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Establishment of a recombinase polymerase amplification (RPA) assay for the detection of *Brucella spp.* Infection

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ABSTRACT

Brucellosis is a worldwide re-emerging zoonosis. It has an economic impact due to abortion and loss of fertility in livestock. In this study, Real-time recombinase polymerase amplification (RT-RPA-BP26) targeting *Brucella spp.* bp26 gene and Lateral flow dipstick (LFD-RPA-IS711) combined with SYBR- Green recombinase polymerase amplification (RPA) targeting insertion sequence *IS711* region of *Brucella spp.* bp26 gene, was developed to detect *Brucella spp.* from different sample types in domestic animals. The sensitivity and specificity of the two developed RPAs were compared with real-time PCR, PCR, and Rose Bengal Plate Test (RBPT). The analytical sensitivity and detection limit of Real-time RPA and LFD RPA were four and six copies per reaction respectively. The detection of six colony forming units (CFU) of the bacteria-bearing construct with the target sequence was within 20 min at 40 °C for Real-time RPA and 37 °C for LFD RPA. The LFD RPA could work at temperatures between 30 and 35 °C and could be completed within 10–30 min. No significant differences were observed when comparing the results from Real-time RPA and LFD RPA to Real-time PCR and PCR. Both methods showed no cross reactivity with *Chlamydia abortus, Toxoplasma gondii, Salmonella typhimurium, and Escherichia coli.* In conclusion, RPA is a useful and convenient field and point of care test for brucellosis.

1. Introduction

Brucellosis is a zoonotic bacterial disease caused by the genus *Brucella*. It comprises twelve species, *B. melitensis*, *B. abortus, B. suis, B. ovis, B. canis* and *B. neotomae, B. ceti, B. pinnipedialis, B. microti, B. inopinata, B. papionis and B. vulpis* [1–3]. The disease was eradicated in several countries by the implementing eradication programs, disease surveillance and control of disease spread. However the disease remains enzootic in many regions around the world [4]. There are numerous methods to detect brucellosis in animals. Isolation, identification, and bio-typing of *Brucella spp.* is considered the 'gold standard' method, and is useful for epidemiological studies, genotyping and molecular characterization. Serological tests, such as agglutination tests, complement fixation test (CFT) and Enzyme-linked immunosorbent assay (ELISA) are widely used for the diagnosis of animal and human brucellosis in several countries worldwide. These test are also used for international trading purposes [5,6]. Polymerase chain reaction (PCR) has been used for the diagnosis of human and animal brucellosis and for direct detection of the pathogen in contaminated food products [7]. In addition, multiplex PCR methods have been developed to identify six classical species and subtypes isolated from marine mammals and some vaccine strains [8,9]. Restriction Fragment Length Polymorphisms PCR (RFLPs-PCR) has been used for typing *Brucella spp.* for taxonomic and epidemiological studies [9]. Real-time PCR has also been used for the diagnosis of *Brucella spp.* infection in humans and animals from blood, urine and embedded tissues in paraffin wax [10]. Loop-mediated isothermal amplification (LAMP) has been used for the diagnosis of *Brucella spp*., in infected mouse organs and contaminated milk [11].

There is high prevalence of animal brucellosis in the western provinces of China [12]. For this reason, simple and rapid techniques are

Abbreviations: RT-RPA-Bp26, Real-time Recombinase Polymerase Amplification BP26; LFD-RPA-IS711, Lateral flow dipstick RPA-IS711; cPCR-BP26, conventional Polymerase chain reaction-BP26; cPCR-IS711, conventional polymerase chain reaction-IS711; q PCR-IS711, quantitative Polymerase chain reaction-IS711; RBPT, Rose Bengal Plate Test; *B. melitensis*, *Brucella melitensis*; *B. abortus*, *Brucella abortus*; *B. suis*, *Brucella suis*; *E. coli*, *Escherichia coli*; CFU, Colony forming unit

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needed for the detection of *Brucella* in veterinary clinics. For establishment of successful control and eradication programs, reliable diagnostic techniques are needed for surveillance and investigation of disease outbreaks. Current bacterial isolation procedures and identification assays are time-consuming, require experienced technicians and put the laboratory personnel at risk [13]. Furthermore, current assays are affected by the number of viable bacteria in the samples and may be influenced by other bacterial and fungal contaminants [14]. Recombinase polymerase amplification (RPA) is an isothermal amplification method performed at temperatures between 37 and 42 °C. The assay can rapidly detect target nucleic acids without the need for sophisticated laboratory equipment. It can detect very low concentrations of pathogen-specific nucleic acid within 20 min [15].

RPA has been developed for the diagnosis of several biohazardous organisms such as *Francisella tularensis*, *Yersinia pestis*, *Bacillus anthracis*, and variola virus, while reverse transcriptase RPA (RT-RPA) assays have been developed to detect Rift Valley fever virus, Ebola virus, Sudan virus, and Marburg virus [16]. In addition, RPA has been developed to detect *Brucella spp.* in point-of-care diagnosis assays [17]. Additionally, the lateral-flow RPA has been developed to detect several pathogens. It is considered as a rapid and sensitive technique. This type of RPA does not need sophisticated instruments. Using lateral flow, dipstick strips could evaluate the sample after an incubation period of 10–30 min at fixed temperatures [18]. In this study, Real-time and Lateral Flow Dipsticks was used to develop an, RPA assay for the rapid detection of *Brucella spp.*

2. Materials and methods

2.1. Samples collection

126 samples which included vaginal swabs from sheep, aborted sheep and yak fetuses tissue samples, sheep blood, and cattle milk were analyzed using real-time RPA (Table 1). Parallel to vaginal swabs 45 serum samples were collected from same sheep and tested by RBPT (Table 2).

For LFD-RPA, 90 samples which included tissue samples from sheep and cattle aborted fetuses, milk samples from cattle and vaginal swabs from sheep were collected and analyzed (Table 3). The samples were collected aseptically and properly labelled.

2.2. DNA extraction of bacterial strains and samples

DNA was extracted using the universal genomic DNA extraction kit Takara Mini BEST universal genomic DNA extraction kit (TAKARA Clontech, Shiga, Japan). The extraction procedure was performed according to the manufacturer's instructions. DNA samples were stored at -20^{O}C until analyzed.

2.3. Preparation of positive control plasmid DNA for real-time RPA assay

An aliquot of *Brucella spp.* bp26 gene product was used for Real-time RPA and PCR amplification. The reaction volume was 50 µl and primer sequence (cPCR-BP26primer) used are listed in Table .4. The PCR reaction was denatured at 94 °C for 5 min and then underwent 35 cycles of 94 °C denaturation for 1 min, 53.4 °C annealing for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 1 min. The bands were visualized using ultra-violet light *trans*-illuminator (BIORAD, Hercules, California, USA). The bands were then excised, and DNA was extracted using the Axy Prep DNA Gel Extraction kit, (Axygen Bioscience, California, USA). The amplified DNA portion of bp26 sequence was ligated to the pMD-18 plasmid vector and then transformed to DH5α *Escherichia coli* competent cells. *Plasmid* DNA was extracted using the TIAN prep Mini Plasmid kit, (Tiangen Biotech, Beijing, China). Plasmid DNA was quantified by Nano-drop spectrophotometry (Infinite 200 PRO, TECAN, Groedig, Austria), and then the copy number of plasmid molecules was calculated using equation: number of copies = (amount * 6.022×10^{23})/(length * 1×10^{9} * 650). The constructed plasmid were retransformed into DH5α *E. coli* and incubated in one ml of LB broth at 37 °C for 45 min in a shaker incubator. Afterwards, the bacterial culture was diluted 10-fold in LB broth, and 100 µl of each dilution poured onto LB agar plates containing ampicillin. The plates were incubated at 37 °C for 24 h and the next day colonies were counted for each dilution. Plasmids were extracted from each dilution.

2.4. Preparation of positive control plasmid DNA for LFD- RPA assay

A part of *IS711* sequence product was used for LFD-RPA-IS711 and cPCR-IS711 amplification. The PCR reaction conditions as same as mentioned above but with different annealing temperature which was 49.5 °C. PCR primer pair (*cPCR-IS711* primer) was used (Table 4). The sequence was ligated to pMD-19 vector and the subsequent procedure as mentioned above in 2.3.

Table 1
Field samples included in this work and results obtained in RT-RPA-BP26, qPCR-IS711, and cPCR-BP26.

Animal species	Sample type	Number of samples	RT-RPA-BP26		qPCR-IS711		cPCR-BP26	
			P	N	P	N	P	N
Sheep	Vaginal swabs	45	30	15	28	17	27	18
	lung	3	3	0	2	1	3	0
	Intestine	1	1	0	1	0	1	0
	Liver	6	6	0	6	0	6	0
	Spleen	37	2	35	1	36	4	33
	kidney	4	4	0	4	0	4	0
	stomach content	2	2	0	2	0	2	0
	Blood	14	0	14	1	13	0	14
Yak	Liver	1	1	0	1	0	1	0
	Lung	1	1	0	1	0	1	0
	Spleen	1	1	0	1	0	1	0
	Heart	1	1	0	1	0	1	0
	Stomach content	1	1	0	1	0	1	0
	lymph node	1	1	0	1	0	1	0
	Intestine	1	1	0	1	0	1	0
	Kidney	1	1	0	1	0	1	0
Cattle	Milk	6	2	4	2	4	2	4
Total		**126**	**58 (46.0%)**	**68 (54.0%)**	**55 (43.7%)**	**71 (56.3%)**	**57 (45.2%)**	**69 (54.8%)**

P: positive, N: negative.

Table 2
Agreement (Kappa values) between the real-time RPA, real-time PCR, PCR, and RBPT.

(Test 1/Test2)	+/+	+/−	−/+	−/−	Kappa CI 95%	Total
RT- RPA-BP26/RBPT	25	3	0	17	0.8629 (0.7145–1.0114)	45
q PCR-IS711/RBPT	25	3	0	17	0.8629 (0.7145–1.0114)	45
cPCR-BP26/RBPT	25	2	0	18	0.9091(0.7864–1.0317)	45

CI: confidence interval.

2.5. RT-RPA-BP26Candidate primer pair and probe design

For RT-RPA-BP26 primer pair and probe were designed using the *Brucella spp.* bp26 gene (Genbank accession number AF242534.1). The primers and probes were designed using the Twist DX manual [15]. RPA primer length is 30–35 base pair, GC content between 30 and 60%. TwistAmp exo probe has used in Real-time RPA, which should contain at least 30 nucleotide at 5′ end followed by nucleotide analogue Tetrahydrofuran (THF) flanked by a dT-fluorophore (FAM) and a corresponding dT-quencher (BHQ) group followed by 15 nucleotide located at 3' and blocked by Phosphate to block polymerase extension (Table 4). All primers and probes were manufactured by (Sangon Biotech, CO, LTD, Shanghai, China).

2.6. Lateral flow dipstick candidate primer pair and probe design

With regards to LFD RPA, the primer and probe were designed as outlined in Twist DX manual [15], using oligo 6 software (Version 6.31 Molecular biology insight, Inc. USA). The insertion sequence *IS711* region of bp26 gene of *Brucella spp.* (Genbank accession number AF242534.1) has used as target sequence for primer pair and probe design. LFD-RPA primers characteristics are 30–35 nucleotides length, but differ by presence of biotin bound to 5′ end of reverse primer. TwistAmp LF Probe oligonucleotide backbone includes an antigenic label FAM group at 5' end, an internal a basic nucleotide analogue THF spacer and a 3′-polymerase extension blocking group C3-spacer (Table 4). Primer pair and probe were manufactured by (Sangon Biotech, CO, LTD, Shanghai, China).

2.7. Real-time RPA assay

RPA was performed in a total volume of 50 μl using Twist Amp Exo kit (Twist Dx, Cambridge, United Kingdom). The master mix was prepared using 29.5 μL of TwistAmp rehydration buffer, 12.2 μl RNase free water (TAKARA Clontech, Shiga, Japan), 2.1 μl (10 μM) primers, and 0.6 μl (10 μM) probe. The master-mix (45.5 μl) was then added to freeze-dried enzyme pellets in 0.2 ml reaction tubes and mixed by gentle pipetting. 1 μl of DNA template was added to each 0.2 ml tube. 2.5 μl of 280 mM magnesium acetate was then added to the tubes lids and directly centrifuged into reaction tubes. The tubes were then incubated at optimum temperature for 20 min using real-time PCR thermocycler (Agilent Technologies Stratagene Mx3005 P, California, USA). Threshold time by minutes and fluorescence intensity values has recorded by real-time PCR thermocycler. In Real-time RPA reaction, primers and probe will anneal to target sequence, and then the probe will be cleaved by exonuclease III at THF spacer, leading to separation of fluorophore (FAM) from its quencher (BHQ) and generating fluorescence signals. The fluorescence generation can be detected by Real-time PCR thermocycler and exhibited by real-time thermocycler software as a plotting curves with threshold times and fluorescence intensity, as same as in real-time PCR. In RPA positive sample generate plotting curve which cross threshold, but the negative doesn't generate plotting curve [19]. The amplification was measured using threshold time expressed as minutes and fluorescence intensity.

2.8. Optimum temperature of RT-RPA-BP26

For optimal temperature selection, RPA was performed as mentioned above with candidate primer pair and probe. DNA of *Brucella suis* S2 was used as positive control and RNase free was used as negative control. The tubes were then incubated at 37 °C, 38 °C, 39 °C, and 40 °C for 20 min using a real-time PCR thermocycler (Agilent Technologies Stratagene Mx3005 P, California, USA) to monitor amplification every 20 s.

2.9. Lateral flow dipstick- RPA assay (LFD-RPA-IS711))

LFD-RPA-IS711 was performed in a 50 μl volume using a Twist Amp nfo kit (Twist DX, Cambridge, UK). 2.1 μl (10 μM) of RPA primers, 0.6 μl (10 μM) of nfo probe, 2.5 μl (280 mM) magnesium acetate, 29.5 μl of TwistAmp 1 × rehydration buffer and 11.2 μl of RNase-free water. All reagents, except for the DNA template and magnesium acetate was added to the master mix in 1.5 ml tube. The prepared master mix was then aliquoted into 0.2 ml reaction tubes containing the

Table 3
Performances of LFD-RPA-IS711, qPCR-IS711, and cPCR-IS711 assays for detection of suspected *Brucella spp.* infectious field samples.

Species	Sample type	Number of samples	LFD-RPA-IS711		qPCR-IS711		cPCR-IS711	
			P	**N**	**P**	**N**	**P**	**N**
Cattle	Liver	5	4	1	5	0	4	1
	spleen	1	1	0	1	0	1	0
	intestine	3	3	0	3	0	3	0
	lung	6	6	0	5	1	4	2
	stomach	3	3	0	3	0	3	0
	Milk	6	2	4	2	4	2	4
Sheep	Lung	4	4	0	4	0	4	0
	Liver	9	8	1	7	2	7	2
	Spleen	3	3	0	3	0	3	0
	Intestine	2	2	0	2	0	2	0
	Stomach	4	4	0	4	0	4	0
	Kidney	3	2	1	2	1	2	1
	Heart	1	1	0	1	0	1	0
	Vaginal swabs	40	33	7	35	5	36	4
Total		90	**76 (84.4%)**	**14 (16.6%)**	**77 (86.6%)**	**13 (14.4%)**	**76 (84.4%)**	**14 (16.6%)**

Table 4
Primers pairs and probes used in RPA, real-time PCR and PCR.

Primers	Primers and probe sequence (5–3)	Product size (bp)	Reference
RT-RPA-BP26 primers and probe of bp26 gene:	FP: CAATGTTGGAAAAATTTTGGATGAATCCGT RP: TTACTTGATTTCAAAAACGACATTGACCGATA Probe: 5-GGCGGTGATTTGAACCTGGTCAATGATAA(FAM-dt)C(THF)C(BHQ) CCGCCGTGATCAAC-3P.	340	This work
LFD-RPA-IS711primers and probe of IS711:	FP: TCA ATG TTT TCT CGC ATC GCA GCA CAA TAC RP: Biotin-GCA ATC TCA AGG CAA CGG CTC AGA TCA AGG Probe: FAM- AAC TTG TTA AAA AAG CAT TCA ATC TGA TGG-THF- GTT CCT TGT ACA GCC-P	159	This work
cPCR-BP26primers:	FP: ATCCACCAGTCTCACGGTTC. RP: GGCGTCATACCCCAGCTAT.	395	This work
cPCR-IS711 primers:	FP-GATTAGGGCGTGTCTGCATT RP-TCATTGCCAGCAATCTCAAG	240	
qPCR-IS711 primers and probe:	FP: IS241: CGCTCGCGCGGTGGAT. RP: IS511: CTTGAAGCTTGCGGACAGTCACC. ISTq: FAM-ACGACCAAGCTGCATGCTGTTGTCGATG-TAMARA.	178	Bounaadja et al. (2009)

dried enzyme pellet. 2 µl of DNA template was then added to the reaction tube. Magnesium acetate was then added into the tubes lids, and centrifuged briefly into the tubes. The tubes were incubated at optimum temperature for suitable reaction time immediately. After incubation, RPA products were analyzed by lateral flow strips and the RPA products were purified using TIANquick Midi Purification Kit (TIANGEN Biotech, Beijing, China) and analyzed by electrophoresis using 1% agarose gel.

2.10. Optimum temperature and incubation time of LFD-RPA-IS711

To determine the optimum amplification temperature, the RPA reactions were carried out on a heat block (Tiangen Biotech, Beijing, China) set at 30, 37, 39 °C and 45 °C using 8 ng/µl of plasmid DNA as a positive control template. The subsequent RPA products were analyzed by lateral flow dipstick strips and then purified using PCR purification kit (Tiangen Biotech, Beijing, China), and then analyzed by electrophoresis using a 1% agarose gel. To define the optimum incubation time for amplification, the reactions were performed at the specified temperature for 5, 10, 20 and 30 min using plasmid DNA as templates followed by lateral flow dipstick analysis and product purification and agarose gel electrophoresis. All purified products have been sequenced using Sanger dideoxy technology to confirm amplification specificity (Appendix D).

2.11. Endpoint detection by lateral flow dipstick (LFD) and SYBR green assays

During LFD-RPA reaction, after primers and probe annealing to specific sequence, the bound probe is cleaved by the nfo endonucleases IV at the tetrahydrofuran (THF) to generate an extensible 3′-OH group for polymerization. The DNA polymerases extend and displace from 3′-ends of the primers and cleaved probe to produce the minor amplicons (from the forward and reverse primers) and a displaced strand. The displaced strand combines with the labelled reverse primer, and leads to the production of a dual-labelled amplicon bound with FAM at 5′ end and Biotin at 3'end (the major amplicon) also called FAM-Biotin linking amplicons [20]. Lateral flow dipstick strips are made of permeable membrane. It has sample pad to dip in diluted RPA product with appropriate buffer, this pad contains gold nanoparticles ligated with Anti-FAM antibodies. Upper to sample pad there is a line contains another immobilized antibody captures biotin, this forming the test line, and after the test line there is control line contain immobilized anti-species antibodies captures that antibodies ligated to gold nanoparticles. In positive reactions anti-FAM antibodies will bind to FAM in dual labelled amplicons, and then diffuse to test band line. In the test band line anti-biotin will bind biotin and form red line. The remaining gold Nanoparticles will diffuse to control line to be captured by anti-species antibodies and form another red control line. In the positive result, test and control lines will be visible in LF-strip. In negative reactions the double-labelled products are absent, and will not bin with anti-FAM and anti-biotin in test line, and gold nanoparticles will diffuse to control line to form red band only in control line [19]. To detect RPA amplification by LFD, 1 µl of RPA product was added to 99 µl of assay buffer provided in the kit (Milenia Biotec, Giessen, Germany) into a new 1.5 ml tube. Then, the LFD strip (Milenia Biotec, Giessen, Germany) was dipped into 100 µl running buffer for 2 min to visualize the test result. Positive result distinguished by the presence of test line and control line, while in the negative result only control line is visible. The entire LFD assay was performed at room temperature. *2.12. Analytical sensitivity and detection limit of Real-time RPA and LFD-RPA:*

To test the analytical sensitivity of Real-time RPA, the constructed plasmid was diluted 10-fold using plasmid DNA kit elution buffer ranging from 4.56×10^7 and 4.56×10^0 copy number per reaction and then tested by real-time RPA. Every run was repeated three times. The sensitivity of Real-time RPA was further evaluated by testing each plasmid extracted from *E. coli* bearing constructed plasmid (counted to be 6×10^2 to 3 colony forming units). Five independent runs were performed. To determine analytical sensitivity of LFD-RPA 10-fold dilutions of plasmid ranging from 6×10^5 to 6 copy number and then tested by LFD-RPA, and then plasmid extracted from *E. coli* bearing constructed plasmid which ranging from 6×10^2 to 6 colony forming units. Every run was performed separately three times.

2.12. Analytical specificity of real-time RPA and LFD-RPA

Reference DNA samples from *B. melitensis*, *B. suis* and *B. abortus* were analyzed by Real-time and LFD-RPA. In addition DNA samples from *Chlamydia abortus* field strain and vaccine strain, *Salmonella typhimurium* and *Toxoplasma gondii* and *Escherichia coli* were analyzed by RPA using the optimal conditions identified.

2.13. Real-time PCR

Real-time PCR was used to analyzed the samples using primer and probe sequences obtained from a previously published protocol [10]. The reaction consisted of 10 µl Taqman master mix (AceQs qPCR Probe Master Mix, Vanzyme, Nanjing, China), 0.4 µl (10 µM) each forward and reverse primers, 0.2 µl (10 µM) of Probe, 2 µl of DNA template and 7 µl of RNase free water for a total volume of 20 µl. The PCR conditions were 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Real-time PCR was performed using real-time PCR machine (BIORAD, Hercules, California, USA).

Table 5
Different organisms tested for analytical specificity of RPA.

Bacterial species	Source	RPA detection	Ct/min
B. melitensis biovar3	LVRI	+	18.7
B. melitensis biovar3	LVRI	+	15.8
B. melitensis biovar3	LVRI	+	9.8
B. melitensis biovar 3	LVRI	+	15.9
B. suis S2 vaccine	LVRI	+	10.5
B. abortus	LVRI	+	13.85
B. abortus S19	LVRI	+	8.5
Chlamydia abortus	LVRI	–	No ct
Chlamydia abortus vaccine strain	LVRI	–	No ct
Toxoplasma gondii	LVRI	–	No ct
Salmonella typhimurium	LVRI	–	No ct
E. coli	LVRI	–	No ct

-LVRI: Lanzhou Veterinary Research Institute.

2.14. Polymerase chain reaction (PCR)

Two PCR primer pairs were designed using Primer3 software (Table 1). One targeting partial sequence of bp26 gene sequence used to analyze field samples in Table 3 and compared to Real-time RPA. Another PCR targeting portion of insertion sequence *IS711* and used to analyze field samples in Table 5 to be compared with LFD-RPA. These two primer pairs also used in PCR reaction mentioned before in paragraphs 2.3.1 and 2.3.2 respectively. Two PCRs reaction mix used to screen field samples consisted of 12.5 μl Taqman master mix (TAKARA Clontech, Shiga, Japan), 10.5 μl RNase free water, 0.5 μl (1 μM) of forward and reverse primers and 1 μl of DNA template. The reaction conditions were an initial denaturation of 94 °C for 5 min followed by 35 cycles of 94 °C denaturation for 1 min, 53.4 °C (bp 26 primers) and 49.5 °C (IS711 primers) annealing for 1 min and 72 °C extension for 1 min. The final extension was performed at 72 °C for 1min. PCR products were then loaded and run on a 1% agarose gel and then stained with Gel Red stain. Bands were visualized by UV light using the gel documentation system (BIORAD, Hercules, California, USA).

2.15. Rose Bengal Plate test (RBPT)

RBPT was used to analyze forty-five serum samples. Briefly, 30 μl of serum was added to 30 μl of RBPT antigen (Yebio Bioengineering, Qingdao, Shandong, China) and then mixed thoroughly and agitated gently for 4 min. Clump formation denoted the presence of antibodies [13].

2.16. Statistical analysis

Microsoft Excel software was used to perform regression analysis of cycle thresholds of Real time against plasmid copy number and colony forming units to evaluate reproducibility. In addition, the software was used to analyze the results of RPA, PCR, and real-time PCR from field samples. The agreement and differences between the results obtained by RPA, Real-time PCR and PCR were determined using Kappa statistics and chi-square. Calculations were performed using the website calculator: Sergeant, ESG, 2019. Epitools epidemiological calculators. Ausvet Pty Ltd. Available at: http://epitools.ausvet.com.au.

3. Results and discussion

In this study real-time RPA and LFD-RPA were developed, and optimized to detect *Brucella spp.* in different animal samples. The target sequences selected for amplification were *Brucella spp.* bp26 for Real-time RPA amplification and *IS711* for LFD-RPA amplification. These target sequences are conserved between the different species of *Brucella* and are generally used for PCR target gene assays [21].

3.1. Optimum conditions of real-time RPA assay

The reaction temperatures for Real-time RPA had cycle thresholds values of 11.95, 7.16, 5.25, and 4.35 threshold times per minute at 37 °C, 38 °C, 39 °C and 40 °C respectively using same DNA template. The increase in reaction temperature increase reaction velocity of RPA to produce more products detected by fluorescence signal, this signal is detected at short time and demonstrated by low threshold time. Optimal conditions for Real-time RPA were determined to be 40 °C with 20 min incubation.

The optimal temperature range of RPA is between 37°C and 42 °C. In developed Real-time RPA for detection of *Campylobacter coli* and *Campylobacter jejuni,* which performed at a temperature gradient from 35 to 51 °C., the lowest temperature 35 °C produced the highest Ct values from the two targets and the Ct values were reduced as the reaction temperature was increased up to 45 °C, but at 49 °C. The amplifications were retarded and the amplification curves were not detected at 51 °C [22]. The reaction velocity increase with reaction temperature, at low temperature the reaction rate will be reduced, and in very high temperature the reaction will because the enzymatic system of reaction will be compromised [19,23]. Many RPA reactions were inhibited at reaction temperature of 50 °C [22,24,25].

3.2. Optimum conditions of LFD- RPA-IS711 assay

For optimization of LFD-RPA-IS711 incubation temperatures, we found temperature ranges from 30 to 45 were satisfactory Positive results in lateral flow strips were visualized by the presence of control and test lines, and the presence of specific gel bands in the gel (Appendix A Fig. S1C). The optimal temperature was between 37 and 39 °C and had the greatest gel band intensity compared to 30 °C and 45 °C. For optimization of incubation times, 5 min was not sufficient for band detection, while incubation times of 10–30 min produced detectable bands, with incubation times of 20 and 30 min being optimal (Appendix A Fig. S1D). The optimal incubation time was 20 min. The selected incubation temperature for LFD-RPA was set at 37 °C with 20 min of incubation time.

The optimized RPA assay demonstrated that amplification could occur at temperature ranges from 30 °C to 45 °C and incubation times ranging from 10 min to 30 min. This agrees with previous results obtained during the optimization of lateral flow RPA assays for the detection of goatpox virus, sheeppox virus, and ORF virus, bovine ephemeral fever and *Schistosoma hematobium* [25–28].

3.3. Analytical sensitivity, detection limits and reproducibility of real time RPA

Real-time RPA sensitivity and detection limits were determined by serial dilution of plasmid DNA copies between 4.56×10^7 and 4.56×10^0. The test detected 4.56×10^0 copies of the plasmid (Appendix C; Table S2). RPA was able to detect plasmids extracted from 6×10^2 CFU, 6×10 CFU, and six CFUs, however 3 CFU and non-template control (NTC) did not detected (Appendix B; Table S1) (Appendix A Fig. S2). The reproducibility of the assay is shown in Appendix A (Fig. S3 A and B) and Appendix B (Tables S1 and S2). Regression analysis showed that the coefficient of determination (R^2) was 0.8821 of the serially diluted plasmid and its respective time threshold values by RT-RPA-BP26. The mean R^2 of the colony forming units and RPA threshold time values was 0.9892, exhibiting good correlation between the starting templates and threshold time values. R^2 values demonstrated that the efficiency of the assay was the same for different starting template copy numbers.

The developed Real-time RPA assay demonstrated high analytical sensitivity. RPA assays have been developed for the detection of several bacterial pathogens such as Methicillin-resistant *Staphylococcus aureus, Francisella tularensis*, *Bacillus anthracis, Yersinia pestis* and *Mycobacterium*

tuberculosis [16,29,30]. The test can detect copy numbers of less than 10 copies within 7–30 min [19,26,31]. RPA could also detect five color-changing units (CCU ml) and 50 genome copies of *Mycoplasma capricolum subsp. capripneumonae* [32]. Sensitive and specific RPAs have also been developed and optimized for the detection of several pathogens such as *Leptospira spp. also, Listeria monocytogenes,* and other viral and fungal pathogens [33]. Regression analysis of threshold -times against DNA template copy numbers of several Real-time RPA assays, showed good correlation expressed as R^2 values between 0.8 and 0.9 [26,34].

3.4. Analytical sensitivity and detection limit of LFD-RPA

The detection limit of the developed LFD- RPA was six colony forming units (CFU) of *E. coli* bearing the constructed plasmid at 37 °C in 20 min, visualized on lateral flow strips and specific gel bands in agarose gel (Appendix A Fig. S4). For the 10-fold serial dilutions of the constructed plasmid, the detection limit of LFD-RPA was 6 copies of the constructed plasmid as visualized by the presence of the control and test lines in lateral flow strips and presence of green color of SYBR-green I in the RPA product. However for the non-template control, the control line was only observed and the color of SYBR-green I did not change (Appendix A Fig. S5).

The developed lateral flow RPA for the detection of *Borrelia burgdorferi* had analytical sensitivity of one copy of constructed plasmid and 10 fg of DNA [35]. The sensitive LFD-RPA developed for detection of ORF virus with detection limit of 80 copies per reaction [27]. The developed LFD-RPA for detection of urogenital schistosomiasis has ability to detect 100 fg of DNA in 10 min [28].

3.5. Analytical specificity of RT-RPA-BP26 and LFD-RPA-IS711

The reference DNA samples of the different *Brucella spp.* were *B. melitensis, B. abortus,* and *B. suis* gave cycle threshold values in real-time RPA. The amplification reaction demonstrated no cross-reactivity with *Chlamydia abortus*, field and vaccine strain, *Toxoplasma gondii*, *Salmonella typhimurium,* and *E. coli* which did not produce cycle threshold values in RPA (Table 5). LFD-RPA-IS711 could detect *B. abortus*, *B. melitensis* and *B. suis,* distinguished by visual control and test lines in lateral flow strips and presence of specific gel band in 1% agarose gel. Negative results were obtained for *Chlamydia abortus, E. coli, Salmonella typhimurium and Toxoplasma gondii*, as visualized with obvious control lines in lateral flow strips and absence of test line. This demonstrated specificity of this assay (Appendix A Fig. S6).

The two developed RPA assays showed specificity for *Brucella spp*. with, no DNA samples being amplified from other organisms such as *Chlamydia abortus, Salmonella typhimurium, E. coli, and Toxoplasma gondii*. Several Real-time and LFD-RPA assays showed high specificity for detection of target pathogens and doesn't cross-react with other pathogens with the same clinical signs [36]. The specificity of RPA assays due to use of specific probes like Real-time PCR [10].

3.6. Screening of field samples by RT-RPA-BP26, qPCR-IS711, and cPCR-BP26

The results of the screening of the different field samples are presented in Table 3 and Appendix C: Table S1 of the 126 samples tested, 58 (46.0%), 55 (43.7%) and 57 (45.2%) were positive by RT-RPA-BP26, qPCR-IS711, and cPCR-BP26 respectively (Table 3 and Appendix C: Table S1). Kappa statistics analysis of the results of three tests showed almost perfect agreement between RT-RPA-BP26 and qPCR-IS711was 0.9032 (0.8276–0.9788) at confidence interval (CI) 95% and RT-RPA-BP26and cPCR-BP26 was 0.968 (0.9239–1.012) at CI 95%. There was no difference observed between RT-RPA-BP26 and qPCR-IS711and RT-RPA-BP26 and cPCR-BP26 ($p = 0.6831$ and 0.4795 respectively). The RT-RPA-BP26assay worked equally well as qPCR-IS711and cPCR-BP26 for field samples.

Real-time RPA assays have been developed for the diagnosis of sheep and goat pox and Peste des Petits Ruminants virus were found to have similar sensitivity compared to real-time PCR [26,36]. Real-time PCR is faster and more sensitive compared to conventional PCR because it does not require post-amplification handling of PCR products. It also reduces the risk of laboratory contamination and false positive results. RPA assay time is about 20 min, and is shorter compared to PCR and real-time PCR. Real-time RPA could be performed using a small tube scanner(such as Twista) for fluorescence detection instead of qPCR thermocycler, and hence easily handled and used in the field. However, PCR and real-time PCR require sophisticated instruments and are not possible to be used in the field. In our study qPCR thermocycler has used for Real-time RPA incubation because of its availability in the lab.

3.7. Comparison of RT-RPA-BP26, qPCR-IS711, cPCR-BP26 and Rose Bengal Plate test (RBPT)

For the 45 vaginal ewe swabs collected; 28(62.2%), 28 (62.2%), and 27 (60.0%) were positive by RT-RPA-BP26, qPCR-IS711and cPCR-BP26respectively. However, RBPT was only 25 (55.6%) positive in serum samples obtained from the same animals. RPA can detect more positive samples of *Brucella* DNA in negative animals examined by RBPT Table 2. There was high agreement between RT-RPA-BP26 and RBPT (Kappa value = 0.8629).

Serological tests like Rose Bengal Plate test, ELISA and CFT are considered high sensitivity screening methods for brucellosis and have been accredited by international trading organizations. They have been used to test herds/flocks for prevalence, animal infection and used in programs for eradication. However, these methods lack a certain amount of specificity. They can detect antibodies for other cross-reacting bacteria such as *Yersinia enterocolitica* O: 9 and *E. coli* O: 157 [5]. The results from Rose Bengal Plate test from sera and RT RPA-BP26 from vaginal swabs from the same ewes showed that the prevalence is higher byRT RPA-BP26 in vaginal swabs. Hence, Real-time RPA could be used to confirm infection and fill the diagnostic gap of serological tests. Bp26 based PCR was compared to RBPT for detection of *B. melitensis* infection in slaughtered goats. Among 138 animals, 23 (16.6%) were positive to bp26 gene PCR from tissue samples, whereas 15 (10.8%) were positive to RBPT from serum samples [21]. Due to limitations in culture techniques and serologic tests, various molecular methods like PCR and real-time PCR have been developed for the for diagnosis of brucellosis in human and animals [6].

3.8. Performances of LFD-RPA-IS711, qPCR-IS711, and cPCR-IS711 assays for detection of Brucella spp. In field samples

Among the 90 samples tested, 76 (84.4%), 77 (86.6%) and 76 (84.4%) were positive by LFD-RPA-IS711, qPCR-IS711, and cPCR-IS711respectively (Table 5, and Appendix C). There were no significant difference between LFD-RPA-IS711, qPCR-IS711 and cPCR-IS711 ($p = 0.4795$ and $p = 0$ respectively) (Appendix C: Tables S2, S3, and S4) and with almost perfect agreement between LFD-RPA-IS711 and qPCR-IS711 and RPA and cPCR-IS711 (kappa 0.9154 (0.7997–1.0311) at CI 95% and 1.00 (1.00–1.00) at CI 95% respectively). Kappa statistics analysis demonstrated that LFD-RPA-IS711 performed similar to qPCR-IS711 and cPCR-IS711.

Several LFD-RPA assays were developed and showed same performances compared to Real-time PCR, such as LFD-RPA developed for detection of ORF virus, Sheep pox and Goat pox virus, and bovine Corona virus [27,36,37]. The LFD-RPA developed for detection of bovine ephemeral fever was compared with Real-time PCR in 128 samples, the positive samples detected by RPA and Real-time PCR were (96/128)75%, (95/128) 74.22%, respectively [25].

. The results from RT- RPA-BP26 and LFD-RPA-IS711 are promising, however the assays need to be further evaluated and validated using larger sample number of field samples, and with gold standard positive

isolated *Brucella spp*. and negative controls from *Brucella* free herds. In addition, these samples should be from different species. The detection window of two assays should be determined by experimental models. This work is required before its used in routine laboratory and field testing for brucellosis.

4. Conclusions

Real-time RPA and LFD RPA are sensitive and specific techniques for diagnosing animal brucellosis. The diagnostic capabilities of theses assays were acceptable. Real-time and LFD-RPAs gave similar results as Real time PCR for field samples. However, the assays need to be validated by screening larger sample numbers. Despite the high cost of the test kits, this technique could be used in field and point of care with a short turn-around time and could be performed in laboratories that lack specialized instruments. In addition, the kits and instruments to perform RPA could be easily transported and used in the field as well as in mobile veterinary clinics. However, RPA reagents are expensive and are only produced by a single manufacturer [38]. Reducing the cost of the reagents will help increase the availability of the RPA kit to testing centers and veterinary clinics with limited budgets.

Author contributions

Gumaa M. M., Xiaoan Cao, Zhizhang Zhou, and Baoquan Fu designed the study and writing paper. Gumaa M. M., Performed all experiments and Zhijun Zhang participated in RPA experiment. Zhaocai Li in PCR, real-time PCR and results analysis. Zhongzi Lou participated in collection and preparation of samples. Ninazhang Zhang participated in editing and proofreading.

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5. 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).Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcp.2019.101434.

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