NOTE *Bacteriology*

Prevalence of Swine Hemoplasmas Revealed by Real-Time PCR Using 16S rRNA Gene Primers

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ABSTRACT Hemoplasma is a tribal name for epierythrocytic mycoplasmas including *Mycoplasma suis* and *M. parvum* which are currently recognized in pigs as causative of porcine hemoplasmosis. Here, we report a real-time PCR assay for differential detection of these swine hemoplasma species by using allelic primers in the16S rRNA gene, and its application to survey for hemoplasma infections in pigs. Universal primers and species-specific primers were designed and evaluated by using swine blood samples positive in hemoplasmas. *Mycoplasma suis* and *M. parvum* infections were both confirmed by universal primers, and mixed infections were clearly distinguished by species-specific primers. Further, we applied this real-time PCR assay to 120 swine blood specimens from clinically healthy pigs in eleven farms in Japan, and found six (5.0%) were positive for *M. suis* and 18 (15.0%) were positive for *M. parvum*, and three (2.5%) were mixed infection by both hemoplasma species.

KEY WORDS hemoplasma, mycoplasma, swine.

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Hemoplasmas are epierythrocytic procaryotes that include mycoplasma species transferred from the *Eperythrozoon* and *Haemobartonella* genera, based on their similarity of the 16S rRNA sequences, and newly identified hemotropic mycoplasmas [2, 9]. They are uncultivable *in vitro* so far and causative of hemolytic anemia, resulting in icterus and pyrexia in various mammalian species. Two hemoplasma species, *Mycoplasma suis* and *M. parvum*, formerly *Eperythrozoon suis* and *E. parvum*, respectively, are currently known in pigs [14].

Mycoplasma suis is a causative agent of swine hemoplasmosis, previously called porcine eperythrozoonosis, of which symptoms are variable. In the acute form, anemia, icterus and anorexia are observed in sows [5], severe anemia and pyrexia in newborn and weaned piglets [2, 4]. Chronic infection following acute form may depress growth rate and increase susceptibility to other infectious diseases in feeder pigs, resulting to reproductive failures or immunosuppression in sows [4, 9, 18]. The clinical signs of *M. parvum* infection have not well been documented, despite severe anemia along with pyrexia in splenectomised pigs [1, 13].

Diagnosis of swine hemoplasmosis has been based on microscopic observation of the organisms on the surface of erythrocytes in Giemsa-stained blood smears, but this method is limited in acute infection, because the parasites are not always apparent unless the parasitemia is developed [10]. Serological tests including indirect hemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) have also been applied for *M. suis* detection [8, 10], but they are not common because of difficulty in obtaining specific antigens. Polymerase chain reaction (PCR) using specific primers targeting the16S rRNA gene has most widely been used for screening various hemoplasmas [6, 12, 15–17]. Species identification of *M. suis* is usually depending on PCR [3, 7], but the identification of *M. parvum* has long been hampered due to lack of established strains maintained *in vivo* so far. This particular species was detected by real-time and end-point PCR from clinically healthy pigs in a commercial farm, and the nucleotide sequences of almost entire region of the16S rRNA and RNase P RNA genes were recently determined [17].

In the present study, we evaluated universal and species-specific primers for PCR to detect swine hemoplasmas by applying to six swine blood specimens, designated A through F, shown positive in hemoplasma infection in our previous study [17]. To evaluate the primers for real-time PCR, we examined three sets of primers, consisting of an universal primer set Pig 16S and two species-specific primer sets, Parvum 16S specific for *M. parvum* and Suis 16S specific for *M. suis* (Table 1). The six swine blood specimens infected with hemoplasmas were subjected to real-time PCR by using these primers as described previously [17].

All the six blood specimens were positive for hemoplasma infection by the real-time PCR using the universal Pig 16S primer set. The PCR products showed the melting

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Table 1. Primer sets and sequences of primer used in real-time and end-point PCR assays

Primer set	Primer name	Primer sequence (5' to 3')	Position	size
Pig 16S	Pig 16S F	5'-ttt tag tgg caa acg ggc ga-3'	60–79**	247 bp
	Pig 16S R	5'-tca atc cca ttg cgg ctg tt-3'	287–306**	
Parvum 16S	Parvum 16S F	5'-aac aca tat tta act tgc tc-3'	100–119*	137 bp
	Parvum 16S R	5'-cat att cct att cat ccg cg-3'	217–236*	
Suis 16S	Suis 16S F	5'-aac gca tac tta act tac tt-3'	82–101**	138 bp
	Suis 16S R	5'-cat act cct att tac ccg ct-3'	200–219**	
Parvum 16S-2	Parvum 16S F	5'-aac aca tat tta act tgc tc-3'	100–119*	484 bp
	Hemo 16S R	5'-cct acg ctt cct tta cgc cc-3'	546–565**	
Suis 16S-2	Suis 16S F	5'-aac gca tac tta act tac tt-3'	82–101**	483 bp
	Hemo 16S R	5'-cct acg ctt cct tta cgc cc-3'	546–565**	

* Relative to the *M. parvum* Morioka 9 (AB610850) sequence. ** Relative to the *M. suis* Illinois (U88565) sequence.

temperature (*Tm*) at 84.62 ± 0.33°C in the melting experiments. Specimens A, B and C were positive in the real-time PCR using the species-specific Parvum 16S primer set, and the *Tm* was 80.38 ± 0.13°C. All the specimens except B were positive in the real-time PCR using species-specific Suis 16S primer set, with *Tm* at 81.36 ± 0.14°C (Fig. 1).

Then, we performed end-point PCR by using the same primer sets as real-time PCR. The PCR consisted of 5 μl of DNA solution, 5 μl of 10× Buffer for KOD-Plus-Ver.2, 5 μl of 2 mM dNTPs, 3 μl of 25 mM $MgSO_4$, 0.3 μl of forward primer, 0.3 μl of reverse primer (50 pmol/μl each), 1 μl of KOD -Plus- (1 U/μl) and water to a final volume of 50 μl. After the denaturation at 98°C for 2 min, the reaction was carried out 30 cycles with denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The amplified products were fractionated by 2.5% agarose gel electrophoresis with a Quick-Load 100 bp DNA Ladder marker (New England BioLabs Inc., Ipswich, MA, U.S.A.), and photographed by the GelDoc-It Imaging system (UVP, Upland, CA, U.S.A.) after staining with 0.4 μg/ml ethidium bromide.

By using the primer set Pig 16S, all the specimens produced a relevant sized band in the end-point PCR. In the case of Parvum 16S primer set, specimens A, B and C produced a relevant sized band, and in the case of Suis 16S primer set, specimens A, C, D, E and F produced a relevant sized band. All the end-point PCR results were consistent with real-time PCR (Fig. 2). In addition, the PCR products obtained by using species-specific primer sets were too small to determine the nucleotide sequence in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Next, we explored two other primer sets, Parvum 16S-2 and Suis 16S-2, consisting of species-specific forward primers and a common universal reverse primer bracketing about 500 bp portion of the 16S rRNA gene (Table 1), by subjecting all the six blood specimens to end-point PCR as described previously [16]. The amplified products were fractionated by 1.0% agarose gel electrophoresis with a 200 bp ladder DNA size marker (TaKaRa Bio., Otsu, Japan), and photographed as described above.

By using the primer set Parvum 16S-2, specimens A, B and C produced a relevant sized band to *M. parvum* in the end-point PCR. In the case of the Suis 16S-2 primer set, specimens A, C, D, E and F produced a relevant sized band to *M. suis*, though the band of specimen A was faint to compare to others (Fig. 3). These PCR products were

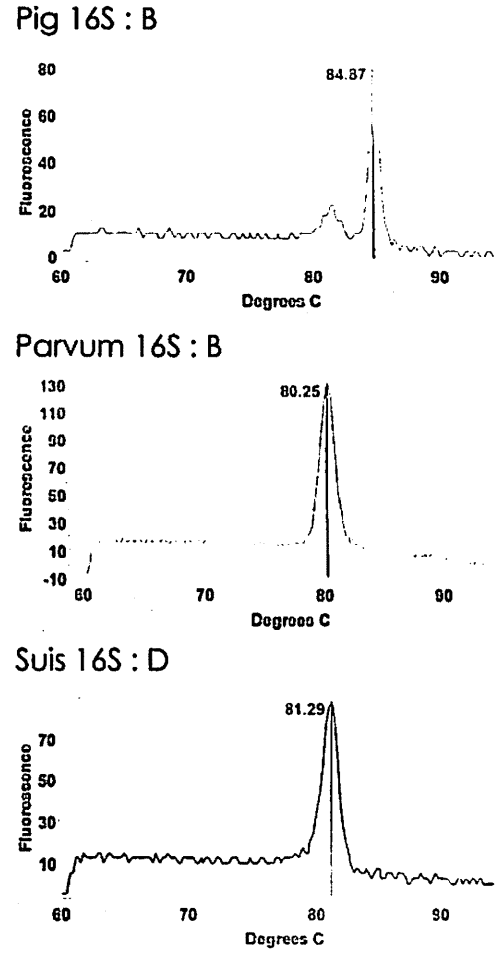

Fig. 1. Representative melting curves using primer sets Pig 16S (top: specimen B), Parvum 16S (middle: specimen B) and Suis 16S (bottom: specimen D).

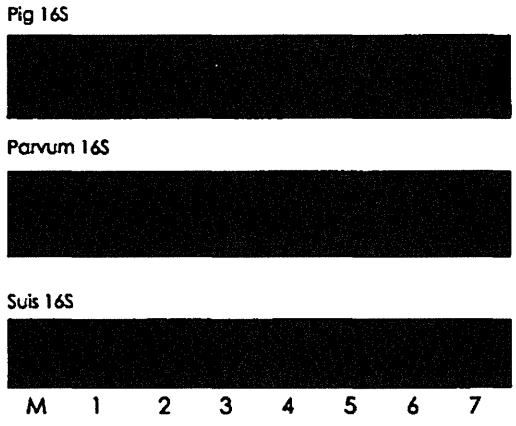

Fig. 2. Agarose gel electrophoresis of the PCR products amplified from six blood samples infected with hemoplasma using three primer sets, Pig 16S, Parvum 16S and Suis 16S. Lane M, DNA size marker (100 bp DNA ladder); lane 1, negative control; lane 2, A; lane 3, B; lane 4, C; lane 5, D 1; lane 6, E; lane 7, F.

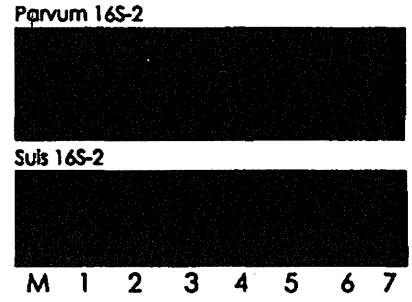

Fig. 3. Agarose gel electrophoresis of the PCR products amplified from six blood samples infected with hemoplasma using two primer sets, Parvum 16S-2 and Suis 16S-2. Lane M, DNA size marker (200 bp DNA ladder); lane 1, negative control; lane 2, A; lane 3, B; lane 4, C; lane 5, D 1; lane 6, E; lane 7, F.

further subjected to direct sequencing as described above. All the nucleotide sequences of PCR products obtained by using Parvum 16S-2 primer set were identical to *M. parvum* sequence. On the other hand, the nucleotide sequences of the PCR products obtained by using Suis 16S-2 were identified as *M. suis*, though sequencing of the specimen A was failed in our experiment. Taken together these results, existence of both *M. parvum* and *M. suis* was confirmed in specimen C, and this indicates the existence of mixed infection of both swine hemoplasmas that was not revealed by the universal primers. End-point PCR using Parvum 16S-2 and Suis 16S-2 primer sets were not sensitive as compared to real-time and end-point PCRs using Parvum 16S and Suis 16S primer sets for detection of swine hemoplasmas.

Our data support that the universal primers allow to detection of hemoplasma infection by real-time PCR as well as end-point PCR, but they were not able to distinguish the mixed infection of swine hemoplasmas.

Each hemoplasma species was detected by both real-time and end-point PCRs by using Parvum 16S or Suis 16S primer sets, but the real-time PCR with melting experiments was much more convenient than the end-point PCR. Thus, the real-time PCR using species-specific primers seemed more useful to evaluate the epidemiology of swine hemoplasmas.

Lastly, we applied real-time PCR in the same condition using species-specific primer sets to investigate the prevalence of swine hemoplasmas in 120 blood samples collected from clinically healthy pigs including 108 feeders and twelve sows raised in eleven different commercial swine farms in Japan. Of them, 18 (15.0%) pigs in six (54.5%) farms were positive for *M. parvum*, and six (5.0%) pigs in one (9.1%) farm were positive for *M. suis*. Mixed infections with both hemoplasma species were detected in three (2.5%) blood samples.

Our results first demonstrated the existence of mixed infection with *M. parvum* and *M. suis* in swine, and also revealed the prevalence of swine hemoplasma infections in Japanese farms nowadays. *Mycoplasma suis* infection causes panleucopenia in both piglets and sows and immunosuppression under the stressed conditions by parturition for sows or weaning for piglets [4]. This may lead to increased infections of respiratory and enteric diseases [6], and also it pertinent to consider this organisms affect as etiological agent with recent swine disease complex associated with emergent infectious diseases due to *Porcine circovirus 2* infection etc. Besides, human infection with *M. suis* has been reported among swine farm workers in China [18] and should be considered in aspects of public hygiene. Although *M. parvum* has been thought relatively less pathogenic to swine [13], a clinical episode has been recorded in Japanese commercial swine farm in 1984 [11]. Thus, an in-depth investigation of swine disease complex is extremely urgent, because of high incidence of hemoplasma infection in the modern swine operation of this country.

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BRIEF COMMUNICATION

Seroepidemiological survey of sheep flocks from Northern Japan for *Mycoplasma ovipneumoniae* and *Mycoplasma agalactiae*

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Abstract Sheep flocks from Hokkaido, Iwate and Aomori, three northern prefectures of Japan, were screened for antibodies to *Mycoplasma ovipneumoniae* and *Mycoplasma agalactiae* by ELISA. Sixty four animals out of 246 (26%) were seropositive to *M. ovipneumoniae*, with positive results obtained from all three prefectures. None of the sera tested were serologically positive to *M. agalactiae*.

Keywords Epidemiology · Enzyme-linked immunosorbent assay · Japan · *Mycoplasma agalactiae* · *Mycoplasma ovipneumoniae* · Sheep

The welfare and economic effect of mycoplasma infections in small ruminants is recognized around the world,

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with sheep production adversely affected by several *Mycoplasma* species. Awareness of the distribution of these diseases is therefore essential for both production and trade. *Mycoplasma agalactiae*, the cause of contagious agalactia, is an OIE listed disease (OIE 2008); it affects sheep and goats and is characterised by mastitis, arthritis, keratoconjunctivitis and, occasionally, abortion. Other mycoplasma pathogens associated with contagious agalactia include: *Mycoplasma capricolum* subsp. *capricolum*, *Mycoplasma mycoides* subsp. *capri* (formerly named *M. mycoides* subsp. *mycoides* Large Colony; Manso-Silván et al. 2009) and *Mycoplasma putrefaciens*. Worldwide, *Mycoplasma ovipneumoniae* is one of the most commonly isolated microorganisms from sheep with respiratory disease. In young lambs, it can be associated with a severe paroxysmal cough leading to rectal prolapses, termed "coughing syndrome." The disease is chronic and persists for several weeks, with variable morbidity and mortality rates between flocks (Nicholas et al. 2008).

Japanese zootechnics is under rapid development, but it has focused mainly on cattle breeding as small ruminant farming remains a marginal sector, with just 21,000 goats and 11,000 sheep in the entire country. Limited information of the various pathogens that affect small ruminants in Japan is available, with only Caprine arthritis encephalitis (CAE) virus, Scrapie and Visna Maedi having been studied by Konishi et al. (2004, 2007), Horiuchi et al. (2002) and Okada and Yonemichi (1982). *M. agalactiae* has not been reported in Japan; however, *M. mycoides* subsp. *mycoides* Large Colony was reported in two goats in 2006 (OIE 2007) from Nakagusuku, Okinawa Prefecture.

Pneumonia associated with *M. ovipneumoniae* infection was reported by Ichihoshi and Matsuda (2003), from 4-month-old Saanen breed goats, in Kyoto Prefecture. To date, neither *M. agalactiae* nor *M. ovipneumoniae* have been reported in sheep in Japan. The purpose of this study was to determine the seroprevalence of these *Mycoplasma* species in Japanese sheep.

Material and methods

A survey was carried out in the northern prefectures in Japan, Hokkaido, Aomori and Iwate, where 4,775 (43%) of Japanese sheep are bred (Japan Livestock Industry Association 2000). Farmers were interviewed regarding flock management, productivity and losses, referring also to previous years, to define possible factors influencing the epidemiology of infection and to explore the impact of disease on sheep productivity.

Thirteen sheep flocks were sampled between September 2007 and January 2008. Nine flocks were sampled in the Hokkaido Prefecture, as approximately 37% of sheep breeding occurs in this region. The sampling was completed with four flocks from the Tohoku area, two from Iwate Prefecture and two from Aomori Prefecture. Details of the flocks sampled are given in Table 1. Up to 20 sheep from each flock were randomly selected for sampling, but took into account the national standard of flock composition (number of rams, ewes and yearlings). The age categories from 1 to 12 years were sampled, but not lambs. Two hundred and forty-six blood serum samples were collected. The sera were stored at −20°C until examination.

Ovine sera were examined for the presence of antibodies to *M. ovipneumoniae* using a whole cell indirect enzyme-linked immunosorbent assay (ELISA) with a Protein G conjugate. Briefly a 1/100 dilution of sera was applied to a microtitre plate coated with washed and optimized whole cells of *M. ovipneumoniae*. After 30 min of incubation, plates were washed thoroughly and protein G conjugate added for 30 min. Following another washing, the substrate tetramethylbenzidine was added and the reaction stopped after approximately 10 min by the addition of citric acid so that the positive control serum had a final OD of 1.0. Plates were read by an ELISA reader at 450 nm. OD values of >0.30 were considered positive, and below this were considered negative.

Screening for anti-*M. agalactiae* antibodies was performed using an ELISA kit (Institut Pourquier, France) following the manufacturer's instructions.

Results

Sixty-four of the 246 (26.01%) samples from 13 flocks were seropositive for *M. ovipneumoniae*. None were seropositive for *M. agalactiae*.

Exposure to *M. ovipneumoniae* infection appears widespread, with antibodies detected in sheep from the three Japanese prefectures. Both farms sampled in the Iwate Prefecture, seven farms from Hokkaido and one farm from Aomori showed seropositive animals (Table 1). The highest overall prevalence was in Hokkaido at 32.35%, but prevalence at flock level varied from 10.00% to 52.63% in ten flocks, whilst three flocks were seronegative.

All sheep breeds tested were affected, although the animals appeared healthy. The proportion of seropositive sheep appeared to increase with age from 2 to 10 years. Evaluation of the possible impact of *M. ovipneumoniae* infection on production did not reveal any correlation with the reported levels of seropositive animals. In the ten seropositive flocks, the average number of lambs surviving per ewe ranged from 0.71 to 1.94 (mean = 1.26), and in the two flocks with no evidence of infection, where data were available, it ranged from 1.15 to 1.23 (mean = 1.19). Furthermore, the annual lamb mortality rate demonstrated

Table 1 Details of flocks sampled for serological testing of antibodies to *M. ovipneumoniae* and *M. agalactiae* in sheep from prefectures of northern Japan

Flock	Prefecture	Flock characteristics		Sampled animals		
		Flock composition (total in flock)	Breeds	Number tested and (%) serologically positive to *M. ovipneumoniae*	Gender	
					Rams	Ewes
1	Hokkaido	R5, E35, Y10 (50)	CB, S, C, Rv, B, SD, Cor	20 (30% positive)	0	20
2	Hokkaido	R4, E47, Y33 (84)	S, SD, B	20 (25% positive)	0	20
3	Hokkaido	R10, E200, Y200 (410)	S	19 (52.63% positive)	0	19
4	Hokkaido	R13, E270, Y30, L287 (600)	PD, CB, S, C	20 (50% positive)	1	19
5	Hokkaido	R1, E80, Y40, L100 (221)	CB, SD, PD	20 (45% positive)	0	20
6	Hokkaido	R2, E29, Y14, L37 (82)	CB, S	20 (50% positive)	0	20
7	Hokkaido	E260, Y440 (700)	CB, Rv, PD, S	20 (25% positive)	0	20
8	Hokkaido	R2, E40, Y58 (100)	CB, S, Rv, F, BWM	20 (0% positive)	12	8
9	Hokkaido	E25, L10 (35)	Cor	11 (0% positive)	8	3
10	Iwate	R2, E24, Y8 (34)	CB, S, Rv, C, Cor	20 (25% positive)	1	19
11	Iwate	R1, E30, Y21 (52)	S	20 (10% positive)	1	19
12	Aomori	R1, E26, Y7 (34)	S	20 (0% positive)	0	20
13	Aomori	E22 (22)	S	16 (12.5% positive)	0	16

The data show the number of samples tested and the percentage positive to *M. ovipneumoniae*

Flock composition: *R* rams, *E* ewes, *Y* yearlings, *L* lambs. Breeds: *B* Black, *BWM* Black Welsh Mountain, *C* Cheviot, *CB* crossbred, *Cor* Corriedale, *F* Friesland, *PD* Poll Dorset, *Rv* Romanov, *S* Suffolk, *SD* South Down

little relationship to seropositivity, showing only a marginally higher mean (13.06% in a range from 1.29% to 21.87%) in affected flocks to a mean of 12.67% (from 0% to 25.35%) in others. In addition, mortality in lambs and variations in average live body weight and annual wool yield did not appear to be related to seropositivity.

Discussion

Antibodies against *M. ovipneumoniae* were detected in all the three northern prefectures of Japan where sera samples were collected. These results confirm the presence of *M. ovipneumoniae* in Japan, reported by Ichihoshi and Matsuda (2003), and we report the first cases of *M. ovipneumoniae* infections in Hokkaido, Aomori and Iwate Prefectures.

The information obtained on reproduction rates, infertility, lambing rate and lamb survivability from the different flocks showed no apparent link to *M. ovipneumoniae* seropositivity. This is in contrast to reports from Australia which describe outbreaks of pneumonia, from which *M. ovipneumoniae* was recorded, in housed lambs in the first year of life with high morbidity and low mortality, poor growth rates and exercise intolerance (Ayling and Nicholas 2007). *M. ovipneumoniae* has also been associated with an adverse affect on animal production, especially in the survival of young animals. This was clearly reported in young goats in Kyoto Prefecture (Ichihoshi and Matsuda 2003) which showed a high mortality (85%). In the present study, the positive rate (from 10% to 52.63%) indicated a low mean pathogenic pressure. However, the infection could not be clearly related to losses in sheep production which are probably due to multifactorial causes.

This study reports for first time the occurrence of *M. ovipneumoniae* in sheep in Japan. Further investigations are required to clarify the exact epidemiological situation of mycoplasma infection in sheep flocks in the region. Taking into account the negative impact on sheep production of the pathogen, the cost effectiveness of control measures should be investigated.

Seropositivity to *M. agalactiae* was not shown in this study and suggests that the disease is currently absent from the country. This situation should encourage the implementation of regulations monitoring animal movements with associated quarantine measures. Furthermore, the application of prophylactic contagious agalactia vaccine is undesirable and should be prevented, thus protecting from iatrogenic accidents which occurred in countries were immunization of sheep against contagious agalactia is required. However, taking into account that *M. mycoides* subsp. *capri* has been reported in goats in the country, monitoring of sheep flocks for contagious agalactia should be implemented.

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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

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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

Sono stati analizzati sieri ovini prelevati nelle prefetture settentrionali di Hokkaido, Iwate e Aomori in Giappone per la ricerca di anticorpi Coxiella burnetii *(febbre Q) utilizzando il test di fissazione del complemento, e* Brucella *spp. utilizzando il test rapido di sieroagglutinazione e il test di fissazione del complemento. Nessuno dei sieri testati sierologicamente è risultato positivo per* Brucella *spp. Su un totale di 243 campioni, 21 animali (8,64%) sono risultati sieropositivi per l'antigene* C. burnetii. *In tutte e tre le Prefetture sono stati osservati livelli di infezione, precisamente in dieci greggi sui quattordici campionati. Pur non essendo state messe in atto misure diagnostiche, l'infezione non può essere correlata alle perdite di*

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produzione ovina, né in particolare alla riduzione della fertilità, o a mortalità negli agnelli. Questi 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 regione.

Parole chiave

Amori, *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).

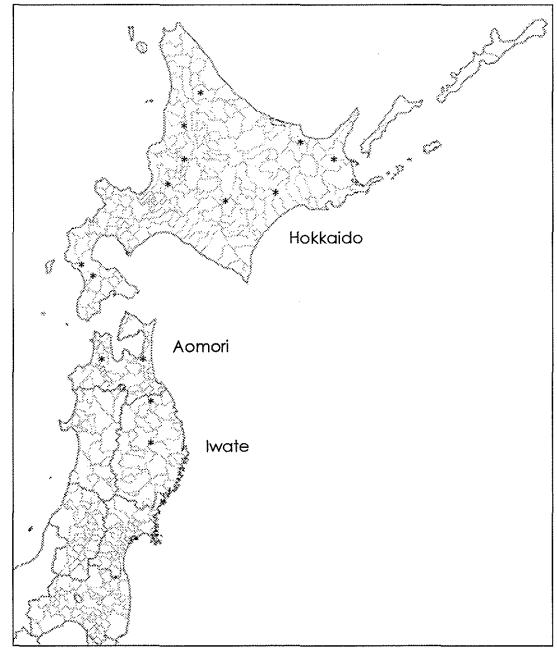
Although *C. burnetii* has not been sufficiently investigated in sheep in Japan, one report on the prevalence of *C. burnetii* in sheep in Japan (9) described a seroprevalence rate of 17.6% in 256 sheep samples that originated from experiment laboratories from 5 prefectures. No indication of the exact origin was provided in this report. Neither eradication measures have been undertaken, nor further investigations have 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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