Development of a rapid test for detection of foot-and-mouth disease virus specific antibodies using gold nanoparticles

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Abstract Foot and mouth disease is an economically important transboundary disease of wildlife and cloven hoofed animals including ruminants. In the absence of vaccination, detection of antibodies against structural proteins (SPs) of foot-and-mouth disease virus is an indicator of infection. In the present study, a rapid dot blot assay using gold nanoparticles was developed for the detection of antibodies against SPs of FMDV. Commercially available FMD vaccine was used as a source of FMD antigen. After the synthesis of gold nanoparticles (GNPs), the GNP-dot blot assay was tested and was found very sensitive, as the detection of antibody was up to 10^{-4} of serum dilution. The GNP-dot assay was found specific as it didn't give dot with normal horse sera, fetal bovine sera and neonatal bovine calf serum samples when tested at 10^{-3} working dilution. When 30 serum samples from post-vaccinated buffaloes were tested at dilution of 10^{-3} , all the samples were found positive with the variable intensity of dot. The synthesized GNPs and conjugated GNPs with antibody were characterized for their absorption limit, for their stability and for their approximate size. These characterized

conjugated and non-conjugated GNPs were also analyzed by Transmission electron microscopy and Scanning electron microscopy. The GNP dot blot assay developed in this work gave promising results using vaccine antigen and can form an important tool for rapid diagnosis of FMD in FMD free countries, zones free of FMD and during last stage of FMD eradication when FMD vaccination will be withdrawn.

Keywords Foot-and-mouth disease virus · Gold nanoparticles · GNP-dot blot · Transmission electron microscopy · Scanning electron microscopy

Introduction

Foot-and-mouth disease (FMD) is an economically significant, clinically acute and contagious vesicular disease of cloven hoofed animals including domestic ruminants, pigs, deer, antelopes, camel and more than seventy wild life species. FMD is caused by Foot-and-mouth disease virus (FMDV) of genus *Aphthovirus* and family *Picornaviridae* [4]. FMDV is a non-enveloped virus with positive sense viral RNA consisting of a single open reading frame (ORF). FMDV has four structural and eight non-structural proteins (NSPs).

In endemic countries FMD is controlled by prophylactic vaccination at every 6 months. FMD free countries do not vaccinate against FMD. However, if FMD outbreak occurs, slaughtering of infected and in-contact animals, restriction in movement of animals and animal products is done. This is followed by emergency vaccination and sero-surveillance for detection of antibodies against non-structural proteins (NSPs). The FMD vaccines consist of FMDV SPs and devoid of NSPs which can be used to differentiate

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infected from vaccinated animals (DIVA). In the absence of vaccination, detection of antibodies against SPs is indicator of FMDV infection. Antibodies against SPs of FMDV can be detected by virus neutralization test (VNT), liquid phase blocking ELISA (LPBE) and Solid phase competitive ELISA (SPC-ELISA). Since the inactivated antigen has variable stability in LPBE, the LPBE has low specificity, SPC-ELISA has been developed [9].

SPC-ELISA is having more sensitivity and specificity. Though FMDV is economically important, there is no 'point of care' or 'pen-side test' available for early diagnosis of this disease. All available tests need sophisticated laboratories, high throughput instruments as well as skilled personnel to perform the test. Therefore, it is essential to have a field level diagnostic tool or a pen side test, which can provide rapid diagnosis, accurately, with high level of sensitivity and specificity. It should be cheap and economical so that the farmers can afford to buy such test. It should not require technical expertise to perform such test. Also, the interpretation of the test should be easy and can be analyzed with naked eye.

Gold nanoparticles (GNPs) based diagnostic tools are one of the tests which fulfill above requirements. The colloidal GNPs have been used for centuries by artists due to the vibrant colors produced by their interaction with visible light. The optical property of GNPs is exhibited through Surface Plasmon Resonance (SPR). This property of GNPs can be exploited for the detection of antibody–antigen complex. These properties are tunable by changing the size and shape of GNPs. Also when a substance is converted to nano scale, their surface to mass ratio is increased, therefore more number of antibodies can be conjugated and also the chance of their interaction with antigen increases and thus resulting in the increased sensitivity of the test. In this work, a GNP based dot blot test using FMDV vaccine antigen was developed which can detect antibodies against the SPs of FMDV present in the field samples.

Materials and methods

Serum samples

30 serum samples were collected from buffaloes which were vaccinated more than two times by FMD vaccine. These animals were vaccinated with conventional trivalent FMD vaccines consisting of antigens from FMDV serotypes O, A and Asia 1. Out of these, 15 serum samples were collected randomly from Haryana state of India and remaining 15 serum samples were obtained from regional FMD lab, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, India. Positive and negative

controls were considered by initially testing with Liquid phase blocking ELISA (LPBE) by using kit provided by Project Directorate Foot and Mouth Disease, India. Sample that scored strong positive in LPBE was taken as positive control and that scored negative as negative control in dot-blot assay.

Preparation of antigen

Trivalent commercial vaccine having antigens from O, A and Asia-1 FMDV serotypes was used as source of antigen. Vaccine oil was removed so that vaccine can be used as an antigen. Briefly, vaccine was centrifuged at 10,000 rpm for 15 min. After removal of supernatant, the filtrate was kept at -20°C for overnight. The unfreezed oil was removed. After removal of oil, commercially available FMD vaccine was used as antigen.

Anti-bovine antibody

The anti-bovine IgG (Sigma-Aldrich, USA) was reconstituted in Phosphate buffer. The concentration of the anti-bovine IgG was estimated by using Bio-Rad protein assay kit as per manufacturer's instructions.

Synthesis of GNPs

The GNPs were synthesized by the chemical reduction method [19]. Before use, all the glasswares and the magnet of magnetic stirrer were washed with the help of aquaregia mixture. After 1 h, glassware were treated with triple glass distilled water and kept at least for 3 days for all the residual fumes to go. In this aquaregia treated beaker, 50 ml distilled water was taken and then gold chloride stock solution was added to dilute it to 0.1%. After this, the solution was kept on heating magnetic stirrer. When the solution started boiling, sodium citrate solution was added slowly. The color change was observed for change from white to purple, then to red and finally to dark wine red. The color was allowed to stabilize for 10 min with boiling. After cooling, the GNPs were stored at 4°C .

Conjugation of GNP with purified antibody

The pellet of GNP was suspended in 10 mM Phosphate buffer (PB) pH 7.4. GNPs were kept on shaking incubator for 2 h after adding Tween 20 (Sigma-Aldrich, USA). Purified IgG were added into the solution at the concentration of 200 $\mu\text{g/ml}$. This was mixed vigorously and kept in the shaking incubator at 37°C for 2 h. The unconjugated IgG were removed by centrifuging at 10,000 rpm at 4°C for 20 min. The pellet of GNP-Antibody was re-suspended in 10 mM PB, pH 7.4.

Characterization of non-conjugated GNPs and GNPs conjugated with antibody

The characterization of GNPs, before and after conjugation was done on the basis of their absorbance by UV–Vis spectrophotometry (BMG labtech, SPECTROstarnano ELISA reader). By measuring the Zeta-potential and approximate size of the GNPs before and after conjugation was done using Zetasizer (Malvern, Zetasizer nano ZS 90) in Guru Jambheshwar University of Science and Technology, Hisar, India. The samples were sent for Transmission electron microscopy (TEM), Scanning electron microscopy (SEM) and Fourier transformed infrared spectroscopy (FTIR) analysis for determination of exact size and the shape of GNPs. For TEM and SEM analysis, the samples were outsourced to the Sophisticated Analytical Instrumentation Facility and Sophisticated Test and Instrumentation Centre, Cochin, Kerala, India (SAIF-STIC, Cochin).

Standardization of dot-blot assay

The vaccine antigen was quantified using NanoDropTM spectrophotometers (Thermo ScientificTM, USA). The test was performed on the Nitro-cellulose membrane (NCM) and Nylon membrane with porosity 0.45 μ . The vaccine antigen was tested at various dilutions and volumes to standardize the dot-blot assay. Briefly, the vaccine antigen was diluted 1:100 and 1 μ l vaccine antigen was coated on nitrocellulose membrane. Incubation was done for 1 h at room temperature. Blocking was done by NHS and Bovine serum albumin (BSA). This was followed by incubation for 1 h at room temperature. After washing with PBS the membrane was allowed to dry. Serum sample was diluted 1:100 followed by addition of 1 μ l serum sample and incubation at room temperature. After incubation, membrane was washed with PBS (Phosphate buffered saline). After drying, anti bovine IgG-HRPO conjugate was added and kept for incubation at room temperature for 1 h. This was followed by washing drying and addition of Diaminobenzidine (DAB) as substrate. Before using DAB, hydrogen peroxide (H₂O₂) was added into it at a final concentration of 0.01%. After 15 min, the membrane was observed for the appearance of visible color.

Standardization of dot-blot assay using GNP conjugation

After standardizing dot-blot assay, dot-blot assay using GNP was standardised using the same vaccine antigen and serum sample. Briefly, 0.8 μ l vaccine antigen was coated on nitrocellulose membrane and dried under table lamp for 15 min. The remaining sites on the membrane were blocked by two percent NHS (Sigma-Aldrich, USA) and

allowed to dry for 15 min. This was followed by washing with PB and drying under lamp. Then 1 μ l of serum sample was added and again the membrane was allowed to dry under table lamp for 15 min. This was followed by washing and drying under lamp for 15 min. Subsequently, 0.7 μ l GNP conjugated with anti-bovine IgG was added. After washing with PB, the membrane was dried and was observed for the appearance of red dot in case of positive sample and no dot in negative sample.

Sensitivity and specificity of the assay

The analytical sensitivity of the assay was measured by testing the serum samples in tenfold serial dilution. The 30 samples collected from field were also tested by the GNP-dot blot assay. The analytical specificity of the assay was detected by testing FBS, NBCS and NHS.

Results

Synthesis and characterization of GNP

The GNPs synthesized by the chemical reduction method when characterized with UV–Vis spectroscopy for their absorption spectrum showed the maximum absorption at 520 nm (Figs. 1a, 2). After the conjugation of GNPs with anti-bovine IgG, the absorption was increased to 524 nm (Figs. 1b, 2). When the stability of GNPs before and after conjugation was compared, GNPs conjugated with antibody were found more stable with the zeta potential of -12.4 mV than GNPs with the zeta potential of -9.43 mV. In TEM analysis, the size range of GNPs before conjugation was 20–28 nm, whereas after conjugation the size range was increased to 25–33 nm (Fig. 3). In SEM, the change in the color intensity (wine red) of the GNPs after conjugation in comparison to normal GNPs was observed.

Immuno dot-blot assay

The Immuno dot-blot assay was standardized for better quality results by using different concentration of antigen, anti-bovine IgG-HRPO conjugate and by using different blocking agents at various incubation time. The best result was found with the antigen dilution of 1:100 and antibody (serum) dilution of 1:100. When bovine serum albumin (BSA) was used for blocking, a bluish-purple dot was appeared whereas with the NHS as blocking agent no dot appeared.

Fig. 1 UV-visible spectroscopy absorbance recorded for GNPs at 520 nm (a) and conjugated GNPs at 524 nm (b)

Fig. 2 OD values of non-conjugated GNPs and conjugated GNPs. Non-conjugated GNPs showing peak at OD value 520 nm. Conjugated GNPs showing peak at OD value 524 nm. The horizontal lines are joining lines of OD values of non-conjugated GNPs and conjugated GNPs at same wavelength

Standardization of dot-blot assay using GNP conjugation

Antigen dilution determined by dot-blot assay with anti-bovine IgG-HRPO conjugate was then used to standardize the dot-blot assay with GNP conjugation and as a negative control and for blocking NHS was used. Ten fold serial dilutions of antibody (serum) were used to perform the dot-blot assay with

GNP conjugation. At 1:1000 the dot was strong positive and clearly visible. Hence, 1:1000 was chosen as the working dilution for sample. The limit of detection of assay was measured up to 1:10,000 dilutions of serum sample (Fig. 4a). The original undiluted serum sample gave a faint reaction, while increasing the dilutions (tenfold) to certain limits; the reaction became more clear and bright. The GNP Dot-blot assay was also tested for its analytical specificity by using NHS, FBS and NBCS. The assay was found specific as no color developed while testing NHS, FBS and NBCS (Fig. 4b).

Testing of post vaccinated samples

A total of 30 serum samples collected from field after vaccination against FMD were also tested by GNP dot-blot assay. All the thirty samples were found positive with variable intensity of blot. Few samples scored weak positive with faint bands Column 1-Row 2, 3 and 4; Column 2-Row 1 (Fig. 5). Out of 30 serum samples tested, 16 have been shown in Fig. 5.

Discussion

In ruminants, irrespective of vaccination status an asymptomatic, persistent infection can be established following recovery after FMDV infection. The existence of the

Fig. 3 TEM; Non-conjugated GNPs with average size of 20–28 nm (a) and conjugated GNPs with average size of 25–33 nm (b)

Fig. 4 Immuno dot-blot assay showing ten fold dilution of antibody. *N* neat serum (a) dot-blot assay showing –ve result with NHS, FBS, NBCS and +ve control (b)

Fig. 5 Field samples showing positivity in immuno dot-blot assay. Positive and negative controls were positive and negative, respectively. *PC* positive control, *NC* negative control, *NHS* normal horse serum, *NBCS* neonatal bovine calf serum, Column 1–Column 4 and Row 1–Row 4 are field samples

carrier state has a significant impact on control and eradication programs as these carriers pose a threat for new outbreaks of FMD. The duration of carrier status varies between the species, the maximum reported duration of the carrier state in African buffalo, cattle, sheep and goats are 5, 3.5 years, 9 and 4 months, respectively [1, 5].

In OIE Terrestrial Manual, virus neutralization test, liquid-phase blocking ELISA (LPBE) and solid phase competitive ELISA (SPCE) are three serotype specific serological tests for detection of antibody against the SPs of FMDV but these tests require sophisticated laboratories, therefore, it costs a lot and also they consume a lot of time to perform. Hence, these cannot be used at the door step of the farmer as a pen-side test. It is therefore important to have a test which can be simple, robust, sensitive and cheap so that it can be used at the farmer's door step.

The biosensor is an analytical device in which ligand-specific bio-recognition element, such as antibody, enzyme, receptor, nucleic acid, aptamers, peptide/protein, lectin, cells, tissue or whole organisms are immobilized on a sensor surface and then integrated with a signal conversion unit or transducer [2]. Due to the different physico-chemical properties such as electrochemical [12], chemical luminescence [14] and optical [18], nano materials has

contributed significantly for the development of the biosensor technological approach [17].

Ding and co-workers [6] has evaluated usefulness of a bio-barcode assay (BCA) derived highly sensitive GNP improved immuno-PCR technique for detecting clinical samples of FMDV. Also, GNP based test have been developed to detect antibodies against FMDV NSPs [7]. However, a GNP based test to detect antibodies against FMDV SPS is lacking. Therefore, keeping the requirement of a pen-side test for the diagnosis of anti-FMDV antibodies irrespective of the serotype of FMDV and by using the whole FMDV antigen, a GNP based rapid diagnostic test, to detect anti FMDV SPS antibodies was developed in the present study.

The different approaches for the synthesis of GNP includes Turkevich method, Burst method, Perrault method, Martin method, Navarro method [10], Sonolysis method [3], Block copolymer method and green synthesis method [8, 13, 15]. In the present study, the GNPs were synthesized by chemical method using trisodium citrate as the reducing agent to reduce the gold salt tetrachloroaurate as suggested by Turkevich [19]. The changes suggested by Frens in 1970 were followed to obtain the variation in the size of GNPs. After synthesis, GNPs were characterized for their size by

different techniques such as Zeta-sizer, TEM (Transmission electron microscopy) and SEM (Scanning electron microscopy). The results were comparable with the earlier reports as published by Patel and colleagues [11].

The size of GNPs was determined by measuring the diameter of colloidal gold nanoparticle in TEM images. The average diameter of whole GNPs were in the range of 20–28 nm with very few particles of higher and lower size distribution. Due to repulsion among the negatively charged citrate layer from each other, gold colloidal stayed in monodispersed state which accounts the probe preparation and generation of color signal in chromatographic strip test. Moreover, the TEM images show that most of the Gold nanospheres are round or spherical in shape. Spectrometry is another important aspect for characterization of gold nanoparticles. With increase in particle size, the absorption spectra are related to the size distribution range. Generally, gold nanospheres display a single absorption peak in the visible range between 510 and 550 nm, because of SPR and heavy absorption of visible light at 520 nm. This gives brilliant red color to GNPs which varies according to their size. The colloidal gold synthesized in this study showed heavy absorption at 520 nm (OD 1.3572).

The particle size, size distribution as well as for zeta potential measurement for both conjugated and non-conjugated GNPs, zeta study was conducted. In transmission electron microscopy (TEM) study, images show particles with lower and higher size range. Distribution study, peak number and peak area gives important explanation for zeta-potential. The affinity purified IgG dissolved in PBS was desalted by dialysis and also by using centricon. The quantification of IgG was performed using the Bicinchoninic acid (BCA) protocol [16]. The GNPs were conjugated with IgG by electrostatic binding without using any linker molecule [20]. The conjugation was analyzed by using different techniques, such as UV–Vis spectrometry. After conjugation was completed, the absorption was increased from 520 to 524 nm. This showed that conjugation was performed properly. It was also confirmed by sodium chloride precipitation method. By doing this, conjugated GNPs remained in the colloidal state but the un-conjugated GNPs clumped and precipitated immediately. It was also confirmed by doing the TEM and SEM electron microscopy analysis. In TEM, the size of conjugated GNPs was increased and on the other hand, the SEM showed the change in the color intensity of the GNPs after conjugation as compared with normal GNPs.

The GNP based test was found to be very sensitive. The bright pink dot appears when the antigen and antibody concentrations are optimal. In the present study the antibody detection limit was found up to 10^{-4} dilution of serum. Initially, faint dot appeared which became clearly visible at the antibody dilution of 10^{-3} and on further dilution again the dot became faint. Faint dot while testing

with neat serum could be due to antibody excess, thus hindering the interaction between antigen and antibody. Analytical specificity of the test was also tested by using NHS, FBS and NBCS. No visible dot with NHS, FBS and NBCS were observed, although, it gave positive result with BSA. It could be due to binding of anti-bovine IgG with BSA. All the 30 serum samples collected from field after vaccination against FMD were found positive. As India is endemic for FMD, unvaccinated infected samples were unavailable; therefore, vaccinated samples were tested to develop this test. As a proof of principle, this test was able to detect anti FMDV SP antibodies in vaccinated animals. In future, we aim to procure samples from unvaccinated infected animals and further validate the assay.

Since the GNP-dot assay developed in present study detects antibodies against FMDV irrespective of serotype, hence can be used for the screening of animals. This test is useful when serosurveillance is done in countries free of FMD, where vaccination is not followed and also during last stage of FMD eradication when vaccination is withdrawn. Moreover, based on this principle IgA ELISA which detects FMDV serotype specific anti-SPs antibodies from the nasal and saliva of carrier can also be developed to detect the FMDV carriers.

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