Specific Detection and Differentiation Between \textit{Brucella melitensis} and \textit{Brucella abortus} by a Duplex Recombinase Polymerase Amplification Assay

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Brucellosis is a highly contagious zoonosis caused by a species under the genus \textit{Brucella}. A duplex recombinase polymerase amplification (Duplex RPA) assay for the specific detection of \textit{Brucella melitensis} and \textit{Brucella abortus} was developed in this study. Primers were designed targeting hypothetical protein genes and membrane transporter genes of \textit{B. melitensis} and \textit{B. abortus}, respectively. The newly developed assay was validated for its analytical sensitivity and specificity. Different samples were collected from the Qinghai, Inner Mongolia, and Xinjiang provinces. After DNA extraction, the samples were analyzed by Duplex RPA, real-time PCR, and multiplex AMOS PCR to estimate the prevalence of brucellosis in sheep and yak in West China. The analytical sensitivities of Duplex RPA were $9 \times 10^2$ plasmid copies of \textit{B. melitensis} and $9 \times 10^1$ plasmid copies of \textit{B. abortus}, but by mixing the reaction tubes after 4 min of incubation, the sensitivities were $4 \times 10^0$ and $5 \times 10^0$ copies of \textit{B. melitensis} and \textit{B. abortus}, respectively. There was no cross-reactivity with \textit{Brucella suis}, \textit{Chlamydia abortus}, \textit{Salmonella typhimurium}, \textit{Escherichia coli}, and \textit{Toxoplasma gondii}. The screening of field samples by Duplex RPA revealed that the prevalence of \textit{B. melitensis} in sheep and yak was 75.8% and the prevalence of \textit{B. abortus} was 4.8%. Multiplex AMOS PCR showed that the prevalence of \textit{B. melitensis} was 19.3%, and that of \textit{B. abortus} was 4.8%. It was concluded that the developed Duplex RPA is sensitive and specific to the detection of and differentiation between \textit{B. melitensis} and \textit{B. abortus} which will be useful in epidemiological surveillance and in the clinical settings.

Keywords: Duplex RPA, \textit{B. melitensis}, \textit{B. abortus}, real-time PCR, AMOS multiplex PCR
INTRODUCTION

Brucellosis is a contagious zoonotic disease caused by different species under the genus Brucella (1). Brucella melitensis, Brucella abortus, and Brucella suis are responsible for severe human infection in addition to economic losses in livestock due to abortions and loss of fertility (2). Two species B. melitensis and B. abortus are considered the most important species that cause illness in humans and domestic animals in many countries throughout the world. Three biovars of B. melitensis mainly infect sheep and goats, while the preferred hosts of seven biovars of B. abortus are cattle and buffaloes (3). The main clinical manifestations of B. melitensis infection in sheep and goats and B. abortus in cattle are abortion and stillbirth in females and orchitis and loss of fertility in males (4). The distribution of B. melitensis has long been associated with the Mediterranean littoral, however, it is now known to be more widely distributed, with only North America, North Europe, South-East Asia and Oceania being spared (5). B. abortus is distributed in many African, European, Asian, and American countries (6). Identification of Brucella organisms at the species and biotype level, mainly relies on culture, isolation, and subsequent identification by morphology, biochemical tests, reaction with monospecific antisera, sensitivity to dyes, and phage lysis (7). Several molecular methods have been developed to detect Brucella at the species level despite the high sequence homology between different species (8). The first species-specific PCR to be developed was multiplex AMOS PCR which can detect B. melitensis, B. abortus biovar 1, 2, and 4, B. ovis, and B. suis biovar 1 (9). Later Bruce-Ladder multiplex PCR was developed to identify 10 species in addition to vaccine strains of B. abortus and B. melitensis (10). Real-time PCR and multiplex PCR microarrays have been established to identify species (11, 12). Real-time and lateral flow dipstick RPA’s have been developed for the detection of Brucella spp. (13). Because of the high prevalence of B. melitensis and B. abortus in many countries throughout the world, their economic impact is due to losses of productive livestock. In addition to their zoonotic potential, we developed a novel Duplex RPA for the species specific detection of B. melitensis and B. abortus and to differentiate between them, and then validated the assay in field samples compared to real-time PCR and multiplex AMOS PCR. Another target of this work was to estimate the prevalence of brucellosis outbreaks in the Qinghai, Inner Mongolia, and Xinjiang provinces in West China by cross-sectional molecular detection by Duplex RPA, real-time PCR, and multiplex AMOS PCR.

MATERIALS AND METHODS

Bacterial Strains

B. melitensis biovar 3, B. melitensis biovar 1 M5 vaccine strain, B. abortus S19, and B. suis biovar 1 S2 vaccine were used as reference strains. The Reference DNA was obtained from Lanzhou Veterinary Research Institute and Harbin Veterinary Research Institute and was confirmed by AMOS PCR (9). The concentration of B. melitensis biovar 3 DNA was 10 ng/µL, that of B. melitensis biovar 1 M5 strain was 13 ng/µL, that of B. abortus S19 was 14 ng/µL, and that of B. suis biovar 1 S2 vaccine strain was 11 ng/µL. The purity of DNA samples measured by the ratio A260/A280 was 1.8–2.0.

Collection of Samples and DNA Extraction

Sixty-two different samples from sheep and yak were collected from the Qinghai, Inner Mongolia, and Xinjiang provinces. Tissue samples were collected in sterile plastic bags and milk samples were collected in sterile vials. The number and types of samples from each province are described in Table 1. Genomic DNA was extracted using the TIANamp Genomic DNA kit (TIANGEN Biotech, Beijing, China). Tissue samples were cut into small pieces and ground in a mortar and pestle. Then small portions of ground tissues (>10 mg) were lysed by the addition of 200 µL of GA buffer, 20 µL of proteinase K (provided in the kit), and RNase (100 mg/mL), and were incubated in a water bath at 56°C for 20–30 min until completely lysed. The next steps were carried out following the kit manufacturer’s instructions. Milk samples (10 mL) were centrifuged at 3,000 RPM for 5 min, and fluid between the supernatant and deposit was pipetted and discarded. The supernatant and deposit were mixed together, transferred to 1.5 mL tubes, and then lysed with the addition of 200 µL GA buffer, 20 µL proteinase K, and RNase (100 mg/mL), and were incubated at 56°C for 10 min. The remaining steps were performed as outlined in the kit manual. The extracted DNA samples were quantified using NanoDrop (Infinite 200 PRO, TECAN, Groedig, Austria). DNA samples were stored at −20°C until analysis.

Selection of Specific Sequences and Bioinformatics Analysis

According to the previous studies of (9, 15, 20), the specific sequence regions of B. melitensis (Accession number: CP007763.1 of B. melitensis 16 M strain) of the hypothetical protein gene and B. abortus (Accession number: AE017224.1 of B. abortus strain 9-941) of the membrane transporter gene was selected and retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). The similarities between each sequence and other sequences were determined by the Basic Local Aligned Sequence Tool (BLAST). Multiple sequence alignment of each B. melitensis and B. abortus sequence was carried out by the MegAlign software (DNASTAR Lasergene, Madison, Wisconsin USA).

Primers Design

Primers were designed according to the appendix in the TwistAmp™ reaction kit manuals (http://www.twistdxe.co.uk/images/uploads/docs/Appendix.pdf). Unlike PCR, the RPA primers should be 30–35 nucleotides in length, with GC content between 40 and 60%, no shorter than 30 nucleotides and longer than 45 nucleotides, no long tracks of guanines at the 5’ end, Gibbs free energy (−ΔG) should be between −4 and −5 kcal/mol for both the 5’ and 3’ ends of the primers. The species-specific primer pairs were designed by the Oligo Primers analysis software (Version 6.31 Molecular Biology Insights, Inc., USA), the parameters in the software such as primer
TABLE 1 | The results of Duplex RPA and multiplex AMOS PCR in field samples.

| Province          | Sample types and number | Animal species       | RPA          | AMOS-PCR |
|-------------------|-------------------------|----------------------|--------------|----------|
| Qinghai           | Liver (14)              | Yak (aborted fetus)  | B. melitensis (8) | –        |
|                   | Intestine (3)           | Yak (aborted fetus)  | B. melitensis (3) | –        |
|                   | Stomach (1)             | Yak (aborted fetus)  | B. melitensis (15) | –        |
|                   | Lung (16)               | Yak (aborted fetus)  | B. melitensis (14) | –        |
|                   | Intestine (3)           | Sheep (aborted fetus)| B. melitensis (1) | –        |
|                   | Lung (2)                | Sheep (aborted fetus)| B. melitensis (1) | –        |
|                   | Liver (2)               | Sheep (aborted fetus)| B. abortus (1)   | B. abortus (1) |
|                   | Spleen (1)              | Sheep (aborted fetus)| B. melitensis (1) | –        |
| Inner Mongolia    | Milk (17)               | Sheep                | B. melitensis (18) | B. melitensis (19) |
| Xinjiang          | Liver (20)              | Sheep (aborted fetus)| B. melitensis (23) | –        |
|                   | Lung (2)                | Sheep (aborted fetus)| B. melitensis (2)  | –        |
|                   | Stomach (2)             | Sheep (aborted fetus)| B. melitensis (2)  | –        |
|                   | Kidney (2)              | Sheep (aborted fetus)| B. melitensis (2)  | –        |
|                   | Spleen (2)              | Sheep (aborted fetus)| B. melitensis (2)  | –        |
| Total             | 62                      |                      | B. melitensis (47) | (75.8%)  |
|                   |                         |                      | B. abortus (3) | (4.8%)   |

length, and amplified sequence length range were adjusted, the primers were synthesized by (Tsingke Biological Technology, Xian, China).

Duplex Recombinase Polymerase Amplification (Duplex RPA) Development and Primary Experimentation

Duplex RPA was performed with a volume of 50 μL using a TwistAmp Basic kit (TwistDx, Cambridge, United Kingdom). The master mix was composed of 29.5 μL of RPA rehydration buffer, 1.2 μL (5 μM) of each primer, 11.2 μL of RNase free water (TAKARA Clontech, Shiga, Japan), 2.5 μL (280 mM) of magnesium acetate, and 2 μL of the DNA template. All reagents were prepared in 1.5 mL tubes except magnesium acetate and the DNA template. Then 45.5 μL of master mix was added to freeze-dried enzyme pellets in 0.2 mL reaction tubes each containing dried enzyme pellets. Magnesium acetate was pipetted into the tube lids. Subsequently 2 μL of the standard or genomic DNA template was added to each tube. The tubes were closed, the magnesium acetate was centrifuged into the tubes using a Minispin centrifuge, and the tubes were immediately placed in a dry bath heat block (TIANGEN Biotech, Beijing, China). To select the optimum temperature, the reaction tubes were incubated at 37, 38, and 39°C for 20 min. After incubation, the RPA reaction products were purified by the TIANquick Midi Purification Kit (TIANGEN Biotech, Beijing, China). The purification procedure was carried out according to the steps outlined in the kit manual. Purified RPA products were loaded in a 1.5–2% agarose gel and agarose gel electrophoresis was carried out for 15–30 min. The specific bands were visualized by a gel documentation system (BIORAD, Hercules, California, USA). The steps of RPA are demonstrated in Figure 1. The total time of the RPA reaction was between 45 and 60 min.

To examine candidate primers and optimal temperature, genomic DNA of B. melitensis biovar 3 and B. abortus strain 19 were used as positive control and B. suis biovar 1 S2 vaccine was used as negative control in addition to RNase free water as a non-template control.

Construction of Positive Control Plasmid DNA

The positive control plasmid DNA was constructed by ligation of a specific sequence of B. melitensis and B. abortus to the plasmid vector pMD-19 (TAKARA Clontech, Shiga, Japan). After purification of the RPA product, agarose gel electrophoresis, and the visualization of the bands, specific bands of B. melitensis and B. abortus were cut and subjected to gel DNA extraction by an AxyPrep DNA gel extraction kit (Axygen Bioscience, California, USA). After extraction from the gel, DNA was ligated to the plasmid vector by the addition of 4 μL of DNA to 1 μL of vector and 5 μL of 2× solution (provided with plasmid vector) and then incubated at 16°C overnight. The transformation was performed after the incubation of DNA, plasmid vector, and 2× solution mixtures by the addition of 5 μL of mixture to 50 μL of Escherichia coli DH5α competent cells in a 1.5 mL tube. The tube was then incubated on ice for 30 min, placed in a water bath at 42°C for 60–90 s, and placed again on ice for 3 min. One milliliter of Luria-Bertani (LB) broth was added to the tube which was then incubated in a shaker incubator at 37°C for 45 min. Luria Bertani (LB) agar plates containing 100 μg/mL ampicillin were inoculated with 100 μL of the cultured LB broth. The plates were incubated at 37°C for 18–24 h. Individual colonies (10, 13) were cultured into 1 mL of LB broth and incubated at 37°C in a shaker incubator. The plasmid DNA was extracted from the cultured broth by the TIAN prep Rapid Mini Plasmid Kit (TIANGEN
The extracted plasmid was quantified by NanoDrop (Infinite 200 PRO, TECAN, Groedig, Austria). The copy numbers were calculated by the equation: number of copies = (amount * 6.022 × 10^{23})/(length * 1 × 10^{9} * 650). The quantified plasmid was serially diluted 10-fold with elution buffer supplied in the extraction kit and analyzed by RPA to determine analytical sensitivity (detection limit).

### Analytical Sensitivity of Duplex RPA

To determine the analytical sensitivity (detection limit) of Duplex RPA, serial dilutions of the constructed plasmid of *B. melitensis* ranging from 9 × 10^{7} and 9 × 10^{1} copy numbers per reaction and *B. abortus* ranging from 9 × 10^{6} and 9 × 10^{3} copy numbers per reaction were analyzed at 38°C for 20 min. Two microliters of each dilution were added to a single 0.2 mL tube, and in the last one 2 µL of RNase free water was added instead of the DNA template, which was considered a non-template control. Every run was repeated three times. To increase the sensitivity of Duplex RPA, further evaluation was performed by testing other 10-fold dilutions of the constructed plasmids of *B. melitensis* ranging from 4 × 10^{5} and 4 × 10^{0} copy numbers per reaction and *B. abortus* ranging from 5 × 10^{6} and 5 × 10^{3} per reaction. Each 0.2 mL tube received 2 µL of DNA of each dilution and the last tube received 2 µL of RNase free water added to be considered as a non-template control. The reaction tubes (0.2 mL) were removed from the dry bath heat block after 4 min from the beginning and were mixed by inversion 10 times and then placed back into the dry bath heat block. The reaction tubes were incubated at 38°C for 20 min. Every run was repeated three times.

### Analytical Specificity of Duplex RPA

To determine the analytical specificity, 2 µL of DNA of *B. melitensis* and *B. melitensis M5*, *B. abortus S19*, *B. abortus*, and *B. suis S2* was first confirmed by AMOS PCR and analyzed by Duplex RPA at an optimal temperature for 20 min. Other DNA samples (2 µL) of organisms such as *Chlamydia abortus*, *Salmonella typhimurium*, *E. coli*, and *Toxoplasma gondii* were analyzed by Duplex RPA to determine analytical specificity.

### Real-Time PCR

The primer pair and probe of the real-time PCR assay were published by (14). Real-time PCR was prepared in a total volume of 20 µL. The total mix contained 10 µL of TaqMan Master mix (AceQs qPCR Probe Master Mix, Vanzyme, Nanjing, China), 0.4 µL of each primer, 0.2 µL of probe, 7 µL of RNase free water, and 2 µL of the DNA template. The reaction conditions were...
were 95°C for 5 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The reaction was incubated in a real-time PCR thermocycler (BIORAD, Hercules, California, USA). The primer pair and probe used in the real-time PCR are presented in Table 2.

**Multiplex AMOS PCR**

The AMOS (abortus-melitensis-ovis-suis) (9, 16) PCR was performed using five sets of primers (Table 2). The total reaction mix consisted of: 25 µL of 2XTaq master mix (TIANGEN Biotech, Beijing, China), 0.2 µM of each primer and RNase free water (TAKARA Clontech, Shiga, Japan), and 2 µL of the DNA template. The PCR conditions were as follows: initial denaturation at 96°C for 5 min followed by 40 cycles of 95°C denaturation for 1 min, 55.5°C annealing for 2 min and 72°C extension for 2 min, and a final extension at 72°C for 5 min. Species-specific bands were visualized after agarose gel electrophoresis.

**Screening of Field Samples With Duplex RPA and AMOS PCR**

The collected field samples were simultaneously screened by Duplex RPA, real-time PCR, and AMOS PCR for the species-specific detection of *B. melitensis* and *B. abortus*. RPA and PCR procedures were performed as described above.
Statistical Analysis
The results of the field sample screening by Duplex RPA and real-Time PCR were analyzed by Microsoft Excel version 2010 to estimate the prevalence of brucellosis in aborted and seropositive animals. The upper and lower limits of prevalence at 95% confidence intervals were calculated by Wilson score intervals using the online calculator available on the website: http://epitools.ausvet.com.au/content.php?page=CIProportion.

RESULTS
Detection of Bacterial Strains by AMOS PCR
Bacterial strains showed positive AMOS PCR results characterized by a specific band for each species (Figure 2). B. melitensis yielded a band of 730 bp, B. abortus had a 498 bp band, and B. suis produced a 285 bp band.

Sequence Alignments and Primer Pairs
The results obtained from BLAST revealed the high similarity of each sequence of B. melitensis and B. abortus to different strains of B. melitensis and B. abortus. The selected B. melitensis sequence showed some similarity with Brucella ceti and B. suis. Thus, the selected primers were located in the B. melitensis specific region. The selected sequence region of B. abortus did not show any similarities with other species. The alignment of partial sequences from B. melitensis is demonstrated in Figure 3. The partial sequence of B. abortus and its similar sequence alignments are shown in Figure 4. The primer pairs of B. melitensis and B. abortus are presented in Table 1. Forward and reverse primer locations are indicated in each sequence of B. melitensis and B. abortus by black highlights in Figures 3, 4.

Duplex RPA Optimal Conditions
Duplex RPA can be performed at reaction temperatures ranging from 37 to 39°C. There was no difference in the results between 37, 38, and 39°C. The selected reaction conditions were 38°C.
for 20 min. The selected temperature was arbitrarily selected. The selected primers of *B. melitensis* yielded a 167 bp band, while *B. abortus* yielded a 235 bp band.

### Analytical Sensitivity and Specificity

The analytical sensitivity of Duplex RPA by amplifying different serial dilutions of plasmids bearing target sequences of *B. melitensis* and *B. abortus* was $9 \times 10^2$ copies of the constructed plasmid of *B. melitensis* and $9 \times 10^3$ copies of *B. abortus* plasmid (Figures 5A, 6A). The test could detect *B. melitensis* biovar 3, *B. melitensis* M5, and *B. abortus* A19 (Figures 6, 7A). The mixing of the RPA reaction 4 min after starting increased the sensitivity to $4 \times 10^5$ and $5 \times 10^6$ copies of *B. melitensis* and *B. abortus*, respectively (Figures 5B, 6B). There was no cross-reactivity of the developed RPA with *B. suis* S2 and other bacteria, including *C. abortus*, *S. typhimurium*, *E. coli*, and the parasite *T. gondii* (Figure 7B).

### Results of Screening Field Samples

Different types of sheep and yak samples from the Qinghai, Inner Mongolia, and Xingjian provinces showed a high prevalence of brucellosis, with prevalence rates of 80.6% by duplex RPA and 95.2% by real-time PCR (Table 3). The incidence of *B. melitensis* was 47 (75.8%), and that of *B. abortus* was 3 (4.8%). The incidence results obtained by AMOS PCR were 12 (19.3%) for *B. melitensis* and 3 (4.8%) for *B. abortus* (Table 1). A sheep was found to be infected with *B. melitensis* and *B. abortus*, and yak were found to be infected with *B. abortus* and *B. melitensis* (Table 1). These results reveal the transmission of *B. abortus* to sheep and *B. melitensis* to yak.

### DISCUSSION

Brucellosis is a re-emerging zoonotic disease caused by the closely related species of the genus *Brucella*. *B. melitensis* and *B. abortus* were the first described species in 1887 and 1895 A.D., respectively (22). Livestock species (cattle, sheep, goats, swine, and camel) can be infected with *B. melitensis*, *B. abortus*, and *B. suis*, which are responsible for severe human infection (23). Cattle can be infected by *B. abortus* and transiently infected with *B. suis* and more commonly by *B. melitensis* when they come into direct contact with infected pigs, goats, and sheep.
in common pastures and at shared water sources. *B. abortus*, *B. melitensis*, and *B. suis* can be transmitted by cow’s milk and cause a serious public health threat (24). The newly developed Duplex RPA could detect either *B. melitensis* or *B. abortus* with a reaction time of 20 min; additionally it did not require sophisticated equipment instead employing a water bath or a
heat block dry bath. The previously developed types of PCR, i.e., AMOS PCR, Bruce-Ladder PCR, and multiplex real-time PCR, were successfully capable of detecting and differentiating between different species under the genus *Brucella*. Furthermore, these techniques can differentiate between field strains and vaccine strains (10, 19), but these techniques require 2–4 h of reaction time and require a thermocycler. Similar to PCR and multiplex PCR, Duplex RPA was used for end-point detection by agarose gel electrophoresis, which increased the time of the test. Therefore, the developed Duplex RPA cannot be considered a rapid test. Other types of PCR have been developed for species-specific detection and differentiation between field and vaccine strains (*B. melitensis*, *B. suis*, and *B. abortus*-specific PCR) (18, 21, 25). One of the limitations of developed RPA is the inability to differentiate vaccinated animals from naturally infected animals. Generally, RPA is considered a newly invented technique that can rapidly amplify DNA or RNA within 10–20 min (26). Similar to PCR and real-time PCR, multiplexing in RPA is applicable. Therefore, many sensitive and specific multiplexed RPA assays have been developed.
for the diagnosis of many pathogens (27). The sensitivity of RPA can be increased by mixing during incubation, which is supported by a selection of short fragments of the target sequence that leads to swift amplification (28, 29). In multiplex lateral flow RPA for the detection of many intestinal protozoa, the molecular sensitivities are 403 synthetic gene copies per
of RPA and its ability to amplify DNA in the presence of AMOS PCR, which may be attributed to the higher sensitivity was high variation between the results obtained by RPA and B. abortus can only be used areas in which M5 can be used; in contrast, B. melitensis egg and chicken meat samples (pre-enrichment procedure, and 1 CFU/g from 24 h enriched Campylobacter coli and Campylobacter jejuni for the detection of 10 colony-forming units for methicillin-resistant Salmonella enterica, was 1 CFU/mL from chicken broth, for food applications, the detection limit of C. coli and C. jejuni was 1 CFU/reaction in pure culture. In the case of jejuni and detection of 10 colony-forming units for methicillin-resistant Staphylococcus aureus, Neisseria gonorrhoeae and Salmonella enterica, which are 730 bp and 498 bp, respectively, influencing the sensitivity. The detection sensitivity of AMOS PCR for the detection of Campylobacter coli and Campylobacter jejuni was 1 CFU/reaction in pure culture. In the case of food applications, the detection limit of C. coli and C. jejuni using the RPA assay were 1 CFU/mL from chicken broth, 10^3 CFU/g from egg and chicken meat samples without a pre-enrichment procedure, and 1 CFU/g from 24 h enriched egg and chicken meat samples (17). Similar to the previously developed multiplex RPA assays, the developed Duplex RPA is highly sensitive especially after mixing components after 4 min of incubation. However, the end-point detection of most of the multiplex RPA assays depend on real-time fluorescence detection or lateral flow immunochromatographic strips, which make it a rapid technique.

In western China, bacterial isolation and identification, AMOS PCR and multi-locus variable number tandem repeat analysis found that B. melitensis biovar 3 was the dominant causative agent of sheep brucellosis, while B. abortus biovars 1 and 3 were found in infected yaks (31, 32). This study agrees with a previous study reporting the isolation of B. melitensis from yak in Qinghai province, west of China (33). The importance of Brucella species identification, in addition to the determination of the epidemiological situation, is also essential for vaccine-type selection, for example; if B. melitensis is endemic to a region, B. melitensis vaccines such as B. melitensis Rev.1 or B. melitensis M5 can be used; in contrast, B. abortus strain 19 can only be used areas in which B. abortus is endemic. There was high variation between the results obtained by RPA and AMOS PCR, which may be attributed to the higher sensitivity of RPA and its ability to amplify DNA in the presence of PCR inhibitors (26); conversely, AMOS PCR amplifies long fragments DNA of B. melitensis and B. abortus which are 730 bp and 498 bp, respectively, influencing the sensitivity (34). In addition, AMOS multiplex PCR can only detect all biovars of B. melitensis, biovars 1, 2, and 4 of B. abortus, B. suis biovar 1, and B. ovis (9). The cost estimation of one reaction of RPA is 8 Euros while, that of AMOS PCR is 1 Euro and that of real-time PCR is 3 Euros. The high cost of the RPA reaction is attributed to the production of RPA reagents by one company (35).

From previous studies, the species and biovars isolated from western China were B. melitensis biovars 1 and 3, B. abortus biovars 1 and 3, and B. suis biovar 3 (33), which explains the variation of the results between AMOS-PCR, real-time PCR, and Duplex RPA. Through our work, RPA products can be purified, sequenced, or applied for ligation to plasmid vectors and cloning. This study has limitations, mainly in analyzing an inadequate number of Brucella species and strains and other genetically related bacteria. Therefore, this work must be continued by analyzing other species and biovars of B. melitensis, B. abortus, and B. suis. Further, this study can be considered as preliminary and key for the development of real-time or lateral flow RPA for the detection of two or three species of Brucella or the differentiation between field and vaccine strains. Additionally, RPA can be developed for the simultaneous detection of many bacteria species that can cause abortion, such as C. abortus, Coxiella burnetti, and Brucella spp.

It is concluded that Duplex RPA is an isothermal assay for specific detection and differentiation between B. melitensis and B. abortus that is highly sensitive and specific. The assay takes less time (20 min incubation) than multiplex AMOS PCR and real-time PCR. The test sensitivity can be increased by mixing the reaction components. The developed RPA is more sensitive than multiplex AMOS-PCR. Because the test still has limitations characterized by using agarose gel electrophoresis, this could further be improved by the application of a real-time exoprobe and a lateral flow dipstick for end-point detection. These characteristics demonstrate that Duplex RPA is a sensitive, specific, and direct detection technique for the species-specific detection of brucellosis.

### DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.
ETHICS STATEMENT

All animals were handled, samples collected and processed, and all techniques carried out in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People’s Republic of China. The Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences approved the study (Permit No. LVRIAEC-2014-009). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

GM, XC, JZ, and BF designed the study and wrote the paper. GM performed all the experiments, ZLi performed PCR, realtime PCR, and results analysis. ZLo participated in the collection and preparation of samples. NZ participated in article editing and proofreading. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets. 2020.539679/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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