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Recombinant non-structural polyprotein 3AB-based serodiagnostic strategy for FMD surveillance in bovines irrespective of vaccination

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A B S T R A C T

In India, the proportion of bovines vaccinated against foot-and-mouth disease (FMD) is increasing since the implementation of the Government supported 'FMD Control Programme', and non-structural protein (NSP)-based serological assays for discriminating between antibodies induced by infection or vaccination (DIVA) could be useful. The FMD virus NSP 3AB was expressed in a prokaryotic system and an indirect ELISA ($r3AB_3$ I-ELISA) was developed and validated as a screening assay for detecting virus in vaccinated bovines. The diagnostic sensitivity of the assay was estimated to be 96%, while the diagnostic specificity varied between the naïve and vaccinates as 99.1% and 96.4%, respectively. This assay could detect antibodies to 3AB (3AB-Ab) from 10 to as late as 900 days post-infection in cattle infected experimentally. The "in-house" assay demonstrated higher sensitivity than a commercial 3ABC ELISA kit particularly with samples obtained from the late stages of infection. Transient post-vaccinal 3AB-Ab response could be detected in one of the three commercial vaccines during the six-month vaccination regimen, which emphasizes the fact that for a DIVA-compatible diagnostic strategy to be a realistic option, all vaccines need to be quality checked for the NSP content.

1. Introduction

Foot-and-mouth disease (FMD) poses a serious threat to the international trade in livestock products. Preventive vaccination combined with surveillance has been preferred over culling as the principle means of FMD control in India. The population of multiply-vaccinated bovine in India is expanding since the launch of the Government endorsed 'National FMD Control Programme' (FMD CP) in 2003. Monitoring virus circulation by a discriminatory assay in FMD endemic regions practising vaccination is of the utmost importance. The potential use of the nonstructural proteins (NSPs) for differential detection of animals infected with the FMD virus (FMDV) in the vaccinated herds (DIVA) is well established. Several NSPs such as 2B, 2C, 3A, 3AB, 3B, 3ABC and 3D have been exploited as antigens for this purpose (for review see Clavijo et al., 2004). A reasonable consensus has emerged in favour of the polyprotein 3ABC or its derivatives such as 3AB as the most appropriate antigens for use in the screening tests (De Diego et al., 1997; Mackay et al., 1998; Sorensen et al., 1998).

The literature is replete with NSP ELISAs based on recombinant 3AB protein (Silberstein et al., 1997; Sorensen et al., 1998; Yakovleva et al., 2006; He et al., 2010). It has been demonstrated that the antibody response against polyprotein 3AB (3AB-Ab) could be used as a reliable serological marker for DIVA since the 3AB-Ab in pigs persisted up to 3.5 years post-outbreak and the animals, even after 10 rounds of vaccination, were found negative for 3AB-Ab (Chung et al., 2002). Therefore, the polyprotein 3AB was expressed in *Escherichia coli* ($r3AB_3$) and a simple, serotype independent indirect ELISA (I-ELISA) was developed and validated as a cost-effective import-alternative diagnostic assay for large scale serosurveillance in India.

2. Materials and methods

2.1. Sera

Serum samples examined in this study were derived from either cattle or buffalo and the term 'bovine', wherever it appears, implies both cattle and buffalo. This study complied with the international standards for animal welfare.

2.1.1. Sera from naïve cattle

210 samples were collected (paired sera collected one month apart from 105 cattle) from a geographically isolated, closed, dairy cow herd (age group 6–24 months and non-vaccinated) without a history of FMD for over 15 years. All the serum samples were negative ($<\log_{10}$ 0.9 titre) for anti-FMDV structural protein antibodies (SP-Ab) by liquid phase blocking ELISA (LPBE). The LPBE was

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performed essentially as described earlier (Ranabijuli et al., 2010) except that eight serial twofold dilutions of the test serum (1:4 to 1:512) instead of four dilutions (1:16 to 1:128) were used for better resolution of the end-point titre. Antibody titres were expressed as the $\log_{10}$ of the reciprocal of the highest test serum dilution yielding ≤50% of the absorbance recorded in the antigen control wells (with virus but without serum). Five commercial bovine serum samples (Sigma–Aldrich, St. Louis, MO, USA and Hyclone Laboratories, Logan, UT, USA) originating from FMD-free continents (North America and Australia) were also included. This set of 215 sera was used to determine the cut-off and diagnostic specificity of the I-ELISA.

2.1.2. Sera from uninfected vaccinated bovines

Serum samples (n = 48) were obtained from two bull calves, each vaccinated with the recommended dose of either of the two commercial (Indian Immunologicals Limited, Hyderabad, India and Intervet India Private Limited, Pune, India) trivalent (serotype O, A and Asia 1 components), killed, oil-adjuvanted vaccines (henceforth coded as vaccine A and B, respectively) every six months (5 boosters within 25 months). Samples (n = 24) were derived from two more calves, vaccinated with the same vaccines, but employing a rapid schedule in which every 15 days they received a standard dose (7 doses within 90 days). Samples (n = 356 from 119 cattle) were also collected from an FMD-free dairy cattle herd, vaccinated routinely every six months, on 21, 60 and 180 days post-vaccination (dpv). This herd received a trivalent inactivated vaccine (manufactured by Indian Veterinary Research Institute, Bangalore, India), designated henceforth as vaccine C. From FMD CP areas without any report of clinical FMD for more than five years, where the majority of the adult bovine population has received at least eight rounds of vaccination, samples (n = 372, 208 from buffalo and 164 from cattle) were collected one and six months post-vaccination. 54 samples were obtained at 7, 15 and 21 dpv from 18 cattle following a single vaccination (with a type O monovalent vaccine for which the exact antigen mass was not known) in a vaccine potency trial undertaken by a commercial vaccine manufacturer (Indian Immunologicals Limited, Hyderabad, India). Excluding the set of 24 sera derived from the rapid vaccination regime, the remaining 830 vaccinated samples were used for the I-ELISA cut-off and diagnostic specificity estimation.

2.1.3. Sera from infected bovines with clinical disease

A total of 192 sequential samples were obtained between 3 and 900 days post-infection (dpi) from four non-vaccinated bull calves. Two of the calves were infected experimentally by intradermolingual inoculation with either serotype A (A IND 40/2000) or Asia 1 (Asia1 IND 63/1972) virus. The remaining two calves were contact-infected after being co-housed separately with one of the inoculated animals. All four calves displayed classic vesicular lesions on the tongue within 72 h of infection except the calf contact-infected with type Asia 1 virus, where vesicular lesions were less notable and delayed. All these serum samples were made available from the serum repository of the Project Directorate on Foot and Mouth Disease (PDFMD), Mukteswar and were employed for assessing the post-infection kinetics of the 3AB-Ab and the SP-Ab response. Samples (n = 1158, 900 from cattle and 258 from buffalo) from clinical cases of FMD in field outbreaks (type O, A or Asia 1 involvement confirmed by sandwich ELISA) with or without a history of regular vaccination were included. These samples were collected at various time points, ranging from the acute phase of the outbreak to nearly 1 year post-outbreak. This panel of infected bovine sera (n = 1350) was used for determining the I-ELISA cut-off and diagnostic sensitivity.

2.1.4. Sera from in-contact bovines without clinical disease

Samples (n = 120, 86 from cattle and 34 from buffalo) were collected from outbreak areas from in-contact bovines presenting no symptoms of FMD. The majority of these herds were vaccinated regularly against FMD. This set of samples was used to assess NSP-seroconversion, which could be an underlying indicator of subclinical infection.

2.2. Expression, purification and immunological characterization of $r3AB_3$

The 3AB coding region (672 bp) of serotype Asia 1 virus (IND 491/1997) was amplified by RT-PCR and inserted into the pET-45b (+) vector (Novagen, Darmstadt, Germany). The recombinant plasmids were transformed into *E. coli* BL21 (DE3) pLysS expression host (Novagen, Darmstadt, Germany). Protein expression was induced with varying concentrations (0.1, 0.25, 0.5 and 1 mM) of IPTG at different temperatures (20, 25, 30 and 37 °C) and proceeding for various time intervals up to 12 h post-induction to optimize the maximum protein expression conditions. Both supernatant and the precipitate fractions obtained after sonication of the bacterial pellet were analyzed on 12% SDS-PAGE. Nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatographic purification of the His-tagged target protein was performed using QIAexpress kit (Qiagen, Hilden, Germany). Finally, the bound $r3AB_3$ was eluted in the elution buffer containing 300 mM imidazole. The concentrated protein was stabilized with trehalose (100 mM) before lyophilization. The immunoreactivity of $r3AB_3$ was tested by Western blot using Penta-His horseradish peroxidase (HRP) conjugated antibodies (Qiagen, Hilden, Germany), 29 dpi serum from the calf inoculated experimentally with A IND 40/2000 virus (Section 2.1.3) and a naïve cattle serum (Section 2.1.1).

2.3. Optimization and standardization of $r3AB_3$-based I-ELISA ($r3AB_3$ I-ELISA)

For optimization, pooled high-titred sera collected at 29 dpi {$\log_{10}$ 3.01 and 2.7 titre in LPBE and virus neutralization test, respectively and 110 percent positivity (PP) in the commercial Svanovir 3ABC ELISA (Svanovir FMDV 3ABC-Ab ELISA Kit, Svanova Biotech AB, Sweden)} and pooled low-titred sera collected at 5 and 10 dpi ($\log_{10}$ 1.5 titre by both LPBE and VNT and 49 PP in Svanovir 3ABC ELISA) from calves infected intradermolingually with A IND 40/2000 and Asia 1 IND 63/1972 viruses (Section 2.1.3) and pooled naïve cattle sera (<$\log_{10}$ 0.9 titre in LPBE and virus neutralization and 12 PP in Svanovir 3ABC ELISA) (Section 2.1.1) were employed as strong positive, weak positive and negative working standards, respectively. These sera served as internal controls in each run of the assay. The VNT was performed as described earlier (Mohapatra et al., 2008) with slight modifications to accommodate ten serial twofold dilutions of the serum (1:4 to 1:2048) instead of five dilutions (1:32 to 1:512). The end point titre was calculated as the $\log_{10}$ of the reciprocal of the highest dilution of serum that neutralized 100 $TCID_{50}$ of homologous virus in 50% of the wells. The optimum concentration of purified $r3AB_3$ protein for coating and the dilution of serum were finalized by using a gradient of protein concentrations and serial twofold dilutions of strong positive and negative control sera in a checkerboard titration format.

Briefly, ELISA plates (Nunc, Roskilde, Denmark) were coated with 1 μg/ml of purified $r3AB_3$ protein diluted in carbonate buffer (50 μl/well), pH 9.6 and incubated overnight at 4 °C. Test serum samples, negative, weak and strong positive control sera were diluted (1:20) in the dilution buffer {phosphate buffered saline containing 0.05% Tween 20 (PBST), 3% skim milk, 10% chicken serum and 0.01% *E. coli* sonicate}. After three washes with PBST, diluted serum samples were transferred to the coated plates, and