Letter to the Editor

Advances in the Application of Molecular Diagnostic Techniques to Brucellosis*

Haiwen Liu[1] and Hai Jiang[2,#]

Brucellosis is a zoonotic infectious and allergic disease caused by *Brucella* bacteria. Brucellosis occurs worldwide and has had a huge economic impact on the livestock industry in many countries and regions. It has become a major public health problem. *Brucella* is an endoparasitic, non-motile Gram-negative bacterium capable of surviving within a diverse range of domestic animal hosts. To date, 12 *Brucella* species have been documented, encompassing *B. melitensis, B. suis, B. abortus, B. ovis, B. canis, B. neotomae, B. ceti, B. pinnipedialis, B. microti, B. inopinata, B. vulpis* and *B. papionis.* Those most frequently responsible for human infections are *B. melitensis, B. abortus and B. suis*. *Brucella* has the potential to cause varying degrees of harm to both humans and domestic animals. After being infected with *Brucella*, individuals may experience prolonged fever, joint pain and other symptoms, and severe cases may impar their ability to work. After being infected with *Brucella*, female animals may exhibit symptoms such as abortion, metritis and orchitis, and hind limb dyskinesia is also commonly observed.

Diagnosis of brucellosis has become a research hotspot because it is important to develop a rapid, efficient and accurate detection technology. At present, there are three main types of detection methods for brucellosis. The first is serological detection, including the commonly used Rose Bengal plate test (RBPT), serum agglutination test (SAT), enzyme-linked immunosorbent assay (ELISA) and gold Immunochromatography assay (GICA)[1]. RBPT is characterized by ease of operation and short reaction time, but its specificity is limited, and visual interpretation of results by laboratory personnel can lead to significant errors, resulting in false positive or negative outcomes. The SAT method exhibits high specificity, albeit with prolonged detection time and intricate operational procedures. Serological methods have many limitations, not least that not all animals produce a measurable antibody state after infection and require high antibody titres in samples for tests to be effective. The second is pathogen detection. Culture is considered the 'gold standard' for laboratory diagnosis of brucellosis. However, isolation and culture of *Brucella* is complicated, the separation efficiency is low, there are high requirements for dedicated laboratories and professional technicians, and the biosafety risk is high. The third is molecular biology detection. Since the advent of PCR in 1985, it has been widely used for detection in medicine, food, biological research and other fields due to its rapid, accurate and convenient characteristics. A variety of nucleic acid-based molecular biological methods for detection of pathogenic microorganisms have since been established. Molecular detection techniques have been widely used in the detection of *Brucella* due to their advantages of high sensitivity, good specificity and low risk of laboratory contamination.

Real-time fluorescence quantitative PCR (qPCR) is a rapidly developing and widely utilised detection technology that has been extensively employed for molecular diagnosis of *Brucella* due to its high sensitivity and low biological risk. The sensitivity and specificity of this method surpass those of traditional serological detection methods, thereby compensating for the limitations of conventional brucellosis diagnostic techniques. Moreover, it enables rapid identification and detection of *Brucella* species in samples with positive results. Primers and probes were designed based on *Brucella*-specific genes (*IS711*, *bcsp31*, etc.) to detect the presence of *Brucella* in biological samples such as milk, tissues (placenta, liver, spleen), whole blood and serum, and in environmental samples such as soil[2,3]. The qPCR

doi: 10.3967/bes2024.106

*This study was supported by Major Infectious Diseases Surveillance Projects [Grant No. 33002, 33003, 33055].

1. School of Public Health, Baotou Medical College, Baotou 014040, Inner Mongolia Autonomous, China; 2. National Key Laboratory of Intelligent Tracing and Forecasting for Infectious Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

results hold great significance for hospitalised patients exhibiting clinical symptoms but negative RBPT outcomes, since they enable early and rapid confirmation of human brucellosis. Distinguishing between virulent strains of *Brucella* in milk samples and vaccine strains holds significant importance, as the occurrence of false positive results could erroneously imply the presence of brucellosis in the region, thereby leading to substantial economic losses.

Diverse reaction mechanisms have been utilised by numerous isothermal nucleic acid amplification methods since the early 1990s. This review focuses on the application of loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) techniques in *Brucella* detection. Compared with traditional PCR methods, the LAMP approach demonstrates superior sensitivity and lower detection limits, making it an ideal diagnostic tool for brucellosis due to its simplicity and rapidity. This novel nucleic acid amplification technology can be executed using standard thermostatic equipment for the reaction. At the grassroots level, reaction conditions can also be achieved using simple equipment such as a constant temperature water bath.

Li et al. developed two LAMP methods combined with nanoparticle lateral flow biosensors (LAMP-LFB, *B. abortus*-LAMP-LFIA)[4]. The LAMP-LFB method utilises a simple instrument, maintains a constant temperature of 63, and enables completion of the entire reaction including DNA extraction within 65 min. The detection limit for pure cultures is 100 fg per vessel. However, the main limitation is that its qualitative red band results cannot be quantitatively analysed, making it more suitable for on-site workers to quickly detect *Brucella* in samples.

RPA technology relies on three key enzymes: recombinases, single-stranded DNA-binding proteins (SSB) and strand-displacement DNA polymerases that bind to oligonucleotide primers. The amplification process of RPA is rapid, yielding an amplification product within 30 min. This method has great potential as a replacement for PCR in detection applications. Qin et al. developed an RPA method for detecting *Brucella*. By targeting the *bcsp31* gene of *Brucella*, the sensitivity and specificity of RPA were evaluated using plasmid standards, representative strains of *Brucella*, and non-*Brucella* strains. In 95% of cases, RPA achieved a detection limit of 17 molecules and successfully distinguished 18 representative *Brucella* strains as well as four vaccine strains (A19, S19, S2 and M5). Simultaneous detection of 52 *Brucella* field strains using qPCR and RPA yielded a sensitivity of 94% (49/52) for RPA compared to qPCR. These results demonstrate that RPA is a rapid, sensitive and specific method for detecting *Brucella*[5].

The implementation of droplet digital polymerase chain reaction (ddPCR) typically necessitates the utilization of a thermal cycler, droplet generator, and droplet detector. Unlike traditional fluorescence qPCR, quantitative dPCR technology does not rely on a standard curve, and its sensitivity and accuracy are higher than those of the traditional method. The establishment of a dPCR method is of great significance for the detection of *Brucella*, the prevention and control of brucellosis, the development of epidemiological traceability, and ensuring food safety.

Du et al. developed a multiplex ddPCR method for simultaneous detection of five high-risk bacteria (*Yersinia pestis*, *Bacillus anthracis*, *Brucella*, *Pseudomonas holderia* and *Francisella tularensis*). According to various design primers and probes, it is theoretically feasible to discriminate between wild-type strains and vaccine strains using the ddPCR method. The new ddPCR method necessitates additional refinement and advancement[6].

Clustered regularly interspaced short palindromic repeats (CRISPR) and their proteins (CRISPR-associated, Cas), first reported in 1987, have been developed into a molecular biology tool.

In several Cas family members, including Cas13, Cas12 and Cas14 effectors, cleavage of target nucleic acids can trigger cleavage of unrelated single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA). By adding a single-stranded DNA or single-stranded RNA reporter gene with a fluorescent group and a fluorescence quenching group to label the end of the reaction system, when the Cas effector protein and the crRNA complex specifically recognise the cleavage target gene, trans-activity is stimulated, and the added reporter gene in the system can be cleaved non-specifically. Fluorescein is released, allowing visualisation of the results.

The CRISPR/Cas system has been applied to *Brucella* detection due to its exceptional efficiency, rapidity, sensitivity, and capacity for multiple target nucleic acid detection. Combination of the CRISPR/Cas system with biological amplification techniques such as RPA and LAMP can further improve the detection sensitivity. Because it does not require specific or complicated instruments, dedicated laboratory conditions and technical personnel are not needed, hence it is more suitable

for rapid and immediate detection of *Brucella* in the field.

Xu et al. developed a sensing platform based on RPA-CRISPR/Cas12a for detection of *Brucella* that can output both fluorescence and electrochemical signals[7]. The diagnostic performance of qPCR and CRISPR/Cas12a was compared for milk and blood samples. The results revealed a detection limit of 2 copies per reaction for the RPA-CRISPR/Cas12a-based dual biosensor, which enables rapid and accurate identification of *Brucella* in milk (food) and blood (clinical) samples. This method is comparable to traditional qPCR and serves as a valuable tool for early diagnosis of *Brucella* in milk. Dang et al. integrated the CRISPR reaction system with lateral flow chromatographic test strips, creating a rapid and visually detectable method for Brucella CRISPR/CAST. CRISPR/Cas system demonstrates high accuracy in detecting *Brucella* DNA in infected livestock serum samples within a short duration of 30 minutes. Its detection efficacy is comparable to that of qPCR, while offering the advantages of simplicity, rapidity, exceptional sensitivity, and specificity[8]. The future holds great promise for its application in on-site brucellosis screening at the grassroots level, rendering it an indispensable tool for the prevention and control of this debilitating disease.

Currently, *Brucella* detection is typically carried out in a laboratory. Since the advent of molecular biological techniques for *Brucella* detection, various methods have demonstrated exceptional sensitivity and specificity. qPCR, LAMP, and RPA methods are particularly well-suited for analysing large sample sizes due to their exceptional sensitivity, excellent specificity, and the capability to simultaneously detect multiple samples with a single addition. However, in the course of experiments, aerosols carrying DNA from positive samples can readily contaminate the air, potentially resulting in false-positive outcomes.

Digital PCR offers the benefits of extended detection time, reduced data volume per unit time, slower sequencing speed, and limited applicability to large sample sizes. Despite being in its exploratory stage, CRISPR technology holds great promise. The CRISPR detection system can be integrated with lateral flow chromatography test strips to yield visually interpretable results within a time frame of 30 minutes.

This approach exhibits rapidity, efficacy, and convenience while concurrently streamlining procedural steps and minimizing operational expenses. Consequently, it is well-suited for large-scale field screening of infectious diseases. However, designing crRNAs can be complex because it requires recognition of specific PAM sites to enhance specificity while limiting flexibility. Therefore, further investigation is warranted for the detection of diverse *Brucella* species and vaccine strains. Tables 1 and 2 provide a comprehensive overview of the molecular biological detection methods for *Brucella* discussed in this review, including target genes, detection limits, specificity, *Brucella* species detected, sample types analysed, detection timeframes, and advantages and limitations. Currently, the development of laboratory diagnostic methods that achieve high specificity and sensitivity, are cost-effective, and capable of distinguishing between natural infection and vaccine-induced immunity remains a major challenge in the diagnosis of brucellosis. In the future, *Brucella* nucleic acid detection methods based on molecular biology will continue to evolve towards greater sensitivity, speed, convenience and cost-effectiveness through optimisation and integration.

Detection of *Brucella* is increasingly shifting towards more rapid, convenient, environmentally friendly and cost-effective methods. Recent advancements in molecular biological detection techniques facilitate the swift visual identification of *Brucella*. This article presents a comprehensive review of various molecular biological methods commonly utilised for detection of *Brucella*, and compares their efficacy in terms of sensitivity, specificity, detection time, sample type and other relevant parameters. Molecular biological techniques often outperform traditional methods due to their high sensitivity and specificity, as well as their ability to minimise the risk of laboratory contamination. Through targeted gene sequencing design, specific techniques enable multiple detection of *Brucella* to enhance clinical significance. Coupled with flowmetry chromatography test strips, this approach minimises reliance on laboratory resources and staff while optimising efficiency. This allows prompt visual identification of *Brucella* in the field.

Competing Interests None of the authors have competing interests to declare.

[#]Correspondence should be addressed to Hai Jiang, PhD, Tel: 86-18612892606, E-mail: jianghai@icdc.cn.

Biographical note of the first author: Haiwen Liu, female, born in 1999, Master's Degree, majoring in public health.

Received: August 9, 2023;

Accepted: May 8, 2024

Table 1. Comparison of the merits and challenges associated with *Brucella* molecular detection techniques

Me-thods	Advantages	Limitations	Refe-rences
qPCR	Rapid, specific and sensitive Can be used as a complementary diagnostic strategy to detect false seronegative cases	Assays are time-intensive with a high risk of cross-contamination, requiring highly-trained personnel, ample operating space and expensive equipment and reagents	[2,3]
LAMP	Sensitivity, specificity, rapidity, disposability, facile construction and ease of use Avoids specialised or costly equipment and reagents Results can be directly visualised, rapidly obtained, and indirectly reported using a biosensor	Primer design is complicated Reaction conditions need to be optimised Amplified products pose a risk of sample contamination through aerosols	[4,9]
RPA	Rapid, sensitive and specific Does not require sophisticated equipment Low risk of laboratory contamination and false-positive results	Reagents are expensive and produced by only one manufacturer Requires a long probe and primer set, which is not suitable for short sequence nucleic acid detection Limited sample size per reaction affects the lower limit of detection	[5,10]
ddPCR	Inter-laboratory commutability Less affected by sample inhibitors Better detection of low copy number variants	Instrument reagents are expensive Long operating time High risk of contamination	[6]
CRISPR/Cas	Fast, ultrasensitive, accurate, portable and isothermal	The specific mechanism of action is still partially unknown Off-target effects	[7,8]

Note. qPCR, quantitative PCR; LAMP, loop-mediated isothermal amplification; RPA, recombinase polymerase amplification; ddPCR, droplet digital polymerase chain reaction; CRISPR, clustered regularly interspaced short palindromic repeats.

Table 2. Summary of five molecular biological methods for detection of *Brucella* spp.

Method	Target genes	LOD	Specificity (%)	Bacteria	Sample	Time (min)	References
qPCR	*bcsp31*	–	100	*Brucella* spp.	Whole blood (human)	–	[2]
	IS711	–	100	*Brucella* spp.	Milk (cattle)	–	[3]
LAMP	*bscp31*	100 fg	100	*B. abortus* *B. melitensis* *B. ovis* *B. canis* *B. suis* *B. neotomae*	Whole blood (human and goat)	65	[4]
	BruAb2_0168	100 fg	100	*B. abortus*	Whole blood (cattle)	85	[9]
RPA	*IS711*	40 copies/reaction	100	*Brucella* spp.	Tissue (sheep and yak) Milk (cattle) Vaginal swabs (sheep)	10–30	[10]
	bcsp31	17 copies/reaction	100	*B. abortus* *B. melitensis* *B. suis* *B. neotomae* *B. ovis* *Brucella* vaccine strains (A19, S19, S2 and M5)	–	5–20	[5]
ddPCR	–	0.1 pg/μL	100	*Brucella* spp.	Soil	–	[6]
CRISPR/Cas12a	*omp2a*	2 copies/reaction	100	*B. melitensis* *B. abortus* *B. suis* *B. canis*	Milk (cattle) Blood (human)	60	[7]
	bp26	10 copies/reaction	100	*Brucella* spp.	Serum (sheep and cattle)	60	[8]

Note. LOD, limit of detection; qPCR, quantitative PCR; LAMP, loop-mediated isothermal amplification; RPA, recombinase polymerase amplification; ddPCR, droplet digital polymerase chain reaction; CRISPR, clustered regularly interspaced short palindromic repeats.

REFERENCES

1. Islam MA, Khatun MM, Werre SR, et al. A review of *Brucella* seroprevalence among humans and animals in Bangladesh with special emphasis on epidemiology, risk factors and control opportunities. Vet Microbiol, 2013; 166, 317−26.
2. Liu L, Li MY, Liu GW, et al. A novel, highly sensitive, one-tube nested quantitative real-time PCR for *Brucella* in human blood samples. Microbiol Spectr, 2023; 11, e00582−23.
3. Béjaoui A, Abdallah IB, Maaroufi A. *Brucella* spp. Contamination in artisanal unpasteurized dairy products: an emerging foodborne threat in tunisia. Foods, 2022; 11, 2269.
4. Li SJ, Liu Y, Wang Y, et al. Lateral flow biosensor combined with loop-mediated isothermal amplification for simple, rapid, sensitive, and reliable detection of *Brucella* spp. Infect Drug Resist, 2019; 12, 2343−53.
5. Qin LD, Nan WL, Wang Y, et al. A novel approach for detection of brucella using a real-time recombinase polymerase amplification assay. Mol Cell Probes, 2019; 48, 101451.
6. Du YP, Yan ZH, Song K, et al. Development and evaluation of a multiplex droplet digital polymerase chain reaction method for simultaneous detection of five biothreat pathogens. Front Microbiol, 2022; 13, 970973.
7. Xu JH, Ma JF, Li YW, et al. A general RPA-CRISPR/Cas12a sensing platform for *Brucella* spp. detection in blood and milk samples. Sens Actuators B: Chem, 2022; 364, 131864.
8. Dang S, Sui H, Zhang S, et al. CRISPR-Cas12a test strip (CRISPR/CAST) package: In-situ detection of Brucella from infected livestock. BMC Vet Res, 2023; 19, 202.
9. Yang XG, Wang Y, Liu Y, et al. A label-based polymer nanoparticles biosensor combined with loop-mediated isothermal amplification for rapid, sensitive, and highly specific identification of *Brucella abortus*. Front Bioeng Biotechnol, 2021; 9, 758564.
10. Gumaa MM, Cao XA, Li ZC, et al. Establishment of a recombinase polymerase amplification (RPA) assay for the detection of *Brucella* spp. Infection. Mol Cell Probes, 2019; 47, 101434.