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Development of an immunochromatographic lateral flow strip test for rapid detection of Brucella antigens in clinical specimens

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Abstract

Brucellosis is an endemic, worldwide zoonotic disease of public health and socioeconomic importance. It mainly infects livestock and human population, with potentially pathogenic species including *Brucella abortus*, *Brucella melitensis,* and *Brucella suis*. Serological methods are used for the diagnosis of brucellosis, resulting in false negatives or false positives, which can cause diagnostic uncertainty. Isolation of Brucella species from clinical specimens is the gold standard of diagnosis. Lateral flow immunoassay (LFIA) is a rapid, simple, and economical test that is widely used in the field for fast detection of various analytes. In our study, we generated LFIA strips using anti-smooth Brucella lipopolysaccharides (S-LPS) polyclonal antibodies (pAbs) for direct identification ofthe Brucella antigen. A nanogold lateral flow immunoassay-based technique (LFIA) for the detection of Brucella antigen was developed and evaluated for diagnostic performance characteristics using Brucella and non-Brucella cultures and spiked specimens. LFA strips were fabricated by printing anti-bovine antibodies IgG and anti- S-LPS polyclonal antibodies (pAbs) on the control and test lines, respectively. Brucella isolation, identification and multiplex PCR were used as the gold standard tests. It was found that the limit of detection (LOD) of the developed LFIA test, when evaluated using the Rev-1 vaccinal strain, indicated that the LFIA strip was able to detect $1X10^4$ to $5X10^3$ CFU/ reaction. The developed LFIA did not exhibit any cross-reactivity with non-Brucella pathogens, and it did not react to rough Brucella strains, but gave rise to 100% positive results with all smooth Brucella strains and isolates showing 100% selectivity.

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Introduction

Brucellosis is an endemic zoonotic disease of public health and socioeconomic importance worldwide. It mainly infects livestock and most domestic animals and also infects the human population with potentially pathogenic species, including *Brucella abortus, Brucella melitensis,* and *Brucella suis* **(Ghanbari *et al.,* 2020).** Current serological methods for the diagnosis of brucellosis include the Rose Bengal test and complement fixation test **(OIE, 2018).** The Rose Bengal test is a pilot test that is generally used for primary screening, while CFT is used for confirmatory diagnostic characterization. However, all serological tests produce a certain number of false negatives or false positives, which can cause diagnostic uncertainty **(Bonfini *et al.,* 2018; Bulashev and Eskendirova, 2023).** The isolation of Brucella species from microbiological cultures is the gold standard for the laboratory diagnosis of brucellosis **(Streva *et al.,* 2024).**

Lateral flow immunoassay (LFIA) is a rapid, simple, and economical test that is widely used in the field for fast detection of various analytes **(Ge *et al.*, 2021).** It is a simplified model of IELISA consisting of a nitrocellulose detection strip that contains Brucella antigen as S-LPS or Brucella antibody, as well as a Brucella -specific capture probe, flanked at one end by a reagent pad that consists of a colloidal gold immune conjugate and flanked at the other end by an absorption pad. Few assays were designed for Brucella antigen-based detection. **Byzova *et al.* (2012)** developed a sandwich format immunochromatographic assay for detecting Brucella antigen (smooth Brucella lipopolysaccharide (S-LPS). The LFIA assay is simply carried out by the addition of the sample (serum, specimens, etc.) directly into the sample pad, followed by the addition of test liquid to the buffer pad. The result is visually read within 5 to 15 minutes. The assay is based on the binding of specific antibodies or antigens present in the clinical specimen to the antigen, such as LPS or polyclonal antibodies against the antigen, respectively, and the staining of the bound antibodies or antigens by a colloidal gold-labeled antibody conjugate **(Saavedra *et al.,* 2019).**

Thus, in our proposed study, we generated LFIA strips using anti- smooth Brucella LPS pAbs to detect intact whole Brucella antigen cells for rapid and accurate detection of brucellosis. This LFIA was evaluated and validated for its LOD, sensitivity, specificity, performance, and cross-reactivity using Brucella and non-Brucella cultures and spiked specimens. Brucella isolation and identification, and multiplex PCR were used as the gold standard tests.

Materials and Methods

Bacterial strains and specimen types

Vaccine strains: Strain-19 and RB51 (*B.abortus* biovar 1) and Rev-1 (*B.melitensis* biovar 1) (CZ veterinaria S.A., Pontevedra, Spain).

References strains: 544 (*B. abortus* biovar 1), 16M (*B. melitensis* biovar 1), ETHER (*B.melitensis*biovar3), REO198 (*Brucella ovis*), and *Brucella suis* 1330 (*B. suis* biovar 1) were kindly supplied by **Prof. Dr. J.M. Blasco**, CITA Institute, Zaragoza, Spain. Additionally, S99 (*B. abortus* biovar 1) was kindly supplied by the Veterinary Serum and Vaccine Research Institute (VSVRI).

Field isolates: Twenty-five Egyptian field isolates were recovered from different animal species and kindly supplied by **Prof. Dr. Ashraf Sayour** and **Prof. Dr. Waleed Shell.** Were identified serologically, biochemically, and via a Bruce-Ladder multiplex PCR assay **(López–Goñi *et al.,* 2008, and Shell *et al.,* 2013, OIE, 2018).** Twenty-two of these 25 isolates were *Brucella melitensis* biovar 3, and the other three field isolates were *Brucella abortus*.

Other bacteria: *Yersinia enterocolitica* O9, *Escherichia coli* O157:H7, and Salmonella serovars of Kauffmann-White Group N as cross-reacting bacteria with serological tests used for the diagnosis of Brucella.

Using a colony count equal to the standardized LOD determined in this study, A spiked sample is prepared by adding a Brucella field isolate to Brucella-free heparinized bovine serum and ground Brucella-free bovine lymph nodes in PBS (matrix) **(Dong *et al.*, 2016).** LOD is detected by an initial study, which was performed to determine the detectable range (colony forming unit/LFIA sample = 40µl) in the spiked specimens and pure Brucella culture. The Rev-1 strain was selected and used in the determination of LOD. These spiked samples are used in validation assay experiments to determine the selectivity of the method and identify matrix effects.

Synthesis of nanogold (40 nm): (Aboelqassem *et al*., 2022 and Frens, 1973)
One milliliter of a 1% aqueous HAuCl4 solution was added to 100 mL of deionized water, then boiled with 1.5 mL of 1% sodium citrate while stirring. The mixture turned red within 2 minutes, boiled for 15 minutes, cooled, and stored at 4°C. Additional deionized water was added to reach a total volume of 100 mL, and 0.02% sodium azide was incorporated into the colloidal gold before storage at 4°C. The gold colloids' particle diameter was analyzed using transmission electron microscopy (TEM, H-7650).

Preparation of smooth Brucella lipopolysaccharide (S-LPS)
In-house-prepared smooth lipopolysaccharide (S-LPS, hot Saline Extract) was prepared from *Brucella abortus* S99 according to **Alton *et al.* (1988) and Plackett *et al.*(1976).**

Preparation of Rabbit IgG anti-LPS (Aboul-Ella *et al.,* 2023, Fishman and Berg, 2018b, Dos Santos *et al*., 1989):
Animals were obtained from the laboratory animal house in the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB).

1. Rabbit inoculation of LPS
For priming immunization, a stable S-LPS emulsion antigen was created by combining equal volumes of complete Freund's adjuvant (CFA) and LPS antigens at 2 mg/kg body weight, mixed for 20 minutes until achieving a milky white emulsion, then incubated overnight at 6°C. For boosting immunization, a similar process was used, substituting incomplete Freund's adjuvant (IFA) for CFA. Both emulsions were inoculated under aseptic conditions in two 2 kg white male New Zealand rabbits for the production of LPS pAbs.

2. Rabbit IgG purification:
Rabbit IgG was purified from serum pooled five days post-booster with Brucella S-LPS. After adding caprylic acid and stirring, the mixture was centrifuged, and supernatants were dialyzed overnight against PBS. Immunoglobulin concentrations, measured post-dialysis, ranged from 2 to 2.4 g/dl, with a subsequent dilution to 1 mg/ml using ultrapure water. Brucella-specific polyclonal antibodies (pAbs) were formed by pooling equal volumes of IgG antibodies against LPS. An agar gel precipitation test was then optimized to evaluate these pAbs.

Preparation of IgY against LPS (Amro *et al.,* 2018):

1. Immunization of chicken and egg collection
Five chickens of Single Comb White Leghorns that were 6 months old were inoculated S/C with 0.1 ml of 5 mg of S-LPS. Three doses were administered with a two-week interval between the first and the second vaccination and one week between the second and the third vaccination. To detect the best quantities of IgY antibodies, Eggs were collected from the immunized chickens between specific time intervals of the three doses. Samples were then stored at 4 °C until the extraction of the IgY from the immunized egg yolk.

2. Extraction of chicken IgY antibodies from immunized Single Comb White Leghorns egg yolk
This protocol outlines the extraction of immunoglobulin Y (IgY) using Polyethylene Glycol (PEG 6000) precipitation from egg yolk. Initially, the eggshell is cracked, and the yolk is filtered to remove egg white. After measuring the yolk volume, phosphate-buffered saline (PBS) is added, followed by PEG 6000, and the mixture is vortexed and centrifuged. The process is repeated with an increment in PEG concentration. The final pellet is dissolved in PBS and transferred to a dialysis membrane to eliminate salt, followed by overnight stirring in dialysis buffer. The sample's successful IgY extraction is confirmed through SDS-PAGE and Western blot.

Conjugation of nanogold with rabbit IgG anti-S-LPS (Sayed *et al.*, 2023):
Using 0.02M K_2CO_3, the colloid gold solution was adjusted to pH 8.5. With gentle stirring, 100ul rabbit IgG anti S-LPS (1mg/0.1ml of 0.05% NaCl buffer) were added drop-wise to

10ml of pH-adjusted colloid gold solution. The mixture was gently mixed for 10min and then blocked using 1% (m/v) final concentration of polyethylene glycol (PEG - 20,000 kDa), followed by stirring for an additional 15 min and centrifugation for 30 min at 10,000 g. The pellets were suspended in 1ml dilution buffer [20mM Tris/HCI buffer (pH 8.2) containing 3% (w/v) sucrose, 1% (w/v) BSA, and 0.02% sodium azide then stored at 4°C until used.

Preparation of the immunochromatographic lateral flow kit (Sayed *et al*., 2023 and Ajaikumar *et al*., 2021):

1. Sample pad (Ahlstrom)

Sample pad is made of glass fiber and was saturated with PBS solution of pH 7.2 containing blockers like bovine serum albumin (1%), casein (0.1–0.5%), gelatin (0.05–0.1), and surfactants like Triton X-100 (< 0.05%) and Tween-20 (< 0.05%) and dried at 37ºC. Then it was kept under dry conditions at room temperature until used.

2. The conjugate pad (Ahlstrom):

The conjugate pad was made of glass fiber and was treated with 0.1% Tween-20 for 10 min and dried at 60°C. The prepared glass fiber was cut into sections (4cm×0.5 cm), and then saturated with 150µl of colloidal gold probe consisting of rabbit IgG anti-Brucella S-LPS conjugated with nanogold. The conjugate pad was dried for 1 hour at 37°C and stored under dry conditions at 4°C until used.

3. Nitrocellulose membrane (BIODOT - XYZ-3):

Two lines were dispensed on the nitrocellulose membrane (25mm×300mm) first line was the IgY anti LPS (1.5mg/0.1ml) which was dispensed on the test (T) line (1µl per 1 cm line) and the second line was for goat antirabbit IgG immunoglobulin (1mg/0.1ml) which was dispensed on the control line C (1µl per 1 cm line) using IsoFlow™ Lateral Flow Reagent Dispenser, USA.

4. Cutting the membrane:

After that, the membrane was covered with the top laminate and cut into 0.5-cm-width test strips by using an automated cutter (Guillotino Cutter GCI1800),

Interpretation of the test

This LFIA is coated with two lines (a control line and a test line). If the specimen contains Brucella antigens, they will bind to the Brucella antibodies coated on the test line region (T), generating a colored line, indicating a positive result. If Brucella antigens are not present in the samples, the test line region will not generate any colored line, indicating a negative result. As a control, a colored line will always appear in the control line region (C), either in negative or positive specimens, indicating that the test procedure has been carried out in a proper manner.

In case of positive results, two colored lines will appear, and a colored line should always appear in both the control (C) line and in the test line (T) region. A positive result indicates the detection of Brucella antigen in the specimen, as shown in Figure 1 (A). But in the case of negative results, a colored line appears only in the control region, while a colorless test line appears, as shown in Figure 1 (B). Both A and B are valid results. Invalid results, as shown in Figure 1 (C), occur when a colored control line does not appear. Interpretation and reading of the test were performed within5-10 minutes (Fig. 2

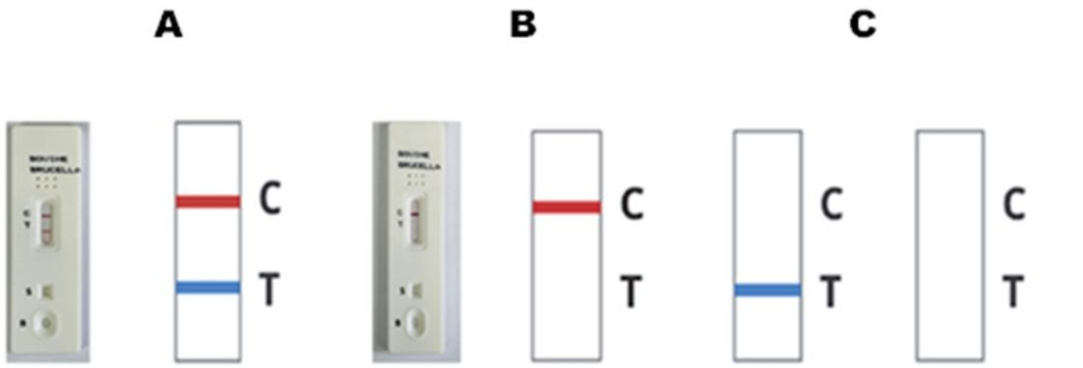

Fig. 1 Interpretation of LFIA: Valid results: (A= negative), (B= positive), Invalid results (C)

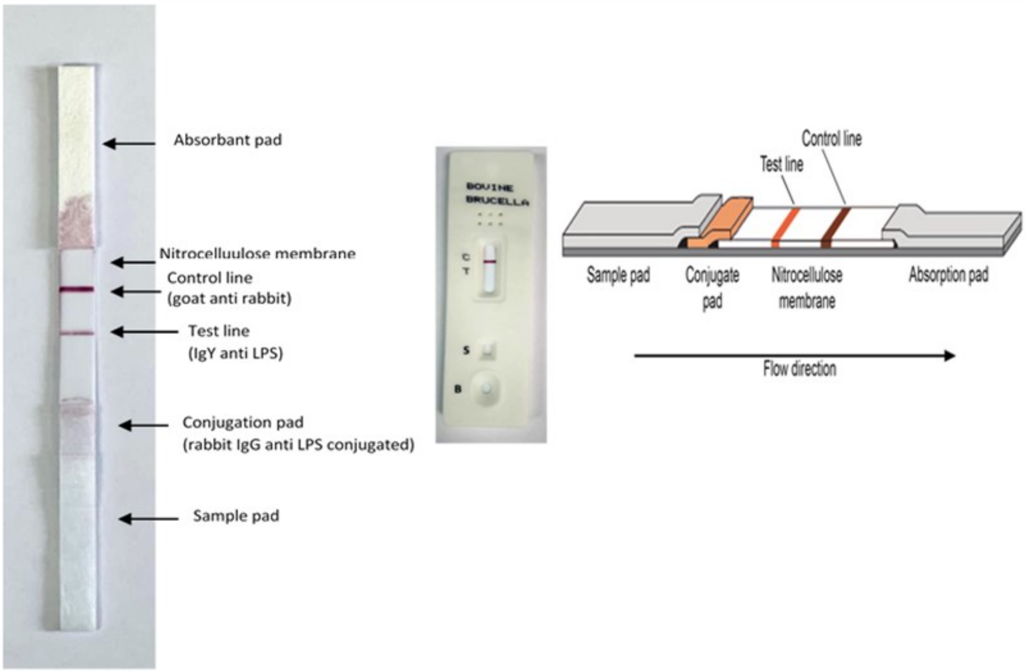

Fig. 2 LFIA test strip illustration.

Gold standard tests

Isolation and detection of Brucella recovered from clinical specimens, and its identification, traditionally and by multiplex PCR assay (BRUCE-LADDER) were used in this study as gold standard tests. All procedures involving Brucella were conducted under biosafety level 2 (BSL-2) conditions due to its zoonotic risk. Laboratory work was performed using a Class II biosafety cabinet, with strict use of personal protective equipment (PPE) and aseptic techniques. All infectious materials and waste were properly disinfected or autoclaved before disposal, and only trained personnel handled the samples to minimize the risk of laboratory-acquired infection **OIE (2018).**

Results

All strains in this study are identified traditionally and by the BRUCE-LADDER assay, as shown in Photo (1)

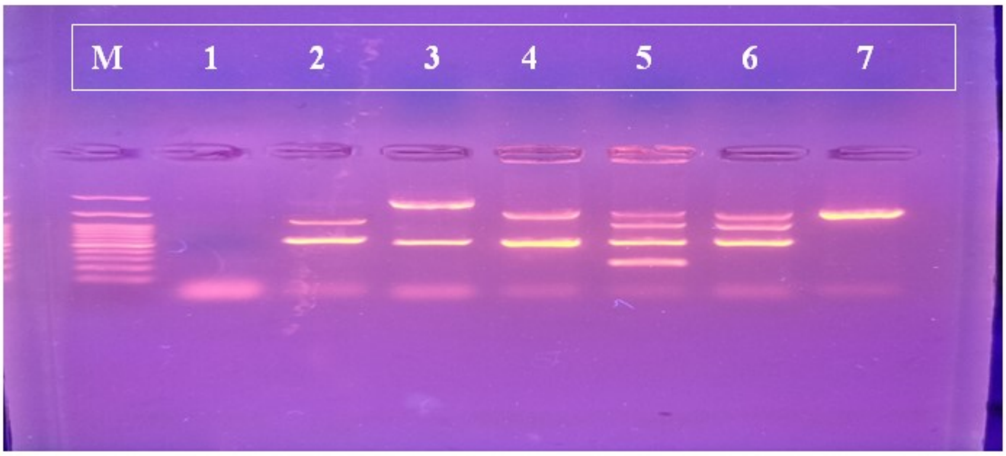

Photo. (1). Bruce-ladder multiplex PCR profile of vaccine, reference and locally-isolated Brucella strains and *Yersinia enterocolitica* O9. M = 100 base pair (bp) DNA ladder (Fermentas). Lane 1 are *Yersinia enterocolitica* O9, Lanes 2: Amplification products of B. ovis REO198, lane 3: *B. abortus* RB51 vaccine strain, lane 4: PCR profile of all *Brucella abortus* isolates, lane 5: *B. melitensis* Rev1 vaccine strain, lane 6: PCR profile of all *Brucella melitensis* isolates, lane 7: *B. abortus* S19 vaccine strain

LOD using different matrices:

The LOD was evaluated using direct bacterial culture of different *Brucella melitensis* biovar 1 (Rev-1) matrices. In this study, the LOD was determined by using eight dilutions of *Brucella melitensis* biovar 1 (Rev-1) with wide range of colony counts ranging from 5 X 10^7 to 5 X 10^1 CFU/40 µl (sample dose) and then with seven dilutions of *Brucella melitensis* biovar 1 (Rev-1) with narrow range of colony counts ranging from 1 X 10^6 to 1 X 10^3 CFU/40 µl prepared by ten and double serial dilution respectively in PBS. The limit of detection of Brucella by this assay ranged from 1 X 10^4 to 5 X 10^3 CFU/40 µl (Figure 3 and 4).

1 2 3 4 5 6 7 8

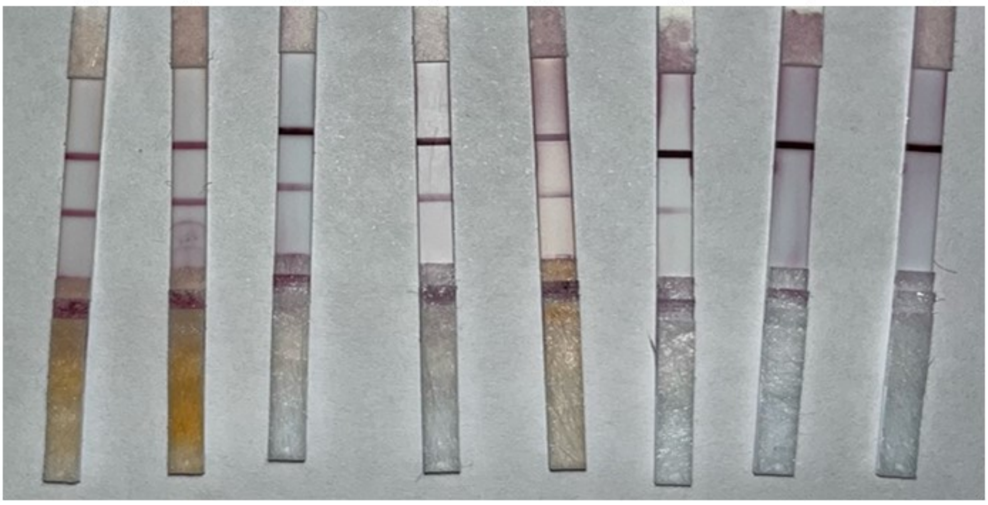

Fig. (3) LOD of LFIA assay using serially diluted culture *Brucella melitensis* biovar 1, vaccine strain (rev. 1). 1–7 represented the colony count of 5 X 10^7, 5 X 10^6, 5 X 10^5, 5 X 10^4, 5 X 10^3, 5 X 10^2, 5 X 10^1 CFU per sample and last strip is Brucella free culture.

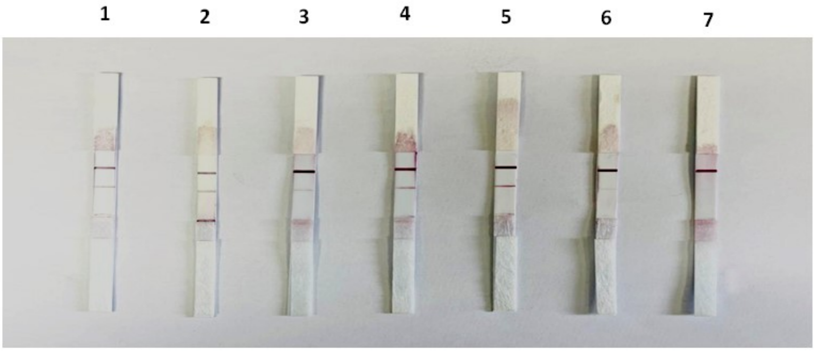

Fig. (4). LOD of LFIA assay using serially diluted culture *Brucella melitensis* biovar 1, vaccine strain (rev. 1). 1–7 represented the colony count of 1 X 10^6, 5 X 10^5, 1 X 10^5, 5 X 10^4, 1 X 10^4, 5 X $10^{3,}$ 1 X 10^3 CFU per sample.

Sensitivity of LFIA to smooth Brucella strains

All Brucella reference strains and field isolates were investigated using the developed LFIA for evaluation of assay sensitivity. Six out of the 31 strains are reference strains, where the other 25 isolates are Egyptian field isolates belong to different Brucella species and biovars, and all of these strains and isolates are smooth in nature. Additionally, 15 specimens spiked with field isolates were used, with 5 clinical specimens free from brucellosis used as a negative control (Figures 5 and 6) and (Table 1).

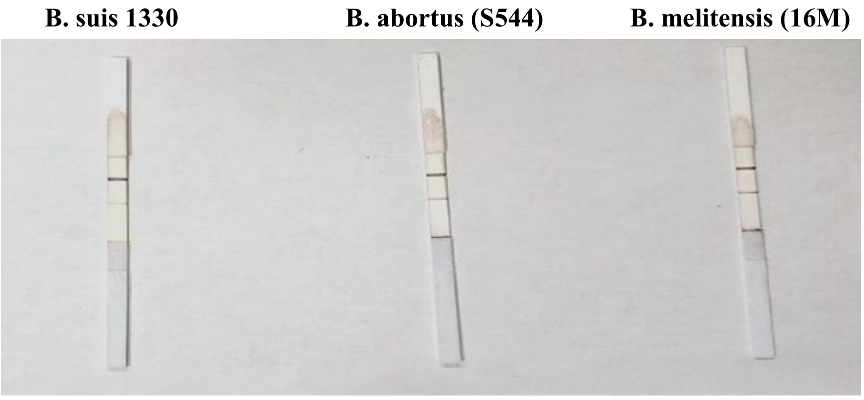

Fig. (5). Sensitivity of LFIA assay using direct culture of smooth Brucella strains: *Brucella abortus* biovar 1 (S544), *Brucella suis* biovar 1 (S1330) and *Brucella melitensis* biovar 1 (S16M). All strains were in a colony count of LOD (1 X 10^4 to 5 X 10^3 CFU / sample).

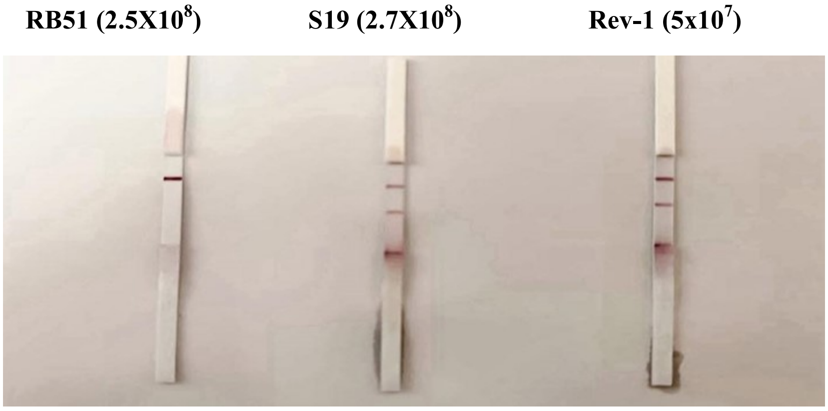

Fig. (6). Sensitivity of LFIA assay using direct culture of smooth and rough Brucella vaccine strains: rough *Brucella abortus* biovar 1 (SRB51), smooth *Brucella suis* biovar 1 (S19) and smooth *Brucella melitensis* biovar 1 (Rev. 1). All strains were in a colony count of 1-5X 10^8 CFU / sample.

Table (1). Sensitivity of the developed LFIA against smooth Brucella strains

Strain/Specimen Type	Number Tested	LFIA Result	Sensitivity
Brucella abortus isolates	3	Positive	100%
Brucella melitensis isolates	22	Positive	100%
Smooth reference strains	6	Positive	100%
Spiked clinical specimens	15	Positive Starting from $1x10^4$ CFU / sample	100%
Negative control specimens	5	Negative	—

Specificity against rough Brucella strains

Specificity was evaluated against rough Brucella strains using two main rough strains, which are *Brucella abortus* biovar 1 (RB51) vaccinal strain and Brucella ovis REO198 in colony count ranging from 1-5X 10^8 CFU/ 40 µl (Figure 7).

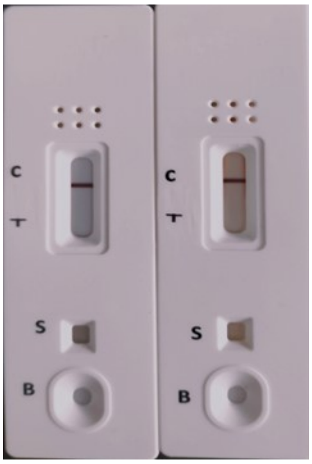

Fig. (7). Specificity of LFIA assay using direct culture of rough Brucella strains: (1) *Brucella abortus* biovar 1 (vaccine strain RB51), (2) B. ovis REO198 both were in a colony count of 1-5x 10^8 CFU/ sample.

Specificity against other Gram-negative bacteria (False positive results of LFIA)

In this assay, specificity was evaluated using cultures of other gram-negative bacteria, especially *Y. enterocolitica* O:9, Salmonella serovars of Kauffmann-White Group Nand *E. coli* O157:H7, with known colony counts ranging from 1-5X 10^8 CFU/ 40 µl to checkthe presence of cross reactions and false positive results (Table 2). These Gram-negative bacteria are the most important cross-reactive bacteria with Brucella. None of the gram-negative bacteria used in this study reacted with anti-Brucella S-LPS polyclonal antibodies (pAbs).This indicates that, based on the analytical specificity determination, no cross reactions to any non-Brucella strains were detected (Figure 8).

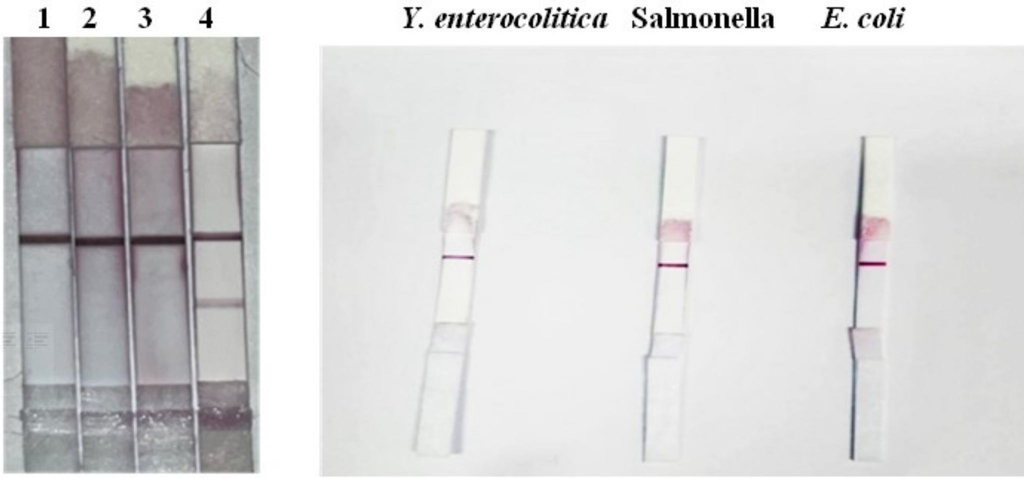

Fig. (8). Specificity of LFIA assay using direct culture of non-Brucella strains: (1) *Y. enterocolitica* O:9, (2) Salmonella serovars of Kauffmann-White Group N and (3) *E. coli* O157:H7 and control positive (4) smooth Brucella strain (S16M).

The results of LFA with non-Brucella strains and rough Brucella strains demonstrated that this LFIA assay displayed high selectivity for the detection of smooth Brucella strains with 100% specificity and 100% sensitivity.

Table (2). Specificity and cross-reactivity of the developed LFIA

Tested Organism	Strain Type	LFIA Result	Cross-reactivity
Brucella abortus RB51	Rough Brucella strain	Negative	None
Brucella ovis REO198	Rough Brucella strain	Negative	None
Yersinia enterocolitica O:9	Non-Brucella bacterium	Negative	None
Salmonella Group N	Non-Brucella bacterium	Negative	None
Escherichia coli O157:H7	Non-Brucella bacterium	Negative	None

Discussion

The present study demonstrated that the developed lateral flow immunoassay (LFIA) strip is capable of detecting smooth Brucella antigens with a detection limit ranging from 5×10^3 to 1×10^4 CFU/40 µl of *Brucella melitensis* biovar 1 (Rev-1). This level of sensitivity indicates that the assay is suitable for detecting moderate bacterial loads in clinical samples and may be useful in early-stage infections where bacterial counts are still limited. The achieved sensitivity can be attributed to the use of specific anti-Brucella S-LPS polyclonal antibodies in combination with colloidal gold nanoparticles, which enhance both antigen–antibody binding efficiency and visual signal detection.

In terms of specificity, the developed LFIA showed no cross-reactivity with non-Brucella gram-negative bacteria included in this study. This high specificity may be explained by the selective targeting of smooth lipopolysaccharide (S-LPS), which is a major antigenic determinant unique to smooth Brucella strains. This finding is in agreement with **Byzova *et al.* (2012),** who reported that their immunochromatographic assay showed no cross-reactions with a wide range of bacterial species even at high concentrations. Similarly, **Prakash *et al.***

(2021) demonstrated that LFIA could specifically detect Brucella isolates without interference from other pathogens. However, in contrast to the present findings, **Lu *et al.* (2023)** reported minor cross-reactivity with *Yersinia enterocolitica* O:9, which may be attributed to structural similarities in LPS antigens. This suggests that assay specificity can be influenced by antigen selection and antibody design.

The diagnostic performance of the LFIA developed in this study is comparable to other serological and molecular diagnostic methods. **Prakash *et al.* (2021)** reported sensitivity values ranging from 78.57% to 80% and specificity values between 93.07% and 94% when LFIA was compared to culture and PCR methods. Likewise, **Amahyel *et al.* (2019)** found that LFIA showed high sensitivity and specificity across different animal species, with values approaching those of established tests such as ELISA and the Rose Bengal Test (RBT). In addition, **Ge *et al.* (2021)** reported no significant difference between LFIA and **ELISA** in diagnostic accuracy, although LFIA demonstrated slightly higher sensitivity in some cases. More recently, **Lu *et al.* (2023)** developed a nanoparticle-based LFIA system that achieved very high sensitivity (98.33%) and specificity (100%), highlighting the potential for further enhancement of LFIA performance through nanotechnology.

One of the major advantages of the LFIA developed in this study is its rapid detection capability. The assay provides results within a short time without requiring complex instrumentation or highly specialized laboratory conditions. This makes it particularly suitable for field applications and routine diagnostic laboratories with limited resources. This observation is consistent with **Amahyel *et al.* (2019),** who emphasized the practicality and ease of use of LFIA for large-scale screening in veterinary settings.

Despite the effectiveness of colloidal gold nanoparticles used in this study, alternative labeling systems such as latex microspheres have been reported to offer improved sensitivity. **Li *et al.* (2019a)** demonstrated that latex-based LFIA systems can produce stronger signals and lower detection limits compared to traditional gold-based assays. However, both systems remain widely used and effective, and the choice between them depends on the required sensitivity and application context.

In addition to immunoassays, molecular diagnostic approaches such as multiple cross displacement amplification (MCDA) have shown promising results for Brucella detection. This technique targets species-specific genes such as Bscp31 and can be combined with lateral flow biosensors to achieve rapid and highly specific detection. **Li *et al.* (2019b) and Huang *et al.* (2020)** reported 100% specificity with no cross-reactions when using this approach. However, despite its high accuracy, MCDA requires more technical expertise and laboratory setup compared to LFIA, which remains a simpler and more accessible method.

Overall, the findings of the present study indicate that the developed LFIA strip is a reliable and efficient diagnostic tool for the detection of smooth Brucella strains. It combines acceptable sensitivity with high specificity and offers significant advantages in terms of speed, simplicity, and ease of use. Future improvements, particularly through the use of more sensitive labeling materials such as latex microspheres or advanced nanoparticles, may further enhance its diagnostic performance and contribute to better control and eradication of brucellosis.

Acknowledgments

The authors acknowledge their respective central laboratory and institute for their cooperation.

Ethics approval and consent to participate

This study was conducted in accordance with guidelines set by the Institutional Animal Care and Use Committee at the Laboratory Animal Resources isolation facilities of Central Laboratory for Evaluation of Veterinary Biologics-Agriculture Research Center. IACUC approval No. (ARC-CLEVB-35-25). No anesthesia or euthanasia protocols were employed for animals involved in this study, as all animal-dependent methodological procedures were categorized as either no-pain or low-pain procedures that can be ethically performed on a conscious and alive animal.

Authors' contributions

The contribution of the authors was as follows; W.S. and M.E; design of experiment and laboratory work, Sh.A.; preparing required strips and writing its data; M.E. and L.F.; preparing of strains for laboratory work,W.S.; writing the original draft, M.D.; review and editing, G.Y.; collecting reviews and interpretation, Sh. A.,W.S., L.F., M.D., G.Y., and M.E. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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