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Specific detection of *Brucella* spp. from slaughter-aged livestock using a dual priming oligonucleotide system

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Abstract

Background *Brucella* spp. are Gram-negative bacteria causing brucellosis, a major zoonotic disease affecting animals and humans. Annually, over 500,000 human cases are reported globally, with many undiagnosed due to nonspecific symptoms and diagnostic challenges. Current methods for *Brucella* detection, such as culture and serology, are time-consuming and lack specificity, hindering effective disease control. This study aims to develop a novel dual priming oligonucleotide (DPO) system-based PCR method for the specific detection of *Brucella* spp. in slaughter-aged livestock. This approach provides a rapid, sensitive, and field-deployable tool to improve early diagnosis and control of brucellosis.

Methods We developed a DPO system-based PCR assay for the specific detection of *Brucella* spp. in slaughter-aged livestock. The method utilizes two sets of primers designed to specifically target unique regions of the *Brucella* genome. The assay was validated for specificity using a panel of 15 non-target bacterial species commonly found in livestock, including *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. Sensitivity was evaluated using DNA extracted from a range of *Brucella* strains, with detection limits assessed using serially diluted samples. The assay's performance was further tested on 500 samples from slaughter-aged sheep to assess its applicability in field conditions.

Results The DPO-based PCR assay demonstrated excellent specificity, with no cross-reactivity observed in any of the 15 non-target bacterial species tested. The assay was able to detect *Brucella* spp. at low DNA concentrations, with a sensitivity limit of approximately 5.3×10^1 CFU/mL of the *Brucella* per reaction. In the field validation, 500 samples from slaughter-aged sheep were tested, and the assay successfully identified *Brucella* infections in animals

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with no false positives or negatives. When compared to conventional PCR, the DPO-PCR method exhibited improved specificity and faster results, with a significantly reduced time to diagnosis.

Conclusions The DPO-based PCR assay provides a highly specific, rapid, and cost-effective tool for the detection of *Brucella* spp. in slaughter-aged livestock. This method is suitable for routine surveillance in slaughterhouses, offering a promising solution for early detection and control of brucellosis in both livestock and public health contexts. The assay's simplicity and robustness make it an ideal candidate for field deployment, particularly in resource-limited settings where timely disease control is crucial.

Clinical trial number Not applicable

Keywords *Brucella* spp, *Omp25*, DPO-based PCR assay, Bacteria detection

Introduction

Brucella species, non-motile, aerobic, Gram-negative cocco-bacilli, are the causative agents of brucellosis, a zoonotic infection with significant implications for both human and animal health [1, 2]. This highly contagious disease is a global public health concern, with the World Health Organization estimating 500,000 new cases annually [3]. Human infections typically occur through direct contact with infected tissues, inhalation of aerosols, or consumption of unpasteurized dairy products [4–6]. Given the serious threat posed by brucellosis, the development of an accurate and specific diagnostic assay for *Brucella* spp. is essential.

Traditional laboratory diagnosis of brucellosis has been based on culture and isolation of the bacteria or serological tests for anti-*Brucella* antibodies [7, 8]. While culture-based assays are considered the "gold standard," they are laborious and time-consuming [9]. Serological tests, on the other hand, struggle with differentiating between *Brucella* spp [10]. To address these limitations, molecular diagnostic techniques, especially PCR-based assays, have been increasingly adopted [11–14]. For instance, the AMOS-PCR assay has shown promise in detecting *Brucella* spp. from impure culture isolates, although it has its own set of limitations, such as the complexity of multiple PCR reactions, risk of contamination, and potential for false positives due to non-specific amplification [15–17].

The Dual Priming Oligonucleotide (DPO) system represents a novel approach to PCR primer design, offering a simpler and more efficient alternative to traditional methods. The DPO primer is characterized by a longer 5'-segment, a shorter 3'-segment, and a polydeoxyinosine (poly I) linker, which together enhance specificity and allow for a broader range of annealing temperatures. This system has been successfully applied to detect a wide range of foodborne pathogens in various studies. For example, a DPO-based multiplex PCR assay was developed for the simultaneous detection of *Salmonella* spp., *Listeria monocytogenes*, *Shigella* spp., *Staphylococcus aureus*, *Campylobacter jejuni*, and *Yersinia enterocolitica* from food samples [18]. The assay demonstrated high specificity, amplifying only the target genes in the presence of 238 target and 83 non-target bacterial strains, with an analytical detection limit of 10^2-10^3 CFU/mL. The successful application of the DPO-PCR system to detect multiple pathogens in food matrices underscores the versatility and reliability of this system for diagnostic purposes. Given its success in detecting various pathogens, the DPO-PCR system is ideally suited for *Brucella* spp. detection due to its high specificity, reduced cross-reactivity, and ability to handle complex sample matrices, such as those encountered in livestock and food samples. The economic and health implications of misdiagnosis or delayed diagnosis of *Brucella* spp. are significant. Misdiagnosis or delayed detection can lead to the spread of the disease among livestock populations, resulting in costly outbreaks that affect animal health, food safety, and the economy. Brucellosis also has serious public health implications, as it is a zoonotic disease that can cause long-term chronic illness in humans, leading to prolonged treatment and increased healthcare costs. Early and accurate diagnosis is essential not only for controlling the disease within animal populations but also for preventing human infections. In particular, the use of a reliable and rapid diagnostic tool can reduce the time to diagnosis, limit disease transmission, and mitigate the broader socio-economic impact.

Similarly, this study introduces a DPO-based PCR assay for the specific detection of *Brucella* spp. in slaughter-aged livestock, targeting the *Brucella Omp25* gene, with the aim of overcoming the limitations of existing diagnostic methods. It is reported that no DPO-PCR method has been employed for the detection of *Brucella* spp. to date, highlighting the novelty and potential impact of this research.

Methods

Bacterial strains

In the present study, 24 bacterial strains were used to evaluate the specificity of the DPO-based PCR assay (Table 1). Bacterial strains, including *Brucella* spp., *Campylobacter*, *Vibrio*, and *Lactobacillus*, were cultured under conditions optimized in our previous study, using

Table 1 The bacterial strains used in the study

Bacterial	Strain uumber	Bacterial	Strain number
Brucella abortus	ATCC 23,448	*Cronobacter sakazakii*	ATCC 51,329
Brucella melitensis	ATCC 23,457	*Bacillus cereus*	ATCC 14,579
Aeromonas hydrophila	ATCC 7966	*Vibrio parahaemolyticus*	ATCC 27,968
Campylobacter jejuni	ATCC 33,560	*Lactobacillus casei*	ATCC 393
Escherichia coli	ATCC 25,922	*Lactobacillus plantarum*	ATCC 8014
Staphylococcus aureus	ATCC 29,213	*Vibrio cholerae*	ATCC 14,035
Klebsiella pneumoniae	ATCC 31,488	*Listeria welshimeri*	ATCC 35,897
Salmonella enteritidis	ATCC 13,076	*Clostridium perfringens*	ATCC 13,124
Shigella flexneri	ATCC 12,022	*Vibrio anguillarum*	ATCC 43,307
Listeria monocytogenes	ATCC 19,111	*Vibrio vulnificus*	ATCC 27,562
Yersinia enterocolitica	ATCC 9610	*Pseudomonas aeruginosa*	ATCC 27,853
Streptococcus haemolyticus	ATCC 21,060	*Proteus vulgaris*	ATCC 49,027

Table 2 DPO primers used in this study

Target gene	Primer type	Sequences (5′→3′)	Amplicon size
Omp25 gene	Conventional	F: CAGGAACAGCCTCCGGTTCCGGCTCCGGTTGA R: AGGTACGGCATAACCGGGTTCAGGTCGTAGCC	320 bp
	DPO	F: *CAGGAACAGCCTCCGGTTC*IIIIICCGGTTGA a b c R: *AGGTACGGCATAACCGGGT*IIIIITCGTAGCC	320 bp

a: The longer 5′-segment of DPO primer; b: The poly I linker; c: The shorter 3′-segment of DOP primer

selective media and temperature-controlled environments specific to each strain [19].

DPO primers

The DPO primers targeting *Brucella* spp. gene *Omp25* were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China), following our established protocol as previously described [19]. Briefly, the primers were optimized for high specificity under varying annealing temperatures, as reported. Details of the DPO primers are summarized in Table 2.

Development of the DPO-based pcr assay for *Brucella* spp

For the DPO-based PCR assay, the optimal reaction conditions were determined by testing various concentrations of Mg^{2+} (0–5.0 µL, 25 mmol/L), rTaq DNA polymerase (0.02–0.2 µL, 5 U/µL), dNTP (0.25-4 µL, 2.5 mmol/L), and DPO primer pair (0.2–1.4 µL, 10 µmol/µL) in a total volume of 25 µL. PCR was performed under the following conditions: 95℃ for 5 min; 35 cycles of 94℃ for 30 s, 50–70℃for 30 s, and 72℃ for 30 s; and a final extension at 72℃ for 10 min. PCR products were analyzed by agarose gel electrophoresis.

Determination of detection limit and specificity of the DPO-based PCR assay

The detection limit of the DPO-based PCR assay for *Brucella* spp. was assessed in both pure cultures and spiked tissue samples, following our established protocol [19]. Serial 10-fold dilutions of *Brucella* cultures were prepared in phosphate-buffered saline (PBS, pH 7.4), and genomic DNA was extracted using the TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit (TaKaRa, Dalian, China). For spiked tissue samples, 100 µL of each dilution was added to 100 mg of *Brucella*-negative lamb liver, and total DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Beijing, China). The detection limit was defined as the last dilution yielding a positive PCR result in triplicate experiments. Bacterial concentrations were confirmed by the standard plate count method. The specificity of the assay was evaluated using the bacterial strains listed in the "Bacterial Strains" section. DNA from each strain was extracted using the TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit and tested by the DPO-based PCR assay. To assess the reproducibility of the DPO-based PCR assay, intra-assay and inter-assay variability were calculated using the coefficient of variation (CV). Each sample was tested in triplicate within the same run (intra-assay variability) and across three independent runs on different days (inter-assay variability).

Practical application of the DPO-based PCR assay

A total of 500 samples including 250 liver samples and 250 blood samples were collected from sheep at livestock slaughter plants. Liver samples were aseptically collected during post-mortem examination, and blood samples were drawn from the jugular vein using sterile vacutainers. All samples were transported to the laboratory on ice and processed within 24 h of collection. Liver tissues were homogenized in sterile PBS, and 100 mg of each homogenate was used for DNA extraction. Whole

blood samples were centrifuged at 3,000 × g for 10 min to separate plasma and buffy coat. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Beijing, China) according to the manufacturer's instructions. The extracted DNA from both liver and blood samples was subjected to the DPO-based PCR assay and under the optimized conditions described above. Liver homogenates and whole blood samples were streaked onto *Brucella*-selective agar plates and incubated at 37℃ under 5% CO_2 for 7–10 days. Colonies suspected to be *Brucella* were identified based on morphology, Gram staining, and biochemical tests. Serum was separated from blood samples by centrifugation at 3,000 × g for 10 min and stored at -20 °C until analysis. The presence of anti-*Brucella* antibodies was detected using a commercial ELISA kit (*Brucella* Competitive ELISA Antibody Detection Kit, Shanghai Huzheng Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions.

Results

Establishment of the DPO-based PCR assay

Following the optimization of the reaction system, the DPO-based PCR assay for specific detection of *Brucella* spp. was successfully developed (Fig. 1). The optimal reaction system of the DPO-based PCR assay in a total volume of 25 μL was as follows: 10×PCR buffer 2.5 μL, Mg^{2+} (25 mmol/L) 2.5 μL (Fig. 1a), r*Taq* DNA polymerase (5U/μL) 0.05 μL (Fig. 1b), dNTP (2.5 mmol/L for each) 1 μL (Fig. 1c), DPO primer pair (10 μmol/μL) 0.4 μL (Fig. 1d), DNA template 0.5 μL and deionized water added up to 25 μL. In this DPO-based PCR assay, the longer 5'-segment of DPO initiates stable annealing and its shorter 3'-segment determines target-specific extension, resulting in unparalleled high specificity. The DPO system enables a wide annealing temperature range of 50–70 °C for effective target gene amplification, comparable to conventional PCR. Through optimization, we selected 61 °C as the optimal annealing temperature for our DPO-PCR assay, showcasing the system's adaptability (Fig. 2) Thus, the DPO system provides a powerful tool for the development of multiplex PCR assay [20, 21].

Detection limit of the DPO-based PCR assay

The detection limit of the DPO-based PCR assay for *Brucella* spp. was determined and result showed that the detection limit of this assay for *Brucella* spp. was 5.3×10^1 CFU/mL (Fig. 3), which was consistently found in pure cultures and spiked animal tissue samples. The assay showed consistent results in repeated experiments. The DPO-based PCR assay demonstrated excellent reproducibility, with intra-assay and inter-assay coefficients of variation (CV) of less than 5% and 10%, respectively. However, there was no significant difference in detection limit between DPO-based PCR assay and conventional PCR assay.

Specificity of the DPO-based PCR assay

Applying the assay to test *B. abortus*, *B. melitensis*, *(A) hydrophila*, *C. jejuni*, *E. coli*, *S. aureus*, *K. pneumoniae*, *S. enteritidis*, *S. flexneri*, *L. monocytogenes*, *Y. enterocolitica*, *S. haemolyticus*, *C. sakazakii*, *(B) cereus*, *V. parahaemolyticus*, *L. casei*, *L. plantarum*, *V. cholerae*, *L. welshimeri*, *(C) perfringens*, *V. anguillarum*, *V. vulnificus*, *P. aeruginosa* and *P. vulgaris*, only *Brucella* spp. were positive in this DPO-based PCR assay without non-specific results (Fig. 4a), while conventional primer-based PCR assay

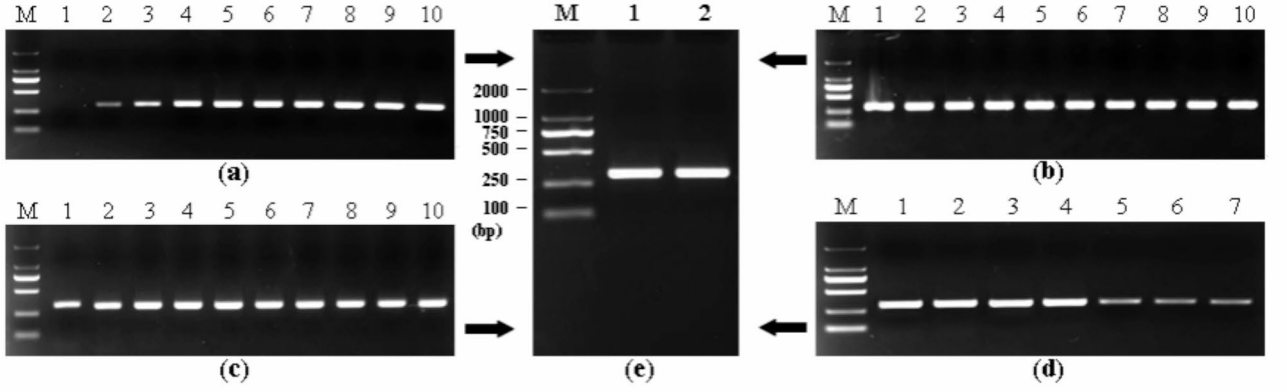

Fig. 1 Development of the DPO-based PCR assay for *Brucella* spp. (**a**) Optimization of Mg^{2+} concentration: Lanes 1–10 represent Mg^{2+} concentrations of 0 mM, 0.5 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM, 4 mM, and 5 mM, respectively. The optimal concentration was determined to be 2.5 mM (lane 6). (**b**) Optimization of r*Taq* DNA polymerase: Lanes 1–10 represent r*Taq* concentrations of 0.1 U, 0.15 U, 0.2 U, 0.25 U, 0.3 U, 0.35 U, 0.4 U, 0.45 U, 0.5 U, and 1 U, respectively. The optimal concentration was determined to be 0.25 U (lane 4). (**c**) Optimization of dNTP: Lanes 1–10 represent dNTP concentrations of 0.025 mM, 0.05 mM,0.075 mM,0.1 mM,0.125 mM,0.15 mM,0.175 mM,0.2 mM,0.3 mM,0.4 mM, respectively. The optimal concentration was determined to be 0.1 mM (lane 4). (**d**) Optimization of DPO primer: Lanes 1–7 represent DPO primer concentrations of 0.08 μM, 0.16 μM,0.24 μM,0.32 μM,0.4 μM,0.48 μM,0.56 μM, respectively. The optimal concentration was determined to be 0.16 μM (lane 2). (**e**) Successful development of the DPO-based PCR assay: This panel illustrates the assay's performance under optimal conditions, demonstrating efficient and specific amplification of the target *Brucella* DNA

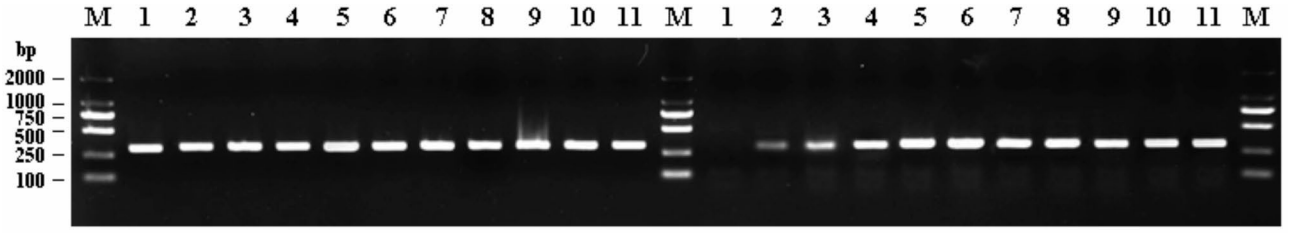

Fig. 2 Comparison of effective annealing temperature between the DPO-based PCR assay and conventional primer-based PCR assay. The DPO-based PCR assay demonstrates a wider range of annealing temperatures (50–70℃) for efficient target gene amplification, showing a nearly identical pattern across left lanes 1–11. In contrast, the conventional primer-based PCR assay exhibits amplification primarily at a specific temperature, highlighting its narrow optimal temperature range, as seen in right lanes 1–11. M: DNA Marker 2000; lanes 1–11: 49.8℃, 51.5℃, 53.4℃, 55.7℃, 58.3℃, 61.0℃, 63.7℃, 66.1℃, 68.0℃, 69.4℃ and 70.0℃, respectively

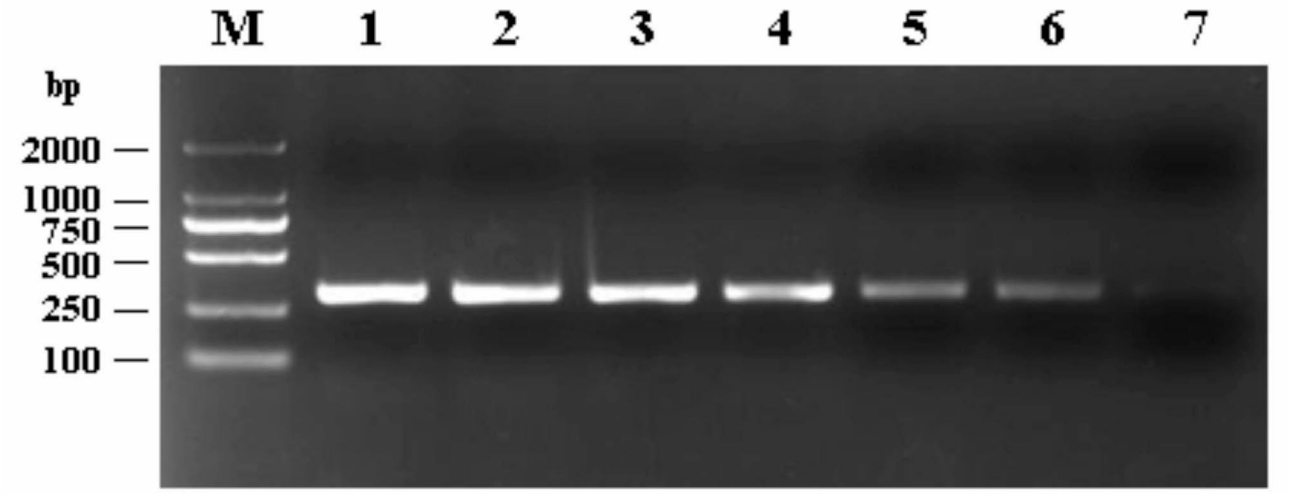

Fig. 3 Detection limit of the DPO-based PCR assay for *Brucella* spp. M: DNA Marker 2000; 1: 5.3×10^{6} CFU/mL; 2: 5.3×10^{5} CFU/mL; 3: 5.3×10^{4} CFU/mL; 4: 5.3×10^{3} CFU/mL; 5: 5.3×10^{2} CFU/mL; 6: 5.3×10^{1} CFU/mL; 7: 5.3×10^{0} CFU/mL

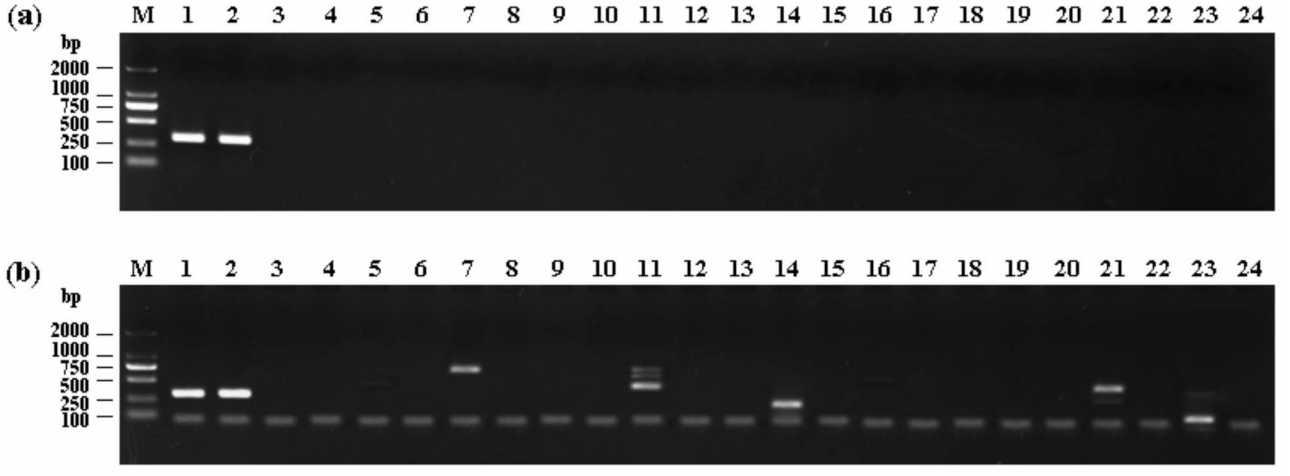

Fig. 4 Specificity of the DPO-based PCR assay for *Brucella* spp. The DPO-based PCR assay developed in this work has high diagnostic specificity for *Brucella* spp. (**a**), while conventional primer-based PCR assay shows a low specificity (**b**). M: DNA Marker 2000; Lanes 1–24 were *B. abortus*, *B. melitensis*, *A hydrophila*, *B cereus*, *C jejuni*, *C. sakazakii*, *C. perfringens*, *E. coli*, *K. pneumoniae*, *L. monocytogenes*, *L. welshimeri*, *L. casei*, *L. plantarum*, *P. aeruginosa*, *P. vulgaris*, *S. aureus*, *S. enteritidis*, *S. flexneri*, *S. haemolyticus*, *V. parahaemolyticus*, *V. cholerae*, *V. anguillarum*, *V. vulnificus* and *Y. enterocolitica*, respectively

Table 3 Practical application of the DPO-based PCR assay

Code of livestock slaughter plants	Samples[a]	No.	DPO-PCR assay		Conventional assay		ELISA[b]	
			P[c]	*N*[d]	*P*	*N*	*P*	*N*
LSP1	Liver	50	11	39	11	39	-	-
	Blood	50	11	39	11	39	8	42
LSP2	Liver	50	4	46	4	46	-	-
	Blood	50	4	46	4	46	4	46
LSP3	Liver	50	9	41	9	41	-	-
	Blood	50	9	41	9	41	7	43
LSP4	Liver	50	3	47	3	47	-	-
	Blood	50	3	47	3	47	2	48
LSP5	Liver	50	9	41	9	41	-	-
	Blood	50	9	41	9	41	5	45

a: Fifty slaughter-aged sheep at livestocks randomly selected from each slaughter plant were used to collect liver and blood samples, respectively; b: ELISA was only used to detect blood samples in this work. c: Total number of positive samples; d: Total number of negative samples

showed a lower specificity (Fig. 4b). The results indicated that the DPO-based PCR assay showed no cross-reactivity with non-target species under the tested conditions, while the conventional PCR assay exhibited non-specific amplification in several lanes (Fig. 4b, lanes 7, 11, 14, 21, and 23). This suggests that the DPO system, due to the bubble-like structure of the poly I linker, may offer improved specificity compared to conventional primers.

Diagnostic capability of the DPO-based PCR assay in practice

Applying the assay to 500 samples collected from sheep at livestock slaughter plants revealed that 72 samples were positive detected by the DPO-based PCR assay (Table 3), which were consistent with the results of conventional culture-based assay. Both the DPO-based PCR assay and the conventional culture-based assay showed a detection rate of 14.4% (72/500), while the ELISA method exhibited a significantly lower detection rate of 10.4% (26/250) in blood samples. This discrepancy may be attributed to the low production rate of antibodies in the early stages of the disease, which limits the sensitivity of serological tests. By contrast, the DPO-based PCR assay was more rapid, time-saving, and sensitive, showing a better diagnostic capability and practicality.

Discussion

The microbiological diagnosis of brucellosis is based on three key approaches: culture, serology, and nucleic acid amplification tests [7]. This study used liver and blood samples available from sheep at slaughter-aged livestocks to investigate brucellosis, which incuded bacteriology, serology, and molecular detection. Specifically, the conventional culture-based assay, ELISA to detect *Brucella* antibodies in serum, and a DPO-based PCR assay were used.

According to the literature, the detection limits of *Brucella* in blood by isolation and culture methods were within the range of 10^3-10^4 CFU/mL, while the detection limits of the same specimens by real-time PCR were 10^4-10^5 CFU/mL. The main factors affecting the detection limits of real-time PCR were fat and protein [22]. To address these limitations, another study developed a propidium monoazide-quantitative PCR (PMA-qPCR) method to detect viable *Brucella* by targeting its BCSP31 gene. This novel, rapid method has a higher sensitivity of 10^3 CFU/mL, utilizing PMA, a DNA-binding dye that can form covalent cross-links with DNA upon photolysis, to selectively amplify DNA from viable cells and inhibit amplification of DNA from dead cells [23]. In our own study, the detection limit of the DPO-based PCR assay for *Brucella* spp. was even lower, at 5.3×10^1 CFU/mL. Moreover, droplet digital PCR (ddPCR), as a novel PCR technique that partitions the sample into thousands of nanoliter-sized droplets, allowing for absolute quantification of target nucleic acids without the need for standard curves or reliance on amplification efficiency. The ddPCR assay developed in another recent study demonstrated a limit of detection of 1.87 copies per reaction with high repeatability, and exhibited promising diagnostic performance, detecting *Brucella* DNA in 88.5% of SAT positive patients and 57.6% of suspected sero-negative samples, suggesting its potential as a valuable diagnostic and prognostic tool for brucellosis [24].

Considering *Brucella* may infect any body organ or tissue, blood cultures is the most common clinical specimen. The bacteriology and serology detection were used as the consistency examination. According to the results, the positive rate was 14.4% by the traditional bacterial culture method on liver and blood samples. The ELISA method was used to detect antibodies in serum, and the positive detection rate was 10.4% in blood samples. We hypothesize that the low serological positive detection rate is due to the ability of blood culture to detect *Brucella* organisms during the early stage of the disease, when serological test results may still be negative or show low antibody titers [25]. While the DPO-based PCR assay demonstrated superior performance compared to

ELISA, a more meaningful comparison would involve other molecular methods, such as conventional PCR or real-time PCR. Future studies should include these methods to provide a comprehensive evaluation of the DPO assay's diagnostic capabilities.

Although the DPO-based PCR assay demonstrates high sensitivity and specificity, there remains a limitation in its inability to differentiate between *Brucella* spp. Further optimization of primer design is necessary to accurately distinguish between subtypes of *Brucella*, such as *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis*, in cattle, sheep, goats, and pigs.

While the DPO-based PCR assay demonstrated high specificity against a panel of bacterial strains, it should be noted that potential cross-reactivity with other clinically relevant pathogens, such as *Mycobacterium* spp. or *Coxiella burnetii*, was not evaluated. These pathogens share environmental niches with *Brucella* spp. and may pose a risk of cross-reactivity [26, 27]. Future studies should include these pathogens to further validate the specificity of the assay.

In conclusion, the development of a dual priming oligonucleotide (DPO)-based PCR detection system for *Brucella* spp. in slaughter-aged livestock, specifically targeting *B.abortus* and *B.melitensis*, has shown promising results. To the best of our knowledge, this is the first report of using a DPO-based PCR detection system for detecting *Brucella* spp.The optimized DPO-based PCR assay exhibited a wide range of annealing temperatures for specific detection, with a low analytical detection limit and high specificity. The high specificity and sensitivity demonstrated by the DPO-based PCR assay suggest its utility in enhancing the diagnosis and surveillance of brucellosis, offering a valuable contribution to the field of veterinary and public health. Although the DPO-based PCR assay showed improved performance over ELISA, further studies are needed to compare its diagnostic accuracy with other molecular methods, such as conventional PCR and real-time PCR.

Author contributions

LD, XW, HS and AW developed the DPO-based PCR detection system for the identification of Brucella spp. CZ and ZF optimized the reaction conditions. AJ and YJ collected the clinical samples. GS detected the clinical samples. CL and XG conceived the project. LD, CL, LY and XG was the grant holder and drafted the manuscript. All authors read, revised, and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are publicly available and included in this published article.

Declarations

Ethics approval and consent to participate

Non-applicable.

Consent for publication

Non-applicable.

Competing interests

The authors declare no competing interests.

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