

## Development of gold nanoparticles-lateral flow test as a novel field diagnostic assay for detecting foot-and-mouth disease and lumpy skin disease viruses

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### ABSTRACT

**Background and Objectives:** Rapid diagnosis is a cornerstone for controlling and preventing viral disease outbreaks. The present study is aimed to develop a rapid field diagnostic test based on gold nanoparticles for the detection of lumpy skin diseases (LSD), and foot and mouth diseases (FMD) in animals with high sensitivity and specificity.

**Materials and Methods:** FMD and LSD vaccines were used as a source of viruses' antigens for preparing monoclonal antibodies and conjugated with gold nanoparticles that characterized using various techniques such as UV-visible spectrometry, and transmission electron microscopy (TEM). Monoclonal antibodies (mAbs) for each serotype produced in experimental rats and used to capture antibodies for FMDV and/or LSDV. ELISA was used to screen 469 milk samples and 1165 serum samples from naturally infected cattle, buffaloes, sheep, and goats for validation of the lateral flow test (LFT). LSDV DNA was extracted from 117 blood and skin biopsy samples collected from naturally infected cattle during the 2019 outbreak.

**Results:** The specificity and sensitivity of GNP-LFT were evaluated and compared to Ag-ELISA, Western blot tests (WB), and PCR. A total of 95 FMDV positives out of 469 (20.25%) milk samples and 268 FMDV positives out of 1165 (23.3%) serum samples from natural infected cattle, buffaloes, sheep, and goats examined by ELISA to valid GNPS-LFT Viral LSDV DNA was detected in 60/117 (51.5%) and 31/60 (52.9%). While the GNPS-LFT assay results were 49/117 (41.9%) and 29/60 (48.3%) blood and skin biopsy samples, respectively. The diagnostic sensitivity and specificity of the GNP-LFT test were 72% and 82%, respectively. All vesicular fluid and epithelium samples collected from infected animals were identified as positive by the GNP- LFT and Ag-ELISA. Ag-ELISA, on the other hand, was 90% and 100%. While the developed GNP-LFT used LSDV polyclonal antibodies were similar to ELISA and IgG-WB with a sensitivity of 72.8% and a specificity of 88.8%, respectively.

**Conclusion:** The GNPS-LFT is a novel immunoassay based on mono or polyclonal antibodies conjugated with gold nanoparticles that provides an accurate, rapid, specific, and sensitive tool for field rapid diagnosis of FMDV and LSDV.

**Keywords:** Foot-mouth disease; Gold nanoparticles; Lateral flow test; Lumpy skin disease virus; Rapid diagnostic tests; Skin lesion biopsy

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## INTRODUCTION

Viral infectious diseases are mainly caused by remarkable pathogens, including lumpy skin diseases (LSD), and foot and mouth diseases (FMD), which cause prominently increasing morbidity, and mortality. Detection of antigens and/or antibodies is a cornerstone for controlling and preventing disease outbreaks (1-3).

Many viral diseases exist in animals and their environments. Most of those viruses are non-pathogenic and important for maintaining the life ecosystem on earth (4). Infectious diseases are the major reasons for death, especially in low income countries world (5). Viral infections affect millions of animals and cause major public health problems worldwide. FMDV spreads via direct or indirect contact (6). FMDV infection in ruminants could be recovered from esophageal-pharyngeal fluids during the convalescent phase of FMD (7).

The globalization of international trade leads to increase risk of emerging infectious diseases, due to easy transportation for people, goods, and animals around the world; unfortunately, this time frame is shorter than the incubation periods of infectious diseases (8). The danger of viral infection is not limited to low resource area, but, lack accurate, rapid viral diagnostic capacity in many regions of the world and undetected outbreaks may stay for a long time and spread through population in the affected area (9).

Lumpy skin disease (LSD) is a highly contagious viral disease endemic in several regions of Africa and the Middle East, including Palestine, Iraq, Jordan and Egypt (10). LSD in cattle is characterized by sudden fever, nodular skin lesions distributed along animals skin, internal organs, and lymph nodes due to lack LSDV antibody and cross-protection leading to infection in vaccinated cattle (11). Buffaloes and sheep remained clinically normal. However, close related with infected wild ruminants, cattle, like wild buffaloes that can harbour infections without clinical signs (12). However, infectivity and distinguishing between vaccinated and infected animals (13). Lumpy skin disease virus (LSDV) rapid accurate diagnostic tools for confirmation of viral diseases, by isolation in tissue culture, and serological diagnosis (agar gel immunodiffusion (AGID), indirect ELISA, IFAT, and western immunoblotting (WB) are keystones and essential for control. Although they have many disadvantages, they cannot differentiate

between infected and vaccinated animals (14). Furthermore, they require skilled manpower, more time, sophisticated labs, and a high cost (15). PCR-based assays have been established, which are associated with high sensitivity and efficiency (16). However, viral diagnosis requires 24 hrs for detection, including direct collection, viral transport media, highly trained personnel, expensive instruments, and complicated protocols (16, 17).

The lateral flow test (LFT) is rapid diagnostic technique based on specific immune responses involving antigens and antibodies that does not necessitate any special. An antibody labeled with gold nanoparticles (GNP) placed in a dried state on fiber glass with the great sensitivity (18).

The complex reacts with the immobilized antibody and gives colour in proportion to the analyzed concentration (19). The LFT was effortless, generate their result within 15-30 min (20, 21). Therefore, the present study was targeted at an antibody-based rapid diagnostic method, which is quite promising for detecting the virus at the field level with high sensitivity and specificity. The current study was aimed at creating a rapid field diagnostic test based on nanotechnology application development of an GNPs-LFT with FMDV and LSDV specific antibodies, as well as field-level detection validation.

## MATERIALS AND METHODS

**Ethical approval.** All research steps includes samples collection and laboratory animals handling according to standard procedure without causing any stress or harm to different animals. This work was done according to guidelines of National Research Centre ethical guide under certificate number # 19-148.

**Samples collection.** Samples collected from different animals with or without signs included, high fever (40°C-41°C), tongue with excessive profuse salivation, vesicles, ulcerations on the gum, dorsum of plate, foot lesions in inter-digital space with ulcerations leading to lameness, in dairy animals, vesicles and ulcerations on the udder and ranged from severe, moderate (20). A total of 1178 samples from suspects were collected. A total of 469 milk samples and 1165 serum samples from natural infected (cattle (n = 325), buffaloes (n = 265), sheep (n = 305), and goats

(n = 175) in different governorates (Beni-suef, Giza, Monufia, El Fayoum, Alexandria, Sharqia, and New Valley Siwa in Egypt from 2019 to 2021. All FMD samples were kept and stored at -20°C until they were used. While, suspected cases clinically showed LSD signs of affected cattle showed 40-41°C raised animal body temperatures, depression, loss of appetite; naso-ocular discharge, pneumonia severe edema in the shoulder, brisket, and circumscribed nodules on the skin with different size which covering over the entire animal's skin included head, neck, trunk, perineum, udder. Skin biopsies and whole blood samples were obtained from animals with or without clinical signs of LSD at different governorates in Egypt. Each biopsy (vesicles, ulcerations on the gum, dorsum of plate, tongue) for FMD or skin biopsy sample for LSD was ground using a sterile mortar and pestle, then suspended in PBS containing 10% antibiotic solution (5). Each of tissue homogeneity was centrifuged at 3000 rpm for 10 min at 4°C. The clear supernatant fluid was collected, and frozen at -20°C.

**Reference virus strains.** The Veterinary Research Division and Animal Health Research Institute, Dokki, Giza, Egypt, provided FMDV strains A, O, and SAT-2, as well as Lumpy skin disease virus strain (Neethling strain) and sheep pox virus vaccine Romanian strain (TCID 50/dose, and FMDV and LSDV anti-sera.

**Production and purification of anti-FMDV and LSDV antibodies: preparation of inactivated LSD and FMD viruses.** Reference LSDV strain was inoculated in Madin-Darby bovine kidney (MDBK) cells. After 48 hrs of virus inoculation, the harvested cells were centrifuged and disrupted with lysis buffer and sonication to obtain a clear. Each FMDV genotypes A, SAT-2 and O were inoculated in baby hamster kidney-21 (BHK) cells supplement with Eagle's medium (EMEM; Canada) and 0.05 % Fetal calf serum (FCS). After, 48 hr harvested infected cell for complete cytopathic effect (CPE). The harvested cell culture disrupted with freezing and thawing three successive time and sonication (21). To obtain a clear lysate after inactivation with 2-Bromoethylamine Hydrobromide (BEI, Sigma-Aldrich, USA) and purified as previously described used for immunization (22).

**Animal experiments for monoclonal antibodies production.** All experimental procedures for animal

inoculations and care were performed following the National Research and Center (NRC) in Egypt animal care guidelines. A total of twenty-five specific pathogen-free (SPF) Group (G1 to G5) /5 rats (4-8 week olds, female and male), were grown SPF animal house at National Research and Center (NRC) Egypt. Rats were divided into 5 groups (5 rats/group) for the inactive FMDV genotypes O, A, and SAT-2 immunization. The rats were isolated and kept under controlled conditions after one week prior to the experiment. Experimental groups 3 groups (5 rats / group) were administered with inactive FMDV genotypes as follows G1 to G3 were injected with FMDV different genotypes O, A, and SAT-2. While G4 was injected with LSDV; 0.5 ml of each was subcutaneous injection (S/C) and intraperitoneal (I/P). The control group rats were inoculated with PBS at PH 7.2. The rats were immunized three times with a dose of the inactive FMDV (40-50 µg) by two routes, S/C, and I/P. The examination of antibody response from blood and ascites fluid collected from the experimental rats over a 7-day period before inoculation, and at 2 or 3-week intervals after infection. The rats' ascites fluids (give clear rich fluid with antibody) were harvested after 28-30 days and centrifuged at 3000 for 15 min anti-FMDV O, A, SAT-2 and LSDV were separated from the whole uncoagulated blood samples and ascetic peritoneal from experimental animals for assay of antibody weekly post vaccination for 4 weeks then stored at -80°C.

**Bio-conjugation of colloidal gold nanoparticles.** The size of gold-nanoparticles ranged from 5 to 30 nm. They were prepared by adding 1% disodium citrate solution (w/v) added to 0.01% aqueous chloroauric acid solution (w/v) at 100°C for 5 min according to the protocol described by Abdalhamed et al. (18), then gradually cooled, the pH of the solution was adjusted to 8.4, and then the solution was stored at 4°C in a dark bottle. Monoclonal antibody (Ab) (100 mg/ml) was added drop wise into a 10 ml solution. After stirring the mixture for 30 minutes, 2.5 ml of 10% bovine serum albumin (BSA) was added to 30 µl/ml of gold suspension to block the gold colloids' excess reactivity. The mixture was stirred on the magnetic stirrer for 30 minutes and stored at 4°C for 2 h. After the mixture was centrifuged at 3000 rpm for 30 min, the supernatant was centrifuged at 12,000 rpm at 4°C for 30 min, and the conjugate pellet was suspended in 10 mM buffer (pH 8.0) containing 2% (wt/ vol), BSA

and 0.05% sodium azide.

**Gold nanoparticles conjugated.** The particle size and shaped diameter of Au NPs were determined by transmission electron microscopy (TEM), the gold-antibodies spray onto a glass-fiber membrane (conjugate pad) and dispensed at a rate of  $10^{-1}$ /cm (approximately 1.5 g/cm) dried in air drier.

**Gold nanoparticles lateral-flow test (GNPs-LFT) preparation and construction.** Gold particle suspensions were adjusted to pH 7.2 with 50 mM potassium carbonate (pH 9.6). The monoclonal antibodies were added dropwise to a 10 to 20-nm gold solution while stirring to achieve a final concentration of 10 g/ml, and the solution was stirred for 15 minutes. Antibodies (2 mg/ml) anti-mouse Ig dissolved in phosphate-buffered saline (PBS) at pH 7.4 was loaded onto the test and control lines on the nitrocellulose membrane in laminar flow and then dried for an hour at 37°C before being stored at 4°C. After drying for 2 h at 37°C, the membrane strips were blocked by incubating with PBS (pH 7.4) containing 2% (w/v) bovine serum albumin (BSA) for 30 min and washed three times with PBS containing 0.1% (v/v), Tween-20 for 3 min each time. The member was dried for 2 h at 37°C and stored at 4°C. The immobilized nitrocellulose membrane and the absorbent pad were adhered to a backing plate (300 mm × 70 mm) in the proper order as Fig. 1. The plates was then cut into four-mm-wide strips using a CM-4000 cutter. The strips were stored in aluminum foil sachet packaging bags at 4°C until used.

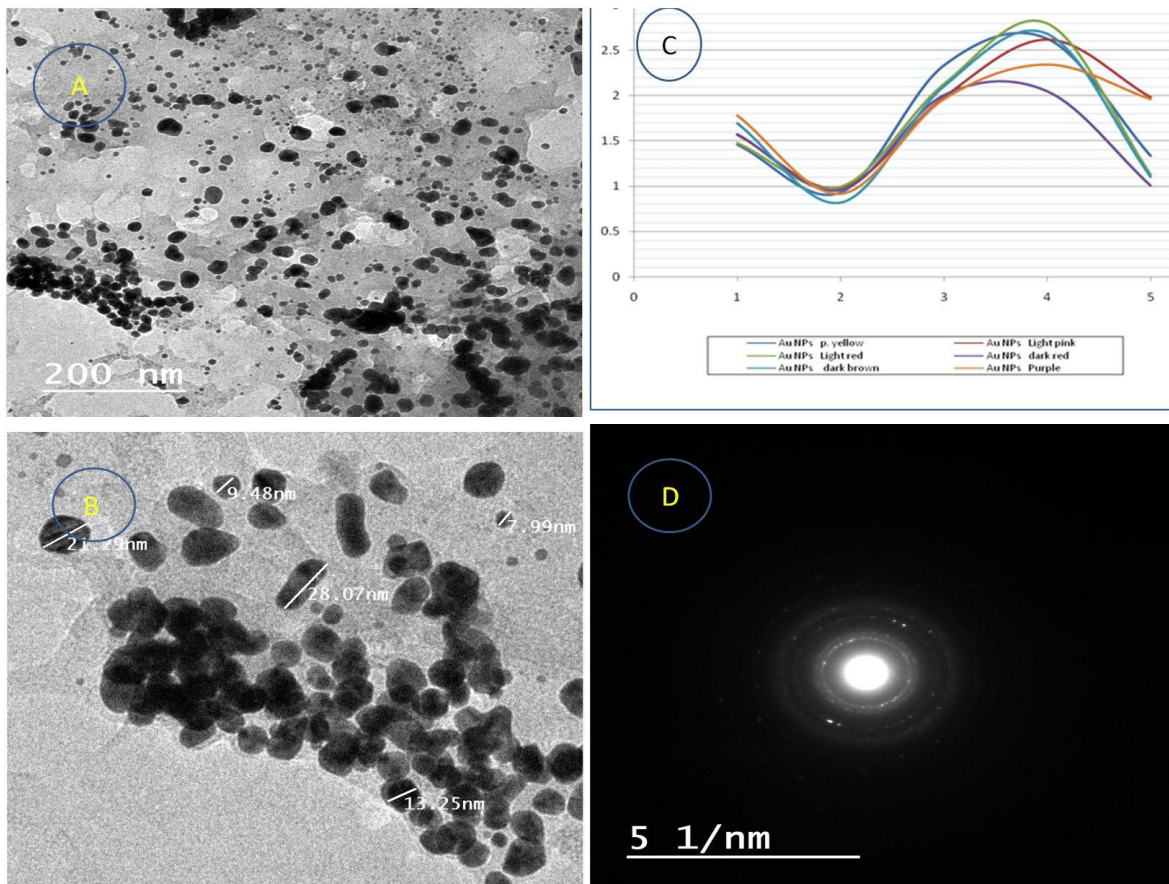
**Evaluation of GNPs- LFT with naturally infected samples.** The developed GNP-LFT was validated by positive and negative sample. The results were classified as true positive (TP), true negative (TN), false positive (FP), or false negative (FN) and were assigned to designate the intensity of detection. The following formulas were used to calculate the sensitivity and specificity of the GNP-LFT diagnostic efficiency. The sensitivity of the assay was determined by testing with serially diluted FMDV suspensions ( $10^{3.5}$  and  $10^6$  TCID<sub>50</sub>/ 100 µL), with the limit of detection (LOD) of the developed GNPS-LFT strip being ascertained as the least dilution of FMV and/or LSDV suspension that showed the positive reaction. The specificity of the strip was assessed using GNPS-LFT against the following poultry viruses: Egg Drop Syndrome (EDS-76), Newcastle Disease virus (NDV)100 µL of

each virus suspension was used.

$$\text{Sensitivity (\%)} = \frac{\text{TP}}{\text{TP} + \text{FN}} \times 100 \quad \text{Specificity (\%)} = \frac{\text{TN}}{\text{TN} + \text{FP}} \times 100$$

**Sandwich enzyme-linked immunosorbent assay (ELISA).** Sandwich-ELISA (S-ELISA), [Brescia, Italy for LSDV and Pirbright, UK for FMDV], was used to detect the presence of antigens and serotypes in infected biopsy samples of different animals (tongue tissue biopsies). Supernatants of the homogenized clinical tissue materials were tested to detect the virus serotype involved in the outbreaks. Coating sera was diluted for each serotype as per manual instruction with 0.05 mole (mol) of carbonate buffer (pH 9.6). A 96-well flat-bottom plate was coated with 50 µl/well of each antiserum at 4°C overnight. The plates were washed three times in the washing buffer. The tested samples were added to the wells and incubated for 1 h at 37°C. The plates were washed three times with phosphate-buffered saline (PBS) and dispensed with 50 µl of blocking buffer and positive and negative controls. The wells were filled with 50 µl of peroxidase-labeled water and incubated for 45 minutes at 37°C. Afterward, the plates were washed three times, and 50 µl of o-phenylenediamine-H<sub>2</sub>O<sub>2</sub> (OPD) substrate was added to the plates, which were then incubated in the dark for 15 minutes at room temperature, and the optical density (OD) was measured with an ELISA reader at 406 nm.

**Indirect ELISA.** It was performed according to by dilution of sera, and whole antigens were checked by checkerboard for optimal amounts of antigen and serum dilution, and optical density values. ELISA was used to characterize the mAbs' binding epitopes. The ELISA and virus denaturation was (20 µg/ml, denatured or trypsin treated virus protein at 0.3 µg/ml) in 0.1 M bicarbonate, pH 8.3 were coated onto Microtitre plates performed as previously described (15). Briefly, microtitre plates were coated with purified and denatured FMDV/A, O and SAT-2 diluted in 0.1 M bicarbonate, pH 8.3 and incubated overnight at 4°C. The plates were blocked with 5% skim milk in a washing buffer (0.05% Tween20 in 0.01 M phosphate-buffered saline (PBS-T) at 37°C. After incubation, supernatants were added to the plates. Then a horse-radish peroxidase (HRP) conjugated goat anti-rat IgG (1:2000, USA) was added at 405 nm was measured using a plate reader.



**Fig. 1.** (A, B and D): Electron microscopy micrograph of gold nanoparticles was showed (spherical particles with particle size diameter < 30 nm, characterized by TEM and UV-Vis spectroscopy showed 10 to 20 nm sizes with maximum absorption at 520 nm, and larger size with Mabs conjugation, the size range from 25-30 nm the color intensity (wine red).

**3ABC-enzyme-linked immunosorbent assay (3ABC-ELISA).** 3ABC-ELISA kit (IDEXX FMD 3ABC Bovine, Spain) for detection of non-structural polyprotein of FMD antigen antibody in cattle and buffaloes serum samples were used and followed by the manufacturer instructions. Whole blood samples without anticoagulants were collected from suspected animals after serum separation, stored at -40°C or examined after appropriate dilution by 3ABC-FMD antigen using. The 3ABC-ELISA was developed under standard laboratory conditions, with all incubation steps at 37°C with gentle shaking. Plates were washed three times between incubation steps with washing buffered (phosphate-buffered saline pH 7.2 tween 0.05%). Briefly, a 96-well coated with poly FMDV 3ABC nonstructural polyprotein, 50 µL of the test serum samples or control serum samples were added at a 1: 50 dilutions (in blocking buffer) and the plate was incubated for 30 min. Control serum and the plate incubated for an additional 30min.

After washing, anti-bovine horse radish peroxidase conjugate was added (50 µL/well in blocking buffer) and incubated for 1 h. After washing, 50 µL/well of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate were added and the reaction stopped after 10 min at room temperature by the addition of 50 µL /well of 1 mol /L H<sub>2</sub>SO<sub>4</sub> and the absorbance of each well at 450 nm measured on an ELISA reader after blanking. Serum samples were tested duplicate or triplicate, and the final result was expressed as the mean value. Each plate contained four replicates of the positive and negative controls. Results were presented with percentage and values ≥30% were considered positive, < 20% and < 30% were considered suspicious.

**Rapid slide agglutination test with staphylococcus protein A (SAT-SPA).** It was prepared according to (23, 24). Samples collected from naturally infected animals and mixed with equal amounts of Staphylococcus protein A (SPA) particles (prepared by mixing

staphylococci suspension with specific mono/polyvalence antiserum against viral, bacterial, or parasite on a glass slide surface), and the mixtures were gently rotated at room temperature for 5-10 min. The sample was tested in triplicate with the control.

**Immune-chromogenic (IC) test.** It was prepared according to Mahapatra and Parida (25). Tissue biopsy and saliva for FMDV samples were added to a micro-immune-tube previously coated with specific anti-viral FMDV antibody and / or LSDV antibody. Incubation time was 2 hrs. Anti-species conjugated with horse radish peroxidase were added to incubate for 15 min at RT. Then they will add TMB and stop the reaction after 5 minutes. Discard the suspension and wash it three successive times. The test is considered positive when the colour changes compared to the negative tube.

**Western blot test (WB-IgG).** It detects pathogen-specific antibodies and/or antigens, by separating native antigen mixtures using electrophoresis or isoelectric focusing as described by Li et al. (27). Briefly, electrophoresis was performed in polyacrylamide gel under reducing conditions. LSDV and /or FMDV (10 µl of each 10× concentrated culture supernatants) were mixed 1:1 with sample buffer and incubated at 95°C for 5 min. FMDV proteins were separated by 10-20% SDS-PAGE using Vertical electrophoresis apparatus and transferred onto nitrocellulose membrane. The membranes were blocked and then incubated with 1:20 diluted hybridoma culture supernatants in dilution buffer 0.25% skim milk in PBS with 0.05% Tween 20. Antibody binding was detected by incubation with alkaline phosphatase-conjugated goat anti-rat IgG (1:30,000 dilutions). The membrane was washed three times for 5 min with washing buffer 0.05% Tween 20 in PBS between each step.

**DNA extraction.** DNA was extracted from homogenised tissue samples and blood samples using DNA mini extraction kit according to the manufacturer's instructions and their modification (28). The reference strains were used as positive control, and sterile deionized water used as negative control. In all cases, DNA was eluted in 50 µl of elution buffer and stored at 20°C until further analysis.

**Polymerase chain reaction (PCR).** PCR was per-

formed according to the procedures of Poonsuk et al. (29). The PCR primers were developed from the gene for viral attachment protein with the following sequences; forward primer: 20 pmol of oligonucleotide primers for LSDV F and R 5' TTTCCTGATTTTCT-TACTAT 3' and 5' AAATTATATA C GTAAATAAC 3' sets were used in the PCR Master Mix. 25 µl volumes were used with 2.5 of the extracted viral genome, 0.2 µl of each primer (50 nM), 12.5 µl of master mix, and 9.6 µl water, the amplicon size of the PCR product is 192 bp. To amplify a specific fragment of the attachment gene primers, The PCR was performed for according to (25, 26). Amplification was done under the following conditions: initial denaturation cycle at 95°C for 2 min, 40 cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min), followed by a final extension cycle at 72°C for 10 min. 5 µl of each PCR product was analysed by gel electrophoresis with 1.5% agarose gel at 100 V for 30 min. The DNA ladder of 100 bp was used as a size standard and the gel was visualized using an ultraviolet light transilluminator.

**RNA extraction.** Viral RNA was extracted QIAamp® Viral RNA Mini Kit (Qiagen, UK) using according to manufacturer's guidelines. RNA was extracted from 140 µl from blood and tissue samples (50-100 mg) according to the manufacturer's instructions (using the spin column protocol) and eluted in a final volume of 60 µl.

One-step reverse transcription polymerase chain reaction (RT-PCR) was carried out in 25 µL reaction mixture as described by Poonsuk et al. (29) using primers forward and backward as in Table (1). The reaction was run at 50°C for 30 minutes for reverse transcription and 15 minutes for initial denaturation at 95°C, followed by 35 cycles (94°C for 1 minute followed by annealing for 1 minute at 52°C for serotypes O and SAT 2, and 54°C and 55°C for serotype A). The extension temperature was set to 72°C for 1 minute, followed by a final 10-minute extension step at 72°C. The amplicons were analysed by 1.5% agarose gel electrophoresis.

**Statistical analysis.** The Statistical Package for the Social Sciences (SPSS) software (Version 17.0; SPSS, Inc, USA) was used for the analysis of the collected data. The McNemar test was used to compare the sensitivity of the GNPS-LFT assay compared with PCR. Receiver operating characteristic (ROC) curve

**Table 1.** Oligonucleotide primers used to detect the foot and mouth disease virus using reverse transcription polymerase chain reaction (RT-PCR)

Primer	Sequence (5' to 3')
FMDV-All serotypes	F- GCC TGG TCT TTC CAG GTC T R- CCA GTC CCC TTC TCA GAT C
FMDV-O	F AGC TTG TAC CAG GGT TTG GC R ' GCT GCC TAC CTC CTT CAA
FMDV- SAT	F CCA CAT ACT ACT TTT GTG ACC TGG A R ACA GCG GCC ATG CAC GAC AG
FMDV-A	F TAC CAA ATT ACA CAC GGG AA ' R GAC ATG TCC TCC TGC ATC TG

analysis and area under the curve (AUC) were used to determine sensitivity and specificity.

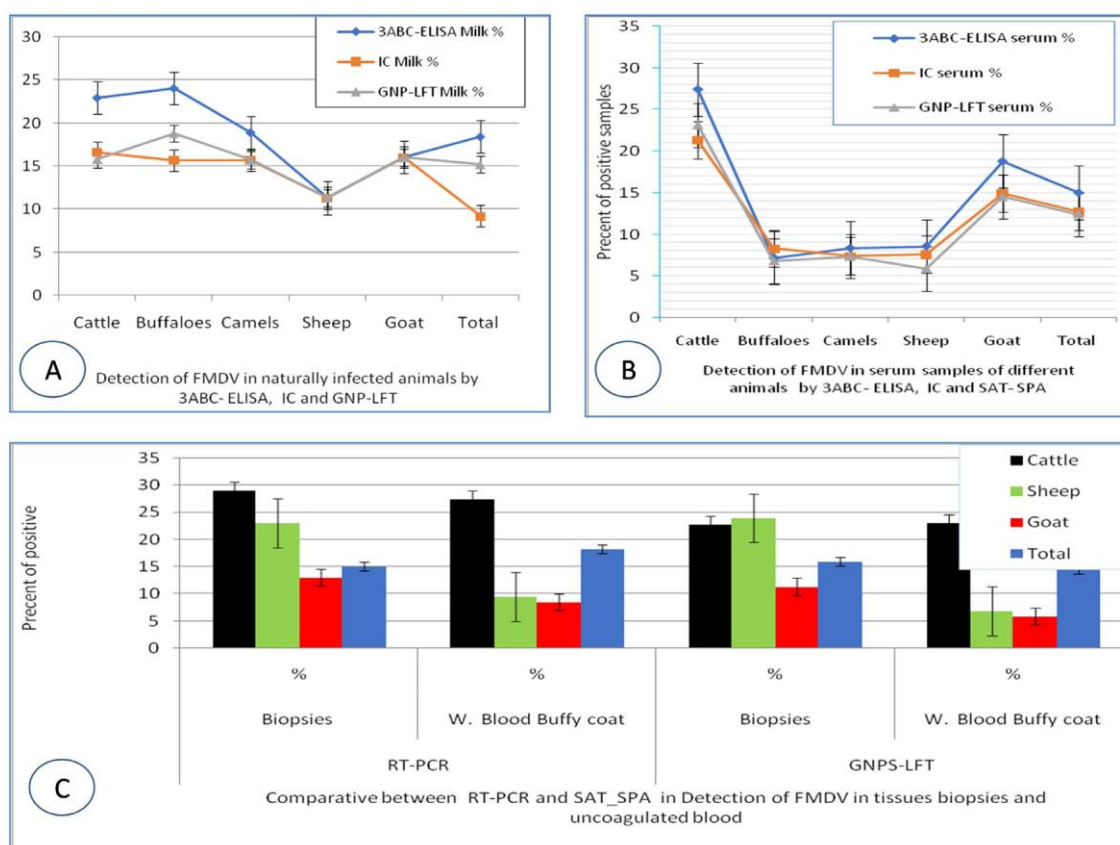
## RESULTS

**Synthesis and characterization of GNPs.** The biosynthesis of formation gold nanoparticles (Au-NPs) was observed by color mixture changed from red to light ruby red after 20 min and then to a purple color after 2 h of incubation, which signified the completed formation of Au-NPs. Formation of gold nanoparticles can be easily detected by ultraviolet-visible spectroscopy (UV-VS), giving rise to an absorption band at 510-540 nm. The UV-vs spectra of the reaction mixture obtained after 30 min of reaction was presented in Fig. 1. Absorption spectra of Au-NPs exhibited a well-defined SPR band centered on 530 nm. We observed that the increasing intensity of colloid color was owing to the increasing number of gold nanoparticles formed. The reduction of hydrogen tetrachloroaurate (HAuCl<sub>4</sub> 3H<sub>2</sub>O) by citrate produced gold nanoparticles (Au NPs), the light yellow color of 0.1 mM hydrogen tetrachloroaurate (HAuCl<sub>4</sub>, 3H<sub>2</sub>O) solution after dissolving in deionized water. The observed colour changed after gold nanoparticles (Au NPs) colloid gradually formed a 1 h reaction mixture to a purple colour solution was observed.

The rapid field diagnostics tests with high sensitivity, specificity, reliability, and cost-effectiveness; lateral flow test strips positive has two red lines. While, lateral flow test strips negative has one red line, interpreted result by naked eyes as field test, it showed deep yellow color revealed strong positive, while pale yellow color means weak positive results and colorless observed in negative results.

**Detection of FMDV in clinical samples collected from naturally infected animals.** Anti-FMDV antibodies type O, A, and SAT-2 strains had high reactivity against FMDV antigen detected by 3 ABC-ELISA and indirect ELISA. FMDV was detected in milk samples of different animals: 22.8% (29/127) in cattle, 23.9% (23/96) in buffaloes, 18.75% (6/32) in camels, 11.3% (13/115) in sheep, and 15.9% (15/ 94) in goats. While, serum samples were 27.4% (89/325) positive for FMDV in cattle, 7.2% (19/265) in camels, 8.4% (9/108), and 17.71% (26/305) in sheep and goats, indicating active FMDV infection and negative serum samples mean no infection or animals may have been previously vaccinated with poly FMDV vaccines, as shown in Fig. 2 ( A and B).

Fig. 2 showed detection of FMDV antigen and/or antibody in serum and milk samples collected from naturally infected animals during the FMD outbreak by GNPT-LFT compared to different assays IC, SAT-SPA, and 3 ABC-ELISA. GNPs- LFT showed a sensitivity of 72% and specificity of 88.8 with an accuracy of 91.3%. While, IC was sensitive 72.7%, and a specificity of 65.2% with an accuracy of 86.9%, compared to the sensitivity of ELISA was 99.9% and specificity of 96.8% with an accuracy of 97.8%. The results of GNP-LFT were nearly similar detection limits in comparison with 3ABC-ELISA and IC tests for antigen and /or antibodies in serum and milk collected from different animals. Fig. 2C showed Comparative between RT-PCR and GNP-LFT in detection of FMDV in tissue biopsy and whole blood from infected animals'. GNPs- LFT standardized for detection of FMDV antigen in milk, and tissue biopsy samples (vesicular fluid and tongue epithelium tissue). GNPs-LFT used for detection of FMDV in tongue epithelium and/or FMDV animals gave positive results similar to



**Fig. 2.** 3ABC-ELISA: WB: Western blot test, GNPs-LFT, IC: immune-chromogenic test 3ABC-enzyme-linked immunosorbent assay

those obtained by RT-PCR.

The FMDV antibodies were detected in milk ( $n = 469$ ), and in serum samples ( $n = 1165$ ) of infected animals by different classical serological assays (IC and SAT-SPA with 3ABC-ELISA) was 95 out of 469 (20.25%) and 268 out of 1165 (23.3%), respectively. Also, positive and negative serum and milk samples were used for rapid test GNPS-LFT validated. The average of three 3ABC-ELISA, IC, and GNPs-LFT significant differences ( $p < 0.05$ ) between control (ELISA) and, IC, and GNPs-LFT. Detection of FMDV antigen and/or antibody in serum and milk samples collected from naturally infected animals during the FMD outbreak by GNPT-LFT compared to different assays IC, SAT-SPA, and 3ABC-ELISA. GNPs-LFT showed sensitivity 72% and specificity 88.8 with an accuracy of 91.3%. While, IC was showed sensitivity 72.7%, and specificity of 65.2% with an accuracy of 86.9%, compared to sensitivity of ELISA was 99.9% and specificity 96.8% with an accuracy of 97.8%. The results of GNP-LFT was nearly similar detection limit

in comparison with 3ABC-ELISA and IC test for antigen and /or antibodies in serum and milk collected from natural infected animals. GNPs-LFT standardized for detection of FMDV antigen in milk, and tissue biopsy samples (vesicular fluid and tongue epithelium tissue). GNPs-LFT used for detection of FMDV in tongue epithelium and/or FMDV animals gave positive results similar to those obtained by RT-PCR.

Table 2 showed that the 136 samples were examined by Sandwich ELISA, which found three different FMDV serotypes (O, two A, and SAT-2) circulating in different animals in Egypt. Three of O, A, and SAT2 were detected in cattle, buffaloes, sheep, and goats. While, biopsy samples collected from camels had no detectable FMDV strain by sandwich typing ELISA. S-ELISA detected 22 (16.2%), 18 (13.3%), 19 (14%), and 8 (6.7%) positive samples, while SAT-SPA detected only 17 (12.5%) positive samples. All the tests that used specific FMD virus type antiserum polyvalence for valid GNP-LFT were similar. The GNPs LFT, Ag-ELISA, or 3 ABC ELISA, and PCR were per-



**Table 2.** Detection of FMDV in tongue biopsy samples collected from naturally infected animals by sandwich-ELISA

Species	No biopsy samples	A		O		SAT2		Mixed infection	
		+ve	%	+ve	%	+ve	%	+ve	%
Cattle	51	12	23.6	9	16.7	8	15.7	2	4
Buffaloes	12	3	25	1	8.4	2	16.8	2	16.8
Sheep	52	6	11.6	5	9.7	6	11.6	4	7.7
Goat	21	1	4.8	3	14.3	3	14.3	1	4.8
Total	136	22	16.2	18	13.3	19	14	8	6.7

formed with FMDV serotypes O, A, and SAT-2. Results in the LFT were scored compared with a mean optical density (ODs) values  $\geq 0$  compared to S-ELISA. The overall sensitivity and specificity of the LFT were 72% and 82%, respectively, low than Ag-ELISA were 90% and 100%, respectively. Two assays have different sensitivity for each of the three FMDV serotypes: O, A, and SAT-2. The sensitivity of the GNPs LFT was 82% when it was tested with type O, while SAT-2 gives only weak positive reactions in the LFT. The sensitivity of the GNPs LFT was 80% with type A and O samples, SAT-2 samples were weakly positive and needed more qualitative and improve their sensitivity.

The results that were obtained with the GNP-LFT in comparison with Ag-ELISA. They were showed high similar, sensitivity and specificity. Both assays had similar sensitivities for each of the 3 FMDV serotypes O, A, and SAT-2. As shown in Table 3.

**Detection of LSDV by different assays.** Cattle (n = 325) from different governorates (Beni-suef, Giza, Monufia, Alexandria, Sharqia, New valley Siwa, El Fayoum) in Egypt during 2019 were clinically examined. The cattle different breed, ages and sex only showed typical LSD clinical signs, such as nodules

on the limbs, neck, head and trunk with fever ranged from (39 to 41°C), oedema or lymphadenopathy in brisket, perineum, genitalia, belly or legs. Typical necrotic lesions (sit- fast lesion) were observed .

Detection of LSDV-DNA by PCR used primers RPO-30 in biopsy and blood samples compared with positive control. Furthermore, the rapid immune-chromogenic tests (IC), Ag-ELISA and WB-confirmed the presence of a viral antigen. Positive and negative samples used for validation of GNPs-LFT as shown in Fig. 3.

The compatibility of results obtained by PCR and GNP-LFT was compared to detect of LSDV-DNA by PCR used primers RPO-30 in biopsy and blood samples compared with positive control, with overall agreement between PCR and GNP-LFT was achieved in large positive and negative samples as shown in Fig. 3. Furthermore, the rapid immune-chromogenic tests (IC), Ag-ELISA and WB confirmed the presence of a viral antigen. Positive and negative samples used for validation of GNPs-LFT.

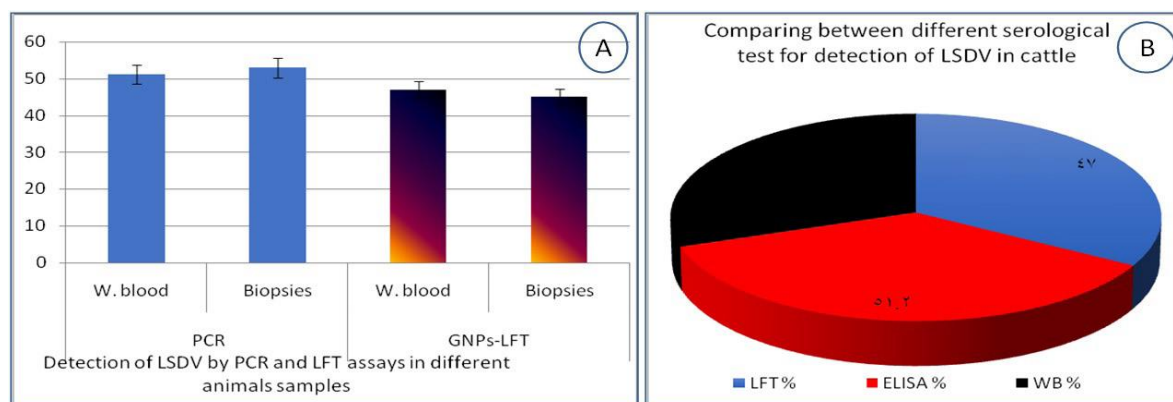
**DISCUSSION**

Diagnostic assays to confirm the initial clinical determination of viral infection are component of any viral disease control measure (30). For the diagnosis of viral diseases, several laboratory methods such as enzyme-linked immunosorbent assay (ELISA), slide agglutination test (SAT), and molecular assay PCR are used (31). Most of these diagnostic methods require the availability of a laboratory facility, highly trained personnel, and multistep sample handling preparation (31). In particular, viral isolation requires a laboratory cell culture facility that can be difficult and expensive to maintain, besides requiring 4 to 6 days for test completion, and logistical considerations associated with sample collection and transport are required (32).

**Table 3.** Comparative between different assays sensitivity and rapid test for detection of FMDV

Species	S-ELISA	GNPs-LFT		IC		WB-IgG	
	+ve	+ve	%	+ve	%	+ve	%
Cattle	10	8	80	7	70	7	70
Buffaloes	5	3	60	4	80	4	80
Sheep	5	4	60	3	60	3	60
Goat	5	3	40	3	60	3	60
Total	25	18	72	17	68	17	68

S-ELISA: Sandwich ELISA, GNPs-LFT: Gold nanoparticles- Lateral flow test IC: immunochromogenic WB-IgG: Western blot



**Fig. 3.** Detection of LSDV by different molecular assays in whole blood and skin biopsy samples collected from cattle in different governorates in Egypt.

A rapid and easy-to-perform test that performed on-site diagnosis would be developed in the case of a suspected disease outbreak, circumventing problems associated with the transportation of samples to the laboratory (33). The GNPs-LFT is an appropriate assay on which to base a rapid assay or pen-side test. The rapid diagnosis allows time for the early implementation of treatment and control measures to reduce the possibility of the spread of infectious viruses. The GNPs-LFT has been widely developed to support the clinical diagnosis of different viral diseases, including FMD and LSD. Rapid development tests without serotyping of different genotypes (SAT-SPA, IC and LFT) as routine methods for diagnosis of FMD outbreaks (34).

The difference in the obtained values of the particle size of the Au- NPs is based on the difference between the observable particle edges, mono-disperse could be owing to the fact that the existence of some significant bio-organic molecules in the plant extract seems to act as a legend which efficiently stabilizes the formed gold nanoparticles and responsible for reducing the Au<sup>+</sup> to Au<sup>0</sup> as shown in Fig 1, and this explanation was agreed with (35). The protein conjugated with an antibody, was found to be more stable as shown in Fig 1. This observation is in agreement with (36). GNPs-LFT interactions almost simultaneously; when two capture lines for the same virus are present, cross-reacting and the type-specific. However, GNPs- LFT-reactive test line had surprisingly relative sensitivity. Starting from the experimental observation that the position of the LFT reactive line dramatically influenced the sensitivity, the effect was exacerbated by distancing the capture line from the sample application point, as the longer the path, the

longer the time available for the detector-antigen complex formation as shown in Fig. 2. The gold standard test used for comparison of the analytical and diagnostic performances of Ag-ELISA kit is implemented with the same Abs; both methods detect the same analyses, polymerase chain reaction, which is therefore intrinsically more sensitive. The moderate sensitivity of the SAT 2 type-specific test line, which was necessarily samples application point compared to the SAT 2 lines (37). The novel LFT was evaluated on clinical samples as well as in the field. Anti-FMDV polyclonal antibody had high reactivity against the FMDV antigen by ELISA assay used for preparation of lateral flow strip tests based on each of Anti-FMDV polyclonal sera were evaluated (capture and conjugated antibodies) as in Table 2. Detection of FMDV antibodies in milk, and serum samples by different classical serological assays (IC and SAT-SPA with 3ABC-ELISA) were 95 positive antiFMDV out of 469 (20.25%) and 268 out of 1165 (23.3%), respectively. Also, positive and negative serum and milk samples were used for rapid test GNPS-LFT validation as shown in Table 3 and Fig. 2 and this result is in agreement with (38). Detection of FMDV antigen and/or antibody in serum and milk samples collected from naturally infected animals during the FMD outbreak) by GNPT-LFT compared to different assays (IC, SAT-SPA, and 3ABC-ELISA) revealed that the GNPs-LFT gave nearly the same results as the assays, as shown in Fig. 3, and this validation is agreed with (37).

Conventional RT-PCR detected the FMDV gene in 80 out of 432 and 172 out of, 1070 tissue biopsies and un-coagulated blood samples collected from different animals, respectively as shown in Fig. 3 and this

result is consistent with [28, 38, 44], they used a molecular assay for detection of different FMDV strains during the 2012 outbreak in Egypt. The different FMDV serotypes A, type O, type SAT2 and mixed were detected in 22 (16.2%), 18 (13.3%), 19 (14%) and 8 (6.7%) tissue biopsy, saliva and fluid vesicles examined by the Sandwich-ELISA assay. Positive and negative samples were used to prepare and validate rapid tests for FMDV different serotypes A, O, AT-2, these results are in agreement with many authors (36-39). GNPs-LFT showed sensitivity 72% and specificity 88.8 with an accuracy of 91.3%. While, IC was showed sensitivity 72.7%, and specificity of 65.2% with an accuracy of 86.9%, compared to sensitivity of ELISA was 99.9% and specificity 96.8% with an accuracy of 97.8%. GNPs- LFT standardized for detection of FMDV antibody and /or antigen in milk, serum, and tissue biopsy samples (vesicular fluid and tongue epithelium tissue). GNPs- LFT used for detection of FMDV in tongue epithelium and/or FMDV antibodies from vaccinated and non-vaccinated animals gave positive results similar to those obtained by ELISA as Fig. 3, those results are agreed with (39). It was noticed that the intense of color was observed with high titers of virus antigen or antibodies, and the explanation of the reactivity of GNPs LFT with immunoglobulin has potential use in the serological survey (36). Viral LSDV DNA was detected in 60/117 (51.5%) and 31/60 (52.9%) in blood and skin biopsy samples, respectively as shown were supported by many authors (22-34).

It is explained that the differences between both assays for the detection of FMDV may be due to a difference in the affinity of the antibody assays (polyclonal antibodies are used in the ELISA and in house polyclonal antibodies in the LFT for the detection of FMDV within 30 min). Since the LFT is simple and easy to use, it can be adopted in underdeveloped countries where there are limited diagnostic facilities. GNPs-LFT serves as an alternative means of sample testing and helps in the implementation of appropriate control measures. However, with the introduction of FMDV of different serotypes to different regions, future efforts will focus on the failure of the GNPs-LFT for detection of FMDV in nearly 15%-20% of ELISA positive samples, and this observation is agreed with (36-38).

Lumpy skin disease virus (LSDV) was isolated from skin biopsy homogenates collected from infected cattle based on the appearance of characteristic

pock lesions on the CAM of ECE identified by PCR with a specific primer set amplifying a 192 bp DNA fragment equal to the expected amplification product size from LSDV. Viral LSDV DNA was detected in 60/117 (51.5%) and 31/60 (52.9%). While, GNPS-LFT assay results were 49/117 (41.9%) and 29/60 (48.3%) blood and skin biopsy samples, respectively. Assays confirmed that the outbreaks were caused by LSDV, as shown in Fig. 3 and these results were supported by many authors (39-40).

The highest number of positives by ELISA was not unexpected and had already been reported, but the mismatch of positive and negative samples seen between ELISA, GNPS-LFT, and WB in different samples was agreed with (19). The same nonconformity of serological tests (22). That is explained by detection of different anti-capripoxvirus antibodies with different test. The strong correlation between GNPs-LFT developed and WB modified indicates the suitability of GNPs-LFT for the detection of the LSDV-specific neutralizing antibody was agreed with (40). GNPs- LFT assays, several hundred samples, are needed for validation.

## CONCLUSION

Rapid Gold nanoparticles-lateral flow test and IC tests showed detection limits nearly similar to those obtained by ELISA for detection of different viral diseases (FMDV, & LSDV) in different animals. The gold nanoparticles-lateral flow test has the potential to solve many diagnostic problems related to animal health. GNPs-LFT is used for FMDV and LSDV antibodies to detect viruses at the field site of suspicion. The development of an on-field one-site GNPs-LFT to detect FMDV and LSDV infection in blood, biopsy tissue, and milk samples within 15-30 min. The developed strip is economical, time-saving, and sensitive and does not demand any professional expertise. Our study provided IC and GNPs-LFT rapid tests, which provide numerous opportunities for accurate and timely diagnosis.

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