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Diagnostic application of recombinant non-structural protein 3A to detect antibodies induced by foot-and-mouth disease virus infection

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ABSTRACT

Detection of antibodies to the non-structural proteins (NSPs) of FMD virus (FMDV) is the preferred differential diagnostic method for identification of FMD-infected animals in the vaccinated population. Nevertheless, due to the observed variability in the antibody response to NSPs, the likelihood of screening or confirming the FMD infection status in animals is increased if an antibody profile to multiple NSPs is considered for diagnosis. In order to develop and evaluate an additional NSP-based diagnostic assay, in this study, the recombinant 3A protein of FMDV was expressed in *Escherichia coli* and used as an antigen for detection of FMD infection specific antibodies. At the fixed cut-off value of 45 percentage of positivity, the diagnostic sensitivity and specificity of 3A indirect-ELISA (I-ELISA) were found to be 95.7% and 96.3%, respectively. In FMD naturally infected cattle, about 85% of clinically infected and 75% of asymptomatic in-contact populations were found positive at 13 months post-outbreak. The 3A I-ELISA was further evaluated with the bovine serum samples collected randomly from different parts of the country. Furthermore, the performance of newly developed 3A I-ELISA was compared with the extensively used in-house r3AB3 I-ELISA, and the overall concordance in test results was found to be 93.62%. The r3A I-ELISA could be useful as a screening or confirmatory assay in the sero-surveillance of FMD in India irrespective of extensive bi-annual vaccination.

1. Introduction

Foot-and-mouth disease (FMD) is the most contagious vesicular viral disease of cloven footed livestock species. The disease is endemic in many parts of the world, where it causes significant socio-economic losses due to international trade restriction, livestock productivity and livelihood. In India, the 'National FMD Control Programme' (FMD-CP) is mainly implemented by adopting prophylactic bi-annual mass-vaccination of domesticated large ruminants [1]. In this context, it is essential to have information on the level of FMDV exposure in large ruminants irrespective of their vaccination status. Detection of serum antibodies against FMD virus (FMDV) non-structural protein (NSP) in vaccinated and subsequently infected animals has been used as differential marker of infection, as vaccination with purified vaccine induces antibodies only against the structural proteins of FMDV [2]. Antibody against FMDV 3ABC poly-protein has been considered to be the most reliable indicator of infection [2,3]. However, not all the infected animals can be assured to seroconvert against a particular NSP, and therefore, in an international NSP ELISA validation workshop at Brescia, Italy, it was suggested to use more than one NSP assay in order to enhance the overall sensitivity and specificity of the determination of FMDV infection status [4,5]. Considering that recommendation, immunodiagnostic assays using different NSP proteins such as, 3AB3, 3ABC, 3B, 2C, 2B have been developed and evaluated at Indian Council of Agricultural Research (ICAR)-Project Directorate on foot-and-mouth disease, Mukteswar, which oversees the FMD sero-surveillance activities in the country [6]. However, vaccine preparations, depending upon their sources, can contain traces of NSPs, which makes unequivocal detection of infection in populations which have been repeatedly vaccinated, difficult [7–9].

In cells infected with FMDV, NSP 3A has been shown to co-localize with the intracellular membrane components [10], and this membrane binding activity could be due to the hydrophobic property 3A NSP [11]. Further, earlier report indicates that most of the 3A NSP can be removed after collection of cell debris during the production of FMD vaccine [12]. These findings, form a theoretical foundation for the development of 3A NSP based immunodiagnostic assay for efficient detection of FMDV infection specific

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antibodies. In the present study, FMDV NSP 3A was expressed as a soluble recombinant protein in *Escherichia coli* (*E. coli*), and its diagnostic application to distinguish infected from vaccinated bovine population was studied by optimising an indirect-ELISA (I-ELISA). Further, we compared the performance of the developed recombinant 3A I-ELISA (r3A I-ELISA) to those of the previously validated r3AB3 I-ELISA.

2. Materials and methods

2.1. Serum samples

A panel of bovine (both cattle and buffalo) serum samples collected from naïve, non-infected vaccinated and FMDV infected animals were obtained from the serum repository maintained at ICAR-Project Directorate on FMD, Mukteswar, India. This study complied with the international standards for animal welfare.

A total of 120 serum samples collected from clinically healthy animals and found negative for anti-FMDV structural antibodies in the in-house liquid phase blocking ELISA were used in this study.

Serum samples ($n = 300$) were collected sequentially from an FMD free dairy cattle herd that was vaccinated routinely at six-month intervals with trivalent inactivated vaccine. These samples were collected at 28 and 180 days post-revaccination. Serum samples ($n = 170$) from FMD-CP areas without any report of FMD for the last eight years were also included in the study. The majority of the bovine population in the FMD-CP areas had received at least eight rounds of vaccination. Samples ($n = 60$) were also collected at 21 days post-vaccination (dpv) from cattle that were used in FMD vaccine potency studies. All these 530 serum samples collected from vaccinated non-infected animals along with the serum samples from a naïve bovine population ($n = 120$), were used for determination of cut-off value and diagnostic specificity of 3A I-ELISA.

Bovine serum samples ($n = 1000$) from clinical cases of FMDV field outbreaks were also included in this study. These samples were collected at different time points during the outbreaks, ranging from one-week to nearly 13-months post-index case. Furthermore, serum samples ($n = 96$) were also collected sequentially until 13-months post-index case from cattle ($n = 16$) that remained asymptomatic during the course of an outbreak, but remained in-contact with clinically infected animals. The serum samples from asymptomatic bovines were used to find out the efficiency of r3A I-ELISA in detecting viral activity in the absence of clinical signs.

Bovine serum samples ($n = 1800$) that had been collected at random from different parts of the country were also analysed in r3A I-ELISA in order to determine the prevalence of 3A antibodies in bovines.

2.2. Molecular cloning, expression and purification of recombinant 3A NSP

2.2.1. Construction of r3A gene expression vector

The 3A coding region (459 bp) of FMDV O IND R2/1975 virus was amplified by RT-PCR. Oligo $d(T)_{20}$ primer (Invitrogen, CA,USA) was used for cDNA synthesis, while 3A-F (5′-**CGCGAACAGATTGGAGGT**ATCTCAATTCCCTCCCAAAAATCC-3′) and 3A-R (5′-**GTGGCGGCCGCTCTATTA**TTCAGCTTGTGGTTGTTCCTC-3′) primers were used for PCR amplification. In order to perform enzyme free cloning with the pETite™ plasmid vector (Lucigen, Middleton, USA), the upstream and downstream primers contained an 18 nucleotide sequence (bold and underlined) that add sequences identical to the ends of the cloning vector adjacent to the cloning site. Subsequently, the agarose gel purified 3A amplicon was mixed with the pETite N-6xHis-SUMO plasmid vector and transformed directly into chemically competent HI-Control 10G *E. coli* cells (Lucigen, Middleton, USA). Homologous recombination within the host *E. coli* cells seamlessly joined the 3A insert with the vector to generate the recombinant plasmid pETite-N-His-SUMO-3A. The positive recombinant clones were re-transformed into HI-Control BL21 (DE3) cells (Lucigen, Middleton, USA) for expression of the cloned 3A gene from the T7 promoter. Nucleotide sequence confirmed positive clones were subsequently subjected to protein expression screening.

2.2.2. Purification and immunological characterisation of r3A protein

Expression and affinity purification of the recombinant 6xHis-SUMO-3A fusion protein was performed according to a method described earlier [13]. The purity of the r3A protein was assessed by SDS-PAGE [14]. Differential immunoreactivity of the purified r3A protein was analysed by Western blot using the bovine convalescent serum (BCS) collected from known FMDV infected cattle and naïve serum diluted 1:100 in blocking buffer.

2.3. Development of recombinant 3A I-ELISA

During the development of the recombinant 3A I-ELISA, the concentrations of the various components of the assay were optimised by the checker-board titration method. Briefly, 96-well, flat-bottom polystyrene plates (Nunc, Denmark) were coated with 50 μl of purified recombinant 3A protein diluted in carbonate-bicarbonate buffer and incubated at 4 °C overnight. Plates were washed three times with PBS containing 0.05% Tween-20 (PBST) and test serum samples, positive and negative control sera were diluted (1:20) in the dilution buffer (PBST, 3% skim milk, 10% normal horse serum and 0.02% *E. coli* sonicate) and 50 μl were added to the plates in duplicate. The positive and negative sera were included as internal controls, while dilution buffer without any serum was included as a conjugate control to determine any background activity. After incubating the plates at 37 °C for 1 h, the plates were washed with PBST, rabbit anti-cow immunoglobulin/HRP conjugate (DAKO, Denmark) diluted 1:2000 in the dilution buffer (final volume 50 μl) was added, and the ELISA plates were incubated at 37 °C for 1 h. Finally, 50 μl of substrate solution containing orthophenylene diamine/hydrogen peroxide was added and allowed to stand for 12 min for colour to develop. The reaction was stopped by using 1 M H_2SO_4 (50 μl/well). The optical density (O.D) values were measured at 492 nm using the ELISA plate reader (Tecan, Switzerland).

The corrected mean OD values of the positive control (mOD_{Pos}), the negative control (mOD_{Neg}), and the test samples (mOD_{Sample}) were determined after subtracting the mean OD value of the background control wells (mOD_{Bg}). The OD for each test serum sample was expressed as a percentage of the positive control using the following formula:

$$\text{Percent of positive control (PP)} = \left[mOD_{Sample}\right] \times 100 \Big/ \left[mOD_{POS}\right]$$

2.4. Determination of the precision of r3A I-ELISA

For the precision analysis, coefficients of variation (CVs) were calculated based on the PP values from intra-plate replicates (four replicates per sample), and inter-plate replicates (three plates per day), and inter-day replicates (between five different days) of five