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First Isolation of *Brucella canis* from Pet Dogs in Sichuan Province, China: Molecular Characterization, Pathogenicity and Antigen Location Analysis

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YG, YW and QX collected the samples and analyzed the data. ZZ, RT and XW wrote the article. ZZ, YT, RT, JD and BW performed the experiment. ZZ and GP helped in preparation of the article.

Key words
***Brucella canis*, Pet dog, Molecular diagnostics, Pathology, Immunohistochemistry.**

ABSTRACT

Brucellosis is a worldwide zoonosis that is primarily caused by *Brucella melitensis, Brucella abortus, Brucella suis*, *and Brucella canis*. However, in China, information regarding brucellosis in pet dogs caused by *B. canis* is limited. In the present study, we conducted a comprehensive molecular, pathological, and immunohistochemical analysis to detect this pathogen in pet dogs. Molecular methods, combining three types of PCR assays, identified two strains isolated from two pet dogs as *Brucella canis*. Histopathological changes revealed extensive inflammation and necrosis in the liver, lung, spleen, kidney, testicle, and lymph nodes, among which changes in the spleen were the most serious. Immunohistochemistry results demonstrated the detection of *B. canis* antigens in the lesions of all examined tissues. Strong positive staining was primarily found in the spleen, liver, and testicle. In conclusion, this study was the first to report the isolation of two *B. canis* strains from pet dogs in Sichuan province, southwestern China, and to further evaluate *B. canis* antigen location in tissues. Our study will contribute to the understanding of *B. canis* pathogenicity in naturally-infected pet dogs.

INTRODUCTION

Brucellosis, caused by gram negative and aerobic, facultative bacteria of the genus *Brucella*, is regarded as one of the most important zoonotic diseases worldwide (Zhong *et al*., 2013). In recent years, an increasing number of reports have described brucellosis caused by *Brucella canis*, suggesting that this disease might be increasing in incidence (Agudelo-Flórez *et al*., 2012; Gyuranecz *et al*., 2011b; Hofer *et al*., 2012; Holst *et al*., 2012; Kang *et al*., 2011; Keid *et al*., 2017; Kulakov, 2012; Purvis *et al*., 2017; Sayan *et al*., 2011); however, *B. canis* infection in dogs has not conventionally been considered a major problem. This species was first isolated from dogs in 1966 and has since been considered a threat to animal and human health (Krueger *et al*., 2015; Makloski, 2011). To date, dogs infected with *B. canis* has been primarily reported in European countries such as Sweden, Italy, Germany, Poland, Great Britain, France, Spain, Russia, Hungary, Austria; in addition, it is also prevalent in Asian countries such as Korea, Japan, China, Turkey, India, the Philippines, and Malaysia, as well as in the Americas including the United States, Canada, Brazil and Columbia (Brennan *et al*., 2008; Holst *et al*., 2012; Keid *et al*., 2017; Purvis *et al*., 2017). However, information regarding *B. canis* infection in pet dogs in China is scant.

In China, *B. canis* was first isolated from Beagle dogs in Shanghai (Deqiu *et al*., 2002). Subsequently, more strains were isolated in different provinces such as Guangxi, Henan, Inner Mongolia, Beijing, Guangxi, Anhui, Hubei, Jiangsu, Xinjiang, Shanxi, and Fujian, indicating that the prevalence of *B. canis* infection in dogs is increasing in China (Deqiu *et al*., 2002). This species is not only highly pathogenic for dogs, but it is also able to infect humans and eventually cause severe diseases (Krueger *et al*., 2015; Lucero *et al*., 2005; Marzetti *et al*.,

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2013). Humans become infected through close contact with infected dogs or abortion-related materials (Lucero *et al*., 2010). In the last decade, there has been a rising trend of human brucellosis caused by *B. canis* (Lucero *et al*., 2010; Nomura *et al*., 2010). Sichuan province is the second most populated province in China and contains a large amount of pet dogs. Therefore, infected pet dogs could become a major threat to dog owners. However, little information is available regarding *B. canis* infection of pet dogs in Sichuan province. Considering the high-density feeding environment and the increase in reports of *B. canis* infection in pet dogs in recent years, canine brucellosis might become an emerging challenge to animals and public health in China.

In this study, we combined the advantage of AMOS-PCR and BcSS-PCR to distinguish *B. canis* from other *Brucella* species. Then, pathologic and immunohistochemical (IHC) techniques were employed to detect pathological changes and *B. canis* antigens in tissues from a naturally-infected dog. This is the first study reporting the isolation of *B. canis* strains from pet dogs, in addition to the use of IHC methods to detect *B. canis* antigen locations in a naturally-infected pet dog, in Sichuan province, China.

MATERIALS AND METHODS

Clinical samples

In December 2015, one male dog (golden retriever), aged two years and five months, presented with low fever, enlarged lymph nodes, and unilateral testis, indicating the possibility of canine brucellosis. In October 2016, another dog, a male poodle, aged three years, presented with obviously enlarged testicle and undulating fever, and was sent to Veterinary Medical Teaching Hospital of Sichuan Agricultural University.

Serological tests

The rapid slide agglutination test (RSAT) and Rose Bengal plate test (RBPT) were performed to detect *Brucella* antibodies in blood samples, and were performed as previously reported (Ali *et al*., 2017). For further detection of rough *Brucella* antibodies, we adopted the Rose Bengal plate test (RBAT) with rough antigen.

Bacteriological studies

Blood samples were plated on tryptic soy agar (Beijing Selarbio Science and Technology Co., Ltd, Beijing, China) and streaked for isolation. Plates were inoculated at 37 °C in 5% CO_2 for greater than 5 days. Smooth or rough colony phenotypes of isolates were confirmed by crystal violet staining and auto agglutination reactions as described previously (Alton *et al*., 1998). Additional bacteriological identification studies were performed by assessing agglutination with monospecific sera against A and M antigens, hydrolysis of urea, H_2S production, and growth in the presence of CO_2. The strains mentioned in this study included three *Brucella* reference strains (544A, 16M, and S19) and the two isolates (W5 and Y4).

DNA extraction

Before DNA extraction, specimens were boiled for 15 min. *Brucella* DNA was extracted according to the manufacturer's instructions using, TIANamp Bacteria DNA extraction kit (TIANGEN Biotech Corporation, Beijing, China).

Polymerase chain reaction assays

Three different PCR protocols were used to identify the two *Brucella* isolates. All primers are listed in Table I. The products (6 µl from each reaction mixture) were analyzed by electrophoresis using a 1.5% agarose gel, after which the1 gel was stained with ethidium bromide and photographed.

Brucella *genus-specific PCR*

DNA from all isolates was amplified using *BCSP31* gene, producing a 224-bp amplicon. Primers used are listed in Table I, and were previously described by Imaoka *et al*. (2007). Amplification conditions consisted of an initial denaturation at 99 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min.

Table I.- Primers used in this study.

Primer	Sequence (5'-3')
BCSP31	F: TGG-CTC-GGT-TGC-CAA-TAT-CAA
	R: CGC-GCT-TGC-CTT-TCA-GGT-CTG
BcSS	F: CCA-GAT-AGA-CCT-CTC-TGG-A
	R: TGG-CCT-TTT-CTG-ATC-TGT-TCT-T
Specific primer	
IS711	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT
B. abortus	GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC
B. melitensis	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA
B. ovis	CGG-GTT-CTG-GCA-CCA-TCG-TCG
B. suis	GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG

AMOS-PCR

The *Brucella* PCR diagnostic assay primer cocktail was composed of the five primers listed in Table I (Bricker and Halling, 1994). The reaction mixture consisted of 12.5 µl Taq PCR MasterMix (0.1U Taq Polymerase/µl,

500 µM dNTP each, 20 mM Tris-HCl, 100 mM KCl, 3 mM $MgCl_2$), 2 µl of template DNA, 7.9 µl ddH2O, and the five-primer cocktail (0.4 µl *B. abortus-, B. melitensis-, B. suis-, B. ovis-*, and 1 µl of IS711-specific primer). The PCR conditions consisted of an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min (Chen *et al*., 2006).

BcSS-PCR

B. canis was detected by BcSS-PCR amplification of a 300-bp nucleotide fragment of the *BCAN* gene. The PCR amplification primers and conditions were previously described by Kang *et al*. (2014). PCR reactions were performed using a 20-µl reaction mixture containing 10 µl Taq PCR MasterMix, 2 µl of template DNA, 2 µl of each of the primers (10 pmol), and 4 µl ddH_2O.

Histopathology and immunohistochemistry

Based on serological tests, the two dogs (golden retriever and poodle) were diagnosed with canine brucellosis. Due to the cost of treatment, the owner of one dog (golden retriever) opted against treatment and signed a consent form for euthanasia and post-mortem examination, according to the recommendations of the Sichuan Agricultural University ethics committee. Humane euthanasia was performed and tissue samples (liver, spleen, kidney, lung, lymph node, and testicle) were removed immediately. Tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned into 5-µm thick sections, and stained with hematoxylin and eosin (HE) following standard procedures (Gyuranecz *et al*., 2011a). These tissues were further analyzed by IHC analysis following the method previously described by Saglam *et al*. (2008). The primary antibody was a polyclonal goat antiserum (anti-*Brucella* Positive Control Serum, China Institute of Veterinary Drug Control, China) at a dilution of 1:50. For various medical reasons, the other dog (poodle) was sent to a different hospital for further treatment, and thus, we could not obtain further treatment information.

RESULTS

Serological and bacteriological tests

RSAT and RBPT were all showed positive for the two pet dog's blood samples. Blood samples from two pet dogs (golden retriever and poodle) yielded colonies that were culturally confirmed to be *Brucella*. Tests for urease, hydrogen sulfide, and reactions against monospecific sera A and M indicated that the two isolates (W5 and Y4) were *B. canis*. Bacteriological results are summarized in Table II.

Molecular diagnostics

Three types of PCR assays identified the two strains isolated from two pet dogs as *Brucella canis* (Table III).

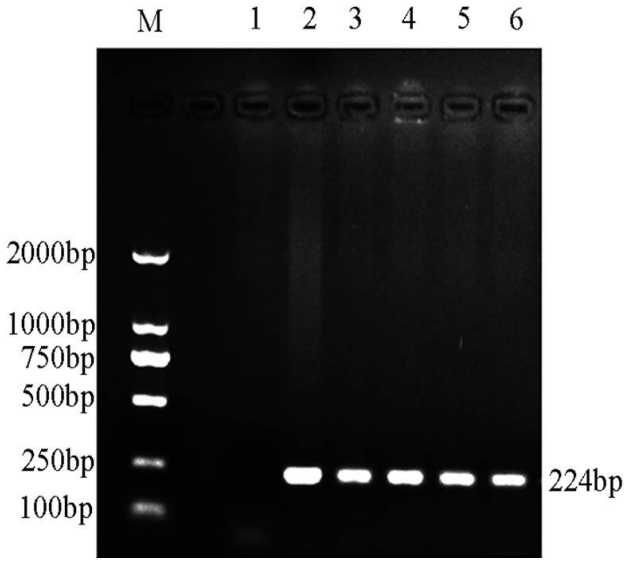

Fig. 1. *Brucella* genus-specific PCR results of the strains isolated from five reference strains and two isolates from two pet dogs. Lane M, molecular weight marker; Lane 1, negative control with no DNA added; Lane 2, positive control with DNA extracted from the *B. melitensis* strain (16M); Lane 3, DNA extracted from isolate *B. abortus* (544A); Lane 4, DNA extracted from the *B. suis* (S2); Lane 5, DNA extracted from isolate strain W5; Lane 6, DNA extracted from isolate strain Y4.

Table II.- Results of bacteriological findings for the two isolates from dogs.

Isolates	Colony morphology	CO_2 requirement	H_2S production	Hydrolysis of urea	Agglutination in sera		Auto agglutination with acraflavin	Crystal violet staining
					A	M		
W5	Rough	-	-	+	-	-	+	+
Y4	Rough	-	-	+	-	-	+	+

Table III.- Results of three PCR assays for amplification of *Brucella* isolated from five strains in this study.

Species	Strains	PCR results		
		***Brucella* genus specific PCR**	**AMOS-PCR**	**BcSS-PCR***
B. abortus	544A	+	+	-
B. melitensis	16M	+	+	-
B. suis	S2	+	+	-
B. canis	W5	+	-	+
B. canis	Y4	+	-	+

**B. canis* species-specific PCR performed in this study; + indicates application by PCR; - indicates no application by PCR.

Brucella genus-specific PCR, using the five strains (two isolates and three reference strains), successfully produced a 224-bp PCR amplicon (Fig. 1), indicating that all tested strains belong to the *Brucella* genus. Upon performing AMOS PCR, DNA from isolates Y4 and W5 was not amplify (Fig. 2), indicating that the two isolates do not belong to *B. abortus* biovar 1, 2, or 4, *B. melitensis*, *B. ovis*, or *B. suis*. BcSS-PCR assays using the two isolates (Y4 and W5) resulted in a specific 300-bp amplicon (Fig. 3), suggesting that the isolates were *B. canis*.

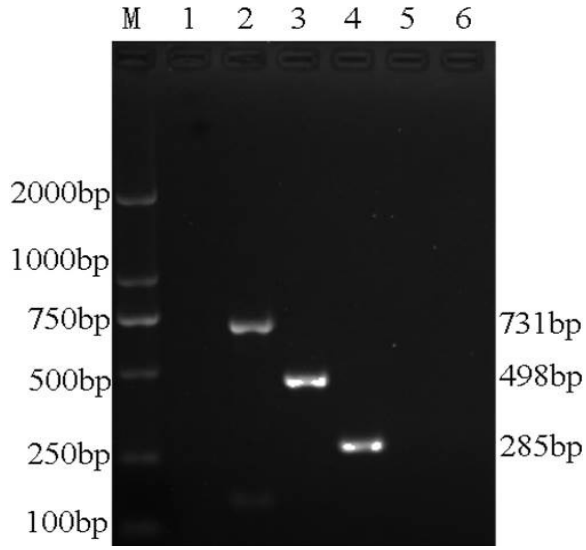

Fig. 2. AMOS-PCR results of the strains isolated from three reference strains and two isolates from two pet dogs. Lane M, molecular weight marker; Lane 1, negative control with no DNA added; Lane 2, DNA extracted from *B. melitensis* (16M); Lane 3, DNA extracted from *B. abortus* strain (544A); Lane 4, DNA extracted from *B. suis* (S2); Lane 5, DNA of isolate strain W5; Lane 6, DNA of isolate strain Y4.

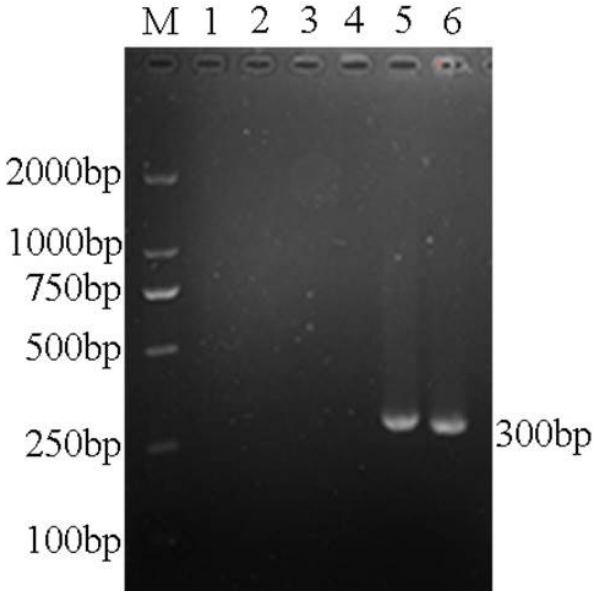

Fig. 3. BcSS-PCR results of the strains isolated from three reference strains and two isolates from two pet dogs. Lane M, molecular weight marker; Lane 1, negative control with no DNA added; Lane 2, DNA extracted from the reference strain *B. abortus* (544A); Lane 3, DNA extracted from the reference strain *B. melitensis* (16M); Lane 4, DNA extracted from the reference strain *B. suis* (S2); Lane 5, DNA extracted from isolate strain W5; Lane 6, DNA extracted from isolate strain Y4.

Histopathology

Extensive inflammatory and necrotic lesions in the liver, spleen, testicle, lymph node, lung, and kidney were observed. Histopathological changes in the liver were mainly located in the portal tracts, where severe infiltration of abundant lymphocytes and neutrophils was detected. In addition, hepatocellular necrosis and extensive vacuole degeneration in hepatocytes were also observed (Fig. 4A). Microscopic examination of the spleen showed the presence of many granulomas with central necrotic areas in red pulp. Necrotic foci were encircled by several different types of cells, consisting of a large number of necrotic neutrophils, a few plasma cells, and occasional macrophages (Fig. 4B). The principle lesions mainly found in the testicle were severe necrosis of spermatogenic cells and damaged seminiferous tubule structures accompanied by massive neutrophil, lymphocyte, and macrophage accumulation in interstitial tissue (Fig. 4C), resulting in orchitis or epididymitis. The principle alterations observed in the lymph nodes were proliferation of lymphocytes and reticuloendothelial cells along with deposition of fibrinous material. In addition, a bacterial bluish discoloration was found adjacent to the necrotic foci (Fig. 4D).

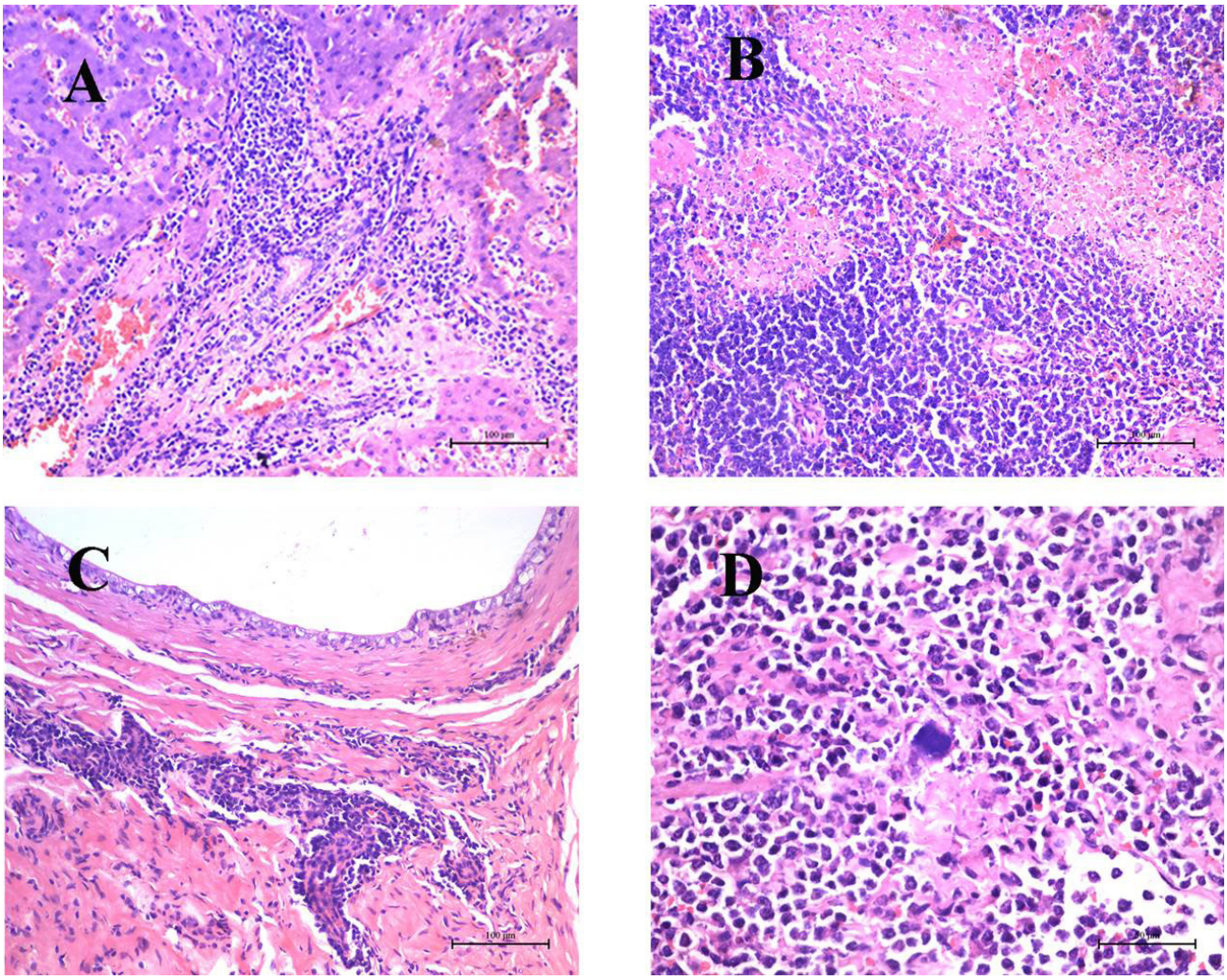

Fig. 4. Pathologic lesions in liver, spleen, testicle and lymph nodes from the dog naturally infected with *B. canis*. A, liver showing severe infiltration of abundant lymphocytes and neutrophils in addition to hepatocellular necrosis and extensive vacuole degeneration of hepatocytes. HE Bar, 100 μm; B, spleen showing the presence of many granulomas with central necrotic areas in red pulp. HE Bar, 100 μm; C, testicle showing severe necrosis of spermatogenic cells and damaged seminiferous tubule structures accompanied by massive neutrophil, lymphocyte, and macrophage accumulation in interstitial tissue. HE Bar, 100 μm. D, lymph nodes showing proliferation of lymphocytes and reticuloendothelial cells along with deposition of fibrinous material; a bacterial bluish discoloration was found adjacent to the necrotic foci. HE Bar, 50 μm.

However, changes in the lung and kidney were much milder compared to those of the other four tissues. The main lesions in the lung were low-to-moderate alveolar septum and mesenchyme infiltration by lymphocytes. Changes in the kidney were necrosis and exfoliation of renal tubular epithelial cells accompanied by focal infiltration of inflammatory cells.

Immunohistochemistry

IHC staining showed bacterial antigens in the lesions of various organs. *B. canis* antigens were primarily located in the cytoplasm of macrophages and neutrophils in portal infiltrates of the liver (Fig. 5A). *Brucella* antigens were also detected in the cytoplasm of macrophages in the red splenic pulp (Fig. 5B), cytoplasm of epithelial cells of cortical and medullar tubules, and macrophages and neutrophils of the renal interstitium. The cytoplasm of spermatogenic cells, macrophages and neutrophils of the testicle (Fig. 5C), and macrophages of the lymph nodes (Fig. 5D) were all positive for *B. canis* staining. Positive staining was also found in cellular debris from the alveolar septum of the lung.

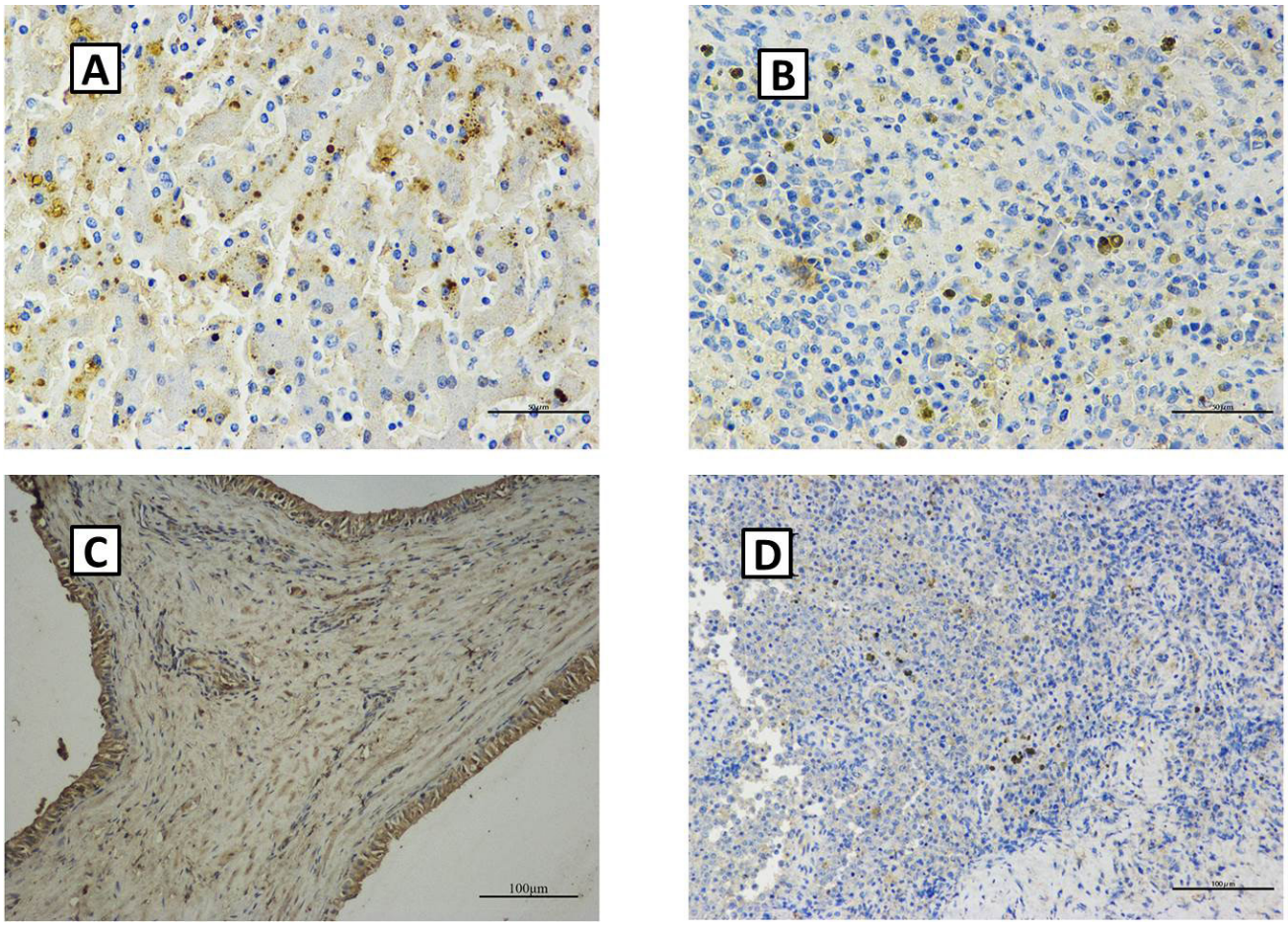

Fig. 5. IHC staining of *B. canis* antigens in the lesions of four organs (liver, spleen, testicle, and lymph nodes). A, positive staining in the cytoplasm of macrophages, neutrophils and Kupffer cells of the liver (200×); B, *Brucella* antigens were detected in the cytoplasm of macrophages in the red splenic pulp (200×); C, *B. canis* antigens in the cytoplasm of interstitial macrophages and spermatogenic cells of the testicle (200×); D, positive staining in macrophages of the lymph nodes (200×).

DISCUSSION

Canine brucellosis is one of the most serious zoonotic diseases, and not only endangers dogs but is also a threat to public health. Dogs can be infected by four *Brucella* species including *B. canis*, *B. abortus, B. melitensis and B. suis* (Hollett, 2006). Among the four species, *B. canis* is the predominant pathogen causing canine brucellosis (Gyuranecz *et al*., 2011b). Since the first isolation of *B. canis* strains from dogs in China, nationwide research regarding the infection caused by *B. canis* has been carried out in 25 provinces, and more than 300 strains have been isolated in 20 provinces with the infection rate varying from 0.3% to 42.7% (Deqiu *et al*., 2002). Epidemic regions are mostly located in the north, south, and southeast of China, for example Beijing, Shanghai, Tianjin, and Inner Mongolia (Di *et al*., 2014). In 2013, a study conducted in Inner Mongolia indicated that the *B. canis* infection rate in dogs reached 38.37% (Gao, 2013). In 2015, studies in Beijing suggested that this incidence is on an upward trend (Di *et al*., 2014). Published information suggests that canine brucellosis is more prevalent in China. However, no report is available regarding pet dogs infected with *B. canis* in Sichuan province, southwest China. In this study, we first isolated this species from two naturally-infected pet dogs in Sichuan, China. Because of the rapid growth of pet and companion animal industries in this region, *B. canis* infections pose a substantial risk to individuals and public health.

In the present study, the two strains were confirmed by biochemical and molecular methods. It is noteworthy that, a BcSS-PCR assay was employed to identify the two *B. canis* isolates. This assay was established by Kang *et al*. (2014)and consists of *B. canis* species-specific

amplification of a 300-bp fragment of the *BCAN* gene. Therefore, BcSS-PCR should be strongly recommended for the rapid detection of *B. canis* stains. In addition, we further conducted pathologic and IHC techniques to identify pathology changes and antigen locations in an infected dog (a male golden retriever). Although no obvious clinical signs other than enlarged lymph nodes and unilateral testis were found, pathological examination (in our study) revealed mild-to-severe inflammatory and necrotic lesions in all tested tissues, among which lesions in the liver, kidney, and lymph nodes were similar to those of published reports (Brennan *et al*., 2008; Carmichael and Kenney, 1970; Gleiser *et al*., 1971; Gyuranecz *et al*., 2011b). Previous reports demonstrated that pathologic differences between male and female dogs are remarkable in the spleen except for their own reproductive system (Brennan *et al*., 2008). In this study, significant necrotic changes were observed in the splenic red pulp with few hyperplastic lesions being observed in white pulp, which corresponds to the findings of Brennan *et al*. (2008). According to that report, hyperplasia of the splenic white pulp is prominent in females and not in males (Brennan *et al*., 2008). In addition to sex, there are also differences in the lungs between adult dogs and aborted fetuses. Our study showed that changes in the lung were much less prominent, in agreement with findings described for adult dogs (Brennan *et al*., 2008; Gleiser *et al*., 1971; Gyuranecz *et al*., 2011b). Accordingly, previous studies showed that histological alterations in the lung are the most significant lesions in aborted fetus (Brennan *et al*., 2008; Hofer *et al*., 2012; Xavier *et al*., 2009). The reason for this might be that fetuses are in close contact with vaginal discharges, abortion materials, and fluids with high bacterial loads. IHC techniques have been widely used for the detection of *B. abortus*, *B. suis*, and *B. melitensis* antigens in many animals such as cows, sheep, goats, bovine and ovine aborted fetuses, and hares (Gyuranecz *et al*., 2011a; Osburn and Kennedy, 1966; Saglam *et al*., 2008; Xavier *et al*., 2009). However, information regarding the detection of *B. canis* antigens in naturally-infected pet dogs is scant, especially in China. In our study, immunolabeling of *B. canis* antigens was stronger in the spleen, testicle, and liver than in the kidney and lymph nodes, and this was associated with the severity of inflammatory and necrotic lesions in those tissues. The detection is characterized by the observations on histopathology and IHC from a single study case. Yet to get more insight of *B. canis* pathogenicity in naturally-infected pet dogs, it is important to include more study cases in the future.

Canine brucellosis is an increasing zoonotic risk, with *B. canis* infections in pet dogs showing an upward trend in recent years (Holst *et al*., 2012). Given that therapy is not completely effective and the serious zoonotic implications of these infections, euthanasia of infected dogs should be advocated by professional agencies. If treatment must be adopted, infected dogs should be sterilized first. However, the treatment fee is relatively high and the period is lengthy. Additionally, disease relapse is still possible after ending therapy (Makloski, 2011).

CONCLUSION

We reported the first isolation of *B. canis* strains from pet dogs in Sichuan province, with an analysis of pathogenicity and antigen locations in a naturally-infected animal. This will benefit the epidemiologic, pathologic, and immunohistochemical study of *B. canis* infection in pet dogs. Future studies are needed to fully elucidate the epidemiology of such infections in China. In addition, the lack of a canine *Brucella* vaccine might imply a significant threat to animal and public health, which necessitates the future development of a safe and efficient vaccine against *B. canis*.

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Ethics statement

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University under permit number DYY-S20157034. Prior to the collection of specimens from dogs, permission was obtained from owners.

Statement of conflict of interest

The authors declare that they have no competing interests.

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