# Comparison of BP26, Omp25 and Omp31 and a Multi-Epitope Based Fusion Protein in Serological Detection of Canine Brucellosis

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**Research Article**

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## Abstract

# Background

Brucellosis is one of the most important zoonotic diseases in the world. Canine brucellosis caused mainly by *B. canis* is seriously neglected and there is lack of accurate diagnostic tools.

# Methods

In this study, using 34 brucellosis positive dog sera and 62 negative control sera, the *Brucella* outer membrane protein of Omp31, BP26, Omp25 and a multi-epitope based fusion protein were evaluated by iELISA for their potential use as antigens in serological diagnosis for canine brucellosis.

# Results

The result showed that multi-epitope based fusion protein performed best in distinguishing brucellosis positive and negative dog sera, with positive predictive value (PPV) was 100% and the negative predictive value (NPV) was 98.41%. BP26 and Omp31 showed excellent sensitivity in detecting brucellosis positive dog sera, but their cross reaction to sera infected with *Vibrio parahaemolyticus* and *Listeria monocytogenes* may hinder their application as diagnostic reagent. While Omp25 was lack of sufficient sensitivity and just showed limited ability in distinguishing positive and negative dog sera.

# Conclusion

The multi-epitope based fusion protein could accelerate the development of diagnostic kit for canine brucellosis currently urgently needed in China.

## Background

Brucellosis, caused by members of genus *Brucella*, is one of the most important zoonotic diseases in the world. Of the twelve classified species in genus *Brucella*, *B. abortus*, *B. melitensis* and *B. suis* are the main pathogenic species, causing cattle, small ruminant and pig brucellosis respectively [1]. These three species are also the main causative agent for human brucellosis. *B. canis* is a relatively newly identified species and primarily infectious to dogs, but its pathogenicity to human was also confirmed shortly after first identification [2]. Due to the huge number of dogs raised across the world, a healthy dog population free from *Brucella* infection would be great significant to public health, especially for the owners who have intimate contact with their companion dogs in daily life.

The prevalence of canine brucellosis differs among the countries and higher prevalence was usually found in rural area of developing countries [3–6]. In China, the canine brucellosis is recognized as a

seriously neglected disease. Sporadic serological surveys suggested that the prevalence of canine brucellosis in capital city Beijing was 1.40%, while in northwest city Urumqi, the prevalence was as high as 25.5% [7, 8]. Human brucellosis cases caused by *B. canis* was just sporadically reported [9], but more cases could be overlooked due to the none-specific signs and unreliable diagnostic tools. In current China, there is no reliably and commercially available diagnostic reagents for canine brucellosis. The Oligopolysaccharide (OPS) based serological diagnosis for smooth *Brucella* (*B. abortus*, *B. melitensis* and *B. suis*) caused brucellosis are not applicable for *B. canis* caused disease, as OPS is missing from *B. canis*. The traditional rapid slide agglutination tests (RSAT and ME-RSAT) using whole cell of rough *Brucella* failed to show sufficient specificity [10]. The recently developed molecular techniques (PCR and qPCR) have high sensitivity and specificity, but they are only limitedly used in laboratories equipped with expensive instruments [11, 12]. Thus, accurate and easy-to-perform serological methods are still needed for diagnosis of canine brucellosis.

Over the past years, the study on diagnostic antigens for canine brucellosis was focused on outer membrane antigens and cytoplasmic proteins extracted from *Brucella* [13–15]. Some functional enzymes in *Brucella*, such as lumazine synthase (LS) and superoxide dismutase (SOD), were also evaluated for their potential competency in serological diagnosis for canine brucellosis [14, 16]. More recently, we developed a novel fusion protein containing multiple B cell epitopes derived from *Brucella* main outer membrane proteins (OMPs), which displayed considerable ability in identifying smooth *Brucella* infected animal sera [17]. In this study, the potential use of this fusion protein in serological diagnosis of canine brucellosis was evaluated with a collection of dog sera positive for brucellosis and control sera collected from healthy dogs. At the same time, its efficacy as diagnostic antigen was compared to other important out membrane proteins such as Omp31, BP26 and Omp25. Hopefully, the data in this paper could accelerate the development of diagnostic kit for canine brucellosis urgently needed in China.

# Materials And Methods

### Canine sample collection and testing

From 2019 to 2020, we collected 1, 220 canine serum samples from pet clinics and kennels in three cities of Tianjin, Qingdao and Chongqing, China. Firstly, these sera were screened for brucellosis using RSAT method. The R-type Rose Bengal reagent was provided by China Institute of Veterinary Drug Control (CIVDC). RSAT positive samples (n=110) were further investigated by tracing back to original hosts, from which blood and swab (urethral or virginal) samples were collected for bacteriological detection by PCR and bacteriological isolation [11, 12]. *Brucella* isolation was carried out in biosafety level □ laboratory in China Animal Health and Epidemiology Center (CAHEC). Thirty four dogs were confirmed to be infected by *Brucella*, other *Brucella* specific DNA fragments were amplified (n=32) or *B. canis* was isolated (n=2) from blood and swab materials. The backgrounds of 34 dogs confirmed with *Brucella* infection were listed in **Supplementary table 1**. A total of 62 sera were chosen as negative control from three Beagle kennels (n=37) with no reported brucellosis case in past two years and fifteen pet raising households (n=25) which are clinically and superficially healthy. PCR result also showed that these dogs were

negative for brucellosis. These brucellosis positive and negative sera were used in following iELISA to evaluate the efficacy of protein antigens in diagnosing canine brucellosis.

### Preparation of multi-epitopes based fusion protein and main OMPs

The multi-epitopes based fusion protein was prepared according to our previously described method [18]. Briefly, 22 B cell epitopes were predicted from five *Brucella* OMPs (BP26, Omp16, Omp2b, Omp25 and Omp31) using BepiPred Linear Epitope Prediction software. These peptides were then concatenated by GGGGS linker to form a fusion protein with 495 amino acids. The fusion protein was prokaryotically expressed in BL21 cells using pET30a vector. Whole Omp25 and BP26 proteins were expressed and purified with prokaryotic expression system, and the detail procedure can be found in our published paper [19]. For Omp31 (WP_006133751.1), truncated protein without signal peptide (1-19 amino acids) was expressed in BL21 cells using pET28a (+) vector. All these prokaryotically expressed proteins were purified by nickel column affinity chromatography according to the instruction of manufacturer (Takara, China).

### Canine sera testing

An indirect ELISA method was used to test canine sera. In antigen coating step, 100μL protein solution properly diluted in carbon buffer solution (CBS, pH = 9.6) was added to each well of 96-well plate (NUNC, Denmark) and incubated overnight at 4 °C. After washing three times with PBST (PBS containing 0.05% Tween 20), 200μL of 5% skimmed milk powder was added to each well to block plate. After another round of washing, 100μL diluted serum (1:10, in 4% BSA) was added to each well of the plate and incubated at 37 °C for 1 h. Washing plate three times with PBST, then HRP-labeled protein G (Bersee Science and Technology, China) was added (1:8000 dilution, in PBST) to well and incubated at room temperature for 30 min. In coloring step, 100μL TMB substrate (Sangon Biotech, China) was added to well and incubated at room temperature for 15 min. Coloring was terminated by adding 50μL stopping buffer, then plate was measured immediately using an ELISA plate reader (BioTek, USA).

### Statistical analysis

Dot plot and receiver operating characteristic curve (ROC) analyses were performed using GraphPad Prism version 6.05. The significance of gray intensity differences was determined by Student's t-test (unpaired t-test). Differences were considered statistically significant when $P< 0.05$

# Results

# Optimization of antigen concentration and serum dilution for iELISA

The optimal conditions for antigen coating and serum dilution in iELISA assay were determined by chessboard titration. The brucellosis positive serum used in this assay was collected from a poodle

confirmed by bacteriological isolation, and the negative serum was collected from a healthy beagle dog. In the experiment using Omp31 as antigen, the maximum ratio of P/N was 17.49, under which the optimal antigen coating concentration was 0.625μg/mL and the optimal dilution of serum was 1:50 (Table 1). For Omp25, the maximum ratio of P/N was only 3.22, with optimal antigen coating concentration as 1.25μg/mL. Fusion protein antigen and BP26 showed similar ability in discerning canine brucellosis positive and negative sera, with maximum ratio of P/N as 4.33 and 4.67 respectively. However, for fusion protein antigen, higher concentration (2.5μg/mL) was needed to coat plate and more serum (1:25) was used in iELISA.

Table 1
Optimization of antigen concentration and serum dilution for iELISA

| Antigen | Maximum P/N value | Average OD (postive serum) | Average OD (negative serum) | Antigen coating concentration (ug/mL) | Serum dilution |
|---|---|---|---|---|---|
| Fusion protein | 4.33 | 0.5689 | 0.1321 | 2.5 | 1:25 |
| Omp31 | 17.49 | 1.7582 | 0.1005 | 0.625 | 1:50 |
| BP26 | 4.67 | 1.7408 | 0.3725 | 1.25 | 1:50 |
| Omp25 | 3.22 | 0.7593 | 0.236 | 1.25 | 1:50 |

## Comparison of BP26, Omp25 and Omp31 and fusion protein in serological detection of canine brucellosis

Using above optimized conditions and 96 canine brucellosis positive and negative sera, the efficiency of multi-epitope based fusion protein, Omp31, BP26 and Omp25 in serologically detecting canine brucellosis was evaluated and compared. According to result of iELISA, the average $OD_{450}$ values of 34 positive sera were 1.034, 1.317, 0.935 and 0.325 for fusion protein, Omp31, BP26 and Omp25 respectively, while average $OD_{450}$ values of 62 negative sera were 0.189, 0.300, 0.310 and 0.184 respectively. Dotplot demonstrated that Fuison protein, Omp31 and BP26 performed better than Omp25 in distinguishing canine brucellosis positive and negative sera (Figure 1).

The ROC curves were also obtained for these four antigens (Figure 2). The fusion protein had the biggest area under the ROC curve (AUC=0.9991; 95% CI= 0.9965 to 1.002), followed by Omp31 (AUC=0.9692; 95% CI= 0.9215 to 1.017) and BP26 (AUC=0.9355; 95% CI= 0.8824 to 0.9886). While, the Omp25 showed the least area under the ROC curve (AUC=0.9099; 95% CI= 0.8505 to 0.9692).

Based on Youden index, the optimal cutoff value for fusion protein antigen was 0.4855, under which the sensitivity was 97.06% (95% CI= 0.8467 to 0.9993) and the specificity was 100% (95% CI=0.9422 to 1.000). At the optimal cutoff value, 33 out of 34 positive samples were accurately diagnosed, and all

negative samples were correctly identified. The positive predictive value (PPV) was 100% and the negative predictive value (NPV) was 98.41%, making the fusion protein the best performing antigen in serological diagnosis for canine brucellosis (Table 2). Correspondingly, the fusion protein antigen showed the highest accuracy (98.96%) in serological detecting canine brucellosis. The accuracy of Omp31 and BP26 were 95.83% and 90.63% respectively. For Omp25, the accuracy was only 82.29%.

Table 2
Predictive values for positive and negative calculated at different cutoff values

| Cutoff value | Positive | | Negative | | PPV (%) | NPV (%) | Accuracy (%) |
|---|---|---|---|---|---|---|---|
| | TP | FN | TN | FP | | | |
| >0.4855 (Fusion protein) | 33 | 1 | 62 | 0 | 100.0 | 98.41 | 99 |
| >0.4130 (Omp31) | 33 | 1 | 59 | 3 | 91.67 | 98.33 | 95.8 |
| >0.6565 (BP26) | 26 | 8 | 61 | 1 | 96.30 | 88.41 | 90.6 |
| >0.2165 (Omp25) | 32 | 2 | 47 | 15 | 68.09 | 95.92 | 82.3 |

TP, true positives; TN, true negatives; FP, false positives; FN, false negatives; PPV, positive predictive value (TP/TP+FP)×100; NPV, negative predictive value (TN/TN+FN) ×100; Accuracy, (TP+TN)/(TP+FN+TN+FP)×100.

# Discussion

Canine brucellosis is a neglected disease mainly caused by *B. canis*. Since its first identification in 1960s, human brucellosis case with *B. canis* infection was intermittently reported, arousing increased concern on its zoonotic feature [20–22]. In China, canine brucellosis was first reportedly in 1984 and endemic in 20 provinces before 1990s [23]. Canine brucellosis reemerged again and showed a trend of spread in the past decade. In 2011, a brucellosis outbreak was reported in a beagle dog breeding farm in Beijing and *B. canis* infection was confirmed by bacteriological isolation [24]. In the same year, a 45-year-old woman was diagnosed as *B. canis* infection in Zhejiang provinces [9]. These cases revealed the human populations at high risk for exposure to *B. canis*, which are kennel workers and pet owners, especially immunosuppressed patients, children and pregnant women. As data displayed in this study, prevalence of canine brucellosis in some cities could be at least 2.78% (34/1, 220). But for most areas of China, the prevalence of canine brucellosis is still unknown.

The unknown to prevalence of canine brucellosis, in a great extent, is due to the lack of accurate and commercially available testing reagents. The RSAT and 2ME-RSAT using rough *Brucella* as antigen was the first method to detect canine brucellosis [25, 26], and it is still popularly used in some countries [27, 28]. But in China, there is no officially authorized RSAT antigen and homemade reagents was only limitedly available, making large scale screening test for canine brucellosis impossible. Even though RSAT can be easily accessible, the result with this method should be carefully interpreted. Our data

showed that the RSAT has a strong cross reaction to rabbit serum infected with *Vibrio Parahaemolyticus*. In China, *Vibrio Parahaemolyticus* is a major food borne pathogen to human [29], especially in coastal cities, where pet dog can be infected with this pathogen in household and misdiagnosed as brucellosis. We once isolated *Vibrio Parahaemolyticus* from dog blood collected in coastal city of Tianjin. Moreover, cross reaction to *Bordetella*, *Pseudomonas* and *Moraxella* was also reported in RAST using rough *Brucella* cell antigen [10]. Thus, the RAST positive dog should be further diagnosed with more specific method.

PCR and real time PCR have high sensitivity in detecting pathogen specific nucleic acid fragment. Primer sets have been proposed to detect *B. canis* using blood, swab and tissue samples, including ITS66 and ITS279 directed to 16S-23S rRNA interspace region and BcSS-PCR primers for *B. canis* species-specific fragment [30–32]. We also used these two sets of primers to determine the truly *Brucella* infected sera. As shedding of *B. canis* is undulated, PCR testing using blood and swab samples may lead to false negative result. So, the actual prevalence of brucellosis may be higher than 2.78% as displayed in this study. In our practical application, the animal host DNA can be easily amplified with ITS66 and ITS279 primers, and the size of product is equal to the targeting *Brucella* fragment in agarose gel. To avoid false positive result, PCR product by this primer set has to be sequenced and compared to *Brucella* reference sequence. The Real time PCR targeting for *Brucella* genus was also used in this study [33], which showed high sensitivity and specificity in detecting blood and swab samples and can be a useful tool to replace PCR methods.

The oligo-polysaccharide (OPS) antigen is not applicable in diagnosing rough *B. canis* caused brucellosis, so a quite number of protein antigens have been assessed for their potential use in detection for canine brucellosis [13, 15, 16]. These protein antigens demonstrated considerable efficiency in diagnosing dog sera, but their cross reaction to other pathogen infected sera were not evaluated, especially the pathogens often isolated from dog like *Escherichia coli*, *Salmonella* and *Vibrio parahaemolyticus*. Previous study showed that BP26 and Omp31 have strong reactivity to sera of animals infected by both smooth and rough *Brucella* [19, 34, 35], in consistent with the data in this paper. However, the cross reaction of BP26 to *Listeria monocytogenes* positive serum and Omp31 to *Vibrio parahaemolyticus* positive serum were prominent [19]. Multi-epitope based fusion protein showed a bit less sensitivity than BP26 and Omp31, but it had the least cross reaction to sera infected with other pathogens [17]. Based on all these results, the fusion protein is the best candidate antigen for serological diagnosis for canine brucellosis.

Canine brucellosis is principally caused by *B. canis* but it can be infected with *B. melitensis* or *B. abortus* in rural and pastoral area where dogs were closely contact with small ruminants and cattle. We cannot guarantee that all of 34 brucellosis positive sera used in this study were solely from *B. canis* infected dogs, as the primers used for PCR testing were not *B. canis* specific. The ITS66 and ITS279 primers can amplify the genomic DNA of *B. canis*, *B. melitensis*, *B. abortus* and *B. suis*, while BcSS primers can detected *B. canis* and *B. melitensis* strains. We successfully isolated one *B. canis* strain from these brucellosis positive samples, and subsequent MLVA16 typing revealed a MLVA profile as 2-3-9-11-3-1-5-2-

5-40-9-6-7-9-4-3. This MLVA16 genotype was closely related to a *B. canis* strain isolated from Beijing in 2011 [9, 24]. Bacteriological isolation suggested that most of dogs raised in cities were *B. canis* infected. Even if some dogs were infected with smooth *Brucella*, they can still be detected by this fusion protein, as the epitopes contained in this antigen are shared almost by all *Brucella* species.

# Conclusion

The multi-epitope based fusion protein displayed sufficient sensitivity and specificity than other outer membrane proteins in serological diagnosis of canine brucellosis. Using this novel antigen, accurate and easily available diagnostic kit can be developed to meet the need for reliable diagnostic for canine brucellosis.

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used or analyzed during the study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.


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### Authors' contributions

MS and DY conceived and designed the study. MY and ML performed the assays and drafted the manuscript. XC, JL, RW, YL, LK and XD were responsible for collecting sera and swab materials. XS, XF and LT provided technical support. SS reviewed and made improvements for the manuscript. All authors approved the final version of the paper.

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# References

1. Godfroid J, Nielsen K, Saegerman C. Diagnosis of brucellosis in livestock and wildlife. Croatian Med J. 2010;51(4):296–305.
2. Kauffman LK, Petersen CA. Canine Brucellosis: Old Foe and Reemerging Scourge. The Veterinary clinics of North America Small animal practice. 2019;49(4):763–79.
3. Chinyoka S, Dhliwayo S, Marabini L, Dutlow K, Matope G, Pfukenyi DM. Serological survey of Brucella canis in dogs in urban Harare and selected rural communities in Zimbabwe. J S Afr Vet Assoc. 2014;85(1):e1–5.
4. Ayoola MC, Ogugua AJ, Akinseye VO, Joshua TO, Banuso MF, Adedoyin FJ, Adesokan HK, Omobowale TO, Abiola JO, Otuh PI, et al. Sero-epidemiological survey and risk factors associated with brucellosis in dogs in south-western Nigeria. The Pan African medical journal. 2016;23:29.
5. Buhmann G, Paul F, Herbst W, Melzer F, Wolf G, Hartmann K, Fischer A. Canine Brucellosis: Insights Into the Epidemiologic Situation in Europe. Frontiers in veterinary science. 2019;6:151.
6. Jamil T, Melzer F, Khan I, Iqbal M, Saqib M, Hammad Hussain M, Schwarz S, Neubauer H. **Serological and Molecular Investigation of Brucella Species in Dogs in Pakistan**. *Pathogens* 2019, 8(4).
7. Qi HZH, Deng X, Zhang X, Cao Y. **Dog and Cat Brucellosis Epidemiology**. *Beijing Agriculture* 2012, 18.
8. Wang TZY, Wang L, Li A, Yang Q, Wang W, Ma W, Xu M, Yang X, Fang X, Zhang Q. **Epidemiological Investigation of Canine Brucellosis in Urumqi City of Xinjiang**. *China Animal Health Inspection* 2018, 35(6).
9. Piao D, Wang H, Di D, Tian G, Luo J, Gao W, Zhao H, Xu W, Fan W, Jiang H. MLVA and LPS Characteristics of Brucella canis Isolated from Humans and Dogs in Zhejiang, China. Frontiers in veterinary science. 2017;4:223.
10. Hollett RB. Canine brucellosis: outbreaks and compliance. Theriogenology. 2006;66(3):575–87.

11. Keid LB, Soares RM, Vieira NR, Megid J, Salgado VR, Vasconcellos SA, da Costa M, Gregori F, Richtzenhain LJ. Diagnosis of canine brucellosis: comparison between serological and microbiological tests and a PCR based on primers to 16S-23S rDNA interspacer. Vet Res Commun. 2007;31(8):951–65.
12. Kang SI, Lee SE, Kim JY, Lee K, Kim JW, Lee HK, Sung SR, Heo YR, Jung SC, Her M: **A new Brucella canis species-specific PCR assay for the diagnosis of canine brucellosis**. *Comparative immunology, microbiology and infectious diseases* 2014, **37**(4):237-241.
13. Baldi PC, Wanke MM, Loza ME, Fossati CA. Brucella abortus cytoplasmic proteins used as antigens in an ELISA potentially useful for the diagnosis of canine brucellosis. Veterinary microbiology. 1994;41(1-2):127–34.
14. Wanke MM, Delpino MV, Baldi PC. Comparative performance of tests using cytosolic or outer membrane antigens of Brucella for the serodiagnosis of canine brucellosis. Veterinary microbiology. 2002;88(4):367–75.
15. Barrouin-Melo SM, Poester FP, Ribeiro MB, de Alcantara AC, Aguiar PH, Nascimento IL, Schaer RE, Nascimento RM, Freire SM. Diagnosis of canine brucellosis by ELISA using an antigen obtained from wild Brucella canis. Res Vet Sci. 2007;83(3):340–6.
16. Tsogtbaatar G, Tachibana M, Watanabe K, Kim S, Suzuki H, Watarai M. Enzyme-linked immunosorbent assay for screening of canine brucellosis using recombinant Cu-Zn superoxide dismutase. J Vet Med Sci/Jpn Soc Vet Sci. 2008;70(12):1387–9.
17. Yin DBQ, Wu X, Li H, Shao J, Sun M, Zhang J. **A Multi-epitope Fusion Protein-Based p-ELISA Method for Diagnosing Bovine and Goat Brucellosis**. *Front Vet Sci* 2021, 8.
18. Yin D, Bai Q, Wu X, Li H, Shao J, Sun M, Jiang H, Zhang J. Paper-based ELISA diagnosis technology for human brucellosis based on a multiepitope fusion protein. PLoS Negl Trop Dis. 2021;15(8):e0009695.
19. Bai Q, Li H, Wu X, Shao J, Sun M, Yin D. Comparative analysis of the main outer membrane proteins of Brucella in the diagnosis of brucellosis. Biochem Biophys Res Commun. 2021;560:126–31.
20. Wallach JC, Giambartolomei GH, Baldi PC, Fossati CA. Human infection with M- strain of Brucella canis. Emerg Infect Dis. 2004;10(1):146–8.
21. Lucero NE, Corazza R, Almuzara MN, Reynes E, Escobar GI, Boeri E, Ayala SM. Human Brucella canis outbreak linked to infection in dogs. Epidemiol Infect. 2010;138(2):280–5.
22. Nomura A, Imaoka K, Imanishi H, Shimizu H, Nagura F, Maeda K, Tomino T, Fujita Y, Kimura M, Stein G. Human Brucella canis infections diagnosed by blood culture. Emerg Infect Dis. 2010;16(7):1183–5.
23. Shang DLL, Cheng Y. R.Z: **The investigation of Brucella canis in China**. Chin J Epidemiol. 1989;10:24–30.
24. Jiang H, Mao LL, Zhao HY, Li LY, Piao DR, Tian GZ, Di DD, Lei L, Cui BY. Reemergence and genetic comparison of Brucella canis in China, using a multiple-locus variable-number tandem-repeat assay. Veterinary microbiology. 2012;154(3-4):419–21.

25. George LW, Carmichael LE. A plate agglutination test for the rapid diagnosis of canine brucellosis. American journal of veterinary research. 1974;35(7):905–9.
26. Badakhsh FF, Carmichael LE, Douglass JA. Improved rapid slide agglutination test for presumptive diagnosis of canine brucellosis. J Clin Microbiol. 1982;15(2):286–9.
27. Johnson CA, Carter TD, Dunn JR, Baer SR, Schalow MM, Bellay YM, Guerra MA, Frank NA. Investigation and characterization of Brucella canis infections in pet-quality dogs and associated human exposures during a 2007-2016 outbreak in Michigan. J Am Vet Med Assoc. 2018;253(3):322–36.
28. Whitten TV, Brayshaw G, Patnayak D, Alvarez J, Larson CM, Root Kustritz M, Holzbauer SM, Torrison J, Scheftel JM: **Seroprevalence of Brucella canis antibodies in dogs entering a Minnesota humane society**, **Minnesota**, 2016-**2017**. *Preventive veterinary medicine* 2019, **168**:90-94.
29. Xie T, Yu Q, Tang X, Zhao J, He X. **Prevalence, antibiotic susceptibility and characterization of Vibrio parahaemolyticus isolates in China**. *FEMS microbiology letters* 2020, 367(16).
30. Keid LB, Soares RM, Vasconcellos SA, Chiebao DP, Salgado VR, Megid J, Richtzenhain LJ. A polymerase chain reaction for detection of Brucella canis in vaginal swabs of naturally infected bitches. Theriogenology. 2007;68(9):1260–70.
31. Aras Z, Ucan US. Detection of Brucella canis from inguinal lymph nodes of naturally infected dogs by PCR. Theriogenology. 2010;74(4):658–62.
32. Batinga MCA, Dos Santos JC, Lima JTR, Bigotto MFD, Muner K, Faita T, Soares RM, da Silva DAV, Oliveira T, Ferreira HL, et al. Comparison of three methods for recovery of Brucella canis DNA from canine blood samples. J Microbiol Methods. 2017;143:26–31.
33. Probert WS, Schrader KN, Khuong NY, Bystrom SL, Graves MH. **Real-time multiplex PCR assay for detection of Brucella spp., B. abortus, and B. melitensis**. J Clin Microbiol. 2004;42(3):1290–3.
34. Cassataro J, Pasquevich K, Bruno L, Wallach JC, Fossati CA, Baldi PC. Antibody reactivity to Omp31 from Brucella melitensis in human and animal infections by smooth and rough Brucellae. Clin Diagn Lab Immunol. 2004;11(1):111–4.
35. Chaudhuri P, Prasad R, Kumar V, Gangaplara A. Recombinant OMP28 antigen-based indirect ELISA for serodiagnosis of bovine brucellosis. Mol Cell Probes. 2010;24(3):142–5.

# Figures

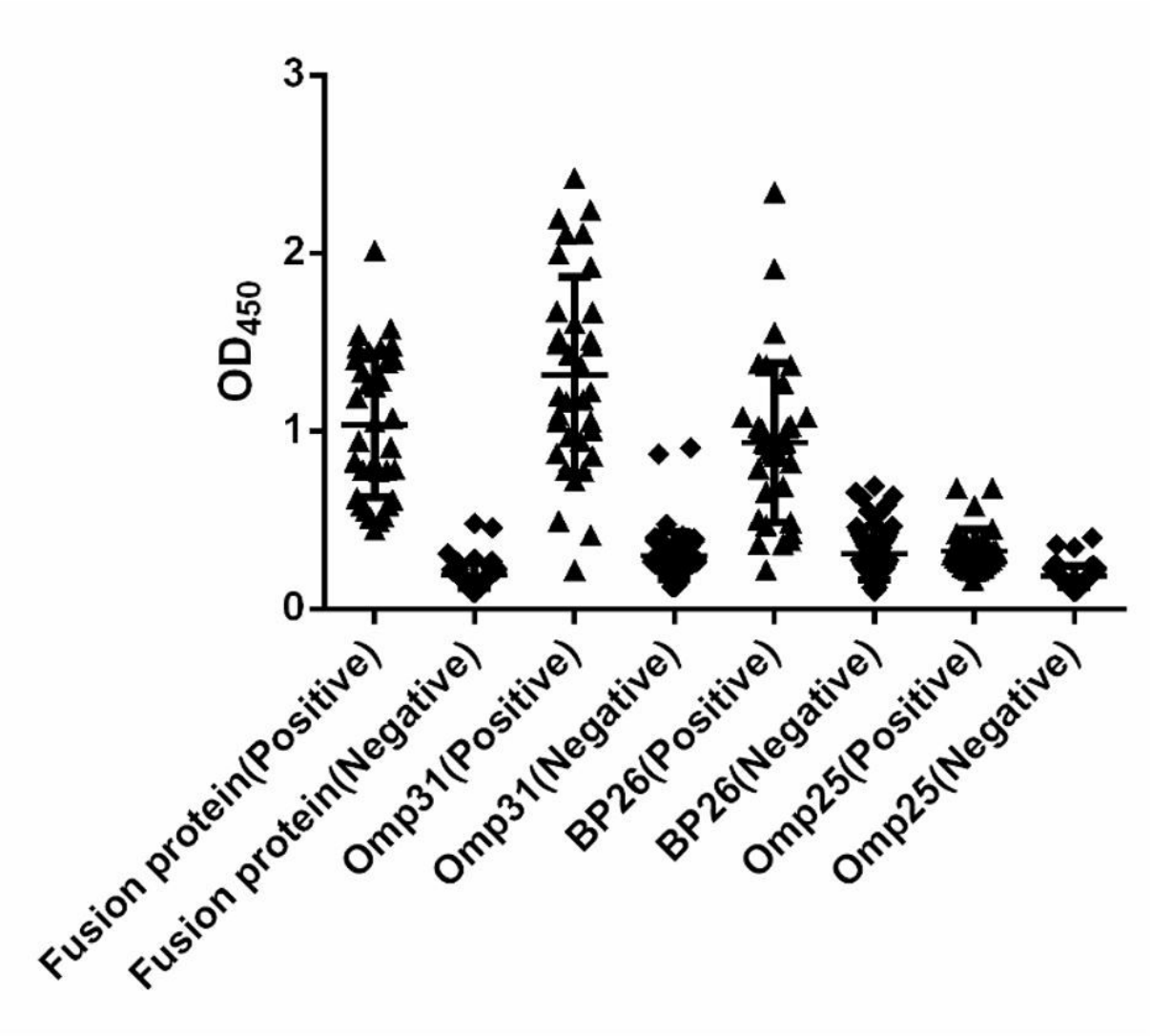


**Figure 1**

Dotplot of iELISA assays using fusion protein, Omp31, BP26 and Omp25 as antigen to detect canine brucellosis positive and negative sera.

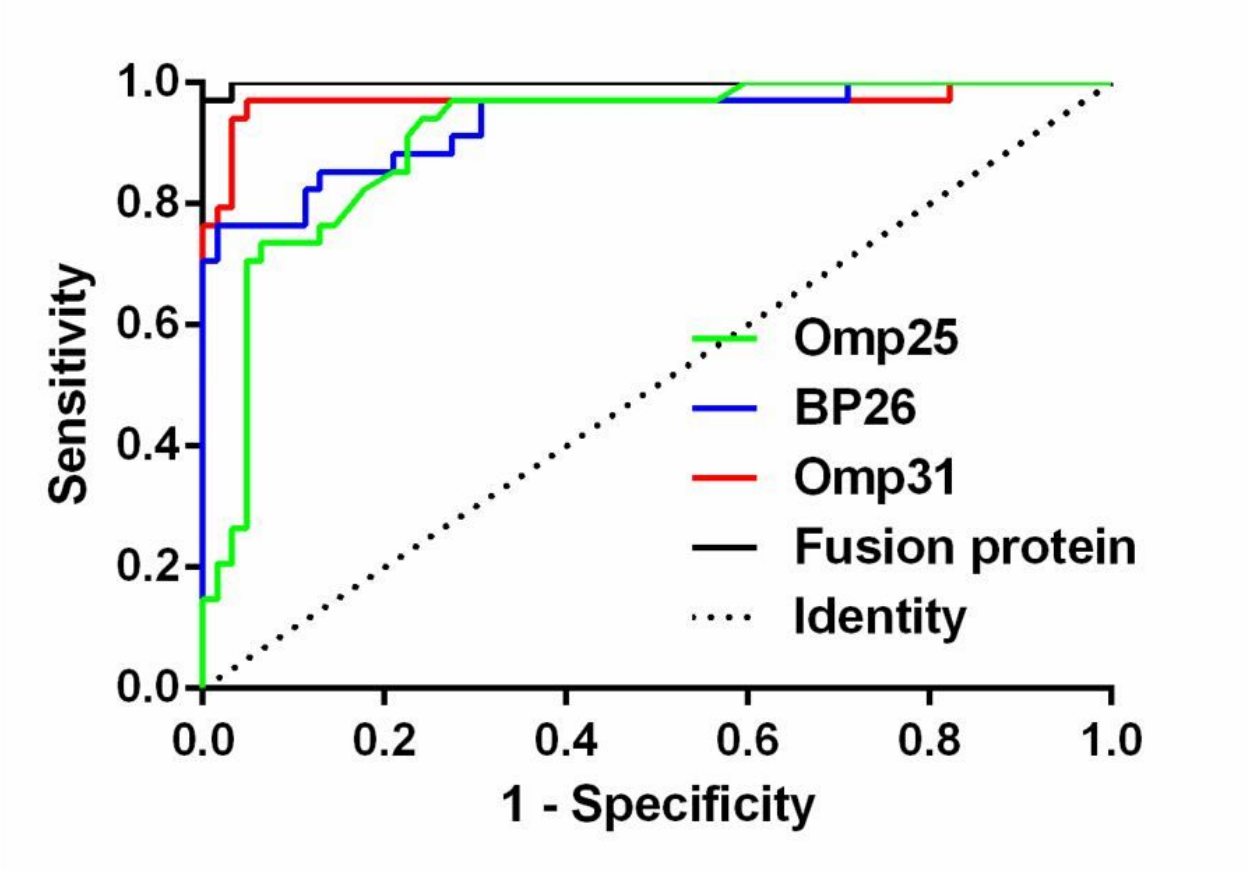


**Figure 2**

ROC analysis of fusion protein, Omp31, BP26 and Omp25 in detecting canine brucellosis.

## Supplementary Files

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- Supplementarytable.xlsx