A quantum dot fluorescent microsphere based immunochromatographic strip for detection of brucellosis

yufang kong
Chinese Academy of Inspection and Quarantine

Huiyu Wang
Chinese Academy of Inspection and Quarantine

Shaoqiang Wu
Chinese Academy of Inspection and Quarantine

Jizhou LV
Chinese Academy of Inspection and Quarantine

Lin Mei
Chinese Academy of Inspection and Quarantine

Huifang Zhou
People's Hospital of Jiaxiang

Xueqing Han (✉ 1422355351@qq.com)
https://orcid.org/0000-0003-3779-5786

Xiangmei Lin
Chinese Academy of Inspection and Quarantine

Research article

Keywords: Brucellosis, quantum dots fluorescent microspheres, immunochromatographic strip test

DOI: https://doi.org/10.21203/rs.3.rs-39026/v2

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background: Brucellosis is a serious zoonosis disease that frequently causes significant economic loss in animal husbandry and threatens human health. Therefore, we established a rapid, accurate, simple and sensitive fluorescent immunochromatographic test strip (ICTS) based on quantum dots (QDs) for detection the antibodies of Brucella infection animals serum.

Result: The test strips were successfully prepared by quantum dot fluorescent microspheres (QDFM) as tracers, which were covalently coupled to an outer membrane protein of Brucella OMP22. The outer membrane protein OMP28 and monoclonal antibodies of OMP22 were separately dispensed onto a nitrocellulose membrane as test and quality control lines, respectively. The detection results were achieved by using the ratio of the fluorescence signals of the test and control lines (H<sub>T</sub>/H<sub>C</sub>) with a threshold of 0.0492. The repeatability was excellent with an overall average CV of 8.78%. Under optimum conditions, the limit of detection was 1.05 ng/mL (1:512 dilution). With regard to the detection of brucellosis in 150 clinical samples, the total coincidence rate of ICTS and Rose Bengal plate test (RBPT) was 97.3%, the coincidence rate of positive samples was 98.8%, the coincidence rate of negative samples was 95.3%, and no cross reaction with the sera of other related diseases was observed.

Conclusion: In our present study, the QDFM has promising application for on-site screening of brucellosis owing to its high detection speed, high sensitivity, high specificity and low cost.

Background

Brucellosis is a highly infectious zoonosis and poses serious threats to human health [1]. Brucella can infect humans in many ways, for example, contact with infected livestock and wildlife, and consumption of meat products and milk products infected with Brucella or incidental exposure to live attenuated vaccine and so on [2, 3, 4, 5]. Brucella contributes to abortions, infertility, placenta retention, still birth or weak offspring, and poor reproductive performance of animals, which results in huge economic losses for livestock farmers [6]. Currently, there is no effective method to prevent this disease, therefore, early diagnosis and monitoring are very essential.

Traditional detection methods of Brucella are pathogen isolation identification, serological diagnosis and molecular biology, which have a few defects [7]. The pathogen isolation identification method produces qualitative and quantitative results, but the method requires strict laboratory conditions and poses potential exposure risk to performers. The serological diagnostic methods including the Rose Bengal plate test (RBPT), standard tube agglutination test (SAT) and enzyme-linked immunosorbent assay (ELISA) need the whole cell or whole smooth lipopolysaccharides (S-LPS) as the antigen. Moreover, these methods may cause false positives and cross-reactivity with other Gram-negative bacteria [8, 9, 10]. Molecular biology methods such as polymerase chain reaction (PCR) [11], real time PCR (qPCR) [12], provide qualitative and quantitative results with good accuracy and sensitivity. However, these methods require expensive instruments and professional operators. They are time consuming and easy to produce
aerosol pollution [13]. Therefore, it is extremely important to establish a rapid, accurate and sensitive method to detect brucellosis [7].

In previous studies, the colloidal gold test strip method showed low sensitivity and species limitation. Dmitriy et al established Brucella colloidal gold antibody test strip can only detect bovine serum and the serum dilution limit of detection was only 1:250 [14]. Recently, a new labelled and more sensitive method was developed with fluorescent microspheres [15, 16]. So, in present study, we established and developed an immunochromatography brucellosis diagnostic method labelled with QD florescent microspheres.

Results

Optimization of the coating concentration for the NC membrane

The optimal coating concentration of the test line and control line was selected as 1 mg/mL and 0.5 mg/L, respectively. As shown in Fig. 2, there was good correlation between $H_T/H_C$ (x) and the sample concentration (y). The linear regression equation was $y = 37.882x - 3.3625$ and the correlation coefficient was 0.9777, which suggests the feasibility of the ICTS for the detection of brucellosis.

Limit of detection

The standard curve was established with serial 2-fold dilutions of Brucella positive serum from 1:4 to 1:1024, which were detected by. As shown in Fig. 3, the results can be read with naked eyes using a UV lamp and the fluorescent intensity of the test line gradually decreased. The fluorescent intensity of the test line was weaker than the control line for low antibody samples, which became weaker at the 1:512 dilution with a detection limit of 1.05 ng/mL. The fluorescent line disappeared at the dilution of 1:1024. As shown in Table 1, the value can also be accurately detected by the Fluorescence Reader.

Threshold and specificity testing

To determine the threshold, 50 healthy serum samples (30 bovine serum, 20 sheep serum) were tested by the ICTS and their results suggested that the ICTS threshold is 0.0492. The $H_T/H_C$ value $\geq$ 0.0492 (Table 2) indicated a positive assay of the ICTS. The brucellosis samples displayed $H_T/H_C$ values greater than the threshold, indicating the positive results. The tuberculosis, bluetongue disease, viral diarrhea, foot-and-mouth disease, bovine leukemia and peste des petits ruminants samples all displayed $H_T/H_C$ values less than the threshold, indicating the negative results and there was no cross reaction with the sera of other diseases. (Table 3).

Detection of brucellosis in clinical samples using ICTS and RBPT

The results of 150 samples were detected by the Fluorescence Reader. The coincidence rate was calculated by comparing with RBPT results. The detection results are shown in Table 4. For brucellosis detection, the total coincidence rate of ICTS and RBPT was 97.3% [(85+61)/150]. Compared with RBPT,
the positive coincidence rate of ICTS was 98.8% \([85/(85+1)]\) and the negative coincidence rate of ICTS was 95.3% \([61/(61+3)]\).

**Repeatability assay of the ICTS for brucellosis**

The results suggested that the ICTS maximum CV of 8.78% with an average of 6.16% was obtained for all samples. All the CV values of the above results are less than 10%, illustrating the brucellosis diagnosis procedure based on the QDFM detection technology is repeatable (Table 5).

**Discussion**

At present, it has been more than 100 years to develop effective methods for diagnosis of brucellosis, but brucellosis is still a recurring disease and is prevalent again in many parts around the world [17]. *Brucella* infections are easily transmitted to humans, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculoskeletal, cardiovascular, and central nervous systems [OIE Terrestrial Manual chapter 3.01.04]. The most rational approach for preventing human brucellosis is the control and elimination of the infection in animals [https://www.who.int/zoonoses/diseases/brucellosis/en/]. So selecting the dominant diagnostic antigen for brucellosis and establishing a rapid diagnostic method are important to preventing and treating the disease.

Classical immunological detection technology mainly relies on LPS antigen, however, related studies have shown the high cross-reactivity of LPS antigen with several Gram-negative bacteria. This may result in false-positive results, so they are not appropriate for accurate diagnosis of brucellosis [18, 19, 20]. Therefore, many researchers are looking for a better diagnostic antigen such as outer membrane proteins (OMPs) to replace LPS and improve the sensitivity and specificity of immunological detection technology [21, 22, 23, 24]. OMPs are necessary for the complete virulence of brucellosis strains [25]. Lindler et al identified one non-LPS group of immunogens with OMPs for vaccine and diagnostic purposes [26]. One of these OMPs, OMP22, has many advantages, such as it being highly conserved in all *Brucella* species and it having nearly identical amino acid sequences with OMP25. In a previous clinical study, the absence of OMP25 or OMP22 proteins was demonstrated to lead to a striking decrease in the virulence of *B. ovis* PA in mice [27]. Another OMPs, OMP28, is a conserved protein present in at least four *Brucella* species, including. *B. melitensis, B. abortus, B. suis,* and *B. ovis*. The protein is well studied both as a vaccine candidate and as an antigen for serodiagnosis [28, 29, 30]. The studies showed that the rOMP28-based I-ELISA had high sensitivity and specificity in the diagnosis of brucellosis in bovine sera [31, 32, 33, 34]. Lim et al established rOMP28 ELISA to detect bovine brucellosis and the sensitivity, specificity, and accuracy were 96.7%, 95.4%, and 96.2% respectively [28]. In our present study, we also used the OMP22 and OMP28 as the diagnostic antigen to test brucellosis by immunochromatography because the specificity and sensitivity is higher than LPS.

*Brucella* OMPs are generally expressed in the form of inclusion bodies in *E. coli*, and the refolding rate of inclusion bodies is low, which cannot meet the requirements of this test. Therefore, the pCold-TF DNA
vector containing a 48kD fusion tag was used in this study to express OMP22 and OMP28 in the supernatant in *E. coli*. Considering that the large fusion tag will affect the immunogenicity of the protein, we remove the fusion tag by HRV 3C Protease and combine it with his-Tag containing medium to purify the target protein. In the end, we obtained relatively good quality protein for the subsequent testing process.

Many researchers have recently focused on developing quantum dot fluorescent microsphere (QDFM) immunochromatography and they have been widely used in the field of biological detection such as microbiological detection, analytical chemistry and disease diagnosis. These immunoassays have many advantages, compared with other detection techniques, such as fast detection speed, accurate, efficient, strong specificity, high sensitivity and simple operation [35]. For example, Taranova *et al.* established a QD-based immunochromatographic assay used for detection of several antibiotics in milk [36]. This labelled technology forms hundreds or even thousands of particles by encapsulating or connecting to other materials to form nanoparticles. It has the unique properties of good light stability and biocompatibility, long fluorescence lifetime, wide excitation spectrum, a narrow emission spectrum and size-tunable. With these advantages, the QDFM have potential as a new labeling technology immunoassay [37, 38]. The microspheres amplified the optical signal of specific antigen-antibody binding and the sensitivity is improved compared with other labelled technologies [39, 40]. This method avoids shortcomings of time consumption and the use of accurate and expensive instruments, with advantages of rapid on-site detection, low cost, and no special operation requirements [41, 42].

QD immunochromatographic test strips have strict requirements for the immunogenicity of labeling and coating proteins. Dan *et al.* developed a detection of *Brucella* with QDs and magnetic beads conjugating with different polyclonal antibodies and this method takes 105 min with a detection limit of $10^3$ CFU/mL [43]. The method we established only need 10-15 min to obtain final results with a limit of 1.05 ng/mL. It indicated that the sensitivity of QDFM is higher and can be used for semi-quantitative detection. This QDFM detecting *Brucella* antibodies is safer than that of antigen detection and can reduce the risk to the detection personnel. The $H_T/H_C (x)$ shows good correlation with the sample concentration ($y$) (Fig.2), after we optimized the coating concentrations for the NC membrane.

As is shown in Table 3, QDMF can be used for *Brucella* in real samples with high specificity. The brucellosis samples displayed $H_T/H_C$ values greater than the threshold which indicating a positive test result, and all the other samples of related diseases displayed $H_T/H_C$ values less than the threshold. As Table 4 shows, ICTS shows high feasibility in the 150 brucellosis clinical serum samples assay and the total coincidence rate of ICTS and RBPT was 97.3%. Compared with RBPT, the positive coincidence rate of ICTS was 98.8% and the negative coincidence rate of ICTS was 95.3%. It further shows that this detection method can achieve ideal results in *Brucella* real sample detection.

**Conclusion**
In this study, we presented QDFMs tagged OMP22 to facilitate detection of *Brucella* antibodies in standard and clinical samples of only a few microliters using ICTS. The limit of detection was 1.05 ng/mL (1:512), the total coincidence rate of ICTS and RBPT was 97.3%, the positive coincidence rate was 98.8%, the negative coincidence rate was 95.3%, the repeatability was good with an overall average CV of 8.78%, and no cross reaction with the sera of other related diseases was observed. However, the quantitative research of this method needs to be further studied.

**Methods**

**Materials and reagents**

Quantum dot fluorescent microspheres were purchased from Invitrogen Corp (Carlsbad, CA, USA). A Rose Bengal plate test (RBPT) was obtained from the China Institute of Veterinary Drug Control. Bovine serum albumin (BSA), tween-20, polyvinyl pyrrolidone (PVP), sodium azide, tris base (TB), 2-(4-Morpholino)ethanesulfonic acid (MES), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The test strip materials, including nitrocellulose (NC) membranes (Millipore Hiflow-95) and glass cellulose membranes (Product number 8951), were purchased from Shanghai Jiening Biotechnology Co., Ltd (Shanghai, China).

**Apparatus**

The BioDot XYZ dispensing platform (BioDot, Richmond, CA, USA) was used to dispense reagents to conjugate pad, nitrocellulose membrane, and an automatic cutter was used to cut the strip. A fluorescent strip reader JN615 was purchased from Shanghai Jie Ning Biological Co., Ltd (Shanghai, China). A 365-nm hand held UV lamp (American Precision Co., Ltd., USA) was used to test strip.

**Samples and biological materials**

Bovine *Brucella* negative and positive standard sera were purchased from the China Institute of Veterinary Drug Control. Positive sera of tuberculosis, bluetongue disease, viral diarrhea, foot-and-mouth disease, bovine leukemia, peste des petits ruminants and 50 healthy negative bovine and sheep were acquired from the Chinese Academy of Inspection and Quarantine. A total of 150 clinical serum samples was kindly provided by the Animal Husbandry Bureau of Ningxia Hui Autonomous Region. *Brucella* OMP22 and OMP28, and monoclonal antibodies of OMP22 were prepared by our laboratory.

**Preparation of QDFM-protein conjugates**

Protein was coupled to the QDFMs by carboxyl activation. In brief, the protein-conjugated QDFMs were prepared using EDC and NHS as cross linkers. The surface carboxyl groups of the QDFM were bound with the amino groups of the antigen under catalysis of EDC and NHS. A commercial QDFM solution (100 μL) was pipetted into centrifuge tubes and activated with EDC and NHS. The mixture solution was dissolved in MES buffer to yield a final concentration of 0.5 mg/L EDC and 0.2 mg/mL NHS. The solution was
mixed by vortex for 30 min, followed by reaction at 37°C for 15 min. Then, 100 μL of OMP22 (0.1 mg/mL) was added, and the mixture was reacted for 2–4 h at room temperature under gentle agitation. 50 μL of 10% BSA was added and the mixture was incubated for 30 min at room temperature. The resulting QDFM conjugates were washed three times by centrifugation at 8000 g for 20 min. The purified functional QDFM-OMP22 was resuspended in 1 mL of 20 mM Tris base (TB, pH 8.5) containing 0.5% BSA, 2% source, 0.2% Tween-20, and Triton 405-X and stored at 4°C until use.

**Assembly of the QDFM test strip**

The strip consisted of four parts: sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad. The sample pad was saturated with a 20 mM TB (pH 8.5) buffer containing 5% sucrose, 0.5% BSA, 0.01% PVP-40, 2% Tween-20 and 0.02% NaN₃ and then the pad was dried at 70°C for 2 h and stored at room temperature. The components of the test strip were sequentially laminated and pasted to a PVC backing pad with appropriate 2-mm overlap to ensure the testing sample solution could migrate through the whole assembled test strip. In our present study, QDFM labeled *Brucella* OMP22 was dispensed onto the conjugate pad and then the pad was dried at 37°C overnight and stored at 4°C. 0.03 mL of OMP28 (1.5, 1, 0.75 mg/mL) and 0.3 mL of McAb OMP22 (1, 0.75, 0.5 mg/mL) were dispense onto the nitrocellulose membraneas test and control lines, respectively, and the strip was dried at 37°C for 2 h. Finally, the whole assembled strip was cut into a 5-mm width and 80-mm length using a BIO-DOT strip cutting machine (Fig. 1). According to the Sotnikov *et al* describe three schemes of analysis to detect antibodies, our research plan is similar to the author’s second scheme. The outer membrane protein OMP22 combine with the antibodies in serum samples and captured by OMP28 on the detection line to form an OMP22-Ab-OMP28 immune complex [44].

**Sensitivity, threshold, feasibility, repeatability and specificity testing**

To improve the sensitivity of the diagnostic procedure, eight brucellosis positive serum samples with different concentrations were tested to determine the NC membrane coating concentrations. The corresponding concentrations of the samples were 0.169 ng/μL, 0.666 ng/μL, 1.35 ng/μL, 2.11 ng/μL, 3.06 ng/μL, 27.06 ng/μL, 45.2 ng/μL, 64.2 ng/μL, respectively. The standard cure was established with serial 2-fold dilutions of *Brucella* positive serum from 1:4 to 1:1024, and ICTS was used determine the limit of detection.

The test strip was applied to detect positive and negative serum samples, and the test strip was detected using a 365-nm handheld UV lamp and a Fluorescence Reader connected to a laptop. The ICTS was used to detect 50 healthy *Brucella* negative bovine and sheep serum samples using a Fluorescent Reader and the Hₜ/Hₐ values were recorded as negative controls. The ratio of the signals of the test line to that of the control line (Hₜ/Hₐ) provides a qualitative detection for QDFM. The Hₜ/Hₐ threshold values of ICTS were calculated as the following equation: threshold value = mean ± 3× standard deviation.

To evaluate the feasibility of the strip for detection of antibodies against brucellosis, and to test the reader accuracy, 150 brucellosis clinical serum samples (68 bovine serum, 82 sheep serum) were
collected from the Animal Husbandry Bureau of Ningxia Hui Autonomous Region. All the samples were pretreated with 0.01 M Tris-HCl (pH 9.5) buffer containing 0.9% NaCl and 0.05% Tween-20 for 15 min. The 150 clinical samples were tested using ICTS and the coefficient of the detection results were compared with a commercial RBPT.

The repeatability of ICTS was tested by 11 serially diluted standard brucellosis positive serum samples concentrations ranging from 50 ng/mL to 1 ng/mL and 1 negative serum. Each sample was detected for three times to calculated coefficient of variation (CV). The CV was calculated by dividing the mean of three measurements by the standard deviation to determine the repeatability.

**Abbreviations**

ICTS: immunochromatographic test strip  
RBPT: Rose Bengal plate test  
QDFM: quantum dot fluorescent microspheres  
SAT: standard tube agglutination test  
ELISA: enzyme-linked immunosorbent assay  
S-LPS: smooth lipopolysaccharides  
PCR: polymerase chain reaction  
qPCR: real time PCR  
RV: rotavirus  
AdV: adenovirus  
OMPs: outer membrane proteins  
BSA: bovine serum albumin  
MES: 2-(4-Morpholino) ethanesulfonic acid  
EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride  
NHS: N-hydroxysuccinimide  
NC: nitrocellulose  
CV: coefficient of variation  
PVP: polyvinyl pyrrolidone  
TB: tris base

**Declarations**

_Ethics approval and consent to participate_

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, this article.

_Research involving human participants and/or animals_  
This article does not contain any studies with humans. All animal experiments in this study were approved and conducted under the supervision by Ethics Committee on Scientific Research on Animal Pathogenic Microorganisms, Institute of Animal Quarantine, Chinese Academy of Inspection and Quarantine (ECSRPM0626001). Orally permissions were obtained from the owners before collection of the specimens. Ethics Committee on Scientific
Research on Animal Pathogenic Microorganisms (ECSRPM) approved the procedure for verbal consent. ECSRPM felt the need for written consent was not necessary for this study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was financed by the Research Program of Chinese Academy of Inspection and Quarantine (2018JK012). The funding sources had no involvement in the design of the research, the collection, analysis, and interpretation of data, and the writing of the manuscript.

Authors' contributions

YFK and HYW collected and analyzed data and drafted the first version of the manuscript. SQW, JZL, LM and HFZ performed experiments and analyzed the data. XML and XQH participated in the conception and design the experiments and critically revised the manuscripts. All authors read and approved the final manuscript.

Acknowledgements

We are grateful to all the members of the Laboratory of Institute of Animal Quarantine, Chinese Academy of Inspection and Quarantine for their helpful discussions, encouragements, and support.

References

1. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos The new global map of human brucellosis. Lancet Infect Dis. 2006;6:91-99.

2. Godfroid J, Cloeckaert A, Liautard JP, Kohler S, Fretin D, Walravens K, Garin-Bastuji B, Letesson JJ. From the discovery of the Malta fever’s agent to discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet Res. 2005;36:313-336.

3. Zinsstag J, Roth F, Orkhon D, Chimed-Ochir G, Nansalmaa M, Kolar J, Vounatsou P. A model of animal-human brucellosis transmission in Mongolia. Prev Vet Med. 2005;69:77-95.
4. Falenski A, Mayer-Scholl A, Filter M, Göllner C, Appel B, Nöckler K. Survival of *Brucella* spp. in mineral water, milk and yogurt. Int J Food Microbiol. 2011;145:326-330.

5. Franco MP, Mulder M, Gilman RH, Smits HL. Human brucellosis. Lancet Infect Dis. 2007;7:775-86.

6. de Oliveira MZ, Vale V, Keid L, Freire SM, Meyer R, Portela RW, Barrouin-Melo SM. Validation of an ELISA method for the serological diagnosis of canine brucellosis due to *Brucella canis*. Res Vet. Sci. 2011;90(3):425-431.

7. Li L, Yin D, Xu K, Liu Y, Song D, Wang J, Zhao C, Song X, Li J. A sandwich immunoassay for brucellosis diagnosis based on immunemagnetic beads and quantum dots. J PHARMACEUT BIOMED. 2017;141:79-86.

8. Patra KP, Saito M, Atluri VL, Rolán HG, Young B, Kerrinnes T, Smits H, Ricaldi JN, Gotuzzo E, Gilman RH, Tsolis RM, Vinetz JM. A protein-conjugate approach to develop a monoclonal antibody-based antigen detection test for the diagnosis of human brucellosis. PLoS Negl Trop Dis. 2014;6(8):1-13.

9. Ahmed IM, Khairani-Bejo S, Hassan L, Bahaman AR, Omar AR. Serological diagnostic potential of recombinant outer membrane protein (rOMPs) from *Brucella melitensis* in mouse model using indirect enzyme-linked immunoabsorbent assay. BMC Vet Res. 2015;11:275.

10. Corrente M, Desario C, Parisi A, Grandolfo E, Scaltrito D, Vesco G, Colao V, Buonavoglia D. Serological diagnosis of bovine brucellosis using melitensis strain B115. J Microbiol Methods. 2015;119:106-109.

11. Gupta VK, Shivasharanappa N, Kumar V. Diagnosis evaluation of serological assays and different gene based PCR for detection of *Brucella melitensis* in goat. Small Rumin Res. 2014;117:94-102.

12. L.D. Orzil, I.S. Preis, I.G. de Almida, P.G. de Souza, P.M. Soares, F.B. Jacinto, A.A. Fonseca. Validation of the multiplex PCR for identification of *Brucella spp.* Cienc Rural. 2016;5(46):847-852.

13. Tabibnejad M, Alikhani MY, Arjomandzadegan M, Hashemi SH, Naseri Z. The optimization of molecular detection of clinical isolates of *Brucella* in blood cultures by eryD transcriptase gene for confirmation of culture-negative samples. Iran Red Crescent Med J. 2016;18(4):e23879.

14. Dmitriy V S, Nadezhda A B, Anatoly V Z, Saule Z E, Kairat K B, Kasim K M, Erlan M R, Elchin G S, Boris B D. Express immunochromatographic detection of antibodies against *Brucella abortus* in cattle sera based on quantitative photometric registration and modulated cut-off level. 2015;36:80-90.

15. Song C, Zhi A, Liu Q, Yang J, Jia G, Shervin J, Tang L, Hu X, Deng R, Xu C, Zhang G. Rapid and sensitive detection of beta-agonists using a portable fluorescence biosensor based on fluorescent nanosilica and a lateral flow test strip. Biosens Bioelectron. 2013;50:62–65.

16. Xu W, Chen X, Huang X, Yang W, Liu C, Lai W, Xu H, Xiong Y. Ru (phen) 3 (2+) doped silica nanoparticle based immunochromatographic strip for rapid quantitative detection of beta-agonist residues in swine urine. Talanta. 2013;114:160–166.

17. McGiven JA. New developments in the immunodiagnosis of brucellosis in livestock and wildlife. Rev Sci Tech. 2013;32(1):163-176.

18. Nielsen K, Smith P, Yu WL, Halbert G. Salmonella enterica serotype urbana interference with brucellosis serology. J Immunoassay Immunochem. 2007;28:289-296.
19. Vahedi F, Talebi AF, Ghorbani E, Behroozikah AM, Ahmadi FS. Isolation, cloning and expression of the \textit{Brucella melitensis} OMP31 gene. Iran J Vet Res. 2011;12:156–162.

20. Weynants V, Tibor A, Denoel PA, Saegerman C, Godfroid J, Thiange P, Letesson JJ. Infection of cattle with \textit{Yersinia enterocolitica} O:9 a cause of the false positive serological reactions in bovine brucellosis diagnostic tests. Vet Microbiol. 1996;48:101–112.

21. Büyüktanir Ö, Genç O, Yurdusev N. Production, Purification and Characterization of the Recombinant \textit{Brucella abortus} rP17 Protein. Kafkas Univ Vet Fak. 2011;17:135–140.

22. Farahi F, Asli E, Mobarez AM, \textit{et al}. Recombinant \textit{Brucella abortus} outer membrane protein 19 (rOMP19) significantly stimulates splenic lymphocytes of immunized BALB/c mice. Afr J Microbiol Res. 2012;6:4128–4131.

23. Thavaselvam D, Kumar A, Tiwari S, Mishra M, Prakash A. Cloning and expression of the immunoreactive \textit{Brucella} melitensis 28 kDa outer-membrane protein (OMP28) encoding gene and evaluation of the potential of OMP28 for clinical diagnosis of brucellosis. J Med Microbiol. 2010;59:421–428.

24. Eoh H, Jeon BY, Kim Z, Kim SC, Cho SN. Expression and validation of D-erythrulose 1-phosphate dehydrogenase from \textit{Brucella abortus}: a diagnostic reagent for bovine brucellosis. J Vet Diagn Invest. 2010;22:524–530.

25. Martín-Martín AI, Caro-Hernández P, Orduña A, Vizcaíno N, Fernández-Lago L. Importance of the Omp25/Omp31 family in the internalization, and intracellular replication of virulent \textit{B. ovis} in murine macrophages and HeLa cells. Microbes and Infection. 2008;10(6): 706-710.

26. Lindler LE, Hadfield TL, Tall BD, Snellings NJ, Rubin FA, Van De Verg LL, Hoover D, Warren RL. Cloning of a \textit{Brucella melitensis} group 3 antigen gene encoding Omp28, a protein recognized by the humoral immune response during human brucellosis. Infect Immun. 1996;64:2490-2499.

27. Caro-Hernández P, Fernández-Lago L, de Miguel MJ, Martín-Martín AI, Cloeckaert A, Grilló MJ, Vizcaíno N. Role of the Omp25/Omp31 family in outer membrane properties and virulence of \textit{Brucella} ovis. Infect Immun. 2007;75 (8):4050-4061.

28. Lim JJ, Kim DH, Lee JJ, Kim DG, Min W, Lee HJ, Rhee MH, Chang HH, Kim S. Evaluation of recombinant 28 kDa outer membrane protein of \textit{Brucella abortus} for the clinical diagnosis of bovine brucellosis in Korea. J Vet Med Sci. 2012;74 (6):687-691.

29. Chaudhuri P, Prasad R, Kumar V, Gangaplara A. Recombinant Omp28 antigen based indirect ELISA for serodiagnosis of bovine brucellosis. Mol Cell. Probes. 2010;(24): 142-145.

30. Patricia S M, JeanM V, Maggy G, Axel C, Clara M. M, Michel S Z, Luis F L, Nieves V. Epitope Mapping of the \textit{Brucella melitensis} BP26 Immunogenic Protein: Usefulness for Diagnosis of Sheep Brucellosis. 2003;10(4):647-651.

31. Manat Y, Shustov AV, Evtuhova E, Eskendirova SZ. Expression, purification and immunochemical characterization of recombinant OMP28 protein of \textit{Brucella} species. Open Vet J. 2016;6(2):71-77.

32. Cloeckaert A, Baucheron S, Vizcaíno N, Zygmunt MS. Use of recombinant BP26 protein in serological diagnosis of \textit{Brucella melitensis} infection in sheep. Clin Diagn Lab Immunol. 2001;8(4):772-775.
33. Liu WX, Hu S, Qiao ZJ, Chen WY, Liu LT, Wang FK, Hua RH, Bu ZG, Li XR. Expression, purification, and improved antigenic specificity of a truncated recombinant bp26 protein of *Brucella melitensis* M5-90: a potential antigen for differential serodiagnosis of brucellosis in sheep and goats. Biotechnol Appl Biochem. 2011;58(1):32-38.

34. Cha SB, Rayamajhi N, Lee WJ, Shin MK, Jung MH, Shin SW, Kim JW, Yoo HS. Generation and envelope protein analysis of internalization defective *Brucella* abortus mutants in professional phagocytes, RAW 264.7. FEMS Immunol Med Microbiol. 2012;64(2):244-254.

35. Yang Q, Gong X, Song T, Yang J, Zhu S, Li Y, Cui Y, Li Y, Zhang B, Chang J. Quantum dot-based immunochromatography test strip for rapid, quantitative and sensitive detection of alpha fetoprotein. Biosens Bioelectron. 2011;30:145-150.

36. Taranova NA, Berlina AN, Zherdev AV, Dzantiev BB. ‘Traffic light’ immunochromatographic test based on multicolor quantum dots for the simultaneous detection of several antibiotics in milk. Biosens. Bioelectron. 2014; 63:255-261.

37. Di Nardo F, Anfossi L, Giovannoli C, Passini C, Gofman VV, Goryacheva IY, Baggiani C. A fluorescent immunochromatographic strip test using quantum dots for fumonisins detection. Talanta. 2016;150:463–468.

38. Xie Y, Zhang L, Yang X, Le T. Development of a quantum dot-based immunochromatography test strip for rapid screening of oxytetracycline and 4-epioxytetracycline in edible animal tissues. Food Addit Contam A. 2017;34(3):371–378.

39. Qiu L, Bi Y, Wang C, Li J, Guo P, Li J, He W, Wang J, Jiang P. Protein a detection based on quantum dots antibody bioprobe using fluorescence coupled capillary electrophoresis. International Journal Molecular Science. 2014;15(2):1804-1811.

40. Mulder WJ, Castermans K, van Beijnum JR, Oude Egbrink MG, Chin PT, Fayad ZA, Löwik CW, Kajzel EL, Que I, Storm G, Strijkers GJ, Griffioen AW, Nicolay K. Molecular imaging of tumor angiogenesis using alphavbeta3-integrin targeted timodal quantum dots. Angiogenesis. 2009;12(1):17-24.

41. Zhu MY, Tang YJ, Wen QQ, Li J, Yang PH. Dynamic evaluation of cell-secreted interferon gamma in response to drug stimulation via a sensitive electro-chemiluminescence immnosensor based on a glassy carbon electrode modified with graphene oxide, polyaniline nanofibers, magnetic beads, and gold nanoparticles. Microchim Acta. 2016;183:1739-1748.

42. Min H, Jo SM, Kim HS. Efficient capture and simple quantification of circulating tumor cells using quantum dots and magnetic beads. Circulating Tumor Cells. 2015;21(11):2536-2542.

43. Song D, Qu X, Liu Y, Li L, Yin D, Li J, Xu K, Xie R, Zhai Y, Zhang H, Bao H, Zhao C, Wang J, Song X, Song W. A rapid detection method of *Brucella* with quantum dots and magnetic beads conjugated with different polyclonal antibodies. Nanoscale Res Lett. 2017:12:179.

44. Dmitriy V R, Anna N B, Anatoly V Z, Saule Z E, Kassym K M, Yerlan M R, Kannatbek N M, Boris B D. Comparison of Three Schemes of Quantum Dots-Based Immunochromatography for Serodiagnosis of Brucellosis in Cattle J.Eng. Applied Sci. 2019;14(11):3711-3718.
### Table 1. Sensitivity assay of ICTS testing of brucellosis

| Dilution | $H_T$ | $H_C$ | $H_T/H_C$ |
|----------|------|------|---------|
| 1:4      | 65376 | 12324 | 5.3048  |
| 1:8      | 56124 | 13632 | 4.1171  |
| 1:16     | 55735 | 18346 | 3.0380  |
| 1:32     | 47775 | 20434 | 2.3380  |
| 1:64     | 34152 | 33784 | 1.0109  |
| 1:128    | 26789 | 31892 | 0.8400  |
| 1:256    | 16995 | 31145 | 0.5457  |
| 1:512    | 8667  | 31972 | 0.2711  |
| 1:1024   | 56    | 32754 | 0.0017  |

### Table 2. The threshold assay of ICTS with 50 healthy *Brucella* negative serum samples from bovine and sheep

| Samples                      | $H_T/H_C$     |
|-------------------------------|---------------|
| Negative serum samples        | 0.0154 0.0004 0.0156 0.0151 0.0145 |
|                              | 0.0123 0.0269 0.0287 0.0269 0.0238 |
|                              | 0.0208 0.0212 0.0260 0.0332 0.0127 |
|                              | 0.0143 0.0037 0.0101 0.414 0.0011 |
|                              | 0.0187 0.0116 0.0070 0.0303 0.0123 |
|                              | 0.0362 0.0312 0.019 0.0116 0.0103 |
|                              | 0.0244 0.0128 0.0125 0.0117 0.0201 |
|                              | 0.0316 0.0146 0.0281 0.0219 0.0018 |
|                              | 0.0151 0.0267 0.00148 0.0327 0.0333 |
|                              | 0.0007 0.0139 0.0044 0.0312 0.0031 |
| Mean                          | 0.0177       |
| Standard deviation            | 0.0105       |
| Threshold                     | 0.0492       |

### Table 3. Specificity of the ICTS for brucellosis

| Sample                  | $H_T/H_C$ | Result |
|-------------------------|-----------|--------|
| Brucellosis             | 1.2648    | +      |
| Tuberculosis            | 0.0329    | -      |
| Bluetongue              | 0.0156    | -      |
| Viral diarrhea          | 0.0317    | -      |
| Foot-and-mouth          | 0.0448    | -      |
| Bovine leukemia         | 0.0118    | -      |
| Peste des petits ruminants | 0.0463  | -      |

### Table 4. Clinical sample detection with ICTS and RBPT
| ICTS | RBPT | Total     |
|------|------|-----------|
|      |      | Positive  |
|      |      | Negative  |
| Positive | 85  | 3  | 88 |
| Negative | 1   | 61 | 62 |
| Total   | 86  | 64 | 150 |

Table 5. Repeatability of the ICTS for brucellosis

| C (ng/mL) | \( \frac{H_T}{H_C} \) | Repeat1 | Repeat2 | Repeat3 | Mean | CV(%) |
|-----------|----------------------|---------|---------|---------|------|-------|
| 0         | 0.0171               | 0.0175  | 0.0177  | 0.0174  | 1.42%|
| 1         | 0.0478               | 0.0455  | 0.0484  | 0.0472  | 2.65%|
| 5         | 0.0641               | 0.0626  | 0.0697  | 0.0654  | 4.60%|
| 10        | 0.1684               | 0.1661  | 0.1449  | 0.1598  | 6.62%|
| 15        | 0.6964               | 0.6316  | 0.6802  | 0.6694  | 4.11%|
| 20        | 1.1364               | 1.1056  | 1.0006  | 1.0809  | 5.38%|
| 25        | 1.9127               | 1.5876  | 1.7925  | 1.7643  | 7.61%|
| 30        | 1.9263               | 1.7422  | 1.6239  | 1.7641  | 7.05%|
| 35        | 2.0472               | 1.8752  | 2.3045  | 2.0756  | 8.50%|
| 40        | 2.3329               | 2.1006  | 2.1923  | 2.2086  | 4.33%|
| 45        | 2.9876               | 2.5969  | 2.4273  | 2.6706  | 8.78%|
| 50        | 3.0015               | 3.0314  | 3.1425  | 3.0585  | 1.98%|

Figures
Figure 1

Schematic diagram of the QDFM based multiplex ICTS. The control line was coated with McAb against OMP22 and the test line was coated with OMP28. The conjugate pad was QDFM functional OMP22.

Figure 2

Optimization of the coating concentration for the NC membrane

\[ y = 37.882x - 3.3625 \]

\[ R^2 = 0.9777 \]
Figure 3

The extreme detection limit of ICTS for Brucella standard positive serum 1. Sensitivity assay of ICTS testing of brucellosis 2. The threshold assay of ICTS with 50 healthy Brucella negative serum samples from bovine and sheep 3. Specificity of the ICTS for brucellosis 4. Clinical sample detection with ICTS and RBPT 5. Repeatability of the ICTS for brucellosis