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Monitoring the course of *Brucella* infection with qPCR-based detection

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ABSTRACT

Objectives: To determine blood *Brucella* DNA loads between brucellosis patients and those without brucellosis.
Methods: The patient group included 350 brucellosis patients. The control was composed of 200 subjects without brucellosis. The extracted DNA from blood was tested by quantitative polymerase chain reaction (qPCR). The cutoff value was determined by receiver operating characteristic curve analysis. A portion of the brucellosis patients were monitored by qPCR during therapy.
Results: The detection limit of qPCR was between 1E+01 cfu/μL and 1E+08 cfu/μL. The standard curve R^2 reached 0.998. The cutoff value was 4E+01 cfu/μL, which was determined by comparison of the patient group and the control. The qPCR assay had a specificity of 100% and a sensitivity of 93.14%. The monitoring results showed that the *Brucella* DNA load decreased in most patients during the first 4 weeks of treatment. One patient with bad treatment compliance showed a rebound.
Conclusions: The qPCR results were in accordance with the course of brucellosis in the clinic. The DNA load often reflects the situation of the *Brucella*-infected patient. The cutoff value provides an important reference of infection. This qPCR-based method can be used to assist in the diagnosis of brucellosis and to adjust the therapy.

Introduction

Brucella is a facultative anaerobic Gram-negative bacillus. It resembles *Mycobacterium tuberculosis* and establishes infection intracellularly. *Brucella* infection shows a strong tissue preference for the reproductive system, bone, and fetal ectoderm (de Figueiredo et al., 2015). Early manifestations of brucellosis consist of fever, sweating, joint pain, symptoms of poisoning, chronic spine arthritis, testis or ovarian inflammation, and neurological complications. The prognosis of brucellosis tends to be poor and can result in disability if an early diagnosis was missed and/or no timely treatment was initiated. Thus, an early diagnosis and treatment are required for achieving satisfactory outcomes.

The pathogenic diagnosis of brucellosis still relies on traditional serological methods and blood or bone marrow culture (Ariza et al., 1992; Young, 1991). Blood/bone marrow culture is the standard assay for the diagnosis of brucellosis, but it takes an average of 5 days; in addition, the positive rate of detection is low at the acute phase of infection (Pappas et al., 2005), so a proper diagnosis can be missed easily. The positive rate can reach nearly 50% after the acute phase (Queipo-Ortuno et al., 2005), but it delays the timely initiation of antibacterial treatment. Furthermore, the lack of a rapid and reliable diagnostic assay endangers biosecurity.

Brucella infection in humans induces an antibody response by the production of both IgM and IgG antibodies at 1–2 weeks after infection, which persists for 1 year or longer. Thus, antibody detection cannot effectively distinguish current or recurrent

Abbreviations: qPCR, quantitative polymerase chain reaction; PCR, polymerase chain reaction; PAT, plate agglutination test; SAT, standard agglutination test; PAMP, pathogen-associated molecular pattern; DAMP, danger-associated molecular pattern.

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infection from a resolved or recessive infection (Corbel, 1997). Moreover, the induced antibodies can cross-react with *Escherichia coli* O157:H7, *Stenotrophomonas maltophilia*, *Pseudomonas*, *Vibrio cholerae*, *Yersinia enterocolitica*, and *Bartonella* after *Brucella* infection (Pappas et al., 2005). The diagnosis based on blood-based assays likely leads to misdiagnosis when a fever is caused by these pathogens.

Polymerase chain reaction (PCR) is an important method that is used to diagnose brucellosis rapidly and classify species effectively (Adone et al., 2001; Amin et al., 2001; Garshasbi et al., 2014). Its sensitivity and specificity are much higher than those of serology and blood culture (Halling et al., 1993). Also, PCR consumes a short amount of time and small quantities of materials (Yu and Nielsen, 2010). Quantitative PCR (qPCR) is a more developed method originating from PCR. qPCR can determine bacterial load over time and provides a valuable tool to assess the infection status and treatment response.

In this study, we constructed a qPCR method to test the DNA load in blood samples of brucellosis patients. Then, we calculated the cutoff value in line with the results of the clinical diagnosis. Furthermore, we monitored the DNA load in blood samples from brucellosis patients during the course of therapy. Our work provides criteria for a laboratory diagnosis of brucellosis and also supplies the groundwork for evaluation of the treatment program.

Materials and methods

Subjects in this study

This study included 550 subjects admitted to the Department of Infectious Diseases and Physical Examination Center at The First Hospital of Jilin University from January 2014 to January 2016. The included subjects consisted of 350 brucellosis patients who were diagnosed if they met item 1, 2 and plus one of item 3, 4 and 5 listed in Table 1 and 200 control cases (managed by Physical Examination Center), including those with a fever of unknown origin (30 cases), tuberculosis (20 cases), positive blood cultures for *Escherichia coli* (12 cases), *Staphylococcus aureus* (1 case), *Streptococcus viridans* (1 case), *Micrococcus lutea* (1 case), *Girard rose Pseudomonas aeruginosa* (1 case), *Klebsiella pneumoniae* (1 case), *Pseudomonas aeruginosa* (1 case), and *maltotrophomonas* (1 case), and healthy people (131 cases). Among the patient group, 10 cases received combination therapy with rifampicin combined with doxycycline. All patients and control subjects had their blood samples monitored at 3 days, 7 days, 14 days, 28 days, 3 months, 6 months, and 1 year after admission, respectively.

Plate agglutination test (PAT) and standard agglutination test (SAT)

The plasma samples were prepared after centrifugation. PAT and SAT kits were supplied by The Centers for Disease Control and Prevention (CDC) of China (Beijing, China)and were performed per the manufacturer's instructions. Briefly, in PAT, 0.03 ml plama sample and 0.03 ml antigen were mixed on the plate; the result was observed after 5 min in room temperature; any agglutination indicated positive reaction. In SAT, serial diluted plasma samples at 1:12.5, 1:25, 1:50 and 1:100 were mixed with equal volume antigen, respectively, and incubated at 37 °C overnight. The results were read using standard serial turbidimetry. A positive reaction was considered if turbid growth was extended in 1:100 diluted sample.

Preparation of standards

The standards were prepared using the cultured bacteria from a brucellosis patient at the acute phase of infection. Briefly, once the bacterial identifier(VITEK, Mérieux, France) results were confirmed as the Maltese (sheep) species, a small amount of liquid was removed from the *Brucella* blood culture bottle (Becton, Dickinson Company, USA) and streaked onto a trypticase soy broth (TSB) blood plate, which was incubated at 37 °C in an incubator for 5 days. Single colonies were inoculated into 10% calf plasma TSB medium and incubated at 37 °C for 4 days. The culture product was counted by the flat colony counting method. Next, a quantity equivalent to 10^8 colony-forming units (cfu) of culture product was inactivated at 60 °C for 30 min, and the DNA was extracted per the manufacturer's instructions (Takara Bio. Inc., Japan). The quality and quantity of the DNA extract were measured by a multiscan-spectrum SynergyH1 instrument (Bio-Tek Instruments, Inc. USA). The standards were aliquoted and kept at −40 °C for further use.

Preparation of blood samples

A 200-μL volume of blood was used for DNA extraction, according to the manufacturer's instructions (E.Z.N. ATM Blood DNA Kit, Omega Bio-Tek, Inc., USA); the DNA was eluted in 200 μL of elution buffer, and the DNA concentration was measured by a SynergyH1 instrument.

PCR and primers

The PCR amplification cycle consisted of denaturation at 94 °C for 10 min, followed by 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min for 30 cycles. The final extension was at 72 °C for 1 min. The PCR products were visualized after 1% agarose gel electrophoresis. All primers used in this experiment are listed in Table 2.

Quantitative PCR

Standards containing 10^8 to 10 copies per reaction were prepared by diluting the standard bone marrow DNA in water. qPCRs were performed in 20-μL final volumes in capillary tubes by a One step instrument (Roche Diagnostics, Mannheim, Germany). The reaction mixtures contained 10 μL of DNA master mix for SYBR Green I (Roche Diagnostics), 0.5 μL of each primer (IS711 forward and reverse primer, Table 2), and 5 μL of template DNA. All capillaries were sealed, centrifuged at 500 *g* for 5 s, and then amplified by a One step instrument, with activation of polymerase (95 °C for 5 min), followed by 40 cycles of 15 s at 95 °C and 45 s at 60 °C. The temperature transition rate was 30 °C/s for all steps. The

Table 1
Qualification of recruited patients with clinical brucellosis.

①	Investigate the contact history;
②	Typical clinical manifestations of fever accompanied by fatigue, sweating, joint muscle pain, or enlarging or swelling in the liver and spleen, lymph nodes and testicles;
③	Plate agglutination test (PAT) positive and standard agglutination test (SAT) ≥1:100;
④	Blood culture and bone marrow culture positive;
⑤	Response to anti-epididymal disease treatment.

Brucellosis was suggested if patients met both ① and ②, and one of ③, ④, or ⑤.

Table 2
Primers used in this experiment.

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
Brucellamelitensis	TGCCGATCACTTAAGGGCCTTCA	AAATCGCGTCCTTGCTGGTCTGA	733
Brucellasuis		GCG CGG TTT TCT GAA GGT TCA GG	285
Brucella abortus		GACGAACGGAATTTTTCCAATCCC	498
bcsp31	CAATCTCGGAACTGGCCATCTCGAACGGTAT	ATGTTATAGATGAGGTCGTCCGGCTGCTTGG	208
16SrRNA	TCAACTTGAGAGTTTGATCCTGG	CCGGTCGCTGACCCTACCGTGGT	904
16SrRNA	TGAAGATAATGACGGTAACCGG	CAACGCTAGCCCCCTTGTAT	82
IS711	AAGTTTCTTGTCGTGAATCGCCT	TATTACTGCTCTACCTTCTGTGG	95

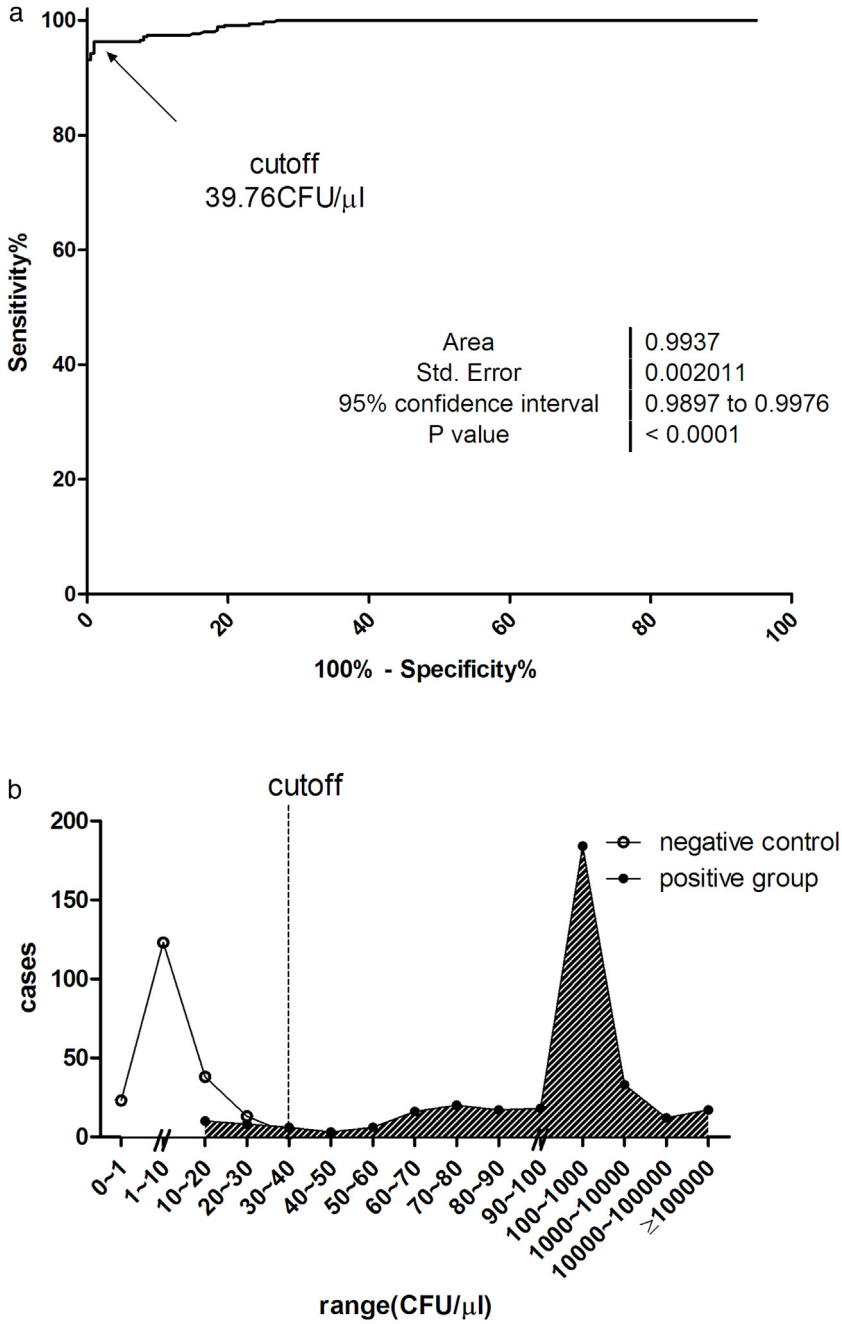

Figure 1. (a) The receiver operating characteristic curve. When the specificity was 100%, the maximum sensitivity was 93.14%, and the cutoff value was 39.76 CFU/μL. (b) The DNA load in patients and controls.

double-stranded PCR product was measured during the 60 °C extension step by detection of fluorescence associated with the binding of SYBR Green I to the product. Fluorescence curves were analyzed with the One step instrument. Melting curve analysis was performed immediately after the amplification protocol. The melting peak generated represented the specific amplified product. The crossing point was defined as the maximum of the second derivative of the fluorescence curve.

Statistical analysis

The diagnostic accuracy was assessed by calculating the area under the receiver operating characteristic curve. Data were analyzed by SPSS software, version 11.0 for Windows (SPSS). Sensitivity, specificity, positive and negative predictive values, likelihood ratios, and 95% confidence intervals were calculated using the SigmaPlot12.5 analyzer program.

Ethics

This study was approved (Number 2014-324) by the Ethics Committee of The First Hospital, Jilin University. The patients were recruited with informed consent.

Results

The standard is a Brucella melitensis *isolate*

The standard originating from a patient blood sample was determined by multiple-PCR and sequencing to be a *Brucella melitensis* isolate (Supplementary Figure S1). The *Brucella* isolate was 100% identical to *Brucella melitensis* isolate 1, sequence ID: LT963350.1 (Supplementary Figure S2).

Primers based on the insert sequence are specific to Brucella

The primers selected could amplify *Brucella* specifically. The agarose gel electrophoresis results showed that *Brucella melitensis, Brucella abortus*, and *Brucella suis* but not a non-*Brucella* template could be amplified with the selected primers (Supplementary Table S1). The IS711 forward and reverse primers are identical for *Brucella melitensis, Brucella abortus*, and *Brucella suis.*

The quantity of Brucella *in blood samples and the cutoff value*

The detection limit of qPCR was between 1E + 01 cfu/μL and 1E + 08 cfu/μL. The dissolution curve showed a single peak at a melting temperature value of 75.89 °C, suggesting no primer dimer. The standard curve R^2 reached 0.998 (Supplementary Figure S3). The qPCR value of the patient group ranged from 1E + 01 cfu/μL to 3E + 07 cfu/μL, while the maximum value of the control group was no more than 3.8E + 01 cfu/μL. The best cutoff was 4E + 01 cfu/μL. The specificity was 100%, and the sensitivity was 93.14% (Figure 1a,b).

The true positive rate by means of qPCR, SAT, and blood culture

When the cutoff value was 40 cfu/μL, qPCR had the highest true positive rate among the three methods. Of the 350 brucellosis patients, 326 of them tested positive by the qPCR method, 310 tested positive by SAT, and only 41 tested positive by blood culture. (Supplementary Table S3)

Comparison of qPCR, PAT, and SAT using 41 positive blood culture cases

We chose 41 *Brucella*-positive blood culture cases from the patient group and tested them by qPCR, PAT, and SAT, respectively.

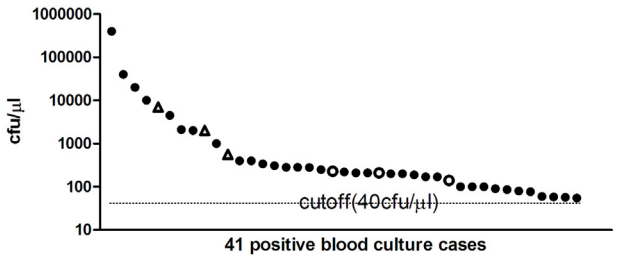

Figure 2. qPCR for 41 *Brucella*-positive blood culture samples.

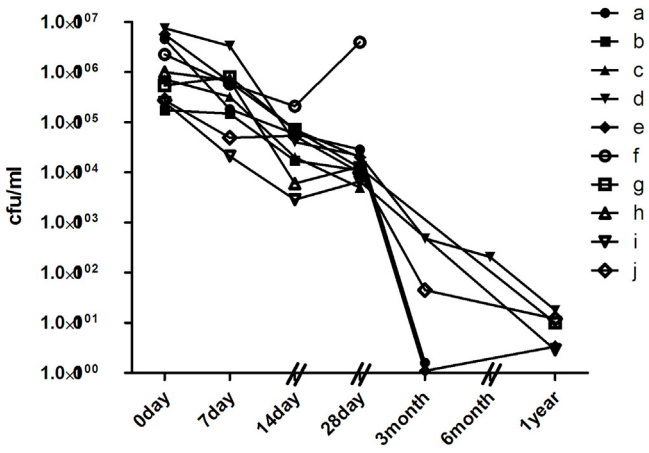

Figure 3. The DNA load decreased during the course of therapy for brucellosis.

The qPCR results showed that all samples had a higher *Brucella* DNA load than the cutoff value. Among these cases, three of them tested negative by PAT and SAT (≤1:50), and three of them were only SAT-negative (Figure 2).

Brucella *DNA load monitoring during therapy*

The bacterial loads in blood from 10 brucellosis patients were monitored during the treatment and follow up by qPCR. These patients received doxycycline, rifampicin, and/or gentamicin treatment for at least 4 weeks. The blood samples were collected at day 0 (before treatment) as well as 7 days, 14 days, 28 days, 3 months, 6 months, 1 year, and 3 years after treatment. All patients except for patient F improved significantly. The *Brucella* DNA load in patient F rose again after the 4-week treatment. However, we were unable to obtain more samples for analysis from patient F (Figure 3). PAT and SAT results show that the change was too small to administer the drug and guide the therapy program (Supplementary Table S2).

Discussion

Brucella can penetrate through the skin or mucous membranes into the body after the successful establishment of infection in a local spot that may involve the nearby lymph nodes, producing local symptoms. When the bacteria spread into the blood and replicate in additional organs/tissues, more bacteria are released into the blood, which increases the risk of complications (Nimri, 2003; Sofian et al., 2008). Without timely therapy or an incomplete cure, brucellosis will turn into a chronic disease and the *Brucella* are extremely difficult to clear (Franco et al., 2007). Clinicians are urgently looking for a reliable laboratory method to help diagnose brucellosis. To date, only blood culture methods are accepted techniques for brucellosis diagnosis. However, these methods

require a long period of time to complete, causing burden and pain for patients. As brucellosis is a complex clinical syndrome, a high index of suspicion is needed for prompt laboratory testing (Navarro et al., 2006; Queipo-Ortuño et al., 2005; Vrioni et al., 2008). A pathogen-associated molecular pattern (PAMP) or a danger-associated molecular pattern (DAMP) is a crucial break point for clinicians to determine the best treatment program. Up to now, there is still no effective means to distinguish whether the syndrome is caused by PAMP or DAMP after *Brucella* infection. Therefore, a more helpful and suitable laboratory diagnostic method should be investigated as soon as possible (Al Dahouk and Nöckler, 2011).

On the other hand, a long period of drug administration requires exact terms for laboratory diagnosis. Doxycycline and rifampicin are commonly used in brucellosis therapy but not in most other infectious diseases. These drugs have more severe side effects than other popular medicines used clinically (Covic et al., 1998; Lochhead and Elston, 2003). The recommended treatment period is at least 6 weeks (Bayindir et al., 2003; Skalsky et al., 2008). It is important to develop a method with a high sensitivity and specialty to assist in the clinical diagnosis so that the patients can receive antibiotics properly for a short treatment period.

Researchers have paid close attention to diagnostic methods based on qPCR because they are rapid, specific, and sensitive. Some species of *Brucella* are not harmful to human health, such as *Brucella canis* and *Brucella suis*, but veterinarians have developed PCR-based assays for their detection (Boeri et al., 2018; Sabrina et al., 2018). However, the course of brucellosis and the endpoint of the treatment are not the primary issues to veterinarians. Therefore, clinicians are confronted with how to transfer this effective method to applications in clinical brucellosis diagnosis.

It is difficult to develop a standard protocol and definitive diagnostic criteria for *Brucella* detection owing to differences in conditions and operators, together with the complexity of brucellosis (Sabrina et al., 2018; Thakur et al., 2018; Wareth et al., 2014). Many commercial DNA extraction kits can be used to treat clinical blood samples (Hull et al., 2018). They are useful for acquiring qualified DNA for qPCR. Also, numerous primers targeting *Brucella* have been designed (Boggiatto et al., 2018). To date, most studies have focused on the following target genes: *bcsp31*, *16SrRNA*, *IS711,* and *per* (Bounaadja et al., 2009; Pappas et al., 2005). In our experiments, the IS711 primer showed excellent performance. A drawback of this method is that a high-level barrier system is necessary to ensure biosafety when *Brucella* needs to be separated from clinical samples.

To find a regular pattern of the *Brucella* DNA load in blood samples between brucellosis patients and those without brucellosis, we extracted total DNA from 350 brucellosis patients and 200 nonbrucellosis control subjects and then drew a standard curve to quantify the *Brucella* DNA load in these samples. In addition, we collected follow-up data from 10 patients for one year. The results showed that the cutoff value was 4E + 01 cfu/μL, which was determined by comparing the patient group and the control group. The qPCR assay had a specificity of 100% and a sensitivity of 93.14%. The patient monitoring results showed that the *Brucella* DNA load decreased within 4 weeks of treatment. One patient with bad compliance had a *Brucella* DNA load that rebounded. We found that the most obvious changes of *Brucella* DNA load occurred among the brucellosis samples during the acute phase of infection. In contrast, there were only slight changes during the chronic phase of disease.

Conclusions

In conclusion, the qPCR results for blood *Brucella* DNA load were in accordance with the course of brucellosis in the clinic. The DNA load often reflects the stage of *Brucella* infection in the patient. The cutoff value provides an important reference to determine the infection status. Our work focused on the diagnosis of brucellosis as well as on the effectiveness of therapy. The qPCR-based methods described in this work can be used to assist in the diagnosis of brucellosis as well as to adjust the therapy program.

Conflict of interest

The authors declare no conflicts of interest.

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Access to data

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Contributions

LC, ND, and LG performed the experiments; CQ and WB designed the experiments, analyzed the results, and wrote the manuscript; YL and KZ provided expertise and feedback. JL and XJ provided clinical and other necessary support. All authors have critically reviewed the manuscript and agree with the final version.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.09.013.

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