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Immunodiagnosis of foot-and-mouth disease using mutated recombinant 3ABC polyprotein in a competitive ELISA

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Differentiation of infected from vaccinated animals (DIVA) is essential for effective control of foot-and-mouth disease (FMD) by vaccination. The antibody response against FMD viral non-structural proteins (NSPs) has been used widely for this purpose. Among all the NSPs, the 3ABC polyprotein has been recognized as the most appropriate indicator for DIVA. In this study, mutated full-length 3ABC polyprotein was expressed in a prokaryotic system and monoclonal antibody against the recombinant protein was developed. A competitive ELISA (C-ELISA) for DIVA was standardized for different species of livestock animals using recombinant 3ABC and monoclonal antibodies. The diagnostic sensitivity and specificity of the assay were estimated by testing a panel of known serum samples consisting of sera from naive, vaccinated and infected animals as 86.9% with 66.4–97.2 (95%) confidence interval and 97% with 89.6–99.6 (95%) confidence interval respectively at 40% inhibition cut-off. The assay was validated further by testing sera from different livestock species collected at random from different parts of the country. The assay will provide a common method for testing sera from different species of livestock and wild animals. The C-ELISA is a sensitive and specific DIVA assay for FMD and can be used as a method for FMD control programme with vaccination.

1. Introduction

Foot-and-mouth disease (FMD) is a highly infectious and contagious disease of cattle, buffalo, pig, sheep, goat and wild ruminant species including elephants. There are seven distinct serotypes of FMD virus (FMDV), designated as O, A, C, South African Territories (SAT) 1–3 and Asia1. The economic impacts of FMD reflect mainly on production losses while the indirect losses are associated with the restrictions of international and regional trade. To date, the adopted policies by FMD free nations are to prevent this infection by destroying "infected and in-contact" animals together with a ban on the import of animal and animal products from the endemic countries. Slaughtering large number of animals is not economical hence the consensus for emergency vaccination using inactivated viral antigens in preference to testing and slaughter is increasing. Accordingly "vaccinate-to-live" policy is now emerging as a realistic alternative to large scale slaughter for control of the disease (Parida, 2009), at the same time in endemic countries, control by vaccination is a practical option. However, vaccination of susceptible herds raises some other critical issues such as differentiation of infected from vaccinated animals (DIVA) and the development of carrier animals due to vaccination (Uttenthal et al., 2010) hence active surveillance of the disease is required to control the disease. Serology helps in retrospective diagnosis and surveillance of FMD where animals are screened with very sensitive enzyme linked immunosorbent assay (ELISA) which detects antibodies against the structural proteins of FMDV. Vaccination with inactivated vaccines elicits antibody response against the structural proteins of the virus similar to the infection, making discrimination of infected and vaccinated herd difficult. To overcome this problem, different methods have been proposed such as virus isolation, reverse transcriptase-polymerase chain reaction (RT-PCR), virus neutralization test, mucosal IgA ELISA and non-structural proteins (NSPs) based ELISAs. Among all these methods, NSPs based ELISAs are the most promising because of their high sensitivities and specificities. Purified preparations of vaccines consist of inactivated virions which almost exclusively induce antibodies against the structural proteins and not to the NSPs (Clavijo et al., 2004a). These purified FMD vaccines can be considered as the marker vaccines for DIVA (Parida, 2009). Among all the NSPs, detection of antibodies against 3ABC polyprotein of FMDV has been shown to be early and reliable indicator of infection, since this polyprotein is cell associated

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and does not come in antigen fragment during vaccine purification (Bergmann et al., 1993; Mackay et al., 1998b).

The aim of this study was to develop and validate a competitive ELISA (C-ELISA) for DIVA using recombinant mutated 3ABC polyprotein and monoclonal antibodies (mAbs) which can be used for wide range of species.

2. Materials and methods

2.1. Serum samples

A panel of the sera was prepared as per the standard guidelines for standardization and statistical validation of immunodiagnostic assays (Jacobson, 1998; Parida et al., 2007). The serum samples from FMD naive, vaccinated and infected animals were obtained from the serum repository maintained at Project Directorate on Foot and Mouth Disease (PD-FMD), Mukteswar, India. A total of 3465 bovine, 128 pig, 42 sheep and 159 goat serum samples were included in the study. The status of 120 bovine samples was confirmed by testing in 3ABC antigen based commercial indirect ELISA kit (Svanovir FMDV 3ABC-Ab ELISA Kit, Svanova Biotech, Sweden), while rest of the sera were tested in house recombinant 3AB indirect ELISA (Mohapatra et al., 2011) and in house liquid phase blocking ELISA (LPBE). This study complied with the international standards for animal welfare.

2.1.1. FMD negative bovine sera

Bovine sera of known negative status ($n = 55$) were included in the panel. Sera were collected at zero day from 27 animals used in FMD vaccine potency testing. Four adult bovine cattle sera of FMD free origin were procured commercially (Hyclone Laboratories, Logan, UT, USA; Sigma–Aldrich, St. Louis, MO, USA; Life Technologies, NY, USA and PAA Laboratories, Ontario, Canada). Another 24 sera were collected from naive animals from a farm having no history of FMD in last five years and located at an isolated geographical location.

2.1.2. FMD positive bovine sera

Bovine sera of known to be positive for FMD ($n = 65$) were included in the panel. Bovine sera ($n = 23$) from cattle and buffalo with clinical disease were obtained from the serum repository. These animals were found to be infected with either FMDV serotypes O, A or Asia1 as confirmed by virus isolation and neutralization assays. Serum samples ($n = 42$) were collected from cattle with a recent history of FMD and included in the panel. Sera from challenged animals were obtained from the commercial organization engaged in FMD vaccine potency testing. These samples were collected from four cattle on 0 days post-infection (DPI), 3 DPI, 5 DPI, 14 DPI and 21 DPI to assess the earliest detection limit of the ELISA.

2.1.3. FMD pre- and post-vaccinated bovine sera

Bovine sera ($n = 1150$) were obtained from the areas where FMD control programme (FMD-CP) with vaccination has been implemented and animals were immunized (at least 10 vaccinations/animal) with different commercial vaccines available in the country. Out of these, 694 samples were collected immediately before the vaccination and another 456 samples after one month post vaccination. Bovine sera ($n = 2175$) collected at random from different parts of the country were included in the panel for validation of the assay.

2.1.4. Sera from other livestock animals

Sera from goats ($n = 159$) and sheep ($n = 42$) were obtained from the FMD endemic regions where small ruminants are usually not vaccinated. Samples were classified as FMD negative or positive based upon the FMDV structural protein and NSP responses in LPBE and recombinant 3AB3 indirect ELISA respectively (Ranabijuli et al., 2010). Pig sera ($n = 128$) were obtained from the serum repository of which 22 samples were collected from a farm which had recent history of FMD outbreak. The clinical samples collected from the farm were confirmed for FMDV by viral antigen trapping ELISA and multiplex PCR. The remaining of the samples were collected from healthy animals with unvaccinated status.

Table 1
List of primers with restriction enzyme site reorganization for amplification of whole 3ABC gene along with inserted site directed mutagenesis.

S. No.	Primer name	Primer sequence	No. of bases
1	3ABCF	5′ TTC AAG GAT CCA ATT CCT TCC CAA AAG	27
2	3ABCR	5′ TGT CAG CGG CCG CGT GGT GTG GTT CGG	27
3	3C163R	5′ CTC CTC CGC CGT AGC CAG CCT TA	23
4	3C163F	5′ TAA GGC TGG CTA CGG CGG AGG AG	23
5	3C46F	5′ CGT ACC TCG TTA TCT TTT CGC	22
6	3C46R	5′ TGC GAA AAG ATA ACG AGG TAC G	22

2.2. Virus strain and 3ABC cDNA template

The supernatant from baby hamster kidney (BHK-21) cell monolayers infected with IND 491/1997 (Asia1 serotype) FMDV isolate was used for RNA extraction for 3ABC gene amplification. RNA was extracted using Qiagen RNA easy kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The extracted RNA was quantified in Nanodrop (Thermo, Wilmington, DE, USA) and reversed transcribed using oligo dT reverse primer (Qiagen, Hilden, Germany) and M-MLV reverse transcriptase enzyme (Promega, Madison, USA) as described earlier (Mohapatra et al., 2011). The synthesized cDNA was stored at −20 °C till further use.

2.3. Primers

A set of six primers were designed for amplification of the full length 3ABC gene with substitution of two key amino acids in the protease active sites of $3C_{Pro}$. The amino acid cystedine-163 (GGC) and histidine-46 (CAT) were substituted by glycine-163 (TGC) and tyrosine-46 (TAT), respectively in $3C_{Pro}$ gene by site directed mutagenesis. The details of primers along with their amplification target are listed in Table 1. Primers 3ABCF and 3ABCR were modified at 5′ ends to insert recognition sites for restriction enzymes *Bam*H 1 and *Not* 1 respectively. All the primers were synthesized by the Metabion (Martinsried, Germany).

2.3.1. In vitro amplification of the 3ABC gene

The full length 3ABC gene with amino acid substitutions was obtained by an overlap PCR technique as described previously (Ho et al., 1989). The 3ABC gene was amplified in three segments designated as segment I, II and III. Three fragments were combined in the subsequent fusion reactions by PCR to obtain the full length 3ABC gene. Briefly, segment I was amplified using 2 μl cDNA, 5 mM each of 3ABCF and 3C46R primers, 1.5 mM $MgCl_2$, 5 mM dNTPs, and 1 U Pfu polymerase enzyme (Fermentas, Ontario, Canada) in PCR buffer. The reaction mixture was placed under initial denaturation at 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min. The DNA extension was continued for another 10 min. Segment II was amplified from the cDNA using the 3C46F and 3C163R primers and reaction was placed under initial denaturation of 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and final extension for 10 min. Similarly, segment III was amplified using 3C163F and 3ABCR primers