aetiological agent of Q fever, a worldwide zoonotic pathogen (Woldehiwet 2004). There has been a recent upsurge of reported cases in humans, domestic ruminants and wildlife in many parts of the world, in particular in the Netherlands, and also in the UK (Amitai et al. 2010; Enserink 2010; Koch et al. 2010; Lemos et al. 2010; Ruiz-Fons et al. 2008;van der Hoek W. et al. 2010;Wallensten et al. 2010).

C. burnetii has a very wide host range and has been found in many host species, including vertebrate and invertebrate taxa (Babudieri 1959), and is unique among the family Rickettsiaceae in its non-dependence on arthropod transmission. Although it can be found in ticks and other arthropods, the main source of infection for domestic animals and humans is exposure to parturient secretions by inhalation of contaminated aerosols (Angelakis and Raoult 2010;Woldehiwet 2004). The major mode of infection is via aerosol (inhalation), but infection by ingestion of contaminated dairy products is also possible (Woldehiwet 2004), and ingestion of infected prey species has been implicated as a means of infection in cats (Komiya et al. 2003). The organism is found in the blood, lungs, spleen and liver, and is shed in urine and faeces. During pregnancy, in both humans and other animals, there is massive contamination of the placenta with *C. burnetii* which can lead to abortion or low foetal birth weight (Babudieri 1959;Carcopino et al. 2009). The mammary glands are also infected and large numbers of bacteria can be found in milk. The extracellular form of *C. burnetii* is resistant to killing by desiccation, low or high pH, or by disinfectants such as 0.5% sodium hypochlorite or UV radiation. Therefore it can be persistent in the environment for several weeks, and can also be spread by the wind, so direct contact with animals is not necessary for infection to occur (Maurin and Raoult 1999).

Domestic ruminants (cows, sheep, and goats) are the main source of infection for humans, but the wide range of host species comprising arthropods, birds and mammals, including small rodents (Burgdorfer 1963;Woldehiwet 2004), dogs and horses (Roest 2013), suggests that complex reservoir systems may exist. *C. burnetii* has been isolated from hares (Marrie et al. 1993), wild mice and rabbits, and Q fever pneumonia in humans has been linked to exposure to wild rabbits (Marrie et al. 1986). In man, exposure to wildlife has been identified as a risk factor for seropositivity to Q fever in a recent survey of US veterinarians (Whitney et al. 2009). In the UK, antibodies to *C. burnetii* have been detected in wild brown rats on farms (Webster et al. 1995). Recent studies in the Netherlands have also indicated that wild brown rats, especially those near farms, may act as true reservoirs for *C. burnetii* (Reusken 2011) and may be implicated in the recent outbreaks in livestock and humans in the Netherlands.

Cats are also now recognised as an important host species and have been implicated in human outbreaks of Q fever (Kosatsky 1984). *C. burnetii* DNA is found in vaginal and uterine samples from healthy cats (Cairns et al. 2007), and thus parturient cats are a zoonotic risk (Woldehiwet 2004). A Japanese study found a higher seroprevalence to *C. burnetii* in stray cats (41.7%) compared with pet cats (14.2%) (Komiya, Sadamasu, Kang, Tsuboshima, Fukushi, & Hirai 2003), with a greater consumption of wild prey (rodents, birds) suggested as an explanation for the higher seroprevalence in the stray animals. However, in a search for additional reservoirs for human Q fever in the outbreak affecting the Netherlands for 2007-2010, no *C. burnetii* DNA was isolated from the placentas of domestic cats and the study provided no indication for major reservoirs of *C. burnetii* in cats, goats or pigs in this outbreak (Roest 2013). In the same study, *C. burnetii* DNA was detected in both dogs and horses (Roest 2013). Dogs are a known domestic animal reservoir (Woldehiwet 2004) of *C. burnetii* and human infection has been directly linked to pet dogs (Komiya et al., 2003b).

Evidence of infection has also been found in wild canids, such as grey fox and coyote (Enright et al. 1971).

Being an intracellular pathogen, serological testing is mainly relied upon for detection (OIE 2008). However, commercially available ELISA kit tests for serological testing of *C. burnetii* are designed for use in domestic ruminants and are not validated for wildlife species. Common difficulties encountered with serological testing of wildlife species using indirect ELISA methodologies are the lack of species-specific secondary antibodies and the lack of species-specific reference sera (positive and negative controls) which both hamper the validation and assessment of performance of the assay used. Therefore there is a need to develop and validate ELISA testing methodologies to enable their use on multiple species including wildlife. Use of an identical test on multiple species is likely to be more cost-efficient and effective use of resources when undertaking serological studies for *C. burnetii* than using a specific test for each individual species.

The aim of this study was to investigate the seroprevalence of *C. burnetii* in three species of wild rodents (bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*) and wood mice *Apodemus sylvaticus*)), red foxes (*Vulpes vulpes*) and domestic cats in the UK using a modified indirect ELISA kit adapted for use in multiple species.

Materials and methods

Sample collection

Serum samples were collected from wild rodents, foxes and domestic cats in three rural study sites of varying habitat in northern England (Cumbria) and Scotland (Pentlands and Borders) over a twenty-nine month period (April 2007- October 2009) (For details of sampling dates see Figure 1 (Meredith 2013).

Wild rodents were trapped and blood sampled as described in Meredith et al (2013). Rodent trapping was carried out over four seasons (April-August 2007; November 2007 - March 2008; April - August 2008; November 2008-December 2008).

Foxes were shot as part of routine pest control operations and blood collected from the thoracic or abdominal cavity within 24 hours of death. Cat blood samples were obtained via veterinary surgeons from animals presented for veterinary examination where a blood sample was routinely taken, and surplus serum was submitted for the study. An owner questionnaire was used to confirm that the cats submitted for the study were known to hunt and consume wild rodents. The sampling methodology was approved by the University of Edinburgh's Ethical Review Committee.

For all samples, serum was separated by centrifugation at 4000rpm within 24 hours of collection and stored at -70C. Foxes and cat samples were collected opportunistically throughout the study period. Location (grid reference), age class (adult/ non-adult), and sex were recorded for all animals.

Modified indirect ELISA

Serum was tested using a modified commercial indirect ELISA kit adapted for the purposes of this study by the manufacturer for use in multiple non-ruminant species (IDVet, Montpellier, France). This was achieved by replacing the species- specific (ruminant) conjugate with a mixture of protein A and G peroxidase conjugate to detect bound serum (LeCoq, IDVet, personal communication). 96-microwell plates were supplied pre-coated with phase I and phase II antigens, from a _C. burnetii_ strain isolated in France from an aborted bovine placenta. All reagents and solutions were supplied directly by the manufacturer. Briefly, 90 µl of dilution buffer was added to each microwell and 10 µl of each test serum and a positive and negative control serum added in duplicate to the wells and

incubated for 45 minutes at room temperature. Wells were washed with approximately 300 µl wash solution three times. 100 µl conjugate (protein A and G) was added to each well, and the plate incubated for 30 minutes at room temperature. Wells were washed with approximately 300 µl Wash Solution three times 100 µl substrate solution was added to each well and the plate incubated for 15 minutes at room temperature in the dark. The reaction was stopped by adding 100 µl stop solution (H_2SO_4) to each well and the optical densities measured on a Model 550 microplate reader (Bio-Rad Laboratories, Hemel Hempstead, UK) reader at 450nm. The test results were deemed valid as per manufacturer's instructions if the mean optical density (OD) of the 2 positive controls (OD_{PC}) was > 0.350 and the ratio of the mean OD values of the 2 positive and 2 negative controls was > 3. The sample:positive control (S/P) percentage was calculated using the formula

$$S/P = \frac{OD_{sample} - OD_{nc}}{OD_{pc} - OD_{nc}} \times 100$$

Threshold determination

The modified ELISA developed for this study could not be validated prior to deployment, as known confirmed seronegative and seropositive samples for the wildlife species tested and cats were not available and the commercial ELISA on which this was based has only been validated for livestock, which may have substantially different immune responses to *C. burnetii* compared to the target species. In the absence of any validation against a gold standard, we used a statistical method of ELISA threshold selection in these species as outlined in Opsteegh *et al.* 2010. Briefly, a bi-modal normal distribution was fitted to the observed ELISA values to obtain estimates for the mean and standard deviation of the two distributions representing negative and positive results. This model was fitted using Bayesian Markov chain Monte Carlo methods (Gilks 1998), implemented using the JAGS software

(Plummer 2013) interfaced using the 'runjags' package (Denwood 2013) from R (R Core Team 2013). Nine chains with over-dispersed starting values were used, and trace plots visually assessed to ensure convergence. The posterior distributions were output from this simulation and subsequently used to calculate the optimum threshold value (defined as the highest total sensitivity and specificity of the test) for each iteration of parameter values, using two cumulative distribution functions to calculate the probability of observing an ELISA value above and below the given threshold for the negative and positive groups respectively. Uncertainty regarding the true parameter values representing negative and positive groups was reflected in a full posterior distribution for optimised threshold, with the mean of this distribution taken as the best estimate for the new test threshold. The data was split into prey (the 3 rodent species) and predator (cat and fox) data to reflect possible differences in the threshold, and the process above repeated on each dataset.

Data analysis

Generalised linear mixed-effect models with binomial errors (*GLMEb*) were used to consider the variation in seroprevalence (based on the calculated thresholds as described above) between host species and within host species. For the main analyses the study area the samples came from was entered as a random effect to take account of area related infection pressures. In addition, individual study sites within areas were entered as a random effect nested within study area for rodent species. Sex and age of host species and sampling season were incorporated as fixed effects in different univariate models. For age, animals were classed as either adult or non-adult (juvenile and subadult). In addition, differences between the seroprevalences of the 3 rodent species within each study site were also considered in separate *GLMEb* with just study site as the random effect. All these analyses were carried out in R (R Core Team 2013). The spatial clustering of seropositive and negative animals in each

study area in both rodents and predator species was investigated using Bernouilli statistical

spatial models of the number of positive animals at a particular location (Kulldorff 1997) in

SaTScan (v9.1.1 www.satscan.org). Statistical significance level was placed at $P<0.05$.

Results

A total of 924 serum samples were tested for antibodies to <u>C. burnetii</u>, from 793 rodent species (180 bank voles (BV), 309 field voles (FV), 307 wood mice (WM)), 26 cats and 102 foxes (**Table 1**). The majority of samples for both rodents and foxes were collected in seasons 1 to 3, and in season 4 only the Cumbria study area was sampled for rodent species. Most of the cat samples were obtained after season 3, with cat samples from the Borders and Pentlands study areas collected up to over a year after the last rodent sampling in those areas. Fox samples were collected throughout the study. All 3 rodent species were found in the 3 study areas.

Threshold determination

The MCMC mixed model converged upon two distinct distributions for both the predator and prey species, with median estimates of 6.9 (95% credible intervals: 6.8-7.0) and 22.7 (20.8-22.7) representing the mean of the negative and positive prey distributions, and median estimates of 11.4 (10.4-12.8) and 24.5 (21.0-28.3) representing the mean of the negative and positive predator distributions. These fitted distributions are shown along with the observed data in Figure 1. While it did not make any qualitative difference to the final results, the S/P of 98.1 in the predator data and 58.9 in the prey data were excluded from the threshold determination procedure as this improved convergence and reduced the spread of the posteriors, for the loss of 0.78% and 0.13% of the values. Based on the posterior estimates for the distributional parameters obtained, the threshold optimisation procedure described produced best estimates for the prey and predator thresholds of 10.27 and 16.31, respectively. Based on these thresholds and distributions fitted to the observed data, our modified ELISA test has a median sensitivity of 93.5% (95% credible interval 88.4-97.6%) and specificity of 99.2% (98.8-99.6) for prey, and a median sensitivity of 89.0% (76.6-100) and specificity of

97.9% (86.7-100) for predators.

Rodent species

The overall seroprevalence in the three rodent species was 17.3% (**Table 1**). Within individual rodent species, there was no significant difference in seroprevalence (15.6-19.1%, $P>0.403$). In addition, there was no significant difference in seroprevalence in bank voles (13.1-19.6%), field voles (6.1-28.9%), or wood mice (1.8-19.0%) between study sites ($P>0.137$), though only 17 bank voles were obtained from the Pentlands site. Furthermore, no significant difference in seroprevalence was found between rodent species within any of the study areas ($P>0.301$, **Table 1**).

There was a significantly greater seroprevalence in female rodents (90/383; 23.6%) compared to male rodents (48/404; 11.9%, $P=0.005$). The greater prevalence in females was observed in all 3 rodent species, significantly so for wood mice (F=22.8%, M=10.9%, $P=0.002$; bank voles F=23.3%, M=13.3%, $P=0.254$; field voles F=24.2%, M=12.1%, $P=0.350$). Only 1/21 non-adult rodent (wood mouse) was seropositive (4.7%), compared to 137/795 (17.9%) adults..

Seasonality and spatial patterns

There was a significance difference in seroprevalence between seasons ($P<0.001$), with an increase in seroprevalence from an average of <3% at the start of the study (Season 1) to ~50% at the end ($P<0.001$, **Figure 2**). This increase was observed in all 3 rodent species separately ($P<0.001$, **Supp Figure 1**) with no difference in the rate of increase between prey species ($P>0.051$). However, this pattern was not observed in all 3 study areas – with no increase observed in the Borders study area overall ($P=0.959$) or individual prey species ($P>0.305$). In contrast, an increase was observed both in the Cumbrian and Pentlands study areas overall ($P<0.001$, **Supp Figure 1**) and in individual prey species (Cumbria $P<0.0498$,

Pentlands P<0.010).

All rodent trapping locations in the Pentlands site included at least one seropositive rodent (seroprevalence range 6.4-60% **Figure 3**), as did all Cumbrian locations with >7 rodents trapped (11/15 locations, range 4.8-38%). In contrast, only 3/7 of Border rodent trapping locations had rodents with a seroprevalence >0% and levels were in general lower (range 3.6-21%). This variation in seroprevalence with trapping location was reflected in 2 statistically significant spatial clusters in the Borders study area (P<0.001), one associated with the 2 most easterly rodent trapping locations with the greatest seroprevalence (>16%) and the other with the 4 negative trapping locations. In addition, 3 clusters were identified in the Pentlands study area (P<0.001) with the 2 trapping locations with seroprevalences >57% considered as separate spatial clusters of high seroprevalence and the 2 most southerly trapping locations a cluster of low seroprevalence. No such spatial clusters for low or high seroprevalence were detected in the Cumbrian study site rodent data (P>0.369, **Figure 3**).

Predators

The overall seroprevalence in predators was 45.3% (**Table 1**), and while the seroprevalence in cats (61.5%) was greater than that of the foxes (41.2%) this was not significant (P=0.066). There was also no significant difference in seroprevalence in cats between the study areas (18.2-72.7%, P>0.278), though only <12 samples from cats were obtained from any single study area. In contrast, the seroprevalence in samples obtained from foxes in the Borders area (64.1%) was significantly higher than those obtained from Cumbria (26.1%, P=0.002) and higher than samples obtained from the Pentlands study area (29.4%, P=0.052). There was also a significantly higher seroprevalence in samples from cats in Cumbria (66.7%) compared to the foxes (26.1%, P=0.027). No such significant difference was observed between the predator species in the Borders (72.7 vs. 64.1%, P=0.595) and Pentlands (33.3

vs. 29.4%, P=0.858).

Seroprevalence was not significantly different between sexes ($F=22/41=53.7\%$, $M=33/62=53.2\%$, P=0.893), nor was any differences within cats (F=75.0%, M=62.5%, P=0.676) or foxes (F=48.5%, M=50.0%, P=0.946). Only adult cats were sampled, and no difference in seroprevalence was observed between adult (25/46, 44.6%) and non-adult foxes (14/24, 58.3%, P=0.768).

Seasonality and spatial patterns

While there was no overall seasonal pattern in seroprevalence of the predator species combined (P=0.460) or if foxes were considered alone (P=0.083), there was a change in seroprevalence in cats with time (P=0.032), from 96.8% in the autumn of 2008 down to 28.3% by spring 2009. There was no statistical evidence of spatial clustering of infection, either when predators were considered overall, or if just foxes were considered within each study area (P>0.133, **Figure 3**).

Predator and rodent comparison

Seroprevalence was statistically significantly higher in predators compared to rodents for the study overall (P<0.001, **Table 1**, **Figure 3**). Furthermore the seroprevalence in both foxes and cats were statistically significantly higher than all individual rodent species (P<0.001). However, there was no statistically significant correlation between the overall levels of seroprevalence observed in the different rodent sample seasons in prey and predator species in the 3 study areas (P=0.072). In addition, if individual prey species were considered or if foxes were considered separately, this result did not change (P>0.063).

Discussion

This study is the first report of seroprevalence to _C. burnetii_ in bank voles, field voles, wood mice, foxes and cats in the UK. In other species of rodents, a seroprevalence of 9.7% (3/31) has been reported in wild rats and 3.2 % (1/31) in wild mice in Northern Ireland (McCaughey et al. 2010) but in the rest of the UK this pathogen has only been reported in wild brown rats in England (Webster, Lloyd, & Macdonald 1995). Seropositive cats and wild carnivores have been reported before, including foxes in the US (McQuiston and Childs 2002), but not in the UK.

In this study, all species investigated were seropositive in all seasons, with seroprevalence varying from 1.8-72.7% depending on species and study area. The year round detection of seropositive rodents and foxes in all study areas indicates that these species could maintain _C. burnetii_ as reservoir hosts, as indicated for brown rats in previous studies (Reusken 2011; Webster, Lloyd, & Macdonald 1995). Seasonal and area differences in levels of seropositivity could be due to many possible factors, including the intrinsic differences in study areas selected in terms of habitat type, species population density, distribution and home range size, and presence and degree of direct or indirect contact with other possible sources of _C. burnetii_ infection such as domestic livestock. In studies of other pathogens, such as hantavirus in wild rodents population density and seroprevalence have been shown to be highly dependent on habitat type (Heyman et al. 2009; Olsen 1975). Seroprevalence in rodents was highest (21.9%) in the Pentlands area with evidence of spatial clustering. This was the most agricultural of the study areas, and the cluster associated with the very high seroprevalence (>60%, **Figure 3**) was adjacent to farmland on which sheep were grazed and lambed. Although clinical disease due to _C. burnetii_ had not been reported in sheep or cattle on this farm, and no routine testing for exposure was been carried out, it is possible that these rodents could have been exposed to higher levels of _C. burnetii_ in this site from close contact

with ruminants or a contaminated environment, allowing "spillover" of _C. burnetii_ to occur from livestock to wildlife (Reusken 2011). However, the finding that this high seroprevalence in wild rodents was not associated with disease incidence in livestock in this site could be due to many factors, such as insufficient levels of direct or indirect contact due to rodent or livestock density for infection by aerosol or ingestion, insufficient levels of viable extracellular pathogen, or that infected rodents were not excreting _C. burnetii_ at times of year or in areas of pasture where livestock were being grazed in close proximity to enable a "spillback" effect to occur. The infection dynamics and route by which transmission of infection from wild rodents to livestock may occur is unclear, and greater understanding of this is necessary to determine the factors involved where a high prevalence in rodents might correspond to a higher risk of disease in livestock. For example, in addition to the possibility of vertical transmission or shedding during parturition, it is thought that arthropod vectors such as ticks may play a significant role in the transmission of _C. burnetii_ among wild rodents and lagomorphs (Babudieri 1959, Marrie 1986), so more complex routes of transmission may be involved in maintenance of infection in rodent reservoirs and rodent to livestock transmission.

It was interesting to note the overall increase in seroprevalence during the duration of the study (**Figure 2**), which was present in all three rodent species and in 2 of the 3 study areas. A direct explanation for this is not apparent, but could be related to cyclical fluctuations in rodent population density (Lambin et al. 2000). However, the concurrent decline in seroprevalence in domestic cats means the factors behind these change remain to be elucidated.

The finding that seroprevalence was significantly higher in predator species than in prey in all study areas also provides some evidence that predator species (foxes and cats) could act as

indicators for the presence of _C. burnetii_ in rodents, presumably by ingestion of these prey species, however the lack of correlation between seroprevalence levels in predator and prey species indicates that infection dynamics in these multi-host systems are likely to be complex and a single seroprevalence value in predator or prey species may be difficult to interpret in terms of assessing the degree of livestock or human disease risk. Exposure of predators to infected prey could also be occurring by other routes in addition to ingestion, such as via aerosols of parturient secretions (_e.g._ by exploration of rodent nesting areas), or via arthropod vectors; there may also be intraspecies maintenance of infection, and direct or indirect transmission from other wildlife species or livestock.

A common approach for determining thresholds for ELISA tests is to analyse the optical densities of serum from known positive and negative populations, then placing the cut-off at an S/P between these two populations where overlap is minimised (Gardner et al. 1996;Greiner et al. 1994). This is the approach adopted by the manufacturers of the current test for testing for Q fever in domestic ruminants (A. Lecoq, IDVet Ltd., pers. comm.). An alternative approach is to use the mean value from a known negative reference population and add a two-or three-fold standard deviation to the mean to this value to determine the threshold (Richardson et al. 1983). However, serological testing and interpretation of results in wildlife species is challenging because the majority of serologic tests designed for human or livestock pathogens, such as the one used in this study, have not been validated for use in non-target species (Greiner and Gardner 2000) and tend just to be directly transposed from use in domestic livestock species (Gardner, Hietala, & Boyce 1996).

ELISA tests have been used in wildlife species previously, including black bears, various deer species, hares, monkeys, wild rats, raccoon dogs, serow, wild pigs and palm civets in Japan, where species-specific conjugated antibodies were also not available and were

replaced with conjugated Protein A and G (Ejercito et al. 1993). Ejercito et al. (1993) used a distribution analysis approach to determine threshold values of an ELISA test for a serosurvey of a variety of species of wild animals for antibodies against *C. burnetii* in Japan using a qualitative (titre) rather than quantitative (S/P) approach to the interpretation of results in the absence of known species specific thresholds. In their study, Ejercito et al determined a threshold value for considering a species positive by titrating serial dilutions (1:100 to 1:3,200) of serum for each animal and looking at the bimodal distribution of antibody titre per species. They report that, usually, two peaks of antibody titre distribution were observed in every species with a peak in the lower dilution and a peak in the higher one, and considered the tapering end of the first peak, which was the start of the second peak, to be the cut-off for seropositive samples. Species with only one peak at the low dilution were considered *Coxiella*-antibody negative. They found that species with a high antibody prevalence, such as black bears, deer and hares, usually had a 1:100 cut-off titre, whereas those with a low antibody prevalence such as monkeys and nutria (a rodent species) had a cut-off titre of 1:400. In another study, Webster and Macdonald (1995) used a direct IgG ELISA to test wild brown rats for antibodies to *C. burnetii* in addition to IFA testing and also used serial dilutions to determine a titre of >1:100 as positive (Webster and Macdonald 1995).

However, the lack of species-specific reference sera can make interpretation of test results in wildlife species difficult, particularly if there are very low proportions of suspected seropositive or seronegative animals and the two populations are not distinct. Furthermore, reports of ELISA test use cannot be found in species of rodents other than rats or nutria (*Myocastor coypus*), nor in foxes or domestic cats. This means that for many organisms and their hosts there is a requirement for a robust methodology that can be used to determine and interpret the seroprevalence in wild and/or novel species.

The methods employed within this paper attempt to address this issue, by employing a mixed-model analysis to separate the observed data into separate distributions assumed to represent negative and positive individuals, and selecting a new test threshold for the target species based on the fitted distributions. This approach has been used by others (e.g. to determine seroprevalence of _Toxoplasma. gondii_ in sheep (Opsteegh 2010)), is statistically more rigorous than simply drawing a line on observed data 'by eye', and also provides a degree of precaution against misidentification of separate distributions by providing no prior information about the separation in means or relative variances of these two distributions.

Although this methodology does assume that infected and uninfected animals would be represented by 2 distributions of ELISA values, as opposed to a more uniform continuous distribution, we would advocate this approach for any future studies involving a diagnostic test that has not been validated in the target species. While we adopted the standard approach to determining our thresholds via optimisation of the total sensitivity and specificity of the test, the approach could easily be modified to select a threshold based on a more specific optimisation criterion – for example, the highest test sensitivity with a specificity of 99%, or any other combination of characteristics. However, even with the approach we adopted very high specificities were obtained (>97%) indicating that our seroprevalence estimates are likely to be conservative, and therefore if anything an underestimation of true seroprevalence.

In the present study, as in the studies by Ejercito _et al._ (1993) and Webster and Macdonald (1995) described above, the threshold value for being considered positive also varied between species - 10.3% for the three rodent species and 16.3% for cats and foxes. Sensitivity and specificity found in the present study were 93.5% and 99.2% respectively for prey, and 89.0% and 97.9% respectively for predators. In human studies, the ELISA also has a high sensitivity and good specificity. For example in the diagnosis of acute Q fever in man,

the ELISA has a reported sensitivity of 80% for anti-phase II IgG and 84% for anti-phase II IgM and a specificity of >99% in one study (Waag et al. 1995), and in a other study a specificity of 97.7% and sensitivity of 95.65% for anti-phase II IgM (Frangoulidis et al. 2006).

One way of increasing confidence in the results found and interpretation of thresholds would have been to compare the results of the ELISA test used in this study with results using another serological test method such as CF or IFA. However, due to intrinsic differences between tests, interpretation can be difficult – for example the positive control in the ELISA test used was likely to be negative by CF (A LeCoq, IDVet, personal communication), because complement fixing antibodies appear later after exposure to *C. burnetii*, although they persist for long periods after illness (Murphy and Field 1970). Due to the limited amounts of serum available from the species tested in this study, use of another test was not undertaken but would be highly desirable.

Overall, this study confirms that wildlife species have the potential to contribute significantly to reservoirs of Q-fever infection for both livestock and humans, and wildlife surveillance may be a useful tool in monitoring patterns of infection and potential disease risk. The high seroprevalence in domestic cats also highlights the potential zoonotic risk of this species.

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Acknowledgements

Table 1. Seroprevalence for <u>*C. burnetii*</u> (with exact binomial 95% confidence intervals) for rodents, cats and foxes in each study area, with number positive for *C. burnetii* as determined by threshold analysis

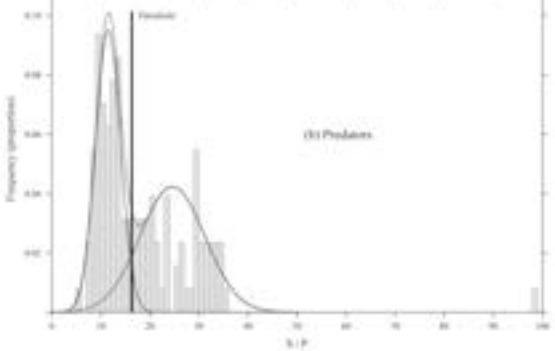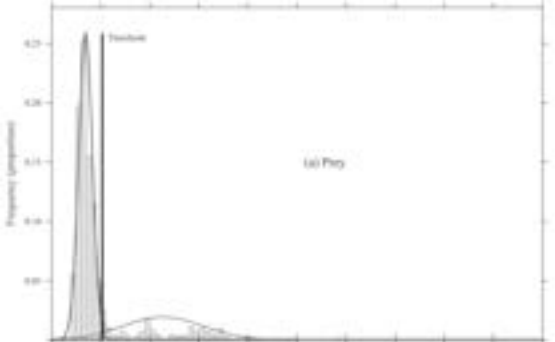| Species			*C. burnetii* results	
	Area	Total	Positive	Seroprevalence (95% CI)
Prey				
Bank vole	Pentlands	102	20	19.6 (12.4-28.7)
	Borders	17	3	17.7 (3.8-43.4)
	Cumbria	61	8	13.1 (5.8-24.2)
	Total	180	31	17.2 (12.0-23.6)
Field vole	Pentlands	97	28	28.9 (20.1-39.0)
	Borders	99	6	6.1 (2.3-12.7)
	Cumbria	113	25	22.1 (14.9-30.9)
	Total	309	59	19.1 (14.9-23.9)
Wood mouse	Pentlands	153	29	19.0 (13.1-26.1)
	Borders	55	1	1.8 (0.1-9.7)
	Cumbria	99	18	18.2 (11.8-27.2)
	Total	307	48	15.6 (11.8-20.2)
All	Pentlands	352	77	21.9 (17.7-26.6)
	Borders	171	10	5.9 (2.8-10.5)
	Cumbria	273	51	18.7 (14.2-23.8)
	Total	796	138	17.3 (14.8-20.2)
Predator				
Cat	Pentlands	6	2	33.3 (4.3-77.7)
	Borders	11	8	72.7 (39.0-94.0)
	Cumbria	9	6	66.7 (29.9-92.5)
	Total	26	16	61.5 (40.6-79.8)
Fox	Pentlands	17	5	29.4 (10.3-56.0)
	Borders	39	25	64.1 (47.2-78.8)
	Cumbria	46	12	26.1 (14.3-41.1)
	Total	102	42	41.2 (31.5-51.4)
All		128	58	45.3 (36.5-54.4)

Figure legends

Figure 1. Distribution of S/P values for (a) prey and (b) predator species (light grey bars). Solid curves are the fitted distributions using median estimates of the mean and variance of the two distributions from the MCMC analysis, with the compound distribution derived from these constituent normal distributions added on top. The best estimate threshold associated with the optimised maximum sum for specificity and sensitivity is shown as a vertical solid line.
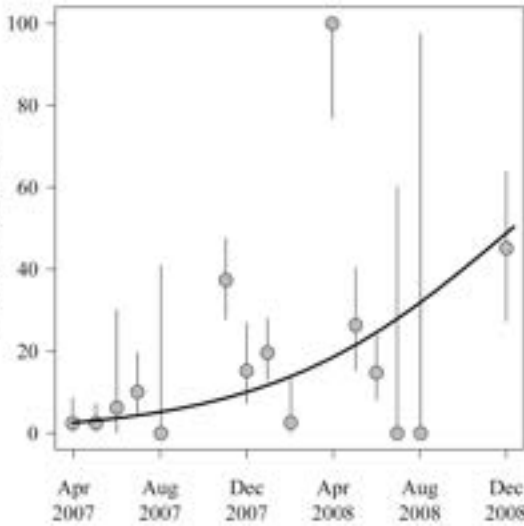
Figure 2. Plot of the seroprevalence observed in prey species with sample month. Vertical lines indicate the exact binomial 95% confidence interval and the solid thick line the fitted line from the *GLMEb* statistical model.

Figure 3. Summary map of the seroprevalences of the rodent and fox and cat samples for the 3 study areas (Pentlands, Borders and Cumbria). The seroprevalence of samples positive at a sampling site indicated by the proportion of each pie chart darkly shaded. The large pie charts are for rodents and range from 1-82 rodents and for the predators 1-10 foxes and 1-5 cats. The smaller pie charts with semi-transparent circles underneath are fox pie charts with fox territories of approximately 10km^2 also indicated. For the Borders area a sample was also obtained from one cat (positive) where the owner lived 8km north of the area in map (not shown), and for the Cumbria area 2 cat samples (1 positive, 1 negative) were also obtained where the owners lived either lived over 4km north of the area in map (not shown).

Supplementary Figure 1. Plots of the seroprevalence observed in prey species with sample month subdivided by rodent species (a-c) or by study area (d-f). Vertical lines indicate the exact binomial 95% confidence interval and the solid thick line the fitted line from the *GLMEb* statistical model

(a) Prey

(b) Predators

(Pentlands)

(Borders)

(Cumbria)

Prey seroprevalence

seronegative

seropositive

Rodent sampling site

Predator seroprevalence

Cats - seronegative

Cats - seropositive

Foxes - seronegative

Foxes - seropositive

Fox territories