BMC Veterinary Research

RESEARCH

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Establishment and application of a qPCR method for differential detection of *Brucella* S2 vaccine strain

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Abstract

Background Brucellosis is one of the most serious zoonotic bacterial diseases in the world. The disease has caused serious harm to people and livestock, hindered the healthy development of the breeding industry, and led to serious economic losses.At present, the prevention and control of this kind of disease is still based on vaccine immunization. However, after the widely used vaccine is inoculated to livestock, there is no widely used differential diagnosis method to distinguish vaccine immune antibodies from natural infection antibodies. Quarantine and purification work is difficult to carry out. In addition, there are few studies using real-time PCR(qPCR) methods in the differential diagnosis of natural virulent strains and vaccine strains of brucellosis.The purpose of this study is to establish a rapid, sensitive and accurate differential diagnosis method for *Brucella* S2 vaccine strain, and to solve the problem of lack of identification of *Brucella* S2 vaccine strain and natural virulent strain in clinical detection.It avoids the killing of some livestock due to the positive antibody of the *Brucella* S2 vaccine strain, and can also identify sick animals from immune herds, reducing the economic losses of farms, and providing certain technical support for the quarantine and purification of epidemic diseases.

Results In this study, combined with TaqMan probe-based qPCR technology, specific primers and probes were designed according to the specific deletion genes of the *Brucella* S2 vaccine strain,which could be used as marker genes.The qPCR and duplex qPCR detection methods of *Brucella* were successfully established.The method has good specificity, sensitivity and repeatability, the lowest limit of detection can reach 1×10^{1} copies/μL, the sensitivity is about 100 times higher than that of conventional PCR, and there is no cross-reaction with *Escherichia coli*,*Salmonella*,*streptococcus* and other common strains.The coefficient of variation between groups was less than 0.6%, and the coefficient of variation within groups was less than 0.55%.Subsequently, this method was used to monitor the antibody levels in goat inoculated with different doses of *Brucella* S2 vaccine strain, and the method could also detect the corresponding nucleic acid signals in goat milk samples, and the clinical samples were detected. In summary, this method has good specificity, sensitivity and repeatability, and can be used for the differential diagnosis of clinical brucellosis.

Conclusions This study successfully established a duplex qPCR detection method for the differential diagnosis of the *Brucella* S2 vaccine strain. From the establishment of the method to the clinical application of the method, it shows that the method can be used for the differential diagnosis of clinical brucellosis.

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Keywords qPCR, Duplex qPCR, *Brucella* S2 vaccine, Sensitivity, Specificity

Introduction

Brucella is the pathogen of brucellosis. Brucellosis is a global zoonotic disease affecting a wide range of mammals, including livestock and humans [1]. In 1905, *Brucella melitensis* was successfully isolated from goat milk in Malta, which proved the zoonotic nature of brucellosis [2]. *Brucella* can be transmitted through the digestive tract, respiratory tract and damaged skin mucosa. Humans are usually infected with the disease by contact with diseased livestock, products and contaminants (such as feces and aborted fetuses).After infection, symptoms such as repeated high fever, fatigue and joint pain can occur.*Brucella* is susceptible to almost all terrestrial animals and mammals in the ocean. Among terrestrial livestock, sheep, cattle and pigs are most susceptible. *Brucella* infection can affect the reproductive system of livestock, such as abortion and orchitis, thus reducing the milk production and dairy quality of livestock, and may even lead to the spread and spread of diseases [3, 4]. Compared with male livestock, female livestock are more likely to be infected with brucellosis, and adult livestock are also more likely to be infected than young livestock [5]. In marine mammals, *Brucella* can affect the reproductive tract and cause abortion, but this pathogen may also cause fatal complications, including meningoencephalitis, spleen and liver necrosis. Due to the ability of bacteria to spread throughout the body, patients may have symptoms such as liver and spleen enlargement and lymph node enlargement [6].

In recent years, due to the widespread epidemic of brucellosis among humans and animals, the annual number of new cases has remained high. Human brucellosis is mostly transmitted through animals.The prevention of human brucellosis depends on the prevention and purification of livestock brucellosis.At present,the main prevention and control measures for brucellosis in China are based on the combination of vaccination and quarantine purification.Vaccines have played an important role in the prevention and control of brucellosis, but they have also brought some new problems.The current serological diagnostic methods are generally used to detect the presence of *Brucella* antibodies,Such as Rose-Bengal plate-agglutination test(RBPT) and tube agglutination test(SAT),etc.But they cannot distinguish vaccine immune antibodies from natural infection antibodies,nor can they distinguish diseased animals from immune groups, which makes it difficult to carry out quarantine and purification work effectively. This is not only a potential threat to humans, but also a danger to fellow animals.so it is of great significance to study the differential diagnosis method of brucellosis. The common *Brucella* vaccines on the market in China include *Brucella suis* S2 vaccine, *Brucella abortus* A19 vaccine and *Brucella melitensis* M5,Rev.1 vaccine,etc. Among them, the *Brucella* S2 vaccine was isolated from the aborted fetus of sows by the China Veterinary Drug Supervision Institute and put into use after weakening.Compared with *Brucella* S19 and *Brucella* Rev.1, *Brucella* S2 has the advantages of low residual virulence, low production cost and relatively easy oral immunization [7]. However, after inoculation with *Brucella* S2 vaccine, it will interfere with the diagnosis of brucellosis. Ordinary detection methods cannot distinguish *Brucella* S2 vaccine strain infection from natural virulent strain infection.This also means that it is imperative to develop a diagnostic method for differential diagnosis of *Brucella* S2 vaccine strain infection and natural virulent strain infection.

Real-time PCR can monitor the whole PCR process in real time according to the synchronous accumulation of fluorescence signal and PCR amplification products [8]. It has the advantages of high sensitivity, high specificity and short time, and has been widely used in the diagnosis of many pathogens [9]. It is divided into two methods: dye method and probe method. Among them, SYBR Green I dye based qPCR method is more likely to form hairpin structure and primer dimer, resulting in false positive results, and the specificity and sensitivity are lower than TaqMan probe method [10]. TaqMan-MGB probe was first reported in 2000. MGB stabilizes the A/T-rich double strands, allowing the use of shorter probes and higher melting temperatures than conventional DNA probes, and MGB probes are highly specific,easy to operate, and suitable for most mutations [11]. At present, new diagnostic techniques and early screening of newly introduced animals are crucial for the prevention and control of brucellosis.Brucellosis diagnostic techniques include etiological diagnostic techniques,serological diagnostic techniques,and molecular biological diagnostic techniques [12]. The methods for the detection of brucellosis have certain limitations,Etiological diagnosis is considered to be the 'gold standard' for the diagnosis of brucellosis.But it has a certain risk.Therefore, etiological diagnosis is not suitable for routine clinical testing [13]. The RBPT can be used for the primary screening of brucellosis, with high specificity, low sensitivity and high false negative rate [14]. The results of SAT are also affected by human subjective factors, and it takes a long time.Therefore, it is not suitable for large-scale clinical testing [15]. Many PCR-based detection methods have been developed, such as conventional PCR based on *Brucella BCSP31 gene* and 16S ± 23S rRNA operon, PCR

that can identify *Brucella* species,such as AMOS-PCR (based on *IS711*),Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR–RFLP) (e.g.the *omp2* locus),arbitrary-primed PCR,and ERIC-PCR,Loop-Mediated Isothermal Amplification (LAMP) and so on [16]. In 2000, Notomi developed a new molecular detection technology,called LAMP [17], Mujiaming designed three sets of primers based on the *Brucella* S2 vaccine strain specific gene *GL_0002189* and *TrbJ* sequences, and five sets of LAMP primers based on the *eryC* gene deletion sequence of *Brucella* S19 vaccine. The results showed that the LAMP method based on the *Brucella* S2 specific gene of *Brucella* could effectively distinguish *Brucella* S2 vaccine strain from *Brucella abortus* and *Brucella melitensis*.The LAMP method established by the specific gene of *Brucella* S19 can effectively distinguish the *Brucella* S19 vaccine strain from the natural virulent strain of *Brucella abortus* and *Brucella melitensis* [18]. But its sensitivity is the same as that of conventional PCR.PCR–RFLP is based on PCR amplification of the target region containing the mutation site of the studied species, followed by restriction enzyme digestion and gel electrophoresis to visualize the RFLP map [19]. However, it also needs agarose gel electrophoresis to observe the results after PCR amplification, and there is a risk of contamination.While qPCR can ensure its sensitivity, it can also be monitored in real time. Therefore, it is imperative to study the detection methods that can identify *Brucella* natural infection and vaccine immunized animals in China [20].

Materials and methods

Source of test samples

In the part of identification and detection of *Brucella* S2 vaccine strain by duplex qPCR method, the sample used was a farm in Hohhot, Inner Mongolia. This sample is a goat sample.

In the clinical sample detection part, the sample used was a farm in Bayannur City, Inner Mongolia.This sample is a sheep sample.

Screening of differential fragments and PCR amplification

Screening of differential fragments

SnapGene software was used to compare the whole genome sequences of the *Brucella* S2 vaccine strain and *Brucella suis* natural virulent strain 1330, and the differential sequences between the two strains were screened. Then the differential sequences were compared with other *Brucella* strains to find out the specific gene fragments with certain coverage and can distinguish *Brucella* S2 vaccine strain from other *Brucella* strains as candidate molecular target genes for differential detection. Then the differential fragments were compared with other *Brucella* strains by BLAST function on the NCBI website, and the differential sequences with certain coverage and specificity were screened out. Primers were designed according to the gene sequence information of the specific deletion site of the *Brucella* S2 vaccine strain.

PCR amplification

Primers were designed according to the gene sequence information of the specific deletion site of the *Brucella* S2 vaccine strain (Table 1).

As the nucleic acid identification sequence for screening is the common difference between *Brucella* S2 attenuated vaccine and other types of *Brucella* (including common *Brucella* attenuated vaccine strains and natural strong strains), the whole genome DNA of *Brucella* A19 attenuated vaccine strain and *Brucella* S2 attenuated vaccine strain was used as a template to carry out PCR amplification and sequencing of nucleic acid marker sequence genes for genetic difference verification.The whole genome DNA of *Brucella* A19 attenuated vaccine strain and *Brucella* S2 attenuated vaccine strain was used as a template to carry out PCR amplification and sequencing of nucleic acid marker sequence genes for genetic difference verification.The reaction system is shown in Supplementary File 5.

Establishment of two qPCR detection methods for *Brucella*

TaqMan probe-based qPCR is a nucleic acid quantitative technology developed on the basis of PCR qualitative technology, which has been widely used in nucleic acid detection and quantification [21]. It has the advantages of high sensitivity, strong specificity, direct detection of viral nucleic acid and quantitative detection of nucleic acid concentration [22]. And high-throughput TaqMan probe genotyping method has been used to detect single nucleotide polymorphism (SNP) [23]. In order to develop a new molecular diagnostic method, this study carried out TaqMan probe-based qPCR detection method research based on the specific deletion site of the *Brucella* S2 vaccine strain, in order to provide a new choice for qPCR nucleic acid detection of *Brucella*.

Table 1 3 pairs of primer sequence information

Primer pair number	sequence	Purpose fragment length
1	F: AAACCGACGACGCAGGAC	193 bp
	R: CGAGATAACCCAGATTGACC	
2	F: CGCTGGGCAATCAGGAAC	134 bp
	R: CGCTGGACCGATACGATAA	
3	F: ATATTGCGCAGCTTCACACC	133 bp
	R: CATCATAGTCGAGATAACCCAGATTG	

Table 2 Screened primer and probe sequences

Gene name	Sequence	Tm value
primer(193 bp)	F: AAACCGACGACGCAGGAC R: CGAGATAACCCAGATTGACC	58.2 ℃ 53.2 ℃
S2-HEX-1probe	TGATCGGACGGTCTC	69.0 ℃
S2-HEX-2probe	GAGACCGTCCGATCA	71.0 ℃
NS2-ROXprobe	TTGATCGGACGGGTCT	70.0 ℃

Table 3 The reaction procedure of qPCR

reaction stage	Reaction temperature	reaction time
Pre-denaturation	95℃	30s
PCR amplification	95℃	5s
	60℃	30s
Number of 40 cycles		

Design and synthesis of qPCR probe

Primer Express 3.0 was used to design the probe. After the target fragment was introduced, the Tm value of the probe was about 10 °C higher than that of the primer. Two S2-HEX probes and one NS2-ROX probe were designed and synthesized (Table 2).

Preparation of recombinant plasmid standard

Construction of *Brucella* S2 and *Brucella* A19 recombinant plasmid standards: The target fragment was amplified according to the designed primers. The PCR reaction procedure was: 95 °C 5 min, 95 °C 50 s, 60 °C 50 s, 72 °C 30 s, 30 cycles, 72 °C extension 10 min. The PCR reaction system is shown in Supplementary File 6.Subsequently, the gel recovery and cloning expression were carried out, and a single colony was selected for culture 3 μL bacterial solution was used as the template for PCR amplification, and a negative control was set up. The PCR reaction procedure was the same as above. The reaction system of bacterial solution PCR is shown in Supplementary File 7.Finally, 2 mL bacterial solution was taken to extract the plasmid, and the plasmid concentration was measured by NanoDrop nucleic acid concentration meter. According to the formula: copy number = ($6.02\times10^{23}\times$concentration$\times10^{-9}$) / [(clone vector length + product size) $\times$ 660] unit converted to copies / μL, and the initial concentration of the two plasmids was adjusted to 1×10^{9} copies / μL.

Optimization of qPCR reaction conditions

The primer and probe are diluted to a working concentration of 10 μmol/L, and the reaction system is shown in Supplementary File 8, the reaction procedure is shown in the Table 3. Keeping the reaction program unchanged, primers and probes were added on the basis of 10 μmol / L, and the addition amount was set to 0.2 μL, 0.4 μL, 0.6 μL, 0.8 μL, and 1 μL. The reaction system was optimized by the checkerboard method to determine the optimal primer and probe addition amount. Finally, the primer and probe addition amount with the lowest Ct value under the same concentration of positive template was the best reaction amount.

Establishment of standard curve

The concentrations of *Brucella* A19 and *Brucella* S2 recombinant plasmids were diluted to 1×10^{8} copies / μL, 1×10^{7} copies / μL, 1×10^{6} copies / μL, 1×10^{5} copies / μL, 1×10^{4} copies / μL by DNA Dilution Buffer, respectively. The recombinant plasmid was used as a template, and three replicates were made for each dilution. The initial dilution concentration was set to 1.00E + 04 (corresponding to the template concentration). Two qPCR amplification curves were obtained for *Brucella* S2 and *Brucella* A19 vaccine sequences using the optimized reaction system.

Specificity experiment

The *Beucella* S2 vaccine strain DNA was used as the positive control, the genomic DNA extracted from *Escherichia coli,Salmonella,Pediococcus acidilactici* and *Streptococcus* was used as the template of the reaction group, and the RNase-free Water was used as the negative control group. Each group was set up three holes to observe whether the qPCR reaction had amplification curve and to evaluate whether the detection method was specific.

Sensitivity experiment

The two recombinant plasmids were diluted 10 times from 10^{5} copies / μL to 10^{1} copies/μL, and the diluted recombinant plasmid was used as a template.Three replicate wells were set for each concentration gradient of the two qPCR reactions, and RNase-free Water was used as a negative control to evaluate the sensitivity of the two qPCR reactions.The two recombinant plasmids were diluted from 10^{6} copies / μL to 10^{1} copies / μL, and the diluted recombinant plasmids were used as templates for conventional PCR. The sensitivity of qPCR and conventional PCR was compared.

Repeatability experiment

The concentration of each recombinant plasmid was 1×10^{6} copies/μL, 1×10^{5} copies/μL, and 1×10^{4} copies/μL respectively as positive templates. The optimized

single reaction condition. After replacing different batches of Premix Ex Taq (Probe qPCR), the experiment was repeated again. The coefficient of variation of Ct values within and between groups was calculated to evaluate the repeatability of single qPCR.

Establishment of duplex qPCR differential diagnosis method for *Brucella* S2 vaccine strain

Two probes were added to the reaction system at the same time to optimize the duplex qPCR reaction system and reduce the mutual interference between primers and probes to obtain a stable and reliable duplex qPCR differential diagnosis method, and realize the purpose of differential diagnosis of *Brucella* S2 vaccine strain and natural virulent strain by single tube qPCR.

Optimization of duplex qPCR reaction conditions

The reaction system was optimized by the checkerboard method. The qPCR reaction conditions and the concentration of the two primers and probes were fixed at 10 μmol/L, and the upstream and downstream primers and probes of the duplex qPCR reaction systems of S2-HEX and NS2-ROX were adjusted respectively.12.5μL Premix Ex Taq (Probe qPCR) was added to the 25μL reaction system, and the amount of primers was adjusted to 0.2μL, 0.4μL, 0.6μL, 0.8μL and 1.0μL, respectively, to optimize the amount of primers. The volumes of the two probes (S2 probe and NS2 probe) were adjusted to 0.2μL, 0.4μL, 0.6μL, 0.8μL, and 1.0μL, respectively, to optimize the probe amount. At the same template concentration, the reaction amount with the smallest Ct value is the best reaction amount.

Establish standard curve

The concentration of the two recombinant plasmids was adjusted to 1×10^9 copies / μL, and then mixed in equal proportion. After 10 times of equal proportion dilution, the mixed standard recombinant plasmids of 1×10^9 copies / μL, 1×10^8 copies / μL, 1×10^7 copies / μL, 1×10^6 copies / μL, 1×10^5 copies / μL and 1×10^4 copies / μL were obtained after dilution. Two mixed plasmids were used as templates, and 2 μL was added to each well. In addition, RNase-free Water was used as the negative control group, and each concentration was repeated three times. The target fragment amplification and fluorescence signal detection were performed with the optimized reaction system and reaction procedure, and the standard curve of duplex qPCR amplification was obtained.

Specificity experiment and evaluation of identification effect

Two standard positive mixed plasmids were used as the positive control group, and RNase-free Water was used as the negative control group. DNA of *Brucella* A19 vaccine strain, DNA of *Brucella* S2 vaccine strain, DNA of *Brucella* Rev.1 vaccine strain and genomic DNA extracted from *Escherichia coli*,*Salmonella*,*Pediococcus acidilactici* and *Streptococcus* were used as templates, respectively. Three replicate wells were made for each template, and the specificity and identification effect of the established duplex qPCR detection method were evaluated.

Sensitivity experiment

Two kinds of 1×10^9 copies / μL standard positive recombinant plasmids were mixed in equal proportion, and then ten-fold gradient diluted to $1\times10^9\sim1\times10^1$ copies / μL, a total of 9 concentrations of double mixed standard positive plasmids. The mixed recombinant plasmids of 5 concentrations of $1\times10^5\sim1\times10^1$ copies / μL with ten-fold gradient dilution were selected as templates. Each concentration gradient is set to 3 multiple holes, and RNase-free Water was used as a negative control to evaluate the sensitivity of the duplex qPCR reaction. Six mixed recombinant plasmids with a concentration of $1\times10^6\sim1\times10^1$ copies / μL were selected, and ten-fold gradient dilution was used as a template.The negative control group with RNase-free Water as a template was subjected to conventional PCR. The sensitivity of duplex q PCR and conventional PCR was compared.

Repeatability experiment

Two equal proportion mixed plasmids with three concentrations of 1×10^6 copies / μL, 1×10^5 copies / μL and 1×10^4 copies / μL were selected as templates, and each concentration was repeated three times. After 7 days, the experiment was repeated again. After 21 days, different batches of Premix Ex Taq (Probe qPCR) repeated experiments were replaced to evaluate the repeatability of this method.

Application of duplex qPCR method for differential detection of *Brucella* S2 vaccine strain

Study on the bacterial retention of Brucella S2 vaccine in the whole blood of dairy goats

Thirty goat were divided into three groups, 10 goat in the recommended dose group, 10 goat in the double dose group and 10 goat in the negative control group. Blood samples were collected before immunization and 3,5,7,15 ,21,30,60,90,120,150,180,210 and 240 days after immunization. The serum was separated and detected by RBPT, SAT and iELISA.After the nucleic acid was extracted from the whole blood, the *Brucella* S2 vaccine antibody or other vaccine strain and natural strain antibody were detected by the established *Brucella* S2 vaccine strain duplex qPCR differential diagnosis method. It can not only verify the sensitivity of this research method, but

also provide a reference for the dose and time of *Brucella* S2 vaccine inoculation in dairy goats.

Rose-Bengal plate-agglutination test and tube agglutination test The non-anticoagulant blood collected at different time points was placed in a refrigerator at 4 °C overnight, and the serum was separated the next day.

Rose-Bengal plate-agglutination test: After the Rose-Bengal plate-agglutination test antigen was balanced to room temperature, the Rose-Bengal plate-agglutination test was performed. The results were observed within 4 min. At the same time, standard negative serum and standard positive serum were set as controls. When the standard negative serum did not agglutinate and the standard positive serum agglutinated, the serum to be tested could be determined.

Tube agglutination test: 0.5% carbonic acid solution was prepared before the test, and 121 °C high pressure for 30 min. The turbidity tube was prepared according to Table 4, and the standard negative and positive controls were set up. Finally, the added test tube was placed in a constant temperature and humidity incubator at 37° C for 18–24 h, and the results were observed and recorded.

Brucellosis kit antibody detection The packaged serum was ablated and thawed, and 2 negative control holes and 2 positive control holes were set up in each plate, and 50 μL was added to each hole. Then 50 μL of serum sample was added to the remaining wells, and 100 μL of horseradish peroxidase-labeled detection antibody was added to the control wells and sample wells, and incubated at 37° C for 60 min. After drying time, each hole was filled with 350 μL of detergent. After standing for 1 min, the detergent was removed and the absorbent paper was dried. Wash the board 5 times. After that, 50 μL of substrates A and B were added to each well and incubated in the dark at 37 °C for 15 min. Finally, 50 μL termination solution was added to each hole. After the above operation is completed, as soon as possible with microplate reader detection, measurement of absorbance (OD value), with reference to the instructions, the negative control OD value + 0.25, the sample OD value is greater than the threshold, determined as positive, otherwise negative.

Table 4 Turbidity tube configuration

Pipe number	Diluted antigen solution/μL	0.5% carbolic acid solution/μL	Brightness / %	label
1	0	1000	100	+ + + +
2	250	750	75	+ + +
3	500	500	50	+ +
4	750	250	25	+
5	1000	0	0	—

Extraction and detection of genomic DNA from whole blood of Brucella Thirty whole blood samples were collected at different time points. The red blood cell lysis method was used to operate according to the instructions of the blood genomic DNA extraction kit. Firstly, 900 μL of red blood cell lysis solution was added to 300 μL of blood for sample pretreatment. Finally, 50 μL of TE elution buffer was used to elute DNA, which was packaged in a PCR tube and frozen at-20 °C for subsequent use. The extracted whole blood genomic DNA at different time points was detected by the established duplex qPCR differential diagnosis method of *Brucella*. When the amplification results had a typical amplification curve and the Ct value ≤ 35, the results were judged to be positive.

Study on the bacterial residence of Brucella S2 vaccine in dairy goat milk after immunization

Ten goat milk samples with positive *Brucella* antibody and ten goat milk samples with negative *Brucella* antibody were selected. The established duplex qPCR detection method of the *Brucella* S2 vaccine strain was used to detect whether the milk contained *Brucella* S2 vaccine or other vaccines and natural virulent strain nucleic acids. The detection of bacterial residence in dairy goat milk, it can provide a reference for timely screening of *Brucella*-infected goat milk samples, solving the problem of artificial harm caused by live bacteria discharged from goat milk, and providing a reference for the prevention of brucellosis.

Extraction of Brucella genomic DNA from goat milk The collected 20 portions of goat milk were operated according to the instructions of the bacterial genomic DNA extraction kit. 1000 μL of goat milk was centrifuged at 10,000 r / min for 1 min for pretreatment. Finally, 50μL of TE elution buffer was used to elute the DNA,which was packaged in a PCR tube and frozen at-20 °C for subsequent use.

Sample detection The 20 goat milk genomic DNA extracted above was detected by the established duplex qPCR differential diagnosis method of the *Brucella* S2 vaccine strain. When the amplification result had a typical amplification curve and the Ct value was ≤ 35, the result was positive.

Results

PCR amplification and recombinant plasmid preparation results

After PCR reaction, 1% agarose gel electrophoresis was used for detection.The results are shown in Fig. 1. The PCR products were recovered and sequenced.

The pMD-S2 and pMD-NS2 recombinant plasmids were constructed respectively, and the recombinant bacterial liquid was identified by bacterial liquid PCR. The results are shown in Fig. 2, and the sizes of the two target bands are consistent with the expected results. The recombinant bacterial liquid was sequenced and identified. The sequencing results showed that the recombinant bacterial liquid was consistent with the original gene sequence after sequencing, and the homology was 100%. The recombinant plasmid was successfully constructed.

Single qPCR optimization results

The optimization results of qPCR reaction conditions

In the qPCR reaction, the concentration of the primer–probe was fixed at 10 μmol / L, and the amount of the primer–probe was optimized. It was concluded that both S2-HEX and NS2-ROX showed the best amplification curve when the primer amount was 0.8 μL (Fig. 3). When the amount of fixed primer was 0.8μL, the amplification curve showed that the optimal amplification curve of S2-HEX appeared when the amount of probe was 1.0μL, and the optimal amplification curve of NS2-ROX appeared when the amount of probe was 0.8μL (Fig. 4).

Establishment of standard curve

The recombinant plasmids pMD-S2 and pMD-A19 of 1×10^9copies / μL, 1×10^8copies / μL, 1×10^7copies / μL, 1×10^6copies / μL, 1×10^5copies / μL and 1×10^4copies / μL were used as templates to construct qPCR standard curve with optimized reaction conditions. The amplification efficiency of S2-HEX standard curve was 103.1%, and R^2 was 0.998. The amplification efficiency of NS2-ROX standard curve was 103.6%, and R^2 was 0.998. The values are within the effective range and can be used for subsequent quantitative detection. The standard curve is shown in Fig. 5.

Results of specificity experiment

The DNA of *Brucella* A19 vaccine strain, *Brucella* Rev.1 vaccine strain, *Brucella* S2 vaccine strain and common

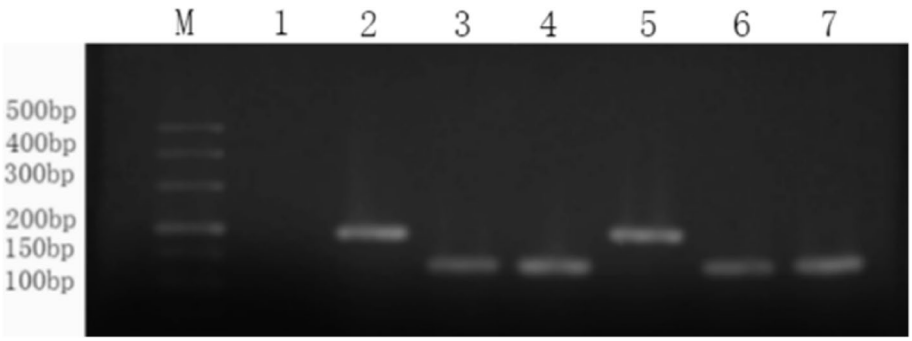

Fig. 1 PCR primer specificity experiment.M,DL500 DNA Marker,lane 1,negative control;lane 2,Primer S2-1;lane 3,Primer S2-2;lane 4,Primer S2-3;lane 5,A19-1;lane 6,A19-2;lane 7,A19-3

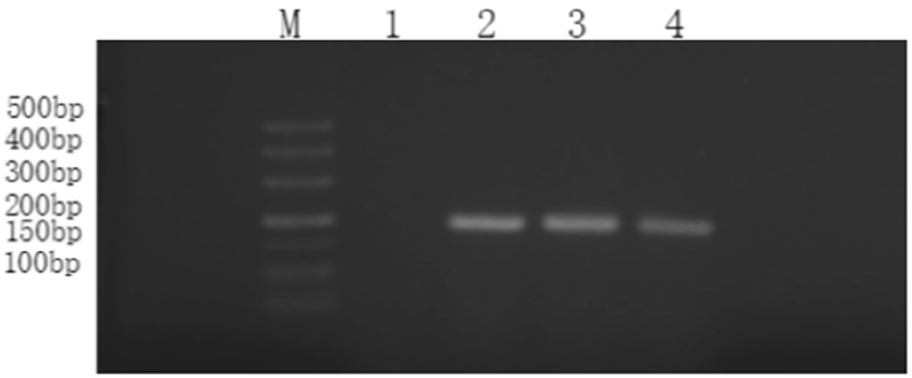

Fig. 2 Identification of recombination plasmid by PCR.M DL 500 DNA Marker,lane 1,negative control;lane 2, positive control;lane 3,Primer pMD-S2;lane 4,Primer pMD-NS2

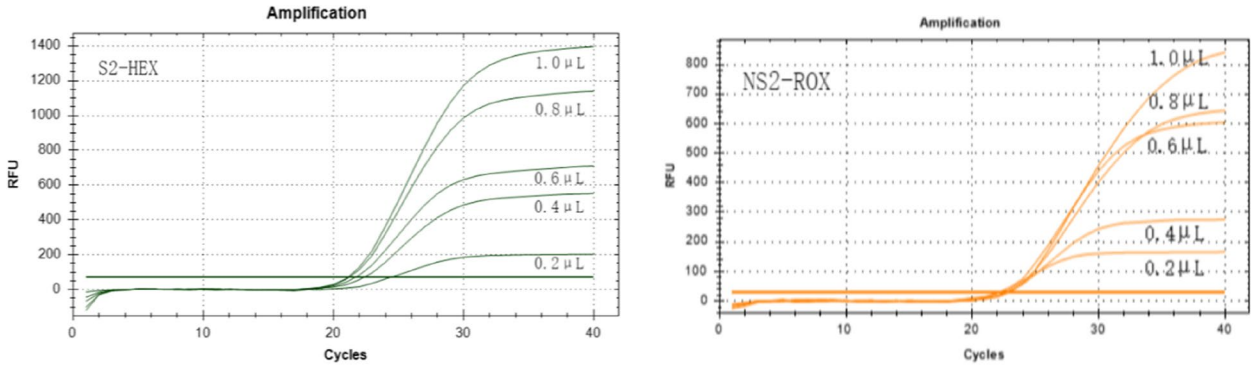

Fig. 3 Two single qPCR primer concentration optimization results.The left figure shows the optimization results of S2-HEX primer concentration: the amount of primers was 0.2μL,0.4μL,0.6μL,0.8μL and 1.0μL, respectively.The right figure is the optimization result of NS2-ROX primer concentration: the amount of primers was 0.2μL,0.4μL,0.6μL,0.8μL and 1.0μL, respectively

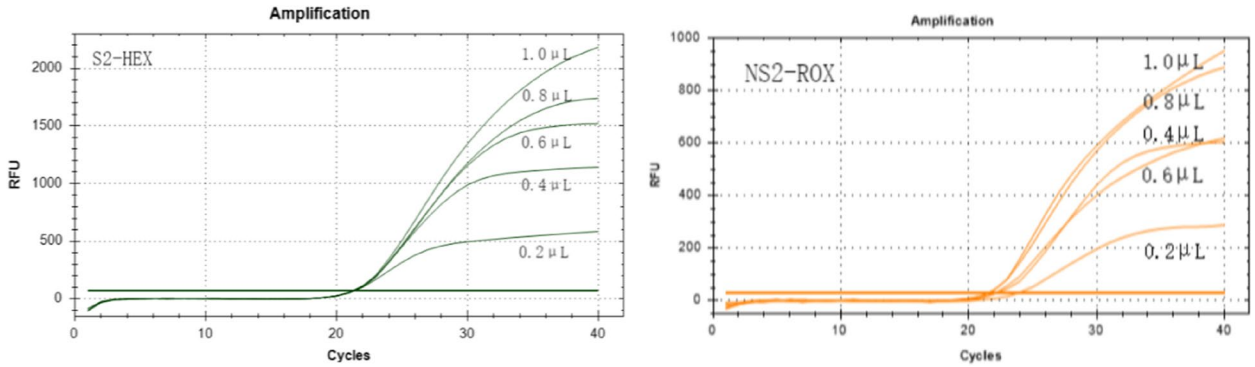

Fig. 4 Two single qPCR probe concentration optimization results.The left image shows the optimization results of S2-HEX probe concentration: the amount of probe was 0.2μL,0.4μL,0.6μL,0.8μL and 1.0μL, respectively. The figure on the right is the optimization result of NS2-ROX probe concentration: the amount of probe was 0.2μL,0.4μL,0.6μL,0.8μL and 1.0μL, respectively

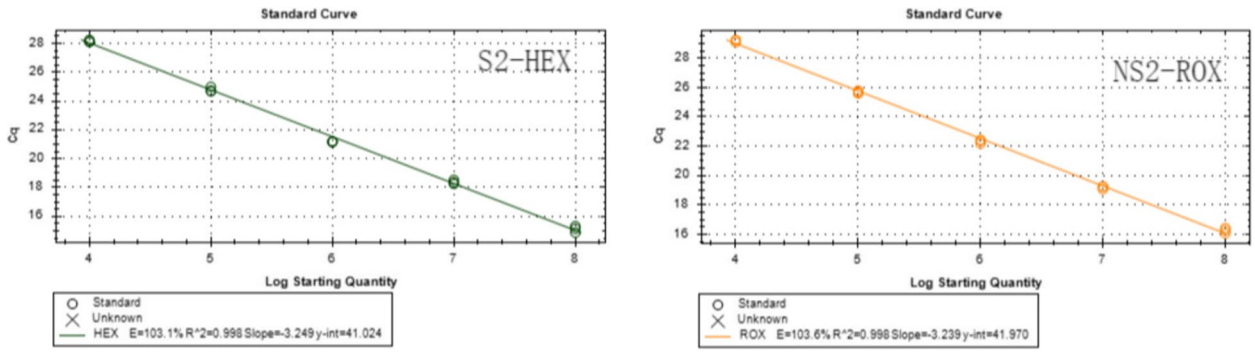

Fig. 5 Results of establishment of standard curves of two single qPCR.The amplification efficiency of S2-HEX standard curve was 103.1%, and R^2 was 0.998. The amplification efficiency of NS2-ROX standard curve was 103.6%, and R.^{2} was 0.998

Escherichia coli, *Salmonella*, *Streptococcus*, and *Pediococcus acidilactici* DNA were used as templates for qPCR amplification to verify the specificity of qPCR. The results showed that the positive amplification curve appeared only when the *Brucella* S2 vaccine strain DNA was used as the amplification template in the S2-HEX group. In NS2-ROX group, the positive amplification curve appeared when *Brucella* A19 vaccine strain and

Brucella Rev.1 vaccine strain were used as amplification templates. There was no amplification curve in the negative control of the two primer probes, and there was no cross reaction with *Escherichia coli*, *Salmonella*, *Streptococcus* and *Pediococcus acidilactici*. The specificity results are shown in Fig. 6.

Sensitivity experiment

The results of sensitivity test showed that the amplification curves of S2-HEX and NS2-ROX groups showed that the amplification curves were generated in each multiple hole at $10^5 \sim 10^1$ copies / μL, and S2-HEX had two holes in three parallel holes with 10^1 copies / μL template concentration to produce effective results ($Ct \leq 35$). NS2-ROX can produce effective results ($Ct \leq 35$) in three parallel holes with a template concentration of 10^1 copies / μL. The sensitivity test results are shown in Fig. 7.

The primers were amplified by conventional PCR with the same template concentration, and the sensitivity was detected by gel electrophoresis. The results showed that the minimum limit of detection of S2 and NS2 was 1×10^3 copies / μL, and the sensitivity of the qPCR method was higher than that of conventional PCR about 100 times, the electrophoresis results are shown in Fig. 8.

Results of repetitive experiments

The recombinant plasmids of 1×10^6 copies / μL, 1×10^5 copies / μL and 1×10^4 copies / μL were used as templates, and different batches of Premix Ex Taq (Probe qPCR) were used for repeated experiments. The results showed that the coefficient of variation of the two single qPCR groups was about 0.5%, and the coefficient of variation between groups was less than 0.6%. The amplification curve is shown in Fig. 9. Repeated experimental data are shown in Table 5.

Duplex qPCR optimization results

Optimization results of duplex qPCR reaction system

The chessboard method was used to optimize the amount of duplex qPCR primers and probes, and the amplification curve and Ct value were observed. The results showed that when the amount of primers was 0.8μL, the Ct values of S2-HEX and NS2-ROX amplification curves were the smallest. When the amount

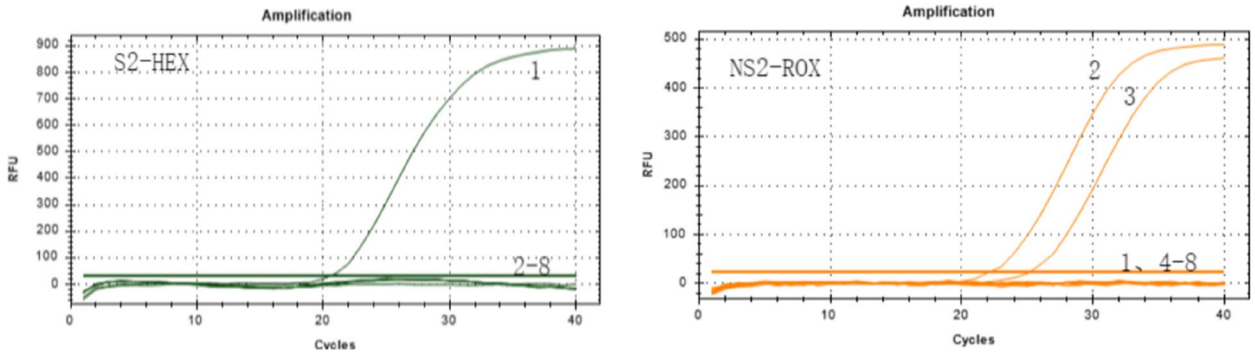

Fig. 6 Results of specificity of two single qPCR.1,*Brucella* S2 vaccine strain DNA;2,*Brucella* A19 vaccine strain DNA;3,*Brucella* Rev.1 vaccine strain DNA;4–7,*Escherichia coli* DNA, *Salmonella* DNA, *Pediococcus acidilactici* DNA, *Streptococcus* DNA;8, Negative control

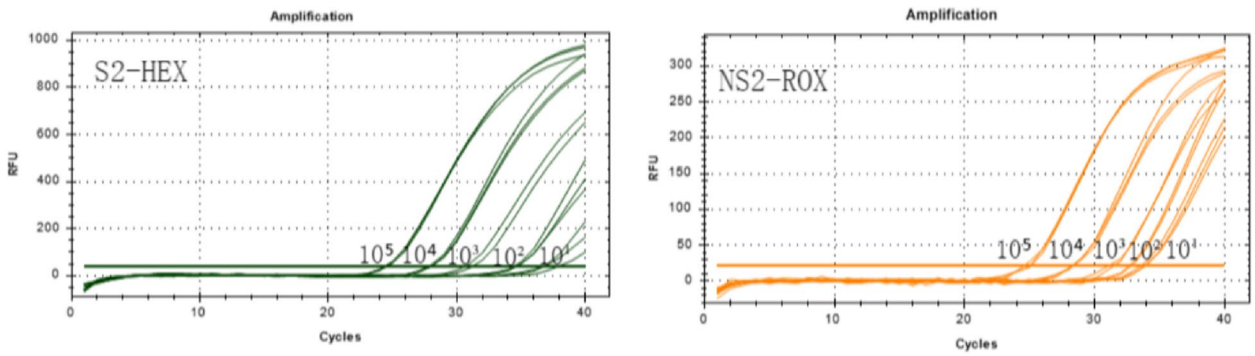

Fig. 7 Results of sensitivity experiment of two single qPCR.The left figure shows the sensitivity results of S2-HEX: The template concentrations were 10^5–10^1 copies/μL, respectively.The right figure shows the sensitivity results of NS2-ROX:The template concentrations were 10^5–10^1 copies/μL, respectively

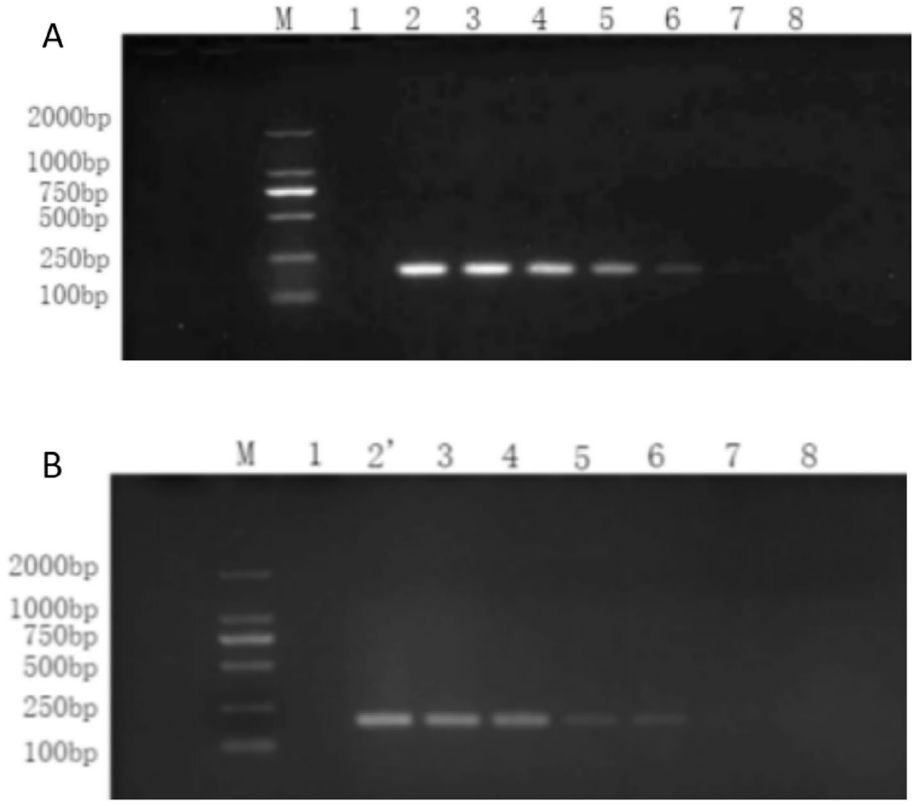

Fig. 8 Results of sensitivity experiment of PCR.M**,**DL 2000 DNA Marker, **A** lane 1, negative control,lane2,DNA positive control of S2 vaccine strain,3 ~ 8,S2 template concentration1 × 10^6 copies/μL、1 × 10^5 copies/μL、1 × 10^4 copies/μL、1 × 10^3 copies/μL、1 × 10^2 copies/μL、1 × 10^1 copies/μL; **B** lane 1, negative control**,**lane **2',**A19 vaccine strain positive control,3 ~ 8, A19 template concentration1 × 10^6 copies/μL、1 × 10^5 copies/μL、1 × 10^4 copies/μL、1 × 10^3 copies/μL、1 × 10^2 copies/μL、1 × 10^1 copies/μL

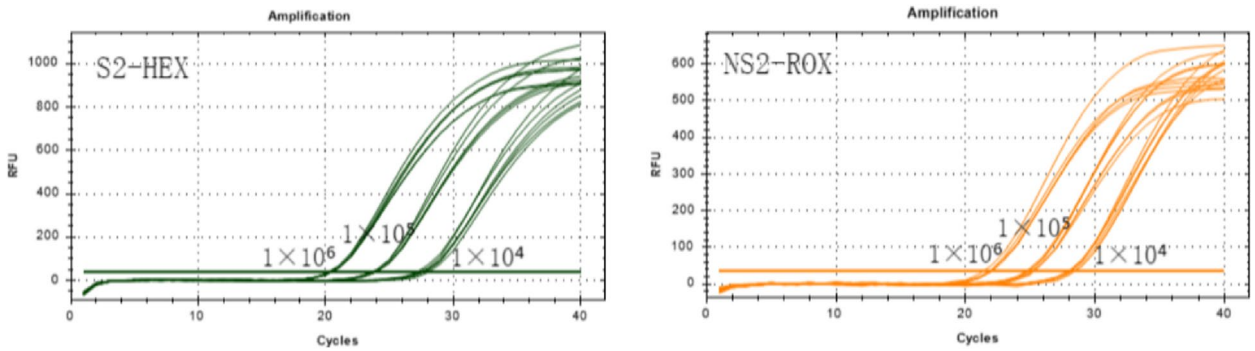

Fig. 9 Results of repeatability experiment of two single qPCR.The left image shows the S2-HEX repeatability results:The template concentrations were 1 × 10^6 copies /μL, 1 × 10^5 copies /μL and 1 × 10^4 copies /μL; The right image shows the S2-HEX repeatability results:The template concentrations were 1 × 10^6 copies /μL, 1 × 10^5 copies /μL and 1 × 10^4 copies /μL

of S2 probe and NS2 probe was 0.8μL, the Ct values of S2-HEX and NS2-ROX amplification curves were the smallest. The optimization results of duplex qPCR reaction conditions are shown in Fig. 10, and the optimized reaction system is shown in Supplementary File 9.

Results of duplex qPCR standard curve establishment

With the optimized reaction conditions, the duplex qPCR standard curve was obtained by repeated experiments with two different concentrations of mixed plasmids as DNA templates (Fig. 11). The results of

Table 5 Repeatability experimental data of two single qPCR

probe	template concentration(copies/μL)	Intragroup coefficient of variation		Intergroup coefficient of variation	
		Average number	Coefficient of variation (%)	Average number	Coefficient of variation (%)
S2-HEX	1×10^4	27.64	0.51	28.49	0.44
	1×10^5	24.35	0.54	25.18	0.57
	1×10^6	20.38	0.42	21.91	0.39
NS2-ROX	1×10^4	28.45	0.45	27.82	0.46
	1×10^5	24.87	0.48	25.00	0.57
	1×10^6	21.61	0.43	20.79	0.43

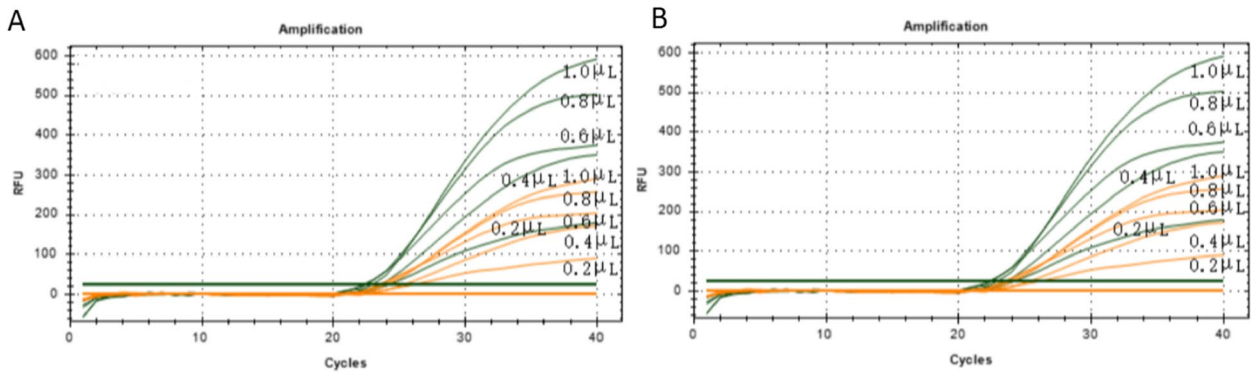

Fig. 10 Optimization of duplex qPCR reaction conditions.A,Primer concentration optimization: the amount of primers was 0.2μL,0.4μL,0.6μL,0.8μL and 1.0μL, respectively;B,Probe concentration optimization: the amount of probe was 0.2μL,0.4μL,0.6μL,0.8μL and 1.0μL, respectively. The S2-HEX group was the green curve and the NS2-ROX group was the orange curve

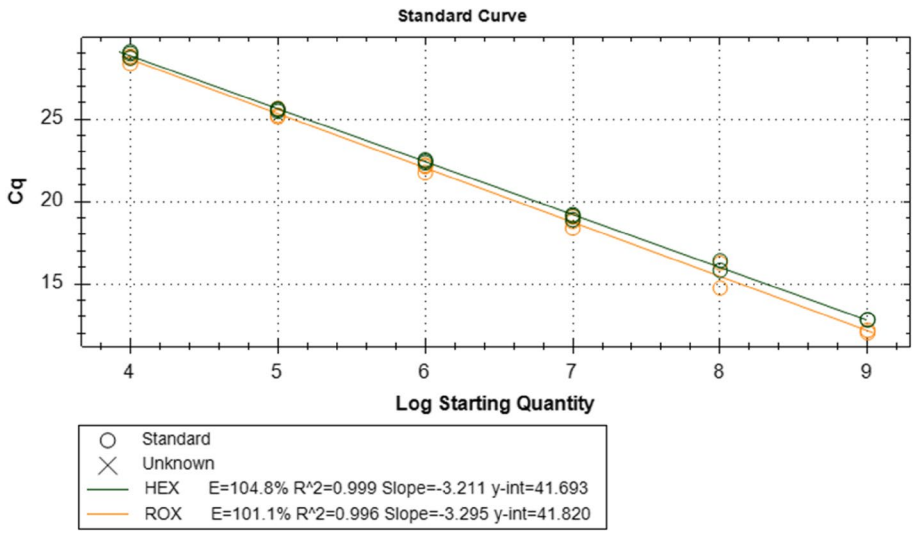

Fig. 11 Duplex qPCR standard curve.the amplification efficiency of S2-HEX standard curve was 104.8%, and R^2 was 0.999. The amplification efficiency of NS2-ROX standard curve was 101.1%, and $R.^2$ was 0.996

standard curve showed that the amplification efficiency of S2-HEX standard curve was 104.8%, and R^2 was 0.999. The amplification efficiency of NS2-ROX standard curve was 101.1%, and R^2 was 0.996. The results of duplex qPCR showed that the repeatability was high and the linear relationship was good, which could be used for the identification and analysis of subsequent samples.

Experimental results of specificity and identification effect of duplex qPCR

Two standard positive mixed plasmids were used as the positive control group, and RNase-free Water was used as the negative control group. The *Brucella* A19 vaccine strain DNA,*Brucella* S2 vaccine strain DNA,*Brucella* Rev1 vaccine strain DNA and genomic DNA extracted from *Escherichia coli*, *Salmonella*, *Pediococcus acidilactici* and *Streptococcus* were used as templates for duplex qPCR detection. The results showed that the *Brucella* S2 vaccine strain DNA had an amplification curve and Ct value at S2-HEX, but no amplification curve at NS2-ROX. The DNA of *Brucella* A19 and *Brucella* Rev.1 vaccine strains showed amplification curve and Ct value at NS2-ROX, but no amplification curve and Ct value at S2-HEX. The genomic DNA of *Escherichia coli*, *Salmonella*, *Pediococcus acidilactici* and *Streptococcus* did not show amplification curves, showing negative results (Fig. 12). The results showed that the method had good specificity and could identify *Brucella* S2 vaccine strain and natural virulent strain.

Results of duplex qPCR sensitivity assay

The optimized reaction conditions were used to detect mixed recombinant plasmids with template concentrations of 1×10^5 copies / μL, 1×10^4 copies / μL, 1×10^3 copies / μL, 1×10^2 copies / μL, and 1×10^1 copies / μL. The sensitivity of the duplex qPCR was evaluated, and the amplification curve was shown in Fig. 13. The experimental results showed that the minimum limit of detection for S2-HEX and NS2-ROX could reach 1×10^1 copies / μL, and the sensitivity was high, which proved that the established duplex qPCR differential diagnosis method could meet the requirements of clinical sample detection.

The same mixed template concentration was used for conventional PCR amplification, and gel electrophoresis was used for sensitivity detection. The results showed that the minimum limit of detection was 1×10^3 copies / μL, and the sensitivity of qPCR method was about 100 times higher than that of conventional PCR, as shown in Fig. 14.

Duplex qPCR repeatability test results

The mixed recombinant plasmids with concentrations of 1×10^6 copies / μL, 1×10^5 copies / μL, and 1×10^4 copies / μL were used as templates to perform repetitive intragroup and intergroup experiments and replace different batches of reaction enzymes. The experimental results showed that the CV of Ct value within the group was less than 0.5%, and the CV of Ct value between groups was less than 0.7%, indicating that the duplex qPCR detection method established in this study had good repeatability. The results are shown in Table 6.

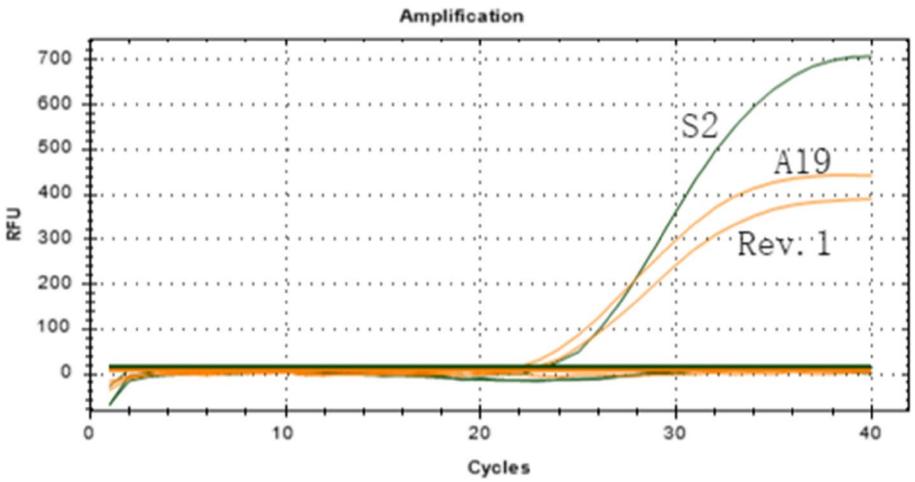

Fig. 12 Duplex qPCR amplification curve of specificity and identification effect.The DNA of the *Brucella* S2 vaccine strain had an amplification curve at S2-HEX and no amplification curve at NS2-ROX. The DNA of *the Brucella* A19 and *Brucella* Rev.1 vaccine strains had amplification curves at NS2-ROX and no amplification curves at S2-HEX. There was no amplification curve in the genomic DNA of *Escherichia coli, Salmonella, Pediococcus acidilactici and Streptococcus*

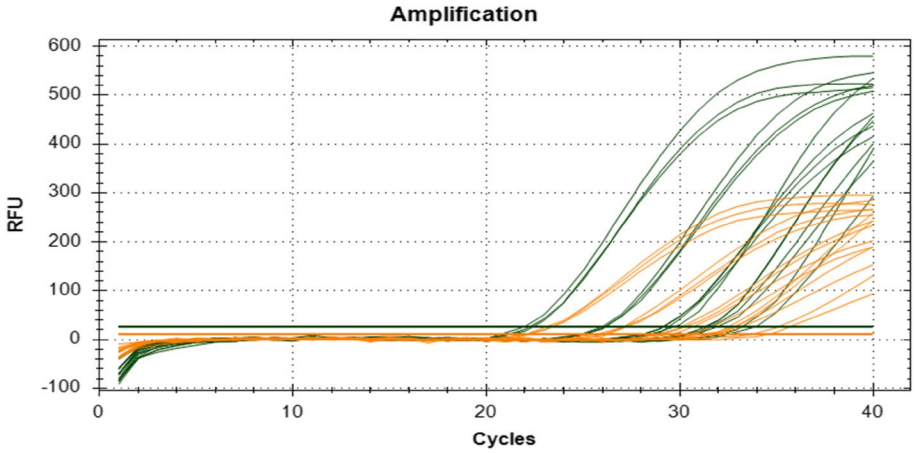

Fig. 13 Duplex qPCR amplification curve of sensitivity experiment.Note: The two mixed plasmids were 1×10^5–1×10^1 copies / μL from left to right. The S2-HEX group was the green curve and the NS2-ROX group was the orange curve

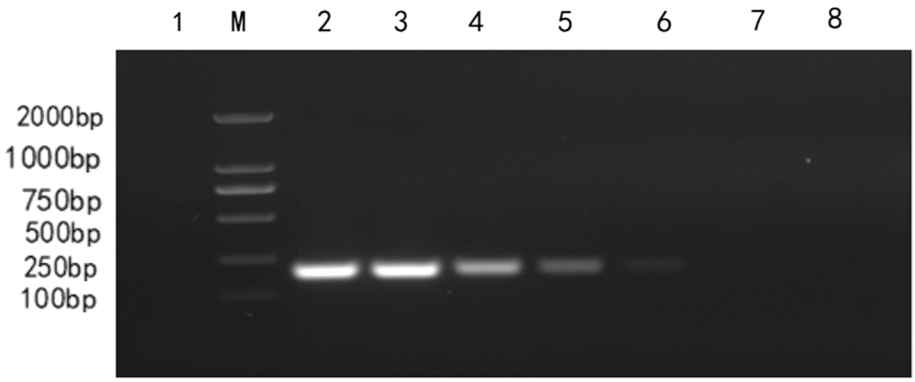

Fig. 14 The results of PCR sensitivity test of mixed template. M:DL 2000 DNA Marker, lane 1:negative control, lanes 2:mixed DNA template positive control, 3~ 8: mixed DNA template concentration 1×10^6copies / μL, 1×10^5copies/ μL, 1×10^4copies / μL, 1×10^3copies / μL, 1×10^2copies/ μL, 1×10^1copies / μL

Application results of duplex qPCR method for differential detection of Brucella S2 vaccine strain

The results of Brucella in whole blood bacteria resident rule

The results of Rose-Bengal plate-agglutination test, tube agglutination test and iELISA of 30 selected goat were all negative.

Based on the established duplex qPCR differential diagnosis method for the *Brucella* S2 vaccine strain, the bacterial residence rule of the recommended dose group after immunization with the *Brucella* S2 vaccine was shown in Figs. 15 and 17. The Ct values of bacterial residence are shown in Tables 7 and 8. The rule of bacterial residence in the double dose group: as shown in Figs. 16 and 17 and the Ct value of bacterial residence is shown in Tables 9 and 10.

By Tables 7 and 8, it can be seen that *Brucella* nucleic acid can be detected by this method after 3 days of oral recommended dose *Brucella* S2 vaccine immunization, and the Ct value is the lowest after 15 days of immunization. *Brucella* nucleic acid can still be detected at 180 days and 210 days after immunization, and *Brucella* nucleic acid is not detected at 240 days after immunization. Tables 9 and 10 show that *Brucella* nucleic acid can also be detected by this method after 3 days of oral double dose *Brucella* S2 vaccine immunization, and the Ct value is the lowest after 15 days of immunization. *Brucella* nucleic acid can still be detected at 180 days after immunization, and *Brucella* nucleic acid is not detected at 210 days and 240 days after immunization.

Detection results of Brucella vaccine bacteria residence in goat milk

Using the established duplex qPCR differential diagnosis method of *Brucella* S2 vaccine strain, 5 samples were

Table 6 Duplex qPCR repeated experimental results within and between groups

probe	template concentration (copies/μL)	Intragroup coefficient of variation		Intergroup coefficient of variation	
		Mean (Ct value)	Coefficient of variation (%)	Mean (Ct value)	Coefficient of variation (%)
S2-HEX	10^4	28.75	0.47	29.25	0.61
	10^5	25.46	0.32	25.88	0.53
	10^6	22.15	0.34	22.66	0.52
NS2-ROX	10^4	28.46	0.14	28.81	0.30
	10^5	25.44	0.31	25.80	0.49
	10^6	22.12	0.39	22.67	0.44

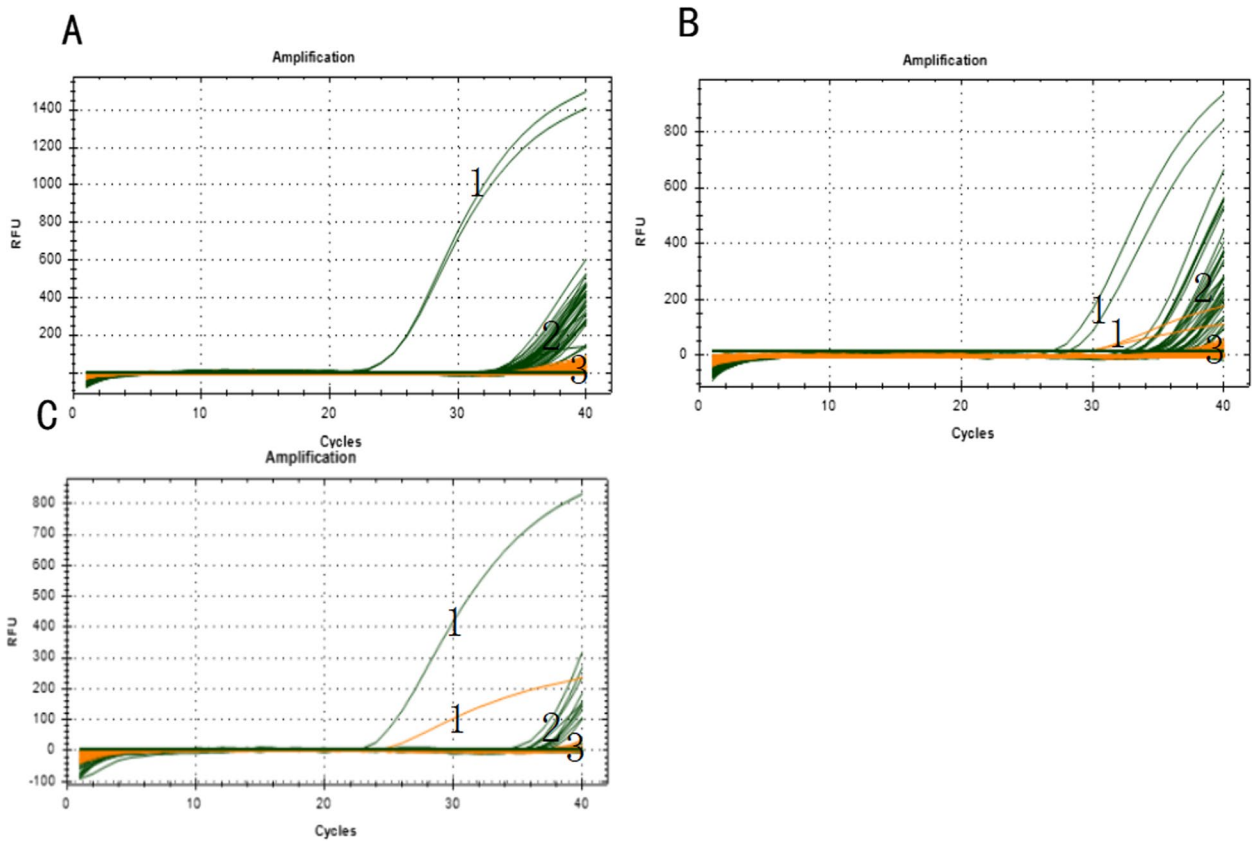

Fig. 15 **A** 0~60 d recommend dose amplification curve1, **B** 0~60 d recommend dose amplification curve2, **C** 90 d and 120 d recommend dose amplification curve.1:Positive control,2:Partial samples,3:Partial samples andnegative controls

detected to be positive for *Brucella* S2 vaccine strain in 10 samples of *Brucella* antibody-positive goat milk, and 2 samples were detected to be positive for *Brucella* S2 vaccine strain mixed with wild strain. The amplification curve results are shown in Fig. 18(A), and the Ct value is shown in Table 11. Among the selected 10 negative goat milk controls, 2 samples were detected to be positive for the *Brucella* S2 vaccine strain, and 1 sample was detected to be positive for the *Brucella* S2 vaccine strain mixed with the wild strain. The results of the amplification curve are shown in Fig. 18(B), and the results of the Ct value are shown in Table 12.

Clinical sample detection

A total of 64 sheep blood samples were detected by the established duplex qPCR differential diagnosis method for the *Brucella* S2 vaccine strain. A total of 64 *Brucella* positive samples were detected, including 44 *Brucella* S2 vaccine positive samples and 20 mixed infection samples. The results are shown in Fig. 19.

Table 7 Recommend dose bacterial residual Ct value1

Days	Recommend dose									
	1		2		3		4		5	
	HEX	ROX	HEX	ROX	HEX	ROX	HEX	ROX	HEX	ROX
Immunization 0 d	-	—	38	—	38.07	—	—	—	36.38	—
Immunization 3 d	32.65	—	34.42	—	35.14	—	32.95	—	34.27	—
Immunization 5 d	33.76	—	32.62	—	33.02	—	32.08	—	31.87	—
Immunization 7 d	33.29	—	32.44	—	34.52	—	32.75	—	34.27	—
Immunization 15 d	32.65	—	31.28	—	31.64	—	32.75	—	28.55	—
Immunization 21 d	34.09	—	33.66	—	33.98	—	33.03	—	32.93	—
Immunization 30 d	33.53	—	34.41	—	31.95	—	32.97	—	34.10	—
Immunization 60 d	33.14	—	34.48	—	35.04	—	34.8	—	34.38	—
Immunization 90 d	—	—	36.14	—	35.34	—	36.16	—	36.53	—
Immunization 120 d	—	—	35.31	—	37.21	—	36.26	—	—	—
Immunization 150 d	37.77	—	38.57	—	38.57	39.91	37.82	—	—	—
Immunization 180 d	37.98	—	38.01	37.30	—	—	38.12	—	36.52	—
Immunization 210 d	—	—	—	—	—	—	—	—	37.88	—
Immunization 240 d	—	—	—	—	—	—	—	—	—	—

Table 8 Recommend dose bacterial residual Ct value 2

Days	Recommend dose									
	6		7		8		9		10	
	HEX	ROX	HEX	ROX	HEX	ROX	HEX	ROX	HEX	ROX
Immunization 0 d	—	38.59	—	36.78	—	—	—	—	—	—
Immunization 3 d	34.92	35.44	34.61	38.98	33.42	—	36.45	—	33.30	—
Immunization 5 d	34.67	35.04	36.56	39.91	34.54	—	—	—	36.10	—
Immunization 7 d	35.25	36.10	35.72	39.19	34.96	—	32.41	—	34.40	—
Immunization 15 d	31.72	37.39	33.35	—	30.02	—	31.06	—	33.54	—
Immunization 21 d	33.53	36.97	32.51	38.13	34.20	—	34.58	—	36.57	—
Immunization 30 d	34.96	37.28	34.54	—	36.13	—	37.33	—	—	—
Immunization 60 d	37.54	—	34.43	—	38.12	—	35.77	—	35.76	—
Immunization 90 d	35.89	38.59	36.16	—	35.36	—	36.23	—	32.85	—
Immunization 120 d	37.09	—	—	—	34.26	—	—	—	38.2	—
Immunization 150 d	—	—	35.43	—	36.32	—	36.47	—	38.85	—
Immunization 180 d	—	—	34.53	—	—	—	38.46	—	—	38.57
Immunization 210 d	—	—	35.02	—	—	—	—	—	—	—
Immunization 240 d	—	—	36.98	—	—	—	—	—	—	—

Discussion

Brucellosis is an important zoonotic infectious disease that endangers public health security and the healthy development of animal husbandry [24]. It is widely popular around the world, especially in countries and regions with more developed agriculture and animal husbandry. Because the symptoms of brucellosis are complex and diverse, and similar to many other diseases, the diagnosis requires laboratory testing [25]. At present, the laboratory diagnosis methods of brucellosis mainly include etiological detection and serological detection. Bacterial culture is considered to be the gold standard for diagnosis. Molecular biology method, nucleic acid amplification detection, can detect *Brucella* within a few hours, with high sensitivity and specificity. The methods of nucleic acid amplification detection include conventional PCR technology, nested PCR, real-time PCR, multiple real-time PCR and so on [26]. At present, the vaccines that can be used to prevent sheep brucellosis in China include *Brucella melitensis* Rev.1 strain, *Brucella melitensis* M5 /

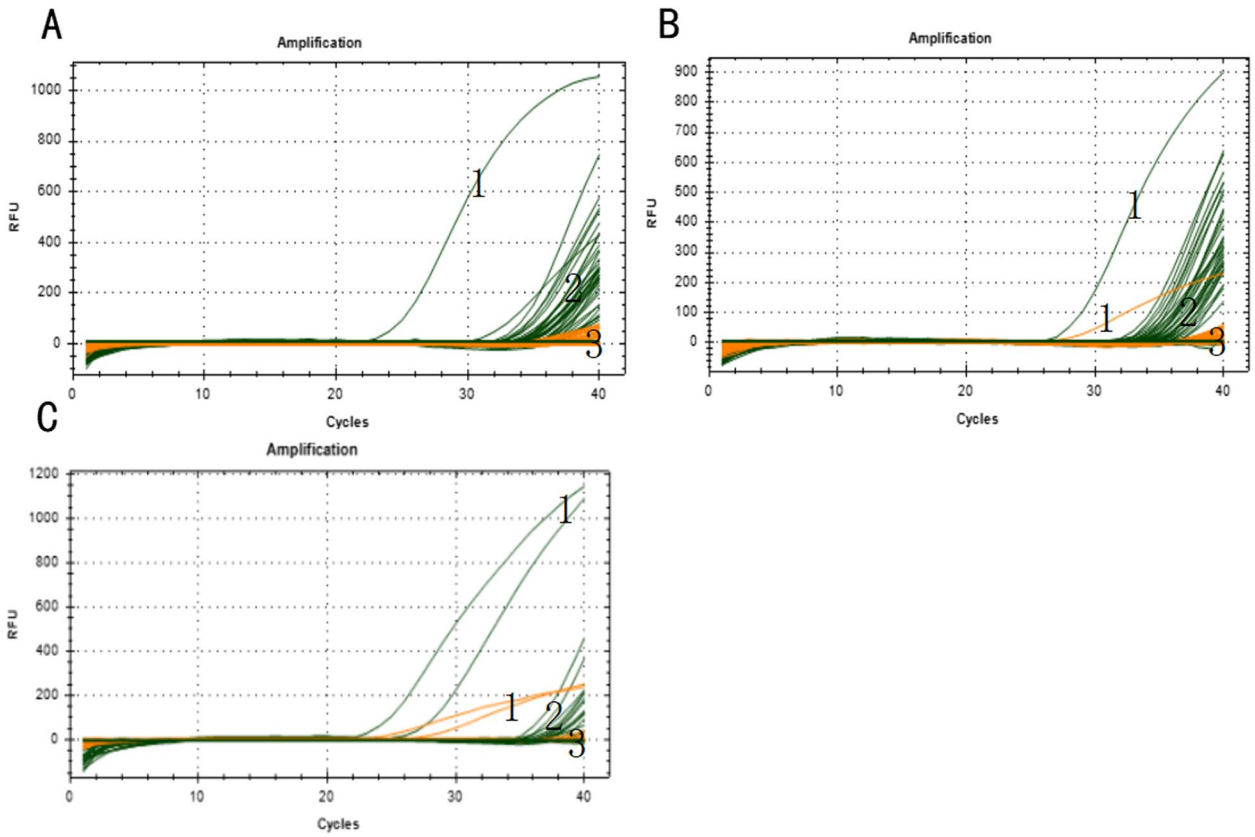

Fig. 16 **A** 0~60 d double dose amplification curve1, **B** 0~60 d double dose amplification curve2, **C** 90 d and 120 d double dose amplification curve.1:Positive control,2:Partial samples,3:Partial samples and negative controls

M5-90 strain, *Brucella suis* S2 strain and other conventional attenuated vaccines. The shortcomings of these vaccines are as follows: First, the safety is not enough, and the injection of pregnant animals will cause abortion; second, the vaccine strain and the wild strain are both smooth, which interferes with clinical diagnosis after immunization [27]. Therefore, the prevention and control of the disease is of great significance for ensuring the safety of meat and dairy products [28].

TaqMan qPCR is a nucleic acid quantitative technology based on PCR qualitative technology. It is a method that has been widely used in nucleic acid detection and quantification. The TaqMan qPCR method is based on the use of specific primers and probes to bind to the target DNA sequence and generate a fluorescent signal during the amplification process. Compared with the qPCR directly added with fluorescent dyes, the specificity is stronger.Compared with conventional TaqMan probes, MGB probes have two major characteristics: one is that the 3' end of the probe is labeled with a quencher molecule that does not emit itself to replace conventional fluorescent markers such as TAMRA that can emit light. This reduces the fluorescence background and greatly improves the fluorescence spectral resolution. Second, MGB was added to the 3' end of the probe. MGB can stabilize the hybridization between the probe and the template and increase the annealing temperature of the probe. On the one hand, the length of the probe is shortened, the distance between the fluorescent group and the quenching group is closer, and the quenching effect is better. On the other hand, it also improves the specificity of the probe, making the results more accurate and higher resolution.Chang et al.established the TaqMan qPCR detection method based on *Brucella IS711 gene* sequence, with a limit of detection 3.8 copy/μL, which is characterized by high sensitivity, strong specificity, and simple operation, and can be used as a reliable clinical detection method for *Brucella* [21, 29]. Liu et al.designed primers and probes with *Brucella Bcsp31* gene as the target gene to evaluate the sensitivity, repeatability and specificity of the droplet digital PCR (ddPCR) method for detecting *Brucella*. The results showed that the established ddPCR method for detecting *Brucella* had high sensitivity, the minimum limit of detection was 100 copies / μL, and the detection of *Brucella* showed good specificity and accuracy [30]. Suman et al.developed

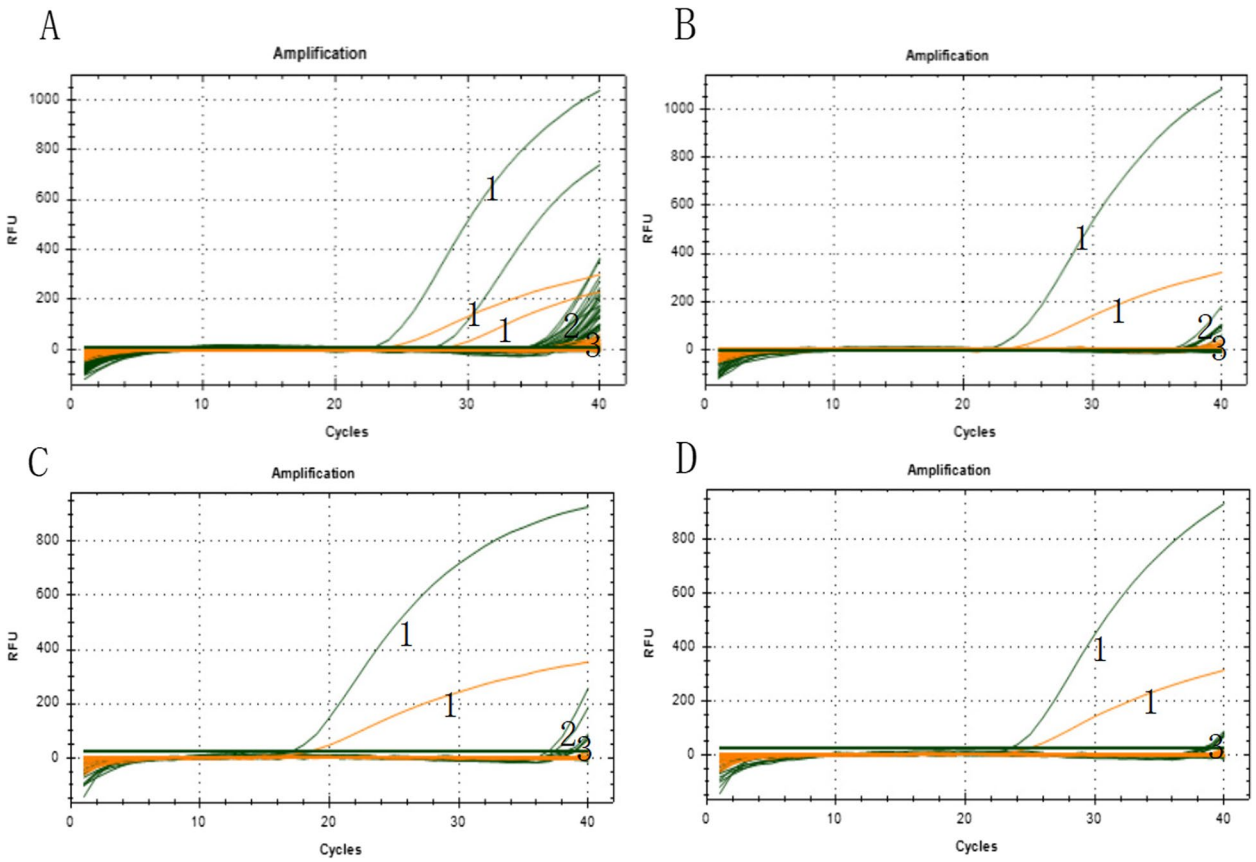

Fig. 17 **A** 150 d recommend and doubled dose amplification curve, **B** 180 d recommend and doubled dose amplification curve, **C** 210 d recommend and double dose amplification curve, **D** 240 d recommend and double dose amplification curve**.**1:Positive control,2:Partial samples,3:Partial samples and negative controls. Note : The S2 vaccine strain (HEX) is a green curve, and the wild strain (ROX) is an orange curve

Table 9 Double dose bacterial residual Ct value 1

Days	Double dose									
	16		17		18		19		20	
	HEX	ROX	HEX	ROX	HEX	ROX	HEX	ROX	HEX	ROX
Immunization 0 d	—	—	—	—	—	38.97	—	37.92	—	—
Immunization 3 d	34.42	—	34.43	—	33.97	37.21	33.18	—	34.74	38.80
Immunization 5 d	33.81	38.63	34.09	—	33.68	—	33.27	—	35.21	—
Immunization 7 d	33.12	—	34.35	—	33.48	37.76	33.13	37.52	33.98	—
Immunization 15 d	32.47	—	31.77	—	30.16	38.28	31.98	39.89	32.20	—
Immunization 21 d	34.45	—	32.28	—	32.33	38.59	33.89	—	34.28	—
Immunization 30 d	34.75	—	35.75	—	34.25	—	34.41	39.30	35.57	37.26
Immunization 60 d	36.55	—	36.10	—	34.61	—	36.18	—	35.27	—
Immunization 90 d	36.25	—	35.15	—	36.38	—	35.98	—	35.48	—
Immunization 120 d	—	—	36.21	37.46	37.15	—	—	—	36.64	—
Immunization 150 d	—	39.91	37.39	—	—	—	36.32	39.76	38.94	—
Immunization 180 d	—	—	38.09	—	37.91	—	34.72	—	37.11	—
Immunization 210 d	—	—	—	—	—	—	—	—	—	—
Immunization 240 d	38.71	—	—	—	38.03	—	38.55	—	—	—

Table 10 Double dose bacterial residual Ct value 2

Days	Double dose									
	21		22		23		24		25	
	HEX	ROX	HEX	ROX	HEX	ROX	HEX	ROX	HEX	ROX
Immunization 0 d	—	37.98	—	—	38.75	37.26	—	—	37.51	38.13
Immunization 3 d	34.53	38.81	33.18	—	36.16	37.47	34.22	—	38.44	—
Immunization 5 d	33.36	—	31.27	—	34.90	—	34.11	38.81	39.98	—
Immunization 7 d	32.93	—	33.01	—	34.28	37.98	34.49	37.91	—	—
Immunization 15 d	33.03	—	31.79	—	31.61	—	33.42	—	—	—
Immunization 21 d	34.59	—	30.59	—	32.25	—	34.38	—	—	—
Immunization 30 d	33.65	—	32.25	37.30	33.30	—	35.01	—	—	—
Immunization 60 d	34.14	—	32.15	—	33.45	—	35.85	38.01	—	—
Immunization 90 d	38.19	—	—	—	37.51	—	37.54	—	36.04	—
Immunization 120 d	37.29	—	36.72	—	—	—	36.32	—	—	—
Immunization 150 d	36.87	—	36.19	—	36.74	—	39.76	38.42	39.64	—
Immunization 180 d	36.73	—	37.40	—	37.71	—	—	—	—	—
Immunization 210 d	—	—	—	—	—	—	—	39.01	—	—
Immunization 240 d	—	—	38.28	—	—	—	—	—	—	—

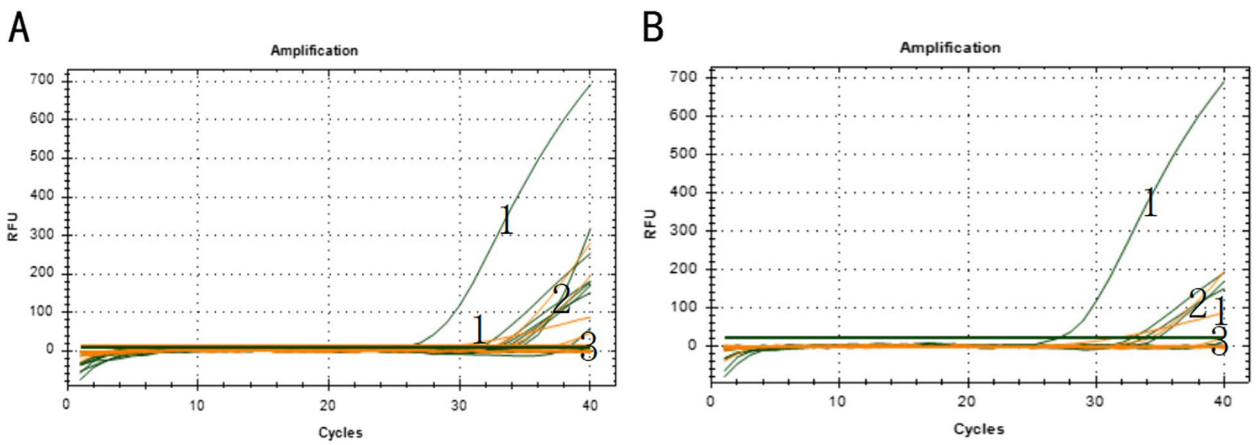

Fig. 18 **A** Residual amplification curves of bacteria in positive goat milk, **B** Residual amplification curves of bacteria in negative goat milk.1:Positive control,2:Partial samples,3:Partial samples and negative controls

Table 11 Bacteria-resident Ct values in positive goat's milk

Number	HEX	ROX
1	34.11	32.78
2	32.99	—
3	32.45	36.38
4	33.08	32.36
5	32.57	—
6	38.51	—
7	—	—
8	34.38	—
9	31.40	—
10	37.46	—

Table 12 Bacteria-resident Ct values in negative goat's milk

Number	HEX	ROX
Y1	34.64	30.81
Y2	—	—
Y3	—	—
Y4	—	—
Y5	—	—
Y6	33.59	—
Y7	—	—
Y8	32.72	—
Y9	—	—
Y10	—	—

qPCR primers targeting the *Omp31 gene* and designed primers using the discontinuous conserved sequence of the *Omp31 gene*. The *Omp31* probe was designed by connecting a 6-FAM reporter dye at the 5 ' end and a BHQ-1 quencher at the 3 ' end. The TaqMan ® probe real-time detection method of *Omp31 gene* has the advantages of simplicity, rapidity, sensitivity and specificity. As low as 100 fg of *Brucella* nucleic acid can be detected [31]. Nan Wenlong et al.designed *IclR* primers based on the deletion of 25 bases (bp) in the genome of *Brucella suis* S2. In the published papers (Bricker et al., 1994), *IS711* primers were screened, *IS711* primers were used to identify wild strains of *Brucella suis* type 1, *IclR* primers and *IS711* primers were used to identify *Brucella suis* S2 vaccine strains. It was verified that DNA can be detected at a concentration of as low as 60 fg. The duplex PCR method is sensitive and specific. It can distinguish *Brucella suis* biotype *S2 and Brucella suis* biotype 1 from other common species and biotypes of *Brucella*, four *Brucella* vaccine strains (104 M, BA19, S19 and M5) and four non-*Brucella* (*Escherichia coli K99*, *Pasteurella multocida C48-1*, *Streptococcus suis ST171*, *Pseudomonas aeruginosa DI-1*) [32].

In this study, the whole genome sequence of *Brucella* S2 vaccine strain and the whole genome sequence of natural virulent strain 1330 were compared by SnapGene software. Firstly, the differential sequences between the two *Brucella* strains were found. A total of 25 differential gene fragments were screened out. The 25 differential gene fragments were subjected to BLAST specificity verification on NCBI, and 23 of them had low coverage and no specificity. Only the 25 bp deletion at 246,928 on chromosome I of the *Brucella* S2 vaccine strain and the 1 bp deletion at 1,064,715 on chromosome II of the *Brucella* S2 vaccine strain have a high coverage, and can distinguish the *Brucella* S2 vaccine strain from other *Brucella* strains, of which the 25 bp deletion fragment has been reported.Therefore, in this experiment, the 1 bp deletion on chromosome II of *Brucella* S2 vaccine strain was selected as the nucleic acid marker sequence. After PCR amplification and sequencing analysis, it was determined that it could be used as the marker sequence of *Brucella* S2 vaccine strain for subsequent differential diagnosis. This paper can provide a method for the differential diagnosis of *Brucella* S2 vaccine strain. By designing primers and probes for the specific sequence of the *Brucella* S2 vaccine strain, two S2-HEX probes were designed. After the detection of S2-HEX-2 probe by qPCR, the *Brucella* S2 vaccine strain could not be distinguished from other strains, and it was not specific. Therefore, S2-HEX-1 probe was selected for subsequent experiments.Firstly, a single qPCR detection method for

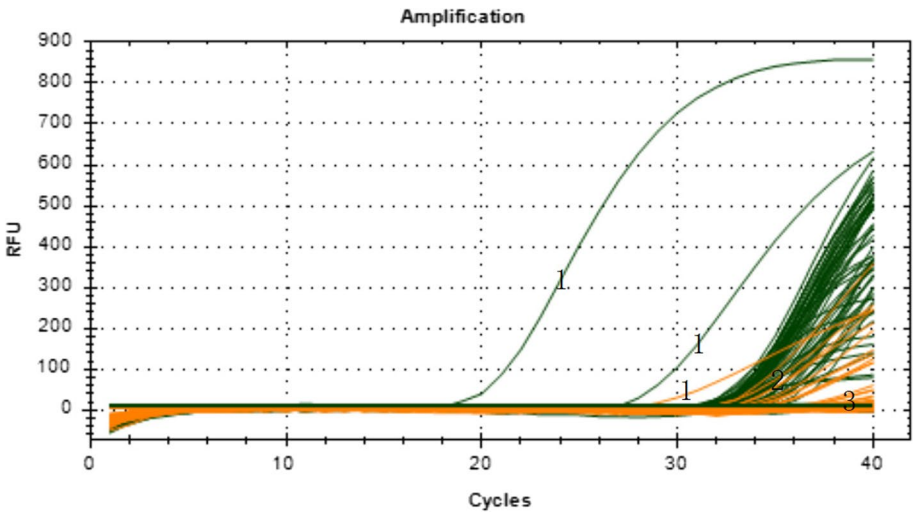

Fig. 19 The qPCR amplification curve of clinical samples.1:Positive control,2:Partial samples,3:Partial samples and negative controls

Brucella was established. After verifying its feasibility through experiments, two single qPCR detection methods for *Brucella* were combined to verify the compatibility and matching effect between primers and probes. Finally, a duplex qPCR differential diagnosis method for specific deletion sites of *Brucella* S2 vaccine strain was optimized and established.

The established duplex qPCR detection method of *Brucella* S2 vaccine strain can effectively identify whether the positive samples detected by Rose-Bengal plate-agglutination test and tube agglutination test belong to S2 vaccine immune antibody or other vaccine antibody and wild virus infection antibody, which provides a certain reference for quarantine and purification of positive livestock.By immunizing goat with normal dose and double dose of *Brucella* S2 vaccine, it can be shown that *Brucella* nucleic acid can be detected in the blood three days after immunization, and the Ct value is the smallest on the 15 th day, indicating that the bacterial content in the body is the largest, and then the amount of bacteria begins to decrease, and the retention of *Brucella* nucleic acid in the animal body is not detected on the 240 th day after immunization.The presence of the *Brucella* S2 vaccine strain and other vaccine and wild strains in goat milk was detected, indicating that *Brucella* can exist in the mammary gland tissue of infected dairy goats and then enter the goat milk. In the negative samples, some goat were detected by *Brucella* S2 vaccine duplex qPCR differential diagnosis method to detect *Brucella* S2 vaccine nucleic acid or other vaccine and wild virus nucleic acid. It is speculated that the experimental goat may be orally administered *Brucella* S2 vaccine, resulting in aerosol pollution in the air, so that the non-immunized goat showed *Brucella* S2 vaccine positive, while the wild strain infection may be caused by vertical transmission of the goat by the female livestock through the birth canal infection or horizontal transmission. Because the *Brucella* antibody positive herd was not raised in time, the negative goat was infected. This indicates that immunized animals and non-immunized animals should be kept in isolation, and the enclosure of immunized animals should pay attention to the environment, disinfection of utensils, etc. To prevent aerosol pollution.From the establishment of *Brucella* S2 vaccine strain differential diagnosis method to clinical sample detection, the establishment of the method to the application of the method was realized. It can solve the technical problems of the lack of differential diagnosis methods of brucellosis in production practice, provide technical support for the effective control of the disease, and is of great significance for the purification and prevention and control of the disease.

Conclusions

In this study, TaqMan probe-based qPCR technology was used to study the molecular biological identification method of *Brucella* by combining the differential genes between the *Brucella* S2 vaccine strain and the natural virulent strain.Compared with serological detection and conventional PCR detection,this method has the characteristics of less DNA consumption, 100 times higher sensitivity, short detection time, and about one and a half hours from the configuration of the system to the end of the program operation. And Only one pair of primers and two probes can be detected. The minimum limit of detection can reach 10 copies/μL.and it can effectively identify *Brucella* S2 vaccine strain and other *Brucella* strains.It provides a new method for the differential diagnosis of the *Brucella* S2 vaccine strain, which can contribute to the prevention and control of *Brucella*.After immunizing dairy goats with the *Brucella* S2 vaccine,the results showed that the nucleic acid of vaccine bacteria could be detected in the recommended dose group for 3 days, and the bacterial content was the largest at 15 days. The bacterial content began to decrease at 30 days, and all nucleic acid tests were negative at 240 days. The nucleic acid of vaccine bacteria could be detected in the double dose group at 3 d, the bacterial content was the highest at 15 d, the bacterial content began to decrease at 21 d, and the nucleic acid detection was negative at 210 d and 240 d. *Brucella* can also be detected in goat milk by this method, and *Brucella* can be detected in clinical samples.

Abbreviations

PCR	Polymerase chain reaction
qPCR	Quantitative Polymerase Chain Reaction
RBPT	Rose-Bengal plate-agglutination test
ELISA	Enzyme linked immunosorbent assay
cELISA	Competitive enzyme linked immunosorbent assay
iELISA	Indirect enzyme linked immunosorbent assay
GICA	Gold Immuno-chromatographic Assay
LAMP	Loop-Mediated Isothermal Amplification
MRT	Whole milk ring agglutination test
FPA	Fluorescence polarization assay
SAT	Tube agglutination test
RPA	Recombinase polymerase amplification
CFT	Complement fixation test
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
ERIC-PCR	Enterobacterial repetitive intergenic consensus
RT-LAMP	Reverse Transcription Loop-Mediated Isothermal Amplification
AMOS-PCR	Abortus melitensis ovis Suis polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04635-w.

Supplementary Material 1.

Acknowledgements
Thank you for the samples provided by the breeding sites and the strong support of the staff, and thank all the authors for their contribution to this article.

Authors' contributions
WL and CX designed the research; XR conducted data analysis and wrote manuscripts and clinical sample detection. XR and ZL searched for differential sequences and designed primers and probes. Finally, the diagnostic method was established.YT was responsible for the detection of bacterial retention.YY were responsible for the detection of milk samples.WG instructed the writing. All authors read and approved the final manuscript. Note : XR, ZL, YT contributed equally to this work and are co-first authors of this manuscript.

Funding
This work was funded by the Inner Mongolia Autonomous Region First Class Discipline Research Special Project (YLXKZX-NND-012),the Key technologies of dairy goat germplasm innovation and intensive precision breeding(2021ZD0023) and the Comparative genomics study of *Brucella* virulent strains and vaccine strains and establishment of diagnostic methods(NJZZ19041).

Data availability
The datasets supporting the results of this document are contained within the article. Any additional data may be requested to the corresponding author.

Declarations

Ethics approval and consent to participate
The experimental animals used in this project were treated in strict accordance with the Guidelines for Animal Ethics and Experimentation of thePeople's Republic of China. The experimental animals were used in accordance with the protocols approved by The Scientifc Ethics Committee of Inner Mongolia Agricultural University (Approval code: NND2022112).Before collecting samples, we have obtained the informed consent of the animal owner.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 21 December 2024 Accepted: 28 February 2025
Published online: 02 April 2025

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