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Research Article

Immunochromatographic Serodiagnosis of Brucellosis in Cattle Using Gold Nanoparticles and Quantum Dots

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ABSTRACT

The article considers formats of immunochromatographic serodiagnosis with the use of gold nanoparticles and quantum dots as labels. The developed systems are designed for rapid (10 min) detection of brucellosis in cattle directly at the sampling site. The advantage demonstrated for the serodiagnosis of brucellosis in cattle with quantum dots is the detection of low titers of specific antibodies. Prototypes of the test systems were tested on a panel of 53 sera of cows infected with brucellosis and 20 sera of healthy animals. It is shown that the registration of the quantum dots' fluorescence makes it possible to achieve, on average, a more than fourfold increase in the signal intensity, compared with the colorimetric detection of gold nanoparticles. In the system with quantum dots, the diagnostic sensitivity in this group of samples was increased to 100%, compared to 94% in the system with gold nanoparticles due to the correct detection of specific antibodies in weakly positive sera. When testing sera of healthy cows, the specificity of the two systems was 100%.

Key words: Brucellosis, Serodiagnosis, Rapid testing, Immunochromatography

INTRODUCTION

Brucellosis is a recognized global problem in veterinary medicine because of the significant economic damage it causes in agricultural livestock. In addition, there is a risk of transmitting the disease to humans, which makes brucellosis a dangerous socially significant infection (Yasmin and Lone, 2015; Beeching and Erdem, 2016; Ducrotoy *et al*., 2018). Brucellosis is among the most widespread diseases in the world, with a high concentration of infection foci in Mediterranean countries, Asia, Africa and Central and South America (Junior *et al*., 2017). Conducting effective anti-brucellosis measures is impossible without timely diagnostics. The rapid and reliable assays are crucial for preventing the spread of the infection (Kumar *et al*., 2016; Junior *et al*., 2017;). For this reason, the time of diagnosis determines the effectiveness of measures to combat the disease.

One of the main methods of screening diagnostics of brucellosis is serodiagnosis-the detection of specific antibodies against antigens of the brucellosis pathogen in the blood of infected animals (Abdoel *et al*., 2008; Kumar *et al*., 2016). Particularly promising is the implementation of serodiagnosis in the format of immunochromatographic assay (ICA), which allows diagnostics to be carried out directly at the sampling site by untrained personnel (Bronsvoort *et al*., 2009; Mdluli *et al*., 2014). The ICA principle is based on the progression of a liquid sample along membranes (forming a test strip) under the action of capillary forces, which leads to the sequential interaction of reagents in different parts of the membranes and the staining of the functional sections of the test strip. With this implementation of the assay, the number of manipulation steps is minimized, which makes ICA an ideal solution for field diagnostics.

The ICA scheme for serodiagnosis is based on the interaction of immunoglobulins in the tested serum with a labeled immunoglobulin-binding reagent. The subsequent detection of specific antibodies that have formed a complex with the label is carried out via their interaction with the immobilized antigen in the analytical zone of the test strip, which leads to a concentration of the label in the analytical zone and the formation of a stained band (Sajid *et al*., 2015; Sotnikov *et al*., 2017a).

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It should be noted that the use of immuno-chromatography for serodiagnostic purposes is difficult because of the need to identify the fraction of specific immunoglobulins against the background of a manyfold excess (by two or more orders of magnitude) of total immunoglobulins. Moreover, the assay is carried out in one step without separating the reagents. Therefore, immunochromatography is often inferior in sensitivity to alternative methods of serodiagnosis (Chandler *et al.*, 2000).

The gold nanoparticle (GNP) is the traditional ICA marker (Abdoel *et al*., 2008; Goryacheva *et al*., 2013). The overwhelming majority of immunochromatographic systems for serodiagnosis of brucellosis is based on the use of GNPs (see (Smits *et al*., 2003; Kim *et al*., 2007; Abdoel *et al*., 2008; McGiven, 2013; Sotnikov *et al*., 2015; Shome *et al*., 2015) as well as commercially available tests from Quicking Biotech Co. Ltd. (China), BioNote Inc. (Korea), Life Assay Ltd. (South Africa), ubio Biotechnology Systems Pvt. Ltd. (India), etc.). One of the ways to increase the sensitivity of ICA is to use alternative markers that generate a stronger assay signal (Qu *et al*., 2009; Juntunen *et al*., 2012). Very promising in this respect are quantum dots (QDs), fluorescent nanoparticles that can be detected at a concentration by an order of magnitude lower than that of GNPs (Goldman *et al*., 2006; Yang *et al*., 2010; Berlina *et al*., 2013a; Bruno, 2014; Wang *et al*., 2015). Water-soluble QDs have a surface coating containing carboxyl or amino groups, which helps simplify the process of conjugation with biomolecules (Liu *et al*., 2007; Wang *et al*., 2011). In comparison with organic fluorescent markers, QDs are more stable, have a narrow symmetrical emission peak and are resistant to photofading (Alivisatos, 1996). At the same time, QDs are characterized by a wide peak of light absorption, which makes it possible to excite their fluorescence using ordinary polychromatic LEDs instead of lasers and monochromators. All these properties make QDs the most attractive marker for fluorescent ICA.

Our study's aims included the development of immunochromatographic test systems for the detection of specific class G immunoglobulins against the lipopolysaccharide antigen (LPS) of *Brucella abortus* (the pathogen of cattle brucellosis). The LPS of *Br. abortus* is the main antigen used for serodiagnosis of this disease since it contains the immunodominant O-antigen (Poester *et al*., 2010). The test systems were developed in two versions that used either GNPs or QDs as markers. These test systems were tested on a selection of bovine sera with a confirmed diagnosis, and a comparative characterization was performed of the assays on the effectiveness of serodiagnosis for animals with low titers of antibodies against the LPS of *Br. abortus*.

MATERIALS AND METHODS

Preparation of gold nanoparticles by Frens method

A total of 200 µl of 5% tetrachloroauric acid (Sigma, USA) was added to 97.5 ml of deionized water. The mixture was brought to boiling while stirring. Then 1.5 ml of 1% sodium citrate solution (Sigma, USA) was added. The mixture was boiled for 30 min and cooled to room temperature (Frens, 1973).

Determination of GNP sizes by transmission electron microscopy

GNP preparations were applied to 200 mesh hexagonal copper grids coated with formvar film (SPI Supplies, USA). Microscopic analysis was performed on a JEM–100 CX/SEG instrument (Jeol, Japan). The obtained microphotographs were scanned to produce 1200 dpi black and white images, and 117 images of particles were processed using the Image Tool program (University of Texas Health Science Center at San Antonio, USA). From the obtained data, the average sphere diameter, the dispersion of values and the degree of ellipticity were calculated.

Preparation of conjugates of GNPs with streptococcal protein G

Protein G (Imtec, Russia) was diluted in a solution of GNPs (optical density at 520 nm: OD_{520} = 1) to a concentration of 10 µg/ml. It was incubated for 30 min while stirring at room temperature and then stabilized with a bovine serum albumin (BSA) solution (MP Biomedicals, USA) that was added to a final concentration of 1%. The resulting solution was centrifuged for 30 min at 10,000 g. The supernatant was decanted, and the precipitate was brought to a volume of 1 ml with 10% BSA solution. The optical density of the prepared conjugates was measured on a spectrophotometer at 520 nm.

Conjugation of proteins with QDs

Protein G was conjugated with QDs with an emission wavelength of 625 nm (core CdSe / shell ZnS) (Invitrogen, USA) as described in (Berlina *et al*., 2013b) with a slight modification. A total of 300 µL of protein G in 10 mM borate buffer solution (pH 8.6) at a concentration of 144 µg/mL was mixed with 25 µL of QDs with carboxyl groups on the surface (Qdot 625 ITK, Invitrogen, USA, 8 µM concentration) at a molar ratio of 10:1. Then, 50 µL each of freshly prepared solutions of N-(4-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (Fluka, Switzerland) and sodium N-hydroxysulfosuccinimide (Sigma, USA) at concentrations of 308 µg/mL and 348 µg/mL, respectively, was added to the mixture of protein G and QDs. The reaction was performed in the dark at room temperature by stirring on an Intelli-Mixer RM-2 shaker (Elmi, Latvia) for 120 min. After this, the resulting conjugate was concentrated and simultaneously dialyzed against 10 mM borate buffer solution (pH 8.6) with the use of Amicon Ultracel 30K filters (Millipore, USA) at 10,000 g for 15 min to remove excess activators and unbound protein. After centrifugation, the resulting conjugate was concentrated by 10 times compared to the reaction volume and kept at +4°C before application.

Preparation of LPS of *Br. abortus*

Br. abortus cell cultures were grown on erythritol agar (NPO Microgen, Russia). The cells were washed with sterile saline solution (pH 7.0–7.2), filtered and heated at 80°C for 1 h to inactivate. The resulting slurry was centrifuged at 3,000 g for 15 min. The supernatant was separated, and the precipitate was used to obtain the LPS of the *Br. abortus* cell wall.

A total of 20 g (dry weight) of bacterial cells were carefully resuspended in 350 mL of water at 65–68°C. Then, 350 mL of phenol melt was heated to a temperature of 65–68°C and added to the cell suspension. The mixture was vigorously stirred, kept for 15 min at 65°C and, then, cooled in an ice bath to 10°C. The emulsion was centrifuged for 30 min at 10,000 g. As a result, three layers were formed: aqueous, phenolic and precipitate. The upper aqueous layer was removed and stored separately. The substances at the phase boundary were removed and discarded. Another 350 ml of water was added to the phenolic layer and precipitate and was thoroughly mixed while maintaining the temperature at 65–68°C for 15 min. The mixture was again centrifuged, and the upper aqueous layer was combined with the aqueous layer obtained after the first centrifugation. The aqueous suspension was dialyzed overnight against distilled water to remove phenol and low molecular weight impurities. The dialyzed solution was concentrated to 5 mL using a rotary evaporator at 37°C. The concentrated solution was centrifuged at 3,000 g for 10 min to remove insoluble substances. The LPS comprised half of the organic matter in the supernatant fraction. From 20 g (dry weight) of the biomass of bacteria, 200 mg of the LPS was prepared.

The concentration of the LPS in the prepared fractions was determined by the phenol-sulfur method. For calibration, glucose solutions of 1 mg/mL to 125 μg/mL were prepared. A total of 200 μL of 5% phenol was added to 200 μL of the material under study. Then, 1 mL of concentrated sulfuric acid was added, and the concentration of carbohydrates was determined based on the optical density (λ=488 nm) of the obtained solution (Westphal and Jann, 1965).

Panel of bovine blood sera

A characterized panel of blood sera of cows infected with *Br. abortus* (53 samples) and healthy animals (20 samples) was provided by the Republican State Enterprise "National Center for Biotechnology" at the Science Committee of Ministry of Education and Science of the Republic of Kazakhstan. The diagnosis of brucellosis was made on the basis of ELISA testing of sera using a commercial kit produced by Bicentre, Stepnogorsk, Kazakhstan.

ELISA detection of specific antibodies against LPS of *Br. abortus* in bovine sera

Sorption of the LPS preparation in the wells of a 96-well Greiner microplate was carried out overnight at 4°C from 100 μl of the LPS solution at a concentration of 1 μg/mL in 50 mM carbonate buffer (pH 9.6). The microplate was washed four times with 50 mM potassium phosphate buffer (pH 7.4), containing 0.1 M NaCl and 0.05% Triton X-100 (PBST). After that, 100 μl of sera diluted with PBST from $1:10^2$ to $1:10^5$ were added to the wells in increments of two, and the microplate was incubated for 1 h at 37°C. Then, the microplate was washed again; 100 μl of the solution of anti-bovine IgG monoclonal antibodies labeled with horseradish peroxidase (Imtec, Russia) in PBST was added at a concentration of 160 ng/mL; and the microplate was incubated for 1 h at 37°C. After washing the microplate (three times with PBST and once with distilled water), the peroxidase activity of the enzyme label bound to the carrier was determined. To do this, 100 μL of 0.4 mM solution of 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma, USA) substrate with 0.01% H_2O_2 was added to the wells, incubated for 15 min at room temperature and measured at OD_{450} on a Zenyth 3100 flatbed spectrophotometer (Anthos Labtec Instruments, Austria).

Making immunochromatographic assay systems

For making immunochromatographic assay systems, we used a set of MDI Easypack membranes (Advanced Microdevices, India) that includes working nitrocellulose membranes on polyester substrate CNPH90, pads for conjugate PT-R5, membranes for applying sample FR1(0.6) and final adsorbing membranes AP045. To apply the reagents, we used an IsoFlow dispenser (Imagene Technology, USA).

Conjugates of GNPs (OD_{520} = 20; application volume = 2 μL/mm) and QDs (concentration = 34 μM; application volume = 1 μL/mm) were applied onto the pad for the conjugate. For the formation of the analytical zone, the LPS preparation at a concentration of 1 mg/mL was applied onto the nitrocellulose membrane 90CNPH with an application volume of 0.1 μL/mm. The control zone was formed on a polyester backed nitrocellulose membrane using rabbit anti-bovine IgG antibodies (Imtek, Russia); 2 μL of the antibodies (5 mg/mL in 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl) were applied per centimeter of the strip. After applying the reagents, the membranes were air-dried at 20–22°C for at least 20 h. A multi-membrane composite was assembled, from which 3.5 mm wide strips were prepared using an Index Cutter-1 automatic guillotine cutter (A-Point Technologies, USA). Cutting and packing were carried out at 20–22°C in a special room with a relative humidity of not more than 30%. Packed test strips were stored at 20–22°C (Berlina *et al*., 2013a).

Immunochromatographic assay

ICA was carried out at room temperature. One drop of blood serum and, then, three drops of PBS containing 1% Tween-20 were added to an Eppendorf tube, and the test strip was vertically placed into the tube. In 10 min, the ICA result was visually controlled. The registration of the results of immunochromatography with QDs was performed in a fluorescent chamber by irradiation with light at a wavelength of 365 nm. To quantify the assay results, images of the test strips were photographed and digitized using a TotalLab TL120 software package (Nonlinear Dynamics, UK).

RESULTS AND DISCUSSION

Characterization of marker nanoparticles

One of the key factors influencing the sensitivity of the immunochromatographic assay system is the marker properties. To achieve the best assay characteristics, it is necessary to use stable non-aggregating particles with a high degree of uniformity in size and shape.

GNPs were synthesized by the Frens method (Frens, 1973). An analysis of the obtained preparations using transmission electron microscopy demonstrated a high

homogeneity of the sizes of the synthesized nanoparticles: the length of the major axis was 29±6 nm, and that of the minor axis was 25±4 nm. Thus, the average diameter of the prepared nanoparticles was 27±6 nm, and the degree of ellipticity was 0.93. An example of a microphotograph of the GNPs and a histogram of the distribution of particles by size are shown in Fig. 1.

QDs have a unified size that, according to the manufacturer's passport, is 6 nm (without polymer coating).

The absorption spectrum of the synthesized GNPs with a characteristic peak at 520 nm (Fig. 2A) corresponds to the spectrum of dispersed particles without significant aggregates and indicates the stability of the obtained preparation. The fluorescence spectrum of QDs and synthesized conjugate with protein G is shown in Fig. 2B. An important property of the conjugate of QDs with protein is the preservation of the fluorescence properties of the marker. The fluorescence spectra demonstrate a 10% decrease in the fluorescence intensity of the conjugate relative to that of free QDs. Moreover, the peaks of fluorescence of free QDs and the conjugate of QDs coincide. Experimental values of the fluorescence's relative quantum yields also show a decrease in fluorescence from 0.88±0.04 to 0.78±0.04. These data suggest that the covalent crosslinking of QDs with protein G does not cause significant changes in the fluorescent properties of the marker, which makes it possible to effectively use the synthesized conjugate in the assay.

ELISA testing of bovine sera

Serum from cows infected with *Br. abortus* (53 samples) and healthy cows (20 samples) were tested by the ELISA scheme to determine the titers of specific immunoglobulins. The results of ELISA testing on a limited panel of sera are shown in Fig. 3. The tested sera were divided into three groups according to the signal in ELISA:

1. negative sera that, at 500-fold dilution, produced a signal in the ELISA below 0.2 optical units-20 sera;
2. weakly positive sera that were characterized by the lowest antibody titers but significantly exceeded the threshold level for healthy animals (quantitative criterion: the optical density in ELISA is from 0.2 to 0.4 at a 500-fold serum dilution)-5 sera; and
3. strongly positive sera, the signal of which exceeded 0.4 optical units according to the above criterion-48 serums.

Immunochromatographic assay of sera

ICA was implemented according to the scheme that assumes the interaction of the IgG with the marker-protein G conjugate and the subsequent detection of specific antibodies in the complex with the marker (GNP or QD) by means of their binding with the immobilized LPS preparation in the analytical zone. Streptococcal protein G was chosen as the immunoglobulin-binding protein since this protein has a lower molecular weight than anti-species antibodies (26 kDa vs. 150 kDa) and

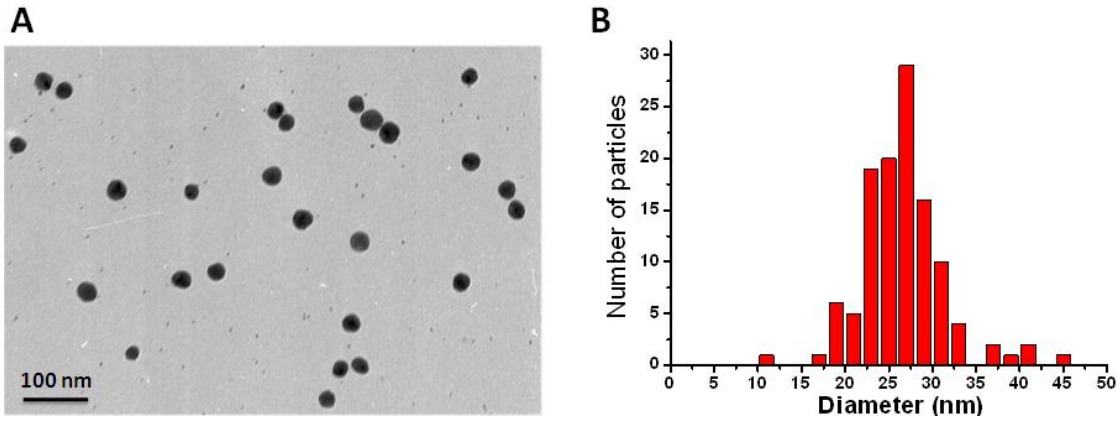

Fig. 1: (A) Microphotograph of GNPs and (B) histogram of particle size distribution.

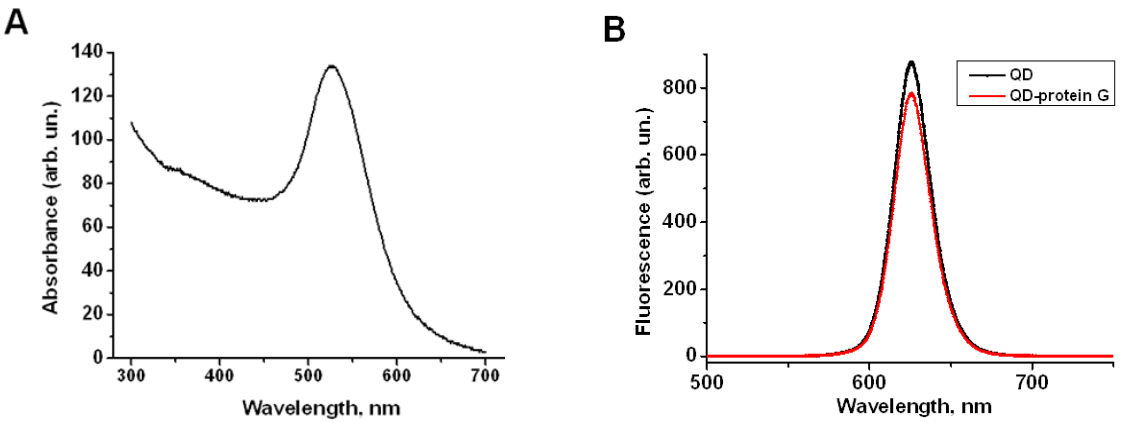

Fig. 2: (A) Absorbance spectrum of synthesized GNPs. (B) Fluorescence spectrum of QDs and QDs conjugated with protein G dissolved in 25 mM PBS. Excitation wavelength is 365 nm.

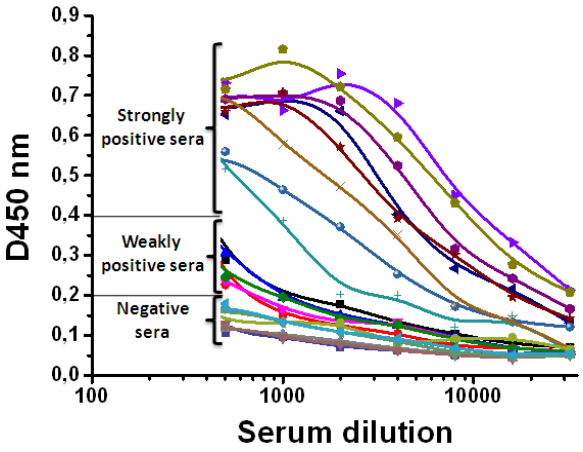

Fig. 3: Detection of specific immunoglobulins against the LPS of *Br. abortus* in bovine blood serum by ELISA.

therefore, can provide a higher density of immunoglobulin-binding sites. In addition, as shown in (Quintero *et al*., 2018), immunochromatographic serodiagnosis of brucellosis in cattle using streptococcal protein G is characterized by higher sensitivity than that using staphylococcal protein A.

Upon contact of the sample with the marker conjugate, all IgG interact with the protein G while only specific antibodies interact with the immobilized LPS. That is, most of the labeled IgG complex does not participate in the interaction with the antigen in the analytical zone. In addition, the serum contains about 10 mg/ml IgG while the binding capacity of the labeled conjugate is two orders of magnitude lower (based on the maximum amount of immunoglobulin-binding protein on the surface of the marker). The potential of marker concentration is also limited since its concentration facilitates the aggregation of the preparation and reduces the specificity of the assay (Sotnikov *et al*., 2017b). Consequently, most specific immunoglobulins will not be in a complex with the marker. Thus, there will be a competition in the analytical zone between free and labeled immunoglobulins for binding to the LPS. All these factors reduce the amount of the detectable complex in the analytical zone of the test strip. For this reason, the marker detection limit is crucial for the sensitivity of immunochromatographic serodiagnosis.

In our case, GNPs and QDs were used as markers. With the exception of the marker conjugate, all other components and reagent concentrations were identical for the two assay systems. Serum samples that were classified as strongly positive by ELISA demonstrated positive results in testing by ICA as well (all 48 samples). However, for an immunochromatographic system with GNP, testing results for five samples from the weakly positive group were either negative or questionable. At the same time, all sera of infected cows gave positive results in testing by ICA with QDs.

The results of testing a panel of seven sera, including five weakly positive sera, are presented in Fig. 4. To assess the maximum and minimum intensity of staining of the analytical zone of the test strips, strongly positive and negative sera were included in the panel. The presented photographs demonstrate that the use of QDs as a label gives a clearer visualization of the immune complex in the analytical zone.

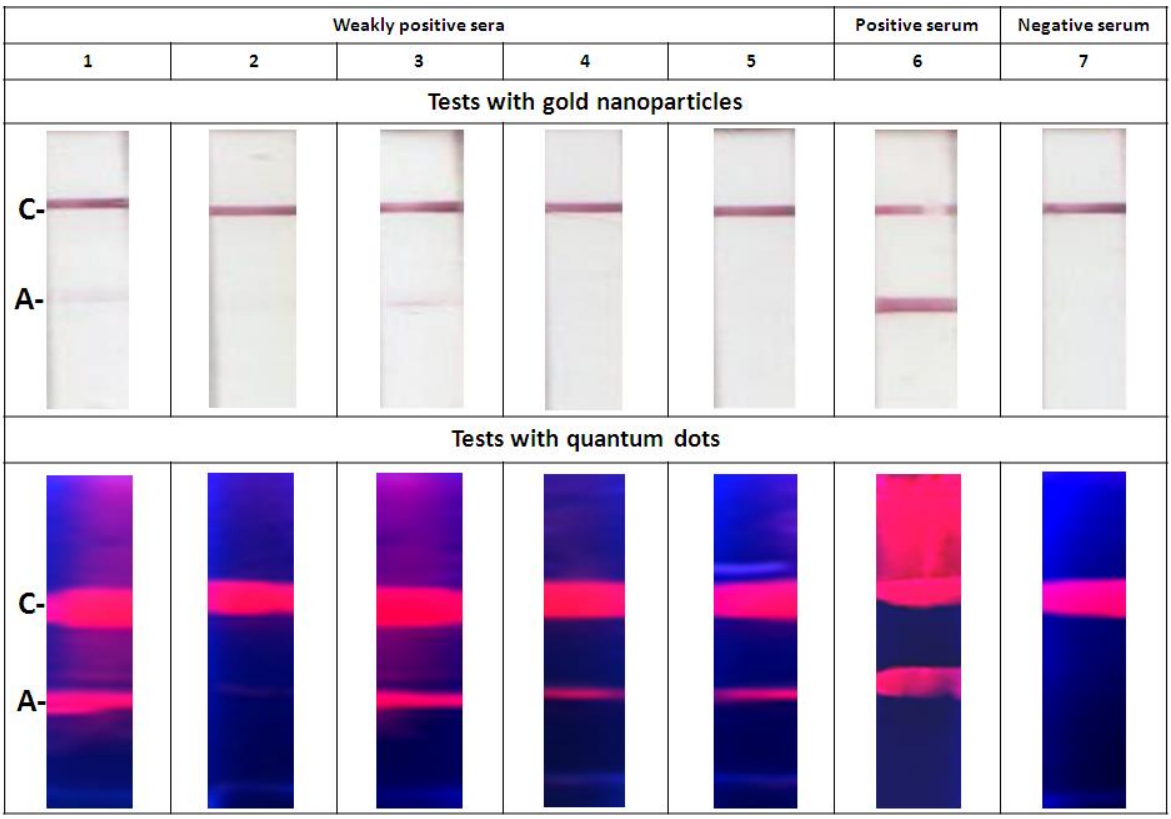

Fig. 4: Testing bovine blood sera by the immunochromatography method using GNPs and QDs as labels. A = analytical zone; C = control zone.

Table 1: Results of digitization of assay system images in the TotalLab TL120 program-intensities of staining of analytical zones and qualitative result of testing

Serum	Gold nanoparticles		Quantum dots	
	Staining of analytical zone, arbitrary units	Testing result	Staining of analytical zone, arbitrary units	Testing result
No. 1	2.6	±	12.5	+
No. 2	0.4	-	0.8	+
No. 3	2.2	±	9.3	+
No. 4	0.0	-	3.3	+
No. 5	0.3	-	3.1	+
No. 6	17.2	+	14.4	+
No. 7	0.0	-	0.0	-

Digitization of assay system images using the TotalLab TL120 program (Nonlinear Dynamics, UK) provides indicators of the relative intensity of the staining of the analytical zones of the test strips summarized in Table 1. The obtained data demonstrate that the use of QDs as a label allows increasing the signal intensity in weakly positive samples by more than four times. As a result, the diagnostic sensitivity of the assay on the selected panel increased from 94% for GNPs to 100% for QDs. The specificity of the assay when testing sera of healthy cows was 100% for both versions of the assay systems.

Conclusion

A comparative evaluation of the use of GNPs and QDs in immunochromatographic serodiagnosis of brucellosis in cattle was performed. The results of testing assays with two types of markers demonstrate an increase in the sensitivity in detecting specific antibodies against the LPS of *Br. abortus* when using QDs. Thus, the use of QDs in immunochromatographic serodiagnosis appears to be an effective way to improve the reliability of ICA for specific antibodies.

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