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Serological screening of *Coxiella burnetii* (Q fever) and *Brucella* spp. in sheep flocks in the northern prefectures of Japan in 2007

Massimo Giangaspero[(1)], Takeshi Osawa[(2)], Barbara Bonfini[(3)], Riccardo Orusa[(4)], Serena Robetto[(4)] & Ryô Harasawa[(1)]

Summary

Ovine sera collected from the northern Prefectures of Hokkaido, Iwate and Aomori in Japan, were examined for the presence of antibodies against *Coxiella burnetii* (Q fever) using the complement fixation test and, against *Brucella* spp., using both the rapid serum agglutination test and the complement fixation test. None of the sera tested were serologically positive to *Brucella* spp. A total of 21 animals (8.64%) out of 243 samples tested were seropositive to the *C. burnetii* antigen. Levels of infection were observed in all of the three Prefectures and in ten flocks of the fourteen sampled. Although no diagnostic measures were in place, the infection could not be linked to losses in sheep production or to the decreased fertility in ewes, a lower lambing rate and mortality in lambs. These data confirmed that Q fever is widespread in the sheep population in the area studied. Considering the zoonotic potential of the disease, further studies to investigate the epidemiology of Q fever in this region are required.

Keywords

Aomori, *Brucella* spp., *Coxiella burnetii*, Hokkaido, Iwate, Japan, Q fever, Serology, Sheep.

Screening sierologico di *Coxiella burnetii* (febbre Q) e *Brucella* spp. in greggi di pecore nelle prefetture settentrionali del Giappone nel 2007

Riassunto

Nello studio sono stati analizzati sieri ovini prelevati nelle prefetture settentrionali di Hokkaido, Iwate e Aomori, in Giappone, per identificare la presenza di anticorpi Coxiella burnetii *(febbre Q), utilizzando il test di fissazione del complemento, e per anticorpi* Brucella *spp., impiegando il test rapido di sieroagglutinazione e il test di fissazione del complemento. Su 243 campioni, 21 animali (8,64%) sono risultati sieropositivi per l'antigene* C. burnetii. *Nessuno dei sieri testati è risultato positivo per* Brucella *spp. Nelle tre prefetture sono stati osservati livelli di infezione in 10 gruppi sui 14 campionati. Pur in assenza di misure diagnostiche, l'infezione non può essere correlata alle perdite di produzione ovina né, in particolare,*

(1) Department of Veterinary Microbiology, School of Veterinary Medicine, Faculty of Agriculture, Iwate University, Ueda 3 Chome, Morioka, Iwate 020-8550, Japan
giangasp@gmail.com
(2) Laboratory of Theriogenology, Department of Veterinary Sciences, Faculty of Agriculture, University of Miyazaki, Gakuen-kibanadai Nishi 1-1, Miyazaki, Miyazaki 889-2192, Japan
(3) Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise 'G. Caporale', National reference laboratory for brucellosis, Via Campo Boario, 64100 Teramo, Italy
(4) National Reference Centre for Wild Animal Diseases (CeRMAS), S.C., VdA, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, rue Amérique 7G, 11020 Quart (AO), Italy

alla riduzione della fertilità o ad una più bassa percentuale di mortalità negli agnelli. I dati hanno confermato che nella zona studiata la febbre Q è molto diffusa nella popolazione ovina. Considerando il potenziale zoonotico della malattia, sono opportuni ulteriori studi per indagare l'epidemiologia della febbre Q in questa zona del Giappone.

Parole chiave
Aomori, *Brucella* spp., *Coxiella burnetii*, Febbre Q, Giappone, Hokkaido, Iwate, Pecora, Sierologia.

Introduction

Coxiella burnetii is a species of intracellular, pathogenic bacteria and is the causative agent of Q fever that affects humans and animals. The genus *Coxiella* is morphologically similar to *Rickettsiae*, but has a variety of genetic and physiological differences. *C. burnetii* are small Gram-negative bacteria with two growth phases, as well as a spore form which lies idle in soil (15). In contrast to other *Rickettsiae* which are highly sensitive and easily killed by chemical disinfectants and changes in their surroundings, *C. burnetii* is highly resistant. The organism is resistant to heat, drying, and it can survive standard disinfectants (12). These features enable the bacteria to survive for long periods in the environment. The bacterium is so virulent that a single organism is able to cause an infection (11).

This organism is uncommon but may be found in cattle, sheep, goats and other domestic mammals, including cats and dogs. The common path of infection is inhalation of endospores, contact with contaminated milk, meat, wool, urine, faeces and particularly birthing products. Shedding of *C. burnetii* into the environment occurs mainly during parturition; over 10^9 bacteria per gram of placenta are released at the time of delivery (2). Milk may also contain large quantities of *C. burnetii*, although this is probably a minor route of Q fever infection. The disease is rarely tick-borne. Cattle, sheep and goats are the primary reservoirs of *C. burnetii*. Infection is usually clinically unapparent in these animals, although it may cause abortion in sheep and goats.

Humans are often very susceptible to the disease, although many infections are unapparent. Only about one-half of all people infected with *C. burnetii* show signs of clinical illness. Acute Q fever is the primary infection and, in specific hosts, may become chronic (11, 14). The major clinical manifestations of acute Q fever are pneumonia and hepatitis (14). Less common clinical manifestations are aseptic meningitis and/or encephalitis and pancreatitis. Chronic Q fever, characterised by infection that persists for more than six months, is rare but is a much more serious disease. The main clinical manifestation of the chronic form is endocarditis, generally involving the aortic heart valves and, less commonly, the mitral valve. Infections of vascular grafts or aneurysms, hepatitis, osteomyelitis and prolonged fever have also been described (11, 14). Only 1%-2% of patients with acute Q fever die of the disease. Up to 65% of patients with chronic Q fever may die of the disease.

The pathogenic agent is to be found in different parts of the world, except New Zealand (5). However, the incidence of this disease is largely unknown, especially in Asia (16). Q fever has been previously reported in humans in Japan (10, 17) as well as in domestic and wild animals (7, 8, 9, 10, 18).

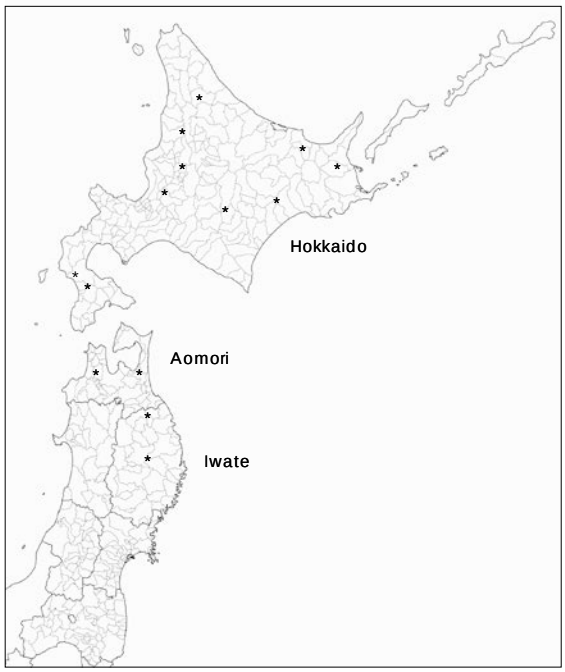
Although *C. burnetii* has not been sufficiently investigated in sheep in Japan, one report on the prevalence of *C. burnetii* in sheep in Japan (9) described a seroprevalence rate of 17.6% in 256 sheep samples that originated from experiment laboratories from 5 prefectures. No indication of the exact origin was provided in this report. Neither eradication measures have been undertaken, nor further investigations been reported. According to the World Animal Health Organisation (*Office International des Épizooties*: OIE) world animal health information system, no information has been provided from Japan in the last decade concerning the occurrence of the disease in animals. Official reports have been made available to OIE only on the occurrence of Q fever in humans, namely: eight cases and two cases in 2005 and 2009, respectively (23).

Concerning brucellosis, the well-known serious debilitating disease in humans and an important cause of abortion and sterility in animals, the country is officially free from the disease in domestic animals. Only rare cases of *B. abortus* have been reported in cattle in 1992, 2002 and 2008 (23). *B. suis* has never been reported. In 2006, the first serological survey in wild animals revealed a positive rate of 7.8% for antibodies against *Brucella* spp. in Japanese wild boar (*Sus scrofa leucomystax*) from the Shikoku region of southern Japan (19). However, the authors could not exclude cross-reactivity with infection from other pathogens. With reference to small ruminants, the last occurrence of *B. melitensis* in Japan was reported in 1949. *B. abortus* has never been reported in sheep and goats. Five zookeepers in the city of Kawasaki developed brucellosis in 2001 after attending the delivery of a baby moose (*Alces alces*). Subsequent investigations confirmed the infection in a goat in the zoo, but no information on isolation or characterisation has been provided (1). No data are available on *B. ovis*.

In order to examine the presence of the Q fever infection and to obtain a preliminary picture of its epidemiology 15 years after the first report (9) and to confirm the absence of brucellosis in sheep, a serological survey conducted to identify antibodies to *C. burnetii* and *Brucella* spp. This was to determine the prevalence of the infections in the three northern Prefectures in Japan (Hokkaido, Aomori and Iwate), where the majority of the Japanese sheep, a total of 4 775 sheep (43%), are bred. The survey was performed on sheep raised both commercially and traditionally.

Material and methods

Fourteen sheep flocks from the three northern Prefectures in Japan (Hokkaido, Iwate and Aomori), were sampled from September 2007 until January 2008, before the lambing season. The number of flocks was chosen according to the animal population of each prefecture and is representative of the livestock production systems in the country. A total of 10 flocks were sampled in the Hokkaido Prefecture, as approximately 37% of sheep breeding in Japan is concentrated in this region. The sampling was completed with four flocks from Tohoku area, of which two were from the Iwate Prefecture and two from the Aomori Prefecture (Fig. 1).



* sampling locations in Hokkaido, Aomori and Iwate prefectures
Dark grey line: prefecture boundaries
Light grey line: municipality boundaries

Figure 1
Northern prefectures of Japan

Farmers were interviewed regarding flock productivity and losses, and were also asked to refer to previous years to explore the potential impact of disease on sheep productivity. No diagnostic measures were performed. Concerning flock production, the annual lambing rate was calculated as the number of lambs born per ewe exposed to a ram, and was based on the lambing season that takes place from February to April. Pearson's correlation coefficients were calculated for a possible relationship between the prevalence of *C. burnetii* antibodies and production parameters, such as annual lambing rate, annual lamb mortality rate, and annual adult mortality rate. Differences were considered to be significant at $p<0.05$.

A total of 20 sheep from each flock were selected for sampling, depending on the flock composition on the national level (number of rams, ewes and yearlings). All age categories, from one year to 12 years of age, were sampled. Lambs were not sampled to avoid interpretation difficulties due to the potential presence of maternal antibodies. Overall 267 serum samples were collected. All sera were stored at –20°C prior to examination. The collected ovine sera were subsequently transported to laboratories in Italy, namely: to the National Reference Centre for Wild Animal Diseases (*Centro di Referenza Nazionale per le Malattie degli Animali Selvatici*: CeRMAS) in Quart and at the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'* (*Istituto 'G. Caporale'*) in Teramo, fulfilling regulatory restrictions, for further analyses.

One of the tests prescribed by the OIE, a complement fixation test (CFT), was used for the detection of antibodies against *C. burnetii* (3). The CFT is specific but less sensitive than the enzyme-lined immunosorbent assay (ELISA) or the indirect immunofluorescence assay (IFA) and provides excellent results for routine diagnosis at the flock level for abortive diseases. Seroconversion is detected later by the CFT than by the IFA or ELISA but CF antibodies can persist for long periods after illness (22).

Briefly, the test was performed in microplate format. All sera were heat inactivated for 30 min at 60°C before use and diluted in a two-fold series to cover a dilution range of 1/8-1/128. A total of 25 µl of diluted sera, equivalent aliquots of antigen (Q fever antigen, Siemens, Munich) diluted 1:10 according to the instructions of the manufacturer and 2U complement were added to the plate wells and incubated at 37°C for 30 min. After incubation, 25 µl of haemolytic system 2U was added and the plate, after further incubation at 37°C for 30 min, was centrifuged for 4 min at 1 500 g. Positive and negative reference controls (*Istituto 'G. Caporale'*, Teramo) were included in the test. Samples with at least 100% of fixation at the first dilution were considered positive (3); sera showing less than 100% of fixation at the first dilution were considered negative.

Screening for anti-*Brucella* spp. antibodies was performed using a rapid serum agglutination test (Rose Bengal plate test: RBPT) and CFT using *B. abortus* biovar 1 strain 99 antigen covering *B. melitensis* and *B. abortus* and *Brucella ovis* strain REO 198 antigen covering *B. ovis*, respectively, in accordance with the *Manual of diagnostic tests and vaccines for terrestrial animals* of the World Organisation for Animal Health (*Office International des Épizooties*: OIE) (20, 21). Antigen and control sera were obtained from the *Istituto 'G. Caporale'* in Teramo.

Results

Of 267 collected samples, 243 sera underwent serological tests for Q fever. The other samples showed an anti-complement reaction ($n = 21$) due to the persistence of endogenous complement despite inactivation at 60°C, or they were not tested for insufficient serum quantity ($n = 3$). Using the CFT, 21 animals were positive for anti-*C. burnetii* immunoglobulins from 243 sera examined (Table I); this corresponds to a prevalence of 8.64%. Infection was detected in ten flocks out of fourteen sampled in all of the three prefectures. The prevalence of infection was found to vary between prefectures. Positive sera originated from eight flocks from Hokkaido and one flock from both Iwate and Aomori Prefectures. The percentage of positive sheep was 7.86% and 2.78% in Hokkaido and Iwate Prefectures, respectively. In Aomori Prefecture, the percentage was higher: 17.24%. The average incidence of seropositive animals in individual herds ranged from 5% to 29% in the ten sampling groups from affected flocks. Titres obtained using the CFT were 1:8 in all positive sera tested, suggesting latent infection (22) that could refer to new, as well as old, infections. The seropositive sheep were all females and one ram; they were of different breeds, namely: Suffolk, Cheviot, Corriedale and crossbreeds. The animals were apparently healthy. The age of the infected sheep ranged from 1 to 10 years of age. Infection could not be linked to losses in sheep production or indeed to the reduced reproduction levels (Table II).

Table I
Details of flocks sampled for serological testing of antibodies to *Coxiella burnetii* in sheep from prefectures of northern Japan
The data shows the number of samples tested and the percentage positive to *Coxiella burnetii*
Seropositive sheep were all females and one ram

Flock	Prefecture	Characteristics of flocks		Animals sampled		
		Flock composition	Breeds	No. tested (% serologically positive animals)	Gender	
					Rams	Ewes
1	Hokkaido	Rams:5 Ewes: 35 Yearlings: 10 Total: 50	Crossbred, Suffolk, Cheviot, Romanov, Black, South Down, Corriedale	20 (26%)	0	20
2	Hokkaido	Rams: 4 Ewes: 47 Yearlings:33 Total: 84	Suffolk, South Down, Black	20 (6%)	0	20
3	Hokkaido	Rams: 10 Ewes: 200 Yearlings: 200 Total: 410	Suffolk	20 (10%)	0	20
4	Hokkaido	Rams: 4 Ewes: 80 Yearlings: 94 Total: 178	Suffolk	20 (25%)	3	17
5	Hokkaido	Rams: 13 Ewes: 270 Yearlings: 30 Lambs: 287 Total: 600	Poll Dorset, Crossbred, Suffolk, Cheviot	20 (6%)	1	19
6	Hokkaido	Rams: 1 Ewes: 80 Yearlings: 40 Lambs: 100 Total: 221	Crossbred, South Down, Poll Dorset	20 (5%)	0	20
7	Hokkaido	Rams: 2 Ewes: 29 Yearlings: 14 Lambs: 37 Total: 82	Crossbred, Suffolk	20 (5%)	0	20
8	Hokkaido	Ewes: 260 Yearlings: 440 Total: 700	Crossbred, Romanov, Poll Dorset, Suffolk	20 (5%)	0	20
9	Hokkaido	Rams: 2 Ewes: 40 Yearlings: 58 Total: 100	Crossbred, Suffolk, Romanov, Friesland, Black Welsh Mountain	20 (0%)	12	8
10	Hokkaido	Ewes: 25 Lambs: 10 Total: 35	Corriedale	11 (0%)	8	3
11	Iwate	Rams: 2 Ewes: 24 Yearlings: 8 Total: 34	Crossbred, Suffolk, Romanov, Cheviot, Corriedale	20 (5%)	1	19
12	Iwate	Rams: 1 Ewes: 30 Yearlings: 21 Total: 52	Suffolk	20 (0%)	1	19
13	Aomori	Rams: 1 Ewes: 26 Yearlings: 7 Total: 34	Suffolk	20 (29%)	0	20
14	Aomori	Ewes: 22 Total: 22	Suffolk	16 (0%)	0	16

All 267 samples collected were tested for *Brucella* spp. antibodies. Three samples (H8/17, H9/1 and H9/9) were positive when tested with the RBPT that was used as qualitative screening test. Sera originated from two different flocks from Hokkaido. CF was used to confirm results. None of the sera were confirmed positive, neither for *B. ovis* nor for *B. melitensis* or *B. abortus* that yielded titres <50 UI and <20 UI, respectively.

Discussion

None of the sera tested gave positive serological results for *Brucella* spp. The three RBPT-positive samples revealed non-specific reactions and were negative when re-tested with the CFT. This confirms the absence of the disease in sheep in the area studied in 2007 and corroborates the official free status of the country for brucellosis. However, further epidemiological studies are required and sheep flocks have to be included in general surveillance for the prevention of the disease.

Antibodies against *C. burnetii* were detected in the three northern Prefectures of Japan where serum samples were collected. These results confirm previous studies on the presence of Q fever in sheep in the northern Prefectures of Japan, first reported by Htwe *et al*. (9) in 1992 and infection may still persist in these Prefectures. The positive rate (8.64%) in our study showed a low pathogenic pressure in 2007 that was lower than the previously reported level (17.6%) of infection (9). The lower prevalence rate reported was probably due to the use of CFT instead of the more sensitive IFA test previously used by Htwe *et al*. (9). However, by using more sensitive tests, such as ELISA and IFA, higher prevalence rates may have been detected in this study.

In Japan, investigations on prevalence of coxiellosis in animal populations have been focused on cattle, particularly in dairy cattle with reproductive disorders (18), showing prevalence rates that are relatively high (58.9% and 60.4%). Raw milk contamination by *C. burnetii* was also reported in 16.8% and 24.6% of samples that originated from dairy cattle with reproductive disorders (8, 18). Isolations were positive also from mammary gland samples (8%) that originated from healthy dairy cattle (8). Furthermore, reports also indicated serological evidence in wild

Table II
Flock average production and reduction of rate of survivors, 2006 and 2007
Data refer to means from two years prior to sampling

Flock	Total number of sheep per flock	Annual lambing rate (lambs/ ewes)	Infertility of ewes	Annual mortality rate (lambs)	Average no. of lambs/ ewes that survived	Annual culling rate	Annual mortality rate (adults)	*Coxiella burnetii* positive animals (%)
1	50	NR	NR	NR	NR	NR	5%	26.31
2	84	0.72	22.89%	1.29%	0.71	0%	4.76%	6.25
3	410	1.62	1.61%	3.46%	1.57	14.77%	9.2%	10.00
4	178	1.10	5.62%	12.78%	0.96	2.97%	0%	25.00
5	600	1.61	NR	20.00%	1.29	11.73%	8.33%	5.88
6	221	1.48	5.06%	17.09%	1.22	10.33%	9.09%	5.55
7	82	1.58	0.00%	16.92%	1.31	6.66%	2.22%	5.00
8	700	2.44	1.02%	20.53%	1.94	NR	NR	5.00
9	100	1.23	30.15%	0.00%	1.23	0.00%	0%	0.00
10	35	NR	NR	NR	NR	NR	10%	0.00
11	34	1.61	0.00%	6.89%	1.50	0.00%	11.76%	5.55
12	52	1.38	9.09%	9.83%	1.25	24.03%	4.8%	0.00
13	34	1.54	4.34%	25.35%	1.15	17.64%	2.94%	29.41
14	22	1.14	3.12%	21.87%	0.89	0.00%	9.09%	0.00

NR not recorded

animals (7). In eight Prefectures in Japan, 26% of sera from different wild animal species had antibody titres to *C. burnetii* when tested with the ELISA. High levels of serological prevalence were observed in the Japanese black bear (*Ursus thibetanus*) (78%), Hokkaido deer (*Cervus nippon yesoensis*) (69%), Japanese hare (*Lepus brachyurus*) (63%), Japanese deer (*Cervus nippon centralis*) (56%). The results of isolation suggested high prevalence of *Coxiella* infection in dairy cattle with reproductive problems and in some wild animal species in Japan. Based on the high prevalence, they are possibly one of the important reservoirs of *C. burnetii* and are responsible for infection in human populations in Japan. Furthermore, cats are considered a significant source of infection caused by *C. burnetii* that is responsible for human outbreaks in association with the presence of infected parturient cats (13). However, the epidemiology of Q fever in Japan remains to be elucidated and the exact modes of transmission are still unproven (13).

In our study of the different flocks, production records from 2006 and 2007 (prior to sampling) revealed levels of reduction in fertility (including rarely reported abortions), lambing rate, number of offspring, and survival rates among lambs. In addition to stillbirth, lamb mortality was reported to occur generally during the first week of life, followed by a second peak when lambs were separated from their mothers at three months of age. Causes of losses were not elucidated, but this appeared not to be influenced by *C. burnetii* infections (Table II).

The major concern with Q fever is the zoonotic potential of the disease, as *C. burnetii* is a highly infectious agent for humans. In Japan, various studies have demonstrated the infection in the human population and the disease is notifiable since 1999, with between 7 and 46 clinical cases of Q fever reported each year (13). No marked episode of Q fever in humans has been reported in Japan and no relationship between Q fever in humans and *C. burnetii* infection in sheep has been revealed. Atypical pneumonia due to *C. burnetii* among children was reported, showing a positive rate of 39.7% among patients suffering from respiratory diseases (17). The prevalence of *C. burnetii* antibodies in samples from adult humans revealed overall seroconversion rates that ranged from 8.6% to 16.5%. Although no statistical evaluation is available, the rates differed for healthy humans and patients suffering from respiratory disorders. Antibody prevalence was high for healthy humans who lived in close contact with animals (e.g. veterinarians and meat-processing workers). The authors concluded that Q fever poses an occupational risk to humans who live in close contact with animals and that serological data showed that *C. burnetii* infection occurred in Japan, often subclinically (10).

Recent reports from the Netherlands of serious outbreaks in humans indicate the potential of emerging or re-emerging Q fever (6). Subsequently, the application of more effective control and preventive measures needs to be considered. A vaccine for use in animals has been developed. Preliminary conclusions indicate that vaccination reduces the chance of an abortion considerably. At the same time, there are indications that a considerable reduction in shedding of *C. burnetii* is achieved by fully and timely vaccination of the animals. The vaccine used in the Netherlands is not (yet) registered in the European Union, but has, in the meanwhile, received temporary approval in France (4).

However, the general prevention and control efforts should be directed primarily towards the protection of risk groups and environments. The following measures should be used to prevent and control Q fever:

- appropriate disposal of placenta, birth products, foetal membranes and aborted foetuses at facilities housing sheep and goats
- access to barns used in housing potentially infected animals should be restricted
- only pasteurised milk and milk products should be used
- imported animals should be quarantined
- holding facilities for sheep should be located away from populated areas
- animals should be routinely monitored for *C. burnetii*
- measures should be implemented to prevent airflow to other occupied areas.

Of utmost importance is the awareness of 'at risk' categories, such as veterinarians, meat-processing plant workers, sheep and dairy workers, livestock farmers, animal transporters and other workers who are in close contact with potentially infected animals. The public too needs to be informed of the sources of infection. Advice should be provided to people at greatest risk of developing chronic Q fever, especially those with cardiac valvular disease or individuals with vascular grafts. In particular, flock owners should be careful not to neglect Q fever or to ignore the potential zoonotic role of the pathogen and the serious threat that this represents for human health. Furthermore, producers and consumers should be aware of the problem and the potential contamination of unpasteurised milk and milk products with *C. burnetii*. The ovine milk and milk products sector is practically inexistent and only rare and specific trials are at the embryonic stage. In our study, an annual milk yield over a period of lactation of 150 days was reported from only one farm, which is a rare example in Japan for ovine dairy production. Sheep milk, with 6% fat and 10% proteins, with a total yield of 17 tons (production of 1 litre per day for 150 days based on early weaning at 21 days), was commercialised for human consumption. Milk was not submitted for *Brucella* spp. testing considering that the disease had not been reported in Japan since 1949. On this farm, one animal gave a positive result for *Brucella* spp. using the RBPT, but confirmatory testing revealed the non-specificity of the immune reaction. However, the flock gave positive results for *C. burnetii* (Q fever) serology. Therefore, it is essential that staff members are adequately informed on the potential of contracting the infection and that the systems of sterilisation and pasteurisation of milk and milk products released for human consumption are effectively monitored.

These data confirm that Q fever is widespread in the sheep population in the area studied. However, considering the preliminary nature of this study, further investigations on the epidemiology of Q fever in sheep population in the country are required. Such studies should be based on isolation techniques and genomic evaluation of isolates. If combined with investigation into other domestic and wild animal species, they will contribute to the improvement of surveillance among humans. Considering the zoonotic potential of the disease and the fact that in severe cases, especially those with myocarditis, the disease could be fatal, professionals should regularly monitor and report the occurrence of the pathogen both in animals and in humans as this disease is notifiable according to the World Organisation for Animal Health (OIE: *Office International des Épizooties*).

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