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Development of point-of-care lateral flow immuno-chromatographic assay for foot and mouth disease diagnosis

M. Anil[1,2*], P. Navaneetha[1,2], P. V. Janardhan Reddy[2], M.Prudhvi Chand[1,2] A. Vijay Kumar[3], P. B. Kavi Kishor[6], K.R.S. Sambasiva Rao[5] P. Rathnagiri[1,2,4*]

[1]Department of Medical Microbiology, Genomix Molecular Diagnostics Pvt. Ltd., Hyderabad-500072, Telangana, India
[2]Department of Veterinary Microbiology, Genomix CARL Pvt. Ltd., Pulivendula-516390, Andhra Pradesh
[3]Department of Veterinary Public Health and Epidemiology, P.V.N.R Telangana Veterinary University Hyderabad 500 030, India
[4]Genomix Biotech Inc, Atlanta, GA 30345, USA
[5]Department of Biotechnology, Mizoram University (A Central University), Aizawl-796004, Mizoram, India
[6]Department of Biotechnology, Vignan's Foundation for Science, Technology and Research, Vadlamudi, Guntur 522 213, India
*Corresponding author: anilbio2010@gmail.com

Abstract

Foot and mouth disease (FMD) is highly infectious, contractable disease of ungulates and is associated with cattle which causes high economic losses in livestock industry. Virtual control of FMD requires specific and sensitive point-of-care diagnostic tools to eradicate the disease spreading. So far, the diagnostic tools used for Foot and Mouth Disease Virus (FMDV) are molecular based assays which are more expensive. The main objective of this research study is to develop and evaluate point-of-care test for rapid detection of FMDV in animals. For this study, a highly specific and sensitive FMD non-structural protein (NSP) antibody rapid immuno-diagnostic assay was developed using recombinant 3ABC (r3ABC) protein of FMDV for the detection of antibodies against FMDV and compared with commercial ELISA. The FMDV *3ABC* gene was cloned into pET28a (+) vector and the gene product was expressed in *E. coli* BL21 cells. The expressed r3ABC protein was detected by SDS-PAGE analysis which resulted in a protein band with approximate molecular weight of 60 kDa. Purified r3ABC antigen was used as a detection reagent in rapid Lateral Flow assay (LFA). The diagnostic Assay was performed with 33 reference and 380 field samples and results showed that 94% sensitivity and 98.9% specificity. This study revealed that successful expression of *3ABC* gene and diagnostic assay development lead to the identification of diseased animals. It further demonstrated that LFA as potential diagnostic tool for the point-of-care diagnosis of FMDV in large herds within limited time.

Key words: Foot and Mouth Disease, Sensitivity, Specificity, Lateral Flow Assay, ELISA, Point-of-Care.

Introduction

Foot and Mouth Disease (FMD) is the most economically important disease of animals and endemic in majority of the Asian countries. FMD is a highly contagious viral disease in livestock and has serious economic impact in cloven footed animals. The disease spreads in cattle, buffaloes, goat, swine and more than 70 species including domestic and wild animals (1). FMD's causative

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agent is Foot and Mouthdisease virus (FMDV) and it belongs to the family *Picornaviridae* and genus *Apthovirus*. FMD virus exists as seven immunologically distinct serotypes O, A, C, Asia 1 and SAT 1, SAT 2, SAT 3, (Southern African Territories) and multiple subtypes in each serotype (2,3). All these serotypes are recorded so far in India since 1995 onwards whereas serotype C was not reported in India (4,5). Due to the infectious nature, FMD is classified as a list "A" disease by the World Organization for Animal Health (OIE) (FAO/OIE/WHO (1995) (6). FMD causes the lameness and vesicular lesion on tongue, teats, feet, besides huge weight loss and milk reduction. Many developed and undeveloped countries obtained FMD free status by making mass vaccination and by strict trade policy. FMDV is having RNA as genetic material and encodes various structural and non-structural proteins (7). Mostly, inactivated FMD whole virus vaccines were used for prevention of disease all over the world. This form of vaccine consists of killed viral particles and are supposed to evoke antibodies against the structural proteins of FMDV. Majority of the vaccine makers remove NSPs while producing the vaccines and animals probably produce antibodies to non-structural proteins (NSPs) (8,9,10). The animals naturally infected with the FMD virus are supposed to express NSP proteins and elicit the immune response that can be detected using a diagnostic prospective. These particular approaches of diagnostic methods are meant to differentiate the infected animals from vaccinated herds. Majority of the testing methods used for the detection of FMD infected animals were produced by a combination of NSPs 2A, 2B, 2C, 3A, 3B, 3AB, 3D and 3ABC which in-turn used to develop immuno diagnostic assays. Among all NSPs, 3ABC protein is highly immunogenic and described (11) as one with high amount of antibodies against 3ABC antigen in animal sera. Currently FMD serological diagnosis was carried out using Complement Fixation Test (CFT) and Enzyme Linked Immunosorbent Assays (ELISAs), but these assays need more time to execute and trained laboratory personnel. But, screening large number of animals in less time at field level requires point-of-care tests. At present, lateral flow assays have more importance for detection of pathogenic antigen in clinical specimens.

The main objective of the present study is production of FMDV recombinant 3ABC antigen using a bacterial expression system and evaluation of lateral flow immuno-chromatographic assay using FMDV NSPs (r3ABC) to determine the diagnostic efficacy of lateral flow immunoassay.

Materials and Methods

Production of recombinant 3abc protein of FMDV: A 1326 bp long fragment whole *3ABC* gene (from the viral nucleic acid at the repository of Genomix CARL) was cloned into pET-28a(+) vector and transformed into *E.coli* BL21 competent cells as per standard protocol (12) and grown on Luria Bertani (LB) agar plate containing 100 mg/ml kanamycin. For the expression and purification of proteins from *E.coli* BL21 cells containing pET-28a (+)/3ABC, construct was cultured overnight at 37°C in 5ml LB broth containing 100 mg/ml kanamycin. The overnight culture was transferred into 250 ml fresh LB kanamycin medium and allowed to grown till the OD reaches 0.7 to 0.8. The cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h. After the cells were harvested, the cell pellets were lysed in lysis buffer (8 M urea, 0.1M NaH_2PO_4, 10 mM Tris-HCl, pH 8.0) and the suspension was sonicated. Then the lysate was transferred into a Ni-NTA column. The column was saturated with 4-5 ml volumes of lysis buffer followed by adding 50 ml 1M imidazole and the protein was allowed to bind the column for 2 h. After binding, the lysate was passed through the column. The column was then washed with 10 volumes of wash buffer (8 M Urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl, pH 6.3) and the protein was eluted with elution buffer (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl, pH 4.5) in fractions of 500 ml. Purified protein concentrations were determined by Bradford method and protein

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concentrations were analyzed with SDS-PAGE gel electrophoresis followed by Coomassie brilliant blue staining. Protein fractions were aliquoted and stored at -80°C till further use.

Sample collection and study area: The present study was majorly conducted at the Genomix CARL Pvt.Ltd., (Pulivendula, AP, India), Dodla Dairy (Pulivendula, AP,India) and Genomix Molecular Diagnostics Pvt. Ltd., (Hyderabad, India). The third-party evaluation study was carried out at the Department of Veterinary Public Health and Epidemiology, P.V.N.R. Telangana Veterinary University, Hyderabad. Analysis of the study was carried out in 180 vaccinated bovine serum samples, 200 non-vaccinated bovine serum samples and 33 positive and 20 negative reference serum samples. The positive and negative reference serum samples were provided by P.V.N.R. Telangana Veterinary University, Hyderabad. Blood samples of approximately 6 ml volumes were drawn from jugular vein of each animal in plain vacationer tubes (BD) and serum was collected from the tubes after clotting the blood by centrifuging the tubes at 4000 x *g* for 7 minutes.

Development of Lateral Flow Assay (LFA)

Components of the LFA: The LFA test strip used in the development of immuno chromatographic assay kit contained four key components; sample pad (for sample application), nitrocellulose membrane (contains test and control lines), conjugate pad and absorbent pad. To prepare whole card, all 4 pads are assembled in a side by side manner on self-adhesive card with a backing card support. After proper assembling, the card was cut into strips, then strips were housed inside a plastic cassette. The casettes have openings on two windows at sample pad and nitrocellulose membrane where test and control lines are visible. Purified r3ABC protein, biotin BSA and protein G gold colloid were used as reagents in making lateral chromatographic assay. The nitrocellulose membrane was coated with r3ABC protein at test area and biotin-BSA was coated as control line by using Bio-jet apparatus (BioDotQuanti-200). A fiber mat containing 40 mm diameter protein G Gold Colloidal Nanoparticles (Genomix, USA) were used as detecting reagent. At last the test cassette was sealed in a moisture resistant pouch along with silica gel and dropper for sample application. The assay optimization was carried out by step-wise procedure with a reference panel of positive and negative samples. The assay execution was performed by placing test cassette on a clean surface and adding 5 ml of serum sample along with 50 ml of sample dilution buffer (1X PBS) to the sample pad. After application of sample, the sample will pass through the device along the conjugate pad, nitrocellulose membrane and at last the absorbent pad. The results were recorded within 20 min on the basis of appearance of colored line on test and control lines. The positive test results were analyzed by the presence of red or brown colored band in test and control lines, negative results by a colored band observed at only control line and invalid result by a clear band at test line and no clear line at control line (Fig. 2).

The specificity and sensitivity of FMDV lateral flow assay was evaluated by testing 33 reference control sera samples and 20 negative control samples. After testing 380 field samples (all the samples tested in duplicates) with LFA and ELISA and the LFA test was compared with the commercial FMDV ELISA assay and sensitivity and specificity of the assay was analyzed by employing the below formula.

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

Results

A fragment of 1326 bp of FMDV *3ABC* gene was cloned into the expression plasmid pET-28a (+) and transformed into *E. coli* BL21 cells for the production of r3ABC protein. The produced recombinant 3ABC protein having His-tag was purified using Ni-NTA column chromatography and during purification processes, the elutes were fractionated. The fractionated elutions resulted a

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60 kDa distinct band on SDS PAGE analysis and Coomassie brilliant blue staining (Fig.1). Purified recombinant protein was used as antigen for the LFA and validated the assay with reference sera obtained by third-party. While the positive reaction was observed with FMD infected sera samples,

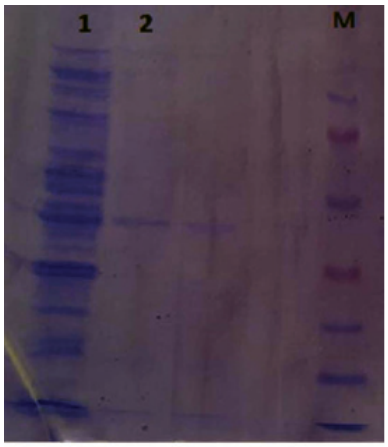

Fig. 1. SDS-PAGE gel analysis of purified recombinant FMDV 3ABC protein M. Molecular weight marker, Lane 1: Flow through lysate, Lane 2: Recombinant 3ABC protein

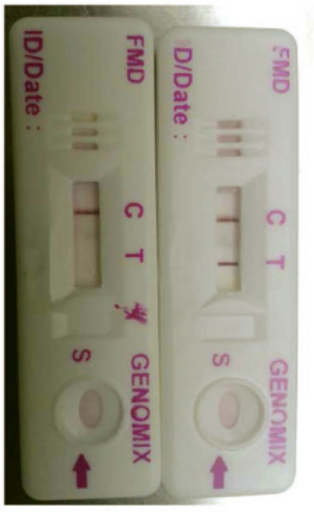

Fig. 2. LFA tests depicting the results of the assay
A – Negative; B – Positive
C – Control Line; T – Test line (with r3ABC)

the colored bands noticed at test and control lines appeared with negative sera bands at control line only. Among the 33 positive reference sera, 31 showed positive reaction with LFA and 33 with ELISA assay. Among the 20 negative controls, 19 samples showed negative results with both LFA and ELISA assay and 1 sample displayed positive with LFA. Also, 380 field samples were tested with LFA and the results were compared with the commercial ELISA results (Table 1). Among 180 vaccinated animal sera, 26 were positives with LFA and 29 with ELISA. Among the 200 non-vaccinated samples, 8 were positive with LFA and 8 with ELISA. The specificity of the LFA was 95.23% and the sensitivity was 94.28% (Table 2).

Discussion

In the current study, we have evaluated FMD r3ABC antibody detection test for rapid antibody detection in FMDV-infected animals. This FMD LFA test allows the rapid antibody detection in field and is useful for differentiating vaccinated from infected animals. FMD is a highly contagious viral disease of domestic ungulates and causes severe economic loss for livestock industry due to losses in milk production (13), and increased risk of abortion and cause of mortality among young animals. Presently, the FMDV infections are detected by virus capture in sandwich ELISA, virus isolation, neutralization assays, and PCR-based assays. These assays are reliable but need trained laboratory persons and also take more time to obtain test results (14). At present, FMD vaccination program is only the more effective medicament against the foot and mouth disease, but there is a problem in differentiating infected animals from vaccinated animals. Vaccination-based disease controlling operational program is effective in India with regular six-month vaccination programs and monitoring immuno-reactive antibody levels in the herds. Detection of infected animals from vaccinated animals is the important disease controlling measurement, NSP-based immunoassays (15) are the sensitive techniques to detect infected from vaccinated herd. For getting good results, researchers developed ELISA test

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to detect the antibodies to NSPs with Differentiating Infected from Vaccinated Animals (DIVA) strategies as per OIE recommended non-structural protein group, 3ABC polyprotein or individual proteins like 3A, 3B, 2C (OIE, 1995) (6). The similar profiling immunoassay using NSPs have been developed in India for FMDV. Previous studies (16) used individual antigens and developed an indirect ELISA to detect diseased animals from vaccinated animals. Eventually multiple antigens were used for FMD control. In South Africa, 3ABC polyprotein was used as an antigen in ELISA development for obtaining better sensitivity and specificity (17). DIVA test was developed for FMD using recombinant protein 3AB C for the surveillance of infection in vaccinated herd in India in the year 2009. Usage of DIVA test would be helpful for identification of disease-free areas in India where vaccination was performed. In India, the northern states are becoming disease-free with the regular vaccination programs and sero-monitoring. But, screening of animals with the ELISA assay for FMDV requires well-trained laboratory personnel, sophisticated labs and takes more time and high cost to perform the test. For a large number of populations, this type of test is not affordable. Therefore, it is important to have a pen-side test which can be performed at door step. The lateral flow immunoassay is a diagnostic device which incorporates immunoassay technique with rapid chromatographic principle and coating highly specific antibodies and antigens on nitrocellulose membrane. The lateral flow assay has been developed extensively to instigate immunological diagnosis of a vast number of diseases, including FMD (**18**). In the past few years, higher demand was noticed for pen-side tests with multiplex test lines which permit quick and simultaneous detection of different biological components present in the sample (19). Besides, lateral flow assay does not require specific expertise and special equipment and also no refrigeration for storage of the device (20). Hence, in the current study, LFA was evaluated with ELISA. The results illustrate that a pen-side, point-of-care LFA for the detection of FMDV antibodies to r3ABC in animals has been successfully developed. The test results

Table 1. Comparative results of FMD LFA and ELISA tests

S. No	Sample names	No. of Samples	LFA Positive	LFA Negative	ELISA Positive	ELISA Negative
1	Vaccinated sera samples	180	26	154	29	151
2	Non-vaccinated serum samples	200	8	192	8	192
3	Reference positive controls	33	31	2	33	0
4	Negative control sera	20	1	19	0	20

Table 2. Sensitivity and Specificity of FMD 3ABC Lateral Flow Assay

No. of Specimen		LFA		
ELISA	Positive	Negative		
	Positive	66	70	
	Negative	367	363	
	Total samples: 433			
Sensitivity: 66/(66+4)x100=94.3% Specificity: 363/(4+363)=98.9%				

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can be available within 15 minutes and has 94% sensitivity and 98% specificity. The newly developed LFA has high specificity and can be the most useful rapid immunodiagnostic tool to detect infected animals in vaccinated herds.

Conclusions

In the current study, lateral flow FMDV antibody detection assay has been developed and sensitivity comparable to the ELISA test was accomplished and the results could be produced with in 15 min. The LFA has the ability to give rapid results and more specificity to detect r3ABC of FMDV at pen-side. The newly developed lateral flow rapid antibody assay of FMDV NSPs was ideal for the point-of-care diagnosis, reliable, quick, easy and most preferable test to screen the vaccinated animals and can be used to control and prevent the FMDV disease eradication in endemic regions.

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