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

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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 to detect serum antibodies of brucellosis.

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 against *Brucella* were separately dispensed onto a nitrocellulose membrane as test and quality control lines, respectively. Antibodies specific for brucellosis was detected qualitatively using the ratio of the fluorescence signals of the test and control lines (H_T/H_C). The threshold of assay was determined as a H_T/H_C value 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 diseaseswas 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 pose threatens that seriously to human health, and brucellosis leads to several billion dollars of loss annually [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 birth of weak off spring, and poor reproductive performance of animals, which results in huge economic losses for livestock farmers [6]. Currently, there is no effective method to prevent such pathophoresis, contributing to the increased attention on the rapid detection of Brucella.

Traditional detection methods of Brucella are pathogen isolation identification, serological diagnosis, and molecular biology, which all have a few defects. The pathogen isolation identification method produces qualitative and quantitative results, but the method requires strict laboratory conditions, is time consuming and there is potential exposure risk to those performing the experiments. Serological diagnostic methods mainly include the Rose Bengal plate test (RBPT), standard tube agglutination test (SAT) and enzyme-linked immunosorbent assay (ELISA). However, these methods both use the whole cell and whole smooth lipopolysaccharides (S-LPS) as the antigen to detect Brucella serum antibodies, which can cause false positives and cross-reactivity with other Gram-negative bacteria such as Yersinia

entercolitica [7, 8, 9]. Molecular biology methods, such as polymerase chain reaction (PCR) [10], real time PCR (qPCR) and multiplex PCR [11], provide qualitative and quantitative results with good accuracy and sensitivity. However, these methods require expensive instruments and professional operators, are time consuming and they produce aerosol pollution [12]. Therefore, it is extremely important to establish a rapid, accurate and sensitive method to detect brucellosis [13].

In previous studies, the colloidal gold test strip method was shown to have low sensitivity, and it may be difficult to detect low titer rotavirus (RV) and enteric adenovirus (AdV) [14]. An emerging detection technique uses fluorescent microspheres [15, 16] instead of colloidal gold particles [17]. Many researchers have recently focused on developingquantum dot fluorescent microsphere 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, such as fast detection speed, accurate ,efficient , strong specificity ,high sensitivity and simple operation, for compared with other detection techniques [18]. For example, Taranova et al. established a QD-based immunochromatographic assayuse for detection of several antibiotics in milk [19]. In present study, we selected a QD immunochromatography technology for labeling an outer membrane proteins (OMPs) in samples. Lindler et al. identified one non-LPS group of immunogens with OMPs for vaccine and diagnostic purposes [20]. This labeling technology forms hundreds or even thousands of particles by encapsulating or connecting to other materials to form nanoparticles, and it has their own unique nature of good light stability , long fluorescence lifetime, wide excitation spectrum and a narrow emission spectrum, good biocompatibility , size-tunable, indicated that the QD fluorescent microspheres (QDFM) have potential as a new labeling technology immunoassay [21, 22]. The microspheres mainly transmit the optical signal from specific antigen-antibody binding, making the original extremely weak and complex antigen-antibody reaction signal easy to detect. The optical signal amplifies the signal and the sensitivity is improved compared with other labelling technologies [23, 24]. The 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 requirements forspecial operation [25, 26].

OMPs are necessary for the complete virulence of brucellosis strains [27]. 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 [28]. OMP28 is a conserved protein present in at least four Brucella species and is well studied both as a vaccine candidate and as an antigen for serodiagnosis [29, 30]. In present study, we developed anew brucellosis diagnostic method using OMP22 functionalized QD florescent microspheres to form immune complexes with samples conjugated to OMP28.

Results

Optimization of the coating concentration for the NC membrane

To improve the sensitivity of the diagnostic procedure, eight brucellosis positive serum samples with different concentrations of NC membrane coating concentrations were tested, and 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 HT/HC (x) and the sample concentration (y). The linear regression equation was y = 37.882× - 3.3625 and the correlation coefficient was 0.9777, which suggests the feasibility of the ICTS for the determination of brucellosis.

Limit of detection

The standard curve was established with a serial dilution Brucella positive serum of of 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, and was used ICTS for detection. As shown in Fig. 3, the results can be read with an unaided eye 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 limit of detection 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 setting, 50 healthy bovine and sheep serum samples were tested and found a threshold level of 0.0492. The HT/HC value of ≥ 0.0492 (Table 2) indicated a positive specificity assaying the cross-reactivity of the ICTS. The tuberculosis, bluetongue disease, viral diarrhea, foot-and-mouth disease, bovine leukemia and peste des petits ruminants samples all displayed HT/HC values less than the threshold, indicating a positive test result, while the brucellosis samples displayed HT/HC values greater than the threshold indicating there was no cross reaction with the sera of other related disease in the strip (Table 3).

Detection of brucellosis in clinical samples using ICTS and RBPT

To evaluate the feasibility of the strip for detection of antibodies against brucellosis, and to test the reader accuracy, 150 brucellosis clinical serum samples were collected from the Animal Husbandry Bureau of Ningxia Hui Autonomous Region. All of 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 fluorescence reader was used to detect 150 samples and compare with RBPT to calculate the coincidence rate of the two detection methods. 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

To test the repeatability of the ICTS, standard brucellosis positive serum samples were serially diluted to generate 12 concentrations ranging from 0 to 50 ng/mL and three sets of repeated experiments were performed at each concentration. A maximum CV of 8.78% with an average of 6.16% was obtained for all

samples. The CV values of the above results are all less than 10%, illustrating the stability and repeatability of the brucellosis diagnosis procedure based on the QDFM detection technology was very good (Table 5).

Discussion

At present, the effective method for diagnosis of brucellosis has a history of more than 100 years, but brucellosis is still a recurring disease, which is rampant again in many parts around the world [31]. Brucella infection is readily transmissible to humans, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculo-skeletal, 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, it is selecting the dominant diagnostic antigen for brucellosis, and establishing a rapid diagnostic method is the key to preventing and treating the disease. Classical immunological detection technology mainly relies on LPS antigen, and related studies have shown that the high cross-reactivity of LPS antigen with several Gram-negative bacteria is lead to the reason for false-positive results creating difficulties in accurate diagnosis of brucellosis [32, 33, 34]. Therefore, many researchers are looking for a diagnostic antigen such as outer membrane proteins (OMPs) to replace LPS, in order to make up for the shortcomings of LPS antigen, improve the sensitivity and specificity of immunological detection technology[35, 36, 37, 38]. Lim et al. established rOMP28 ELISA to detection of bovine brucellosis and the sensitivity, specificity, and accuracy of 96.7, 95.4, and 96.2%, respectively[30]. In our present study, we also used the OMP28 as the diagnostic antigen to test of brucellosis by immunochromatography because of the specificity and sensitivity is higher than LPS. Brucella OMPs are generally expressed in the form of inclusion bodies in E. coli, and the renaturation 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 E. coli in supernatant form. Considering that the larger fusion tag will affect the immunogenicity of the protein, this experiment uses HRV 3C Protease to cut off the fusion tag and combine it with his-Tag containing medium to purify the target protein. In the end, we obtained relatively satisfactory results in the subsequent testing process. Quantum dot immunochromatographic test strips have higher 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 limt of detection of 103 CFU/mL [39].Compared with Dan detection method, we developed a rapid, accurate and high sensitive method to detection of the antibodies against brucellosis. As is shown in Fig.3 only takes 10-15min to be read with an unaided eye across a UV lamp which became weaker at the 1:512 dilution with a limit of detection 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 antigen detection and can reduce the risk to the detection personnel. When we optimized the coating concentration for the NC membrane, and as is shown in Fig.2 there was good correlation between HT/HC (x) and the sample concentration (y). As is shown in table.3, the

brucellosis samples displayed HT/HC values greater than the threshold which indicating a positive test result, and the tuberculosis, bluetongue disease, viral diarrhea, foot-and-mouth disease, bovine leukemia and peste des petits ruminants samples all displayed HT/HC values less than the threshold, demonstrating that this QDMF can be used for Brucella in real samples with high specificity. To further evaluate the feasibility of the strip for detection of antibodies against brucellosis, it was applied to detect 150 brucellosis clinical serum samples.As Table.4 shows, 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 be used for Brucella detection in real samples with achieved ideal results.

Conclusion

In this study, we presented antigen tagged QDFMs to facilitate detection of Brucella antibodies in standards and clinical samples of only a few microliters using ICTS. The limit of detection of this functional QDFM was many orders of magnitude lower than currently employed in colloidal gold test strips and is comparable to conventional immunoassays. 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 expanded.

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), 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) and an enzyme-linked immunosorbent assay (ELISA) was purchased from IDEXX (Westbrook, ME, 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 toconjugate 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 handheld UV lamp (American Precision Co., Ltd., USA) was used to test strip.

Samples and biological materials

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 crosslinkers. 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 concentrations of 0.5 mg/L EDC and 0.2 mg/mL NHS. The solution was mixed by vortexing 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. Fifty microliters 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 8000g for 20 min. The purified functional QDFM-OMP22 was resuspended in 1 mL of 20 mM Trise 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% NaN_3 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. The final OMP22 concentration was 1 mg/mL. 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). The assembled strips were examined using the serum samples. Eight Brucella serum samples were selected for the determination of the optimal coating concentration for the NC membrane, 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 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/HC values were recorded as negative controls. The ratio of the signals of the test line to that of the control line (HT/HC) provides a qualitative detection for QDFM. The HT/HC threshold values of ICTS were calculated as the following equation: threshold value = mean ± 3× standard deviation. The standard cure was established by use of a serial dilution of Brucella positive serum : 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, and ICTS was used determine the limit of detection. The coefficient of variation (CV) was calculated by dividing the mean of three measurements by the standard deviation to determine the reproducibility and stability of ICTS. The 150 clinical samples were tested using ICTS and the coefficient of the detection results were compared with a commercial RBPT to evaluate the feasibility of the strip for detection of antibodies against brucellosis.

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

Declarations

Ethics approval and consent to participate

Disclosure of potential conflicts of interest 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(ECSRAPM0626001). Orally permissions were obtained from the owners before collection of the specimens. Ethics Committee on Scientific

Reseach on Animal Pathogenic Microorganisms(ECSRAPM) approved the procedure for verbal consent. ECSRAPM 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.

Authors' contributions

Yufang Kong and Huiyu Wang collected and analyzed data and drafted the first version of the manuscript. Shaoqiang WU, Jizhou Lv, Lin Mei and Huifang Zhou performed experiments and analyzed the data. Xiangmei Lin and Xueqing Han participated in the conception and design the experiments and critically revised the manuscripts. All authors read and approved the final manuscript.

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Tables

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	0.0414	0.0011
	0.0187	0.0116	0.0070	0.0303	0.0123
	0.0362	0.0312	0.0109	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)	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

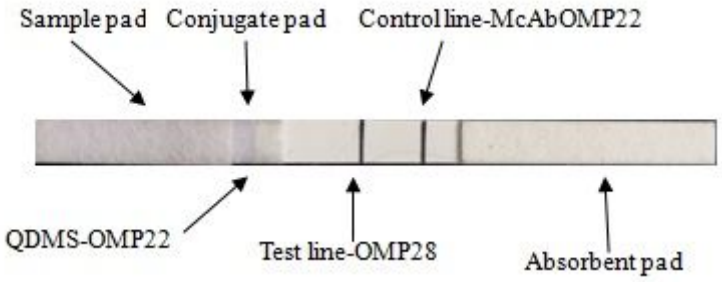

Figure 1

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

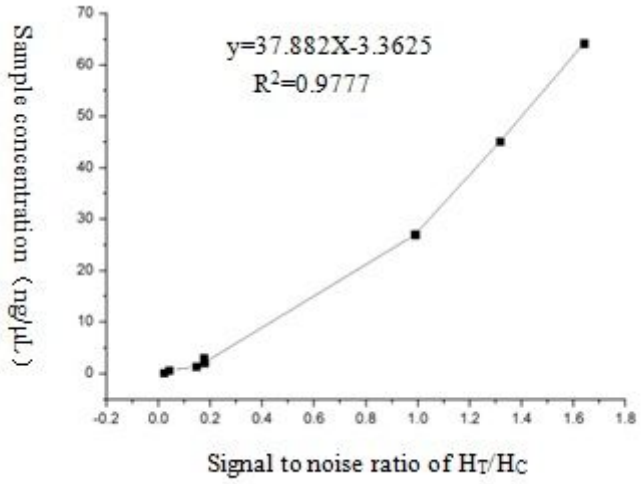

Figure 2

Test result of the coating concentration of the NC membrane.

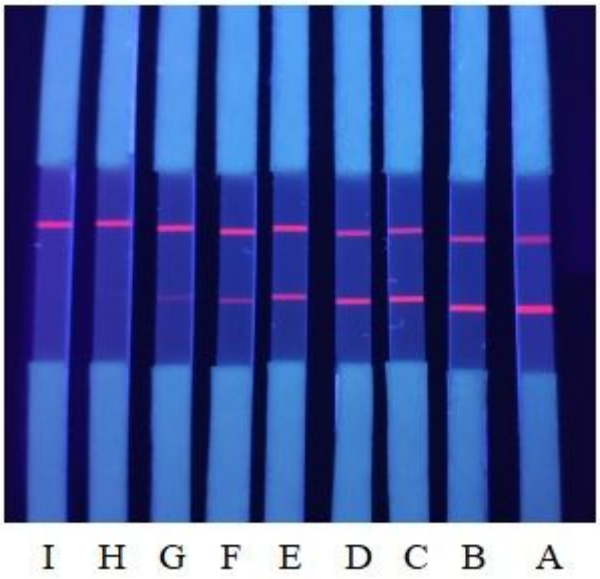

Figure 3

The standard positive serum of Brucella was established as a dilution series and was used ICTS for detection 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