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Serological and molecular evidence of *Coxiella burnetii* in samples from humans and animals in China

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Abstract

Introduction. *Coxiella burnetii* is the agent of Q fever, a worldwide zoonosis. To add to the available knowledge of the disease in China, *C. burnetti* infections were investigated in convenience samples from five animal species and humans from Yangzhou, Jiangsu province, eastern China.

Materials and methods. Commercial ELISA kits were used to detect antibodies to phase I and II *C. burnetii*. A FRET-qPCR targeting the outer membrane protein *com1* gene was also developed to detect *C. burnetii* DNA in blood samples from animals and humans, and bovine milk samples.

Results. Seropositive cattle (44/150; 29%), goats (33/150; 22%), humans (45/180; 25%) and pigs (4/130; 3%) were found, while dogs (0/136; 0%) and cats (0/140; 0%) were seronegative. Seropositivity in humans was associated with increasing age, but there was no gender difference. DNA was amplified from two milk samples (2/150, 1.3%), while none of the blood samples were positive. The sequences of the obtained amplicons were identical to those of the *com1* gene of the universal *C. burnetii* RSA 493 strain and other stains from China.

Conclusions. The findings indicaten that *C. burnetii* is endemic in Yangzhou, China, and therefore human and animal health workers should be aware of the possibility of infections and the occurrence of outbreaks of Q fever.

Key words

Coxiella burnetii, Q fever, seroprevalence, FRET-qPCR, China

INTRODUCTION

Q fever is an important yet under-reported and under-diagnosed zoonosis with almost worldwide distribution. The causative agent is the obligate intracellular Gram-negative category B biothreat proteobacterium *Coxiella burnetii* [1]. While the organism is maintained in nature in various mammals, birds and ticks, it is domestic animals, especially ruminants that are the most important sources of human infections [2, 3, 4]. Although infections do not usually cause clinical signs in domestic animals, they can sometimes result in late-stage abortions, stillbirths and delivery of weak offspring. Even following subclinical infections, however, there is often contamination of the environment by disseminating highly resistant organisms in birth products, milk, faeces and vaginal discharges [2, 5]. Numerous disease outbreaks have been reported in animals and humans around the world [6] and in a recent outbreak in the Netherlands involving 3,700 people exposed to infected goats has led to renewed interest in Q fever worldwide [7]. Humans acquire infection primarily through inhalation of aerosols from animal birth products, infectious dust particles and contaminated wool [8, 9]. Most infections are asymptomatic (60%) but there can also be signs including atypical pneumonia, granulomatous hepatitis, meningioencephalitis and endocarditis [10, 11].

In China, Q fever was first reported in 1950 [12]; thereafter, five outbreaks amongst abattoir workers were investigated [13]. During the last two decades, a variety of serosurveys of humans (N=19) and animals (N=15) have been reported. Many were performed using outdated and less specific and sensitive methods, and only five of them involved molecular and /or modern serological techniques [14]. Availability of data on *C. burnetii* infections in different animal species and humans is important to enable animal and human health workers to formulate policies on education and preventive measures that might lessen the chances of infections and outbreaks. To provide further data on *C. burnetii* infections in people and domestic animals in China, the authors carried out a serological and PCR survey of convenience samples of people and animals from Yangzhou, a city in eastern China where *C. burnetii* infections have not previously been studied.

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MATERIALS AND METHOD

Ethics statement. Protocols for the collection of samples were reviewed and approved by the Institutional Animal Care and Use Committee and the Institutional Review Board of Yangzhou University College of Medicine.

Sample collection. Whole blood was collected into EDTA from convenience samples of apparently healthy dairy cattle, goats, pigs, dogs and cats from a dairy, a goat farm, a piggery and a small animal veterinary practice, respectively. Human whole blood samples (n=180) were collected from consenting apparently healthy people attending the Subei People's hospital in Yangzhou for routine health examinations. Demographic data on age and gender were also collected.

Also at the dairy, convenience milk samples (n=150) were randomly collected. The milk samples (around 10ml) were collected into sterile tubes after the teats had been wiped with 70% ethyl alcohol and the first milk fractions obtained by hand-milking were discarded.

Following collection, samples were transported on ice to the College of Veterinary Medicine at Yangzhou University, where whole blood samples were centrifuged, aliquots of buffy coats (200µl) used for DNA extraction as below, and plasma stored at -80 °C for ELISAs.

DNA extraction. DNA from whole blood samples of the animals and humans were extracted with the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Milk samples (10 ml) were centrifuged at 3,220×g for 30 minutes at 10 °C, and the resulting pellet washed twice with PBS before being resuspended in 400µl PBS and DNA extracted with the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA).

Serological assay. The IDEXX Q-fever indirect ELISA kit (Checkit® IDEXX Laboratories, Switzerland) was used to detect antibodies against phase I and phase II *C. burnetii,* according to the manufacturer's instructions. The ID SCREEN® Q Fever Indirect Multi-Species ELISA kit (IDVet, Montpellier, France) was used to detect antibodies to phase I and phase II *C. burnetii* in the human, pig, dog and cat plasma, according to the manufacturer's instructions.

FRET-qPCR. The Clustal Multiple Alignment Algorithm was used on nucleotide sequences of *C. burnetii* strains (Z11828, AB004712, AF318147) to identify a highly conserved region in the *com1* gene for primers and probes to be used in the FRET-qPCR (Fig. 1). *Coxiella burnetii* Nine Mile strain RSA 493 (kindly provided by Dr. Carsten Heydel, Giessen University, Germany) was used as a positive control to validate our PCR system in the LightCycler® 480II Real-time PCR platform (Roche, Germany), as described previously [15]. For quantitative standards, the concentration of the PCR product of the positive control purified from an electrophoresis gel was determined by the Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen®, Eugene, OR, USA), and diluted in 1× $T_{10}E_{0.1}$ to give solutions containing 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 DNA copies/10µl.

The *C. burnetii* FRET-qPCR was performed with 10µl of extracted DNA in a 20µl final reaction volume with double-distilled DNAase and RNAase free water serving as a negative control. The PCR products were verified by sequencing (BGI, Shanghai, China) of amplicons purified by electrophoresis (2.0% agarose gels, BIOWEST®, Hong Kong, China) and the QIAquick PCR Purification Kit (QIAgen, Valencia, CA, USA).

Statistical analysis. A two-tailed Chi-squared test was performed to analyze Q fever antibody positivity between various groups. Differences were considered statistically significant if the P value is < 0.05.

RESULTS

Seroprevalence of anti-*C. burnetii* antibodies. Overall, a total of 886 whole blood samples from dairy cattle (n=150), goats (n=150), pigs (n=130), dogs (n=136), cats (n=140) and humans (n=180) were tested. The seroprevalences in dairy cattle (44/150, 29%), goats (22%; 33/150) and humans (25%,

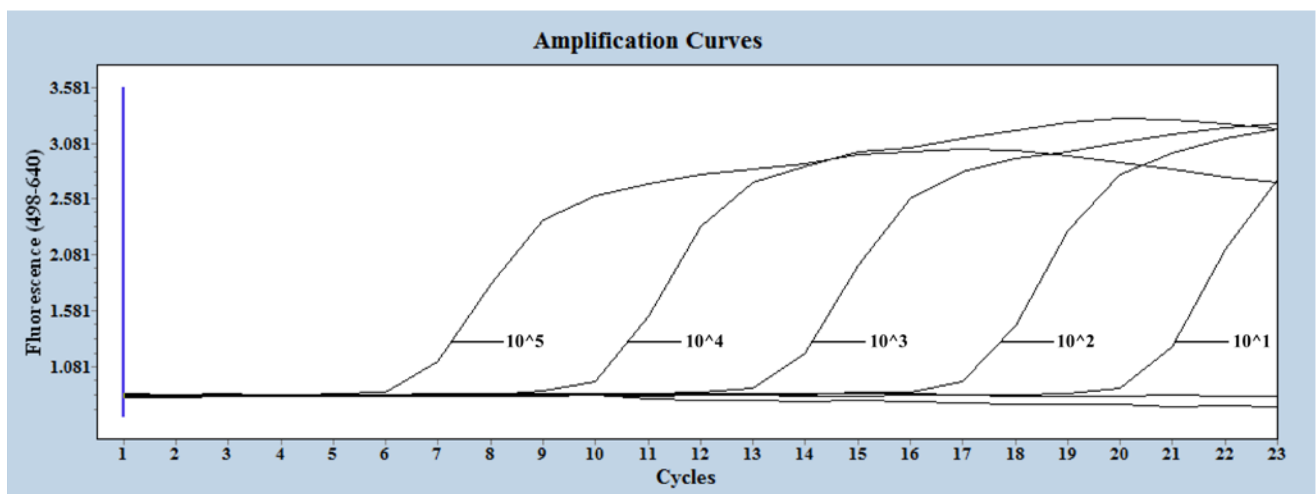

Figure 1. Sensitivity of *C. burnetii* FRET-qPCR performed with quantitative standard solutions. The primers and probes for *C. burnetii* FRET-qPCR established in this study were: upstream primer: 5'-TTAACGACCCTGCATCACCAGTGG-3'; downstream primer: 5'-GCCAAAAATGGGCAGTTCTTTGAAGAC-3'; 6-FAM probe: 5'-TTGATAATCGAAAAATTCAACCAATG-6-FAM-3'; LCRed640 probe: 5'-LCRed640-AACATTGCCATGAGGATTGCCT-phosphate-3'. The quantitative standard dilutions (10^5, 10^4, 10^3, 10^2, 10^1, 10^0 DNA copies/10µl) containing PCR products of *C. burnetii* Nine Mile strain RSA 493 and PCR negative control were detected by the FRET-qPCR established in this study. The detection limit of the *C. burnetii* FRET-qPCR was 10 copies per 20µl PCR reaction volume

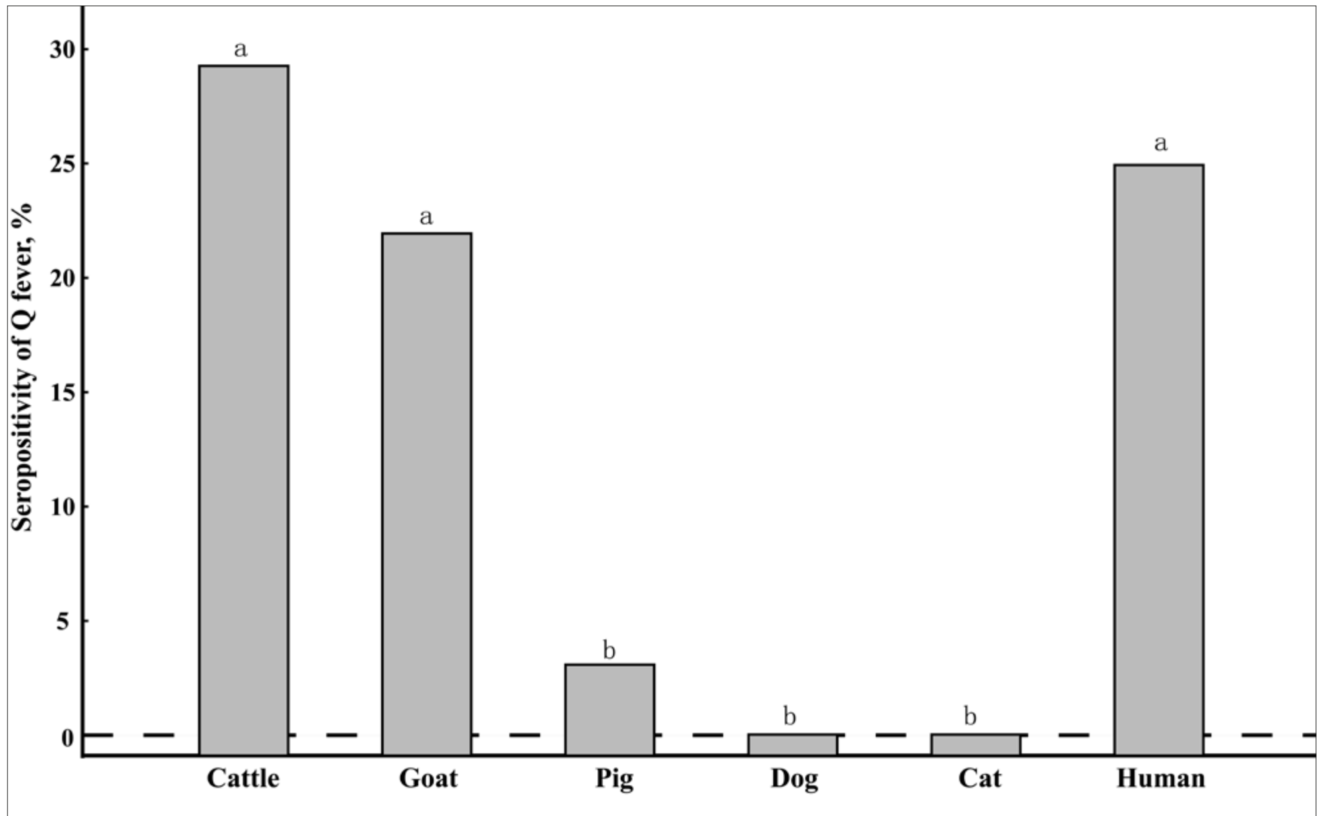

Figure 2. Distribution of *C. burnetii* seropositivity among animals and humans by ELISA.
The seroprevalences in dairy cattle (44/150, 29.3%), goats (22.0%; 33/150) and people (25.0%, 45/180) were significantly higher than in pigs (3.1%; 4/130), dogs (0/136) and cats (0/140) ($p>10^{-4}$). Different letters (a,b) indicates significant difference between groups

45/180) were significantly higher than in pigs (3%; 4/130), dogs (0%/136) and cats (0%/140) ($p>10^{-4}$) (Fig. 2). In humans, the presence of anti-*Coxiella* antibodies was positively associated with increasing age: no reactive antibodies were found in youths under 20 years of age, while they positivity increased from 7% in 21–30 year olds to 29% in adults over 30 years of age. There was no significant difference in the seropositivity in men (21/91) and women (22/89) (Fig. 3).

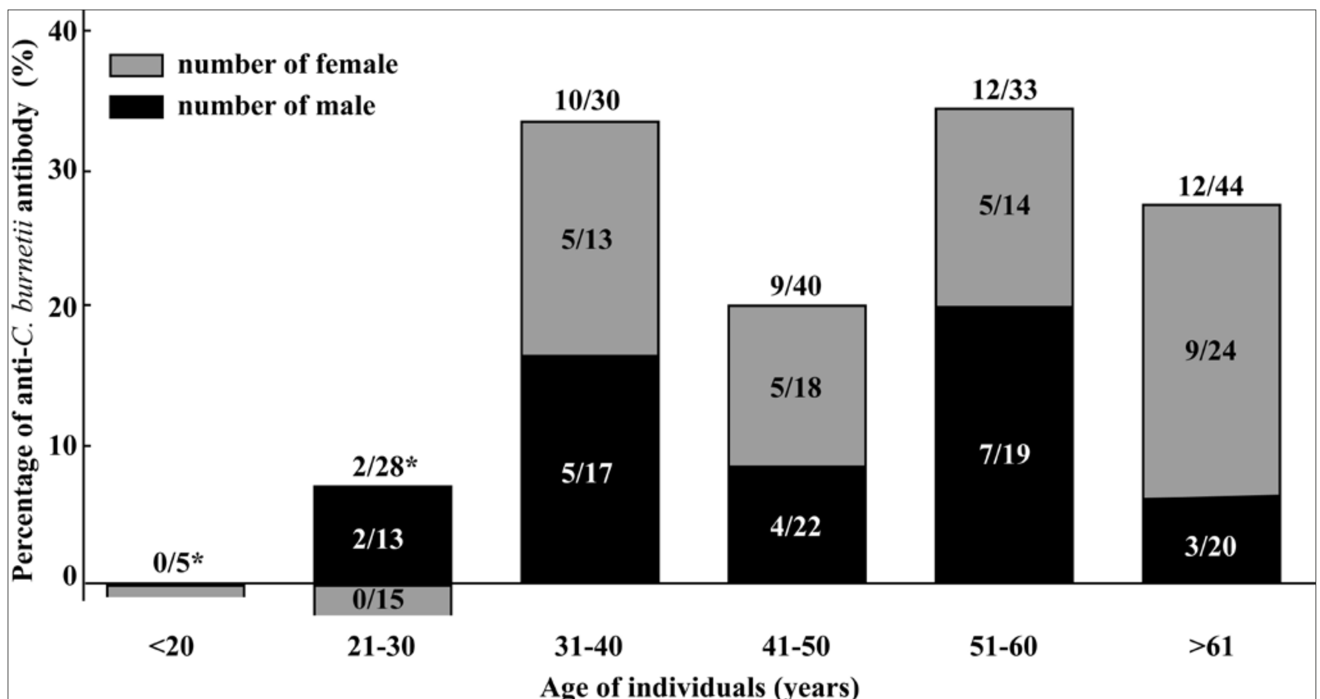

Figure 3. Seroprevalence of Q fever in humans by age and gender. Seropositivity was positively associated with increasing age but not with gender. Individuals over 30 years of age were significantly (*) more likely to have a higher seroprevalence than those below 30 years old

Establishment of FRET-qPCR. When the sequences of the primers and probes selected for the PCR were tested with the BLAST programme in GenBank, they were found to be highly specific for *C. burnetii* and recognized all strains of the organism. Using DNA from *C. burnetii* Nine Mile strain RSA 493 as a quantitative standard, a detection limit of the *C. burnetii* FRET-qPCR was found to be 10 copies per 20μl PCR reaction system (Fig. 1).

Molecular detection of *C. burnetii*. Overall *C. burnetii* DNA could be detected using FRET-qPCR in milk (1.3%; 2/150), while all the tested animal and human blood samples were negative. The obtained *com1* amplicon sequences were identical to one another and to sequences of *C. burnetii* available in GenBank database, mainly the universal *C. burnetii* RSA 493 strain (GenBank No. CP007555.1) and a strain found in China (GenBank No. AF318149).

DISCUSSION

Although *C. burnetii* occurs in most parts of the world, the prevalence of infections in humans as well as animals is often underestimated because definitive testing is not carried out and signs are generally subclinical or confused with more common diseases such as influenza. The presented study is the first in Yangzhou, and the results indicate that *C. burnetii* is present the in city with humans and domestic livestock being exposed. A previous study in five areas of Jiangsu Province, 150 – 450 km distant fromo Yangzhou, also identified seropositive dairy cattle (11%, 17/158) [16], indicating that infections are widespread among dairy cattle in the Province. Infections also appear to be widespread in cattle across China, with infected animals identified in eight of the 32 Provinces, Autonomous Regions and Municipalities of China where serosurveys have been performed to-date [14].

Goats have recently been noted as important reservoirs of *C. burnetii* after they were implicated in an outbreak of Q fever in the Netherlands [7]. In the current study, seropositive goats were found in Yangzhou, which is consistent with reports of positive animals in 10 of the 32 Provinces, Autonomous Regions and Municipalities of China where serosurveys have been performed [14]. Although infections of goats with *C. burnetii* seem to be widespread in China there appear to be some unexposed herds (0/150 animals) with five seronegative farms described in a previous study in Jiangsu [16].

Pigs were uncommonly exposed to *C. burnetii* on a farm invbestigated for the present study, which was also the case in the other two studies of pigs in China where a low seroprevalence was shown in one Province (10%; 10/100) and pigs from two other Provinces were seronegative [17, 18]. In the current study, no evidence was found of exposure to *C. burnetii* in dogs and cats, which is probably because the animals were pets and therefore not exposed to domestic livestock which are the main reservoirs of infection. While this study is the first serosurvey of cats for exposure to *C. burnetii* in China, previous serosurveys of dogs have also failed to find evidence of infections in Henan [19], and demonstrated only low levels of seropositive animals in Jiangsu and three other Provinces [16, 18, 20]. Dogs and cats are generally regarded to be unimportant in the epidemiology of Q fever [2] and this also appears to be the case in China.

The relatively high seroprevalence (25%) found in the 180 people in this study is consistent with that reported in other studies in China to-date, mainly 9% – 56% of people from four sites in Anhui [17, 21], and 23% to 60.0% (overall 39%) of animal husbandry workers from nine sites in Beijing [22]. A previous study conducted at five sites in Jiangsu province, however, showed only 0.3% of the 2,560 people tested were positive [16]. The variations in the seroprevalences most likely reflect differences in the exposure of the study populations to domestic animals and their products which are generally regarded to be the main sources of infection for people [2].

Although no effect of gender on seroprevalence was fiound in the current study, it has been reported in China [21] and related to the role of women in rural activities, such as tea picking and managing livestock. Seroconversion rates were lowest among young people, and increased with age, indicating cumulative exposure over time which has been reported in other studies [23, 24].

Although the FRET-qPCR used in the prersented study has been shown to be highly sensitive, detecting as few as 10 gene copies per reaction, none of the blood samples tested in this study were positive. This is most likely because circulating *C. burnetii* are usually only present in the early phase of acute infections in humans and animals, and rapidly disappear as an antibody response develops [25, 26, 27, 28]. While this study is the first molecular survey of humans in China for circulating *C. burnetii* DNA there have been PCR surveys of blood samples from livestock which were also negative in the three provinces studied [29]. Although blood is seldom found to contain organisms, *C. burnetii* is not uncommonly shed in cows' milk and a high percentage of bulk milk tanks can be infected [30, 31, 32]. In the current study, positive FRET-qPCR results were obtained for two milk samples (1.3%; 2/150). Although *C. burnetii* can survive for long periods in milk, it appears that in most cases of ingestion of infected milk, the result in seroconversion without disease [33]. Further studies are underway in the authors' laboratory to determine the extent to which milk supplies from around China contain *C. burnetii,* and the implications they might have for consumers and farm workers.

CONCLUSIONS

The current study shows that livestock and humans in Yangzhou, eastern China, are commonly exposed to *C. burnetii* and this finding should alert human and animal health workers to the possibility of infections in their patients and for the possibility of outbreaks of Q fever. Further studies of representative samples of humans and animals appear to be warranted to more precisely define the epidemiology and clinical relevance of *C. burnetii* in China.

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