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Europium-based fluorescent immunochromatographic test for brucella antibody detection

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Abstract

This study presents the development and validation of a novel fluorescent immunochromatographic test (F-ICT) using Europium-labeled conjugates for the detection of Brucella antibodies in serum and milk samples. The assay utilizes purified Brucella lipopolysaccharide (S-LPS) as the capture antigen, which was extracted with an optimized protocol yielding 15 µg/mg dry biomass, a seven-fold improvement over previous methods. The extracted S-LPS demonstrated high purity and retained its structural and immunological properties, as confirmed by SDS-PAGE, Western blot, and endotoxin testing. The F-ICT showed 100% sensitivity and specificity when tested against a French national brucellosis standard panel of bovine sera, significantly outperforming two commercially available gold-labeled immunochromatographic tests. In artificially spiked milk samples, the F-ICT demonstrated a detection limit of 1×10^{-5} dilution, two orders of magnitude more sensitive than conventional ELISA (1×10^{-3}). The fluorescent signal remained stable for at least five days at room temperature, with no significant photobleaching observed. These results establish the F-ICT as novel a highly sensitive, specific, and stable diagnostic tool for Brucella antibody detection in both serum and milk samples, offering potential advantages for large-scale surveillance programs.

Keywords: Fluorescent immunochromatography (F-ICT); Brucella diagnostics; lipopolysaccharide (LPS) extraction; Immunoassay sensitivity; *In vitro* diagnostics (IVD); Point-of-care testing (POCT)

1. Introduction

Brucellosis, is a zoonotic disease caused by bacteria of the genus Brucella that poses a significant threat to both animal health and public health globally. It primarily affects livestock such as cattle, sheep, goats, and pigs, leading to reproductive failure, economic losses, and potential transmission to humans through direct contact or consumption of contaminated products. One of the primary means of human infection is through the consumption of unpasteurized dairy products, where the bacteria can reside [1, 2].

The detection of Brucella-specific antibodies in milk samples offers a non-invasive, practical approach to diagnosing brucellosis in dairy animals. Milk is readily available and routinely collected, making it an ideal sample for large-scale surveillance. Several immunological assays, such as enzyme-linked immunosorbent assay (ELISA), have been developed to detect antibodies against Brucella antigens in milk. These methods enable early diagnosis, which is crucial for controlling the spread of the disease in both animals and humans [3].

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Infection with Brucella in cattle is usually caused by biovars (bv.) of Brucella abortus. In some countries, particularly in southern Europe, Africa and western Asia, where cattle are kept in close association with sheep or goats, infection can also be caused by B. melitensis [4]. Occasionally, B. suis may cause infections in cattle. Young animals and non-pregnant females usually show no signs of the disease. Following infection with B. abortus or B. melitensis, pregnant adult females develop a placentitis usually resulting in abortion between the fifth and ninth month of pregnancy. The mammary gland and associated lymph nodes may also be infected, and brucella's germ may be excreted in the milk. Colostrum originating from infected dams is a source of infection in the newborn population. In acute infections, the organism is present in most major body lymph nodes. Adult male cattle may develop orchitis/epididymitis and brucellosis may be a cause of infertility in both sexes. Brucella abortus can be shed in semen, seminal fluid and urine. Hygromas, usually involving leg joints, are a common manifestation of brucellosis in some tropical countries and may be the only obvious indicator of infection; the hygroma fluid is often infected with Brucella [5]. Accurate and timely detection of antibodies in milk samples can assist in the identification of infected herds, allowing for better management and control measures, including culling infected animals and improving hygiene practices in dairy production. This approach not only helps in reducing economic losses in the livestock industry but also contributes to preventing zoonotic transmission to humans.

A fluorescence polarization assay (FPA) for detection of antibody to Brucella abortus in individual milk samples was developed and validated. The relative sensitivity value obtained with milk from cattle of infected herds and the specificity values of the indirect ELISA were 98.5 and 99.9%, respectively [6]. For the diagnosis of brucellosis, a low molecular weight fragment (average 22 kDa) of the lipopolysaccharide (LPS) from Brucella abortus strain 1119-3 Smooth-Lipopolysaccharide (S-LPS) was labeled with fluorescein isothiocyanate (FITC) and utilized as the antigen in a homogeneous immune assay. Following the initial blank/background reading, FITC-LPS was added to the diluted serum/milk samples. The antibody content was measured approximately 2 minutes after the addition of the antigen using a fluorescence polarization analyzer (FPA) [7]. Unlike other homogeneous methods (e.g., Rose Bengal Test), a blank/background reading is required for each sample prior to the addition of the antigen, making the fluorescence polarization assay (FPA) a two-step process [5].

LPS as a potent antigen, is known to induce specific humoral response in infected hosts by activating B lymphocytes and inducing the production of antibodies without the apparent contribution of T cells, thus LPS is traditionally described as a T cell-independent antigen [8]. LPS plays a vital role in the integrity of Gram-negative bacterial outer membranes. It comprises three regions: a Lipid A moiety composed of a disaccharide backbone linked to up to seven hydrophobic acyl chains that are embedded in the outer membrane, a species-conserved core polysaccharide linker, and the external most O-polysaccharide chain [9]. The O-polysaccharide is also the outermost part of the LPS molecule expressed on bacteria and is therefore the major antigen targeted by host antibody responses. These responses can be highly O-chain specific, and for this reason the O-chain is often also referred to as the O-antigen. As a result of this, serology of O-antigens has become a useful tool in typing strains of organisms and LPS [10].

The objective of our study was to develop fluorescent lateral flow immunochromatography tests for detection of brucella's specific antibodies in serum and spiked milk samples and compare its sensitivity with indirect ELISA and immunogold lateral flow immunochromatography tests, commercially available on the Chinese market.

2. Material and methods

2.1. Brucellosis LPS antigen isolation and characterization

LPS was isolated from brucellosis live vaccine strain A19 using hot phenol method, according [11] with our modifications. Bacterial cells cultivated in Brucella Broth medium (Bacto tryptone 20g/L, Yeast extract 5g/L, Dextrose 1g/L, Sodium Chloride 5g/L and Sodium Citrate 10 g/L, pH 7.5) at 37°C under aeration (250 rpm) overnight. Bacterial pellet after centrifugation (10,000xg for 15 min) washed twice with cold PBS to remove any media residues. Finally, equal volume (1:1) of preheated 90% phenol to the cell suspension, vortexed briefly to mix, and then incubated at 65°C in a water bath for 20 minutes with frequent shaking. Phase separation was carried out by centrifugation at 10,000xg for 15 min at 4°C. Aqueous phase (contains LPS) carefully collected and dialyzed against distilled water for 48 hours to remove residual phenol and small contaminants. LPS was precipitated from dialyzed solution by addition of equal volume of cold ethanol and incubation overnight at -20°C. Precipitated LPS was pelleted by centrifugation (10,000xg for 15 min), washed twice with methanol and dried at 60C in heating oven. Dried LPS pellet was reconstituted in distilled water with 0.05% Tween-20.

LPS concentration was measured by phenol-sulfuric acid method, according [12]. We adopted this method for microscale format, the final reaction volume was adjusted to 350 µL (50 µL sample, 50 µL 5% Phenol and 250 µL Sulfuric

Acid). The LPS concentration was determined using a calibration curve with a 1% dextrose solution as the reference standard, after absorbance measuring at 490 nm.

LPS purity and structural integrity was estimated by it separation in 12% SDS-PAGE at 100 mA for 1h, with Silver staining, according [13]. All procedures with Brucella live vaccine were carried out in BSL2 condition with personal safety rules compliance.

2.2. Estimation of Endotoxin Potency Using the Fluoro-LAL Method

The endotoxin potency of brucellosis lipopolysaccharide (LPS) was evaluated using the Fluoro-LAL assay, a fluorescence-based kinetic method established in our laboratory. Limulus Amebocyte Lysate (LAL) reagent (Charles River) was reconstituted in endotoxin-free water according to the manufacturer's instructions. A control standard endotoxin *E. coli* (CSE) with a potency of 10 Endotoxin Units (EU) was dissolved in 1.0 mL of distilled water containing 0.05% Tween-20 to ensure accurate dispersion and stability.

The substrate peptide FITC-Acp-VPRSK(Dnp), synthesized by Apeptide Co., Ltd. (China) with a confirmed HPLC purity of 98.55%, was dissolved in DMSO to prepare a stock solution at 10 μM. A working concentration of 1 μM was optimized through preliminary titration experiments (data not shown). The reaction mixture was assembled in 200 μL PCR tubes, where 8 μL of LAL reagent was combined with 2 μL of the peptide substrate solution (1 μM) and mixed thoroughly. The mixture (10 μL per well) was then dispensed into PCR tube wells, followed by the addition of 10 μL of either CSE dilutions, brucellosis LPS samples, or endotoxin-free water as a blank control. This resulted in a final reaction volume of 20 μL.

The assay was conducted using a 7500 Fast Real-Time PCR System (Applied Biosystems) with the following conditions: 40 cycles at 37°C, with fluorescence measurements recorded every 60 seconds in the FAM channel (520 nm). Calibration curves were prepared using serial dilutions of *E. coli* CSE and were generated by plotting fluorescence intensity against endotoxin concentration in EU. Brucellosis LPS samples were prepared by dissolving purified LPS in endotoxin-free water at concentrations that fell within the detection range of the assay.

Fluorescence data for the brucellosis LPS samples were extrapolated to the *E. coli* CSE calibration curve to determine endotoxin potency.

2.3. Goat immunization with purified Brucella's LPS

A 9-month-old male goat used in this study was maintained and handled in accordance with the Experimental Animal Regulation Ordinances established by the China National Science and Technology Commission. The experimental protocol was approved by the Animal Ethics Committee of the company board (Protocol No. 8, dated 20 March 2023). The goat received three subcutaneous injections of 250 μg of purified Brucella lipopolysaccharide (LPS) preparation at 10-day intervals. The first injection was administered with complete Freund's adjuvant (Sigma, Cat. No. F5881), while the subsequent two injections were performed using incomplete Freund's adjuvant (Sigma, Cat. No. F5506). Blood samples were collected from the ear vein prior to immunization and 30 days following the initial injection.

2.4. ELISA

LPS purified preparation was coated into ELISA plate (Nunc, Maxysorp) at 10 μ/mL in 50 mM Carbonate bicarbonate buffer (pH 9.6), plates incubated at 4°C overnight. After coating plates were blocked 1 h at 37°C with 1% BSA (Protease free, IgG free, Biogradetech, Cat. No. A-CSH006) in phosphate buffered saline with 0.05% Tween-20 (BSA-PBS-T). Serum samples (Table 1) were diluted in BSA-PBS-T 1.0 $x10^{-2}$ and incubated 1 h at 37C, washed with PBS-T using ELISA plate washer (Wellwash 4 MK 2, Thermo Scientific). As secondary enzyme labelled reagent, we used horse radish peroxidase (HRP) conjugated recombinant protein G (BBI, Cat. D 111128) in 1.0 x 10^{-3} dilution in PBS-T, incubation time 30 min at 30°C. After plate washing HRP chromogenic substrate Tetramethylbenzidine (TMB, Solarbio life science, Cat. No. PR1200) was added into plate wells and incubated 10 min at room temperature in the dark. Color development was stopped by addition 2M Sulfur Acid and plate absorbance was read at λ 450 nm, using ELISA plate reader (AMR-100, Allsheng).

Table 1 Serum samples used in this work

Code	Description	Supplier
SR 01	Bovine Brucellosis French National Negative Standard serum panel	ANSES, France
SR 02		
SR 03		
SR 04		
SR 05		
SR 06	Bovine Brucellosis French National Positive Standard serum panel	ANSES, France
SR 07		
SR 08		
SR 09		
SR 10		
SR 11		
SR 12		
EU G BSS	European Union Goat Brucellosis Standard serum	ANSES, France
EU S BSS	European Union Sheep Brucellosis Standard serum	
EU P BSS	European Union Pig Brucellosis Standard serum	
EU Bo SS	European Union Brucella ovis Standard serum	
EU D BSS	European Union Dog Brucellosis Standard serum	
RBP	Brucellosis Positive Rabbit Serum	Egypt*
CBP	Brucellosis Positive Camel Serum	SA**
GBN	Goat Brucellosis Negative before immunization	This study
GBP	Goat Brucellosis Positive 30 dpi	

* Personal gift Dr. Magdy Elsayed, MEVAC, Egypt; ** Personal gift Dr. Osman Abdel Hameed, ARTAT Enterprise, Saudi Arabia

2.5. Western blot

Different quantities of extracted LPS were separated by SDS-PAGE, as mentioned above and wet transferred to PVDF-membrane (Thermo Scientific, Cat. No. 88520) in Transfer buffer (Tris base 3g/L, Glycine 14.4 g/L, Methanol 100 g/L) 1h at 100A. After electro transfer membrane was blocked in 1% BSA in PBS-T for 1h at room temperature and probed with Goat Brucellosis Positive serum (30 dpi) $1.0x10^{-2}$ dilution for 1h at room temperature, after membrane washing 3 times, the bound antibodies were detected by horse radish peroxidase (HRP) conjugated recombinant protein G (BBI, Cat. No. D 111128) in 1.0×10^{-3} dilution while incubated 1 h at room temperature, following washing membrane 3 times with PBS-T. Bound immune complexed were detected using Enhanced Chemiluminescence kit (Genview, Cat. No. GE2301) and fixed on the X-ray film (Carestream, Cat. No. 6549380).

2.6. Milk spiking with Goat Brucellosis Positive serum

Pure milk from the local market, labelled for Protein content 3.2%, Fat - 3.8%, Carbohydrate - 4.7%, Na^+ - 0.06%, and Ca^{2+} - 0.1%, was used for spiking with Goat Brucellosis Positive Serum sample (30 dpi). For ELISA testing we used 10-fold spiking levels from 10^{-2} to 10^{-5}. For Fluorescent immunochromatographic testing we used spiking levels from 10^{-2} to 10^{-6}.

2.7. Secondary regent (SPG-Eu PC) fluorescent labelling

Recombinant Streptococcal protein G, produced in-house following the protocol described by [14], was utilized as the secondary immunological component due to its well-documented ability to interact with immunoglobulins from

multiple milk-producing cattle species. For fluorescent labeling, Europium-impregnated carboxyl-modified polystyrene microspheres (200 nm in diameter; Suzhou Vdo Biotech, Cat. No. FT0200CA) were employed. The conjugation of protein G to the microspheres was performed using standard EDC/NHS chemistry, following the manufacturer's instructions.

2.8. Immunochromatographic tests fabrication

Equipment used for ICT fabrication from Kinbio Tech.Co., Ltd. are mentioned in table 2:

Table 2 ICT production equipment

Model Name	Description
YZ Platform Dispenser HM3035	Used for membrane Test-line, Control-line dispensing and conjugate spraying,
Programmable Strip Cutter ZQ2002	Used for cutting immunoassay uncut sheet into test strips with specific width
Assembly Roller YK725	Assembly roller is designed for the plastic cassette's compression
Programmable Sheet Cutter CTS300	Used to cut the absorbent paper, sample sheets, conjugated sheets and membranes

Materials used for ICT fabrication are mentioned in table 3:

Table 3 ICT production consumable materials

Materials Name	Description	Supplier
NC membrane Cat. No.: MPFC010025	Nitrocellulose membrane, used for LPS antigen coating (Test-line) and bovine IgG (Control-line)	Mdi, India
Conjugate pad Cat. No.: VHC06001	Glass fibre membrane, used for spraying secondary labelled reagent (SPG-Europium Polystyrene nanoparticles conjugate)	Suzhou Vdo Biotech, China
Absorbent Pad Cat. No.: H5072	Thick filter paper, used for liquid lateral flow direction and absorbance	JY BIOTECH, China
Sample Pad Cat. No.: XQ-Y2	Glass fibre membrane, used for sample application	JY BIOTECH, China
Plastic base card DB-6	Plastic base card for ICT strips preparation, size 6.0cm x 30cm	JY BIOTECH, China
Plastic cassettes Cat. No.: 2103000506	Housing for ICT strips, size: 70 mm x 20 mm	Yuyang, Chin

Immunological reagents were dispensed on the NC membrane, for T-line we used Brucellosis LPS antigen (0.5 mg/mL) 0.8 μL/cm and for C-line we used bovine IgG (5.0 mg/mL) 0.8 μL/cm). NC membrane vacuum dried used for ICT strips assembling.

Fluorescent labelled secondary reagent (SPG-Eu PC) was sprayed on the conjugate pad 5 μL/cm, vacuum dried and used for ICT strips assembling.

Sample pad was pretreated first with 20 mM Phosphate buffer (pH 7.4), 0.2% PEG 6000, 0.1%BSA (IgG free), 0.1%Tween-20 and 0.3% S9 (Surfactant Tetronic 1307), air dried and used for ICT strips assembling.

Absorbent pad was used without any modification.

All ICT components were assembled using plastic card as a base, cut into strips with 3 mm width and put inside of the plastic cassettes, packaged into aluminum foil bags with silica gel as desiccants. Final products until use were kept at room temperature with controlled humidity ~30%.

2.9. Immunochromatographic samples testing

Standard bovine reference panel samples were diluted first 10^{-2} times with Running buffer: PBS, 0.1% Tween-20, 0.2% S9 and 3 drops (~10 μL) were applied on the sample pad cassette hole, waited 10-15 minutes and fluorescence data were read out using compact immunochromatographic fluorescence reader device, model FIC-H0 (Suzhou Helmen Precision Instrument) (Figure 1, A) using 365/610 nm excitation/detection wavelength, respectively. For comparison we have tested samples with two immunogold lateral flow immunochromatography tests for detection Brucella's antibodies, commercially available on the Chinese market (company A and company B).

2.10. Statistical analysis

All statistical analyses, including the calculation of means, standard deviations, and comparative tests, were conducted using Microsoft Excel (version 2021). Statistical significance was determined based on a threshold of $P<0.05$, indicating that the probability of the observed results occurring by chance was less than 5%.

3. Results

3.1. Yield and Characterization of Brucella's smooth Lipopolysaccharides (S-LPS)

The yield of the smooth Brucella's lipopolysaccharides (S-LPS) was quantified from a 1.0 L overnight bacterial culture, resulting in a total LPS concentration of 12.0 mg/L. The bacterial wet biomass was measured at 80 g per liter of culture, with a corresponding dry biomass of 8 g/L. This translates to an LPS yield of 15 μg/mg of dry biomass, representing a significant 7-fold increase compared to the previously reported maximum yield of 2.5 μg/mg dry biomass [15]. This improved yield underscores the efficiency of our cultivation and extraction protocols, highlighting their potential for optimizing LPS production in similar bacterial systems.

To validate the robustness of the extraction protocol, LPS was also successfully extracted from *Pasteurella multocida* and *Salmonella enteritidis* (data not shown). The purity of the extracted LPS from *Brucella* was analyzed by SDS-PAGE and silver staining (Figure 1, B). The analysis revealed major bands ranging between 25–45 kDa, representing the O-polysaccharide chain (O-antigen), the primary antigenic molecule of *Brucella*. The O-antigen is a crucial target for immune response and antibody production.

Purity assessments for protein and nucleic acid contamination were conducted by measuring absorbance at 260 nm (for nucleic acids) and 280 nm (for proteins). Spectral analysis confirmed minimal contamination, with impurity levels below 0.1%.

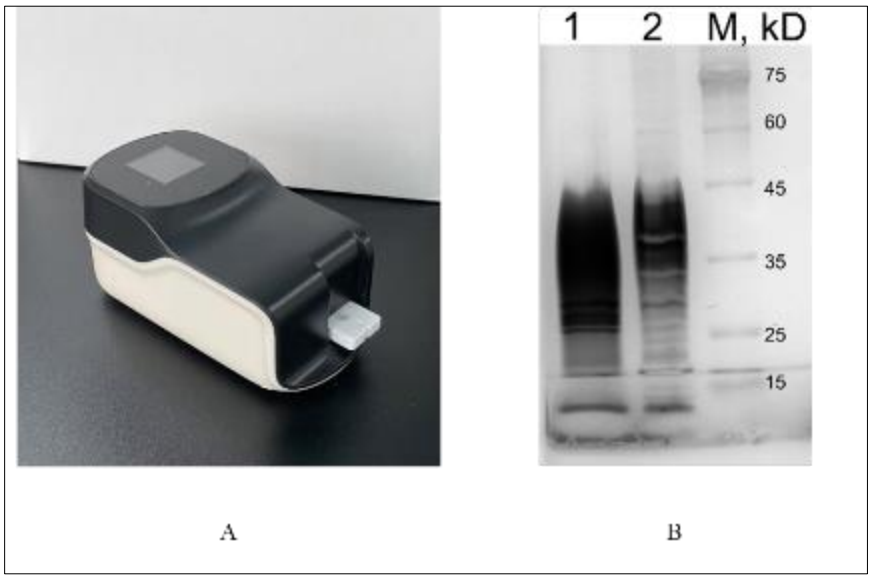

Figure 1 A - Immunochromatographic fluorescence reader, model FIC-H0 (Suzhou Helmen Precision Instrument); B - Silver SDS-PAGE to check purity and integrity of Brucella LPS. 1 – LPS extraction, 2 – Brucella whole cell lysate

3.2. Endotoxin potency of the extracted Brucella's S-LPS

Further characterization of the extracted LPS focused on evaluating its endotoxin potency. A novel approach was developed, integrating fluorescence-based detection with precise temperature regulation using a real-time PCR instrument. This method enables the measurement of endotoxin potency in extremely low volumes (10 µL) through kinetic fluorescence readings at 550 nm (FAM channel) under isothermal conditions at 37°C. Figure 2 illustrates the kinetic fluorescence curves for the Brucella LPS antigen preparation and the Control Standards Endotoxin (CSE). A 1/1000 dilution of the Brucella LPS preparation (initial concentration: 0.5 mg/mL) exhibited an endotoxin potency equivalent to 0.01 EU/mL. Based on this, the endotoxin activity of the Brucella LPS was calculated to be 20 EU per milligram of extracted LPS.

Our Fluoro-LAL method demonstrated a Limit of Quantification (LOQ) as low as 0.00125 EU/mL, representing a significant improvement in sensitivity (Fig. 2, A, B).

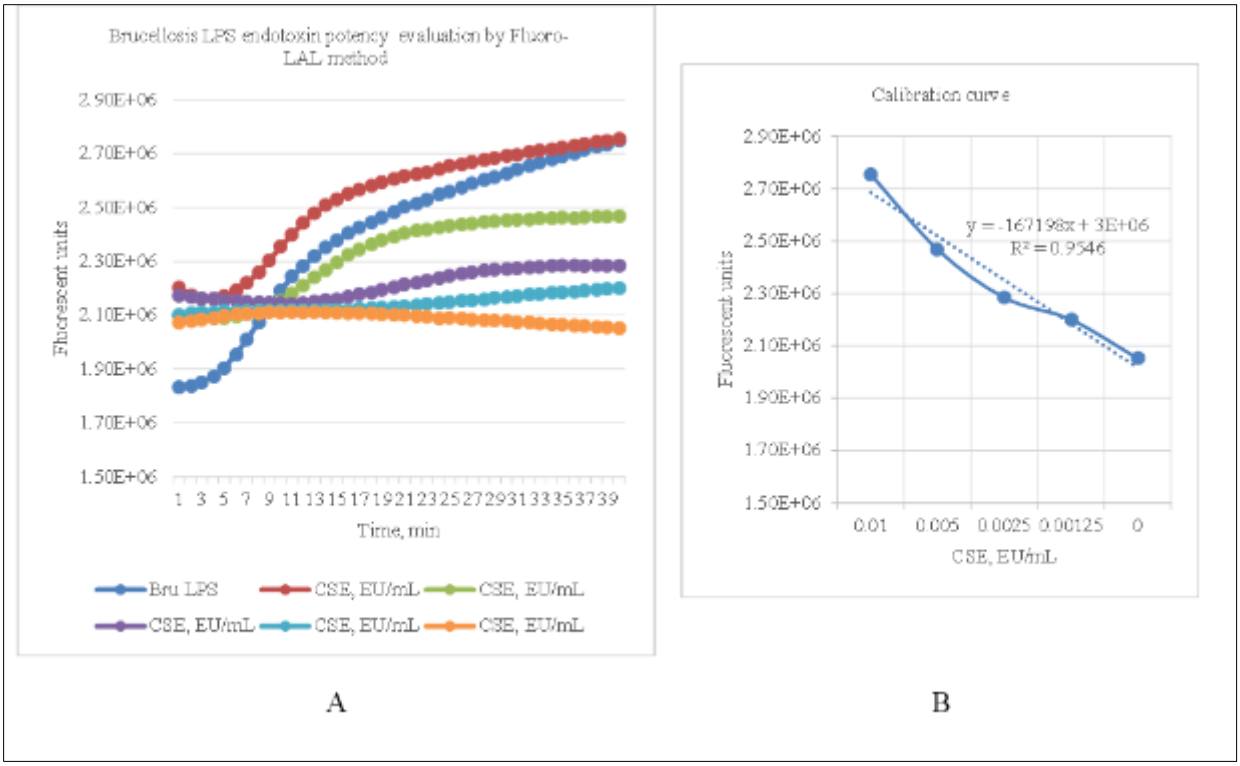

Figure 2 Estimation of endotoxin potency of extracted brucella's LPS preparation using Fluoro-LAL method, A – kinetic curves; B – Calibration curve

By comparison, commercially available chromogenic LAL tests, such as the Pierce™ Chromogenic Endotoxin Quant Kit (cat. No. A39553), typically claim a sensitivity range of 0.01 EU/mL to 1 EU/mL. Thus, our Fluoro-LAL approach enhances endotoxin detection sensitivity by an order of magnitude, offering a powerful tool for ultra-sensitive endotoxin quantification.

The assay demonstrated good linearity in the calibration curve ($R^2 > 0.95$), and the brucellosis LPS fluorescence data aligned well with the calibration range (Fig. 2 B). This facilitated the precise quantification of endotoxin activity in the *Brucella* LPS samples, highlighting its distinct potency relative to the *E. coli* endotoxin standard. These findings underscore the applicability of the Fluoro-LAL method for endotoxin quantification and its potential utility in assessing the pathogenicity of *Brucella* LPS.

The reduced volume requirement for Limulus Amebocyte Lysate (LAL) reagents not only conserves natural resources, such as the hemolymph of horseshoe crabs, but also enables laboratories equipped with qPCR instruments to perform endotoxin contamination quality control in a sustainable and cost-effective manner. This innovative approach has significant potential to advance bacterial endotoxin testing in both research and industrial settings.

3.3. Immunological characterization of the Brucella's S-LPS

To evaluate the immunological properties of the extracted Brucella S-LPS, experimental goat was immunized with the purified LPS. Anti-Brucella S-LPS antibodies reached detectable levels by 30 days post-immunization (dpi), as confirmed using a commercial gold-labeled immunochromatographic assay (Fig. 3). This indicates the immunogenicity of the extracted S-LPS and its potential to elicit a specific antibody response.

The specificity of the elicited antibodies was further verified through Western blot analysis. Gradual decreases in signal intensity were observed with decreasing quantities of the LPS preparation, confirming the dose-dependent reactivity and specificity of the raised antibodies to Brucella S-LPS in goat serum (Fig. 4).

Additionally, Western blot analysis allowed us to identify the characteristic molecular sizes of Brucella's O-antigen, consistent with literature values. The O-antigen was observed as a prominent band ranging from 25 to 60 kDa, with the core polysaccharide showing an average molecular weight of 40 ± 2 kDa. These findings further validate the structural and antigenic integrity of the extracted S-LPS.

Together, these results demonstrate that the extracted Brucella S-LPS retains its immunological and structural properties, making it a valuable target for further diagnostic and immunological studies.

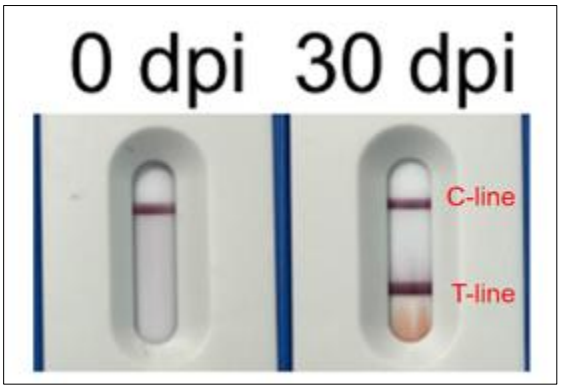

Figure 3 Gold ICT testing experimental goat blood testing before immunization (0 dpi) and 30 days after immunization (30 dpi)

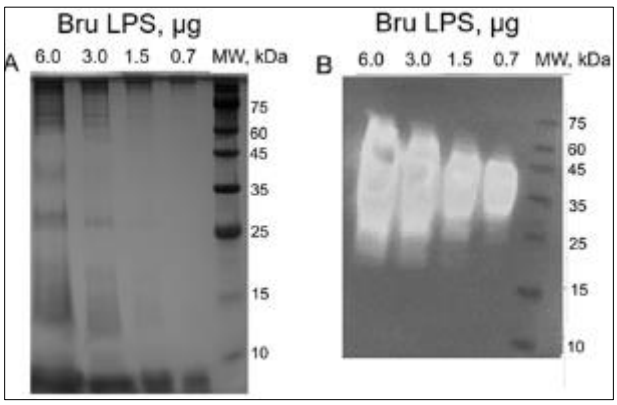

Figure 4 Silver PAGE separation of different quantities of Brucella's S-LPS and Western blot membrane evaluation using ECL, signal development after immunological evaluation, A - Silver-stained PAGE separation of LPS; B - Western blot signal evaluation

3.4. Validation Brucella S-LPS preparations

To validate the quality of the extracted Brucella lipopolysaccharide (LPS), an ELISA assay was performed using a panel of French national standard bovine sera. The panel comprised five negative and seven positive sera for brucellosis, encompassing a range of antibody signal intensities from low positive to high positive. The negative sera consistently exhibited signal levels below the cutoff threshold (0.2 AU), confirming the high specificity of the extracted LPS. In

contrast, all positive sera were correctly identified, with signal outputs ranging from a minimum of 0.5 AU (sample P6) to a maximum of 1.2 AU (samples P10 and P12), figure 5 A.

To further assess the sensitivity of the extracted LPS, we conducted a titration of experimental goat anti-LPS sera using ELISA. The detection limit was estimated at a serum dilution of 1×10^{-3}, yielding a signal of 0.22 AU. This result demonstrates the robust sensitivity of the extracted Brucella LPS and the elicited goat anti-LPS antibodies (Figure 9, A).

These findings confirm the suitability of the extracted LPS as a reliable antigen for brucellosis serological testing, ensuring both specificity and sensitivity across varying conditions. The compatibility with standard and experimental sera underscores its potential for diagnostic applications.

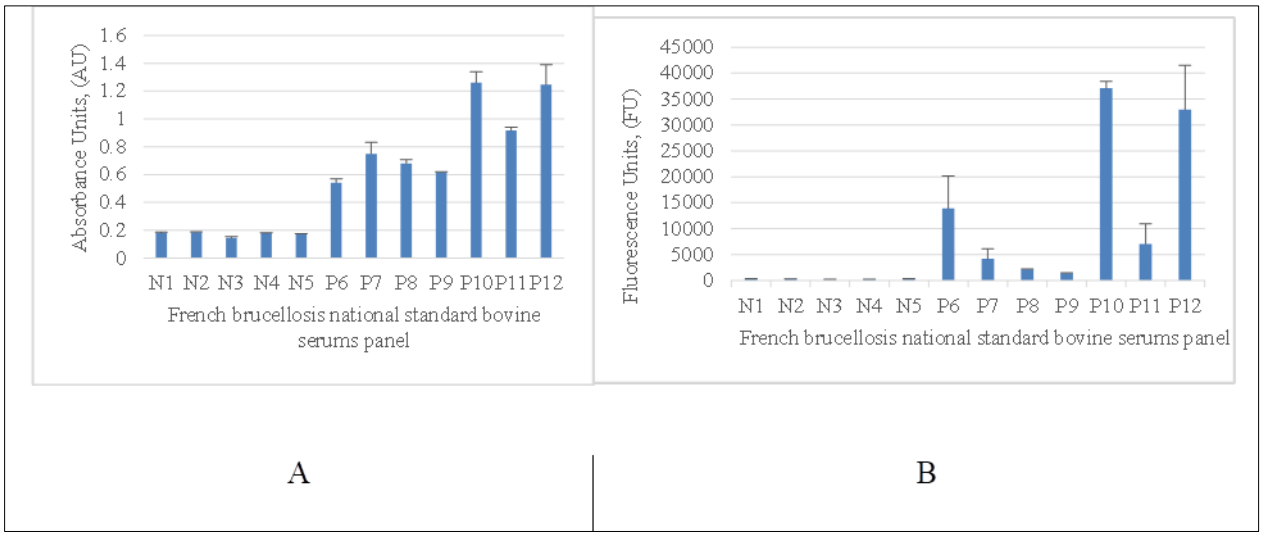

Figure 5 Validation brucellosis LPS in ELISA (A), and Fluorescence Lateral Flow Immunichromatography (B), using French national standard panel serum, SD bar shown

The ELISA validation of specificity demonstrated high cross-reactivity of extracted Brucella S-LPS across different animal species. EU sera from goat, sheep, pig, and camel showed strong positive reactions (OD > 0.9), indicating conserved immunogenic epitopes. EU Ovis sera showed moderate positivity (OD = 0.464), while rabbit sera from Egypt exhibited weak positivity (OD = 0.237). Only EU dog sera tested negative (OD = 0.147), suggesting species-specific differences in antibody recognition (Rought-LPS, lack O-antigen), table 4.

Table 4 ELISA validation S-LPS using reference panel sera (Dilution $1x10^{-2}$)*

Sample name	Mean OD	SD OD	Result
N1	0.1815	0.00495	Negative
N2	0.1865	0.002121	Negative
N3	0.145	0.009899	Negative
N4	0.179	0.002828	Negative
N5	0.1755	0.000707	Negative
P6	0.543	0.02687	Positive
P7	0.7485	0.082731	Positive
P8	0.6815	0.027577	Positive
P9	0.618	0.002828	Positive
P10	1.26	0.077782	Positive
P11	0.916	0.024042	Positive
P12	1.2465	0.143543	Positive
EU Goat	0.942	0.028284	Positive
EU Sheep	0.9105	0.038891	Positive
EU Ovis	0.464	0.024042	Positive
EU Pig	1.2065	0.126572	Positive
EU Dog	0.1475	0.003536	Negative
Egypt, Rabbit	0.237	0.018385	Positive
Saudi Arabia, Camel	0.952	0.027882	Positive
Background Control	0.07775	0.012473	Accepted

* - cutoff value 0.2 AU

To evaluate the performance of the Brucella lipopolysaccharide (LPS) antigen and validate it in detecting brucellosis specific antibodies, a Receiver Operating Characteristic (ROC) curve analysis was conducted. The ROC curve is a graphical representation of a diagnostic test's ability to discriminate between diseased and non-diseased states.

As depicted in Figure 6, the ROC curve demonstrated 100% sensitivity and 100% specificity. The area under the ROC curve (AUC) was calculated to be 1.0, indicating a perfect match between the test results and the actual disease status. This AUC value of 1.0 confirms that the Brucella LPS antigen assay has excellent diagnostic accuracy, with no false positives or false negatives.

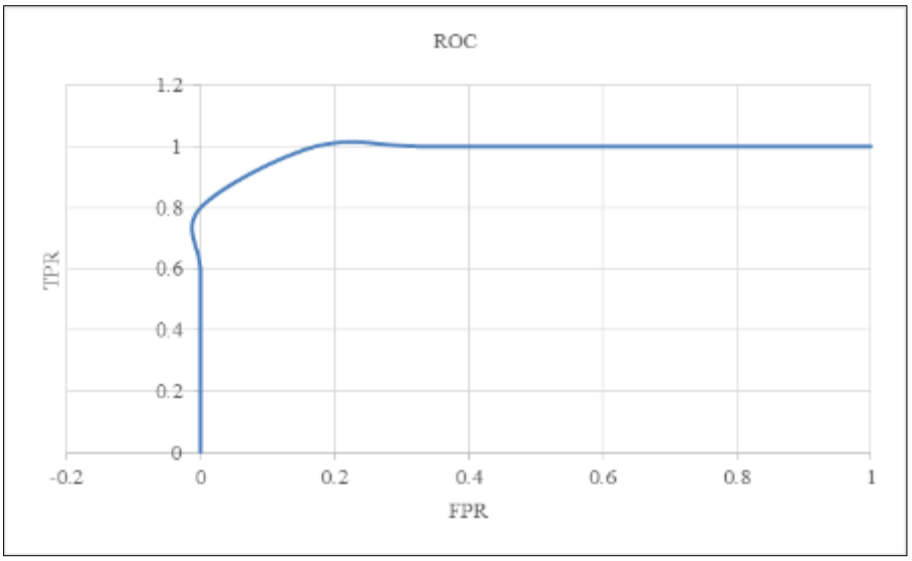

Figure 6 Receiver operation curve (ROC) analysis to test brucella's LPS diagnostic performance, FPR – false positive rate, TPR – true positive rate

3.5. Gold-labelled Immunochromatographic testing

Following the ELISA validation, using reference panel, which included bovine brucellosis negative (n=5) and positive (n=7) serum, we conducted additional analyses using two commercially available gold-labeled lateral flow immunoassays (from Companies A and B). Detailed data are presented in Table 5 and Figure 7.

The results demonstrate that only three positive samples (No 6, 10, and 12) were correctly identified as positive by the gold-labeled immunochromatographic test (ICT) from Company A, albeit with low signal intensity. In contrast, the kit from Company B failed to detect any of the seven positive samples. These findings indicate that the gold-labeled ICT kits lack sufficient sensitivity to reliably detect Brucellosis-positive bovine serum samples from the French national brucellosis reference panel.

Table 5 Gold labelled immune assay performance testing French national brucellosis reference panel

Sample code	Company A			Company B		
	T-line	C-line	Result	T-line	C-line	Result
SR 01	-	++++	Negative	-	++++	Negative
SR 02	-	++++	Negative	-	++++	Negative
SR 03	-	++++	Negative	-	++++	Negative
SR 04	-	++++	Negative	-	++++	Negative
SR 05	-	++++	Negative	-	++++	Negative
SR 06	+	++++	Positive	-	++++	Negative
SR 07	-	++++	Negative	-	++++	Negative
SR 08	-	++++	Negative	-	++++	Negative
SR 09	-	++++	Negative	-	++++	Negative
SR 10	+	++++	Positive	-	++++	Negative
SR 11	-	++++	Negative	-	++++	Negative
SR 12	+	++++	Positive	-	++++	Negative

Figure 7 Testing French national standard panel serum using commercial gold labelled immunoassays Brucellosis kits, Upper panel company A test results, lower panel – company B test results

3.6. Fluorescent Immunochromatographic testing (F-ICT)

The reference serum panel was evaluated using our developed fluorescent-labeled immunoassay with signal detection performed via a reader (Figure 1, A). All positive serum samples were accurately identified, exhibiting sufficient signal intensity for reliable detection, while all negative samples showed no signal at the T-line zone (Figures 8 and 9, and Table 6). To simplify data interpretation and ensure correct discrimination between brucellosis-positive and negative samples, a cutoff value was calculated for this specific lot of tests. The cutoff value was determined to be **410 FU** (Fluorescence Units). However, it is important to note that additional negative samples need to be tested to refine this value further and establish a more precise threshold for broader applicability.

Our findings demonstrate that the fluorescent immunochromatographic test (ICT) is significantly more sensitive compared to commercially available assays utilizing colloidal gold conjugates when analyzing bovine brucellosis-positive and -negative sera. This increased sensitivity highlights the superior performance of the fluorescent ICT for detecting brucellosis.

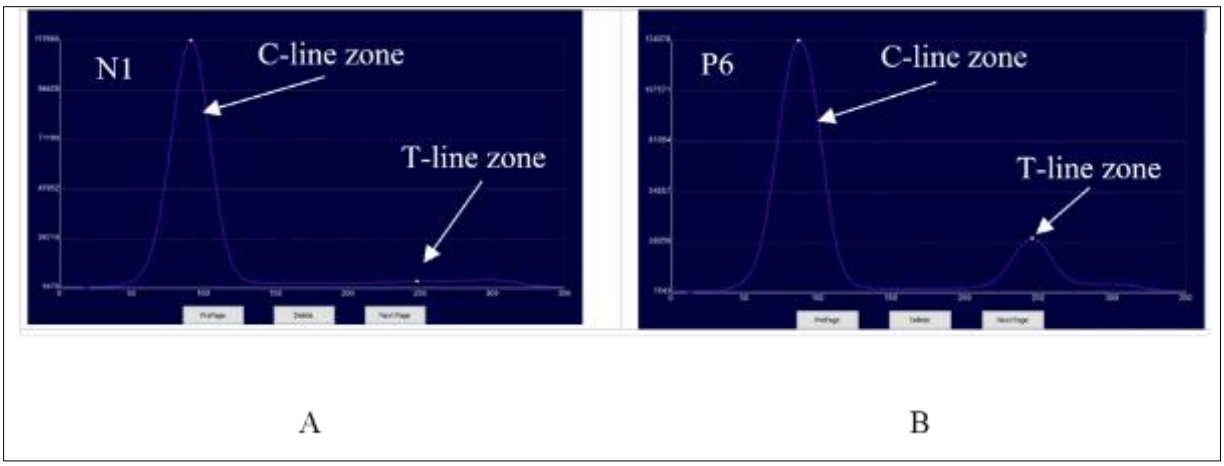

Figure 8 Fluorescence derived peaks after ICT testing bovine brucellosis reference panel serum (example), Negative serum N1 (panel A) and Positive serum P6 (panel B)

Table 6 Fluorescent data from reader testing French national brucellosis reference panel

Serum number	Fluoresces Units (FU)		Result
	T-line*	C-line	
N1	293	82093	Negative
N2	273	81972	Negative
N3	179	90453	Negative
N4	209	78313	Negative
N5	140	83026	Negative
P6	18300	97543	Positive
P7	5546	90501	Positive
P8	2202	26093	Positive
P9	1436	53489	Positive
P10	36189	57751	Positive
P11	9769	82536	Positive
P12	39026	64165	Positive

* Cutoff value was calculated as 410 FU

3.7. Comparison sensitivity F-ICT and ELISA

Next, we aimed to evaluate and compare the sensitivity of the fluorescent immunochromatographic test (F-ICT) and ELISA for the detection of brucellosis antibodies in milk samples artificially spiked with varying concentrations of high-titer goat brucellosis serum (30 days post-infection, dpi).

As shown in Figure 5 B and 9, B, the F-ICT demonstrated exceptional sensitivity, detecting brucellosis antibodies at a dilution as low as 1×10^{-5} (8652.5 ± 1647 FU, with a cutoff of 410 FU). In contrast, the ELISA could only detect antibodies at a dilution of 1×10^{-3} (0.22 ± 0.01 AU, with a cutoff of 0.2 AU).

These results highlight that the F-ICT is two orders of magnitude more sensitive than the ELISA. This significantly improved sensitivity establishes a new standard for brucellosis antibody detection in milk and serum samples, providing a more reliable and robust tool for diagnostic applications.

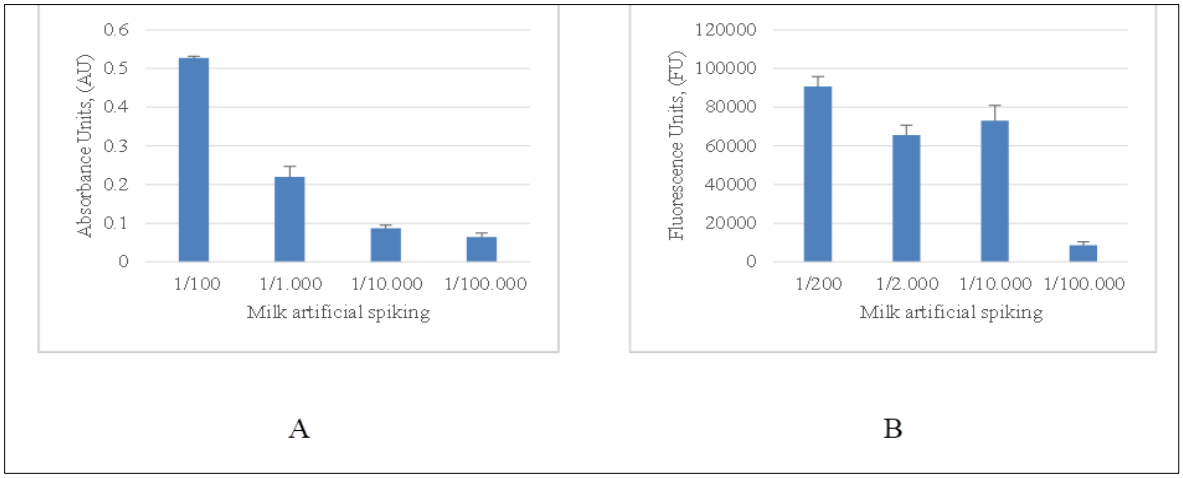

Figure 9 Comparison sensitivity of brucellosis antibodies detection between ELISA (panel A) and F-ICT (panel B), using artificially spiking milk samples with Brucellosis positive goat serum (30 dpi)

3.8. Evaluation of the fluorescent signal photobleaching during test-cassettes storage at room temperature

We also evaluated the stability of fluorescence signal intensity over time by measuring the signal after several days of storage at room temperature. The results demonstrated stable signal intensity with no noticeable decrease over a 5-day period (Figure 10). This confirms the robustness of the fluorescent signal when Europium, a rare-earth metal element, is used as a label for immunochromatography. Unlike conventional fluorescent dye labels, Europium-based fluorescence does not exhibit photobleaching, ensuring signal stability over time.

Even a slight increase in fluorescence intensity was observed after 5 days of test cassettes storage after testing, likely due to the drying of immune components in the T-line zone, which led to a localized increase in the concentration of the fluorescent-labeled conjugate within the immune complexes.

Figure 10 Fluorescent signal stability test, A – repeat 1; B – repeat 2

4. Discussion

Our study demonstrates the successful development of a highly sensitive fluorescent immunochromatographic test for detecting Brucella antibodies, with several notable achievements and implications for diagnostic applications.

The improved extraction protocol for Brucella S-LPS represents a significant advancement, achieving a seven-fold increase in yield compared to previous methods. The extracted S-LPS maintained its structural integrity and immunological properties, as evidenced by SDS-PAGE analysis showing characteristic bands between 25-45 kDa and Western blot confirmation of specific antibody recognition. The endotoxin potency of 20 EU per milligram, measured using our novel Fluoro-LAL method, confirms the biological activity of the extracted LPS.

The F-ICT demonstrated superior performance compared to existing diagnostic methods. When tested against the French national standard panel of bovine sera, our assay achieved 100% sensitivity and specificity, with an AUC of 1.0 in ROC analysis. This performance notably surpassed two commercially available gold-labeled immunochromatographic tests, which showed limited sensitivity in detecting positive samples. The commercial tests from Company A detected only three out of seven positive samples, while Company B failed to detect any positive samples, highlighting the significant sensitivity advantages of our fluorescent approach.

A particularly noteworthy finding is the exceptional sensitivity of the F-ICT in milk testing. The ability to detect Brucella antibodies at dilutions as low as 1×10^{-5} represents a hundred-fold improvement over traditional ELISA methods, which only detected antibodies at 1×10^{-3} dilution. This enhanced sensitivity could prove crucial for surveillance programs, potentially allowing earlier detection of infection in dairy herds and improving disease control strategies.

The stability of the Europium-based fluorescent signal addresses a common limitation of fluorescent immunoassays. The observed signal stability over five days at room temperature, with no significant photobleaching, suggests practical

advantages for field applications. The slight increase in signal intensity noted after five days, attributed to the concentration of immune complexes during drying, does not impact the test's diagnostic interpretation given the clear separation between positive and negative results.

These findings have important implications for brucellosis surveillance programs. The high sensitivity in milk samples suggests potential applications in bulk milk testing for herd-level screening, while the improved detection in serum samples could enhance individual animal testing accuracy. The stability of results allows for delayed reading of tests, which could facilitate batch processing in laboratory settings.

Future research should focus on validating the F-ICT with a larger sample set, particularly under field conditions, and establishing precise cutoff values for different sample types and species. Additionally, investigation of the test's performance with samples from different geographical regions and Brucella species would further validate its broad applicability

5. Conclusion

In conclusion, our developed F-ICT represents a significant advancement in Brucella antibody detection, offering improved sensitivity, specificity, and stability compared to existing methods. The high cross-reactivity in ruminants (bovine, goat, sheep, camel) and pigs suggests shared Brucella-specific epitope recognition, while lower reactivity in dogs indicates potential limitations for canine brucellosis diagnosis. These characteristics, combined with the practical advantages of the immunochromatographic format, suggest potential applications in both laboratory and field settings for enhanced brucellosis surveillance and control programs.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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