CRISPR-Cas12a Test Strip (CRISPR/CAST) Package: Poverty Family Pasture Health Patron Saint for Livestocks Free from Brucella Infection

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Abstract

Brucellosis is a common zoonotic disease caused by *Brucella*, which causes enormous economic loss and public burden to the epidemic areas. Earlier and precise diagnosis and timely culling of infected animals are crucial to prevent the infection of *Brucella* and the spread of the disease. In recent years, RNA-guided CRISPR/Cas12a nucleases have shown great promise in nucleic acid detection. This research aims to develop a CRISPR/CAST (CRISPR/Cas12a Test strip) package that can rapidly detect *Brucella* nucleic acid on-site screening, especially on the remote family pasture. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and its associated protein 12a (Cas12a), the CRISPR/Cas12a system combined with recombinase polymerase amplification(RPA), and lateral flow read-out. The CRISPR/CAST package can complete the assay of *Brucella* nucleic acid within 30 min under isothermal temperature conditions, with a sensitivity of 10 copies/μl, and no antigen cross-reacting against *Yersinia enterocolitica* O:9 , *Escherichia coli* O157 , *Salmonella enterica serovar* Urbana O:30 , and *Francisella tularensis*. The serum samples of 398 sheep and 100 cattle were tested by CRISPR/CAST package, of which 31 sheep and 8 cattle were *Brucella* DNA positive. The detection rate was consistent with the qPCR and higher than the Rose Bengal Test (RBT, 19 sheep, and 5 cattle were serum positive). CRISPR/CAST package can accurately detect the infected livestock's *Brucella* DNA and accomplish within 30 min, which has the advantages of simple, fast, high sensitivity, and strong specificity, with no window period. Besides, the package needs no expensive equipment, standard laboratory, or professional operators. It is an effective tool for field screening and earlier, rapid diagnosis of *Brucella* infection. A package is an efficient tool for epidemic prevention and control.

CRISPR-Cas12a Test Strip (CRISPR/CAST) Package: Poverty Family Pasture Health Patron Saint for Livestocks Free from *Brucella*Infection

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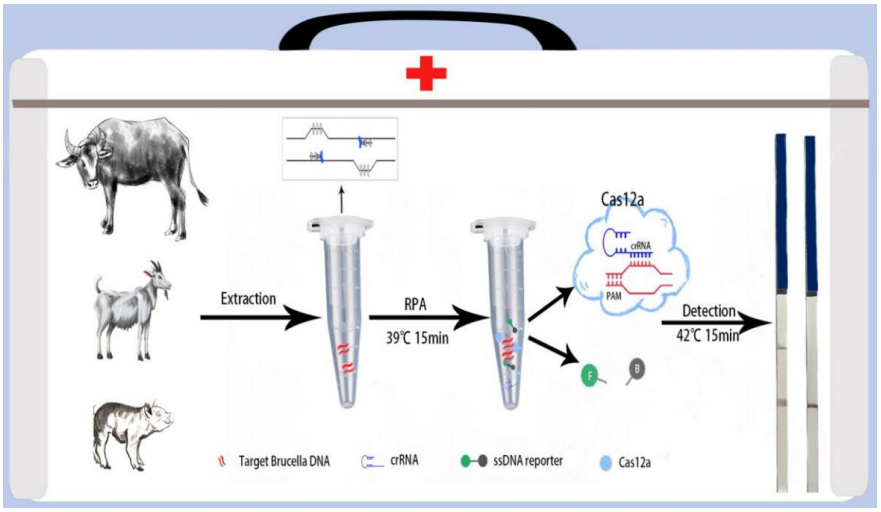

SUMMARY

Brucellosis is a common zoonotic disease caused by *Brucella* , which causes enormous economic loss and public burden to the epidemic areas. Earlier and precise diagnosis and timely culling of infected animals are crucial to prevent the infection of *Brucella* and the spread of the disease. In recent years, RNA-guided CRISPR/Cas12a nucleases have shown great promise in nucleic acid detection. This research aims to develop a CRISPR/CAST (CRISPR/*Cas* 12a *T* est strip) package that can rapidly detect *Brucella* nucleic acid on-site screening, especially on the remote family pasture. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and its associated protein 12a (Cas12a), the CRISPR/Cas12a system combined with recombinase polymerase amplification(RPA), and lateral flow read-out. The CRISPR/CAST package can complete the assay of *Brucella*nucleic acid within 30 min under isothermal temperature conditions, with a sensitivity of 10 copies/μl, and no antigen cross-reacting against *Yersinia enterocolitica* O:9*, Escherichia coli* O157*, Salmonella enterica serovar* Urbana O:30*,* and *Francisella tularensis* . The serum samples of 398 sheep and 100 cattle were tested by CRISPR/CAST package, of which 31 sheep and 8 cattle were*Brucella* DNA positive. The detection rate was consistent with the qPCR and higher than the Rose Bengal Test (RBT, 19 sheep, and 5 cattle were serum positive). CRISPR/CAST package can accurately detect the infected livestock's *Brucella* DNA and accomplish within 30 min, which has the advantages of simple, fast, high sensitivity, and strong specificity, with no window period. Besides, the package needs no expensive equipment, standard laboratory, or professional operators. It is an effective tool for field screening and earlier, rapid diagnosis of*Brucella* infection. A package is an efficient tool for epidemic prevention and control.

KEYWORDS: *Brucella* infection, CRISPR-Cas12a, RPA, Test strip, CRISPR/CAST package

INTRODUCTION

The global burden of *Brucella* infection in livestock is substantial, and conservative estimates are that 300 million of the 1.4 billion worldwide cattle population are infected with the organism (de Figueiredo, Ficht, Rice-Ficht, Rossetti, & Adams, 2015) It threatens the breeding industry and human health and seriously affects the import and export trade of livestock and meat, causing huge economic loss and social burden. Even in developed countries, *Brucella* infection fails to be ultimately eradicated because the infected wildlife can infect persistently and spread to domestic animals (Al Dahouk & Nöckler, 2011; Godfroid, 2018). Currently, serological detection is the primary method to diagnose brucellosis, which depends on the detection of the infected livestock-specific antibodies, indirectly proving the existence of this pathogen. Because the history of *Brucella* exposure and animal immune system, immune response, and the resulting patterns of the antibodies generation and dynamics are variable among individuals, thus can not be the direct evidence to prove the existence of the pathogen (Al Dahouk & Nöckler, 2011). RBT is a card agglutination test with simple operation, rapidity, and high sensitivity. It is suitable for field assay and is usually used as a screening test for brucellosis (Díaz, Casanova, Ariza, & Moriyón, 2011). Nonetheless, *Brucella* has cross-reacting antigens with *Yersinia enterocolitica* O:9*, Escherichia coli* O157*, Salmonella enterica serovar* Urbana O:30*,* and *Francisella tularensis* easily emerging false positives. Therefore, the serological test of brucellosis is not the objective, direct diagnostic evidence (Yagupsky, Morata, & Colmenero, 2019).

Moreover, RBT cannot detect *Brucella* of infected livestock during the window period. PCR is a practical method that can amplify nucleic acid for detecting sample infected microorganisms (Wang & Cui, 2020; Lazcka, Del Campo, & Muñoz, 2007), and PCR has a high sensitivity (Kaden, Ferrari, Alm, & Wahab, 2017). Whereas PCR-based methods have many limitations, such as expensive instruments, matching standard reagents, relying on high-standard laboratories and professional technicians, and the diagnostic standards are not yet unified. Developing countries and rural areas with high morbidity of brucellosis have difficulties implementing PCR as they lack laboratory equipment and professional technicians. Furthermore, PCR is time-consuming and not suitable for rapid on-site screening (Yang & Rothman, 2004). Collectively, finding faster and more effective methods for field nucleic acid assay is urgent.

In recent years, RNA-based guide CRISPR/Cas nucleases have shown great promise in nucleic acid detection with high sensitivity and rapidity. This system has been transformed into an efficient gene-editing tool widely applied in gene editing in eukaryotes (Jinek et al., 2012). Lately, scientists have found that some class II Cas proteins, such as Cas12a (Cpf1), which have the activity of accessory cleaving single-stranded DNA (ssDNA) and applying the CRISPR-Cas system to the nucleic acid assay field (Chen et al., 2018). Zhang et al. discovered the CRISPR-Cpf1 system (Zetsche et al., 2015), which is mediated by a single Cas protein with cleavage activity similar to the CRISPR-Cas9 system in 2015. Compared to Cas9, Cpf1 is a single RNA-guided endonuclease lacking tracrRNA, recognizing T-rich PAM (protospacer-adjacent motif), and Cpf1 introduces a staggered DNA double-stranded break with a 4 or 5-nt 5' overhang. While Cas12a, guided by crRNA, binds to the target sequence and cleaves the target double-stranded DNA, it shows the activity of arbitrary cleavage of single-stranded DNA in the system; According to this discovery, Doudna et al. developed a diagnostic system named DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter) in 2018 (Chen et al., 2018), this system can quickly and instantly detect trace amounts of DNA in samples. The DETECTR is a nucleic acid detection system that combines isothermal amplification, CRISPR-Cas12a, crRNA, and fluorescent reporter groups, with which human papillomavirus (HPV) has been successfully detected. The visualization is essential for the wild application of nucleic acid assay. The same year, Zhang et al. developed "SHERLOCKv2" (Specific High-sensitivity Enzymatic Reporter un-LOCKing Version 2) (Gootenberg et al., 2018), which uses lateral flow strips by observing color changes with naked eyes to interpret the results. Several CRISPR-Cas-based methods have been developed to detect and diagnose infectious and non-infectious diseases (such as cancer) (Wang et al., 2018; Pomeroy et al., 2020; Otten & Sun, 2020) Compared to PCR, DETECTR and SHERLOCKv2 provide another level of ultrasensitive detection method (Mustafa & Makhawi, 2021). Recombinase Polymerase Amplification (RPA) is a new nucleic acid isothermal amplification technology developed in 2006 (Piepenburg, Williams, Stemple, & Armes, 2006), which can realize exponential amplification of template DNA in a short time at an isothermal temperature. Therefore, RPA can get rid of the dependence on the PCR instrument. Just like PCR, RPA also showed non-specific amplifi-

cation (Li, Macdonald, & von Stetten, 2018). Thus, by combining the RPA with the CRISPR-Cas12a system, the single-stranded DNA containing a reporter group is added to the system, target fragment amplification through RPA, Cas12a recognizes and binds to the amplification product, then cleaves the single-stranded probe within the system to release the fluorescent reporter group, target DNA can be detected by capturing the fluorescent signal. The nucleic acid detection test strip adopts a chromatographic double-antibody sandwich method to detect the probes cleavaged by Cas12a. When designing probes, one end of ssDNA is labeled with biotin and the other with 6-carboxyfluorescein (6-FAM) or fluorescein isothiocyanate (FITC). The target fragment of*Brucella* DNA is amplified through isothermal amplification (e.g., RPA, LAMP, RAA), then the amplification products and the labeled ssDNA with the fluorescent report group are cleaved by Cas12a simultaneously. As a result, the test strip can detect the fluorescent signal to identify the *Brucella* infection. The method has the characteristics of high sensitivity and strong specificity, which provides a new way for earlier and rapid home-based on-site diagnosis of*Brucella* .

This study refers to the principle of DETECTR and SHERLOCKv2 system, combining CRISPR-Cas12a system and RPA, through observing lateral flow strip color to assay the result, building *Brucella* rapid CRISPR/CAST package. The package not only has the advantages of simplicity, high sensitivity, and strong specificity, the process of*Brucella* nucleic acid assay only takes about 30 min, but also realizes the rapid detection of *Brucella* nucleic acid on-site screening, especially on the remote family pasture (Figure 1).

Materials and Methods

Materials

Brucella strain is *B. melitensis* from Kailu county of Tongliao city, Inner Mongolia autonomous region, China (GenBank No. CIT21: CP025819, CP025820). The positive quality control plasmid from T-Vector pMD19 inserted target sequence fragments were synthesized in our laboratory and extracted with TIANprep Mini Plasmid Kit purchased from Tiangen (Beijing, China). *Brucella* infection Pretreatment Kit (Zhai et al., 2017). The TwistAmp Basic kit was purchased from TwistDx Ltd (Hertfordshire, AL, U.K.). Cas12a protein, lateral flow strip purchased from Bio-Lifesci (Guangzhou, China). RNase inhibitor was purchased from TaKaRa Bio Inc. (Dalian, China). *Yersinia enterocolitica* O:9*, Escherichia coli* O157*, Salmonella enterica serovar* Urbana O:30*,* and *Francisella tularensis*plasmids were stored in our laboratory.

1.2 Instruments

Applied Biosystems (ABI) StepOnePlus^{TM}Real-Time PCR Systems, Thermo Fisher Company; spectrophotometer SimpliNano (GE Health Bio-Sciences (USA)).

1.3 Methods

1.3.1 RPA primer design

In this study, the highly conserved sequence IS711 (Genebank No. AF242532) gene was selected as the target sequence from the National Center for Biotechnology Information (NCBI), then used Premier 5.0 software to design RPA forward and reverse primers (Gumaa et al., 2019) based on primer design principles (Table 1). Through the BLAST (The Basic Local Alignment Search Tool) to compare the primer sequences with the genomes of the cross strains *Yersinia enterocolitica*O:9*, Escherichia coli* O157*, Salmonella enterica serovar*Urbana O:30*,* and *Francisella tularensis* to determine the primer specificity; Primers was synthesized by Sangon Biotech (Shanghai, China).

1.3.2 crRNAs and probes design

According to the LbCas12a protein corresponding to the crRNA stem-loop structure and the target gene sequence, referring to the crRNA design requirements (Zhu & Liang, 2019), four groups of specific crRNA (Table 2) were designed on the IS711 gene sequence of *Brucella.* We referred to Chen et al for the principles of reporter probe design (Chen et al., 2018). The fluorescent probe (yg-probe) sequence is 6-FAM-TTATT-BHQ. The test strip probe refers to the principal design of nucleic acid test strip detection, the sequencing probe

(szt-probe) is 6-FAM-TTATT-Biotin. crRNAs and probes were synthesized by Sangon Biotech (Shanghai, China).

1.3.3 CRISPR/CAST Package reaction

RPA reaction system (50 μl) : 2.4 μl of RPA forward-and-reverse-direction primers F and R (10 μM), 29.5 μl of primer-free rehydration buffer, 2.5 μl of MgOAc (280 mM), 1 μl of target DNA, RNase-free water was replenished to 50 μl, place it in a 39°C temperature box for the reaction for 15min. Cas12a digestion reaction system (25 μl): 3 μl of 10 × LbCas12a buffer, 1 μl of LbCas12a (500 pmol), 1 μl of crRNA (10 μM), 0.5 μl of RNase inhibitor (5U/ μl), 0.5 μl of szt-Probe (yg-Probe) (10 μM), The RPA amplified product, 3 μl, refilling to 25 μl with RNase-free water; The prepared reaction tube was centrifuged at 12,000 r/min for several seconds, at 42°C temperature for 10min. Test strip detection: transferred to a safe area after the Cas12a cleavage reaction, makeup to 50 μl with RNase-free water, and insert the test strip below the liquid level. After the test strip is infiltrated completely, take out the test strip and observe the result within 10min with naked eyes.

1.3.4 Screening of the crRNA

The constructed *Brucella* IS711 plasmid as the template, applying the RPA-Cas12a detection method mentioned in 1.3.3, and screening the four groups of crRNA (IS711-crRNA-1-4), respectively. The Cas12a cleavage products will be checked by electrophoresis on 2% agarose gel to observe the cleavage efficiency. Furthermore, observe the endpoint fluorescence value with a real-time PCR instrument. Select the most efficient crRNA by the above two approaches.

1.3.5 CRISPR/CAST package sensitivity test

The IS711 positive plasmid concentration was quantified by spectrophotometer, SimpliNano (GE Health Bio-Sciences (USA)). The copy number of the plasmid was calculated by the following equation: Copy number = $(M \times 6.022 \times 10^{23})/(n \times 650 \times 1 \times 10^{9})$, where M is the amount of DNA in nanograms, n is the length of the plasmid in the base pair, and the average weight of a base pair is 650 Daltons. The plasmids gradient dilution was from 10^8 to 10^0 copies/μl. RPA sensitivity was measured by RPA-Cas12a mentioned in 1.3.3, with each diluted concentration positive plasmid as a template and RNase-free water as a negative control. The Real-Time PCR system measured the CRISPR/CAST package sensitivity by collecting fluorescent signals, and the nucleic acid test strip observed the color changes with naked eyes. Each concentration had three replicates.

1.3.6 CRISPR/CAST package specificity test

Compare primers and crRNA sequences with *Brucella* (GenBank No. CP025819, CP025820) and its cross-strains (*Yersinia enterocolitica* O:9*, Escherichia coli* O157*, Salmonella enterica serovar* Urbana O:30*,* and *Francisella tularensis*) by BLAST function. Using the CRISPR/CAST package method as described in 1.3.3. Simultaneous assay of *Brucella* , *Yersinia enterocolitica* O:9*, Escherichia coli* O157*, Salmonella enterica serovar* Urbana O:30*,* and *Francisella tularensis* , RNase-free water as a negative control for specificity test.

1.3.7 Field assay of CRISPR/CAST package for serum samples

The total number of serum samples was 498, including 398 samples of sheep serum and 100 cattle serum from the Tongliao Zarut Banner area (April 2022), and *Brucella* DNA was extracted with a*Brucella* infection pretreatment kit (Zhai et al., 2017) at 100°C for 5 min, and using the method CRISPR/CAST package as mentioned in 1.3.3, referring to the Bio-Lifesci Cas12/13 special nucleic acid test note instructions to interpreting the results. The results were also compared with the Taqman probe-based real-time PCR (Liu et al., 2021) and RBT. The forward primer (bcsp31-F, ACCTTGCCCTTGCCATCAT) and reverse primer (bcsp31-R,AGTCCGGCTTTACGCAGTCA), and probe (bcsp31-P, FAM-TGCCGTTATAGGCCCAATAGGCAACG- BHQ1) targeting bcsp31 of*Brucella* . Primers and probe were used at a final concentration of 0.2 μM with 2xPremix Ex Taq Probe qPCR (TaKaRa Bio Inc. Dalian, China) and 2 μl blood DNA in a 25 μl qPCR reaction. The qPCR cycling conditions consisted of 95 °C for 30 s, followed by 45 cycles at 95 °C for 15 s and 60 °C for 30 s.

1.3.8 Statistical Methods

SPSS 17.0 was employed to process the data. The data was analyzed using the homogeneity test of variance and normality test. Measurement data were expressed as mean ± SEM. Univariates between groups were analyzed using the t-test. Data among groups were analyzed by ANOVA. Heterogeneity of variance was analyzed using a non-parametric test.P <0.05 was considered statistically significant.

2 Results

2.1 crRNA screening

Four groups of crRNAs (IS711-crRNA-1-4) (Table 2) were compared by the method of RPA-Cas12a, and show four groups of crRNA binding sites are indicated below (Figure 2A). The Cas12a cleavage products will be checked by electrophoresis on 2% agarose gel to exhibit the cleavage efficiency (Figure 2B). Moreover, Figure 2C exhibited the endpoint fluorescence value with a real-time PCR instrument. The highest efficient crRNA was selected by the above two approaches. The results are presented below (Figure 2B). The gel electrophoresis of IS711-crRNA-1,2 and 3 Cas digestion products showed complete cleavage compared to IS711-crRNA-4. The fluorescence quantification showed the highest fluorescence value at the IS711crRNA-2 endpoint. Therefore, all subsequent experiments were performed by adopting IS711-crRNA-2 (uaauuucuacuaaguguagauGAUGAAUCCGUCACGCUCGG).

2.2 CRISPR/CAST package sensitivity test

The RPA sensitivity test that prepared a series of diluted positive quality controls with the number of copies was 10^8-10^0 copies/μl as the template and RNase-free water as the negative control. The CRISPR/CAST package sensitivity test that prepared a series of diluted positive quality controls with the number of copies was 10^7-10^0 copies/μl as the template and RNase-free water as the negative control. The results are exhibited in Figure 3. The lowerdetection limit for RPA is 1000 copies/μl (Figure 3A), and the CRISPR/CAST package sensitivity test is 10 copies/ μl (Figure 3B, 3C).

2.3 CRISPR / CAST package specificity test

Compare primers and crRNA sequences with *Brucella* and its cross-strains(*Yersinia enterocolitica* O:9, *Escherichia coli* O157, *Salmonella enterica serovar* Urbana O:30, and*Francisella tularensis*) by BLAST function. The RPA primers and four groups of crRNA alignments were single copies of the*Brucella* gene, and cross strains have no matching fragments. As mentioned in 1.3.3, the *Brucella* DNA samples test was positive, negative controls (no RNA enzyme water) and *Yersinia enterocolitica* O:9*, Escherichia coli* O157*, Salmonella enterica serovar* Urbana O:30*,* and *Francisella tularensis*samples were all negative (Figure 4). The above results showed that the CRISPR/CAST package could specifically assay target genes in the*Brucella* DNA samples without cross-reacting with other bacterial nucleic acids, indicating that the assay showed high specificity and that the samples of *Yersinia enterocolitica* O:9*, Escherichia coli* O157*, Salmonella enterica serovar* Urbana O:30*,* and *Francisella tularensis* showed no false-positive results.

2.4 Effect evaluation of the serum samples test

The blood samples of 398 sheep and 100 cattle from the Tongliao Zarut Banner area were tested by RBT. Through extracting DNA of serum samples, then adopting the CRISPR/CAST package method as mentioned in 1.3.3 and the qPCR to assay *Brucella* DNA, the results in Table 3, of which 31 sheep and 8 cattle were *Brucella* DNA positive. The detection rate was consistent with the qPCR and higher than RBT's (19 sheep and 5 cattle serum were positive).

3. Discussion

Infections caused by *Brucella* have emerged as a considerable threat worldwide, particularly in the vast amount of livestock. It is essential to eradicate and control livestock infection with *Brucella* from the source. Meanwhile, earlier detection and timely culling are equally important. Consequently, the screening used for livestock must be accurate, sensitive, specific, simple, and fast. At present, the methods of brucellosis

diagnosis mainly include agglutination test, Real-Time PCR, ELISA, semi-quantitative PCR, colloidal gold test strip, and polarized light technology. No single diagnostic method can meet the required sensitivity and specificity criteria. Some methods have low specificity and sensitivity, such as the agglutination and colloidal gold test strip. Some require special equipment, complex procedures, and professional personnel; Therefore, they can only be carried out in professional laboratories, unsuitable for on-site testing herders at home, such as the Real-Time PCR. RBT is simple, rapid, and highly sensitive, which is the primary method currently used for screening brucellosis in livestock groups, and it is also the designated test for brucellosis in cattle, sheep, and pigs in international trade (Godfroid, Nielsen, & Saegerman, 2010). However, the interpretation of the results is subjective, and there are cross-antigens among *Brucell* a, *Yersinia enterocolitica*O:9, *Escherichia coli* O157, *Salmonella enterica serovar*Urbana O:30, and *Francisella tularensis* , and cross-agglutination reaction with brucellosis-specific antibodies, prone to false positives. Therefore, RBT cannot be used as an objective and direct diagnostic evidence of brucellosis and cannot assay *Brucella* infection during the window period. That is, the antibodies were not produced at the initial infection stage. RPA is carried out under isothermal conditions, realizing nucleic acid detection independent of professional instruments and personnel. Although RPA is simple to operate and has high amplification efficiency, non-specific amplification is also inevitable.

As reported, a single RPA cannot assay low levels of targets (Gootenberg et al., 2018). These studies have confirmed Cas12a accessory splicing ssDNA activity for nucleic acid detection. The ternary complex consists of *Brucella* DNA, Cas12a, and crRNA. Cas12a possesses a RuvC domain that can exert activity to cleave the ssDNA labeled with fluorescent signal arbitrarily. As for the Real-Time PCR and RPA detection, the probe corresponds to the template one by one. Theoretically, under identical circumstances, we can hypothesize that the fluorescent signals produced by the CRISPR-Cas12a reaction are higher than those of Real-Time PCR and RPA detection. However, the studies have been ambiguous regarding how much higher they are, so we need to conduct additional experiments. By detecting *Brucella* DNA with the fluorescence signal, we can determine whether livestock have ever been infected. The proposed method has high sensitivity and strong specificity. The test strip is portable and convenient for field assay. When designing the probe, one end of ssDNA was labeled with biotin, and the other was labeled with 6-FAM. Nucleic acid detection of*Brucella* infection can be achieved without relying on large-scale instruments through lateral flow chromatography detection (Figure 1). This study developed a new, rapid, sensitive, and specific nucleic acid detection package (CRISPR/CAST package), which can be used in grassroots veterinary stations and farms at home. It is vitally significant for the earlier diagnosis of *Brucella* infection, comprehensive prevention and control, and eliminating the threat of infected animals to environmental biosecurity.

The CRISPR/CAST package combines RPA, CRISPR-Cas12a, and nucleic acid detection test strips. The assay can complete within 30 min under isothermal conditions, with a sensitivity of 10 copies/μl (Figures 2B, 2C). In addition, the CRISPR/CAST package can detect *Brucella*without antigenic cross-reacting to *Yersinia enterocolitica* O:9,*Escherichia coli* O157, *Salmonella enterica serovar* Urbana O:30, and *Francisella tularensis* (Figure 4). The high specificity is due to the specific primers designed in the RPA reaction, followed by the specific binding of crRNA to the target sequence. This dual-specific base-complementary binding allows detection even if non-specific amplification occurs in the RPA reaction; The second-step crRNA cannot complementarily pair with the target sequence, which leads to the cleavage reaction of Cas12a can not generate so that no fluorescent signal can be collected and illuminated. Cas9 needs to form a complex with two small RNA (sgRNA and tracrRNA), both of which are required for cleavage activity; However, Cas12a requires only one crRNA to form a complex (Yao et al., 2018), which is more highly efficient, flexible in binding to the target sequence and decrease the off-target probability. Logistically, Cas12a presents a more minimalistic system than Cas9 (Paul & Montoya, 2020).

CRISPR/CAST Package has unique advantages in nucleic acid detection. On the one hand, the assay, which takes a shorter time of around 30 min, is rapid. On the other hand, the package is portable; that is to say, the package requires no large instrument, professional laboratory, or professional and technical personnel, and the package attaches to strong specificity of *Brucella* nucleic acid assay with no cross-reacting to other organisms. Not only are the specific primers used during isothermal amplification, but the complex formed by

crRNA and Cas proteins can be accurately located in the target sequence (Li, Li, Wang, & Liu, 2019). Such dual localization ensures the high specificity of the CRISPR/CAST package (Figure 4). The lower detection limit for the CRISPR/CAST package sensitivity experiment was 10 copies/μl (Figure 3B, 3C), while the one of RPA was 1000 copies/μl (Figure 3A). The strip facilitated naked-eye observation to determine the result (Figure 3C, 4A).

Thus, the CRISPR/CAST package is superior to the conventional serological methods and PCR for detecting *Brucella* infection. Field serum samples of 398 sheep and 100 cattle were tested by CRISPR/CAST package, of which 31 sheep and 8 cattle were *Brucella*DNA positive. The detection rate was consistent with the qPCR and higher than that of the RBT (19 sheep, 5 cattle were serum positive). CRISPR/CAST package as a patron saint for livestock health enables nucleic acid detection technology to enter pastoral households successfully. The package can realize the earlier screening of infected animals, timely culling, and cleaning up the infected environment.

Meanwhile, it can control the incidence effectively, reduce the spread of *Brucella* infection and suppress large-scale infection, improving the quality of meat and dairy products. Furthermore, it ensures the safety of individual farmers' transactions, and it can protect the livestock herd's safety and reduce the probability of abortion in pregnant animals in the livestock. In other words, reduce farmers' losses. Improving the quality of meat and dairy products can also promote the import and export trade and protect human health. CRISPR/CAST package with high sensitivity, strong specificity, and portability facilitates farmers and herders who live in remote areas to complete the test whenever and wherever. CRISPR/CAST package provides a tool for field screening, earlier and rapid detection of *Brucella*infection, and new ideas for establishing rapid nucleic acid assays for other pathogens. We believe that the "Patron Saint" will prevent livestock free from *Brucella* infection and safeguard the herders' benefits and health eternally. The CRISPR-Cas system is leading to a new technological revolution. This new diagnostic tool will rewrite future diagnostic technologies, especially in developing countries with relatively poor sanitation and a high incidence of animal diseases.

4. Conclusion

The CRISPR/CAST package can detect *Brucella* DNA of infected livestock within 30 min. It is simple and rapid, has high sensitivity and strong specificity, has no window period, and does not require expensive equipment, standard laboratories, or professional personnel. A package is an efficient tool for epidemic prevention and control.

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Declarations

Ethics approval and consent to participate

In this paper, the requirement for ethics approval was waived by the Research Ethics Committee of Inner Mongolia Minzu Univeristy.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure 1. The CRISPR / CAST package detection principle.

Figure 1 shows the CRISPR/CAST package detection principle, and the detection process is divided into three steps. The first step is to use the RPA isothermal amplification technology to amplify the*Brucella* target DNA; In the second step, the 3 μl RPA amplification products are added to the reaction solution containing Cas12a, crRNA, and the ssDNA with fluorescent report group at 42°C for 10min; The third step is to fill the second reaction solution with pure water to 50 μl and then the test strip inserts into the solution. After the test strip is infiltrated thoroughly, observe the result within 10min with naked eyes. The detection line color is displayed, and the result is positive. The result is negative if the detection line has no color and the quality control line is displayed. The result is invalid if the quality control and detection line color are displayed.

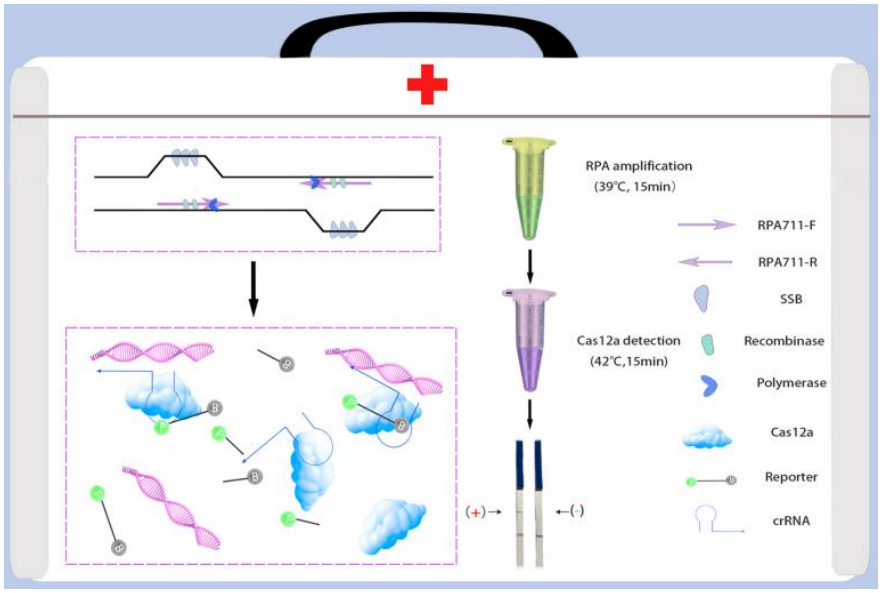
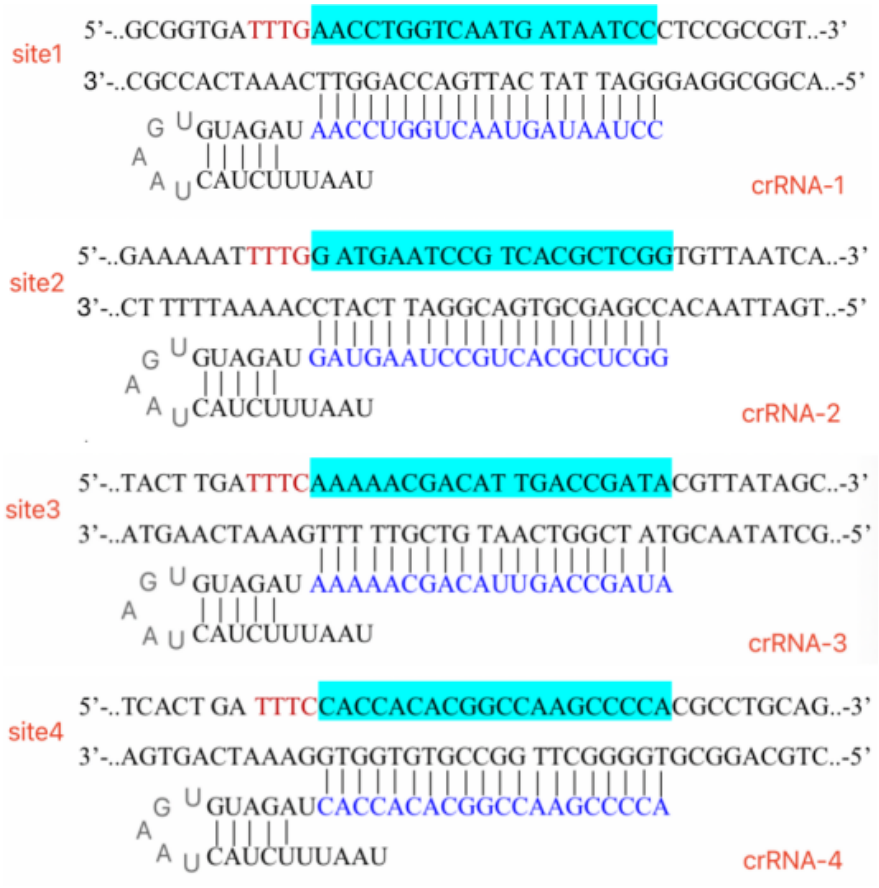
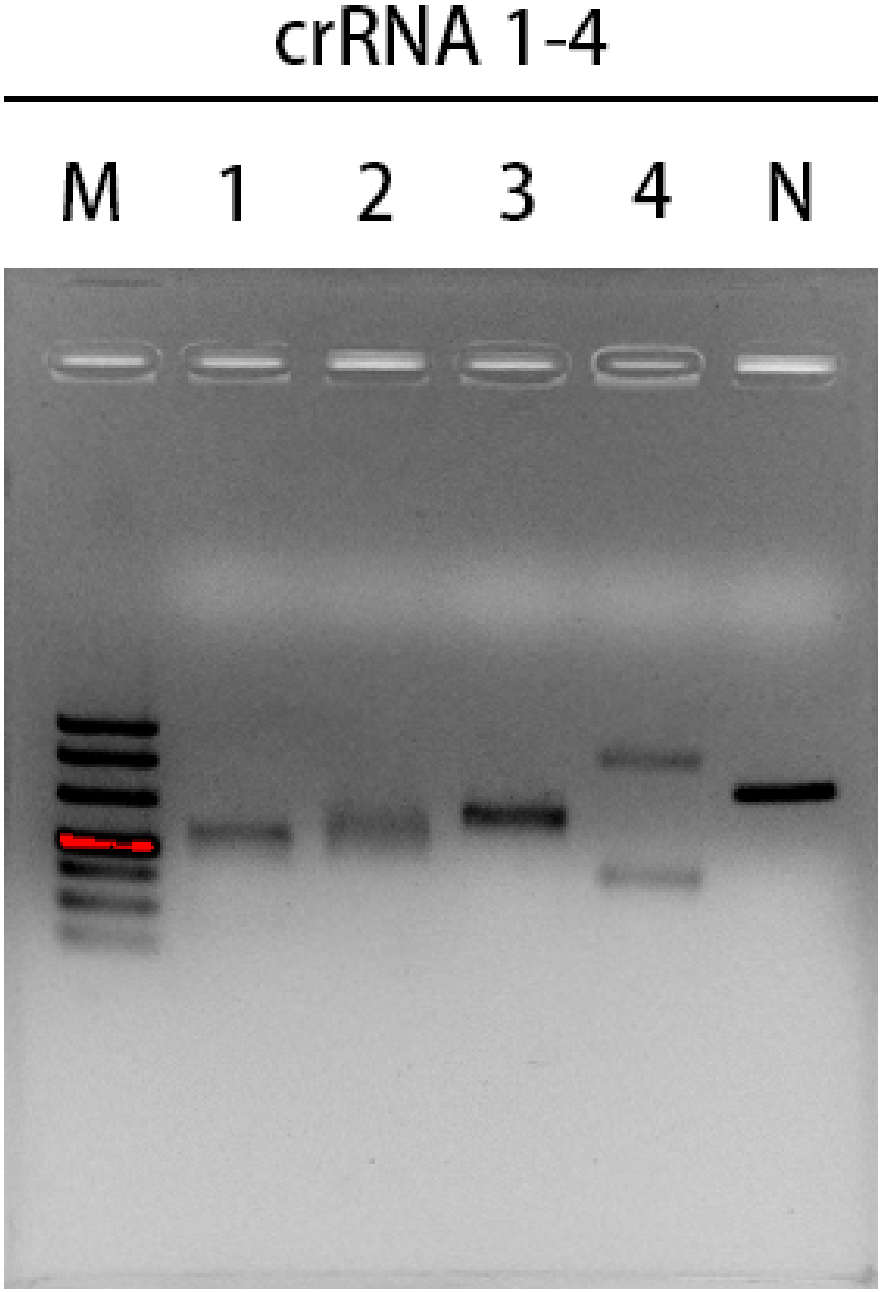
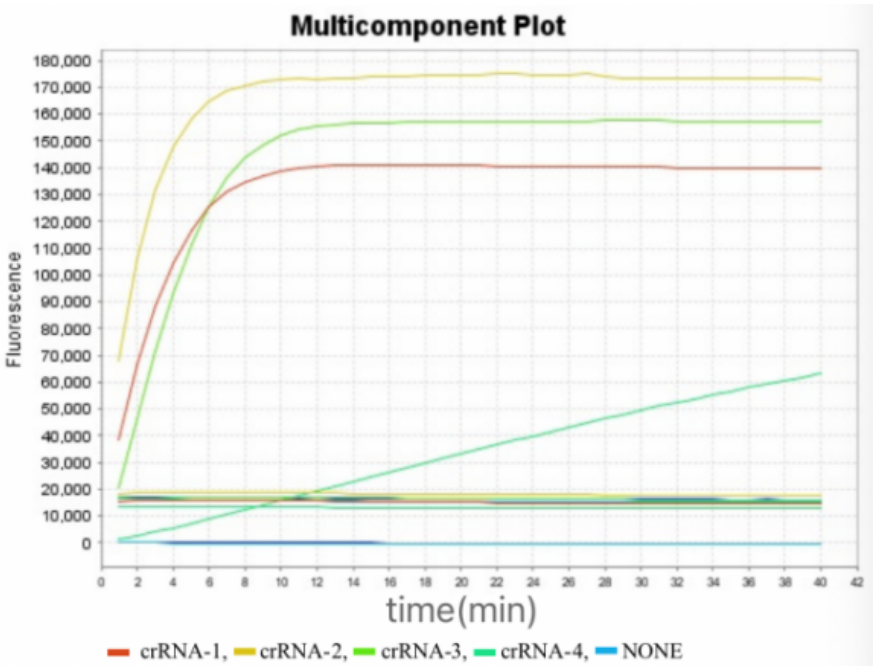


Figure 2. crRNA screen. A. The IS711-crRNA-1-4 structure and the corresponding target sequences. The target sites are highlighted in blue, and PAM sequences are marked red. B. Product of Cas12a cleavage, 20 μl of each reaction was electrophoresed (2% agarose), M: DL 500 DNA marker (Takara, Code No.3590Q), 1-4: crRNA-1-4, (RPA amplification products were 340 bp, 261/79 for crRNA-1, 303/37 for crRNA-2, 310/30 for crRNA-3, 174/166 for crRNA-4), N: negative control. Figure 2C Four groups of crRNAs were screened by the quantitative fluorescence method. The reaction was performed at 42°C for 40min, and the signal was collected every minute. The reaction entered the plateau phase at 8-10min. As shown in the figure, the endpoint fluorescence value of crRNA-2 was slightly higher than that of crRNA-1 and crRNA-3, significantly higher than crRNA-4.



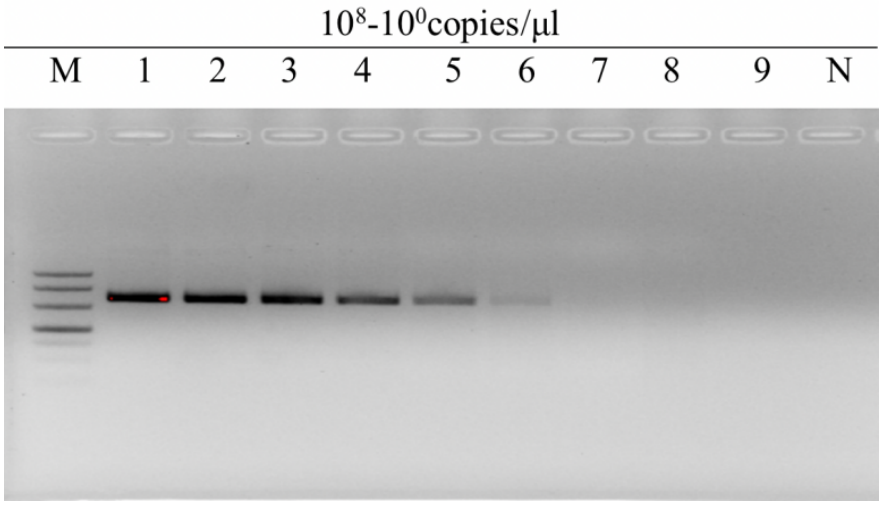


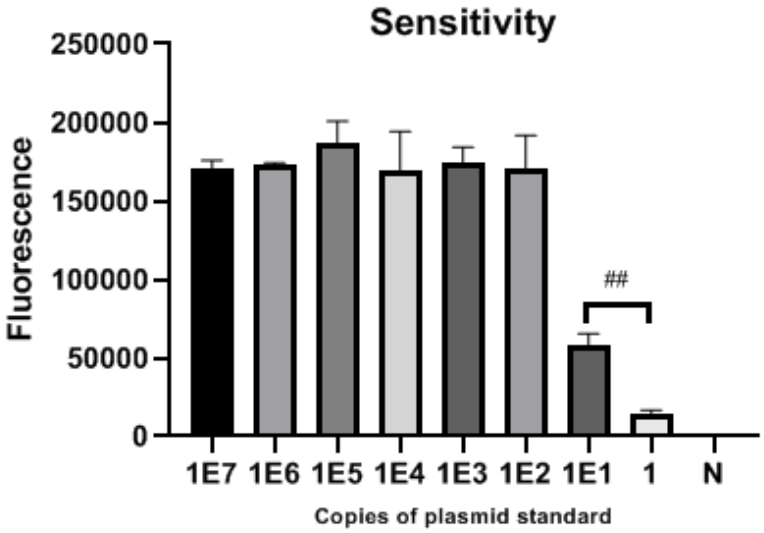
A **B**



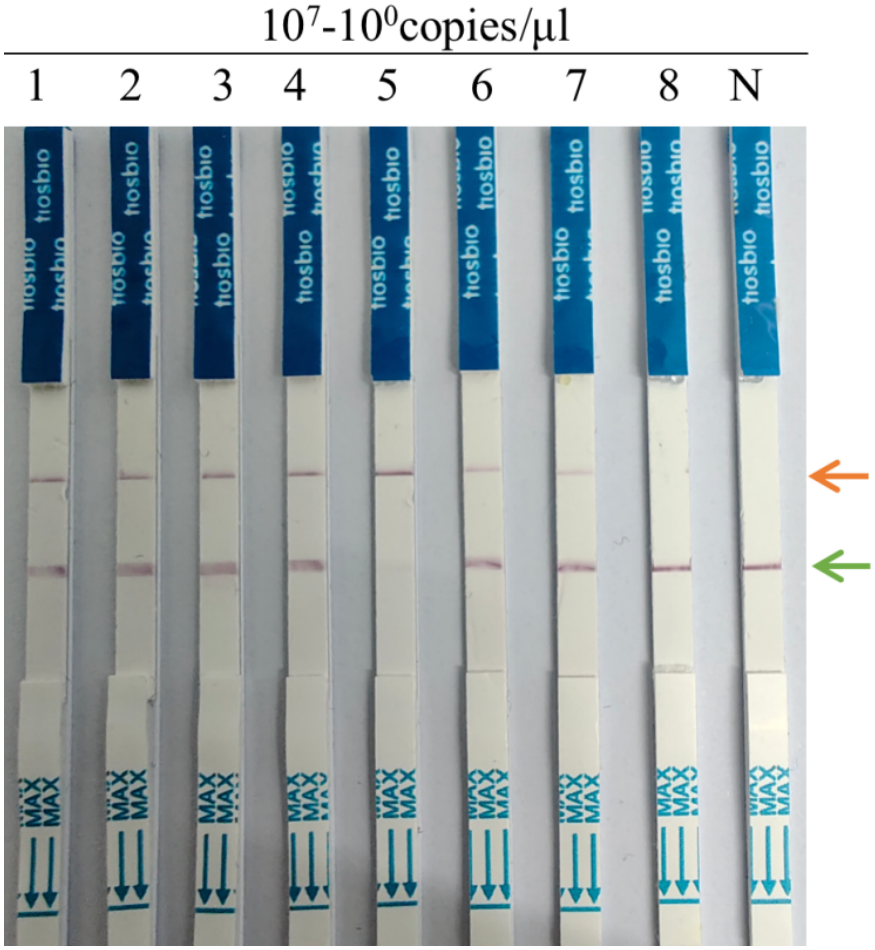
C

Figure 3. Sensitivity of three methods. A. Sensitivity of the standard RPA for nucleic acid detection. After amplification at 39°C for 15min, 5 μl of each reaction was electrophoresed (2% agarose), M: 500 bp Marker (Takara, Code No.3590Q), 1-8 corresponds to the copies of plasmid standard were 10^8-10^0copies/μl, N: a negative control. B. Sensitivity of the DETECTR method. A volume of 3 μl of standard RPA product was added to the Cas12a cleavage system, and maximum fluorescent signals were recorded (n = 3, error bars showed mean ± SEM). C. Sensitivity of the CRISPR/CAST package, 1-8 corresponding to the plasmid copy number is 10^7-10^0 copies/μl, N: negative control, the green arrow was the quality control line, and the orange arrow was the detection line.



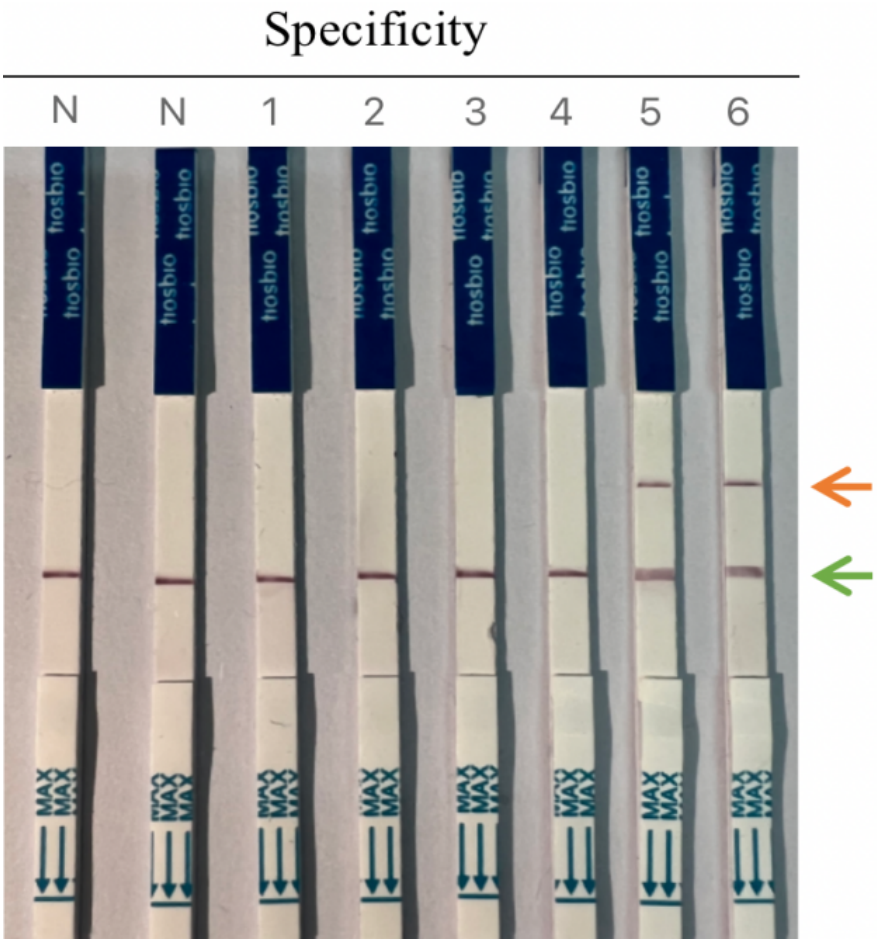


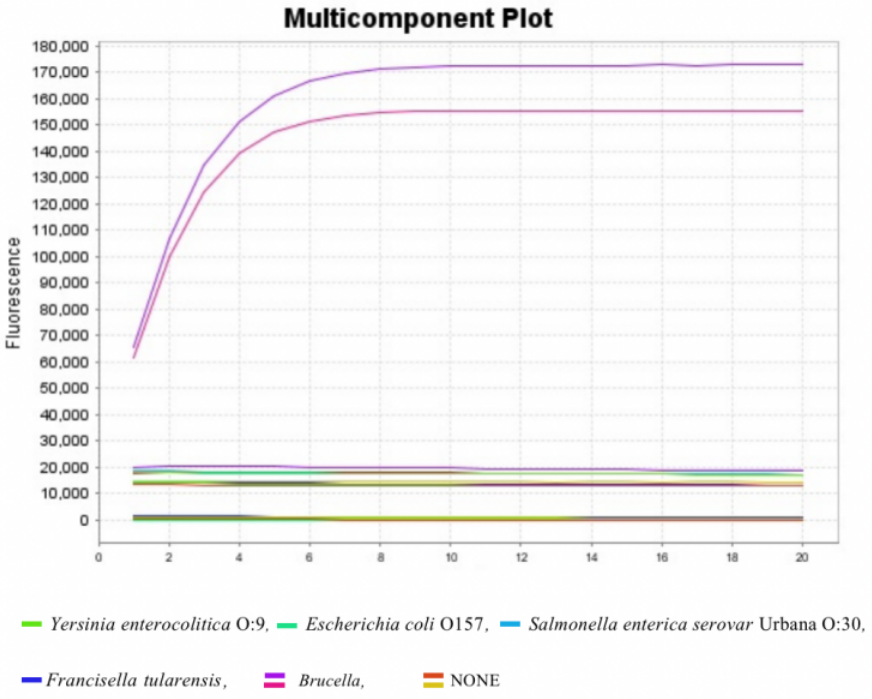
A B



C

Figure 4. The specificity test. A. Specific test strip test: N negative control, 1-4 are *Yersinia enterocolitica* O:9, *Escherichia coli* O157, *Salmonella enterica serovar* Urbana O:30, and *Francisella tularensis* , 5,6 *Brucella* ; green arrow was the quality control line, and orange arrow was the detection line. B. Specificity was detected by the fluorescence quantification method.





A B

Table 1. RPA primers sequence.

Gene	Primers name	Primers sequence (5'-3')
IS711	RPA711 F	CAATGTTGGAAAAATTTTGGATGAATCCGT
	RPA711 R	TTACTTGATTTCAAAAACGACATTGACCGATA

Table 2. The crRNAs Sequence.

Category	crRNAs name	crRNAs sequence (5'-3')
IS711	IS711-crRNA-1	uaauuucuacuaaguguagauAACCUGGUCAAUGAUAAUCC
	IS711-crRNA-2	uaauuucuacuaaguguagauGAUGAAUCCGUCACGCUCGG
	IS711-crRNA-3	uaauuucuacuaaguguagauAAAAACGACAUUGACCGAUA
	IS711-crRNA-4	uaauuucuacuaaguguagauCACCACACGGCCAAGCCCCA

Sample type	Serum of 398 sheep	Serum of 398 sheep	Serum of 398 sheep	Serum of 100 Cattle	Serum of
test method	CRISPR/CAST package	qPCR	RBT	CRISPR/CAST package	qPCR
Positive	31	31	19	8	8
Negative	367	367	379	92	92

Table 3. Serum samples test.